Functional Insights into Antimicrobial Effectors Driving Niche Adaptation of the Soil-Borne Plant Pathogenic Fungus *Verticillium dahliae*

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Table of Contents

Thesis Summary

Plants are colonized throughout their entire life by a complex community of microorganisms, termed the plant microbiota. Together, the plant and its microbiota form a functional unit, the holobiont, reflecting the idea that optimal plant performance depends on interactions with its microbial partners. While some microbes are vertically transmitted via seed endophytes, most are recruited from the environment, with soil as the primary source. The composition of the plant microbiota is shaped by both biotic and abiotic factors, including plant genotype, root exudates, environmental conditions, and agricultural practices. Members of these communities can interact with the plant in ways ranging from commensalism and mutualism to parasitism, and crucially, some can protect the host from pathogens, for example by secreting antibiotic compounds to antagonize the pathogen. In turn, emerging research has shown that plant pathogens deploy effector proteins not only to suppress host immune responses, but also to manipulate the host microbiota to their advantage and facilitate host colonization. This thesis investigates the roles of such antimicrobial effector proteins in plant pathogenic fungi, focusing on the broad host-range vascular wilt pathogen *Verticillium dahliae* and how these functions vary across environmental contexts.

In this thesis I describe the development and application of a gnotobiotic system designed to thoroughly investigate the complex interactions among plants, their microbiota, and the fungal pathogen *V. dahliae*. I outline the establishment of reliable infection protocols within this sterile environment and demonstrate how a synthetic microbial community can effectively disrupt fungal disease progression. Importantly, the results show that specific antimicrobial effector proteins from *V. dahliae* contribute to fungal virulence in distinct ways. Some function in a microbiota-dependent context, while others have additional roles beyond manipulating the microbiota, suggesting a dual functionality of particular effectors.

Further, I characterize a novel *V. dahliae* antimicrobial effector protein, called Av2. Building on *in silico* predictions suggesting antimicrobial properties of Av2, this thesis confirms its antimicrobial activity *in vitro*. By using a combination of microbiota sequencing, microbial co-cultivation assays, and experiments conducted within a gnotobiotic plant cultivation system, I demonstrate that Av2 serves as a microbiota-dependent virulence factor during host colonization. Specifically, these results show that Av2 is exploited to counteract the plant's recruitment of antagonistic *Pseudomonas* species during host infection thereby facilitating successful fungal invasion.

Additionally, in this thesis I explore underlying principles of microbiota assembly and how the antimicrobial effector protein Ave1, secreted by *V. dahliae*, affects pathogen virulence and microbial

communities during infections of plants with diverse microbiota. I assembled an extensive collection of natural soil samples and showed, across three plant species, that root-associated bacterial and fungal communities are predominantly shaped by soil type, while the phyllosphere microbiota is largely determined by plant species. Utilizing this soil collection and microbiota profiling of *V. dahliae*-infected tomato plants, I reveal that the contribution of the antimicrobial effector Ave1 to fungal virulence varies depending on soil type. Although Ave1 consistently modifies host microbiota in all tested soils, the changes in microbial composition caused by the effector are strongly by the original soil's microbial composition. These results indicate that while Ave1-driven manipulation of the microbiota is a general phenomenon, its effect on fungal virulence is shaped by the specific soil-derived microbial communities assembled by the plant.

Collectively, the results presented in this thesis support the view that fungal antimicrobial effectors are important tools for establishment across diverse environments. These effectors however are not universally acting virulence determinants with the same function in every environment. Rather, they are key components of the fungal secretome whose contribution to niche establishment is tightly linked to the environmental and microbial conditions in which infections occur. Understanding these functions and the mechanisms underlying their variability may not only deepen our understanding of fungal niche adaptation but also inform the future development of more robust microbiota-based disease control strategies for agriculture.

Zusammenfassung

Pflanzen werden während ihres gesamten Lebens von einer komplexen Gemeinschaft von Mikroorganismen besiedelt, die als Pflanzenmikrobiom bezeichnet wird. Zusammen bilden die Pflanze und ihre Mikrobiom eine funktionelle Einheit, den Holobionten, was die Vorstellung widerspiegelt, dass die optimale Leistungsfähigkeit einer Pflanze von den Wechselwirkungen mit ihren mikrobiellen Partnern abhängt. Während einige Mikroben vertikal als Samenendophyten übertragen werden, stammen die meisten Mikroben des pflanzlichen Mikrobioms aus der Umwelt, wobei der Boden die Hauptquelle ist. Die Zusammensetzung des Pflanzenmikrobioms wird sowohl von biotischen als auch von abiotischen Faktoren beeinflusst, darunter der Genotyp der Pflanze, Wurzelausscheidungen, Umweltbedingungen und landwirtschaftliche Praktiken. Die Mitglieder dieser mikrobiellen Gemeinschaften können mit der Pflanze auf verschiedene Weise interagieren, von Kommensalismus und Mutualismus bis hin zu Parasitismus, und vor allem können sie den Wirt vor Krankheitserregern schützen, beispielsweise können Pflanzen nützliche Mikroben rekrutieren, die in der Lage sind, den Krankheitserreger zu bekämpfen. Im Gegenzug haben verschiedene Studien der letzten Jahre gezeigt, dass Pflanzenpathogene während der Infektion so genannte Effektorproteine nicht nur einsetzen, um die Immunantwort des Wirts zu unterdrücken, sondern auch, um das Mikrobiom des Wirts gezielt zu ihrem Vorteil zu manipulieren. Diese Arbeit untersucht die Rolle solcher antimikrobiellen Effektorproteine in pflanzenpathogenen Pilzen, wobei der Schwerpunkt auf *Verticillium dahliae* liegt, einem Pathogen mit breitem Wirtsspektrum, und darauf, wie sich die Funktionen dieser Proteine in verschiedenen Umweltkontexten unterscheiden.

In dieser Arbeit beschreibe ich die Entwicklung und Anwendung eines gnotobiotischen „Flowpot"-Systems, das dazu dient, die komplexen Wechselwirkungen zwischen Pflanzen, ihres Mikrobioms und dem Pathogen *V. dahliae* gründlich zu untersuchen. Ich präsentiere die Etablierung zuverlässiger Infektionsprotokolle in dieser sterilen Umgebung und zeige, wie ein synthetischer Mix aus verschiedenen Mikroorganismen das Fortschreiten der Pilzkrankheit wirksam unterbrechen kann. Wichtig ist, dass unsere Ergebnisse zeigen, dass bestimmte antimikrobielle Effektorproteine von *V. dahliae* auf unterschiedliche Weise zur Virulenz des Pilzes beitragen, wobei einige in einem Mikrobiom-abhängigen Kontext funktionieren, andere hingegen zusätzliche Rollen über die Manipulation des Mikrobioms hinaus haben, was auf eine doppelte Funktionalität einer Untergruppe von Effektoren hindeutet.

Darüber hinaus charakterisiere ich ein neuartiges antimikrobielles Effektorprotein von *V. dahliae* namens Av2. Aufbauend auf *In-silico*-Vorhersagen, die auf antimikrobielle Eigenschaften von Av2 hindeuten, bestätigt diese Arbeit dessen antimikrobielle Aktivität *in vitro*. Mithilfe einer Kombination

aus Mikrobiom-Sequenzierung, mikrobiellen Co-Kultivierungsassays und Experimenten, die in einem gnotobiotischen Pflanzenkultivierungssystem durchgeführt wurden, zeige ich, dass Av2 während der Besiedlung des Wirts als Mikrobiom-abhängiger Virulenzfaktor fungiert. Insbesondere zeigen diese Ergebnisse, dass Av2 genutzt wird, um der Rekrutierung antagonistischer *Pseudomonas* Bakterien durch die Pflanze während der Wirtsinfektion entgegenzuwirken und so eine erfolgreiche Pilzinvasion zu ermöglichen.

Des Weiteren untersuche ich in dieser Arbeit die zugrunde liegenden Prinzipien der Mikrobiom-Bildung und wie das von *V. dahliae* sekretierte antimikrobielle Effektorprotein Ave1 die Virulenz des Krankheitserregers und mikrobielle Gemeinschaften während Infektionen von Pflanzen mit unterschiedlicher Mikrobiomen beeinflusst. Diese Studie begann mit der Zusammenstellung einer umfangreichen Sammlung natürlicher Bodenproben und zeigte anhand von drei Pflanzenarten, dass die mit den Wurzeln assoziierten Bakterien- und Pilzgemeinschaften überwiegend vom Bodentyp geprägt sind, während das mit oberirdischen Pflanzengeweben assoziierte Mikrobiom überwiegend von der Pflanzenart geprägt wird. Mittels dieser Bodensammlung und der Mikrobiom-Analysen von mit *V. dahliae* infizierten Tomatenpflanzen zeige ich, dass der Beitrag von Ave1 zur Virulenz von Pilzen je nach Bodentyp variiert. Obwohl Ave1 das Pflanzenmikrobiom in allen getesteten Böden modifiziert, hängen die von diesen Veränderungen beeinflussten mikrobiellen Taxa stark von der ursprünglichen mikrobiellen Zusammensetzung des Bodens ab. Diese Ergebnisse deuten darauf hin, dass die durch Ave1 verursachte Manipulation der Mikrobioms zwar ein allgemeines Phänomen ist, ihre Wirkung auf die Virulenz von Pilzen jedoch von den spezifischen, vom Boden stammenden mikrobiellen Gemeinschaften geprägt wird, die von der Pflanze gebildet werden.

Insgesamt stützen die in dieser Arbeit vorgestellten Ergebnisse die Ansicht, dass antimikrobielle Effektoren von Pilzen wichtige Werkzeuge für die Etablierung in verschiedenen Umgebungen sind. Diese Effektoren sind jedoch keine universell wirkenden Virulenz-Faktoren mit derselben Funktion in jeder Umgebung. Vielmehr sind sie Schlüsselkomponenten im Sekretom der Pilze, deren Beitrag zur Nischenetablierung eng mit den Umwelt- und mikrobiellen Bedingungen verbunden ist, unter denen Infektionen auftreten. Das Verständnis dieser Funktionen und der Mechanismen, die ihrer Variabilität zugrunde liegen, kann nicht nur unser Verständnis der Nischenanpassung von Pilzen vertiefen, sondern auch die zukünftige Entwicklung robusterer Mikrobiom-basierter Strategien zur Krankheitsbekämpfung in der Landwirtschaft beeinflussen.

Chapter 1: General Introduction

The plant microbiota

Throughout their life cycle, plants associate with complex and diverse microbial communities that form the so-called plant microbiota (Trivedi *et al*., 2020). These communities are predominantly composed of bacteria, followed by fungi, with other groups such as protists, nematodes, algae, and archaea also being considered integral members (Trivedi *et al*., 2020; Sokol *et al*., 2022). Together with their host, these microbial communities form a complex biological unit often referred to as the holobiont (Vandenkoornhuyse *et al*., 2015). Plant microbiota are commonly categorized according to the specific plant compartments they inhabit. The rhizosphere microbiota comprises microbes that establish in the soil zone directly influenced by root exudates, while the phyllosphere microbiota includes microorganisms associated with above-ground plant surfaces (Trivedi *et al*., 2020). Further, endophytic microorganisms, collectively termed the endosphere microbiota, live within plant tissues, including roots and shoots, thus spanning the rhizosphere and phyllosphere.

The assembly of the plant microbiota is not a random process but the result of a complex interplay between numerous biotic and abiotic factors (Trivedi *et al*., 2020; Mesny *et al*., 2023). While some members of the plant microbiota are vertically transmitted across generations via seed endophytes, a substantial portion originates from environmental sources. Airborne transmission contributes to this recruitment, but the bulk of microbial colonizers is derived from the surrounding bulk soil microbiota (Chialva *et al*., 2022). Soil can harbor an enormous number of microbes, as a single gram of soil can host more than 10^9 bacterial cells, 2x 10^8 fungal hyphae, 10^{12} viruses and 10^4 protists (Sokol *et al*., 2022). The composition of these bulk soil microbiota, and thus the pool of microbes plants recruit a substantial part of their microbiota from, is heavily influenced by soil physicochemical properties like pH, organic carbon content, nutrient availability, temperature, and redox status (Fierer, 2017). As a result, soil properties play a key role in shaping the plant microbiota, as evidenced by the distinct microbial communities associated with plants grown on different soils (Bulgarelli *et al*., 2012; Thiergart *et al*., 2020).

Beyond environmental factors, the host plant itself exerts selective pressure on microbial colonization, influencing which microbes become established within the plant microbiota (Bulgarelli *et al*., 2012; Lundberg *et al*., 2012; Wagner *et al*., 2016). This is illustrated by the concept of the core microbiota, which revolves around the observation that particular microbial taxa consistently colonize plants across different environments (Lundberg *et al*., 2012; Almario *et al*., 2022). Additionally, host selection is also evident in the compartment-specific microbiota within an individual plant, where a general decline in microbial diversity is observed from the surrounding soil to the rhizosphere- and

phyllosphere-associated microbiota (Trivedi *et al*., 2020). Besides all these deterministic factors, microbial community assembly and structure is also affected by different stochastic processes (Zhou and Ning, 2017). For example, the order of microbial arrival, also known as a priority effect, can significantly influence the final composition of the plant microbiota (Carlström *et al*., 2019; Debray *et al*., 2022; Wippel *et al*., 2021).

Plant immunity and the protective role of the microbiota

Microbes that establish in the plant microbiota can interact with their host according to a range of different interactions. While most microbes establish commensal interactions with their host, some microbes establish beneficial interactions with the plant, whereas others establish pathogenic interactions that lead to disease (Hassani *et al*., 2018). To detect pathogens and to initiate immune responses plants harbor an immune system (Jones and Dangl, 2006; Cook *et al*., 2015; Jones *et al*., 2024).

An important model of the plant immune system, termed the "Zig-Zag"-model proposed that plants can detect pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) with particular transmembrane pattern-recognition receptors (PRRs) to initiate an immune response termed pattern triggered immunity (PTI). Host-adapted pathogens are capable of secreting effector molecules to interfere with this PTI response and causing disease. The resulting susceptibility is then termed effector-triggered susceptibility (ETS). Plants, in turn, can possess an additional layer of their defense system, harboring nucleotide-binding, leucine-rich repeat (NB-LRR) receptors that recognize pathogen effectors and initiate so-called effector-triggered immunity (ETI). ETI can then be overcome by pathogens by losing or mutating the effector or acquiring new ones in order to suppress ETI responses (Jones and Dangl, 2006). Subsequently, a modified model was proposed based on the observations that ETI and PTI cannot be separated, but are rather highly connected. Taking this into account, the "Invasion"-model of the plant immune system proposes that plants utilize invasion pattern receptors (IPRs) to detect invasion patterns (IPs), which are molecules that are produced by the invading pathogen or result for the invasion procedure itself (Cook *et al*., 2015). This results in initiating an invasion pattern triggered response (IPTR), which again may be manipulated by an invading pathogen using effector molecules (Cook *et al*., 2015). Upon pathogen perception plants can induce a range of defense mechanisms, including the production of reactive oxygen species (ROS), ion influxes, activation of protein kinases, biosynthesis of defense hormones, induction of defense related genes and cell wall fortifications (Peng *et al*., 2018; Jones *et al*., 2024).

Plants can also in part rely on their microbiota in the defense against invading pathogens (Du *et al*., 2025). A key strategy is the so-called "cry-for-help" recruitment of beneficial microbes upon pathogen

attack, which aids the plant to cope with the invader (Rolfe *et al*., 2019). For example, during infections by the soil-borne pathogen *Fusarium oxysporum* f. sp. *cucumerinum*, cucumber plants can recruit *Bacillus amyloliquefaciens* bacteria to reduce disease severity (Liu *et al*., 2017). Under favorable conditions, ultimately such recruitment-based defense mechanisms can lead to the formation of disease-suppressive soils. Disease suppressive soils are soils with a virulent pathogen and a susceptible host, but not outbreak of disease (Spooren *et al*., 2024). For instance, several years of monoculturing wheat plants in the same field has been demonstrated to under favorable conditions to lead to the reduction of the take-all-disease, caused by the soil borne pathogen *Gaeumannomyces graminis* var. *tritici*. This decline was attributed to a plant-mediated recruitment of Pseudomonas species, capable of producing 2,4-diacetylphloroglucinol or phenanzine-1-carboxylic acid antibiotics, which antagonize the pathogen and diminish further disease establishment (Raaijmakers and Weller, 1998; Spooren *et al*., 2024). Importantly, microbial-mediated protection extends beyond direct pathogen antagonism through the secretion of antibiotic compounds. Certain beneficial microbes enhance plant immunity by triggering systemic defense responses. For instance, *Arabidopsis thaliana* infected with the foliar pathogen *Hyaloperonospora arabidopsidis* selectively enrich three bacterial species in its rhizosphere, boosting systemic resistance against the pathogen, improving plant growth and even benefiting future plant generations by promoting a more protective microbiota (Berendsen *et al*., 2018).

Gnotobiotic systems and synthetic communities in plant microbiota research

Research on plant-microbiota interactions is often complicated by the sheer complexity of host-associated microbial communities. The multitude of plant-microbe and microbe-microbe interactions, constantly influenced by various environmental factors, make mechanistic studies challenging. To circumvent this issue, gnotobiotic plant growth systems have been developed, that enable controlled experiments either with defined microbial communities or in complete sterility (Kremer *et al*., 2021; Vorholt *et al*., 2017; Liu *et al*., 2019; Ma *et al*., 2022). Over the past decades, various gnotobiotic systems have been developed using different substrates, each with specific advantages and limitations. Agar-based systems allow precise control over nutrient availability but create highly artificial growth conditions (Innerebner *et al*., 2011). Clay-based systems, such as those using calcined clay, better mimic soil structure and are easily sterilizable, yet lack organic carbon and make it difficult to regulate key nutrients like nitrogen and phosphorus (Carlström *et al*., 2019). In contrast, peat-based systems provide a source of organic carbon and a more natural matrix but offer only limited control over nutrient composition (Liu *et al*., 2019; Kremer *et al*., 2021). Consequently, the choice of substrate and system is always depending on the specific research question of interest.

Gnotobiotic systems are frequently used in reductionist approaches, particularly when combined with synthetic microbial communities (SynComs), which are defined, relatively low-complexity microbiotas derived from microbial culture collections. These allow researchers to track the behavior and function of specific microbes or microbial consortia in a reproducible and controlled manner (Vorholt *et al*., 2017; Novak *et al*., 2024). For example, a calcined clay-based gnotobiotic system combined with a SynCom of *Arabidopsis thaliana*-associated bacteria revealed the importance of priority effects in shaping phyllosphere microbiota assembly (Carlström *et al*., 2019). Another study using SynComs derived from *A. thaliana* and *Lotus japonicus* in a peat-based system demonstrated host-specific preferences among commensal bacteria (Wippel *et al*., 2021). Gnotobiotic systems have also proven valuable for investigating the role of the host microbiota in plant pathogen interactions. For example, a peat-based gnotobiotic system demonstrated that *Arabidopsis thaliana* plants grown in the absence of a microbiota exhibit impaired immunity against *Pseudomonas syringae* and *Botrytis cinerea*, a defect that can be restored by introducing a synthetic microbial community (SynCom) derived from healthy plants (Paasch *et al*., 2023). Another example is a systematic screen of 224 bacterial isolates from an *Arabidopsis thaliana* phyllosphere culture collection, which found that approximately 10% conferred protection against *Pseudomonas syringae*, highlighting the disease-suppressive potential of natural microbiota members (Vogel *et al*., 2021).

The biology of the fungal plant pathogen *Verticillium dahliae*

Fungal pathogens pose significant threats, impacting both food production and/or human health directly (Stukenbrock and Gurr, 2023). A highly important fungal pathogen that can cause severe problems in food production is the soil-borne fungal pathogen *Verticillium dahliae*, the causal agent of Verticillium wilt disease (Inderbitzin *et al*., 2011; Fradin and Thomma, 2006). *V. dahliae* is an asexual, ascomycete fungus that is capable of causing disease on hundreds of dicotyledonous plant species, including many important crops like tomato, olive, sunflower and cotton. Verticillium wilt symptoms can vary on the respective host, but often include necrosis, stunting, wilting, chlorosis and vascular discoloration (Fradin and Thomma, 2006).

The *V. dahliae* infection cycle begins with the germination of microsclerotia, fungal resting structures that persist in the soil and respond to root exudates. The resulting hyphae grow through the soil to reach the roots of the host plants, typically entering the plant at the root tip or sites of lateral root emergence. After penetrating the root cortex and crossing the endodermis, the fungus invades xylem vessels. Within the xylem, *V. dahliae* sporulates, producing conidia that are passively transported with the transpiration stream, thereby disseminating the pathogen throughout the plant. Once the spores become trapped within the vasculature, they germinate and penetrate neighboring vessels. Upon host

tissue necrosis or senescence, the fungus exits the xylem and enters a saprophytic phase, during which it forms new microsclerotia. These structures are eventually released into the soil as plant tissues decompose (Fradin and Thomma, 2006).

Plant protection against *Verticillium* wilt is challenging for several reasons. First, the broad host range of the pathogen, combined with the long-term survival of its microsclerotia in soil, makes crop rotation ineffective unless extended over long periods with non-host species. However, the latter is complicated given the extremely wide host range of the pathogen. Additionally, fungicide treatments are largely ineffective once the fungus has entered the plant's vascular system (Fradin and Thomma, 2006). Although monogenic resistance offers a potential avenue for control, such resistance remains scarce. One well-characterized example is the *Ve1* locus in tomato, which encodes a receptor-like protein that can recognize the Ave1 effector and trigger an immune response (Kawchuk *et al*., 2001; de Jonge *et al*., 2012). Strains that express *Ave1*, and are thus recognized by Ve1, are classified as race 1. In contrast, race 2 strains have evolved to evade detection by losing the *Ave1* gene (de Jonge *et al*., 2012; Faino *et al*. 2016). Interestingly, *Ave1* also contributes to fungal virulence in plants that lack *Ve1* (de Jonge *et al*., 2012). Another resistance locus, *V2*, was identified in wild tomato species and confers resistance against race 2 strains (Usami *et al*., 2017). Comparative genomic approaches between race 2 strains that are contained by *V2* and race 3 strains that overcome *V2*-mediated resistance led to the identification of the *Av2* effector gene. The Av2 effector activates resistance responses in *V2*-carrying tomato plants (Chavarro-Carrero *et al*., 2021). Notably, like *Ave1*, *Av2* also contributes to virulence on plants that lack the corresponding resistance locus (Kraege *et al*., 2025).

Manipulation of host immunity and microbiota by effector proteins

Intensive research over the past decades has led to a solid understanding of the molecular biology of infection strategies of microbial plant pathogens, as well as the colonization strategies of other kinds of microbes that establish symbiotic interactions with plant hosts. Intriguingly, pathogens and other kinds of microbial symbionts, including bacteria, fungi, and oomycetes, have evolved to secrete so-called effector molecules during host colonization; a diversity of molecules, some of which remain in the apoplast while others have intracellular destinations where they ultimately function to promote host colonization (Jones and Dangl, 2006; Cook *et al*., 2015).

Initially, effectors were proposed to interfere with host immune responses, at the level of pathogen perception by immune receptors, downstream immune signalling, or at the level of the execution of immunity (King *et al*., 2014; Jonge *et al*., 2010; Bozkurt *et al*., 2011). For example, whereas the effector Ecp6 is secreted by the fungal tomato pathogen *Cladosporium fulvum* to sequester chitin

oligosaccharides that are released from its cell walls to prevent recognition by chitin immune receptors (Jonge *et al*., 2010), the Cmu1 effector is produced by *Ustilago maydis* to interfere with host salicylic acid biosynthesis (Djamei *et al*., 2011), and the AVRblb2 effector is discharged by *Phytophtora infestans* to suppress the release of host defense proteases (Bozkurt *et al*., 2011). Later it was realized that besides modulating plant immune responses directly, effectors can also function in self-protection to undermine plant immune responses in an indirect manner. For instance, while *Cladosporium fulvum* secretes the chitin-binding effector Avr4, the fungal wheat pathogen *Mycosphaerella graminicola* secretes three chitin-binding LysM effectors that can shield chitin in their cell-walls from the activity of plant-derived chitinases (van den Burg *et al*., 2006; Marshall *et al*., 2011; Sánchez-Vallet *et al*., 2020). Although effectors were initially implicated in suppression of host immune responses, it became evident, that effectors may target other host physiological processes as well. For example, *Pseudomonas syringae* secretes the effectors HopM1 and AvrE to affect ABA signaling, leading to stomatal closure which contributes to water-soaking of the tissue in turn (Hu *et al*., 2022; Roussin-Léveillée *et al*., 2022). The aqueous living space that is generated in this manner is crucial for bacterial virulence (Xin *et al*., 2016), presumably also because the hydrated apoplast becomes enriched in metabolites that are exploited by the bacteria (Gentzel *et al*., 2022). Similarly, also *Xanthomonas* bacteria utilize effectors to trigger the release of nutrition from host cells, as they secrete transcription activator-like effector proteins into host cells to modulate the expression of host sugar transporters that secrete sugars into the apoplast (Chen *et al*., 2010).

The hypothesis for yet another type of function for effector proteins was inspired by findings for the *Zymoseptoria tritici* effector Zt6 (Kettles *et al*., 2018). This effector is expressed during spore germination and possesses ribonuclease activity that leads not only to phytotoxicity, but also to antimicrobial activity. In this manner, Zt6 was hypothesized to eliminate microbial competitors or antagonists near germinating spores to support the early stages of leaf colonization (Kettles *et al*., 2018). However, as a role for Zt6 in *Z. tritici* disease establishment could not be demonstrated, and effects of Zt6 secretion on phyllosphere microbiota compositions were not investigated, solid evidence for this hypothesis has been lacking. Nevertheless, this finding, combined with the increasing body of evidence that host plants rely on their microbiota to withstand pathogen attack, led to the hypothesis that pathogens may exploit effector proteins to support host colonization through the manipulation of host microbiota compositions (Snelders *et al*., 2018).

Evidence for this hypothesis has been obtained for the soil-borne fungal plant pathogen *Verticillium dahliae*. Incubation of the *V. dahliae* effector protein Ave1 with a selection of plant-associated microbes revealed selective antibacterial activity of the effector. Experiments that focused on the rhizosphere microbiota of tomato and cotton plants inoculated either with the wild type or an *Ave1*

deletion mutant, displayed significant shifts in the microbiota, including a suppression of Sphingomonadales bacteria in the presence of Ave1. Subsequent *in planta* assays revealed that pre-treatment of tomato seeds with Sphingomonadales bacteria reduced the severity of Verticillium wilt symptoms. At the same time, secretion of Ave1 significantly impaired the growth of these bacteria, arguing that Sphingomonadales can act antagonistically against *V. dahliae* and that the pathogen exploits the Ave1 effector to act against these antagonists during host colonization (Snelders *et al*., 2020).

Notably, Ave1 is not the only *V. dahliae* effector protein possessing antibacterial activity. A search for additional effectors within the *V. dahliae* secretome that display homology to known antimicrobial proteins yielded the AMP2 effector that is exclusively expressed in soil extract, mimicking conditions that *V. dahliae* encounters in soil. AMP2 revealed complementary activity to Ave1, suggesting that *V. dahliae* exploits different effectors to cope with the diversity of competitors in soil (Snelders *et al*., 2020). *In planta*, *V. dahliae* also secretes additional antimicrobial effectors, such as the antimicrobial effector protein Ave1L2. This effector that displays 65% sequence identity to Ave1, manipulates the host microbiota through the direct suppression of antagonistic Actinobacteria (Snelders *et al*., 2023). The activities of *V. dahliae* antimicrobial effector proteins are not limited to bacteria. For example, the defensin-like effector AMP3, which was identified based on its potential antimicrobial fold, was found to target the fungal component of the plant microbiota also called mycobiome. Intriguingly, and in contrast to Ave1, AMP2 and Ave1L2, the AMP3 effector is exclusively expressed at late stages of the infection when novel resting structures are formed in decaying plant tissue. At these stages, host immune responses fade and opportunists and decay organisms, including many fungi, start to colonize the host tissues, so AMP3 is likely to provide protection against these fungal competitors (Snelders *et al*., 2021). Over the last years several other studies have characterized antimicrobial effector proteins also in different pathogens like *Rosellinia necatrix*, *Ustilago maydis*, *Fusarium oxysporum*, and *Albugo candida* (Chavarro-Carrero *et al*., 2024; Gómez-Pérez *et al*., 2023; Chang *et al*., 2021; Ökmen *et al*., 2023). Notably, the machine learning tool AMAPEC, capable of predicting antimicrobial effectors from fungal secretomes, identified 349 putative antimicrobial effectors in *V. dahliae*, suggesting that a substantial portion of the fungal secretome is devoted to microbiota manipulation (Mesny and Thomma, 2024). Interestingly, large numbers of antimicrobial effectors were also predicted in the secretomes of the saprotrophic fungus *Coprinopsis cinerea* and the arbuscular mycorrhizal fungus *Rhizophagus irregularis*, with 457 and 558 effectors, respectively, indicating that these antimicrobial effector proteins are important also for non-pathogenic fungi that utilize them to manipulate their environment in their favor (Mesny and Thomma, 2024). This further supports the hypothesis that antimicrobial proteins are fundamental for the biology of fungi (Snelders *et al*., 2022).

Harnessing the microbiota for enhancing agriculture

In recent years, the plant microbiota has emerged as a key determinant of plant health, development, and productivity (Trivedi *et al*., 2020; Chialva *et al*., 2022). Growing insights into the processes and mechanisms by which these microbial communities enhance plant fitness have fueled increasing interest in harnessing the plant microbiota to sustainably improve agricultural outcomes (French *et al*., 2021; Escudero-Martinez and Bulgarelli, 2023). Reflecting this trend, the global market for microbial products in agriculture has grown significantly, reaching a valuation of over 14 billion USD by 2023. These products are broadly categorized as either biofertilizers, which promote nutrient uptake and plant growth, or biocontrol agents, which help protect plants from pathogens (French *et al*., 2021). Commonly used microbial agents include arbuscular mycorrhizal fungi, fungal endophytes, and nitrogen-fixing bacteria, some of which form nodules, while others are free living (French *et al*., 2021; Cassán and Diaz-Zorita, 2016). For example, Trichoderma species have been utilized for their protective effects against a variety of fungal pathogens, including *Rhizoctonia solani* and *Botrytis cinerea* (Harman *et al*., 2004). Similarly, *Azospirillum* spp. are widely employed as plant growth-promoting bacteria in legumes and cereal crops, enhancing nitrogen fixation and root development (Lugtenberg & Kamilova, 2009). Notably, field trials have reported significant yield increases in up to 70% of cases following inoculation with *Azospirillum* (Cassán and Diaz-Zorita, 2016). Often beneficial microbial strains can be combined in a synthetic community with other beneficial microbes and applied to plants. For instance, application of 12-member bacterial SynComs led to soybean plants grown on agricultural fields significantly increased soybean yield (Wang *et al*., 2021). Despite such promising results, a major challenge remains as the effects of the microbes can be highly inconsistent across different environments. Beneficial microbes that perform well under laboratory or greenhouse conditions often fail to replicate these outcomes in field settings, and positive effects can strongly vary between different fields (Sessitsch *et al*., 2019; French *et al*., 2021; Vaccaro *et al*., 2022). This variability arises from a range of abiotic and biotic factors that differ across receiving environments, influencing the ability of introduced bioinoculants to establish and function effectively (Weller, 1988; Sessitsch *et al*., 2019; French *et al*., 2021; Vaccaro *et al*., 2022).

Aside from the application of individual or smaller communities of biofertilizers or control strains understanding the microbiota can be harnessed to facilitate crop productivity predictions (Sessitsch *et al*., 2019; Song *et al*., 2025). One example is potato seed tuber transplantation from one field to another. The microbiota associated with these seed tubers has been shown to play a crucial role in

plant health and productivity. Notably, a recent study demonstrated that a model based on the seed tuber microbiota could accurately predict the growth potential of potato seedlings (Song *et al*., 2025).

Additionally, integrating knowledge on plant-microbiota interactions into plant breeding programs may ultimately pave away to enhance crop productivity in an environmentally sustainable way (Araujo *et al*., 2025; Escudero-Martinez and Bulgarelli, 2023). Adjusting root exudate composition may not only enhance recruitment of beneficial microbes but could also substantially improve the efficiency of strains exploited for biocontrol and biofertilization, enhancing sustainable agriculture (Kawasaki *et al*., 2021). For example, plants engineered to secrete octopine into the rhizosphere were found to selectively enrich for octopine-degrading microbial taxa, providing a proof-of-concept for adjusting host genetics in order to ultimately shape host microbial communities (Mondy *et al*., 2014).

Thesis aims and chapter outline

With this PhD thesis, I aim to overcome previous experimental limitations and develop novel gnotobiotic tools to investigate whether antimicrobial effectors act solely as antimicrobials or whether they also exert additional functions directly on host physiology. I aim to further broaden our understanding on antimicrobial effectors of *V. dahliae* and how they aid the pathogen to overcome microbiota-mediated plant defense mechanisms. Lastly, I aim to build on previous research to now investigate the role and virulence contributions of antimicrobial effectors during infections of plants that are grown in different environments and consequentially harbor different microbiota. By elucidating the multifaceted roles of antimicrobial effectors, this work advances our understanding of plant-microbe-microbiota interactions which may ultimately support the development of innovative microbiota-assisted disease control strategies in agriculture.

Chapter 2 (Figure 1a):

In this chapter called **"A gnotobiotic system reveals multifunctional effector roles in plant-fungal pathogen dynamics"**, I present the development and application of a refined gnotobiotic system optimized to mechanistically investigate the intricate interactions between plants, their microbiota, and the fungal pathogen *Verticillium dahliae*. I detail the establishment of robust infection protocols within this sterile system and demonstrate the efficacy of a synthetic microbial community (SynCom) in interfering with fungal disease progression. Crucially, the findings reveal that specific *V. dahliae* antimicrobial effector proteins differentially contribute to virulence, with some, such as Ave1L2, operating in a microbiota-dependent manner, while others like Ave1, appear to exert functions beyond microbial manipulation.

Chapter 3 (Figure 1b):

In this chapter called **"Undermining the cry for help: The phytopathogenic fungus *Verticillium dahliae* secretes an antimicrobial effector protein to undermine host recruitment of antagonistic Pseudomonas bacteria"**, I functionally characterize Av2, an effector protein from *V. dahliae*, as a novel suppressor of the plant's "cry for help" defense mechanism. Following *in silico* predictions of Av2 being an antimicrobial, I demonstrate antimicrobial activity of Av2 *in vitro*. Through a combination of microbiota sequencing, microbial co-cultivation assays, and experiments within a gnotobiotic plant cultivation system, I reveal that Av2 functions as a microbiota dependent-virulence factor during host

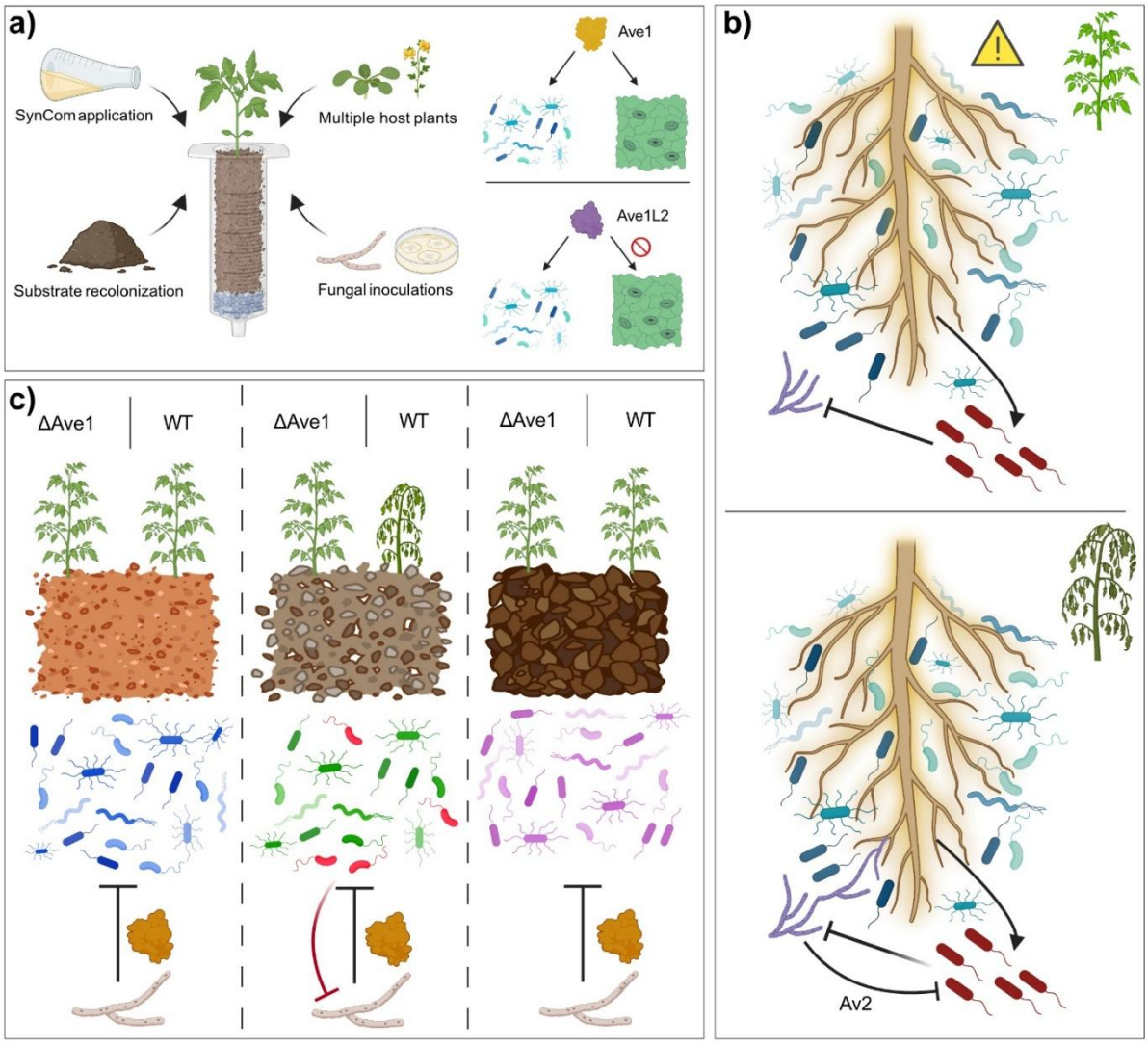

Figure 1 Schematic overview of each thesis chapter. a) Establishment of a novel gnotobiotic system to conduct mechanistic research on plants, their microbiota and fungal pathogens. The system was utilized to demonstrate that the antimicrobial effector Ave1L2 contributes to virulence in a microbiota dependent manner, whereas Ave1 possess additional virulence functions in host manipulation. **b)** Upon *V. dahliae* infections plants utilize a "cry for help" approach to recruit Pseudomonas bacteria into their microbiota that are capable of antagonizing the pathogen. In turn *V. dahliae* harbors the antimicrobial effector protein Av2 to undermine this recruitment and target the beneficial microbes, ultimately keeping the microbiota vulnerable and facilitating host colonization. **c)** The *V. dahliae* effector Ave1 alters tomato microbiota composition across diverse natural soils and contributes variably to fungal virulence on tomato plants, arguably due to the presence or absence of particular antagonists.

colonization. More specifically, these findings indicate that Av2 undermines the plant's recruitment of antagonistic *Pseudomonas* spp. upon *V. dahliae* infection, directly inhibiting *Pseudomonas* spp. growth and thereby paving the way for successful fungal invasion.

