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Eva F. Leifheit

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Eva F. Leifheit

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Prof. Dr. Matthias C. Rillig

Institut für Biologie

of Freie Universität Berlin, Germany.

Supervisor: Prof. Dr. M. Rillig

Co-Supervisors: Dr. E. Verbruggen & Dr. E.K. Morris

1st reviewer: Prof. Dr. Matthias Rillig

2nd reviewer: Prof. Dr. Jana Petermann

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Foreword

This dissertation is a cumulative work of the following published and submitted manuscripts or manuscripts in preparation for submission:

I Leifheit, E.F., Veresoglou, S.D., Lehmann, A., Morris, E.K., Rillig, M.C., 2014. Multiple factors influence the role of arbuscular mycorrhizal fungi in soil aggregation-a meta-analysis. Plant and Soil 374, 523-537.

II Rillig, M.C., Mardatin, N.F., Leifheit, E.F., Antunes, P.M., 2010. Mycelium of arbuscular mycorrhizal fungi increases soil water repellency and is sufficient to maintain water-stable soil aggregates. Soil Biology & Biochemistry 42, 1189-1191.

III Leifheit, E.F., Verbruggen, E., Rillig, M.C., Rotation of hyphal in-growth cores has no confounding effects on soil abiotic properties – Short communication (submitted).

IV Leifheit, E.F., Verbruggen, E., Rillig, M.C., Arbuscular mycorrhizal fungi reduce decomposition of woody plant litter while increasing soil aggregation (in preparation).

Contents

Chapter 1
General Introduction

"Soil degradation causes a shrinking of arable land resources, and the persistence of starvation and malnutrition." (Lal, 1998)

The preservation of a high soil quality is a key factor for sustainability of agroecosystems. This applies also to nonagricultural ecosystems where enhancing soil quality can help to restore disturbed sites, reduce the risk of erosion and store carbon in the soil (Rillig and Mummey, 2006). Two important aspects of soil quality are the soil structure and the soil fertility, parameters that are often strongly influenced by the presence of arbuscular mycorrhizal fungi (AMF). The main goal of this dissertation was to increase our understanding of the ecology of soil aggregation and of the interaction of AMF with associated microbiota in the context of soil stability and soil C cycling.

Soil aggregation

Soil structure is defined as the size, shape and arrangement of soil particles and pores (Bronick and Lal, 2005). Soil aggregates are clusters of particles that adhere to each other more strongly than to surrounding particles (Kemper and Rosenau, 1986). The stability of soil aggregates can be assessed by various laboratory methods such as wet sieving, rainfall simulation or dry sieving and is usually expressed as water stable aggregates, mean weight diameter or geometric mean diameter (Diaz-Zorita et al., 2002). The formation and stabilization of soil aggregates influences the pore continuity, water holding capacity and infiltration, and thus the growth conditions for plant roots and soil organisms and together these control biogeochemical cycling processes (e.g. of nitrogen and carbon) (Bronick and Lal, 2005; Diaz-Zorita et al., 2002; Miller and Jastrow, 1992; Oades, 1984). Soil structure is therefore of pivotal importance for ecosystem functioning, and can contribute to ecological land restoration, erosion prevention and carbon stabilization in the soil (Rillig and Mummey, 2006). Soil erosion is one of the most severe problems in agriculture on a global scale (Daily, 1995; Pimentel et al., 1995) and thus a better understanding of the process of soil aggregation holds promise for increasing

sustainability in agriculture. Aggregate breakdown contributes to erosion in the following way: first, smaller aggregates are produced that are more easily transported by runoff and splash (LeBissonnais, 1996). Second, the release of soil particles causes crusting and surface sealing of the soil, which closes soil pores and cracks in the soil that otherwise would discharge water from the surface. In the past, aggregate stability has proven to be a relevant indicator of soil susceptibility to runoff and erosion (Barthes and Roose, 2002). An improved soil structure can increase water infiltration into the soil and a higher soil stability can mitigate raindrop impact through resistance to slaking and reduced particle detachment (Barthes and Roose, 2002). Additionally, soil erosion plays a role in the global carbon cycle as it removes soil organic carbon (SOC) from the site of formation and deposits SOC containing material at downhill sites (Van Oost et al., 2007).

Soil carbon storage is a key component of the global carbon cycle since two thirds of the earth's carbon are stored in terrestrial ecosystems (Amundson, 2001; Jobbagy and Jackson, 2000). Aggregates can physically protect soil organic matter and therefore contribute to soil carbon storage (Six et al., 2004; Tisdall and Oades, 1982). However, the relationship between organic matter (OM) and soil aggregation is variable and depends on factors such as the mineralogy of the soil, the chemical properties of the soil (pH) and the type of organic matter involved (Six et al., 2004). According to their life span Tisdall and Oades (1982) divide organic binding agents into transient, temporary and persistent, which determine age, size and stability of aggregates. Transient binding agents are mainly polysaccharides, which are rapidly decomposed. Temporary binding agents are roots and fungal hyphae, which can persist for months or years. Among the temporary binding agents arbuscular mycorrhizal fungi are of particular importance. The persistent binding agents consist of organomineral complexes, which probably derive from resistant fragments of temporary binding agents. This OM is thought to be located in the center of aggregates covered with particles of clay (Tisdall and Oades, 1982). This enclosed OM represents the most stable form of OM (Six et al., 2004).

Arbuscular mycorrhizal fungi and soil aggregation

The evidence for the role of arbuscular mycorrhizal fungi (AMF) in soil aggregation dates back to the 1950s (Martin et al., 1955; see also review of Six et al., 2004) and meanwhile AMF are well known to play a major role in soil aggregate formation and stabilization (Rillig and Mummey, 2006). Arbuscular mycorrhizal fungi (Glomeromycetes) are widespread obligately biotrophic symbionts that can associate with up to 80 % of the land plant species (Smith and Read, 2008). They penetrate the cortical cells of a root and form unique tree-shaped structures within these cells called arbuscules and storage organs called vesicles that can be identified microscopically. Furthermore, their extraradical mycelium can extensively proliferate in the soil to reach nutrient patches (Hodge et al., 2001). Their most prominent function is the supply of phosphorus and nitrogen to the host plant. In exchange, AMF receive up to 20 % of the host plants assimilates (Parniske, 2008). Additional to their contribution in plant nutrition, AMF are able to improve soil structure and mitigate plant stress by pathogens, drought or salinization (Gianinazzi et al., 2010; Smith and Read, 2008). AMF thus play a crucial role in many ecosystems and can contribute to many ecosystem functions such as the maintenance of plant biodiversity (Rillig and Mummey, 2006; van der Heijden et al., 1998).

AMF can influence soil aggregation at various scales: they are able to influence plant community composition and diversity, for instance by providing variable benefits to their hosts (van der Heijden et al., 1998). Plant species differentially affect soil aggregation, and their productivity determines how much carbon enters the soil, which is an important determinant in soil aggregation (Rillig and Mummey, 2006). AMF can also influence the individual host plant, especially root associated parameters (e.g. rhizodeposition or root architecture). Plants themselves stabilize soil by entangling particles with their fine roots and by excreting carbohydrates that serve as resources for microorganisms. Finally, AMF can influence soil aggregation via their mycelium (Rillig and Mummey, 2006). Oades (1993) described the enmeshment of soil particles by AM hyphae as "sticky string bag" because they entangle and align soil particles and thus physically stabilize soil particles. Additionally, AM hyphae exude gluing substances such as glomalin, and they influence the neighboring microbial community (Rillig 2004; Rillig et al. 2005). Rillig and Mummey (2006) hypothesized that hydrophobic substances might also be related to AMF and soil aggregation, as these

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compounds were shown to occur in ectomycorrhizal species and they help fungi to attach to surfaces. Hydrophobins are a widespread class of fungal proteins, which play various roles for filamentous fungi such as barrier to microbial attack or alteration of surface tension and thus contact to water (Rillig, 2005; Wessels, 1997).

The extent to which soil aggregation is promoted is furthermore influenced by the AMF species identity involved, as more and less beneficial species for soil aggregation have been observed (e.g. Piotrowski et al., 2004; Schreiner and Bethlenfalvay, 1997). Increased AMF species richness generally positively affects plant productivity - probably through enhanced hyphal growth and P transfer (Jansa et al., 2008; Klironomos, 2003; van der Heijden et al., 1998) - but it is not known if the same is true for soil aggregation. Additionally, the specific combination of fungal species and host plant can be important for effects on soil aggregation (Piotrowski et al., 2004).

The extent of extraradical hyphal length is usually correlated with soil stability parameters and is probably the key property of AMF for soil aggregation (Barto et al., 2010; Rillig et al., 2010; Wilson et al., 2009). Members of different AMF families might therefore have divergent effects on soil aggregation as they can have various hyphal lengths (Hart and Reader, 2002). Apart from fungal identity, hyphal proliferation depends on environmental conditions such as nutrient levels, soil pH or water content (Helgason and Fitter, 2009; Johnson et al., 2003; Parniske, 2008; Pietikäinen et al., 2009). Particularly acidic soils appear to have a detrimental effect on the development of the extraradical mycelium and the number of spores (Helgason and Fitter, 2009). In soils with low nutrient levels, especially with low P concentrations, AMF abundance in the soil and in the root is usually increased (Johnson et al., 2003; Parniske, 2008). Therefore, effects of AMF on soil aggregation are probably more easily detected in nutrient poor soils with neutral or alkaline soil pH.

AMF influences on soil C sequestration

The relationship between soil aggregation and soil C cycling and the influence of AMF on this relationship are important current research topics (Cotrufo et al., 2013; Fuentes et al., 2012; Verbruggen et al., 2013; Yu et al., 2012). Contrary to the well-established role AMF play in soil aggregation, their role in soil C sequestration is less clear. AMF do not have saprotrophic capabilities,

but there is increasing evidence that AM hyphae can acquire substantial amounts of nitrogen from decomposing litter and that they can transport the nitrogen to the host plant (Herman et al., 2012; Hodge and Fitter, 2010; Koller et al., 2013). AMF probably stimulate the decomposition of organic material, and thus liberate mineral nutrients, via the supply of carbon containing exudates to the surrounding decomposer microbial community (Herman et al., 2012; Nuccio et al., 2013). The composition of hyphal exudates ranges from low-molecular-weight sugars and organic acids to high-molecular-weight polymeric compounds, substances that can both enhance and reduce bacterial growth in the soil (Toljander et al., 2007).

In the context of climate change there is increasing interest in carbon sequestration in the soil and the role of AMF therein. It is known that a rise in atmospheric CO_2 stimulates the growth of AMF through increased allocation of plant photosynthates to AMF (e.g. Drigo et al., 2010) and it is possible that AMF enhance soil C storage via their stabilizing effect on soil aggregates. Cheng at al. (2012) recently published a study showing increased decomposition of organic C under elevated atmospheric CO_2 conditions and N amendment. However, this study applied a sudden rise in CO_2, which can induce responses of the fungal community that disappear when CO_2 is gradually increased (Klironomos et al., 2005). Furthermore, there might be long-term gains in recalcitrant compounds that originate from increased decomposition where organic matter was released and the growth of plants and microbes stimulated. This process thus increases litter input and the release of recalcitrant metabolic products that can be physically protected within soil aggregates (Cotrufo et al., 2013; Verbruggen et al., 2013).

Interactions of AMF and associated microbiota

The evidence for effects of AMF on soil processes such as nutrient transport, pathogen protection or soil aggregation generally originates from experiments using non-sterile inocula that contain a non-AM microbial community. It is not clear however, how associated microorganisms interact with AMF and whether they contribute to the observed processes.

The narrow zones around roots (rhizosphere) and around hyphae (hyphosphere) have their own specific microbial community (Andrade et al., 1997). Additionally, different species of AMF differentially influence the microbial community (Andrade et al., 1998; Artursson et al., 2005; Rillig et al., 2005).

The differential effect of AMF species on the microbial community composition in the soil can have decisive consequences for a number of processes in the soil such as phosphate solubilization, decomposition of organic matter, fungal and nematodal pathogen protection and results of each of these on plant growth (Barea et al., 2002; Koller et al., 2013; Veresoglou and Rillig, 2012), which can all influence soil aggregation.

AMF do not only directly influence the composition but also the activity of the bacterial community via the exudation of mycelial products that serve as a resource (Filion et al., 1999). Microorganisms such as bacteria or other non-AM fungi also play a role in soil aggregation (Tisdall, 1994). Polysaccharides excreted by bacteria are thought to stabilize microaggregates (< 250 μm) (Tisdall 1994). Macroaggregates (> 250 μm) are mainly stabilized by fungal hyphae, which entangle and enmesh soil particles (Tisdall and Oades 1982). Furthermore, AMF indirectly influence the microbial community via effects on rhizodepositions (qualitatively and quantitatively) that alter the bacterial community. The abundance of non-AM microbes also depends on the size and distribution of soil pores, which determines nutrient and water availability, oxygen diffusion and the relation to bacterivores. The pore structure is strongly influenced by the aggregating activities of AMF (Rillig and Mummey, 2006).

The interactions of AMF with their associated microorganisms and the consequences for soil aggregation still remain fairly unclear. Rillig et al. (2005) showed that associated microbiota of different AMF species vary in their composition and that these differences are important for soil aggregation. However, this experiment was performed in absence of AMF and further research is needed to elucidate interactions between AMF, soil microbes and soil aggregation.

Methodological aspects

As the study of AMF generally requires the presence of a host plant, effects of roots and hyphae need to be disentangled. The use of rotated hyphal in-growth cores was proposed by Johnson et al. (2001) and is now a common tool in research on AMF (e.g. Achatz et al., 2014; Babikova et al., 2013; Barto et al., 2011; Nottingham et al., 2013). Despite the broad application however, it is unknown if this experimental design has side-effects that could hinder the detection of effects caused by AMF.

Especially in small compartments the water content of the soil could be altered if water flow and hyphal connections, both contributing to water movement, are frequently disrupted.

Thesis outline

The main objective of this dissertation is to increase our understanding of the ecology of soil aggregation and the interaction of AMF with associated microbiota in the context of soil stability and soil C cycling.

Hoeksema et al. (2010) recently showed in a meta-analysis that AMF effects on plant growth are strongly context-dependent. However, such a quantitative review for effects of AMF on soil aggregation does not exist and we therefore performed a meta-analysis, in which we first tested the general assumption that AMF positively influence soil aggregation (**chapter 2**). As it is known that AMF functions depend on various biotic and abiotic factors such as host plant identity or soil pH (Johnson et al., 1997; Klironomos, 2003; Smith and Read, 2008) we additionally tested 13 factors that potentially influence effects of AMF on soil aggregation. Meta-analyses have the advantage of synthesizing data quantitatively, with the possibility of weighing effect sizes and simultaneously testing effects of categorical and numerical variables on the selected effect size (Borenstein et al., 2009).

Chapter 3 tries to answer two separate questions. First, we asked whether AMF alone - in absence of other microorganisms – are sufficient to maintain soil aggregate stability and whether AM hyphae affect water repellency of the soil. We performed a sterile laboratory experiment using small *in vitro* bioreactors, in which we grew mycelium of *Glomus intraradices* in absence of roots and other living microorganisms. In the second part we tested if the broadly applied method of rotated hyphal in-growth cores has any side-effects that would hinder attributing effects to AMF. For this part we performed a greenhouse experiment with the presence/absence of AMF and a non-AMF microbial community, where each pot contained a rotated and a non-rotated soil core. We analyzed hyphal lengths, soil water

content, carbon and nitrogen concentrations, soil pH and electrical conductivity and water stable aggregates for differences between rotated and static cores.

In **chapter 4** we analyzed interrelationships between AMF and associated microbiota and their effects on soil aggregation and C cycling. We performed a 2 x 2 factorial experiment in a climate-chamber using a sterile spore inoculum of *Rhizophagus irregularis* (Schenck & Smith, DAOM 197198) and a microbial wash as treatment. A hyphal compartment was installed to disentangle effects of roots and hyphae. Additionally, we inserted small wooden sticks into the soil to test for treatment effects on the decomposition of organic matter. Our main response variables were water stable aggregates and the weight loss of the wood sticks.

In **chapter 5** I synthesized the results in a General Discussion.

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Chapter 2
Multiple factors influence the role of arbuscular mycorrhizal fungi in soil aggregation - a meta-analysis

Abstract

Background and aims

Soil aggregation is a crucial aspect of ecosystem functioning in terrestrial ecosystems. Arbuscular mycorrhizal fungi (AMF) play a key role in soil aggregate formation and stabilization. Here we quantitatively analyzed the importance of experimental settings as well as biotic and abiotic factors for the effectiveness of AMF to stabilize soil macroaggregates.

Methods

We gathered 35 studies on AMF and soil aggregation and tested 13 predictor variables for their relevance with a boosted regression tree analysis and performed a meta-analysis, fitting individual random effects models for each variable.

Results and conclusions

The overall mean effect of inoculation with AMF on soil aggregation was positive and predictor variable means were all in the range of beneficial effects. Pot studies and studies with sterilized sandy soil, near neutral soil pH, a pot size smaller than 2.5 kg and a duration between 2.2 and 5 months were more likely to result in stronger effects of AMF on soil aggregation than experiments in the field, with non-sterilized or fine textured soil or an acidic pH. This is the first study to quantitatively show that the effect of AMF inoculation on soil aggregation is positive and context dependent. Our findings can help to improve the use of this important ecosystem process, e.g. for inoculum application in restoration sites.

Introduction

Arbuscular mycorrhizal fungi (AMF) are widespread and multifunctional members of the soil biota. In addition to their generally acknowledged functions of nutrient transfer and amelioration of abiotic and biotic stresses of their plant hosts (Auge 2001; Sikes et al. 2010; Veresoglou and Rillig 2011), AMF play a key role in soil aggregate formation and stabilization (Rillig and Mummey 2006; Tisdall and Oades 1982). Soil aggregates are defined as particles that adhere to each other more strongly than to surrounding particles (Kemper and Rosenau 1986). They are a main component of soil structure, which is a term used to describe the size, shape and arrangement of solids and pores, hence affecting pore continuity, water holding capacity and infiltration (Bronick and Lal 2005). AMF hyphae entangle soil particles and thus mainly stabilize macroaggregates ($> 250\ \mu m$) (Tisdall and Oades 1982), whereas bacteria, polysaccharides and organo-mineral complexes mainly stabilize microaggregates ($< 250\ \mu m$) (Tisdall 1994). Because soil structure influences physical, biological and chemical parameters of the soil (reviewed in Diaz-Zorita et al. 2002; Six et al. 2004), it is a crucial aspect of sustainability in agriculture and ecosystem functioning. In nonagricultural ecosystems a favorable soil structure can contribute to the restoration of disturbed lands, erosion prevention and soil carbon storage (Rillig and Mummey 2006).

The functioning of AMF can differ based on a number of biotic and abiotic factors (Johnson et al. 1997; Klironomos 2003; Smith and Read 2008).The context-dependency in the effects of AMF on host growth was recently revealed by a meta-analytical approach (Hoeksema et al. 2010). However, no such analysis exists for the AMF function of soil aggregation. We thus examined here a number of biotic and abiotic parameters with likely effects on mediating the influence of AMF on soil aggregation.

It is commonly assumed that AMF have a positive effect on soil aggregation (Amézketa 1999; Lynch and Bragg 1985; Rillig and Mummey 2006; Six et al. 2004; Tisdall and Oades 1982). We first tested the potential overall effect of AMF on aggregation and then we addressed more detailed hypotheses, which we develop in the following sections.

It has been shown that AMF species differ in their root colonization rate and, depending on the AMF family, colonization may take up to 8 weeks (Hart & Reader 2002). We expect that the development

of the extraradical mycelium (ERM) takes at least the same time as the colonization of the root does and that only after the ERM has been developed AMF may affect soil aggregation. Depending on the pot size and experiment duration the roots can grow to such an extent that they are pot-bound or the roots become very crowded in the soil (Poorter et al. 2012). If roots are crowded, effects of AMF on soil aggregation might be covered by the roots. An overall optimal time span for experiments does not exist because potential soil aggregation depends on the development of extraradical mycelium, and thus on the AMF species used; and it depends on root growth and therefore on the plant host used and the space available for root growth (see below) (Piotrowski et al. 2004). We therefore tested the following hypothesis: i) Experiments with intermediate duration will have more pronounced effects of AMF on soil aggregation than experiments with short or long durations.

In addition to the experimental duration, the biotic and abiotic experimental settings can have an important effect on the functional performance of AMF. Several studies demonstrated lower spore and extraradical mycelium development in lower pH, with varying response among isolates (Helgason and Fitter 2009). Varying soil pH can change species richness and community composition (Clark 1997; Toljander et al. 2008). However, in many studies the interpretation of pH effects on AMF functioning is difficult due to confounding factors. AMF functions and abundance (soil hyphal length, percent root colonization) are more pronounced in soils with low nutrient availability, especially under P limiting conditions, (Johnson et al. 2003; Parniske 2008; Pietikäinen et al. 2009). Because nutrient availability in sandy soils is typically low, AMF hyphae will have more pronounced effects on soil aggregation in coarsely textured soils (Six et al. 2004). This effect, however, can be mitigated by the presence of organic matter (OM) or clay. Both OM and clay can be major components of soil aggregation either as direct binding agent or as components of an abiotic process where aggregates are formed through shrink and swell cycles, respectively (Oades 1993; Tisdall and Oades 1982). We therefore tested the following hypothesis: ii) The influence of AMF on soil aggregation should be stronger for nutrient poor soils that have low intrinsic aggregation capacity, e.g. low clay content or low content of OM, and favorable conditions for AMF growth such as optimal soil pH.

