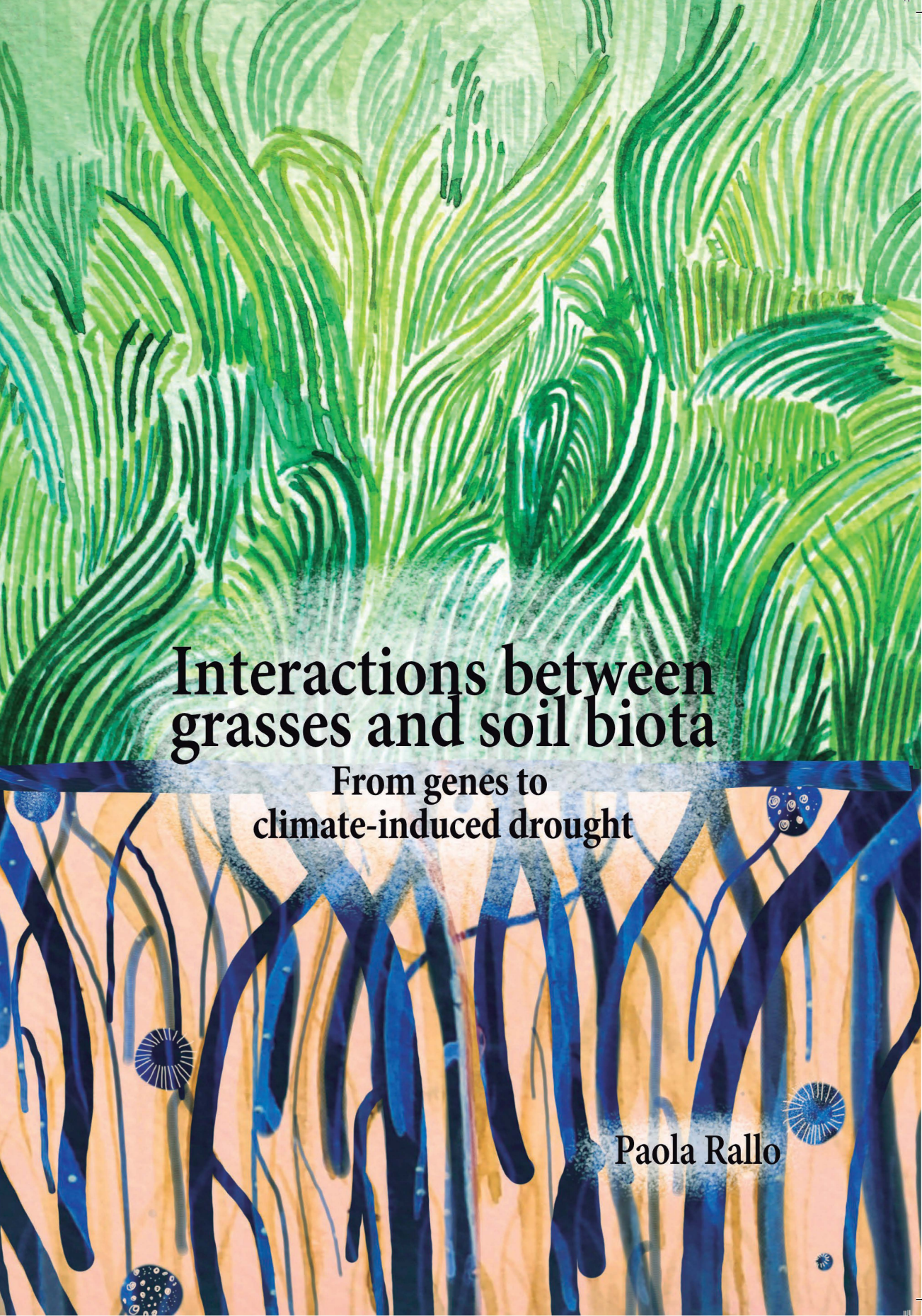

Interactions between grasses and soil biota

From genes to climate-induced drought

Paola Rallo

Propositions

1. In grasses, the overall impact of soil microbes is dominated by pathogens.
   (this thesis)
2. The beneficial effects of arbuscular mycorrhizal fungi on grass growth under drought are overstated.
   (this thesis)
3. Embracing mistakes as opportunities for innovation promotes a culture of seeking breakthroughs in scientific understanding.
4. The traditional hiring process overlooks individuals who possess scientific potential but lack conventional markers of success.
5. Cultural context is a prime responsible for gender discrimination and inequality.
6. Society prioritizes loud voices, overshadowing the importance of active listening and meaningful communication.

Paola Rallo

Wageningen, 25 September 2023

Interactions between grasses and soil biota

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Paola Rallo

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Interactions between grasses and soil biota

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Paola Rallo

Thesis

Submitted in fulfillment of the requirements for the degree of doctor
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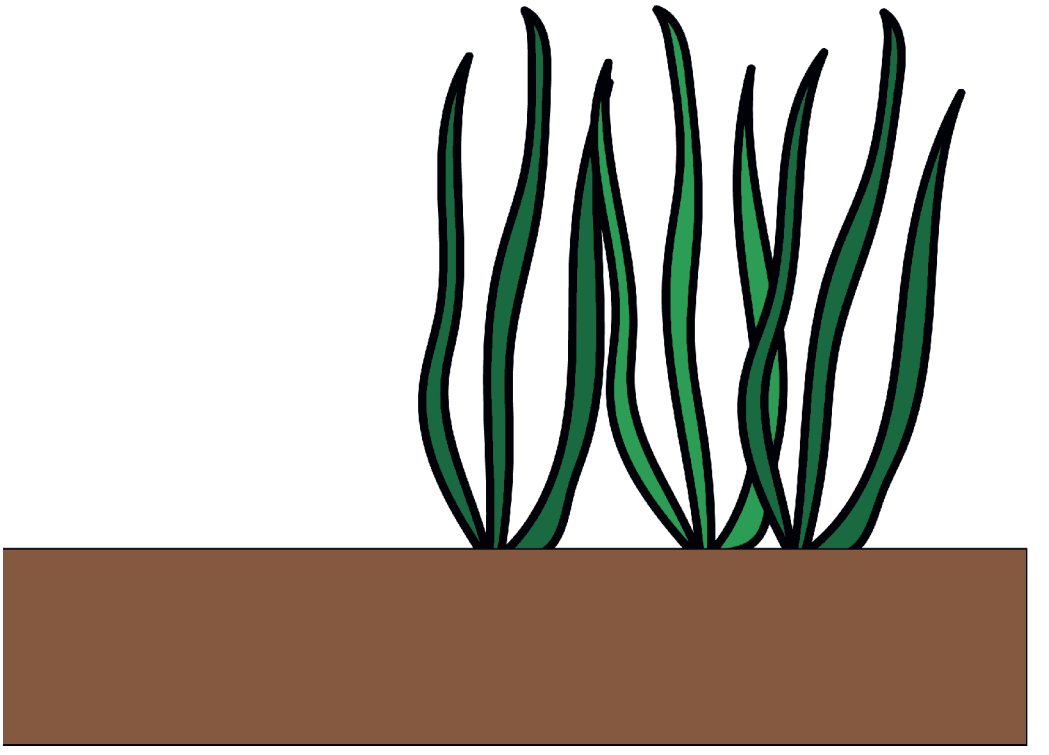
Chapter 1

General introduction



Introduction

Ever since plants colonized land, plants and microorganisms have co-evolved over millions of years. This co-evolution has led to the development of highly specialized and intricate relationships. These relationships are of great ecological importance because of their potential beneficial and harmful effects with the potential to influence plant fitness (Figure 1.1). Through association with beneficial bacteria and fungi, for instance, plants can express increased growth via improved nutrient uptake and disease resistance (Mendes, Garbeva, and Raaijmakers 2013; Harman et al. 2021). Importantly, certain microbes such as arbuscular mycorrhizal fungi (AMF) can increase a plant's abiotic stress tolerance (Lenoir, Fontaine, and Lounès-Hadj Sahraoui 2016; Begum et al. 2019). On the other hand, abiotic stress can increase a plant's susceptibility to disease with often detrimental consequences for the plant (Martínez-Arias et al. 2022). Consequently, the overall effect of soil biota on plant health will likely vary between environments (Lau and Lennon, 2012).

Indirect effects of the environment on plant health and fitness via microbes are crucial to understand plant ecological adaptation and to create stress-resilient crops. However, natural or artificial selection by breeding can only shape these traits if genetic variation exists for the ability to engage with beneficial microbes. Therefore, to fully understand the impact of plant-microbe interactions on plant fitness, it is necessary to consider both the genetic and environmental factors that shape plant-microbe interactions. In this thesis, I aim to understand both the role of plant genetics on their associated microbiome and soil microbiome-mediated effects on plant growth under drought stress. My PhD study therefore focused on three main themes i) Ecology of plant-soil biota

interactions, ii) Responses of plant-soil biota interactions to drought and iii) Genetic regulation of plant-microbe interactions.

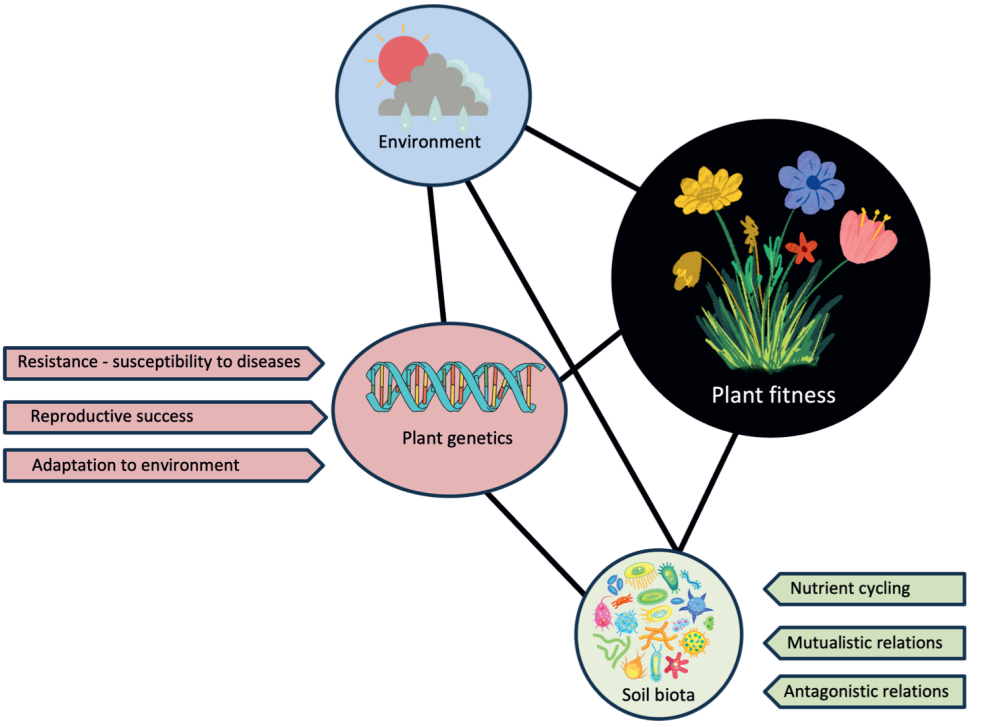


Figure 1.1. Plant fitness, defined as the ability of a plant to survive, reproduce, and persist in its environment, is the result of the complex interplay between plant genetics, soil biota, and other biotic or abiotic environmental factors that determine the success and adaptation of plants in their respective habitats. **Soil biota** can affect plant fitness via mutualistic or antagonistic relationships or via nutrient cycling. **Plant genetics** can affect a plant's ability to resist to disease, to tolerate environmental stressors or simply can affect the success of reproduction. The direction of the outcome between soil biota and plant genetics can differ between **environments.**

Ecology of plant-soil biota interactions

The rhizosphere and root microbiome

Plant-associated microbiomes exhibit variations in composition across different parts of plant tissues. The belowground portion comprises the rhizosphere, root surface (rhizoplane), and the root endosphere (Turner, James, and Poole 2013; Bulgarelli et al. 2013). The rhizosphere refers to the thin layer of soil that is in direct contact with plant roots, inhabited by a diverse array of organisms, including viruses, archaea, bacteria, fungi, nematodes, protists, algae, and arthropods (De Deyn et al. 2003). These organisms engage in extensive interactions that are primarily influenced by the release of exudates from plant roots, which are a major food source, fueling their population density and influencing their activities (Lynch and Whipps 1990; Kent and Triplett 2002; Raaijmakers et al. 2009; Shi et al. 2011; Sasse, Martinoia, and Northen 2018). There is evidence that plants have the ability to manipulate the composition of the rhizosphere microbiome in a way that benefits their own growth and health (Cook et al. 1995; Mendes, Garbeva, and Raaijmakers 2013; Cordovez et al. 2019; Rizaludin et al. 2021). Therefore, plant root exudates can attract allies and activate defense mechanisms that help them survive under stress via releasing chemical signals.

Bacteria, fungi, and other microorganisms can also colonize the surface of plant roots creating complex and intimate mutualistic-symbiotic associations. Examples are endophytes (microbes that live inside plant tissues) or rhizobia (bacteria that form mutualistic associations with legume roots). The microorganisms colonizing the rhizoplane (root epiphytic microbiome) and the interior part of the roots (endophytic microbiome) together make up the root microbiome (Bai et al. 2022).

Many of the microbes growing inside the roots (endophytic) have beneficial roles, as they can enhance plant growth by protection against pathogens, or against drought. Many of these endophytes have co-evolved metabolic mutualisms with their host (Gaiero et al. 2013; H. Lu et al. 2021; Rani et al. 2022). One of the best-described endophytic interaction is the rhizobia-legume symbiosis where the bacterial endosymbiont is pivotal for the plant's need for nitrogen (Oldroyd et al. 2011). Similarly, the colonization of plants by fungi such as arbuscular mycorrhizal fungi (AMF) has been associated with enhanced plant tolerance to stressors like drought and salinity (Dastogeer et al. 2020; Madouh and Quoreshi 2023).

While there is good understanding of how individual microbes interact with plants and influence their growth, it is important to note that in the real world, most effects of single microbial taxa on plants are often the result of multiple microorganisms that may influence each other's effects. Therefore, rather than solely relying on the impact of a single microbe, it is the combined effect of diverse microbial communities that ultimately will affect plant growth and overall fitness.

Plant-soil feedbacks: the net effect

One approach to study the effects of soil microbiomes on plant fitness is via plant-soil feedback (PSF) approach. Plant-soil feedbacks (PSFs) refer to the reciprocal interactions between plants and the soil, wherein plants affect soil properties and processes (in experimental studies this is named the 'conditioning phase'), and in turn, soil conditions steer the growth and consequently the performance of plants (this is named the 'feedback phase') (Bever, Westover, and Antonovics 1997; van der Putten et al. 2013). Plant-soil feedbacks can lead to either positive, negative, or

neutral outcomes for plant growth based on the balance between harmful and beneficial microorganisms, other soil biota, the properties of the soil (i.e., nutrient availability, moisture level, soil structure), and plant traits (Cong et al. 2015; Cavagnaro 2016; De Deyn, Quirk, and Bardgett 2011; Metcalfe, Fisher, and Wardle 2011; van der Putten et al. 2013). Positive feedbacks occur when plants enhance soil conditions, promoting as a result, their growth. This enhanced productivity can have several consequences, such as long-term persistence and dominance of certain plant species in an ecosystem. Positive feedbacks can also have cascading effects on other trophic levels and thus support diverse communities of organisms that contribute to plant performance. Negative feedbacks, on the other hand, occur when plants deplete resources or modify soil conditions in a way that is detrimental to their own growth. This can hinder their growth, and even facilitate the growth of competing plant species (Van der Putten and Peters 1997).

Negative PSFs promote plant diversity by preventing the dominance of a single plant species in a specific area (Klironomos 2002). Moreover, negative PSFs play a crucial role in ecological succession where they promote species turnover by facilitating the establishment of later successional plant species (Reynolds et al. 2003; Kardol, Martijn Bezemer, and van der Putten 2006). Unlike positive and negative PSFs that have direct effects on plant growth or performance, neutral feedbacks indirectly affect the overall complexity and functioning of ecosystems. In fact, the absence of strong positive or negative feedbacks might facilitate other ecological processes, such as species coexistence (in ’t Zandt et al. 2021). The study of these feedbacks provides insights into the mechanisms driving plant community dynamics, nutrient cycling, and the resilience of ecosystems in the face of environmental change.

Soil nematodes

In addition to the complex dynamics of plant-soil feedback, soil fauna, such as nematodes, also may play a role in shaping plant community composition. Nematodes are the most abundant soil animals within belowground food webs (Yeates et al., 1993; Bardgett and Van Der Putten 2014). Based on their feeding habits and ecological role, nematodes are classified in different groups. Bacterial-feeding nematodes have a small size and high reproduction rates that allows them to thrive in soil habitats where bacteria are abundant (Van Den Hoogen et al. 2019). By consuming and breaking down bacterial biomass, bacterial-feeding nematodes release nutrients that become available to plants and other organisms in the ecosystem (Russell E. Ingham et al. 1985; Djigal et al. 2004a). Due to their abundance and ecological significance, bacterial-feeding nematodes are often used as indicators of soil health and ecosystem functioning (Neher 2002).

Along with bacterial-feeding nematodes, fungal-feeding nematodes may also be highly abundant in the soil. These nematodes specialize in feeding on fungal hyphae in the soil, thus controlling fungal populations and influencing the composition and diversity of fungal communities (De Ruiter, Neutel, and Moore 1995; Hua et al. 2014). Interactions of fungivorous nematodes with fungi can have cascading effects on other soil organisms and plant health. Nematodes from higher trophic levels, such as predators and omnivores, are central in soil food webs, due to their ability to feed on nearly all smaller sized organisms (Wilschut and Geisen 2021). Lastly, root feeders specialize in feeding on plant roots, and their role in natural systems is less well studied than in agricultural systems (Nicol et al. 2011). Overall, most types of nematodes may

contribute to vegetation dynamics through direct negative effects on plants (i.e., root-feeders), and indirect positive effects through top–down control of plant-associated organisms.

Responses of plant-soil biota interactions to drought

Climate change-induced drought

As a result of climate change, drought events are increasing in frequency and intensity (Snyder and Harmon-Threatt 2019). These extreme climatic events may decrease plant performance and thus productivity, also resulting into massive plant die-offs (Wu et al. 2011; Snyder and Harmon-Threatt 2019). Droughts not only directly affect plant performance through changes in water and nutrient availability (Farooq et al. 2012), but also indirectly via changes in the structure and functioning of the soil microbiome associated with plants (Kardol et al. 2010a; McLaughlin 2011; Dai 2013; Putten et al. 2016a; de Vries et al. 2018; Ochoa-Hueso et al. 2018; Hari et al. 2020). There is increasing awareness of the negative effects of drought on the diversity and abundance of soil microbial communities, with bacteria typically considered more sensitive than fungi (Evans & Wallenstein, 2012; Fry et al., 2016). Because microbial communities and soil functionality are closely related, any variation in microbial community composition due to drought could influence soil functionality and, in turn, the provisioning of ecosystem services. (Bellard et al., 2012; McLaughlin, 2014). Therefore, drought and other human-induced environmental changes require differently adapted plants, as well as increase the need for food and feed crops that are more capable of growing under the different types of new environmental stresses.

Microbially mediated plant traits

The selection of crops has primarily been focused on plant traits that enhance crop quality, yield, and tolerance to pathogens and environmental stressors, such as drought and salinity among others. However, it is increasingly recognized that part of these plant traits is mediated by interactions with beneficial microbes. Several studies have pointed at the ability of plant-associated microbiomes to shape plant traits including disease resistance, growth, and abiotic stress tolerance (Zhang et al. 2021). Fungi such as *Trichoderma* species are well-studied because of their ability to improve plant growth potential, resistance to disease, and tolerance to abiotic stresses (Verma et al. 2021). Moreover, AMF can improve the growth of host plants by promoting nutrient and water uptake, which alleviates abiotic stresses, such as drought (Baum, El-Tohamy, and Gruda 2015; Zhao et al. 2015; Bowles, Jackson, and Cavagnaro 2018; Li et al. 2019; de Vries et al. 2020).

Along with fungi, plant growth-promoting rhizobacteria (PGPRs) have been extensively studied for their ability to enhance plant nutrient acquisition, plant growth and host resistance against pathogen infections (Lugtenberg and Kamilova 2009; Lakshmanan, Ray, and Craven 2017; H. Zhang, Sun, and Dai 2022). For instance, inoculation of maize with the bacterial endophyte *Azospirillum lipoferum* resulted in increased plant drought resistance via enhanced production of plant hormones (i.e., abscisic acid and auxin) (Cohen et al. 2009). Moreover, studies have indicated that exogenous application of hormones, nutrients, and antioxidants, may enhance crop drought resistance (Latif, Akram, and Ashraf 2016; Vaidya et al. 2019; H. Zhang, Sun, and Dai 2022).

Research on the positive impacts of soil microbes on plant resilience during drought has been primarily focused on the inoculation of plants with specific groups of microbes. However, there is limited empirical evidence that supports the beneficial role of microbes under drought when using inocula of whole natural microbial communities instead of adding specific taxa in isolation (Lau and Lennon 2011; Friesen et al. 2011; Valliere et al. 2020). Insights into how the microbial community, rather than single species, might affect plants when challenged by extreme environmental effects can be expected to provide a more comprehensive understanding of how microbiomes function with respect to affecting plant health and resilience. This knowledge may be applied to develop innovative strategies for improving crop productivity, enhancing stress tolerance, and mitigating the impact of environmental change on plants.

Genetic regulation of plant-microbe interactions.

Is there intraspecific variation for plant-microbe interactions?

There is increasing awareness of the effects of plant genetics on the associated microbiome (Lau and Lennon 2011; Horton et al. 2014; Wagner et al. 2016; Brachi et al. 2022; Wood et al. 2022; Quiza et al. 2023). This plant intraspecific variation in the ability to interact with microbes plays a crucial role in natural ecosystems, because it can fuel rapid adaptation in plant populations. In addition, it has important implications for artificial crop selection in agriculture, as intraspecific variation for plant-microbe interactions is the basis for selection that could lead to improved crop productivity. Evidence for such intraspecific variation comes from crop domestication, where the capacity of plants to 'use' their microbiome may have been reduced, or even lost, during breeding (Pérez-Jaramillo, Mendes, and Raaijmakers 2016). Therefore,

learning from plant-microbiome interactions in natural plant populations can help us to understand possible mechanisms that have become lost, at least partially, in crop varieties that have been bred for high yields. By considering the microbiome as a complex plant trait (an 'extended' phenotype), we can apply quantitative genetic tools to investigate if and how some parts of the microbiome are determined by plant genes. These genetic tools (i.e., genome-wide association studies -GWAS-, and quantitative trait locus analysis) can expose a genetic basis of variation in the plant-associated microbiome and have the potential to pinpoint molecular mechanisms underlying these interactions (Whitham et al. 2006; 2003; Wagner et al. 2016).

Genomic tools to unravel the genetic basis of plant-microbiome interactions.

Genome-wide association studies (GWAS) is a genomic analysis approach for mapping and identifying plant genetic variation associated with plant phenotypes (Beilsmith et al. 2019). GWAS scan marker loci, such as single nucleotide polymorphisms (SNPs) across the entire genome in a diverse panel of genotypes for association with the trait of interest, overcoming several limitations of traditional cross-based gene mapping (QTL) such as the limited genomic resolution (Brachi, Morris, and Borevitz 2011). However, only a few studies to date have adopted GWAS to examine the role of host plants in shaping variation in the microbiome. One of the first studies on the model species *Arabidopsis thaliana* showed that plant genetic variation shaped the composition of the leaf microbiome at the community level (Horton et al. 2014). Moreover, it has been shown that host genetics influenced fungal communities more strongly than bacterial communities (Bergelson, Mittelstrass, and Horton 2019). This highlights the importance of focusing on multiple kingdoms in the analysis of the soil microbiome.

Mapping plant loci associated with microbiome interactions will be the first step to identify genes engaged in the recruitment of specific microorganisms. The characterization and identification of the genes involved are essential to understanding the mechanisms by which plants might 'control' their microbiome.

Learning from grasses

My PhD study was focused on studying interactions between grass species and soil biota. Grasses (family *Poaceae*) are among the most dominant plant families on earth, comprising a significant portion of terrestrial ecosystems (Jacobs, Kingston, and Jacobs 1999). With 780 genera and appr. 12,000 species, *Poaceae* are the fifth-largest plant family (Christenhusz and Byng 2016). Grass species are often resilient to environmental stressors (i.e., drought, heat) (Vogel, Scherer-Lorenzen, and Weigelt 2012). Thus, studying their interactions with soil biota in response to climate change is expected to provide insights into the role of soil microbiomes in affecting plant resilience. Such studies are expected to contribute to developing grass varieties with enhanced resilience.

In natural ecosystems, grasses frequently create or experience negative plant soil feedbacks that are driven, at least to some extent, by pathogenic microorganisms. However, grasses also engage in beneficial interactions, which could enable rapid adaptation in nature, or allow artificial selection via breeding (e.g., in the turf and forage grass industry). Breeding of many grass species has been focused on biomass production, tolerance to abiotic stresses (i.e., drought, frost) and association with beneficial microbes (i.e., *Epichloë festucae*) or tolerance to pathogens (i.e., *Puccinia coronata*) (Barth and Milbourne 2013; Von Cräutlein et al. 2021; Sustek-Sánchez et al. 2023). However, there is still

a knowledge gap concerning the plant microbiome as a whole and its potential role in mediating the traits that are useful for breeding purposes.

Variation within grass species

I have investigated variation within grass species using accessions of *Lolium perenne* (perennial ryegrass) and cultivars of *Lolium perenne*, *Poa pratensis* (Kentucky bluegrass), and *Schedonorus arundinaceus* (tall fescue; formerly known as *Festuca arundinacea*). I assessed the molecular mechanisms underlying plant intraspecific variation for microbial communities in *L. perenne*. *Lolium perenne* is a temperate perennial grass species (*Poaceae* family) predominantly used as a forage and turf grass. It has broad, dark green leaves and forms dense, sod-forming clumps. It is known for its rapid establishment, high productivity, and tolerance to grazing and mowing. (Figure 1.2 A). *Lolium perenne* possesses several characteristics that makes it an interesting model species to study intraspecific variation in the ability to engage with microorganisms. *Lolium perenne*, firstly, shows high genetic diversity possibly due to large population sizes and a self-incompatible outcrossing mating system (Balfourier, Charmet, and Ravel 1998). Moreover, *L. perenne* closely interacts with various microorganisms, such as AMF (Omacini et al. 2001; Van Der Heijden et al. 2006). Since *L. perenne* is often grown in monocultures, it could be an example on how genetic variation of microbiome recruitment works in low-diversity systems. Species that are known to grow well in monocultures might be good candidates for identifying interactions with soil microbes that promote plant growth, because monoculture persistence can be promoted by overall beneficial interactions with soil microbiomes. Overall negative effects of soil microbiomes, in contrast, would prevent monoculture

persistence. The second studied species is *Poa pratensis*. It is a temperate perennial grass species characterized by fine-textured, deep green leaves and forms dense, spreading tufts (Figure 1.2 B). The third studied species is *Schedonorus arundinaceus*, a temperate perennial grass species that is widely cultivated as a forage grass, particularly in areas with hot summers and drought conditions. It has broad, coarse leaves and forms clumps with tall, sturdy stems (Figure 1.2 C).

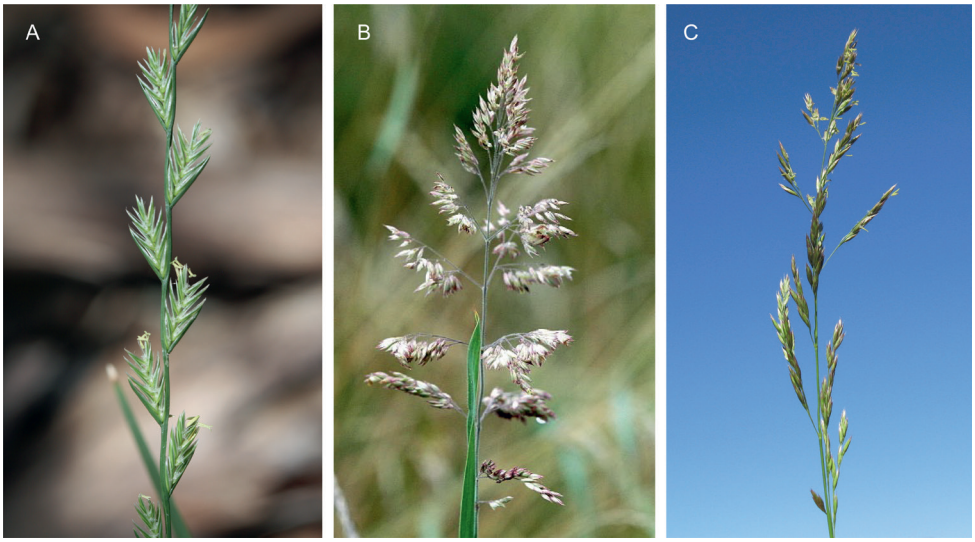


Figure 1.2. Grass species used to study intraspecific variation for plant-microbe interactions. (A) *Lolium perenne* (perennial ryegrass), (B) *Poa pratensis* (Kentucky bluegrass), C) *Schedonorus arundinaceus* (tall fescue).

Variation between grass species

To examine variation between species I used both perennials such as *Agrostis capillaris* (common bentgrass), *Alopercurus pratensis* (meadow foxtail), *Anthoxanthum odoratum* (sweet vernal grass), *Festuca ovina* (sheep's fescue), *Phleum pratense,* and *Nardus stricta)* and annuals such as *Apera-spica venti* (silky bentgrass), *Poa annua* (bluegrass) grass species. These grass species belong to the *Poaceae* family and their

interactions with soil microbes partially determines their abundance along ecological successional gradients (Kardol, Martijn Bezemer, and van der Putten 2006). *Agrostis capillaris* is mostly found in meadows, lawns, and open habitats. It has fine leaves and forms dense tufts or mats (Figure 1.3 A). *Alopecurus pratensis* is an important forage grass and is also valued for its ornamental qualities and thrives in moist meadows and grasslands. It has cylindrical flower heads and narrow leaves (Figure 1.3 C) and it is often found in meadows, grasslands, and open habitats. While it is not typically used as a forage grass, its aromatic qualities make it a popular choice for ornamental purposes. *Anthoxanthum odoratum* is known for its pleasant fragrance, reminiscent of vanilla. It has slender leaves and produces dense, cylindrical flower spikes (Figure 1.3 D). *Apera spica-venti*, is typically found in disturbed habitats such as agricultural fields, gardens, and waste areas. It has a loose, open inflorescence and slender leaves (Figure 1.3 F). *Festuca ovina* is widespread in grasslands, heaths, and rocky slopes. It forms dense tussocks and has narrow, bristle-like leaves (Figure 1.3 H). It is well-adapted to dry, nutrient-poor soils and is often used in erosion control and habitat restoration projects. *Phleum pratense* (timothy grass) is widely cultivated for hay and forage purposes. Timothy grass has flat, narrow leaves and produces compact, cylindrical flower spikes (Figure 1.3 G) and it is known for its high nutritional value and is commonly used in livestock feed. *Poa annua* is often found in lawns, disturbed areas, and agricultural fields. It has flattened, boat-shaped leaves and produces small, inconspicuous flowers (Figure 1.3 B) and it is known for its ability to quickly colonize bare ground and is considered a weed in some contexts. *Nardus stricta* (matgrass), is a low growing, tufted grass species

well-adapted to acidic, nutrient-poor soils and contributes to the biodiversity of heathland ecosystems (Figure 1.3 E).

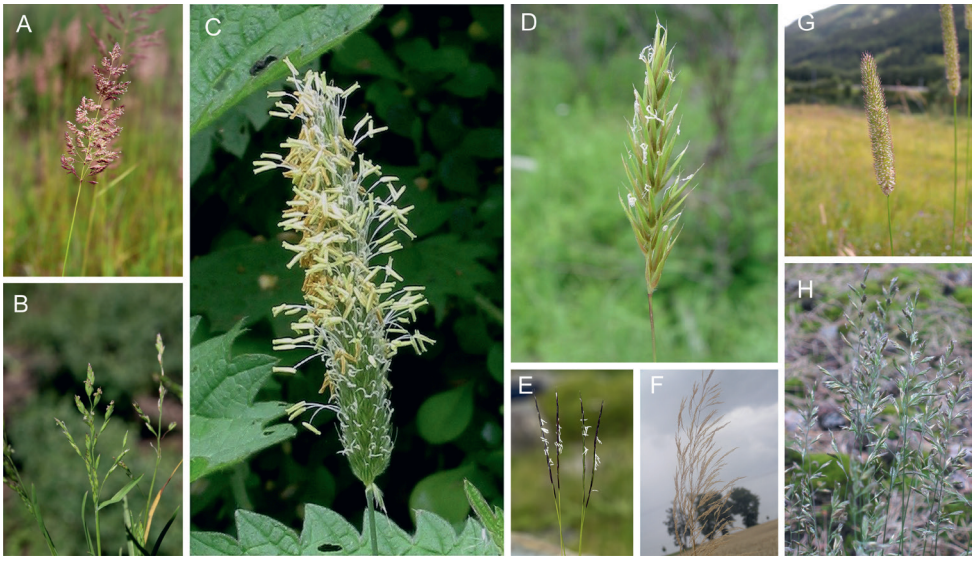


Figure 1.3. Grass species used to study interspecific variation for plant-microbe interactions. (A) *Agrostis capillaris* (bentgrass). (B) *Poa annua* (bluegrass). (C) *Alopercurus pratensis* (meadow foxtail). (D) *Anthoxanthum odoratum* (sweet vernal grass). (E) *Nardus stricta* (matgrass). (F) *Apera-spica venti* (silky bentgrass). (G) *Phleum pratense* (timothy grass). (H) *Festuca ovina* (sheep's fescue).

Overall aims and thesis outline

Interactions that plants establish with beneficial microorganisms are of great ecological importance because of their contribution to increased growth via improved nutrient uptake, disease resistance, and abiotic stress tolerance. Microbial-mediated plant traits might be crucial for ecological adaptation and crop enhancement. However, for natural or artificial selection to shape these traits, it is necessary that there is genetic variation in the plant's ability to interact with specific beneficial microbes. In this PhD thesis, I focus on both plant *genetic effects* on the

soil microbiome and *microbiome-mediated effects* on plants fitness. I investigate *intraspecific variation* in plant-microbiome interactions. I identify loci for plant control over its microbiome composition, diversity and for specific microbial taxa both in endosphere and rhizosphere. In addition, I study whether plant genetic variation affects the overall microbial communities and whether this has ecological consequences for plant fitness (plant-soil feedbacks). In the second part of this thesis, I examine the microbial effects on plant growth under drought stress. I focus on microorganisms inhabiting the rhizosphere, and also consider soil fauna both in rhizosphere and endosphere, particularly nematodes and their potential effects on plant performance under drought.

The aims stated are addressed in 4 **Chapters** (Figure 1.4) whereas **Chapter 6** contains the general discussion.

In Chapter 2, I use a genome-wide association study (GWAS) approach and examine potential impact of plant genetics on its associated microbiome. I determine where this effect is most pronounced: in the rhizosphere, or in the root endosphere. To gain mechanistic understanding of plant genetic control over the associated microbiome, I examine which plant genes or molecular pathways associate with microbiome composition.

In Chapter 3, I investigate intra- and interspecific variation of plant-soil feedbacks. Specifically, I test whether 3 grass species, and cultivars within these species, accumulate different soil microbial communities (plant-effects on overall microbiome). In turn, I examine how the soil community composition affects the performance of the plants growing later in the soil (microbiome-effects on plant growth). To identify potential microbial candidates as drivers of PSFs, I test how specific

microbial fungal and bacterial taxa are correlated with plant biomass production.

After gaining a better understanding of the plant effects on its associated microbiome, and on the microbiome's effect on grasses, I focus on the microbiome-mediated effects on plant biomass production with and without drought (**Chapters 4 and 5**). My main focus is on rhizosphere bacteria and fungi, but I also consider soil fauna, particularly nematodes that might also affect plant performance under drought.

I**n Chapter 4**, I examine how the presence of natural soil microbiomes affects plant growth under drought. In a greenhouse experiment, I used two different soil inocula derived from field soils from different successional stages and eight native grass species occurring along a secondary successional gradient. To explore potential causes, I focus on AMF and fungal plant pathogens and test how their abundance relates to plant biomass under drought.

In Chapter 5, I investigate the effect of drought on nematodes in the soil and in the roots of two grass species, using inoculations of two natural nematode communities derived from soils from different successional stages (as used in **Chapter 4**). In addition, I relate these drought effects on nematodes to plant biomass production.

In Chapter 6, I discuss the findings of this thesis and how they relate to overall aims of the PhD study. Finally, I propose future research directions.

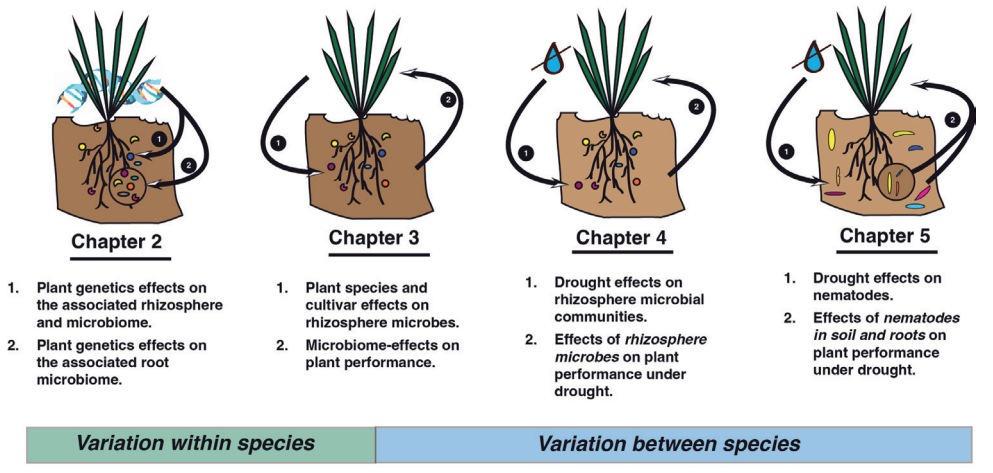
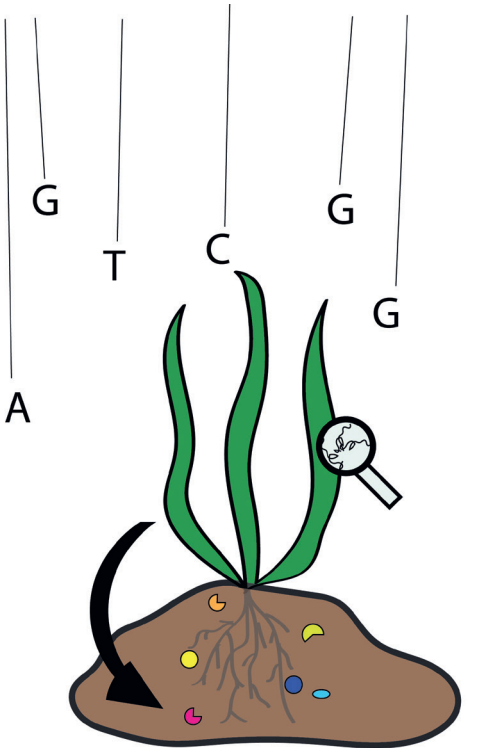


Figure 1.4. Overview of the main aims described in each Chapter.

Chapter 2

Genome-wide association study pinpoints plant loci associated with rhizosphere and root endosphere microbiome of *Lolium perenne*

Paola Rallo, Bram van Eijnatten, Freddy C. ten Hooven, Mattia Fois, Torben Asp, Basten Snoek, Wim H. van der Putten, Jan Kammenga, S. Emilia Hannula, Koen J.F. Verhoeven



Abstract

Plants can benefit from interacting with their soil microbiomes. To harness these interactions for improved plant performance, it is necessary to understand the genetic basis of plant effects on microbiome composition. To date, only few studies have aimed to pinpoint plant genetic variants that affect soil microbiome composition, and for grasses this issue remains largely unexplored. Using 16S and ITS amplicon sequencing, here we characterized the microbiome in the rhizospheres and root endospheres of 154 genotypes of *Lolium perenne*, an important turf and forage grass species. Using available single nucleotide polymorphisms (SNPs) data, we used a genome-wide association study (GWAS) to identify candidate genes associated with variation in the plant microbiomes. Our results suggest that bacterial and fungal communities are modulated by similar biological processes, of which pathways related plant defenses and root development are strongly implicated. Genes related to root development and root lateral formation seem particularly relevant for shaping plant-fungal interactions. In root endosphere microbiomes, *Lolium perenne* genetic effects had a stronger influence on plant-fungal communities than plant-bacterial communities. Our study adds to growing evidence that genetic variation within plant species modulates their associated microbiome and provides insight in the plant pathways and mechanisms by which plants can control microbiome composition.

Introduction

Soil microorganisms can influence plant growth and health by increased nutrient uptake (Jacoby et al. 2017), disease resistance (Van Wees, Van der Ent, and Pieterse 2008), stress tolerance (Meena et al. 2017), and other effects. Such microbe-mediated plant traits are relevant for ecological adaptation and for crop improvement, as the selection of plants that have desirable microbiome-mediated traits may lead to more productive and resilient agricultural systems. However, natural, or artificial selection can only shape these traits if there is plant intraspecific variation for the ability to engage with specific microbes.

There is substantial evidence for plant intraspecific variation in associations with soil microbes (Liu et al. 2019; Brown et al. 2020). Identifying the plant genes that govern these interactions could revolutionize plant breeding and biotechnology because of their potential of optimizing plant microbiome functions in crops. However, pinpointing such genes is challenging, because plant soil microbiomes are very diverse and are sourced from the soil environment. As a result, the genetic effects on microbiome composition may be weak compared to environmental effects, and only a few studies so far have revealed candidate genes that affect associations with soil microbes (Horton et al. 2014; Bergelson, Mittelstrass, and Horton 2019; Deng et al. 2021).

Genome-wide association studies (GWAS) have been recently adopted to identify genes associated with microbiome composition. Unlike other genetic approaches, such as cross-based QTL analysis (Oyserman et al. 2022), GWAS has the potential to provide high genomic resolution, which is important for identifying candidate genes for a variety of plant traits (Hua et al. 2022; Beilsmith et al. 2019). GWAS-based approaches have

recently focused on plant genetic variants associated with the root and phyllosphere microbiome in *Arabidopsis thaliana* (Horton et al. 2014; Bergelson, Mittelstrass, and Horton 2019), with root bacteria in *Panicum virgatum* (Singer et al. 2019), and with rhizosphere bacteria in *Sorghum bicolor* (Deng et al. 2021). These studies suggest that plant genes related to plant defense, cell wall integrity, plant physiology and immunity have effects on the composition of microbial communities. The increasing availability of plant genomes and densely genotyped accession panels is opening the application of GWAS approaches to a wide range of species of ecological or agronomic interest.

While rhizosphere microbiome composition may be affected by root exudate composition (Badri et al., 2009; Lakshmanan et al., 2012; Carvalhais et al., 2013; Lebeis et al., 2015), the root endosphere of plants is less exposed to the environment. Plant traits may affect the way microbes are filtered from the environment to attract or filter the microbes from the rhizosphere to become part of the endosphere microbiome (Edwards et al., 2015; Naylor et al., 2017). As such, plant genetic control over microbiomes might be stronger in the endosphere compared to rhizosphere. However, empirical evidence for this assumption is mixed. For example, in *Medicago truncatula*, host genotype effects were stronger in the endosphere compared to the rhizosphere (Brown et al. 2020) whereas other species (including rice, switchgrass, and sorghum) showed the opposite pattern (Edwards et al. 2015; Singer et al. 2019; Bergelson, Mittelstrass, and Horton 2019). The reasons for such differences between species, and the mechanisms by which the species may affect their associated microbiomes, remain poorly understood.

Here, we aim to unravel the host-genetic effects on bacterial and fungal microbiome composition in the rhizosphere and root endosphere of the grass *Lolium perenne*, which is a widely cultivated grass species used for turf and forage purposes and it is frequently grown as a monoculture (Boller, Posselt, and Veronesi 2010). Moreover, *Lolium perenne* is known to closely interact with various microorganisms, including symbiotic fungi and bacteria, affecting its performance (Tannenbaum et al. 2021). In such low-diversity systems the impact of highly specific plant-microbe interactions can be more important than in more diverse systems (Shipton 1977). In fact, in such systems there is an increased reliance on microbes for essential functions such as nutrient acquisition, disease suppression, and stress tolerance and thus disruptions can impact plant health and system productivity (Sanguin et al. 2009).

We characterized rhizosphere and endosphere microbiomes using amplicon sequencing in a panel of *L. perenne* accessions. We linked this information to dense single-nucleotide polymorphisms (SNP) information that is available for these accessions (Fois et al. 2021), in order to identify genetic loci affecting microbiome composition using a GWAS approach. To our knowledge, this is the first study to use GWAS to study both bacteria and fungi in the different compartments in a grass species. We aim to identify plant genes that are associated with *L. perenne* microbiomes, to gain insight in the functions and pathways that might control microbiome composition in this species. We further evaluate if evidence for plant genetic control over microbiome composition is different in the endosphere versus the rhizosphere, in fungal from bacterial microbiomes. We also examine if plant genetic effects extend beyond associations with individual microbial taxa to higher-level

microbiome community traits such as microbiome diversity and composition.

Material and methods

Plant material

From a population of 239 diploid perennial ryegrass (*Lolium perenne L.)* accessions that have been recently resequenced and for which dense genotyping information is available (Fois et al. 2021), we included 154 accessions in our study. The 154 plants were maintained prior to our study in a common garden field plot at the DLF Research Center, 4660 Store Heddinge, Denmark (Fig. 2.1 A). We collected cuttings from these plants and grew plants individually in pots at the Netherlands Institute of Ecology (Wageningen, Figure 2.1 B) greenhouse for six months prior to the common garden experiment (see below). The *Lolium perenne* population that we examined exhibited a total of 14288946 single nucleotide polymorphisms (SNPs) spread across seven distinct chromosomes. SNP accessions information and genomic locations on the *L. perenne* reference genome were taken from (Byrne et al. 2015).

Common garden experiment

A common garden experiment was conducted in the experimental garden at the Netherlands Institute of Ecology (NIOO-KNAW, Wageningen, The Netherlands), in which we exposed the 154 *L. perenne* accessions to the same soils and let them grow for five months. Plant accessions were grown as individual plants in 10L pots using a mixture of 90% commercial river sand and 10% sandy soil collected from three grassland fields (N52° 01.718' E5° 47.928', N52° 00.893' E5° 47.241', N52° 04.312' E5° 44.168'). The soil from the three grassland fields was mixed prior to

the experiment and was added as an inoculum to each pot to provide the same diverse, natural soil microbiome to all plants at the start of the experiment. In addition, we collected 35g of soil from each pot where these plants were growing before the start of the experiment which was homogenized and mixed with the grassland inoculum. The experiment followed a constant water regime through an automatic drip irrigation system, and 5.6 g of slow-release nutrients (Osmocote Extract Mini 3-4) were added to each pot to avoid nutrient deficit. Weeds and flowers were removed periodically from the experimental pots.

