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Decreasing relatedness among mycorrhizal fungi in a shared plant network increases fungal network size but not plant benefit

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Abstract

Relatives are expected to cooperate more and compete less. In symbiotic partnerships, hosts may benefit from interacting with highly-related symbionts because there is less conflict. This has been difficult to test empirically. We used the arbuscular mycorrhizal symbiosis to study the effects of fungal relatedness on host and fungal benefits, creating fungal networks varying in relatedness (self vs. non-self) between two host plants, in both soil and in-vitro systems. To determine how fungal relatedness affected overall transfer of nutrients to host roots, we fluorescently tagged phosphorus and quantified resource distribution. We imaged fungal networks, and related fractal dimension to fungal growth strategies. We found that decreased relatedness was associated with increased fungal growth and complexity, lower transport of nutrients across the fungal network, and lower plant benefit, likely an outcome of increased fungal competition. More generally, we demonstrate how relatedness among symbionts can mediate the overall benefits of symbiotic partnerships.

Introduction

A key prediction in evolutionary biology is that relatives will cooperate more and compete less (Griffin & West 2002; West 2002; West *et al.* 2007). Kin selection emerges because relatives share high proportions of their genes, and by cooperating, more of these genes are passed to the next generation. Kin selection has been applied to understand a diversity of cooperative phenomena, from cooperating RNA molecules (Levin & West 2017a, b) to cooperation among human beings (dos Santos & West 2018). However, kin selection can also be vulnerable to competition among relatives, especially in cases where there is high spatial structuring in the population. Under these cases, increased competition among relatives can totally negate benefits of cooperation (Queller 1992; West 2002).

While there is an increasing understanding of when and why relatives cooperate, it is unknown how these dynamics affect organisms interacting with a group of relatives. In symbiotic partnerships, for example, a host interacts with communities of microbes, which can vary in levels of relatedness (Foster *et al.* 2017). The host may benefit from interacting with highly related strains because of reduced conflict and competition within the community (Frank 1996a, 2003; West 2002). However, interacting with less related strains may not always entail a cost for the host, and could even be beneficial. Specifically, if there is a greater relative difference among the symbiont species in their ability to acquire different, or complementary resources, the host could benefit from interacting with non-relatives (Jansa *et al.* 2008; Wagg *et al.* 2011). Likewise, if competition drives an underbidding scenario, which results in symbionts providing more benefits, for less in

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return, the host could benefit from interacting simultaneously with competing symbiotic strains (Wyatt *et al.* 2014; Noë & Kiers 2018).

Manipulating relative relatedness in symbiotic communities has historically been challenging, making direct tests of these ideas difficult. Here, we use the arbuscular mycorrhizal symbiosis to study the effects of symbiont relatedness on host plants interacting via competing or cooperating mycorrhizal fungal networks. The vast majority of land plants are colonized by arbuscular mycorrhizal fungi. The fungi trade soil bound nutrients such as phosphorus and nitrogen for photosynthetic carbon from the host plant (Jiang *et al.* 2017; Keymer *et al.* 2017; Luginbuehl *et al.* 2017). The fungi form underground networks that can connect roots of different plant individuals. Hyphal fusion, otherwise known as anastomosis, can occur among closely-related arbuscular mycorrhizal fungi (Giovannetti *et al.*2004; Jakobsen 2004). This has the potential to increase resource sharing across the fungal network (Johansen & Jensen 1996; Walder *et al.* 2012), which could increase the fitness of the fungi (Giovannetti *et al.* 2015) and potentially their hosts (Roger *et al.* 2013). However, when fungi are genetically less related, the hyphae can be vegetative incompatible and fusion will not occur (Giovannetti *et al.*2003; Croll *et al.* 2009). Direct antagonism among competing arbuscular mycorrhizal strains has been shown to lead to negative outcomes for fungal abundance and plant growth (Engelmoer *et al.*2014), and can also influence fungal co-existence within host roots (Roger *et al.* 2013). For example, past work has shown how competition between distantly related arbuscular mycorrhizal fungal isolates resulted in almost complete exclusion of one isolate by the other, whereas more related isolates shared the roots space in an almost 50:50 proportion (Roger *et al.* 2013). While this suggests that level of relatedness can affect fungal competitive dynamics within a root, it is unknown how relatedness affects the functioning of the hyphal network, especially when the hyphae connect multiple plants.

