Order of Arrival Structures
Arbuscular Mycorrhizal Colonisation of Plants

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Abstract

Priority effects - the impact of a species' arrival on subsequent community development - have been shown to influence species composition in many organisms. Whether priority effects among arbuscular mycorrhizal fungi (AMF) structure fungal root communities is not well understood. Here, we tested if priority effects influence the success of two closely related AMF species (*Rhizophagus irregularis* and *Glomus aggregatum*), hypothesising that (i) a resident AMF suppresses invader success, (ii) this effect is time-dependent and (iii) a resident will experience reduced growth when invaded. We performed two glasshouse experiments using modified pots, which permitted direct inoculation of resident and invading AMF on the roots. We quantified intraradical AMF abundances using quantitative PCR and visual colonisation percentages. We found that both fungi suppressed the invading species and that this effect was strongly dependent on the time lag between inoculations. In contrast to our expectations, neither resident AMF was negatively affected by invasion. We show that order of arrival can influence the abundance of AMF species colonising a host. These priority effects can have important implications for AMF ecology and the use of fungal inocula in sustainable agriculture.

Introduction

Most terrestrial plant species in nature are colonised by multiple species of arbuscular mycorrhizal fungi (AMF). These fungi act as symbionts, exchanging inorganic nutrients from the soil for host photosynthate (Parniske, 2008). AMF communities show considerable diversity at various scales: hundreds of taxa are found globally (Opik *et al.*, 2010) and dozens can be found in a single ecosystem (Opik *et al.*, 2008). Interesting patterns in AMF community composition in ecosystems are starting to emerge. Recent advances in large-scale sampling and sequencing efforts have revealed that seasonal and temporal effects (Husband *et al.*, 2002; Dumbrell *et al.*, 2011; Davison *et al.*, 2011), local adaptation (Ji *et al.*, 2010, 2013), host specificity (Vandenkoornhuyse *et al.*, 2002, 2003; Santos-González *et al.*, 2007; Öpik *et al.*, 2009) and environmental factors like soil type (Oehl *et al.*, 2010), soil management (Jansa *et al.*, 2002; Oehl *et al.*, 2010) and nutrient concentration (Gosling *et al.*, 2013) play a role in structuring AMF community composition.

However, despite this major progress in describing and understanding AMF community composition at the ecosystem level, we still know little about the factors determining the root composition of individual plants. One important driver of intraradical AMF community structure is competitive interactions among AMF species themselves (Wilson, 1984; Hepper *et al.*, 1988). AMF species competition has been studied across a range of systems and species and is known to be mediated by environmental factors like soil nutrients (Pearson *et al.*, 1994), soil disturbance (Verbruggen *et al.*, 2012), host plant species (Jansa *et al.*, 2008; Ehinger *et al.*, 2009) and AMF competitor species identity (Wilson & Trinick, 1983; Jansa *et al.*, 2008; Janousková *et al.*, 2009; Hart *et al.*, 2012). As obligate biotrophs, AMF are fully dependent on plant hosts for their carbon supply (Parniske, 2008). Such dependence can drive competition for access to root resources. *In-vitro* studies using root organ cultures have revealed strong competition among AMF species for intraradical colonisation of plant roots (Cano & Bago, 2005; Engelmoer *et al.*, 2014); and the ability to intensely colonise roots is a leading factor in determining the success of an AMF species (Bennett & Bever, 2009; Maherali & Klironomos, 2012).

An important question is whether the order of arrival of AMF species on a plant root system is an important factor in its subsequent colonisation success. Priority effects - the impact of the arrival of a species on subsequent community development - have been shown to structure species composition in many organisms, including nectar yeast (Peay *et al.*, 2012), wood-decomposing fungi (Fukami *et al.*, 2010; Weslien *et al.*, 2011; Dickie *et al.*, 2012), amphibians (Alford & Wilbur, 1985; Wilbur & Alford, 1985), and plant communities (Facelli & Facelli, 1993; Körner *et al.*, 2008; Ladd &

Facelli, 2008). Because AMF are horizontally transmitted, germinating seedlings are initially uncolonised by AMF. The first AMF to colonise a seedling may therefore gain a significant advantage because it will be competitor-free. Since competition among AMF over root space is intense (Cano & Bago, 2005; Engelmoer *et al.*, 2014) and some AMF species can exclude others from colonisation (Hepper *et al.*, 1988), priority effects could play a large role in structuring intraradical AMF communities, particularly early in a plant's life cycle.