Chapter 4 (Figure 1c):

In this chapter called **"An antimicrobial effector from *Verticillium dahliae* differentially contributes to virulence and differentially impacts tomato microbiota across natural soils."** I investigate how the antimicrobial effector protein Ave1, secreted by *V. dahliae*, influences pathogen virulence and microbial communities across infections on plants harboring distinct microbiota. I first established a comprehensive collection of natural soil samples to demonstrate across three plant species that root-associated bacterial and fungal communities are primarily shaped by soil type, whereas the phyllosphere microbiota is mainly determined by plant species. Using the same soil collection, and through microbiota profiling of *V. dahliae*-inoculated tomato plants, I demonstrate that the contribution of Ave1 to fungal virulence varies across different soil types**.** Although Ave1 consistently alters host microbiota across all tested soils, the specific microbial taxa affected by these shifts are highly dependent on the original soil composition. These findings suggest that while Ave1-mediated microbiota manipulation occurs across soils, its impact on fungal virulence is influenced by the specific, soil-derived microbial community assembled by the plant host.

Chapter 5:

In this general discussion of my thesis I provide an overall discussion of the findings obtained during my PhD research and place these findings into a broader context.

Chapter 2: A gnotobiotic system reveals multifunctional effector roles in plant-fungal pathogen dynamics

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Abstract

Plants host diverse microbiota that influence physiological processes and can enhance resilience against invading pathogens that, in turn, evolved effector proteins to manipulate host microbiota in their favor. However, the complexity of microbial communities and their interactions complicates mechanistic research on processes governing microbiota assembly and function. Gnotobiotic systems are valuable tools to study plant microbiota by reducing complexity and enabling controlled microbiota reconstitution experiments. Despite their utility, no gnotobiotic systems have been established to investigate the role of antimicrobial effector proteins in the interactions between plants, their microbiota, and fungal pathogens. Here, we present a refined gnotobiotic system designed to study these interactions, establishing protocols for infections with the fungal pathogen Verticillium dahliae across multiple host plants under sterile conditions. We demonstrate that a synthetic microbial community (SynCom) derived from a culture collection generated for this study can be applied in this system where it interferes with fungal infections. Additionally, using our gnotobiotic system we reveal that specific antimicrobial effectors of V. dahliae, like Ave1L2, contribute to fungal virulence in a microbiota-dependent manner, whereas other antimicrobial effectors, such as Ave1, seem to possess functions beyond microbiota manipulation.

Introduction

During their life, plants functions as holobionts, an integrated unit consisting of the plant with its associated microbial communities, also known as the plant microbiota (Vandenkoornhuyse *et al*., 2015). This complex microbiota is associated with all plant parts and its composition depends on the interplay of various biotic and abiotic factors (Trivedi *et al*., 2020; Mesny *et al*., 2023). Microbes that establish in the plant microbiota can interact with the plant host in various ways, ranging from beneficial and growth-promoting to pathogenic and disease-inducing (Mesny *et al*., 2024). Consequently, the composition and functions of the microbiota contribute to plant health and productivity. Importantly, plants possess the ability to actively shape their microbiota to alleviate biotic or abiotic stresses. For instance, upon invasion of the "take-all-disease" pathogen Gaeumannomyces graminis var. tritici, wheat plants in particular fields in the USA were demonstrated to actively recruit beneficial Pseudomonas bacteria into their microbiota. These Pseudomonas species antagonize G. graminis via the secretion of 2,4-diacetylphloroglucinol or phenanzine-1-carboxylic acid antibiotics, leading to "take-all decline"; a reduction of disease severity over time (Spooren *et al*., 2024). Thus, microbiota have been described as an additional layer of plant defense against pathogen invasion (Mendes *et al*., 2011; Carrión *et al*., 2019). Microbiota not only serve as crucial defense barrier against invading pathogens, but also help plants mitigate the pathogenic potential of certain microbial community members. This is evident in cases where some microbes severely inhibit plant growth when inoculated individually, yet this negative impact disappears when they are introduced within a community context (Durán *et al*., 2018).

During host invasion, plant pathogens secrete so-called effectors, a diversity of molecules some of which remain in the apoplast while others have intracellular destinations where they function to promote host colonization (Jones and Dangl, 2006; Cook *et al*., 2015). Initially, effectors were proposed to interfere with host immune responses, by interfering with pathogen perception by plant immune receptors, downstream immune signalling, or the execution of immunity (de Jonge *et al*., 2010; Bozkurt *et al*., 2011; King *et al*., 2014). However, later it was realized that besides direct modulation of plant immune responses, effectors can also function in self-protection to undermine plant immune responses in an indirect manner (van den Burg *et al*., 2006; Jonge *et al*., 2010). Although effectors were initially implicated in suppression of host immune responses, manipulation of other host physiological processes could also be demonstrated, including for instance the induction of water-soaking by manipulation of stomatal closure, or sugar release into the apoplast through the manipulation of host sugar transporter expression (Chen *et al*., 2010; Hu *et al*., 2022; Roussin-Léveillée *et al*., 2022). Notably, several effectors were described to possess multiple functions, contributing to host colonization through different mechanisms (Liu *et al*., 2016; Lin *et al*., 2023).

Recently it was shown that pathogens exploit effector proteins to target host microbiota, and manipulate their composition to breach the protective defense layer that the microbiota provides which ultimately promotes host colonization (Kettles *et al*., 2018; Snelders *et al*., 2020; Snelders *et al*., 2021; Chang *et al*., 2021; Snelders *et al*., 2023; Ökmen *et al*., 2023; Gómez-Pérez *et al*., 2023; Chavarro-Carrero *et al*., 2024; Mesny *et al*., 2024). For example, the effector protein Ave1 from the fungal plant pathogen *Verticillium dahliae* exhibits selective antibacterial activity, suppressing Sphingomonadales bacteria in the rhizosphere microbiota of infected cotton and tomato plants. Since Sphingomonadales can reduce Verticillium wilt severity, *V. dahliae* exploits Ave1 to inhibit these beneficial bacteria and facilitate host colonization (Snelders *et al*., 2020). Building on this discovery, the effector protein Ave1L2 was identified through sequence similarity to Ave1, and similarly exhibits selective antibacterial activity, albeit with a distinct activity spectrum. In planta assays showed that Ave1L2 manipulates host microbiota by targeting Actinobacteria in the tomato rhizosphere. Antibiotic-induced depletion of Actinobacteria in the plant microbiota increased plant susceptibility to *V. dahliae* while reducing the virulence impact of Ave1L2 (Snelders *et al*., 2023). In both cases, the contribution of antimicrobial effector proteins to virulence of *V. dahliae* could only be tested in the presence of plant-associated microbiota, making it impossible to exclude the simultaneous occurrence of virulence contributions through direct manipulation of host targets. Considering that several antimicrobial effectors seem to have an ancient origin, and likely acted in intermicrobial competition before land plant evolution, they may have acquired additional functions in host manipulation during fungal co-evolution with their host plants (Snelders *et al*., 2021; Snelders *et al*., 2022; Mesny and Thomma, 2024).

Research on plant-microbiota interactions, including the role and contribution of antimicrobial effector proteins, is often complicated by the sheer complexity of host-associated microbial communities. The numerous plant-microbe and intermicrobial interactions, which constantly respond to various environmental cues, make mechanistic studies challenging. Gnotobiotic plant growth systems, which allow for controlled experiments in the presence or absence of particular microbiota, offer a powerful tool to address these challenges (Vorholt *et al*., 2017; Liu *et al*., 2019; Ma *et al*., 2022). Over the past decades, various gnotobiotic plant growth systems have been developed based on diverse substrates, including agar-based (Innerebner *et al*., 2011), clay-based (Carlström *et al*., 2019), and peat-based substrates (Kremer *et al*., 2021), each with distinct advantages and limitations. Agar-based systems provide precise control over nutrient availability but generate highly artificial conditions. Clay-based systems offer a soil-like substrate structure and are easily sterilizable, but lack organic carbon, and the substrate itself makes it challenging to regulate nitrogen and phosphorus levels. In contrast, peat-based systems comprise organic carbon, yet lack control over nutrient composition (Liu *et al*., 2019;

Kremer *et al*., 2021). Ultimately, the choice of gnotobiotic system depends on the specific research question that is addressed. Gnotobiotic systems are frequently used in reductionist experiments and can be particularly powerful when combined with synthetic microbial communities (SynComs). Such SynComs typically are communities with reduced complexity when compared with natural communities, generated from microbial culture collections, allowing to monitor the impact of defined microbial communities on a particular trait of interest (Vorholt *et al*., 2017; Novak *et al*., 2024). For example, experiments utilizing a calcined clay-based gnotobiotic system and a culture collection of Arabidopsis thaliana-associated bacteria were used to demonstrate the role of priority effects during assembly of the A. thaliana phyllosphere microbiota (Carlström *et al*., 2019). Additionally, a repopulation study using A. thaliana plants grown in a peat-based gnotobiotic system with a 106-member multi-kingdom SynCom identified evolutionary conserved genetic determinants for bacterial root colonization (Vannier *et al*., 2023). Several studies have utilized gnotobiotic systems to study the impact of the microbiota on plant defense against pathogens (Vogel *et al*., 2021; Paasch *et al*., 2023). For instance, in a systematic approach, screening of 224 bacterial isolates from an A. thaliana phyllosphere culture collection for protection against the bacterial pathogen Pseudomonas syringe revealed that 10% of the bacteria can prevent bacterial speck disease (Vogel *et al*., 2021).

Due to the lack of suitable inoculation protocols, no gnotobiotic system has been available to determine whether antimicrobial effector proteins from the soil-borne fungal pathogen V. dahliae contribute to virulence also in the absence of host-associated microbiota, and therefore have additional host targets. In this study, we describe a peat-based gnotobiotic system for plant inoculations with V. dahliae and its use to investigate the role of antimicrobial effector proteins in fungal virulence.

Results

Establishment of a Flowpot-system tailored for Verticillium wilt development

We aimed to modify a previously published Flowpot-system to study the behavior of the fungal pathogen *Verticillium dahliae* during disease development (Kremer *et al*., 2021). Our adapted Flowpot-system relies on plants grown in commonly available 50 ml syringes that are filled with a blend of peat substrate and vermiculite (Figure 1a). The substrate mixture is sterilized in three consecutive autoclaving steps. To assess sterility, sterilized substrate was plated onto various growth media. Following four days of incubation, no signs of microbial growth were observed, indicating successful substrate sterilization (Suppl. Figure 1a). Next, we aimed to generate a non-sterile control substrate that underwent a similar treatment through triple sterilization. For this purpose, we recolonized sterilized substrate by mixing sterilized and non-sterilized substrate in a 9:1 ratio (Figure 1b). To verify

substrate recolonization, we again made use of plating, revealing substantial microbial growth on all media (Suppl. Figure 1a).

Considering the impact of autoclaving on the physicochemical properties of peat-based substrates and to try and eliminate potentially toxic compounds that might have been released during the autoclaving

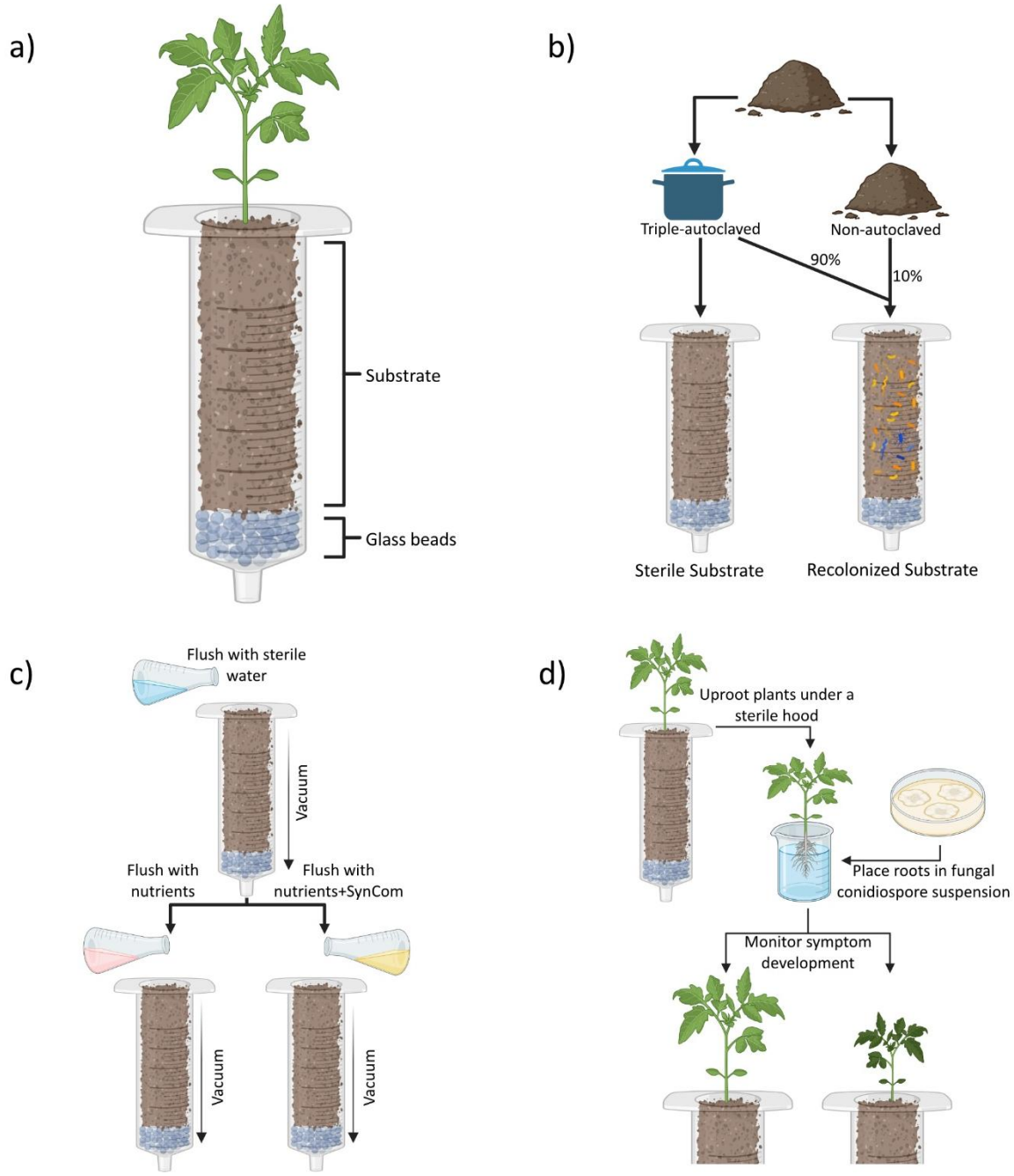

Figure 2 Technical set-up of the Flowpot-ystem. a) Schematic overview of an individual Flowpot-unit. **b)** Substrate preparation procedure. Triple autoclaved substrate is mixed with 10% untreated soil to create a substrate that is recolonized by microbes. **c)** Flowpot-flushing procedure. Water is flushed through each Flowpot using vacuum, followed by another flushing step with a nutrient solution that can be supplemented with a SynCom. **d)** *Verticillium dahliae* inoculation procedure. Plants are uprooted in a sterile hood and placed into *V. dahliae* conidiospore-suspension for several minutes before replanting into the substrate and monitored for symptom development at 14 dpi.

procedure that may affect plant growth, we applied substrate washes. To this end, vacuum was applied to each Flowpot and the substrate was rinsed with sterilized water. Moreover, the flushing mechanism was utilized to supply nutrients to the substrate (Figure 1c).

Gnotobiotic systems can be used for microbiota reconstitution experiments, conducted through the application of single microbial species or SynComs to study the role of defined microbiota on a particular trait. To enable inoculation of the substrate, our Flowpot protocol utilizes the vacuum flushing mechanism to supplement substrate with microbial suspensions, thereby enabling substrate colonization by a defined microbial inoculum. In this manner, our modified Flowpot-system provides a versatile platform for experiments to be conducted either in the absence or in the presence of non-defined or defined microbial communities.

The Flowpot-system is suitable for *Verticillium* infections on various host plants

Thus far, the role and impact of antimicrobial effector proteins of the fungal plant pathogen *V. dahliae* has been studied mostly on tomato. Research on these proteins can be facilitated substantially with suitable gnotobiotic systems, allowing for fungal infections in otherwise sterile environments. Therefore, we aimed to establish infections of *V. dahliae* on tomato plants in our Flowpot-system. To test if the Flowpot-system is suitable to maintain tomato plants under gnotobiotic conditions, we germinated surface-sterilized seeds on sterilized substrate and assessed plant growth. Despite a low germination rate of only 20% under these conditions, tomato plants grown in gnotobiotic conditions appeared healthy after 24 days of growing, indicating that the conditions are suitable for growth (Figure 2a). Since plants recruit a substantial part of their microbiota from the surrounding bulk soil, but also from endophytes that already reside within the plant seed, we also assessed to what extent growth in the sterilized substrate leads to a less diverse plant microbiota. To this end, we conducted 16S amplicon sequencing of tomato stems of plants grown on sterilized or recolonized substrate. As expected, plants grown on sterilized substrate carried communities with a significantly lower Shannon index when compared with plants grown on recolonized substrate, indicating that plants grown on sterilized substrate harbor significantly less diverse microbial communities (Suppl. Figure 1b). This reduction in microbial diversity correlated with notable effects on plant growth, as tomato plants grown on recolonized substrate revealed substantially higher germination rates (76%) and produced significantly more biomass when compared with plants grown on sterilized substrate, demonstrating the growth-promoting ability of diverse microbiota (Figure 2a).

Next, we aimed to establish *V. dahliae* infections on tomato plants grown in our gnotobiotic system. To this end, tomato seedlings grown on either sterilized or recolonized substrate were uprooted in a sterile hood, inoculated with a *V. dahliae* conidiospore suspension, and replanted in the Flowpots

(Figure 1d). At 14 days post inoculation, *V. dahliae*-inoculated plants grown in recolonized substrate displayed significantly less growth when compared with mock-inoculated plants. Similarly, also in sterilized substrate, plants treated with *V. dahliae* revealed significantly reduced growth when compared with mock-inoculated plants, indicating successful *V. dahliae* infection under the gnotobiotic

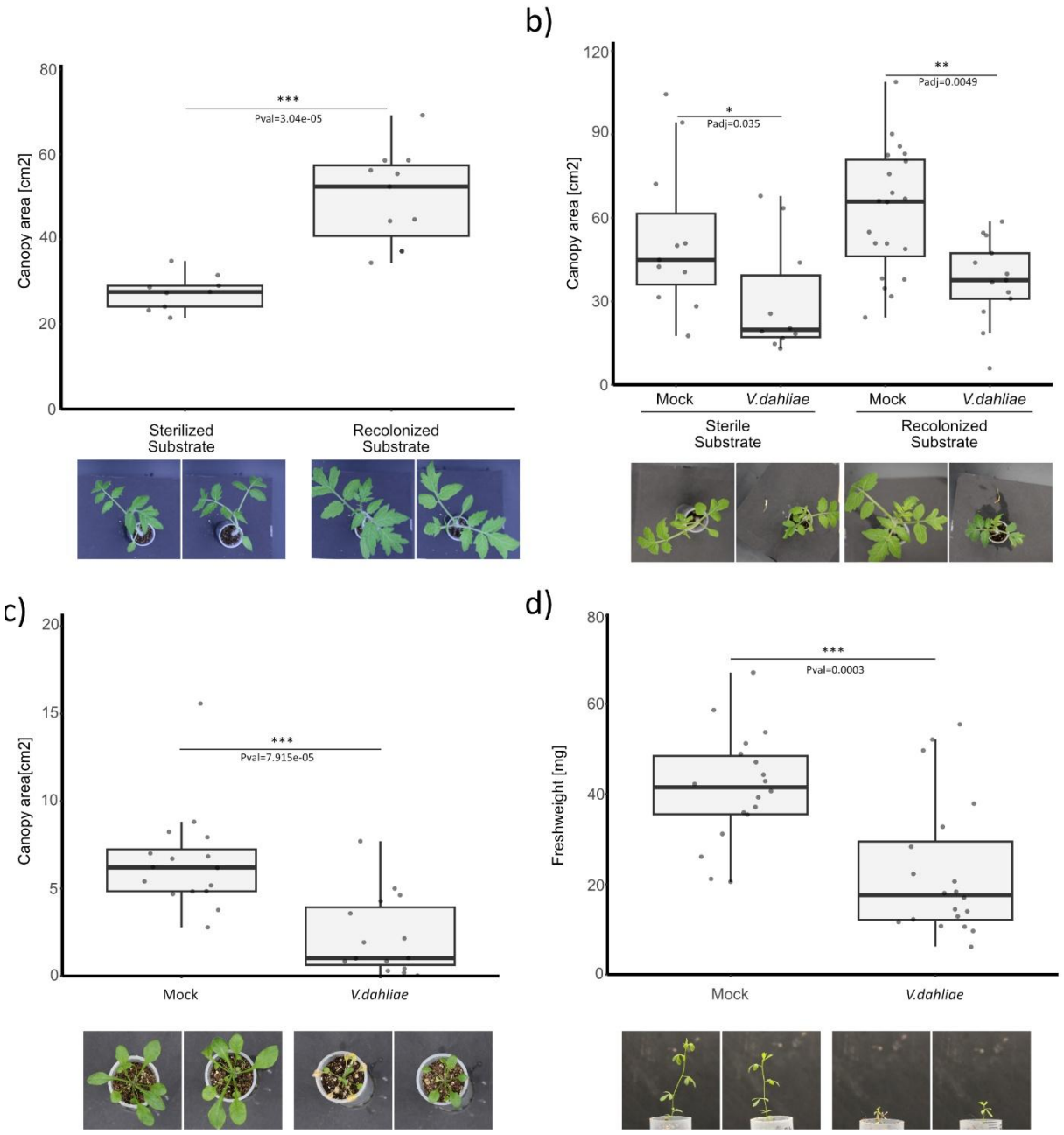

Figure 2 The Flowpot-system accommodates interactions of *V. dahliae* with diverse host plants. a) Canopy area of tomato plants grown on either sterilized or recolonized substrate. Pairwise comparison was performed using the Wilcoxon Rank Sum test (*** = Pval < 0.001). **b)** Canopy area of tomato plants grown on recolonized or sterilized substrate at 14 dpi with *V. dahliae*. Pairwise comparison was performed using the Wilcoxon Rank-Sum test, followed by multiple testing correction using Benjamini-Hochberg (FDR) corrections. (* = Padj < 0.05; **= Padj < 0.01). **c)** Canopy area of *Arabidopsis thaliana* Col-0 plants grown in sterilized substrate at 14 dpi of V. dahliae. Pairwise comparison was performed using the Wilcoxon Rank Sum test (*** = Pval < 0.001). **d)** Fresh weight of *Lotus japonicus* cv. Gifu plants grown on sterilized substrate at 14 dpi of *V. dahliae*. Pairwise comparison was performed using the Wilcoxon Rank Sum test (*** = Pval < 0.001).

conditions (Figure 2b). Considering the broad host range of *V. dahliae*, we investigated whether our inoculation protocol is also suitable for other plant hosts. To this end, we tested *Arabidopsis thaliana* and *Lotus japonicus*, as both plant species were previously grown in the Flowpot-system (Kremer *et al.,* 2021; Wippel *et al*., 2021). Interestingly, both *A. thaliana* and *L. japonicus* plants displayed significantly reduced growth upon *V. dahliae* inoculation when compared with mock-inoculated plants, indicating that our *V. dahliae* inoculation protocol is suitable to study diverse *V. dahliae*-host interactions under gnotobiotic conditions (Figure 2c, d).

Application of a protective SynCom prevents Verticillium wilt symptoms

To enable reconstitution experiments on tomato plants using host-associated bacteria, we generated a bacterial culture collection of tomato-associated bacteria. To this end, two commercially purchased tomato plants were separated into phyllosphere and root samples, which served as starting material for a colony picking approach. In total, 374 colonies were picked and identified using Sanger sequencing followed by BLAST searches to the NCBI database, which led to a total number of 132 unique bacterial isolates. Further, we confirmed the results from the BLAST identification by performing whole- genome sequencing on 75 of the species with Oxford Nanopore Technology (ONT) Sequencing. The culture collection comprises 100 distinct isolates that belong to 48 genera isolated from root tissue, and 48 distinct isolates that belong to 31 genera isolated from phyllosphere tissues (Figure 3a).

Next, we compared the species isolated by our colony picking approach to the microbiota of the input tomato material. To this end, we conducted 16S amplicon sequencing on the plant material and determined the most abundant bacterial genera. The 20 most abundant genera compose 55% of the tomato root microbiota, with *Pseudomonas*, *Flavobacterium* and *Cellvibrio* as the most abundant genera. In the tomato phyllosphere microbiota, the 20 most abundant genera make up 50% of the input microbiota with *Pseudomonas*, *Streptomyces* and *Ohtaekwangia* as most abundant. The root-associated culture collection contains at least one isolate from eight of the 20 most abundant genera, with 74 isolates belonging to less abundant genera. The phyllosphere culture collection captured at least one isolate from 5 out of the 20 most abundant genera, with 37 isolates belonging to other, less abundant genera (Figure 3b). Thus, our culture collection captured a wide diversity of microbes from the tomato microbiota.

To employ this culture collection within our Flowpot-system, we generated a SynCom composed of 26 isolates in an attempt to reduce the impact of *V. dahliae* infection on tomato plants. The selection of isolates was focused on the root-associated collection, to enhance the likelihood of successful substrate colonization. We selected one representative isolate from each family that is present in the

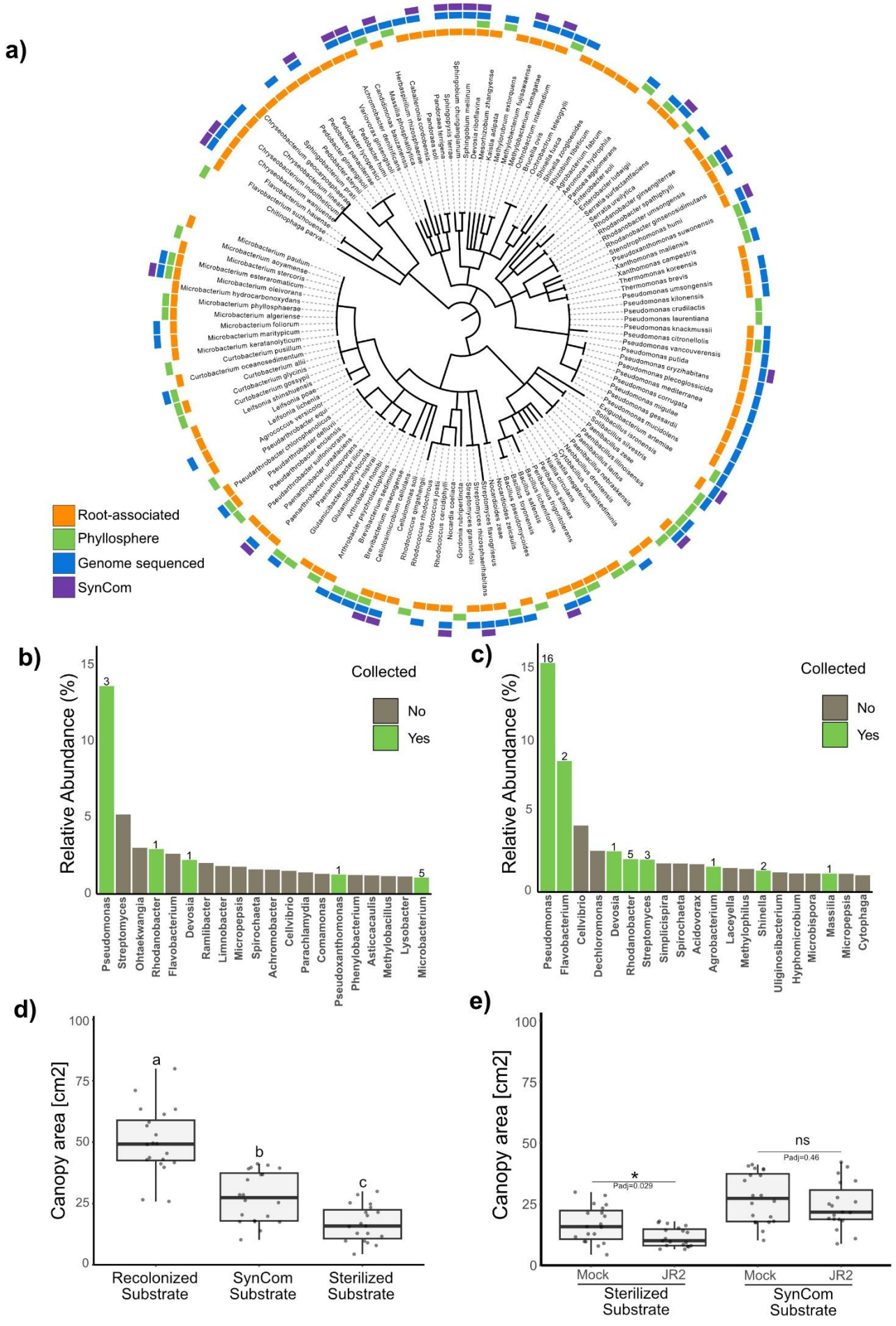
a)
Root-associated
Phyllosphere
Genome sequenced
SynCom
b)
Relative Abundance (%)
Collected
No
Yes
Pseudomonas
Streptomyces
Ohtaekwangia
Rhodanobacter
Flavobacterium
Devosia
Ramlibacter
Limnobacter
Micropepsis
Spirochaeta
Achromobacter
Cellvibrio
Parachlamydia
Comamonas
Pseudoxanthomonas
Phenylobacterium
Asticcacaulis
Methylobacillus
Lysobacter
Microbacterium
c)
Relative Abundance (%)
Collected
No
Yes
Pseudomonas
Flavobacterium
Cellvibrio
Dechloromonas
Devosia
Rhodanobacter
Streptomyces
Simplicispira
Spirochaeta
Acidovorax
Agrobacterium
Laceyella
Methylophilus
Shinella
Uliginosibacterium
Hyphomicrobium
Microbispora
Massilia
Micropepsis
Cytophaga
d)
Canopy area [cm2]
a
b
c
Recolonized Substrate
SynCom Substrate
Sterilized Substrate
e)
Canopy area [cm2]
*
Padj=0.029
ns
Padj=0.46
Mock
JR2
Sterilized Substrate
SynCom Substrate

Figure 3 Tomato-derived synthetic communities can suppress Verticillium wilt disease. a) Phylogenetic tree of the tomato-associated culture collection. Orange boxes indicate strains isolated from the rhizosphere, while green boxes indicate strains isolated from the phyllosphere. Blue boxes indicate strains that were subjected to whole genome sequencing. Purple boxes indicate strains that were used to compose the SynCom. **b)** Relative abundance in % of the 20 most abundant genera in the tomato phyllosphere microbiota. Green bars indicate genera of which at least one member was isolated, whereas grey bars indicate genera of which no member was isolated. Numbers above the bars indicate the number of strains isolated per genus. **c)** Relative abundance in % of the 20 most abundant genera in the tomato rhizosphere microbiota. Green bars indicate genera of which at least one member was isolated, whereas grey bars indicate genera of which no member was isolated. Numbers above the bars indicate the number of strains isolated per genus. **d)** Canopy area of plants grown on either sterilized, SynCom-treated or recolonized substrate. Different letters indicate statistical differences based on One-Way-Anova (Tukey HSD-Test pval < 0.05). **e)** Canopy area of mock- or *V. dahliae*-inoculated plants grown on sterilized or SynCom-treated substrate. Pairwise comparisons are performed using Wilcoxon Rank-Sum test, followed by multiple testing correction using Benjamini-Hochberg (FDR) correction

suppress disease or promote growth. First, we assessed if the application of the SynCom leads to rescue of the growth depletion phenotype we observed for plants grown in sterilized substrate. To this end, we grew plants on sterilized, recolonized and on sterilized substrate that was treated with the SynCom, respectively. Although tomato growth on SynCom-treated substrate was significantly reduced when compared with growth on recolonized substrate, tomato plants grown on SynCom-treated substrate produced significantly more biomass than plants grown on sterile substrate, suggesting that the SynCom partially restored the growth reduction observed in the absence of a substrate microbiota (Figure 3d). Next, we tested if pre-treatment of the substrate with the SynCom represses *V. dahliae* symptom development. Whereas plants grown on sterilized substrate revealed significant stunting upon *V. dahliae* inoculation in absence of the SynCom treatment, SynCom-treatment of the substrate eliminated *V. dahliae* symptom development, demonstrating that the SynCom successfully prevented disease development (Figure 3e).

Discrimination of microbiota modulation from host target manipulation

Antimicrobial effector proteins of *V. dahliae* contribute to fungal virulence. However, due to the lack of suitable gnotobiotic systems it remained elusive if this virulence contribution is solely through microbiota manipulation, or rather relies on additional activities on plant virulence targets. If these effectors primarily function to manipulate host-associated microbiota, no contribution to fungal virulence should occur in plants that are devoid of an associated microbiota. To address this hypothesis, we grew tomato plants on sterilized or recolonized substrates and inoculated with *V. dahliae* wild-type strains or a deletion mutant for the gene encoding the antimicrobial effector protein Ave1L2. We previously showed that *V. dahliae* secretes Ave1L2 to facilitate host colonization of tomato plants by suppression of Actinobacteria. Reducing the abundance of Actinobacteria in the plant microbiota through antibiotic application led to increased plant sensitivity to *V. dahliae*, while reducing the virulence contribution of Ave1L2, suggesting that the effector is secreted to target plant-protective Actinobacteria (Snelders *et al*., 2023). In the Flowpot-system, plants that were grown in sterilized

substrate revealed no difference in disease development when inoculated with wild-type *V. dahliae* or the Ave1L2 deletion mutant. In contrast, when grown on recolonized substrate, plants inoculated with the *Ave1L2* deletion mutant showed significantly reduced disease development when compared with plants that were inoculated with wild-type *V. dahliae*. Thus, Ave1L2 only contributes to virulence in the presence of a plant-associated microbiota, suggesting that this effector does not have additional virulence targets in the plant (Figure 4a).

Next, we assessed the virulence contribution of the *V. dahliae* Ave1 effector in sterilized substrate. This effector protein is utilized by *V. dahliae* to facilitate host colonization of tomato and cotton plants by suppression of Sphingomonad bacteria. We previously showed that pre-treatment of surface-sterilized

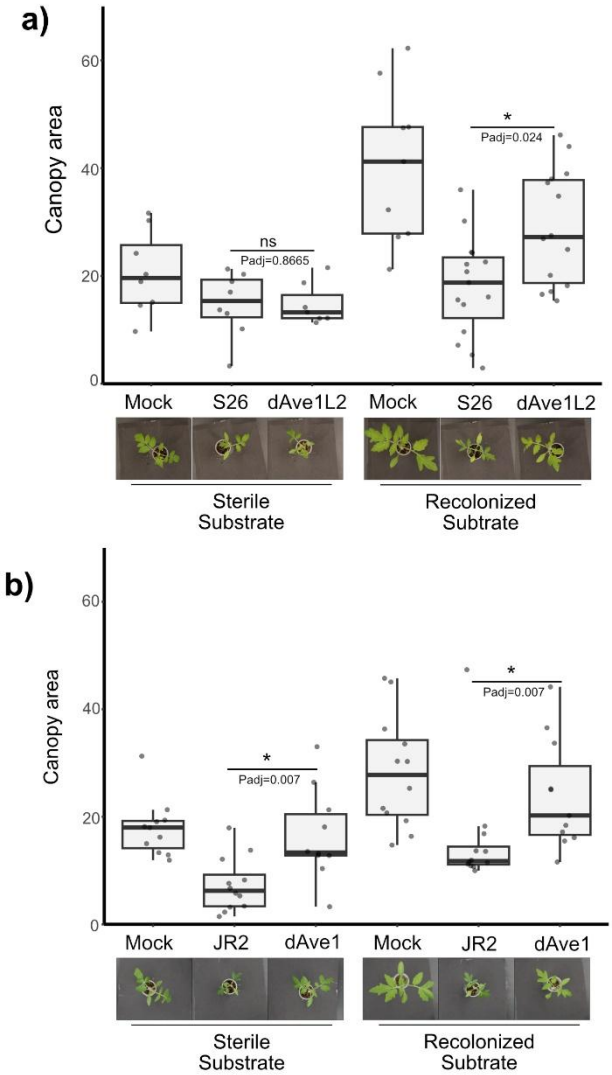

Figure 4. Antimicrobial effectors of *V. dahliae* differentially contribute to virulence in gnotobiotic conditions. **a)** Canopy area of tomato plants grown on either sterilized or recolonized substrate at 14 dpi of wild-type *V. dahliae* or an *Ave1L2* deletion mutant. Pairwise comparisons are performed using the Wilcoxon Rank-Sum test, followed by multiple testing correction using Benjamini-Hochberg (FDR) correction. **b)** Canopy area of tomato plants grown on either sterilized and recolonized substrate at 14 dpi of wild-type *V. dahliae* or an *Ave1* deletion mutant. Pairwise comparisons are performed using Wilcoxon Rank-Sum test, followed by multiple testing correction using Benjamini-Hochberg (FDR) correction.

tomato seeds with *Sphingomonad bacteria* reduced Verticillium wilt disease development, and that Ave1 secretion by *V. dahliae* significantly reduced Sphingomonad proliferation *in planta* (Snelders et al. 2020). In contrast to our observations for Ave1L2, tomato plants that were grown in sterilized substrate and inoculated with the *V. dahliae* wild-type strain JR2 were more stunted when compared with plants that were inoculated with an *Ave1* deletion mutant, indicating a clear virulence contribution of the effector protein on plants grown in sterile substrate. This contribution was also observed on plants grown on recolonized substrate, overall indicating that Ave1 may contribute to virulence in the absence of a host-associated microbiota too, which may rely on modulation of a host virulence target (Figure 4b).

Ave1 affects host physiology

The *V. dahliae* effector Ave1 has numerous homologs in plants and in several other microbes, including *A. thaliana* PNP AtPNP-A and *Xanthomonas citri* pv. *citri* PNP XacPNP (Jonge *et al*., 2012). Most plant homologs of Ave1 are annotated as plant natriuretic peptides (PNPs), mobile molecules that are released under biotic and abiotic stress conditions and have been implicated in several responses important for plant growth and homeostasis (Gehring and Irving, 2003; Ruzvidzo *et al*., 2011). Multiple sequence alignment of the protein domains that have previously been implicated in PNP activity of AtPNP-A and XacPNP with Ave1 revealed high sequence similarity at the PNP site, a 12 amino acid long stretch that was previously reported to confer biological activity (Gottig *et al*., 2008). Notably, this similarity was much lower for Ave1L2 (Figure 5a). Previously PNPs were shown to be able to induce stomatal opening (Gottig *et al*., 2008). To investigate if Ave1 also possess such PNP-activity, we measured its ability to promote stomatal opening. Treatment of tomato leaf epidermis with purified Ave1 resulted in significantly enhanced stomatal opening as observed upon treatment with XacPNP and the synthetic auxin analogue indole-3-acetic acid (IAA) (Figure 5b). In contrast, addition of Ave1L2 did not affect stomatal opening. Consistent with previous reports demonstrating that PNP-induced responses are dependent on cyclic guanosine monophosphate (cGMP) signaling (Pharmawati *et al*., 2001; Turek and Gehring, 2016), aperture changes caused by Ave1 were partially repressed by the guanylate cyclase inhibitor methylene blue (Supplementary Figure 2). Collectively, these findings demonstrate that Ave1 possess PNP activity, suggesting that beyond its antimicrobial function, Ave1

may also contribute to virulence through an additional function that involves the manipulation of host physiology.

