Charles University

Faculty of Science

Plant Anatomy and Physiology

Mgr. Alena Voříšková

Functioning of arbuscular mycorrhiza in relation to fungal community composition and environmental conditions

Fungování arbuskulární mykorhizy ve vztahu ke složení společenstva hub a podmínkám prostředí

Ph.D. Thesis

Supervisor: **Mgr. Martina Janoušková, Ph.D.**

Institute of Botany, CAS

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Declaration

I declare that the PhD thesis was drafted independently and that I cited all the information sources and literature used. This work or its substantial portion has not been submitted to obtain another academic degree or its equivalent.

Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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ABSTRACT

Arbuscular mycorrhizal (AM) symbiosis, a widespread plant-fungal relationship, is based on reciprocal resource exchange. The functioning of this fragile relationship balances on the scale from mutualism to parasitism, depending on the specific context. The thesis aims to interlink the functioning of AM symbiosis both with the composition of AM fungal communities and with different abiotic conditions. The thesis is divided into a methodological and a factual part and consists of three publications and one manuscript.

All experiments were conducted in greenhouse conditions with medic (*Medicago sp.*) as host plant. Host plants were inoculated with single AM fungal species in Paper I and II, and with a synthetic AM fungal community of five species in Paper III and IV. The host plant identity, the amount of phosphorus (P) in substrate and the type of substrate played an important role for the achievement of mutualistic AM symbiosis, as demonstrated in Paper I. Paper II showed that mitochondrial and nuclear qPCR markers can be used alternatively for the quantification of particular AM fungal species. However, intraradical fungal biomass was better related to copy numbers of nuclear DNA than of mitochondrial DNA.

The functioning of AM symbiosis was modulated by the availability of P, light and water, though different abiotic conditions had a very limited influence on the quantitative composition of AM fungal communities, as shown in Paper III. The mycorrhizal benefits were linked to contrasting P demands of the plants across all the abiotic contexts. Paper IV showed that mycorrhizal benefits were mainly related to intraradical fungal biomass and partially also to the abundance of a particular AM fungal species. The composition of communities establishing form differently composed inoculum pools initially differed in their composition, but tended to converge in time.

In conclusion, the functioning of AM symbiosis was found to be influenced by different abiotic conditions and by the quantitative composition of AM fungal communities via the traits of particular AM fungi related to spread and symbiotic functioning. The composition of the AM fungal synthetic communities was affected by the root colonization traits, temporarily also by the amount of propagules in the inoculation pools and a only little by abiotic conditions.

KEY WORDS: mycorrhizal response, abiotic context, quantitative composition, plant growth, P uptake

ABSTRAKT

Arbuskulárně mykorhizní (AM) symbióza, velmi rozšířený vztah mezi rostlinami a houbami, je založena na vzájemném výměnném obchodu. Tento křehký vztah funguje na široké škále od mutualismu po parazitismus v závislosti na konkrétních podmínkách. Disertační práce si klade za cíl propojit fungování AM symbiózy se složením společenstev AM hub v kořenech rostlin a s podmínkami prostředí. Práce je rozdělena na metodickou a faktickou část a je složena ze tří publikací a jednoho manuskriptu.

Všechny čtyři experimenty byly založeny ve skleníkových podmínkách s vybranou hostitelskou rostlinou (*Medicago sp*.). Hostitelské rostliny byly inokulována jediným druhem AM houby v článcích I a II, a syntetickým společenstvem složeným z pěti druhů AM hub ve článcích III a IV. Druh hostitelské rostliny, množství fosforu v substrátu a typ substrátu hrály důležitou roli pro dosažení mutualistické symbiózy v článku I. Výsledky z článku II ukázaly, že mitochondriální a jaderné qPCR markery mohou být použity alternativně pro kvantifikaci konkrétních druhů AM hub, avšak vnitrokořenová biomasa AM hub lépe souvisela s počtem kopií jaderné DNA než mitochondriální DNA.

Fungování AM symbiózy bylo výrazně modulováno dostupností fosforu, zastíněním a nedostatkem vody, nicméně tyto rozdílné abiotické podmínky měly jen velmi omezený vliv na kvantitativní složení společenstev AM hub, jak je uvedeno v článku III. Přínosy AM symbiózy byly určeny rozdílnými požadavky rostlin na fosfor napříč všemi abiotickými podmínkami. Článek IV ukázal, že přínosy AM symbiózy byly určeny zejména množstvím vnitrokořenové houbové biomasy a částečně také abundancí určitých druhů AM hub. Složení společenstev AM hub založených z různých inokulačních poolů se zpočátku lišilo, ale v čase se rozdílnost postupně snižovala.

Závěrem lze říci, že fungování AM symbiózy bylo ovlivněno abiotickými podmínkami a kvantitativním složením společenstev AM hub zejména prostřednictvím vlastností jednotlivých AM hub souvisejících s jejich symbiotickým fungováním a kolonizačními schopnostmi. Složení syntetických společenstev AM hub bylo ovlivněno především kolonizačními schopnostmi jednotlivých AM hub, dočasně také množstvím propagulí v inokulačních poolech a pouze málo abiotickými podmínkami.

KLÍČOVÁ SLOVA: mykorhizní odpověď, abiotický kontext, kvantitativní složení, růst rostlin, příjem fosforu

CONTENTS

ABBREVIATIONS

AM	arbuscular mycorrhizal
C	carbon
LSU	large subunit
MGR	mycorrhizal growth response
mtDNA	mitochondrial ribosomal DNA
N	nitrogen
nrDNA	nuclear ribosomal DNA
P	phosphorus
Pi	orthophosphate
qPCR	quantitative real-time PCR

1. INTRODUCTION

Symbiosis is a very common and important phenomenon, which pervades all ecosystems and modulates the life in the biosphere of the Earth. In a broader context, symbiosis can be described as an intimate association between organisms, spanning a wide range of relationship from mutualism to parasitism. This phenomenon also includes the interaction between plants and soil fungi, the so-called mycorrhizal symbiosis or shortly mycorrhiza, which is in the focus of many biologists and ecologists due to its broad impact on plant diversity and productivity. The term mycorrhiza was firstly introduced by Frank (1885) and means simply fungus-root. Mycorrhizal symbiosis belongs to the most widespread and ancient symbioses found on Earth and had been already present at the time of the transition of plants from water to land almost 420 million years ago. At the present time, about 90% of all species of vascular plants developed mycorrhizal symbiosis with certain fungi.

Over the span of the evolution, several different types of mycorrhizal symbiosis have developed. The most common types are arbuscular mycorrhizal (AM) symbiosis, ectomycorrhiza, ericoid and orchideoid mycorrhizal symbiosis. These types of mycorrhizal association can be distinguished based on the structures which are formed inside or outside of roots. The host plants involved in mycorrhizal symbiosis and their symbiotic fungi differ among the mycorrhizal types. Fungal symbionts belonging to phyla Mucoromycota, Basidiomycota and Ascomycota can be facultatively or obligately dependent on their host plants.

Mycorrhizal symbiosis is spread worldwide throughout almost all ecosystems, even though some mycorrhizal types occur more frequently in specific ecosystems, which corresponds to the occurrences of their host plants. Ectomycorrhiza and ericoid mycorrhiza dominate in boreal forests as compared to AM symbiosis, which is the dominant type of grasslands and tropical forests.

The functioning of the mycorrhizal association quite differs among mycorrhizal types, though, in general, mycorrhiza works as an (not necessarily mutually beneficial) exchange trade, where plants supply fungi with organic carbon (C) from photosynthesis and fungi provide nutrients or other non-nutritional benefits to plants. The most widespread type is arbuscular mycorrhiza which forms symbiosis with almost 80% of all vascular plant

species. Moreover, important agriculture crops belong among plant species forming this type of symbiosis, and thus arbuscular mycorrhiza soon became a popular subject of research.

1.1. Arbuscular mycorrhizal symbiosis

Arbuscular mycorrhiza or, with the same meaning, AM symbiosis is the oldest type of the mycorrhiza. Structures reminiscent of the present day AM symbiosis in roots were found in fossils dated to be 400-450 million years old (Remy *et al.*, 1994; Strullu-Derrien *et al.*, 2016). The early origin of AM symbiosis could be a reason for the observed low host specificity and a large expansion of this type of mycorrhizal symbiosis. AM symbiosis forms relationship with fungi from the phylum Mucoromycota, subphylum Glomeromycotina (Spatafora *et al.*, 2016), further called AM fungi, which were earlier classified as independent phylum Glomeromycota. In total, up to approximatelly 310 species (http://www.amf-phylogeny.com/amphylo_species.html) of AM fungi were described.

1.1.1. Principles of AM symbiosis establishment and functioning

Unlike other mycorrhizal fungi, AM fungi are not able to grow for a longer period of time without their plant hosts and are characterized as obligate biotrophs (Bonfante & Genre, 2010). AM fungi form spores, which can germinate in favorable conditions and develop into asymbiotic mycelium. As obligate symbionts, AM fungi in early stage acquire C only from the C storage in the spores and explore the soil for the signal in the form of root exudates, e.g. strigolactones (Besserer *et al.*, 2006; Nagahashi & Douds, 2011). Fungal symbionts also send molecular signals in form of lipochitooligosaccharides called Myc factors to their host plants (Maillet *et al.*, 2011). The hyphae start to branch and their growth follows the gradient of the concentration of the root exudates and then reaches root tips. The symbiotic stage starts after the hyphae contact the wall of epidermal cells and adhere on the wall. In this stage, a complicated process of signal exchange occurs between the host plant and the fungal partners. Subsequently, hyphae penetrate through the cell wall, enter the inner cortex and get into the host cells. The hyphae penetrate only the cell wall, not the plasmatic membrane. The periarbuscualar space is formed between the fungal plasmatic membrane and the plant plasmatic membrane (periarbuscular membrane). The active exchange of nutrients and

C between the symbionts is mediated by the arbuscules, i.e. branching fungal structures, which are enclosed by the periarbuscular space and periarbuscular membrane. Other fungal structures localized in roots are the vesicles, which carry out the storage function. Extraradical mycelium is formed by a network of hyphae growing outside of the colonized roots and expanding to a large volume of soil. Extraradical hyphae take up nutrients and transfer them through the intraradical mycelium into plant tissues. The ability of nutrient transportation from a large amount of soil, through fungi and directly into plants has a great ecological importance.

1.1.2. Specific features of AM fungi

No obvious sexual structures have been observed in this group of fungi up to now, and also due to their low variability in morphology, AM fungi used to be generally considered as strictly asexual fungi (Monier *et al.*, 2017). However, some kind of cryptic sexuality may exist because genes for meiosis and sexuality were discovered in some AM fungi (Riley & Corradi, 2013).

AM fungi lack septa in hyphae and form the so-called coenocytic or aseptic mycelium, which contains many nuclei. AM fungal hyphae have the ability to anastomose, the interconnected hyphae form the mycelial network, within which the cytoplasmic flow and nuclei freely move (Giovannetti *et al.*, 2001). Anastomosis are formed not only among hyphae of one individual growing from one spore but also among different individuals within the same isolate of AM fungi (Giovannetti *et al.*, 1999). Moreover, different isolates of *Rhizphagus irregularis* from one site had the ability to form anastomosis among each other (Croll *et al.*, 2009), whereas geographically distinct isolates of *Funneliformis mosseae* did not have this capability (Giovannetti *et al.*, 2003). In the presence of anastomosis, genetic exchange can occur among different AM fungal genotypes (Croll *et al.*, 2009). Furthermore, extraradical hyphae growing from roots and forming the complex mycelial network can interconnect many host plants of the same or different species in the ecosystem (Barreto De Novais *et al.*, 2017).

Spores of AM fungi are multicellular and contain hundreds to thousands of nuclei (Viera & Glenn, 1990) and genetic divergence among nuclei was observed even within individual spores (Kuhn *et al.*, 2001). The coenocytic mycelium of AM fungi is assumed to

be heterokaryotic as the assemblage of genetically different nuclei (Sanders & Croll, 2010; Ehinger *et al.*, 2012). Though, a homokaryotic theory suggesting variation among copies of ribosomal DNA within nuclei has also been proposed (Hosny *et al.*, 1999; Pawlowska & Taylor, 2004). As a consequence, it is problematic to consider AM fungi as individuals, when even as basic a unit as one spore consists from populations of genetically different nuclei (Kuhn *et al.*, 2001; Corradi *et al.*, 2004). Species of AM fungi were conventionally delimited based on the morphological characterization of spores, but a huge genetic variability at the intraspecific level was discovered with the expansion of molecular approaches (Stockinger *et al.*, 2010). This fact complicates the identification and delimitation of AM fungal species. In addition, DNA sequences have been assigned only to several morphologically described species due to problematic cultivation of AM fungi. Moreover, different regions of nuclear ribosomal DNA (nrDNA) such as the internal transcribed spacer, the small and large subunit (LSU) of ribosomal RNA genes, each possesses different power to distinguish AM fungal taxa and is used for their delimitation (Kohout *et al.*, 2014; Hart *et al.*, 2015). These circumstances complicate the development of a united sequence-based taxonomy of AM fungi (Öpik *et al.*, 2014; Hibbett, 2016).

1.2. Reciprocal benefits in AM symbiosis

For a proper functioning of a relationship such as arbuscular mycorrhiza, both partners have to somehow contribute into the relationship. AM fungi provide mineral nutrients and non-nutritional benefits to plants. In return, AM fungi as obligate biotrophs are fully supplied with C in the form of hexoses, especially glucose, from plant photosynthesis, which they need for their growth and metabolism. The C transferred to the fungal symbionts could, however, also be considered as a certain loss for the host plants. It has been stated, that from 5% up to 20 % of total C obtained by photosynthesis is distributed to AM fungi (Jakobsen & Rosendahl, 1990; Grimoldi *et al.*, 2006; Lendenmann *et al.*, 2011; Calderón *et al.*, 2012). Even though C losses to AM symbiosis are not negligible, plants are capable to compensate these losses by increasing the rate of photosynthesis under suitable conditions (Kaschuk *et al.*, 2009).

1.2.1. Nutrient benefits of AM symbiosis

One of the most important benefits of fungal symbionts is the improvement of mineral nutrition of host plants. AM fungi have the ability to transfer nutrients, particularly phosphorus (P), from soil to plants. The extensive extraradical mycelium of AM fungi can achieve a size of more than 100 meters per cm^3 of soil (Miller *et al.*, 1995; Hart & Reader, 2002b) and thus explore considerably higher amount of soil as compared to plant roots, whose length can be hundreds of times shorter (George *et al.*, 1995). Simultaneously, the thin hyphae with size of few μm in diameter (Friese & Allen, 1991; Drew *et al.*, 2003) can easily explore the soil pores of comparable dimensions and thus provide transport of nutrients from diminutive cavities, otherwise inaccessible to plant roots.

1.2.1.1. Phosphorus acquisition by AM fungi

Phosphorus is the most important nutrient element transported by the AM network. This nutrient is essential for the growth and development of plants as it takes part in the energy metabolism and features in the cell structure, and thus appears with a high concentration (about 0.5 % of dry weight) in plant tissues (Saito & Ezawa, 2017). However, low availability of P in soil is common in natural conditions due to the very low solubility of phosphates of iron, aluminum and calcium (Pierzynski *et al.*, 2005). Low availability together with low mobility and the high demand of this element leads to the formation of depletion zones around the roots and frequent plant P limitation (Schachtman *et al.*, 1998).

The uptake of P in the form of orthophosphate (Pi) into mycorrhizal plants can occur either directly by root epidermis or via the mycorrhizal pathway (Smith *et al.*, 2003). A switching off or a decrease in the direct uptake pathway can be observed when roots become colonized by AM fungi (Smith *et al.*, 2009). In the extraradical hyphae, transporter genes are expressed such as discovered H^+/Pi (Harrison & Van Buuren, 1995) and Na^+/Pi symporters (Tisserant *et al.*, 2012; Kikuchi *et al.*, 2014), which are responsible for the active transport of Pi from the soil across the plasma membrane into the cytosol of hyphae. Received Pi inside of the fungal cytosol immediately polymerase into polyphosphate and then polyphosphate is possibly stored and transported using the vacuolar system (Saito & Ezawa, 2017). Transfer of P between the fungal cell and the plant cell occurs in arbuscules, from

which P enters into the periarbuscular space (Parniske, 2008; Smith & Smith, 2011). Before the transfer of P into the plant cell, the polyphosphate is probably depolymerized either already in the arbuscules or in the periarbuscular space (Saito & Ezawa, 2017). Afterwards Pi in the periarbuscular space is transported using Pi transporters localized in the periarbuscular membrane (Harrison *et al.*, 2002; Kobae & Hata, 2010; Pumplin *et al.*, 2012). Pi transporters play an essential role in the P transportation via the mycorrhizal pathway as the knockout of the genes for the Pi transporters causes almost complete interruption of P transportation from the fungal symbionts to the plant host (Javot *et al.*, 2007; Yang *et al.*, 2012).

1.2.1.2. Other nutrients provided by AM fungi

Although P is the most commonly reported nutrient provided to host plants, AM fungi also acquire and deliver to their host plants other nutrients like nitrogen (N), zinc, copper, magnesium, potassium and calcium (Gnekow & Marschner, 1989; Siqueira *et al.*, 1990; Lambais & Cardoso, 1993; Bürkert & Robson, 1994). However, only small emphasis is put on the AM fungal uptake of these elements with the exception of N, which is the second most reported nutrient in AM research.

N is the primary component of plants, which forms many plant structures and is involved in metabolic activities. The inorganic N in soil is considered as a relatively mobile element, though the mobility of N can be reduced by specific conditions like drought (Tinker & Nye, 2000). The ammonium form of N is less mobile than the nitrate form and is also more acquired by AM fungi (Marschner & Dell, 1994). The dominant form of N in soil is the organic form, which is also discussed in AM research (Hodge, 2003; Leigh *et al.*, 2009; Whiteside *et al.*, 2009). Although it is known that AM fungi are able to supply host plants by N (Govindarajulu *et al.*, 2005; Leigh *et al.*, 2009; Fellbaum *et al.*, 2012), some studies concluded that AM fungi have no effect on the N nutrition of their host plants (Ames *et al.*, 1983; Hawkins *et al.*, 2000; Reynolds *et al.*, 2005), whereas other studies showed that AM fungi provide benefits to their host plants via N uptake (George *et al.*, 1995; Cavagnaro *et al.*, 2006). Therefore, the role of AM fungi in supplying plants with N is still debatable. Different effects of AM fungi on the host plant supply can be explained e.g. by the high

demand of AM fungi themselves for N (Hodge *et al.*, 2010), different forms of N and availability of N in soil.

1.2.2. Non-nutritional benefits of AM symbiosis

Non-nutritional benefits of AM fungi are not easy to separate from the nutritional benefits, because they are often not independent of each other (Delavaux *et al.*, 2017). Non-nutritional factors (e.g. drought conditions, pathogens etc.) influence the fitness of plants and AM fungi can improve it directly or indirectly – by the way of improving mineral nutrition. The example of the direct and indirect effects of AM fungi on the fitness of plants can be demonstrated in the conditions of soil water deficiency. Soil is very often depleted and water remains in smaller pores i.e. with diameter < 30 μm (Allen, 2007), which are inaccessible to roots and root hairs in drought conditions. Nevertheless, the tiny hyphae of AM fungi can penetrate into these micropores and mediate water to plants. AM fungi can also alter the stomatal conductance in drought conditions (Augé *et al.*, 2015). The effects and mechanisms of water redistribution by AM fungi and the water balance of mycorrhizal plants were described in a review of Augé (2001). However, plants under soil water deficiency deal also with P acquisition and are often P limited (Allen, 2007). Plant P uptake from the soil solution and the lack of water leads to the fact that also undepleted and available P becomes inaccessible for plants (Jansa *et al.*, 2011). Under these conditions, the P uptake and transfer to plants can occur almost exclusively through the mycorrhizal pathway (Glassop *et al.*, 2005). Thus, AM fungi can improve the fitness of water deficient plants not only by direct distribution of water, but also by improved supply of mineral nutrients. However, clear non-nutritional benefits of AM fungi were demonstrated in some studies. Stimulating plants to express defense genes, altering root morphology, competing with pathogens for the root niche are some ways how AM fungi are able to enhance the resistance to pathogens (Azcón-Aguilar & Barea, 1996; Pozo & Azcón-Aguilar, 2007; Facelli *et al.*, 2009). Another example of non-nutritional benefits is the alleviation of the negative effects of allelochemicals by AM fungi (Džafić *et al.*, 2013).

1.3. Mycorrhiza as a balanced relationship

AM fungi as obligate biotrophs always benefit from the mycorrhizal association in contrast to plants, and therefore, the mycorrhizal relationship is asymmetric (Johnson *et al.*, 2015). The net benefit of plants from the mycorrhizal relationship is determined based on the ratio between the obtained benefits from AM fungi and the costs in form of C. Based on this evidence, mycorrhizal symbiosis can have a positive, neutral or negative effect on the growth of plants and can be considered as a relationship on the continuum from mutualism to parasitism (Johnson *et al.*, 1997). The term 'mycorrhizal phenotype' refers to the fitness outcome for both partners in the relationship and to the location of the mycorrhizal relationship on the continuum (Johnson *et al.*, 2015). The fitness of the host plant is an appropriate, but difficultly measurable parameter. The measurement of plant biomass is widely used as a proxy for fitness, which is less correct but practical.

Mycorrhizal response, or in other words the effectiveness of mycorrhizal symbiosis or plant benefit, was introduced into mycorrhizal research for the purposes of better interpretation and easier comparison of profits from mycorrhizal symbiosis. Several formula have been established for the calculation of mycorrhizal response (Janos, 2007), but the most widely used is the equation $MR\ [\%] = 100*(M - NM)/NM$ (e.g. Baon *et al*., 1993), where *MR* is the mycorrhizal response, *M* expresses the biomass of mycorrhizal plants and *NM* the biomass of non-colonized plants. Mycorrhizal response calculated this way is oftentimes called the mycorrhizal growth response (MGR), though the mycorrhizal response in nutrient uptake can be easily calculated by replacing the plant biomass values by the amount of a nutrient in plant biomass (Zhu *et al.*, 2001).

The mycorrhizal response is based on the interaction of a particular host plant and a mycorrhizal fungus under certain environmental conditions, whereas plant species differ in the dependency on mycorrhizal symbiosis. The mycorrhizal dependency is a given characteristic of a particular species and can be determined using the definition of Janos (2007) as the continuum from obligately dependent species to facultatively dependent species. The obligately dependent species are not able to finish their reproductive cycle without mycorrhiza at the highest fertility of soil present in nature, while facultatively dependent species can reach the reproductive stage without mycorrhiza at least at the highest soil fertility in nature. Based on the classification of a plant species to a particular functional

or phylogenetic group, we can partly predict the mycorrhizal response of this species (Hoeksema *et al.*, 2010; Reinhart *et al.*, 2012). The so called non-mycotrophic plants such as e.g. species from families *Brassicaceae*, *Caryophyllaceae* and *Cyperaceae* either do not form mycorrhizal structures in their roots or possibly existing mycorrhizal colonization in their root system is not functional (Tester *et al.*, 1987; Regvar *et al.*, 2003; Püschel *et al.*, 2007).

The function of mycorrhizal symbiosis in the sense of the net effect of mycorrhizal symbiosis on the fitness of both partners (Johnson & Graham, 2013) is notably dependent on the environmental context (Johnson *et al.*, 1997). Nutrient availability in soil has been suggested as one of the most important determinants of the mycorrhizal phenotype on the scale from parasitism to mutualism (Mosse, 1973; Johnson, 2010). Mycorrhizal symbiosis enhances the growth of plants especially in P limited soil (Koide, 1991), on the other hand, in soils with high P availability as e.g. fertilized arable fields, plants can be sufficient in the P uptake by their own root system and do not necessarily need the fungal symbionts for P acquisition, which can result in the reduction of mycorrhiza development in their roots (Youpensuk *et al.*, 2008; Jansa *et al.*, 2009).

The situation in nutrient uptake via the mycorrhizal pathway can be also completely different in the case of C deficiency (e.g. in the conditions of low light). Plants need C mainly for themselves, when the growth of plants is primary limited by light, not by mineral nutrition, and therefore host plants should not supply AM fungi with C (Konvalinková & Jansa, 2016). The reduction of mycorrhiza development in roots in low light conditions was previously described (Heinemeyer *et al.*, 2003; Schreiner & Pinkerton, 2008; Liu *et al.*, 2014; Mao *et al.*, 2014). It is not clear yet, whether the host plants can actively participate at the regulation of their mycorrhizal colonization levels in unfavorable conditions or the decrease in root colonization is caused passively by low C content in plant tissues. However, AM fungi are usually not completely eliminated from root systems of host plants (Schubert *et al.*, 1992; Konvalinková & Jansa, 2016). The decrease in MGR or reduced P content of inoculated host plants can be the result of a light reduction (Marschner & Timonen, 2005; Johnson *et al.*, 2015; Konvalinková *et al.*, 2015; Zheng *et al.*, 2015) and can be related to observed lower P inflow into mycorrhizal roots in these conditions (Smith & Gianinazzi-Pearson, 1990; Fellbaum *et al.*, 2014).

An equally important factor for a balanced relationship is the N availability in soil (Johnson, 2010). AM fungi have high N concentrations in their tissues (Hodge & Storer, 2014), and therefore also a high demand for N resulting in different relationship between the host plants and AM fungi depending not only on the availability of N is soil, but also on P availability (Johnson *et al.*, 2010). The same study demonstrated the trade balance model, which can predict the functioning of AM symbiosis by the availability of P and N on C supply. Thus, a fragile balance in the stoichiometry of P, C and N may determine the functioning of mycorrhizal symbiosis (Johnson *et al.*, 2015) and the mycorrhizal phenotype expressed as the location on the continuum from parasitism to mutualism is given by a complex set of circumstances like the interaction among plants, fungi and environmental contexts (Johnson *et al.*, 1997).

1.4. AM fungi in communities

In nature, root systems of host plants are almost always colonized by multiple taxa of AM fungi and it has been suggested that a community of AM fungi enhances the plant growth more than colonization with only a single fungus (van der Heijden *et al.*, 1998; Jansa *et al.*, 2008; Thonar *et al.*, 2014). The individual AM fungal taxa may complement each other and more diverse communities can improve the growth of plants better than less diverse communities, but the growth also depends on the community composition (Sharma *et al.*, 2009). The observed improvement of plant growth can also be caused by the so called 'sampling effect', where the increasing richness of AM fungal species in the community may increase the chance for the occurrence of very effective AM fungal taxa. It was also demonstrated that increasing richness of species in the community did not necessarily increase the growth of plants (Gosling *et al.*, 2016) or even had a negative effect on mycorrhizal responses as compared to inoculation with single AM fungal taxa (Violi *et al.*, 2007).

AM fungal communities can affect also the biodiversity and productivity of plant community (van der Heijden *et al.*, 1998). The study of van der Heijden *et al*. (1998) suggested a higher efficiency of resource utilization with a species-rich community of AM fungi as a consequence of the specific combination between host plants and fungal symbionts. Vogelsang et al. (2006) showed that although the diversity and productivity of

plant community increased with increasing species richness of the AM fungal community, the effect of the rich AM fungal community on plant diversity and productivity was small relative to the 'sampling effect'.

The importance of AM fungal communities and their diversity on plant benefits and plant communities in nature is unquestionable, although we still have limited knowledge about the establishment of AM fungal communities and about the link between the functioning of mycorrhizal symbiosis and AM fungal community structure.

1.4.1. Functional diversity in AM fungi

Symbiotic efficiency of AM fungi i.e. the degree of ability to provide benefits to plants in specific conditions, can be affected by colonization with different AM fungal species (Klironomos, 2003; Antunes *et al.*, 2008) or isolates of same species (Munkvold *et al.*, 2004; Koch *et al.*, 2006; Börstler *et al.*, 2010; Mensah *et al.*, 2015). As a result of different symbiotic efficiency, the mycorrhizal responses of plants therefore vary depending on the colonization with specific AM fungi. Phenotypic characteristics and growth traits given by the phylogenetic classification of AM fungi may partly explain differences in symbiotic efficiency. Individual families of AM fungi differ in the range of extraradical and intraradical mycelium and growth rates (Hart & Reader, 2002b). Avio *et al*. (2006) showed that the size of the extraradical mycelium can vary also at the species level. Different AM fungal species (Thonar *et al.*, 2011; Lendenmann *et al.*, 2011) and also isolates of one species (Munkvold *et al.*, 2004) vary in the P uptake and therefore the amount of delivered P to host plant may depend on the fungus identity (Avio *et al.*, 2006). The ability of fungi to provide benefits may be related to the size of their mycelium, where the more expanded mycelium may provide more nutrients to plants, but on the other hand, larger mycelium can also drain more C from the plants. By contrast, Koch *et al*. (2017) showed with 56 AM fungal isolates that even though growth traits and morphology were conserved among isolates of the same species or among species within a family, mycorrhizal responses were highly variable. It was suggested that the growth of host plants is not exactly related to the AM fungal growth traits and morphology and therefore to taxonomical classification (de Novais *et al.*, 2014; Mensah *et al.*, 2015; Koch *et al.*, 2017). Equally, the combination of different contexts e.g. particular host plants, pH of soil, P availability etc. are equally important factors for the determination

of mycorrhizal response (Hayman & Tavares, 1985; van der Heijden *et al.*, 1998; Klironomos, 2003; Vogelsang *et al.*, 2006).

1.4.2. Establishment, coexistence and composition of AM fungal communities

The presence, diversity and community composition of AM fungi are affected by the whole range of abiotic factors on the site, but also by biotic factors and the interaction with other organisms. Specialists, which are able to occur only in the specific conditions, and generalists, tolerating wide range of conditions, exist in this phylum. An important factor determining the AM fungal community is the amount of nutrients in the soil (Jumpponen *et al.*, 2005; Toljander *et al.*, 2008), e.g. increased N in soil may select for *Glomus* species (Egerton-Warburton & Allen, 2000). The AM fungal community is also influenced e.g. by soil pH (An *et al.*, 2008), concentration of heavy metals (Zarei *et al.*, 2010) or soil type (Oehl *et al.*, 2010). Agricultural practice as a whole includes many different factors and has a great impact on AM fungal communities. It has been suggested that, generally, increasing intensity of agricultural practices decreases the AM fungal diversity (Boddington & Dodd, 2000) and leads to the selection for AM fungal taxa with boarder ecological valence (Johnson & Pfleger, 1992; Jansa *et al.*, 2002). Even though AM symbiosis is generally consider as a non-specific interaction (Smith & Read, 2008), the interaction with host plants partly determine the AM fungal community composition. It was found that AM fungal communities can differ among different plant host species on one site (Wubet *et al.*, 2006; Sýkorová *et al.*, 2007) and thus, increasing diversity of host plants may increase the diversity of AM fungi (Burrows & Pfleger, 2002). Other important factors determining the community composition of AM fungi are the seasonal succession (Dumbrell *et al.*, 2011; Varela-Cervero *et al.*, 2016) and competitive and facilitative interaction among AM fungi (Vandenkoornhuyse *et al.*, 2003; Lekberg *et al.*, 2007, 2011; Dumbrell *et al.*, 2010). Without doubt, AM fungal communities are affected by the interaction with other organisms like e.g. bacteria (Vivas *et al.*, 2006) and nematodes (Rodríguez-Echeverría *et al.*, 2009). Thus AM fungal communities can dynamically form and change under the influence of many different factors.

Competition among AM fungi is probably less intense in soil than in roots, where space limitation occurs (Engelmoer *et al.*, 2014). However, AM fungi not only compete for space, but also for C, where the host plant's involvement is substantial (Pearson *et al.*, 1994).

It has been demonstrated that plants are able to direct their C preferably to those AM fungal species which provide them with more nutrients (Kiers *et al.*, 2011; Fellbaum *et al.*, 2012). In this manner, plants support perhaps the most efficient AM fungi in their roots and shape the AM fungal community. Still, less efficient AM fungal species are not completely eliminated but persist at a low abundance (Kiers *et al.*, 2011), which is consistent with the usually observed composition of intraradical AM fungal communities, where one species dominates, while other species have a low but stable abundance (Dumbrell *et al.*, 2010; Janoušková *et al.*, 2013). However, this host plant involvement is certainly not the only mechanism of modulating the AM fungal communities. Niche differentiation among AM fungi including functional differences and different growth traits and life strategies of AM fungi are other important aspects, which enable the coexistence of multiple AM fungi in one root system (Maherali & Klironomos, 2007; Dumbrell *et al.*, 2011; Thonar *et al.*, 2011).

