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Research

# Host diversity affects the abundance of the extraradical arbuscular mycorrhizal network

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## Summary

- Arbuscular mycorrhizal fungi (AMF) can form complex networks in the soil that connect different host plants. Previous studies have focused on the effects of these networks on individual hosts and host communities. However, very little is known about how different host species affect the success of the fungal network itself.
- Given the potentially strong selection pressure against hosts that invest in a fungal network which benefits their competitors, we predict that the presence of multiple host species negatively affects the growth of the extraradical network.
- We designed an experiment using an *in vitro* culture approach to investigate the effect of different hosts (carrot, chichory and medicago) on the formation of a common mycelial network. *In vitro* root cultures, each inoculated with their own fungal network, were grown in a double split plate design with two host compartments and a common central compartment where fungal networks could form.
- We found that the size of fungal networks differs depending on the social environment of the host. When host species were propagated in a mixed species environment, the fungal abundance was significantly reduced compared to monoculture predictions. Our work demonstrates how host-to-host conflict can influence the abundance of the fungal partner.

## Introduction

In today's interconnected world, there is a growing interest in understanding how networks are formed and controlled (Bebber *et al.*, 2007; Tero *et al.*, 2010). Some of the largest and most complex natural networks can be found in the soil (Heaton *et al.*, 2012). Among the most abundant are the hyphal networks formed in the symbiosis between plants and arbuscular mycorrhizal fungi (AMF), which can extend for kilometres underground. AMF networks are critical for movement of nutrients through the soil ecosystem, providing interconnected plant hosts with essential inorganic nutrients, and even facilitating the transmission of host signals (Johnson *et al.*, 2002; Simard & Durall, 2004; Mikkelsen *et al.*, 2008; Barto *et al.*, 2011; Babikova *et al.*, 2013). Besides transporting nutrients and signals, AMF networks have been shown to influence the competitive dynamics between host plants. Studies have demonstrated how common fungal networks and their species composition can affect competitive outcomes among different host species, ultimately affecting host plant community composition and diversity (Hartnett & Wilson, 1999; van der Heijden & Horton, 2009; Wagg *et al.*, 2011a; Walder *et al.*, 2012).

Thus far, the majority of research on common mycorrhizal networks (CMN) has focused on aboveground effects, namely what is the effect of the network on the plant host and plant community? In contrast, relatively little is known about how host dynamics affect the success of the fungal network itself. In an experiment designed to look at the effects of host genetic relatedness, File *et al.* (2012) showed higher hyphal length in an AMF network established between a population of sibling host plants compared to a population of more distantly related host plants of the same species. This was attributed to an increased investment into the common mycorrhizal network by related kin. In contrast, Derelle *et al.* (2012) showed that seedlings of different species, integrating into an existing fungal network stimulated AMF growth to a greater extent than when two seedlings of the same species were present, but the exact relatedness of the conspecific pairing of hosts was not measured. One of the major differences in these experiments is the method by which the hosts were connected into the network. The majority of studies have focused on plants connecting into an existing network, either as seedling or as adult plants (Voets *et al.*, 2009; Derelle *et al.*, 2012), a situation that simulates undisturbed environments. However, hyphal networks are subject to continuous reconfiguration. In some cases, networks extending from different hosts can form a single network. The formation of these single networks can be formed through hyphal fusion (i.e. anastomosis) of genetically similar fungi (Giovannetti *et al.*, 2001; Croll *et al.*, 2009). The fusion of these smaller networks allows the fungal partner to rapidly increase in size, and potentially increase the access to inorganic nutrients for the host (Giovannetti *et al.*, 2004; Simard & Durall, 2004).

A major outstanding question is whether fungal networks benefit from being connected to different host species (Hart *et al.*, 2013). From a mycocentric point of view, a fungal species connected to different hosts has a more diverse choice of partners and can theoretically obtain a better carbon (C) 'payback' for their nutrient exchange (Kiers *et al.*, 2011; Wyatt *et al.*, 2014). Although increasing the number of host partners could provide a trade advantage for the fungal partner, this would not necessarily be beneficial for the host. This is because the host plant is faced with a dilemma of whether to invest into a network that is shared, and potentially benefits, competing hosts. Ultimately, we are interested in understanding whether hosts or fungi have evolved mechanisms to mediate network size or connections in the presence of competing plants.

