Impact of arbuscular mycorrhizal fungi on nutrient cycling in agroecosystems

Invloed van arbusculaire mycorrhiza schimmels op de nutriëntenkringloop in agro-ecosystemen

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Invloed van arbusculaire mycorrhiza schimmels op de nutriëntenkringloop in agro-ecosystemen

(met een samenvatting in het Nederlands)

Proefschrift

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door

Luise Köhl

geboren op 4 september 1981 te Kyritz, Duitsland

Promotor: Prof. dr. ir. M.G.A. van der Heijden

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Contents

Chapter 1

General introduction

In the year 2050 the global population will top 9 billion, a dramatic increase in the number of people that need to be fed (Godfray et al. 2010, Gerland et al. 2014). This will be a challenge, as agricultural productivity needs to increase, while reducing the impact on natural resources for future generations (Tilman et al. 2011). Developing sustainable agriculture practices requires innovative strategies based upon applied research. In addition to advances in pest management, plant breeding, harvest, storage and transportation of food, improved nutrient management will be an essential pillar of creating sustainable food production.

There is an increasing awareness that mineral fertilizers, which have been ubiquitously and excessively applied in agriculture, are in finite supply. Until now food production has been totally dependent on mined phosphate rock. Numerous researchers have suggested phosphate rock resources and reserves and will be depleted in the 21st century (Cordell et al. 2009, Rosemarin et al. 2009, Vaccari 2009), and although nitrogen (N) for fertilizer use can be synthesized, the production process demands nonrenewable fossil fuel resources (Erisman et al. 2008). Thus, averting a nutrient crisis requires that alternative management strategies will be established. As microbiota are essential for nearly all soil nutrient cycling processes it is logical to involve them in agricultural management. Besides approaches to reduce fertilizer input by enhancing beneficial soil microbes, the reduction of nutrient losses from soils can be part of lessening the ecological impact of agriculture. Here, microbes could play key roles as well.

In this thesis, I will focus on phosphorus and nitrogen, the two main elements that mostly limit plant productivity (Elser et al. 2007, Erisman et al. 2008). Here, I will first provide a general introduction about soil P and soil N. Then I focus on arbuscular mycorrhizal fungi, an abundant group of soil fungi that influence plant nutrient uptake and which also might influence N and P availability in soil and N and P losses through leaching.

Soil phosphorus

Both plants and soil microbes need phosphorus in relatively high amounts, which is opposed to the low P availability in the soil solution (typically 0.5-10µM). Unfortunately, the quantity of total P in soil (0.005-0.15%) does not reflect the amount of P available to plants (Smith and Read 2008).

Besides the categories of inorganic (P_i) and organic (P_o), soil P can be thought of existing in three different pools: solution P, labile P and nonlabile P (Havlin et al. 2005). The solution P pool (mainly orthophosphate and some organic P) is the reservoir for plant P uptake. P from the labile P pool readily exchanges with solution P in an equilibrium controlled by plant P uptake and P immobilization. The solution P and the labile P pool are thought to be the plant available P pools. P_i in the soil solution that is not immobilized by plants or microbes can be adsorbed to clay mineral surfaces (labile P) or precipitated in largely insoluble forms like Ca, Fe and Al phosphates (nonlabile P). These processes are summarized as P fixation. The extent of P fixation depends largely on the soil pH (Havlin et al. 2005). Whereas in acid soils P_i precipitates as Fe/Al-P or is adsorbed to surfaces of Fe/ Al oxide, in neutral and alkaline soils P_i precipitates as minerals of Ca/ Mg-P and is adsorbed to $CaCO_3$. P_i is best available at a pH of 6.5. P deficiency of soils is mainly caused by strong adsorption of phosphate. Due to the generally low quantity of soluble soil P, its susceptibility to leaching is low (Domagalski and Johnson 2011).

Organic P can make up 20-80% of total soil P (Dalal 1977) and is mainly present as orthophosphate monoesters, including inositol phosphates (phytate), phospholipids, and nucleic acids (Condron et al., 2005). Up to 40% of P_o can be represented by microbial biomass P (Turner et al. 2013). Whereas the liberation of P_i in soil is primarily chemically driven, biological processes mainly contribute to mineralization of P_o, which is catalyzed by phosphatases and phytases.

Soluble P added to the soil by mineral fertilization or following hydrolysis (mineralization) will temporarily increase available orthophosphate in the soil solution. However, soluble P is constantly removed from the solution by fixation involving sorption on soil surfaces, precipitation of mineral phosphates, and immobilization of P in biomass. As a result, much of the P applied as fertilizer is generally not plant available and leads to a P enrichment of agricultural soils. For example, it has been estimated that the accumulation of 0.7 to 57.2 kg P ha^{-1} yr^{-1} in 25 European countries was the result of P fertilization (Runge-Metzger 1995).

Soil nitrogen

In contrast to P in the soil solution, which is mainly controlled by soil chemical reactions, the N cycle is almost entirely driven by microbial processes and strongly affected by plant-microbe interactions (Craine et al. 2007). Nitrogen enters the soil as a result of biological N-fixation of atmospheric N, N deposition, or fertilization and is subsequently transformed into inorganic and organic compounds. In agricultural top soils in the temperate zone, total N is between 0.7 and 2 g per kg soil (Blume et al. 2010). More than 90% of soil nitrogen is of organic nature and highly dependent on the activity of soil organisms (Schulten and Schnitzer 1997, McNeill and Unkovich 2007, Blume et al. 2010). Organic N compounds are oxidized to ammonium ions (NH_4^+) in the processes of N mineralization or ammonification by soil bacteria. NH_4^+ is consequently assimilated by microbes or plants, fixed by clay minerals, or transformed into nitrate (NO_3^-) (nitrification). During microbially facilitated denitrification nitrate can further be reduced to molecular nitrogen (N_2) through a series of intermediate gaseous nitrogen oxide products like the greenhouse gas N_2O. Plant available nitrogen is primarily nitrate, which is highly soluble and thus very mobile in soil. In contrast, ammonium is bound by adsorption and thus less likely to be leached. The proportion of NH_4^+ in ventilated soils of Central Europe is typically less than 1% and is usually only increased by NH_4^+-fertilization or slurry application. Plants are also able to utilize small organic N-compounds like amino acids but these are usually assimilated by competing rhizobacteria.

Because N only occurs in small amounts in the parent rock material and soil mineral matter, it has to be supplemented in agricultural soils by the addition of organic or mineral fertilizer. This addition of N can increase crop yields significantly, as plants need 10 times more N than P, and N-availability is often a limiting factor in plant growth (Chapin III et al. 2011). The constant application of excessive nitrogen fertilizer during the last decades has resulted in higher nitrate leaching from soil leading to contamination of ground and surface water, as well as increased emissions of the greenhouse gases N_2O and NH_3 to the atmosphere (Cameron et al. 2013).

Nutrient leaching

Nutrient leaching is the downward movement of nutrients, with percolating water, through the soil profile and beyond the rooting zone (Lehmann and Schroth 2003, Blume et al. 2010). In addition to this vertical flow, nutrients can be further lost through erosion or surface run-off. Nutrients leached beyond the rooting zone are temporarily unavailable to the system and have to be replaced by fertilization if necessary. Besides this financial loss, leaching can furthermore pose –depending on many factors– an environmental threat. Although surface run-off has a much larger effect on eutrophication of aquatic ecosystems, vertical leaching can contribute to the contamination of groundwater and downstream surface water systems, which is one of the most serious environmental problems throughout the world (Diaz et al. 2003, Salvia-Castellví et al. 2005). While nitrogen runoff or leaching from agricultural systems can harm coastal marine productivity, eutrophication in many freshwater aquatic systems is dominated by agricultural P-input (Schindler 1977, Matson et al. 1997, Correll 1998). High concentrations of nitrate in drinking water can cause methemoglobinemia in infants, and have been linked to cancer (Gulis et al. 2002, Richard et al. 2014). Grizzetti et al. (2011) estimated that a significant part of the European population could potentially be exposed to high nitrate concentrations in drinking water if adequate treatments were not in place.

When rainfall or irrigation amounts exceed that lost due to evaporation, which is more prevalent in humid areas, the water content of the soil can rise about its field capacity and leaching can occur. Besides the leaching of excess precipitation, water can also move rapidly downwards by preferential flow through macropores. These macropores can result from root growth, faunal activity, or the cracking of clay soils (like Vertisols) at the start of the rainy season (Smaling and Bouma 1992).

The leaching volume is mainly determined by the amount of precipitation, the water holding capacity of the soil, and the water uptake by plants (Blume et al. 2010). In areas with deep silty-loamy soils with a high water holding capacity like boulder clay or loess, in arid areas, or in areas with a year-round ground cover, less water leaches through the soil profile. In areas with sandy soils and high precipitation leaching can be enhanced. In Central Europe leaching usually occurs during fall and winter, when precipitation is high and plant water absorption and nutrient uptake is low (Cameron et al. 2013).

As mentioned previously, soil nutrients differ in their mobility in soil. Phosphate is rather immobile in most soils resulting in low leaching losses of < 1kg P $ha^{-1}yr^{-1}$ (Sharpley and Menzel 1987, Blume et al. 2010). In sandy soils, where continuous P fertilization exceeds the P-binding capacity of the soil and in soils with high preferential flow, P leaching in colloid or soluble form can be up to 6.5 kg P $ha^{-1}yr^{-1}$ (Blume et al. 2010). In bog soil, which contains a low quantity of mineral sorbents, P losses via leaching can be up to 15 kg P $ha^{-1}yr^{-1}$ (Blume et al. 2010). N is mainly leached as nitrate, as it is more mobile than ammonium, and ammonium is readily transformed into nitrate by microbial processes. Nitrate losses via leaching can be up to 100 kg N $ha^{-1}yr^{-1}$(Low and Armitage 1970, Blume et al. 2010). In addition to the previously discussed influences of climate conditions and soil type, the agricultural management system can also determine the amount of nitrate leached. Under comparable soil and climate conditions, N leaching differed between conventional and organic farming systems with 99 and 26 kg N $ha^{-1}yr^{-1}$ leached, respectively (Blume et al. 2010). Organic farms that include a high rate of legumes in the crop rotation and apply high amounts of organic fertilizers can increase their N losses significantly.

One reason for increased leaching in agroecosystems is thought to be the pulsed fertilization of mobile forms of N, which is often not applied based on plant demand. Furthermore, because soil organic matter in agroecosystems is often reduced and the functioning of the soil biological community disturbed, interception, immobilization, and transfer of nutrients is decreased. This, in turn, can result in increased nutrient losses after fertilization. It has been estimated that only 40 to 60% of fertilizer N can be used by the crop, with the rest remaining in the soil or being lost via diverse pathways (Paustian et al. 1992, Parton and Rasmussen 1994, Smil 1999). In contrast, one study indicated that high N leaching over winter is less affected by high N fertilization in spring, than by a high mineralization rate of organic N (Macdonald et al. 1989). Consequently, even a drastic reduction in N fertilizer use would have little effect on nitrate leaching. Additionally, biological N input (e.g,. by symbiotic N-fixation) can increase the risk of nitrogen leaching. For example, it has been repeatedly reported that clover abundance is positively correlated with N leaching (Loiseau et al. 2001, Scherer-Lorenzen et al. 2003, Bouman et al. 2010).

Arbuscular mycorrhizal fungi

Among the variety of beneficial soil microbes, arbuscular mycorrhizal fungi (AMF) are widely discussed for the use in low-input agriculture. These soil fungi form symbiotic associations with 80% of land plants – among them many crop species – and have a worldwide distribution. AMF can account for up to 50% of the microbial biomass in the soil (Olsson et al. 1999, Ryan and Graham 2002) and comprise their own phylum, the *Glomeromycota* (Schüssler et al. 2001), which dates back to the origin of land plants 480 million years ago (Wang and Qiu 2006). There is increasing evidence that this co-development is not a coincidence, but that AMF enabled plants to successfully colonize terrestrial ecosystems (Read et al. 2000, Brundrett 2002).

The co-development of AMF and land plants is based on the substantial exchange of assimilated carbon for soil nutrients. AMF form an extensive network of fine hyphae in the soil, which helps to scavenge for nutrients that are out of reach of the plant root. In exchange, the plant is the only carbon source of the obligate biotrophic fungus and transfers up to 22% of the assimilated carbon to the fungal symbiont (Wright et al. 1998). Up to 100% of a plant's P uptake can occur via the mycorrhizal pathway (Smith et al. 2003, 2004). Additionally, AMF can supply micronutrients like Zn and Cu (Liu et al. 2000), contribute up to 74% of the total nitrogen (Ames et al. 1983, Toussaint et al. 2004, Tanaka and Yano 2005), and 10% of the total K uptake (George et al. 1992) of mycorrhizal plants. Furthermore, AMF can provide non-nutritional benefits to the plant by enhancing plant resistance to several abiotic (drought, salinity, heavy metals) and biotic stressors, like soil-borne plant pathogens (Galli et al. 1994, Azcón-Aguilar and Barea 1996, Bothe 2012). As ecosystem engineers, AMF affect plant (van der Heijden et al. 1998a) and soil microbial community assembly (Amora-Lazcano et al. 1998, Marschner and Baumann 2003, Mechri et al. 2014), as well as soil aggregation (Rillig 2004). Due to the reciprocal nature of the relationship between the symbionts, the growth responses of the plant can vary between positive and negative within a mutualism to parasitism continuum (Johnson et al. 1997, Johnson and Graham 2012, Smith and Smith 2013). The biomass response – positive, neutral, or negative – is dependent on many factors (plant species, fungal species, soil and nutrient availability) and is often difficult to predict, especially under field conditions.

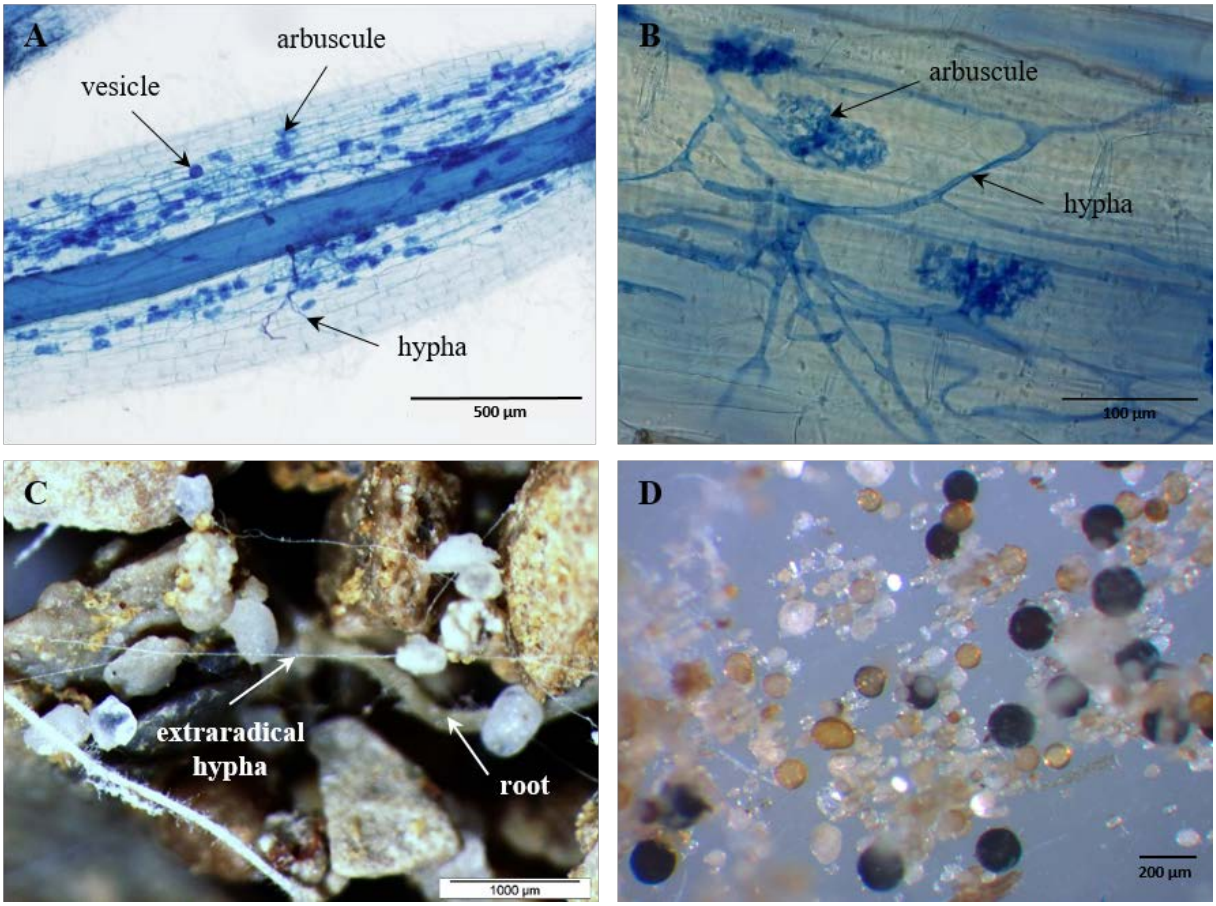

Figure 1. A) Stained flax root with characteristic AMF structures (Florian Walder), **B)** arbuscules form within cortex root cells and are considered as major site of exchange between the fungus and host (Django Hegglin), **C)** extraradical hyphae can form an extensive network outside the plant root (Florian Walder), **D)** spore community with different AMF species isolated from fields under no-tillage management.

Although AMF were said to be of low host specifity (Marschner and Timonen 2005), recent research has revealed a growing evidence of host specific variation in the plant response, as well as in the fungal response, to the host (Helgason et al. 2002, Scheublin et al. 2004, Smith et al. 2004, Tisserant et al. 2013). So far, approximately 270 AMF species have been described (Schüssler, 2015) with high inter- and even intraspecific variation in the genome (Jansa et al. 2002a, Munkvold et al. 2004), as well as morphology and consequently in their colonization strategies and nutrient acquisition efficiency (Jakobsen et al. 1992a, Ravnskov and Jakobsen 1995, Smith et al. 2000, Smith et al. 2004, Jansa et al. 2005). The functional diversity of AMF is reflected in the plant response. The AMF taxa can determine the plant P uptake (Jakobsen et al. 1992a, Taylor and Harrier 2000, Pellegrino et al. 2011), the biomass production (Owusu-Bennoah and Mosse 1979, Schenck and Smith 1982, Newsham et al. 1995, Smith et al. 2000, Taylor and Harrier 2000), the clonal reproduction (Streitwolf-Engel et al. 2001), or even the plant community composition (van der Heijden et al. 1998a) and the competitive relationship between plant species (Scheublin et al. 2007). As natural occurring plants are usually colonized by several different AMF, the AMF community structure will be an essential predictor for the plant response (Köhl et al. 2014) and plant species coexistence (van der Heijden et al. 1998b, Wagg et al. 2011b). The benefit plants can receive from certain AMF species and AMF

communities can be deliberately stimulated in agroecosystems by choosing the adequate management practice to manipulate the inherent AMF (Barber et al. 2013, Köhl et al. 2014, Figure 1D) or by adding an efficient AMF inoculum (Köhl et al. 2015).

Soil phosphorus and AMF

By forming an extensive network of extraradical hyphae (Figure 1C), AMF can enlarge the absorbing surface of the root system of their host plants beyond the nutrient depletion zone (Figure 2). Furthermore, the small hyphae (< 10µm) can reach nutrients in macro $ha^{-1}yr^{-1}$s that plant roots (mm scale) cannot. P uptake by AMF hyphae is much more efficient than by plant roots, as phosphate ions diffuse faster into the hyphae (Bolan 1991). As AMF and plants likely get P from the same soil sources (orthophosphate absorbed as H2PO4-, Bolan 1991, Yao et al. 2001), the extended soil volume that AMF can exploit, and the efficient P uptake and transfer, are the driving mechanism behind an enhanced P uptake. Up to 100% of the absorbed P can be of mycorrhizal origin, and the mycorrhizal pathway can contribute to P uptake even in non-mycorrhiza-responsive plants (Smith et al. 2003, Li et al. 2006).

Because the low amount of soluble (labile) P in the soil contrasts the high P uptake efficiency of AMF, it is increasingly assumed that AMF can mobilize P from the non-labile P fraction in soil. So far, evidence for mycorrhizal solubilization of insoluble inorganic P forms is lacking. Some studies have shown an increase in P uptake of mycorrhizal plants upon fertilization with rock phosphate or other non-labile P forms in contrast to non-mycorrhizal controls (Pairunan et al. 1980, Bolan et al. 1987, Shibata and Yano 2003). But others did not find any effects of adding non-labile P (Barea et al. 1980, Ngwene et al. 2010). So far, there is no definitive experimental evidence of direct P solubilization by AMF via secretion of chelating agents by hyphae (Allen et al. 1996, Antunes et al. 2007). Using root-organ cultures of carrots inoculated with or without *Glomus intraradices* and different rock phosphate sources, Antunes et al. (2007) did not detect any differences in the mycorrhizal treatment or localized changes in pH in proximity of *G. intraradices*. It is more likely that AMF increase the utilization of the chemically dissociated ions drawn into solution when P is depleted (Powell 1979, Javaid 2009). Furthermore, synergistic action between AMF and P solubilizing microorganisms can be another underlying mechanism (Barea et al. 2005, Figure 2). For mineralization of organic P, the production and secretion of phosphatases is required. It is still debated if AMF exudate phosphatases from their extraradical hyphae in sufficient amounts for P mineralization, as the origin of phosphatase enzymes in the hyphal zone of mycorrhizal crops is difficult to determine (Joner et al. 2000, Joner and Johansen 2000, Elbon and Whalen 2015). Other microorganisms in the rhizosphere, as well as plant roots themselves, also produce phosphatases. Nevertheless, several studies have indicated that AMF can obtain P from organic sources (Koide and Kabir 2000, Feng et al. 2003a).

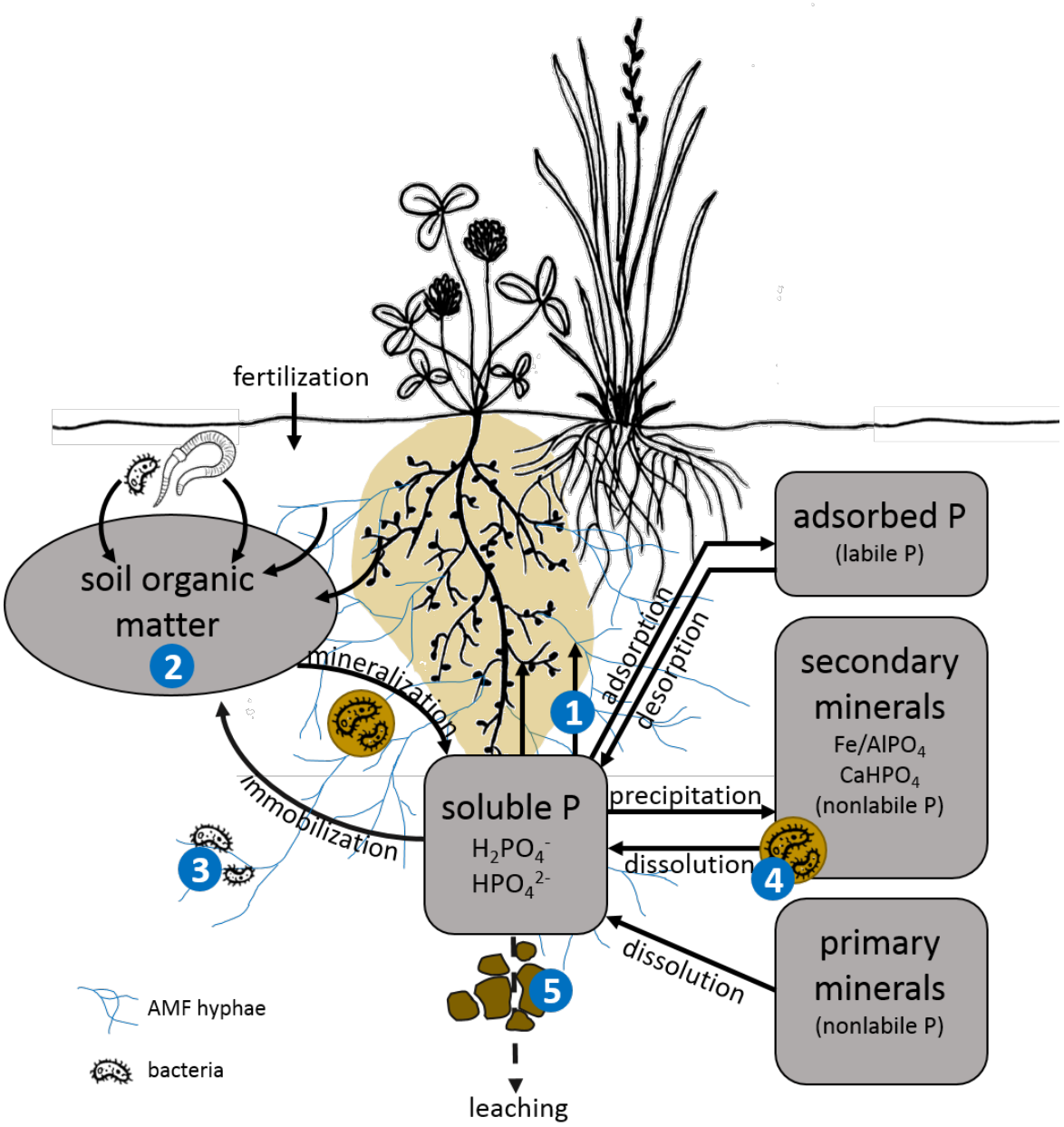

Figure 2. The P-cycle in soil with focus on mycorrhizal interactions. **1) P-uptake:** up to 100% of a plant's P uptake can occur via the mycorrhizal pathway (Smith et al. 2003, 2004), AMF hyphae can absorb P beyond the P depletion zone (shaded), **2) organic P:** AMF exudates increase labile soil organic matter, and AMF contribute up 50% to soil microbial biomass (Olsson et al. 1999), **3) fungal highway:** fungal mycelia can function as bridge between air-filled pores and enable bacteria to spread in soil (Wick et al. 2007, Nazir et al. 2010), **4) phosphate solubilization:** synergistic effects with phosphate solubilizing microorganisms (Kim et al. 1998, Souchie et al. 2010), **5) soil aggregation**: mycorrhizal fungi can influence soil aggregation and water relations (Augé 2004, Rillig and Mummey 2006, Leifheit et al. 2014).

Soil nitrogen and AMF

Positive effects of AMF on P uptake are well known. Unlike Pi, inorganic forms of N are mobile in soil, and thus the rhizosphere is less likely to be N depleted. Whereas a widespread network of extraradical hyphae beyond the P depletion zone is advantageous for plant P uptake, it does not necessarily extend the uptake of N resources in soil. The mycorrhizal contribution to the N nutrition of their host plants is still widely discussed. That AMF can transfer N to their hosts is widely accepted, although some authors propose that improved N nutrition is due to an increased P supply to the host (Reynolds et al. 2005). In contrast, positive effects of AMF on N nutrition independent of P supply have been reported (Azcon-Aguilar et al. 1993, Mensah et al. 2015). The impacts of AMF on plant N nutrition are variable and can be negative (George et al. 1995), neutral (Hawkins and George 1999) or positive (Saia et al. 2014, Hodge and Storer 2015, Mensah et al. 2015). Furthermore, AMF stimulate growth, nodulation, and symbiotic N-fixation of many legumes by increasing P uptake (Hayman) and thus can indirectly enhance plant N nutrition.

Although nitrate is generally the predominant form of plant and AMF available N (Tobar et al. 1994, Hawkins et al. 2000), the extraradical hyphae prefer to take up ammonium (Ames et al. 1983, Barea et al. 1987, Frey and Schüepp 1993), because it is energetically more efficient (Hawkins et al. 2000, Toussaint et al. 2004, Jin et al. 2005, Figure 3). Like plants roots, the extraradical hyphal mycelium can also take up simple organic nitrogen compounds like amino acids in addition to inorganic N (Whiteside et al. 2012). Although some studies have demonstrated that AMF can transfer N from organic patches to their host (Leigh et al. 2009, Hodge and Fitter 2010, Thirkell et al. 2015), so far no saprophytic capabilities in AMF have been shown (Hodge and Fitter 2010). Instead, the fungus acquires N from these organic patches as decomposition products (Hodge and Fitter 2010) and can indirectly increase N uptake from organic N sources by accelerating N mineralization. By shaping the mycorrhizosphere - the region around a mycorrhizal fungus- as a unique ecological niche with nutritionally favorable conditions for many microbes, it is likely that AMF play an important role in community assembly and activity during decomposition processes (Herman et al. 2012, Nuccio et al. 2013, Finzi et al. 2015, Figure 3).

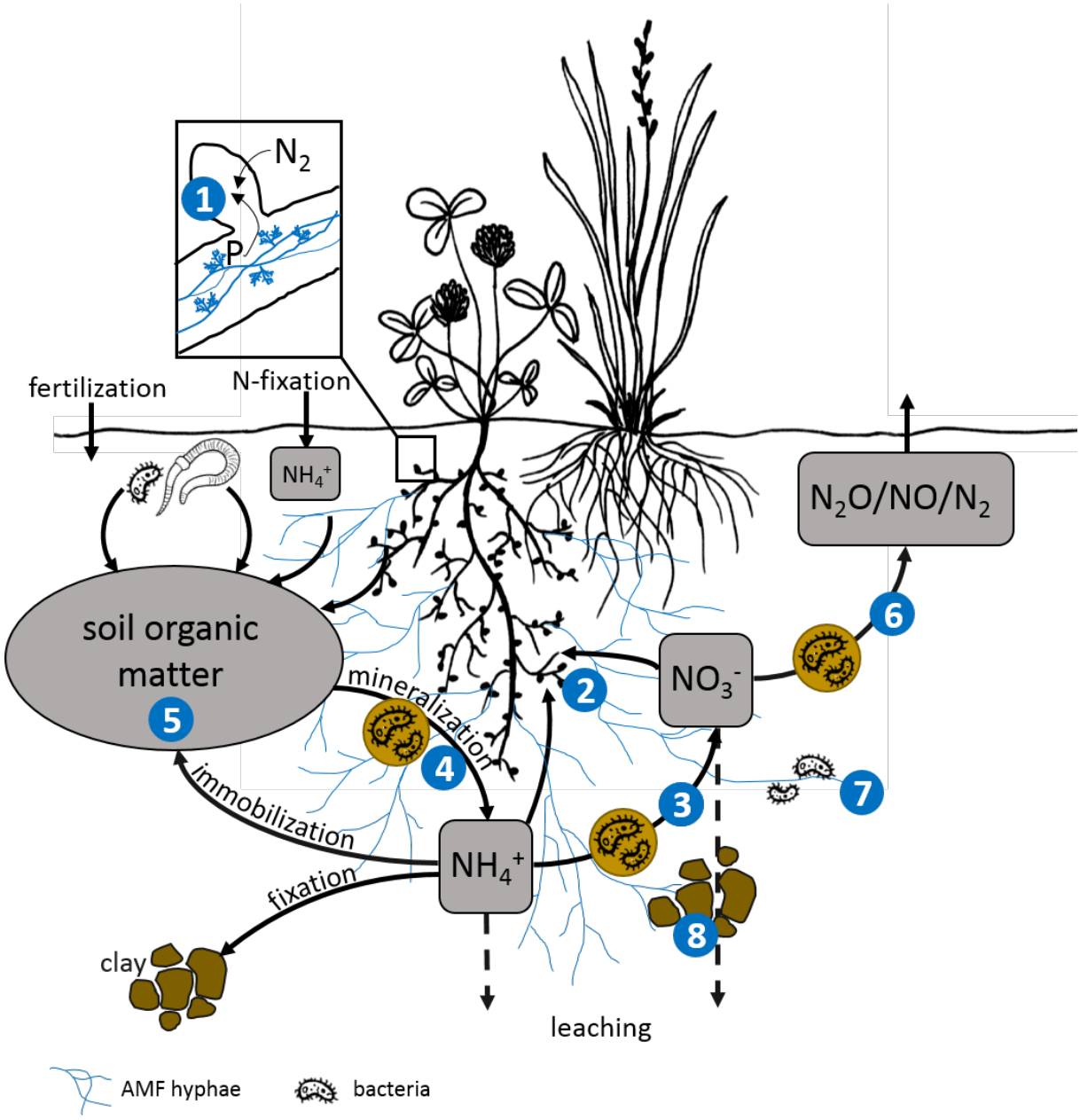

Figure 3. The N-cycle in soil with focus on mycorrhizal interactions. **1) symbiotic N-fixation:** synergistic effects with rhizobia on symbiotic N-fixation (Barea et al. 1992, Mortimer et al. 2008), **2) N-uptake:** NH_4^+ and NO_3^- (and partly amino acid) uptake and transfer to the host (Whiteside et al. 2012, Hodge and Storer 2015), **3) nitrification:** populations of autotrophic ammonium oxidizers are affected by AMF (Amora-Lazcano et al. 1998), **4) mineralization/ ammonification:** community composition of decomposers is affected by AMF (abundance of ammonifying bacteria is decreased, Amora-Lazcano et al. 1998), the transport of mineralized N to the plant is increased (Atul-Nayyar et al. 2009, Nuccio et al. 2013), **5) organic N:** AMF exudates increase labile soil organic matter, AMF contribute up to 50% of soil microbial biomass (Olsson et al. 1999), **6) denitrification:** AMF affect communities of denitrifying bacteria (Amora-Lazcano et al. 1998, Veresoglou et al. 2012, Bender et al. 2014), **7) fungal highway:** fungal mycelia can function as bridge between air-filled pores and enable bacteria to spread in soil (Wick et al. 2007, Nazir et al. 2010), **8) soil aggregation**: mycorrhizal fungi can influence soil aggregation and water relations (Augé 2004, Rillig and Mummey 2006, Leifheit et al. 2014).

AMF and nutrient leaching

Considering the immense importance of AMF for nutrient interception, immobilization, and transfer, AMF likely play a role in nutrient leaching. Indeed, recent research using artificial microcosms has shown that AMF presence can alter the amount of nutrients lost via leaching (Asghari et al. 2005, van der Heijden 2010, Asghari and Cavagnaro 2012, Köhl et al. 2014, Bender et al. 2015, Köhl and van der Heijden 2016). Mechanisms that underlie a mycorrhizal effect on nutrient leaching are not fully understood, but research indicates that efficient nutrient uptake and transfer to the plant contributes to nutrient immobilization in the soil (Cavagnaro et al. 2015). As AMF improve soil structure and soil water retention, AMF could also impact the volume of water draining through the soil and leaching valuable nutrients (Augé 2004). The impact of AMF on the composition and activity of microbial communities is known and increasingly being investigated (Amora-Lazcano et al. 1998, Marschner and Baumann 2003, Miransari 2011, Nuccio et al. 2013). Thus, indirect effects of AMF on nutrient retention and cycling via other microbes are likely but to date have not been well described. Besides direct and indirect effects of AMF on leaching losses, improving AMF management in the field can help to reduce fertilizer input, the main driver of nutrient leaching, and consequently decrease nutrient losses.

Previous experiments conducted in this area have focused on the question: “Are there any AMF driven effects on nutrient leaching?” and have relied on sterile experimental systems with similar experimental factors that favor high nutrient leaching losses (sandy soil, high fertilization rates). The context dependency of the results as well as a critical evaluation of the quantity of nutrients leached have seldom been discussed. In this thesis I investigate this question using artificial microcosms under controlled greenhouse conditions (see Figure 4), but manipulate important abiotic and biotic parameters like AMF species, host plant, sand content, and soil type. Furthermore, I go a step further and test if increased AMF abundance in unsterile field soil will also affect nutrient leaching.

Figure 4. Microcosms and rain simulator used for the experiments. During a rain event water was drip irrigated on the grassland plants, percolated through the microcosms with a permeable bottom into a funnel and was collected in a bottle.

Thesis outline

The research described in this thesis (schematic overview in Figure 5) was built upon the general question: "Can AMF reduce nutrient losses via leaching?" The aim of this work is to evaluate the mycorrhizal potential to reduce nutrient losses from the farmer's point of view. Thus, we applied two approaches to manipulate AMF in the field: inoculation of different AMF species and shaping inherent AMF communities by management practices.

In **chapter 2**, we address the functional diversity within AMF species as well as potential effects of the host plants on nutrient losses. Using three different AMF species, *Claroideoglomus claroideum*, *Rhizoglomus irregulare*, and *Funneliformis mosseae*, in sterile microcosms we observed differences in leaching effects depending on the isolate present. The two different host plants used, a non-responsive grass (*Lolium multiflorum*) and a N-fixing mycorrhizal legume (*Trifolium pratense*), show the partly extreme difference in leaching effects depending on the host. By manipulating the two main biotic components of this complex issue, we show that effects are highly host and fungus dependent.

To extrapolate the relevance of mycorrhizal leaching effects to the ecosystem, especially agroecosystem, level – which is our objective – we conducted an experiment with eight different unsterile soil types. **Chapter 3** addresses the question if one potent AMF isolate inoculated into field soil can establish within a given AMF community and can enhance plant performance (biomass production, nutrient uptake). Successful inoculum establishment is monitored with a species specific real-time PCR. Inoculation of eight different field soils emphasizes the influence of edaphic factors on AMF effectiveness.

In **chapter 4**, leaching results in unsterile field soil will be evaluated. We pose the question: "Can farmers reduce leaching losses by promoting a high AMF abundance in soil?" Additionally, we address the abiotic aspect of mycorrhizal leaching with an additional leaching experiment. A range of sterile substrates with varying sand content, including one unsterile soil, will be evaluated for their effects on the mycorrhizal impact on nutrient leaching.

Chapter 5 addresses the fact that AMF in the field can not only be efficiently managed by direct inoculation, but also by varying management practices like the tillage regime. We show that AMF communities isolated from fields under tillage and no-till management result in different effects on plant biomass, nutrient content, and nutrient leaching.

In the final discussion (**chapter 6**) I assess the ecological and agronomical significance of AMF for the reduction of leaching losses and provide recommendations for the direction of future research. As a conclusion, I aim to answer the question: "Can a farmer reduce nutrient leaching by managing AMF in the field?".

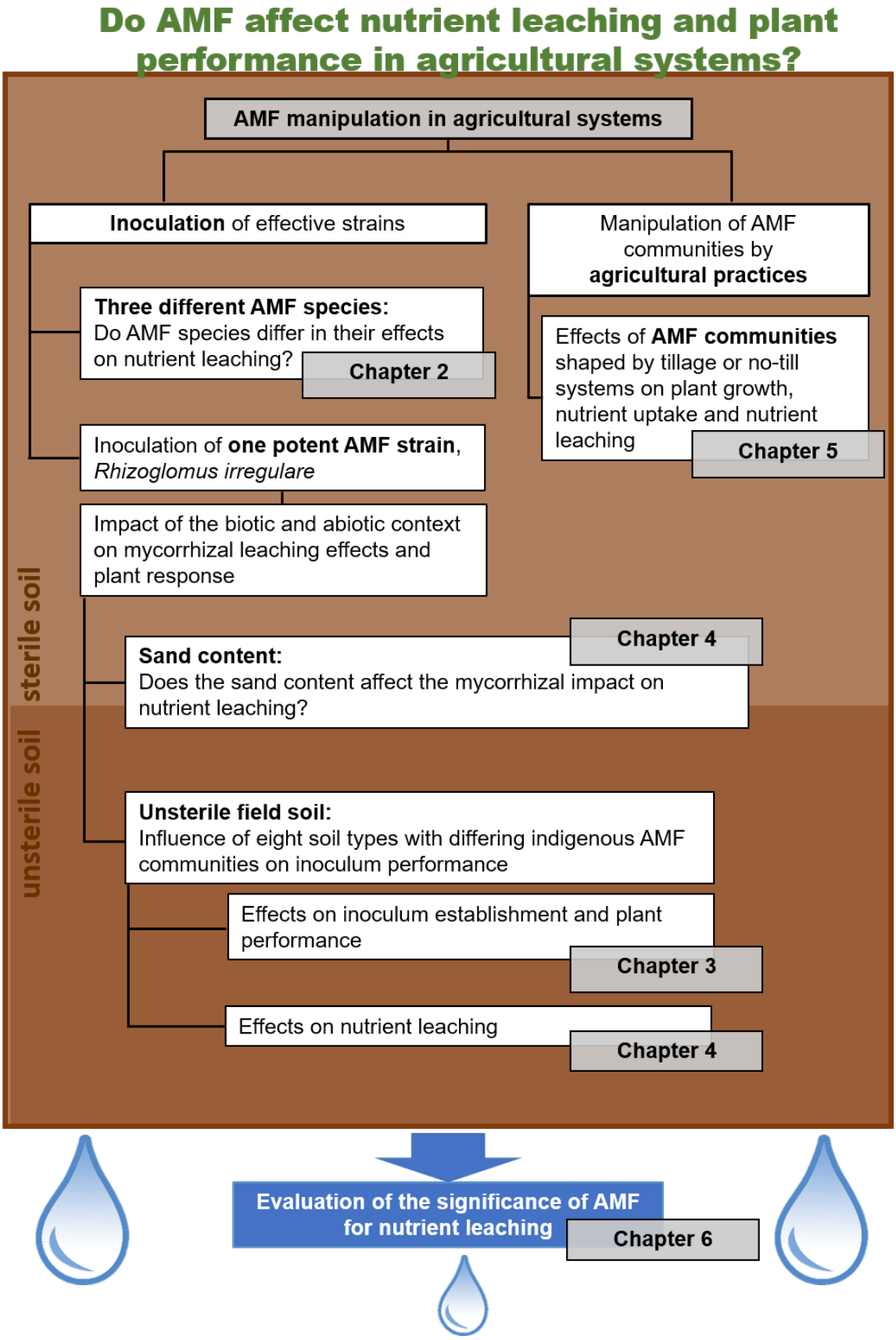

Figure 5. Schematic representation of the research described in this thesis.

Chapter 2

Arbuscular mycorrhizal fungal species differ in their effect on nutrient leaching

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Abstract

Arbuscular mycorrhizal (AM) fungi have been shown to play a crucial role in nutrient cycling and can reduce nutrient losses after rain induced leaching events. It is still unclear whether nutrient leaching losses vary depending on the AM fungal taxa that are present in soil. Using experimental microcosms with one of two different host plants (the grass *Lolium multiflorum*, or the legume *Trifolium pratense*) and inoculated with one of three different AM fungal species (*Claroideoglomus claroideum*, *Rhizoglomus irregulare*, and *Funneliformis mosseae*), we tested whether AM fungal species vary in their effects on nutrient leaching and plant productivity.

AM fungi reduced nitrogen leaching, and the effects varied depending on host plant species and the identity of the AM fungal species present in soil. The reduction of nitrogen leaching losses was strongest in microcosms planted with *Trifolium*. The effects of AM fungi on phosphorus leaching losses were relatively small, and in most cases not significant, although a significant negative correlation between root colonization and phosphate leaching was observed in microcosms planted with *Lolium*. AM fungi enhanced plant P uptake for both plant species, and different AM fungi varied in their effects on plant biomass and nutrient acquisition.

Our results demonstrate, for the first time, that AM fungal species differ in their effect on nutrient leaching. This indicates that agricultural practices that alter AM fungal communities also indirectly change nutrient cycling and nutrient leaching losses.

Highlights

- The impact of different arbuscular mycorrhizal (AM) fungal species on nutrient leaching was studied in grassland microcosms.
- AM fungi reduced nitrogen leaching compared to a non-mycorrhizal control.
- The mycorrhizal effect on the leached nutrients depended on the identity of the AM fungal species.
- Plant biomass and nutrient uptake varied with AM fungal species and fungus/host combination.

Keywords

Arbuscular mycorrhizal fungi, nutrient leaching, nutrient uptake, phosphorus, nitrogen, sustainability, nutrient use efficiency, legume, grass

Introduction

In many ecosystems substantial amounts of nutrients can be lost due to rain induced leaching events. Up to 160 kg of nitrogen (N) and 30 kg of phosphorus (P) per hectare can be leached annually (Sims et al. 1998, Herzog et al. 2008). Leaching losses pose environmental and economic problems because they contribute to the eutrophication of aquatic ecosystems (Carpenter et al. 1998). At the same time, nutrients lost from agro-ecosystems have to be replaced by the farmer with costly fertilizer, which also poses a problem due to the expected depletion of phosphorus deposits in the next 50-100 years (Cordell et al. 2009) and the high energy costs of N fertilizer production (Vance 2001). The amount of nutrients lost varies widely and depends on factors such as climate, land use, soil type and vegetation type (Jung 1972, Scholefield et al. 1993, Simmelsgaard 1998, Di and Cameron 2002b). Recently it has been observed that soil biota such as arbuscular mycorrhizal (AM) fungi can reduce nutrient leaching losses and enhance nutrient retention in soil (Asghari et al. 2005, van der Heijden 2010, Corkidi et al. 2011, Asghari and Cavagnaro 2012, Verbruggen et al. 2012, Bender et al. 2015).

AM fungi are a group of soil fungi that form symbiotic associations with the majority of land plants (Smith and Read 2008, van der Heijden et al. 2015). The fungus forms extensive hyphal networks in soil and forages efficiently for nutrients, primarily for P, but also for Zn, N and other nutrients that are delivered to their host plants in exchange for carbon (Smith and Read 2008, Lehmann et al. 2014, Watts-Williams and Cavagnaro 2014, Walder and van der Heijden 2015). AM fungi have recently been reported to reduce nutrient leaching losses from soil (Asghari et al. 2005, van der Heijden 2010, Asghari and Cavagnaro 2012, Bender et al. 2015), but the underlying mechanisms are not fully understood (Cavagnaro et al. 2015). Exploration of a larger soil volume by extensive hyphal networks and efficient nutrient uptake and immobilization in plant and fungal biomass is considered one of the key mechanisms for the reduction of P and N leaching through AM fungi (Jakobsen et al. 1992a, Cavagnaro et al. 2015). As AM fungi improve soil structure (Rillig and Mummey 2006) and soil water retention (Augé 2004). AM fungi could also impact the leachate volume. But evidence for this mechanism is weak, as not always an AM fungal mediated reduction in leaching volume was reported (Asghari and Cavagnaro 2012).

So far, only few studies investigated effects of AM fungi on nutrient leaching losses, and it is still unclear whether the reported effects are a general characteristic of the mycorrhizal symbiosis and are relevant under a wide range of conditions, or dependent on soil and ecosystem type, or host species. Moreover, while it is well established that different AM fungi have different effects on plant growth and nutrient uptake (Owusu-Bennoah and Mosse 1979, Schenck and Smith 1982, Jakobsen et al. 1992a), it is still unclear whether different AM fungi also vary in their ability to influence nutrient leaching losses from soil. We expect that those AM fungal taxa that acquire large amounts of nutrients for their host plants or fungal taxa that form extensive hyphal networks and store nutrients in their mycelium are better able to reduce nutrient leaching losses compared to AM fungi that have marginal effects on plant nutrient uptake. In the later situation, nutrients are not biologically bound, freely available in soil and, thus, more prone to be lost due to rain or irrigation induced leaching events.

In this study we tested whether 1.) AM fungi can indeed reduce nutrient leaching losses from experimental grassland microcosms planted with two different host plant species, and 2.) whether AM fungal species vary in their effects on nutrient leaching. We investigated these questions using microcosms planted with a grass, *Lolium multiflorum*, or a legume, *Trifolium pratense*. The microcosms were inoculated with one of three different AM fungal species

(*Rhizoglomus irregulare* (formerly known as *Rhizophagus irregularis / Glomus intraradices*), *Funneliformis mosseae* (formerly named *Glomus mosseae*) or *Claroideoglomus claroideum* (formerly known as *Glomus claroideum*)) or a non-mycorrhizal control inoculum. Effects on nutrient leaching were tested with a rain simulation after microcosms were fertilized.

Material and methods

Plant species, substrate and mycorrhizal inoculum

In this study we present two similar experiments using different host plants, one with *Lolium multiflorum* Lam. cv. ORYX, Italian ryegrass, (experiment 1) and one with *Trifolium pratense* L. cv. Formica, red clover (experiment 2). We focused on both species as they are widespread in natural grasslands and are often the dominant plant species in pastures in Switzerland (Nyfeler 2009, Suter et al. 2015). Moreover, both plant species represent different plant functional types (a grass and a nitrogen fixing legume) and respond differently to AM fungi. The grass, *Lolium*, is usually unresponsive to AM fungi (Wagg et al. 2011b, Köhl et al. 2014), whereas the legume *Trifolium* is highly mycotrophic (Köhl et al. 2014, Köhl et al. 2015). All seeds were surface sterilized with 5% sodium hypochlorite for 5 min, 70% ethanol for 10 min and rinsed thoroughly with dH_2O. Plants were germinated on 1.5% sterile water agar.

Soil for the substrate originated from a permanent grassland at Research Station Agroscope in Zurich, Switzerland (47° 25’ 38.71’’ N, 8° 31’ 3.91’’ E). The soil, a calcaric cambisol, was sieved through a 3 mm sieve, dried, and mixed with quartz sand at a ratio of 1:1 (v/v). The mixture was gamma-sterilized using a dose of 30 kGy and stored for two (experiment 1) or three months (experiment 2) at room temperature.

The sterilized substrate including the inoculum had a pH of 7.1 and contained 1.0% Humus, 8.7% clay, 6.3% silt and 84% sand. The substrate was phosphate poor with plant available P_2O_5 (extracted with CO_2-saturated water) of 0.36 mg/kg. Due to mineralization and nitrification processes during the storage and different inoculum substrates, mineral N content differed between the experiments. In experiment 1 the substrate initially contained 12.9 mg NH_4^+/kg and 0.7 mg NO_3^-/kg, in experiment 2 it contained 4.9 mg NH_4^+/kg and 19.3 mg NO_3^-/kg.

Experiment 1 (with *Lolium* as host plant) and experiment 2 (with *Trifolium* as host plant) consisted each of four treatments, plants were either inoculated with one of three AM fungi or received a non-mycorrhizal control treatment. Fungal species used were *Claroideoglomus claroideum* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüssler (formerly named *Glomus claroideum*), *Rhizoglomus irregulare* (Błaszk., Wubet, Renker & Buscot) Sieverd., G.A. Silva & Oehl (formerly known as *Rhizophagus irregularis*/ *Glomus intraradices* (Sieverding et al., 2014)), and *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüssler (formerly known as *Glomus mosseae*). We applied isolate HG 181/ SAF4 of *C. claroideum* in experiment 1 and isolate HG 281a/ SAF6 in experiment 2, isolate SAF22 of *R. irregulare* (van der Heijden et al., 2006) in experiment 1 and isolate BEG75/ SAF16 (Jansa et al., 2002) in experiment 2, and isolate HG 505/ SAF10 of *F. mosseae* in both experiments.

All isolates are deposited in the Swiss Collection of Arbuscular Mycorrhizal Fungi (www.agroscope.ch/saf) and were propagated in the greenhouse on *Zea mays* L. (experiment 1) or *Plantago lanceolata* L. (experiment 2) in an autoclaved substrate made of 15% grassland soil and 85% hydrated lime or sand respectively. After four (experiment 1) and eight months (experiment 2) of growth, pots were left to dry out and the aboveground biomass was discarded.

The roots were then cut into small pieces and mixed thoroughly with the rest of the substrate to serve as soil inoculum. Non-mycorrhizal controls were prepared analogously to the AM fungal inoculum. *R. irregulare* (=Ri), *F. mosseae* (Fm) and *C. claroideum* (Cc) colonized 95%, 62% and 16% of the root length of *Z. mays* and 81.5%, 33% and 21% of the root length of *P. lanceolata*. Both control inocula did not contain any AM fungal propagules.

