Plant-Dependent Microbiome Variation in Natural *Lotus corniculatus* Populations

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Declaration of author contributions

The thesis entitled “Plant dependent microbiome variation in natural *Lotus corniculatus* populations” is based on the work I did during my PhD at the University of Tübingen, supervised by Prof. Dr. Oliver Bossdorf and Prof. Dr. Eric Kemen. I collaborated with Katrina Lutap, Dr. Madalin Parepa, Jun Hee Jung and Dr. Walter Durka. Chapter II – V in this thesis includes four independent scientific manuscripts. Each with co-authorship, that have been or will be published. The contribution of the authors for each chapter is stated as following:

Chapter II — Understanding plant microbiomes requires a genotype × environment framework

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Chapter III — Organ-specific microbiomes in natural *Lotus corniculatus* populations*:* Metacommunity dynamics in the plant endosphere

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Chapter IV — Plant age and genotype explain variation in the microbiome of natural *Lotus corniculatus* populations

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OB, EK, FR and KL designed the study. FR and KL planned and conducted the sampling and processing of the samples. FR and KL performed the lab work and data analysis, with WD performing the ddRAD sequencing library preparation and the first analysis of the raw data. FR performed the follow up experiment and analyzed the data with the help of JHJ performing the green pixel analysis. FR, KL and OB wrote the manuscript with input from all authors. All authors read and approved the final manuscript.

Chapter V — Natural variation in disease resistance of *Lotus corniculatus*

Frank Reis, Jun Hee Jung, Madalin Parepa, Eric Kemen, Oliver Bossdorf

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OB, MP, EK and FR designed the study. FR performed the experiment and data analysis with the input from MP and help of JHJ performing the green and yellow pixel analysis. FR, MP and OB wrote the manuscript with input from JHJ.

Note: The manuscript in Chapter II is also part of co-author Jun Hee Jung's dissertation. The manuscripts in Chapter III and IV are also part of co-author Katrina Lutap's dissertation.

Summary

Like all eukaryotes, plants are colonized by a diverse community of microorganisms which collectively form the plant microbiome. While some can cause diseases, the majority are either harmless or beneficial to the plant. These beneficial effects can range from enhanced growth, improved nutrient uptake, increased tolerance to stress, or resistance to pathogens. Although these effects have been studied intensely in recent years, we still have a poor understanding of which factors shape the complex plant microbiome, particularly under natural conditions. Improving our understanding of the plant microbiome and its impacts on plants will be important not only for advancing fundamental science but also for agriculture and crop protection. In the first chapter of my thesis, I introduce two main perspectives for studying the plant microbiome interactions. One focuses on how the microbiome affects the fitness and survival of plants, and their interactions with the environment. The other perspective shifts attention towards the microbiome itself, exploring which factors shape its composition and diversity, and how these factors interact. In the following chapters, I focused on the microbiome perspective, specifically investigating how host factors such as plant organ, plant age, and intraspecific genetic variation influence the composition and diversity of the plant microbiome. I worked with *Lotus corniculatus*, a widely distributed perennial legume that is commonly used as livestock forage, and as a nitrogen-fixer it impacts the nutrient dynamics of its ecosystems. Over four years, I sampled plants across multiple natural populations in southern Germany and sequenced their microbiomes (bacteria, fungi and eukaryotes) separately for the roots, shoots, flowers and seeds of each plant. I also genotyped all plants using ddRAD sequencing and determined their ages through herb chronology. Chapter III focuses on how plant-associated microbial communities differ across plant organs, with a gradual reduction in diversity from roots to shoots, and further into flowers and seeds. Building on this, Chapter IV shows that plant genotype further shapes both the diversity and composition of these communities, with certain taxa associated with specific genotypes. The genotype effects tended to be strongest and most consistent for plant-associated bacteria, with the largest plant genotype differences in the microbiome diversity of flowers and seeds. In contrast to plant genotype effects, I found less but still some evidence for an effect of plant age on microbiome diversity. The age of plants explained variation in fungi diversity, and it was associated with the abundance of several microbial taxa. To test specifically for genetic variation in pathogen resistance, I conducted a controlled growth chamber experiment in which I infected 20 natural accessions of *L. corniculatus* with the two pathogenic fungi *Fusarium* and *Uromyces* under different temperature and shade conditions. In Chapter V I present the results of this experiment, showing that the effects of both pathogens varied significantly across plant genotypes. Furthermore, while the interactions between plant genotypes and pathogens remained

largely independent of the abiotic stressors, I observed a strong overall influence of environmental conditions on infection outcomes. These results show that intraplant variation among organs, or age, as well as intraspecific genetic variation influence the diversity and composition of plant-associated microorganisms, even in complex natural environments. In the future, this knowledge could support breeding programs not only in enhancing pathogen resistance but also in selecting plant genotypes that support beneficial microbe combinations. Such approaches could contribute to more effective strategies for managing both abiotic and biotic stress, increasing plant health and resilience in a rapidly changing world.

Zusammenfassung

Wie alle Eukaryoten sind auch Pflanzen von einer vielfältigen Gemeinschaft von Mikroorganismen besiedelt, die zusammen das Pflanzenmikrobiom bilden. Während einige von ihnen Krankheiten verursachen können, ist die Mehrheit entweder harmlos oder nützlich für die Pflanze. Diese positiven Wirkungen reichen von der Förderung des Wachstums, der Verbesserung der Nährstoffaufnahme, der Erhöhung der Stresstoleranz bis hin zur Stärkung der Resistenz gegen Krankheitserreger. Obwohl diese Wirkungen in den letzten Jahren intensiv untersucht wurden, wissen wir immer noch nicht genau, welche Faktoren das komplexe pflanzliche Mikrobiom prägen, insbesondere unter natürlichen Bedingungen. Ein besseres Verständnis des pflanzlichen Mikrobioms und seiner Auswirkungen auf die Pflanzen kann nicht nur für den Fortschritt in der Grundlagenforschung, sondern auch für die Landwirtschaft und den Pflanzenschutz von Bedeutung sein. Im ersten Kapitel meiner Dissertation stelle ich zwei Hauptperspektiven für die Untersuchung der Wechselwirkungen zwischen Pflanzen und dem Mikrobiom vor. Die eine konzentriert sich darauf, wie das Mikrobiom die Fitness, das Überleben und im Allgemeinen die Integration der Pflanze mit ihrer Umgebung beeinflusst. Die andere Perspektive verlagert die Aufmerksamkeit auf das Mikrobiom selbst und untersucht, welche Faktoren seine Zusammensetzung und Vielfalt prägen und wie diese Faktoren zusammenwirken. In den nachfolgenden Kapiteln habe ich mich auf die Perspektive des Mikrobioms konzentriert und insbesondere untersucht, wie Wirtsfaktoren wie Gewebetyp, Pflanzenalter und intraspezifische genetische Variation die Zusammensetzung und Vielfalt des Pflanzenmikrobioms beeinflussen. Ich arbeitete mit *Lotus corniculatus*, einer weit verbreiteten mehrjährigen Hülsenfrucht, die häufig als Viehfutter verwendet wird und als Stickstofffixierer die Nährstoffdynamik ihrer Ökosysteme beeinflusst. Über einen Zeitraum von vier Jahren sammelte ich Pflanzen aus mehreren natürlichen Populationen in Süddeutschland und sequenzierte ihre Mikrobiome (Bakterien, Pilze und Eukaryoten) für Wurzeln, Spross, Blüten und Samen jeder Pflanze separat. Darüber hinaus habe ich mithilfe von ddRAD-Sequenzierung den Genotypen jeder Pflanze bestimmt und durch das Zählen der Jahresringe im ältesten Teil der Wurzel das Alter identifiziert. Kapitel III befasst sich damit, wie sich pflanzenassoziierte mikrobielle Gemeinschaften in den verschiedenen Pflanzenorganen unterscheiden, wobei die Diversität von den Wurzeln zum Spross und weiter zu den Blüten und Samen allmählich abnimmt. Darauf aufbauend wird in Kapitel IV gezeigt, dass der Pflanzengenotyp sowohl die Vielfalt als auch die Zusammensetzung dieser Gemeinschaften beeinflusst, wobei bestimmte Taxa mit bestimmten Genotypen assoziiert sind. Die Auswirkungen des Genotyps waren tendenziell am stärksten und konsistentesten bei pflanzenassoziierten Bakterien, wobei die größten Unterschiede zwischen den Genotypen der Pflanzen in der Mikrobiomvielfalt von Blüten und Samen zu verzeichnen waren. Im

Gegensatz zu den Auswirkungen des Pflanzengenotyps fanden sich weniger, aber immer noch einige Hinweise auf eine Auswirkung des Pflanzenalters auf die Vielfalt des Mikrobioms. Das Alter der Pflanzen hatte Einfluss auf die Vielfalt der mikrobiellen Pilze, und beeinflusste die Häufigkeit mehrerer mikrobieller Taxa. Um speziell die genetische Variation bei der Pathogenresistenz zu testen, führte ich ein Experiment in kontrollierten Bedingungen durch, bei dem *L. corniculatus* aus 20 natürlichen Herkünften mit den beiden pathogenen Pilzen *Fusarium* und *Uromyces* unter verschiedenen Temperatur- und Schattenbedingungen infizierte. In Kapitel V werden die Ergebnisse dieses Experiments vorgestellt und gezeigt, dass die Auswirkungen beider Pathogene stark vom Pflanzengenotyp abhängig sind. Während die Wechselwirkungen zwischen Pflanzengenotypen und Krankheitserregern weitgehend unabhängig von den abiotischen Stressfaktoren waren, konnte man einen starken Gesamteinfluss der Umweltbedingungen auf die Infektionsergebnisse feststellen. Diese Ergebnisse zeigen, dass intrapflanzliche Variationen zwischen den Organen oder das Alter sowie intraspezifische Genetische Variation die Vielfalt und Zusammensetzung der pflanzenassoziierten Mikroorganismen beeinflussen, selbst in komplexen natürlichen Umgebungen. In Zukunft könnten diese Erkenntnisse Züchtungsprogramme nicht nur bei der Verbesserung der Pathogenresistenz unterstützen, sondern auch helfen Pflanzengenotypen auszuwählen, die ein vorteilhafte Mikrobiom begünstigen. Solche Ansätze könnten zu wirksameren Strategien für die Bewältigung von abiotischem und biotischem Stress beitragen und die Gesundheit und Widerstandsfähigkeit von Pflanzen in einer sich schnell verändernden Welt verbessern.

Chapter I

General Introduction

The plant microbiome

Microorganisms are a fundamental part of all ecosystems on earth. They thrive in diverse habitats, from the soil beneath our feet to the most extreme conditions such as hot springs (Massello et al., 2020), deep extensive cave systems (Barton et al., 2004), and even extremely cold landscapes of Antarctica (Cary et al., 2010). Microbes perform critical metabolic functions and are key players in maintaining nutrient cycles within ecosystems (Madsen, 2011; Rappuoli et al., 2023). But they can also form close relationships with other organisms.

Every eukaryotic life form hosts a complex community of microbes, collectively known as the microbiome. These microorganisms reside on body surfaces or within internal tissues and can have strong effects on the host metabolism. While some microbes such as pathogens can negatively impact their hosts and cause disease, the vast majority are not harmful (Rappuoli et al., 2023) and can even have beneficial effects, contributing positively to the health and function of their hosts (Berendsen et al., 2012; Leser and Mølbak, 2009; Reis et al., 2020; Sampayo et al., 2008). In such mutualistic relationships both sides benefit from the relationship.

Also in plants many of these beneficial interactions with microorganisms are well understood. Some of the most prominent examples are nitrogen-fixing nodule bacteria which allow plants to convert atmospheric nitrogen into a usable form enabling growth in nutrient-poor soils (Udvardi and Poole, 2013). Another key example is mycorrhizal fungi, which enhance the plant’s ability to absorb water and nutrients from the soil (Parniske, 2008). In recent years however, it has become increasingly evident that beyond these well-known effects plant-associated microbiomes can influence the plant fitness and ecology beneficial in many ways. For instance the microbiome can enhance plant growth (Lugtenberg and Kamilova, 2009) and improve tolerance to abiotic stressors such as drought, salinity, and heat (Parasar et al., 2024; Rodriguez et al., 2008; Rolli et al., 2015). In addition the microbiome also plays an important role in the plant resistance to pathogens. It can suppress harmful microbes through competition for nutrients and space, production of antimicrobial compounds or activation of the plant’s immune system (Du et al., 2025; Pereira et al., 2023). Together these effects highlight the significant role of the plant microbiome in supporting long-term plant health.

The microbiome consists of a diverse range of microorganisms from different biological kingdoms. Bacteria which form the largest group, have a wide range of influences on plant health and development and have been extensively studied. However, fungi also play a crucial role within the microbiome impacting plant function in numerous ways. While many fungi are pathogenic and contribute to plant diseases (Avery et al., 2019), others can improve the plants fitness and health (Adedayo and Babalola, 2023). In addition to bacteria and fungi, eukaryotic microorganisms, though less abundant, can also influence plants and the broader microbiome. Many previous studies have focused on just one or a subset of these groups. However, to gain a comprehensive understanding of plant-microbiome interactions, it is important to consider all three groups in research.

Given its significant impact on plant fitness and survival, the microbiome plays a crucial role in shaping interactions within ecosystems and can’t be ignored in ecological studies. Understanding the microbiome and its influence on plants is important not only for advancing fundamental science but also for practical applications in agriculture and crop protection. For instance, managing beneficial plant microbiomes could enhance crop yield, stress tolerance, and resistance to pathogens. This is particularly critical, as pathogens present a major threat to global food security (Strange and Scott, 2005). Thus it’s important to understand the factors that shape the microbiome. This knowledge can help us better comprehend the interaction between plants and their microbial communities, which will support the management of plant populations in an era of rapidly changing environments.

Microbiome variation

There are two main perspectives when studying the plant microbiome. One focuses on how the microbiome affects the plants fitness, survival, and overall interaction with the environment. The other shifts attention to the microbiome itself, exploring which factors shape its composition and diversity, and how these factors interact with each other. For both approaches it is important to not only focus on plant-microbiome interactions but also consider external factors that may influence these interactions. For instance, environmental fluctuations can influence not only the plant but also how the microbiome interacts with the plant (Cheng et al., 2019). Therefore, it is essential to study plant-microbiome interactions within their environmental context. This aspect I will expand in more detail in Chapter II of this thesis. As mentioned in the first paragraph, there are many examples showing that the plant microbiome can have significant effects on plant health and development. However, to better understand and potentially utilize these beneficial effects, it is crucial to identify the factors that influence the composition of the microbiome. Plant microbiome diversity and composition can be influenced by a wide range of factors. First, of course the microbial community present in the plant’s surrounding environment plays a role, especially the soil microbes which are one of the most important sources for plant colonization (Berlow et al., 2024). Accordingly, environmental conditions also play a major role, not only by affecting the structure of the surrounding microbial communities, but also by influencing which microbes are able to successfully colonize and persist within the plant. Soil composition is a major factor influencing the plant microbiome (Tkacz et al., 2015), but land use practices (Estendorfer et al., 2017; Gaube et al., 2021) and climate conditions (Chen et al., 2019; Trivedi et al., 2022) can also have significant effects. In addition to these external factors, the host plant itself can also strongly affect its associated microbiome.

Plant dependent factors

Numerous studies have shown that microbiome composition is strongly influenced by the plant species (Bonito et al., 2014; Knief et al., 2010) and even within a single species, genetic variation can lead to differences in the associated microbial communities (Leopold and Busby, 2020; Liu et al., 2019; Wagner et al., 2016). Genetic variation, both within and between species can alter the plant's chemical and physiological properties, influencing which microbes are favored or suppressed. These genetic differences may also affect the plant's needs, leading to variation in how plants select and interact with microbial partners. Plants are not passive in this process. They can actively recruit specific microbes based on environmental conditions by releasing secondary metabolites that certain microbes can detect, prompting them to move toward the plant roots and initiate colonization (Su et al., 2023). The plant immune system also plays an important role in the formation of the microbiome. The presence and absence of different plant resistance genes (R genes) can affect the plant's ability to defend against different pathogens (Dangl and Jones, 2001; DeYoung and Innes, 2006; Periyannan et al., 2013). But also some genes can have an opposing effect reducing the resistance and making it more vulnerable to infections (Gorshkov and Tsers, 2022). The interaction of these factors shapes the plant's immune response and determines which microbes are able to successfully colonize the plant. In addition to genetic variation within the plant genome, developmental processes may also drive variations in plant traits and needs, which can in turn influence microbiome composition. For instance, several studies showed that the microbiome differs between different plant life stages (Edwards et al., 2018; Sugiyama et al., 2014). Furthermore, plants are not homogeneous organisms; various tissue types exhibit distinct chemical and physiological properties that shape microbial communities. Earlier studies found significant microbiome variation between roots and above-ground plant tissues (Bernard et al., 2021). In addition to vegetative tissues, plants also produce flowers and seeds, each with unique physiological characteristics (Junker et al., 2011). Pollinators, visiting flowers, can introduce new microbes, thereby influencing the microbiome (Hietaranta et al., 2023). Seeds, which develop from flowers, may also carry microbes that are passed on to the next generation through vertical transmission.

How can we study the plant microbiome

Since microbiome composition is influenced by numerous factors making its study highly complex, there are several approaches to managing this complexity. One common method is to examine the microbiome under controlled conditions to minimize external variables. This often involves keeping the plant in sterile conditions and inoculating it only with individual microbes or a predefined artificial microbiome, a so-called synthetic community or SynCom (Bodenhausen et al., 2014; Lebeis et al., 2015). A SynCom can range from a few to over a hundred well-characterized microbes. While this approach is well suited for studying the specific effects of individual microbes or groups of microbes, it does come with certain limitations. It is often difficult to translate results from controlled laboratory conditions to natural environments (Yu et al., 2016). Additionally, these studies are limited to microbes that can be cultured and propagated in the lab. Furthermore, environmental factors and their natural variability in the field are typically not considered. A clear example of this issue can be seen in a study on *Arabidopsis thaliana* where a in the lab identified pathogenic *Pseudomonas* strain showed no negative effect in the plants of natural populations (Karasov et al., 2018). Another limitation is that these studies often focus on a small number of model plant species which can reduce the applicability of the findings to a wider variety of plants. Nevertheless, as already explained, the relationships between microbiomes and plants are extremely complex and can be influenced by an extremely wide range of factors. As such, studies with reduced complexity are essential for understanding the underlying processes. A contrasting approach is to study the microbiome under natural conditions which has the advantage of observing organisms in their native environments providing a particularly high level of ecological realism. However, as previously discussed the multitude of interacting factors that shape the microbiome in nature can be highly complex and difficult to disentangle. Ecological experiments can’t cover all three aspects of precision, generalism, and realism at the same time and scientists have to choose where they want to focus (Guisan and Zimmermann, 2000; Levins, 1966; van Kleunen et al., 2014), as illustrated by the precision-generalism-realism trade-off triangle (Fig. 1). Each approach comes with its own strengths and limitations and to get the best possible understanding of all the interactions in plant-microbe interactions it is essential to integrate findings from all these perspectives.

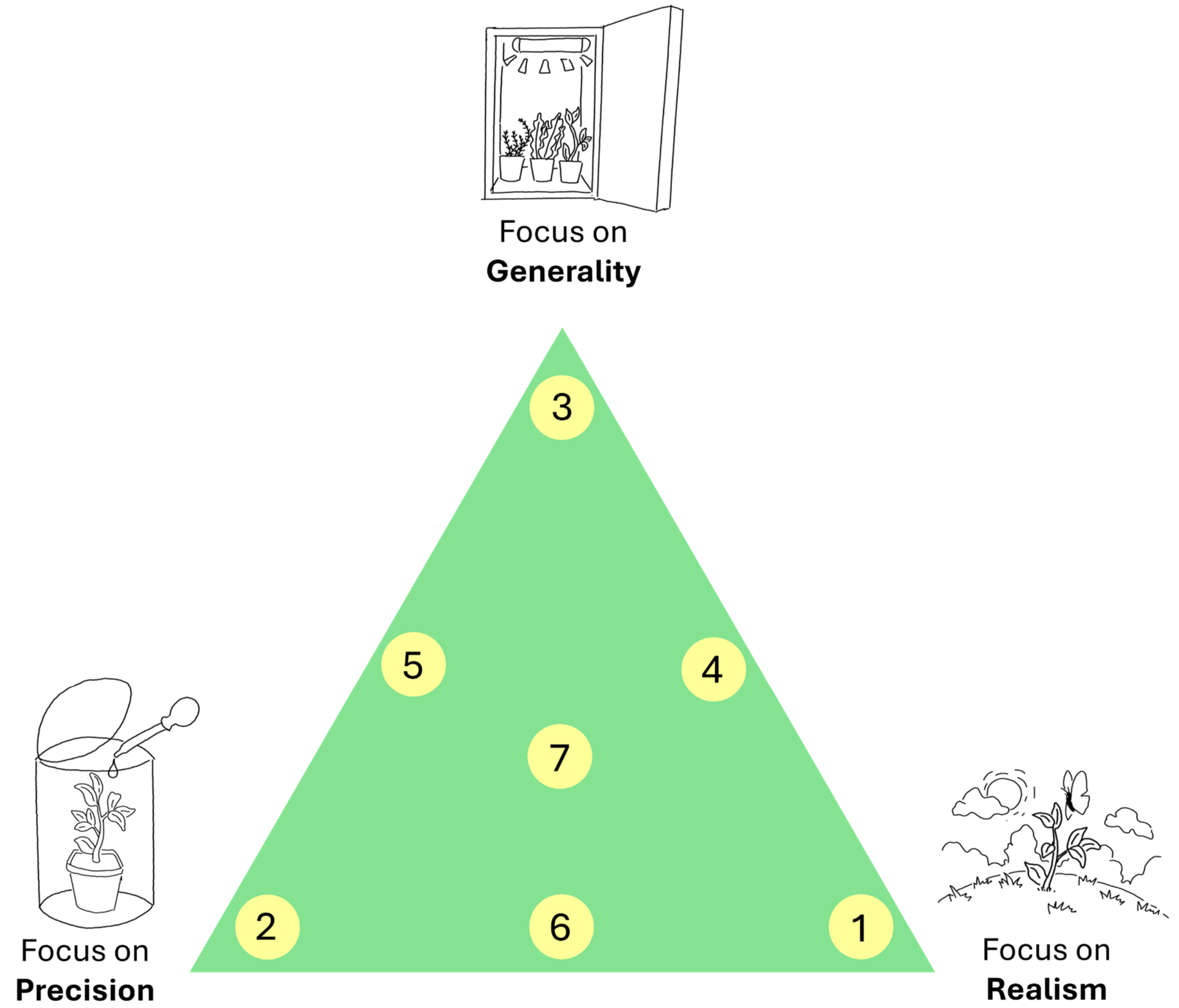

Figure 1: The trade-off between precision, generalism, and realism. These terms can represent different factors depending on the experimental context. However, if we define precision as the level of control over the microbes used, generalism as the number of plant species or genotype studied, and realism as whether the experiment uses field samples or is conducted in controlled growth conditions, studies can focus on different combinations of these aspects: 1. One host species studied, natural microbiome, samples from the field; 2: One host species, SynCom in otherwise sterile conditions, growth chamber; 3: Many species, natural microbiome, growth chamber; 4: Several host species, natural microbiome, garden; 5: Several host species, SynCom, growth chamber; 6: One host species, SynCom, garden; 7: Several host species, SynCom, garden. Illustrations by S. Nicolai Rühl.

Lotus corniculatus as a study organism

Lotus corniculatus commonly known as bird's-foot trefoil is a widespread perennial legume found in a variety of environments across temperate Eurasia including grasslands, quarries and rock crevices or roadsides. *L. corniculatus* plant serves as an important food source for many herbivorous insects and pollinators including bumblebees, honeybees, wild bees, butterflies, flies, and beetles (Hennig and Ghazoul, 2011; Weiner et al., 2014) and a nitrogen fixer. *L. corniculatus* plays a key role in the plant-microbe symbiosis that influences the nutrient dynamics of grassland ecosystems (van der

Heijden et al., 2016). For this reason it plays an important role in many ecosystems but also in agriculture where it is widely used as a fodder plant on poor soils. *L. corniculatus* has a broad lifespan extending at least up to 15 years (Roeder et al., 2017; Anna Roeder & Christiane Roscher, unpublished data) and displays considerable intraspecific genetic diversity (Abraham et al., 2015; Durka et al., 2025). It flowers from early summer through late autumn with mature seeds forming while new flowers are still present. This makes it possible to study both flowers and seeds of the same plant at the same time. These characteristics make *L. corniculatus* an ideal system for addressing the research questions investigated in this thesis.

Objectives of this thesis

The interactions between host plants and their microbiomes are highly complex and as previously discussed shaped by a wide range of factors. In Chapter II I introduce the distinction between plant-focused and microbiome-focused perspectives and propose a framework for studying both within the context of variable environmental conditions. In the following part of the thesis I will then focus on the microbiome perspective. As numerous studies have demonstrated the microbiome's impact on plant health and stress tolerance it becomes increasingly important to understand how microbiome composition is formed. While many valuable insights have been gained through precision focused highly controlled studies using SynCom or individual microbial strains we also need more research that focuses on realism and study these processes in natural environments. In Chapters III and IV I explored which influence the host plant has on the microbiome variation in natural conditions. Therefore, I analyzed the microbiome composition of seven natural *Lotus corniculatus* populations. Chapter III focuses on how different plant tissue types or organs such as roots, shoots, flowers and seeds shape the microbiome. In Chapter IV I examined how other plant related factors such as plant age and intraspecific genetic variation affect microbiome diversity and composition across the different tissue types. Building on the insights from these field-based studies I complemented the findings with a more controlled experimental approach shifting the focus in the precision direction. Chapter V presents an experiment designed to test how different *L. corniculatus* genotypes respond to an infection with the pathogenic fungi *Fusarium* and *Urmocyes* in different environmental conditions. This experiment served to take a closer look at the interactions between the environment and plant genotype and also helped to confirm part of the results from Chapter IV under standardized conditions. Since realism-focused studies in natural environments often rely on statistical associations, this controlled experiment allows for a more direct test of causal relationships and helps validate the patterns observed in the natural populations.

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Chapter II

Understanding plant microbiomes requires a genotype × environment framework

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ON THE NATURE OF THINGS: ESSAYS

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Understanding plant microbiomes requires a genotype × environment framework

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Plant microbiomes have become one of the hottest topics in plant biology. Driven by the increased availability of microbiome sequencing methods, analyses of plant-associated microbiomes have been skyrocketing during the last decade. They have generally taken one of two main perspectives: (1) a focus on the microbiome itself, where researchers describe microbiome diversity and attempt to understand its drivers (Figure 1A), or (2) a focus on the consequences of microbiomes, where researchers analyze effects of microbiomes on plants (Figure 1B). Below, we briefly discuss these two perspectives, and we argue that for both a genotype-by-environment (G × E) framework will be key for achieving a deeper and more general understanding of plant microbiomes.

TWO PERSPECTIVES IN PLANT MICROBIOME RESEARCH

Studies with a microbiome focus typically describe the diversity and composition of the root or leaf microbiomes of plants, and they often test influences of plant characteristics or environmental conditions on the microbiomes (Figure 1A). For instance, previous studies have shown that microbiome composition varies within and among plant species, with significant influences of plant genotype (Agler et al., 2016; Wagner et al., 2016; Bowen et al., 2017; Bergelson et al., 2019) and phylogeny (Fitzpatrick et al., 2018) and that not only plant tissue but also plant age and developmental stage (Chaparro et al., 2014; Wagner et al., 2016) influence plant microbiomes. In addition to plant characteristics, environmental conditions also play a role in microbiome development. For instance, soil conditions and root exudates strongly influence root microbiomes (Fitzpatrick et al., 2018; Hu et al., 2018; Sasse et al., 2018), and leaf microbiomes vary predictably among different habitats (Agler et al., 2016; Wagner et al., 2016).

Microbiome studies with a plant focus, in contrast, are interested in how the microbiome of a plant affects its growth or environmental tolerances. They test—through experiments or association patterns—how plant performance is influenced by the presence or composition of microbiota, sometimes also taking plant or environmental characteristics into account (Figure 1B). Studies with individual microbial taxa have demonstrated that these can promote growth and stress tolerance of plants (Lugtenberg and Kamilova, 2009) and influence pathogen and herbivore resistance (Pieterse et al., 2014; Hu et al., 2018). More recent studies with complex microbiomes have confirmed these effects: inoculation with diverse microbiota altered plant growth and physiology (Fitzpatrick et al., 2019; Belimov et al., 2020), phenology (Panke-Buisse et al., 2015), and pathogen resistance (Berendsen et al., 2018; Berg and Koskella, 2018), sometimes in a genotype- or environment-dependent fashion (Berg and Koskella, 2018; Belimov et al., 2020).

In summary, recent research has demonstrated the ubiquity and importance of plant microbiomes, but this work has also shown that microbiomes are complex and influenced by a range of plant and environmental factors. Another challenge is that drivers of microbiome variation often interact. For instance, in a multi-site field experiment with different genotypes of *Boechera stricta*, Wagner et al. (2016) found that genotype and age effects on bacterial microbiomes were often

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Jun Hee Jung and Frank Reis contributed equally.

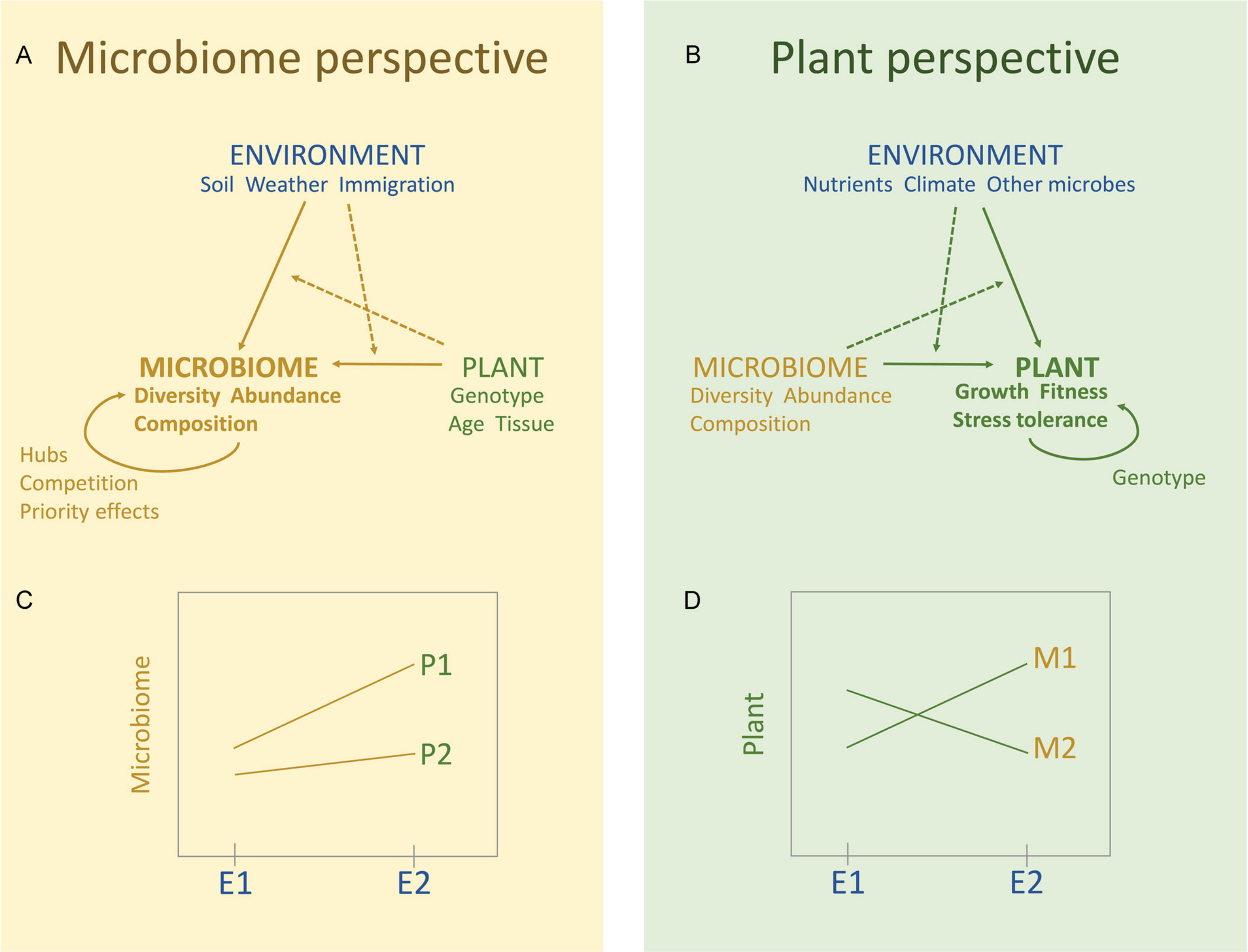

FIGURE 1 Two main perspectives in plant microbiome research, and reaction norm plots illustrating possible G × E interactions tested in each. With a microbiome perspective, researchers usually study community-level characteristics of the microbiome and test effects of plant genotype or other plant characteristics (P1/P2) and environmental conditions (E1/E2). With a plant perspective, the dependent variables are measures of plant performance, and experiments test influences of microbiomes (M1/M2), environmental conditions, and their interactions. The dashed lines in the upper graphs indicate indirect effects where some drivers of plant or microbiome variation alter plant or microbiome responses to others. Note that, while the bottom graphs display only categorical variables, the G × E framework can be equally applied to continuous explanatory variables

site-specific. In an experiment with natural accessions of *Arabidopsis thaliana*, Fitzpatrick et al. (2019) found that the effects of a natural soil microbiome on plant fitness depended not only on the plant genotype but also on the ecological context. Similarly, Petipas et al. (2020) showed that the effects of soil microbes on the growth and fitness of *Hypericum perforatum* were habitat- and plant-genotype-specific.

In spite of the many and often interacting drivers of microbiome diversity and microbiome effects, the vast majority of previous studies focused on one or few drivers, often in reductionist laboratory settings, and there have been few solid multifactorial studies to date that allowed testing for interactions between different factors. As a result, the generality of many previous results, and their transferability to more natural environmental conditions, remains uncertain, and we are still far from truly understanding natural plant microbiomes. A significant step forward could be to embrace an important conceptual framework from evolutionary ecology: that of genotype-by-environment interactions.

ADOPTING A G × E FRAMEWORK

Genotype-by-environment (G × E) interactions are statistical interactions between the effects of genotypes and environment on phenotypes, that is, when phenotypic differences among genotypes depend on the environment in which they are tested, or when phenotypic responses to environment depend on the genotype. The G × E concept has long been central to plant evolutionary ecology (Nicotra et al., 2010), and a large body of research has often found strong G × E interactions in many plant species, to the extent that genotype effects may be strong in some but absent in other environments, and phenotypic responses to environment are sometimes opposite for different genotypes. Similar results in animal research confirmed that G × E interactions are the rule in natural populations and that caution is needed with generalizing from single-factor studies. G × E experimental designs are essential for understanding and documenting local adaptation, and they are also relevant for adjusting agricultural and forestry practices to changing environments (Nicotra et al., 2010).

The classic experimental approach to testing G × E interactions is a common garden experiment where multiple genotypes are replicated across different environments in a multifactorial design, so that the generality of genotype and environment effects, as well as their interactions, can be statistically tested. The results of such experiments are often visualized through reaction norm plots that show genotype-specific responses to environment or other relevant interactions (Figure 1C, D). We can easily apply these concepts and experimental approaches to a multifactorial study of plant microbiomes, both for the microbiome and the plant perspective.

Microbiome-focused studies with a G × E character essentially treat the microbiome as an "extended phenotype" of the plant that is subject to the same complex influences as other plant phenotypes. Such studies will, for example, test the influence of plant genotype on plant microbiomes under different environmental conditions, or they will examine environmental effects on plant microbiomes across multiple plant genotypes (Figure 1C). For instance, field experiments can transplant multiple plant genotypes into different habitats and test the interactive effects of genotype and habitat on spontaneous microbiome development (Wagner et al., 2016). In laboratory experiments, different plant genotypes can be inoculated with identical microbial communities and their divergence followed under different growth conditions. An extension of the G × E concept may also include plant factors other than genotype (e.g., plant tissue, age, or even species). More complex studies may include several environmental factors and/or additional microbial drivers of microbiome composition (Figure 1A).

Plant-focused studies with a G × E framework generally test microbiome effects on plant performance or stress tolerance not only for one narrow type of experimental set-up but across a range of different environments and/or multiple plant genotypes (Figure 1B). For instance, beneficial or pathogenic effects of different microbial inoculates can be studied under several, controlled levels of resource availability or abiotic stress (Fitzpatrick et al., 2019). For more realistic tests, seedlings can be inoculated with different microbial communities and planted in a range of natural habitats (Petipas et al., 2020). Depending on one's perspective and strategy of data analysis, these approaches will examine how microbiome effects on plants are modulated by environmental influences or, equally important, how plant responses to the environment (phenotypic plasticity; environmental tolerances) are modulated by microbes (Figure 1D). Finally, the environmental component in such experiments may also include additional biotic factors such as competitors (Fitzpatrick et al., 2019), herbivores, or other (background) microbiota, which will allow for testing microbe–microbe interactions or other complex biotic interactions.

CONCLUSIONS

The study of plant microbiomes is an important frontier in current plant biology, with many open questions, particularly from an ecological-evolutionary (Koskella et al., 2017; Fitzpatrick et al., 2020) and agricultural perspective (Toju et al., 2018). Irrespective of whether the focus is on the plant or the microbiome, studies that are too simple may overlook important interactions between different factors, and they run the risk of overestimating or overgeneralizing results. A more thorough understanding of plant microbiomes will require working with a broader range of plant genotypes and nonmodel species, and to take a G × E perspective and explicitly test the generality of plant–microbiome interactions across multiple interacting drivers. Such studies will not only be relevant for researchers with an ecological-evolutionary mindset, but they will ultimately also allow those with a more reductionist focus on functional and molecular details to better understand the robustness of their insights and refine their models of plant–microbe interactions.

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All authors jointly developed the ideas in this essay and contributed to writing the manuscript.

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Chapter III

Organ-specific microbiomes in natural *Lotus corniculatus* populations: Metacommunity dynamics in the plant endosphere

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Abstract

The structure of plant microbial communities varies due to a broad range of factors such as host and environmental factors, abiotic and biotic perturbations, and various assembly processes occurring at multiple tempo-spatial scales. In natural environments plant microbial communities are constantly exposed to such perturbations and processes. Thus, to attain a systemic understanding of the ecology of plant microbiomes, it is essential to study assembly processes that influence patterns of microbial community structures in natural environments. In this study we examined bacterial, fungal, and eukaryotic communities in plant organs of *Lotus corniculatus* in natural populations at seven grassland sites for four years. We used the framework of metacommunity theory of ecology to understand assembly processes that shape community structures and variations by defining microbial communities associated with the roots, shoots, flowers, and seeds as distinct communities linked by dispersal. In this study we show the organ-specificity of plant endophytic communities. Our findings suggest that selective filtering by plant organs, microbial interactions, as well as abiotic and biotic factors at tempo-spatial scales result in distinct core microbiomes of plant organs. In addition, transmission of microorganisms from within and outside the plant hosts accounts for the distinct yet overlapping organ microbiomes. We could provide a comprehensive knowledge of the stochastic and deterministic assembly processes that shape plant microbial communities in natural conditions. Understanding these ecological processes is essential for harnessing beneficial effects of plant-associated microbial communities on plant productivity, resilience, and pathogen defense.

Key words

plant microbiomes, organ-specific microbiomes, metacommunity dynamics, core microbiomes, microbial networks, microbial dispersal, plant-microbe interactions

Introduction

Plants are associated with diverse microbial communities of beneficial, neutral, and pathogenic microorganisms from across different kingdoms of archaea, bacteria, fungi, protists, and viruses. These plant-microbe interactions can be beneficial for both plant hosts and associated microorganisms - while microorganisms function in plant growth and development, nutrient acquisition, stress tolerance, and pathogen defense, host plants provide habitats and resources for growth and survival of microorganisms (Trivedi et al., 2020). These microbes that live within (i.e. endophytes) or on the surface (i.e. epiphytes) of plants are acquired from seeds via vertical transmission and from biotic and abiotic environments comprising air or wind, rain, soil, insects, and animals via horizontal transmission (Abdelfattah et al., 2021; Allard et al., 2020; Ambika Manirajan et al., 2016; Maignien et al., 2014; O'Rorke et al., 2017; Walsh et al., 2021). The structure of plant microbial communities vary across plant age and genotypes, geographic locations, soil properties and land use types, developmental stages, and plant compartments (Bernard et al., 2021; Lundberg et al., 2012; Moroenyane et al., 2021; Schlatter et al., 2020; Wagner et al., 2016). While many studies on plant microbiomes described these variations in microbial community structures, there remains a need for comprehensive understanding on assembly processes and microbial interactions that account for these community patterns. Current research on plant microbiomes revealed complex associations between plant hosts, microorganisms, and environment, however there are remaining challenges on our understanding of mechanisms that govern community assembly, community structure variations, and intricate interactions in plant microbial communities, especially in natural conditions. Given the high diversity and complexity of microbial communities in their natural habitats, the ecology of microbiomes in situ can only be partially conveyed by in vitro studies under laboratory conditions (Yu et al., 2016). To address these challenges, it is crucial to study plant microbiomes in natural environments, with constant exposure to natural perturbations and interactions, biotic and abiotic elements, and natural microbial sources.

Plant microbiomes are shaped by factors acting at multiple scales - from populations to individual plants and plant organs, across time and space (Cordovez et al., 2019; Fitzpatrick et al., 2020). Plant microbial communities are also interconnected via transmission of microbes at different levels, from microbial sources outside plant hosts (i.e. other plants, animals, and environment) to transmission within plant tissues (Compant et al., 2019; Müller et al., 2016). To incorporate simultaneously the community assembly processes occurring at multiple spatial and temporal scales and consequently to attain a more complete understanding of the variations and interactions in plant microbial communities, we examined community dynamics of plant microbiomes in the framework of metacommunity theory of ecology. Metacommunity theory is the study of distinct communities that are linked by dispersal (Leibold et al., 2004). This concept consolidates the effect of different ecological processes and their interactions across space and time scales on community structures (Adair and Douglas, 2017; Leibold and Chase, 2017). The assembly processes defined in Vellend's conceptual synthesis of community ecology are integrated into the metacommunity theory - selection, drift, and diversification are shaping separately each of the distinct communities, which are linked by dispersal (Nemergut et al., 2013; Vellend, 2010). Studies on microbial communities in the context of metacommunity ecology are relatively recent. Traditionally, metacommunity theory is used in the study of spatially distinct macrobial communities, such as marine and freshwater habitats, forest communities, moss patches, and insect and aquatic invertebrate metacommunities (Bush et al., 2020; Logue et al., 2011). Application of the metacommunity concept in microbiome research allows for synthesis of both the deterministic and stochastic processes in their roles on shaping microbial communities at multiple temporal and spatial scales. Microbial communities in different ecosystems or sites (i.e. soil and freshwater ecosystems), as well as in plant and animal populations, were analyzed as metacommunities (Adams et al., 2014; Christian et al., 2015; Hovatter et al., 2011; Jiao et al., 2020; Sorensen and Shade, 2020). Research on host-microbiome systems as metacommunities accounts for the interactions of assembly processes and microbial routes within hosts and their outside environments. A few studies on host-microbiomes and their interactions with their environments have been conducted with communities of insects, aquatic and terrestrial animals, and humans (Brown et al., 2020; Buttimer et al., 2021; Cleary et al., 2019; Costello et al., 2012; Escalas et al., 2017; Miller et al., 2018; Sarkar et al., 2020). Studying plant microbiomes as metacommunities takes into account varied plant host characteristics as well as diverse environmental factors and microbial sources influencing at scales ranging from individual plants to populations. Plant-microbiome systems as metacommunities is a fairly new concept (Borer et al., 2016). Moreover, studies focusing on microbial communities in plant organs in the context of metacommunity ecology are currently few. Investigation of microbial communities associated with roots, flowers, and seeds as discrete communities interacting with their biotic and abiotic environment were recently presented (Bergmann and Leveau, 2022; Keller et al., 2021; Rebolleda Gómez and Ashman, 2019; Toju et al., 2018).

Studying plant organ-associated microbial communities as distinct communities linked with other microbial communities within and outside the plant host holds advantages towards better understanding of complex assembly processes and microbial interactions. Factors influencing the interconnected microbial communities at multiple tempo-spatial scales - from plant populations to individual plants and tissues - are simultaneously integrated in one ecological framework. In the metacommunity context, the plant host encompasses heterogeneous habitats for microbes, in which each of the plant organs are influenced by different assembly processes, biotic interactions, and microbial transmission, shaping distinct plant organ-specific microbial communities(Bodenhausen et al., 2013; Junker et al., 2011; Trivedi et al., 2020). Different resources or microhabitat conditions can cause varied growth and survival responses of microorganisms within plant organs, resulting in organ-specific selection for or against a set of microorganisms (Compant et al., 2019; Fitzpatrick et al., 2020). The abundance-persistence concepts in ecology can be utilized to determine well-adapted microorganisms consistently interacting with host organs across time and space (Massoni et al., 2020; Nemergut et al., 2013; Rahel, 1990; Stopnisek and Shade, 2021). Aside from these host-microbe interactions, distinct microbe-microbe interactions can emerge from compartment-specific environments (Durán et al., 2018; Hassani et al., 2018). These microbe-microbe interactions can be inferred through correlation networks and consequently microbial hubs that potentially shape microbial community structures can be identified (Agler et al., 2016; Banerjee et al., 2018; Faust and Raes, 2012). Biotic and abiotic factors acting at tempo-spatial scales ranging from plant organs to populations are simultaneously influencing plant microbiomes (Almario et al., 2022; Compant et al., 2019; Fitzpatrick et al., 2020; Müller et al., 2016; Sánchez-Cañizares et al., 2017). By inspecting these biotic and abiotic elements at the level of microbial scales, individual plant scales, to population scales (i.e. at levels of plant organs, below- and above-ground, and between sites, respectively), we can identify factors that significantly contribute to observed community patterns in plant organ microbiomes. While plant organ microbiomes are distinct, they are interconnected via transmission of microorganisms among plant organs and from outside of plant hosts (Compant et al., 2019; Cordovez et al., 2019; Fitzpatrick et al., 2020; Müller et al., 2016; Sánchez-Cañizares et al., 2017; Trivedi et al., 2020). Dispersal events can be inferred by estimating potential microbial sources of plant organ microbiomes (Shenhav et al., 2019). The influence of dispersal on microbial communities via priority effects can also be explored by predicting candidate early-arriving microbes which altered community composition (Debray et al., 2022).