Our aim was to understand how fungal relatedness affects the physical formation and nutrient transfer in a fungal network formed between host plants. To study phosphorus distribution and transfer, we employed a recently developed technique in which we tag phosphorus rock (apatite) with fluorescent quantum-dot nanoparticles (van 't Padje *et al.*in press; Whiteside *et al.* 2019; van't Padje *et al.* 2020). Quantum-dots fluoresce in bright and pure colors when excited with UV-light. We used a class of quantum-dots that were highly fluorescent, stable and well characterized in terms of toxicity, uptake and transfer by fungal hyphae, with accumulation patterns in root (and leaf) tissue as expected (Whiteside*et al.* 2009; Gustafsson *et al.* 2015). This allowed us to determine how much phosphorus was transferred across the fungal network per unit of fungal biomass.

We grew a host root colonized by a single focal strain. The arbuscular mycorrhizal hyphae of this focal plant were allowed to interact with a fungal network of a second host plant that was either the same fungal strain ("selfing") or two genetically less-related fungal strains (both "non-selfing"). In order of highest to lowest relatedness, these treatments included: (i) the same fungal strain (selfing), (ii) a different fungal strain within the same species (non-selfing), or (iii) a fungal strain of a different species in the same genus (non-selfing). We grew these plant and fungal treatments as both whole plants in soil and as *in-vitro* root organ cultures in petri plates. The latter allowed us to determine where phosphorus was distributed across the network using our quantum-dot tagging technique, as well as to quantify the physical fungal network structure using imaging techniques (Boddy 1999; Heaton*et al.* 2012a, b). We determined how varying relatedness in fungal networks between the two host plants influenced: (i) host growth, (ii) fungal colonization inside root tissue (intraradical colonization), (iii) network formation outside the root tissue (extraradical colonization), and (iv) transfer of nutrients across the network to the host root.

Materials and methods

Experimental design

In both whole plant greenhouse and *in-vitro* root organ culture experiments, we employed a basic three-compartment setup (Olsson *et al.*2014). One compartment contained the focal plant or root, which was then consistently inoculated with the model strain *Rhizophagus irregularis* strain A5 (Sanders Lab, hereafter A5). The second compartment contained a second root inoculated with one of three fungal treatments,

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one selfing: *R. irregularis* A5, and two non-selfing fungi: *R. irregularis* strain B12 (Sanders Lab, hereafter B12) or *R. aggregatum* (hereafter Agg), listed in order of decreasing relatedness to the focal strain (Roger *et al.* 2013). These strains were chosen because they allowed us to test three levels of relatedness in a genetically well-characterized genus (Roger *et al.* 2013). In both the whole plant and *in-vitro* setup, the roots compartments were physically separated by a 'fungus-only' compartment in which the fungi from the two hosts could directly interact (Fig. 1a&b). To study the physical structure of fungal networks in *in-vitro*root organ cultures, we covered the fungus-only compartment with a cellophane sheet to restrict network growth to 2D top layer (Crawford *et al.*1993; Hitchcock *et al.* 1996; Ritz *et al.* 1996) (Fig. 1c). To determine the nutrient transport from the fungal network into the host roots, we added quantum-dot tagged apatite to the partner compartment of the *in-vitro* root organ cultures, and determined how much was transferred to the focal root (Fig. 1d).

Whole plant greenhouse experiment

Germination and growing conditions

We first performed whole plant greenhouse experiments. We used*Medicago truncatula* as a host (genotype Jemalong A17, courtesy of dr. Bettina Hause, Leibniz Institute of Plant Biochemistry, Halle, Germany), as previously (Kiers *et al.* 2011; Whiteside *et al.* 2019). We scarified *M. truncatula* seeds by submerging them in 95% sulfuric acid for 5-10 minutes, after which we rinsed the seeds with excess ddH_2O. We stored the seeds in petri dishes on moist filters, first two days in the dark at 5 @C, then one day at 20 @C in the dark, followed by two days in the light at 20 @C. We planted the germinated seeds in autoclaved germination soil. After 11 days, we selected seedlings of 3-4 cm with at least three leaves to transplant to three-compartment boxes with a 6 L capacity (Garcia *et al.*2006). The boxes were divided into three equal compartments with a 50 µm pore size nylon mesh (Cell Micro Sieves, Gentaur) glued onto a PVC window. This limited the plant roots to the outermost compartments, but allowed the fungal hyphae to grow in the central fungus-only compartment. We filled each compartment with autoclaved quartz sand and supplemented the central fungus-only compartment with 1 g hydroxyapatite per kilogram quartz sand as a phosphorus source (Pel *et al.*2018). We planted one*M. truncatula* seedling in each of the two outer compartments of the three-compartment box.