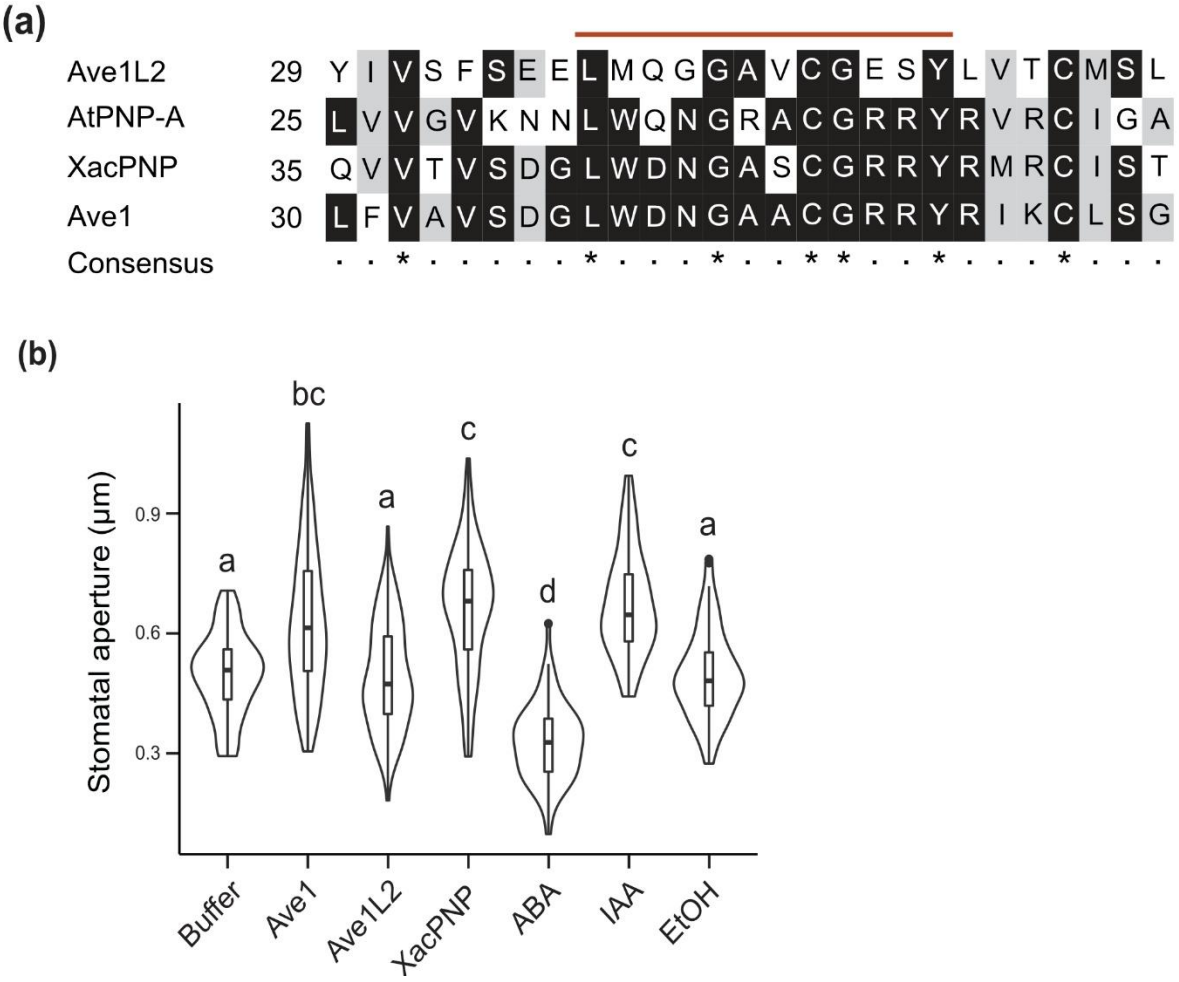

Figure 5. The *Verticillium dahliae* effector Ave1 contains an active PNP site, while Ave1L2 does not. a) Multiple sequence alignment of a short peptide stretch of Ave1 (amino acid 30 to 56) containing the PNP site (red line) with Ave1L2 and homologous sequences from *Xanthomonas citri* pv. *citri* (Xac) and *Arabidopsis thaliana* (At). **b)** Stomatal opening in tomato epidermis following treatment with 5 µM purified protein. Indole-3-acetic acid (IAA; 1 µM) was used as positive control, whereas 50 µM abscisic acid (ABA) and EtOH served as negative controls. Data are from one representative experiment are shown. Letters represent statistically significant differences in stomatal opening index (width/length) according to one-way ANOVA (F (7,626) = 47.06, p<2e-16) and Tukey's post-hoc test. Error bars represent the mean ± SD (n>60).

Discussion

Over the past decades, research has established that plant-associated microbiota play crucial roles in plant health by suppressing the pathogenic potential of resident microbes and forming a barrier against invading pathogens (Mesny *et al*., 2023; Trivedi *et al*., 2020; Mesny *et al*., 2024). However, pathogens have evolved to overcome this additional layer of defense by secreting effector proteins with antimicrobial properties that manipulate host microbiota to their advantage (Kettles *et al*., 2018; Snelders *et al*., 2020; Snelders *et al*., 2021; Chang *et al*., 2021; Snelders *et al*., 2023; Ökmen *et al*., 2023; Gómez-Pérez *et al*., 2023; Chavarro-Carrero *et al*., 2024; Mesny *et al*., 2024). While these effectors exhibit antimicrobial activity, it remains unclear whether they additionally perform other

functions, such as manipulation of host physiology. Using a gnotobiotic system, we now demonstrate that it is possible to address this question by testing virulence contributions separately, in the absence and in the presence of plant-associated microbiota. We show that the antimicrobial effector Ave1L2, which was previously shown to facilitate host colonization through suppression of Actinobacteria (Snelders *et al*., 2023), does not markedly contribute to virulence during infections on tomato plants that were grown in sterilized substrate. Notably, it contributes to virulence in the presence of a plant-associated microbiota, suggesting that Ave1L2 enhances fungal virulence through its antimicrobial activity, and furthermore that it lacks significant other virulence functions, and thus host targets. These findings contrast with those for the *V. dahliae* effector Ave1 that was previously shown to facilitate host colonization through suppression of Sphingomonad bacteria (Snelders *et al*., 2020), as we now reveal that Ave1 also significantly contributes to virulence on tomato plants grown under sterile conditions. This finding points towards a dual role for the Ave1 effector, contributing to microbiota manipulation as well as to host manipulation.

Multifunctionality has previously been observed for other fungal effectors as well. For instance, *Parastagonospora nodorum* secretes Snf1, which induces cell death to promote nutrient release while also protecting the fungus from wheat chitinases (Liu *et al*., 2016). Likewise, *Ustilago maydis* utilizes the effector UmPr-1La to suppress host immunity while simultaneously sensing plant-derived compounds to guide hyphal growth (Lin *et al*., 2023). The Ave1 effector has widespread plant homologs and was likely acquired via horizontal gene transfer from plants (de Jonge *et al*., 2012). Notably, most plant homologs of Ave1 are annotated as plant natriuretic peptides (PNPs), systemically mobile molecules released during biotic and abiotic stress that play key roles in plant growth and homeostasis (Gehring and Irving, 2003; Ruzvidzo *et al*., 2011; Jonge *et al*., 2012). In this study, we demonstrate that Ave1 induces stomatal opening in the tomato epidermis, confirming its PNP activity *in vitro*, whereas Ave1L2 lacks this activity. Consequently, we speculate that Ave1 may contribute to virulence *in planta* by manipulating plant physiology, possibly through PNP-activity, thereby promoting host colonization. This mode of action is reminiscent of a strategy employed by the biotrophic bacterial plant pathogen *Xanthomonas citri* subsp. *citri*, which exploits the PNP homologue XacPNP to alter host physiology to promote infection and bacterial proliferation (Nembaware *et al*., 2004; Gottig *et al*., 2008; Garavaglia *et al*., 2010a; Garavaglia *et al*., 2010b).

Several studies have indicated that PNPs play roles in host defense against invading pathogens (Breitenbach *et al*., 2014; Ficarra *et al*., 2018). For example, expression of the Arabidopsis *AtPNP-A* gene is induced upon infection with *Pseudomonas syringae* pv. *syringae*, and *AtPNP-A* deletion mutants exhibit increased susceptibility to the pathogen (Ficarra *et al*., 2018). Notably, previous studies also reported that AtPNP-A, similar to Ave1, possess an antibacterial activity against *Bacillus*

subtilis (Snelders *et al*., 2020). Thus, PNPs may similarly display dual activities and exert, antimicrobial effects besides their PNP activity that could contribute to shaping the plant microbiota as well as to antagonizing invading pathogens (Snelders *et al*., 2020). Future studies investigating the direct impact of PNPs on host microbial communities and pathogen susceptibility will be crucial to validate this hypothesis.

To facilitate mechanistic research on plant-microbiota interactions, which includes research into plant-pathogen interactions, various gnotobiotic systems have been developed (Innerebner *et al*., 2011; Carlström *et al*., 2019; Kremer *et al*., 2021; Ma *et al*., 2022). Among these, peat-based systems, such as the Flowpot-system, have emerged as valuable tools due to their ability to mimic natural conditions by providing a soil-like substrate structure, shielding roots from light, and supplying organic carbon (Kremer *et al*., 2021; Ma *et al*., 2022). To leverage the advantages of the Flowpot-system for studying plant-microbiota-pathogen interactions, we report inoculation protocols that allow to inoculate plants with *V. dahliae* in sterile settings. While *Arabidopsis thaliana* and *Lotus japonicus* were previously shown to be compatible with the Flowpot-system (Kremer *et al*., 2021; Wippel *et al*., 2021), we now also successfully established tomato growth in this system and established *V. dahliae* infections across all three host species. Additionally, we supplemented our gnotobiotic system by assembling a collection of 133 unique bacterial strains from tomato plants that were commercially grown on potting soil, capturing a wide diversity of tomato-associated microbiota. By providing full genome sequences for over half of our bacterial collection, we offer a valuable resource for mechanistic investigations into the tripartite interaction of plants, their microbiota and *V. dahliae*.

Collectively, our findings reveal that antimicrobial effectors can serve dual functions for fungal virulence, both as antimicrobial agents but also at the same time as modulators of host physiology. Notably, many antimicrobials are ancient proteins, widely distributed across the fungal kingdom, and likely functioned in microbial competition long before the evolution of land plants (Snelders *et al*., 2021; Snelders *et al*., 2022; Mesny and Thomma, 2024). It is therefore conceivable that some of these effectors have adapted novel functions throughout co-evolution with host plants, potentially contributing to plant manipulation. This suggests that dual functionality may be a common feature among antimicrobial effector proteins. Furthermore, the development of *V. dahliae* inoculations in a tomato-compatible Flowpot-system combined with a tomato-associated bacterial collection provides a robust platform for future research on the role and contribution of antimicrobial effector proteins for fungal pathogens. Understanding the precise molecular mechanisms underlying the role of antimicrobial effector proteins to the biology of fungal plant pathogens may ultimately open up novel strategies for microbiota-based disease control in agriculture.

Materials and Methods

Preparation and assembly of the Flowpot-system

Flowpot substrate was prepared by sieving potting soil (Balster Einheitserde, Frödenberg, Germany) through a 1 cm x 1 cm mesh, using only the material that passed through. Vermiculite with a kernel size ranging from 0.1 mm - 0.3 mm (LIMERA Gartenbauservice, Geldern-Walbeck, Germany) was sieved through a 1 mm x 1 mm mesh, retaining only the material left in the sieve. The two components were mixed in a 1:1 ratio, followed by the addition of 150 ml of water per liter of substrate and thorough mixing, and autoclaved on a liquid cycle at 121°C for 20 minutes (Systec, Linden, Deutschland). After 16 hours of incubation in darkness, the substrate was mixed thoroughly in a sterile hood before 50 ml of water per liter of substrate were added and the substrate was autoclaved again on a liquid cycle. To assemble individual Flowpot units, truncated (at the 45-ml mark) and autoclaved 50 ml luer-lock syringes (Terumo Europe, Leuven, Belgium) were filled with an autoclaved 250 µm pore-size polyamide filter mesh (Biologie-Bedarf Thorns, Deggendorf, Germany), and a 3 cm layer of autoclaved 3 mm silica glass beads (Roth, Karlsruhe, Germany). Assembled Flowpots containing sterile substrate were autoclaved again on a liquid cycle. For re-colonized substrate, sterile substrate was mixed with 10% non-autoclaved substrate and incubated overnight at room temperature, after which the mixture was used to assemble Flowpots. To remove toxic compounds released during autoclaving, the substrate was flushed using a vacuum system. To this end, Flowpots were placed onto a Qiavac 24 plus system with luer-lock adapters (Qiagen, Venlo, The Netherlands). Vaccuum was applied and 30 ml sterile MQ water was poured into each Flowpot. Subsequently, the substrate was enriched by flushing with 30 ml nutrient solution. For tomato and Arabidopsis plants, half-strength Murashige & Skoog (MS) medium (Duchefa, The Netherlands) was added whereas for Lotus plants 0.25x B&D (Broughton and Dilworth, 1971) solution was added. For SynCom treatments, the SynCom was added to the medium prior to flushing.

To assess sterility of the substrate, 500 mg substrate were suspended in 10 ml 100 mM $MgCl_2$ and shaken at 300 rpm for 1 hour at room temperature. Following 1.000-fold dilution, the samples were plated onto lysogenic broth agar (LB), tryptic soy agar (TSA) and Reasoner's 2A agar (R2A) and incubated at RT for up to 4 days.

Plant material and seed sterilization

Plants used in the Flowpot-system were tomato (*Solanum lycopersicum* L.) cultivar MoneyMaker, *Arabidopsis thaliana* Col-0 and *Lotus japonicus* Gifu. Tomato and Arabidopsis seeds were surface sterilized as described previously (Schlesier *et al*., 2003). Following sterilization, the seeds were stratified at 8°C for 24 hours and then sown into each Flowpot unit. Lotus seeds were rubbed with

sand paper and sterilized by 20 minutes incubation in 10 ml of MQ water with 200 µl NaCOl on a rotary shaker at 185 rpm. Subsequently, seeds were washed 5 times with sterile MQ water. Sterilized Lotus seeds were germinated on 0.8% plant agar at 22℃ for 5 days before seedlings were transferred into Flowpot units. In total, five individual Flowpot units were placed into a Microbox container with four air-filters (SacO2, Deinze, Belgium) and placed in a greenhouse chamber (17 hours of light at 23℃ followed by 7 hours of darkness at 22℃).

Fungal inoculation assays

For *Verticillium dahliae* inoculations, conidiospores were harvested from wild-type or effector deletion strains of *V. dahliae* (Snelders *et al*., 2020; Snelders *et al*., 2023).after growth on potato dextrose agar (PDA; Carl Roth, Karlsruhe, Germany) for 10 days. Conidiospores were washed three times by centrifugation at 10.000 rpm for 10 minutes followed by pellet resuspension in sterile MQ water. Conidiospores were counted using a Neubauer Chamber and the concentration of the final conidiospore-suspension was adjusted to 10^6 conidiospores/ml. Inoculation was performed on plants that were grown for 14 days in the Flowpot-system. To this end, microboxes with Flowpots were opened in a sterile hood and plants were carefully uprooted from the Flowpots. Roots were rinsed with sterile MQ water and subsequently placed into the *V. dahliae* conidiospore suspension for minimum 8 minutes. Subsequently, plants were placed back into the original Flowpots and the boxes were placed back into the greenhouse. Verticillium wilt symptom development was monitored at 14 days post inoculation. Symptoms were monitored by measuring shoot fresh weight and canopy areas were calculated from overhead pictures using ImageJ (Schneider *et al*., 2012).

Colony picking-based collection of tomato-associated bacteria

To assemble a collection of tomato-associated bacteria, root and stem samples from two commercially purchased tomato plants grown in a potting soil, were separated and cut into 2 mm long pieces and washed in 100 mM $MgCl_2$. Subsequently, 3 mm metal beads were added and samples were homogenized for 3x 45 seconds at 30 Hz in a tissue-lyzer (Retsch, Haan, Germany). Samples were diluted 1/10;1/100;1/1.000 and plated on agar plates containing either TSA, LBA, M9 minimal medium, R2A or R2A supplemented with 0.5% v/v . Plates were incubated in darkness at room temperature for 5 days and individual colonies were picked and transferred onto fresh plates. Following 2 rounds of single colony streaking, material of each colony was added to 1 ml of sterile 25% glycerol and stored at -80℃. To identify the bacteria, a 5 µl loop of bacteria was transferred into 1 ml of $MgCl_2$ and vortexed for 5 minutes. DNA was extracted as described previously (Zhang *et al*., 2021) and used to amplify the 16s rRNA gene using the 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGWTACCTTGTTACGACT) primers. PCR was conducted using Phusion HF polymerase (New England

Biolabs, Ipswich, USA) at 98℃ for 30 s followed by 30 cycles of 98℃ for 10 s, 56℃ for 30 s, 72℃ for 45 s and a final extension at 72℃ for 8 min. PCR products were examined on 1.5% agarose gel and purified using the ExoSAP-IT™ Express Kit (Thermo Fisher Scientific, Waltham, USA). Following purification, the samples were collected in 96-well plates and send for Sanger sequencing (Microsynth Seqlab, Göttingen, Germany). Sequences were trimmed using CLC workbench (Qiagen, Venlo, The Netherlands) and blasted against the NCBI rRNA/ITS database. Identified strains were used to construct a phylogenetic tree of the collection with the ETE-Toolkit (V3.1.3, Huerta-Cepas *et al*., 2016). Visualization of the tree was conducted using iTOL (V6.9.1, Letunic and Bork, 2024)

SynCom preparation

To generate a disease-suppressive SynCom, all isolated Sphingomonadales strains from the collection of root-associated tomato bacteria were selected. Additionally, one representative strain from each other bacterial family in the bacterial culture collection was selected, with preference for plant-beneficial strains; if none were identified, a random strain was chosen. All selected bacterial strains were cultured in R2A broth for 2 days at room temperature and 160 rpm. Bacterial cells were collected by 10-minute centrifugation at 4500 rpm. Cells were washed twice with 10 mM $MgSO_4$ and resuspended in half-strength MS to an OD_{600} of 0.5. All cultures were combined in equal amounts and the overall OD_{600} of the final SynCom was adjusted to 0.02. Next, the SynCom was applied to the Flowpot-system by flushing the soil.

Tomato microbiota sequencing

Flowpot tomato plants were harvested in a sterile hood and ground to powder using a tissue lyzer (Retsch, Haan, Germany). DNA was extracted using the Power Soil Pro Kit (Qiagen, Venlo, The Netherlands). DNA was further purified using the Monarch PCR&DNA Clean up Kit (New England Biolabs, Ipswich, United States). All kit-solutions were filter-sterilized before use. Purified DNA was used to amplify the V3-V4 region of the 16S gene in the presence of the pPNA and mPNA blocking clamps (PNABio, Newbury Park, United States). Amplicons were sequenced using 16S sequencing on an Illumina MiSeq Platform (BGI-Genomics, Shenzhen, China). To sequence the input material for the culture collection, root and stem samples of the tomato plant were manually ground. Subsequently DNA was extracted as described previously (Chavarro-Carrero *et al*., 2021). Extracted DNA was used to amplify the V5-V7 region of the 16S gene using 799F and 1193 primers as described previously (Wippel *et al*., 2021). Purified amplicons were submitted for sequencing on an Illumina sequencing platform (Cologne-Center for Genomics, Cologne, Germany). Data analysis was conducted as described previously (Callahan *et al*., 2016; Snelders *et al*., 2020).

Nanopore Sequencing and bacterial genome assembly

Bacteria were cultured in R2A-broth for 48 hours and pelleted through centrifugation. Bacterial pellets were resuspended in TEN-Buffer (10 mM Tris-HCL pH 8.0, 10 mM EDTA, 150 mM NaCl), supplemented with 20 µl lysozyme (20 mg/µl) and incubated at 37℃ for 20 minutes. Next, 3 µl RNase A (20 mg/µl) were added and the samples were incubated at 65℃ for 5 minutes. Subsequently, 550 µl of a reduced TEN-buffer (10 mM Tris/HCL, 1 mM EDTA, 50 nM NaCl), supplemented with 2 µl of proteinase K (20 mg/µl) and 40 µl SDS (10% w/v) were added followed by incubation for 2 hours at 60℃. Subsequently, phenol washing was performed twice and the aqueous phase was further cleaned by two chloroform washing steps. Next, DNA was precipitated by adding 10 volumes of ice-cold 100% EtOH and incubation at 4℃ overnight. Precipitated DNA was collected and washed with 70% EtOH and resuspended in MQ water. DNA quality and quantity were assessed using Qubit, Nanodrop and agarose gel assays. Full genome sequencing was carried out on a Nanopore MinION device using R10 Flowcells (Oxford Nanopore Technologies, Oxford, UK). The sequencing library was prepared using the ligation sequencing gDNA-Native Barcoding Kit 96 V14 (SQK-NBD114.96; Oxford Nanopore Technologies, Oxford, UK). Bacterial genomes were assembled using the uncorrected sequenced reads in Flye (2.9.5) with default settings and the --nano-hq input option (Kolmogorov et al. 2019). The assembled genomes were annotated using Prokka (1.14.6) and completeness of the genome assemblies was assessed with BUSCO (5.3.2) (Manni *et al*., 2021; Seemann, 2014).

Protein production and stomatal opening assay

Protein sequence alignment was performed using MAFFT (Version 7.271; Katoh et al. 2002). The sequences encoding mature Ave1 and XacPNP were cloned into the pET-15b expression vector for N-terminal His_6 tagging (Novagen, Madison, WI, USA) (for primer sequences see Supplementary Table 1). Heterologous proteins were produced as described previously (Snelders *et al*., 2020) and purified from inclusion bodies under denaturing conditions using His60 Ni^{2+} Superflow Resin (Clontech, Mountain View, CA, USA). Purified proteins were stored in 0.25 M ammonium sulphate with 0.1 M BisTris, pH 5.5. Final concentrations were determined using the BioRad Protein Assay (BioRad, Veenendaal, The Netherlands). Stomatal aperture was tested as described previously (Gottig *et al.,* 2008) using tomato leaf tissue.

Author contributions

W.P., K.W. and B.P.H.J.T. conceived the project. W.P., H.R., K.W. and B.P.H.J.T. designed the experiments. W.P., J.P., A.K., N.S., J.W., N.C.S., G.L.F., E.A.C.-C., A.L.M., G.C.P. and N.S. performed the experiments. W.P., A.K., H.R. and B.P.H.J.T. analyzed the data. W.P. and B.P.H.J.T. wrote the manuscript. All authors read and approved the final manuscript.

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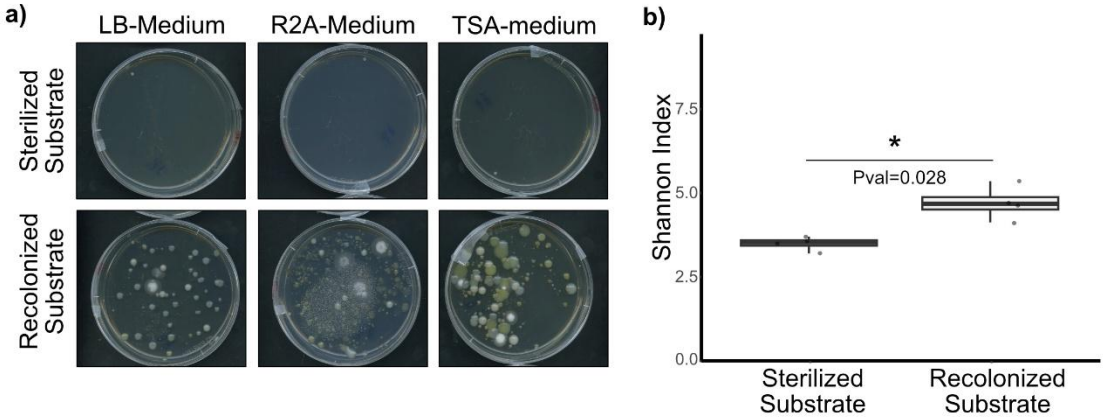
Competing Interests

The authors declare no competing interest exists.

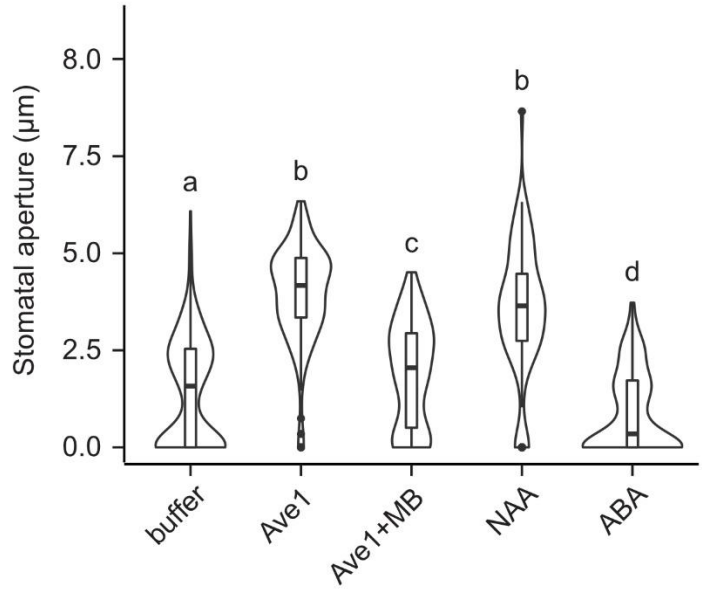
Data availability

Genomes from the bacterial culture collection are available via the European Nucleotide Archive (ENA) under the study accession number PRJEB87606. The genome annotation outputs as well as the scripts used in this study are available on https://github.com/antonkraege/BacterialTomatoCultureColection_Genomes/

Supplementary Figures



Supplementary Figure 1 a) Microbial growth from sterilized or recolonized Flowpot substrate on three growth media after four days of incubation. **b)** Bacterial alpha diversity in stem tissue of tomato plants grown on sterilized or recolonized Flowpot substrate. Pairwise test using Wilcoxon rank sum test (Pval < 0.05).



Supplementary Figure 2. PNP activity of VdAve1 is mediated by cGMP signaling. Stomatal opening in tomato epidermis following treatment with 5 µM VdAve1 with or without the cGMP signaling inhibitor methylene blue (MB). Indole-3-acetic acid (IAA; 1 µM) and 50 µM abscisic acid (ABA) were used as positive and negative controls, respectively. Data are from one representative experiment. Experiments were performed twice. Letters represent statistically significant differences in stomatal opening according to one-way ANOVA ($F(5,824) = 124.8$, $p<0.001$) and Tukey's post-hoc test. Error bars represent the mean ± SD ($n>70$).

Chapter 3: Undermining the cry for help: The phytopathogenic fungus Verticillium dahliae secretes an antimicrobial effector protein to undermine host recruitment of antagonistic Pseudomonas bacteria

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SUMMARY

•During pathogen attack, plants recruit beneficial microbes in a ”cry for help” to mitigate disease development. Simultaneously, pathogens secrete effectors to promote host colonization through various mechanisms, including targeted host microbiota manipulation.

•Inspired by *in silico* antimicrobial activity prediction, we investigated the antimicrobial activity of Av2 in vitro. Furthermore, its role in *V. dahliae* virulence was assessed through microbiota sequencing of inoculated plants, microbial co-cultivation assays, and inoculations in a gnotobiotic plant cultivation system.

•We show that Av2 inhibits bacterial growth, and acts as a virulence factor during host colonization. Structural prediction revealed that Av2 is not only sequence but also structural a unique. Microbiota sequencing revealed involvement of Av2 in suppression of Pseudomonas spp. recruitment upon plant inoculation with V. dahliae, indicating that Av2 suppresses the cry for help. We show that several Pseudomonas spp. are antagonistic to V. dahliae and sensitive to Av2 treatment.

•We conclude that *V. dahliae* secretes Av2 to suppress the plant’s cry for help by inhibiting the recruitment of antagonistic Pseudomonas spp. to pave the way for successful plant invasion.

INTRODUCTION

Plants associate with a plethora of microbes above and below ground, collectively called the microbiota, that can positively impact plant productivity and health (Berendsen *et al*., 2018; Trivedi *et al*., 2020). Through the secretion of root exudates plants shape their microbiota and actively recruit beneficial microbes to mitigate biotic and abiotic stresses (Berendsen *et al*., 2018; López *et al*., 2008). Under pathogen attack, plants can modify these exudates to selectively attract protective microbes in order to limit disease progression. This targeted recruitment in response to pathogen infection is known as the plant's "cry for help" (Berendsen *et al*., 2018; Liu *et al*., 2024; Spooren *et al*., 2024; Yuan *et al*., 2018). For example, cucumber plants increase the exudation of tryptophan during *Fusarium oxysporum* infection, which promotes the recruitment of beneficial *Bacillus amyloliquefaciens* that can mitigate disease progression (Liu *et al*., 2017).

Ultimately, the cry for help, which results in the recruitment of beneficial microbes, may have a legacy effect in cases when it leads to an increased population of these microbes in the soil, resulting in the establishment of disease-suppressive soils that protect future plants grown in the same soil (Mesny *et al*., 2024; Rolfe *et al*., 2019). However, the development of such a legacy effect typically requires years and many plant generations to fully establish (Rolfe *et al*., 2019). Arguably, the most famous example of such legacy effect concerns the decline of take-all disease, caused by the fungal plant pathogen *Gaeumannomyces tritici*, in wheat over years of monoculture that has been associated with the recruitment of 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. (Raaijmakers and Weller, 1998).

To detect pathogens, plants have evolved a complex immune system that recognises a multitude of microbe-derived molecules to activate appropriate defence responses (Jones and Dangl, 2006). Initial immune responses are triggered upon recognition of conserved microbe-associated molecular patterns (MAMPs), such as chitin or flagellin, by plant membrane-localised MAMP recognition receptors that activate pattern-triggered immune (PTI) responses (Cook *et al*., 2015; Jones and Dangl, 2006). In response, host-adapted pathogens have evolved strategies to suppress or overcome such PTI responses, which includes the secretion of virulence factors, also known as effectors (Rovenich *et al*., 2014). In turn, particular host genotypes evolved to recognize effectors, or their activities, by resistance (R) proteins that include cell surface and cytoplasmic receptors that activate effector-triggered immunity (ETI) (Cook *et al*., 2015; Jones and Dangl, 2006).

Most effectors characterized to date deregulate host immune responses or target other aspects of host physiology through various biochemical activities and mechanisms (Rovenich *et al*., 2014). For example, the effector Ecp6 is secreted by *Cladosporium fulvum* to sequester chitin oligosaccharides

that are released from its cell walls to prevent recognition by chitin immune receptors (Sánchez-Vallet *et al*., 2013). Intriguingly, several research groups have recently uncovered a novel function of effectors besides the modulation of host physiology, by showing that several pathogens secrete effectors that target host-associated microbiota through the display of selective antimicrobial activity in order to promote host colonisation (Gómez-Pérez *et al*., 2023; Snelders *et al*., 2020).

Several antimicrobial effectors have been functionally characterized in the soil-borne fungus *Verticillium dahliae*, a presumed asexual filamentous fungus that causes vascular wilt disease on hundreds of host plants (Fradin and Thomma, 2006). The fungus generates genetic diversity through largescale chromosomal rearrangements and segmental duplications, leading to hypervariable regions between *V. dahliae* strains that are called adaptive genomic regions (AGRs) (Cook *et al*., 2020; de Jonge *et al*., 2013; Faino *et al*., 2016). These AGRs are enriched in repeats and in effector genes, and display a unique chromatin profile that sets these regions apart from core genomic regions (Cook *et al*., 2020). Interestingly, despite being dispersed across the genome, these AGRs were found to physically interact in the nucleus, possibly contributing to their differential behaviour (Torres *et al*. 2024). Overall, similar to other filamentous pathogens, *V. dahliae* has a compartmentalised genome containing AGRs with increased plasticity when compared with core genomic regions, an observation often referred to as a "two-speed genome" (Raffaele and Kamoun 2012; Torres *et al*., 2021).

The first *V. dahliae* effector for which antimicrobial activity was shown is the AGR-encoded lineage-specific effector Ave1 that was identified by comparative genomics between *V. dahliae* strains that are controlled by *Ve1*-mediated resistance in tomato and resistance breaking strains that are virulent towards *Ve1* (de Jonge *et al*., 2012). Besides being recognized by the tomato Ve1 immune receptor as an avirulence factor, Ave1 contributes to fungal virulence on plants lacking Ve1 by targeting antagonistic bacteria of the Sphingomonadales order (Snelders *et al*., 2020). Notably, Ave1 is not the only *V. dahliae* effector protein with antibacterial activity, as a search for effectors with homology to known antimicrobial proteins within the *V. dahliae* secretome yielded the AMP2 effector that is expressed in soil extract. AMP2 revealed complementary activity to Ave1, suggesting that *V. dahliae* exploits different effectors to cope with the diversity of microbial competitors in soil (Snelders *et al*., 2020). The antimicrobial activity of *V. dahliae* effector proteins is not restricted to bacteria, as the defensin-like effector AMP3 was found to target the mycobiota (Snelders *et al*., 2021). Intriguingly, and in contrast to Ave1 and AMP2, AMP3 is exclusively expressed at late infection stages when resting structures are formed in decaying plant tissue while host immune responses fade and opportunists and fungal decay organisms invade host tissues (Snelders *et al*., 2021).

Over the years only two *R* loci were identified that confer resistance against *V. dahliae* in tomato. Besides the recognition of Ave1 by the Ve1 receptor, the fungal effector Av2 is recognised in *V2* tomato plants, although the corresponding *R* gene has not yet been cloned (Chavarro-Carrero *et al*., 2021; Usami *et al*., 2017). Similar to Ave1, Av2 is a small (73 amino acid mature protein; net charge +1.8) secreted protein, produced only by a subset of *V. dahliae* strains. Apart from homologues found in other *Verticillium* spp., the only homologues of this effector were found in the *Fusarium* genus (Chavarro-Carrero *et al*., 2021). *V. dahliae Av2* occurs in two allelic variants that differ in one non-synonymous single nucleotide polymorphism (SNP) that are both recognised in *V2* plants, and so far its intrinsic function for the pathogen has remained enigmatic (Chavarro-Carrero *et al*., 2021). In this study we aim to characterise the virulence function of *Av2* through a combination of *in silico* and functional analysis.

RESULTS

Av2 selectively inhibits bacterial growth *in vitro*

Most functionally characterised effectors target host physiology and are strictly *in planta* expressed, while microbiota-manipulating effectors can be expressed *in planta* as well as during fungal life cycle stages outside the plant host (Snelders *et al*., 2020, 2021). In order to functionally characterize *Av2*, its expression was analysed by querying previously generated RNA sequencing datasets (Cook *et al*., 2020; de Jonge *et al*., 2012), revealing that *Av2* is not only expressed during host colonisation (1,695 transcripts per million (TPM), 16, de Jonge *et al*., 2012) but also during *in vitro* growth on PDA (3,256 TPM, 4 day old, Cook et al., 2020). Furthermore, *Av2* is expressed in conditions mimicking soil colonisation (Figure S1). A similarly broad expression pattern, including expression in soil, has previously been observed for the *V. dahliae Ave1* effector gene (Figure S1, Snelders et al., 2020), suggesting that Av2 may act as an antimicrobial too. Interestingly, *in silico* analysis using the Antimicrobial Peptide Scanner (vr.2; Veltri *et al*., 2018) predicted antimicrobial activity for Av2 with a probability of 99.6%.

To validate the predicted antimicrobial activity of Av2 *in vitro*, the two previously identified variants, Av2 and Av2^{V73E}, were expressed heterologously in *E. coli*, purified, and used in antimicrobial activity assays. Additionally, Av2 homologues from two *Fusarium* spp. were produced, purified and tested for antimicrobial activity as well. To this end, a panel of ten phylogenetically diverse plant-associated bacteria was incubated with either of the Av2 variants at a concentration of 8 µM, or buffer as a control, and bacterial growth was assessed. Interestingly, three out of ten bacteria showed reduced growth when incubated with either of the two *V. dahliae* Av2 variants, namely *Bacillus drentensis*, *Pseudoxanthomonas suwonensis* and *Devosia riboflavina* (Figure 1). A subset of bacteria was also

tested with the Av2 homolouges from *Fusarium*, which showed activity against *Bacillus drentensis*, *Pseudoxanthomonas suwonensis* while *Devosia riboflavina* was not tested (Figure 1). Importantly, no differences in inhibitory activity were observed between any of the Av2 variants, including the

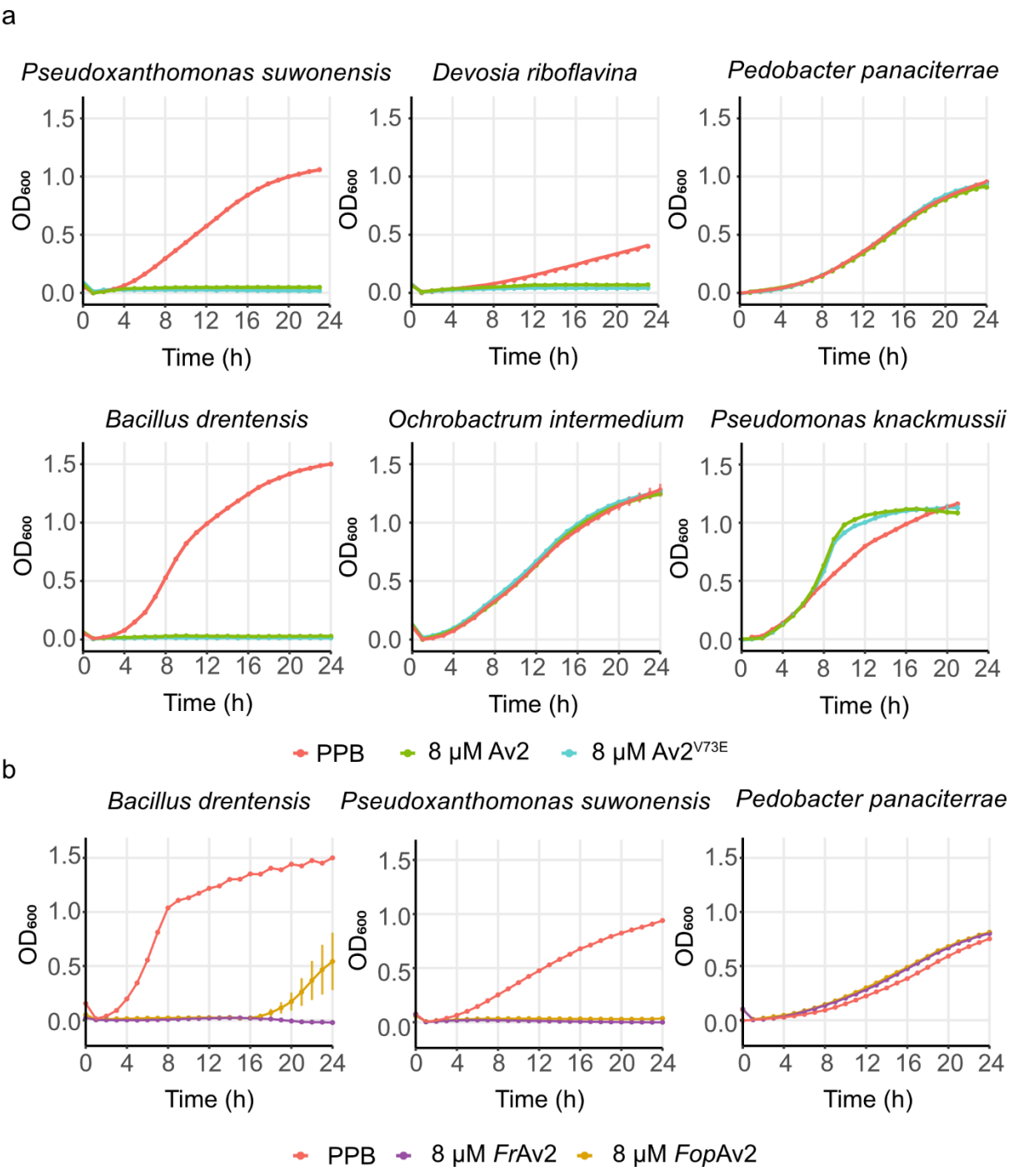

Figure 1 Av2 effector variants from *Verticillium dahliae* and homologs from *Fusarium* spp. display selective antibacterial activity. (a) The Av2 effector as well as the effector variant Av2^{V73E} selectively inhibits growth in a panel of phylogenetically diverse plant-associated bacteria *in vitro*. **(b)** Av2 homologues from *Fusarium redolans* (FrAv2) and *F. oxysporum* f. sp. *pisi* (FopAv2) display an overlapping activity spectrum with the *V. dahliae* Av2 variants. Phosphate buffer (PPB) was used as control. Graphs display time-course measurements of bacterial densities in the presence or absence of effector proteins with 15 min intervals over 24 h and display the average OD_{600} of three biological replicates ± standard deviations

homolouges, suggesting they have overlapping activity spectra. Thus, all Av2 proteins display selective antimicrobial activity against bacteria *in vitro*.