In this study, we investigated the diversity and community composition of microbiomes associated with *Lotus corniculatus* in natural populations. *L. corniculatus*, a common legume species that is widespread in European grasslands, is an ideal system to study assembly processes and variations of microbial communities in natural environments. *L. corniculatus* is a perennial flowering plant that can adapt to a broad range of natural environments - it can grow on different types of soils, is resistant to grazing and mowing, and is host to various insects and bees (Gutiérrez et al., 2001; Hewett, 1985; Jones and Turkington, 1986). *L. corniculatus* is also known to host diverse microbes, including nitrogen-fixing rhizobia bacteria and arbuscular mycorrhiza fungi which enhance the plant host's ability to adapt in poor habitats, as well as non-rhizobial endophytes that promote plant growth (Knežević et al., 2021; Pawlik et al., 2017; van der Heijden et al., 2016). In this study, we also examined the assembly processes that account for the community structures and variations in *L. corniculatus*-associated endophytic communities in the framework of metacommunity theory in ecology by defining the microbial communities associated with roots, shoots, flowers, and seeds as distinct communities linked by transmission of microorganisms from within and outside the plant hosts. Specifically, we aimed (I) to establish that plant organs host distinct microbial communities by using diversity measures and predictive models in machine learning (i.e. organ-specificity of plant microbial communities); (II) to identify well-adapted microorganisms selectively filtered by each host plant organ by utilizing abundance-persistence concepts in ecology and machine learning approaches (i.e. host selection); (III) to infer important microbe-microbe interactions in plant organs by constructing correlation networks and predicting hubs in the organ-specific microbial communities (i.e. microbial interactions); (IV) to explore at different levels of tempo-spatial scales the factors that contribute to plant organ-specific patterns of microbial communities by using diversity measures across sampling years and sites (i.e. biotic and abiotic factors); and finally (V) to predict dispersal events that link distinct plant organ communities by estimating transmission of microorganisms from various microbial sources and predicting microorganisms that potentially have roles in priority effects phenomena (i.e. microbial transmission and priority effects). To address these aims, we collected *L. corniculatus* from seven grassland sites in the Swabian Alps, Germany for four years. We performed amplicon sequencing of microbial 16S rRNA, ITS2, and 18S rRNA genes targeting endophytic communities in plant organs. Results are synthesized in the framework of the metacommunity concept to establish a comprehensive understanding of ecological processes that shape plant microbial community dynamics.

Methods

Collection of L. corniculatus samples in Swabian Alps

To study the ecology of plant microbiomes in natural environments, we collected *Lotus corniculatus* from seven grassland sites in the region of Swabian Alps, Germany for four years (Fig. 4a). These grassland sites of the Biodiversity Exploratories project in southwest Germany have different land use types including unfertilized, mown pastures (AEG3, AEG8, AEG43), fertilized, mown pastures (AEG10, AEG40), and fertilized, mown meadows (AEG17, AEG22) (Blüthgen et al., 2012; Fischer et al., 2010; Ostrowski et al., 2020; Vogt et al., 2019). We collected plant samples every August-September from 2018 to 2021 when plants are flowering and producing fruits. We sampled from each site six plants that were randomly distributed throughout the area. We also sampled soil from where plants were uprooted and then pooled together for each site. To characterize endophytic communities in *L. corniculatus* organs, we separated plant samples into roots, shoots, flowers, and seeds and then surface-sterilized them sequentially with sterile water, epiphyte wash (1X TE + 0.1 % Triton X-100), 80 % ethanol, bleach (2 % NaOCl), and finally sterile water. We stored sterilized samples at -20 0C until processing for DNA extraction.

Amplicon sequencing of L. corniculatus-associated bacteria, fungi, and eukaryotes

We sequenced a total of 700 samples of soil and *L. corniculatus* roots, shoots, flowers, and seeds. We homogenized frozen samples of soil and surface-sterilized roots, shoots, flowers, and seeds in Precellys 24 Tissue Homogenizer (Bertin Technologies) before we extracted DNA using FastDNATM Spin Kit for Soil (MP Bio) as described in the manufacturer's protocol. Extracted DNA, along with blank samples (i.e. water and blank DNA extraction), were used as templates for two-step PCR amplification of bacterial 16S rRNA V5-V7 region, fungal ITS2 region, and eukaryotic 18S rRNA V9 region using primers 799F/1192R, fITS7/ITS4, and F1422/R1797, respectively (Tab. S1) (Agler et al., 2016). We designed blocking oligos using R package “AmpStop” to minimize amplification of mitochondrial and chloroplast 16S rRNA, ITS, and 18S rRNA from *L. corniculatus* (Tab. S1) (Mayer et al., 2021). Amplification products randomized in eight sequencing batches were pooled in equimolar concentrations and purified via magnetic bead clean-up before sequencing on Illumina MiSeq with PhiX control using MiSeq Reagent Kit v3 (600-cycle).

Sequence data processing

We processed amplicon sequence data of microbial 16S rRNA, ITS2, and 18S rRNA using Mothur as described in Almario et al. (Method S1) (Almario et al., 2022; Schloss et al., 2009). We taxonomically classified bacterial 16S rRNA, fungal ITS2, and eukaryotic 18S rRNA sequences based on Greengenes database (13_8_99 release), UNITE database (02.02.2019 release), and PR2 database (version 4.12.0), respectively, with the PhiX genome included in the databases (DeSantis et al., 2006; Guillou et al., 2013; Nilsson et al., 2019). For 16S rRNA and 18S rRNA data, Cutadapt was used to remove primer sequences, and for ITS2 data, ITSx was used to remove non-ITS sequences (Bengtsson-Palme et al., 2013; Martin, 2011).

Diversity and community composition analysis

For diversity analysis and relative abundance calculations, we used R packages phyloseq, vegan, microbiome, and microeco to analyze OTU tables outputted from Mothur pipeline (Lahti and Shetty, 2017; Liu et al., 2021; McMurdie and Holmes, 2013; Oksanen et al., 2025). We used Shannon's diversity and observed species indices to assess alpha-diversity of samples. To check if alpha-diversity measures between samples are significantly different, we tested data whether they are normally distributed via Shapiro-Wilk normality tests and then analyzed using parametric test ANOVA (for normally distributed data) or nonparametric Kruskal-Wallis rank sum test (for non-normal data). We conducted post-hoc analysis using Dunn's test (for Kruskal-Wallis test) or Tukey's HSD (for ANOVA test). For beta-diversity analyses, we used OTU relative abundance tables for Principal Coordinate Analysis (PCoA) ordination of Bray-Curtis dissimilarities between samples. We used PERMANOVA analysis of Bray-Curtis distances to assess significant explanatory variables (i.e. plant organ, years of sampling, sampling sites) affecting microbial community structures.

Identification of key organ-specific microbes in L. corniculatus

To determine abundant endophytes in *L. corniculatus* roots, shoots, flowers, and seeds, we identified OTUs with highest relative abundances in each organ (≥1 % relative abundance). To determine persistent core microbes in organs, we identified OTUs that occur in at least 90 % of the samples across seven sampling sites for four years (≥90 % occurrence). To determine hub bacteria, fungi, and eukaryotes in plant organs, we computed correlation networks for each plant organ using the SparCC algorithm, as described in Almario et al. (Method S2) (Almario et al., 2022; Friedman and Alm, 2012). We used Cytoscape (v.3.9.1) to visualize networks and to calculate network features such as number of nodes and edges, node connectedness (degree), betweenness centrality, and closeness centrality (Shannon et al., 2003). We assigned OTUs that are top 5 % in betweenness centrality and closeness centrality scores as hub microbes. We used representative sequences of abundant, core, or hub OTUs for multiple sequence alignment in MUSCLE (Web Form) using default parameters (Edgar, 2004). We used resulting alignments in ClustalW format to build neighbor-joining phylogenetic trees in the online tool iTOL (v.6.7.3) (Letunic and Bork, 2024).

Machine learning classification for diagnosing multi-organ involvement and predicting organ-specific microbes in L. corniculatus

To explore the possibility of a predictive pattern for separation of *L. corniculatus* organs based on microbial composition, we employed a linear classification machine learning model using relative abundance data of bacteria, fungi, and eukaryotes. We utilized one-vs-one approach on the support vector machine with a linear kernel (OneVsOneClassifier(svm.SVC) function in Scikit-learn) to train a binary classification model for separating each organ from others (Pedregosa et al., n.d.). Using Scikit-learn, we randomly selected 67 % of samples (n=467) for training and reserved the remaining 33 % (n=231) for independent testing and evaluation using the classification_report function, then we visualized confusion matrices using the plot_confusion_matrix function. To identify microbial species that can discriminate each organ from the rest, we employed recursive feature elimination with cross-validation (RFECV function). We calculated the accuracy of the model using K-fold cross-validation (K=10).

Transmission of microbes in L. corniculatus

To determine how *L. corniculatus* organ microbiomes can be linked and influenced by dispersal, we used Sankey diagrams to visualize potential flow of microbes across the soil and plant organs (Method S3). To determine potential origins of organ-associated microbial communities, we used FEAST (Fast Expectation-mAximization microbial Source Tracking) to estimate contribution of potential microbial sources, such as soil, the different plant compartments, or the environment, to each plant organ microbiome (Method S3) (Shenhav et al., 2019). To statistically predict potential priority effects phenomena from roots, shoots, flowers, to seeds in *L. corniculatus*, we identified taxa of interest that are potentially involved in such phenomena in plant organs, as described in Debray et al. (Method S3) (Debray et al., 2022).

Results and Discussion

Endophytic bacterial, fungal, and eukaryotic communities in natural L. corniculatus populations are organ-specific

We surveyed endophytic communities associated with *L. corniculatus* in natural populations by amplicon sequencing of bacteria, fungi, and eukaryotes in 700 samples of soil and plant roots, shoots, flowers, and seeds (Tab. S2, Tab. S3). We used blocking oligos which decreased the number of nontarget plant DNA reads (i.e. chloroplast, mitochondria, plant ITS2, and plant 18S rRNA) by 90-100 % and significantly increased the number of microbial 16S rRNA, ITS2, and 18S rRNA reads (Fig. S1). After processing raw reads and removing unknown and plant sequences, we identified a total of 4,225 16S rRNA, 2,027 ITS2, and 1,773 18S rRNA OTUs clustered based on 97 % sequence similarity and were classified into 113 phyla and 1,542 genera (Tab. S3). Among the plant compartments, roots have the greatest number of OTUs followed by shoots, while there are less OTUs detected in flowers and seeds (Fig. S2a). In all plant compartments, phyla Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Acidobacteria, Ascomycota, Basidiomycota, and unclassified Fungi and Eukaryota are the most abundant groups (Fig. 1a). In addition, in roots phyla Chloroflexi and Nematoda are abundant, while in shoots, flowers, and seeds, Arthropoda and unclassified Bacteria dominate. Soil samples have more observed OTUs compared with all plant organs, with groups Actinobacteria, Proteobacteria, Ascomycota, Basidiomycota, and unclassified Fungi as the most abundant phyla (Fig. 1a, Fig. S2a).

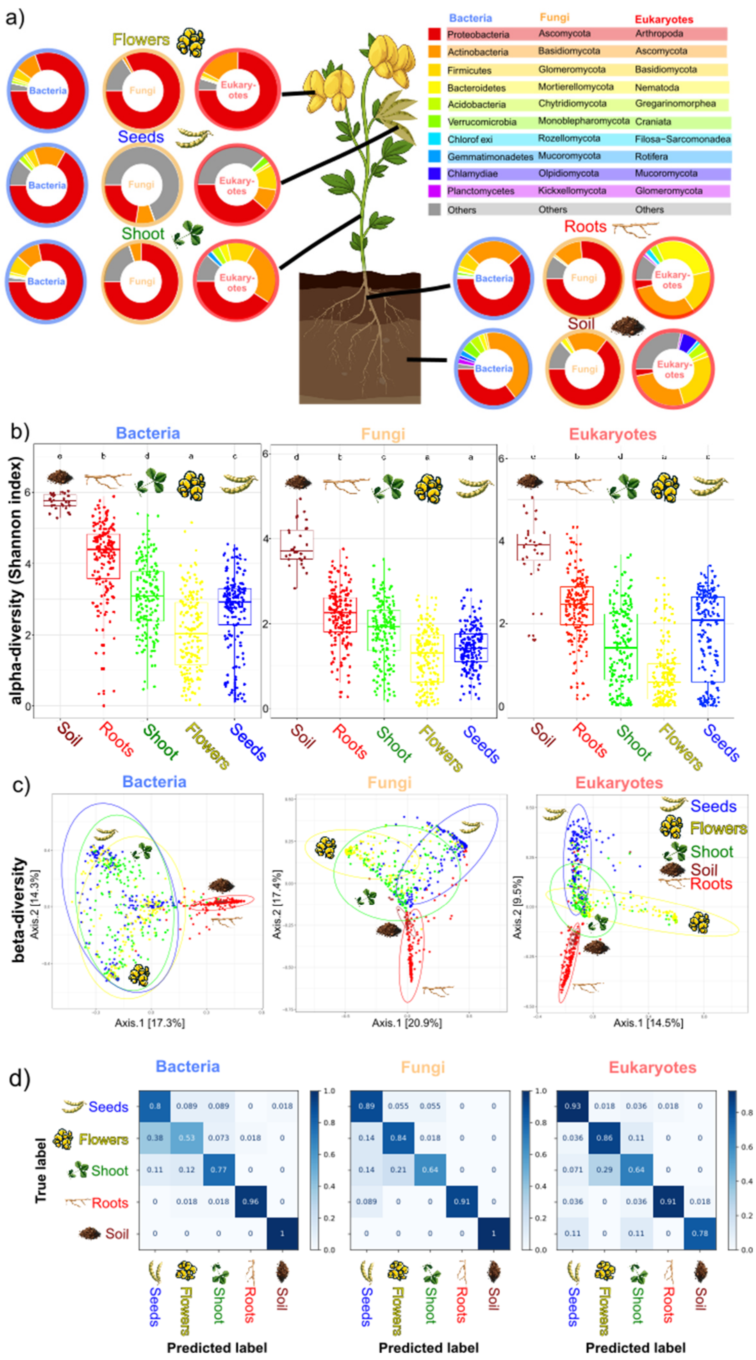

Figure 1: Organ-specificity of plant microbial communities. (a) Relative abundance profiles showing the top ten most abundant bacterial, fungal, and eukaryotic phyla in soil and plant organ samples collected from seven grassland sites for four years. The relative abundance of the OTUs for each compartment (*i.e.* soil and plant organs) were aggregated at the phylum level. (b) Boxplots of Shannon's α-diversity measurements of bacterial, fungal, and eukaryotic microbial communities associated with soil and plant organs. Shapiro-Wilk normality tests indicated that datasets have non-normal distribution ($p<0.05$) and Kruskal-Wallis rank sum tests were used to test significant differences of α-diversity measurements between soil and plant organ samples. Post-hoc analysis via Dunn's tests indicates that groups are significantly different if letters are not similar (Supplementary Table 4). (c) Principal coordinate plots based on Bray-Curtis dissimilarities between bacterial, fungal, and eukaryotic microbial communities associated with all soil and plant organ samples. (d) Performance of the multi-class support vector machine (SVM) model on the test set showing high accuracy in separating bacterial, fungal, and eukaryotic communities in organs and soil samples.

Alpha-diversity analysis showed that endophytic communities in roots are the most diverse compared with communities in aboveground plant organs. Shannon diversity index showed that endophytic communities progressively become less diverse from roots to shoots and flowers, and then an increase in bacterial and eukaryotic diversity is observed in seeds (Fig. 1b, Tab. S4b). Soil microbial communities are more diverse than microbial communities associated with *L. corniculatus* (Fig. 1b, Tab. S4b). Beta-diversity analysis revealed that the diverse and stable microbial communities in soil and roots are distinct from the less diverse and more variable communities in shoots, flowers, and seeds. Bray-Curtis dissimilarities-based PCoA showed that soil and root microbial communities are similar in community structures and are distinct from the overlapping endophytic communities in shoots, flowers, and seeds (Fig. 1c). The PCoA plots showed a main separation (axis 1) of bacterial communities between above and belowground communities while fungal and eukaryotic communities separate the aboveground organs. Both PCoA and relative abundance profiles of individual samples across sampling sites and years also showed that soil and root microbial communities are more clustered and less dispersed compared with the variable microbial communities in shoots, flowers, and seeds (Fig. S3). PERMANOVA indicated that significant separation of microbial communities was largely explained by plant organ, while sampling years and sites contributed relatively lower to microbial community variations (Fig. S4; Tab. S4c).

We trained a multi-class support vector machine (SVM) model to determine whether relative abundances of bacterial, fungal, and eukaryotic microbial communities could distinguish between each organ from other organs (all binary possibilities) and soil samples. The performance of the model on the test set showed high accuracy in separating organs and soil samples (accuracy = 77-83 %; Tab. S5). In bacterial and fungal communities, roots and soil samples separated from other groups with higher prediction accuracy compared with aboveground organs, while in eukaryotic communities, seeds are most accurately predicted among all the groups (Fig. 1d). We trained an SVM classifier with recursive feature elimination and cross-validation to identify OTUs that discriminate each organ from the others (without soil samples). Results revealed a subset of 57-166 OTUs that could separate roots, shoots, flowers, and seeds from other organs (Fig. S5).

Based on diversity analyses and predictive models, results show the organ-specificity of endophytic communities in natural *L. corniculatus* populations. Root microbial communities are the most diverse among the plant organ communities. There is significant separation of community structures between aboveground and belowground bacterial communities, while shoot, flower, and seed communities are mainly separated by fungi and eukaryotes. The aboveground endophytic communities are more variable compared with the root communities. Soil microbial communities, which are more diverse than all plant organ communities, have overlapping community composition with the root communities.

L. corniculatus core microbiomes are shaped by host selection

To identify key microbes which can explain the identified organ-specific patterns in community structures of *L. corniculatus* microbiomes, we examined species abundance, an ecological pattern that is observed in macrobial and microbial communities (Fig. 2, Tab. S6). Except for *Pantoea* and *Pseudomonas*, which are abundant in both above- and below-ground plant organs, the most abundant OTUs (relative abundance (RA) > 1 %) in root microbial communities are distinct from the set of abundant OTUs in aboveground microbial communities. Similarly in microbial communities of shoots, flowers, and seeds, the most abundant OTUs (RA > 1 % in at least one plant organ) are mostly the same and enriched specifically in the aboveground microbial communities. The observed similarity in the sets of abundant taxa accounted for the observed overlapping community structures of the aboveground plant organ communities despite high variability throughout sampling years and sites (Fig.1c, Fig. S3). Thus, the overlapping community structure in shoots, flowers, and seeds, as well as the distinction between aboveground and belowground plant organ community structure, can be largely explained by the most abundant species.

Persistence of species over time and space is also an ecological pattern that is important in structuring macrobial and microbial communities. We identified persistent organ-specific ‘core’ microbes that can be detected in 90 % of *L. corniculatus* organ samples collected across all seven sites and four years (Fig. 2, Tab. S7). In roots we found 56 bacterial, seven fungal, and seven eukaryotic core OTUs, which consists 1 % of all 7,504 root OTUs. Many of the core species identified in roots are also abundant species in roots. In shoots there are seven bacterial, four fungal, and four eukaryotic core OTUs (0.3 % of 5,223 total shoot OTUs), while in flowers and seeds there are seven (0.2 % of 3,706 total flower OTUs) and eight (0.3 % of 3,706 total seed OTUs) core OTUs, respectively. Likewise, most of the core species in aboveground communities are also abundant in their respective plant organ microbial communities. The smaller core communities in shoots, flowers, and seeds suggest fluctuating aboveground microbial communities, in contrast with the more stable root communities with a relatively larger core community. Meanwhile, *Pseudomonas* and *Cladosporium* are persistent over time and space in all plant organs, suggesting stable associations and high adaptation to the plant endophytic environment.

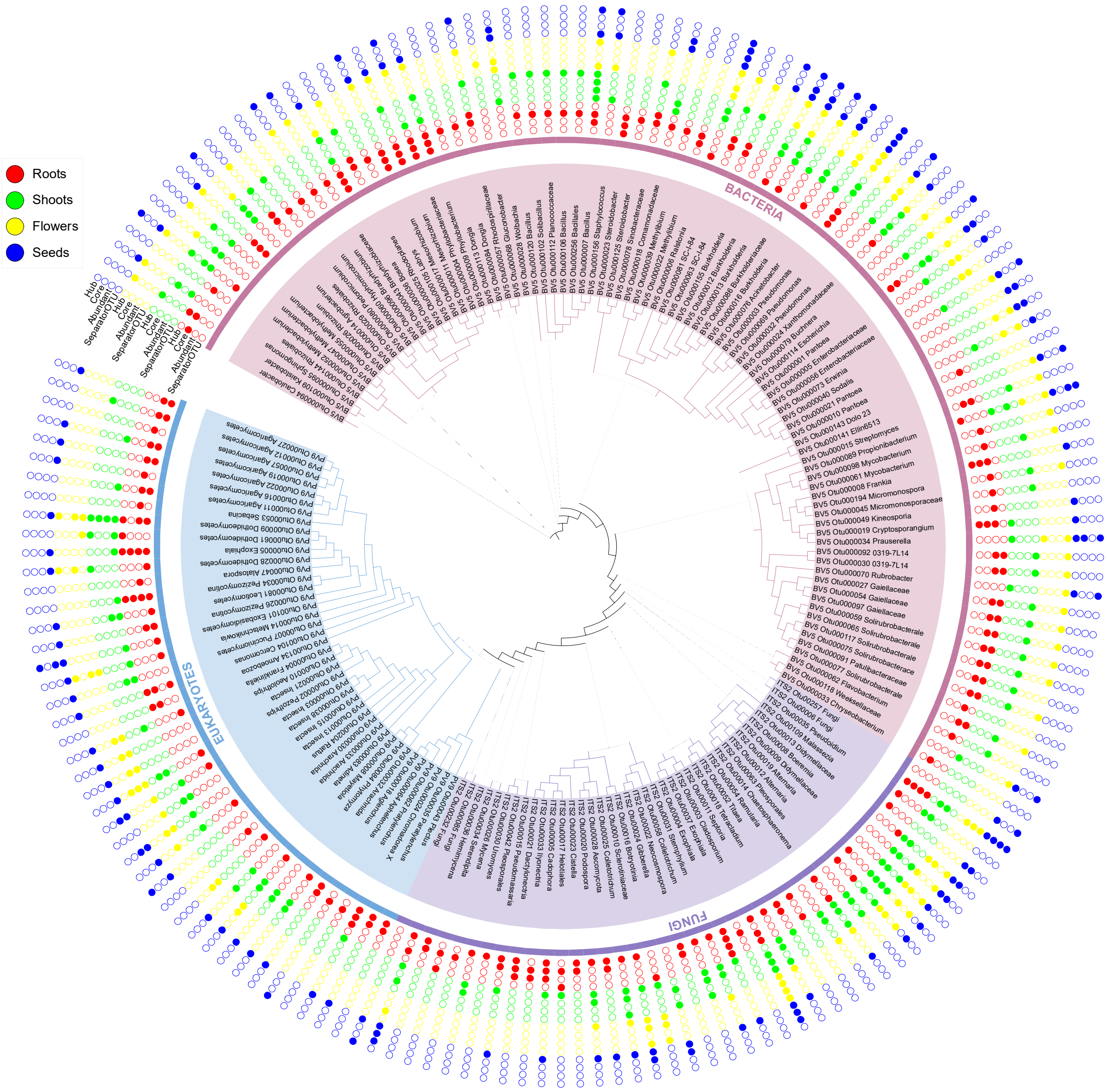

Figure 2: Organ-specific microbes identified via abundance, persistence, and predictive machine learning models. Neighbor-joining phylogenetic tree of top abundant OTUs (relative abundance in the plant organ microbial communities > 1 %) and core OTUs (detected in 90 % of the organ samples). Abundant and core OTUs that are either separator OTUs (all identified separator OTUs using multi-class support vector machine (SVM) model are detailed in Supplementary Fig. 5) or hubs (hubs identified using microbial networks are detailed in Fig. 3 and Supplementary Fig. 6) are also indicated.

To determine microbes that distinguish between *L. corniculatus* organs, we used multi-class support vector machine (SVM) model that predicts OTUs that are highly associated with each organ (Fig. S5). In root microbial communities, 84 separator OTUs were identified with the root compartment. Among these OTUs are abundant species in roots and are persistent throughout years and sites of collection (Fig. 2, Tab. S7). *Pseudomonas*, *Phyllobacterium*, *Frankia*, *Mesorhizobium*, *Cryptosporangium*, *Steroidobacter*, *Rhizobium*, and *Bosea* are abundant and core bacteria that showed high association with roots. Abundant and core fungi such as *Exophiala*, *Cadophora*, and *Dactylonectria* are also distinctive of root microbial communities. In shoot microbial communities there are 102 separator OTUs identified, among these OTUs are core and abundant species *Bacillus*, *Agrobacterium* and *Alternaria* (Fig. 2, Tab. S7). There are 57 OTUs that are highly associated with the flower compartment including abundant and core fungi *Cladosporium* and insects *Pezothrips* (Fig. 2, Tab. S7). There are 166 OTUs that are distinctive of seed microbial communities (Fig. 2, Tab. S7). *Pseudomonas*, *Ralstonia*, and *Cladosporium* are abundant and persistent species that are highly associated with seeds. *Ralstonia* are known pathogens of various plants (Hayward 1991). Different resources or habitat conditions in plant organs could result in the filtering of different groups of microorganisms adapted to organ-specific features, consequently resulting in organ-specific distinctive microbial communities (Aschehoug et al., 2014; Bizabani and Dames, 2015; Donati et al., 2018; Gelvin, 2009; Hamayun et al., 2009; Laranjo et al., 2014; Lindow and Suslow, 2003; Lopes et al., 2018; Navarro-Campos et al., 2012; Prasannath et al., 2021; Rivas et al., 2009; Santos et al., 2021; Thomma, 2003). These microorganisms that are selectively filtered by each of the plant organs are likely specialized microbes that are able to thrive in the unique physical microstructures of the organs while improving plant growth, nutrient uptake, and resistance to stress and diseases They could supply nutrients while in turn benefitting from resources in particular organs. Alternatively they could be pathogens that hijack the host's genetic resources.

Community structures of L. corniculatus microbiomes are influenced by microbial interactions

Species interactions are also important in maintaining structures of macrobial and microbial communities. Microbial species uniquely adapted to a particular organ environment that can significantly affect microbial interactions consequently can also affect organ-specific microbial community structuring. To infer potential microbe-microbe interactions and to identify hubs in the plant organ communities, we built correlation networks based on species abundances (Fig. 3). In general, root microbial community networks are more complex than networks of aboveground organs. In addition, bacterial networks are more complex compared with fungal and eukaryotic networks in all plant organs. The number of nodes is consistently highest in roots in all bacterial, fungal, and eukaryotic networks, followed by microbial community networks of shoots, flowers, and seeds. (Fig. 3b). Root networks also have the highest number of edges, which correspond to significant correlations between microbes, compared with aboveground organ networks (Fig. 3c). Based on ANOVA and Tukey's HSD, the nodes of root microbiome networks have the highest number of interactions among all the organ microbiome networks (Fig. 3d). Aboveground, shoot microbiome networks have higher node connectedness compared with flower and seed networks, which have node connectedness that are not significantly different. There are more nodes that are shared between shoots and flowers in bacterial and fungal networks compared with other organ-to-organ networks, while in eukaryotic networks the root-to-shoot networks have the highest number of shared nodes (Fig. 3e).

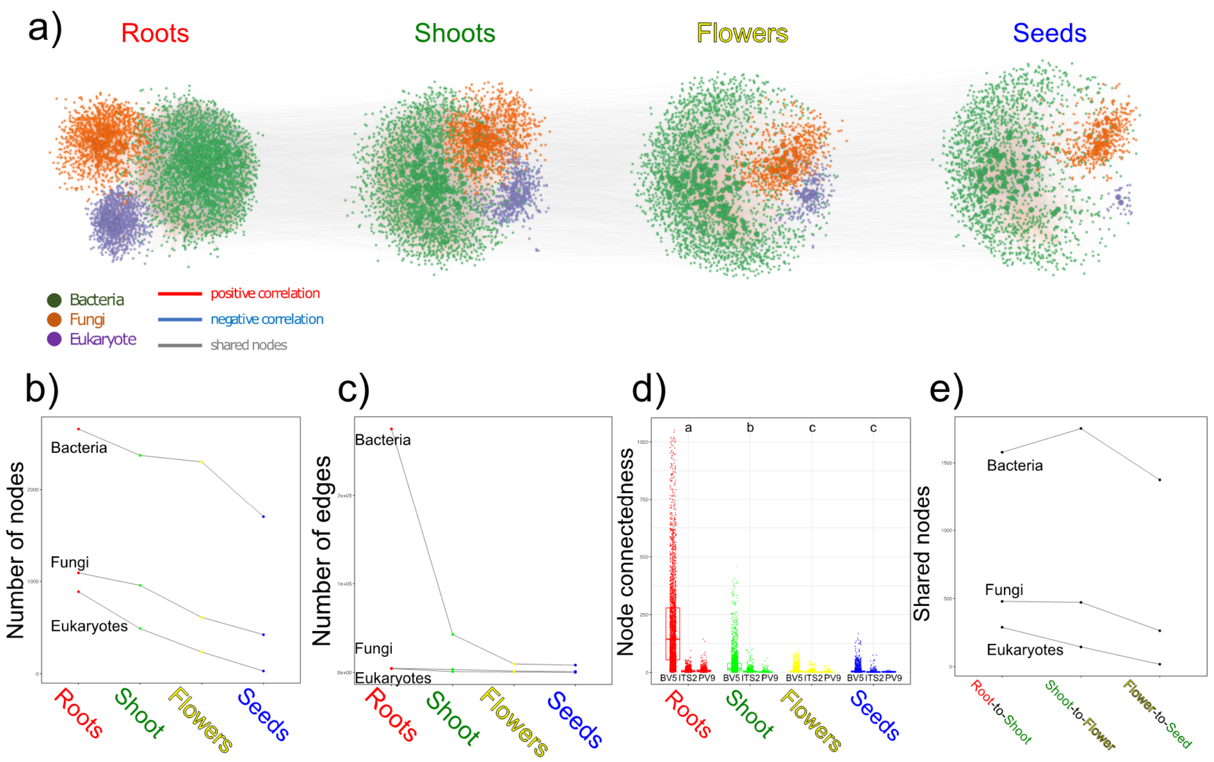

Figure 3: Microbial interactions in organ-specific plant microbiomes. (a) Correlation networks based on species abundance of bacterial (green), fungal (orange), and eukaryotic (purple) microbes of each plant organ. Nodes are OTUs and edges represent potential microbe-microbe interactions. Significant positive interactions are colored red, while negative interactions are blue (P<0.001). Hub microbes of each organ microbial communities are represented by bigger and bordered nodes. Grey lines that connect each organ microbiome network show connections of nodes/OTUs that are shared with the next organ. Network characteristics such as number of (b) nodes and (c) edges, (d) node connectedness (degree), and (e) nodes shared between organs are used to describe the networks. Overall (d) node connectedness (node degree of all bacterial (BV5), fungal (ITS2), and eukaryotic (PV9) networks) were compared between organ microbiome networks via ANOVA and Tukey's HSD.

Hub microbes, which are highly connected with other microbes in the network, potentially have essential roles in plant-microbe and microbe-microbe interactions and thus shape the community structure (Agler et al., 2016). We identified hub microbes which can significantly influence microbial community structures in each plant organ based on betweenness centrality and closeness centrality scores (Fig. 2, Fig. S6, Tab. S7). Many among the hub microbes are abundant and persistent across seven sampling sites throughout four years. In root microbial communities there are 101 bacterial, 39 fungal, and 32 eukaryotic hubs, which comprise 2 % of total root OTUs. Many of the inferred hubs are also machine learning-predicted separator microbes in roots, including the hub fungi Exophiala that are both abundant and persistent species in roots. Shoot microbial communities comprise 97 bacterial, 33 fungal, and 13 eukaryotic hubs (3 % of all shoot OTUs). Separator microbes for the shoot compartment *Bacillus*, *Alternaria*, and Dothideomycetes are hub microbes that are both persistent and abundant in shoots. Flower-associated microbial communities have 90 bacterial, 23 fungal, and 8 eukaryotic hubs, which consists 3 % of total flower OTUs. Expectedly, most of the hubs are separator microbes, including *Cladosporium*, species that are also abundant and persistent in flower compartments. In seed microbial communities, there are 61 bacterial, 14 fungal, and 4 eukaryotic hubs, which is 2 % of total seed OTUs. *Pseudomonas* and *Ralstonia* are hub microbes that are both core, abundant, and seed separator microbes. Among the predicted hubs in the plant organs are microbes that are abundant, or persistent across sampling years and sites, and highly associated with the respective organ compartments and are thus important in diversity, stability, and organ-specificity of the microbial communities. These hub microbes either as pathogens or beneficial endophytes play important roles in microbial interactions and can be important in shaping microbial communities of each plant organ (Aschehoug et al., 2014; Donati et al., 2018; Hamayun et al., 2009; Lindow and Suslow, 2003; Lopes et al., 2018; Prasannath et al., 2021; Santos et al., 2021; Thomma, 2003).

Variation in L. corniculatus microbiomes are driven by biotic and abiotic factors across time and space

Beta-diversity analysis of microbiomes in *L. corniculatus* natural populations revealed that plant organs largely contributed to microbial community variation, while years of collection and sampling sites showed smaller effects (Fig.1c, Fig. S4, Tab. S4c). To further dissect other factors that can influence community structure in *L. corniculatus* microbiomes, we analysed diversity and community composition on each plant organ across sampling years and sites. Throughout sampling sites and years, *L. corniculatus* plants as a whole maintained significantly different levels of microbiome alpha-diversity (except the fungal community), and at least one of the bacterial, fungal, or eukaryotic communities of the plant organs exhibited significantly different diversities (Fig. S7, Fig. 4b-g). Relative abundance profiles of the most abundant bacterial, fungal, and eukaryotic groups in the plants also vary across all sampling sites and years (Fig. S8). Specifically, in roots the diversity of microbial communities is similar across all sampling plots, and while the diversity of fungal and eukaryotic communities are also similar across all sampling years, root bacterial communities exhibited different alpha-diversities throughout the years (Fig. 4b-g). In contrast, microbial communities in aboveground plant organs have significantly different alpha-diversities throughout the years, except in fungal communities of all aboveground organs and in shoot eukaryotic communities (Fig. 4e-g). At least one of the bacterial or fungal communities associated with shoots, flowers, and seeds showed significantly different alpha-diversities across different sampling sites, while eukaryotic communities are similar (Fig. 4b-d).

Beta-diversity analysis of *L. corniculatus* microbial communities indicated that years of collection accounted more on the observed variation in bacterial communities compared to sampling sites (Fig. S4, Tab. S4c). Specifically, while in roots both variables have almost equal effects on variation, the influence of years of collection is higher in the aboveground microbial communities (Fig. 4h, Fig. S9a). These observations show that bacterial communities in roots are relatively more stable across all years and sites, while aboveground communities varied through years of collection, consistent with the relative abundance profiles of bacterial communities in plant organs throughout all years and sites (Fig. S3a-b). *L. corniculatus* are perennial plants, hence the roots could have maintained stable bacterial communities over the years, while in aboveground plant organs the communities are more variable due to consistent perturbations in the sampling sites such as mowing or animal grazing. The recurrent emergence of shoots, flowers, and seeds amidst such perturbations potentially contributed to the variation in bacterial communities in these organs (Shade et al., 2013; Smets et al., 2022). On the other hand, fungal and eukaryotic community structures in all plant organs except seeds are influenced to a larger extent by sampling sites (Fig. 4i-j, Fig. S9b-c). The larger effect of sampling sites suggests that local environmental conditions and soil microbial communities of the different grassland sites have larger influence on the variation of fungal and eukaryotic communities associated with *L. corniculatus* roots, shoots, and flowers. Bacterial, fungal, and eukaryotic community variations in the soil are also influenced largely by sampling sites (Fig. 4h-j, Fig. S9), hence soil microbiomes could have contributed to the beta-diversity patterns observed in the plant organs. Disturbances in the local environments like temperature fluctuations, wind, UV levels, and precipitation, and consequently plant adaptations to these changes, can also potentially augment to the variations of fungal and eukaryotic communities in roots, shoots, and flowers. Insect visitors present in the sampling sites, especially pollinators, can also affect microbial community composition in plants (Vannette, 2020). Thus, observed patterns of diversity and composition could be attributed to abiotic factors and biotic interactions acting at different spatial and temporal scales, that is, at the level between roots and aboveground plant organs to the level of sampling locations or years.

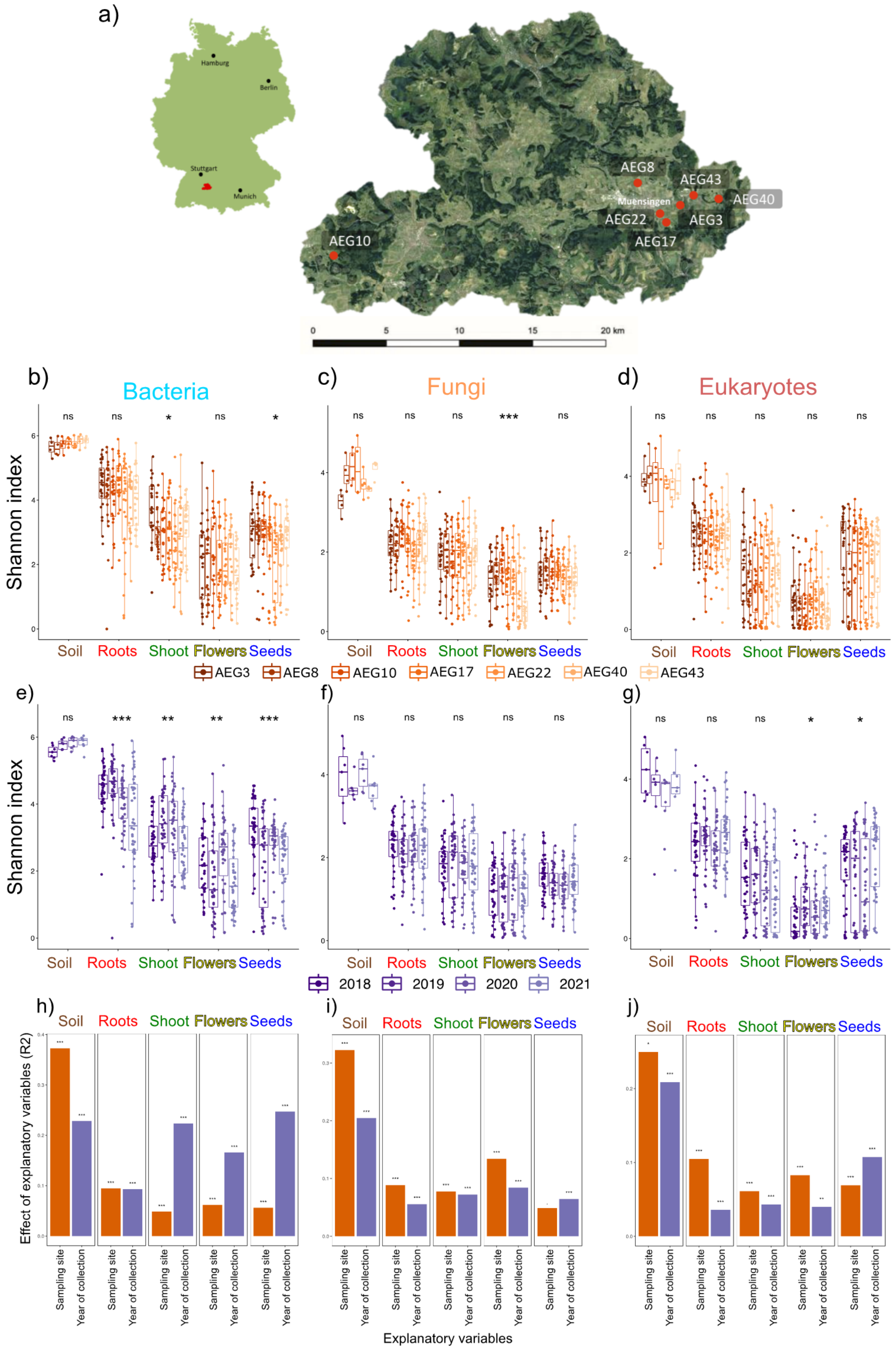

Figure 4: Diversity and community composition of plant organ-associated microbiomes in *Lotus corniculatus* populations in seven grassland sites for four years. (a) Map of the seven grassland plots in the Swabian Alps, Germany where samples were collected from years 2018-2021. α-diversity (Shannon index) of the bacterial, fungal, and eukaryotic microbial communities associated with soil and plant organs were compared between (b-d) sampling sites or (e-g) collection years using Kruskal-Wallis significance test. The effect of explanatory variables (*i.e.* sampling sites, year of collection) on the β-diversity of soil- and plant organ-associated microbial communities were also assessed using (h-j) PERMANOVA.

L. corniculatus microbiomes are distinct communities linked and influenced by dispersal

To investigate how *L. corniculatus* recruits microbes to assemble into organ-specific communities, we compared OTUs detected across soil and plant organs (Fig. 5a). The distinct but overlapping plant organ microbiomes share a subset of their communities. Most OTUs that are present in plant organs are also detected in soil samples. With increasing distance from the soil, soil-detected OTUs in the plant organs decrease (Fig. S10). Only a small proportion of the core communities in roots are transmitted to upper plant organs. Core microbes *Pseudomonas* and *Cladosporium* are potentially good disperser microbes that are consistently transmitted and maintained throughout the interconnected plant organs (Fig. 2). Mostly, the OTUs in plant organs are transmitted from neighboring compartments and a lesser proportion are from other sources, presumably from the local environment. These observations suggest that there are similarities between plant organ microbial communities due to initial colonizer microbes from soil, while variations in community composition are accumulated through various microbial sources from the environment as well as from other plant compartments. Thus, plants establish their organ-specific microbiomes by recruitment and selecting microbes from soil and their environment as well as by transmission of microbes from other plant compartments.

To further verify these observations, we used FEAST to determine potential origins of the organ-specific microbiomes (Fig. 5b). FEAST was used to estimate the contribution of potential microbial sources, such as soil, the different plant compartments, or the environment, to each plant organ microbiome. Since in FEAST the source and sink communities are preassigned and consequently the direction of transmission is not determined, we tracked the transmission at multiple directions (Fig. S11). We observed that there is potential dispersal of microbes from various microbial sources and multiple directions. A large proportion of plant microbes are dispersed between plant compartments (13 %-92 % of microbes in sink organs are from other plant compartments). Soil microbes are transmitted to all plant organs, and aboveground plant organs tend to have lesser soil microbes compared with roots. Certain fractions of plant organ microbiomes were assigned by FEAST to "unknown sources", which can be other potential microbial sources from the environment such as insects, pollinators, animals, rain, wind, or soil splashes. These calculations showed that dispersal occurs between plant organ microbiomes as well from soil and environment and thus influences community structures of these organ-specific microbiomes.

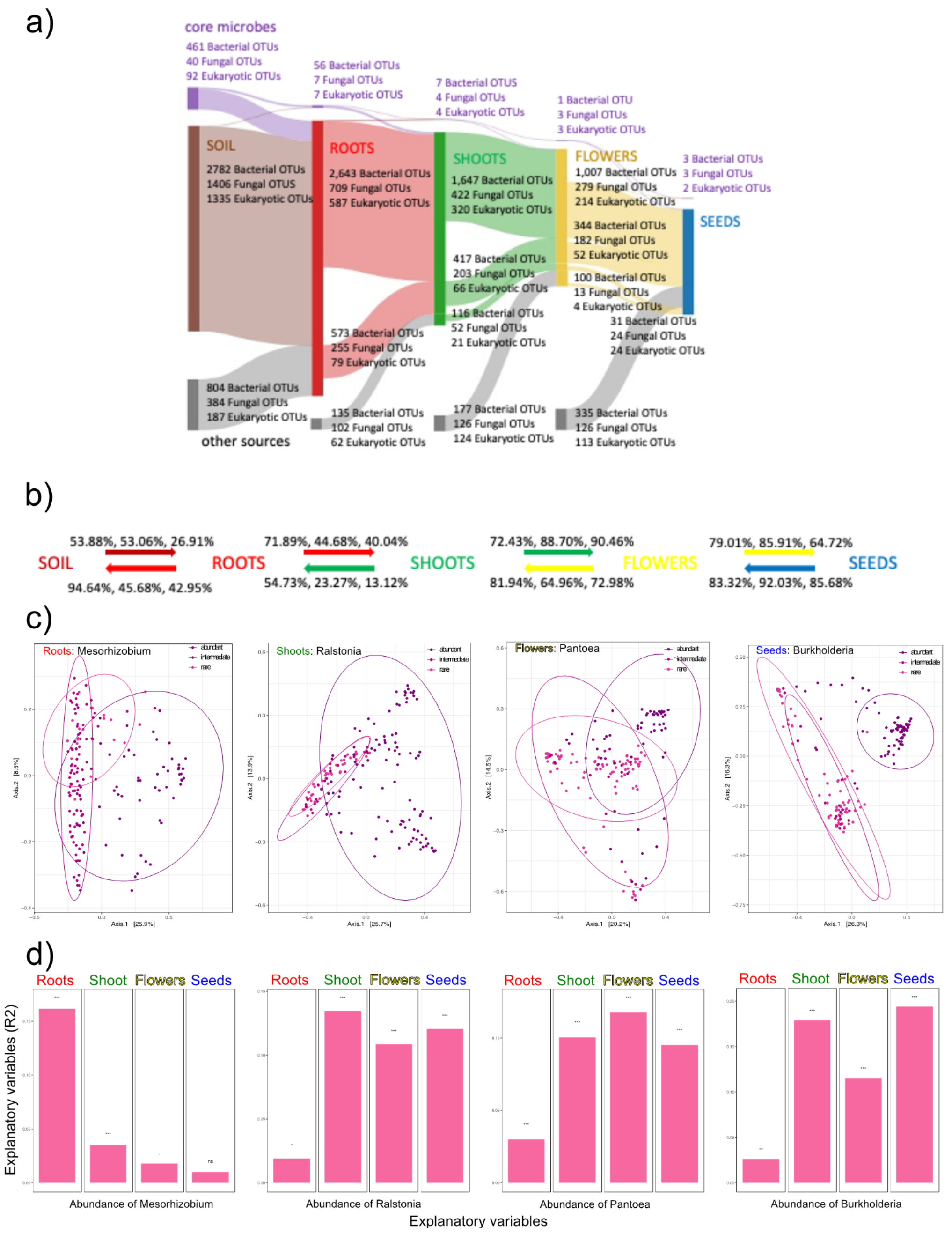

Figure 5: Recruitment of microbes from soil and environment to establish organ-specific microbiomes in *Lotus corniculatus*. (a) Sankey diagram of shared microbes between neighboring plant compartments as well as from other sources (*i.e.* from local environment). Nodes of the diagram represent different potential microbial sources (*i.e.* soil, plant organs, and environment/others) and arcs correspond to the number of bacterial, fungal, and eukaryotic OTUs shared between nodes. The number of core OTUs in each plant organ are displayed in purple nodes and arcs in the diagram. (b) FEAST was also used to estimate the contribution (% bacterial, fungal, and eukaryotic contribution, respectively) of potential microbial sources to each plant organ microbiome. In FEAST the direction of microbial transmission is preassigned, thus calculation of microbial contribution was tracked at both directions. For more details of transmission at multiple directions, see Supplementary Fig. 11. (c) PCoA of Bray-Curtis dissimilarities of plant organ microbial communities and (d) PERMANOVA using abundance of candidate early-arriving OTU as explanatory variable of community composition variation. (c) and (d) show that candidate early-arriving OTUs (BV5_OTU11_*Mesorhizobium*, BV5_OTU6_*Ralstonia*, BV5_OTU1_*Pantoea*, BV5_OTU16_*Burkholderia*) potentially altered the microbial community composition of roots, shoots, flowers, and seeds, respectively, based on their abundance in the plant organ microbial communities. (Explanatory variables: abundant OTU: Relative abundance in each plant organ microbial community (RA) >= 0.01; intermediate OTU: RA >= 0.001 and < 0.01; rare OTU: RA < 0.001).