2. OBJECTIVES

The dissertation deals with the functioning of AM symbiosis in relation to different environmental conditions and composition of AM fungal communities. Experiments in the study were conducted in greenhouse conditions with *Medicago* sp. as host plant and selected AM fungal isolates. In order to understand the topic, the thesis addresses the following specific objectives:

- methodical aims
    - to characterize experimental conditions for a mutualistic functioning of AM symbiosis
    - to compare different approaches to quantification of AM fungi and contribute to the consolidation of quantitative real-time PCR (qPCR) markers

- factual aims
    - to determine how abiotic conditions affect the functioning of AM symbiosis
    - to describe how the quantitative composition of AM fungal communities influences the functioning of mycorrhizal symbiosis
    - to compare how different factors affect the composition of AM fungal communities

3. PUBLICATIONS AND MANUSCRIPT

The dissertation contains three publications and one manuscript:

PAPER I

Püschel, D., Janoušková, M., Voříšková, A., Gryndlerová, H., Vosátka, M., Jansa, J., 2017. **Arbuscular mycorrhiza stimulates biological nitrogen fixation in two *Medicago* spp. through improved phosphorus acquisition.** Frontiers in Plant Science 8, 390.

PAPER II

Voříšková, A., Jansa, J., Püschel, D., Krüger, M., Cajthaml, T., Vosátka, M., Janoušková, M., 2017. **Real-time PCR quantification of arbuscular mycorrhizal fungi: does the use of nuclear or mitochondrial markers make a difference?** Mycorrhiza 27, 577–585.

PAPER III

Voříšková, A., Jansa, J., Püschel, D., Vosátka, M., Šmilauer, P., Janoušková, M., 2019. **Abiotic contexts consistently influence mycorrhiza functioning independently of the composition of synthetic arbuscular mycorrhizal fungal communities.** Mycorrhiza 29, 127–139.

PAPER IV

Voříšková, A., Jansa, J., Püschel, D., Vosátka, M., Janoušková, M. **Are mycorrhizal benefits influenced by the quantitative composition of arbuscular mycorrhizal fungal communities?** Manuscript

3.1. Paper I: Püschel *et al*. (2017)

Arbuscular mycorrhiza stimulates biological nitrogen fixation in two *Medicago* spp. through improved phosphorus acquisition

David Püschel[1,2], Martina Janoušková[1], **Alena Voříšková**[1], Hana Gryndlerová[2], Miroslav Vosátka[1], Jan Jansa[2]

1) Department of Mycorrhizal Symbioses, Institute of Botany, Czech Academy of Sciences, Průhonice, Czechia
2) Laboratory of Fungal Biology, Institute of Microbiology, Czech Academy of Sciences, Prague, Czechia

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Contribution statement

AV participated in the establishment, carrying out and harvesting of the experiment. Subsequently, she was involved in the mineral nutrient analyses of plant biomass. AV also contributed to the interpretation of the results and provided comments on the manuscript during its preparation.

Arbuscular Mycorrhiza Stimulates Biological Nitrogen Fixation in Two *Medicago* spp. through Improved Phosphorus Acquisition

David Püschel[1,2,*], Martina Janoušková[1], Alena Voříšková[1], Hana Gryndlerová[2], Miroslav Vosátka[1] and Jan Jansa[2]

[1] Department of Mycorrhizal Symbioses, Institute of Botany, Czech Academy of Sciences, Průhonice, Czech Republic

[2] Laboratory of Fungal Biology, Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic

* Corresponding author: david.puschel@ibot.cas.cz

Abstract

Legumes establish root symbioses with rhizobia that provide plants with nitrogen (N) through biological N fixation (BNF), as well as with arbuscular mycorrhizal (AM) fungi that mediate improved plant phosphorus (P) uptake. Such complex relationships complicate our understanding of nutrient acquisition by legumes and how they reward their symbiotic partners with carbon along gradients of environmental conditions.

In order to disentangle the interplay between BNF and AM symbioses in two *Medicago* species (*M. truncatula* and *M. sativa*) along a P-fertilization gradient, we conducted a pot experiment where the rhizobia-treated plants were either inoculated or not inoculated with AM fungus *Rhizophagus irregularis* 'PH5' and grown in two nutrient-poor substrates subjected to one of three different P-supply levels. Throughout the experiment, all plants were fertilized with ^{15}N-enriched liquid N-fertilizer to allow for assessment of BNF efficiency in terms of the fraction of N in the plants derived from the BNF ($\%N_{BNF}$). We hypothesized (1) higher $\%N_{BNF}$ coinciding with higher P supply, and (2) higher $\%N_{BNF}$ in mycorrhizal as compared to non-mycorrhizal plants under P deficiency due to mycorrhiza-mediated improvement in P nutrition.

We found a strongly positive correlation between total plant P content and $\%N_{BNF}$, clearly documenting the importance of plant P nutrition for BNF efficiency. The AM symbiosis generally improved P uptake by plants and considerably stimulated the efficiency of BNF under low P availability (below 10 mg kg^{-1} water extractable P). Under high P availability (above 10 mg kg^{-1} water extractable P), the AM symbiosis brought no further benefits to the plants with respect to P nutrition even as the effects of P availability on N acquisition via BNF were further modulated by the environmental context (plant and substrate combinations). As a response to elevated P availability in the substrate, the extent of root length colonization by AM fungi was reduced, the turning points occurring at about

8 and 10 mg kg^{-1} water extractable P for *M. sativa* and *M. truncatula*, respectively. Our results indicated competition for limited C resource between the two kinds of microsymbionts and thus degradation of AM symbiotic functioning under ample P supply.

Keywords**: legumes, root symbioses, rhizobia, arbuscular mycorrhiza, nitrogen acquisition, phosphorus uptake, competition, synergies**

Introduction

Legumes form two different types of root symbioses with soil microorganisms. Rhizobial symbiosis, exclusive to legumes, is established with soil diazotrophic bacteria that induce formation of nodules in host plants' roots. Rhizobia fix atmospheric dinitrogen (N_2) and provide it to the plants in the ammonium form that can easily be assimilated by the plant. Biological nitrogen fixation (BNF) thus contributes significantly to the nitrogen (N) budget of legumes. Its share in total N uptake by the plants is estimated to reach as high as 65–95% (Bolger et al., 1995). Arbuscular mycorrhizal (AM) symbiosis is by far more widespread among plant taxa. This association is established between the majority of terrestrial vascular plants and AM fungi from the phylum Glomeromycota (Smith and Read, 2008). AM fungi colonize plant roots and then their hyphae radiate into the surrounding soil, creating extensive networks of mycelium reaching to soil volume up to two orders of magnitude greater than what is accessible by plants alone (Raven and Edwards, 2001) and thus well beyond the depletion zone of the roots of poorly mobile nutrients such as phosphorus (P). The pivotal role of AM symbiosis occurs in enhancing plants' uptake of such poorly mobile nutrients as P and/or zinc (Jansa et al., 2011, 2003a; Kiers et al., 2011).

In both rhizobial and AM symbioses, plants reward their microbial partners with photosynthetically assimilated carbon (C). The flows of C, N and P in plants hosting both symbionts thus becomes rather complex (Figure 1). Each symbiosis may consume around 3–20% of recently fixed C to maintain the growth and activity and to build up energy reserves of the participating microbes (Jakobsen and Rosendahl, 1990; Kaschuk et al., 2009; Slavíková et al., 2016). The plants can partly compensate for C needs of their symbionts by increased CO_2 assimilation (Paul and Kucey, 1981), either due to C sink stimulation or indirectly through the nutritional benefits received from the symbioses (Kaschuk et al., 2009; Řezáčová et al., 2017).

Although the functioning of either of these symbioses alone has been studied in depth through past decades, their interaction remains insufficiently explored. Synergistic effects on plants of rhizobial and mycorrhizal symbioses have been described (e.g., Kaschuk et al., 2010; Larimer et al., 2014; van der Heijden et al., 2016), but the interaction of the two symbionts may also reduce plant growth (e.g., Ballhorn et al., 2016; Bethlenfalvay et al., 1982). As pointed out in a review by Larimer et al. (2010), there is a need for more experimental studies relating the interaction of the symbionts to abiotic conditions because

nutrient availability and other environmental factors may influence the outcome. For example, Saia et al. (2014) observed that AM symbiosis enhanced BNF and total plant biomass under drought stress but not under water-sufficient conditions. Ballhorn et al. (2016) reported interactive effects of AM and rhizobial symbioses depending on light availability. Surprisingly, however, there is only limited and inconclusive information on how the interaction of the two symbionts changes along the P-availability gradients (see Bethlenfalvay et al., 1982; Larimer et al., 2014).

Maintenance of rhizobial symbiosis imposes great P demand on the host plants (Jakobsen, 1985). This is because nodules have high sink-strength for P, probably due to considerable nitrogenase demand for ATP and because P concentration in microbial tissue is substantially higher than in plant cells (Jakobsen, 1985). Under low P availability to the plants, the efficiency of BNF thus often decreases (Kleinert et al., 2014). This effect is thought to be merely indirect, through intensifying P deficiency of the host plant and, as a consequence, impairing the photosynthetic capacity of the host plant, and not directly affecting nodule formation or function (Jakobsen, 1985). As AM fungi usually improve their host plants' P status under low P availability, AM symbiosis is expected to support rhizobial activity and increase BNF. At high P availability, AM symbiosis usually does not further improve the host plant's P budget (Smith and Read, 2008) and is therefore unlikely to increase BNF through improved P nutrition. In contrast, the host plant may become C-limited under P-sufficient conditions (Johnson et al., 1997) with the consequence that synergy of the two symbionts changes to antagonism as the two compete for C as the system-limiting resource (Bethlenfalvay et al., 1982; Reinhard et al., 1993).

To achieve a better understanding of the role of P availability in the functional interplay between rhizobial and mycorrhizal symbioses, a factorial pot experiment with two soil types and three P-fertilization levels was conducted using mycorrhizal and non-mycorrhizal individuals of the species *Medicago truncatula* and *M. sativa* inoculated with their compatible rhizobia. The plants were grown in substrates differing in pH and P availability, and fertilized with ^{15}N-labeled ammonium nitrate to allow assessment of BNF contribution to the plants' N uptake. We hypothesized that (1) the efficiency of BNF would positively correlate with P nutrition of the plants, and (2) under low P availability in the substrate, mycorrhizal plants would acquire relatively more N from BNF than would non-mycorrhizal plants due to the functional synergy between the two symbioses.

Material and methods

Experimental design

In a greenhouse pot experiment, two model plant species, *M. truncatula* and *M. sativa*, were planted in two different substrates amended or not with mineral P fertilizer to reach three levels of P availability for each of the substrates. All plants were inoculated with rhizobia compatible with the respective host plant species. Half of the plants were further inoculated

with AM fungal isolate *Rhizophagus irregularis* 'PH5', whereas the other plants grew without the AM fungus. The experiment was conducted in a fully factorial experimental design with five biological replicates per treatment, and thus it comprised 120 pots.

Substrate and initial P fertilization

A mixture (1:1, v:v) of autoclaved (at 121°C for 30 min) quartz sand (grain size < 4 mm) and autoclaved zeolite (grain size 1–2.5 mm; Zeopol s.r.o., Břeclav, Czech Republic; www.zeopol.com) provided the basis of the substrate used in this study. To this sand–zeolite mixture, 10% (of final volume) of γ-irradiated (>25 kGy) soil was added. Two soils of different origins and with different physicochemical properties were used. The first soil originated from Litoměřice, Czech Republic (GPS coordinates 50.532°N, 14.110°E) and the second soil was obtained from Tänikon, Switzerland (47.489°N, 8.919°E). The two soils differed in pH and calcium content and thus were assumed to have different P saturation kinetics, effectively resulting in a 6-point P-availability gradient obtained as a combination of 2 substrates and 3 P-supply levels (see Table 1 for selected physicochemical properties of the different substrates).

The substrates (further referred to as "LT" or "Tän" substrate depending on the identity of the soil component) were filled into tall, 2 L plastic pots (11 × 11 × 20 cm). First, the bottom third of each pot's volume was filled. Prior to filling the upper two thirds of the pots, the respective volume of the substrate was subjected to initial fertilization and/or mycorrhizal inoculation, as required by the specific experimental treatment (details described below).

To prevent plant growth limitation due to lack of potassium (K), magnesium (Mg) and/or calcium (Ca), these nutrients were uniformly added into all pots as initial fertilization of the substrate. The doses of 60 mg of K, 30 mg of Mg, and 30 mg of Ca (per pot) were provided by means of two separate nutrient solutions that were prepared by dissolving either 13.372 g of K_2SO_4 together with 30.423 g of $MgSO_4 \cdot 7H_2O$, or 11.005 g of $CaCl_2 \cdot 2H_2O$ per 1 L of distilled water. Both solutions were applied in doses of 10 mL per pot and thoroughly mixed into the upper two thirds of the substrate filled into each pot.

The gradient of P supply comprised three levels, hereafter referred to as "P0", "P10" and "P40", with either 0, 10 or 40 mg of P added to the pots (Table 1). This was achieved by applying one of two P solutions, prepared by dissolving either 11.563 or 46.251 g of $Na_2HPO_4 \cdot 12H_2O$ per 1 L of distilled water. The 10 mL dose of the respective P solution or distilled water in case of P0 level was applied and mixed into the upper two thirds of the substrate filled into each pot simultaneously with the application of cations as described earlier. For P availability measured in the different substrates, please see Table 1.

Mycorrhizal inoculation

Sixty pots were inoculated with the AM fungal isolate *R. irregularis* 'PH5'. The isolate is maintained in the AM fungal collection of the Department of Mycorrhizal Symbioses

(Institute of Botany, Czech Academy of Sciences, Průhonice, Czech Republic) in sand–zeolite–LT soil (2:2:1, v:v:v) mixture. The AM fungal inoculum cultures, established with *Zea mays* as the initial and *Desmodium* sp. as the follow-up, long-term host plant, were 16 months old when used as inoculum source. Inspection under a stereomicroscope had confirmed very abundant intraradical and extraradical sporulation of *R. irregularis*, as well as an absence of contamination by other AM fungal morphospecies. To prepare the AM fungal inoculum, the host plants' shoots were removed and the roots were cut into ca 0.5 cm pieces and mixed back into the substrate. The material was subsequently dried at room temperature for 1 week. After thorough homogenization by mixing, the complex AM fungal inoculum (substrate+roots) was weighed into 50 g aliquots, stored temporarily in plastic bags, and then mixed into the upper two thirds of the substrate filled into each mycorrhiza-inoculated pot. This was done simultaneously with the application of cations and P (if applicable) described earlier.

To obtain an appropriate control (non-mycorrhizal, NM) treatment, a "mock" inoculum was produced in exactly the same manner as described above but using NM cultures: the same host plants were grown in the same substrate and under the same conditions as the AM fungal inoculum, but without the AM fungi. Visual inspection of the mock-inoculum cultures under a stereomicroscope confirmed the absence of AM fungal spores and/or mycelium clumps. The mock inoculum was processed and applied into the experimental pots in exactly the same manner as was the AM fungal inoculum (see above).

Plants and rhizobia

The seeds of *M. truncatula* J5 and *M. sativa* cv. Vlasta were surface-sterilized (10% sodium hypochlorite; 10 min) and thereafter rinsed with sterilized tap water. The plants were germinated on moist filter paper in sterilized glass Petri dishes. Those seedlings with developed cotyledon leaves were transplanted into the pots, four seedlings per pot. During transplantation, the plants were inoculated with their compatible rhizobia. *M. truncatula* was inoculated with *Sinorhizobium meliloti* strain LT10, indigenous to LT soil, which was previously selected amongst several rhizobium strains isolated from the Litoměřice field site as the most beneficial rhizobium compatible with *M. truncatula* (unpublished observation). *M. sativa* was inoculated with strain 740 (Rhizobial collection, Crop Research Institute, Prague, Czech Republic), which had been recommended for *M. sativa* plants by Lenka Kabátová (Crop Research Institute, Prague, Czech Republic, personal communication). Both of the bacterial strains were grown in TY liquid medium (Somasegaran and Hoben, 1994) on a shaker at 24°C for 3 days. The bacteria were washed with 0.5% (w:v) aqueous $MgSO_4$ solution and the suspension was then adjusted to the optical density of 0.7 at 600 nm (which corresponded to approximately 2×10^9 cells mL^{-1}). One mL of this suspension was applied to each planting pit of individual seedlings during planting. After one week, the plants were thinned to two plants per pot.

Additionally, two control pots were established, one with LT and the other with Tän substrate, both of which were added with the AM fungal inoculum and fertilized with 40 mg P per pot. These pots were then planted with an isogenic mutant TRV25 (Morandi et al., 2005) of *M. truncatula* with suppressed ability to form both mycorrhizal and rhizobial symbioses. The plants were treated with the LT10 rhizobial strain as were the other experimental pots planted with *M. truncatula*. These pots were important for estimating the amount of N taken up from the substrate by P-sufficient plants in the absence of BNF. Due to spatial limitations and low availability of seeds of the mutant plant genotype, such control treatment could not have been established for each P-supply level and in a fully replicated manner.

Plant cultivation and ^{15}N labeling

The experiment was begun at the end of September and conducted for 9 weeks in a heated greenhouse (where temperature did not drop below 18°C at night). Natural light was supplemented with 400 W metal halide lamps set to 14 h photoperiod such that the photosynthetically active radiation flux at plant level ranged between 370 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at midday and a minimum of 85 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at dawn or dusk. The positions of the pots were fully randomized. The plants were watered with 25, 50 or 100 mL of distilled water per day (all pots received always the same amount of water that progressively increased with plant age).

The plants were regularly fertilized with N provided as NH_4NO_3 solution. N-fertilization was first applied in the third week after planting (to prevent potential suppression of nodulation at early stages of plant development) and then repeated weekly, thus totaling six applications per pot. With each application, the plants were provided with 20 mg of N per pot (1.14 g of NH_4NO_3 was dissolved per 1 L of distilled water, and 50 mL of this solution were applied per pot).

To distinguish N uptake by plants via the root/mycorrhizal and the BNF pathways, the ammonium nitrate applied in the pots was enriched with $^{15}NH_4{}^{15}NO_3$ (>98% ^{15}N; Cambridge Isotope Laboratories, Inc., Andover, MA, USA) to reach $\delta\ ^{15}N$ = +4491‰, corresponding to fractional abundance of ^{15}N of 0.01979 (calculated value using isotopic abundance of the unlabeled and ^{15}N-enriched ammonium nitrate and their molar ratio in the liquid fertilizer).

Harvest and sampling

The shoots were cut at the substrate surface level, pooled per pot, dried at 65°C to constant weight and weighed to obtain shoot dry weight (SDW). The compact root system with substrate was removed from the pot and the roots were shaken off to remove most of the substrate.

The roots were then carefully washed of the remaining substrate with water. Mycorrhizal colonization was assessed on roots sampled throughout the zone originally

laying approximately in the 4–8 cm depth. The sampled roots were cut into ca 1 cm pieces, immersed into 10% KOH and then stained using the modified method of Koske and Gemma (1989). In brief, the roots were first macerated in 10% KOH (overnight at room temperature, then 50 min at 90°C), washed with tap water, neutralized in 2% lactic acid (20 min at 90°C), and stained with 0.05% Trypan blue in LG (lactic acid–glycerol–water, 1:1:1, v:v:v) for 30 min at 90°C plus overnight at room temperature. The next day, roots were washed with tap water and further stored in LG. Colonization was evaluated microscopically using an Olympus SZX12dissecting microscope at 100× magnification and quantified according to the gridline intersection method (Giovannetti and Mosse, 1980) while observing at least 100 intersections per sample.

The remaining roots were also weighed fresh and then reweighed after drying at 65°C to constant weight. Root dry weight (RDW) of the entire root system per pot was then calculated. Plants' total dry weight (TDW) was calculated as the sum of SDW and RDW. To compare plants' growth response to inoculation in different substrate treatments, mycorrhizal growth response (MGR) of individual mycorrhizal pots was calculated from the TDW values according to the equation $MGR = (M - NM_{mean})/NM_{mean} \times 100\%$ (Gange and Ayres, 1999), where M is the TDW recorded for a given mycorrhizal pot and NM_{mean} is the mean TDW of pots in the corresponding NM treatment (i.e., the same substrate and P level).

Elemental analyses

Prior to analyses of P and N concentrations in plant tissues, the dried samples of shoots and roots were ground to powder using a ball mill (MM200, Retsch, Haan, Germany). To determine the P concentration in plant tissues, milled samples of shoots and roots (100 mg each) were incinerated in a muffle furnace at 550°C for 12 h. The resulting ash was combined with 1 mL of concentrated (69%) HNO_3 and briefly heated to 250°C on a hot plate. The materials was then transferred to volumetric flasks through a filter paper and brought up to 50 mL with ultrapure (18 MΩ) water. Phosphorus concentration in the extracts was then measured by colorimetry at 610 nm using a Pharmacia LKB Ultrospec III spectrophotometer by the malachite green method (Ohno and Zibilske, 1991).

The N concentrations and N isotopic composition in shoots and roots were measured using a Flash EA 2000 elemental analyzer coupled with a Delta V Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

Total N and P contents were calculated from SDW and RDW data and the concentrations of the corresponding elements in shoot and root biomass, respectively. Additionally, mycorrhizal P-uptake response (MPR) and mycorrhizal N-uptake response (MNR) were calculated from the P contents of the plants (shoots and roots combined) similarly as described above for the MGR.

Calculation of BNF efficiency

Assuming very similar isotopic composition of aerial N_2 and total N in the potting substrates (fractional abundance of ^{15}N in those two pools being 0.00364 and 0.00368, respectively,

with the latter being the grand mean of 12 measurements of the two potting substrates amended with different levels of P before the experiment), the fraction of plant N derived from the ^{15}N-labeled fertilizer (Ndff) was calculated separately for the shoots ($Ndff_S$) and the roots ($Ndff_R$) as follows:

$Ndff_S$ (mg N) = shoot N content (mg) * (^{15}N-AT%$_S$ − 0.368)/(1.979 − 0.368) (Equation 1)

$Ndff_R$ (mg N) = root N content (mg) *(^{15}N-AT%$_R$ − 0.368)/(1.979 − 0.368) (Equation 2),

where ^{15}N-AT%$_S$ and ^{15}N-AT%$_R$ represent isotopic composition of N in the shoots and roots expressed as ^{15}N atom percent, respectively, and were measured by isotope ratio mass spectrometry. From these values, efficiency of the BNF was calculated, here defined as the fraction of the plant N derived from biological N fixation (%N_{BNF}) as follows:

%N_{BNF} (% of the plant N) = ((shoot N content − $Ndff_S$) + (root N content − $Ndff_R$))/(shoot N content + root N content) (Equation 3)

This calculation effectively ignored the contribution of seed N (likely to be very small due to the small seed size of the two experimental plant species) as well as the contribution of N contained in the components of the potting substrates. This simplification was necessary because we had not established replicated non-fixing controls for each and every combination of the potting substrate and P amendment to experimentally measure N acquisition from the differentially P-amended substrates by non-fixing plants. Inasmuch as we have experimental evidence that the substrate contribution to N acquisition by the plants is generally not very large, the simplifications described above are justifiable. Indeed, the measured contribution of substrate N to N uptake of the asymbiotic *M. truncatula* mutants supplied with the highest P level (and assuming this saturated their P demand) reached only 12.0 or 11.2 mg N for the LT and Tän substrates, respectively. This was only about 19% and 18% of their total N content when grown in the LT and Tän substrates, respectively, meaning the plants relied to a large extent for their N supply on N uptake from the liquid fertilizer (which was the only remaining N source for these plants lacking BNF as well as AM symbiosis). If extrapolated to our symbiotic experimental plants, N acquisition from the substrates would only cover about 12% of their N budget. The actual values were most likely even lower than that 12% of the plant N budget due to the functional BNF.

Statistical analyses

The data were analyzed using STATISTICA 12 (StatSoft Inc., USA). None of the presented data deviated significantly from the normal distribution and thus the data sets were not transformed for the statistical analyses. Data for two pots were removed from the subsequent statistical analyses: one pot from the NM treatment whose roots were colonized heavily by AM fungi (i.e., due to contamination) and one mycorrhizal pot with unusually high P content in the plant (more than twice the average of the treatment). Therefore, at least four biological replicates were retained per each treatment combination and five were included in most of them. The data were first subjected to general linear model analyses using the factors “plant”,

"substrate", and "AM fungal inoculation" as categorical predictors and "P addition" as continuous predictor in order to determine the contributions of individual factors and/or their interactions to explaining the variability in the data set (Table S1). Individual parameters were analyzed by *t*-test to find differences between mycorrhizal and NM plants in every combination of substrate and fertilizer. A *t*-test also was used to analyze general differences in mycorrhizal colonization, MGR, MPR, and MNR between *M. truncatula* and *M. sativa* plants. The differences in mycorrhizal colonization, MGR, and MPR between P fertilization treatments were analyzed with ANOVA followed by Tukey's HSD test for separating the means. Correlation analyses between plant P nutrition and $\%N_{BNF}$ were carried out using a linear regression model. The slopes of regression lines for mycorrhizal and non-mycorrhizal plants were further compared in Statgraphics Plus 3.1 (Statistical Graphics Corp., USA).

Results

Development of the symbiotic microorganisms

All plants, excluding the two pots planted with the TRV25 mutant of *M. truncatula*, had nodules well developed in their root systems. The roots of all mycorrhizal plants were also highly (>65% of the root length) colonized with AM fungi (Figure 2), whereas those of NM and the mutant plants remained free of AM fungal colonization (data not shown). Mycorrhizal *M. truncatula* plants had their roots colonized to a significantly greater extent than did the *M. sativa* plants (87% vs 77% of root length, respectively; *t*-test, $p < 0.0001$). The levels of mycorrhizal colonization were generally significantly lower in the P40 treatment compared to the less-fertilized treatments (Figure 2A, B), the exception being for *M. truncatula* in LT substrate (Figure 2B).

Plant growth and mycorrhizal P uptake

In general, the plants responded to the presence of AM fungi in terms of their biomass either positively (*M. truncatula*, except for the P40 treatment in both substrates) or else no significant effect was recorded. No case of significant negative effect of AM symbiosis on plants' TDW was found within the individual treatments (Figure 3A). The calculation of MGR nevertheless did show negative values in some cases, and particularly for the P40 treatments (Figure 4A, B). MGR of plants, analyzed for the whole data set covering four combinations of plant species and substrates, was significantly negatively correlated with the increasing P inputs ($R^2 = 0.1663$, $p = 0.013$). This general trend was driven, however, by a very strong correlation ($R^2 = 0.901$, $p < 0.0001$) recorded for *M. truncatula* planted in Tän substrate, whereas correlations for the other plant–substrate combinations were not significant (data in Figure 4B). Highly significant differences in MGR were found between *M. truncatula* and *M. sativa* plant species, the former responding much more strongly to mycorrhiza than the later (*t*-test, $p < 0.0001$).

Mycorrhizal symbiosis significantly increased P uptake by both *Medicago* species in all substrate and fertilization treatments, except for the P40 treatment in Tän substrate (Figure 3B). MPR was significantly higher in *M. truncatula* plants than in *M. sativa* plants (*t*-test, $p = 0.0413$). A strong negative correlation ($R^2 = 0.4566$, $p < 0.0001$) between MPR and P fertilization was found for the whole data set (Figure 4C). Individual plant–substrate combinations followed this trend (Figure 4D). Only in the case of *M. truncatula* planted in LT substrate was the correlation not significant (data in Figure 4D).

Biological nitrogen fixation

With the exception of the P40 treatment in Tän substrate, mycorrhizal symbiosis increased total plant N content and $\%N_{BNF}$ in all *M. truncatula* plants (Figure 3C, D). In *M. sativa* plants, by contrast, the presence of AM fungus had no effect whatsoever on N content (Figure 3C), but it increased $\%N_{BNF}$ in the LT substrate irrespective of the P input level (Figure 3D). Also, highly significant differences in MNR (*t*-test, $p < 0.0001$) evidenced the more important role of mycorrhiza in N acquisition by *M. truncatula* plants than by *M. sativa* plants.

The $\%N_{BNF}$ was strongly and positively correlated with P content in plant biomass. This was manifest not only for the data set as a whole (Figure 5A), but it was confirmed also when smaller data sets were tested separately (Figure 5B–D). While in the case of *M. truncatula* plants the slopes of regression lines for mycorrhizal and NM plants differed significantly ($p = 0.005$), and with the regression line for NM plants being steeper than that for mycorrhizal plants (Figure 5C), in the case of *M. sativa* plants the slopes of the regression lines for mycorrhizal and NM plants were not statistically different (Figure 5D). Likewise, the slopes of regression lines for mycorrhizal and NM plants pooled across the two plant species (Figure 5B) did not differ significantly ($p > 0.05$).

Discussion

Using two different substrates and three levels of P supply allowed establishing a wide range of experimental conditions (P availabilities) for examining the symbiotic functioning of *Medicago* spp. along a P-fertilization gradient. The two *Medicago* species differed in their response to AM symbiosis, with *M. truncatula* being substantially more responsive to mycorrhiza formation than *M. sativa* in terms of growth, P acquisition, as well as N uptake. The two soils employed in this study as substrate components caused the P-sorption kinetics to differ between the two substrates (Table 1). Presumably, P was more efficiently immobilized in the calcareous LT soil with pH 7.88 (Püschel et al., 2016) than in the acidic Tän Luvisol with pH 6.2 (Jansa et al., 2003b). If the Tän substrate was fertilized with 40 mg kg^{-1} P, the water extractable P levels exceeded 10 mg kg^{-1}, thereby resulting in P-sufficient conditions even for the NM plants (Table 1 and Figure 3).

Are BNF and plant P nutrition related?

Considering the high P demand of the symbiotic BNF (Divito and Sadras, 2014), we had hypothesized that leguminous plants better supplied with P would, consequently, also show higher $\%N_{BNF}$. Not only was this hypothesis clearly confirmed for both *Medicago* species in association with their own compatible rhizobia, this general trend was also valid for both mycorrhizal and NM plants (Figure 5). These results confirmed previous observations (Ankomah et al., 1996; Kuang et al., 2005; Vadez et al., 1999) made with different leguminous plant species, although the range of environmental conditions (such as P availabilities) was usually more restricted in the previously published case studies than in our current research. Duplication of BNF efficiency due to massive P fertilization in a mixed clover–grass sward was previously reported from a mesocosm experiment by Edwards et al. (2006). That study indirectly confirmed that the increase of BNF efficiency from 25% to 50% observed in our study due to removal of P limitation for the plants – either through AM symbiosis establishment or P fertilization – is comparable to the effects observed under other (field-relevant) settings.

We also had expected to observe functional synergy between the two root symbionts (Barea et al., 2005), particularly if the plants were exposed to P deficiency. Our experimental evidence fully supports this second hypothesis for M. truncatula plants but only partly so for M. sativa plants. In the case of M. truncatula, mycorrhizal plants in all treatments with low P availability (i.e., below 10 mg kg^{-1}) had significantly higher $\%N_{BNF}$ than did their respective non-mycorrhizal counterparts. This was not the same, however, for M. sativa plants. Although AM symbiosis still provided M. sativa plants with more P in exactly the same combinations of substrate and fertilization as in the case of M. truncatula plants, this advantage was reflected in higher $\%N_{BNF}$ in LT substrate only but not in any of the P treatments within the Tän substrate (Figure 3). This indicates that different functional traits of plants, the rhizobia, or the interaction between the two can respond to the outer environment in a context-specific manner. It seems, in fact, that M. sativa with its rhizobia particularly liked the Tän substrate, as it maintained BNF levels high in this substrate irrespective of the P-supply levels.

