Unravelling mechanisms linking plant diversity to plant-disease suppression

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„Ein Gelehrter in seinem Laboratorium“… „ist vor den Naturgesetzen wie ein Kind vor der Märchenwelt.“

Marie Curie

I | General Introduction

1.1 Economic importance of soil-borne phytopathogens

Soil-borne plant pathogens, in particular fungi, pose a global threat to crop production (Péchy-Tarr *et al.* 2008; Raaijmakers *et al.* 2009; Philippot *et al.* 2013). Plant species can be affected by various diseases such as root rot, root blackening, wilt, stunting or seedling damping-off and new diseases constantly appear (Haas & Defago 2005). An average yield loss of 7-15% was estimated for the most important food-crops maize, potato, rice, soybean and wheat (Oerke 2006). Well known aggressive pathogens such as Fusarium, Pythium or Rhizoctonia can even cause losses up to 20-35% (Cook 1987; Cook, Schillinger & Christensen 2002; Smiley *et al.* 2005), underlining the necessity for soil-borne disease control.

Since pathogenic fungi are susceptible to heat, heat-steaming was a common practice in greenhouses to improve vegetable yields, but this technique got restricted due to increasing energy-costs (Raaijmakers *et al.* 2009). Chemical pesticides are rarely effective in controlling root diseases sustainably and besides high costs, concern for the environment and human health restricts their application (Baehler *et al.* 2005; Lugtenberg & Kamilova 2009). However, some more harmless pesticides are still in use, while their application has uncontrolled side-effects on the whole microbial community (both, harmful and beneficial microorganisms are affected), e.g., their composition and development (Raaijmakers *et al.* 2009). Consequently, the interest in former cultural-practices such as crop rotation and tillage became renewed in order to reduce disease severity in a more sustainable and environmentally friendly way (Janvier *et al.* 2007). Crop rotation with non-hosts is effective for the suppression of rather specialist pathogens with a low saprophytic survival capacity (Cook *et al.* 2002; Janvier *et al.* 2007). Further, specific tillage practices (Sturz, Carter & Johnston 1997; Pankhurst *et al.* 2002), intercropping (Trenbath 1993) as well as residue destruction (Baird, Watson & Scruggs 2003) and organic amendments (Tilston, Pitt & Groenhof 2002) have decreased disease severity. Interestingly, also long-term mono-cropping accompanied by severe disease outbreaks can result in increased pathogen suppression (Weller *et al.* 2002; Postma, Scheper & Schilder 2010; Mendes *et al.* 2011). However, the link between cultural practices and disease suppression is

rarely made and it still remains challenging to unravel mechanisms linking crop management to the disease suppressive potential of soils (Janvier *et al.* 2007; addressed in Chapter 2.4).

1.2 The potential of soils to suppress phytopathogens

Suppressive soils are soils in which plant-disease severity is reduced or plants even resist certain diseases, although disease-causing pathogens are present (Haas & Defago 2005). In those soils, pathogens have low fitness, grow poorly and thus cause little damage to plants (Baker & Cook 1974; Weller *et al.* 2002). Virtually all soils have the potential to suppress diseases to some extent, which is due to the overall activity of the autochthonous soil microbial community. Whereas, specific pathogen suppression is rather caused by a distinct biological mechanism (Weller *et al.* 2002).

Most prominently, plant-pathogen antagonistic rhizobacteria have been discussed in respect of their potential to suppress pathogens (Garbeva, van Veen & van Elsas 2004; Janvier *et al.* 2007; van der Heijden, Bardgett & van Straalen 2008). Further, in addition to biotic factors, abiotic factors have been shown to alter disease suppressiveness of soils (Garbeva *et al.* 2004; Berg & Smalla 2009; Philippot *et al.* 2013). Pathogenic as well as pathogen antagonistic microbial organisms have specific pH-optima and therefore react specifically sensitive to changes in soil pH (Rousk *et al.* 2010). Further, soil-water content affects microorganisms, with some being more resistant to drought or, on the other extreme, oxygen-deficiency then others (Hinsinger *et al.* 2009; Bardgett & van der Putten 2014). In addition, macro- and micronutrients as well as physicochemical soil characteristics have been shown to influence soil suppressiveness (Janvier *et al.* 2007). However, the importance of abiotic factors is far from being clear, which might be due to the fact that they are often intercorrelated, and the complexity of mechanisms driving soil properties (Janvier *et al.* 2007; addressed in Chapter 2.3).

1.3 The rhizosphere as habitat of microorganisms

The rhizosphere is the narrow soil-zone around the root-surface that is influenced by the plant (Bais *et al.* 2006; Lugtenberg & Kamilova 2009; Raaijmakers *et al.* 2009). Here, the abundance

and activity of soil microorganisms is much higher compared to surrounding bulk-soil (Hiltner 1904). Plants provide a carbon-rich environment, 5–21% of the fixed carbon is secreted as root exudates or enters the soil as other root-derived resources summarized as rhizodeposits (Marschner 2011). Root exudates provide a cocktail of amino-acids, fatty-acids, nucleotides, organic-acids, sugars and phenolic compounds and other nutritional compounds that attract microorganisms (Uren 2007; Lugtenberg & Kamilova 2009). Rhizodeposits also contain toxic compounds that restrict microbial colonization (Bais *et al.* 2006). Further, soil pH, which is an important driver of soil microbial communities, can change up to two units due to root-secretion or uptake of ions (Hinsinger *et al.* 2009). Interestingly, electric potentials in plant-roots have been shown to attract spores of pathogenic oomycetes to swim towards hosts (van West *et al.* 2002). Plants further affect microbial growth in the rhizosphere via influencing a soils oxygen status which might be indirectly driven by soil properties such as soil aggregation and soil-water contend (Hinsinger *et al.* 2009). Thereby, plants essentially form the habitat of plant-beneficial as well as plant-pathogenic microorganisms (Raaijmakers *et al.* 2009; addressed in Chapters 2.1 and 2.3).

1.4 Phytopathogens and plant-pathogen antagonistic microbes

Phytopathogens and plant-pathogen antagonistic microbes are natural competitors for nutrients and niches. Therefore, rhizosphere competence is one of the most important issues for plant-infection by pathogens as well as suppression of pathogens by antagonistic microbes (Haas & Defago 2005; Lugtenberg & Kamilova 2009). Rhizosphere microorganisms have evolved strategies to improve their competitiveness, some grow in tight biofilms thereby preventing competitors to capture a site (Rudrappa *et al.* 2008), others show high mobility which enables them to rapidly colonize new sites (Czaban, Gajda & Wroblewska 2007). Some bacteria have evolved ingenious strategies to take-up resources more quickly, thereby outcompeting other root-colonizers (e.g., iron-chelators; Schippers, Bakker & Bakker 1987). Interestingly, some bacteria are able to interfere communication of their competitors (AHL-signal degredation; Dong & Zhang 2005). Further, exoenzymes such as chitinases and proteases that damage fungal cell-walls contribute significantly to the antifungal activity of bacteria (Haas & Keel 2003; Kielak *et al.* 2013). However, the most effective strategy in pathogen suppression presumably is the

synthesis of antibiotic compounds (Haas & Defago 2005). *Streptomyces* spp., *Bacillus* spp. and *Pseudomonas* spp. are known to produce highly effective antibiotics (Weller *et al.* 2002). In particular, *Pseudomonas* spp. have been intensively studied (Weller 2007). Pseudomonads are ubiquitous rhizosphere colonizers, easy to isolate and cultivate, and can be modified genetically. Thus, Pseudomonads represent ideal model organisms for investigating mutualistic interactions between plants and rhizosphere bacteria (Lugtenberg, Dekkers & Bloemberg 2001). Their ability to suppress pathogens is mainly ascribed to the production of antibiotic compounds including phloroglucinols, phenazines, pyoluteorin, pyrrolnitrin, lipopeptides, and hydrogen cyanide (Haas & Keel 2003). These antibiotics present antihelminthic, antifungal and antibacterial activities (Raaijmakers, Vlami & de Souza 2002), and contribute to plant protection by directly inhibiting pathogens (Haas & Defago 2005) and eliciting plant defenses (induced systemic resistance; Iavicoli *et al.* 2003). Despite their capability to suppress pathogens, in most cases, bacteria have been shown to be ineffective when applied to the field, arguing for the existence of additional mechanisms and regulators of the suppressive-ability of bacteria (Lugtenberg & Kamilova 2009; addressed in Chapters 2.1 and 2.3).

1.5 The role of predation in disease suppression

To be a successful root-colonizer, bacteria do not only need to compete with other microorganisms but also to escape predation by indigenous microfauna (Lugtenberg & Kamilova 2009). Predation by protozoa exerts a high pressure on bacterial communities in the rhizosphere, and causes up to 50% productivity loss (Foissner 1999). Since grazing on bacteria is selective, protozoa feed mainly medium-sized bacterial cells that lack defense-mechanisms, they essentially shape microbial communities and their functioning (Rønn *et al.* 2002; Jousset *et al.* 2009). Especially nutrient cycling and the ability to suppress pathogens by bacteria are affected by protozoan grazing (Bonkowski 2004; Müller, Scheu & Jousset 2013). However, the role of predation in plant-growth and health promotion by bacteria is only beginning to be understood (Dubuis, Keel & Haas 2007; addressed in Chapter 2.4).

1.6 The role of plant diversity in shaping microbial communities

Diverse plant communities are generally more productive than monocultures, an effect that strengthens through time (Cardinale *et al.* 2007). This phenomenon has been extensively studied over the last decades, but the underlying mechanisms are not yet fully resolved (van der Heijden *et al.* 2008; Reich *et al.* 2012; Ebeling *et al.* 2014; Tilman, Isbell & Cowles 2014). The increasing probability of including and becoming dominated by species with a high impact on a given function with increasing diversity levels (sampling-effect; Aarssen 1997; Tilman, Lehman & Thomson 1997; Huston 1997) and a more complementary resource-use or species facilitation (complementarity-effect; Loreau *et al.* 2001) have been most prominently discussed. To predict complementarity effects between species, functional traits, i.e. morphological, phenological or physiological characteristics affecting the performance of organisms, have been used (Ebeling *et al.* 2014). However, it remains unresolved which trait differences matter, if species number can be used to summarize those differences, or if functional group affiliation or phylogenetic relatedness is providing additional information (Tilman *et al.* 2014).

Plant community composition and diversity shape belowground microbial communities (Kowalchuk, de Souza & van Veen 2002; Zul *et al.* 2007). Generally, plant diversity increases the diversity, abundance and activity of soil microorganisms (Stephan, Meyer & Schmid 2000; Zak *et al.* 2003; Liu *et al.* 2008; Eisenhauer *et al.* 2010a). Effects of soil microorganisms on plant growth, such as positive effects through nutrient provisioning or negative effects through pathogen pressure, have been suggested to be plant diversity dependent (Mitchell 2003; Schnitzer *et al.* 2011; Eisenhauer, Reich & Scheu 2012). Soil enzyme activities that reflect the ability of microorganisms to contribute to soil health and quality are increased in diverse grasslands (Bandick & Dick 1999). In addition, bacteria involved in pathogen suppression are more abundant in diverse plant communities and likely contribute to decreased disease severity observed associated with diverse grassland systems (Garbeva *et al.* 2006; Weller 2007; Latz *et al.* 2012).

Generally, effects of plant diversity on microbial communities have been suggested to be due to increased plant productivity accompanied by increased resource quantity (Spehn *et al.* 2000; Zak *et al.* 2003; de Deyn, Quirk & Bardgett 2011). On the other hand, specific ecosystem functions are assumed to depend on resource composition and the quality of specific

resources, driven by variation in plant species richness and identity (de Deyn *et al.* 2011; Latz *et al.* 2012, 2015). However, the exact patterns driving plant diversity effects on rhizosphere microbial communities need further investigation (addressed in Chapters 2.1 and 2.3).

1.7 Plant identity and soil suppression

The importance of plant species identity in shaping rhizosphere microbial communities is receiving increased attention. Some microbial species are highly specific in their association with specific plant groups or even single plant species. Rhizobia-legume as well as pathogen-host interactions represent examples of high specificity (Long 2001; Bais *et al.* 2006).

Root exudates that are highly plant-species specific may be the driving force for this phenomenon, but the role of single compounds is only beginning to be understood (Berg & Smalla 2009). Certain root exudates prevent colonization by pathogenic microorganism while attracting others, or attract a couple of microorganisms with differing consequences to the plant (Bais *et al.* 2006). For example, flavonoids in soybean root exudates attract a pathogen as well as its antagonist (Tomasi *et al.* 2008). Plants growing in nutrient-poor environments have been shown to attract nutrient-acquiring microorganisms (Dakora & Phillips 2002). Further, plants shape the rhizosphere microbial community via exudation of toxic compounds. As a consequence, specific microbes resistant to the toxin(s), or microbes that are able to degrade the toxin(s) colonize the roots (Kowalchuk, Hol & van Veen 2006; Bressan *et al.* 2009).

In addition, root morphology and plant biomass effects have been shown to be plant species specific and drive rhizosphere microbial communities (de Deyn *et al.* 2011; Philippot *et al.* 2013). Notably, the root surface is only fragmentally colonized by bacteria. Bacterial populations preferentially grow in root-zones that are especially rich in root exudates (Bais *et al.* 2006). Borders between epidermal cells and side-root bifurcations are popular sites for bacterial colonization (Dubuis *et al.* 2007).

Interestingly, growth and resource allocation of plants growing without competitors (for resources and niches) differ markedly from plants growing in competition with other species (Semchenko, Hutchings & John 2007). In the presence of competitors, plants invest more in root exudates provide certain functions, such as nutrient foraging, facilitative plant-plant communication and allelopathy, which on the other hand likely shape the rhizosphere microbial

community (Bais *et al.* 2006).

Indeed, not only plant induced shifts in the microbial community (Garbeva *et al.* 2004; Bakker *et al.* 2012; Mendes, Garbeva & Raaijmakers 2013), but also plant induced changes in the activity of bacteria in producing antifungal compounds (de Werra *et al.* 2008; Rochat *et al.* 2010) alters soil suppressiveness. However, if the production of antibiotics observed in dependence on specific plant species also occurs in multi-species plant communities, remains to be investigated (Latz *et al.* 2015; addressed in Chapter 2.1).

1.8 Objectives and chapter outline

In this thesis, I investigated the role of plant diversity in driving disease suppression (Figure 1). First, I analysed whether plant diversity *per se* or plant functional group affiliation, plant identity or interaction effects are the main drivers of biocontrol bacteria and their activity in producing antifungal compounds. Second, I investigated abiotic factors and protozoan predators as mediators of plant community composition effects on plant-disease suppression.

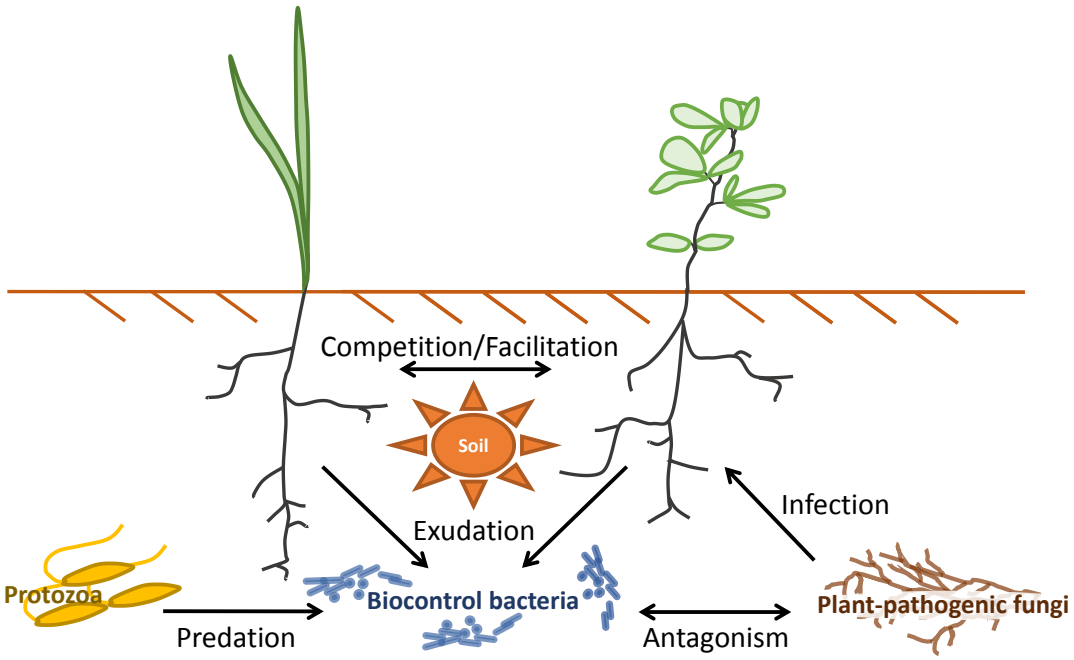

Figure 1. Interactions taking place in the rhizosphere of plants. Plant-pathogenic fungi cope with the antagonism of biocontrol bacteria. Bacteria are influenced by plants and their community composition is driven by protozoan-predation. The whole system is influenced by the plant-roots surrounding soil.

In *RESEARCH CHAPTER 1* the role of plant diversity in driving the expression of antifungal traits by biocontrol bacteria is evaluated. I measured the effect of plant diversity on the expression of genes responsible for the production of the most important antifungal compounds (2,4-diacetylphloroglucinol [DAPG], Pyrrolnitrin [PRN] and hydrogen cyanide [HCN]) in strain *Pseudomonas protegens* CHA0 grown in gnotobiotic cultures. Further, I related the expression of genes to biomass effects that I expected to increase with plant diversity and in turn increase bacterial activity in producing antifungal compounds. I hypothesised plant diversity to increase plant productivity and assumed this effect to increase bacterial abundances that in turn increase their activity and thereby contribute to increased disease suppression. Results of this study showed expression to be driven mainly by plant identity, an effect that persisted along the plant diversity gradient for all tested genes. While the expression of the gene coding for DAPG showed to be primarily directly driven by plant identity effects, expression of the genes coding for PRN and HCN were more pronounced indirectly driven by high density microbial communities. Interestingly, the effect-direction of certain plant identities varied between the three tested genes, indicating a selective impact of plant species on bacterial gene expression. This study shows that the presence of certain plant species within a community disproportionately impact biocontrol traits expressed by rhizosphere bacteria, providing new insight into our understanding of the patterns driving plant health and productivity.

In *RESEARCH CHAPTER 2* a newly developed two-pathogen infection model is presented. Bioassays are a standard method to evaluate plant-breeding or pesticide-application efficacy, and further are used to estimate the potential of natural-soils to suppress soil-borne plant diseases. Generally, in such assays, plants are exposed to a pathogen and infections are rated over time. Using natural soils causes the problem that additional infections might be caused by a naturally occurring pathogen, which might bias the exact estimation of infection rates. The developed model enables to evaluate disease suppression in natural soils by allowing to incorporate infection rates of control treatments. Further, the model makes infection start and infection rate measures in different soils comparable.

In *RESEARCH CHAPTER 3* the role of plant diversity in driving the composition of microbial communities with antifungal activity is evaluated and their abundances are related to disease suppression. I used cultivation dependent methods to investigate if plant diversity promotes

the abundance of *Pseudomonas*, *Streptomyces* and *Bacillus* spp, the three main groups of biocontrol bacteria in natural soil. Further, I related the abundances of the biocontrol groups to abiotic soil properties and investigated their impact on disease suppression by conducting a standardized infection assay and determined the infection start by using the two-pathogen infection model (*CHAPTER 2.2*). I hypothesised plant diversity to increase plant productivity and assumed this effect to increase bacterial abundances thereby contributing to increased disease suppression. Further, I assumed plant diversity to affect abiotic soil parameters that in turn influence the biocontrol microbial community and thereby the disease suppressive potential of soils. The results indicate that among an important indirect role of plant communities in shaping soil-disease suppression (e.g., via changes in root biomass, soil pH and the abundance of important biocontrol groups), plants interactively, directly affect the suppressive potential of soils. This study represents an important step in understanding the complexity of mechanisms linking plant community composition and disease suppression.

In *RESEARCH CHAPTER 4* the role of intercropping and predation pressure by protozoa in driving plant-growth promoting enzyme activities and therewith plant productivity is assessed.
In cooperation with the Universidad Nacional de Quilmes (Buenos Aires, Argentina) I used a soybean-maize intercropping system and scrutinized the links between crop richness, soil enzymes involved in pathogen suppression and nutrient supply, and yield. Further, I related enzymatic activities to abundances of the three main taxa of protozoan-predators amoeba, ciliates and flagellates. I hypothesized intercropping to increase protozoan abundance and to improve enzyme activity, thereby increasing crop yield. I observed that indeed mixing soybean and maize increased crop yield. In addition, this study provides first evidences that cropping regime interactively with soil protozoa shapes soil functioning by influencing soil enzyme activities and thereby crop yield.

AIM OF THIS THESIS

This thesis aimed at a mechanistic understanding of the effect of plant diversity and plant community structure on the abundance and activity of soil bacteria responsible for soil-borne plant disease suppression.

II | Research Chapters

2.1 Research Chapter 1

PLANT IDENTITY DRIVES THE EXPRESSION OF BIOCONTROL FACTORS IN A RHIZOSPHERE BACTERIUM ACROSS A PLANT DIVERSITY GRADIENT

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2.1.1 | Summary

Plant performance is influenced by root-associated bacteria that provide important services to the host plant, such as pathogen suppression. Suppression of pathogens is known to be context dependent and to vary between plant species, yet, the significance of plant identity in shaping rhizosphere bacterial functioning in multi-species communities is largely unknown.

We questioned whether the activity of a rhizosphere bacterium in producing biocontrol compounds varies with plant identity in a plant diversity gradient. We set up a gnotobiotic microcosm experiment with the model rhizosphere bacterium *Pseudomonas protegens* CHA0, an important biocontrol agent, and investigated the effects of plant identity and diversity on its production of biocontrol compounds. Using GFP-based reporter fusions, we assessed gene expression linked to the production of the biocontrol compounds 2,4-diacetylphloroglucinol, pyrrolnitrin, and hydrogen cyanide.

The expression of genes coding for biocontrol compounds was driven to a large extent by plant identity and persisted along the plant species richness gradient for all tested genes. Notably, the effect of certain plant identities varied between the three tested genes, indicating a selective impact of plant species on bacterial gene expression. However, some plant species, such as *Lolium perenne*, consistently stimulated bacterial gene expression irrespective of the diversity of the plant community.

Our results indicate that the presence of certain plant species within a community disproportionately impact biocontrol traits expressed by rhizosphere bacteria, providing new insight in the patterns driving plant health and productivity.

2.1.2 | Introduction

Plant performance is driven to a large degree by plant-microbe interactions. Soil microorganisms influence plant nutrient acquisition (Chapman *et al.* 2006; van der Heijden *et al.* 2008; Bardon *et al.* 2014), enhance plant immune responses (Pineda *et al.* 2013) and stress tolerance (Schardl, Leuchtmann & Spiering 2004), and directly as well as indirectly enhance plant growth (Bais *et al.* 2006; Raaijmakers *et al.* 2009). In agriculture, a special group of rhizosphere bacteria has gained particular attention due to their potential to protect plants against soil-borne fungal pathogens (Weller *et al.* 2002). Fluorescent pseudomonads form a dominant and one of the best characterized groups of bacteria responsible for natural plant protection against pathogens (Haas & Defago 2005), and are particularly relevant in agricultural systems as well as grasslands (Mendes *et al.* 2011; Latz *et al.* 2012). Several strains produce broad spectrum antibiotic compounds, such as 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (PRN), and hydrogen cyanide (HCN; Raaijmakers *et al.* 2002; Haas & Keel 2003; Haas & Defago 2005; Weller 2007). However, the expression of the factors that promote plant health are subject to complex regulation, and respond to microbe-microbe interactions, numerous environmental factors, as well as plant-derived cues (Dubuis *et al.* 2007).

The selectivity of plant species in shaping microbial communities in the rhizosphere has been studied intensively. So far, effects of plants on the efficacy of biocontrol bacteria have been shown to be mediated by 1) plant-induced shifts in the rhizosphere microbial community (Garbeva *et al.* 2004; Bakker *et al.* 2012; Mendes *et al.* 2013) and 2) plant-induced changes in the activity of biocontrol bacteria in producing antifungal compounds (de Werra *et al.* 2008; Rochat *et al.* 2010). However, information on plant-microbe interactions is mainly based on plant monocultures, and plant identity effects have rarely been studied in multi-species plant communities.

In grasslands, high plant diversity is associated with increased primary production (Hooper *et al.* 2005; Cardinale *et al.* 2012), but the mechanisms underlying this relationship are not yet fully resolved (van der Heijden *et al.* 2008; Reich *et al.* 2012; Ebeling *et al.* 2014). Diverse communities are suggested to be able to capture limiting resources in a complementary and thereby more efficient way (Loreau *et al.* 2001).