Dispersal can also affect microbial community diversity through arrival history. A set of early-arriving species can impact assembly of communities by changing resources or environmental conditions, in a historically-contingent community assembly called priority effects. Debray et al. presented an approach to statistically predict from a natural microbiome dataset which taxa are potentially involved in influencing succession during community assembly (Debray et al., 2022). Based on such approach, we identified candidate early-arriving OTUs that may have inhibited or facilitated establishment of other OTUs by examining if their abundance in plant organs correlated with altered community composition. Changes in relative abundance of some of the key OTUs in plant organs consequently showed changes in community structure (Fig. 5c-d, Fig. S12). For instance, relative abundances of *Phyllobacterium* and *Mesorhizobium* contributed to the variation of microbial communities in roots, while relative abundances of *Pantoea*, *Ralstonia*, and *Burkholderia* accounted for the microbial community variations in aboveground organs. *Phyllobacterium* and *Mesorhizobium* are abundant, persistent, and highly associated microbes in roots (Fig. 2). *Pantoea* are abundant throughout the whole plant and are core microorganisms in shoots, while *Ralstonia* and *Burkholderia* are key microbes (*i.e.* either abundant, core, hub, or separator microbes) in most of aboveground organs (Fig. 2). The prediction of these taxa and their potential roles during priority effects phenomenon enables future experimental manipulation of arrival history in complex natural microbial communities.

Conclusion

We examined the diversity and community composition of *Lotus corniculatus* microbiomes in natural populations in the framework of metacommunity theory to gain broader insight on the ecology of plant microbiomes in situ. In this study we showed the organ-specificity of endophytic communities of *L. corniculatus* and established an overview of the assembly processes at tempo-spatial scales that account for the community patterns observed in plant microbiomes in natural populations (Fig. 6). Analysis of community composition and diversity of *L. corniculatus* microbiomes revealed that plant organs are the main source of variation in microbial community structure, while sampling years and sites contributed less. It has been shown that plant compartments contribute more in shaping microbial community composition than geographical locations or sampling times (Hamonts et al., 2018; Smets et al., 2022; Zheng and Gong, 2019). Other studies found that plant compartments account more for associated bacterial community composition, while geographical locations of host plants rather determine fungal community composition (Bernard et al., 2021; Bulgarelli et al., 2012). Plant organs select for a group of microorganisms that developed adaptive traits to successfully inhabit their unique microenvironments, and in *L. corniculatus* organs abundant and persistent microorganisms are beneficial microbes that enhance growth and fitness, or pathogens that utilize host's genetic and physiological resources. In the same way, hub microbes in *L. corniculatus* organs are abundant and persistent microbes that are either known plant pathogens or beneficial to plant hosts.

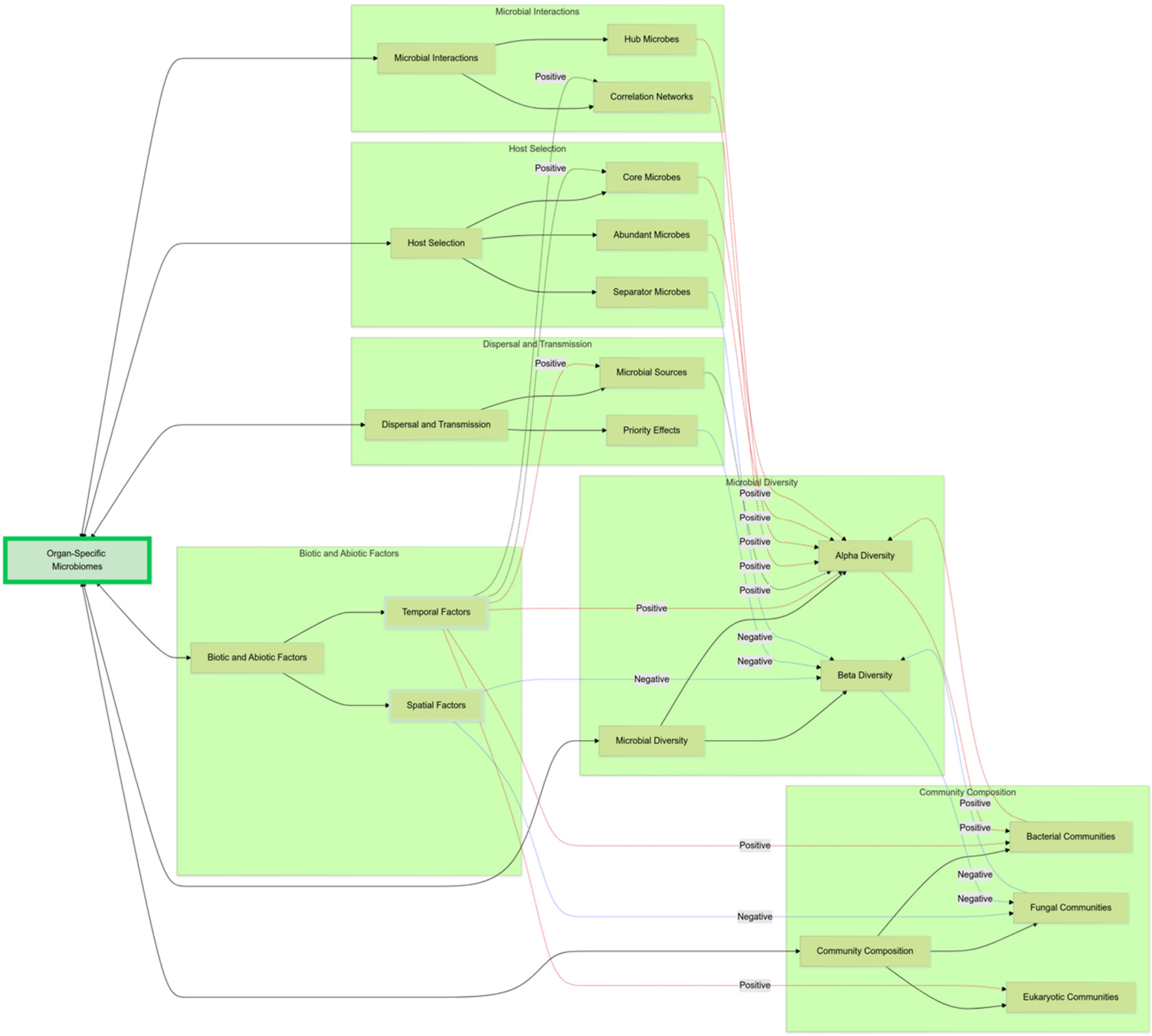

Figure 6: Ecological processes at different tempo-spatial scales that shape metacommunity dynamics in organ-specific *Lotus corniculatus* microbial communities. This figure integrates results from multiyear, multisite sequencing of bacterial, fungal, and eukaryotic communities in *L. corniculatus*, summarizing how spatial, temporal, and ecological processes shape organ-specific microbiomes within a metacommunity framework. Arrows indicate effects supported by results in the manuscript. Solid lines represent hierarchical relationships in the framework (*e.g.*, microbial diversity as a component of organ-specificity), while dotted lines indicate functional effects extracted from observed data: red for positive influences and blue for negative influences. For instance, temporal factors positively influenced alpha diversity in aboveground plant parts, whereas spatial factors had a stronger effect on the beta diversity of fungal and eukaryotic communities. Transmission processes such as microbial sourcing and priority effects further shaped organ-specific microbiomes by introducing stochastic variation and assembly history-dependent patterns.

L. corniculatus organs host distinct but overlapping microbial communities linked via transmission of microorganisms within the plant host and the outside environment, which signifies that the organs are discrete ecological niches that are interconnected with each other and with the environment. *L. corniculatus* organ-associated microorganisms can also potentially influence community composition during dispersal via priority effects. Consistent with previous observations, the root microbiomes are the most diverse communities among the plant organ communities and have distinct but overlapping community composition with soil microbiomes (Bodenhausen et al., 2013; Bulgarelli et al., 2012; Hamonts et al., 2018). The overlapping and less diverse aboveground microbiomes that are distinct from root and soil microbiomes demonstrated the compositionally-nested characteristic of microbial communities observed in several plant species, where the aboveground communities are subsets of the more diverse belowground communities (Bernard et al., 2021; Coleman-Derr et al., 2016; Hamonts et al., 2018). While the main source of microbiome variation is plant compartment, biotic and abiotic factors from the environment also contribute to patterns of community structures in *L. corniculatus* microbiomes. Plant microbial community structure is also shaped by environmental gradients such as local site conditions, land use, and soil properties, as well as biotic elements like pollinators, insects, and local fauna (Pirttilä et al., 2023; Schlatter et al., 2020; Vannette, 2020; Whitaker et al., 2018; Zarraonaindia et al., 2015). Abiotic and biotic elements in the environment acting at multiple temporal and spatial scales affect *L. corniculatus* microbiomes - while different sampling times and locations affected plant microbial community composition, environmental factors acting at different plant compartments also attributed to the more diverse and stable root microbial communities that are distinct from the less diverse and more variable but overlapping endophytic communities in shoots, flowers, and seeds.

In this study we present bases for future experimentation to explore mechanisms on how key members of *L. corniculatus* organ microbiomes influence community dynamics and species interactions. Variations in community diversity and composition observed at tempo-spatial scales (*i.e.* from differences between root and aboveground plant microbiomes to between year/site variations) provided basis for more in-depth investigation of the crucial roles of abiotic environmental factors, such as soil properties like land use history and soil chemistry, climate and temperature differences in soil and aboveground, or elevation gradients, in shaping these community patterns. This study also provided a requisite basis to test predictions on important agents of horizontal transmission in the environment such as pollinators or on seed microbiomes as initial colonizers during vertical transmission. While in this study we focused on selective filtering by plant organs, microbial interactions, and environmental factors, as well as on stochastic transmission of microorganisms from various sources, other factors such as plant genotype diversity, wider range of geographic locations, or other ecological processes such as genetic drift and diversification of microbiome members additionally cause variations in community dynamics of plant-associated microbiomes. Given the functions of plant-associated microbial communities in plant growth, stress tolerance, and protection, reconstruction of plant microbiomes offers prospects to maximize their beneficial effects for plant productivity, resilience, and pathogen defense. To successfully control plant microbiomes in the field, there remains a need for a comprehensive knowledge of the ecological processes and microbial interactions that shape microbial community dynamics and assembly in natural environments.

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Supplementary material

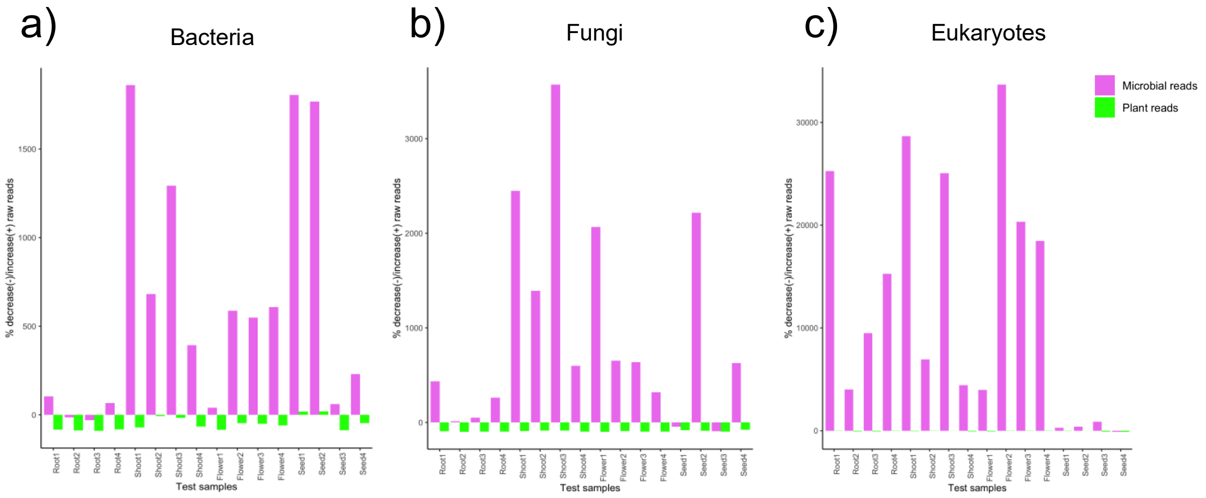

Figure S1: Increase of target (a) bacterial 16S rRNA, (b) fungal ITS2, and (c) eukaryotic 18S rRNA reads and decrease of corresponding reads of nontarget host *Lotus corniculatus* chloroplast, mitochondria, plant ITS2, and plant 18S rRNA when blocking oligos are used during library preparation of test sequencing samples.

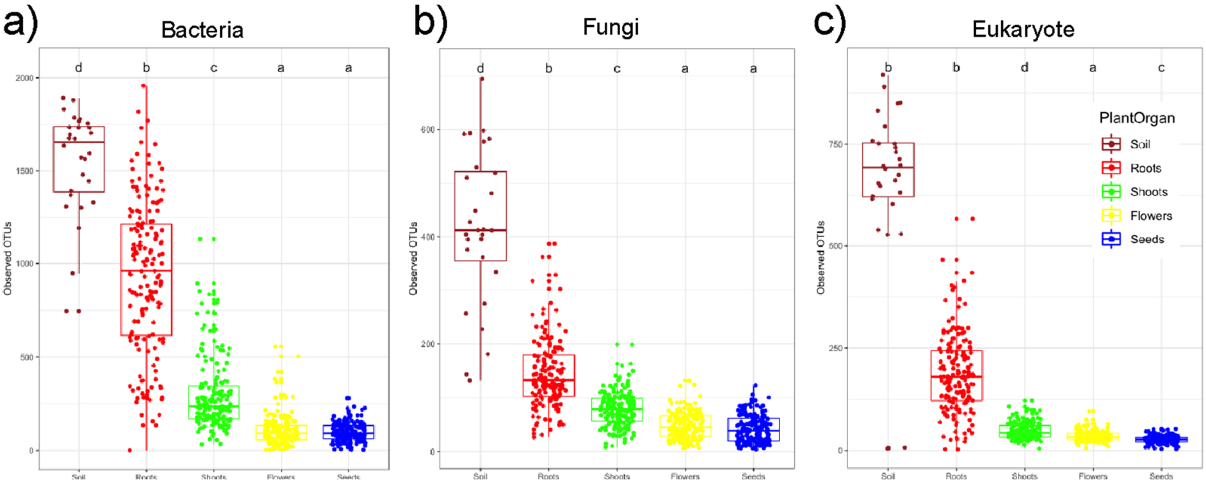

Figure S2: Boxplots of Observed OTUs of **(a)** bacterial, **(b)** fungal, and **(c)** eukaryotic microbial communities associated with soil and *Lotus corniculatus* plant organs. Datasets have non-normal distribution based on Shapiro-Wilk normality tests ($p<0.05$) thus Kruskal-Wallis rank sum tests were used to test significant differences of α-diversity measurements between soil and plant organ samples. Compartments are significantly different if letters are not similar based on post-hoc analysis via Dunn's (Supplementary Table 4).

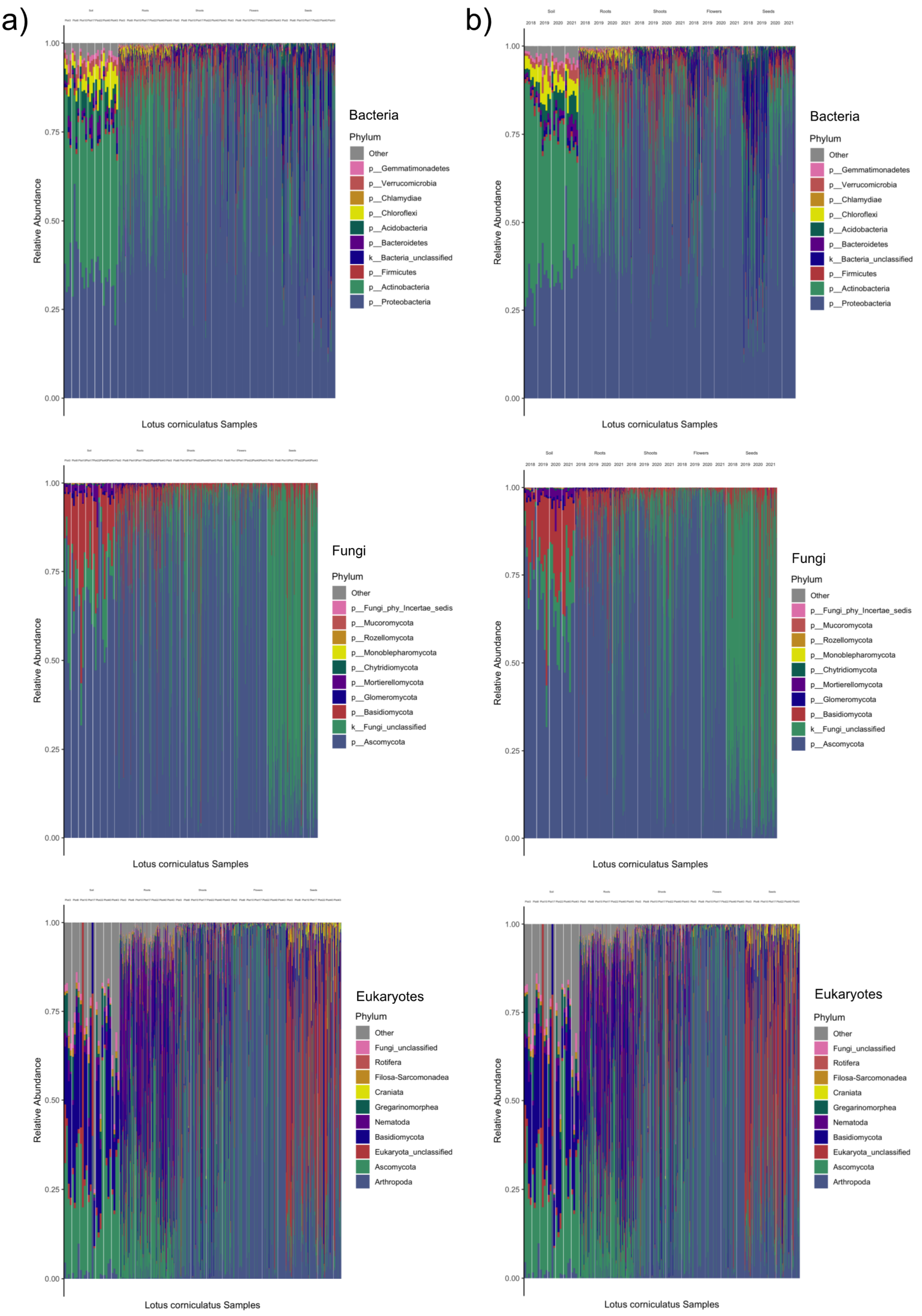

Figure S3. Relative abundance of the top ten most abundant bacterial, fungal, and eukaryotic phyla detected in all soil and plant samples collected from **(a)** seven grassland sites for **(b)** four years. The relative abundance of the OTUs for each sample were aggregated at the phylum level.

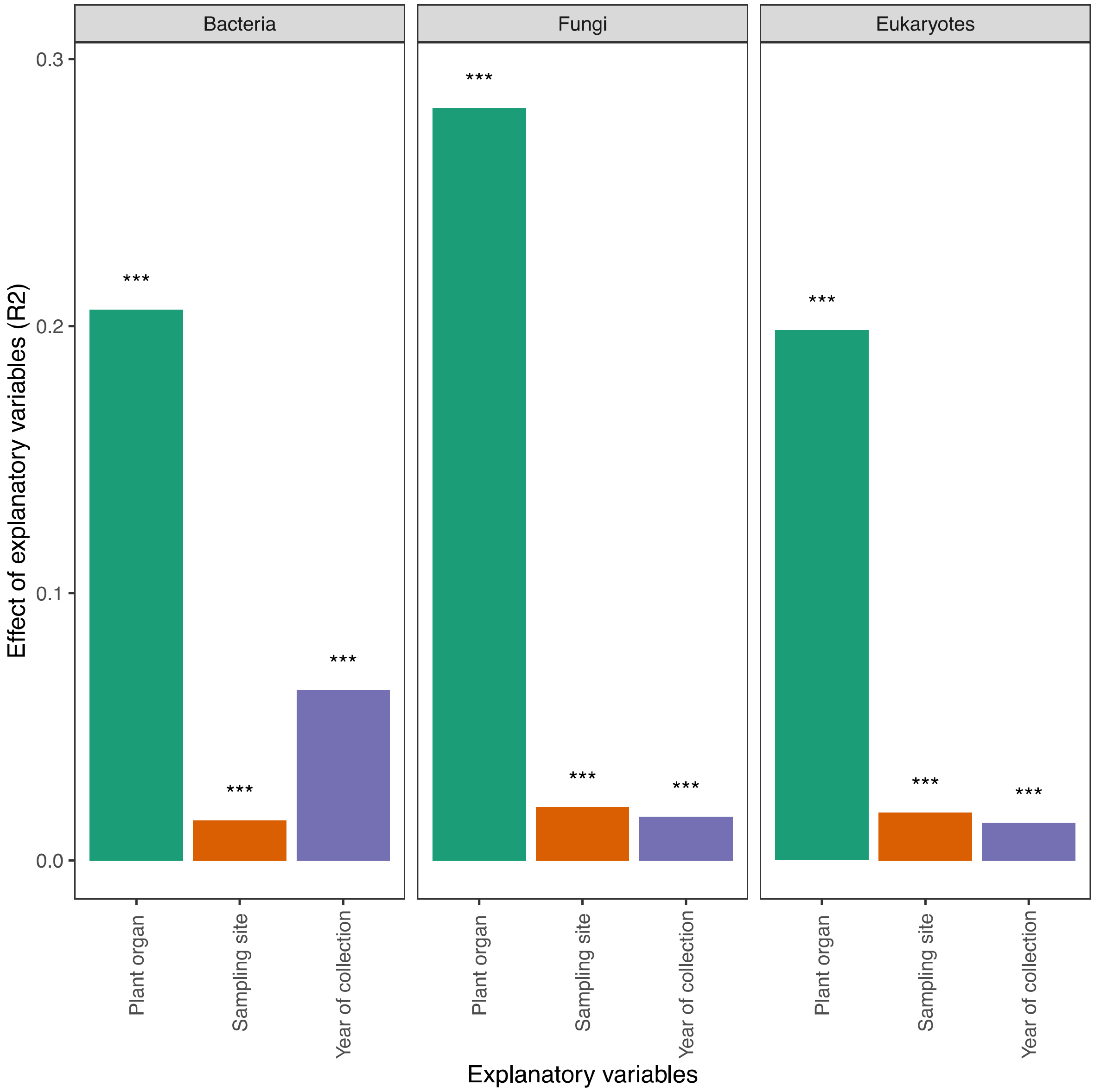

Figure S4: Barplots of R^2 statistic from PERMANOVA showing the percentage of variance that can be explained by factors such as plant organ, year of collection, and sampling sites (Supplementary Table 4).

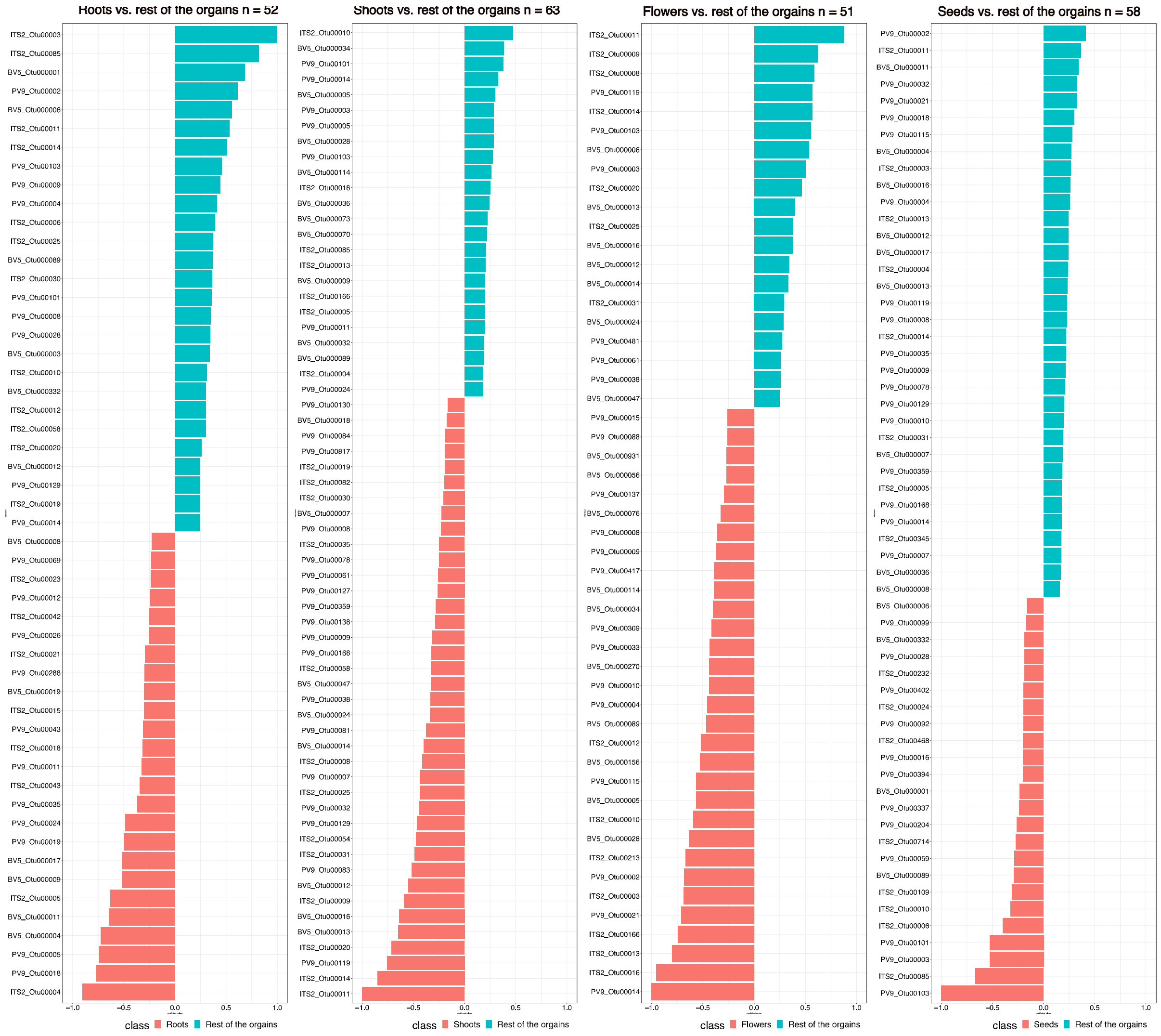

Figure S5: Discriminatory OTUs that distinguish each organ from others. The OTUs were identified using an SVM classifier with recursive feature elimination and cross-validation. A total of 84, 102, 57, and 166 OTUs for roots, shoots, flowers, and seeds, respectively, were identified. The x-axis values represent the coefficient value of each OTU in separating a specific organ (values < 0) from the rest (values > 0). Only OTUs with absolute coefficient values greater than 0.5 are included.

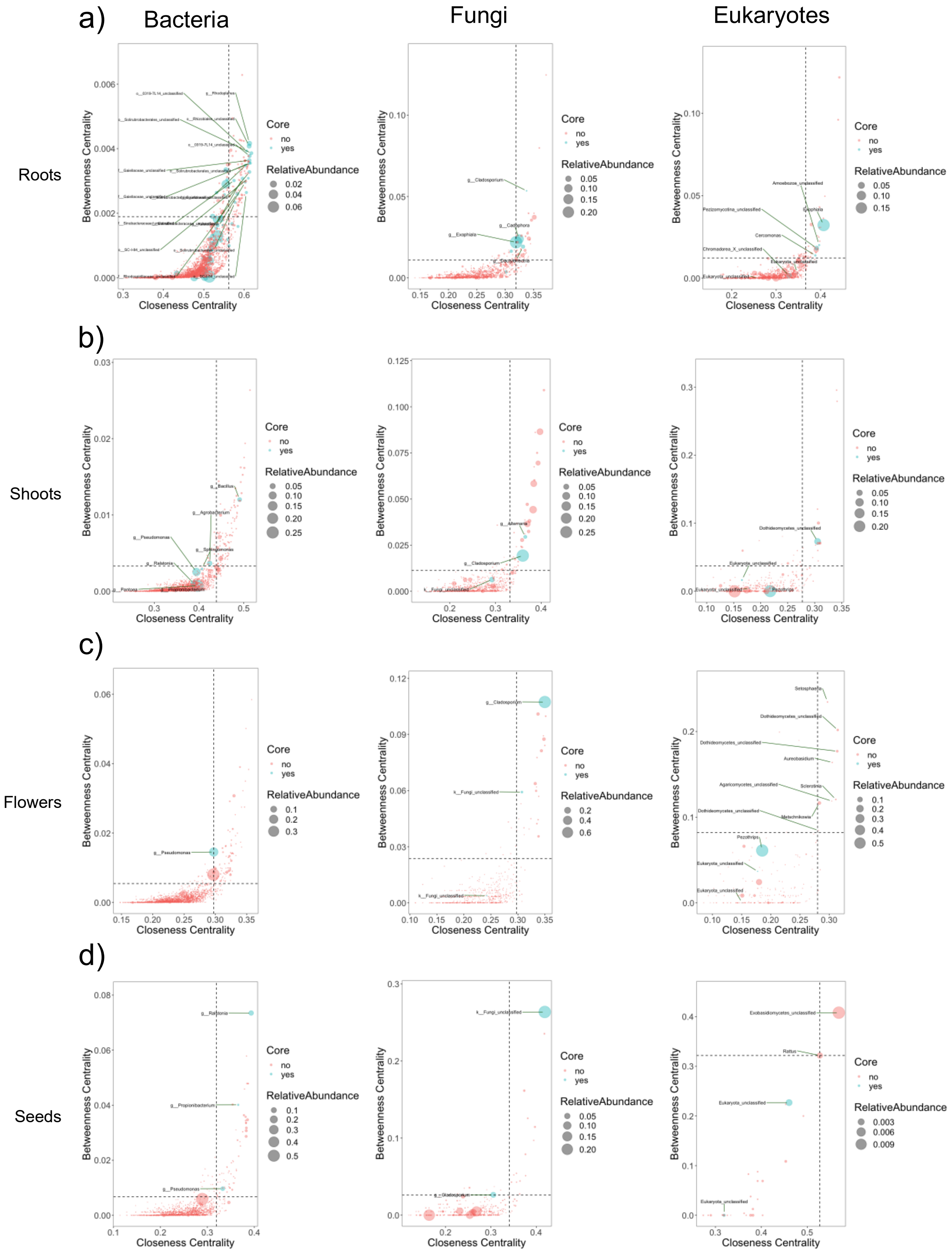

Figure S6: Microbial hubs of (a) root, (b) shoot, (c) flower, and (d) seed microbiomes. Bacterial, fungal, and eukaryotic hubs were identified based on their high betweenness centrality and closeness centrality in correlation networks calculated with FastSpar (Fig. 3). Dotted lines mark the OTUs that are top 5 % in betweenness centrality and closeness centrality scores and thus assigned as hub microbes. Size of the circles represent the OTU abundance and green circles are the core microbes.

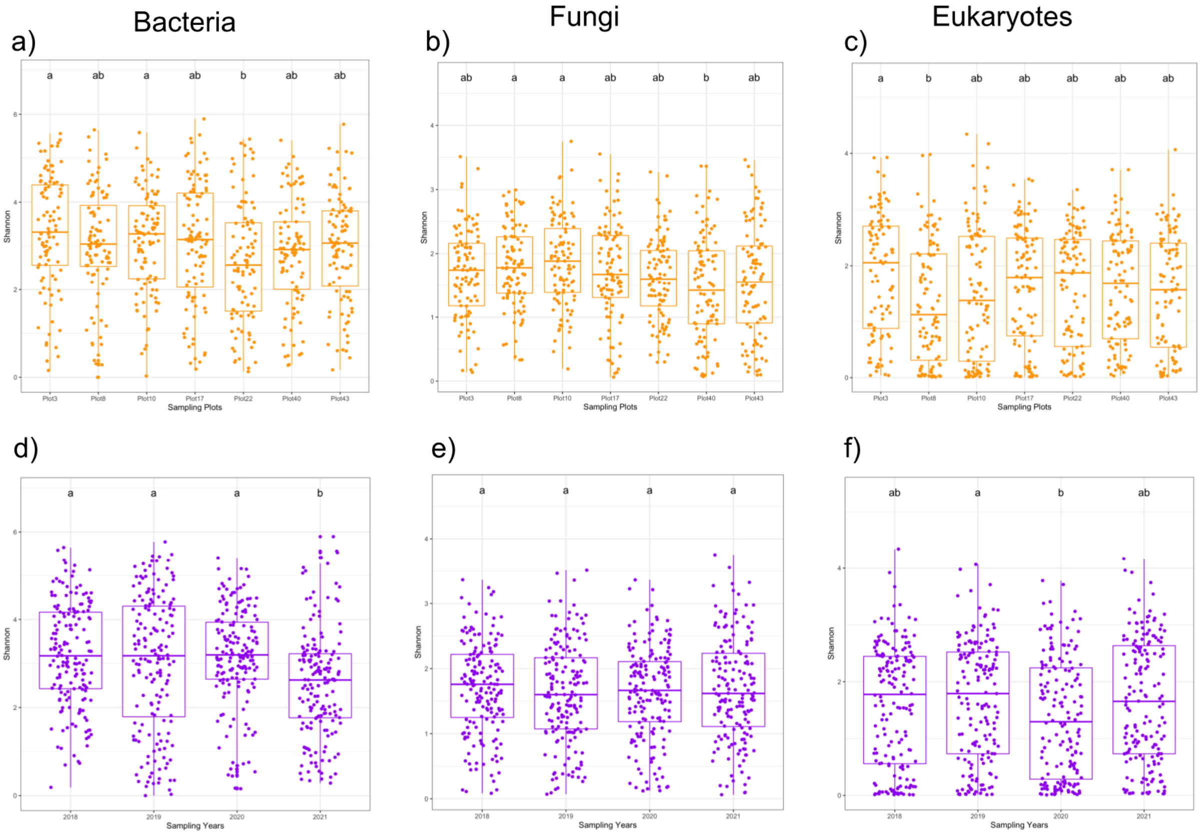

Figure S7: Boxplots of Shannon's α-diversity measurements of bacterial, fungal, and eukaryotic microbial communities associated with whole plants. Shapiro-Wilk normality and Kruskal-Wallis rank sum tests were used to test significant differences of Shannon measurements between (a-c) sampling sites and (d-f) sampling years. Post-hoc analysis via Dunn's tests indicate that groups are significantly different if letters are not similar.

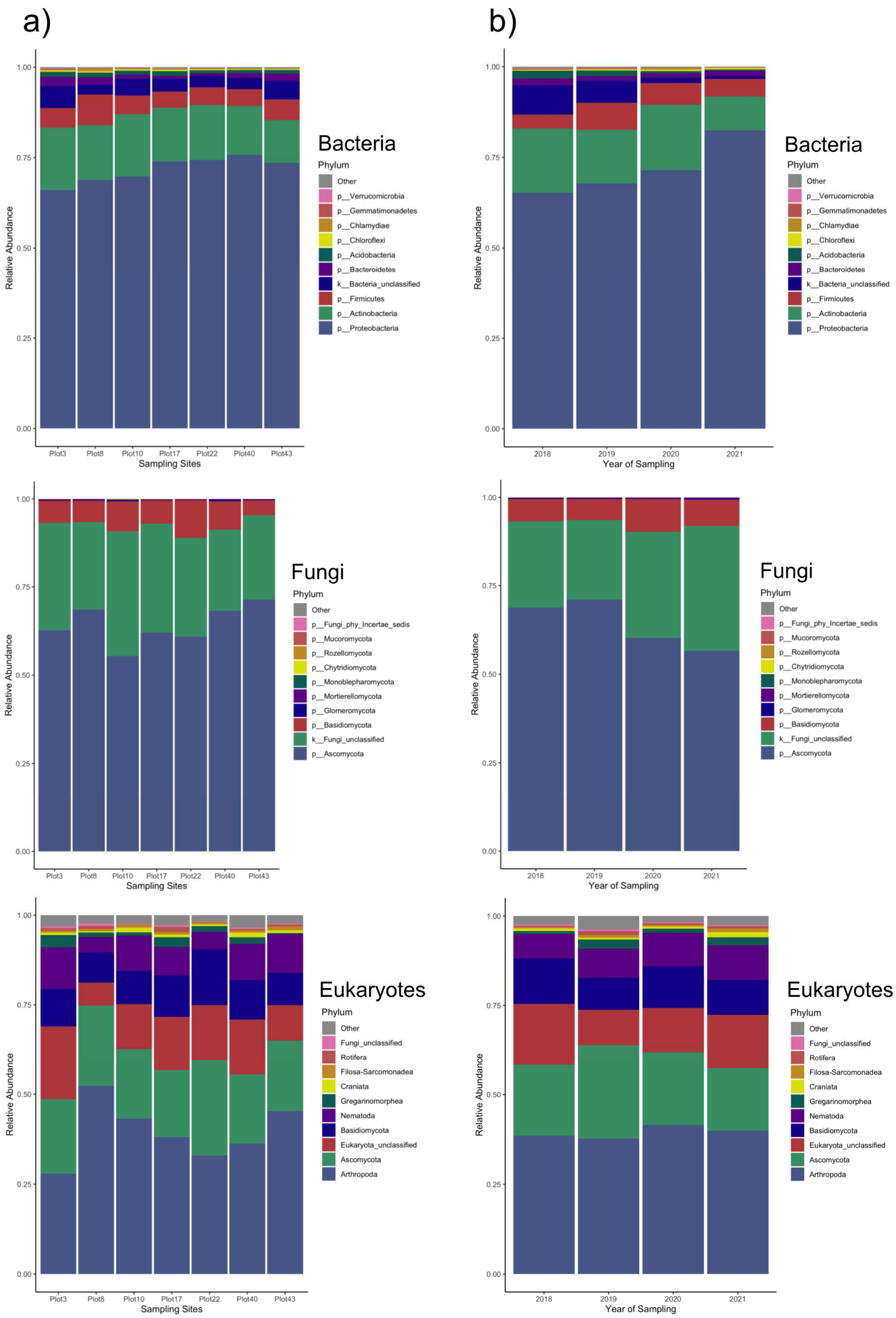

Figure S8: Relative abundance profiles showing the top most abundant bacterial, fungal, and eukaryotic phyla in plant samples collected from (a) seven grassland sites for (b) four years. The relative abundance of the OTUs for each site or year were aggregated at the phylum level.

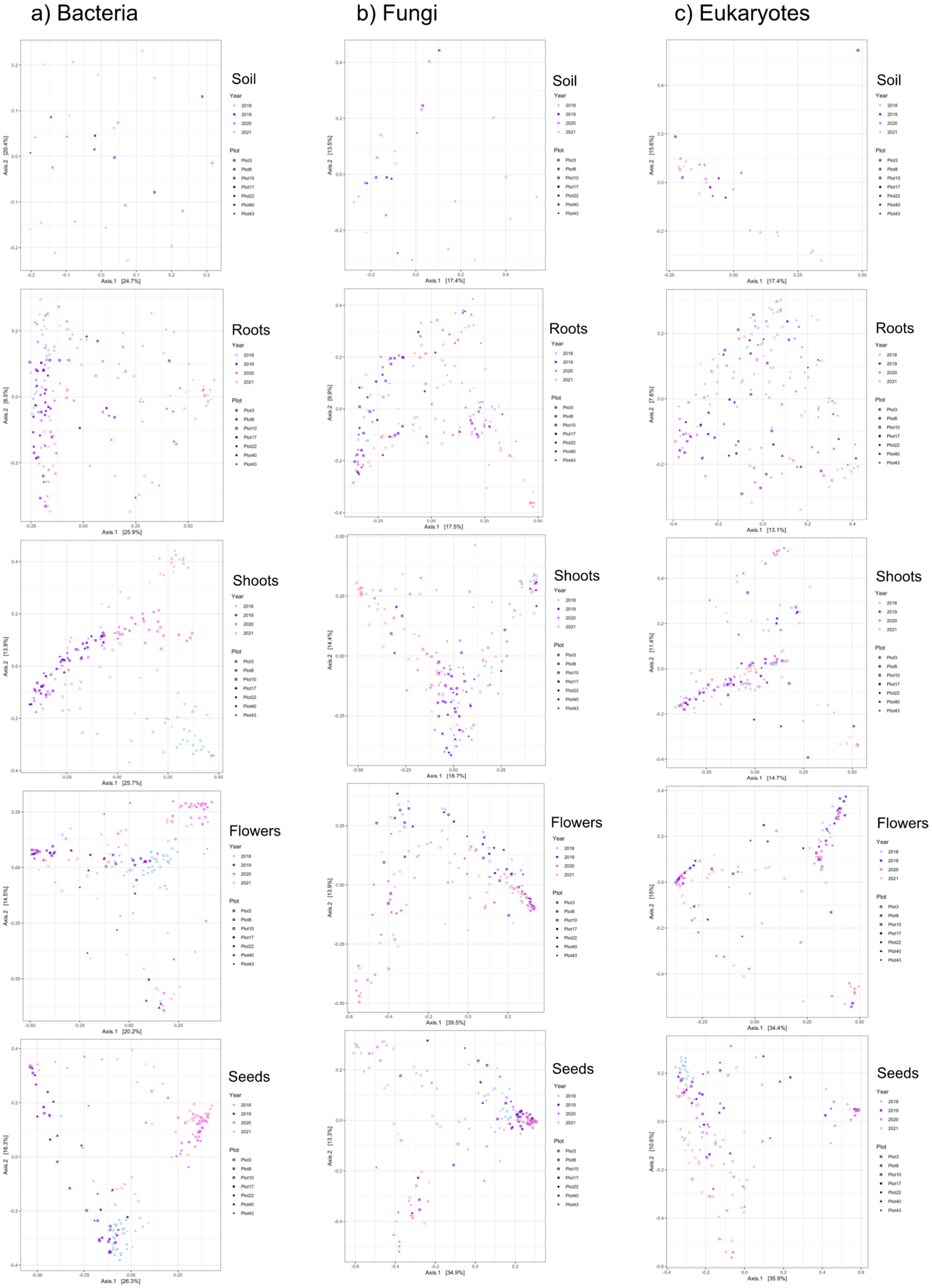

Figure S9: Principal coordinate plots based on Bray-Curtis dissimilarities between (a) bacterial, (b) fungal, and (c) eukaryotic microbial communities associated with soil and plant organ samples.

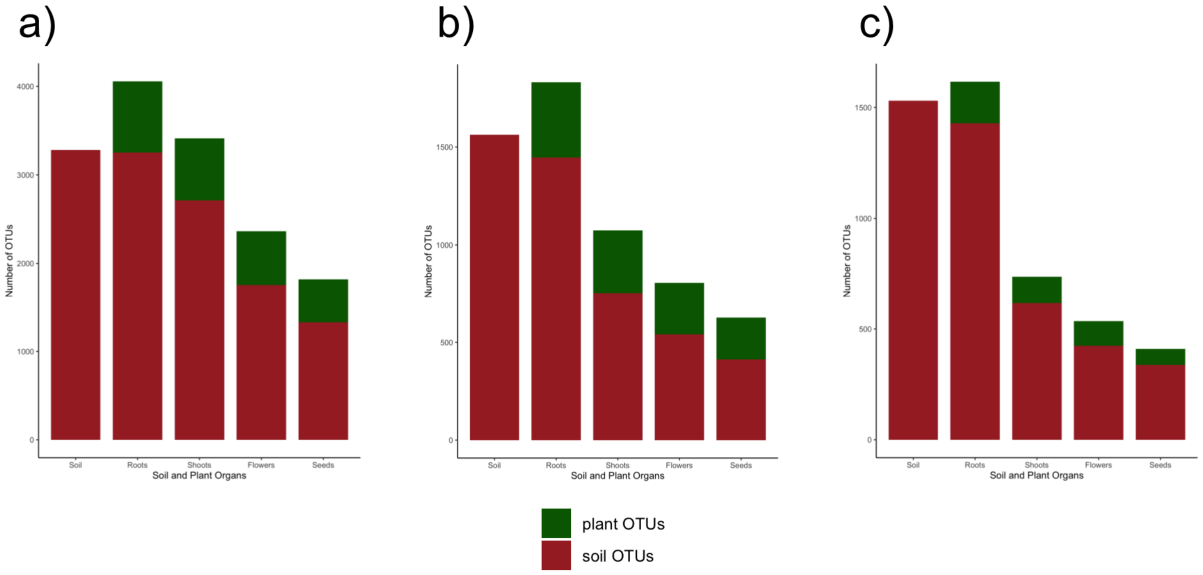

Figure S10: Distribution of soil and plant (a) bacterial, (b) fungal, and (c) eukaryotic OTUs in soil and plant organs. OTUs are designated as soil OTUs if they are found in soil samples, and the rest of non-soil OTUs are designated as plant OTUs.

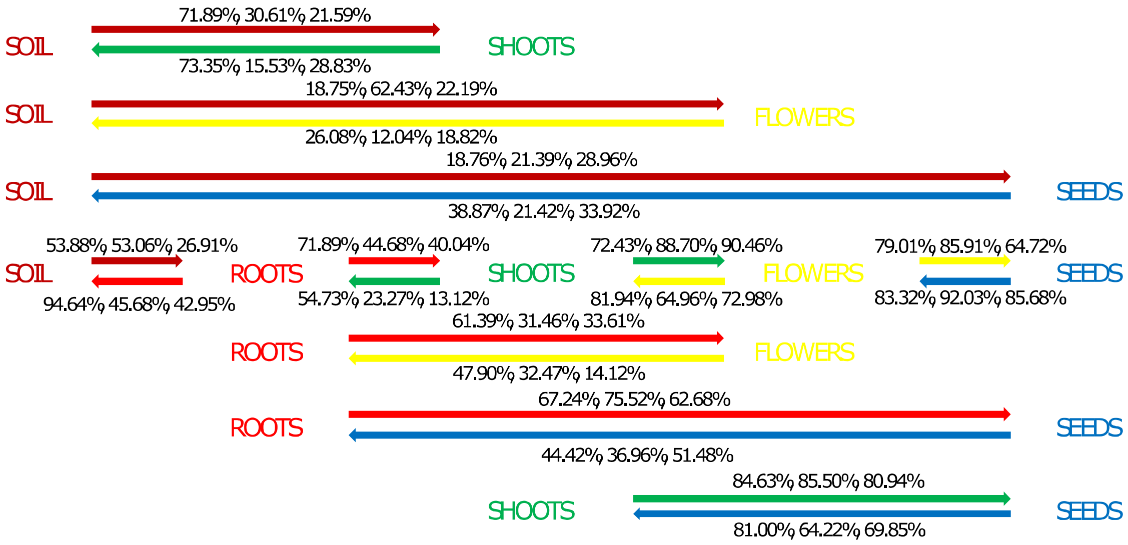

Figure S11: FEAST calculations of source contribution to the sink microbiomes. Microbial transmission was tracked at multiple directions of sources and sinks. The contribution of the source to the sink microbiomes are in percentages of the total bacterial, fungal, and eukaryotic sink microbiomes, respectively.

