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Research

Order of arrival structures arbuscular mycorrhizal colonization of plants

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Summary

- 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 investigated whether priority effects influence the success of two closely related AMF species (*Rhizophagus irregularis* and *Glomus aggregatum*), hypothesizing that a resident AMF suppresses invader success, this effect is time-dependent and 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 colonization 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 colonizing 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 colonized 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; Davison *et al.*, 2011; Dumbrell *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 such as 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 such as 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 colonization of plant roots (Cano & Bago, 2005; Engelmoer *et al.*, 2014). The ability to intensely colonize 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 an AMF species on a plant root system is an important factor in its subsequent colonization 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 uncolonized by AMF. The first AMF to colonize a

seedling may therefore gain a significant advantage because it will be competitor-free. As competition among AMF over root space is intense (Cano & Bago, 2005; Engelmoer *et al.*, 2014) and some AMF species can exclude others from colonization (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 symbiont 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 asked if priority effects (i.e. sequence of arrival) influence the colonization success of two closely related AMF species on young, uncolonized plants. We hypothesized that: (1) the first species to arrive will have an advantage, and this resident AMF will reduce the subsequent colonization success of an invading AMF; (2) 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 (3) 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 & Schüßler, 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 2, 4 and 10 wk and quantified intraradical abundances of both species. All three time treatments were replicated in eight plants. This experiment allowed us to determine the intraradical root abundances these two species achieve when they colonize host plant roots simultaneously.

In the second experiment, which we called the 'priority experiment', we used partitioned pots (Fig. 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 2 or 4 wk and harvested after 10 wk. The full experimental design of the priority experiment included 11 treatments (Table 1), ensuring that, for both invasion sequences (*G. aggregatum* invaded by *R. irregularis* and the reverse) and for both head starts (2 or 4 wk time difference), we could compare the abundance of the invader in treatments with a prior resident with the abundance in treatments without a resident. This addressed our first two hypotheses. By comparing the abundances of uninvaded residents and invaded residents, we addressed our third hypothesis.

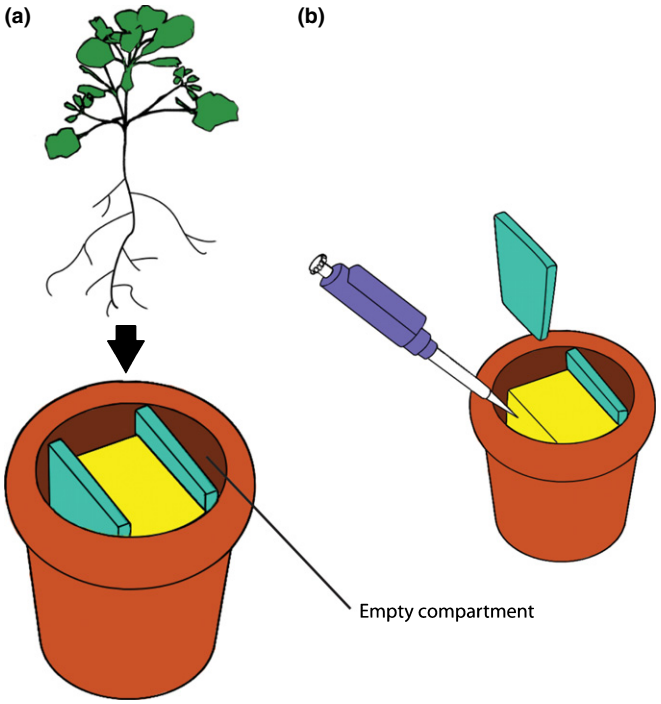

Fig. 1 Schematic drawing of the modified pots used in this study. (a) Polyethylene partitions (turquoise) were used to keep a portion of the pots free from soil upon planting. The two compartments on the outside of these partitions were left empty during the experiment, and a seedling was planted in 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 subsequent inoculation with an invading arbuscular mycorrhizal fungus (AMF) after the time lag period.

Table 1 All treatments in the priority experiment

Resident species	Invading species	Head start (wk)	*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

N, number of replicates. Arbuscular mycorrhizal fungal (AMF) species used are *Glomus aggregatum* and *Rhizophagus irregularis*.