Effects of soil microorganisms on plant growth, such as positive effects through nutrient provisioning or negative effects through pathogen pressure, are suggested to be plant diversity-

dependent (Schnitzer *et al.* 2011; Eisenhauer *et al.* 2012). In case of plant-pathogen interactions, biocontrol bacteria play a crucial role in promoting plant productivity by reducing pathogen pressure, an effect likely to be more pronounced in species rich plant communities (Garbeva *et al.* 2006; Weller 2007; Latz *et al.* 2012). Plant diversity has been shown to increase plant root biomas (Craine *et al.* 2003; Reich *et al.* 2012; Mueller *et al.* 2013; Ravenek *et al.* 2014), and it has been suggested that plant diversity drives microbial density via increasing plant biomass (Spehn *et al.* 2000; Zak *et al.* 2003; de Deyn *et al.* 2011), thereby increasing habitat and resources of root-associated bacteria. Since cell-cell signalling becomes more important at high population densities (Dubuis *et al.* 2007; Pierson & Pierson 2007; Lapouge *et al.* 2008), the production of biocontrol compounds by bacteria is likely to be enhanced in more diverse plant communities. In order to understand the functioning of plant communities and improve intercropping as well as break-cropping strategies (Li *et al.* 2014), it is essential to link plant community composition and the activity of biocontrol bacteria. However, it has not yet been investigated if the expression of antifungal genes in biocontrol bacteria is modified by the presence of neighbouring plant species in multi-species plant communities.

In addition to plant diversity, the presence of certain functional groups, such as grasses and legumes, has been shown to affect primary productivity in grassland and pasture systems (Spehn *et al.* 2000; Hedlund *et al.* 2003; Zak *et al.* 2003; Milcu *et al.* 2008). Grasses have highly branched roots with a higher biomass than other plant functional groups (Bessler *et al.* 2009, 2012; Pérès *et al.* 2013), thereby providing more habitat for root-associated bacteria (Lange *et al.* 2014), but also increasing the area for the attack by pathogenic fungi (Sikes, Cottenie & Klironomos 2009; Kulmatiski *et al.* 2014). Therefore, grasses are likely to rely heavily on the activity of bacteria antagonistic to fungal pathogens and to benefit from dense bacterial populations protecting their roots. Indeed, in experiments with gnotobiotic systems *Pseudomonas* spp. reached up to ten times higher densities on the roots of monocots than on those of dicots (Lugtenberg, Dekkers & Bloemberg 2001). However, whether the expression of genes coding for biocontrol compounds is increased in multi-species plant communities containing grasses remains to be investigated.

We investigated the impact of plant diversity and identity on the expression of genes coding for biocontrol compounds in a root-associated bacterium in a gnotobiotic microcosm experiment. To separate plant diversity and identity effects we set up a full factorial species richness gradient including two grass and two legume species. Plant seedlings were inoculated

with the representative biocontrol strain *Pseudomonas protegens* CHA0. Three different GFP reporter fusions were used for measuring the expression of the genes *phlA*, *prnA*, and *hcnA*, responsible for the production of DAPG, PRN, and HCN, respectively (Baehler *et al.* 2005; Rochat *et al.* 2010). Bacterial gene expression was determined via flow cytometry, after three weeks of plant growth. In addition to linear regression analyses, we used structural equation modelling (Grace 2006) to be able to unravel direct and indirect pathways driving the expression of genes coding for bacterial biocontrol compounds.

According to the observations mentioned above, we expected bacterial gene expression to increase with plant diversity. We expected grasses to increase and legumes to decrease the expression of genes coding for biocontrol compounds. Further, we expected plant species to differ in their effects on bacterial gene expression.

2.1.3 | Materials and Methods

Bacterial strains, plasmids, and culture conditions. By using GFP-based reporter fusions, we integrated bacterial response over the duration of the experiment (de Werra *et al.* 2008). The expression of DAPG, PRN, and HCN biosynthetic genes has been shown to adequately reflect the production of these biocontrol compounds in *P. protegens* (Baehler *et al.* 2005; Rochat *et al.* 2010). Bacteria and plasmids used in this study are listed in Table 1. All chemicals were purchased from Merck (Darmstadt, Germany), unless noted otherwise. Pseudomonad strain stocks were stored in glycerol at -80°C and pre-grown on Luria Broth (LB) agar plates with the appropriate antibiotics (125 µg ml^{-1} tetracycline hydrochloride). Subsequently, bacteria were grown at 22°C in 5 ml 1/3 King´s B (KB; BactoTM Protease Peptone No. 3, BD, Le Pont de Claix, France) liquid medium (King, Ward & Raney 1954; McSpadden Gardener *et al.* 2001) with agitation for 12 h, harvested by centrifugation (4500 g, 4°C, 10 min) and washed twice in 0.85% NaCl. We used slowly grown (low temperature) early exponential phase cultures to avoid GFP accumulation in the cells prior to microcosm inoculation (Rochat *et al.* 2010). 200 µl of the overnight culture were grown in 30 ml KB liquid medium at 25°C to an optical density (OD) of 0.1-0.15. Bacterial cells were harvested, washed twice in 0.85% NaCl and adjusted to an OD of 0.1 in 1⁄5 Long Ashton nutrient solution (Hewitt 1966).

Table 1. Strains and plasmids.

Strain / plasmid	Properties	Reference
Strains		
Pseudomonas protegens	CHA0 Wild type, biocontrol strain	Voisard *et al.* (1994)
Plasmids		
pME7100	*phlA*-gfp transcriptional fusion; reporter for 2,4-diacetylphloroglucinol biosynthetic gene expression; Tc^r	Baehler *et al.* (2005)
pME7116	*prnA*-gfp transcriptional fusion; reporter for pyrrolnitrin biosynthetic gene expression; Tc^r	Baehler *et al.* (2005)
pME7156	*hcnA*-gfp transcriptional fusion; reporter for hydrogen cyanide biosynthetic gene expression; Tc^r	Rochat *et al.* (2010)

Tc^r, tetracycline resistant

Plants. Four plant species, two from each functional group, grasses and legumes, were used. Upscaling the maximum of four plants species per 0.077 m x 0.077 m equals approximately 60 species per 20 m x 20 m (according to species area relationships for generalist European grassland species; Krauss *et al.* 2004), which is the maximum diversity per area in The Jena Experiment (Roscher *et al.* 2004). Grasses included *Lolium perenne* L. (*Lolium*) and *Dactylis glomerata* L. (*Dactylis*), and legumes *Lotus corniculatus* L. (*Lotus*) and *Trifolium repens* L. (*Trifolium*; Appels Wilde Samen GmbH, Darmstadt, Germany). It has been shown that nutrient uptake strategies and other functional traits differ considerably between these species (Roscher *et al.* 2004).

Grass seeds were incubated in 62% H_2SO_4 on a magnetic stirrer (approximately 250 rpm) to remove the husks (*Lolium*: 3.8 g seeds in 50 ml acid for 35 min; *Dactylis*: 3 g seeds in 60 ml acid for 32 min), washed in 50 ml distilled water under reduced speed for 15 min and subsequently sterilized by softly swivelling in 50 ml 2% $AgNO_3$ solution on an orbital shaker for 10 min. Silver ions were removed by washing nine times for 10 min alternating sterile distilled water and 1% NaCl solution (adapted from Henkes *et al.* 2008). Legume seeds (2 g each) were shaken in 10 ml 70% ethanol for 2 min, surface sterilized with 10 ml 1% NaClO for 15 min and washed six times in sterile water by orbital shaking (adapted from Hensel *et al.* 1990). All seeds were vernalised overnight at 4°C and pre-incubated until germination (40 g $liter^{-1}$) on moisturised, 2% water agar at 25°C (grasses up to 30 h; legumes up to 6 h). Germinated seeds were stored at 4°C until use on the same day.

Microcosm construction and inoculation. Magenta boxes (0.077 x 0.077 x 0.097 m) were filled with 50 g of 2-5 mm prewashed expanded clay (FiboExClay Deutschland GmbH, Lamstedt, Germany), watered with 15 ml 1⁄5 Long Ashton nutrient solution and autoclaved. Plant communities of twelve germinated seeds per microcosm were planted and each seed was inoculated with 50 µl bacterial suspension.

Experimental setup. We set up a substitutive, full factorial plant species richness gradient ranging from one to four plant species. Two blocks per bacterial treatment (*P. protegens* inhabiting plasmid pME 7100, pME 7116 and pME 7156, respectively) were set up, each with two replicates per plant species richness level, resulting in four replicates. Additionally, four plant monocultures inoculated with the *P. protegens* wild-type strain per block served as control (Supplementary Figure 1). The microcosms were incubated at 18-22°C with a 12 h photoperiod (photon flux density: 150 µmol m^{-2} s^{-1}) and randomized every 2-3 days over the total experimental time of 20 days.

Sampling. The microcosms were destructively sampled at the end of the experiment. Bacteria were extracted by horizontally shaking total roots in 6 ml of cold phosphate-buffered saline for 30 min (PBS; Sambrook & Russell 2001) and immediately examined by flow cytometry (see below). Total root biomass (fresh weight) was measured for each microcosm.

Bacterial quantification. Bacterial counts were performed with a C6 flow cytometer (Accuri, Ann Harbor, MI, USA) in 100 µl of the root-wash suspension (25,000 events in a maximum time of 2 min were recorded; threshold on FSC-H 5,000; threshold on SSC-H 2,000). By gating on the basis of log-scaled fluorescence signals (FL1-A x FL2-A), we measured the density of active bacteria (counted events) and bacterial per capita gene activity (emitted fluorescence per bacterium). In addition to the measured variables, we calculated total bacterial activity as related to the plant communities, but irrespective of the bacterial treatment (*P. protegens* CHA0 with either *phlA*, *prnA*, or *hcnA* gene reporter fusions) by normalizing the measures of the different reporter fusions (from each bacterial per capita gene activity measure we subtracted its mean and divided this by its variance).

Statistical analyses. Data were analysed using the statistical software R (R Core Team 2014) with

the packages **nlme** (Pinheiro *et al.* 2014) and **lavaan** (Rosseel 2012). To test whether the block had an effect on investigated variables, we used linear models with each measure as explained by plant diversity (continuous) and block (factorial). To measure plant identity effects independent of diversity, we used a sequential statistical approach (Bell *et al.* 2009). In a first step, we used linear mixed effect models with block as a random effect for analysing the effect of plant diversity on bacterial density [$\log_{10}$ (active bacteria g_{root}^{-1})], total bacterial activity [$\log_{10}$ (total gene expression g_{root}^{-1})], and *hcnA* gene activity [$\log_{10}$ (gene expression bacterium^{-1})]. Due to estimated Pearson´s product moment correlations between root biomass and bacterial density (cor = 0.33; $P < 0.001$), as well as root biomass and bacterial activity (cor = 0.31; $P < 0.001$), bacterial density and activity were normalized by root biomass. As the block had no effect on gene expression of *phlA* and *prnA* [$\log_{10}$ (gene expression bacterium^{-1})], these variables were fitted using linear models. In a second step, the residuals of the first step in the analyses were fitted against the explanatory variables *Lolium*, *Dactylis*, *Lotus*, and *Trifolium* (presence/absence coded; factorial) using linear models. In a third step, the residuals of the second step were fitted against plant interaction effects. The significance of slopes was determined via t-tests.

To separate plant community effects via root biomass and bacterial density from other mechanisms, we used structural equation modelling, which allows the analysis of variables in a multivariate approach (Grace 2006). We used plant identities (exogenous variables), as well as the explanatory variables root biomass and bacterial density (endogenous variables), as potential indirect pathways affecting bacterial gene activities. Models were performed with the respective residuals of linear models including the explanatory variables as influenced by block. After fitting the full model, the most parsimonious model was derived by removing non-significant pathways using Bayesian information criterion (BIC; Burnham & Anderson 2004) as well as χ^2 tests ($P > 0.05$; Grace 2006).

2.1.4 | Results

Sequential analysis. The density of active bacteria (bacterial density), as well as total bacterial gene expression (bacterial activity), increased with plant diversity (Figure 2 a, b; Table 2); however, the per capita gene expression was not significantly affected by plant diversity (Figure

2 c-e). The presence of the grass *Lolium* significantly increased the density of active bacteria, total bacterial gene expression and per capita gene expression of all tested genes (Figure 2 f-j; Table 2). On the other hand, the presence of the grass *Dactylis* significantly decreased bacterial density, total bacterial gene expression and per capita expression of *phlA* and *prnA* and marginally affected the expression of *hcnA* (Figure 2 f-j; Table 2). Fitting *Lolium* before diversity in the sequential analyses eliminated the marginally significant plant diversity effect on active bacterial density (slope -0.01, P = 0.303) as well as its significant effect on bacterial gene expression (slope-0.03, P = 0.278), suggesting that the observed plant diversity effects were due to the presence of *Lolium* and the increased probability of including this species in more diverse communities. The legume *Trifolium* significantly decreased the density of active bacteria, total bacterial gene expression and per capita expression of all tested genes (Figure 2 f-j; Table 2). The legume *Lotus* significantly decreased active bacterial density and marginally impacted total bacterial gene expression (Figure 2 f-g; Table 2). As indicated by the third step of the sequential analyses, plant-plant interactions did not significantly affect bacterial gene expression (all $P > 0.1$).

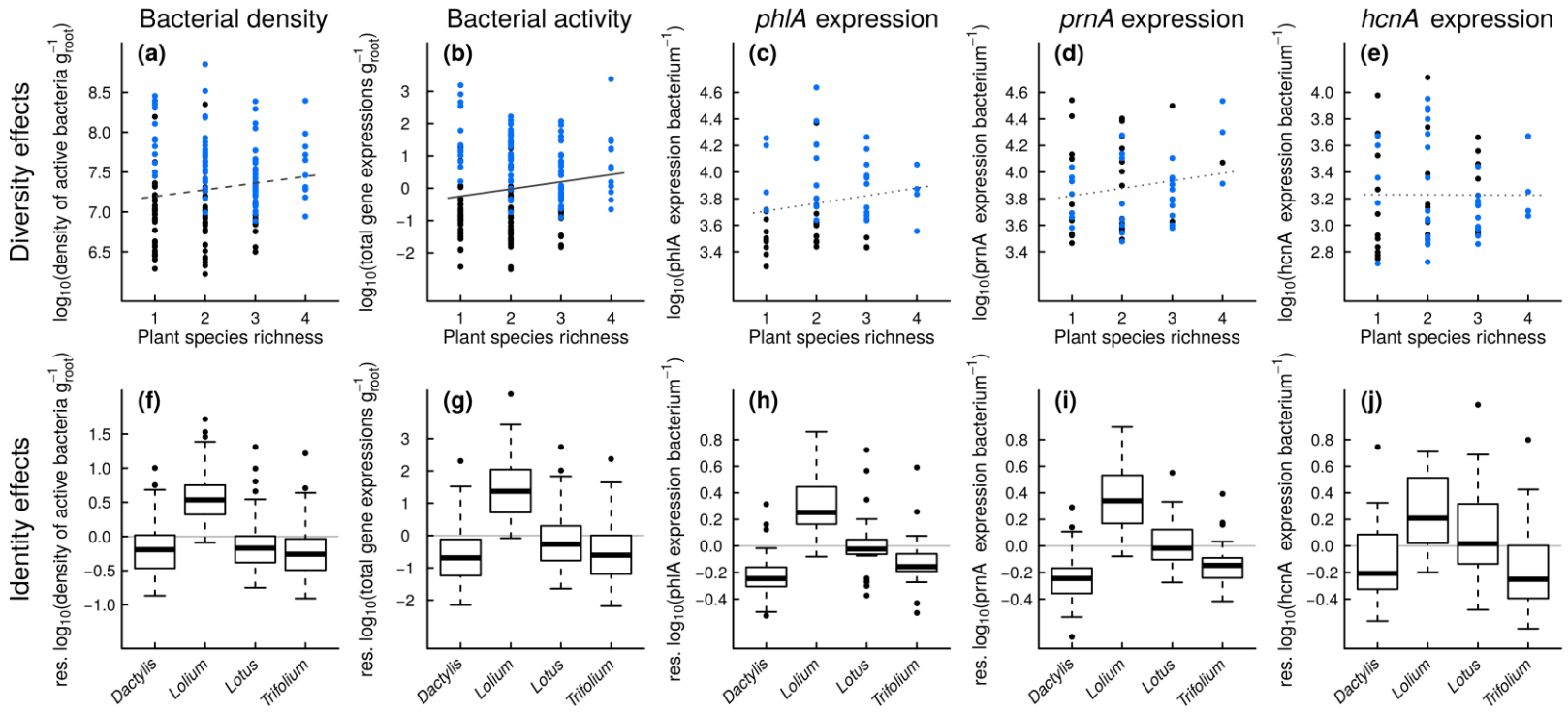

Figure 2. Effect of plant species richness on log_{10}-transformed (a) density of active bacteria per root biomass, (b) total bacterial gene expression per root biomass, (c) *phlA* expression per bacterium, (d) *prnA* expression per bacterium, and (e) *hcnA* expression per bacterium (upper panel), and the effect of plant identity on log10-transformed (f) density of active bacteria per root biomass, (g) total bacterial gene expression per root biomass, (h) *phlA* expression per bacterium, (i) *prnA* expression per bacterium, and (j) *hcnA* expression per bacterium [lower panel; analysis based on partial residuals (res.) after fitting of species richness]. Solid regression line: $P \leq 0.05$; dashed regression line: $P \leq 0.1$; dotted regression line: not significant. Blue dots represent microcosms containing *Lolium*.

Table 2. Effects of plant community composition on biocontrol bacteria (*Pseudomonas protegens*) as indicated by sequential analyses (see text for details).

	Bacterial density			Bacterial activity			*phlA expression*			*prnA expression*			*hcnA expression*		
1. step	Estimate	S.E.	t-value	Estimate	S.E.	t-value	Estimate	S.E.	t-value	Estimate	S.E.	t-value	Estimate	S.E.	t-value
Intercept	7.112	0.22	31.83 ***	-0.467	0.34	-1.39	3.649	0.10	36.08 ***	3.765	0.10	36.07 ***	3.231	0.24	13.74 ***
Plant diversity	0.083	0.04	1.86 .	0.222	0.10	2.16 *	0.058	0.04	1.32	0.056	0.05	1.23	-0.001	0.05	-0.02
DF/R^2	167/-			167/-			56/0.03			53/0.03			58/-		
2. step															
Lolium	0.582	0.05	11.62 ***	1.430	0.11	13.38 ***	0.305	0.05	6.26 ***	0.367	0.05	7.65 ***	0.245	0.07	3.41 **
Dactylis	-0.195	0.05	-3.91 ***	-0.640	0.11	-6.02 ***	-0.211	0.05	-4.37 ***	-0.238	0.05	-5.05 ***	-0.140	0.07	-1.94 .
Trifolium	-0.239	0.05	-4.82 ***	-0.573	0.11	-5.41 ***	-0.118	0.05	-2.44 *	-0.135	0.05	-2.89 **	-0.179	0.07	-2.48 *
Lotus	-0.144	0.05	-2.86 **	-0.204	0.11	-1.89 .	0.013	0.05	0.26	0.022	0.05	0.46	0.083	0.07	1.15
DF/R^2	170/0.46			170/0.54			54/0.48			51/0.58			57/0.25		

The tables were generated with the R-function summary(), showing the probability of a variable to be zero (two-tailed t-test). Asterisks denote the level of significance: P ≤ 0.1; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. DF, degrees of freedom; R^2, coefficient of determination.

Structural equation models. The structural equation model explained 49% of the variance in *phlA* gene expression. The initial model (BIC = 816.26; Figure 3 a) could be improved by removing non-significant paths (BIC = 797.37; Figure 3 b). The chi-square test indicated that our model adequately represents the data (χ^2_6 = 5.48; P = 0.484). Expression of *phlA* was directly increased by the presence of *Lolium* and decreased by the presence of *Dactylis*, while it was not significantly influenced by bacterial density. Bacterial density increased in presence of *Lolium*, but was negatively affected by the presence of *Trifolium*. Root biomass increased significantly in the presence of *Lolium* and *Lotus*, respectively, and decreased in the presence of *Trifolium* (Figure 3 b; Supplementary Table 1).

The structural equation model explained 77% of the variance in *prnA* gene expression. The initial model (BIC = 731.03; Figure 3 a) could be improved by removing non-significant paths (BIC = 709.51; Figure 3 c). The chi-square test indicated that our model adequately represents the data (χ^2_7 = 6.53; P = 0.480). In the final model, expression of *prnA* was directly increased by the presence of *Lolium* and *Lotus*, but decreased in the presence of *Dactylis*. In addition, it significantly increased with the density of active bacteria. Active bacterial density was positively related to the presence of *Lolium* and was significantly driven by root biomass. Root biomass increased in the presence of *Lolium* and decreased in the presence of *Trifolium* (Figure 3 c; Supplementary Table 1).

The structural equation model explained 62% of the variance in *hcnA* gene expression. The initial model (BIC = 899.24; Figure 3 a) could be improved by removing non-significant paths (BIC = 777.14; Figure 3 d). The chi-square test indicated that our model adequately represents the data (χ^2_4 = 3.02; P = 0.554). In contrast to the expression of *phlA* and *prnA*, *hcnA* expression was only affected by the presence of *Lolium*, but here the grass decreased gene expression. As in the case of *prnA*, the expression of *hcnA* significantly increased with the density of active bacteria. Active bacterial density increased in the presence of *Lolium* and decreased in the presence of *Trifolium*. As in the case of *phlA*, root biomass significantly increased in the presence of *Lolium* and *Lotus*, and decreased in the presence of *Trifolium*. As *Dactylis* presence played no appreciable role, it was removed from the model (Figure 3 d; Supplementary Table 1).

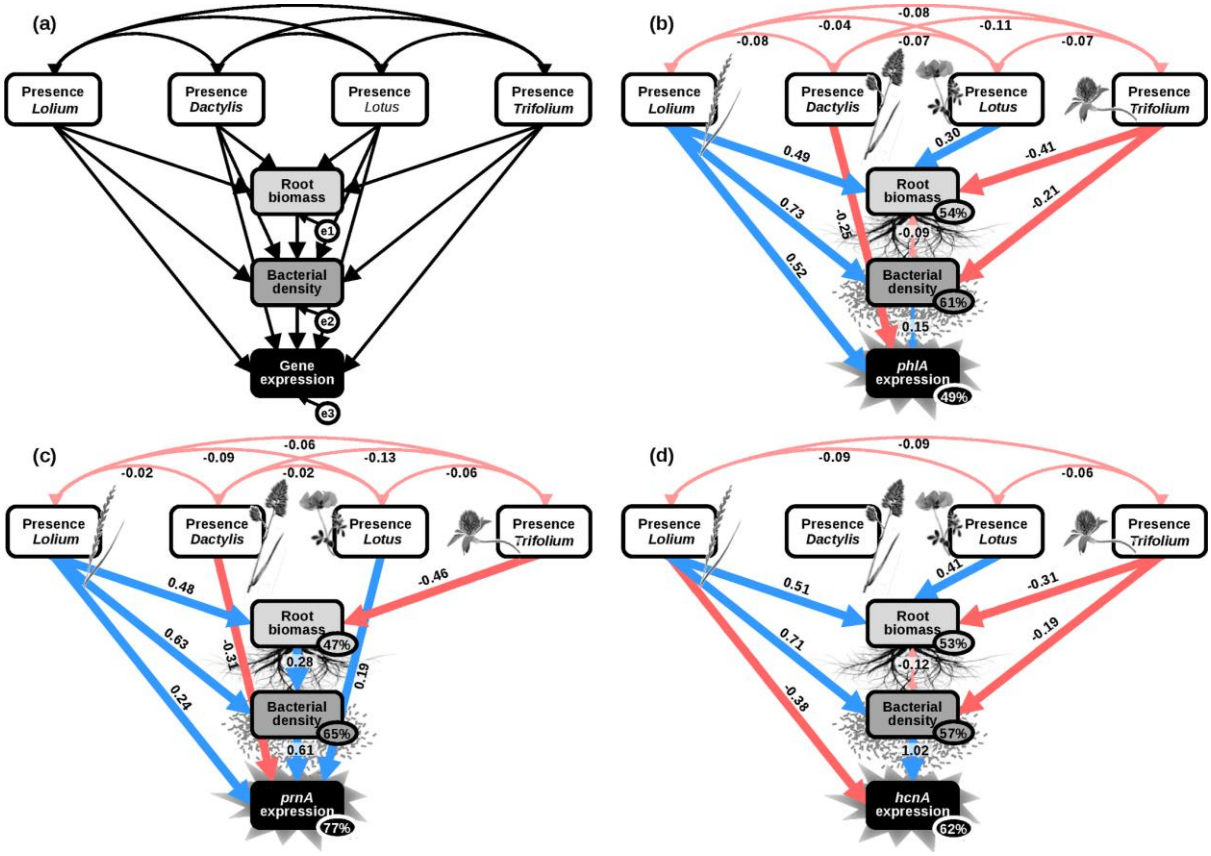

Figure 3. Structural equation models (see (a) for initial model) of direct and indirect (through changes in root biomass and bacterial density) effects of plant identity on (b) *phlA* expression, (c) *prnA* expression, and (d) *hcnA* expression. Exogenous variables (plant identity) are given in white boxes, endogenous, explanatory variables in grey and black boxes. The data did not significantly deviate from the respective models (see main text for model fits). Normal arrows represent causal relationships, and double-headed arrows indicate undirected correlations. Numbers on arrows are standardized path coefficients. Blue arrows indicate positive and red negative estimates; bold arrows indicate significant ($P \leq 0.05$) and thin arrows non-significant ($P > 0.05$) estimates. Circles in (a) indicate error terms (e1 – e3). Percentages close to endogenous variables indicate the variance explained by the model (R2).