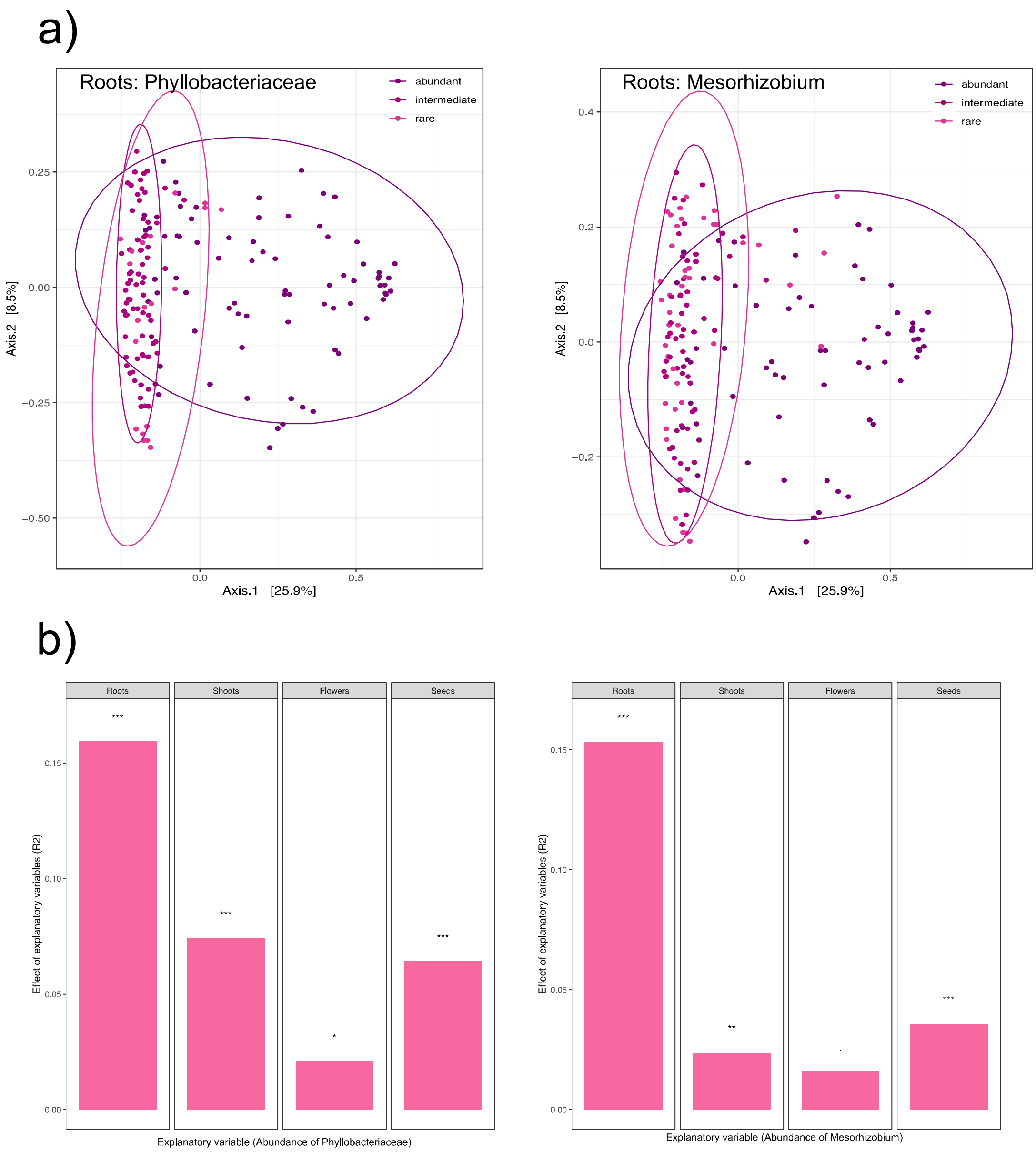

Figure S12: (a) PCoA of Bray-Curtis dissimilarities of root microbial communities and (b) PERMANOVA using abundance of candidate early-arriving OTU as explanatory variable of community composition variation. (a) and (b) show that candidate early-arriving OTUs (BV5_OTU4_*Phyllobacteriaceae,* BV5_OTU17_*Mesorhizobium*) potentially altered the microbial community composition in the roots based on their abundance. (Explanatory variables: abundant OTU: Relative abundance in the root microbial community (RA) >= 0.01; intermediate OTU: RA >= 0.001 and < 0.01; rare OTU: RA < 0.001).

Table S1: Primers and blocking oligos used in this study.

Primer name	Primer sequence (5’-to-3’ orientation)
799F	AACMGGATTAGATACCCKG
1192R	ACGTCATCCCCACCTTCC
fITS7	GTGARTCATCGAATCTTTG
ITS4	TCCTCCGCTTATTGATATGC
F1422	ATAACAGGTCTGTGATGCCC
R1797	TGATCCTTCTGCAGGTTCACCTAC
clamp1_BV5_mitoF	GATGAGTGTTCGCCCTTGGTCTACGTGGAT
clamp1_BV5_mitoR	CTGCTCAGGGTTCCAAACTCAACGTTGGCA
clamp1_ITS2_F	AACCATTAGGTCGAGGGCACGTCTGCCTGG
clamp1_ITS2_R	TGAGMGYGGTTACACCACGCATGCGGGTCT
clamp9_PV9_F	GATGTATTCAACGAGTCTATAGCCTTGGCC
Clamp15_PV9_R	TCTCACAACGTCGCAGGCAGCGAACCGCCC

Table S2: Basic statistics of the Illumina sequencing data. Amplicon sequencing of the microbial 16S rRNA, ITS2, and 18S rRNA genes in a total of 700 samples of soil and *Lotus corniculatus* roots, shoots, flowers, and seeds, along with blank samples collected from seven grassland sites in the Swabian Alps, Germany for four years.

Sequencing runs	Sequence reads	Sequence length	%GC
RunLotus1	15,367,467	300	52
RunLotus2	19,049,132	300	52
RunLotus3	19,931,059	300	52.5
RunLotus4	18,326,536	300	52.5
RunLotus6	21,112,668	301	53
RunLotus7	20,429,103	301	52.5
RunLotus8	20,404,891	301	53
RunLotus9	21,088,582	301	52.5
	155,709,438 (total)		

Table S3: Summary of sequence data after processing by mothur and phyloseq.

Locus	Domain	Subdomain	Kingdom	Phylum	Subphylum	Class	Order	Family	Genus	Species	OTUs
16S rRNA	-	-	Bacteria	25	-	95	182	335	586	671	4,225
ITS2	-	-	Fungi	15	-	51	129	280	531	788	2,027
18S rRNA	Eukaryota	10	26	73	122	197	-	-	425	486	1,773

Table S4: Statistical analysis of alpha- and beta-diversity measurements

(a) Shapiro-Wilk normality tests

α-diversity index	Statistic (W)	p-value	normality
16S rRNA_Shannon	0.98747	1.091e-05	non-normal distribution
16S rRNA_Observed	0.75878	< 2.2e-16	non-normal distribution
ITS2_Shannon	0.98424	7.343e-07	non-normal distribution
ITS2_Observed	0.70022	< 2.2e-16	non-normal distribution
18S rRNA_Shannon	0.95252	3.048e-14	non-normal distribution
18S rRNA_Observed	0.56119	< 2.2e-16	non-normal distribution

(b) Kruskal-Wallis rank sum tests by soil/plant organs and post-hoc analysis via Dunn’s test

α-diversity index	Statistic (K-W chi-squared)	p-value	significance
16S rRNA_Shannon	295.25	< 2.2e-16	significant differences
16S rRNA_Observed	498.33	< 2.2e-16	significant differences
ITS2_Shannon	237.31	< 2.2e-16	significant differences
ITS2_Observed	397.04	< 2.2e-16	significant differences
18S rRNA_Shannon	258.82	< 2.2e-16	significant differences
18S rRNA_Observed	423.6	< 2.2e-16	significant differences

(c) Permutational multivariate analysis of variance (PERMANOVA) of variables plant organ, collection year, and sampling sites.

Permutation test for adonis under reduced model

Terms added sequentially (first to last)

Permutation: free

Number of permutations: 999

		16S rRNA			
	Df	SumOfSqs	R2	F	Pr(>F)
PlantOrgan	4	55.372	0.20622	49.3158	0.001 ***
Year	3	17.103	0.06370	20.3100	0.001 ***
Plot	6	4.029	0.01501	2.3923	0.001 ***
Residual	684	192.001	0.71507		
Total	697	268.506	1.00000		
		ITS2			
	Df	SumOfSqs	R2	F	Pr(>F)
PlantOrgan	4	76.128	0.28167	70.9434	0.001 ***
Year	3	4.441	0.01643	5.5178	0.001 ***
Plot	6	5.408	0.02001	3.3598	0.001 ***
Residual	687	184.303	0.68190		
Total	700	270.280	1.00000		
		18S rRNA			
	Df	SumOfSqs	R2	F	Pr(>F)
PlantOrgan	4	60.755	0.19845	44.2882	0.001 ***
Year	3	4.310	0.01408	4.1894	0.001 ***
Plot	6	5.475	0.01788	2.6606	0.001 ***
Residual	687	235.610	0.76959		
Total	700	306.151	1.00000		

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Abbreviations: Df ‘degrees of freedom’; SumsOfSqs ‘Sums of Squares’; F ‘F statistic’; R2 ‘R2 statistic’; Pr(>F) ‘p-values’

Table S5: Performance tests of the SVM models.

Taxa	Organ		precision	recall	f1-score	support
Bacteria	Seeds	0	0.625	0.80357143	0.703125	56
Bacteria	Flowers	1	0.69047619	0.52727273	0.59793814	55
Bacteria	Shoots	2	0.81132075	0.76785714	0.78899083	56
Bacteria	Roots	3	0.98148148	0.96363636	0.97247706	55
Bacteria	Soil	4	0.9	1	0.94736842	9
Bacteria		accuracy	0.77489177	0.77489177	0.77489177	0.77489177
Bacteria		macro avg	0.80165569	0.81246753	0.80197989	231
Bacteria		weighted avg	0.78134907	0.77489177	0.77254389	231
Taxa			precision	recall	f1-score	support
Fungi	Seeds	0	0.7	0.89090909	0.784	55
Fungi	Flowers	1	0.75806452	0.83928571	0.79661017	56
Fungi	Shoots	2	0.9	0.64285714	0.75	56
Fungi	Roots	3	1	0.91071429	0.95327103	56
Fungi	Soil	4	1	1	1	9
Fungi		accuracy	0.82758621	0.82758621	0.82758621	0.82758621
Fungi		macro avg	0.8716129	0.85675325	0.85677624	232
Fungi		weighted avg	0.84634316	0.82758621	0.82807477	232
Taxa	Organ		precision	recall	f1-score	support
Eukaryote	Seeds	0	0.85	0.92727273	0.88695652	55
Eukaryote	Flowers	1	0.73846154	0.85714286	0.79338843	56
Eukaryote	Shoots	2	0.76595745	0.64285714	0.69902913	56
Eukaryote	Roots	3	0.98076923	0.91071429	0.94444444	56
Eukaryote	Soil	4	0.875	0.77777778	0.82352941	9
Eukaryote		accuracy	0.83189655	0.83189655	0.83189655	0.83189655
Eukaryote		macro avg	0.84203764	0.82315296	0.82946959	232
Eukaryote		weighted avg	0.8353256	0.83189655	0.8304252	232

Table S6: Abundant OTUs (**relative abundance > 1 %**) in *Lotus corniculatus* organs.

		Relative abundance			
OTU	Genus	Roots	Shoots	Flowers	Seeds
Bacteria					
BV5_Otu000001	*Pantoea*	**0.01498769**	**0.25475602**	**0.39135219**	**0.53456217**
BV5_Otu000003	*Pseudomonas*	**0.01419121**	**0.10772585**	**0.20539426**	**0.0531343**
BV5_Otu000004	*Phyllobacteriaceae*	**0.07248534**	0.00054067	0.00086442	0.00054539
BV5_Otu000005	*Enterobacteriaceae*	2.45E-05	0.00237893	**0.12226135**	**0.01422907**
BV5_Otu000006	*Ralstonia*	0.0005517	**0.06358809**	**0.01562879**	**0.07815603**
BV5_Otu000007	*Bacillus*	**0.03395148**	**0.02838181**	0.00241461	0.00069461
BV5_Otu000008	*Frankia*	**0.04906623**	0.00023149	3.93E-05	1.57E-05
BV5_Otu000009	*Phyllobacterium*	**0.03919923**	0.00068449	0.00030655	7.77E-05
BV5_Otu000010	*Pantoea*	0.00091647	**0.0124075**	**0.01680126**	**0.02267523**
BV5_Otu000011	*Mesorhizobium*	**0.02558929**	0.00063515	0.00072956	7.94E-05
BV5_Otu000012	*Burkholderia*	0.00164728	**0.03042322**	0.00972065	**0.01644205**
BV5_Otu000013	*Burkholderia*	0.00083817	**0.03141899**	0.00876367	**0.01736878**
BV5_Otu000014	*Agrobacterium*	0.00501434	**0.03865919**	0.00136009	0.00281248
BV5_Otu000015	*Streptomyces*	**0.02373183**	0.00045353	9.95E-05	0.00011431
BV5_Otu000016	*Burkholderia*	0.00074167	**0.0303996**	0.00767331	**0.01618725**
BV5_Otu000017	*Mesorhizobium*	**0.02286487**	0.00031075	0.00065989	0.00025132

OTU	Genus	Roots	Shoots	Flowers	Seeds
BV5_Otu000018	*Comamonadaceae*	0.0062342	**0.01769185**	0.00033791	**0.02457407**
BV5_Otu000019	*Cryptosporangium*	**0.02155104**	0.00015905	0.00010301	2.01E-05
BV5_Otu000021	*Pantoea*	0.00052872	0.00695622	**0.01308926**	**0.01592197**
BV5_Otu000022	*Methylibium*	**0.019637**	0.00022047	7.02E-05	1.83E-05
BV5_Otu000023	*Steroidobacter*	**0.01899183**	0.00025301	5.92E-05	4.80E-05
BV5_Otu000024	*Xanthomonadaceae*	0.00477739	**0.02151746**	0.00025778	0.00059513
BV5_Otu000028	*Wolbachia*	1.01E-06	0.0014283	**0.01519235**	**0.01760352**
BV5_Otu000032	*Pseudomonas*	0.00045571	0.00352115	**0.01337044**	0.00700109
BV5_Otu000040	*Sodalis*	0.00184406	1.26E-05	5.47E-06	**0.02810647**
BV5_Otu000056	*Enterobacteriaceae*	0.00108648	0.00033332	**0.01385366**	3.49E-05
BV5_Otu000068	*Gluconobacter*	2.27E-06	5.25E-06	**0.0128056**	1.75E-06
Fungi					
ITS2_Otu00003	*Cladosporium*	0.00108977	**0.2671789**	**0.76240753**	**0.04540433**
ITS2_Otu00004	*Exophiala*	**0.23861407**	0.00042552	0.00037056	0.00037517
ITS2_Otu00005	*Cadophora*	**0.12629519**	0.00027117	0.0001305	0.00026054
ITS2_Otu00006	*Fungi*	**0.01084811**	**0.04378309**	**0.01783285**	**0.24165332**
ITS2_Otu00008	*Boeremia*	0.00022675	**0.06855963**	0.00358989	**0.15808927**
ITS2_Otu00009	*Didymellaceae*	0.00791914	**0.0633385**	**0.01207996**	0.00837013
ITS2_Otu00010	*Sclerotiniaceae*	1.36E-05	2.38E-05	**0.03687987**	**0.20319659**
ITS2_Otu00011	*Septoria*	4.2685E-05	**0.09075922**	5.94E-05	0.0002119
ITS2_Otu00012	*Alternaria*	4.83E-05	**0.02015062**	**0.03302806**	**0.0907913**
ITS2_Otu00013	*Didymellaceae*	0.00023233	**0.02319715**	**0.03025786**	**0.03560474**
ITS2_Otu00014	*Chaetosphaeronema*	0.00059344	**0.07000393**	4.40E-05	0.00016153
ITS2_Otu00015	*Pseudomassaria*	**0.02789404**	4.14E-05	3.9402E-05	2.95E-05
ITS2_Otu00016	*Botryotinia*	2.97E-05	0.00139567	**0.04266852**	0.00042554
ITS2_Otu00017	*Helotiales*	**0.02200804**	0.00236582	1.64E-05	9.90E-05
ITS2_Otu00018	*Tetracladium*	**0.02145356**	1.11E-05	1.59E-05	1.74E-05
ITS2_Otu00019	*Alternaria*	4.93E-05	**0.01773128**	**0.01641507**	**0.03986536**
ITS2_Otu00020	*Podospora*	1.12E-05	**0.04191523**	0.00152255	3.47E-05
ITS2_Otu00021	*Dactylonectria*	**0.01881724**	1.4032E-05	1.59E-05	4.86E-05
ITS2_Otu00022	*Neocosmospora*	**0.0187555**	2.68E-05	4.10E-06	1.39E-05
ITS2_Otu00023	*Cistella*	**0.01668841**	8.16E-06	5.79E-05	3.65E-05
ITS2_Otu00024	*Gibberella*	0.00182659	**0.01768527**	0.00076435	**0.07204492**
ITS2_Otu00025	*Colletotrichum*	0.00125693	**0.03415043**	0.00052657	0.00015979
ITS2_Otu00026	*Mycena*	**0.0148273**	6.79E-05	1.25E-05	1.74E-05
ITS2_Otu00028	*Ascomycota*	**0.01283799**	0.00064285	0.00170248	5.21E-05
ITS2_Otu00030	*Uromyces*	1.29E-06	**0.03014028**	0.00010317	2.78E-05
ITS2_Otu00031	*Stemphylium*	0.00164826	**0.02476839**	4.30E-05	0.00101609
ITS2_Otu00085	*Fungi*	0.00087376	0.00188744	0.00146493	**0.01086953**
ITS2_Otu00109	*Malassezia*	8.0501E-05	0.0006931	0.0005539	**0.02153931**
Eukaryotes					
PV9_Otu00002	*Pezothrips*	0.00127649	**0.22407626**	**0.5935281**	0.00544734
PV9_Otu00003	*Insecta*	0.0001378	0.00840221	0.00466225	**0.9106856**
PV9_Otu00004	*Frankliniella*	0.00015456	**0.09482595**	**0.12677873**	0.00044784
PV9_Otu00005	*Exophiala*	**0.18068931**	0.00018327	6.80E-05	9.03E-05
PV9_Otu00007	*Pucciniomycetes*	0.00063188	**0.23242558**	0.0020743	0.00016626
PV9_Otu00008	*Mayetiola*	0.00012911	**0.07492251**	**0.0488326**	0.00012514
PV9_Otu00009	*Dothideomycetes*	**0.02789218**	**0.06098462**	**0.01528368**	0.00410025
PV9_Otu00010	*Aeolothrips*	2.76E-05	0.00940713	**0.05010144**	3.66E-05
PV9_Otu00011	*Agaricomycetes*	**0.05681411**	6.27E-05	2.94E-05	3.22E-05
PV9_Otu00012	*Agaricomycetes*	**0.04652027**	0.00029151	1.51E-05	5.99E-05
PV9_Otu00013	*Insecta*	1.18E-05	**0.03836716**	**0.02424527**	0.00065611
PV9_Otu00014	*Metschnikowia*	1.24E-05	0.00027306	**0.0343287**	5.10E-05
PV9_Otu00015	*Insecta*	1.64E-05	**0.01191882**	**0.02390365**	**0.02006059**
PV9_Otu00016	*Agaricomycetes*	**0.03859289**	0.0001064	5.70E-06	0.00031286
PV9_Otu00018	*Aglenchus*	**0.03959254**	3.69E-05	1.12E-05	0.00012067
PV9_Otu00019	*Agaricomycetes*	**0.02896228**	0.00206027	1.56E-05	1.07E-05
PV9_Otu00021	*Insecta*	1.64E-05	0.00815929	**0.01215686**	1.79E-05
PV9_Otu00022	*Agaricomycetes*	**0.03146715**	9.84E-06	4.61E-06	4.92E-05
PV9_Otu00024	*Chromadorea*	**0.02882231**	5.66E-05	1.51E-05	2.41E-05
PV9_Otu00026	*Pezizomycotina*	**0.02743875**	1.23E-05	8.78E-06	2.32E-05
PV9_Otu00032	*Arachnida*	4.03E-06	**0.04404551**	0.00080172	1.34E-05

OTU	Genus	Roots	Shoots	Flowers	Seeds
PV9_Otu00033	*Arachnida*	3.26E-05	7.38E-06	**0.01642066**	1.79E-05
PV9_Otu00038	*Insecta*	6.52E-06	**0.0440246**	1.54E-06	2.86E-05
PV9_Otu00081	*Leotiomycetes*	3.57E-05	**0.01225707**	0.00010707	9.83E-06
PV9_Otu00101	*Exobasidiomycetes*	0.00018094	0.00033456	0.00011497	**0.01129961**

Table S7: Key microbes (abundant OTUs: relative abundance > 1 %; core OTUs: persistent in 90 % of samples; hub OTUs; separator OTUs: predicted by SVM model) in *Lotus corniculatus* organs.

(a) Roots

	Separator	Abundant	Core	Hub
BV5_Otu000001_Pantoea	yes	yes	no	no
BV5_Otu000003_Pseudomonas	yes	yes	yes	no
BV5_Otu000004_Phyllobacteriaceae	yes	yes	yes	no
BV5_Otu000005_Enterobacteriaceae	no	no	no	no
BV5_Otu000006_Ralstonia	yes	no	no	no
BV5_Otu000007_Bacillus	no	yes	yes	no
BV5_Otu000008_Frankia	yes	yes	yes	no
BV5_Otu000009_Phyllobacterium	yes	yes	yes	no
BV5_Otu000010_Pantoea	no	no	no	no
BV5_Otu000011_Mesorhizobium	yes	yes	yes	no
BV5_Otu000012_Burkholderia	yes	no	no	no
BV5_Otu000013_Burkholderia	yes	no	no	no
BV5_Otu000014_Agrobacterium	no	no	no	no
BV5_Otu000015_Streptomyces	no	yes	yes	no
BV5_Otu000016_Burkholderia	yes	no	no	no
BV5_Otu000017_Mesorhizobium	yes	yes	yes	no
BV5_Otu000018_Comamonadaceae	no	no	yes	no
BV5_Otu000019_Cryptosporangium	yes	yes	yes	no
BV5_Otu000021_Pantoea	no	no	no	no
BV5_Otu000022_Methylibium	no	yes	yes	no
BV5_Otu000023_Steroidobacter	yes	yes	yes	no
BV5_Otu000024_Xanthomonadaceae	no	no	yes	no
BV5_Otu000025_Rhodoplanes	no	no	yes	yes
BV5_Otu000026_Rhizobium	yes	yes	yes	no
BV5_Otu000027_Gaiellaceae	no	no	yes	yes
BV5_Otu000028_Wolbachia	no	no	no	no
BV5_Otu000029_Rhizobiales	no	no	yes	yes
BV5_Otu000030_0319-7L14	no	no	yes	yes
BV5_Otu000032_Pseudomonas	no	no	no	no
BV5_Otu000033_Chryseobacterium	no	yes	no	no
BV5_Otu000034_Prauserella	no	no	no	no
BV5_Otu000036_Bosea	yes	yes	yes	no
BV5_Otu000039_Methylibium	no	no	yes	no
BV5_Otu000040_Sodalis	no	no	no	no
BV5_Otu000043_Bradyrhizobiaceae	no	no	yes	no
BV5_Otu000045_Micromonosporaceae	no	no	yes	no
BV5_Otu000047_Methylobacterium	no	no	no	no
BV5_Otu000049_Kineosporia	no	no	yes	no
BV5_Otu000052_Methylobacterium	yes	no	no	no
BV5_Otu000054_Gaiellaceae	no	no	yes	yes
BV5_Otu000055_Methylobacterium	no	no	no	no
BV5_Otu000056_Enterobacteriaceae	no	no	no	no
BV5_Otu000057_Rhodospirillaceae	no	no	yes	yes
BV5_Otu000059_Solirubrobacterales	no	no	yes	yes
BV5_Otu000060_Hyphomicrobium	no	no	yes	no
BV5_Otu000061_Mycobacterium	no	no	yes	no
BV5_Otu000062_Flavobacterium	no	no	yes	no
BV5_Otu000063_SC-I-84	no	no	yes	yes
BV5_Otu000065_Solirubrobacterales	no	no	yes	yes
BV5_Otu000068_Gluconobacter	no	no	no	no

	Separator	Abundant	Core	Hub
BV5_Otu000069_Pseudomonas	no	no	no	no
BV5_Otu000070_Rubrobacter	no	no	no	no
BV5_Otu000073_Erwinia	no	no	no	no
BV5_Otu000075_Solirubrobacteraceae	no	no	yes	yes
BV5_Otu000076_Acinetobacter	no	no	no	no
BV5_Otu000077_Solirubrobacterales	no	no	yes	yes
BV5_Otu000078_Sinobacteraceae	no	no	yes	yes
BV5_Otu000079_Buchnera	no	no	no	no
BV5_Otu000080_Pedomicrobium	no	no	yes	no
BV5_Otu000081_SC-I-84	no	no	yes	yes
BV5_Otu000084_Dongia	no	no	yes	no
BV5_Otu000086_Burkholderiaceae	no	no	no	no
BV5_Otu000089_Propionibacterium	yes	no	no	no
BV5_Otu000091_Patulibacteraceae	no	no	yes	no
BV5_Otu000092_0319-7L14	no	no	yes	yes
BV5_Otu000094_Caulobacter	no	no	yes	no
BV5_Otu000095_Sphingomonas	no	no	no	no
BV5_Otu000096_Bradyrhizobium	no	no	yes	no
BV5_Otu000097_Gaiellaceae	no	no	yes	yes
BV5_Otu000098_Mycobacterium	no	no	yes	no
BV5_Otu000102_Solibacillus	no	no	yes	no
BV5_Otu000105_Labrys	no	no	yes	no
BV5_Otu000106_Bacillus	no	no	yes	no
BV5_Otu000109_Kaistobacter	no	no	yes	yes
BV5_Otu000112_Planococcaceae	no	no	yes	no
BV5_Otu000113_Dongia	no	no	yes	no
BV5_Otu000114_Escherichia	no	no	no	no
BV5_Otu000117_Solirubrobacterales	no	no	yes	yes
BV5_Otu000118_[Weeksellaceae]	no	no	no	no
BV5_Otu000120_Bacillus	no	no	yes	no
BV5_Otu000125_Steroidobacter	no	no	yes	no
BV5_Otu000141_Ellin6513	no	no	no	no
BV5_Otu000143_Dolo	no	no	yes	no
BV5_Otu000144_Rhizobiales	no	no	yes	no
BV5_Otu000155_Burkholderia	no	no	no	no
BV5_Otu000156_Staphylococcus	no	no	no	no
BV5_Otu000194_Micromonospora	no	no	yes	no
BV5_Otu000256_Bacillales	no	no	yes	no
BV5_Otu000713_Bacteria	no	no	no	no
ITS2_Otu00003_Cladosporium	yes	no	yes	yes
ITS2_Otu00004_Exophiala	yes	yes	yes	yes
ITS2_Otu00005_Cadophora	yes	yes	yes	no
ITS2_Otu00006_Fungi	yes	yes	yes	yes
ITS2_Otu00008_Boeremia	no	no	no	no
ITS2_Otu00009_Didymellaceae	no	no	no	no
ITS2_Otu00010_Sclerotiniaceae	yes	no	no	no
ITS2_Otu00011_Septoria	yes	no	no	no
ITS2_Otu00012_Alternaria	yes	no	no	yes
ITS2_Otu00013_Didymellaceae	no	no	no	yes
ITS2_Otu00014_Chaetosphaeronema	yes	no	no	yes
ITS2_Otu00015_Pseudomassaria	yes	yes	no	no
ITS2_Otu00016_Botryotinia	no	no	no	yes
ITS2_Otu00017_Helotiales	no	yes	no	yes
ITS2_Otu00018_Tetracladium	yes	yes	no	no
ITS2_Otu00019_Alternaria	yes	no	no	no
ITS2_Otu00020_Podospora	yes	no	no	no
ITS2_Otu00021_Dactylonectria	yes	yes	yes	no
ITS2_Otu00022_Neocosmospora	yes	yes	no	no
ITS2_Otu00023_Cistella	yes	yes	no	no
ITS2_Otu00024_Gibberella	no	no	no	no
ITS2_Otu00025_Colletotrichum	yes	no	no	no
ITS2_Otu00026_Mycena	no	yes	no	no

	Separator	Abundant	Core	Hub
ITS2_Otu00028_Ascomycota	yes	yes	no	no
ITS2_Otu00030_Uromyces	yes	no	no	no
ITS2_Otu00031_Stemphylium	yes	no	no	no
ITS2_Otu00033_Ilyonectria	no	yes	yes	no
ITS2_Otu00034_Serendipita	no	yes	no	yes
ITS2_Otu00035_Pseudoidium	yes	no	no	no
ITS2_Otu00036_Hemimycena	yes	yes	no	no
ITS2_Otu00037_Exophiala	no	yes	no	no
ITS2_Otu00042_Pleosporales	yes	no	no	no
ITS2_Otu00052_Titaea	yes	no	no	yes
ITS2_Otu00054_Ramularia	no	no	no	no
ITS2_Otu00058_Colletotrichum	yes	no	no	no
ITS2_Otu00063_Pleosporales	no	no	no	no
ITS2_Otu00085_Fungi	yes	no	yes	no
ITS2_Otu00109_Malassezia	yes	no	no	no
ITS2_Otu00232_Fungi	no	no	no	no
ITS2_Otu00257_Fungi	no	no	no	no
PV9_Otu00002_Pezothrips	yes	no	no	no
PV9_Otu00003_Insecta	yes	no	no	no
PV9_Otu00004_Frankliniella	yes	no	no	no
PV9_Otu00005_Exophiala	yes	yes	yes	yes
PV9_Otu00007_Pucciniomycetes	no	no	no	no
PV9_Otu00008_Mayetiola	yes	no	no	no
PV9_Otu00009_Dothideomycetes	yes	yes	no	yes
PV9_Otu00010_Aeolothrips	no	no	no	no
PV9_Otu00011_Agaricomycetes	yes	yes	no	no
PV9_Otu00012_Agaricomycetes	yes	yes	no	no
PV9_Otu00013_Insecta	yes	no	no	no
PV9_Otu00014_Metschnikowia	yes	no	no	no
PV9_Otu00015_Insecta	no	no	no	no
PV9_Otu00016_Agaricomycetes	no	yes	no	no
PV9_Otu00018_Aglenchus	yes	yes	no	no
PV9_Otu00019_Agaricomycetes	yes	yes	no	no
PV9_Otu00021_Insecta	no	no	no	no
PV9_Otu00022_Agaricomycetes	yes	yes	no	no
PV9_Otu00024_Chromadorea_X	yes	yes	yes	no
PV9_Otu00026_Pezizomycotina	yes	yes	no	no
PV9_Otu00027_Agaricomycetes	yes	yes	no	no
PV9_Otu00028_Dothideomycetes	yes	no	no	no
PV9_Otu00030_Arachnida	no	yes	no	no
PV9_Otu00032_Arachnida	yes	no	no	no
PV9_Otu00033_Arachnida	yes	no	no	no
PV9_Otu00034_Pezizomycotina	yes	yes	yes	yes
PV9_Otu00035_Paratylenchus	yes	yes	no	no
PV9_Otu00038_Insecta	no	no	no	no
PV9_Otu00043_Plectus	yes	yes	no	no
PV9_Otu00047_Alatospora	no	yes	no	no
PV9_Otu00053_Sebacina	no	yes	no	no
PV9_Otu00057_Agaricomycetes	no	yes	no	no
PV9_Otu00061_Dothideomycetes	no	no	no	yes
PV9_Otu00062_Pratylenchus	no	yes	no	no
PV9_Otu00064_Aphelenchus	no	yes	no	no
PV9_Otu00081_Leotiomycetes	yes	no	no	no
PV9_Otu00083_Adineta	no	no	no	no
PV9_Otu00084_Phytomyza	no	no	no	no
PV9_Otu00101_Exobasidiomycetes	yes	no	no	no
PV9_Otu00103_Eukaryota	yes	no	yes	no
PV9_Otu00104_Cercomonas	yes	no	yes	yes
PV9_Otu00134_Amoebozoa	no	no	yes	yes
PV9_Otu00204_Rattus	no	no	no	no
PV9_Otu00337_Eukaryota	no	no	yes	no

(b) Shoots

	Separator	Abundant	Core	Hub
BV5_Otu000001_Pantoea	no	yes	yes	no
BV5_Otu000003_Pseudomonas	no	yes	yes	no
BV5_Otu000004_Phyllobacteriaceae	yes	no	no	no
BV5_Otu000005_Enterobacteriaceae	yes	no	no	no
BV5_Otu000006_Ralstonia	no	yes	yes	no
BV5_Otu000007_Bacillus	yes	yes	yes	yes
BV5_Otu000008_Frankia	no	no	no	no
BV5_Otu000009_Phyllobacterium	yes	no	no	yes
BV5_Otu000010_Pantoea	no	yes	no	no
BV5_Otu000011_Mesorhizobium	no	no	no	yes
BV5_Otu000012_Burkholderia	yes	yes	no	no
BV5_Otu000013_Burkholderia	yes	yes	no	yes
BV5_Otu000014_Agrobacterium	yes	yes	yes	no
BV5_Otu000015_Streptomyces	no	no	no	yes
BV5_Otu000016_Burkholderia	yes	yes	no	no
BV5_Otu000017_Mesorhizobium	no	no	no	no
BV5_Otu000018_Comamonadaceae	yes	yes	no	no
BV5_Otu000019_Cryptosporangium	yes	no	no	no
BV5_Otu000021_Pantoea	no	no	no	no
BV5_Otu000022_Methylibium	no	no	no	no
BV5_Otu000023_Steroidobacter	no	no	no	no
BV5_Otu000024_Xanthomonadaceae	yes	yes	no	yes
BV5_Otu000025_Rhodoplanes	no	no	no	yes
BV5_Otu000026_Rhizobium	no	no	no	no
BV5_Otu000027_Gaiellaceae	no	no	no	yes
BV5_Otu000028_Wolbachia	yes	no	no	no
BV5_Otu000029_Rhizobiales	no	no	no	yes
BV5_Otu000030_0319-7L14	no	no	no	yes
BV5_Otu000032_Pseudomonas	yes	no	no	no
BV5_Otu000033_Chryseobacterium	no	no	no	no
BV5_Otu000034_Prauserella	yes	no	no	no
BV5_Otu000036_Bosea	yes	no	no	yes
BV5_Otu000039_Methylibium	no	no	no	no
BV5_Otu000040_Sodalis	no	no	no	no
BV5_Otu000043_Bradyrhizobiaceae	no	no	no	no
BV5_Otu000045_Micromonosporaceae	no	no	no	yes
BV5_Otu000047_Methylobacterium	yes	yes	no	no
BV5_Otu000049_Kineosporia	no	no	no	no
BV5_Otu000052_Methylobacterium	yes	yes	no	yes
BV5_Otu000054_Gaiellaceae	no	no	no	yes
BV5_Otu000055_Methylobacterium	yes	no	no	yes
BV5_Otu000056_Enterobacteriaceae	no	no	no	no
BV5_Otu000057_Rhodospirillaceae	no	no	no	yes
BV5_Otu000059_Solirubrobacterales	no	no	no	yes
BV5_Otu000060_Hyphomicrobium	no	no	no	no
BV5_Otu000061_Mycobacterium	no	no	no	yes
BV5_Otu000062_Flavobacterium	no	no	no	yes
BV5_Otu000063_SC-I-84	no	no	no	yes
BV5_Otu000065_Solirubrobacterales	no	no	no	yes
BV5_Otu000068_Gluconobacter	no	no	no	no
BV5_Otu000069_Pseudomonas	no	no	no	no
BV5_Otu000070_Rubrobacter	yes	no	no	no
BV5_Otu000073_Erwinia	yes	no	no	no
BV5_Otu000075_Solirubrobacteraceae	no	no	no	yes
BV5_Otu000076_Acinetobacter	yes	no	no	no
BV5_Otu000077_Solirubrobacterales	no	no	no	yes
BV5_Otu000078_Sinobacteraceae	no	no	no	yes
BV5_Otu000079_Buchnera	no	no	no	no
BV5_Otu000080_Pedomicrobium	no	no	no	yes
BV5_Otu000081_SC-I-84	no	no	no	yes

	Separator	Abundant	Core	Hub
BV5_Otu000084_Dongia	no	no	no	no
BV5_Otu000086_Burkholderiaceae	yes	no	no	no
BV5_Otu000089_Propionibacterium	yes	no	yes	no
BV5_Otu000091_Patulibacteraceae	no	no	no	no
BV5_Otu000092_0319-7L14	no	no	no	yes
BV5_Otu000094_Caulobacter	no	no	no	no
BV5_Otu000095_Sphingomonas	yes	no	yes	no
BV5_Otu000096_Bradyrhizobium	no	no	no	no
BV5_Otu000097_Gaiellaceae	no	no	no	yes
BV5_Otu000098_Mycobacterium	no	no	no	no
BV5_Otu000102_Solibacillus	no	no	no	yes
BV5_Otu000105_Labrys	no	no	no	no
BV5_Otu000106_Bacillus	no	no	no	yes
BV5_Otu000109_Kaistobacter	no	no	no	yes
BV5_Otu000112_Planococcaceae	no	no	no	yes
BV5_Otu000113_Dongia	no	no	no	no
BV5_Otu000114_Escherichia	yes	no	no	no
BV5_Otu000117_Solirubrobacterales	no	no	no	yes
BV5_Otu000118_[Weeksellaceae]	yes	no	no	no
BV5_Otu000120_Bacillus	no	no	no	yes
BV5_Otu000125_Steroidobacter	no	no	no	no
BV5_Otu000141_Ellin6513	no	no	no	no
BV5_Otu000143_Dolo	no	no	no	no
BV5_Otu000144_Rhizobiales	no	no	no	no
BV5_Otu000155_Burkholderia	no	no	no	no
BV5_Otu000156_Staphylococcus	yes	no	no	no
BV5_Otu000194_Micromonospora	no	no	no	yes
BV5_Otu000256_Bacillales	no	no	no	yes
BV5_Otu000713_Bacteria	no	no	no	no
ITS2_Otu00003_Cladosporium	no	yes	yes	yes
ITS2_Otu00004_Exophiala	yes	no	no	no
ITS2_Otu00005_Cadophora	yes	no	no	no
ITS2_Otu00006_Fungi	no	yes	yes	no
ITS2_Otu00008_Boeremia	yes	yes	no	yes
ITS2_Otu00009_Didymellaceae	yes	yes	no	yes
ITS2_Otu00010_Sclerotiniaceae	yes	no	no	no
ITS2_Otu00011_Septoria	yes	yes	no	yes
ITS2_Otu00012_Alternaria	no	yes	no	yes
ITS2_Otu00013_Didymellaceae	yes	yes	no	no
ITS2_Otu00014_Chaetosphaeronema	yes	yes	no	yes
ITS2_Otu00015_Pseudomassaria	yes	no	no	no
ITS2_Otu00016_Botryotinia	yes	no	no	no
ITS2_Otu00017_Helotiales	yes	no	no	no
ITS2_Otu00018_Tetracladium	yes	no	no	no
ITS2_Otu00019_Alternaria	yes	yes	yes	yes
ITS2_Otu00020_Podospora	yes	yes	no	no
ITS2_Otu00021_Dactylonectria	no	no	no	no
ITS2_Otu00022_Neocosmospora	no	no	no	no
ITS2_Otu00023_Cistella	no	no	no	no
ITS2_Otu00024_Gibberella	no	yes	no	yes
ITS2_Otu00025_Colletotrichum	yes	yes	no	yes
ITS2_Otu00026_Mycena	no	no	no	no
ITS2_Otu00028_Ascomycota	no	no	no	no
ITS2_Otu00030_Uromyces	yes	yes	no	no
ITS2_Otu00031_Stemphylium	yes	yes	no	no
ITS2_Otu00033_Ilyonectria	no	no	no	no
ITS2_Otu00034_Serendipita	no	no	no	no
ITS2_Otu00035_Pseudoidium	yes	yes	no	no
ITS2_Otu00036_Hemimycena	no	no	no	no
ITS2_Otu00037_Exophiala	no	no	no	no
ITS2_Otu00042_Pleosporales	no	no	no	no
ITS2_Otu00052_Titaea	no	no	no	no

	Separator	Abundant	Core	Hub
ITS2_Otu00054_Ramularia	yes	yes	no	no
ITS2_Otu00058_Colletotrichum	yes	yes	no	yes
ITS2_Otu00063_Pleosporales	yes	yes	no	yes
ITS2_Otu00085_Fungi	yes	no	yes	no
ITS2_Otu00109_Malassezia	no	no	no	no
ITS2_Otu00232_Fungi	no	no	no	no
ITS2_Otu00257_Fungi	no	no	no	no
PV9_Otu00002_Pezothrips	no	yes	yes	no
PV9_Otu00003_Insecta	yes	no	no	no
PV9_Otu00004_Frankliniella	no	yes	no	no
PV9_Otu00005_Exophiala	yes	no	no	no
PV9_Otu00007_Pucciniomycetes	yes	yes	no	no
PV9_Otu00008_Mayetiola	yes	yes	no	no
PV9_Otu00009_Dothideomycetes	yes	yes	yes	yes
PV9_Otu00010_Aeolothrips	no	no	no	no
PV9_Otu00011_Agaricomycetes	yes	no	no	no
PV9_Otu00012_Agaricomycetes	no	no	no	yes
PV9_Otu00013_Insecta	no	yes	no	no
PV9_Otu00014_Metschnikowia	yes	no	no	no
PV9_Otu00015_Insecta	no	yes	no	no
PV9_Otu00016_Agaricomycetes	no	no	no	no
PV9_Otu00018_Aglenchus	no	no	no	no
PV9_Otu00019_Agaricomycetes	yes	no	no	no
PV9_Otu00021_Insecta	no	no	no	no
PV9_Otu00022_Agaricomycetes	no	no	no	no
PV9_Otu00024_Chromadorea_X	yes	no	no	no
PV9_Otu00026_Pezizomycotina	no	no	no	no
PV9_Otu00027_Agaricomycetes	no	no	no	no
PV9_Otu00028_Dothideomycetes	no	no	no	yes
PV9_Otu00030_Arachnida	no	no	no	no
PV9_Otu00032_Arachnida	yes	yes	no	no
PV9_Otu00033_Arachnida	no	no	no	no
PV9_Otu00034_Pezizomycotina	yes	no	no	no
PV9_Otu00035_Paratylenchus	no	no	no	no
PV9_Otu00038_Insecta	yes	yes	no	no
PV9_Otu00043_Plectus	no	no	no	no
PV9_Otu00047_Alatospora	no	no	no	no
PV9_Otu00053_Sebacina	no	no	no	no
PV9_Otu00057_Agaricomycetes	no	no	no	no
PV9_Otu00061_Dothideomycetes	yes	no	no	yes
PV9_Otu00062_Pratylenchus	no	no	no	no
PV9_Otu00064_Aphelenchus	no	no	no	no
PV9_Otu00081_Leotiomycetes	yes	yes	no	no
PV9_Otu00083_Adineta	yes	no	no	yes
PV9_Otu00084_Phytomyza	yes	yes	no	no
PV9_Otu00101_Exobasidiomycetes	yes	no	no	no
PV9_Otu00103_Eukaryota	yes	no	yes	no
PV9_Otu00104_Cercomonas	no	no	no	no
PV9_Otu00134_Amoebozoa	no	no	no	no
PV9_Otu00204_Rattus	no	no	no	no
PV9_Otu00337_Eukaryota	no	no	yes	no

(c) Flowers

	Separator	Abundant	Core	Hub
BV5_Otu000001_Pantoea	no	yes	no	no
BV5_Otu000003_Pseudomonas	no	yes	yes	yes
BV5_Otu000004_Phyllobacteriaceae	no	no	no	yes
BV5_Otu000005_Enterobacteriaceae	yes	yes	no	no
BV5_Otu000006_Ralstonia	yes	yes	no	yes
BV5_Otu000007_Bacillus	yes	no	no	yes

	Separator	Abundant	Core	Hub
BV5_Otu000008_Frankia	no	no	no	no
BV5_Otu000009_Phyllobacterium	no	no	no	yes
BV5_Otu000010_Pantoea	no	yes	no	yes
BV5_Otu000011_Mesorhizobium	no	no	no	yes
BV5_Otu000012_Burkholderia	yes	no	no	yes
BV5_Otu000013_Burkholderia	yes	no	no	yes
BV5_Otu000014_Agrobacterium	yes	no	no	no
BV5_Otu000015_Streptomyces	no	no	no	yes
BV5_Otu000016_Burkholderia	yes	no	no	yes
BV5_Otu000017_Mesorhizobium	no	no	no	yes
BV5_Otu000018_Comamonadaceae	no	no	no	no
BV5_Otu000019_Cryptosporangium	no	no	no	no
BV5_Otu000021_Pantoea	no	yes	no	yes
BV5_Otu000022_Methylibium	no	no	no	no
BV5_Otu000023_Steroidobacter	no	no	no	no
BV5_Otu000024_Xanthomonadaceae	yes	no	no	no
BV5_Otu000025_Rhodoplanes	no	no	no	yes
BV5_Otu000026_Rhizobium	no	no	no	yes
BV5_Otu000027_Gaiellaceae	no	no	no	yes
BV5_Otu000028_Wolbachia	yes	yes	no	no
BV5_Otu000029_Rhizobiales	no	no	no	yes
BV5_Otu000030_0319-7L14	no	no	no	yes
BV5_Otu000032_Pseudomonas	yes	yes	no	yes
BV5_Otu000033_Chryseobacterium	no	no	no	no
BV5_Otu000034_Prauserella	yes	no	no	yes
BV5_Otu000036_Bosea	yes	no	no	no
BV5_Otu000039_Methylibium	no	no	no	no
BV5_Otu000040_Sodalis	no	no	no	no
BV5_Otu000043_Bradyrhizobiaceae	no	no	no	yes
BV5_Otu000045_Micromonosporaceae	no	no	no	no
BV5_Otu000047_Methylobacterium	yes	no	no	no
BV5_Otu000049_Kineosporia	no	no	no	no
BV5_Otu000052_Methylobacterium	no	no	no	yes
BV5_Otu000054_Gaiellaceae	no	no	no	yes
BV5_Otu000055_Methylobacterium	no	no	no	yes
BV5_Otu000056_Enterobacteriaceae	yes	yes	no	no
BV5_Otu000057_Rhodospirillaceae	no	no	no	yes
BV5_Otu000059_Solirubrobacterales	no	no	no	yes
BV5_Otu000060_Hyphomicrobium	no	no	no	no
BV5_Otu000061_Mycobacterium	no	no	no	yes
BV5_Otu000062_Flavobacterium	no	no	no	no
BV5_Otu000063_SC-I-84	no	no	no	yes
BV5_Otu000065_Solirubrobacterales	no	no	no	yes
BV5_Otu000068_Gluconobacter	no	yes	no	no
BV5_Otu000069_Pseudomonas	no	yes	no	no
BV5_Otu000070_Rubrobacter	no	no	no	yes
BV5_Otu000073_Erwinia	no	no	no	yes
BV5_Otu000075_Solirubrobacteraceae	no	no	no	yes
BV5_Otu000076_Acinetobacter	yes	yes	no	no
BV5_Otu000077_Solirubrobacterales	no	no	no	yes
BV5_Otu000078_Sinobacteraceae	no	no	no	yes
BV5_Otu000079_Buchnera	no	yes	no	no
BV5_Otu000080_Pedomicrobium	no	no	no	no
BV5_Otu000081_SC-I-84	no	no	no	yes
BV5_Otu000084_Dongia	no	no	no	no
BV5_Otu000086_Burkholderiaceae	no	no	no	yes
BV5_Otu000089_Propionibacterium	yes	no	no	yes
BV5_Otu000091_Patulibacteraceae	no	no	no	no
BV5_Otu000092_0319-7L14	no	no	no	yes
BV5_Otu000094_Caulobacter	no	no	no	no
BV5_Otu000095_Sphingomonas	no	no	no	yes
BV5_Otu000096_Bradyrhizobium	no	no	no	yes