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 sterilized using 95% H_2SO_4 for 6.5 min and rinsed six times in an excess of

demineralized water to remove all traces of acid. The scarified seeds were cold-treated at 4°C for 5 d and then planted in autoclaved peat-based germination mix. After 10 d, seedling roots were carefully washed with demineralized water to remove germination mix, and seedlings were transferred to sterilized modified pots (Fig. 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 13-h light cycle. The sand had a gravimetric water-holding capacity of 25%; we maintained a 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% of standard solution every 2 wk.

Modified pots

We used round polypropylene 750-ml pots (Greiner Pots; Greiner, Kremsmünster, Austria) fitted with two plastic polyethylene partitions and filled with *c.* 450 ml of sand per pot (Fig. 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 2 or 4 wk 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 (Fig. 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 4 months (for details of *in vitro* culture conditions, see Engelmoer *et al.*, 2014). We then suspended the cultures in demineralized H_2O, and standardized 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°C in the meantime) and distributed the 500 spores evenly over the roots on each side of the root system to ensure homogenous colonization 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 1000 spores) immediately upon planting.

Harvest

Plants were destructively harvested 2, 4 or 10 wk after planting, depending on the treatment. We clipped the aboveground plant at the soil surface, dried it for a minimum of 5 d at 60°C and then determined dry biomass. We extracted the full belowground plant root system from each pot and carefully washed it using demineralized 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 into small fragments (*c.* 1 cm) and randomized these fragments. We divided the randomized root fragments into two subsets: one subset was frozen at –20°C and used for future molecular analyses and determination of colonization percentages, and the second subset was weighed again and dried at 60°C for a minimum of 5 d before determination of 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 2 wk had such small root systems that we used the full root system for molecular analysis and determination of colonization 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 biomasses. For one replicate in a priority experiment treatment, the fresh root mass of a subsample was not recorded during harvest and thus we have no full root biomass data. This replicate was omitted from our analysis of plant weights.

Intraradical AMF abundance and colonization

We used quantitative PCR (qPCR) to determine intraradical abundance and root staining to visually determine colonization levels for both AMF species. We freeze-dried a subset of the frozen randomized plant root fragments for 48 h. We subsequently weighed the subset and used a bead-beater to fully homogenize 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 (iTaq Universal Probes Supermix; Bio-Rad) 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 quantification cycle (*Cq*) value to calculate AMF species-specific gene (mtLSU; Mitochondrial large subunit) copy numbers in our DNA extract. This copy number is a metric for the abundance of mitochondrial DNA in both species and therefore a measure of the overall AMF abundance (Kiers *et al.*, 2011; Thonar *et al.*, 2012, 2014; Engelmoer *et al.*,

2014). We calculated 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 used mtLSU 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. As this makes it more difficult to observe repression of AMF invader colonization by an already present resident AMF, setting low values for 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 the roots of the negative controls.

To visually score colonization percentages in the simultaneous experiment, we also used the magnified intersections method. The magnified intersections method cannot be used to discriminate closely related and morphologically identical AMF species such as *R. irregularis* and *G. aggregatum*. However, these data allowed us to determine the correlation between AMF abundances in terms of qPCR copy numbers and colonization percentages. Visual colonization 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 2 wk, the root systems were so small that not enough material remained following the molecular analyses to make microscopy slides and score colonization 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 the R package *lme4* (Bates *et al.*, 2013), using the logarithm of copy number mg^{-1} root mass as the dependent variable, AMF species, harvest date and the interaction between these two factors as fixed effects, and plant as a random effect. This takes into account nonindependence of measurements of different AMF species taken from the same root material (Behm *et al.*, 2013; Engelmoer *et al.*, 2014). For colonization data, we used a linear model with the harvest date as the explanatory variable to test for differences in colonization percentages between the three plant growth periods. We used Pearson's product-moment correlation to test for a correlation between AMF abundance measured in copy numbers and that measured in colonization percentages.

