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# Intense competition between arbuscular mycorrhizal mutualists in an *in vitro* root microbiome negatively affects total fungal abundance

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## Abstract

The root microbiome is composed of an incredibly diverse microbial community that provides services to the plant. A major question in rhizosphere research is how species in root microbiome communities interact with each other and their host. In the nutrient mutualism between host plants and arbuscular mycorrhizal fungi (AMF), competition often leads to certain species dominating host colonization, with the outcome being dependent on environmental conditions. In the past, it has been difficult to quantify the abundance of closely related species and track competitive interactions in different regions of the rhizosphere, specifically within and outside the host. Here, we used an artificial root system (*in vitro* root organ cultures) to investigate intraradical (within the root) and extraradical (outside the root) competitive interactions between two closely related AMF species, *Rhizophagus irregularis* and *Glomus aggregatum*, under different phosphorus availabilities. We found that competitive interactions between AMF species reduced overall fungal abundance. *R. irregularis* was consistently the most abundant symbiont for both intraradical and extraradical colonization. Competition was the most intense for resources within the host, where both species negatively affected each other's abundance. We found the investment ratio (i.e. extraradical abundance/intraradical abundance) shifted for both species depending on whether competitors were present or not. Phosphorus availability did not change the outcome of these interactions. Our results suggest that studies on competitive interactions should focus on intraradical colonization dynamics and consider how changes in investment ratio are mediated by fungal species interactions.

*Keywords*: competitive interactions, cooperation, *Glomus aggregatum*, mutualism, *Rhizophagus irregularis*, root organ cultures, symbiosis

## Introduction

The microbial diversity associated with plant roots is tremendous: the soil microbial community likely represents the greatest reservoir of biological diversity known in the world so far (Curtis *et al.* 2002; Torsvik *et al.* 2002; Gams 2006; Roesch *et al.* 2007). The collective genome of this rhizosphere microbial community – which is typically much larger than that of the host plant (Berendsen *et al.* 2012) – is composed of a distinct community of interacting microbes that compete and cooperate with each other and the host plant (Berg & Smalla 2009; Mendes *et al.* 2011; Denison *et al.* 2013). This complex network of species can benefit hosts by boosting immunity and stimulating growth via provisioning of nutrients, increased stress resistance and pathogen exclusion (Redman *et al.* 2002; van der Heijden *et al.* 2008; Lugtenberg & Kamilova 2009).

As in human microbiome research, a major question in rhizosphere research is how species in the root microbiome interact with each other and with their host

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(Dennis *et al.* 2010; Bakker *et al.* 2012; Denison *et al.* 2013; Gehring *et al.* Special Issue). The majority of species in the rhizosphere compete for root exudates. However, some species have evolved more direct mechanisms to access host resources, including those organisms involved in mutualistic interactions where resources are exchanged to the benefit of both the plant and the microbe (Denison & Kiers 2011). One of the most prominent rhizosphere mutualisms is that between 60% and 80% of all plant species and arbuscular mycorrhizal fungi (AMF). This symbiosis mainly involves the exchange of carbohydrates from plants for phosphorus, nitrogen and trace nutrients from the fungal partner (Parniske 2008). Other beneficial functions of partnering with mycorrhizal fungi, such as pathogen protection and increased drought resistance, have also been described (Ellis *et al.* 1985; Ozgonen & Erkilic 2007; Abdel-Fattah *et al.* 2011).

Plants engaging in symbiosis with AMF can gain a growth advantage under poor nutrient conditions (Hoeksema *et al.* 2010). However, not all AMF species are of equal quality to the plant. Whereas some fungal species can increase plant growth such as those with low costs of carbon per unit phosphorous (P) transferred, others can cause growth depressions (Klironomos 2003; Munkvold *et al.* 2004; Hart *et al.* 2013). Differences in benefits conferred to hosts are generally associated with different life history strategies employed by AMF species (e.g. Maherali & Klironomos 2012). For example, AMF species differ in the amount of carbon they extract from their host (Zhu & Miller 2003; Li *et al.* 2008; Olsson *et al.* 2010), their ability to acquire phosphorus (P) (Smith *et al.* 2000; Drew *et al.* 2003) and their nutrient storage strategies (Kiers *et al.* 2011). These differences in life history strategies likely dictate the nature of competition inside and outside the host. Specifically, AMF strains will compete intraradically for host-derived carbon (Herrera Medina *et al.* 2003), but also extraradically for available mineral nutrients (Johnson *et al.* 2003; Parniske 2008).