	Separator	Abundant	Core	Hub
BV5_Otu000097_Gaiellaceae	no	no	no	yes
BV5_Otu000098_Mycobacterium	no	no	no	yes
BV5_Otu000102_Solibacillus	no	no	no	no
BV5_Otu000105_Labrys	no	no	no	no
BV5_Otu000106_Bacillus	no	no	no	no
BV5_Otu000109_Kaistobacter	no	no	no	yes
BV5_Otu000112_Planococcaceae	no	no	no	no
BV5_Otu000113_Dongia	no	no	no	no
BV5_Otu000114_Escherichia	yes	no	no	yes
BV5_Otu000117_Solirubrobacterales	no	no	no	no
BV5_Otu000118_[Weeksellaceae]	no	no	no	yes
BV5_Otu000120_Bacillus	no	no	no	no
BV5_Otu000125_Steroidobacter	no	no	no	no
BV5_Otu000141_Ellin6513	no	no	no	yes
BV5_Otu000143_Dolo	no	no	no	no
BV5_Otu000144_Rhizobiales	no	no	no	no
BV5_Otu000155_Burkholderia	no	no	no	no
BV5_Otu000156_Staphylococcus	yes	no	no	no
BV5_Otu000194_Micromonospora	no	no	no	yes
BV5_Otu000256_Bacillales	no	no	no	no
BV5_Otu000713_Bacteria	no	no	no	no
ITS2_Otu00003_Cladosporium	yes	yes	yes	yes
ITS2_Otu00004_Exophiala	no	no	no	no
ITS2_Otu00005_Cadophora	no	no	no	no
ITS2_Otu00006_Fungi	no	yes	yes	yes
ITS2_Otu00008_Boeremia	yes	no	no	yes
ITS2_Otu00009_Didymellaceae	yes	yes	no	yes
ITS2_Otu00010_Sclerotiniaceae	yes	yes	no	yes
ITS2_Otu00011_Septoria	yes	no	no	no
ITS2_Otu00012_Alternaria	yes	yes	no	yes
ITS2_Otu00013_Didymellaceae	yes	yes	no	yes
ITS2_Otu00014_Chaetosphaeronema	yes	no	no	no
ITS2_Otu00015_Pseudomassaria	no	no	no	no
ITS2_Otu00016_Botryotinia	yes	yes	no	yes
ITS2_Otu00017_Helotiales	no	no	no	no
ITS2_Otu00018_Tetracladium	no	no	no	no
ITS2_Otu00019_Alternaria	no	yes	no	yes
ITS2_Otu00020_Podospora	yes	no	no	no
ITS2_Otu00021_Dactylonectria	no	no	no	no
ITS2_Otu00022_Neocosmospora	no	no	no	no
ITS2_Otu00023_Cistella	no	no	no	no
ITS2_Otu00024_Gibberella	no	no	no	no
ITS2_Otu00025_Colletotrichum	yes	no	no	no
ITS2_Otu00026_Mycena	no	no	no	no
ITS2_Otu00028_Ascomycota	no	no	no	no
ITS2_Otu00030_Uromyces	no	no	no	no
ITS2_Otu00031_Stemphylium	yes	no	no	no
ITS2_Otu00033_Ilyonectria	no	no	no	no
ITS2_Otu00034_Serendipita	no	no	no	no
ITS2_Otu00035_Pseudoidium	no	no	no	no
ITS2_Otu00036_Hemimycena	no	no	no	no
ITS2_Otu00037_Exophiala	no	no	no	no
ITS2_Otu00042_Pleosporales	no	no	no	no
ITS2_Otu00052_Titaea	no	no	no	no
ITS2_Otu00054_Ramularia	yes	no	no	no
ITS2_Otu00058_Colletotrichum	no	no	no	no
ITS2_Otu00063_Pleosporales	no	no	no	no
ITS2_Otu00085_Fungi	no	no	yes	no
ITS2_Otu00109_Malassezia	no	no	no	no
ITS2_Otu00232_Fungi	no	no	no	no
ITS2_Otu00257_Fungi	no	no	no	no
PV9_Otu00002_Pezothrips	yes	yes	yes	no

	Separator	Abundant	Core	Hub
PV9_Otu00003_Insecta	yes	no	no	no
PV9_Otu00004_Frankliniella	yes	yes	no	no
PV9_Otu00005_Exophiala	no	no	no	no
PV9_Otu00007_Pucciniomycetes	no	no	no	no
PV9_Otu00008_Mayetiola	yes	yes	no	no
PV9_Otu00009_Dothideomycetes	yes	yes	no	yes
PV9_Otu00010_Aeolothrips	yes	yes	no	no
PV9_Otu00011_Agaricomycetes	no	no	no	no
PV9_Otu00012_Agaricomycetes	no	no	no	no
PV9_Otu00013_Insecta	no	yes	no	no
PV9_Otu00014_Metschnikowia	yes	yes	no	yes
PV9_Otu00015_Insecta	yes	yes	no	no
PV9_Otu00016_Agaricomycetes	no	no	no	no
PV9_Otu00018_Aglenchus	no	no	no	no
PV9_Otu00019_Agaricomycetes	no	no	no	no
PV9_Otu00021_Insecta	yes	yes	no	no
PV9_Otu00022_Agaricomycetes	no	no	no	no
PV9_Otu00024_Chromadorea_X	no	no	no	no
PV9_Otu00026_Pezizomycotina	no	no	no	no
PV9_Otu00027_Agaricomycetes	no	no	no	no
PV9_Otu00028_Dothideomycetes	no	yes	no	yes
PV9_Otu00030_Arachnida	no	no	no	no
PV9_Otu00032_Arachnida	no	no	no	no
PV9_Otu00033_Arachnida	yes	yes	no	no
PV9_Otu00034_Pezizomycotina	no	no	no	no
PV9_Otu00035_Paratylenchus	no	no	no	no
PV9_Otu00038_Insecta	yes	no	no	no
PV9_Otu00043_Plectus	no	no	no	no
PV9_Otu00047_Alatospora	no	no	no	no
PV9_Otu00053_Sebacina	no	no	no	no
PV9_Otu00057_Agaricomycetes	no	no	no	no
PV9_Otu00061_Dothideomycetes	yes	no	no	no
PV9_Otu00062_Pratylenchus	no	no	no	no
PV9_Otu00064_Aphelenchus	no	no	no	no
PV9_Otu00081_Leotiomycetes	no	no	no	no
PV9_Otu00083_Adineta	no	no	no	no
PV9_Otu00084_Phytomyza	no	no	no	no
PV9_Otu00101_Exobasidiomycetes	no	no	no	no
PV9_Otu00103_Eukaryota	yes	no	yes	no
PV9_Otu00104_Cercomonas	no	no	no	no
PV9_Otu00134_Amoebozoa	no	no	no	no
PV9_Otu00204_Rattus	no	no	no	no
PV9_Otu00337_Eukaryota	no	no	yes	no

(d) Seeds

	Separator	Abundant	Core	Hub
BV5_Otu000001_Pantoea	yes	yes	no	no
BV5_Otu000003_Pseudomonas	yes	yes	yes	yes
BV5_Otu000004_Phyllobacteriaceae	yes	no	no	yes
BV5_Otu000005_Enterobacteriaceae	no	yes	no	no
BV5_Otu000006_Ralstonia	yes	yes	yes	yes
BV5_Otu000007_Bacillus	yes	no	no	yes
BV5_Otu000008_Frankia	yes	no	no	no
BV5_Otu000009_Phyllobacterium	yes	no	no	no
BV5_Otu000010_Pantoea	no	yes	no	no
BV5_Otu000011_Mesorhizobium	yes	no	no	no
BV5_Otu000012_Burkholderia	yes	yes	no	yes
BV5_Otu000013_Burkholderia	yes	yes	no	yes
BV5_Otu000014_Agrobacterium	no	no	no	yes
BV5_Otu000015_Streptomyces	no	no	no	no

	Separator	Abundant	Core	Hub
BV5_Otu000016_Burkholderia	yes	yes	no	yes
BV5_Otu000017_Mesorhizobium	yes	no	no	yes
BV5_Otu000018_Comamonadaceae	yes	yes	no	no
BV5_Otu000019_Cryptosporangium	yes	no	no	no
BV5_Otu000021_Pantoea	yes	yes	no	no
BV5_Otu000022_Methylibium	no	no	no	no
BV5_Otu000023_Steroidobacter	no	no	no	no
BV5_Otu000024_Xanthomonadaceae	yes	no	no	no
BV5_Otu000025_Rhodoplanes	no	no	no	no
BV5_Otu000026_Rhizobium	yes	no	no	no
BV5_Otu000027_Gaiellaceae	no	no	no	no
BV5_Otu000028_Wolbachia	yes	yes	no	no
BV5_Otu000029_Rhizobiales	no	no	no	no
BV5_Otu000030_0319-7L14	no	no	no	no
BV5_Otu000032_Pseudomonas	yes	no	no	yes
BV5_Otu000033_Chryseobacterium	no	no	no	no
BV5_Otu000034_Prauserella	yes	yes	no	yes
BV5_Otu000036_Bosea	yes	no	no	no
BV5_Otu000039_Methylibium	no	no	no	no
BV5_Otu000040_Sodalis	yes	yes	no	no
BV5_Otu000043_Bradyrhizobiaceae	no	no	no	yes
BV5_Otu000045_Micromonosporaceae	no	no	no	no
BV5_Otu000047_Methylobacterium	no	no	no	no
BV5_Otu000049_Kineosporia	yes	no	no	no
BV5_Otu000052_Methylobacterium	yes	no	no	yes
BV5_Otu000054_Gaiellaceae	no	no	no	no
BV5_Otu000055_Methylobacterium	no	no	no	no
BV5_Otu000056_Enterobacteriaceae	no	no	no	no
BV5_Otu000057_Rhodospirillaceae	no	no	no	yes
BV5_Otu000059_Solirubrobacterales	no	no	no	no
BV5_Otu000060_Hyphomicrobium	no	no	no	no
BV5_Otu000061_Mycobacterium	no	no	no	no
BV5_Otu000062_Flavobacterium	no	no	no	no
BV5_Otu000063_SC-I-84	no	no	no	no
BV5_Otu000065_Solirubrobacterales	no	no	no	no
BV5_Otu000068_Gluconobacter	no	no	no	no
BV5_Otu000069_Pseudomonas	no	no	no	no
BV5_Otu000070_Rubrobacter	yes	no	no	yes
BV5_Otu000073_Erwinia	yes	no	no	yes
BV5_Otu000075_Solirubrobacteraceae	no	no	no	no
BV5_Otu000076_Acinetobacter	no	no	no	no
BV5_Otu000077_Solirubrobacterales	no	no	no	no
BV5_Otu000078_Sinobacteraceae	no	no	no	no
BV5_Otu000079_Buchnera	no	no	no	no
BV5_Otu000080_Pedomicrobium	no	no	no	no
BV5_Otu000081_SC-I-84	no	no	no	no
BV5_Otu000084_Dongia	no	no	no	no
BV5_Otu000086_Burkholderiaceae	yes	no	no	yes
BV5_Otu000089_Propionibacterium	yes	no	yes	yes
BV5_Otu000091_Patulibacteraceae	no	no	no	no
BV5_Otu000092_0319-7L14	no	no	no	no
BV5_Otu000094_Caulobacter	no	no	no	no
BV5_Otu000095_Sphingomonas	yes	no	no	no
BV5_Otu000096_Bradyrhizobium	no	no	no	yes
BV5_Otu000097_Gaiellaceae	no	no	no	no
BV5_Otu000098_Mycobacterium	no	no	no	no
BV5_Otu000102_Solibacillus	no	no	no	no
BV5_Otu000105_Labrys	no	no	no	no
BV5_Otu000106_Bacillus	no	no	no	no
BV5_Otu000109_Kaistobacter	no	no	no	no
BV5_Otu000112_Planococcaceae	no	no	no	no
BV5_Otu000113_Dongia	no	no	no	no

	Separator	Abundant	Core	Hub
BV5_Otu000114_Escherichia	no	no	no	yes
BV5_Otu000117_Solirubrobacterales	no	no	no	no
BV5_Otu000118_[Weeksellaceae]	yes	no	no	yes
BV5_Otu000120_Bacillus	no	no	no	no
BV5_Otu000125_Steroidobacter	no	no	no	no
BV5_Otu000141_Ellin6513	yes	no	no	yes
BV5_Otu000143_Dolo	no	no	no	no
BV5_Otu000144_Rhizobiales	no	no	no	no
BV5_Otu000155_Burkholderia	yes	no	no	yes
BV5_Otu000156_Staphylococcus	yes	no	no	yes
BV5_Otu000194_Micromonospora	no	no	no	no
BV5_Otu000256_Bacillales	no	no	no	no
BV5_Otu000713_Bacteria	yes	no	no	yes
ITS2_Otu00003_Cladosporium	yes	yes	yes	no
ITS2_Otu00004_Exophiala	yes	no	no	no
ITS2_Otu00005_Cadophora	yes	no	no	no
ITS2_Otu00006_Fungi	yes	yes	yes	yes
ITS2_Otu00008_Boeremia	yes	yes	no	no
ITS2_Otu00009_Didymellaceae	yes	no	no	no
ITS2_Otu00010_Sclerotiniaceae	yes	yes	no	no
ITS2_Otu00011_Septoria	yes	no	no	no
ITS2_Otu00012_Alternaria	no	yes	no	no
ITS2_Otu00013_Didymellaceae	yes	yes	no	no
ITS2_Otu00014_Chaetosphaeronema	yes	no	no	no
ITS2_Otu00015_Pseudomassaria	yes	no	no	no
ITS2_Otu00016_Botryotinia	no	no	no	no
ITS2_Otu00017_Helotiales	no	no	no	no
ITS2_Otu00018_Tetracladium	no	no	no	no
ITS2_Otu00019_Alternaria	no	yes	no	no
ITS2_Otu00020_Podospora	yes	no	no	no
ITS2_Otu00021_Dactylonectria	no	no	no	no
ITS2_Otu00022_Neocosmospora	no	no	no	no
ITS2_Otu00023_Cistella	no	no	no	no
ITS2_Otu00024_Gibberella	yes	yes	no	no
ITS2_Otu00025_Colletotrichum	yes	no	no	no
ITS2_Otu00026_Mycena	no	no	no	no
ITS2_Otu00028_Ascomycota	yes	no	no	no
ITS2_Otu00030_Uromyces	no	no	no	no
ITS2_Otu00031_Stemphylium	yes	no	no	no
ITS2_Otu00033_Ilyonectria	no	no	no	no
ITS2_Otu00034_Serendipita	no	no	no	no
ITS2_Otu00035_Pseudoidium	no	no	no	no
ITS2_Otu00036_Hemimycena	no	no	no	no
ITS2_Otu00037_Exophiala	no	no	no	no
ITS2_Otu00042_Pleosporales	no	no	no	no
ITS2_Otu00052_Titaea	no	no	no	no
ITS2_Otu00054_Ramularia	no	no	no	no
ITS2_Otu00058_Colletotrichum	no	no	no	no
ITS2_Otu00063_Pleosporales	no	no	no	no
ITS2_Otu00085_Fungi	yes	yes	yes	no
ITS2_Otu00109_Malassezia	yes	yes	no	no
ITS2_Otu00232_Fungi	yes	no	no	yes
ITS2_Otu00257_Fungi	yes	no	no	yes
PV9_Otu00002_Pezothrips	yes	no	no	no
PV9_Otu00003_Insecta	yes	yes	no	no
PV9_Otu00004_Frankliniella	yes	no	no	no
PV9_Otu00005_Exophiala	yes	no	no	no
PV9_Otu00007_Pucciniomycetes	yes	no	no	no
PV9_Otu00008_Mayetiola	yes	no	no	no
PV9_Otu00009_Dothideomycetes	yes	no	no	no
PV9_Otu00010_Aeolothrips	yes	no	no	no
PV9_Otu00011_Agaricomycetes	no	no	no	no

	Separator	Abundant	Core	Hub
PV9_Otu00012_Agaricomycetes	no	no	no	no
PV9_Otu00013_Insecta	yes	no	no	no
PV9_Otu00014_Metschnikowia	yes	no	no	no
PV9_Otu00015_Insecta	yes	yes	no	no
PV9_Otu00016_Agaricomycetes	yes	no	no	no
PV9_Otu00018_Aglenchus	yes	no	no	no
PV9_Otu00019_Agaricomycetes	yes	no	no	no
PV9_Otu00021_Insecta	yes	no	no	no
PV9_Otu00022_Agaricomycetes	yes	no	no	no
PV9_Otu00024_Chromadorea_X	yes	no	no	no
PV9_Otu00026_Pezizomycotina	yes	no	no	no
PV9_Otu00027_Agaricomycetes	yes	no	no	no
PV9_Otu00028_Dothideomycetes	yes	no	no	no
PV9_Otu00030_Arachnida	no	no	no	no
PV9_Otu00032_Arachnida	yes	no	no	no
PV9_Otu00033_Arachnida	no	no	no	no
PV9_Otu00034_Pezizomycotina	no	no	no	no
PV9_Otu00035_Paratylenchus	yes	no	no	no
PV9_Otu00038_Insecta	no	no	no	no
PV9_Otu00043_Plectus	no	no	no	no
PV9_Otu00047_Alatospora	no	no	no	no
PV9_Otu00053_Sebacina	no	no	no	no
PV9_Otu00057_Agaricomycetes	no	no	no	no
PV9_Otu00061_Dothideomycetes	no	no	no	no
PV9_Otu00062_Pratylenchus	no	no	no	no
PV9_Otu00064_Aphelenchus	no	no	no	yes
PV9_Otu00081_Leotiomycetes	yes	no	no	no
PV9_Otu00083_Adineta	yes	no	no	no
PV9_Otu00084_Phytomyza	yes	no	no	no
PV9_Otu00101_Exobasidiomycetes	yes	yes	no	yes
PV9_Otu00103_Eukaryota	yes	no	yes	no
PV9_Otu00104_Cercomonas	yes	no	no	no
PV9_Otu00134_Amoebozoa	no	no	no	no
PV9_Otu00204_Rattus	yes	no	no	yes
PV9_Otu00337_Eukaryota	yes	no	yes	no

Method S1: Sequence data processing using Mothur.
We processed amplicon sequence data of microbial 16S rRNA, ITS2, and 18S rRNA using Mothur as described in Almario *et al*. (Almario et al., 2022; Schloss et al., 2009). In brief, processing of amplicon reads include forming contigs by pairing single-end reads (*make.contigs*), quality filtering for paired reads that are 100-600 bases long with at least 5 bases overlap (*screen.seqs*), demultiplexing (*rename.seqs*), dereplication (*unique.seqs, count.seqs*), detecting and removing chimera using VSEARCH (*chimera.vsearch, remove.seqs*), classifying sequences *(classify.seqs*), OTU clustering at 97 % sequence similarity threshold using dgc method (*cluster*), abundance filtering for OTUs with more than 50 reads (*split.abund*), classifying OTUs (*classify.otu*), creating OTU tables (*make.shared*), removing OTUs classified as chloroplast, mitochondria, *Arabidopsis*, Embryophyceae, unknown, and PhiX sequences (*remove.lineage*), and identifying OTU representative sequences based on abundance (*get.oturep*).

Method S2: Identification of hub microbes in *L. corniculatus*.
To determine hub bacteria, fungi, and eukaryotes in plant organs, correlation networks for each plant organ were computed using SparCC algorithm, as described in Almario *et al*. (Almario et al., 2022; Friedman et al., 2012). In brief, OTU absolute count tables with low abundance OTUs removed were used as input for FastSpar, a parallelizable implementation of SparCC algorithm, to calculate correlations (Watts et al., 2019). P-values were calculated from 1,000 bootstraps and resulting correlations with $P<0.001$ were used for constructing networks.

Method S3: Transmission of microbes in *L. corniculatus*.

To determine how *L. corniculatus* organ microbiomes can be linked and influenced by dispersal, we used Sankey diagrams to visualize potential flow of microbes across the soil and plant organs. Nodes of the diagrams represent different potential microbial sources (*i.e.* soil, plant organs, and environment/others) and arcs correspond to the number of OTUs shared between nodes.

To determine potential origins of organ-associated microbial communities, we used FEAST (Fast Expectation-mAximization microbial Source Tracking) to estimate contribution of potential microbial sources, such as soil, the different plant compartments, or the environment, to each plant organ microbiome (Shenhav et al., 2019). In the FEAST analysis we used multidirectional approach where microbiomes from plant organs or soil samples can be both sources or sinks. We implemented FEAST in R using default parameters for 1,000 iterations and we tested sinks with the same group of sources (*i.e.* all source microbiomes throughout seven sites for four years). In FEAST a proportion of the sink microbiomes is also potentially transmitted from "unknown sources", which can be other microbial sources that are not the assigned source in the analysis.

To statistically predict potential priority effects phenomena from roots, shoots, flowers, to seeds in *L. corniculatus*, we identified taxa of interest that are potentially involved in such phenomena in plant organs, as described in Debray *et al*. (Debray et al., 2022). We examined abundant, core, hub, or machine learning-predicted OTUs if changes in their relative abundance in plant organs correlate with changes in microbial community structure. We used PCoA of Bray-Curtis dissimilarities of plant organ microbial communities and PERMANOVA to assess the influence of these OTUs on community structure when they are abundant or rare (*i.e.* abundant OTUs: Relative abundance in each plant organ (RA) >= 0.01; intermediate OTUs: RA >= 0.001 and < 0.01; rare OTUs: RA < 0.001).

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Chapter IV

Plant age and genotype explain variation in the microbiome of natural *Lotus corniculatus* populations

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Abstract

In natural populations, plants are associated with a huge diversity of bacteria, fungi and other microbes. There is usually substantial microbiome variation between different plant individuals and populations, and the drivers of this variation are still poorly understood, particularly in wild plants. Here, we were interested in the potential for plant genotype and plant age to explain intraspecific variation in the plant microbiome. In seven natural populations, we genotyped a total of 168 plants over four years, determined their ages through herb chronology, and then sequenced their root, shoot, flower and seed microbiomes. We found that plant genotypes differed both in the diversity and composition of microbes, and that some microbial taxa were associated with particular plant genotypes - a result that we experimentally confirmed for one of the observed genotype-microbe associations. The genotype effects tended to be strongest and most consistent for plant-associated bacteria, with the largest plant genotype differences in the microbiome diversity of flowers and seeds. In contrast to the widespread plant genotype effects, we found less evidence for an effect of plant age on microbiome diversity: the age of plants explained variation in fungi diversity, and it was associated with the abundance of several microbial taxa. Our study indicates that the genotype of a plant and - to a lesser degree its age - influence the diversity and composition of plant-associated microbiota, even in complex natural environments.

Key words

bacteria, fungi, herb chronology, metagenomics, microbial ecology, plant-microbe interactions

Introduction

Plants are colonized by a wide range of bacterial, fungal and eukaryotic microorganisms collectively called the plant microbiome. The diversity and composition of this microbiome is strongly shaped by the plant species (Bonito et al., 2014; Knief et al., 2010) and the surrounding microbial community, especially the soil microbiome (Bulgarelli et al., 2012; Schlaeppi et al., 2014), but there are many other biotic and abiotic factors that are known to influence plant microbiome variation among and within species, including climate (Chen et al., 2019; Trivedi et al., 2022), soil characteristics (Tkacz et al., 2015), land use (Estendorfer et al., 2017; Gaube et al., 2021), herbivory (Malacrinò et al., 2021a, 2021b) or plant disease (Cui et al., 2021; Diskin et al., 2017; Kusstatscher et al., 2019; Solís-García et al., 2021; Wen et al., 2020). Besides this plethora of external factors, the plant microbiome can also be influenced by the plant itself. Variations in gene expression and plant traits can result in changing physical and chemical properties of the plants, and thus habitat conditions for the microbes, which in turn can alter microbiome diversity and composition. Previous studies have shown that the plant microbiome indeed varies across different plant tissue types such as roots, shoots or flowers (Bernard et al., 2021; Junker et al., 2011; Trivedi et al., 2020) and also between the inside (endophytic) and outside (epiphytic) microbiome (Agler et al., 2016; Trivedi et al., 2020). Two other potentially important factors creating microbiome variation among plants of the same species are their genetic differences, and their differences in age or developmental stage.

It is well established that the genetic variation in plants can change the pathogen resistance (Deng et al., 2020; Karasov et al., 2020; Thrall and Burdon, 2000), and there is also good evidence that it can influence the overall composition of the plant microbiome, e.g. in soybean (Liu et al., 2019), potato (Malacrinò et al., 2021a) and black cottonwood (Leopold and Busby, 2020). While the studies mentioned above were all under controlled lab conditions, there is also evidence from more natural common garden experiments, e.g. for plant genotype effects on the microbiome of *Medicago truncatula* (Brown et al., 2020) and *Boechera stricta* (Wagner et al., 2016). So far the research on these questions has been largely restricted to crops and model species, and to - usually short-term - experiments, whereas studies on wild plants and natural populations remain rare. Still, extrapolating lab results about plant-microbe interactions to natural environments is often challenging (Yu et al., 2016) because in natural populations plant microbiomes are influenced by a much larger complexity of biotic and abiotic factors. A true understanding of plant genotype effects, and their relative importance therefore, requires to also study plant genotype-microbiome relationships in natural populations.

Another potentially important but so far very little studied intrinsic factor creating intraspecific variation in plant microbiomes is the age or developmental stage of a plant. As a plant grows and develops, it undergoes physiological and biochemical changes that influence important functional traits, such as defense against herbivory (Boege et al., 2007; Yang et al., 2020) or immune responses to pathogens (Develey-Rivière and Galiana, 2007). These changes across different life stages will inevitably also influence plant-microbiome interactions (Bressan et al., 2009). For example, the root-associated microbiome of rice varies between the vegetative phase and later life stages (Edwards et al., 2018), and very similar results were found for soybean (Sugiyama et al., 2014) and sugarcane (Hamonts et al., 2018). For annual plants like rice or soybean such a comparison between vegetative and reproductive phase covers their entire life cycle. However, many plants, in particular wild ones, are perennial, which creates additional possibilities for age-related microbiome variation e.g. through slower successional or competitive replacement processes. So far, there are hardly any studies of perennial plants examining relationships between plant age and plant microbiome. An exception is the study by Wagner et al. (2016) who found substantial changes in root (and to a lesser extent leaf) bacterial communities when comparing two- and four-year-old *Boechera stricta* plants in the common garden, suggesting that significant microbiome changes can also occur at later life stages of perennials. Considering that most plants are perennial, we clearly need more studies relating plant age to plant microbiome in longer-lived plants.

Understanding the influences of plant age and genotype on microbiome composition may be important not only from a fundamental scientific perspective but also for agriculture and plant conservation, for instance if it helps to better manage beneficial crop microbiomes that increase yield production, stress tolerance or pathogen resistance. The last point is particularly important given that pathogens are a huge threat to global food security (Strange and Scott, 2005). More generally, understanding the drivers and consequences of plant microbiome diversity may support the management of plant populations in rapidly changing environments, and the effects of plant age and genotype on plant microbiomes - particularly under realistic conditions - are important elements of this.

Here, we combined amplicon-based metagenomics with herb chronology and plant genotyping to study relationships between plant age and genotype, and the diversity and composition of plant microbiomes within and among natural plant populations. Our study organism was *Lotus corniculatus*, a widely distributed perennial legume that grows naturally in a broad range of environments in temperate Eurasia and is an important food source for many pollinators, such as bumblebees, honeybees, and many wild bees as well as butterflies, flies and beetles (Hennig and

Ghazoul, 2011; van der Kooi et al., 2016; Weiner et al., 2014). As a nitrogen fixer it is part of a complex plant-microbe symbiosis influencing the nutrient dynamics of grassland ecosystems (van der Heijden et al., 2016), and it therefore plays an important role in agriculture. As *L. corniculatus* is known to have a broad life span range at least up to 15 years (Roeder et al., 2017; Anna Roeder & Christiane Roscher, unpublished data), and it is known to harbor significant intraspecific genetic diversity (Abraham et al., 2015; Durka et al., 2025), the species is a suitable system for asking questions about effects of plant age and genotype. Specifically, we wanted to answer the following questions:

1. Do plant age and genotype affect the diversity of the plant microbiome, and if yes how do these effects vary between different plant tissues and microbial groups?
2. Are plant age and genotype related to the composition (beta diversity) of the plant microbiome, and how are these relationships influenced by the plant tissue and microbial groups?
3. Which specific microbial taxa are most differentiated between plants of different ages and genotypes, and can we confirm some of these findings experimentally?

To answer these questions, we collected *L. corniculatus* plants from seven semi-natural grasslands in the Swabian Jura region in Germany over four consecutive years, and we then determined the ages and genotype of all plants, and analyzed their bacterial, fungal and eukaryotic microbes separately for roots, shoots, flowers and seeds.

Methods

Plant material

We collected *Lotus corniculatus* plants from seven extensively managed meadows in the Swabian Alb region of southern Germany (Fig. 1a, Tab. S1) for four consecutive years from 2018 to 2021. All collections were done in the late summer (August/September), when the plants were still flowering but already carried some ripe fruits, so that both flowers and seeds could be sampled. In each of the seven populations, we carefully excavated six entire plants, resulting in a total of 4 years x 7 populations x 6 plants = 168 plants. In the lab, we divided the plants into roots, shoots, flowers and seeds, and surface sterilized all plant parts. Therefore, we washed them by shaking in Falcon tubes (50 ml for roots and shoots; 25 ml for flowers) and 1.5 ml Eppendorf tubes for seeds for 30 s with autoclaved ultrapure water (UPW), for 60 s with epiphyte wash solution (0.1 % Triton X-100 in 1x TE buffer), for 30 s with 80% EtOH and for 30 s with 2 % NaOCl. After that, we rinsed the samples three times (seeds: five times) for 10 s with UPW and froze them.

Determination of plant ages

To determine the ages of the sampled plants, we cut 3 cm of the root crown just below the hypocotyl (Fig. 1b), the oldest part of the plant root, from all plants and preserved them in 70% ethanol. In the lab, we placed these root samples into a microtome (Reichert, Vienna, Austria) and sliced them into transverse sections. Very small roots with a diameter smaller than 3 mm were clamped using a piece of wood. To harden the specimens and prevent them from drying out and cracking, all specimens and the blade were kept moist with 99% ethanol during the cutting process. The root crown slices were then fixed on a microscope slide in a 1:1 mixture of water and glycerol and covered with a cover glass. We used a microscope to count the number of annual rings, with ring boundaries defined by marginal parenchyma, semi-ring porosity or both (Schweingruber and Poschlod, 2005) (Fig. 1b). Where necessary we used polarized light to improve the visibility of the annual rings. Since the annual rings were not equally distinct in all specimens, we classified the distinctness of the rings into 1 = very good, 2 = intermediate, 3 = poor and 4 = no annual rings visible (Tab. S2) and used only classes 1 to 3 for our analyses. For the statistical analysis we decided to group the plants into 5 age groups since the low number of replicates for the very young (2–3 years) and very old plants (6–13 years). Therefore, we grouped the plants into age categories: younger than 4, 4, 5, 6, and older than 6 years (Fig. 1d).

Plant genotyping

To genotype the sampled plants, and assess their relatedness, we employed ddRAD Peterson et al. (2012), a reduced-representation sequencing approach. We extracted plant DNA from all shoot samples using the QIAGEN DNeasy Plant Mini Kit (QIAGEN, 2019). We then double-digested the DNA and followed the library preparation protocol of Peterson et al. (2012) with small modifications as given in Durka et al. (2025). Multiplexed samples were sequenced paired-end (PE 150) on an Illumina Novaseq 6000 platform. After sequencing, we demultiplexed the reads using process_radtags from the Stacks 2.0 pipeline (Rochette et al., 2019) resulting in an average of 6.31 million reads retained per sample. We assembled reads, including three outgroup samples of *L. tenuis* and *L. pedunculatus* from Durka et al. (2025) and called SNPs using dDocent 2.7.8 (O'Leary et al., 2018; Puritz et al., 2014) with default parameters, except of using 0.90 as ClusteringSimilarity, filtered according to O'Leary et al. 2018 introducing a mean depth threshold of 5, and a minor allele frequency threshold of 0.05 and keeping only 1 SNP per contig. The resulting VCF file was converted into a fasta file (containing only the SNPs for each sample) with PGDSpider version 2.1.1.5 (Lischer and Excoffier, 2012). To construct a Maximum Likelihood tree, we used the program IQ-TREE release 2.0.3 (Minh et al., 2020). We used ModelFinder to select the best-fitting substitution model based on the Bayesian Information Criterion (BIC) (Kalyaanamoorthy et al., 2017) which identified the General Time Reversible (GTR) model with empirical base frequencies (F), a proportion of invariable sites (I), and gamma-distributed rate heterogeneity with four categories (G4) as the best-fit model (GTR+F+I+G4). To assess branch support, we also performed ultrafast bootstrap approximation (UFBoot) with 1,000 replicates (Hoang et al., 2018). Finally, we used TreeCluster version 1.0.4 (Balaban et al., 2019) to classify the SNP-based genotypes into a more restricted number of genetic groups (later used in our analyses), based on the total branch length of all leaves in the cluster (Sum Branch) with a threshold of 3.1. We visualized the phylogenetic tree using Interactive Tree of Life (iTOL) (Letunic and Bork, 2024) and colored the samples by their genetic group assignments. From here on we will refer to these nine genetic groups as genotypes.

Microbiome sequencing

To prepare the plant samples for microbiome sequencing, we first homogenized all frozen samples of soil, roots, shoots, flowers, and seeds with a Precellys 24 Tissue Homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France), and then extracted the DNA using the FastDNA Spin Kit for Soil (MP Biomedicals, Irvine, CA, USA). We then performed a two-step PCR targeting the bacterial 16S rRNA V5-V7 region, the fungal ITS2 region, and the eukaryotic 18S rRNA V9 region, using the primers 799F/1192R, fITS7/ITS4, and F1422/R1797, respectively (Tab. S3) (Agler et al., 2016). As controls we used blank samples (UPW and blank DNA extraction). To reduce the amplification of mitochondrial and chloroplast rRNA sequences from *L. corniculatus*, we incorporated blocking oligos designed with the R package *AmpStop* (Tab. S3) (Mayer et al., 2021) (Lutap et al., 2025 / Chapter III). We pooled the amplified products in equal concentrations, purified them with magnetic bead clean-up, and randomly assigned each to one of eight sequencing batches. We sequenced all pools on a Illumina MiSeq platform with PhiX control, using the MiSeq Reagent Kit v3 (600-cycle), and then processed all microbial 16S rRNA, ITS2, and 18S rRNA amplicon sequences using Mothur (Almario et al., 2022; Schloss et al., 2009; Lutap et al., 2025 / Chapter III). To remove the primer sequences from 16S rRNA and 18S rRNA data we used Cutadapt, and to remove it from ITS2 data we used ITSx (Bengtsson-Palme et al., 2013; Martin, 2011). For the taxonomic classification of the bacterial 16S rRNA reads, we used the Greengenes database (13_8_99 release), for fungal ITS2 reads the UNITE database (02.02.2019 release), and for eukaryotic 18S rRNA the PR2 database (version 4.12.0), all of which included the PhiX genome (DeSantis et al., 2006; Guillou et al., 2013; Nilsson et al., 2019).

Data analysis

We carried out all data analyses in R Studio 2024.12.1 (RStudio Team, 2024), and we used *dplyr* for structuring and manipulating data frames (Wickham et al., 2023) *ggplot2* package (Wickham, 2016) for creating figures. We used the *vegan* package (Oksanen et al., 2025) for calculating the alpha diversity (Shannon index) of bacterial, fungal or eukaryotic microbiomes of each tissue in each plant, and then linear models to test for the effects of plant age or genotype. To account for variation in other factors, all models included the plant organ, and its respective interaction with plant genotype or plant age as well as sample location and year.

In addition to variation in microbial alpha diversity, we also analyzed the compositional turnover (beta diversity) between different samples, using the R packages *vegan*, *phyloseq*, and *microbiome* (Liu et al., 2021; McMurdie and Holmes, 2013; Oksanen et al., 2025). We first used the OTU relative abundances for a Principal Coordinate Analysis (PCoA) of the Bray-Curtis dissimilarities between samples, and then a PERMANOVA to test how much of the variation in microbial community composition could be explained by plant age, plant genotype, or their interaction. We also visualized the relative abundance profiles of the ten most abundant classes of bacteria, fungi or eukaryotes for the different *L. corniculatus* genotypes or age groups, using the *microeco* package (Liu et al., 2021). To further understand the abundance patterns of the three different main groups of microbes, we also created graphs of the frequencies of different levels of commonness of OTUs, occurring in one to nine genotypes, or in one to five age groups.

To identify specific bacterial, fungal or eukaryotic taxa that were associated with particular plant genotypes or plant age groups, we used the R packages *microbiomeMarker* and *phyloseq* (Cao et al., 2022; McMurdie and Holmes, 2013). We used linear discriminant analysis effect size (LEfSe) (p-value < 0.05, LDA score ≥ 2) (Segata et al., 2011) to identify microbial genera that were significantly associated with particular *L. corniculatus* genotypes or age groups, either separately for each plant organ, or across organs.

Genotype-specificity experiment

While LEfSe analyses can suggest a role of host genotype for specific microbial taxa, formal proof of genotype specificity is only possible with infection experiments. Although a comprehensive test of our LEfSe results was beyond our means, we exemplarily tested one LEfSe result for the fungal pathogen *Gibberella*/*Fusarium* that showed a strong genotype association. We isolated *Fusarium* strains from *L. corniculatus* shoots collected around Tübingen using standard surface sterilization and plating on *Fusarium*-selective medium (Jung et al., 2013). The detailed protocol can be found in the Supplementary Methods (S1). We used the isolated *Fusarium* strain to experimentally inoculate daughter plants of our genotype 1 - associated with high *Fusarium* abundances in LEfSe - versus plants of the (presumably) less susceptible genotype 8. To break seed dormancy, we submerged the seeds of the two genotypes in liquid nitrogen for five minutes and then germinated them on wet tissue paper at 21°C/18°C with a 16 h/8 h light/dark cycle in a growth chamber. After seven days we transplanted the germinated seeds into individual pots filled with standard potting soil. For each genotype, we used six plants for the infection treatment and six plants as controls. For spore production, we transferred five 1 cm^2 pieces of Potato Dextrose Agar media with the isolated *Fusarium* in 250 ml of liquid mung bean media (García-Bastidas et al., 2019) and incubated them for four days in the dark at 28°C at 150 RPM. For the plant infection, we filtered the suspension through autoclaved cheesecloth, counted the spores using a counting chamber and standardized the suspension to 10^6 spores/ml. To infect the plants, we dipped the entire shoots of the infection treatment plants in the spore suspension, whereas the shoots of the control plants were dipped in UPW. Two weeks after the infection we harvested all plants and quantified infection intensities through image analysis. For each plant, we disassembled all shoots and leaves and scanned them with a photo scanner (Epson Perfection V600) at high resolution (~ 35 MP). We used ImageJ (Schneider et al., 2012) to mask the plant stems and restrict analyses to the leaf area, and used the OpenCV2 package (Bradski, 2000) in Python v3.17 (Van Rossum, G., & Drake Jr, F. L., 1995) to filter all green (HSV thresholds of 5,70,70 and 75,250,215) and yellow pixels (HSV thresholds of 0,70,71 and 30,234,234) in these images. We used the number of yellow and green pixels as a proxy for the plant health, since leaf wilt and chlorosis are the most common symptoms of a *Fusarium* infection (Ekwomadu and Mwanza, 2023). We tested the genotype specificity of the *Fusarium* infection with a linear model that used the number of yellow pixels as a response variable and included infection treatment, plant genotype and their interaction as categorical explanatory variables, and the number of green pixels as a covariate. To improve the model fit, we log-transformed the yellow to green pixel ratio prior to the analysis.

Results

The ddRAD sequencing identified a total of 5739 SNPs across all plant individuals. The Maximum Likelihood tree with best score (LL score = -733814, not shown) and consensus tree (LL score = -733810; Fig. S1) agree in all major branches. Both trees are robust, with most bootstrap values above 50%. Based on the branch lengths of the ML tree, the TreeCluster algorithm grouped the plants into nine genotype groups, each represented by 14 to 26 samples (Fig. 1b, Fig. S1). While six of these groups were significantly overrepresented in some sites (Fig. 1b, χ2 test, *P* < 0.001), all groups occurred across multiple sites (3-6 sites, average 4.4 sites), usually with multiple replicates per site, and the use of the genotype groups therefore allowed us to test for their microbiome associations at least partly independently from site variation. The estimated ages of the same plant individuals ranged from two to 13 years, with most plants four to six years old (Fig. 1d). We found no between plant age and sampling year (χ2 test, *P* = 0.076), but there was a statistical association between plant age and sampling site (χ2 test, *P* < 0.001), with an overrepresentation of the younger than four-year-old plants in one of the sites. Thus, plant age was only to a small extent confounded with site, allowing us to test for its (largely) independent association with microbiome.

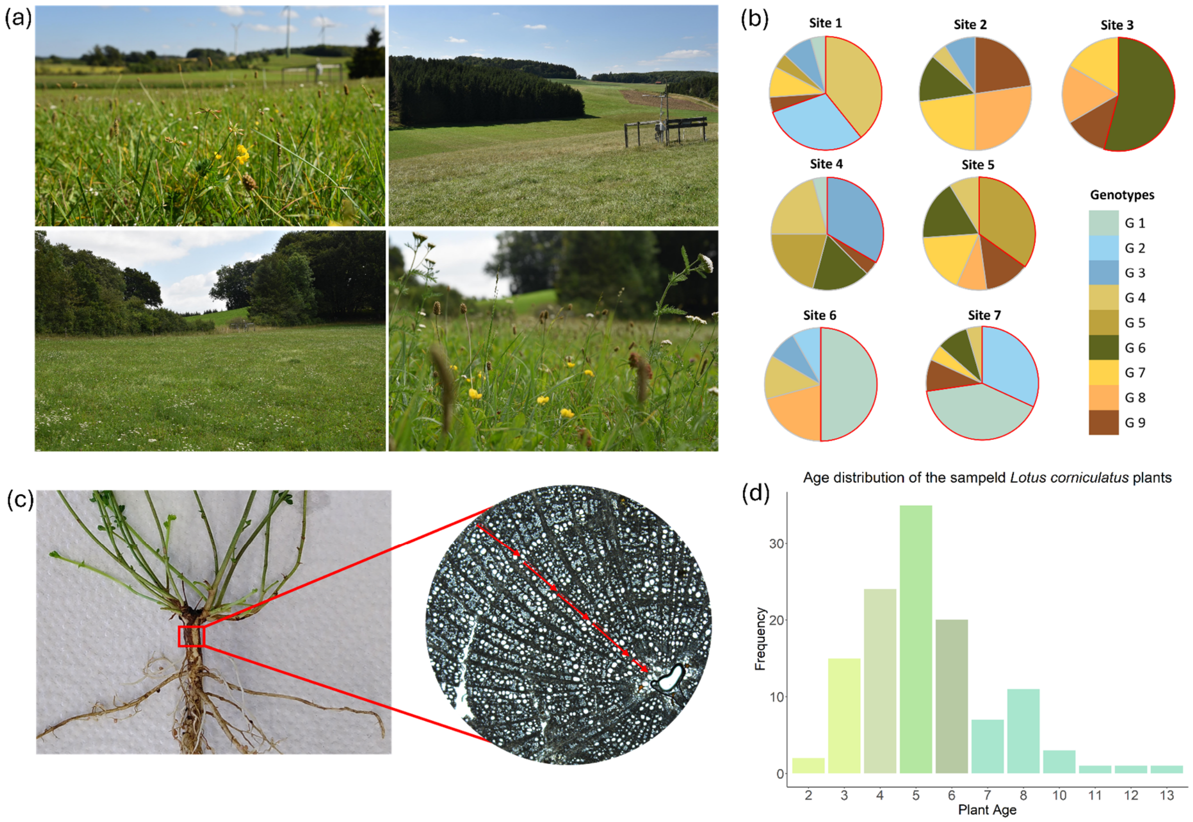

Figure 1. Sampling region, genotypic diversity, and determination of plant age. **a)** Photos of four of the sampling sites in southwest Germany. **b)** Frequencies of *Lotus* genotypes in the seven studied populations. The red borders indicate cases where genotypes are significantly overrepresented in specific populations. **c)** The root crown of a *Lotus* plant, and its cross-section, with the growth rings used to assess plant age. **d)** Age distribution of all sampled plants based on herb chronology. The colors indicate the five age groups we created for the statistical analysis.

Microbiome diversity differs between plant genotypes and ages

Across all sampling sites, years, plant individuals and plant organs (672 samples), the metagenome sequencing identified a total of 4,225 bacterial (16S rRNA), 2,027 fungal (ITS2 rRNA), and 1,773 eukaryotic (18S rRNA) OTUs, clustered at 97% sequence similarity and classified into 113 phyla and 1,542 genera. The most abundant bacterial classes we detected in the *Lotus* plants were Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, Actinobacteria, and Bacilli (Fig. 3a). The most abundant fungal classes are Dothideomycetes, Leotiomycetes, Eurotiomycetes, Sordariomycetes, and the most abundant eukaryotic classes Insecta, Dothideomycetes, Chromadorea_X, and Agaricomycetes (Fig. 3b,c).

Part of the observed microbial diversity differed systematically between plant organs, genotypes and age groups. Specifically, the variation in Shannon diversity (= within-sample) was highly significant between the different plant organs of all genotypes and age groups. In all cases, root diversity was substantially higher than that of other organs, followed by shoot and flower diversity. Notably, seed diversity was higher than that of flowers (Fig. 2a-f, Fig. S2). Both bacterial and fungal communities differed significantly among plant genotypes, with some genotypes harboring consistently higher bacterial or fungal diversity than others, across plant organs (Fig. 2a,b). We also found a significant interaction between genotype and plant organ for bacterial diversity: genotype differences were much more pronounced in *Lotus* flowers and seeds than in shoots and roots (Fig. 2a). A similar, albeit not statistically significant, pattern was visible for fungal diversity, where also the genotype differences were greatest in flowers (Fig. 2b). In contrast to the flower and seed microbiome, the microbial diversity of roots and shoots was much less variable between plant genotypes, and there were also no genotype differences in the diversity of eukaryotes (Fig. 2c). When comparing the microbiome diversity of different plant age groups, we found a significant age effect for fungal diversity, with younger plants harboring a higher diversity of fungal microbes than older ones (Fig. 2e). However, there were no plant age effects on bacterial or eukaryote diversity (Fig. 2d,f).