We then analysed the AMF abundance data from the priority experiment (expressed as the logarithm of copy number 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 addressed our first two hypotheses. In the second linear model, we used a full factorial design with the factors AMF species and invasion time to test for differences in resident abundances. This addressed our third hypothesis. We did not use LMMs for these analyses because in both models one measurement is analysed per plant, thus avoiding nonindependence of measurement. For all models analysing qPCR data, we performed post hoc tests in the 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 (the sum of aboveground and belowground biomasses). In the simultaneous experiment, we used plant growth period (2, 4 or 10 wk) 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 in Dryad (provisional doi: 10.5061/dryad.08c2k).

Results

Intraradical AMF abundance and colonization increase 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 4- and 10-wk-old plants, *R. irregularis* consistently achieved higher abundances than *G. aggregatum* (Fig. 2). This suggests that, after initial comparable infectivity of the two species, subsequent intraradical growth of *R. irregularis* was higher.

We also determined AMF intraradical abundance (mixture of the two species) in the simultaneous experiment by visually scoring colonization percentages. We found a strong positive correlation between colonization percentages and the mean AMF abundance in terms of gene copy numbers ($r = 0.58$; $P < 0.01$). We found that colonization percentages significantly increased over the three harvests ($F_{2,19} = 78.22$; $P < 0.001$), from 29.8% (± 5.8%) after 2 wk and 68.9% (± 3.5%) after 4 wk to 94.1% (± 1.0%) after the full 10-wk growth period (Supporting Information Fig. S1).

Longer lag time is a disadvantage for the invading species

We then asked how a lag time between the inoculations would affect the root colonization 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

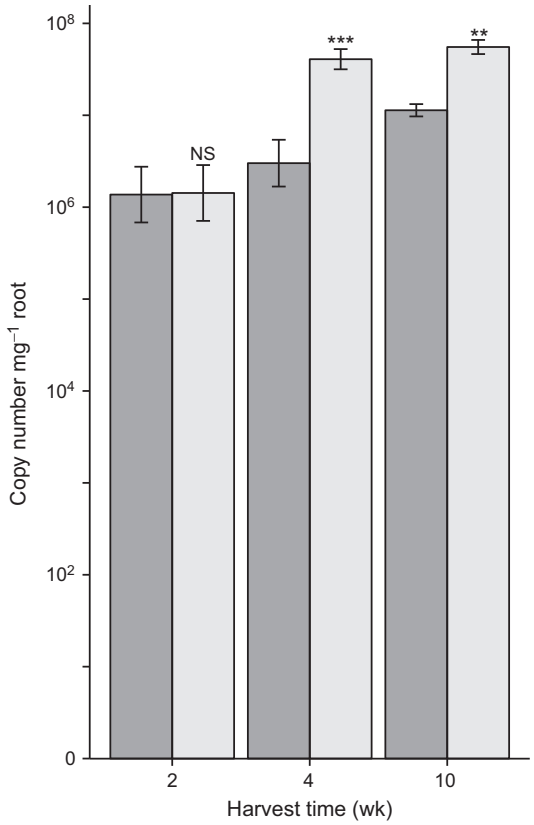

Fig. 2 Arbuscular mycorrhizal fungal (AMF) growth under simultaneous inoculation. Intraradical AMF abundance (copy number mg^{-1} freeze-dried root mass; mean ± SE) was determined for three harvest dates and two AMF species (*Glomus aggregatum* (dark grey bars) and *Rhizophagus irregularis* (light grey bars)) in the simultaneous experiment. Statistical significance of the copy number difference between the two AMF species within each harvest date: NS, not significant; **; $P < 0.01$; ***, $P < 0.001$.