As fungal inoculum, we homogenized *in-vitro* Ri T-DNA *Daucus carota* L. transformed root organ cultures containing each fungus and added 16 mL of the mixture to the roots (˜700 spores). We randomly assigned one plant as the "focal plant". This focal plant was consistently inoculated with the strain 'A5'. The other root was designated as the partner plant, and inoculated with either A5 (n =8), B12 (n =9) or Agg (n =9) (Fig. 1a). After inoculation, we added 10 mL water to the roots and we fertilized the plants once with an adjusted Hoagland solution with 25% phosphorus (5.5 mM KNO_3; 4.0 mM $CaCl_2{\cdot}2H_2O$; 7.25 mM NH_4NO_3; 0.5 mM KH_2PO_4; 1.0 mM 20 mM $MgSO_4{\cdot}6H_2O$; Fe(Na)EDTA; 1.0 mL/L micronutrients). We placed all experimental units in a randomized grid, and we rotated them every two weeks to avoid local effects. After a week, we covered the sand with a one cm layer of sterile low-density polyethylene beads (Fardem Packaging, Edam, the Netherlands) to limit evaporation. Temperature in the greenhouse fluctuated between 20 @C and 30 @C. We watered the plants twice a week with dH_2O keeping the water content between 10 and 12.5 % and fertilized the plants every fourth day with 35 mL of an adjusted Hoagland solution, containing no phosphorus but extra nitrogen (5.5 mM KNO_3; 4.0 mM $CaCl_2{\cdot}2H_2O$; 7.25 mM NH_4NO_3; 0.5 mM KCl; 1.0 mM $MgSO_4{\cdot}6H_2O$; 20 mM Fe(Na)EDTA; 1.0 mL/L micronutrients). To confirm that our three fungal strains did not differ significantly in their mutualistic quality, we also grew single *M. truncatula* plants in standard pots of 880 mL, filled with sterile quartz sand. We inoculated host plants with either A5, B12, or Agg, and grew and fertilized plants as above. We found no statistically significant difference in either root or shoot biomass of plants grown with our three strains, confirming that they did not significantly differ in their nutrient provisioning strategy (Fig. S3).

Harvest

We harvested the plants after eight weeks and separated the shoot from the root by cutting the stem just

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below the rosette formation. We stored the shoots in paper bags and dried the material at 70 @C. We washed the sand from the roots and homogenized them. We weighed root material, and took subsamples for DNA isolation. We stored the subsamples at -20 @C and dried the remaining roots material in paper bags at 70 @C.

In-vitro **root organ cultures**

Inoculation and growing conditions

We then performed *in-vitro* root organ culture experiments. To create a three compartment *in-vitro* system, we modified squared a 4-well compartment system by removing the central barrier, creating a plate with a large central fungal compartment and two smaller root compartments (Olsson*et al.* 2014). We filled each compartment with Modified Strullu Romand (MSR) media (0.4 % phytogel, pH 5.5, 55 nM sucrose, 3980 µM N, 30 µM P, Fortin *et al.* 2002). To each focal and partner root compartment, we transplanted a branching, two cm long, section of *in-vitro* Ri T-DNA*Daucus carota* L. transformed root organ culture. We inoculated the roots with an 1x1 cm^2 agar plug containing ˜700 fungal spores. We again randomly designated one root as the "focal root", and inoculated it with *R. irregularis*A5. The partner root was inoculated with either A5, B12 or Agg (Fig. 1b). In the A5-A5 treatment, the two compartments were randomly assigned as focal or partner. We sealed the plates with parafilm and stored them in the dark at 25 @C. We placed any roots crossing into the central compartment back into the root compartment using sterile equipment.

Image analysis

To image and quantify the growth of the extraradical fungal network, we covered the central compartment of a random subset of plates (A5:n =12, B12: n =12, Agg: n =17) with a sterile cellophane sheet to facilitate 2D imaging (Fig. 1c). We monitored plates for fungal growth in the focal and partner compartment and checked weekly for fungal cross-over into the central compartment. After approximately 20 days, the first hyphae crossed the plastic barrier to the central compartment. We then imaged the entire fungal network in the central compartment using a 5x objective on a Leica Wild M8 preparation microscope, taking images with an Olympus SC180 camera.