After five months, we harvested a quarter of each plant's root system from which we collected the soil attached to the roots (rhizosphere) and roots samples for endosphere analysis. Rhizosphere samples were collected by manually shaking roots to remove excess sand and then collecting the sand attached to the roots. Samples were kept on dry ice and then stored at −80 °C until further processing. To sample roots for endosphere analysis we used the protocol described by (Hannula et al. 2019). Specifically, plant roots were placed on a wire screen with 350 µm mesh size and gently washed with water to remove soil aggregates attached to the root surface. We then sampled fine-roots using sterile forceps and then transferred them to a 15 ml falcon tube filled with 10 ml autoclaved Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, Darmstadt, Germany). The root samples were then sonicated in a BRANSONIC ultrasonic cleaner for 10 min (ten 30s bursts followed by ten 30s rests). After sonication, the roots were rinsed with demi-water three times and then stored at -80 °C until processing.

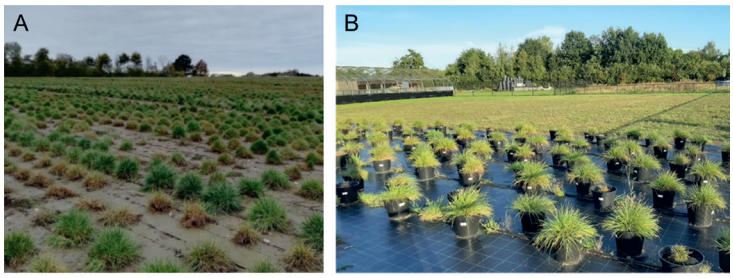


Figure 2.1. (A) *Lolium perenne* genotypes growing in the field in Denmark (B) Experimental Garden experiment located at The Netherlands Institute of Ecology.

Isolation of microbial DNA and sequencing

DNA was isolated from 0.25 g of sand (rhizosphere) and 0.3 g of roots (endosphere) using the Power Soil DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration and purity were measured via a Nanodrop spectrophotometer (Thermo Scientific, Hudson, NH, USA). Library preparation and sequencing were performed at McGill University and the Génome Québec Innovation Centre (Canada). To target the V4 region of the bacterial 16S the primers 515FB and 806RB were used (Caporaso et al. 2011; Amy Apprill et al. 2015; Parada, Needham, and Fuhrman 2016) while for fungi ITS4ngs and ITS3mix targeting the ITS2 region were used (Tedersoo et al. 2015a). Libraries were sequenced using the Illumina MiSeq 300PE platform (Illumina inc., San Diego, USA).

Bioinformatics microbiome

Bacterial and fungal sequences were analyzed using DADA2 (v. 1.12;) using SILVA (v.132) as reference database for bacteria and PIPITS (v. 2.3;) (Gweon et al. 2015) with UNITE (v. 8.0;) (Abarenkov et al. 2010) as reference database for fungi, respectively. Fungal OTUs were parsed against the FunGuild (v1.1) database to assign putative life strategies.

All reads from other than bacterial or fungal origin (i.e., plant material, mitochondria, chloroplasts) were removed from the datasets. Fungal samples with less than 1000 reads were discarded; the average read depth for the remaining 105 samples in endosphere and 151 samples in rhizosphere was respectively 7253.981 and 15137.03 reads. Bacteria with low read depth were also excluded for further analysis; the average read depth for the remaining 151 samples in endosphere and 147 samples in rhizosphere was respectively 11659.5 and 18493.75 reads.

Mantel tests were performed to determine the correlation between host genetic distances and rhizosphere and endosphere microbiome distances using the mantel function in the R package vegan (Oksanen et al. 2013), with 9999 permutations and using Spearman's correlations to reduce the impact of outliers. Pairwise genetic distances were calculated based on all SNPs using correlation matrices based on dissimilarity. Pairwise microbiome distances were calculated using Bray-curtis dissimilarity.

We characterized the microbiomes of each individual accession in three different ways. First, relative abundance of individual microbial taxa was calculated using total sum scaling (TSS) and classified at the genus level. We excluded from further analysis all taxa with a mean relative abundance < 0.01. Second, using these relative abundances we performed PCoA analysis based on Bray-Curtis's distance and extracted projections on PC1, PC2 and PC3 as quantitative descriptors of a plant's microbiome community composition. Third, we calculated the Shannon diversity index as a measure of alpha diversity of each plant's microbiome community.

Genome-wide association study

We used the lme4qtl R package to perform GWAS (Ziyatdinov et al. 2018). We used the covariance between the SNPs as a kinship matrix which we included in the GWAS model to correct for population structure. For each SNP, we model the phenotype (relative abundance of individual microbial taxa, PCoA axis scores, and Shannon index) using a mixed linear model with population structure as a random effect and the SNP genotype as a fixed effect. For GWAS on the Shannon index we included the samples' read depth as cofactor in the model, because read depth can create bias in diversity estimates. We pre-filtered the SNPs correlating them with the phenotype and only selecting those SNPs with a correlation coefficient with the phenotype of $r > 0.2$. For the relative abundance we only used SNPs with a minor allele frequency (MAF) > 0.1 (Kido et al. 2018) (so only SNPs where at least 10% of genotypes had the reference allele and at least 10% had the alternative allele). In the case of the Shannon/PCoA, for which the issue of samples with missing data is less severe as for OTU-level analysis, we used a MAF of 0.05. We used a heuristic p value significance threshold of $-\log10(P) > 7$. For GWAS on the individual OTU abundances and the PCoA axis scores, the number of SNPs tested (after filtering for correlation with the phenotype; see above) never exceeded ~70k SNPs. For this number of tests, the heuristic p value threshold corresponds to a Bonferroni correction at a nominal alpha of approximately 0.01.

Gene annotation

To determine which biological processes underlie variation in the composition of *L.perenne* microbiomes, we evaluated significant SNPs from GWAS (significance threshold of $-\log10(p) > 7$), including only

phenotypes that were detected in > 100 samples. The genome assembly was read using the *readDNAStringSet* function from the Biostrings package (H. Pagès 2017). To avoid redundancy, we selected only the entries in the gff (Byrne et al. 2015) marked as "gene" or "ncRNA". For each peak we took an interval of 0.1 Mb on both sides (0.2 in total) of the SNP with the most significant p-value, and selected all elements predicted "gene" and "ncRNA". Specifically, we selected all elements starting before the start of the 0.2 Mb interval but ending after the start of the interval, all elements that fell cleanly in the interval and all elements that started inside the interval but ended outside of the end of the interval. We extracted all the sequences from the *readDNAStringSet* object, and wrote them to a fasta using the write.fasta() function from the seqinr package (Charif and Lobry 2007). We blasted the sequences using blast x (so nucleotide to protein blast) against the *Arabidopsis* proteins in the nr (non-redundant) protein database. Blast outputs for each sequence match an NCBI database accession number. For each gene/ncRNA we chose the top 10 sequences (if there were more than 10). Using the *entrez_fetch(rettype = "txt")* function from the rentrez package (Winter 2017), we read in all the raw text in the html of the NCBI database page corresponding to the accession numbers of the hit to get TAIR gene names. For each hit with an annotation, we recorded the E-value which is a measure of the quality of the hit (an E-value of 0 means an exact hit). For each gene, we recorded the start and end position in bp and the chromosome, the microbial phenotype that mapped to it, and the position of the peak SNP. Moreover, we calculated the marker effect of the peak SNP by performing an anova using the *aov()* function on a linear model of phenotype ~ SNP genotype. All unknown genotypes were coded as heterozygous. We then extracted the sum of squares explained by the

marker (SSM) and the residual sum of squares RSS. We then calculated the eta squared (SSM/(SSM+RSS)) to quantify the strength of the marker effect. For the Shannon phenotypes, which had read depth as cofactor in the GWAS model, we calculated the partial eta squared of the marker effect, using aov() on a linear model of phenotype ~ genotype top SNP + total read count.

Results

Plant population structure

We used a population of 154 perennial ryegrass (*Lolium perenne L.*) genotypes that has been recently resequenced (Fois et al. 2021). This population is characterized by a total of 14288946 SNPs distributed over seven chromosomes with an average of one SNP every 171 bp. Based on their SNP profiles, plant accessions were correlated, and genetic similarity was visualized using a heatmap (Figure 2.2). No clear structure of the plant population was visible, indicating the absence of genetically differentiated groups of samples within the overall population. The absence of population structure makes the material particularly suitable for GWAS analysis, as population structure can cause false positive GWAS results (Marchini et al. 2004).

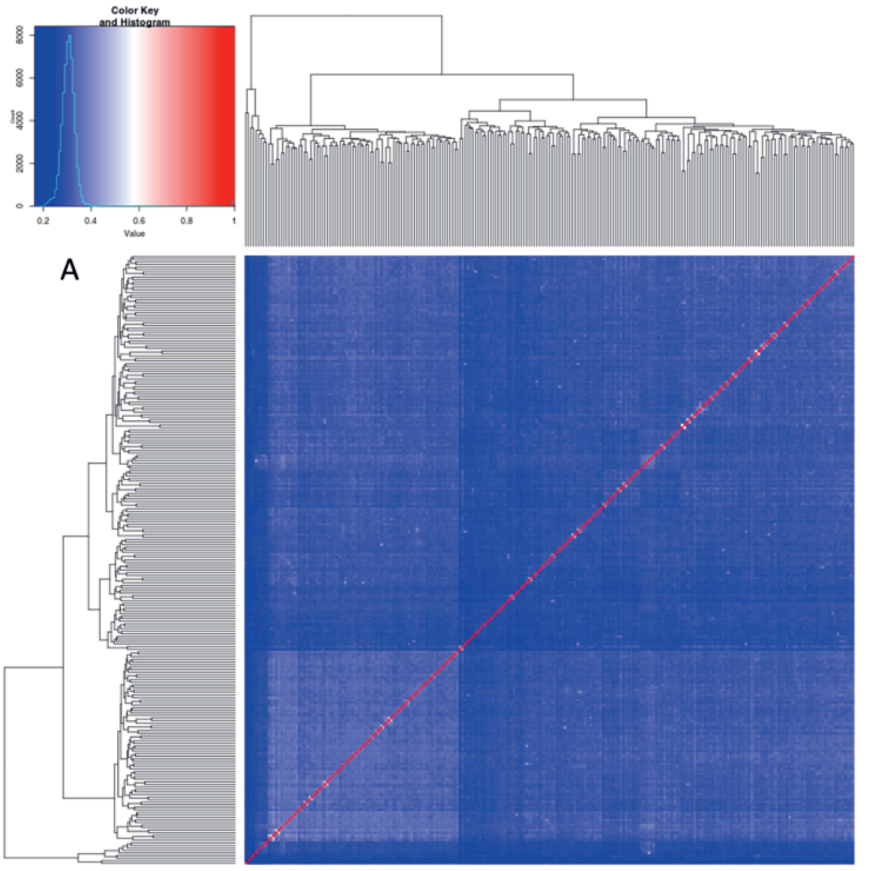


Figure 2.2: Heatmap of pairwise correlations based on SNP profiles of the entire plant population (239 accessions). A correlation matrix of the entire genome (all chromosomes) was used; every entry in the matrix is a correlation between two accessions based on their SNP profile.

Microbiome structure and diversity in rhizosphere and root endosphere

Genotypes of *L. perenne* recruited different bacterial ($R^2 = 0.44$; $p < 0.001$; Figure 2.3B) and fungal ($R^2 = 0.15$; $p < 0.001$; Figure 2.3A) microbial communities in their rhizosphere than in their root endosphere. Divergence in bacterial community composition between different *L. perenne* accessions was more pronounced in the rhizosphere than in the endosphere, whereas the accessions showed highest divergence in fungal communities in the endosphere. The fungal microbiome of *L. perenne*

exhibited minimal overlap between the endosphere and rhizosphere compartments (Figure 2.3 E), except for the *Slopeiomyces* and *Alternaria* genera, which are well-known grass pathogens. Fungal genera enriched in the endosphere included *Drechsler*, *Clohesyomyces* and *Plenodomus* genera. The rhizosphere, however, was more diverse and was dominated by *Penicillum*, *Vishniacozyma*, *Valsonectria*, *Pseudoppithomyces*, *Lecythophora*, *Pyrenophora* and *Stachybotrys* genera (Figure 2.3E). When examining bacteria, genera with relative abundance > 0.01 showed no overlap between rhizosphere and endosphere compartments. However, the rhizosphere contained the genera *Gemmata*, *Polycyclovorons,* whereas in the root endosphere the most abundant genera were *Ohtaekwangia, Haliangium, Caldimonas* and *Streptomyces* (Figure 2.3E). The levels of microbial diversity differed significantly among the plant compartments. We observed high levels of microbial diversity within both root endosphere and rhizosphere samples, but bacterial communities from the rhizosphere showed the highest Shannon index (Figure 2.3C-D).

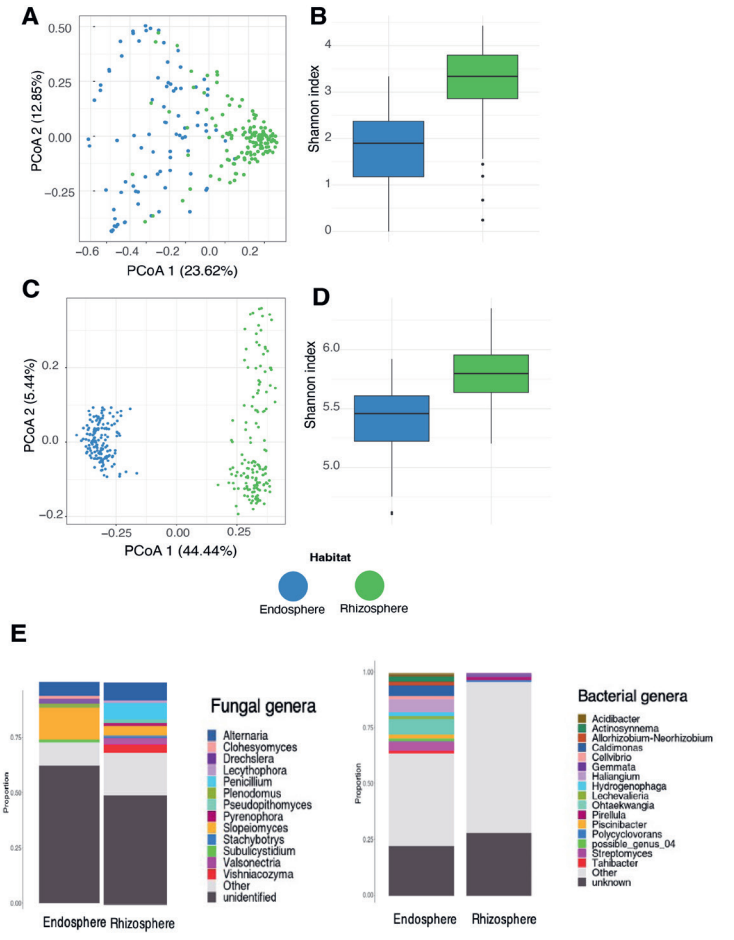


Figure 2.3. (A) PCoA of fungi (genus level) using Bray-Curtis distance. (B) Alpha diversity expressed using Shannon index of fungi. (C) PCoA of bacteria (genus level) using Bray-Curtis. (D) Alpha diversity expressed using Shannon index of bacteria. E) Relative abundances of fungal and bacterial genera in the endosphere and rhizosphere. Others denotes genera with a relative abundance < 0.01.

Host genetic variation affects root endosphere and rhizosphere microbiomes

Mantel tests

Independent Mantel tests (9999 permutations) were used to assess the degree of correlation between host genotypic distance in each chromosome and microbial distance for rhizosphere and root endospheric microbiomes using Bray Curtis distance. At the chromosome level, evidence for a correlation between genetic distance and microbiome distance was strongest for host genotypic distance on chromosome 2 and bacterial distance ($R^2 = 0.08$, $p < 0.01$, Figure S2.1) in the endosphere. We found no significant correlations between genetic distance and fungal distance neither in root endosphere, nor in the rhizosphere (Figure S2.1).

GWAS

For GWAS analysis, where microbiomes were considered as phenotypes that are potentially affected by genetic variation at specific genomic loci, we used microbial traits at three different levels: the relative abundances of each single OTU (classified at the genus level), microbiome diversity as estimated by the Shannon index, and microbiome community composition as estimated by principal coordinate analysis (PCoA) scores.

For single-OTU GWAS, we tested microbial genera present in > 30 % of the samples which resulted in 8 fungal OTUs from root endosphere microbiomes and 51 fungal OTUs from the rhizosphere. For bacteria, we tested a set of 58 OTUs from roots and 125 OTUs from the rhizosphere. For both fungi and for bacteria, and in both compartments, GWAS revealed significant SNP-OTU associations for 17% - 37% of the OTUs tested (Table 2.1). In the endosphere, the plant genetic effect on OTU

abundance was stronger for fungi than for bacteria (three out of eight significant OTUs in fungi versus 15 out of 58 in bacteria; and an average peak SNP effect size of 0.252 in fungi versus 0.16 in bacteria; Table 2.1) This difference was not observed in the rhizosphere (Table 2.1). Overall, our findings suggest that *L. perenne* effects have a stronger influence on plant-fungal communities than plant-bacterial communities.

Significant SNP associations were identified for 3 fungal OTUs belonging to the genera *Alternaria* (chromosome 3), *Plenodomus* (chromosome 5), and *Visniacozyma* (chromosomes 5 and 7) in the root endosphere (Table S2.5). In the rhizosphere we identified 9 genera including various grass pathogens such as *Alternaria*, and *Slopeiomyces* along chromosome 3, 4, 5 and 6 (Table S2.7). For bacteria, we found that variation in abundance of 15 genera in the endosphere (Table S2.1) and 43 genera in the rhizosphere significantly associated with SNP variation (with significant SNPs distributed over all 7 chromosomes; Table S2.3). Significant GWAS results were not only observed for single-OTU microbial phenotypes, but effects of genetic variation were also detectable at community-level. GWAS of fungal diversity (Shannon index) in the rhizosphere showed associations with SNPs located on chromosomes 2 and 4 (Table S2.8), whereas the top results from GWAS of bacterial diversity were detected on chromosome 7 (Table S2.4). The GWAS analysis performed for bacterial and fungal community composition (PC1, PC2, PC3 scores of PCoA) revealed associations with genetic variants on chromosomes 4 and 7 with bacterial PC2 in the rhizosphere (Table S2.4).

We observed pleiotropy in the rhizosphere, where a single genetic locus was associated with multiple microbial phenotypes as well as with a community-level phenotype (Figure 2.4). For instance, one genetic

variant at chromosome 7 associated with relative abundances of the bacterial genera *Acidothermus*, *RB4,* and *Tumebacillus* (Table S2.3). Such pleiotropy indicates that a single plant mechanism is responsible for variation in relative abundance of different microbes.

Table 2.1. Number of significant microbiome phenotypes (single OTUs, diversity index, and compositional index) in endosphere and rhizosphere. N refers to the total number of phenotypes used in the analysis whereas S indicate the number of significant phenotypes (at least one SNP with -log(p) >7). ME refers to the average of the marker effect of the GWAS peaks. ME per each phenotype are reported in Supplementary Table S1-8.

Phenotype	**Bacteria**						**Fungi**					
	Endosphere			**Rhizosphere**			**Endosphere**			**Rhizosphere**		
	N	S	ME	N	S	ME	N	S	ME	N	S	ME
Single OTUs	58	15	0.160	125	43	0.184	8	3	0.252	51	9	0.177
Diversity	1	1	NA	1	1	0.164	1	1	0.218	1	1	0.175
Composition	3	1	NA	3	1	0.181	3	2	NA	3	3	NA

Genes associated with rhizosphere and endosphere microbiome

After confirming that host accessions play a significant role in shaping microbial communities, we explored the potential plant processes shaping plant-microbe interactions. For each significant peak from the GWAS analysis, we identified homologous genes of *A.thaliana* located along an interval of 0.1 Mb on both sides of the peak SNP. We described the biological processes using gene ontology (GO) categories descriptors associated with some of the peaks, emphasizing the most significant ones (mostly -log(p)>8) and focusing on microbial phenotypes that were detected in minimally 100 samples (Tables 2.2 and 2.3).

When examining bacteria, we detected candidate genes that regulate metabolic pathways such as calcium signaling and growth regulation

(Table 2.3). This process may be linked to plant defense against pathogens. For instance, genes related to calcium signaling pathways (i.e., *NAMT1* and *At3g57880*) have been reported of plants to responding to biotic and abiotic stresses and in processes related to plant immunity (Galon, Finkler, and Fromm 2010; Teixeira et al. 2019). These genes were identified at a pleiotropic locus associated with relative abundance of the bacterial genera *Acidothermus*, *RB41*, *Tumebacillus* of *L. perenne's* rhizosphere (Figure 2.4). At the same locus we identified the *FTIP1* gene which regulates phloem transport and is linked to defence against pathogens (Corbesier et al. 2007; L. Liu et al. 2012; Koenig and Hoffmann-Benning 2020). Another gene related to plant defense was *WRKY20*, which regulates antimicrobial defense reactions in host plants (Hahlbrock et al. 2003; Eulgem and Somssich 2007) and in *N. benthamiana* conferred elevated resistance to bacterial and fungal infections (Shi et al. 2011). We also detected a candidate gene related to root lateral formation (*LRS1)* near a SNP on Chromosome 5 (104673660 Mb) (Table S2.3).

Similar to bacteria, also for fungi we identified several candidate genes involved in plant defense mechanisms (Table 2.3). A single locus on chromosome 3 that associated with the relative abundance of the pathogen *Alternaria* in the endosphere contained several candidate genes (Figure 2.5) that have previously been implicated in plant defenses. For instance, the gene *GSTU13* regulates mechanisms in the response against fungi (Gullner et al. 2018; Piślewska-Bednarek et al. 2018). Interestingly, also in *A. thaliana* the overexpression of *BcGSTU* resulted in increased resistance against *Alternaria* (Gullner et al. 2018). At the same locus we detected genes related to response to ethylene (i.e., ERF73, ERF71, EER5), which is known to regulate various processes such as

plant defense and growth. For fungi, a relatively higher number of genes involved in root development was found (Table 2.3). Moreover, we found genes involved in the process of lateral root formation (Table 2.3). These genes were found near loci associated with fungal diversity in plant endosphere (*SRD2*, Chromosome 5, 231606346 Mb; Table S2.6) and rhizosphere (*EDF3, RAV1*; Chromosome 4; 336409963 Mb; Table S.2.8).

Although our data suggest that bacterial and fungal communities are modulated by similar biological processes such as plant defense and root development, we found little overlap between bacteria and fungi in the actual plant candidate genes that were identified. We found only one gene (*SERPININ1)* that is a potential candidate for regulating *L. perenne*'s interaction with bacteria (*Candidatus udaeobacter*, Chromosome 3, 258467457 Mb) and fungi (*Lecanicillium*, Chromosome 4, 139354746 Mb), both in the rhizosphere (Table S2.3, S2.7).

Table 2.2: Overview of the number of genes observed within a 0.2 Mb window around the top SNPs of all significant GWAS peaks. From the top SNPs, we filtered based on occurrence of the phenotype in the samples (> 100) and reported the total number of genes (which include genes and ncRNA) and number of genes with annotation.

Kingdom	**Habitat**	**GWAS peaks**	**GWAS peaks** (sample occurrence >100)	**N Genes** (total)	**N Genes** (with annotation)
Fungi	Endosphere	22	5	70	25
Fungi	Rhizosphere	18	9	145	24
Bacteria	Endosphere	29	3	78	8
Bacteria	Rhizosphere	54	20	260	36
Total		123	37	553	93

Table 2.3: Biological processes observed for a subset of the genes identified using gene ontology (GO) categories descriptors. The full list of the genes identified is reported in Table S2.1 – S2.8.

Biological process	Genes	Kindom	Habitat	Phenotype
Flowering regulation (GO:0009911)	*FTIP1* *At3g57880*	Bacteria Bacteria	Rhizosphere Rhizosphere	*Acidothermus, RB41,* *Tumebacillus, PC2*
Nicotinate metabolism (GO:1901847)	*NAMT1*	Bacteria	Rhizosphere	*Acidothermus, RB41,* *Tumebacillus, PC2*
Response to wounding (GO:0009611)	*WRKY20*	Bacteria	Rhizosphere	*Subgroup 10 (Thermoanaerobaculaceae)*
Response to ethylene (GO:0071369) (GO:0009873)	*ERF73* *ERF13*	Fungi Fungi	Endosphere Endosphere	*Alternaria* *Alternaria*
Response to fungus (GO:0050832) (GO:0050832)	*GSTU13* *At2g20950*	Fungi Fungi	Endosphere Rhizosphere	*Alternaria* *Trichoderma*
Root development (GO:0048364) (GO:2000280) (GO:0010015)	*EER5* *ERF71* *EDF3*	Fungi Fungi Fungi	Endosphere Rhizosphere Rhizosphere	*Alternaria* *Alternaria* *Diversity*
Lateral root formation (GO:0010386) (GO:0010311) (GO:0048527)	*LRS1* *SRD2* *RAV1*	Bacteria Fungi Fungi	Rhizosophere Endosphere Rhizosphere	Mycobacterium Diversity Diversity

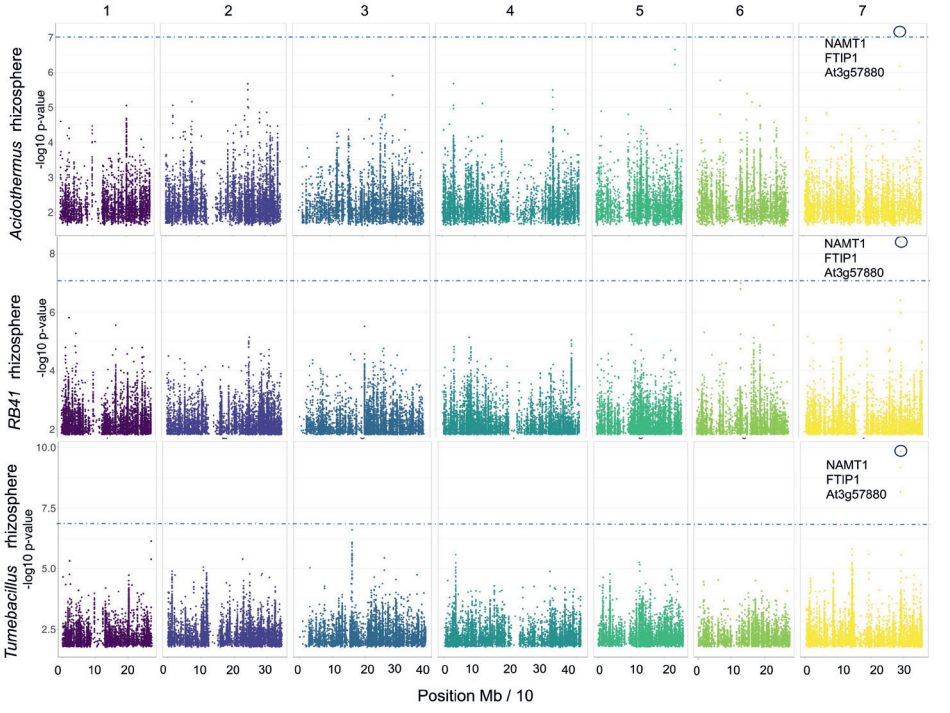


Figure 2.4: Manhattan plots showing a pleiotropic locus on chromosomes 7 (290459937 Mb) associated with the bacterial genera *Acidothermus*, *Tumebacillus* and *RB41* identified in the rhizosphere. In the same locus, three candidate genes (NAMT1, FTIP1, At3g57880) were detected.

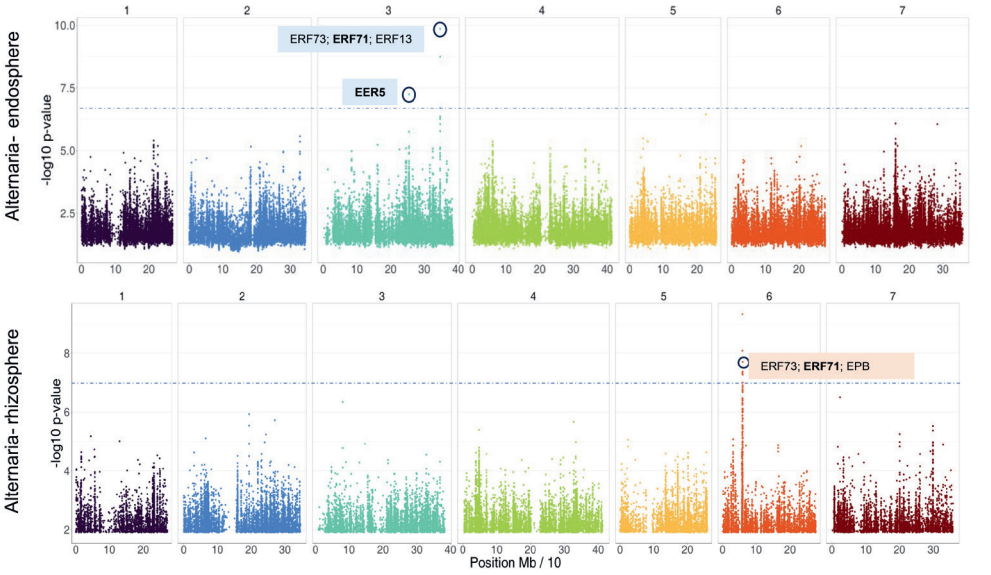


Figure 2.5: Manhattan plots showing candidate genes related to root development. On chromosome 3, the genes *ERF73*, *ERF71* and *ERF13* were identified close to a locus (347085118 Mb) associated with the genus *Alternaria* in the endosphere. On the same chromosome, another gene related to root development (*EER5*) was detected close to the locus 254129747 Mb). On chromosome 6, the genes *ERF73*, *ERF71* and *EPB* were found close to the locus 59614588 Mb associated with the genus *Alternaria* isolated from the rhizosphere.

Discussion

GWAS approaches have begun to unravel the genetic basis of how plants engage with, and potentially can manipulate, their associated microbiomes. While initial efforts have used the model species *A. thaliana* (Horton et al. 2014; Bergelson, Mittelstrass, and Horton 2019), the broad availability of high-resolution genomic analysis has now opened up similar GWAS opportunities for other species (Deng et al. 2021; J. A. Edwards et al. 2023). Here, we use GWAS to link intraspecific genetic variation in the grass *Lolium perenne* to the composition of the plants' rhizosphere and root endosphere microbiomes. Our study

provides evidence that the relative abundance of single OTUs, but also community-level descriptors of the microbiome such as structure and diversity of the rhizosphere and root microbiome, are shaped by genetic differences among accessions of *L. perenne.* Our results contribute to growing evidence for a plant genetic component to rhizosphere and endosphere microbiome composition. Depending on the effects of the affected microbes on plant traits, such genetic determination implies that there is scope for breeding programs and natural selection to improve plant performance via selection on microbiome-mediated traits.

Our results indicate that bacterial and fungal communities are modulated by similar biological processes, mostly by pathways related to plant defenses and root development. These biological pathways described here are in line with previous research in other plant species such as *Arabidospsis thaliana*, *Sorghum bicolor*, and *Panicum virgatum* (Horton et al. 2014; Bergelson, Mittelstrass, and Horton 2019; Deng et al. 2021; J. A. Edwards et al. 2023). Interestingly, it appears that candidate genes involved in root development and root lateral formation might more strongly shape plant-fungal interactions than plant-bacterial interactions. When studying fungi, genes related to the regulation of the ethylene hormone were more frequently observed than for bacteria. Ethylene serves crucial functions in both plant defense and root development and elongation (Le et al. 2001; Mao et al. 2007; H. Yang et al. 2021). There is evidence that ethylene gene regulators affect interactions with pathogens such as the necrotrophic fungus *Botrytis cinerea* (Berrocal-Lobo, Molina, and Solano 2002). However, genes that we detected that are involved in regulating the ethylene pathways could have an impact also on root development as well. For instance, the gene *ERF71* has been reported to regulate root development in *A.thaliana* and

soybean (Mao et al. 2016). Interestingly, these genes were found in close proximity to significant SNPs associated with the relative abundance of plant pathogens, specifically the fungal pathogen *Alternaria*. One speculative interpretation of this finding is that *L. perenne* accessions exhibit distinct root structures that effectively provide better resistance against pathogens like *Alternaria*. Furthermore, we detected a correlation between fungal diversity and the gene *SRD2*, which is known to be involved in root lateral formation. It is possible that variations in lateral root density and spacing could create microhabitats with unique nutrient gradients and oxygen levels, thereby establishing niches for different microbial taxa and promoting microbial diversity.

We hypothesized that evidence of plant genetic determination of microbiome composition is stronger in the endosphere than in the rhizosphere. There is currently mixed evidence for this hypothesis from studies in other species. However, it is important to note that this evidence is based primarily on bacteria, while there is limited information regarding fungi (but see (Bergelson, Mittelstrass, and Horton 2019). Our results in *L. perenne* showed that the impact of plant genetic variation on the abundance of fungal OTUs was stronger in the endosphere. The strongest effect was observed specifically with the fungal genus *Alternaria*, which is a well-known pathogen. However, the pattern of stronger effects in endosphere compared to the rhizosphere was not observed for bacterial OTUs. Thus, differences in plant genetic control over endosphere versus rhizosphere microbiomes may be species- and kingdom-specific, and there may not be a clear, general difference in the level of plant genetic control over endosphere versus rhizosphere microbiomes. Our observation that both types of microbiomes are

regulated by similar plant functions (including defense and root development processes) supports this suggestion.

Genome-Wide Association Studies represent a valuable tool for targeting microbiome traits in breeding programs. While there are various factors driving the exploration of the plant microbiome, a primary motivation lies in its potential to enhance plant health and productivity. However, when it comes to plants that are frequently cultivated in monoculture systems, such as grasses, it becomes particularly relevant to understand how to select for traits that can improve plant tolerance to pathogens. In monoculture settings, the reduced genetic diversity can make plant communities more susceptible to diseases and infections (King and Lively 2012). Therefore, identifying and selecting traits that enhance the plant's ability to withstand pathogenic pressures becomes crucial to enhance the overall resilience and productivity of these cultivated species.

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Supplementary material

Table S2.1. List of candidate genes for each **bacterial** phenotype in the **endosphere.** For each gene we recorded the start and end position in bp and the chromosome, the phenotype that mapped to it, and the position of the SNP with the peak SNP that the gene is close to. Another column contains the names of all the blast hits to that gene that had an annotation of gene name or tair ID. The name/ID of the hits are separated by |. E-value is an indicator of the quality of the peak. For each blast hit with a gene ID, we reported the accession number of the NCBI database page. Moreover, we reported the marker effect (ME). See Methods for more information.

Phenotype	Sart/End	C	SNP	*p*	AT_homologs	E_value	Accession_nr	ME
Ohtaekwangia	S:92569295 E:92571164	6	92598564	7.13	AOS|CYP74B2	4.11e-137|1.16e-94	NM_123629|Q9ZSY9	0.157
Ohtaekwangia	S:92617556 E:92618347	6	92598564	7.13	F28K20.4|F17A8.60|M3E9.210	0.000245|0.015|0.018	AC004793|AL049482|AL022223	0.157
Ohtaekwangia	S:92618533 E:92619518	6	92598564	7.13	At2g31080|At2g14430|T18B22.50|dl3275w|T4C9.20|At2g07730	0.000274|0.00047|0.001|0.002|0.005|0.02	AC005311|AC006067|AL138652|Z97336|AL080318|AC004483	0.157
Ohtaekwangia	S:92621522 E:92627975	6	92598564	7.13	THY-2|THY-2|THY-2|THY-2|THY-2	3.32e-75|3.32e-75|3.32e-75|3.32e-75|3.32e-75	NM_001342289|NM_001342289|NM_001342289|NM_001342289|NM_001342289|NM_001342289	0.157
Ohtaekwangia	S:92628159 E:92630680	6	92598564	7.13	At5g66930|At5g66930	5.76e-20|5.76e-20	AY056211|AY056211	0.157
Ohtaekwangia	S:92630876 E:92636222	6	92598564	7.13	SPT16|SPT16|T4F9.130|GTC2	0|0|1.57e-102|2.34e-100	NM_001340681|NM_001340681|AL049523|NM_117135	0.157

Pir4_lineage	S:136802224 E:136822426	5	136886118	7.10	At2g15410|At2g13330|At2g13330|At2g14400|At2g14400	0|2.05e-121|2.05e-121|5.71e-89|5.71e-89	AC006920|AC006446|AC006446|AC006067|AC006067	0.158
Pir4_lineage	S:136822725 E:136824413	5	136886118	7.10	CAT8	6.03E-07	NM_101572	0.158
Pir4_lineage	S:136841895 E:136844731	5	136886118	7.10	At2g45230|At2g45230|At2g45230|At2g01840	6.48e-79|6.48e-79|6.48e-79|6.53e-72	AC002387|AC002387|AC002387|AC007069	0.158
Pir4_lineage	S:136865735 E:136868317	5	136886118	7.10	ABCG17|ABCG17|ABCG17|ABCG19	2.73e-59|2.73e-59|2.73e-59|1.77e-58	NM_001339720|NM_001339720|NM_001339720|NM_115371	0.158
Pir4_lineage	S:136877796 E:136881759	5	136886118	7.10	FRS3|FRS3|FRS3|FRS3	3.36e-34|3.36e-34|3.36e-34|3.36e-34	NM_001036351|NM_001036351|NM_001036351|NM_001036351	0.158
Pir4_lineage	S:136885022E:136887417	5	136886118	7.10	At1g23390|At1g23390|At1g23390|At1g23390|At1g23390|At1g23390|At1g23390|At1g23390|At1g23390	9.31e-22|9.31e-22|9.31e-22|9.31e-22|9.31e-22|9.31e-22|9.31e-22|9.31e-22	FM995321|FM995321|FM995321|FM995321|FM995321|FM995321|FM995321|FM995321	0.158
Pir4_lineage	S:136901841E:136912292	5	136886118	7.10	At2g10780|At2g10780|At2g04670|At2g04670|T32B20.f|T21B14.24|F23H6.1	0|0|0|0|0|0|0	AC006570|AC006570|AC006955|AC006955|AF262041|AC069473|AC011621	0.158

Pir4_lineage	S:136941353E:136943338	5	136886118	7.10	NRT2.5|NRT2:1|NRT2.4|ACH2|NRT2.2|NRT2.3|NRT2.6	0|4.73e-174|8.44e-168|7.68e-167|9.23e-167|1.9e-160|2.88e-159	NM_101165|NM_100684|NM_125470|AF019749|NM_100685|NM_125471|NM_114375	0.158
Pir4_lineage	S:177536996E:177539283	2	177589671	8.17	dl4485c|At2g10780|At2g07660|F5K24.7|At2g04670|At2g14650|T32B20.f|F9D12.11	5.65e-148|6.81e-148|1.51e-139|1.07e-134|6.54e-129|3.33e-119|1.32e-116|9.32e-111	Z97342|AC006570|AC007730|AF128395|AC006955|AC005398|AF262041|AF077407	0.172
Pir4_lineage	S:177589102E:177590894	2	177589671	8.17	MSS1	1.97E-141	NM_122535	0.172
Pir4_lineage	S:177634832E:177637060	2	177589671	8.17	At2g12210|At2g14980|T25H8.2|At2g13000|F28J9.11	2.47e-179|2.19e-133|2.22e-125|9.27e-125|1.72e-123	AC005897|AC005957|AF128394|AC006437|AC007918	0.172

Table S2.2. List of candidate genes for each **community-level bacterial** phenotype observed in the **endosphere**. For each gene we recorded the start and end position in bp and the chromosome (C), the phenotype that mapped to it, and the position of the SNP with the peak SNP that the gene is close to. Another column contains the names of all the blast hits to that gene that had an annotation of gene name or tair ID. The name/ID of the hits are separated by |. E-value is an indicator of the quality of the peak. For each blast hit with a gene ID, we reported the accession number of the NCBI database page. Moreover, we reported the marker effect (ME). See Methods for more information.

Phenotype	Start/End (gene)	C	SNP	*p*	AT_homologs	E_value	Accession_nr	ME
Shannon	S:10714 7213 E:1071 55836	7	1072169 44	11.0 9	At2g10780| T28A8_120| T5L23.15|F 7M19_100|T 32B20.f	1.27e-19|2.03e-15|2.25e-13|9.14e-13|1.09e-12	AC006570|AL16 2691|AC005142 |AL138643|AF2 62041	0.1 64
Shannon	S:10716 3265 E:1071 68501	7	1072169 44	11.0 9	UBP1|UBP 1	1.63e-11|1.63e-11	NM_128838|NM _128838	0.1 64
Shannon	S:10718 9235 E:1071 94345	7	1072169 44	11.0 9	UBP2|UBP 2|UBP2	2.61e-14|2.61e-14|2.61e-14	NM_100364|NM _100364|NM_10 0364	0.1 64
Shannon	S:10722 9494 E:1072 34674	7	1072169 44	11.0 9	T7M24.7|F5 K24.8	1.14e-13|1.35e-07	AF077408|AF12 8395	0.1 64
PC2	S:29044 2112 E:2904 45987	7	2904599 19	7.15	At3g57880	0	AK229856	0.1 77
PC2	S:29047 1151 E:2904 74297	7	2904599 19	7.15	FTIP1	0	NM_120768	0.1 77
PC2	S:29048 5633 E:2904 87874	7	2904599 19	7.15	NAMT1|NA MT1|NAMT 1|NAMT1	4.49e-26|4.49e-26|4.49e-26	NM_120519|NM _120519|NM_12 0519|NM_12051 9	0.1 77
PC2	S:33535 9948 E:3353 63338	4	3353536 81	7.77	At2g12210| F28J9.11|T 25H8.2|At2 g13000|At2 g14980|T4I 21.6	7.85e-180|2.73e-137|4.81e-134|6.36e-131|2.68e-130|6.73e-120	AC005897|AC00 7918|AF128394 |AC006437|AC0 05957|AC02245 6	0.1 86
PC2	S:33539 9362 E:3354 04466	4	3353536 81	7.77	F11O6.6|F1 1O6.6	2e-154|2e-154	AC018460|AC01 8460	0.1 86
PC2	S:33556 0396 E:3355 60761	4	3356559 20	8.23	FRS3|FRS3 |dl3590w|F AR1	2.79e-18|2.79e-18|5.96e-18|1.22e-17	NM_128269|NM _128269|Z97337 |NM_001340994	0.1 82

PC2	S:33556 2034 E:3355 66989	4	3356559 20	8.23	At2g12210| At2g12210| At2g12210	8.31e-120|8.31e-120|8.31e-120	AC005897|AC00 5897|AC005897	0.1 82
PC2	S:33572 3302 E:3357 26347	4	3356559 20	8.23	At2g13330| At2g15410| At2g15410| At2g15410| At2g14400| At2g14400| At2g14400	3.52e-124|5.89e-118|5.89e-118|5.89e-118|1.14e-96|1.14e-96|1.14e-96	AC006446|AC00 6920|AC006920 |AC006920|AC0 06067|AC00606 7|AC006067	0.1 82

Table S2.3. List of candidate genes for each bacteria phenotype in the **rhizosphere.** For each gene we recorded the start and end position in bp and the chromosome (C), the phenotype that mapped to it, and the position of the SNP with the peak SNP that the gene is close to. Another column contains the names of all the blast hits to that gene that had an annotation of gene name or tair ID. The name/ID of the hits are separated by |. E-value is an indicator of the quality of the peak. For each blast hit with a gene ID, we reported the accession number of the NCBI database page. Moreover, we reported the marker effect *(ME)*. See Methods for more information.