Despite some pioneering work studying the effect of invasion sequence on the success of ectomycorrhizal symbionts species (Kennedy & Bruns, 2005; Kennedy *et al.*, 2007, 2009), there have been no empirical studies investigating the dynamics of priority effects among AMF. One problem has been our inability to determine the abundance of morphologically similar AMF species. However with the advent of molecular markers, we can now quantify the abundance of fungi found on the same root system (Kiers *et al.*, 2011; Thonar *et al.*, 2012, 2014; Engelmoer *et al.*, 2014).

Here, we ask if priority effects (*i.e.* sequence of arrival) influence the colonisation success of two closely related AMF species on young, uncolonised plants. We hypothesise that: (i) the first species to arrive will have an advantage, and this resident AMF will reduce the subsequent colonisation success of an invading AMF; (ii) this suppression will depend on the head start (*i.e.* time difference) of the resident fungus with an increasing head start causing a bigger reduction in invader abundance; and (iii) despite having an advantage, the resident AMF species will experience reduced intraradical growth as a result of being invaded.

Materials and Methods

Experimental Design

We performed two experiments, both using the two closely related AMF species *Glomus aggregatum* (N.C. Schenck & G.S. Sm.) and *Rhizophagus irregularis* (Blaszk., Wubet, Renker & Buscot; Walker & Schü ler, 2010), the latter formerly known as *Glomus intraradices* (Krüger *et al.*, 2012)). In the first experiment, which we called the 'simultaneous experiment' we inoculated the roots of host seedlings with a 50:50 mixed inoculum of both AMF species upon planting (t = 0). We then destructively harvested the plants after two, four and ten weeks and quantified intraradical abundances of both species. All three time treatments were replicated in 8 plants. This experiment allowed us to determine the intraradical root abundances these two species achieve when they colonise host plant roots simultaneously.

Table 1: All treatments in the priority experiment. N indicates number of replicates. AMF species used are *Glomus aggregatum* and *Rhizophagus irregularis*.

Resident species	Invading species	Head Start (weeks)	N
-	-	-	8
G. aggregatum	-	Not Invaded	12
R. irregularis	-	Not Invaded	12
-	*G. aggregatum*	2	12
R. irregularis	*G. aggregatum*	2	12
-	*G. aggregatum*	4	12
R. irregularis	*G. aggregatum*	4	12
-	*R. irregularis*	2	12
G. aggregatum	*R. irregularis*	2	12
-	*R. irregularis*	4	12
G. aggregatum	*R. irregularis*	4	12

In the second experiment, which we called the 'priority experiment', we used partitioned pots (see description below and Figure 1) to inoculate plant roots with a single AMF species (the resident) at t = 0. Subsequently, these same plant roots were inoculated with the other AMF species (the invader) after two or four weeks and harvested after ten weeks. The full experimental design of the priority experiment includes 11 treatments (Table 1), ensuring that for both invasion sequences (*G. aggregatum* invaded by *R. irregularis* and the reverse) and for both head starts (two or four weeks time difference), we could compare the abundance of the invader in treatments with a prior resident to without a resident. This addresses our first two hypotheses. By comparing uninvaded residents to invaded residents' abundances, we can address our third hypothesis.

Plant growth conditions

We used *Medicago truncatula* Gaertn. (courtesy of Prof. B. Hause, Leibniz Institute of Plant Biochemistry, Halle, Germany) as a host plant. Seeds were scarified and sterilised using 95% H_2SO_4 for 6.5 minutes and rinsed six times in an excess of demineralised water to remove all traces of acid. The scarified seeds were cold-treated at 4ºC for five days and then planted in autoclaved peat-based germination mix. After 10 days, seedling roots were carefully washed with demineralised water to remove germination mix, and seedlings were transferred to sterilised modified pots (Figure 1) containing autoclaved nutrient-poor dune sand (pH 7.2; 0.2% organic matter; 0.3 mg kg-1 P($CaCl_2$-extracted) and 190 mg kg-1 total N (Kiers *et al.*, 2011)). Plants were grown in a semi-controlled glasshouse with a 13h light cycle. The sand had a gravimetric water holding capacity of

25%; we maintained gravimetric moisture content of 12.5%. We added 14 ml per pot of Hoagland solution (Hoagland & Arnon, 1950) with N and P content reduced to 75% percent of standard solution every two weeks.
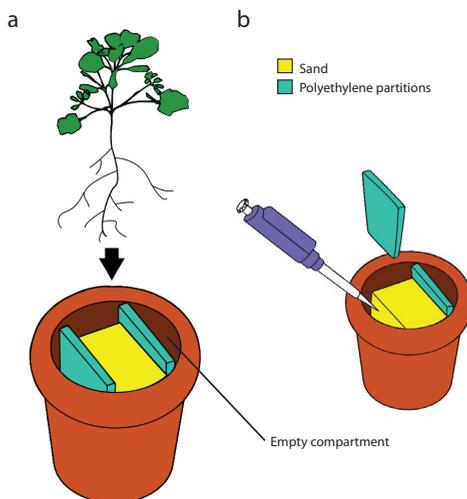
Modified pots

Figure 1: Schematic drawing of the modified pots used in this study.