Experimental setup and artificial rain

Experiment 1: Effects of different AM fungal species on the grass *Lolium multiflorum*

Lolium microcosms were established in PVC tubes with a diameter of 15.2 cm and a height of 40 cm (Figure S1 A). A total of 9.25 kg sterilized substrate including 11% (w/w) thoroughly intermixed inoculum was added to each microcosm to a height of 35 cm. The bottom of each microcosm consisted of a 500 µm PP mesh, which permitted excess water to leach through. For better drainage a 3 cm layer of autoclaved sand was added to the bottom of the tubes. In each microcosm 33 *Lolium* seedlings were planted equally spaced apart.

Each microcosm received 77 ml of a microbial wash to correct for differences in the non-mycorrhizal microbial communities between the inocula (Ames et al. 1987, Koide and Li 1989). For this, 90 g of each inoculum including the non-mycorrhizal control, and 90 g of fresh field soil were mixed with 4.2 L dH_2O and filtered through filter paper (N°598, Schleicher and Schuell, Dassel, Germany). All microcosms were arranged in a complete randomized block design with each of the four different treatments replicated ten times.

The plants were grown in a greenhouse with an average daily temperature of at least 24 °C, a night temperature of at least 18 °C and 16 hours of light per day. Supplemental light was provided by 400 W high-pressure sodium lights when natural irradiation was lower than 300W. Plants were kept in the greenhouse for 20 weeks between March and August 2010. *Lolium* plants were watered with deionized water 3 times a week to 80% field capacity. Blocks were rotated randomly in the greenhouse when pots were watered. The microcosms were fertilized 11 weeks after planting with 100 ml of a nutrient solution (6mM KNO_3, 4mM $Ca(NO_3)2*4H_2O$, 2mM $NH_4H_2PO_4$, 1mM $MgSO_4*6H_2O$ and micronutrients (50µM KCl, 25µM H_3BO_3, 2µM $MnSO_4*4H_2O$, 2µM $ZnSO_4*7H_2O$, 0.5µM $CuSO_4*5H_2O$, 0.5µM $(NH_4)6Mo_7O_{24}*4H_2O$, 20µM Fe(Na)EDTA)) and 17 weeks after planting with 100 ml of the same fertilizer reduced in P (same as before, but 0.5 mM $NH_4H_2PO_4$ and 0.75 $(NH_4)_2SO_4$ instead of 2mM $NH_4H_2PO_4$). This corresponded to a nutrient addition of 24.7 kg N/ha and 4.3 kg P/ha. Pest management was applied when necessary and according to Swiss regulations for organic farming (predatory mites *Amblyseius swirskii* against thrips and Cu/S against powdery mildew, ladybugs against aphids).

The ability of different AM fungal species to reduce nutrient leaching was investigated after 20 weeks of plant growth using a rain simulator (Knacker et al. 2004). For this purpose, microcosms were fertilized with 200 ml of fertilizer (6mM KNO_3, 4 mM $Ca(NO_3)2*4H_2O$, 1 mM $NH_4H_2PO_4$, 0.5 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4*6H_2O$ and micronutrients (50µM KCl, 25µM H_3BO_3, 2µM $MnSO_4*4H_2O$, 2µM $ZnSO_4*7H_2O$, 0.5µM $CuSO_4*5H_2O$, 0.5µM $(NH_4)_6Mo7O_{24}*4H_2O$, 20µM Fe(Na)EDTA)) corresponding to 24.7 kg N/ha and 3.4 kg P/ha. After 48 hours, the microcosms were watered to 100% field capacity and exposed to 2 L artificial rain applied with the rain simulator following the same procedure as in Köhl et al. (2014). The leachate draining off the microcosms was collected, weighed and analyzed. The pots were harvested five hours after the raining started.

Experiment 2: Effects of different AM fungal species on the legume *Trifolium pratense*

The second experiment, using *Trifolium pratense* as a host plant, was performed in 3 L pots (upper ∅ 16 cm, lower ∅ 12.5 cm, height 19.3 cm, Figure S1 B). Pots were modified to contain a polypropylene mesh (500 µm) instead of a solid bottom, and 3 cm layer of an autoclaved sand-gravel was added to improve drainage. The sterilized substrate was thoroughly intermixed with 8.7% inoculum (w/w), and the resulting 3.5 kg soil mixture was used to fill each pot. 55 ml of a microbial wash was added to each pot to equalize the non-mycorrhizal microbial community between treatments. To prepare this microbial wash, 40 g of each inoculum and 80 g of fresh grassland soil were suspended in 2.4 L of dH_2O and filtered through a filter paper (N°598, Schleicher and Schuell, Dassel, Germany) to exclude mycorrhizal propagules. In each microcosm 33 *Trifolium* seedlings were planted equally spaced apart.

All microcosms were arranged in a complete randomized block design in the greenhouse with each of the four different treatments replicated eight times. Greenhouse conditions, watering and pest management were regulated as described for the first experiment. *Trifolium* plants grew in the greenhouse for 21 weeks between May and September 2010. *Trifolium* received a lower amount of nutrients compared to *Lolium* because *Trifolium* fixes nitrogen and usually enhances N availability. In addition, it is recommended not to fertilize legume crops with nitrogen in Switzerland (Flisch et al. 2009). After 14 weeks of plant growth 10 ml of a fertilizer with low P was added (0.5 mM KH_2PO_4, 1 mM $MgSO_4$ and micronutrients (50µM KCl, 25µM H_3BO_3, 2µM $MnSO_4*4H_2O$, 2µM $ZnSO_4*7H_2O$, 0.5µM $CuSO_4*5H_2O$, 0.5µM $(NH_4)_6Mo_7O_{24}*4H_2O$, 20µM Fe(Na)EDTA).

Analogously to experiment 1, leaching from *Trifolium* pots was determined after 21 weeks of plant growth using a rain simulator. In contrast to experiment 1, 100 ml of fertilizer (2 mM Ca(NO3)2*4H2O, 2 mM NH4H2PO4, 1 mM MgSO4*6H2O, 3 mM K2SO4), corresponding to 4.8 kg N/ ha and 3.5 kg P/ha, were added to each pot 48 hours before raining. Each pot received a simulated rain of 925 ml (equal to 100% field capacity). The leachate was collected for three hours and subsequently weighed before pots were harvested.

Harvest and analyses

After 9 weeks for experiment 1 and 7 weeks for experiment 2 shoots were cut 5 cm aboveground to simulate hay making or grazing. Because of low plant growth in the *Trifolium* control treatment of experiment 2, the intermediate harvest was not done. After the simulated rain at the final harvest (20 and 21 weeks respectively for experiment 1 and 2), shoots were cut at the soil surface. Shoots were dried at 60°C for 48 hours and weighed. Microcosms were emptied and larger roots were collected, washed and weighed. In order to obtain the remaining fine roots, the soil substrate was homogenized and a weighed soil sample was taken and washed by repeatedly decanting the watered subsamples onto a 250 µm mesh. Weighed subsamples of both root samples were dried at 60°C for 48 hours and total root biomass per microcosm was calculated. Subsamples of both root samples were cut into pieces <1cm, mixed in water and stored in 50% ethanol for mycorrhizal root colonization analysis. In addition to this, soil samples were collected for nutrient and microbial biomass analysis (stored at 4°C) and mineral N analysis (stored at -20°C). Soil water content was determined gravimetrically to standardize the results for all microcosms.

Analyses

Microbial parameters

Mycorrhizal root colonization was determined using the ink-vinegar method described by Vierheilig et al. (1998). For this purpose, roots were cleared with 10% KOH and stained with 5% ink-vinegar. Percentage of root length colonized and frequency of hyphae, arbuscules and vesicles was quantified microscopically at a magnification of 200× with the intersect method (McGonigle et al. 1990) using 100 intersections. Soil microbial biomass was estimated by chloroform-fumigation-extraction (CFE) according to Vance et al. (1987). CFE was done in duplicates with 20 g (dry matter) fresh subsamples that were extracted with 80 ml of a 0.5M K_2SO_4. Organic C (TOC) was quantified using infrared spectrometry after combustion at 850°C (DIMATOC® 2000, Dimatec, Essen, Germany). Using the same sample, total microbial N was subsequently determined by chemoluminescence (TN_b, Dimatec, Essen, Germany). Soil microbial biomass C was then calculated according to Joergensen (1996) and microbial N according to Joergensen and Mueller (1996).

Plant nutrient analysis

Shoots were pooled across the two harvests for each species. Shoots and roots were ground for nutrient analysis. Total shoot nitrogen concentration was determined using a CHNSO analyzer (Euro EA, HEKAtech GmbH, Wegberg, Germany). For plant P determination, ground biomass was ashed at 600°C and digested using 6M HCl. Digests were diluted and P was quantified colorimetrically according to the molybdenum blue method (Watanabe and Olsen 1965).

Leachate analysis

The collected leachates were very clear and were not filtered before analysis. Leached phosphate and nitrate were quantified using a Dionex DX500 anion chromatograph (Dionex Corporation, Sunnyvale, CA) with an IonPac AG4A-SC guard column, an IonPac AS4A-SC analytical column (both 4mm) and 1.8mM Na2CO3/1.7mM NaHCO3 as eluent. Ammonium was determined spectrophotometrically using the Berthelot reaction method (Krom 1980). The absorption of the resulting coloured complexes was quantified with the continuous flow analyzer SAN++ (Skalar Analytical B.V., Breda, Netherlands). The total amount of dissolved P was determined colorimetrically according to the molybdenum blue ascorbic acid method (Watanabe and Olsen 1965) after oxidation with Oxisolv® (Merck, Darmstadt, Germany). The difference between total dissolved P and phosphate was defined as unreactive P. This fraction comprises all compounds not directly available to plants such as soluble and particulate organic P compounds, polyphosphates and particulate inorganic material like clays (Daniel and DeLaune 2009). As leached volumes differed between treatments, leached nutrients are presented as total amount leached. To calculate this, the volume of the leachate was multiplied with the particular nutrient concentration.

Soil analyses

All soil analyses were conducted by Agroscope, Institute for Sustainability Sciences, Zurich, Switzerland according to the Swiss reference methods for soil analyses (Forschungsanstalt Agroscope Reckenholz-Tänikon ART and Forschungsanstalt Agroscope Changins-Wädenswil ACW 1996). Plant available soil P was quantified colorimetrically analogously to the total P in the leachate after extraction with CO_2 saturated water (6 mMol

CO2 per 75 ml). Soil NO_3^- and NH_4^+ were determined colorimetrically after extraction with 0.01M CaCl2. No NO_3^- was detected at the end of the experiments (except for the *Trifolium* control). Total nitrogen was assessed by first reducing nitrate and organic N to NH_4^+, followed by quantifying the NH_4^+ by distillation and titration.

Statistical analysis

Statistical analyses were conducted using the software R version 3.0.1 (R Core Team 2013). Experiment 1 (*Lolium*) and experiment 2 (*Trifolium*) were analyzed separately, as the two experiments cannot be compared directly (e.g. the soil volume and fertilization varied between the two experiments and different fungal isolates were used in experiment 1 and 2). In order to assess whether the non-mycorrhizal control differed from the three treatments with fungal inoculation, a contrast was created separating the control from the mycorrhizal treatments. The contrast and the inoculum identity (4 levels) as well as the block as error term were used as factors in an ANOVA to analyze all response variables. A t-test or a Wilcoxon rank sum test (when errors were non-normal) was subsequently performed to specifically test whether the control treatment differed from the individual mycorrhizal treatments. The effect of the fungal identity was tested with an ANOVA analysis with block and inoculum identity as factors while excluding the control treatment from the data set. A Tukey HSD test was performed to specifically test which treatments differed from each other. Correlations between two variables were assessed using Pearson's correlation. In the text, all figures and tables presented show estimates of the means with their standard error (SEM). There was one missing value in root biomass as well as root N content (*F. mosseae*, experiment 1).

Results

Mycorrhizal colonization and microbial biomass

All mycorrhizal isolates successfully colonized *Lolium* and *Trifolium* roots and each of the isolates formed arbuscules and vesicles, structures specific for AM fungi. The non-mycorrhizal control treatments remained largely uncolonized (total root colonization <1%) in both experiments showing that we successfully eliminated AM fungi. Interestingly, mycorrhizal isolates differed in their colonization rate of *Trifolium* and *Lolium* roots (*Lolium*: $F_{2,23}$=1075.18, p<0.001, *Trifolium*: $F_{2,18}$=160.20, p<0.001, Figure 1). The highest colonization was observed in roots inoculated with Ri (= *Rhizoglomus irregulare*), ranging from 84 to 99%. Ri also produced significantly more vesicles and arbuscules than the other two fungi (Figure 1). Colonization performance of Fm (= *Funneliformis mosseae*) and Cc (=*Claroideoglomus claroideum*) was host plant dependent, as Fm colonized *Trifolium* roots to a greater extent than Cc (Fm 58-74%, Cc 31-53%), whereas in *Lolium* roots a greater colonization by Cc (30-39%) compared to Fm (5-25%) was observed (Figure 1).

The microbial biomass C and N did not change due to mycorrhizal inoculation in *Lolium* microcosms (C: $F_{1,32}$=0.54, p=0.47, Figure 1), but significantly increased by 99% (C) and 177% (N) respectively upon addition of AM fungi, compared to the non-mycorrhizal control in *Trifolium* pots (C: $F_{1,25}$=126.27, p<0.001, N: $F_{1,25}$=156.45, p<0.001). Microbial biomass C and N was significantly influenced by fungal identity (*Lolium* C: $F_{2,23}$=53.53, p<0.001, *Trifolium* C: $F_{2,18}$=19.33, p<0.001, Table S1).

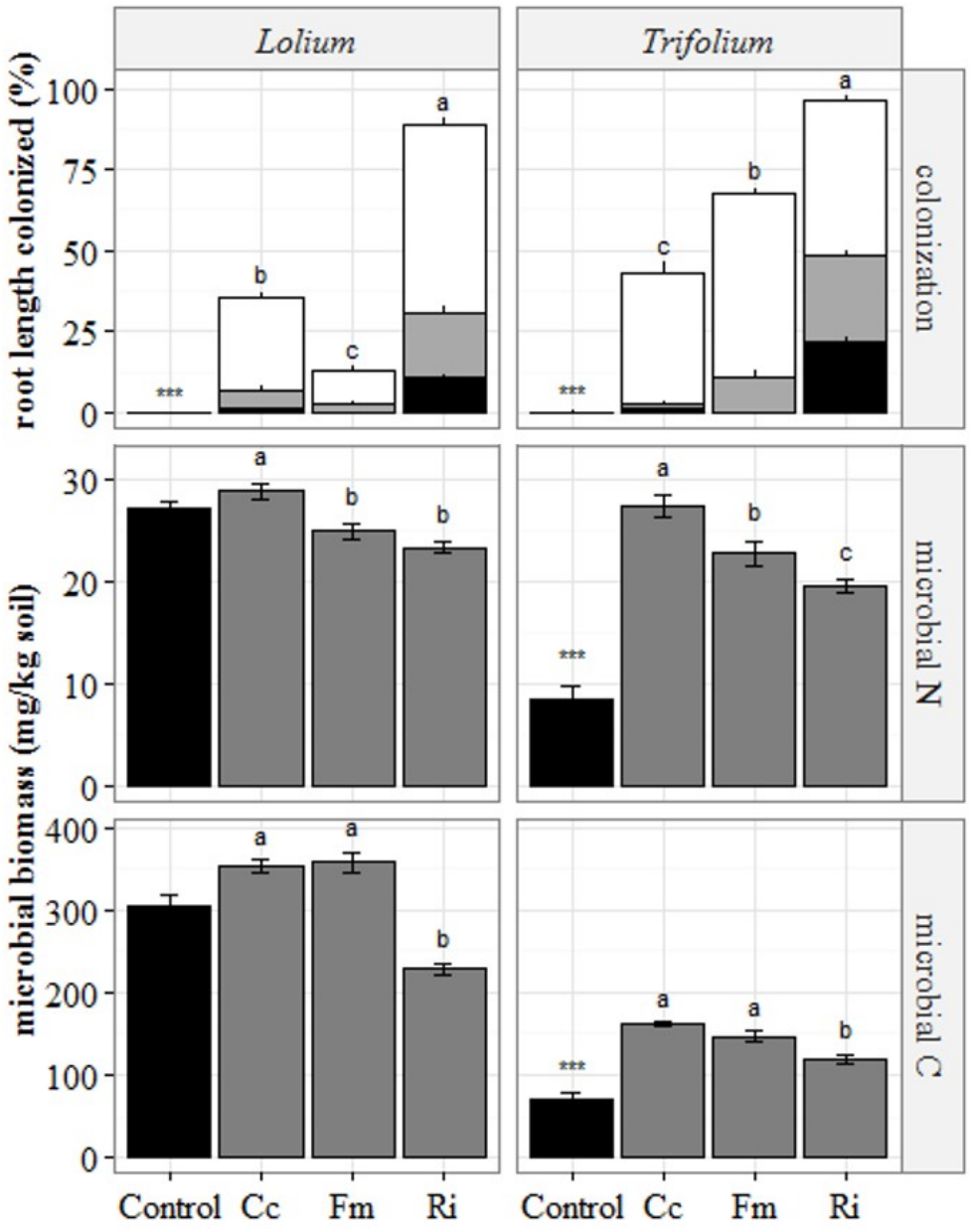

Figure 1. Percentage of total root length colonized (%) by AM fungi and nitrogen and carbon of the microbial biomass (mg per kg of dry soil) of microcosms planted with *Lolium* or *Trifolium* and inoculated with a non-mycorrhizal control inoculum or three different AM fungal species: Cc= *Claroideoglomus claroideum*, Fm=*Funneliformis mosseae*, Ri=*Rhizoglomus irregulare*. Total root length colonized by AM fungi (%) is presented as the sum of the percentages of root length colonized by vesicles (black), arbuscules (grey) and hyphae (white). Bars are means of eight (*Trifolium*) and ten (*Lolium)* replicates ± SEM. Asterisks represent significant differences between the non-mycorrhizal control and mycorrhizal plants ($p<0.001$***,< 0.01**,< 0.05*). Means of the mycorrhizal treatments with the same letter are not significantly different at the 0.05 level using Tukey HSD test.

Biomass production

The biomass of the highly mycotrophic *Trifolium* increased significantly by 1228.4% in response to AM colonization ($F_{1,25}$=3091.20, p<0.001, Figure 2). In contrast, the biomass of *Lolium* was not affected by AM fungal inoculation ($F_{1,32}$=2.84, p=0,10, Figure 2). Effects on biomass for both host plants were dependent on the AM fungal species present. Similar to the effect on root colonization, Ri increased *Trifolium* biomass more than the other two isolates, while *Lolium* growth was actually decreased relative to the non-mycorrhizal control by Ri inoculation. Interestingly, percentage of root length colonized by AM fungi correlated to an extent with the total biomass produced: The higher the colonization level of *Trifolium* roots the more biomass was gained (r=0.7, p<0.001) and vice versa for *Lolium* plants (r=-0.68, p<0.001).

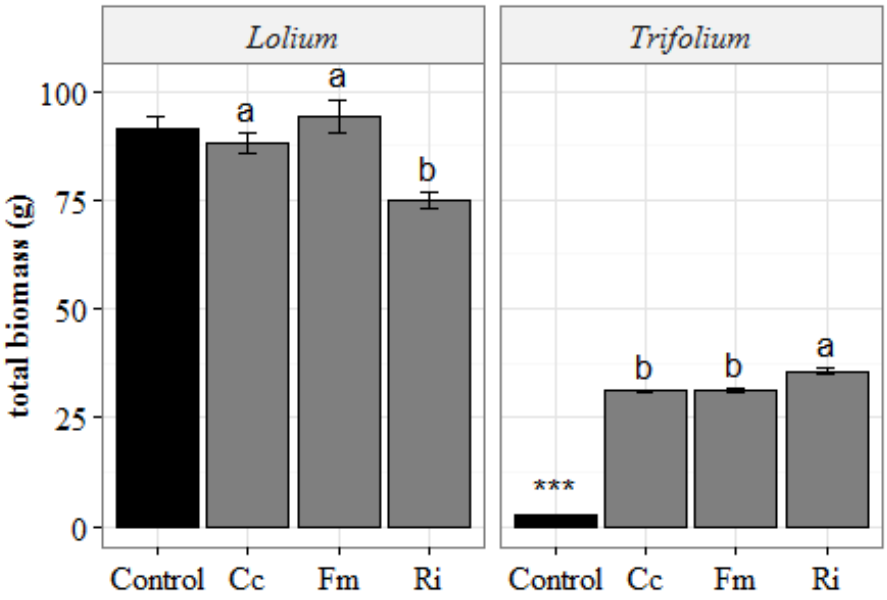

Figure 2. Total biomass (roots and shoots) (g) of *Lolium* and *Trifolium* inoculated with a non-mycorrhizal control inoculum or three different AM fungal species: Cc= *Claroideoglomus claroideum*, Fm=*Funneliformis mosseae*, Ri= *Rhizoglomus irregulare*. Means of eight (*Trifolium*) and ten (*Lolium*) replicates ± SEM are shown. Asterisks indicate that the control treatment is significantly different from the mycorrhizal treatments (p<0.001***,< 0.01**,< 0.05*). Mycorrhizal treatment means with the same letter are not significantly different at the 0.05 level using Tukey HSD test.

Nutrient uptake

Colonization by AM fungi significantly increased P and N content of *Trifolium* (P: $F_{1,25}$=3374.66, p<0.001, N $F_{1,25}$=1566.97, p<0.001, Figure 3). Moreover, the three different AM fungal isolates differed in their effects on *Trifolium* N and P content (P: $F_{2,18}$=42.65, p<0.001, N: $F_{2,18}$=36.24, p<0.001). P and N content of microcosms inoculated with Fm were lower compared to plants inoculated with Ri and Cc indicating that Fm was less effective in nutrient uptake than the other two isolates.

The P content of *Lolium* plants inoculated with each of the three AM fungal isolates was significantly higher than in the non-mycorrhizal control plants ($F_{1,32}$=40.44, p<0.001, Figure 3). Interestingly, even though *Lolium* plants grown in microcosms inoculated with Ri had the lowest biomass, they did not contain lower amounts of P compared to the other isolates. *Lolium* N shoot content was decreased by mycorrhizal inoculation ($F_{1,32}$=5.72, p=0.023) and the extent of the effect was dependent on the fungal species ($F_{2,23}$=5.38, p=0.012). *Lolium* root N content was affected neither by inoculation ($F_{1,32}$=0.01, p=0.93) nor by fungal identity ($F_{2,22}$ =2.67, p=0.09, Table S1).

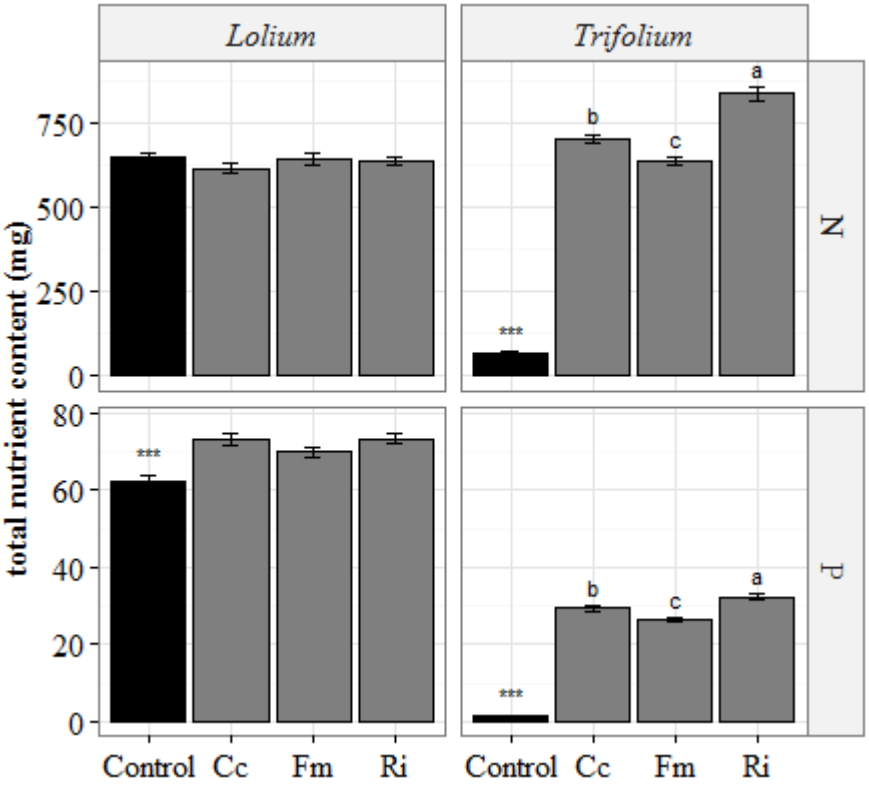

Figure 3. Total nutrient content (mg) of *Lolium* and *Trifolium* plants (roots and shoots) inoculated with a non-mycorrhizal control inoculum (Control) or three different AM fungal species: Cc= *Claroideoglomus claroideum*, Fm=*Funneliformis mosseae*, Ri= *Rhizoglomus irregulare*. Means of eight (*Trifolium*) and ten (*Lolium*) replicates ± SEM are shown. Asterisks indicate that the control treatment is significantly different from the mycorrhizal treatments (p<0.001***,< 0.01**,< 0.05*). Mycorrhizal treatment means with the same letter are not significantly different at the 0.05 level using Tukey HSD test.

Nutrient leaching

Phosphorus

Phosphorus leaching was not affected by inoculation with mycorrhizal fungi with no significant differences between inoculated and uninoculated plants both for *Lolium* (PO_4^{3+}: $F_{1,32}$=1.03, p=0.32 and unreactive P: $F_{1,32}$=0.29, p=0.59) and *Trifolium* (PO_4^{3-}: $F_{1,25}$=1.29, p=0.27, Figure 4). An exception was the leaching of unreactive P in *Trifolium* microcosms which was significantly increased in the mycorrhizal treatments compared to the non-mycorrhizal control ($F_{1,25}$=21.55, p<0.001, Figure 4). Comparing each fungal strain individually with the control, Cc reduced the unreactive P fraction in the leachate of *Lolium* microcosms by

13% (t_{18}=2.50, p=0.022, Table 1), whereas Fm increased PO_4^{3-}: leaching by 46% (t_{18}=-2.74, p=0.013). The identity of the fungus used for inoculation determined the amounts of nutrients leached (*Lolium* PO_4^{3-}: $F_{2,23=}$14.51, p= p<0.001 and unreactive P: $F_{2,23}$=8.01, p=0.002, *Trifolium* unreactive P: $F_{2,18}$=11.39, p<0.001, Table S1) except for PO_4^{3-} leached from *Trifolium* microcosms ($F_{2,18}$=0.16, p=0.86). Phosphate leaching from *Lolium* microcosms was positively correlated with total biomass production (r=0.5, p=0.005, Table S2) and negatively with mycorrhizal colonization level (r=-0.65, p<0.001, Figure S3). Increasing microbial carbon also enhanced phosphate leaching from *Lolium* pots as well (r=0.56, p=0.001). In contrast, *Trifolium* biomass production correlated negatively with the amount of unreactive P leached (r=-0.53, p=0.008, Table S2), as well as *Trifolium* P content (r=-0.64, p<0.001, Figure S4).

Nitrogen

Lolium and *Trifolium* microcosms differed in their effects on nitrogen leaching from microcosm due to the mycotrophic and N-fixing nature of *Trifolium* (Figure 4, Figure S2). Nitrogen leaching from *Trifolium* pots was highly affected by mycorrhizal inoculation. Ammonium losses were 3.3 times higher in the presence of AM fungi, whereas NO_3^- losses were 22 times lower in mycorrhizal treatments compared to the control. In *Trifolium* microcosms the fungal identity did not affect nitrogen leaching (NH_4^+: $F_{2,18=}$1.44, p=0.26, NO_3^-: $F_{2,18=}$1.92, p=0.18). Ammonium and nitrate leaching from microcosms planted with *Lolium* were, in contrast to *Trifolium*, not affected by mycorrhizal inoculation in general (NH_4^+: $F_{1,32}$=3.41, p=0.07, NO_3^-: $F_{1,32}$=0.51, p=0.48), but NH_4^+ and NO_3^- losses were influenced by fungal identity and reduced in microcosms with Cc (NH_4^+: t_{18}=2.75, p=0.013) and Ri (NH_4^+: W=22, p=0.04, NO_3^-: W=13, p=0.005, Table 1) compared to the non-mycorrhizal control. Fungal identity only affected ammonium leaching ($F_{2,23}$=13.68, p<0.001) with Cc and Ri having the highest reduction in ammonium losses.

Neither plant biomass, nor root colonization or plant nutrient uptake could explain differences in leaching effects between mycorrhizal species (Table S2, analyses without control treatment). Only the total root length colonized by AM fungi in inoculated *Trifolium* plants correlated positively with the total amount of NH_4^+ leached (r=0.41, p=0.045). The remaining mineral nitrogen in the soil at the end of the experiment reflected the amount of nitrogen that was leached (Table S3): The more nitrogen leached the more N was available in the soil at the end of the experiment. Exceptions were the NH_4^+ level in the *Trifolium* control treatment as well as NO_3^- in the soil of Gc and Ri inoculated *Lolium* microcosms. Here, the amount of N in the soil was comparable to the other treatments and much higher than the amount of N leached (Table S3).

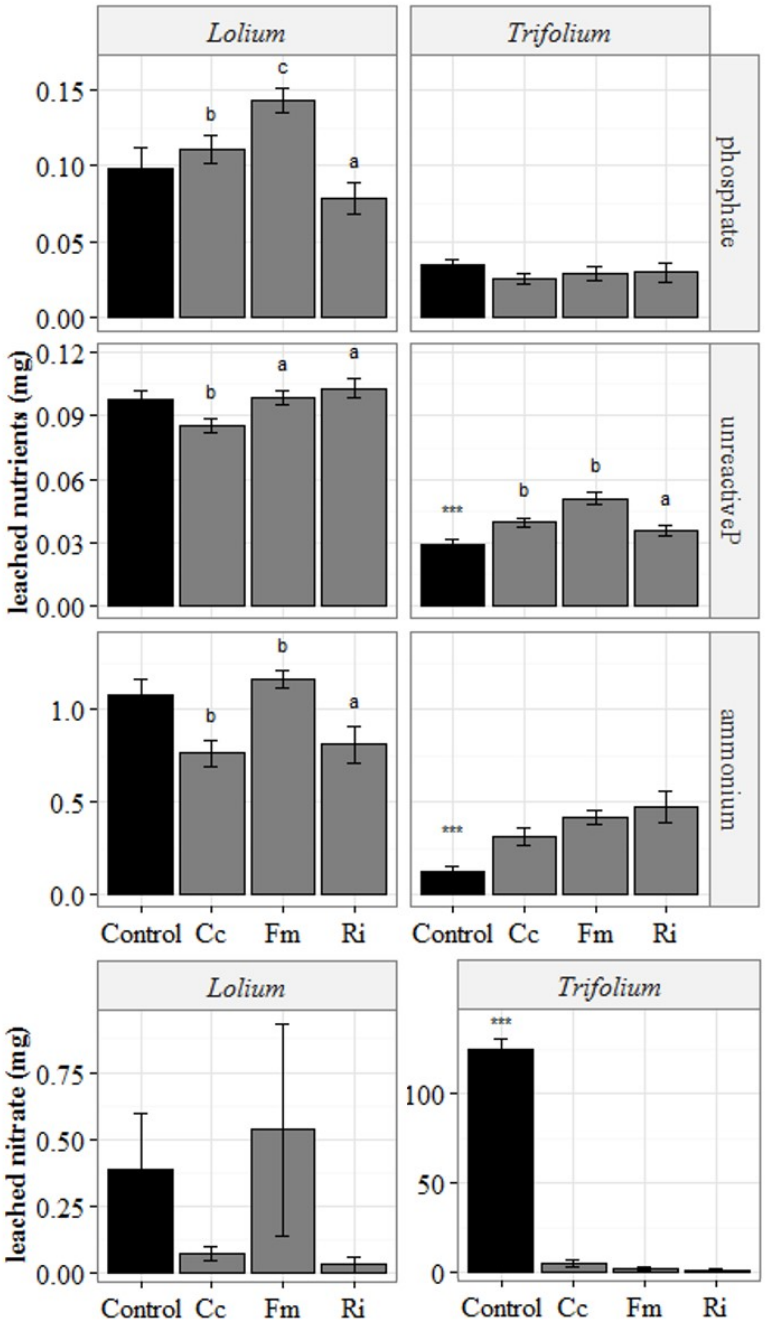

Figure 4. Nutrients leached from pots planted with *Lolium* or *Trifolium* after a leaching inducing rain simulation. All P fractions besides phosphate in the leachate are summarized as "unreactive P". Pots were inoculated with a non-mycorrhizal control inoculum or three different AMF species: Cc= *Claroideoglomus claroideum*, Fm=*Funneliformis mosseae*, Ri=*Rhizoglomus irregulare*. Means of eight (*Trifolium*) and ten (*Lolium*) replicates ± SEM are shown. Asterisks indicate that the control treatment is significantly different from the mycorrhizal treatments ($p<0.001$***,< 0.01**,< 0.05*). Mycorrhizal treatment means with the same letter are not significantly different at the 0.05 level using Tukey HSD test.

Table 1. Results of t-tests or Wilcoxon rank sum test ([a]) (if errors were not normal distributed) comparing leaching results of the non-mycorrhizal control with results of mycorrhizal microcosms for each AM fungal species separately. *Lolium* (df=18) and *Trifolium* (df=14) microcosms were analyzed separately. Values in bold are significantly different ($P < 0.05$).

	Lolium						***Trifolium***					
Response	**Cc**		**Fm**		**Ri**		**Cc**		**Fm**		**Ri**	
	t	**p**	**t**	**p**	**t/W[a]**	**p**	**t**	**p**	**t**	**p**	**t**	**p**
total mineral N	**3.23**	**0.005**	-0.51	0.614	**2.97**	**0.008**	**19.47**	**0.000**	**20.76**	**0.000**	**20.87**	**0.000**
NH_4^+ [a]	**2.75**	**0.013**	-0.84	0.410	**22**	**0.035**	**-3.48**	**0.004**	**-6.07**	**0.000**	**-3.87**	**0.002**
NO^{3-} [a]	1.47	0.160	-0.33	0.745	**13**	**0.005**	**19.54**	**0.000**	**20.86**	**0.000**	**21.01**	**0.000**
total dissolved P	-0.03	0.978	**-2.67**	**0.015**	0.69	0.499	-0.47	0.643	-2.03	0.062	-0.29	0.774
PO_4^{3-}	-0.75	0.462	**-2.74**	**0.013**	1.07	0.299	1.69	0.114	0.96	0.352	0.60	0.556
unreactive P	**2.50**	**0.022**	-0.21	0.837	-0.85	0.405	**-3.11**	**0.008**	**-5.60**	**0.000**	-1.76	0.101
leachate volume	**-2.65**	**0.016**	-0.67	0.513	**-3.23**	**0.005**	1.27	0.225	1.11	0.284	1.55	0.145

Discussion

The positive effects of AM fungi on plant growth and nutrition are well known. However, the effects of AM fungi on other ecosystem functions, such as effects on nutrient retention in soils are less well explored (for review see Cavagnaro et al. 2015). This study, together with other recent studies (Asghari and Cavagnaro 2012, Bender et al. 2015, Bender and van der Heijden 2015) demonstrates that AM fungi can reduce N losses from soil, sometimes resulting in a substantial reduction of nitrogen leaching. Other studies showed that the effects of AM fungi on nutrient leaching depend on host plant species (van der Heijden 2010, Corkidi et al. 2011) and soil type (Bender et al. 2015). This study, using two different host plants and three different AM fungal species, partly confirms these results and puts the leaching effects in a more context dependent perspective. It shows, for the first time, that nutrient leaching is also influenced by the identity of the AM fungal species colonizing the roots and on the host plant/ AM fungal species combination.

AM fungi affect nutrient leaching

In *Trifolium* microcosms, a reduction in total leached nitrogen of 60.53 kg/ha was achieved by AM fungal inoculation compared to the non-mycorrhizal control. In *Lolium* microcosms the reduction was very low with 0.18 kg N/ha. We assume that differences in the growth response of *Lolium* and *Trifolium* explained effects of AM fungi on nitrogen leaching losses. *Trifolium* was highly dependent on the presence of AM fungi, and nitrogen uptake by AM fungi and plant roots and its subsequent immobilization in fungal and plant biomass is probably the main mechanism for a reduction of nitrogen leaching losses by AM fungi in association with *Trifolium*. In contrast, AM fungi had a minor effect on *Lolium* biomass and did not influence the plant N content of *Lolium*, probably explaining why effects on nitrogen leaching losses were relatively small for this plant species. Similarly, Asghari and Cavagnaro

(2012) showed greater biomass production and 40 times less N lost to leaching in mycorrhizal tomato plants compared to non-mycorrhizal mutants. In contrast, van der Heijden (2010) could not detect any effect of *G. irregulare* on nitrate leaching in a grassland similar to the system we used, with grass species having the same biomass with and without AM fungi.

We did not find any overall differences in P leaching between the mycorrhizal treatments and the non-mycorrhizal control in *Lolium* and *Trifolium* microcosms (only unreactive P was increased in clover pots with AM fungi present; Figure 4). The absence of an effect on total P leaching is surprising, as significantly more P was transferred to the plant biomass in mycorrhizal treatments, even in *Lolium* plants. Furthermore, the microbial biomass C in *Trifolium* microcosms was higher in the mycorrhizal treatments indicating a higher microbial P storage as well. The removal of P into *Trifolium subterraneum* and fungal biomass was shown by Asghari et al. (2005) to be one reason for a reduced P leaching in AM fungi presence. The substrate used in this study was very sandy (84 %) and thus should favor higher P leaching losses (Weaver et al. 1988, Atalay 2001). We assume that the soil substrate used in this study, a calcaric cambisol, has a strong P-fixing ability, and thus very small amounts of P were found in the leachate. Bender et al. (2015) used a similar substrate based on the same pasture soil and observed only minor P leaching losses compared to a heath soil, confirming our results. Phosphate is usually immobile and strongly fixed to soil particles or immobilized when complexes with iron, aluminum or calcium are being formed, and as a consequence phosphorus leaching losses are usually low. In contrast nitrate is much more mobile in soil and, therefore, prone to leaching (Havlin et al. 2005).

General conclusions about the effects of AM fungi on nutrient leaching losses should be carefully formulated. A close examination of the reported benefits of AM fungi by a number of studies suggests that these could be largely dependent on biotic and abiotic factors of the experiment. Differences in host plant identity, soil type, fertilization treatment, inoculum identity and soil nitrogen and phosphorus pools and availability could explain why results vary so strongly across studies. While this does not challenge the validity of previous findings, future studies need to focus on examining the precise mechanisms that influence leaching effects of AM fungi. Moreover, AM fungi also influence two other sources of N loss, namely leaching of dissolved organic nitrogen and the loss of N_2 and N_20 through denitrification (Bender et al. 2014, Bender et al. 2015). In most studies, including this one, these factors were not investigated.

AM fungal species dependent effect on ecosystem services

Earlier work showed that different AM fungal taxa differentially influenced plant biomass and nutrient uptake (Ravnskov and Jakobsen 1995, Taylor and Harrier 2000, Hart and Reader 2002, Jansa et al. 2005). This study confirms that different AM fungal taxa vary in their effects on plant biomass production and P content. The results show that these effects were, at least in part, explained by species specific differences in root colonization. The AM fungus with the highest levels of root colonization (Ri) had the strongest effects on plant biomass (resulting in the greatest growth stimulation (+1170%) for the mycotrophic plant species (*Trifolium*) and the greatest growth suppression (-18%) for the grass species (*Lolium*)).

While earlier work focused on the effects of different AM fungi on plant biomass and nutrient uptake, it was still unclear whether different AM fungi could also influence nutrient leaching losses. Here, we demonstrate, for the first time, that different AM fungi can vary in their effect on nutrient leaching. It confirms a correlative study by Verbruggen et al. (2012) who demonstrated that the abundance of specific AM fungal taxa, as determined by terminal-

RFLP, correlated well with plant productivity and PO_4^{3-} leaching from microcosms. The present study, together with the one by Verbruggen et al. (2012), thus indicates that the composition of the AM fungal community can influence nutrient leaching losses from soil.

The precise mechanisms by which AM fungi reduce nutrient leaching are unclear (Cavagnaro et al. 2015). Effects of AM fungi on plant nutrient uptake could, in part, be related to their effects on nutrient leaching losses. Ri developed the highest root colonization level among the three AM fungal species and plants inoculated with Ri took up the largest amount of P. At the same time, Ri microcosms planted with *Lolium* leached the least amount of P (negative correlation between root length colonized and phosphate leaching, Figure S3). However, such an effect was not found for *Trifolium* indicating that other factors must be involved as well.

It has been observed that AM fungi alter root and hyphae associated bacterial communities involved in N (Amora-Lazcano et al. 1998, Veresoglou et al. 2012, Bender et al. 2014) and P cycling (Kim et al. 1998, Villegas and Fortin 2001, 2002). Such changes in microbial communities may influence nutrient leaching losses. Moreover, AM fungi exude nutrient binding glycoproteins (Rillig and Mummey 2006), and these may also play an additional role in explaining differences in nutrient losses from soil cores. Mycorrhizal impact on soil structure and soil water retention can provide further explanation for altered nutrient losses in presence of AM fungi (Augé, 2004; Rillig and Mummey, 2006), although an AM-mediated reduction in nutrient leaching was not always shown (Asghari et al., 2012; Figure S6).

Effects on host plants

Two different host plants were chosen for their agronomic importance and their different responses to AM fungi. *L. multiflorum*, like many grasses, is colonized by AM fungi, but its biomass does not respond strongly to AM fungi (Wagg et al. 2011a, Bender et al. 2014, Köhl et al. 2014). By using an unresponsive grass, we intended to uncover the proportion of the mycorrhizal effect on nutrient leaching that is not related to increased nutrient storage in the plant biomass.

In contrast, the legume *T. pratense* is highly mycotrophic, and it usually benefits greatly in terms of biomass production and plant nutrient content from mycorrhizal infection (Wagg et al. 2011a, Köhl et al. 2014, Köhl et al. 2015). As a consequence, the soil nutrient concentrations in pots with *Trifolium* also differed between mycorrhizal and non-mycorrhizal treatments at the end of the experiment, and it is therefore much more difficult to separate effects of AM fungi on plant growth from those on nutrient leaching.

The most evident difference in nutrient leaching between *Lolium* and *Trifolium* microcosms was the amount of nitrate leached. With both hosts, nitrate leaching was reduced by AM fungal inoculation compared to the non-mycorrhizal control (Figure 4). But comparing the two plant systems, NO_3^- amounts leached per ha were 292 times higher in the *Trifolium* control compared to the *Lolium* control (0.21 kg/ha vs. 62.31 kg N/ha) and 14 times higher when AM fungi were present (0.12 kg/ha vs. 1.65 kg/ha). This observation is consistent with other studies reporting that clover abundance is positively correlated with N leaching (Loiseau et al. 2001, Scherer-Lorenzen et al. 2003, Bouman et al. 2010). Grass systems usually have a high N efficiency and thus lower nitrogen losses via leaching (Simmelsgaard 1998). Scherer-Lorenzen et al. (2003) detected only very low rates of N leaching in pure grass monocultures and mixtures (<1kg NO_3-N $ha^{-1}*yr^{-1}$), whereas low diversity grasslands containing *Trifolium* had equally high N losses as bare ground plots (100 kg NO_3-N $ha^{-1}*yr^{-1}$). The higher N leaching from *Trifolium* microcosms, despite the lower N fertilization (*Lolium* 29.4 kg N/ ha, *Trifolium*

4.8 kg /ha) can be attributed to low *Trifolium* biomass in microcosms without AM fungi (see above) and the symbiotic N-fixing activity of the legume. The nitrogen fixation can range from 50-250 kg N $ha^{-1}*yr^{-1}$ (Ledgard and Giller 1995), which would exceed the amount of N fertilized in grass microcosms.

The high N availability in *Trifolium* microcosms was also shown by the high plant N:P ratio (>16), which indicates that the plants were P limited, especially in the control treatment (Koerselman and Meuleman 1996) (Figure S5). In contrast, *Lolium* growth was N limited in all treatments (N:P ratio < 14). As all microcosms received an AM fungi free filtrate of fresh grassland soil, we assume that N-fixing, decomposing, denitrifying and nitrifying microbes were equally present in all treatments, although AM fungi will have a certain impact on the microbial background (Marschner and Baumann 2003). Furthermore, the experimental soil at the start of the experiment contained more nitrate in the *Trifolium* experiment than in the *Lolium* experiment. This difference disappeared by the end of the greenhouse trials.

Conclusion

Here, we demonstrate that AM fungi not only influence plant growth and nutrient uptake but also ecosystem services such as nutrient retention. We demonstrate, for the first time, that AM fungal species differ in their effect on nutrient leaching. In view of the urgent need for a more sustainable, low-input agriculture, these properties of AM fungi might be utilized to reduce fertilizer input and environmental pollution through fertilizer runoff. As different AM fungal species differ in the quantity and quality of ecosystem services they provide (Ravnskov and Jakobsen 1995, Smith et al. 2000), it has to be considered that the AM fungal community structure in an ecosystem will be of importance for its functioning. The AM fungal community can be intentionally manipulated by different agricultural management systems like fertilization, tillage practices and crop rotation (Douds and Millner 1999, Köhl et al. 2014, Säle et al. 2015). Field inoculation can systematically introduce powerful strains (like the *Rhizoglomus irregulare* in this study) (Köhl et al. 2015) to reduce nutrient losses from the field while decreasing the fertilizer input. Here, we have shown that the outcome of the mycorrhizal symbiosis is host plant dependent. Furthermore, nutrient leaching is highly dependent on soil type (Bender et al. 2015). Thus, more studies, especially under field conditions with various host plants, have to be conducted to reveal the practical relevance of AM fungi and their community structure for the prevention of nutrient losses. In our study, we have shown that the mechanisms underlying the mycorrhizal effects on nutrient leaching are diverse and not fully explained. As nutrient availability in the soil strongly depends on microbial activity, more emphasis should be placed on untangling the interdependent relationship between mycorrhiza and soil microbes and on how AM fungi shape the soil microbial community.

Acknowledgements

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Supporting information

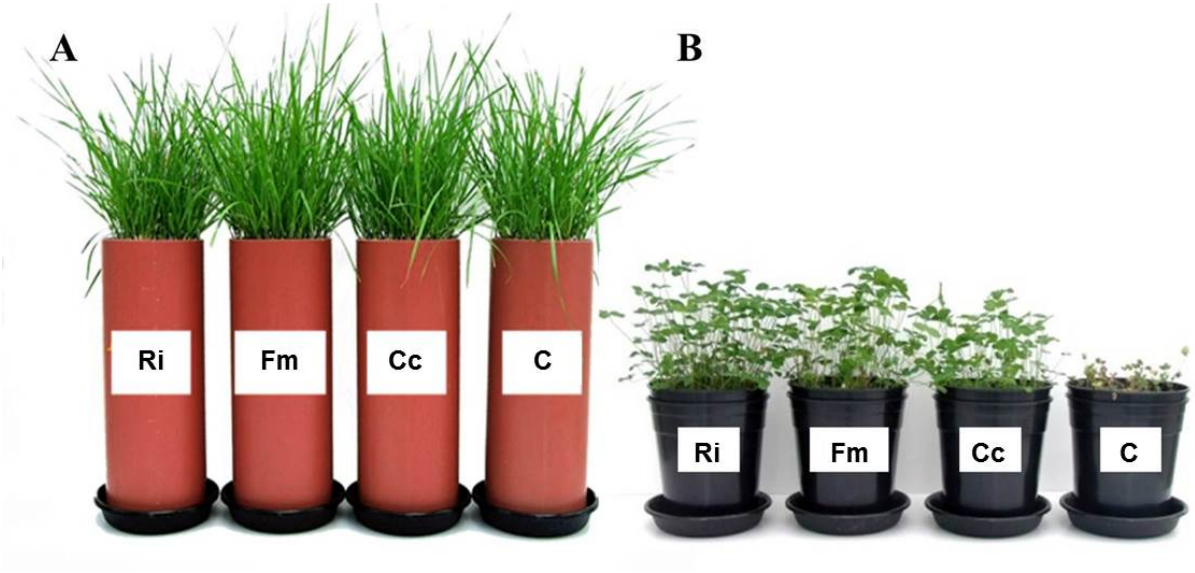

Figure S1. Microcosms with **A)** *Lolium multiflorum* twelve weeks after planting and with **B)** *Trifolium pratense* seven weeks after planting. Microcosms were inoculated with Ri= *Rhizoglomus irregulare*, Fm= *Funneliformis mosseae*, Cc= *Claroideoglomus claroideum* or with a non-mycorrhizal control inoculum (C).

Table S1. Statistics for the assessment of microbial, plant and leaching parameters. All parameters were analyzed with ANOVA with block included as an error term. The effect of the mycorrhizal inoculation was assessed by contrasting the non-mycorrhizal control against the remaining treatments (control vs. AM fungal inoculum). The effect of the different AM fungal species was assessed by excluding the control from the analysis ($p<0.001$***,<0.01**,< 0.05*).

	Lolium						***Trifolium***					
Response	**control vs. AM fungal inoculum**			**AM fungi identity**			**control vs. AM fungal inoculum**			**AM fungi identity**		
	df	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**	**df**	**F**	**p**
Microbial response												
total root colonization	1, 32	1447.74	***	2, 23	1075.18	***	1, 25	1046.87	***	2, 18	160.2	***
microbial biomass C	1, 32	0.54		2, 23	53.53	***	1, 25	126.27	***	2, 18	19.33	***
microbial biomass N	1, 32	3.06		2, 23	15.17	***	1, 25	156.45	***	2, 18	19.49	***
Plant response												
shoot biomass	1, 32	16.44	**	2, 23	28.39	***	1, 25	7922.88	***	2, 18	11.41	***
root biomass	1, 32	0.98		2, 23	7.00	**	1, 25	448.98	***	2, 18	16.52	***
total biomass	1, 32	2.84		2, 23	12.10	***	1, 25	3091.20	***	2, 18	22.93	***
total N content	1, 31	1.01		2, 22	1.39		1, 25	1566.97	***	2, 18	36.24	***
total P content	1, 32	40.44	***	2, 23	2.06		1, 25	3374.66	***	2, 18	42.65	***
Leaching												
total mineral N	1, 32	1.92		2, 23	4.57	*	1, 25	1088.27	***	2, 18	1.68	
NH_4^+	1, 32	3.41		2, 23	13.68	***	1, 25	18.16	***	2, 18	1.44	
NO_3^-	1, 32	0.51		2, 23	1.57		1, 25	1103.76	***	2, 18	1.92	
total dissolved P	1, 32	0.58		2, 23	10.24	***	1, 25	1.65		2, 18	2.89	
PO_4^{3-}	1, 32	1.03		2, 23	14.51	**	1, 25	1.29		2, 18	0.16	
unreactive P	1, 32	0.29		2, 23	8.01	**	1, 25	21.55	***	2, 18	11.39	***
leachate volume	1, 32	8.11	**	2, 23	5.74	**	1, 25	4.68	*	2, 18	1.37	

Table S2. Correlation coefficients (r) for Pearson's correlation between plant and microbial parameters, respectively and total amount of nutrients leached from microcosms. Analyses were done with and without the non-mycorrhizal control treatment and were performed for *Lolium* and *Trifolium*. Significant correlations are depicted in bold.