In pot experiments most confounding factors are excluded but there are edge effects of the pots that include exposure to higher temperatures and impedance. These edge effects can represent unfavorable

environmental conditions that could negatively impact the growth of the plant and alter the behavior of microorganisms (Poorter et al. 2012). Field experiments typically are not subject to edge effects (no container boundaries) but there are many confounding factors such as changing precipitation, irradiation, temperature and small scale soil properties (Six et al. 2004). Sterilizing the soil eliminates living organisms in the soil and thus enables the researcher to create the treatments with only the desired organisms present in the soil. This can be particularly important in experiments with AMF to establish an AMF free control (Endlweber and Scheu 2006). In the field, mycorrhizal treatments are established by either increasing AMF abundance through AMF inoculation procedures in the AMF treatments or through controlling AMF abundance in the non-mycorrhizal treatment through, e.g. fungicides (e.g. Alguacil et al. 2008; Wilson et al. 2009). In these field experiments the controls are not completely AMF-free, which represents a confounding factor for the analysis of the treatment differences. We therefore formulated the following hypothesis: iii) In laboratory pot experiments aggregation will be higher than in field experiments because controlled experiments exclude most confounding factors and thus effects of AMF on soil aggregation are easier to detect.

In small pots nutrient availability may be limited and therefore plant growth can be smaller, but root density might be higher due to scavenging (Herold and McNeil 1979; Poorter et al. 2012), Mycorrhizal colonization decreases with increasing root length in the pot (Baath and Hayman 1984) and hence the AMF effect on plant growth decreases when pot size decreases or root density increases (Koide 1991; Kucey and Janzen 1987). The reduced effect of AMF on plant growth at small rooting volumes (pot sizes) might also extend to the effect on soil aggregation. Furthermore, comparatively low root densities in large pots or in the field might again return small effects of AMF on soil aggregation as hyphal density declines with increasing distance from the roots (Jakobsen et al. 1992). We formulated the following hypothesis: iv) AMF effects on soil aggregation in pots will depend on the space available for root growth. AMF effects on soil aggregation might be absent or difficult to detect in small pots which result in high root densities as well as in large pots where root and hyphal density can be comparatively low.

The degree to which single AMF species contribute to the various functions can vary but increased AMF species richness generally has positive effects on the promotion of plant productivity, probably

mediated by increases in hyphal length and P transfer (Jansa et al. 2008; Klironomos 2003; van der Heijden et al. 1998). Whether functional complementarity among AMF species also induces synergistic effects on soil aggregation has not been broadly studied (Rillig and Mummey 2006). A number of studies on soil aggregation addressed the influence of single fungal species and the role of fungal diversity (Enkhtuya 2005; Klironomos et al. 2005). Schreiner and Bethlenfalvay (1997) found that a mix of three species was more beneficial to plant growth promotion and soil aggregation than the single species alone. We therefore tested the following hypothesis: v) Some single AMF species will have more pronounced effects on soil aggregation than others and higher levels of AMF richness will have more beneficial effects than single species.

Moreover, the identity of the fungal species, the community composition, as well as the combination of fungal species and host plant, appear to be important for the extent to which soil aggregation is promoted (e.g. Piotrowski et al. 2004; Schreiner et al. 1997). Plant roots themselves are important for soil aggregation. Roots create biopores, exude soil binding compounds and fine roots enmesh soil particles and thereby stabilize soil aggregates (Milleret et al. 2009; Oades 1993). Aggregation tends to increase with increasing root length density and aggregation is higher in rhizosphere soil than in non-rhizosphere soil. Plant species can differ in their effects on soil aggregation as root properties such as root architecture or number of fine roots can differ substantially between different plant groups like grasses and shrubs or trees for example (Oades 1993). The colonization of a host plant by AMF can change root properties such as the root physical force, rhizodeposition, root entanglement of soil particles and also the soil water regime; parameters that are all related to soil aggregation (Rillig and Mummey 2006). Leguminous host plants, for example, tend to have higher microbial biomass and water stable aggregates compared to non-legumes where aggregation is instead related to root mass (Bronick and Lal 2005). Woody species have less fine roots and a slower root growth than grasses or herbaceous species, thus providing less opportunity for infection with AMF. This could slow the progress of colonization (Smith and Read 2008) and potentially also the development of extraradical hyphae, which would be important for soil aggregation. We formulated the following hypothesis: vi) The host plant identity will be important for the effect of AMF on soil aggregation as it can affect AMF hyphal growth and functioning via various mechanisms. The association of AMF with trees will

produce smaller effects than the association with herbaceous or leguminous plants.

Soil aggregate stability can be assessed by several methods: dry sieving, wet sieving and rainfall simulation (Diaz-Zorita et al. 2002; Lax et al. 1994). If a rewetting step is included in the method, this step can be slow (by vapor or capillary rewetting) or fast (by slaking), in which fast rewetting represents the maximum level of disruption (Diaz-Zorita et al. 2002). The macroaggregate size classes considered in the literature vary substantially from 0.25 mm up to 10 mm (Bearden and Petersen 2000; Garcia-Cruz et al. 2007) and the chosen fraction can be small, e.g. 0.25-0.5 mm or encompass a larger range from 0.25 – 4 mm (Enkhtuya et al. 2003; Kohler et al. 2009a). However, beneficial effects of AMF on soil aggregation have been found in numerous studies across a broad range of laboratory procedures (e.g. Alguacil et al. 2004; Auge et al. 2001; Bethlenfalvay and Barea 1994; Siddiky et al. 2012). We formulated the following hypothesis: vii) The detection of a potential effect of AMF on soil aggregation will not be influenced by the chosen laboratory procedures such as the method of aggregation assessment, including rewetting of samples, and the chosen aggregate size fraction.

The effectiveness of AMF at aggregating soil can therefore depend on a range of factors, whose individual effects cannot always be disentangled. To date there are several narrative reviews addressing AMF and soil aggregation (e.g. Oades 1993; Rillig and Mummey 2006; Six et al. 2004; Tisdall 1994). Here we aim to quantitatively synthesize the importance of experimental settings and multiple biotic and abiotic factors for the effect of inoculation with AMF on soil macroaggregates.

Methods

Data acquisition

We conducted a literature search with the Web of Knowledge platform on the 29th of November 2012 with the search terms 'soil AND (aggregat* or structur*) AND (mycorrhiz* OR Glom* OR Giga*)'. We additionally traced back citations from review papers on soil aggregation (see Introduction) and altogether retrieved 1935 papers. We found articles from the years 1986 to 2012. Articles were screened according to the following inclusion criteria:

i)   Studies needed to have a treatment with one or more AMF species present. These could be pot or field studies with single species or a mix of species applied (as spores, mixed inoculum with spores, roots and hyphae or whole soil inoculum) or field experiments where AMF host plants were used.

ii)   Studies needed to have a non-inoculated control. For inclusion of a study the AMF percent root colonization or hyphal length (m per g soil) in the treatment group needed to be at least twice as high as in the non-inoculated control. Studies that applied fungicides were eligible as control if they met this criterion.

iii)   The AMF treatment and the control had to be directly comparable in that either root growth had been inhibited in the assayed compartment or not in both treatments. Studies with a plant present in the AMF treatment but not in the control (e.g. fallow fields) were excluded.

iv)   Studies needed to report their response variable referring to aggregate stability as percent water stable aggregates (WSA), mean weight diameter (MWD) or geometric mean diameter (GMD) in the size class of macroaggregates (> 250 μm). Studies on microaggregates were excluded.

Studies on soil aggregation and sole inoculation with AMF are comparatively rare; this is why our dataset only included 35 studies. With the abovementioned search terms we found many studies that did not explicitly use AMF (but used the whole soil microbial community or other single non-AMF

microorganisms e.g. ectomycorrhizae) in the treatment and were therefore excluded. From the studies that did report values for aggregate stability many did not have a non-mycorrhizal control and were therefore excluded as well. For the majority of the included studies more than one trial could be extracted if, for example, results of parallel experiments were presented in one paper. Multiple trials within each publication were treated as independent when they were drawn from systems differing in at least one of the moderators chosen (see below). Responses from multiple trials sharing a common control were not independent and thus merged to one adjusted value for the response and the variance respectively (Lajeunesse 2011). The dataset of 35 studies yielded a total of 175 trials.

The effect size calculated for all statistical analyses was the log response ratio of the soil aggregate stability in experimental vs. control group. It was calculated as: $\ln(R) = \ln(X_i/X_n)$, where X_i is the soil aggregation in the inoculated AMF treatment group and X_n is the soil aggregation in the non-inoculated control group. This effect size is positive for a beneficial effect of inoculation with AMF and negative for a detrimental effect on soil aggregation. It was chosen because it is an effect size that requires only mean values, but can be expanded to include additional statistics depending on their availability (Hedges et al. 1999; Lajeunesse and Forbes 2003).

Variance was calculated as in Hedges et al. (1999, eqn (1)). Where not given, the variance was back-calculated from ANOVA results. The median of the given calculated variances (variance of $\ln(R)$) was determined and used as surrogate for those studies with missing data on variance and where a back-calculation was not possible (Corrêa et al. 2012). The inverse of the variance was used for weighting of the studies.

In addition to soil aggregation responses we collected information on 13 factors that could potentially affect soil aggregation. These were used either as continuous or categorical explanatory variables. To maximize the statistical power of our tests we reduced the number of levels of the categorical moderators to a minimum. This was done through either merging information on related categories or, alternatively, excluding poorly represented trials (see below).

The moderators used as covariates were:

Setting: The location of the experiment had two levels: 'field' and 'pot'.

Duration of the experiment: The duration of the experiment was included as a continuous variable. The mean experimental duration of studies with only a single harvest was 6 months. If there was more than one harvest, the sampling closer to 6 months was chosen in order to keep the span of duration of experiments as narrow as possible. For an improved data distribution the duration was ln transformed.

Pot size: The pot size was included as a continuous variable using the soil weight (kg) added to the pots as the parameter. Alternatively we used pot size in liters and converted the value to a weight using the bulk density of the soil. For this moderator analysis all field studies were excluded.

Sterility: The variable had two levels: 'yes' and 'no' and reported whether or not the soil of pot studies was sterilized prior to the experiment.

AMF richness: The number of AMF species present in the experiment. Due to the poor representation, information on experiments with more than one fungus was merged into a single category. The variable thus had two levels: 'one' and 'more'.

AMF species: A single AMF species used for inoculation in the experiment. There were two levels: *Gl. intraradices* and *Gl. mosseae*. All other species were excluded from the analysis because they were only poorly represented.

Plant: The variable had three groups: 'legume', 'herbaceous' and 'tree'. Other plant groups were poorly represented. The poorly represented groups and hyphal compartments were excluded from this analysis.

Soil content of organic carbon: This continuous variable described the content of soil organic carbon in the experimental soil in percent.

Sand content: Values for sand content in the experimental soil were either directly reported or deduced from the information given on texture using the 'texture triangle' according to the classification of the United States Department of Agriculture (Juma 1999). The continuous data were converted to categories with three approximately equally sized classes (in terms of number of trials): 'low', 'medium' or 'high' sand content. These classes correspond to proportions of sand of 7 – 40 %, 41 % (all soil textures designated as just "loam" in the specific experiment) and 42 – 82 %, respectively.

Soil pH: Because we expected highest responses for intermediate pH values we converted the continuous data of soil pH to a categorical variable. The data were grouped based on their ranking into three classes with equal sample size: 'low', 'medium' and 'high', corresponding to pH values of 5.05 – 6.7, 6.8 – 8 and 8.1 – 8.9, respectively.

Method of soil aggregation assessment: The variable had two levels: 'wet sieving' (as described by Kemper and Rosenau (1986)) and 'artificial rainfall' (as in Lax et al. (1994)).

Rewetting: This categorical variable described the rewetting step during the measurement of soil aggregate stability. It had two levels: 'fast' (slaking) or 'slow' (capillary rewetting or vapor rewetting).

Aggregate size fraction: There was a large heterogeneity of macroaggregate size classes in the studies. In order to detect differences between size classes we summarized them in the order of size: 'small' (0.25-1 mm), 'medium' (1-2 mm) and 'large' (> 2 mm). Aggregate size classes that did not fit in these categories were excluded from this analysis, e.g. size classes encompassing 0.25-4 mm.

Data exploration: Boosted regression trees

Boosted regression tree analysis (BRT) is a relatively new technique of machine learning that combines regression trees with boosting, a method for improving model accuracy by additively fitting and combining many simple models (trees) in a forward step-wise procedure (De'ath 2007). For a detailed

description of the application of this method in ecological contexts see Eltih et al. (2008). The BRT was performed with the packages 'dismo' and 'gbm' (Hijmans et al. 2013; Ridgeway 2012) in the R software (R Development Core Team 2012).We used this method as an exploratory tool that allowed us to make statistical inferences while relaxing the assumptions of meta-analysis, including the simultaneous assessment of more than one predictor variable and their interactions as well as the fitting of complex nonlinear relationships. This step may reveal whether single moderator models could sufficiently explain variability in the dataset. In order to identify the optimal settings for the model we performed a 10 x cross-validation for every setting of the 'gbm.step' function using suggestions by Elith et al. (2008). The error distribution was set to "Gaussian" (Ridgeway 2012). The setting with the highest predictive performance was selected. The criterion we used to assess performance of the settings was their ability to minimize residual deviance (Eltih et al. 2008); among all settings increasing the proportion of data that was selected at random had the largest influence on model performance. The deviance of the initial model was lowest with the following settings: number of interactions (*tree complexity) = 6*, contribution of each tree to the model (*learning rate) = 0.01* and random proportion of data (*bag fraction) = 0.75*. The average number of trees was 1150. This model was simplified using the 'gbm.simplify' function suggested by Elith et al. (2008). Non-informative variables were dropped one at a time in a backward selection starting with the least important using a 10 x cross-validation. This process was repeated until the model deviance could not be reduced further.

Meta-Analysis

The meta-analysis was performed through fitting random-effects models separately for each moderator using the statistical software Metawin v. 2.1 (Borenstein et al. 2009; Rosenberg 2000). Significance of comparisons was based on a permutation procedure (3999 iterations) and means and confidence intervals (CI) were calculated based on a bootstrapping procedure (Adams et al. 1997). To correct for errors due to small sample size (classes with a small number of studies) we used the bias-corrected bootstrap CI, which corrects for distributions where more than half of the bootstrap replicates are above or below the observed mean. Categorical moderator levels were considered statistically different if the

significance level of the random probability value of the Q statistics was < 0.05. For continuous data the significance was tested using the significance level of the probability value for the slope of the regression. For significant moderators we checked the proportional influence of single studies with a sensitivity analysis to identify studies that had a disproportionally high impact on the effect size (Copas and Shi 2000) (see Supplementary Material). To better visualize the relationship between the moderator 'duration of the experiment' and the effect size we additionally implemented a loess regression on unweighted data (Zuur et al. 2009). This specific model was fitted in the statistical software R (R Development Core Team 2012) with the 'gam' package (Hastie 2011).

Results

Data exploration: boosted regression trees analysis

The simplified model included the four most influential moderators and ranked them according to their relative contribution to the explanation of variation in the effect size (Fig. 1). The experiment duration (N = 172) had the largest influence on the effect size, explaining 31.6 % of variation in the data. The effect size started to increase at 2.2 months, had a peak at approximately 4 months and strongly declined after 5 months (back calculated from ln transformed values). The moderator 'soil pH' explained 27.6 % of variation in the data. 'Medium' pH values (range 6.8 to 8, N = 51) clearly induced the highest responses compared to both lower (N = 55) and higher (N = 54) values.
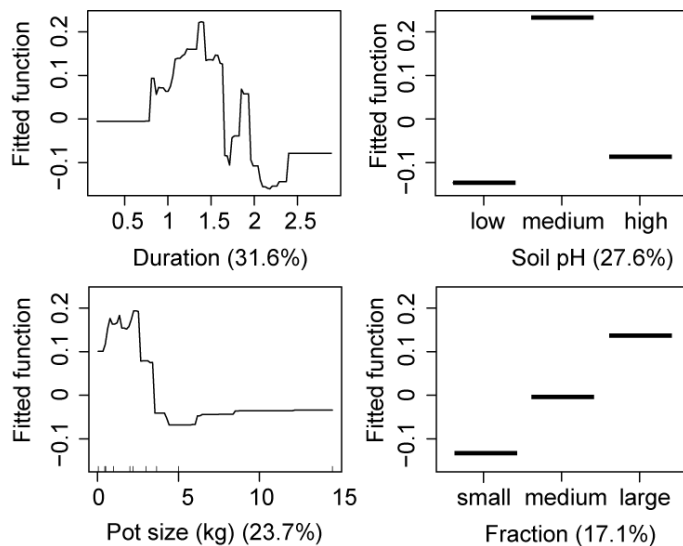

Fig. 1 Partial dependence plots for the four most influential variables in the model for the effect size. Y-axes represent the effect size ln(R) on a natural logarithmic scale where zero means no effect of AMF on soil aggregation and values above zero show a beneficial effect of AMF on soil aggregation. For explanation of categorical variables and their units see Methods Section. The X-axis for the experiment duration is on a ln transformed scale. Numbers in parentheses refer to the percent of variation in effect size explained by this variable

The moderator 'pot size' (N = 136) explained 23.7% of variation. Smaller pot sizes gave higher effect sizes than large pot sizes with a peak between 0.6 and 2.5 kg. The moderator 'fraction' explained 17.1 % of variation in the data. There seemed to be a decreasing trend from larger aggregate sizes (N = 32),

inducing higher responses, to smaller aggregate sizes inducing lower responses (medium: N = 26, small: N = 32).

Meta-Analysis

There was a positive overall effect of inoculation with AMF on soil aggregation (ln(R) = mean effect 0.2028 (bias-corrected CI: 0.1645 to 0.2425) for N = 175).

For the continuous moderator 'duration of the experiment' a significant negative relationship was detected (*p*-value of 0.0094 for the slope of the linear regression, N = 172). To better visualize the relationship between the effect size and this moderator we present the scatterplot for the unweighted average model with first and second order fits, which did not appropriately represent the data distribution, and additionally the result of the loess regression, which clearly showed a better fit to the data (Fig. 2). The smoothing function of the loess regression (span width 0.65) produced a graph with a non-monotonic function that showed a clear increase in the effect size until a peak at 3.7 months in duration (back calculated from ln transformed values) was reached. Afterwards the smoothing line decreased. There was a negative linear relationship between the effect size and the pot size (Fig. 3).
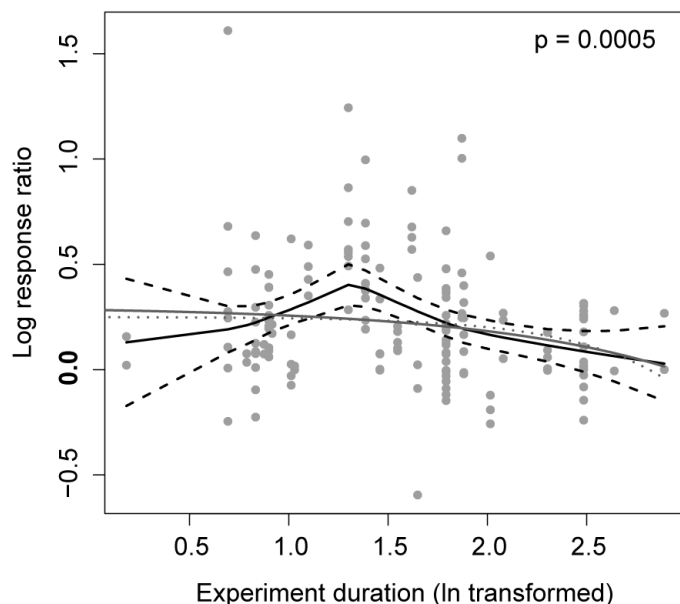

Fig. 2 Scatterplot for data points of ln transformed 'duration of experiment' ((N = 172), original unit: months) and the effect size (log response ratio). Dotted and solid grey lines represent linear fits of first and second order, respectively. The solid black line represents the smoothed line of the loess regression with ± standard errors as dashed black lines. The reported *p*-value was obtained from the loess regression
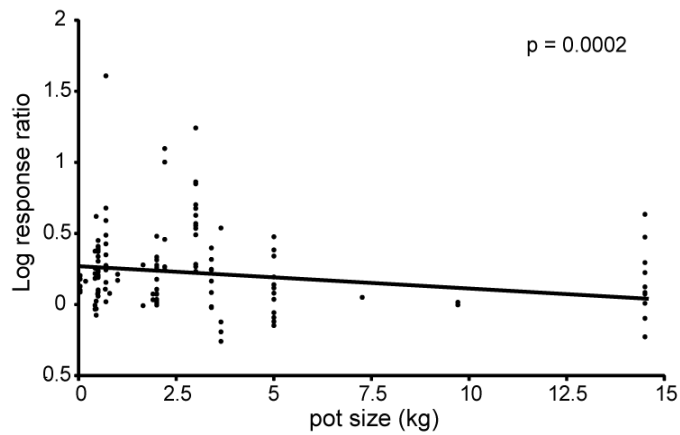
Fig. 3 Scatterplot for data points of the pot size (kg) (N = 136) and the effect size (log response ratio) and linear regression line. The reported p-value was obtained from the permutation test