Under ample P supply, mycorrhizal benefits in terms of improved plant P acquisition were reduced or vanished completely (Figures 3 and 4). This is consistent with the general consensus that mineral fertilization may render root symbionts dispensable (Morgan et al., 2005). Yet, the efficiency of BNF did not necessarily follow the same trend. Careful inspection of the regression lines plotted in Figure 5 reveals that there were different slopes of regression lines describing how P content of M. truncatula related to the $\%N_{BNF}$ of the same plants ($p = 0.005$). A similar observation (though only marginally significant, with $p = 0.088$) was made also for the data set as a whole, but the slopes were not significantly different between mycorrhizal and non-mycorrhizal plants of M. sativa ($p = 0.30$). These results indicate that, at least in the case of M. truncatula (Figure 5C), to achieve the same BNF efficiency, mycorrhizal plants had to have substantially greater P content than did the

NM plants, and the maximum $\%N_{BNF}$ values were achieved with greater difficulty or more slowly for the mycorrhizal as compared to the NM plants. These results indicate that with increasing P supply, the AM fungi and rhizobia increasingly competed for another limiting resource (at least in M. truncatula that also showed greater root colonization levels than did M. sativa). It is conceivable, based on the evidence of previous research, that the elusive limiting resource for BNF under sufficient P supply is the plant C (Morgan et al., 2005; Mortimer et al., 2009). If the metabolic energy to fix atmospheric N_2 is in short supply due to significant mycorrhizal C sink, which could be as high as 20% of the gross photosynthetic production (Jakobsen and Rosendahl, 1990), the benefits conferred to the host by rhizobia are actually hampered by the AM fungi. Although we do not have unequivocal evidence to show that this is happening, it is highly plausible, based also on previous experimental evidence showing additivity of C costs of the two microsymbionts in tripartite root symbioses (Ballhorn et al., 2016; Millar and Ballhorn, 2013; Mortimer et al., 2008; Paul and Kucey, 1981). Under high P availability or low light conditions, the coexistence of two root symbionts becomes a burden for the plant host (Ballhorn et al., 2016). Indirect support for this theory can be observed in the suppression of root colonization by AM fungus in most of the plant–substrate treatments with increasing P fertilization (Figure 2), which is consistent with the preferential allocation hypothesis (Bever, 2015).

Relatives, yet functionally different

Although using two different species of the genus *Medicago* yielded strong evidence here for a common underlying mechanism with respect to the functional interactions between mycorrhizal symbiosis and the BNF along a P-availability gradient, there were also some notable differences. One important issue that needs to be emphasized here is that both the plant and the rhizobial genotypes differed for the two plant species treatments. This was intentionally established in this manner to achieve the highest functional compatibility of the plant–bacterial partners. Thus, we were actually comparing two plant–rhizobial (biological) systems rather than two plant species per se.

Non-mycorrhizal *M. sativa* plants cultivated in the Tän substrate yielded surprisingly high $\%N_{BNF}$ despite that their P content under low P supply was significantly smaller than that of their mycorrhizal counterparts (Figure 3). It is possible that the rhizobia associated with *M. sativa* were either less P-demanding, more P-efficient, or generally more adapted to specific conditions of Tän substrate than were the bacterium used to inoculate *M. truncatula*. Such differences have been described previously and have been argued to be the result of plant–bacterial coevolution (Garau et al., 2005). Interestingly, the N contents and biomass of mycorrhizal and NM *M. sativa* plants were surprisingly similar in all substrate treatments, even though the BNF efficiency and P uptake obviously varied markedly (Figure 3). We therefore assume that *M. sativa* might actually better compensate for the missing symbiotic benefits through more dynamic root traits such as greater plasticity of root branching (Lynch, 2007; Nibau et al., 2008) and/or root exudation (Rao et al., 2016). Inasmuch as these traits

were not recorded in our study, however, this remains a matter of speculation, although it does point to possible mechanisms accounting for why different plants vary in their response and/or dependency on mycorrhizal and other symbioses (Jakobsen et al., 2005; Linderman and Davis, 2004).

Conclusions

Working with a large environmental (P availability) gradient established by using two different kinds of substrates in combination with three levels of mineral P inputs, we show here that AM symbiosis clearly promotes BNF efficiency, particularly in the case of low P supply. This effect was most likely mediated by improved P acquisition of the mycorrhizal as compared to the NM plants under conditions of low P. With increasing P inputs, however, the costs of the AM symbiosis (at least in the more heavily colonized *M. truncatula*) become more and more apparent, resulting in a lower P use efficiency (or in luxurious P uptake) of the mycorrhizal plants as compared to the NM plants and without concomitant increases in plant biomass production. Based upon Liebig's law of the minimum (Johnson et al., 2015), therefore, we conclude that there was strong competition between the symbionts and the plants for another resource, thereby preventing the occurrence of a significant positive growth response in the plants at higher P-supply levels. Most likely, this competition was for carbon (Ballhorn et al., 2016). In response to sufficient (or even luxurious) P supply at higher P-fertilization levels, mycorrhizal root colonization levels were reduced. Although this was in accordance with previous reports (Treseder, 2004), this obviously was not effective enough to counteract the C drain to the AM fungus, at least not in the *M. truncatula*. Although general reduction of root colonization at higher P-fertilization levels was true for both *Medicago* species (each in association with its compatible rhizobia), notable differences were observed between the two plant species. These could be due either to the plants or the rhizobial strains used and reflect such factors as inherent tolerance of the particular rhizobia to deviation from their pH optima, different architecture of the root systems, differential efficiency of plant genotypes in mineral nutrient use and/or redistribution, root exudation patterns, or other mechanisms. Disentangling these factors would require further experimental efforts, and particularly with respect to quantifying the C costs of the two root symbioses under a range of environmental conditions.

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Author Contributions

DP, MJ, and JJ designed the experiment, which was then carried out mainly by DP. HG conducted the P and N analyses, and JJ calculated the BNF efficiency. DP conducted the statistical analyses. All authors contributed to interpreting the results. DP and JJ did most of the writing, whereas MJ, AV, and MV critically commented on earlier versions of the manuscript.

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Table 1 | Selected physico-chemical properties of sand–zeolite (1:1, v:v) substrates with 10% volumetric content of either Tänikon (Tän) or Litoměřice (LT) soil, and supplemented with either 0, 10 or 40 mg of P per pot (P0, P10 and P40, respectively). The pH was measured in 1:2.5 (w:v) water slurry following 30 min shaking at room temperature by pH meter FE20 (Mettler Toledo). Available (water extractable) phosphorus (P) concentrations were measured in supernatant of a water slurry (1:1, w:v, shaken for 18h at room temperature) by malachite green method (Ohno and Zibilske, 1991). Total P concentrations were measured by the same method in acid (HNO_3, 69%) extracts of the samples following incineration at 550°C for 20h. Reported values for P concentrations are means of two separate extractions performed 9 and 19 days after application of the mineral P fertilizer. The carbon (C) and nitrogen (N) concentrations in the samples were analyzed by Flash EA 2000 elemental analyzer (Thermo Fisher Scientific). All presented data are means of two technical replicates.

Substrate	**pH $_{H2O}$**	**C [%]**	**N [%]**	**Available P [mg kg^{-1}]**	**Total P [mg kg^{-1}]**
Tän P0	7.52	0.186	0.012	2.06	73.06
Tän P10	7.51	0.230	0.018	5.00	85.09
Tän P40	7.43	0.226	0.015	12.35	125.96
LT P0	8.28	0.260	0.010	3.03	108.52
LT P10	8.19	0.307	0.012	5.49	158.68
LT P40	8.33	0.214	0.008	8.32	161.81

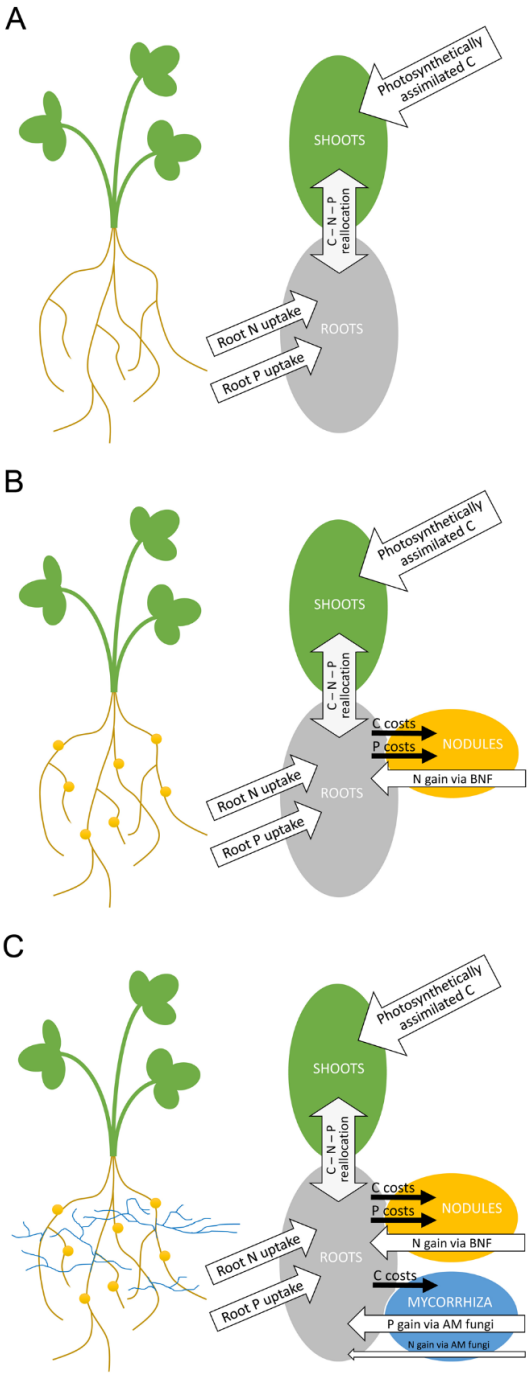

FIGURE 1 | Schematic representation of increasing complexity of carbon (C), nitrogen (N), and phosphorus (P) flows in a model leguminous plant growing either without any symbiosis (A), with only rhizobial symbionts mediating the biological nitrogen fixation (BNF) (B), or with both rhizobial and mycorrhizal symbioses established (C). Rhizobial nodules are shown as yellow circles on the roots, whereas the hyphae of arbuscular mycorrhizal (AM) fungi are represented by blue lines radiating from the roots. White arrows indicate plant acquisition pathways, gray arrows reallocation, and black arrows loss of C, N and P, respectively.

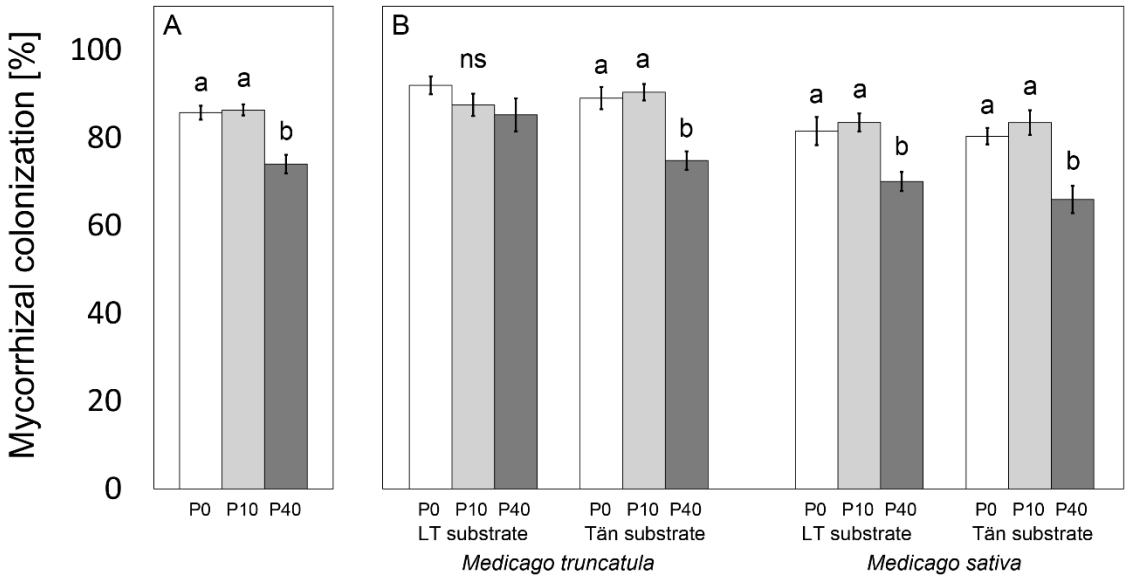

FIGURE 2 | Mycorrhizal colonization of plants inoculated with *Rhizophagus irregularis* 'PH5' affected by the addition of 0, 10 or 40 mg pot^{-1} of phosphorus (P0, P10, P40, respectively). The results are shown either for the whole data set **(A)**, or separately for *Medicago truncatula* or *M. sativa* plants grown in sand–zeolite substrate with 10% of either Litoměřice or Tänikon soil (LT or Tän substrate, respectively) **(B)**. Colonization is expressed as percentage of root length occupied by mycorrhizal fungal structures such as hyphae, arbuscules, and/or vesicles. Means ± standard errors are shown (n = 19 or 20 for A, n = 4 or 5 for B). Different letters indicate significant differences between different P levels within each plant–substrate combination according to Tukey's HSD test ($p < 0.05$); ns, $p \geq 0.05$. The roots of control non-mycorrhizal plants remained free of mycorrhizal fungal structures (data not shown).

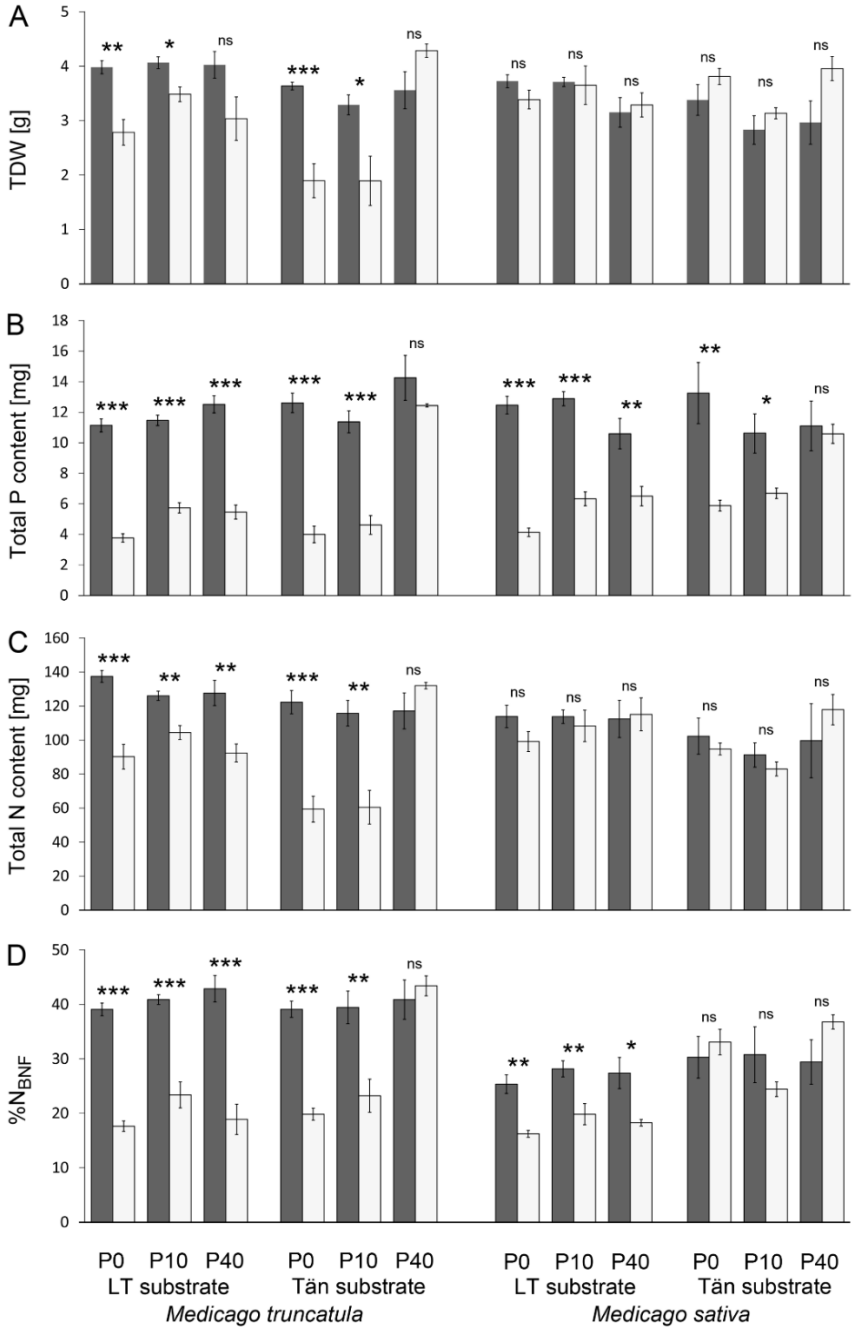

FIGURE 3 | Total plant dry weight (TDW) (A), total phosphorus (P) content (B), total nitrogen (N) content (C), and fraction of plant N derived from biological N fixation (%N_{BNF}) (D) of mycorrhizal (dark columns) or non-mycorrhizal (light columns) *Medicago truncatula* or *M. sativa* plants grown in sand–zeolite substrate with 10% of either Litoměřice or Tänikon soil (LT or Tän substrate, respectively). The substrate was amended with 0, 10 or 40 mg pot^{-1} of phosphorus (P0, P10, P40, respectively). Bars represent means ± standard errors (n = 4 or 5). Asterisks indicate significant differences between mycorrhizal and non-mycorrhizal plants according to *t*-test (* $0.01 \leq p < 0.05$; ** $0.001 \leq p < 0.01$; *** $p < 0.001$; ns, $p \geq 0.05$).

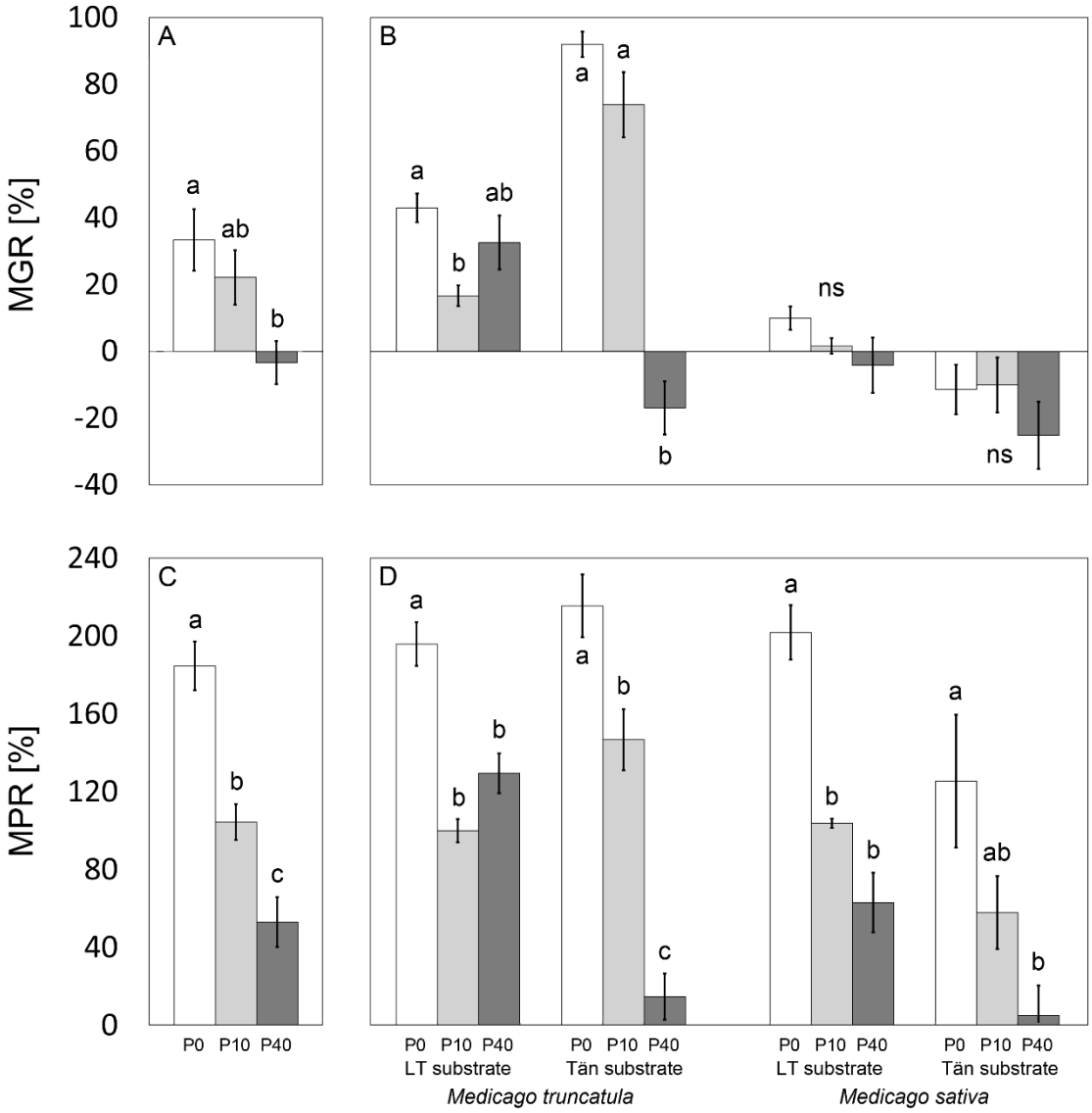

FIGURE 4 | Mycorrhizal growth response (MGR) and mycorrhizal phosphorus-uptake response (MPR) of plants along a phosphorus fertilization gradient consisting of three input levels (0, 10 and 40 mg P added per pot; P0, P10 and P40, respectively). The results are shown either for the whole data set (A, C), or separately for *Medicago truncatula* or *M. sativa* plants grown in sand–zeolite substrate with 10% of either Litoměřice or Tänikon soil (LT or Tän substrate, respectively). Means ± standard errors are shown (n = 19 or 20 for A and C, n = 4 or 5 for B and D). Different letters indicate significant differences between different P levels within each plant–substrate combination according to Tukey's HSD test ($p < 0.05$); ns, $p \geq 0.05$.

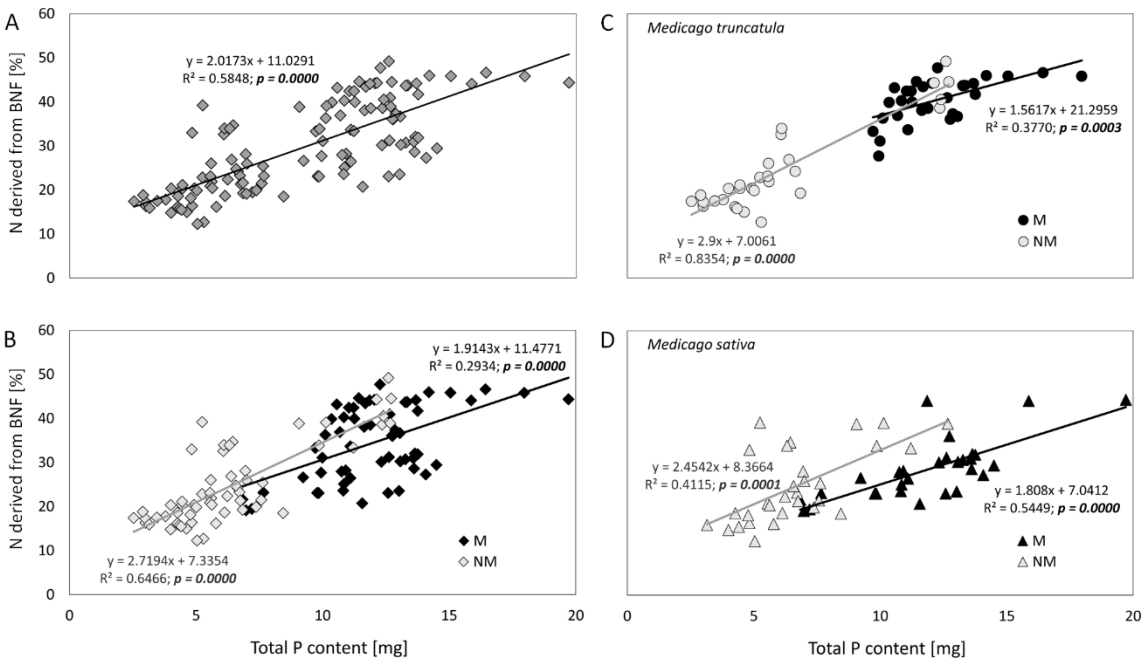

FIGURE 5 | Correlation between total phosphorus (P) content of the plants and percentage of nitrogen (N) derived from biological N fixation (BNF). Shown either for the whole data set **(A)**, for the data set split into mycorrhizal (M) and non-mycorrhizal (NM) plants of both species **(B)**, or further separately for *Medicago truncatula* **(C)** or *M. sativa* **(D)** plants. Equations along with associated R^2- and *p*-values are provided for each of the linear regression models. Statistical significance (*p*-) values were derived from a goodness-of-fit test of the linear regression models.

3.2. Paper II: Voříšková *et al.* (2017)

Real-time PCR quantification of arbuscular mycorrhizal fungi: does the use of nuclear or mitochondrial markers make a difference?

Alena Voříšková[1,3], Jan Jansa[2], David Püschel[1,2], Manuela Krüger[1,4], Tomáš Cajthaml[2,5], Miroslav Vosátka[1,3], Martina Janoušková[1]

1) Institute of Botany, The Czech Academy of Sciences, Zámek 1, 252 43 Průhonice, Czech Republic
2) Institute of Microbiology, The Czech Academy of Sciences, Vídeňská 1083, 142 20 Prague, Czech Republic
3) Department of Experimental Plant Biology, Faculty of Science, Charles University, Viničná 5, 128 44 Prague, Czech Republic
4) Institute of Experimental Botany, The Czech Academy of Sciences, Rozvojová 263, 165 02 Prague, Czech Republic
5) Institute for Environmental Studies, Faculty of Science, Charles University, Benátská 2, 128 01 Prague, Czech Republic

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Contribution statement

AV participated in the development of experimental design and in the establishment/carry out/harvesting of the experiment. She also participated in the conducting of molecular analyses and contributed to the consolidation of the qPCR markers. AV performed all statistical analyses and interpreted results together with her supervisor MJ. AV did most of the writing with a contribution of MJ and consultation with other co-authors.

Real-time PCR quantification of arbuscular mycorrhizal fungi: Does the use of nuclear or mitochondrial markers make a difference?

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[1)] Institute of Botany, The Czech Academy of Sciences, Průhonice, Czech Republic
[2)] Institute of Microbiology, The Czech Academy of Sciences, Prague, Czech Republic
[3)] Department of Experimental Plant Biology, Faculty of Science, Charles University, Czech Republic
[4)] Institute of Experimental Botany, The Czech Academy of Sciences, Prague, Czech Republic
[5)] Institute for Environmental Studies, Faculty of Science, Charles University, Prague, Czech Republic

Corresponding author: Alena Voříšková, Institute of Botany, The Czech Academy of Sciences, Lesní 322, CZ-252 43 Průhonice, Czech Republic; e-mail address: alena.voriskova@ibot.cas.cz

Abstract

Root colonization by arbuscular mycorrhizal fungi (AMF) can be quantified by different approaches. We compared two approaches that enable discrimination of specific AMF taxa and are therefore emerging as alternative to most commonly performed microscopic quantification of AMF in roots: quantitative real-time PCR (qPCR) using markers in nuclear ribosomal DNA (nrDNA) and mitochondrial ribosomal DNA (mtDNA). In a greenhouse experiment, *Medicago truncatula* was inoculated with four isolates belonging to different AMF species (*Rhizophagus irregularis*, *Claroideoglomus claroideum*, *Gigaspora margarita* and *Funneliformis mosseae*). The AMF were quantified in the root samples by qPCR targeted to both markers, microscopy and contents of AMF-specific phospholipid fatty acids (PLFA).

Copy numbers of nrDNA and mtDNA were closely related within all isolates; however, the slopes and intercepts of the linear relationships significantly differed among the isolates. Across all isolates, a large proportion of variance in nrDNA copy numbers was explained by root colonization intensity or contents of AMF-specific PLFA, while variance in mtDNA copy numbers was mainly explained by differences among AMF isolates. We propose that the encountered inter-isolate differences in the ratios of mtDNA and nrDNA copy numbers reflect different physiological states of the isolates. Our results suggest that nrDNA is a more suitable marker region than mtDNA for the quantification of multiple AMF taxa as its copy numbers are better related to fungal biomass across taxa than are copy numbers of mtDNA.

Key words

Arbuscular mycorrhizal fungi; Real-time PCR; PLFA; Mitochondrial DNA; Molecular genetic quantification; Nuclear ribosomal DNA; Microsymbiont screening; Isolate discrimination

Introduction

Arbuscular mycorrhiza (AM) is an almost ubiquitous symbiosis formed between the roots of most terrestrial plant species and arbuscular mycorrhizal fungi (AMF) from the phylum Mucoromycota, subphyllum Glomeromycotina (Spatafora et al. 2016). The degree of root colonization by AMF is an important parameter of the association as it is often (but not always, see e.g. Sawers et al. 2017) positively correlated with the functioning of AM in terms of plant growth promotion and/or improved plant nutrition (Lekberg and Koide 2005). A commonly used method to quantify AMF colonization in roots is microscopy after non–vital staining of the intraradical fungal structures (Vierheilig et al. 2005). Alternatively, AMF can be quantified in roots or soil by analysis of AMF-specific lipid biomarkers of the neutral or phospholipid fatty acid (PLFA) fractions. PLFA are key structural elements of membrane lipids and their contents therefore may provide a proxy of the amount of AM fungal biomass (Sharma and Buyer 2015). A substantial disadvantage of the microscopic and PLFA-based approaches, however, is their low resolution, namely the inability to distinguish among AMF taxa. In contrast, DNA-based methods, such as quantitative real-time PCR (qPCR), allow quantification of specific AMF taxa in roots and soil.

Nuclear ribosomal DNA (nrDNA) has been the most commonly used targeted DNA region for the development of markers for specific amplification of AMF taxa. Particularly, nucleotide variability in the large subunit (LSU) of nrDNA allows design of primers suitable to discriminate taxa at the species-level (Pivato et al. 2007). Sequence polymorphism among multiple gene loci of nuclear ribosomal genes, however, is an important disadvantage of this marker region which complicates designing species-specific qPCR assays, especially for discriminating among closely related species (Thonar et al. 2012). Often primers designed for one isolate cannot be used for another isolate of the same species, which may limit broad application of nrDNA-targeted qPCR assays. Moreover, nrDNA is not polymorphic enough to discriminate isolates within one AMF species. In contrast, mitochondrial genomes are usually homogenous within isolates of AMF (Lee & Young, 2009; Pelin *et al.*, 2012; Beaudet *et al.*, 2013; De la Providencia *et al.*, 2013; Nadimi *et al.*, 2015; Nadimi *et al.*, 2016) and at the same time, the variability of some regions allows for discrimination of closely related AMF species (Kiers et al. 2011) and isolates of *Rhizophagus irregularis* (Börstler et al. 2008; Couillerot et al. 2013). For this reason, the LSU of mitochondrial ribosomal DNA (mtDNA) has emerged as an alternative discriminatory marker for AMF quantification (Kiers et al. 2011) or to track specific isolates of *R. irregularis* (Krak et al. 2012; Badri et al. 2016).

Information still is limited about how copy numbers of mtDNA correlate with other quantification approaches for intraradical colonization by AMF. The copy number of nrDNA has been reported to correlate with quantification of AMF root colonization by microscopy (Alkan et al. 2004; Thonar et al. 2014), but this is not always the case (Gamper et al. 2008; Jansa et al. 2008). Although mtDNA copy numbers have been suggested to more closely approximate metabolic activity than nrDNA copy numbers (Tamasloukht et al. 2003; Gamper et al. 2010), a comparison of both approaches found a close correlation of nrDNA and mtDNA copy numbers within isolates (Krak et al. 2012) indicating that nrDNA-based quantification and mtDNA-based quantification may provide a similar picture of AMF abundance in roots. Nevertheless, mtDNA-based quantification of AMF in roots has been limited to *R. irregularis* or its close relatives, which calls for analyses on members of other phylogenetic lineages of AMF.

Therefore, the aims of our study were to determine (1) how copy numbers of mtDNA relate to nrDNA copy numbers in four different AMF species' isolates, and (2) how DNA-based quantification approaches relate to microscopic and PLFA-based quantification.