To obtain representative images of each of the fungal strains, we selected three spatial locations with a dimension of 5x5 mm^2 (640x640 px^2) across the central fungus-only compartment in each of the treatments. The locations ranged across the space connecting the partner compartment barrier to the center of the central compartment (Fig. 1b). Using MATLAB, we applied morphological operations to the images, binarized the images, removed isolated cluster (background noise) and extracted the network skeleton of the extraradical fungi. We calculated the mass fractal dimension (D_m) of every spatial area using the box-counting technique (Hitchcock*et al.* 1996; Falconer 2003; Boddy & Donnelly. 2008; Bouda*et al.* 2016), with a square grid size ranging from 8 to 64 pixels, i.e. from $^1/_{10}$ to$^1/_{80}$ times the total square area. We then estimated the fractal dimension by:

$N(s) \propto s^{-Dm}$

Where s corresponds to the grid size and $N(s)$ the total number of boxes that contain fungal hyphae. We calculated the density of the network (surface percentage) as the ratio between the surface occupied by the network and the total square area.

Nutrient transfer

To determine nutrient transport across the fungal network and into the root growing in the focal compartment, we used a second subset of the*in-vitro* plates (A5: n =12, B12: n =8, Agg:n =12) in which we injected quantum-dot tagged apatite as a fluorescently labeled phosphorus source in the partner root compartment. We constructed green (490 nm) quantum-dot apatite by conjugating hydroxyapatite with fluorescent quantum-dots following the technique described in Whiteside*et al.* (2019). We injected 500 µL of a 126 mM phosphorus solution to the partner compartment of each replicate (Fig. 1d), and harvested these

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plates two weeks after quantum-dot apatite injection. This allowed us to quantify phosphorus transfer from one root compartment to another via the mycorrhizal network.

Harvest, fluorescent analysis and molecular analysis

We harvested all plates three months after inoculation. We discarded contaminated plates and plates in which the fungal network did not cross into the central compartment. We removed roots from the plates and dried them in paper bags and extracted extraradical hyphae from the MSR medium as described in Whiteside*et al.* (2019). We weighed the dried root and fungal material and subsampled the roots for fluorescent analysis (˜7 mg) and DNA extraction (˜20 mg). We measured phosphorus transfer to the roots by measuring the quantum-dot apatite fluorescence in the focal root systems with a Bio-Tek Synergy MX plate reader as described in Whiteside*et al.* (2019). To measure intraradical fungal colonization in the whole plant greenhouse and the *in-vitro* root organ culture experiment, and extraradical fungal abundance in the *in-vitro*root organ culture experiment, we first isolated fungal DNA using the DNeasy Plant Mini kit (Qiagen, Hombrechtikon, Switzerland), and then analyzed the fungal abundance with real time (qPCR) on DNA as described in Whiteside *et al.*2019. qPCR allowed us to obtain total copy numbers of intra- and extraradical colonization in all replicates. It also allowed us to distinguish between *R. irregularis* and R.*aggregatum* when grown in combination. In contrast*, R.irregularis* strain A5 and *R. irregularis* B12 are too genetically similar to use qPCR to differentiate their abundances. In those cases, only total abundance was measured.

Statistical analyses

We performed all statistical analysis in R version 3.3.1. We tested all data for normality with a Shapiro test and transformed data if necessary. We analyzed the data using linear models, with the independent variable as the partner strain (A5, B12 or Agg). We tested the homogeneity of the variances with a Leneve's test and checked the distribution of the residuals by eye with a normal QQ plot. We produced ANOVA type II tables with the R package car (Fox *et al.* 2016). To assess the statistical differences between the groups, we used a Tukey HSD test as post-hoc test. We calculated the ratio of intraradical colonization of focal/partner plant in the whole plant experiment by dividing the intraradical colonization of the focal root over the intraradical colonization of the partner root, (Engelmoer *et al.*2014) we then analyzed the logarithm of the ratio. For the*in-vitro* root organ culture experiment, we analyzed the logarithm of the intraradical and the extraradical colonization because the residuals were not normally distributed. We then calculated the investment ratio as a metric to quantify fungal investment into intraradical growth versus extraradical growth (Engelmoer *et al.*2014), by dividing the total extraradical copy number per plate over the total amount of intraradical copy number per plate. To calculate the network efficiency, we calculated the amount of quantum-dot apatite per total focal root over the extra-radical hyphal abundance in the focal compartment.