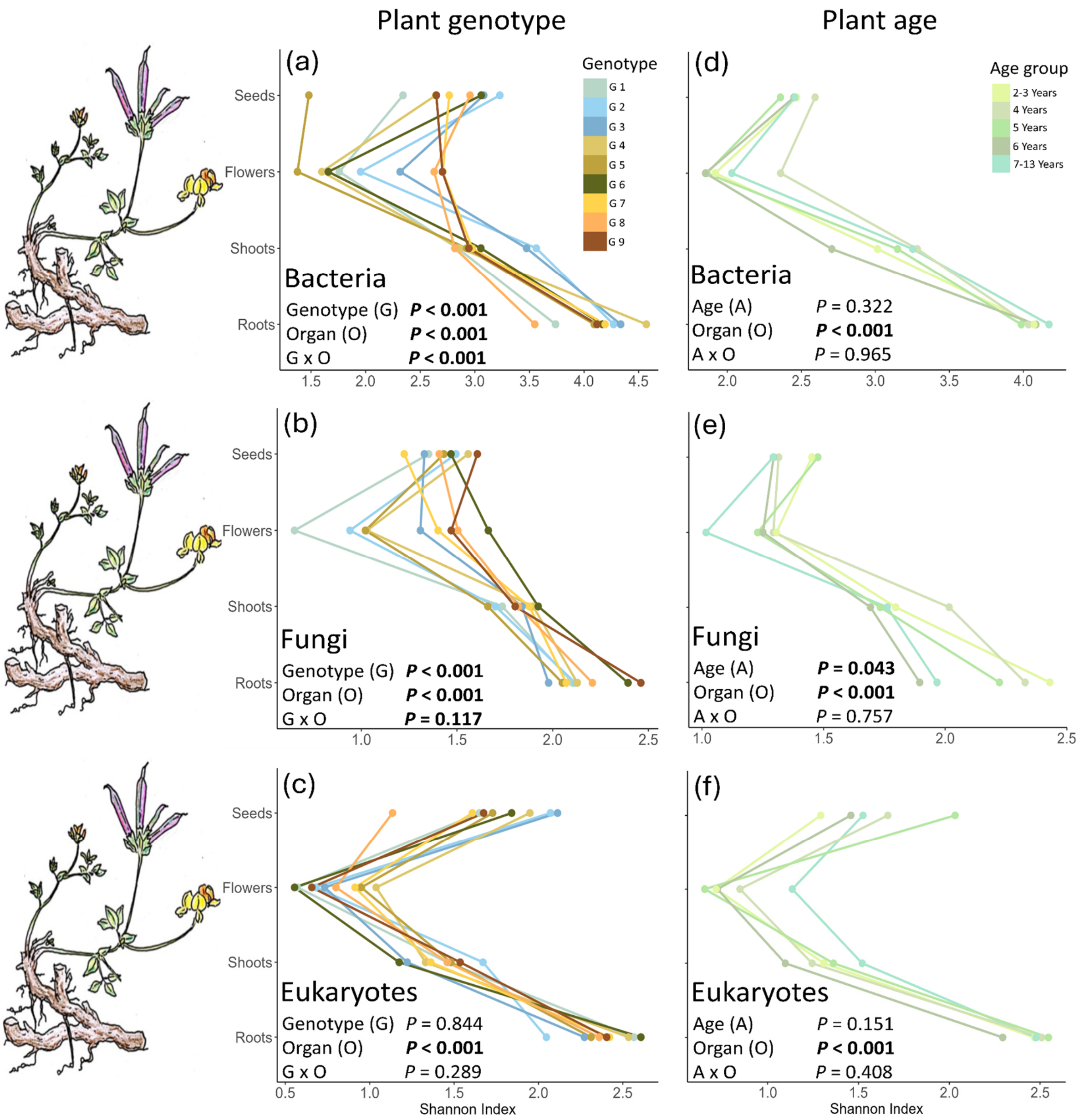

Figure 2: The average alpha diversity of bacterial, fungal and eukaryotic microbial communities associated with different genotypes (panels a - c) or different age groups (panels d - f) of *Lotus corniculatus* across seven semi-natural grasslands. Illustrations by S. Nicolai Rühl.

Plant genotype and age explain variation in microbiome composition

The age and genotypes of plants were not only associated with the diversity but also the composition of their microbiome. A large number of taxa associated with *L. corniculatus* were shared among all genotypes and age groups, but we also found taxa that were less widely distributed or even occurred in only one genotype or age group. Interestingly, the patterns of microbial commonness and rarity strongly differed between bacteria on the one side, and fungi and eukaryotes on the other side. In bacteria the majority of OTUs (2572 out of 4225) occurred in all nine genotypes, while OTUs that occurred in only one or few genotypes were rare. In fungi and eukaryotes, such less widely distributed taxa were much more frequent (Fig. 3g). The results were similar when we examined OTU distribution across age groups: almost 80% of all bacterial OTUs occurred in all age groups, and such that occurred in only one or few age groups were rare, whereas the patterns were much more even in fungi and eukaryotes (Fig. 3h).

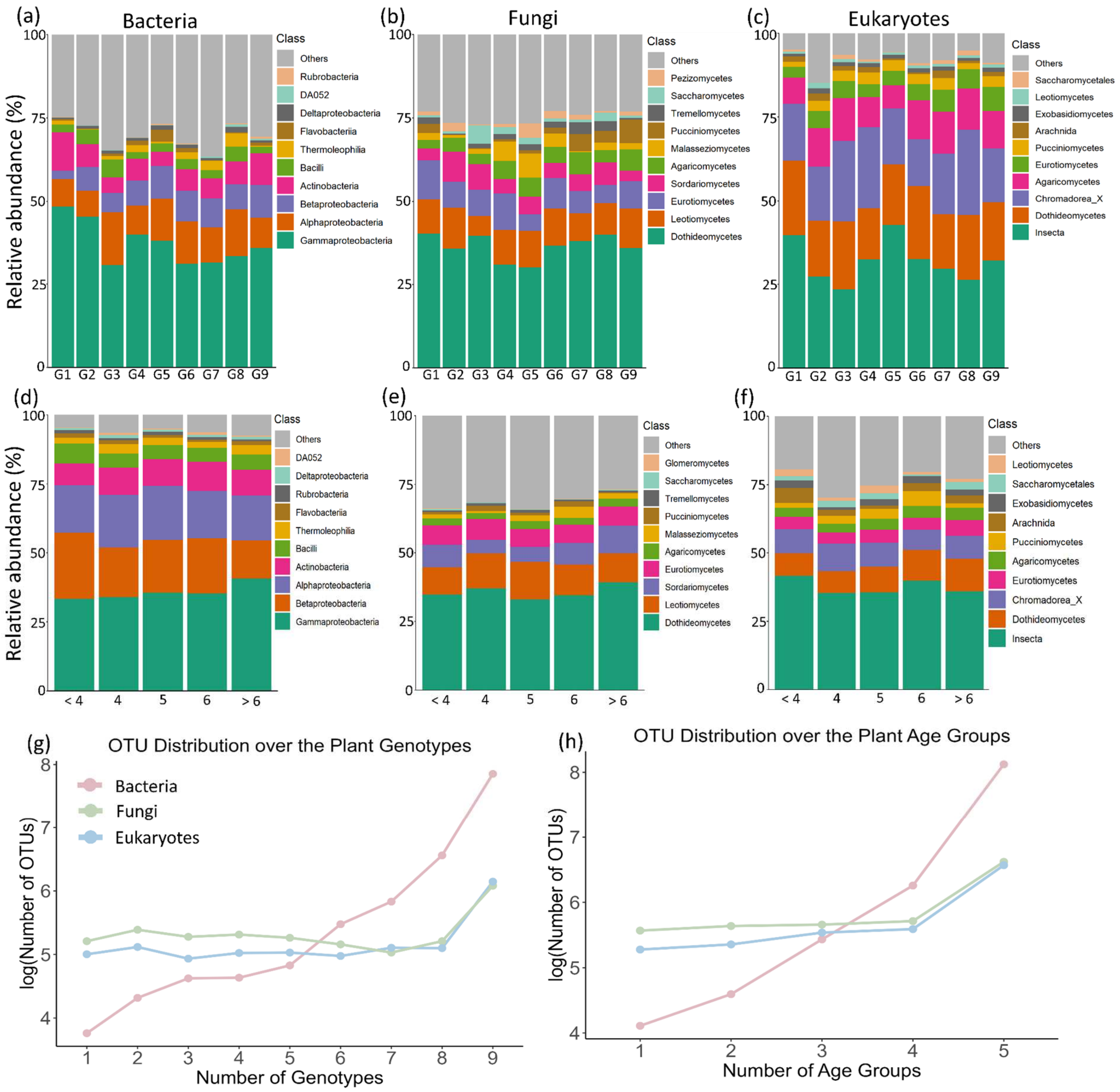

Figure 3: Relative abundance profiles of microbial communities associated with different genotypes (G1 - G9; panels a-c) or ages (panels d-f) of *Lotus corniculatus*, separately for bacteria, fungi and eukaryotes. We restricted the plots to the ten most abundant classes in each microbial group. Panels (g) and (h) summarize the frequencies of different levels of commonness across genotypes or ages in the three microbial groups.

The most consistent genotype effects were in roots and shoots, where the PERMANOVA identified significant plant genotype effects in the composition of all three microbial groups, with around 10% of the variation in community composition explained by plant genotype. In addition, there were significant genotype effects for bacterial communities in flowers and seeds, and for fungal communities in flowers (Tab. 1, Fig. S3). Interestingly, these genotype effects in flowers and seeds were even stronger, with over 15% variance explained.

We found no significant main effects of plant age on the composition of bacteria, fungal and eukaryotic communities in any of the four plant organs (Tab. 1). However, there were significant plant age by genotype interactions for fungal communities in roots and shoots and eukaryotic communities in roots, indicating a complex interplay between plant genotype and age in explaining microbial community composition in these cases.

Table 1: Results of PERMANOVA analyses testing the effects of plant genotype, plant age, or their interaction, on the Bray-Curtis dissimilarities of microbial communities associated with the roots, shoots, flowers, and seeds of *Lotus corniculatus* across seven semi-natural grasslands in SW Germany.

	Bacteria											
	Roots			Shoots			Flowers			Seeds		
	R^2	*F*	*Pr(>F)*	*R^2*	*F*	*Pr(>F)*	*R^2*	*F*	*Pr(>F)*	*R^2*	*F*	*Pr(>F)*
Plant genotype	0.102	1.53	**0.009**	0.103	1.57	**0.004**	0.174	2.67	**0.001**	0.160	2.59	**0.001**
Plant age	0.033	0.99	0.455	0.043	1.3	0.107	0.026	0.81	0.732	0.049	1.55	0.064
Genotpye x Age	0.234	1.04	0.309	0.231	1.04	0.277	0.180	0.82	0.956	0.204	0.98	0.549
Residual	0.632			0.623			0.620			0.588		

	Fungi											
	Roots			Shoots			Flowers			Seeds		
	R^2	*F*	*Pr(>F)*	*R^2*	*F*	*Pr(>F)*	*R^2*	*F*	*Pr(>F)*	*R^2*	*F*	*Pr(>F)*
Plant genotype	0.092	1.41	**0.005**	0.108	1.61	**0.001**	0.173	3.02	**0.001**	0.070	1.04	0.385
Plant age	0.029	0.90	0.71	0.034	1.06	0.351	0.042	1.48	0.103	0.025	0.74	0.844
Genotpye x Age	0.264	1.21	**0.012**	0.248	1.15	**0.036**	0.239	1.23	0.079	0.258	1.12	0.385
Residual	0.615			0.615			0.545			0.646		

	Eukaryotes											
	Roots			Shoots			Flowers			Seeds		
	R^2	*F*	*Pr(>F)*	*R^2*	*F*	*Pr(>F)*	*R^2*	*F*	*Pr(>F)*	*R^2*	*F*	*Pr(>F)*
Plant genotype	0.107	1.66	**0.001**	0.090	1.38	**0.008**	0.082	1.21	0.139	0.073	1.13	0.235
Plant age	0.035	1.07	0.264	0.037	1.13	0.257	0.029	0.85	0.641	0.046	1.41	0.076
Genotpye x Age	0.246	1.13	**0.021**	0.245	1.11	0.067	0.244	1.07	0.308	0.264	1.20	**0.043**
Residual	0.612			0.628			0.645			0.617		

Specific microbial taxa associated with plant genotypes and age groups

While a large number of taxa are shared among the plant genotypes and age groups, the LEfSe analyses revealed that some microbe taxa were significantly associated with particular individual plant genotypes or age groups (Fig. 4). Interestingly, these patterns of differential abundance were generally stronger in bacteria and fungi than in eukaryotic microbes, and they also tended to be more frequent with regard to plant genotypes (Fig. 4a) than for plant age groups (Fig. 4b), corroborating the PERMANOVA results with more frequent plant genotype than plant age effects (Tab. 1). Moreover, the LEfSe analyses also found that some plant genotypes and age groups showed much larger numbers of specific microbial taxa associations (e.g. genotypes 2, 3, 7 and 8, and age groups 2/3 and 5) than others. When analyzed separately for each plant organ, the LEfSE results differed significantly between organs, both for plant genotypes and plant age groups (Tab. S4), indicating that the observed microbial taxa associations are to some degree organ-specific.

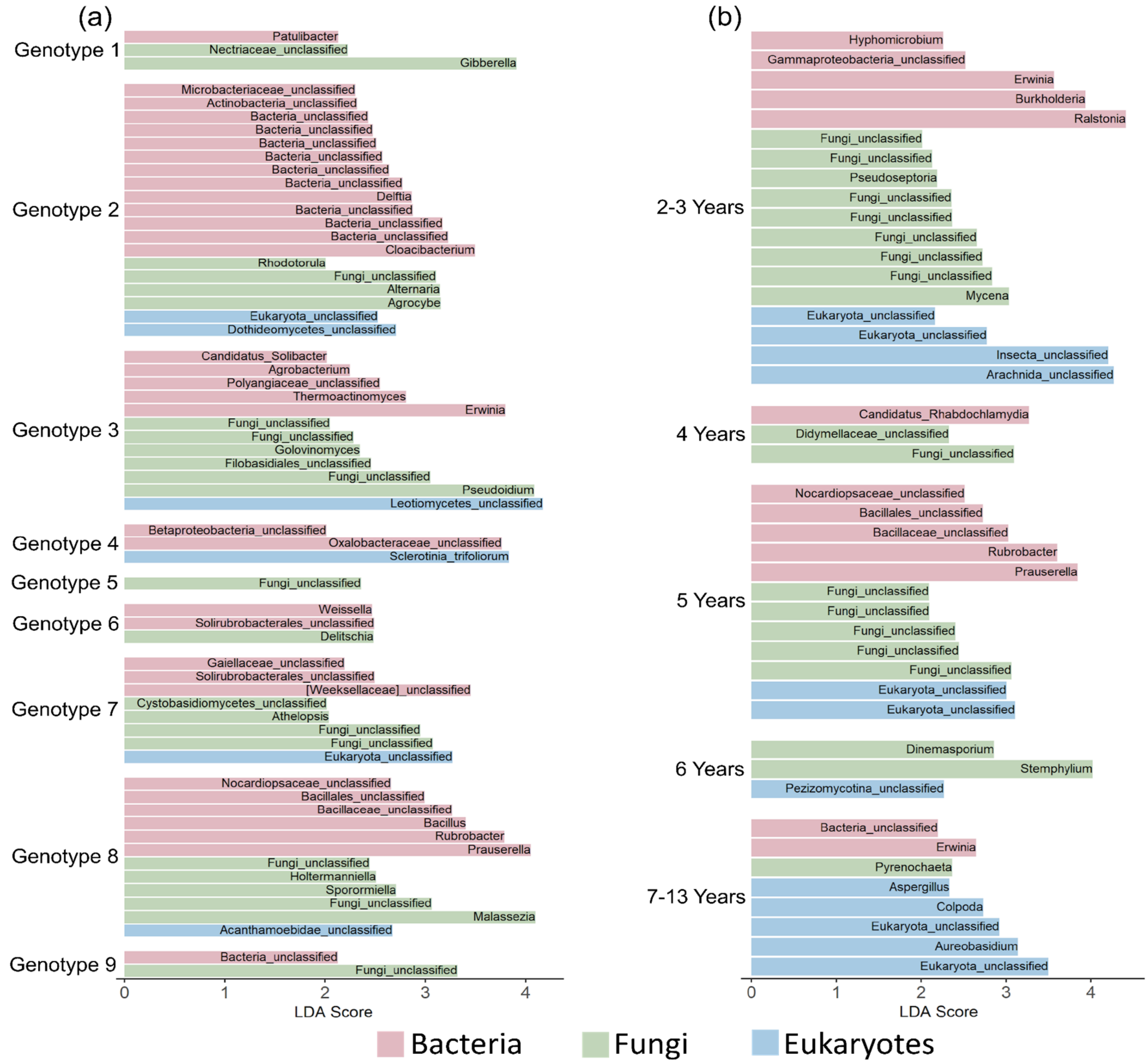

Figure 4: Microbial taxa significantly associated with specific genotypes (a) or age groups (b) of *Lotus corniculatus*, as identified through LefSe analyses. The three colors represent separate LefSe analyses conducted for bacteria, fungi, and eukaryotes, which were analyzed independently. These groups are presented together to provide a clearer overview of microbial variation across genotypes and age groups; however, the LDA score of taxa from different groups cannot be directly compared with each other. The raw results can be found in the supplementary material (Fig. S4).

Our experimental test of one of the observed LEfSe results, the overabundance of *Gibberella/Fusarium* associated with *Lotus* genotype 1 confirmed the results from the field. In both tested genotypes the chlorotic area (Fig. 5b) increased after infection with *Fusarium* (Fig. 5a) compared to the control treatment. However, the strength of this effect strongly varied between the genotypes. In the more susceptible genotype 1 the chlorotic area increased on average over 10-fold, whereas in genotype 8 the average chlorosis only doubled in infected plants compared to control plants (Fig. 5c). These differences in effect sizes were also confirmed by a significant genotype by treatment effect in the linear model (see Fig. 5 for details).

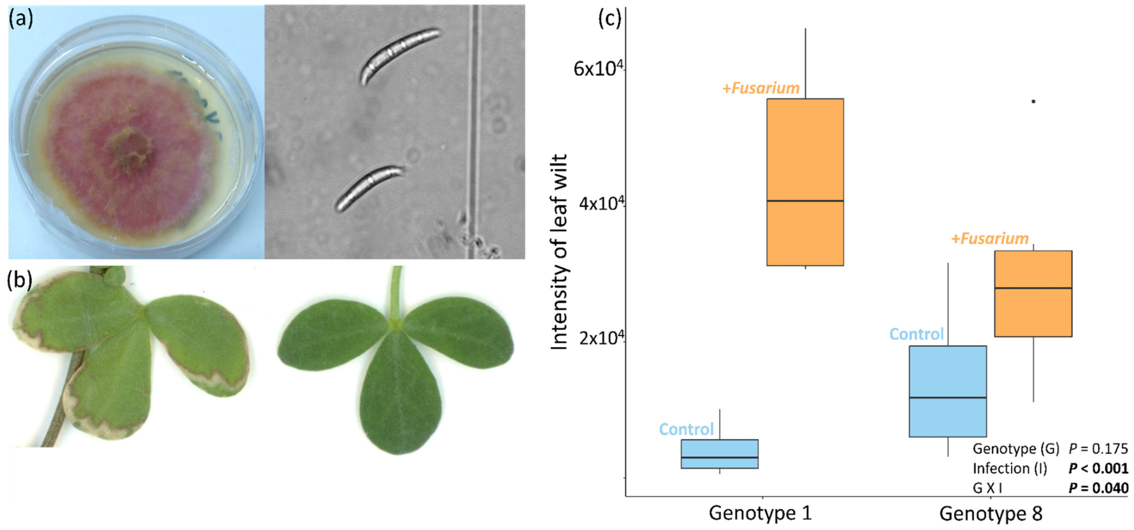

Figure 5: Experimental test of genotype-specificity of *Fusarium* infection of *Lotus corniculatus*. **a)** Isolated *Fusarium* in plate cultivation and microscopic image of *Fusarium* spores. **b)** Typical diseased and healthy leaves of *Lotus corniculatus*. **c)** Average intensities of leaf wilt of two *Lotus* genotypes, with or without experimental *Fusarium* infection.

Discussion

Plants harbor an astonishing diversity of microbes, but the factors shaping these complex communities throughout a plant's life, particularly in natural populations, remain poorly understood. Here, we show that in natural *Lotus corniculatus* populations both the age and genotype of plants is associated with changes in the diversity and composition of their associated microbiome. This is the case for the entire microbiome but also for individual microbial taxa that are significantly associated with specific genotypes or age groups.

Effect of plant age and genotype on microbiome diversity

We generally found large differences in the average microbiome diversity of different plant organs, a result that has already been described in detail elsewhere (Lutap et al., 2025 / Chapter III), and that likely reflects the different properties of plant organs as habitats for microbes, and their different modes of connectedness with the environment (Huang et al., 2024). Because of this large variation, we included the plant organs as factors in our analysis or ran analyses separately for different organs.

The alpha diversity of bacterial and fungal microorganisms differed significantly among the studied *Lotus corniculatus* genotypes. Interestingly, this effect was particularly strong in the flowers where we observed the strongest among-genotype variation in bacteria and fungi, whereas the microbiome diversity of roots and shoots was much more stable across genotypes. A possible explanation for this could be that flower traits are generally thought to be under stronger genetic control than vegetative plant traits (Campbell et al., 2022), and that these traits can influence the microbiome, particularly if they affect the abundance and diversity of flower visitors (Genung et al., 2010), which are known to influence the flower microbiome (Keller et al., 2021; Zemenick et al., 2021). For example, nectar secretion can vary among *L. corniculatus* genotypes (Murrell et al., 1982), potentially influencing the rate of flower visitation. In the study area, the flower visitors of *L. corniculatus* include various bee species, bumblebees, honeybees, and wild bees, but also butterflies (primarily Lycaenidae) and sometimes flies and beetles (Weiner et al., 2014). This broad range of pollinators may play a role in the observed flower microbiome variation between different plant genotypes. Moreover, the flower microbiome is closely linked to the seed microbiome, particularly for bacteria (Compant et al., 2011; Dutta et al., 2015; Lessl et al., 2007; Mitter et al., 2017), which may explain why we also found considerably among-genotype variation for bacteria in seeds.

Among-genotype variation in microbiome was generally lower in plant roots and shoots than in flowers and seeds. It is well known that the root microbiome is strongly influenced, in part through affecting root traits, by different environmental factors such as soil type, structure and pH, and by the surrounding soil microbiome (Bonito et al., 2014; Bulgarelli et al., 2012; Ristova and Busch, 2014; Schlaeppi et al., 2014; Schreiter et al., 2014). Together, this may ‘override’ the influence of host genetics and reduce its relative impact on root microbiomes and, as a consequence, also the neighboring shoot microbiomes.

Compared to the variation among genotypes, there was less variation in microbiome diversity between plants of different ages. Only for the Shannon diversity of fungi there were significant differences between age groups. This is interesting, as many fungi play important roles as plant symbionts or pathogens (Fisher et al., 2020, 2012; Martin and van der Heijden, 2024), and their interactions with plants are strongly influenced by the plant immune system. The immune response of plants changes throughout their development (Develey-Rivière and Galiana, 2007; Hu and Yang, 2019), and this could explain plant age-related variation in fungal microbiome diversity. Our results show that in principle plant age can also influence plant microbiome diversity, although to a lesser degree than plant genotype, and our evidence is limited to fungal microorganisms.

We should point out that comparisons of plant ages and genotypes in natural populations are not without challenges. In a field study, variation in plant age and genotype is inevitably confounded with variation in environment. We tried to account for this by aggregating both genotypes and ages into broader groups, which allowed 'replication' across populations. However, there was still statistical overrepresentation of some genotypes and age groups in some populations. Thus, despite our efforts both factors have not been fully independent of the sampling population, and we therefore cannot rule out that some of the observed genotype or age effects are overestimates.

Effect of plant genotype and age on microbiome composition

We found that different *L. corniculatus* genotypes were also associated with variation in the composition of the microbiome, i.e. genetic variation in the host not only affected the alpha diversity but also the beta diversity of the microbes. Again, the extent of these effects strongly depended on the plant organ: in roots and shoots the composition of all three major microbial groups (bacteria, fungi and eukaryotes) was affected by plant genotype, whereas in flowers only the composition of bacteria and fungi, and in the seeds only that of bacteria, showed significant plant genotype associations. A plethora of genetically variable plant traits are known to affect the plant microbiome and could thus be underlying these observed composition changes. For example, root exudates are an important determinant of the rhizosphere microbiome (Hu et al., 2018; Zhalnina et al., 2018), and can vary across plant genotypes (Micallef et al., 2009; Pacheco-Moreno et al., 2024). Intraspecific variation in leaf morphology and leaf chemistry has been shown to influence microbial communities in the phyllosphere (Hunter et al., 2010; Kniskern et al., 2007), and variation in plant defense genes is strongly affecting plant colonization by specific pathogens (Glander et al., 2018; Ronald et al., 1992).

In contrast to plant genotype, we found little evidence for effects of plant age on the composition of microbial communities. Only in fungi and eukaryotes, and mainly in roots and shoots, we found a significant plant age by genotype interaction, i.e. age effects were inconsistent and differed among genotypes, or were restricted to particular genotypes. This was counter to our expectation of age-related changes in species composition because of gene expression and functional trait changes during plant development (Hu and Yang, 2019). One possible reason for our findings could be that most species composition changes happen at early plant ages, and that the main factors shaping the microbiome such as root exudates (Chaparro et al., 2014) stabilize after this initial phase, so that the microbial communities reach an equilibrium fairly early. Unfortunately our youngest sampled plants were two years old, so we could not compare with one-year old plants, to understand the early-stage dynamics better. Clearly, a better understanding of these questions, and of the observed plant age by genotype interactions, requires controlled experiments with longer-term observation of the colonization dynamics of different plant genotypes under standardized environmental conditions.

When looking at the overall frequency patterns of microbes, we found that although many taxa were shared across all plant genotypes and ages, there were still significant numbers of taxa that occurred only in a subset of plant genotypes or age groups, and there was an intriguing difference between bacteria on the one hand, and fungi and eukaryotes on the other hand. While in bacteria the level of generalism was particularly high, i.e. a large fraction of taxa occurred everywhere, frequency distributions of taxa were much more even in fungi and eukaryotes, showing greater dispersal rates of bacteria than of non-bacteria microbes. Bacteria generally possess dispersal mechanisms unavailable to other microorganisms, allowing them to spread more efficiently. For instance, they can rapidly disperse through fungal hyphae in soil (Vieira et al., 2025) and, in general, exhibit much higher dispersal rates than fungal microbes (Larsen et al., 2023). In contrast, fungal dispersal is more strongly constrained by potential dispersal limitation (Zhang et al., 2021), which may further contribute to the broader distribution of bacterial taxa.

Associations of microbial taxa with specific plant genotypes and age groups

The differential abundance analysis identified a number of significant associations of microbial taxa with specific plant genotypes. Interestingly, these associations were not randomly distributed, but some plant genotypes harbored many more specific microbial taxa than others. Plant genotypes can differ in their production of secondary metabolites, which influence the recruitment or inhibition of specific microbes (Su et al., 2023). Additionally, modifications in root morphology can limit microbial attachment and interaction, reducing the presence of specialized microbial associations in the root (Robertson-Albertyn et al., 2017). Changes in these chemical and morphological traits can require greater levels of specialization of associated microbes.

If we look at the genotype associations in more detail, we find that several mutualistic bacteria, such as *Bacillus* (Idriss et al., 2002; Lozo et al., 2022; Tiwari et al., 2010), primarily soil-associated bacteria like *Cloacibacterium*, *Prauserella* and *Rubrobacter*, and some growth-promoting fungi like *Agrocybe* (Albrechtova et al., 2012; Wu and Kawagishi, 2020) were overrepresented in some plant genotypes. Similar host genotype effects on *Bacillus* have been observed in other studies, e.g. in maize cultivars resistant to corn stalk rot versus non-resistant cultivars (Xia et al., 2024), or in sweet pepper cultivars (Lozo et al., 2022). While plant interactions with the soil-associated bacteria are not yet fully understood, previous studies have shown that the plant genotype can influence the abundance of specific soil microbes in the rhizosphere (Yang et al., 2022), and that plants actively recruit genotype-specific beneficial soil microbes (Dilla-Ermita et al., 2021). We also found several pathogens with differential abundance among plant genotypes, e.g. *Erwinia* bacteria responsible for soft rot (Toth et al., 2003) and the fungal pathogens *Sclerotinia*, *Alternaria*, *Pseudoidium*, *Golovinomyces* and *Gibberella*. Our results corroborate previous studies with different plant species that also found genetic variation in plant resistance to these pathogens (Abe et al., 2010; Sobiczewski et al., 2015; Wegulo et al., 1998; Yang et al., 2010). In general, plant genetic effects on pathogen resistance are a well-known phenomenon, and a key topic in agriculture and crop management (Crute and Pink, 1996). They also play an important role in natural populations, and are a cornerstones of the geographic mosaic of coevolution observed in many wild species (Laine, 2009; Thompson, 2005; Thrall et al., 2012).

We also found that some microbial taxa were significantly overrepresented in particular plant age groups, e.g. bacteria from the genera *Burkholderia, Erwinia and Ralstonia* and fungi from the genus *Mycena* in the youngest plant group. *Burkholderia* bacteria are known for their growth-promoting effects (Dilla-Ermita et al., 2021) and have been shown to benefit maize plants during germination and seedling development (dos Santos et al., 2022). Although the studied *Lotus* plants were already beyond these initial stages of development, the observed higher abundances of *Burkholderia* in young plants suggest that these bacteria also play a role in early *Lotus* growth and development. *Mycena* is a saprotrophic fungus opportunistically invading plant roots (Harder et al., 2023), and *Erwinia* and *Ralstonia* are bacterial pathogens causing wilt in various plant species. The increased abundances of these taxa in young *Lotus* plants suggests these may have a less well developed immune system (Lemessa and Zeller, 2007; Liu et al., 2018) or other, e.g. root physiological, differences that make them more susceptible to invasion (Ando et al., 2015). There were also microbial taxa that were particularly abundant in older plants, e.g. the pathogenic fungus *Pyrenochaeta* which is known to cause root lesions (Hilton et al., 2013), and which we found overrepresented in plants older than six years. Our results confirm a previous study with oilseed rape that also observed a higher abundance of *Pyrenochaeta* in later plant developmental stages (Bennett et al., 2020). In general, our data show that while plant age does not have strong effects on the overall composition of microbes, there are some significant influences at the level of individual microbial taxa, even in a long-lived plant such as *Lotus corniculatus*.

It is important to keep in mind that all of the results discussed above are statistical associations between microbial taxa and plant genotypes/age groups in the field, but no formal proof of causal relationships. The latter require manipulative experiments, and to obtain such formal proof at least for one example of an observed significant genotype-microbe association, we conducted a small lab experiment testing the susceptibility of two of the sampled *Lotus corniculatus* genotypes to the fungal pathogen *Fusarium* (teleomorph *Gibberella*) under controlled conditions. As expected, the genotype with overabundance of *Fusarium* in the LEfSe analysis developed significantly greater leaf wilt when infected with *Fusarium* in the lab than another plant genotype that had not shown the same abundance of *Fusarium* in the field. This strongly indicates that the observed plant genotype association of *Fusarium* is indeed true and reflects genetically-based plant variation.

Conclusion

Our findings show the host genotype is an important driver of natural microbiome diversity of *L. corniculatus*. This influence is evident not only in overall microbial diversity and composition but also in the abundance of specific microbial genera across different genotypes. We also found evidence of plant age influencing the fungal diversity. Additionally, the abundance of specific microbial genera varies between the different host age groups. With varied genetics or age, the physiology and requirements of the plants change, and due to the close interaction between the microbiome and the host, this can result in an altered optimal microbiome composition. Therefore, it is valuable to combine insights from controlled laboratory experiments with data from natural environments to gain a more comprehensive understanding of the impact of genotype and age on the microbiome. Overall, our results show that genotype, and to a lesser extent age, play important roles in shaping the microbiome diversity and composition of natural *L. corniculatus* populations. This knowledge can also help optimize plant health, improve crop yields, and enhance ecosystem resilience which is crucial for effective ecosystem management and plant breeding practices.

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Supplementary material

Supplementary Methods (S1): We surface-sterilized shoot tissue from plants in different health conditions by washing it for 30 seconds with 2 % sodium hypochlorite and rinsing it three times with UPW. The washed shoots were then homogenized in UPW and plated on *Fusarium*-selective medium (Jung et al., 2013) slightly modified to 20 g/L dextrose, 15 g/L agar, 4 g/L potato extract, 50 mg/L kanamycin, 20 mg/L toxoflavin per 10 µl of the solution. After incubation at 30°C for 18h, we transplanted the hyphal tips of all growing fungi to fresh plates, extracted the DNA of these pure cultivations using the FastDNA^{TM} Spin Kit for Soil (MP Biomedicals), and Sanger sequenced it at the ITS2 marker for identification.

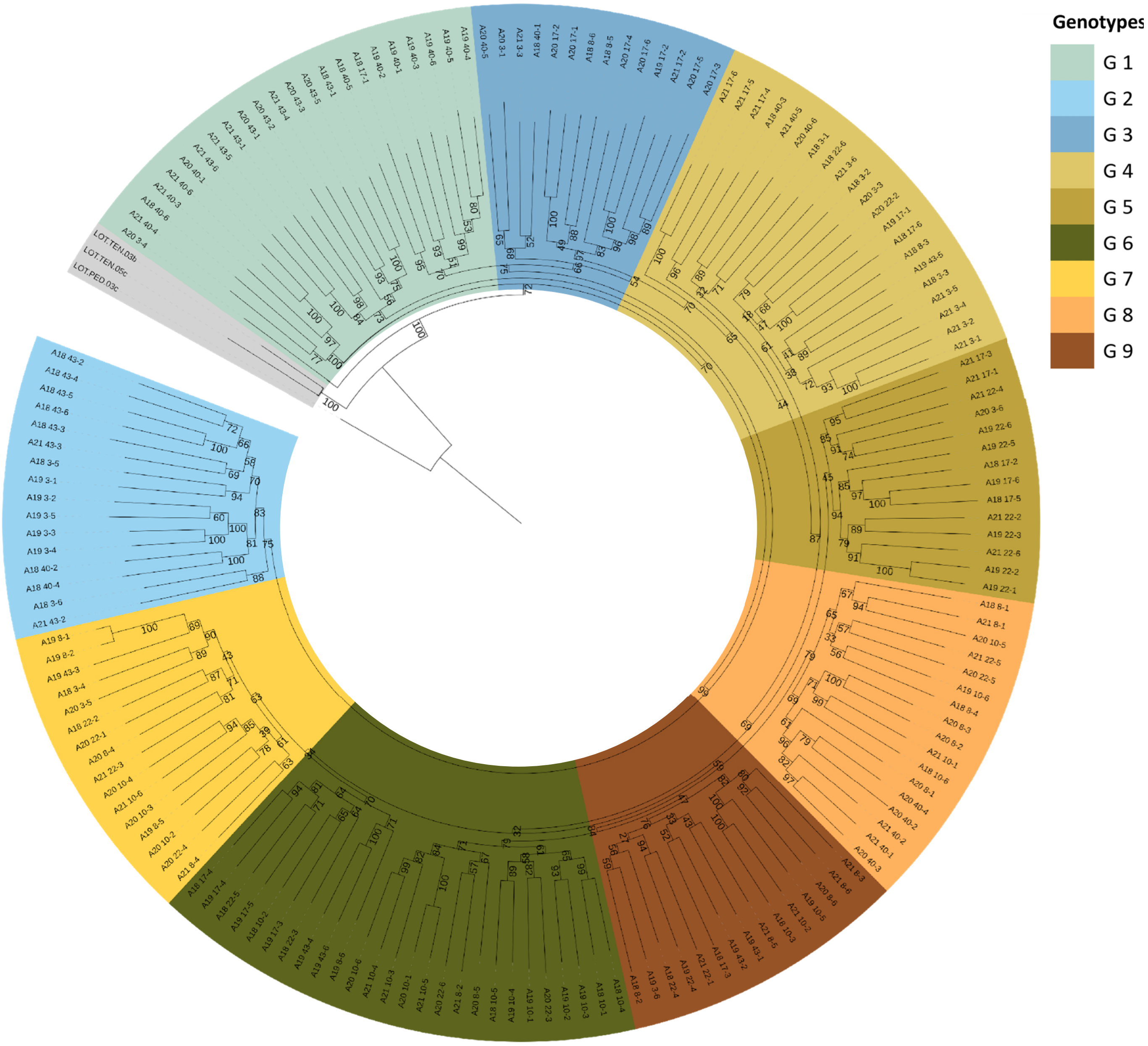

Figure S1: Bootstrap consensus tree derived from 1,000 replicates using IQ-TREE. Branches are supported if present in ≥50% of bootstrap replicates. Coloured by the different genotypes identified using IQ-tree.

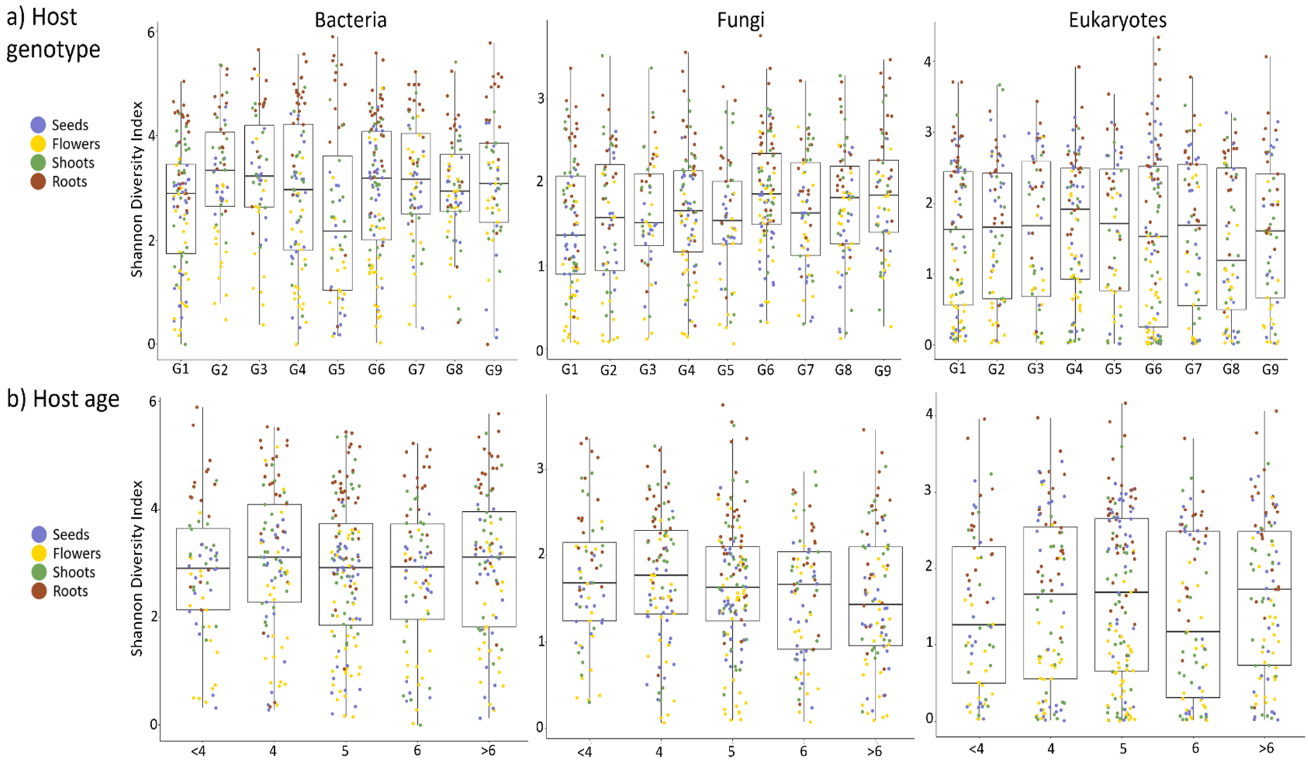

Figure S2: a) Box plots of the alpha diversity of bacterial, fungal and eukaryotic microbial communities associated the nine host genotypes colored by plant organs. **b)** Box plots of the alpha diversity of bacterial, fungal and eukaryotic microbial communities associated the five host age groups colored by plant organs.

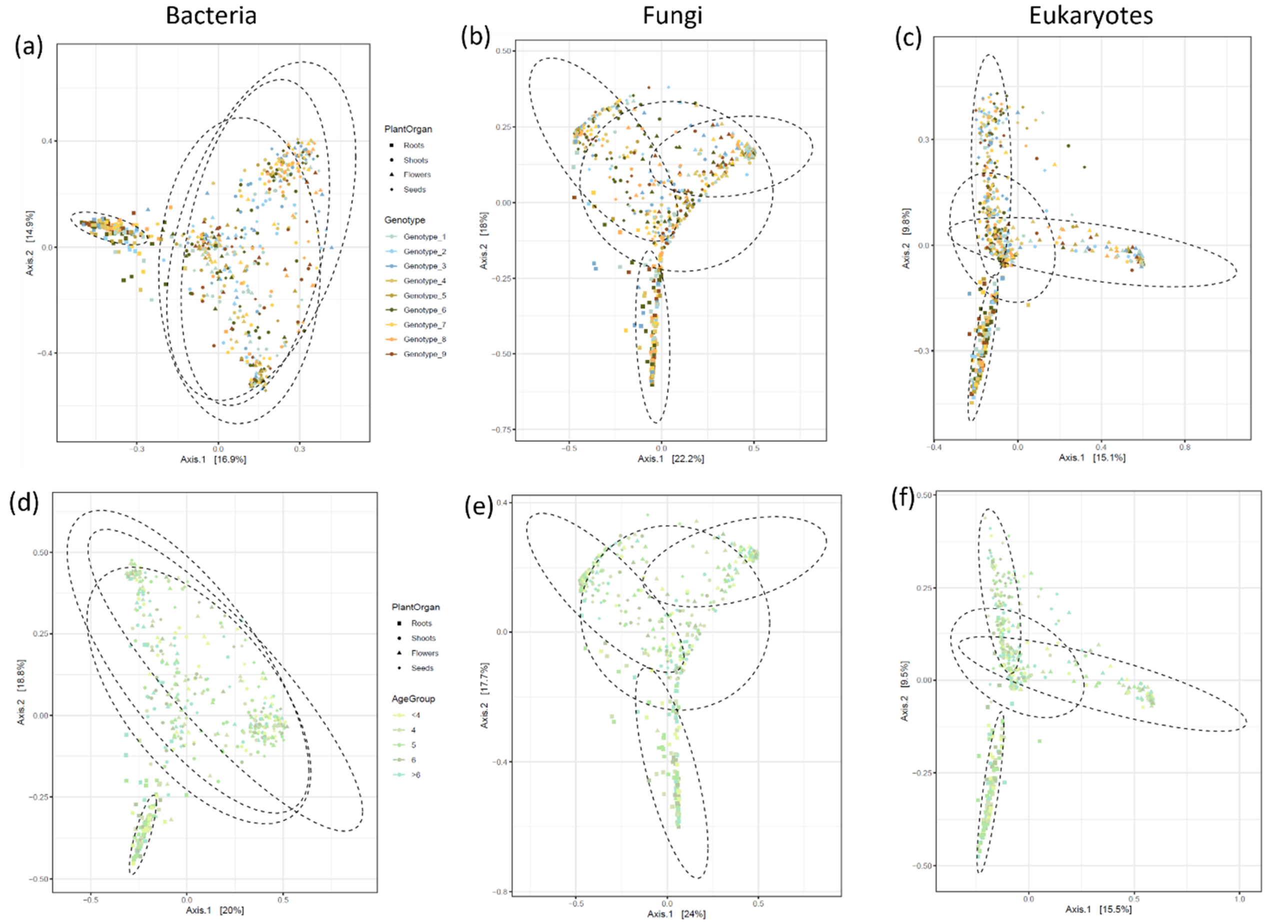

Figure S3: PCoA based on Bray-Curtis dissimilarities of the bacterial **(a,d)**, fungal **(b,e)** and eukaryotic **(e,f)** communities associated the different plant genotypes and age groups.

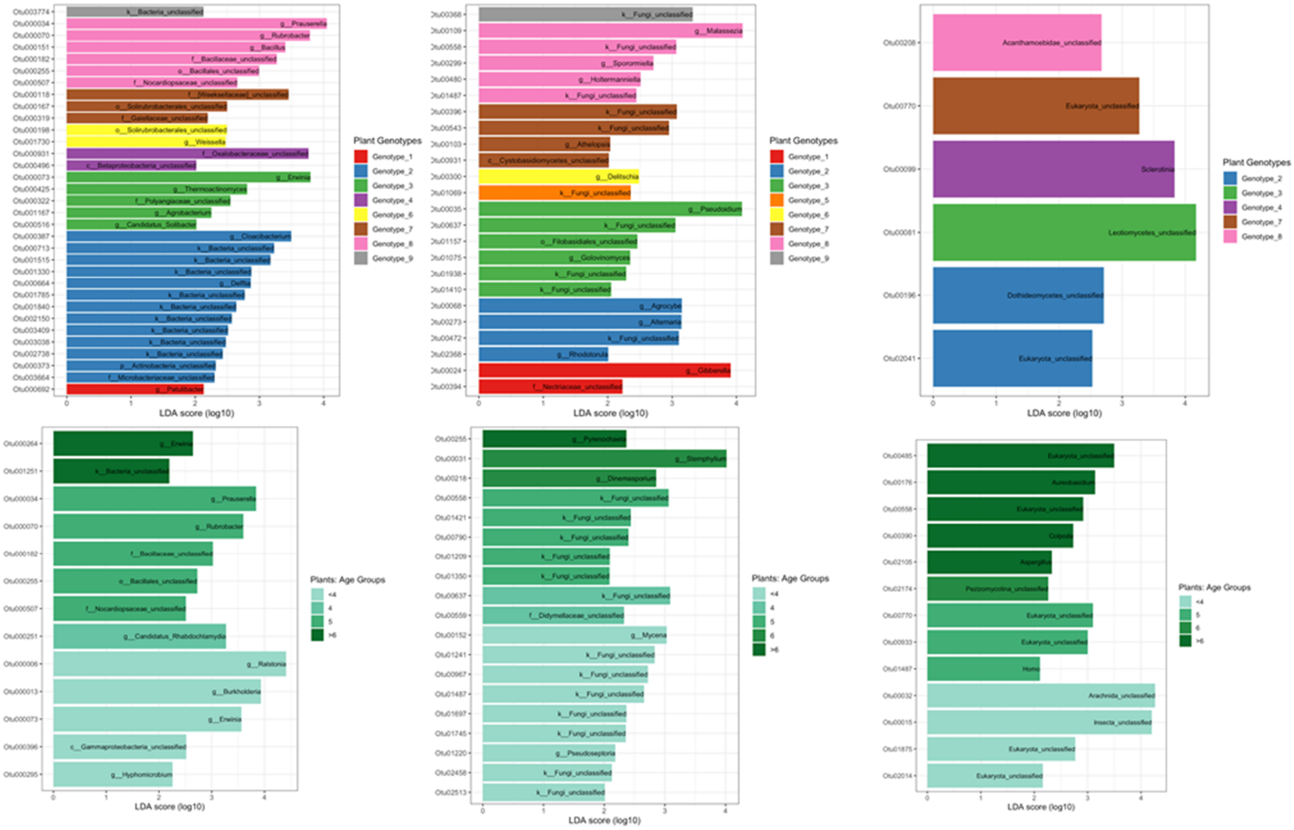

Figure S4: LefSe to identify differentially abundant OTUs in different genotypes of *Lotus corniculatus* plants

Table S1: Coordinates of the seven sampling locations

Site	Latitude	Longitude
1	48.41	9.53
2	48.42	9.49
3	48.38	9.21
4	48.40	9.52
5	48.40	9.51
6	48.41	9.57
7	48.41	9.54

Table S2: Tab of all Age determination results including Quality score

Sampled 2019			Sampled 2020			Sampled 2021		
Plant-ID	Age	Quality score	Plant-ID	Age	Quality score	Plant-ID	Age	Quality score
Lot19_3-1	4	2	Lot20_3-1	4	1	Lot21_3-1	5	2
Lot19_3-2	5	3	Lot20_3-2	5	2	Lot21_3-2	3	2
Lot19_3-3	5	2	Lot20_3-3	5	2	Lot21_3-3	4	1
Lot19_3-4	7	3	Lot20_3-4	4	3	Lot21_3-4	3	1
Lot19_3-5	11	2	Lot20_3-5	4	1	Lot21_3-5	4	1
Lot19_3-6	4	2	Lot20_3-6	5	2	Lot21_3-6	8	2
Lot19_8-1	4	2	Lot20_8-1	3	3	Lot21_8-1	4	3
Lot19_8-2	8	1	Lot20_8-2	2	2	Lot21_8-2	3	3
Lot19_8-3	N/A	4	Lot20_8-3	2	2	Lot21_8-3	4	3
Lot19_8-4	4	1	Lot20_8-4	4	3	Lot21_8-4	3	3
Lot19_8-5	N/A	4	Lot20_8-5	6	2	Lot21_8-5	3	2
Lot19_8-6	6	2	Lot20_8-6	3	1	Lot21_8-6	4	3
Lot19_10-1	5	1	Lot20_10-1	5	3	Lot21_10-1	8	2
Lot19_10-2	4	1	Lot20_10-2	5	2	Lot21_10-2	3	2
Lot19_10-3	N/A	4	Lot20_10-3	6	3	Lot21_10-3	5	1
Lot19_10-4	6	3	Lot20_10-4	7	2	Lot21_10-4	5	3
Lot19_10-5	8	2	Lot20_10-5	5	3	Lot21_10-5	5	1
Lot19_10-6	6	2	Lot20_10-6	4	2	Lot21_10-6	7	2
Lot19_17-1	3	1	Lot20_17-1	5	1	Lot21_17-1	3	3
Lot19_17-2	4	3	Lot20_17-2	4	2	Lot21_17-2	5	2
Lot19_17-3	3	1	Lot20_17-3	4	3	Lot21_17-3	4	1
Lot19_17-4	N/A	4	Lot20_17-4	5	3	Lot21_17-4	6	1
Lot19_17-5	8	3	Lot20_17-5	5	2	Lot21_17-5	6	1
Lot19_17-6	6	2	Lot20_17-6	5	1	Lot21_17-6	5	2
Lot19_22-1	5	1	Lot20_22-1	5	1	Lot21_22-1	5	2
Lot19_22-2	5	2	Lot20_22-2	5	2	Lot21_22-2	5	2
Lot19_22-3	5	3	Lot20_22-3	6	2	Lot21_22-3	6	3
Lot19_22-4	8	3	Lot20_22-4	3	2	Lot21_22-4	4	2
Lot19_22-5	5	2	Lot20_22-5	6	1	Lot21_22-5	4	1
Lot19_22-6	6	2	Lot20_22-6	5	2	Lot21_22-6	4	3
Lot19_40-1	6	3	Lot20_40-1	3	1	Lot21_40-1	6	1
Lot19_40-2	10	3	Lot20_40-2	7	3	Lot21_40-2	8	2
Lot19_40-3	12	3	Lot20_40-3	5	1	Lot21_40-3	N/A	4
Lot19_40-4	10	3	Lot20_40-4	6	3	Lot21_40-4	7	2
Lot19_40-5	8	2	Lot20_40-5	7	2	Lot21_40-5	N/A	4
Lot19_40-6	6	2	Lot20_40-6	6	2	Lot21_40-6	3	2
Lot19_43-1	8	3	Lot20_43-1	6	3	Lot21_43-1	8	1
Lot19_43-2	8	2	Lot20_43-2	6	1	Lot21_43-2	3	1
Lot19_43-3	6	2	Lot20_43-3	5	3	Lot21_43-3	5	1
Lot19_43-4	4	2	Lot20_43-4	5	3	Lot21_43-4	10	2
Lot19_43-5	13	2	Lot20_43-5	7	2	Lot21_43-5	5	2

Lot19_43-6	5	2	Lot20_43-6	4	2	Lot21_43-6	5	2

Table S3: Primers and blocking oligos used for the microbiome sequencing.