(a) Polyethylene partitions (turquoise) were used to keep a portion of the pots free from soil upon planting. The two compartments on the outside of these partitions were left empty during the experiment, a seedling was planted in the middle sand (yellow) compartment. **(b)** Lifting the partitions permitted direct access from the side to the root system of the plant (plant omitted for clarity), allowing for subsequent inoculation with an invading AMF (arbuscular mycorrhizal fungus) after the time lag period.

We used round polypropylene 750 ml pots (Greiner Pots) fitted with two plastic polyethylene partitions and filled with ~450 ml of sand per pot (Figure 1a). The side partitions allowed us to create a sand-filled compartment in the centre of the pot, in which the plant was grown, and two empty compartments to both sides. During the initial growth period (*i.e.* the two or four weeks time lag), plant roots had grown against the partition plastic. By briefly lifting the partitions, we could directly access the plant root system from the empty compartments (Figure 1b). Using this set-up, we were able to apply the resident AMF inoculum directly to the roots upon initial planting of the seedlings (the resident AMF) while also applying the second AMF inoculum (the invader) directly to the roots after the lag period.

AMF inoculation

To produce AMF inocula, we grew *in vitro* cultures of *R. irregularis* isolate 09 and G. *aggregatum* isolate 0165 on *Daucus carota* L.-transformed root organ cultures for four months (for details of in vitro culture conditions, see: Engelmoer *et al.*, 2014). We then suspended the cultures in demineralised H_2O, and standardised spore densities using a custom-made spore counter. The resulting inocula contained AMF-infected root fragments and 350-450 AMF spores ml^{-1}. We then applied a suspension volume corresponding to 500 spores of the AMF treatment directly to the root system of each plant, either immediately upon planting (for the resident AMF), or after the appropriate lag period (for the invader). For the invaders, we used these same suspensions as for the residents (stored at 4 ºC in the mean time) and distributed the 500 spores evenly over the roots on each side of the root system to ensure homogenous colonisation of the plant. For the treatments that did not receive an invading AMF, partitions were similarly lifted and inoculated with a comparable volume (1.25 ml) of H_2O divided over both partitions, to mimic the same disturbance of the plant root system. In the simultaneous experiment with no lag time, we applied a mixed suspension volume corresponding to 500 spores of each of both AMF species (*i.e.* a total of 1,000 spores) immediately upon planting.

Harvest

Plants were destructively harvested two, four or ten weeks after planting, depending on the treatment. We clipped the aboveground plant at the soil surface, dried it for a minimum of five days at 60 ºC and then determined dry biomass. We extracted the full belowground plant root system from each pot and carefully washed it using demineralised H_2O to remove sand. We then blotted the root system dry using paper towels and immediately determined the fresh weight. Subsequently we cut the root system in small fragments ($\sim$ 1 cm) and randomised those fragments. We divided the randomised root fragments in two subsets: one part was frozen at -20 ºC and used for future molecular analyses and colonisation percentages, the second subset was weighed again and dried at 60 ºC for a minimum of five days prior to determining its dry mass so that the ratio of dry mass to fresh mass could be used to determine the full dry belowground biomass for each plant. Plants that were harvested after two weeks had such small root systems that we used the full root system for molecular analysis and colonisation percentages. For these plants, we used the average dry mass to fresh mass ratio in the other treatments to calculate plant belowground biomass. Full plant biomass was obtained by summing belowground and aboveground biomass. For one replicate in a priority experiment treatment, a subsample of fresh root mass was not recorded during harvest and thus we have no full root biomass date. This replicate was omitted from our analysis of plant weights.

Intraradical AMF abundance and colonisation

We used qPCR to determine intraradical abundance and root staining to visually determine colonisation levels for both AMF species. We freeze-dried a subset of the frozen randomised plant root fragments for 48 hours. We subsequently weighed the subset and used a bead-beater to fully homogenise the fragments. We extracted DNA with the Plant DNeasy kit (Qiagen), using the manufacturer's instructions but after the lysis step we added a known copy number of a plasmid containing a fragment of cassava mosaic virus as an internal standard. This allows us to quantify the efficiency of DNA extraction, and correct for variation in this efficiency among samples (Kiers *et al.*, 2011; Engelmoer *et al.*, 2014).