While there is high variation in AMF species traits, we still know little about how this variation drives fungal community composition (Kummel & Salant 2006; Jansa *et al.* 2008). Some studies have shown that competition can lead to the complete exclusion of particular species (Abbott & Robson 1983; Hepper *et al.* 1988). However, more often, competitors remain present in the population, often at reduced densities, and are not fully excluded (Pearson *et al.* 1993; Jansa *et al.* 2008; Bennett & Bever 2009; Bever *et al.* 2009; Janousková *et al.* 2009; Kiers *et al.* 2011). The outcome of these competitions has been linked to differences in life history strategies (Bennett & Bever 2009). In addition, environmental conditions can also affect competitive interactions, making the outcome of competition context dependent (Pearson *et al.* 1994; Bever *et al.* 2009; Verbruggen *et al.* 2012). For example, addition of mineral nutrients seems to strongly reduce the number of AMF strains that can successfully colonize a host (Johnson *et al.* 2003; Toljander *et al.* 2008). The role of nutrients in driving competitive dynamics is particularly important because nutrient deposition is increasing under global change (Galloway *et al.* 2008), and this has the potential to shift competitive dynamics (Carney *et al.* 2007; Van Diepen *et al.* 2011) and mediate which fungal species dominate terrestrial ecosystems (Johnson *et al.* 2013).

Previous studies of AMF competition have been limited by two factors. First, researchers are unable to distinguish closely related AMF species based on morphological characters and thus have not been able to accurately follow their relative abundances in a mixed population across time (Hepper *et al.* 1988; Pearson *et al.* 1993; Cano & Bago 2005). However, with the development of suitable genetic markers, we are now in a prime position to track abundances of closely related species, a situation in which competition is likely to be the strongest (Roger *et al.* 2013; Jansa *et al.* 2008; Janousková *et al.* 2009; Kiers *et al.* 2011; Gorzelak *et al.* 2012). Second, researchers have not been able to accurately compare the intensity of competition inside and outside the root because of the difficulty in collecting extraradical fungal mycelium in soil rhizospheres. Therefore, it is unknown where competition among AMF species is the most intense: Are AMF species predominately competing for space and carbon within host roots or for resources in the rhizosphere? We address this limitation using *in vitro* root organ cultures (ROC) to study the intensity of intraradical and extraradical competition and how changes in external phosphorous conditions affect competitive dynamics. Although ROCs create an artificial environment, they allow us to strictly monitor and control nutrient and culture conditions and to precisely separate the intraradically colonized host and the extraradically colonized rhizosphere from the agar medium for downstream analysis.

We studied the competitive dynamics between two closely related AMF species, *Rhizophagus irregularis* (previously known as *Glomus intraradices*) and *Glomus aggregatum*. The competitive dynamics of these (or any other) AMF species on artificial root systems have yet to be quantified; thus, our study is also an opportunity to test whether ROCs are a useful medium for competition studies. The growth of these two species has been well characterized on several plant species under greenhouse conditions (Kiers *et al.* 2011; Hart *et al.* 2013), and their specific nutrient exchange strategies have been studied in single-species cultures using *in vitro* isotope-labelling approaches (Kiers *et al.* 2011; Fellbaum *et al.*

2012). From this previous work, we know that *R. irregularis* transfers more P per unit carbon to the host, while *G. aggregatum* tends to hoard P in a form that is inaccessible for the host (Kiers *et al.* 2011). Competitive studies in a whole-plant system found that *R. irregularis* consistently outcompetes *G. aggregatum* (Kiers *et al.* 2011). However, it is unknown how external resource conditions mediate competition, nor where this competition is manifested. We therefore focused on these two species in mixed and monoculture (single species) treatments, quantifying competition under two different phosphorus concentrations. Our aim was to (i) determine the effect of competition on the abundance of each fungal species, (ii) determine whether competitive pressures were equal for intraradical colonization or extraradical colonization and (iii) identify whether the outcome of competition was modified by nutrient availability.