Primer name	Primer sequence (5’-to-3’ orientation)
799F	AACMGGATTAGATACCCKG
1192R	ACGTCATCCCCACCTTCC
fITS7	GTGARTCATCGAATCTTTG
ITS4	TCCTCCGCTTATTGATATGC
F1422	ATAACAGGTCTGTGATGCCC
R1797	TGATCCTTCTGCAGGTTCACCTAC
clamp1_BV5_mitoF	GATGAGTGTTCGCCCTTGGTCTACGTGGAT
clamp1_BV5_mitoR	CTGCTCAGGGTTCCAAACTCAACGTTGGCA
clamp1_ITS2_F	AACCATTAGGTCGAGGGCACGTCTGCCTGG
clamp1_ITS2_R	TGAGMGYGGTTACACCACGCATGCGGGTCT
clamp9_PV9_F	GATGTATTCAACGAGTCTATAGCCTTGGCC
clamp15_PV9_R	TCTCACAACGTCGCAGGCAGCGAACCGCCC

Table S4: LefSe Lefse to identify differentially abundant OTUs in different genotypes of *Lotus corniculatus* plants by plant organs.

Genotype

OTU	**enrich_genotype**	**organ**	**ef_lda**	**pvalue**	**padj**	**Locus**	**Genus**
Otu000030	Genotype_4	roots	3.589	<0.001	<0.001	16S	o__0319-7L14_unclassified
Otu000074	Genotype_8	roots	3.739	0.003	0.003	16S	f__Sinobacteraceae_unclassified
Otu000075	Genotype_3	roots	3.346	<0.001	<0.001	16S	f__Solirubrobacteraceae_unclassified
Otu000098	Genotype_3	roots	3.184	<0.001	<0.001	16S	g__Mycobacterium
Otu000103	Genotype_4	roots	3.052	0.004	0.004	16S	g__Kribbella
Otu000107	Genotype_1	roots	3.638	0.037	0.037	16S	f__Sphingomonadaceae_unclassified
Otu000110	Genotype_7	roots	3.423	0.000	0.000	16S	o__Solirubrobacterales_unclassified
Otu000129	Genotype_4	roots	2.890	<0.001	<0.001	16S	f__Gaiellaceae_unclassified
Otu000133	Genotype_1	roots	3.399	0.001	0.001	16S	g__Agrobacterium
Otu000162	Genotype_5	roots	2.747	0.001	0.001	16S	f__[Entotheonellaceae]_unclassified
Otu000167	Genotype_6	roots	2.895	0.016	0.016	16S	o__Solirubrobacterales_unclassified
Otu000193	Genotype_5	roots	2.737	0.000	0.000	16S	f__EB1017_unclassified
Otu000215	Genotype_5	roots	2.596	0.002	0.002	16S	f__Gaiellaceae_unclassified
Otu000244	Genotype_5	roots	2.659	<0.001	<0.001	16S	g__Amaricoccus
Otu000254	Genotype_2	roots	2.981	<0.001	<0.001	16S	c_Gammaproteobacteria_unclassified
Otu000260	Genotype_4	roots	2.683	<0.001	<0.001	16S	o__Solirubrobacterales_unclassified
Otu000263	Genotype_4	roots	2.507	<0.001	<0.001	16S	f__C111_unclassified

OTU	enrich_genotype	organ	ef_lda	pvalue	padj	Locus	Genus
Otu000273	Genotype_5	roots	2.203	0.016	0.016	16S	c__PAUC37f_unclassified
Otu000282	Genotype_6	roots	2.485	0.001	0.001	16S	f__Micromonosporaceae_unclassified
Otu000283	Genotype_2	roots	2.648	0.008	0.008	16S	f__Gaiellaceae_unclassified
Otu000289	Genotype_1	roots	2.539	0.001	0.001	16S	g__Marmoricola
Otu000338	Genotype_9	roots	2.966	0.006	0.006	16S	f__Haliangiaceae_unclassified
Otu000351	Genotype_4	roots	2.310	<0.001	<0.001	16S	o__Acidimicrobiales_unclassified
Otu000364	Genotype_3	roots	2.408	<0.001	<0.001	16S	f__AKIW874_unclassified
Otu000368	Genotype_4	roots	2.385	<0.001	<0.001	16S	o__Acidimicrobiales_unclassified
Otu000370	Genotype_3	roots	2.479	0.001	0.001	16S	c__S085_unclassified
Otu000373	Genotype_2	roots	2.852	<0.001	<0.001	16S	p__Actinobacteria_unclassified
Otu000386	Genotype_5	roots	2.358	<0.001	<0.001	16S	f__EB1017_unclassified
Otu000423	Genotype_5	roots	2.425	<0.001	<0.001	16S	g__Balneimonas
Otu000425	Genotype_3	roots	2.514	0.000	0.000	16S	g__Thermoactinomyces
Otu000436	Genotype_3	roots	2.303	0.002	0.002	16S	g__Coprococcus
Otu000467	Genotype_3	roots	2.656	0.002	0.002	16S	f__Comamonadaceae_unclassified
Otu000474	Genotype_9	roots	3.100	<0.001	<0.001	16S	g__Sphingomonas
Otu000516	Genotype_4	roots	2.096	<0.001	<0.001	16S	g__Candidatus_Solibacter
Otu000536	Genotype_8	roots	2.365	0.035	0.035	16S	f__Dolo_23_unclassified
Otu000545	Genotype_8	roots	2.650	<0.001	<0.001	16S	g__Actinoallomurus
Otu000567	Genotype_2	roots	2.038	<0.001	<0.001	16S	f__EB1017_unclassified
Otu000573	Genotype_5	roots	2.085	<0.001	<0.001	16S	o__Acidimicrobiales_unclassified
Otu000592	Genotype_9	roots	2.088	0.016	0.016	16S	f__Conexibacteraceae_unclassified
Otu000644	Genotype_5	roots	2.362	<0.001	<0.001	16S	f__Cystobacterineae_unclassified
Otu000656	Genotype_5	roots	2.133	<0.001	<0.001	16S	o__Micrococcales_unclassified
Otu000669	Genotype_8	roots	2.470	0.006	0.006	16S	f__Caulobacteraceae_unclassified
Otu000690	Genotype_7	roots	2.575	0.002	0.002	16S	c_Gammaproteobacteria_unclassified
Otu000734	Genotype_5	roots	2.029	0.014	0.014	16S	o__NB1-j_unclassified
Otu000764	Genotype_2	roots	2.011	0.004	0.004	16S	f__Haliangiaceae_unclassified
Otu000812	Genotype_2	roots	2.423	<0.001	<0.001	16S	f__Polyangiaceae_unclassified
Otu000974	Genotype_2	roots	2.077	0.026	0.026	16S	f__Beijerinckiaceae_unclassified
Otu001053	Genotype_8	roots	2.559	0.002	0.002	16S	c__SJA-4_unclassified
Otu001075	Genotype_2	roots	2.479	<0.001	<0.001	16S	g__Rahnella
Otu001147	Genotype_3	roots	2.025	<0.001	<0.001	16S	o__Bacillales_unclassified
Otu001210	Genotype_5	roots	2.091	0.024	0.024	16S	g__Candidatus_Protochlamydia
Otu00004	Genotype_5	roots	5.014	0.027	0.027	ITS2	g__Exophiala
Otu00035	Genotype_7	roots	2.227	0.007	0.007	ITS2	g__Pseudoidium
Otu00038	Genotype_1	roots	4.110	0.017	0.017	ITS2	f__Bionectriaceae_unclassified
Otu00068	Genotype_2	roots	3.920	<0.001	<0.001	ITS2	g__Agrocybe
Otu00139	Genotype_3	roots	3.419	0.007	0.007	ITS2	g__Leohumicola
Otu00300	Genotype_1	roots	3.000	0.013	0.013	ITS2	g__Delitschia
Otu00774	Genotype_1	roots	2.588	<0.001	<0.001	ITS2	g__Dendryphion
Otu01037	Genotype_5	roots	2.037	0.002	0.002	ITS2	g__Gremmenia
Otu01062	Genotype_9	roots	2.006	0.002	0.002	ITS2	g__Microdochium
Otu00023	Genotype_6	roots	2.829	0.006	0.006	18S	Penicillium
Otu00024	Genotype_1	roots	4.438	0.004	0.004	18S	Chromadorea_X_unclassified

OTU	enrich_genotype	organ	ef_lda	pvalue	padj	Locus	Genus
Otu00129	Genotype_4	roots	2.948	0.004	0.004	18S	Gregarinidae_unclassified
Otu00154	Genotype_3	roots	2.636	0.012	0.012	18S	Chytridiomycotina_unclassified
Otu00196	Genotype_2	roots	3.218	<0.001	<0.001	18S	Dothideomycetes_unclassified
Otu00208	Genotype_8	roots	3.401	0.022	0.022	18S	Acanthamoebidae_unclassified
Otu00306	Genotype_6	roots	2.922	0.002	0.002	18S	Assulina
Otu00314	Genotype_2	roots	2.355	0.015	0.015	18S	Trichosporon
Otu00505	Genotype_6	roots	2.313	0.037	0.037	18S	Ramicandelaber
Otu00510	Genotype_9	roots	2.552	0.005	0.005	18S	Eukaryota_unclassified
Otu00770	Genotype_3	roots	3.275	0.020	0.020	18S	Eukaryota_unclassified
Otu000009	Genotype_3	shoots	2.987	0.010	0.010	16S	g__Phyllobacterium
Otu000017	Genotype_2	shoots	2.617	<0.001	<0.001	16S	g__Mesorhizobium
Otu000034	Genotype_3	shoots	4.025	0.001	0.001	16S	g__Prauserella
Otu000062	Genotype_2	shoots	3.000	0.030	0.030	16S	g__Flavobacterium
Otu000070	Genotype_3	shoots	3.739	0.002	0.002	16S	g__Rubrobacter
Otu000073	Genotype_3	shoots	3.857	<0.001	<0.001	16S	g__Erwinia
Otu000118	Genotype_8	shoots	3.148	0.011	0.011	16S	f__[Weeksellaceae]_unclassified
Otu000151	Genotype_3	shoots	3.351	0.001	0.001	16S	g__Bacillus
Otu000466	Genotype_2	shoots	2.792	0.002	0.002	16S	g__Massilia
Otu000938	Genotype_5	shoots	2.025	0.001	0.001	16S	f__Sporichthyaceae_unclassified
Otu001023	Genotype_2	shoots	2.012	0.013	0.013	16S	f__Xanthomonadaceae_unclassified
Otu001029	Genotype_1	shoots	2.250	0.000	0.000	16S	g__Microbacterium
Otu001174	Genotype_9	shoots	2.080	0.012	0.012	16S	f__Planococcaceae_unclassified
Otu001270	Genotype_1	shoots	2.387	<0.001	<0.001	16S	f__Nocardiaceae_unclassified
Otu001782	Genotype_2	shoots	2.658	0.000	0.000	16S	g__Buchnera
Otu001914	Genotype_2	shoots	2.352	<0.001	<0.001	16S	f__Oxalobacteraceae_unclassified
Otu002076	Genotype_2	shoots	2.022	<0.001	<0.001	16S	f__Actinosynnemataceae_unclassified
Otu003094	Genotype_6	shoots	2.137	<0.001	<0.001	16S	g__Pseudomonas
Otu00004	Genotype_2	shoots	3.176	0.003	0.003	ITS2	g__Exophiala
Otu00026	Genotype_3	shoots	2.411	<0.001	<0.001	ITS2	g__Mycena
Otu00103	Genotype_2	shoots	2.376	0.000	0.000	ITS2	g__Athelopsis
Otu00122	Genotype_2	shoots	3.164	<0.001	<0.001	ITS2	g__Plectosphaerella
Otu00312	Genotype_7	shoots	3.324	0.006	0.006	ITS2	g__Dissoconium
Otu00381	Genotype_9	shoots	2.886	<0.001	<0.001	ITS2	g__Cystofilobasidium
Otu00386	Genotype_2	shoots	2.021	0.005	0.005	ITS2	f__Lentitheciaceae_unclassified
Otu00562	Genotype_1	shoots	2.729	<0.001	<0.001	ITS2	o__Xylariales_unclassified
Otu01017	Genotype_2	shoots	2.427	0.001	0.001	ITS2	g__Desmococcus
Otu01075	Genotype_7	shoots	2.665	0.003	0.003	ITS2	g__Golovinomyces
Otu01359	Genotype_5	shoots	2.390	0.000	0.000	ITS2	o__Pleosporales_unclassified
Otu01433	Genotype_2	shoots	2.005	0.002	0.002	ITS2	f__Stachybotryaceae_unclassified
Otu01689	Genotype_2	shoots	2.096	0.000	0.000	ITS2	g__unclassified_Verrucariaceae
Otu01708	Genotype_5	shoots	2.227	0.000	0.000	ITS2	g__Puccinia
Otu01710	Genotype_2	shoots	2.235	0.000	0.000	ITS2	o__Hypocreales_unclassified
Otu01762	Genotype_3	shoots	2.072	<0.001	<0.001	ITS2	g__Thanatephorus
Otu02224	Genotype_2	shoots	2.039	<0.001	<0.001	ITS2	g__Hypoxylon
Otu00043	Genotype_2	shoots	3.314	0.032	0.032	18S	Plectus

OTU	**enrich_genotype**	**organ**	**ef_lda**	**pvalue**	**padj**	**Locus**	**Genus**
Otu00308	Genotype_9	shoots	3.054	<0.001	<0.001	18S	Microdochium
Otu02170	Genotype_2	shoots	2.556	0.000	0.000	18S	Klebsormidium
Otu000016	Genotype_8	flowers	4.265	<0.001	<0.001	16S	g__Burkholderia
Otu000024	Genotype_3	flowers	2.793	0.043	0.043	16S	f__Xanthomonadaceae_unclassified
Otu000025	Genotype_1	flowers	3.196	0.035	0.035	16S	g__Rhodoplanes
Otu000034	Genotype_8	flowers	4.140	<0.001	<0.001	16S	g__Prauserella
Otu000070	Genotype_8	flowers	3.843	<0.001	<0.001	16S	g__Rubrobacter
Otu000073	Genotype_8	flowers	3.893	<0.001	<0.001	16S	g__Erwinia
Otu000082	Genotype_8	flowers	3.730	<0.001	<0.001	16S	p__Proteobacteria_unclassified
Otu000086	Genotype_8	flowers	3.599	<0.001	<0.001	16S	f__Burkholderiaceae_unclassified
Otu000096	Genotype_7	flowers	2.767	0.009	0.009	16S	g__Bradyrhizobium
Otu000115	Genotype_2	flowers	3.033	0.029	0.029	16S	g__Massilia
Otu000151	Genotype_8	flowers	3.536	<0.001	<0.001	16S	g__Bacillus
Otu000291	Genotype_8	flowers	2.941	0.004	0.004	16S	g__Methylotenera
Otu000404	Genotype_6	flowers	2.339	0.003	0.003	16S	f__Sinobacteraceae_unclassified
Otu001113	Genotype_2	flowers	2.511	0.001	0.001	16S	c_Gammaproteobacteria_unclassified
Otu001383	Genotype_7	flowers	2.113	<0.001	<0.001	16S	o__MIZ46_unclassified
Otu004213	Genotype_2	flowers	2.629	<0.001	<0.001	16S	g__Deinococcus
Otu00273	Genotype_2	flowers	3.742	<0.001	<0.001	ITS2	g__Alternaria
Otu00543	Genotype_8	flowers	3.115	<0.001	<0.001	ITS2	k__Fungi_unclassified
Otu01410	Genotype_7	flowers	2.273	<0.001	<0.001	ITS2	k__Fungi_unclassified
Otu02368	Genotype_2	flowers	2.398	<0.001	<0.001	ITS2	g__Rhodotorula
Otu00099	Genotype_8	flowers	4.061	0.011	0.011	18S	Sclerotinia
Otu000126	Genotype_4	seeds	2.395	0.024	0.024	16S	g__Rhizobium
Otu000141	Genotype_2	seeds	4.070	<0.001	<0.001	16S	o__Ellin6513_unclassified
Otu000332	Genotype_2	seeds	4.604	0.011	0.011	16S	k__Bacteria_unclassified
Otu000493	Genotype_5	seeds	2.306	<0.001	<0.001	16S	g__Pantoea
Otu000630	Genotype_5	seeds	2.115	0.003	0.003	16S	g__Pantoea
Otu000713	Genotype_6	seeds	3.654	<0.001	<0.001	16S	k__Bacteria_unclassified
Otu001330	Genotype_2	seeds	3.344	<0.001	<0.001	16S	k__Bacteria_unclassified
Otu001840	Genotype_2	seeds	3.150	0.001	0.001	16S	k__Bacteria_unclassified
Otu002150	Genotype_2	seeds	3.025	0.001	0.001	16S	k__Bacteria_unclassified
Otu00321	Genotype_8	seeds	4.259	0.024	0.024	ITS2	g__Malassezia
Otu00410	Genotype_2	seeds	3.392	0.005	0.005	ITS2	k__Fungi_unclassified
Otu00472	Genotype_2	seeds	3.583	<0.001	<0.001	ITS2	k__Fungi_unclassified
Otu00603	Genotype_2	seeds	3.529	0.009	0.009	ITS2	k__Fungi_unclassified
Otu00990	Genotype_3	seeds	3.127	0.005	0.005	ITS2	k__Fungi_unclassified

Age

OTU	**enrich_age**	**organ**	**ef_lda**	**pvalue**	**padj**	**Locus**	**Genus**
Otu000093	>6	roots	3.680	0.002	0.002	16S	f__Xanthobacteraceae_unclassified
Otu000295	<4	roots	2.716	0.015	0.015	16S	g__Hyphomicrobium
Otu000396	<4	roots	3.184	0.020	0.020	16S	c__Gammaproteobacteria_unclassified
Otu000421	>6	roots	2.634	0.003	0.003	16S	g__Steroidobacter
Otu000575	>6	roots	2.282	0.029	0.029	16S	f__C111_unclassified

OTU	enrich_genotype	organ	ef_lda	pvalue	padj	Locus	Genus
Otu000889	<4	roots	2.184	0.013	0.013	16S	g__Bacillus
Otu001115	<4	roots	2.086	0.046	0.046	16S	o__B07_WMSP1_unclassified
Otu001130	>6	roots	2.007	0.036	0.036	16S	o__Phycisphaerales_unclassified
Otu001174	<4	roots	2.045	0.048	0.048	16S	f__Planococcaceae_unclassified
Otu001384	6	roots	2.061	0.047	0.047	16S	f__Flavobacteriaceae_unclassified
Otu001569	<4	roots	2.164	0.043	0.043	16S	g__Bdellovibrio
Otu001627	>6	roots	2.164	<0.001	<0.001	16S	g__Criblamydia
Otu00152	<4	roots	3.717	0.008	0.008	ITS2	g__Mycena
Otu00246	4	roots	2.817	0.032	0.032	ITS2	p__Ascomycota_unclassified
Otu00255	>6	roots	2.964	0.006	0.006	ITS2	g__Pyrenochaeta
Otu00816	>6	roots	2.285	0.002	0.002	ITS3	g__Rhizophagus
Otu00072	6	roots	4.353	0.018	0.018	18S	Helicotylenchus
Otu00223	4	roots	3.070	0.044	0.044	18S	Pezizomycotina_unclassified
Otu00413	>6	roots	2.628	0.004	0.004	18S	Pezizomycotina_unclassified
Otu00469	5	roots	2.097	0.030	0.030	18S	Syncephalis
Otu00577	<4	roots	2.292	0.037	0.037	18S	Chromadorea_X_unclassified
Otu01077	<4	roots	2.005	<0.001	<0.001	18S	Paulinella
Otu000033	>6	shoots	3.370	0.045	0.045	16S	g__Chryseobacterium
Otu000043	4	shoots	2.542	0.001	0.001	16S	f__Bradyrhizobiaceae_unclassified
Otu000070	4	shoots	3.672	0.007	0.007	16S	g__Rubrobacter
Otu000186	>6	shoots	2.484	0.034	0.034	16S	c__S085_unclassified
Otu000191	>6	shoots	2.835	0.046	0.046	16S	f__Gaiellaceae_unclassified
Otu000370	>6	shoots	2.106	0.020	0.020	16S	c__S085_unclassified
Otu000511	>6	shoots	2.798	0.013	0.013	16S	g__Sphingomonas
Otu000560	>6	shoots	2.203	0.030	0.030	16S	g__Nocardioides
Otu000568	4	shoots	2.360	0.028	0.028	16S	g__Streptomyces
Otu000977	>6	shoots	2.130	0.013	0.013	16S	f__Gaiellaceae_unclassified
Otu001155	<4	shoots	2.117	0.040	0.040	16S	g__Paenibacillus
Otu001242	>6	shoots	2.368	0.003	0.003	16S	g__Tissierella_Soehngenia
Otu001934	>6	shoots	2.766	0.015	0.015	16S	g__Actinoplanes
Otu00007	6	shoots	2.734	0.017	0.017	ITS2	g__Acremonium
Otu00008	>6	shoots	4.517	0.009	0.009	ITS2	g__Boeremia
Otu00090	6	shoots	3.435	0.025	0.025	ITS2	g__Nectriella
Otu00559	4	shoots	2.710	0.018	0.018	ITS3	f__Didymellaceae_unclassified
Otu01220	<4	shoots	2.739	0.002	0.002	ITS3	g__Pseudoseptoria
Otu00294	>6	shoots	3.251	0.008	0.008	18S	Panagrolaimus
Otu01388	>6	shoots	2.312	0.002	0.002	18S	Panagrolaimus
Otu000112	>6	flowers	3.019	<0.001	<0.001	16S	f__Planococcaceae_unclassified
Otu000140	4	flowers	2.855	0.002	0.002	16S	g__Frigoribacterium
Otu000276	4	flowers	2.684	0.010	0.010	16S	g__Nocardioides
Otu001033	4	flowers	2.284	0.001	0.001	16S	g__Chryseobacterium
Otu001273	4	flowers	2.237	0.032	0.032	16S	k__Bacteria_unclassified
Otu002511	6	flowers	2.041	<0.001	<0.001	16S	k__Bacteria_unclassified
Otu00007	>6	flowers	2.733	0.006	0.006	ITS2	g__Acremonium
Otu00488	6	flowers	2.924	0.032	0.032	ITS2	k__Fungi_unclassified

OTU	enrich_genotype	organ	ef_lda	pvalue	padj	Locus	Genus
Otu00610	5	flowers	3.457	0.039	0.039	ITS2	g__Cenococcum
Otu00558	>6	flowers	3.549	0.011	0.011	18S	Eukaryota_unclassified
Otu00770	4	flowers	2.837	0.049	0.049	18S	Eukaryota_unclassified
Otu000074	4	seeds	2.651	0.024	0.024	16S	f__Sinobacteraceae_unclassified
Otu000122	4	seeds	2.185	0.015	0.015	16S	f__Gaiellaceae_unclassified
Otu000275	>6	seeds	3.189	0.022	0.022	16S	f__Burkholderiaceae_unclassified
Otu000289	<4	seeds	2.131	0.018	0.018	16S	g__Marmoricola
Otu000819	<4	seeds	2.032	<0.001	<0.001	16S	o__Bacillales_unclassified
Otu001353	6	seeds	2.621	0.020	0.020	16S	g__Cupriavidus
Otu001638	>6	seeds	3.030	0.036	0.036	16S	k__Bacteria_unclassified
Otu002021	>6	seeds	2.835	0.003	0.003	16S	k__Bacteria_unclassified
Otu002029	<4	seeds	2.063	0.037	0.037	16S	g__Mesorhizobium
Otu00058	5	seeds	2.276	0.010	0.010	ITS2	g__Colletotrichum
Otu01315	<4	seeds	2.558	0.036	0.036	ITS2	k__Fungi_unclassified
Otu01406	>6	seeds	2.772	0.008	0.008	ITS2	k__Fungi_unclassified
Otu01771	<4	seeds	2.377	0.002	0.002	ITS3	k__Fungi_unclassified

Chapter V

Natural variation in disease resistance of *Lotus corniculatus*

Frank Reis, Jun Hee Jung, Madalin Parepa, Eric Kemen, Oliver Bossdorf

Abstract

Natural variation is an important driver of evolution, allowing plant populations to adapt to shifting environmental conditions and withstand herbivores and pathogens. However, the expression of genetic variation, reflected in the extent of phenotypic variation between genotypes and their relative performance, can also depend on environmental conditions. In this study we experimentally tested how genetic variation in *Lotus corniculatus* plants affects their pathogen resistance under different environmental conditions. In a growth chamber experiment we exposed *L. corniculatus* plants from 20 natural geographic origins to the two fungal pathogens *Fusarium* and *Uromyces*, isolated from natural *L. corniculatus* plants, under different heat and shade conditions. Our results show that the infection severity of both pathogens varied significantly among plant genotypes, confirming the influence of natural genetic variation on pathogen resistance. Furthermore, while this genotype-specificity remained largely independent of the environmental conditions, we observed a strong overall influence of both heat and shading on infection outcomes. Our findings highlight the role of natural genetic diversity in providing plant resistance to pathogens, and they demonstrate that pathogen success is shaped not only by plant defense mechanisms but also by environmental factors that can enhance or suppress infection.

Key words

natural variation, plant pathogen resistance, fungi, environmental conditions, microbial ecology, plant-microbe interactions

Introduction

Intraspecific natural variation is a fundamental driver of evolution, enabling plant populations to adapt to changing environmental conditions and resist pressures from herbivores and pathogens. Genetic variation in plants shapes their responses to drought, shade, temperature fluctuations, and pathogen infections, influencing their overall fitness. (Su et al., 2023; Tailor and Bhatla, 2024; Vassileva et al., 2011; Zhao et al., 2025). Especially pathogen resistance is a crucial trait that directly impacts plant survival and ecosystem stability. Genetic variation in resistance allows some plants to fend off infections more effectively, limiting disease spread and supporting both population health and ecological resilience (Ennos, 2015; Karasov et al., 2020). The plant reaction to pathogens depends on a number of factors including morphological adaptations that can act as physical barriers against pathogens (Munzert and Engelsdorf, 2025). Of course, the plant immune system also plays a crucial role. Most prominent the presence and absence of different plant resistance genes (R genes) can change how well the plant can fight off a pathogen infection (Dangl and Jones, 2001; DeYoung and Innes, 2006; Periyannan et al., 2013). On the other hand, certain genes can increase a plant's susceptibility to pathogens, making it more vulnerable to infection (Gorshkov and Tsers, 2022). The interaction of all these plant-dependent factors determines whether a pathogen infection is successful and how severely it impacts the plant.

In addition to pathogens, plants are commonly exposed to variation in multiple other environmental conditions. Abiotic factors such as temperature, water, and sunlight play a crucial role in plant health and survival. Heat stress, for example, has multiple negative effects, including reduced growth and yield, early cell death, and decreased nutrient uptake (Angon et al., 2024; Giri et al., 2017). One of the main challenges of heat stress is the increased misfolding of newly synthesized and the denaturation of existing proteins (Bita and Gerats, 2013; Ellis, 1990). This can have severe consequences for cellular processes, such as the inactivation of Rubisco activase (Allakhverdiev et al., 2008; Salvucci and Crafts-Brandner, 2004), resulting in reduced photosynthesis efficiency. To counteract protein misfolding, plants produce heat shock proteins, such as chaperones, which help stabilize and protect proteins (Ellis, 1990). The production of such proteins and other stress-related proteins is regulated by heat shock factors. These are transcription factors that can enhance the plant stress tolerance to temperature stress (Duan et al., 2019; Ohama et al., 2017) as well as other environmental stresses such as drought and salt (Chauhan et al., 2013; Li et al., 2014), and even the pathogen defense (Kumar et al., 2009). Previous studies have shown that variations in the plant genome can influence the expression of these transcription factors (Andrási et al., 2021; Hu et al., 2020), indicating that natural variation in plants can alter their tolerance to heat and other environmental stressors. Sunlight, as the primary source of energy for plants, is another critical factor that can limit growth and survival. Especially small herbaceous plants in dense vegetation face challenges related to variation in light availability. When light is limited and shade avoidance is not an option, plants can respond with morphological changes to increase shade tolerance. These responses include a larger leaf area, reduced leaf thickness (Carabelli et al., 2007; Gommers et al., 2013), or adjustments in the ratio of chlorophyll a to chlorophyll b (Beneragama and Goto, 2011), and intraspecific natural variation can influence their intensity.

Plant responses to environmental changes may interact with their resistance to pathogens. Environmental conditions can modulate the effectiveness of plant defenses, e.g. high temperatures have been shown to suppress certain immune responses, making plants more vulnerable to pathogen attacks (Alcázar and Parker, 2011; Cohen and Leach, 2020; Prasad et al., 2022). Also drought can reduce plant defense mechanisms (Majeed et al., 2025), but excessive moisture, on the other hand, can create favorable conditions for pathogen proliferation, particularly for fungal infections, overwhelming the plant's immune response (Velásquez et al., 2018). Also, light availability affects plant immunity. Shading or low light conditions can alter hormone signaling, reducing the effectiveness of resistance genes (Ballaré, 2014a; Cerrudo et al., 2012a; de Wit et al., 2013a). These interactions between the influences of environmental conditions and plant defects are also reflected in molecular mechanisms. For example, it has been shown that the signaling pathways of the stress hormone abscisic acid (ABA) and defense hormones such as jasmonic acid (JA) antagonize each other, which can lead to a reduced immune response under biotic stress (Anderson et al., 2004). Since all these factors interact, an important question arises: how constant is plant resistance, and in particular the variation among different genotypes, in changing environments? Does a plant with strong genetic resistance to a particular pathogen lose that resistance under abiotic stress, ultimately performing similarly to a non-resistant genotype? Alternatively, could resistance be enhanced under certain environmental conditions that are less favorable for the pathogen? To answer such questions, it is important to incorporate environmental variation in studies of plant-pathogen interaction.

In a previous study on wild *Lotus corniculatus* populations, we found a significant association between specific genetic groups of plants and the abundance of several plant pathogens in the field, suggesting that genetic variation influences susceptibility to specific pathogens (Reis et al. unpublished data / Chapter IV). Our observation is supported by a recent study that examined the natural diversity of worldwide *L. corniculatus* accessions and found significant genetic variation among populations, including differences in the accumulation of the defense compound cyanogenic glycosides (Chen et al., 2023). These findings suggest that *L. corniculatus* exhibits varying levels of pathogen resistance across different populations. To test whether the statistical associations found in natural conditions reflect true genetically-based differences in susceptibility, we selected two pathogens, *Fusarium* and *Uromyces,* which were overabundant in specific plant genotypes, for experimental confirmation.

Both pathogens are widespread in natural environments, affecting a diverse range of plant species and posing a serious threat to crops. Understanding the genetic variation in resistance to these pathogens is thus crucial for enhancing crop resilience and developing effective disease management strategies. Plant pathogens are among the greatest threats to global food security (Strange and Scott, 2005), especially since a changing climate can increase the proportion of soil-borne pathogens (Delgado-Baquerizo et al., 2020).

In nature, pathogens are not an isolated threat to plants. As mentioned earlier, environmental conditions also influence plant fitness and affect their response to pathogen infections. To better understand natural variation in plant response to biotic and abiotic challenges and how these stresses interact we therefore conducted a combined stress experiment. Specifically, this study aims to answer the following main questions:

1. Do *Lotus corniculatus* plants of different genotypes respond differently to *Fusarium* and *Uromyces* infections?
2. Does the plant's response to pathogens depend on abiotic conditions?

We worked with *L. corniculatus* plants from 20 different worldwide origins, and infected plants with either *Fusarium* or *Uromyces* spores, and subjected them to heat, shade, or a combination of both in a growth chamber experiment.

Methods

Study system

The focus plant of our study was *Lotus corniculatus*, a perennial legume with a wide natural distribution across temperate regions of Eurasia. The species is often used as food for livestock, and as a nitrogen-fixing plant it forms a complex plant-microbe symbiosis, impacting nutrient dynamics within its ecosystems (van der Heijden et al., 2016). It also serves as a food source for numerous pollinators, including bumblebees, honey bees, various wild bees, butterflies, flies, and beetles (Hennig and Ghazoul, 2011; Weiner et al., 2014). With considerable intraspecific genetic variation

(Abraham et al., 2015; Durka et al., 2025), *L. corniculatus* provides an excellent system for investigating natural variation in pathogen resistance.

In our experiment we worked with *L. corniculatus* genotypes from 20 different natural origins. 14 of these genotypes came from different countries around the world, stored in the seed collection of the federal ex-situ Genbank at Gatersleben (IPK). The remaining six genotypes came from different regions within Germany, purchased from Saaten Zeller GmbH & Co. KG. This company collects seeds from natural plant populations in different regions within Germany and propagates them for ecological restoration projects (Tab. S1). The 20 genotypes from geographically distinct regions, each characterized by different climates and environmental conditions, allowed us to test our experimental questions across a broad range of the species' genetic diversity.

To test for variation in pathogen resistance in *L. corniculatus*, we used two common fungal pathogens: *Fusarium* and *Uromyces*. *Fusarium* is a genus of common fungal plant pathogen causing significant diseases in a wide range of plants, including crops like tomatoes, wheat, bananas and many others (Ma et al., 2013; Ploetz, 2015) but also wild plants. *Fusarium* infections can lead to wilting, blight, necrosis, and death of the entire plant (Aoki et al., 2003; Ma et al., 2013). Given the substantial impact of *Fusarium* on natural plant populations and crops, understanding how different plant genotypes respond to *Fusarium* infection can help to understand and manage *Fusarium* outbreaks. *Uromyces* is a well-known genus of rust fungi that causes diseases in a wide range of plants, particularly in the legume family (Graham and Vance, 2003). Under favorable conditions, *Uromyces* can spread rapidly within and between plant populations, traveling long distances through wind-dispersed spores (Gautam et al., 2022). As an obligate biotroph, *Uromyces* lives and reproduces by feeding on living plant tissue, while avoiding and suppressing the plant immune response. It infects its host through specialized infection, primarily entering the plant through the stomata. Once inside, it forms an intricate network of intercellular mycelium, extracting nutrients while keeping the plant cells alive (Staples, 2000; Voegele and Mendgen, 2003). During this stage, *Uromyces* produces urediniospores and can spread and infect other plants. Over time, infections can weaken the plant, reducing growth, photosynthetic capacity, and ultimately leading to yield loss (Gautam et al., 2022). Due to its obligate biotrophic lifestyle, *Uromyces* interacts with the plant and its immune system differently than *Fusarium*, making the two an interesting comparison. Both pathogens are known to infect *L. corniculatus* and can cause severe damage to the plant. Infected individuals are easily identifiable, as *Fusarium* typically leads to leaf wilt and chlorosis, while *Uromyces* infection is marked by characteristic red rust pustules (Fig.1).

Pathogen isolation and identification

To isolate the pathogens, we sampled *L. corniculatus* plants with visible infection signs for both pathogens around Tübingen, Germany. The *Uromyces* infected plants were washed with 50 ml of sterile ultrapure water (UPW) mixed with two drops of silwet L-77 (Momentive Performance Materials Inc., Niskayuna, USA) to help dissolve the hydrophobic rust spores. We used this suspension to infect five-week-old *L. corniculatus* plants to further propagate the pathogen. After ten days the plants showed first signs of infection. From them we harvested the spores every three to five days by shaking the plant gently over aluminum foil, dried the spores at room temperature in silica gel for 8 h and stored them for later use at -80 °C. To isolate the *Fusarium* pathogen, we sampled *L. corniculatus* plants with signs of leaf wilt and chlorosis from the field, separated the shoot from the other plant organs and surface-sterilized them by washing for 30 seconds with 2 % sodium hypochlorite and rinsing three times with ultrapure water (UPW). We then grounded the shoots in UPW and used 10 µl of the suspension to infect *Fusarium*-selective media (Jung et al., 2013) modified to 20 g/L dextrose, 15 g/L agar, 4 g/L potato extract, 50 mg/L kanamycin, 20 mg/L toxoflavin, and incubated them at 30 °C. After 18 h all growing fungi were transplanted several times on clean plates until we obtained pure cultures. For identification we extracted the DNA of these, as well as the harvested *Uromyces* spores, using the FastDNA^{TM} Spin Kit for Soil (MP Bio) as described in the manufacturer's protocol and sequenced at the ITS2 marker with Sanger sequencing to confirm pathogen identity (Tab. S2). For spore production, we transferred five 1 cm^2 pieces of Potato Dextrose Agar media with the isolated *Fusarium* to 250 ml of liquid mung bean media (García-Bastidas et al., 2019) and shaker-incubated them for 4 days at 28 °C and 150 RPM.

Experimental design and setup

To test the effect of natural variation in pathogen resistance we performed an experiment in which we subjected our 20 *L. corniculatus* genotypes to a full-factorial combination of pathogen infection with three levels *(Fusarium*, *Uromyces*, control) with two environmental factors, heat (two levels) and shade (two levels). We had two replicates in each genotype by environment combination, which resulted in a total of 480 plants (20 genotypes x 3 pathogen treatments x 2 shade treatments x 2 heat treatments x 2 replicates; see also Fig. 1).

For the experimental setup we germinated the *L. corniculatus* seeds after we submerged them in liquid nitrogen for 5 minutes to break the dormancy (Baskin and Baskin, 2014). After stratification we placed the seeds in wet potting soil at a 16:8 h light:dark cycle at 21 °C, 18 °C for germination. Four days later, we transplanted the seedlings into individual pots filled with a 1:3 mixture of sand and potting soil and grouped them for the different environmental treatments (see Fig. 1). We used two climate chambers, one for each temperature treatment and created four blocks in each chamber, two with shade, and two without. Within each environmental treatment, we randomized the genotypes. Two weeks after transplanting we measured the plant height as a reference point for the plant development before the treatment started. Then we infected the plants with the different pathogens. For *Fusarium* we filtered the suspension through autoclaved cheesecloth, added two drops of silwet L-77 per 50 ml to the suspension and counted the spores using a counting chamber. For the infection with *Uromyces* we suspended the frozen-stored *Uromyces* uredospores in UPW, mixed with two drops of silwet L-77 per 50 ml, and counted the uredospores using a counting chamber. We then standardized both pathogen spore suspensions to 10^6 spores/ml, and prepared a UPW with two drops of silwet L-77 per 50 ml for the control treatment. For the infection we dipped the plants into the respective inoculation suspension until the entire shoot was submerged. Before each dipping, the inoculation suspension was mixed to ensure homogenous distribution of the spores. After infection we let the plants dry for 30 minutes and incubated them in darkness for 18 h at 21 °C in 100 % humidity. After this incubation time we exposed the plants to a combination of two environmental stressors, shade and heat. For this, we divided the plants between two climate chambers, one at ambient temperatures, 21 °C during the day and 18 °C at night, and one with heat conditions, 38 °C during the day and 35 °C at night. In both chambers the day-night cycle was set to 16 h day and 8 h night. For the shade treatments we shaded two of the four blocks in each climate chamber using frames with 50% shade cloth, creating a full factorial design for the two environmental treatments (Fig.1).

After two weeks of environmental treatment, we harvested all plants. We cut all shoots and separated the individual leaves from the shoots, and then spread the shoots and leaves on a transparent foil, ensuring they did not overlap (Fig. S1). Then we scanned the total shoot and leaves area on an Epson Perfection V600 Photo Scanner at a resolution of 4962 x 7013 pixels. After the scanning we transferred the samples in paper bags, dried them for three days at 80 °C and determined the dry weight of each. To estimate resistance to *Fusarium*, we measured the percentage of yellow leaf area, as leaf wilt and chlorosis are the most common symptoms of *Fusarium* infection (Ekwomadu and Mwanza, 2023). We used the OpenCV2 package (Bradski, 2000) in Python v3.17 (Van Rossum, G., & Drake Jr, F. L., 1995) and kept all green and yellow pixels, while setting all non-green or non-yellow pixels to black (Fig. S1). We used the HSV scale thresholds of 5, 70, 70 and 75, 250, 215 for green pixels and 0, 70, 71 and 30, 234, 234 for yellow pixels for each plant image. To determine the effect of *Uromyces* on the plants we also counted all red rust pustules per sample using the Multi-point Tool in ImageJ (Schneider et al., 2012).

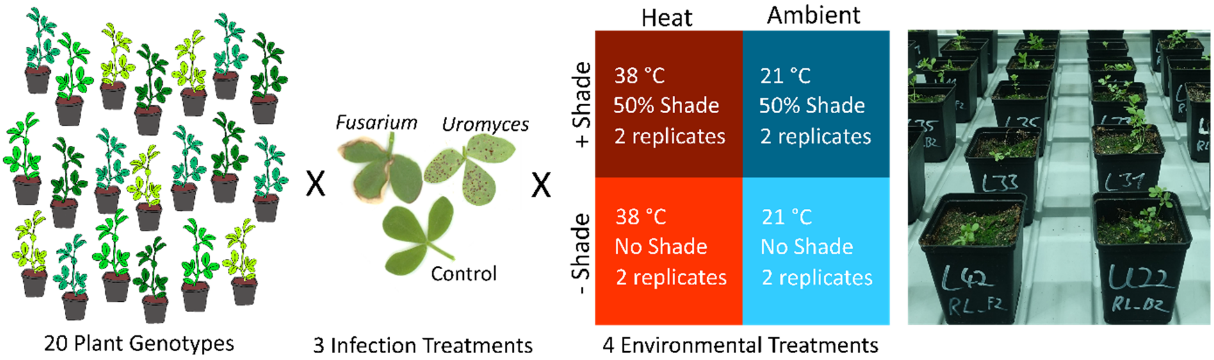

Figure 1. Overview of the experimental design, with 20 Lotus corniculatus genotypes and three pathogen treatments under factorial combination of heat and shade conditions, for a total of 480 plants. Illustrations by S. Nicolai Rühl.

Data analysis

For the data analysis we used R Studio 2024.12.1 (RStudio Team, 2024). First, we looked at changes in plant performance in response to infection and abiotic stress, and we tested for the interactive effects of plant genotype, microbial treatment and environmental conditions on plant dry weight using a linear model that included initial plant height as a covariate, to correct for variation in plant development before the start of the treatments. To meet the assumptions of the linear model, we applied a square root transformation to the dry weight data prior to analysis. In a second model, we analyzed *Fusarium* infection severity using the ratio of yellow to green pixels as the response variable. To meet the model assumptions, we applied a natural log transformation to the yellow to green pixel ratio. Finally, in a third model we analyzed the intensity of *Uromyces* infection and compared the numbers of rust pustules between genotypes and treatments. Due to the high number of zero values in the dataset, as many plants did not show any signs of infection, we were not able to analyze these data with standard linear models. We therefore used a zero-inflated negative binomial model to account for the excess zeros, while comparing plant genotypes and treatments. We used the R package *pscl* (Zeileis et al., 2008), specifying plant genotype as the factor influencing the number of pustules, and infection treatment and heat stress as the factors generating the structural zeros. All heat-treated plants had zero pustules, leading to an infinite odds ratio (OR = Inf, 95% CI: 19.64 to Inf), strongly suggesting that heat completely prevented infection. A Fisher's Exact Test confirmed that heat treatment had a highly significant negative effect on pustule formation. Since these data were incompatible with a zero-inflated model, and to avoid perfect separation issues, we removed the heat treated plants from the final model. To test for an effect of plant genotype on variation in rust pustules, we then compared two versions of the zero-inflated model: one including genotype as a factor and one that did not. A Likelihood Ratio test then allowed us to test whether genotype significantly improves the explanatory power of the model. We also investigated how infection severity impacted plant performance by analyzing the correlation between plant biomass and yellow pixel ratio for *Fusarium*, and number pustules for *Uromyces*. For all models, we used the *ggeffects* package (Lüdecke, 2018) to extract and plot parameter estimates and their confidence intervals.

Results

Overall, the environmental stress and microbial treatments strongly affected the plants. Both heat and shade treatment reduced plant biomass, and the combination of both treatments had the strongest negative effect (Fig.2a, Tab.1). The effects of abiotic stressors also varied significantly among the plant genotypes (Tab.1). For the pathogen treatments, an infection with *Fusarium* significantly reduced the plant biomass, but infection with *Uromyces* did not (Fig. 2b, Tab.1). When analyzing the severity of infection symptoms, we found that plants infected with *Fusarium* exhibited a significantly higher percentage of yellow leaf area compared to uninfected plants (Tab. 1, Fig. S2). The negative impact the leaf wilt caused by *Fusarium* is also confirmed by the significant negative correlation between biomass and the percent of yellow area (r = -0.146, P < 0.001; Fig. 2c, Tab. S3). Interestingly, plants infected with *Uromyces* also displayed greater leaf yellowing (Tab. 1). On the other hand, the occurrence of rust pustules was restricted to plants inoculated with *Uromyces* spores (Fig. 3c), with only two exceptions likely due to contamination.