Control included	***Lolium***				***Trifolium***			
	PO_4^{3-}-P	**unreactive P**	**NH_4^+-N**	**NO_3^--N**	**PO_4^{3-}-P**	**unreactive P**	**NH_4^+-N**	**NO_3^--N**
root colonization	**-0.36**	0.12	**-0.41**	-0.26	-0.13	**0.40**	**0.69**	**-0.84**
microbial N	0.07	-0.18	0.05	0.22	-0.26	**0.44**	**0.46**	**-0.82**
microbial C	**0.45**	**-0.32**	0.20	0.23	-0.30	0.51	**0.49**	**-0.80**
total biomass	0.23	-0.24	0.18	0.28	-0.17	**0.48**	**0.63**	**-0.98**
plant P content	-0.05	-0.13	**-0.34**	-0.06	-0.18	**0.44**	**0.62**	**-0.97**
plant N content	-0.14	-0.06	0.01	0.04	-0.13	**0.40**	**0.64**	**-0.96**

Control excluded	***Lolium***				***Trifolium***			
	PO_4^{3-}-P	**unreactive P**	**NH_4^+-N**	**NO_3^--N**	**PO_4^{3-}-P**	**unreactive P**	**NH_4^+-N**	**NO_3^--N**
root colonization	**-0.65**	0.23	**-0.39**	-0.28	0.09	-0.14	**0.41**	-0.35
microbial N	0.23	-0.31	0.02	0.10	-0.16	-0.07	-0.22	0.24
microbial C	**0.56**	-0.34	0.27	0.25	-0.19	0.13	-0.08	0.30
total biomass	**0.50**	-0.21	0.30	0.26	0.24	**-0.53**	0.17	-0.27
plant P content	-0.31	-0.06	-0.33	0.00	0.14	**-0.64**	0.11	-0.15
plant N content	-0.20	0.04	-0.08	0.15	0.30	**-0.68**	0.24	-0.08

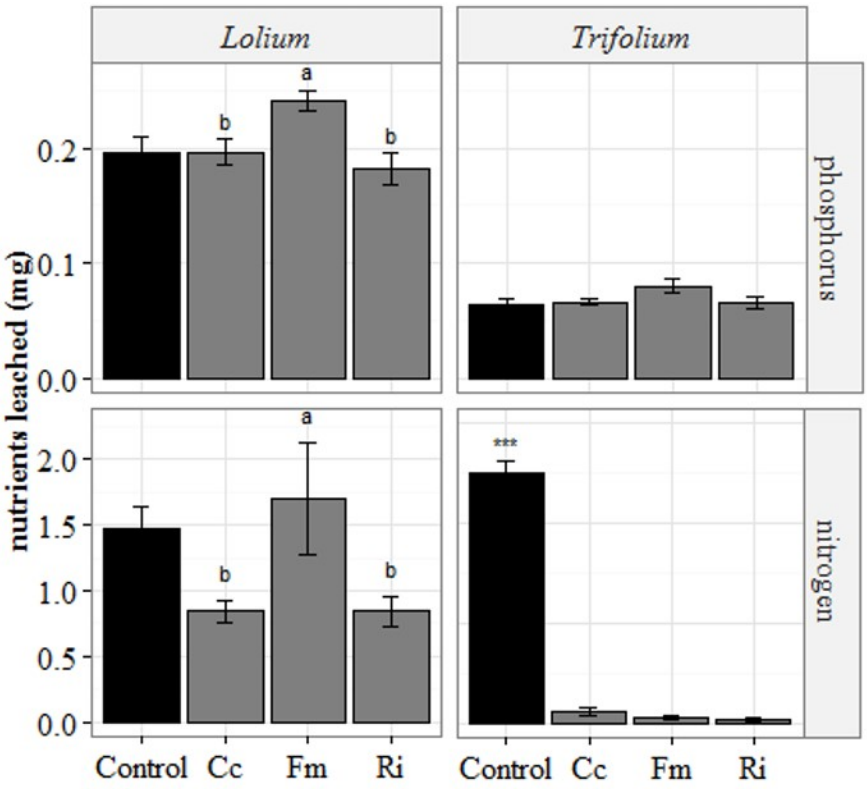

Figure S2. Total P and total mineral N leached from pots planted with *Lolium* or *Trifolium* after a leaching inducing rain simulation. Pots were inoculated with a non-mycorrhizal control inoculum or three different AM fungal species: Cc= *Claroideoglomus claroideum*, Ri= *Rhizoglomus irregulare*, Fm=*Funneliformis mosseae*. Means of eight (*Trifolium*) and ten (*Lolium*) replicates ± SEM are shown. Asterisks indicate that the control treatment is significantly different from the mycorrhizal treatments ($p<0.001$***,< 0.01**,< 0.05*). Mycorrhizal treatment means with the same letter are not significantly different at the 0.05 level using Tukey HSD test. Individual results for ammonium, nitrate, phosphate and unreactive P leaching are shown in the supplements.

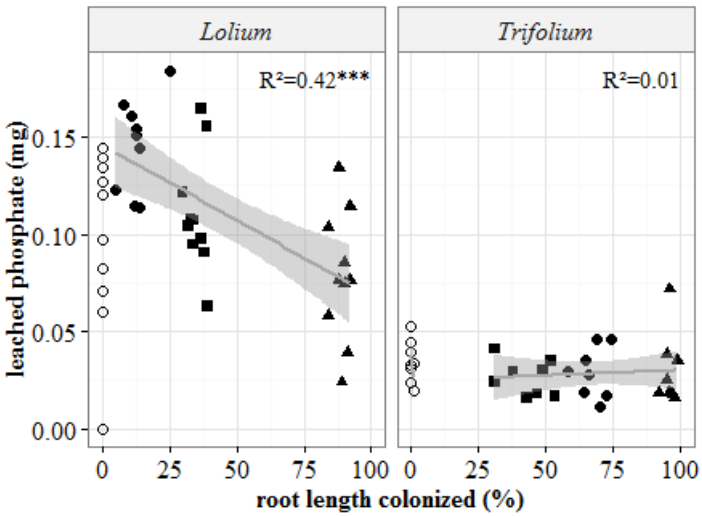

Figure S3. Correlation between phosphate leached from microcosms (mg) and total root length colonized of *Lolium* (n=40) or *Trifolium* plants (n=32) which have been inoculated with a non-mycorrhizal control inoculum (○) or three different AM fungal species: Cc= *Claroideoglomus claroideum* (■), Ri=*Rhizoglomus irregulare* (▲), Fm=*Funneliformis mosseae* (●). Regression analysis shown was performed without the non-mycorrhizal control treatment.

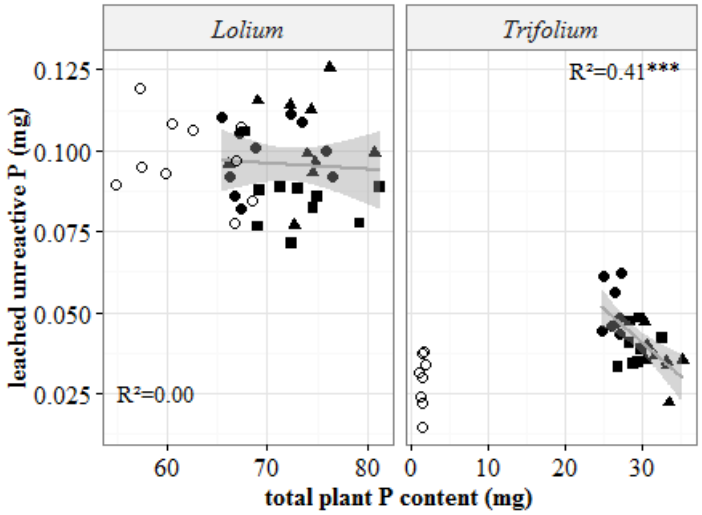

Figure S4. Correlation between unreactive P leached from microcosms (mg) and total plant P content (mg, roots and shoots) of *Lolium* (n=40) or *Trifolium* plants (n=32) which have been inoculated with a non-mycorrhizal control inoculum (○) or three different AM fungal species: Cc= *Claroideoglomus claroideum* (■), Ri=*Rhizoglomus irregulare* (▲), Fm=*Funneliformis mosseae* (●). Regression analysis was performed without the non-mycorrhizal control treatment.

Table S3. Total nutrient balance of *Lolium* and *Trifolium* microcosms. All data are depicted as means ± SEM in mg per microcosm (*Lolium*: N=10, *Trifolium*: N=8). Cc= *Claroideoglomus claroideum*, Ri=*Rhizoglomus irregulare*, Fm=*Funneliformis mosseae*

	Location of nutrients		*Lolium*			
			Control	**Cc**	**Ri**	**Fm**
N	plant	plant uptake	651.67 ± 13.4	617.07 ± 14.16	639.41 ± 13.05	646.97 ± 17.33
	soil	N total	5958.29 ± 262.4	5786.22 ± 124.98	5900.94 ± 140.08	6273.42 ± 150.42
		nitrate	0.00 ± 0	0.00 ± 0	0.00 ± 0	0.00 ± 0
		ammonium	3.68 ± 1.77	0.49 ± 0.17	3.48 ± 1.77	4.88 ± 1.48
		microbial N	241.00 ± 7.39	258.03 ± 6.41	208.52 ± 5.36	222.62 ± 6.51
	leaching	NO_3^-	0.39 ± 0.21	0.08 ± 0.02	0.03 ± 0.03	0.54 ± 0.4
		NH_4^+	1.08 ± 0.09	0.76 ± 0.07	0.81 ± 0.1	1.16 ± 0.05
	fertilization[a]	NO_3^-	78.44			
		NH_4^+	11.20			
P	plant	plant uptake	62.22 ± 1.55	73.27 ± 1.38	73.47 ± 1.24	70.01 ± 1.31
	soil	plant available[b]	2.47 ± 0.04	2.39 ± 0.05	2.39 ± 0.11	2.39 ± 0.07
	leaching	PO_4^{3-}	0.10 ± 0.01	0.11 ± 0.01	0.08 ± 0.01	0.14 ± 0.01
		unreactive P	0.10 ± 0	0.09 ± 0	0.10 ± 0	0.10 ± 0
	fertilization[a]	PO_4^{3-}	13.93			

	Location of nutrients		*Trifolium*			
			Control	**Cc**	**Ri**	**Fm**
N	plant	plant uptake	67.20 ± 3.91	706.54 ± 12.24	838.34 ± 20.57	639.29 ± 12.79
	soil	N total	2251.49 ± 71.96	2298.48 ± 35.04	2609.46 ± 203.09	2374.59 ± 26.75
		nitrate	41.22 ± 4.22	0.00 ± 0	0.00 ± 0	0.00 ± 0
		ammonium	3.00 ± 0.4	0.74 ± 0.15	2.88 ± 0.68	0.54 ± 0.18
		microbial N	28.28 ± 4.64	92.75 ± 3.56	71.61 ± 4.79	77.23 ± 4.24
	leaching	NO_3^-	125.29 ± 5.8	5.53 ± 1.98	1.89 ± 0.92	2.50 ± 1
		NH_4^+	0.12 ± 0.03	0.32 ± 0.05	0.47 ± 0.09	0.42 ± 0.04
	fertilization[c]	NO_3^-	5.6			
		NH_4^+	2.8			
P	plant	plant uptake	1.46 ± 0.08	29.35 ± 0.59	32.38 ± 0.63	26.37 ± 0.35
	soil	plant available[b]	2.12 ± 0.04	1.06 ± 0.03	1.29 ± 0.1	1.13 ± 0.02
	leaching	PO_4^{3-}	0.03 ± 0	0.03 ± 0	0.03 ± 0.01	0.03 ± 0
		unreactive P	0.03 ± 0	0.04 ± 0	0.04 ± 0	0.05 ± 0
	fertilization[d]	PO_4^{3-}	6.34			

[a] sum of three fertilization events
[b] extraction with CO2-saturated water
[c] one fertilization event
[d] sum of two fertilization events

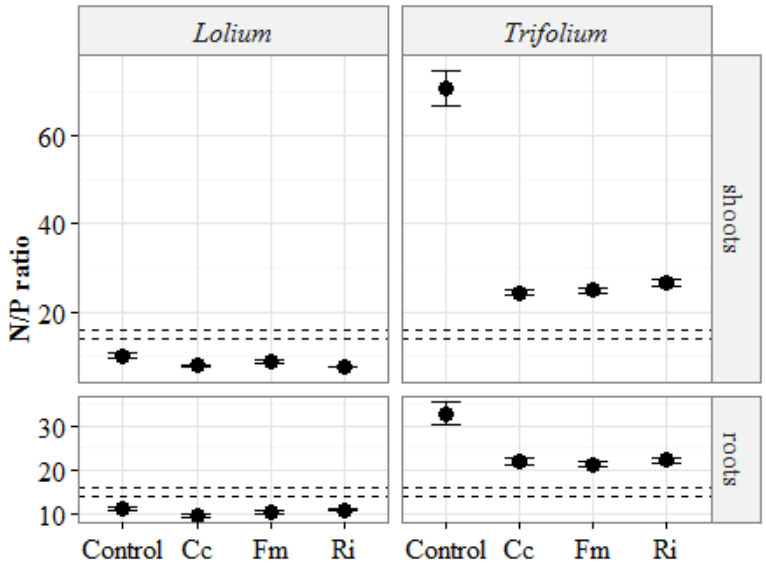

Figure S5. Shoot and root N/P ratio of *Lolium* and *Trifoliu*m inoculated with a non-mycorrhizal control inoculum or three different AMF species: Cc= *Claroideoglomus claroideum*, Fm=*Funneliformis mosseae*, Ri= *Rhizoglomus irregulare*. Means of eight (*Trifolium*) and ten (*Lolium*) replicates ± SEM are shown. A N/P ratio below 14 indicates that plants are N limited, a N/P ratio above 16 indicates P limitation (Koerselman and Meuleman 1996). These threshold values are highlighted in the graph as dashed lines.

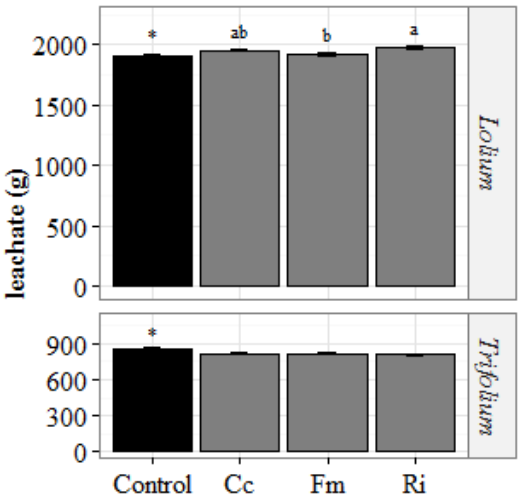

Figure S6. Amount of leachate (g) drained from *Lolium* and *Trifolium* microcosms inoculated with a non-mycorrhizal control inoculum or three different AMF species: Cc= *Claroideoglomus claroideum*, Fm=*Funneliformis mosseae*, Ri=*Rhizoglomus irregulare*. The microcosms were watered to field capacity and subsequently received a heavy rain shower by adding 2L (microcosms with *Lolium*) or 925 ml (microcosms with *Trifolium*) with a rain simulator. Means of eight (*Trifolium*) and ten (*Lolium*) replicates ± SEM are shown. Asterisks indicate that the control treatment is significantly different from the mycorrhizal treatments ($p<0.001$***,< 0.01**,< 0.05*). Mycorrhizal treatment means with the same letter are not significantly different at the 0.05 level using Tukey HSD test.

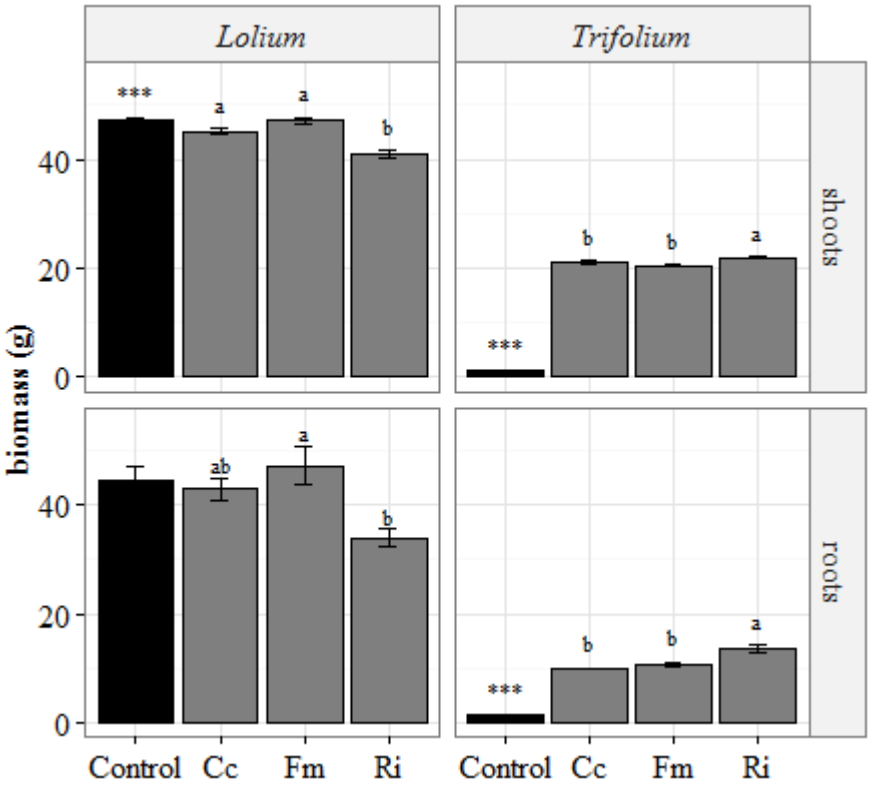

Figure S7. Shoot and root biomass (g) of *Lolium* and *Trifolium* in microcosms inoculated with a non-mycorrhizal control inoculum or three different AMF species: Cc= *Claroideoglomus claroideum*, Fm=*Funneliformis mosseae*, Ri= *Rhizoglomus irregulare*. Means of eight (*Trifolium*) and ten (*Lolium*) replicates ± SEM are shown. Asterisks indicate that the control treatment is significantly different from the mycorrhizal treatments ($p<0.001$***,<0.01**,<0.05*). Mycorrhizal treatment means with the same letter are not significantly different at the 0.05 level using Tukey HSD test.

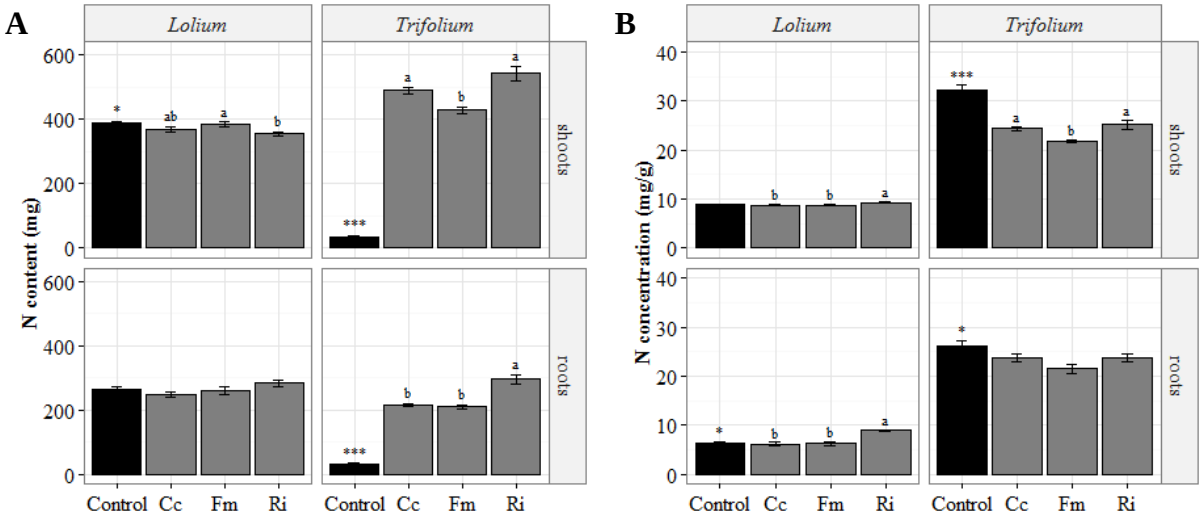

Figure S8. Shoot and root N **A)** content (mg) and **B)** concentration (mg/g) of *Lolium* and *Trifolium* in microcosms inoculated with a non-mycorrhizal control inoculum or three different AMF species: Cc= *Claroideoglomus claroideum*, Fm=*Funneliformis mosseae*, Ri= *Rhizoglomus irregulare*. Means of eight (*Trifolium*) and ten (*Lolium*) replicates ± SEM are shown. Asterisks indicate that the control treatment is significantly different from the mycorrhizal treatments (p<0.001***,< 0.01**,< 0.05*). Mycorrhizal treatment means with the same letter are not significantly different at the 0.05 level using Tukey HSD test.

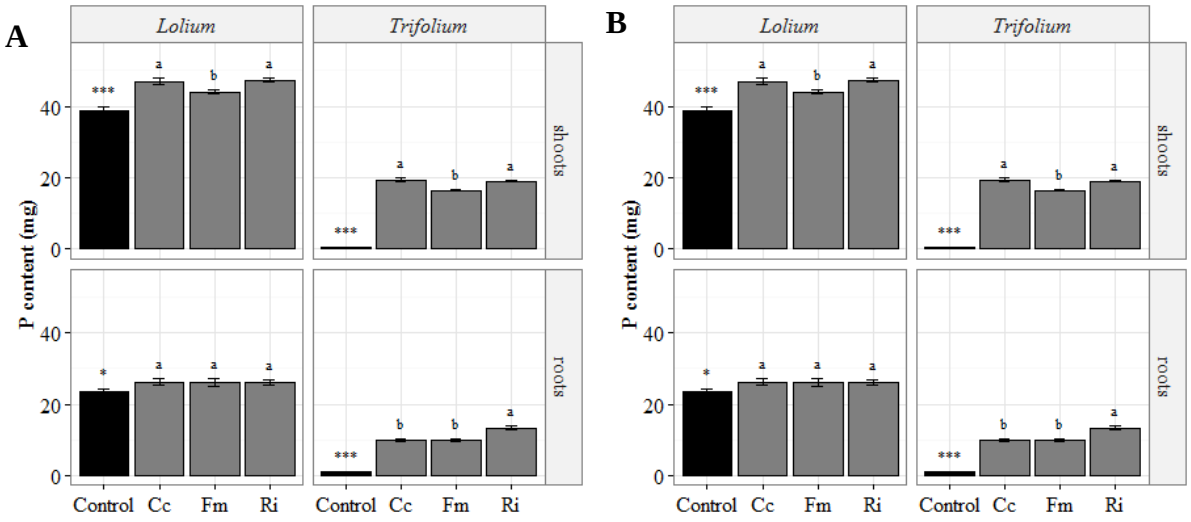

Figure S9. Shoot and root P **A)** content (mg) and **B)** concentration (mg/g) of *Lolium* and *Trifolium* in microcosms inoculated with a non-mycorrhizal control inoculum or three different AMF species: Cc= *Claroideoglomus claroideum*, Fm=*Funneliformis mosseae*, Ri= *Rhizoglomus irregulare*. Means of eight (*Trifolium*) and ten (*Lolium*) replicates ± SEM are shown. Asterisks indicate that the control treatment is significantly different from the mycorrhizal treatments (p<0.001***,< 0.01**,< 0.05*). Mycorrhizal treatment means with the same letter are not significantly different at the 0.05 level using Tukey HSD test.

Chapter 3

Establishment and effectiveness of inoculated arbuscular mycorrhizal fungi in agricultural soils

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Abstract

Arbuscular mycorrhizal fungi (AMF) are promoted as biofertilizers for sustainable agriculture. So far, most researchers have investigated the effects of AMF on plant growth under highly controlled conditions with sterilized soil, soil substrates or soils with low available P or low inoculum potential. However, it is still poorly documented whether inoculated AMF can successfully establish in field soils with native AMF communities and enhance plant growth.

We inoculated grassland microcosms planted with a grass-clover mixture (*Lolium multiflorum* and *Trifolium pratense*) with the arbuscular mycorrhizal fungus Rhizoglomus irregulare. The microcosms were filled with eight different unsterilized field soils that varied greatly in soil type and chemical characteristics and indigenous AMF communities. We tested whether inoculation with AMF enhanced plant biomass and *R. irregulare* abundance using a species specific qPCR.

Inoculation increased the abundance of *R. irregulare* in all soils, irrespective of soil P availability, the initial abundance of *R. irregulare*, or the abundance of native AM fungal communities. AMF inoculation had no effect on the grass but significantly enhanced clover yield in 5 out of 8 field soils. The results demonstrate that AMF inoculation can be successful, even when soil P availability is high and native AMF communities are abundant.

Keywords

arbuscular mycorrhizal fungi, *Rhizoglomus irregulare*, inoculation, field soil, *Lolium*, *Trifolium*, qPCR

Introduction

There is an increased interest to utilize beneficial soil biota as a tool to enhance plant nutrition and plant productivity (Barrios 2007). The presence of beneficial soil biota can be stimulated by altering agricultural practices such as crop rotation or tillage intensity that favor particular groups of microorganisms (Altieri 1999, Köhl et al. 2014). In addition, beneficial soil biota can also be deliberately introduced into agroecosystems through inoculation or seed coating in order to add a desired function or enhance an already existing one (Vessey 2003, Berg 2009).

Within the soil microbial community, arbuscular mycorrhizal fungi (AMF) are well known for their ability to enhance plant nutrient uptake, improve plant growth, and influence ecosystem functioning (Smith and Read 2008). Up to 50% of the soil microbial biomass consists of AMF (Olsson et al. 1999). AMF form a symbiosis with over 80% of the land plants including many important crops (Smith and Read 2008). AMF can provide a range of soil nutrients to plants in exchange for carbohydrates. In addition, AMF can also contribute to soil aggregate formation (Leifheit et al. 2014), protect their hosts against abiotic (Galli et al. 1994, Bothe 2012) and biotic stresses (Azcón-Aguilar and Barea 1996) influence nutrient cycling (Cavagnaro et al. 2006, Bender and van der Heijden 2015) and reduce the production of the greenhouse gas N_2O (Bender et al. 2014).

AMF are native to all terrestrial ecosystems and can be found in almost every soil (Abbott and Robson 1982, Öpik et al. 2006, Jansa et al. 2009). Several studies report reduced AMF diversity upon land use intensification (Helgason et al. 1998, Verbruggen et al. 2010). The reduction of mycorrhizal abundance and species diversity is due to factors related to intensive agricultural management such as high fertilization, intensive tillage, fallow, and crop sequence with non-host crops (Jansa et al. 2006, Koide and Peoples 2012, Säle et al. 2015). It has been shown in microcosms that this loss of fungal diversity in soil can disrupt a range of soil ecosystem services (van der Heijden et al. 1998b, Maherali and Klironomos 2007, Wagg et al. 2014). Moreover, some studies indicate that intensive agriculture selects for inferior mutualists (Johnson 1993, Scullion et al. 1998).

Soil inoculation with beneficial AMF has been proposed to overcome this limitation, and contribute to more efficient nutrient use. Inoculation with beneficial AMF is increasingly considered for species-poor and often sterile soils in nurseries (Azcón-Aguilar and Barea 1997) and in tropical crop production where soils are low in plant available phosphorus and AMF abundance (Sieverding 1991, Ceballos et al. 2013). The hesitant application of AMF in commercial agriculture in the temperate zone might be due to high application costs, the perception that AMF are not very beneficial when P-availability is high, and that AMF may even lead to plant growth depression in some crops (Ryan and Graham 2002). Despite these concerns, meta-analyses have revealed that biomass production and P-uptake can indeed be increased by inoculation of soil with AMF (McGonigle 1988, Lekberg and Koide 2005, Hoeksema et al. 2010).

One of the crucial biotic soil factors determining the success of the fungal inoculant is the indigenous mycorrhizal community. If the strain is compatible with a particular soil, it still needs to outcompete the indigenous AMF community, and AMF already established in the field may be competitively superior (priority effect) compared to introduced ones (Verbruggen et al. 2013). Furthermore, it is thought that ecosystems can only support AMF populations to a certain quantity (carrying capacity) preventing further establishment if this carrying capacity has already been reached. Thus, it seems questionable, if inoculation can be successful in fields

with high fungal abundance. Despite numerous inoculation studies, only a few attempts using molecular tools have been made to assess, if a foreign strain can successfully colonize host plants and persist in field soil despite of other AMF being present (Farmer et al. 2007, Ceccarelli et al. 2010, Pellegrino et al. 2012, Sýkorová et al. 2012). Moreover, all these studies focused on one particular field, and it has not yet sufficiently been tested whether a particular inoculant can establish in a wide range of soils. It is also still unclear, whether the same fungal isolate as it often occurs in commercial inoculum can successfully established in a broad range of field sites. Such a broad applicability is one pre-condition for commercial AMF inocula.

In this study, we introduced *Rhizoglomus irregulare* to a range of agriculturally managed field soils. *R. irregulare* (formerly named *Rhizophagus irregularis/ Glomus irregulare/ Glomus intraradices*, (Sieverding et al. 2014) is a widespread AMF present in almost any ecosystem investigated (Öpik et al. 2006), and is especially abundant in agricultural soils (Jansa et al. 2003, Oehl et al. 2010). Earlier studies with this isolate have shown that it has a positive impact on the growth and nutrition of a range of plant species, when added to sterilized soil (van der Heijden et al. 2006, Scheublin et al. 2007, Wagg et al. 2011a). Here we specifically test whether 1.) the introduced AMF can establish in a wide range of field soils, 2.) the AMF is able to establish and compete with different resident AMF communities and 3.) whether AMF inoculation enhances plant productivity and nutrient uptake. In order to test this, we inoculated or mock inoculated the AMF *R. irregulare* into microcosms planted with a grass-clover mixture. The microcosms were filled with unsterilized field soil originating from eight agriculturally managed fields that differed strongly in soil type and chemical characteristics.

Materials & methods

Field soil

Eight different soils from tilled fields distributed across Switzerland were used as experimental soil. We specifically chose field sites that differed strongly in soil type and chemical characteristics (Table 1). These different soils also varied in cropping history and agricultural management, like fertilization. All soils are representative for the temperate zone. Soils were taken from the tilled layer before fertilization in the spring. As a control, we selected a field soil with a very low mycorrhizal inoculum potential that had been stored long term at the research station Agroscope (Figure 1C). Soils were sieved to 5 mm for homogenization and to remove larger fragments and stones.

Soil physical and chemical properties (Table 1) were analyzed by lbu (Thun, Switzerland). The initial total N content in the dried soils was quantified with an elemental analyzer Euro EA 3000 (HEKAtech, Wegberg, Germany). Available soil ammonium and nitrate concentrations were determined according to the Swiss reference methods of the research station Agroscope (Forschungsanstalt Agroscope Reckenholz-Tänikon ART and Forschungsanstalt Agroscope Changins-Wädenswil ACW 1996) using the Berthelot reaction (Krom 1980) and the cadmium reduction method (van Staden 1982) followed by a Griess assay (Griess 1879) respectively. The absorption of the resulting colored complexes was quantified with the continuous flow analyzer SAN++ analyzer (Skalar Analytical B.V., Breda, Netherlands).

Table 1. Soil type, origin, physical and chemical properties of the soils used in the greenhouse inoculation experiment.

Soil	Soil type[a]	Location	pH	Clay	Loam	Humus	AAE10-Ex P[b]	CO2-Ex P[c]	total N	NO_3-N	NH_4-N	CEC[d]
				%	%	%	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	meq/100 g
A	Fluvisol	Paradislihof, Rietheim	7.8	16.0	31.0	1.6	52.8	5.3	1295.0	20.2	0.19	9.3
B	cambic Stagnosol	Laubbergerhof, Rietheim	7.3	21.0	31.0	3.8	109.3	3.0	2540.0	77.4	0.24	23.4
C	Regosol	Hardhof, Tegerfelden	8.0	21.0	41.0	2.1	210.1	18.8	1660.0	24.8	0.22	13.5
D	Histosol	Riedmatt, Rümlang	8.0	21.0	51.0	10.5	63.9	2.9	6610.0	32.3	0.23	46.9
E	Gleysol	Gordola, Ticino	6.2	16.0	41.0	2.3	56.2	1.6	1300.0	7.8	3.85	10.9
F	Cambisol	Agroscope, Zürich	6.6	16.0	31.0	2.5	62.1	3.8	2030.0	19.3	0.56	14.2
G	eutric Stagnosol	Agroscope, Zürich	7.6	26.0	41.0	5.5	118.2	3.7	4400.0	53.0	0.31	35.4
H	control soil	Agroscope, Zürich	5.6	11.0	31.0	1.0	7.8	0.3	1160.0	29.2	25.71	45.9

[a] according to IUSS Working Group WRB (2006)
[b] ammonium acetate EDTA extraction; cannot be interpreted when pH>6.8
[c] extraction with CO_2-saturated water
[d] cation exchange capacity

AMF Inoculum

Soils were either inoculated with the AM fungus *Rhizoglomus irregulare* (Błaszk., Wubet, Renker & Buscot) Sieverd., G.A. Silva & Oehl (formerly known as *Rhizophagus irregularis*/ *Glomus irregulare/ Glomus intraradices* (Sieverding et al. 2014), isolate BEG21 (accession number DQ377990, SAF22, Swiss Collection of Arbuscular Mycorrhizal Fungi, Agroscope, Zurich, www.agroscope.ch/saf) or received a non-mycorrhizal control inoculum. *R. irregulare* is a common AMF with a worldwide distribution (Öpik et al. 2006), and is very abundant in a wide range of ecosystems, including many agricultural fields in Switzerland (Sýkorová et al. 2007b, Oehl et al. 2010). *R. intraradices* is reported to be a good root colonizer (Pellegrino et al. 2011) and very resistant to intensive agricultural management practices (Oehl et al. 2010). Furthermore, this AM fungus is commonly used in commercially available biofertilizers (Faye et al. 2013). The isolate was propagated in the greenhouse on *Plantago lanceolata* in an autoclaved substrate made of 15% grassland soil and 85% quartz sand. After eight months of growth, the pots were left to dry out, and aboveground biomass was discarded. The roots were then cut into small pieces and mixed thoroughly with the rest of the substrate to serve as the soil inoculum. A non-mycorrhizal control was prepared analogously to the AMF inoculum, but without AMF added. The *R. irregulare* inoculum contained roots that were at least 72% colonized by AMF and 75 spores per g of inoculum. No AMF spores or root colonization was observed in the control inoculum.

Set-up of the AMF inoculation trial in the greenhouse

The experiment was set up as a full factorial block design and consisted of two factors: “soil type” (eight different field soils, A-H; where H served as control soil with low abundance of AMF; Table 1) and “mycorrhizal inoculation” (microcosms inoculated with AM fungi (I) or inoculated with a non-mycorrhizal control (C)). Each of the 16 treatments was replicated six times resulting in 96 microcosms.

Grassland microcosms were established in PVC tubes with a diameter of 15.2 cm (surface corresponding to 1.8e-06 ha) and a height of 40 cm. For better drainage 1040 g of an autoclaved gravel mixture was added to the bottom of the tubes. A total of 5.125 L sieved field soil was added to each microcosm and 5.1% v/v soil inoculum (275 ml in total) was mixed with

the upper 450 ml of soil. Each microcosm was covered with 175 ml of the corresponding soil on the top to prevent cross contamination.

The microcosms were planted with a model grassland community consisting of *Trifolium pratense* L. 'Formica' (red clover) and *Lolium multiflorum* Lam. 'Oryx' (Italian ryegrass), plant genotypes often planted in Swiss pastures (Boller et al. 2002, Frick et al. 2008). A grass-clover mixture was chosen as it is widespread in both agricultural and natural grassland ecosystems where these species commonly coexist (Nyfeler 2009). Moreover, the two plant species belong to different functional groups (a legume and a grass) and show different mycorrhizal growth responses (*Trifolium* is a highly responsive species (Köhl et al. 2014, van der Heijden et al. 2016) and *Lolium* is an unresponsive species (Wagg et al. 2011a)). Before planting, seeds (propagated by Agroscope, Zurich, Switzerland) were surface sterilized with 5% household bleach for 5 min, 70% ethanol for 10 min, and then rinsed thoroughly with sterilized water. Plants were germinated on 1.5% sterile water agar. Twelve individuals of each plant species were planted into the microcosms according to a predefined design. During the first two weeks non-surviving seedlings were replaced. All microcosms were kept in the greenhouse (see supporting information for growth conditions). In Switzerland temporary grass-clover ley is often sown in summer and fertilized for the first time in spring, when the pasture has established. As our experiment lasted only 13 weeks, we choose not to fertilize and give the plant community and mycorrhizal fungi time to establish.

All microcosms received 5 ml of an AMF-free filtered washing of the two different inocula. This was done to equalize differences in the non-mycorrhizal microbial communities between the two soil inocula (Ames et al. 1987). The microbial wash was prepared by suspending 100 g of each inoculum together in 1 L deionized water. The suspension was filtered through several sieves (25-250 µm) and finally through filter paper (N°598, ∅210 mm, Schleicher and Schuell, Dassel, Germany).

Harvest and analysis

After 8 weeks, shoots were cut 6 cm aboveground to simulate hay making or grazing, which is typical for most grasslands in Switzerland. After 13 weeks the microcosms were harvested and shoot dry weight, root dry weight, shoot N and P content, and AMF root colonization levels for each plant species were determined (see supporting information for details).

Quantification of R. irregulare in the roots by qPCR

A subsample of the root sample, containing roots of both species, was selected randomly, frozen, and lyophilized. DNA was extracted, and quantitative PCR was conducted using primers and a hydrolysis probe, which are specific for the nuclear large ribosomal subunit (nLSU) of *R. irregulare*, following Thonar et al. (2012) (see supporting information for details).

Statistical analysis

Statistical analyses were conducted using the software R 2.14.1 (R Development Core Team 2011).

The effect of soil type and AMF inoculation on plant responses (biomass, shoot nutrient content, mycorrhizal structures in the roots) was analyzed separately with mixed-effect models (Pinheiro and Bates 2000) using the function lme in the library nlme. Soil type, inoculation treatment and their interaction were used as fixed effect, whereas block functioned as random effect. When homoscedasticity was not guaranteed, the varIdent() function was used to allow

each treatment to have different variances. Plant biomass was combined for two harvests. As plant mortality had a significant effect on *Lolium* biomass, the plant biomass was standardized by the number of survived individuals for both *Lolium* and *Trifolium*. nLSU copy numbers were log10 transformed before all analyses. The control soil H accounted for a significant amount of variance in the biomasses, as well as the root colonization. As this soil served as control (no fresh field soil, low inoculum potential), a separate analysis was performed without this soil to estimate the effects of field soils and AMF inoculation.

To assess the effect of the AMF inoculation on plant growth, growth responses were calculated as effect size of the inoculation treatment relative to the non-inoculated control for each soil type. The effect size of the mycorrhizal inoculation on the root colonization was evaluated as difference between the total colonization of the inoculated (I) and the mean of the uninoculated soil (C_{mean}) (Lekberg and Koide 2005) for each soil separately (Equation 1).

$$\Delta AMF = I - C_{mean} \quad \text{(Eqn 1)}$$

I — mycorrhizal root colonization (%) of plants growing in microcosms with mycorrhizal inoculum

C_{mean} — mean mycorrhizal root colonization (%) of plants growing in microcosms that were not uninoculated (average of six replicates for each soil type)

The same was done for the total root length colonized by AMF and *R. irregulare* LSU copy number determined by qPCR.

The mycorrhizal growth response (MGR) (Veiga et al. 2011) was used to express the effects of AMF inoculation on biomass production for each soil type. To calculate the MGR two equations are required, one for plants which perform better with AMF ($I > C_{mean}$) and one for plants growing better without AMF ($I < C_{mean}$) (Equation 2 and 3).

$$\text{if } I > C_{mean}, \text{ then MGR} = \left(1 - \left(\frac{C_{mean}}{I}\right)\right) \times 100\% \quad \text{(Eqn 2)}$$

$$\text{if } I < C_{mean}, \text{ then MGR} = \left(-1 + \left(\frac{I}{C_{mean}}\right)\right) \times 100\% \quad \text{(Eqn 3)}$$

I — biomass of plants growing in microcosms with mycorrhizal inoculum

C_{mean} — mean biomass of plants growing in microcosms that were not uninoculated (average of six replicates for each treatment)

The effect of mycorrhizal inoculation on plant P and N content in the experiment was evaluated analogously to the MGR of the biomass. Means and SEMs of the raw data can be found in the supporting information (Table S1-S3).

The calculated effect sizes were assessed with a one sample t-test to determine, if the difference between the inoculated an uninoculated soil was different from zero. Differences between mycorrhizal and non-mycorrhizal treatments in N:P ratios of aboveground nutrient concentrations were assessed with a two sample t-test for each soil.

Correlations between two variables were assessed using Pearson's correlation.

All figures and tables presented show estimates of the means with their standard error (SEM). Two DNA samples for qPCR were below the detection limit of 9.6e+09 copies/µl. Due to the given detection limit we cannot state that these samples were free of *R. irregulare* DNA, thus we assigned them a value of 1% of the detection limit. One sample (soil A-I) was not quantifiable due to PCR-inhibitors and was excluded from the analysis. One microcosm (soil B-C) was discarded from biomass and qPCR analysis as only 4 out of 12 *Lolium* plants survived in the course of the experiment despite repeated replanting.

Results

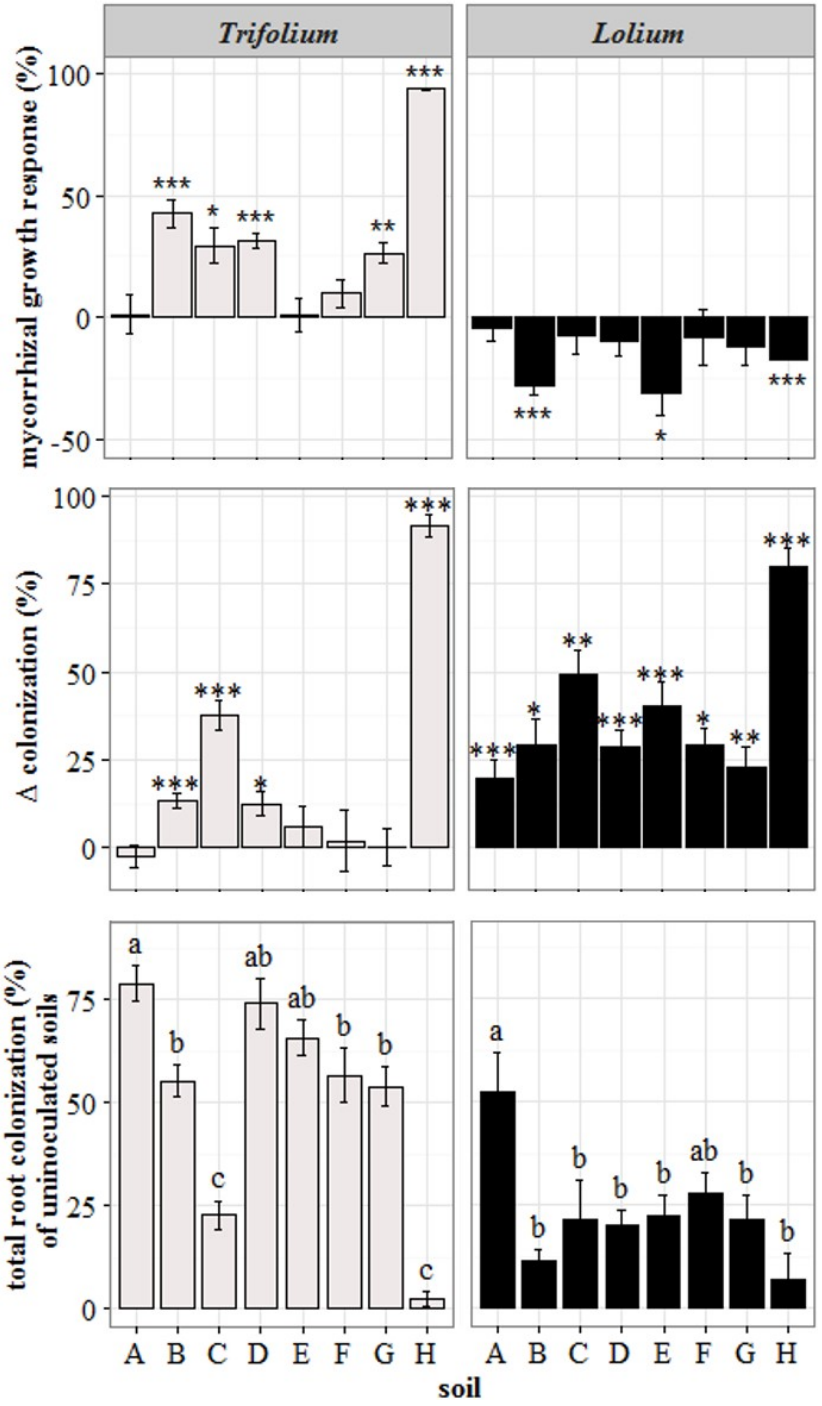

Figure 1. A) Mycorrhizal growth response of *Lolium* and *Trifolium* plants (%), **B)** change in total root colonization (%) due to inoculation with *R. irregulare* and **C)** total root colonization (%) of uninoculated treatments in microcosms with eight different unsterile field soils (A-H). Bars depict means ± SEM of 6 replicates. Asterisks indicate that the effect size is significantly different from zero ($p<0.05$ *, $p<0.01$ **, $p<0.001$ ***) according to a one-sample t-test. Means with the same letter do not differ significantly (Tukey test, $p<0.05$).

Mycorrhizal colonization

Total root colonization of *Lolium* was enhanced by *R. irregulare* inoculation in all soil types while root colonization of *Trifolium* was enhanced in four out of eight soil types (Fgure 1B, Table 2). Effects of inoculation on *Trifolium* root colonization depended on soil type as indicated by a significant "soil type" x "mycorrhizal inoculation" interaction ($F_{6, 63}$ = 4.06, p=0.0017). The effect of inoculation on root colonization of *Lolium* was superior over the effect of soil identity (Table 2). Such a hierarchy could not be detected for *Trifolium* root colonization.

The change in total *Trifolium* root colonization upon inoculation was significant for soil B (total: t_5=7.07, p=0.0009), C (total: t_5=8.97, p=0.0003), D (total: t_5=3.78, p=0.013) and control soil H (total: t_5=28.68, p<0.0001, supporting information Table S4). The inoculation driven change in total *Lolium* root colonization was significant for all soils (Figure 1B). Total colonization was enhanced by up to 166% in *Trifolium* roots (soil C) and up to 232% in *Lolium* roots (soil C) (soil H: 4217% *Trifolium*, 1194% *Lolium*). In soil H, no arbuscules were detected without inoculation.

R. irregulare was present in all field soils and in soil H before inoculation, as detected with qPCR. nLSU copy number in the uninoculated treatments varied between soils with soil A having the highest amount of *R. irregulare* and the control soil H (followed by soil C) with the lowest (supporting information Table S2). nLSU copy number increased in all soils after inoculation (Figure 2), but the response depended on soil type ($F_{6, 27}$ = 29.91, p<0.0001). The highest increase in *R. irregulare* nLSU copy number was observed in the control soil H (1e+4.26 more nLSU copies), followed by soil C (1e+2.32). Total root length colonized was positively correlated with *R. irregulare* nLSU copy number (Figure 3, *Lolium*: t_{92}=7.70, r=0.63, p<0.0001, *Trifolium*: t_{92}=7.55, r=0.62, p<0.0001, supporting information Table S5).

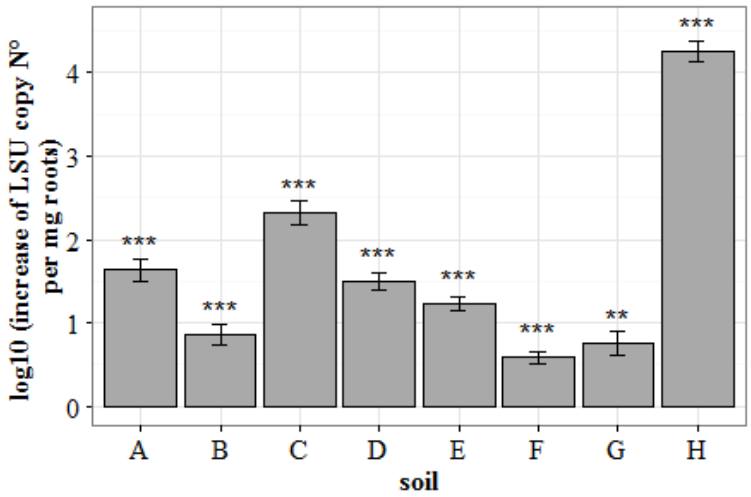

Figure 2. Increase of intraradical *R. irregulare* nLSU copy number (log10) upon inoculation with *R. irregulare* for eight different unsterile field soils (A-H). nLSU copy number was determined by *R. irregulare* specific qPCR. According to qPCR results *R. irregulare* was naturally present in all soils (10^{12} – 10^{15} nLSU copies, supporting information Table S2). Bars depict means ± SEM of 6 replicates (soil A N=5). Asterisks indicate that the change in LSU copy number due to inoculation is significantly different from zero (**p<0.01, ***p<0.001) according to a one-sample t-test.

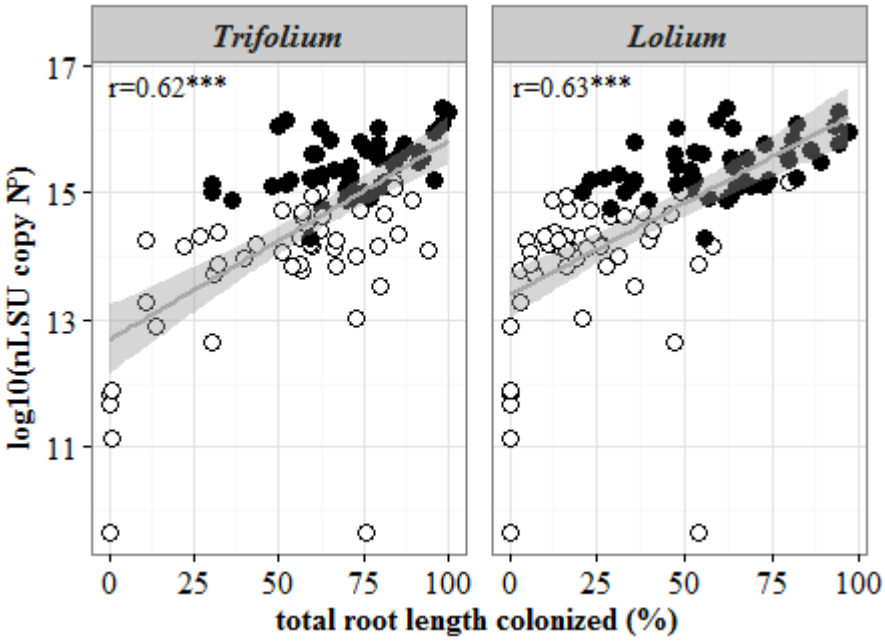

Figure 3. Relation between total root length colonized per plant species and nLSU copy number of *R. irregulare* in the roots (N=94) for inoculated (black) and uninoculated soils (white). All soils were included in the regression analysis (without control soil H: *Lolium* r=0.50***, *Trifolium* r=0.31**). Grey shades visualize the confidence interval. When correlations were performed for each inoculation treatment separately, total root length colonized was still positively correlated with nLSU copy number of *R.irregulare* (*Lolium*: uninoculated r=0.31*, inoculated r=0.47***, *Trifolium*: uninoculated r=0.55***, inoculated r=0.41**). Visualization by soil type is shown in the supporting information (Fig. S2). (p<0.05 *, p<0.01 **, p<0.001 ***)

Biomass production

Trifolium

Trifolium biomass generally increased with AMF inoculation, the effect varying with soil type (significant "soil type" x "mycorrhizal inoculation" interaction: $F_{6,62}$=3.17, p=0.0089, Table 2). The growth response to the inoculation (MGR) was significantly influenced by the soil type, regardless of whether the control (H) was included or not (without H: $F_{6,28}$=9.07, p<0.0001, Table 3).