## Methods

### Culture conditions and inoculum preparation

We grew *in vitro* cultures of the two focal AMF species, *Rhizophagus irregularis* isolate 09 (F.K.A. *Glomus intraradices*) and *Glomus aggregatum* isolate 0165 on a Ri T-DNA *Daucus carota L.*-transformed ROC using MSR medium, supplemented with glycine (3 mg/L) and myo-inositol (50 mg/L) (Declerck *et al.* 2005). We maintained cultures at 25 °C in the dark. Prior to inoculation, we grew uncolonized sterile root fragments for 1 week on Petri plates (Ø 9 mm). This pregrowth period allowed us to select for root systems of equal size across treatments. We then produced inoculum, which was composed of ddH$_2$O and a spore suspension from mature (4 month old) single-species cultures. We determined spore densities from our inoculum using a standard volume on a custom-made spore counter.

### Competition setup

Our competition experiment consisted of three AMF treatments: single-species treatments for each species and one mixed treatment, crossed by two phosphorous (P) concentrations. We used 10 replicated root systems per treatment inoculated with 100 spores for either monoculture treatment or a 1:1 mix (50 spores each) of both species for the mixed treatment. We confirmed that this inoculum contained equal copy numbers of the fungal species using the quantitative PCR (qPCR) techniques described below. Treatments were grown under either normal (i.e. standard culture environment with 30 μM P (Declerck *et al.* 2005)) or high P conditions in which we increased the P concentration from 30 μM to 700 μM. We compensated osmotic differences between

the two environments by an equimolar reduction of KCl in the high P treatment. We grew the experimental cultures for 13 weeks.

At harvest, we first carefully removed the roots from the plate using fine tweezers and determined the weight of each root system after oven drying (at 60 °C for 3 days). We homogenized the root systems and took a subsample for DNA isolation. We then isolated the extraradical mycelium from the remaining agar fraction of the plate by dissolving the agar in 10 mM sodium citrate at 65 °C and then collected the hyphae and spores on a 0.45-μm filter membrane via vacuum filtration. The extraradical mycelial sample was subsequently freeze-dried for 24 h before DNA isolation.

### Phenotypic assay

In a second set of experiments, we collected roots from monoculture treatments (*R. irregularis* and *G. aggregatum*, *n* = 5 for each species) grown under standard P (30 μM) conditions to determine whether there was a correlation between mycorrhizal colonization percentage using trypan blue staining and the magnified intersection method (McGonigle *et al.* 1990; 100 intersections per replicate) and intraradical abundance determined by qPCR (details described below). Using these same plates, we determined extraradical mycelium dry weight and correlated this with extraradical mycelium abundance, as calculated via qPCR methods, after freeze drying the samples for 24 h. While measuring total fungal colonization percentage, we also calculated the intersections that contained specific fungal structures (i.e. vesicle and/or arbuscules) so that the relative abundances of these structures could be compared between fungal species.

### DNA isolation

For DNA isolation from both the mycelial and root samples, we used the standard protocol of the Plant Dneasy mini kit by Qiagen, with the exception that directly after the lysis step, we spiked each sample with a fixed copy number of internal standard, a plasmid containing a fragment of cassava mosaic virus. This step allowed us to determine the actual fraction of the DNA that is extracted in each sample. We then use this to calculate the theoretical copy numbers present in a 100% efficient extraction. Because the efficiency of the DNA isolation step varies among sample, this allows for a more accurate comparison.