We used TaqMan probe-based qPCR (Bio-Rad iTaq Universal Probes Supermix) and the CFX96 Real-Time PCR Detection System (Bio-Rad) to determine AMF copy number in each DNA isolate. We used primers specific for *G. aggregatum* and *R. irregularis* that were previously described by Kiers et al. (2011). Standard curves for these primers on this analysis system were calibrated and described by Engelmoer *et al.* (2014). With these standard curves, we can use the Cq-value to calculate AMF species-specific gene copy (mtSLU) numbers in our DNA extract. This copy number is a metric for the abundance of mitochondrial DNA in both species and thereby a measure of the overall AMF abundance (Kiers *et al.*, 2011; Thonar *et al.*, 2012, 2014; Engelmoer *et al.*, 2014). We expressed copy numbers per mg freeze-dried root mass, correcting for the DNA extraction efficiency of each sample, as determined using the qPCR copy number of the internal standard. We use mtSLU copy number per unit root mass as our metric for AMF intraradical abundance, unless otherwise indicated. For samples in which Cq-values were below the limit for reliable detection (Engelmoer *et al.*, 2014), copy numbers were set to the detection limit. Since this makes it more difficult to observe repression of AMF invader colonisation by an already present resident AMF, setting low values to the detection limit is the most conservative option, given our experimental question. Using the magnified intersections method (McGonigle *et al.*, 1990), we found no contamination in any of roots of the negative controls.

To visually score colonisation percentages in the simultaneous experiment, we also used the magnified intersection method. The magnified intersections method cannot be used to discriminate closely related and morphologically identical AMF species like *R. irregularis* and *G. aggregatum.* However, these data allowed us to determine the correlation between AMF abundances in terms of qPCR copy numbers and colonisation percentages. Visual colonisation analysis is also useful to help explain to what extent the observed effects can be linked to space limitation in the roots. For each plant, we scored the presence of any AMF structures (hyphae, vesicles or arbuscules) in 100 random intersections. For two plants harvested after two weeks, the root systems were so small

that not enough material remained following the molecular analyses to make microscopy slides and score colonisation percentages.

Analysis

We performed all our analyses in R 3.1.0. (R Core Team, 2014). We first analysed the qPCR root abundance data in the simultaneous experiment with linear mixed models (LMMs) in R-package *lme4* (Bates *et al.*, 2013)*,* using the logarithm of copy numbers mg^{-1} root mass as dependent variable, AMF species, harvest date and the interaction between both of these factors as fixed effects, and plant as a random effect. This takes into account non-independence of measurements of different AMF-species taken from the same root material (Behm *et al.*, 2013; Engelmoer *et al.*, 2014). For colonisation data, we used a linear model with the harvest date as explanatory variable to test for differences in colonisation percentages between the three plant growth periods. We used Pearson's product-moment correlation to test for correlation between AMF abundance measured in copy numbers and in colonisation percentages.

We then analysed the AMF abundance data from the priority experiment (expressed as the logarithm of copy numbers mg^{-1} root mass) in two separate linear models to address our three hypotheses. In the first linear model, we used a full factorial design with the factors AMF species, resident presence and head start time to test for differences in invader abundance. This addresses our first two hypotheses. In the second linear model, we used a full factorial design with the factors AMF species, invasion time to test for differences in resident abundances. This addresses our third hypothesis. We did not use LMMs for these analyses because in both models one measurement is analysed per plant, thus avoiding non-independence of measurement. For all models analysing qPCR data, we performed post-hoc tests in R-package *phia* using Holm's method to adjust for multiple comparisons (De Rosario-Martinez, 2013).

For the plant data, we analysed plant full plant biomass (sum of aboveground and belowground biomass). In the simultaneous experiment, we used plant growth period (two, four or tens weeks) as an explanatory variable. In the priority experiment, we used treatment (Table 1) as an explanatory variable. We used Tukey Honest Significant Difference tests for post-hoc differences in our analyses of plant data.

We checked for major deviations from normal distribution of residuals for all models. All values reported are ± SE, unless otherwise indicated. The full data analysed in this study as well as the R-script for analysis have been deposited on Dryad (provisional doi: 10.5061/dryad.08c2k).