Significant categorical moderators were 'soil pH', 'sand content', 'setting' and 'sterility' (see Fig. 4 next page). As in the exploratory BRT model, the bootstrapping procedure produced the highest effects for 'medium' soil pH values compared to 'low' and 'high' values. The analysis of the texture classes showed that inoculation with AMF had the highest effects on soil aggregation in soils with a high sand content (42 - 82 %) (N = 58), compared to soils with 'low' (N = 29) or 'medium' (N = 54) sand content. Laboratory studies using pots (N = 151) had higher effect sizes than field studies N = 24). The field studies were the only class having a CI that overlaps zero. Sterilization of the soil prior to an experiment (N = 89) resulted in higher effect sizes compared to non-sterilized soils (N = 71).
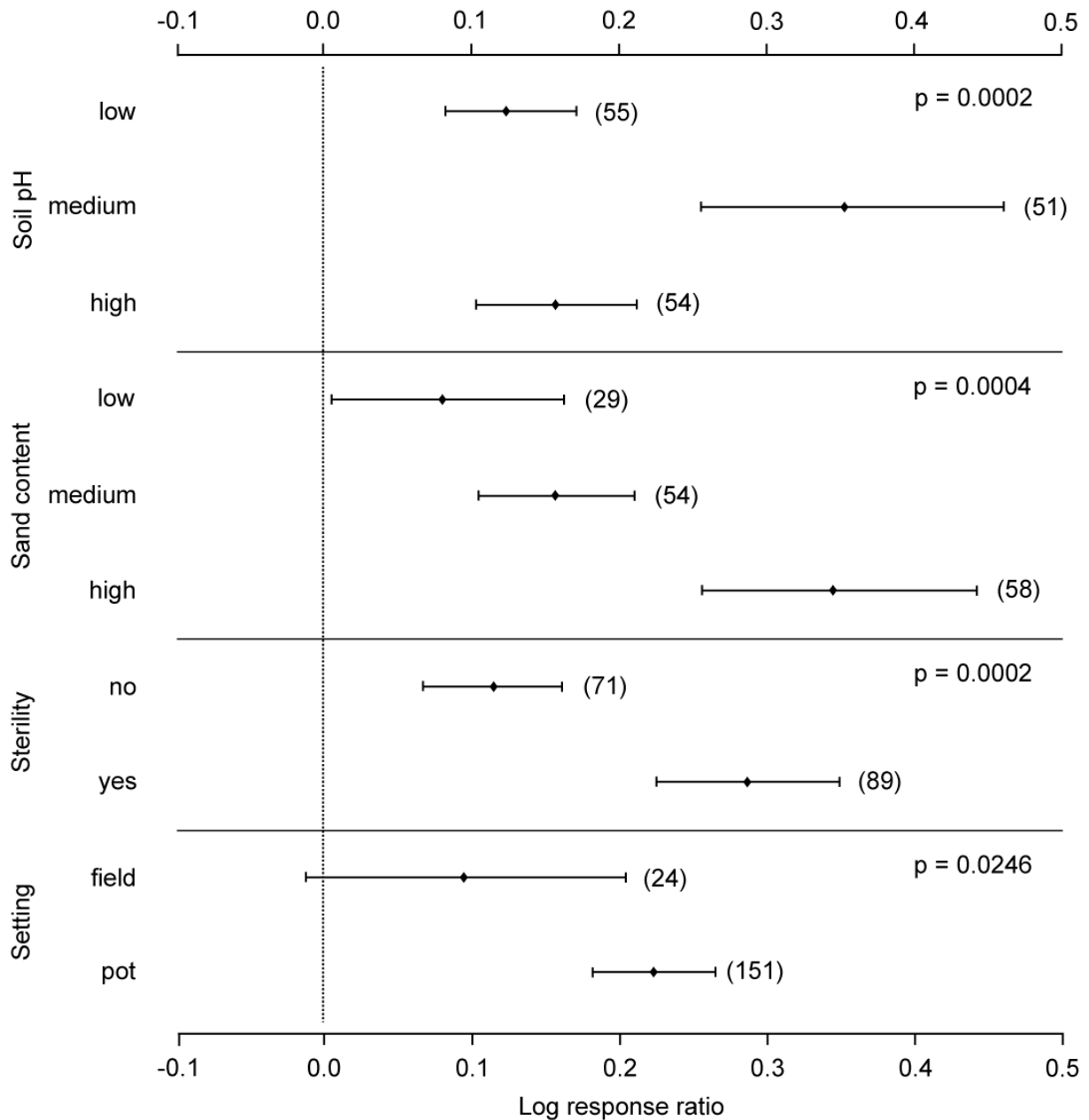
Fig. 4 Means and bias-corrected CIs of the effect size for the levels of the moderators 'soil pH', 'sand content', 'sterility' and 'setting'. Numbers in parentheses refer to the number of trials present in the class. For the definition of the classes see 'Materials and Methods'. The *p*-values were obtained from the permutation test

The permutation test showed no significance for the moderators 'AMF richness', 'AMF species', 'aggregate size fraction', 'soil organic carbon', 'method of aggregation assessment', 'rewetting' and 'plant' (see Table 1). In order to compare only woody plants (trees) and non-woody plants (legumes and herbaceous plants) we merged the two levels legume and herbaceous of the moderator 'plant' into

one group. The permutation test returned a significant *p*-value of 0.0343 for the difference between the two groups, where non-woody plants had a higher estimated mean effect size (see Table 1). The conversion of the moderator 'plant' did not change the result of the BRT analysis.

Table 1: Results of permutation tests which yielded non-significant *p*-values ($\alpha = 0.05$; for significant moderator effects please refer to figures) and for the additional analysis of the moderator 'plant'. For the moderators 'plant' and 'aggregate size fraction' see also Fig. S13. Moderator levels are followed by the number of trials present in the level. For definition of the classes see 'Materials and Methods'

Moderator	*p*-value
Level (N)	
Plant	
'tree' (N=44), 'legume' (N=67), 'herbaceous' (N=29)	0.0778
Plant	
'woody' (N=44), 'non-woody' (N=96)	0.0343
AMF richness	
'one' (N=110), 'more' (N=65)	0.3826
AMF species	
'*Gl. intraradices*' (N=34), '*Gl. mosseae*' (N=48)	0.8624
Organic amendment (% added organic C) (N=36)	0.3034
Soil organic carbon (%) (N=86)	0.1244
Method of soil aggregation assessment	
'wet sieving' (N=125), 'artificial rainfall' (N=50)	0.1244
Rewetting	
'slow' (N=97), 'fast' (N=50)	0.4564
Aggregate size fraction	
'small' (N=32), 'medium' (N=26), 'large' (N=32)	0.8112

Discussion

This is the first study to quantitatively synthesize effects of AMF inoculation on soil aggregation. Our finding of an overall positive mean effect of AMF corroborates previous narrative reviews. Moderator means and CIs were - with one exception - all in the range of beneficial effects, i.e. above zero. Our results provide insight into how the experimental outcome depends on specific experimental settings and abiotic and biotic factors. We found support for four of our hypotheses but there was no support for two of them.

Hypothesis (i): Experiments with intermediate duration will have more pronounced effects than short or long experiments.

Our analyses suggest that there is a minimum time span of approximately two months after which positive effects of AMF on soil aggregation are more likely to occur. We could additionally show that there is also a maximum time span for aggregation experiments of about 5 months, after which positive effects of AMF on soil aggregation become less likely to occur. There was a non-monotonic relationship between the duration of an experiment and the effect size with the highest response after approximately 3.7 months (Fig. 3, N = 171). The first part of the smoothed curve is rather flat and can likely be attributed to the time it takes the fungus to colonize the plant and to subsequently develop extraradical mycelium (Hart and Reader 2002). After this initial phase the effect size increases, probably parallel to changes in hyphal or root proliferation. After the peak, the response progressively decreases, showing a reduced influence of AMF, possibly as a result of the decomposition and degradation of the hyphae or increased root growth that gradually overwhelms the AMF effect. The BRT analysis (Fig. 1) supports this pattern. These results support our hypothesis and we therefore conclude that the duration of an experiment appears to be of exceptional importance for the detection of AMF effects on soil aggregation.

Hypothesis (ii): The influence of AMF on soil aggregation should be stronger in nutrient poor soils that have favorable conditions for AMF growth.

To test our hypothesis (ii) we used the moderators 'sand content', 'soil pH' and 'soil content of organic carbon'.

Our tests highlight the role of abiotic settings in experiments such as texture and soil pH. The preference of AMF for a near neutral or alkaline soil pH (Helgason and Fitter 2009; Marschner et al. 2005), reflected in a more extensive extraradical network, could be directly correlated with an increase in soil aggregation. Likewise, AMF effects are more pronounced in nutrient poor soils, as are coarsely textured soils or soils low in OM (Parniske 2008). For sandy soils we could show an increased effect size, giving strong support for our hypothesis. For soil OM there was no significant trend on the effect size. This may suggest that AMF effects on soil aggregation are more strongly influenced by texture than by OM. Soil aggregation was previously found to be positively influenced by SOM, but also neutral effects have been reported (Chaudhary et al. 2009; Six et al. 2004; Tisdall and Oades 1982). In the literature, both increases and neutral effects of the presence of OM on AMF hyphal length have been found (e.g. Atul-Nayyar et al. 2009; Herman et al. 2012; Hodge 2001). The influence of OM on AMF may depend on the amount of OM added to the soil, the composition of the OM and the different methods of assessing organic Carbon in the soil (Barto et al. 2010). The complexity of interactions between microbes such as AMF, the soil fauna, roots, inorganic binding agents (e.g. metal oxides) and environmental factors (e.g. soil pH) may also lead to the variability of OM effects on AMF and soil aggregation (Six et al. 2004).

Hypothesis (iii): In laboratory pot experiments aggregation will be higher than in field experiments.

To test our hypothesis (iv) we used the moderators 'setting' and 'sterility'. The meta-analysis revealed a higher effect size for pot experiments (N = 156) compared to field experiments (N = 29), corroborating our hypothesis. Potential edge effects of pots (Poorter et al. 2012) do not appear to affect the positive effect of AMF on soil aggregation. The 'field' group is the only moderator level having a CI overlapping zero, indicating that detrimental effects are more likely to occur in field studies. There

are multiple possible reasons that could affect the functional performance of the inoculated AMF species in the field: a) increased competition among AMF species or with saprotrophic decomposers (Verbruggen et al. 2012), b) greater variability due to small-scale heterogeneity in soil properties such as texture, water holding capacity, nutrient levels or pH (Chen et al. 2006; Kumar et al. 2006; Zhou et al. 2008) or c) the lack of controlled conditions (possible plant neighbors that affect aggregation, changing precipitation and irradiation). The naturally higher heterogeneity in the field, but also the low sample size in the 'field' group causes differences in variance between this and the 'pot' group. We conclude that the location of an experiment is crucial for soil aggregation experiments that include AMF. The result of more beneficial effects of AMF in pot experiments corroborates findings of a meta-analysis on plant growth in the field vs. laboratory studies, where more beneficial effects of AMF on plant growth were reported for greenhouse or growth chamber experiments (Lekberg and Koide 2005), It is important to note that both settings have drawbacks: results from pot experiments have reduced reality while field experiments include confounding factors that both limit the interpretation and extrapolation of results. More research in the field could improve our knowledge on the quantification of the effects of AMF on soil aggregation under realistic conditions, which would be important for practical applications of AMF inoculum such as in the restoration of degraded areas or erosion prevention (Rillig and Mummey 2006).

Sterilized soils (N = 93) yielded higher effect sizes than non-sterile (N = 77) soils. Sterilizing the soil prior to an experiment removes any priority effects of other AMF species, leaving only the newly inoculated species (Mummey et al. 2009). The species added can proliferate without competing species and are more likely to produce an effect. Non-sterilized pot or field experiments include priority effects (Verbruggen et al. 2012), and measure only the effect of the addition of propagules; the effect size would thus automatically be expected to be smaller due to less pronounced treatments. This result shows that sterilizing the experimental soil is important to clearly disentangle effects of an inoculated AMF species and other microorganisms and that the detection of an effect is more likely in sterilized soils. For future pot experiments studying effects of interactions with other microbiota or effects of diversity it is inevitable to use sterilized soil, especially when molecular techniques or other techniques to assess diversity are involved (see below).

Hypothesis iv) AMF effects on soil aggregation in pots will depend on the space available for root growth.

In smaller pots the development of a hyphal network throughout the whole soil is faster compared to large pots. Thus it is not surprising to see a significant impact of the moderator 'pot size' on the effect size. However, it is interesting to observe that this relationship is non-monotonic. There seems to be a plateau with an "optimal" pot size yielding the highest effect sizes. One reason could be that in very small pots (< 0.6 kg) the effect of plant roots aggregating soil more rapidly covers up the direct hyphal effect. Root density was found to correlate negatively with mycorrhizal infection (Baath and Hayman 1984). Therefore, a high root density in a small pot may lead to low infection rates with AMF and small effects of AMF on soil aggregation. In very large pots (> 3.5 kg) on the contrary, hyphal proliferation may not be fast enough to yield comparable results in soil aggregation and root mass could be relatively smaller (as root mass per rooting volume) due to increased available amounts of nutrients and water (Poorter et al. 2012). For future soil aggregation experiments the pot size or rooting volume, in conjunction with experiment duration, should carefully be taken into account to avoid pot-bound roots or extremely high root densities, which might impair the interpretation of AMF effects on soil aggregation.

Hypothesis (v): Some single AMF species will have more pronounced effects on soil aggregation than others and higher levels of AMF richness will have more beneficial effects than single species.

In the literature both positive and negative effects on soil aggregation have been found for *Gl. intraradices* and *Gl. mosseae* (e.g. Alguacil et al. 2004; Ambriz et al. 2010; Enkhtuya et al. 2003). However, in our meta-analysis these species yielded similar positive mean responses. We had high replication for *Gl. intraradices* (N = 34) and *Gl. mosseae* (N = 48) and the permutation test produced a high p-value (non-significance: $p = 0.8624$). We can therefore confidently state that there is no difference in soil aggregation capability between *Gl. intraradices* and *Gl. mosseae*. These two *Glomus* species are commonly used in AM experiments and often showed positive effects on soil aggregation (e.g. Auge 2001; Bethlenfalvay and Barea 1994; Kohler et al. 2008), but AMF functional effects

generally depend on the combination of fungus and host plant species (Klironomos 2003). Studies yielding negative effect sizes were mostly field experiments, which are less likely to detect AMF effects on soil aggregation, as discussed above. Therefore, we think that the setting is a more important factor influencing soil aggregation than the AMF species identity. Furthermore, the lack of species of other genera (*Gigaspora*, *Scutellospora*), which can have more dissimilar lengths in extraradical hyphae than *Glomus* (Hart and Reader 2002), might be the reason why we did not find support for our hypothesis.

The analysis for the moderator 'AMF richness' returned no effect, i.e. no difference between the groups 'one' and 'more'. Studies on effects of different levels of diversity or richness of AMF on soil aggregation are rare as this is a relatively new topic (Rillig and Mummey 2006). To improve our statistical power it was necessary to merge studies with more than one fungus into a single group, necessitating that studies with only two AMF species and studies with the entire AMF community adapted to the specific soil were classified together. Also the field studies were included in this group, which tend to have lower aggregation values (see hypothesis iv). This might have caused variability in the group 'more' that reduced the probability of finding effects.

However, the overall replication level in the group was high (N = 65) and the analysis returned a highly non-significant p-value (p = 0.3826). Hence, we lack support for our hypothesis and for the existence of synergistic effects in the soil aggregation process as was suggested by Rillig and Mummey (2006) and Schreiner and Bethlenfalvay (1997). There are occasions when AMF identity, as well as selection mechanisms, soil type and the specific combination of AMF and the host, may have more pronounced effects on ecosystem functioning than AMF richness (Hoeksema et al. 2010; Koide and Kabir 2000; Vogelsang et al. 2006; Wagg et al. 2011). For the studies considered in our meta-analysis we also found evidence that other factors might be more important for soil aggregation than AMF richness. To gain a better insight into the role of AMF richness on soil aggregation, more single studies on different levels of richness or diversity that incorporate experimentation with variable contexts (location, host plant and soil type) are needed. Knowledge on this topic could help us to improve the use of the ecosystem function soil aggregation (in terms of inoculum application) in, for example, restoration sites (Miller and Jastrow 1992; Sikes et al. 2010).

Hypothesis (vi): The host plant identity will be important for the effect of AMF on soil aggregation. The association of AMF with trees will produce smaller effects than the association with herbaceous or leguminous plants.

Root systems among the analyzed plant groups differed considerably (herbaceous including grasses compared to trees for example) and it could be expected to see differences in their influence on soil aggregation. There is evidence in the literature that the combination of AMF with a single plant species – including herbaceous plants and legumes – is important for the influence on AMF functions (Klironomos 2003). Although we observed a trend for the effect size in plant groups in the expected order herbaceous>legumes>trees (N = 29, N = 67, N= 44, respectively; Fig. S13), this was not significant. If we only analyzed woody (N = 44) and non-woody (N = 96) plants the difference between the moderator levels was significant (p = 0.034). This second analysis corroborates the trend that we found in the first analysis. However, we think that by merging legumes and herbaceous plants that include grasses, we lose information on plant traits such as the number of fine roots (Oades 1993), which probably has an important influence on the result. When keeping more detailed information on plant traits, it seems that the aggregation effect is detectable independently of the host plant type. As AMF functions including soil aggregation usually depend on the specific combination of host and fungus (Piotrowski et al. 2004), the overall influence of a certain plant (root) type or fungus becomes indifferent when meta-analyzed. As the BRT analysis showed, the moderator 'plant' was not influential on the data and therefore we conclude, contrary to our hypothesis, that the differences caused by the host plant identity are not strong enough to have an impact on the effect of AMF on soil aggregation.

Hypothesis (vii): The detection of a potential effect of AMF on soil aggregation will not be influenced by the chosen laboratory procedures.

To test this hypothesis we used the moderators 'aggregate size fraction', 'method of soil aggregation assessment' and 'rewetting'. For the moderator 'aggregate size fraction' the permutation test showed a pattern (higher effect sizes for larger aggregate size fractions, Fig. S13), but it was not significant and the CIs widely overlapped. The moderators 'method of aggregation assessment' and 'rewetting' had high replication levels, and thus there probably was a high power to find any effects. It appears

that the magnitude of the aggregate stabilizing effect of AMF is not strongly influenced by the method used to measure water stability. However, we only compared water stability of aggregates including rainfall simulation and wet sieving; no studies using dry sieving were included in the analysis. This might be a reason for the similarity in results for the moderator 'method of soil aggregation assessment'.

These results show that the effect of AMF on soil aggregation can be detected in small, medium and large macroaggregates with various methods, corroborating results of previous single studies where positive effects have been shown for various aggregate size fractions and different methods to determine aggregate stability (e.g. Andrade et al. 1998b; Auge et al. 2001; Kohler et al. 2009b; Schreiner et al. 1997; Siddiky et al. 2012). We therefore find support for our hypothesis. Future studies might consider that the entire macroaggregate size range from 0.25 – 10 mm is positively affected by AMF rather than single narrow macroaggregate range classes. They may also use wet sieving and simulation of rainfall interchangeably to determine the water aggregate stability, but - for our dataset we can state that - wet sieving seems to be more commonly used (wet sieving: N = 125, artificial rainfall: N = 50).

Direct vs. indirect effects of AMF

The majority of results on soil aggregation experiments included in this meta-analysis are from mycorrhizosphere soils, i.e. soils that contain AMF hyphae and roots. The inoculation with AMF can alter the host plant roots, e.g. root morphology and rhizodeposition (Rillig and Mummey 2006), which can hence indirectly affect soil aggregation. The calculated effect size – the log response ratio that compares the inoculated with the non-inoculated control group - cannot account for these indirect effects. However, we were able to detect a positive effect size across all trials, showing a beneficial effect of AMF inoculation on soil aggregation (ln(R) = 0.2028, N = 175). If we consider only hyphal compartments (excluding root effects), the overall effect across these trials is even higher with ln(R) = 0.3039 (N = 13), supporting the assertion that AMF hyphae are (at least partly) directly responsible for the increase in aggregation. To clearly show direct AMF mycelium effects, more studies with sterilized soil (AMF free control) are needed that also include hyphal compartments or studies with split root

designs that enable us to distinguish between root, hyphae and AMF altered root effects (as in Andrade et al. 1998a).

Conclusions

There is a clear overall positive effect of AMF on soil aggregation when considering the evidence available to date. With our meta-analysis we showed that the selection of experimental parameters can have an important influence on the outcome of any study testing for AMF effects on water stable aggregates. Pot studies with sterilized sandy soil, near neutral soil pH, a pot size smaller than 2.5 kg and a duration between 2.2 and 5 months are more likely to result in positive effects of AMF on soil aggregation than experiments in the field, with non-sterilized fine textured soil or with an acidic soil pH. The extent to which AMF promote soil aggregation seems to be independent of the number of fungal species present in the system or the soil organic carbon content, and the experimental outcome does not seem to be altered by the selected laboratory procedures for measuring soil aggregation. Differences in soil aggregation caused by host plant identity do not seem to be strong enough to generally influence the effect by AMF, when information on trees, herbaceous plants and legumes is conserved. Future research should include experiments with sterile soil and hyphal compartments or split root designs to clearly distinguish between root and hyphal effects as well as studies on AMF richness and diversity in differing contexts such as location (field vs. laboratory) and soil type.

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Chapter 3.1

Mycelium of arbuscular mycorrhizal fungi increases soil water repellency and is sufficient to maintain water-stable soil aggregates

Abstract

Using an *in vitro* bioreactor system in which the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* was grown in a soil devoid of detectable living microbes, we could show that the mycelium of this fungus contributed to the maintenance of water-stable soil aggregates and increased soil water repellency, as measured by water-drop penetration time. This is to our knowledge the first demonstration of a causal link between AM fungal growth and water repellency of soil aggregates. Our results also place AM fungal contributions to soil aggregation on a firm mechanistic footing by showing that hyphae are sufficient to produce effects, in the absence of other soil biota, which have always been included in previous studies.