2.1.5 | Discussion

Plants rely on their associations with soil microbes, and positive plant-soil microbe interactions are suggested to be more pronounced in species-rich plant communities (Schnitzer *et al.* 2011; Latz *et al.* 2012; Eisenhauer *et al.* 2012). On the other hand, root-associated microbial composition and functioning in diverse plant communities is suggested to be driven by specific plant species within a community (Kowalchuk *et al.* 2002; Eisenhauer *et al.* 2010a; de Deyn *et al.* 2011). Unfortunately, however, the mechanisms involved in the association and facilitation of root-associated bacteria with a specific function like plant growth promotion are virtually unknown in species-rich plant communities. We demonstrate that grassland plant species

selectively impact the expression of genes coding for biocontrol compounds in *P. protegens* CHA0, and that this effect persists in more diverse plant communities, highlighting the importance of plant species identity for soil microbial functioning. Especially *Lolium perenne* played a key role by significantly influencing each of the three measured genes coding for important biocontrol compounds. We suggest that the presence of specific plant species within a community may be essential for sustainable and environmentally friendly plant protection, which is one of the today's biggest issues in agricultural management.

Effect of plant diversity. The results showed that increasing plant diversity not only enhanced the density of active bacteria, but further enhanced the expression of genes coding for biocontrol compounds protecting plants from fungal pathogens. This supports reports of higher suppressive abilities against a soil-borne fungal pathogen in species-rich grasslands than in crop monocultures (Garbeva *et al.* 2006). Interestingly, effects of plant diversity were not pronounced when considering the bacterial genes separately, possibly because of the reduced sample size. However, the results may also indicate that more diverse plant communities are able to support higher activities of multiple genes respectively higher ecosystem multifunctionality (Hector & Bagchi 2007; Isbell *et al.* 2011). Nonetheless, the observed diversity effect likely was due mainly to a sampling effect; that is, the greater chance of a species (*Lolium*) with a disproportionate effect on ecosystem function properties being present at higher diversity levels (Huston 1997). Supporting this conclusion, the diversity effect disappeared when fitting the presence of *Lolium* before plant diversity, but remained when separately fitting the other species before plant diversity in the sequential analyses. Therefore, this study does not support a general positive effect of plant diversity on gene expression of biocontrol bacteria due to plant-plant interactions. However, it has been suggested that diversity effects are more pronounced in the long-term (Eisenhauer *et al.* 2012); whether this also applies to bacterial gene expression patterns remains to be investigated.

Effects of plant identity. Generally, our results highlight the importance of plant species identity in eliciting bacterial gene expression. This is in line with studies where soil microbial functioning was attributed to the role of single plant species (Stephan *et al.* 2000; Eisenhauer *et al.* 2010a; de Deyn *et al.* 2011).

The grass *Lolium* exerted the strongest positive effect, increasing the density of active bacteria

as well as the total and per capita expression of the three investigated genes as seen in the sequential analyses. Interestingly, although the number of *Lolium* individuals decreased with increasing plant diversity due to the substitutive design, the positive effect of *Lolium* remained throughout the diversity gradient. The effect, however, was dampened at higher plant diversity, but it remains unclear to what extend this was driven by the presence of the other species or by a lower number of *Lolium* individuals.

Structural equation models showed that *Lolium* increased root biomass as well as the density of active bacteria in each of the models. Notably, in contrast to the other two genes, *hcnA* was additionally directly negatively affected by *Lolium*, probably explaining its less pronounced positive effect in the sequential analyses.

In contrast to the overall strong positive effect of *Lolium*, the presence of the grass *Dactylis* decreased the density of active bacteria and total gene expression. The observed decrease in the per capita expression of *phlA* and *prnA* in the sequential analysis was reflected in the structural equation models, where *Dactylis* presence directly decreased *phlA* and *prnA* expression, but not that of *hcnA*. The contrasting effects of the two grass species are in line with results from experimental grasslands (Latz *et al.* 2012), where the presence of grasses in plant communities positively affected PRN producers, but this effect vanished with increasing grass species richness, suggesting that only specific species were responsible for the observed effect.

In the structural equation model, the expression of *hcnA* was not directly increased by any plant species and down-regulated in the presence of *Lolium*. It was previously shown that plants up-regulate the production of biocontrol compounds in bacteria in the presence of pathogens (Barret *et al.* 2009; Jousset *et al.* 2011). Due to the potential phytotoxic side effects of biocontrol compounds, such as DAPG and HCN (Rudrappa *et al.* 2008; Brazelton *et al.* 2008), susceptible plants may only benefit from enhancing their production in the presence of pathogens and may therefore suppress their production when pathogens are absent. Future studies are needed to understand how the observed plant community-induced bacterial gene expression patterns are affected by the presence of pathogens or other soil organisms.

The two legume species also had contrasting effects on biocontrol gene expression. In the sequential analyses, the presence of *Trifolium* decreased all tested parameters, whereas *Lotus* did not affect the per capita gene expression by the bacteria. In the structural equation models, the presence of *Lotus* increased root biomass in the models on *phlA* and *hcnA* gene expression.

It remains unclear why *Lotus* did not significantly influence root biomass in the model on *prnA* expression, where root biomass directly positively influenced gene expression. The effect of *Trifolium* was mainly driven by its negative effect on root biomass, in addition to a less pronounced negative effect on the density of active bacteria in the case of the *phlA* and *hcnA* model. Interestingly, *Trifolium* was the only plant species that did not directly affect the expression of any of the studied genes, and future studies should investigate the dependence of this legume species on biocontrol bacteria.

The overall negative effect of legumes on both the density of active bacteria and per capita gene expression matches well with observed decreases in the abundance of DAPG and PRN producers in the presence of legumes in grassland plant communities (Latz *et al.* 2012). These authors speculated that their results may be due to the production of biocontrol compounds by the plant, such as saponins and coumarin, providing plant-derived defence against root diseases and inhibiting DAPG producers, respectively (Djordjevic *et al.* 1987; Landa *et al.* 2002; Bergsma-Vlami, Prins & Raaijmakers 2005a). Consequently, the general positive effects of legumes on plant community productivity due to N fixation may be counterbalanced to some extent by a reduction of soil suppressiveness.

Effect of root biomass. In the structural equation models, root biomass increased the density of active bacteria in the case of *prnA*. Interestingly, in this case, root biomass did not increase in the presence of *Lotus*. This in turn suggests that an enhanced proportion of *Lotus* roots that occurred in the models on *phlA* and *hcnA* expression negatively affected the density of active bacteria. This may explain the observed negative effect of the legume on bacterial densities in the sequential analyses (see above). The results support the conclusion that root biomass is an important determinant of the density of active bacteria (Spehn *et al.* 2000), but they furthermore suggest that the strength and direction of effects depend on the presence of certain plant species.

Effect of active bacterial density. As bacterial gene expression is driven by cell population density-dependent patterns (Lapouge *et al.* 2008), it is not surprising that the density of active bacteria strongly increased the per capita expression of *prnA* and *hcnA* in the structural equation models. Interestingly, although an auto-inducing function is known for DAPG (Schnider-Keel *et al.* 2000; Baehler *et al.* 2005), the density of active bacteria did not increase

the per capita expression of *phlA* in our study. Auto-induction of DAPG can be counteracted by bacterial and fungal metabolites (Schnider-Keel *et al.* 2000), and our results suggest that unknown plant metabolites may also be involved in modifying the expression of biocontrol compounds in bacteria.

Conclusion. This study emphasizes the importance of plant identity for the expression of genes coding for biocontrol compounds in bacteria associated with multi-species plant communities. Expression patterns varied between genes, but some plant species, such as *Lolium* consistently impacted the expression of biocontrol traits. The results suggest that plants can steer specific microbial activities in the rhizosphere with important consequences for plant health. Similar to legumes improving plant productivity via symbiosis with nitrogen-fixing bacteria, we propose that certain plant species may contribute to community productivity by stimulating biocontrol gene expression in root-associated bacteria. Increased plant biomass production in more diverse communities might therefore in part be due to reduced pathogen load caused by the presence of certain plant species. Further studies are needed to evaluate the magnitude of the effect of single plant species in dampening pathogen pressure on plant communities. We conclude that management systems might benefit from taking the biocontrol-enhancing capabilities of certain plant species within a community into account.

2.1.6 | Acknowledgements

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2.2 Research Chapter 2

ASSESSING PLANT PATHOGEN INFECTION RATES IN NATURAL SOILS: USING bbmle AND deSolve

Björn Christian Rall & Ellen Latz

2.2.1 | Summary

Pathogen infection assays are a standard method for estimating plant resistance to a specific pathogen. In natural soils, however, alternative pathogens might also simultaneously infect plants of the experiment hindering the estimation of the focal pathogen's infection rate. Here we present a method in R correcting for these unwanted effects by developing a two pathogen monomolecular infection model. We fit the model to data using an integrative approach by combining a numerical simulation of the two pathogen monomolecular infection model and an iterative maximum likelihood fit. Our method will be particularly useful for exploring resistance of natural soils (e.g., biodiversity experiments) from different sites because it allows for different naturally occurring pathogens while estimating comparable infection parameters.

2.2.2 | Introduction

Pathogen infection assays (bioassays) are a standard method for estimating plant resistance to pathogens, induced systemic resistance in plants, the effect of artificial or natural plant protectants (e.g., plant beneficial bacteria), and the suppressive potential of soils. Generally, bioassays are performed using just a single point in time (Maurhofer *et al.* 1994; Pierson & Weller 1994; Postma *et al.* 2008) or multiple points in time (Postma *et al.* 2008; Hanse *et al.* 2011; Latz *et al.* 2012). In the latter case, often only one single point in time is chosen for evaluation (e.g., Postma *et al.* 2008; Hanse *et al.* 2011; Latz *et al.* 2012), or the increase from one to the next point in time is evaluated (Kushalappa & Ludwig 1982). However, disease progression is more precisely described by classical growth curve models (Neher & Campbell 1992). Out of the plethora of growth models (Paine *et al.* 2012), the monomolecular model has often been used to describe bioassays with soil-borne pathogens (Stanghellini *et al.* 2004; Wilson *et al.* 2008). The monomolecular infection model describes the disease progression (the change of infections over time) with an initial linear increase of infections (the infection rate), followed by a saturation (given by the maximum number of infectable plants, also known as carrying capacity or asymptotic growth).

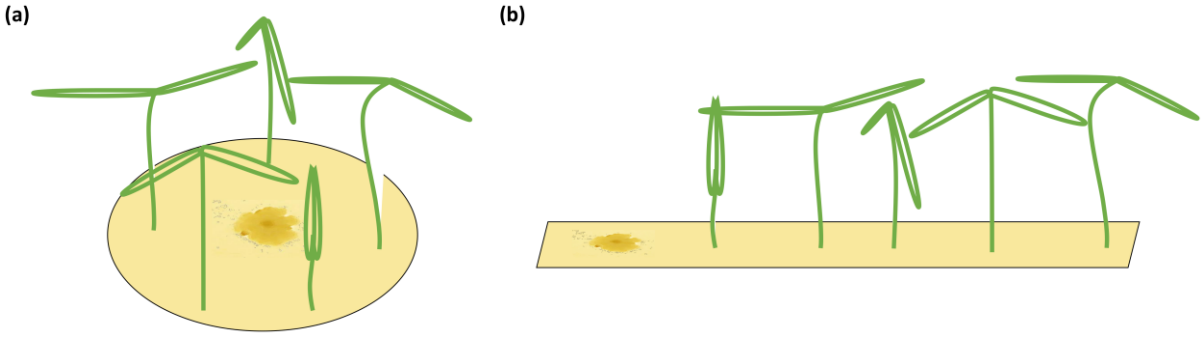

Figure 4. Two different possible setups for infection treatments. The circular setup with a centered pathogen surrounded by plants (a) may lead to a steep linear infection scenario as all plants are probably infected by the source pathogen at more or less the same time. Only the linear spatial assembly (b) allows for a consecutive infection of plants resulting in a linear increase that can be modelled by the monomolecular infection model.

The infection rate seems to be the most important parameter for determining pathogenicity (Raaijmakers *et al.* 2009). However, when estimating the suppressive potential of soils, the time

until infections occur (resistance time) might be even more important since pathogen inhibition occurs largely during pathogen growth. Actually, only a few experimental setups allow the investigation of both, infection rate and resistance time. To measure an infection rate it is necessary to use a system with multiple plant individuals (Figure 4) where plants can be infected one after another (i.e. measuring a time-series). In such experiments, the pathogen inoculant can be applied in different ways: (i) equally distributed application, i.e. homogeneously mixed in the soil or growth-substrate, or (ii) single point application (where pathogen spread can be assessed; Figure 4). If a pathogen is homogeneously distributed in the plant growth substrate, it is possible to measure the number of infected plants over time. The measured infection rate, however, would not represent the infection rate per se but rather the resistance variance of the plant community to the pathogen. The same problem occurs if a pathogen is applied to one location in the substrate and plants are planted at equal distances around the inoculum (Figure 4 a). Linear spatial designs (Figure 4 b) have the potential to estimate the correct infection rate in addition to the resistance time, whereas the further mentioned approaches solely allow to estimate the resistance time. Hence, it is important to keep in mind that the design determines the hypothesis that can be tested.

Another difficulty in performing pathogen infection assays occurs if natural field soils are used as substrate (e.g., Mendes *et al.* 2011; Latz *et al.* 2012). Here, in addition to the applied pathogen, other unknown pathogens may already exist in the soil and may increase infection in the plants. To cope with this problem, control treatments may be used to reveal the occurrence of natural soil inhabiting pathogens. If controls show infections, (i) these infections might be ignored if they are evaluated as statistically not relevant, (ii) the treatments where the corresponding controls showed infections could be excluded from further analyses, (iii) the treatments may be linearly corrected by simply subtracting the total amount of infectable plants by the infections that occurred in the control, which may lead to erroneous results in non-linear analyses as shown for functional response models (McCoy, Stier & Osenberg 2012). None of these approaches are desirable as they may lead to a bias in single infection rate measures (due to ignoring or wrongly correcting infections of a naturally occurring pathogen) and the loss of data (exclusion of treatments where the corresponding control was infected).

Here, we present an alternative approach that incorporates infections caused by any additional pathogens in the system by using a two-pathogen monomolecular infection model inspired by the competition model for logistic growth (Lotka 1925; Volterra 1926). This two pathogen

monomolecular model is an ordinary differential equation system with two equations. Systems with two equations are hardly analytically integrable to a single equation describing the progress of infections over time, therewith preventing the use of standard non-linear fitting functions in R, (e.g., `nls()`). To overcome this limitation, we applied a numerical integration routine (Soetaert, Petzoldt & Setzer 2010) combined with a maximum likelihood optimizer (Bolker & R Development Core Team 2014) to fit our model to data.
Our method allows for the use of natural soils (i) already contaminated with naturally occurring pathogens, and (ii) from different origins and habitats, while allowing for accurate evaluation of pathogenicity and plant resistance patterns in the field.

2.2.3 | The Model

The monomolecular infection model (Raaijmakers *et al.* 2009; Paine *et al.* 2012) describes the increase of infections in a (plant) community over time, $dI\ dt^{-1}$, by:

$$\frac{dI}{dt} = r(I_{max} - I) \qquad \text{Equation 1}$$

with r [time^{-1}] being the infection rate and I_{max} [Infected (Plants) Area^{-1}] being the maximum number of potentially infectable plants. The infection of the first plant is not necessarily instantaneous, but depends on the resistance of the soil and the plants to the pathogen, leading to a lag phase at the beginning of the experiment. To account for this mechanism, we extend the monotonic infection model by the time to the first infection, t_0:

$$\frac{dI}{dt} = \begin{cases} 0, & t < t_0 \\ r(I_{max} - I), & t \geq t_0 \end{cases} \qquad \text{Equation 2}$$

Below t_0 new infections are zero whilst above the occurrence of new infections follows the monomolecular infection model. In experiments using natural soils, alternative pathogens may be responsible for infections in the bioassay. To correct for these "wrong" infections, we extend the monomolecular model, inspired by the two-species competition growth model (Lotka 1925; Volterra 1926), to a two-species monomolecular infection model:

$$\frac{dI_p}{dt} = \begin{cases} 0, & t < t_{0,p} \\ r_p(I_{max} - (I_p + I_c)), & t \geq t_{0,p} \end{cases}$$ Equation 3a

$$\frac{dI_c}{dt} = \begin{cases} 0, & t < t_{0,c} \\ r_c(I_{max} - (I_p + I_c)), & t \geq t_{0,c} \end{cases}$$ Equation 3b

where I_p is the number of infected plants due to the pathogen, I_c are the number of infected plants in the control; r_p and r_c are the infection rates of the pathogen and the control treatment, respectively; and $t_{0,p}$ and $t_{0,c}$ are the resistance time of the pathogen and the control treatment, respectively.

2.2.4 | Application

Before starting the analyses, the packages **deSolve** (Soetaert *et al.* 2010) and **bbmle** (Bolker & R Development Core Team 2014) must be loaded using `library()` or `require()`; the source files (infection.models.r and infection.nll.r) must be loaded using `source()`.

Parameter estimation. First, the control treatment (Figure 5 a) must be fitted using the monomolecular model including a lag phase (Eqn. 2) using the `mle2()` function for general maximum likelihood fits from the package **bbmle** (Bolker & R Development Core Team 2014). The `mle2()` is used to minimize the negative likelihood function `mon.inf.lag.1p.nll()` from the infection.nll.r source file (see Bolker 2014 for detailed information on using the `mle2()` function). We need to supply the arguments `start`, containing a list of `r` and `t0` (the start values of the optimization process); `data`, a list with the headers `nI` (the number of infected plants in the experiment), `tps` (the **t**ime**p**oint**s** from the experiment), and `Imax` (if the number of total plants differs between experimental units); `fixed`, a list containing the headers `steps` (the length of a time step that should be simulated by the integration routine; this is set to 0.1 as default and does not have to be supplied), `Imax`, (a single value for the maximum of potentially infectable plants in the experiment, only needed if not supplied as data), and `tracing` (if set to 1, the trace including the negative likelihood and the parameters is displayed in the console, useful for error checking, default to 0):

```
R> fit.control = mle2(mon.inf.lag.1p.nll,
+    start = list(r = 1, t0 = 1),
+    data = list(nI = y, tps = x1, Imax = x2),
+    fixed = list(steps = .1, Imax = x2, tracing = 0)
+  )
```

Note that the initial values are just placeholders and might be adapted, but see section *Examples* for details. `Imax` must only appear once, either in the data list or in the fixed list in dependence if `Imax` is only a fixed single value or a set of data of the same length as `y` and `x1`.

Second, after estimating the infection parameters `r` and `t0` for the control, the two-pathogen monomolecular infection model can be fitted to the data with the experimentally added pathogen (Eqn. 3). Again we use the `mle2()` function, but now using the `mon.inf.lag.2p.nll()` function to be minimized. The estimates of the control treatment are assigned to `rc` and `t0c` in the fixed argument list. The target infection parameters are still placed in the start argument but now called `rp` and `t0p`:

```
R> fit.treatment = mle2(mon.inf.lag.2p.nll,
+    start = list(rp = 1, t0p = 1),
+    data = list(nI = y, tps = x1, Imax = x2),
+    fixed = list(steps = 1,
+      Imax = x2,
+      rc = coef(fit.control)[[1]],
+      t0c = coef(fit.control)[[2]]
+    )
+  )
```

One can investigate the fitting results and statistics using the generic `summary()` function applied on the object containing fitting results (here: `fit.control` & `fit.treatment`).

2.2.5 | Examples

R code and data files are provided as supplementary information on CD; how to use the R code

is discussed below.

The files infection.frontend1.r and infection.frontend2.r are the front-end files containing the adaptable examples we will discuss below. These files load the source files, infection.models.r and infection.nll.r, containing all underlying functions. We begin with a detailed description of the front-end files which should be sufficient to apply our fitting method to data. Moreover, we kindly invite you to continue reading the descriptions of the source files later in section *Underlying Functions*.

Please extract all required files from the zip-folder into one folder and keep the provided folder structure. If you prefer different organization of data and files you have to adapt the paths described below in the code.

Before getting started, use e.g. `install.packages()` to install the required packages deSolve (Soetaert *et al.* 2010) and bbmle (Bolker & R Development Core Team 2014), but see introductory R books and manuals for details (Bolker 2008; Crawley 2012). We first discuss the case of a constant number of plants in the experimental units (infection.frontend1.r).

Required packages and data. First, the required packages, the source files and the data must be loaded:

```
R> library("deSolve")
R> library("bbmle")
R> setwd("/path/to/your/folder/") #please replace this according to your path
R> source("source/infection.models.r")
R> source("source/infection.nll.r")
R> sample.data = read.csv("data/sample.data.csv")
```

To investigate the data structure we use the generic `str()` function:

```
R> str(sample.data)
'data.frame':     40 obs. of  3 variables:
 $ treatment      : Factor w/ 2 levels "control","treatment": 1 1 1 1 1 ...
 $ time.days      : int  1 2 3 4 5 ...
 $ number.infected: int  0 0 1 1 3 ...
```

The data comprises three variables, (1) the factorial variable treatment determining if the data

belongs to the control or the experimental treatment. The variable `time.days` contains the time of the measurement in days (you can switch the temporal resolution, the unit here determines the unit of the infection rate), and the variable `number.infected` contains the information of how many plants are infected (note that this must be an integer as we apply a binomial distribution later on; but see [Crawley 2012] and [Bolker 2008] for a detailed description on this topic). The data represents single experimental units (independent replicates, each data point represents the last measurement of a time series). Before continuing, we separate the data into two sub data sets containing just the control or just the treatment data using the subset function.

```
R> data.control = subset(sample.data, treatment == "control")
R> data.treat = subset(sample.data, treatment == "treatment")
```

Subsequently, we investigate the data graphically. For a better overview we set the graphical device to display two plotting regions using the `par()` function (the argument `mfrow` is set to `c(1,2)`, generating 2 horizontal adjacent plotting regions). Within the `plot()` functions we fix the y-axes ranges to create comparable plots (`ylim = c(0,10)`) and display the number of infections (y-axis) depending on the experimental time (x-axis):

```
R> par(mfrow=c(1,2))
R> plot(data.control$time.days,
+    data.control$number.infected,
+    ylim=c(0,10),
+    xlab="days",
+    ylab="infections control")
R> plot(data.treat$time.days,
+    data.treat$number.infected,
+    ylim=c(0,10),
+    xlab="days",
+    ylab="infections treatment")
```

The control without an experimentally added pathogen shows an early increase (day 3) of infected plants over time (Figure 5 a), but not all plants are infected during the experimental trial. The treatment with the experimentally added pathogen shows the first infections at day

4, but the increase in new infections is much steeper than in the control, and all plants might be infected (Figure 5 b).

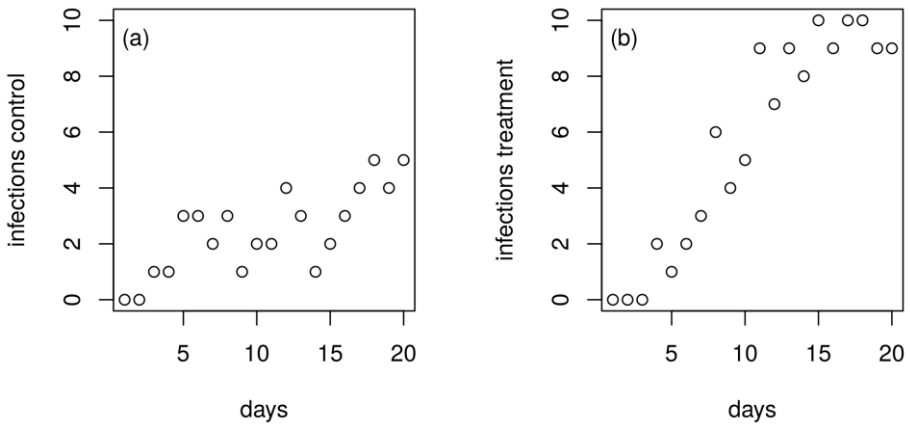

Figure 5. Simulated data of an infection bioassay. The data is simulated based on equations 2 and 3 using the functions `mon.inf.lag.1p()` and `mon.inf.lag.2p()`. After simulating the numeric average we applied a random number generator (`rbinom()`) using `Imax` as size of the binomial distribution and the floating average of the simulation divided by by `Imax` as probability. The control data (a) was simulated using `r` = 0.025, `Imax` = 10 and `t0` = 1 and the experimental treatment (b) was simulated using `rp` = 0.19, `rc` = 0.025, `t0p` = 5.5, `t0c` = 1 and `Imax` = 10.