Here, we use an *in vitro* AMF culture approach (e.g. Hammer *et al.*, 2011) to test whether fungal networks benefit from being formed in the presence of host monocultures compared to host mixed cultures. We utilized petri plates that were split into three compartments, using custom-made aluminum barriers covered by a fine mesh that could only be crossed by the fungal partner. In each of the outer compartments, a host was inoculated with a fungal partner. Subsequently, the emerging AMF network colonized a common central compartment that was free of host roots (Fig. 1). The *in vitro* approach allowed us to accurately assess both internal root colonization and extraradical fungal abundance for each host and of the central fungal network (Engelmoer *et al.*, 2014). Using combinations of three different *in vitro* host species roots (carrot, medicago, chicory), we tested for network investment (biomass, fungal abundance) to determine if the fungal abundance of the common central compartment was smaller when the host compartments contained different host species.

## Material and Methods

### Experimental procedure

Our network experiment was conducted in large petri dishes (ø 140 mm) in which we created three separate compartments. We formed these compartments using pairs of custom-made aluminium barriers (15 × 120 × 3 mm with tapered ends) covered with 50 μm nylon mesh. These were placed at each side of a plate directly after pouring the liquid adjusted MSR-medium. This approach allowed us to construct two equal sized outer host compartments at either side of the petri dish, and a central 'fungus only' compartment (Fig. 1).

In order to establish hosts, we used cultures of Ri tRNA-transformed root organ cultures (ROC) of three plant species: carrot (*Daucus carrota*), medicago (*Medicago truncatula*) and chicory (*Cichorium intybus*). Although ROCs do not produce any shoot biomass, they have similar nutrient and resource transfer and metabolic characteristics to whole plants (Pfeffer *et al.*, 2004). Hosts were grown on an adjusted MSR-medium (Declerck *et al.*, 2005; Engelmoer *et al.*, 2014). The C source in the medium is absorbed only by the host, and therefore the obligate biotrophic fungal partner is fully dependent on the host for carbohydrates. We substituted the P-sources of MSR-medium with 8.5 μM hydroxyapatite. Hydroxyapatite is a poorly soluble P-source, which increases ROC growth in the presence of AMF (see Supporting Information Fig. S1). In each host compartment we placed a fresh 2 cm root fragment from the appropriate plant species together with a 1.5 $cm^2$ agar plug from a donor culture of arbuscular mycorrhizal species *Rhizophagus irregularis* A5 Sanders Lab (FKA. *Glomus intraradices*), originally isolated from a field in Switzerland (Koch *et al.*, 2004), that had been cultivated on a carrot ROC. We maintained the experimental cultures at 25°C in the dark for 9 weeks. With this setup, we were able to create a symbiotic network in each host compartment before these networks crossed into the root-free central compartment. This separate compartment allowed us to simulate and study the interaction between two independent networks.

In total, there were six treatments. Three treatments were 'monocultures' where both host compartments contained only host species (i.e. either carrot, chicory or medicago). The other three treatments were 'mixed' cultures in which each host compartment contained a different host species. The result was a full factorial design of the three host species, each replicated ten times. After 9 weeks of growth we harvested each of the three compartments independently, as described earlier (Engelmoer