Results

Intraradical AMF abundance and colonisation increases over the plant growth period

In plants that were inoculated with mixes of both AMF species simultaneously, we found that intraradical abundance was dependent on AMF species identity ($F_{1,42}$ = 21.92, P < 0.001), harvest date (*i.e.* plant growth period) ($F_{2,42}$ = 12.87, P < 0.001) and their interaction ($F_{2,42}$ = 6.13, P < 0.01). Two weeks after inoculation, both AMF species had the same intraradical root abundance (in terms of copy number per unit root mass), but in four and ten weeks old plants, *R. irregularis* consistently achieved higher abundances than *G. aggregatum* (Figure 2). This suggests that after initial comparable infectivity of the two species, subsequent intraradical growth of *R. irregularis* was higher.
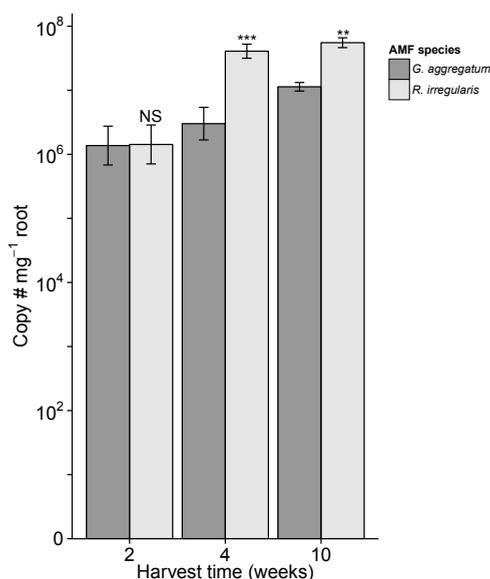

Figure 2: AMF growth under simultaneous inoculation.

Intraradical AMF abundance (copy number mg^{-1} freeze-dried root mass, ±SE) for three harvest dates and two AMF species (*Glomus aggregatum* and *Rhizophagus irregularis*) in the simultaneous experiment. Statistical significance of the copy number difference between the two AMF species within each harvest date is indicated (NS: Not Significant, *: p <0.05, **: p < 0.01, ***: p <0.001).

We also determined AMF intraradical abundance (mixture of two species) in the simultaneous experiment by visually scoring colonisation percentages. We found a strong positive correlation between colonisation percentages and the mean AMF abundance in terms of gene copy numbers (r=0.58, P<0.01). We found that colonisation percentages significantly increased over the three harvests ($F_{2,19}$ = 78.22, P < 0.001), from 29.8 % (± 5.8%) after two weeks and 68.9% (± 3.5%) after four weeks to 94.1% (± 1.0%) after the full ten week growth period (Figure S1).

Longer lag time is a disadvantage for the invading species

We then asked how a lag time between the inoculation would affect root colonisation of both AMF species. To address our first two hypotheses, we studied the intraradical abundances of invading AMF (Table 1), comparing their abundances in root systems containing a resident AMF species to their abundances after invading previously uncolonised plants. We found that both the presence of a resident species ($F_{1,88} = 16.54$, $P < 0.001$) and the time lag to the second inoculation ($F_{1,88} = 29.63$, $P < 0.001$) decreased the abundance of the invading AMF significantly (Figure 3).
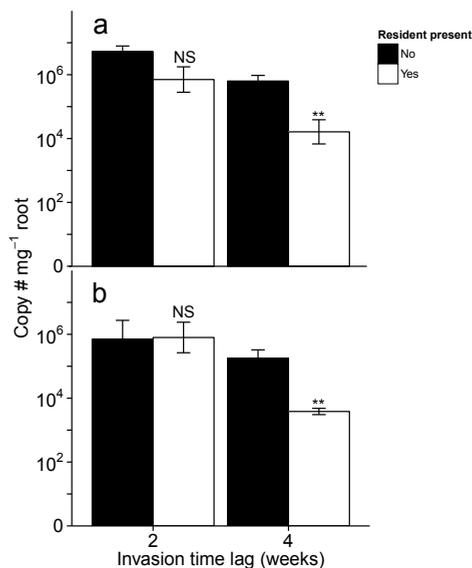

Figure 3: AMF invasion success after a time lag.

Intraradical AMF abundance (copy number mg^{-1} root dry mass, ±SE) of (A) *Glomus aggregatum* as invader and (B) *Rhizophagus irregularis* as invader after two and four weeks of head start in the absence and presence of a resident AMF. Plants were harvested ten weeks after planting. Statistical significance of the difference in copy number for invading AMF species in the presence and absence of a resident within each head start duration is indicated (NS: Not Significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

This finding holds regardless of which AMF species is the resident, and confirms our first hypothesis that a resident AMF suppresses invader colonisation success. Consistent high invader abundances in plants without resident AMF confirmed that successful invasion was possible using our inoculation method (Figure 3).

The invader disadvantage varies with time (Lag Time * Resident Presence, $F_{1,88}$ = 5.87, P = 0.02): a four weeks resident head start results in a roughly ~97% lower abundance (in terms of absolute copy numbers) for both invading AMF when compared to an invader without resident present. In contrast, a two week head start results in a 86.7% lower (but statistically insignificant) invader abundance for *G. aggregatum* compared to an invader without resident present and does not decrease the success of *R. irregularis* as an invader (11.8 % increase with resident compared with no resident). This confirms our second hypothesis that the suppression of invading AMF by a resident community depends on the head start experienced by that resident.