Analysis of the data "wrong" approach. First, we analyse the experimental data using the standard monomolecular growth model (Raaijmakers *et al.* 2009; Paine *et al.* 2012). This model ignores the fact that not only the experimentally added pathogen but, in addition, other soil inherent pathogens may infect plant individuals and will be named "wrong method" (`fit.treatment.wrong()`, note that this method is valid if the medium chosen for the bioassay is sterile or does not contain any alternative pathogens). To fit the model to the data we use the `mle2()` function from the **bbmle** package by Ben Bolker (Bolker 2008; Bolker & R Development Core Team 2014). The `mle2()` function requires (1) a negative log-likelihood function, here the function `mon.inf.lag.1p.nll()` discussed in section *Underlying Functions*; (2) a list containing the model parameters that should be estimated (here the infection rate, `r`, and the resistance time, `t0`); (3) a list containing the data the model should be fitted to, here the first element of the list must be named `nI` (the number of infected plants) and the second element must be named `tps` (**t**ime**p**oint**s**, the time of the experimental data); (4) we also submit a fixed value to `mle2()`, again in a list object, containing the maximum

number of potentially infected plants (all plants in the experimental unit). Note that `Imax` does not have to be a fixed value but can also be assigned in the data list if the different replicates contain different numbers of plants (see subsection “Analysing data with multiple Imax”). Here, we start the fitting optimization with the initial guessed values of `r = 1` and `t0 = 1` (note that these values are just place-holders and might be adapted by the user, see subsection "What to do if…" below, moreover these values will be changed during the fitting algorithm by the optimizer `mle2()`; note also that the initial `t0` must be equal or smaller than the experimental resistance time, which according to Figure 5 is at day 4); we set `Imax = 10` (the maximum number of plants in the experiment).

```
R> fit.treatment.wrong = mle2(minuslogl = mon.inf.lag.1p.nll,
+    start = list(r = 1, t0 = 1),
+    data = list(nI = data.treat$number.infected,
+      tps = data.treat$time.days),
+    fixed = list(Imax = 10)
+  )
```

After the fitting procedure, we investigate the data using the generic `summary()` function:

```
R> summary(fit.treatment.wrong)

Maximum likelihood estimation

Call:
mle2(minuslogl = mon.inf.lag.1p.nll, start = list(r = 1, t0 = 1),
    fixed = list(Imax = 10), data = list(nI = data.treat$number.infected,
        tps = data.treat$time.days))

Coefficients:
   Estimate Std. Error z value     Pr(z)
r  0.170595   0.021636  7.8849 3.148e-15 ***
t0 3.461684   0.392051  8.8297 < 2.2e-16 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

-2 log L: 49.99375
```

The output provides information on the estimates of the model, as well as statistical indicators such as standard errors and p-values (but see Bolker 2008 for a detailed discussion of the underlying statistics). The infection rate, r, is estimated to be ~0.171 +/- 0.022 infections per day, and the resistance time, $t0$, is estimated to be ~3.46 +/- 0.39 days. These values underestimate both model parameters compared to the simulation parameters ($t0_{simulation}$ = 5.5 days, $r_{simulation}$ = 0.19 infections per day).

Analysis of the data- correct approach. Next, we apply the two-pathogen approach to the data, taking the infections of the control treatment into account. In the first step we analyze the control data similarly to the treatment data shown above only with exchanging the data supply from the experimental to the control data and decreasing the starting values for the model parameters (we already saw that the first infections occurred earlier and the slope is less step than for the treatment data).

```
R> fit.control = mle2(mon.inf.lag.1p.nll,
+    start = list(r = 0.5, t0 = 0.5),
+    data = list(nI = data.control$number.infected,
+      tps =  data.control$time.days),
+    fixed = list(Imax = 10)
R> )
R> summary(fit.control)

Maximum likelihood estimation

Call:
mle2(minuslogl = mon.inf.lag.1p.nll, start = list(r = 0.5, t0 = 0.5),
    fixed = list(Imax = 10), data = list(nI = data.control$number.infected,
        tps = data.control$time.days))

Coefficients:
    Estimate Std. Error z value     Pr(z)
r  0.0292603  0.0075671  3.8668 0.0001103 ***
t0 0.4666304  2.0573508  0.2268 0.8205705
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
-2 log L: 56.46962
```

The fitting algorithm revealed that the infection rate, `r`, is ~0.029 +/-0.0076, and the resistance time, `t0`, is ~0.47 +/-2.057. Both values are not significantly different from the simulated value. We will use the infection rate and the resistance time of the control to parameterize the two-pathogen model using the `mon.inf.lag.2p.nll()` function (see section *Underlying Functions* for details). Using `mon.inf.lag.2p.nll()` requires some reformulation of the code, the infection rate, `r`, and the resistance time, `t0`, for the treatment "pathogen" are now called `rp` and `t0p` and both appear in this spelling in the list of starting parameters. In addition to `Imax`, the list of fixed parameters contains here the infection rate of the control pathogen, `rc`, and the resistance time of the control, `t0c`. We call this values directly using the `coef()` function.

```
R> fit.treatment = mle2(mon.inf.lag.2p.nll,
+    start = list(rp = 0.5, t0p = 5),
+    data = list(nI = data.treat$number.infected,
+      tps = data.treat$time.days),
+    fixed = list(Imax = 10,
+      rc = coef(fit.control)[[1]],
+      t0c = coef(fit.control)[[2]])
+  )
R> summary(fit.treatment)

Maximum likelihood estimation

Call:
mle2(minuslogl = mon.inf.lag.2p.nll,
      start = list(rp = 0.5, t0p = 5),
      fixed = list(Imax = 10, rc = coef(fit.control)[[1]],
                t0c = coef(fit.control)[[2]]),
      data = list(nI = data.treat$number.infected,
                tps = data.treat$time.days))

Coefficients:
    Estimate Std. Error z value     Pr(z)
rp  0.193377   0.035434  5.4574 4.831e-08 ***
```

```
t0p 5.831930   0.614306  9.4935 < 2.2e-16 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

-2 log L: 48.21475
```

The values estimated by the two pathogen monomolecular model match the simulated values better than the results from the uncorrected fit. The estimated infection rate is measured to be ~0.193 +/- 0.035 ($rp_{simulation}$ = 0.19) and the first infection time is estimated to be ~5.83 +/- 0.61 ($t0p_{simulation}$ = 5.5).

Adding lines to the plot. The graphical representation of data and model fits is common practice. Simple linear regressions can be added as line to an existing plot using e.g. the generic `abline()` function. Our example is slightly more complex, but not much. First we have to create a vector containing values for the x-axis (time in days in our case) which will later be used to display a line in the plot. Note that non-linear lines need many values to create a smooth appearance of the line. Here we choose to create 100 x-values ranging from `t=0` to `t=20`, the end of our experiment using the `seq()` function with the third argument set to `length = 100` (which creates 100 evenly distributed values ranging from the minimum to the maximum value). To simulate the corresponding y-values we use the `lsoda()` function from the package **deSolve** (Soetaert *et al.* 2010). The `lsoda()` function builds a complex object including background information on the simulation run not needed for our purpose. To get rid of this information, we save the object created by `lsoda()` as a data frame by applying the `data.frame()` function on the `lsoda()` function. `lsoda()` requires the starting density of the infected plants as first argument, `y`, here `c(I = 0)`; the second argument is the sequence of time points the number of infected plants should be calculated for; the third argument requires the ordinary differential equation model that should be applied (`mon.inf.lag.1p()`, see below for detailed discussion); and fourth, the parameters of the model must be supplied, here the results of our model fit.

```
R> xvalues = seq(0,20,length=100)
R>
R> control.est =  data.frame(lsoda(y = c(I = 0),
+    times = xvalues,
```

```
+     func = mon.inf.lag,
+     parms = c(r = coef(fit.control)[[1]],
+       t0 = coef(fit.control)[[2]],
+       Imax = 10)
+  ))
```

To simulate the regression line for the correct treatment fit, we must extend the code described above to incorporate both the control as well as the treatment pathogen parameters. We again use the `lsoda()` function, but now call the `mon.inf.lag.2p()` function that allows to model two pathogens. Also, we have to provide two starting densities for the infected plants (zero infected plants by the treatment pathogen, `Ip = 0`, and zero infected plants by the natural occurring (control) pathogens, `Ic = 0`). Moreover, the parameter list must now contain five parameters, both infection rates (`rp` and `rc`), both resistance times (`t0p` and `t0c`) and again the maximum reachable number of infected plants, `Imax`.

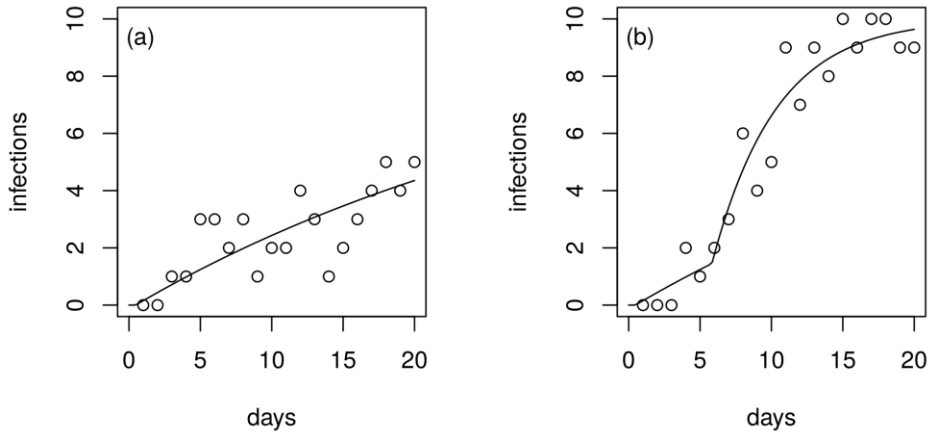

Figure 6. Infection bioassay as in Figure 5. The control (a) shows less infections starting earlier in the time series compared to the treatment with the experimentally added pathogen (b). This leads to a low infection rate combined with a low resistance time visualized by the black line in (a). The early infections in the treatment are due to infections by the natural pathogen, leading to a slight increase of the fitted curve (b), at t ~ 6, the treatment pathogen leads to a steep increase of the infection curve.

```
R> treat.est =  data.frame(lsoda(y = c(Ip = 0, Ic = 0),
+     times = xvalues,
+     func = mon.inf.lag.2p,
+     parms = c(rp = coef(fit.treatment)[[1]],
+       t0p = coef(fit.treatment)[[2]],
```

```
+       rc = coef(fit.treatment)[[3]],
+       t0c = coef(fit.treatment)[[4]],
+       Imax = 10)
+  ))
```

To add the lines to the plot, we again call `plot()` from above and subsequently `lines()`. Note that we have to sum both densities of infected plants (due to the treatment pathogen and the natural occurring (control) pathogen) to plot correct line (Figure 6) for the treatment data.

```
R> par(mfrow=c(1,2))
R> plot(data.control$time.days,
+    data.control$number.infected,
+    xlim=c(0,20),
+    ylim=c(0,10),
+    xlab="days",
+    ylab="infections")
R> mtext(side=3,"(a)",line=-1.5,adj=0.03)
R> lines(control.est$time, control.est$I)
R>
R> plot(data.treat$time.days,
+    data.treat$number.infected,
+    xlim=c(0,20),
+    ylim=c(0,10),
+    xlab="days",
+    ylab="infections")
R> mtext(side=3,"(b)",line=-1.5,adj=0.03)
R> lines(treat.est$time, treat.est$Ip+treat.est$Ic)
```

What to do if...

... I get the

```
Warning message:
In dbinom(x = nI, prob = Isim/Imax, size = Imax, log = TRUE) :
  NaNs produced
```

Keep calm, this is just a warning message, not an error and the fitting algorithm still succeeded.

Let us perform an example using the control treatment from the infection.frontend1.r (line 107 ff). We change the initial infection rate to 3, a rather high starting value:

```
R> fit.control.warning = mle2(mon.inf.lag.1p.nll,
+    start = list(r = 3, t0 = 0.5),
+    data = list(nI = data.control$number.infected,
+      tps =  data.control$time.days),
+    fixed = list(Imax = 10)
+ )

Warning messages:
1: In dbinom(x = nI, prob = Isim/Imax, size = Imax, log = TRUE) :
  NaNs produced
2: In dbinom(x = nI, prob = Isim/Imax, size = Imax, log = TRUE) :
  NaNs produced

R> summary(fit.control.warning)

Maximum likelihood estimation

Call:
mle2(minuslogl = mon.inf.lag.1p.nll, start = list(r = 3, t0 = 0.5),
     fixed = list(Imax = 10),
     data = list(nI = data.control$number.infected,
        tps = data.control$time.days))

Coefficients:
    Estimate Std. Error z value     Pr(z)
r  0.0292636  0.0075801  3.8606 0.0001131 ***
t0 0.4656128  2.0637430  0.2256 0.8215003
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

-2 log L: 56.46962
```

The warning message appears twice, but the fit still succeeded. But we can have a closer look at the problem. To do so, we can have a look at the trace log of the fit.

... I want to see the trace of the integrative fitting process?

We continue the example by adding the element `tracing` to the fixed list. This allows to track the iteration steps in which the `NaNs` occurred and the values of the infection parameters that have been used in these steps:

```
R> fit.control.tracing = mle2(mon.inf.lag.1p.nll,
+    start = list(r = 3, t0 = 0.5),
+    data = list(nI = data.control$number.infected,
+      tps =  data.control$time.days),
+    fixed = list(Imax = 10, tracing = 1)
+  )
```

Running this code a list of negative likelihood values and infection parameters appears in the console, getting longer the more the fitting advances (to save space here in the text we truncated the output, you might have to scroll up a little in your console output to see the same):

```
...
[1] "negLL:  28.2629342646186 r:  0.0277248134455506 t0:  0.248536802570284"
[1] "negLL:  28.2627221727419 r:  0.0277248134455506 t0:  0.246536802570284"
[1] "negLL:  NaN r:  54.3011142347463 t0:  0.141490864217889"
[1] "negLL:  NaN r:  10.8824026977057 t0:  0.226327614899805"
[1] "negLL:  2897.85108207059 r:  2.19866039029758 t0:  0.243294965036188"
[1] "negLL:  582.147373888558 r:  0.461911928815957 t0:  0.246688435063465"
...
```

Clearly, the `NaNs` are produced when extremely high infection rates (`r` ~ 50.3 and `r` ~ 10.88) are tested by the optimization algorithm. This causes the `lsoda()` to fail as the increase in new infected plants is much larger than the step size of the numeric integration routine (we set the default to 0.1, which corresponds here to 0.1 days). Therefore (1) the warning message can be ignored as it applies only to unlikely high infection rates; a change in the starting parameters may avoid that the optimization algorithm picks randomly these high rates (the main example did not show any warnings); or the step size of the numerical integration routine can be decreased to increase the temporal resolution of the simulation.

... I want to change the step size of the numerical integration routine?

```
R> fit.control.steps = mle2(mon.inf.lag.1p.nll,
+    start = list(r = 3, t0 = 0.5),
+    data = list(nI = data.control$number.infected,
+      tps =  data.control$time.days),
+    fixed = list(Imax = 10, steps = 0.01)
+  )
R> summary(fit.control.steps)

Maximum likelihood estimation

Call:
mle2(minuslogl = mon.inf.lag.1p.nll,
      start = list(r = 3, t0 = 0.5),
      fixed = list(Imax = 10, steps = 0.01),
      data = list(nI = data.control$number.infected,
                  tps = data.control$time.days))

Coefficients:
    Estimate Std. Error z value     Pr(z)
r  0.0292641  0.0076073  3.8468 0.0001197 ***
t0 0.4656282  2.0725455  0.2247 0.8222400
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

-2 log L: 56.46962
```

By decreasing the temporal resolution of the underlying integration of the infection model, we got rid of the warning messages, but at the cost of speed (it will take much longer to run the model function with this settings).

Analysing data with multiple Imax. In bioassays using plant seeds instead of plants not all seeds may germinate. This leads to different `Imax` in the different replicates. You find the example in infection.frontend2.r. As for the above mentioned example, we first have to set the working directory, load the required packages, source files and data:

```
R> library("deSolve")
R> library("bbmle")
R> source("source/infection.models.r")
R> source("source/infection.nll.r")
R> sample.data2 = read.csv("data/sample.data2.csv")
R> str(sample.data2)

'data.frame':     60 obs. of  4 variables:
 $ treatment      : Factor w/ 2 levels "control","treatment": 1 1 1 1 1 ...
 $ time.days      : int  1 2 3 4 5 ...
 $ number.infected: int  0 0 0 1 2 ...
 $ total.number   : int  10 10 10 12 9 ...
```

You might notice that the data set now includes the additional variable `total.number` containing the information which experimental unit has which `Imax`. We continue with separating the data sets as above:

```
R> data2.control = subset(sample.data2, treatment == "control")
R> data2.treat = subset(sample.data2, treatment == "treatment")
```

As above we begin by fitting the control treatment as discussed above. Additionally we set the step length of the numerical integrator to 0.01 to avoid `NaNs`:

```
R> fit.control = mle2(mon.inf.lag.1p.nll,
+    start = list(r = 0.02, t0 = 0.5),
+    data = list(nI = data2.control$number.infected,
+      tps =  data2.control$time.days,
+      Imax = data2.control$total.number),
+    fixed = list(steps = 0.01)
+  )
R> summary(fit.control)

Maximum likelihood estimation

Call:
mle2(minuslogl = mon.inf.lag.1p.nll,
      start = list(r = 0.02, t0 = 0.5),
      fixed = list(steps = 0.01),
```

```
      data = list(nI = data2.control$number.infected,
                  tps = data2.control$time.days,
                  Imax = data2.control$total.number))

Coefficients:
    Estimate Std. Error z value   Pr(z)
r  0.0060132  0.0024244  2.4803 0.01313 *
t0 2.8417380  6.3979722  0.4442 0.65693
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

-2 log L: 47.61573
```

The most important change is that we supply the information for `Imax` to the `data` list instead of the `fixed` list. In total the fit takes longer as in the example above. This is due to the fact that a numerical integration of a time series for each `Imax` must be calculated which is rather time consuming. We provide more details on this below.

After fitting the controls we follow the procedure explained above and fit the treatments:

```
R> fit.treatment = mle2(mon.inf.lag.2p.nll,
+    start = list(rp = 1, t0p = 1),
+    data = list(nI = data2.treat$number.infected,
+      tps = data2.treat$time.days,
+      Imax = data2.treat$total.number),
+    fixed = list(steps = 0.01,
+      rc = coef(fit.control)[[1]],
+      t0c = coef(fit.control)[[2]])
+  )
R> summary(fit.treatment)

Maximum likelihood estimation

Call:
mle2(minuslogl = mon.inf.lag.2p.nll,
      start = list(rp = 1, t0p = 1),
      fixed = list(steps = 0.01,
                  rc = coef(fit.control)[[1]],
                  t0c = coef(fit.control)[[2]]),
```

```
      data = list(nI = data2.treat$number.infected,
                  tps = data2.treat$time.days,
                  Imax = data2.treat$total.number))

Coefficients:
    Estimate Std. Error z value    Pr(z)
rp   0.60307    0.26499  2.2758  0.02286 *
t0p  8.27764    1.15717  7.1533 8.47e-13 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

-2 log L: 9.043275
```

2.2.6 | Underlying Functions

Negative Likelihood Functions (infection.nll.r). The fitting algorithm used above, `mle2()`, requires a negative log-likelihood (nll) function that will be minimized (see Bolker 2008 for a detailed introduction). The `nll()`-function for the monomolecular infection model can be written as:

```
R> mon.inf.lag.1p.nll = function(nI,tps,r,t0,Imax,steps=0.1,tracing=0){
+    if(r <= 0 || t0 <= 0 || t0 >= min(tps[nI>0])){ return(Inf) }
+    Isim = sim.inf.1p(tps, r, Imax, t0, steps)
+    negLL = -sum(dbinom(x = nI,
+      prob =  Isim / Imax,
+      size = Imax,
+      log = TRUE))
+    if(tracing == 1) print(paste("negLL: ",negLL,"r: ",r,"t0: ",t0))
+    return(negLL)
+ }
```

The function includes seven arguments: the experimentally measured number of infected plants, `nI`; the corresponding time estimates from the experiment, `tps` (**t**ime**p**oint**s**); the infection rate, `r`; the resistance time, `t0`; the maximum number of infections possible, `Imax`, (i.e. the maximum number of host plants in the experimental unit); the desired step length of the numerical integration, `steps`, by default set to 0.1; and the tracing argument which is

deactivated by default. First the function checks if either `r` or `t0` are below zero or `t0` is greater than or equal to the minimum time of an infection in the real data. If any of these queries is true, the function returns infinity. Next, the model predictions for each experimentally measured value are simulated using `sim.inf.1p()` (see subsection "ODE functions"). Finally, the function calculates the negative likelihood using the binomial density function `dbinom()` and returns it. We choose the binomial distribution as our data is binomially distributed (integer values for infections have a clear defined minimum and maximum number of infections, but see Bolker (2008) for a detailed introduction on this topic).

The two-pathogen variant, `mon.inf.lag.2p.nll()`, is similar to the above described function and we will only discuss the differences. As this function is created to estimate the negative log-likelihood of a two pathogen system, the model parameters consist of `rp`, `rc`, `t0p` and `t0c`. Moreover, the function `sim.inf.2()` is used to calculate the number of infections from the model simulation. Also a more complex if-statement is added to ensure that at least one of `t0p` or `t0c` falls below the time the first infection occurred in the experiment (otherwise `NaNs` may be produced):

```
R> mon.inf.lag.2p.nll = function(nI, tps, rp, t0p, rc,
+    t0c, Imax, steps = 0.1, tracing = 0){
+    if(rp <= 0 || t0p <= 0 || rc <= 0 || t0c <= 0){ return(Inf) }
+    if(t0p >= min(tps[nI>0]) && t0c >= min(tps[nI>0])){ return(Inf) }
+    Isim = sim.inf.2p(tps, rp, rc, Imax, t0p, t0c, steps)
+    negLL = -sum(dbinom(x = nI,
+      prob = Isim / Imax,
+      size = Imax,
+      log = TRUE))
+    if(tracing == 1) print(paste("negLL: ",negLL, "r: ",r, "t0: ",t0))
+    return(negLL)
+ }
```

The function `sim.inf.1()` first creates an empty numeric vector (`Iout`) to store the calculated values for infection in. If `Imax` is only a single value we apply the `lsoda()` function to simulate a single time series of plant infections according to the assigned parameter values (`parms = c(r = r, Imax = Imax, t0 = t0)`) and a starting density of infected plants of unity (`y = c(I = 0)`). The numerical simulation of the integration process needs

a vector of consecutive points in time. This vector consists of a sequence of consecutive values from zero to the maximum time value (`seq(0, max(tps), steps)`), and the experimental time values, `tps`. As no duplicate values should appear in the vector and the time vector should increase consecutively, we first apply the `unique()` function on the vector to delete duplicates and second sort the vector by the `sort()` function. The ordinary differential equation system that should be integrated is given by the function `mon.inf.lag.1p()`. Please read into Soetaert and Herman (2008) to get a general introduction into the topic "solving ordinary differential equation systems in R using deSolve". After the integration the number of estimated infections are saved according to their appearance in the experimental time series using a for-loop.