A significant increase in *Trifolium* biomass following inoculation was observed in five out of eight soils (Figure 1A). A significant growth increase was observed for soil B (t_5=7.64, p=0.0006), soil C (t_5=4.00, p=0.01), soil D (t_5=10.98, p=0.0001), soil G (t_5=6.43, p=0.0013) and the control soil H (t_5=250.78, p<0.0001). The control soil (H) yielded the highest biomass increase (1477%). In the other responsive soils, *Trifolium* biomass increased in the range of 33 to 51%, equivalent to an additional yield of 1,240 to 2,215 kg *Trifolium* shoots per ha.

Lolium

Lolium biomass was decreased by AMF inoculation in all soils, with the effect size of the inoculation depending on the soil type (significant "soil type" x "mycorrhizal inoculation" interaction: $F_{6,62}$=4.34, p=0.001, Figure 1A, Table 2). The growth response to inoculation (MGR) was significantly affected by the soil type, regardless of whether the control (H) was

included or not (without H: $F_{6,28}$=6.21, p=0.0003, Table 3). Three soils showed a statistically significant growth reduction of *Lolium*. These soils were B (t_5=-7.23, p=0.0008), E (t_5=-3.39, p=0.02) and the control H (t_5=-28.83, p<0.0001). Biomass reduction corresponded to 371 to 607 kg per ha (soils B, E).

Correlation analysis revealed that the change in root colonization by inoculation explained 59% of the change in aboveground *Trifolium* biomass (MGR), when all soils (including soil H) were included in the correlation (t_{46}=8.32, p<0.0001, r=0.77, Table S6). When the control soil H was excluded the correlation was still significant but only explained 10% of the variation in *Trifolium* biomass (t_{40}=2.15, p=0.016, r=0.32). The increase in nLSU copy number after inoculation did not explain any variation in MGR of *Trifolium* (excluding H: t_{39}=0.25, p=0.8, r=0.04). The decrease in *Lolium* biomass correlated with the increase in arbuscules in *Lolium* roots (t_{40}=-2.09, p=0.043, r=-0.31). Correlation analysis (using the means per treatment) revealed that the mycorrhizal growth response of both species to inoculation could not be explained by soil pH (MGR *Trifolium*: t_6=1.48, r=0.52, p=0.19, MGR *Lolium*: t_6=-1.10, r=-0.41, p=0.31) or soil phosphorus concentration (MGR *Trifolium*: t_6=1.21, r=0.44, p=0.27, MGR *Lolium*: t_6=-0.44, r=-0.18, p=0.68). *Lolium* growth response was partly explained by initial ammonium content in the soil (t_6=3.47, r=0.82, p=0.013), but this correlation has to be interpreted with caution as only means were used (N=8).

Total biomass

The effect of AMF inoculation on total plant biomass (*Trifolium* and *Lolium*) depended on soil type, as revealed by a significant “soil type” x “mycorrhizal inoculation” interaction term ($F_{6,62}$=3.23, p=0.0079, Table 2). Upon inoculation, total biomass of the microcosms increased significantly in soil G (t_5=2.64, p=0.046, Table S4) and in the control soil H (t_5=5.33, p=0.0031), and it decreased in soil E (t_5=-2.98, p=0.031). The biomass increase in soil G and H corresponded to 265 and 916 kg more biomass per ha compared to uninoculated soils, which had a biomass production of 9,969 and 13,042 $kg*ha^{-1}$.

Plant nutrient content

Plant nitrogen and phosphorus content was influenced by soil type (N: $F_{6,62}$=61.55, p<0.0001, P: $F_{6,62}$=62.00, p<0.0001, Table 2). AMF inoculation only affected N content ($F_{1,62}$=14.53, p=0.0003), while no effect on P content was found ($F_{1,62}$=0.07, p=0.8). N content was significantly increased by inoculation in soil D (t_5=5.50, p=0.0027, see supporting information Figure S1). All plants, except those growing in soil H, had N:P ratios below 14, indicating that all soils were N limited (Figure S1, Koerselman & Meuleman, 1996).

Table 2. Results for the mixed effect models using soil type and inoculation treatment as well as their interaction as fixed effect and block as random effect. All analyses excluded the control soil H (analyses including H see supporting information Table S7). Asterisks indicate significance levels ($p<0.05$ *, $p<0.01$ **, $p<0.001$ ***).

Source of variation		Soil			Inoculation			Inoculation:Soil		
		df	F		df	F		df	F	
Biomass (g)										
Trifolium		6, 62	5.76	***	1, 62	37.92	***	6, 62	3.17	**
Lolium		6, 62	32.50	***	1, 62	27.03	***	6, 62	4.34	**
total biomass		6, 62	19.67	***	1, 62	0.89		6, 62	3.23	**
roots		6, 62	3.99	**	1, 62	2.18		6, 62	0.65	
nutrient content (mg)										
N[v]		6, 62	61.55	***	1, 62	14.53	***	6, 62	1.76	
P		6, 62	62.00	***	1, 62	0.07		6, 62	0.70	
nutrient concentration (mg/g)										
N[v]		6, 62	13.21	***	1, 62	8.01	**	6, 62	0.62	
P		6, 62	19.76	***	1, 62	8.39	**	6, 62	2.41	*
AMF colonization (%)										
arbuscular	*Trifolium*	6, 63	9.52	***	1, 63	9.11	**	6, 63	1.63	
arbuscular[v]	*Lolium*	6, 63	2.96	*	1, 63	11.96	**	6, 63	1.94	
total	*Trifolium*	6, 63	16.39	***	1, 63	15.14	***	6, 63	4.06	**
total	*Lolium*	6, 63	9.26	***	1, 63	113.14	***	6, 63	1.73	
log10 (nLSU)		6, 61	0.21		1, 61	70.71	***	6, 61	2.31	*

[v] Because of heterogeneity in the variance structure the varIdent() function was used.

Discussion

Our study showed that I.) *R. irregulare* can successfully establish in a wide range of soil types and compete effectively with other AMF to colonize plant roots, II.) clover yield in field soil can be enhanced by AMF inoculation, even when the soil P availability and initial inoculum potential are very high and III.) effects of AMF inoculation on plant productivity depend on soil type.

R. irregulare can be successfully introduced and established in a wide range of soil types

R. intraradices is known to be globally distributed (Öpik et al. 2006) and well adapted to intensive agricultural practices (Oehl et al. 2004). The presence of this fungus in all our uninoculated soils, as demonstrated by qPCR, confirms its general abundance in agricultural soils in Switzerland. This ubiquitous occurrence indicates that *R. intraradices* is compatible with a wide range of soil conditions varying in pH (5.6 to 8.0), P availability (0.3 to 18.8 mg/kg CO_2-extracted P), sand content (17.5 to 57.0%), and humus content (1.0 to 10.5%). This compatibility with the environment (soil conditions, host plant) is a crucial factor determining successful establishment of an AMF inoculant and an important characteristic for commercial application (Verbruggen et al. 2013).

Successful establishment of the *R. irregulare* strain in this study was shown by a significant increase in nLSU abundance after inoculation in all eight soils (Figure 2). This indicates that the investigated *R. irregulare* can be considered a favorable inoculant for a wide range of soils. Moreover, the tested soils were all non-sterile and contained a native AMF community as evidenced by total root colonization levels between 2 and 79% in the non-inoculated soils (Figure 1C). The fact that the abundance of *R. irregulare* increased upon inoculations confirms other studies that it can successfully establish when being introduced in an existing AMF community (Alkan et al. 2006, Janoušková et al. 2013). While these other studies focused on one single field site or a specific soil substrate, the results from this study are valid for a much broader range of conditions. The ability of *R. irregulare* to quickly colonize the host (Hepper et al. 1988, Jansa et al. 2008) might have enabled the fungus to colonize unoccupied niches sooner than competing indigenous AMF species. As a result, the addition of *R. irregulare* caused a shift in AMF community structure because in all treatments the nLSU copy number was significantly increased by inoculation, while in several soil types, root colonization was not enhanced.

Table 3. Assessment of the effect sizes with mixed effect models using soil type as fixed effect and block as random effect. Analyses were conducted with and without the control soil H. Asterisks indicate significance levels ($p<0.05$ *, $p<0.01$ **, $p<0.001$ ***).

Source of variation		including soil H			excluding soil H		
		df	F		df	F	
MGR biomass							
Trifolium		7, 33	33.51	***	6, 28	9.07	***
Lolium		7, 33	4.66	**	6, 28	6.21	***
total biomass		7, 33	7.35	***	6, 28	9.03	***
roots		7, 33	1.24		6, 28	1.25	
MGR nutrient content							
N		7, 33	4.60	**	6, 28	5.61	***
P		7, 33	2.11		6, 28	2.63	*
MGR nutrient concentration							
N		7, 33	3.18	*	6, 28	2.29	
P		7, 33	5.60	***	6, 28	6.76	***
delta AMF colonization							
arbuscular	*Trifolium*	7, 33	28.37	***	6, 28	4.98	**
total		7, 33	56.04	***	6, 28	9.75	***
arbuscular[v]	*Lolium*	7, 33	4.23	**	6, 28	3.30	*
total		7, 33	11.80	***	6, 28	3.26	*
delta log10 (nLSU)		7, 32	132.11	***	6, 27	29.91	***

[v] Because of heterogeneity in the variance structure the varIdent() function was used.

Plant responses to AMF inoculation

AMF inoculation significantly enhanced clover biomass in five out of eight soils tested (averaged across all sites inoculation resulted in a growth increase of 41%, Figure 1A), confirming other studies that clover is responsive to AMF (Drew et al. 2003, Köhl et al. 2014, van der Heijden et al. 2015). This study now also shows that AMF inoculation in a range of

field soils can enhance clover biomass even if a resident AMF community is already present. In contrast to *Trifolium*, the biomass of the grass *Lolium* declined upon inoculation in all soils (on average with 14%, Figure 1A), confirming other studies that *Lolium* is an unresponsive species or is even suppressed by AMF (Tawaraya 2003, Köhl et al. 2014). Our observation that the grass (*Lolium*) and the legume (*Trifolium*) responded differently to inoculation, confirms results from Hoeksema et al. (2010) that plant functional group is an important determinant in predicting the plant growth responses to inoculation.

Although, both *Lolium* and *Trifolium* were influenced by AMF inoculation, the total biomass was not influenced by AMF inoculation (on average 3% biomass increase). Similarly, AMF did not improve the nutritional status of the grassland mixture. Instead AMF reduced the competitive inequality between the two plant species by reducing the growth suppression of the AMF responsive legume by the non-responsive grass. The direct effect of mycorrhizal colonization on the biomass of the individual species as observed in monocultures (Veiga et al. 2011, Wagg et al. 2011b) is thus additionally influenced by competitive interactions in mixture.(Hall 1978, Hartnett et al. 1993, Wagg et al. 2011b). Although, the average net biomass of the grassland communities did not increase significantly, the composition of the aboveground biomass shifted towards a better forage quality with more biomass of the nitrogen fixing legume.

Colonization response to inoculation

Inoculation with *R. irregulare* increased root colonization by 39% in clover and by 163% in the grass (Figure 1B). This is in agreement with meta-analyses of various inoculation trials (McGonigle 1988, Lekberg and Koide 2005) that showed that inoculation usually enhances root colonization by AMF. This also indicates that the carrying capacity for successful establishment was not reached in the investigated field soils, despite high P availability and high AMF abundance at some of the investigated sites.

Interestingly, qPCR revealed that *R. irregulare* abundance in the roots was successfully increased by inoculation in all soils regardless of the initial inoculum potential (colonization in uninoculated treatments). However, this did not necessarily lead to an enhancement in clover biomass. Root colonization was a much better predictor of plant production than *R. irregulare* abundance (nLSU copy number) in this study (Table S6), although root colonization correlated well with nLSU copy number (Figure 3). Discrepancies between DNA quantification and staining as measures of fungal biomass have been reported before (Pivato et al. 2007, Gamper et al. 2008, Jansa et al. 2008). We assume in case of *R. irregulare* that intraradically produced spores contribute much more to the pool of DNA than intraradical hyphae (Gamper et al. 2008). Furthermore, vital hyphae can be devoid of nuclei leading to a considerable heterogeneity in nuclear distribution (Gamper et al. 2008). It is important to stress that the qPCR assay in this study only focused on *R. irregulare*, while root colonization encompasses all fungal structures, regardless of fungal species identity. With this in consideration, it is not surprising that root colonization is a better predictor of plant growth response in this study. Thus, a change in *R. irregulare* abundance does not necessarily lead to an altered overall root colonization, but does indicate a substitution of AMF species within the mycorrhizal community (Gazey et al. 2004, Janoušková et al. 2013). An altered mycorrhizal community will consequently affect the plant response, as the identity of the fungi colonizing a plant root is important because AM fungi vary in their ability to provide nutrients to plants (Ravnskov and Jakobsen 1995, Smith et al. 2000) and plant species respond differently to different AM fungal species (Newsham et al. 1995, Taylor and Harrier 2000, Streitwolf-Engel et al. 2001, Scheublin et al. 2007).

Biotic and abiotic soil factors are determining the inoculum response

Generally, it has been observed that the importance of AMF for plants is inversely related to P availability (Stribley et al. 1980, Marschner and Dell 1994, Treseder 2004). Our study did not confirm this. Inoculation success (in terms of biomass stimulation and establishment success of *R. irregulare*) depended on the field soil selected. As expected the control soil H with the lowest plant available P and low initial AMF abundance showed the strongest biomass reaction to additional AMF. However, plant available P of soil C was enriched, but nevertheless showed a strong biomass response in clover. Furthermore, clover did not respond to inoculation in soil E, a soil with a moderate P availability, which would be favorable for inoculation. As soil nutrient status could not predict mycorrhizal inoculation success in this study, we assume that other factors such as initial AMF abundance and AMF community composition influence establishment success.

For successful application it is necessary to develop a mechanistic model that can predict under which conditions and for which crops application is feasible and commercially attractive. In this sense, it is important to mention that the amount of inoculum we added to the pots was large (corresponding to $1.4x10^5$ L per ha). Adding such a large amount is expensive and possibly unrealistic for successful commercial application, despite of substantial progress which has been made in inoculum production over the last years (Jolicoeur et al. 1999, IJdo et al. 2011, de Santana et al. 2014). Other inoculation techniques that use smaller amounts of inoculum (e.g. seed coating or pre-inoculation of seedlings) are likely to be more promising (Vosátka et al. 2012).

Conclusions

Unlike former observations that AMF are not beneficial in agricultural fields (Ryan and Graham 2002, Ryan and Kirkegaard 2012), our results demonstrate that AMF inoculation in field soils can enhance growth of clover irrespective of initial soil P availability and AMF abundance. We have also shown that our tested AMF strain can successfully establish in a wide range of soils with highly variable chemical characteristics suggesting that it has a broad niche and is able to compete successfully with indigenous AMF.

Acknowledgements

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Supporting information

Supporting Materials and methods

Growth conditions

Plants were grown with 16 h days under natural light with a day temperature of at least 24°C and 8 h night with a temperature of at least 18°C. If light levels fell below 300W/m2 additional lightning was provided with 400W high pressure sodium lights. Plants were kept in the greenhouse for 13 weeks between April and July 2012.

Microcosms were watered with deionized water 3 times a week to 80% field capacity. Blocks were rotated randomly in the greenhouse when microcosms were watered. Pest management was applied when necessary and according to Swiss regulations for organic farming.

Harvest and analysis

After 8 weeks, shoots were cut 6 cm aboveground to simulate hay making or grazing, which is typical for most grasslands in Switzerland. After 13 weeks at final harvest, shoots were cut at soil surface. For each harvest, plants were separated per species, dried at 60°C for 48 hrs and weighed. At final harvest of each plant species, three individuals per pot were pulled out of the soil together with the roots before being cut. This was done to determine plant species specific root colonization. These roots were washed under tap water, paper dried and weighed. The roots were cut into pieces <1cm and stored in 50% ethanol.

After aboveground harvesting, all roots that could easily be separated by hand from the soil were sampled and weighed. The remaining soil was thoroughly mixed and a weighed subsample to determine the amount of the remaining roots was taken. Roots from both samples were washed by repeatedly decanting the watered subsamples onto a 250 µm mesh. Washed roots were dried with paper towels and fresh weight was determined. A mixed subsample being representative for both plant species was taken for molecular analysis and frozen. The remaining roots were weighed and dried for 48 hrs at 60°C, after which the root dry weight was quantified. The total amount of root dry weight per pot was calculated by relating the dry weight of the corresponding soil sample and adding it up for the whole pot. Soil water content was determined gravimetrically to standardize the results for all microcosms.

AM fungal parameters

For the analysis of mycorrhizal root colonization, roots separated for each plant species, were cleared with 10% KOH and stained with 5% ink-vinegar (Vierheilig *et al.*, 1998). Percentage of root length colonized and frequency of hyphae, arbuscules and vesicles was quantified microscopically at a magnification of 200x with the line intersection method (McGonigle *et al.*, 1990) using 100 intersections. Total root colonization was defined as added percentage of hyphae, arbuscules and vesicles, whereas for the arbuscular root colonization only arbuscules were considered.

Plant nutrient analysis

Shoots were pooled across the two harvests for each species and grinded for nutrient analysis. Total nitrogen shoot content was determined using a CHNSO analyzer (Euro EA, HEKAtech GmbH, Wegberg, Germany). For plant P determination grinded biomass was ashed

at 600°C and digested using 6M HCl. Digests were diluted and P was quantified colorimetrically according to the molybdenum blue method (Watanabe & Olsen, 1965).

Quantification of *R. irregulare* in the roots by qPCR

A subsample of the pooled root sample, containing roots of both species, was selected randomly, frozen and lyophilized. Approximately 20 mg (exact weight was recorded) of lyophilized root material was homogenized with a mixture of Tungsten and glass beads using the TissueLyser II (Qiagen, Hilden, Germany). DNA extraction of the resulting powder was accomplished by the DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA-extracts were purified before PCR analysis with the NucleoSpin® gDNA Clean-up Kit (Macherey-Nagel, Düren, Germany). Quantitative PCR was conducted using the Bio-Rad CFX96 real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Reaction volume was 25µl (12.5µl 2x Qiagen QuantiTect Probe PCR Master Mix, 1µl of each 10µM primer, 0.5µl 10µM Probe, 5 µl bidest water, 5µl undiluted template). Cycling conditions were optimized as follows: initial denaturation and polymerase activation 95°C for 15min, then 45 cycles each with denaturation at 94°C for 15s and a combined annealing and elongation step at 60°C for 1min. Primer and hydrolysis probe, which were specific for the nuclear large ribosomal subunit (nLSU) of *R. irregulare* were used following Thonar *et al.* (2012). The HPLC-purified oligonucleotides were synthesized at Microsynth (Balgach, Switzerland). The probe was labeled with fluorescein and BHQ-1 quencher at the 5' and the 3' end, respectively. Forward primer sequence was 5'-TTCGGGTAATCAGCCTTTCG-3', reverse primer sequence was 5'-TCAGAGATCAGACAGGTAGCC-3' and the sequence of the hydrolysis probe was 5'-TTAACCAACCACACGGGCAAGTACA-3'. An amplicon of the size of 250 bp was produced. The calibration of the qPCR analysis was done as described before by Jansa *et al.* (2008) using DNA extracted as described above from spores of *R. irregulare*, isolate BEG21. LSU copy numbers were calculated as described in Jansa *et al.* (2008).

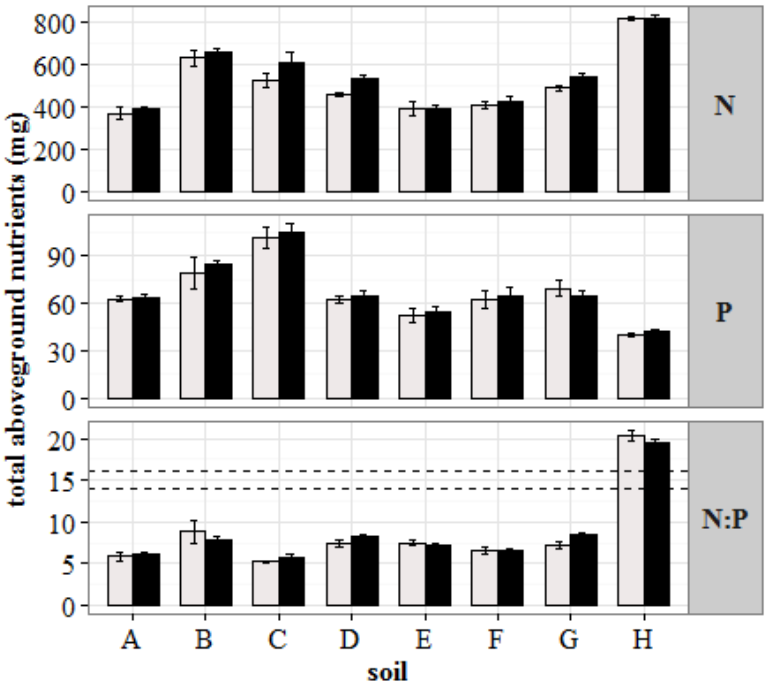

Figure S1. Shoot N and P content (mixture of *Lolium* and *Trifolium*) for eight different field soils inoculated with *R. irregulare* (black) or left uninoculated (grey). Additionally, N:P ratios of shoot nutrient concentrations are shown. N:P ratios below 14 indicate N-limitation whereas N:P ratios above 16 imply P-limitation (Koerselman and Meuleman 1996). Depicted are means ± SEM of 6 replicates. Asterisks indicate significant differences between the inoculated and not inoculated field soil according to a two-sample t-test (*p<0.05,**p<0.01).

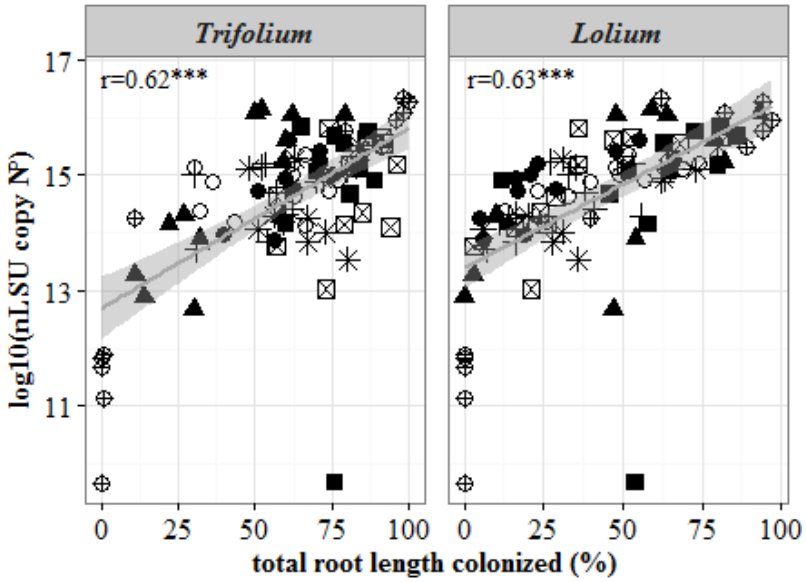

Figure S2. Relation between total root length colonized per plant species and the number of copies of the nLSU for *R. irregulare* in the roots (N=94). All soils were included in the regression analysis (without control soil H: *Trifolium* r=0.31**, *Lolium* r=0.50***). Grey shades visualize the confidence interval. (p<0.05 *, p<0.01 **, p<0.001 ***)

Table S1. Means and SEMs (6 replicates) of the different above and belowground biomasses (in g per individual plant) for 8 soil types (A-H) and two inoculation treatments C=control, I=mycorrhizal inoculation).

Soil type	Inocu-lation	biomass (g/ plant)			
		Trifolium	***Lolium***	**total biomass**	**roots**
A	C	0.59 (0.06)	0.85 (0.06)	1.43 (0.05)	0.54 (0.06)
	I	0.60 (0.05)	0.81 (0.04)	1.41 (0.02)	0.46 (0.03)
B	C	0.42 (0.04)	1.59 (0.06)	2.01 (0.04)	0.43 (0.05)
	I	0.77 (0.07)	1.15 (0.06)	1.92 (0.06)	0.40 (0.04)
C	C	0.61 (0.05)	1.17 (0.06)	1.78 (0.09)	0.35 (0.04)
	I	0.93 (0.11)	1.10 (0.09)	2.02 (0.12)	0.33 (0.03)
D	C	0.58 (0.06)	0.86 (0.08)	1.44 (0.03)	0.31 (0.02)
	I	0.86 (0.04)	0.78 (0.05)	1.63 (0.07)	0.29 (0.02)
E	C	0.59 (0.06)	0.90 (0.18)	1.50 (0.24)	0.50 (0.12)
	I	0.61 (0.05)	0.62 (0.08)	1.23 (0.09)	0.39 (0.05)
F	C	0.64 (0.02)	0.75 (0.1)	1.39 (0.1)	0.36 (0.03)
	I	0.73 (0.06)	0.70 (0.1)	1.43 (0.14)	0.40 (0.06)
G	C	0.71 (0.06)	0.73 (0.08)	1.44 (0.06)	0.32 (0.04)
	I	0.97 (0.05)	0.65 (0.06)	1.62 (0.07)	0.33 (0.04)
H	C	0.03 (0)	1.91 (0.05)	1.93 (0.05)	0.70 (0.05)
	I	0.44 (0.03)	1.58 (0.01)	2.02 (0.02)	0.70 (0.07)

Table S2. Means and SEMs (6 replicates) of the total root length colonized and colonized by arbuscules as well as the copy number of nLSU (presented as log10) specific *for R. irregulare* for 8 soil types (A-H) and two inoculation treatments C=control, I=mycorrhizal inoculation).

Soil type	Inoculation	root length colonized (%)				log10 nLSU copy N°
		Trifolium		*Lolium*		
		arbuscular	total	arbuscular	total	
A	C	42.00 (7.26)	79.00 (4.18)	1.50 (0.62)	52.50 (9.36)	13.94 (0.87)
	I	42.83 (7.19)	76.67 (3.33)	2.50 (1.18)	72.33 (5.19)	15.58 (0.12)
B	C	26.60 (4.96)	53.00 (3.27)	1.20 (0.89)	12.60 (2.69)	14.36 (0.19)
	I	44.33 (2.7)	68.67 (2.22)	1.83 (0.79)	40.33 (7.61)	15.21 (0.12)
C	C	10.49 (2.99)	22.67 (3.52)	0.17 (0.17)	21.33 (9.49)	13.53 (0.28)
	I	28.33 (6.03)	60.33 (4.2)	5.33 (2.68)	70.83 (6.81)	15.85 (0.15)
D	C	47.33 (7.99)	74.17 (6.12)	1.67 (0.67)	19.83 (3.82)	14.01 (0.23)
	I	65.67 (4.81)	86.67 (3.3)	4.50 (0.72)	48.50 (4.9)	15.50 (0.11)
E	C	32.97 (5.61)	65.67 (4.36)	2.67 (0.49)	22.50 (4.6)	14.01 (0.12)
	I	38.50 (6.39)	71.83 (5.8)	10.83 (3.09)	62.67 (6.87)	15.24 (0.09)
F	C	35.17 (9.07)	56.67 (6.46)	2.33 (1.36)	27.67 (5.02)	14.52 (0.14)
	I	35.00 (7.01)	58.67 (8.63)	3.83 (1.35)	56.67 (5.25)	15.11 (0.07)
G	C	33.00 (5.46)	53.83 (4.7)	1.67 (0.49)	21.50 (5.87)	14.20 (0.15)
	I	31.17 (4)	54.00 (5.47)	1.67 (0.92)	44.33 (5.89)	14.96 (0.14)
H	C	0.00 (0)	2.17 (1.78)	0.00 (0)	6.67 (6.67)	11.74 (0.61)
	I	72.17 (3.78)	93.67 (3.19)	13.17 (3.46)	86.33 (5.32)	15.99 (0.13)

Table S3. Means and SEMs (6 replicates) of the aboveground nutrient analysis and N:P concentration ratio for 8 soil types (A-H) and two inoculation treatments C=control, I=mycorrhizal inoculation).

Soil type	Inoculation	shoot nutrients				
		content (mg)		concentration (mg/g)		N:P
		N	P	N	P	
A	C	397.03 (29.94)	68.11 (1.99)	23.65 (1.77)	4.06 (0.08)	5.86 (0.49)
	I	417.89 (11.5)	69.24 (2.34)	24.60 (0.25)	4.08 (0.14)	6.06 (0.22)
B	C	706.73 (29.96)	94.78 (4.92)	33.08 (2.84)	4.37 (0.15)	7.57 (0.52)
	I	708.44 (12.16)	90.67 (3.1)	30.90 (1.16)	3.93 (0.06)	7.86 (0.3)
C	C	560.48 (35.67)	108.55 (7.01)	25.78 (0.4)	5.00 (0.12)	5.18 (0.15)
	I	648.19 (52.61)	112.07 (7.1)	26.90 (0.73)	4.68 (0.15)	5.78 (0.25)
D	C	487.69 (10.01)	67.02 (2.47)	28.73 (1.11)	3.93 (0.1)	7.35 (0.42)
	I	569.73 (16.95)	69.89 (3.28)	29.82 (0.8)	3.64 (0.07)	8.20 (0.24)
E	C	421.71 (35.93)	56.68 (4.49)	28.42 (0.8)	3.83 (0.12)	7.45 (0.34)
	I	418.91 (18.8)	59.08 (3.6)	28.85 (1.02)	4.04 (0.05)	7.14 (0.26)
F	C	438.15 (18.12)	67.76 (5.59)	28.47 (1.43)	4.33 (0.11)	6.61 (0.39)
	I	458.31 (21.24)	70.23 (5.68)	28.40 (1.02)	4.29 (0.09)	6.62 (0.24)
G	C	526.57 (13.95)	74.90 (5.48)	29.42 (0.41)	4.16 (0.19)	7.16 (0.36)
	I	579.17 (24.3)	69.73 (4.13)	31.48 (0.78)	3.78 (0.1)	8.37 (0.33)
H	C	875.26 (8.79)	43.14 (1.26)	37.40 (1.12)	1.84 (0.05)	20.38 (0.69)
	I	878.03 (11.43)	45.10 (1.26)	35.00 (0.77)	1.80 (0.05)	19.52 (0.36)

Table S4. t-values of one sample t-tests with the effect size of biomass and mycorrhizal parameters for each of the eight different field soils. Degrees of freedom were 5 except for the change in nLSU copy numbers for soil A (df=4).

Response		Soil															
		A		**B**		**C**		**D**		**E**		**F**		**G**		**H**	
MGR biomass																	
Trifolium		0.13		7.64	***	4.00	*	10.98	***	0.14		1.62		6.43	**	250.78	***
Lolium		-0.92		-7.23	***	-0.99		-1.66		-3.39	*	-0.72		-1.44		-28.83	***
net productivity		-0.93		-1.61		1.82		2.56		-2.98	*	0.05		2.64	*	5.33	**
roots		-2.55		-0.89		-1.19		-1.27		-2.14		0.19		0.09		-0.25	
MGR nutrient content																	
N		1.77		0.09		1.58		5.50	**	-0.27		0.83		2.15		0.21	
P		0.39		-1.34		0.34		0.76		0.50		0.21		-1.39		1.48	
MGR nutrient concentration																	
N		3.97	*	-1.89		1.46		1.28		0.33		-0.14		2.93	*	-3.13	*
P		0.10		-6.74	**	-2.15		-4.17	**	4.38	**	-0.41		-3.80	*	-0.82	
N:P ratio		-0.38		-0.47		-2.10		-1.76		0.72		-0.03		-2.48	*	1.12	
ΔAMF colonization																	
arbuscular	*Trifolium*	0.12		6.56	**	2.96	*	3.81	*	0.86		-0.02		-0.46		19.09	***
total	*Trifolium*	-0.70		7.07	***	8.97	***	3.78	*	1.06		0.23		0.03		28.68	***
arbuscular	*Lolium*	0.85		0.80		1.93		3.94	*	2.64	*	1.11		0.00		3.81	*
total	*Lolium*	3.82	*	3.65	*	7.27	***	5.85	**	5.84	**	5.52	**	3.88	*	14.97	***
log10 (nLSU)		12.07	***	7.00	***	15.96	***	14.07	***	14.05	***	8.14	***	5.35	**	32.64	***

Table S5. Results of the correlation between mycorrhizal root length colonized (in %, arbuscular and total) and log10 of the nLSU copy number of *R. irregulare* per mg root. Significance levels are indicated as asterisks (p<0.05 *, p<0.01 **, p<0.001 ***).

AMF root length colonized (%)		**log10 (nLSU copy number/mg root)**			
Plant	**AMF structures**	excluding H (df=80)		including H (df=92)	
		t	**r**	**t**	**r**
Trifolium	arbuscular	2.65 **	0.28	6.02 ***	0.53
	total	2.87 **	0.31	7.55 ***	0.62
Lolium	arbuscular	2.71 **	0.29	3.85 ***	0.37
	total	5.11 ***	0.50	7.70 ***	0.63

Table S6. Correlations between the plant response to mycorrhizal inoculation (expressed as MGR) and the AMF response to inoculation (expressed as difference between inoculated and uninoculated soils). Analyses were done with and without control soil H and were performed for *Trifolium* and *Lolium* and with total root colonization and root colonization with arbuscules. Significance levels are indicated by asterisks (p<0.05 *, p<0.01 **, p<0.001 ***).

AMF responses	plant growth response (MGR)							
	Trifolium				*Lolium*			
	excluding H		including H		excluding H		including H	
	t	**r**	**t**	**r**	**t**	**r**	**t**	**r**
arbuscular[a]	2.67 *	0.39	8.48 ***	0.78	-2.09 *	-0.31	-1.83	-0.26
total[a]	2.15 *	0.32	8.32 ***	0.77	-0.34	-0.05	-0.49	-0.07
log10(nLSU)[b]	0.25	0.04	6.40 ***	0.69	1.73	0.27	0.81	0.09

[a] root length colonized in %

[b] log10 of nLSU copy numbers per mg of root

Table S7. Results for the mixed effect models using soil type (eight levels: A-H) and inoculation treatment as well as their interaction as fixed effect and block as random effect. Asterisks indicate significance levels (p<0.05 *, p<0.01 **, p<0.001 ***). One microcosm was taken out of the analysis except for the plant specific root colonization.

Source of variation		Soil			Inoculation			Inoculation:Soil		
		df	**F**		**df**	**F**		**df**	**F**	
Biomass (g)										
Trifolium[v]		7,72	83.83	***	1,72	286.04	***	7,72	8.68	***
Lolium		7,72	88.81	***	1,72	37.43	***	7,72	3.99	**
total biomass		7,72	24.12	***	1,72	1.37		7,72	2.66	*
roots		7,72	14.67	***	1,72	1.52		7,72	0.50	
nutrient content (mg)										
N		7,72	123.66		1,72	10.86	**	7,72	1.65	
P		7,72	71.85	***	1,72	0.16		7,72	0.56	
nutrient concentration (mg/g)										
N[v]		7,72	28.89	***	1,72	4.73	*	7,72	1.57	
P[v]		7,72	593.85	***	1,72	7.84	**	7,72	2.90	**
AMF colonization (%)										
arbuscular[v]	*Trifolium*	7,73	9.52	***	1,73	248.99	***	7,73	31.66	***
arbuscular	*Lolium*	7,73	4.03	***	1,73	34.82	***	7,73	5.42	***
total	*Trifolium*	7,73	17.90	***	1,73	76.90	***	7,73	23.50	***
total	*Lolium*	7,73	7.76	***	1,73	174.68	***	7,73	5.98	***
log10 (nLSU)		7,71	2.08	.	1,71	115.4	***	7,71	7.67	***

[v] Because of heterogeneity in the variance structure the varIdent() function was used.

Chapter 4

The role of arbuscular mycorrhizal fungi for nutrient losses is highly dependent on the experimental conditions

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Abstract

Arbuscular mycorrhizal fungi (AMF) are well known for the key role they play in nutrient cycling. Despite this knowledge, surprisingly little is known about the influence of these symbiotic soil fungi on nutrient leaching. We used experimental grassland microcosms (planted with the grass, *Lolium multiflorum*, and the legume *Trifolium pratense*) to evaluate the effect of AMF inoculation with *Rhizoglomus irregulare* on nutrient leaching under varying experimental conditions. In one experiment, five different sterile substrates and one unsterilized substrate were tested. In a second experiment, eight different unsterile field soils were inoculated to evaluate AMF effects on leaching under more natural conditions. Our results show that AMF can substantially reduce nitrogen leaching losses from sterile microcosms. Depending on sand content, up to 46 kg N (primarily nitrate) per ha was retained by AMF presence. We observed that biomass production in substrates with less sand was higher upon AMF inoculation, and thus more N was immobilized. Only a negligible amount of ammonium was lost via leaching and was generally not affected by AMF inoculation. The total amount of P leached was also negligible, and we do not consider the observed effects to be agroecologically relevant. In contrast to sterile substrates, we could not detect mycorrhizal effects on N or P leaching in unsterile field soil. Surprisingly, despite successful inoculation, large effects on N leaching in sterile soil vanished when the same unsterilized soil was inoculated. In conclusion, we have shown that leaching effects are highly dependent on experimental conditions and extrapolating results to field conditions has to be done with caution.

Highlights

- Experimental conditions influence the effect of AMF on nutrient leaching.
- AMF can substantially reduce nitrate leaching in sterile soil.
- Ammonium and phosphorus leaching is not affected by AMF abundance.
- No leaching effects of AMF were observed in unsterile field soil.

Introduction

Agricultural intensification, through high rates of mineral fertilizer application, has constantly increased the productivity of agroecosystems to meet the food demands of a continuously growing world population. But these human activities have disturbed natural nutrient cycling in a variety of ways and have had unintended environmental consequences. Due to over fertilization many agroecosystems are saturated with excess phosphorus and nitrogen which cannot be taken up by crops or otherwise processed in biogeochemical cycles (Barberis et al. 1995, Frossard et al. 2000, Liu et al. 2010). The likelihood that these superfluous nutrients are lost by leaching is high, and the consequences include eutrophication of surface water and contamination of groundwater (Sims et al. 1998). Furthermore, losing valuable nutrients via leaching processes is costly and not sustainable. Phosphate mines suitable for fertilizer production are predicted to be exhausted within the next century (Cordell et al. 2009, van Vuuren et al. 2010), while the Haber process for synthesis of nitrogen fertilizer depends on non-renewable fossil fuel resources (Vance 2001).

To meet the growing demand for agricultural products, while reducing nutrient leaching and preserving biotic and geological resources, innovative management practices have been suggested to reduce leaching. These include a rapid installation of vegetation cover and small pulses of fertilizer in several applications (Di and Cameron 2002b, Havlin et al. 2005). Because microbial activity is a major driver of nutrient cycling in soil, managing soil biota should be considered as potential management tool for reducing nutrient losses. Soil biota is responsible for liberating soil nutrients and making them available to plants (Ingham et al. 1985, Hassink et al. 1993b). Furthermore, recent research has shown that microbial activity affects nutrient losses via leaching or as a gas (Plante 2007, Philippot et al. 2009, Bender et al. 2014, Wagg et al. 2014, Bender et al. 2015). Among the microbial groups that are active in soil nutrient cycling, arbuscular mycorrhizal fungi (AMF), obligate plant root symbionts, are especially potent (Smith and Read 2008). AMF can efficiently scavenge for nutrients in the soil, thereby increasing plant nutrient availability and the P uptake of their host plant (Li et al. 1991, Smith et al. 2003). A greater P supply for the plant is likely to lead to a higher N use efficiency. Additionally, up to 25% of plant N is taken up by the mycorrhizal pathway (Ames et al. 1983). As 80% of all land plants worldwide can live in symbiosis with AMF, these soil fungi likely have a great significance in soil nutrient cycling and have the ability to reduce nutrient leaching.

Indeed, recent research using artificial microcosms has shown that AMF presence can alter the amounts of nutrients that are leached (Asghari et al. 2005, van der Heijden 2010, Asghari and Cavagnaro 2012, Köhl et al. 2014, Bender et al. 2015, Köhl and van der Heijden 2016). Mechanisms that underlie a mycorrhizal effect on nutrient leaching are not fully understood, but research has indicated that efficient nutrient uptake and transfer to the plant contributes to nutrient immobilization in the soil (Cavagnaro et al. 2015). As AMF improve soil structure and soil water retention, AMF could also impact the volume of water draining through the soil and leaching valuable nutrients (Augé 2004).

Amounts of nutrients leached are not only dependent on the soil biota present, but also on soil type (Perry et al. 1988, Havlin et al. 2005), management factors like fertilization and cropping system (Di and Cameron 2002b), and climate or seasonal conditions, more specifically the water balance (Scholefield et al. 1993, Di et al. 1999). The sand content of the soil plays an especially key role in determining leaching amounts. Excessive leaching often occurs in coarse-textured soils, as water drains easily through sandy soil with low-activity clays and low organic matter. Furthermore, net mineralization of soil organic matter, and consequently nutrient availability, was found to be more rapid in sandy soils than in clay soils (Hassink et al. 1993a, Sogn and Haugen 2011).

Among the agriculturally relevant nutrients, nitrate (NO_3^-) is most soluble and mobile in soil. The concentration of ammonium (NH_4^+) in most soils is very low, as it is readily converted by soil microbes into nitrate (Jackson et al. 2008). Thus, nitrogen is generally leached as NO_3^- (Havlin et al. 2005). P in soil is strongly adsorbed to clays and thus is not very prone to leaching (Rodríguez and Fraga 1999). However, P leaching can be high in soils with low P adsorption capacity, like sandy soils and soils in which P pools are enriched by excessive fertilization (Havlin et al. 2005).

Although several publications indicate that AMF presence can affect the amount of nutrients lost by leaching, so far only little information exists on how different environmental conditions like soil type (Bender et al. 2015) or leaching ability of the soil will influence the observed effects, and if they can be enhanced by AMF inoculation in the field.

Therefore, we conducted two greenhouse experiments to answer the following research questions: (1) Is the mycorrhizal effect on nutrient leaching affected by the sand content (and consequently the nutrient availability and nutrient holding capacity) of the substrate? (2) Differs nutrient leaching between sterilized and unsterilized soil) and, (3) Does an increased AMF abundance reduce nutrient losses via leaching in different unsterilized agriculturally managed soils?

Methods

In this study we investigated if AMF have a general tendency to reduce nutrient losses or if the effect is highly context dependent. Therefore, two greenhouse experiments were conducted. In ***experiment one***, five different sterile sand-soil mixtures were inoculated with *Rhizoglomus irregulare* to test the effect of sand content on the mycorrhizal leaching response. The treatments were complemented with one unsterilized grassland soil with no added sand to compare the results to natural field conditions (6 different substrates in total). In ***experiment two***, eight different agriculturally managed field soils were inoculated with *R. irregulare* or left uninoculated to test, if mycorrhizal abundance affects nutrient leaching under natural soil conditions. Mycorrhizal effects on plant biomass for this experiment are already published in Köhl et al. (2015).

AMF Inoculum and plant species

In both experiments, *Rhizoglomus irregulare* (N.C. Schenck & G.S. Sm.) Sieverd., G.A. Silva & Oehl (formerly known as *Glomus intraradices* (Sieverding et al. 2014)), isolate BEG21 (accession number DQ377990, SAF22 Swiss Collection of Arbuscular Mycorrhizal Fungi, Agroscope, Zurich, Switzerland) was used as an inoculant. *R. irregulare* was chosen as it is a common species with a worldwide distribution (Öpik et al. 2006) and can be found in a wide range of ecosystems, including agricultural fields in Switzerland (Jansa et al. 2003, Oehl et al. 2010). This fungus is resistant to a range of intensive agricultural management systems (Oehl et al. 2010) and aggressively colonizes roots (Pellegrino et al. 2011). As a result, *R. irregulare* is commonly used in commercially available biofertilizers (Faye et al. 2013).

The inoculum for experiment one was propagated in a greenhouse on *Zea mays* ‘Gavott Bio’ in an autoclaved substrate comprising 15% grassland soil and 85% agricultural lime. After three months of growth, pots were left to dry out, aboveground biomass was discarded, and roots were cut in small pieces and mixed thoroughly with the rest of the substrate to serve as soil inoculum. Inoculum for experiment two was prepared as described in Köhl et al. (2015). In short, *Plantago lanceolata* was grown in a sterile mixture of 15% grassland soil and 85% quartz sand together with the AMF isolate for eight months before roots and substrate were taken as inoculum. Non-mycorrhizal controls were prepared analogously to the AMF inocula.

R. irregulare colonized 90% of the root length of *Z. mays* and 72% of the root length of *P. lanceolata*. The mycorrhizal inoculum for experiment one contained 316 spores in 10 ml substrate, and the inoculum for experiment two contained 75 spores per g. Neither control inocula contained any AMF propagules.

For both experiments a model grassland community consisting of *Trifolium pratense* L. ‘Formica’ and *Lolium multiflorum* Lam. ‘Oryx’ was used. This plant community was chosen as it is widespread in both agricultural and natural grassland ecosystems of Switzerland where these species commonly coexist (Nyfeler 2009). Moreover, the two plant species belong to different functional groups (a legume and a grass) and show different mycorrhizal growth responses. *Trifolium* is a highly responsive species (Hart and Reader 2002, Köhl et al. 2014), while *Lolium* is an unresponsive or negatively responding species (Bender et al. 2014). Before planting, seeds (propagated by Agroscope, Zurich, Switzerland) were surface sterilized with 5% household bleach for 5 min, 70% ethanol for 10 min, and rinsed thoroughly with sterilized water. Plants were germinated on 1.5% sterile water agar before 12 individuals of each plant species were planted into the microcosms according to a predefined design. Seedlings that did not survive were replaced up to two weeks after planting.

Experimental setup

Experiment 1: Mycorrhizal effect on nutrient leaching in soil with different sand contents

Six different substrates with varying sand contents and sterilization treatments were used for experiment one (Figure 1). The substrates were composed of increasing proportions of quartz sand and field soil from a long-term grassland that included native *Lolium* and *Trifolium* species (Agroscope, Zurich, Switzerland, 47°25'38.71''N, 8°31'3.91''E). The field soil was sieved to 3 mm and mixed with quartz sand to obtain the following substrate mixtures: 0, 25, 50, 75 and 100% sand content. Substrates were autoclaved at 121°C for 90 min. Additionally, as a positive control treatment, the remaining field soil was left unsterilized. Substrate characteristics were analyzed by Agroscope (Zürich, Switzerland) and are presented in Table 1. The amount of quartz sand added was highly correlated with the nutrient content; thus increasing the sand content can be also interpreted as decreasing the nutrient availability in the substrate. Plant available P (CO_2-extracted) did not change with sand content (0-75% sand content: R^2=0.03) except for the 100% sand treatment where P was increased (0-100% sand content: R^2=0.52) which we did not expect. However, as available P in all substrates was very low (P deficient substrates), we assumed that this would have negligible effects on AMF and leaching.

Table 1. Physical and chemical characteristics of the six different field soil/sand mixtures (n=1). The amount of quartz sand added was highly correlated with the nutrient content.

Sand added (%)	**Steri-li-zation**	**pH**	**soil physical characteristics (%)**					**main nutrients** (mg/kg)					
			C_{org} [a]	**Humus**	**Clay**	**Silt**	**Sand**	**P** [b]	**K** [b]	**Mg** [c]	**NO_3-N**	**NH_4-N**	**N_{total}** [d]
0	-	7	1.04	1.8	17.5	20.6	60.1	0.50	9	108	19.5	1.5	1420
0	+	7.4	0.84	1.5	16.2	19.4	63	0.28	9	99	5.3	19.5	1230
25	+	7.7	0.47	0.8	12.9	15.9	70.4	0.18	8	68	2.6	13.6	790
50	+	7.9	0.31	0.5	7.7	8.7	83.1	0.25	7	43	1.5	9.9	470
75	+	8.2	0.2	0.3	7.2	3	89.5	0.28	6	21	0	6.4	290
100	+	8.9	0.01	0	2.3	0.5	97.2	0.89	5	7	0	0.7	40
Pearson correlation with sand amendment													
r		0.97	-0.97	-0.97	-0.98	-0.99	0.99	0.72	-1.00	-0.99	-0.95	-1.00	-0.99

[a] percentage of organic C
[b] extraction with CO_2-saturated water, ratio soil: extractant=1:2.5, extraction time 1 hr
[c] extraction with 0.0125 M $CaCl_2$, ratio soil: extractant=1:10, extraction time 2 hr
[d] total amount of N (mg/kg) quantified with the elemental analyzer Euro EA 3000 (HEKAtech, Wegberg, Germany)

Figure 1. Pots inoculated with *Rhizoglomus irregulare* with decreasing sand content from left to right.

Each substrate was inoculated with 8% (v/v) *R. irregulare* inoculum or with a non-mycorrhizal control inoculum. Furthermore, all substrates received 9 ml/ kg of a filtered, AMF-free washing of the two different inocula. This was done to equalize differences in the non-mycorrhizal microbial communities between the two soil inocula (Ames et al. 1987). The microbial wash was prepared by suspending 1k g of fresh grassland soil in 5 L deionized water, filtering suspension through progressively smaller sieves (250-25 µm), and finally through filter paper (N°598, Ø 210 mm, Schleicher and Schuell, Dassel, Germany). The experiment was conducted in 3L pots (upper radius 8 cm, lower radius 6.25 cm, height 19.3 cm, radius used for kg/ha calculation r= 7.14 cm) each containing a piece of 500 µm synthetic mesh and an autoclaved sand/gravel mixture at the bottom to accelerate the leaching process. Each pot received 2.3L of substrate (dry weights: 100% sand - 3291 g, 75% sand - 3202 g, 50% sand - 3023 g, 25% sand - 2875 g, 0% sand (sterile) - 2658 g, 0% sand (unsterile) - 2689 g) and a final additional layer of inoculum free substrate (150 g) to prevent cross contamination between pots.

After planting, the pots were arranged in a complete randomized block design in a greenhouse. The factorial design consisted of six soil treatments (five sterile substrates with different sand amendments and the unsterile field soil) combined with two mycorrhizal treatments (microcosms inoculated with *R. irregulare* (M) or inoculated with a non-mycorrhizal control (NM)) each replicated 6 times, for a total of 72 pots.

The plants were grown in the greenhouse with an average daily temperature of 24°C, nightly temperature of 18°C, and 16 hours of light per day. Supplemental light was provided by 400 W high-pressure sodium lights when natural light fell below 300 W/m^2. Plants were kept in the greenhouse for 56 days between September and November 2010 and watered three times a week to 50% field capacity. One time per week (seven times in total) each pot was fertilized with 3 mL of a phosphorus free fertilizer (6 mM KNO_3, 4 mM $Ca(NO_3)_2$*$4H_2O$, 0.75 mM NH_4NO_3, 0.25 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$*6 H_2O, 50 µM KCl, 25 µM H_3BO_3, 2 µM $MnSO_4$*$4H_2O$, 2 µM $ZnSO_4$*$7H_2O$, 0.5 µM $CuSO_4$*$5H_2O$, 0.5 µM $(NH_4)_6Mo_7O_2$, 20 µM Fe(Na)EDTA) corresponding to a total of 2.7 kg N/ ha.