To explore the potential mode of action of Av2, InterProScan was used to identify functional domains, and the structure of Av2 was predicted using AlphaFold2. No protein domains were annotated by InterProScan. The resulting structural model had a low confidence score (pLDDT = 53.8), indicating limited reliability of the predicted structure (Figure S2a). Nevertheless, FoldSeek was used to search for structural similarities between Av2 and previously characterized proteins. No significant structural homologs were identified. These results suggest that, in addition to sharing sequence similarity only with *Fusarium* homologs, Av2 lacks detectable structural similarity to any known protein in the AlphaFold Protein Structure Database. To further investigate whether any compositional features could provide functional insight, the amino acid composition of Av2 was compared to that of other secreted proteins in *V. dahliae* (Figure S2c). Av2 displayed a net positive charge of +2.33, in contrast to the average net charge of –10.1 among the rest of the secretome. Consistent with this, the structural model revealed positively charged surface regions (Figure S2b). Although the overall model confidence is low, the observed surface charge could point to a potential membrane-interacting function, a mechanism previously described for certain antimicrobial peptides (G. Oliveira Júnior *et al*., 2025). However, given the limited reliability of the structural prediction, this interpretation remains highly speculative.

***Av2* contributes to *V. dahliae* virulence through microbiota manipulation**

Next, we hypothesised that *Av2* is utilized by *V. dahliae* for microbiota manipulation during host colonization as well as during soil-colonizing stages. To investigate this hypothesis, we pursued microbiota sequencing through bacterial 16S ribosomal DNA profiling of tomato plants. To this end, tomato plants were inoculated with either wild-type *V. dahliae* strain TO22 or the corresponding *Av2* deletion strain (Chavarro-Carrero *et al*., 2021), while water treatment was used as control. Interestingly, while tomato plants inoculated with wild-type *V. dahliae* showed severely stunted growth by ten days after inoculation when compared with mock-inoculated plants (Figure 2a), plants inoculated with the *Av2* deletion strain only showed mild symptoms of disease, and significantly less stunting occurred than in plants inoculated with the wild-type fungus. Importantly, significantly more fungal biomass was recorded in tomato plants inoculated with the wild-type fungus than in plants inoculated with the *Av2* deletion strain (Figure 2b), showing that *Av2* contributes to *V. dahliae* virulence during host colonisation.

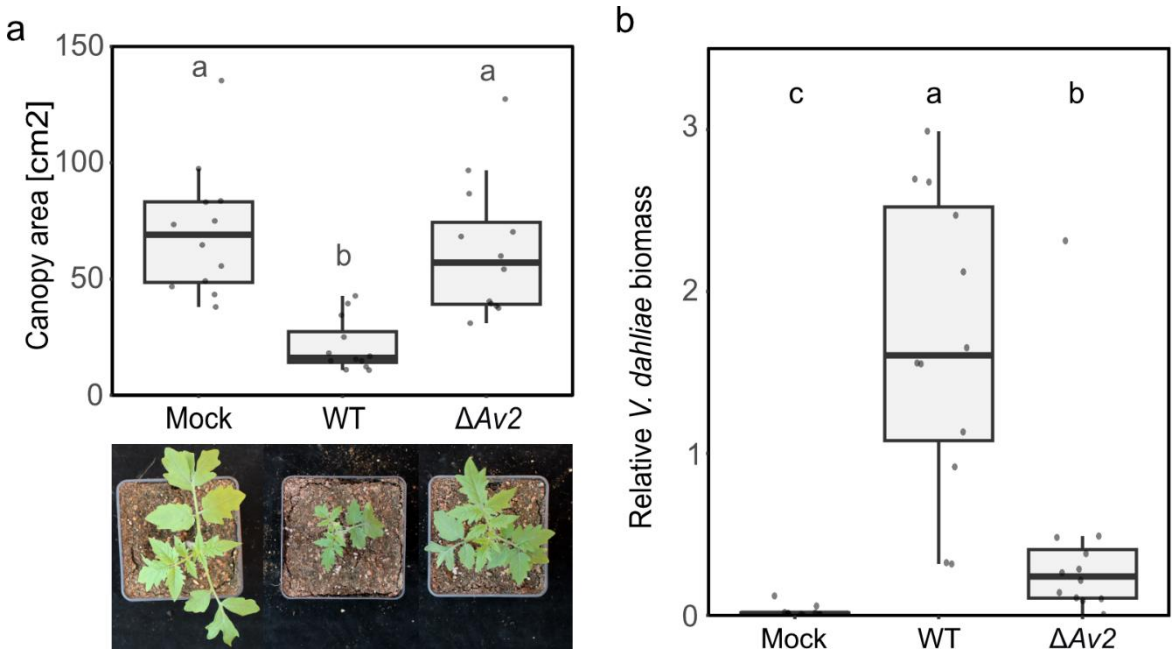

Figure 2. Av2 contributes to *Verticillium dahliae* virulence on tomato. (a) Av2 contributes to virulence of *V. dahliae* in tomato. The canopy area measurements of inoculated plants show stronger stunting upon inoculation with wild-type *V. dahliae* strain TO22 (WT) when compared with the corresponding *Av2* deletion strain (ΔAv2). Mock inoculated plants were treated with sterile water. Different letters represent significant differences (one-way ANOVA and Tukey's post hoc test; $P < 0.05$). **(b)** *V. dahliae* biomass in tomato stems was quantified with real-time PCR and normalised to Rubisco abundance. Different letters represent significant differences (one-way ANOVA and Tukey's post hoc test; $P < 0.05$).

To address the hypothesis that *Av2* contributes to virulence through microbiota manipulation, tomato plants were inoculated in a peat-based gnotobiotic system (Punt *et al*., 2025). If microbiota manipulation is the genuine function of the effector, *Av2* should not contribute to fungal virulence when plants are grown axenically, in the absence of microbes, while its contribution should become noticeable upon microbial reintroduction. To reintroduce microbes into sterile soil while maintaining physico-chemical properties similar to the sterilized substrate, 10% unsterilized soil was mixed with 90% sterilized soil. Importantly, plating confirmed that sterilization effectively removed the microbial population from the substrate, whereas reintroduction resulted in microbial colonization of the originally sterilized substrate (Figure S3). Next, tomato seedlings were inoculated with wild-type *V. dahliae* strain TO22 or the corresponding *Av2* deletion strain and cultivated in the two substrates. Importantly, at two weeks after inoculation, tomato plants inoculated with wild-type *V. dahliae* were significantly smaller than the mock-inoculated plants while plants inoculated with the *Av2* deletion strain developed similarly to tomato plants grown in potting soil (Figure 3, Figure 2a), showing that *V. dahliae* can establish infections on tomato plants also in a gnotobiotic system on sterilized substrate. As previously observed for other plant species, tomato plants grown axenically generally developed slower than those grown in the presence of a microbiota on recolonized substrate (Kremer *et al*., 2021; Punt *et al*., 2025). However, when tomato plants were grown on sterile substrate, no difference could be observed between tomato plants inoculated with wild-type *V. dahliae* or with the *Av2* deletion

strain, showing that *Av2* only contributes to virulence in the presence of a microbiota. This finding suggests that microbiota manipulation is the genuine virulence function of the Av2 effector, and that the effector lacks plant virulence targets.

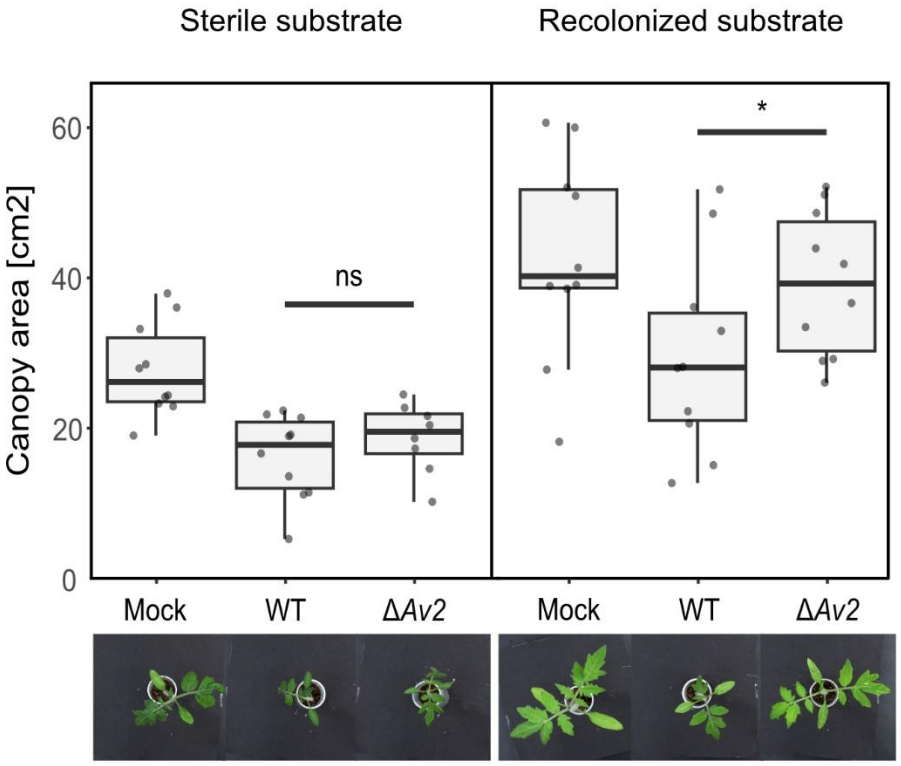

Figure 3. Av2 contributes to *V. dahliae* virulence on tomato plants solely in the presence of microbes. Canopy area measurements of inoculated tomato plants grown in Flowpots show stronger stunting upon inoculation with wild-type *V. dahliae* strain TO22 (WT) when compared with the corresponding *Av2* deletion strain (ΔAv2) in recolonized substrate but not in sterile substrate. Mock inoculated plants were treated with sterile water. Statistical analyses were performed for each of the substrates, and the star indicates significant differences (unpaired two-sided student's t-test; $p < 0.05$). Photographs display phenotypes of representative plants for each of the treatments at 14 days past infection.

Av2 suppresses the recruitment of Pseudomonadales

To perform microbiota sequencing through bacterial 16S ribosomal DNA profiling, tomato stem samples were collected at ten days post *V. dahliae* inoculation, before the onset of wilting symptoms, and the V5-V7 region of the bacterial 16S rDNA was amplified and sequenced. Subsequent analysis did not reveal major changes in microbial diversity (α-diversity) between plants inoculated with *V. dahliae* wild-type and mock-inoculated plants (Figure 4a). Interestingly, however, plants inoculated with the *V. dahliae Av2* deletion strain showed a significant reduction in microbial diversity that coincided with a strong increase in the relative abundance of Proteobacteria (Figure 4c). Principal component analysis based on weighted unifrac distance revealed differential grouping of the tomato stem endosphere microbiota for the three different treatments (PERMANOVA, $P < 0.001$; Figure 4b). To investigate which bacterial orders drove the separation of the samples in the principal component analysis, pairwise bacterial abundance comparisons were performed between plants inoculated with *V. dahliae* wild-

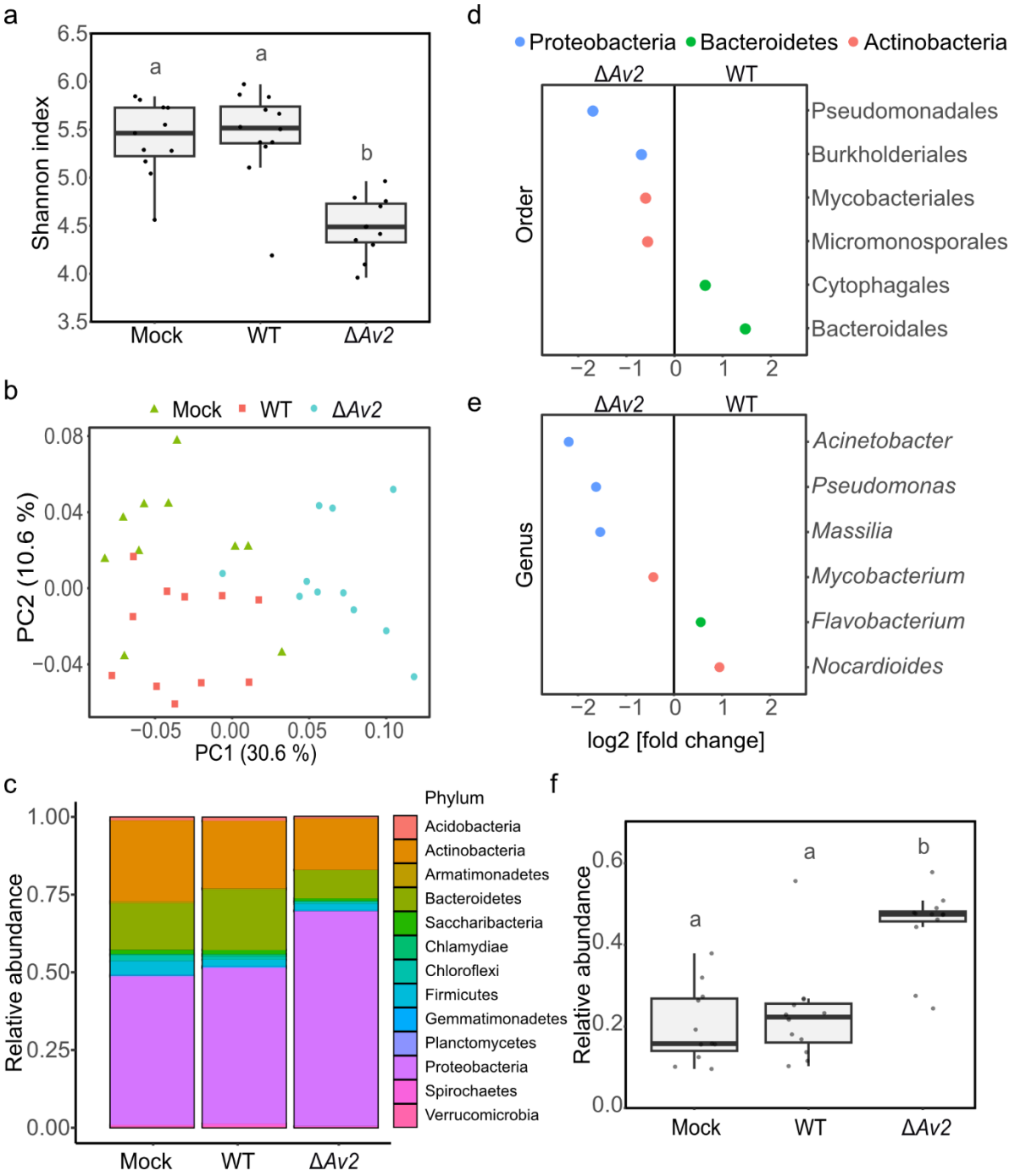

Figure 4. *Verticillium dahliae* Av2 suppresses Pseudomonas recruitment during host colonisation. (a) α-diversity of tomato endosphere microbiota ten days after inoculation as determined with 16S ribosomal DNA profiling. The α-diversity is significantly lower for microbiomes of plants inoculated with the *Av2* deletion strain (ΔAv2) when compared with the other treatments. Different letters represent significant differences (one-way ANOVA and Tukey's post hoc test; $P < 0.05$). **(b)** Principal component analysis based on weighted unifrac distance reveals separation of tomato stem endosphere microbiota upon inoculation with either water (Mock), wild-type *V. dahliae* or the *Av2* deletion strain (PERMANOVA, $p < 0.001$). **(c)** Relative abundance of bacterial phyla show increased Proteobacteria abundance in plants inoculated with the Av2 deletion strain. **(d)** Differentially abundant bacterial orders in the stem endosphere of tomato plants upon inoculation with either wild-type *V. dahliae* or the *Av2* deletion strain (Wald test, adjusted $P < 0.05$). **(e)** Differential abundance analysis of bacteria at the genus level in the tomato stems upon inoculation with either wild-type *V. dahliae* or the *Av2* deletion strain. **(f)** Relative abundance comparison of *Pseudomonas* in tomato stems upon inoculation with either water, wild-type *V. dahliae* or the *Av2* deletion strain. Different letters represent significant differences (one-way ANOVA and Tukey's post hoc test; $P < 0.05$).

type and the *Av2* deletion strain. Several bacterial orders were significantly more abundant in plants

inoculated with the *Av2* deletion strain, namely Pseudomonadales, Burkholderiales, Mycobacteriales, Micromonsporales (Figure 4d). Of these bacterial orders, the Pseudomonadales displayed the largest increase in abundance (log2-fold change 1.67). Only a few genera appeared to drive the differential abundance of these bacterial orders. Within the Pseudomonadales only the genera *Pseudomonas* and *Acinetobacter* were significantly more abundant upon inoculation with the *Av2* deletion strain, while within the order of Burkholderiales only the genus *Massilia* showed a significant increase (Figure 4e). The genus *Pseudomonas* especially caught our attention because of its high relative abundance in the tomato microbiota, with around 20% and 50% in plants inoculated with the wild-type *V. dahliae* and the *Av2* deletion strain, respectively. Intriguingly, while we anticipated a reduction in *Pseudomonas* abundance in plants inoculated with wild-type *V. dahliae* when compared with mock-inoculated plants, we observed no difference in *Pseudomonas* abundance between the two treatments (Figure 4f, Figure S5). This significant increase of *Pseudomonas* in plants inoculated with the *Av2* deletion strain also explains the decrease in alpha diversity of this treatment (Figure 4a). Given that we only saw a strong recruitment of *Pseudomonas* during the infection by the *Av2* deletion strain, we conclude that this effector is utilised by *V. dahliae* to suppress the recruitment of this bacterial genus by the host upon pathogen invasion.

***V. dahliae* utilises Av2 to inhibit antagonistic *Pseudomonas* spp.**

The targeted recruitment of *Pseudomonas* by tomato plants upon *V. dahliae* colonization, and the role of *Av2* in prevention of such recruitment, suggests that *Pseudomonas* acts as antagonist of the fungus. To investigate whether the interaction between *V. dahliae* and *Pseudomonas* involves direct antagonism, and to elucidate the role of *Av2* in this interaction, competition assays were performed between *V. dahliae* and *Pseudomonas* strains isolated from tomato plants (Punt *et al*., 2025). To this end wild-type *V. dahliae* strain TO22 and the corresponding *Av2* deletion strain were incubated with a panel of 15 *Pseudomonas* species. Interestingly, wild-type *V. dahliae* grew significantly better than the *Av2* deletion strain in presence of any of the four *Pseudomonas* species *P. crudilactis, P. laurentiana*, *P. plecoglossicida*, or *P. vancouverensis* (Figure 5a, Figure S4). No difference in growth between the two *V. dahliae* strains could be observed when co-cultured with the remaining *Pseudomonas* species under these conditions. The reduced growth of the *Av2* deletion strain when co-cultured with particular *Pseudomonas* species demonstrates that several *Pseudomonas* spp. are antagonists of *V. dahliae* growth and suggests that *Av2* is utilised by the fungus to counter these antagonists.

To test whether Av2 inhibits the growth of antagonistic *Pseudomonas* spp., their sensitivity towards Av2 was assessed *in vitro*. Interestingly, all antagonistic *Pseudomonas* spp. that showed reduced antagonism in the presence of *Av2* were inhibited when incubated with 8 µM Av2 or Av2^{V73E} (Figure

5b). In contrast, most of the *Pseudomonas* spp. for which no difference in antagonism was recorded in

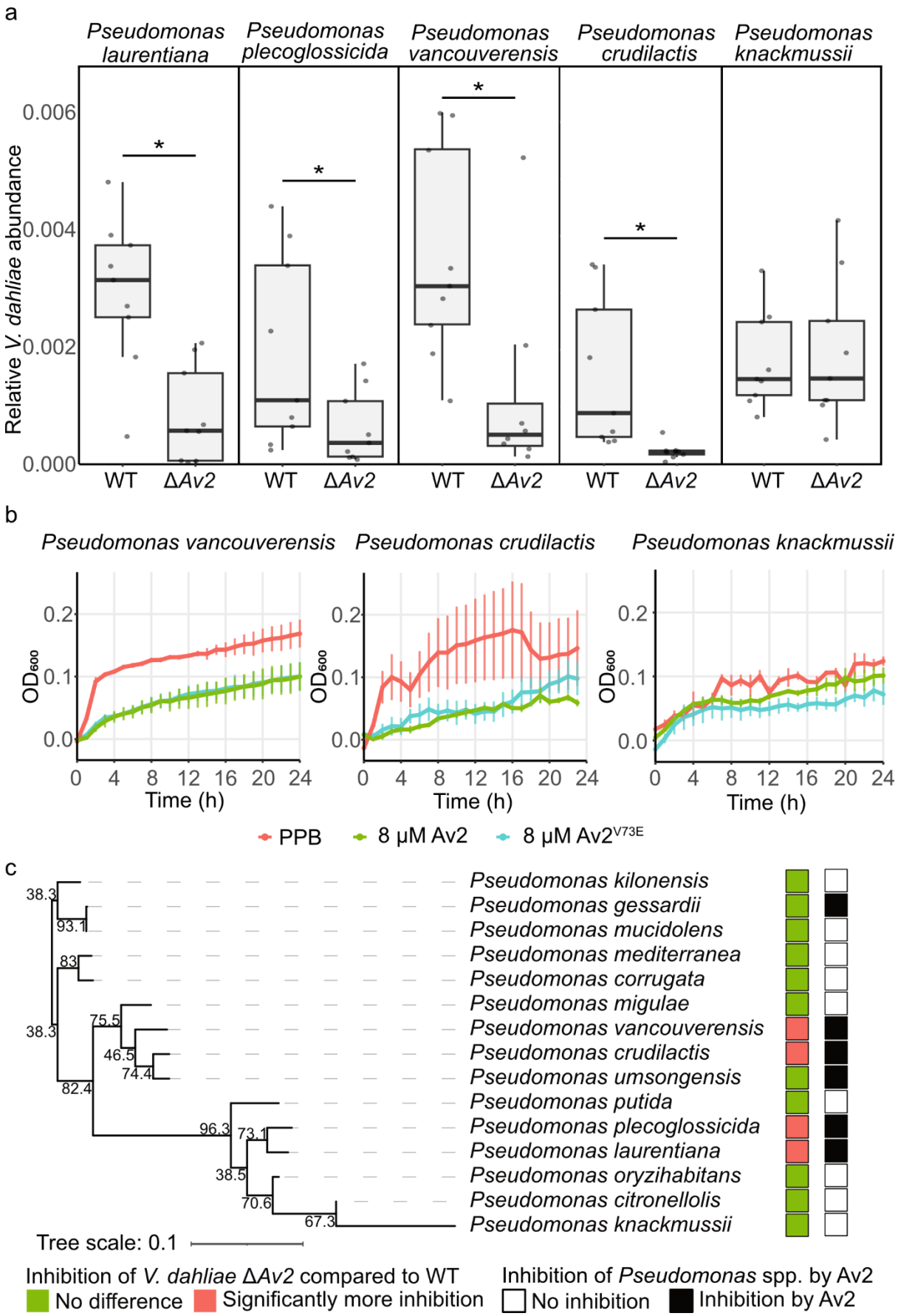

Figure 5 Av2 is used by *V. dahliae* for direct growth inhibition of antagonistic *Pseudomonas* spp. (a) Relative biomass of wild-type *V. dahliae* strain TO22 (WT) and the corresponding *Av2* deletion strain (ΔAv2) was quantified with real-time PCR after co-cultivation with a panel of Pseudomonadales in half-strength Murashige and Skoog medium for 48 h. The *V. dahliae* biomass was normalised against abundance of spike-in DNA, added during DNA extraction. **(b)** *Pseudomonas* spp. are differentially inhibited by Av2 and Av2^{V73E} *in vitro*. Phosphate buffer (PPB) was used as control. Graphs display time-course measurements with 60 min intervals over 24 h and display the average OD_{600} of three biological replicates ± standard deviations. **(c)** *Pseudomonas* spp. that display stronger antagonism towards the *Av2* deletion strain than towards wild-type *V. dahliae* do not group in a phylogenetic tree that was generated based on 2,495 orthologous genes present in all species. Inhibition of *Pseudomonas* spp. by Av2 and Av2^{V73E} *in vitro* is largely overlapping with that pattern.

the co-cultivation with *V. dahliae* were unaffected by Av2 or Av2^{V73E}, suggesting that *V. dahliae* co-opted Av2 to selectively suppress antagonistic *Pseudomonas* spp. (Figure 5c). To investigate the phylogenetic placement of the diverse *Pseudomonas* spp. isolates, and assess potential clustering of the species that act as *V. dahliae* antagonists and are inhibited by Av2, 2495 orthologous genes present in all species were extracted from their genomic sequences and used to infer a phylogenetic tree. Interestingly, *Pseudomonas* spp. that showed increased antagonism towards the *Av2* deletion strain when compared with wild-type *V. dahliae* do not seem to cluster, but appear in two clades (Figure 5c). Further insight into the molecular function of Av2 could reveal whether this phylogenetic split is caused by the evolution of resistance against Av2 within the *Pseudomonas* genus or is due to physiological similarities among the inhibited antagonistic species. In conclusion, our findings suggest that *V. dahliae* exploits Av2 to suppress the cry for help recruitment of beneficial *Pseudomonas* spp. during plant colonisation.

DISCUSSION

The plant microbiota has been shown to be crucial for plant health and to act as an additional layer of defense against invading pathogens (Trivedi *et al*., 2020). In a phenomenon known as the "cry for help", plants respond to pathogen invasion by dynamically altering their microbiota through modulating the composition of their root exudates to selectively recruit beneficial, disease-suppressing microorganisms and thereby mitigate disease progression (Rolfe *et al*., 2019). Here, we characterise the *V. dahliae* effector *Av2* as an antimicrobial effector that actively suppresses the plant's cry for help. We show that in tomato, *Av2* suppresses the recruitment of antagonistic *Pseudomonas* spp. into the rhizosphere. As a result, plants inoculated with wild-type *V. dahliae* exhibit *Pseudomonas* spp. levels comparable to mock-inoculated controls, whereas infection with an *Av2*-deletion mutant leads to strong *Pseudomonas* spp. enrichment that correlates with significantly reduced fungal colonization. This activity is distinct from previously characterized antimicrobial effectors such as Ave1 and Ave1L2, which promote pathogen virulence by depleting antagonistic Sphingomonadales and Actinobacteria from the host plant microbiota (Snelders *et al*., 2020), or the suite of antimicrobial proteins secreted by *Albugo candida*, which collectively target core members of the *Arabidopsis thaliana* microbiota to facilitate host colonization (Gómez-Pérez *et al*., 2023). Although the overall structural model

confidence is low, we observed positively charged surface areas which could point to a potential membrane-interacting function, a mechanism previously described for certain antimicrobial peptides (Oliveira Júnior *et al*., 2025). However, given the limited reliability of the structural prediction, this interpretation remains highly speculative. Our findings reveal a further sophisticated level of pathogen interference, demonstrating that pathogens can not only respond to and reshape the plant microbiome, but also sabotage microbiota-mediated host defense responses by compromising the cry for help recruitment during infection. *Pseudomonas* species are well known for their role in plant disease suppression and are frequently enriched during plant cry for help responses (Wang and Song, 2022). For example, beneficial *Pseudomonas* spp. are recruited in response to take-all disease in wheat, where they protect the host through direct antagonism against the pathogen (Raaijmakers and Weller, 1998). We observed antagonism by *P. crudilactis*, *P. laurentiana, P. plecoglossicida,* and *P. vancouverensis* against the *V. dahliae Av2*-deletion mutant *in vitro*, indicating that these *Pseudomonas* spp. are capable of suppressing *V. dahliae* during infection. Furthermore, the same *Pseudomonas* spp. that exhibited enhanced antagonism toward the *Av2* deletion mutant were directly inhibited by Av2. This reciprocal antagonism aligns with previous findings showing that antimicrobial effectors target beneficial bacteria that are able to antagonise the pathogen (Chavarro-Carrero *et al*., 2024; Snelders *et al*., 2020, 2023). Interestingly, *Pseudomonas* species inhibited by Av2 span two distinct phylogenetic groups, suggesting that some *Pseudomonas* species have evolved resistance to overcome suppression by Av2. This may suggest that a co-evolutionary arms race takes place between *V. dahliae* and host-associated microbiota members reminiscent of the development of antibiotic resistance. Given the abundance of microbes that produce antimicrobial molecules (Mesny and Thomma, 2024; Mullis *et al*., 2019), the resistance of *Pseudomonas* spp. to Av2 may be part of a broader antimicrobial resistance developed through diverse microbial interactions, with *V. dahliae* playing only a minor role in this process. Elucidating how particular *Pseudomonas* species have overcome Av2 sensitivity may provide valuable insight into the mode of action of the effector and selective pressures shaping pathogen–microbe interactions in the rhizosphere. Within the *V. dahliae* population, two closely related homologues of the *Av2* effector have been identified, differing by only a single amino acid (Chavarro-Carrero *et al*., 2021). Since this variation does not seem to affect recognition by the V2 immune receptor (Chavarro-Carrero *et al*., 2021), we hypothesized that it might affect the antimicrobial activity that is exerted by the effector protein. However, our *in vitro* activity assays revealed no significant differences in antimicrobial activity between the two variants, suggesting that the amino acid substitution does not affect this function. *Av2* homologues have furthermore been reported in other species of the *Verticillium* genus, and in *Fusarium* (Chavarro-Carrero *et al*., 2021). Intriguingly, recent evidence indicates that *V. dahliae* acquired *Av2* via horizontal gene transfer from *Fusarium* species

(Sato et al. 2025). Although sequence variation exists among these homologues, our assays did not reveal any functional differences in antimicrobial activity. It is important to note, however, that only a limited panel of bacterial strains was tested, and the possibility remains that sequence variation modulates activity against untested microbial targets. The conservation of the antimicrobial function observed for Av2 is reminiscent of Ave1, which was also horizontally acquired by *V. dahliae*, in this case from plants (de Jonge *et al*., 2012; Snelders *et al*., 2020). Interestingly, plant homologues of *Ave1*, known as plant natriuretic peptides (PNP), likely exhibit similar antimicrobial activity *in vitro*, as both *A. thaliana* PNP-A and Ave1 inhibit the growth of *Bacillus subtilis* (Snelders *et al*., 2020). These parallels raise the possibility that conserved antimicrobial effectors, regardless of their evolutionary origin, fulfil similar ecological roles in shaping microbial communities. Since both *Fusarium* spp. and *V. dahliae* are soil-borne fungal pathogens that infect plants via the roots and disperse within their hosts via the vasculature (Di Pietro *et al*., 2003; Fradin and Thomma, 2006), further investigation into the role of *Av2* in *Fusarium* spp. could help clarify whether its conserved antimicrobial activity similarly contributes to the colonization strategy shared by these pathogens.

Taken together, our findings broaden the understanding of how pathogens manipulate their hosts by revealing that antimicrobial effectors can actively suppress the pathogen-induced cry for help response. By blocking the recruitment of protective microbes, pathogens undermine a critical layer of microbiota-mediated immunity. This adds to growing evidence that the plant microbiota is a strategic battleground in host–pathogen interactions (Mesny *et al*., 2024). As more antimicrobial effectors are identified and characterized (Chang *et al*., 2021; Chavarro-Carrero *et al*., 2024; Gómez-Pérez *et al*., 2023; Kettles *et al*., 2018; Ökmen *et al*., 2023; Snelders *et al*., 2020, 2021, 2023) it will become increasingly clear how deeply the molecular arms race between plants and pathogens extends into the plant's microbial sphere. Finally, given that the cry for help recruitment of beneficial microbes may ultimately lead to the establishment of disease-suppressive soils (Mesny *et al*., 2024), future research will have to reveal whether Av2 suppresses such long-term legacy effects in the soil microbiome.

MATERIALS AND METHODS

Detection of *V. dahliae Av2* expression in soil extract

For each treatment, 10^6 conidiospores of *V. dahliae* strain JR2 were added to 10 mL potato dextrose broth (PDB) and incubated while shaking with 130 rpm at 28°C for 2 days (Ecotron, Infors-HT, Bottmingen, Swizerland). Subsequently, the mycelium was collected using sterilized miracloth (Merck, Darmstadt, Germany) and washed with sterilized water. Next, the mycelium was transferred to new

flasks containing 10 mL of soil extract that was prepared by adding 40 g of potting soil (Balster Einheitserde, Frödenberg, Germany) to 200 mL of sterilized water followed by incubation at room temperature for 2 days, after which soil particles were pelleted by centrifugation for 30 min at 4,000 x g and the supernatant was collected. The flasks were then incubated while shaking with 130 rpm at 28°C for 5 days (Ecotron, Infors-HT, Bottmingen, Swizerland). Next, mycelium was recollected using sterilized miracloth and washed with sterilized water. RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) of which 1 µg was transcribed into cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Bio USA, San Jose, CA, USA). Real-time PCR was performed using SsoAdvance Universal SYBR Green Supermix (BioRad, Hercules, California, USA) on a CFX Opus Real-Time PCR System (BioRad, Hercules, California, USA)and the expression of effector genes was normalized using the *V. dahliae* glyceraldehyde 3-phosphate dehydrogenase gene (*VdGAPDH*) as a reference.

Heterologous expression of Av2 homologs

The *Av2* alleles encoding *V. dahliae* Av2 and Av2^{V73E} (from strains TO22 and JR2, respectively) and their homologs from *Fusarium oxysporum* f. sp. *pisi* and *F. redolens* (*Fop*Av2 and *Fr*Av2, respectively), were codon-optimized for expression in *E. coli* and cloned into the pET15b vector (Merck, Darmstadt, Germany) such that the proteins are produced without a signal peptide and as a fusion protein with an N-terminal His_6 tag. All vectors were ordered from BioCat GmbH (Heidelberg, Germany). While *Vd*Av2 and Av2^{V73E} were produced in *E. coli* strain BL21 (Thermo Fisher Scientific, Waltham, Massachusetts, USA), *Fop*Av2 and *Fr*Av2 were produced in *E. coli* strain SHUFFLE T7 (New England Biolabs, Ipswich, Massachusetts, USA). A pre-inoculum of bacterial cultures was incubated overnight in Lysogeny broth (LB) supplemented with 50 µg/mL ampicillin at 37°C for BL21 and at 30°C for SHUFFLE T7 while shaking at 170 rpm (Ecotron, Infors-HT, Bottmingen, Swizerland). Subsequently, the pre-inoculum was transferred to 1 L of LB supplemented with ampicillin (50 µg/mL) and incubated at 37°C (BL21) or 30°C (SHUFFLE T7) until the optical density at 600 nm (OD_{600}) reached 0.6-0.8. Next, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM and the culture was incubated for 4 h at 37°C (BL21) or 30°C (SHUFFLE T7). Next, cells were pelleted through centrifugation (21,000 x g) at 4°C and resuspended in 6 M guanidine, 10 mM TRIS-HCl and 10 mM β-mercaptoethanol (pH 8.0) and incubated overnight at 4°C while rotating continuously. After centrifugation at 21,000 x g for 30 min, proteins were purified from the supernatant by immobilized metal affinity chromatography (IMAC) on a custom packed 5 mL Ni^{2+} CYTIVIA column (XK16/20 Column, Cytiva, Marlborough, USA) with His60 Ni Superflow Resin (Takara Bio USA, San Jose, CA, USA). Fractions containing the protein of interest were identified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, combined and dialysed in a stepwise fashion. To this end, the

protein was dialyzed in a stepwise manner over several 18-hour intervals. Initially, dialysis was performed against 4 M guanidine, 50 mM BIS-TRIS, 10 mM reduced glutathione, and 2 mM oxidized glutathione (pH 7.0). This was followed by dialysis against 3 M guanidine, 50 mM BIS-TRIS, 10 mM reduced glutathione, and 2 mM oxidized glutathione (pH 6.5). Subsequently, the protein was dialyzed against 2 M guanidine, 100 mM BIS-TRIS, 250 mM ammonium sulfate, 10 mM reduced glutathione, and 2 mM oxidized glutathione (pH 6.5), followed by 1 M guanidine, 100 mM BIS-TRIS, 125 mM ammonium sulfate, 10 mM reduced glutathione, and 2 mM oxidized glutathione (pH 5.8). The final dialysis step was performed in 100 mM BIS-TRIS, 125 mM ammonium sulfate, 10 mM reduced glutathione, and 2 mM oxidized glutathione (pH 5.8). Ultimately, the protein was dialyzed against potassium phosphate buffer (pH 6.5).Final protein concentrations were determined with Nanodrop (Thermo Fisher Scientific, Waltham, Massachusetts, USA) based on absorbance at 280 nm and adjusted to a final concentration of 16 µM.

