

Journal of Visualized Experiments

Investigation of plant interactions across common mycorrhizal networks using rotated cores

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59338R2
Full Title:	Investigation of plant interactions across common mycorrhizal networks using rotated cores
Keywords:	Common mycorrhizal networks, competition, extraradical mycelium, facilitation, mycorrhizal fungi, plant interactions, plant-soil feedback, rotated core
Corresponding Author:	Joanna Weremijewicz, PhD North Central College Naperville, IL UNITED STATES
Corresponding Author's Institution:	North Central College
Corresponding Author E-Mail:	jweremi@gmail.com
Order of Authors:	Joanna Weremijewicz, PhD David P. Janos
Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the city, state/province, and country where this article will be filmed . Please do not use abbreviations.	Naperville, IL, United States

TITLE:

Investigation of Plant Interactions Across Common Mycorrhizal Networks Using Rotated Cores

AUTHORS AND AFFILIATIONS:

Weremijewicz, Joanna^{1,2}, David P. Janos²

¹Current Address: Department of Biology, North Central College, Naperville, IL, USA

²Department of Biology, University of Miami, Coral Gables, FL, USA

Corresponding author:

Joanna Weremijewicz

JWeremijewicz@noctrl.edu

Email Addresses of Co-authors:

David P. Janos DavidJanos@miami.edu

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 vitro root organ culture work has found that CMNs preferentially exchange mineral nutrients with host roots that provide the most carbon to the fungi^{11,12}. 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 fungi^{16,17}, 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 soil^{22,23}. 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 mycelia^{18,24,25}. 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 tetradonta* 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 microcosms^{20,30}. 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

1.1. Modify commercial tubular seedling containers (subsequently called ‘containers’; **Table of Materials**) to have 19 mm wide x 48 cm length openings.

1.1.1. 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.

1.1.2. Cut the remaining thin piece of plastic between the holes with scissors, a wire cutter, or tin snips (for rigid plastic use a sabre-saw) to make one elongated opening about 2 cm wide and 5 cm long.

1.1.3. Repeat steps 1.1.1 – 1.1.2 on the opposite side of the container.

1.2. Cover the slots with nylon mesh and/or hydrophobic membrane covered slots (**Figure 1A**).

1.2.1 Cut nylon mesh with 40 µm pores into 9.5 cm x 8.5 cm pieces. Cut as many pieces as there are containers.

1.2.2 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.

1.2.3 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 membrane^{31,32} (**Table of Materials**) that allows AM fungus hyphae to pass, but only the movement of water vapor and not liquid water.

1.2.4 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.

1.2.5 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.

1.3 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.

1.4 To prevent soil loss while providing drainage, place a glass marble into the bottom of each container.

1.5 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

2.1 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.

2.2 Preparation of polystyrene foam

2.2.1 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.

2.2.2 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.

2.2.3 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**).

2.2.4 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

3.1 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.

3.2 Position the filled containers in the drilled foam or microcosm bottom and fill the interstitial space with an infertile substrate.

3.3 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

4.1 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

4.2 When all containers have established seedlings, remove shoots by clipping so that only one individual remains in each container.

4.3 Allow 2-3 months for plant growth and CMN establishment.

5. Establishment of experimental plants and treatments

5.1 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.

5.2 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.

5.3 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

6.1 Fertilize neighboring plants with 0.5% ¹⁵N enriched KNO₃ and NH₄Cl.

6.2 Fertilize the target individual with a ¹⁴N fertilizer of equal concentration.

7. Monitoring and maintenance of experiment

7.1 Regularly (at least monthly) re-randomize the positions of the pots or microcosms over the course of the experiment.

7.2 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

8.1 Clip all aboveground tissue and place individual plants into labeled envelopes that identify their treatment, pot or microcosm, and position.

8.2 Dry aboveground tissues at 60 °C to constant weight. Measure the dry weight of each plant tissue.

8.3 Allow the soil to dry before extracting the containers and harvesting the roots.

8.4 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.

8.5 Allow the roots to air-dry and weigh the whole root system.

8.6 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.

8.7 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:

$$\text{Whole Dry Weight} = \frac{\text{Whole Fresh Weight} \times \text{Clipped Dry Weight}}{\text{Clipped Fresh Weight}}$$

9. Mineral nutrient and stable isotope analyses

9.1 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.