### Molecular analysis

We analysed all samples using TaqMan probe-based qPCR (iTaq universal probes supermix), a LightCycler

(CFX96) and analysis software (CFX manager) from Bio-Rad. We ran this analysis using primers and reaction conditions that were designed to target the lesser subunit of the mitochondrial DNA previously described by Kiers *et al.* (2011). Before qPCR analysis, we diluted all samples 15-fold. We then quantified the presence of *R. irregularis, G. aggregatum* and internal standard for each sample. We produced standard curves based on a dilution series of a plasmid containing the LSU/virus fragments as the target in the qPCR. We used these standard curves to transform Cq values into raw copy numbers. We then determined DNA isolation efficiency by dividing the copy number of the internal standard in the sample by the initial copy number of the internal standard at the moment of spiking. We used the DNA isolation efficiency to normalize the raw *R. irregularis* and *G. aggregatum* copy numbers. From these copy numbers, we determined the copy number per gram dry weight of root for each root sample and mycelial sample. Finally, we determined the investment ratio (i.e. the relative investment of intraradical colonization to extraradical colonization) of the fungus by taking the ratio of mycelial copy number to root copy number.

*Statistical analysis*

We performed all statistical analyses in R (R Core Team 2012). Before analyses, we ln-transformed all qPCR data to comply with the normality assumption of future tests. We compared copy numbers of the two species at the start of the experiment for both the mixed treatment and monoculture treatment using a *t*-test. We used a linear model to test for the effects of competition treatment and P concentration on host biomass. We used Pearson correlations to test for a relationship between copy number and extraradical mycelium biomass, host sample biomass and colonization percentage by trypan blue staining. To compare the two fungal species for differences in fungal colonization rate and specific colonization structures (arbuscules and vesicles), we used a *t*-test. We used separate linear mixed effects models (lmer function from lme4 package (Bates *et al.* 2001)) to investigate differences in intraradical species-specific abundances, extraradical species-specific abundances, and the investment ratio with species, competition treatment and P concentration as fixed factors and plate nested within species as a random factor. The latter term was used to take into account the nonindependence of the responses of the two species from the same experimental replicate (Behm *et al.* 2013). The mixed treatments contained half the spore density of each species compared with the monoculture treatments. Therefore, the numbers of the monoculture treatments were halved before ln-transformation. This standardization is required in experiments with a substitutive design, which are often used in competition studies, to be able to compare the abundance of a species in mixtures to monocultures (e.g. Jolliffe 2000). To compare the investment ratios in the mixed model, we tested for an investment 'bias', which compared the investment ratio to 0 (i.e. equal investment; 0 because data were ln-transformed) using independent contrasts. We followed the mixed model analysis of the species-specific intraradical and extraradical abundances with planned contrasts of treatment means to explore the nature of competition among the species (Behm *et al.* 2013). The first set of contrasts compares the abundance of *R. irregularis* to *G. aggregatum* in the mixed treatment and then in the monoculture. The second set of contrasts quantified competition strength. Here, we compared the difference in abundance in the presence of the other species versus alone in both the mixed and monoculture treatments. We used a Bonferroni correction to adjust the *P*-values in the four sets of independent contrasts to account for multiple testing. Plates showing contamination were excluded from all analyses.

## Results

*Effects of phosphorus and competition treatments on host biomass and fungal abundances*

We found no significant effect of AMF treatment on the biomass of the host ($F_{2,17} = 0.458$, $P = 0.64$) when inoculated with either *Rhizophagus irregularis* (Mean ± SD: 0.047 g ± 0.018), *Glomus aggregatum* (Mean ± SD: 0.057 g ± 0.020) or mixture (Mean ± SD: 0.053 g ± 0.016). Likewise, P concentration in the media had no significant effect on host biomass ($F_{1,17} = 0.209$, $P = 0.65$).

We found that mean colonization percentages, as calculated via visual microscopy, were equal between the two species (Table 1; $t = 0.883$, d.f. = 8, $P = 0.40$). However, the number of arbuscules (typically indicative of nutrient transfer) and vesicles (storage structures) differed significantly between *R. irregularis* and *G. aggregatum*: *R. irregularis* produced more arbuscules (Table 1;

**Table 1** Mean fungal colonization and specific structure percentages for *Rhizophagus irregularis* and *Glomus aggregatum* monocultures under standard P conditions

|  | Colonization% | Arbuscule% | Vesicle% |
| --- | --- | --- | --- |
| *R. irregularis* | 50 (±1.2) | 31 (±2.5) | 2 (±0.6) |
| *G. aggregatum* | 54 (±4.5) | 18 (±3.1) | 7 (±1.3) |

N = 5 for each species. Numbers in parentheses are the standard error of the mean.