Materials and methods

Four AMF isolates, representing different species, were used for the purpose of this quantification study: *Rhizophagus irregularis* PH5, *Claroideoglomus claroideum* BEG96, *Gigaspora margarita* BEG34 and *Funneliformis mosseae* BEG95. Fungal inoculum of each isolate was produced in multi-spore pot cultures with *Allium porrum* as the host plant. *Medicago truncatula* J5 was chosen as the host plant for the experiment. Surface-sterilized seeds (10 min in 0.5% NaClO) were pre-germinated at room temperature on moist filter paper in sterile Petri dishes for 3 days.

The experiment were setup in Ray Leach Cone-tainers SC10 Super Cells (Stuewe & Sons, Tangent, Oregon, USA), each with a volume of 164 ml. The cultivation substrate was a mixture of 45% autoclaved sand, 45% autoclaved zeolite and 10% γ-irradiated soil from Tänikon, Switzerland (pH 7.52, C [%] 0.186, N [%] 0.012, available (water-extractable) P [mg kg^{-1}] 2.06 (the analytical methods are described in Püschel et al. 2017). For each AMF isolate, seven inoculation doses were used to achieve a range of intensity levels in root colonization. Air-dried substrate from the cultures containing spores, extraradical mycelium and roots cut to 1-cm-fragments, was weighted to achieve inoculation doses of 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 % (w:w) for *R. irregularis*, *C. claroideum* and *F. mosseae.* For *G. margarita*, doses of 50, 100, 250, 500, 1000, 2500 and 5000 spores per Super Cell were picked from the cultures and used for inoculation of transplanted seedlings because spores are the main infective propagules of *Gigaspora* spp. (Klironomos and Hart 2002). The spores and the air-dried inocula were placed directly below transplanted seedlings at the establishment of the experiment. Additionally, several Super Cells with non-inoculated plants were prepared to obtain root material of *M. truncatula* without AMF, which was later used as a negative control in AMF quantification by qPCR and PLFA analysis. Two germinated seedlings of

M. truncatula were planted into each Cell. Two replicate Cells were established per dose and AMF isolate, resulting in a total of 56 inoculated Cells. All seedlings were inoculated at planting with 1 ml suspension (optical density adjusted to 0.8 at 600 nm) of *Sinorhizobium* strain LT10, which had been prepared in the same way as described in Konvalinková et al. (2015).

After 10 weeks of cultivation in the greenhouse under supplementary light (400 W metal halide lamps providing a minimum photosynthetic flux density of 85 $\mu mol \cdot m^{-2} \cdot s^{-1}$ during 14 hours), the root systems of the plants were rinsed and dried on filter paper. The roots of replicate Cells per AMF isolate and inoculum dose were pooled and handled as one sample for all further analyses. The fresh weights of the pooled root systems ranged between 1.29 and 3.09 g. The roots were cut into 1 cm fragments and homogenized. Aliquots of 100 mg fresh weight (for molecular analyses) and ca. 1 g fresh weight (for the PLFA analysis) were frozen in liquid nitrogen and stored at – 80 °C. Root length was determined in five randomly selected replicates of 100 mg root fresh weight per inoculation treatment according to Newman (1966).The root length did not differ among the inoculation treatments and had a mean of 129 cm (SEM 2.794). This corresponds to specific root length of 87.81 m g^{-1} root dry weight, based on the average ratio of root dry weight to root fresh weight in a previous experiment with *M. truncatula* (Püschel et al. 2017).

The remaining parts of the root systems were placed into 10 % KOH and stained with 0.05 % Trypan Blue in lactoglycerol (Koske and Gemma 1989) for the microscopy. Root colonization was evaluated on 30 root segments (each 1 cm long) per sample as described in Trouvelot (1986). This approach is based on the categorization of root colonization into five classes of intensity and the abundance of arbuscules/vesicles into three classes. Thus, it enables estimation of the abundance of fungal structures independently of root thickness and/or heterogeneity of mycorrhizal colonization within the analyzed root segments. Among the parameters that can be calculated by the program Mycocalc (https://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html), we chose the intensity of mycorrhizal colonization in the root system (M%) as the main indicator of root colonization, because it gives a better proxy for the fungal biomass in roots than frequency of mycorrhizal colonization in the root segments (F%). Arbuscule abundance in mycorrhizal parts of the root system (a%) also is presented as it provides information on the physiological state of the fungus independent of root colonization extent. Furthermore, vesicle abundance relative to the entire root system (V%) is presented, as it may better approximate the fungal DNA content in roots than M% (Gamper et al. 2008). The roots of the non-inoculated control plants were confirmed to contain no AMF structures.

For the PLFA analysis, lipids were extracted from lyophilized root samples (100 mg dry weight), and fractioned as described previously (Šnajdr et al. 2008). The PLFA were converted to methyl esters using mild alkaline methanolysis and analyzed using tandem gas chromatography–mass spectrometry (GC–MS; 450-GC, 240-MS Varian, Walnut Creek, CA, USA) as reported previously in Covino *et al.* (2016). The concentrations of the AMF-specific fatty acids C16:w5 for Glomerales and C20:1w9 for Gigasporaceae were quantified in the

different samples, using appropriate external standards and expressed as microgram per gram. For statistical analysis, their relative content (subsequently called "the relative PLFA content") was calculated using the equation: $C_{PLFA} = C_M / C_{NM}$, where C_M is the concentration of AMF-specific fatty acid in mycorrhizal samples and C_{NM} is the mean of concentrations of AMF-specific fatty acid in non-mycorrhizal samples. This was done to standardize the concentrations of the Glomerales-specific and Gigasporaceae-specific fatty acids.

For the qPCR analyses, frozen root samples were ground in liquid nitrogen and DNA was extracted using the DNA Plant Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, and eluted into 80 µl of 10mM Tris. The concentration of each DNA extract was measured in two replicates using a Qubit dsDNA BR Assay Kit on the Qubit Fluorimeter (ThermoFisher, Scientific), and the means of the two replicates were used for the calculation of the numbers of gene copies. DNA extracts were stored in – 80 °C. *Taq*Man-based qPCR assays with PCR amplicons as standards were used for quantifying nrDNA LSU. SYBR Green-based qPCR assays with plasmid standards were used for quantifying mtDNA LSU on a LightCycler 480 II Real-Time PCR Instrument (Roche). The DNA extracts were diluted 1:10 for the reactions to ensure absence of PCR inhibition (Janoušková et al. 2015). The details of the protocols are given in the Supplementary Material (Text S1, Table S1, Table S2, and Fig. S1). Arbuscular mycorrhizal fungal abundance was expressed as the numbers of gene copies per nanogram of template DNA.

The evaluation of the pooled root sample inoculated with the 0.1 % (*w*:*w*) inoculation dose of *F. mosseae* rendered M% = 0 and negative qPCR results with both markers. This sample was excluded from further analyses.

Linear regression models were constructed to test relevant pair wise relationships between the different parameters of root colonization. The models included AMF isolate as a categorical predictor and the interaction of the categorical and continuous predictor. When the interaction term was non-significant in a model, the model was simplified by removing the interaction term in order to capture the full amount of variance explained by the main effects. In a first step, a%, V% or the relative PLFA content (as response variable) were related to M% (as predictor) which is the most commonly used estimate of fungal development in roots. In a second step, the two genetic estimates of fungal abundance in roots (mtDNA and nrDNA copy numbers) were related to each other. NrDNA copy numbers were set as predictor and mtDNA copy numbers as the response variable because nrDNA copy numbers are the more commonly used parameter and it is more logical to test how mitochondrial abundance depends on nuclear abundance than vice versa. In a third step, relationships were tested between the genetic estimates on one hand (nrDNA copy numbers, mtDNA copy numbers, and their ratio) and the other parameters (M%, relative PLFA content, a%) on the other hand. The genetic parameters were set as the response variable in the models. V% was not included because of pronounced variation in vesicle formation among species (especially no vesicle formation by *Gigaspora margarita*). Additionally, the *varpart* function in the 'vegan' package of R (Oksanen et al. 2012) was used to partition the variance

in each of the two genetic parameters between AMF isolate and each of the other parameters (M%, relative PLFA content, a%).

To complement the overall picture of the relationships among the parameters, linear relationships also were tested for each isolate separately by standardized major axis (SMA) regression using SMATR package (version 3.4-3; http://bio.mq.edu.au/ecology/SMATR/). The slopes and intercepts of the linear relationships of nrDNA and mtDNA copy numbers were compared among the AMF isolates. For these parameters, the regressions additionally were performed with data centered on nrDNA copy numbers in order to compare the intercepts at mean nrDNA copy numbers. Differences in means of each parameter among isolates were determined by non-parametric Kruskal-Wallis test (at $P = 0.05$). All statistical analyses were performed in R (version 3.3.2; https://www.r-project.org/).

Results

Microscopic parameters and relative PLFA content

The achieved ranges of root colonization differed among the four isolates (see Fig. 1a). While the M% values of *C. claroideum* and *F. mosseae* were well differentiated (7 – 66 % and 15 – 60 %, respectively), the root colonization of *R. irregularis* was relatively high at all inoculum doses (M% ranging from 44 % to 90 %) and *G. margarita* produced a narrow range of M% (20 % to 44 %).

Arbuscule abundance a% (Fig. 1b) depended on AMF isolate, but not on root colonization intensity M% (Table S3). In contrast to M%, the highest mean a% was recorded in *G. margarita* and the lowest in *C. claroideum* (compare Fig. 1a and 1b). Vesicle abundance V% was significantly related to M% and differed among the AMF isolates (Table S3): Vesicles were abundant in *C. claroideum* and *R. irregularis* and nearly or entirely absent in *F. mosseae* and *G. margarita*, respectively (Fig. S2a). Within isolates, a significant positive relationship of M% and a% was encountered in *C. claroideum* (Table S4, Fig. S3), and a significant positive relationship of M% and V% was encountered in both vesicle-forming species, *R. irregularis* and *C. claroideum* (Table S4, Fig. S3).

Relative PLFA content (Fig. S2b) was significantly affected by M%, AMF isolate and the interaction of both factors according to the linear regression model (Table S3). Relative PLFA content was significantly related to M% within *F. mosseae* and *C. claroideum*, but not within *G. margarita* and *R. irregularis* (Table S4, Fig. S3).

Copy numbers of nrDNA and mtDNA

The ranges of nrDNA and mtDNA copy numbers are shown in Fig. S2c and S2d. The copy numbers of mtDNA were significantly explained by AMF isolate ($df = 3$, $F = 32.70$, $P < 0.001$), nrDNA copy numbers ($df = 1$, $F = 46.08$, $P < 0.001$) and the interaction of both predictors ($df = 3$, $F = 7.50$, $P = 0.002$). The copy numbers also were associated significantly within all four isolates (Fig. 2), but, in accordance with the significant interaction of AMF

isolate and nrDNA, the slopes and intercepts of the relationships significantly differed among the AMF isolates, with the highest slope encountered for *G. margarita* and the lowest for *R. irregularis* (Table 1). The intercepts at mean nrDNA copy numbers of each isolate were higher for *G. margarita* and *F. mosseae* than for *C. claroideum* and *R. irregularis*. Resulting from these relationships, copy numbers of mtDNA were always several-fold higher than copy numbers of nrDNA. The mean ratio of mtDNA to nrDNA copy numbers (mt/nr) differed significantly among isolates, with the highest mean value recorded for *G. margarita* and the lowest for *R. irregularis* (Fig. S2e).

Relationship between copy numbers, microscopy and relative PLFA contents

Copy numbers of mtDNA or nrDNA were predicted by AMF isolate and M%, but the interaction of these predictors was not significant (Table 2). The variance explained by the two factors differed, however, between the two markers (Table 3): For nrDNA, variance partitioning revealed a pure significant effect of M% only, explaining 44% of total variance, while the pure effect of AMF isolate was not significant. For mtDNA, both AMF isolate and M% had pure significant effects, but AMF isolate explained a considerably higher proportion of total variance (62%) than M% (14%). The same pattern was found for the relationship of each of the two genetic parameters with relative PLFA contents (Table 2, Table 3). In testing the relationship of nrDNA or mtDNA copy numbers with a%, neither of the two parameters was predicted by a% while the effect of AMF isolate was significant for mtDNA copy numbers and merely close to significance for nrDNA copy numbers (Table 2). Variance partitioning revealed no significant pure effect of either a% or AMF isolate (Table 3). Within isolates, linear relationships between M%, PLFA, a% or V% on one hand, and nrDNA or mtDNA copy numbers on the other hand, were mostly non-significant, (see Table S4 and Fig. S3).

The mt/nr ratio was significantly affected by AMF isolate and the interaction of AMF isolate and M%, and the same result was obtained for the relationship of mt/nr with relative PLFA content (Table 2). With a% as continuous predictor, mt/nr was affected by AMF isolate only. Variance partitioning revealed pure significant effects of AMF isolate in all three relationships, explaining 50%, 68% and 71% of total variance (in the models with M%, relative PLFA content and a%, respectively) while the pure effect of the continuous parameter was either not significant or explained a low amount of variance only (M%, see Table 3). Relationships of the parameters within isolate were mostly non-significant (Table S4, Fig. S3).

Discussion

This study revealed a close linear relationship between mtDNA and nrDNA copy numbers in the intraradical structures of four isolates belonging to four AMF species. The linear regression lines, however, differed significantly among the AMF isolates in both intercept and slope, pointing at isolate-specific factors influencing the relationship between copy

numbers. Different linear relationships between the numbers of nrDNA and mtDNA copies were reported previously for two *Rhizophagus* sp. isolates (Krak et al. 2012). The new primers targeted to the mtDNA LSU of other AMF species enabled us to expand those previous findings to other taxonomic lineages including Gigasporaceae, a family with a distinct root colonization pattern (Klironomos and Hart 2002). Nevertheless, our findings suggest that linear relationships between mtDNA and nrDNA copy numbers might be found for AMF isolates in general.

Krak et al. (2012) proposed that different mtDNA to nrDNA ratios among two isolates may result from inter-isolate variability in nrDNA copy numbers per nucleus (Corradi et al. 2007). This assumption would explain the different slopes of the relationships, but is not consistent with our finding that variance in nrDNA copy numbers was significantly explained by M% or relative PLFA content (both proxies of fungal biomass), while AMF isolate had no pure effect. In contrast, AMF isolate explained a high proportion of variance in mtDNA copy numbers, suggesting that isolate-specific relationships between nrDNA and mtDNA copy numbers should be attributed to isolate-specific levels of mtDNA relative to fungal biomass.

Mitochondrial rDNA is present in one copy only per mitochondrial genome in AMF (Lee and Young 2009; Pelin et al. 2012; Beaudet et al. 2013; Nadimi et al. 2016), while the number of mitochondrial genomes per mitochondrion varies in relation to cell cycle in fungi (Kuroiwa et al. 1994; Sasaki et al. 1994; Hausner 2003). Inherent differences in average number of mtDNA copies per mitochondrion among AMF species could explain the different slopes we found for different isolates, independently of species-specific energy metabolism regulation at the cytological level. Limited knowledge of mitochondrial structure and dynamics in filamentous fungi, however, does not enable any conclusion in this regard.

On the other hand, evidence is available for mitochondria proliferating in growing hyphae (Tamasloukht et al. 2003; Levina and Lew 2006). A high abundance of mitochondria can be therefore assumed in young than in old stages of AMF root colonization, where fungal vitality declines (Isayenkov et al. 2004). AMF species differ in the dynamics of the root colonization process independently of inoculum doses (Wilson 1984; Thonar 2009; Thonar et al. 2014) and therefore it is possible that our sampling encountered the isolates in different physiological states. This assumption is supported by inter-isolate differences in arbuscule abundance, which is a good marker of fungus vitality (Smith and Dickson 1991; Isayenkov et al. 2004). The highest abundance of arbuscules was found in *G. margarita*, which is a slow root colonizer (Boddington and Dodd 1998; Thonar 2009), the lowest in *C. claroideum* and *R. irregularis*, which were previously shown to rapidly decline in the vitality of intraradical structures (Thonar 2009; Janoušková et al. 2013). This pattern is consistent with the encountered differences in the relationships of mtDNA and nrDNA copies among isolates (slopes, intercepts at mean nrDNA copy numbers, mt/nr ratios). The values of mt/nr were surprisingly high in *G. margarita* (ranging from 10 to 29) and *F. mosseae* (14 to 21) as compared to values reported by Krak et al. (2012), which ranged between 0.4 and 4 in two isolates of *R. irregularis*. In their study, the ratios were about three times higher in 3-week-

old mycorrhizas than in 6-week old mycorrhizas, which is consistent with the assumption that this parameter may be related to fungal vitality.

The patterns indicating a relationship between mtDNA copy numbers and arbuscule abundance at the inter-isolate level, however, were not confirmed within individual isolates: the mt/nr copy number ratio was influenced by isolate only and not by a% in the linear regression model. We argue that this is because of relatively little differentiation of a% within each isolate and is consistent with the assumption of homogenous fungus vitality within isolate independent of the amount of initial inoculum. Further studies using material of different ages, therefore are needed to elucidate to what extent mtDNA copy numbers (or their ratios to nrDNA copy numbers) reflect fungal vitality. It should be noted in this context that nrDNA copy numbers relative to fungal biomass also have been reported to decrease during cultivation, which was interpreted as reflecting decreasing fungus vitality (Jansa et al. 2008, Thonar et al. 2009, Janoušková et al. 2013). This is consistent with reports that germ tube growth of AMF is accompanied by replication of nuclear DNA (Bécard and Pfeffer 1993; Bianciotto et al. 1995).

In contrast to some previous studies (e.g. Alkan et al. 2004; Isayenkov et al. 2004; Wagg et al. 2011), significant linear relationships between copy numbers on one hand and fungal biomass estimates (M%, PLFA) on the other hand were found inconsistently and with probabilities ranging from 0.03 to 0.05. This primarily should be attributed to the low number of observations in our study and probably also to the variable range of root colonization levels achieved with the different fungus species. Therefore, we only can state that within species, the two genetic measures were more closely related to one another than either of them was related to the fungal biomass estimates. Notably, vesicle abundance V%, which may be a better proxy of fungal nuclear content than M% (Gamper et al. 2008), was not correlated with copy numbers for the vesicle-forming species *R. irregularis* and *C. claroideum*.

Badri et al. (2016) developed a highly reliable toolkit for the quantification of *R. irregularis* spores by mtDNA-based qPCR, and previous studies suggest that this may be possible also with nrDNA (Filion et al. 2003; Gamper et al. 2008) despite that *R. irregularis* spores vary in nuclear content by up to one order of magnitude (Marleau et al. 2011). Intraradical structures, however, were reported to be well correlated (Alkan et al. 2004; Isayenkov et al. 2004; Wagg et al. 2011; Thonar et al. 2014) or not correlated at all (Pivato et al. 2007; Jansa et al. 2008) with the results of microscopy. In addition to the previously discussed interference of differences in fungus vitality, the detailed study of Gamper et al. (2008) suggested patchy root colonization as a probable cause for imprecision in genetic quantification of AMF in roots and consequently, for mismatches between microscopically and genetically determined AMF abundance in roots. Based on the sample sizes used in our study, however, imprecision was most likely for microscopy, which was based on ca. 30 cm of roots, while qPCR was based on ca. 130 cm. Determining the (relative) precision of the two methods, however, would require many samples and rigorous testing of replicate samples from one root system. Such testing also should explicitly address potential sources of imprecision in the qPCR approach, such as the assumption that non-target DNA in samples

is constant. The DNA content of roots may be influenced by many factors such as endoreduplication of plant nuclei in colonized roots (Bainard et al. 2011) or the density of rhizoplane bacteria. In our study, DNA yields were not affected by AMF species or root colonization (data not shown), so that factor did not introduce a systemic error into our data, but we cannot exclude that variability in the content of non-target DNA among samples increases the imprecision of measurements.

In conclusion, our results provide important methodologic information for the assessment of the suitability of nrDNA and mtDNA copy numbers for the quantification of AMF in roots. We show that within AMF isolates, both markers generate well-correlated abundance data, which suggests they can be used alternatively for the detection of particular isolates. The high inter-isolate differences in mtDNA copy numbers (and consequently, loose relationship of this parameter with intraradical biomass estimates across multiple fungus species), however, questions the suitability of this parameter for the quantification of AMF taxa higher than isolates, or even for whole AMF communities with general primers. It has been suggested that qPCR could become a useful tool for routine screening of root colonization e.g. in nurseries or plant-breeding programs. In such applications, however, at least a rough correlation of the qPCR-based estimates with microscopic determination of AMF colonization in roots is desirable, and according to our results, is likely to be achieved only with nrDNA-based markers if roots are colonized by multiple AMF taxa. Our results therefore show that markers based in nrDNA, despite disadvantages related to the sequence polymorphism of this region and to the low ability to discriminate among closely related species or intra-specific isolates of AMF, are more suitable than mtDNA-based markers for the development of tools for routine screening of AMF in roots.

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Table 1 Linear regressions of copy numbers of nuclear ribosomal DNA (nrDNA CN) and mitochondrial ribosomal DNA (mtDNA CN) in each arbuscular mycorrhizal fungal isolate: *C. claroideum* BEG96 (CC), *F. mosseae* BEG95 (FM), *G. margarita* BEG34 (GI), *R. irregularis* PH5 (RI). Intercept-centered was calculated based on centered nrDNA CN and thus reports mtDNA copies at mean nrDNA CN. Different letters indicate significant differences among AMF species in slope or intercepts ($P < 0.05$)

	Adj. R^2	*P* value	slope	intercept	intercept-centered
CC	0.993	< 0.001	10.732 b	-5448 b	224242 b
FM	0.858	0.008	20.879 ab	-94487 c	704755 a
GM	0.672	0.024	42.762 a	-976735 c	884361 a
RI	0.759	0.011	3.491 c	27295 a	184709 b

Table 2 Linear regression models of copy numbers of nuclear ribosomal DNA (nrDNA CN), mitochondrial ribosomal DNA (mtDNA CN) and their ratio (mt/nr) with arbuscular mycorrhizal fungal isolate (AMF) as a categorical predictor and root colonization intensity (M%), relative content of AMF-specific phospholipid fatty acids (PLFA) or arbuscule abundance in mycorrhizal parts of root systems (a%) as the continuous predictor. n.d.: the interaction term was not included in the model. Significant effects are highlighted in bold

		nrDNA CN		mtDNA CN		mt/nr	
	df	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value
AMF	3	**5.04**	**0.008**	**10.57**	**< 0.001**	**35.60**	**< 0.001**
M%	1	**21.02**	**0.000**	**6.32**	**0.020**	0.16	0.693
AMF * M%	3	n.d.	n.d.	n.d.	n.d.	**4.04**	**0.023**

		nrDNA CN		mtDNA CN		mt/nr	
	df	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value
AMF	3	**3.98**	**0.021**	**15.17**	**< 0.001**	**87.86**	**< 0.001**
PLFA	1	**11.96**	**0.002**	**13.86**	**0.001**	2.70	0.118
AMF * PLFA	3	n.d.	n.d.	n.d.	n.d.	**18.00**	**< 0.001**

		nrDNA CN		mtDNA CN		mt/nr	
	df	*F* value	P value	*F* value	*P* value	*F* value	*P* value
AMF	3	2.91	0.057	**9.55**	**< 0.001**	**25.49**	**< 0.001**
a%	1	2.81	0.108	3.56	0.072	0.67	0.422
AMF * a%	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table 3 Pure and shared effects of arbuscular mycorrhizal fungal isolate (AMF) and root colonization intensity (M%), relative content of AMF-specific phospholipid fatty acids (PLFA) or arbuscule abundance in mycorrhizal parts of root systems (a%) on copy numbers of nuclear ribosomal DNA (nrDNA CN), mitochondrial ribosomal DNA (mtDNA CN) or the ratio of both parameters (mt/nr) according to variance partitioning. Significant factors are highlighted in bold

	Pure AMF	Pure M%	Shared	Residuals
	(*F* value, *P* value)	(*F* value, *P* value)	AMF + M%	
nrDNA CN	0.095 (2.68, 0.104)	**0.435 (15.46, 0.002)**	-0.013	0.482
mtDNA CN	**0.615 (12.81, 0.001)**	**0.144 (6.22, 0.020)**	-0.202	0.443
mt/nr	**0.496 (56.30, 0.001)**	**0.02 (5.23, 0.043)**	0.408	0.076
	Pure AMF	Pure PLFA	Shared	Residuals
	(*F* value, *P* value)	(*F* value, *P* value)	AMF + PLFA	
nrDNA CN	-0.017 (0.77, 0.467)	**0.281 (8.05, 0.004)**	0.100	0.637
mtDNA CN	**0.644 (14.46, 0.001)**	**0.18 (8.08, 0.006)**	-0.231	0.407
mt/nr	**0.676 (59.08, 0.001)**	-0.003 (0.57, 0.455)	0.227	0.099
	Pure AMF	Pure a%	Shared	Residuals
	(*F* value, *P* value)	(*F* value, *P* value)	AMF + a%	
nrDNA CN	0.022 (1.20, 0.334)	-0.019 (0.67, 0.421)	0.061	0.936
mtDNA CN	0.130 (3.08, 0.072)	0.053 (2.60, 0.125)	0.283	0.533
mt/nr	**0.706 (65.52, 0.001)**	0.003 (1.571, 0.240)	0.198	0.093

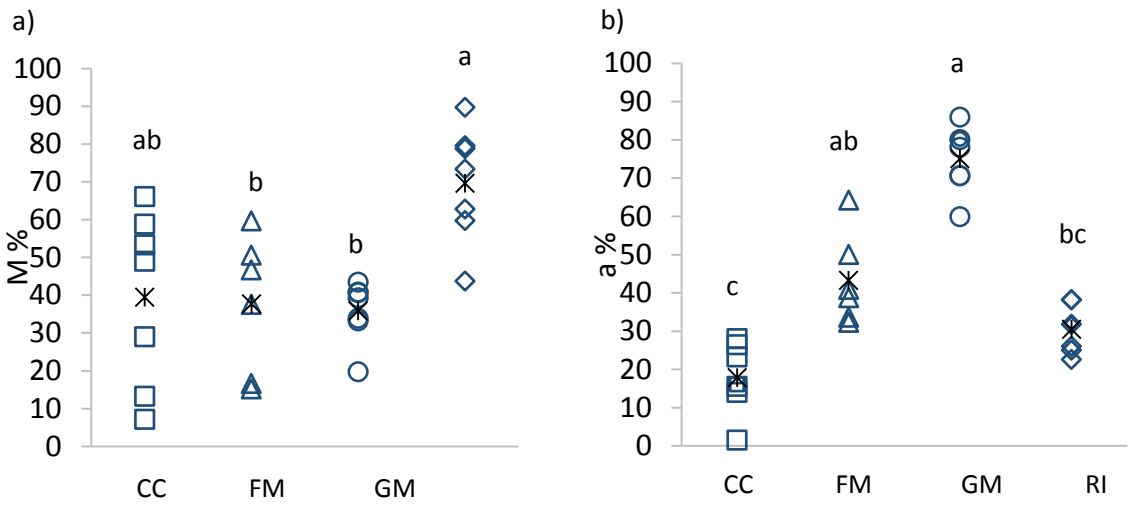

Figure 1 a Range of mycorrhizal colonization intensity in root systems (M%) and **b** arbuscule abundance in mycorrhizal parts of root systems (a%) as determined for each arbuscular mycorrhizal fungal isolate: *C. claroideum* BEG96 (CC, squares), *F. mosseae* BEG95 (FM, triangles), *G. margarita* BEG34 (GI, circles), *R. irregularis* PH5 (RI, diamonds). Each symbol represents one root sample, asterisks show means. Different letters indicate significant differences among AMF species according to non-parametric Kruskal-Wallis tests ($Chi^2 = 8.41$ and 12.93, $P < 0.05$ and 0.01 for M% and a%, respectively)

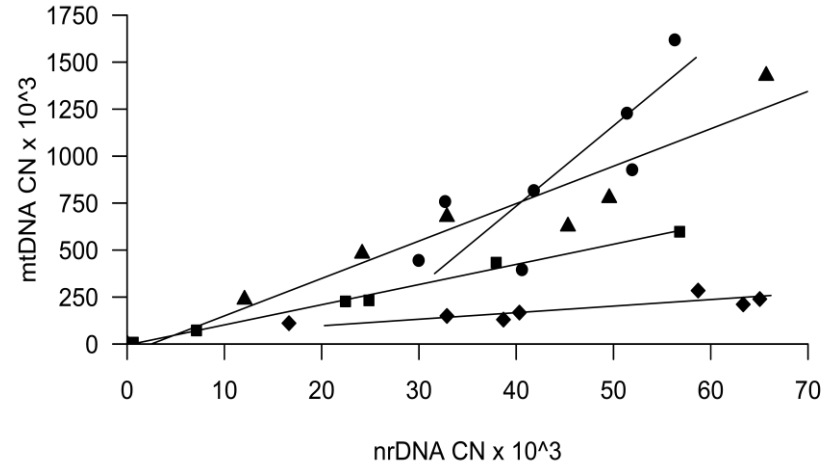

Figure 2 Linear relationships of copy numbers of nuclear ribosomal DNA (nrDNA CN) and mitochondrial ribosomal DNA (mtDNA CN) in four arbuscular mycorrhizal fungal isolates: *C. claroideum* BEG96 (CC, squares), *F. mosseae* BEG95 (FM, triangles), *G. margarita* BEG34 (GI, circles), *R. irregularis* PH5 (RI, diamonds). Each point represents one root sample. Parameters of the regression lines are given in Table 1

3.3. Paper III: Voříšková *et al.* (2019)

Abiotic contexts consistently influence mycorrhiza functioning independently of the composition of synthetic arbuscular mycorrhizal fungal communities

Alena Voříšková[1,2], Jan Jansa[3], David Püschel[1,3], Miroslav Vosátka[1], Petr Šmilauer[4], Martina Janoušková[1]

1) Institute of Botany, The Czech Academy of Sciences, Zámek 1, 252 43 Průhonice, Czech Republic
2) Department of Experimental Plant Biology, Faculty of Science, Charles University, Viničná 5, 128 44 Prague, Czech Republic
3) Institute of Microbiology, The Czech Academy of Sciences, Vídeňská 1083, 142 20 Prague, Czech Republic
4) Department of Ecosystem Biology, Faculty of Science, University of South Bohemia, Branišovská 1760, 370 05 České Budějovice, Czech Republic

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Contribution statement

AV participated in the establishment, carrying out and harvesting of the experiment. AV evaluated root colonization and took part in the molecular work. AV carried out most of the statistical analyses and interpreted the results in consultation with other co-authors. AV wrote the manuscript with a contribution from her supervisor MJ and other co-authors.

Abiotic contexts consistently influence mycorrhiza functioning independently of the composition of synthetic arbuscular mycorrhizal fungal communities

Alena Voříšková[1,3*], Jan Jansa[2], David Püschel[1,2], Miroslav Vosátka[1,3], Petr Šmilauer[4], Martina Janoušková[1]

[1)] Institute of Botany, The Czech Academy of Sciences, Zámek 1, 252 43 Průhonice, Czech Republic
[2)] Institute of Microbiology, The Czech Academy of Sciences, Vídeňská 1083, 142 00 Prague, Czech Republic
[3)] Department of Experimental Plant Biology, Faculty of Science, Charles University, Viničná 5 128 00, Prague, Czech Republic
[4)] Department of Ecosystem Biology, Faculty of Science, University of South Bohemia, Branišovská 1760, 370 05 České Budějovice, Czech Republic

[*]Corresponding author, e-mail: alena.voriskova@ibot.cas.cz, tel.: +420 271 015 332

Abstract

The relationship between mycorrhiza functioning and composition of arbuscular mycorrhizal (AM) fungal communities is an important, but experimentally still rather little explored topic. The main aim of this study was thus to link magnitude of plant benefits from AM symbiosis in different abiotic contexts with quantitative changes in AM fungal community composition. A synthetic AM fungal community inoculated to the model host plant *Medicago truncatula* was exposed to four different abiotic contexts, namely drought, elevated phosphorus availability, and shading, as compared to standard cultivation conditions, for two cultivation cycles. Growth and phosphorus uptake of the host plants was evaluated along with the quantitative composition of the synthetic AM fungal community. Abiotic context consistently influenced mycorrhiza functioning in terms of plant benefits, and the effects were clearly linked to the P requirement of non-inoculated control plants. In contrast, the abiotic context only had a small and transient effect on the quantitative AM fungal community composition. Our findings suggest no relationship between the degree of mutualism in AM symbiosis and the relative abundances of AM fungal species in communities in our simplified model system. The observed progressive dominance of one AM fungal species indicates an important role of different growth rates of AM fungal species for the establishment of AM fungal communities in simplified systems such as agroecosystems.