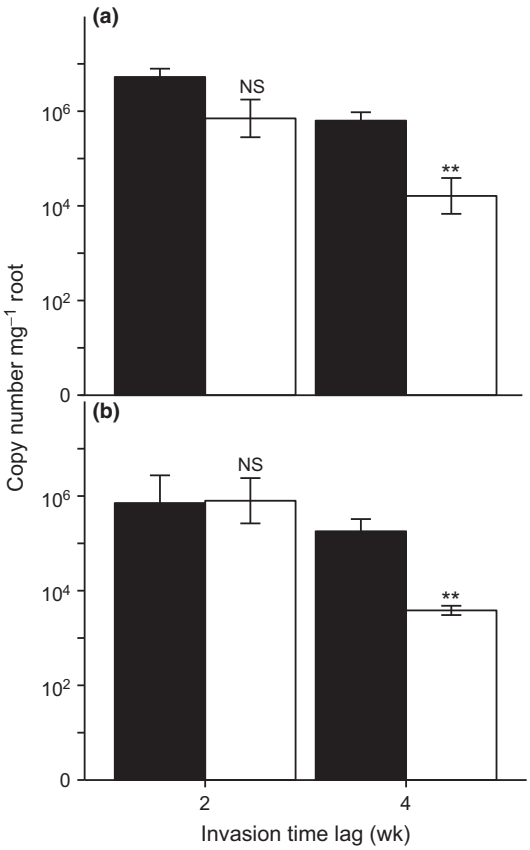

Fig. 3 Arbuscular mycorrhizal fungal (AMF) invasion success after a time lag. Intraradical AMF abundance (copy number mg^{-1} root dry mass; mean ± SE) of (a) *Glomus aggregatum* as the invader and (b) *Rhizophagus irregularis* as the invader was determined after 2 and 4 wk of head start in the absence (closed bars) and presence (open bars) of a resident AMF. *Medicago truncatula* plants were harvested 10 wk 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: NS, not significant; **; $P < 0.01$;

after invading previously uncolonized plants. We found that both the presence of a resident species ($F_{1,88} = 16.54$; $P < 0.001$) and a time lag to the second inoculation ($F_{1,88} = 29.63$; $P < 0.001$) decreased the abundance of the invading AMF significantly (Fig. 3). This finding held regardless of which AMF species was the resident, and confirms our first hypothesis that a resident AMF suppresses invader colonization success. Consistent high invader abundances in plants without resident AMF confirmed that successful invasion was possible using our inoculation method (Fig. 3).

The invader disadvantage varied with time (lag time × resident presence: $F_{1,88} = 5.87$; $P = 0.02$): a 4-wk resident head start resulted in a roughly *c.* 97% lower abundance (in terms of absolute copy numbers) for both invading AMF when compared with an invader without a resident present. By contrast, a 2-wk head start resulted in a 86.7% (but statistically insignificant) lower invader abundance for *G. aggregatum* compared with an invader without a resident present and did not decrease the success of *R. irregularis* as an invader (11.8% increase with resident compared with no resident). This confirmed our second hypothesis that the suppression of invading AMF by a resident community depends on the head start experienced by that resident.

To determine whether there were differences in the ability of 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.

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 did not result in a significant reduction in the resident (Fig. 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: $F_{2,65} = 0.42$; $P = 0.66$).

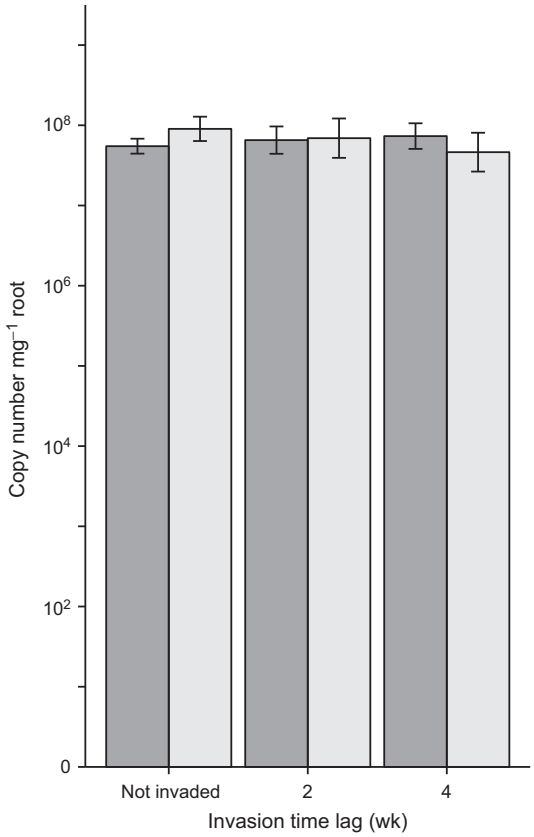

Fig. 4 Resident arbuscular mycorrhizal fungal (AMF) abundance. Intraradical AMF abundance (copy number mg^{-1} root dry mass; mean ± SE) of both resident species (*Glomus aggregatum* (dark grey bars) and *Rhizophagus irregularis* (light grey bars)), either not invaded or invaded after 2 or 4 wk, was determined. Main effects (invasion time and AMF species) did not significantly affect resident intraradical root abundance.