Results

Fungal colonization

First, we analyzed how level of fungal relatedness affected plant growth and fungal colonization in the whole plant experiment. We found that level of fungal relatedness affected the intraradical colonization of host roots: intraradical colonization of the focal plant root was significantly higher in treatments of non-selfing fungal combinations – meaning that decreased relatedness of the fungi was associated with higher colonization of the focal root. Intraradical colonization of the focal plant connected to a fungal network with high genetic relatedness (i.e. network containing selfing A5 only) was half of that observed when the partner plant was inoculated with the non-selfing partner strain B12, and a fifth of that when the partner plant was inoculated with Agg (One-way ANOVA: $F_{2,23}$=5.732, p<0.001) (Fig. 2a). The intraradical colonization of the partner plant roots showed the opposite pattern, with higher colonization of partner roots inoculated with A5 versus with B12 or Agg (One-way ANOVA: $F_{2,21}$=5.617, p=0.011) (Fig. S1 in Supporting Information). We then converted these numbers to a ratio of fungal abundance in focal roots/partner roots so we could compare colonization dynamics within replicates. A ratio of 1:1 means equal fungal growth in both roots. As expected, we found a ratio of ˜1:1 in the plants inoculated with only A5. However, as relatedness decreased, this ratio increased (˜10 times higher), meaning a bias in favor of colonization in the focal root when relatedness

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decreased (one-way ANOVA: $F_{2,21}$=15.812, p<0.0001) (Fig. 2b).

In the *in-vitro* experiment, we quantified both extraradical and intraradical fungal abundance. We found a similar trend as in the whole plant experiment: decreased relatedness (non-selfing) was associated with increased total fungal abundance. Specifically, we found that the extraradical abundance of highly related A5-A5 networks were roughly eight times lower than when the partner plant was inoculated with the non-selfing partner strain B12 or Agg (one-way ANOVA: $F_{2,37}$=9.0833, p<0.001) (Fig. 3a). However, we found intraradical fungal colonization did not differ statistically among the treatments (Focal roots: one-way ANOVA: $F_{2,38}$=0.418, p= 0.662; partner roots: $F_{2,38}$=0.68, p=0.513) (Fig. S2a&b). We also found that partner roots inoculated with Agg, were also colonized by fungal strain A5 ($7.09 \cdot 10^5$ $\pm 3.2 \cdot 10^4$ copy numbers, Fig. S2c). Since A5 and Agg are not known to fuse, this indicates that the fungus of the focal root crossed the central compartment and into the partner root compartment. We then tested whether level of relatedness affected investment ratio of the fungus, defined as fungal allocation to intraradical versus extraradical growth. We found that low fungal relatedness resulted in a bias towards more extraradical growth (one-way ANOVA: $F_{2,37}$=12.343, p<0.001) (Fig. 3b).

Fungal network architecture

Network architecture qualitatively differed with varying levels of fungal relatedness. We quantified complexity, measured as fractal dimension, D_m of the three relatedness treatments. Measuring from the focal root compartment toward the central fungus-only compartment, we found that a network composed of only A5 (i.e. selfing) increased in complexity fromD_m ˜1.1 toD_m ˜1.2 moving toward the center of the central fungus-only compartment. This was accompanied by an increase in surface area covered from 4 to 8% (Fig. 4a-c). However, when the partner root was inoculated with the non-selfing partner strain B12 (Fig. 4d-f) or Agg (Fig. 4g-i), the complexity and density of the network showed the opposite pattern, with both decreasing towards the center of the central compartment. The fractal dimensions decreased from D_m ˜ 1.3 toD_m ˜1.2 in B12 and fromD_m ˜1.5 toD_m ˜1.1 in Agg, and the surface area from 10 to 5% in B12 and from 19 to 4% in Agg.

Nutrient transfer and host plant benefit

Level of relatedness affected the amount of quantum-dot apatite per mg of fungal network transferred to the focal host root in *in-vitro*root organ cultures (one-way ANOVA: $F_{2,29}$=3.351, p=0.049) (Fig. 5a). Specifically, selfing fungal networks formed between A5-A5, transferred on average 36% more quantum-dot apatite per mg of network to the focal host roots compared to when the partner plant was inoculated with the non-selfing partner strain B12, and 28% more than when the partner plant was inoculated with Agg.