Keywords

pre-conditioning, mycorrhizal functioning, community, qPCR, phosphorus, *Medicago truncatula*

Introduction

Plants have developed a range of mechanisms to overcome unfavorable environmental conditions, one of the most notable being their relationship with symbiotic microorganisms. Arbuscular mycorrhizal (AM) fungi, obligate plant symbionts from the phylum Glomeromycota (Tedersoo et al. 2018), are a key group of root-associated symbionts. They form symbioses with most terrestrial plant species and provide them with mineral nutrients and non-nutritional benefits (Smith and Read 2008). In return, they receive a proportion of assimilates from plant photosynthesis, which constitutes the cost of the symbiosis to the host plant (Smith and Read 2008). The net benefit of plants from the mycorrhizal association depends on the ratio of the mycorrhizal benefits and costs, and is modulated by abiotic context (Johnson et al. 1997). That is defined by the combination of many abiotic factors, the most important ones being available nutrient levels in soil, intercepted light and water availability.

The abiotic context is therefore an important determinant of the location of a particular AM symbiosis on the continuum from mutualism to parasitism (Johnson et al. 2015). Abiotic stresses to plants that are alleviated by the symbiosis conserve or even reinforce its mutualistic character: Carbon (C) flow from the plant into the fungal partner is counterbalanced by improved ability of C fixation leading to better growth and higher demand for (nutritional) benefits provided by the fungi (Kaschuk et al. 2009). On the other hand, abiotic context can also disturb the fragile balance of element flow between the symbionts needed for a mutualistic functioning of mycorrhiza (Johnson et al. 2015). Most prominent examples of such contexts are high phosphorus availability in soil (Johnson 1993) and shading of the host plant (Zheng et al. 2015), both reducing, directly or indirectly, the need of the plant to rely on mycorrhizal uptake to meet its phosphorus demand as well as the C pool available to the fungal partner, While not necessarily imposing stress on the plant, they represent a stress to the mycorrhizal symbiosis.

Root systems of individual plants are often colonized by many AM fungal species (Fitter et al. 2005; Öpik et al. 2008), which compete for root space and plant-derived reduced C. The AM fungi directly respond to abiotic conditions such as soil temperature (Barrett et al. 2014; Řezáčová et al. 2018) and drought (Augé 2001; Klironomos et al. 2001). However, the effect of abiotic context on AM fungal community composition is also plant-mediated. For example, high P availability alters the plant's allocation of reduced C to members of the AM fungal community (Ji et al. 2016). Shading has been shown to modulate the outcome of competition among AM fungi (Knegt et al. 2016, Zheng et al. 2015). Consequently, the

relative abundances of AM fungal species within communities also can be influenced by the degree of mutualism in the mycorrhizal symbiosis.

The effects of different abiotic contexts on the plant's mycorrhizal benefits and AM fungal community composition have only rarely been addressed within a single experiment. Yet, different environmental stresses induced similar changes in ectomycorrhizal communities that subsequently improved their mutualistic potential (Gehring et al. 2014). Changes of rhizospheric microbial communities induced by drought have been interpreted as adaptive after just three plant generations (Lau and Lennon 2012). In the long-term, abiotic contexts that represent stress to mycorrhizal functioning can select for specific communities of adapted AM fungi that may be better mutualists (or less parasitic) under the given environmental conditions (Doubková et al. 2012; Ji et al. 2013). These results indicate that the functioning of AM symbiosis and composition of the plant-associated AM fungal community may well be interlinked and represent two complementary aspects of the mycorrhizal symbiosis. This linkage can be due to long-term evolutionary mechanisms, but also due to short-term ecological mechanisms (Millar and Bennett 2016).

The main aim of the research presented here therefore was to link mycorrhiza functioning and quantitative changes in the composition of the AM fungal community in different abiotic contexts, either those promoting mutualism or those representing stress to the AM symbiosis. To this end, we designed a two-stage experiment with a synthetic AM fungal community exposed to different abiotic contexts: 1) reference standard cultivation conditions enabling the establishment of a mutualistic symbiosis (Püschel et al. 2017); 2) drought stress, expected to increase plant mycorrhizal benefits (Augé 2001); 3) P surplus and 4) light shortage, both expected to decrease plant benefits from mycorrhiza formation (Johnson et al. 1993, 1997, 2015; Kiers and van der Heijden 2006). We hypothesized that abiotic contexts leading to highly mutualistic mycorrhizal functioning would increase the proportions of the less abundant fungus species within communities as compared to contexts in which mycorrhizas are less beneficial or even suppress plant growth (Knegt et al. 2016). Subsequently, AM fungal communities established within each abiotic context in the first experimental stage were exposed (in a factorial manner) to all the abiotic contexts in a second stage of the experiment. The aim of this second stage was to determine the degree of progressivity and/or reversibility of the context-dependent changes in the composition of the AM fungal communities. We hypothesized that changes to AM fungal communities induced by a specific environmental context would be fully reversible in another context, but progressive under continuing environmental pressure (i.e., under the same conditions).

Material and Methods

Experimental design

The two-stage greenhouse pot experiment was established with *Medicago truncatula* as host plant and a synthetic AM fungal community consisting of five species. In both stages, plants

were cultivated in four abiotic contexts: Standard conditions (full watering, i.e. water saturation fluctuating between 75 and 100%, full light intensity, no additional P), low water (water saturation of the substrate maintained between 40 % and 50 %), low light (35% of full light), and high P (addition of 40 mg P per pot). In the first stage, plants were inoculated with mixed inoculum of the five AM fungal species or left non-inoculated (non-mycorrhizal controls) and cultivated in the four abiotic contexts for 12 weeks. After the harvest of the first stage, the substrates from the different conditions containing AM fungal communities of presumably conditions-specific composition were used as inocula for the second stage of the experiment (AM fungi – standard, AM fungi – high P, AM fungi – low light, AM fungi – low water). Plants inoculated with one of the four inocula or left non-inoculated were subjected to the same set of conditions in a cross-factorial experimental design for 9 weeks (Fig. 1). The experiment was conducted with 6 replicates per treatment, i.e. 48 pots in the first stage and 120 pots in the second stage.

Material

The substrate for the plant cultivation was a mixture of 45 % autoclaved sand, 45 % autoclaved zeolite and 10 % γ-irradiated (>25 kGy) soil from Tänikon, Switzerland (pH 7.52, C [%] 0.186, N [%] 0.012, available (water extractable) P [mg kg^{-1}] 2.06, determined by the malachite green method (Ohno and Zibilske 1991). At the beginning of each experimental stage, the substrate of each pot was once fertilized with nutrient solutions supplying (per pot) 60 mg of K (as K_2SO_4), 30 mg of Mg (as $MgSO_4.7H_2O$) and 30 mg of Ca (as $CaCl_2.2H_2O$). Each pot of the 'high P' conditions received once 40 mg of P (as $Na_2HPO_4.12H_2O$) at the beginning of the respective experimental stage before planting the pots. The nutrient solutions were applied in amounts of 10 ml per pot and thoroughly mixed into the upper two thirds of the substrate.

The synthetic AM fungal community of five species was prepared so as to contain representatives of different taxonomic lineages of AM fungi (five genera in four Glomeromycotan families), which also represent different functional lineages (Hart and Reader 2002; Powell et al. 2009): *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) Walker & Schüßler isolate PH5, *Claroideoglomus claroideum* (Schenck & Sm.) Walker & Schüßler isolate BEG23, *Acaulospora tuberculata* Janos & Trappe isolate BEG41, *Gigaspora margarita* Becker & Hall isolate BEG34, and *Funneliformis mosseae* (Nicolson & Gerd.) Walker & Schüßler isolate BEG95. The *R. irregularis* isolate was originally obtained from a heavy metal contaminated grassland site in the Czech Republic (Rydlová and Vosátka 2003), the other isolates are registered in The International Bank of the Glomeromycota (https://www.i-beg.eu/). All the isolates had been maintained in inert sand-zeolite mixture and identical greenhouse conditions with regular passaging for at least 10 years, and were selected for the experiment based on their high compatibility with these cultivation conditions. Based on previous experience with synthetic AM fungal communities, we expected one of the three Glomerales isolates to dominate the community (Janoušková et

al. 2013; Jansa et al. 2008; Thonar et al. 2014), and the degree of dominance and the abundance of the other isolates to differ according to the abiotic conditions.

Inoculum of each AM fungal isolate was produced in multi-spore pot cultures with *Allium porrum* as a host plant. The multi-spore pot cultures were established simultaneously and harvested after seven months of growth. Air-dried substrate from the cultures containing extraradical mycelium, spores and 1 cm-long root fragments was used for the inoculation. 40 g of dried inocula of each AM fungal isolate were mixed to achieve 200 g of mixed inoculum in total, which was then applied to each pot. The 'blank' inoculum for the non-inoculated controls was prepared from non-inoculated pot cultures planted with *Allium porrum* and cultivated as above and then applied to the experimental pots in the same way as the AM fungal inoculum.

Surface-sterilized (10 min in 0.5 % solution of NaClO) seeds of *M. truncatula* J5 were pre-germinated on moist filter paper in sterilized Petri dishes at room temperature for three days. The germinated seedling were planted into multipots, each with the volume of 100 ml, filled with the sterilized substrate detailed above, and cultivated in a growth chamber (14 - hour photoperiod) for four weeks. All seedlings were inoculated with 1 ml suspension (optical density adjusted to 0.8 at 600 nm) of *Sinorhizobium* strain LT10, which had been prepared as described in Konvalinková et al. (2015).

The first stage of the experiment

Plastic 2 L pots (11 × 11 cm, height 20 cm) were first filled with 600 ml of the sterilized substrate. Subsequently, 800 ml of the sterilized substrate, 200 g of inoculum or 'blank' inoculum and fertilizing solutions containing the nutrients as specified above were homogenized and filled on top of the bottom layer. *M. truncatula* seedlings were transplanted from the multipots into the experimental pots (one plant per pot) and the pots were filled with a further 250 ml of the sterilized substrate. All plants were watered daily with the same volume of distilled water to full substrate saturation and cultivated with supplemental light (400 W metal halide lamps providing a minimum photosynthetic flux density of 85 $\mu mol \cdot m^{-2} \cdot s^{-1}$ during a 14-hour photoperiod). Two weeks after the establishment of the experiment, the low water and low light conditions were induced in the corresponding treatments. Plants of the low light conditions were shaded by a raschel shelter (knitted mesh) which reduced the incoming light intensity to 35 % (Konvalinková et al. 2015). All pots of the low water treatment were weighed and watered twice a day to maintain the substrate saturation between 40 and 50 % of the full water-holding capacity. Pots of other treatments were watered daily to full water-holding capacity of the substrate and occasionally controlled by weighting to assure that water availability did not drop below 75% of the full water-holding capacity. The experiment was harvested after 12 weeks of cultivation in total, before the onset of flowering. Plant shoots were removed and the root systems with substrate were lifted from the pots. The substrates were shaken off the roots, homogenized within treatments (including remaining root fragments) and air-dried. Shoots were dried at 65 °C for three days to constant weight,

and then weighed to obtain the shoot dry weight (SDW). Root systems were washed, dried with paper towels and weighed. Samples for molecular analyses and determination of root colonization were randomly collected from the upper two thirds of the root systems after cutting the roots to 2 cm fragments and homogenizing. Aliquots of 100 mg fresh weight of roots for molecular analyses were frozen in liquid nitrogen and stored at – 80 °C. Samples of about 1 g fresh weight of roots were placed into 10 % KOH and stained with 0.05 % Trypan Blue in lactoglycerol (Koske and Gemma 1989) for the microscopic estimation of AM fungal root colonization. Hyphal, arbuscular and vesicular root colonization were estimated using the magnified intersection method (McGonigle et al. 1990), scoring 100 intersections per sample within 30 root segments, each about 2 cm long, at 100× magnification (Olympus BX60). The remaining roots were weighed, dried at 65 °C for three days and weighed again. The root dry weight (RDW) of the whole root system was then calculated.

The second stage of the experiment

M. truncatula seedlings and pots were prepared as described above for the first stage of the experiment. Plants were inoculated with four conditions-specific inocula (200 g of inoculum per pot), i.e. with the homogenized substrates from the four abiotic conditions of the first stage. Substrate of the non-inoculated controls from the standard conditions was used as a 'blank' inoculum for the non-inoculated controls of the second stage (200 g per pot). Additionally, a second non-inoculated control with inoculum from non-inoculated cultures of *A. porrum* (200 g per pot) was established to control for the effects of possible accumulation of plant pathogens in the system during the first experimental stage with *M. truncatula* as host plant. As none of the measured plant parameters significantly differed between the two non-inoculated controls, only data from the first control are included in the subsequent analyses. Subsequently, plants of each inoculation treatment and the non-inoculated controls were cultivated under the same four abiotic conditions as in the first stage (Fig. 1). Plants were harvested three weeks earlier (i.e., after 9 weeks of cultivation) in the second stage as compared to the first stage due to earlier onset of flowering, in order to have both experimental stages harvested in comparable ontogenetic stages. The harvest and sample handling proceeded in the same way as in the first experimental stage.

Quantification of the AM fungal community composition in roots

Frozen root samples were ground in liquid nitrogen and DNA was extracted using a DNA Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and eluted into 80 µl of 10mM Tris buffer (pH 8.0). DNA concentration was quantified and the quality of the extracts assessed using a Nanodrop spectrophotometer (ThermoFisher, Scientific, Waltham, MA, USA). The DNA extracts were stored at – 80 °C. We quantified the abundance of each AM fungal species using quantitative PCR (qPCR). *Taq*Man-based qPCR assays with PCR amplicons as standards were used for the quantification of copy

numbers of the large subunit (LSU) of nuclear ribosomal DNA (nrDNA) as described in Voříšková et al. (2017) and detailed in Online Resource 1. Nuclear ribosomal DNA of each of the five species was quantified using *Taq*Man-based qPCR with hydrolysis probes labelled with fluorescent reporter dye 6-carboxyfluorescein (6-FAM) at the 5′ end and the Black Hole Quencher (BHQ1) at the 3' end. The qPCR was performed in 10 µl reaction volume with the following reagents and qPCR conditions: 1 x LightCycler 480 Probes Master (Roche, Penzberg, Germany), taxon-specific primers (for primers and concentrations see Online Resource 1: Table S1), 50 nM of TaqMan taxon-specific probes and 2.5 µl of 1:9 diluted DNA extract or the corresponding dilution of a standard. The thermal cycling program was the following: pre-incubation at 95 °C for 10 min, 40 cycles of 95 °C for 10 s, annealing for 30 s with primer–specific temperature (see Online Resource 1: Table S1) and elongation at 72 °C for 1 s. DNA concentrations of the amplified target region in the DNA samples were calculated based on calibration curves derived from the serially diluted standards in the LightCycler 480 software version 1.5 (Roche). The five-fold dilution series of the standards were amplified in technical triplicates, samples in technical duplicates. DNA concentrations of each species were calculated to copy numbers (CN) of the nrDNA LSU region according to Krak et al. (2012): $CN[\mathrm{ng}^{-1}\mathrm{DNA}] = (6.022 * 10^{23})/(L * 10^{9} * 660)$, where L is the length in bp of the amplified fragment.

Analyses of P and N in shoot biomass

Shoot biomass samples were ground to powder using a ball mill (MM200, Retsch, Haan, Germany). Milled samples were used to determine P and N concentrations in shoots as described in Püschel et al. (2017). The P concentration was measured by colorimetry at 610 nm using a Pharmacia LKB Ultrospec III spectrophotometer by the malachite green method (Ohno and Zibilske 1991). The N concentration was measured using a Flash EA 2000 elemental analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Total amount of P [mg] in shoots (i.e., the P content) was calculated by multiplying the P concentration by SDW.

Statistical analyses

The data on SDW, RDW, P content, P concentration and N:P ratio from both stages of the experiment met the assumptions of homogeneity of variances and normality, and were therefore analyzed by two-way ANOVAs. For the parameters from the first stage experiment, the effects of the factors abiotic conditions (4 levels: standard, high P, low light, low water), inoculation (2 levels: inoculated or non-inoculated) and their interaction were determined. In the second stage, the effect of inoculum history (5 levels: non-inoculated + 4 inocula with different history) was determined separately within each abiotic treatment, and significant differences among the different inocula obtained by Tukey's test at $p < 0.05$ are summarized in Online Resource 2: Table S1. Subsequently, the treatments receiving inocula of different histories were merged into one inoculated treatment per abiotic conditions, and the effects of abiotic conditions, inoculation (inoculated or non-inoculated) and their interaction were

determined as for the first stage. In both stages, growth of non-inoculated and inoculated plants in low water, high P and low-light conditions was compared to the standard conditions using planned contrasts in order to determine the general effect of the "stress" conditions on plant growth.

The parameters mycorrhizal growth response (MGR) and mycorrhizal response in P uptake (MPR) were calculated as $\log(\frac{M}{NM})$, where M is the value (shoot biomass or P content) of an inoculated plant and NM is the mean value of the non-inoculated treatment of the corresponding abiotic conditions. These parameters provided a standardized estimate of mycorrhizal benefits and enabled us to combine MGR and MPR data from both stages to test the effect of inoculum history (5 levels: one level – *none* for the first stage and the 4 history types for the second stage), abiotic conditions (4 levels: standard, high P, low light, low water) and the interaction of both factors on the mycorrhizal responses using two-way ANOVAs. Significant differences among abiotic conditions were identified by Tukey's test at $p < 0.05$. Subsequently, we used data only from the second stage to test the effect of pre-conditioning (inoculum from the same abiotic conditions vs. inocula from different conditions) together with the effect of abiotic conditions and their interaction on mycorrhizal responses using two-way ANOVAs.

Root colonization by hyphae, arbuscules and vesicles were arcsine transformed before statistical analyses. We tested the effect of inoculum history, abiotic conditions and the interaction of both factors on root colonization using two-way ANOVAs as described for the mycorrhizal responses. Due to an overall significant effect of inoculum history on root colonization by arbuscules and vesicles, we subsequently tested the differences between the first and second stage of the experiment using planned contrast between the *none* inoculum history and the average of the other history types within these two parameters. The effect of inoculum pre-conditioning on root colonization in the second stage also was tested in the same way as for the mycorrhizal responses.

The effects of the predictors on AM fungal community composition in each experimental stage (abiotic conditions in the first stage; abiotic conditions and inoculum history in the second stage) were tested using a multivariate GLM model with negative binomial error structures in the *mvabund* package (Wang et al 2012) in R. Subsequently, we used univariate tests to further examine the responses of each species to the abiotic conditions.

In a next step, we examined the effect of predictors on each AMF species separately in order to compare their abundances among the experimental treatments. Copy numbers of nrDNA of *F. mosseae*, *C. claroideum* and *Gi. margarita* from the first stage were square-root transformed and data of *R. irregularis* from the second stage were reciprocal square-root transformed. The effects of abiotic conditions on the abundances of each AM fungal species in the first stage were determined using one-way ANOVAs. The effects of the factors abiotic conditions, inoculum history and their interaction on the abundance of *R. irregularis* in the second stage were determined using a two-way ANOVA. The tested full model was reduced

to a model with abiotic conditions as the only factor based on AIC (Akaike Information Criterion) and non-significant effects of the factor inoculum history and the interaction.

The zero-inflated negative binomial (ZINB) model (the 'pscl' package in R, Zeileis et al. 2008, Jackman 2017) was applied to determine the effect of abiotic conditions and inoculum history on the copy numbers of *F. mosseae*, *C. claroideum*, *A. tuberculata* and *Gi. margarita* in the second stage, which were dominated by zeroes (absences in samples). The ZINB model is a two-part model with the count part explaining the non-zero counts and the zero-inflation part explaining the frequency of zeroes. The effect of each factor was tested in both parts of the model. Multiple comparisons among the levels of a significant factor were performed as pairwise comparisons between all the levels followed by an adjustment of the obtained *p* values using Holm's correction.

All statistical analyses were performed in R (version 3.3.2, R Core Team, 2016).

Results

Shoot biomass and P content in the first stage

Shoot biomass was significantly affected by the abiotic conditions, but not by inoculation with AM fungi. However, a highly significant interaction of both predictors (Table 1) showed that the effect of abiotic conditions depended on inoculation. Non-inoculated plants produced significantly more shoot biomass than inoculated plants under the high P conditions and less under the low water and low light conditions (Fig. 2a) as compared to the standard conditions. Shoot biomass of inoculated plants significantly differed from the standard conditions at low water only. The responses of root biomass to the experimental factors followed a similar pattern as described for shoot biomass (Online Resource 2: Fig. S1).

The P content in shoots was significantly affected by abiotic conditions, inoculation and their interaction (Table 1). In both the non-inoculated and inoculated plants, it was significantly lower under the low water conditions and higher at high P and at low light as compared to the standard conditions (Fig. 2). Inoculated plants had higher P content than non-inoculated plants in all the abiotic conditions, but the difference depended upon the abiotic conditions (see Fig. 2). The effects of abiotic conditions and inoculation on the P concentration and N:P ratio in shoot biomass are shown in Online Resource 2: Figs. S2 and S3, respectively. Most importantly, the low water conditions had contrasting effects on both parameters in the non-inoculated and inoculated plants: P concentration was decreased in the former and increased in the latter as compared to the standard conditions. The effect on N:P ratio was exactly opposite, i.e. it was increased by low water in the non-inoculated plants and decreased in the inoculated plants.

Shoot biomass and P content in the second stage

Shoot biomass was unaffected by inoculum history in the second stage of the experiment (see Online Resource 2: Table S1). All the inoculated treatments were therefore merged and

compared to the non-inoculated control. Then, the abiotic conditions and the interaction between abiotic conditions and inoculation significantly influenced shoot biomass, while inoculation itself had no significant effect, similarly as in the first stage (Table 1). The shoot biomass of non-inoculated plants was affected by abiotic context similarly as in the first stage (Fig. 2a), while the response of inoculated plants differed from the first stage: they had higher shoot biomass at high P and lower shoot biomass at low light and low water, as compared to the standard conditions (Fig. 2b).

The P content of the plant shoots was significantly affected by inoculum history ($F_{3,20} = 4.90$, $p = 0.010$) in the low water conditions only, where it was significantly higher with inoculum originating from the standard conditions than with inocula from the low light and low water conditions (see Online Resource 2: Table S1). When the treatments receiving inocula of different origin were merged into one inoculation treatment, abiotic conditions, inoculation and the interaction of these factors significantly affected the P content in shoots (Table 1). The effects were similar to those in the first stage (Fig. 2b) with the exception of significantly lower P content at low light than in the standard conditions in both inoculated and non-inoculated plants (Fig. 2).

The responses of root biomass, P concentration and N:P ratio to the experimental factors also displayed similar patterns to those in the first stage (Online Resource 2: Table S1, Fig. S1, Fig S2 and Fig. S3).

Mycorrhizal responses and the effect of inocula pre-conditioning

Analysis of merged data from both stages of the experiment revealed a significant effect of abiotic context on MGR, while inoculum history (encoded for the first stage as a separate level *none*) had no effect on MGR (Table 2). The MGR was consistently highly positive under the low water conditions, neutral to slightly positive at low light, slightly negative to neutral under the standard conditions and consistently negative at high P (Fig. 3a). Though the interaction of both factors also affected MGR, there was no significant effect of inoculum pre-conditioning to the same abiotic conditions (Table 3).

MPR was significantly influenced by abiotic conditions and inoculum history (Table 2). Multiple comparisons of the different levels of the latter factor, however, did not reveal any significant differences among the inocula of different history. Establishment of mycorrhizal symbiosis most efficiently promoted P uptake of the plants under low water conditions (Fig. 3b), where the P content of inoculated plants was approximately twelve to fifteen times higher than that of non-inoculated plants (Fig. 2). In the other abiotic conditions, MPR was considerably lower, but still consistently positive (Fig. 3b). As for MGR, the interaction of both factors also affected MPR (Table 2), but there was no significant effect of inoculum pre-conditioning to the same abiotic conditions (Table 3).

Root colonization

Roots of inoculated plants were intensively colonized by AM fungi in all conditions of both experimental stages, with hyphal colonization ranging between 87.8 % and 99.7 % (Table 4). No AM fungal structures were found in the roots of the non-inoculated plants. All three root colonization parameters were affected by abiotic conditions, while inoculum history significantly affected root colonization by arbuscules and vesicles, but not by hyphae (Table 2). Root colonization by hyphae and arbuscules was significantly decreased by low light and low water as compared to the standard conditions and high P. Vesicular colonization was significantly lower at low light than in all the other abiotic conditions. Overall differences between the first and the second stage of the experiment were tested for arbuscular and vesicular colonization, and both parameters were significantly higher in the second stage than in the first stage (t_{70} = -8.54, $p < 0.001$ and t_{70} = -3.28, $p = 0.001$, respectively) (Table 4). Interaction of both factors (conditions and inoculum history) also affected root colonization by hyphae, arbuscules and vesicles (Table 2), however, inoculum pre-conditioning in the same conditions had no significant effect on any of the root colonization parameters in the second stage of the experiment (Table 3).

AM fungal communities

The AM fungal communities were progressively dominated by *R. irregularis*. Across all cultivation conditions, 92 % of the AM fungal gene copy numbers belonged to this species in the first experimental stage and 99 % in the second stage (see Fig. 4a and Fig. 4b for an overview of the communities in the different conditions).

In the first stage, the composition of AM fungal communities significantly differed in different abiotic conditions (likelihood-ratio test (LRT) = 37.4, $p = 0.019$) using the multivariate GLM model. However, the univariate test did not show significant response of any AM fungal species to abiotic conditions. When the effect of abiotic conditions was evaluated for each AMF species separately, the abundance of *R. irregularis* did not significantly differ among the abiotic conditions (Online Resource 2: Table S2), but differences were found for the less abundant AM fungal species (Online Resource 2: Table S3). The abundances of *F. mosseae* and *A. tuberculata* were significantly higher at low light as compared to the other cultivation conditions, while the abundance of *Gi. margarita* was significantly higher at high P than in the standard conditions and at low light (Online Resource 2: Fig. S4).

In the second stage, the composition of the AM fungal communities significantly differed among the different abiotic conditions (LRT = 51.1, $p = 0.001$), but not among the inocula of different history, and the interaction of both factors was also non-significant according to the multivariate GLM model. The univariate test revealed significant effects of abiotic conditions on the abundance of *R. irregularis* (LRT = 19.4, $p = 0.001$), *F. mosseae* (LRT = 7.2, $p = 0.020$) and *A. tuberculata* (LRT = 19.8, $p = 0.001$). When the effect of abiotic conditions and inoculum history was evaluated for each AMF species separately, the

inoculum history did not influence the abundance of any them (Online Resource 2: Table S2, Table S4). Abiotic conditions affected the abundance of *R. irregularis* ($F_{3,92}$ = 6.84, p < 0.001, for the full model see Online Resource 2: Table S2), which was significantly less abundant in the standard condition as compared to the conditions with high P and low light (Online Resource 2: Table S2). The remaining AM fungi constituted only 1 % of the AM fungal communities (Fig. 4b). *Gi. margarita* was detected in two samples only and *A. tuberculata* was found almost exclusively in the low light conditions (Online Resource 2: Fig. S5b). The abundances of *C. claroideum* and *F. mosseae* were significantly affected by cultivation conditions in the binomial portion of the zero – inflated model in that their frequency was highest in the low light conditions and lowest in the high P conditions (for details see Online Resource 2: Table S4, Fig. S5a and S5c).

Discussion

Our study demonstrates a consistent effect of abiotic context on the mycorrhizal benefits to the plants. In contrast, AM fungal community composition was affected rather inconsistently by the abiotic context, and changes in species abundances found in the first stage had no effect on the second-stage plant benefits. Thus, the changes in the community composition could not be related to the functioning of the AM symbiosis along the continuum between mutualism and parasitism.

Despite improved P uptake in all the tested abiotic contexts by mycorrhiza, the symbiosis was, with respect to promoting plant biomass production, highly mutualistic in the low water conditions, around neutral in standard and low light conditions and (apparently) parasitic at high P. Improvement of plant growth by mycorrhiza under water deficient conditions has been well documented previously (e.g. Cho et al. 2006; Allen 2007, 2011), but the contributing mechanisms still are not well understood (Augé 2001; Ruiz-Lozano 2003). In our experiment, the highly positive mycorrhizal growth response for the low water conditions can clearly be linked to improved P uptake. The non-inoculated plants were less satisfied in their P demand at low water than in the standard cultivation conditions, as evident in the N:P ratio of their shoot biomass, and this P deficiency was effectively alleviated by the mycorrhizal symbiosis. AM fungi can directly improve P uptake of plants due to penetration of hyphae into pores of small size or indirectly contribute to water redistribution in the substrate (Augé 2001). Even if P is present in larger soil pores, soil water deficiency causes P, normally dissolved in soil solution prior to plant uptake (Jansa et al. 2011), to not be accessible to the roots in dry soils. Thus, our results are in agreement with previously demonstrated dependency of plant P nutrition on the mycorrhizal symbiosis in water-limited conditions (Allen 2011).

The lower degree of mutualism of mycorrhizal symbiosis encountered in the other abiotic contexts corresponds to limitation by other resources than P. The N:P ratio of shoot biomass of non-inoculated plants cultivated under standard and high P conditions indicates N limitation (Koerselman and Meuleman 1996), especially in the second experimental stage,

most probably due to N depletion in the substrates. In N-limited conditions, mycorrhiza usually does not improve plant growth due to the high N demand of AM fungi, and the improved P uptake of inoculated plants cannot be translated into more biomass (Johnson et al. 2015, Püschel et al. 2016). The N demand of *M. truncatula* is partly covered by biological nitrogen fixation (BNF) carried out by symbiotic rhizobia. This was accounted for at the establishment of the experiment by rhizobial inoculation and no additional N fertilization in order to support the establishment of the rhizobial symbiosis (Püschel et al. 2017). At harvest, we observed formation of nodules by all plants, but evidently BNF was insufficient to cover the plants' requirement for N in most treatments including the standard conditions. Interestingly, however, mycorrhiza also improved plant growth at low light in the first experimental stage, which is counter-intuitive as C limitation is assumed to decrease the degree of mutualism in mycorrhiza (Konvalinková and Jansa 2016). The relatively high N:P ratio in the shoots of the non-inoculated plants indicates comparable need for P as in the low water treatment, which was likely stronger than the C limitation due to shading. The improved P uptake of inoculated plants can also increase C assimilation and thus compensate for the C cost of the symbiosis, allowing for a positive mycorrhizal growth response even in conditions of C limitation (Konvalinková and Jansa 2016).

The AM fungal communities of all abiotic contexts were strongly dominated by *R. irregularis* in the first stage, and even more so in the second stage. Although it was expected that the AM fungal community would be dominated by one species (Dumbrell et al. 2010), we did not expect such strong dominance. Isolates of *R. irregularis* are fast colonizers with highly infective mycelium and massive formation of spore clusters in root fragments (Oehl et al. 2011). Their fast colonization rate allows them to preferentially occupy the root niche and exclude other AM fungal species (Alkan et al. 2006; Janoušková et al. 2013; Engelmoer et al. 2014; Symanczik et al. 2015), which may lead to progressive dominance within plant life cycle or systems consisting of adult plants and seedlings (Janoušková et al. 2017). Additionally, *R. irregularis* may have been favored by a high proportion of infective propagules in the initial inoculum pool after having formed a higher density of infective propagules than the other species in the standardized inoculum preparation procedure. Standardization of the initial inoculum pool to equal proportions of infective propagules of the different species may have led to lower dominance of *R. irregularis*, but it probably would have not principally altered the community composition (see Janoušková et al. 2013). The same holds true for the homogenization of the substrate between the two cultivation stages: This step probably disfavored *G. margarita*, whose soil infectivity is sensitive to the disturbance of extraradical mycelium (Boddington and Dodd 2000, Klironomos and Hart 2002). However, *F. mosseae*, which is assumed to be tolerant of disturbance (Chagnon et al. 2013), also displayed declining relative abundances in the second experimental stage.