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 g) at 2 wk to 0.56 g (± SE 0.03 g) after 10 wk.

In the priority experiment, we found that AMF treatment significantly affected plant biomass ($F_{10,116} = 4.24$; $P < 0.001$) (Fig. 5). Although mycorrhizal treatments did not consistently increase plant mass compared with the negative control plants, nonmycorrhizal 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 (nonsignificant) trends in our plant biomass data (Fig. 5): plants invaded by a second AMF had a higher average dry mass compared with those treatments with only a resident AMF; and 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 with inoculation of a single species.

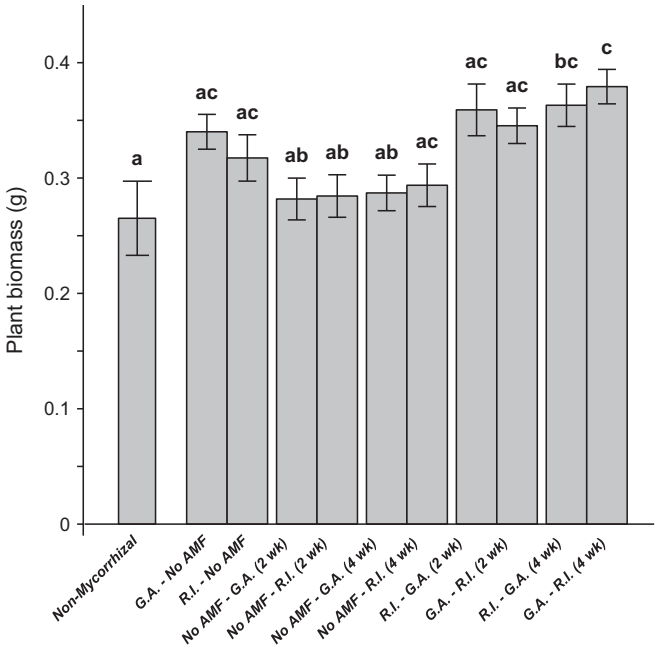

Fig. 5 Full dried plant (*Medicago truncatula*) biomasses (mean ± SE) in the priority experiment. Different letters indicate a significant difference at the $\alpha = 0.05$ level, using a post hoc Tukey test. Treatment codes indicate the first inoculated arbuscular mycorrhizal fungus (AMF) (resident), followed by the second inoculated AMF (the invader) followed by the time lag in weeks. G.A, *Glomus aggregatum*; R.I, *Rhizophagus irregularis*.

Discussion

Here we demonstrate that arrival order is important in structuring AMF colonization of seedlings. We found that *R. irregularis* reached higher abundances than *G. aggregatum* when they were inoculated simultaneously (Fig. 2); however, priority effects were able to outweigh those effects. A resident AMF with a 4-wk head start could effectively suppress invader root colonization, regardless of the resident species (Fig. 3). This supports our first hypothesis that an earlier arriving AMF dominates colonization and can suppress subsequent invaders, and shows that 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 (Fig. 3): *G. aggregatum*, the species with lower abundance under simultaneous inoculation (Fig. 2), had no impact on invading *R. irregularis* colonization after a 2-wk head start. However, a 4-wk head start allowed *G. aggregatum* to substantially reduce *R. irregularis* invader colonization. By contrast, *R. irregularis* as a resident reduced *G. aggregatum* colonization at both time-points, but much more so after 4 wk than after 2 wk. These observations confirm our second hypothesis that suppression by a resident is not absolute but time-dependent, and suggest that there is a species-dependent minimum head start for priority effects to be important. Correspondingly, from 2 to 4 wk the intraradical abundance of both AMF strains (but particularly of the strongest suppresser *R. irregularis*) still increased (Fig. 2). This is consistent with previous work on ectomycorrhizal fungal symbionts, where

successful inhibition of invader colonization required a minimum colonization 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 (Fig. 4). The initial resident managed to maintain its colonization advantage despite colonization by the invading AMF. This was also the case when we considered total abundance per plant, confirming that in this system colonization by an invading AMF species does not reduce resident colonization.