Phenotype	**Start/End (gene)**	**C**	**SNP**	***p***	**AT_homologs**	**E_value**	**Accession nr**	**ME**
Acidothermus	S:290442112 E:29044 5987	7	29045 9937	7.2 2	At3g57880	0	AK229856	0.17 9
Acidothermus	S:290471151 E:29047 4297	7	29045 9937	7.2 2	FTIP1	0	NM_120768	0.17 9
Acidothermus	S:290485633 E:29048 7874	7	29045 9937	7.2 2	NAMT1|NAMT 1|NAMT1|NA MT1	4.49e-26|4.49e-26|4.49e-26|4.49e-26	NM_120519|N M_120519|N M_120519|N M_120519	0.17 9
Candidatus_ Udaeobacter	S:190462880 E:19046 6744	6	19047 3382	7.1 9	At2g12210|At2g 12210|F 28J9.11 |F28J9. 11	7.24e-163|7.24e-163|8.76e-128|8.76e-128	AC005897|AC 005897|AC00 7918|AC0079 18	0.15 3
Candidatus_ Udaeobacter	S:258476224 E:25847 7629	3	25846 7457	8.8 1	SERPIN 1|SERP IN1|CC P3	2.66e-49|2.66e-49|5.94e-48	NM_103664|N M_103664|N M_128081	0.19 5
Candidatus_ Udaeobacter	S:258500998 E:25850 8323	3	25846 7457	8.8 1	At4g375 60|At4g 37560|A t4g3756 0|At4g3 7560|At 4g37560	6.07e-88|6.07e-88|6.07e-88|6.07e-88|6.07e-88	AY035000|AY 035000|AY03 5000|AY0350 00|AY035000	0.19 5

Candidatus_ Udaeobacter	S:258514038 E:258516123	3	25846 7457	8.8 1	UNE14|UNE14|CML30|CML30	7.96e-19|7.96e-19|5.54e-18|5.54e-18	NM_117355|NM_117355|NM_127129|NM_127129	0.195
Candidatus_ Udaeobacter	S:258516931 E:258517317	3	25846 7457	8.8 1	TCH2|TCH2|AGD11|UNE14	1.92e-21|1.92e-21|4.15e-20|5.54e-20	NM_001344218|NM_001344218|NM_111627|NM_117355	0.195
Candidatus_ Udaeobacter	S:258528915 E:258531180	3	25846 7457	8.81	T7M24.7|T7M24.7|T7M24.7|T7M24.7|T7M24.7|T7M24.7|T7M24.7|T7M24.7	1.9e-125|1.9e-125|1.9e-125|1.9e-125|1.9e-125|1.9e-125|1.9e-125|1.9e-125	AF077408|AF077408|AF077408|AF077408|AF077408|AF077408|AF077408|AF077408	0.195
Candidatus_ Udaeobacter	S:258552577 E:258553630	3	25846 7457	8.8 1	At3g50770|CML41	3.72e-21|6.17e-21	AY099647|NM_114937	0.195
Candidatus_ Udaeobacter	S:258552914 E:258553510	3	25846 7457	8.8 1	At3g50770|CML41	1.56e-22|2.45e-22	AY099647|NM_114937	0.195
Candidatus_ Udaeobacter	S:258562921 E:258567649	3	25846 7457	8.8 1	KIN11|KIN11|KIN11|KIN11|KIN11|At5g10930|At5g10930|At5g10930|At5g10930	4.1e-32|4.1e-32|4.1e-32|4.1e-32|5.11e-32|5.11e-32|5.11e-32|5.11e-32	NM_202646|NM_202646|NM_202646|NM_202646|NM_202646|BT001234|BT001234|BT001234|BT001234	0.195
Dongia	S:169603519 E:169606187	3	16964 6136	8.2 2	ENODL18|ENODL18	4.87e-28|4.87e-28	NM_100723|NM_100723	0.19
Dongia	S:169641695 E:169645976	3	16964 6136	8.2 2	TUB6|TUB6|TUB6|At5g12250/MXC9_21|At5g12250/MXC9_21|At5g12250/MXC9_21|TUB8|TUB8|TUB8|At5g23860; MRO11.10	3.09e-134|3.09e-134|3.09e-134|3.12e-134|3.12e-134|3.12e-134|3.89e-133|3.89e-133|3.89e-133|3.93e-133	NM_121263|NM_121263|NM_121263|AK117925|AK117925|AK117925|M84705|M84705|M84705|AY054693	0.19

Dongia	S:169671681 E:169674284	3	16964136	8.22	PAP15|PAP15|PAP15|PAP15	0|0|0|0	NM_001337692|NM_001337692|NM_001337692|NM_001337692	0.19
Dongia	S:169707197 E:169713833	3	16964136	8.22	At1g19580|At1g19580|At1g19580|At1g19580|At1g19580	1.06e-29|1.06e-29|1.06e-29|1.06e-29|1.06e-29	AK175388|AK175388|AK175388|AK175388|AK175388	0.19
Dongia	S:169718809 E:169721265	3	16964136	8.22	RBL14|RBL14|RBL14|RBL14|At3g17611	4.52e-47|4.52e-47|4.52e-47|4.52e-47|3.91e-09	NM_180275|NM_180275|NM_180275|NM_180275|AK175222	0.19
Dongia	S:169737583 E:169740246	3	16964136	8.22	RBL14|RBL14|RBL14|RBL14|At3g17611|F14P22.50	1e-67|1e-67|1e-67|1.95e-22|1.08e-09	NM_180275|NM_180275|NM_180275|NM_180275|AK175222|AL137082	0.19
Hirschia	S:137812671 E:137820421	3	13791201	8.36	F15D2.30|F15D2.30|F15D2.30|F15D2.30|F15D2.30|F15D2.30	4.09e-30|4.09e-30|4.09e-30|4.09e-30|4.09e-30	AC068667|AC068667|AC068667|AC068667|AC068667	0.153
Hirschia	S:137822529 E:137823650	3	13791201	8.36	BPM2|BPM1|BPM3|BPM2|BPM1	3.98e-71|3.25e-67|4.51e-64|3.98e-71|3.25e-67	NM_111494|NM_001343580|NM_129534|NM_111494|NM_001343580	0.153
Hirschia	S:137824350 E:137829367	3	13791201	8.36	ML4|ML4|ML4|ML4	1.8e-26|1.8e-26|1.8e-26|1.8e-26	NM_120811|NM_120811|NM_120811|NM_120811	0.153
Hirschia	S:137831430 E:137840102	3	13791201	8.36	BPM2|BPM2|BPM1|BPM1	6.18e-65|6.18e-65|2.01e-61|2.01e-61	NM_111494|NM_111494|NM_001343580|NM_001343580	0.153
Hirschia	S:137831756 E:137838380	3	13791201	8.36	BPM2|BPM2|BPM1|BPM1	4.54e-65|4.54e-65|1.48e-61|1.48e-61	NM_111494|NM_111494|NM_001343580|NM_001343580	0.153

Hirschia	S:137880489 E:137882678	3	13791201	8.36	T15M6.14|T15M6.14|F11I4_21|F11I4_21|T28P6.8|T28P6.8|T20K12.230|T20K12.230	4.57e-174|4.57e-174|1.57e-173|1.57e-173|1.74e-171|1.74e-171|2.11e-171|2.11e-171	AC079604|AC079604|AC073555|AC073555|AC007259|AC007259|AL137898|AL137898	0.153
Hirschia	S:137954613 E:137955500	3	13791201	8.36	At1g10160|F2J7.11|At2g01840	4.23e-16|5.3e-14|3.54e-13	AK229033|AC079281|AC007069	0.153
Hirschia	S:137999265 E:138002306	3	13791201	8.36	At2g10780|At2g10780|At2g04670|At2g04670|T4F9.40|T4F9.40	0|0|0|0|0|0	AC006570|AC006570|AC006955|AC006955|AL049523|AL049523	0.153
Mycobacterium	S:104584766 E:104588079	5	10467660	8.17	At2g06890|At2g06890|At2g06890|F5K24.1|F5K24.1|T21B14.24|F23H6.1	0|0|0|0|0|0|5.11e-174|2.49e-165	AC005561|AC005561|AC005561|AF128395|AF128395|AF128395|AC069473|AC011621	0.184
Mycobacterium	S:104597161 E:104598662	5	10467660	8.17	T6B12.3|At2g05200	1.8e-34|1.98e-31	AC079679|AC007018	0.184
Mycobacterium	S:104649874 E:104652305	5	10467660	8.17	T21B14.24|T21B14.24|T21B14.24	1.27e-115|1.27e-115|1.27e-115	AC069473|AC069473|AC069473	0.184
Mycobacterium	S:104657725 E:104672336	5	10467660	8.17	LRS1|LRS1|LRS1|LRS1|LRS1|LRS1|LRS1|LRS1	9.04e-58|9.04e-58|9.04e-58|9.04e-58|9.04e-58|9.04e-58|9.04e-58|9.04e-58	NM_001337562|NM_001337562|NM_001337562|NM_001337562|NM_001337562|NM_001337562|NM_001337562|NM_001337562	0.184
Mycobacterium	S:104708453 E:104731938	5	10467660	8.17	T2O9.150	4.06E-93	AL138658	0.184
Mycobacterium	S:104737946 E:104746678	5	10467660	8.17	At2g16680	2.65E-08	AC005825	0.184

Mycobacterium	S:104750139 E:104752086	5	104673660	8.17	T18F15.5|T18F15.5|AT4g27210|AT4g27210	1.35e-137|1.35e-137|2.61e-136|2.61e-136	AC084807|AC084807|AL030978|AL030978	0.184
Nitrospira	S:267891879 E:267897979	7	267892798	7.89	AT4G38510	6.93E-56	AK317596	0.183
Nitrospira	S:267919336 E:267923782	7	267892798	7.89	TMN7|TMN7|TMN7|TMN7|TMN7	0|0|0|0|0	NM_112228|NM_112228|NM_112228|NM_112228|NM_112228	0.183
Nitrospira	S:267929075 E:267930843	7	267892798	7.89	T29M8.13|T29M8.13	2.14e-116|2.14e-116	AC069143|AC069143	0.183
Ohtaekwangia	S:140554626 E:140555317	3	140647693	7.57	At2g10780|At2g10780|At2g14650|At2g14650|F9D12.11|F9D12.11|At2g04670|At2g04670	1.76e-42|1.76e-42|2.15e-42|2.15e-42|1.07e-41|1.07e-41|1.56e-40|1.56e-40	AC006570|AC006570|AC005398|AC005398|AF077407|AF077407|AC006955|AC006955	0.178
Ohtaekwangia	S:140653643 E:140656954	3	140647693	7.57	ADCL|ADCL	1.79e-105|1.79e-105	NM_125170|NM_125170	0.178
Ohtaekwangia	S:140688754 E:140691993	3	140647693	7.57	T18A20.5|F28K20.17|ATRE1|RF28	2.22e-24|2.08e-23|6.18e-23|7.41e-23	AC009324|AC004793|AB021265|AB028223	0.178
Ohtaekwangia	S:140730456 E:140735042	3	140647693	7.57	At2g10780|At2g04670|At2g04670|T32B20.f	0|0|0|0	AC006570|AC006955|AC006955|AF262041	0.178
OLB12	S:267891879 E:267897979	7	267928328	7.20	AT4G38510	6.93E-56	AK317596	0.172
OLB12	S:267919336 E:267923782	7	267928328	7.20	TMN7|TMN7|TMN7|TMN7|TMN7	0|0|0|0|0	NM_112228|NM_112228|NM_112228|NM_112228|NM_112228	0.172
OLB12	S:267929075 E:267930843	7	267928328	7.20	T29M8.13|T29M8.13	2.14e-116|2.14e-116	AC069143|AC069143	0.172

OLB12	S:268005078 E:268005436	7	26792832 8	7.20	T7M24.7|At2g19840|At2g13930|F28L22.3|T26N6.5|At2g15700|T32O22.19	2.1e-39|4.18e-33|2.68e-31|3.32e-31|7.09e-31|1.05e-30|2.08e-29	AF077408|AC005169|AC006528|AC007505|AF076243|AC006248|AC079028	0.172
OLB12	S:268014277 E:268017954	7	26792832 8	7.20	F1P2.120|F1P2.120|At3g47570|At3g47570	0|0|0|0	AL132955|AL132955|AY064013|AY064013	0.172
Phaselicystis	S:109577325 E:109581922	3	10958 4947	9.53	TTL1|TTL1|TTL1	1.4e-81|1.4e-81|1.4e-81	NM_104208|NM_104208|NM_104208	0.217
Phaselicystis	S:109609053 E:109611035	3	10958 4947	9.53	At2g10780|dl4485c|At2g04670|F5K24.7|T32B20.f|At2g07660|At2g07660	0|0|0|4.3e-172|2.54e-162|2.48e-135|2.48e-135	AC006570|Z97342|AC006955|AF128395|AF262041|AC007730|AC007730	0.217
Phaselicystis	S:109640291 E:109642506	3	10958 4947	9.53	T7M24.7|F5K24.8|F5K24.8|At2g17490|T15M6.14|T20K12.230|T28P6.8|F11I4_21|T16L24.270	2.6e-174|1.57e-97|1.57e-97|2.59e-77|1.53e-75|5.15e-75|5.2e-75|2.63e-74|2.65e-74	AF077408|AF128395|AF128395|AC007584|AC079604|AL137898|AC007259|AC073555|AL138659	0.217
Phaselicystis	S:109662640 E:109666400	3	10958 4947	9.53	At2g12210|T1E4.4|T17A2.5|At2g10630|At2g14980|T25H8.2	7.98e-53|1.25e-51|3.67e-48|7.88e-48|8.36e-47|2.13e-46	AC005897|AC069299|AF160183|AC006250|AC005957|AF128394	0.217
Phaselicystis	S:109666908 E:109667152	3	10958 4947	9.53	ndhB|ndhB|ndhB|ndhB|ndhB|ndhB|ndhB1|ndhB	7.7e-19|7.7e-19|7.7e-19|7.7e-19|7.7e-19|1.74e-17|7.7e-19	AJ971670|AJ971670|AJ971670|AJ971670|AJ971670|P0CC32|AJ971670	0.217

Phaselicystis	S:109679155 E:109684342	3	109584947	9.53	F28J9.11|T7O23.23|F1K3.9|F14G16.1|F28J9.10	3.37e-18|1.4e-15|2.13e-14|4.27e-10|6e-10	AC007918|AC074228|AC006266|AF147260|AC007918	0.217
Polycyclovorans	S:294507017 E:294509584	7	294578684	8.03	RLK4	1.77E-81	NM_116257	0.181
RB41	S:290442112 E:290445987	7	290459937	8.51	At3g57880	0	AK229856	0.191
RB41	S:290471151 E:290474297	7	290459937	8.51	FTIP1	0	NM_120768	0.191
RB41	S:290485633 E:290487874	7	290459937	8.51	NAMT1|NAMT1|NAMT1|NAMT1	4.49e-26|4.49e-26|4.49e-26|4.49e-26	NM_120519|NM_120519|NM_120519|NM_120519	0.191
Subgroup_10	S:74233400 E:74236651	2	74253240	8.18	At5g53940|At5g53940	4.26e-08|4.26e-08	AK176141|AK176141	0.179
Subgroup_10	S:74274824 E:74281012	2	74253240	8.18	WRKY20|WRKY20|WRKY20|WRKY20|WRKY20|WRKY20|WRKY20|WRKY20|WRKY20|WRKY20	3.43e-31|3.43e-31|3.43e-31|3.43e-31|3.43e-31|3.43e-31|3.43e-31|3.43e-31|3.43e-31|3.43e-31	NM_001341822|NM_001341822|NM_001341822|NM_001341822|NM_001341822|NM_001341822|NM_001341822|NM_001341822|NM_001341822|NM_001341822	0.179
Subgroup_10	S:74281569 E:74285997	2	74253240	8.18	SCL5|At1g50600|SCL5|SCL5|PAT1	1.13e-173|1.83e-173|1.13e-173|1.13e-173|3.76e-173	NM_001333446|AY034929|NM_001333446|NM_001333446|NM_001344761	0.179
Subgroup_10	S:74299078 E:74309498	2	74253240	8.18	T7M24.7|T7M24.7|T7M24.7|T7M24.7|T7M24.7|T7M24.7|T7M24.7|T7M24.7|T7M24.7	0|0|0|0|0|0|0|0|0	AF077408|AF077408|AF077408|AF077408|AF077408|AF077408|AF077408|AF077408|AF077408	0.179

Subgroup_10	S:74311162 E:74312473	2	74253240	8.18	At3g15310|At3g15310|At3g15310|At2g13770	1.08e-79|1.08e-79|1.08e-79|2.1e-55	AY050876|AY050876|AY050876|AC006436	0.179
Subgroup_10	S:74318628 E:74322782	2	74253240	8.18	T18F15.5|T18F15.5|dl4465c|dl4465c|dl4465c	2.42e-122|2.42e-122|7.38e-111|7.38e-111|7.38e-111	AC084807|AC084807|Z97342|Z97342|Z97342	0.179
Subgroup_10	S:249229997 E:249231908	2	249256748	7.06	dl4485c|dl4485c|dl4485c|At2g10780|At2g10780|At2g10780|At2g10780|At2g07660	1.97e-82|1.97e-82|1.97e-82|2.02e-82|2.02e-82|2.02e-82|2.02e-82|1.05e-81	Z97342|Z97342|Z97342|AC006570|AC006570|AC006570|AC006570|AC007730	0.169
Tumebacillus	S:290442112 E:290445987	7	290459937	9.96	At3g57880	0	AK229856	0.223
Tumebacillus	S:290471151 E:290474297	7	290459937	9.96	FTIP1	0	NM_120768	0.223
Tumebacillus	S:290485633 E:290487874	7	290459937	9.96	NAMT1|NAMT1|NAMT1|NAMT1	4.49e-26|4.49e-26|4.49e-26|4.49e-26	NM_120519|NM_120519|NM_120519|NM_120519	0.223

Table S2.4. List of candidate genes identified for community-level bacterial phenotype observed in the rhizosphere (Shannon index and PC). For each gene we recorded the start and end position in bp and the chromosome (C), the phenotype that mapped to it, and the position of the SNP with the peak SNP that the gene is close to. Another column contains the names of all the blast hits to that gene that had an annotation of gene name or tair ID. The name/ID of the hits are separated by |. E-value is an indicator of the quality of the peak. For each blast hit with a gene ID, we reported the accession number of the NCBI database page. Moreover, we reported the marker effect *(ME)*. See Methods for more information.

Phenotype	Start/ End (gene)	C	SNP	*p*	AT_homologs	E_value	Accession_nr	ME
Shannon	S:107147213 E:107155836	7	107216944	11.08	At2g10780|T28A8_120|T5L23.15|F7M19_100|T32B20.f	1.27e-19|2.03e-15|2.25e-13|9.14e-13|1.09e-12	AC006570|AL162691|AC005142|AL138643| AF262041	0.164
Shannon	S:107163265 E:107168501	7	107216944	11.08	UBP1|UBP1	1.63e-11|1.63e-11	NM_128838|NM_128838	0.164
Shannon	S:107189235 E:107194345	7	107216944	11.08	UBP2|UBP2|UBP2	2.61e-14|2.61e-14|2.61e-14	NM_100364|NM_100364|NM_100364	0.164
Shannon	S:107229494 E:107234674	7	107216944	11.08	T7M24.7|F5K24.8	1.14e-13|1.35e-07	AF077408|AF128395	0.164
PC2	S:290442112 E:290445987	7	290459919	7.15	At3g57880	0	AK229856	0.177
PC2	S:290471151 E:290474297	7	290459919	7.15	FTIP1	0	NM_120768	0.177
PC2	S:290485633 E:290487874	7	290459919	7.15	NAMT1|NAMT1|NAMT1|NAMT1	4.49e-26|4.49e-26|4.49e-26|4.49e-26	NM_120519|NM_120519|NM_120519|NM_120519	0.177
PC2	S:335359948 E:335363338	4	335355368 1	7.767	At2g12210|F28J9.11|T25H8.2|At2g13000|At2g14980|T4I21.6	7.85e-180|2.73e-137|4.81e-134|6.36e-131|2.68e-130|6.73e-120	AC005897|AC007918|AF128394|AC006437|AC005957|AC022456	0.186
PC2	S:335399362 E:335404466	4	335355368 1	7.767	F11O6.6|F11O6.6	2e-154|2e-154	AC018460|AC018460	0.186

PC2	S:3355 60396 E:3355 60761	4	3356 5592 0	8.22 7	FRS3|FRS3|dl3 590w|FAR1	2.79e-18|2.79e-18|5.96e-18|1.22e-17	NM_128269|NM _128269|Z97337 |NM_001340994	0.18 2
PC2	S:3355 62034 E:3355 66989	4	3356 5592 0	8.22 7	At2g12210|At2g 12210|At2g1221 0	8.31e-120|8.31e-120|8.31e-120	AC005897|AC00 5897|AC005897	0.18 2
PC2	S:3357 23302 E:3357 26347	4	3356 5592 0	8.22 7	At2g13330|At2g 15410|At2g1541 0|At2g15410|At 2g14400|At2g14 400|At2g14400	3.52e-124|5.89e-118|5.89e-118|5.89e-118|1.14e-96|1.14e-96|1.14e-96	AC006446|AC00 6920|AC006920 |AC006920|AC0 06067|AC00606 7|AC006067	0.18 2

Table S2.5. List of candidate genes for each **fungal** phenotype in the **endospheree.** For each gene we recorded the start and end position in bp and the chromosome (C), the phenotype that mapped to it, and the position of the SNP with the peak SNP that the gene is close to. Another column contains the names of all the blast hits to that gene that had an annotation of gene name or tair ID. The name/ID of the hits are separated by |. E-value is an indicator of the quality of the peak. For each blast hit with a gene ID, we reported the accession number of the NCBI database page. Moreover, we reported the marker effect *(ME)*. See Methods for more information.

Phenotype	**Start/ End (gene)**	**C**	***SNP***	***p***	**AT_homologs**	**E_value**	**Accession_ nr**	**ME**
Alternaria	S:2540 45385 E:2540 48680	3	2541 2974 7	7.25	At2g15650|T18 A20.5|F28K20.1 7|F11O6.6|T18 F15.5|F20P5.25 |T4F9.150|F9K 21.100	0|0|6.36e-179|8.28e-170|9.75e-170|2.87e-168|6.58e-166|3.23e-161	AC006248| AC009324| AC004793| AC018460| AC084807| AC002062| AL049523|A L138657	0.21 2
Alternaria	S:2541 36929 E:2541 41833	3	2541 2974 7	7.25	At2g19560|EER 5|EER5	0.01|0.015| 0.015	AC005917| NM_127514 |NM_12751 4	0.21 2
Alternaria	S:2542 23627 E:2542 28244	3	2541 2974 7	7.25	SULTR3;5|SUL TR3;5|SULTR3; 5	3.99e-82|3.99e-82|3.99e-82	NM_121965 |NM_12196 5|NM_1219 65	0.21 2
Alternaria	S:3470 11602 E:3470 18006	3	3470 8511 8	9.85 7	KUP3|KUP3|K UP3|KUP3|KU P3|KUP3|KUP 3	7.93e-168|7.93e-168|7.93e-168|7.93e-168|7.93e-168|7.93e-168	NM_111071 |NM_11107 1|NM_1110 71|NM_111 071|NM_11 1071|NM_1 11071|NM_ 111071	0.28 2

Alternaria	S:347083863 E:347085664	3	34708511 8	9.857	GSTU8|GSTU7|GSTU1|GSTU13|GSTU5	3.67e-46|1.65e-45|1.92e-44|3.93e-43|5.26e-42	NM_111761|NM_128496|NM_128503|NM_102475|NM_128499	0.282
Alternaria	S:347107604 E:347109108	3	34708511 8	9.857	At2g31080|At2g31080|At2g23880|F12K8.9|F12K8.9	4.25e-32|4.25e-32|4.76e-31|1.1e-30|1.1e-30	AC005311|AC005311|AC005170|AC006551|AC006551	0.282
Alternaria	S:347180165 E:347180689	3	34708511 8	9.857	ERF73|ERF71|ERF13|ERF73|ERF73	6.52e-11|6.71e-11|8.87e-11|6.52e-11|6.52e-11	NM_105895|NM_130320|NM_130048|NM_105895|NM_105895	0.282

Table S2.6: List of candidate genes for each ***c*ommunity-level fungal** phenotype observed in the **endosphere**. For each gene we recorded the start and end position in bp and the chromosome(C), the phenotype that mapped to it, and the position of the SNP with the peak SNP that the gene is close to. Another column contains the names of all the blast hits to that gene that had an annotation of gene name or tair ID. The name/ID of the hits are separated by |. E-value is an indicator of the quality of the peak. For each blast hit with a gene ID, we reported the accession number of the NCBI database page. Moreover, we reported the marker effect *(ME)*. See Methods for more information.

Phenotype	**Start/End (gene)**	**C**	**SNP**	***p***	**AT_homologs**	**E_value**	**Accession_nr**	**ME**
Shannon	S:231513647 E:231519565	5	231606346	7.11	F15A17_130	2.22E-09	AL163002	0.201
Shannon	S:231513717 E:231519070	5	231606346	7.11	T21B14.24|T9I1.13|F23H6.1|At2g10780|At2g04670|T32B20.f|At2g14650	0|0|0|0|0|0|0	AC069473|AC069160|AC011621|AC006570|AC006955|AF262041|AC005398	0.201
Shannon	S:231567188 E:231567931	5	231606346	7.11	T21B14.24|T9I1.13|F23H6.1|At2g10780|At2g04670|T32B20.f|At2g14650	0|0|0|0|0|0|0	AC069473|AC069160|AC011621|AC006570|AC006955|AF262041|AC005398	0.201
Shannon	S:231570407 E:231573773	5	231606346	7.11	T18B22.40|T18B22.40	2.56e-14|2.56e-14	AL138652|AL138652	0.201
Shannon	S:231597118 E:231599514	5	231606346	7.11	T18B22.40	1.17E-32	AL138652	0.201
Shannon	S:231603520 E:231607904	5	231606346	7.11	At4g38900|At4g38900	2.31e-17|2.31e-17	AY080839|AY080839	0.201

Shannon	S:231612604 E:231618668	5	231606346	7.11	SRD2|SRD2	1.62e-36|1.62e-36	NM_102624|NM_102624	0.201
Shannon	S:231639692 E:231642413	5	231606346	7.1145	At2g06890|At2g06890|At2g06890|F5K24.1|F5K24.1|F5K24.1|F5K24.1|T21B14.24	0|0|0|0|0|0|0|0	AC005561|AC005561|AC005561|AF128395|AF128395|AF128395|AF128395|AC069473	0.201
Shannon	S:231680382 E:231683945	5	231606346	7.1145	T7M24.7|T7M24.7|T7M24.7|T32O22.19|T32O22.19|T32O22.19	0|0|0|4.22e-101|4.22e-101|4.22e-101	AF077408|AF077408|AF077408|AC079028|AC079028|AC079028	0.201
Shannon	S:231689108 E:231690289	5	231606346	7.114	At2g10780|At2g04670|T32B20.f|T4F9.40|T4F9.40|T21B14.24|F23H6.1	0|0|0|0|0|0|0	AC006570|AC006955|AF262041|AL049523|AL049523|AC069473|AC011621	0.201
Shannon	S:236359785 E:236362901	5	231606346	7.114	At3g15310|At2g13770|At2g07520	1.42e-100|2.51e-65|1.38e-45	AY050876|AC006436|AC007662	0.201
Shannon	S:236389766 E:236390945	7	236361575	9.048	At3g48980	1.12E-138	AK227663	0.246
Shannon	S:236389772 E:236390972	7	236361575	9.048	acyb-2|ACYB-2|ACYB-2|AT4g25570|CYB-1	6.16e-37|7.44e-37|7.44e-37|1.07e-35|1.6e-29	AB049628|NM_118689|NM_118689|AL161563|NM_123224	0.246
Shannon	S:236440249 E:236440812	7	236361575	9.048	acyb-2|ACYB-2|ACYB-2|AT4g25570|CYB-1	6.87e-37|7.63e-37|7.63e-37|1.07e-35|2.49e-29	AB049628|NM_118689|NM_118689|AL161563|NM_123224	0.246
Shannon	S:236441256 E:236445155	7	236361575	9.048	YUP8H12R.23	0.007	AC002986	0.246
Shannon	S:236448134 E:236448796	7	236361575	9.048	At2g45230|At2g31520|At2g25550|T6B12.3|F17A8.60|F8M12.22|At2g05200	5.39e-144|2.39e-141|8.56e-141|2.9e-138|6.63	AC002387|AC007071|AC006300|AC079679|AL049482|AF080118|AC007018	0.246

						e-129|7.12e-128|1.56e-126		
Shannon	S:236453714 E:236455757	7	236361575	9.048	At2g01550	0.023	AC005560	0.246
Shannon		7	236361575	9.048	OFP13|OFP15|At2g36050|OFP18|OFP14	6.28e-29|1.45e-12|2.08e-12|4.97e-11|3.58e-08	NM_120564|NM_129164|AK118491|NM_115114|NM_106645	0.246

Table S2.7: List of candidate genes for each **fungal** phenotype in the **rhizosphere.** For each gene we recorded the start and end position in bp and the chromosome (C), the phenotype that mapped to it, and the position of the SNP with the peak SNP that the gene is close to. Another column contains the names of all the blast hits to that gene that had an annotation of gene name or tair ID. The name/ID of the hits are separated by |. E-value is an indicator of the quality of the peak. For each blast hit with a gene ID, we reported the accession number of the NCBI database page. Moreover, we reported the marker effect *(ME)*. See Methods for more information.

Phenotype	**Start/End (gene)**	**C**	***SNP***	***p***	**AT_homologs**	**E_value**	**Accession_nr**	**ME**
Alternaria	S:58739854 E:58746773	6	58807906	9.32	NRPD2A|NRPD2A|NRPD2A	2e-102|2e-102|2e-102	NM_001203028|NM_001203028|NM_001203028	0.205
Alternaria	S:58753375 E:58753878	6	58807906	9.32	T7M24.7|T9G5.7|At2g15650|T28P6.8|T20K12.230|T15M6.14	2.29e-18|2.13e-17|2.13e-17|8.35e-17|8.43e-17|9.27e-17	AF077408|AC055769|AC006248|AC007259|AL137898|AC079604	0.205
Alternaria	S:58841762 E:58850399	6	58807906	9.32	F4M19_60|F10O5.11|At2g05640|At2g05080|At2g14470|F14G24.23	0|0|0|0|0|0	AL356013|AC027032|AC006220|AC007211|AC006067|AC019018	0.205
Alternaria	S:58870891 E:58874483	6	58807906	9.32	At2g15650|T2O9.150|T15M6.14|F11I4_21|T28P6.8|T20K12.230	1.22e-141|1.32e-137|1.34e-135|5.09e-134|2.06e-133|1.06e-132	AC006248|AL138658|AC079604|AC073555|AC007259|AL137898	0.205

Alternaria	S:58893436 E:58895280	6	58807906	9.32	T24M8.8|At2g28980|F21E10.5|F2J7.11	1.25e-71|1.52e-70|9.5e-70|4.57e-65	AF077409|AC005315|AF058914|AC079281	0.205
Alternaria	S:59521292 E:59524774	6	59614588	7.70	At2g12210|At2g14980|F28J9.11|At2g13000|T25H8.2	4.56e-131|2.1e-100|4.82e-99|6.68e-94|6.75e-93	AC005897|AC005957|AC007918|AC006437|AF128394	0.175
Alternaria	S:59527370 E:59532739	6	59614588	7.70	At2g32230|At2g32230|PRORP1|PRORP1	7.63e-73|7.63e-73|3.18e-70|3.18e-70	AC006223|AC006223|NM_001336377|NM_001336377	0.175
Alternaria	S:59626990 E:59627496	6	59614588	7.70	EBP|ERF73|ERF71|ERF73|ERF73	7.77e-06|1.83e-05|2.05e-05|1.83e-05|1.83e-05	NM_112550|NM_105895|NM_130320|NM_105895|NM_105895	0.175
Alternaria	S:59628753 E:59632029	6	59614588	7.70	At2g12210|F28J9.11|T25H8.2|At2g13000|At2g14980|T4I21.6	1.58e-179|3.86e-142|5.99e-134|1.27e-133|2.29e-130|1.78e-120	AC005897|AC007918|AF128394|AC006437|AC005957|AC022456	0.175
Alternaria	S:59693290 E:59696137	6	59614588	7.70	At3g03360|At3g03360	7.84e-08|7.84e-08	AK229563|AK229563	0.175
Alternaria	S:59701655 E:59702637	6	59614588	7.70	SDC	0.007	NM_127323	0.175
Aspergillus	S:106264311 E:106266692	3	106357256	7.01	T18F15.5|T18F15.5	4.1e-143|4.1e-143	AC084807|AC084807	0.156
Aspergillus	S:106276880 E:106277516	3	106357256	7.01	F28M20.130|REM9	5.89e-07|2.32e-05	AL031004|O81778	0.156
Aspergillus	S:106337997 E:106351431	3	106357256	7.01	F9D12.11	0.000761	AF077407	0.156
Aspergillus	S:106389026 E:106391241	3	106357256	7.01	At3g15310|F8D11.12|At2g13770|F3A14.5	2.75e-26|8.76e-16|1.3e-11|0.001	AY050876|AC035249|AC006436|AC066690	0.156
Aspergillus	S:106445494 E:106449635	3	106357256	7.01	ARF12|ARF12|ARF12|ARF12|ARF12|F23M19.4|F23M19.4|F23M19.4|F23M19.4|F23M19.4	1.74e-52|1.74e-52|1.74e-52|1.74e-52|1.74e-52|1.86e-52|1.86e-52|1.86e-52|1.86e-52	NM_103153|NM_103153|NM_103153|NM_103153|NM_103153|AC007454|AC007454|AC007454|AC007454|AC007454	0.156

Lecanicillium	S:139240476 E:139244451	4	139334984	8.06	RPM1|RPM1|RPM1|RPM1|RPP13	1.02e-25|1.02e-25|1.02e-25|1.02e-25|1.02e-25|3.96e-24	KC211311|KC211311|KC211311|KC211311|KC211311|DQ465440	0.18
Lecanicillium	S:139296794 E:139299087	4	139334984	8.06	At2g12210|At2g14980|T9I1.5|T1E4.4|F27M3_21|F27M3_21	4.12e-87|7.32e-80|1.34e-74|2.32e-73|1.04e-71|1.04e-71	AC005897|AC005957|AC069160|AC069299|AC074360|AC074360	0.18
Lecanicillium	S:139331134 E:139352012	4	139334984	8.06	T24M8.8|At2g45230|At2g45230|At2g25550|At2g25550|At2g31520|At2g31520|At2g05200	6.68e-91|6.68e-86|6.68e-86|7.67e-85|7.67e-85|2.06e-84|2.06e-84|8.73e-84	AF077409|AC002387|AC002387|AC006300|AC006300|AC007071|AC007071|AC007018	0.18
Lecanicillium	S:139352138 E:139354746	4	139334984	8.06	SERPIN1|SERPIN1|SERPIN1	1.31e-73|1.31e-73|1.31e-73	NM_103664|NM_103664|NM_103664	0.18
Lecanicillium	S:139370015 E:139384336	4	139334984	8.06	T21B14.24|T21B14.24|F23H6.1|F23H6.1|F23H6.1|T9I1.13|T9I1.13|At2g04670|At2g04670|T32B20.f	0|0|0|0|0|4.36e-173|4.36e-173|9.01e-165|9.01e-165|1.83e-153	AC069473|AC069473|AC011621|AC011621|AC011621|AC069160|AC069160|AC006955|AC006955|AF262041	0.18
Slopeiomyces	S:237634703 E:237636388	5	237729812	8.03	F12G12.9|F12G12.9|T2L5.9|T2L5.9|AT4g27210|AT4g27210	1.83e-105|1.83e-105|4.24e-100|4.24e-100|8.41e-100|8.41e-100	AC015446|AC015446|AF096371|AF096371|AL030978|AL030978	0.186
Slopeiomyces	S:237719978 E:237726084	5	237729812	8.03	RABA3|RABA3|RABA4a|RABA4a	4.27e-44|4.27e-44|6.25e-39|6.25e-39	NM_100002|NM_100002|NM_125925|NM_125925	0.186
Slopeiomyces	S:237724740 E:237728916	5	237729812	8.03	ndhD|ndhD|ndhD|ndhD	2.7e-14|2.7e-14|2.7e-14|2.7e-14	MK380720|MK380720|MK380720|MK380720|MK380720	0.186
Slopeiomyces	S:237729416 E:237730443	5	237729812	8.03	AT4g24080|T19F6.1|ALL1	1.28e-56|3.7e-55|2.38e-54	AL109619|AC002343|NM_118540	0.186
Slopeiomyces	S:237731799 E:237733062	5	237729812	8.03	RMR1|RMR1	0.006|0.006	NM_203272|NM_203272	0.186
Slopeiomyces	S:237753267	5	237729812	8.03	At4g24090|At4g24090|AT4g24	1.56e-27|1.56e-	BT004223|BT004223|AL109619|AL109619	0.186

	E:237759305			090|AT4g24090	27|3.85e-26|3.85e-26		
Slopeiomyces	S:237766144 E:237768348	5	23772981 2	8.03	EMB2024	8.64E-55	NM_122348	0.186
Slopeiomyces	S:237777024 E:237779629	5	23772981 2	8.03	EMB2024|EMB2024	3.51e-48|3.51e-48	NM_122348|NM_122348	0.186
Trichoderma	S:246427580 E:246435119	5	24652995 4	7.11	T7M24.7|T7M24.7|T7M24.7|T7M24.7|T7M24.7	0|0|0|0|0	AF077408|AF077408|AF077408|AF077408|AF077408	0.164
Trichoderma	S:246483621 E:246486030	5	24652995 4	7.11	AT2G20930|AT2G20930	2.01e-13|2.01e-13	AK318632|AK318632	0.164
Trichoderma	S:246486395 E:246488898	5	24652995 4	7.11	At2g20950	3.55E-05	AC006264	0.164
Trichoderma	S:246495360 E:246495989	5	24652995 4	7.11	At1g10160	5.91E-12	AK229033	0.164
Trichoderma	S:246496695 E:246498464	5	24652995 4	7.11	At2g23880|F19C24.27|At2g28980|T6B12.3	4.18e-49|3.1e-48|3.11e-47|1.53e-45	AC005170|AC025294|AC005315|AC079679	0.164
Trichoderma	S:246499124 E:246502738	5	24652995 4	7.11	At5g66090/K2A18_17|At5g66090/K2A18_17|At5g66090/K2A18_17|At5g66090/K2A18_17	9.45e-22|9.45e-22|9.45e-22	AK118734|AK118734|AK118734|AK118734	0.164
Trichoderma	S:246524478 E:246525629	5	24652995 4	7.11	At2g31080|At2g07650|T24M8.8|At2g31520|At2g41580	5.32e-20|3.82e-18|6.75e-17|7.03e-17|8.24e-17	AC005311|AC007730|AF077409|AC007071|AC002510	0.164
Trichoderma	S:246577359 E:246578727	5	24652995 4	7.11	At2g04230	4.06E-09	AC007213	0.164

Table S2.8. List of candidate genes for each **community-level fungal** phenotype observed in the **rhizosphere.** For each gene we recorded the start and end position in bp and the chromosome (C), the phenotype that mapped to it, and the position of the SNP with the peak SNP that the gene is close to. Another column contains the names of all the blast hits to that gene that had an annotation of gene name or tair ID. The name/ID of the hits are separated by |. E-value is an indicator of the quality of the peak. For each blast hit with a gene ID, we reported the accession number of the NCBI database page. Moreover, we reported the marker effect (ME). See Methods for more information.

Phenotype	Start/ End (gene)	C	SNP	*p*	AT_homologs	E_value	Accession_nr	ME
Shannon	S:26972071 E:26973369	2	27025048	7.58	At2g15410|At2g15100|At2g13230|At2g14400|At2g13020	1.35e-70|1.71e-64|5.12e-52|6.6e-45|4.72e-41	AC006920|AC005957|AC006446|AC006067|AC006437	0.168
Shannon	S:26981796 E:26986343	2	27025048	7.58	At2g10780|At2g10780|At2g10780|At2g10780|At2g10780|dl4485c|At2g04670|At2g04670|At2g04670|At2g04670	0|0|0|0|0|0|0|0|0|0	AC006570|AC006570|AC006570|AC006570|AC006570|Z97342|AC006955|AC006955|AC006955|AC006955	0.168
Shannon	S:27009698 E:27025626	2	27025048	7.58	At2g13370|At2g13370|At2g13370|At2g13370|At2g13370|At2g13370|At2g13370|At2g13370|At2g13370	3.94e-65|3.94e-65|3.94e-65|3.94e-65|3.94e-65|3.94e-65|3.94e-65|3.94e-65|3.94e-65	AK221299|AK221299|AK221299|AK221299|AK221299|AK221299|AK221299|AK221299|AK221299	0.168
Shannon	S:33363698 E:33365214	4	33640 9963	7.25	EDF3|ERF71|RAV1|ERF73|ERF73|ERF73	2.4e-06|3.3e-06|1.16e-05|1.26e-05|1.26e-05|1.26e-05	NM_113472|NM_130320|NM_101197|NM_105895|NM_105895|NM_105895	0.168
Shannon	S:33659695 E:33663930	4	33640 9963	7.25	At2g31520|At2g25550|At2g45230|At2g01840|F8M12.22|T6B12.3|At2g05200	2.99e-162|1.63e-161|2.31e-154|2.42e-144|1.01e-140|3.49e-	AC007071|AC006300|AC002387|AC007069|AF080118|AC0	0.168

						138|1.17e-137	79679|AC007018	
Shannon	S:336474566 E:336483046	4	336409963	7.25	T16L1.260|T16L1.260|T16L1.260|T16L1.260	5.83e-22|5.83e-22|5.83e-22|5.83e-22	AL031394|AL031394|AL031394|AL031394	0.168
Shannon	S:336494242 E:336495139	4	336409963	7.25	T16L1.260|T16L1.260|T16L1.260	2.24e-21|2.24e-21|2.24e-21	AL031394|AL031394|AL031394	0.168
Shannon	S:342593127 E:342598014	2	342620055	8.13	At2g17420|At2g17420|NTRA|NTRA|NTRA|NTRA	4.51e-105|4.51e-105|4.66e-105|4.66e-105|4.66e-105|4.66e-105	AK226480|AK226480|NM_127297|NM_127297|NM_127297|NM_127297	0.185
Shannon	S:342677537 E:342679140	2	342620055	8.13	At2g05960|At2g05960|T15M6.14|T15M6.14	7.21e-59|7.21e-59|2e-57|2e-57	AC005970|AC005970|AC079604|AC079604	0.185
Shannon	S:342690521 E:342711284	2	342620055	8.13	T7M24.7|T32O22.19|F19K19.5|At2g16000|F28L22.3|F28L22.3	0|2.82e-160|5.1e-128|7.82e-126|2.42e-122|2.42e-122	AF077408|AC079028|AC011808|AC007134|AC007505|AC007505	0.185
Shannon	S:342712663 E:342717378	2	342620055	8.13	T4P3.8|T4P3.8|ARP6|ARP6|ARP6|ARP6|At3g33520|At3g33520	4.72e-77|4.72e-77|4.82e-77|4.82e-77|4.82e-77|4.82e-77|8.02e-77|8.02e-77	AC009992|AC009992|AF507914|AF507914|AF507914|AF507914|AY050786|AY050786	0.185
Shannon	S:342718193 E:342721183	2	342620055	8.13	ACBP1|ACBP1|ACBP1|ACBP1	1.37e-24|1.37e-24|1.37e-24|1.37e-24	NM_124726|NM_124726|NM_124726|NM_124726	0.185

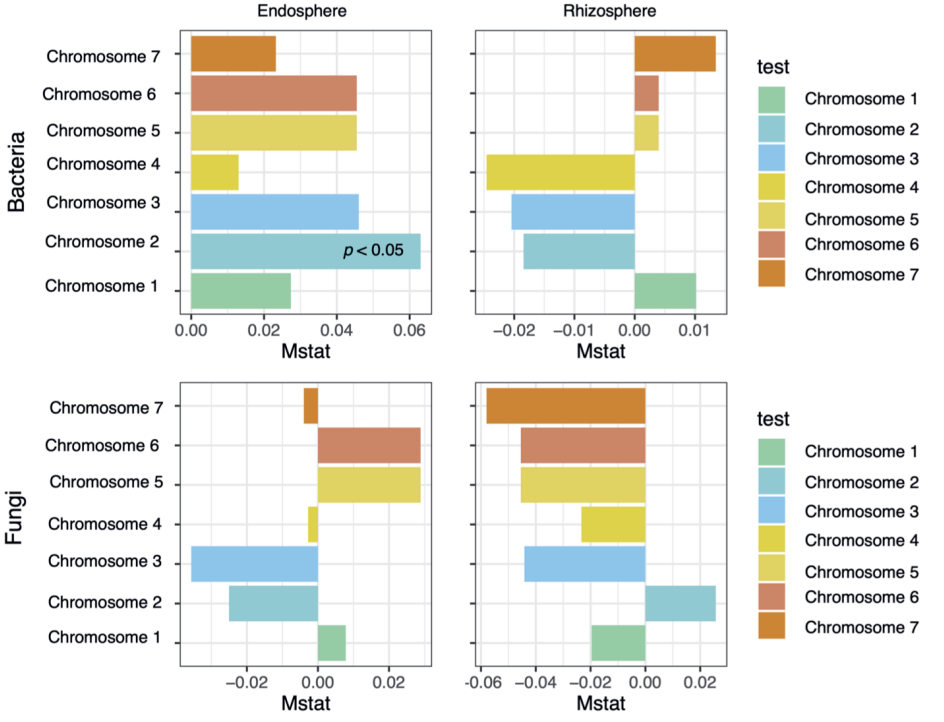

Figure S2.1. Mantel's R statistic plotted for each chromosome across all 154 accessions indicating the degree of correlation between host genotypic distance and bacterial and fungal distance in root endosphere and rhizosphere.

Chapter 3

Inter- and intraspecific plant-soil-feedbacks of grass species

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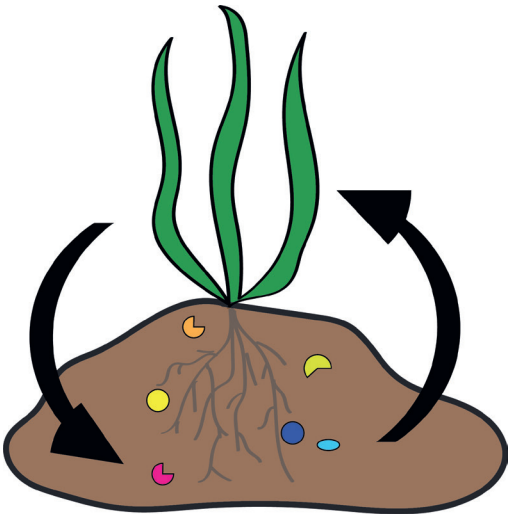

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Abstract

Plants continuously interact with soil microbiota. These plant-soil feedbacks (PSFs) are considered a driving force in plant community dynamics. However, most PSF information comes from inter-family studies, with limited information on possible causes. We studied the variation of PSFs between and within grass species and identified the soil microbes that are associated with the observed PSFs effects. We grew monocultures of ten cultivars of three grass species (*Lolium perenne, Poa pratensis, Schedonorus arundinaceus*) using a two-phase PSF experiment. We measured plant total biomass to determine PSFs between and within species and correlated it with sequenced rhizosphere bacteria and fungi.In the soil conditioning phase, grass species developed microbial legacies that affected the performance of other grass species in the feedback phase. We detected overall negative interspecific PSFs. While we show that *L. perenne* and *P. pratensis* increased their performance respectively in conspecific and heterospecific soils, *S. arundinaceus* was not strongly affected by the legacies of the previous plant species. Contrary to our expectation, we found no evidence for intraspecific variation in PSFs. Bacterial taxa associated with PSFs included members of *Proteobacteria, Firmicutes, Verrucomicrobia* and *Planctomycetes* whereas fungal taxa included members of *Ascomycota.* Our results suggest differences in PSF effects between grass species, but not between cultivars within species. Thus, in the studied grass species, there might be limited potential for breeding on plant traits mediated by PSFs. Furthermore, we point out potential microbial candidates that might be driving the observed PSF effects that could be further explored.

Introduction

Interactions between plants and soil microorganisms are important determinants of plant performance. The influence that plants exert on microorganisms generates microbial legacies in the soil that, in turn, affect the performance of other plants growing later in that soil, which is called plant-soil feedback (PSF) (Bever, Westover, and Antonovics 1997; Ehrenfeld, Ravit, and Elgersma 2005; Kulmatiski and Kardol 2008; van der Putten et al. 2013). Plant-soil feedbacks can be considered positive when plant growth is promoted, negative when plant growth is reduced, and neutral when there is no impact on plant growth. Plant-soil feedback effects are net effects, and their direction and strength depend on the ratio between pathogenic and beneficial microorganisms and the physicochemical properties of the soil, including nutrient availability, soil moisture level, and soil structure (Cong et al. 2015; Cavagnaro 2016; De Deyn, Quirk, and Bardgett 2011; Metcalfe, Fisher, and Wardle 2011; van der Putten et al. 2013). Negative PSFs play key roles in plant community dynamics such as maintaining plant species diversity and rarity by reducing the abundance of dominant and subordinate plant species (Klironomos 2002). The specificity of PSF effects is most often studied at the species level, comparing how soil microorganisms associated with one plant species affect growth of the same (conspecific) or of different (heterospecific) species. However, PSFs may also be influenced by the genetic variation within species, named intraspecific variation (Lau and Lennon 2011; Wagg et al. 2015; Allen et al. 2017; Bukowski and Petermann 2014).

Intraspecific variation in PSFs may drive the selection of microbiome-mediated plant traits and determine consequent adaptation of natural populations (Maron et al. 2011, 201; Bolnick et al. 2011; Maron et al. 2016). Furthermore, these interactions may be of agronomic interest and utilized for selective breeding. For instance, negative feedbacks caused by a build-up of soil pathogens, could lead to selection of traits that diminish negative feedbacks, such as the promotion of mutualistic associations with soil organisms or resistance to soil-borne pathogens. Interest in PSFs within species has recently increased and a growing body of literature has shown intra-specific differences in PSFs effects. For instance, studies on the model species *Arabidopsis thaliana* showed that negative PSF effects depended on which accession had previously occupied the soil (Aguilera et al. 2011; Bukowski and Petermann 2014). Differences in PSFs within species have been documented also in *Plantago lanceolata* (Kirchhoff et al. 2019) and *Trifolium pratense* (Wagg et al. 2015). However, studies have mainly focused on forbs, and the intraspecific variation for plant-soil feedbacks in other plant taxonomic groups remain still unresolved.

Similar to PSFs between species, the degree of variation within species may differ among plant taxonomic groups (Bukowski and Petermann 2014; Cortois et al. 2016; Heinen et al. 2018; De Long et al. 2021). Thus, the patterns observed in forbs might very well differ from, for example, grasses. So far, little is known about the specificity of PSFs within grass species. Recent studies have investigated this in wetland grasses. For instance, studies on *Phragmites australis* showed that invasive plants may have an advantage over other plant species driven by soil legacies (Allen et al. 2017; 2018; 2020). A better understanding of the role of plant genetic diversity in grass-soil-microbiome interactions might help to slow

down the accumulation of soil-borne pathogens since grasses, especially in current livestock farming systems, are often grown in monocultures.