To test if there were differences in the ability of a species to invade roots, we included species identity as a factor in our model and found a marginally significant effect ($F_{1,88}$ = 3.92, P = 0.051), with *G. aggregatum* generally (but not in all cases) reaching higher abundances.
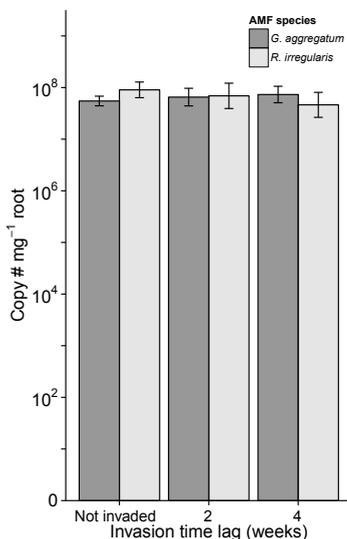

Figure 4: Resident AMF abundance.

Intraradical AMF abundance (copy number mg^{-1} root dry mass, ±SE) of both resident species (*Glomus aggregatum and Rhizophagus irregularis*), either not invaded, or invaded after two or four weeks. Main effects (Invasion time and AMF species) did not significantly affect resident intraradical root abundance.

Being invaded does not decrease the success of the resident

To answer our third hypothesis, we studied intraradical abundances of the resident AMF (Table 1). In contrast to our expectation of decreased root abundances of the invaded resident AMF, we found that being invaded does not result in a significant reduction

in the resident (Figure 4). This result was consistent across species and invasion time (AMF Species: $F_{1,66} = 0.01$, $P = 0.93$; Invasion time: $F_{2,66} = 0.11$, $P = 0.90$, AMF Species*Invasion time: $F_{2,66} = 0.63$, $P = 0.53$). These results were also confirmed when we considered the total AMF abundance per plant, rather than the abundance per unit root mass (AMF Species: $F_{1,65} = 0.01$, $P = 0.91$; Invasion time: $F_{2,65} = 0.06$, $P = 0.95$, AMF Species*Invasion time: $F_{2,65} = 0.42$, $P = 0.66$).

AMF treatment and harvest time affect plant growth

In the simultaneous experiment, we found that full plant biomass increased significantly with plant growth period ($F_{2,21} = 42.91$, $P < 0.001$), from an average of 0.12 g (± SE 0.02) at two weeks to 0.56 g (± 0.03 SE) after ten weeks.
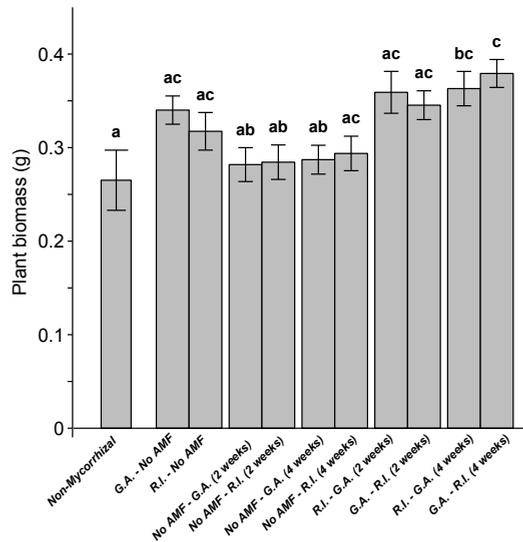

Figure 5: Full dried plant (*Medicago truncatula*) biomasses (± SE) in the priority experiment.

Letters indicate significant difference at the α = 0.05 level, using a post-hoc Tukey test. Treatment codes indicate first inoculated AMF (resident), followed by the second inoculated AMF (the invader) followed by the time lag in weeks. G.A = *Glomus aggregatum* and R.I = *Rhizophagus irregularis*.

In the priority experiment, we found that AMF treatment significantly affected plant biomass ($F_{10,116} = 4,24$, $P < 0.001$) (Figure 5). Although mycorrhizal treatments did not consistently increase plant mass compared to the negative control plants, non-mycorrhizal plants were smallest and mycorrhizal plants were an average (over all

mycorrhizal treatments) 21.4% bigger, indicating a generally positive effect of AMF on plant growth. Furthermore, we found two (non-significant) trends in our plant biomass data (Figure 5): (i) plants invaded by a second AMF had a higher average dry mass compared to those treatments with only a resident AMF and (ii) plants with a resident and an invader were on average bigger than plants with only an invader. This suggests a trend that inoculation with a second AMF generally had positive effects on plant growth, compared to inoculation with a single species.