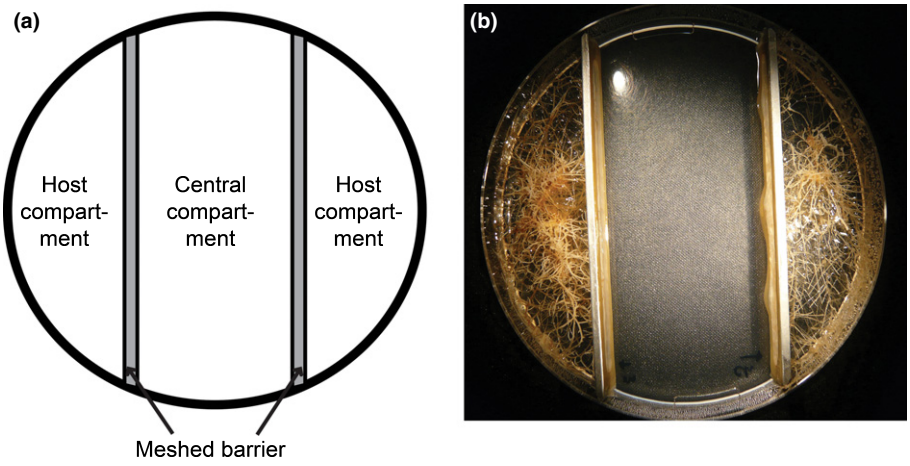


**Fig. 1** Experimental setup. (a) Schematic drawing of the experimental setup of the *in vitro* cultures. (b) Photograph of the setup with medicago cultures. Three compartments were created by custom-made aluminum barriers with a fine mesh that only allowed fungal hyphae to pass. In each host compartment a host root was inoculated with a mycelium plug resulting in the growth of two equal fungal networks, which would meet in the central compartment.

*et al.*, 2014). Briefly, we used tweezers to remove the colonized roots (intraradical fraction of the fungus) from each host compartment, while leaving the extraradical hyphae imbedded in the agar. We then collected the agar containing all fungal hyphae and spores (extraradical fraction of the fungus). Thus, together with the central compartment, there were in total five samples per plate. We stored all samples at −20°C until further analysis.

### DNA isolation

In order to isolate and quantify fungal DNA, we first removed the plate agar by treating samples with 10 mM sodium citrate at 65°C for 1 h. We then collected the extraradical mycelium on a 45-μm cellulose filter, from which we removed the extraradical mycelial samples with tweezers. We stored the samples in an Eppendorf tube at −20°C before freeze-drying. We carefully cut and homogenized the root samples before splitting each sample for DNA-isolation and backup storage. We stored the root samples at −20°C. We freeze-dried both extraradical and root samples for 48 h, after which we immediately weighed them. We then pulverized the samples with a glass bead in a bead-beater. From the pulverized samples, we isolated DNA following the standard protocol of the Qiagen Plant Dneasy Mini kit, with a slight modification at the lysis step. During this step, we spiked the samples with a known quantity of a previously described internal standard, which allowed us to normalize for DNA isolation efficiency later in the analysis (Engelmoer *et al.*, 2014).

### AMF quantification

We used Taqman probe-based qPCR following a previously described method to determine AMF abundance (Kiers *et al.*, 2011), based on Biorad's iTaq Universal Probes Supermix in their CFX96 Lightcycler for all qPCR reactions. We analysed both the abundance of *R. irregularis* and the internal standard for each sample. We determined the Cq-values, that is, the threshold value at which point illumination in the qPCR reaction starts to increase exponentially, with the CFX-manager software from Biorad using a regression approach. From the Cq-values, we calculated the copy numbers, which can be directly related to fungal biomass (Engelmoer *et al.*, 2014), present in the sample using standard curves generated from samples with a known quantity of target DNA. We then used the values of the internal standard to calculate DNA isolation efficiency (i.e. (the copy number determined by qPCR/quantity added to the DNA isolation) × 100%). Subsequently, we normalized the abundance of *R. irregularis* for the DNA isolation efficiency (Kiers *et al.*, 2011; Engelmoer *et al.*, 2014). We expressed the intraradical abundance and extraradical abundance in the host compartment as copy number per gram host DW. For the extraradical sample, this is the total sample copy number divided by the total host DW in grams. The AMF abundance in the communal central compartment was expressed as the total copy number in the sample. After we removed replicates that were contaminated from the analysis, we had 9 replicates for the medicago monoculture; 8 replicates for the chicory and medicago mix; 6 replicates for the carrot monoculture, chicory monoculture, and carrot and medicago mix; and 5 replicates for the carrot and chicory monoculture.