A significant challenge in studying the specificity of PSFs, is to identify the microbial taxa that are responsible for the feedback effects. High-throughput amplicon sequencing has enabled the characterization of the composition of microbial communities and derived functional approaches such as FUNGuild can be used to classify microbial species into functional categories (Nguyen et al. 2016; Zanne et al. 2020). Moreover, studying individual microbial taxa may also be relevant to identify which microbes may contribute most to the observed effects (Cortois and De Deyn 2012; Putten et al. 2016b). Most of this research has focused on differences in microbial community composition *between* plant species, whereas little is known on differences *within* plant species. To our knowledge, there are hardly any studies that have focused on differences within terrestrial grasses with respect to their effects on soil microbial community composition.

We measured the strength and direction of PSFs between and within three grass species: *Lolium perenne, Poa pratensis*, and *Schedonorus arundinaceus*. These are all perennial grass species that grow in a wide range of habitats and are known to engage in interactions with beneficial microorganisms (Saikkonen et al. 2016). All three species are widely used by grass breeding companies as forage and turf grasses due to their high nutritional properties, high productivity, and tolerance to abiotic stressors. To examine variations in PSFs within each species, we conducted a PSF experiment using multiple commercial cultivars of each grass species, and correlated the variation in PSFs to variations in the soil microbes based on sequencing. We then tested: (1) whether

rhizosphere bacterial and fungal community composition differ between grass species and intraspecific cultivars of each of the species (2) whether there are differences in PSFs between species and between cultivars of the same species and in what direction (3) what are the microbes that are associated with the observed PSFs effects.

Material and methods

Plant material and germination

We used ten commercial cultivars used for turf for each of the three perennial grass species (*Lolium perenne, Poa pratensis*, and *Schedonorus arundinaceus*). The plant material was provided by the Barenbrug grass seed company research facility (Wolfheze, The Netherlands) (Table S1). The cultivars used for *L. perenne* and *S. arundinaceus* show some level of segregating genetic variation within cultivars whereas cultivars used for *P. pratensis* are single genotypes, owing to apomictic reproduction of *P. pratensis*. Seeds were surface-sterilized by washing them for one min in commercial bleach (< 5%) and 0.1 % Tween-20 solution. After rinsing three times for one minute with demi-water, seeds were germinated on sterilised glass beads in a germination cabinet at 20°C (*L. perenne* and *S. arundinaceus*) and 15°C (*P. pratensis*). One week after germination, the seedlings were stored at 4 °C for a week under continuous light conditions until the start of the experiment.

Experimental design

The PSF experiment consisted of two phases, conditioning, and feedback phase (Figure 1). The conditioning phase was started from a mixture of 90% sterilised background soil and 10% live inoculum soil. The background soil was sandy loam soil collected from a former agricultural

field abandoned in 1995 close to Barenbrug research facility (Mossel, The Netherlands; N 52.06141, E 5 ° 75.266). It was sterilised by gamma-sterilization (25 KGray, Syngenta by Ede, The Netherlands). The inoculum soil was collected from field plots where the same species and 30 cultivars were growing in monocultures at the Barenbrug grass seed company research facility (Wolfheze, The Netherlands). To capture all microbial diversity, the sampling consisted of 150 sub-samples (5 soil samples for each of 30 cultivars monocultures) that were all pooled together and homogenised to generate a single inoculum soil mixture. The conditioning phase followed a randomised block design with five replicated blocks (each block has 1 replicate per variety). Per replicate block, 38 pots of 4L were filled with a mixture of 3.78 kg of sterilised background soil and 0.42 kg of sieved (1cm diameter) of inoculum soil. In each pot, 15 plants were planted in monoculture, and for each replicate block, eight pots were left unplanted to be used as controls, (called hereafter "unconditioned soil"). The experiment was performed in a climatised greenhouse at 16/8 h light/dark and 20/15 °C day/night conditions. During the last four weeks, all pots received a weekly amount of 10 ml 5% Hoagland solution to avoid nutrient deficiency. Pots were weighted two times per week to adjust soil moisture to 15% (w/w). After 20 weeks of growth, the above-ground biomass was clipped, dried at 60 °C until constant weight, and weighed, whereas roots were chopped in ~ 2cm pieces and homogenised with the soil and thus used as a source of microbial inoculum.

In the feedback phase, soil from each conditioning phase pot was individually transferred to four 1L pots filled with 920 g soil on a dry weight basis. Every pot, irrespective of having conditioned or unconditioned soil, was planted with three cultivars of one of the three

species in monoculture following a partial factorial design in a replicated block design with five replicates. Every cultivar was tested on the following soil treatments: (1) unconditioned soil (conditioning phase control soil), (2) conditioned by a different cultivar of the same species, (3) conditioned by the same cultivar, (4 and 5) conditioned by a cultivar of either one or the other grass species. For treatments 2, 4, and 5, we tested each cultivar on only one of the available cultivar-conditioned soils and not on all possible soils. For instance: to test the growth of a cultivar in soil that was conditioned by another cultivar of the same species, nine possible soils could be used; we choose only one of those (Figure S3.1). The design that we used for pairing conditioned soils to feedback plants ensured that all 30 cultivars contributed equally to soil feedback effects in the overall experiment. The experiment was performed in a climatised greenhouse at 16/8 h light/dark and 20/15 °C Day/night conditions, and the same watering regime was applied as in the conditioning phase to maintain a moisture content of maximally ~ 15% (w/w). Every week, all pots received 10 ml of 5% Hoagland. After eight weeks of plant growth, shoots were clipped, dried at 60 °C until constant weight, and weighed, whereas roots were first washed and then dried at 60 °C, and weighed to determine biomass.

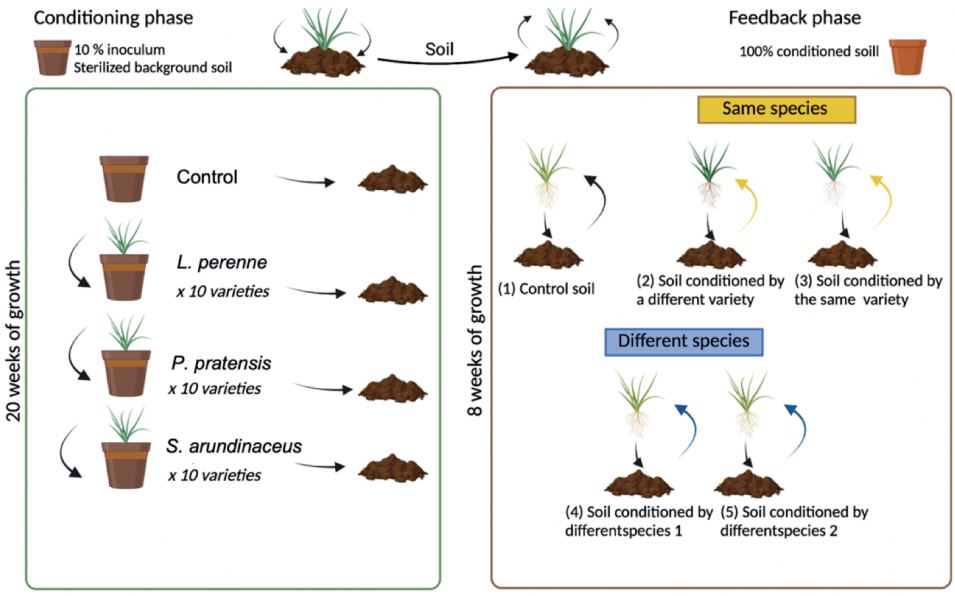


Figure 3.1. Experimental design. In the conditioning phase, 10 cultivars of each of *L. perenne*, *P. pratensis* and *S. arundinaceus* species were grown in soil composed of 90% sterilized background and 10% live inoculum soil. In the control treatment, pots were filled with the soil mixture, but no seedlings were planted. After 20 weeks, rhizosphere samples were collected for the sequencing of bacterial and fungal communities. The aboveground biomass was collected whereas the roots were left in the soil after chopping. In the feedback phase, new seedlings were planted into unconditioned (control) soil (1), soil conditioned by different cultivars of the same species (2), soil conditioned by the same cultivars of the same species (3), soil conditioned by a different species 1 (4) and, soil conditioned by different species 2 (5). After 8 weeks of growth, total biomass was determined and used to calculate feedback effects.

Microbial DNA extraction and sequencing

DNA was extracted from 0.25 g of rhizosphere soil collected at the end of the conditioning phase using the Power Soil DNA extraction kit (Qiagen, Hilden, Germany). Bacterial and fungal DNA was amplified using respectively the primers 515F/806R targeting the V4 region of the 16S rRNA gene (Caporaso et al. 2012; A Apprill et al. 2015). For fungi, ITS4ngs and ITS3mix targeting the ITS2 region of fungi were used

(Tedersoo et al. 2015b). Preparation of libraries and sequencing (Illumina MIseq PE 250) were performed at McGill University and the Génome Québec Innovation Centre (Canada).

Data analysis

The variation of PSFs was analysed by subjecting the biomass data from the feedback phase to a two-way ANOVA. To fulfil requirements of normality, plant biomass was log-transformed prior to analyses. To test if soil legacies generated in the conditioning phase affected the responding plants in the feedback phase, we used a linear mixed model [lmer function in R - lme4 package (Bates et al. 2015)] to model the effects of plant species, plant cultivars, soil treatments (unconditioned soil, conditioned by a different cultivar, conditioned by the same cultivar, conditioned by different species 1 and conditioned by different species 2), block, and the interactions between soil treatments and cultivars and soil treatment and plant species on plant biomass. Because a single pot in the conditioning phase contributed soil for four pots in the feedback phase, conditioning phase pot was included in the model as a random factor. The factors soil treatments, cultivar, their interactions, and block were considered fixed effects. Tukey's HSD post hoc tests were performed to guide interpretation of significant main effects and interactions. To address how soil microbial legacies generated in the conditioning phase affected the responding grass species in the feedback phase, we excluded the unconditioned soil and used a linear mixed model per each species (lmer in R) to model the effects of plant cultivars, soil treatments, block, conditioning phase pot, interactions between soil treatments (conditioned by a different cultivar, conditioned by the same cultivar, conditioned by different species 1 and conditioned by different species 2) and cultivars

on plant biomass. The aboveground biomass produced in the conditioning phase was used as a cofactor to account for possible effects of conditioning plant size differences on conditioned soils, which might have contributed to nutrient depletion. Tukey's HSD post hoc tests were performed for biomass data and soil treatments to highlight significant differences between treatments.

The raw 16S and ITS sequence reads were analysed using Dada2 (v. 1.12) (Callahan, 2016) and Pipits (v. 2.3) pipelines (Gweon et al. 2015). The SILVA (v.132) database was used to classify bacteria whereas the UNITE (v. 8.0;) database (Abarenkov et al. 2010) was used for the identification of fungi, and the ITSx extractor was used to extract fungal ITS regions. FUNGuild (Nguyen et al. 2016) was used to classify fungal operational taxonomic units (OTUs) into potential functions, and the assignment was further curated using an in-house database (Hannula et al. 2017). The OTUs were grouped into saprotrophs, plant pathogens, plant endophytes, arbuscular mycorrhizal (AMF), and others (i.e., fungal/animal-plant pathogens). In the case of uncertain fungal guilds, combination assignments (such as saprotroph – plant pathogen) were done. All reads not belonging to bacterial or fungal kingdoms were excluded from the datasets. To normalize our data, we followed a compositional approach (Gloor et al. 2017) using the Total-Sum Scaling (TSS). To test the central hypothesis of the effects of plant species and the plant cultivar on soil microbial community structure, a PERMANOVA model was constructed using Bray-Curtis distances [vegan package in R (Oksanen et al. 2013)]. To assess the total variation explained by a variable in the model, we used the R^2 values derived from the model. Non-metric multidimensional scaling (NMDS) ordination was used to visualise the effects of plant species and cultivar within species on microbial community structure. To

explore possible effects of relative abundance of microbial taxa on variation in PSFs effects, linear mixed models (lmer in R) were used to predict total plant biomass at the end of the feedback experiment from the relative abundance of each fungal and bacterial family quantified at the end of the conditioning phase. The model was built for each grass species where soil treatments, cultivar and block were set as fixed factors, whereas conditioning phase pot was used as a random factor and the relative abundance of each fungal and bacterial family as a covariate. To achieve normality of residuals, a Hellinger transformation was used. To maintain a low chance of making type I errors and therefore avoid false discoveries, we implemented the false discovery rate (FDR) approach with an FDR threshold for significance of 0.05 (pdjust function with FDR method in R) (Benjamini and Hochberg 1995; Verhoeven, Simonsen, and McIntyre 2005).

Results

Rhizosphere community composition between and within grass species

NMDS visualised whether the three grass species accumulated different bacterial (16S rRNA) and fungal (ITS) rhizosphere communities. The structure of both bacterial and fungal communities was shaped by plant species that explained approximately 4% of the variation in the bacterial community and 6% of the variation in fungal community structure. When only fungi assigned to major guilds were included, 9% of the variation was explained (Figure 3.2). However, plant cultivar did not significantly explain compositional differences of bacteria (R^2 = 0.19; p >0.05 in *L. perenne*, R^2 = 0.27, p >0.05 in *P. pratensis*, R^2 = 0.22, p >0.05 in *S. arundinaceus*), and fungi (R^2 = 0.19; p >0.05 in *L. perenne*, R^2 = 0.28, p >0.05 in *P. pratens,* R^2 = 0.19, p >0.05 in *S. arundinaceus*) (Figure S3.5). Therefore,

overall soil microbial communities in general were not differently conditioned by individual cultivars of the same grass species.

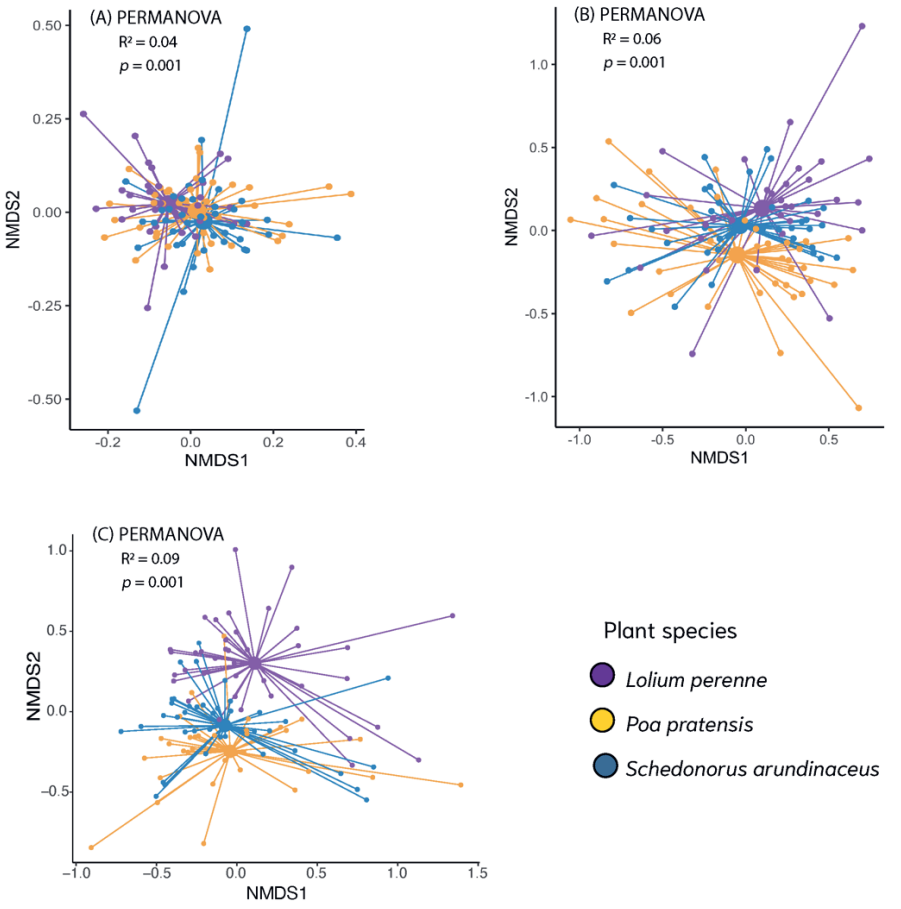


Figure 3.2. NMDS based on Bray Curtis distances visualize the effects of plant species on (A) bacterial and (B) fungal community structure, and (C) on fungal guilds. Centroids are shown as large dots and the individual plants are displayed with small dots.

Interspecific plant- soil feedbacks

To test whether soil legacies generated in the conditioning phase affected the responding plants in the feedback phase, we compared plant biomass generated in conditioned soils versus unconditioned soils. We detected overall negative PSFs (Figure S3.2). Specifically, biomass production was dramatically decreased when growing in conditioned soils compared to unconditioned soils both when plants were tested on soil conditioned by the same or by a different species. To address how soil microbial legacies generated in the conditioning phase affected the responding grass species in the feedback phase, we compared plant biomass production in conditioned soils and detected an overall significant soil treatment effect on plant biomass (Table S3.2). Further study indicated that these effects are not caused by intraspecific soil differences (no significant differences between soil conditioned by different or same cultivar in any of the species; see Figure 3.3). Instead, the strongest effects were caused by interspecific PSFs. *Lolium perenne* plants produced more biomass in conspecific soils compared to heterospecific soils (Figure 3.3A). The pattern of enhanced performance in conspecific soils was not observed in *P. pratensis* and *S. arundinaceus*. In fact, *P. pratensis* produced most biomass in heterospecific soils and particularly in soil conditioned by *L. perenne* (Figure 3.3B) whereas *S. arundinaceus* did not show a strong preference between conspecific and heterospecific soils (Fig. 3.3C).

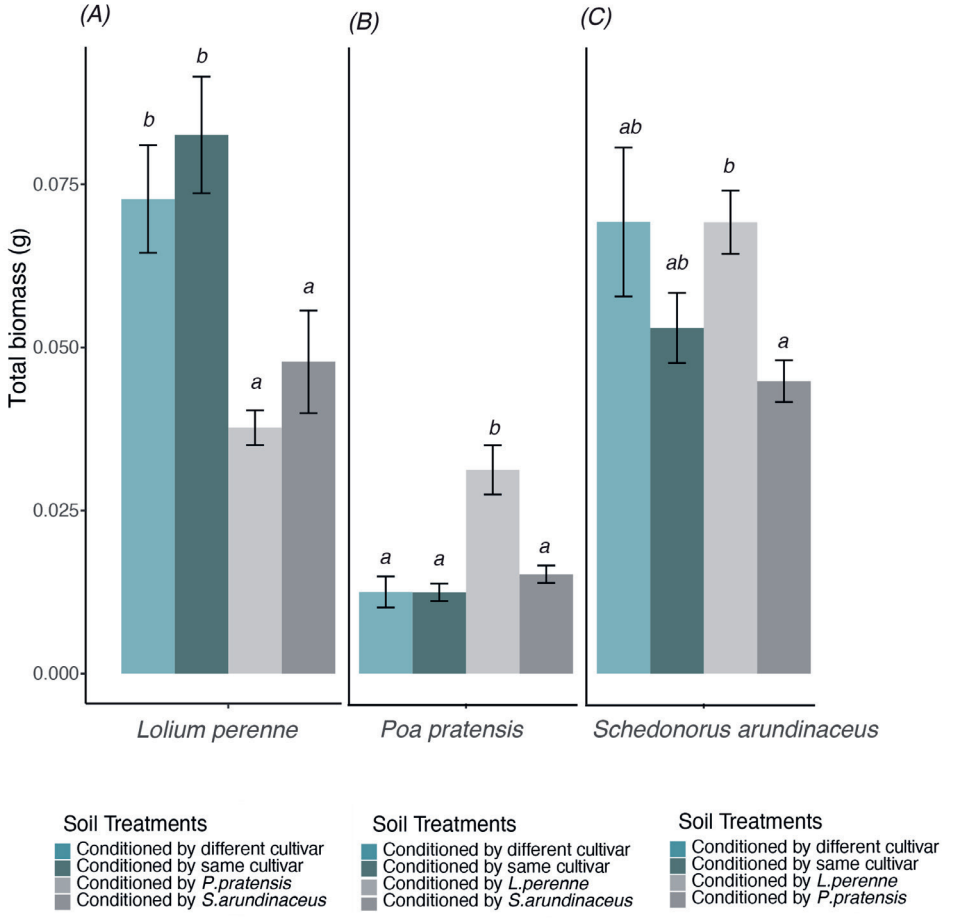


Figure 3.3. Total biomass (g) in the feedback phase of (A*) Lolium perenne*, (B) *Poa pratensis*, and (C) *Schedonorus arundinaceus* grass species when grown on conditioned soils: conditioned by a different cultivar of the same species (light blue), conditioned by same cultivar (dark blue), conditioned by different species 1 (light grey), conditioned by different species 2 (dark grey). Bars and whiskers represent log10 transformed biomass + SE using a linear mixed model. Tukey's HSD post hoc tests are performed for biomass data of each species and soil treatments.

Intraspecific plant-soil feedbacks

Within species, different cultivars did not cause significant differences on each other's biomass production through soil conditioning (soil treatments x plant cultivar p >0.05; Table S3.2). To test if the disproportionate biomass in the unconditioned soil might have influenced findings of the treatment comparisons, we also did our tests without the unconditioned soils. Nevertheless, also when using a statistical model per each species where the unconditioned soil had been excluded, no soil treatments x cultivar effect was detected (Table S3.3). Thus, we have no evidence for intraspecific specificity in PSFs.

Correlations between plant performance and microbial taxa.

We explored the relationship between the relative abundances of individual microbial taxa at the end of the conditioning phase and plant growth in the feedback phase. When examining bacteria, we found significant correlations only in *P. pratensis*, and identified mainly positive correlations between plant biomass and relative abundance of the *Opituraceae* family (*Verrucomicrobia* phylum), CPla.3_termite_group family (*Planctomycetes* phylum), *Rhodospirillaceae* and *Steroidobacteraceae* families *(Proteobacteria* phylum). We observed a negative correlation between plant biomass and relative abundance of *Planococcaceae* family (*Firmicutes* phylum) (Figure S3.3). When examining fungi, we found significant correlations between plant total biomass of each grass species and the relative abundance of families belonging to the *Ascomycota* phylum. Specifically, in *L. perenne* we found a negative correlation with the relative abundance of *Nectriaceae* fungal family, in *P. pratensis* we found a positive correlation with the relative abundance of the fungal family *Magnaportaceae,* and in *S. arundinaceus*

the relative abundance of the *Didymosphaeriaceae* and *Leptosphaeriaceae* families was positively correlated with plant biomass.

Discussion

Our results confirm that grass species generate microbial legacies in the soil that, in turn, affect the performance of other grass species growing later in these soils. Thus, plant-soil feedbacks (PSFs) occur among members of the grass family. We detected strong negative PSF effects between species. Furthermore, while comparing the effects of conditioned soils on plant biomass production in each species, we demonstrate that *L. perenne* enhanced its performance on conspecific soils, *P. pratensis* produced most biomass in heterospecific soils, whereas *S. arundinaceus* was not strongly impacted by the legacies of the previous plant species. Within species, the composition of the microbiome did not vary across cultivars during the conditioning phase, and we did not find evidence for intraspecific variation in PSFs.

Lolium perenne, P. pratensis and, S. arundinaceus created specific rhizosphere microbiomes that contributed to variations in the performance of the plants in the feedback phase. Specifically, we showed that the presence of live soil communities led to strong negative feedbacks in each of the three grass species compared to the unconditioned soils. Plant biomass was substantially reduced in conditioned compared to the unconditioned soil, irrespective of which plant species had conditioned the soil. These results suggest that negative interactions may have affected plant performance more strongly than positive interactions. This is in line with previous research that has shown that in similar grassland ecosystems negative interactions dominated by pathogenic microorganisms drive plant community

dynamics (Heinen et al. 2020; Hannula et al. 2021). However, in our approach the substantial difference between the unconditioned and the conditioned soils overwhelmed all other effects. Therefore, the question of whether soil microbes or nutrients might have caused the negative effect when compared to the unconditioned treatment cannot be answered unequivocally. While we aimed to minimize different availabilities of nutrients by providing external nutrients during plant growth in the conditioning phase, it cannot be excluded that nutrient depletion of the soils in the conditioning phase has contributed to a negative growth effect of conditioned soils relative to unconditioned soils, which received nutrients as well. Therefore, it appears that differences between unconditioned and conditioned soils will have been caused by a combination of nutrient limitation and negative biotic interactions. However, when using the plant biomass of the conditioning phase as covariate in our statistical model, we observe no significant effect suggesting that nutrient depletion might not be a dominant factor.

The consequences for the performance of a specific plant species may depend on whether the soil has a predominant legacy of its own (conspecific), or of other (heterospecific) plant species. There is increasing awareness that most plant species show enhanced performance when growing in soil with a legacy of heterospecific plants relative to soil with a legacy of conspecific plants (Kulmatiski et al. 2008). This appears to be especially true for grass species. However, in the present study, the biomass production of *P. pratensis* was promoted only by the soil of *L. perenne* (heterospecific), whereas biomass production was negatively affected by conspecific soils. This suggest that within a grassland community, *P. pratensis* may be advantaged not only by PSFs from the other grasses (positive heterospecific feedback), but also by generating

negative feedbacks towards other grass species (negative heterospecific feedback). Nevertheless, *P. pratensis* was less productive than the other species because it created soils that decreased its own growth (negative conspecific feedback), which might not be suitable for breeding purposes. While negative conspecific feedbacks maintain plant diversity in grassland ecosystems, they could also reshuffle plant spatial distribution (in 't Zandt et al. 2021). In fact, it had been shown that some grass species may escape soil-borne pathogens overcoming negative effects on plant growth by occupying different soil patches overtime (Vincenot et al. 2017; Real and McElhany 1996; Thakur et al. 2021).

Interestingly, while the feedbacks observed in *P. pratensis* support previous findings on other grass species, *L. perenne* species did not support this general pattern, as it produced most biomass in conspecific soils. This might suggest that in *L. perenne*, beneficial microorganisms such as mutualists or plant growth-promoting rhizobacteria had a relatively greater impact on plant performance than pathogens. These findings suggest that the PSFs might allow *L. perenne* to thrive in monoculture. However, we cannot exclude that *L. perenne* could be disadvantaged in a diverse community as its microbiome might enhance biomass production of other grass species. In order to assess consequences of PSFs for grass community dynamics in more detail, further studies are needed comparing plant-soil community effects to individual effects (Van der Putten and Peters 1997). Similar studies may also help to test consequences for plant community overyielding in grass mixtures compared to a single grass species (Maron et al. 2011).

Schedonorus arundinaceus did not exhibit a strong preference towards conspecific or heterospecific soils, suggesting potential neutral PSFs. As

a result of neutral PSF, *S. arundinaceus* species may be effective competitors in grassland ecosystems, which may eventually lead to a plant's tolerance towards a wide range of biotic environmental factors, such as damage by aboveground herbivores (Fraser and Grime 1998). However, when comparing heterospecific soils, it matters what species had previously conditioned the soil. In fact, *S. arundinaceus* produced more biomass in soil conditioned by L. *perenne* than in soil conditioned by *P. pratensis.*

To explore the potential underlying causes, we correlated the relative abundances of individual microbial taxa at the end of the conditioning phase with the plant biomass production of the feedback phase. Such correlations between relative abundance and plant biomass might identify microbial taxa that are candidates for driving the observed PSF effects. We demonstrated that the relative abundance of the *Necritriaceae* fungal family, which include well known plant pathogens, is negatively correlated with plant biomass production of *L. perenne* but not with the other two species. Possibly, *Necritriaceae* could be specialized pathogens of *L. perenne* that hardly interfere with plant growth of other plant species. In the other two grass species we observed positive correlations between the relative abundance of specific fungal families and plant biomass production. Some of the families, such as the *Magnaporthaceae* family, have been identified in previous studies (Hannula et al. 2021). Although *Magnaporthaceae* include many pathogenic members it is possible that non-target pathogens have relatively positive effects on plant growth (Cortois et al., 2016), or that the plants were protected by symbionts which allowed the pathogens to multiply without harming the plants.

We also observed positive correlations between bacterial members of *Proteobacteria* phylum and the biomass of *P. pratensis*. The *Rhodospirillaceae* family, for example, include members with the ability to colonize the roots and promote plant growth and development (Chabot et al. 1996; Antoun et al. 1998). We identified members of the *Firmicutes* phylum which are known to be part of the plant growth promoting rhizobacteria (PGPR) community and although several studies show their involvement in promoting plant growth, we found a negative correlation with the plant biomass production. We cannot exclude that these bacterial families include members that could indirectly generate negative effects on plant performance. We acknowledge that correlations do not imply causality and therefore further tests with the bacterial and fungal taxa identified are needed to tease biological effects apart from nutrient depletion and unravel further potential microbial candidate of PSFs.

Grass cultivars did not create a clear distinct rhizosphere microbiome and consistently, the soil communities generated by different grass cultivars at the end of the conditioning phase did not differentially affect the growth of the cultivars in the feedback phase. This is in contrast with previous studies that showed, for instance in the model species *A. thaliana*, that genotypes differed in the direction and strength of feedback due to genotype-specific soil communities. Here, we used ten cultivars per each species, and despite this being a relatively high number compared to other studies, the genetic differences between the cultivars might not have been large enough to cause differences in plant growth. To minimize possible bias in assessing PSF effects within species relative to the PSF effects between species, we did not select the cultivars

based on previous existing knowledge of growth difference between cultivars.

One factor that might have affected our results is that commercial grass cultivars are not always genotypes; in fact, cultivars of *S. arundinaceus* and *L. perenne* are a mix of different genotypes and therefore they contain substantial amounts of segregating variation which makes it difficult to pinpoint genotypes-level effects. However, *P. pratensis* had a much narrower genetic profile than the other two species, yet we did not find evidence for intraspecific variation in PSFs. Nevertheless, even when PSFs within species might exist in these species, the effects were clearly not strong enough to be expressed in our experimental design. Therefore, our results suggest that in the studied grass species, there may be limited scope for breeding on plant traits that are mediated by PSFs.

Acknowledgements

We thank Barenbrug grass seed company research facility (Wolfheze, The Netherlands) for providing plant material and soil, and Piet Arts for discussing our approach in various stages of the work. We thank Slavica Ivanovic, Tanja Bakx, Eke Hengeveld, Gregor Disveld and Renske Jongen for the assistance with the field and greenhouse work. We thank Sophie van Rijssel and Luis Merloti for support with microbial analysis and Keli Li, Morgane van Antro, Cristian Pena, Haymanty Saha, and Vera Hesen for the help in the field and in the greenhouse. Figure 3.1 has been created with BioRender.com. The sequencing was done by McGill University and Génome Québec Innovation Centre.

Supplementary material

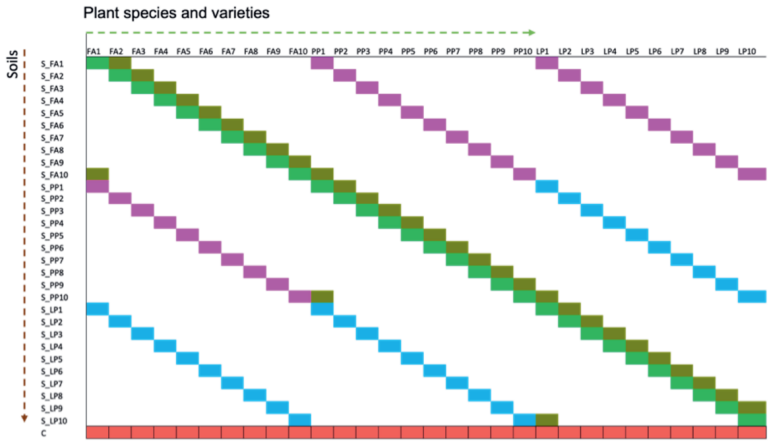


Figure S3.1. Partial factorial design of feedback phase. Every cultivar (FA1-LP10) was tested on the following soil treatments (S_FA1-C): unconditioned soil (red), soil conditioned by a different cultivar of the same species (light green), soil conditioned by the same cultivar (dark green), and soil conditioned by a cultivar of either one or the other grass species (blue - fuchsia). For soil conditioned by a different cultivar of the same species (light green), and soil and conditioned by a cultivar of either one or the other grass species (blue – fuchsia) we tested each cultivar on only one of the available cultivar-conditioned soils and not on all possible soils. Empty cells indicate that plants were not tested in that soil. See ID in Table S1 for further information.

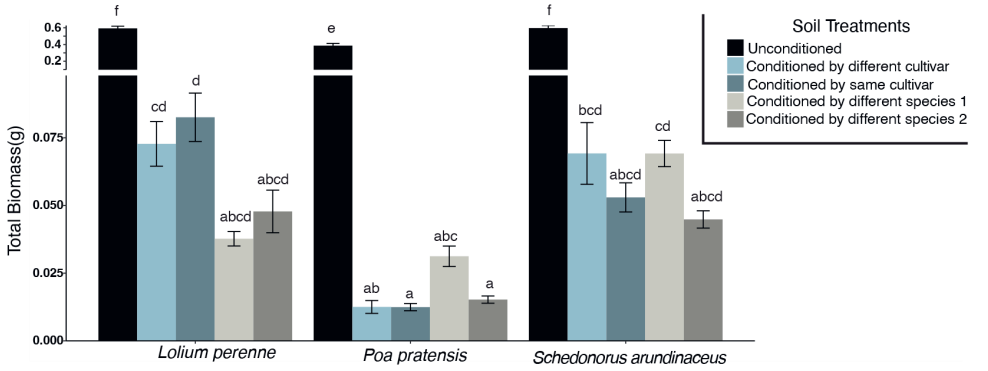


Figure S3.2: Total biomass of *Lolium perenne*, *Poa pratensis* and *Schedonorus arundinaceus* grass species when grown on soils with different conditioning treatments: unconditioned (black), conditioned by a different cultivar of the same species (light blue), conditioned by same cultivar (dark blue), conditioned by different species 1 (light grey), conditioned by different species 2 (darl grey). Bars and whiskers represent $\log_{10}$ transformed biomass + SE using a linear model.

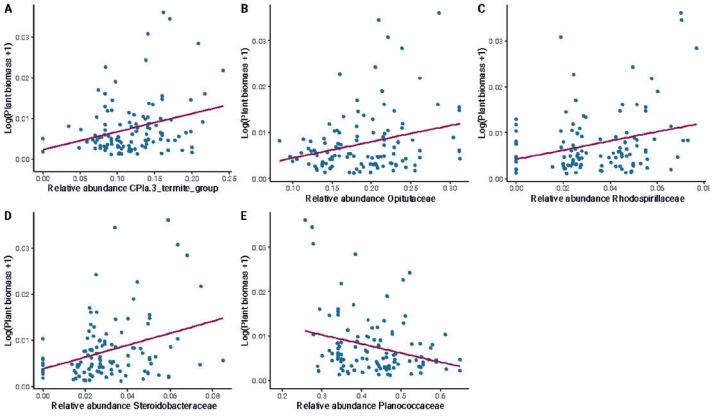


Figure S3.3. Linear model plots of relative abundances of bacterial families at the end of the conditioning phase and plant total biomass ($\log_{10}$) at the end of the feedback phase in *Poa pratensis*. Dots represent data points, and a linear trendline was fitted (with a 95% confidence interval). Summary statistics are presented in Table S5.

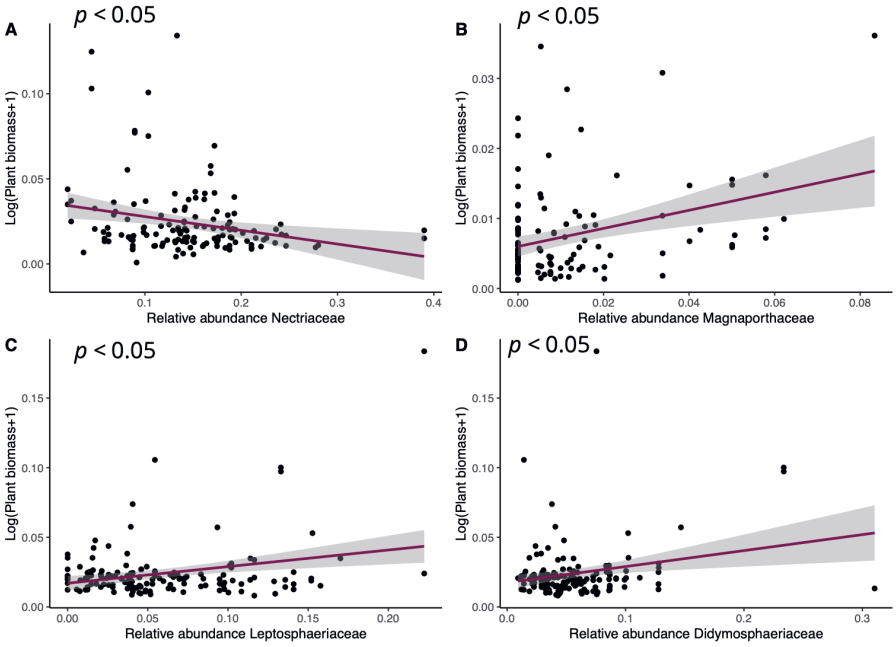


Figure S3.4. Linear model plots of relative abundances of fungal families at the end of the conditioning phase and plant total biomass (log_{10}) at the end of the feedback phase in (A) *Lolium perenne*, (B) *Poa pratensis* and (C-D) *Schedonorus arundinaceus*. Dots represent data points, and a linear trendline was fitted (with a 95% confidence interval). Summary statistics are presented in Table S3.7, S3.8 and Table S3.9)

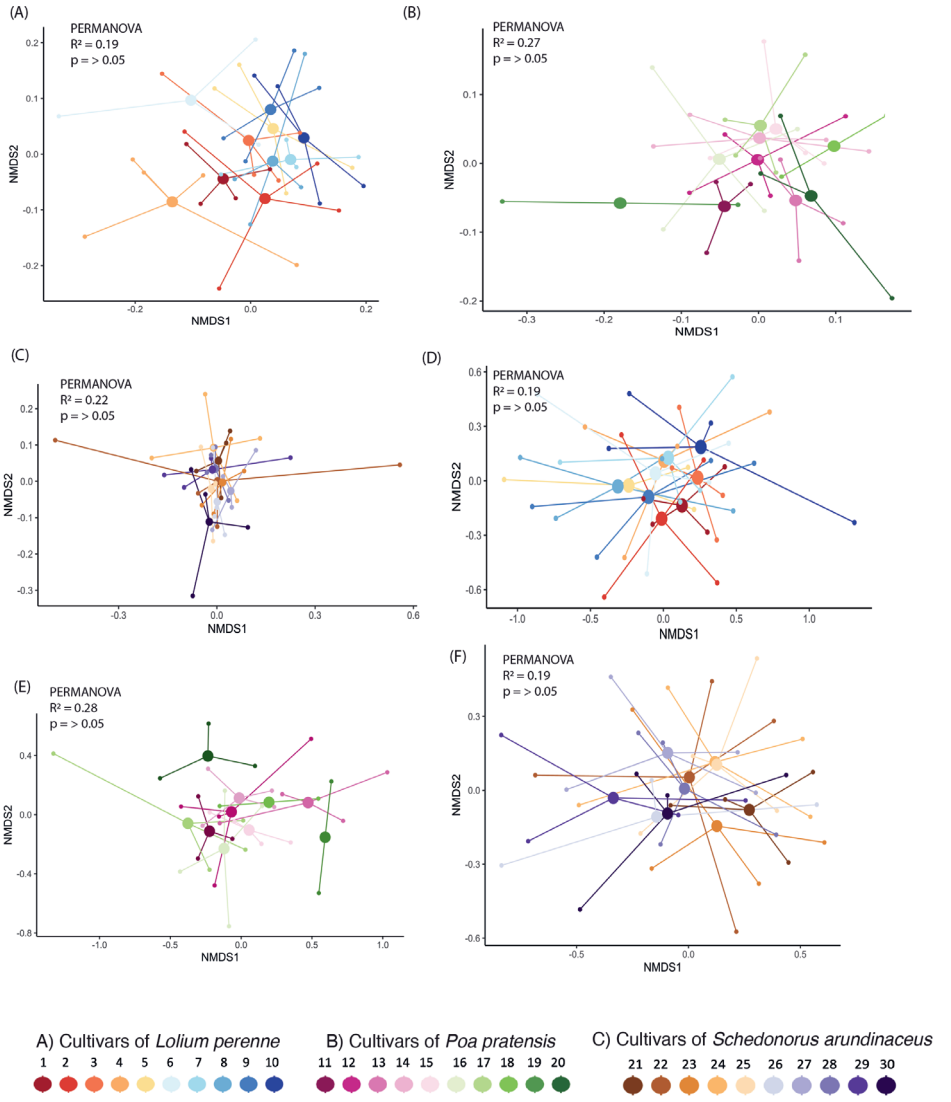


Figure S3.5. NMDS using Bray Curtis distance on the effects of plant cultivars on bacterial community composition in *L. perenne* (A), *P. pratensis* (B), *S. arundinaceus* (C) and fungal community compostion in *L. perenne* (D), *P. pratensis* (E), *S. arundinaceus* (F). Each colour corresponds to a cultivar.

Table S3.1. List of grass species and commercial cultivars used in the study.

ID	Plant species	Cultivar
FA1	*Schedonorus arundinaceus*	Bardesta
FA2	*Schedonorus arundinaceus*	Barepar
FA3	*Schedonorus arundinaceus*	Firecracker
FA4	*Schedonorus arundinaceus*	Barmesh
FA5	*Schedonorus arundinaceus*	Baraline
FA6	*Schedonorus arundinaceus*	Justice
FA7	*Schedonorus arundinaceus*	RGT Nuance
FA8	*Schedonorus arundinaceus*	Palladio
FA9	*Schedonorus arundinaceus*	Barnoble
FA10	*Schedonorus arundinaceus*	Barcesar
PP1	*Poa pratensis*	Barclaren
PP2	*Poa pratensis*	Limousine
PP3	*Poa pratensis*	Baron
PP4	*Poa pratensis*	Barserati
PP5	*Poa pratensis*	Barrister
PP6	*Poa pratensis*	Barvette
PP7	*Poa pratensis*	Barhelene
PP8	*Poa pratensis*	Barimpala
PP9	*Poa pratensis*	Baronial
PP10	*Poa pratensis*	Bariris
LP1	*Lolium perenne*	Barlancia
LP2	*Lolium perenne*	Bargold
LP3	*Lolium perenne*	Bareuro
LP4	*Lolium perenne*	Eventus
LP5	*Lolium perenne*	Eurodiamond
LP6	*Lolium perenne*	Pinnacle III
LP7	*Lolium perenne*	Baromario
LP8	*Lolium perenne*	15ER 18
LP9	*Lolium perenne*	Barprium
LP10	*Lolium perenne*	Barprecious

Table S3.2. Linear mixed model of the effect of soil treatments (T), plant species (S), plant cultivars (C) and interactions on total biomass of feedback phase. Plant cultivar (V) was nested in Plant Species (S). Pot of conditioning phase was used in the model as random factor.

Factors	df	F-value	*p*
Soil Treatments (T)	4, 671	172.23	< 0.001 ***
Plant Species (S)	2, 671	6.60	< 0.01 **
Block (B)	4, 671	2.45	< 0.05 *
Plant Species:cultivar (C)	26, 671	1.33	> 0.05
T x S	8, 671	2.02	< 0.05 *
T x V	104, 671	0.85	> 0.05

Table S3.3. Linear mixed models per each species of the effect of soil treatments (T), plant cultivars (C), interactions and blocks on total biomass of feedback phase. Pot of conditioning phase was used in the model as random factor whereas the biomass of the conditioning phase was used as cofactor.

Plant species	Fixed-Factors	df	F-value	*p value*
L. perenne	Biomass conditioning	1, 186	3.18	> 0.05
	Soil Treatments (T)	3, 186	6.99	< 0.01 ***
	Plant cultivar (C)	9, 186	1.19	> 0.05
	Block (B)	4, 186	1.97	> 0.05
	T x V	27, 186	0.72	> 0.05
P. pratensis	Biomass conditioning	1, 152	0.25	> 0.05
	Soil Treatments (T)	3, 152	12.58	< 0.001 ***
	Plant cultivar (C)	8, 152	3.79	< 0.001 ***
	Block (B)	4, 152	3.87	< 0.01 **
	T x V	24, 152	0.10	> 0.05
S. arundinaceus	Biomass conditioning	1.192	0.065	> 0.05
	Soil Treatments (T)	3, 192	3.18	< 0.05 *
	Plant cultivar (C)	9, 192	1.54	> 0.05
	Block (B)	4, 192	0.20	> 0.05
	T x V	27, 192	0.96	> 0.05

Table S3.4. Relationship between the abundance of bacterial families (M) and the growth of *Lolium perenne*. Significant values derived from LME model are marked in bold. The model included the factors soil treatments (T), plant cultivars (V), interactions and blocks (B) as fixed effects. Pot of conditioning phase was used in the model as random factor. Due to high presence of zeros, we excluded observations that had more that had >50 zeros. Relative abundances have been subjected to Hellinger transformation and p value of relative abundance have been corrected using fdr method.