A rain induced leaching event was performed 54 days after planting. First, each pot received 100 ml of a nutrient solution (5.7 mM $NH_4H_2PO_4$, 5.7 mM KNO_3, 32.1 mM NH_4NO_3, 1 mM $MgSO_4$*6 H_2O, 2 mM $CaCl_2$, 40 mM KCl, 25 µM H_3BO_3, 2 µM $MnSO_4$*$4H_2O$, 2 µM $ZnSO_4$*$7H_2O$, 0.5 µM $CuSO_4$*$5H_2O$, 0.5 µM $(NH_4)_6Mo_7O_2$, 20 µM Fe(Na)EDTA) corresponding to 66 kg N and 11 kg P per ha. Two days after fertilization pots were watered to 100% field capacity using a rain simulator (Knacker et al. 2004). The rain volume equaled 100% of the field capacity of each substrate and differed between the different

sand-soil mixtures (100% sand: 579 g, 75% sand: 625 g, 50% sand: 722 g, 25% sand: 753 g, 0% sand (sterile): 879 g, 0% sand (unsterile): 970 g). Leached water was collected for three hours and stored at 4°C for further analysis. Final harvest started immediately after the leachate was collected.

Experiment 2: Effect of mycorrhizal abundance on nutrient leaching for eight different agricultural soils

Experiment two was part of a larger study assessing the effect of mycorrhizal soil inoculation and mycorrhizal abundance on ecosystem services in agriculturally managed soils. Plant responses to mycorrhizal inoculation are documented in Köhl et al. (2015). Here we focus on the effects of AMF on nutrient leaching in natural soil.

Sixteen treatments were applied using a completely randomized factorial design with six replicates for each treatment combination, resulting in 96 microcosms. The treatments were (1) "soil type" with eight different levels (field soils A-H) and (2) the "AMF" treatment consisting of two levels (microcosms inoculated with *R. irregulare* (M) or inoculated with a non-mycorrhizal control (NM)).

The eight different experimental soils were collected from tilled fields distributed across Switzerland. We specifically selected field sites that strongly differed in soil type and chemical characteristics (Table 2). Additionally, the different soils also varied in their cropping history and management regime, like amount of fertilizer applied. All soils are representative of those commonly found in the temperate zone and were collected from the tilled layer before the spring fertilizer application. As a control, we selected a field soil with a very low mycorrhizal inoculum potential that had been stored at Agroscope for a long period of time (Köhl et al. 2015). Soils were sieved to 5 mm for homogenization and to remove larger fragments and stones. Soil physical and chemical properties were analyzed by lbu (Thun, Switzerland) and are summarized in Table 2.

Table 2. Physical and chemical characteristics of the eight different field soils used for experiment two (modified after Köhl et al. 2015).

			soil physical characteristics					**main nutrients** (mg/kg)					
Soil	**Soil type**[a]	**pH**	**SOM**[b] (g/kg)	**Humus** (%)	**Clay** (%)	**Silt** (%)	**CEC**[c] meq/kg	**P**[d]	**K**[d]	**Mg**[e]	**NO_3-N**	**NH_4-N**	**N_{total}**[f]
A	Fluvisol	7.8	3.1	1.6	16	31	93	5.3	45	51	20.2	0.19	1295
B	Cambic-Stagnosol	7.3	4.4	3.8	21	31	234	3.0	44	124	77.4	0.24	2540
C	Regosol	8.0	1.1	2.1	21	41	135	18.8	112	77	24.8	0.22	1660
D	Histosol	8.0	1.4	10.5	21	51	469	2.9	61	126	32.3	0.23	6610
E	Gleysol	6.2	6.1	2.3	16	41	109	1.6	37	56	7.8	3.85	1300
F	Cambisol	6.6	1.1	2.5	16	31	142	3.8	17	110	19.3	0.56	2030
G	eutric Stagnosol	7.6	2.1	5.5	26	41	354	3.7	30	116	53.0	0.31	4400
H	control	5.6	2.0	1.0	11	31	459	0.3	42	57	29.2	25.7	1160

[a] according to IUSS Working Group WRB (2006)
[b] soil organic matter
[c] cation exchange capacity
[d] extraction with CO_2-saturated water, ratio soil: extractant=1:2.5, extraction time 1 hr
[e] extraction with 0.0125 M $CaCl_2$, ratio soil: extractant=1:10, extraction time 2 hr
[f] total amount of N (mg/kg) quantified with the elemental analyzer Euro EA 3000 (HEKAtech, Wegberg, Germany)

For experimental setup and maintenance see Köhl et al. (2015). A rain induced leaching event was performed twelve weeks after planting. First, microcosms were watered to 80% field capacity less 300 ml, and 20 ml of a nutrient solution (194.33 mM NH_4NO_3, 29.29 mM KH_2PO_4, 2 mM $CaCl_2*2H_2O$, 1 mM $MgSO_4*7H_2O$) was added (corresponding to 60 kg N and 10 kg P per ha). Microcosms were further watered to 80% field capacity. Four days after fertilization microcosms were watered to 100% field capacity and exposed to 500 ml of artificial rain using a rain simulator. In contrast to experiment one, we chose to add a standardized amount of water across all eight soils during the rain simulation, as this approach better replicates conditions experienced in the field. Leached water was collected for two hours and stored at 4°C for further analysis.

Harvest and analyses

At the final harvest, after 8 weeks for experiment 1 and 13 weeks for experiment 2 shoots, were cut at the soil surface. Plants were separated by species, dried at 60°C for 48 h, and weighed. Microcosms were emptied and larger roots were collected, washed, and weighed. In order to obtain remaining fine roots, the soil substrate was homogenized and a weighed soil sample was taken and washed by repeatedly decanting the watered subsamples onto a 250 µm mesh. Weighed subsamples of both root samples were dried at 60°C for 48 h, and total root biomass per microcosm was calculated. Subsamples of both root samples were cut into pieces <1cm, mixed in water, and stored in 50% ethanol for root colonization analysis. Soil water content was determined gravimetrically to standardize the results for all microcosms.

AM fungal parameters

In experiment two, to determine plant species specific root colonization, three individuals of each plant species per pot (including roots) were removed from the substrate before being cut. The roots were washed under tap water, paper dried, and weighed. The roots were cut into pieces <1cm and stored in 50% ethanol. In experiment one, mixed root samples (roots of both plant species mixed) were taken, cleared with 10% KOH, and stained with 5% ink-vinegar (Vierheilig et al. 1998). Percentage of root length colonized and frequency of hyphae, arbuscules, and vesicles was quantified microscopically at a magnification of 200× with the intersect method (McGonigle et al. 1990) using 100 intersections.

Leachate analysis

Leached phosphate, nitrite, and nitrate were quantified using a Dionex DX500 anion chromatograph (Dionex Corporation, Sunnyvale, CA) with an IonPac AG4A-SC guard column, an IonPac AS4A-SC analytical column (both 4mm) and 1.8mM Na_2CO_3/1.7mM $NaHCO_3$ as eluent. Ammonium was determined spectrophotometrically using the Berthelot reaction method (Krom 1980). The absorption of the resulting colored complexes was quantified with a continuous flow analyzer SAN++ analyzer (Skalar Analytical B.V., Breda, Netherlands). The total amount of dissolved P was determined colorimetrically according to the molybdenum blue method (Watanabe and Olsen 1965). The difference between total dissolved P and phosphate was defined as unreactive P. This fraction comprises all compounds not directly available to plants, such as soluble and particulate organic P compounds, polyphosphates, and particulate inorganic material like clays (Daniel and DeLaune 2009). As leachate volumes differed between treatments, leached nutrients are presented as the total amount leached, calculated by multiplying the volume of the leachate times the nutrient concentration.

Statistical analysis

Statistical analyses were conducted using R version 3.0.1 (R Core Team 2013).

In experiment one, we investigated the mycorrhizal effect on nutrient leaching as a function of the sand content of the soil. Because the six substrates had different water holding capacities and consequently were given different amounts of water, we chose to analyze the effect size (see formulas I-IV) to compare nutrient leaching responses between different the sand contents. In experiment two, we chose to add a standardized amount of water across all eight soils during the rain simulation, as this approach better replicates conditions experienced in the field.

For experiment 1, the effect of soil type and AMF inoculation on plant biomass was analyzed with a two-way ANOVA, using AMF, soil type, and their interaction as factors and block as the error term. To fulfill model assumptions all biomass data were log-transformed. For experiment 2, the total amount of leached nutrients was assessed using the same statistical method on log-transformed data values.

To assess the effect of the AMF inoculation, responses were calculated as relative effect of the inoculation treatment compared to the non-inoculated control for each substrate type. The effect size of the mycorrhizal inoculation on the root colonization was evaluated as the difference between the total colonization of the inoculated (M) and the mean of the uninoculated substrate (NM_{mean}) (Lekberg and Koide 2005) for each substrate separately (formula I).

$$\Delta\mathrm{AMF} = \mathrm{M} - \mathrm{NM}_{mean} \quad \text{(I)}$$

The mycorrhizal growth response (MGR) (Veiga et al. 2011) was used to express the effects of AMF inoculation on biomass production for each substrate. To calculate the MGR two equations are required, one for plants that perform better with AMF (formula I) and one for plants that perform better without AMF (formula II):

$$\text{if } \mathrm{M} > \mathrm{NM}_{mean}, \text{ then MGR} = \left(1 - \left(\frac{\mathrm{NM}_{mean}}{\mathrm{M}}\right)\right) \times 100\% \quad \text{(II)}$$

$$\text{if } \mathrm{M} < \mathrm{NM}_{mean}, \text{ then MGR} = \left(-1 + \left(\frac{\mathrm{M}}{\mathrm{NM}_{mean}}\right)\right) \times 100\% \quad \text{(III)}$$

M biomass of the plant growing with mycorrhizal inoculum

NM_{mean} mean biomass of the plant growing in the uninoculated substrate

The effect of the mycorrhizal inoculation on plant P and N content in experiment two was evaluated analogously to the MGR of the biomass.

The effect size of the mycorrhizal inoculation on the amount of leached nutrients was expressed as the difference between the total amount of nutrients leached from the inoculated treatments (M) and the mean of the total amount of nutrients leached from the non-inoculated treatment (NM_{mean}) for each substrate separately (formula IV).

$$\Delta leaching = M - NM_{mean} \quad \text{(IV)}$$

The calculated effect sizes were assessed with a one sample t-test to determine if the difference between the inoculated and the uninoculated soil were different from zero. Furthermore, differences in effect size between substrates were evaluated using a one-way ANOVA with “soil” as a factor and “block” as the error term. Correlations between two effect sizes were assessed using Pearson’s correlation and excluded the unsterile control soil

(experiment 1) and soil H (experiment 2) from the analysis. In experiment 1, differences in effect size between the sterile and the unsterile substrate with no sand amendment were analyzed by excluding all other substrates from the analysis and performing a one-way ANOVA with substrate as a factor and block as the error term.

In the text, all figures and tables presented show estimates of the means with their standard error (SEM) or confidence interval (CI, confidence level = 95%). In some cases in experiment 1, total dissolved P was smaller than the phosphate measured in the leachate resulting in negative values for unreactive P. These values were set to zero for statistical analysis and visualization. Additionally, the root biomass data contained three missing values (100% sand: M and NM, 25% sand: M). In experiment 2, one microcosm (soil B-control) was discarded from all analyses because only 4 out of 12 *Lolium* plants survived over the course of the experiment despite repeated replanting.

Results

Experiment 1: Effect of sand content on the mycorrhizal effect on nutrient leaching

Mycorrhizal colonization

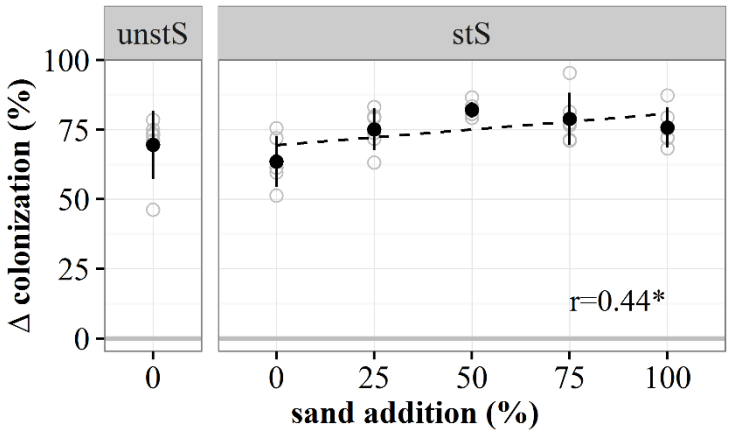

Figure 2. Difference in mycorrhizal root length colonized (%) between mycorrhizal (M) and non-mycorrhizal (NM) microcosms for five different sand-soil mixtures which were sterilized (stS) or left unsterilized (unstS). Means (black points) and 95% confidence intervals (CI) are depicted as well as single values (grey circles). The M and the NM treatment differ significantly from each other when CIs do not cross the base line (0). The effect of *R. irregulare* inoculation increases with increasing sand content (r=0.44, p=0.016).

Roots in the non-mycorrhizal control treatments with sterile substrate remained uncolonized (<1.5%) during the course of the experiment, whereas plant roots in uninoculated unsterile soil showed a mean total root colonization of 7.8% (supplements Table S1). Inoculation with *R. irregulare* significantly increased total root colonization by 63.5 to 82.1% in all substrates (Figure 2). The effect of inoculation on mycorrhizal colonization slightly increased with increasing sand content (and decreasing nutrient availability) (r=0.44, p=0.016). Root colonization was increased by inoculation to the same extent in sterilized and unsterilized field soil ($F_{1,5}$=1.33, p=0.3).

Growth responses

Analysis of the aboveground biomass showed that both *Lolium* and *Trifolium* growth were highly affected by mycorrhizal inoculation and sand content (significant "AMF" x "sand content" interaction: *Trifolium* $F_{4,45}$=3.96, p= 0.008, *Lolium* $F_{4,45}$=94.28, p<0.0001, supplements Table S2). *Trifolium* growth was highly dependent on mycorrhizal presence in both substrates, but the extent of the positive response was affected by the sand content ($F_{4,20}$=13.76, p<0.0001, Figure 3). An increasing sand content intensified the AMF induced growth response (r=0.64, p=0.00014, supplements Table S3) of *Trifolium*. Further evidence of the strong mycorrhizal dependency of *Trifolium* is demonstrated by the positive correlation between *Trifolium* shoot biomass and root colonization (r=0.97, p<0.0001, only sterile substrates). Mycorrhizal growth responses of *Lolium* highly varied between substrates with different sand contents ($F_{4,20}$=275.09, p<0.0001, Figure 3) ranging from 51.4±1.6% in the no sand substrate to -47.2±2.9% in 100% sand. These results are indicative of a significant relationship between MGR and sand content (r=-0.94, p<0.0001). *Lolium* biomass was higher than *Trifolium* in every treatment except for the inoculated substrate consisting of 100% sterile sand (supplements Table S1). Mycorrhizal inoculation enhanced the relative abundance of

Trifolium compared to *Lolium* in all substrates, indicating that AM fungi improved interspecific plant competition of *Trifolium*.

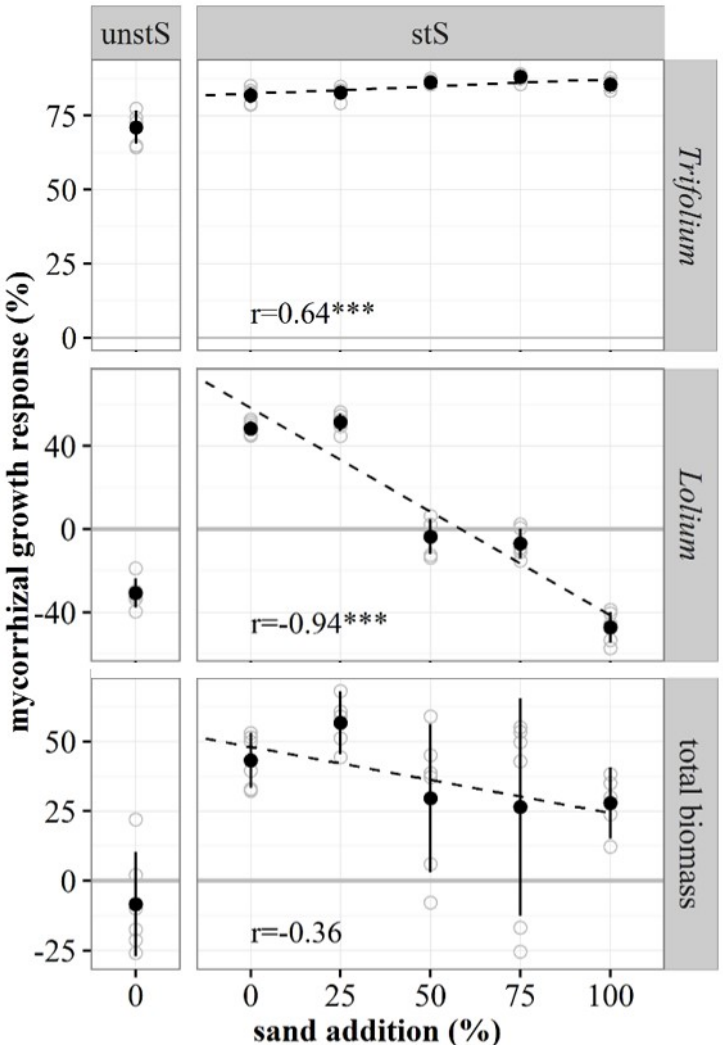

Figure 3. Mycorrhizal growth response (%) of *Trifolium*, *Lolium* and total biomass (shoots and roots) for five different sterilized (stS) or unsterilized (unstS) sand-soil mixtures. Means (black points) and 95% confidence intervals (CI) are depicted, as well as single values (grey circles). Negative values show that the presence of AMF reduce biomass production while positive values indicate that biomass is higher when AM fungi are present. The mycorrhizal growth response differs significantly from zero when CIs do not cross the base line (0). The relationship between the sand content of the substrate and the mycorrhizal growth response is indicated by the dashed line and expressed with the correlation coefficient *r* (* p<0.05, ** p<0.01, *** p<0.001). Total biomass differed significantly between sand treatments ($F_{4,18}$=3.77, p=0.02).

Leaching responses

Phosphorus

The effect of mycorrhizal inoculation on the total amount of dissolved P leached from the microcosms was highly dependent on the sand content of the substrate ($F_{4,20}$=19.11, p<0.0001, Figure 4, supplements Table S4) In general, there was a significant correlation between increasing sand content and a greater mycorrhizal reduction of P leaching (r=-0.51,p=0.0044, supplements Table S3). The substrate effect on total dissolved P was mainly

driven by the response of phosphate leaching. The effect of mycorrhizal presence on the amount of leached phosphate varied between sand contents ($F_{4,20}$=17.81,p<0.0001, Figure 4). Low sand content tended to increase phosphate leaching after inoculation compared to the non-mycorrhizal control (25% sand: t_5=9.17, p=0.0003, supplements Table S5), whereas in higher sand content substrates AMF presence reduced the loss of phosphate compared to the non-mycorrhizal control (100% sand: t_5=-4.29, p=0.0078, r=-0.70, p<0.0001). On a field scale, the change in phosphate leaching ranged from an increase of 0.03±0.003 kg PO_4^{3-} per ha to a reduction of 0.05±0.01 kg PO_4^{3-} per ha. Leaching losses of dissolved unreactive P tended to be reduced by AMF presence compared to the non-mycorrhizal control, but this effect varied with the sand content ($F_{4,20}$=22.96,p<0.0001). The mycorrhizal effect on unreactive P in the leachate ranged from a reduction of 0.024±0.003 kg/ha to an increase of 0.016±0.005 kg/ha. The microbial complexity of the soil (sterilized vs. unsterilized) determined the P leaching response after mycorrhizal inoculation compared to the control. In natural soil, no mycorrhizal effect on unreactive P leaching was detected, whereas it was reduced in the sterilized counterpart ($F_{1,5}$=24.83,p=0.0042). Phosphate leaching was significantly reduced in natural soil by mycorrhizal presence but was slightly increased in the sterilized substrate ($F_{1,5}$=47.70,p=0.0012).

Nitrogen

Nitrogen leaching was highly affected by mycorrhizal presence. In general, total mineral N (NO_2^-, NO_3^-, NH_4^+) losses via leaching were reduced when *R. irregulare* was present, but the amount of N retained in the substrate depended on the sand content ($F_{4,20}$=261.98,p<0.0001, Figure 4, supplements Table S4). A higher percentage of sand reduced the amount of mineral N retained in the soil by AMF presence (r=0.89, p<0.0001, supplements Table S3). The largest mycorrhizal effects were obtained in sterilized soil with no sand added (46 kg N/ ha less were leached with AMF inoculation). The effects on total mineral N leaching were consistent with nitrite and nitrate leaching. In the sterilized soil with no sand amendment, *R. irregulare* inoculation reduced nitrate leaching by 36.5 kg/ ha and nitrite leaching by 9.5 kg/ha compared to the non-mycorrhizal control. However, with increasing sand content, the amounts of nitrate and nitrate lost in the mycorrhiza treatments approached those of the control. The mycorrhizal effect on ammonium leaching was not influenced by sand content ($F_{4,20}$=1.89,p=0.15, Figure 4). Mycorrhizal inoculation only significantly reduced ammonium losses compared to the control in substrates with no sand addition. In the other substrates the mycorrhizal treatments did not differ from each other. Similar to nitrate and nitrite leaching, increasing sand content in the substrate resulted in more ammonium leached from the AMF treatment compared to the mycorrhizal control (r=0.42, p=0.021).

Interestingly, soil sterilization by autoclaving highly affected the mycorrhizal effect on N leaching (Figure 4). In natural soil, AMF inoculation did not change the amount of nitrate and nitrite leached compared to the non-mycorrhizal control. However, in the sterilized substrate, agriculturally significant amounts of nitrate and nitrate were retained by AMF presence. AMF inoculation significantly reduced ammonium leaching in both substrates (sterilized and unsterilized), but 138% more ammonium was retained in the sterilized soil compared to the unsterilized soil.

The effects on nitrate and nitrite leaching were correlated with total root colonization (NO_3^-: r=0.49, p=0.0057, NO_2^-: r=0.62, p=0.00025, supplements Table S3) and *Trifolium* (NO_3^-: r=0.69, p<0.0001, NO_2^-: r=0.74, p<0.0001) and *Lolium* (NO_3^-: r=-0.89, p<0.0001, NO_2^-: r=-0.84, p<0.0001, Figure 5) mycorrhizal growth response. In conclusion, the higher the aboveground ratio of *Trifolium* to *Lolium*, the smaller the mycorrhizal reduction in nitrate and nitrite leaching (NO_3^-: r=0.45, p=0.014, NO_2^-: r=0.45, p=0.013). The mycorrhizal effect on

ammonium leaching was correlated with the mycorrhizal growth response of *Lolium* (r=0.42, p=0.021).

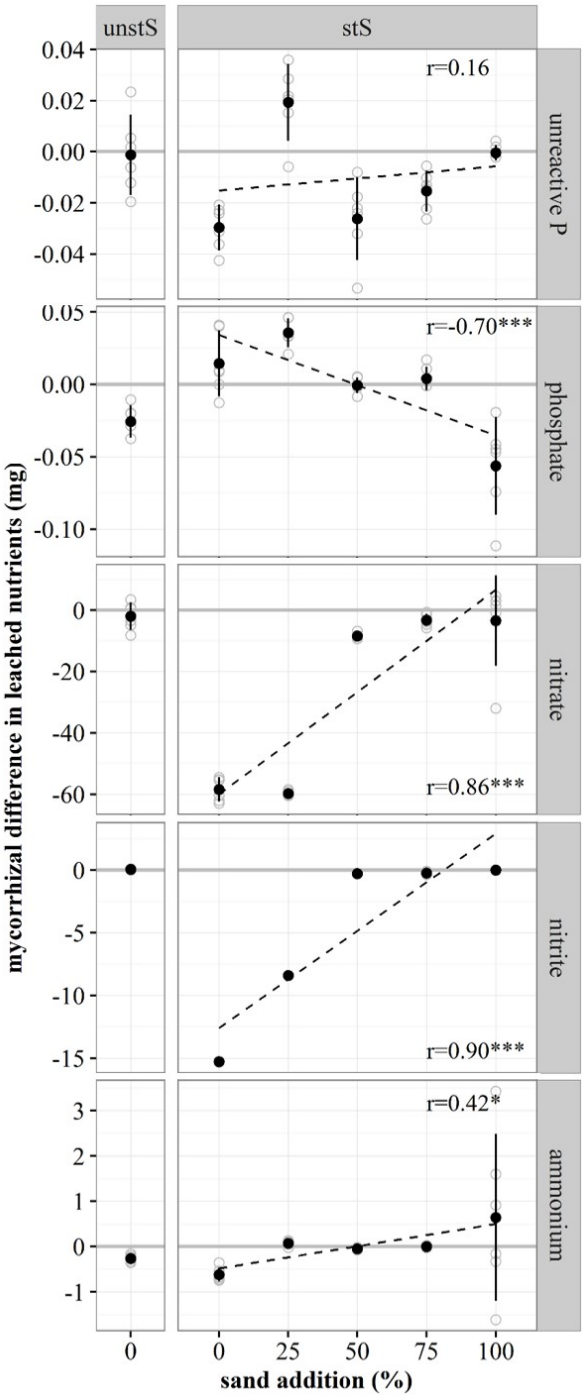

Figure 4. The difference in the total amount nutrients leached (mg) between mycorrhizal and non-mycorrhizal microcosms for five different sterilized (stS) or unsterilized (unstS) sand-soil mixtures. Negative values show that the presence of AMF reduce nutrient leaching while positive values indicate that leaching is higher when AM fungi are present. Means (black points) and 95% confidence intervals (CI) are depicted, as well as single values (grey circles). NM and M treatments differ significantly in nutrient leaching when CIs do not cross the base line (0). The relation between the sand content and the mycorrhizal nutrient leaching response is depicted by the dashed line and expressed with the correlation coefficient r (*** p<0.001, ** p<0.01, * p<0.05).

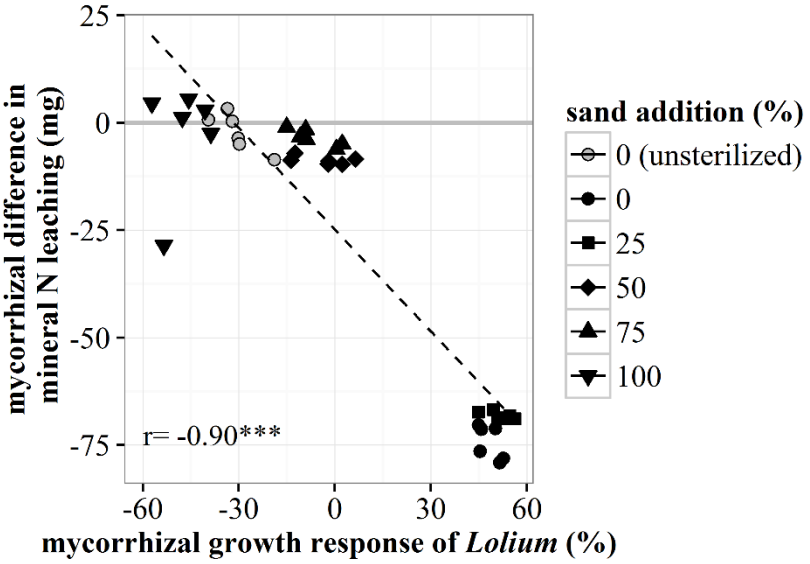

Figure 5. Relationship between the mycorrhizal effect size of total mineral N leached (mg) and the mycorrhizal growth response of *Lolium* (%).

Experiment 2: Effect of mycorrhizal abundance on nutrient leaching for eight different natural soils

Inoculation effects of *R. irregulare* on mycorrhizal root colonization, biomass production and nutrient uptake for the eight field soils are published in Köhl et al. (2015).

Leaching responses

No interaction between inoculation treatment and soil type was detected for any of the analyzed nutrients, indicating that the soil type did not affect the nutrient leaching response to inoculation (supplements Table S6). Inoculation with *R. irregulare* had no effect on the overall leaching response in the different soils, whether the control soil H was included in the analysis or not. However, soil type significantly affected the total amount of nutrients leached. Total amounts of nutrients leached from microcosms ranged from 0.3 to 5.2 kg/ha for total mineral N and from 0.1 and 0.4 kg/ha for total dissolved P.

The inoculation effect on the leaching response of each soil type was further examined using the effect size, e.g., the difference in the total amount of nutrients leached between the inoculation treatment and the non-inoculated control for each field soil (Figure 6). A one-way ANOVA using soil type as a factor revealed that the difference in nutrient leaching between the inoculation treatment and the non-inoculated control is determined by the soil type for NH_4^+ ($F_{6,28}$=2.88, p=0.026) and NO_3^- ($F_{6,28}$=5.16, p=0.001). Inoculation driven changes in leaching were highest for nitrate, ranging from –1.94 to 1.97 kg/ ha. Conversely, differences in ammonium leaching were very low, ranging from -0.01 to 0.04 kg/ha.

Individual t-tests within the different soil types revealed that leaching of unreactive P in soil E was increased with inoculation (t_5=4.55, p=0.006, 0.03 kg unreactive P/ ha, supplements Table S9), while nitrate leaching was decreased (t_5=-2.58, p=0.049, -1.93 kg NO_3^-/ha). Furthermore, AMF inoculation reduced NH_4^+ leaching in soils A (t_5=-3.00, p=0.03, -0.01 kg NH_4^+/ha) and D (t_5=-2.60, p=0.048, -0.01 kg NH_4^+/ha), as well as and NO_2^- leaching in soil D (t_5=-5.39, p=0.003, -0.01 kg NO_2^-/ha).

As a result of AMF inoculation, mycorrhizal root colonization was significantly increased in *Lolium* roots in all soils and in *Trifolium* roots in four out of eight soils (B, C, D,

H) (for results see Köhl et al. (2015)), but correlation analysis did not reveal a causal relationship between the root length colonized by AMF and the total amount of nutrients leached (supplements Table S8). The growth response of *Lolium* to mycorrhizal inoculation partly explained the amount of nutrients leached from the microcosms, with increasing biomass production correlating with less nitrate ($r=-0.52$, $p=0.0004$) and phosphate ($r=-0.41$, $p=0.0073$) leaching. Surprisingly, this correlation was reversed for unreactive P leaching ($r=0.43$, $p=0.0047$). We observed a similar trend for the correlation between the mycorrhizal response in P uptake and P leaching. Phosphate leaching decreased ($r=-0.47$, $p=0.0018$), while unreactive P leaching increased ($r=0.39$, $p=0.01$), as a result of increased plant P uptake due to AMF inoculation.

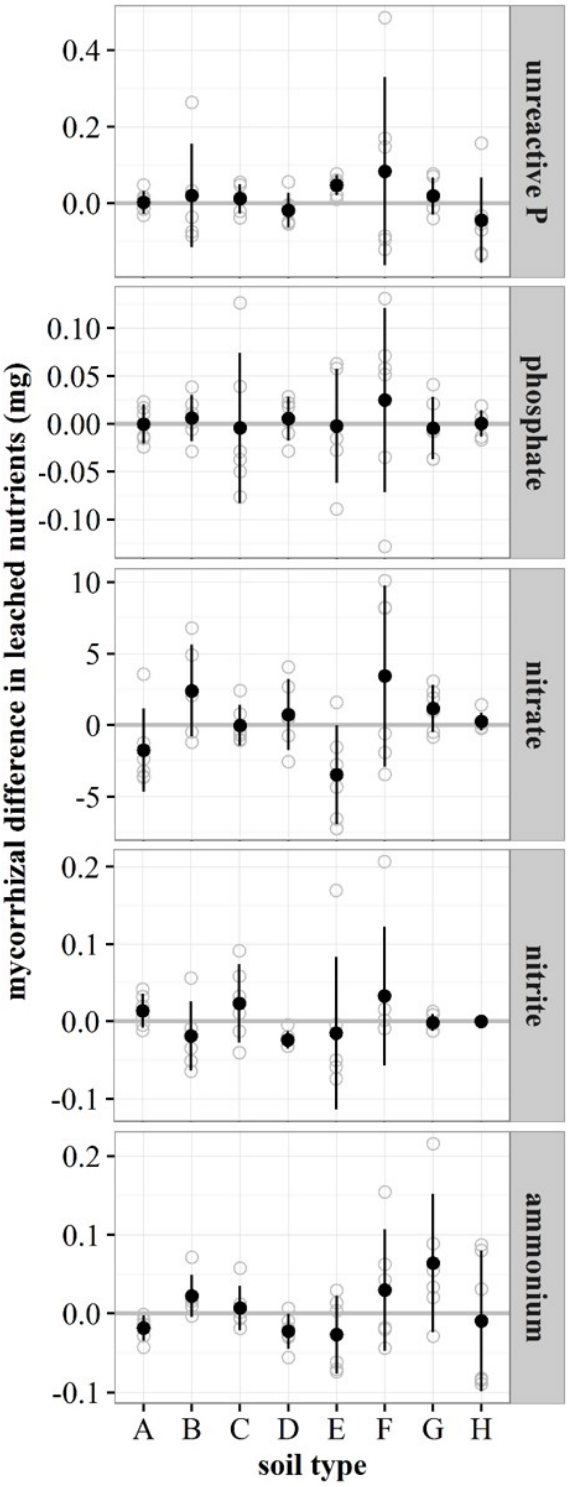

Figure 6. The difference in the total amount nutrients leached (mg) between microcosms *R. irregulare* inoculated or non-inoculated microcosms. Means (black points) and 95% confidence intervals (CI) are depicted, as well as single values (grey circles). Negative values show that the presence of AMF reduce nutrient leaching while positive values indicate that leaching is higher when AM fungi are present. NM and M treatments differ significantly in nutrient leaching when CIs do not cross the base line (0).

Discussion

Our study demonstrates that mycorrhizal presence can substantially reduce nitrogen losses from experimental soil microcosms. Furthermore, we have shown that the mycorrhizal effect on nutrient leaching in sterile systems is dependent on the sand content of the substrate and thus, presumably on its leaching ability, nutrient availability, soil organic matter content, and other correlated parameters. On the contrary, in unsterile field soils an increase in AMF abundance does not necessarily affect nutrient losses via leaching; although positive effects on mycorrhizal root colonization and plant biomass can be observed. Generally, in the two experiments performed in this study, we could not detect remarkable changes in nutrient leaching due to AMF inoculation of unsterile soil. These considerable differences between sterile and unsterile soil emphasize the great impact soil sterilization can have on the experimental outcome and conclusions from sterile systems should be carefully drawn. Additionally, we have demonstrated that mycorrhizal inoculation of sterile and unsterile soils can greatly affect biomass production and plant community structure and, in confirmation with the available literature, that these plant responses are highly substrate dependent.

AMF affect nitrate leaching from experimental soil microcosms

The most pronounced AMF effects on nutrient leaching in sterile substrates were observed for nitrate, in which mycorrhizal inoculation significantly reduced nitrate leaching up to 37 kg N/ ha. This observation is congruent with other studies demonstrating a mycorrhizal driven reduction of nitrogen leaching of up to 30 kg $N\text{-}NO_3$/ha (Bender 2014) and 60.4 kg $N\text{-}NO_3$/ha (Köhl and van der Heijden 2016) compared to a non-mycorrhizal control. However, the complex nature of this issue is reflected in opposed studies reporting no effect of AMF on nitrate leaching (van der Heijden 2010), an increase in nitrate losses in the presence of mycorrhizal (Köhl et al. 2014) or even an increase and a decrease within the same study (Bender et al. 2015). These contradictions about the relationship between AMF and nitrate leaching stress the necessity to identify the factors that explain context dependent leaching responses and the mechanisms behind them. Compared to nitrate, only a negligible amount of ammonium was lost via leaching and was not generally affected by AMF inoculation, except for a small reduction in the non-sand amended substrate (0.39 kg N-NH4/ha, Figure 4). This is not surprising, as ammonium has a low soil mobility (Havlin et al. 2005) and can be readily transformed into nitrate (Jackson et al. 2008). As a result of this rapid conversion, an early mycorrhizal interception of ammonium (Tanaka and Yano 2005) could reduce the available amount of mobile nitrate and further limit nitrogen losses. Data on the effects of AMF on ammonium leaching are as conflicting as those for nitrate. For example, a study with grasses did not reveal a notable effect of AMF inoculation on ammonium losses (van der Heijden 2010), and a study with a grassland mixture observed a reduction of 0.05 to 0.17 kg $N\text{-}NH_4$/ha upon AMF addition (Köhl et al. 2014). When *Trifolium* was used as host plant, ammonium leaching was increased by up to 0.17 kg $N\text{-}NH_4$/ha (Köhl and van der Heijden 2016), whereas ammonium losses from pots with a grass were partly reduced.

We assume that enhanced soil N immobilization resulting from increased nitrogen interception by the mycorrhizal root system is a major mechanism for AMF effects on N leaching. Through their widespread mycelium, AMF can increase the absorptive surface of the host plant's root system, forage a larger soil volume for nutrients, and store substantial amounts

of N in their biomass for transfer to the host plant (Johansen et al. 1992, Hodge and Fitter 2010). As microbial and plant N from sterile substrates was not determined in this study, we can only hypothesize that storage of N in plant and microbial biomass reduced nitrogen losses. However, in the two substrates with the greatest mycorrhizal reduction in N leaching (grassland soils with 0% or 25% sand amendment), plant available soil N was also greatly reduced (supplements Table S1), suggesting that more nitrate was immobilized and not thus available for leaching (Asghari and Cavagnaro (2012)).

Leaching of individual phosphorus compounds as well as of total P was both increased and decreased by AMF addition, but these trends were mostly not significant, especially not in unsterile soil. Because the total amount of P that was leached was very small, we do not consider the observed effects to be agroecologically relevant. The highest P leaching reduction by AMF compared to the non-mycorrhizal control was 40 g/ha after a heavy rainfall event on a substrate at water holding capacity. Several other greenhouse studies have investigated how AMF influence P losses (Asghari et al. 2005, van der Heijden 2010, Corkidi et al. 2011, Köhl et al. 2014, Bender et al. 2015, Köhl and van der Heijden 2016). The scope of observations in these studies ranges from positive to negative effects of AMF on P leaching without a clear tendency towards one direction. Differences in the total amount of P leached per ha in previous studies, including this one, are usually very small (e.g. 10 g/ha in van der Heijden, 2010 and 170 g/ha in Asghari et al., 2005) and seem to be agronomically insignificant in comparison to the typically high fertilizer input in these experiments and the total amount of P that can be lost due surface run off in some areas (Sims et al. 1998). Vertical leaching of P, especially in amounts like those reported in previous studies, likely plays a minor role in the eutrophication of surface waters. As phosphate binds to most soils and sediments, P is usually introduced by surface flows (Correll 1998, Daniel et al. 1998). We assume that P amounts in the leachate affected by AMF will be less relevant for surface water eutrophication. In conclusion, a number of studies imply that AMF are of little importance for a direct reduction of P leaching in agro-ecosystems. Nevertheless, AMF are of utmost importance for plant P nutrition and can be a valuable tool to reduce fertilizer input, therefore contributing indirectly to a general reduction in P losses.

The mycorrhizal effect on nutrient leaching in sterile systems is dependent on the sand content

Effects of mycorrhizal inoculation were not universal, but highly dependent on the composition of the substrate. Whereas the mycorrhizal effect on nitrate leaching was reduced with increasing sand content, AMF retained more phosphate from being leached as the sand content increased (Figure 4).

Soils with low water and nutrient retention capacity, like sandy soils, are especially prone to nutrient leaching (Havlin et al. 2005). Although we cannot directly compare the amount of nutrients leached in our experiment across the different substrates, as different raining volumes were used (corresponding to 100% field capacity), we noted a trend towards higher leaching in the 100% sand substrate.

Our data suggest that the observed substrate effects are partly indirect, as the sand content and thus the soil N:P ratio determined the mycorrhizal growth response. Plants growing in substrates with less sand produced more shoot and root biomass upon AMF inoculation (Figure 3). Especially *Lolium* growth was enhanced by the mycorrhizal symbiosis in the substrates with the lowest sand content. Thus, we suspect the absorptive surface of the root system was increased via the mycorrhizal pathway resulting in enhanced nutrient uptake and immobilization in plant biomass. In the substrates with the highest mycorrhizal reduction in N

leaching, inoculation with AMF reduced soil N at the end of the experiment remarkable compared to the non-mycorrhizal control. This observation further supports the explanation of an increased N immobilization in mycorrhizal treatments. In addition to biomass production, especially of *Lolium*, the plant community composition in both experiments was influenced by AMF inoculation and substrate type as well. Nitrogen, especially nitrate, leaching seems to be particularly sensitive to the identity and functional group of the host plant, which has been reported before (Phoenix et al. 2008). Studies on the mycorrhizal effect on nitrogen leaching have so far been conducted with non-N-fixing monocultures like grasses (van der Heijden 2010) and tomatoes (Asghari and Cavagnaro 2012, Bender 2014). Here, we used a model grassland system with a grass and a N-fixing legume which adds further complexity, as the biomass of the two plants is differentially and often oppositely influenced by AMF abundance (Wagg et al. 2011b, Köhl and van der Heijden 2016). We have observed in the unsterile substrate, that the mycorrhizal effect on nutrient leaching is dependent on the shoot biomass ratio of *Trifolium* to *Lolium*, with a higher proportion of *Trifolium* resulting in greater nitrate leaching (experiment 2: $r=0.5$, $p<0.0001$). Independent of AMF abundance, a grass dominated system will leach less nitrogen compared to a legume dominated system. In the presence of AMF, this reduction can be many times greater. Our observation is consistent with other studies reporting that clover abundance is positively correlated with N leaching (Loiseau et al. 2001, Scherer-Lorenzen et al. 2003, Bouman et al. 2010). Grass systems usually have a higher N efficiency, higher nutrient retention, and thus lower nitrogen losses via leaching (Simmelsgaard 1998, Aerts and Chapin Iii 1999). Scherer-Lorenzen et al. (2003) detected only very low rates of N leaching in pure grass monocultures and mixtures (<1kg NO_3-N $ha^{-1}*yr^{-1}$), whereas low diversity grasslands containing *Trifolium* had high N losses equivalent to bare ground plots (100 kg NO_3-N $ha^{-1}*yr^{-1}$). Higher N leaching from *Trifolium* dominated microcosms can also be attributed to the symbiotic N-fixing activity of the legume, which can range from 50-250 kg N $ha^{-1}*yr^{-1}$ (Ledgard and Giller 1995). Besides N_2 fixation, legume dominated plant communities usually have a higher microbial activity and, because of a low C:N ratio, faster litter decomposition rate (Swift et al. 1979, Hobbie 1992, Scherer-Lorenzen et al. 2003). Effects on the community composition of NH_3 oxidising bacteria consequently lead to an increase in nitrification rates (Hickman et al. 2010, Malchair et al. 2010). This further increases the plant and leaching available nitrogen pool of the soil.

Effect of an increased AMF abundance on nutrient leaching in unsterile soil

Over all, increasing the mycorrhizal abundance in the different unsterile soils tested in the two experiments did not reduce leaching losses of P and N, although mycorrhizal inoculation significantly increased clover biomass in six out of the nine unsterile soils (experiment 1 and 2). So far, most studies on the influence of AMF on nutrient leaching have been conducted by adding AMF inoculum to sterile substrates (Corkidi et al. 2011, Köhl et al. 2014, Bender et al. 2015, Bender and van der Heijden 2015, Köhl and van der Heijden 2016).

Growing a mycorrhiza defective tomato mutant and its mycorrhizal wildtype, only Asghari and Cavagnaro (2012) and Bender (2014) used an unsterile soil:sand mixture or pure soil substrate to avoid effects of soil sterilization. In contrast to our findings with unsterile soil, nitrate leaching was reduced by the presence of a mycorrhizal root system. NH_4-N (Asghari and Cavagnaro 2012, Bender 2014) and PO_4-P and total P (F. Bender, personal communication) leaching were unaffected by AMF presence in these studies. Whereas the mentioned studies compare to a non-mycorrhizal control, we investigated natural field soil containing an inherent AMF community and the same soil with an additional, potent AMF inoculum. Thus the studies

are only partially comparable. In a large scale correlation study it was reported that AMF may contribute to reduced N leaching (de Vries et al. 2013), as the authors showed that N leaching decreased with increasing biomass of AMF across 60 sites in Europe. In contrast, de Vries et al. (2011) concluded that even an enhanced fungal biomass is not the direct cause of better N retention when comparing high to low fungal biomass treatments. In agricultural grasslands it has been demonstrated that N leaching decreased with increasing soil fungal biomass (de Vries et al. 2006), although fungal abundance seemed to be more of an indicator than a cause for different N retention. These studies evaluated not specifically AMF biomass, but fungal biomass in general and thus, are just an indication for AMF involvement in N leaching.

The comparison of the same soil sterilized and unsterilized is surprising: nitrogen leaching between the sterilized and the unsterilized grassland soil in experiment one (Figure 4) clearly demonstrates that soil sterilization will affect the results. Whereas AMF addition did not affect nitrogen leaching in natural grassland soil, adding AMF to the same, but sterilized, soil reduced nitrogen losses by 46.4 kg N/ha. As AMF are present in most soil ecosystems, establishing non-mycorrhizal controls is a major challenge in mycorrhiza research. Sterilizing soil by autoclaving or gamma radiation is common practice but can alter soil chemical properties, in particular soil nitrate and ammonium levels (Salonius et al. 1967, McNamara et al. 2003). For example in experiment 1, autoclaving the pasture soil decreased nitrate content of the substrate and increased ammonium content (see Table 1). A much greater impact of sterilization on nutrient availability and cycling is caused by a reduction of the soil biota, although a so-called microbial wash (Ames et al. 1987, Koide and Li 1989) is often applied to reintroduce microorganisms smaller than fungal spores. Nevertheless, the remaining microbial diversity will be greatly reduced and devoid of most soil fauna like, collembola, nematodes, mites, earthworms, and others which are important for nutrient cycling and other ecosystem services (Wagg et al. 2014). We assume that the complex microbial background present in unsterile soil has a much greater impact on nutrient immobilization and will obscure mycorrhizal effects on nutrient leaching. Soil microbes determine processes of N loss and retention by controlling the form and availability of N in soil. Numerous processes related to soil nutrient cycling like N-fixation, mineralization and solubilisation of nutrients, denitrification and nitrification, and nutrient distribution are performed by bacteria, fungi, and soil-dwelling animals, most of which are excluded from artificial greenhouse experiments. In general, bacterial-dominated microbial communities are linked to rapid decomposition and nutrient mineralization and consequently high nutrient supply rates, whereas fungal-dominated microbial communities typically decelerate rates of N cycling (Wardle et al. 2004), with consequences for ecosystem N losses and retention. Mycorrhizal controls established via sterilization and a microbial wash will assumingly be bacterial-dominated compared to the corresponding mycorrhizal treatment (Wagg et al. 2014) and might have a higher nutrient turnover.

Conclusion

In recent years, an increasing number of studies have addressed the question if AMF can reduce nutrient losses from soil and have provided a generally positive outlook (for review see Cavagnaro et al. 2015). Similar to the results of other studies (Asghari and Cavagnaro 2012, Bender 2014, Köhl and van der Heijden 2016), we found that AMF greatly reduce nitrate leaching in sterilized grassland compared to a non-mycorrhizal control. However, mycorrhizal effects on leaching appear to be dependent upon experimental conditions. The addition of AMF to sterilized soil clearly emphasizes their nutrient retention abilities. In contrast, increasing the

mycorrhizal abundance in unsterile field soil did not result in reduced nutrient losses, eventhough biomass production was enhanced in several cases. Furthermore, we have shown that leaching effects are dependent upon host plant species and functional group, sand content, and soil type. Phosphorus leaching appears to be minorly affected by AMF and usually in ecologically and agriculturally negligible amounts. Mechanisms underlying the impacts of AMF on nutrient leaching are only partly understood. To further investigate this simplified greenhouse experiments will be indispensable. However, to quantify the contribution of AMF to the sustainability of agro-ecosystems more field-applied, and thus complex, trials have to be conducted. This is especially important, as many farming practices as tillage, crop rotation, and fertilization affect mycorrhizal abundance and diversity (Oehl et al. 2010, Säle et al. 2015). Therefore, knowledge about the contribution of the corresponding AMF communities to nutrient cycling is essential when evaluating the sustainability of agricultural management practices.

Supporting information

Table S1. Means and SEM (in brackets) of response variables measured in experiment one (n=6).

Response	sterility	unsterile		sterile									
	sand content %	0		0		25		50		75		100	
	AMF	NM	M	NM	M	NM	M	NM	M	NM	M	NM	M
volume (mL)		834.00	882.67	772.17	774.33	672.33	632.50	659.17	663.83	603.67	601.17	584.00	569.83
		(13.24)	(4.79)	(4.14)	(6.37)	(7)	(1.45)	(9.32)	(4.24)	(4.94)	(5.42)	(5.92)	(5.86)
total dissolved P (mg)		0.06	0.03	0.19	0.17	0.07	0.12	0.08	0.06	0.04	0.03	0.19	0.13
		(0.01)	(0.00)	(0.03)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0)	(0)	(0.04)	(0.01)
unreactive P (mg)		0.02	0.02	0.13	0.10	0.06	0.08	0.07	0.04	0.04	0.03	0.002	0.002
		(0.00)	(0.01)	(0.01)	(0.00)	(0.01)	(0.01)	(0.01)	(0.01)	(0.00)	(0.00)	(0.00)	(0.00)
phosphate (mg)		0.04	0.01	0.05	0.07	0.00	0.04	0.02	0.01	0.001	0.005	0.19	0.13
		(0.01)	(0.00)	(0.03)	(0.01)	(0.00)	(0.00)	(0.01)	(0.00)	(0.00)	(0.00)	(0.04)	(0.01)
total mineral N (mg)		22.99	20.86	80.61	6.24	69.65	1.55	10.30	1.57	8.29	4.81	38.41	35.61
		(8.15)	(1.78)	(5.88)	(1.6)	(4.53)	(0.36)	(4.71)	(0.38)	(0.32)	(0.8)	(1.14)	(5.28)
nitrate (mg)		22.43	20.50	64.22	5.75	60.93	1.17	9.73	1.31	7.71	4.47	31.96	28.52
		(8.06)	(1.76)	(7.04)	(1.55)	(5.19)	(0.34)	(4.66)	(0.37)	(0.31)	(0.78)	(1.08)	(5.75)
nitrite (mg)		0.06	0.12	15.30	0.02	8.41	0.01	0.33	0.05	0.46	0.22	0.03	0.03
		(0.02)	(0.02)	(3.41)	(0.01)	(1.25)	(0.00)	(0.08)	(0.01)	(0.03)	(0.04)	(0.01)	(0.01)
ammonium (mg)		0.50	0.24	1.09	0.47	0.30	0.37	0.25	0.20	0.12	0.12	6.41	7.05
		(0.11)	(0.03)	(0.34)	(0.06)	(0.02)	(0.02)	(0.03)	(0.01)	(0.02)	(0.01)	(0.27)	(0.72)
Lolium shoots (g)		3.42	2.37	2.69	5.22	2.03	4.21	3.19	3.07	2.62	2.44	0.45	0.24
		(0.32)	(0.09)	(0.16)	(0.14)	(0.08)	(0.14)	(0.26)	(0.11)	(0.08)	(0.07)	(0.01)	(0.01)
Trifolium shoots (g)		0.27	0.97	0.13	0.75	0.16	0.92	0.18	1.34	0.13	1.13	0.15	1.06
		(0.02)	(0.07)	(0.01)	(0.04)	(0.01)	(0.04)	(0.01)	(0.03)	(0.00)	(0.05)	(0.01)	(0.05)
root biomass (g)		4.87	4.59	4.59	7.38	3.65	8.91	5.03	8.84	5.25	9.36	1.06	1.07
		(0.51)	(0.67)	(0.82)	(0.88)	(0.57)	(1.31)	(0.78)	(1.87)	(1.32)	(2.16)	(0.13)	(0.12)
total root colonization (%)		7.78	77.29	0.00	63.52	0.25	75.37	0.08	82.15	0.00	78.84	0.00	75.76
		(1.57)	(4.77)	(0.00)	(3.6)	(0.25)	(2.93)	(0.08)	(1.07)	(0.00)	(3.67)	(0.00)	(2.78)
arbuscular root colonization (%)		0.97	6.82	0.00	3.72	0.00	6.33	0.00	3.89	0.00	6.18	0.00	10.72
		(0.44)	(1.94)	(0.00)	(1.12)	(0.00)	(1.25)	(0.00)	(1.34)	(0.00)	(1.26)	(0.00)	(5.34)
soil NH_4-N		4.27	4.23	2.13	1.14	2.31	1.31	1.64	1.25	0.40	0.33	5.13	4.87
		(0.27)	(0.24)	(0.18)	(0.23)	(0.25)	(0.21)	(0.43)	(0.15)	(0.13)	(0.16)	(0.15)	(0.4)
soil NO_3-N		1.53	1.94	16.92	1.49	8.47	0.93	2.19	1.26	2.62	1.93	2.33	1.54
		(0.81)	(0.42)	(1.29)	(0.53)	(1.2)	(0.25)	(0.35)	(0.29)	(0.14)	(0.09)	(0.32)	(0.18)

Table S2. Statistical results of assessing the plant and mycorrhizal parameters using two-way ANOVA with soil, AMF treatment and their interaction as factors and Block as the error term for experiment one. Analyses were conducted with and without the data from the nonsterile control substrate. Biomass data were log-transformed, and colonization data were asin(sqrt/100)-transformed.