As widespread and abundant members of the soil biota, the obligately biotrophic arbuscular mycorrhizal (AM) fungi are a generally acknowledged biotic factor in the formation and maintenance of soil aggregates (Tisdall and Oades, 1982; Six et al., 2004; Rillig and Mummey, 2006). Evidence for the involvement of these fungi comes from a variety of field observational or experimental studies (Wilson et al., 2009) and controlled pot experiments in which AM fungal presence or isolate identity have been specifically modified (*e.g.*, Piotrowski et al., 2004). In none of these studies have AM fungi been present as the only component of the soil microbiota; it is generally accepted experimental practice to equilibrate microbial communities in controls and AM fungus treatments. Thus, the interaction between AM fungal hyphae and other soil biota may have contributed to the observed effects on soil aggregation. Rillig et al. (2005) documented significant effects of microbial communities associated with different AM fungal isolate cultures on soil aggregation in the absence of live AM fungi. This raises the questions: a) to what extent are microbiota associated with certain AM fungi responsible for effects on soil aggregation attributed to AM fungi?; b) is the AM fungal mycelium able to directly mediate the formation of water-stable soil aggregates (*i.e.*, without the interaction with associated microbiota)? We set out to test the latter question in the present study. Additionally, we test the hypothesis that AM fungal mycelium increases soil water repellency (Rillig, 2005); a putative factor contributing to aggregate stabilization.

We utilized a recently developed *in vitro* bioreactor system (described in detail in Gadkar et al., 2006), which was modified to include a sterilized soil / expanded clay mixture (Fig. 1). In these bioreactors (Fisher Scientific, Pittsburgh, PA), consisting of cylindrical chambers (150 mm x 100 mm) with tightly closing lids, the AM fungal culture is placed on a removable mesh (5-6 μm) assembly above the substrate without touching it. The roots are retained by the mesh and the substrate becomes the hyphal growth compartment. The soil was an Albic Luvisol from a local meadow in Berlin with the following properties: 73.6% sand, 18.8% silt and 7.6% clay; 6.9 mg/100 g P; 5.0 mg/100 g K (analyses conducted by LUFA Rostock Agricultural Analysis and Research Institute, Germany). The soil was wet-sieved to 212 μm, mixed with expanded clay (spheres up to 1 cm diam.) in a 2:1 ratio (vol:vol) to provide aeration, and 180 g of this mixture were added to each bioreactor. The bioreactors were then autoclaved (121°C; 20 min) three times on three consecutive days. The experimental treatment

consisted of presence / absence of AM fungus, with 8 replicates per treatment. We used the AM fungus *Glomus intraradices* (DAOM 197198) propagated on *Daucus carota* (wild carrot). *In vitro* cultures of this isolate were obtained from Premier Tech Biotechnologies (Rivière-du-Loup, Quebec, Canada). To the control we added non-colonized cultures. In each case, the entire intact contents of one Petri dish (9 cm diam.) were added to each bioreactor. All cultures were of the same age.
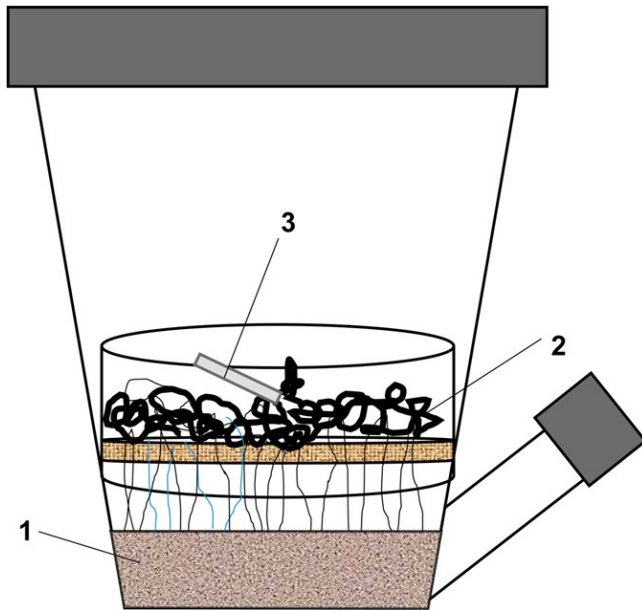

Fig. 1 Diagram showing set-up of the modified bioreactors. The substrate mix (1) is in the bottom of the bioreactor, above which the tray (2) holding the root organ culture and AM fungal inoculum is located. The root cultures are fed with cotton rolls containing glucose (3).

The bioreactors were incubated at 25°C in the dark and the substrate was watered with 20 ml of sterile water four days after inoculation. We added 4 ml glucose (50 mg mL^{-1}) using sterile cotton rolls three times during the study, as in Gadkar et al. (2006). At each of those times, aeration was provided by opening the container for approximately 10 min in the sterile work bench. During the experiment we observed hyphal growth within pore spaces at the edge of the containers. After 12 weeks, the experimental units were destructively harvested. No visible fungal contamination was evident during the experiment in the bioreactors. Additionally, the substrate was tested for living microbial propagules by plating out aliquots on Nutrient Agar (1.5%; Sigma-Aldrich, Steinheim, Germany; N4019). No microbial growth was visible after 7-10 days. Using the ink and vinegar staining method (Vierheilig et

al., 1998), AM fungal root colonization was 75% in the inoculated roots, whereas no colonization was found in the control roots. In one AM-treated bioreactor the culture failed to establish, hence n = 7 for this treatment. Upon harvest the substrate was air-dried (60°C; 48 hrs), and the soil, separated from the expanded clay by gently dry-sieving, used for further analyses.

We extracted extraradical hyphae using an aqueous filtration extraction method and quantified AM fungal mycelium according to the morphological criteria described by Rillig et al. (1999). We did this in the control as well, where all AM fungal hyphae will be dead (the obligate biotrophic AM fungi did not colonize host roots in the control), but – owing to the absence of a microbial community – did not decompose / disintegrate. To quantify the presence of fungal hyphae on macroaggregate surfaces, we determined the frequency of surficial hyphal presence / absence on each of 20 randomly selected aggregates (1-2 mm diam.) per sample using a stereoscope (magnification 60-80X). We used stacked sieves (0.212 mm, 0.5 mm, 1.0 mm, and 2.0 mm) to measure the soil aggregate distribution (starting mass of 50 g soil) and to calculate the mean weight diameter (Kemper and Rosenau, 1986). Additionally, to measure the amount of water-stable macroaggregates, we used a wet-sieving apparatus (sieving time 5 min; total soil used 4.0 g) as described in Kemper and Rosenau (1986), after a 10 min capillary rewetting period. Weights were corrected for coarse matter (> 0.25 mm). For both the soil and water-stable macroaggregate fraction we measured the water drop penetration time (WDPT) in seconds. Using ground samples, the time to absorption of an 8µL droplet of deionized water placed on the smoothed surface was measured for three sub-samples per bioreactor. On both the water-stable macroaggregate fraction and whole soil we also measured the percentage of total C and N using a Euro EA analyzer (HEKAtech GmbH, Wegberg, Germany). Means were compared using one-way analyses of variance.

At the end of the experiment there was about a three-fold increase in AM fungal mycelium in the inoculated bioreactors compared to the control (Table 1); additionally, a much higher proportion (5-fold) of observed aggregate surfaces carried fungal mycelium in the inoculated bioreactors compared to control. While dry sieving revealed no differences between the treatments, using water as a disintegrating force resulted in a significant difference, with increased water-stable macroaggregate mass in the AM fungal inoculated bioreactors. There was a trend towards an increase in soil water

repellency, and there were no differences in C and N content. For the stable macroaggregate fraction (Table 1), there was a highly significant increase in water drop penetration time, while C and N content were again not different between the treatments.

Table 1 Differences in soil parameters between sterile bioreactor systems inoculated or not with the AM fungus *Glomus intraradices*. Values are means and standard errors (n = 8 for control and n = 7 for inoculated). Significant *p* values (< 0.05) are bolded.

Response variable	Non-inoculated (control)	Inoculated	F value (*p* value)
	Whole soil		
AM fungal extraradical hyphal length (m g^{-1} soil)	4.12 (0.33)	12.20 (1.12)	43.8 **(<0.0001)**
Proportion of aggregates with surficial hyphae (%)	10.0 (4.7)	47.9 (5.0)	29.4 **(0.0006)**
Mean weight diameter from dry-sieving (mm)	0.93 (0.06)	0.91 (0.05)	0.09 (0.77) ns
Water-stable macroaggregates > 0.250 mm (% of soil weight)	61.8 (2.6)	72.9 (2.7)	8.75 **(0.011)**
Water drop penetration time (seconds)	9.6 (0.7)	11.9 (0.9)	4.0 (0.058) ns
Total C (%)	2.22 (0.06)	2.25 (0.06)	0.11 (0.74) ns
Total N (%)	0.19 (0.005)	0.19 (0.006)	0.11 (0.75) ns
	Stable macroaggregates		
Water drop penetration time (seconds)	12.2 (1.0)	26.3 (2.2)	37.5 **(<0.0001)**
Total C (%)	2.51 (0.03)	2.49 (0.04)	0.17 (0.8) ns
Total N (%)	0.21 (0.002)	0.21 (0.002)	1.46 (0.24) ns

There are three key points to be taken from the study: (a) we demonstrated the feasibility of using our previously described *in vitro* culture system for carrying out highly controlled, targeted studies of AM fungal mycelium effects on soil properties; and (b) we conclusively showed, to our knowledge for the first time, that AM fungi alone can be sufficient to form and / or maintain water-stable soil

macroaggregates; (c) we demonstrated that AM fungal mycelium can affect soil water repellency, particularly of macroaggregates.

In vitro culture systems of AM fungi have been used successfully for an array of research topics, including systematics, fungal life cycles and structures, as well as physiological and biochemical properties of fungi and the symbiosis (Declerck et al., 2005). The use of soil medium in *in vitro* culture systems is unusual, possibly because of the difficulty to avoid contamination and the inability of direct observation of hyphae in the opaque medium; however, our completely autoclaveable container system has illustrated the potential for also using this approach to study effects on the soil substrate, with the caveat that it is impossible to conclusively prove the absence of residual microbial contamination.

While there is ample evidence for the involvement of fungi in soil aggregation in general (e.g. Gupta and Germida, 1988) and for AM fungi in particular, and good conceptual integration of AM effects in aggregate hierarchy theory (Tisdall and Oades, 1982), only with this study have we established clear causality for a direct AM fungal mycelium effect by excluding other players. This came, like with all mechanistic studies that employ high experimental control, at the cost of ecological realism. Carrying out greenhouse experiments on soil aggregation in which the growth medium is kept sterile is typically neither desired nor technically feasible. Even when defined AM culture material is being used in studies on soil aggregation (*e.g.,* Piotrowski et al., 2004), and care is taken to equilibrate microbial communities by adding a microbial 'wash', it should be realized that microbes could influence the observed effects. While establishing causality in terms of AM mycelium involvement in the formation of water-stable macroaggregates, our study could not address the relative importance of the interaction with soil microbiota compared to the main effects of the mycelium itself; clearly this should be a focus of future research.

Previous work has shown the existence of a link between soil hydrophobicity (for example on aggregate surfaces) and aggregate stability (Sullivan, 1990; Goebel et al., 2005). However, our study is the first to experimentally demonstrate a direct causal link between the growth of AM fungal mycelium and soil water repellency. Rillig (2005) had previously hypothesized that hydrophobins, ubiquitous proteins present in filamentous fungi but which have not yet been demonstrated to be

present in AM fungi, may be responsible for some of the (sub-critical) water repellency phenomena observed in the field. Hallett et al. (2009) could, however, not find a relationship between AM fungi and water repellency in their study system. We do not know what substance(s) were responsible for the observed increase in hydrophobicity in our study. It would be exciting to utilize this bioreactor experimental system to test the idea that hydrophobins were involved; additionally, glomalin-related soil protein (GRSP; Rillig and Mummey, 2006) could also have contributed to the observed effects, even though it has likely no resemblance to hydrophobins (Gadkar and Rillig, 2006). Irrespective, with this result we can add the mechanism of increased water repellency as a potential contribution of AM fungal hyphae to aggregate water stability.

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Chapter 3.2
Rotation of hyphal in-growth cores has no confounding effects on soil abiotic properties

Abstract

To disentangle effects of fungal hyphae and plant roots hyphal in-growth cores have become a common tool in research on arbuscular mycorrhizal fungi (AMF). However, it is unknown if the frequent rotation of a compartment has any side-effects that may hinder attributing findings to AMF. We set up an experiment with the presence/absence of a non-AMF microbial community, where each pot contained a rotated and a non-rotated soil core. The results show that within our rotation design soil parameters such as water content, soil structure, pH, and C and N concentrations are not influenced by the regular rotation in the absence of AMF. Our study therefore clearly underlines the validity of the rotated hyphal in-growth core as an experimental control for AMF growth and activity.

Arbuscular mycorrhizal fungi (AMF) associate with ~ 80 % of the land plants species and are able to improve plant nutrition, soil structure and mitigate plant stress by pathogens or drought (Rillig and Mummey, 2006; Smith and Read, 2008; van der Heijden et al., 1998). As AMF are obligate biotrophic symbionts, hyphal proliferation requires a connection to a host. To disentangle the unique effects of hyphae in the absence of plant roots, hyphal in-growth cores have become a common tool to study the contribution of mycorrhizal fungi to nutrient and allelopathic compound translocation, plant growth, litter decomposition and carbon cycling (Johnson et al., 2001; Wallander et al., 2013). A cylindrical core with windows is covered with a mesh of a pore size that allows hyphae to grow inside the core but excludes the roots (usually 25-40 µm, e.g. Babikova et al., 2013; Cheng et al., 2008; Nottingham et al., 2013). One hyphal in-growth core is static while the other is rotated or moved up and down to sever the hyphae (Barto et al., 2011), and thus serves as a reduced (or non-) AMF control (Johnson et al., 2001). However, despite the broad application of this method, it is unknown if the frequent rotation of a compartment has any side-effects that may hinder attributing findings to AMF. Especially in small compartments the water content could be influenced by the repeated movement of the core, which may also have consequences for a number of processes such as decomposition, soil aggregation and nutrient transfer.

In order to test for confounding effects of the rotated core design, we set up a greenhouse experiment in either presence or absence of a natural soil microbial community, where each pot contained two hyphal in-growth cores. The soil was a loamy sand with the following properties: pH 7.1 (CaCl$_2$), 6.9 mg P / 100 g soil (calcium-acetate-lactate), 0.12% N (total) and 1.87% C (total) (for analytical methods see Rillig et al. (2010)). Soil was autoclaved twice and filled in pots (3 l) where we inserted two plastic tubes (15 cm length, 32 mm diameter), which had a grid structure with 72 openings per tube of a size of 7 x 8.5 mm. The tubes (cores) were covered with a 38 µm mesh that was attached to the core with silicone glue. One soil core was left stable (static compartment), while the second soil core was rotated 3 times per week for 2-3 mm horizontally (rotated compartment) to keep the disturbance as small as possible (see also Fig. 1).
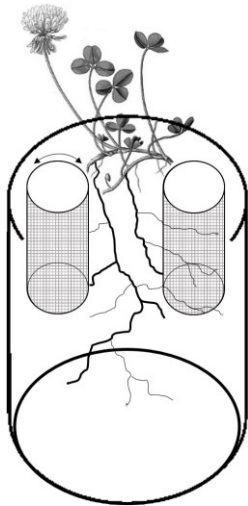
Fig. 1: Rotated core design with a 38 µm mesh: pot with plant, roots and hyphae, rotated compartment (indicated by the arrow) without roots and hyphae and static hyphal compartment (Original image of *T. repens* by Thomé 1885).

Seeds of *Trifolium repens* L. were sterilized in 10% commercial bleach and sown directly into the center of the pots next to the hyphal in-growth cores. After seed emergence plants were thinned to one plant per pot. One treatment received no inoculum and was used to test for side-effects of the rotated core design (N = 10). For five replicate pots each plant was inoculated with a microbial filtrate of fresh field soil that was collected at a meadow of the Freie Universität Berlin. The filtrate was prepared by sieving a soil suspension through a 20 µm sieve and collecting the filtrate, thus excluding larger sized spores such as those of AMF. Half of the filtrate was autoclaved and added to non-inoculated pots. This treatment was used to test whether potential effects of the core rotation may be found in presence of natural saprobic fungi and bacteria but in absence of AMF. Additionally, we inoculated in a similar fashion another five replicates with an AMF spore extract: a soil suspension was sieved through a 38 µm sieve and what remained on the sieve was collected and surface sterilized in commercial bleach (10 %). The primary purpose of this treatment was to check the success of the rotation in reducing AMF hyphal length. Full results of this treatment are therefore presented in Supplementary Table S1. The greenhouse had an average temperature of 26 C° / 20 C° (day / night). During the experiment plant leaves were cut once per week to prevent excessive growth of leaves (to reduce contamination) and roots (to avoid pot bound roots).

After 3.5 months plants and soil were harvested, dried at 40 °C, and stored at room temperature until laboratory analysis. All laboratory analyses were performed for both soil cores, except the water content was analyzed for the soil of the pot as well. Ball milled soil was analyzed for percentages of

total C and N as above. Soil pH and electrical conductivity (EC) were analyzed according to ISO 10390:2005 and ISO 11265:1997, respectively. Water stable soil aggregates (WSA) were assessed by wet sieving in a sieving apparatus modified after Kemper and Rosenau (1986): 4.0 g of dried soil were rewetted by capillary action and sieved for 5 minutes using a 250 μm sieve. We separated the coarse matter by crushing the aggregates that remained on the sieve and pushing the soil through. Coarse matter and soil were collected and dried at 80 °C for 36 hrs. We calculated the amount of total WSA and corrected the calculations for coarse matter. Hyphae were extracted from 4.0 g of soil (Jakobsen et al., 1992), stained with Trypan blue, and hyphal length (m · g^{-1}) soil was measured according to Rillig et al. (1999). Water content of the soil was analyzed gravimetrically by weighing at harvest and after drying. All statistical analyses were conducted using the software R, version 3.0.2 (R Core Team 2013). Soil parameters were analyzed by linear mixed-effects models with the pot number as random factor using the package 'nlme' (Pinheiro et al., 2013).

Our results show that in the absence of AMF there is only slight variation in the measured abiotic soil parameters between the rotated and static compartment (see Table 1). The only significant difference was a slightly lower EC in the rotated cores in the microbial filtrate treatment, which we will discuss below. This suggests that - within our rotation regime - the design is generally suitable for studying specific effects of AMF without confounding abiotic effects. However, compared to the cores, the water content was significantly lower in the rest of the pot (10.2 ± 2.0 %; p = 0.001), indicating that water was removed by the roots and that the mesh may have obstructed the water movement between the pot and the soil cores. Such an effect could be strengthened by a more severe rotation regime than we applied here, and could cause differences to arise between rotated and static cores even though they were absent in our experiment. In the study by Johnson et al. (2001) for instance, where the hyphal in-growth cores were presented for the first time, the cores were rotated by 45 °C around the vertical axis every week.

Table 1: Soil analyses of the non-inoculated treatment and the treatment with microbial filtrate, means and standard errors in parentheses

	Non-inoculated treatment			Treatment with microbial filtrate		
	Rotated	Static	p-value	Rotated	Static	p-value
AMF hyphae ($m \cdot g^{-1}$)	0.33 (0.02)	0.35 (0.03)	0.28	0.28 (0.05)	0.31 (0.03)	0.65
Non-AMF hyphae ($m \cdot g^{-1}$)	1.14 (0.04)	2.05 (0.09)	< 0.001	1.23 (0.09)	2.83 (0.14)	< 0.001
Water content (%)	12.8 (1.6)	13.6 (1.7)	0.23	11.6 (2.2)	11.2 (2.3)	0.39
EC ($\mu S \cdot cm^{-1}$)	346 (5)	347 (8)	0.98	313 (13)	338 (7)	0.03
pH ($CaCl_2$)	6.20 (0.02)	6.16 (0.01)	0.19	6.03 (0.01)	6.03 (0.01)	0.69
C (%)	3.20 (0.13)	3.29 (0.21)	0.67	2.80 (0.63)	2.82 (0.67)	0.93
N (%)	0.26 (0.01)	0.26 (0.02)	0.75	0.23 (0.06)	0.23 (0.06)	0.85
WSA (%)	65.0 (3.7)	64.0 (4.0)	0.70	51.4 (2.3)	59.4 (3.4)	0.09

In other designs rotation was as much as 180 °C once per week or two complete turns twice a week (Nottingham et al., 2013; Weremijewicz and Janos, 2013). The latter represent a considerable disturbance that might cause comparatively stronger effects of the rotating such as the creation of air gaps between the core and surrounding soil, which might hamper the movement of water and nutrients.

As can be seen in Table 1, rotating cores also caused a reduction of non-AM fungal hyphae. These filamentous fungi might have entered the soil with the spore extract, and in the case of the non-inoculated treatment as airborne propagules. The presence of these fungi is therefore unsurprising, as greenhouse experiments generally do not remain sterile. This does however indicate that processes within this experimental design attributed to AMF in the past might have been partly caused by saprobic fungi, at least to the extent that they are affected through rotating cores as we show here. This effect of core rotation on non-AMF may be related to the formation of branched networks that are also severed, which reduces their proliferation efficiency (Barto et al., 2011).