Bacterial family	**F-value; p-value (M)**	**F-value; p-value (B)**	**F-value; p-value (T)**	**F-value; p-value (C)**	**N 0**	***p*-value (M) fdr**
Acetobacteraceae	7.47;0.01	0.22; 0.64	2.74;0.05	0.48;0.49	0	0.484
Bdellovibrionaceae	8.46;0	0.04; 0.84	2.7;0.05	0.43;0.51	2	0.484
Geobacteraceae	3.62;0.06	0.1; 0.75	1.31;0.28	0.24;0.63	42	0.87
Magnetospiraceae	3.69;0.06	0.33; 0.57	2.82;0.05	0.47;0.5	43	0.87
Opitutaceae	4.39;0.04	0.25; 0.62	3.04;0.03	0.67;0.41	0	0.87
Sphingomonadaceae	4.95;0.03	0; 0.95	3.28;0.03	0.77;0.38	0	0.87
Spirochaetaceae	4.63;0.03	0.02; 0.87	2.74;0.05	0.85;0.36	6	0.87
TRA3.20	3.85;0.05	0.6; 0.44	2.38;0.08	0.54;0.46	2	0.87
Verrucomicrobiaceae	3.83;0.05	0.28; 0.59	2.08;0.11	0.64;0.43	0	0.87
X67.14	2.02;0.16	0.18; 0.67	2.48;0.07	0.67;0.42	0	0.921
A0839	2.98;0.09	0.13; 0.71	3.02;0.04	0.39;0.53	26	0.921
AKYG1722	2.36;0.13	0.38; 0.54	2.59;0.06	0.72;0.4	10	0.921
Bacillaceae	1.54;0.22	0.22; 0.64	2.19;0.1	0.39;0.53	0	0.921
Burkholderiaceae	1.82;0.18	0.22; 0.64	2.74;0.05	0.42;0.52	0	0.921
Chthoniobacteraceae	0.98;0.33	0.08; 0.78	2.78;0.05	0.58;0.45	0	0.921
CPla.3_termite_group	2.61;0.11	0.01; 0.92	1.82;0.15	0.6;0.44	1	0.921
Desulfarculaceae	1.01;0.32	0.19; 0.66	2.61;0.06	0.47;0.5	14	0.921
Devosiaceae	1.32;0.25	0.12; 0.73	2.68;0.05	0.48;0.49	0	0.921
env.OPS_17	1.25;0.27	0.13; 0.72	2.71;0.05	0.64;0.42	4	0.921
Family_XVIII	1.4;0.24	0.1; 0.75	2.35;0.08	0.48;0.49	37	0.921
Fibrobacteraceae	1.25;0.27	0.22; 0.64	2.74;0.05	0.56;0.46	2	0.921
Fimbriimonadaceae	2.24;0.14	0.3; 0.59	2.62;0.06	0.64;0.42	8	0.921
Geminicoccaceae	1.89;0.17	0.3; 0.59	2.68;0.05	0.52;0.47	39	0.921
Gemmatimonadaceae	0.99;0.32	0.22; 0.64	2.56;0.06	0.38;0.54	0	0.921

Geodermatophilaceae	1.52;0.22	0.02; 0.89	2.53;0.06	0.45;0.5	0	0.921
Haliangiaceae	1.66;0.2	0.14; 0.71	2.27;0.09	0.72;0.4	0	0.921
Hyphomonadaceae	1.38;0.24	0.46; 0.5	2.17;0.1	0.42;0.52	0	0.921
Ilumatobacteraceae	0.82;0.37	0.26; 0.61	2.48;0.07	0.49;0.49	26	0.921
JG30.KF.CM45	0.93;0.34	0.17; 0.69	2.9;0.04	0.53;0.47	0	0.921
KD3.10	1.16;0.28	0.14; 0.71	2.99;0.04	0.55;0.46	38	0.921
KF.JG30.B3	2.3;0.13	0.26; 0.61	2.79;0.05	0.42;0.52	0	0.921
Kineosporiaceae	1.14;0.29	0.15; 0.7	2.98;0.04	0.5;0.48	2	0.921
Legionellaceae	1.07;0.3	0.09; 0.77	2.26;0.09	0.55;0.46	29	0.921
Limnochordaceae	1.43;0.23	0.14; 0.71	2.49;0.07	0.37;0.54	10	0.921
Methyloligellaceae	1.05;0.31	0.16; 0.69	2.86;0.04	0.68;0.41	2	0.921
Micrococcaceae	1.13;0.29	0.07; 0.79	2.25;0.09	0.54;0.46	0	0.921
Microscillaceae	1.63;0.2	0.08; 0.78	2.74;0.05	0.54;0.47	0	0.921
Nitrosomonadaceae	0.82;0.37	0.06; 0.8	2.84;0.04	0.45;0.5	0	0.921
P3OB.42	0.86;0.36	0.14; 0.71	2.96;0.04	0.68;0.41	41	0.921
Phycisphaeraceae	0.87;0.35	0.26; 0.61	2.43;0.07	0.43;0.51	0	0.921
Planococcaceae	1.01;0.32	0.19; 0.66	2.17;0.1	0.49;0.48	0	0.921
Pseudonocardiaceae	1.32;0.25	0.11; 0.74	2.98;0.04	0.59;0.45	0	0.921
Rhodocyclaceae	1.28;0.26	0.1; 0.75	1.8;0.16	0.36;0.55	39	0.921
Rhodospirillaceae	1.55;0.22	0.14; 0.71	2.53;0.06	0.58;0.45	14	0.921
SM2D12	2.04;0.16	0.04; 0.85	3.06;0.03	0.32;0.57	8	0.921
Sphingobacteriaceae	2.36;0.13	0.05; 0.83	2.81;0.05	0.58;0.45	34	0.921
Sporolactobacillaceae	1.98;0.16	0.03; 0.86	2.56;0.06	0.39;0.53	2	0.921
Streptomycetaceae	0.82;0.37	0.09; 0.76	2.76;0.05	0.5;0.48	0	0.921
Streptosporangiaceae	1.59;0.21	0.27; 0.6	2.47;0.07	0.54;0.46	0	0.921
Unknown_Family	1.85;0.18	0.35; 0.56	3.17;0.03	0.31;0.58	0	0.921
Vermiphilaceae	0.97;0.33	0.09; 0.76	2.78;0.05	0.71;0.4	22	0.921
Xanthomonadaceae	1.57;0.21	0.04; 0.84	2.8;0.05	0.33;0.56	0	0.921
Iamiaceae	0.77;0.38	0.07; 0.79	2.43;0.07	0.45;0.51	42	0.929
Nakamurellaceae	0.76;0.39	0.13; 0.72	2.52;0.06	0.65;0.42	10	0.929
AKIW781	0.69;0.41	0.19; 0.67	2.88;0.04	0.42;0.52	24	0.967

A21b	0.03;0.87	0.17; 0.68	2.66;0.05	0.5;0.48	0	0.99
A4b	0.06;0.81	0.2; 0.66	2.67;0.05	0.53;0.47	0	0.99
Acidobacteriaceae.Subgroup1	0.23;0.63	0.09; 0.76	2.72;0.05	0.51;0.48	0	0.99
Acidothermaceae	0.3;0.58	0.19; 0.67	2.36;0.08	0.5;0.48	0	0.99
AKYH767	0.08;0.77	0.17; 0.68	2.62;0.06	0.56;0.45	5	0.99
Alicyclobacillaceae	0.54;0.46	0.22; 0.64	2.67;0.05	0.65;0.42	12	0.99
Anaerolineaceae	0.02;0.89	0.18; 0.67	2.67;0.05	0.5;0.48	0	0.99
Archangiaceae	0.25;0.62	0.14; 0.71	2.49;0.07	0.51;0.48	0	0.99
B1.7BS	0.29;0.59	0.12; 0.73	2.59;0.06	0.46;0.5	24	0.99
Beijerinckiaceae	0.34;0.56	0.25; 0.62	2.71;0.05	0.44;0.51	5	0.99
BIrii41	0.17;0.68	0.21; 0.64	2.51;0.07	0.5;0.48	0	0.99
Blastocatellaceae	0.04;0.84	0.2; 0.66	2.64;0.06	0.5;0.48	0	0.99
Caldilineaceae	0.07;0.79	0.2; 0.66	2.62;0.06	0.49;0.49	18	0.99
Chitinophagaceae	0.07;0.8	0.17; 0.68	2.64;0.06	0.52;0.47	0	0.99
Chthonomonadaceae	0.06;0.8	0.21; 0.65	2.56;0.06	0.47;0.5	9	0.99
Clostridiaceae_1	0.08;0.78	0.18; 0.67	2.61;0.06	0.53;0.47	2	0.99
Dongiaceae	0.07;0.79	0.16; 0.69	2.66;0.06	0.51;0.48	0	0.99
Elsteraceae	0.38;0.54	0.12; 0.73	2.58;0.06	0.48;0.49	32	0.99
Frankiaceae	0.47;0.5	0.17; 0.68	2.27;0.09	0.51;0.48	0	0.99
Gaiellaceae	0.06;0.81	0.16; 0.69	2.66;0.05	0.54;0.46	0	0.99
Gemmataceae	0.36;0.55	0.13; 0.72	2.62;0.06	0.51;0.48	0	0.99
Gimesiaceae	0.23;0.63	0.12; 0.73	2.68;0.05	0.49;0.49	38	0.99
Halanaerobiaceae	0.04;0.84	0.18; 0.67	2.67;0.05	0.51;0.48	34	0.99
Heliobacteriaceae	0.61;0.44	0.27; 0.61	2.27;0.09	0.64;0.43	24	0.99
Holophagaceae	0.53;0.47	0.1; 0.75	2.06;0.11	0.42;0.52	1	0.99
Intrasporangiaceae	0.02;0.88	0.15; 0.7	2.65;0.06	0.49;0.49	0	0.99
Isosphaeraceae	0.1;0.75	0.22; 0.64	2.68;0.05	0.52;0.47	0	0.99
JG30.KF.AS9	0.02;0.9	0.19; 0.66	2.6;0.06	0.48;0.49	0	0.99
Ktedonobacteraceae	0.27;0.6	0.21; 0.65	2.64;0.06	0.52;0.47	0	0.99
Labraceae	0.03;0.87	0.18; 0.67	2.66;0.05	0.49;0.48	15	0.99
Methylacidiphilaceae	0.14;0.71	0.15; 0.7	2.52;0.07	0.56;0.46	15	0.99

Micavibrionaceae	0.47;0.5	0.17; 0.68	2.63;0.06	0.36;0.55	23	0.99
Microbacteriaceae	0.15;0.7	0.23; 0.63	2.64;0.06	0.47;0.49	10	0.99
Micromonosporaceae	0.02;0.9	0.17; 0.68	2.66;0.05	0.5;0.48	1	0.99
Mycobacteriaceae	0.03;0.87	0.14; 0.71	2.64;0.06	0.48;0.49	0	0.99
Myxococcaceae	0.04;0.84	0.17; 0.68	2.64;0.06	0.52;0.47	37	0.99
Nocardioidaceae	0.46;0.5	0.1; 0.76	2.66;0.05	0.53;0.47	0	0.99
NS9_marine_group	0.42;0.52	0.35; 0.56	2.61;0.06	0.53;0.47	38	0.99
Omnitrophaceae	0.51;0.48	0.23; 0.63	2.58;0.06	0.49;0.49	2	0.99
Paenibacillaceae	0.52;0.47	0.18; 0.67	2.43;0.07	0.46;0.5	0	0.99
Pedosphaeraceae	0.08;0.77	0.17; 0.68	2.68;0.05	0.53;0.47	0	0.99
Phaselicystidaceae	0.04;0.85	0.17; 0.68	2.63;0.06	0.51;0.47	26	0.99
Pirellulaceae	0.36;0.55	0.18; 0.67	2.77;0.05	0.46;0.5	0	0.99
Polyangiaceae	0.07;0.79	0.15; 0.7	2.69;0.05	0.5;0.48	0	0.99
Pyrinomonadaceae	0.24;0.63	0.16; 0.69	2.71;0.05	0.45;0.51	2	0.99
Reyranellaceae	0.14;0.71	0.18; 0.67	2.71;0.05	0.45;0.5	0	0.99
Rhizobiales_Incertae_Sedis	0.25;0.62	0.18; 0.67	2.75;0.05	0.61;0.44	0	0.99
Rhodanobacteraceae	0.62;0.43	0.07; 0.79	2.64;0.06	0.43;0.51	0	0.99
Roseiflexaceae	0.16;0.69	0.13; 0.71	2.71;0.05	0.44;0.51	0	0.99
Rubinisphaeraceae	0.15;0.7	0.17; 0.68	2.68;0.05	0.42;0.52	1	0.99
Sandaracinaceae	0.03;0.87	0.16; 0.69	2.65;0.06	0.51;0.48	0	0.99
SC.I.84	0.15;0.7	0.18; 0.67	2.7;0.05	0.56;0.46	0	0.99
Solibacteraceae_.Subgroup_3.	0.21;0.65	0.17; 0.68	2.64;0.06	0.51;0.48	0	0.99
Solirubrobacteraceae	0.18;0.67	0.1; 0.75	2.55;0.06	0.49;0.49	0	0.99
Steroidobacteraceae	0.29;0.59	0.2; 0.65	2.58;0.06	0.44;0.51	9	0.99
Thermoactinomycetaceae	0.32;0.58	0.06; 0.81	2.33;0.08	0.42;0.52	2	0.99
Thermoanaerobaculaceae	0.14;0.71	0.19; 0.66	2.4;0.07	0.5;0.48	0	0.99
Thermomicrobiaceae	0.32;0.57	0.14; 0.71	2.49;0.07	0.47;0.5	31	0.99
Thermomonosporaceae	0.07;0.79	0.17; 0.68	2.67;0.05	0.51;0.47	10	0.99
URHD0088	0.05;0.82	0.2; 0.66	2.68;0.05	0.48;0.49	24	0.99
WD2101_soil_group	0.17;0.68	0.18; 0.67	2.52;0.07	0.48;0.49	0	0.99
Xanthobacteraceae	0.48;0.49	0.03; 0.85	2.8;0.05	0.64;0.43	0	0.99

Xiphinematobacteraceae	0.04;0.85	0.17; 0.68	2.65;0.06	0.49;0.48	4	0.99
BSV26	0.01;0.92	0.17; 0.68	2.66;0.05	0.49;0.48	41	0.998
Caulobacteraceae	0;1	0.18; 0.68	2.52;0.07	0.5;0.48	1	0.998
Cryptosporangiaceae	0;0.98	0.18; 0.68	2.65;0.06	0.5;0.48	24	0.998
Diplorickettsiaceae	0;0.98	0.18; 0.68	2.59;0.06	0.5;0.48	12	0.998
Hydrogenedensaceae	0;0.99	0.18; 0.68	2.65;0.06	0.49;0.48	32	0.998
Hyphomicrobiaceae	0;1	0.18; 0.68	2.65;0.06	0.5;0.48	0	0.998
Koribacteraceae	0;0.97	0.17; 0.68	2.66;0.05	0.5;0.48	9	0.998
Micropepsaceae	0;0.95	0.17; 0.68	2.66;0.06	0.5;0.48	0	0.998
Nitrospiraceae	0.01;0.94	0.17; 0.68	2.65;0.06	0.51;0.48	0	0.998
Peptostreptococcaceae	0;1	0.17; 0.68	2.54;0.06	0.5;0.48	19	0.998
Rhizobiaceae	0;0.95	0.18; 0.67	2.66;0.06	0.49;0.48	0	0.998
Solimonadaceae	0;0.99	0.18; 0.68	2.6;0.06	0.5;0.48	1	0.998

Table S3.5. Relationship between the abundance of bacterial families (M) and the growth of *Poa pratensis*. Significant values derived from LME model are marked in bold. The model included the factors soil treatments (T), plant cultivars (C), interactions and blocks (B) as fixed effects. Pot of conditioning phase was used in the model as random factor. Due to high presence of zeros, we excluded observations that had more that had >50 zeros Relative abundances have been subjected to Hellinger transformation.

Bacterial Family	**F-value; p-value (M)**	**F-value; p-value (B)**	**F-value; p-value (T)**	**F-value; p-value (C)**	**N 0**	***p* value (M) fdr**
A0839	2.09;0.15	2.02;0.16	1.09;0.3	0.36;0.55	22	0.49
A21b	0.04;0.84	2.19;0.14	1.35;0.25	0.48;0.49	0	0.94
A4b	0.86;0.36	2.71;0.1	1.42;0.24	0.56;0.46	0	0.76
Acetobacteraceae	1.9;0.17	2.2;0.14	1.72;0.19	0.46;0.5	0	0.51
Acidiferrobacteraceae	0.08;0.78	2.32;0.13	1.4;0.24	0.52;0.48	37	0.93
Acidobacteriaceae.Sub1	2.52;0.12	1.65;0.2	0.91;0.34	0.19;0.66	0	0.44
Acidothermaceae	2.61;0.11	2.97;0.09	1.08;0.3	0.43;0.51	0	0.44
AKIW781	0.22;0.64	2.51;0.12	1.3;0.26	0.45;0.51	18	0.86
AKYG1722	1.64;0.2	2.41;0.12	1.12;0.29	0.22;0.64	9	0.54
AKYH767	6.75;0.01	4.92;0.03	0.28;0.6	0.9;0.34	4	0.13
Alicyclobacillaceae	0.06;0.81	2.33;0.13	1.37;0.24	0.52;0.47	15	0.94
Anaerolineaceae	1.15;0.29	2.67;0.11	1.07;0.3	0.57;0.45	0	0.67
Archangiaceae	0.67;0.42	2.68;0.1	1.06;0.31	0.55;0.46	0	0.79
B1.7BS	3.53;0.06	1.91;0.17	1.48;0.23	0.99;0.32	21	0.33
Bacillaceae	5.57;0.02	3.25;0.07	1.41;0.24	0.37;0.54	0	0.20
Bdellovibrionaceae	0.44;0.51	2.54;0.11	1.25;0.27	0.42;0.52	1	0.79
Beijerinckiaceae	0.34;0.56	2.53;0.11	1.28;0.26	0.59;0.44	5	0.81
BIrii41	0.47;0.5	2.65;0.11	1.4;0.24	0.62;0.43	0	0.79
Blastocatellaceae	1.11;0.29	2.73;0.1	1.31;0.25	0.21;0.65	0	0.67
BSV26	0.5;0.48	2.54;0.11	1.27;0.26	0.51;0.48	32	0.79
Burkholderiaceae	2.37;0.13	2.54;0.11	1.45;0.23	0.36;0.55	0	0.46
Caldilineaceae	0.11;0.74	2.32;0.13	1.32;0.25	0.47;0.5	14	0.91
Catenulisporaceae	0.42;0.52	2.54;0.11	1.42;0.24	0.59;0.45	42	0.79
Caulobacteraceae	1.37;0.25	1.9;0.17	1.02;0.31	0.56;0.46	2	0.60

Chitinophagaceae	0.61;0.44	2.37;0.13	1.13;0.29	0.5;0.48	0	0.79
Chthoniobacteraceae	1.85;0.18	2.86;0.09	1.64;0.2	0.84;0.36	0	0.52
Chthonomonadaceae	0.12;0.73	2.3;0.13	1.29;0.26	0.56;0.46	12	0.91
Clostridiaceae_1	0.68;0.41	1.92;0.17	1.55;0.22	0.59;0.44	3	0.79
CPla.3.termite_group	**10.94;0**	**3.04;0.08**	**1.13;0.29**	1.39;0.24	**2**	**0.04**
Cryptosporangiaceae	0.96;0.33	2.17;0.14	1.14;0.29	0.4;0.53	19	0.73
Desulfarculaceae	0.42;0.52	2.56;0.11	1.47;0.23	0.47;0.5	10	0.79
Devosiaceae	4.76;0.03	3.65;0.06	1.15;0.29	1.18;0.28	0	0.24
Diplorickettsiaceae	4.83;0.03	1.85;0.18	1.11;0.29	0.91;0.34	10	0.24
Dongiaceae	0;0.95	2.26;0.14	1.33;0.25	0.48;0.49	0	0.98
Elsteraceae	0.5;0.48	2.29;0.13	1.24;0.27	0.35;0.55	30	0.79
env.OPS_17	0.74;0.39	2.21;0.14	1.12;0.29	0.58;0.45	4	0.79
Family_XVIII	0.27;0.6	2.19;0.14	1.31;0.25	0.54;0.47	30	0.83
Fibrobacteraceae	0.19;0.66	2.29;0.13	1.18;0.28	0.48;0.49	1	0.86
Fimbriimonadaceae	0.38;0.54	2.26;0.14	1.52;0.22	0.55;0.46	8	0.80
Frankiaceae	5.01;0.03	1.3;0.26	0.88;0.35	0.38;0.54	0	0.24
Gaiellaceae	0.12;0.73	2.3;0.13	1.4;0.24	0.46;0.5	0	0.91
Geminicoccaceae	0.28;0.6	2.41;0.12	1.3;0.26	0.47;0.5	28	0.83
Gemmataceae	0.05;0.82	2.27;0.14	1.36;0.25	0.52;0.47	0	0.94
Gemmatimonadaceae	0.03;0.87	2.26;0.14	1.26;0.26	0.5;0.48	0	0.96
Geobacteraceae	**11.49;0**	**2.6;0.11**	**2.1;0.15**	0.48;0.49	**35**	**0.04**
Geodermatophilaceae	3.54;0.06	1.88;0.17	1.88;0.17	1.24;0.27	0	0.33
Gimesiaceae	0.35;0.56	2.51;0.12	1.37;0.24	0.29;0.59	28	0.81
Halanaerobiaceae	2.19;0.14	2.77;0.1	1.32;0.25	0.5;0.48	27	0.49
Haliangiaceae	6.92;0.01	2.06;0.15	0.91;0.34	0.91;0.34	0	0.13
Heliobacteriaceae	4.56;0.04	2.19;0.14	0.8;0.37	0.36;0.55	21	0.25
Holophagaceae	2.76;0.1	2.59;0.11	1.41;0.24	0.78;0.38	1	0.42
Hydrogenedensaceae	0.23;0.63	2.52;0.12	1.26;0.26	0.54;0.46	25	0.86
Hyphomicrobiaceae	0.04;0.85	2.27;0.14	1.36;0.25	0.47;0.5	0	0.94
Hyphomonadaceae	0;0.97	2.26;0.14	1.33;0.25	0.45;0.5	0	0.98
Iamiaceae	1.45;0.23	2.84;0.1	1.93;0.17	1.05;0.31	36	0.60
Ilumatobacteraceae	0.45;0.5	2.65;0.11	1.41;0.24	0.6;0.44	28	0.79

Intrasporangiaceae	0.69;0.41	2.5;0.12	1.28;0.26	0.42;0.52	0	0.79
Isosphaeraceae	2.85;0.1	3.66;0.06	1.84;0.18	1.29;0.26	0	0.42
JG30.KF.AS9	1.76;0.19	2.52;0.12	1.27;0.26	0.05;0.83	0	0.53
JG30.KF.CM45	0.08;0.78	2.34;0.13	1.3;0.26	0.43;0.51	0	0.93
KD3.10	0.01;0.94	2.28;0.13	1.32;0.25	0.48;0.49	29	0.98
KF.JG30.B3	2;0.16	2.22;0.14	1.07;0.3	0.64;0.43	0	0.50
Kineosporiaceae	2.13;0.15	3.13;0.08	1.37;0.25	0.31;0.58	2	0.49
Koribacteraceae	0.21;0.64	2.48;0.12	1.29;0.26	0.46;0.5	10	0.86
Ktedonobacteraceae	2.39;0.13	3.39;0.07	1.16;0.28	0.24;0.62	0	0.46
Labraceae	3.32;0.07	1.76;0.19	1.6;0.21	0.68;0.41	11	0.34
Legionellaceae	0.77;0.38	1.97;0.16	1.41;0.24	0.76;0.38	19	0.79
Leptospiraceae	0.09;0.77	1.84;0.18	1.23;0.27	0.49;0.49	44	0.93
Limnochordaceae	0.04;0.84	2.26;0.14	1.36;0.25	0.49;0.49	8	0.94
Magnetospiraceae	0.55;0.46	2.56;0.11	1.5;0.22	0.34;0.56	31	0.79
Methylacidiphilaceae	0.76;0.39	1.99;0.16	1.16;0.28	0.5;0.48	8	0.79
Methyloligellaceae	0.26;0.61	2.51;0.12	1.12;0.29	0.49;0.49	2	0.84
Micavibrionaceae	0.09;0.77	2.2;0.14	1.27;0.26	0.55;0.46	14	0.93
Microbacteriaceae	0.01;0.91	2.29;0.13	1.33;0.25	0.43;0.52	7	0.98
Micrococcaceae	1.36;0.25	1.81;0.18	1.37;0.24	0.68;0.41	0	0.60
Micromonosporaceae	2.11;0.15	2.81;0.1	1.6;0.21	0.52;0.47	2	0.49
Micropepsaceae	2.13;0.15	2.39;0.13	0.87;0.35	0.44;0.51	0	0.49
Microscillaceae	6.71;0.01	3.75;0.06	1.62;0.21	1.72;0.19	0	0.13
Mitochondria	3.12;0.08	0.55;0.46	0.53;0.47	0.15;0.7	48	0.38
Mycobacteriaceae	0.03;0.86	2.23;0.14	1.36;0.25	0.51;0.48	0	0.95
Myxococcaceae	1.08;0.3	2.3;0.13	1.68;0.2	0.46;0.5	27	0.68
Nakamurellaceae	0.93;0.34	2.53;0.12	1.28;0.26	0.68;0.41	5	0.74
Nitrosomonadaceae	2.78;0.1	3.18;0.08	1.4;0.24	1.04;0.31	0	0.42
Nitrospiraceae	0.01;0.93	2.3;0.13	1.27;0.26	0.49;0.49	0	0.98
Nocardioidaceae	1.94;0.17	3.12;0.08	1.24;0.27	0.93;0.34	0	0.51
NS11.12_marine_group	1.14;0.29	1.81;0.18	0.86;0.36	0.19;0.66	39	0.67
NS9_marine_group	1.41;0.24	2.6;0.11	1.31;0.26	0.94;0.34	38	0.60

Omnitrophaceae	0.76;0.39	2.88;0.09	1.24;0.27	0.6;0.44	2	0.79
Opitutaceae	**11.5;0**	**5.76;0.02**	**0.43;0.51**	0.73;0.4	**0**	**0.04**
P3OB.42	0.56;0.46	2.26;0.14	1.39;0.24	0.46;0.5	26	0.79
Paenibacillaceae	4.07;0.05	2.73;0.1	1.33;0.25	0.47;0.49	0	0.27
Pedosphaeraceae	3.62;0.06	1.91;0.17	1.53;0.22	0.5;0.48	0	0.33
Peptostreptococcaceae	1.67;0.2	1.72;0.19	1.02;0.32	0.87;0.35	10	0.54
Phaselicystidaceae	3.46;0.07	2.15;0.15	1.6;0.21	0.36;0.55	15	0.33
Phycisphaeraceae	0.52;0.47	2.41;0.12	1.31;0.26	0.44;0.51	0	0.79
Pirellulaceae	0.52;0.47	1.93;0.17	1.25;0.27	0.52;0.47	0	0.79
Planococcaceae	10.33;0	5.03;0.03	1.37;0.24	0.35;0.56	0	0.05
Polyangiaceae	5.99;0.02	3.11;0.08	1.44;0.23	0.6;0.44	0	0.17
Pseudonocardiaceae	0.64;0.43	1.96;0.16	1.35;0.25	0.37;0.54	0	0.79
Pyrinomonadaceae	0;0.96	2.23;0.14	1.32;0.25	0.49;0.49	3	0.98
Reyranellaceae	0.33;0.57	2.36;0.13	1.45;0.23	0.45;0.51	0	0.81
Rhizobiaceae	0.02;0.9	2.3;0.13	1.34;0.25	0.46;0.5	0	0.97
Rhizobiales,Incert, Sedis	0;0.98	2.1;0.15	1.33;0.25	0.48;0.49	0	0.98
Rhodanobacteraceae	0.06;0.8	2.33;0.13	1.38;0.24	0.53;0.47	0	0.94
Rhodocyclaceae	7.75;0.01	2.77;0.1	1.72;0.19	0.54;0.46	33	0.12
Rhodospirillaceae	**11.1;0**	**3.9;0.05**	**0.66;0.42**	1.42;0.24	**13**	**0.04**
Roseiflexaceae	0.06;0.8	2.14;0.15	1.21;0.27	0.46;0.5	0	0.94
Rubinisphaeraceae	0.12;0.73	2.24;0.14	1.42;0.24	0.47;0.5	1	0.91
Sandaracinaceae	0;0.98	2.27;0.13	1.33;0.25	0.48;0.49	0	0.98
SC.I.84	0.59;0.45	2.67;0.11	1.32;0.25	0.54;0.46	0	0.79
SM2D12	0.58;0.45	1.85;0.18	1.47;0.23	0.63;0.43	7	0.79
Solibacteraceae.Sub 3	0.14;0.71	2.19;0.14	1.31;0.25	0.48;0.49	0	0.91
Solimonadaceae	0.19;0.66	2.47;0.12	1.23;0.27	0.47;0.5	2	0.86
Solirubrobacteraceae	6.62;0.01	4.48;0.04	1.28;0.26	2.22;0.14	0	0.13
Sphingobacteriaceae	0.15;0.7	2.34;0.13	1.36;0.25	0.38;0.54	29	0.90
Sphingomonadaceae	4.4;0.04	3.9;0.05	1.35;0.25	1.27;0.26	0	0.25
Spirochaetaceae	4.85;0.03	3.69;0.06	0.89;0.35	0.83;0.37	6	0.24
Sporolactobacillaceae	7.64;0.01	4.62;0.03	1.66;0.2	1.39;0.24	1	0.12

Steroidobacteraceae	**13.05;0**	**1.74;0.19**	**0.71;0.4**	1.8;0.18	**13**	**0.04**
Streptomycetaceae	0.32;0.57	2.48;0.12	1.36;0.25	0.56;0.46	0	0.81
Streptosporangiaceae	1.82;0.18	2.38;0.13	1.09;0.3	0.56;0.46	0	0.52
Tepidisphaeraceae	0.67;0.42	2.03;0.16	1.47;0.23	0.6;0.44	47	0.79
Thermoactinomycetaceae	4.12;0.05	2.01;0.16	2.14;0.15	1.75;0.19	2	0.27
Thermoanaerobaculaceae	2.53;0.12	1.97;0.16	0.89;0.35	0.55;0.46	0	0.44
Thermomicrobiaceae	0.04;0.84	2.23;0.14	1.29;0.26	0.44;0.51	14	0.94
Thermomonosporaceae	0.38;0.54	2.47;0.12	1.4;0.24	0.43;0.51	12	0.80
TRA3.20	1.71;0.19	3.11;0.08	1.44;0.23	0.21;0.64	1	0.53
Unknown_Family	1.3;0.26	1.18;0.28	0.99;0.32	0.78;0.38	0	0.62
URHD0088	0.47;0.49	1.97;0.16	1.2;0.28	0.62;0.43	21	0.79
Vermiphilaceae	0.38;0.54	2.52;0.12	1.31;0.25	0.43;0.52	17	0.80
Verrucomicrobiaceae	4.08;0.05	2.16;0.15	1.03;0.31	0.48;0.49	0	0.27
Vulgatibacteraceae	0.02;0.9	2.31;0.13	1.32;0.25	0.49;0.48	46	0.97
WD2101_soil_group	0.63;0.43	1.65;0.2	1.22;0.27	0.52;0.47	0	0.79
Xanthobacteraceae	0.54;0.46	2;0.16	1.02;0.31	0.72;0.4	0	0.79
Xanthomonadaceae	0;0.95	2.28;0.13	1.32;0.25	0.49;0.49	0	0.98
Xiphinematobacteraceae	0;0.95	2.29;0.13	1.34;0.25	0.47;0.49	2	0.98

Table S3.6. Relationship between the abundance of bacterial families (M) and the growth of *Schedonorus arundinaceus*. Significant values derived from LME model are marked in bold. The model included the factors soil treatments (T), plant cultivars (V), interactions and blocks (B) as fixed effects. Pot of conditioning phase was used in the model as random factor. Due to high presence of zeros, we excluded observations that had more that had >50 zeros. Relative abundances have been subjected to Hellinger transformation.

Bacterial Family	**F-value; p-value (M)**	**F-value; p-value (B)**	**F-value; p-value (T)**	**F-value; p-value (V)**	**N 0**	***p*-value *(M) fdr***
Pseudonocardiaceae	11.65;0	4.41;0.04	2.6;0.11	0.45;0.5	0	0.12
Acidobacteriaceae_.Subgroup_1.	8.05;0.01	5.02;0.03	3.57;0.06	0.12;0.72	0	0.24
Micropepsaceae	8.06;0.01	5.51;0.02	3.33;0.07	0.34;0.56	0	0.24
CPla.3_termite_group	5.7;0.02	7.14;0.01	2.91;0.09	1.67;0.2	1	0.30
Fibrobacteraceae	5.74;0.02	5.94;0.02	3.76;0.05	0.78;0.38	1	0.30
Planococcaceae	5.78;0.02	8.35;0	2.47;0.12	0.94;0.33	0	0.30
Verrucomicrobiaceae	6.31;0.01	6.22;0.01	2.93;0.09	0.7;0.41	0	0.30
WD2101_soil_group	5.51;0.02	2.88;0.09	3.53;0.06	0.82;0.37	0	0.30

Xanthobacteraceae	6.9;0.01	4.1;0.04	3.7;0.06	2.32;0.13	0	0.30
Haliangiaceae	4.6;0.03	5.98;0.02	3.29;0.07	0.93;0.34	0	0.44
Caldilineaceae	3.93;0.05	5.16;0.02	2.5;0.12	1.05;0.31	22	0.56
Gemmatimonadaceae	3.75;0.06	4.91;0.03	3.45;0.07	0.87;0.35	0	0.56
Paenibacillaceae	3.82;0.05	6.02;0.02	2.71;0.1	0.64;0.42	0	0.56
Holophagaceae	3.22;0.07	6.84;0.01	2.45;0.12	1.15;0.29	1	0.70
Polyangiaceae	3.08;0.08	6.06;0.02	3.07;0.08	0.93;0.34	0	0.71
Spirochaetaceae	2.84;0.09	6.96;0.01	3.47;0.06	1.03;0.31	7	0.72
Streptomycetaceae	2.88;0.09	4.54;0.03	3.3;0.07	0.45;0.51	0	0.72
Nitrosomonadaceae	2.66;0.11	6.43;0.01	2.59;0.11	1.19;0.28	0	0.76
Acidothermaceae	1.26;0.26	4.1;0.04	3.04;0.08	0.83;0.37	0	0.82
AKYG1722	1.9;0.17	5.65;0.02	3.2;0.08	0.75;0.39	9	0.82
Archangiaceae	1.68;0.2	6.34;0.01	2.73;0.1	1.12;0.29	0	0.82
B1.7BS	1.3;0.26	5.46;0.02	2.48;0.12	1.24;0.27	26	0.82
Bacillaceae	1.45;0.23	6.2;0.01	2.57;0.11	0.69;0.41	0	0.82
Bdellovibrionaceae	2.17;0.14	4.29;0.04	3.09;0.08	1.01;0.32	2	0.82
Caulobacteraceae	1.42;0.24	5.06;0.03	3.2;0.08	0.83;0.36	1	0.82
Devosiaceae	1.44;0.23	6.43;0.01	2.77;0.1	0.9;0.34	0	0.82
env.OPS_17	2.39;0.12	5.86;0.02	3.73;0.06	0.92;0.34	8	0.82
Iamiaceae	1.87;0.17	6.5;0.01	2.27;0.13	1.24;0.27	40	0.82
JG30.KF.CM45	1.4;0.24	5.68;0.02	2.68;0.1	0.61;0.44	0	0.82
Ktedonobacteraceae	1.31;0.26	3.99;0.05	3.07;0.08	1.03;0.31	0	0.82
Myxococcaceae	1.37;0.24	5.51;0.02	2.31;0.13	0.85;0.36	35	0.82
Opitutaceae	2.34;0.13	6.97;0.01	3.41;0.07	0.63;0.43	0	0.82
Pedosphaeraceae	1.69;0.2	5.3;0.02	2.7;0.1	0.76;0.39	0	0.82
Phycisphaeraceae	1.75;0.19	5.75;0.02	2.55;0.11	0.53;0.47	0	0.82
Rhodanobacteraceae	1.86;0.17	4.71;0.03	3.17;0.08	0.46;0.5	0	0.82
SC.I.84	2.18;0.14	4.69;0.03	2.46;0.12	0.92;0.34	0	0.82
Sporolactobacillaceae	1.28;0.26	5.95;0.02	2.35;0.13	1.05;0.31	1	0.82
Steroidobacteraceae	1.58;0.21	5.7;0.02	3.1;0.08	1.08;0.3	12	0.82
Thermoactinomycetaceae	1.39;0.24	5.45;0.02	2.36;0.13	1.4;0.24	1	0.82
Thermoanaerobaculaceae	1.3;0.26	5.2;0.02	3.29;0.07	0.69;0.41	0	0.82
Thermomonosporacea e	1.67;0.2	4.68;0.03	3.15;0.08	1.07;0.3	9	0.82
Unknown_Family	1.4;0.24	3.83;0.05	3.07;0.08	1.02;0.31	0	0.82
Reyranellaceae	1.18;0.28	5.38;0.02	2.21;0.14	0.73;0.4	0	0.85
Labraceae	1.11;0.29	5.26;0.02	2.8;0.1	0.9;0.34	14	0.87
Geminicoccaceae	1;0.32	4.68;0.03	2.32;0.13	0.96;0.33	36	0.91
Ilumatobacteraceae	0.98;0.32	5.97;0.02	2.96;0.09	0.94;0.33	30	0.91
Clostridiaceae_1	0.85;0.36	5.33;0.02	3.05;0.08	0.74;0.39	4	0.93
Frankiaceae	0.88;0.35	5.14;0.02	2.78;0.1	0.93;0.34	0	0.93
Koribacteraceae	0.76;0.39	4.67;0.03	2.9;0.09	0.75;0.39	7	0.93
Microscillaceae	0.91;0.34	6.02;0.02	2.78;0.1	0.94;0.33	0	0.93

Phaselicystidaceae	0.76;0.38	5.19;0.02	2.22;0.14	0.84;0.36	20	0.93
Rhodocyclaceae	0.77;0.38	5.93;0.02	2.4;0.12	0.96;0.33	43	0.93
Rubinisphaeraceae	0.83;0.36	5.09;0.03	2;0.16	0.71;0.4	1	0.93
TRA3.20	0.82;0.37	6.1;0.01	2.59;0.11	0.56;0.46	3	0.93
Heliobacteriaceae	0.72;0.4	5.52;0.02	3.11;0.08	0.53;0.47	25	0.94
X0319.6G20	0.37;0.55	5.51;0.02	2.47;0.12	0.68;0.41	0	0.94
X67.14	0.55;0.46	5.83;0.02	2.82;0.1	0.79;0.38	0	0.94
AKIW781	0.38;0.54	4.59;0.03	2.87;0.09	0.83;0.37	26	0.94
Blastocatellaceae	0.52;0.47	4.73;0.03	2.98;0.09	1.03;0.31	0	0.94
Chthonomonadaceae	0.3;0.58	5.48;0.02	2.36;0.13	0.64;0.42	13	0.94
Diplorickettsiaceae	0.3;0.58	5.3;0.02	2.89;0.09	0.85;0.36	14	0.94
Dongiaceae	0.7;0.4	5.33;0.02	2.59;0.11	0.81;0.37	0	0.94
Family_XVIII	0.33;0.56	5.4;0.02	2.58;0.11	0.69;0.41	38	0.94
Gaiellaceae	0.31;0.58	5.52;0.02	2.63;0.11	0.72;0.4	0	0.94
Isosphaeraceae	0.44;0.51	4.36;0.04	2.9;0.09	0.66;0.42	0	0.94
JG30.KF.AS9	0.5;0.48	5.02;0.03	3.01;0.08	1.07;0.3	0	0.94
KD3.10	0.3;0.58	5.49;0.02	2.75;0.1	0.78;0.38	39	0.94
Limnochordaceae	0.59;0.45	5.55;0.02	2.55;0.11	0.86;0.35	8	0.94
Micrococcaceae	0.48;0.49	5.38;0.02	2.78;0.1	0.92;0.34	0	0.94
Mycobacteriaceae	0.48;0.49	4.62;0.03	2.83;0.09	0.57;0.45	0	0.94
Nocardioidaceae	0.58;0.45	5.81;0.02	2.72;0.1	0.93;0.34	0	0.94
NS9_marine_group	0.47;0.49	5.07;0.03	2.78;0.1	0.41;0.52	46	0.94
P3OB.42	0.38;0.54	5.5;0.02	2.62;0.11	0.8;0.37	35	0.94
Pirellulaceae	0.32;0.57	5.44;0.02	2.6;0.11	0.77;0.38	0	0.94
Rhizobiaceae	0.33;0.56	5.25;0.02	2.53;0.11	0.79;0.38	0	0.94
Rhodospirillaceae	0.43;0.51	5.75;0.02	2.87;0.09	0.91;0.34	17	0.94
Solibacteraceae_.Subgroup_3.	0.58;0.45	5.31;0.02	2.73;0.1	0.77;0.38	0	0.94
Solirubrobacteraceae	0.41;0.52	5.83;0.02	2.6;0.11	0.99;0.32	0	0.94
Thermomicrobiaceae	0.32;0.57	5.12;0.03	2.91;0.09	0.66;0.42	23	0.94
Xanthomonadaceae	0.45;0.5	5.65;0.02	2.22;0.14	0.89;0.35	0	0.94
Xiphinematobacteraceae	0.32;0.57	5.06;0.03	2.61;0.11	0.79;0.38	2	0.94
X27F.1492R	0.13;0.72	5.49;0.02	2.73;0.1	0.75;0.39	44	0.98
AKYH767	0.15;0.7	5.44;0.02	2.85;0.09	0.84;0.36	8	0.98
Anaerolineaceae	0.11;0.74	4.96;0.03	2.61;0.11	0.82;0.37	0	0.98
Burkholderiaceae	0.16;0.69	5.42;0.02	2.52;0.11	0.8;0.37	0	0.98
Cryptosporangiaceae	0.11;0.74	5.34;0.02	2.72;0.1	0.83;0.36	24	0.98
Elsteraceae	0.15;0.7	5.46;0.02	2.52;0.11	0.9;0.34	34	0.98
Gemmataceae	0.18;0.67	5.41;0.02	2.86;0.09	0.85;0.36	0	0.98

Legionellaceae	0.17;0.68	4.96;0.03	2.72;0.1	0.88;0.35	26	0.98
Microbacteriaceae	0.14;0.71	5.45;0.02	2.64;0.11	0.72;0.4	12	0.98
Nakamurellaceae	0.13;0.72	5.52;0.02	2.85;0.09	0.82;0.37	6	0.98
Peptostreptococcaceae	0.11;0.74	5.08;0.03	2.8;0.1	0.88;0.35	18	0.98
Pyrinomonadaceae	0.2;0.65	5.57;0.02	2.71;0.1	0.82;0.37	3	0.98
Rhizobiales_Incertae_Sedis	0.13;0.71	5.46;0.02	2.78;0.1	0.73;0.39	0	0.98
Roseiflexaceae	0.2;0.66	4.91;0.03	2.88;0.09	0.77;0.38	0	0.98
Sandaracinaceae	0.22;0.64	5.23;0.02	2.83;0.09	0.89;0.35	0	0.98
Solimonadaceae	0.18;0.67	5.55;0.02	2.8;0.1	0.76;0.38	1	0.98
Sphingobacteriaceae	0.15;0.7	5.34;0.02	2.79;0.1	0.87;0.35	39	0.98
Sphingomonadaceae	0.15;0.7	5.52;0.02	2.8;0.1	0.88;0.35	0	0.98
A0839	0;0.95	5.35;0.02	2.69;0.1	0.79;0.38	30	0.99
A21b	0.03;0.87	5.21;0.02	2.63;0.11	0.82;0.37	0	0.99
A4b	0;0.95	4.97;0.03	2.71;0.1	0.8;0.37	0	0.99
Acetobacteraceae	0.01;0.92	5.36;0.02	2.67;0.1	0.8;0.37	0	0.99
Alicyclobacillaceae	0.08;0.78	5.38;0.02	2.56;0.11	0.86;0.36	11	0.99
Beijerinckiaceae	0.05;0.82	5.33;0.02	2.77;0.1	0.84;0.36	3	0.99
BIrii41	0.02;0.9	5.35;0.02	2.64;0.11	0.82;0.37	0	0.99
Chitinophagaceae	0.08;0.78	5.46;0.02	2.72;0.1	0.8;0.37	0	0.99
Chthoniobacteraceae	0.04;0.85	5.39;0.02	2.76;0.1	0.81;0.37	0	0.99
Desulfarculaceae	0;0.99	5.32;0.02	2.71;0.1	0.8;0.37	15	0.99
Fimbriimonadaceae	0.01;0.91	5.38;0.02	2.68;0.1	0.82;0.37	7	0.99
Geodermatophilaceae	0.06;0.81	5.31;0.02	2.69;0.1	0.85;0.36	0	0.99
Gimesiaceae	0.03;0.87	5.23;0.02	2.73;0.1	0.83;0.36	39	0.99
Halanaerobiaceae	0.02;0.88	5.4;0.02	2.73;0.1	0.8;0.37	33	0.99
Hydrogenedensaceae	0;0.99	5.05;0.03	2.7;0.1	0.81;0.37	44	0.99
Hyphomicrobiaceae	0.02;0.88	5.39;0.02	2.72;0.1	0.8;0.37	0	0.99
Hyphomonadaceae	0;0.97	5.37;0.02	2.71;0.1	0.8;0.37	0	0.99
Intrasporangiaceae	0.04;0.84	5.41;0.02	2.76;0.1	0.71;0.4	0	0.99
KF.JG30.B3	0;0.95	5.37;0.02	2.68;0.1	0.8;0.37	0	0.99
Kineosporiaceae	0;0.95	5.17;0.02	2.73;0.1	0.81;0.37	1	0.99
Magnetospiraceae	0.01;0.93	5.23;0.02	2.73;0.1	0.81;0.37	49	0.99
Methylacidiphilaceae	0;0.96	5.25;0.02	2.67;0.1	0.79;0.38	13	0.99
Methyloligellaceae	0;0.98	5.25;0.02	2.71;0.1	0.8;0.37	4	0.99
Micavibrionaceae	0.02;0.89	5.4;0.02	2.48;0.12	0.81;0.37	23	0.99
Micromonosporaceae	0.04;0.84	5.17;0.02	2.75;0.1	0.84;0.36	1	0.99
Nitrospiraceae	0;0.96	5.38;0.02	2.67;0.1	0.79;0.38	0	0.99
Omnitrophaceae	0.04;0.85	5.29;0.02	2.72;0.1	0.82;0.37	4	0.99
SM2D12	0.04;0.84	5.42;0.02	2.76;0.1	0.76;0.39	8	0.99
Streptosporangiaceae	0.08;0.78	5.29;0.02	2.67;0.1	0.83;0.36	0	0.99

URHD0088	0.02;0.9	5.22;0.02	2.73;0.1	0.82;0.37	20	0.99
Vermiphilaceae	0;0.99	5.35;0.02	2.72;0.1	0.8;0.37	21	0.99

Table S3.7. Relationship between the abundance of fungal families (M) and the growth of *Lolium perenne*. Significant values derived from LME model are marked in bold. The model included the factors soil treatments (T), plant cultivars (V), interactions and blocks (B) as fixed effects. Pot of conditioning phase was used in the model as random factor. Due to high presence of zeros, we excluded observations that had >50 zeros. Relative abundances have been subjected to Hellinger transformation and p value of relative abundance have been corrected using fdr method.