***In vitro* antimicrobial activity assays**

All bacteria used in the assays originated from a tomato culture collection (Punt *et al*., 2025). After growth of bacterial isolates on tryptone soy agar (TSA) at 28°C, single colonies were selected and grown overnight in tryptone soy low salt broth (TSB LS) at 28°C while shaking at 200 rpm (Ecotron, Infors-HT, Bottmingen, Swizerland). Subsequently, optical density was measured at 600 nm (OD_{600}) was adjusted to 0.05 by dilution with TSB LS. One hundred µL of bacterial culture were mixed with 100 µL of Av2 protein variants (16 µM) in clear 96-well plates (BRAND SCIENTIFIC GMBH, Wertheim, Gemany) with three replicates for each treatment. The plates were incubated in a CLARIOstar® plate reader (BMG LABTECH, Ortenberg, Germany) at 28°C with double orbital shaking every 15 min (10 s at 300 rpm) after which the $OD_{600}$was measured (Snelders *et al*., 2020).

Plant disease assays

Inoculation of tomato plants to determine the virulence of *V. dahliae* was performed as described previously (Fradin *et al*., 2009). Briefly, conidiospores of *V. dahliae* wild-type or *Av2* deletion strain (Chavarro-Carrero *et al*., 2021) were harvested after ten days of cultivation on potato dextrose agar (PDA). The conidiospore suspensions were centrifuged at 10,000 x g for 10 min and the pellets were resuspended in water. This washing step was repeated twice before spores were counted and the concentration was adjusted to 10^6 conidiospores/mL. For the inoculation, ten-day-old tomato (*Solanum lycopersicum* L.) MoneyMaker plants were carefully uprooted, roots were rinsed in water, and placed into the inoculum for 6 min. Next, plants were placed back into soil, and placed in the greenhouse at 22°C during 16h/8h day/night periods with a maximum 80% relative humidity, and symptoms were monitored at 14 days post inoculation (dpi). To this end canopy areas were measured

and fungal biomass inside the tomato stem was determined. For the latter, samples were frozen in liquid nitrogen, ground to a fine powder, and DNA was isolated using phenol-chloroform extraction (Chavarro-Carrero *et al*., 2021). *V. dahliae* biomass was quantified through real-time PCR using *V. dahliae*-specific primers targeting the internal transcribed spacer (ITS) region of the ribosomal DNA (Snelders *et al*., 2020). The tomato *Rubisco* gene was used for sample calibration (Snelders *et al*., 2020).

Tomato stem microbiota sequencing

Tomato stem samples were collected, washed with sterile water, frozen in liquid nitrogen and manually ground using mortar and pestle. Total DNA was extracted following a phenol-chloroform-based extraction procedure (Chavarro-Carrero *et al*., 2021) and DNA concentrations were determined using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Sequence libraries were prepared following amplification of the V5-V7 region of the bacterial 16S rDNA (799F and 1139R) as described previously (Wippel *et al*., 2021) and sequenced (paired-end 300 bp) on an Illumina MiSeq V3 Platform (Cologne Center for Genomics, Cologne, Germany). Sample barcoding was done as described previously (Fadrosh *et al*. 2014).

Microbiota analysis

Sequencing data were processed using R v.4.2.0. as described previously (Callahan *et al*., 2016; Snelders *et al*., 2020). Briefly, reads were demultiplexed using cutadapt (v4.1; Martin 2011). Afterwards reads were trimmed and filtered to an average paired read length of 412 bp with the Phred score of 30. From the trimmed reads, OTUs were inferred using the DADA2 method (v 1.24; Callahan *et al*., 2016). Taxonomy was assigned using the Ribosomal Database Project (RDP,v 18; Cole *et al*., 2014). The pyloseq package (v1.40.0; McMurdie and Holmes 2013) was used to calculate β-diversity (weighted unifrac distance) after the data was normalised with the metagenomeseq package (v.1.38.0; Paulson et al. 2013)using cumulative sum scaling. PERMANOVA was performed with the vegan (v2.6-4; Oksanen *et al*., 2004)package. Differential abundance analysis was done using the DESeq2 package (v1.36.0; Love *et al*., 2014) using a negative binomial Wald test and a significance P adjusted threshold < 0.05.

***In vitro* competition assay**

Conidiospores of *V. dahliae* strain TO22 and the *VdAv2* deletion strain were harvested from a PDA plate using sterile water and diluted to a concentration of 2 x 10^6 conidiospores/mL in half-strength Murashige and Skoog (MS) medium (Duchefa, Haarlem, The Netherlands). Plant-associated *Pseudomonas* spp. (Punt *et al*., 2025) were cultured overnight in half-strength MS medium. Next, overnight cultures were adjusted to OD_{600} of 0.05 in half-strength MS and added to the conidiospores,

and 500 µL of the microbial mixture was added into 12-well flat bottom polystyrene tissue culture plate (Sarstedt, Nümbrecht, Germany). Following 48 h of incubation at RT with shaking at 150 rpm (Ecotron, Infors-HT, Bottmingen, Swizerland), genomic DNA was extracted using the SmartExtract DNA kit (Eurogentec, Maastricht, The Netherlands), and *V. dahliae* biomass was quantified through real-time PCR using *V. dahliae*-specific primers targeting the ITS region of the ribosomal DNA (Snelders *et al*., 2020). A spike-in DNA sequence was added during DNA extraction for sample calibration (Guo *et al*., 2020). Genomic sequences of the tested *Pseudomonas* spp. (Punt *et al*., 2025) were used to infer rooted species trees based on single-copy orthologous genes (Emms and Kelly, 2019).

Gnotobiotic tomato cultivation for *V. dahliae* inoculations

For tomato cultivation, a previously developed Flowpot-system was used *(Kremer et al., 2021; Punt et al*., 2025). A 1:1 blend of potting soil (Balster Einheitserdewerk, Fröndenberg, Germany) and vermiculite (LIMERA Gartenbauservice, Geldern-Walbeck, Germany) were autoclaved three times on a liquid cycle and filled into 50 mL syringes (Terumo Europe, Leuven, Belgium). To check for substrate sterility, 500 mg of substrate was added to 10 mL of 100 mM $MgCl_2$ and shaken at 300 rpm at room temperature for 1 h (Ecotron, Infors-HT, Bottmingen, Swizerland). Subsequently, the samples were diluted 1,000-fold and 50 µL of the dilution was plated onto Reasoner's 2A agar (R2A), TSA and LB agar (LBA), and incubated in darkness at room temperature for 4 days before microbial growth was assessed. The substrate-filled syringes were flushed with 40 mL of sterile H_2O followed by 40 mL of half-strength MS. Next, surface-sterilized tomato seeds (MoneyMaker) were placed into each syringe and six syringes were placed into an autoclaved Microbox container (SacO2, Deinze, Belgium) and placed in the greenhouse at 22°C during 16-h/8-h day/night periods with a maximum of 80% relative humidity. After two weeks, tomato plants were carefully uprooted under sterile conditions and inoculated with 10^6 conidiospores/mL of either wild-type TO22 or the corresponding *Av2* deletion strain. Subsequently, the plants were placed back into the syringe and the syringes into the container in the greenhouse. Symptoms of disease were scored at 14 days post inoculation. For biomass quantification, stems of the plants were frozen in liquid nitrogen and ground to a fine powder. DNA was isolated using phenol-chloroform extraction (Chavarro-Carrero *et al*., 2021). *V. dahliae* biomass was quantified through real-time PCR using *V. dahliae* specific primers targeting the ITS region of the ribosomal DNA. The tomato *Rubisco* gene was used for sample calibration.

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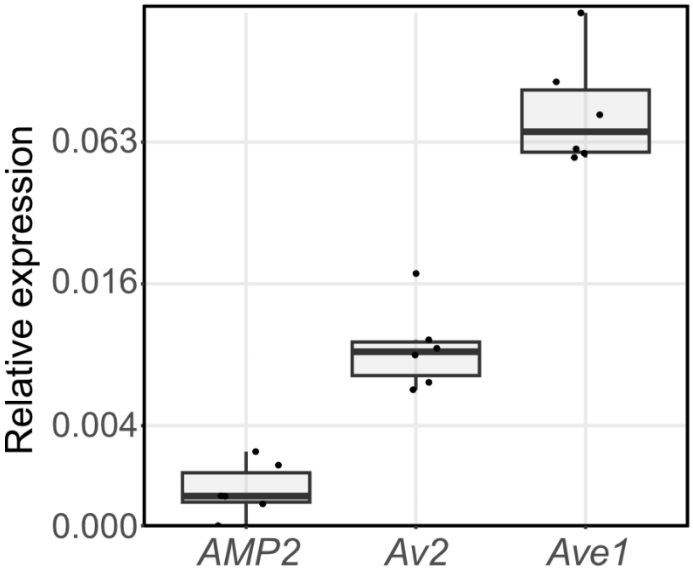
AUTHOR CONTRIBUTIONS

A.K., W.P., N.C.S. and B.P.H.J.T. conceived the project. A.K., W.P., J.Z., N.C.S. and B.P.H.J.T. designed the experiments. A.K., W.P., A.D., J.Z. and N.S. performed the experiments. A.K., W.P., A.D., J.Z., N.S. and B.P.H.J.T. analyzed the data. A.K., W.P. and B.P.H.J.T. wrote the manuscript. All authors read and approved the final manuscript.

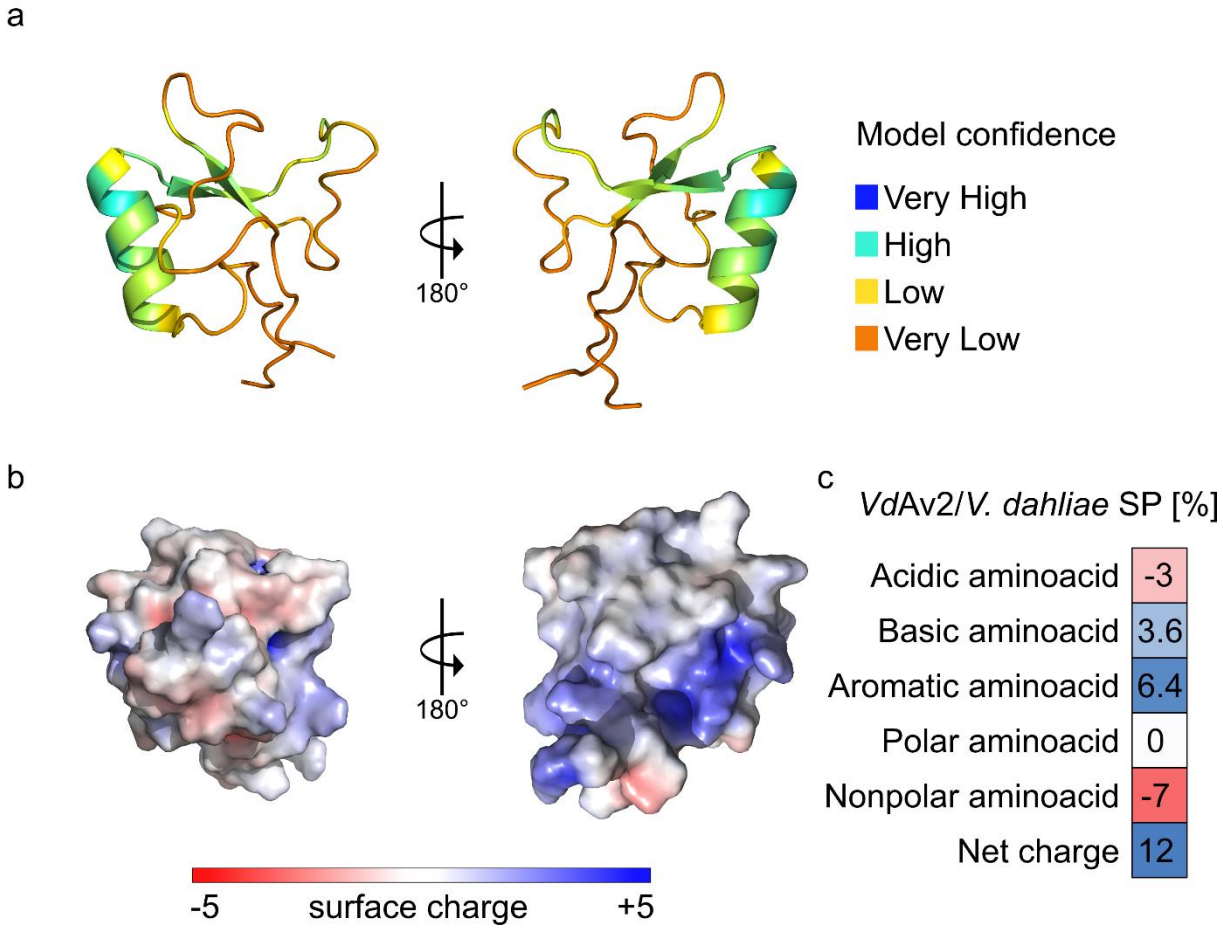
DATA AVAILABLILITY

The 16S profiling data have been deposited in the NCBI GenBank database under BioProject PRJEB90267

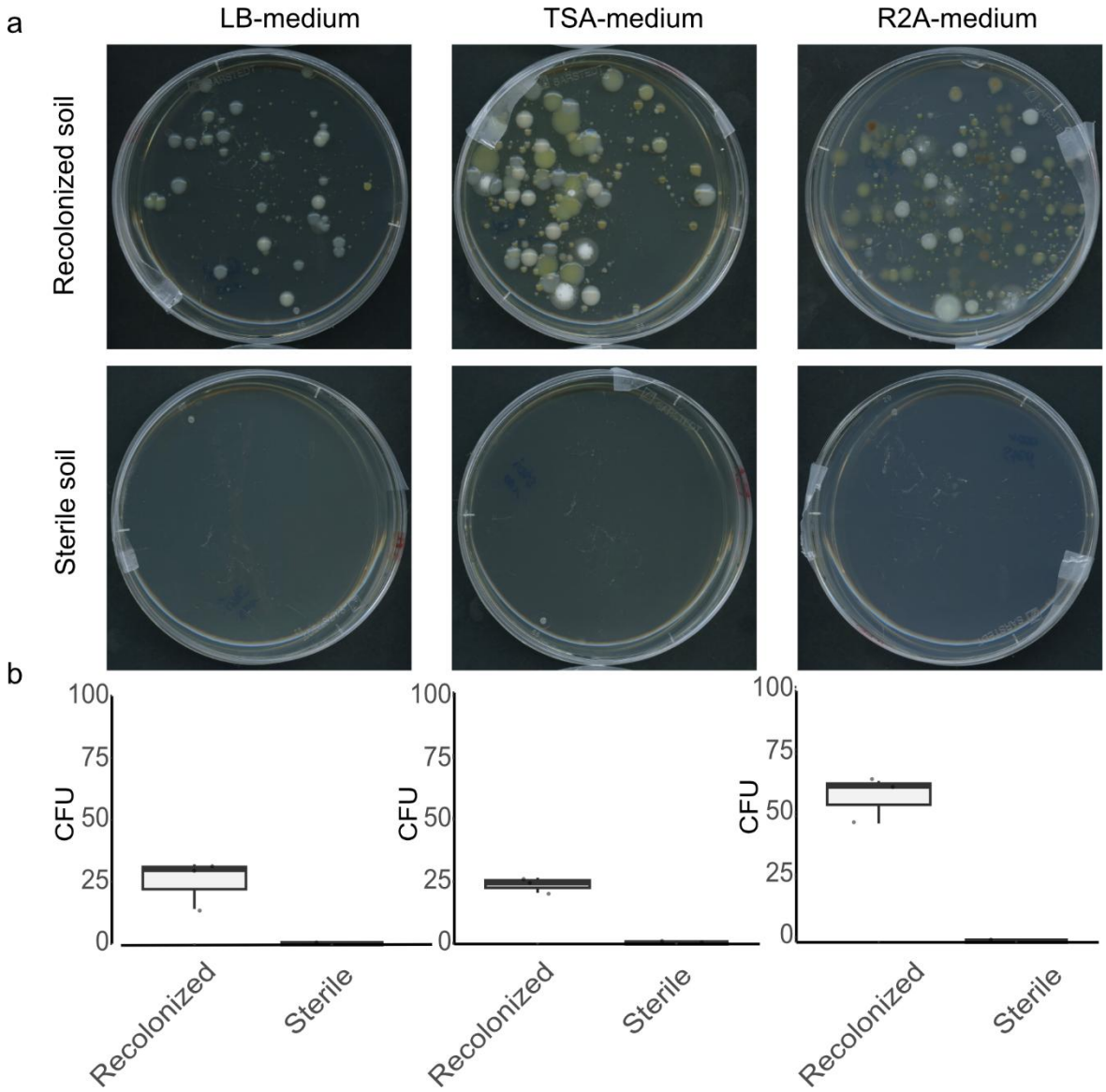
Supplementary Figures



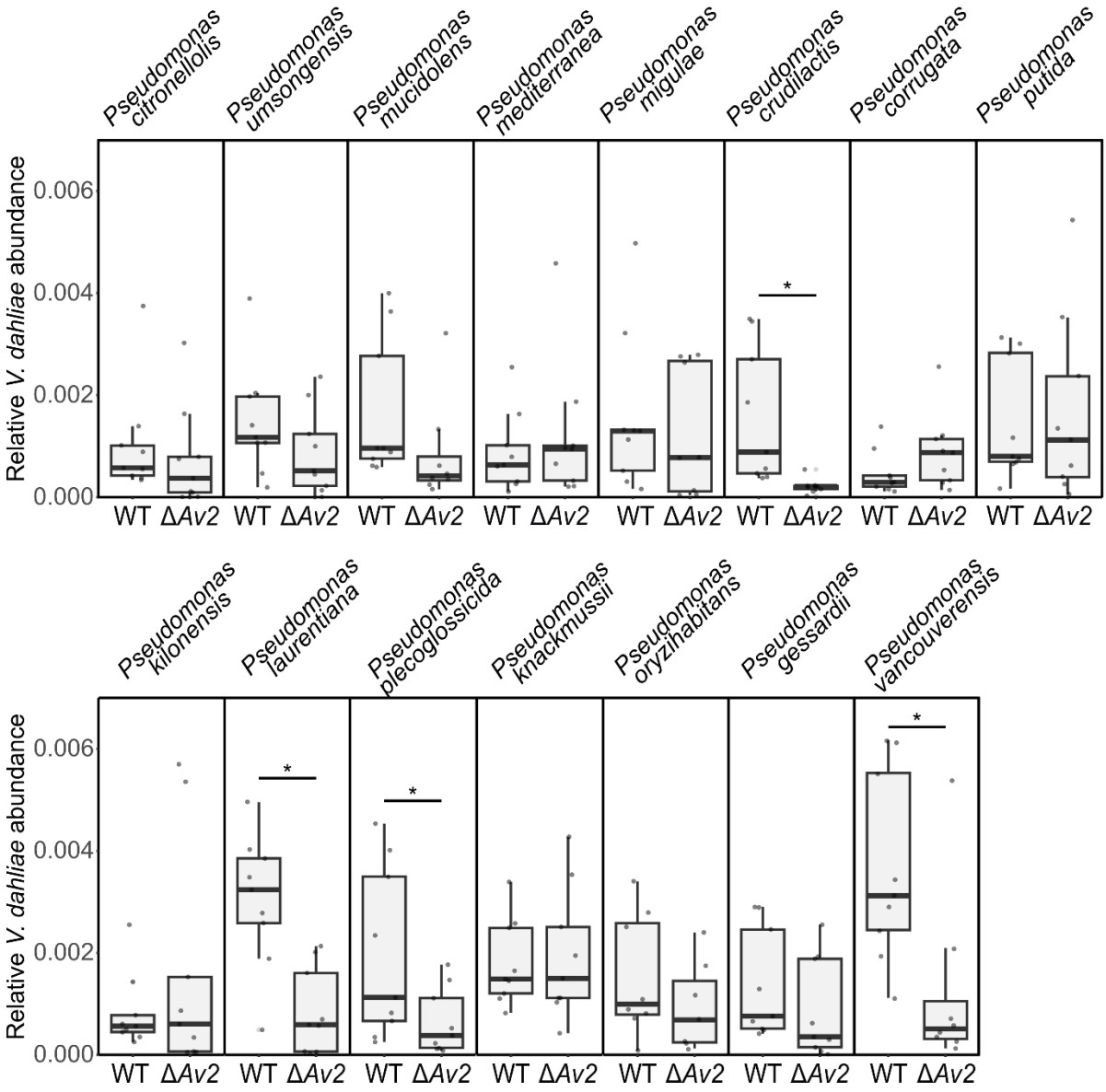
Supplementary Fig. 1. *Verticillium dahliae Av2* is expressed in soil extract. Expression of *V. dahliae* effectors after seven days of growth in soil extract when normalised to glyceraldehyde 3-phosphate dehydrogenase (*VdGAPDH*) expression.



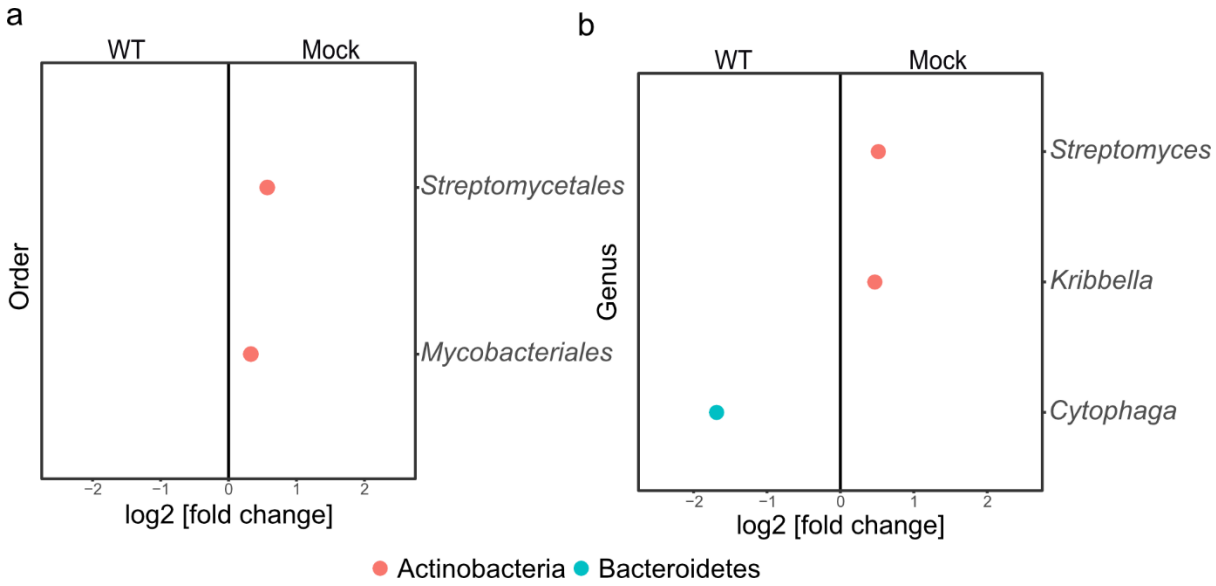
Supplementary Fig. 2. The predicted structure of the antimicrobial effector Av2 shows positively charged surface residues. **(a)** Structural prediction of Av2 using Alphafold2 resulted in a low confidence structure with an overall pLDDT score of 53.8. Coloring of the individual amino acids in the structure was done according to the Alphafold Protein Structure Database, where amino acids with a pLDDT score above 90 are colored blue, between 90 and 70 cyan, between 70 and 50 yellow and blow 50 orange. While local stretches have higher pLDDT scores the majority of Av2 is predicted with a score below 50. **(b)** Surface charge of the predicted structure was calculated using the APBS plugin for PyMOL. **(c)** To assess differences in amino acid composition between Av2 and the *V. dahliae* secretome, the proportion of chemically distinct amino acids in Av2 was subtracted from their expected proportions in the overall secretome.



Supplementary Fig. 3. Microbes were successfully reintroduced into sterile flowpot substrate with 10% non-autoclaved soil. a) Either recolonized or sterile Flowpot substrate were resuspended in $MgCl_2$ and streaked out on three different media, namely Lysogeny broth agar (LB), Tryptic soy agar (TSA) and Reasoner's 2A agar (R2A). There was growth on all plates containing recolonised substrate while no growth was observed on plates with sterile substrate. Photographs display agar plates after the plating of a 100x diluted substrate-$MgCl_2$ suspension and a 4-day incubation in darkness at room temperature. **b)** Boxplots displaying the number of colony-forming units (CFU) on three different growth media after plating a 100x substrate-$MgCl_2$ suspension and 4 days of incubation in darkness at room temperature. Substrate suspension from recolonized substrate showed significant more colonies compared to sterile substrate (unpaired two-sided student's t-test; $p < 0.05$).



Supplementary Fig. 4. Growth of a *Verticillium dahliae Av2* deletion strain is selectively impaired when co-cultured with *Pseudomonas* spp. Relative biomass of wild-type *V. dahliae* strain TO22 (WT) and the corresponding *VdAv2* deletion strain (ΔAv2) was quantified with real-time PCR after co-cultivation with a panel of Pseudomonadales in half-strength Murashige and Skoog medium for 48 h. *V. dahliae* biomass was normalised against abundance of spike-in DNA added during DNA extraction.



Supplementary Fig. 5. Differentially abundant bacterial between mock and *V. dahliae* WT inoculated plants. a) Differentially abundant bacterial orders in the stem endosphere of tomato plants upon inoculation with either wild-type *V. dahliae* or mock treatment (Wald test, adjusted P < 0.05). **b)** Differential abundance analysis of bacteria at the genus level in the tomato stems upon inoculation with either wild-type *V. dahliae* or mock treatment.

Chapter 4: An antimicrobial effector from *Verticillium dahliae* differentially contributes to virulence and differentially impacts tomato microbiota across natural soils

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Abstract

Throughout their life cycle plants associate with diverse and complex microbial communities, known as their microbiota. These microbiota contribute to plant performance and health, in part by providing a microbial barrier against invading plant pathogens. To colonize plant hosts, pathogens not only have to overcome host immune responses, but also breach the microbial barrier, for which they secrete so-called effector proteins. Accordingly, the soil-borne fungal plant pathogen *Verticillium dahliae* secretes the antimicrobial effector Ave1 to suppress antagonistic microbes and facilitate host colonization. Notably, many pathogens, including *V. dahliae*, have life stages outside their host plants, for instance in soil, where they encounter diverse microbial communities. Yet, how antimicrobial effectors support establishment across these environments remains poorly understood. To address this, we established a collection of natural soil samples with diverse physicochemical properties and microbiota compositions. Using this collection, we show for three plant species, barley, tomato and cotton, that root-associated bacterial and fungal communities are primarily shaped by soil type, whereas the phyllosphere microbiota is mainly determined by plant species. On tomato, we furthermore show that Ave1 differentially contributes to virulence on diverse soils, as Ave1 altered the tomato microbiota on all soils tested, but the taxa affected by these shifts varied depending on the specific soils. Our findings suggest that while Ave1-mediated microbiota manipulation occurs across soils, its impact on fungal virulence is influenced by the specific composition of the soil-derived microbiota assembled by the host.

Introduction

Plants host diverse microbial communities, known as the plant microbiota, which mainly include bacteria, fungi, and protists (Trivedi *et al*., 2020). These microorganisms colonize all plant parts, and together with the host plant, form a unified biological entity often referred to as the holobiont (Vandenkoornhuyse *et al*., 2015). Apart from seed-borne microbes inherited from the mother plant in the previous plant generation, the majority of microbes that make up the plant microbiota are recruited from environmental niches. While some microbes are transmitted through the air, the surrounding soil serves as the primary reservoir from which plants acquire most of their microbiota (Chialva *et al*., 2022). Soil properties such as pH, nutrient availability, organic carbon content, temperature and redox status shape the pool of microbes available for recruitment into the plant microbiota (Fierer, 2017). Consequently, the physicochemical properties of soil have a strong influence on plant microbiota assembly, as evidenced by the distinct microbial communities found in plants grown on different soils (Bulgarelli *et al*., 2012; Thiergart *et al*., 2020). At the same time, host genetics exert selective pressure on which taxa colonize and persist in the plant microbiota (Bulgarelli *et al*., 2012; Lundberg *et al*., 2012; Wagner *et al*., 2016). This is particularly evident in the formation of the core microbiota, which is a consistent subset of microbial taxa that reliably establish within the microbiota of a plant, even when plants are grown in diverse soils (Lundberg *et al*., 2012; Almario *et al*., 2022).

To date, numerous studies have separately demonstrated the importance of the bulk soil on the one hand, and of host genetics on the other hand, in structuring plant microbiota (Bulgarelli *et al*., 2012; Lundberg *et al*., 2012; Wagner *et al*., 2016; Fitzpatrick *et al*., 2018; Walters *et al*., 2018; Thiergart *et al*., 2020; Simonin *et al*., 2020; Tkacz *et al*., 2020). These studies have examined plant-associated microbes in diverse natural environments, where abiotic factors like local climate and weather can influence microbiota assembly, or have compared microbiota of different plant species grown in the same soil at a single location (Ofek-Lalzar *et al*., 2014; Wagner *et al*., 2016; Walters *et al*., 2018). However, studies that simultaneously evaluate the contribution of plant genetics and differential bulk soil microbiota on plant microbiota assembly, for instance by using various plant species in diverse natural soils while controlling for environmental influences, remain scarce (Tkacz *et al*., 2020).

Microbes that establish in the plant microbiota interact with the host plant in various ways. Many microbes interact with plants as neutral commensals, while other microbes can be beneficial to the plant, or can be pathogenic and cause disease (Hassani *et al*., 2018). The community balance and composition of the microbiota plays an important role in plant health and performance, particularly by contributing to defense against pathogens (Du *et al*., 2025). Notably, plants have the ability to

actively recruit beneficial microbes in response to pathogen attack. For instance, cucumber plants infected by the soil-borne pathogen *Fusarium oxysporum* f. sp. *cucumerinum* recruit *Bacillus amyloliquefaciens* to reduce disease severity (Liu *et al*., 2017). Over longer timescales, such plant-driven recruitment of beneficial microbes can result in the formation of disease-suppressive soils, where susceptible plants can grow in the presence of pathogens without experiencing severe disease symptoms (Du *et al*., 2025). A well-documented example, is the response of wheat plants to infection by *Gaeumannomyces graminis* var. *tritici*, the causal agent of "take-all" disease In this case, wheat recruits beneficial *Pseudomonas* species that antagonize the pathogen through the secretion of antimicrobial compounds, ultimately contributing to disease suppression over successive planting cycles in particular fields (Raaijmakers and Weller, 1998; Spooren *et al*., 2024). Importantly, protection via microbial recruitment is not limited to direct antagonism of pathogens. Some beneficial microbes enhance plant immunity through the induction of systemic defense responses (Pieterse *et al*., 2014). For example, *Arabidopsis thaliana* plants infected with the foliar pathogen *Hyaloperonospora arabidopsidis* (Hpa) selectively promote the growth of three bacterial species in the rhizosphere. This recruitment boosts systemic resistance to Hpa, improves overall plant growth, and can even benefit subsequent plant generations by fostering a protective microbiome (Berendsen *et al*., 2018). In this way, the plant microbiota has also often been considered as an additional layer of the immune system against pathogens by both inducing immune responses and directly antagonizing pathogens (Mendes *et al*., 2011; Carrión *et al*., 2019; Du *et al*., 2025).

While colonizing their hosts, plant pathogens secrete so-called effector molecules to promote host colonization by manipulating host physiology, including immunity (Jones and Dangl, 2006; Cook *et al*., 2015). Recently, several studies have demonstrated that pathogens exploit effector proteins that possess antimicrobial activity to manipulate the host microbiota, and thus facilitate colonization (Snelders *et al*., 2020; Snelders *et al*., 2021; Snelders *et al*., 2023; Chavarro-Carrero *et al*., 2024; Ökmen *et al*., 2023; Kettles *et al*., 2018; Chang *et al*., 2021; Gómez-Pérez *et al*., 2023; Mesny *et al*., 2024; Kraege *et al*., 2025). For example, the soil-borne fungal plant pathogen *Verticillium dahliae* exploits the antimicrobial effector protein Ave1 to suppress antagonistic Sphingomonadales bacteria during host colonization of tomato and cotton plants (Snelders *et al*., 2020). Interestingly, predictions from a machine learning tool suggest that 349 secreted *V. dahliae* effectors possess antimicrobial activity, indicating that *V. dahliae* may devote a substantial proportion of its secreted proteins to microbiota manipulation (Mesny and Thomma, 2024).

Fungal pathogens such as *V. dahliae* occupy a range of ecologically distinct niches throughout their life cycle (Fradin and Thomma, 2006; Guerreiro and Stukenbrock, 2025). While they infect host plants

during specific life stages, many also persist outside the host for extended periods, particularly in the soil (Fradin and Thomma, 2006; Katan, 2017). Soil microbial communities are generally more diverse than those associated with plants and vary substantially depending on the physicochemical properties of the soil (Fierer, 2017; Sokol *et al*., 2022). Accordingly, many pathogens are exposed to diverse microbial environments and must interact with a wide range of microbial taxa over time (Snelders *et al*., 2022). This is particularly relevant for broad host range pathogens like *V. dahliae*, which are adapted to numerous hosts and habitats and are thought to rely on antimicrobial effectors that facilitate interactions with different microbial communities (Trivedi *et al*., 2020; Snelders *et al*., 2022). Building on previous studies that explored antimicrobial effector functions using a single soil type (Snelders *et al*., 2020), we hypothesize that the virulence contribution of antimicrobial effectors like Ave1, as well as their impact on microbial communities, may vary depending on the host-associated microbiota, which is largely determined by the bulk soil microbial community.

Here we report the establishment of a collection of natural soils that are diverse in both physicochemical characteristics and microbiota composition. We use this resource to simultaneously assess the contributions of the diverse soil types and the plant genotype to plant microbiota assembly under controlled greenhouse conditions by analyzing fungal and bacterial communities associated with barley, cotton and tomato plants grown on each soil. Additionally, we utilize the soil collection to investigate the impact of the antimicrobial effector protein Ave1 on tomato microbiota composition and its role in *V. dahliae* virulence during infection of tomato plants harboring distinct microbiota.

Results

Composing a collection of diverse natural soil samples

To study microbiota assemblies and the role of antimicrobial effector proteins of fungal plant pathogens in diverse soils we composed a collection of natural soil samples. We collected our soil samples in the Netherlands given the well-documented soil types and the opportunity to sample a wide range of distinct soil types on a relatively short geographical distance (Hartemink and Sonneveld, 2013; Figure 1a). In total we collected samples from nine different natural soils which can be classified into the five major soil types: river clay, sea clay, sand, peat and loam (Suppl. Table 1). Sampling sites were selected to avoid agricultural usage. In order to eliminate weeds and the majority of roots, the top 10 cm of soil was removed and the subsequent 30 cm of soil was collected. Besides the nine Dutch soils, we included the well-characterized and intensively studied Cologne agricultural soil (Bulgarelli *et al*., 2012).

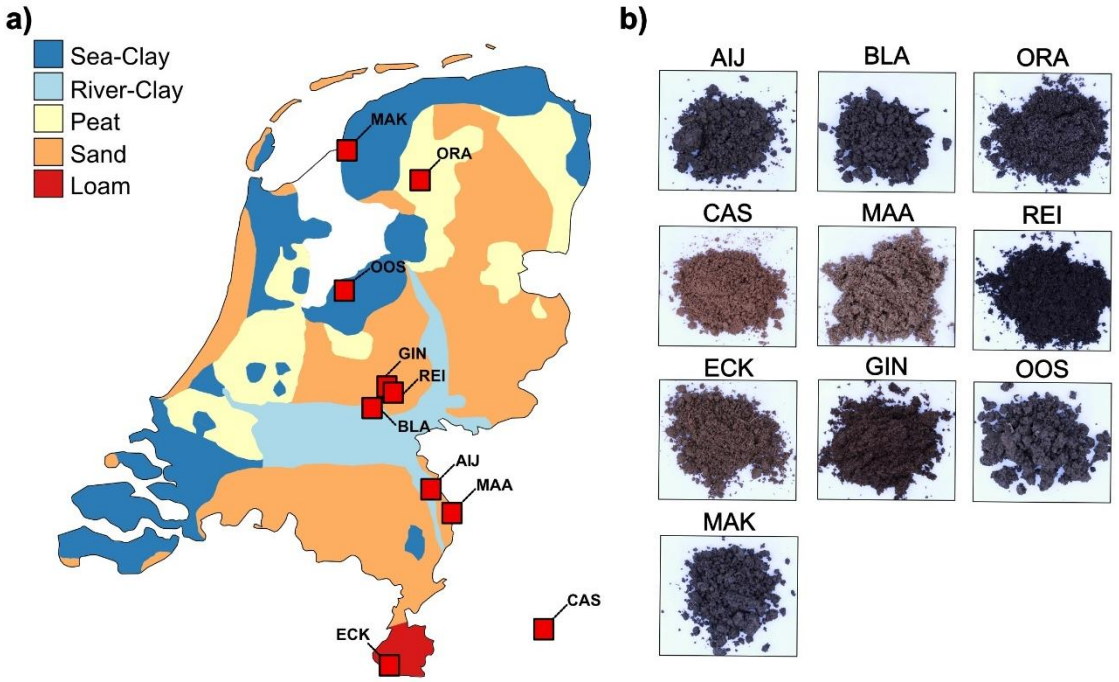

Figure 1. Establishment of a natural soil collection. a) Soil collection sites in the Netherlands. The map is colored according to major soil types in the Netherlands. Sampling locations are indicated by red squares. **b)** Pictures of each soil from the soil sample collection.

The diversity of our soil sample collection is apparent from visible differences in soil texture and appearance (Figure 1b). To determine differences in physicochemical properties of our soil samples, we measured pH, the amount of total organic carbon and nitrogen, as well as element levels for all soil samples. The sandy soils (sand, peat, loam) displayed relatively low pH values, between 4.0 and 5.6, while the clay soils (river clay, sea clay) displayed higher pH values ranging from 6.2 to 7.7 (Figure 2a). With respect to carbon content, particularly the two river clay soils collected in Aijen (AIJ) and Blauwe Kamer (BLA) stood out with the highest carbon content of 4,83% and 7,01% respectively. The lowest carbon content was measured for the Cologne agricultural soil (CAS) with 0,26% and the sand soil collected in Maasduinen (MAA) with 0,21% (Figure 2b). A similar pattern was observed for the nitrogen content, as the highest value was measured for the river clay BLA with 0,35%, while lowest values were again determined for MAA at 0,006% and CAS at 0,02% (Figure 2C). Further, we also performed a total element analysis by conducting a HNO_3-based element extraction followed by inductively coupled plasma mass spectrometry (ICP-MS) measurement. The elemental profiles of our soil samples were dominated by iron, calcium and aluminum (Figure 2d). Notably, when computing a principal component analysis (PCA) of the measured elemental profiles we observed separation according to soil type, as the clay soils separated from the sandy soils and the CAS-soil (Figure 2e).