The only parameter that was significantly different between compartments in our study was the electrical conductivity in the treatment with microbial filtrate (see Table 1). The conductivity was higher in the static core, suggesting that the hyphae (more abundant in the static core; Table 1) retained some of the ions, possibly directly within their biomass or indirectly through the stimulation of associated microbes that incorporated those ions, which were then released upon soil drying and measurement of EC (Singh et al., 1989). Replication level in the treatment with microbial filtrate was lower (N = 5) than in the non-inoculated treatment (N = 10), which might have reduced the statistical power to detect more differences between rotated and static compartments. Given the relative subtlety of the effect of rotating on EC, and the fact that it only occurred in the presence of a soil microbial filtrate, we believe it is unlikely to compromise the rotated core design for attributing effects to AMF. In our experiment non-AMF hyphae tended to be reduced in the presence of AMF, which would make any slight modifications in soil properties caused by non-AMF even less severe, as AMF are generally found to have stronger effects on these same properties (e.g. Babikova et al. 2013; Bago et al. 1996; Barto et al. 2011; Johnson et al. 2001) (See also Supplementary Table S1).

In the treatment with AMF spore inoculation AMF were abundant in the static compartment (3.01 (0.19) m · g^{-1}) and successfully reduced in the rotated core (0.43 (0.03) m · g^{-1}) (means and standard error, see Supplementary Table S1). In both treatments not inoculated with AMF we also found AMF hyphae in a comparatively low amount and similar in rotated and static cores (see Table 1). These hyphae were probably dead and not yet decomposed by the microbes present in the soil.

In conclusion, our results clearly underline the validity of the rotated hyphal in-growth core, as pioneered by Johnson et al. (2001), as an experimental control for AMF growth and activity. Besides studies with AMF, it would be interesting to use this experimental design in future studies for saprobic fungi as well, as these were found to also be affected by the rotation of cores. Differing abundance of saprobic fungi can indirectly contribute to levels of various parameters of interest such as OM mineralization or nutrient transport. Generally, a careful control of the water regimes in the cores and the pot soil is necessary if parameters are to be compared between these compartments.

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Chapter 4
Arbuscular mycorrhizal fungi reduce decomposition of woody plant litter while increasing soil aggregation

Abstract

The decomposition of plant organic matter and the stability of soil aggregates are important determinants of soil carbon storage, and the relationship between decomposition rate and arbuscular mycorrhizal fungi (AMF) has recently received considerable attention. In a laboratory pot experiment we simultaneously tested the single and combined effects of AMF and a non-AMF microbial community on the decomposition of small wooden sticks and on soil aggregation. To disentangle effects of hyphae and roots we placed a mesh bag as root exclusion compartment into the soil. The decomposition of the wooden sticks in the hyphal compartment was significantly reduced in the presence of AMF, while aggregation was increased in all inoculated treatments. Both effects were independent of the additional inoculation of other microbes. We suggest that AMF inhibited the activity of decomposers of recalcitrant material, or provided an alternative preferred carbon source, leading to different levels of plant litter degradation under our experimental settings.

1. Introduction

The decomposition of organic matter in soil is an important factor determining soil carbon storage, and the relationship between decomposition of plant litter and arbuscular mycorrhizal fungi (AMF) has received considerable attention in the past few years (e.g. Cheng et al., 2012; Drigo et al., 2013; Herman et al., 2012). Until recently AMF were thought to contribute to soil C storage mainly through the input of plant photosynthates in combination with stabilization of C within soil aggregates (Six et al., 2004; Talbot et al., 2008). This stimulative effects on soil C levels can however be offset if AMF simultaneously promote decomposition of plant litter: even though AMF do not have saprotrophic capabilities, they can enhance decomposition of OM through the stimulation of other soil microorganisms such as bacteria and protozoa (Hodge et al., 2001; Koller et al., 2013). Cheng et al. (2012) showed that plant litter decomposed faster in the presence of AMF, especially under conditions of elevated CO_2 and nitrogen (N) concentrations. Hodge et al. (2001) showed increased plant capture of N and a reduction of C from a patch containing plant leaf litter in the presence of AMF and hypothesized that AMF promoted decomposition by stimulating the activity of hyphosphere bacteria. Meanwhile, a long-term field study (17 and 6 years) found carbon stocks to positively correlate with AMF extraradical hyphae (Wilson et al., 2009), indicating that soil carbon levels do not necessarily decrease in response to AMF. Decomposition of organic matter (OM) appears to generally depend on nutrient availability in the soil and on litter quality (recalcitrant vs. labile) (Cotrufo et al., 2013; Milcu et al., 2011). Recalcitrant litter in the form of lignified plant material is a major contributor to soil OM and the decomposition of woody material depends on a number of factors including humidity, size of the wood piece and the organisms involved (Boddy and Watkinson, 1995). In the soil environment fungi are the main decomposers of recalcitrant litter and the relationship between numerous saprotrophic fungi and wood decomposition has been studied intensely (e.g. Boddy and Watkinson, 1995; Clinton et al., 2009; Worrall et al., 1997). However, to our knowledge the effect of AMF on decomposition of woody material has not yet been studied.

Organic matter can, beyond intrinsic recalcitrance, further be physically protected within soil aggregates, and thus a higher soil aggregate stability can contribute to OM stabilization in the soil

(Rillig, 2004; Six et al., 2004). The role of AMF in soil aggregate formation and stabilization is well documented (see review of Rillig and Mummey, 2006) and we recently showed in a meta-analysis that there generally is a beneficial effect of AMF across a wide range of studies (Leifheit et al., 2014). While it is clear that AM fungi are usually part of a natural microbial community with numerous organism interactions, e.g. with bacteria and non-AM fungi that may also play a role in soil aggregation (Tisdall, 1994), the significance of these interactions for soil aggregation (and for soil C cycling) is fairly unknown. Rillig et al. (2005) showed that various AMF species differentially affect the microbial community composition and that these differences are important for soil aggregation. Furthermore, the combination of fungal species and host plant, the characteristics of the fungal species and the microbial community composition can be highly influential on effects on soil aggregation (Piotrowski et al., 2004; Schreiner et al., 1997). Soil aggregation additionally depends on factors such as the mineralogy of the soil, the chemical properties of the soil (such as pH) and the type of OM involved (Six et al., 2004).

Here, we aimed at simultaneously testing the effect of the single and combined inoculation of AMF and a natural microbial community on OM decomposition and soil aggregation. We tried to answer the question whether the decomposition of small wood pieces would be influenced by the presence of AMF and if potential effects would be enhanced or reduced by the presence of a more natural microbial food web. For soil aggregation we expected a positive, additive effect of AMF and associated microorganisms as both groups are capable of forming and stabilizing soil aggregates.

2. Materials and Methods

2.1. Experimental design and setup

In a 2 x 2 factorial experiment we tested the effects of presence/absence of AMF and a natural microbial community, and interactions between those two factors. Each treatment combination was replicated 10 times for a total of 40 pots. The experiment was located in a climate chamber with an average day/night temperature of 20/16 °C. The soil was a loamy sand collected from an experimental field of Freie Universität Berlin, which had the following properties: pH 7.1 ($CaCl_2$), 6.9 mg P / 100 g soil (calcium-acetate-lactate), 0.12 % N (total) and 1.87 % C (total) (for analytical methods see Rillig et al. (2010)). The soil was autoclaved twice on two consecutive days with at least 24 hours in between in order to ensure absence of viable microbial propagules. The soil was dried in the autoclaving bags at 60 °C and subsequently smashed with a rubber mallet to reduce the soil aggregation level. The soil was then sieved using a 2 mm sterilized sieve and simultaneously mixed with 20 % autoclaved sand in order to partially compensate for nutrient release during sterilization, resulting in a sand content of 79 %. One and a half liters of the soil-sand-mixture were transferred into 3 L pots with a 2 cm layer of sterilized sand on the bottom and on the top. Close to the center of the pot a mesh bag (120 ml volume, 38 μm pore size) was installed to allow the penetration of hyphae while excluding roots (further referred to as hyphal compartment). Pots were re-randomized regularly throughout the experiment.

Seeds of *Plantago lanceolata* L. were sterilized in 10% bleach for 10 minutes and in 70 % ethanol for 30 seconds, rinsed in deionized water after each step, and sown directly into the soil. After seedling emergence plants were thinned to one plant per pot (further referred to as planted compartment). During the growing period plant leaves were cut twice to a height of approximately 15 cm to prevent excessive growth of leaves (to avoid contamination) and roots (to avoid pot bound roots). Plants were watered as needed three times per week.

Control pots were left non-inoculated. For the AMF treatment we inoculated approximately 1000 spores per pot of *Rhizophagus irregularis* (Schenk & Smith, DAOM197198, Symplanta GmbH & Co. KG). The sterile spores were carried in a rock flour material that was mixed with deionized water and pipetted into a cut-off pipette tip that was positioned under the plant towards the roots. The controls received

sterilized carrier material. Fresh field soil collected at the same field site as above was used to produce a microbial wash from a soil filtrate (200g of soil in 2 liter of tap water) where the smallest sieve had a size of 20 μm, thus excluding larger sized spores such as those of AMF. This microbial wash represents the non-AMF microbial community. We inoculated the microbial wash ('MW') to the seedlings of the treatments and autoclaved microbial wash to controls. Inoculation with both AMF and MW will later be referred to as the 'AMF + MW' treatment.

To measure microbially mediated decomposition we used wooden sticks that were inserted in the soil and determined their mass loss rate at the end of the experiment (Arroita et al., 2012; Sinsabaugh et al., 1993). Linden (*Tilia sp.* L.) wood sticks (4 x 2 mm width, Meyer & Weigand GmbH, Germany) were cut into pieces of ca. 30 mm length, autoclaved and weighed (154.43 mg ± 0.35, N = 30). One stick was introduced into the soil of each compartment (hyphal and planted) using a spatula. Each stick contained approximately 76 mg C and 32 mg N (see also Table 1). We used wood as OM because it is a relatively easy assessment that allowed us to simultaneously test effects of AMF on decomposition of an organic material and on soil aggregation, which are two important processes that relate to soil C storage. Experiments studying effects of AMF on soil aggregation usually require a duration of several months (Leifheit et al., 2014) and we thus needed a material that allowed us to measure potentially meaningful differences over a longer period. Because our primary focus was on direct decomposition of the OM (but not necessarily nutrient transport by AMF mycelia) and the wood sticks can be readily recovered, it was not necessary to use isotopically labeled plant materials.

2.2. Harvest and sample storage

The hyphal compartment was harvested 5 months after inoculation. The newly formed holes were filled with a plastic tube to prevent the surrounding soil from collapsing. In order to extend the plant growth period for as long as possible we continued watering the plants until they would have needed another cut (see above), which was 6 weeks after the harvest of the hyphal compartment (6.5 months after inoculation). All materials were dried at 40 °C, weighed and stored at room temperature until laboratory analysis. Soil subsamples were immediately frozen in liquid N and stored at -80 °C.

2.3. Lab analyses

The wooden sticks were removed from the dried soil of the hyphal and planted compartment, cleaned with scalpel and brush and dry-weight was determined. One half of each wooden stick was ball milled and analyzed for percentages of total C and N content (EuroEA, HekaTech, Germany).

All soil analyses were performed for the hyphal compartment only. Ball milled soil was analyzed for percentages of total C and N with an Elemental Analyzer (EuroEA, HekaTech, Germany). Hyphae were extracted from 4.0 g of soil (Jakobsen et al., 1992), stained with ink and vinegar and hyphal length in m · g^{-1} soil was measured according to Rillig et al. (1999). On six randomly selected samples we additionally determined the amount of blue-stained and 'light brown' colored non-AM fungal hyphae as a rough indication of differences in fungal communities.

The dried soil was sieved through a 4 mm sieve and water stable soil aggregates were assessed for eight subsamples by wet sieving with a series of stacked sieves of the size 2 mm, 1 mm, 0.5 mm and 0.212 mm with the smallest sieve at the bottom (modified from Kemper and Rosenau 1986). We immersed the stack of sieves in a bucket of water (40 cm high, 30 cm diameter). 50.0 g of soil were rewetted by capillary action, placed on top of the uppermost sieve and moved up and down for 4 minutes (30 strokes per minute), while the soil on the uppermost sieve was completely immersed in water the entire time. We separated the coarse matter (mainly sand particles) by crushing the aggregates and pushing the soil through the respective sieve. Coarse matter and soil were collected and dried at 80 °C for 36 hrs. All calculations were corrected for coarse matter. The fraction of water stable aggregates (WSA) in each size class was calculated as described in Barto et al. (2010). Here, we only report the data for the total amount of water stable aggregates. Data on the different size classes are reported in the Supplementary Material (Table S3).

For a subsample of five replicates the following parameters were analyzed. Plant available phosphate in 5.0 g of dry soil was extracted with 100 ml of a 0.05 M calcium-acetate-lactate-solution while shaking for two hours. The supernatant was filtered through a P-free filter and phosphate concentration was determined photometrically (Analytical Continuous Flow Analyzer SAN Plus, Skalar, The Netherlands). Ammonium was extracted from a 5.0 g sample of frozen soil with a 0.01 M CaCl$_2$ solution

and measured photometrically (ISO 14255, Analytical Continuous Flow Analyzer SAN Plus, Skalar, The Netherlands). Soil pH and electrical conductivity were analyzed in 0.01 M $CaCl_2$ and deionized water, respectively, according to ISO 10390:2005 and ISO 11265:1997. Roots were washed, dried at 40 °C and weighed. To demonstrate the success of inoculation we determined the percent root colonization in each treatment: roots were stained with ink and vinegar (as described in Vierheilig et al., 1998) and percentage of AMF structures was determined microscopically at 200 x magnification with the gridline intersect method (100 intersects per sample) (McGonigle et al., 1990; Rillig et al., 1999).

2.4. Statistics

All statistical analyses were conducted in the statistical software R version 2.15.2 (R Core Team 2013). For the analysis of single and combined effects of inoculation with AMF and MW we used two-way ANOVAs with AMF and MW as factors and the water content at harvest as covariate, as this variable can strongly influence several parameters that were important in our experiment such as decomposition, microbial community composition and activity or soil aggregation. Where appropriate we applied a Tukey Honest Significant Difference post-hoc test (in case of a significant interaction term). Data were log transformed if necessary to meet the assumptions of normality of residuals. If conditions for homoscedasticity were not met we used a generalized least squares model (gls) using the package 'nlme' (Pinheiro et al., 2013). AMF hyphal length residuals were not normally distributed and treatment effects were therefore analyzed using a Kruskal-Wallis rank sum test. To test whether C and N concentrations of the wood changed during the experiment, concentrations at the start of the experiment and at harvest were compared using linear models. The weight loss of the wood sticks of the hyphal compartment was analyzed with an ANOVA with the full number of replicates (N = 10) and we repeated the analysis excluding three samples for which the recovery of the whole stick was uncertain. The results of these two analyses were the same and we report the results excluding those three samples as outliers from our statistics. The recovery of wood sticks from the planted compartment was more difficult compared to the hyphal compartment and we therefore report statistics for eight replicates, for which we could fully recover the sticks.

3. Results

3.1. Wood stick decomposition

The weight loss (%) of the wooden sticks in the hyphal compartment (HC) was significantly reduced in the presence of AMF (p = 0.0001) (see Fig. 1). Overall weight loss in the hyphal compartment was approximately 47 % in the control and the MW treatment. The presence of AMF reduced the weight loss to an average of 31 %. Overall weight loss in the planted compartment was approximately 25 % in the control and MW treatment, while the presence of AMF reduced the weight loss to an average of 13 % (p = 0.003, see Table S2, Supplementary Material). The water content of the soil did not have a significant influence on decomposition in both cases (p > 0.4 for both).
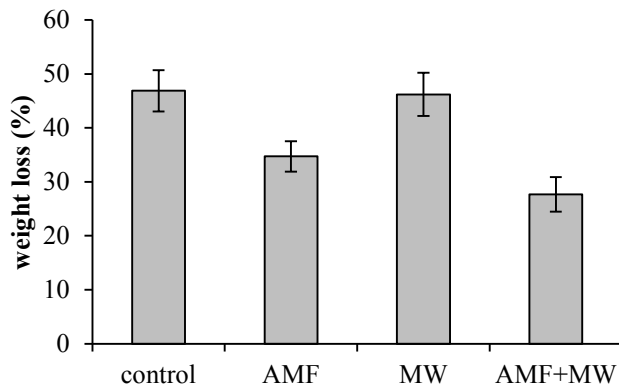

Fig. 1: Average (± se) gravimetric weight loss (%) of the wood sticks in the hyphal compartment after the experiment. Control = non-inoculated treatment, AMF = treatment with *R. irregularis*, MW = treatment with microbial wash, AMF + MW = treatment with combined inoculation of AMF and MW.

The C : N ratio of the wood sticks in the AMF + MW treatment was significantly higher compared to the AMF and MW treatment (p = 0.005, p = 0.004, respectively) (see Table 1). This increase was caused by a significant reduction in N concentration in the AMF + MW treatment compared to the AMF and the MW treatment (p = 0.02, p = 0.004, respectively). The total carbon concentration was not different between the treatments (p ≥ 0.08 for all treatments), and did not change compared to the start concentration. Concentrations of N in the wood sticks all increased during the experiment (p ≤ 0.03 for all treatments), while C : N ratios decreased during the experiment (p < 0.02 for all treatments).

Table 1: Mean (± se) carbon and nitrogen concentrations (%) in the wood sticks and C : N ratio at the start and after the experiment.

Treatment	C (%)	N (%)	C : N ratio
Start	48.7 (0.15)	0.10 (0.01)	473 (40)
Control	48.0 (0.65)	0.31 (0.06)	215 (45)
AMF	47.9 (0.42)	0.31 (0.09)	166 (31)
MW	49.2 (0.44)	0.38 (0.06)	163 (24)
AMF + MW	47.9 (0.24)	0.17 (0.02)	336 (44)

Soil nutrient concentrations (ammonium, phosphate, C, N) and electrical conductivity were not significantly different between the treatments (see Table S1, Supplementary Material). For pH there was a slight increase in all inoculated treatments compared to the control ($p < 0.02$ for all treatments), but all values were in the range of a neutral soil pH: 6.80 - 7.02 (Table S1, Supplementary Material).

R. irregularis successfully colonized the host plant roots to around 59 (± 11) % in the AMF treatment and 75 (± 4) % in the AMF + MW treatment, while AMF root colonization was absent in the control. AMF soil hyphal length was significantly higher in the AMF-inoculated treatments ($p < 0.0001$) (see Table 2). Non-AMF hyphae were significantly higher in the MW treatment and the AMF + MW treatment ($p \leq 0.03$ for both treatments). The length of non-AMF 'light brown' hyphae in the soil was significantly influenced by the AMF and MW treatment ($p < 0.01$ for both treatments). Non-AMF blue-stained hyphae increased in the presence of AMF ($p < 0.05$).

Table 2: Mean (± se) hyphal length (m · g^{-1} soil)

Treatment	AMF hyphae	Blue non-AMF hyphae	Light brown non-AMF hyphae	Total non-AMF hyphae
Control	0.12 (0.04)	0.15 (0.04)	0.89 (0.17)	1.73 (0.17)
AMF	5.02 (0.13)	0.45 (0.16)	0.65 (0.06)	1.98 (0.12)
MW	0.11 (0.03)	0.12 (0.05)	1.42 (0.65)	3.25 (0.38)
AMF + MW	5.09 (0.54)	0.44 (0.25)	0.87 (0.13)	2.05 (0.31)

3.2. Soil aggregation

The formation of water stable aggregates in the hyphal compartment was significantly increased in all inoculated treatments compared to the control (AMF: $p < 0.001$, MW: $p < 0.01$, AMF + MW: $p < 0.05$), but there were no differences between the inoculated treatments (see Fig. 2). Root dry weight in the plant compartment and water content in the hyphal compartment were not significantly different between the treatments (see Table S1 and S2, Supplementary Material).
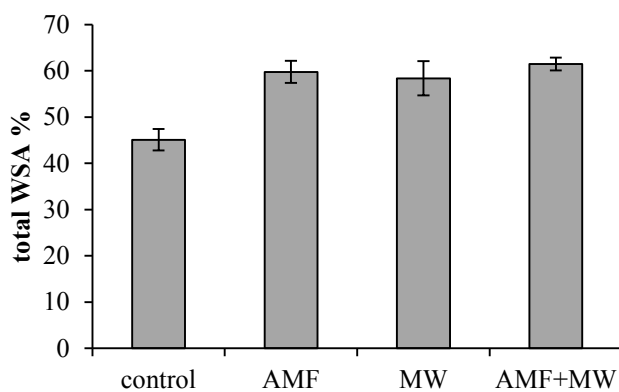

Fig. 2: Average (± se) percentage of total water stable aggregates (%) in the hyphal compartment. Control = non-inoculated treatment, AMF = treatment with *Rh. irregularis*, MW = treatment with microbial wash, AMF + MW = treatment with combined inoculation of AMF and MW.