$t = -3.124$, d.f. $= 8$, $P = 0.01$), while roots colonized by *G. aggregatum* contained more vesicles (Table 1; $t = 3.635$, d.f. $= 8$, $P < 0.01$). We analysed the relationship between colonization percentage in roots as calculated using microscopy and copy numbers as calculated by qPCR and found no correlation (Pearson's $r = 0.006$, $P = 0.99$). We then determined the extraradical biomass, which was equal between species ($t = -2.175$; d.f. $= 7$; $P = 0.07$). We found a strong positive correlation between extraradical mycelium biomass and copy number of extraradical mycelium as calculated by qPCR (Pearson's $r = 0.807$, $P < 0.01$).

We then analysed the effects of competition on fungal abundance. We found a significant main effect of the competition treatment for intraradical colonization: total fungal abundances were lower in the mixed treatment compared with the monoculture treatment (Fig. 1A; $F_{1,32} = 4.51$, $P = 0.04$). However, we found no differences in total fungal abundances in extraradical colonization between mixed and monoculture treatments (Fig. 1B; $F_{1,32} = 3.50$, $P = 0.07$).

We found a significant main effect of species identity on fungal abundances for both the intraradical colonization (Fig. 1A; $F_{1,32} = 55.61$, $P < 0.01$) and the extraradical colonization (Fig. 1B; $F_{1,32} = 4.44$, $P = 0.04$), with *R. irregularis* reaching higher abundances. Planned contrasts used to compare fungal abundances in mixed and monoculture treatments separately showed that in monocultures, both AMF species reached equal abundances for both intraradical colonization (Fig. 1A; $P = 0.96$) and extraradical colonization (Fig. 1B, $P = 0.99$). However, in the mixed treatments, the abundance of *R. irregularis* was higher than *G. aggregatum* for both intraradical colonization (Fig. 1A, $P < 0.01$) and extraradical colonization (Fig. 1B; $P = 0.02$). We found no main effect of P concentration on fungal abundances for intraradical colonization ($F_{1,32} = 1.61$, $P = 0.21$) nor for extraradical colonization ($F_{1,32} = 0.33$, $P = 0.57$). We found no significant interactions among these factors.
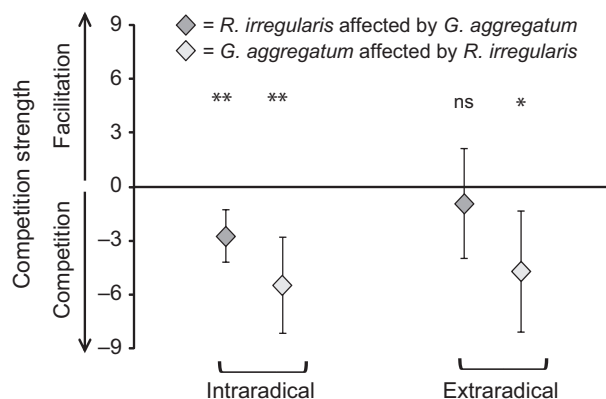
We then calculated the competition strengths (i.e. the difference in abundance in the presence of the other species minus abundance in monoculture) of both species to determine where (intraradically or extraradically) competition was the most intense for each species. We found that *G. aggregatum* experienced significant interspecific competition from *R. irregularis* for both intraradical colonization (Fig. 2, $P < 0.01$) and extraradical colonization (Fig. 2, $P < 0.01$). However, the reverse was not true: *R. irregularis* only experienced significant interspecific competition from *G. aggregatum* for intraradical colonization (Fig. 2, $P = 0.03$), but not for extraradical colonization (Fig. 2, $P = 0.99$).
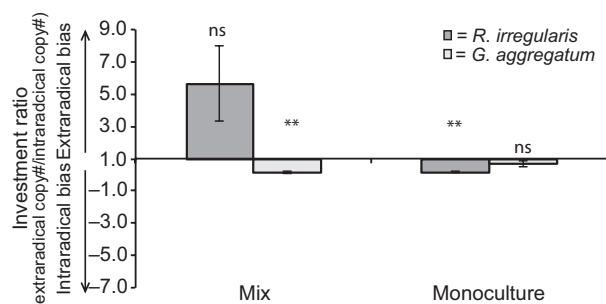
### Effects on investment ratio