If the experimentally data consist of more than one single value for `Imax`, the else part of the `if/else`-statement is activated. First, we create three empty numeric vectors to store the number of infected plants, the time, and the maximum number of infectable plants in (`mres.I`, `mres.time`, `mres.Imax`). Second we use a `for`-loop to calculate the number of infected plants for each `Imax`. Third, we save the results to `Iout` as described above using a `for`-loop, with additionally separating for each `Imax`.

```
R> sim.inf.1p = function(tps, r, Imax, t0, steps) {
+    Iout = numeric()
+    if(length(Imax) == 1){
+      mres = data.frame(lsoda(y = c(I = 0),
+      times = sort(unique(c(seq(0, max(tps), steps), tps))),
+      func = mon.inf.lag.1p,
+      parms = c(r = r,
+        Imax = Imax,
+        t0 = t0)))
+      for(tps.i in 1:length(tps)){
+        Iout[tps.i] = mres$I[mres$time == tps[tps.i]]
+      }
+    } else {
+      mres.I = numeric()
+      mres.time = numeric()
+      mres.Imax = numeric()
+      for(Imax.i in 1:length(unique(Imax))){
+        mres = data.frame(lsoda(y = c(I = 0),
+          times = sort(unique(c(seq(0, max(tps), steps), tps))),
```

```
+          func = mon.inf.lag.1p,
+          parms = c(r = r, Imax = unique(Imax)[Imax.i], t0 = t0)))
+        mres.I = c(mres.I,mres$I)
+        mres.time = c(mres.time,mres$time)
+        mres.Imax = c(mres.Imax,
+          rep(unique(Imax)[Imax.i], length(mres$time)))
+      }
+      for(tps.i in 1:length(tps)){
+    Iout[tps.i]=mres.I[mres.time == tps[tps.i] & mres.Imax == Imax[tps.i]]
+      }
+    }
+    return(Iout)
+  }
```

The function `sim.inf.2()` is similar to the function `sim.inf.1()` but models the two-pathogen system. The differences are: model parameters consist are `rp`, `rc`, `t0p` and `t0c`; `lsoda()` needs two starting values for infections at time = 0 (`c(Ip = 0, Ic = 0)`); the ordinary differential equation system is given by the function `mon.inf.lag.2p()`. The results for the total infected plants, estimated by the model, are now calculated by `(mres$Ic+mres$Ip)`.

```
R> sim.inf.2p = function(tps, rp, rc, Imax, t0p, t0c, steps) {
+    Iout = numeric()
+    if(length(Imax) == 1){
+      mres = data.frame(lsoda(y = c(Ip = 0, Ic = 0),
+        times = sort(unique(c(seq(0, max(tps), steps), tps))),
+        func = mon.inf.lag.2p,
+        parms = c(rp = rp,
+          rc = rc,
+          Imax = Imax,
+          t0p = t0p,
+          t0c = t0c)))
+        for(tps.i in 1:length(tps)){
+          Iout[tps.i] = (mres$Ip+mres$Ic)[mres$time == tps[tps.i]]
+        }
+      } else {
+        mres.I = numeric()
+        mres.time = numeric()
```

```
+        mres.Imax = numeric()
+        for(Imax.i in 1:length(unique(Imax))){
+          mres = data.frame(lsoda(y = c(Ip = 0, Ic = 0),
+            times = sort(unique(c(seq(0, max(tps), steps), tps))),
+            func = mon.inf.lag.2p,
+            parms = c(rp = rp,
+              rc = rc,
+              Imax = unique(Imax)[Imax.i],
+              t0p = t0p,
+              t0c = t0c)))
+          mres.I = c(mres.I, mres$Ip+mres$Ic)
+          mres.time = c(mres.time, mres$time)
+          mres.Imax = c(mres.Imax,
+            rep(unique(Imax)[Imax.i],
+            length(mres$time)))
+        }
+        for(tps.i in 1:length(tps)){
+    Iout[tps.i]=mres.I[mres.time == tps[tps.i] & mres.Imax == Imax[tps.i]]
+        }
+      }
+    return(Iout)
+  }
```

ODE Functions (infection.models.r). The monomolecular infection model (Raaijmakers *et al.* 2009; Paine *et al.* 2012) with an additional lag phase can be written as:

```
R> mon.inf.lag.1p = function(t, x, parms){
+    with(as.list(parms),{
+      if (t < t0) { dI = 0
+      } else { dI = r * (Imax - x[1]) }
+      return(list(dI))
+    })
+  }
```

The function contains three arguments, the first argument is the time `t`, the second argument, `x`, is a list of densities that occur in the differential equation system and the third argument, `parms`, is a list of constant parameters. We activate the headers of the `parms` list by using the `with()` function. Within the `with()` function the change of infections over time is

calculated. We use an `if/else`-statement to discriminate between zero growth (before the first infections occur, `t < t0`) and positive new infections above this boundary. Lastly, the function returns a list containing the change of infected plants, `dI`.
The two pathogen monomolecular infection model can be written as:

```
R> mon.inf.lag.2p = function(t, x, parms){
+    with(as.list(parms),{
+      if (t < t0p) {dIp = 0
+      } else { dIp = rp * (Imax - (x[1]+x[2])) }
+      if (t < t0c) {dIc = 0
+      } else { dIc = rc * (Imax - (x[1]+x[2])) }
+      return(list(c(dIp,dIc)))
+    })
+  }
```

The differences to `mon.inf.lag.1p()` are: Within the `with()` function, the change of infections over time is calculated following the two pathogen infection model the function returns a list containing the change in infected plants over time of the experimentally pathogen, `dIp`, and the naturally (control) occurring pathogen, `dIc`.

2.2.7 | Acknowledgements

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2.3 Research Chapter 3

MECHANISTIC LINKS BETWEEN PLANT COMMUNITY COMPOSITION AND THE PATHOGEN-SUPPRESSIVE POTENTIAL OF SOILS

Ellen Latz, Nico Eisenhauer, Björn Christian Rall, Stefan Scheu and Alexandre Jousset

2.3.1 | Summary

Soil-borne plant diseases cause dramatic yield losses worldwide. Current disease control strategies can be deleterious for the environment and human health and foster the need for alternative disease control. Some soils harbour microorganisms that can efficiently suppress pathogens. The main taxa are well characterised, but uncovering mediators driving their functioning in the field still remains challenging.

We set up plant microcosms to experimentally test the *Rhizoctonia*-suppressive potential of soils in dependence on previous plant community composition. Our results indicate that plant communities shape soil-disease suppression via changes in root biomass, soil pH, and the abundance of the bacterial groups *Actinomyces, Bacillus* and *Pseudomonas*, and further stress the significance of plant-plant interactions for the suppressive potential of soils.

Using structural equation modelling, we provide a mechanistic framework showing how the complex interactions between plants, soil and microorganisms jointly shape soil suppressiveness. Our results stress the importance of plant community composition in affecting abiotic and biotic rhizosphere properties, suggesting that plant community composition is an important predictor of the disease suppressive potential of soils.

2.3.2 | Introduction

Soil-borne plant pathogens, in particular fungi, cause important yield losses all over the world (Weller *et al.* 2002; Raaijmakers *et al.* 2009). An average yield loss of 7-15% was estimated for the most important crops maize, potato, rice, soybean and wheat (Oerke 2006), and some especially aggressive pathogens such as *Fusarium, Pythium* and *Rhizoctonia* can cause losses of up to 20-35% (Cook 1987; Cook *et al.* 2002; Smiley *et al.* 2005). Current control methods are based on heavy pesticide application, which, beside of being highly polluting, provide only partial protection (Weller *et al.* 2002; Haas & Defago 2005).

Many soil microorganisms have the potential to suppress diseases to some extent, and pathogen suppression occurring in disease suppressive soils operates through distinct biological mechanisms (Weller *et al.* 2002). Bacteria of the genera *Pseudomonas*, *Actinomyces* and *Bacillus* are particularly important for the suppressiveness of soils (Weller *et al.* 2002; Mendes *et al.* 2011), and their targeted application is offering the opportunity for environmentally friendly control of plant diseases (Weller *et al.* 2002; Haas & Defago 2005). However, often disease suppressive bacteria perform poorly when applied to the field (Lugtenberg & Kamilova 2009; Raaijmakers *et al.* 2009). Despite of extensive research on the molecular mechanisms involved in disease suppression by bacteria (Mazzola, Funnell & Raaijmakers 2004; Haas & Defago 2005; Berg & Smalla 2009; Mendes *et al.* 2011), there is still a lack of knowledge on drivers affecting their survival and functioning in the soil (Philippot *et al.* 2013).

Plant community composition, soil abiotic properties, and pathogen antagonistic microbial communities are linked, and thought to jointly determine the suppressive potential of soils (Garbeva *et al.* 2004; Berg & Smalla 2009; Philippot *et al.* 2013). Soil-moisture is an abiotic component that varies with plant communities and shapes soil microbial communities (Hinsinger *et al.* 2009). Further, plants specifically impact plant-pathogenic as well as pathogen-antagonistic microorganisms via rhizodeposits (Bais *et al.* 2006; Bardgett & van der Putten 2014), and their composition shapes nutritional and pH conditions in the rhizosphere (Uren 2007; Hinsinger *et al.* 2009). However, the importance of abiotic factors is far from being clear, which might be due to their correlative relationships, and the complexity of mechanisms driving soil properties (Janvier *et al.* 2007).

Plant diversity affects a variety of ecosystem functions and services (Hooper *et al.* 2005;

Cardinale *et al.* 2012), and drives the composition of bacterial communities antagonistic to plant pathogens (Garbeva *et al.* 2006; Latz *et al.* 2012). Plant biodiversity further affects the expression of bacterial traits linked to pathogen suppression (Latz *et al.* 2015). Beside plant diversity, the functional composition of plant communities is important for the structure and functioning of biocontrol bacteria. For instance, legumes and grasses have been shown to affect biocontrol bacteria, and the pathogen suppressive potential of the soil (Latz *et al.* 2012).

Generally, effects of plant diversity on microbial communities are suggested to be due to increased plant productivity, accompanied by increased resource quantity exudated by plant-roots (Spehn *et al.* 2000; Zak *et al.* 2003; de Deyn *et al.* 2011). On the other hand, specific ecosystem functions are suggested to be dependent on resource composition and the quality of specific resources, driven by variation in plant species richness and identity (de Deyn *et al.* 2011; Latz *et al.* 2012, 2015). The link between plant diversity and belowground microbial community composition is little studied (Lange *et al.* 2014), and to our knowledge, studies on mechanisms linking plant community composition with the functionality of soil microbes, such as plant pathogens and plant pathogen antagonists, are lacking entirely.

We hypothesised that abiotic and biotic properties of the rhizosphere jointly shape the pathogen suppressive potential of soils. We assumed changes in the rhizosphere environment (root biomass, soil C/N ratio, pH, soil moisture) to vary with plant community composition and in turn affect the abundance and composition of biocontrol bacterial communities, thereby altering pathogen suppression (Supplementary Table 1). We tested this hypotheses by setting up an experimental grassland plant diversity gradient and investigated plant effects on abiotic and biotic soil properties at close to natural conditions. To allow unravelling mechanistic linkages we used a structural equation modelling approach (see Figure 7 and figure legend for details).

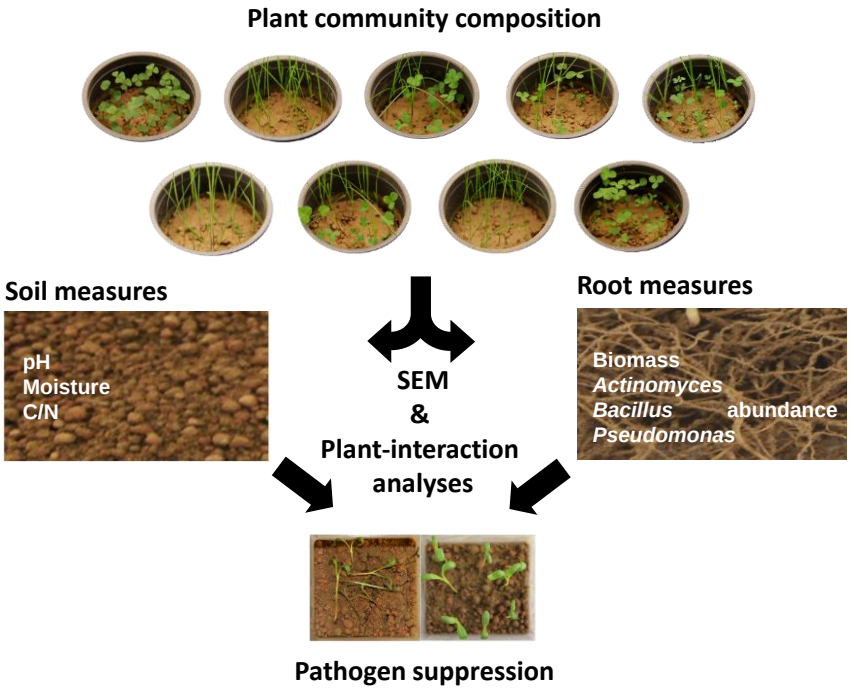

Figure 7. Grassland plant communities consisting of one to eight species were set up in a substitutive diversity gradient. To increase plant community effects on soil parameters, plant succession was simulated in growth cycles. After the fifth growth cycle, soil parameters were measured, plant roots were weighed and biocontrol bacteria (Actinomyces, Bacillus and Pseudomonas) enumerated. Subsequently, the soil was planted with sugar beet seedlings and infested with the model pathogen Rhizoctonia solani, and pathogen suppression was assessed. Mechanisms linking plant community composition and pathogen suppression were unravelled via structural equation modelling. In addition, plant-plant interaction effects on pathogen suppression were assessed (see methods for details).

2.3.3 | Materials and Methods

Plants. We used a total of eight plant species, four from the two functional groups grasses and legumes that are representatives of central European mesophilic grassland Arrhenatherion communities (Roscher *et al.* 2004). Grasses included *Bromus erectus* Huds. (*Bromus*), *Dactylis glomerata* L. (*Dactylis*), *Festuca pratense* Huds. (*Festuca*), *Lolium perenne* L. (*Lolium*), and the legume species were *Lotus corniculatus* L. (*Lotus*), *Medicago lupulina* L. (*Medicago*), *Trifolium pratense* L. (*Trifolium p.*), and *Trifolium repens* L. (*Trifolium r.*; Appels Wilde Samen GmbH, Darmstadt, Germany). It has been shown that nutrient uptake strategies and other functional traits differ considerably between these species (Roscher *et al.* 2004).

Microcosm construction. Microcosms (PVC tubes; diameter 10 cm, height 18 cm) were filled with 680 g of fresh soil obtained from a bare ground area close to the field site of the Jena Experiment (Roscher *et al.* 2004). Prior to plantation, the soil was sieved (2 mm) to remove macrofauna, roots, and stones. Subsequently, the soil was mixed with 170 g 2- 5 mm expanded clay; 20% of total volume (Fibo ExClay Deutschland GmbH, Lamstedt, Germany) to ensure constant humidity. Upscaling the maximum of 8 plants species per 0.00785 m^2 equals approximately 60 species per 20 m x 20 m (according to species area relationships for generalist European grassland species; Krauss *et al.* 2004), which is the maximum diversity per area in the Jena Experiment (Roscher *et al.* 2004). For each plant to be established three seeds were placed per sowing-spot; superfluous plant seedlings were removed after emergence.

Experimental setup. Plant diversity was varied independently of functional group affiliation in a substitutive gradient ranging from one to eight species by using the random partitions design (Bell *et al.* 2009). Every species was drawn at random from the species pool without replacement, such that each species was selected once at each level of diversity. Drawing was replicated three times resulting in three partitions, each containing of eight plant monocultures, eight two-species mixtures, four four-species mixtures, and one eight-species mixture. One microcosm without plants per experimental block served as control (Supplementary Figure 2). We used a well-established accelerated cycle design, in which plants were harvested and the microcosms planted again with the same plant communities in a three week cycle with five cycles in total. This design allowed simulating plant succession cycles in reduced time course, and has been used to investigate the effect of plants on the structure of bacterial communities before (Landa *et al.* 2003; Mazzola *et al.* 2004; Bergsma-Vlami *et al.* 2005b). Plant communities were grown in a climatic chamber (18- 22°C; photoperiod 12 h; 150 µmol m^{-2} s^{-1} photon flux density), and watered and randomized twice a week.

Sampling and measurements. Plant communities were harvested after the completion of the fifth growth cycle. Roots of plant communities were weighed and the soil was homogenized and stored at 4°C until further use. To quantify cultivable bacteria with biocontrol function, total bacteria were recovered from the root systems by horizontally shaking in 20 ml cold 1/10 phosphate-buffered saline for 0.5 h (PBS; Sambrook & Russell 2001). We focused on the bacterial genera *Actinomyces*, *Bacillus*, and *Pseudomonas*, known to have high suppressive

potential against the chosen model pathogen *Rhizoctonia solani* (Garbeva *et al.* 2006; Mendes *et al.* 2011). Starch Casein Agar (SCA) containing 100 µg ml^{-1} cycloheximide (Hirsch & Christensen 1983) was used for the enumeration of actinomycetes in diluted rhizosphere-soil suspensions (2×10^4 - 2×10^6 -fold). *Bacillus* spp. were isolated by incubating the rhizosphere-soil suspension at 85°C for 0.5 h, and dilutions (2×10^3 - 2×10^4 -fold) were plated on 1/10 Tryptic Soy Agar (TSA; Stevenson & Segner 1992). Pseudomonads were isolated by dilution-plating (2×10^4 - 2×10^6 -fold) on 1/3 King´s B agar containing 40 µg ml^{-1} ampicillin, 13 µg ml^{-1} chloramphenicol, and 100 µg ml^{-1} cycloheximide (Simon & Ridge 1974; McSpadden Gardener *et al.* 2001). Bacterial colonies were counted after four and additional colonies after six days (*Actinomyces*), two and three days (*Bacillus*), and three and four days (*Pseudomonas*) of growth at 20°C. For further analyses plate counts from soil dilutions resulting in 50 - 500 bacterial colonies per plate were chosen.

The pH of 2 g soil was determined in a 1:10 dilution with 0.01 M $CaCl_2$. The gravimetric water contend was measured by drying soil at 65°C for three days. Thereafter, dried soil samples were ball-milled (MM 400; Retsch GmbH, Haan, Germany) for analysis of total carbon (C) and nitrogen (N) concentrations in an element analyser (Vario EL III, Elementar, Hanau, Germany).

Soil suppressiveness assay. In order to analyse the effects of previous plant community composition on pathogen suppression in the following crop, we carried out a standardized infection assay with sugar beet seedlings (*Beta vulgaris* L.; variety BELINDA, *Rhizoctonia* susceptible, KWS SAAT AG, Einbeck, Germany) and the model pathogen *Rhizoctonia solani* Kühn (AG 2–2 IIIB; IfZ, Göttingen, Germany), as described elsewhere (Postma *et al.* 2008; Mendes *et al.* 2011; Latz *et al.* 2012). Briefly, four Magenta boxes per experimental plot (7.7 × 7.7 × 9.7 cm; Sigma-Aldrich, St. Louis, MO, USA) were filled each with 100 g of sieved soil. One barley corn infested with *R. solani* was placed in the centre of three boxes, the fourth box remained inoculum as control. Eight sugar beet seeds (germination rate 93%) were added to each box about 0.5 cm below soil surface. The jars were incubated at 21°C and 12 h photoperiod (photon flux density: 120 µmol m^{-2} s^{-1}) and randomised every two days over a total experimental time of ten days. Dead seedlings were counted at day 2, 4, 6, and 10, and pathogen suppression was calculated as the time span until the first infection of sugar beet seedlings occurred (see statistical analyses for details).

Statistical analyses. To estimate the disease suppressive potential of the soils after being exposed to different plant community compositions, we analysed every experimental unit separately using a monomolecular infection model (Raaijmakers *et al.* 2009; Paine *et al.* 2012 Rall & Latz, *in prep.*) describing the change of infected plants (*dI*) over time (*dt*) by an infection rate, *r*, and first infection occurrence, t_0:

$$\frac{dI}{dt} = \begin{cases} 0, & t < t_0 \\ r(I_{max} - I), & t \geq t_0 \end{cases} \qquad \text{Equation 4}$$

If controls were not infected by any pathogen being present in the soil, we estimated infection parameters according to the classic monomolecular model (Eqn. 4). Whereas, to correct for the occurrence of pathogens whose presence was detected in the control, we fitted the monomolecular model (Eqn. 4) to the control data, and subsequently used the results of this fitting for parameterization of a two pathogen monomolecular infection model (Rall & Latz, *in prep.*):

$$\frac{dI_p}{dt} = \begin{cases} 0, & t < t_{0,p} \\ r_p(I_{max} - (I_p + I_c)), & t \geq t_{0,p} \end{cases} \qquad \text{Equation 5a}$$

$$\frac{dI_c}{dt} = \begin{cases} 0, & t < t_{0,c} \\ r_c(I_{max} - (I_p + I_c)), & t \geq t_{0,c} \end{cases} \qquad \text{Equation 5b}$$

The differential equation includes two types of infected plants, plants infected by the experimentally added pathogen, *p*, and plants infected by pathogens in the control treatment, *c* (Eqn. 5).

The models were fitted to the data by using a numerical integration routine (`lsoda()` from the **deSolve** package in R [Soetaert *et al.* 2010; R Core Team 2014]) combined with the maximum likelihood optimizer mle2() from the package **bbmle** (Bolker & R Development Core Team 2014). To ensure the estimation of high infection rates, we chose a step size for the numerical integration routine (`lsoda()`) of 0.025 (default is 0.1) and allowed the maximum likelihood optimizer (`mle2()`) to try at maximum 10,000 iteration steps (`control = list(maxit=10000)`; default is 100). Integrative maximum likelihood estimations of non-linear models may result in local optima that lead to biased results or, in dependence of the starting parameters, fail completely (Bolker 2008). To cope with this problem, we repeated each

analyses 100 times with starting parameters randomly sampled out of a uniform distribution with: $0.0001 \leq r \leq 3$; $0.0001 \leq t_0 \leq 0.8 \times \min(t_{Infectedcontrol>0})$; $0.01 \leq r_p \leq 15$; $0.001 \leq t_{0p} \leq 0.8 \times \min(t_{Infectedtreatment>0})$.

We checked for the quality of the fitted parameters in each experimental unit by (1) selecting all model fits out of the hundred fittings where the ΔAIC was below 2, and (2) we calculated the coefficient of variation for t_0 of the remaining model fits. Only if the coefficient of variation was below 0.05, we rated the fit as trustful. Subsequently, we chose the value for t_0 of the fit with the lowest AIC for further analyses.

Subsequent analyses were performed using the statistical software R (R Core Team 2014) using the packages **car** (Fox & Weisberg 2011), **lavaan** (Rosseel 2012) and **semTools** (Pornprasertmanit *et al.* 2014).

In order to unravel mechanisms responsible for plant community effects on soil suppression, we used structural equation modelling, which allows the analyses of variables in a multivariate approach (Grace 2006). All variables were continuously coded. The initial model contained the exogenous variables plant diversity, presence of grasses, and presence of legumes in addition to the endogenous variables root biomass (g fresh weight; $\log_{10}$-transformed), the abiotic factors pH, total C and N content, and soil moisture (% data; logit-transformed) as well as the abundance of *Actinomyces*, *Bacillus*, and *Pseudomonas* (colony forming units [cfu] per root system; $\log_{10}$-transformed) as potential variables explaining soil suppression against *R. solani* (initial infection occurrence (t_0); $\log_{10}$-transformed;

Supplementary **Table *2***; Figure 8 a). This model was improved by: (i) separately analysing each endogenous variable and its dependencies in a linear regression and selecting the most parsimonious models via using the stepAICc() function (Scherber 2009), respectively. Subsequently, each of those separately predefined paths were used to create a second SEM. (ii) checking model modification indices for potential additional paths and undirected correlations that might not have been considered in the second model (iii) deriving the most parsimonious model by removing non-significant pathways. Model selection was conducted by comparative fitting (Eisenhauer *et al.* 2015) and using corrected Akaike´s Information Criterion (AICc; Akaike 1974; Burnham & Anderson 2004) and absolute goodness of fit was determined by using χ^2 tests ($P > 0.05$; Grace 2006).

To account for additional plant effects, we performed a linear model with the residuals of the SEM fit (after fitting of abundance of *Actinomyces*, *Bacillus,* and *Pseudomonas*, pH, plant diversity and legume presence) as being dependent on the presence and 2nd order interactions of the plant species. We selected the most parsimonious model via AICc. Significance of slopes were determined via t-tests.

2.3.4 | Results

Structural equation model (SEM). Structural equation modelling revealed pathogen suppression to be affected by multiple mechanisms that are shaped by plant community composition. The initial model (χ^2_{11} = 65.30; P < 0.001; Figure 8 a; Supplementary Table 2) could be improved by (i) using linear models to separately predefine each endogenous variable and its main dependencies to set up a second SEM (AICc = -1640.10; χ^2_{22} = 27.64; P = 0.130), (ii) checking model modification indices, and (iii) removing non-significant pathways (AICc = -1649.73; χ^2_{21} = 17.22; P = 0.698). The final model explained 32% of the variance in pathogen suppression Figure 8 b; Supplementary Table 3).

Plant diversity increased root biomass production as well as soil pH, thereby indirectly increasing the abundance of *Bacillus* and thereby increasing pathogen suppression (although the effect being small). In addition, the abundance of *Bacillus* increased in presence of grasses, while grasses slightly decreased root biomass. Root biomass, in turn, indirectly decreased the abundance of *Bacillus* via decreasing soil moisture. Further, soil pH decreased pathogen suppression. Despite the identified indirect pathways, a direct positive effect of plant diversity on pathogen suppression remained in the final model. The presence of legumes increased the abundances of *Pseudomonas* and *Actinomyces* and furthermore, directly decreased pathogen suppression. While being positively correlated, the abundance of *Pseudomonas* and *Actinomyces* decreased with root biomass and in presence of grasses. Pathogen suppression increased with increasing abundance of *Actinomyces*, whereas it marginally decreased with increasing abundance of *Pseudomonas* (Figure 8 b, Supplementary Table 3).