4. Discussion

4.1. Decomposition

Our results show a decreased decomposition of linden wood sticks in treatments inoculated with AMF, independent of whether a natural soil microbial community was present or not. This suggests that AMF may have directly or indirectly inhibited the activity of microbiota capable of plant litter decomposition. Such a reduction is commonly found in ectomycorrhizal fungi, where it was first described by Gadgil and Gadgil (1971). The so-called 'Gadgil-effect' has been proposed to be caused by effects of mycorrhizae on soil nutrient availability, moisture, and/or direct inhibition of saprotrophic fungi (Bending, 2003). We think it is likely that in our experiment AMF influenced the microbial community in a similar manner and thus induced changes in decomposition. There is a large body of evidence showing that the presence of AMF alters the microbial community composition (e.g. Andrade et al., 1997; Artursson et al., 2005; Rillig et al., 2005; Toljander et al., 2007). In our experiment AMF might have influenced other microorganisms either through competition for nutrients, water, space, exudation of inhibitory or stimulatory substances, or by the excretion of mycelial compounds that served as a carbon resource (Nottingham et al., 2013; Rillig, 2004; Rillig and Mummey, 2006). As can be seen in Table 2, the abundance of non-AMF hyphae is different between AMF inoculated and non-inoculated treatments. Abundance of 'light brown' hyphae tended to be lower in the treatment with AMF only and non-AMF blue hyphae were increased in the presence of AMF (AMF and AMF + MW treatment). These alterations in the fungal community may have resulted in a community shift towards fungi with slower decomposition rates, different metabolisms or food preferences (Clinton et al., 2009; Schmidt et al., 2011). The provision of carbohydrates by AMF is likely to affect the feeding and thus abundance of surrounding microorganisms, as the amount of exudated C can be substantial. The transfer of plant C to the hyphae can be up to 20 % of the assimilated C and can become available to microbes through exudation and hyphal turnover (Jakobsen and Rosendahl, 1990). In our experiment, this could have enhanced the transport of C into the soil of the hyphal compartment and microorganisms might have preferred the labile C source to the wood stick.

Besides direct influences of AMF on other fungi through mycelial exudates AMF can also have indirect effects on other microbes, for instance through the removal of nutrients. Litter decomposition and soil OM formation depend on the nutrient availability in the soil and are context dependent (Milcu et al., 2011). Reduction of nutrient levels by mycorrhizae can inhibit saprotrophic fungi, which could be an important process in soil C accumulation (Orwin et al., 2011). In our experiment nutrient concentrations, pH and EC of the hyphal compartment were not different in AMF inoculated treatments, which would have indicated changed chemical conditions in the soil that could have altered microbial activity or proliferation (Baath and Anderson, 2003; Marschner et al., 2005). Another parameter of paramount importance for decomposer activity is the water content of the soil, which was equal in all treatments at harvest and was added as a covariate in the statistical models. If translocation of soil nutrients or water had been responsible for the reduced decomposition, it likely was a very localized effect where AMF scavenge nutrients exactly from patches where they are released or removed water on a micropore scale, without strong effects on overall nutrient or water levels.

Contrary to nutrient levels in the soil, nutrient concentrations in the wood sticks at harvest differed between the treatments. A common effect in wood decomposition studies is that carbon content in the decayed wood can vary substantially depending on the type of wood or the fungi present, whereas it is generally observed that nutrients, especially N, increase during time of decomposition (Boddy and Watkinson, 1995; Clinton et al., 2009; Preston et al., 2012). In this case, growth of microorganisms causes nutrients to be translocated into the wood, where they are retained in the wood-mycelium-biomass, while carbon is mineralized or respired (Boddy and Watkinson, 1995). Mineralized C can be incorporated into microbial biomass and can eventually lead to increased soil C storage. Final N concentration increased in all wood sticks compared to the starting wood, but differed markedly between the treatments: in the AMF + MW treatment the concentration was on average only half of that in all other treatments. A potential reason for this could be a higher microbial turnover promoting N removal from localized OM patches by AMF in the presence of a more complex microbial food web, which was recently demonstrated in presence of protozoa (Koller et al., 2013).

Another parameter important for the rate of decomposition is the litter quality (Cotrufo et al., 2013; Knorr et al., 2005). Berg (2000) argues that in early stages of plant litter decomposition water soluble substances and sugars are decomposed, leading to an increased lignin concentration towards later stages. In advanced stages of decomposition there is a negative relationship between N concentration in litter and lignin mass loss, probably caused by a repression of ligno-lytic enzymes or the formation of recalcitrant compounds of lignin degradation products with ammonia or amino acids (Berg, 2000). More in general he proposed that N availability is the factor that is similarly responsible for increased initial decomposition and decreased subsequent decomposition. Considering these phenomena, the presence of AMF could have both: a stimulating effect in early stages of decomposition and an inhibitory effect on decomposition in later stages of decomposition (or on recalcitrant OM more generally). This hypothesis could explain opposing accounts in the literature and is an exciting topic for future research.

The results of our study contrast with other studies that found enhanced decomposition of OM in the presence of AMF (Cheng et al., 2012; Hodge et al., 2001). However, most studies that tested effects of AMF on litter decomposition used relatively short-term experiments (4 – 10 weeks) and a comparatively large amount of (ground) leaf litter as organic substrate (0.42 – 4.44 % C in the organic patch/compartment) (Cheng et al., 2012; Hodge et al., 2001; e.g. Koller et al., 2013), while we employed a small amount of intact, recalcitrant plant material (0.04 % wood C in the soil of the hyphal compartment) and measured decomposition after approximately 23 weeks. Some of the studies that found increased decomposition in presence of AMF added N to their treatments (e.g. Cheng et al., 2012), which can favor bacterial dominance in the soil, leading to increased turnover rates of high quality OM (Manning, 2012), while we did not fertilize the soil of our experiment. Therefore, the experimental conditions clearly contribute to differences between findings among studies.

Although the control treatment was not inoculated, the decomposition rate was among the highest of our treatments and approximately the same as in the MW treatment. The presence of decomposing microorganisms in the control is unsurprising as experiments in greenhouses or climate chambers generally do not remain sterile when airborne propagules of bacteria or fungi can enter the soil. Absolute decay rates of wood can be highly variable as decomposition depends heavily on the presence of

particular decomposers and the type of wood. Worrall et al. (1997) observed weight loss of two different wood types between 20 and 30 % on average for typical decay fungi (Aphyllophorales) after 12 weeks of incubation, but other fungi caused mass losses up to > 80 %. Therefore, mass loss rates found in our experiment cannot be categorized as comparatively high or low.

4.2. Soil aggregation

By using a hyphal compartment that excluded roots we could show that there are direct positive effects of AMF, a natural soil microbial community excluding AMF, and the combination of both on soil aggregate formation. However, the single effects of AMF and microbial wash were non-additive, contrary to our hypothesis, as the soil aggregation level was not higher when these two treatments were combined compared to either treatment alone. It is possible that the relatively high sand content of the soil-sand-mixture (79 %) did not allow more soil aggregation because of low amounts of clay or organic matter, which are soil components that usually enhance aggregation (Tisdall and Oades, 1982). It is well established that AMF can strongly influence the neighboring microbial community (see above) but our knowledge of the complex interactions between these groups is still limited. The combined inoculation of a single AMF species and a whole soil microbial community in our experiment might have induced strong interspecific competition processes (Smith and Read, 2008). In AMF inoculated treatments the competition might have suppressed non-AMF species with a high soil aggregating ability such as other filamentous fungi or bacteria that excrete sticky polysaccharides (Tisdall, 1994). In absence of AMF, i.e. without the competition, these species could proliferate more freely and thus contribute to soil aggregation.

5. Conclusions

To our knowledge this is the first study that shows reduced decomposition of a recalcitrant plant litter in presence of AMF. Moreover, even when a natural soil microbial community (excluding AMF) is present, the reduction of decomposition entirely depends on whether AMF are present as well. Numerous studies have shown that AMF influence the resident soil microbial community and we suggest that these changes can play a crucial role in the decomposition of plant litter, as we believe was the case in our experiment. To further improve our knowledge of the role of AMF in litter decomposition future research should test plant litter types of differing qualities for their decomposition rates and fate of C in terms of loss or incorporation in microbial biomass or soil OM. The addition of different AMF species, a natural soil microbial community, the use of different soil types and fertilizer levels would be an exciting addition to those experiments.

The findings of reduced decomposition of woody plant litter and increased soil aggregation in the presence of AMF and AMF teamed with a natural soil microbial community could indicate an important role for AMF in soil C storage. However, the lack of an additive effect of AMF in combination with associated microbes on soil aggregation still needs to be validated, given the context dependency of AMF effects. To elucidate this question further experimental investigation with different soil textures and different AM fungal species involved in the presence and absence of a non-AM microbial community will be necessary.

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Chapter 5
General Discussion

Arbuscular mycorrhizal fungi (AMF) have a wide range of functions that are important for soil sustainability and that contribute to ecosystem services such as the improvement of soil structure, plant nutrition and plant resistance to drought and pathogens (Gianinazzi et al., 2010). The soil structure can be indirectly assessed as the stability of soil aggregates and is an important aspect of soil quality with possible benefits for plant growth, water infiltration, microbial activity and soil C storage (Rillig & Mummey, 2006). In this dissertation, I focused on effects of AMF and their interaction with associated microbiota on soil aggregation and soil C cycling.

In chapter 2 we performed a meta-analysis on effects of AMF on soil aggregation and could show that AMF indeed generally increase soil aggregation. This result is corroborated by our findings of chapter 3.1 where we experimentally showed that there is a direct causal link between AMF hyphae alone - in absence of other microorganisms – and soil aggregate stability. In chapter 3.2 we experimentally validated the method of the rotated hyphal in-growth core design. In chapter 4 we studied effects of AMF and a natural microbial community on soil aggregation and litter decomposition.

Evidence from experiments on the interaction of AMF and associated microbiota in the context of soil aggregation and litter decomposition

After I focused on the role of just AMF in soil aggregation and methodological issues in chapter 2 and 3, I used an experimental approach to study the interrelationships of AMF and associated microbiota and their effects on soil aggregation and decomposition of woody plant litter (chapter 4). I showed that AMF can increase soil aggregate formation and that this effect persists when a natural soil microbial community is present simultaneously. However, effects of the combined application of AMF and microbes were not additive. A non-additive effect on soil aggregation was also observed for collembola and AMF (Siddiky et al., 2012). However, as we showed in chapter 2, effects of AMF on soil aggregation depend on a number of abiotic factors such as soil texture, pH, pot size, experiment duration

and location (field vs. pot) and soil sterilization. Such context dependency had been shown before for various other AMF functions and AMF abundance (Helgason and Fitter, 2009; Hoeksema et al., 2010; Johnson et al., 2003; Smith and Read, 2008). Therefore, the interaction of AMF and associated microbes on soil aggregation likely also depends on the specific conditions of a study. Our knowledge of the relationship between these two organism groups is still limited and requires future research in different contexts such as varying soil properties (e.g. texture, pH, organic matter content), different plant hosts and AMF species.

The context is also of pivotal importance when studying effects of AMF on litter decomposition, as the direction and magnitude of effects strongly depend on parameters such as litter quality, nutrient levels in the soil or the atmospheric CO_2 concentration (see also the discussion section in chapter 4). The role of AMF in plant litter decomposition still needs to be further elucidated: mechanisms inducing increased decomposition, such as the stimulation of neighboring microorganisms, were only postulated but experimental evidence is lacking (e.g. Hodge et al. 2001). Additionally, it is questionable how evidence from laboratory experiments can be transferred to more realistic conditions in the field. The majority of experiments uses conditions that are not realistic for the field, such as adding finely ground OM substrates or thoroughly mixing the OM substrate with the soil (e.g. Koller et al., 2013). While these experimental setups allow relatively fast comparisons and generalizations among treatments, field conditions are far more heterogeneous and might thus show more variable decomposition. Although natural amounts of litter in the field can be large (e.g. $3 - 9$ t ha^{-1} year^{-1} from grassland to forests; (Carlisle et al., 1966; Ewel, 1976; Gosz et al., 1972; Tilman et al., 2001)), the availability of the OM differs as it consists of material of differing quality such as stems, branches and fresh broad leaves. The incorporation of the litter in the soil is usually heterogeneous as soil properties can vary on a small scale (Chen et al., 2006; Kumar et al., 2006) and the climatic conditions vary throughout a full growing period. Additionally, there is a number of disturbances in the field such as ploughing. There is a compelling body of evidence showing that organic farming and reduced tillage favor the abundance of AMF and that these systems have higher soil C concentrations as well as higher soil aggregation levels (Bossuyt et al., 2002; Caesar-TonThat et al., 2011; Helgason et al., 2010; Six et al., 2006). Agricultural sites cover 12 % of the earth's land surface (FAO, 2013). The careful management of these sites, favoring AMF

abundance and C accumulation, is therefore of crucial importance for the sustainability of agroecosystems and global C cycling.

Another promising aspect for future research in soil C cycling is the influence of temperature on AMF and litter decomposition. Increasing temperature is known to have a stimulating effect on litter decomposition (Conant et al., 2011; Kirschbaum, 2004; Pendall et al., 2004). There is also evidence for positive effects of increasing temperature on AMF abundance, probably through increasing C allocation to AMF, but negative effects of increased temperature on soil aggregation have been observed (Rillig et al., 2002), thus potentially affecting soil C storage. It is important to note, however, that effects of increased temperature can be confounded with i) changes in soil moisture, which affect microbial activity (Allison and Treseder, 2008; Kirschbaum, 2004) and ii) effects of substrate quality: effects of temperature can be overruled by the addition of labile OM inducing microbial growth and priming of bulk soil OM (Kirschbaum, 2013; Thiessen et al., 2013). Klironomos et al. (2005) showed that effects of AMF after sudden rises in CO_2 disappear if CO_2 is increased gradually. It is not known, however, if this is also true for the influence of temperature on AMF and AMF-mediated decomposition. This topic is therefore highly interesting for future research as it links global warming and soil C cycling. It would be exciting to study effects of AMF on litter decomposition at different temperatures with sudden and gradual rises, including OM of different quality and studying the interaction of AMF with other microorganisms and their effects on the incorporation of OM in soil aggregates.

Methodological issues arising in research on AMF and their interaction with other microorganisms

The number of studies on effects of different species or richness levels on soil aggregation is small. One reason for this could be the methodological difficulties that are involved in cultivation of AMF. Most AMF are cultured as trap cultures in the presence of a host, which is laborious and requires a considerable amount of space and time (Declerck et al., 2005). Spores are extracted from the substrate

usually by sieving and can be surface sterilized if necessary for experimental purposes. However, contamination of surface sterilized spores can be as high as 20 %, as the sterilization success depends on the sterilization time and agents, and the use of antibiotics (Budi et al., 1999). Until recently only a few species (e.g. *Glomus intraradices*) could be cultured in a monoxenic manner *in vitro* (Declerck et al., 2005), but the cultivation of more AMF genera is developing and today *in vitro* produced inoculum of *Scutellopsora* and *Gigaspora* is commercially available in large amounts (i.e. 1 million spores, e.g. Symplanta GmbH & Co. KG). The advantage of the availability of sterile *in vitro* inocula in the context of soil aggregation experiments - that are usually not performed under sterile conditions – is that it enables future research to study the effect of single species without prior attached microorganisms such as bacteria, thus reducing priority effects (Verbruggen et al., 2012). The establishment of species initially present can heavily affect the resulting microbial community composition, which is important for processes such as aggregation and decomposition (see also discussion in chapter 4). Hence, the inoculation of sterile AMF spores is important, because it allows the AM fungus, but also other inoculated organisms such as microbiota of a microbial wash, to proliferate before other microbes enter the soil as airborne propagules and might influence the community composition.

Greenhouse or climate chamber experiments with open pots do generally not remain sterile. In our pot experiment presented in chapter 4 we used autoclaved soil and spores of an *in vitro* culture as inoculum, but nevertheless the non-inoculated controls were contaminated. Nevertheless, we think that our control is still valid for comparison with AMF treatments because i) the length of non-AMF hyphae was significantly lower in the control and ii) we could detect significant effects of AMF on soil aggregation and litter decomposition. The maintenance of sterile conditions throughout an entire experiment of 3 -5 months – the time span usually necessary to see effects of AMF on soil aggregation (see chapter 2) – is difficult to achieve and would require a sophisticated and expensive equipment, i.e. closed containers that allow regular watering and that are large enough to fit soil and a plant for this duration. I think that the use of sterile inoculum, and thus preventing priority effects, is more important than the maintenance of sterile conditions, as our experiment of chapter 4 shows that we can nevertheless detect effects on two important processes that are clearly attributable to the presence of AMF.

Further investigation addressing questions on the influence of different species or richness levels on soil aggregation is thus constrained by methodological difficulties, but appears to be very promising. We are currently performing an experiment with 3 different AMF species, inoculated with sterile spores, in presence/absence of a microbial filtrate (representing a natural non-AMF microbial community), where we will test single and joint effects on soil aggregation. We expect a differential development of the associated microbial community composition between the three AMF species with possible differential effects on soil aggregation.

Rotated hyphal in-growth cores as promising tool in AMF research

In soil aggregation experiments effects of roots and hyphae are often not clearly distinguished, i.e. the effect of treatments in presence of mycorrhizal roots is compared to treatments in presence of non-mycorrhizal roots and the evidence for effects of AMF hyphae on aggregate stabilization in absence of roots is missing (e.g. Bethlenfalvay et al., 1997; Cavagnaro et al., 2006; Kohler et al., 2009). To show effects of AMF hyphae on any process while excluding influences by the root, the use of rotated hyphal compartments was proposed by Johnson et al. (2001). The rotation of a hyphal in-growth core severs the AMF hyphae and can thus control for effects of AMF associated microorganisms in (local) absence of the symbiotic partner. However, if rotating cores has additional effects on other soil parameters independent of AMF, this would hinder attributing effects to the presence of these fungi. We tested if the rotation of the cores has any side effects and could show the validity of the method (see chapter 3.2). I think the use of the rotated core design is especially interesting for litter decomposition studies, as interest in the role of AMF in soil C cycling is rising (Cheng et al., 2012; Drigo et al., 2013; Herman et al., 2012). It is usually assumed that AMF stimulate the activity of neighboring microorganisms, thus promoting decomposition (Hodge et al., 2001), but organism interactions in decomposition processes are still not yet fully understood. In this context, rotated cores could help disentangle direct effects of AMF hyphae (in the absence of other microbes), AMF in the presence of other microbes and effects of AMF associated microbes in the absence of AMF hyphae.

Conclusions

The results presented in this dissertation show a great potential of AMF for soil management practices in e.g. erosion control or land restoration. The ample existing evidence for the importance of AMF in soil aggregation was further supported by our meta-analysis that gave quantitative evidence of the generality of beneficial effects of AMF on soil aggregation and further underlined the context dependency of this effect. We could also show that AMF in the absence of other microbes are able to maintain soil stability. These are important contributions to our understanding of the ecology of soil aggregation. Our study on the design of the rotated hyphal in-growth core helps to validate research on AMF hyphal effects of the past and of the future. Nevertheless, there are still a number of open questions in this field of research. For instance, we still do not fully understand the interaction between AMF and their associated microorganisms and their role in soil aggregation.

Furthermore, this dissertation presented results that showed a potential role of AMF in reducing the decomposition of recalcitrant OM, which could have wide implications for soil C storage. The role of associated microbiota and other non-AMF microorganisms and their interaction with AMF in this process is especially interesting and clearly merits our attention in future research.

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Summary

The main goal of this dissertation was to increase our understanding of the ecology of soil aggregation and the interaction of AMF with associated microbiota in the context of soil stability and soil C cycling.

In **Chapter 2** we performed a quantitative data synthesis (meta-analysis), in which we tested the general assumption that AMF positively influence soil aggregation and additionally tested 13 factors, which potentially influence effects of AMF on soil aggregate stabilization. For the meta-analysis we fitted individual random effects models for each variable. The overall mean effect of inoculation with AMF on soil aggregation was positive and predictor variable means were all in the range of beneficial effects. Pot studies and studies with sterilized sandy soil, near neutral soil pH, a pot size smaller than 2.5 kg and a duration between 2.2 and 5 months were more likely to result in stronger effects of AMF on soil aggregation than experiments in the field, with non-sterilized or fine textured soil or an acidic pH. This is the first study to quantitatively show that the effect of AMF inoculation on soil aggregation is positive and context dependent.

In the first part of **Chapter 3** we performed a sterile laboratory experiment using small *in vitro* bioreactors, in which we grew mycelium of *Glomus intraradices* in absence of roots and other living microorganisms. We showed that there is a direct causal link between AMF hyphae alone and the maintenance of soil aggregate stability. In the second part we tested if the broadly applied method of rotated hyphal in-growth cores has any side-effects that would hinder attributing effects to AMF. We set up an experiment with the presence/absence of AMF and a non-AMF microbial community, where each pot contained a rotated and a non-rotated soil core. The results showed that soil parameters such as water content, soil structure, pH, and C and N concentrations are not influenced by the regular rotation in the absence of AMF. Our study therefore clearly underlines the validity of the rotated hyphal in-growth core as an experimental control for AMF growth and activity.

In **Chapter 4** we experimentally tested the single and combined effects of AMF and a non-AMF microbial community on the decomposition of small wooden sticks and on soil aggregation. A hyphal compartment was installed to disentangle effects of roots and hyphae. While aggregation was increased

similarly in all inoculated treatments, this study showed for the first time, that the presence of AMF can reduce the decomposition of recalcitrant plant litter. Both effects were independent of the additional inoculation of other microbes. We suggest that AMF inhibited the activity of decomposers of recalcitrant material, or provided an alternative preferred carbon source, leading to differential litter degradation. These findings contribute to understanding the interaction of AMF with other microorganisms in soil aggregation and could indicate an important role for AMF in soil C storage.

Zusammenfassung

Das Ziel dieser Dissertation war es unser Verständnis der Ökologie der Bodenaggregation im Allgemeinen und der Interaktion von arbuskulären Mykorrhizapilzen (AM-Pilze) mit anderen Mikroorganismen im Besonderen im Kontext der Bodenaggregation und des Bodenkohlenstoffkreislaufes zu erhöhen.