Fungal family	**F-value; p-value (M)**	**F-value; p-value (B)**	**F-value; p-value (T)**	**F-value; p-value (V)**	**N 0**	***p* value (M) fdr**
Ascobolaceae	3.34;0.07	2.2;0.09	2.94;0.04	3.06;0.01	39	0.23
Aspergillaceae	1.13;0.29	2.43;0.07	2.39;0.08	2.9;0.01	0	0.53
Bionectriaceae	2.76;0.1	2.1;0.11	3;0.04	3.02;0.01	0	0.30
Cantharellales_fam_Incertae_sedis	0.34;0.56	2.16;0.1	2.88;0.04	2.87;0.01	27	0.72
Cephalothecaceae	2.17;0.14	2.29;0.08	2.56;0.06	2.96;0.01	42	0.35
Chaetomiaceae	5.05;0.03	2.15;0.1	3.37;0.02	2.9;0.01	0	0.17
Chaetosphaeriaceae	0.38;0.54	1.93;0.13	2.6;0.06	2.84;0.01	0	0.70
Chrysozymaceae	5.7;0.02	3.09;0.03	2.37;0.08	2.87;0.01	8	0.17
Cladosporiaceae	1.6;0.21	1.96;0.13	3;0.04	2.94;0.01	7	0.45
Claroideoglomeraceae	0.89;0.35	2.16;0.1	3.01;0.04	2.89;0.01	18	0.56
Clavicipitaceae	0.84;0.36	2.35;0.08	2.74;0.05	2.93;0.01	0	0.56
Coniochaetaceae	3.28;0.07	2.75;0.05	2.77;0.05	2.88;0.01	2	0.23
Cucurbitariaceae	4.33;0.04	2.25;0.09	2.57;0.06	3;0.01	7	0.17
Didymellaceae	0.95;0.33	2.21;0.09	2.51;0.07	2.93;0.01	22	0.56
Didymosphaeriaceae	1.19;0.28	2.28;0.08	2.67;0.06	2.88;0.01	0	0.53
Entolomataceae	0.24;0.63	2.16;0.1	2.61;0.06	2.85;0.01	36	0.76
Glomeraceae	2.49;0.12	2.4;0.07	2.3;0.09	2.93;0.01	0	0.31
Helotiales_fam_Incertae_sedis	0.51;0.48	2.01;0.12	2.69;0.05	2.84;0.01	44	0.70
Herpotrichiellaceae	3.82;0.05	2.47;0.07	2.92;0.04	3.03;0.01	0	0.20
Hyaloscyphaceae	0.01;0.93	2.03;0.11	2.74;0.05	2.87;0.01	39	0.95
Hydnodontaceae	4.46;0.04	2.15;0.1	2.44;0.07	2.95;0.01	21	0.17
Hypocreaceae	0.39;0.53	2.21;0.09	2.69;0.06	2.9;0.01	0	0.70
Hypocreales_fam_Incertae_sedis	0.91;0.34	2.28;0.08	2.69;0.05	2.91;0.01	0	0.56

Lasiosphaeriaceae	1.09;0.3	2.12;0.1	2.42;0.08	2.92;0.01	0	0.53
Leptosphaeriaceae	0.01;0.93	2.08;0.11	2.78;0.05	2.87;0.01	13	0.95
Lindgomycetaceae	5.87;0.02	1.89;0.14	3.24;0.03	2.95;0.01	1	0.17
Lipomycetaceae	0.31;0.58	2.06;0.11	2.71;0.05	2.91;0.01	35	0.72
Lycoperdaceae	3.61;0.06	2.71;0.05	2.5;0.07	2.97;0.01	11	0.21
Microascaceae	0.12;0.73	2.04;0.11	2.78;0.05	2.87;0.01	40	0.79
Microdochiaceae	1.12;0.29	2.34;0.08	2.61;0.06	2.94;0.01	27	0.53
Mortierellaceae	3.85;0.05	2.94;0.04	2.48;0.07	3.03;0.01	0	0.20
Mucoraceae	1.06;0.3	2.33;0.08	2.72;0.05	2.95;0.01	3	0.53
Myxotrichaceae	4.44;0.04	2.55;0.06	2.81;0.05	2.88;0.01	8	0.17
Nectriaceae	**12.54;0**	**4.8;0**	**3.08;0.03**	2.89;0.01	**0**	**0.03**
Ophiocordycipitaceae	0.04;0.85	2.08;0.11	2.58;0.06	2.88;0.01	1	0.89
Orbiliaceae	0.12;0.73	2.1;0.11	2.78;0.05	2.88;0.01	0	0.79
Paraglomeraceae	0.4;0.53	2.13;0.1	2.03;0.12	2.89;0.01	12	0.70
Phacidiaceae	0;0.99	2.08;0.11	2.75;0.05	2.87;0.01	9	0.99
Piskurozymaceae	4.75;0.03	3.27;0.02	2.62;0.06	2.97;0.01	0	0.17
Pleosporaceae	5.12;0.03	2.61;0.06	3.48;0.02	2.77;0.01	0	0.17
Psathyrellaceae	0.42;0.52	2.16;0.1	2.66;0.06	2.91;0.01	10	0.70
Pseudeurotiaceae	0.62;0.43	2;0.12	2.62;0.06	2.94;0.01	0	0.66
Pyronemataceae	0.17;0.68	2.14;0.1	2.81;0.05	2.87;0.01	40	0.78
Schizoporaceae	2.41;0.12	2.91;0.04	2.57;0.06	2.79;0.01	2	0.31
Sordariales_fam_Incertae_sedis	4.99;0.03	2.23;0.09	3.31;0.03	2.97;0.01	15	0.17
Spizellomycetaceae	0.19;0.67	2.15;0.1	2.58;0.06	2.84;0.01	34	0.78
Sporormiaceae	0.22;0.64	2.11;0.1	2.85;0.05	2.89;0.01	32	0.76
Sympoventuriaceae	2.48;0.12	2.41;0.07	2.49;0.07	2.78;0.01	34	0.31
Teratosphaeriaceae	5.3;0.02	2.54;0.06	2.3;0.09	2.97;0.01	7	0.17
Terramycetaceae	1.36;0.25	1.87;0.14	2.6;0.06	2.88;0.01	49	0.51
Trichocomaceae	0.45;0.51	2.11;0.1	2.68;0.06	2.85;0.01	2	0.70
Tricholomataceae	0.11;0.74	2.06;0.11	2.7;0.05	2.82;0.01	37	0.79
TrichosporoNAceae	5.81;0.02	3.39;0.02	2.84;0.05	3.23;0	8	0.17
Umbelopsidaceae	1.95;0.17	2.29;0.08	2.81;0.05	3;0.01	0	0.39
unidentified	1.87;0.17	1.88;0.14	2.57;0.06	2.9;0.01	0	0.39
Vibrisseaceae	6.51;0.01	2.48;0.07	2.82;0.05	3.04;0.01	4	0.17

Table S3.8. Relationship between the abundance of fungal families (M) and the growth of *Poa pratensis*. Significant values derived from LME model are marked in bold. The model included the factors soil treatments (T), plant cultivars (V), interactions and blocks (B) as fixed effects. Pot of conditioning phase was used in the model as random factor. Due to high presence of zeros, we excluded observations that had >50 zeros. Relative abundances have been subjected to Hellinger transformation and p value of relative abundance have been corrected using fdr method.

Fungal family	**F-value; p-value (M)**	**F-value; p-value (B)**	**F-value; p-value (T)**	**F-value; p-value (V)**	**N 0**	***p* value M fdr**
Ascobolaceae	0.43;0.51	0.55;0.46	2.25;0.14	1.25;0.27	29	0.84
Aspergillaceae	0.97;0.33	0.71;0.4	2.31;0.13	1.62;0.21	0	0.72
Bionectriaceae	0.09;0.77	0.51;0.48	2.28;0.13	1.38;0.24	0	0.89
Bulleraceae	0.56;0.46	0.38;0.54	2.18;0.14	1.29;0.26	47	0.83
Cantharellales_fam_Incertae_sedis	0.96;0.33	0.54;0.46	0.97;0.33	1.07;0.3	18	0.72
Cephalothecaceae	0.35;0.56	0.53;0.47	2.21;0.14	1.44;0.23	30	0.86
Ceratobasidiaceae	0.1;0.75	0.55;0.46	2.28;0.13	1.23;0.27	49	0.89
Chaetomiaceae	0;0.95	0.48;0.49	2.24;0.14	1.35;0.25	0	0.95
Chaetosphaeriaceae	8.73;0	0.32;0.57	1.76;0.19	0.42;0.52	0	0.15
Chrysozymaceae	0.13;0.72	0.5;0.48	2.37;0.13	1.44;0.23	5	0.89
Cladosporiaceae	0.2;0.66	0.4;0.53	2.25;0.14	1.28;0.26	8	0.88
Claroideoglomeraceae	0.27;0.6	0.33;0.57	2.29;0.13	1.51;0.22	20	0.88
Clavariaceae	0.01;0.93	0.49;0.49	2.24;0.14	1.35;0.25	45	0.95
Clavicipitaceae	0.01;0.92	0.46;0.5	2.16;0.14	1.25;0.27	0	0.95
Coniochaetaceae	2.12;0.15	0.08;0.78	1.75;0.19	2.09;0.15	1	0.63
Cucurbitariaceae	2.73;0.1	0.34;0.56	2.35;0.13	1.4;0.24	3	0.63
Cunninghamellaceae	0.44;0.51	0.4;0.53	2.17;0.14	1.33;0.25	38	0.84
Debaryomycetaceae	2.55;0.12	1.31;0.26	1.89;0.17	1.32;0.25	47	0.63
Didymellaceae	0.55;0.46	0.28;0.6	2.38;0.13	1.22;0.27	19	0.83
Didymosphaeriaceae	0.01;0.91	0.49;0.49	2.24;0.14	1.34;0.25	0	0.95
Entolomataceae	2.44;0.12	0.54;0.47	2.28;0.13	1.15;0.29	25	0.63
Eremomycetaceae	0.03;0.86	0.49;0.49	2.19;0.14	1.38;0.24	40	0.95
Glomeraceae	2.25;0.14	0.33;0.57	3.13;0.08	2.15;0.15	0	0.63
Helotiales_fam_Incertae_sedis	0.85;0.36	0.39;0.54	1.67;0.2	1.68;0.2	27	0.73
Herpotrichiellaceae	0.13;0.72	0.46;0.5	2.33;0.13	1.45;0.23	0	0.89
Hyaloscyphaceae	0.13;0.72	0.54;0.46	2.2;0.14	1.35;0.25	30	0.89
Hydnodontaceae	1.98;0.16	0.38;0.54	2.8;0.1	1.81;0.18	15	0.63
Hypocreaceae	2.73;0.1	0.33;0.57	2.84;0.1	2.08;0.15	0	0.63
Hypocreales_fam_Incertae_sedis	0.95;0.33	0.69;0.41	1.9;0.17	1.17;0.28	0	0.72
Lasiosphaeriaceae	2.91;0.09	0.83;0.36	2.5;0.12	1.11;0.29	0	0.63
Leptosphaeriaceae	0.47;0.5	0.67;0.42	2.38;0.13	1.4;0.24	9	0.84
Lindgomycetaceae	0.27;0.6	0.46;0.5	2.45;0.12	1.3;0.26	0	0.88
Lipomycetaceae	7.03;0.01	0.18;0.67	3.03;0.08	1.51;0.22	26	0.21
Lycoperdaceae	1.9;0.17	0.16;0.69	2.18;0.14	1.66;0.2	4	0.63

Magnaporthaceae	**13.58;0**	**0.3;0.59**	**3.85;0.05**	1.5;0.22	**48**	**0.03**
Microascaceae	3.36;0.07	0.46;0.5	1.71;0.19	1.03;0.31	32	0.63
Microdochiaceae	0.26;0.61	0.51;0.48	2.27;0.13	1.51;0.22	21	0.88
Mortierellaceae	2.09;0.15	0.28;0.6	2.98;0.09	1.82;0.18	0	0.63
Mucoraceae	2.82;0.1	0.21;0.64	3.12;0.08	1.5;0.22	2	0.63
Myrmecridiaceae	1.08;0.3	0.35;0.56	1.97;0.16	1.23;0.27	49	0.72
Myxotrichaceae	1.57;0.21	1.1;0.3	1.84;0.18	2.13;0.15	5	0.66
Nectriaceae	0.19;0.66	0.46;0.5	2.35;0.13	1.49;0.22	0	0.88
Ophiocordycipitaceae	1.73;0.19	0.75;0.39	1.39;0.24	1.17;0.28	1	0.63
Orbiliaceae	0.42;0.52	0.66;0.42	2.64;0.11	1.49;0.23	0	0.84
Paraglomeraceae	1.71;0.19	0.27;0.6	1.33;0.25	1.2;0.28	7	0.63
Phacidiaceae	0.02;0.89	0.47;0.49	2.26;0.14	1.29;0.26	8	0.95
Piskurozymaceae	1.77;0.19	0.1;0.75	2.33;0.13	1.61;0.21	0	0.63
Pleosporaceae	0.11;0.74	0.43;0.52	2.26;0.14	1.44;0.23	0	0.89
Psathyrellaceae	0.91;0.34	0.29;0.59	2.25;0.14	1.63;0.2	5	0.72
Pseudeurotiaceae	1.43;0.23	0.74;0.39	2.58;0.11	1.66;0.2	0	0.69
Pyronemataceae	0.63;0.43	0.32;0.57	2.28;0.13	1.55;0.22	38	0.82
Saccharomycetales_fam_Incertae_sedis	0.07;0.8	0.56;0.46	2.28;0.13	1.35;0.25	47	0.91
Schizoporaceae	0.12;0.73	0.56;0.46	2.17;0.14	1.22;0.27	2	0.89
Sordariales_fam_Incertae_sedis	0.98;0.32	0.5;0.48	2.32;0.13	1.6;0.21	10	0.72
Spizellomycetaceae	0.24;0.63	0.51;0.48	1.95;0.17	1.48;0.23	25	0.88
Sporormiaceae	0.22;0.64	0.52;0.47	1.9;0.17	1.47;0.23	23	0.88
Sympoventuriaceae	0.65;0.42	0.43;0.51	1.77;0.19	1.49;0.23	23	0.82
Teratosphaeriaceae	2.22;0.14	0.75;0.39	2.06;0.15	1.37;0.25	2	0.63
Terramycetaceae	0.93;0.34	0.63;0.43	1.81;0.18	1;0.32	43	0.72
Trichocomaceae	1;0.32	0.52;0.47	2.46;0.12	1.61;0.21	1	0.72
Tricholomataceae	0.03;0.86	0.48;0.49	2.18;0.14	1.28;0.26	34	0.95
TrichosporoNAceae	0.02;0.89	0.48;0.49	2.26;0.14	1.35;0.25	5	0.95
Umbelopsidaceae	1.21;0.27	0.61;0.44	2.44;0.12	1.69;0.2	0	0.72
unidentified	2.73;0.1	0.82;0.37	3.42;0.07	1.37;0.24	0	0.63
Vibrisseaceae	0.38;0.54	0.64;0.42	2.25;0.14	1.37;0.24	1	0.85

Table S3.9. Relationship between the abundance of fungal families (M) and the growth of *Schedonorus arundinaceus*. Significant values derived from LME model are marked in bold. The model included the factors soil treatments (T), plant cultivars (V), interactions and blocks (B) as fixed effects. Pot of conditioning phase was used in the model as random factor. Due to high presence of zeros, we excluded observations that had >50 zeros. Relative abundances have been subjected to Hellinger transformation and p value of relative abundance have been corrected using fdr method.

Fungal family	**F-value; p-value (M)**	**F-value; p-value (B)**	**F-value; p-value (T)**	**F-value; p-value (V)**	**N 0**	***p*-value (M) fdr**
Ascobolaceae	0.43;0.51	0.55;0.46	2.25;0.14	1.25;0.27	29	0.84
Aspergillaceae	0.97;0.33	0.71;0.4	2.31;0.13	1.62;0.21	0	0.72
Bionectriaceae	0.09;0.77	0.51;0.48	2.28;0.13	1.38;0.24	0	0.89
Bulleraceae	0.56;0.46	0.38;0.54	2.18;0.14	1.29;0.26	47	0.83
Cantharellales_fam_Incertae_sedis	0.96;0.33	0.54;0.46	0.97;0.33	1.07;0.3	18	0.72
Cephalothecaceae	0.35;0.56	0.53;0.47	2.21;0.14	1.44;0.23	30	0.86
Ceratobasidiaceae	0.1;0.75	0.55;0.46	2.28;0.13	1.23;0.27	49	0.89
Chaetomiaceae	0;0.95	0.48;0.49	2.24;0.14	1.35;0.25	0	0.95
Chaetosphaeriaceae	8.73;0	0.32;0.57	1.76;0.19	0.42;0.52	0	0.15
Chrysozymaceae	0.13;0.72	0.5;0.48	2.37;0.13	1.44;0.23	5	0.89
Cladosporiaceae	0.2;0.66	0.4;0.53	2.25;0.14	1.28;0.26	8	0.88
Claroideoglomeraceae	0.27;0.6	0.33;0.57	2.29;0.13	1.51;0.22	20	0.88
Clavariaceae	0.01;0.93	0.49;0.49	2.24;0.14	1.35;0.25	45	0.95
Clavicipitaceae	0.01;0.92	0.46;0.5	2.16;0.14	1.25;0.27	0	0.95
Coniochaetaceae	2.12;0.15	0.08;0.78	1.75;0.19	2.09;0.15	1	0.63
Cucurbitariaceae	2.73;0.1	0.34;0.56	2.35;0.13	1.4;0.24	3	0.63
Cunninghamellaceae	0.44;0.51	0.4;0.53	2.17;0.14	1.33;0.25	38	0.84
Debaryomycetaceae	2.55;0.12	1.31;0.26	1.89;0.17	1.32;0.25	47	0.63
Didymellaceae	0.55;0.46	0.28;0.6	2.38;0.13	1.22;0.27	19	0.83
Didymosphaeriaceae	0.01;0.91	0.49;0.49	2.24;0.14	1.34;0.25	0	0.95
Entolomataceae	2.44;0.12	0.54;0.47	2.28;0.13	1.15;0.29	25	0.63
Eremomycetaceae	0.03;0.86	0.49;0.49	2.19;0.14	1.38;0.24	40	0.95
Glomeraceae	2.25;0.14	0.33;0.57	3.13;0.08	2.15;0.15	0	0.63
Helotiales_fam_Incertae_sedis	0.85;0.36	0.39;0.54	1.67;0.2	1.68;0.2	27	0.73
Herpotrichiellaceae	0.13;0.72	0.46;0.5	2.33;0.13	1.45;0.23	0	0.89
Hyaloscyphaceae	0.13;0.72	0.54;0.46	2.2;0.14	1.35;0.25	30	0.89

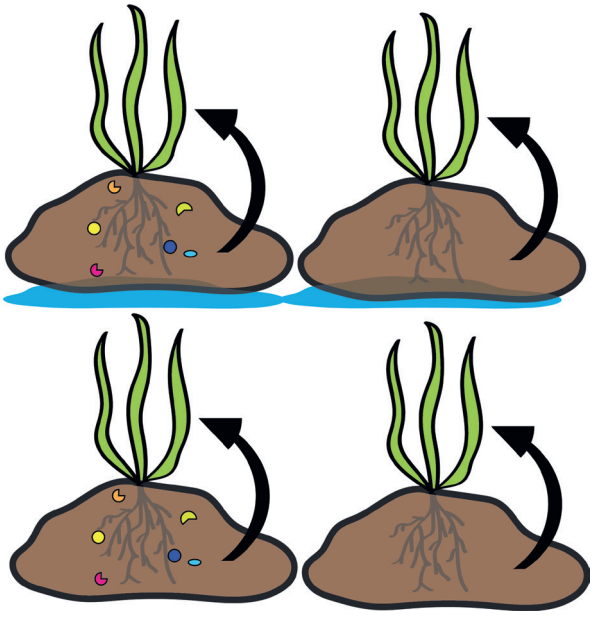
Hydnodontaceae	1.98;0.16	0.38;0.54	2.8;0.1	1.81;0.18	15	0.63
Hypocreaceae	2.73;0.1	0.33;0.57	2.84;0.1	2.08;0.15	0	0.63
Hypocreales_fam_Incertae_sedis	0.95;0.33	0.69;0.41	1.9;0.17	1.17;0.28	0	0.72
Lasiosphaeriaceae	2.91;0.09	0.83;0.36	2.5;0.12	1.11;0.29	0	0.63
Leptosphaeriaceae	0.47;0.5	0.67;0.42	2.38;0.13	1.4;0.24	9	0.84
Lindgomycetaceae	0.27;0.6	0.46;0.5	2.45;0.12	1.3;0.26	0	0.88
Lipomycetaceae	7.03;0.01	0.18;0.67	3.03;0.08	1.51;0.22	26	0.21
Lycoperdaceae	1.9;0.17	0.16;0.69	2.18;0.14	1.66;0.2	4	0.63
Magnaporthaceae	**13.58;0**	**0.3;0.59**	**3.85;0.05**	1.5;0.22	**48**	**0.03**
Microascaceae	3.36;0.07	0.46;0.5	1.71;0.19	1.03;0.31	32	0.63
Microdochiaceae	0.26;0.61	0.51;0.48	2.27;0.13	1.51;0.22	21	0.88
Mortierellaceae	2.09;0.15	0.28;0.6	2.98;0.09	1.82;0.18	0	0.63
Mucoraceae	2.82;0.1	0.21;0.64	3.12;0.08	1.5;0.22	2	0.63
Myrmecridiaceae	1.08;0.3	0.35;0.56	1.97;0.16	1.23;0.27	49	0.72
Myxotrichaceae	1.57;0.21	1.1;0.3	1.84;0.18	2.13;0.15	5	0.66
Nectriaceae	0.19;0.66	0.46;0.5	2.35;0.13	1.49;0.22	0	0.88
Ophiocordycipitaceae	1.73;0.19	0.75;0.39	1.39;0.24	1.17;0.28	1	0.63
Orbiliaceae	0.42;0.52	0.66;0.42	2.64;0.11	1.49;0.23	0	0.84
Paraglomeraceae	1.71;0.19	0.27;0.6	1.33;0.25	1.2;0.28	7	0.63
Phacidiaceae	0.02;0.89	0.47;0.49	2.26;0.14	1.29;0.26	8	0.95
Piskurozymaceae	1.77;0.19	0.1;0.75	2.33;0.13	1.61;0.21	0	0.63
Pleosporaceae	0.11;0.74	0.43;0.52	2.26;0.14	1.44;0.23	0	0.89
Psathyrellaceae	0.91;0.34	0.29;0.59	2.25;0.14	1.63;0.2	5	0.72
Pseudeurotiaceae	1.43;0.23	0.74;0.39	2.58;0.11	1.66;0.2	0	0.69
Pyronemataceae	0.63;0.43	0.32;0.57	2.28;0.13	1.55;0.22	38	0.82
Saccharomycetales_fam_Incertae_sedis	0.07;0.8	0.56;0.46	2.28;0.13	1.35;0.25	47	0.91
Schizoporaceae	0.12;0.73	0.56;0.46	2.17;0.14	1.22;0.27	2	0.89
Sordariales_fam_Incertae_sedis	0.98;0.32	0.5;0.48	2.32;0.13	1.6;0.21	10	0.72
Spizellomycetaceae	0.24;0.63	0.51;0.48	1.95;0.17	1.48;0.23	25	0.88
Sporormiaceae	0.22;0.64	0.52;0.47	1.9;0.17	1.47;0.23	23	0.88
Sympoventuriaceae	0.65;0.42	0.43;0.51	1.77;0.19	1.49;0.23	23	0.82
Teratosphaeriaceae	2.22;0.14	0.75;0.39	2.06;0.15	1.37;0.25	2	0.63
Terramycetaceae	0.93;0.34	0.63;0.43	1.81;0.18	1;0.32	43	0.72
Trichocomaceae	1;0.32	0.52;0.47	2.46;0.12	1.61;0.21	1	0.72
Tricholomataceae	0.03;0.86	0.48;0.49	2.18;0.14	1.28;0.26	34	0.95
TrichosporoNAceae	0.02;0.89	0.48;0.49	2.26;0.14	1.35;0.25	5	0.95
Umbelopsidaceae	1.21;0.27	0.61;0.44	2.44;0.12	1.69;0.2	0	0.72
unidentified	2.73;0.1	0.82;0.37	3.42;0.07	1.37;0.24	0	0.63

Vibrisseaceae	0.38;0.54	0.64;0.42	2.25;0.14	1.37;0.24	1	0.85

Chapter 4

Drought mitigates negative effects of natural microbiomes in grasses

Paola Rallo, Tanja Bakx-Schotman, Jan Kammenga, Wim H. van der Putten, S. Emilia Hannula, Koen J.F. Verhoeven



Abstract

A growing number of studies suggest that soil microbes mitigate plant drought stress. However, little is known about such beneficial microbial effects in grasses. We examined how soil microbial communities influence plant performance in response to drought. We expected drought to reduce plant performance less in the presence of soil microbes. We conducted a greenhouse experiment using microbial inocula and eight native grass species from different positions along a secondary succession gradient. We show that under optimal water supply conditions, inoculation with live soil communities substantially decreased grass biomass production, suggesting net pathogenic effects of the microbiome. However, under drought, the net microbial effect on plant growth was relatively less negative compared to ambient water supply. To explore potential underlying causes, we used amplicon sequencing to identify rhizosphere microbes. Drought affected more strongly the community structure of fungi than bacteria. Furthermore, we found no evidence that the relative abundance of arbuscular mycorrhizal fungi, which are prime candidates for mitigating drought effects on plants, is associated with alleviation of drought stress in grasses. We conclude that drought may reduce net negative microbial effects on grass performance and that this effect is consistent for plants of different successional positions. As such, drought weaken the negative effect of the soil biota in plant-soil interactions which might have major implications for plant community dynamics, and thus ecosystem functioning.

Introduction

As a result of climate change, drought events are increasing in frequency and intensity (Snyder and Harmon-Threatt 2019). These extreme climatic events may decrease plant performance and productivity, also resulting into massive plant die-offs (Snyder and Harmon-Threatt 2019; Wu et al. 2011). Droughts not only directly affect plant performance through changes in water and nutrient availability (Farooq et al. 2012) but also indirectly via changes in the structure and functioning of the soil microbiome associated with plants (Kardol et al. 2010a; McLaughlin 2011; Dai 2013; Putten et al. 2016a; de Vries et al. 2018; Ochoa-Hueso et al. 2018; Hari et al. 2020).

The roles of rhizosphere and endosphere microbes in plant health are widely acknowledged. In addition to altering nutrient cycling, soil microbes may affect plant performance through both mutualistic and antagonistic interactions (Wardle et al. 2004; Putten et al. 2016a). Beneficial microbes include mycorrhizal fungi and plant-growth promoting bacteria, while other fungi and bacteria may act as pathogens. However, climate change-induced extreme weather events may alter the strength and direction of these interactions. A major challenge therefore is to understand how extreme weather events, such as drought and severe rainfall, may affect interactions between plants and their associated microbiota, in order to further understand consequences for plant community dynamics and ecosystem productivity.

There has been a particularly growing interest in the possible beneficial effects of soil microbes on plant performance under drought (de Vries et al. 2018). Considerable attention has been given to mutualistic organisms such as arbuscular mycorrhizal fungi (AMF), as these can be

trustworthy allies for the plant due to their role in improving plant water and nutrient uptake (Mena-Violante et al., 2006; Ruiz-Lozano et al., 2015; Yooyongwech et al., 2016; Moradtalab et al., 2019). However, not all plant species equally rely on mutualists such as AMF, affecting consequently whether plants experiencing drought may have an advantage over other plants that do not (Augé 2001; Hawkes and Keitt 2015); Li et al. 2019). Research on beneficial effects of soil microbes on plant performance under drought has mainly focused on experimental inoculation with specific microbial taxa. This approach can be helpful particularly in agricultural settings to understand and enhance the role of specific microbes on plant health. However, there is limited empirical evidence that supports the beneficial role of microbes under drought when using inoculation of whole natural microbial communities instead of specific taxa (Lau and Lennon 2011; Valliere et al. 2020).

While there is considerable evidence about beneficial effects of soil microbes on plant performance under drought, consequences of drought for plant pathogens and especially their effects on plants have been less well resolved. Pathogens are thought to be better at adapting to drought events than other microbes (Newton, Johnson, and Gregory 2011) and as such, it is expected that soil pathogenicity potentially increases under drought (Van der Putten, Macel, and Visser 2010). However, pathogens are highly diverse and several of them rely more on water than others which might make their effectiveness depending on drought (Meisner and De Boer 2018). This is the case of some oomycetes (i.e. Phytium and Phytophtora) that can be find active under wet conditions (Granke and Hausbeck 2010; Velásquez, Castroverde, and He 2018). As a result, when analyzing soil communities as a whole, it can be challenging to predict the overall effects of soil-borne pathogens on plant health under drought,

as the various positive and negative components may have unpredictable and potentially opposing outcomes.

Secondary successions provide a valuable ecological context for testing hypotheses on the effects of different soil microbial communities on plant growth under drought. During secondary successions, the soil microbial communities are known to change in terms of composition and abundance from one successional stage to the next. For instance, pathogenic effects are known to dominate early stages of secondary succession whereas symbiotic mutualists becoming more dominant in mid to later stages (Kardol et al. 2013; Hannula et al. 2017; Elly Morriën et al. 2017). As a result, the interaction between these soil microorganisms and plants can differ depending on the plant's position along a successional gradient (Wubs et al. 2019). For instance, we have evidence that late successional plants are more responsive to beneficial microbes such as AMF (Cheeke et al. 2019) which could result in a better tolerance against drought.

In a greenhouse experiment, we tested how the presence of natural soil microbiomes affects plant performance under drought. We used two different microbial inocula derived from soils from different successional stages and 8 native grass species occurring along the secondary successional gradient. We tested the following hypotheses:

(i) Plant performance under drought will be enhanced by the presence of live soil microbial inocula compared to plants growing in sterilized soil.

(ii) Later-successional plants will be less susceptible to drought than earlier-successional as a result of stronger relationship with beneficial microbes (i.e., AMF).
(iii) AMF relative abundance is related to improved plant's tolerance to drought.

Material and methods

Study system

Based on their known occurrence along the successional gradient, 8 grass species were selected from a list of native grass species that would germinate from commercially available seed. Seeds were supplied by Weberseeds (Vaals, The Netherlands) and Cruydt-Hoeck (Nijberkoop, The Netherlands). We tested our hypotheses using four earlier-successional (*Alopercurus pratensis, Apera spica-venti*, *Phleum pratense, Poa annua)*, and four later-successional grass species (*Agrostis capillaris, Anthoxantum odoratum, Festuca ovina, Nardus stricta).* Seeds were surface sterilized using a 2.5% hypochloride solution for one minute and then rinsed three times with demineralized water. Subsequently, seeds were placed on sterile glass beads and let germinate for two to three weeks in a growth cabinet (16h light/8h dark, T 20°C day/16°C night).

We collected soil from the Veluwe, a central region of the Netherlands, from sites that represent a secondary succession chronosequence of abandoned agricultural fields. The fields have been used as a framework for previous studies on plant secondary succession in relation to changes in abiotic and biotic properties of the soil (Kardol, Bezemer, and van der Putten 2006; Hannula et al. 2017; Morriën et al. 2017). We used an inoculum approach consisting of 85% sterilized background that was collected from a former agricultural field abandoned in 1995 (Mossel, The

Netherlands; N 52.06141, E 5 ° 75.266) and 15% inoculum soil. The inoculum approach allowed us to largely avoid possible nutrient effects (Brinkman et al. 2010).

The inocula used were collected from 6 locations, (three agricultural and three late-successional fields - Table S4.1). To maximize microbial diversity within succession stage, the inocula collected were pooled and homogenized to generate a single inoculum soil mixture per successional stage. We took this mixed soil sampling (MSS) approach (Gundale et al. 2019), because we were primarily interested in testing how early versus mid/late succession grasses were express their responses to drought under two contrasting soil biological compositions of early versus later secondary succession characteristics. The background soil and half of the soil inocula of the different successional stage was first sieved through a 10-mm sieve to remove stones and other large particles and then γ-sterilized (25 KGray, by Syngenta, Ede, The Netherlands). We stored the other half of the inoculum soil (*live soil*) at 4 °C until the start of the experiment.

Experimental design

The experiment was performed in a climatised greenhouse at 16/8 h light/dark and 20/15 °C Day/night conditions. In each pot, three plants were planted in monocultures according to a full randomized block design with eight blocks, 2 watering levels (control, drought), 2 inoculum types (agricultural, late-successional), 2 microbial conditions (sterilized, live) and 8 grass species (Figure S4.1). Each block contained one replicate of the treatment groups for a total of 64 pots per block. During the first four weeks of the experiment, the soil moisture content of all pots was maintained at 15% (w/w) by watering the pots to compensate unequal

losses. After four weeks, we applied the drought treatment where half of the pots were maintained at 7.5% (w/w) by watering the pots to weight for three weeks. Pots were fertilized to avoid nutrient deficiencies using 5% Hoagland nutrient solution on weekly basis for 5 weeks. After seven weeks of plant growth, shoots were clipped, dried at 60 °C until constant weight, and weighed, whereas roots were first washed and then dried at 60 °C, and weighed to determine biomass.

Microbial DNA extraction and sequencing

Rhizosphere soil samples for microbial analysis were collected from each pot at the end of the drought treatment. The roots were first shaken to remove the soil surrounding the roots (bulk soil) and then the soil attached to the roots was collected. Soil was stored at -80°C prior to extraction. DNA was extracted from 0.25 g of soil using the Power Soil DNA extraction kit (Qiagen, Hilden, Germany). Bacterial and fungal DNA was amplified using respectively the primers 515F/806R (5′-GTGCCAGCMGCCGCGGTAA-3′/5′-GGACTACHVGGGTWTCTAAT-3′) targeting the V4 region of the 16S rRNA gene (Caporaso et al. 2012) For fungi, the primers ITS4/ITS9 (5′TCCTCCGCTTATTGATATGC-3′/5′-GAACGCAGCRAAIIGYGA-3′) targeting intergenic transcribed spacer (ITS2) region were used (Ihrmark et al. 2012). Amplicon library preparation with Nextera tags and sequencing (Illumina MIseq PE 250) were performed at McGill University and the Génome Québec Innovation Centre (Canada).

Statistical analyses

The effects of the presence of microbes on plants under drought stress growing in different soils was measured subjecting the biomass data to ANOVA. Plant biomass was log-transformed prior to analyses to fulfil

ANOVA assumptions. A linear mixed model [lmer function in R- (Bates et al. 2015)] was used to model the effects of grass species, plant successional stage, inoculum type, inoculum treatment (sterilization or live), water treatment, block, and all possible interactions on plant biomass. The factors microbes, inoculum, plant successional stage, plant species, drought were considered fixed effects in the models whereas block was considered as random factor. In the model, the variable "plant species" was nested in “plant successional stage”. Tukey's HSD post hoc tests were performed for biomass data and the interaction between water and inoculum treatments to highlight significant differences.

The raw 16S and ITS sequence reads were processed using Dada2 (v. 1.12) (Callahan, 2016) and Pipits (v. 2.3) pipelines (Gweon et al. 2015). The SILVA (v.132) database was used to classify bacteria whereas the UNITE (v. 8.0;) database (Abarenkov et al. 2010) was used for the identification of fungi, and the ITSx extractor was used to extract fungal ITS regions. The classification of fungal operational taxonomic units (OTUs) into potential functions was done using FUNGuild (Nguyen et al. 2016) and the assignment was further curated using an in-house database (Hannula et al. 2017). The OTUs were grouped into saprotrophs, plant pathogens, plant endophytes and others (i.e., fungal/animal-plant pathogens). Multiple assignments were included in case of uncertain fungal guilds. All reads not belonging to bacterial or fungal kingdoms were excluded from the datasets. To normalize our data, we followed a compositional approach (Gloor et al. 2017) using the Total-Sum Scaling (TSS). A PerMANOVA model was constructed using Bray-Curtis distances [vegan package in R (Oksanen et al. 2013)] to determine the effects of grass species, plant successional stage, inoculum type, inoculum treatment (sterilization or live), water treatment, block, and all

possible interactions on soil microbial community structure. To determine the total variation explained by a variable in the model, we used the R^2 values derived from the model whereas visualization was performed via Principal coordinate analysis (PCoA). To explore possible effects of the relative abundance of AMF on the variation in plant biomass production, linear mixed models (lmer function in R) were built separately for control and drought conditions. The relative abundances of AMF and fungal plant pathogens were fit as a cofactor in the models.

Results

Effect of live microbiome on plant performance under drought

To test whether the soil microbiome affects grass performance under drought, we compared the plant biomass produced in sterilized and live soil inocula. Under ambient water supply (control), all plant species had decreased biomass production in live soil compared to sterilized soil, suggesting a net pathogenic soil microbiome effect. Under drought conditions, these negative effects were less strong suggesting that drought might reduce net pathogenic microbial effects on plant performance (Table 4.1, water treatment x microbial treatment $p < 0.05$; Figure 4.1a). Irrespective of the presence of a live microbiome, there was a strong interactive effect between water treatment and plant species (Table 4.1; water treatment x plant species $p < 0.001$, Figure S4.2) and plant successional stage (Table 4.1; water treatment x plant succession $p < 0.001$), indicating differences in the responses grass successional stage and of grass species to drought. For example, the late successional *N. stricta* was least affected by drought, whereas the early-successional *A. pratensis* and *P. pratense* and the later-successional *A. capillaris* and *A. odoratum* are more strongly affected by drought than other grass species

(Table 4.1; water treatment x plant species $p < 0.001$, Figure S4.2). However, we found no evidence that the effect of microbes on plant response to drought is different between plant successional stages (Table 4.1, water treatment x microbes x plant stage, $p > 0.05$) or to plant species identity (water treatment x microbes x plant species, $p > 0.05$). In fact, all grass species with the exception of *A. spica-venti* and *A. pratensis* consistently produced less biomass in presence of soil microbes both under optimal water conditions and drought (Figure 4.1 B-C).

Table 4.1. Linear mixed model of the effect of water treatment (W), Microbial treatment(M), plant successional stage (P), Inoculum type (I) and plant species (S) and interactions on total biomass of feedback phase. Plant species (S) was nested in plant successional stage (P). was used in the model as random factor.

Factors	df	F value	*p* value
Water treatment (W)	1,509	1905.17	$p < 0.001$ ***
Microbial treatment (M)	1,509	68.03	$p < 0.001$ ***
Plant succession (P)	1,509	977.54	$p < 0.001$ ***
Inoculum type (I)	1,509	27.48	$p < 0.001$ ***
Plant species (S)	6,509	265.83	$p < 0.001$ ***
W x M	1,509	8.020	$p < 0.01$ **
W x P	1,509	62.11	$p < 0.001$ ***
Mx P	1,509	17.49	$p < 0.001$ ***
W x I	1,509	0.19	$p > 0.05$
I x M	1,509	0.33	$p > 0.05$
P x I	1,509	15.08	$p < 0.001$ ***
W x S	6,509	25.32	$p < 0.001$ ***
M x S	6,509	2.33	$p < 0.05$ *
W x M x P	1,509	1.36	$p > 0.05$
W x I x M	1,509	0.11	$p > 0.05$
S x I	6,509	2.85	$p < 0.01$ **
W x P x I	1,509	4.25	$p < 0.05$ *
I x P x M	1,509	0.65	$p > 0.05$
W x M x S	6,509	1.22	$p > 0.05$
W x S x I	6,509	1.75	$p > 0.05$
I x S x M	6,509	0.78	$p > 0.05$
W x I x P x M	1,509	0.09	$p > 0.05$
W x I x S x M	6,509	1.18	$p > 0.05$

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

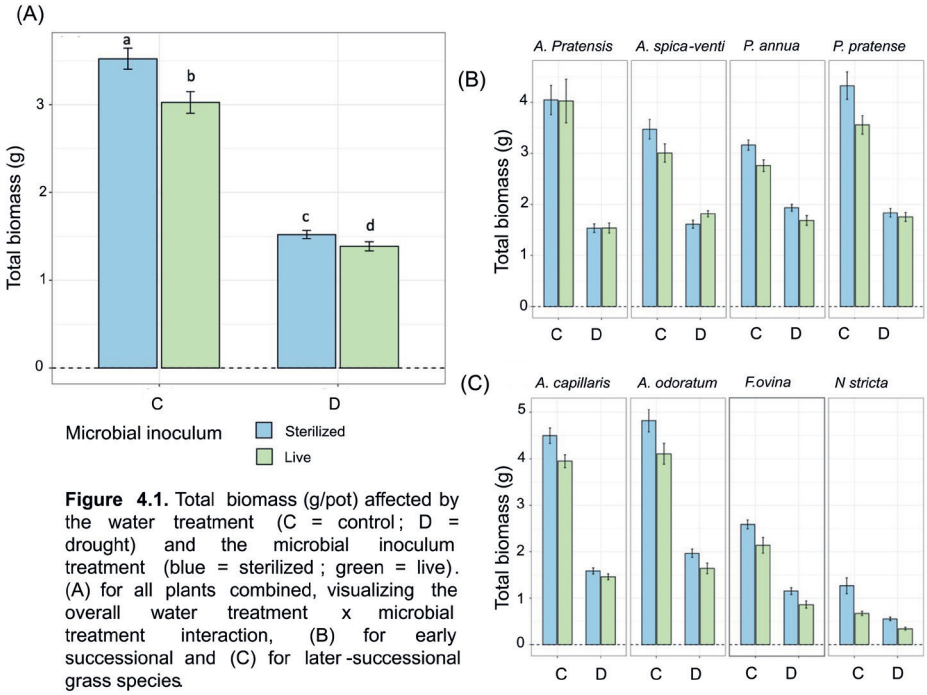


Figure 4.1. Total biomass (g/pot) affected by the water treatment (C = control; D = drought) and the microbial inoculum treatment (blue = sterilized; green = live). (A) for all plants combined, visualizing the overall water treatment x microbial treatment interaction, (B) for early successional and (C) for later-successional grass species.

4

Effects of water treatment and inoculum type on bacterial and fungal communities

We assessed the effects of water treatment and inoculum type on the soil microbial community structure using Bray-Curtis distance based PCoA of amplicon sequencing data (Figure 4.2). The strongest effect was observed between the two different inocula in shaping the structure of rhizosphere soil communities, explaining 25% of the variation for fungi (PerMANOVA, $p < 0.001$, Table S4.2) and 17% for bacterial community structure ($p < 0.001$, Table S4.2). Water treatment significantly altered the composition of the microbial community with stronger effects for fungi (R^2=0.02 $p < 0.01$; Table S1) than bacteria (R^2=0.008, $p < 0.001$; Table S4.1). Plant successional stage significantly explained community composition of bacteria (R^2=0.007, $p < 0.01$, Table S4.2) but not of fungi

(R^2=0.004, p > 0.05, Table S4.2). Plant species did not significantly affect the microbial composition of either bacteria or fungi (bacteria: R^2=0.038, p > 0.05, Table S4.2; fungi: R^2=0.023, p > 0.05). Furthermore, we also detected an interaction between inoculum type and water treatment, which suggests that the two microbial communities were differentially affected by drought (bacteria: R^2=0.007, p < 0.05; fungi: R^2=0.01, p < 0.05;). When examining soil fungi, we detected a significant interaction between the type of inoculum and the plant successional stage, which suggest that the successional position of plants affects the type of microbial communities in a community-specific manner (R^2=0.01, p < 0.05; Table S4.2).

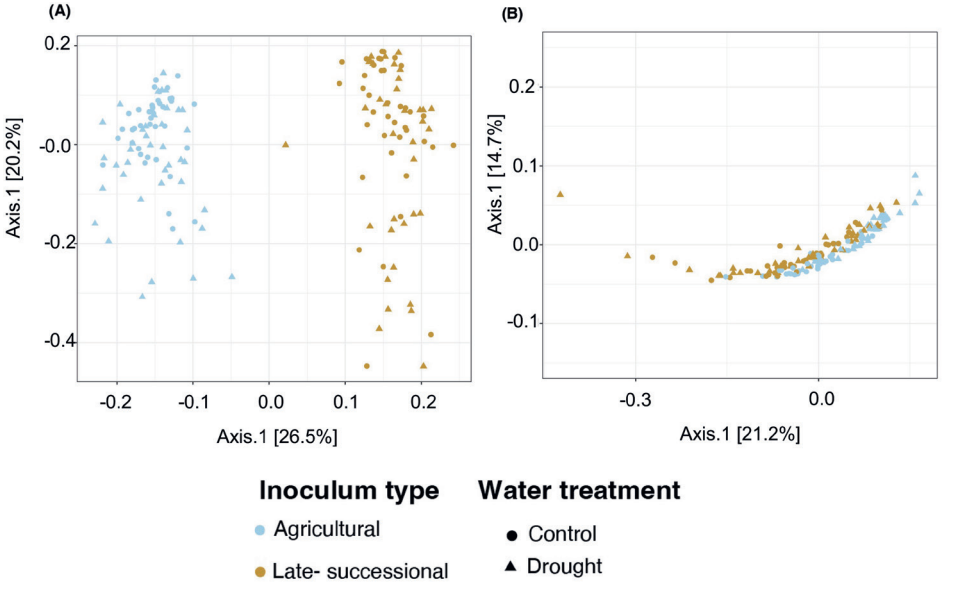


Figure 4.2. PCoA of fungal (A) and bacteria (B) communities using Bray Curtis distance, in rhizosphere soil samples after inoculation with different inoculum types and under different watering treatments. Statistics are shown in Table S4.2.

AMF and plant pathogens

To explore potential causes of the microbial effects observed on plant biomass production under drought, we decided to focus on two of the main fungal groups that affect plant performance: AMF and fungal plant pathogens. To test our hypothesis (iii) on the potential beneficial role of AMF in plant's tolerance to drought, we tested of the relative abundance of AMF was associated with plant biomass production under drought. No such effect was detected, which suggests that AMF did not play a role in influencing plant growth under drought ($p > 0.05$, Table S4.4). However, when testing the effects of drought on the relative abundance of AMF and plant pathogens, a substantial decrease of the relative abundance of AMF under drought appeared ($F = 65.47$, $p < 0.001$, Table S4.3). The reduction of AMF between control and drought was stronger in the agricultural inoculum than in the late inoculum where no AMF were detected under drought conditions (Figure 4.3). Moreover, the relative abundance of AMF was also affected by the interaction between water treatment and plant successional stage ($F = 6.35$, $p<0.001$, Table S4.3) and by the interaction between water treatment and plant species within succession ($F = 2.58$, $p<0.05$, Table S4.3). These findings thus indicate that the relative abundance of AMF might respond differently to water treatment depending on the plant successional position (and plant species within succession). When examining the relative abundance of pathogens, we detected a strong effect of the type of inoculum (inoculum type $F=15.32$, $p < 0.001$, Table S4.3). Specifically, plant pathogens were relatively more abundant in late-successional soils than in agricultural soils (Figure 4.3) and their relative abundance decreased under drought although the effect was not strong ($F=6.29$, $p < 0.05$). Like for AMF, we detected no significant association between plant biomass and relative abundance of plant pathogens under drought ($p > 0.05$, Table S4, Figure S4.3).

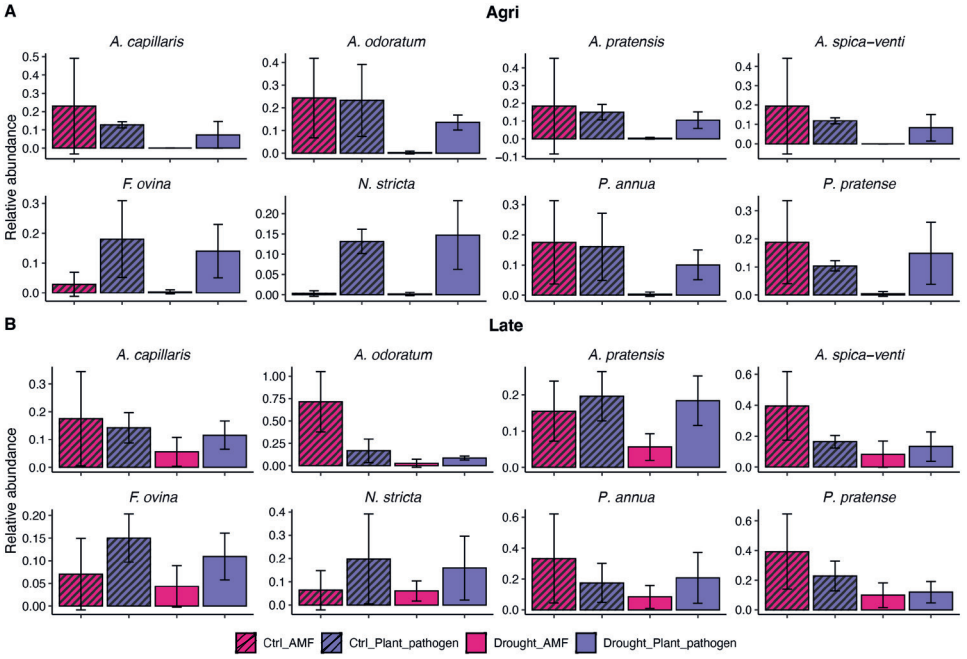


Figure 4.3. (A) Relative abundance of AMF (pink) and plant pathogen (purple) in agricultural soils and (B) in late-successional soils, both under drought (striped) and control ("Ctrl", not striped). Statistics are shown in Table S4.3.

Discussion

The main question of this study was to investigate whether early and late successional grasses harness soil microbes for better resistance to drought. We found that under optimal water conditions, inoculation with natural live soil communities leads to a decrease in plant biomass production. A key finding of this study is that, under drought, these negative effects are less severe. The microbial effects observed on plant growth under drought could not be related to an increased relative abundance of AMF, as we had expected. In addition to the proposed 'harnessing of plants against drought' effect of the microbiome, we propose an alternative hypothesis, which is that drought may

disproportionally reduce the growth-inhibiting effects of soil-borne pathogens.

