**TITLE:**

Investigation of Plant Interactions Across Common Mycorrhizal Networks Using Rotated Cores

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**KEYWORDS:**

common mycorrhizal networks, competition, extraradical mycelium, facilitation, mycorrhizal fungi, plant interactions, plant-soil feedback, rotated core

**SUMMARY:**

Most plants within communities likely are interconnected by arbuscular mycorrhizal (AM) fungi, but mediation of plant interactions by them has been investigated primarily by growing plants with versus without mycorrhizas. We present a method to manipulate common mycorrhizal networks among mycorrhizal plants to investigate their consequences for plant interactions.

**ABSTRACT:**

Arbuscular mycorrhizal (AM) fungi influence plant mineral nutrient uptake and growth, hence, they have the potential to influence plant interactions. The power of their influence is in extraradical mycelia that spread beyond nutrient depletion zones found near roots to ultimately interconnect individuals within a common mycorrhizal network (CMN). Most experiments, however, have investigated the role of AM fungi in plant interactions by growing plants with versus without mycorrhizal fungi, a method that fails to explicitly address the role of CMNs. Here, we propose a method that manipulates CMNs to investigate their role in plant interactions. Our method uses modified containers with conical bottoms with a nylon mesh and/or hydrophobic material covering slotted openings, 15N fertilizer, and a nutrient-poor interstitial sand. CMNs are left either intact between interacting individuals, severed by rotation of containers, or prevented from forming by a solid barrier. Our findings suggest that rotating containers is sufficient to disrupt CMNs and prevent their effects on plant interactions across CMNs. Our approach is advantageous because it mimics aspects of nature, such as seedlings tapping into already established CMNs and the use of a suite of AM fungi that may provide diverse benefits. Although our experiment is limited to investigating plants at the seedling stage, plant interactions across CMNs can be detected using our approach which therefore can be applied to investigate biological questions about the functioning of CMNs in ecosystems.

**INTRODUCTION:**

Arbuscular mycorrhizal (AM) fungi assisted plants in the colonization of land 460 million years ago1 and today, they are ubiquitous symbionts of most plants2, providing them with vital mineral nutrients for growth. The thin, thread-like hyphae of AM fungi forage for mineral nutrients beyond nutrient depletion zones near roots, often encountering and colonizing root systems of neighboring plants in a “common mycorrhizal network” (CMN). Common mycorrhizal networks also may form when fungal germlings join established networks3, or when AM hyphae fuse (anastomose) with conspecific hyphae4-7. The extent of these extraradical hyphae in the soil is enormous, with extraradical hyphae constituting 20% to 30% of total soil microbial biomass in prairie and pasture soils8 and stretching for 111 m·cm-3 in undisturbed grassland9.

Common mycorrhizal networks partition mineral nutrients among interconnected neighboring plants10-13. Plants may receive up to 80% of their phosphorus and 25% of their nitrogen requirements from AM fungi, while providing up to 20% of their total fixed carbon to the fungi in return14. Recent in vitroroot organ culture work has found that CMNs preferentially exchange mineral nutrients with host roots that provide the most carbon to the fungi11,12. Furthermore, different species of AM fungi may differ in their quality as symbiotic partners, with some fungi exchanging more phosphorus for less carbon than others15. Although root organ cultures are beneficial models for studying the AM symbiosis because they present carefully controlled environments and the ability to directly observe hyphal interconnections, they do not include photosynthesizing shoots, which affects important physiological processes such as photosynthesis, transpiration, and diurnal changes, as well as constituting carbon and mineral nutrient sinks.

In nature, seedlings most likely tap into already established CMNs. Until recently, however, scientists have only examined the impact of AM fungi on plant nutrition by growing plants with and without AM fungi, often with a single species of AM fungus. Although this work has been tremendously informative to our understanding of arbuscular mycorrhizas, this method has overlooked the potentially crucial role that CMNs may have in interactions among interconnected host plants. In particular, plants that are highly dependent on AM fungi for growth interact minimally without AM fungi16,17, possibly confounding our interpretation AM fungus-mediated interactions when used as ‘controls’ for baseline reference.

We propose a rotated-core approach for investigation of the role of CMNs in plant interactions and population structuring. Our approach mimics components of the AM symbiosis in nature because whole plants join established CMNs and all plants are grown with AM fungi. By removing root interactions, our methodology specifically focuses on interactions mediated by AM fungi while also tracking mineral nutrient movement within CMNs. Our approach builds on previous work that has used rotated cores both in the field and in the greenhouse to understand AM functioning realistically.