Response		**all sand treatments**			**without unsterilized substrate**		
		Df	**F**	**p**	**Df**	**F**	**p**
Trifolium	Block	5	0.43	0.8240	5	0.23	0.9468
	AMF	1	2688.23	**<0.0001**	1	2726.42	**<0.0001**
	Soil	5	17.75	**<0.0001**	4	15.54	**<0.0001**
	Soil:AMF	5	12.62	**<0.0001**	4	3.96	**0.0077**
	residuals	55			45		
Lolium	Block	5	3.08	0.0161	5	3.21	0.0145
	AMF	1	3.55	0.0649	1	25.06	**<0.0001**
	Soil	5	714.82	**<0.0001**	4	1161.38	**<0.0001**
	Soil:AMF	5	64.65	**<0.0001**	4	94.28	**<0.0001**
	residuals	55			45		
Roots	Block	5	10.54	<0.0001	5	11.16	<0.0001
	AMF	1	23.64	**<0.0001**	1	30.83	**<0.0001**
	Soil	5	50.12	**<0.0001**	4	63.70	**<0.0001**
	Soil:AMF	5	3.21	**0.0135**	4	2.09	0.0991
	residuals	55			45		
total root colonization	Block	5	4.25	0.0025	5	2.90	0.0235
	AMF	1	4244.50	**<0.0001**	1	4466.87	**<0.0001**
	Soil	5	14.05	**<0.0001**	4	5.72	**0.0008**
	Soil:AMF	5	10.30	**<0.0001**	4	4.97	**0.0021**
	residuals	55			45		
arbuscular root colonization	Block	5	5.47	0.0004	5	3.45	0.0101
	AMF	1	179.74	**<0.0001**	1	172.47	**<0.0001**
	Soil	5	2.00	0.0936	4	1.47	0.2282
	Soil:AMF	5	1.79	0.1298	4	1.47	0.2282
	residuals	55			45		

Table S3. Correlation coefficient r of correlations between nutrient leaching effect sizes and plant and mycorrhizal effect sizes, as well as the sand content of experiment one. Significant correlations are depicted in bold (*** p<0.001, ** p<0.01, * p<0.05).

Response	**delta colonization**	**biomass MGR**			**sand content**
		Lolium	*Trifolium*	roots	
including unsterile substrate					
total dissolved P	-0.07	**0.68 *****	-0.02	**0.45 ****	**-0.35 ***
unreactive P	-0.07	-0.03	-0.17	0.01	0.05
phosphate	-0.04	**0.81 *****	0.08	**0.52 ****	**-0.44 *****
total mineral N	**0.34 ***	**-0.91 *****	0.00	**-0.42 ***	**0.55 *****
nitrate	0.31	**-0.90 *****	-0.01	**-0.44 ****	**0.53 *****
nitrite	**0.44 ****	**-0.85 *****	0.03	-0.34	**0.58 *****
ammonium	0.13	**-0.33 ***	0.23	-0.06	**0.43 ****
MGR *Lolium*	-0.21				**-0.55 *****
MGR *Trifolium*	**0.38 ***	0.08			**0.65 *****
MGR roots	0.04				-0.09
delta colonization					**0.43 ****
excluding unsterile substrate					
total dissolved P	-0.10	**0.69 *****	-0.36	**0.46 ***	**-0.51 ****
unreactive P	0.01	0.05	-0.19	0.17	0.16
phosphate	-0.13	**0.80 *****	-0.32	**0.45 ***	**-0.70 *****
total mineral N	**0.52 ****	**-0.90 *****	**0.72 *****	-0.34	**0.89 *****
nitrate	**0.49 ****	**-0.89 *****	**0.69 *****	-0.35	**0.86 *****
nitrite	**0.62 *****	**-0.84 *****	**0.74 *****	-0.26	**0.90 *****
ammonium	0.12	**-0.42 ***	0.28	-0.14	**0.42 ***
MGR *Lolium*	**-0.37 ***				**-0.94 *****
MGR *Trifolium*	**0.57 ****	**-0.62 *****			**0.64 *****
MGR roots	-0.13				-0.36
delta colonization					**0.44 ***

Table S4. Statistical results of assessing the effect size of the nutrient leaching response (difference between inoculated and non-inoculated treatments) using ANOVA with soil as a factor and Block as the error term for experiment one. Analyses were conducted with and without the data from the nonsterile control substrate. Additionally, the difference between the sterile and the unsterile substrate without the addition of sand was assessed.

Difference in nutrient leaching	all sand treatments			without unsterilized substrate			unsterile vs. sterile substrate		
	Df	**F**	**p**	**Df**	**F**	**p**	**Df**	**F**	**p**
total dissolved P	5, 25	19.33	<0.0001	4, 20	19.11	<0.0001	1, 5	1.36	0.2960
unreactive P	5, 25	19.81	<0.0001	4, 20	22.96	<0.0001	1, 5	24.82	0.0042
phosphate	5, 25	18.92	<0.0001	4, 20	17.81	<0.0001	1, 5	45.70	0.0011
total mineral N	5, 25	267.70	<0.0001	4, 20	261.98	<0.0001	1, 5	917.55	<0.0001
nitrate	5, 25	152.49	<0.0001	4, 20	145.32	<0.0001	1, 5	580.13	<0.0001
nitrite	5, 25	135800	<0.0001	4, 20	156200	<0.0001	1, 5	596800	<0.0001
ammonium	5, 25	1.94	0.1240	4, 20	1.89	0.1510	1, 5	56.95	0.0006
volume	5, 25	50.25	<0.0001	4, 20	19.78	<0.0001	1, 5	60.55	0.0006

Table S5. t- and p-values of one sample t-tests (df=5) with the effect size of the nutrient leaching response (difference between inoculated and non-inoculated treatments) for each of the six different substrate treatments of experiment one. Significant t-tests are depicted in bold.

Effect size		sand content (%)					
		0		**25**	**50**	**75**	**100**
		unsterile	sterile				
total dissolved P	t	-5.51	-1.41	**5.73**	**-4.54**	**-6.12**	**-4.45**
	p	**0.003**	0.218	**0.002**	**0.006**	**0.002**	**0.007**
unreactive P	t	-0.20	**-8.48**	**3.32**	**-4.15**	**-4.89**	-0.35
	p	0.850	**0.0004**	**0.021**	**0.009**	**0.004**	0.739
phosphate	t	-5.85	1.63	9.17	-0.31	1.23	-4.29
	p	**0.002**	0.163	**0.000**	0.766	0.274	**0.008**
total mineral N	t	-1.20	**-46.62**	**-188.22**	**-23.04**	**-4.36**	-0.53
	p	0.285	**<0.0001**	**<0.0001**	**<0.0001**	**0.007**	0.618
nitrate	t	-1.10	**-37.72**	**-174.92**	**-22.55**	**-4.15**	-0.60
	p	0.321	**<0.0001**	**<0.0001**	**<0.0001**	**0.009**	0.576
nitrite	t	**3.66**	**-2464.49**	**-3313.01**	**-25.44**	**-6.01**	0.39
	p	**0.015**	**<0.0001**	**<0.0001**	**<0.0001**	**0.002**	0.713
ammonium	t	**-8.71**	**-10.26**	**3.21**	**-5.05**	0.01	0.89
	p	**0.0003**	**0.0002**	**0.024**	**0.004**	0.990	0.413
volume	t	**10.15**	0.34	**-27.38**	1.10	-0.46	-2.42
	p	**0.000**	0.748	**<0.0001**	0.321	0.664	0.060

Table S6. Statistical results of assessing the nutrient leaching responses using two-way ANOVA with soil, inoculation and their interaction as factors and Block as error term experiment 2. Analyses were conducted once including and once excluding the control soil H from the data. Leached nutrients were log-transformed before analysis, leached volume was analyzed without transformation.

Response		A-H			A-G (without H)		
		Df	**F**	**p**	**Df**	**F**	**p**
total dissolved P	Block	7	3.34	0.0039	7	4.66	0.0003
	AMF	1	0.81	0.3705	1	1.63	0.2068
	Soil	7	60.34	**<0.0001**	6	69.22	**<0.0001**
	Soil:AMF	7	0.73	0.6502	6	0.64	0.6987
	residuals	72			62		
unreactive P	Block	7	1.06	0.3980	7	1.96	0.0748
	AMF	1	1.59	0.2120	1	2.31	0.1335
	Soil	7	21.92	**<0.0001**	6	12.40	**<0.0001**
	Soil:AMF	7	0.65	0.7160	6	0.56	0.7636
	residuals	72			62		
phosphate	Block	7	7.16	<0.0001	7	7.92	<0.0001
	AMF	1	0.32	0.5760	1	0.30	0.5860
	Soil	7	75.33	**<0.0001**	6	60.97	**<0.0001**
	Soil:AMF	7	0.09	0.9990	6	0.12	0.9930
	residuals	72			62		
total mineral N	Block	7	3.56	0.0024	7	4.66	0.0003
	AMF	1	1.07	0.3051	1	0.69	0.4090
	Soil	7	14.20	**<0.0001**	6	9.25	**<0.0001**
	Soil:AMF	7	1.09	0.3797	6	1.21	0.3137
	residuals	72			62		
nitrate	Block	7	3.32	0.0041	7	4.45	0.0005
	AMF	1	1.02	0.3169	1	0.51	0.4798
	Soil	7	15.17	**<0.0001**	6	10.03	**<0.0001**
	Soil:AMF	7	0.87	0.5360	6	0.96	0.4593
	residuals	72			62		
nitrite	Block	7	1.93	0.0769	7	1.39	0.2243
	AMF	1	0.20	0.6563	1	0.24	0.6273
	Soil	7	6.92	**0.0000**	6	2.25	**0.0497**
	Soil:AMF	7	1.39	0.2240	6	1.41	0.2235
	residuals	72			62		
ammonium	Block	7	1.05	0.4070	7	1.47	0.1950
	AMF	1	1.01	0.3180	1	1.40	0.2410
	Soil	7	14.15	**<0.0001**	6	9.74	**<0.0001**
	Soil:AMF	7	1.10	0.3710	6	1.22	0.3090
	residuals	72			62		
volume	Block	7	5.59	<0.0001	7	4.66	0.0003
	AMF	1	7.37	0.0083	1	6.84	0.0112
	Soil	7	227.30	**<0.0001**	6	88.99	**<0.0001**
	Soil:AMF	7	0.39	0.9064	6	0.28	0.9424
	residuals	72			62		

Table S7. Statistical results of assessing the effect size of the nutrient leaching response (difference between inoculated and non-inoculated treatments) using ANOVA with soil as factor and Block as error term experiment 2. Analyses were conducted once including and once excluding control the soil H from the data.

Difference in	**A-H**			**A-G (without H)**		
nutrient leaching	**Df**	**F**	**p**	**Df**	**F**	**p**
total dissolved P	7,33	1.04	0.42100	6,28	1.02	0.43400
unreactive P	7,33	0.73	0.64800	6,28	0.82	0.56600
phosphate	7,33	0.48	0.84530	6,28	0.46	0.82950
total mineral N	7,33	4.783	0.00085	6,28	5.299	0.00093
nitrate	7,33	4.65	0.00104	6,28	5.16	0.00111
nitrite	7,33	0.98	0.46000	6,28	1.00	0.44400
ammonium	7,33	2.14	0.06710	6,28	2.88	0.02590

Table S 8. Correlation coefficient r of correlations between nutrient leaching effect sizes and plant and mycorrhizal effect sizes for experiment 2. Significant correlations are depicted in bold (*** p<0.001, ** p<0.01, * p<0.05).

Effect size	**delta colonization**		**biomass MGR**			**shoot nutrient uptake MGR**	
	Lolium	*Trifolium*	*Lolium*	*Trifolium*	total	P	N
including soil H							
total dissolved P	-0.13	**-0.3***	0.23	-0.26	0.15	0.18	0.05
unreactive P	-0.09	**-0.3***	**0.39****	-0.19	**0.33***	**0.36***	0.24
phosphate	-0.08	0.05	**-0.41****	-0.11	**-0.43****	**-0.46****	**-0.44****
total mineral N	-0.08	0.00	**-0.52*****	0.12	**-0.32***	**-0.48*****	-0.18
nitrate	-0.08	0.00	**-0.52*****	0.12	**-0.33***	**-0.49*****	-0.19
nitrite	-0.11	-0.07	0.15	-0.12	-0.07	0.06	0.03
ammonium	-0.15	-0.19	0.03	0.05	0.2	0.07	0.21
excluding soil H							
total dissolved P	0.07	-0.18	0.26	-0.12	0.19	0.19	-0.02
unreactive P	0.10	-0.23	**0.43****	-0.03	**0.39***	**0.39***	0.21
phosphate	-0.08	0.14	**-0.41****	-0.15	**-0.44****	**-0.47****	**-0.46****
total mineral N	-0.11	0.00	**-0.52*****	0.21	**-0.33***	**-0.50*****	-0.19
nitrate	-0.11	0.01	**-0.52*****	0.21	**-0.33***	**-0.50*****	-0.20
nitrite	-0.14	-0.12	0.15	-0.19	-0.07	0.06	0.03
ammonium	-0.12	-0.22	0.05	0.24	**0.35***	0.08	0.20

Table S9. t- and p-values of one sample t-tests (df=5) with the effect size of the nutrient leaching response (difference between inoculated and non-inoculated treatments) for each of the eight different field soils for experiment 2. Significant t-tests are depicted in bold.

Effect size		**soils**							
		A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
total dissolved P	t	0.26	0.48	0.30	-0.63	2.23	1.67	1.15	-0.95
	p	0.806	0.652	0.778	0.558	0.076	0.157	0.301	0.384
unreactive P	t	0.27	0.39	0.82	-1.03	**4.55**	0.88	1.05	-1.01
	p	0.799	0.711	0.451	0.350	**0.006**	0.421	0.340	0.360
phosphate	t	-0.05	0.66	-0.14	0.61	-0.09	0.66	-0.35	0.05
	p	0.963	0.540	0.893	0.568	0.929	0.538	0.740	0.959
total mineral N	t	-1.57	1.94	0.02	0.71	-2.54	1.44	1.88	0.97
	p	0.178	0.110	0.984	0.508	0.052	0.209	0.119	0.376
nitrate	t	-1.55	1.92	-0.03	0.76	**-2.58**	1.39	1.82	1.08
	p	0.181	0.113	0.974	0.482	**0.050**	0.223	0.129	0.330
nitrite	t	1.60	-1.06	1.19	**-5.39**	-0.39	0.94	-0.30	NA
	p	0.170	0.336	0.286	**0.003**	0.709	0.391	0.774	NA
ammonium	t	**-3.00**	2.14	0.64	**-2.60**	-1.38	0.99	1.88	-0.27
	p	**0.030**	0.085	0.552	**0.048**	0.226	0.366	0.119	0.796
volume	t	1.60	1.45	0.83	2.00	**3.13**	2.19	0.84	0.00
	p	0.170	0.208	0.446	0.102	**0.026**	0.080	0.440	0.997

Table S10. Means and SEM (in brackets) of six replicates of leaching response variables measured in experiment two.

Response	A		B		C		D		E		F		G		H	
	control	**inoc**	**control**	**inoc**	**control**	**inoc**	**control**	**inoc**	**control**	**inoc**	**control**	**inoc**	**control**	**inoc**	**control**	**inoc**
volume (mL)	986.80	1003.52	1243.87	1274.74	940.31	967.72	1403.43	1457.47	1268.33	1340.28	1238.58	1271.14	1346.49	1366.31	606.77	606.85
	(30.84)	(10.42)	(30.43)	(21.36)	(51.25)	(33.14)	(17.12)	(27)	(32.18)	(23.02)	(18.66)	(14.89)	(8.94)	(23.66)	(12.7)	(17.68)
total dissolved P	0.19	0.19	0.38	0.40	0.79	0.80	0.22	0.21	0.47	0.52	0.47	0.58	0.37	0.38	0.51	0.46
	(0.02)	(0.01)	(0.04)	(0.06)	(0.04)	(0.03)	(0.01)	(0.02)	(0.02)	(0.02)	(0.07)	(0.07)	(0.02)	(0.01)	(0.04)	(0.05)
unreactive P (mg)	0.12	0.12	0.29	0.31	0.18	0.19	0.15	0.14	0.20	0.24	0.29	0.37	0.22	0.24	0.49	0.44
	(0.02)	(0.01)	(0.03)	(0.05)	(0.04)	(0.01)	(0.01)	(0.02)	(0.03)	(0.01)	(0.08)	(0.10)	(0.02)	(0.02)	(0.04)	(0.04)
phosphate (mg)	0.07	0.07	0.08	0.09	0.61	0.61	0.07	0.07	0.28	0.28	0.18	0.21	0.15	0.14	0.02	0.02
	(0.02)	(0.01)	(0.02)	(0.01)	(0.05)	(0.03)	(0.01)	(0.01)	(0.02)	(0.02)	(0.04)	(0.04)	(0.01)	(0.01)	(0.01)	(0.01)
total mineral	4.03	2.27	2.36	4.77	1.23	1.24	4.11	4.80	8.16	4.64	5.91	9.40	4.31	5.54	0.60	0.85
	(1.76)	(1.12)	(1.02)	(1.24)	(0.92)	(0.55)	(1.40)	(0.97)	(2.00)	(1.39)	(1.31)	(2.42)	(0.64)	(0.65)	(0.13)	(0.26)
nitrate (mg)	3.91	2.16	2.22	4.62	1.11	1.09	3.92	4.66	7.89	4.41	5.79	9.22	4.22	5.39	0.38	0.64
	(1.76)	(1.13)	(0.99)	(1.25)	(0.93)	(0.56)	(1.38)	(0.97)	(1.96)	(1.35)	(1.31)	(2.46)	(0.63)	(0.64)	(0.11)	(0.24)
nitrite (mg)	0.03	0.04	0.06	0.05	0.05	0.08	0.03	0.01	0.07	0.06	0.01	0.04	0.01	0.01	0.00	0.00
	(0.01)	(0.01)	(0.04)	(0.02)	(0.02)	(0.02)	(0.01)	(0.00)	(0.03)	(0.04)	(0.00)	(0.03)	(0.00)	(0.00)	(0.00)	(0.00)
ammonium (mg)	0.09	0.07	0.07	0.10	0.06	0.07	0.16	0.13	0.19	0.17	0.11	0.14	0.08	0.14	0.22	0.21
	(0.01)	(0.01)	(0.01)	(0.01)	(0.00)	(0.01)	(0.02)	(0.01)	(0.06)	(0.02)	(0.01)	(0.03)	(0.01)	(0.03)	(0.03)	(0.03)

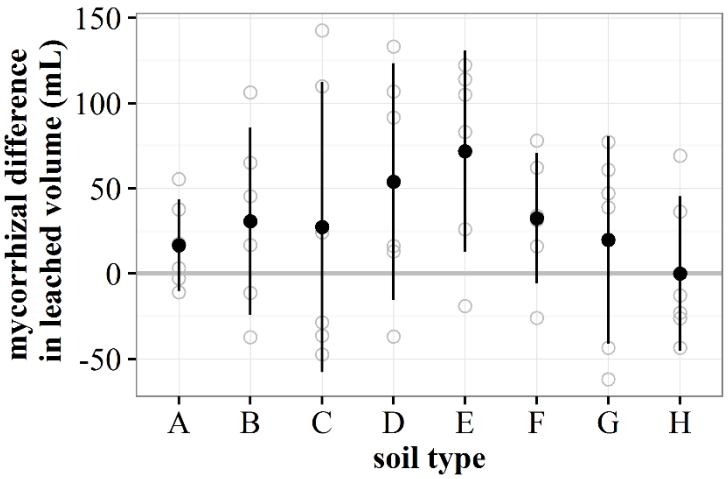

Figure S1. The difference in the total volume of leachate (mL) between microcosms inoculated with *R. irregulare* or left uninoculated for eight different soil types (A-H) for experiment 2. Means (•) and 95% confidence intervals (CI) are depicted as well as single values (o). NM and M treatments differ significantly in nutrient leaching when CIs do not cross the base line (0).

Chapter 5

Agricultural practices indirectly influence plant productivity and ecosystem services through effects on soil biota

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Abstract

It is well established that agricultural practices alter the composition and diversity of soil microbial communities. However, the impact of changing soil microbial communities on the functioning of the agroecosystems is still poorly understood. Earlier work showed that soil tillage drastically altered microbial community composition. Here we tested, using an experimental grassland (*Lolium*, *Trifolium*, *Plantago*) as model system, whether soil microbial communities from conventionally tilled (CT) and non-tilled (NT) soils have different influences on plant productivity and nutrient acquisition. We specifically focus on arbuscular mycorrhizal fungi (AMF), as they are a group of beneficial soil fungi which can promote plant productivity and ecosystem functioning and are also strongly affected by tillage management.

Soil microbial communities from CT and NT soils varied greatly in their effects on the grassland communities. Communities from CT soil increased overall biomass production more than soil communities from NT soil. This effect was mainly due to a significant growth promotion of *Trifolium* by CT microorganisms. In contrast to CT soil inoculum, NT soil inoculum increased plant phosphorus concentration and total plant P content, demonstrating that the soil microbial communities from NT fields enhance P uptake. Differences in AM fungal community composition, resulting for instance in two fold greater hyphal length in NT soil communities when compared to CT, are the most likely explanation for the different plant responses to CT and NT soil inocula.

A range of field studies have shown that plant P uptake increases when farmers change to conservation tillage or direct seeding. Our results indicate that this enhanced P uptake results from enhanced hyphal length and an altered AM fungal community. Our results further demonstrate that agricultural management practices indirectly influence ecosystem services and plant community structure through effects on soil biota.

Keywords

agricultural practice, arbuscular mycorrhiza, *Glomeromycota*, soil management, grassland, ecosystem services, soil microbial communities, tillage, conservation tillage

Introduction

The increasing need for more environmental friendly and sustainable agriculture is driving the search for alternative strategies to reduce the use of fertilizer. One way to enhance agricultural sustainability is by using management practices to manipulate soil microbial communities for increased service provisioning. But thus far, it is an option that has seldom been discussed and much less utilized (Verbruggen and Kiers 2010, Barber et al. 2013). This is surprising, as soil microorganisms facilitate numerous soil-related processes like nutrient uptake, soil aggregate stability, organic matter formation and decomposition, and water regulation, all of which are of fundamental importance for agroecosystem functioning and plant productivity.

An increasing number of studies demonstrate that agricultural practices, such as tillage regime, fertilization, crop rotation, intercropping and management type have a significant impact on the diversity, activity and abundance of soil biota (Altieri 1999, Brussaard et al. 2007, Postma-Blaauw et al. 2010, Verbruggen et al. 2010, Mulder et al. 2011, de Vries et al. 2013). However, until now, the consequences of agricultural induced shifts in soil communities have rarely been investigated (Corkidi et al. 2002, Verbruggen et al. 2012, Barber et al. 2013).

A farming practice that has a big impact on soil microbial communities is soil tillage (Feng et al. 2003b, Mathew et al. 2012). Typically two contrasting tillage regimes can be considered. Conventional tillage (CT), the most widespread tillage regime, disrupts the upper 20-35 cm soil layer and it causes a change in the physical and chemical soil conditions (Peigné et al. 2007). Consequently, the soil habitat for microorganisms is changed and thus, the microbial communities are altered. In contrast, in no-till farming (NT) the soil is not disturbed as the seeds are inserted directly into the soil without tillage. In order to enhance the sustainability of agricultural systems, no-tillage has recently been promoted to reduce soil erosion and energy use as well as increase organic matter content (Peigné et al. 2007). Several reports have shown that soil microbial biomass and activity is higher in NT fields (for review see Andrade et al. 2003, Miura et al. 2008) and the abundance of several functionally important groups of soil organisms, such as earthworms (Jossi et al. 2011, Castellanos-Navarrete et al. 2012) and arbuscular mycorrhizal fungi (AMF) (Borie et al. 2006), is enhanced in absence of soil tillage.

Analogously to the nature of the tillage practice applied, effects on the soil biota will differ with soil depth. Negative impacts of tillage in soil layers below the tillage zone can be smaller for some microbes (Miura et al. 2008). For example, mycorrhizal populations change with both soil depth and tillage practice (Douds et al. 1995). Oehl et al. (2005) observed that AMF communities in deeper soil layers vary from communities in the topsoil. As tillage only disturbs the upper 20-35 cm, a compensational effect of AMF in soil layers below the tillage zone could be considered.

In this paper we focus on arbuscular mycorrhiza fungi (AMF). These fungi form symbiotic associations with the majority of land plants, including many crops (Smith and Read 2008). AMF facilitate nutrient uptake in return for plant carbon (Smith and Read 2008). Up to 80% of plant P can be derived from AMF (Li et al. 1991), showing that they are important for plant nutrition, especially if soil nutrient availability is low. Mycorrhizal diversity and abundance has been shown to be highly affected by agricultural management such as fertilization, crop sequence, fallow periods (for review see Douds and Millner 1999) and tillage practices (for review see Kabir 2005, Alguacil et al. 2008).

Soil tillage is not tolerated by all AMF species equally and several reports have shown that a number of AMF species are highly sensitive to soil tillage and disappear in tilled fields (Boddington and Dodd 2000, Jansa et al. 2003, Castillo et al. 2006, Yang et al. 2012). As a consequence, tillage induced changes in AMF communities result in AMF community structures specific to each soil practice and often lead to a reduced mycorrhizal diversity in tilled fields (Boddington and Dodd 2000, Schnoor et al. 2011, Brito et al. 2012, Yang et al. 2012). The soil tillage induced shift of the mycorrhizal community structure may have consequences for their functioning, as AMF functional traits differ considerably among and within species (Raju et al. 1990, McGonigle et al. 2003, Smith et al. 2003). A prevalent characteristic of no-till AMF communities is that they produce more extraradical hyphae (Kabir et al. 1997, Kabir et al. 1998b, Borie et al. 2006, Curaqueo et al. 2011) and usually colonize the roots of their host plants to a greater extent than those AMF exposed to soil disturbance (Miller et al. 1995, McGonigle and Miller 1996, Galvez et al. 2001).

Thus, while it is well known that tillage influences AMF abundance and the composition and diversity of AMF communities, the functional consequences of such altered AMF communities have not been investigated so far. Soil disturbance created by tillage may select fast-growing AMF species that are less mutualistic and less efficient in improving host plant nutrients uptake (Smith and Smith 1996, Johnson et al. 1997, Scullion et al. 1998). Moreover, soil tillage destroys mycorrhizal hyphal networks, the main structures for nutrient uptake by AMF. As a consequence, soil tillage may select for AMF taxa that are resistant to disturbance and which acquire lower amounts of nutrients. As a result, we expect that nutrient uptake is reduced when plants are colonized by AMF communities from tilled soils rather than communities from non-tilled soils.

In this study we tested whether soil communities from tilled and non-tilled soil have different impacts on plant productivity and nutrient uptake, two soil ecosystem services being of key importance for agricultural productivity. Our main research questions were: 1.) are there differences in biomass production and nutrient uptake in the presence of soil communities from tilled and non-tilled fields?, and 2.) do soil communities isolated from 30-40 cm soil depth have different effects on biomass production and nutrient uptake than soil communities isolated from 0-10 cm soil depth? These research questions were tested using model grassland systems which were inoculated with soil communities originating from tilled and non-tilled fields from 0-10 and 30-40 cm soil depth. Specific attention was given to AMF as these soil fungi are known to be strongly affected by tillage management. We hypothesized that soil communities from non-tilled fields enhanced biomass production and nutrient uptake compared to soil communities from tilled fields. Furthermore, we expected that soil communities from deeper soil layers will substitute for functional losses by ploughing. The aim of this study was to investigate the microbial driven consequences of different soil management practices for agro-ecosystem functioning.

Methods

Soil inoculum

Soil inoculum was sampled from a long-term field trial where conventional tillage (CT) and no-tillage (NT) systems have been compared since 1991. This trial (named “Oberacker”), is located at the Inforama Ruetti in Zollikofen (46° 59' 17.19" N, 7° 27' 47.80" O, 527 m above MSL, Switzerland) and is performed on a Cambisol with 15% clay and 3% organic matter (for

a description of the trial and soil tillage practices see Sturny et al. (2007) and Nemecek et al. (2011)). Soil cores from four tilled and four non-tilled plots were taken in 2009 from plots cultivated with winter barley. For each tillage treatment, soil cores were taken at two soil depths (0-10 and 30-40 cm). These soil depths were chosen, as we wanted to include a soil layer which is affected by tillage and one below the ploughing zone. As ploughing homogenizes the soil and AMF propagules, we did not expect distinct differences associated with the ploughing regime in the zones below the tillage zone (e.g. at 30 – 40 cm soil depth). AMF trap cultures were set up as described in Oehl et al. (2005). Briefly, largely undisturbed soil pieces (approx. 4 cm in diameter) were taken from the soil cores and used as inoculum for the AMF trap cultures. The soil core pieces were placed at four defined locations in pots with a sterile 3:1 w/w mixture of Terragreen (American aluminum oxide, Lobbe Umwelttechnik, Iserlohn, Germany) and Loess (Tegerfelden, Switzerland). The soil inoculum comprised 5% of the total substrate weight. Four trap plant species (*Lolium perenne*, *Trifolium pratense*, *Plantago lanceolata* and *Hieracium pilosella*) were sown in the pots above the added soil core pieces. Additionally, four pots were set up as a control and received a sterilized mix of "Oberacker" soil. The trap cultures were established in April 2009 and maintained during 20 months in the greenhouse under natural ambient light and temperature conditions. During this period, the perennial trap plants were cut repeatedly 3 cm above the ground.

After 20 months, the four pot culture substrates obtained per soil depth and tillage system were air-dried and pooled together in order to use them as inocula. This approach enables us to propagate AM fungal communities characteristic for specific soil or management practices (Oehl et al. 2009).

Three 25 g soil samples were collected from each pooled inoculum and AM fungal spores were isolated using wet sieving and sugar gradient-centrifugation procedures of Sieverding (1991). AM fungal spores were subsequently identified morphologically and counted per species on prepared slides (Hawksworth 2011, Oehl et al. 2011) (for spore communities see appendix, Table B1). No AMF sporulation was detected in the non-mycorrhizal controls over 20 months.

Soil substrate

Soil was collected from a permanent grassland at Agroscope in Zürich, Switzerland (47° 25' 38.71'' N, 8° 31' 3.91'' E). The soil was 5 mm sieved and mixed with quartz sand to a ratio of 1:1 (v/v). The mixture was autoclaved at 121°C for 99 min and was subsequently stored at room temperature for 6 weeks. The autoclaved substrate had a pH of 6.9 and contained 1% humus, 1% clay and 11% silt. The substrate was phosphate poor with plant available P2O5 (extracted with CO2-saturated water) of 0.68 mg/kg and it contained 3.88 mg NH_4^+/kg and 2.93 mg NO_3^-/kg. The cation exchange capacity of the substrate was low (2.87 meq/100g) while the base saturation was classified as saturated (81.78%).

Experimental system

Grassland microcosms were established in PVC tubes with a diameter of 15.2 cm and a height of 40 cm (appendix, Fig. A1). A total of 8.6 kg sterilized substrate (dry weight) was added to each microcosm, covering 32 cm of the height (5.8 L). The substrate contained 3.5% v/v soil inoculum which was placed in two layers, 3 cm and 20 cm below surface. Each microcosm was terminated by a 500 µm PP mesh allowing excess water to leach through. For better drainage 675 g of an autoclaved sand-gravel mixture was added to the bottom of the tubes.

The microcosms were inoculated with soil microbial communities from tilled or non-tilled soil from 0-10 and 30-40 cm soil depth respectively, or microcosms received soil inoculum from non-mycorrhizal control pots. Each treatment was replicated 8 times adding up to a total of 40 microcosms (experimental units).

The microcosms were planted with a model grassland community consisting of *Trifolium pratense* L. 'Formica'(red clover), *Lolium multiflorum* Lam. 'Oryx' (Italian ryegrass) and a Swiss eco type of *Plantago lanceolata* (ribwort plantain). We chose this plant community as it is widespread in both agricultural and natural grassland ecosystems where these species commonly coexist (Nyfeler 2009). Moreover, these three plant species belong to three different functional groups (a legume, a grass, and a forb) and they respond differently to AMF (with *Trifolium* and *Plantago* as highly responsive species (Hart and Reader 2002, Wagg et al. 2011a) and *Lolium* as an unresponsive species (Wagg et al. 2011a)). Before planting, plant seeds (propagated by Agroscope, Zürich, Switzerland) were surface sterilized with 5% household bleach for 5 min and 70% ethanol for 10 min and rinsed thoroughly with sterilized water. Plants were germinated on sterile 1.5% water agar. Of each plant species 6 individuals were planted adding up to a total of 18 seedlings per microcosm.

To equalize differences in non-fungal microbial communities between the different soil inocula, and to include microbes from natural grassland, a microbial wash was added to each microcosm. The microbial wash was created from the same fresh grassland soil used for the experimental substrate and from the soil inocula used in the experiment. 900 g of the fresh field soil and 120 g of each soil inoculum including the control inoculum were suspended in 6 L deionized water and filtered through filter paper (N°598, Schleicher and Schuell, Dassel, Germany) via vacuum filtration. Every microcosm received 100 ml of the microbial wash.

Growth conditions

The plants were grown in a greenhouse with an average daily temperature of 24 ºC, nightly temperature of 18 ºC and 16 hours of light per day. Supplemental light was provided by 400 W high-pressure sodium lights when natural irradiation was lower than 300 W. Plants were kept in the greenhouse for 35 weeks between April and December 2011.

Plants were watered with deionized water 3 times a week to 80% field capacity. Blocks were rotated randomly in the greenhouse when pots were watered. Grasslands were fertilized 11, 13, 19 and 21 weeks after planting with 30 ml of a nutrient solution devoid of phosphorus (6 mM KNO_3, 4 mM $Ca(NO_3)_2*4H_2O$, 4 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4*6H_2O$ and micronutrients (50 µM KCl, 25 µM H_3BO_3, 2 µM $MnSO_4*4H_2O$, 2 µM $ZnSO_4*7H_2O$, 0.5 µM $CuSO_4*5H_2O$, 0.5 µM $(NH_4)_6Mo_7O_{24}*4H_2O$, 20 µM Fe(Na)EDTA)). This was equivalent to a nitrogen fertilization of 20 kg/ha in total. Pest management was applied when necessary and according to Swiss regulations for organic farming (predatory mites *Amblyseius cucumeris* and *A. swirskii* against thrips and Cu/S against powdery mildew).

The effect of soil microbial communities on nutrient leaching was investigated twice using a rain simulator (Knacker et al. 2004, van der Heijden 2010). In short, after six and eight months of plant growth, 20 ml fertilizer (323.9 mM NH_4NO_3, 1 mM $MgSO_4*7H_2O$, 29.3 mM KH_2PO_4, 2 mM $CaCl_2*2H_2O$) was added to each microcosm corresponding to 100 kg N and 10 kg P per ha for each fertilization event. Two days after fertilization microcosms were exposed to 1 L artificial rain provided by a rain simulator. The leachate draining off the microcosms was collected, weighed and analyzed (see appendix E).

Harvest

After 2, 4 and 6 months shoots were cut 5 cm aboveground to simulate hay making or grazing, which is typical for most grasslands in Switzerland. After 8 months at final harvest, shoots were cut at soil surface. Plants were separated per species, dried at 60°C for 48 hrs and weighed. Microcosms were emptied and roots were collected and weighed. In order to obtain finer roots, the soil substrate was homogenized and a 500 g sample was taken and washed by repeatedly decanting the watered subsamples onto a 250 µm mesh. Weighed subsamples of both root samples were dried at 60°C for 48 hrs and total root biomass per microcosm was calculated. Subsamples of both root samples were cut into pieces <1 cm, mixed in water and stored in 50% ethanol for root colonization analysis. In addition to this, soil substrate samples were collected for nutrient analysis and hyphal length quantification. Soil water content was determined gravimetrically to standardize the results for all microcosms.

Analyses

AM fungal parameters

For the analysis of mycorrhizal root colonization, roots were cleared with 10% KOH and stained with 5% ink-vinegar (Vierheilig et al. 1998). Percentage of root length colonized and frequency of hyphae, arbuscules and vesicles was quantified microscopically at a magnification of 200× with the intersect method (McGonigle et al. 1990) using 100 intersections. Extraradical hyphae in the substrate were extracted with water on a filter membrane (Jakobsen et al. 1992a) for three replicates per microcosm and hyphal length was quantified using the modified Newman formula (Tennant 1975).

Plant nutrient analysis

Shoots were pooled across harvests for each species and grinded for nutrient analysis. Total nitrogen shoot concentration was determined using a CHNSO analyzer (Euro EA, HEKAtech GmbH, Wegberg, Germany). For plant P determination grinded biomass was ashed at 600°C and digested using 6 M hydrochloric acid. Digests were diluted and the total amount of P was quantified colorimetrically according to the molybdenum blue method (Watanabe and Olsen 1965).

Experimental design and statistical analysis

The experiment was set up as a complete randomized block design in the greenhouse where each soil inoculation treatment was replicated eight times. Each replicate was assigned to one block, making a total of eight blocks. There were two factors “TILLAGE” (soil communities from CT or NT plots) and “SOIL DEPTH” (soil communities from 0-10 or 30-40 cm depth). The soil depth factor was nested within the tillage treatment. In addition to this, a non-mycorrhizal control treatment was included as a fifth treatment.

Statistical analyses were conducted using the software R 2.14.1 (R Development Core Team 2011). For each variable (shoot and root biomass, shoot nutrient content, mycorrhizal root colonization, hyphal length and N:P-ratio) an analysis of variance (ANOVA) with inoculum identity as factor was performed to test whether treatments varied from each other. Moreover, in order to test whether the non-mycorrhizal control treatment varied from the four treatments with soil inocula, a contrast was created and tested using ANOVA. The effect of “TILLAGE” and “SOIL DEPTH” was evaluated with a nested ANOVA excluding the control from the data set. In all analyses “BLOCK” was included as an error term. *Plantago* biomass

was log transformed to fulfill model assumptions. Correlations between two variables were assessed using Pearson's correlation. In the text, all figures and tables presented show estimates of the means with their standard error (SEM). *Plantago* and *Trifolium* shoot P content contained one missing value each due to insufficient biomass amount for analysis. Root biomass data comprise one missing value.

Results

Mycorrhizal communities, root colonization and hyphal length

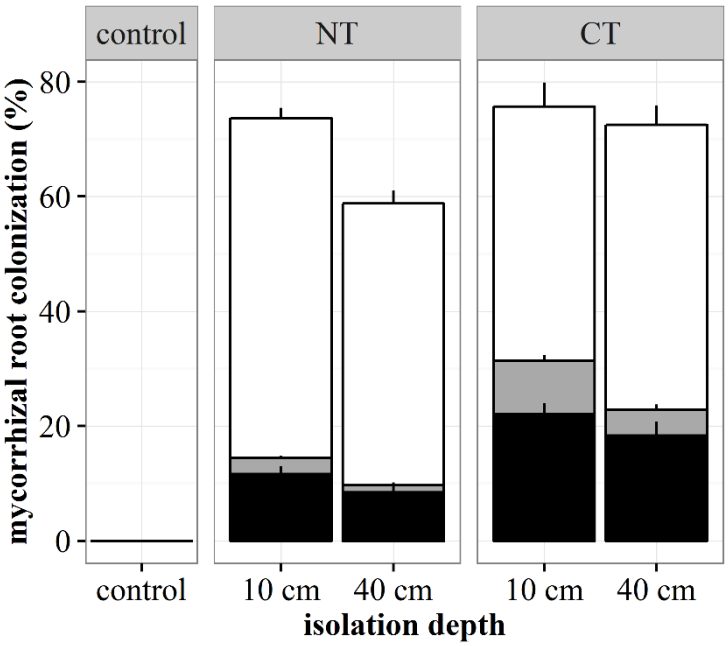

Figure 1. Percentage of root length colonized by AMF in control microcosms without AM fungi (control) or in microcosms inoculated with soil microbes from tilled (CT) or non-tilled (NT) plots for two different isolation soil depths (0-10 and 30-40 cm). Total root length colonized is depicted as the sum of the percental root length colonized by arbuscules (black), vesicles (grey) and hyphae (white). Bars are means of 8 replicates ± SEM.

Funneliformis mosseae, *Claroideoglomus claroideum* and *Glomus intraradices* showed an increased spore density in inoculum from CT compared to soil inoculum from NT (appendix, Table B1). Moreover, *Funneliformis caledonius* was only detected in CT inoculum. In contrast *Diversispora celata* was specific for NT plots. In addition to this, *Septoglomus constrictum* and a *Glomus sp. BE12.* resembling *Gl. microaggregatum* were also much more abundant in NT inoculum compared to CT inoculum. The abundance of all AMF species in tillage treatment and soil depth combinations can be found in the supplemental material (appendix, Table B1).

The degree to which the plant roots were colonized by AMF varied among the treatments ($F_{4,28}$=149.6, p<0.0001; Fig. 1). Plant roots in microcosms inoculated with soil inoculum from CT plots had a higher total root colonization as compared to those with soil inoculum from NT plots ($F_{1,21}$=7.44, p=0.013). Root colonization by arbuscules and vesicles was also significantly higher in microcosms with soil inoculum from CT plots than those from NT soils (vesicular: $F_{1,21}$=60.41, p<0.0001, arbuscular: $F_{1,21}$=30.68, p<0.0001). The soil depth

from where the soil inoculum was generated also affected the percentage of root length colonized. Total root colonization was decreased by soil depth ($F_{1,21}$=6.9, p=0.005). AM fungal communities from 30-40 cm produced significantly less intraradical hyphae ($F_{1,21}$=5.52, p=0.012) and vesicles ($F_{1,21}$=16.68, p<0.0001) than communities from 0-10 cm soil depth. After 8 months, the non-mycorrhizal control treatment had no root colonization and remained uncontaminated by AMF. Root nodules were observed in all treatments.

The length of the extraradical hyphae varied among the different treatments ($F_{4,28}$=20.33, p<0.0001; Fig. 2). Hyphal length in microcosms inoculated with soil communities from NT soils was two-fold greater than in microcosms inoculated with soil communities from CT soils ($F_{1,21}$=29.49, p<0.0001). Microcosms inoculated with soil communities from deeper soil layers (30-40 cm) produced more extraradical hyphae than AMF from the surface (0-10 cm) ($F_{1,21}$=4.13, p=0.031). Hyphal length in the non-mycorrhizal control microcosms was lowest and probably reflects dead hyphae or hyphae from non-mycorrhizal soil fungi. The extent of the extraradical hyphal network was significant negatively correlated with the percentage of root length colonized (r=-0.42, t_{30}=-2.56, p= 0.016, control excluded from analysis).

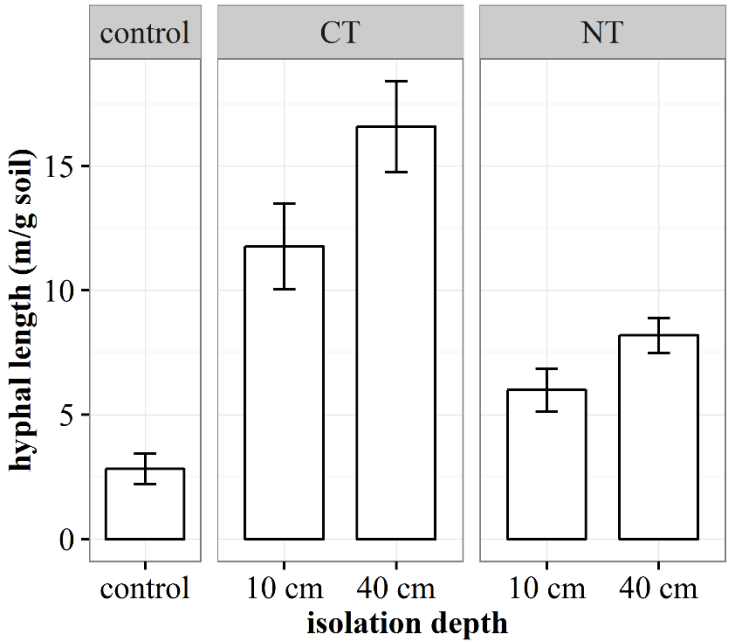

Figure 2. Length of extraradical hyphae per g of soil dry matter in control microcosms without AM fungi (control) or in microcosms inoculated with soil communities from tilled (CT) or non-tilled (NT) plots for two different isolation soil depths (0-10 and 30-40 cm). Bars depict means of 8 replicates ± SEM.

Plant biomass

Total aboveground biomass production of the grassland microcosms varied significantly among the soil inoculation treatments ($F_{4,28}$= 17.57, p<0.0001; Fig. 3). Most of the variance (87.98%) in the total aboveground biomass was explained by the control treatment differing from the other treatments ($F_{1,28}$=61.85, p<0.0001). Aboveground biomass production was highest in grassland microcosms inoculated with soil inoculum from CT plots and lowest in non-inoculated control microcosms (reduction of 22.75%). The biomass of *Plantago* and

Trifolium was on average respectively 4.96 and 21.14 fold higher in microcosms receiving soil inoculum compared to non-inoculated microcosms. In contrast, *Lolium* growth was suppressed by soil inoculation and it was highest in non-inoculated microcosms. Aboveground biomass production in grassland microcosms inoculated with soil communities from CT fields was significantly higher compared to inoculation with inoculum from NT soil ($F_{1,21}$= 5.97, p=0.024). This effect resulted mainly from increased *Trifolium* biomass in grassland microcosms inoculated with soil communities from CT plots ($F_{1,21}$=5.56, p=0.028). Biomass of *Lolium* and *Plantago* did not differ significantly between microcosms receiving soil inoculums from tilled or non-tilled plots (*Lolium*: $F_{1,21}$=0.37, p=0.55; *Plantago*: $F_{1,21}$=2.70, p=0.12). The soil depth from which the soil communities were isolated, did not explain variation in the total aboveground biomass ($F_{2,21}$=2.11, p=0.15) nor in the shoot biomass of the different species. The biomass of each plant species for each individual harvest are given in Fig. C1 of the appendix.

Root biomass differed significantly between inocula ($F_{4,27}$=7.04, p=0.0005) with lower root biomass in the treatment with NT inoculum compared to CT inoculum ($F_{1,20}$=16.61, p=0.0006). Soil depth did affect the root biomass with higher root growth with inoculum from 40 cm soil depth as compared to those from the 10 cm soil depth ($F_{2,20}$=4.86, p=0.019).

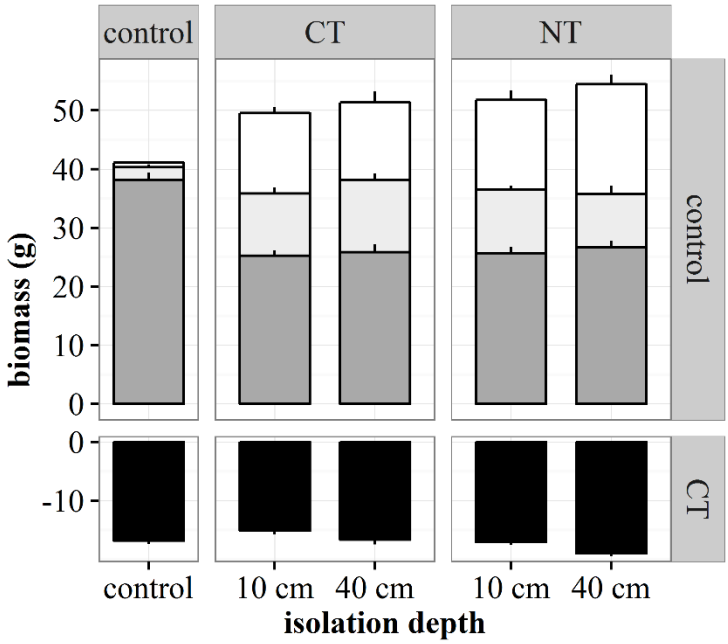

Figure 3. Aboveground and belowground plant biomass (dry weight in g) in control microcosms without AM fungi (control) or in microcosms inoculated with soil microbial communities from tilled (CT) or non-tilled (NT) plots for two different isolation soil depths (0-10 and 30-40 cm). Aboveground biomass is depicted for *Lolium* (dark grey), *Plantago* (light grey) and *Trifolium* (white). Means + SEM of 8 replicates are shown (root biomass of *Trifolium*: N=7). Total aboveground biomass is significantly higher in the CT treatment compared to the NT treatment ($F_{1,21}$=5.97, p=0.023). Less roots are produced in the NT treatment compared to the CT treatment ($F_{1,21}$=16.61, p=0.0006).

Plant P and N uptake

Inoculum source had a significant effect on total aboveground plant P and N content (P: $F_{4,26}$=101.3, p<0.0001, N: $F_{4,28}$=6.92, p=0.0005; Fig. 4 and 5). Foliar P and N content was significantly increased in grassland microcosms receiving soil inoculum (P: $F_{1,26}$=376.9, p<0.0001, N: $F_{1,28}$=19.74, p=0.00013) as compared to those in the non-mycorrhizal control. *Lolium* accumulated the highest amount of P and N due to the highest biomass production. In contrast, *Plantago* showed the highest P concentration and *Trifolium* the highest N concentration (appendix, Table C1). The effect of soil inoculum on the P and N content was highly dependent on the plant species (significant "Soil inoculum" x "plant species" interaction: P: $F_{8,96}$= 5.92, p<0.0001, N: $F_{8,98}$= 35.07, p<0.0001). Plant P and N content of *Plantago* and *Trifolium* as well as P of *Lolium* was increased when inoculated compared to the control, *Lolium* N content decreased compared to the control. Regardless of plant species, the P concentration was always increased by soil inoculum.