Lastly, we tested for effect of relatedness on plant biomass in both whole-plant and *in-vitro* experiments. In the whole-plant experiment, we found no significant difference among total plant biomass across relatedness treatments in the time frame of our experiment (Fig. S4). However, we did see this effect in the *in-vitro* experiment. Total root biomass of *in-vitro* roots decreased when roots were connected to non-selfing fungal strains (one-way ANOVA: $F_{2,38}$= 5.396, p= 0.009). Specifically, total root biomass was 1.5% lower when the partner root was inoculated with B12 and 11% lower when the partner root was inoculated with Agg (Fig. 5b).

Discussion

Our aim was to study the effects of varying fungal relatedness on nutrient transfer and network formation between host roots using both whole-plants and *in-vitro* root culture setups. Using a whole-plant system, we found that increased intraradical colonization of the focal plant was associated with a partner plant inoculated with a non-selfing, less related fungal strain (Fig. 2a). While this difference did not significantly affect overall plant biomass (Fig. S4), it suggests that fungal competition underground may be stimulating increased fungal colonization. This is in line with past work showing an increase in intraradical fungal abundance when a plant is inoculated simultaneously with several mycorrhizal fungal species (Jin *et al.* 2013).

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Because accurately quantifying extraradical hyphal abundance in soil-based systems is notoriously difficult (Fortin *et al.*2002), we further tested this idea using a three-compartment *in-vitro* setup in which we could analyze the architecture of the extraradical network and harvest it in its entirety. Here, we also found that a decrease in fungal relatedness between non-selfing fungal strains was associated with an increase in extraradical fungal growth (Fig. 3a). Specifically, we found that less related, non-selfing fungal strains formed larger extraradical networks between plants compared to networks of the same strain (Fig. 3a). We did not find a statistically significant effect of relatedness on intraradical colonization in the *in-vitro* system (Fig. S2a&b).

We also found that changing relatedness changed the growth strategy of the fungi. Non-selfing fungal combinations were associated with higher investment in extraradical growth compared to intraradical growth (Fig. 3b). Similar results have been found in competition assays using both different species of arbuscular mycorrhizal fungi (Engelmoer *et al.*2014) and different species of ectomycorrhizal fungi (Hortal *et al.*2016). In these cases, it was suggested that allocation to growth in the soil, rather than inside the root, could help maintain a competitive edge of fungi. More generally, theory predicts that low genetic relatedness among parasites in hosts, for example, increases competition, and favors faster growth and higher virulence (Frank 1996b; West *et al.* 2002). We found that inoculation of roots with different species, increased the competition between the arbuscular mycorrhizal fungi, which favored an investment ratio with a bias towards extraradical (Fig. 3b), especially dense near the partner root (Fig. 4d-i).

An open question is whether a higher investment in extraradical growth changes the functionality of the network in transferring nutrients. We studied network efficiency by quantifying the transfer of quantum-dot tagged apatite from the fungal network into host roots. We added quantum-dot tagged apatite as a phosphorus source to the partner root compartment, and determined how much was transferred from the fungal network into the focal root. Unlike 'pulse' techniques, this new approach allowed us to quantify cumulative patterns of phosphorus transfer from the network to the host root using visual florescence in hoot roots (van 't Padje *et al.* in press.; Whiteside *et al.* 2019; van't Padje *et al.* 2020). We found that more quantum-dot apatite was transferred per mg extraradical fungal hyphae when the two roots were inoculated the same strain (selfing) versus transferred between the two roots in the non-selfing treatments (Fig. 5a). This increased efficiency is likely the result of anastomosis, i.e. fusion of individual hyphae of the same strain (Giovannetti & Sbrana 2001; Croll *et al.* 2009), in the central compartment of the selfing treatment, between A5-A5. By fusing, fungi can tap into resources of already existing mycorrhizal fungal networks, increasing the nutrient flow (Sbrana *et al.* 2011; Giovannetti *et al.*2015; Pepe *et al.* 2016; Novais *et al.* 2017). It has also been suggested that by means of fusion, arbuscular mycorrhizal fungi could create indefinitely large networks (Giovannetti *et al.*2004), potentially allowing for higher nutrient transport across the network per unit fungus (Fig. 5a). While only a qualitative comparison, we could visually document differences in growth strategies of the fungal network by extracting descriptive architecture data. These analyses confirmed that less related strains were characterized by dense, and more complex, fungal growth in partner root compartments, while A5-A5 networks formed denser networks in the central fungal compartment (Fig. 4d-i). This increased density in the central compartment could be the result of increased anastomosis, but more work is needed to confirm this idea.