The rotated core method has been established in the literature as a method to manipulate extraradical hyphae18-21, and it has had several reincarnations depending on its purpose over the past two decades. Initially, mesh bags or barriers allowing in-growth of hyphae were used to provide root-free compartments to quantify the amount of arbuscular mycorrhizal hyphae in the soil22,23. Then, cylindrical cores of soil enclosed in rigid water pipes or plastic tubing with slots covered in a nylon mesh penetrable by hyphae, but not roots, were developed. These could easily be rotated to disrupt extraradical mycelia18,24,25. The rotated cores were placed between plants, and soil hyphal lengths per gram of soil18, 13C fluxes to extraradical mycelia24, or phosphorus uptake from plant-free cores were quantified18. Another use of such cores was to grow plants within them in the field to reduce colonization of roots by AM fungi through frequent hyphal disruption as an alternative to sterilization or the application of fungicides, both of which have indirect effects on soil organic matter and other microbes18.

The hyphal mesh barrier approach has been used to investigate nutrient partitioning and plant interactions across CMNs, but in rectangular microcosms rather than with rotated cores. Walder et al.26 investigated interactions between *Linum usitatissimum* (flax) and *Sorghum bicolor* (sorghum) by tracing mineral nutrient for carbon exchange using isotopes across CMNs of either of the AM fungi *Rhizophagus irregularis* or *Funneliformis mosseae*26. The microcosms in their study comprised plant compartments separated by mesh barriers, hyphal compartments only accessible to mycorrhizal hyphae, and labeled hyphal compartments that contained radioactive and stable isotopes. As controls, the study used treatments without mycorrhizal fungi. Song et al.27 used a similar approach to find that plant signals could be carried only among established CMNs of *F. mosseae* when one plant was infected by a fungal pathogen. Also, similarly to Walder et al.26, Merrild et al.28 grew plants in individual compartments separated by mesh to investigate plant performance of *Solanum lycopersicum* (tomato) seedlings linked by CMNs to a large *Cucumis sativus* (cucumber) plant that represented an abundant carbon source. They also used treatments without mycorrhizal fungi instead of severing CMNs28. In a second, related experiment, carbon for phosphorus exchange was examined using mesh bags labeled with 32P. Microcosms with hyphal mesh barriers and CMN severing as a treatment were used by Janos et al.29, who investigated competitive interactions between seedlings of the savanna tree species *Eucalyptus tetrodonta* and transplants of the rain forest tree, *Litsea glutinosa*. In that study, Janos et al.29 lifted compartments containing seedlings a few centimeters, sliding layers of mesh against one another to break hyphal interconnections29.

The final step in the evolution of the rotated core method has been to grow plants inside cores that are within pots or microcosms20,30. Wyss30 used rotated cores to ascertain if extraradical AM mycelium could colonize *Pinus elliottii* seedlings when spreading from a donor or ‘nurse’ AM host plant, *Tamarindus indica*,and how extraradical mycelium of ectomycorrhizal fungi influences seedling performance. Large commercial tubular seedling containers (**Table of Materials**) within microcosms were either solid plastic (no CMNs) or slotted and covered with a hydrophobic membrane. Slotted seedling containers were either not rotated (intact CMNs) or rotated to sever established CMNs. Rotated cores with different mesh barrier sizes were used by Babikova et al.20 to investigate belowground signaling through CMNs among *Vicia faba* (bean) plants. In their study, a central donor plant in 30 cm diameter mesocosms was interconnected either by roots and hyphae (no barrier) or only by CMNs established through a 40 μm mesh. Central plants were severed from interactions with neighboring plants through rotation of the mesh-enclosed cores, or CMNs were prevented by a fine 0.5 μm mesh enclosing the core.

Here, we present a method that combines aspects of prior rotated-core approaches to examine the influence of CMNs on direct plant interactions combined with stable isotope tracing. Our method uses a ‘target plant’ approach, in which the central plant of interest is surrounded by neighboring plants. Plants are grown inside rotatable seedling containers that are slotted and covered with nylon silk-screen mesh, hydrophobic membrane, or are non-modified solid plastic. Common mycorrhizal networks are severed once a week or kept intact, and 15N stable isotopes trace the movement of nitrogen from neighbors’ rotated cores to the central target plant. By comparing plant size with mineral nutrient and stable isotope uptake, we assess which plants may benefit or suffer from CMNs in interactions among host plants.