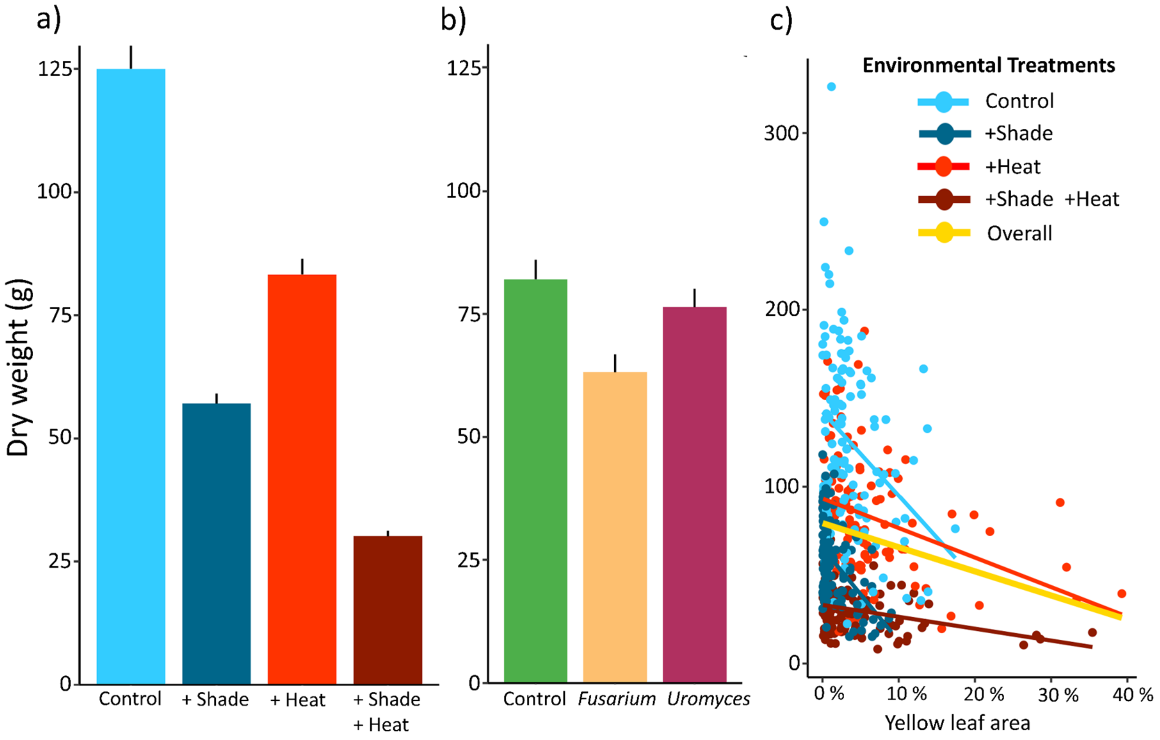

Figure 2. The average aboveground biomass (+ SE) of *Lotus corniculatus* under a) different environmental conditions and b) in different pathogen treatments. c) Correlations between yellow leaf area and plant biomass, separately for environmental treatment, as well across all treatments.

The 20 plant genotypes differed in their resistance to both pathogens. Although the overall effect of *Fusarium* infection on plant biomass was detrimental, it varied significantly among plant genotypes, with some genotypes affected much weaker than others, or even unaffected (Fig. 3a, Tab.1). Likewise, the extent of leaf wilt caused by *Fusarium* also varied among plant genotypes. Although wilting was observed in all genotypes, the severity of this symptom differed substantially, from a few percent leaf area in some genotypes to 10-15% in others (Fig. 3b, Tab.1). Because of the high number of plants without pustules (Fig. S3a,b), we analyzed the effect of plant genotypes on rust pustules with a zero-inflated model. The model detected significant variation in rust pustule counts among plant genotypes, showing that some developed more pustules after infection than others (Fig. 3). The Likelihood ratio test confirmed that the plant genotype explained a significant amount of the red pustules variation ($P < 0.001$).

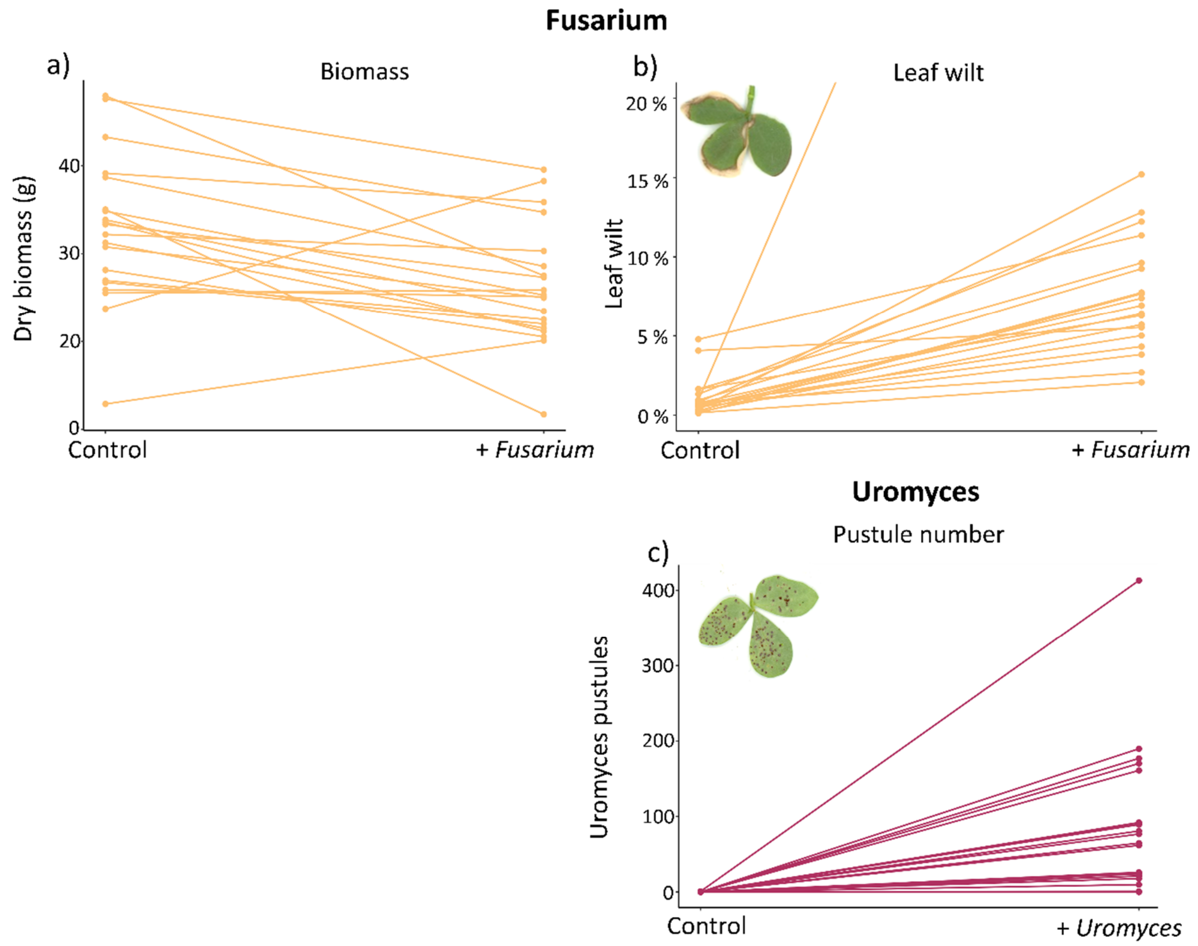

Figure 3. The variation among 20 *Lotus corniculatus* genotypes in a) the effect of *Fusarium* infection on the plant biomass, b) the effect of *Fusarium* infection on the percentage of plant leaf wilt, and c) the effect of *Uromyces* infection on the numbers of rust pustules. In all three cases, the variation among genotypes is significant (genotype x Fusarium interaction in a) and b); LR test comparing models with and without genotype in c).

We found that both environmental treatments influence the pathogen's success or impact. The shade treatment significantly increased the amount of leaf wilt but only in *Fusarium* infected plants (Tab.1, Fig. S4). In the heat treatment, no plants at all developed rust pustules, and a Fisher's Exact Test ($P < 0.001$) confirmed that heat had a highly significant negative effect on pustule formation. Shade too, reduced the number of rust pustules on plants, but to a much lesser degree (Fig. S3a). However, we found no significant three-way interactions between genotype, a pathogen and either heat or shade for biomass or infection severity, indicating that the genetic variation in plant resistance tended to be rather stable across the different environments (Tab.1).

Table.1: Results of linear models testing the effects of pathogen infection (*Fusarium* or *Uromyces*), plant genotype, heat, shade, and their interactions on the biomass of *Lotus corniculatus*, and on the amount of yellow leaf area for biomass and yellow leaf area.

	Biomass		**Yellow Leaf Area**	
Factor	***F***	***Pr(>F)***	***F***	***Pr(>F)***
Plant Genotype	5.93	**0.001**	5.66	**0.001**
Heat	257.85	**0.001**	77.50	**0.001**
Shade	768.87	**0.001**	117.17	**0.001**
Fusarium	57.89	**0.001**	278.94	**0.001**
Uromyces	1.26	0.263	10.70	**0.001**
Heat x Shade	0.72	0.340	13.37	**0.001**
Heat x *Fusarium*	2.01	0.157	0.01	0.917
Heat x *Uromyces*	0.24	0.626	0.3	0.854
Shade x *Fusarium*	3.46	0.064	43.38	**0.001**
Shade x *Uromyces*	0.67	0.415	1.90	0.170
Plant Genotype x Heat	1.29	0.186	2.06	**0.007**
Plant Genotype x Shade	1.86	**0.017**	2.94	**0.001**
Plant Genotype x *Fusarium*	1.75	**0.029**	1.70	**0.037**
Plant Genotype x *Uromyces*	0.54	0.945	0.63	0.881
Plant Genotype x Heat x Shade	1.01	0.447	1.69	**0.038**
Plant Genotype x Heat x *Fusarium*	1.11	0.338	0.92	0.560
Plant Genotype x Heat x *Uromyces*	0.81	0.700	0.85	0.644
Plant Genotype x Shade x *Fusarium*	1.21	0.246	1.29	0.187
Plant Genotype x Shade x *Uromyces*	0.30	0.999	1.45	0.102

Discussion

In this study, we explored genetic variation in *Lotus corniculatus* resistance against two common plant pathogens *Fusarium* and *Uromyces*. We used 20 genotypes and tested their disease susceptibility under different environmental conditions, in particular heat and shade stress, or a combination of both. To evaluate the impact of the pathogens on plants we quantified the biomass and the infection severity by assessing the percentage of leaf wilt for *Fusarium* and the number of rust pustules for *Uromyces*. Both pathogens affected all plant genotypes, although the strength of the effects significantly varied among genotypes. Moreover, both impact and infection severity were influenced by environmental stress.

Variation between plant genotypes

Plant genotypes varied in both plant biomass and yellow leaf area, across all treatments. This aligns with our expectations, as numerous studies have shown that intraspecific genetic diversity can influence traits related to plant fitness, such as growth rate, development speed, and stress resistance (Booy et al., 2000).

More importantly, we also see genetic differences in pathogen resistance, comparing the pathogen effect on the different genotypes, as the impact of *Fusarium* infection on plant biomass varies between plant genotypes. This suggests that the negative effect of *Fusarium* on plant fitness is genotype-dependent, with some plants being less affected by the infection than others. To examine the strength of the *Fusarium* infection we used the amount of leaf wilt and chlorosis as visible signs of infection, the main symptoms of a *Fusarium* infection (Ekwomadu and Mwanza, 2023). Similarly, to the effect on biomass, leaf wilt also differs among genotypes, showing differences not only as reduction of plant fitness, but also in the strength of the infection. A correlation analysis also showed that leaf wilt and plant biomass are negatively correlated, indicating that increased leaf wilt and consequently *Fusarium* infection indeed lowers the plant fitness. *Fusarium* as a hemibiotrophic pathogen, transitions to a necrotrophic lifestyle in the later phase of the infection (Ma et al., 2013). In this phase it mainly relies on killing and feeding on the host tissue. During infection, *Fusarium* mycelium spreads through the xylem, reducing water and nutrient availability, which leads to leaf wilt. Additionally, *Fusarium* produces toxins that disrupt plant physiology and ultimately cause cell death (Ekwomadu and Mwanza, 2023; Gurdaswani and Ghag, 2020). This leads to extensive tissue damage and a severe reduction in water and nutrient uptake, significantly reducing plant fitness.

To measure the infection strength of *Uromyces* infection, we quantified the number of rust pustules on the ventral side of the leaves. Unlike leaf wilt, these pustules are formed by matured uredinia filled with urediniospores and are a direct indicator of pathogen presence (Gautam et al., 2022), making them a specific measure of infection. Here the model shows the plant genotype does influence the infection strength, indicating a variation in the susceptibility of the different plant genotypes to *Uromyces*. The plant biomass on the other hand was not affected by *Uromyces* during the experiment. As an obligate biotroph, *Uromyces* relies on living plant cells to extract nutrients while ensuring the cells remain alive, minimizing the negative effect on the plant (Staples, 2000).

Therefore, the harmful effects of nutrient loss and leaf damage develop gradually and become apparent over a longer period. The experiment time frame of only two weeks of infection was likely not long enough to see these effects. Over a longer time, *Uromyces* infection can still lead to a reduced growth rate due to the nutrient depletion by the fungus, decreased photosynthesis and the eventual cell death of the exploited cells leading to leaf damage and the loss of infected leaves (Gautam et al., 2022; Giordano and Anderson, 2021).

The results for both pathogens align with previous field studies on *L. corniculatus*, where *Uromyces* and *Fusarium* were stronger associated to specific genotypes than to others (Reis et al. unpublished data / Chapter IV), confirming that susceptibility to these pathogens varies among plant genotypes. Similarly, research on other species, such as common bean and pea, has shown that resistance to *Uromyces appendiculatus* and *Uromyces fabae* differs between host genotypes. Showing that variation in QTLs or even individual genes can increase this resistance (Leitão et al., 2023; Omara et al., 2022; Rai et al., 2016). Also, the impact of a *Fusarium* infection can depend on intraspecific genetic variation in the host plant. For example, variations in only one gene influence resistance of maize plants to different *Fusarium* species (Song et al., 2024). Plant genotypes can differ in defense mechanisms and physiological traits. Genetic variation influences the strength of mechanical barriers, as well as the pathogen detection and the immune response (Andersen et al., 2018; Dolatabadian and Fernando, 2022). All of these factors can contribute to resistance against pathogens. Variation of specific defense genes or genes that increase the susceptibility to pathogen infections can change the efficiency of the plant immune response (Dolatabadian and Fernando, 2022; Gorshkov and Tsers, 2022). But also, structural variations such as stomatal density, cell wall thickness, and overall leaf morphology could also affect pathogen entry and progression (Dutton et al., 2019; Munzert and Engelsdorf, 2025). In particular *Uromyces* uses specifically the stomata to enter the plant leaves (Gautam et al., 2022), so variations here can have a strong effect on the infection frequency of *Uromyces*. These genotype-dependent differences in susceptibility remain consistent across the environmental treatments, suggesting that genetic variation between the genotypes plays a dominant role in determining resistance and doesn't change because of the effects of the environmental stresses on the plant pathogen interaction. This is very interesting since environmental stress can affect the immune response of the plant and the efficiency of the pathogens. And also, in this experiment we saw an overall effect of the environmental conditions on the pathogen infection.

Environmental effect on the pathogen infection

Over all genotypes leaf wilt and chlorosis caused by *Fusarium* increased in shade. One potential reason for this is that shade can influence the plant immune system, since the activation of shadow avoidance strategies or the general reduced energy supply, can weaken the plant immune response (Ballaré, 2014b; Cerrudo et al., 2012b; de Wit et al., 2013b). In addition, variation of microclimate in shade can favor the development of *Fusarium*. Both natural and artificial shaded areas experience reduced air flow and lower water evaporation due to the structures creating the shade. This can favor a more humid microclimate (Smith et al., 1987; Valladares et al., 2016), which improves conditions for fungal pathogens such as *Fusarium* (Velásquez et al., 2018).

Also, *Uromyces* was strongly influenced by the environmental conditions. Under heat stress, none of the plants in the experiment developed rust pustules, suggesting that *Uromyces* is unable to infect the host or survive long enough to complete its reproductive cycle under high-temperature conditions. *Uromyces* outbreaks are most common in moderately warm, humid environments. The urediniospores germinate optimally at around 20 °C, but show poor or no development at temperatures above 30 °C (Joseph and Hering, 1997; Singh and Gupta, 2019). These findings are consistent with our experiment, where heat stress completely prevented *Uromyces* spores from infecting the plants, with no variation between plant genotypes or shade treatments. The shade treatment was less hostile to *Uromyces* than heat stress, it however still led to a reduction in rust pustules compared to non-stressed conditions. Although the shade stress can influence the plant immune response (Ballaré, 2014a; Cerrudo et al., 2012a; de Wit et al., 2013a), there are no known cases where shade stress enhances plant immunity. A more likely explanation than an increased immune reaction in the shade is that reduced photosynthetic capacity under shade limits the availability of nutrients for pathogen exploitation, thereby restricting *Uromyces* spread in shade-exposed plants.

Although our experiments showed strong effects of environmental conditions on plant-pathogen interactions, the variation in genotype responses to pathogen infection was independent of these conditions. In none of our models we found an interaction between genotype, pathogen, and either heat or shade. These findings suggest that plant resistance is less influenced by the environmental conditions tested than initially expected, highlighting the significant role of genetic variability in determining pathogen resistance.

Overall effect of environmental stressors on the plant.

Overall, we see that both environmental stressors, heat and shade, had negative effects on plant biomass. As expected, increased temperatures can disrupt metabolic processes, including reducing photosynthetic efficiency (Allakhverdiev et al., 2008; Salvucci and Crafts-Brandner, 2004), while reduced light availability limits the plant's primary energy source, further inhibiting growth (Lu et al., 2021; Qin et al., 2024). Both factors contribute to a decreased growth rate and other negative impacts on plant health. Consequently, the combination of heat and shade resulted in the strongest negative effect on plant fitness.

When examining the effects of environmental factors on leaf wilt, our infection parameter for the *Fusarium* infection, we found that heat stress also increases the extent of wilting. In general, leaf wilt and chlorosis results from the loss of structural integrity in plant cells and can be triggered by various stress factors such as water loss and turgor reduction (Rascio et al., 2023). Although the plants were not drought stressed, heat increases evaporation and can affect the water content of the cells. High temperatures, especially over a longer time can also cause general injury and trigger ferroptosis leading to an increase in dead cells (Distéfano et al., 2025, 2017). In contrast, shade stress, if no *Fusarium* was present, reduces the amount of leaf wilt observed in plants. As mentioned before, shade changes the microclimate by providing protection from radiation and reducing airflow beneath shadow-casting structures (Smith et al., 1987; Valladares et al., 2016). This helps minimize water loss in leaves and prevents natural wilting, even in healthy plants. While shade reduces the leaf wilt and heat stress, contributes to an increase in leaf wilt, the model shows that *Fusarium* had by far the strongest effect on yellow leaf area. Also, across all treatment groups, *Fusarium*-infected plants experienced the highest levels of leaf wilt, reinforcing leaf wilt as a reliable indicator of *Fusarium* infection severity.

The results also show that the variation in leaf wilt caused by heat or shade depends on plant genotype, confirming that different genotypes vary in their tolerance to environmental stressors such as heat and shade (Chaudhary et al., 2020; Su et al., 2024). Overall, while the environmental treatments influenced pathogen development and its impact on the plants, they did not alter the variation in pathogen effects between genotypes.

Conclusion

In this study, we investigated the effects of the pathogens *Fusarium* and *Uromyces* on different *Lotus corniculatus* genotypes under varying environmental conditions. Despite their differing infection strategies, our results show that genetic diversity among plants influences the severity of infection by both fungi. While environmental factors influence the overall plant health and pathogen efficiency, the variation in resistance among genotypes remained independent of heat and shade stress.

These results align with previous studies, primarily on crop species, which have identified plant accession providing resistance to *Fusarium* and *Uromyces*. As both pathogens pose significant threats to agricultural crops, research efforts have focused on developing resistant cultivars in for example tomatoes, wheat, and bananas for *Fusarium* (Chitwood-Brown et al., 2021; Ploetz, 2015; Zhang et al.,

2024) and beans for *Uromyces* (Hurtado-Gonzales et al., 2017). Our findings show that natural genetic diversity in wild plant populations also offer potential resistance to these pathogens. Moreover, our study highlights the crucial role of natural variation in shaping plant-pathogen interactions, demonstrating that genotype-dependent resistance remains consistent across different environmental conditions. Additionally, our results confirm that pathogen success depends not only on plant defense mechanisms but also on environmental factors that can either enhance or reduce infection.

Understanding these dynamics is essential for predicting disease outcomes in both natural ecosystems and agricultural contexts. Our findings show the potential of natural resistance as a tool for ecosystem management and crop protection. This knowledge could contribute to breeding programs aimed at developing pathogen resistant plant varieties and establishing strategies to mitigate disease outbreaks. Given the ongoing challenges posed by climate change, such as shifting temperatures and humidity levels, which can alter pathogen prevalence (Delgado-Baquerizo et al., 2020), these insights are particularly relevant for future agricultural sustainability and biodiversity conservation.

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Supplementary material

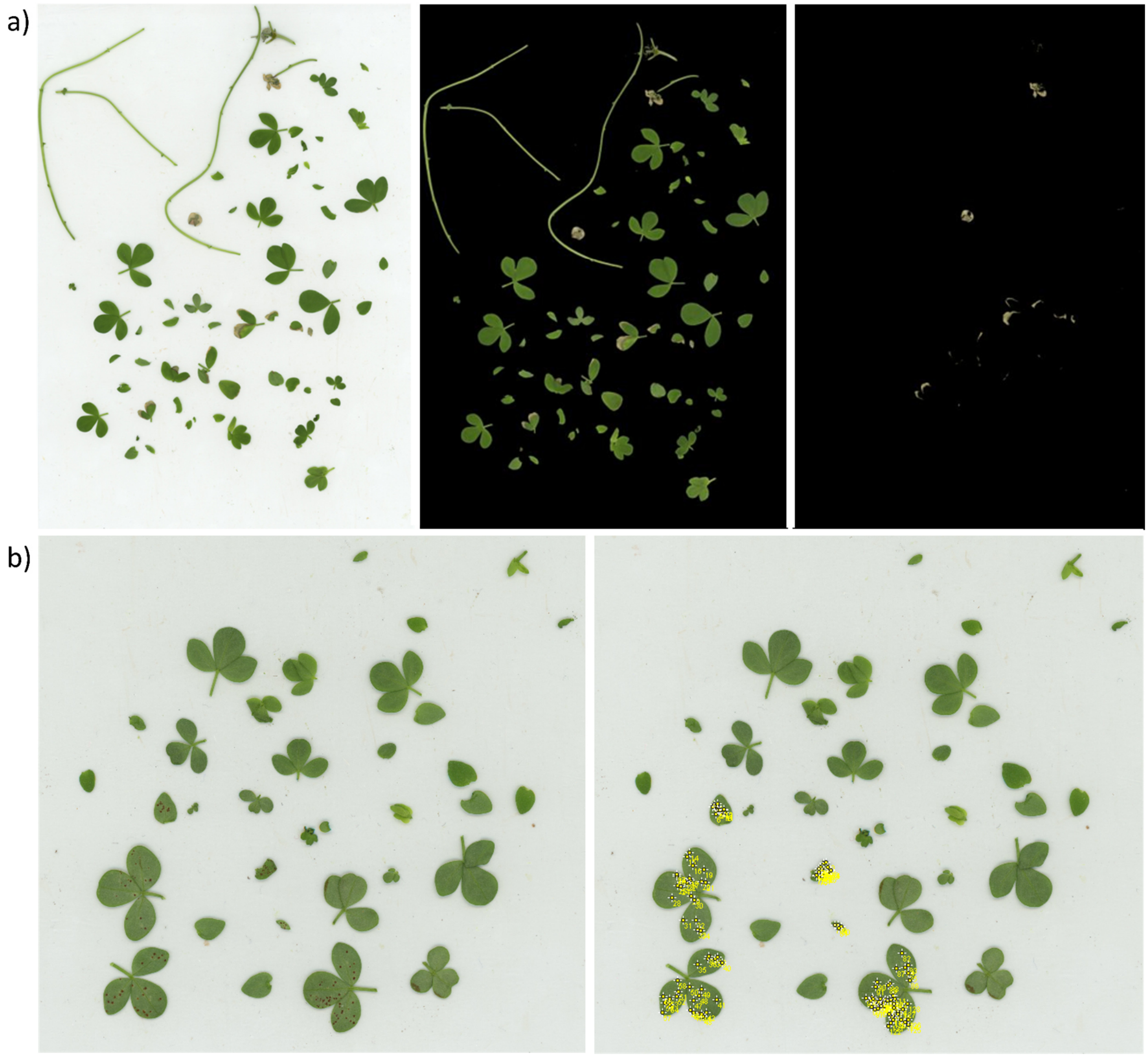

Figure S1: Infection severity estimations of the *L. corniculatus* plants. We spread the shoots and leaves of the plants out to avoid overlapping and scanned them. **a)** To estimate the sickness of the plant we then determine the number of all plant pixels (green and yellow) and the number of only yellow pixels. **b)** To measure severity of the Uromyces infection we counted the rust pustules on the scanned plant using the multi point function in ImageJ.

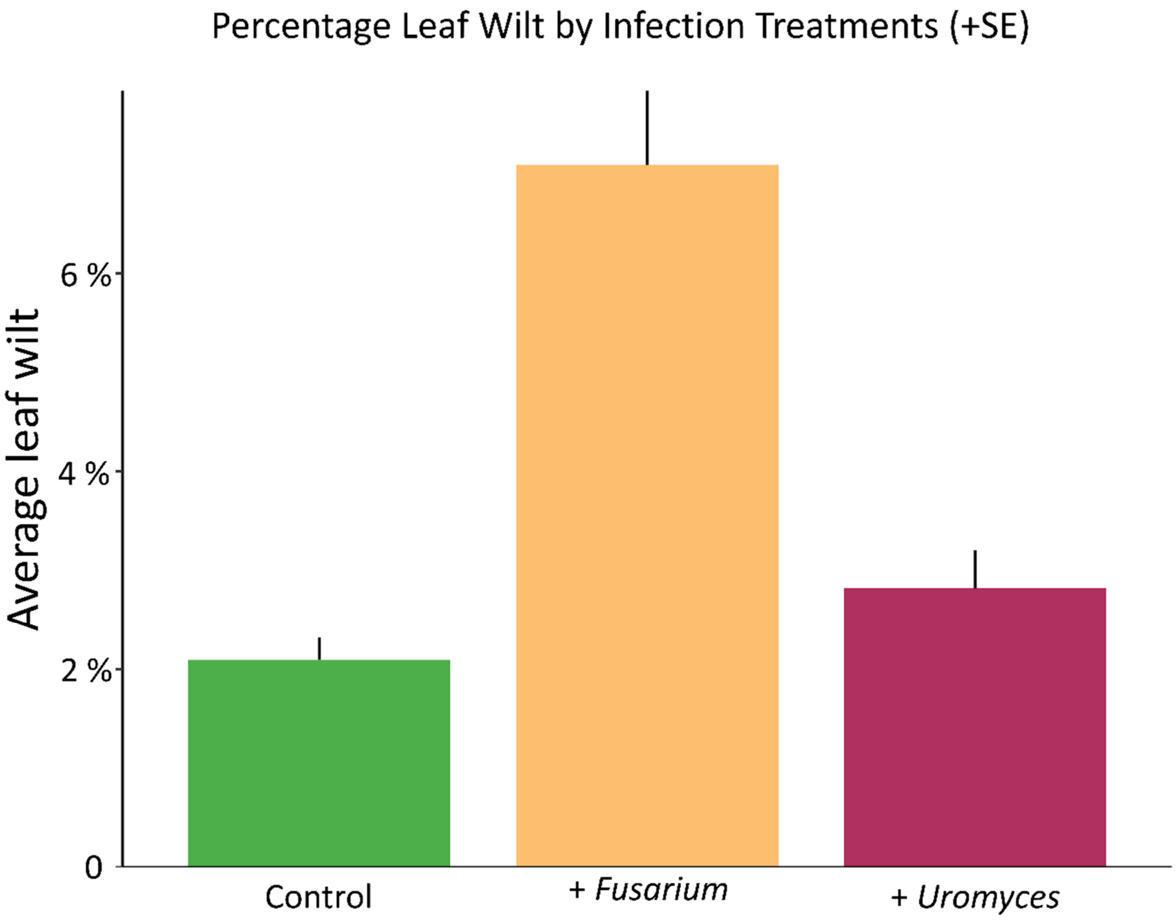

Figure S2: Effect of the infection with *Fusarium* and *Uromyces* on the percentage of yellow leaf area in *L. corniculatus* plants

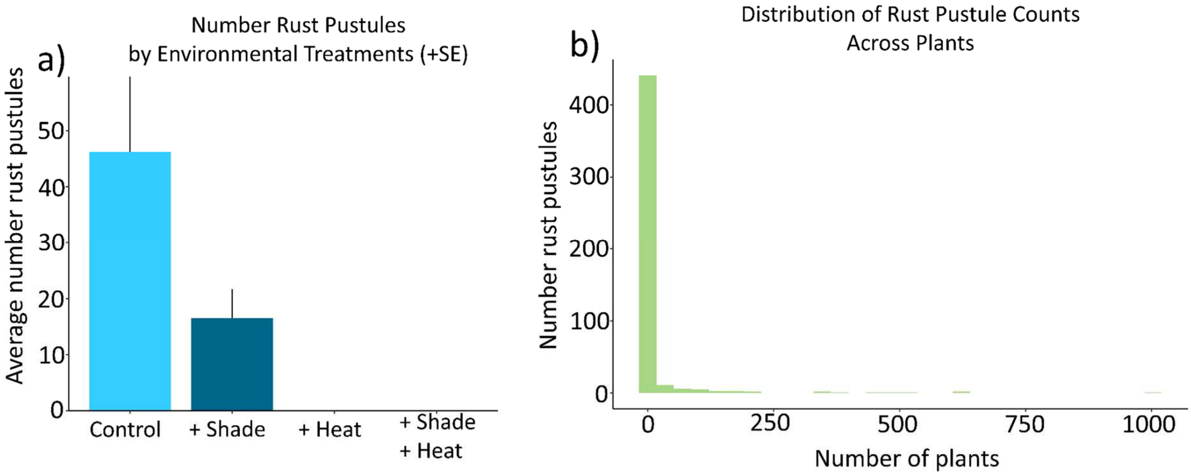

Figure S3: a) Number of rust pustules of *L. corniculatus* plants by environment threatens. **b)** Histogram of number of rust pustules per plant showing the amount of zero inflation in the data structure.

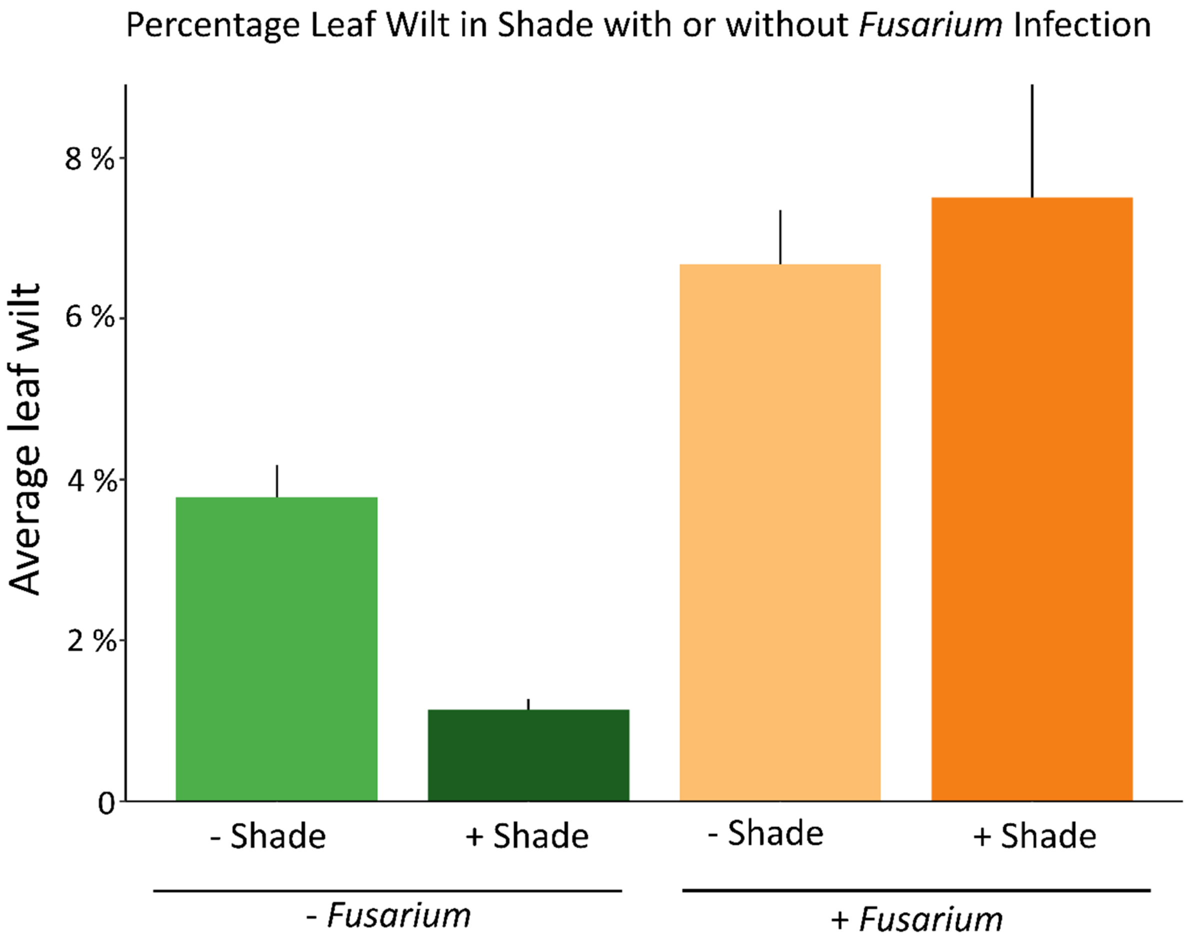

Figure S4: Comparison of the average amount of leaf wilt in *L. corniculatus* plants in shade conditions with and without the infection with the infection with *Fusarium.*

Table S1: List of all 20 *L. corniculatus* genotypes used in the experiment including origin

Genotype	**Origin**	**Donor**
L22	Czech Republic	IPK - Leibniz Institute of Plant Genetics and Crop Plant Research
L25	East Germany	IPK - Leibniz Institute of Plant Genetics and Crop Plant Research
L31	USA	IPK - Leibniz Institute of Plant Genetics and Crop Plant Research
L33	Ukraine	IPK - Leibniz Institute of Plant Genetics and Crop Plant Research
L35	Hungary	IPK - Leibniz Institute of Plant Genetics and Crop Plant Research
L40	USA	IPK - Leibniz Institute of Plant Genetics and Crop Plant Research
L42	Armenia	IPK - Leibniz Institute of Plant Genetics and Crop Plant Research
L49	Poland	IPK - Leibniz Institute of Plant Genetics and Crop Plant Research
L57	Spain	IPK - Leibniz Institute of Plant Genetics and Crop Plant Research
L58	Spain	IPK - Leibniz Institute of Plant Genetics and Crop Plant Research
L67	North Germany	IPK - Leibniz Institute of Plant Genetics and Crop Plant Research
L71	Czech and Slovak Federative Republic	IPK - Leibniz Institute of Plant Genetics and Crop Plant Research
L73	Canada	IPK - Leibniz Institute of Plant Genetics and Crop Plant Research
L74	Democratic People's Republic of Korea	IPK - Leibniz Institute of Plant Genetics and Crop Plant Research
U11	Southwest Germany	Saaten Zeller GmbH & Co. KG
U12	South Germany	Saaten Zeller GmbH & Co. KG
U16	South Germany	Saaten Zeller GmbH & Co. KG
U22	Nort East Germany	Saaten Zeller GmbH & Co. KG
U5	Center Germany	Saaten Zeller GmbH & Co. KG
U9	West Germany	Saaten Zeller GmbH & Co. KG

Table S2: Sanger sequencing results of both fungi isolates

Identified as	**ITS2 Sequence**
Fusarium	AAGGGTTAACTCCCAAACCCCTGTGAACTACCTCTATGTTGCCTCGGCGGATCAGCCCGTTCCTCACGGAACGGCCCGCCGCAGGACCCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAAAAACAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCAGCTTGGTGTTGGGAGCTGTTTTAGTTAACACTCCCCAAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAATTTACACATCGTTACTGGTAATCGACGCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCATAAAGC
Uromyces	CCTGCAGAAGGATCATTATTTAAAAAAATGAGTGCACTTCATTGTGGCTCAAACTTTTTTTTATTATAAACACATATTGAACTTAAGAATGTAAAAACCTTTTATTTTGAAAATAACTTTTAACAATGGATCTCTTGGCTCTCATATCGATGAAGAACACAGTGAAATGTGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATTGAATCTTTGAACGCACCTTGCACCTTTTGGTATTCCAAAAGGTACACCTGTTTGAGTGTCATGAAAATCTCTCATCAAATTAATTTTTGGTGGATGTTGAGTGCTGCTGTTATCTAGCTCACTTTAAATATATAAGTTCTTTCTTGTGTTGTTGGATTGACTTGATGTGATATTTAACTTTGTTTTCATCAAGGAAAGTAGCAATACTTGCCAACATCTTTTTAAGTAAAGGACTCCTAAAACAAATCGTTATTTTATTTTTAGACCTCAAATCAGGT

Table S3: Results of the correlation between biomass and the percent yellow area of the *L. corniculatus* plants

Environmental treatment	**Correlation Coefficient**	***Pr(>F)***
Control	-0.321	**< 0.001**
+Shade	-0.485	**< 0.001**
+Heat	-0.333	**< 0.001**
+Shade / + Heat	-0.330	**< 0.001**
Overall	-0.146	**0.001**

Chapter VI

Synthesis

In this thesis I explored drivers of plant microbiome variability. In Chapter II I first discussed the two general perspectives of looking at plant-microbiome interactions. In the remaining chapters I then focused specifically on the microbiome perspective. As countless studies have shown, the microbiome has a major influence on plant stress resistance (Parasar et al., 2024; Rodriguez et al., 2008; Rolli et al., 2015) and can increase the health, fitness and yield of the host plant (Backer et al., 2018; Berendsen et al., 2012). It is therefore important to understand the factors that influence microbiome composition not only for a fundamental understanding of plant biology but also to benefit from the positive effects of this microbiome in ecosystem management and agriculture. My main focus here was to investigate the effects of the host plant on its microbiome. I examined how factors such as plant organ (Chapter III) as well as plant age and intraspecific genetic variation (Chapter IV) affected the plant microbiome. To address these questions, I sampled *Lotus corniculatus* plants from natural populations and analyzed the microbiomes of individual organs (roots, shoots, flowers, and seeds) using metabarcoding techniques. In addition to the microbiome analysis, I genotyped the plants using ddRAD sequencing and determined their ages using herb chronology. Although the research questions in Chapters III and IV focus on host-related factors, I considered it important to study the plants under natural conditions where plants are exposed to a wide range of different environmental conditions, e.g. in climate and soil composition. These factors as well as interactions with other organisms such as herbivores (Malacrinò et al., 2021a, 2021b), pollinators (Keller et al., 2021; Zemenick et al., 2021), or pathogens (Cui et al., 2021; Diskin et al., 2017; Kusstatscher et al., 2019), also play a significant role in shaping the microbiome (Chen et al., 2019; Tkacz et al., 2015; Trivedi et al., 2022). To better understand these natural processes, it is essential to study plants in their natural environment (Yu et al., 2016). However, experiments under controlled conditions also offer important advantages and have already provided many valuable insights into plant-microbe interactions (Bodenhausen et al., 2014; Lebeis et al., 2015). In Chapter V, I therefore aimed to confirm some of the findings from Chapter IV through a less realistic but more precise experiment under controlled conditions. I selected two pathogens that were significantly associated with specific plant genotypes in Chapter IV. I then tested experimentally how genetic variation among 20 natural *L. corniculatus* genotypes influences the resistance to these pathogens. To understand the potential influences of abiotic factors on these plant-pathogen interactions, I performed a fully factorial experiment including the two stressors heat and shade. In the following, I will discuss the results of the individual chapters, and the overall knowledge that can be drawn from them.

The plant has a major influence on its microbiome

Chapter III shows a selective filtering of the microbiome through the plant organs. The roots, which acquire a large portion of their microbes from the surrounding soil (Berlow et al., 2024), hosted the most diverse microbiome. As microbes move from the roots through to the above ground biomass of the shoots and the generative tissues of flowers and seeds, this filtering process reduces biodiversity and shapes the microbiome composition within each organ.

However, the microbiome does not only vary depending on the organ, but also on plant age. The results of Chapter IV show that host age can alter the diversity of at least some parts of the microbiome, particularly the fungal microbiome, and shows significant associations of individual microbial taxa with specific host ages. Plant organs vary in their physiological and chemical properties (Junker et al., 2011), and throughout its development, the plant can exhibit changes in certain traits, such as defense against herbivory (Boege et al., 2007; Yang et al., 2020) or the expression of specific immune genes (Develey-Rivière and Galiana, 2007; Lemessa and Zeller, 2007; Liu et al., 2018). These changes create micro-environmental variation, as well as temporal variation, within plants, selecting for microorganisms best adapted to these conditions.

However, the microbiome does not only vary within a single plant. In Chapter IV, I demonstrated the effects of natural variation in the host species on the microbiome. The results show that both the diversity and composition of the microbiome differ significantly between natural *L. corniculatus* genotypes. Due to genetic variation, plants can differ in their physiological and chemical traits as well as their immune responses (Glander et al., 2018; Kniskern et al., 2007; Micallef et al., 2009; Pacheco-Moreno et al., 2024; Ronald et al., 1992), which can lead to the promotion or suppression of different microbial taxa. Interestingly, the strength of this genotype effect varies depending on the organ, indicating that plant organ and genotype interact and shape the composition of the microbiome together.

Our results from both chapters clearly demonstrate that, despite the high diversity of microbes in the environment, and the environmental variation between sampling locations and years, the plant has a consistent influence on its microbiome diversity and composition. While some studies have shown external factors play an important role in plant colonization, the plant is clearly not merely passive. Instead, it actively shapes its microbial community, even under varying environmental conditions. My experiment presented in Chapter V supports this conclusion, showing a significant effect of plant genetic variation on resistance to both tested pathogens. Although environmental stressors strongly influenced the overall impact of the pathogens, the variation in pathogen resistance among *L. corniculatus* genotypes remained fairly consistent across all tested environments.

The environment always plays a role

Despite the strong influence of the plant on microbiome composition, the environment also plays a significant role. Environmental factors can have a strong impact on the plant itself influencing its health, fitness and survival (Angon et al., 2024; Beneragama and Goto, 2011; Carabelli et al., 2007; Giri et al., 2017). As discussed in Chapter II, environmental factors can change plant-microbiome interactions in two ways, by changing the effect the microbiome can have on the plant (Alcázar and Parker, 2011; Majeed et al., 2025) but also by changing the microbiome itself (Chen et al., 2019; Gaube et al., 2021; Tkacz et al., 2015). With such strong effects, it is important to also consider the environment when studying microbiome dynamics. In Chapters III and IV, I analyzed the microbiomes of natural *L. corniculatus* populations from seven different sites, each with different abiotic and biotic conditions. In both chapters the analyses showed that both the sampling sites and years had a major influence on microbiome composition, alongside the already discussed plant-dependent factors. This suggests that variation in abiotic and biotic factors such as weather, soil composition, moisture levels, or herbivores may contribute to microbiome differences observed across locations and years.

My results indicate that environmental factors play a crucial role in colonization processes and plant microbiome development. In Chapter V, I examined this aspect in greater detail by specifically including the effects of heat and shade in a controlled experiment. I found that, in addition to plant genotype, the abiotic stresses significantly influenced not only the plant itself but also the development and effectiveness of both plant pathogens. Given that plants in nature are continuously exposed to a broad range of environmental conditions, it is essential to consider these factors when aiming to understand the dynamics of the natural plant microbiomes.

From fundamental insights to application

These results of Chapter III to V demonstrate that the plant has a significant influence on microbiome diversity and composition. Beyond the fundamental scientific insights, these findings can be used for plant breeding and agriculture. Classical breeding programs often focus on specific changes in the plant phenotype, such as stress tolerance and yield (Sinclair, 2011), or the addition of resistance genes (Chitwood-Brown et al., 2021; Hurtado-Gonzales et al., 2017; Zhang et al., 2024). But changes in plant genetics can also lead to less beneficial microbe combinations and indirectly reduce the plant performance. But the knowledge of plant microbiome dynamics gives us another tool to equip plants with resistance to biotic or abiotic stressors. Therefore, future breeding projects should not only focus on beneficial plant genes but also identify plant genotypes that support a beneficial microbiome (Gopal and Gupta, 2016; Mueller and Linksvayer, 2022; Nerva et al., 2022).

These implications are particularly important in a rapidly changing world. Climate change is expected to increase environmental stress such as heat and alter global water distribution, while also contributing to an increase in soil-borne pathogens (Delgado-Baquerizo et al., 2020). Together, these changes present serious challenges to agriculture and threaten global food security (Strange and Scott, 2005; Wheeler and von Braun, 2013). The beneficial potential of the plant microbiome could play an important role in reducing the impact of these problems.

Outlook

Despite the immense progress in plant microbiome research in recent years, many aspects remain not completely understood. One problem of previous microbiome studies is that many focus on only one or a small number of model species. However, the generality of the findings can benefit from increasing the number of species studied (van Kleunen et al., 2014). In the future I would like to shift my focus toward more general approaches within the precision, generalism and realism triangle discussed in Chapter I. General studies are important to make sure the results apply beyond single species and help us to better understand plant-microbe interactions overall. Previous research comparing different plant species has shown that microbiome composition can vary substantially between species (Bonito et al., 2014; Knief et al., 2010), highlighting the limited transferability of results derived from single-species studies.

At the same time, I believe it’s essential to study plant microbiome interactions under conditions that are as close to nature as possible, in order to truly understand how these processes work in real ecosystems. Moving forward, I hope to identify a meaningful compromise between realism and generality. For instance, studies that include a broader range of plant species could provide valuable insights into how plants influence the microbiome across species. However, I would also like to include environmental factors in future research. In several chapters of this thesis, I have highlighted the important role of these factors in shaping the plant microbiome. However, my data did not have the power to identify the precise relationships between different environmental factors and specific microbial taxa. I would like to address this more directly in the future. The Biodiversity Exploratories, where I sampled the plants for this thesis, would be an ideal research platform for such a project. With their wide range of forest and grassland sites across different regions of Germany, the extensive environmental data for each plot, and diverse natural plant populations, they offer excellent conditions for exploring the plant microbiome over a larger range of plant species and environmental conditions.

Lastly, it would be very interesting to learn more about the dispersal pathways of plant-associated microbes. As discussed in this thesis, a substantial proportion of the microbiome is acquired from the soil, which is known to host a diverse microbial community. However, other routes of microbial transmission likely play important roles as well. For instance, pollinators can act as vectors, introducing microorganisms to plants during floral visits (Keller et al., 2021; Zemenick et al., 2021). Exploring how such interactions contribute to the plant microbiome alongside plant dependent and environmentally driven factors could further enhance our understanding of natural plant microbiome dynamics.

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