We utilized a linear mixed model to examine the effects of competition on the investment ratios (i.e. the ratio of extraradical colonization to intraradical colonization) of each fungal species in mixed and monoculture treatments. We found that the two fungal species exhibited significantly different investment ratios compared with each other (Fig. 3; $F_{1,32} = 351.00$, $P < 0.01$) and that these investment ratios were significantly affected by whether the fungal species were grown in mixed or monocultures (Fig. 3; $F_{1,32} = 5.93$, $P = 0.02$). For example, *R. irregularis* shifted from a ratio in which it invested more in root abundance in monoculture (Fig. 3; $P < 0.01$) to a ratio with equal investment in both extraradical colonization and intraradical colonization in the competitive mixed culture (Fig. 3; $P = 0.18$). *G. aggregatum* displayed the opposite pattern: investment in intraradical and extraradical colonization was equal in monocultures (Fig. 3; $P = 0.99$), but did show a significant bias in mixed cultures, favouring intraradical colonization (Fig. 3; $P < 0.01$). This was highlighted by the significant interaction term for the fungal species × culture treatment (i.e. mixed versus monoculture) term at both P concentrations (Fig. 3; $F_{1,32} = 46.08$, $P < 0.01$). However, we found no significant effect of
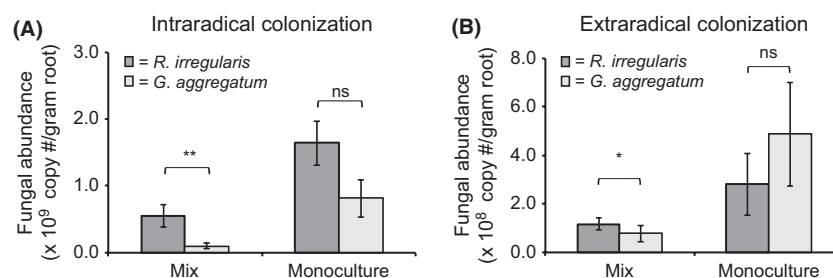


**Fig. 1** Mean abundance (i.e. copy number) of *Rhizophagus irregularis* and *Glomus aggregatum* per gram root in mixed and monoculture in (A) roots and (B) the extraradical mycelial network. The P treatments are pooled for this figure. Significance of independent comparisons are indicated by * = $P < 0.05$, ** = $P < 0.01$ and ns = not significant. N = 11 for mixed cultures and N = 10 for monocultures. Error bars represent standard error of the mean.

**Fig. 2** Competition strength in the mixed cultures for both intraradical and extraradical fractions, as calculated by the difference in fungal abundance in the presence of the other species versus alone. Difference from 0 is indicated by * = $P < 0.05$, ** = $P < 0.01$ and ns = not significant. N = 11 for mixed cultures and N = 10 for monocultures. Error bars are 95% confidence intervals from the mean.



**Fig. 3** Mean investment ratios of each fungal species as calculated by the ratio of fungal abundance in the extraradical fraction to the fungal abundance in the intraradical fraction. The P treatments are pooled for this figure. Significant ratio bias away from 1 is indicated by ** = $P < 0.01$ and ns = not significant. N = 11 for mixed cultures and N = 10 for monocultures. Error bars represent standard error of the mean.

changing P concentration on investment ratios (Fig. 3; $F_{1,32} = 0.48$, $P = 0.49$).

## Discussion

The aim of our study was to employ an *in vitro* root organ culture system to study competitive dynamics between two previously characterized fungal species (Kiers *et al.* 2011; Hart *et al.* 2013). Understanding the processes driving competition among AMF is important because researchers have found positive effects (i.e. complementary function) for hosts when colonized by multiple AMF species (Jansa *et al.* 2008; Wagg *et al.* 2011). However, other studies have found that similar host benefits can be realized in plants with a single

high-quality partner (e.g. Verbruggen *et al.* 2012). We were interested in examining these dynamics from a fungal centric point of view, asking what the effects of co-inoculation are on the AMF themselves. Specifically, we wanted to determine whether there was an effect of competition on the abundance of the fungal species, determine whether competitive pressures were more intense inside the root or outside the root and test whether the outcome of competition was modified by nutrient availability.