**PROTOCOL:**

1. **Construction and assembly of rotatable cores** 
   1. Modify commercial tubular seedling containers (subsequently called ‘containers’; **Table of Materials**) to have 19 mm wide x 48 cm length openings.
      1. Using a drill-press with a 19 mm hole saw without a central, pilot twist drill, cut two holes, one above the other, in the sides of a container (2.5 cm diameter x 12.1 cm length) so that the holes are about 1 cm apart. Hold the container against a fence on the drill press and have a stop with a short dowel that will fit inside the container to help hold it in place while drilling. Use a container with flexible plastic to prevent cracking.
      2. Cut the remaining thin piece of plastic between the holes with scissors, a wire cutter, or tin snips (for rigid plasti,c use a sabre-saw) to make one elongated opening about 2 cm wide and 5 cm long.
      3. Repeat steps 1.1.1 – 1.1.2 on the opposite side of the container.
   2. Cover the slots with nylon mesh and/or hydrophobic membrane covered slots (**Figure 1A**).
      1. Cut nylon mesh with 40 μm pores into 9.5 cm x 8.5 cm pieces. Cut as many pieces as there are containers.
      2. Glue the nylon mesh externally onto the containers to cover both openings with some slight overlap in the fabric using high strength, industrial hot glue.
      3. If the prevention of water movement is needed, such as when using water soluble nutrients or stable isotopes, cover the nylon mesh layer with a hydrophobic membrane31,32 (**Table of Materials**) that allows AM fungus hyphae to pass, but only the movement of water vapor and not liquid water.
      4. Place the hot glue around the openings on the container and along the long edges of the nylon mesh. Roll the container onto the fabric to avoid burning fingers. Add a vertical layer of glue where the mesh edges overlap. Press the edge onto some cardboard to firmly seal it. Always roll consistently in one direction that will be the same direction of the rotation of the finished containers within pots or microcosms so that the overlapped mesh edge will not be pushed to potentially dig into the substrate.
      5. Once the glue has cooled, tape the top and bottom ends of the fabric to the container to prevent loose edges and ripping using a flexible tape, such as electric tape.
   3. Using the same tape as in step 1.2.3, cover the small holes on the sides of the conical end (not the hole at the tip of the bottom) of each container to prevent root growth out of the container into the rest of the pot/microcosm.
   4. To prevent soil loss while providing drainage, place a glass marble into the bottom of each container.
   5. For a control treatment that does not involve any potential for a CMN to form between plants, use solid, unmodified containers (**Figure 1A**).
2. **Assembly of pots or microcosms to fit the conical ends of the containers** 
   1. To ensure containers stand upright in a fixed position and have proper drainage, flip a pot over so that the bottom is facing up. Cut around the bottom of the pot, leaving a small lip for support, using a sabre saw.
   2. Preparation of polystyrene foam
      1. Cut polystyrene foam, about 36 mm thick, to the same diameter as the bottom of the pot using a bandsaw with a circle-cutting jig.
      2. Drill holes into the foam using a drill press and 19 mm hole saw (without a central twist drill) in the pattern in which the containers will be positioned.
      3. For a target plant experiment, drill a central hole with equally spaced holes for neighboring individuals surrounding it. For a pot with a 15.5 cm diameter, space six holes 12 mm apart around the circumference of an 11 cm diameter circle (**Figure 1B**).
      4. Lay out the holes hexagonally or in a square array (**Figure 1C, D**) for a microcosm experiment.
3. **Filling of the containers and pots with soil and sand mixtures**
   1. Select a desired soil mixture and add AM fungus field-collected or pot-cultured inoculum to the soil by uniformly mixing chopped root pieces (1 – 2 cm long) thoroughly with the soil. Mix the desired soil with an infertile silica sand or glass beads to decrease the concentration of mineral nutrients available to plants.
   2. Position the filled containers in the drilled foam or microcosm bottom and fill the interstitial space with an infertile substrate.
   3. Fill the interstitial space between containers with nutrient-poor silica sand mixture using a funnel to assist in filling small spaces. To ensure adequate drainage and mimic the texture of the soil, mix medium particle size sand, such as 6-20 grade, with small particle size sand, such as 30-65 grade, in a cement mixer.
4. **Establishment of CMNs throughout pots/microcosms** 
   1. Plant pretreatment ‘nurse’ plants of the desired species into each container to sustain AM fungi so that they can spread among the containers and establish CMNs
   2. When all containers have established seedlings, remove shoots by clipping so that only one individual remains in each container.
   3. Allow 2-3 months for plant growth and CMN establishment.
5. **Establishment of experimental plants and treatments**
   1. Sow experimental plants by seeding or transplanting into containers. If seeding, wait until all containers have germinated seedlings before removing pre-treatment nurse plants by clipping their shoots. If transplanting, clip all pre-treatment plants before transplanting experimental seedlings to prevent unintended competitive effects.
   2. Establish CMN treatments by either leaving the containers not moved for the duration of the experiment (intact CMNs) or rotating them weekly to physically sever hyphae extending among the modified containers (severed CMNs; **Figure 1**). When severing CMNs, rotate each container through one full rotation to avoid unintentionally altering aboveground interactions, particularly for heliotropic plants.
   3. Heavily water all pots or microcosms immediately after rotation to reestablish contact between the interstitial substrate and the sides of containers.
6. **Tracing of mineral nutrient movement across CMNs**
   1. Fertilize neighboring plants with 0.5% 15N enriched KNO3 and NH4Cl.
   2. Fertilize the target individual with a 14N fertilizer of equal concentration.
7. **Monitoring and maintenance of experiment**
   1. Regularly (at least monthly) re-randomize the positions of the pots or microcosms over the course of the experiment.
   2. Weekly measure growth, such as height or longest leaf length (for grasses) to monitor when growth begins to slow, because it is important to harvest before the plants become root-bound.
8. **Harvest of the experiment** 
   1. Clip all aboveground tissue and place individual plants into labeled envelopes that identify their treatment, pot or microcosm, and position.
   2. Dry aboveground tissues at 60 °C to constant weight. Measure the dry weight of each plant tissue.
   3. Allow the soil to dry before extracting the containers and harvesting the roots.
   4. Delicately brush off as much soil as possible from the root systems and wash them in a pan of water or under a gentle stream of water on a sieve.
   5. Allow the roots to air-dry and weigh the whole root system.
   6. Clip the root system haphazardly and store the root fragments in 50% ethanol. After being stained33, use these fragments for quantification of root colonization using the gridline intersection method34.
   7. Re-weigh the remaining root system and store it in a labeled paper envelope to dry at 60 °C for assessment of dry weight. Use the following equation to calculate the weight of the entire root system:
9. **Mineral nutrient and stable isotope analyses**
   1. Group the seedlings by biomass into “deciles” or 10 groups, “octiles” or 8 groups, “quartiles” or four groups, etc. after rank-ordering them by weight if the tissue quantity is too low for minimum requirements for digestion to determine mineral nutrient concentrations.
   2. Send foliar samples to a contracted lab for mineral nutrient and stable isotope analyses (**Table of Materials**).
      1. Describe isotopic abundance using the following customary expression:

where *R* represents the 15N/14N ratio of a sample or of the standard which is atmospheric N.

* + 1. Use the non-modified, solid container treatment to serve as a control for background 15N ratios in the following mass balance equation when quantifying the amount of 15N taken up by a target plant in severed or intact CMN treatments:

δ15NTarget = *x·*δ15NNeighbors + (1 – *x*) δ15NNo CMN

where δ15N represents the isotopic abundance of targets, neighbors, and target plants in the no CMN treatment, and x represents (as a decimal fraction) the percent nitrogen obtained by the target plant from neighbor containers to which label was added. Values for δ15NNeighbors are obtained for each target plant’s composited neighbors.

**REPRESENTATIVE RESULTS:**

To determine how CMNs may influence plant performance through nutrient partitioning, we grew *Andropogon gerardii* Vitman, a dominant prairie grass, in a target plant experiment with 6 equally spaced neighbors and intact, severed, or no CMNs. We found that severing or preventing CMNs diminished targets’ aboveground dry weights (**Figure 2**), suggesting that intact CMNs promoted plant growth. Plants with severed CMNs and prevented CMNs responded notably similarly to their treatments, suggesting that the rotation of containers once a week was successful in mitigating the effects of CMNs. The severed CMN treatment, however, may be preferred as a control because the nylon mesh (which was overlain by a hydrophobic membrane in this experiment) in both intact and severed treatments may affect water dynamics such as the soil of slotted containers (rotated or not) drying more quickly than that in non-modified containers.