Although the abiotic context did not affect the overall *R. irregularis* dominance, it influenced the abundance of some of the other AM fungal species within the synthetic community. Surprisingly, *F. mosseae* and *A. tuberculata* were promoted mainly by the low light conditions, which is in contrast to the expectation that shaded conditions instead should

suppress less-abundant AM fungal species (Knegt et al. 2016). In our study, the low light conditions were indeed less favorable for the development of mycorrhiza than the standard conditions, as shown by the significantly lower hyphal, arbuscular and vesicular root colonization. It is possible that *R. irregularis* root colonization developed slower in these less-favorable conditions and left more root space for colonization by the species with slower growth rates (Johnson et al. 2004; Mummey et al. 2009). However, their higher root colonization in the low light treatment was not sufficient to propagate into the second cultivation cycle as evident in the progressive suppression of the less abundant species in the second cultivation cycle by *R. irregularis*, regardless inoculum history.

Synthetic communities composed of cultivable AM fungal species are highly simplified in comparison with natural AM fungal communities, and the results of our experiment thus cannot easily be generalized to more complex native communities. However, we have to bear in mind that the taxonomic and trait diversity of AM fungal communities also is diminished in a range of habitats, most notably in agroecosystems (Verbruggen and Kiers 2010), as compared to anthropogenically less-affected ecosystems. For such conditions, our results suggest progressive overdominance of the fastest root colonizer and a decrease of AM fungal diversity even if the less abundant species, presumably slower root colonizers, are principally compatible with the given conditions.

In conclusion, plant mycorrhizal benefits were clearly linked to the P requirements of non-mycorrhizal plants in the different abiotic contexts: Mycorrhiza improved plant growth in conditions where plants were P-limited, while the enhanced P uptake of N-limited plants led to excess P storage. The effect of abiotic context on the relative abundances of the AM fungal species was, however, only small and transient, and the composition of the AM fungal community was probably determined by the differential ability of the AM fungal species to colonize plant roots. These results suggest that inherent AM fungal species traits such as mycelial growth rate and ability to quickly produce infective propagules are decisive for the realized AM fungal community composition in simplified systems, while mycorrhiza functioning on the continuum between mutualism and parasitism has only a limited feedback on AM fungal community structure. It is vital to test this assumption in more complex conditions including more diverse and balanced AM fungal communities, and with spatial and temporal variation.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Contribution Statement

MJ, JJ, DP and MV designed the study. AV and DP performed the research. AV and PŠ analyzed data. PŠ contributed new models. AV wrote the paper with a substantial contribution from MJ, JJ, DP and MV.

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Table 1 Effects of abiotic conditions, inoculation and their interaction on the shoot dry weight (SDW) and phosphorus (P) content in the shoots of plants of each experimental stage, as determined by two-way ANOVA. Significant *p* values are shown in bold. Degrees of freedom were: (*3*,*40*) for conditions, (*1*,*40*) for inoculation and (*3*,*40*) for the interaction (first stage); (*3*,*112*) for conditions, (*1*,*112*) for inoculation and (*3*,*112*) for the interaction (second stage)

		Conditions (A)		Inoculation (B)		A x B	
		F value	*p* value	*F* value	*p* value	*F* value	*p* value
First stage	SDW	**342.17**	**< 0.001**	0.76	0.386	**27.9**	**< 0.001**
	P content	**461.95**	**< 0.001**	**120.3**	**< 0.001**	**6.03**	**< 0.001**
Second stage	SDW	**209.99**	**< 0.001**	1.4	0.239	**10.07**	**< 0.001**
	P content	**589.27**	**< 0.001**	**720.21**	**< 0.001**	**36.23**	**< 0.001**

Table 2 Effects of inoculum history (first experimental stage encoded as *none* level) and abiotic conditions on the mycorrhizal growth response (MGR), mycorrhizal phosphorus uptake response (MPR) and fractional root colonization by hyphae, arbuscules and vesicles for combined data from both stages as determined by two-way ANOVA. Significant *p* values are in bold. Degrees of freedom were: (*4*,*100*) for inoculum history, (*3*,*100*) for conditions and (*12*,*100*) for the interaction

	Inoculum history (A)		Conditions (B)		A x B	
	F value	*p* value	*F* value	*p* value	*F* value	*p* value
MGR	0.41	0.802	**218.05**	**< 0.001**	**2.48**	**0.007**
MPR	**2.76**	**0.032**	**951.10**	**< 0.001**	**5.21**	**< 0.001**
Hyphae	1.63	0.122	**55.36**	**< 0.001**	**2.35**	**0.011**
Arbuscules	**28.22**	**< 0.001**	**27.04**	**< 0.001**	**5.86**	**< 0.001**
Vesicles	**4.24**	**0.003**	**57.17**	**< 0.001**	**2.33**	**0.011**

Table 3: Effects of pre-conditioning in the same abiotic conditions, abiotic conditions and their interaction on mycorrhizal growth response (MGR), mycorrhizal phosphorus response (MPR) and fractional root colonization by hyphae, arbuscules and vesicles as determined by two-way ANOVA. Significant *p* values are in bold. Degrees of freedom were: (*1*,*88*) for pre-conditioning, (*3*,*88*) for conditions and (*3*,*88*) for the interaction

	Pre-conditioning (A)		Conditions (B)		A x B	
	F value	*p* value	*F* value	*p* value	*F* value	*p* value
MGR	0.10	0.753	**134.21**	**< 0.001**	0.40	0.750
MPR	1.60	0.210	**524.95**	**< 0.001**	2.13	0.103
Hyphae	0.42	0.519	**54.53**	**< 0.001**	1.04	0.380
Arbuscules	0.55	0.460	**34.47**	**< 0.001**	2.09	0.107
Vesicles	1.44	0.233	**50.49**	**< 0.001**	1.73	0.167

Table 4: The effect of abiotic conditions on fractional root colonization by hyphae, arbuscules and vesicles in both stages of the experiment. Values are means (± SE), n = 6 for the first stage, n = 24 for the second stage. Different letters indicate significant differences in root colonization among the abiotic conditions according to Tukey's test at $p < 0.05$ for combined data from both stages.

	Hyphae (%)			Arbuscules (%)			Vesicles (%)		
	First stage	Second stage		First stage	Second stage		First stage	Second stage	
Standard	95.5 (± 2.9)	99.5 (± 0.2)	a	77.0 (± 3.8)	95.2 (± 0.8)	a	9.0 (± 0.9)	16.5 (± 1.0)	a
High P	98.8 (± 0.3)	99.7 (± 0.1)	a	72.5 (± 2.4)	97.9 (± 0.4)	a	10.7 (± 1.5)	18.8 (± 1.3)	a
Low light	87.8 (± 2.7)	92.8 (± 1.1)	b	62.3 (± 3.5)	87.5 (± 1.5)	b	4.0 (± 1.1)	4.5 (± 0.6)	c
Low water	95.3 (± 1.3)	90.3 (± 1.4)	b	86.0 (± 4.2)	84.1 (± 1.9)	b	10.0 (± 1.9)	9.8 (± 0.9)	b

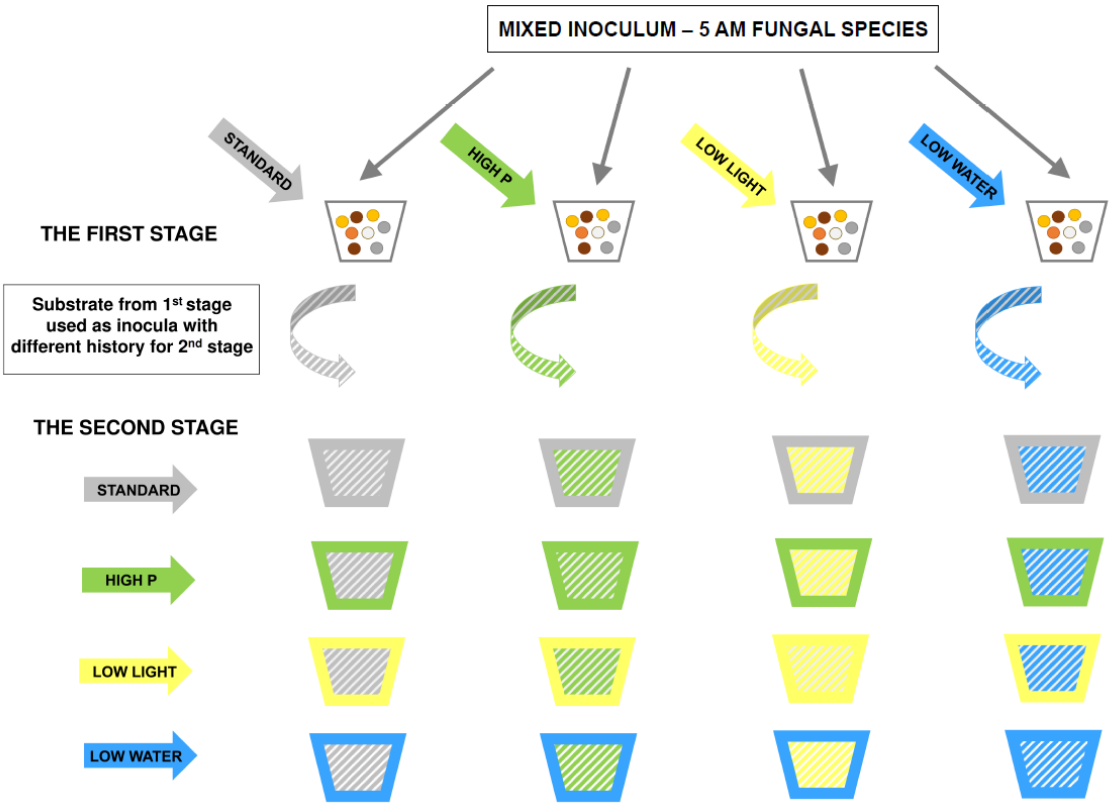

Fig. 1 Outline of the two-stage greenhouse experiment. In the first stage, each treatment was inoculated with the same mixture of arbuscular mycorrhizal fungi and cultivated in different abiotic conditions (indicated by straight arrows of different colors). The substrates from the first stage were used as inocula with different histories (indicated by curved hatched arrows of different colors) for the second stage of the experiment. In the second stage, each inoculum was cultivated in the same abiotic conditions (indicated by straight arrows of different colors) as in the first stage, resulting in sixteen different treatments (hatched color interior of pots indicates inoculum of different histories, color outline of pots indicates different abiotic conditions). Non-inoculated controls were included for all abiotic conditions of both experimental stages (not displayed in this outline)

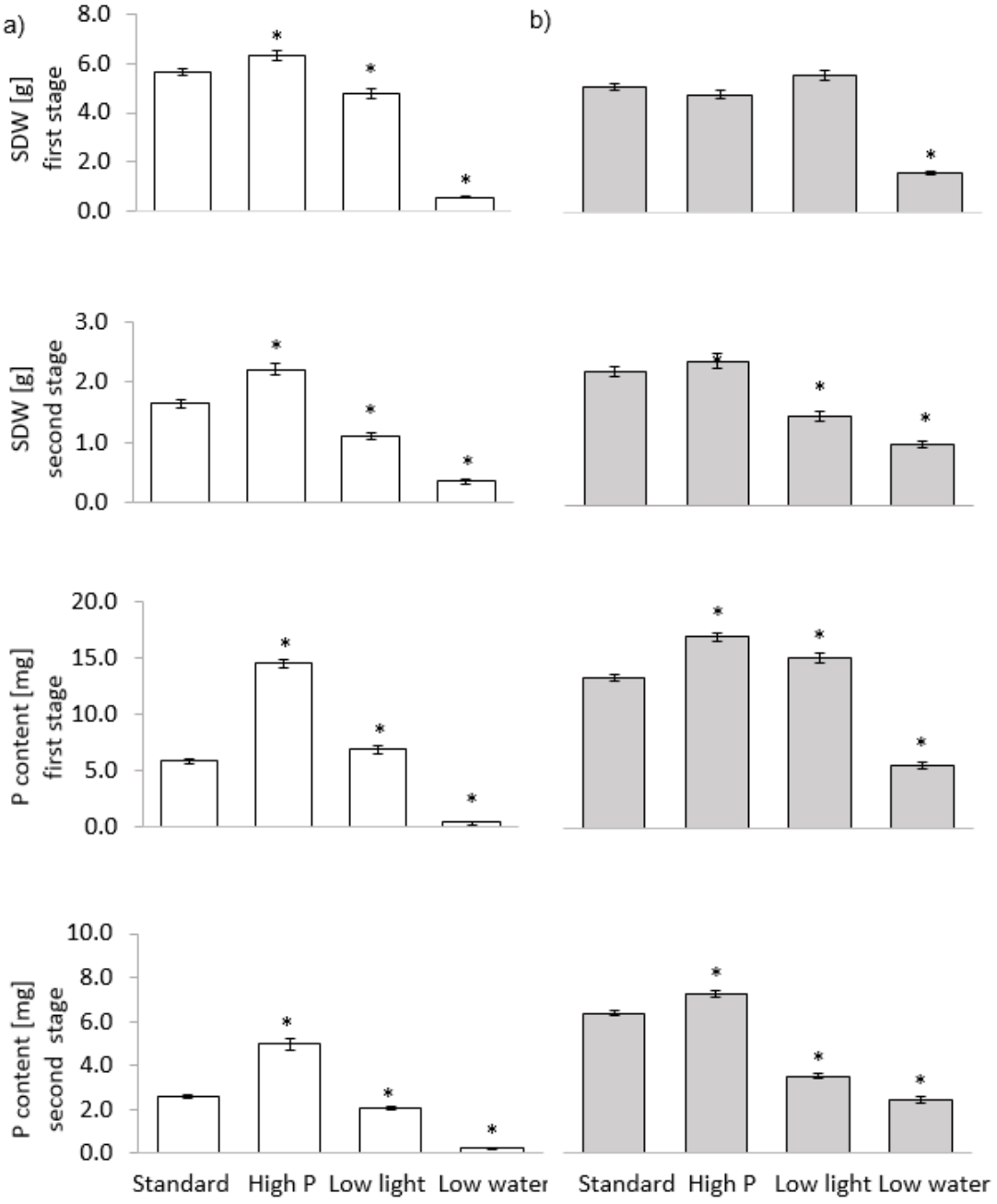

Fig. 2 Shoot dry weights (SDW) and phosphorus (P) content in the shoot biomass of plants in both stages of the experiment. Bars are means (± standard errors) of a) SDW and P content in the shoot biomass of non-inoculated plants and b) SDW and P content in the shoot biomass of inoculated plants in the first and second stages of the experiment under different abiotic conditions. Asterisks (*) indicate significant differences from standard conditions at $p \leq 0.05$, as determined by planned treatment contrasts

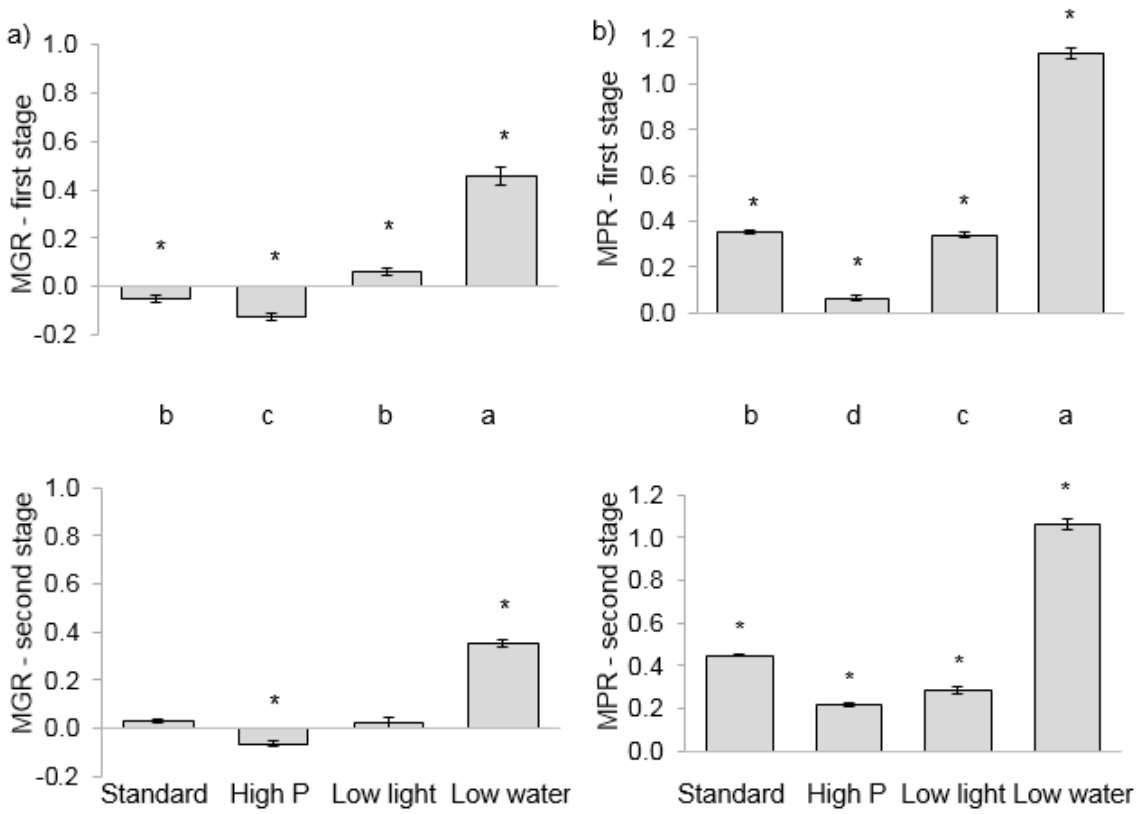

Fig. 3 Mycorrhizal growth response (MGR) and mycorrhizal phosphorus uptake response (MPR) in both stages of the experiment as affected by different abiotic conditions. Bars are means (± standard errors) of a) MGR of the plants and b) MPR of the plants in the first and second stages under different abiotic conditions. Different letters indicate significant differences in the mycorrhizal responses among abiotic conditions according to Tukey's test at $p < 0.05$ for combined data from both stages of the experiment. Asterisks indicate significant differences ($p \leq 0.05$) between non-inoculated and inoculated plants

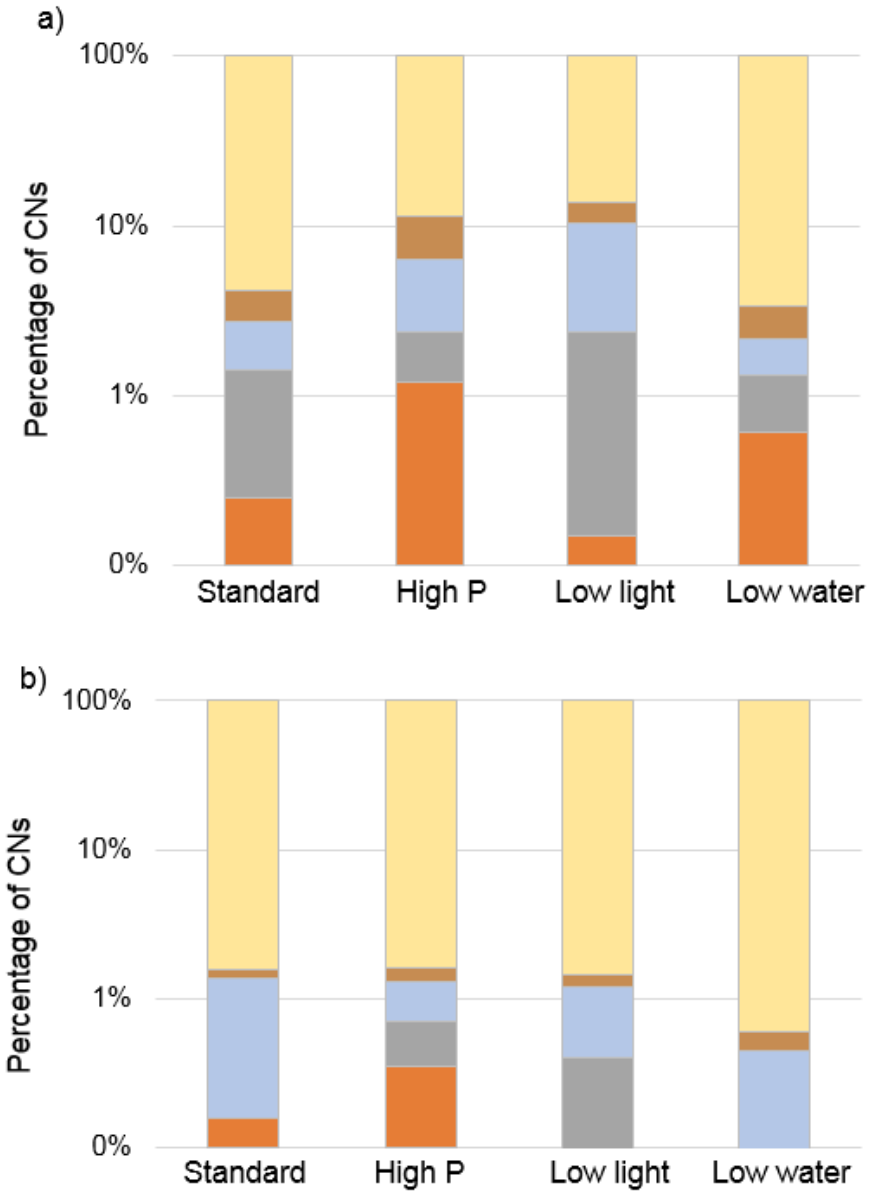

Fig. 4 Relative abundances (percentage of copy numbers of large ribosomal subunit RNA genes of the respective fungal species) of all arbuscular mycorrhizal fungal species in the communities established in different abiotic conditions a) in the first stage and b) in the second stage of the experiment, on log scales. Different colors mark the different species: yellow – *Rhizophagus irregularis*, brown – *Claroideoglomus claroideum*, blue – *Funneliformis mosseae*, grey – *Acaulospora tuberculata*, orange – *Gigaspora margarita*

3.4. Paper IV: Voříšková *et al.* (manuscript)

Are mycorrhizal benefits influenced by the quantitative composition of arbuscular mycorrhizal fungal communities?

Alena Voříšková[1,2], Jan Jansa[3], David Püschel[1,3], Miroslav Vosátka[1], Martina Janoušková[1]

1) Institute of Botany, The Czech Academy of Sciences, Zámek 1, 252 43 Průhonice, Czech Republic
2) Department of Experimental Plant Biology, Faculty of Science, Charles University, Viničná 5, 128 44 Prague, Czech Republic
3) Institute of Microbiology, The Czech Academy of Sciences, Vídeňská 1083, 142 20 Prague, Czech Republic

Manuscript

Contribution statement

AV participated in the establishment, carrying out and harvesting of the experiment. AV evaluated root colonization and participated in the conducting of the molecular analyses. AV performed all statistical analyses and interpreted the results in consultation with other co-authors. AV wrote the manuscript with a contribution from her supervisor MJ and other co-authors.

Are mycorrhizal benefits influenced by the quantitative composition of arbuscular mycorrhizal fungal communities?

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[1)] Institute of Botany, The Czech Academy of Sciences, Zámek 1, 252 43 Průhonice, Czech Republic
[2)] Institute of Microbiology, The Czech Academy of Sciences, Vídeňská 1083, 142 00 Prague, Czech Republic
[3)] Department of Experimental Plant Biology, Faculty of Science, Charles University, Viničná 5 128 00, Prague, Czech Republic

Abstract

The identity and diversity of arbuscular mycorrhizal (AM) fungal symbionts is known to influence the functioning of mycorrhiza, but the role of the fungal species' relative abundances within communities remains little explored. We hypothesized that the quantitative composition of a root-associated AM fungal community would influence the host plant's benefits and that the highest benefits would be conferred with spontaneously established relative abundances of the community members.

To test these hypotheses, we established an experiment with *Medicago truncatula* as host plant, and a synthetic AM fungal community of five isolates, whose quantitative composition was manipulated by the composition of the inoculum pool. One inoculum was produced by the co-cultivation of the isolates.

Plant mycorrhizal benefits were influenced by the abundances of particular AM fungal isolates in the early stage of plant growth and by the total intraradical biomasses of the fungal communities. The community composition established from the spontaneously established inoculum was more beneficial than most, but not all of the alternatively compositions.

It is concluded that mycorrhizal benefits are influenced by the absolute rather than by the relative abundances of particular AM fungal symbionts, and the spontaneously established quantitative community composition represents only one of the possible "good solutions".

Key words

quantitative real-time PCR, phosphorus uptake, carbon cost, *Funneliformis mosseae*, *Gigaspora margarita*

Introduction

Symbiosis with arbuscular mycorrhizal (AM) fungi is an ancient and almost ubiquitous adaptation of plants to the terrestrial environment (Bonfante & Genre, 2008). Improved uptake of nutrients via the associated AM fungi, particularly of the low-available phosphorus (P), is the key benefit of plants from the symbiosis, paid with transfer of photosynthates to the obligately biotrophic fungi. Most plant species are facultative mycotrophs (Janos, 2007): Mycorrhiza formation is favored in conditions of low nutrient availability and partly restricted or even completely eliminated, when the plant has sufficient access to P (Carbonnel & Gutjahr, 2014; Nouri *et al.*, 2014). Mycorrhiza formation is thus part of the adaptive plasticity of plants - its formation and contribution to plant nutrient uptake and growth depends on the abiotic context (Johnson *et al.*, 1997).

Another important factor influencing plant benefits from mycorrhiza is the community composition of the root-colonizing AM fungi. As AM fungal taxa differ in their ability to provide benefits to the host plant (e.g. (Klironomos, 2003; Munkvold *et al.*, 2004), more diverse AM fungal communities are usually more beneficial than less diverse ones or even single isolates due to functional complementarity and/or sampling effect (Maherali & Klironomos, 2007; Wagg *et al.*, 2011). AM fungi colonize roots from a soil inoculum pool composed of colonized roots, extraradical mycelia and spores. The composition of the root-colonizing AM fungal community is influenced by the abundances of AM fungal species in the soil inoculum pool and their inherent root colonization traits (Wilson & Trinick, 1983; Wilson, 1984; Alkan *et al.*, 2006; Thonar *et al.*, 2014). However, there is also a significant role of the host plant identity, as demonstrated by the establishment of compositionally different AM fungal communities in the roots of coexisting plant species or ecological groups (Helgason *et al.*, 2002; Vandenkoornhuyse *et al.*, 2003; Sýkorová *et al.*, 2007; Chagnon *et al.*, 2015). A physiological mechanism, which may regulate the spread of different AM fungi is preferential allocation of carbon (C) to those species that are more efficient in providing nutrients: Though less beneficial symbionts were not completely eliminated, the ones that provide more nutrients became relatively more abundant (Bever *et al.*, 2009; Kiers *et al.*, 2011; Werner *et al.*, 2018).

The ability to discriminate between low-quality and high-quality fungal partners and to reward the latter is an important mechanism of evolutionary stability of symbiotic associations with multiple partners (Bever, 2015). From the ecophysiological perspective, it suggests that plants may increase the profits from arbuscular mycorrhiza by supporting those fungal partners that provide the highest benefits. Then, AM fungal species ratios within communities would be functionally relevant and represent a component of the adaptive plasticity of plants to specific abiotic conditions (Lekberg & Koide, 2013). Such a "functional optimization" of AM fungal communities by the host plant is in line with previously reported selectivity for the most efficient fungal symbiont out of the soil inoculum pool (Helgason *et al.*, 2002). However, studies that would link AM fungal community composition with functional characterization of the AM fungal community members are rare (Lekberg &

Koide, 2013). Even more generally, the relationship between the quantitative composition of AM fungal communities and mycorrhiza functioning has still received surprisingly little attention (Powell & Rillig, 2018).

Basically, "functional optimization" of AM fungal communities is most probable in environmental contexts favoring mutualistic mycorrhiza. Conditions that decrease the mutualism also reduce the ability of plants to preferentially allocate C to high-quality fungal symbionts (Zheng *et al.*, 2015; Ji & Bever, 2016; Werner *et al.*, 2018). Furthermore, "functionally optimized" communities are likely to evolve in stable environmental conditions with consistent differential C allocation to the fungal symbionts (see Lekberg and Koide, 2013). We therefore addressed the relationship of quantitative AM fungal community composition and mycorrhiza functioning in a model greenhouse experiment with *Medicago truncatula* as host plant and a synthetic community of five AM fungal isolates provided in different ratios in the inoculum pools.

We hypothesized that 1) different species ratios in the inoculum pools would lead to the establishment of compositionally and functionally different AM fungal communities, and 2) AM fungal community with spontaneously established species ratios, i.e. developed from an inoculum pool obtained by co-cultivation of the species, would be more beneficial than AM fungal communities of divergent species ratios. In this experiment, we simultaneously monitored plant growth and P uptake, fungal biomass in roots, C flow into the AM fungal communities and abundances of all AM fungal community members in three sequential cultivation times.

Materials and methods

Experimental design and material

The greenhouse experiment was conducted with *Medicago truncatula* J5 as host plant and synthetic communities of five AM fungal isolates, each representing one AM fungal species: *Rhizophagus irregularis* PH5 (RI), *Claroideoglomus claroideum* BEG23 (CC), *Gigaspora margarita* BEG34 (GM), *Funneliformis mosseae* BEG95 (FM) and *Acaulospora tuberculata* BEG41 (AT). In addition to a non-inoculated control treatment, the experiment comprised six treatments inoculated with inoculum pools of differently composed communities of the five AM fungal species. One treatment represented the spontaneously established AM fungal community (MIX), which was prepared by co-cultivation of the five AM fungal isolates. The other five AM fungal communities, termed artificially composed, were obtained by blending pure inocula of the five AM fungal species in different ratios, whereby each inoculum pool was dominated by the inoculum of one isolate (RI+, CC+, FM+, GM+, AT+), see subchapter *Preparation of inocula* for details. Each inoculation treatment comprised 18 replicates, which were sequentially harvested after 3, 6 and 9 weeks of cultivation (six replicates per cultivation time).

The experiment was conducted in tall 2L-plastic pots with sterilized substrate. The substrate was a mixture of 45 % autoclaved sand, 45 % autoclaved zeolite and 10 % γ-irradiated (>25 kGy) soil from Tänikon, Switzerland, and had the following main characteristics: pH 7.52, C [%] 0.186, N [%] 0.012, available P [mg kg^{-1}] 2.06.

Preparation of the inoculum pools

The AM fungal inocula used for the preparation of the inoculum pools were produced with the same host plant and in the same substrate and cultivation conditions as the subsequent experiment. Five pure cultures of each of the five AM fungal isolates were established for the preparation of the artificial communities, four mixed cultures of the five isolates for the inoculation of the MIX treatment. Additionally, four non-inoculated pots (blank cultures) were established for the blank inoculation of the non-inoculated control, i.e. for introducing comparable communities of other soil microorganisms than AM fungi. All the cultures were established simultaneously and cultivated for seven months. Thus, the inocula of all five isolates (and the MIX community) were produced by a standardized procedure, which assured that each AM fungal isolate developed its isolate-specific densities of infective propagules in the substrate.

The pure cultures were inoculated with 200 g of air-dried substrate containing propagules (spores, extraradical mycelium and colonized root fragments) of the corresponding AM fungal isolate. The mixed cultures were inoculated with 40 g of substrate of each AM fungal species. The air-dried substrates used to initiate the cultures originated from sporulating multi-spore cultures of each species with *Allium porrum* as host plant. Surface-sterilized (10 min in 0.5 % solution of NaClO) seeds of *M. truncatula* were pre-germinated on moist filter paper in sterilized Petri dishes at room temperature for three days. The 200 g of treatment-specific AM fungal-colonized substrate was mixed with 800 g of the sterilized cultivation substrate and a fertilization solution supplying (per pot) 60 mg of K (as K_2SO_4), 30 mg of Mg (as $MgSO_4.7H2O$), and 30 mg of Ca (as $CaCl_2.2H2O$).

The mixture was filled into the pots on top of ca. 600 ml of sterilized cultivation substrate and covered by further 250 ml. One germinated seedling was planted into each pot. All seedlings were inoculated with 1 ml suspension (optical density adjusted to 0.8 at 600 nm) of *Sinorhizobium* strain LT10, which had been prepared as described in Konvalinková et al. (2015).