9.2 Send foliar samples to a contracted lab for mineral nutrient and stable isotope analyses (Table of Materials).

9.2.1 Describe isotopic abundance using the following customary expression:

$$\delta^{15}\text{N} (\text{‰}) = \left[\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right] \times 1000$$

where R represents the $^{15}\text{N}/^{14}\text{N}$ ratio of a sample or of the standard which is atmospheric N.

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

$$\delta^{15}\text{N}_{\text{Target}} = x \cdot \delta^{15}\text{N}_{\text{Neighbors}} + (1 - x) \times \delta^{15}\text{N}_{\text{No CMN}}$$

where $\delta^{15}\text{N}$ 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 $\delta^{15}\text{N}_{\text{Neighbors}}$ 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 inequality^{10,35,36}. Size inequalities are affected by competition within populations, particularly when large individuals dominate resource acquisition, thereby disproportionately suppressing the growth of small individuals, also known as asymmetric competition^{37,38}.

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 N^{39,40}. 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 oval 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 be 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 (\pm 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 $\alpha = 0.05$. 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 ($\mu\text{g}\cdot\text{g}^{-1}$) 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. $\delta^{15}\text{N}$ (‰) \pm 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 $\alpha = 0.05$. These figures are modified from Weremijewicz et al.¹⁰.

Figure 6. Mean (\pm 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 $\alpha = 0.05$. 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.

REFERENCES

- 1 Remy, W., Taylor, T. N., Hass, H. & Kerp, H. Four hundred-million-year-old vesicular arbuscular mycorrhizae. *Proceedings of the National Academy of Sciences of the United States of America*. **91** (25), 11841-11843, (1994).
- 2 Smith, S. E. & Read, D. J. *Mycorrhizal Symbiosis*. 3 edn, (Academic Press, 2008).
- 3 Giovannetti, M., Avio, L. & Sbrana, C. in *Mycorrhizal Networks* 41-67 (Springer, 2015).
- 4 Giovannetti, M. & Sbrana, C. in *Cell Biology of Plant and Fungal Tip Growth* Vol. 328 *Nato Science Series, Sub-Series I: Life and Behavioural Sciences* (ed A. Cresti M. Heath I. B. Geitmann) 221-231 (2001).
- 5 Giovannetti, M., Sbrana, C., Avio, L. & Strani, P. Patterns of below-ground plant interconnections established by means of arbuscular mycorrhizal networks. *New Phytologist*. **164** (1), 175-181, (2004).
- 6 Avio, L., Pellegrino, E., Bonari, E. & Giovannetti, M. Functional diversity of arbuscular mycorrhizal fungal isolates in relation to extraradical mycelial networks. *New Phytologist*. **172** (2), 347-357, (2006).
- 7 Giovannetti, M. *et al.* At the root of the wood wide web: self recognition and nonself incompatibility in mycorrhizal networks. *Plant Signaling & Behavior*. **1** (1), 1-5, (2006).
- 8 Miller, R., & Kling, M. J. The importance of integration and scale in the arbuscular mycorrhizal symbiosis. *Plant and Soil*. **226** (2), 295-309, (2000).
- 9 Miller, R., Jastrow, J. & Reinhardt, D. R. External hyphal production of vesicular-arbuscular mycorrhizal fungi in pasture and tallgrass prairie communities. *Oecologia*. **103** (1), 17-23, (1995).
- 10 Weremijewicz, J., Sternberg, L. d. S. L. O. & Janos, D. P. Common mycorrhizal networks amplify competition by preferential mineral nutrient allocation to large host plants. *New Phytologist*. (2016).