Many of the physicochemical properties are known to influence soil microbiota composition (Fierer, 2017). To determine the bulk soil microbiota, we conducted 16S amplicon sequencing and analyzed

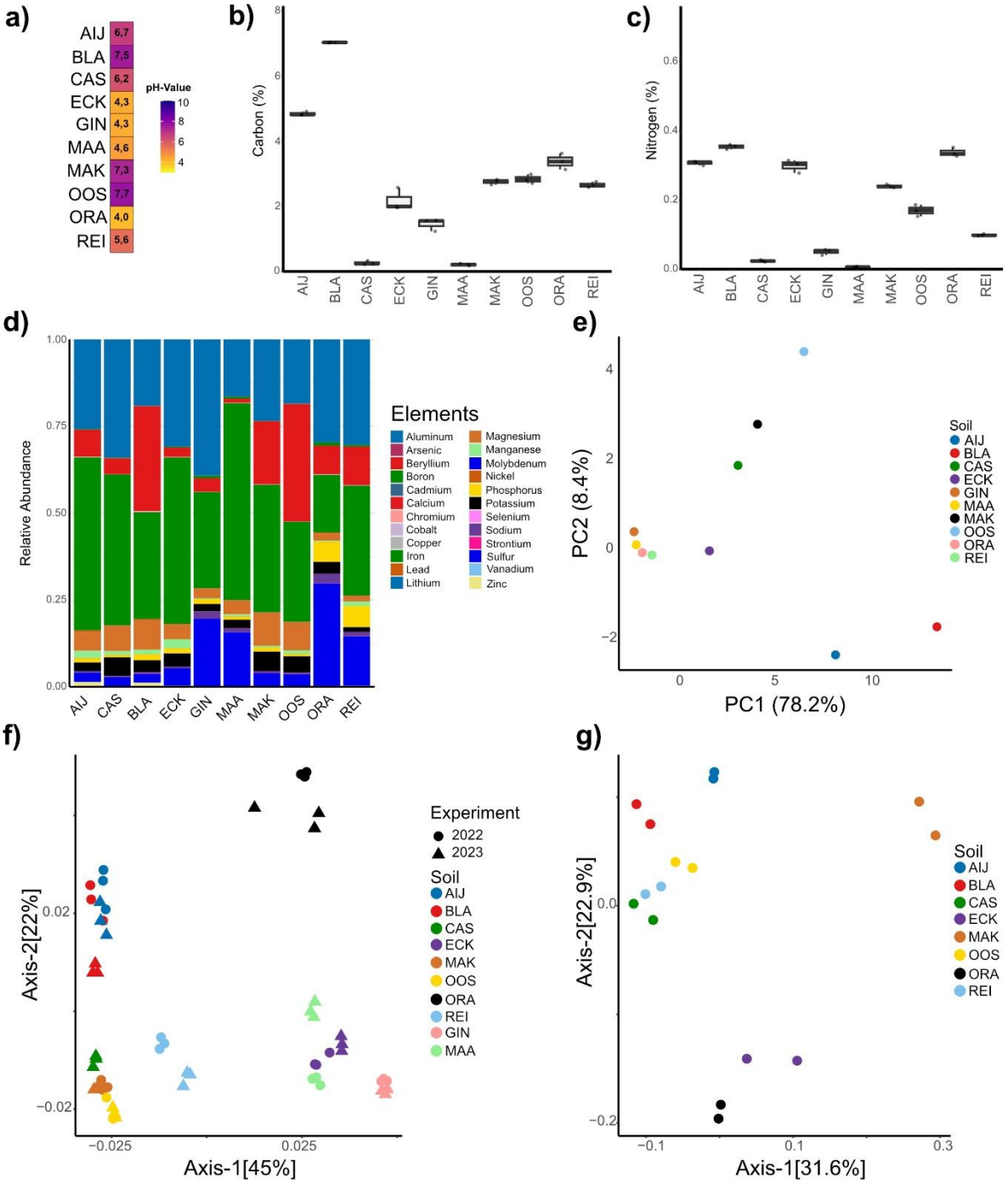

Figure 2. Physicochemical and microbiota analysis of the natural soil collection. a) Heatmap of pH-values. **b)** Boxplots displaying soil carbon contents. **c)** Boxplots displaying soil nitrogen contents. **d)** Relative abundance barplot for elements measured with ICP-MS. **e)** Principal component analysis (PCA) of the elemental profiles measured with ICP-MS. **f)** Principal coordinate analysis (PCoA) using weighted unifrac distances displaying bacterial bulk soil microbiota. Datapoints are shaped according to collection timepoint. **g)** PCoA using weighted unifrac distances displaying fungal bulk soil microbiota.

the β-diversity by computing a principal coordinate analysis (PCoA) using the weighted Unifrac distance. As expected, we observed separation of the microbiota according to the soil type. Notably, we observed that apart from Reijerscamp (REI) the sandy soils collected from de Ginkelse Heide (GIN), Maasduinen (MAA), Oranjewoud (ORA) and ECK separate from the clay soil samples.

To investigate the consistency of the bulk soil microbiota, we compared the bulk soil microbiota of soil samples that were collected in two consecutive years; 2022 and 2023. In the PCoA, soils collected in the different years clustered, demonstrating a high degree of stability of these natural bulk soil microbiota (Figure 2f). Collectively, our data characterize the diversity of our natural soil sample collection with respect to physicochemical properties and bulk soil microbiota.

Drivers of bacterial community assembly in roots and phyllosphere microbiota

Several studies have demonstrated that the soil as well as plant genetics influence plant microbiota assemblies (Bulgarelli *et al*., 2012; Lundberg *et al*., 2012; Wagner *et al*., 2016; Fitzpatrick *et al*., 2018; Walters *et al*., 2018; Thiergart *et al*., 2020; Simonin *et al*., 2020; Tkacz *et al*., 2020). These investigations typically involved plants collected from diverse natural environments, where microbiota assembly may additionally be affected by various abiotic factors, such as local climate and weather conditions, or they involve different plant species grown in the same soil at the same site (Ofek-Lalzar *et al*., 2014; Wagner *et al*., 2016; Walters *et al*., 2018). However, studies that simultaneously assess the contributions of different soils and of the plant genetics to microbiota assembly by examining diverse plant species grown in diverse natural soils while eliminating the impact of environmental factors remain scarce. Thus, we used our soil sample collection to investigate how plant-associated microbiota assemble across different plant species when grown under controlled conditions in a greenhouse. Specifically, we grew tomato (*Solanum lycopersicum*), cotton (*Gossypium hirsutum*), and barley (*Hordeum vulgare*) on the ten soils of our soil sample collection.

We first assessed how the diverse properties of the natural soils influence plant growth, by measuring plant canopy areas at three weeks after sowing. Cotton, tomato and barley plants grew on all soils except on the GIN and MAA soil samples, while tomato additionally failed to grow on ECK. Significant growth differences were observed across soils for each plant species. Generally, the highest plant growth was observed on clay soil. For cotton the highest plant growth was determined on the MAK soil samples with an average canopy area of 39,76 cm^2. Barley and tomato plants displayed highest plant growth on the BLA soil with barley plants reaching an average canopy area of 10,89 cm^2 and tomato 22,23 cm^2. Lowest plant growth for all three plants species was observed on the ORA soil, with average canopy areas of 23,44 cm^2 for cotton, 1,8 cm^2 for tomato plants and 1,42 cm^2 (Suppl. Figure 2). These results highlight the influence of the different soils on plant growth.

Next, we assessed the bacterial root and phyllosphere microbiota of the diverse plants grown on the soil collection by performing 16S rRNA sequencing. Bacterial communities in the root-associated microbiota were dominated by Proteobacteria, Actinobacteria, Acidobacteria, and Bacteroidetes

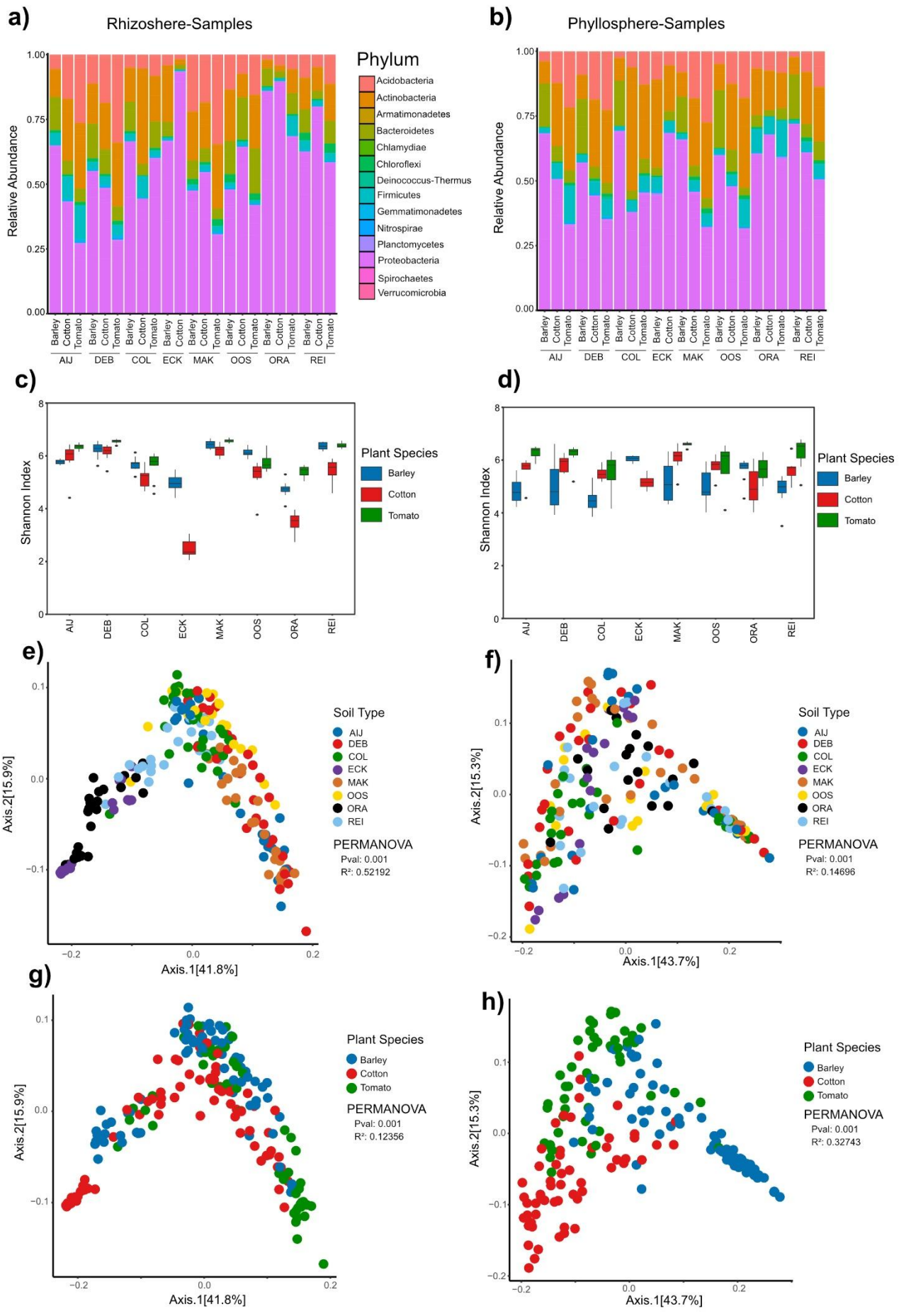
a)
Rhizoshere-Samples
Relative Abundance
Phylum
Acidobacteria
Actinobacteria
Armatimonadetes
Bacteroidetes
Chlamydiae
Chloroflexi
Deinococcus-Thermus
Firmicutes
Gemmatimonadetes
Nitrospirae
Planctomycetes
Proteobacteria
Spirochaetes
Verrucomicrobia
Barley
Cotton
Tomato
AIJ
DEB
COL
ECK
MAK
OOS
ORA
REI
b)
Phyllosphere-Samples
c)
Shannon Index
Plant Species
d)
e)
Soil Type
PERMANOVA
Pval: 0.001
R²: 0.52192
Axis.1[41.8%]
Axis.2[15.9%]
f)
Pval: 0.001
R²: 0.14696
Axis.1[43.7%]
Axis.2[15.3%]
g)
Pval: 0.001
R²: 0.12356
h)
Pval: 0.001
R²: 0.32743

Figure 3. Bacterial composition of root and phyllosphere associated microbiota of barley, cotton and tomato plants grown on the different natural soils. a) Relative abundance in percentage on phylum level of the bacterial root microbiota. **b)** Relative abundance in percentage on phylum level of the bacterial phyllosphere microbiota. **c)** Shannon index of root microbiota. **d)** Shannon index of phyllosphere microbiota. **e)** Principal coordinate analysis (PCoA) based on weighted Unifrac distance of root microbiota. **f)** PCoA based on weighted Unifrac distance of phyllosphere. **g)** PCoA based on weighted Unifrac distance of root microbiota. **h)** PCoA based on weighted Unifrac distance of phyllosphere microbiota. All PERMANOVAs are performed with 9999 permutations.

of the same species grown in the same soil, despite prior homogenization. This may result from heterogeneity that persists in the natural soils samples even after mixing (Suppl. Figure 3). Nevertheless, as expected, we observed strong differences in bacterial community composition between plant species grown on the same soils. For instance, on the river clay soil AIJ, over 50% of the bacterial community in the barley root microbiota consisted of Proteobacteria, compared to only 25% of Proteobacteria in the tomato root microbiota. Rather, the tomato root microbiota on AIJ harbored higher proportions of Acidobacteria and Actinobacteria (Figure 3a). To assess the diversity of the root-associated microbial communities, we investigated microbial alpha diversities by calculating the Shannon index for each bacterial community sample. Notably, Shannon indices for the root microbiota varied across plant species and soils, with no plant species consistently exhibiting higher or lower diversity compared to the other species across the soils (Figure 3c). The lowest Shannon index was measured for cotton plants grown on ECK (2,49), whereas the most diverse communities were assembled by tomato plants grown on MAK (6,57).

To further disentangle the contributions of soil type and plant species to microbial diversity in the root microbiota, we analyzed β-diversities by conducting Principal Coordinate Analyses (PCoAs) based on weighted UniFrac distances. In the root-associated microbiota, bacterial communities grouped primarily according to soil type, with sandy soils (ORA, ECK, REI) separating from clay soils (AIJ, BLA, OOS, MAK, CAS). The soil type accounted for 52,2% of the observed variation within the microbiota, suggesting a dominant contribution to shaping root-associated bacterial communities (Figure 3e). Although also plant species significantly contributed to root-associated microbiota differentiation, it explained only 12,4% of the variation (Figure 3 g; Suppl. 4b). Thus, root-associated microbiota are primarily structured according to soil type, and furthermore by plant species.

Next, we assessed if the patterns observed for root microbiota similarly hold true for phyllosphere microbiota. Similar to root microbiota, phyllosphere microbiota were dominated by Proteobacteria, Actinobacteria, Acidobacteria, and Bacteroidetes across all soils and plant species (Figure 3b). Also, for the phyllosphere microbiota we observed considerably variation between individual plants of the same species when grown in the same soil (Suppl. Figure 3). Notably, we observed strong differences between phyllosphere microbiota of different plant species grown in the same soil. Interestingly,

these differences were similar across soils. For example, the tomato phyllosphere microbiota consistently exhibiting the lowest levels of Acidobacteria, followed by cotton and then barley in seven of the eight soils tested, with ECK as exception (Figure 3b). Next, we assessed community diversity in the phyllosphere microbiota by calculating Shannon indices. In the phyllosphere, the lowest Shannon indices were determined for barley plants grown on CAS (4,51) and AIJ (4,84), whereas highest values were again observed for tomato plants grown on MAK (6,59) and REI (6,27). Notably, the alpha diversity of bacterial phyllosphere microbiota displayed a more structured pattern when compared with the alpha diversity in the root microbiota, as barley consistently exhibited the lowest alpha diversity across six out of the eight soil samples, followed by cotton and then tomato (Figure 3d). This suggests that the plant species has a more pronounced influence on community diversity in the phyllosphere microbiota when compared with root-associated microbiota. We also analyzed β-diversities by conducting Principal Coordinate Analyses (PCoAs) based on the weighted UniFrac distances of the phyllosphere microbiota. Like the root-associated microbiota, the phyllosphere microbiota exhibited significant separation based on the soil type, albeit that this explained substantially less variation (14,7%). Rather, plant species was the strongest determinant of the phyllosphere community composition, accounting for approximately 32,7% of the observed variation (Figure 3f, Suppl. Figure 3).

Collectively, our findings indicate that the soil is the strongest driver of bacterial microbiota diversity in the root microbiota, while plant species plays a more significant role in shaping bacterial phyllosphere communities.

Drivers of fungal community assembly in root-associated and phyllosphere microbiota

To assess whether patterns observed for bacterial microbiota across plant species grown on our soil collection also apply to the fungal component of the microbiota, we conducted ITS sequencing. First, we examined the fungal communities in the bulk soil microbiota of the eight soil samples used for the plant microbiota assembly study. This analysis revealed that the sand-like soils ECK and ORA separate from the clay soil. The REI soil, although also a sandy-soil, grouped with the clays. This indicates that the soil sample collection harbors distinct fungal communities (Figure 2g).

Analysis of the fungal communities in the root-associated microbiota revealed that fungal communities across plant species and soil types were dominated by fungal species from the phyla Ascomycota, with Basidiomycota and Mortierellomycota (Figure 4a). Notably, the fungal composition of the root microbiota is also influenced by plant species across soil samples. For instance, on ECK, the fungal communities in the barley root microbiota contained more than 80% Ascomycota, while the fungal

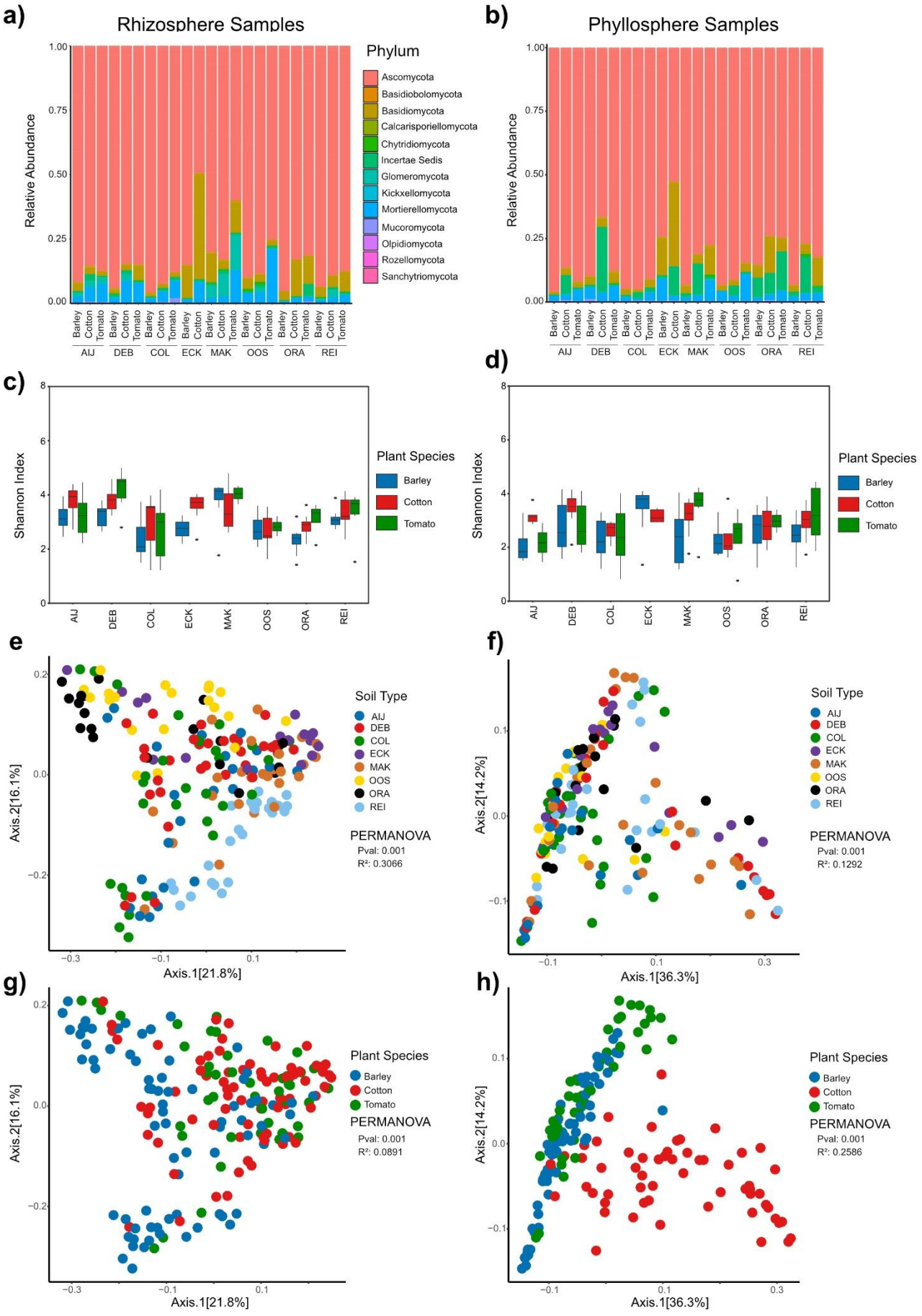
a)
Rhizosphere Samples
Relative Abundance
Phylum
Ascomycota
Basidiobolomycota
Basidiomycota
Calcarisporiellomycota
Chytridiomycota
Incertae Sedis
Glomeromycota
Kickxellomycota
Mortierellomycota
Mucoromycota
Olpidiomycota
Rozellomycota
Sanchytriomycota
AIJ DEB COL ECK MAK OOS ORA REI
b)
Phyllosphere Samples
c)
Shannon Index
Plant Species
Barley
Cotton
Tomato
d)
e)
Soil Type
AIJ
DEB
COL
ECK
MAK
OOS
ORA
REI
PERMANOVA
Pval: 0.001
R²: 0.3066
Axis.1[21.8%]
Axis.2[16.1%]
f)
PERMANOVA
Pval: 0.001
R²: 0.1292
Axis.1[36.3%]
Axis.2[14.2%]
g)
PERMANOVA
Pval: 0.001
R²: 0.0891
h)
PERMANOVA
Pval: 0.001
R²: 0.2586

Figure 4. Composition of the fungal root and phyllosphere associated microbiota of barley, cotton and tomato plants grown on the different natural soils. a) Relative abundance in percentage on phylum level of the fungal root microbiota **b)** Relative abundance in percentage on phylum level of the fungal phyllosphere microbiota **c)** Shannon index of root microbiota. **d)** Shannon index of phyllosphere microbiota. **e)** Principal coordinates analysis based on weighted Unifrac distance of root microbiota. **f)** PCoA based on weighted Unifrac distance of phyllosphere **g)** PCoA based on weighted Unifrac distance of root microbiota **h)** PCoA based on weighted Unifrac distance of phyllosphere microbiota All PERMANOVAs are performed with 9,999 permutations.

abundance of Basidiomycota (Figure 4a; Suppl. Figure 6). Shannon index calculations revealed lower alpha diversities of the root-associated fungal communities when compared with bacterial communities, with no consistent patterns of alpha diversity based on the plant species emerging across soil samples. Analysis of the β-diversity by performing a PCoA using the weighted Unifrac distance matrix revealed that root-associated fungal communities separate based on the soil sample in which the plants were grown, explaining 31% of the variation observed in the fungal microbiota (Figure 4e). Root-associated fungal communities also displayed weak separation according to plant species, which explained 9% of the variation (Figure 4g; Suppl. Figure 6b). Overall, these findings suggest that fungal communities in the root-associated microbiota are primarily shaped by soil type. As expected, also in the phyllosphere microbiota the fungal communities were dominated by Ascomycetes, followed by Basidiomycetes and Mortierellomycetes (Figure 4b; Suppl. Figure 5). Similar as for the alpha diversity in the root-associated fungal microbiome we did not observe any alpha diversity patterns based on plant species or soil type in the fungal phyllosphere microbiota (Figure 4d). The β-diversity analysis of the fungal community in the phyllosphere microbiota revealed weak separation based on soil type, which explained 13% of the variation (Figure 4f). Notably, similar as for the bacterial phyllosphere microbiota, we observed strong separation of the fungal phyllosphere community based on plant species, which explained 26% of the variation (Figure 4h; Suppl. Figure 5b). Collectively, our dataset reveals that fungal communities in the root-associated microbiota are more strongly influenced by soil type than by plant species, while the plant species acts as the primary driving factor for fungal communities in the phyllosphere microbiota.

Differential contribution of antimicrobial effectors to fungal virulence across soil types

The plant microbiota plays an important role in plant health, fitness and defense against plant pathogens (Trivedi *et al*., 2020). To colonize their hosts, plant pathogens have evolved antimicrobial effector proteins to manipulate host-associated microbiota (Mesny *et al*., 2024). For instance, *V. dahliae* uses the antimicrobial effector Ave1 to suppress antagonistic microbes during host colonization. Ave1 was demonstrated to facilitate host colonization of cotton and tomato plants grown in potting soil through targeting, amongst others, antagonistic Sphingomonadales bacteria (Snelders *et al*., 2020). As a globally distributed soil-borne pathogen with a broad host range, *Verticillium dahliae* successfully colonizes host plants across diverse soil types, which likely harbor distinct microbial

communities (Klimes *et al*., 2015; Singh *et al*., 2025). We hypothesized that the outcome of effector-mediated microbiota manipulation may vary depending on the host-associated microbiota, which is largely assembled from the surrounding bulk soil microbiota. To address this, we assessed the virulence contribution of the antimicrobial effector Ave1 by growing tomato plants on our soil collection and inoculating them with either wild-type *V. dahliae* or an *Ave1* deletion mutant (de Jonge *et al*., 2012; Snelders *et al*., 2020). We observed a significant reduction in biomass of tomato plants inoculated with the wild type strain when compared with plants inoculated with the *Ave1* deletion strain on AIJ, BLA, ORA and MAK, indicating that Ave1 contributes to fungal virulence on these soils. In contrast, no such difference was observed for plants grown in OOS and REI, suggesting that Ave1 differentially contributes to fungal virulence across soils (Figure 5a). Previous work demonstrated that Ave1 also negatively impacts the abundance of other taxa, including Verrucomicrobiales, Chitinophagaceae, Flavobacteriales and Burkholderiales during infections of cotton and tomato plants grown on potting soil (Snelders *et al*., 2020). We then asked whether variation in the abundance of these bacteria in the root-associated microbiota of tomato plants could explain the differences in virulence contribution of Ave1 across soils. To test this, we measured their relative abundance in tomato plants grown in the different natural soil samples. Of the tested taxa, Sphingomonadales, Flavobacteriales, and Burkholderiales showed no significant differences in relative abundances across soils. While significant variation in relative abundance was observed for the Verrucomicrobiales and Chitinophagaceae on several soils, these differences did not correlate with the observed Ave1-related virulence phenotype (Suppl. Figure 7a).

To assess the impact of Ave1 on the tomato root-associated microbiota we investigated the microbiota composition of tomato plants that were mock-inoculated, or inoculated with *V. dahliae* strain JR2 or the *Ave1* deletion mutant. By computing a PCoA based on UniFrac distances we observed that the tomato microbiota from plants inoculated with the wild type and the deletion mutant consistently separated across all soils, except for the ORA soil. Notably, we also observe such separation in the microbiota of plants grown on REI and OOS , even though we did not detect a virulence contribution of Ave1.

Further, to investigate the bacterial taxa affected by Ave1 on the natural soils we conducted differential abundance analysis between the microbiota of plants inoculated with *V. dahliae* strain JR2 or the *Ave1*-deletion mutant. This analysis revealed significant shifts in microbiota composition at the genus level across all soil samples, including OOS and REI, even though no virulence contribution of Ave1 was observed on these soils. Notably, on each of the soils the effector causes distinct shifts in the microbiota (Suppl. Figure 7b). Collectively, our data indicates that the outcome of effector-mediated

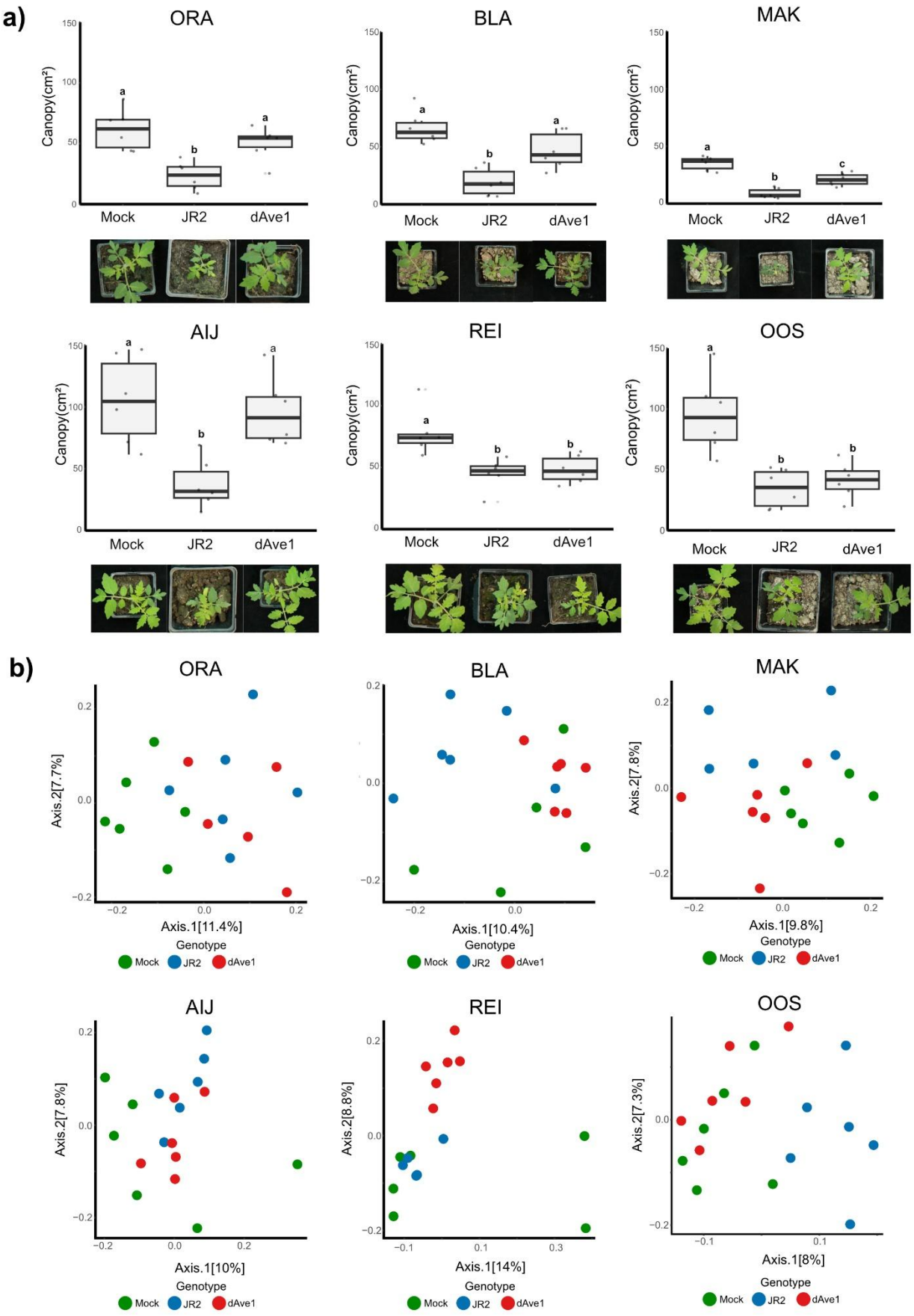
a)
ORA
BLA
MAK
AIJ
REI
OOS
Canopy(cm²)
Mock
JR2
dAve1
b)
Axis.1[11.4%]
Axis.2[7.7%]
Axis.1[10.4%]
Axis.2[7.8%]
Axis.1[9.8%]
Axis.2[7.8%]
Axis.1[10%]
Axis.2[7.8%]
Axis.1[14%]
Axis.2[8.8%]
Axis.1[8%]
Axis.2[7.3%]
Genotype

Figure 5. Antimicrobial effector Ave1 differentially contributes to virulence of *Verticillium dahliae* depending on the soil. **a)** Canopy area in cm2 of tomato plants grown on the different natural soils at 14 dpi with wild-type *V. dahliae* (JR2) or an *Ave1* deletion mutant (dAve1). Different letters indicate statistical differences based on One-Way-Anova (Tukey HSD-Test pval < 0.05). Pictures display a representative plant per treatment. **b)** Principal coordinate analysis (PCoA) based on Unifrac distances of the root microbiota of tomato plants grown on different soils at 14 dpi with wild-type *V. dahliae* (JR2) or an *Ave1* deletion mutant (dAve1).

microbiota which, in turn, is influenced by the surrounding soil.

Discussion

Plant microbiota contribute substantially to plant productivity, in part by serving as an additional barrier against invading pathogens (Mesny *et al*., 2024; Du *et al*., 2025). Over recent years, it has become evident that plant pathogens manipulate host microbiota through the secretion of antimicrobial effector proteins in turn, thus facilitating niche establishment and host colonization (Snelders *et al*., 2020; Chavarro-Carrero *et al*., 2024; Snelders *et al*., 2021; Snelders *et al*., 2023; Ökmen *et al*., 2023; Kettles *et al*., 2018; Chang *et al*., 2021; Gómez-Pérez *et al*., 2023; Mesny *et al*., 2024; Kraege *et al*., 2025). Notably, many pathogens spend parts of their life cycles outside their hosts, where they encounter diverse microbial communities. However, how antimicrobial effectors aid fungal establishment across these diverse environments is still poorly understood. Here, we present a collection of natural soils that we thoroughly characterized in terms of their physicochemical properties as well as their microbiota compositions. Using this soil collection, we reveal that the antimicrobial effector protein Ave1 from soil-borne fungal plant pathogen *Verticillium dahliae,* which was previously demonstrated to facilitate host colonization through the suppression of antagonistic Sphingomonadales bacteria (Snelders *et al*., 2020)*,* contributes to fungal virulence on tomato plants only in a subset of these soils. Our finding suggests that the virulence contribution of this effector is determined by the soil on which the host plant grows. Interestingly, differential virulence contributions have similarly been reported for another antimicrobial effector from *V. dahliae*, called Av2. While initially no contribution to fungal virulence was recorded (Chavarro-Carrero *et al*., 2021), a subsequent study using a different growth substrate, likely with a distinct microbiota, revealed that Av2 interfered with the host plant's 'cry for help' recruitment of beneficial *Pseudomonas* bacteria, leading to a clear virulence contribution of the effector (Kraege *et al*., 2025). These differences in virulence contributions of antimicrobial effectors are likely due to variation in soil microbiota, which impacts the composition of plant-associated microbial communities encountered by the pathogen during infection in turn. Interestingly, our microbiota analyses revealed that the Ave1 effector significantly altered the tomato microbiota on all tested soils. This implies that microbiota manipulation by the effector does not necessarily translate into measurable contributions to fungal virulence and thus, that this effector does not solely target antagonists of *V. dahliae* growth. We therefore infer that the presence or absence of

antagonistic microbes that can be impacted by an antimicrobial effector will determine whether that effector contributes to fungal virulence during host infection. This hypothesis is supported by observations made for the *V. dahliae* antimicrobial effector protein Ave1L2. A previous study investigating Ave1L2 demonstrated that in communities artificially depleted of antagonistic Actinobacteria, described as a crucial target of the effector, the protein still impacted community composition albeit without a measurable virulence contribution (Snelders *et al*., 2023).

Notably, the observed impact that Ave1 caused on the plant microbiota substantially differed across soils. Many antimicrobial effector proteins do not specifically act on a single antagonistic microbe, but rather act on multiple plant microbiota members, thus exerting broader, system-level impacts on microbial communities (Snelders *et al*., 2020; Snelders *et al*., 2021; Chavarro-Carrero *et al*., 2024; Kraege *et al*., 2025). Since plant microbiota function as networks of interdependent species (van der Heijden and Hartmann, 2016), changes affecting one member can cascade through the community. Thus, removal or suppression of particular microbes by fungal effectors may trigger cascading shifts in community structure and function due to these intermicrobial interactions. This interconnectedness implies that the effects of antimicrobial effector activity on the microbiota can vary substantially between microbial communities, driven by the unique web of intermicrobial interactions in each environment.

Our study additionally provides a controlled comparison of how both soil type and plant genotype influence microbiota assembly across different plant compartments. While previous studies have independently demonstrated that rhizosphere communities are primarily shaped by soil and phyllosphere communities by host genotype (Bulgarelli *et al*., 2012; Lundberg *et al*., 2012; Wagner *et al*., 2016; Fitzpatrick *et al*., 2018; Walters *et al*., 2018; Thiergart *et al*., 2020; Simonin *et al*., 2020; Tkacz *et al*., 2020) these insights were often derived from field studies conducted in divergent natural environments, where additional abiotic factors such as climate and weather may influence microbiota composition, or from experiments that varied either soil or plant species, but rarely both. Tkacz *et al*. (2020) assessed microbiota assembly across four plant species grown in two distinct soils and demonstrated that soil has a stronger influence than plant species on shaping rhizosphere microbiota. In our study, we extend these findings by using a different set of plant species and a broader collection of ten diverse, well-characterized soils, including the Cologne agricultural soil (Bulgarelli *et al*., 2012; Bai *et al*., 2015) and Reijerscamp soil (Berendsen *et al*., 2018; Poppeliers *et al*., 2024) under highly controlled greenhouse conditions. We not only confirm that soil type plays a dominant role in rhizosphere microbiota assembly, but also show simultaneously that, in contrast, phyllosphere communities are primarily shaped by plant species rather than soil type.

Taken together, our findings support the view that antimicrobial effector proteins are context-dependent components of fungal secretomes, rather than universally acting virulence factors with consistent effects across environments. Notably, a recent machine-learning analysis predicted that, for several fungi, at least one-third of effector proteins possess antimicrobial activity (Mesny and Thomma, 2024), suggesting that fungi may deploy large repertoires of such antimicrobial effectors to establish themselves in diverse environments. Deeper insight into their functions and the mechanisms underlying this environmental variability will not only advance our understanding of fungal niche adaptation but may also inform the development of more robust, microbiota-based disease control strategies for agriculture.

Materials & methods

Soil collection and storage

Natural soils were collected Three soil collections were performed, in January 2022, February 2023 and February 2024. at nine sites in the Netherlands: Makkum (53°05'09.8"N 5°26'20.3"E), Oranjewoud (52°57'11.7"N 5°57'45.6"E), Ginkelse Heide (52°02'10.7"N 5°43'38.9"E), Eckelrade (50°47'57.7"N 5°44'42.5"E) Maasduinen (51°28'34.3"N 6°11'34.9"E), Oostvaardersplassen (52°27'50.0"N 5°25'10.8"E), Reijerscamp (52°00'37.7"N 5°46'25.0"E), Blauwe Kamer (51°56'34.4"N 5°37'12.9"), Aijen (51°34'55.0"N 6°02'27.3"E)in three consecutive years in February from 2022-2024. For collection, the top 10 cm of soil was removed and the subsequent 30 cm of soil was collected. After collection, soil samples were homogenized and rocks and pieces of plant material were removed before the soil was stored in sealed buckets at 8℃ until further use. Further, Cologne agricultural soil (50°57'27.8"N 6°51'22.4"E; Bai *et al*., 2015) was included.