### Statistical analysis

We used the SPSS 21.0 package to analyse the data (IBM Corp., Armonk, NY, USA). All copy number data were natural log ($\log_e$) transformed before analysis and tested for normality with a Shapiro–Wilk test. All data followed a normal distribution after transformation. We used a one-way ANOVA with a Tukey *post hoc* test for comparisons of different host monocultures. We also used a one-way ANOVA approach to analyse fungal abundance in the host compartment of the mixed cultures compared to monocultures for each host species independently. We used a Tukey *post hoc* test to determine specific differences between cultures. For the central compartment, we made a comparison between an expected fungal abundance value and the observed AMF abundance. We calculated the expected value from the AMF abundance in the central compartment of host monocultures by the following method: Expected value = ½ fungal abundance host species 1 + ½ fungal abundance host species 2. This calculation of an expected AMF value is needed because large variation among monocultures prevents a direct comparison. As a result it is not possible to detect a difference with a mixed culture value that lies between the average of the two monocultures. Our expected value approach is based on the grand centred mean technique, and results in three different expected AMF abundance values, one for each mix of host species. We then compared these expected values with the observed AMF abundance in a one-sample *t*-test. We assumed that each root system in the monoculture contributed equally to the fungal abundance in the central compartment and that the average we found for each monoculture was close to the true mean because of the small amount of variation we found in each monoculture.

## Results

### Single hosts – establishing the baseline

We first analyzed host growth and fungal abundance in the host monocultures. We found that the host biomass differed significantly between host species ($F_{2,41} = 4.749$, $P = 0.014$). *Post hoc* testing showed that chicory hosts reached the greatest weight, whereas carrot hosts were the lowest (Fig 2a). We then investigated the fungal abundance in the host monocultures. We found a significant difference in intraradical fungal abundance among monocultures ($F_{2,41} = 6.851$, $P = 0.003$), with chicory hosting the highest fungal abundance within the roots, and medicago with the lowest (Fig. 2b). We next examined the extraradical colonization in the host compartments for the monocultures. The extraradical abundance in the host compartment differed significantly between host species (Fig. 2c; $F_{2,41} = 8.595$, $P = 0.001$). *Post hoc* testing showed that chicory hosts consistently had the highest extraradical abundance, whereas the fungal abundance did not differ between medicago and carrot hosts (Fig. 2c). Finally, we analysed the fungal abundance in the central

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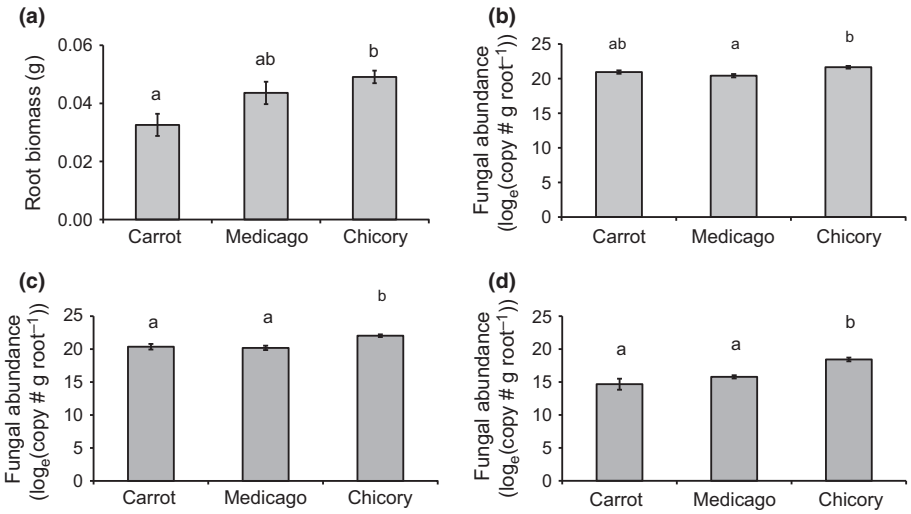


**Fig. 2** Baseline behaviour within monocultures of each host species. (a) host biomass; (b) intraradical colonization of the host; (c) extraradical colonization in the host compartment; (d) fungal abundance in the central compartment. Error bars are SE of the mean. Letters indicate significant differences following *post hoc* testing ($P<0.05$)

compartment of the monocultures and found that fungal abundance differed significantly between hosts (Fig. 2d; $F_{2,41} = 14.638$, $P < 0.001$). *Post hoc* testing again showed that chicory hosts had the highest fungal abundance in the central compartment, whereas medicago and carrot hosts maintained a similar fungal abundance. The fungal abundances in monocultures were then used as baseline values to compare against fungal abundances in mixed host cultures.