We found that there was a negative effect of competition on intraradical colonization, with both fungal species being less abundant in the mixed treatment than the monoculture treatment (Fig. 1). However, we did not find a negative effect of competition on fungal abundance for extraradical colonization (Fig. 1). In the mixed treatment, *Rhizophagus irregularis* was consistently the dominant symbiont (Fig. 1). We found that the presence of *R. irregularis* negatively affected *Glomus aggregatum* in both the roots and the extraradical mycelial network (Fig. 2). This is in agreement with a previous whole-plant study that found *G. aggregatum* abundance, as measured by copy number, decreased by approximately 35% when co-inoculated with *R. irregularis* compared with monocultures (Kiers *et al.* 2011). Here in an *in vitro* system, we found that *G. aggregatum* was reduced 300% when *R. irregularis* was present. Conversely, we found that *R. irregularis* experienced significant interspecific competition from *G. aggregatum* for intraradical colonization, leading to a reduced abundance, but not in the extraradical fraction (Fig. 2). That competition is more intense for intraradical than extraradical competition, agrees with earlier studies of other AMF species (e.g. Wilson & Trinick 1983; Cano & Bago 2005). This is because intraradical growth is more likely to saturate due to space constraints than extraradical colonization in the rhizosphere (Herrera Medina *et al.* 2003). We had also expected competition to be most intense for intraradical colonization because these fungal species are members of the Glomaceae family, which are typically characterized by higher intraradical than extraradical colonization rates (Hart & Reader 2002).

We found that both partners grew equally well on the host in the absence of competition: the abundance of *G. aggregatum* was equal to that of *R. irregularis* in the single-species monoculture treatments in both root and mycelium fractions (Fig. 1). What then allows *R. irregularis* to be competitively dominant in mixed cultures? Previous work has suggested that the success of *R. irregularis* is largely host-mediated: *R. irregularis* has been shown to be preferentially enriched with host carbon when co-inoculated with *G. aggregatum*, and this has been linked to the higher nutrient transfer and

lower carbon costs of *R. irregularis* compared with *G. aggregatum* (Kiers *et al.* 2011). Here, we did not see a difference in cost for the host: host biomass was equal in monocultures of both *R. irregularis* and *G. aggregatum*, suggesting that *R. irregularis* was not a better partner under these conditions. Thus, we cannot explain the higher abundances of *R. irregularis* by a host-mediated preferential allocation of resources. We did find that roots colonized by *R. irregularis* had significantly more arbuscules, generally indicative of higher nutrient transfer (Verbruggen *et al.* 2012), but this did not translate into higher root biomass in the time frame tested here. More work is needed to test whether the success of *R. irregularis* is host-mediated and due to preferential carbon enrichment (Bever *et al.* 2009; Kiers *et al.* 2011; Verbruggen *et al.* 2012) or the result of direct competitive interactions between fungal partners (Jansa *et al.* 2008), for example antagonism as they compete for limited space. However, the obligate nature of the symbiosis for the fungal partner means this question will be difficult to tease apart.

Our work is the first to document changes in investment ratio as a result of competition in an artificial root system. In monoculture treatments, species of the Glomeraceae family, such as *R. irregularis* and *G. aggregatum*, tend to bias towards intraradical investment (Hart & Reader 2002). Here, *R. irregularis* showed a stronger bias towards investment in intraradical colonization in monocultures, but this pattern disappeared in mixed cultures (Fig. 3). It has been suggested that differences in investment ratios of fungal species can help explain patterns of community structure, for example what species are likely to coexist in a particular ecosystem (Maherali & Klironomos 2012). However, our data suggest that these ratios can be plastic and change as a result of co-inoculation. It therefore is important to consider how fungal species interactions can mediate investment ratios when trying to predict patterns of community structure.