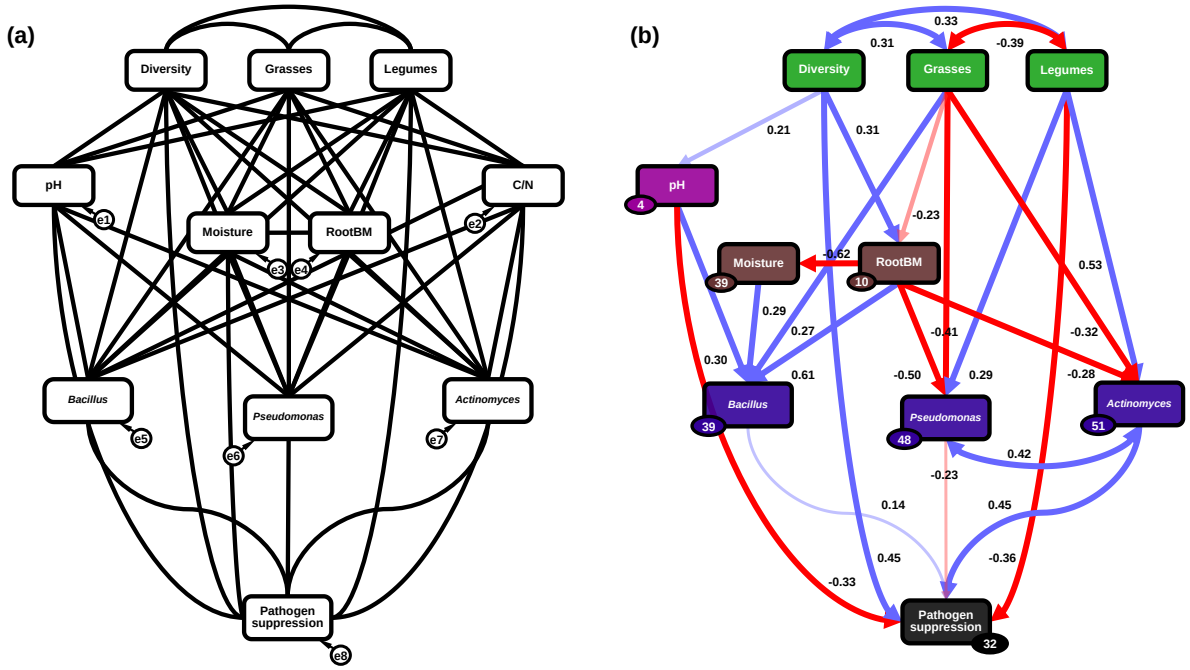

Figure 8. Structural equation model (see (a) for initial model) of (b) direct and indirect (through changes in soil pH, root biomass, soil moisture, and bacterial abundances) effects of plant community composition on pathogen suppression. Exogenous variables (plant diversity and functional group affiliation) are given on top, endogenous variables below. The data did not significantly deviate from the respective models (see main text for model fits). Single-headed arrows represent causal relationships and double-headed arrows indicate undirected correlations. Numbers on arrows give standardized path coefficients. Blue arrows indicate positive and red negative relationships; bold arrows indicate significant ($P \leq 0.05$), medium size arrows indicate marginally significant ($P \leq 0.1$), and thin arrows non-significant ($P > 0.1$) estimates. Circles indicate error terms (e1 – e8). Numbers close to endogenous variables indicate the variables variance explained by the model (R^2; percent).

Plant-plant interaction analyses. Analysing the residuals of the final SEM fit revealed that beyond plant diversity and legume presence, specific plant-plant interactions play an important role in influencing the pathogen suppressive potential of soil (Figure 9). Here, the most parsimonious model included the species pairs *Medicago-Lolium* and *Dactylis-Festuca* that increased pathogen suppression (Figure 9 a, d), and *Medicago-Dactylis* and *Lolium-Festuca* that decreased pathogen suppression (Figure 9 b, c). Further, the species pair *Festuca-Trifolium r.* remained in the most parsimonious model and slightly decreased pathogen suppression (Figure 9 e). Interestingly, the positive effect of *Medicago-Lolium* and *Dactylis-Festuca* was most obvious at plant diversity level 2, whereas the negative effect of the species pair *Festuca-Lolium* and *Festuca-Trifolium r.* was most pronounced at diversity level 4 (Figure 9 f). Interactions explained additional 32% of the remaining variance (after fitting the SEM) in pathogen suppression, resulting in 64% explained variance in total.

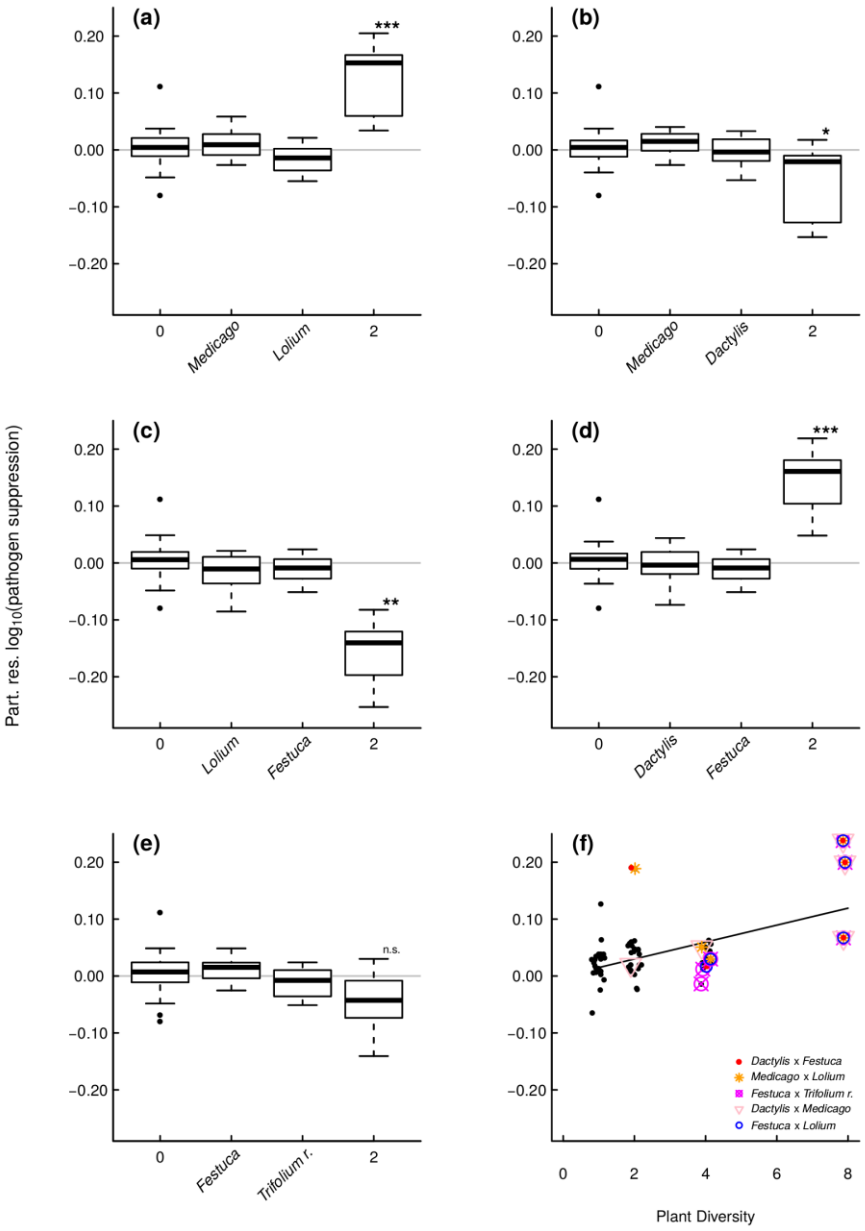

Figure 9. Partial residuals of log_{10}-transformed pathogen suppression (according to the most parsimonious model of the interaction analyses) as affected by (a) Medicago and Lolium, (b) Medicago and Dactylis, (c) Lolium and Festuca, (d) Dactylis and Festuca, (e) Festuca and Trifolium r.. First box per graph indicates both plant species being absent "0"; second and third box indicate named plant species being present and the other being absent; fourth box indicates both plant species being present "2" (left to right). Interaction effects were tested against zero (two-tailed t-test). Asterisks denote the level of significance: *P ≤ 0.05, **P ≤ 0.01, ***P < 0.001. (f) Partial residuals of log_{10}-transformed pathogen suppression (according to the most parsimonious SEM fit) as affected by plant diversity.

Additional analyses. Interestingly, when investigating whether the plant diversity effect was due to the presence of single species (sampling-effect; Aarssen 1997; Tilman *et al.* 1997; Huston 1997) by fitting the presence of *Bromus*, *Dactylis*, *Festuca*, *Lolium, Lotus*, *Medicago*, *Trifolium*

p. and *Trifolium r.* separately in a linear regression and fitted the residuals of the respective analyses against plant diversity (Latz *et al.* 2015; Weidner *et al.* 2015), the plant diversity effect only remained when fitted after the presence of some legume species (Supplementary Table 4). Further, the diversity effect disappeared when fitted after both the number of legume and the number of grass species (Supplementary Table 4).

2.3.5 | Discussion

Sustainable agriculture aims at optimizing crop yield while minimizing deleterious impacts on the environment and human health. Microbial communities that inhibit plant pathogens represent a promising tool to achieve this goal (Mendes *et al.* 2013; Philippot *et al.* 2013). Soil microbial consortia are driven by soil properties and plant community composition (Garbeva *et al.* 2004; Berg & Smalla 2009; Philippot *et al.* 2013). However, so far research neglected the complex linkages taking place in the rhizosphere when predicting or manipulating the suppressive potential of soils. In the present study we provide a mechanistic framework showing how the complex interactions between plants, soil and microorganisms jointly shape soil suppressiveness.

In the present study, pathogen suppression is influenced by a complex set of abiotic as well as biotic rhizosphere properties that are in turn linked, directly or indirectly, to plant community composition. Plant community composition affected pH and the abundance of *Actinomyces* (positively correlated with *Pseudomonas* abundance), that both significantly affected the suppression against *R. solani*. Further, certain interactions between plant species explained a large proportion of pathogen suppression in addition to the presence of plant functional groups and plant diversity *per se*. This suggests that plant community effects on soil abiotic and biotic properties alter microbial consortia in the rhizosphere and interactions therein, which need to be taken into account for predicting and manipulating the disease suppressive potential of soils.

Effects of plant diversity. Generally, our results underline the importance of plant diversity as an important driver of soil suppression. This is supported by studies showing (1) soil suppressiveness to rapidly vanish during the conversion of grasslands to monocultures, which was ascribed to biocontrol microbes being associated to diverse grasslands (Garbeva *et al.*

2006); (2) soils from species-rich grasslands to host high abundances of bacteria associated with pathogen suppression, which in turn improved disease suppression (Latz *et al.* 2012), and (3) diverse plant communities supporting higher abundances of bacteria being active in producing antifungal compounds (Latz *et al.* 2015).

The SEM approach revealed that part of the plant diversity effect on the community structure and functioning of biocontrol bacteria was mediated by increased root biomass and soil pH. This is in line with a recent field study on experimental grassland, showing that plant diversity increases root biomass and soil pH, and thereby microbial biomass in soil (Eisenhauer *et al.* 2013).

Effects of plant functional groups. The presence of functional groups (grasses and legumes) also predicted suppressiveness and again the effects were partly mediated by changes in root biomass and microbial communities. Generally, plant functional groups inconsistently affected the three groups of biocontrol bacteria. While grasses increased the abundance of *Bacillus*, they decreased the abundance of *Pseudomonas* and *Actinomyces*. Interestingly, via decreasing root biomass the presence of grasses in parallel also increased the abundance of *Pseudomonas* and *Actinomyces*. In addition, the abundance of *Pseudomonas* and *Actinomyces* was increased in presence of legumes. In an earlier study legumes and grasses also shaped biocontrol *Pseudomonas* communities, but legumes detrimentally and grasses beneficially affected their abundance (Latz *et al.* 2012). However, effects of legumes and grasses also have been shown to be species specific (Latz *et al.* 2015), and functional group effects on biocontrol bacteria therefore might be dependent on the respective species pool.

Effects of root biomass associated by effects of soil moisture. Root biomass increased the abundance of *Bacillus* but decreased that of *Pseudomonas* and *Actinomyces*. Root morphology differs considerably between plant species and shapes rhizosphere microbial communities (Berg & Smalla 2009). In accordance, inconsistent results of root biomass effects on bacterial abundances in diverse grassland communities were recently suggested to be driven by species identity (Latz *et al.* 2015). Species-specific analyses showed strong effects of the presence of *Medicago* on root biomass (data not shown), suggesting that a higher proportion of *Medicago* roots might foster *Bacillus* while decreasing the abundance of *Pseudomonas* and *Actinomyces*. Further, via decreasing soil moisture root biomass also decreased the abundance of *Bacillus*,

but this neither affected the abundance of *Pseudomonas* and *Actinomyces* nor soil suppression. Generally, soil moisture is an important driver for soil microbial communities (Eisenhauer *et al.* 2013; Lange *et al.* 2014), and our results show that different microbial groups differ in their sensitivity to soil moisture.

Effect of soil pH. Soil pH significantly increased the abundance of *Bacillus* and further directly decreased pathogen suppression. The lack of effects of soil pH on the abundances of *Actinomyces* and *Pseudomonas* is not surprising since variations in pH were small (7.60- 7.85) and close to the optimum of most bacterial consortia (Rousk *et al.* 2010). However, the increased abundance at higher pH suggests that *Bacillus* has a slightly higher pH optimum then the other two bacterial groups. The decreasing effect of pH with higher pH levels on pathogen suppression might have been due to the low pH optimum of *R. solani* AG3 (Ritchie, Bain & McQuilken 2009).

Effects of biocontrol bacterial communities. *Bacillus* abundance only marginally increased pathogen suppression. This supports the observation that *Bacillus* diversity rather than their abundance drives suppression against *R. solani* (Garbeva *et al.* 2006). Nevertheless, other pathogens than *R. solani* might have been affected by *Bacillus* abundance, and therefore the importance of this path in driving pathogen suppression should not be underestimated.
Interestingly, *Pseudomonas* and *Actinomyces* were positively correlated, and *Actinomyces* were the only bacterial group significantly increasing pathogen suppression. In a study on crop management effects on soil bacterial populations of *Actinomyces*, *Pseudomonas* and *Bacillus*, pseudomonads and *Bacillus* turned out to be the most important biocontrol agents associated with *Rhizoctonia*-suppressive soil (Garbeva *et al.* 2006). Further, another study identified *Pseudomonas* as main drivers of suppression, but other taxa, such as *Actinomyces,* were also associated to disease suppressiveness against *R. solani* (Mendes *et al.* 2011). These findings underline that soil pathogen suppression likely is not only due to the presence of certain antagonistic bacterial groups, but to facilitative interactions among bacterial groups or taxa (Mendes *et al.* 2011). This is also supported by the observation that bacterial strains can gain antagonism against pathogens when growing in bacterial consortia (Garbeva *et al.* 2011).

Effects of plant-plant interactions. In addition to the SEM approach, we evaluated the role of

plant species interactions as drivers of pathogen suppression. Identified plant interactions explained additional 32% of the remaining variance (after fitting the SEM) in pathogen suppression, showing that pathogen suppression is strongly influenced by plant-plant interactive effects. Further, sequential analyses suggest that the direct positive diversity and the negative legume effect on pathogen suppression, we observed in the SEM, were driven by those plant-plant interactions (Supplementary Figure 3). In presence of competitors plants increase root exudation and alter exudate composition, thereby affecting rhizosphere microbial communities (Bais *et al.* 2006; Semchenko *et al.* 2007). In addition, different plant species are suggested to use resources in a complementary way, thereby contributing to ecosystem functioning (Loreau *et al.* 2001; Eisenhauer 2012). Signaling adaptations in plants to pathogens results in differential responses in root-associated bacteria (de Werra *et al.* 2008), and may require adequate resource availability. Therefore, an importance of resource complementarity in plant community resistance to pathogens is likely. However, whether the observed plant-plant interaction effects on pathogen suppression were due to complementary resource acquisition, or plant competition driven changes in root exudation, will need further evaluation. Remarkably, in the sequential approach, we observed high pathogen suppressiveness at diversity levels 2 and 8. Interestingly, these positive effects likely were due to synergistic effects of plant species being in close proximity, i.e. were arranged side by side. Further, in each of the 4 species treatments and in one 8 species treatment positive interacting plant individuals were either not arranged side by side or accompanied by negatively interacting species, which might explain their low pathogen suppression (Supplementary Figure 2). Unfortunately, due to design-limitations we were not able to directly test for 3rd order interactions. Microbial communities generally are suggested to respond with a time lag to plant community changes (Eisenhauer *et al.* 2010a). Our design, in which each plant community composition was harvested and the same plant community planted again (but differentially spatially arranged), might have uncovered that plant-plant interaction effects on specific soil functions, such as soil suppression, are rather short-term effects. Further studies are needed to disentangle spatial and temporal effects of plant communities on the rhizosphere-environment and their implications for specific microbial functions.

Conclusion. Results of our study support the assumption that plant community composition, soil abiotic properties, and microbial communities being antagonistic to soil pathogens are

linked and interactively shape the suppressive potential of soils (Garbeva *et al.* 2004; Berg & Smalla 2009; Philippot *et al.* 2013). In addition to plant community induced changes in soil pH, root biomass, and abundances of biocontrol bacteria, plant-plant interactions were of major importance in driving the disease suppressive potential of soils. Therefore, to mechanistically understand the functioning of microbial communities involved in pathogen suppression and enabling to predict and manipulate the suppressive potential of soils, it is necessary to take the environment shaping potential of a plant community into account. The results represent an important step in understanding the complexity of mechanisms linking plant community composition and plant disease suppression.

2.3.6 | Acknowledgements

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2.4 Research Chapter 4

INTERCROPPING AND SOIL PROTOZOA INTERACTIVELY SHAPE SOIL FUNCTION AND CROP YIELD

Ellen Latz, Simone Weidner, Patricio Sobrero, Juan Galantini, Santiago Lorenzatti, Alexandre Jousset and Claudio Valverde

2.4.1 | Summary

Intercropping systems, in which different crop species are sown together in the same field, are increasingly being investigated as a sustainable, high yield agricultural practice. A better understanding of soil processes linked to higher productivity of mixed cultures is an important tool to apply intercropping in an efficient way.

We used a soybean-maize intercropping system and scrutinized the links between crop richness, soil enzymatic profiles and yield. We gave a special attention to protozoa, a group of microbial predators exerting a strong control on the structure and function of bacterial communities but often overlooked in agricultural research.

We observed that mixing soybean and maize increased crop yield. Further, we provide first evidences that cropping regime interactively with soil protozoa shapes soil functioning by essentially influencing soil enzyme activities and thereby influences yield.

We suggest that resident key soil biota (protozoa) that regulate important soil functions should be taken into account in order to predict the effect of agricultural practices on soil fertility and plant yield.

2.4.2 | Introduction

The growing human population and dwindling resources call for new strategies to secure agricultural yields while reducing fertilizer input and thereby minimizing negative impacts on the environment (Tilman *et al.* 2002). Current agricultural systems usually rely on monocultures, with or without crop rotation. While optimizing the use of mechanized management strategies, monocultures tend to be vulnerable to diseases and require high fertilizer input to reach a given yield (Tilman *et al.* 2002). The use of intercropping, in which multiple crop plants are sown on the same field, appears as a promising method to sustainably increase yields (Li *et al.* 2014). Mixed plant communities often show a higher yield than monocultures, an effect that might be due to promotion of beneficial soil (Eisenhauer et al., 2012).

Intercropping cereal fields with legumes that, due to their association with rhizobia increase nitrogen supply, results in an increased cereal yield (Chu, Shen & Cao 2004). Monocots on their side enhance global yield by supporting beneficial bacteria, such as biocontrol pseudomonads, which suppress phytopathogens (Mazzola 2007; Latz *et al.* 2012). Further, some grasses, support aerial fungal endophytes that protect neighboring legumes from aphid herbivory (García Parisi, Grimoldi & Omacini 2014). Thus, setting up appropriate plant mixtures promoting the activity of beneficial soil organisms may help to reach high agricultural yields while reducing agrochemical input.

Soybean and maize are two of the most relevant crops in temperate and subtropical areas. In Argentina, a total of 25 million hectares have been devoted to soybean and maize crops in the 2012/2013 campaign (SIIA 2014). The productivity of maize and soybean together reached 81 million of tons in 2013, with 32 million of tons of maize and 49 million of tons of soybean (SIIA 2014). Interestingly, first studies on maize and soybean intercropping reported to increase yields in terms of land-use efficiency (Verdelli, Acciaresi & Leguizamon 2012; Ariel *et al.* 2013) and enhanced soil-quality (Regehr 2014). However, the short-term impact of maize-soybean intercropping on soil biota and microbial functionality as well as their impact on yield have not yet been explored.

In this study, we followed the effect of intercropping soybean and maize on soil functioning and crop yield. We measured enzymatic activity patterns to estimate microbial traits involved in nitrogen, carbon and phosphorus cycling as well as suppression of soil borne pathogens. Soil

enzymes are important bioindicators of soil health (Burns & Strauss 2012) as they reflect the ability of soil microorganisms to transform nutrients from the soil organic material into forms available for uptake by plants. In addition, lytic exoenzymes such as chitinase or proteases play an important role in pathogen suppression (Haas & Defago 2005; Kielak *et al.* 2013). In addition, we measured the abundance of protozoa, a key soil taxon that structures soil microbial community composition and functioning (Rosenberg *et al.* 2009; Jousset 2012), particularly in relation to nutrient cycling and pathogen suppression (Bonkowski 2004; Müller *et al.* 2013). We expected intercropping to increase protozoa abundance and improve enzymatic activity either by a sampling effect, that is, increasing the chance that at least one of the species will be particularly supportive to a given function (Huston 1997). Alternatively, interaction between the two sown plant species may result in a higher belowground population growth and activity (Eisenhauer *et al.* 2010a, 2013), which may positively affect fertility. Interplay between plant composition, soil communities and subsequent soil fertility are often difficult to separate (Eisenhauer *et al.* 2010b). Thus, we used a structural equation modelling approach (Grace 2006) that enables to unravel direct and indirect pathways driving the effect of intercropping on soil functioning and yield.

2.4.3 | Materials and Methods

Field site. The field site is located near the city of Monte Buey (Córdoba province, Argentina). The climate in the region is characterised as Pampean Temperate (subhumid), with an average temperature of 23°C and a total of 445 mm of rainfall during the period of the experiment (13 November 2012 to 16 May 2013). Soil is characterized as a Typic Argiudoll of silt loam texture. Prior to the experiment, maize has been grown at the whole field site. Soybean (DM3810) and maize (DK692RR) seeds were sown in November 2012. Planting was done mechanically using a John Deere 1740 planter. Triplicate plots of 65 × 18 m each were sown with soybean alone (40 seeds per m^2), with maize alone (7.6 seeds per m^2), or with soybean and maize as alternating individual sowing lines (20 seeds per m^2 for soybean and 3.8 seeds per m^2 for maize). In all plots, the distance between rows was 52 cm. Maize was fertilized at the moment of sowing with 90 kg/ha of mono ammonium phosphate and re-fertilized with 235 kg/ha urea-ammonium nitrate (32% of N) at the V5 stage. Soybean seeds were coated with a commercial

inoculant containing Bradyrhizobium japonicum in addition to fungicides (thiram and carbendazim) according to the instructions of the supplier (Rhizopack 101; Rizobacter Argentina S.A.)

Sampling. Soil samples were taken in March 2013. At each plot, three sites (subplots) were randomly chosen excluding each the outer 2 meters to prevent border effects of neighboring fields. At each site we sampled three soil cores to a depth of 5 cm using a metal corer (inner diameter 7 cm) within one row, within the neighboring row, and in-between the two rows (resulting in three positions per subplot), respectively. The three cores per position were pooled, homogenized and sieved (2 mm) to remove macrofauna, roots and stones and stored at 4°C until analyses.

Harvest. Plants were harvested mechanically in May 2013 with John Deere 9650 harvest equipment. In intercropping plots, maize was harvested in a first pass at 50 cm height, and then soybean was harvested in a second pass at 3 cm height. As this procedure leads to soybean grain loss due to machine trampling, the observed yield is lower than the real one. Thus, soybean yield from intercropping plots was corrected by multiplying by 1.33, a correction factor previously determined by comparison to manual harvesting (Romagnoli, J.; pers. comm.). Yield was calculated as g applied seed^{-1}.

Enzyme tests. Extracellular chitinase, ß-glucosidase and phosphatase were measured in a microplate reader (BMG LABTECH POLARstar Omega; Ortenberg, Germany) via fluorogenic assays (Marx, Wood & Jarvis 2001), using the software associated with the equipment (BMG LABTECH MARS data analysis). For each sample, 0.1 g of soil (fresh weight) were dissolved in 10 ml 0.1 M MES buffer (2-[N-Morpholino]ethanesulfonic acid, pH 6.1) and homogenized with a IKA Ultra Turrax Tube Drive (Staufen im Breisgau, Germany). Enzyme activity was measured at a substrate concentration of 40 µM, corrected for quenching effects of soil particles on the fluorescence intensity of MUB and calculated as enzyme activity min^{-1} g_{soil}^{-1}.