Effects of soil microbiome on plant performance under drought

We compared effects of live versus sterilized soil microbial inocula on plant biomass production. Under optimal water conditions (control), we observed a substantial decrease of plant total biomass production when confronted with living microbiome This finding suggests that pathogenic microorganisms present in the natural environment where these grass species grow may have overwhelmed possible positive effects of AMF and other growth-promoting microbes. Consequently, plant biomass production was substantially decreased. This is consistent with previous studies that have used soils along a similar successional gradient and have found that pathogenic interactions dominate (Heinen et al. 2020; Hannula et al. 2020; 2021). Moreover, our inoculation approach prevents influences of nutrient flushes that may confound comparisons between live and sterilized soil treatments (Troelstra et al. 2001). While our experimental approach prevents nutrient limitation to be a major factor driving the negative effects observed under control, we cannot fully rule out the possibility that nutrients might have played minor roles. Interestingly, under drought conditions these negative effects on plant biomass production caused by the presence of microbes were substantially reduced. This observation can have different underlying causes such as relatively less effects of pathogens, or, by contrast, relatively more effects of beneficial microbes under drought than under control watering conditions.

Rhizosphere microorganisms

To further explore possible causes of our findings, we studied the rhizosphere microbes via amplicon sequencing. We show that water treatment significantly altered the composition of the microbial community with stronger effects for fungi than bacteria. This is in contrast with previous suggestions that fast-growing organisms, such as bacteria tend to be more vulnerable to stress than slow-growing soil organisms such as fungi (de Vries and Shade 2013; de Vries et al. 2012; Barnard, Osborne, and Firestone 2013; Ochoa-Hueso et al. 2018). Fungi and bacteria differ in their resilience and resistance (Hestrin et al. 2022; de Vries et al. 2018) and duration and severity of drought plays a role. The strongest effect on both bacterial and fungal community composition was observed for the type of inoculum.

Due to our MSS approach (Gundale et al. 2019) we cannot unequivocally say that rhizosphere microbial communities are generally different between agricultural and late-successional soils. However, our main aim was to learn about microbial effects on plant growth under drought. We tested this using two different soil inocula, without aiming to provide conclusive answers about how agricultural and late-succesional soils differ in this capacity. Moreover, as effects of drought on bacterial and fungal community structure vary depending on the type of inoculum, we propose that different soil microbial communities respond differently to drought. Fungal community structure was affected by an interactive effect between type of inoculum and plant succession stage, which suggests that there is a potential for different soil feedback effects.

Fungal plant pathogens

To further explore whether the effects observed under drought could be attributed either to a weaker effect of pathogens or to effects driven by beneficial microbes, we combined information on fungal community composition and functions. We detected a decrease in abundance of plant pathogens under drought, which was surprisingly uniform across species. This result is in line with the knowledge that some pathogens (i.e., oomycetes group) depend more on water than others and thus it is possible that the activity of drought-sensitive pathogens is reduced. As such, negative microbial effects on plant performance might be mitigated rather than favored under drought. Given that pathogens dominate the net microbiome effect, and that their abundance decrease under drought, we argue that the interactive effect between water treatment and microbial treatment on plant growth might have been caused by reduced pathogenic effects under drought. We suggest that such possibility might operate next to possible harnessing effects of microbes towards plants.

Effects of drought on relative abundance of pathogens were not plant species specific. This result might suggest that soil-borne fungi present in natural environments might not influence plant diversity and productivity, which is in contrast to previous work showing that fungal pathogens drive ecosystem biodiversity (Mommer et al. 2018). Nevertheless, drought can have profound effects on plant physiology, promoting for example the synthesis of plant defense compounds against pathogens, which could potentially provide an advantage for plant (Fang and Xiong 2015; J. Yang, Kloepper, and Ryu 2009; Fuchslueger et al. 2016; Poudel et al. 2021). As such, plants could shift from being strongly affected to being less or not at all affected by pathogens under ambient

versus drought conditions, while the pathogens could range from being able to cause severe disease to being only weakly pathogenic. Such scenario suggests that drought might weaken the role of the soil biota in plant-soil interactions. That possibility would have major implications for understanding how climate change-induced extreme weather events might influence plant community dynamics and thus ecosystem functioning. (Pugnaire et al. 2019; De Long et al. 2019).

AMF

We examined AMF via rhizosphere sequencing using the fungal extraradical hyphae as a proxy to detect drought effects. We show reduced relative abundance of AMF compared to other fungi under drought, and this effect was highly dependent on the plant species. Surprisingly, the relative abundance of AMF in agricultural soils was almost zero in some of the species. This result could be a potential consequence of agricultural management processes (i.e., long lasting use of fungicide and inorganic fertilizers and reduced host crop diversity) which can reduce AM fungal abundance and diversity (Abbott and Robson 1991; Johnson 1993; Helgason et al. 1998). This result contrasts previous findings from well-established grasslands that show that the abundance of AMF tends to increase under drought with potential benefits for plant tolerance to stressful condition, such as dried soils (de Vries et al. 2018). However, new evidence about the sensitivity of soil AM fungal communities to drought, and its association with plant community dynamics emerged (Fu et al. 2022). Our results suggest the possibility that under stressful conditions, these grass species may not strongly rely on mutualistic interactions. Furthermore, grass species are charactered by thin roots, which is a trait that often not favoured by AMF.

At the individual plant level, having more AMFs was not associated with enhanced growth under drought. In fact, we found no correlation between AMF relative abundance in the rhizosphere and total plant biomass production under drought, suggesting that AMF did not alleviate the negative effects of drought. We cannot fully rule out the possibility that the reduced negative effects could be driven by other beneficial microorganisms (i.e., plant-growth promoting bacteria). Nevertheless, our results might be to some extent related to indirect effect of AMF via for instance root-shoot ratio rather than a direct effect, (Lozano et al. 2022; 2021; Wilschut and van Kleunen 2021). Together, the lack of correlation between plant biomass and the relative abundance of AMF, as well as the AMF decrease in relative abundance under drought, suggest that AMFs are not counteracting the negative effects of soil pathogens on grass growth observed under control conditions. Therefore, our results contrast the conclusions of prior studies showing that microbes can harness plants to drought (Valliere and Allen 2016; Kannenberg and Phillips 2017; de Vries et al. 2020; Prudent et al. 2020) but are more in line with the idea that drought might neutralize plant-soil feedbacks (Fry et al. 2018; Gao et al. 2022).

Grass-successional position

Plants can interact differently with soil microbes based on their successional positions (De Deyn et al. 2003; Kardol, Martijn Bezemer, and van der Putten 2006) and this could lead to improved tolerance to drought stress. This is especially true in late-successional species due to their stronger relationship with beneficial microbes and to their growing strategies and better ability to preserve resources in comparison with early-successional plants (Kardol et al. 2013) Contrary to our

expectation, we found that biomass responses to water treatment were surprisingly homogeneous across grass successional position and between species. Although we do not know the exact underlying mechanisms, we observed a significant shift in fungal community composition in response to drought and a substantial decrease in relative abundance of AMF, which makes it highly likely that drought may weaken grass interactions with AMF. As such, later-successional grass might lose their potential advantage under drought. Another possible explanation is that the grass species used in this study may not depend as much as other plant species do on mutualistic relationships under drought. Such suggestion may mean that drought effects would be more evident for forbs and other plant species, or for grasses that rely more on AMF.

Conclusions

Our work provides evidence that drought mitigates the negative microbial effects of natural soil microbiomes on grass growth and that this effect is consistent for grasses of different successional stages. The reduced severity of negative microbial effects did not appear to be driven by AMF, which contrasts the idea that AMF improve plant tolerance to drought. These results provide a new perspective on drought effects on plant-soil interactions, as they suggest that grass species and associated natural microbiomes might become uncoupled under drought, instead of grasses relying on the beneficial effects of their soil-borne microbiomes. These findings have major implications for understanding and predicting the roles of grasses in plant community dynamics and thus ecosystem functioning under ongoing climate change.

Acknowledgments

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Supplementary material

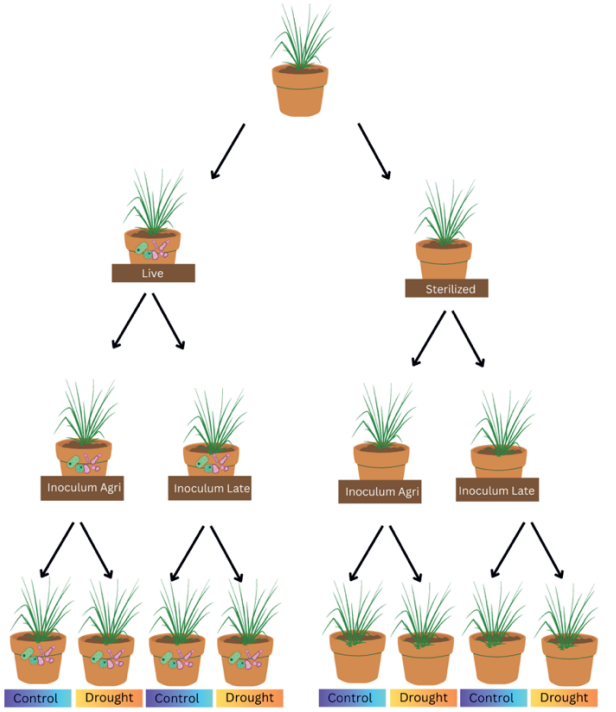


Figure S4.1. Experimental design. Each plant was first growing in soil with a live soil inoculum and sterilized inoculum. The inocula were originating from either agricultural or late-successional fields. After 4 months of growth, grasses were subjected to two water treatments (control and drought) for three weeks. See methods for more information.

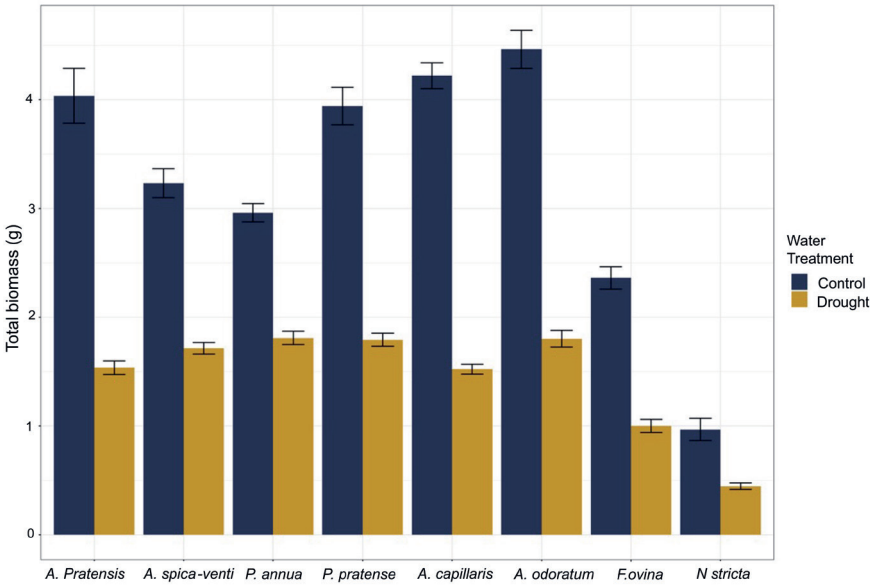


Figure S4.2. Total biomass (g) of early successional (*Alopercurus pratensis, Apera-spica venti, Poa annua, Phleum pratense*) and later-successional (*Agrostis capillaris, Anthoxanthum odoratum, Festuca ovina, Nardus stricta*) grass species when grown under control (blue) and drought (gold) treatments. Bars and whiskers represent log10 transformed biomass + SE using a linear mixed model. Statistics is shown in Table 4.1.

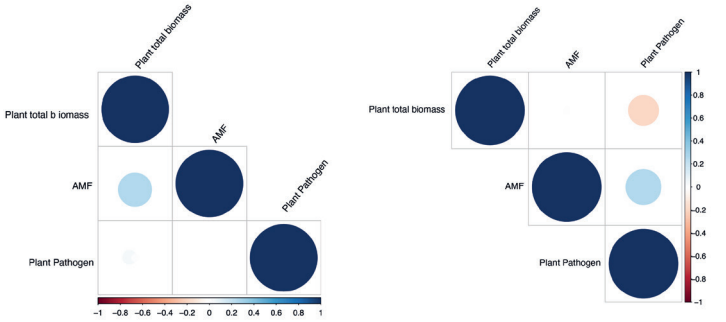


Figure S4.3. Correlations between plant total biomass log10 transformed and relative abundance of AMF and Plant pathogens. Statistics s shown in Table S4.4.

Table S4.1. List of fields used in the experiment.

Field type	Field name	abandoned since	Coordinates
Agricultural soil (inoculum)	Akker Reijerskamp 1 (AR1)	In production	N52° 00.893' E5° 47.241'
	Akker Reijerskamp 3 (AR3)	In production	N52° 00.996' E5° 47.357'
	Akker Ginkel (AG)	In production	N52°00.50118 E5.731445'
Late successional soil (inoculum)	Mossels Veld (MV)	1985	N52° 04.312' E5° 44.168'
	Nieuw Reemst (NR)	NA	N52° 02.563' E5° 46.530'
	Dennenkamp (DK)	1982	N52° 01.718' E5° 47.928'
background soil	Mossel	1995	N 52.06141, E 5 ° 75.266

Table S4.2. PerMANOVA analysis of bacterial and fungi.

	Fungi				*Bacteria*			
Factors	df	R^2	F	*p*	df	R^2	F	*p*
Inoculum type (I)	1,154	0.254	53.24	***p* <0.001**	1,159	0.17	2.73	***p* < 0.001**
Water treatment (W)	1,154	0.021	4.42	***p* < 0.01**	1,159	0.008	1.33	***p* < 0.001**
Plant succession (P)	1,154	0.004	1.10	$p > 0.05$	1,159	0.007	1.17	***p* < 0.01**
Plant Species (S)	6, 154	0.023	0.79	$p > 0.05$	6, 159	0.038	1.02	$p > 0.05$
I x W	1,154	0.010	2.11	***p* < 0.05**	1,159	0.007	1.17	***p* < 0.05**
I x P	1, 154	0.012	2.46	***p* < 0.05**	1, 159	0.006	1.06	$p > 0.05$
W x P	1, 154	0.005	0.99	$p > 0.05$	1, 159	0.006	0.99	$p > 0.05$
I x W x P	1, 154	0.004	0.92	$p > 0.05$	1, 159	0.006	1.03	$p > 0.05$
I x S	6, 154	0.022	0.76	$p > 0.05$	6, 159	0.038	1.03	$p > 0.05$
W x S	6, 154	0.029	1.01	$p > 0.05$	6, 159	0.037	0.99	$p > 0.05$
I x W x S	6, 154	0.028	0.97	$p > 0.05$	6, 159	0.038	1.04	$p > 0.05$

Table S4.3. Linear mixed model of the effect of Inoculum type (I), Water treatment (W), Plant succession (P), Plant Species (S), and interactions on relative abundance of AMF and Plant pathogens. Plant species (S) was nested in Plant Succession (P). Block was used as random factor. Relative abundances have been subjected to Hellinger transformation.

	AMF			Plant pathogens		
Factors	df	F	*p*	df	F	*p*
Inoculum type (I)	1,154	17.08	***p* < 0.001**	1,154	3.00	*p* > 0.05
Water treatment (W)	1, 154	64.83	***p* < 0.001**	1, 154	6.30	***p* < 0.05**
Plant succession (P)	1,154	1.91	*p* > 0.05	1,154	0.00	*p* > 0.05
Plant Species (S)	6, 154	5.18	***p* < 0.001**	6, 154	0.84	*p* > 0.05
I x W	1,154	2.32	*p* > 0.05	1,154	0.02	*p* > 0.05
I x P	1, 154	0.78	*p* > 0.05	1, 154	0.16	*p* > 0.05
W x P	1, 154	6.71	***p* < 0.05**	1, 154	0.08	*p* > 0.05
I x W x P	1, 154	0.49	*p* > 0.05	1, 154	0.54	*p* > 0.05
I x S	6, 154	2.02	*p* > 0.05	6, 154	0.64	*p* > 0.05
W x S	6, 154	5.47	***p* < 0.001**	6,154	0.33	*p* > 0.05
I x W x S	6, 154	2.13	*p* > 0.05	1, 154	0.93	*p* > 0.05

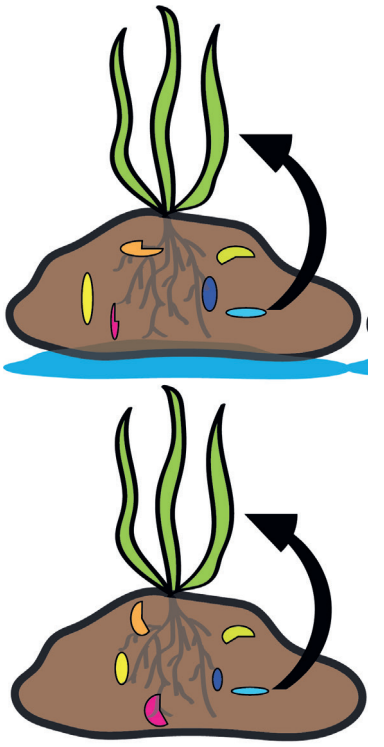
Table S4.4. Relationship between the abundance of AMF and Plant pathogens with the total biomass of grass species. Significant values derived from LME model are marked in bold. The model included the factors Inoculum type (I), Water treatment (W), Plant succession (P), Plant Species (S) as fixed effects. Plant species (S) was nested in Plant Succession (P). Block was used in the model as random factor. Relative abundances have been subjected to Hellinger transformation.

	control			Drought		
Factors	df	F	*p*	df	F	*p*
AMF	1, 79	0.36	$p > 0.05$	1,75	0.98	$p > 0.05$
Inoculum type (I)	1, 79	3.58	$p > 0.05$	1, 75	0.29	$p > 0.05$
Plant succession (P)	1, 79	151.70	***p* < 0.001**	1, 75	104.08	***p* < 0.001**
Plant Species (S)	6, 79	48.16	***p* < 0.001**	6, 75	22.53	***p* < 0.001**
AMF x P	1, 79	0.001	$p > 0.05$	1, 75	0.04	$p > 0.05$
I x P	1, 79	0.19	$p > 0.05$	1, 75	0.52	$p > 0.05$
I x S	6, 79	1.26	$p > 0.05$	6, 75	0.21	$p > 0.05$
Plant pathogens	1, 79	0.40	$p > 0.05$	1, 75	0.55	$p > 0.05$
Inoculum type (I)	1, 79	7.19	***p* < 0.05**	1, 75	1.45	$p > 0.05$
Plant succession (P)	1, 79	86.10	***p* < 0.01**	1, 75	71.02	***p* < 0.001**
Plant Species (S)	6, 79	67.88	***p* < 0.001**	6, 75	20.85	***p* < 0.001**
Plant pathogens x P	1, 79	2.33	$p > 0.05$	1, 75	0.03	$p > 0.05$
I x P	1, 79	0.52	$p > 0.05$	1, 75	0.23	$p > 0.05$
I x S	6, 79	2.01	$p > 0.05$	6, 75	0.28	$p > 0.05$

Chapter 5

Drought effects on fungivorous and bacterivorous nematodes in soil and roots, and possible consequences for grass biomass

Paola Rallo, Nyasha Chikwature, Rutger Wilschut, Arjen de Jonge, S. Emilia Hannula, Koen J.F. Verhoeven, Wim H. van der Putten, Jan Kammenga



Abstract

Most work on the effects on nematodes on plants has focused on root feeders under controlled conditions. However, in the real world, nematode communities also include other feeding types, such as bacterivores and fungivores, and their activities may depend on variable abiotic conditions, such as extreme droughts and rainfall. Here, we tested the impact of drought on different natural nematode communities in the soil and roots of two grass species, *Agrostis capillaris* and *Alopecurus pratensis* and assessed potential consequences for grass growth. Drought reduced nematode communities in the soil, whereas it substantially promoted them in the roots of the two grass species. Nematode communities were mainly comprised of fungivores and bacterivores, whereas root herbivores and omnivores were less abundant. Fungivorous nematodes were more sensitive to drought than bacterivores. Under drought stress, we observed negative relations of total grass biomass with both fungivores in the soil and bacterivores in the roots. These findings indicate that the growth of the studied grass species may be influenced, to some degree, by changes in nematode communities and their distribution, due to drought.

Introduction

Nematodes form an abundant and functionally diverse part of soil biodiversity, and have critical roles in nutrient cycling, decomposition, and other biogeochemical processes (Bardgett et al. 1999; Yeates and Bongers 1999; Wilschut and Geisen 2021). Changes in nematode populations could have far-reaching effects on ecosystem functioning, including plant growth, community-dynamics. Most attention has been given to root feeders because they represent a major threat to plant performance and productivity especially in grasses, due to alteration of plant uptake of water and nutrients (Nicol et al. 2011; Viketoft and van der Putten 2015; Russel E. Ingham and Detling 1990; Franco et al. 2019). However, nematodes can have indirect positive effects on plant performance via predation on plant-associated microorganisms (Russell E. Ingham et al. 1985) (Neher 2001). For instance, bacterivores can reduce bacterial biomass with the side effect of increasing nutrient availability for plant uptake and growth. Likewise, feeding of fungivorous nematodes on fungi which can influence plant growth, for example by suppression of plant pathogens (REF). While the functional role of plant-feeding and other types of nematodes on plant performance has been well studied, their interactions with plants under drought conditions is not well understood.

Nematodes exhibit varying responses to changes in water availability, which may differ between trophic groups and the type of ecosystem in which they reside (Toddet al., 1999). Overall, evidence suggests that drought events can reduce the complexity of nematode communities in the soil, both directly and indirectly through changes in microbiome composition and abundance. Higher trophic level nematodes tend to have

larger body sizes, which can make them more sensitive to changes in soil moisture levels (Wallace 1968). During drought conditions, the water potential of the soil decreases, making it more difficult for larger nematodes to move through the soil and access their food sources. In contrast, lower trophic level nematodes, such as bacterivorous and fungivorous nematodes are characterized by smaller body sizes and can therefore move through soil pores more easily. Moreover, they rely on the soil microbiome as a food source, which may provide a more consistent supply of nutrients even during drought conditions. (Bouwman and Zwart 1994; Jones, Larbey, and Parrott 1969). Although it is currently understood that drought can reduce nematode diversity, the impact of this reduction on plant performance remains largely unclear.

Here, we investigated drought effects on feeding groups of nematodes in the soil and in the roots of two grass species, *Agrostics capillaris* and *Alopecurus pratensis*. We used two natural nematode communities that were inoculated into sterilized soil. These communities represent part of the diversity of agricultural and natural late-successional fields along a secondary ecological succession located in the Veluwe, The Netherlands. Previous research has shown that agricultural soils are more likely to harbor root-feeding nematodes, which can have direct negative effects on plant growth. However, the impact of nematodes on plant growth in natural diverse systems such as late-successional fields is still unclear. These soils typically contain many predatory nematodes, which may result in more effective top-down control. Moreover, we tested whether the effects of drought on nematodes were related to the growth of two grass species.

Methods

Plant species and soil ecological context

We used two grass species (*Agrostis capillaris* and *Alopecurus pratensis)* that naturally co- exist and are widely distributed across The Netherlands. Specifically, *Alopecurus pratensis* is a fast-growing grass mostly found in early successional ecosystems. It favours high moisture levels and becomes semi-dormant and performs less in drought conditions (Bazzaz, 1974). In contrast, *A. capillaris* is a slow-growing grass mostly found in late successional ecosystems. Its ability to efficiently utilize resources results in a better tolerance to drought. Seeds were supplied by Cruydt-Hoeck (Nijberkoop, The Netherlands) that collects plant seeds from wild populations and sterilized using 2.5% commercial bleach for one minute and rinsed three times using demineralised water. The sterilized seeds were then germinated in a growth cabinet at 20°C and under a 16/8-hour light/dark cycle for 10 days.

Soil was collected from a secondary succession chronosequence located in a central region of the Netherlands (Veluwe, The Netherlands) (Figure 1A). The chronosequence consists of former agricultural fields abandoned at different times and it has been used in previous studies as a framework for plant secondary succession in relation to changes in abiotic and biotic properties of the soil (Kardol et al., 2006; Van der Wal et al., 2006; Holtkamp et al., 2008; Van de Voorde et al., 2011; Hannula et al. 2017, Morrien) (Kardol, Martijn Bezemer, and van der Putten 2006; Hannula et al. 2017; Elly Morriën et al. 2017). To avoid possible nutrient effects (Pernilla Brinkman et al. 2010), we used an inoculum approach consisting of 15% inoculum soil and 85% sterilized background soil that

was collected from a former agricultural field abandoned in 1995 (Mossel, The Netherlands; N 52.06141, E 5 ° 75.266). Both the background soil and the inoculum soil were sieved through a 3 mm sieve to remove stones and other large particles and afterwards mixed. The background soil was γ-sterilized (25 KGray, by Syngenta, Ede, The Netherlands) prior to mixing. To maximize microbial diversity within succession stage, the inocula collected were pooled and homogenized to generate a single inoculum soil mixture per successional stage. This mixed soil sampling (MSS) approach (Gundale et al. 2019), allow us to test plant-nematode interaction under drought using two contrasting soil biological compositions of early versus later secondary succession characteristics.

Experimental Design

To assess the relationship between the growth of grass species and the effects of drought on nematodes, we experimentally exposed the grass species to the live soil inocula and subjected them to either control or drought conditions. The experiment was conducted in a climatised greenhouse at 16/8 h light/dark and 20/15 °C day/night conditions. We used a randomised block design with 6 blocks, 2 watering levels (control and drought), 2 inoculum types (agricultural and late-successional soil), and 2 grass species. Each block contained one replicate of the treatment groups for a total of 8 pots and each pot contained 4 seedlings in monoculture. The soil water content of all pots was maintained at 15% (w/w) for four weeks, by watering the pots 3 times per week, to compensate unequal losses. After four weeks, we applied the drought treatment where half of the pots were maintained at 7.5% (w/w) by watering the pots to weight for three weeks. To minimize nutrient deficiency, pots were fertilized using 5% Hoagland nutrient solution on a

weekly basis for 5 weeks. After seven weeks of plant growth, shoots were clipped, dried at 60 °C until constant weight, and weighed, whereas roots were first washed with high pressure to remove possible nematodes that were attached to the root surface and then were separated in two parts. One part of the roots then was dried at 60 °C until constant weight, whereas the other half was cut into pieces of 1–2 cm and placed in a mistifier for 48h to extract nematodes from the inside of the root. After nematodes extraction, the root samples were dried and added to the total root weight.

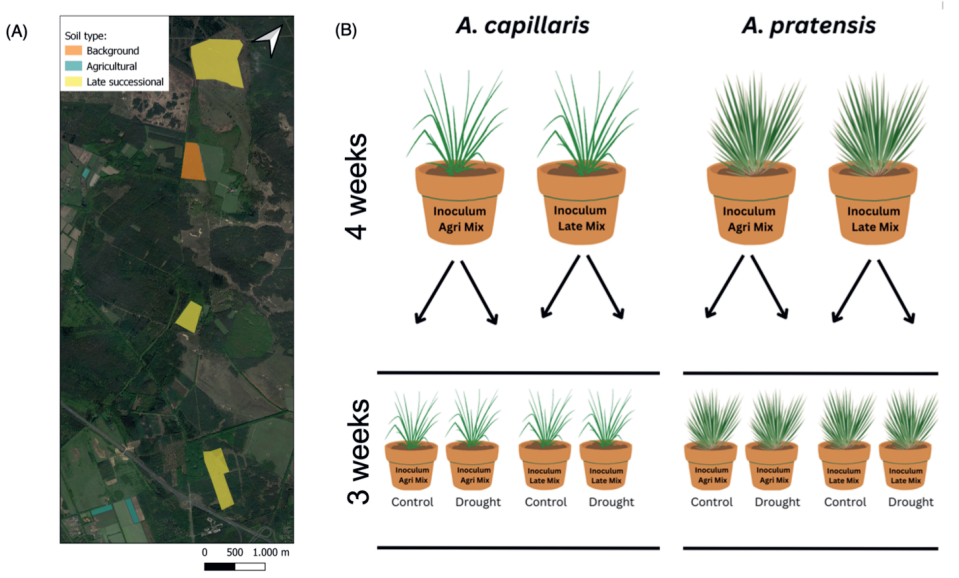


Figure 5.1. (A) Map of fields used to collect the soil inocula, (B) experimental design.

Nematode extraction, counts and identification

Soil from each pot was individually bagged and stored at 4 °C until nematode extraction. Nematodes were subsequently extracted from 100g of soil (wet weight) from each pot using an Oostenbrink elutriator (Oostenbrink, 1960; Verschoor et al., 2002). After nematodes were extracted, we concentrated the nematode suspension to 10ml. Additionally, soil samples were collected to determine soil moisture content so that the number of nematodes could be determined per dry weight of soil. Subsequently, roots were cut into pieces of 1–2 cm and placed in a mistifier for 48 hours to extract nematodes from the inside of the roots (Funnel-spray method; Oostenbrink, 1960). Nematode suspensions were harvested from the mistifier after 48h and concentrated to 10 ml. Using the total dry weight, total nematode numbers (density) inside the roots were estimated. To preserve their morphological structure, soil and root samples were placed in formalin. Both nematode samples were then counted and identified using an inverted light microscope (AxioCam MRc5 light microscope (Zeiss, Germany) at 200x magnification). Nematodes were grouped according to their feeding behaviour based on morphological differences (Yeates et al., 1993). The abundances were expressed individually per 100g dry soil. To standardize the number of nematodes per gram root, the number of nematodes in each root was divided by total dry weight of the roots from which nematodes were extracted.

Statistical analyses

Prior to all analysis, data were log-transformed to fulfil ANOVA assumptions. We examined drought and inoculum effects on plant biomass by constructing a linear model (lm function in R) with the factors

inoculum type (mix agricultural, mix late-successional), and drought treatments (control, drought), block, and all possible interactions on total biomass. To test the effects of drought on nematode density, we generated statistical models for the total density of nematodes in soil (N/100g dry soil) and roots (N/ g dry roots) and each individual feeding group using linear models (lm in R). Residual plots and Shapiro-Wilk normality tests were used to confirm that the model assumptions were not violated. Densities of root feeders and omnivores could not be reliably analyzed due to high numbers of zeroes. To examine the potential impact of nematode abundance on total grass biomass, linear models were utilized, with nematode total abundance included as a cofactor. We built separate models for the abundance of nematode in the soil (total numbers in 100 g soil) and in the roots (total numbers in g roots). Linear models included the fixed factors plant species, inoculum, drought, block, and the interaction between nematode absolute abundance and plant species, nematode absolute abundance and inoculum, nematode absolute abundance and drought treatment.

Results

Drought effects on soil nematodes

The effects of drought on the total abundance of soil nematodes depended on the plant species and the type of inoculum used (Plant species x Inoculum x Drought $p < 0.05$; Table S5.2). While the total abundance of soil nematodes of *A. capillaris* decreased significantly under drought conditions in both agricultural and late-successional soil inocula, the effect of drought on the total number of soil nematodes in *A. pratensis* was different between the two types of inoculum. Specifically, when grown with agricultural mix inoculum, the total density of nematodes

increased under drought in *A. pratensis* soil, whereas the total density of nematodes substantially decreased in pots with late-successional inoculum.

Morphological identification of nematodes revealed that the total number of nematodes was mainly comprised of fungivores and bacterivores. The number of omnivores and root feeders was found to be relatively low, and no predator nematodes were identified (Figure S1). Fungivores were the most abundant in late-successional inocula and they substantially decreased under drought in both grass species. However, fungivorous nematodes from agricultural mix inocula were only negatively affected by drought in *A. capillaris*, but not in *A. pratensis* (Figure 5.2A, Table S5.2). The density of bacterivorous nematodes decreased under drought in late-successional inocula, but not in pots with the agricultural inoculum for both species. While in *A. capillaris* soil bacterivorous nematodes were not strongly affected by dry conditions, the number of soil bacterivores increased under drought in *A. pratensis* (Figure 5.2B); however, this different effect between species was not statistically significant.

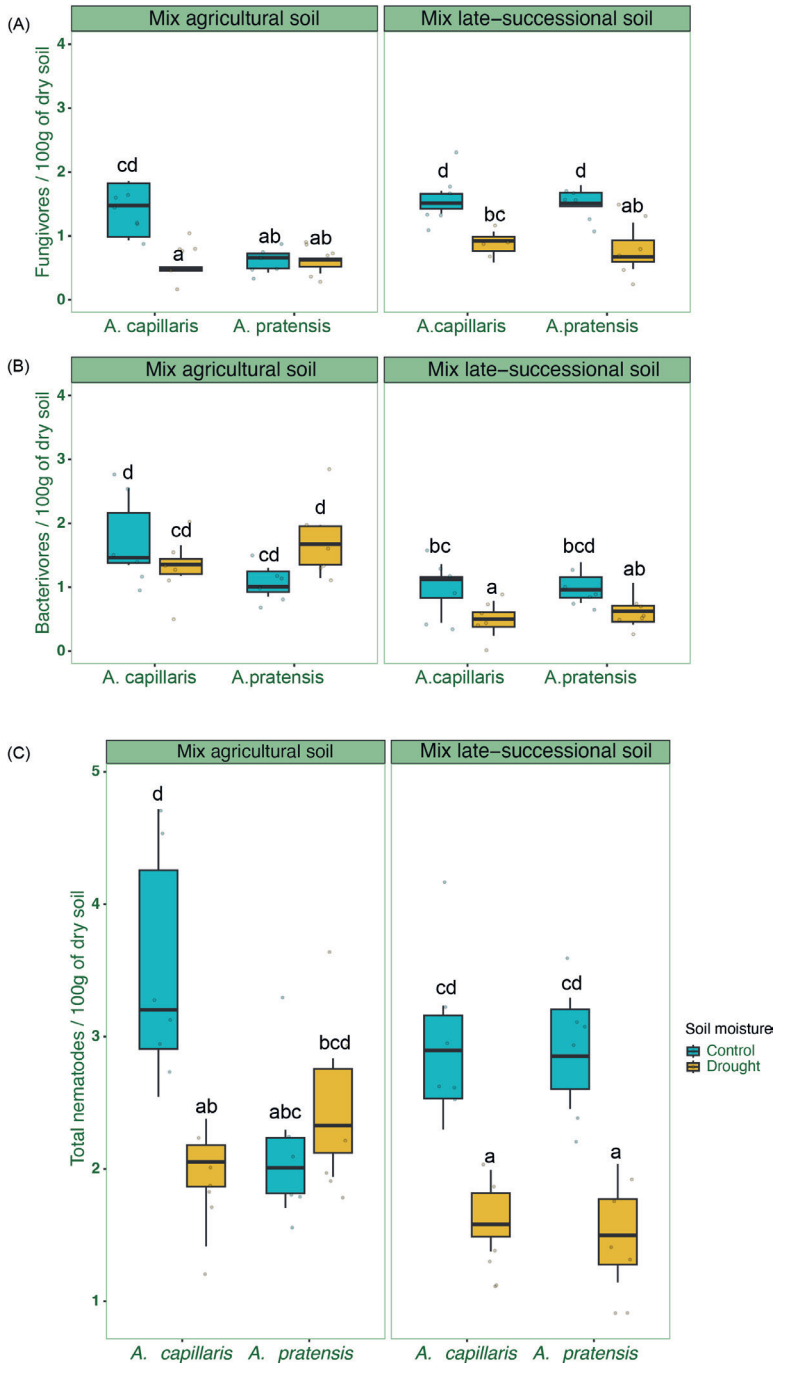


Figure 5.3. A) Density of fungivorous nematodes in the soil B) bacterivores C) total nematodes. Statistics is shown in Table S5.2.

Drought effects on nematodes in the roots

Contrary to the patterns observed in soils, the total abundance of nematodes in the roots substantially increased under drought (Figure 5.3C; Table S5.2). The strongest drought effect was observed for *A. capillaris* growing in pots with late-successional inocula, whereas nematodes in the roots of *A. pratensis* were more common in agricultural mix inocula. Fungivorous nematodes were the most abundant group in the roots of both grass species, and their numbers in late-successional mix inocula substantially increased under drought, with a stronger effect for *A. capillaris* than for *A. pratensis* (Figure 5.3A*)*. Overall, bacterivores were less abundant than fungivores inside the roots and were also less affected by drought. However, nematodes in *A. capillaris* roots growing in agricultural mix inocula were significantly promoted under drought (Figure 5.3B).

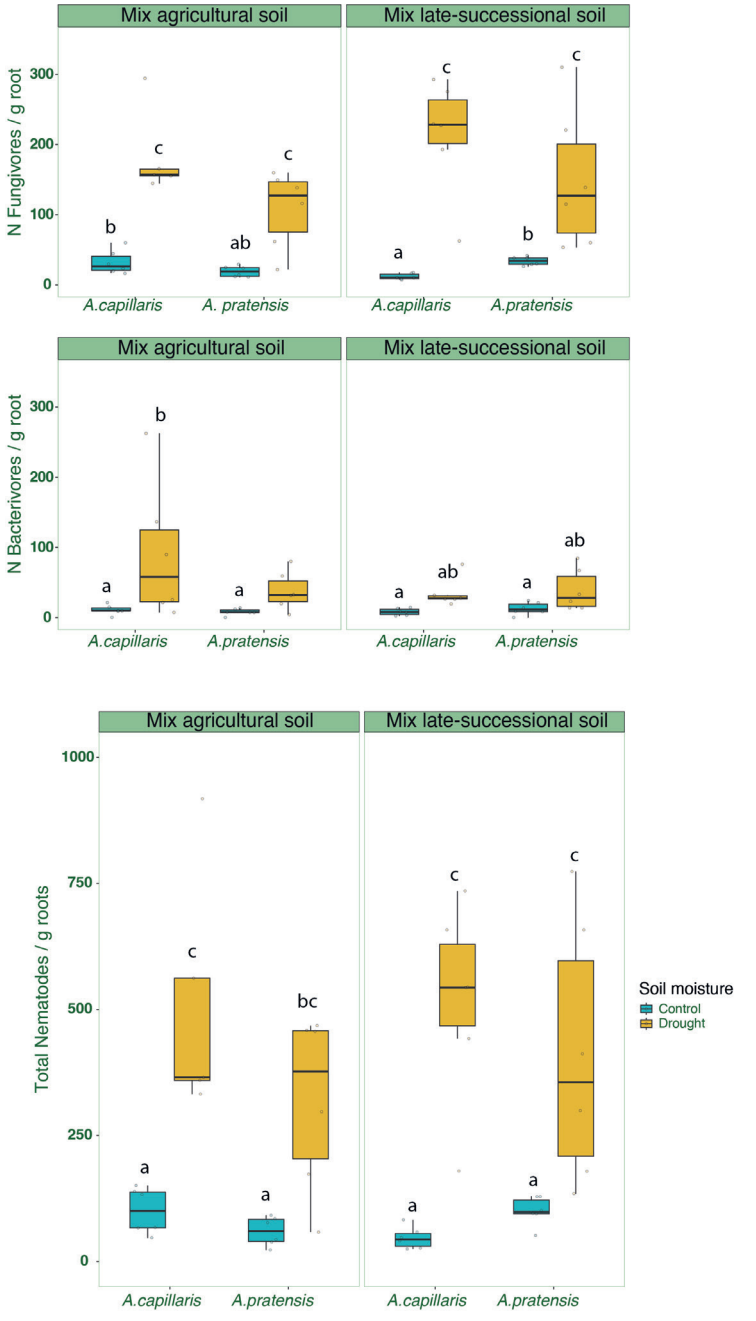


Figure 5.4. A) Density of fungivore in the roots B) bacterivores C) total nematodes. Statistics is shown in Table S5.2

Relation between nematodes abundance and plant growth

We found that both grass species experienced a reduction in total biomass under drought conditions, but the extent of the effect was different between the two species (Figure 5.4B, Table S5.1). Specifically, *A. capillaris* was more strongly affected by drought than *A. pratensis*, regardless of the type of inoculum used. However, the different soil inocula used in this study did not have a significant effect on plant growth under drought (inoculum*drought $p > 0.05$; Table S5.1), suggesting that nematode communities did not differentially affect plant total biomass under drought conditions. Nevertheless, we found an association between the total number of nematodes and grass total biomass under drought conditions (Figure 5.5). Specifically, when grasses were growing under normal watering conditions (control), there was a positive relation between their total biomass and the number of bacterivores in the roots. However, under drought, we observed a negative relation between grass biomass and the total number of bacterivores in the roots (bacterivores*drought $p < 0.05$; Table S5.3). Furthermore, fungivores in the soil were negatively associated with plant biomass with stronger effects under drought conditions than under control conditions (fungivores * drought, $p < 0.05$; Table S5.3).

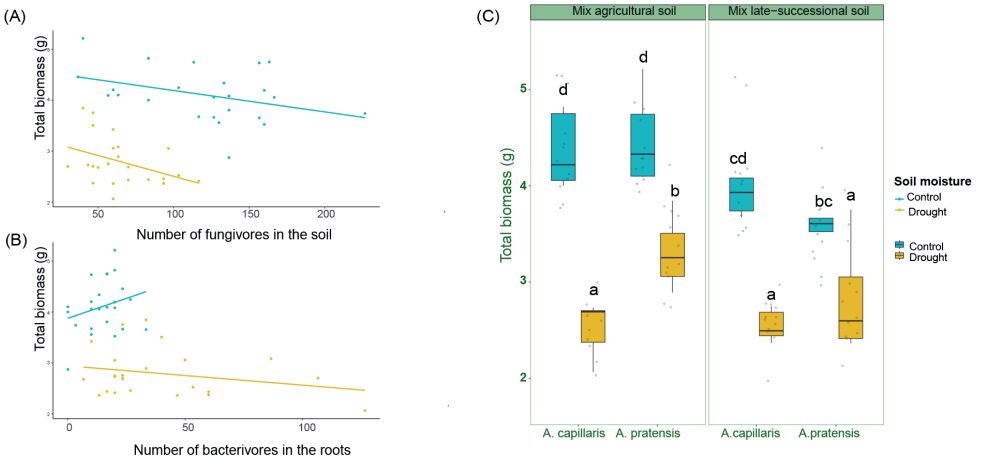


Figure 5.5. Relation between plant total biomass (g) and (A) number of fungivores isolated from soil and (B) plant roots. C) Plant total biomass produced under control (blue) and drought (yellow).

Discussion

Soil fauna, particularly nematodes, are facing increasing threats from more intense and frequent droughts. Despite the critical roles nematodes play in regulating plant growth, and maintaining soil health and ecosystem functioning, we have limited understanding of the effects of drought on nematodes and how they might impact plant performance and consequently plant community composition.

Nematodes migrate into plant roots under drought.

Among the nematodes observed, bacterivore and fungivore feeding groups were the most prevalent, accounting for the majority of the total number of nematodes. Interestingly, our study suggests that free-living nematodes, particularly bacterivores and fungivores in the soil, may migrate into the roots where the impact of drought is less severe. Therefore, our results suggest that nematodes might migrate and seek shelter in plant roots upon drought. Although this hypothesis has not

been previously tested, it is possible that drought might lead to a root system that is more prone to fragmentation and could potentially facilitate the entry of non-root feeding nematodes. While fungivorous nematodes possess morphological features that enable them to penetrate roots using their stylets, bacterivores cannot actively enter roots. One possible explanation for this phenomenon could be that bacterivorous nematodes are able to exploit the weakened status of roots caused by drought and enter passively. Alternatively, they could migrate into plant roots after fungivores. It is also worth noting that we detected some root herbivores, which possess the capacity to break down roots after feeding (Figure S5.1) We acknowledge that there is a possibility that the nematodes were attached to the root surface and thus entered during the process of extraction. To overcome this potential method limitation, we suggest combining other methods such as root staining that might provide further evidence about whether bacterivorous nematodes can be found inside the plant roots. Our study contributed to this gap in knowledge and provided evidence that drought suppresses nematodes in the soils but promotes nematodes inside the roots of two grass species.

Impact of drought on nematodes differs between nematode feedings groups.

Drought had a greater impact on fungivorous nematodes compared to bacterivores, and the extent of this impact depended not only on the plant species but also on the type of inoculum (plant species x drought x inoculum $p < 0.05$). Fungivorous nematodes are recognized for their indirect beneficial effects on plant growth, such as enhancing nutrient cycling and improving soil structure. However, fungivores can also indirectly harm the plant when they feed on beneficial fungi (Zhang et al.

2020). As a result, it is possible that fungivore nematodes may have limited food resources and may be more vulnerable to drought stress. Although less strongly than fungivores, the impact of drought on bacterivores was dependent on plant species. However, bacterivores did not differ significantly between agricultural and natural systems. This has also been suggested in a previous study on the same chronosequence, involving a wider variety of abandoned arable fields (Kardol, Martijn Bezemer, and van der Putten 2006, 200). In that study, it was proposed that nematode abundance re-arranges following land abandonment, but that there were not many new species of bacterivores colonizing the soils. This would mean that bacterivorous nematodes have limited value of acting as indicators of land use change.

Do nematodes mediate drought effects on plant growth?

The effects of drought on nematodes were related to plant total biomass of the two grass species. While plants may be more susceptible to certain nematode trophic groups, such as root feeder nematodes, our results suggest that the number of bacterivores and fungivores might be related, to some extent, to grass growth under drought. Bacterivores in the roots were associated positively with grass growth under control conditions whereas under drought we observed a shift towards a negative relation. This result is in line with the knowledge that bacterivores can increase plant N and P uptake (Russell E. Ingham et al. 1985; Djigal et al. 2004b; M. Lu et al. 2011), which consequently can enhance plant growth. However, under drought it is possible that bacterivorous nematodes have limited food resources, due to high sensitivity of bacteria under drought (de Vries and Shade 2013, 201; de Vries et al. 2012; Barnard, Osborne,

and Firestone 2013; Ochoa-Hueso et al. 2018). Further experiments are required to test this hypothesis.

Fungivores in the soil were negatively associated with total plant biomass, both under drought and control conditions. In a recent study by (Kane et al. 2023) it was found that fungivorous nematodes play a crucial role in driving microbial diversity and carbon cycling in soil. This large group includes members and genera that may feed on beneficial fungi, ultimately harming plants. This study presents initial evidence suggesting that nematodes, including both bacterivores and fungivores, may have indirect harmful effects on grass growth under drought conditions. However, further investigations are required to establish a causal relationship between the various feeding types of nematodes and grass growth during drought. Specifically, studying the effects of less severe drought conditions provides a more accurate representation of the challenges plants face and allows for a more comprehensive understanding of the role of nematodes in plant health during drought. Although many questions about the role of microbivorous nematodes remain, these findings suggest the importance of including microbivores in future studies - both in their interactions with microorganisms and other nematodes - which is crucial for gaining a comprehensive understanding of the functioning of soil subsystems under extreme weather events.

Acknowledgments

We thank Ciska Raaijmakers, Freddy ten Hooven for the help with nematode extraction and identification, Tanja Bakx for the help in the greenhouse, and Ivor Keesmaat in the laboratory. And Vera Hesen and Jonathan De Long for discussions.