## Mixed hosts – host compartment growth

We next examined the growth and colonization of the host compartment in mixed host cultures compared to monocultures. We analysed each host species separately because of the significant differences among host species in monoculture growth. We first compared host biomass and, for each species, we found no significant difference between monoculture growth and the mixed cultures (carrot: $F_{2,22} = 2.838$, $P = 0.082$; medicago: $F_{2,30} = 0.379$, $P = 0.688$; chicory: $F_{2,25} = 0.110$, $P = 0.896$). We then investigated intraradical colonization. We found no significant differences for cultures of medicago and chicory (medicago: $F_{2,30} = 1.897$, $P = 0.169$; chicory: $F_{2,25} = 0.337$, $P = 0.717$). However, intraradical colonization of carrot hosts was significantly different ($F_{2,22} = 4{,}473$, $P = 0.025$). *Post hoc* testing showed that colonization of the carrot host in the carrot–medicago mix was significantly lower than both the carrot monoculture and the carrot–chicory mix. We did not observe a significant difference in intraradical colonization for the carrot–chicory mix compared to the carrot monoculture.

We then examined the extraradical colonization in the host compartment, again with each host species analysed independently. The extraradical colonization of carrot hosts differed significantly between treatments (Fig. 3a; $F_{2,22} = 4.872$, $P = 0.019$). *Post hoc* testing showed that this difference was between the two mixed cultures, and not between the monoculture and either mixed culture. In medicago hosts, the extraradical colonization differed significantly between treatments (Fig. 3b; $F_{2,30} = 4.648$, $P = 0.018$), and again the AMF abundance was significantly different between the two mixed cultures, but not between the

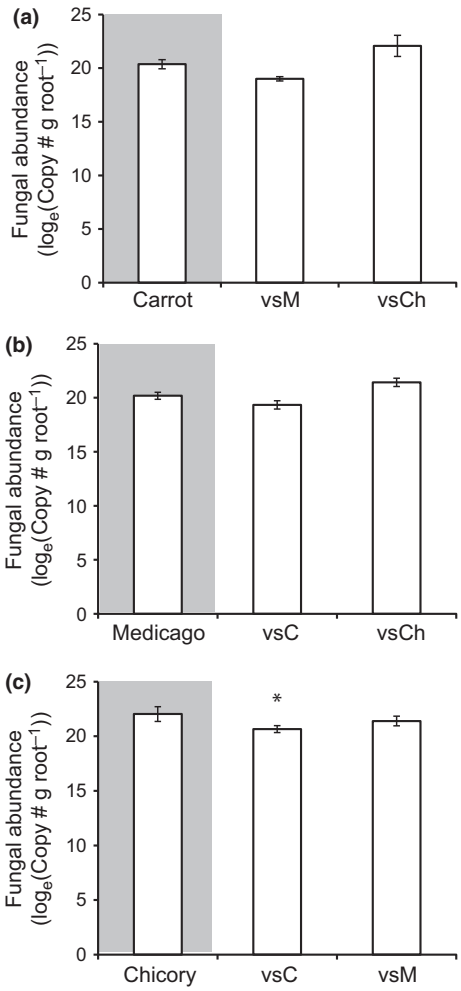


**Fig. 3** Extraradical fungal abundance within the host compartment for each host species. Bar with a grey background is the extraradical abundance found in the monoculture of the focal host species. The other two bars are the extraradical abundance for the same focal host species in a mix with a different host, which is indicated on the *x*-axis. (a) carrot host (*Daucus carrota*); (b) medicago host (*Medicago truncatula*); (c) chicory host (*Cichorium intybus*). vsC is a mix with a carrot host, vsM is a mix with a medicago host, vsCh is a mix with a chicory host. Error bars are the SE of the mean. *, indicates a significant difference from the monoculture.

monoculture and either mixed culture. Also in chicory, we found a significant difference for extraradical colonization between the different treatments (Fig. 3c; $F_{2,25} = 4.691$, $P = 0.020$). *Post hoc* tests showed that this main effect was due to the chicory in the carrot–chicory host mix being significantly lower than the chicory monoculture. However, we could not find a significant difference between the chicory monoculture and the chicory–medicago mix.