Physicochemical soil analysis

For physicochemical analysis, 50 g of each soil was freeze dried and ground to fine powder using a mortar and pestle. To measure soil pH, ground soil powder was suspended with 150 ml of distilled water and incubated for 1 hour. Subsequently the pH was measured using a pH-electrode (Meddler Toledo, Giessen, Germany). Carbon and nitrogen levels were measured using the FLASH2000 CHNS/O analyzer (Thermo Fisher Scientific, Waltham, USA). To measure elemental contents, 100 mg of soil powder was weighed into metal-free centrifugation tubes (VWR, Radnor, USA). Samples were the soaked in 500 µl of 30% nitric acid for 2 hours. Subsequently, the volume was adjusted to 1 ml with 30% nitric acid and the sample was incubated for 14 hours at 65℃. Next, the suspension was incubated at 95℃ for 90 minutes. Samples were cooled to room temperature and 200 µl of hydrogen peroxide were added. Subsequently, the samples were incubated at 95℃ for 30 minutes. Next, the samples

were diluted to 10 ml using MQ-water and centrifuged at 13,000 rpm for 1 hour at 4℃. The supernatant was transferred to a clean metal-free 50 ml centrifugation tube and incubated at 4℃ overnight, followed by centrifugation at 13,000 rpm for 1 hour at 4℃. Finally, 600 µl of supernatant were mixed with 2,4 ml of 2% nitric acid. ICP-MS measurements were carried out on an Agilent 7700 ICP-MS (Agilent Technologies, Waldbronn, Germany) in the Biocenter MS-Platform of the University of Cologne. All measurements were performed in technical triplicates and strictly followed the manufacturer`s instructions using He in the collision cell mode to minimize spectral interference.

Plant growth assays

Tomato (*Solanum lycopersicum L.*) cultivar MoneyMaker, barley (*Hordeum vulgare*) cultivar GoldenPromise, Cotton (*Gossypium hirsutum*) cultivar DDHY642201-AC and Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 were used for all assays. Before sowing, seeds were surface-sterilized using chlorine gas generated by adding 3 mL of hydrochloric acid (HCl) to 100 mL of bleach (sodium hypochlorite) in a 250 mL beaker placed inside a glass container sealed with a lid and parafilm and incubated for 5 hours. After sterilization, the container was vented in a fume hood overnight. Subsequently, surface sterilized seeds were sown on soil and grown for three weeks in a greenhouse chamber with 16 hours of light at 23°C, followed by 8 hours in darkness at 22°C. Plant growth was assessed by calculating canopy areas, for tomato and cotton based on overhead pictures and for barley plants based on side pictures using ImageJ (Schneider *et al.*, 2012). Subsequently, plants were harvested for microbiota analysis. Tomato and cotton phyllosphere samples were collected by harvesting the stem from the soil-line to the cotyledons, while barley phyllosphere samples were collected by harvesting the first 5 cm of plant tissue above the soil line. To collect root microbiota samples, plants were uprooted and loose soil was removed from the root system through gentle shaking.

Microbiota sequencing

Samples were manually ground to fine powder using a mortar and pestle. Subsequently, 400 mg of tissue or soil were used for DNA extraction using the DNeasy PowerSoil Pro Kit (Qiagen, Venlo, The Netherlands). Next, DNA was further purified using the Monarch PCR&DNA Clean Up kit (New England Biolabs, Ipswich, USA). DNA purity and concentration were assessed using the Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, USA) and the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). DNA was used for the amplification of the variable regions 3-4 of the 16S region using primers 341f (ACTCCTACGGGAGGCAGCAG) and 806r (GGACTACHVGGGTWTCTAAT) in the presence of the mPNA (GGCAAGTGTTCTTCGGA) and pPNA (GGCTCAACCCTGGACAG) blocking clamps (PNABio, Newbury Park, USA). Additionally, amplification of the ITS2 region was conducted using the

primers ITS3 (GCATCGATGAAGAACGCAGC) and ITS4 (TCCTCCGCTTATTGATATGC) in the presence of the ITS2 PNA (CGAGGGCACGTCTGCCTGG) blocking clamp (PNABio, Newbury Park, USA). All amplicons were sequenced on an Illumina MiSeq Platform (BGI-Genomics, Shenzhen, China). For the bulk soil microbiota from the 2022 and 2023 collections, the V5-V7 regions were amplified with primers 799F (AACMGGATTAGATACCCKG) and 1139R (ACGTCATCCCCACCTTCC) and amplicons were similarly sequenced on an Illumina Miseq Platform (Cologne Center for Genomics, Cologne, Germany). Only samples with at least 10.000 reads were considered for the analysis. Data analysis was conducted as described previously (Callahan *et al*., 2016; Snelders *et al*., 2020).

***Verticillium* inoculation assays**

Verticillium dahliae inoculations were conducted on 10-day-old tomato plants. Inoculum was prepared by harvesting conidiospores of 10-day-old cultures of *V. dahliae* strain JR2 and an *Ave1* deletion mutant (de Jonge *et al*., 2012; Snelders *et al*., 2020) on potato dextrose agar (PDA; Carl Roth, Karlsruhe, Germany). The collected conidiospores were washed three times in MQ water, each time followed by centrifugation at 10.000 rpm for 10 minutes. Subsequently, the conidiospores were counted using a Neubauer chamber and the inoculum concentration was adjusted to 10^6 conidiospores/ml. For the inoculations, plants were uprooted and the roots were rinsed with MQ-water before being placed into the conidiospore suspension for 8 minutes. Subsequently, plants were planted back into the soil. Disease symptoms were monitored at 14 dpi by measuring the tomato canopy area based on overhead pictures using ImageJ (Schneider *et al*., 2012).

Author contributions

W.P., A.K., N.C.S and B.P.H.J.T. conceived the project. W.P., A.K., N.C.S and B.P.H.J.T. designed the experiments. S.H. provided biological materials. W.P., A.K., J.Z., S.M., M.B., N.S. and N.C.S. performed the experiments. W.P., A.K., N.C.S and B.P.H.J.T. analyzed the data. W.P., A.K. and B.P.H.J.T. wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements

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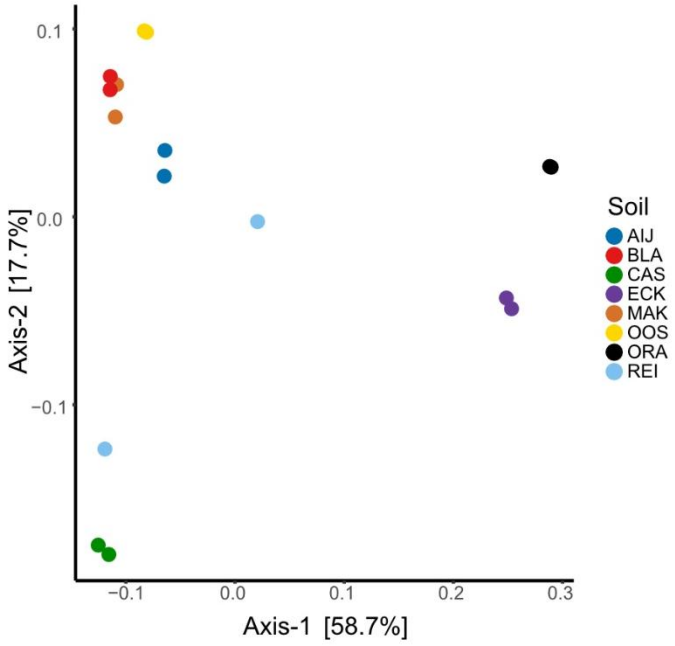
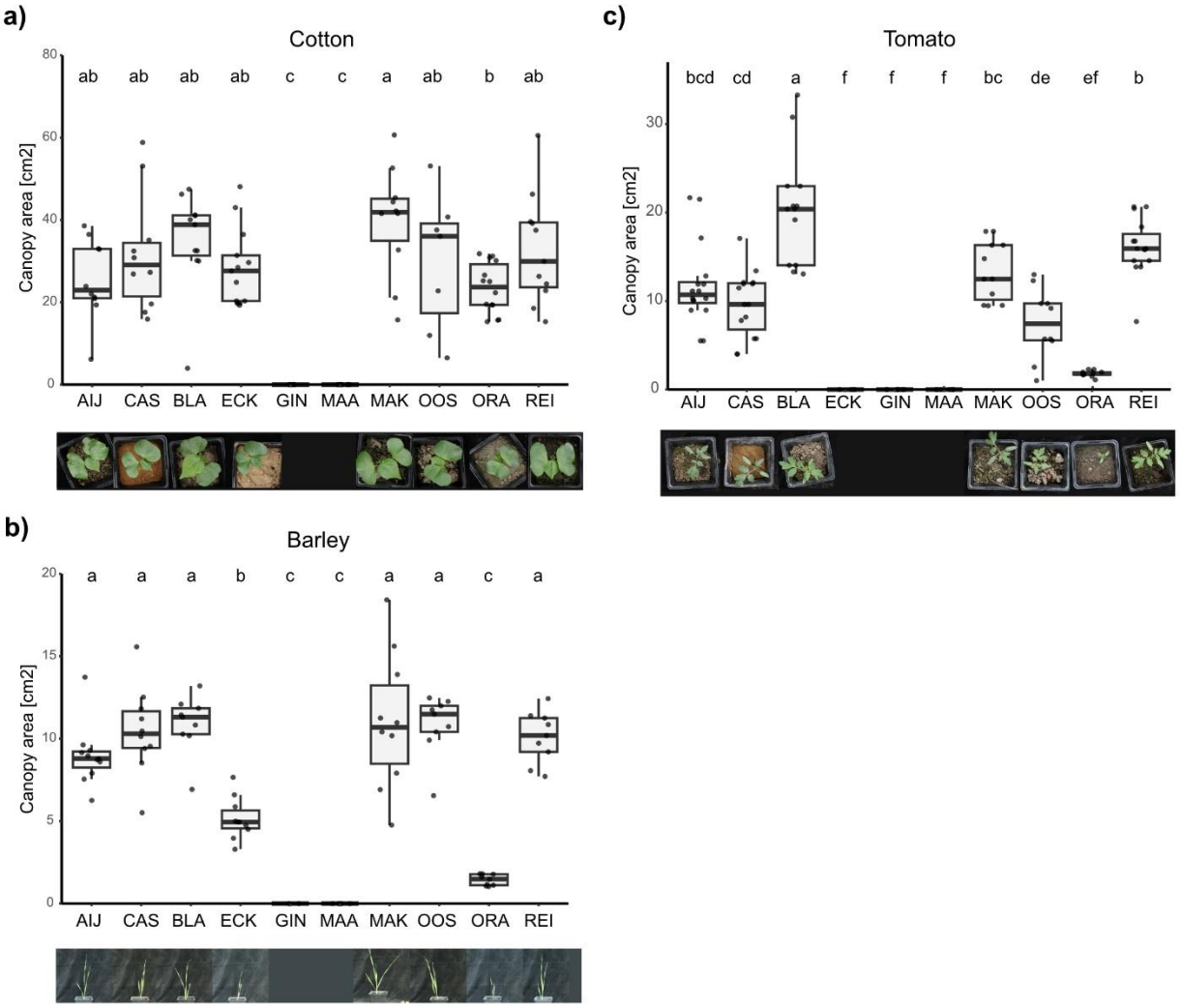
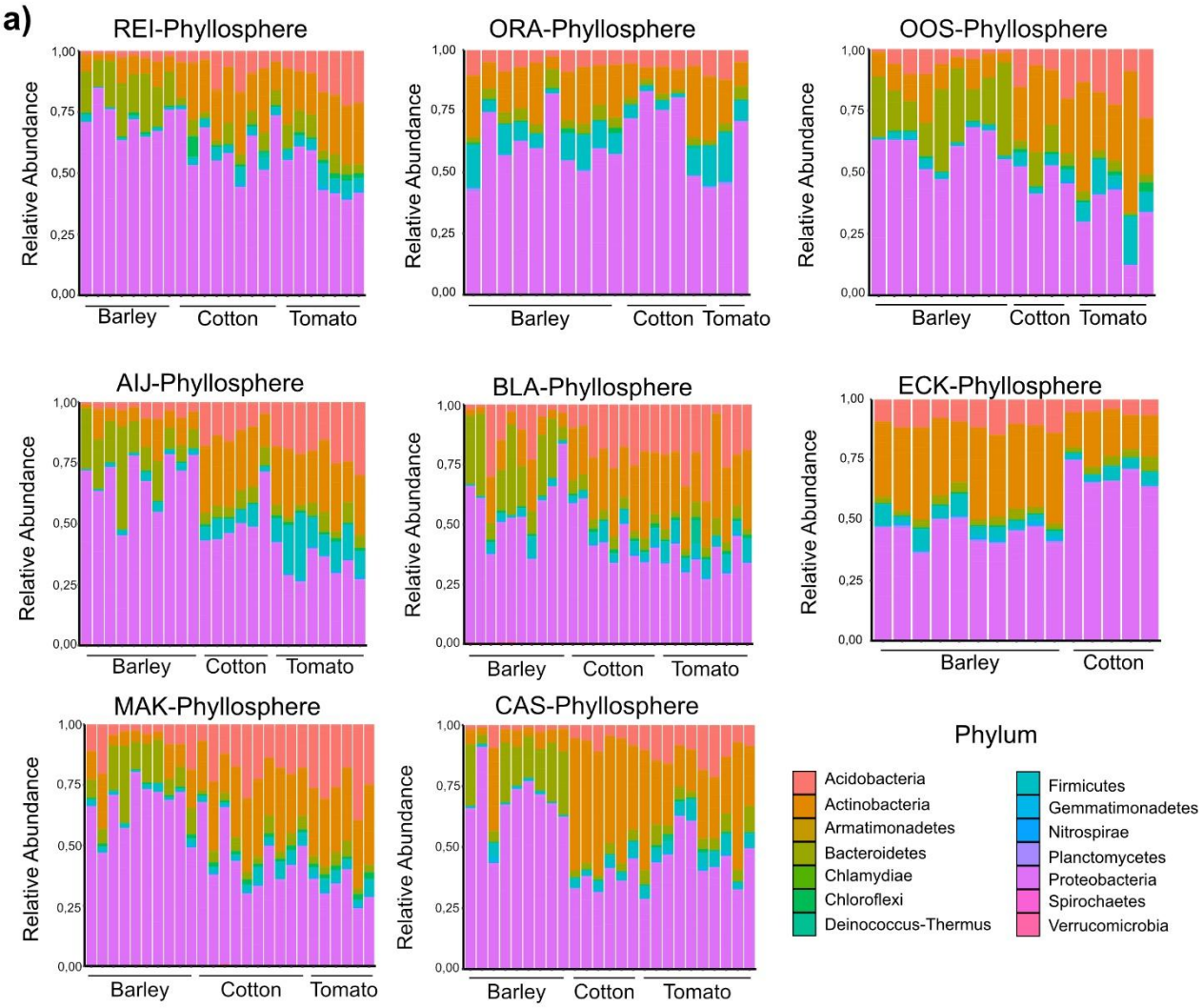
Supplementary Materials



Supplementary Figure 1. Principal coordinate analysis (PCoA) based on weighted unifrac distances of the bacterial bulk soil microbiota from soil samples collected in 2024.



Supplementary Figure 2. Plant growth on natural soils. a) Canopy area of cotton plants grown on natural soils for 21 says. **b)** Canopy area of tomato plants grown on natural soils for 21 says. **c)** Canopy area of barley plants grown on natural soils for 21 says. Different letters indicate statistical differences based on One-Way-Anova (Tukey HSD-Test pval < 0.05) for each panel.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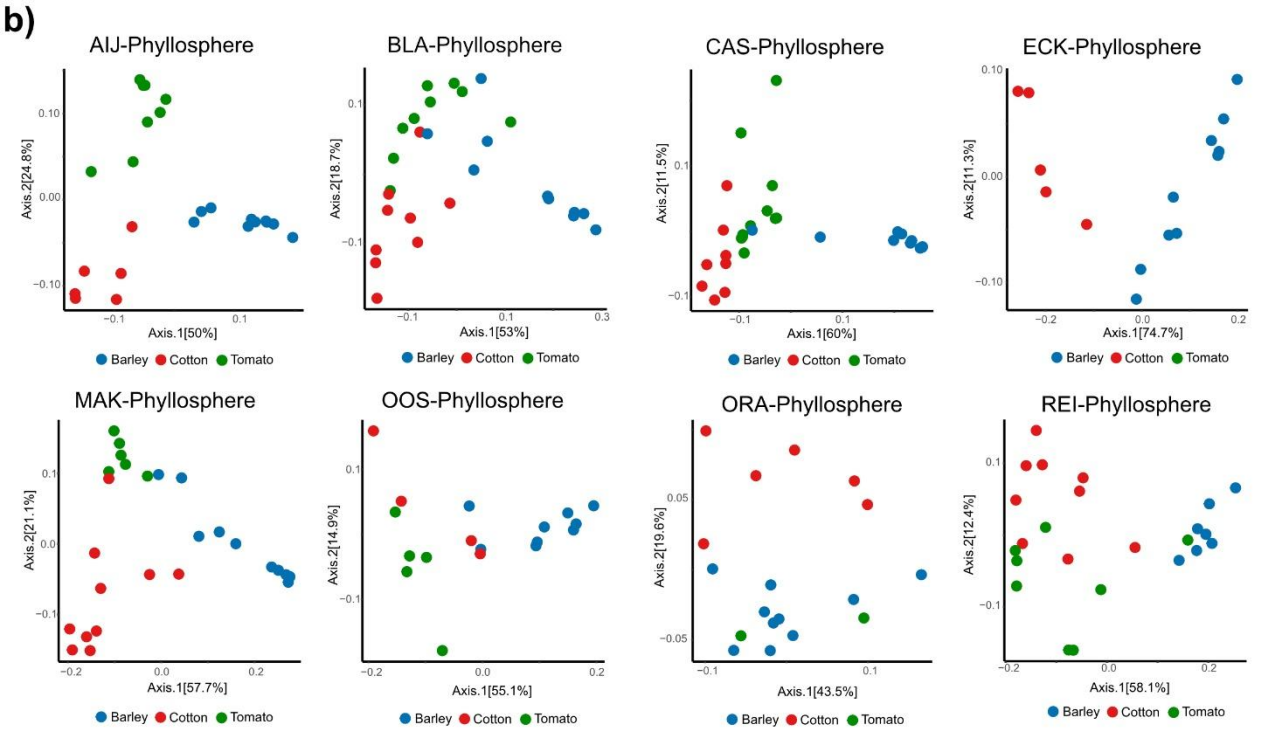
ORA-Phyllosphere
OOS-Phyllosphere
AIJ-Phyllosphere
BLA-Phyllosphere
ECK-Phyllosphere
MAK-Phyllosphere
CAS-Phyllosphere
Relative Abundance
1,00
0,75
0,50
0,25
0,00
Barley
Cotton
Tomato
Phylum
Acidobacteria
Actinobacteria
Armatimonadetes
Bacteroidetes
Chlamydiae
Chloroflexi
Deinococcus-Thermus
Firmicutes
Gemmatimonadetes
Nitrospirae
Planctomycetes
Proteobacteria
Spirochaetes
Verrucomicrobia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Axis.1[60%]
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MAK-Phyllosphere
Axis.2[21.1%]
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OOS-Phyllosphere
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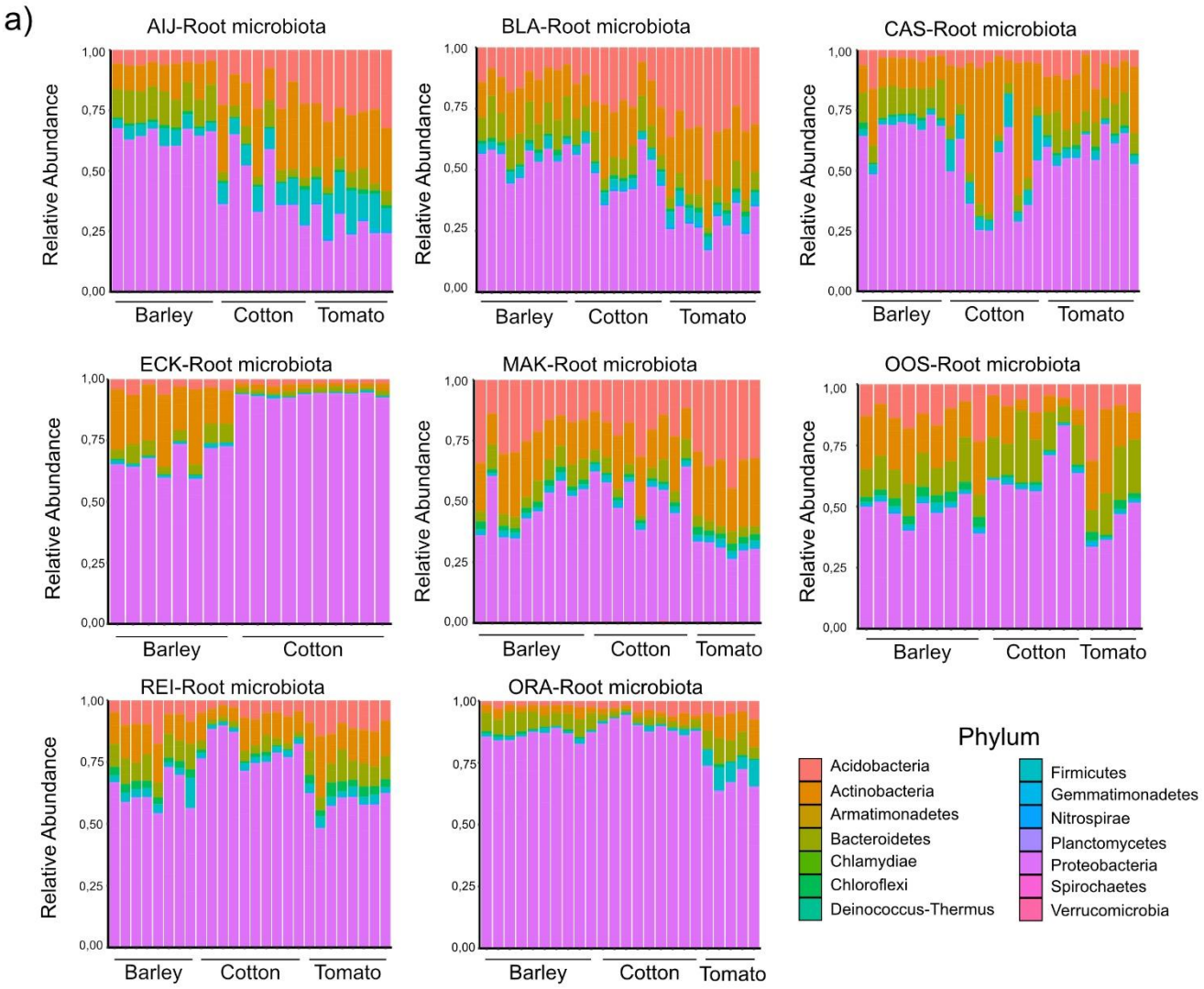
Axis.1[55.1%]
ORA-Phyllosphere
Axis.2[19.6%]
Axis.1[43.5%]
REI-Phyllosphere
Axis.2[12.4%]
Axis.1[58.1%]
Barley
Cotton
Tomato

Supplementary Figure 3. Bacterial phyllosphere microbiota. a) Relative abundance in percentage of the bacterial phyllosphere microbiota from barley, cotton and tomato plants grown for three weeks on different natural soils. **b)** Principal coordinate analysis (PCoA) based on weighted Unifrac distances of bacterial phyllosphere microbiota from barley, cotton and tomato plants grown on the different natural soils.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AIJ-Root microbiota
BLA-Root microbiota
CAS-Root microbiota
ECK-Root microbiota
MAK-Root microbiota
OOS-Root microbiota
REI-Root microbiota
ORA-Root microbiota
Relative Abundance
1,00
0,75
0,50
0,25
0,00
Barley
Cotton
Tomato
Phylum
Acidobacteria
Actinobacteria
Armatimonadetes
Bacteroidetes
Chlamydiae
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Nitrospirae
Planctomycetes
Proteobacteria
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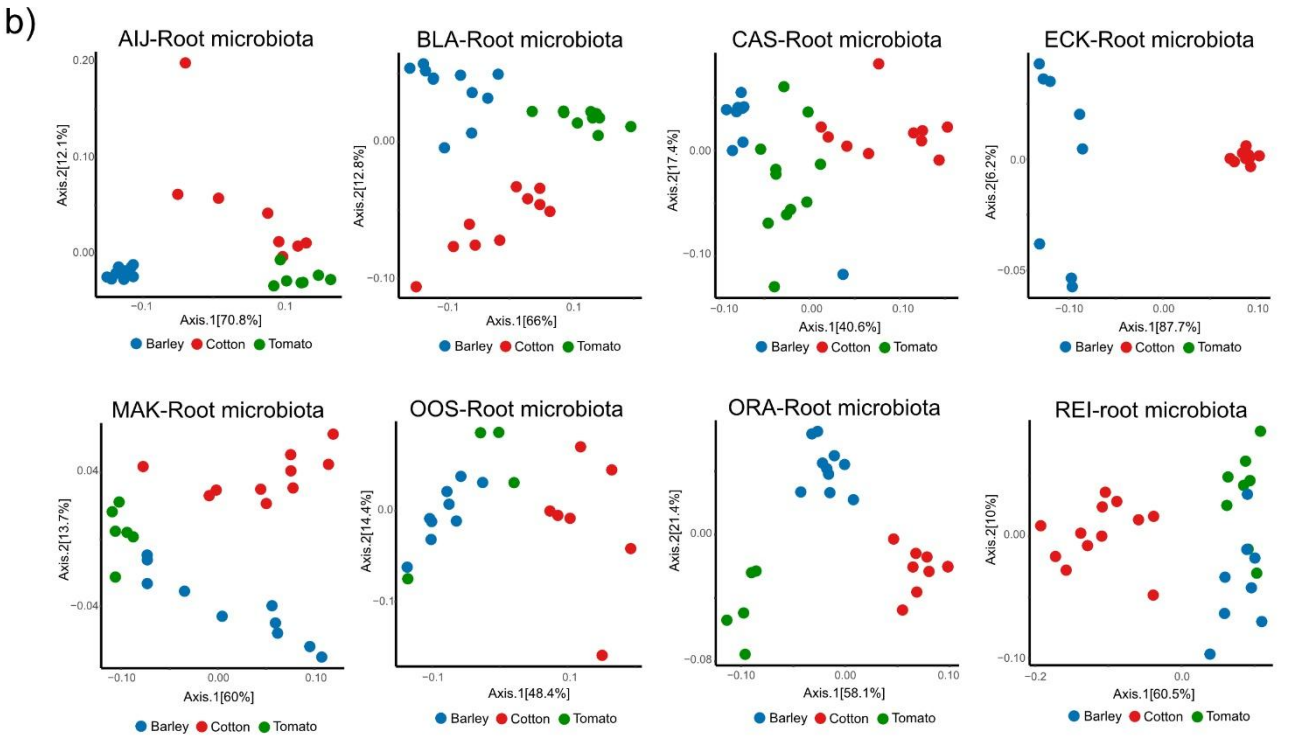
Verrucomicrobia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ORA-Root microbiota
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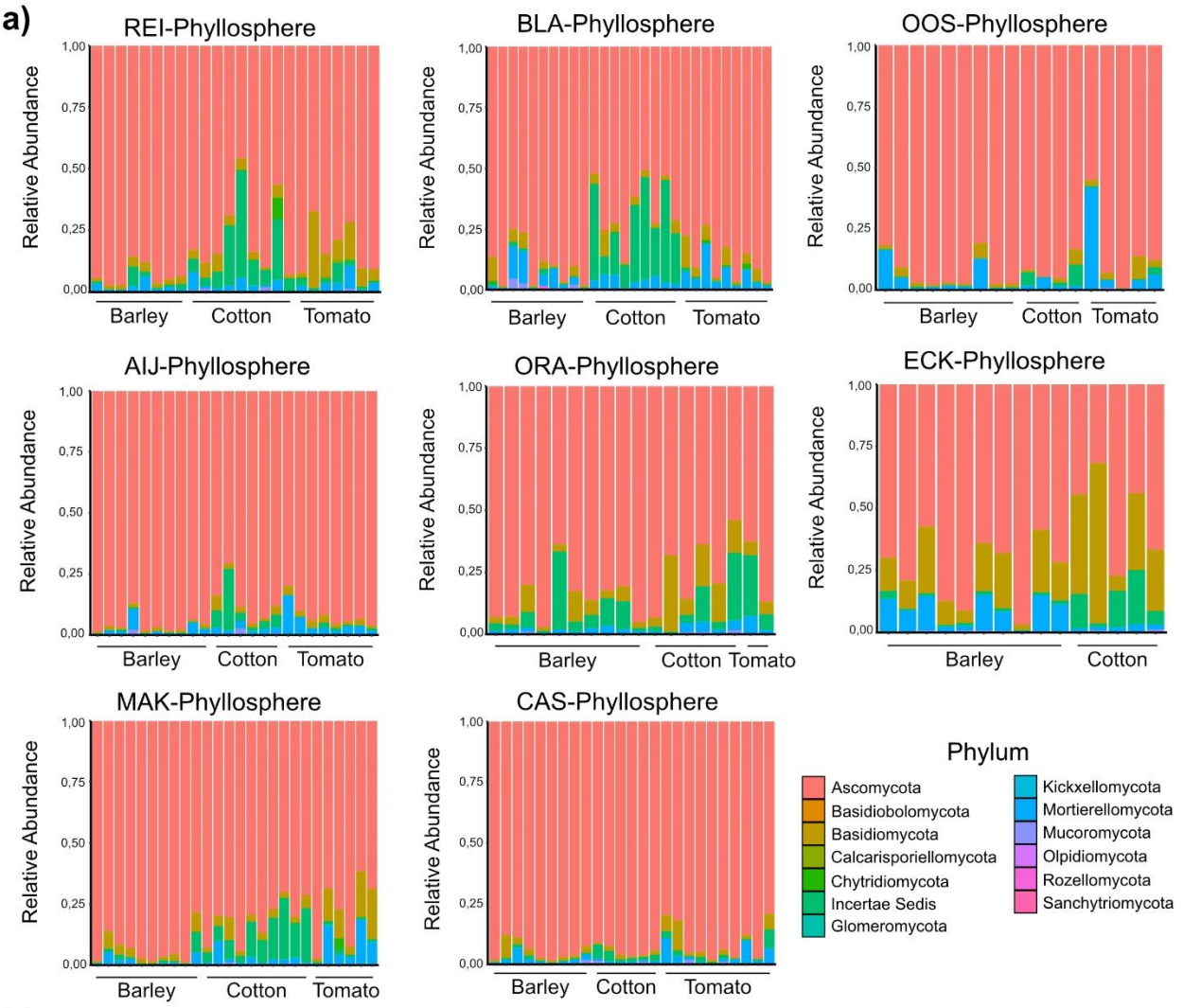
Axis.2[10%]
Barley
Cotton
Tomato

Supplementary Figure 4. Bacterial root microbiota. a) Relative abundance in percentage of the bacterial root microbiota of barley, cotton and tomato plants grown for three weeks on different natural soils. **b)** Principal coordinate analysis (PCoA) based on weighted Unifrac distances of the bacterial root microbiota from barley, cotton and tomato plants grown on the different natural soils.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BLA-Phyllosphere
OOS-Phyllosphere
AIJ-Phyllosphere
ORA-Phyllosphere
ECK-Phyllosphere
MAK-Phyllosphere
CAS-Phyllosphere
Relative Abundance
1,00
0,75
0,50
0,25
0,00
Barley
Cotton
Tomato
Phylum
Ascomycota
Basidiobolomycota
Basidiomycota
Calcarisporiellomycota
Chytridiomycota
Incertae Sedis
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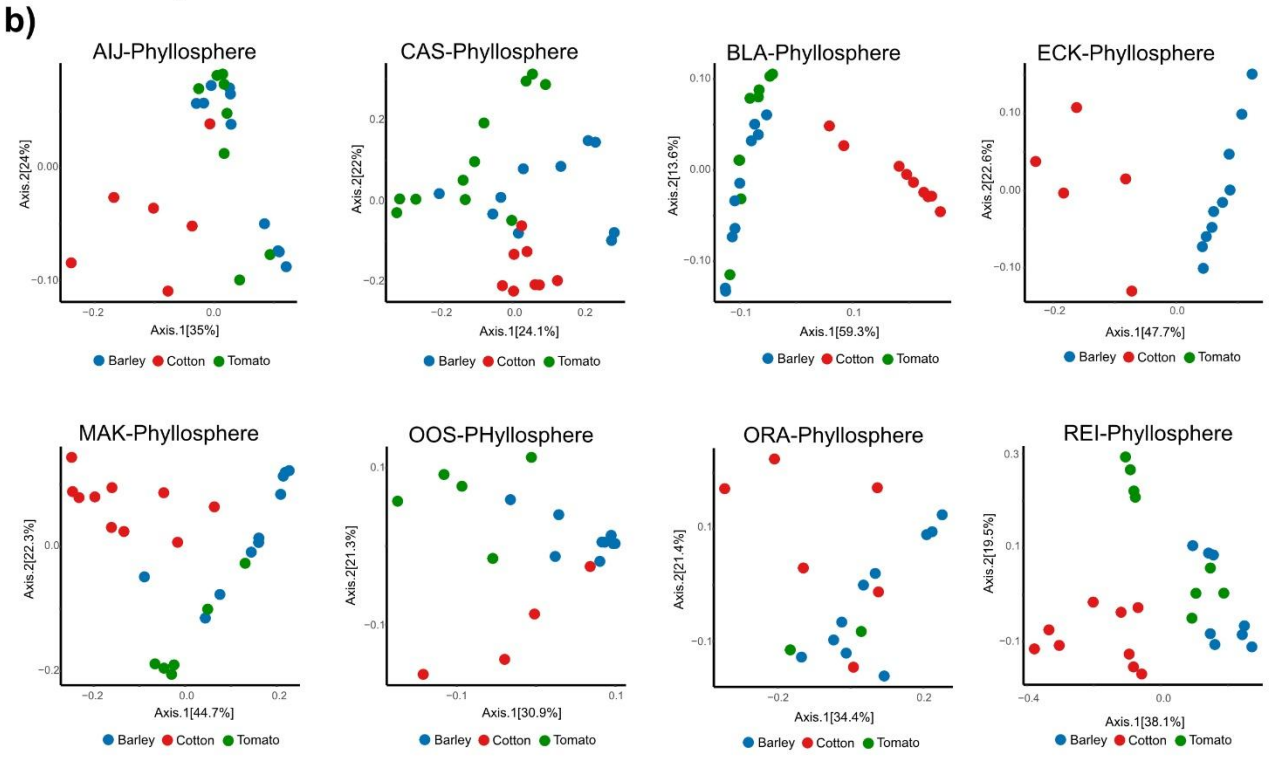
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OOS-PHyllosphere
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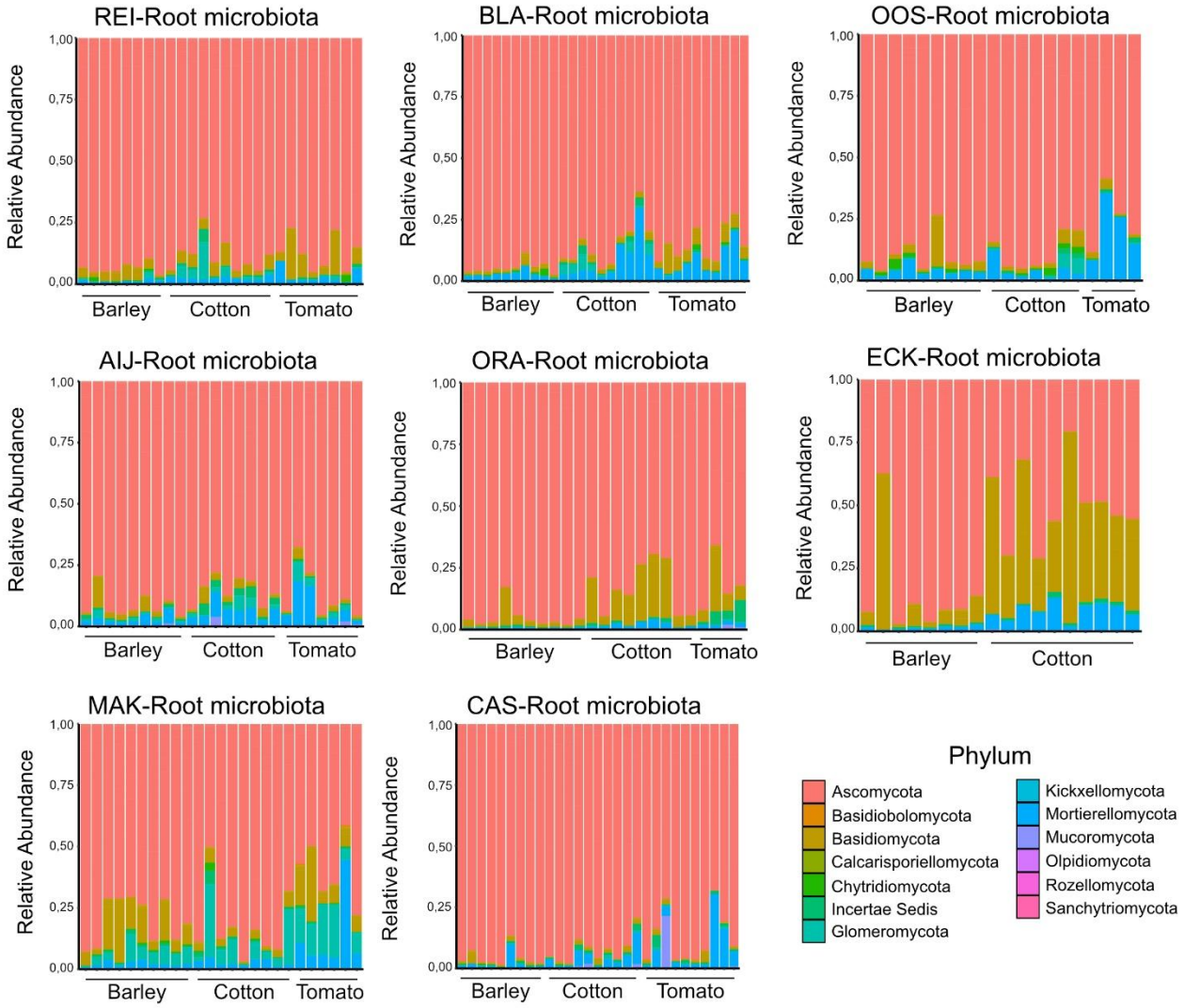
Axis.2[22.3%]
Axis.1[30.9%]
Axis.2[21.3%]
Axis.1[34.4%]
Axis.2[21.4%]
Axis.1[38.1%]
Axis.2[19.5%]
Barley
Cotton
Tomato