- 567 11 Fellbaum, C. R. *et al.* Fungal nutrient allocation in common mycorrhizal networks is
568 regulated by the carbon source strength of individual host plants. *New Phytologist*. **203**
569 (2), 646-656, (2014).
570
- 571 12 Lekberg, Y., Hammer, E. C. & Olsson, P. A. Plants as resource islands and storage units--
572 adopting the mycocentric view of arbuscular mycorrhizal networks. *FEMS Microbiol Ecol*.
573 **74** (2), 336-345, (2010).
574
- 575 13 Jakobsen, I. & Hammer, E. C. in *Mycorrhizal Networks* 91-131 (Springer, 2015).
576
- 577 14 Jakobsen, I. & Rosendahl, L. Carbon flow into soil and external hyphae from roots of
578 mycorrhizal cucumber plants. *New Phytologist*. **115** (1), 77-83, (1990).
579
- 580 15 Kiers, E. T. *et al.* Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis.
581 *Science*. **333** (6044), 880-882, (2011).
582
- 583 16 Hartnett, D. C., Hetrick, B. A. D. & Wilson, G. W. T. Mycorrhizal influence on intra- and
584 interspecific neighbour interactions among co-occurring prairie grasses. *Journal of*
585 *Ecology*. **81** (4), 787-795, (1993).
586
- 587 17 Hetrick, B. A. D., Wilson, G. W. T. & Todd, T. C. Differential responses of C3 and C4 grasses
588 to mycorrhizal symbiosis, phosphorus fertilization, and soil microorganisms. *Canadian*
589 *Journal of Botany*. **68** (3), 461-467, (1990).
590
- 591 18 Johnson, D., Leake, J. R. & Read, D. J. Novel in-growth core system enables functional
592 studies of grassland mycorrhizal mycelial networks. *New Phytologist*. **152** (3), 555-562,
593 (2001).
594
- 595 19 Leake, J. R. *et al.* Networks of power and influence: the role of mycorrhizal mycelium in
596 controlling plant communities and agroecosystem functioning. *Canadian Journal of*
597 *Botany-Revue Canadienne De Botanique*. **82** (8), 1016-1045, (2004).
598
- 599 20 Babikova, Z. *et al.* Underground signals carried through common mycelial networks warn
600 neighbouring plants of aphid attack. *Ecology Letters*. **16** (7), 835-843, (2013).
601
- 602 21 Schüepp, H., Miller, D. D. & Bodmer, M. A new technique for monitoring hyphal growth
603 of vesicular-arbuscular mycorrhizal fungi through soil. *Transactions of the British*
604 *Mycological Society*. **89** (4), 429-435, (1987).
605
- 606 22 Miller, D. D., Bodmer, M. & Schüepp, H. Spread of endomycorrhizal colonization and
607 effects on growth of apple seedlings. *New Phytologist*. **111** (1), 51-59, (1989).
608
- 609 23 Jakobsen, I., Gazey, C. & Abbott, L. K. Phosphate transport by communities of arbuscular
610 mycorrhizal fungi in intact soil cores. *New Phytologist*. **149** (1), 95-103, (2001).

- 24 Johnson, D., Leake, J., Ostle, N., Ineson, P. & Read, D. J. *In situ* $^{13}\text{CO}_2$ pulse-labelling of upland grassland demonstrates a rapid pathway of carbon flux from arbuscular mycorrhizal mycelia to the soil. *New Phytologist*. **153** (2), 327-334, (2002).
- 25 Johnson, D., Leake, J., Read, D. J. Transfer of recent photosynthate into mycorrhizal mycelium of an upland grassland: short-term respiratory losses and accumulation of ^{14}C . *Soil Biology and Biochemistry*. **34** (10), 1521-1524, (2002).
- 26 Walder, F. *et al.* Mycorrhizal networks: Common goods of plants shared under unequal terms of trade. *Plant Physiology*. **159** (June 2012), 789-797, (2012).
- 27 Song, Y. Y. *et al.* Interplant communication of tomato plants through underground common mycorrhizal networks. *Plos One*. **5** (10), e13324, (2010).
- 28 Merrild, M. P., Ambus, P., Rosendahl, S. & Jakobsen, I. Common arbuscular mycorrhizal networks amplify competition for phosphorus between seedlings and established plants. *New Phytologist*. **200** (1), 229-240, (2013).
- 29 Janos, D. P., Scott, J., Aristizábal, C. & Bowman, D. M. J. S. Arbuscular-mycorrhizal networks inhibit *Eucalyptus tetrodonta* seedlings in rain forest soil microcosms. **8** (2), *Plos One*. e57716, (2013).
- 30 Wyss Lozano Hoyos, T. *Pinus elliottii* var. *densa* Seedling Performance Reflects Ectomycorrhizas, Soil Nutrient Availability and Root Competition. (2010).
- 31 Mäder, P., Vierheilig, H., Alt, M. & Wiemken, A. Boundries between soil compartments formed by microporous hydrophobic membranes (GORE-TEX) can be crossed by vesicular-arbuscular mycorrhizal fungi but not by ions in the soil solution. *Plant and Soil*. **152** 201-206, (1993).
- 32 Mäder, P. *et al.* Transport of ^{15}N from a soil compartment separated by a polytetrafluoroethylene membrane to plant roots via the hyphae of arbuscular mycorrhizal fungi. *New Phytologist*. **146** (1), 155-161, (2000).
- 33 Brundrett, M., Bougher, N., Dell, B. & Grove, T. Working with Mycorrhizas in Forestry and Agriculture. (1996).
- 34 McGonigle, T. P., Miller, M. H., Evans, D. G., Fairchild, G. L. & Swan, J. A. A new method which gives an objective-measure of colonization of roots by vesicular arbuscular mycorrhizal fungi. *New Phytologist*. **115** (3), 495-501, (1990).
- 35 Weremijewicz, J. & Janos, D. P. Common mycorrhizal networks amplify size inequality in *Andropogon gerardii* monocultures. *New Phytologist*. **198** (1), 203-213, (2013).