Protozoan abundance. Numbers of protozoa were determined by the most-probable-number (MPN) method based on an established protocol (Rønn, Ekelund & Christensen 1995). Briefly, 0.2 g of air-dried soil were dissolved in 20 volumes of autoclaved distilled water, and then

shaken vigorously at room temperature for 20 min in a 15-ml Falcon tube containing 3 stainless-steel beads (4 mm of diameter), to liberate the protozoa from soil particles. The soil samples were distributed in 96-well microtiter plates (four samples per plate; Cellstar®, Greiner Bio-One, Frickenhausen, Germany) by using four replicates and six threefold dilutions in modified Neff's amoeba saline (Page, 1988) supplemented with 0.3 g L^{-1} tryptic soy broth (Oxoid, Basingstoke, UK). Plates were incubated at 22°C in the dark and examined for the presence of protozoa (flagellates, amoebae and ciliates) after 8 days using an inverted microscope (200× magnification, phase contrast). A freely distributed Excel sheet (Jarvis, Wilrich & Wilrich 2010) was used to convert the microtiter plate patterns to the MPN of fast-growing flagellates and total protozoa (individuals g^{-1} soil).

Statistical analyses. Data were analyzed using the statistical software R (R Core Team 2014) with the packages nlme (Pinheiro *et al.* 2014) and lavaan (Rosseel 2012). To be able to compare soybean and maize yields, both were normalized by dividing each measure by the respective treatments mean (norm. yield). The effect of treatment on norm. yield was analysed in pairwise comparisons by using Tukey´s Honest Significance Difference (Tukey´s HSD). In order to unravel the linkages between crop richness, protozoa, soil enzymatic profiles and yield we used structural equation modelling (SEM), which allows analysing variables in a multivariate approach (Grace 2006). In the initial model we used the exogenous variable crop richness as potential variable explaining norm. yield, presence of amoeba and ciliates, abundance of flagellates as well as chitinase, phosphatase and ß-glucosidase activity. The endogenous variables presence of amoeba and ciliates and abundance of flagellates were used as variables potentially explaining chitinase, phosphatase and ß-glucosidase activity. Further, the endogenous variables presence of amoeba and ciliates, abundance of flagellates as well as chitinase, phosphatase and ß-glucosidase activity were used as variables potentially explaining yield (continuous data were $\log_{10}$-transformed). Model modification indices were checked for potential additional paths that initially have not been considered in the model. Subsequently, model selection was conducted via using Bayesian information criterion (BIC; Burnham & Anderson 2004) as well as χ^2 tests ($P > 0.05$; Grace 2006).

We completed this approach by using linear mixed effect models fitted by restricted maximum likelihood (REML) with subplot and position (factorial) as random effects to account for spatial differences between samples. Here, we estimated effects of protozoa, namely flagellate

abundance (continuous, log_{10}(individuals g^{-1} soil)), amoeba presence (factorial) and ciliates presence (factorial), on chitinase, phosphatase and ß-glucosidase activity (log_{10}(enzyme activity min^{-1} g_{soil}^{-1})) crop independently, in soybean, and maize monocropping-rows and in interrows (here, only subplot was used as random effect), where we expected plant root interactions to take place.

2.4.4 | Results

Tukey´s HSD indicates that intercropping maize with soybean increases maize yield up to 50%, whereas soybean yield was not significantly affected by intercropping (Figure 10). The clear-cut stimulation of maize yield in intercropping with soybean compared with maize alone was not accompanied by overall significant changes in a set of chemical soil indicators, such as total nitrogen content, extractable phosphorus content, total organic carbon content, soluble carbohydrate and total carbohydrate content (Supplementary Table 5).

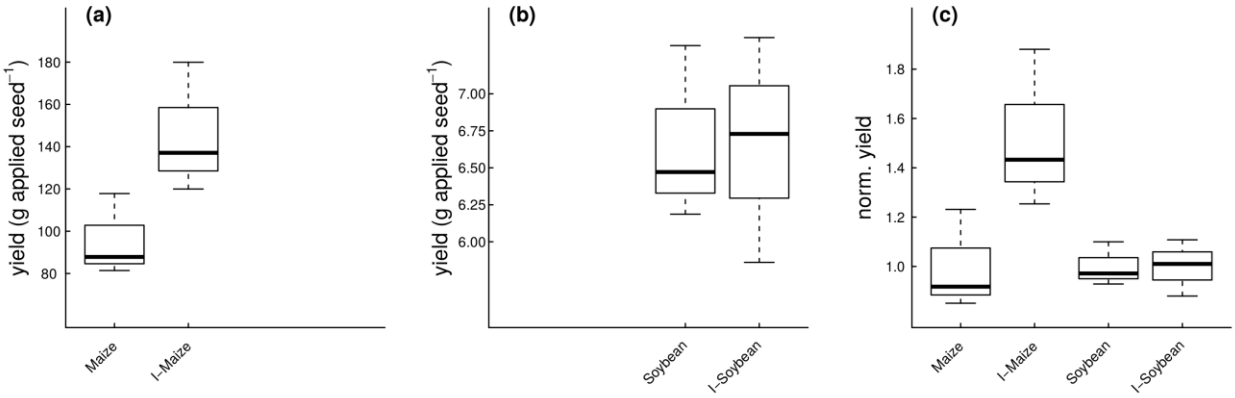

Figure 10. Yield (g applied seed^{-1}) of (a) maize and (b) soybean in monoculture (Maize, Soybean) and in intercropped fields (I-Maize, I-Soybean); (c) normalized yield, which is the respective yield divided by the treatments mean. *yield in I-Maize is significantly higher than in Maize (Tukey´s HSD; $P < 0.05$).

Structural equation modelling (SEM) revealed that yield is affected by multiple mechanisms, including crop richness and enzymes activities that in turn were influenced by protozoa. The initial model (BIC = -215.24; χ^2_6 = 19.99; P = 0.003) could be improved by removing non-

significant paths (BIC = -242.81; χ^2_{14} = 9.82; P = 0.775; Figure 11). The final model explained 76% of the variance in yield.

Crop richness increased the activity of ß-glucosidase and directly increased yield, while protozoa weren't affected. ß-glucosidase activity and phosphatase activity decreased in presence of ciliates, but increased with increasing flagellate abundance. By contrast, chitinase activity, which also increased with flagellate abundance, was decreased in presence of amoeba. Further, amoeba and ciliate presence were correlated and amoeba presence was also correlated to flagellate abundance. In addition, amoeba directly increased yield. Chitinase activity decreased yield as so did ß-glucosidase activity that, although its effect was not significant, remained in the final model. While being marginally significant, phosphatase activity was the only enzyme activity having a positive effect on yield.

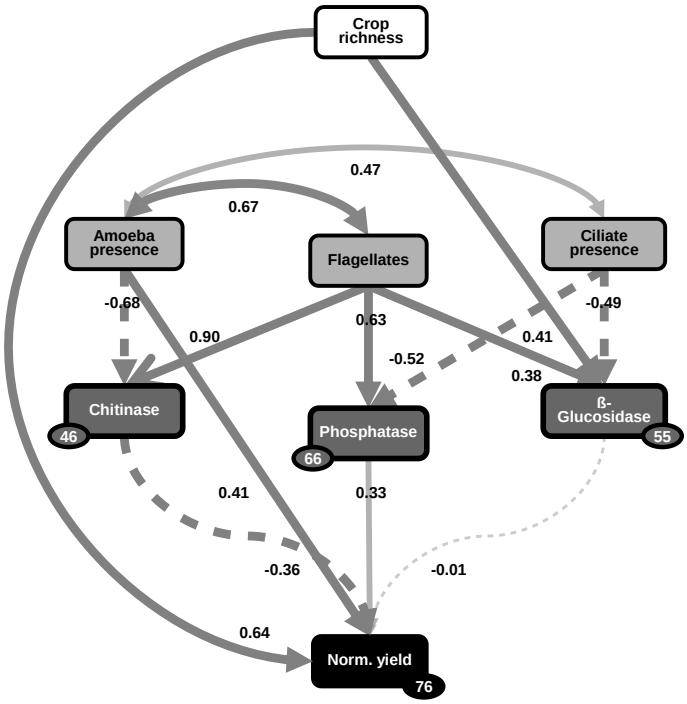

Figure 11. Final structural equation model showing effects of crop richness, protozoa abundance/presence and enzyme activities on normalized yield (see main text for model fit). Normal arrows indicate directed regressions and double-headed arrows indicate undirected correlations. Numbers on arrows indicate standardized path coefficients. Solid arrows indicate positive and dashed negative estimates; bold arrows indicate significant ($P \leq 0.05$) thinner arrows marginally significant ($P \leq 0.1$) and thinnest arrows non-significant ($P > 0.1$) estimates. Percentages close to endogenous variables indicate the variance explained by the model (R^2).

Generally, SEM results could only partially be rediscovered in the mixed effect models (Table 3). While we couldn't underline the observed positive effects of flagellates on enzyme activities, we could observe negative effects of amoeba on chitinase activity (P_F = 0.030) and ciliate presence on phosphatase activity (P_F = 0.032) that were independent of the cropping-regime in the mixed effect models. Further, ciliates decreased chitinase activity, an effect we only observed in maize-rows (P_F = 0.047; Table 3).

Table 3. Effects of Flagellate abundance, Amoeba presence and Ciliate presence on chitinase, ß-glucosidase and phosphatase activities as indicated by Mixed Effects Models.

Chitinase activity

	crop independently					in maize-rows					in soybean-rows					in inter-rows				
	DF	Estimate	S.E.	t-value		DF	Estimate	S.E.	t-value		DF	Estimate	S.E.	t-value		DF	Estimate	S.E.	t-value	
Intercept	36	0.418	0.09	4.59	***	17	0.620	0.31	2.01		17	0.423	0.09	4.50	***	25	0.343	0.29	1.19	
Flagellate abundance	17	0.009	0.01	0.86		8	-0.015	0.04	-0.39		8	0.009	0.01	0.80		25	0.018	0.04	0.51	
	DF	Mean absent	Mean present	F-value		DF	Mean absent	Mean present	F-value		DF	Mean absent	Mean present	F-value		DF	Mean absent	Mean present	F-value	
Ciliates	17	0.510	0.474	1.78		8	0.539	0.436	5.52	*	8	0.488	0.506	0.18		25	0.503	0.468	0.42	
Amoeba	17	0.510	0.432	5.65	*	8	0.522	0.440	2.85		8	0.500	0.463	0.33		25	0.507	0.409	2.25	

beta-Glucosidase activity

	crop independently					in maize-rows					in soybean-rows					in inter-rows				
	DF	Estimate	S.E.	t-value		DF	Estimate	S.E.	t-value		DF	Estimate	S.E.	t-value		DF	Estimate	S.E.	t-value	
Intercept	36	0.756	0.09	8.43	***	17	0.584	0.29	2.05	.	17	0.836	0.11	7.63	***	25	0.416	0.27	1.53	
Flagellate abundance	17	0.009	0.01	0.84		8	0.029	0.03	0.84		8	-0.003	0.01	-0.20		25	0.055	0.03	1.62	
	DF	Mean absent	Mean present	F-value		DF	Mean absent	Mean present	F-value		DF	Mean absent	Mean present	F-value		DF	Mean absent	Mean present	F-value	
Ciliates	17	0.849	0.804	2.95		8	0.838	0.796	0.91		8	0.836	0.789	0.99		25	0.879	0.818	1.38	
Amoeba	17	0.834	0.814	0.34		8	0.827	0.807	0.17		8	0.821	0.774	0.39		25	0.861	0.826	0.27	

Phosphatase activity

	crop independently					in maize-rows					in soybean-rows					in inter-rows				
	DF	Estimate	S.E.	t-value		DF	Estimate	S.E.	t-value		DF	Estimate	S.E.	t-value		DF	Estimate	S.E.	t-value	
Intercept	36	1.524	0.07	21.45	***	17	1.566	0.14	11.14	***	17	1.494	0.12	12.14	***	25	1.558	0.16	9.75	***
Flagellate abundance	17	0.001	0.01	0.16		8	-0.001	0.02	-0.08		8	0.002	0.02	0.12		25	-0.002	0.02	-0.11	
	DF	Mean absent	Mean present	F-value		DF	Mean absent	Mean present	F-value		DF	Mean absent	Mean present	F-value		DF	Mean absent	Mean present	F-value	
Ciliates	17	1.555	1.508	5.45	*	8	1.569	1.528	4.27	.	8	1.537	1.474	1.61		25	1.554	1.523	1.17	
Amoeba	17	1.538	1.526	0.19		8	1.562	1.533	1.47		8	1.514	1.460	0.43		25	1.542	1.538	0.01	

Flagellates: significances of slopes were generated with the R-function summary(), showing the probability of an estimate to be zero (two-tailed t-test). Ciliates and amoeba: significances of factors were determined via analyses of variance (ANOVA). Asterisks denote the level of significance: ˙P ≤ 0.1; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. DF: degrees of freedom.

2.4.5 | Discussion

While intercropping strategies are receiving increasing interest, it remains a challenge to unravel the pathways linking cropping treatment to yield. By using the structural equation modelling approach, we were able to separate direct effects of crop richness, different soil protozoa and soil inherent enzyme activities from indirect pathways connecting the investigated variables to crop yield. Protozoa are considered to be a keystone soil taxon regarding plant growth. As predators of bacterial communities, they select for species and functional genes responsible for plant growth (Bonkowski & Brandt 2002; Müller *et al.* 2013) and enhance nutrient cycling (Uikman, Jansen & van Veen 1991; Bonkowski 2004). Here, we provide first evidences that cropping strategy interactively with soil protozoa shapes soil functioning and thereby influences yield.

In the present study, crop richness had an important positive effect on normalized yield, which was mainly driven by an enhanced yield of maize in intercropping treatments (Figure 10 and Figure 11). We suggest that the maize plants benefitted of the nitrogen fixing property of rhizobia in their association with the legume, which is in line with other experiments where intercropping with legumes increased yields (Chu *et al.* 2004). In addition, different plant species can exploit different soil fractions and have different affinities for nutrients allowing for complementarity between species (Loreau *et al.* 2001; Eisenhauer 2012), an affect already suggested to improve yields in intercropping regimes (Bedoussac *et al.* 2015).

The SEM approach revealed that intercropping further directly increased ß-glucosidase activity, an enzyme linked to carbon cycling (Marx *et al.* 2001). We could, however, not connect the activity of this enzyme to plant yield, suggesting that this enzyme may have more long-term effects on plants.

Our approach revealed that protozoa may play a central role in linking plant diversity to soil fertility, and that the effect may be functional group-specific. Flagellates, ciliates and amoeba, which represent the three main functional types of protozoa in agricultural soils, (Ekelund & Rønn 1994), responded differently to the cropping regime, had contrasting effects on enzymatic activity and plant yield. According to the mixed effect model, amoebae decrease chitinase activity independently of the crop diversity. By contrast, the negative effects of ciliates on chitinase and phosphatase observed in the SEM, might have been driven by the presence of maize as indicated by the mixed effect models and might be powered by specific compounds

exuded by maize roots. Root exudates are suggested to play an important role in driving plant-specific effects on soil microbial communities (Bais *et al.* 2006; Berg & Smalla 2009).

Amoeba, affected both enzymatic activity and directly promoted crop yield, indicating that they may affect plant yield via other mechanisms independent from enzymatic activity. Amoeba are known to support plant-growth-promotion by bacteria (Bonkowski & Brandt 2002), an effect that might also have played a role in the present study.

Flagellates appeared as the functional group with the strongest effect on enzyme activity, stimulating the activity of all tested enzymes. The discrepancies between the SEM and the mixed effect model might be at least partially explained by the correlation effects between the three protozoan groups that we could not account for in the mixed effect models. However, by using mixed effect models in addition to the SEM we were able to account for differences between crop identities that we were not able to address in the SEM (due to the small sample size accompanied by limiting degrees of freedom).

With this study, we provide first evidence that agricultural treatment and soil biota may interactively explain the increased plant yield in intercropping systems. In this study we focused on a restricted number of organisms and functions. Due to the large number of soil organisms shaping soil fertility and plant health, we propose that our approach could be refined by including more functional groups, from bacteria to macrofauna across a higher number of sites to achieve a sufficient explanatory power. We suggest that combining those studies with the SEM approach will enable to disentangle the complex biotic interactions linking cropping regime to soil functioning and yield and may help developing agricultural practices that enhance soil fertility and yield sustainably.

2.4.6 | Acknowledgements

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III | General Discussion

Microbial communities producing antifungal compounds are crucial drivers of naturally occurring disease suppression in soil (Weller *et al.* 2002; Haas & Defago 2005), and soil microbial consortia are interactively driven b
y soil properties and plant community composition (Garbeva *et al.* 2004; Berg & Smalla 2009; Philippot *et al.* 2013). However, there are still significant gaps in our understanding of the mediators that structure rhizosphere microbial functioning that prevent successfully improving plant growth and health (Bakker *et al.* 2012; Mendes *et al.* 2013).

This thesis demonstrated that (1) plant species within a diverse community exert a strong impact on antifungal traits of bacteria, (2) plant community composition affects soil abiotic and biotic properties and thereby drives the suppressive potential of soils, (3) plant-plant interactions exert a strong impact on disease suppression, and (4) cropping regime interactively with soil protozoa shapes soil microbial functioning.

The results underline that the abundance, activity and diversity of root-associated bacteria is affected by biotic and abiotic properties of the rhizosphere environment (Berg & Smalla 2009). Therefore, to mechanistically understand the functioning of microbial communities involved in pathogen suppression and enabling to predict and manipulate the suppressive potential of soils, it is necessary to consider the potential of plant communities to shape microbial communities in soil.

3.1 Effects of plant species diversity

In the experiments presented in Chapters 2.1 and 2.3 plant species richness significantly affected soil microbial functioning. This is in line with studies identifying plant diversity as important driver of the diversity, abundance and activity of soil microorganisms (Stephan *et al.* 2000; Zak *et al.* 2003; Liu *et al.* 2008; Eisenhauer *et al.* 2010a). Further, the importance of plant diversity in driving microbial communities involved in pathogen suppression is highlighted (Garbeva *et al.* 2006; Weller 2007; Latz *et al.* 2012).

Bacterial population density turned out to be a distinct mediator of plant diversity effects on bacterial gene activities (Chapter 2.1; Latz *et al.* 2015). Effects of plant diversity on microbial density were observed in several studies (Chapter 2.3; Garbeva *et al.* 2006; Weller 2007; Latz *et al.* 2012), and density dependent gene activity was also observed previously (Rochat *et al.* 2010). Further, soil pH and plant root biomass mediated plant diversity effects on a plant pathogen and on groups of bacteria antagonistic to plant pathogens (Chapter 2.1 [root biomass], Chapter 2.3 [root biomass and pH]), underlining their role in mediating plant diversity effects on microbial communities (Eisenhauer *et al.* 2013).

However, the observed diversity effects turned out to be mainly driven either by the presence of specific plant species (Chapter 2.1) or specific plant species interactions (Chapter 2.3). Therefore, although species richness measures often cover significant plant community effects on ecosystem functions, they might be not adequate to mechanistically understand plant community driven effects on given ecosystem functions (Loreau et al. 2001; Ebeling et al. 2014).

3.2 Effects of plant functional groups

Effects of plant functional groups on microbial abundances and functioning were non-consistent throughout the experiments of this thesis. Grasses and legumes were previously suggested as key plant functional groups for soil microbial communities (Spehn *et al.* 2000; Milcu *et al.* 2008; Eisenhauer *et al.* 2010a), and also turned out to shape *Pseudomonas* communities producing antifungal compounds (Latz *et al.* 2012). In the experiment presented in Chapter 2.3, grasses mediated effects on pathogen suppression mainly via increasing the abundance of *Bacillus*, and decreasing abundances of *Pseudomonas* and *Actinomyces.* Interestingly, the presence of grasses also partly increased the abundance of the latter two bacterial groups by decreasing root biomass. In addition, legumes mediated effects on pathogen suppression via increasing the abundance of *Pseudomonas* and *Actinomyces*. These results were unexpected, since legumes have been shown to decrease and grasses to increase *Pseudomonas* communities (Latz *et al.* 2012). In the experiment reported in Chapter 2.1, however, effects of the two grass respectively the two legumes species contrasted each other, with one species increasing and the other decreasing *Pseudomonas* density and/or gene expression patterns (Latz *et al.* 2015). Here, plant species identity turned out to be the most

important driver of *Pseudomonas* functioning, suggesting that functional group effects on biocontrol bacteria depend on the respective species pool. Understanding differences in functional properties of plant species may be essential for understanding diversity–ecosystem functioning relationships, but the importance of specific species traits may differ depending on a given ecosystem function (Cadotte, Albert & Walker 2013). Results of this thesis underline that important species differences are not adequately covered by traditional functional group assignments (Ebeling *et al.* 2014), at least regarding differences in drivers of biocontrol microbial functioning.

3.3 Effects of plant species identity

Results of the experiment presented in Chapter 2.1 support the role of plant species identity in eliciting bacterial gene expression. This is in line with studies where the production of antifungal compounds by bacteria has been shown to be plant species and also plant cultivar specific (de Werra *et al.* 2008; Rochat *et al.* 2010). As shown by Rochat *et al.* (2010), the expression of genes coding for DAPG and HCN is associated with higher numbers of *P. protegens* CHA0 (prior known as *P. fluorescens* CHA0) colonizing the roots. Results of the experiment presented in Chapter 2.1, however, unraveled that bacterial density is not the only driving force in shaping bacterial gene expression, but unknown plant mediators (e.g., specific exudates) drove bacterial density and/or gene expression (Latz *et al.* 2015). Interestingly, not higher biomass of plant roots *per se*, which was suggested to increase habitat and resource availability (Spehn *et al.* 2000; Zak *et al.* 2003; de Deyn *et al.* 2011), but an enhanced root-biomass of specific species mediated plant effects on bacterial abundances (Chapter 2.1; Latz *et al.* 2015). This species-specific plant root biomass effect was supported by results of the experiment presented in Chapter 2.3. Here, *Medicago* was primarily accountable for increased root biomass (data not shown) that in turn increased the abundance of *Bacillus*, while it decreased that of *Pseudomonas* and *Actinomyces*. The importance of plant species identity for the community composition of rhizosphere bacteria was also supported by results of the study on intercropping, where ciliates decreased the activity of chitinase in presence of maize plants (an enzyme involved in the suppression of fungal pathogens; Loper *et al.* 2012; Chapter 2.4).

Remarkably, *Lolium perenne* that enhanced bacterial densities and activities in the gnotobiotic

system (where only *P. protegens* CHA0 was present) turned out to non-specifically foster microbial densities in natural soil, as indicated by increased colony forming units on non-selective media in presence of that grass species (Chapter 2.3; data not shown). Since gene activities have been shown to be partly density dependent (Rochat *et al.* 2010; Latz *et al.* 2015), this suggests that observations regarding bacterial gene activities in gnotobiotic systems may not be transferable to natural soil systems and have to be interpreted with care. Gene activities could not be measured in the experiment presented in Chapter 2.3, and possibly specific species drove gene activities. However, the presence of specific species had no important effect at least on the investigated host-pathogen system.

3.4 Plant-plant interaction effects

In the experiment presented in Chapter 2.3, direct effects of specific plant-plant interactions rather than plant species identity effects turned out to be essential drivers of pathogen suppression. It was shown that plant-plant competition increases the amount of exudates released by plant roots, an effect likely influencing microbial communities (Bais *et al.* 2006). Enhanced root exudation might also explain the increased enzymatic activity of the ß-glucosidase (which is involved in sugar degredation; Marx *et al.* 2001) in response to intercropping (Chapter 2.4). In the same experiment, crop species richness increased crop yield, presumably due to a positive effect of soybean on maize. Maize plants likely benefitted from the nitrogen fixing property of rhizobia in association with legumes, which is in line with other experiments where intercropping with legumes increased crop yields (Chu et al., 2004). In addition, different plant species can exploit different soil fractions and have different affinities for nutrients allowing for species complementarity (Loreau *et al.* 2001; Eisenhauer 2012), an effect suggested to improve crop yields in intercropping systems (Bedoussac *et al.* 2015). Signaling adaptations in plants to pathogens, that results in differential responses in root-associated bacteria (de Werra *et al.* 2008), may require adequate resource availability. Therefore, resource complementarity of plant species is likely to play a role in plant community resistance to pathogens. However, the experiment presented in Chapter 2.3 was rather short-termed and effects of complementary resource use or facilitative resource acquisition generally increase with time (Eisenhauer 2012). Whether the effect of plant-plant interactions on

pathogen suppression, observed in the experiment presented in Chapter 2.3, was due to complementary resource acquisition or plant competition driven changes in root exudation needs further investigation.

3.5 Conclusion

The use of microbes for biological control of plant diseases represents an environmentally friendly and promising approach. Biocontrol bacteria are natural antagonists to pathogens and act on a local scale, thereby alleviating environmental pollution that is a problem of artificial, chemical pathogen control (Lugtenberg & Kamilova 2009). However, for developing effective biocontrol agents, bacteria not only need to produce antibiotics in sufficient amounts, but also successfully compete for nutrients and niches on the root surface, and escape predation (Lugtenberg & Kamilova 2009). Soil conditions and plant communities essentially impact the composition, dynamics and functions of rhizosphere microbial communities (Garbeva *et al.* 2004, 2006; Berg & Smalla 2009). However, the extent to which each mediator impacts rhizosphere microbial functioning is not fully understood (Bakker *et al.* 2012; Mendes *et al.* 2013). Further, since many of the driving forces involved in disease suppression are interwoven, disentangling the mechanisms driving rhizosphere microbial functioning is essential (Philippot *et al.* 2013).