While significantly lower compared to selfing treatments, we did find that there was also transfer of nutrients from the partner root to the focal root in the non-selfing treatments. As confirmed by qPCR in the A5-Agg treatment, this transfer is likely explained by the A5 strain from the focal root crossing the fungal compartment and colonizing the partner root compartment (Fig. S2C). By crossing two physical barriers, A5 was able to form a continuous network between the two roots, facilitating movement of phosphorus between root compartments. As past worked has confirmed that the plastic barriers used here prevent the passive diffusion of the quantum-dot tagged apatite across the plate (Whiteside *et al.*2019), any movement of tagged nutrients into the fungus-only and focal root compartments is via the fungal network.

This decrease in efficiency of less-related networks was translated into a growth cost for host roots (Fig. 5b). We found a significantly lower total biomass of roots when inoculated with non-selfing strains. Taken together,

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this suggests that competition among fungi may drive an increase in fungal size, but not in phosphorus transfer benefits to the host. This result is in agreement with past work on these fungal strains suggesting that decreasing genetic relatedness within a single host root can decrease plant growth (Roger *et al.* 2013). It also agrees with work showing that plant productivity does not increase with the addition of more fungal species (Van der Heijden *et al.* 2006; Jin *et al.* 2013; Boyer *et al.* 2015; Lin*et al.* 2015). More fungal species can, depending on the specific plant-fungal combinations, even decrease plant size (Jansa *et al.* 2008; Long *et al.* 2010).

More generally, our data suggest that decreased genetic relatedness in fungal networks can drive changes in the overall effectiveness of the symbiosis. However, as the complexity of the environment increases, such that different strains are better able to acquire different or complementary resources, the benefits of interacting with a network of non-relatives may likewise increase (Koide 2000). Future work should aim to mimic the diverse challenges faced by plants growing in natural ecosystems as a further test of the costs and benefits of variation in symbiont relatedness.

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Figure legends

Fig. 1. Experimental design. (a) We grew two host plants of*Medicago truncatula* in an elongated box divided into three compartments by nylon mesh. The focal plant grew in the focal compartment and was inoculated with *Rhizophagus irregularis*strain A5 (A5). The partner plant grew in the partner compartment and was inoculated with either A5, *R. irregularis* strain B12 (B12) or*Rhizophagus aggregatum* (Agg). Only the central compartment was supplied with phosphorus (P) in the form of apatite. (b) We grew two host roots of *in-vitro Daucus carota* on a rectangular plate. The focal host root grew in the focal compartment,

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and was inoculated with A5. The partner plant grew in the partner compartment and was inoculated with either A5, B12 or Agg. The fungal hyphae from both root systems could cross over the plastic barrier into the central compartment, but the plastic barrier prevented the diffusion of nutrients. We imaged the central compartment in three locations (black squares) to study fungal architecture. (c) Side view of an*in-vitro* plate in which the central compartment was covered with a cellophane sheet to allow for 2D fungal imaging. (d) Side view of an*in-vitro* plate without cellophane sheet. The fungal hyphae could cross over the plastic barrier into the medium of the central compartment. We then added quantum-dot apatite to the partner compartment to quantify the transfer of quantum-dot tagged apatite from partner roots across the fungal network and into focal roots.

Fig. 2. Boxplot of intraradical colonization of focal roots and ratio of intraradical colonization of focal plant to partner plant in the whole plant greenhouse experiment. (a) Lower intraradical colonization of focal roots when the fungal network was composed of less-related, non-selfing fungal strains. (b) Focal and partner were colonized equally when network was composed of one strain (A5-A5), there was higher colonization of the focal root when there was a non-selfing partner fungus. Black dotted line indicates a 1:1 ratio. Box-plots with different letters indicate significant difference ($p < 0.05$), top and bottom of the box indicate the first and third quartile, and the whiskers indicate the minimum and maximum values. $n_{A5} = 8$, $n_{B12}=9$,$n_{Agg}=9$.

Fig. 3. Boxplots of extraradical fungal abundance and extraradical to intraradical investment ratio in the root organ cultures (a) Total extraradical fungal abundance (sum of all three compartments) is significantly influenced by the fungal strain in the partner compartment, with higher fungal abundance when the network is less related. (b) We found a bias towards intracellular growth when networks were selfing (A5-A5), but more extracellular growth when networks were not selfing. Box-plots with different letters indicate significant difference ($p < 0.05$), top and bottom of the box indicate the first and third quartile, and the whiskers indicate the minimum and maximum values. $n_{A5}=12$,$n_{B12}=11$, $n_{Agg}=17$.