655
656 36 Weremijewicz, J., O'Reilly, L. d. S. L. & Janos, D. P. Arbuscular common mycorrhizal
657 networks mediate intra-and interspecific interactions of two prairie grasses. *Mycorrhiza*.
658 1-13, (2017).
659
660 37 Weiner, J. Asymmetric competition in plant populations. *Tree*. **5** (11), 360-364, (1990).
661 38 Damgaard, C. & Weiner, J. Describing inequality in plant size or fecundity. *Ecology*. **81** (4),
662 1139-1142, (2000).
663
664 39 Johnson, C. R., Joiner, J. N. & Crews, C. E. Effects of N, K, and Mg on growth and leaf
665 nutrient composition of 3 container grown woody ornamentals inoculated with
666 mycorrhizae. *Journal of the American Society for Horticultural Science*. **105** (2), 286-288,
667 (1980).
668
669 40 Estrada-Luna, A. A., Davies, F. T. & Egilla, J. N. Mycorrhizal fungi enhancement of growth
670 and gas exchange of micropropagated guava plantlets (*Psidium guajava* L.) during ex vitro
671 acclimatization and plant establishment. *Mycorrhiza*. **10** (1), 1-8, (2000).
672
673 41 Hurlbert, S. H. Pseudoreplication and the design of ecological field experiments.
674 *Ecological Monographs*. **54** (2), 187-211, (1984).
675
676 42 Argüello, A. *et al.* Options of partners improve carbon for phosphorus trade in the
677 arbuscular mycorrhizal mutualism. *Ecology Letters*. **19** (6), 648-656, (2016).