Despite underlining the role of plant diversity in driving soil disease suppression (Garbeva *et al.* 2006; Latz *et al.* 2012, 2015), results of this thesis show that plant diversity (and functional group) effects likely are mediated by specific plant identity and plant-plant interaction effects. Further, results of this thesis proved plant community induced changes in soil pH, root biomass and abundances of biocontrol bacteria to be important determinants of pathogen suppression. The results underline the assumption that plant community composition, soil abiotic properties and microbial communities being antagonistic to soil pathogens are linked and interactively shape the suppressive potential of soils (Garbeva *et al.* 2004; Berg & Smalla 2009; Philippot *et al.* 2013). Choosing specific plant communities may enable to manipulate rhizosphere environmental conditions, thereby fostering microbial establishment in the rhizosphere and increase the disease suppressive potential of soils.

Overall, results of this thesis represent an important step in unravelling the complexity of

mechanisms linking plant community composition and plant disease suppression.

3.6 Perspectives

Although having identified distinct mechanisms and unravelled indirect effects of plant community composition as drivers of the pathogen suppressive potential of soils, there is the need to investigate the rhizosphere system in more detail. In addition to abiotic soil conditions, plant protective bacteria and protozoa, the role of other (micro-) organisms need to be considered.

New methodologies, such as next-generation sequencing or non-invasive two-dimensional imaging, allow the assessment of the roles of organisms within communities thereby uncovering unknown microorganisms and genes and their functioning in rhizosphere interactions (Mendes *et al.* 2013; Philippot *et al.* 2013). This may enable to identify (micro-) organisms needed to fulfil specific ecosystem functions (Mendes *et al.* 2013). However, more important than knowing which microbial consortia are needed to fulfil specific functions might be knowledge on how their functioning can be maintained.

Plants essentially shape the rhizosphere environment (Raaijmakers *et al.* 2009), and the use of specific (sets of) plant traits as promising predictors of plant community effects on soil functions has been proven (Duffy 2008; Ebeling *et al.* 2014). However, the set of traits being important predictors may differ depending on the respective ecosystem function (Cadotte *et al.* 2013), probably explaining why previously used morphological, physiological and phenological traits failed in adequately predicting abundances of biocontrol bacteria (Latz *et al.* 2012). Identifying distinct plant traits is essential in order to predict and manipulate ecosystem functions mediated by plant effects on microbiota.

Root exudates are primarily discussed in mediating plant effects on microbiota in the rhizosphere (Bardgett & van der Putten 2014). Therefore, understanding differences in plant exudation properties, and being able to relate them to environmental conditions, cropping regimes and plant traits is crucial to be able to successfully promote plant growth and health (Philippot *et al.* 2013).

The results of this thesis suggest that setting up plant communities selected for traits that support specific microbial communities in the rhizosphere can be used, in order to prepare the

soil for subsequent crops. This would allow both the promotion of specific functions of microbial communities as well as induction of multifunctionality (since in managed systems multiple functions and services may be of primary importance; Duffy 2008).

I suggest that (1) additional (micro-) organisms involved in pathogen suppression and their drivers need to be identified, and (2) knowledge on plant traits that predict and maintain community functioning is needed, in order to improve environmentally friendly plant protection strategies and ensure the long term stability of crop yield.

IV | References

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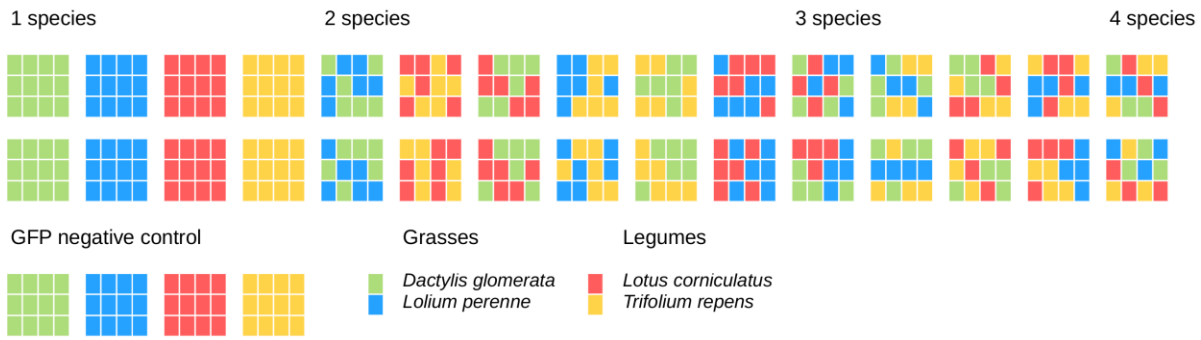
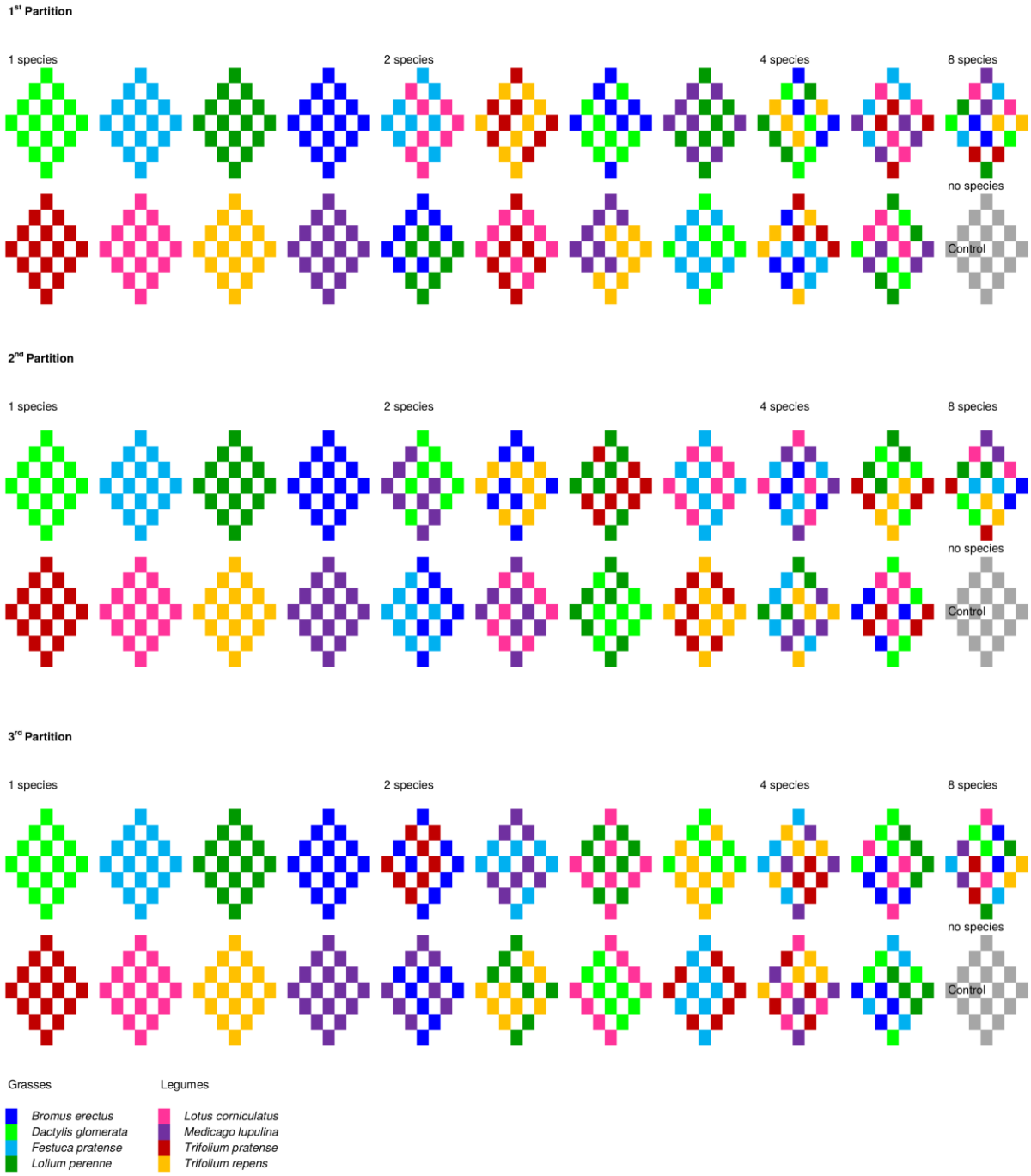
V | Supplementary Material

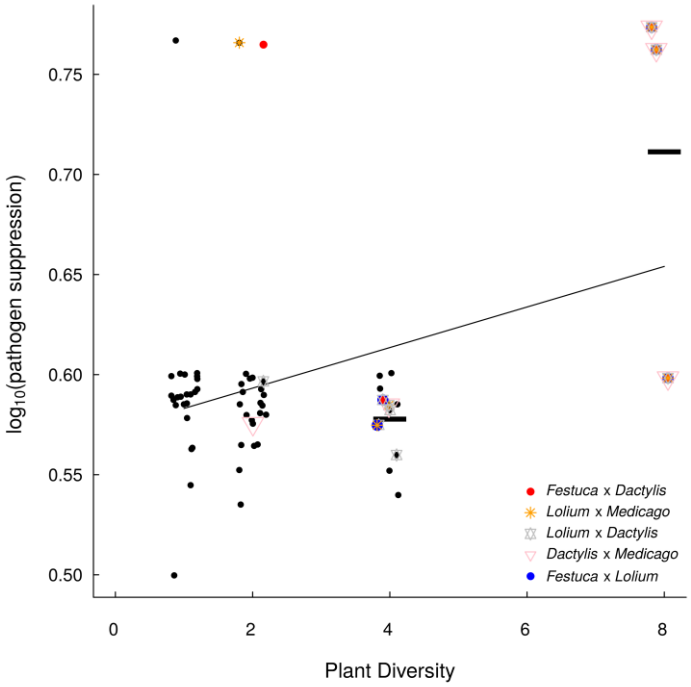
In addition to the complete thesis, published papers and supplementaries for all research chapters, original R data and code files as supplementary information to Research Chapter 2, can be found as digital copy on CD.



Supplementary Figure 1. Experimental setup of the substitutive, full factorial plant species richness gradient. Displayed is one of the six blocks. Each small square represents one plant individual; each main square represents one plant community. We set up two blocks per bacterial treatment (*Pseudomonas protegens* inhabiting plasmid pME 7100, pME 7116 and pME 7156, respectively). Each with two replicates per plant species richness level, resulting in a total of four replicates. Four plant monocultures inoculated with *P. protegens* wild-type strain per block served as control (GFP negative control).



Supplementary Figure 2. Experimental setup of the substitutive plant species richness gradient. Plant diversity was varied independently of functional group affiliation by using the random partitions design (Bell et al. 2009). Displayed is the spatial arrangement of the last plant growth cycle (fifth). Each small square represents one plant individual; each main square represents one plant community.



Supplementary Figure 3. To test for plant identity and interaction effects independent of diversity, we used a sequential statistical approach (Bell *et al.* 2009). In a first step, we used linear regression models to analyse the effect of plant diversity (continuously coded) on pathogen suppression (log_{10} [pathogen suppression]). In a second step, the residuals of the first step in the analyses were fitted against the explanatory variables *Bromus*, *Dactylis*, *Festuca*, *Lolium*, *Lotus*, *Medicago*, *Trifolium p.* and *Trifolium r.* (presence [1] / absence [0], continuously coded; intercept = 0). In a third step, the residuals of the second step were fitted against plant diversity (factorially coded; intercept = 0). Additionally, to identify explanatory interactions, the residuals of the second step were fitted against 2nd order interactions in a linear regression approach. In the interaction fitting, the most parsimonious model was selected using the stepAICc() function (Scherber 2009).
Diversity increased pathogen suppression (slope = 0.010, $P < 0.05$), whereas none of the plant identities showed a significant influence on suppression (all $P > 0.1$). Interestingly, pathogen suppression was decreased on diversity level four (slope =-0.036, $P < 0.05$) and increased on diversity level eight (0.057, $P < 0.05$) indicating that interactive effects of plants drive the diversity pattern.
Analyses (instead of the third step) using interactions up to the 2nd order revealed that the species combinations *Festuca-Dactylis* (red dots) and *Lolium-Medicago* (yellow stars) increased pathogen suppression. The negative effect of the species pair *Festuca-Lolium* (blue-circle) was most obvious at diversity level 4, where it might have hampered the positive effect of *Dactylis-Festuca* (red dot and blue circle) and *Medicago-Lolium* (yellow star and blue circle). In addition, while only being marginally significant, *Lolium-Dactylis* (grey, crossed triangles) and *Dactylis-Medicago* (pink triangle) might have hampered the positive effect of *Lolium-Medicago*.

Supplementary Table 1. Results of path analyses of Dactylis, Lolium, Lotus and Trifolium presence on root biomass, active bacterial density and gene expression.

			Estimate	S.E.	C.R.	
(A) *phlA*						
Root biomass	←	*Lolium* presence	0.852	0.16	5.44	***
	←	*Trifolium* presence	-0.719	0.16	-4.61	***
	←	*Lotus* presence	0.519	0.16	3.34	**
Bacterial density	←	*Lolium* presence	0.690	0.08	8.86	***
	←	*Trifolium* presence	-0.198	0.08	-2.55	*
phlA expression	←	*Lolium* presence	2.994	0.83	3.63	***
	←	*Dactylis* presence	-1.442	0.55	-2.65	**
	←	Bacterial density	0.932	0.87	1.07	
(B) *prnA*						
Root biomass	←	*Lolium* presence	1.022	0.21	4.89	***
	←	*Trifolium* presence	-0.987	0.21	-4.72	***
Bacterial density	←	Root biomass	0.093	0.03	3.04	**
	←	*Lolium* presence	0.444	0.07	6.82	***
prnA expression	←	*Lolium* presence	1.447	0.62	2.34	*
	←	*Lotus* presence	1.129	0.39	2.86	**
	←	*Dactylis* presence	-1.847	0.39	-4.68	***
	←	Bacterial density	5.189	0.87	5.95	***
(C) *hcnA*						
Root biomass	←	*Lolium* presence	0.743	0.13	5.76	***
	←	*Trifolium* presence	-0.452	0.13	-3.53	***
	←	*Lotus* presence	0.601	0.13	4.70	***
Bacterial density	←	*Lolium* presence	1.061	0.13	8.46	***
	←	*Trifolium* presence	-0.285	0.13	-2.27	*
hcnA expression	←	*Lolium* presence	-2.704	0.83	-3.24	**
	←	Bacterial density	4.931	0.56	8.81	***

Given are non standardized path coefficients (estimates), standard error of regression weight (S.E.) and the critical value for regression weight (C.R.; z = estimate/ S.E.). Asterisks denote the level of significance: . P ≤ 0.1; *P ≤ 0.05; **P ≤ 0.01; ***P < 0.001. For more information of exogenous and endogenous variables as well as on model fit see main text.

Supplementary Table 2. Hypotheses liable for the initial structural equation model (see methods for details).

Dependent variables	Expected effect	Independent variables	Reference
Pathogen suppression		*Actinobacteria* / *Bacillus* / *Pseudomonas*	Garbeva *et al.* (2006), Mendes *et al.*(2011), Latz *et al.* (2012)
		Soil moisture	Janvier *et al.* (2007)
		Soil pH	Janvier *et al.* (2007)
		C/N ratio	Janvier *et al.* (2007)
*Microbial Biomass**		Plant diversity	Eisenhauer *et al.* (2010a, 2013), Latz *et al.* (2012)
		Legumes / Grasses	Eisenhauer *et al.* (2010a), Latz *et al.* (2012)
		Root biomass	Spehn *et al.* (2000), Zak *et al.* (2003), de Deyn *et al*. (2011), Eisenhauer *et al.* (2013)
		Soil moisture	Eisenhauer *et al.* (2013), Lange *et al.* (2014)
		Soil pH	Eisenhauer *et al.* (2013), Philippot *et al.* (2013)
		C/N ratio	Kühn *et al.* (2009), Eisenhauer *et al.* (2013), Pérès *et al.* (2013)
Soil moisture		Plant diversity	Eisenhauer *et al.* (2013), Lange *et al.* (2014)
		Legumes / Grasses	Lange *et al.* (2014)
		Root biomass	Eisenhauer *et al.* (2013)
Root biomass		Plant diversity	Spehn *et al.* (2000), Eisenhauer *et al.* (2013), Pérès *et al.* (2013)
		Legumes / Grasses	Pérès *et al.* (2013), Bessler *et al.* (2009, 2012)
Soil C/N		Plant diversity	Steinbeiss *et al.* (2008), Einsenhauer *et al.* (2013)
		Legumes / Grasses	Pérès *et al.* (2013), Lange *et al.* (2014)
		Root biomass	Steinbeiss *et al.* (2008), Einsenhauer *et al.* (2013)
Soil pH		Plant diversity	Eisenhauer *et al.* (2013)
		Legumes / Grasses	no reference found

*likely, equivalently *Pseudomonas*, *Actinomyces*, *Bacillus* and pathogen abundance. Dashed arrows indicates negative, solid arrow indicates positive coherences. Grey arrows indicate assumed but not proofed effects or effects that gave inconsistent results.

Supplementary Table 3. Results of the final structural equation model (X^2_{21} = 17.22; P-value = 0.698), showing effects of direct and indirect (through changes in soil pH, root biomass, soil moisture and bacterial abundances) effects of plant community composition on pathogen suppression.

			Estimate	S.E.	C.R.	
Regressions						
Soil pH	←	Plant diversity	0.077	0.05	1.66	.
Soil moisture	←	Root biomass	-0.207	0.03	-6.19	***
Root biomass	←	Plant diversity	0.478	0.20	2.41	*
	←	Grass presence	-1.333	0.74	-1.79	.
Bacillus	←	Soil pH	0.645	0.22	3.00	**
	←	Soil moisture	0.443	0.19	2.28	*
	←	Root biomass	0.308	0.07	4.73	***
	←	Grass presence	0.797	0.30	2.66	**
Pseudomonas	←	Root biomass	-0.057	0.01	-5.26	***
	←	Grass presence	-0.272	0.07	-4.05	***
	←	Legume presence	0.192	0.07	2.83	**
Actinomyces	←	Root biomass	-0.047	0.02	-3.02	**
	←	Grass presence	-0.323	0.10	-3.33	**
	←	Legume presence	0.529	0.10	5.38	***
Pathogen suppression	←	Plant diversity	0.149	0.04	3.74	***
	←	Legume presence	-0.442	0.18	-2.47	*
	←	Soil pH	-0.291	0.10	-2.86	**
	←	*Pseudomonas*	-0.428	0.27	-1.60	
	←	*Actinomyces*	0.547	0.20	2.71	**
	←	*Bacillus*	0.057	0.05	1.15	
Covariances						
Actinomyces	↔	*Pseudomonas*	0.000	0.00	3.00	**
Plant diversity	↔	Grass presence	0.002	0.00	2.32	*
	↔	Legume presence	0.003	0.00	2.46	*
Grass presence	↔	Legume presence	-0.001	0.00	-2.82	**

Given are non standardized path coefficients (estimates), standard error of regression weight (S.E.) and the critical value for regression weight (C.R.; z = estimate/ S.E.). Asterisks denote the level of significance: . P ≤ 0.1; *P ≤ 0.05; **P ≤ 0.01; ***P < 0.001. For more information of exogenous and endogenous variables as well as on model fit see main text.

Supplementary Table 4. Effects of plant community composition on pathogen suppression as indicated by additional, sequential analyses (see text for details).

	Number of grasses				*Bromus erectus*				*Dactylis glomerata*				*Festuca pratense*				*Lolium perenne*			
1. step	Estimate	S.E.	t-value		Estimate	S.E.	t-value		Estimate	S.E.	t-value		Estimate	S.E.	t-value		Estimate	S.E.	t-value	
Intercept	0.578	0.010	58.74	***	0.591	0.008	71.99	***	0.589	0.008	71.82	***	0.591	0.008	71.13	***	0.589	0.008	72.54	***
Plant number / identity	0.016	0.006	2.50	*	0.018	0.016	1.17		0.026	0.015	1.75	.	0.018	0.015	1.16		0.025	0.015	1.64	
DF/R^2	59/0.10				59/0.02				59/0.05				59/0.02				59/0.04			
2. step																				
Intercept	-0.005	0.114	-0.47		-0.018	0.011	-1.58		-0.016	0.011	-1.39		-0.018	0.011	-1.60		-0.016	0.011	-1.41	
Plant diversity	0.002	0.004	0.58		0.008	0.004	1.96	.	0.007	0.004	1.73	.	0.008	0.004	1.98	.	0.007	0.004	1.74	.
DF/R^2	59/0.01				59/0.06				59/0.05				59/0.06				59/0.05			
	Number of Legumes				*Lotus corniculatus*				*Medicago lupulina*				*Trifolium pratense*				*Trifolium repens*			
1. step	Estimate	S.E.	t-value		Estimate	S.E.	t-value		Estimate	S.E.	t-value		Estimate	S.E.	t-value		Estimate	S.E.	t-value	
Intercept	-0.016	0.011	-1.41		0.593	0.008	70.87	***	0.588	0.008	71.88	***	0.595	0.008	71.67	***	0.594	0.008	70.85	***
Plant number / identity	0.007	0.004	1.74	.	0.011	0.015	0.70		0.027	0.015	1.79	.	0.005	0.016	0.33		0.009	0.015	0.61	
DF/R^2	59/0.05				59/0.01				59/0.05				59/0.00				59/0.01			
2. step																				
Intercept	-0.013	0.012	-1.08		-0.020	0.011	-1.77	.	-0.015	0.011	-1.38		-0.022	0.011	-1.91	.	-0.021	0.011	-1.81	.
Plant diversity	0.005	0.004	1.45		0.009	0.004	2.20	*	0.007	0.004	1.71	.	0.009	0.004	2.36	*	0.009	0.004	2.24	*
DF/R^2	59/0.03				59/0.08				59/0.05				60/0.06				59/0.08			

The tables were generated with the R-function summary(), showing the probability of a variable to be zero (two-tailed t-test). Asterisks denote the level of significance: . $P \leq 0.1$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P < 0.001$. DF, degrees of freedom; R^2 coefficient of determination.

Supplementary Table 5. Chemical soil properties were determined according to Duval *et al.* (2013).

Chemical indicator	Soybean	Maize	Soybean/Maize
Organic carbon content (%w/w)	2.10 ± 0.14	2.24 ± 0.01	2.12 ± 0.08
COPc (%w/w)	0.30 ± 0.03	0.33 ± 0.03	0.30 ± 0.09
COPf (%w/w)	0.26 ± 0.04	0.28 ± 0.03	0.25 ± 0.01
MOC (%w/w)	1.54 ± 0.11	1.63 ± 0.05	1.56 ± 0.06
Nitrogen (%w/w)	0.17 ± 0.02	0.17 ± 0.01	0.17 ± 0.01
Extractable phosphorus (mg/kg)	46.4 ± 8.5	47.3 + 7.1	49.0 ± 7.4
Soluble carbohydrates (mg/kg)	117.48 ± 7.7	111.3 ± 7.5	111.49 ± 17.3
Total carbohydrates (mg/kg)	846.5 ± 29.8	1235.5 ± 119.6*	983.7 ± 19.1

*, total carbohydrate content of maize plots was significantly higher than those of soybean alone or intercropped plots (Tukey´s HSD; $P < 0.05$). POCc: coarse fraction (105-2000 µm) containing coarse particulate organic carbon; POCf: medium fraction (53-105 µm) containing fine particulate organic carbon; MOC: fine fraction (<53 µm) containing mineral associated organic carbon.

VI | Thesis Declarations

7.1 Declaration of the author's own contribution to manuscripts

RESEARCH CHAPTER 1 is published in a peer-reviewed journal. *RESEARCH CHAPTERS 2 and 4* are currently submitted to peer reviewed journals. In all manuscripts except that presented in *RESEARCH CHAPTER 2* I am the first author. In *RESEARCH CHAPTERS 1, 3* and *4* I have collected and analysed the data, developed the main ideas and primarily wrote the manuscripts, created tables, figures and supplementaries and contributed significantly to the study design (in *CHAPTER 4*, data collection was done by Simone Weidner and myself, Claudio Valverde has determined Protozoan abundances and ideas were developed cooperatively). The model presented in *RESEARCH CHAPTER 2* was developed and written by Björn C. Rall. The idea for the model and writing of the manuscript was cooperative work. Generally, all co-authors contributed to the final versions of the manuscripts.

7.2 Plagiarism declaration

I declare that I have written this doctoral thesis independently. All persons contributing to the manuscripts have been named so. All sentences or passages quoted from other people's work have been specifically acknowledged by clear cross-referencing. I have not submitted this thesis in any form for another degree at any university or institution.

Ellen Latz Leipzig, August 2015

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