Inoculum source had contrasting effects on aboveground P and N content. NT soil inoculum increased total, and *Lolium* and *Plantago* P content compared to CT inoculum (total P: $F_{1,21}$=15.6, p=0.0007, *Lolium*: $F_{1,21}$=5.5, p=0.029, *Plantago*: $F_{1,21}$=11.1, p=0.0032). In contrast, total and *Trifolium* N content was higher in pots with CT inoculum (total: $F_{1,21}$=5.69, p=0.027, *Trifolium*: $F_{1,21}$=5.3, p=0.032). Regardless of plant species, the P concentration was always higher in microcosms inoculated with NT soil than those with CT soil (appendix, Table C1).

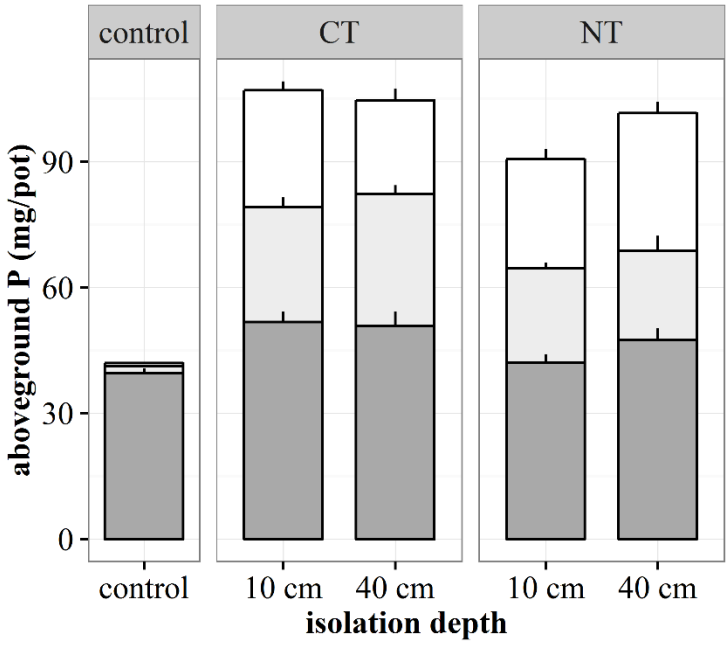

Figure 4. Total aboveground phosphorus (mg) of *Lolium* (dark grey), *Plantago* (light grey) and *Trifolium* (white) in control microcosms without AM fungi (control) or in microcosms inoculated with soil microbial communities from tilled (CT) or non-tilled (NT) plots for two different isolation soil depths. Means + SEM of 8 replicates are shown (for *Plantago* and *Trifolium* only 7 replicates in the control treatment for the P content).

Total P content of the aboveground biomass was higher with inoculum from 30-40 cm soil depth than with inoculum from the surface (nested ANOVA: $F_{2,21}$=5.17, p=0.015). Plant species specific P content as well as N content was not affected by soil depth (Appendix, Table D1).

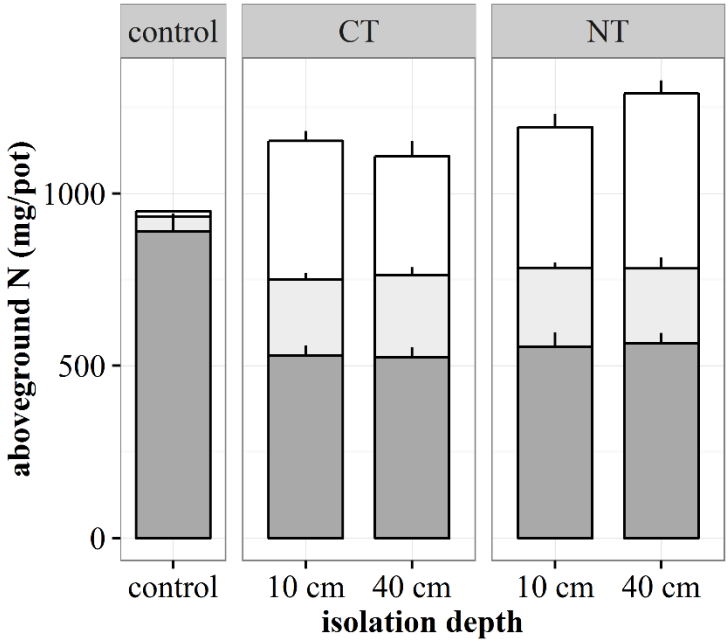

Figure 5. Total aboveground nitrogen (mg) of *Lolium* (dark grey), *Plantago* (light grey) and *Trifolium* (white) in control microcosms without AM fungi (control) or in microcosms inoculated with soil microbial communities from tilled (CT) or non-tilled (NT) plots for two different isolation soil depths. Means + SEM of 8 replicates are shown.

Aboveground P concentration and hyphal length correlated positively in treatments receiving soil inoculum (excluding the control treatment) (r=0.55, t_{30}=3.61, p=0.001). As the total aboveground biomass was negatively correlated with hyphal length (r=-0.38, t_{30}=-2.28, p=0.03), there was no correlation between hyphal length and total aboveground P content (r=0.29, t_{30}=1.67, p=0.11). There was a negative correlation between aboveground N concentration and N content respectively with increasing hyphal length (N concentration: r=-0.48, t_{30}=-2.98, p=0.0057, N content: r=-0.53, t_{30}=-3.41, p=0.002). Total root colonization did not explain any variation in aboveground P and N concentration and content when the control was excluded from the analysis (P concentration: r=-0.14, t_{30}=-0.78, p=0.44, N concentration: r=0.34, t_{30}=1.99, p=0.06).

The shoot N:P ratio can be used as diagnostic tool to evaluate the nature of nutrient limitation (Koerselman and Meuleman 1996). A N:P ratio <14 indicates N limitation whereas a ratio >16 is indicative of P limitation. N:P ratios of the three plant species in non-inoculated control microcosms were higher than 22 suggesting that plant productivity in these microcosms was P limited (Fig. 6). Inoculation with soil communities decreased shoot N:P ratios significantly for each of the investigated plant species (p<0.0001, *Lolium*: $F_{1,28}$=219.56, *Trifolium*: $F_{1,27}$=187.16, *Plantago*: $F_{1,27}$=322.63). Both *Plantago* and *Lolium* were N limited in grassland microcosms receiving soil inoculum (as indicated by an N:P ration below 14), whereas no indications about nutrient limitations were observed for *Trifolium* (which had a N:P

ratio between 14 and 16 in each of the treatments receiving soil inocula from CT or NT soils). NT soil communities intensified N limitation for *Lolium* ($F_{1,21}$=13.71, p=0.0013) and *Plantago* ($F_{1,21}$=44.94, p<0.0001) compared to CT soil inoculum as reflected by a lower N/P ratio in NT soil (Fig. 6).

Nutrient leaching

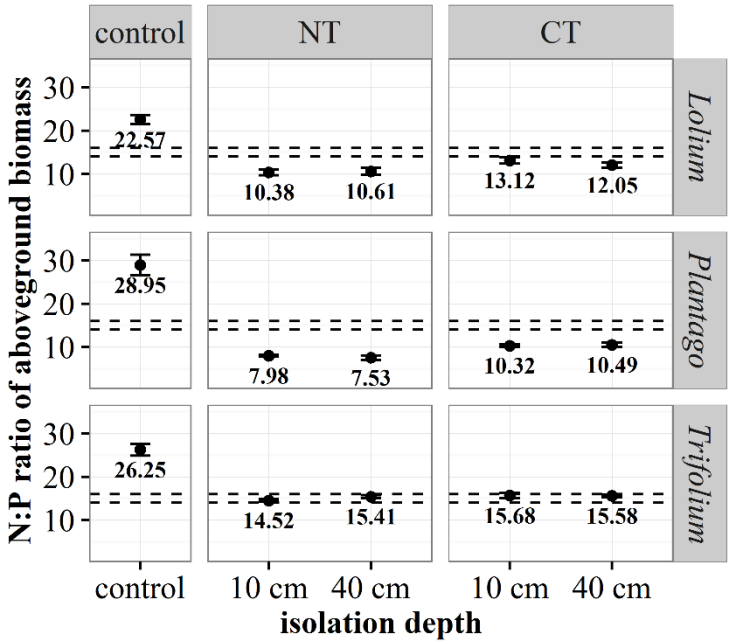

Figure 6. Shoot N:P ratios of *Lolium*, *Plantago* and *Trifolium* in control microcosms without AM fungi (control) or in microcosms inoculated with microbial soil inoculum from tilled (CT) or non-tilled (NT) plots for two different isolation soil depths. Depicted are means ± SEM of 8 replicates (for *Plantago* and *Trifolium* only 7 replicates in the control treatment). Dashed lines indicate threshold values for N-limitation (<14) and P-limitation (>16). At N:P ratios between 14 and 16 either N and P can be limiting or co-limiting for plant growth (Koerselman and Meuleman 1996).

The grassland microcosms received a simulated rain after 6 and 8 months of plant growth. This was done to assess whether the different soil communities varied in their ability to retain nutrients. In this paper we focused on the effects of the different microbial communities on plant productivity and nutrient uptake. Thus, leaching data are presented very shortly. Soil communities from CT soil reduced phosphate losses via leaching by 27.0% (corresponding to 13.09±6.52 g P/ha) compared to the NT soil community ($F_{1,21}$= 4.38, p=0.049, appendix, Fig. E1). No differences were found after 8 months of plant growths ($F_{1,21}$= 1.49, p=0.24). Unreactive P leaching did not differ between NT and CT treatments (six months: $F_{1,21}$= 1.90, p=0.18, eight months: $F_{1,21}$= 0.68, p=0.42, appendix, Fig. E1). Both, NO_3^- and NH_4^+ leaching losses were determined by the origin of the inoculum (appendix, Fig. E2). Soil communities from CT significantly reduced ammonium and nitrate losses after 6 months of plant growth by 66.5% and 37.4% respectively (NO_3^-: $F_{1,21}$= 33.73, p<0.0001, NH_4^+: $F_{1,21}$= 4.96, p=0.037) and by 28.9% and 43.2% after 8 months (NO_3^-: $F_{1,21}$= 22.63, p=0.00011, NH_4^+: $F_{1,21}$= 0.33, p=0.57).

Discussion

A wide range of studies have shown that soil tillage alters the composition and diversity of microbial communities (e.g. Jansa et al. 2002b, Borriello et al. 2012). The effect of this change in community structure on agroecosystem processes has to our knowledge not been investigated so far. The present study shows, for the first time, that soil microbial communities conditioned by distinct tillage practices provide different and partly contrasting ecosystem services. Soil communities from NT field plots increased P uptake of the grassland communities (Fig. 4) as compared to soil communities from tilled plots. In contrast, overall biomass production tended to be higher in grassland microcosms inoculated with soil communities from CT soil than those from NT soil (Fig. 3).

The increase in shoot P content in grassland microcosms inoculated with soil communities from NT soil can be explained by substantially higher amounts of extraradical hyphae in those microcosms. Extraradical hyphae have been identified before to determine the uptake and transport of P to the plant (Jakobsen et al. 1992a, b, Jansa et al. 2005, Avio et al. 2006). The correlation between shoot P concentration and extraradical hyphae in this study provides further evidence that extraradical hyphae are important for P uptake. In this study, increased P uptake did not result in increased productivity, possibly because the grassland microcosms were N-limited, rather than P limited, as indicated by N/P ratios <14 (Koerselman and Meuleman 1996) (Fig. 6). Thus, excessive fungal P facilitation could not be used for further biomass buildup. Other studies have shown that enhanced P uptake (e.g. by mycorrhiza) does not necessarily lead to enhanced plant growth (Smith et al. 2003, Lekberg and Koide 2005), indicating that there can be a sort of superfluous P consumption (Chapin 1980). Furthermore, higher microbial biomass (appendix, Fig. F1) and higher hyphal length in microcosms inoculated with NT soil inoculum indicate that more plant C was allocated belowground but could not be invested in plant shoot biomass.

It has been estimated that global phosphate stocks will be depleted within the next 50-100 years, with the quality of the mined material already decreasing and mining costs rising (Cordell et al. 2009). Hence, in the future, there will be a need to develop new methods of fertilizing crops with phosphorus. Enhanced phosphorus uptake from the soil is one way to increase phosphorus availability, especially because many soils contain large amounts of plant unavailable P (e.g. P adsorbed to mineral clay particles, Fe-, Al- or Ca-phosphates or P in organic complexes; Bünemann and Condron 2007). Moreover, in many soils large amounts of phosphorus have accumulated as a result of long-term inputs of fertilizer. Our study shows that the manipulation of microbial communities (e.g. by adapting agricultural management) can help to enhance phosphorus availability to plants by using inherent phosphorus pools and to reduce fertilizer input.

Ploughing usually affects a soil depth of 20-35 cm (Peigné et al. 2007), thus we hypothesized that soil biotic communities from 30-40 cm soil depth would substitute for soil disturbance effects (Miura et al. 2008). Although spore communities, hyphal length and root colonization levels did differ between 0-10 cm and 30-40 cm, no effect could be detected in various ecosystem functions. This suggests that soil depth is not the dominating factor in determining how soil biotic communities perform.

Our objective was to assess the functioning of soil biotic communities without the influence of existing environmental context. This was achieved by propagating predominantly specific AMF communities for two years in the greenhouse and testing their impact on plant productivity and nutrient uptake in grassland microcosms grown in sterile standard soil

substrate. We attributed the differences between CT and NT soil inoculum mainly to differences in AMF communities and not to other soil biota. We assumed this because 1.) we used relatively small amounts of soil from the field to prepare the inocula (e.g. no earthworms were present), 2.) we applied a microbial filtrate to each microcosm to equalize bacterial and fungal communities (propagules <10 µm), and 3.) our propagation system is specifically designed to propagate AMF from the field (which is also reflected by the high number of AMF species found in the inocula). Moreover plant biomass and P-uptake was correlated with hyphal length and root colonization, providing further evidence that differences in AMF communities, at least in part, explained our results. However, we cannot exclude that soil organisms other than AMF could be responsible for the observed effects.

Trifolium biomass production was most affected by the tillage inoculum treatment (Fig. 3). Furthermore, *Trifolium* was the only plant species where total biomass differed significantly between the CT and the NT treatments (appendix D1). Previous work has shown that legumes can vary in their response to inoculation with different AMF taxa (Owusu-Bennoah and Mosse 1979, Drew et al. 2003, Scheublin et al. 2007). Hence, the differences in AMF community composition between the CT and NT treatments could explain differences in *Trifolium* biomass production. Differences in *Trifolium* performance will, in turn, affect biological nitrogen fixation, as the fixation rate is positively related to biomass production (Carlsson and Huss-Danell 2003, Pimratch et al. 2008). Therefore, the increased plant nitrogen content in treatments harboring CT soil inoculum may be related to enhanced *Trifolium* productivity. This would constitute an indirect mycorrhizal effect on overall nutrient cycling, which cannot be uncoupled from the N-fixing microbial community. Although we applied a standardized microbial wash to each microcosm to reduce variation in the bacterial communities between treatments, it is possible that differences in nitrogen fixing communities associating with *Trifolium* partly explain the varying biomass response between the CT and NT treatments. This is because, plant growth responses can be dependent on the AMF-*Rhizobium* combination (Xavier and Germida 2002). Furthermore, because *Trifolium* exhibited the strongest response to the different microbial communities, the effects of the microbial communities on plant productivity would likely be lower if *Trifolium* was absent. Despite this, differences in P-uptake between CT and NT were also prevalent in *Plantago* and *Lolium* (Figure 4), indicating that microbial communities from CT and NT provided different ecosystem services irrespective of effects on *Trifolium*.

All soils we used to propagate inoculum originated from a long-term replicated field experiment with the same abiotic conditions and the same crop rotation for each plot (Nemecek et al. 2011). Thus, differences between the soil communities are exclusively due to the specific tillage treatment and not due to other factors such as soil type or location. Using our trap culture system, we were able to propagate tillage dependent soil communities. Earlier work showed that AMF community composition in trap cultures can change according to cultivation duration, substrate, and the host plant species (Sýkorová et al. 2007a, Oehl et al. 2009). This might also be the case in our experiment. Despite this caveat, we successfully propagated different spore communities (appendix, Table B1) and found 16 to 20 different AMF species in the inocula from each tillage treatments, which is a representative species number for many AMF communities in the field (Oehl et al. 2005). Moreover, the observed differences in AMF communities between the CT and NT treatments resemble some soil tillage practice dependent species responses which have been described before in the field. For instance, *G. mosseae* was increased in CT (Jansa et al. 2002b, Schalamuk et al. 2006) as well as *G. intraradices* (Jansa et al. 2002). In contrast, in this study and in another study (Borriello et al. 2012), NT enhanced

Diversosporaceae abundance. The number of AMF species present in our inocula is comparable to the species richness often found in the field (Oehl et al. 2003, Oehl et al. 2010). Despite this, it is possible that some none or rarely sporulating AMF taxa, which might be especially abundant under NT conditions (Rosendahl and Stukenbrock 2004), might not have been detected, as we did not use molecular tools to characterize the AMF communities.

In our study, important ecosystem services (plant productivity, biomass production by individual plant species and nutrient uptake) differed between soil communities from tilled and non-tilled fields. These differences are likely to be determined by specific functional traits of the AMF (such as the size of the hyphal network and the intensity of root colonization). Several studies before have shown that different AMF communities alter plant growth (Johnson 1993, Moora et al. 2004) and other ecosystem functions such as nutrient leaching (Verbruggen et al. 2012). The AMF spore communities used in this studies shared a lot of common species. However, a subset of specialists (*Funneliformis mosseae, Claroideoglomus claroideum* and *Glomus intraradices*, *Funneliformis caledonius*, *Diversispora celata*, *Septoglomus constrictum* and a *Glomus sp. BE12)* varied in abundance between the two tillage treatments or were specific for soil inoculum from tilled or non-tilled plots. These specialists were therefore most likely responsible for observed differences in productivity and nutrient uptake. A focus on AM fungal specialists might therefore be of great importance for future inoculation trials, especially in fields with P-deficiency.

In this greenhouse study, we assessed the functioning of soil biotic communities independently of other abiotic and biotic factors. Differences detected in the greenhouse might not persist under field conditions as tillage systems vary not only in the soil microbial communities but also in other parameters. Differences in soil temperature, soil mineralization, vertical nutrient distribution, soil moisture and weed pressure (for review see Peigné et al. 2007) between NT and CT systems will affect biomass production as well as nutrient uptake. These different conditions may, in part, overrule the here described effects. Verbruggen et al. (2012) reported that the effects of soil biota on agro-ecosystems are field site specific. Interestingly though, several field studies confirm our findings that P uptake (Gavito and Miller 1998, Kabir et al. 1998a, Galvez et al. 2001) and fungal hyphal length (Kabir et al. 1997, Boddington and Dodd 2000, Borie et al. 2006) are increased under NT, whereas the biomass production is often reduced (Miller et al. 1995, Gavito and Miller 1998, Galvez et al. 2001). This indicates that the driving mechanism behind tillage effects in the field may be of microbial, and especially of mycorrhizal nature.

Conclusion

In this study we demonstrate that 1.) agricultural management practices in particular tillage, influences the community composition of soil biota and 2.) that such tillage induced changes in soil biota alter a number of ecosystem services (plant productivity, nutrient uptake). The observed differences in ecosystem functioning between NT and CT soil biotic communities imply that belowground soil biodiversity has to be taken into consideration when choosing a soil management system. Beyond that, our results also indicate that soil biota should be deliberately manipulated by agricultural practices to reduce fertilizer input and increase sustainability. As NT soil communities are superior in P supply, NT might be especially interesting for fields with P deficient condition and reduced P fertilizer input. Further studies should investigate the contribution of single specialist AMF within the AMF community,

especially regarding field inoculum application. Long-term effects of specific microbial communities have to be described, as well as field inoculation studies at field sites under more natural conditions.

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Supporting information

Appendix A. Picture of the experimental system (microcosms).

Appendix B. Spore community composition of the different soil inocula determined by morphological AMF spore analysis.

Appendix C. Development of the individual shoot biomasses between the four harvests and a summary table of all means of the responses including the standard error.

Appendix D. Results for statistical analyses of the effect of soil inocula on plant biomass, shoot nutrient content and concentration, and mycorrhizal parameters, as well as correlation matrix for plant responses and mycorrhizal parameters.

Appendix E. Nutrients leached from microcosms after the simulation of rain, including a summary of the statistical analysis and a description of the method.

Appendix F. Microbial biomass C and N, including a summary of the statistical analysis and a description of the method.

Supporting information

APPENDIX A. Picture of the experimental system (microcosms).

Figure A1. Grassland microcosms made of PVC tubes two month after planting. Depicted are microcosms with control inoculum and soil inoculum from 10 cm soil depth from NT and CT (from left to right).

APPENDIX B. Spore community composition of the different soil inocula determined by morphological AMF spore analysis.

Table B1. Mean spore density, species richness, total number of species detected in trap cultures and relative spore density (%) of AMF species isolated from pooled trap cultures from CT and NT fields from 0-10 and 30-40 cm soil depth. Means ± SEM of three technical replicates are shown. No spores were detected in non-mycorrhizal control trap cultures.

Soil management	**no-till**		**tillage**	
Isolation depth [cm]	0-10	30-40	0-10	30-40
AMF spore density g^{-1} inoculum	19.0 ± 1.6	54.9 ± 0.8	12.1 ± 1.3	21.0 ± 0.8
AMF species richness	15.7 ± 0.7	12.3 ± 0.1	15.3 ± 0.3	15.3 ± 0.3
Total N° of AMF species detected in trap cultures	19	16	20	18
Acaulosporaceae				
Acaulospora longula	0.1		0.2	
Acaulospora paulinae	23.8		21.2	19.5
Acaulospora sieverdingii	1.3		0.4	
Racocetraceae				
Cetraspora helvetica	0.1		0.7	
Archaeosporaceae				
Ambispora fennica			0.2	0.6
Archaeospora trappei	0.5	0.4	0.9	3.6
Entrophosporaceae				
Claroideoglomus claroideum	0.8	0.3	6.2	8.6
Claroideoglomus etunicatum	0.8	0.3	2.9	2.1
Claroideoglomus lamellosum				0.9
Claroideoglomus luteum	16.0	7.8	19.6	16.7
Diversisporaceae				
Diversispora celata	8.5	59.3		
Diversispora przewelensis				0.2
Diversispora versiformis	0.4			
Glomeraceae				
Glomus aureum		0.6		1.9
Glomus badium		0.0		
Glomus diaphanum	0.2	0.1	0.2	0.9
Glomus fasciculatum			1.6	0.2
Glomus intraradices	1.4	0.1	14.0	11.1
Glomus invermaium		0.1		
Glomus irregulare	13.1	2.6	15.1	3.2
Glomus sp. BE12[a]	16.1	17.0		
Glomus sp. BR11[b]	1.1	0.6	2.0	2.1
Septoglomus constrictum	11.3	10.6	0.7	
Funneliformis caledonius			4.0	5.6
Funneliformis geosporus			0.7	1.5
Funneliformis mosseae	4.0	0.1	7.3	21.0
Paraglomaceae				
Paraglomus occultum	0.2		1.6	0.2
Paraglomus sp. BE9		0.1		
Scutellosporaceae				
Scutellospora calospora	0.1		0.4	

[a] Resembles *Gl. microaggregatum*

[b] Resembles *Gl. arborense*

APPENDIX C. Development of the individual shoot biomasses between the four harvests and a summary table of all means of the responses including the standard error.

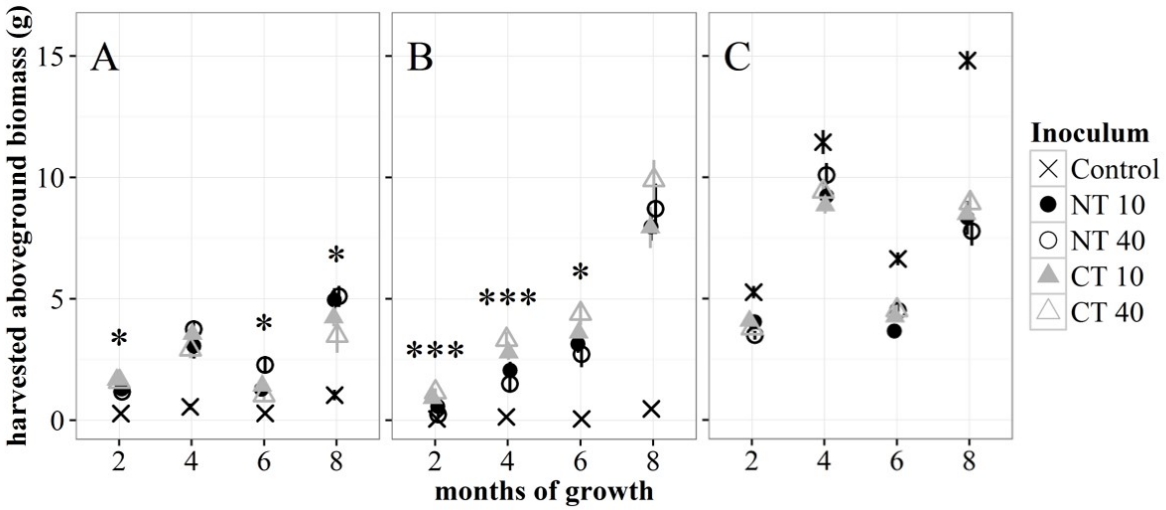


Figure C1. Aboveground biomasses (g) of A) *Plantago*, B) *Trifolium* and C) *Lolium* at each individual harvest in control microcosms without AM fungi (control) or in microcosms inoculated with soil microbial communities from tilled (CT) or non-tilled soil (NT) for 2 different isolation soil depths (10: 0-10 cm and 40: 30-40 cm). Means + SEM of 8 replicates are shown. Significant differences in biomass production between NT and CT microcosms are indicated (significance codes: p<0.001***, p<0.05 *).

Table C1. AM fungal and plant results of the microcosms being inoculated with a non-mycorrhizal control or with soil inoculum from no-till and tillage fields from 0-10 or 30-40 cm isolation depth respectively. Means ± SEM are depicted (N=8, *Trifolium* and *Plantago* shoot phosphorus and root biomass: N=7).

Soil management	**control**	**no-till**		**tillage**	
Isolation depth (cm)		0-10	30-40	0-10	30-40
root colonization (%)					
total	0.0 ± 0.0	73.7 ± 1.8	58.8 ± 2.2	75.7 ± 4.2	72.5 ± 3.4
arbuscular	0.0 ± 0.0	11.6 ± 1.4	8.4 ± 1.6	22.0 ± 1.9	18.4 ± 2.4
vesicular	0.0 ± 0.0	2.9 ± 0.4	1.2 ± 0.5	9.3 ± 0.9	4.5 ± 0.9
hyphal	0.0 ± 0.0	59.3 ± 1.3	49.1 ± 2.7	45.4 ± 3.5	50.2 ± 1.7
extraradical hyphae (m/ g soil)	2.8 ± 0.6	11.8 ± 1.7	16.6 ± 1.8	6.0 ± 0.9	8.2 ± 0.7
shoot biomass (g)					
Plantago	2.2 ± 0.5	10.5 ± 1.1	12.3 ± 1.1	10.9 ± 0.7	9.0 ± 1.5
Trifolium	0.7 ± 0.1	13.7 ± 1.0	13.2 ± 1.9	15.2 ± 1.6	18.8 ± 1.5
Lolium	38.2 ± 1.2	25.3 ± 0.9	25.9 ± 1.3	25.7 ± 1.0	26.7 ± 1.1
total	41.0 ± 1.4	49.5 ± 0.8	51.3 ± 1.4	51.8 ± 1.0	54.5 ± 1.1
root biomass (g)	16.8 ± 0.6	15.1 ± 0.6	16.7 ± 0.7	17.0 ± 0.5	19.0 ± 0.4
shoot phosphorus					
concentration *(mg/g)*					
Plantago	0.8 ± 0.0	2.9 ± 0.1	2.9 ± 0.1	2.3 ± 0.0	2.6 ± 0.0
Trifolium	1.0 ± 0.0	2.2 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.0
Lolium	1.1 ± 0.0	2.3 ± 0.1	2.1 ± 0.0	1.8 ± 0.0	2.0 ± 0.1
mean	1.0 ± 0.0	2.2 ± 0.0	2.1 ± 0.1	1.8 ± 0.0	1.9 ± 0.0
content *(mg/pot)*					
Plantago	1.7 ± 0.4	27.4 ± 2.3	31.5 ± 2.1	22.4 ± 1.4	21.3 ± 3.5
Trifolium	0.7 ± 0.1	27.8 ± 2.1	22.4 ± 2.8	26.1 ± 2.5	32.8 ± 2.8
Lolium	39.6 ± 1.1	51.8 ± 2.5	50.8 ± 3.5	42.1 ± 1.9	47.4 ± 2.8
total	41.4 ± 1.3	107.0 ± 3.0	104.7 ± 2.5	90.6 ± 1.4	101.6 ± 2.5
shoot nitrogen					
concentration *(mg/g)*					
Plantago	21.8 ± 1.1	23.4 ± 1.0	21.6 ± 1.2	23.6 ± 0.9	27.5 ± 1.4
Trifolium	25.8 ± 0.6	32.6 ± 0.9	29.5 ± 0.8	30.0 ± 1.1	30.1 ± 0.9
Lolium	25.6 ± 0.8	23.2 ± 1.4	22.6 ± 1.4	23.8 ± 1.6	23.5 ± 1.1
mean	23.1 ± 0.7	23.2 ± 0.9	21.5 ± 0.9	23.1 ± 0.9	23.7 ± 0.6
content *(mg/pot)*					
Plantago	41.6 ± 9.1	219.7 ± 19.7	238.4 ± 23.6	229.8 ± 14.3	217.1 ± 31.6
Trifolium	16.8 ± 2.8	402.0 ± 29.7	345.2 ± 44.3	408.0 ± 39.5	507.4 ± 38.2
Lolium	889.9 ± 38.9	530.0 ± 28.3	524.0 ± 29.2	554.5 ± 41.6	565.0 ± 29.9
total	948.3 ± 43.4	1151.8 ± 58.2	1107.6 ± 71.5	1192.2 ± 45.2	1289.5 ± 41.3

APPENDIX D. Results for statistical analyses of the effect of soil inocula on plant biomass, shoot nutrient content and concentration and mycorrhizal parameters, as well as correlation matrix for plant responses and mycorrhizal parameters.

Table D1. Statistics for the assessment of AM fungal and plant parameters. All parameters were analyzed with ANOVA with block included as error term. The effect of the soil inoculum was assessed by contrasting the non-mycorrhizal control against the remaining treatments (control vs. soil inoculum). The effect of the soil communities was assessed by excluding the control from the analysis with a nested ANOVA with "soil depth" nested in "tillage".

Response	all treatments			control vs. soil inoculum			without control					
							tillage			tillage:soil depth		
	df	F	p	df	F	p	df	F	p	df	F	p
root colonization												
total	4, 28	149.6	<0.0001	1,28	572.4	<0.0001	1,21	7.4	0.013	2,21	6.9	0.005
arbuscular	4, 28	27.6	<0.0001	1,28	67.8	<0.0001	1,21	30.7	<0.0001	2,21	1.8	0.2
vesicular	4, 28	39.1	<0.0001	1,28	47.8	<0.0001	1,21	60.4	<0.0001	2,21	16.7	<0.0001
hyphal	4, 28	117.6	<0.0001	1,28	447.9	<0.0001	1,21	7.1	0.014	2,21	5.5	0.012
extraradical hyphae	4, 28	20.3	<0.0001	1,28	35.1	<0.0001	1,21	29.5	<0.0001	2,21	4.1	0.031
shoot biomass												
Plantago (log)[a]	4, 28	28.1	<0.0001	1,28	109.2	<0.0001	1,21	2.7	0.12	2,21	2.4	0.12
Trifolium	4, 28	25.5	<0.0001	1,28	91.7	<0.0001	1,21	5.6	0.028	2,21	1.4	0.27
Lolium	4, 28	28.8	<0.0001	1,28	114.3	<0.0001	1,21	0.4	0.55	2,21	0.3	0.72
total	4, 28	17.6	<0.0001	1,28	61.9	<0.0001	1,21	6.0	0.024	2,21	2.1	0.15
root biomass	4, 27	7.0	0.0005	1,27	0.1	0.83	1,20	16.6	0.0006	2,20	4.9	0.019
shoot phosphorus												
concentration												
Plantago	4, 27	124.2	<0.0001	1,27	449.5	<0.0001	1,21	31.1	<0.0001	2,21	4.3	0.028
Trifolium	4, 27	61.2	<0.0001	1,27	221.0	<0.0001	1,21	6.5	0.19	2,21	7.1	0.0045
Lolium	4, 28	60.3	<0.0001	1,28	203.6	<0.0001	1,21	29.1	<0.0001	2,21	2.5	0.11
mean	4, 26	91.4	<0.0001	1,26	306.7	<0.0001	1,21	49.7	<0.0001	2,21	3.7	0.041
content												
Plantago	4, 27	24.9	<0.0001	1,27	85.0	<0.0001	1,21	11.1	0.0032	2,21	0.8	0.45
Trifolium	4, 27	27.9	<0.0001	1,27	100.5	<0.0001	1,21	3.1	0.091	2,21	3.0	0.069
Lolium	4, 28	4.5	0.0061	1,28	9.0	0.0056	1,21	5.5	0.029	2,21	1.0	0.40
total	4, 26	101.3	<0.0001	1,26	376.9	<0.0001	1,21	15.6	0.0007	2,21	5.2	0.015
shoot nitrogen												
concentration												
Plantago	4, 28	4.2	0.0086	1,28	3.0	0.096	1,21	7.2	0.014	2,21	3.5	0.049
Trifolium	4, 28	7.4	0.00033	1,28	22.6	<0.0001	1,21	1.0	0.33	2,21	2.5	0.10
Lolium	4, 28	1.3	0.28	1,28	4.6	0.041	1,21	0.5	0.48	2,21	0.1	0.89
mean	4, 28	1.5	0.28	1,28	0.1	0.73	1,21	2.2	0.15	2,21	1.7	0.21
content												
Plantago	4, 28	16.6	<0.0001	1,28	65.7	<0.0001	1,21	0.1	0.79	2,21	0.3	0.75
Trifolium	4, 28	32.3	<0.0001	1,28	116.5	<0.0001	1,21	5.3	0.032	2,21	2.4	0.11
Lolium	4, 28	26.0	<0.0001	1,28	102.7	<0.0001	1,21	1.2	0.29	2,21	0.0	0.96
total	4, 28	6.9	0.0005	1,28	19.7	0.00013	1,21	5.7	0.027	2,21	1.3	0.29

[a] variables have been transformed before analysis

Table D2. Correlation matrix displaying relationships between assessed plant responses and mycorrhizal parameters. Correlations were determined including all treatments (with control) and excluding the non-mycorrhizal control (without control). Values are represented as r (p-value). Significant correlations are depicted in bold.

	hyphal length (m/ g soil)				**total root length colonized (%)**			
	with control		without control		with control		without control	
Biomass (g)								
Lolium	**-0.37**	**(0.0178)**	0.21	(0.2407)	**-0.85**	**(<0.0001)**	-0.29	(0.1054)
Trifolium	0.24	(0.1329)	**-0.41**	**(0.0214)**	**0.85**	**(<0.0001)**	**0.40**	**(0.0242)**
Plantago	**0.39**	**(0.0121)**	-0.03	(0.8907)	**0.70**	**(<0.0001)**	-0.11	(0.5612)
Total	0.13	(0.1345)	**-0.38**	**(0.0297)**	**0.77**	**(<0.0001)**	0.18	(0.3158)
Roots	-0.29	(0.0715)	**-0.37**	**(0.0429)**	0.03	(0.8329)	0.00	(0.9836)
P content (mg/ pot)								
Lolium	**0.61**	**(<0.0001)**	**0.51**	**(0.0027)**	**0.33**	**(0.0375)**	-0.23	(0.2088)
Trifolium	0.24	(0.1411)	**-0.39**	**(0.0288)**	**0.86**	**(<0.0001)**	**0.46**	**(0.0081)**
Plantago	**0.50**	**(0.0014)**	0.19	(0.3046)	**0.70**	**(<0.0001)**	-0.25	(0.1697)
Total	**0.53**	**(0.0006)**	0.29	(0.1058)	**0.87**	**(<0.0001)**	-0.02	(0.8983)
P concentration (mg/ g)								
Lolium	**0.69**	**(<0.0001)**	**0.54**	**(0.0015)**	**0.81**	**(<0.0001)**	-0.03	(0.8496)
Trifolium	**0.53**	**(0.0006)**	0.18	(0.3306)	**0.86**	**(<0.0001)**	0.09	(0.6169)
Plantago	**0.64**	**(<0.0001)**	**0.56**	**(0.0009)**	**0.83**	**(<0.0001)**	-0.35	(0.0507)
Total	**0.64**	**(<0.0001)**	**0.55**	**(0.0011)**	**0.81**	**(<0.0001)**	-0.14	(0.4389)
N content (mg/ pot)								
Lolium	**-0.55**	**(0.0002)**	-0.28	(0.1196)	**-0.78**	**(<0.0001)**	0.05	(0.7733)
Trifolium	0.26	(0.1085)	**-0.42**	**(0.0174)**	**0.86**	**(<0.0001)**	**0.40**	**(0.0238)**
Plantago	**0.33**	**(0.036)**	-0.19	(0.3059)	**0.76**	**(<0.0001)**	0.03	(0.8851)
Total	-0.07	(0.6876)	**-0.53**	**(0.0019)**	**0.58**	**(<0.0001)**	0.33	(0.0663)
N concentration (mg/ g)								
Lolium	**-0.44**	**(0.0043)**	**-0.40**	**(0.0252)**	-0.17	(0.2814)	0.26	(0.149)
Trifolium	**0.35**	**(0.0277)**	0.05	(0.794)	**0.55**	**(0.0002)**	-0.07	(0.6944)
Plantago	-0.11	(0.4858)	**-0.36**	**(0.0411)**	0.30	(0.0622)	0.24	(0.1804)
Total	**-0.38**	**(0.0165)**	**-0.48**	**(0.0057)**	0.07	(0.6494)	0.34	(0.0552)

APPENDIX E. Nutrients leached from microcosms after the simulation of rain including a summary of the statistical analysis and a description of the method.

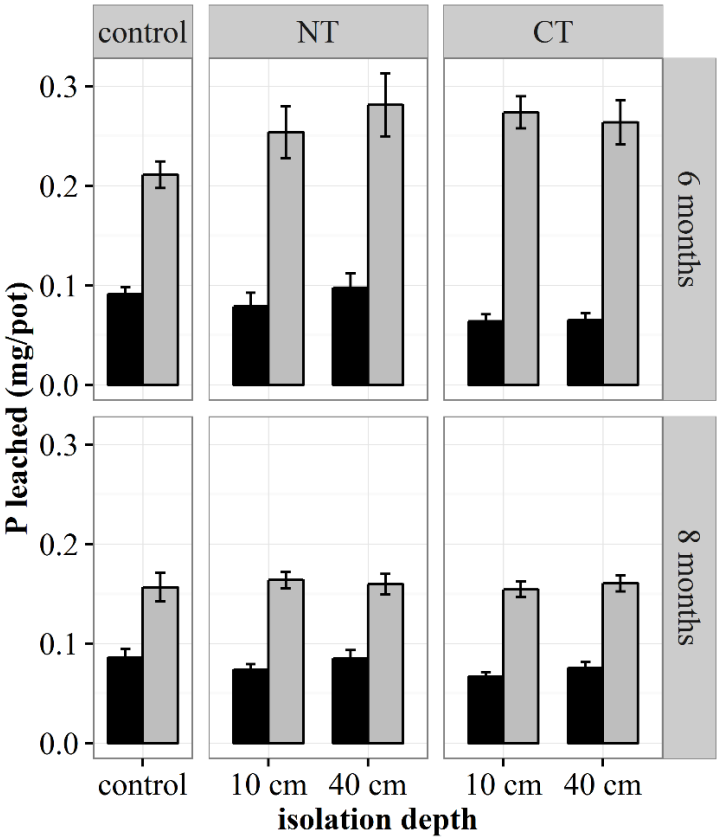


Figure E1. Dissolved PO_4-P (black) and unreactive P (grey) leached from control microcosms without AM fungi (control) or microcosms inoculated with microbial soil communities from tilled (CT) or non-tilled soil (NT) for 2 different isolation soil depths (0-10 and 30-40 cm). Unreactive P is defined as total dissolved P without phosphate. Leaching was induced by a raining event after 6 and 8 months of plant growth after fertilizing with 10kg P/ha and 60kg N/ha (for methods see E4). Means + SEM of 8 replicates are shown. Note: in this paper we focus on the effects of the different microbial soil communities on plant productivity and nutrient uptake. We present the leaching results for sake of being complete. Interpretation of the results will take place in an upcoming paper where we summarize several experiments in which we investigated effects of microbial communities on nutrient leaching.

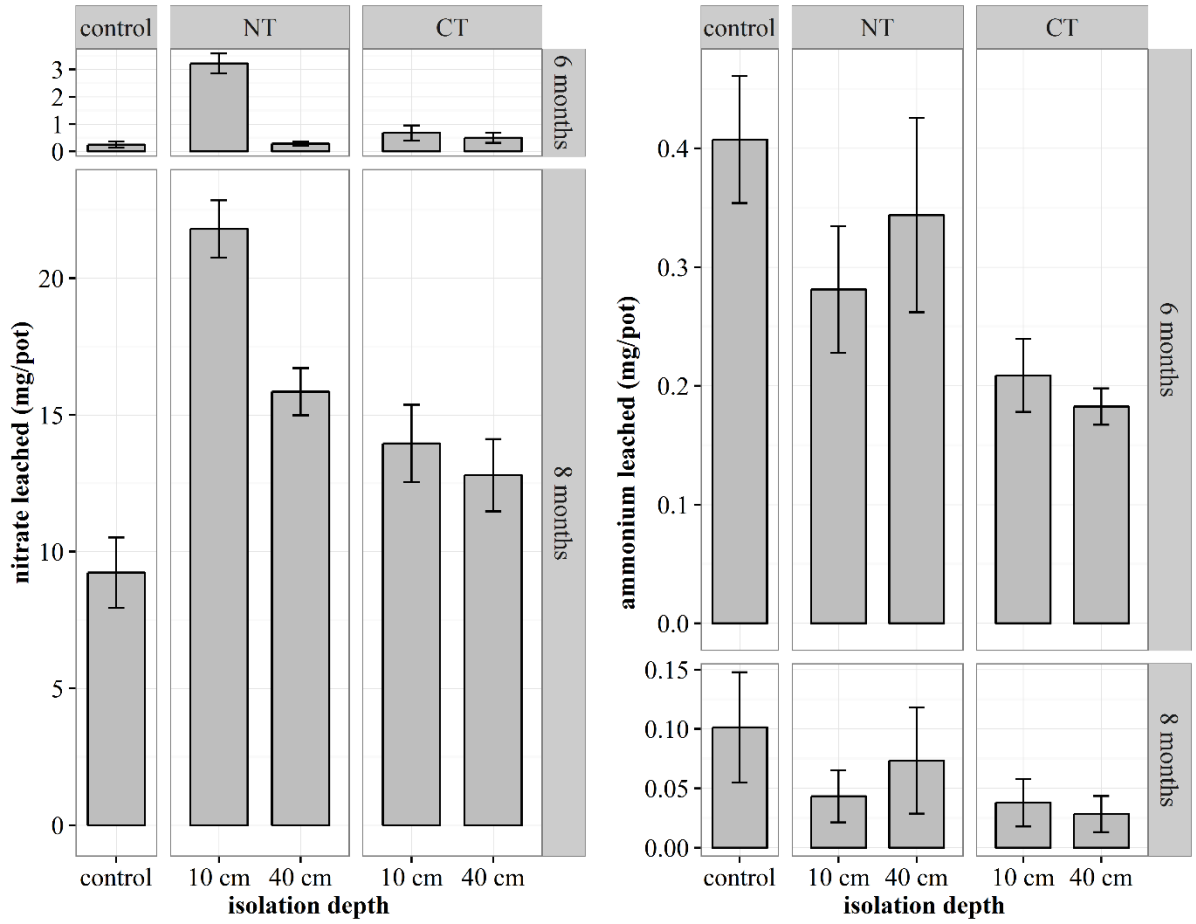


Figure E2. Nitrate and ammonium leached from control microcosms without AM fungi (control) or microcosms inoculated with soil inoculum from tilled (CT) or non-tilled soil (NT) for 2 different isolation soil depths (0-10 and 30-40 cm). Leaching was induced by a raining event after 6 and 8 month of plant growth after fertilizing with 10 kg P/ha and 60 kg N/ha (for methods see E4). Means + SEM of 8 replicates are shown. Note: in this paper we focus on the effects of the different microbial soil communities on plant productivity and nutrient uptake. We present the leaching results for sake of being complete. Interpretation of the results will take place in an upcoming paper where we summarize several experiments in which we investigated effects of microbial communities on nutrient leaching.

Table E1. Statistics for the assessment of nutrients leached from the microcosms. All parameters were analyzed with ANOVA with block included as error term. The effect of the soil inoculum was assessed by contrasting the non-mycorrhizal control against the remaining treatments (control vs. soil inoculum). The effect of the soil communities was assessed by excluding the control from the analysis with a nested ANOVA with "soil depth" nested in "tillage".

Response	all treatments			control vs. soil inoculum			without control					
							Tillage			Tillage:Depth		
	df	F	p	df	F	p	df	F	p	df	F	p
after 6 months												
unreactive P	4, 28	4.16	0.0091	1,28	14.15	0.0008	1,21	1.90	0.18	2,21	0.15	0.86
$(PO_4)^{3-}$ (boxcox)[a]	4, 28	2.93	0.039	1,28	3.90	0.058	1,21	4.38	0.049	2,21	1.19	0.32
NH^{4+}	4, 28	2.89	0.041	1,28	6.25	0.019	1,21	4.96	0.037	2,21	0.42	0.66
NO^{3-}	4, 28	43.00	<0.0001	1,28	18.30	0.0002	1,21	33.73	<0.0001	2,21	53.61	<0.0001
after 8 months												
unreactive P	4, 28	2.74	0.049	1,28	5.82	0.023	1,21	0.68	0.42	2,21	2.39	0.12
$(PO_4)^{3-}$	4, 28	1.19	0.34	1,28	1.66	0.21	1,21	1.49	0.24	2,21	1.03	0.38
NH^{4+} (sqrt)[a]	4, 28	0.86	0.5	1,28	3.02	0.093	1,21	0.33	0.57	2,21	0.06	0.95
NO^{3-}	4, 28	16.25	<0.0001	1,28	28.61	<0.0001	1,21	22.63	0.00011	2,21	7.02	0.0053

[a] variables have been transformed before analysis to fulfill model assumptions

Method E1. Method for the determination of the nutrient concentrations in leachates.

Nutrient concentrations in leachates were determined as follows: NO_3-N and PO_4-P in leachates were determined using a Dionex DX500 anion chromatograph (Dionex Corporation, Sunnyvale, CA) with an IonPac AG4A-SC guard column, an IonPac AS4A-SC analytical column (both 4mm) and 1.8mM Na_2CO_3/1.7mM $NaHCO_3$ as eluent. NH_4-N was determined spectrophotometrically using the Berthelot reaction method (Krom 1980). The absorption of the resulting coloured complex was quantified with the continuous flow analyzer SAN++ analyzer (Skalar Analytical B.V., Breda, Netherlands) according to the reference methods of the Swiss Federal Research Stations (Forschungsanstalt Agroscope Reckenholz-Tänikon ART and Forschungsanstalt Agroscope Changins-Wädenswil ACW 1996). Total dissolved P in the leachate was determined using Oxisolv® (Merck, Darmstadt, DE) oxidation prior to the photometric analysis with a spectrophotometer (Helios Gamma, Thermo Scientific, Digitana AG, Switzerland) using the molybdenum blue ascorbic acid method (Watanabe and Olsen 1965). The difference between total dissolved P and phosphate was defined as unreactive P. This fraction comprises all compounds not directly available to plants such as soluble and particulate organic P compounds, polyphosphates and particulate inorganic material like clays(Daniel and DeLaune 2009). The volume of the leachate was multiplied with the particular nutrient concentration to calculate the total amounts of nutrients leached which are presented in this study. In order to analyze the leachate no additional filtering was necessary as the leachates, which flowed through the sand-gravel mixture at the bottom of the microcosms, were very clear.

APPENDIX F. Microbial biomass C and N including a summary of the statistical analysis and a description of the method.

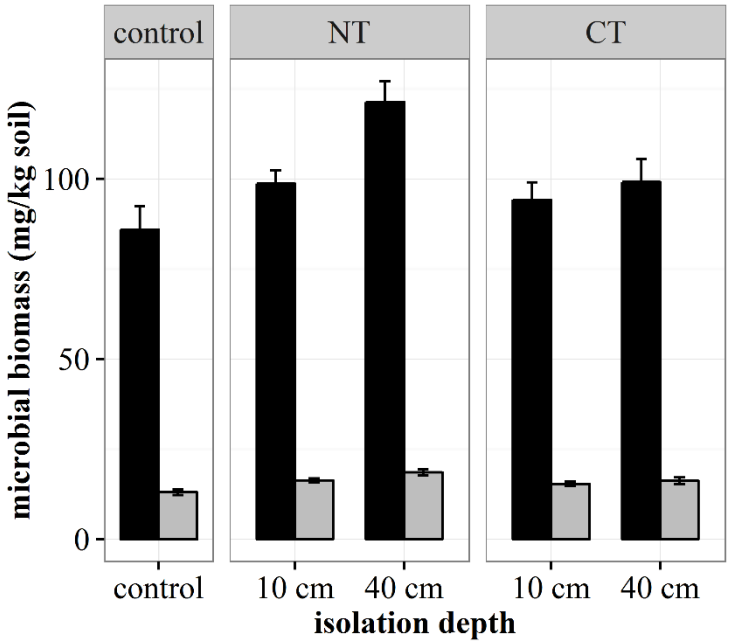


Figure F 1. Microbial biomass C (black) and N (grey) in microcosms without AM fungi (control) or microcosms inoculated with soil inoculum from tilled (CT) or non-tilled soil (NT) for 2 different isolation soil depths. Means ±SEM of 8 replicates are shown.

Table F 1. Statistics for the assessment of microbial biomass N and C in the substrate at the end of the experiment. All parameters were analyzed with ANOVA with block included as error term. The effect of the soil inoculum was assessed by contrasting the non-mycorrhizal control against the remaining treatments (control vs. soil inoculum). The effect of the soil communities was assessed by excluding the control from the analysis with a nested ANOVA with "soil depth" nested in "tillage".

Response	all treatments			control vs. soil inoculum			without control					
							Tillage			Tillage:Depth		
	df	F	p	df	F	p	df	F	p	df	F	p
nitrogen	4, 28	9.91	<0.0001	1,28	25.78	<0.0001	1,21	9.02	0.0068	2,21	4.65	0.021
carbon	4, 28	8.21	0.0002	1,28	11.58	0.002	1,21	10.39	0.0041	2,21	7.87	0.0028

Method F1. Method for determination of the microbial biomass.

Chloroform-fumigation-extraction (CFE) according to (Vance et al. 1987) was used to estimate the microbial biomass. CFE was done in duplicates with 20 g (dry matter) subsamples that were extracted with 80 ml of a 0.5M K_2SO_4. Organic C was determined with infrared spectrometry after combustion at 850°C (DIMATOC® 2000, Dimatec, Essen, Germany). The same sample was used to quantify total N by chemoluminescence (TNb, Dimatec, Essen,

Germany). Soil microbial biomass C was then calculated according to Joergensen (1996) and microbial N according to Joergensen and Mueller (1996).