Discussion

Here we demonstrate how arrival order is important in structuring AMF colonisation of seedlings. We found that *R. irregularis* reached higher abundances than *G. aggregatum* when they were inoculated simultaneously (Figure 2), however priority effects were able to outweigh those effects. A resident AMF with a four weeks head start could effectively suppress invader root colonisation regardless of the resident species (Figure 3). This supports our first hypothesis that an earlier arriving AMF dominates colonisation and can suppress subsequent invaders and shows how order of arrival can affect resulting AMF intraradical community composition.

Our data also reveal that the success of an invader depends upon the head start, and that this effect depends on AMF species (Figure 3): *G. aggregatum*, the species with lower abundance under simultaneous inoculation (Figure 2), had no impact on invading *R. irregularis* colonisation after a two weeks head start. However, a four week head start allowed *G. aggregatum* to substantially reduce *R. irregularis* invader colonisation. In contrast, *R. irregularis* as a resident reduced *G. aggregatum* colonisation at both time points, but much more so after four than after two weeks. These observations confirm our second hypothesis that suppression by a resident is not absolute but time-dependent, and suggest there is a species-dependent minimum head start for priority effects to be important. Correspondingly, from two to four weeks the intraradical abundance of both AMF strains (but particularly of the strongest suppresser *R. irregularis*) still increased (Figure 2). This is consistent with previous work on ectomycorrhizal fungal symbionts, where successful inhibition of invader colonisation required a minimum colonisation threshold (Kennedy *et al.*, 2009).

In contrast to our third hypothesis, we found no evidence that the resident AMF experienced a growth reduction as a result of being invaded (Figure 4). The initial resident managed to maintain its colonisation advantage despite colonisation by the invading AMF. This was also the case when we considered total abundance per plant, confirming that in this system colonisation by an invading AMF species does not reduce resident colonisation.

Mechanistic Basis of AMF priority effects

What mechanisms allow priority effects to emerge in the AMF system? While the exact explanation may differ depending on the plant-fungal combination or conditions, there are at least two non-mutually exclusive hypotheses explaining the documented patterns: (i) space limitation in host roots means all or most available root space is rapidly occupied by the initial coloniser, leaving no room for invading species or (ii) plants actively suppress colonisation of a second invading AMF species.

Past work has found intense competition among AMF for root space (Wilson, 1984; Cano & Bago, 2005; Bennett & Bever, 2009; Engelmoer *et al.*, 2014). Because intraradical growth is more likely to saturate due to space constraints than extraradical colonisation (Herrera Medina, 2003), the species given the head start has the possibility to colonise the majority of root space. A study using spatially separated inoculum sources found that AMF species can physically block each other's colonisation (Hepper *et al.*, 1988). However, the intensity of competition for root space can vary with fungal species: pre-exposure of seedlings to AMF in the *Glomeraceae* reduced the overall number of ribotypes (a measure of diversity) in roots, while pre-exposure to AMF in the *Gigasporaceae* (a family where biomass is predominantly located extraradically) did not have this effect (Mummey *et al.*, 2009). Our fungal species are both in the *Glomaceae* family, which is typically characterised by higher intraradical than extraradical colonisation rates (Hart & Reader, 2002) so we would predict high root space competition. However, we did not see space limitation in our visual colonisation data at the invasion times (Figure S1), which ranged from low (29.8%, after two weeks) to moderately high (68.9%, four weeks, arguing against space limitation as a key factor. Also, the observation that resident AMF abundance is not reduced by invasion (Figure 4), even though the invaders manage to successfully colonise plant roots (Figure 3) seems inconsistent with space limitation as important explanatory factor.

A second hypothesis is that active downregulation by the host following initial colonisation helps establish priority effects. Such effects have been found in split-root experiments in which initial AMF colonisation suppressed subsequent colonisation by different AMF species in the second root compartment (Pearson *et al.*, 1993; Vierheilig *et al.*, 2000; Vierheilig, 2004). This could explain part of the drastic suppression an invading AMF experiences from resident presence (Figure 3), and is consistent with our observation of the resident AMF being unaffected by invading AMF species (Figure 4). The ability of hosts to regulate carbon allocation to specific mycorrhizal partners has been previously documented (Kiers *et al.*, 2011), suggesting that plants have can influence fungal colonisation dynamics. While our data support this top-down hypothesis, work on split-root ectomycorrhizal colonisation suggests that prior fungal colonisation does not reduce subsequent colonisation by a second species (Kennedy *et al.*, 2009). More

work using various plant and fungal combinations is needed to understand if host regulation is an important organising principle across mycorrhizal fungi.