Supplementary Figure 5. Fungal phyllosphere microbiota. a) Relative abundance in percentage of the fungal phyllosphere microbiota from barley, cotton and tomato plants grown for three weeks on different natural soils. **b)** Principal coordinate analysis (PCoA) based on weighted Unifrac distances of fungal phyllosphere microbiota from barley, cotton and tomato plants grown on the different natural soils.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OOS-Root microbiota
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ORA-Root microbiota
ECK-Root microbiota
MAK-Root microbiota
CAS-Root microbiota
Relative Abundance
1,00
0,75
0,50
0,25
0,00
Barley
Cotton
Tomato
Phylum
Ascomycota
Basidiobolomycota
Basidiomycota
Calcarisporiellomycota
Chytridiomycota
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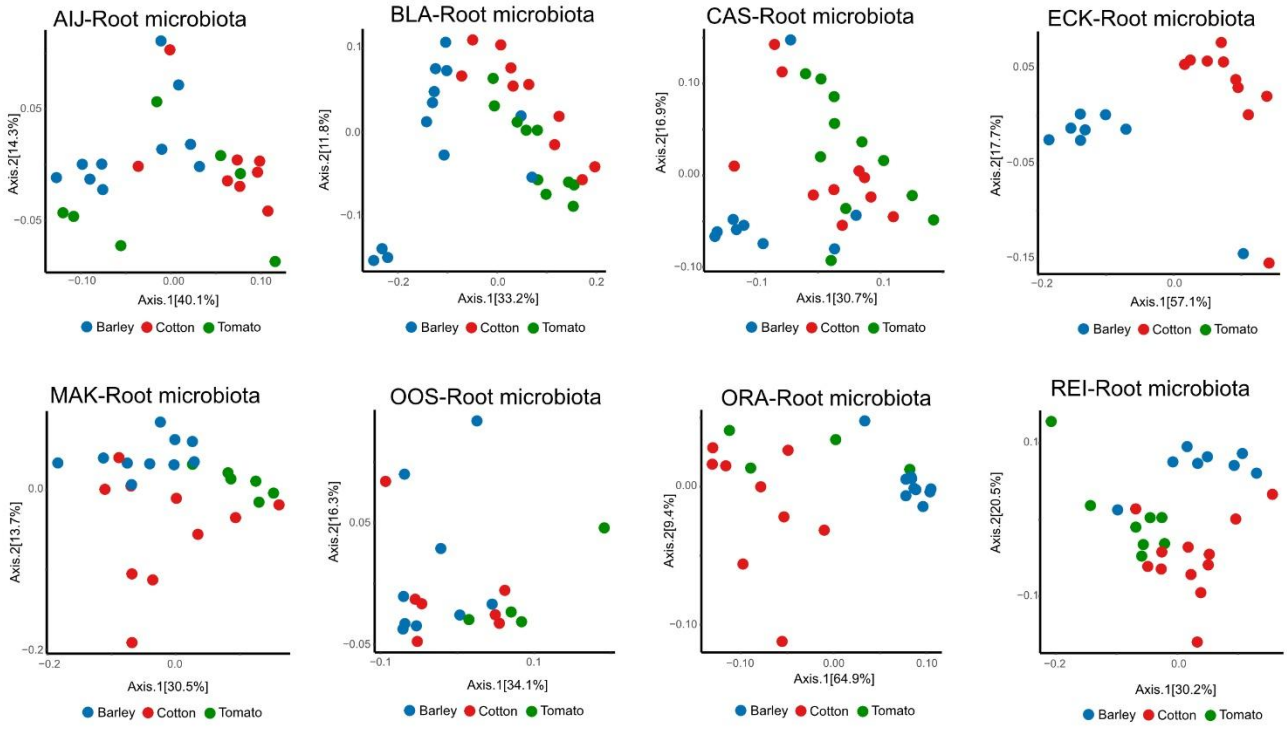
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Axis.1[40.1%]
BLA-Root microbiota
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CAS-Root microbiota
Axis.2[16.9%]
Axis.1[30.7%]
ECK-Root microbiota
Axis.2[17.7%]
Axis.1[57.1%]
MAK-Root microbiota
Axis.2[13.7%]
Axis.1[30.5%]
OOS-Root microbiota
Axis.2[16.3%]
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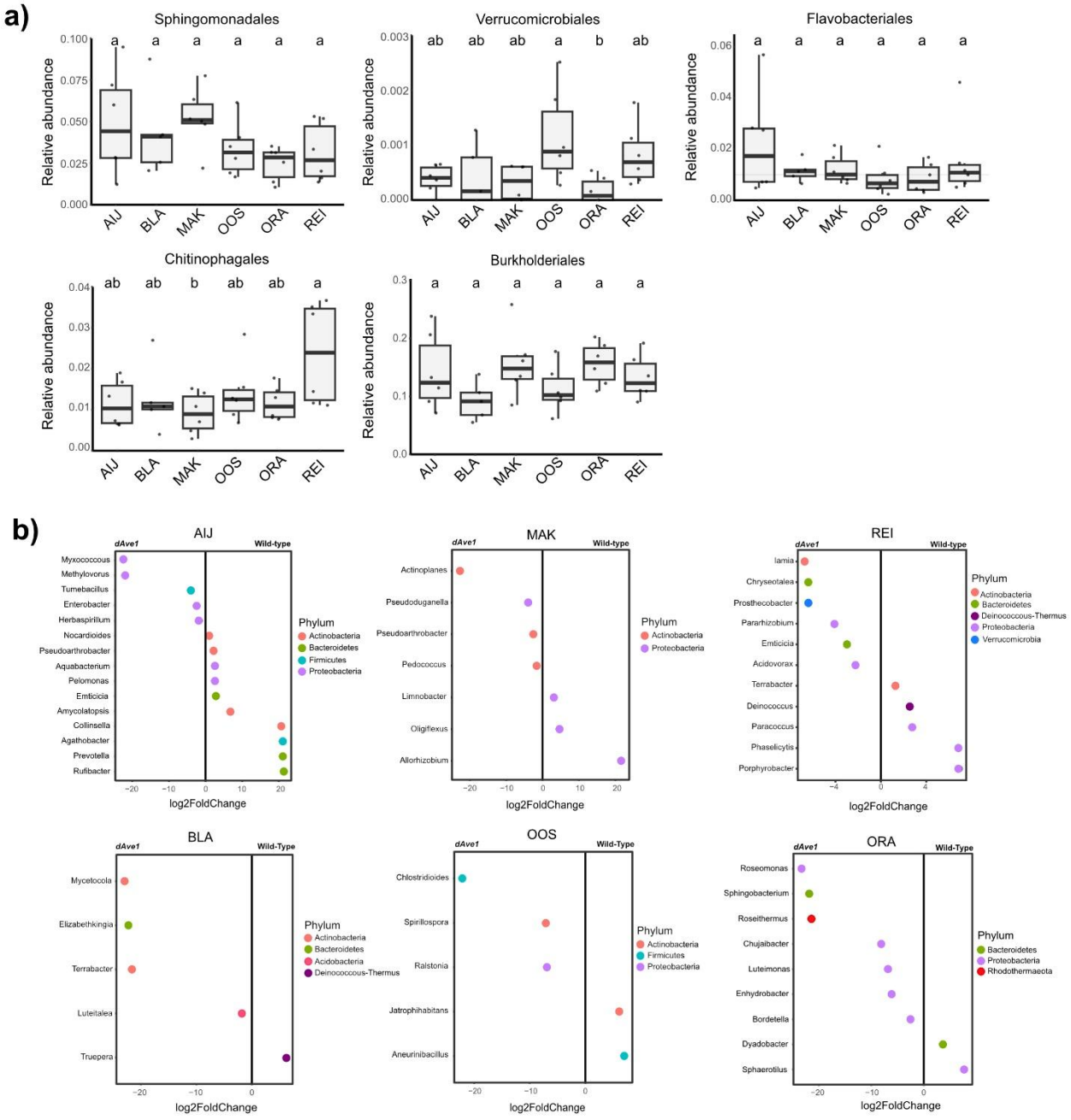
ORA-Root microbiota
Axis.2[9.4%]
Axis.1[64.9%]
REI-Root microbiota
Axis.2[20.5%]
Axis.1[30.2%]
Barley
Cotton
Tomato

Supplementary Figure 6. Fungal root microbiota. a) Relative abundance in percentage of the fungal root microbiota from barley, cotton and tomato plants grown on different natural soils. **b)** Principal coordinate analysis (PCoA) based on weighted Unifrac distances of the fungal root microbiota from barley, cotton and tomato plants grown on the different natural soils.



Supplementary Figure 7. Ave1-mediated microbiota manipulation in the different natural soils. a) Bacterial families previously reported to be impacted by Ave1 during tomato colonization. Relative abundance of each family in the root microbiota of three-week-old mock-inoculated plants. Different letters indicate statistical differences based on One-Way-Anova (Tukey HSD-Test pval < 0.05). **b)** Differential abundance of bacterial genera between root microbiota of tomato plants inoculated with wild-type *V. dahliae* or an *Ave1* deletion strain (Wald test, adjusted P < 0.05).

Supplementary Table 1: Locations of the soil collection sites

LOCATION	COORDINATES	SOIL TYPE	ABBREVIATION
AIJEN	51°34'55.0"N 6°02'27.3"E	River Clay	AIJ
DE BLAUWE KAMER	51°56'34.4"N 5°37'12.9"	River Clay	BLA
MAKKUM	53°05'09.8"N 5°26'20.3"E	Sea Clay	MAK
OOSTVARDERSPLASSEN	52°27'50.0"N 5°25'10.8"E	Sea Clay	OOS
ECKELRADE	50°47'57.7"N 5°44'42.5"E	Loam	ECK
ORANJEWOUD	52°57'11.7"N 5°57'45.6"E	Peat	ORA
REIJERSCAMP	52°00'37.7"N 5°46'25.0"E	Sand	REI
DE GINKELSE HEIDE	52°02'10.7"N 5°43'38.9"E	Sand	GIN
MAASDUINEN	51°28'34.3"N 6°11'34.9"E	Sand	MAA
COLOGNE	50°57'27.8"N 6°51'22.4"E	Clay	CAS

Chapter 5: General discussion

Introduction

Throughout its entire life, a plant is accompanied by a multifaceted consortium of microorganisms, collectively termed the plant microbiota (Trivedi *et al*., 2020). Together, host and microbes operate as a cohesive functional entity, termed the holobiont, based on the concept that optimal plant performance can only be established in concert with its microbial partners (Vandenkoornhuyse *et al*., 2015). While a subset of microbes is passed from parental plants to offspring through seed endophytes, the majority of microbes that establish in the plant microbiota are recruited from the environment, with the surrounding soil being the most important source for microbial recruitment (Chialva *et al*., 2022). The community composition of the plant microbiota is shaped by the interplay of various biotic and abiotic factors (Trivedi *et al*., 2020; Mesny *et al*., 2023). These can include the plant genotype and root exudate composition, as well as environmental conditions and agricultural practices (Compant *et al*., 2019). The microbes that comprise the plant microbiota engage in relationships with the plant that span a wide range of symbiotic relationships that comprise commensalism, mutualism, and parasitism (Hassani *et al*., 2018). Importantly, the plant microbiota plays an important role to protect the host from pathogens, for instance by recruiting beneficial microbes with antagonistic effects against pathogenic microbes (Rolfe *et al*., 2019; Du *et al*., 2025). In turn, research over recent years has shown that plant pathogens exploit effector proteins to facilitate host colonization not only through the manipulation of the plant immune system, but also through manipulation of the host microbiota (de Jonge *et al*., 2010; King *et al*., 2014; Bozkurt *et al*., 2014, Mesny *et al*., 2024). The research described in this thesis aimed to advance our understanding of the roles that antimicrobial effector proteins play in the biology of a fungal pathogen, more particularly the broad host range vascular wilt pathogen *Verticillium dahliae,* across diverse environmental contexts.

Through the research described in this PhD-thesis, I established a novel gnotobiotic experimental system to conduct research on antimicrobial effector proteins of the fungal plant pathogen *Verticillium dahliae* in the absence or the presence of (defined) microbiota (Chapter 2). In particular, this chapter reveals multifunctional roles of antimicrobial effectors, as I demonstrated that the antimicrobial effector Ave1 also functions on host physiology besides its role in manipulating the host microbiota (Chapter 2). By combining gnotobiotic assays with microbiota profiling I functionally characterized a novel antimicrobial effector protein, exploited by *V. dahliae* to undermine the “cry for help” recruitment of antagonistic *Pseudomonas* bacteria by the host plant (Chapter 3). Finally, I established a collection of diverse natural soils from the Netherlands to demonstrate that root-associated

microbiota compositions are primarily driven by soil type, and that phyllosphere microbiota compositions are more determined by plant species (Chapter 4). Additionally, using the soil collection, I reveal that the virulence contribution of antimicrobial effectors and their impact on the microbiota is influenced by soil type (Chapter 4). In the following sections, I will provide a deeper discussion of the findings obtained during my PhD research and place these findings into a broader context.

Antimicrobial effectors as key tools for fungal niche adaptation

During infection, plant pathogens must establish themselves successfully within the host-associated microbiota, where they compete with numerous other microorganisms for space and nutrients (Hassani *et al*., 2018). To gain a competitive advantage and facilitate colonization in these dynamic environments, many pathogens deploy antimicrobial effector proteins that eliminate or suppress microbial niche competitors (Kettles *et al*., 2018; Snelders *et al*., 2020; Chavarro-Carrero *et al*., 2024; Snelders *et al*., 2021; Snelders *et al*., 2023; Ökmen *et al*., 2023; Chang *et al*., 2021; Gómez-Pérez *et al*., 2023 Mesny *et al*., 2024).

The composition of plant-associated microbiota is highly dynamic and shaped by a wide array of biotic and abiotic factors and communities can vary significantly not only between environments and soil types, but also across plant compartments (Trivedi *et al*., 2020; also see Chapter 4). Many fungal pathogens, including *Verticillium dahliae*, occupy multiple, and very diverse, ecological niches throughout their life cycle (Fradin & Thomma, 2006; Guerreiro & Stuckenbrock, 2025). While they infect host plants during certain life stages, many pathogens also persist for extended periods of time outside their host, for instance in the soil (Katan, 2017; Fradin & Thomma, 2006). Compared to plant-associated microbiota, soil microbial communities are typically more diverse and can substantially differ according to the physicochemical properties of the soil (Sokol *et al*., 2022; Fierer, 2017). Consequently, many pathogens have adapted to ecologically diverse environments and interact with a broad spectrum of microbial communities across their entire life cycles (Snelders *et al*., 2022). Broad host range pathogens like *V. dahliae*, in particular, are adapted to numerous hosts and habitats, and are therefore expected to possess tools that allow them to modulate diverse microbial surroundings (Trivedi *et al*., 2020; Snelders *et al*., 2022). This ecological complexity raises the question whether tools for manipulating the environment, like antimicrobial effector proteins, have consistent functions across diverse environments, or whether their contribution to fungal biology varies with distinct microbial and environmental contexts.

In this thesis, I demonstrate that both the impact of antimicrobial effectors on microbial communities, and their contribution to fungal virulence, differ in different environments. Chapter 4 reveals that the

antimicrobial effector Ave1 displays soil-specific functionality, as its contribution to virulence varies depending on the soil in which the host plant is grown. These differences are likely due to distinct soil microbiota that shape the plant-associated microbial communities encountered by the pathogen (Chapter 4; Figure 1). A similar instance of microbiota-dependent virulence contribution was previously described the *V. dahliae* effector Ave1L2, which enhances fungal virulence only in the presence of specific sensitive microbial antagonists. Accordingly, experimental removal of these taxa abolished the effector's contribution to fungal virulence (Snelders *et al*., 2022; Punt *et al*., 2025). Similarly, while the Av2 effector was initially reported not to contribute to virulence (Chavarro-Carrero *et al*., 2020), the findings in Chapter 3 of this thesis clearly demonstrate that, under particular conditions, likely involving a distinct soil microbiota, Av2 significantly enhances *V. dahliae* virulence by undermining the host recruitment of beneficial *Pseudomonas* bacteria (Kraege *et al*., 2025). Together, these observations highlight that the virulence contributions of antimicrobial effectors is context-dependent, likely resulting from the dynamic interactions between the pathogen, the host, and the surrounding microbiota.

Beyond targeting individual antagonistic microbes, many antimicrobial effectors act on multiple members of the microbiota and likely exert broader, system-level impacts on microbial communities (Snelders *et al*., 2020; also see Chapter 3 & 4). Microbiota tend to function as networks of many interdependent species (Van der Heijden and Hartmann, 2016), where changes to one member can ripple throughout the entire community, and the removal or suppression of particular microbes by fungal effectors may trigger cascading shifts in community structure and function due to intermicrobial interactions within the microbiota. This interconnectedness implies that the outcome of effector activity on the microbiota can vary substantially between different communities, driven by the unique web of intermicrobial interactions in each environment. This was clearly observed in Chapter 4, where shifts in the plant microbiota caused by the antimicrobial effector Ave1 varied significantly between plants grown on different soils with distinct microbiota (Punt *et al*., 2025b). This finding demonstrates that the impact of an antimicrobial effector on the host microbiota can also vary significantly depending on the environment.

It has been proposed that particular antimicrobial effector proteins may serve dual functions, modulating the host microbiota as well as host physiology (Snelders *et al*., 2018). However, prior to the work described in this thesis no such effectors had been described. The work described in chapter 2 demonstrates that the antimicrobial effector protein Ave1 contributes to fungal virulence even in the absence of a microbiota, suggesting an additional, direct host target. Moreover, we show that Ave1 can modulate stomatal opening patterns, although at present it remains unclear whether this

activity promotes fungal virulence. Nevertheless, this finding indicates that certain antimicrobial effectors can fulfill multiple roles to support colonization of ecological niches within the plant holobiont (Punt *et al*., 2025a; Figure 1). However, this host-targeting function of Ave1 is not observed under all conditions. As shown in Chapter 4, the contribution to virulence of Ave1 can be lost entirely under different environmental conditions (Punt *et al*., 2025b). This suggests that even microbiota-independent effector functions may be influenced by external factors, such as the extremely high humidity in the FlowPot system. Altogether, these observations further support the notion that the function and importance of antimicrobial effectors for fungal virulence are highly context-dependent and shaped by the surrounding environment (Figure 1).

Including the findings presented in Chapter 3 of this thesis, five antimicrobial effector proteins from *V. dahliae* have been functionally characterized to date (Snelders *et al*., 2020; Snelders *et al*., 2021; Snelders *et al*., 2023; Kraege *et al*., 2025). However, a study using the recently developed machine learning tool "AMAPEC" to predict antimicrobial effectors across fungal secretomes identified 349 candidate antimicrobial effectors in *V. dahliae* (Mesny and Thomma, 2024). This finding suggests that the effectors that have been functionally characterized to date represent only a small fraction of a much larger and diverse arsenal of antimicrobial effector proteins, dedicated to successful colonization of diverse niches. Notably, not all of the previously characterized effectors are ubiquitously expressed (Snelders *et al*., 2020; Snelders *et al*., 2021). Thus, investigating mechanisms by which fungi perceive their environment and regulate the expression of antimicrobial effectors may provide deeper insight into the processes underlying fungal adaptation to different niches. Interestingly, also the saprotrophic fungus *Coprinopsis cinerea* and the arbuscular mycorrhiza fungus *Rhizosphagus irregularis* were predicted to harbor large numbers of antimicrobial effectors in their secretomes, with 457 and 558 predicted effectors, respectively. This suggests that antimicrobial effectors also play a crucial role for non-pathogenic fungi in niche establishment (Mesny and Thomma, 2024; Snelders *et al*., 2022). The observation that fungi with diverse lifestyles likely produce extensive numbers of antimicrobial effectors, combined with the fact that all fungi interact with a multitude of microbes in their respective environments, supports the hypothesis that these effectors are fundamental to fungal biology (Snelders *et al*., 2022), and not limited to fungal pathogens that use these molecules for host colonization.

The fundamental role of antimicrobial effectors suggests that these proteins may have an ancient evolutionary origin. Life is believed to have first emerged around 3.8 billion years ago (Mojzes *et al*., 1996), whereas the earliest fungi appeared approximately 1.02 billion years ago (Lutzoni *et al*., 2018). In contrast, vascular land plants evolved much later, around 480 million years ago (Harrison and Morris

2018). Consequently, fungi have been competing with other microbes, and particularly with prokaryotes, for far longer than plants have existed (Snelders *et al.,* 2022). It is therefore conceivable that parts of these extensive catalogues of fungal antimicrobial proteins are ancient and already served as essential tools for shaping the environments long before the emergence of plants (Snelders *et al*., 2021; Snelders *et al*., 2022; Mesny & Thomma, 2024; Mesny *et al*., 2024). With the evolutionary introduction of plants as a new niche, fungi may have adapted by evolving novel effectors and by co-opting ancient antimicrobial proteins to facilitate colonization of plant hosts. For example, *Verticillium dahliae* has been shown to co-opt the ancient antimicrobial protein AMP3 as an effector to manipulate the fungal component of the host microbiota particularly in senescent host tissues (Snelders *et al*., 2021). This supports the notion that a substantial proportion of host-manipulating effectors may have evolved from ancient antimicrobial proteins. Initially, effectors that once functioned to manipulate the microbial environment could have been co-opted to target the plant-associated microbiota during host colonization. Some of these effectors may have subsequently acquired additional functions, allowing them to also directly modulate host physiology. This resulted in dual-function effectors that influence both the host and the host-associated microbiota. Notably, some of these dual-function effectors may have lost their original antimicrobial activity over the course of evolution. Although the dual functionality of the antimicrobial effector Ave1 discovered in this thesis does not serve as a suitable example for this evolutionary hypothesis because this effector was shown to have been acquired from plants through horizontal gene transfer (de Jonge *et al*., 2012), recent findings lend support to this hypothesis. For instance, the beneficial root endophyte *Serendipita indica* employs two chitinase effectors with distinct functions. One of these, SiCHIT, carries a CBM5 domain that enables antimicrobial activity against competing fungi. Its paralog, SiCHIT2, lacks the CBM5 domain and is capable of suppressing plant immune responses during root colonization. Experimentally adding the CBM5 domain to SiCHIT2 restores its antimicrobial function, conceptually supporting the idea that these host-adapted effectors may have evolved from an antimicrobial ancestor, in this particular case through domain loss (Eichfeld *et al*., 2025).

In conclusion, based on the findings in this thesis, it can be speculated that fungi secrete large effector arsenals in order to establish themselves in different environments, allowing them to overcome plant defenses and microbial competitors both within and outside the host. Consequently, antimicrobial effectors should not be viewed as universally acting virulence determinants that always contribute to fungal niche establishment in the same way. Rather, they appear to be important components of fungal secretomes, whose function is tightly linked to the environmental and microbial context in which infection occurs (Figure 1).

Antimicrobial effectors prevent recruitment of beneficial microbiota

Plants rely on their associated microbiota for protection against pathogens, often through a so-called "*cry-for-help"* mechanism, in which microbial antagonists are selectively recruited upon pathogen infection (Du *et al*., 2025; Spooren *et al*., 2024; Rolfe *et al*., 2019). For example, during infection by the soil-borne pathogen *Fusarium oxysporum* f. sp. *cucumerinum*, cucumber plants recruit the beneficial *Bacillus amyloliquefaciens*, which reduces disease severity (Liu *et al*., 2017). Similarly, *Pseudomonas syringae* pv. *tomato* infections in *Arabidopsis thaliana* trigger the secretion of L-malic acid, which facilitates the recruitment of the beneficial rhizobacterium *Bacillus subtilis*, providing protection against the pathogen (Rudrappa *et al*., 2008).

Notably, over time, sustained microbial recruitment can lead to the formation of *disease-suppressive soils*, which are soils in which susceptible plants remain healthy despite the continued presence of a virulent pathogen (Yin *et al*., 2021; Du *et al*., 2025). The most prominent example of a disease suppressive soil is the so-called "*Take-all decline" soil*, where continuous wheat monoculture leads to a reduction in disease symptoms caused by the soil-borne fungal pathogen *Gaeumannomyces graminis* var. *tritici over time*. This decline has been attributed to the enrichment of beneficial *Pseudomonas* spp. that produce antibiotics antagonizing the pathogen (Raaijmakers & Weller, 1998; Spooren *et al*., 2024). Other well-documented examples of suppressive soils involve protection against pathogens such as *Fusarium oxysporum*, *Rhizoctonia solani*, *Phytophthora infestans*, *Heterodera avenae*, and *Ralstonia solanacearum* (Spooren *et al*., 2024). However, the development of disease suppressiveness is often a slow and gradual process, further influenced by agricultural practices and a several physicochemical processes of the soil including, temperature, pH, and nutrient availability (Schlatter *et al*., 2017; Spooren *et al*., 2024). Given that pathogens are known to manipulate host microbiota via secreted antimicrobial effectors, it has been proposed that such antimicrobial effectors may interfere with the recruitment of beneficial microbes, thereby hindering and delaying the establishment of suppressive soil microbiomes (Mesny *et al*., 2024). The establishment of disease-suppressive soils may therefore be the result from a multigenerational battle between plants and their microbiota on one side, and pathogens with their antimicrobial effectors on the other side. While this hypothesis primarily applies to soil-borne pathogens that engage in direct microbial antagonism with a suppressive soil microbiota, foliar pathogens have also been shown to trigger beneficial microbe recruitment in the rhizosphere through plant-mediated signaling, leading to protection across plant generations (Berendsen *et al*., 2018). In such cases, interference by the pathogen is less likely to occur via direct antagonism of beneficial microbes. Instead, foliar pathogens may disrupt the establishment of suppressive microbiomes by interfering with the plant's signaling mechanisms that mediate

microbial recruitment. However, until now, no direct evidence for such mechanisms has been reported.

In Chapter 3 of this thesis (Kraege *et al*., 2025), we describe how the fungal Av2 effector specifically undermines the "*cry-for-help"* response in tomato. By combining gnotobiotic plant assays in the

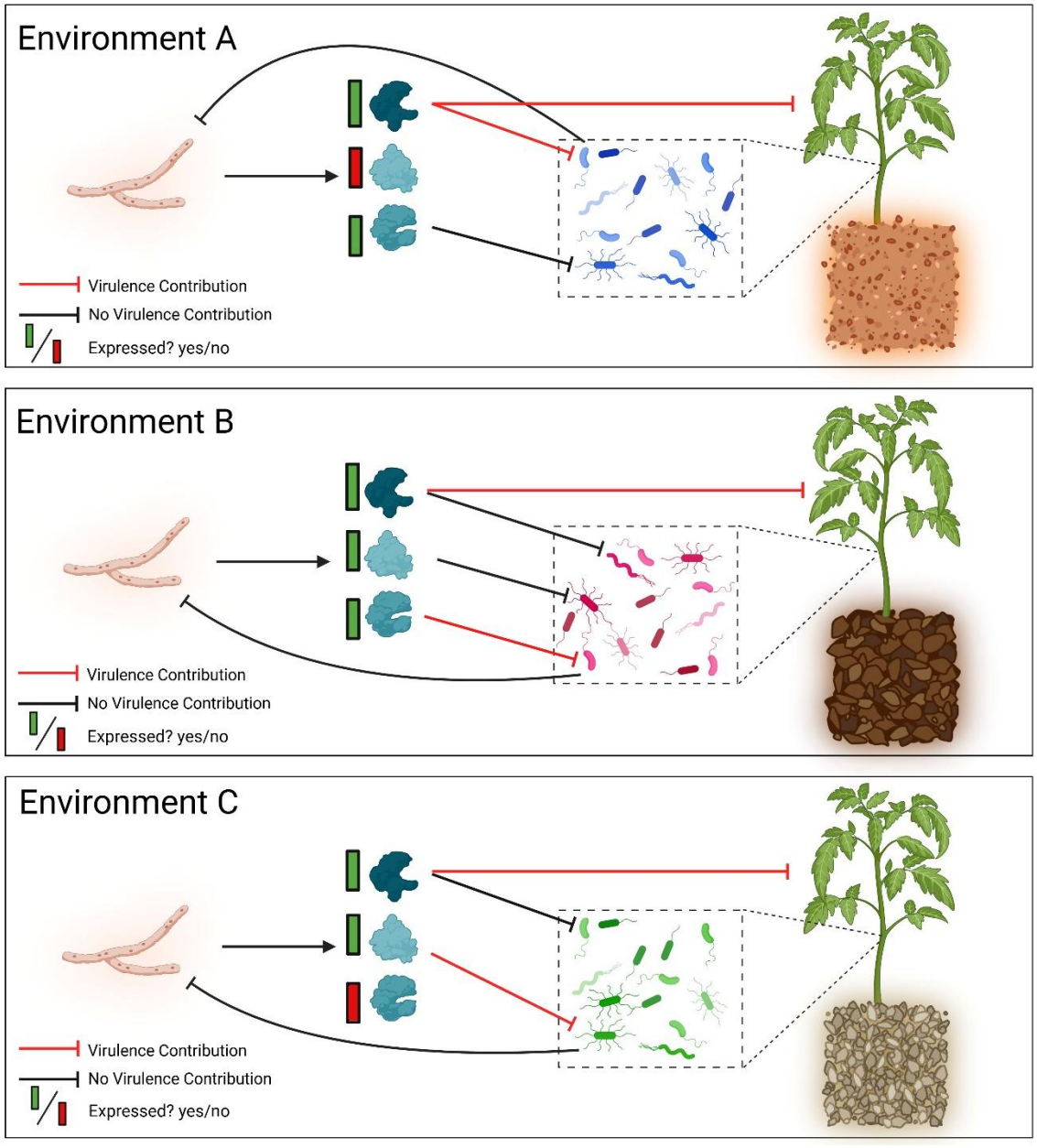


Figure 1 Antimicrobial effectors are key tools for fungal niche adaptation. During colonization, fungi secrete antimicrobial effector proteins to facilitate their establishment within the environment. The impact of antimicrobial effectors on plant-associated microbiota varies with the environment. In environment A, a dual function antimicrobial effector like Ave1 may enhance fungal virulence by targeting fungal antagonists, whereas in another environment, its virulence contribution may instead arise only from direct manipulation of host physiology (see Chapter 1 for details). The role of an antimicrobial effector that targets the host microbiota can depend on the presence of specific antagonistic bacteria. Although such effectors are not microbe-specific and can affect a broader range of microbial taxa, only the elimination of key antagonists contributes to enhanced colonization, while other targeted microbes do not provide a clear benefit to fungal virulence (see Chapter 4 for details).

FlowPot system, established in chapter 2 of this thesis (Punt *et al*., 2025a), with microbiota profiling, we show that Av2 is an antimicrobial effector that targets antagonistic *Pseudomonas* spp. recruited by the host during *Verticillium dahliae* infection. These findings, for the first time provide direct evidence that pathogens can actively interfere with host-mediated recruitment of beneficial microbes. Future experiments exploring whether Av2 disrupts microbial legacy effects should reveal whether this effector also contributes to the suppression or delay of the formation of long-term disease suppressiveness and legacy effects.

Challenges in, and opportunities for, microbiota-based biocontrol

For many years, agritech companies, scientists, and farmers have collaborated to harness the potential of plant-associated microbiota to enhance crop productivity (French *et al*., 2021; Compant *et al*., 2025). A commonly used approach involves the application of biocontrol products, which typically consist of individual microbial strains or small microbial consortia that directly and/or indirectly exert antagonistic effects on specific plant pathogens (Compant *et al*., 2025). For instance, *Trichoderma* species have been widely utilized for their protective effects against various fungal pathogens, including *Rhizoctonia solani* and *Botrytis cinerea* (Harman *et al*., 2004). Despite encouraging outcomes in laboratory settings, biocontrol strategies often show inconsistent performance under field conditions. These inconsistencies are largely attributed to the complexity of environmental variables, both biotic and abiotic, that influence the structure and function of microbiota in agricultural soils and in plants (Sessitsch *et al*., 2019; French *et al*., 2021; Vaccaro *et al*., 2022). Importantly, environmental conditions also affect the plant itself, leading to variable physiological responses that can modulate how plants interact with and respond to microbial colonization, including biocontrol strains. Consequently, many biocontrol strains fail to establish consistently in different host-associated microbiota, which can severely limit their efficacy (Sessitsch *et al*., 2019).

A previously underappreciated challenge contributing to the inconsistent performance of biocontrol strains may lie in the ability of pathogens to actively suppress beneficial microbes through the secretion of antimicrobial effector proteins. As demonstrated in Chapter 3 of this thesis, the fungal plant pathogen *Verticillium dahliae* can deploy the antimicrobial effector Av2 to block the host-mediated recruitment of beneficial microbes (Kraege *et al*., 2025). It is therefore conceivable that such effectors could also severely hinder artificially applied biocontrol strains from establishing within the native microbiota.

Plant pathogens are predicted to encode extensive arsenals of antimicrobial effectors (Mesny and Thomma, 2024), and findings from this thesis reveal that their impact on microbiota and pathogen

virulence can strongly vary in different environments (Punt *et al*., 2025b; Kraege *et al*., 2025). Although biocontrol agents are typically screened in the presence of the pathogen (Kjeldgaard *et al*., 2022; Raymaekers *et al*., 2020), this screening approach may consequently fail to capture important pathogen effectors that are only expressed under certain environmental conditions or possess an environment- or life stage-dependent function. For example, *the V. dahliae antimicrobial effector protein* AMP2 was found to be expressed in soil-like environments, but not on artificial media, *in planta, or* in co-culture with *Bacillus subtilis, Escherichia coli* or *Trichoderma viride* (Snelders *et al*., 2020). Similarly, expression of the antimicrobial effector AMP3 is restricted to hyphae during microsclerotia formation at later stages of the *V. dahliae* infection cycle (Snelders *et al*., 2022). As a result, a pathogen may appear sensitive to a biocontrol strain under particular greenhouse or laboratory conditions but may be insensitive in the field, where environment-specific antimicrobial effector expression patterns or functions could enable it to outcompete or suppress the biocontrol agent.

In the medical field, the concept of a precision microbiota, which refers to the microbiota-informed application of personalized probiotics to treat diseases in humans, has gained substantial attention for overcoming issues with traditional probiotic applications (Fang *et al*., 2025). For example, given the highly variable results obtained from fecal microbiota transplantation (FMT) for the treatment of diseases such as inflammatory bowel disease (Lahtinen *et al*., 2020), more precise approaches are being investigated to help overcome the inconsistencies observed with FMT outcomes. To achieve this, precision microbiota strategies involve analyzing the host environment, such as the patient's gut, to select specific probiotics that can reliably establish in the microbiota, prevent disease like inflammatory bowel disease, and improve overall human health (Fang *et al*., 2025; Pribyl *et al*., 2025). Similarly, also in agriculture, precision microbiota management is gaining increasing interest (French *et al*., 2021). Looking ahead, biological control strategies in agricultural production may benefit from increased precision by tailoring microbial applications to specific field conditions and pathogen genotypes. This could involve screening collections of beneficial microbes not only for their antagonistic activity, but also for their ability to establish and persist in the defined soil environment of the application site. In parallel, pathogen genotyping and analysis of the respective antimicrobial effector repertoires may further support the selection of microbial strains that are naturally insensitive to pathogen-derived antimicrobials. Additionally, experimental evolution, in which biocontrol strains are repeatedly exposed to a specific antimicrobial effector to promote the development of effector-specific resistance, may yield adapted strains that are not artificially genetically modified (non-GMO) with improved efficacy against the corresponding pathogen. Further, as our understanding of the mode of action of antimicrobial effector proteins increases, it may even become possible to rationally

engineer biocontrol strains with resistances towards particular antimicrobial effectors, enhancing their robustness and longevity in field settings.

Future approaches in precision microbiota management, both in the medical and agricultural sectors, will benefit from extensive microbial culture collections (Fang *et al*., 2025; Lima 2025; Raymaekers *et al*., 2020). Effective screening methods will be essential to identify strains with desirable traits, such as the suppression of plant pathogens. To increase the throughput of such screenings, machine learning tools may accelerate the selection of individual candidate strains or beneficial strain combinations (Sun *et al*., 2022; Westfall *et al*., 2021; Biggs *et al*., 2021; Kemen *et al*., 2025). Once candidates are identified, validation should be performed under conditions that allow for controlled experimental manipulation while approximating the intended application environment. In this context, gnotobiotic systems such as the FlowPot system developed in Chapter 2 of this thesis serve as valuable tools to complement conventional *in planta* assays. These systems provide a defined, yet plant-relevant, environment that enables researchers to assess how individual strains or synthetic communities (SynComs) colonize plants and influence disease outcomes (Punt *et al*., 2025a, Vorholt *et al*., 2017). Although the current version of the FlowPot system relies on a potting soil/vermiculite mixture, future adaptations could incorporate natural field soils, allowing screening for potent biocontrol strains under conditions that more closely mimic application sites.

With the identification of Av2 as an antimicrobial effector in this thesis besides the previously characterized Ave1 effector, two of the five functionally characterized antimicrobial effector proteins (AMPs) of *V. dahliae* are now known to be recognized by corresponding immune receptors *in planta* (Kraege *et al*., 2025; Chavarro-Carrero *et al*., 2020; De Jonge *et al*., 2012). Genetic resistance to pathogens often relies on individual resistance genes (*R* genes), which can be rapidly overcome by evolving pathogens (Garcia-Ruiz *et al*., 2021). Given the limited availability of effective *R* genes against pathogens like *V. dahliae* (Vermeulen *et al*., 2022), the loss of efficacy of a single gene can have severe consequences for agriculture. To improve the durability of *R* gene-mediated resistance, combining genetic resistance with specific biocontrol strains offers a promising approach. For example, pairing strong microbial antagonists of *V. dahliae* that the fungus suppresses through Ave1 or Av2, such as certain *Sphingomonadales* species (Snelders *et al*., 2020) or *Pseudomonas* species (Kraege *et al*., 2025), with plants carrying the respective immune receptor could enhance the durability of the resistance. In this scenario, the pathogen cannot overcome detection by simply discarding the effector gene, because this will compromise its ability to suppress these antagonists (Snelders, 2020; Chavarro-Carrero, 2024). This may also explain the efficacy of *Ve1*-mediated resistance in tomato, which recognizes the *V. dahliae* effector Ave1 and has remained effective for decades (Fradin *et al*., 2009;

Jonge *et al*., 2012; Robb and Nazar, 2021). This durability may in part be explained by the widespread presence of Sphingomonadales bacteria across diverse environments and as core members of host microbiota (Lundberg *et al*., 2022), which could constrain *V. dahliae* from losing or mutating Ave1 due to its essential antimicrobial activity.

Ultimately, it's important to note that just as pathogens evolve in response to host immune pressure (Jones & Dangl, 2006), they also have to adapt to microbial competition. The widespread use of effective biocontrol agents may therefore impose selective pressure, driving the evolution of new or modified antimicrobial effectors that specifically target beneficial microbes. Accordingly, pathogens are likely involved in an evolutionary arms-race not only with the plant, but also with members within the plant-associated microbiota. It is increasingly recognized that plant resistance genes are valuable but limited resources that come with a cost, and must be used wisely to avoid losing their effectiveness. This understanding has led to the idea that resistance genes should be deployed in stacks, making it as difficult as possible for pathogens to overcome them. (Zhu *et al*., 2012; Luo *et al*., 2021). Thus, to mitigate the risk of resistance and preserve biocontrol efficacy, deploying microbial consortia composed of functionally diverse and effector-insensitive members may be key. By targeting pathogens through multiple, complementary mechanisms, such consortia could provide more durable disease suppression and reduce the likelihood of pathogen adaptation.

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