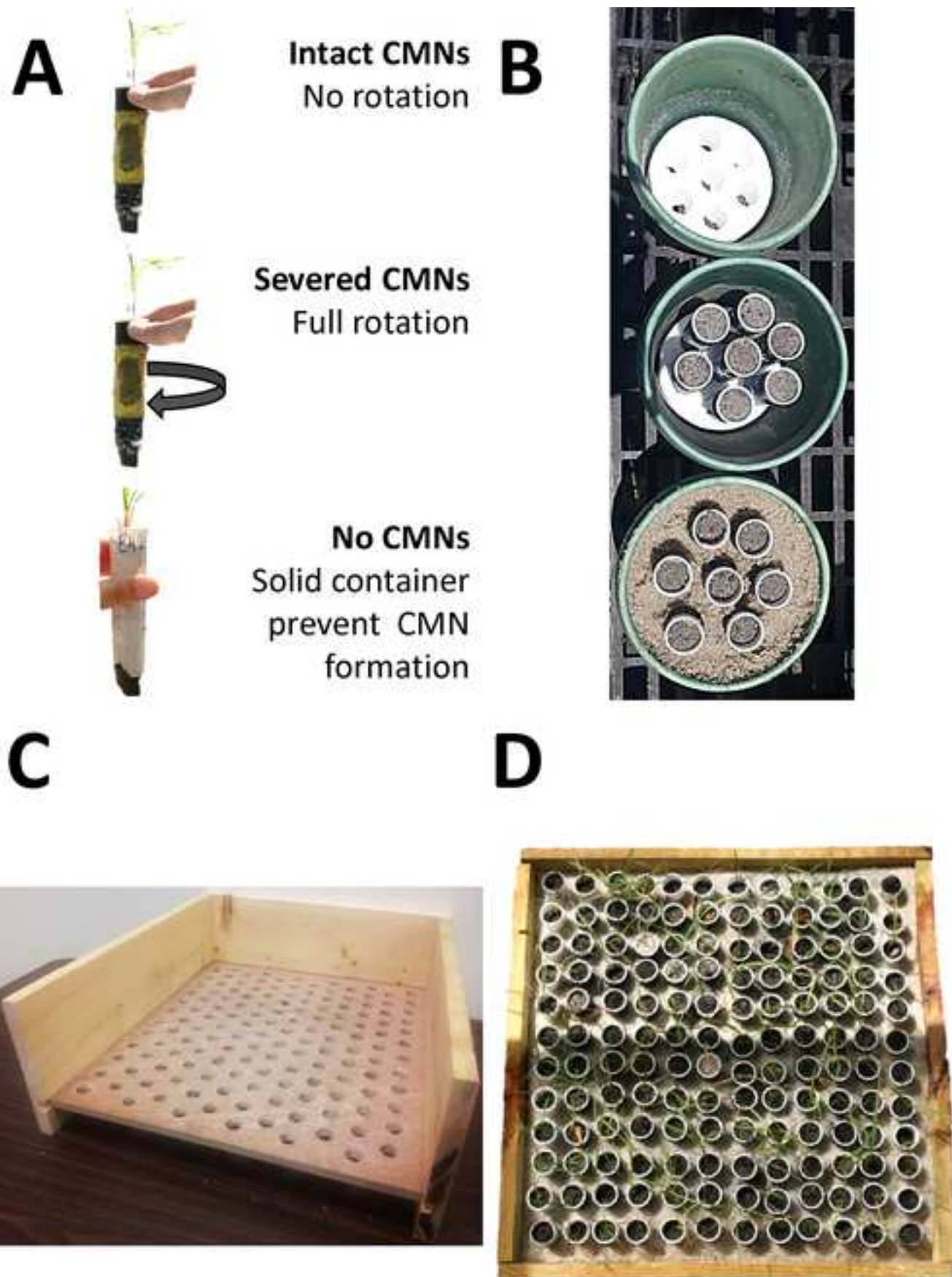


Figure 2