Fig. 4. Physical architecture of extraradical network . Extraradical mycelium in the fungus-only compartment is plotted from the partner compartment barrier (left) (a), (d), (g) to the center (right) (c), (f), (i), for the three partner fungal strains. (a)-(c) A5 grows a denser and more complex network towards the center of the central compartment, increasing the surface area and theD_m . (d)-(f) B12 decreases in density and complexity towards the center of the central compartment. (g)-(i) Agg shows the highest density and complexity near the partner compartment and the least towards the center of the central compartment.

Fig. 5. Boxplots of fungal network efficiency and dry root weight of root organ cultures (a) Quantum-dot-apatite (QD) transfer to the focal roots was less efficient when associated with a less-related fungal network. $n_{A5}=12$,$n_{B12}=8$, $n_{Agg}=12$. (b) We found that the total root mass (focal + partner root) was lower when the fungal network was less related. $n_{A5}=12$,$n_{B12}=12$, $n_{Agg}=17$. Box-plots with different letters indicate significant difference ($p < 0.05$), top and bottom of the box indicate the first and third quartile, and the whiskers indicate the minimum and maximum values.

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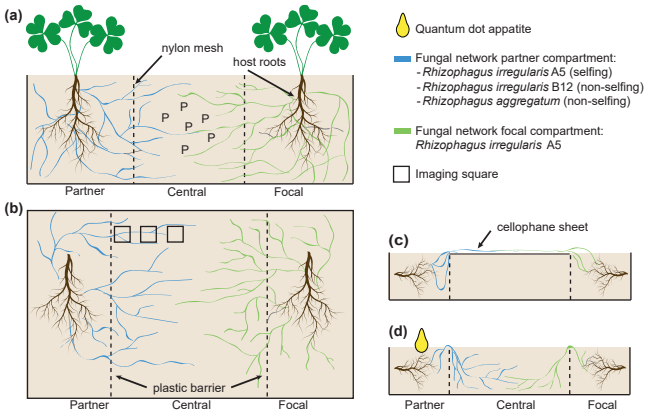

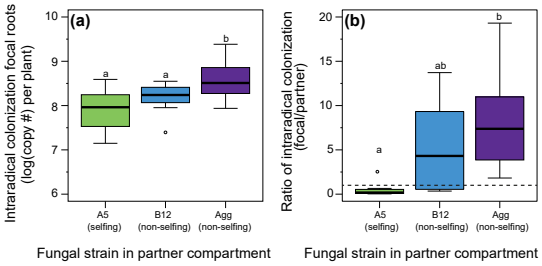

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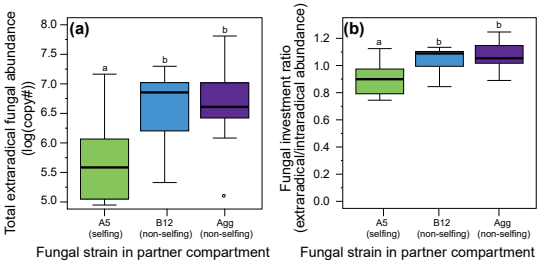

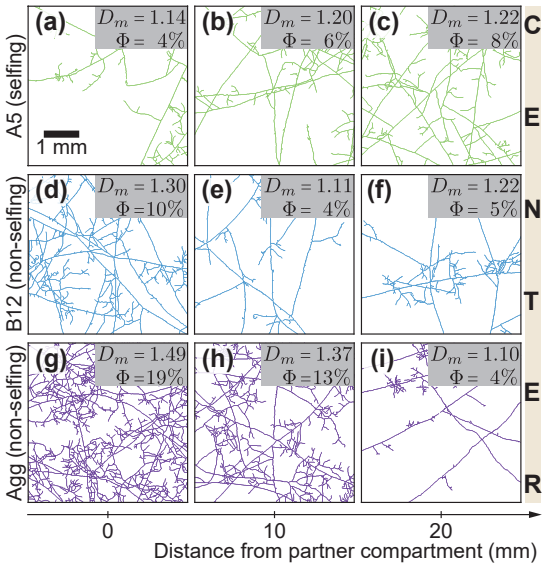

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