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 nonmutually exclusive hypotheses explaining the documented patterns: space limitation in host roots means that all or most available root space is rapidly occupied by the initial colonizer, leaving no room for invading species; or plants actively suppress colonization 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 as a result of space constraints than extraradical colonization (Herrera Medina, 2003), the species given the head start has the possibility to colonize the majority of root space. A study using spatially separated inoculum sources found that AMF species can physically block each other's colonization (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 characterized by higher intraradical than extraradical colonization rates (Hart & Reader, 2002), so we would predict high root space competition. However, we did not see space limitation in our visual colonization data (Fig. S1), which ranged from low (29.8% after 2 wk) to moderately high (68.9% after 4 wk), arguing against space limitation as a key factor. Also, the observation that resident AMF abundance was not reduced by invasion (Fig. 4), even though the invaders managed to successfully colonize plant roots (Fig. 3), seems inconsistent with space limitation as an important explanatory factor.

A second hypothesis is that active down-regulation by the host following initial colonization helps to establish priority effects. Such effects have been found in split-root experiments in which initial AMF colonization suppressed subsequent colonization by different AMF species in the second root compartment (Pearson *et al.*, 1993; Vierheilig *et al.*, 2000; Vierheilig, 2004). This could partly explain the drastic suppression an invading AMF experienced as a result of the presence of a resident (Fig. 3), and is consistent with our observation of the resident AMF being unaffected by the invading AMF species (Fig. 4). The ability of hosts to regulate carbon allocation to specific mycorrhizal partners has been previously documented (Kiers *et al.*, 2011), suggesting that plants can influence fungal colonization dynamics. While our data support this top-down hypothesis, work on split-root ectomycorrhizal colonization suggests that prior fungal colonization does not reduce subsequent colonization 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 organizing 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 colonizer eventually dominated despite an initial disadvantage, suggesting that rapid colonization upon disturbance versus slow colonization but competitive superiority represent two different EMF strategies (Lilleskov & Bruns, 2003). Long-term experiments should explore whether a competitively superior AMF could overcome initial disadvantages resulting from priority effects.

Medicago truncatula is an annual and thus represents a good system in which to study priority effects on shorter time-scales. Native to the Mediterranean, the host can flower within 5 wk (Bucciarelli *et al.*, 2006). After a 10-wk growth period, 94.5% of our plants had already formed seed pods, 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 colonizers under high host turnover.

Our results show that a second factor mediating AMF priority effects is the minimum head start. Under glasshouse conditions, AMF colonization typically occurs within 3–12 d after inoculation, depending on the plant and on the AMF species; however, colonization is thought to be less efficient in field situations (Afek *et al.*, 1990). Field colonization speed is probably influenced by AMF density and by environmental factors, but the minima we found for priority effects to be important are in a similar range (< 2 wk for *R. irregularis*; < 4 wk 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 that *R. irregularis* provided consistently larger plant benefits (Fig. 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 did not find this effect in our study (Fig. 4). It is likely that invader AMF growth (6–8 wk) may have been too short for preferential allocation mechanisms to substantially affect AMF dominance. If, in different hosts or conditions, priority effects (entirely) prevent colonization 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).

Conclusions

We have shown that: priority effects structured the AMF colonization dynamics of young seedlings; the strengths of these effects depended on the length of the head start; and resident AMF species were not affected by later invasion. We found that these effects were 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 affect plant mutualist communities (Larimer *et al.*, 2010, 2014): if host resource needs are already met by another mutualist, host down-regulation of further AMF colonization 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 to maximize soil nutrient uptake and increase yield in some situations (Verbruggen *et al.*, 2013). However, if natural AMF communities colonize before establishment of the inoculum, these priority effects may be hard to undo. By 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 that they might be less likely in agricultural fields. Such dynamics are important to consider as scientists aim for better utilization of microbial mutualisms in agriculture.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Visual colonization percentages in the simultaneous experiment.

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