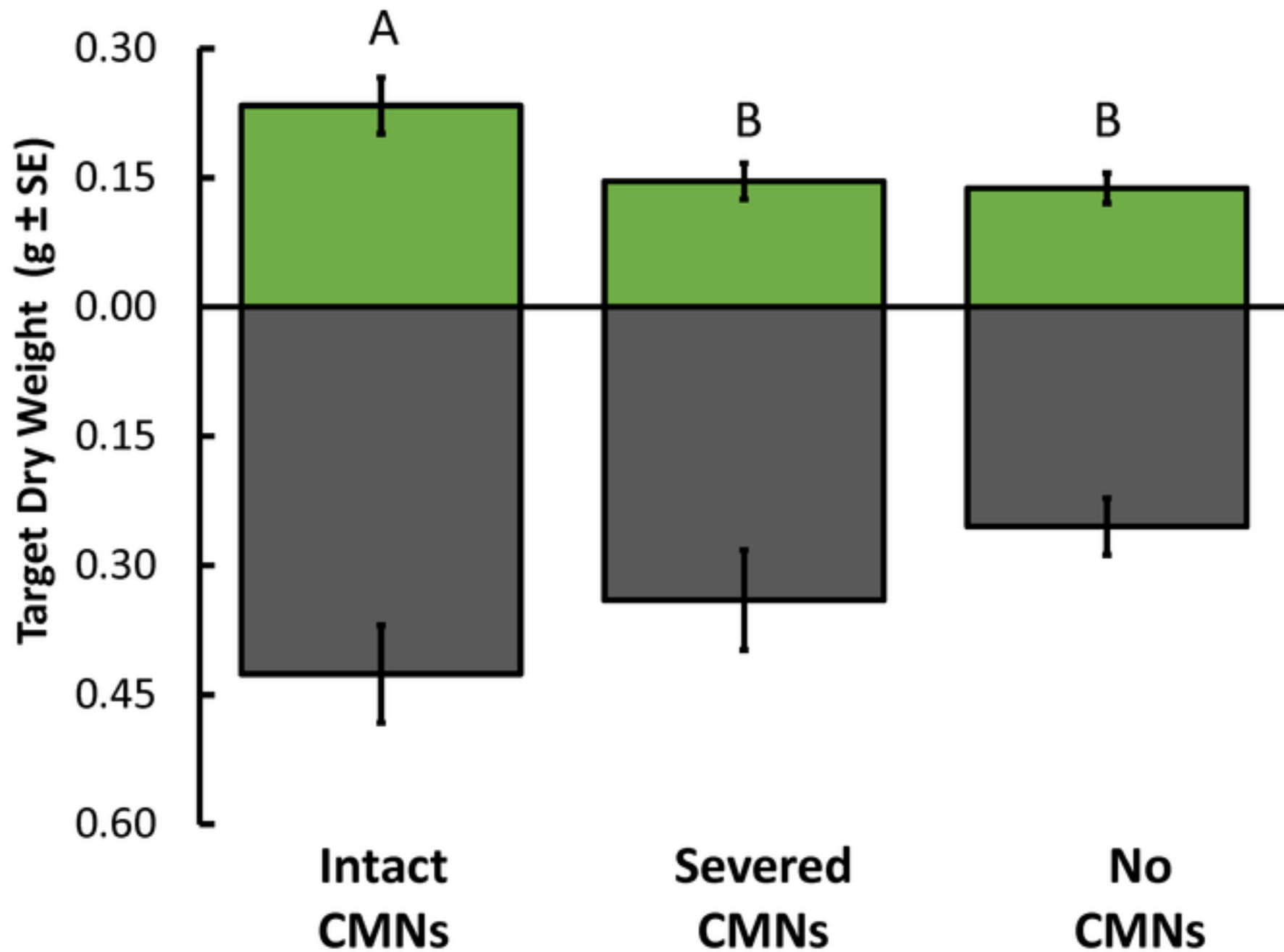


Figure 3

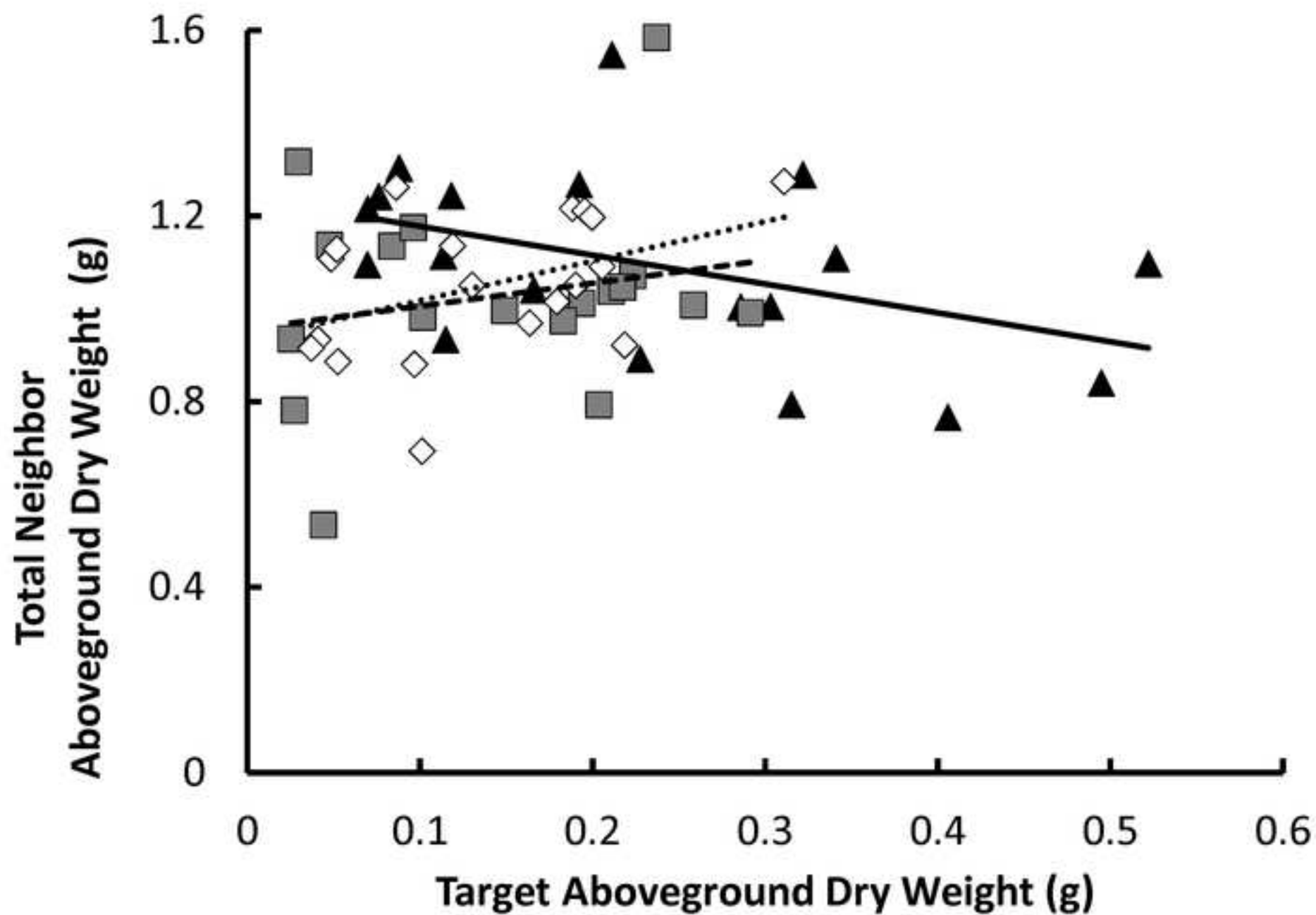