### Mixed hosts – AMF abundance in the central compartment

Following the analysis of the host compartment, we examined the fungal abundance in the common central compartment for mixed host cultures. Here, we calculated a set of expected abundance values for each of the mixed cultures based on the fungal abundance in host monocultures (see M&M). This expected AMF abundance in the central compartment was then compared with the observed AMF abundance under the treatment mixes. Using this method, we found a significantly reduced AMF colonization of the central compartment for the carrot–medicago mix (Fig. 4a; $t = -5.661$, df = 4, $P = 0.005$) and the chicory–medicago mix (Fig. 4b; $t = -5.943$, df = 7, $P = 0.001$). We observed a similar trend suggesting a reduced abundance in the carrot–chicory mix, but this was not statistically significant (Fig. 4c; $t = -1.866$, df = 5, $P = 0.121$).

## Discussion

Although common mycorrhizal networks have been studied in many systems (Simard & Durall, 2004; van der Heijden & Horton, 2009; Babikova *et al.*, 2014), the focus has been almost exclusively on how the network benefits the plant hosts. The effect of hosts on the mycorrhizal network itself is not well understood. Here we investigated the effects of host diversity on the growth of an AMF network originating from two spatially separated host roots. We did this by comparing AMF network abundance in monoculture and mixed host cultures. We expected that the total fungal abundance in the central compartment would be affected by the social environment of the host; specifically we would expect to find a reduced fungal abundance in the central compartment if the fungi originated from mixed host cultures. Such a reduction could be indicative of host–host conflict.

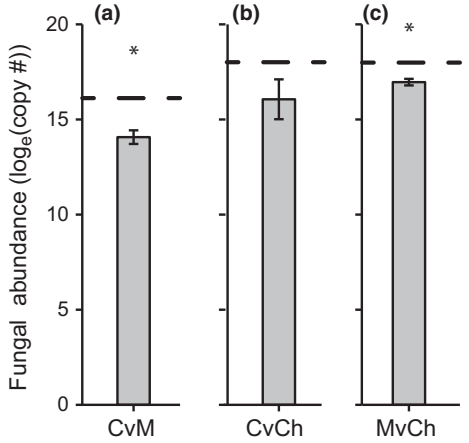


**Fig. 4** Fungal abundance in the central compartment of mixed host cultures. Dashed bars in each graph indicate the expected value of fungal abundance based on fungal abundance in the monocultures of the host species in the mix (see M&M). (a) carrot–medicago mixed host culture, b) carrot–chicory mixed host culture, and (c) medicago–chicory mixed host culture. Error bars are the SE of the mean. *, indicates a significant difference from the expected value.

We find that different ROC hosts support different levels of fungal colonization, both intra- and extraradically, with chicory hosts leading to the high fungal colonization in the host compartments (Fig. 2). We found that mixing host species had no significant influence on the fungal abundance in the host compartment (Fig. 3). This was expected because host colonization in the host compartment happens before extraradical colonization of the common central compartment. In contrast, when we examined the colonization of the common central compartment, there was evidence that the fungal networks suffered in growth when the fungi originated from two different host species: fungal abundance was three to ten times lower than expected based on the monoculture treatments (Fig. 4). Earlier work suggested that growing closely related hosts together will lead to the development of a larger fungal network when compared to distantly related host species (File *et al.*, 2012). Our work supports this finding, suggesting that combining different species of hosts in a network negatively affects fungal abundance. Although this trend may be indicative of host–host conflict in general, there is also a possibility that particular host combinations, for example, those that include medicago (Fig. 4a,b), are more pronounced in driving a reduction in fungal abundance. Although we do not know the exact mechanism behind the negative trend for hosts in combination with medicago, one possibility is that the dependency level of the host on a mycorrhizal partner might play a role (Tawaraya, 2003). We suggest that a whole-plant approach, using a wider variety of hosts and hosts of varying mycorrhizal dependency could help elucidate the generality of this finding.