After six months of greenhouse cultivation, all root systems were sampled by a soil core in order to check the purity of the pure culture and determine the abundance of each AM fungal species in roots by quantitative PCR (qPCR). The cores were wet-sieving and checked on sporulation under a stereo-microscope. All the pure cultures contained abundant spores of the expected morphotype only. Roots were picked with tweezers from the upper sieve and root aliquots of 100 mg fresh weight were grinded in liquid nitrogen. DNA was extracted using DNA Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and eluted into 80 µl of 10mM Tris. DNA concentration was quantified and the

quality of the extracts assessed using a Nanodrop spectrophotometer (ThermoFisher, Scientific, Waltham, MA, USA). *Taq*Man-based qPCR assays with PCR amplicons as standards were used for the quantification of large subunit of nuclear ribosomal DNA (nrDNA LSU) as described in Voříšková et al. (2017). Five-fold dilution series of the standards were amplified in technical triplicates, samples in technical duplicates. The DNA concentrations were calculated to copy numbers of the nrDNA LSU region according to Krak et al. (2012): CN ng^{-1} DNA = (6.022 x 10^{23})/(amplicon length [bp] x 10^9 x 660). The results of the culture screening and parameters of the cultures selected for inoculation are provided in Table S1.

Shoots were cut off from pots which were selected for the preparation of the inocula, the substrates and root systems were homogenized, air-dried and stored in refrigerator until use, i.e. for about three weeks. The artificially composed inocula were prepared for each pot separately directly before the establishment of the experiment by weighing and mixing substrates from the pure cultures as summarized in Table S2. As evident from this table, RI-colonized substrate was added in four-time-smaller amounts than substrates of the other fungi into all the inoculation treatments. This was decided in order to support the establishment of compositionally divergent communities while including RI, which is a strong competitor (Table S1, Voříšková et al., 2019). The inocula for the MIX treatment and the blank inocula for the non-mycorrhizal treatment were prepared by weighing 200 g of the substrate from the mixed cultures and blank cultures, respectively (Table S2).

Establishment and cultivation of the experiment

Surface-sterilized seeds of *M. truncatula* were pre-germinated on moist filter in Petri dishes for three days, the germinated seedling were planted into multipots with sterilized substrate (100 ml of cell volume) and cultivated in the growth chamber (14 - hour photoperiod) for three weeks. Pots were filled with the sterilized cultivation substrate and the treatment specific inocula as described within the *Preparation of inocula* subchapter. One *M. sativa* seedling was planted into each pot and inoculated at planting with 1 ml of suspension (optical density adjusted to 0.8 at 600 nm) of *Sinorhizobium* strain LT10. Plants were cultivated in a heated greenhouse with natural light and light supplement by 400W metal halide lamps.

Two days before each harvest, four randomly selected replicate pots from each inoculation treatment were pulse-labeled with $^{13}CO_2$. The pots with plants were placed inside an air-tight Plexiglass chamber (750 l, 1m2 footprint), and the $^{13}CO_2$ labeling was performed for 1.5 h by exposing plants to $^{13}CO_2$ developed by combining 2.5 g of 99% ^{13}C-enriched calcium carbonate powder (Sigma–Aldrich, Buchs, Switzerland) and 40% phosphoric acid. Concentration of CO_2 and temperature inside the chamber were monitored using Testo435-2 datalogger (Lenzkirch, Germany). These plants were harvested together with the two additional replicate plants per treatment, the latter serving as isotopic controls.

Harvest and laboratory analyses

Shoots of each plant were cut off and dried at 65 °C for three days to constant weight and weighed to obtain the shoot dry weight (SDW).Root systems were washed, dried with kitchen towels, weighed to obtain the root fresh weight (RFW), cut to 1 cm fragments and homogenized. Aliquots of 100 mg fresh weight for the molecular analyses and ca. 0.5 g fresh weight for phospholipid fatty acid C16:1ω5 and neutral lipid fatty acid C16:1ω5 analyses were frozen in liquid nitrogen and stored at – 80 °C. Approximately 100 mg of the fresh roots were sampled for the analyses of C using isotope-ratio mass spectrometer (IRMS). They were weighed to obtain their accurate weight and dried at 65 °C for three days to constant weight.

P concentration in shoots was determined on subsamples (of approx. 50 mg) of ground biomass. They were dried at 60°C and mineralized with 4 ml conc. HNO_3 and 1 ml 30 % H_2O_2 in the microwave mineralization machine Milestone Standard 2500. After vacuum filtering, P concentration was determined using photometric method according to Olsen and Sommers (1982). The absorbance was measured using UV-VIS Spectrophotometer (UV-400 Unicam) at 630 nm. The P content in shoots was calculated by multiplying the P concentration and SDW.

Total C concentrations and isotopic composition of C in shoots and roots of the plants was assessed using Flash EA 2000 elemental analyzer coupled with IRMS as above. Total amount of ^{13}C originating from the $^{13}CO_2$ pulse (i.e., ^{13}C excess, mmol) was calculated using the relevant isotopic controls and calculation framework as described previously (Slavíková *et al.*, 2017).

Mycorrhizal growth/phosphorus response (MGR/MPR) of plants was calculated based on SDW, P content and P concentration values (in shoot biomass) using the equation MGR or MPR [%] = (M – NM)/NM × 100, where M is the SDW or P content or P concentration of the inoculated plant and NM is the mean of the corresponding parameter in the non-mycorrhizal control treatment.

After the second and third harvest, remaining root fragments were placed into 10 % KOH and stained with 0.05 % Trypan Blue in lactoglycerol (Koske & Gemma, 1989) for the microscopic determination of root colonization. No roots were available for this analysis after the first harvest, due to the small size of the root systems. Root colonization levels by hyphae, arbuscules and vesicles were estimated using the gridline intersect method (McGonigle *et al.*, 1990) with scoring of 100 intersections per sample at 100× magnification (Olympus BX60). The abundance of each AM fungal species in roots was quantified after all three harvests using qPCR as described in the *Preparation of inocula* subchapter.

Analyses of neutral fatty acid C16:1ω5 and phospholipid fatty acid C16:1ω5 combined with $^{13}CO_2$ labelling

Total lipids were extracted from freeze-dried root samples as described previously (Konvalinková *et al.*, 2017). Briefly, the root samples (weights between 5 and 67 mg) were spiked with first internal standard (C19:0 fatty acid) and macerated in

chloroform:methanol:citrate buffer (0.15 M, pH 4) mixture (1:2:0.8 v:v:v) overnight. The phase separation of the mixture was then achieved by adding excess citrate buffer. Thereafter, the samples were centrifuged, and the lower (non-polar) phase was recovered and evaporated to dryness at 50°C under a stream of nitrogen. Those lipid extracts were then dissolved in chloroform and fractionated to neutral lipids and phospholipids according to the procedure described by Welc et al. (2012). Briefly, the total lipid extracts were loaded on a silica-column, which was then sequentially eluted with chloroform (resulting in neutral lipids being washed out), acetone and methanol (with the third fraction containing the phospholipids). Each of the fractions was then spiked with a second internal standard (C21:0 fatty acid), evaporated to dryness and transmethylated using a trimethylchlorosilane-methanol mixture as described previously (Konvalinková *et al.*, 2017). The analyses of the fatty acid profiles (recording concentrations and C isotopic compositions of the individual compounds) then proceeded on a Trace 1310 gas chromatograph (GC) coupled with the IRMS via GC isolink (all IRMS components by ThermoFisher Scientific, Waltham MA, USA). The GC was equipped with a DB-5 column (60m, 0.25 mm ID, 2 μm coating), as described previously (López-Mondéjar *et al.*, 2018). Concentrations of C16:1ω5 fatty acid in the neutral (NLFA) and phospholipid fractions (PLFA) were used as proxies for AM fungal abundance in the roots. Measured concentrations in the lipid extracts were converted to concentrations of the signature fatty acid in the root biomass by using the recovery of the two internal standards in the neutral and phospholipid fractions, and root weights entering the lipid extractions. The isotopic composition of the C16:1ω5 in the $^{13}CO_2$ labeled and control pots was used to calculate ^{13}C excess in the AM-signature fatty acid in the $^{13}CO_2$ labeled plants according to the framework presented previously (Konvalinková *et al.*, 2017). ^{13}C allocation to AM fungal lipids was calculated as a ratio of total ^{13}C in PLFA and NLFA to total ^{13}C in the plant biomass.

Statistical analyses

The AM fungal community composition was analyzed using Non-metric Multidimensional Scaling (NMDS) based on Bray-Curtis dissimilarity distances of Hellinger transformed copy numbers of each AM fungal species for each cultivation time separately. A permutational multivariate analysis of variance (PERMANOVA, McArdle and Anderson, 2001) embedded in the function adonis from vegan package in R (Oksanen *et al.*, 2016) tested the effect of inoculation treatments on the AM fungal community composition for each cultivation time separately using the distance matrices mentioned above. The effect of the inoculation treatments on the copy numbers of the three more abundant AM fungal isolates (*FM*, *CC*, *RI*) within each cultivation time was tested by one-way ANOVA following by Tukey's test with a significance level of $p \leq 0.05$. CNs values with non-normal distribution were transformed before the statistic.

The effect of the inoculation treatments on shoot biomass (SDW), P concentration, root colonization, concentration of NLFA C16:1ω5 and PLFA C16:1ω5 was tested using

one-way ANOVA followed by Tukey's test with a significance level of $p \leq 0.05$ within each cultivation time. The mycorrhizal growth response (MGR), mycorrhizal phosphorus responses (MPR) calculated based on the P concentrations and P contents in shoots as well as ^{13}C allocation to AM fungal lipids were analyzed using generalized linear models (GLM) with cultivation time, inoculation treatment and their interaction as explanatory variables. The values of ^{13}C allocation to AM fungal lipids were log transformed before the statistics. Based on the results showing no significant interaction for MGR, the MGR values were pooled 1) across the inoculation treatments within each cultivation time and 2) across the cultivation times within each inoculation treatment, and tested using one-way ANOVA on the effect of 1) cultivation time and 2) inoculation treatment, followed by Tukey's test with a significance level of $p \leq 0.05$. Both MPRs and the ^{13}C allocation to AM fungal lipids were tested using one-way ANOVA followed by Tukey's test with a significance level of $p \leq 0.05$ on the effect of inoculation treatment, for each cultivation time separately.

Correlation analyses between mycorrhizal growth response (MGR) and parameters of AM fungal biomass (root colonization, concentration of NLFA C16:1ω5 and PLFA C16:1ω5) were performed using Pearson's correlation. Subsequently, the effect of inoculation treatments on MGR was tested by analysis of covariance (ANCOVA), where the AM fungal biomass parameters, which were significantly correlated with MGR, were used as covariates at each cultivation time. Additionally, a multiple linear regression was used to predict MGR based on the cultivation time and copy numbers of each AM fungal species. The best model of the multiple linear regression was selected based on the Akaike information criterion.

Results

Composition of the fungal communities and intraradical biomass

Different inoculation treatments lead to the establishment of communities with significantly different proportions of the five AM fungal species according to PERMANOVA ($p = 0.001$; three weeks - *adj.* $R^2 = 0.848$, six weeks - *adj.* $R^2 = 0.717$, nine weeks - *adj.* $R^2 = 0.544$). The communities were mostly dominated by the RI isolated, except for the FM+ and CC+ communities after three and six weeks (Fig. 1, Table S3). Strong dominance of RI was found especially in the MIX and RI+ treatments, where average 97 % of copy numbers belonged to this species at all cultivation times. The FM and CC isolates dominated in the treatments in which they were promoted by higher inoculum doses (FM+ and CC+) after three weeks (Fig. 1a), while RI dominated these treatments after nine weeks owing to its increasing proportion in time (Fig. 1b, c). Consistently, NMDS analysis showed convergence of the AM fungal communities in time (Fig. S1), in accordance with lower adj. R^2 of PERMANOVA after nine weeks than after three or six weeks.

All AM fungal species had higher copy numbers in those inoculation treatments, in which they were promoted, as compared to the other treatments, although the differences

were not always significant due to high variability of the data (Table S3). Their copy numbers did not differ between the RI+ treatment and the MIX treatment at any time. The FM and CC isolates were present in all inoculation treatments throughout the duration of the experiment (Fig 1, Table S3). GM in contrast, was present only in the GM+ treatment throughout the whole experiment, and in low copy numbers also in some other inoculation treatments after six weeks of cultivation (Table S3). The abundance of *A. tuberculata* increased in time: it was found only in the AT+ and GM+ treatments after three weeks and in all inoculation treatments after nine weeks of cultivation (Table S3).

Copy numbers of dominant RI correlated with the concentration of PLFA 16:1ω5 after three weeks (adj. $R^2 = 0.224$, $p < 0.006$), with root colonization after six weeks (adj. $R^2 = 0.413$, $p < 0.001$) and nine weeks (adj. $R^2 = 0.383$, $p < 0.001$), and with the sum of copy numbers of all AM fungal species at all the three harvest (three weeks – adj. $R^2 = 0.537$, $p < 0.001$, six weeks – adj. $R^2 = 0.673$, $p < 0.001$, nine weeks – adj. $R^2 = 0.850$, $p < 0.001$).

Root colonization reached its plateau already after six weeks in all treatments and did not further increase after nine weeks. It was significantly affected by inoculation treatment in both cultivation times (Table 1). The highest root colonization was recorded in the RI+ and MIX treatments (Table 1). Arbuscules and vesicles positively correlated with root colonization after both cultivation times (results not shown).

The concentrations of NLFA 16:1ω5 positively correlated with root colonization at six weeks (adj. $R^2 = 0.124$, $p = 0.037$) and nine weeks (adj. $R^2 = 0.282$, $p = 0.002$), whereas the concentration of PLFA 16:1ω5 did not. The concentrations of NLFA 16:1ω5 was significantly affected by inoculation treatment in all cultivation times and remarkably, was significantly higher in GM+ than in most the other treatments after three weeks (Table 1). The concentration of PLFA 16:1ω5 differed among the treatments only after three weeks with a tendency to highest values in RI+ and MIX, though significant differences were found only between the RI+ and AT+ inoculation treatment (Table 1).

C investment into intraradical fungal structures

^{13}C allocation into AM fungal lipids was significantly affected by cultivation time, inoculation treatment and their interaction (Table 2). ^{13}C allocation into AM fungal lipids was also significantly affected by inoculation treatment at each cultivation time, when analyzed separately (Table S4). ^{13}C allocation into AM fungal lipids was significantly higher in the GM+ treatment than in all the other treatments after three weeks, and remained significantly higher in GM+ than in FM+ after six weeks. After nine weeks, ^{13}C allocation was significantly higher in the MIX and RI+ treatment as compared to AT+ and FM+ (Fig. 2a).

Mycorrhizal effects on plant growth

Mycorrhizal growth response (MGR) was significantly affected by cultivation time and inoculation treatment, but the interaction between these two factors was non-significant

(Table 2). MGR values pooled across the three cultivation times were also significantly affected by inoculation treatment ($F = 14.20$, $p < 0.001$) (Fig. 3a): The RI+ and MIX inocula induced the highest MGR, while GM+ and CC+ the lowest. MGR of the FM+ inoculation treatment was intermediate, and did not significantly differ from the MGR of the MIX treatment. As regards the effect of cultivation time, MGR was significantly higher after six and nine weeks as compared to three weeks ($F = 18.93$, $p < 0.001$) (Fig. 3b). Shoot dry weights as determined in each harvest as well as significant differences from the non-mycorrhizal control treatment, are shown in Fig. S2.

The mycorrhizal responses of P uptake (MPRs) calculated from the P contents and the P concentrations of the experimental plants were significantly affected by cultivation time, inoculation treatment and their interaction (Table 2). The inoculation treatment significantly affected both MPRs after all cultivation times, when the data from each cultivation time were analyzed separately (Table S4). In general, MPR in P content was always higher in the MIX and RI+ treatments as compared to the GM+ and CC+ treatments, but the differences between the inoculation treatments decreased in time (Fig. 2b). MPR in P concentration revealed an early effect of the MIX, RI+ and FM+ inoculation treatments on P concentration in shoot biomass (Fig. 2c): The MPR was significantly higher in these three cultivation treatments than in the AT+, GM+ and CC+ treatments after three weeks (Fig. 2c). Subsequently, after six and nine weeks of cultivation, the response in P concentration gradually declined and became less variable among the inoculation treatments (Fig. 2c). The P shoot concentrations including those of the non-mycorrhizal control plants are shown in Fig. S2.

Relationships of MGR and the fungal community parameters

Correlation analyses of MGR and the parameters of fungal biomass like concentration of NLFA 16:1ω5, PLFA 16:1ω5 and root colonization showed significant correlation of MGR and PLFA after three weeks (Fig. 4a) and of MGR and NLFA after nine weeks (Fig. 4c). Root colonization significantly correlated with MGR after six and nine weeks, i.e. at both cultivation times where it was evaluated (Fig. 4b, c).

Subsequently, the effect of inoculation treatment on MGR was tested using ANCOVA models with those parameters of AM fungal biomass as covariates that were correlated with MGR at the corresponding cultivation time (Table 4). According to these analyses, MGR was affected by inoculation treatment independently of fungal biomass after three and six weeks. After three weeks, MGR was higher in the RI+ and MIX treatments as compared to GM+. After six weeks, it was significantly higher in the RI+ treatment than in AT+ and GM+. After nine weeks, significant effect of inoculation treatment was found only with NLFA as covariate, but the multiple comparison post-hoc test did not show any differences between the treatments.

A multiple regression was calculated to predict MGR based on cultivation time and copy numbers (CNs) of each AM fungal species. The model significantly predicted MGR

($F_{(6,100)}$ = 10.18, p < 0.001) and explained 37.9% of the variance. All tested independent variables contributed significantly to the model with the exception of GM copy numbers, which were sharply non-significant as parameter (Table 5). MGR was positively correlated with cultivation time and the copy numbers of RI and FM, negatively correlated with the copy numbers of CC and AT.

Discussion

As expected, most of the AM fungal communities established from the artificially composed inoculum pools differed compositionally and functionally from the spontaneously established MIX community, which promoted plant growth better than most of the artificially composed communities. A notable exception was the RI+ community, which inoculum pool was dominated by the same isolate as the MIX inoculum. The communities, however, differed not only in composition, but also in their intraradical biomass, which was an important determinant of mycorrhizal benefits.

The encountered positive relationships of mycorrhizal growth response, P uptake and intraradical fungal biomass is in agreement with the importance of AM fungal infectivity for mycorrhiza functioning, demonstrated by the metaanalyses of Treseder (2013) and Lekberg and Koide (2005). In our experiment, the relevant fungal biomass parameters were driven by the abundance of the RI isolate, and consistently, tended to be highest in the MIX and RI+ communities, which received higher amounts of RI-colonized substrate within the inoculum pool as compared to the other communities. The other isolates evidently developed less intraradical biomass than RI from even larger volumes of colonized substrates (see Table S2) either due to their lower infectivity in the substrates or due to different root colonization traits (Thonar; Wilson & Trinick, 1983; Voříšková *et al.*, 2016). In the case of the GM and AT isolates, this is not surprising in view of the phylogenetically conserved life traits of Gigasporaceae and Acaulosporaceae – slow growth rates and life cycles (Chagnon *et al.*, 2013) and their low intraradical biomass (Hart & Reader, 2002; Maherali & Klironomos, 2012). The Glomeraceae species *F. mosseae* and *C. claroideum,* in contrast have a fast life cycle (Oehl *et al.*, 2004, 2009; Chagnon *et al.*, 2013), which should have allowed plateau levels of infectivity to build up within the seven-month inoculum preparation period. But both isolates may have a lower root colonization potential than the RI isolate as root colonization can be highly variable among Glomeraceae (Hart & Reader, 2002; Munkvold *et al.*, 2004). Specifically, *F. mosseae* and *C. claroideum* isolates were previously shown to reach lower plateau levels of root colonization than *R. irregularis*, even if inoculated with the same amount of infective propagules (Thonar; Wagg *et al.*, 2011; Janoušková *et al.*, 2013). Thus, the composition of the soil inoculum pools determined the fungal intraradical biomass (and consequently also the host plant's mycorrhizal benefits) via the traits of the most abundant isolates.

However, our experiment also revealed functional differences among the communities, which were independent of root colonization, in line with the conclusion of

Treseder (2013) that the identity of root-colonizing fungi is another important factor determining the mycorrhizal growth response. The GM+ community induced significantly lower mycorrhizal growth response than the MIX community, after statistically accounting for its lower intraradical fungal biomass, and this was associated with exceptionally high C consumption by the intraradical fungal structures in the early plant growth stage. This suggests that higher GM abundance within this community decreased the net benefits of the mycorrhiza by increasing its C costs, consistently with previously shown high C requirements of *Gigaspora spp.* (Klironomos *et al.*, 2005; Thonar *et al.*, 2011; Lendenmann *et al.*, 2011; Ji & Bever, 2016). However, extensive extraradical mycelium of Gigasporaceae, suggested as a possible cause for the high C demand of *Gigaspora spp.* (Klironomos *et al.*, 2005; Thonar *et al.*, 2011), does not fully explain the high content of fungal storage lipids (the NLFA fraction) in the GM+ colonized roots (Aarle & Olsson, 2003), which was the main cause of the high C consumption. Instead, it indicates that GM induced energy hording in the other members of the AM fungal community, particularly in the most abundant RI isolate. Being GM an "expensive" AM fungal species as regards the amount of P supplied per consumed C (Lendenmann *et al.*, 2011), coexistence with a "cheaper" species may have decrease its C consumption by reducing the "market price" of P (Argüello *et al.*, 2016). But if the high C sink strength of GM is inherent (as indicated by Ji and Bever, 2016) and the host plant is not C limited, the total C flow into the fungal community may increase in order to maintain directly proportional allocation of C to qualitatively different partners (Wyatt *et al.*, 2014).

Another interesting difference was recorded between the functioning of the FM+ and CC+ communities, characterized by similar intraradical biomass and high initial abundances of the isolates that dominated the inoculum pool (FM and CC, respectively). The FM+ community, however, induced significantly higher mycorrhizal growth response than the CC+ community, most probably by efficiently supplying P to the host plant in the early stage of plant growth. This suggests that FM provided more P to the host plants than CC within the communities, which is in agreement with previously described functional traits of *F. mosseae* and *C. claroideum* isolates: While *F. mosseae* was repeatedly reported to efficiently supply P (Burleigh, 2002; Munkvold *et al.*, 2004; Jansa *et al.*, 2005), *C. claroideum* was classified as a rather poor P supplier in earlier experiments (Jansa *et al.*, 2008; Thonar *et al.*, 2011; Lendenmann *et al.*, 2011).

In fact, the FM+ community was functionally very similar to the MIX and RI+ communities, inducing early P supply and high mycorrhizal growth response throughout the experiment. This is important in relation to our assumption of "functional optimization" of AM fungal communities since it shows that a spontaneously established community may represent just one of several "good solutions" with respect to plant P uptake and growth. From the point of view of preferential C allocation, it imposes the question, why FM didn't become more abundant in the communities, being an efficient P provider. The answer is probably related to the fast growth and/or high infectivity of RI, which may have enabled this isolate to capture a large amount of C from the plant's initial investment into the

symbiosis (Bever, 2015; Christian & Bever, 2018). If co-colonizing AM fungal species differ in their growth rates, efficiency of preferential C allocation is expected to decrease (Werner & Kiers, 2015), particularly if the fast root colonizer also satisfies the plant's need for P (Bever, 2015). This mechanism may be reinforced in ruderal host plants, such as the fast growing annual *M. truncatula*, which have been assumed and shown to preferentially associate with ruderal AM fungi (Chagnon *et al.*, 2013; López-García *et al.*, 2014): The AM fungal community member with the fastest life cycle can most efficiently utilize both the high initial C flow (for root and soil colonization) as well as the relatively short C supply period (for the formation of reproductive structures). These assumptions directly call for a test with the same AM fungal community and a host plant classified as competitor or stress-tolerator (Grime, 1977). In a previous study, variation in the C supply from *M. truncatula* due to abiotic context little influenced the quantitative ratios of the same AM fungal community as in this experiment (Voříšková *et al.*, 2019). However, inherently different C supply patterns of plants with a different life strategy may represent a stronger filter and select for a different AM fungal community structure (see Chagnon et al., 2015; Öpik et al., 2009).

Ruderal host plants, which promote the fungus with the fastest life cycle, also profit in the next generation from this fungus' high soil infectivity in being supplied with P in an early growth stage, consistently with our experimental results. However, we have to bear in mind that this positive feedback is just one possible scenario (Johnson, 1993; Bever, 2002; Lekberg & Koide, 2013). Fast root colonizers may also exert a high C demand on the host plant and induce a parasitic mycorrhizal phenotype (Graham & Abbott, 2000; Johnson *et al.*, 2015). Disturbance by tillage, a typical factor that selects for AM fungi with fast life cycles (Oehl *et al.*, 2009) may also select for AM fungi, which are less efficient in promoting P uptake by the host plant (Köhl *et al.*, 2014). The positive feedback of AM fungal community composition demonstrated by our experiment thus requires further testing, possibly by including a range of other fast root colonizers into the AM fungal community.

The significance of our results consists in showing that quantitative ratios of AM fungal isolates in the inoculum pool are important for mycorrhiza functioning by two means: They influence the total intraradical fungal biomass, depending on the abundance of species with different life traits, and they influence the realized abundances of particular, functionally different isolates. In that sense, our results strongly suggest for future studies to pay attention to the absolute abundances of AM fungal species within communities rather than to their relative abundances. Furthermore, our results do not indicate that preferential C allocation to the best mutualist(s) plays an important role in shaping the AM fungal community composition, if its members differ in their root colonization speed – at least not in association with a fast growing annual. This importantly complements previous studies conducted with AM fungal isolates of comparable growth rates (Bever *et al.*, 2009; Kiers *et al.*, 2011; Werner *et al.*, 2018). Further studies of gradually increasing complexity are needed to understand the significance of the observed positive feedback of AM fungal community composition within the context of plant plasticity / adaptation to specific conditions.

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Table 1 The effect of inoculation treatment on root colonization, concentrations of neutral lipid fatty acid (NLFA) 16:1ω5 and phospholipid fatty acid (PLFA) 16:1ω5 within each cultivation time (Time).
Significant effects (* $p \leq 0.05$) are highlighted in bold. Values are means (standard errors).

	Time (weeks)	AT+	GM+	FM+	CC+	RI+	MIX	*F* value *
Colonization [%]	6	43 (4) c	56 (9) abc	46 (5) bc	47 (5) bc	65 (4) ab	77 (3) a	**7.69 ***
	9	44 (7) b	45 (5) b	44 (3) b	43 (6) b	70 (3) a	78 (1) a	**12.33 ***
NLFA [µg/g]	3	112 (33) b	729 (242) a	123 (70) b	189 (46) b	285 (56) ab	133 (24) b	**4.83 ***
	6	101 (24) b	208 (36) ab	127 (42) ab	121 (27) ab	157 (28) ab	244 (34) a	**2.98 ***
	9	44 (17) bc	170 (41) ab	44 (26) c	119 (17) abc	263 (52) a	324 (72) a	**8.66 ***
PLFA [µg/g]	3	34 (9) b	52 (13) ab	39 (12) ab	38 (10) ab	89 (16) a	85 (11) ab	**4.24 ***
	6	16 (5)	27 (4)	15 (6)	22 (4)	24 (5)	18 (5)	1.00 *n.s.*
	9	31 (3)	55 (16)	28 (6)	38 (6)	43 (10)	59 (13)	1.65 *n.s.*

Means followed by the same letter do not significantly differ at $p \leq 0.05$ according to Tukey's test. Degrees of freedom were (*5*,*28*) and (*5*,*30*) for root colonization at six and nine weeks, respectively; (*5*,*23*), (*5*,*24*) and (*5*,*24*) for the fatty acid concentrations in NLFA and PLFA at three, six and nine weeks, respectively

Table 2 Effects of cultivation time, inoculation treatment and their interaction on ^{13}C allocation to AM fungal lipids (^{13}C allocation), mycorrhizal growth response (MGR) and mycorrhizal P response calculated from the P content (MPR - content) and P concentration (MPR - concentration) in shoot biomass, according to GLM.

	Time (A)		Treatment (B)		A x B	
	F value	*p* value	*F* value	*p* value	*F* value	*p* value
^{13}C allocation	**20.45**	**< 0.001**	**11.28**	**< 0.001**	**9.32**	**< 0.001**
MGR	**33.70**	**< 0.001**	**18.16**	**< 0.001**	0.23	0.951
MPR - content	**11.62**	**< 0.001**	**28.16**	**< 0.001**	**10.19**	**< 0.001**
MPR - concentration	**125.88**	**< 0.001**	**14.12**	**< 0.001**	**18.51**	**< 0.001**

Significant *p* values are in bold. Degrees of freedom were: (*1*,*60*) for time, (*5*,*60*) for inoculation treatment and (*5*,*60*) for their interaction in ^{13}C allocation. Degrees of freedom were: (*1*,*95*) for time, (*5*,*95*) for inoculation treatment and (*5*,*95*) for their interaction in MGR, MPR - content, MPR – concentration

Table 3 Effect of inoculation treatment (Treatment) on mycorrhizal growth response (MGR) after different cultivation times (Time) as determined by ANCOVA with different parameters of AM fungal biomass as covariates (root colonization, concentrations of neutral lipid fatty acid (NLFA) 16:1ω5 and phospholipid fatty acid (PLFA) 16:1ω5).

		AM fungal biomass		Treatment	
Time (weeks)	Covariates	*F* value	*p* value	*F* value	*p* value
Three	PLFA	**61.41**	**< 0.001**	**4.54**	**0.005**
Six	Colonization	**25.78**	**< 0.001**	**3.89**	**0.009**
Nine	Colonization	**21.98**	**< 0.001**	1.52	0.215
Nine	NLFA	**11.34**	**0.003**	**2.87**	**0.037**

Significant *p* values are in bold. Degrees of freedom were: (*1*,*22*) for AM fungal biomass and (*5*,*22*) for the treatment in PLFA concentration; (*1*,*23*) for AM fungal biomass and (*5*,*23*) for the treatment in NLFA concentration. Degrees of freedom in root colonization were: (*1*,*27*) for AM fungal biomass and (*5*,*27*) for the treatment after six weeks and (*1*,*29*) for AM fungal biomass and (*5*,*29*) for the treatment after nine weeks

Table 4 The model of multiple linear regression (y-intercept = -199.30) with mycorrhizal growth response (MGR) as the dependent variable, cultivation time (Time) and copy numbers (CNs) of each AM fungal isolates as independent variables.