How important is time scale in AMF priority effects?

We cannot currently exclude the possibility that the legacy of priority effects would disappear if hosts were grown over a long period of time. An open question in ecology is whether historical contingency can lead to multiple alternative stable states, or if eventually a single stable community composition is reached (Chase, 2010; Fukami & Nakajima, 2011). In some systems, long-lasting priority effects were found (Weslien *et al.*, 2011; Plückers *et al.*, 2013), while others faded in strength relatively rapidly (Symons & Arnott, 2014). One possibility is that over longer time, inherently more competitive AMF species would dominate roots regardless of potential disadvantages of priority effects. In two ectomycorrhizal species, the slowest coloniser eventually dominated despite an initial disadvantage, suggesting that rapid colonisation upon disturbance versus slow colonisation but competitive superiority represent two different EMF strategies (Lilleskov & Bruns, 2003). Long-time experiments can explore if a competitively superior AMF could overcome initial disadvantages due to priority effects.

Medicago truncatula is annual and thus represents a good system to study priority effects on shorter time scales. Native to the Mediterranean, the host can flower within five weeks (Bucciarelli *et al.*, 2006). After a ten week growth period, 94.5% of our plants had already formed seed pots, limiting the potential for reversal of priority effects in this host. Priority effects could be particularly important in ecosystems dominated by annual seedlings, and select for AMF to take advantage of early arrival and evolve as rapid colonisers under high host turnover.

Our results show that a second factor mediating AMF priority effects is the minimum head start. Under glasshouse conditions, AMF colonisation typically occurs within 3 to 12 days after inoculation, depending on plant and on AMF species, however colonisation is thought to be less efficient in field situations (Afek *et al.*, 1990). Field colonisation speed is likely influenced by AMF density and by environmental factors, but the minima we found for priority effects to be important are in a similar range (less than two weeks for *R. irregularis*, less than four for *G. aggregatum*).

A final possibility is that over time host plants dilute priority effects by allocating more beneficial AMF more photosynthate (Bever *et al.*, 2009; Kiers *et al.*, 2011). In previous work, the more beneficial AMF *R. irregularis* was preferentially allocated more plant resources when competing with *G. aggregatum* (Kiers *et al.*, 2011), although here we could not confirm *R. irregularis* provided consistently larger plant benefits (Figure 5). If host preferential allocation is strong enough, even later arriving AMF may eventually

reach higher abundances than initially highly competitive strains that provide less benefit (Bennett & Bever, 2009), diluting priority effects and causing more beneficial AMF species to eventually become dominant. We do not find this effect in our study (Figure 4). It is likely that invader AMF growth (six to eight weeks) may have been too short for preferential allocation mechanisms to substantially affect AMF dominance. If, in different hosts or conditions, priority effects (entirely) prevent colonisation of invading AMF, they would reduce the effective number of fungal partners a plant can interact with, limiting the effectiveness of preferential allocation mechanisms (Denison & Kiers, 2011).

Conclusion

We show that (i) priority effects structure AMF colonisation dynamics of young seedlings, (ii) the strengths of these effects depends on the length of the head start and (iii) resident AMF species were not affected by later invasion. We find that these effects are unlikely to be caused by root space limitation in this system. Now research is needed to analyse the factors structuring priority effects. For example, are priority effects influenced by relatedness among AMF strains? Phylogenetic relatedness has been shown to predict priority effects in nectar yeast communities (Peay *et al.*, 2012). A second question is the effect of other plant mutualists on AMF priority effects. Strong interactions between plant root symbionts, particularly between rhizobial bacterial and mycorrhizal fungal mutualists, can effect plant mutualist communities (Larimer *et al.*, 2010, 2014): if host resource needs are already met by another mutualist, host downregulation of further AMF colonisation could potentially produce priority effects across different organisms.

Our study also raises more applied questions. Priority effects on crop seedlings may be important in agricultural settings. Applying AMF inocula to seedlings can help maximise soil nutrients uptake and increase yield in some situations (Verbruggen *et al.*, 2013). However, if natural AMF communities colonise before establishment of the inoculum, these priority effects may be hard to undo. In contrast, when naturally occurring AMF density is low, strains from AMF inocula may successfully establish themselves (Verbruggen *et al.*, 2013). An additional factor is that most agricultural soils are tilled shortly before planting. Tillage disrupts or destroys existing AMF networks; this might reduce competitive advantages of resident communities. In general, priority effects are less likely in variable environments than under constant conditions (Tucker & Fukami, 2014), suggesting they might be less likely in agricultural fields. Such dynamics are important to consider as scientists aim for better utilisation of microbial mutualisms in agriculture.

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