Although *in vitro* root organ cultures offer many advantages for studying fungal networks, such as increasing our ability to accurately measure the abundance of the fungal network outside plant hosts, it is still difficult to document how networks interact physically. For example, we are limited in our ability to quantify hyphal fusion between networks originating from different hosts over entire 9 week long experiments (see Croll *et al.*, 2009). Without knowing if (or to what degree) networks in our study fused in the middle compartment, there are two possible explanations for our observed reduced fungal abundance in the multispecies treatments. First, we could hypothesize that the diversity of the host species reduces the compatibility between symbiotic networks. In this case we would observe less fusion and more incompatibility, similar to what is seen when genetically distant strains interact (Croll *et al.*, 2009; Cardenas-Flores *et al.*, 2010). Alternatively, if a normal fusion frequency did take place, the reduced fungal abundance could instead be mediated by direct antagonistic interactions in the shared network originating from different hosts. Such antagonism can be two-fold: first, it could be based on host-derived compounds hypothesized to move along or in hyphae (Barto *et al.*, 2011; Duhamel *et al.*, 2013; Babikova *et al.*, 2014). Second, hosts detecting network connection with a

competitor could reduce the allocation of carbohydrates to the interconnected fungal partner. In the latter case, colonization by multiple somatically incompatible fungal strains/species could benefit the host because it gives the host the option to switch carbohydrate allocation to a fungal network unconnected to competing plants. Research that specifically focuses on fusion frequency and resource allocation in networks originating from monocultures and mixed species host treatments is now needed.

Do both plant and fungal partners benefit from the formation of a large fungal network? A recent theoretical analysis using an economic framework found that fungal networks that connect several different hosts simultaneously allow the fungal partner to better exploit the nutrient exchange market (Wyatt *et al.*, 2014). Likewise, hosts are also predicted to benefit from interacting with more fungal partners (Wyatt *et al.*, 2014), and verbal arguments for the benefits of large networks in providing increased protection from fungivores in the soil (Duhamel *et al.*, 2013) and general protection from damage via increased interconnectedness (Heaton *et al.*, 2012) have been made. However, if fungal network size was solely fungus-driven, we would expect a similar size or even larger networks in the presence of multiple host species, as predicted by theoretical modelling (Wyatt *et al.*, 2014). Instead we observe the opposite, namely a decrease in fungal abundance in the presence of mixed-plant species. Although we cannot be sure of the mechanism by which host diversity is detected in our experiments (e.g. either through the fungal network or through diffusion of root exudates in the substrate (Biedrzycki *et al.*, 2010)), it is plausible that recognition mechanisms via the fungal network have evolved, given the strong selection pressures against hosts investing in a network that benefits their competitors (File *et al.*, 2012). In contrast, fungal partners may have evolved measures to limit host competitor 'cross-talk' within their networks because AMF, as obligate biotrophs, are totally dependent on the host plant for C. The interplay between such dynamics and the role of root exudates need to be studied across more plant–fungi combinations to see if these patterns of conflict in mixed host cultures consistently arise and what mechanism drives these conflicts.

Although root organ cultures are excellent tools to study the AMF–plant mutualism (Pfeffer *et al.*, 2004), specifically playing an important role in our understanding of C transport in the AMF symbiosis (Olsson *et al.*, 2002; Bucking & Shachar-Hill, 2005; Hammer *et al.*, 2011; Fellbaum *et al.*, 2012), and more recently in studying competitive interactions between different AMF species (Engelmoer *et al.*, 2014), the system is not without flaws. First, it is difficult to quantify host response via biomass measurements because of high variation in individual cultures. Second, the number of hosts available as ROCs is limited, and strongly biased towards agriculturally relevant crops. To better understand these processes in an ecological context, it is important that relevant and co-occurring host species systems are now developed.

## Conclusion

What are the principles that drive self-organized, efficient network building? These principles have potentially wide-ranging applications, from economic markets to communications networks. Here we show that networks formed between multiple plant species are not necessarily beneficial for the abundance of the fungal partner. This work highlights potential conflict between what is optimal for the host and what is optimal for the fungal partner in multi-partner settings. Now we need to understand the physical and chemical mechanisms that determine how shared networks interact.

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## Supporting Information

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

**Fig. S1** Host biomass of *in vitro* cultures grown in MSR-medium with hydroxyapatite in the presence or absence of fungal partner.

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