Competition, in which the growth of one individual suppresses the growth of another nearby individual, was detected in the intact CMN treatment but not in the severed or no CMNs treatments. We found that only when CMNs were intact did target and summed neighbor sizes have a negative relationship as demonstrated by linear regression (**Figure 3**). The severed and no CMNs treatments did not differ from one another, and together, their slopes did not differ significantly from zero. Hence, they significantly differed from the negative slope of the intact CMNs treatment (**Figure 3**). Furthermore, we found that Gini coefficients, a measure of size inequality that ranges from zero to one in which zero reflects a perfectly equal size distribution, differed among treatments. The intact CMNs treatment had the greatest inequality10,35,36. Size inequalities are affected by competition within populations, particularly when large individuals dominate resource acquisition, thereby disproportionally suppressing the growth of small individuals, also known as asymmetric competition37,38.

Whether mineral nutrients were growth-limiting, and if CMNs contributed to intensifying competition for those nutrients was determined through comparisons of mineral nutrient leaf tissue concentrations versus plant size. Of all the mineral nutrients assessed, we found that only Mn leaf tissue concentrations were positively associated with target plant aboveground dry weights over all treatments, with no significant differences among slopes, suggesting that Mn may have limited growth among all treatments (**Figure 4**). Nevertheless, regression line elevations, which suggest differences in mean concentrations among treatments, were affected by CMN severing and prevention. Mean foliar N concentrations were not significantly affected by CMN treatments, but N concentration significantly decreased with aboveground dry weight of target plants, suggesting a potential ‘dilution effect’ of plant size on tissue N39,40. Therefore, N was not likely the growth-limiting mineral nutrient in our experiment. In another similar experiment, mean foliar P was significantly affected by CMN treatment, but also showed a dilution effect when compared to plant size for plants with intact CMNs35.

To examine if CMNs differentially partition mineral nutrients among interconnected individuals, we assessed target plant tissue for 15N in leaf tissues versus plant size. We had added 15N-label only to neighbors’ containers. We found that target plants with intact CMNs had higher 15N concentrations compared to both other treatments, which did not differ from one another (**Figure 5A**). Although target aboveground dry weight was associated with the amount of nitrogen obtained from neighbors’ containers over both intact and severed CMNs treatments, intact CMNs had a strongly positive, significantly different slope from that of the severed CMNs treatment (**Figure 5B**). These results suggest that large plants, potentially with abundant photosynthate, obtained more 15N from CMNs reaching into neighboring containers than small target individuals. Our results also suggest that the hydrophobic membrane successfully prevented free water (and subsequent 15N) movement within pots.

In another rotated-core target plant experiment, guava (*Psidium guajava*) tree seedlings were grown in large seedling containers embedded within large pots, and all (including the large pots) were filled with the same relatively nutrient-rich soil mixture. When CMNs were severed by rotation in the absence of neighbors, plant growth significantly diminished to the same size as plants within solid containers, suggesting that rotated plants simply had reduced access to the full soil volume of the large pots (**Figure 6**). When target plants had any number of neighbors, plant size decreased to similar sizes, and any statistically-detectable effect of severing CMNs disappeared (**Figure 6**).

In a field experiment using rotated-cores made of PVC pipe, one of us investigated the influence of extraradical mycelium on plant performance in a field experiment with Soapberry (*Sapindus saponaria* L.) seedlings (**Figure 7**). Although extraradical mycelium beyond pipes had little effect on plant growth during the thirteen-month experiment, severing it by rotation of pipes reduced foliar N, P and Cu concentrations substantially (by 25% or more).

**FIGURE AND TABLE LEGENDS:**

**Figure 1. Experiment setup of containers in intact, severed, or control treatments (A), pots in a target plant experiment (B), or microcosms with a hexagonal (C) or square (D) layout of containers.** Dark ovular spots on modified containers are an opening into the container covered with a 30 µm nylon mesh for fungal hyphae to penetrate (**A**). Common mycorrhizal networks remain intact with no rotation of the containers, are severed by rotation, or are prevented from establishing with a solid plastic container (**A**). In a target plant pot experiment, containers can be placed into a foam bottom that positions them (**B**). For a microcosm experiment, the bottom can by laid out in a hexagonal array with six equidistant, nearest neighbors for each ‘target’ individual (**C**), or in a square array with four nearest neighbors and another four, slightly more distant, diagonal neighbors for each ‘target’ (**D**). Panel B is modified from Weremijewicz et al.10.