In **Kapitel 2** haben wir eine quantitative Analyse von Primärstudien (Meta-Analyse) durchgeführt, in der wir zunächst die allgemeine Annahme getestet haben, dass sich AM-Pilze positiv auf Bodenaggregation auswirken. Zusätzlich wurden 13 Faktoren getestet, die potentiell AM Effekte auf Bodenaggregatsstabilität beeinflussen können. Nach einer Literaturrecherche haben wir 35 Studien über AM-Pilze und Bodenaggregation einer Meta-Analyse unterzogen, indem wir einzelne Random-Effects-Modelle für jede Variable berechnet haben. Der mittlere Gesamt-Effekt der Inokulation mit AM-Pilzen auf Bodenaggregation sowie die Mittelwerte der Einflussvariablen waren positiv. Topfversuche und Studien mit sterilisiertem sandigen Boden, neutralem Boden-pH, einer Topfgröße kleiner 2,5 kg und einer Versuchsdauer zwischen 2,2 und 5 Monaten führen eher zu stärkeren Effekten von AM-Pilzen auf Bodenaggregation als Feldversuche mit nicht sterilisiertem, feinkörnigen oder saurem Boden. Dies ist die erste Untersuchung die quantitativ zeigt, dass der Effekt von AM-Inokulation auf Bodenaggregation positiv und kontextabhängig ist.

Im ersten Teil von **Kapitel 3** haben wir in einem sterilen Laborversuch in kleinen *in vitro* Bioreaktoren ein Myzel von *Glomus intraradices* räumlich getrennt von Wurzeln und ohne andere lebende Mikroorganismen wachsen lassen. Wir konnten zeigen, dass es einen direkten kausalen Zusammenhang zwischen AM-Pilzhyphen allein, d.h. in Abwesenheit anderer Mikroorganismen, und dem Erhalt von Bodenaggregatsstabilität gibt. Im zweiten Teil des Kapitels haben wir getestet, ob die weitverbreitete Methode der rotierten Hyphenkompartimente Nebeneffekte hat, die sich auf das Zuordnen von Effekten zu AM-Pilzen auswirken könnten. Wir haben ein Experiment mit der Anwesenheit und Abwesenheit von AM-Pilzen und einer mikrobiellen Lebensgemeinschaft ohne AM aufgesetzt, bei dem jeder Topf ein gedrehtes und ein nicht gedrehtes Kompartiment enthielt. Die Ergebnisse haben gezeigt, dass Bodenparameter wie Wassergehalt, Bodenstruktur, pH und C- und N- Konzentrationen in Abwesenheit

von AM-Pilzen nicht durch das regelmäßige Drehen beeinflusst werden. Diese Studie unterstreicht daher die Gültigkeit und Nützlichkeit der Methode als experimentelle Kontrolle für AM-Pilzwachstum und -aktivität.

In **Kapitel 4** haben wir mit einem Experiment die einzelnen und kombinierten Effekte von AM-Pilzen und einer mikrobiellen Lebensgemeinschaft ohne AM auf Bodenaggregation und den Abbau kleiner Holzstäbchen getestet. Zur Unterscheidung von Hyphen- und Wurzeleffekten haben wir ein Hyphenkompartiment eingesetzt. Die Bodenaggregation stieg gleichmäßig in allen inokulierten Behandlungen an. Zum ersten Mal konnte gezeigt werden, dass die Anwesenheit von AM-Pilzen die Zersetzung eines abbauresistenten Pflanzenmaterials reduziert. Beide Effekte waren unabhängig von der Inokulation anderer Mikroorganismen. Wir vermuten, dass die AM-Pilze die Aktivität von Abbauorganismen persistenten Materials unterbunden haben, oder dass die AM-Pilze eine alternative Kohlenstoffquelle zur Verfügung gestellt haben, was zu unterschiedlichem Streuabbau geführt hat. Diese Erkenntnisse tragen zu unserem Verständnis der Interaktion von AM-Pilzen mit anderen Mikroorganismen in der Bodenaggregation bei und könnten darauf hinweisen, dass AM-Pilze eine wichtige Rolle bei der Kohlenstoffspeicherung im Boden spielen.

Contribution to the publications

I Leifheit, E.F., Veresoglou, S.D., Lehmann, A., Morris, E.K., Rillig, M.C., 2014. Multiple factors influence the role of arbuscular mycorrhizal fungi in soil aggregation-a meta-analysis. Plant and Soil 374, 523-537.

Own contribution

EFL performed all the analysis and wrote the manuscript. EKM and SDV mentored the analysis.

All authors reviewed the manuscript.

II Rillig, M.C., Mardatin, N.F., Leifheit, E.F., Antunes, P.M., 2010. Mycelium of arbuscular mycorrhizal fungi increases soil water repellency and is sufficient to maintain water-stable soil aggregates. Soil Biology & Biochemistry 42, 1189-1191.

Own contribution

EFL analyzed the proportion of surficial hyphae in the lab. All authors reviewed the manuscript.

III Leifheit, E.F., Verbruggen, E., Rillig, M.C., Rotation of hyphal in-growth cores has no confounding effects on soil abiotic properties – Short communication (submitted).

Own contribution

EFL designed the experiment together with MCR. EFL performed the experiment and did all the laboratory analysis. EFL statistically analyzed the data (with advice from Dr. S. Hempel), interpreted the results and wrote the manuscript. All authors reviewed the manuscript.

IV Leifheit, E.F., Verbruggen, E., Rillig, M.C., Arbuscular mycorrhizal fungi reduce decomposition of woody plant litter while increasing soil aggregation (in preparation).

Own contribution

EFL designed the experiment together with MCR. EFL performed the experiment and all the laboratory analysis. EFL statistically analyzed the data (with advice from Dr. J. Wehner and EV), interpreted the results and wrote the manuscript. All authors reviewed the manuscript.

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Appendix A
Supplementary Material for Chapter 2

Contents

1. Sensitivity Analysis: Fig. S1 – S12

2. Additional information for the moderators 'plant' and 'aggregate size fraction' (Fig. S13)

3. List of articles used in the meta-analysis

1. Sensitivity Analysis

Methodology:

We performed a sensitivity analysis on our dataset to test whether there have been studies with a disproportionally high impact on the effect size (Copas and Shi 2000). The sensitivity analysis was only conducted for the moderators that showed significance in the permutation test: i) duration of experiment, ii) pot size, iii) soil pH, iv) sand content, v) sterility and vi) setting.

The moderators were meta-analyzed individually while sequentially excluding single studies from the dataset. In cases where the bias-corrected CIs of the retrieved effect did not include the mean effect of the entire dataset, we concluded that the excluded study had a disproportionate impact on the effect size. Results of the sensitivity analysis are presented Fig. S1 – S12.
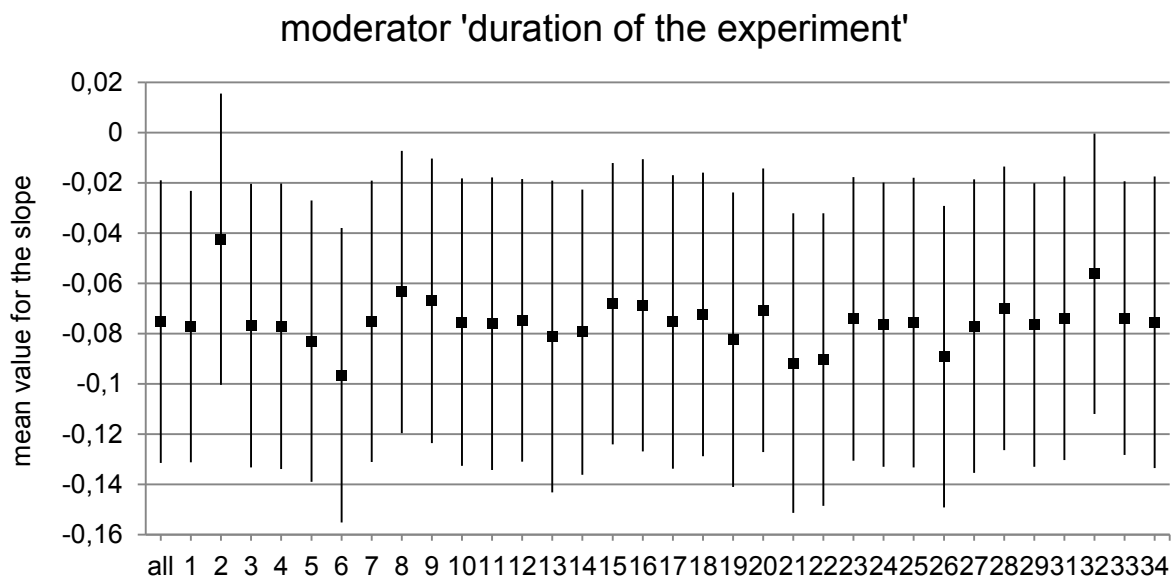
Fig. S1 Sensitivity analysis for the moderator 'duration of the experiment': mean value for the slope and squared standard error. On the x-axis first the mean for the entire dataset is shown. The following values are the respective means for the analysis with one study omitted. Axis labels represent the ID of the omitted study. No study with a disproportionate impact on the effect size was detected.
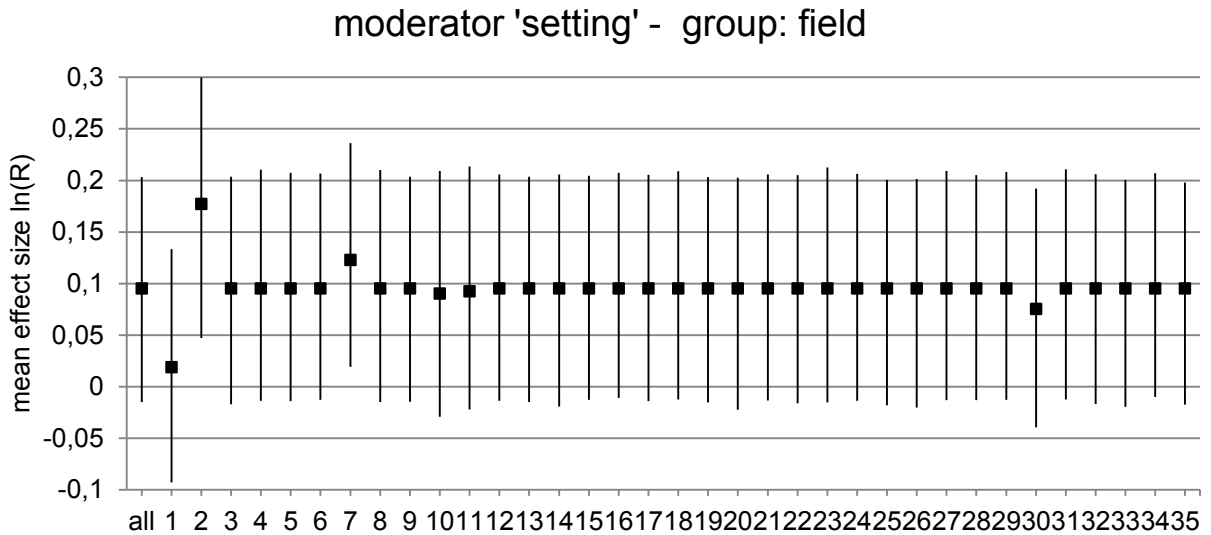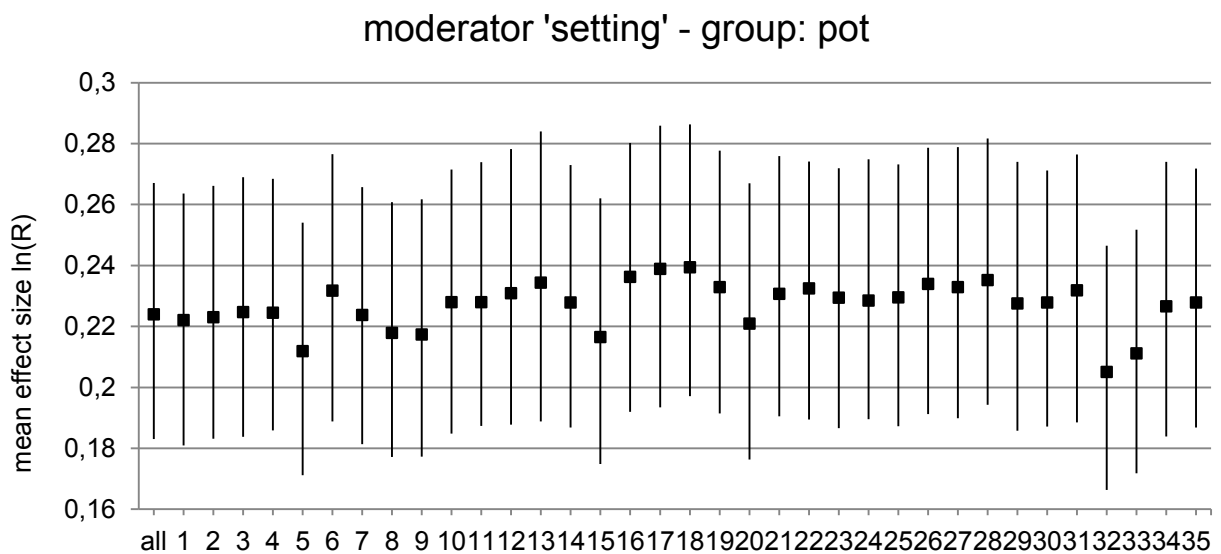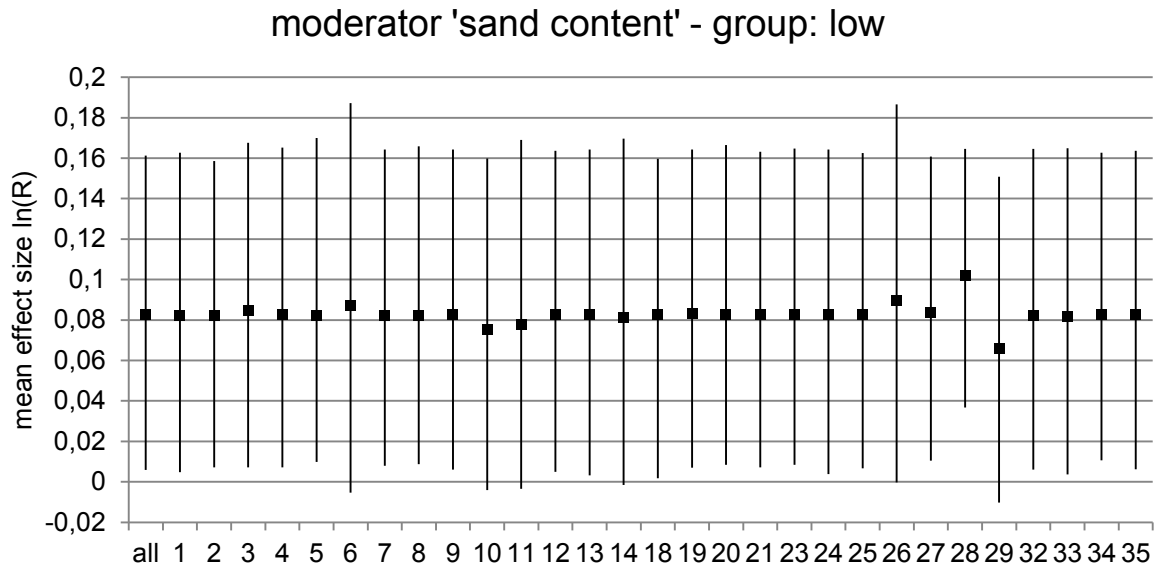
Fig. S2 Sensitivity analysis for the moderator 'setting' - group field, and the effect size (ln(R)): means and bias-corrected CIs. On the x-axis first the mean for the entire dataset is shown. The following values are the respective means for the analysis with one study omitted. Axis labels represent the ID of the omitted study. No study with a disproportionate impact on the effect size was detected.
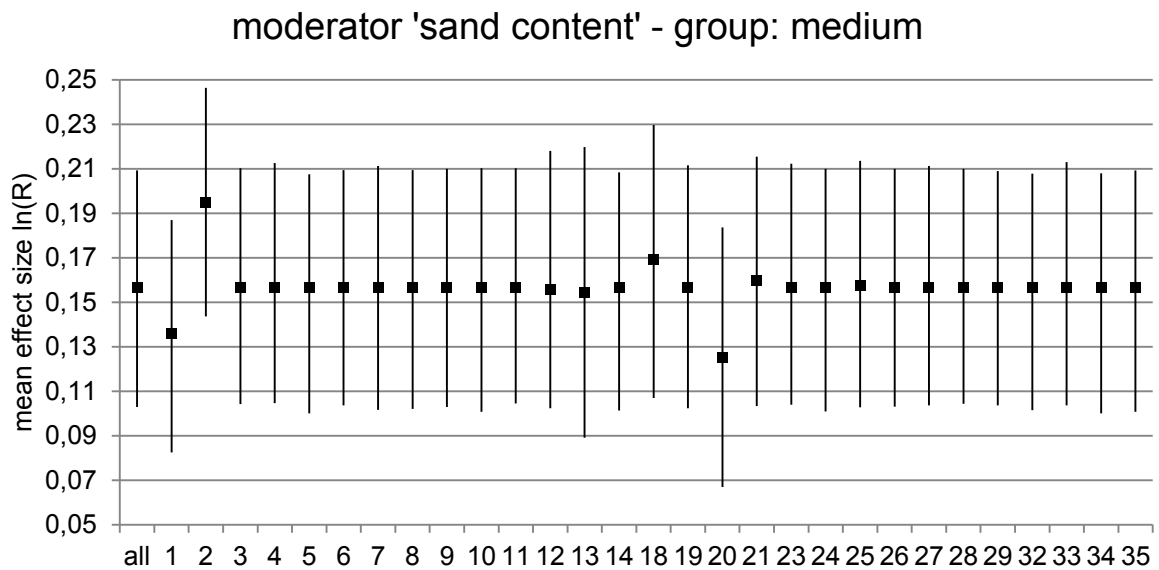

Fig. S3 Sensitivity analysis for the moderator 'setting' - group pot and the effect size (ln(R)): means and bias-corrected CIs. On the x-axis first the mean for the entire dataset is shown. The following values are the respective means for the analysis with one study omitted. Axis labels represent the ID of the omitted study. No study with a disproportionate impact on the effect size was detected.

Fig. S4 Sensitivity analysis for the moderator 'sand content' -group fine texture and the effect size (ln(R)): means and bias-corrected CIs. On the x-axis first the mean for the entire dataset is shown. The following values are the respective means for the analysis with one study omitted. Axis labels represent the ID of the omitted study. No study with a disproportionate impact on the effect size was detected.
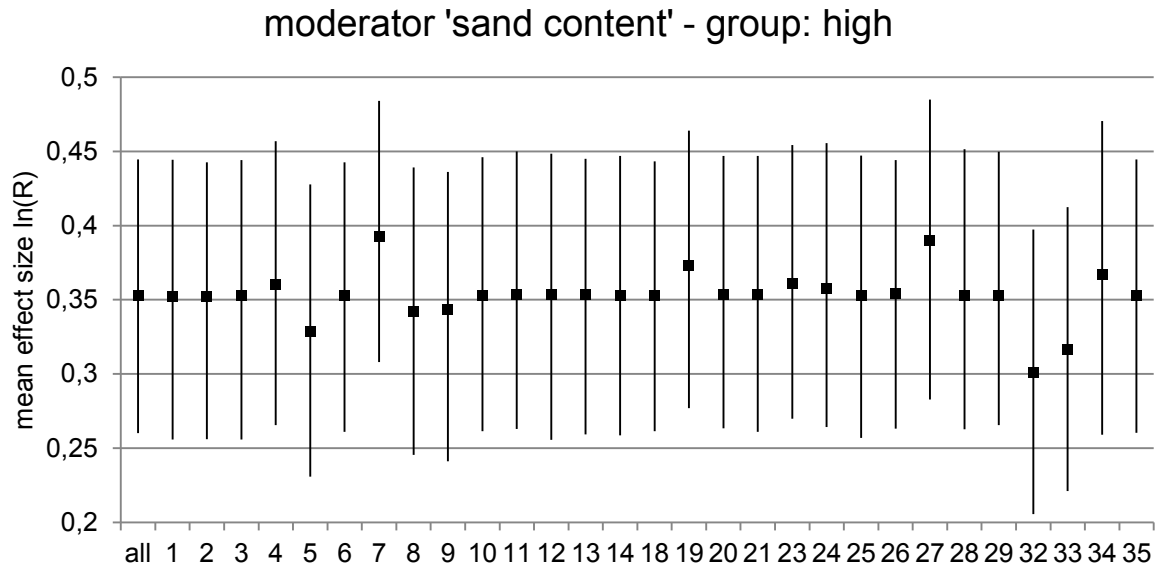

Fig. S5 Sensitivity analysis for the moderator 'sand content' -group medium texture and the effect size (ln(R)): means and bias-corrected CIs. On the x-axis first the mean for the entire dataset is shown. The following values are the respective means for the analysis with one study omitted. Axis labels represent the ID of the omitted study. No study with a disproportionate impact on the effect size was detected.
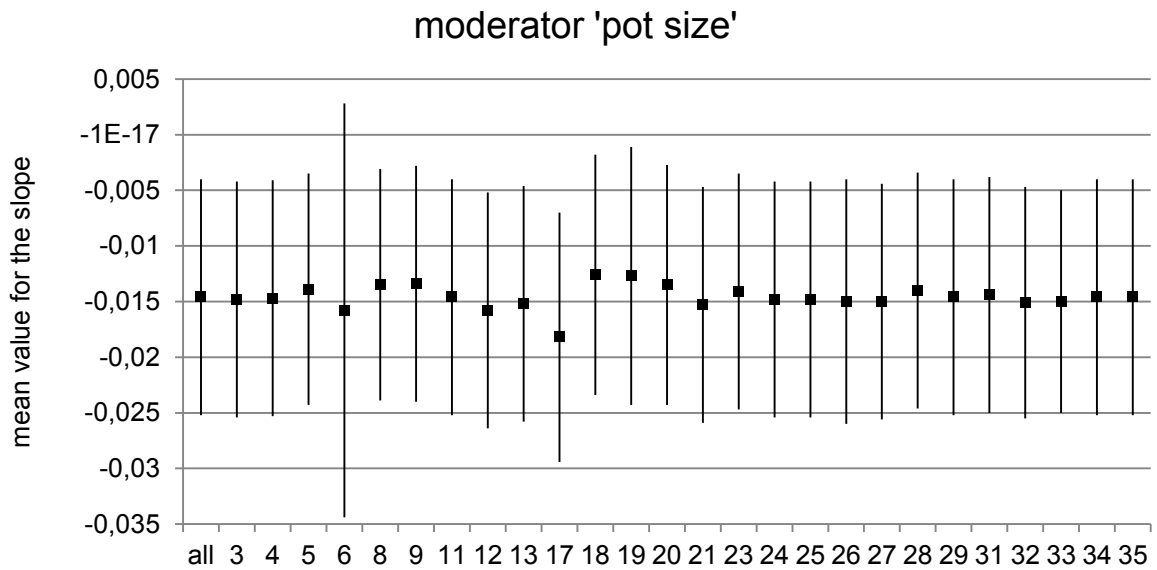
Fig. S6 Sensitivity analysis for the moderator 'sand content' -group coarse texture and the effect size (ln(R)): means and bias-corrected CIs. On the x-axis first the mean for the entire dataset is shown. The following values are the respective means for the analysis with one study omitted. Axis labels represent the ID of the omitted study. No study with a disproportionate impact on the effect size was detected.