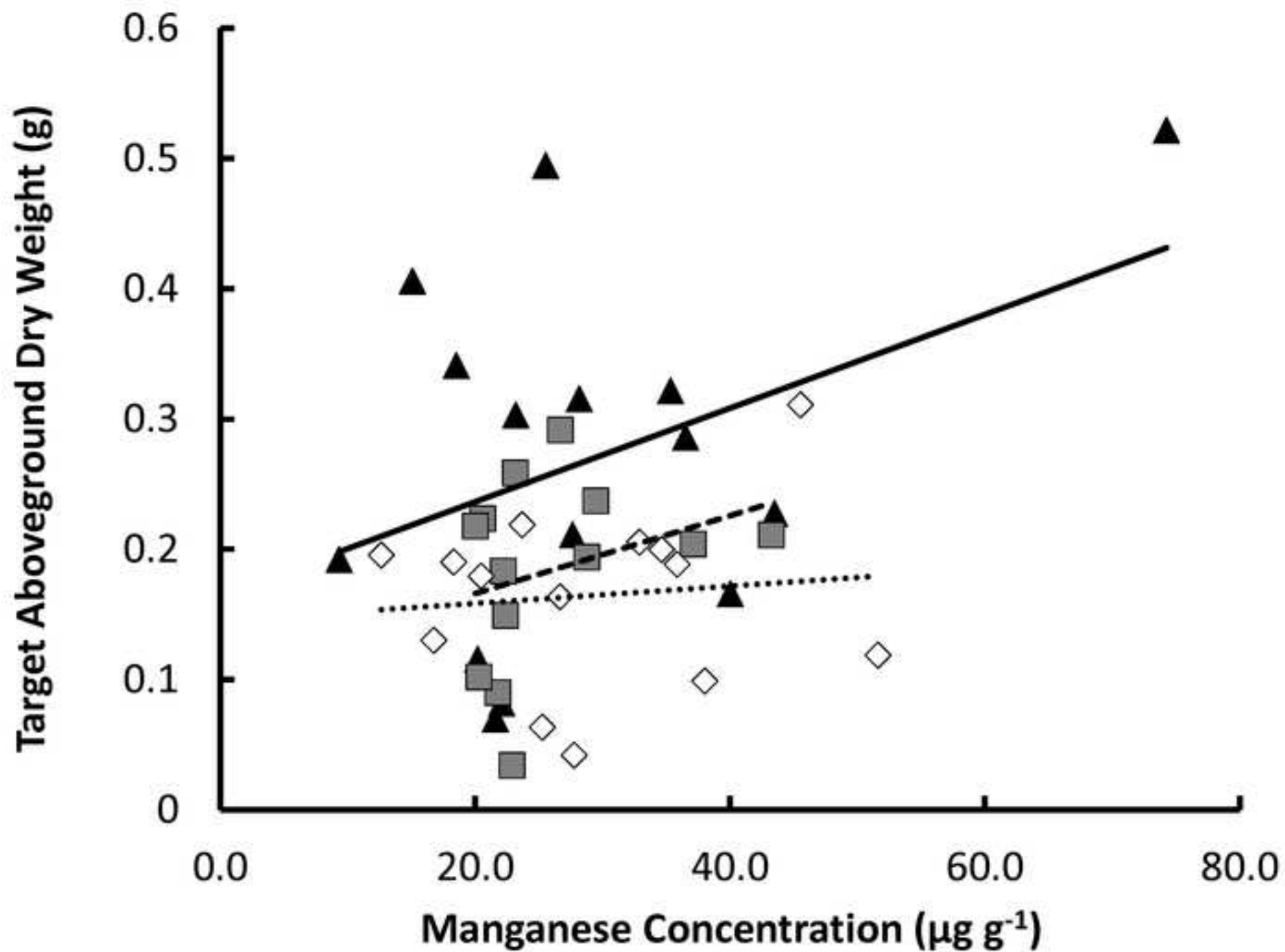