Supplementary material

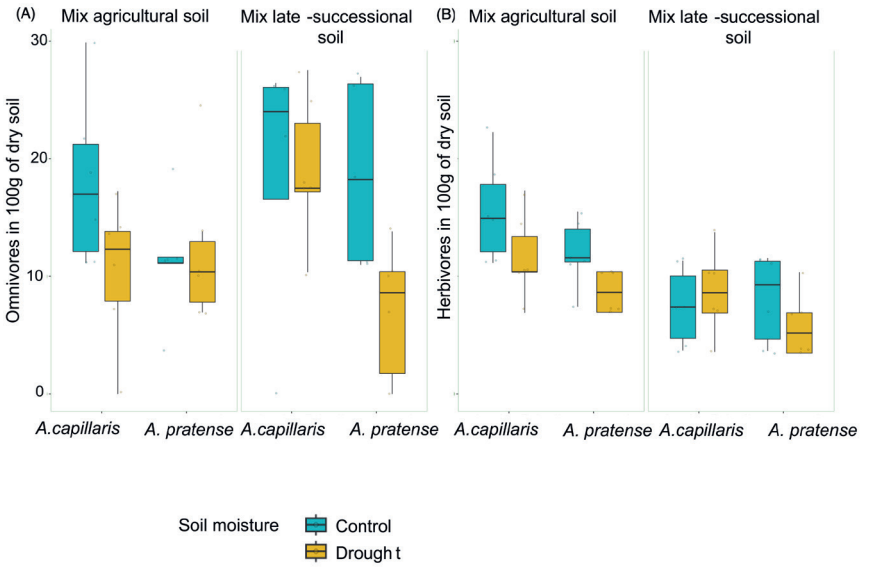


Figure S5.1. Abundance of omnivore and herbivores in the soil under drought (yellow) and control (blue).

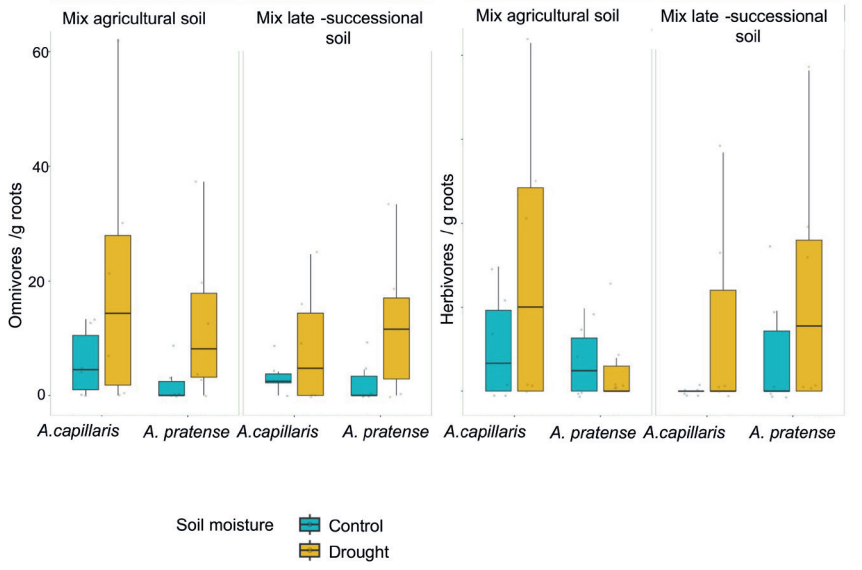


Figure S5.2. Abundance of omnivore and herbivores isolated from the roots under drought (yellow) and control (blue).

Table S5.1: Linear model of the effect of plant species (P), inoculum (I), drought treatment (D), block and possible interactions on total plant biomass (g).

Factors	Df	F value	*p* value
(Intercept)	1,83	30853.06	***p* < 0.001**
Block	5,83	0.87	*p > 0.05*
Plant Species (P)	1,83	8.11	***p* < 0.01**
Drought (D)	1,83	316.11	***p* < 0.001**
Inoculum (I)	1,83	29.96	***p* < 0.001**
P x D	1,83	23.09	***p* < 0.001**
P x I	1,83	12.82	***p* < 0.001**
D x I	1,83	2.62	*p > 0.05*
P x D x I	1,83	0.10	*p > 0.05*

Table S5.2. Linear model of the effects of plant species (P), inoculum (I), drought treatment (D), block and possible interactions on density of nematodes per dry soil and per gram roots.

	soil total nematodes			root total nematodes	
	Df	**F value**	**Pr(>F)**	**F value**	**Pr(>F)**
(Intercept)	1,35	3297.60	***p* < 0.001**	4260.53	***p* < 0.001**
Block	5,35	1.67	$p > 0.05$	2.04	$p > 0.05$
Plant Species (P)	1,35	18.62	***p* < 0.001**	1.95	$p > 0.05$
Inoculum (I)	1,35	1.91	$p > 0.05$	0.02	$p > 0.05$
Drought (D)	1,35	23.75	***p* < 0.001**	125.98	***p* < 0.001**
P x I	1,35	8.57	***p* < 0.01**	8.19	***p* < 0.01**
P x D	1,35	20.81	***p* < 0.001**	5.03	***p* < 0.05**
I x D	1,35	0.02	$p > 0.05$	0.15	$p > 0.05$
P x I x D	1,35	12.15	***p* < 0.01**	1.85	$p > 0.05$
	SOIL FUNGIVORES			**ROOT FUNGIVORES**	
	Df	**F value**	**Pr(>F)**	**F value**	**Pr(>F)**
(Intercept)	1,35	1128.67	***p* < 0.001**	3387.97	***p* < 0.001**
Block	5,35	0.48	$p > 0.05$	2.24	$p > 0.05$
Plant Species (P)	1,35	19.88	***p* < 0.001**	1.64	$p > 0.05$
Inoculum (I)	1,35	1.32	$p > 0.05$	0.02	$p > 0.05$
Drought (D)	1,35	41.45	***p* < 0.001**	185.46	***p* < 0.001**
P x I	1,35	8.92	***p* < 0.01**	11.84	$p < 0.01$
P x D	1,35	15.72	***p* < 0.001**	11.07	***p* < 0.01**
I x D	1,35	3.04	$p > 0.05$	0.92	$p > 0.05$
P x I x D	1,35	9.90	***p* < 0.01**	4.02	$p > 0.05$
	SOIL BACTERIVORES			**ROOT BACTERIVORES**	
	Df	**F value**	**Pr(>F)**	**F value**	**Pr(>F)**
(Intercept)	1,35	990.73	***p* < 0.001**	542.45	***p* < 0.001**
Block	5,35	1.93	$p > 0.05$	3.18	***p* < 0.05**
Plant Species (P)	1,35	5.12	***p* < 0.05**	0.41	$p > 0.05$
Inoculum (I)	1,35	10.50	***p* < 0.01**	0.01	$p > 0.05$
Drought (D)	1,35	2.79	$p > 0.05$	34.92	***p* < 0.001**
P x I	1,35	3.35	$p > 0.05$	0.90	$p > 0.05$
P x D	1,35	7.80	***p* < 0.01**	0.28	$p > 0.05$
I x D	1,35	2.15	$p > 0.05$	0.37	$p > 0.05$
P x I x D	1,35	2.00	$p > 0.05$	0.02	$p > 0.05$

Table S5.3. Linear Regression between total biomass and absolute abundance of total nematodes, fungivores and bacterivore nematodes in soil and roots.

	SOIL TOTAL NEMATODES			ROOTS TOTAL NEMATODES	
FACTORS	**DF**	**F value**	***p* value**	**F value**	***p* value**
(Intercept)	1,31	27.85	$p < 0.001$	63.55	$p < 0.001$
Nematodes (N)	1,31	2.90	*p > 0.05*	0.24	*p > 0.05*
Inoculum (I)	1,31	0.01	*p > 0.05*	1.03	*p > 0.05*
Plant Species (P)	1,31	0.09	*p > 0.05*	0.82	*p > 0.05*
Drought (D)	1,31	3.23	*p > 0.05*	1.34	*p > 0.05*
Block	1,31	1.15	*p > 0.05*	0.16	*p > 0.05*
N X I	1,31	0.10	*p > 0.05*	0.54	*p > 0.05*
N X P	1,31	0.05	*p > 0.05*	1.01	*p > 0.05*
N X D	1,31	4.96	***p < 0.05***	4.15	*p > 0.05*
I x P	1,31	9.65	***p < 0.01***	6.35	***p < 0.05***
P x D	1,31	4.16	***p < 0.05***	1.05	*p > 0.05*
I x D	1,31	0.59	*p > 0.05*	0.05	*p > 0.05*
I x P x D	1,31	0.53	*p > 0.05*	0.44	*p > 0.05*
	SOIL FUNGIVORES			ROOT FUNGIVORES	
FACTORS	**DF**	**F value**	***p* value**	**F value**	***p* value**
(Intercept)	1,31	69.50	$p < 0.001$	64.17	$p < 0.001$
Fungivores (F)	1,31	5.18	***p < 0.05***	0.19	*p > 0.05*
Drought (D)	1,31	0.54	*p > 0.05*	1.59	*p > 0.05*
Plant Species (P)	1,31	0.65	*p > 0.05*	0.90	*p > 0.05*
Inoculum (I)	1,31	2.18	*p > 0.05*	0.28	*p > 0.05*
Block	5,31	0.80	*p > 0.05*	0.21	*p > 0.05*
F X D	1,31	0.45	*p > 0.05*	0.99	*p > 0.05*
F X P	1,31	0.5	*p > 0.05*	1.03	*p > 0.05*
F X I	1,31	4.75	***p < 0.05***	1.97	*p > 0.05*
I x P	1,31	2.22	*p > 0.05*	5.35	***p < 0.05***
P x D	1,31	2.95	*p > 0.05*	0.45	*p > 0.05*
I x D	1,31	2.43	*p > 0.05*	0.27	*p > 0.05*
I x P x D	1,31	1.51	*p > 0.05*	0.50	*p > 0.05*
	SOIL BACTERIVORE			ROOT BACTERIVORES	
FACTORS	**DF**	**F value**	***p* value**	**F value**	***p* value**
(Intercept)	1,31	39.79	$p < 0.001$	610.17	$p < 0.001$
Bacterivores (B)	1,31	0.00	*p > 0.05*	0.01	*p > 0.05*
inoculum (I)	1,31	0.64	*p > 0.05*	1.84	*p > 0.05*
Plant Species (P)	1,31	0.19	*p > 0.05*	0.33	*p > 0.05*
Drought (D)	1,31	0.56	*p > 0.05*	0.14	*p > 0.05*
Block	5,31	0.59	*p > 0.05*	0.29	*p > 0.05*

B X I	1,31	0.31	*p > 0.05*	0.13	*p > 0.05*
B X P	1,31	0.09	*p > 0.05*	0.00	*p > 0.05*
B X D	1,31	1.99	*p > 0.05*	6.60	***p < 0.05***
I x P	1,31	3.61	*p > 0.05*	5.54	***p < 0.05***
P x D	1,31	6.93	***p < 0.05***	7.96	***p < 0.01***
I x D	1,31	0.11	*p > 0.05*	0.28	*p > 0.05*
I x P x D	1,31	0.06	*p > 0.05*	0.01	*p > 0.05*

Chapter 6

General discussion

Plants have the ability to shape their associated belowground microbiome, which can either promote or suppress their growth. In this thesis I focused on exploring both directions of these effects: from plants to microbiome and from microbiome to plants. I examined the influence of grass genetic variation on the associated microbiome, both within and between different grass species. By doing so, I aimed to gain a deeper understanding of the molecular mechanisms that drive plant-microbe interactions. Moreover, I explored the potential effects of microbial communities on grass growth under drought stress. Here, I discuss the findings. Firstly, I compare results obtained using the genomic (Genome-Wide Association Studies – GWAS) and the ecological (Plant-soil feedback – PSF) approaches to study intraspecific variation in plant-microbe interactions. Secondly, I discuss the effects of microbiomes on plant growth, emphasizing the distinctions between single taxa and whole communities. Thirdly, I discuss the impact of drought on grass-microbe and grass-nematode interactions. Finally, I suggest future directions for research based on the findings of my thesis study.

Is there intraspecific variation in plant-microbiome interactions?

Two experiments (**Chapters 2 and 3**) were designed to answer the question how plant genetics may affect associated microorganisms in the rhizosphere. Overall, I observed considerable species- specificity in plant-microbe interactions. However, an emergent question is to what extent these interactions are specific at the level of cultivar and accessions within a plant species. In **Chapter 2**, I used a targeted genomic approach (Genome-Wide Association Studies – GWAS) to pinpoint genes associated with microbiome variation, and thus to gain a better understanding of the molecular mechanisms underlying plant influence

over the associated microbiome. The results of this experiment demonstrate the capability of GWAS to reveal associations between plant single nucleotide polymorphisms (SNPs) and single microbial taxa in the rhizosphere and root endosphere. Moreover, I show that the genetic make-up of grasses also affects the overall structure and diversity of the microbiome.

To further investigate potential ecological consequences of intraspecific variation for plant-microbiome interactions, I adopted the ecological approach of plant-soil feedback experiments (PSFs) in **Chapter 3** (Bever 1994; van der Putten et al. 2013). The PSF approach involves two distinct phases, a soil conditioning, followed by a feedback phase. In the conditioning phase plants influence the composition of the soil community and abiotic soil properties, whereas in the feedback phase the effects of the microbiome on the performance of subsequent plants are determined, either to the same or to another genotype or species. When using this approach, I demonstrated for three grass species that cultivars of the same grass species do not differ in their influence on the rhizosphere microbial community measured at the end of the conditioning phase.

To explain the apparent mismatch between the findings of the two approaches in **Chapters 2 and 3**, it is crucial to highlight their differences in approaches ad experimental set-up. The most important is that PSF analysis detects interactions between *entire plant genomes* and the soil microbiome, whereas GWAS analysis is based on plant-microbiome associations at the level of *individual loci*. Furthermore, other technical aspects, such as the use of controlled (i.e., greenhouse in case of the PSF study) versus uncontrolled (i.e., outdoors in case of the

GWAS study) environments might have played a role as well in the different outcomes of the GWAS and PSF studies. Outdoor experiments, in contrast to greenhouse experiments, offer a closer approximation of field conditions, but the greater environmental fluctuations in, e.g., water availability and temperature might very well have reduced the detectability of genetic effects on the microbiome (de Vries et al. 2020). Interestingly, I did not find any influence of grass genetics on the rhizosphere microbiome under relatively constant environmental conditions in the PSF study (**Chapter 3**), whereas I detected fairly strong genetic control of rhizosphere microbiomes under outdoor conditions in the GWAS study (**Chapter 2**).

Other technical aspects that may have contributed to the discrepancy in results are *sample size* and the *plant material* used. GWAS requires at least 100 samples (Beilsmith et al. 2019; Ziyatdinov et al. 2021; Hua et al. 2022) and this large sample size will have increased the chance to find a genetic signal due to broader genetic variation. In contrast, PSF is often limited by a smaller sample size due to the more elaborate experimental design (two phase experimental set-up with sample numbers rapidly increasing with increased possible pair-wise combinations) (Brinkman et al. 2010). In **Chapter 3**, for each grass species I tested 10 cultivars, which represents a relatively high sample size in comparison to most previous studies (Allen et al. 2017; Bukowski and Petermann 2014; Wagg et al. 2015; Semchenko, Saar, and Lepik 2017). In addition to differences in sample sizes, other differences in the plant material used in both experiments might have led to differences in results. While in **Chapter 2** I used grass accessions, in **Chapter 3** I used grass cultivars that contain substantial amounts of segregating genetic variation within cultivars. This large segregating variation of cultivars makes it more

difficult to detect genotype-level effects (Figure 6.1). Overall, my research shows that there is real genetic variation that affects microbiome taxa and composition. If the affected microbial taxa influence plant traits, my results suggest that there is 'fuel' for selection to drive adaptation (or crop improvement) in the traits that are mediated by the microbiome. As such, this knowledge might take us a step closer to considering microbiome traits (i.e., beneficial interactions or tolerance to pathogens) for breeding purposes.

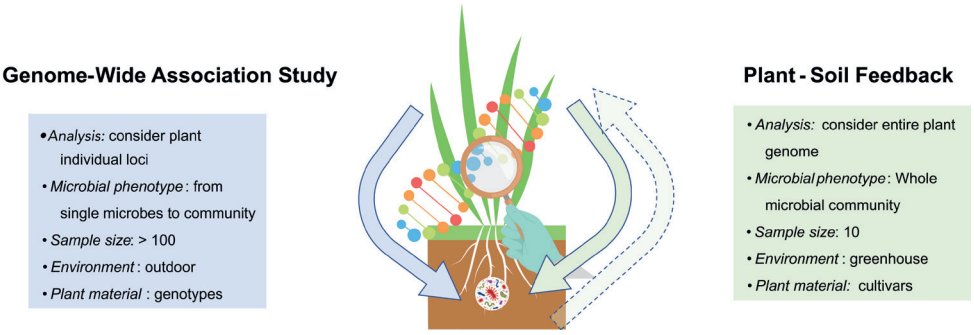


Figure 6.1. Overview of the technical aspects used for genome-wide association study (GWAS) and plant-soil feedbacks (PSFs) approaches in investigating plant genetic effects on the associated microbiome. GWAS analysis is performed at the plant individual loci and can use a various range of phenotypes. In this study three microbial phenotypes have been investigated (single OTUs, microbial diversity and composition). The experiment was performed outdoors using 154 genotypes of *Lolium perenne*. Plant-soil feedback analysis is based on effects determined by the entire plant genome and can detect effects at the level of the whole microbial community. Moreover, PSF assesses the effects of soil on plant growth (dashed arrow). In this study, PSF was conducted in a greenhouse using 10 cultivars and 3 grass species. PSF analysis detects interactions between entire plant genomes and the soil microbiome, whereas GWAS analysis is based on plant-microbiome associations at the level of individual loci.

Interspecific variation in plant-soil feedbacks

I show that plant soil feedbacks affect plants when members within grass family are considered but no differences in PSF effects between cultivars within grass species was detected (**Chapter 3**; Rallo et al. 2023). One of the key aspects of that study was that *Lolium perenne* showed relative higher total biomass when growing in soil inoculated with own microbiome compared to soils inoculated with the microbiomes of other species. Interestingly, this contrasts the reduced biomass production (negative feedbacks) that grass species generally have when growing in soil conditioned by the same species (Kos et al. 2015; De Long et al. 2021; Hannula et al. 2021). The enhanced biomass production (positive feedback) of *L. perenne's* microbiome has important implications not only for plant community dynamics but also for plant breeding. *Lolium perenne* is one the most important forage grass species, providing high-quality feed for livestock. Its adaptability to diverse environments makes it a versatile choice for various climates and soil conditions. Additionally, *L. perenne* is highly valued for its ability to be used in turfgrass, lawns, and erosion control. Furthermore, the species possesses rich genetic diversity, which makes it a valuable resource for breeding programs aiming to develop enhanced cultivars with improved traits for different purposes. (Smith et al., 2001; Baert and Van Waes, 2014). Thus, *Lolium perenne* could be used to increase future crop yield for breeders that currently grow grasses in monocultural settings in temperate agriculture systems.

Microbiome-mediated effects on plant growth in a changing world

After characterizing *plant-mediated* effects on microbiomes **(Chapters 2** and **3**), both at genetic and ecological scales, I assessed *microbiome-mediated* effects on the growth of grasses using a PSF approach combined with drought (**Chapter 4**). Interactions between plants and soil biota are fundamental for grassland ecosystem functioning. Therefore, understanding how climate extremes such as drought might affect plant-soil biota interactions is crucial for developing strategies to enhance plant resilience to drought and promote sustainable agricultural practices (Craine et al. 2013; Cang, Wilson, and Wiens 2016; van der Putten et al. 2016; de Vries and Shade 2013; Kardol et al. 2010). Two experiments were designed (**Chapters 4** and **5**) to assess how drought alters interactions between belowground inhabitants (microorganisms, nematodes) and grasses. Most work to date has focused on the role of single microorganisms on plant fitness under drought (De Long, Semchenko, et al. 2019). While it is important to decipher the effects of single key microorganisms on plants facing drought, plants form associations with complex, highly diverse microbial communities. The *net effect* of such interactions impact on plant biomass production and, ultimately, fitness.

In **Chapter 4**, such a community-level approach was adopted to study whether soil microbes can be harnessed for improved drought tolerance in grasses (de Vries et al. 2020). I found that under optimal water conditions, microbes decreased grass biomass production, suggesting an overall negative effect of the microbiome. This is consistent with the findings of **Chapter 3** that show negative interspecific feedbacks and highlight the potential dominant role of pathogens in grass-soil feedbacks

(Cortois et al. 2016). Under drought, however, there was a reduced negative effect of microbes on plant growth. Does this indicate that soil microbes 'help' the plant to cope better with drought? To understand how drought affects grass-pathogen relations we need to reexamine how causative relationships between microorganisms and plants are defined.

In traditional plant pathology, Koch's postulates state that plant growth should be promoted in the absence of a pathogen, whereas introducing the pathogen to the plant should lead to a reduction in growth (Koch 1884). However, as is increasingly observed, pathogens often operate in multi-species communities, and activities of (potentially) pathogenic microbes could vary from antagonistic to even synergistic interactions. While Koch's postulates were traditionally focused on cases in which a single pathogen affected its host, applying the postulates are less suited for establishing causative relationships between net effects of communities of microorganisms and plants. This will also make it challenging to establish how microbes might influence plants under stressed conditions, such as under drought. Therefore, based on the findings from **Chapter 4**, I will discuss three hypotheses that could explain the reduction of the negative microbial effects observed under drought. Firstly, microorganisms, specifically pathogens, are able to modify their metabolic pathways and nutrient acquisition strategies in response to environmental disturbances, such as drought. Secondly, I discuss the hypothesis that plants modulate their root traits in response to pathogens which made them less vulnerable to drought. Finally, other beneficial soil biota might reduce the negative effects, as can be observed under drought.

Do pathogens modify their nutrient acquisition strategies in response to drought?

The decrease of soil moisture under drought may be critical for soil-inhabiting pathogens and for their infectivity of plants. Many fungal diseases, for instance, require high humidity for spore germination and infection of their host plants (Islam and Toyota 2004; Romero et al. 2022; Singh et al. 2023). However, there are few exceptions in which low soil moisture favors disease development, such as the fungi *Magnaporthe oryzae* in rice, (Bidzinski et al. 2016) and the bacterial scab pathogen *Streptomyces* spp., in potato (Johansen, Dees, and Hermansen 2015). There are hardly any studies in grasses that can explain the interplay between grass-pathogens under drought. The findings of **Chapter 4** suggest that drought might decrease the infectivity of grass pathogens which results in less negative effects on biomass. However, drought has direct (drought-pathogens-plant) and indirect (drought-plant-pathogens) effects on soil biota (Ochoa-Hueso et al. 2018).

To disentangle direct and indirect effects of the reduced pathogenicity in plants under drought (**Chapter 4**), it will be important to consider the strategies that pathogens could employ. (Allison and Martiny 2008; Fuchslueger et al. 2014). As such, I hypothesize that drought changes the lifestyle of facultative pathogens (i.e., from being pathogenic to saprotrophic). In fact, drought can lead to higher plant mortality rates, which can result in a scarcity of resources for pathogens. However, this phenomenon can have a positive outcome for saprotrophs due to the accumulation of dead organic material. In fact, if the pathogen acts in certain conditions as a saprotroph, it can benefit from the increased availability of nutrients from the decaying plant matter (Termorshuizen

and Jeger 2008; van Albada et al. 2022). But what does this switch from pathogen to saprotroph mean for the plant? In the short term the increase of saprophytic activity will promote plant growth due to additional nutrient release (Cairney 2005), which is what is often observed under drought, and especially following rewetting (Birch effect) (Birch 1958; Jarvis et al. 2007). In the long-term, however, grasses could be negatively affected by a facultative saprotrophic lifestyle, as facultative microorganisms are known to be more aggressive as a result of their ability to feed both on dead and living plants (Jarosz and Davelos 1995). These phenomena may need to be better comprehended when trying to understand how drought may influence PSF interactions.

The interplay between pathogens, roots and drought

The reduced negative effect on grass biomass under drought can also be a result of indirect pathogenic effects via modulation of root traits. Roots are the first responders to many kinds of biotic and abiotic stresses (Brunner et al. 2015; Weemstra et al. 2016) and plants are able to modulate them as a defense mechanism. For instance, drought can decrease root length and increase root diameter. We have novel evidence of how drought triggers plants to adjust their roots (Lozano et al. 2022; Wilschut and van Kleunen 2021; Lozano et al. 2021; 2020). We have some evidence that plants can modify their root traits in response to pathogens. A study conducted on marram grass (*Ammophila arenaria*), for instance, showed that in response to harmful soil organisms, the plant developed short and strongly branched roots with few root hairs (Van Der Putten, Van Der Werf-Klein Breteler, and Van Dijk 1989). Moreover, in **Chapter 2**, I detected several candidate genes with roles in root development that affect microbiome composition (i.e., ERF71, ERF73 EER5), especially for

fungal grass pathogens such as *Alternaria*. However, it is unclear whether plants' ability to modify roots in response to pathogens affects their capacity to handle climate extremes like drought. In the study conducted in **Chapter 4** grasses were growing for four weeks before being exposed to drought. As such, it is possible that plants modulate their root traits in response to pathogens which made them less vulnerable to drought, reducing consequently the negative effects observed under optimal conditions.

Do grasses rely on beneficial soil organisms under drought?

The other finding of **Chapter 4** suggests that AMF are not alleviating drought effects. This is in contrast to previous suggestions on the potential increase in abundance of AMF in response to drought with potential benefits for plant tolerance to stressful conditions (Bahadur et al. 2019; Lin, McCormack, and Guo 2015; de Vries et al. 2020; Ruiz-Lozano et al. 2012). Nonetheless, grass species characterized by thin roots could have lower dependence on AMFs for water uptake under drought conditions, due to the ability of thin roots to acquire water efficiently (Lin, McCormack, and Guo 2015). In my experiment, AMF abundance was relatively low not only under drought, but also under controlled conditions, making it unlikely that grasses relied heavily on this association. In support to that, I used the root staining procedure to check AMF root colonization but found no or minimal AMF colonization irrespective of the drought treatment (this result is not presented in the thesis). This raised the question whether these grass species rely on other potential allies under drought including other soil biota (i.e., nematodes) or endophytic fungi or bacteria.

Beyond the rhizosphere microbiome: what is the potential of nematodes?

The findings of **Chapter 4** suggest that under drought the impact of the microbiome is reduced. However, soil also contains other organisms besides microbes. Therefore, in **Chapter 5**, I studied the interplay between grasses and nematodes under drought. My motivation to design this experiment comes from the gap in knowledge about the effects of nematodes on plants experiencing drought. Similarly to **Chapter 2** where I examined microbiomes across different plant compartment, in **Chapter 5** I examined nematode communities in the soil (using the Oostenbrink elutriator) and in the roots (using a mIstifier approach) of two grass species. I tested how nematodes are affected by drought and whether they are related to plant growth under drought. Most of the nematode community was composed of non-parasitic nematodes and specifically fungivorous and bacterivorous nematodes which are known for their contribution to plant growth via nutrient mineralization (Wilschut and Geisen 2021). Surprisingly, I only detected a low number of root feeders in the grass species studied. Interestingly, findings of Chapter 5 suggest that under drought conditions, also these microbial-feeding nematodes might move from the soil into the roots where conditions, such as increased moisture and nutrient availability, are more favorable. In fact, the root zone maintains higher moisture levels compared to the surrounding soil due to the water uptake and transpiration activities of the plant (Kuhlmann et al. 2012). On one hand, these results might bring new insights on bacterivores and fungivores and their potential role in plant growth. In fact, it has not been observed before that fungivores and especially bacterivores move from the soil to the roots under drought. If that is the case, fungivores and bacterivores might negatively affect grass biomass production under drought, as

results in **Chapter 5** suggested. However, I acknowledge that this evidence is not so strong, due to a possible method limitation. In fact, while I aimed at washing plant roots meticulously, I cannot exclude that the nematodes identified as being apparently inside the roots were previously attached to the root surface. The hypothesis that nematodes under drought might move inside plant roots, therefore, needs to be further tested by using for instance root staining, which would provide evidence whether they are inside the roots.

Future directions and concluding remarks.

This thesis presents evidence of genetic variation in grass species that influences their capacity to interact with microbes. Moreover, I used the concept of the microbiome as a community highlighting its *net effect* on plant performance under drought. My findings pave the way for future research on the genetic basis of the functional role of plant-microbe interactions under drought. This could provide insights into ecological adaptation and help breeders to safeguard grass-based food production systems in a changing world. Below, I will propose 3 lines of research based on the findings of this thesis:

1. Using GWAS as a tool to unravel the genetic basis of the role of plant-microbe interactions under drought
2. Studying the whole soil food web under a gradient from ambient conditions to drought
3. Towards a better understanding of microbial specificity and plasticity

1. *GWAS as a tool to unravel the genetic basis of plant-microbe interactions under drought*

In **Chapter 2**, using GWAS, I provide evidence that genetic variation at specific loci determines variation in the composition of the rhizosphere and root endosphere microbiomes in *Lolium perenne*. After confirming the presence of intraspecific variation in the ability of grasses to interact with microorganisms, the next step is to study this phenomenon under stress conditions like drought. A way to do so is to compare *L. perenne* with other grass species that are known for their high drought tolerance, such as *Schedonorus arundinaceus*. This comparison will allow the identification of specific genetic regions associated with traits related to microbial interactions and drought tolerance. Further analysis should be conducted to understand the functional implications of the associated genes, including gene expression studies, pathway analysis, and functional annotation. Validation experiments, such as genetic transformation or knockout studies, may also be performed to confirm the functional relevance of the identified genes. The findings from GWAS can guide future breeding efforts and targeted manipulation of plant-microbe interactions to improve plant resilience and productivity under drought stress.

Furthermore, to improve our understanding of the genetic basis of the microbial net effect on plants, it is crucial to also consider the interconnectedness of microbial communities and their interactions with plants. This could be done by including properties of microbial networks as microbial trait in GWAS (i.e., node degree, network density, network robustness). Microbial networks can enhance the understanding the

environments and its components in an integrated way but also the microbial response to disturbances (Vries and Wallenstein 2017; Thompson and Gonzalez 2017). For instance, there is evidence that ecological networks composed of weak interactions tend to be more stable in comparison with those characterized by strong interactions (Neutel, Heesterbeek, and De Ruiter 2002; Coyte, Schluter, and Foster 2015; de Vries et al. 2018). Therefore, further research could employ GWAS as a tool to unravel the genetic basis of grass-microbe interactions under drought including grass species that are potentially more tolerant to drought (i.e., *Schedonorus arundinaceus*) and different scales of microbial phenotypes (Figure 6.2)

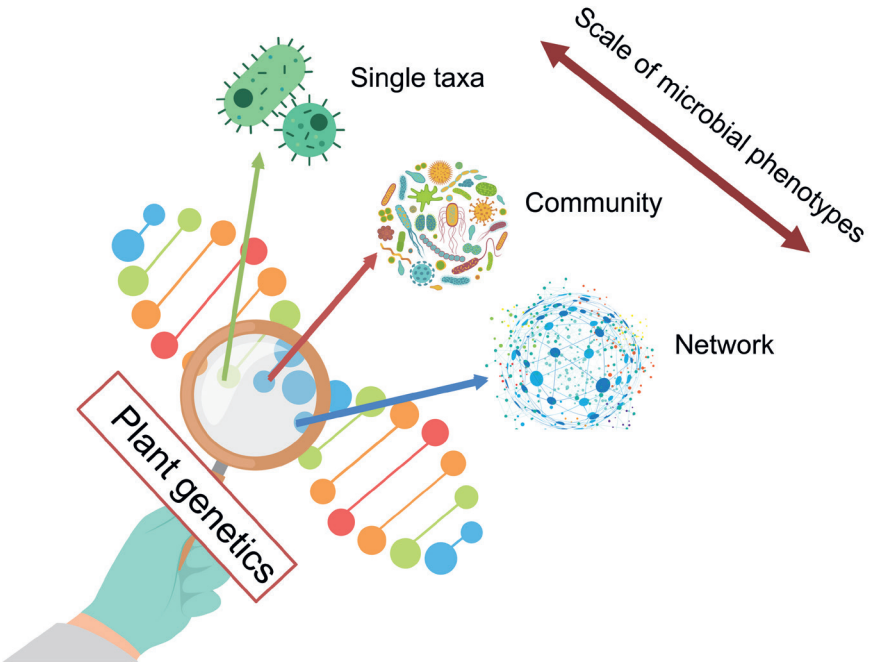


Figure 6.2. Conceptual overview of different scales of microbial phenotypes that can be used in genome-wide association studies. Single taxa are informative of the role of microorganisms in plant-microbe associations. Microbial community composition phenotypes capture the collective population of microorganisms. Microbial networks provide a systems-level understanding of plant-microbe interactions.

2. Soil food web under different gradients of drought

If drought weakens the role of the soil microbiota in plant-soil interactions (**Chapter 4**), who is going to regulate plant growth, and maintain soil health and ecosystem functioning? In **Chapters 4 and 5** I focused separately on microbes and nematodes. The findings of **Chapter 5** shed light on the potential effects of bacterivorous and fungivorous nematodes in grasses. These nematodes use microorganisms as a food source, and their interaction could play a substantial role in the net effect of soil communities on plant performance under drought. We have evidence that nematodes can interact with fungi in causing growth reduction in marram grass (Rooij-Van Der Goes 1995). However, under drought conditions, these interactions become more challenging to understand. In fact, both plants and the soil inhabitants are affected by drought, and because both affect soil C and N availability, the altered plant-soil feedbacks under drought might influence each other's performance in a highly complex way. These feedbacks might be further unraveled by a soil food web approach (Hunt et al. 1987; Morriën 2016). The concept of the *soil food web* emphasizes the importance of biodiversity and functional diversity in maintaining healthy soils (Brussaard 1997; De Ruiter, Neutel, and Moore 1998). While microbial networks allow for a more detailed understanding of the structure and dynamics of the potential interactions within the soil community, the soil food web approach focusses on trophic relationships and feeding interactions within the community. To further understand soil biota and plant sensitivity responses to drought and based on the results of **Chapters 4** and **5**, I suggest using soil food webs under different gradients of drought. In fact, drought conditions can vary in intensity and duration across different regions or even within a specific area, and its

impact on microbes and soil biota can vary depending on the intensity and duration of the drought event. While mild drought could determine shifts in composition of soil biota, severe drought could disrupt microbial interactions with consequences for microbial functionality (i.e., some microbes could become dormant and thus altering nutrient cycling processes) (Xiong et al. 2017).

3. Microbial specificity and plasticity

Based on the findings of this thesis, I suggest that further work is needed to better understand microbial *specificity* and *plasticity*. In my thesis, I provide evidence of specificity of plant-microbe interactions at the grass species and accession-level. Future research could provide a step further including a perspective on the microbial specificity (i.e., specialists versus generalists) (Semchenko et al. 2022). A better understanding of microbial specificity vs plasticity would teach us about how plants and microbes affect each other, especially under the current climatic scenario. In my thesis I detected pathogenic effects in soil communities, which is a common cause of negative feedback to grasses (Cortois et al. 2016; Hannula et al. 2020; Heinen et al. 2020; De Long et al. 2023). However, our understanding of grass pathogens and especially their specificity is still needing further studies. I discussed the possibility that pathogens switch their lifestyle under drought, becoming less pathogenic for the plant as a result. Thus, microbial functions can be affected by climate extremes and their plasticity such as the ability of some microorganisms to change their lifestyle, might play a crucial role in determining their effect on plant performance. Future studies could employ metatranscriptomics or shotgun metagenomics approaches to further unravel both microbial specificity and the plasticity in feeding style.

Having a better understanding of the microbial specificity and the plasticity of feeding style of microbial traits could further elaborate our knowledge of grass-pathogen interactions under drought and could unravel whether and how microbes might harness plants in a changing world.

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Summary

During millions of years, plants and microorganisms have co-evolved intricate and specialized relationships. Understanding how microorganisms influence plant health in response to the environment is crucial for cultivating resilient crops that can withstand stress. The ability to modify plant traits through natural or artificial selection relies on genetic variation within plant species in the ability to engage with beneficial microbes or to prevent pathogenic interactions. Therefore, to fully comprehend the impact of plant-microbe interactions on plant growth and fitness, it is essential to consider both the genetic and environmental factors that shape these interactions.

While there is good understanding of how individual microbes interact with plants and affect plant growth, it is important to recognize that in real-world scenarios, the effects of single microbial taxa on plants are often the outcome of interactions among multiple microorganisms. These microorganisms can influence each other's effects on plants. Consequently, the combined effect of diverse microbial communities ultimately determines plant growth and overall fitness through plant-soil feedbacks, which are net effects of all underlying individual interactions between plants and soil (micro)organisms.

Climate change-induced drought can shape these microbial net effects. As microbial communities and soil functionality are closely linked, any changes in microbial community composition resulting from drought can impact soil functionality and, in turn, the provisioning of ecosystem services. Therefore, the presence of differently adapted plants is crucial in the face of drought and other human-induced environmental changes.

Gaining insights into how the microbiome community affects plants when confronted with extreme environmental conditions will provide a comprehensive understanding of microbiome function in relation to plant health and resilience. This knowledge can be leveraged to develop innovative strategies for improving crop productivity, enhancing stress tolerance, and mitigating the impact of environmental challenges on plants.

Using different cultivars of various grass species, I first aimed at investigating plant intraspecific variation in the ability to interact with belowground microbiomes. I gained insights into the biological processes that plants might apply to modulate their rhizosphere and root microbiomes. Building on this, I compared the intraspecific variation in plant-soil feedbacks (PSFs) with interspecific variation to understand the specificity of PSFs and their implications in natural settings. Additionally, I expanded my study to examine the effects of drought on grass-soil biota interactions across different scales, including microbial bacteria, fungi, and soil fauna, specifically nematodes. By exploring these different levels, I aimed to gain a comprehensive understanding of the impacts of drought on plant-belowground interactions in grass species.

In **Chapter 2**, I characterized the differences in bacterial and fungal rhizosphere and root endosphere microbiomes using amplicon sequencing between 154 different accessions of *L. perenne* growing in outdoor mesocosms in an experimental garden. I then used a genome-wide association study (GWAS) approach to identify genetic loci affecting three microbial phenotypes (microbiome composition, microbiome diversity and abundance of single OTUs). I found associations between genetic variation at specific loci and microbiomes. The results seem to

point at plant defense genes and root developmental processes as important determinants of plant interactions with soil microbes, with stronger effects on fungal interactions. Bacterial and fungal communities are modulated by similar biological processes, such as plant defense, metabolic processes, and root development. However, these processes are regulated by different genes.

In **Chapter 3**, I used a plant-soil feedback approach to understand whether intraspecific variation in PSF effects exists and how large this intraspecific component is relative to the interspecific component of variation in PSF. In a greenhouse experiment, I grew monocultures of ten cultivars from three grass species (*Lolium perenne*, *Poa pratensis*, and *Schedonorus arundinaceus*), that are widely used by breeders as turf and forage grasses. I used plant total biomass of the plants to examine PSFs within and between species and correlated biomass with the abundance of specific bacteria and fungi in the rhizosphere, characterized by amplicon sequencing. Contrary to our expectation, we found no evidence for intraspecific variation in PSF effects. However, when examining interspecific variation in PSFs, overall negative feedbacks were detected. Specifically, during the soil conditioning phase, I observed that each grass species developed microbial legacies that negatively influenced the performance of the other two grass species in the subsequent feedback phase. Interestingly, *Lolium perenne* demonstrated higher biomass production in soils with legacies of conspecifics (the same species) compared to heterospecific (the other two species). In contrast, *Schedonorus arundinaceus* was not strongly affected at all by the legacies of previous plant species.

In **Chapter 4**, I studied how rhizosphere microbial communities mitigated the effects of drought on grasses. I conducted a greenhouse experiment using microbial inocula and eight native grass species from different positions along a secondary succession gradient. The results indicated that under optimal water supply conditions, inoculation with live soil communities led to a substantial decrease in grass biomass production. However, under drought conditions, the negative impact of soil microbes on plant growth was less severe. After identifying and quantifying the microbes present in the rhizosphere, I found no evidence for the popular hypothesis that arbuscular mycorrhizal fungi, a type of beneficial fungi, alleviate the effects of drought in grasses.

In **Chapter 5**, I investigated the impact of drought on nematodes and how changes in nematode populations affect the performance of two grass species, *Agrostis capillaris* and *Alopercurus pratense*. The experiment was conducted in a controlled environment (greenhouse). I hypothesized that drought had differential effects on nematodes in the soil and in the roots. Furthermore, I expected that these effects would influence the growth of the two grass species. The results showed that drought suppressed nematode populations in the soil but significantly increased their presence in the roots of both grass species. The nematode populations consisted mainly of fungivores and bacterivores, while root herbivores and omnivores were less abundant. Fungivores were found to be more sensitive to drought than bacterivores. Based on the observed patterns, I propose that that fungivores and bacterivores in the soil might contribute negatively to plant biomass production under drought conditions.

My work highlighted the potential dominant role of pathogens in grass-soil feedbacks, spanning from candidate genes that might regulate plant-pathogenic interactions to reduced pathogenic effects as a consequence of drought. Specifically, this thesis suggests that genes associated with root development may play a crucial role in regulating the mechanisms employed by grasses to counteract fungal pathogens. In the context of monoculture systems, where grasses are frequently cultivated, the selection of traits that enhance plant tolerance to pathogens becomes crucial. This thesis contributes to the knowledge that plant breeders can leverage to develop grass varieties that possess improved resistance mechanisms, thereby enhancing their overall performance.

This modulation of plant-pathogen interactions could potentially have implications for how plants respond to drought stress and their overall growth. In light of these findings, it becomes evident that a deeper exploration into the intricate relationship among pathogens, drought, and root traits in grasses is warranted, urging the need for future investigations.

List of publications

Rallo, Paola, Hannula, S. Emilia; Ten Hooven, Freddy C.; Verhoeven, Koen J. F.; Kammenga, Jan; Van Der Putten, Wim H. 2023. "Inter- and Intraspecific Plant-Soil Feedbacks of Grass Species." Plant and Soil, February. https://doi.org/10.1007/s11104-023-05893-z.

Birnbaum, Christina; Dearnaley, John; Egidi, Eleonora; Frew, Adam; Hopkins, Anna; Powell, Jeff; Aguilar-Trigueros, Carlos; Liddicoat, Craig; Albornoz, Felipe; Heuck, Meike Katharina; Dadzie, Frederick; Florence, Luke; Singh, Pankaj; Mansfield, Thomas; Rajapaksha, Kumari; Stewart, Jana; **Rallo, Paola**; Peddle, Shawn; Chiarenza, Giancarlo. 2023. "Integrating soil microbial communities into fundamental ecology, conservation, and restoration: examples from Australia". Submitted in New Phytologist.

Rallo, Paola; van Eijnatten, Bram; ten Hooven, Freddy C.; Fois, Mattia; Asp, Torben; Snoek, Basten; van der Putten, Wim H.; Kammenga, Jan; Hannula, S. Emilia; Verhoeven, Koen J.F. *(in prep).* Genome-wide association study pinpoints plant loci associated with rhizosphere and root endosphere microbiome of *Lolium perenne*".

Rallo, Paola; Bakx-Schotman, Tanja; Kammenga, Jan; van der Putten, Wim H.; Hannula, S. Emilia; Verhoeven, Koen J.F. (*in prep*). "Drought mitigates negative effects of natural microbiomesc in grasses".

Rallo, Paola; Chikwature, Nyasha; Wilschut, Rutger; de Jonge, Arjen; Hannula, S. Emilia; Verhoeven, Koen J.F.; van der Putten, Wim H.; Kammenga, Jan. *(in prep).* "Drought effects on fungivorous and bacterivorous nematodes in soil and roots, and possible consequences for grass biomass".

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review/project proposal (4.5 ECTS)

- Genetic basis and ecological relevance of intraspecific variation in grass-microbiome interaction

Post-graduate courses (5 ECTS)

- Multivariate analysis; PE&RC (2021)
- TE soil biodiversity analysis; NIOO (2021-2022)
- Soil ecology; PE&RC (2022)

Deficiency, refresh, brush-up courses (1.5 ECTS)

- Basic statistics; PE&RC (2020)

Laboratory training and working visits (2 ECTS)

- Grass genetic diversity; Flakkebjerg, Aarhus University, Denmark (2019)

Invited review of (unpublished) journal manuscripts (3 ECTS)

- Journal of Ecology: intraspecific variation in plant-microbe interactions (2020)
- New Phytologist: drought effects on soil fungal communities (2021)
- Journal of applied Ecology: effects of pesticide on microbial communities in vineyard (2022)

Competence strengthening/skills courses (2.2 ECTS)

- Scientific writing; EpiGBS (2021)
- On the way to a new job; Centre for Career & Development Royal Netherlands Academy (2023)

Scientific integrity/ethics in science activities (0.3 ECTS)

- Ethics in plant and environmental sciences; WGS (2020)

PE&RC Annual meetings, seminars, and the PE&RC weekend (0.9 ECTS)

- PE&RC Midterm weekend (2021)
- PE&RC Day (2021)

Discussion groups/local seminars or scientific meetings (11 ECTS)

- PhD Discussion group (2019-2023)
- Experimental design maximizing the power of your experiments (2020)
- PoP/QTL Discussion group (2020)
- Coding club (2020-2021)
- Plant microbiome group (2021-2022)

International symposia, workshops, and conferences (9.5 ECTS)

- NAEM; poster presentation; Lunteren, the Netherlands (2019, 2020)
- Ecology of soil microorganisms; poster presentation; Prague, Czech Republic (2022)
- ESA-SCBO; oral presentation; Wollongong, NSW, Australia (2022)
- Network analysis and artificial intelligence workshop; oral presentation; Wageningen, the Netherlands (2022)

Societally relevant exposure (1 ECTS)

- NIOO Open day (2019)

Lecturing/supervision of practicals/tutorials (4.5 ECTS)

- Ecological aspect of biotic interaction (2019)

BSc/MSc thesis supervision of students (5 ECTS)

- Effects of soil microbiome in grasses under drought stress
- Linking microbes, succession, and root architecture in grasses
- Drought effects on nematodes from different compartment

About the author

Paola Rallo was born on the 17th of July 1991 in Castelvetrano, Trapani, Italy 🇮🇹. She grew up on an olive farm and from a young age, her heart was set on becoming an "olive doctor," as she wanted to cure olive trees that developed tumors. During high school, however, she enjoyed the biology classes the most and was specifically fascinated by DNA. At that time, she decided that she wanted to become a "forensic scientist" working with the police to solve cases using DNA evidence.

With that thought in mind, she started a bachelor's degree in **Biotechnology** at Parma University, Italy 🇮🇹 and graduated in 2014. Choosing a masters was a hard task considering her broad interests. She still had not let go of her childhood dream of becoming an "olive doctor". And so, she moved to Perugia, Italy 🇮🇹 where she started a master's degree in **Agricultural and Environmental Biotechnology** at Perugia University. There, she met her first mentor, Prof. Chiaraluce Moretti who inspired her to study bacterial pathogens causing knot disease in olive trees. In 2017, Paola did her Erasmus traineeship at Malaga University, Spain 🇪🇸 where she studied pathogenic bacteria that caused disease in agricultural plants. While in Malaga, Paola started to read about beneficial microorganisms, and the idea that there are also beneficial microorganisms interacting with plants fascinated her. That interest brought Paola to Denmark 🇩🇰 in 2018, at the Flakkebjerg Ecological Institute of Aarhus University. Living in Denmark was a life-changing experience and motivated Paola to pursue a PhD position.

In March 2019, Paola started a PhD at the NIOO-KNAW at the Department of **Terrestrial Ecology** 🇳🇱 under the supervision of Prof. Wim van der Putten, Prof. Jan Kammenga, Dr. Koen Verhoeven and Dr. Emilia Hannula. For her PhD project, Paola investigated grass intraspecific variation in the ability to interact with belowground microbiomes. She gained insight into the biological processes that grasses might apply to modulate their rhizosphere and root microbiomes. Moreover, she gained an overall understanding of the impacts of drought on plant-belowground interactions in grass species.

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