**Figure 2. Mean (± SE) aboveground and belowground dry weights (g) of target *Andropogon gerardii* individuals among common mycorrhizal network treatments.** Aboveground dry weights are shown as positive values above the abscissa and belowground dry weights are positive values below the abscissa. Aboveground dry weight bars topped by the same letter do not differ by Tukey’s Highest Significant Difference *post-hoc* test at ɑ = 0.05. Belowground dry weights did not differ among treatments and thus, are not topped by letters. This figure is modified from Weremijewicz et al.10.

**Figure 3. Total neighbor (g) versus target plant aboveground dry weight (g) for *Andropogon gerardii* plants.** Plants with intact common mycorrhizal networks (CMNs) are represented by dark triangles and a solid line, with severed CMNs by gray squares and a dashed line, and with no CMNs by white diamonds and a dotted line. This figure is modified from Weremijewicz et al.10.

**Figure 4. Target aboveground dry weights (g) versus foliar manganese concentrations (µg·g-1) of *Andropogon gerardii*.** Plants with intact common mycorrhizal networks (CMNs) are represented by dark triangles and a solid line, with severed CMNs by gray squares and a dashed line, and with no CMN by white diamonds and a dotted line. This figure is modified from Weremijewicz et al.10.

**Figure 5. δ15N (‰) ± SE of target *Andropogon gerardii* individuals (A) and percent nitrogen obtained from neighbor container soil versus target aboveground dry weight (g; B) with intact common mycorrhizal networks (CMNs; black bars and triangles), severed CMNs (gray bars and circles), and no CMN (white bars; not shown in B).** Bars topped by the same letter in panel A do not differ by Tukey’s Highest Significant Difference *post-hoc* test at ɑ = 0.05. These figures are modified from Weremijewicz et al.10.

**Figure 6. Mean (± SE) aboveground and belowground dry weights (g) of target *Psidium guajava* individuals among common mycorrhizal networks (CMNs) and neighbor treatments.** Along the abscissa, a no neighbor treatment is represented by “0N”, one neighbor by “1N,” etc., while a control treatment with no CMNs is represented by light shading and the letter “C”. Biomass of plants with intact CMNs are represented by solid bars, while those with severed CMNs are hachured. Aboveground dry weights are shown as positive values above the abscissa and belowground dry weights are positive values below the abscissa. Bars topped by the same letter do not differ by Tukey’s Highest Significant Difference *post-hoc* test at ɑ = 0.05. Aboveground and belowground dry weights of the control treatment were only compared with intact and severed CMN treatments with no neighbors (indicated by Greek letters) because the control did not include neighbors as an additional factor in the treatment.

**Figure 7. Extension of the rotated core method to a field experiment (A) and representative 13-month-old *Sapindus saponaria* L. seedlings with intact (not rotated) and severed (rotated) common mycorrhizal networks.** Polyvinyl chloride pipes (9 cm diameter x 20 cm height) were drilled with a hole-saw to have four 5.3 cm diameter holes in two opposed pairs. The holes were covered with a nylon silk-screen mesh with 30 µm pores through which extraradical mycelium could extend both from and into the cores which were filled with soil from the planting site (**A**). Common mycorrhizal networks were kept intact or severed by rotation using a large pipe-wrench. Positions of paired rotated and non-rotated cores (about 20 cm apart) are marked by flags every 2 m along five transects in the experimental plot located in a Lychee grove (**A**). Evidence of the disruption of extraradical mycelium reducing N, P and Cu uptake is shown by the chlorotic plants labeled “rotated” shown in **B**.