	F value	*p* value	*Slope coefficient*
Time	**30.1**	**< 0.001**	41.62
RI	**11.54**	**< 0.001**	22.37
FM	**8.26**	**0.005**	4.37
CC	**11.27**	**0.001**	-7.20
AC	**4.63**	**0.034**	-4.52
GM	3.73	0.056	-4.72

Significant *p* values are in bold. *RI – Rhizophagus irregularis*, *FM – Funneliformis mosseae*, *CC – Claroideoglomus claroideum*, *AC - Acaulospora tuberculata*, *GM – Gigaspora margarita*

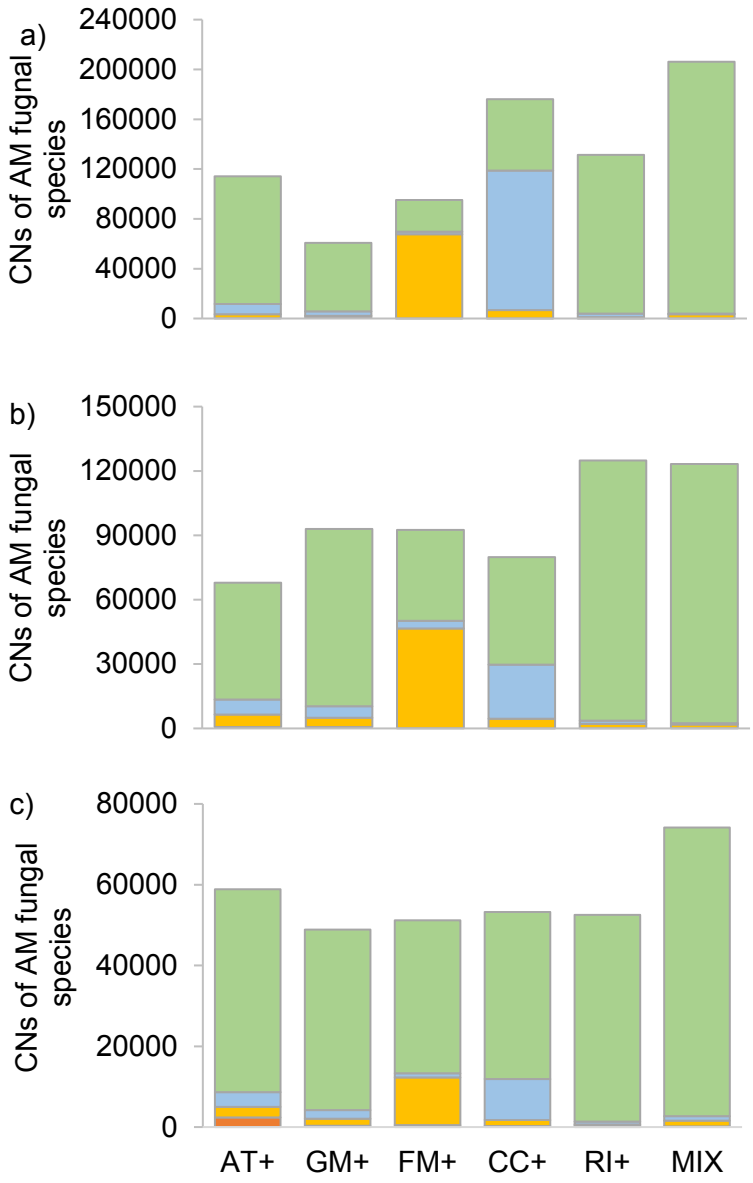

Fig. 1 Abundances (means of CNs) of the AM fungal isolates in the communities established in the different inoculation treatments a) after three weeks, b) after six weeks and c) after nine weeks. Different colors mark different AM fungal species: orange – *Acaulospora tuberculata*, grey – *Gigaspora margarita,* yellow– *Funneliformis mosseae*, blue – *Claroideoglomus claroideum*, green – *Rhizophagus irregularis*

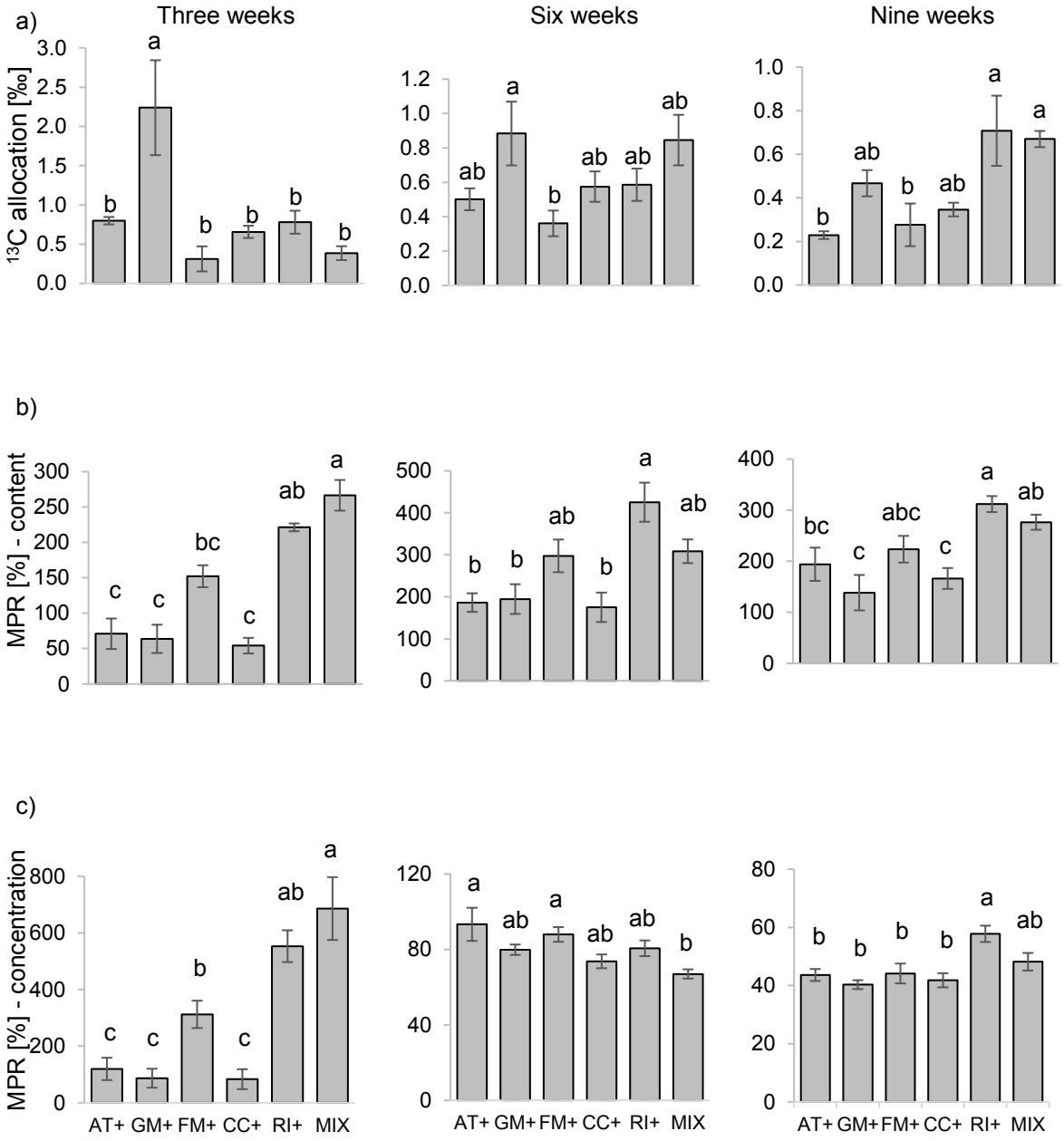

Fig. 2 Means (± standard errors) of a) ^{13}C allocation to AM fungal lipids (^{13}C allocation) and mycorrhizal P response calculated from b) P content (MPR – content) and c) P concentration (MPR – concentration) in shoot biomass in different inoculation treatments after three, six and nine weeks of cultivation. Means followed by the same letter do not differ significantly at $p \leq 0.05$ by Tukey's test.

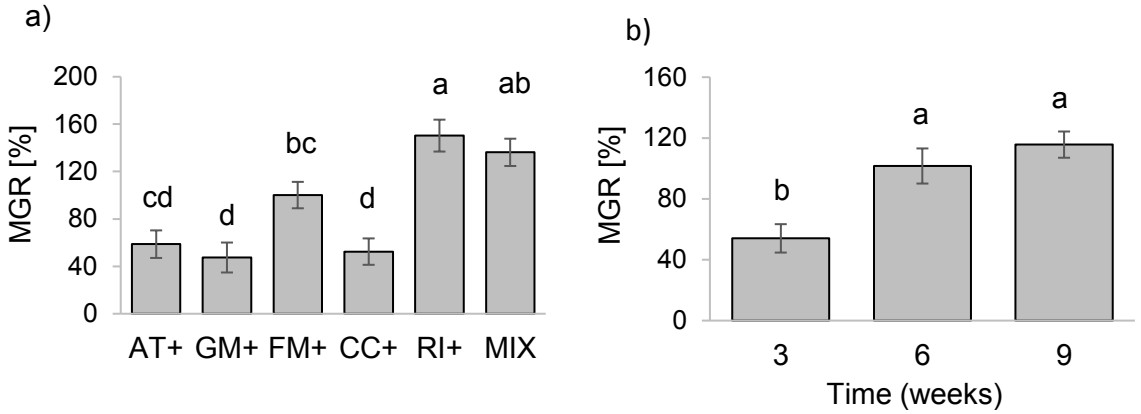

Fig. 3 Means (± standard errors) of mycorrhizal growth response (MGR) in a) different inoculation treatments throughout the cultivation and b) at different cultivation times (Time) across all the inoculated treatments as tested by one – way ANOVA. Means followed by the same letter do not differ significantly at $p \leq 0.05$ by Tukey's test.

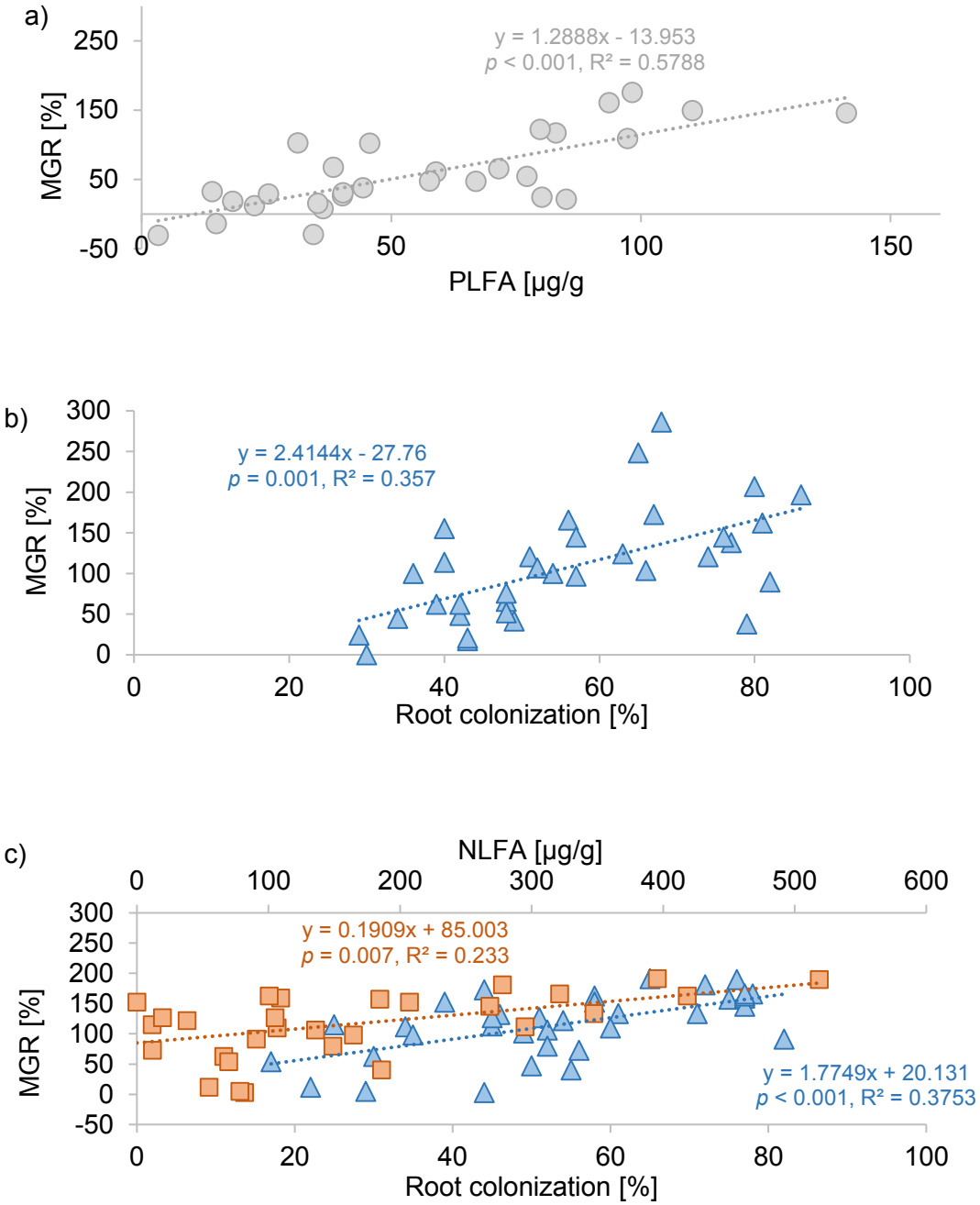

Fig. 4 Correlations of mycorrhizal growth response (MGR) and a) concentrations of phospholipid fatty acid (PLFA) 16:1ω5 after three weeks, b) root colonization by AMF after six weeks and c) concentrations of neutral lipid fatty acid (NLFA)16:1ω5 and root colonization after nine weeks of cultivation. Different symbols and colors indicate different fungal parameters: grey circle – PLFA, blue triangle – root colonization, orange square – NLFA.

4. DISCUSSION

The functioning of AM symbiosis, i.e. the response of host plants to mycorrhiza, is a frequently addressed topic in the field of AM research. Equally, the communities of AM fungi are often investigated primarily at the level of their diversity, but only secondarily on the level of abundances of individual AM species. However, the interconnection of these two important topics, i.e. the structure of AM fungal communities and their symbiotic functioning is often neglected even though it can provide deep insight into the functional importance of the composition of AM fungal communities. The experiments of the dissertation aimed at filling the missing link and connecting the functional aspects of arbuscular mycorrhiza with the abundances of individual AM fungal species in the community.

A unified model experimental system was established and used with variations in subsequent experiments. Based on the results of MGR from the first publication (Paper I, Püschel *et al.*, 2017), the basic settings of the experimental model system were characterized, and the appropriate substrate (mixture of sand, zeolite and Tänikon soil) together with the plant host species (*Medicago truncatula*) were selected for further experiments. The second publication (Paper II, Voříšková *et al.*, 2017) enabled us to compare methods for the quantification of arbuscular mycorrhiza in roots and to complete qPCR markers for quantification of selected AM fungal species. Markers for qPCR in the region of LSU of nrDNA were subsequently used for the quantification of the abundances of each AM fungal species within the community in further experiments. The optimized experimental model system was used for the establishment of complex experiments with synthetic communities of AM fungi consisting of five AM fungal species from four different families in the last publication (Paper III, Voříšková *et al.*, 2019) and in the manuscript (Paper IV, Voříšková *et al*.).

4.1. Brief insight into synthetic AM fungal communities and their quantification

The basis of AM research is the study of the effect of AM fungi on the growth of plants and nutrient uptake. The appropriate and one of the most used ways how to approach this topic is the conducting of experiments in greenhouse conditions. Host plants in some experimental

studies are inoculated with single AM fungus (Hart & Reader, 2002b; Klironomos, 2003; Avio *et al.*, 2006; Lendenmann *et al.*, 2011), which is an acceptable way to evaluate the functioning of the mycorrhizal symbiosis for a specific AM fungus, but it is less relevant for the natural conditions, where plants are colonized by multiple AM fungal species (Jansa *et al.*, 2002; Pivato *et al.*, 2007). This fact is often neglected in experimental conditions because it complicates the conducting and evaluation of the experiments. However, the study of the functional diversity of AM fungi is certainly incomplete without the inclusion of AM fungal communities, which has been reflected by the use of synthetic communities of cultivable AM fungal species in experimental studies (Jansa *et al.*, 2008; Janousková *et al.*, 2013; Thonar *et al.*, 2014; Paper III; Paper IV). The crucial issue in the study of synthetic communities is the quantification of the AM fungal species colonizing roots or soil. Staining roots and microscopy, the traditional and mostly used method, does not allow to distinguish among individual AM fungal species in the community. Therefore the introduction of qPCR into mycorrhizal research greatly advanced this issue, contributed to the expansion of the use of synthetic communities in experimental systems and enabled to link AM fungal community structure with its functioning.

The LSU of nrDNA is mostly used for qPCR quantification (Jansa *et al.*, 2008; Thonar *et al.*, 2012; Wakelin *et al.*, 2012; Janoušková *et al.*, 2013), because it is well-characterized and allows the design of markers suitable to discriminate taxa at the species-level, though it is problematic in distinguishing closely related species (Thonar *et al.*, 2012) due to intraindividual sequence variability (Sanders *et al.*, 1995). The LSU of mitochondrial ribosomal DNA (mtDNA) with the variability of some regions and, simultaneously, no intraindividual sequence variability (Raab *et al.*, 2005; Beaudet *et al.*, 2013; Nadimi *et al.*, 2016), has arisen as an alternative tool with higher-resolution power to distinguish closely related species (Kiers *et al.*, 2011) and even isolates of *R. irregularis* (Krak *et al.*, 2012; Badri *et al.*, 2016). Paper II (Voříšková *et al.*, 2017) compared qPCR in both regions with more traditional approaches (root colonization, lipid biomarkers) and quantified the fungal biomass of four selected AM fungal species. It was found that nrDNA and mtDNA markers generated well-correlated copy numbers for each AM fungal species and, therefore, both markers can be used alternatively for quantification of particular species, which corresponds to the study of Krak *et al*. (2012) with one AM fungal species. However, the copy numbers

of nrDNA were better related to intraradical fungal biomass across different AM fungal species than copy numbers of mtDNA, which indicates better usability of the nrDNA markers in AM fungal communities. The evaluation of copy numbers obtained by qPCR can also impose problems. In the experiment of Paper IV, the copy numbers of individual AM fungal species declined in time after initially high abundances, which was shown also in other time-course experiments (Jansa *et al.*, 2008; Krak *et al.*, 2012; Janoušková *et al.*, 2013). This effect can be related to an initial peak of mycelial activity followed by decreasing AM fungal vitality in later experimental stages (Thonar; Smith & Dickson, 1991; Tisserant *et al.*, 2007; Jansa *et al.*, 2008), even though further investigation on this topic is needed to confirm this assumption.

4.2. Abiotic context as a driver of mycorrhiza functioning

The functioning of AM symbiosis is located on the continuum from parasitism to mutualism, where positive, neutral and negative responses of plants to arbuscular mycorrhiza are observed (Johnson *et al.*, 1997). The net benefit of plants from the mycorrhizal relationship depends on the ratio of the mycorrhizal benefits on one side and on costs in terms of the C supplied to the fungi on the other side. The resulting relationship among host plants and their symbionts is determined by a range of factors, which may be either favorable or unfavorable for the functioning of mycorrhizal symbiosis and shift the plant profit from this relationship (Johnson, 1993; Konvalinková *et al.*, 2015). The availability of P was observed to play a significant role for the functioning of mycorrhiza symbiosis in Paper III, where increased availability of P caused a decrease of the MGR in both stages of the experiment. A situation consistent with the results of Paper III was observed in Paper I, where the MGR of *Medicago truncatula* had a decreasing trend along an increasing P fertilization gradient consisting of three input levels (0, 10, and 40 mg P added per pot). It is generally accepted that the availability of P is one of the most important factor modulating the relationship between host plants and their mycorrhizal symbionts (Mosse, 1973; Koide, 1991; Johnson, 1993; Johnson *et al.*, 2015), although it seems that the identity of the host plants plays an equally important role. Paper I showed that even host plants of the same genus (*Medicago* sp.), but different species, can differently respond to an increase of P availability in the substrate. The MGR of *Medicago sativa* was not affected by the availability of P within the tested range (Paper I).

Paper III studied not only the effect of P availability, but also the effect of other abiotic contexts, namely light and water shortage, on the functioning of AM symbiosis in a two-stage experiment. These three different abiotic conditions, which often appear in nature, can cause stress to the plants (water shortage) or stress the mycorrhizal relationship as a whole (addition of P and light shortage). The stress conditions were intentionally chosen to disrupt the balanced relationship between the host plant and its symbionts and thus to induce different mycorrhizal responses on the scale from parasitism to mutualism (Johnson *et al.*, 2015). An increase of mycorrhizal benefits was expected in drought conditions (Augé, 2001), whereas a decrease of mycorrhizal benefits was expected in P surplus and light shortage conditions (Johnson, 1993; Kiers & van der Heijden, 2006) Results of Paper I allowed us to establish the reference cultivation conditions for the construction of the mutualistic mycorrhizal symbiosis.

The MGR varied among different abiotic conditions, though it was consistent within the two stages of the experiment. The MGR varied from negative (at high P availability), through neutral-mutualistic (in the reference and low light conditions) to a highly mutualistic relationship (at water shortage). P deficiency of plants in the water shortage condition, revealed by the high N:P ratio in the shoot biomass of non-inoculated plants (Koerselman & Meuleman, 1996), was alleviated by the mycorrhizal symbiosis, which was consistent with the high degree of mutualism in these conditions. Conversely, the lower degree of mutualism in the other conditions reflected the limitation by other sources, namely N limitation, as demonstrated by the lower N:P ratio in biomass especially in the second stage of the experiment. However, the mycorrhizal symbiosis consistently increased P content in plant shoot biomass in all conditions. Surprisingly, the beneficial effect of mycorrhiza in P uptake was evident even under the shading conditions, where it could be expected that mycorrhiza would not be beneficial for the C-limited host plants (Konvalinková & Jansa, 2016). An explanation for this unexpected effect of mycorrhiza could be that host plants were more limited by the low relative availability of P (high ratio N:P in their shoot biomass) than by C. Conversely, the higher uptake of P via the mycelial network could compensate the losses of C resulting from the shading (Konvalinková & Jansa, 2016).

An important result of the thesis is the essential role of mycorrhizal symbiosis in drought condition. Improvement of plant growth by arbuscular mycorrhiza at soil water

deficiency has been previously described (Cho *et al.*, 2006; Allen, 2007, 2011), less is however known about the mechanisms of the effect (Augé, 2001; Ruiz-Lozano, 2003). Mycorrhiza was proposed to directly affect the water uptake or P uptake of plants due to the hyphae penetration to pores of small size or to indirectly contribute to redistribution of water through the substrate (Augé, 2001). The results of the experiment presented in Paper III showed that plants inoculated with AM fungi had up to three times higher shoot biomass than non-inoculated plants. Surprisingly, the content of P in shoot biomass was up to fourteen times higher than the P content of the non-inoculated plants. Such a big difference in P content in shoot biomass reveals the extent of reliance of plant P nutrition on mycorrhiza in water limited conditions. Water deficiency in soil can cause that P, ordinarily dissolved in water prior to plant uptake (Jansa *et al.*, 2011), is no longer accessible to plants roots. In this manner, AM symbiosis can play a key role in supplying the host plants by this mineral nutrient at water limited conditions.

4.3. Circumstances affecting the composition of the synthetic AM fungal communities

Different life traits of AM fungi like the colonization strategy and the phenotypic characteristics given by their taxonomic classification can partly predict the symbiotic efficiency of AM fungi (Hart & Reader, 2002b; Avio *et al.*, 2006; Powell *et al.*, 2009). The AM fungal synthetic community in the experiments of the dissertation (Paper III and Paper IV) consisted of five AM fungal species, one reason for the selection of these species having been their different taxonomy. The selected experimental species belonged to two different orders (Glomerales and Diversisporales) and four different fungal families.

The AM fungal community was almost completely dominated by *Rhizophagus irregularis* in the experiments of both studies (Paper III, Paper IV) similarly to the observation in the study of Alkan et al. (2006). The only exception was in the experiment described in Paper IV, where *R. irregularis* dominated together with other members of the Glomales order - *Funneliformis mosseae* and *Claroideoglomus claroideum*. The dominance of these two species was initiated by a high ratio of their propagules in the inoculum pools and lasted only temporarily during the early stage of the experiment, while *R. irregularis* started to dominate in all the treatments in later stages. The non-Glomerales species -

Gigaspora margarita and *Acaulospora tuberculata* - constituted only a very small portion of the intraradical community throughout the experiments in Paper III and in Paper IV, even if supported by higher inoculum doses. These species are members of Gigasporaceae and Acaulosporaceae families, which are usually characterized by a limited root colonization (Hart & Reader, 2002a; Maherali & Klironomos, 2007; Chagnon *et al.*, 2013).

The dominance of *R. irregularis* in the communities is not entirely unexpected. This AM fungal species belonging to family Glomeraceae is considered fast rate colonizer with a highly infective mycelium and forms spore clusters in root fragments (Oehl *et al.*, 2011) which can contribute to fast regeneration and then to the formation of an extensive root colonization (Hart & Reader, 2002a). The fast colonization rate of *R. irregularis* may allow it to preferentially occupy the root niche and thus to exclude the other AM fungal species. *R. irregularis* has been previously often shown to dominate in communities (Alkan *et al.*, 2006; Engelmoer *et al.*, 2014; Symanczik *et al.*, 2015), though not always (Jansa et al., 2008). Symanczik et al. (2015) showed that the introduction of *R. irregularis* into a native AM fungal community may cause a decrease in the abundance of the native species. Progressive overdominance of *R. irregularis* was observed in Paper III and Paper IV.

The dominance of *R. irregularis* was also manifested regardless of the different abiotic contexts indicating the robustness of the species ratio within the synthetic AM community against changing environmental conditions and, consequently, against the functioning of mycorrhiza on the scale from negative to positive mycorrhizal responses (Paper III). However, particular abiotic conditions, especially those unfavorable for mycorrhiza development, enhanced the abundance of some other species in the AM fungal community, probably because they slowed down root colonization by *R. irregularis*.

4.4. The effect of AM fungal community composition on the mycorrhizal functioning

It was reported many times that AM fungal species differ in the extent of benefits provided to their host plants (Klironomos, 2003; Koch *et al.*, 2006) and, therefore, have a different symbiotic efficiency in specific conditions. Our experimental model system with a synthetic community of five AM fungal species, utilizing optimized qPCR markers (Paper II) for the

quantification of the AM fungal species, enabled us to manipulate the AM fungal species ratios in the community (Paper IV). Therefore, we investigated the impact of a spontaneously established inoculum pool and of inoculum pools with different ratios among the species in the community on the symbiotic functioning of arbuscular mycorrhiza (Paper IV).

AM fungal communities in the roots of the experimental plants developing from the different inoculation pools differed in the proportion among AM fungal species, which was particularly evident in the early stage of the experiment (after three weeks of cultivation). In later stages (after six and nine weeks), the proportion among the AM fungal species communities from different inoculum pools tended to converge and one AM fungal species (*R. irregularis*) strongly dominated all the communities. Nevertheless, the early-stage different ratios among the AM fungal species were important for the functioning of the AM symbiosis. The main drivers of providing symbiotic benefits to plants were the abundances of particular AM fungal species and the total intraradical biomass of the fungal community. The positive relationship between plant benefits from mycorrhiza and the amount of fungal biomass in roots was previously described (Lekberg & Koide, 2005; meta-analysis of Treseder, 2013). The intraradical fungal biomass, and therefore also the MGR, were particularly related to the abundance of *R. irregularis* due to the dominance of this species in the communities. However, the functional aspects of other less abundant AM fungal species played a less important, but not negligible role in the functioning of AM symbiosis especially at the early experimental stage.

Interestingly, those communities with artificially reduced abundance of *R. irregularis* in the inoculum pool were symbiotically less efficient while also displaying strong dominance of this species at the early stage. The observed low symbiotic efficiency of these communities can be explained by two complementary reasons. Reduced abundance of *R. irregularis* decreased the symbiotic efficiency of these communities as compared to communities with high abundance of *R. irregularis*. At the same time, another important factor was the abundance of other functionally different AM fungal species in the communities, which could either increase or decrease their symbiotic efficiency. High C requirement of some species during the establishment of the communities could decrease the symbiotic efficiency when they became artificially more abundant, corresponding with the study of Lendenmann *et al*. (2011), which reported high C requirements of *G. margarita*. *F.*

mosseae, which higher abundance increased the symbiotic efficiency of the community as compared to other communities with reduced *R. irregularis*, has been repeatedly reported as efficient supplier of P (Burleigh, 2002; Munkvold *et al.*, 2004; Jansa *et al.*, 2005). This explanation is in line with the fact that *G. margarita* is often considered a less efficient symbiont for the growth of plants or for the P uptake (Hart & Reader, 2002b; Thonar *et al.*, 2011, 2014; Lendenmann *et al.*, 2011) as compared to AM fungal species from family Glomeraceae. Thonar et al. (2017) reported that a community containing both *R. irregularis*, which is considered a symbiotically efficient AM fungal species (Jansa *et al.*, 2008; Thonar *et al.*, 2011), and *G. margarita* had significantly lower symbiotic efficiency than *R. irregularis* inoculated alone (Thonar *et al.*, 2014).

Surprisingly, two communities with artificially promoted species *F. mosseae* and *C. claroideum*, which became more abundant than *R. irregularis* in these communities at least in the early stage of the experiment, were functionally different. Functional differences between these two species were previously reported in the study of Jansa *et al*. (2008), where *F. mosseae* was a less efficient symbiont than *C. claroideum,* apparently due to its lower ability to supply the host plants with P (Thonar *et al.*, 2011; Lendenmann *et al.*, 2011). However, at the end of the experiment, the growth of plants and also the P concentration in shoot biomass were comparable among the different inoculation treatments, possibly due to progressive equalization of *R. irregularis* abundance within the communities.

Paper IV showed that the functioning of AM fungal communities in the provision of benefits to the host plants is obviously affected by the abundances of particular species in the community, which is reflected by intraradical fungal biomass and functional differences among the AM fungal species. It seems that the previously reported importance of high diversity of AM fungal communities for the functioning of mycorrhizal symbiosis (van der Heijden *et al.*, 1998; Sharma *et al.*, 2009) plays only a negligible role in simplified systems like e.g. in commercial inoculum mixtures, where the presence of a single functional and fast root colonizer would be much more important for the promotion of plant growth.

4.5. What should be taken into account in mycorrhizal experimental research?

Still, we should be very careful while generalizing results obtained from model experiments. The investigation of the functioning of AM symbiosis in relation to three abiotic contexts (Paper III) and abundances of particular AM fungal species (Paper IV) with the use of optimized experimental model system (Paper I), one host plant species and selected AM fungal species (Paper II) of course generates a very simplified view intrinsic to the specific context, whereas the true interconnectedness and complexity of a naturally appearing and evolving system is a much harder problem to grasp. Still, the exploration of mycorrhiza in simplified systems is the only way of contributing to the mechanistic understanding of mycorrhizal functioning. The whole range of interactions between abiotic and biotic factors influencing the functioning of mycorrhizal symbiosis appears at natural sites. At least two particular factors are worth mentioning, which could have an important impact on the functioning of mycorrhizal symbiosis and would merit further investigation in the follow-up research of this issue.

Perennial plants have been suggested more responsive to mycorrhiza than annual plants (Boerner, 1992), and simultaneously, the AM fungal community composition in roots of plants can also be affected by different life history traits of plants (Alguacil *et al.*, 2012; Torrecillas *et al.*, 2012a,b). The host plant selected for the purpose of the experiments is a fast growing annual species and therefore, it may favor and also benefit from fast AM fungal colonizers, such as represented by *R. irregularis* in our experimental system, more than from species with slower colonization rates. It would be interesting to set up a long-term experiment with a perennial host plant and the same synthetic community of AM fungi, and focus on how the AM fungal community will change over a longer period time and how would it influence the functioning of mycorrhizal symbiosis. AM fungal species with slow colonization rates like *G. margarita* could possibly appear later in that case.

Moreover, we have to bear in mind that AM fungal communities in the experiments of the thesis are composed of cultivable AM fungal species and formed synthetic communities. The synthetic communities were very simplified in comparison to more complex native communities, therefore, future research should also focus more on native AM fungal communities. The investigation of the functioning of mycorrhizal symbiosis

depending on different abiotic contexts together with the exploration of changes in the composition of native AM fungal communities using next-generation sequencing methods could bring us closer to an understanding of the effect have on the AM fungal communities and mycorrhizal functioning.

5. CONCLUSIONS

The dissertation brought various new insights into the functioning of AM symbiosis in relation to the structure of AM fungal communities and abiotic conditions, specifically:

- Mutualistic functioning of mycorrhizal symbiosis was achieved by specific context, including host plant species, the availability of P in the substrate and the type of substrate (Paper I).

- qPCR markers in the region of mtDNA and nrDNA can be used as alternative tools for quantification of particular AM fungal species, but the nuclear markers are more suitable for the quantification of different AM fungal species within communities (Paper II).

- Different abiotic conditions consistently influenced the functioning of mycorrhizal symbiosis, and the mycorrhizal benefits were clearly linked to the contrasting P demand of the plants depending on the abiotic context (Paper III).

- The quantitative composition of AM fungal communities influenced the functioning of mycorrhizal symbiosis via the fungal root colonization traits and symbiotic functional traits (Paper IV).

- The quantitative composition of synthetic AM fungal communities was influenced mainly by fungal root colonization traits (Paper III, Paper IV), temporarily by the amount of propagules in the inoculum pool (Paper IV) and only little by abiotic conditions (Paper III)

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