Fig. S7 Sensitivity analysis for the moderator 'pot size': mean value for the slope and squared standard error. On the x-axis first the mean for the entire dataset is shown. The following values are the respective means for the analysis with one study omitted. Axis labels represent the ID of the omitted study. No study with a disproportionate impact on the effect size was detected.
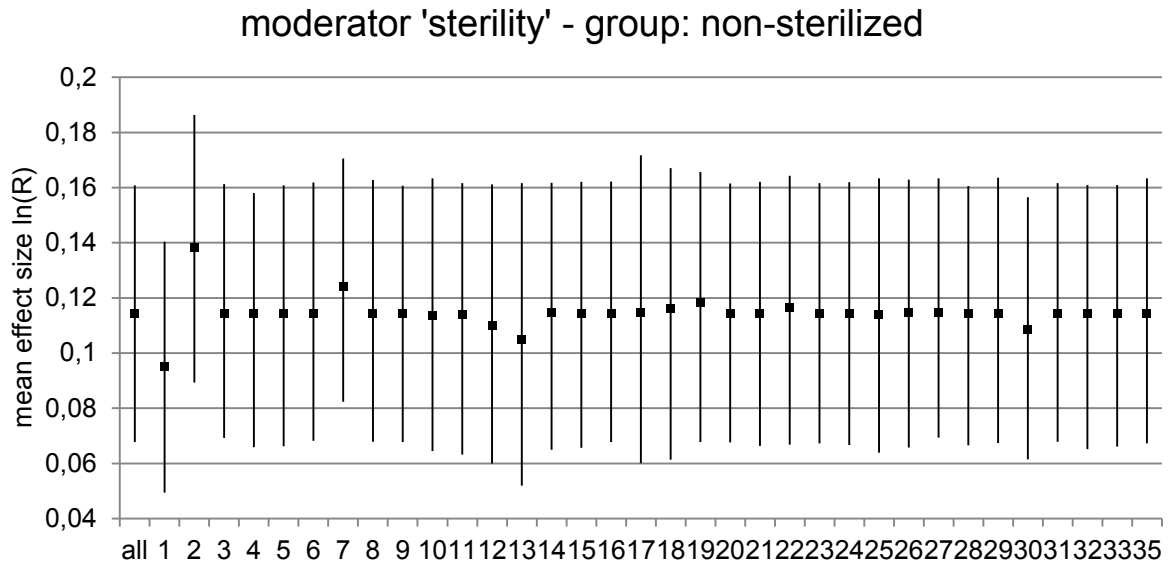
moderator 'sterility' - group: non-sterilized

Fig. S8 Sensitivity analysis for the moderator 'sterility' - group non-sterilized and the effect size (ln(R)): means and bias-corrected CIs. On the x-axis first the mean for the entire dataset is shown. The following values are the respective means for the analysis with one study omitted. Axis labels represent the ID of the omitted study. No study with a disproportionate impact on the effect size was detected.
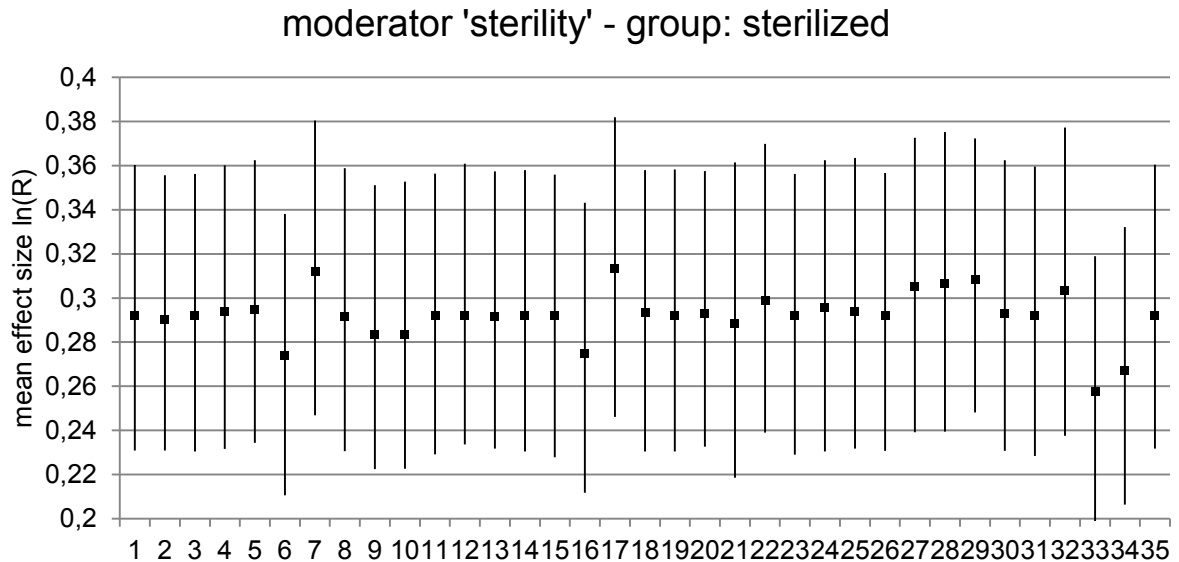

moderator 'sterility' - group: sterilized

Fig. S9 Sensitivity analysis for the moderator 'sterility' - group sterilized and the effect size (ln(R)): means and bias-corrected CIs. On the x-axis first the mean for the entire dataset is shown. The following values are the respective means for the analysis with one study omitted. Axis labels represent the ID of the omitted study. No study with a disproportionate impact on the effect size was detected.
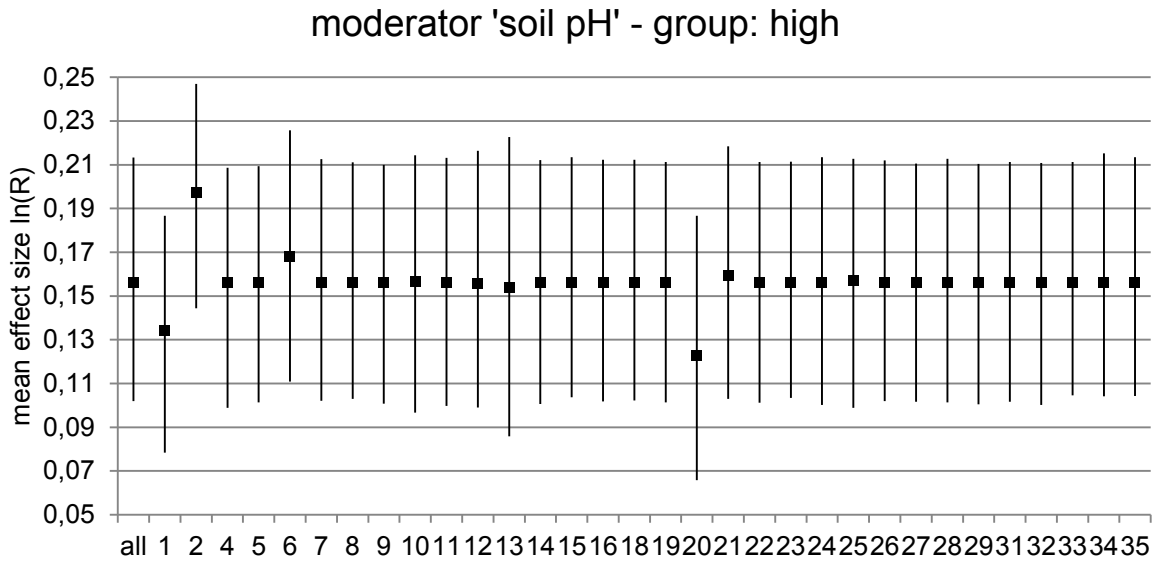
Fig. S10 Sensitivity analysis for the moderator 'soil pH' - group high and the effect size (ln(R)): means and bias-corrected CIs. On the x-axis first the mean for the entire dataset is shown. The following values are the respective means for the analysis with one study omitted. Axis labels represent the ID of the omitted study. No study with a disproportionate impact on the effect size was detected.

Fig. S11 Sensitivity analysis for the moderator 'soil pH' - group low and the effect size (ln(R)): means and bias-corrected CIs. On the x-axis first the mean for the entire dataset is shown. The following values are the respective means for the analysis with one study omitted. Axis labels represent the ID of the omitted study. No study with a disproportionate impact on the effect size was detected.
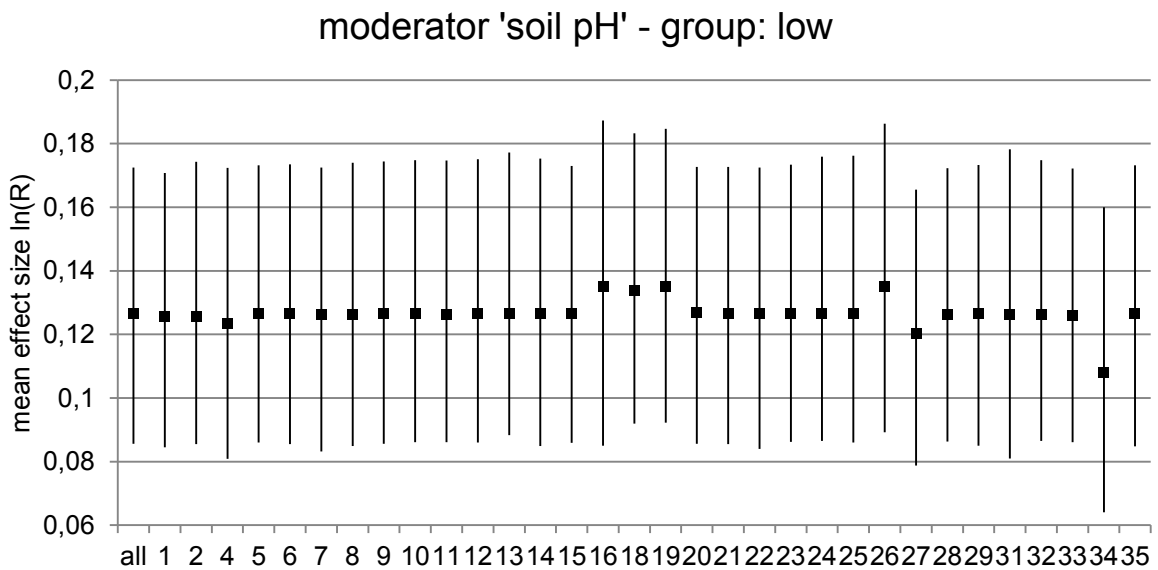
Fig. S12 Sensitivity analysis for the moderator 'soil pH' - group medium and the effect size (ln(R)): means and bias-corrected CIs. On the x-axis first the mean for the entire dataset is shown. The following values are the respective means for the analysis with one study omitted. Axis labels represent the ID of the omitted study. No study with a disproportionate impact on the effect size was detected.
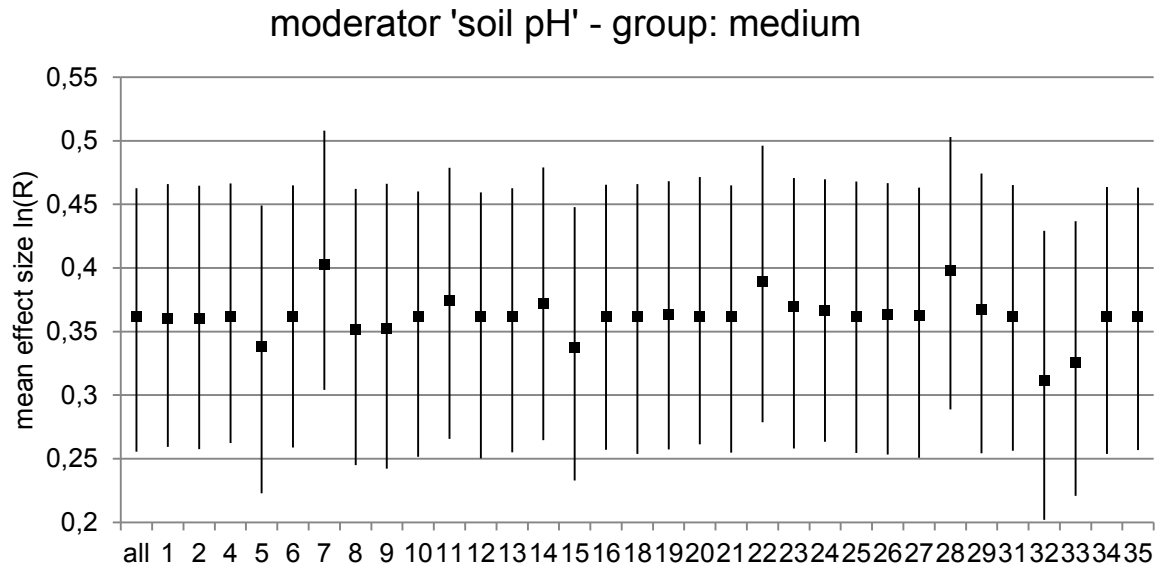
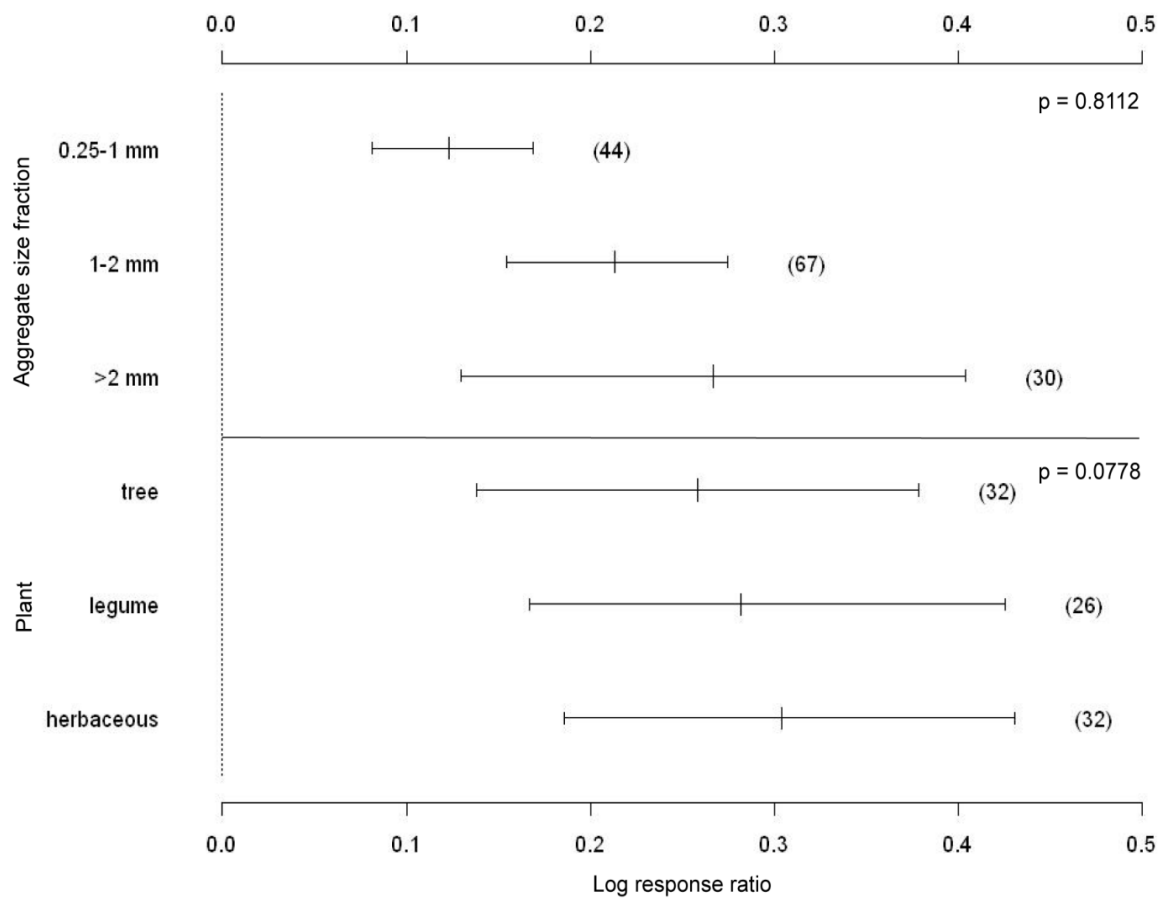**2. Additional information for moderators 'plant' and 'aggregate size fraction'**

Fig. S13 Means and bias-corrected CIs of the effect size for the moderators 'plant' and 'aggregate size fraction'. Numbers in parentheses refer to the number of trials present in the class. The reported p-value was obtained from the permutation test

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Appendix B
Supplementary Material for Chapter 3.2

We used linear mixed-effects models to obtain the p-values, except where conditions for normality were not met we used a Kruskal-Wallis rank sum test.

Table S1: Soil parameters for the treatment with AMF, means and standard errors in parentheses

	Rotated (R)	Static (S)	p-value[1]
AM hyphae (m · g^{-1})	0.43 (0.03)	3.01 (0.19)	0.005
Non-AM hyphae (m · g^{-1})	1.19 (0.11)	2.27 (0.10)	0.009
Water content (%)	9.2 (0.9)	8.8 (0.6)	0.48
EC (μS · cm^{-1})	311 (10)	334 (11)	0.01
pH (CaCl$_2$)	6.08 (0.01)	6.09 (0.02)	0.68
C (%)	3.29 (0.83)	3.38 (0.44)	0.85
N (%)	0.27 (0.07)	0.26 (0.04)	0.89
WSA (%)	59.0 (4.5)	59.4 (3.4)	0.59

[1] p-value for the effect of the rotated vs. static compartment

Appendix C
Supplementary Material for Chapter 4

Table S1: Mean (± se) concentrations of phosphate (calcium-acetate-lactate) and ammonium (calcium-chloride), pH and electrical conductivity (EC) and water content (%) at harvest in the hyphal compartment

Treatment	Phosphate (mg · kg^{-1} soil)	Ammonium (mg · kg^{-1} soil)	Total C (%)	Total N (%)	pH (CaCl$_2$)	EC (µS/cm)	Water content (%)
Control	90.6 (3.9)	21.8 (0.55)	1.68 (0.05)	0.107 (0.002)	6.80 (0.06)	118 (6.9)	9.7 (1.1)
AMF	93.0 (1.2)	22.4 (0.40)	1.69 (0.02)	0.109 (0.002)	7.02 (0.01)	115 (4.2)	10.9 (1.1)
MW	95.5 (2.6)	21.5 (0.47)	1.67 (0.03)	0.108 (0.001)	6.98 (0.01)	117 (4.1)	8.9 (0.9)
AMF + MW	92.5 (1.8)	22.7 (0.66)	1.74 (0.03)	0.113 (0.001)	6.96 (0.02)	115 (1.6)	10.1 (1.3)

Table S2: Mean (± se) weight loss (%) of wooden sticks and mean root dry weight (g) of the planted compartment (6.5 months after inoculation)

Treatment	Weight loss (%)	Root dry weight (g)
Control	25.2 (1.6)	6.3 (0.7)
AMF	16.3 (4.2)	6.8 (1.1)
MW	25.0 (3.7)	7.1 (1.0)
AMF + MW	10.7 (4.0)	6.9 (0.6)

Table S3: Mean (± se) total water stable aggregates (%) in the different size fractions of the soil in the hyphal compartment

Treatment	2 - 4 mm	1 - 2 mm	0.5 - 1 mm	0.212 - 0.5 mm
Control	4.4 (1.4)	6.3 (0.9)	11.9 (0.8)	22.6 (1.1)
AMF	12.2 (1.0)	12.2 (1.1)	14.7 (0.9)	20.6 (1.2)
MW	15.0 (1.3)	13.2 (0.6)	14.6 (0.7)	18.7 (0.8)
AMF + MW	8.5 (1.5)	9.0 (0.6)	17.0 (1.9)	23.8 (2.3)