Chapter 6

Summarizing discussion

Final discussion

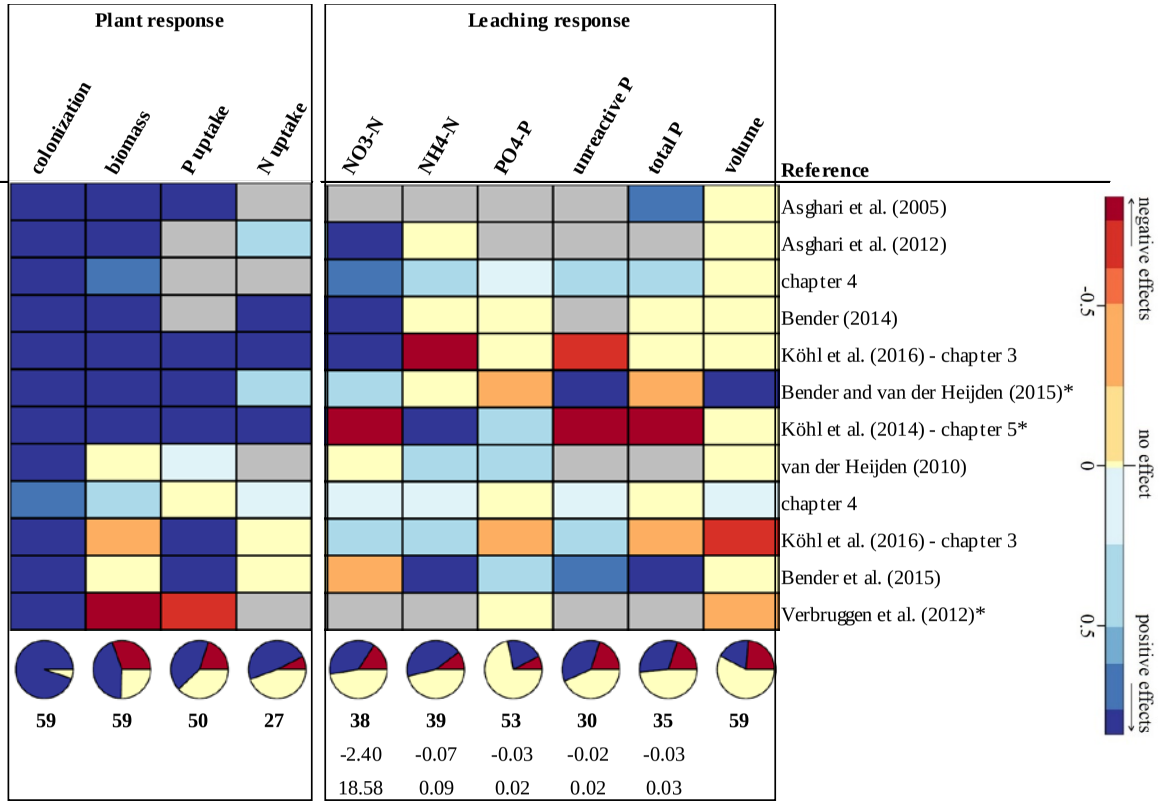
Arbuscular mycorrhizal fungi and nutrient leaching: a critical review of this thesis and other related studies

AMF play a significant role significant role in belowground nutrient cycling for many reasons. First, they efficiently scavenge for nutrients like P and N, but also micronutrients like Zn and Cu and transfer them to their host plants (Parniske 2008). Second, they are themselves, with up to 50% of the soil biomass, a nutrient sink and shape the belowground microbial community which is involved in many geochemical processes (Olsson et al. 1999, Mechri et al. 2014). Third, they affect the soil water balance as well soil aggregation (Augé 2004, Rillig and Mummey 2006) which will indirectly affect soil life, nutrient processing, and nutrient run off. Based on these facts and on preliminary published studies, I hypothesized for my thesis that AMF will reduce nitrogen and phosphorus losses through leaching.

After having conducted five experiments on AMF and nutrient leaching, the hypotheses could not be fully confirmed. In contrast to my hypothesis, AMF did not affect phosphorus leaching in most of the studies. Absolute P losses were low and below 50 g P/ ha (Table 2). This indicates that differences measured are of no direct agricultural or ecological significance. In contrast, considerable amounts of nitrogen, especially nitrate, were reduced, though these effects were highly context dependent.

Studies on nutrient leaching and AMF published so far have suggested that AMF can be important for reducing nutrient losses, and that substantial amounts of nutrients could be saved from leaching (for review see Cavagnaro et al. 2015). After a critical and quantitative review of the entirety of the results, I have to conclude that AMF are important for plant P nutrition, but effects on **P leaching** appear to be small or neglible. First, there are nearly as many cases (non-mycorrhizal control compared to the corresponding mycorrhizal treatment) that suggest a negative effect of AMF on P leaching as there are for a positive effect (Appendix, Table 1). Thus, the data provide as much evidence for an increase in P losses due to mycorrhizal symbiosis as for a decrease. Moreover, most trials did not find any significant effect on P leaching. Second, in all studies the measured P concentrations in the leachates were low and sometimes close to the quantification limit. When extrapolating measured P amounts in the conducted studies (Table 1) to comparable units like kg of P lost per ha (calculated on the basis of the pot surface, but disregarding the total soil volume used), it becomes evident that only small amounts of P are additionally lost or retained (Appendix, Table). On average, the total amount of P lost or retained was between 20 and 30 g per ha. These quantities constitute less than 1% of the applied fertilizer in the examined studies. These small differences in P leaching will likely not be of any agronomical or ecological significance, especially for P contamination of ground and surface water. Despite AMF often enhanced plant P uptake (Table 1), P leaching losses were not affected or sometimes even increased (Bender and van der Heijden 2015, Köhl and van der Heijden 2016). This may be related to the fact that AMF enhance P availability (e.g. by enhancing exudation of phosphates by roots or microbes or promoting phosphorus solubilizing bacteria (Joner and Jakobsen 1995, Singh and Kapoor 1999).

Table 1. Overview of plant and leaching responses in studies dealing with the effect of AMF on nutrient leaching. All individual treatments with an appropriate non-mycorrhizal (or uninoculated) control were counted as one trial and evaluated if AMF inoculation affected the measured responses positively – e.g. increase in biomass and decrease in nutrient leaching - (blue) or negatively (red) or had no effect (yellow). The coding for the heat map which shows the net trend of each publication was calculated as follows: (N° of positive subtrials – N° of negative subtrials)/total N° of subtrials. Effects were counted when they were statistically significant. If no statistic was available, the difference of the mean of mycorrhizal and control treatments was taken as basis for effect calculation. The pie charts show the percentage of the different effects of all sub-trials together.



Plant	Soil sterility	Treatments	N° of sub trials	Reference
Triflolium subterranum	s	P fertilization	4	Asghari et al. (2005)
GM tomato	us	N fertilization	2	Asghari et al. (2012)
grassland	s/us	sand content	6	chapter 4
GM tomato	us		1	Bender (2014)
Trifolium pratense	s	AMF species	3	Köhl et al. (2016) - chapter 3
crop rotation	s	soil biota (yrs)	2	Bender and van der Heijden (2015)*
grassland	s	mycorrhizal communities	4	Köhl et al. (2014) - chapter 5*
3 grasses	s	plant species x fertilization	6	van der Heijden (2010)
grassland	us	soil type	8	chapter 4
Lolium multiflorum	s	AMF species	3	Köhl et al. (2016) - chapter 3
Lolium multiflorum	s	soil type x N fertilization	4	Bender et al. (2015)
Zea mays	s	field site x inoculum strength	16	Verbruggen et al. (2012)*
		total N° of trials	**59**	**59 59 50 27 38 39 53 30 35 59**
Nutrients leached [kg/ha]		mean increase		-2.40 -0.07 -0.03 -0.02 -0.03
		mean decrease		18.58 0.09 0.02 0.02 0.03

*These studies tested soil biota consortia rather than solely mycorrhizal treatments, but focused on the influence of AMF on ecosystem services. The publication by Corkidi et al. (2010) was not included, as data were not available. Asghari et al. (2005) was not considered, as they did not include appropriate

Because the soil volume above aquifers is greater than that in the microcosms typically used for leaching experiments, more P will be immobilized by absorption to soil particles as it travels through the soil column into the water table. Furthermore, input of P into aquatic ecosystems is generally not a result of vertical leaching, but of surface run-off which is not directly affected by AMF (Daniel et al. 1998). The positive picture of the effect of AMF on P leaching was created by previous studies which have not discussed absolute leaching quantities, but rather emphasized positive (van der Heijden 2010, Cavagnaro et al. 2015). As P is rather immobile in soil, it is not surprising that effects on P leaching are weak; although plant P uptake is usually greatly enhanced by AMF regardless of biomass responses (Smith and Smith 2011, Köhl et al. 2014, Köhl and van der Heijden 2016). Because experiments have not yet be conducted at the field scale, my conclusion is based on artificial greenhouse trials and should be treated with care. Nevertheless, including measurements of P losses in mycorrhizal studies will be necessary to augment our knowledge about the relevance of AMF for nutrient cycling.

Results on **nitrogen leaching** appear to be more consistent, although the effects vary depending on the experimental conditions. Generally, **nitrate** losses were reduced by 30 kg N-NO_3/ha (chapter 4, Bender 2014) and even up to 60 kg N/ ha (chapter 2, Köhl and van der Heijden 2016), which is a substantial amount. However, the complex nature of this issue is reflected in opposed studies reporting no effect of AMF on nitrate leaching (van der Heijden 2010), an increase in nitrate losses in the presence of mycorrhiza (Köhl et al. 2014), or even an increase and a decrease within the same study (Bender et al. 2015). These contradictions about the relationship between AMF and nitrate leaching stress the necessity to identify the factors that explain context dependent leaching responses and the mechanisms behind them.

Similar to phosphorus leaching, **ammonium** losses were only marginally affected by AMF. Like phosphate, ammonium has a low soil mobility (Havlin et al. 2005) and can be readily transformed into nitrate (Jackson et al. 2008). Because of this rapid conversion, an early mycorrhizal interception of ammonium (Tanaka and Yano 2005) could reduce the available amount of mobile nitrate and further limit nitrogen losses. Data on the effects of AMF on ammonium leaching are as conflicting as those on nitrate. For example, a study with grasses did not reveal a notable effect of AMF inoculation on ammonium losses (van der Heijden 2010), and in chapter 5 a reduction of only 0.05 to 0.17 kg N-NH_4/ha after the addition of AMF was observed (Köhl et al. 2014). When *Trifolium* was used as host plant, ammonium leaching was increased by up to 0.17 kg N-NH_4/ha, whereas ammonium losses from pots with a grass were partly reduced (Köhl and van der Heijden 2016).

Contrasting results between studies on mycorrhizal leaching effects can be attributed to the **host plant**. Besides the mycorrhizal responsiveness of a plant, its ability to symbiotically fix nitrogen seems to be of importance. Legumes are generally known to be highly dependent upon the mycorrhizal symbiosis for growth (Hayman 1986). Thus, growth promotion due to AMF will theoretically involve enhanced nutrient immobilization in the biomass. However, as legumes are able to fix nitrogen in symbiosis with bacteria, they add another level of complexity to the nutrient cycling of a system. Higher biomass due to mycorrhizal fungi simultaneously results in higher nitrogen fixation and nitrogen, especially ammonium, enrichment in soil. It has been previously observed that clover abundance is positively correlated with N leaching (Loiseau et al. 2001, Scherer-Lorenzen et al. 2003, Bouman et al. 2010). Experiments in this thesis were mainly conducted using a grass-clover mixture. Mycorrhizal presence generally shifted the biomass dominance towards clover. In contrast to other studies investigating effects of AMF on leaching, AMF increased ammonium leaching in these systems. As previously discussed, plant responses to AMF highly depend on the plant species, and thus general

conclusions should be carefully drawn. However, these results imply that host plant is a major factor driving the mycorrhizal effect on nutrient leaching.

In mycorrhizal research, **establishing non-mycorrhizal controls** that are still comparable to the mycorrhizal treatment is a big challenge. Typically, substrates are sterilized, and soil life is reintroduced with or without mycorrhizal fungi. As a result, the control treatment will not necessarily contain the same microorganisms as the mycorrhizal treatment, although a microbial wash devoid of AMF is usually applied (Ames et al. 1987, Koide and Li 1989). As soil biota are involved in numerous nutrient cycling processes in soil, differences in effects measured between control and AMF treatment cannot be fully attributed to the mycorrhizal symbiosis if the background microbial community is not identical. Except for Bender (2014) and Asghari and Cavagnaro (2012), who used non-mycorrhizal GM-tomatoes in unsterile soil, most studies on AMF and nutrient leaching have utilized the sterile system. Using a mycorrhiza-defective mutant tomato genotype (rmc) and its wild-type (76R) can be an elegant way to include a non-mycorrhizal control while keeping a complex biotic soil environment (Barker et al. 1998, Watts-Williams and Cavagnaro 2014, Watts-Williams and Cavagnaro 2015). However, an identical performance (the same biomass and nutrient content) of the two plant genotypes under the given experimental conditions, which is not always shown, would be a mandatory precondition to draw a conclusion about the mycorrhizal role in nutrient leaching (Asghari and Cavagnaro 2012). Marschner and Timonen (2005) observed an impact of the tomato genotype on plant growth and bacterial community composition which might have consequences for nutrient cycling. The studies by Bender (2014) and Asghari and Cavagnaro (2012) using a more natural experimental design have only demonstrated a reduction in nitrate leaching by AMF, not a reduction in P or NH_4-N. This supports my conclusion that only nitrate leaching is significantly affected by the mycorrhizal symbiosis.

So far, most experiments investigating effects of AMF on leaching have been performed with sterile soil or with plant genotypes differing in AMF abundance. Until now, no studies tested the effects of AMF addition in normal field soil on leaching. In my thesis, two experiments included unsterile soil and differed in the amounts of initial AMF abundance. The large effects of autoclaving on the outcome of leaching experiments became apparent in chapter four (experiment one), where differences in P and N leaching between sterile and unsterile grassland soil were large. Soil sterilization by autoclaving releases not only additional nutrients, it furthermore changes in surface charge of the pores of the rock and reduces the surface area of clays available for adhesion of bacteria and nutrients (Jenneman et al. 1986, Trevors 1996). Although a microbial wash was applied to equalize the soil microbial background between treatments, the differing geochemical environment between sterilized and unsterilized substrates will lead to different microbial communities (Bai et al. 2015, Hartman et al., in preparation) and consequently to different nutrient cycling and leaching effects. An increasing AMF abundance in unsterile field soils did not have any effect on the amounts of nutrients leached, although plant responses and root colonization were significantly different (chapter 4, experiment 2). This shows that extrapolating effects of AMF on nitrate leaching in sterile systems to the field scale has to be done with caution. Nevertheless, the use of simplified, sterile systems is necessary to provide insights into pure mycorrhizal effects and underlying mechanisms, whereas field experiments put these effects into a wider context.

A major drawback of using microcosms for leaching experiments is their shallow **depth**. So far, the largest microcosms have had a maximum depth of 40 cm (Asghari and Cavagnaro 2012, Bender et al. 2015, Köhl et al. 2015, Köhl and van der Heijden 2016). Leaching is defined as is the downward movement of nutrients through the soil profile with percolating water

beyond the rooting zone (Lehmann and Schroth 2003, Blume et al. 2010). The roots of many shallow rooting plants, like grasses in intensively managed pastures, are confined the upper 20 cm of soil. However, many plants species, including important crops, like potatoes and barley, can root to a depth of 150 cm, or even up to 200 cm in case of winter wheat and sugar beet (Blume et al. 2010). Therefore, the deeper rooting depths of some plants in the field could mean more nutrients are immobilized and leaching losses reduced. The experimental systems used for evaluating the effect of AMF on leaching losses instead quantify a nutrient translocation through a soil core of a defined depth. This nutrient translocation can indicate effects on nutrient leaching in the field but are not directly comparable.

Nutrient leaching is not necessarily an issue in every soil type. Soils with high water infiltration rates and low nutrient retention capacity, such as sandy soils and soils with low-activity clays and low organic matter contents, are particularly prone to nutrient leaching (von Uexkull 1986, Beaudoin et al. 2005, Blume et al. 2010). Existing studies have used relatively **sandy substrates** to promote leachate formation (van der Heijden 2010, Köhl et al. 2014, Bender et al. 2015, Köhl and van der Heijden 2016). Although sandy soils exist in many areas of the world including e.g. the Netherlands and Northern Germany, this does not reflect the wide range of soil types. Chapter 4 shows that mycorrhizal abundance had no effect on nitrate leaching in different non-sandy soils types from agriculturally managed fields in Switzerland. These results contrast the large amount of nitrate retained by AMF in other trials. This experiment further contextualizes the previous results on AMF and nutrient leaching by introducing the importance of soil type. Furthermore, under natural conditions, soils are **stratified** which makes the role of soil type in nutrient leaching more complex. For example, stagnosols, which are ubiquitous in the temperate zone with humid climate conditions, are characterized by a less permeable geological horizon within the soil profile. This results in an accumulation of precipitation water and consequently reduces leaching losses. Lysimeter trials with different soil types, undisturbed soil horizon, and non-mycorrhizal controls will be difficult to conduct; therefore results from simplified greenhouse trials need to be discussed more critically with the inherently complex nature of soil taken into consideration.

The situation becomes more complex when **climate data** are considered.

When rainfall or irrigation amounts exceed moisture lost to evaporation, which is more prevalent in humid areas, the water content of the soil can rise about its field capacity and leaching can occur. Northwest Switzerland, for example, had an average annual precipitation of 992 mm, with a monthly average of 83 mm between 1980 and 2004 (Bundesamt für Statistik 2015). Furthermore, torrential raining events are rather rare (Jensen et al. 1997). The artificial rain volume used in leaching studies is relatively high, corresponding to a precipitation rate of 33-141 mm (chapter 4, Asghari et al. 2005, Bender et al. 2015, Köhl and van der Heijden 2016) and has resulted in an overestimation of the leaching volume.

Research in the field of AMF effects on nutrient leaching has gained attention during the last 10 years, but so far, few studies have contributed to this topic. Most experiments were simplistic in their design and conducted under highly artificial conditions, as is required to understand mechanisms regulating the functioning of plant-AMF interactions. Conclusions about the mycorrhizal contribution to this ecosystem service have been generally positive. For example, Cavagnaro et al. (2015) stated in their summarizing review, “AM can have a significant role in reducing the loss of N and P in soil.” After having evaluated the existing studies, including those in this thesis, I disagree with that statement. Quantities of NH_4+ and P lost from experiments with high fertilization and precpitation rates designed to induce leaching have been small and of little agronomic significance. AMF-driven reductions in nitrate leaching

were observed, but the results exhibited high inter-, and even intra- experimental variation. Due to the aforementioned drawbacks common among most of the studies I have considered (including this thesis), I conclude that there is substantial variation and no firm conclusions can be made. Despite this, first results of AMF effects on nitrate leaching are promising and need to be validated under different conditions using field-based approaches with minimal soil disturbance, natural rainfall and unsterile soil. For example, lysimeter experiments could be a feasible approach. Bender and van der Heijden (2015) conducted a two year lysimeter experiment comparing a reduced soil-life inoculum (soil biota ≤11 μm) to an enriched soil-life inoculum (soil organisms ≤2 mm, including AMF). In the first year, under a maize/grass mixture, they observed a reduction in nitrate losses of 68 kg N ha^{-1} in the enriched soil life treatment. However, in the second year, they reported an increase in nitrate losses of 18 kg N ha^{-1} in same treatment under a wheat/grass-clover mixture. Field-scale experiments like this, with true non-mycorrhizal controls, are necessary to further assess the relevance of AMF for nitrate losses. Establishing non-mycorrhizal controls in undisturbed field experiments is a challenge but is achievable by using GM-plants to control AMF symbiosis (Watts-Williams and Cavagnaro 2015). Here is to mention that most studies evaluating the mycorrhizal effect on N leaching did not consider organic N which can make up a considerable amount of total N leached (Dijkstra et al. 2007, Ghani et al. 2010). Solely, Bender et al. (2015) analyzed organic N in the leachate and observed a reduction of organic N of up to 24% in one of two soils by AMF.

If AMF can reduce nitrate losses at field scale, an important question is, how, and under what conditions, can we benefit from the fungi? High-input farming systems with high nutrient accumulation are susceptible to nitrate losses but are not favorable ecosystems for AMF fungi. Agricultural intensification, especially high P availability, reduces AMF abundance and diversity (Stribley et al. 1980, Marschner and Dell 1994, Treseder 2004). Fertilization may even select for AMF strains that are inferior mutualists (Johnson 1993, Scullion et al. 1998) and thus, potentially less effective in reducing nitrate leaching. Furthermore, leaching primarily occurs when plant and fungal activity is low, primarily in autumn, winter, and early spring. The mycorrhizal contribution to nutrient retention during these seasons is limited, but the cultivation of mycorrhizal catch crops can reduce winter leaching through enhanced nutrient uptake by roots and possibly also from an increased AMF abundance. The usage of AMF to reduce nitrate losses is especially interesting for horticultural production with sterile substrates and typically high irrigation (Corkidi et al. 2011).

Extensive agricultural management practices have been shown to mitigate nutrient leaching. One possible driving mechanism behind this could be higher functional diversity of soil life, which is generally reduced in intensively managed systems (Tsiafouli et al. 2015). Thus, management practices that enrich soil biodiversity could be promoted as a way to reduce leaching.

- Limiting **fertilizer application** rates can prevent accumulation of unused nutrients in the soil (Constantin et al. 2010). Although AMF may only play a minor role in the reduction of nutrient leaching, they can help to reduce fertilizer application. As “biofertilizers” AMF can efficiently make use of available nutrient reserves in the soil, reducing the need to add further nutrients. More specifically, an enhanced P supply by AMF can stimulate additional N uptake and decrease mobile soil N. Generally, fertilizer rates should be carefully synchronized to plant demand, distributed over the growing season, and calculated according

to the existing N reserve in the soil. Slow releasing fertilizers can thus be a practical solution. The application of nutrients prior to or during high leaching seasons should be limited.

- Cultivating a **catch crop** in the autumn–winter periods can be very effective in reducing nitrate losses during the winter season (Constantin et al. 2010). If sown early, additional nitrogen can be taken up and immobilized, and during autumn and winter the crop provides a ground cover. However, if legumes are cultivated as catch crops, it is critical to keep in mind that they may not decrease nitrate leaching risk but instead increase it (Valkama et al. 2015).
- Delaying the **ploughing** of pasture leys until late autumn or spring reduces the mineralization rate before the high drainage period in winter. Macdonald et al. (1989) even postulate that mineralization of organic N compounds has a higher impact on N leaching than N fertilization. No-till systems are said to reduce less nitrate (Constantin et al. 2010), but results on this issue are contradictory (Di and Cameron 2002a).

Other effects of AMF besides nutrient leaching and how to use them

Although AMF may play less of a role in nutrient leaching than hypothesized, we have clearly shown they have potential to shape sustainable agriculture by providing benefits to the host plant. P-content in the plant biomass was enhanced in three out of four experiments (where P-content was determined) irrespective of the biomass response. Even *Lolium*, which experienced reduced growth, accumulated a surplus of P in the presence AMF, an ecosystem service that has been described before (Jakobsen et al. 1992b, Smith et al. 2003, Li et al. 2006). Furthermore, we observed, even in unsterile field soil, a shift in biomass distribution within the grassland community. Whereas grass was depressed by mycorrhizal presence, legume growth was significantly enhanced. The positive effect of mycorrhiza on biomass production of responsive plant species, as well as the influence of AMF on the competitive ability of plants within a community, has already been well described (Wagg et al. 2011b). Contrary to previous observations that AMF are not beneficial in agricultural fields (Ryan et al. 2002, Ryan and Kirkegaard 2012) the results of chapter 3 (Köhl et al. 2015) demonstrate that AMF inoculation in field soils can enhance the growth of clover, irrespective of initial soil P availability and AMF abundance. To maximize the benefits provided by AMF, a farmer can enhance the mycorrhizal abundance and shape the community by inoculation of specific strains or by adapting AMF-promoting management practices. In chapter 3, we have shown that one potent AMF strain can successfully establish in a wide range of soils with highly variable chemical characteristics, suggesting that it has a broad niche and is able to compete successfully with indigenous AMF. As inoculum production is limited and application rates are high and thus expensive (Jolicoeur et al. 1999, IJdo et al. 2011), field inoculation of AMF in the temperate zone is not yet profitable. The most viable application of AMF is likely in the hobby sector, horticulture, nurseries and for phytoremediation where AMF abundance is generally low (Vosátka et al. 2012). New technologies like seed coating could facilitate a large scale field application under feasible conditions (Malusá et al. 2012, Vosátka et al. 2012). The promotion and maintenance of inherent AMF by a well-adapted agricultural systems has until now been a practical alternative to field inoculation. For example, several reports have shown that a number of AMF species are highly sensitive to soil tillage and disappear in tilled fields (Boddington and Dodd 2000, Jansa et al. 2003, Castillo et al. 2006, Yang et al. 2012). We have shown, for the first time, that AMF communities conditioned by distinct tillage practices provide different and partly contrasting ecosystem services (chapter 5, Köhl et al. 2014). Using no-till soil

systems can enhance P-uptake by promoting potent AMF species with a widespread hyphal network.

Suggestions for future research

As mentioned earlier, the reductionist approaches that have been utilized so far do not reflect the enormous complexity that can be found in the field, where the mycorrhizal symbiosis is influenced by numerous abiotic and biotic factors (Read 2003). The ubiquitous occurrence of mycorrhiza in terrestrial ecosystems makes it difficult to establish non-mycorrhizal control treatments. However, for evaluating the ecological significance of AMF functioning, it is of utmost importance to progress to multi-factor experimental conditions. From the stakeholder and farmer points of view, it is necessary to evaluate AMF as management tool to reduce nutrient losses in comparison to other practical measures like cultivating catch crops or adapting fertilizer application. In addition to lysimeter trials with undisturbed soil cores and quantifying the ecological relevance of AMF for nitrate leaching under a range of different field soils, efforts should be made to develop measures to implement the soil microbiota in a holistic management approach for sustainable agriculture (Schlaeppi and Bulgarelli 2015). For this reason, rapid and objective methods to determine and quantify the inherent mycorrhizal community and other beneficial soil microbes need to be available to facilitate field-based predictions about the rate of return on an increase in AMF abundance or change in the AMF community. For such a prediction, AMF functioning needs to be evaluated under a wide range of biotic and abiotic conditions (varying host, soil type, management system etc). This could result in a prediction model which could then be used by the farmer to assess potential AMF management strategies specifically tailored to his or her situation.

AMF not only interact with their host plant but also with numerous other types of soil life (Miransari 2011). Many of these other microbes are involved in processes relevant for nutrient cycling, for example nitrogen-fixing (Biró et al. 1993, Aryal et al. 2003), phosphate solubilizing (Kim et al. 1998, Souchie et al. 2010) or other plant growth promoting bacteria (Miransari 2011), or bacteria involved in N transformation (Amora-Lazcano et al. 1998, Veresoglou et al. 2012, Bender et al. 2014) or decomposition (Nuccio et al. 2013). These complex interactions need to be further examined to evaluate indirect effects AMF can have on nutrient cycling via other microbes.

Concluding remarks

This thesis demonstrates that AMF can affect nitrate losses. Although the effects were generally strong, it is still too early to propose that AMF can be used to manage nitrate losses in agriculture, as multi-factorial trials on field scale under natural conditions have yet to be conducted. However, the results provide further evidence for a role of AMF in N cycling. In contrast to the rather positive conclusions reached by previous studies (Cavagnaro et al. 2015), our results on P and ammonium leaching do not suggest any agronomical or ecological importance of AMF in reducing leaching losses. The impact of these soil fungi for P leaching should be assessed more critically, and AMF should not generally be promoted as a strategy for P loss prevention. But their role for plant P nutrition is undisputable and has been shown in many experiments (Smith and Read 2008).

In terms of biomass production, P-uptake, and competitive ability, the host plant can benefit from the mycorrhizal symbiosis under various conditions. In agriculture, these benefits can be obtained by adding a potent inoculum, as we have done, or by managing the mycorrhizal community via agricultural practices, with the latter, until now, being more feasible. We have

shown that AMF communities shaped by no-till systems are more efficient in nutrient uptake than fungi from tilled fields, suggesting that management practices can be targeted to promote beneficial soil biota, reduce fertilizer input, and increase sustainability. Furthermore, agricultural management techniques have to be closely examined for their impact on soil biota in order to implement them in a holistic management approach. Finally, this thesis provides new insights on AMF and nutrient cycling and emphasizes their role for the future of agriculture.

Table 2. Overview of leaching responses in studies dealing with the effect of AMF on nutrient leaching. All individual treatments with an appropriate non-mycorrhizal (or uninoculated) control were counted as one subtrial. Differences in leaching between the non-mycorrhizal (or uninoculated) control and the mycorrhizal treatment are calculated on the basis of the mean value and extrapolated to kg/ha (surface area of the pot was used as reference). Differences in bold are statistically significant, if statistics were available. A reduction of nutrient leaching upon AMF inoculation is depicted in blue, an increase in red. DON: organic N, TDN: total dissolved N; unP: unreactive P (=total P – PO_4); ND: not detected

van der Heijden 2010: three different grass species combined with two different fertilization levels (high and low fert); *Bender and van der Heijden 2015*: two-year lysimeter trial with reduced and enriched soil-life and crop rotation; *Bender et al. 2015*: experimental grassland microcosms with two different soils (pasture soil and heath soil) and fertilized with different N forms (NO_{3-} or NH_{4+}); *Bender 2014*: non-mycorrhizal tomato mutant compared to its mycorrhizal wildtype; *Köhl et al. 2014*: see chapter 5; *Köhl et al. 2016*: see chapter 2; *Verbruggen et al. 2012*: maize microcosms inoculated with soils from different organic (O1,O2, O3) and conventional (C1, C2, C3) managed field sites and their mixtures (O123, C123) at two different inoculation levels (4% and 12% inoculum); *Asghari and Cavagnaro 2012*: unsterile microcosms with a mycorrhiza defective tomato mutant and its mycorrhizal wildtype progenitor with three nutrient addition treatments (control, N and P addition); *Asghari et al. 2005*: microcosms with *Trifolium subterraneum* with two P fertilization levels (plus P and no P fertilization) repeated twice (exp. 1 and exp 2).

Publication	**plants**	**subtrials**	**nutrients leached (kg/ha)**						
			NO_3	**NH_4**	**DON**	**TDN**	**PO_4**	**unP**	**total P**
van der Heijden 2010	3 grass species (*Anthoxanthum odoratum* (Ao), *Festuca ovina* (Fo), *Poa pratensis* (Pp))	Ao-low fert.	0.000	0.007			**0.009**		
		Ao-high fert.	-0.010	0.005			0.007		
		Fo-low fert.	0.000	**0.006**			**0.010**		
		Fo-high fert.	-2.507	0.008			0.048		
		Pp-low fert.	0.000	0.005			**0.008**		
		Pp-high fert.	0.006	**0.006**			0.005		
Bender and van der Heijden 2015[a]	maize, grass wheat, grass clover	net over 2 yrs	49.8	**0.09**	**9.31**	**59.3**	**-0.14**	**0.03**	**-0.11**
		1 yr	**67.63**	0.03	**8.51**	**76.18**	**-0.1**	**0.01**	**-0.08**
		2 yr	-17.78	0.06	0.8	-16.9	-0.04	**0.01**	-0.03
Bender et al. 2015	*Lolium*	pasture NH4	0.91	0.20	-1.64	-0.52	0.02	0.01	0.03
		pasture NO3	-0.57	0.40	0.45	0.34	0.00	0.00	0.06
		heath NH4	ND	0.23	2.32	2.49	ND	0.06	0.06
		heath NO3	-1.70	0.51	3.17	2.04	ND	0.06	0.11
Bender 2014	Tomato mutant		**30.16**	0.01			0.00		0.00
Köhl et al. 2014[a]	*Trifolium-Lolium-Plantago*-mix	no till-10 cm	-1.68	0.07			0.01	-0.03	-0.02
		no till-40 cm	-0.02	0.04			0.00	-0.04	-0.04
		tillage-10 cm	-0.24	0.11			0.02	-0.05	-0.04
		tillage-40 cm	-0.14	0.13			0.01	-0.04	-0.03
		no till-10 cm	-7.11	0.03			0.01	-0.01	0.00
		no till-40 cm	-3.74	0.02			0.00	0.00	0.00
		tillage-10 cm	-2.68	0.04			0.01	-0.01	0.00
		tillage-40 cm	-2.02	0.04			0.01	-0.01	0.00
		no till-10 cm	-8.79	0.10			0.01	-0.04	-0.03
		no till-40 cm	-3.76	0.05			0.00	-0.04	-0.04
		tillage-10 cm	-2.91	0.15			0.03	-0.06	-0.03
		tillage-40 cm	-2.16	0.17			0.02	-0.05	-0.03

Publication	plants	subtrials	nutrients leached (kg/ha)						
			NO_3	NH_4	DON	TDN	PO_4	unP	total P
Köhl et al. 2016	*Lolium*	Cc	0.18	**0.18**			-0.01	**0.01**	0.00
		Fm	-0.08	-0.05			**-0.03**	0.00	**-0.03**
		Ri	**0.20**	**0.15**			0.01	0.00	0.01
	Trifolium	Cc	**59.56**	**-0.10**			0.00	**-0.01**	0.00
		Fm	**61.07**	**-0.15**			0.00	**-0.01**	-0.01
		Ri	**61.37**	**-0.17**			0.00	0.00	0.00
chapter 4 experiment 1	grass clover	0% sand (unst)	0.96	**0.13**			**0.01**	0.00	**0.01**
		0% sand	**29.08**	**0.31**			-0.01	**0.01**	0.01
		25% sand	**29.72**	**-0.04**			**-0.02**	**-0.01**	**-0.03**
		50% sand	**4.18**	**0.02**			0.00	**0.01**	**0.01**
		75% sand	**1.61**	0.00			0.00	**0.01**	**0.01**
		100% sand	1.71	-0.32			**0.03**	0.00	**0.03**
chapter 4 experiment 2	grass clover	soil A	0.99	**0.01**			0.00	0.00	0.00
		soil B	-1.36	-0.01			0.00	-0.01	-0.02
		soil C	0.01	0.00			0.00	-0.01	0.00
		soil D	-0.42	**0.01**			0.00	0.01	0.01
		soil E	**1.97**	0.02			0.00	**-0.03**	-0.03
		soil F	-1.94	-0.02			-0.01	-0.05	-0.06
		soil G	-0.66	-0.04			0.00	-0.01	-0.01
		soil H	-0.15	0.01			0.00	0.02	0.02
Verbruggen et al. 2012[a]	maize	4% inok O1					0.10		
		12% inok O1					0.08		
		4% inok O2					0.02		
		12% inok O2					0.06		
		4% inok O3					0.04		
		12% inok O3					0.03		
		4% inok C1					0.03		
		12% inok C1					0.01		
		4% inok C2					0.05		
		12% inok C2					0.06		
		4% inok C3					0.00		
		12% inok C3					-0.04		
		4% inok O123*					-0.02		
		12% inok O123*					0.01		
		4% inok C123*					0.04		
		12% inok C123*					0.00		
Ashgari and Cavagnaro 2012	Tomato mutant	control	**0.31**	0.03					
		N fertilizer	**7.27**	-0.01					
		P fertilizer*	0.00	0.14					
Asghari et al. 2005	*Trifolium*	Exp. 1-plus P							0.17
		Exp. 1-no P							0.01
		Exp. 2-plus P							0.05
		Exp. 2-no P							0.00

*data unpublished

[a]These studies tested soil biota consortia rather than solely mycorrhizal treatments, but focused on the influence of AMF on ecosystem services. The publication by Corkidi et al. (2010) was not included, as data were not available. Asghari et al. (2005) was not considered, as they did not include appropriate controls.

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Summary

The intensification of agricultural production to meet global food demands has led to excessive nutrient leaching from agricultural areas. These losses have negative environmental impacts as they contribute to the eutrophication of aquatic systems and the contamination of groundwater. Furthermore, nutrient leaching wastes valuable fertilizer. Soil biota are essential for nutrient cycling in soil and thus could be considered as a management tool to reduce nutrient input and losses.

Arbuscular mycorrhizal fungi (AMF) are a group of soil fungi that form symbiotic associations with the majority of land plants. The fungus forms extensive hyphal networks in soil and forages efficiently for nutrients, primarily for P, but also for N, Zn, and other nutrients that are delivered to their host plants in exchange for carbon. AMF have recently been reported to reduce nutrient leaching losses from soil, and the formation of extensive hyphal networks, which aid in efficient nutrient uptake and immobilization in plant and fungal biomass, is considered one of the key mechanisms for the reduction of P and N leaching. As AMF improve soil structure and soil water retention, these fungi could also impact the leachate volume.

Because few experiments have been conducted on this aspect of the mycorrhizal symbiosis, many questions remain unanswered. Furthermore, the ecological relevance of this ecosystem service has not yet been assessed. This thesis aims to contribute to the body of knowledge about the impact AMF can have on soil nutrient leaching. For this reason, I conducted several greenhouse experiments in which I filled deep microcosms with sterile or unsterile substrate and planted one or a mixture of grassland plant species. After a growth period, the microcosms were fertilized and exposed to artificial rain. The resulting leachate was collected and analyzed.

I began by testing if AMF species differ in their effect on nutrient leaching. Three different AMF species, *Claroideoglomus claroideum, Rhizoglomus irregulare*, and *Funneliformis mosseae*, and a non-mycorrhizal control were added to microcosms with *Lolium multiflorum* or *Trifolium pratense*. These host plants respond differently to AMF infection. AMF reduced nitrogen leaching, and the effects varied depending on host plant species and the identity of the AMF species present in the soil. The effects of AMF on phosphorus leaching losses were relatively small, and in most cases not significant. AMF enhanced plant P uptake for both plant species, and the different AMF varied in their effects on plant biomass and nutrient acquisition. The results demonstrate, for the first time, that AMF species differ in their effect on nutrient leaching.

The most effective strain from the first experiment was used for an inoculation experiment under more natural conditions. So far, most studies have relied on a sterile experimental system to generate appropriate non-mycorrhizal controls. However, in order to assess ecological relevance, experiments with non-sterile soil are required. Here, we took non-sterile soil from eight different agriculturally managed fields and inoculated them with *Rhizoglomus irregulare* to test if an increase in AMF abundance can improve plant performance and reduce leaching losses in field soil. Using qPCR, we could prove that the inoculated AMF strain was able to establish in all field soils irrespective of soil P availability, the initial abundance of *R. irregulare*, or the abundance of native AM fungal communities. AMF inoculation had no effect on the grass but significantly enhanced clover yield in five out of eight field soils. Generally, nutrient leaching was not affected by an increased AMF abundance. However, in three soils nitrogen leaching was marginally reduced and/or P leaching increased.

Most experiments testing AMF effects on nutrient leaching choose substrates with a high sand content because sandy soils are highly susceptible to leaching losses. Therefore, the importance of soil type, or more specifically of sand content, on the role of AMF play in nutrient leaching was assessed in a third experiment. Sterile grassland soil was amended with varying amounts of sand, planted with a grassland mixture, and inoculated with *Rhizoglomus irregulare* or left uninoculated. We observed that the sand content determines the impact AMF have on nutrient leaching. Whereas P losses were increased by AMF in soils with a low percentage of sand, nitrate losses were greatly reduced, and ammonium leaching was not affected. We suspect that the reduction in nitrate leaching is driven by higher biomass production with AMF inoculation at low sand levels. Additionally, leaching losses from a non-sterile grassland soil were not significantly different from the same soil that had been autoclaved. This experiment in line with my other studies confirms a general observation that results have to be carefully evaluated and conclusions made within the context of the experimental factors like AMF species, host plant and soil type.

The first experiment demonstrated that AMF species differ in the ecosystem services they provide. This indicates that agricultural practices that alter AM fungal communities have the potential to also indirectly influence ecosystem services. Thus, in a final experiment we compared the ecosystem services provided by AMF communities shaped by tillage and no-till practices. We found that the effects of the two different AMF communities varied greatly. Whereas AMF from tilled fields were superior in promoting biomass production in the grassland mixture, AMF from no-till fields enhanced P-uptake to a greater extent due to a higher amount of extraradical hyphae. Generally, inoculation of AMF communities affected nutrient leaching compared to a non-mycorrhizal control. Both nitrate and unreactive P leaching was enhanced in the presence of AMF, whereas ammonium leaching was partly reduced. AMF communities from tilled fields tended to have less nutrient losses via leaching compared to no-till communities. These results demonstrate that agricultural management practices can indirectly influence ecosystem services and plant community structure through effects on soil biota.

This thesis demonstrates that AMF can have both positive and negative effects on nitrate losses. While these effects were partly strong, a critical assessment of the ecological relevance under natural conditions is still necessary. Nevertheless, these results provide further evidence for a role of AMF in N-cycling. In contrast to the rather positive conclusions reached by previous studies, our results on P and ammonium leaching do not suggest any ecological relevance of AMF for reducing leaching losses in agriculture. Therefore, the impact of these soil fungi for P leaching should be assessed more critically, and the manipulation of AMF communities should not generally be promoted as a management strategy for reducing P losses. In terms of biomass production, host plants can benefit from the mycorrhizal symbiosis through enhanced competitive ability and increased P uptake under a variety of growth conditions. Farmers can capitalize on these benefits by adding a potent inoculum or supporting the inherent mycorrhizal community via management practices. We have shown that AMF communities shaped by no-till systems are more efficient in nutrient uptake than fungi from tilled fields suggesting that targeted farming practices can be used to harness the power of beneficial soil biota to reduce fertilizer input and increase sustainability. Furthermore, agricultural management techniques have to be closely examined for their impact on soil biota and implemented in a holistic approach. Finally, this thesis provides new insights on AMF and nutrient cycling and emphasizes their role for the future of agriculture.

Dutch summary – Samenvatting

Om in de globale vraag naar levensmiddelen te voorzien is de landbouw sterk geïntensiveerd, onder andere door gebruik van grote hoeveelheden kunstmest. Dit heeft bijgedragen tot de uitspoeling van grote hoeveelheden nutriënten uit landbouw gebieden. Uitspoeling van nutriënten heeft negatieve effecten op het milieu en draagt bij aan verrijking van aquatische ecosystemen en verontreiniging van het grondwater. Daarnaast gaan er met de uitspoeling waardevolle nutriënten verloren die voor de landbouwkundige productie gebruikt zouden kunnen worden. Bodemorganismen spelen een essentiële rol in de stikstof- en fosfaatkringlopen en een optimaal management van het leven onder de grond kan dus gebruikt worden bij het tegengaan van de uitspoeling van deze nutriënten.

Arbusculaire mycorrhiza-schimmels (AMF) zijn een groep van bodemschimmels die een symbiose vormen met de meerderheid van alle landplanten. De schimmel vormt uitgebreid netwerk van schimmeldraden in de bodem. Met deze schimmeldraden kunnen zeer efficiënt voedingstoffen, vooral fosfaat, maar ook stikstof en zink, opgenomen worden. Deze nutriënten worden vervolgens aan de waardplant afgegeven in ruil voor suikers. Recent werk heeft laten zien dat AMF helpen de uitspoeling van nutriënten uit de bodem te reduceren. Opname van nutriënten en opslag in schimmeldraden en in de plant wordt gezien als een belangrijk mechanisme waarmee de uitspoeling van stikstof en fosfaat gereduceerd kan worden. Daarnaast beïnvloeden AMF de bodemstructuur en de hoeveelheid water die in de bodem vastgehouden kan worden. Dit kan een verder mechanisme zijn waarmee de uitspoeling van nutriënten gereduceerd kan worden.

Tot nu toe zijn er nog nauwelijks experimenten uitgevoerd die de precieze rol van AMF en hun invloed op de uitspoeling onderzocht hebben. Daarom heb ik in een aantal kasexperimenten uitgevoerd, waarbij grote potten (ook wel microcosms) met steriele of niet steriele grond gevuld werden. Deze microcosms werden beplant met verschillende graslandplanten en geënt met AMF. Na een groeiperiode werden de microcosms bemest en beregend, en vervolgens werd de uitspoeling van nutriënten gemeten.

In een eerste experiment heb ik onderzocht of uitspoeling van nutriënten afhangt van de AMF soort die in de bodem aanwezig is. Daartoe heb ik drie verschillende AMF soorten, *Claroideoglomus claroideum*, *Rhizoglomus irregulare*, en *Funneliformis mosseae*, en een negatieve controle zonder AMF aan microcosms met Engels raaigras (*Lolium multiflorum*) of rode klaver (*Trifolium pratense*) toegevoegd. Deze plantensoorten reageren verschillend op AMF. De aanwezigheid van AMF reduceerde de uitspoeling van stikstof. De mate waarin AMF stikstofuitspoeling reduceerde, varieerde afhankelijk van de waardplant en de AMF soort. De effecten van AMF op de uitspoeling van fosfaat waren relatief gering en in de meeste gevallen niet significant. Daarentegen verhoogde AMF de fosfaatopname van beide plantensoorten, waarbij verschillende AMF soorten van elkaar verschilde in de mate waarin de fosfaat opname en biomassproductie beïnvloed werden. Deze resultaten laten, voor de eerste keer, zien dat de uitspoeling van nutriënten daarvan afhangt van welke AMF soorten in de bodem aanwezig is. Verschillen in AMF gemeenschappen (bijvoorbeeld ontstaan door verschillen in landgebruik) kunnen dus invloed hebben op groei van planten en de mate waarin nutriënten uitgespoeld kunnen worden.

De AMF soort (*Rhizoglomus irregulare)*, die de grootste effecten in het eerste experiment had, is vervolgens gebruikt in een tweede experiment onder meer natuurlijke condities. Tot nu toe is het meeste onderzoek uitgevoerd met steriele grond, omdat in dit geval de controle behandelingen geen AMF bevatten. Echter om de ecologische relevantie te

verhogen, zijn experimenten met niet-steriele bodem noodzakelijk. In een vervolg-experiment werd daarom van 8 verschillende velden grond verzameld. Het bodemtype van deze velden varieerde sterk en in elke bodem waren reeds natuurlijke AMF gemeenschappen voorhanden. Vervolgens werd de bodem in microcosms gedaan, beplant met Engels raaigras en rode klaver, en geënt met AMF (*Rhizoglomus irregulare*) of niet geënt (controle). Er werd getest of het mogelijk is het voorkomen van AMF in de grond door inoculatie te verhogen. Met behulp van kwantitatieve PCR heb ik aangetoond dat de geïnoculeerde AMF zich inderdaad in de diverse veldbodems kon vestigen. Het inoculatie-succes was onafhankelijk van: i) de fosfaat concentratie van de bodem, ii) de hoeveelheid natuurlijk aanwezige *Rhizoglomus irregulare* of iii) van de algemeenheid van natuurlijk aanwezige AMF gemeenschappen. Het enten van AMF had geen effect op de biomassa-productie van Engels raaigras. Echter inoculatie verhoogde de biomassa van klaver op 5 van de 8 onderzochte bodems. De uitspoeling van nutriënten werd over het algemeen niet door inoculatie beïnvloed. In 3 bodems was de stikstofuitspoeling iets lager na enting, terwijl de fosfaat-uitspoeling iets toenam.

Eerder onderzoek heeft voornamelijk het effect van AMF op de uitspoeling van nutriënten in zandgrond onderzocht. Dit omdat zandgrond erg gevoelig is voor uitspoeling en zandgrond nutriënten slecht kan binden. Om het belang van AMF en bodemtype, en specifiek de fractie zand, op de uitspoeling verder te onderzoeken, werd in een derde experiment de fractie zand in veldbodem gemanipuleerd (door de veldbodem met verschillende hoeveelheden zand te mengen). De verschillende mengsels werden vervolgens geënt met of zonder mycorrhiza-schimmels. De resultaten laten zien dat de invloed van AMF op de uitspoeling van nutriënten afhangt van de hoeveelheid zand in de bodem. De uitspoeling van fosfaat nam toe, en die van nitraat nam af, wanneer bodem met weinig zand werd geënt met AMF. Inoculatie had een grote invloed op de biomassa- productie en we verwachten dat gereduceerde uitspoeling van nitraat met een verhoogde biomassa-productie (en extra opname van stikstof) samenhangt. Dit experiment, en de beide voorgaande experimenten, laat zien dat de effecten van AMF op de uitspoeling van nutriënten sterk afhangt van de waardplant, de AMF soort en het bodemtype.

Het eerste experiment liet zien dat AMF soorten van elkaar verschillen en verschillende „ecosysteemdiensten“ aanbieden. Dit duidt erop dat teelttechnieken die AMF gemeenschappen veranderen, indirect ook “ecosysteemdiensten” door AMF gemeenschappen beïnvloeden. Om dit te onderzoeken hebben we in een vierde experiment getest of AMF gemeenschappen van een geploegde akker een ander effect hebben op de groei van planten en de opname van nutriënten in vergelijking met AMF gemeenschappen van een niet geploegde akker. We vonden dat deze twee AMF gemeenschappen sterk van elkaar verschilde. De AMF gemeenschappen van de geploegde akker hadden het meeste effect op biomassa productie terwijl de AMF gemeenschappen van de niet geploegde akker vooral een positief effect hadden op de opname van fosfaat. Dit laatste kon verklaard worden door het feit dat de AMF van de niet geploegde akker een veel groter netwerk van schimmeldraden vormde, waarmee meer fosfaat opgenomen kon worden. Inoculatie met AMF gemeenschappen had ook een invloed op de uitspoeling van nutriënten. De uitspoeling van nitraat en organisch fosfaat was hoger wanneer AMF aanwezig waren, terwijl de uitspoeling van ammonium juist wat lager was. De hoeveelheid nutriënten die uitspoelde was wat lager in potten die geïnoculeerd waren met de AMF gemeenschappen uit de niet geploegde akker in vergelijking met de geploegde akker. Deze resultaten laten zien dat verschillende teelttechnieken AMF gemeenschappen kunnen veranderen en zodoende kunnen ze indirect effect hebben op door AMF geleverde diensten.

Dit promotieonderzoek laat zien dat AMF zowel positieve als negatieve effecten hebben op de uitspoeling van nitraat. Hoewel de effecten van AMF in sommige gevallen sterk waren, is een kritische evaluatie van effecten onder natuurlijke omstandigheden (in niet steriele bodem en in het veld) noodzakelijk. Desalniettemin laat dit onderzoek zien dat AMF een rol spelen in de N-cyclus. In tegenstelling tot eerder werk duiden de verkregen resultaten in dit onderzoek er niet op dat AMF de uitspoeling van fosfaat of ammonium sterk beïnvloeden. De invloed van AMF op de uitspoeling van fosfaat en ammonium dient daarom nog kritischer onderzocht te worden. Dit werk laat verder zien dat AMF een positieve invloed heeft op de biomassa-productie van planten en dat AMF onder diverse omstandigheden en in diverse bodems, de P-opname en het concurrentievermogen kunnen verhogen. Boeren kunnen hiervan gebruik maken door het toedienen van AMF inoculum of door het kiezen van teelttechnieken die een positieve invloed op AMF hebben. We hebben laten zien dat AMF gemeenschappen van een niet geploegde akker beter in staat zijn nutriënten op te nemen in vergelijking tot AMF van een geploegde akker. Dit duidt er verder op dat management van AMF gebruikt kan worden om de hoeveel kunstmest te reduceren. Daarmee kan de duurzaamheid van agro-ecosystemen verhoogd worden. Daarnaast is het belangrijk te onderzoeken hoe teelttechnieken het bodemleven beïnvloeden. Tot slot: dit proefschrift toont nieuwe inzichten over de rol van AMF voor de nutriëntenkringloop en het benadrukt dat AMF een rol kan spelen voor de toekomst van de landbouw.

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