We also asked whether competition itself was modified by nutrient availability. When exposed to very high nutrient levels, host plants typically decrease resource allocation to their mycorrhizal partners, resulting in a reduction in AMF colonization (Mader *et al.* 2000). Changes in P availability have been shown to shift the outcome of competition in whole-plant systems (Pearson *et al.* 1994; Bennett & Bever 2009). However in our study, P concentration did not affect the competitive outcome in mixed culture. This may have been due to the direct availability of the nutrients in the Petri plate to both the plant and fungal partners. In nature, nutrients are typically distributed as heterogeneous pockets of resources. Such heterogeneous distributions are difficult to mimic in an *in vitro* environment because the nutrients easily dissolve and become directly accessible for the host. We see this as a clear limitation for the future use of *in vitro* root organ cultures.

While *in vitro* ROC cultures are an artificial system, it has been demonstrated that they possess similar nutrient and resource transfer and metabolic characteristics as whole-plant systems (Pfeffer *et al.* 2004), and their use has been crucial in producing a large body of literature on how nutrient transport and C exchange operates in the AMF symbiosis (Olsson *et al.* 2002; Bücking & Shachar-Hill 2005; Fellbaum *et al.* 2012). However, our inability to measure small changes in host response (e.g. biomass) is a big limitation to the system. If nutrient concentrations are too low, *in vitro* roots fail to grow, making it difficult quantify the benefits of the mutualism over a wide range of conditions (but see Koch *et al.* 2006). A second problem is that the number of host species available in ROC is limited and biased towards agricultural crops. Despite these limitations, we predict that *in vitro* approaches may be useful in further exploring fungal interactions because the fungal component of the mutualism can be easily manipulated (e.g. varying starting densities, staggering inoculation timings, testing a range of species employing different nutrient exchange strategies). Furthermore, antagonism between competing hyphae can potentially be visually identified (Croll *et al.* 2009) and rhizosphere compounds can be trapped and identified easier in sterile media than in soils (Duhamel *et al.* 2013).

As tools to compare relative abundances of competing fungi improve in accuracy and breadth, our understanding of competition in the root microbiome will greatly improve. The use of qPCR to determine AMF abundance in a colonized root system is gaining popularity (Gorzelak *et al.* 2012). Previously, fungal colonization rates were estimated visually. In agreement with other studies, we found no correlation between fungal abundance as measured by qPCR and the colonization percentage measured by the magnified intersection method (Gamper *et al.* 2008; Shi *et al.* 2012). This is not surprising given that the magnified intersection method scores the presence of fungal structures observed at the intersection of a line, but it does not count the actual number of these structures. When fungal structures are visually counted, rather than scored for absence or presence, a high correlation between fungal abundance and qPCR has been found (Alkan *et al.* 2004). As expected, we did find a high correlation between extraradical mycelium biomass and qPCR copy numbers. The use of qPCR is becoming the preferred method for calculating abundance because it both distinguishes AMF species within mixed cultures and also accurately determines the relative abundance of these species. However, to determine and compare differences in specific fungal

structures (Table 1), microscopy techniques will continue to be used.

## Conclusion

Organisms in the root microbiome are in constant competition for access to host resources and soil nutrients. In this system, we found that competition between AMF significantly reduced the abundance of both species. We found that *Rhizophagus irregularis* was the stronger competitor, which is similar to earlier experiments in a whole-plant system (Kiers *et al.* 2011). Competition was especially strong for intraradical colonization, where both species negatively affected each other's abundance. We did not observe an effect of phosphorous concentrations on fungal abundance or investment ratio, and this may be a limitation of using an *in vitro* approach. We argue that *in vitro* approaches in combination with qPCR methods are particularly useful to achieve precise manipulations of the symbiont in mixed populations, such as those needed to investigate the mechanisms driving direct antagonism in mycelial networks. However, *in vitro* approaches are, as of yet, poorly suited to achieve precise manipulations of the host. In the age of high-throughput *omics* tools in biology, we suggest that *in vitro* root organ cultures can provide a platform for investigating ecological questions that compliment molecular ones, allowing us to ask not only 'who is who in the plant root microbiome' (Hirsch & Mauchline 2012) but also 'what were the dynamics allowing them to get there'?

## Acknowledgements

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## Data accessibility

Data have been made available within the Dryad data archiving system: doi:10.5061/dryad.6j9v0.