**DISCUSSION:**

Our results affirm that our rotated core method can sharply focus on the role of CMNs in belowground plant interactions. There are several critical steps in the protocol, however, that if altered, have potential to influence the ability to detect CMN effects. It is critical to fill the interstitial area surrounding containers with a nutrient-poor medium. In our unsuccessful, rotated-core target plant experiment with guava tree seedlings, although there was a marked reduction of target growth in the presence of any number of neighbors, no effects of CMNs on belowground competition were detected, probably because of mineral nutrient availability throughout the pots. In contrast, the use of a nutrient-poor medium between rotated cores ensures that hyphae must reach into neighboring containers which often are filled with both hyphae and roots (especially when working with root-intensive grasses). Extraradical hyphae in neighboring containers thus are in direct competition with root systems and must partition mineral nutrients acquired from such ‘patches’ among plants connected to a CMN. Another critical component in making belowground interactions detectable is to avoid aboveground competition. Our guava experiment revealed that the effect of additional soil volume access evident when the target plants had no neighbors was essentially eliminated when target seedlings were shaded by neighbors. Using grasses which mostly grow vertically, or constraining seedling leaf crowns to prevent overlap would help to mitigate aboveground interactions.

The use of somewhat rigid containers instead of mesh bags, is critical in maintaining a long experiment with ease of severing CMNs through rotation. In an early attempt at a CMN experiment, trying to pull a knife between mesh bags to sever CMNs not only resulted in damaged bags from which roots could protrude but also seemed to favorably increase soil aeration resulting in dramatically enhanced plant growth when CMNs were severed. Because the rotated core approach gently moves each container in an invariant position (thanks to the supporting position holes at the bottom of the pot or microcosm), it minimizes surrounding substrate disruption and potential aeration. It is absolutely critical, however, to thoroughly water the pots after rotation of containers to return low-fertility, interstitial sand substrate to closely contact the containers.

The proposed rotated core method may be modified in a number of ways to answer a variety of questions regarding the functioning of CMNs and extraradical mycelia. For example, the amount of carbon available for host plants to provide CMNs can be reduced by shading10. Shade cloth wrapped around modified seedling protectors to encircle individual containers was successful at reducing carbon provisioning to CMNs and thus, 15N uptake from CMNs10. Additionally, population structure can be investigated in large microcosms (**Figure 1C, D**) comprising many plants, each in an individual, rotated container. It is important to note, however, that care must be taken to avoid pseudoreplication41 when doing so. Individual plants are most certainly not ‘replicates’ because they are not independent of the other plants in a microcosm. Instead, the entire experimental unit (pot or microcosm) is a replicate, which is why we used averaged or totaled neighbor plant sizes per pot prior to running analyses of variance or linear regressions.

Our approach can be modified for field studies to exclude root competition and investigate the influence of intact CMNs. By substituting containers with PVC pipe pieces with large holes covered with nylon silk-screen mesh, rotated cores can withstand harsh field conditions, as in the Soapberry experiment. Similar to our guava pot experiment, however, the effect of severing potential CMNs could not be distinguished from simply restricting the soil volume from which mineral nutrients could be acquired.

Our approach provides a controlled, careful comparison of plants interacting across CMNs versus mycorrhizal plants that are not persistently interconnected (instead of plants entirely lacking mycorrhizas). Hence, it mimics aspects of nature, such as seedlings joining established CMNs as well as the use of a suite of AM fungi. Recent work has demonstrated that different AM fungus species may be different quality partners to plants, and that the presence of a second species of AM fungus on a root system can induce an ‘uncooperative’ fungus species to provide more phosphorus in return for carbon than when alone on the root system42. Moreover, different species of fungi may provide benefits other than mineral nutrient acquisition to plant hosts, such as drought and salinity tolerance or protection from pathogens2. These findings underscore the importance of using a suite of fungi to establish CMNs. Notwithstanding its realism, a conspicuous limitation of our approach is experiment duration. The size of containers or PVC pipes limits the length of time before plants become root bound, and thus tends to restrict focus to only seedlings or young saplings. Nevertheless, we submit that there is considerable flexibility in the design of target-plant rotated-core experiments in which either or both targets and neighbors can be manipulated in a wide variety of ways to understand the roles of CMNs.

**ACKNOWLEDGMENTS:**

We would like to thank the two anonymous reviewers for their suggestions. We also thank the numerous undergraduates who have helped with constructing pots, microcosms, and slotted containers and who have assisted with maintaining and harvesting experiments. We also thank North Central College for startup funds (to JW) and current facilities, as well as Ashley Wojciechowski for obtaining a North Central College Richter Grant supporting an experiment using these methods. Part of this work was funded by a National Science Foundation Doctoral Dissertation Improvement Grant (DEB-1401677).

**DISCLOSURES:**

The authors have nothing to disclose.

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