Figure 4



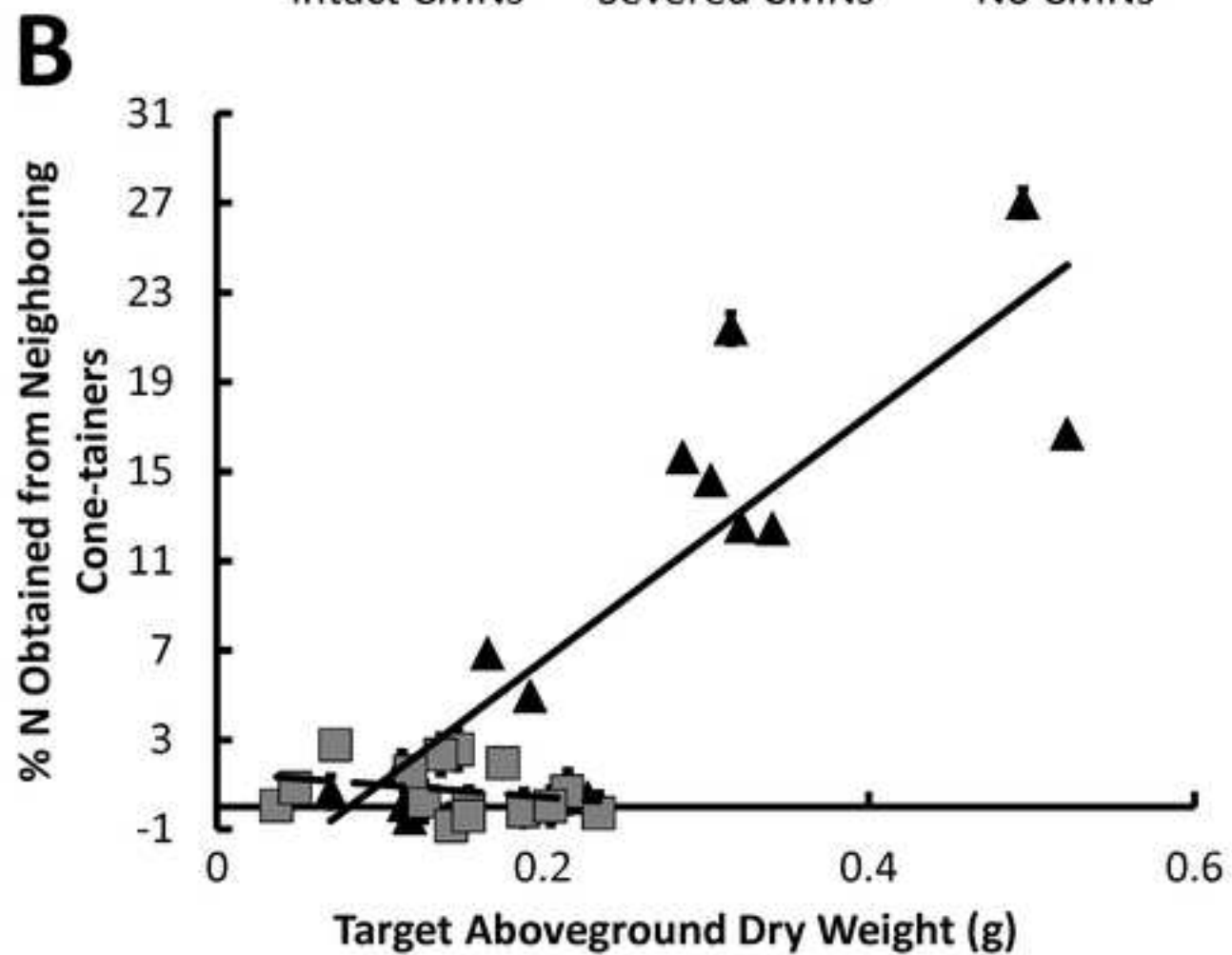
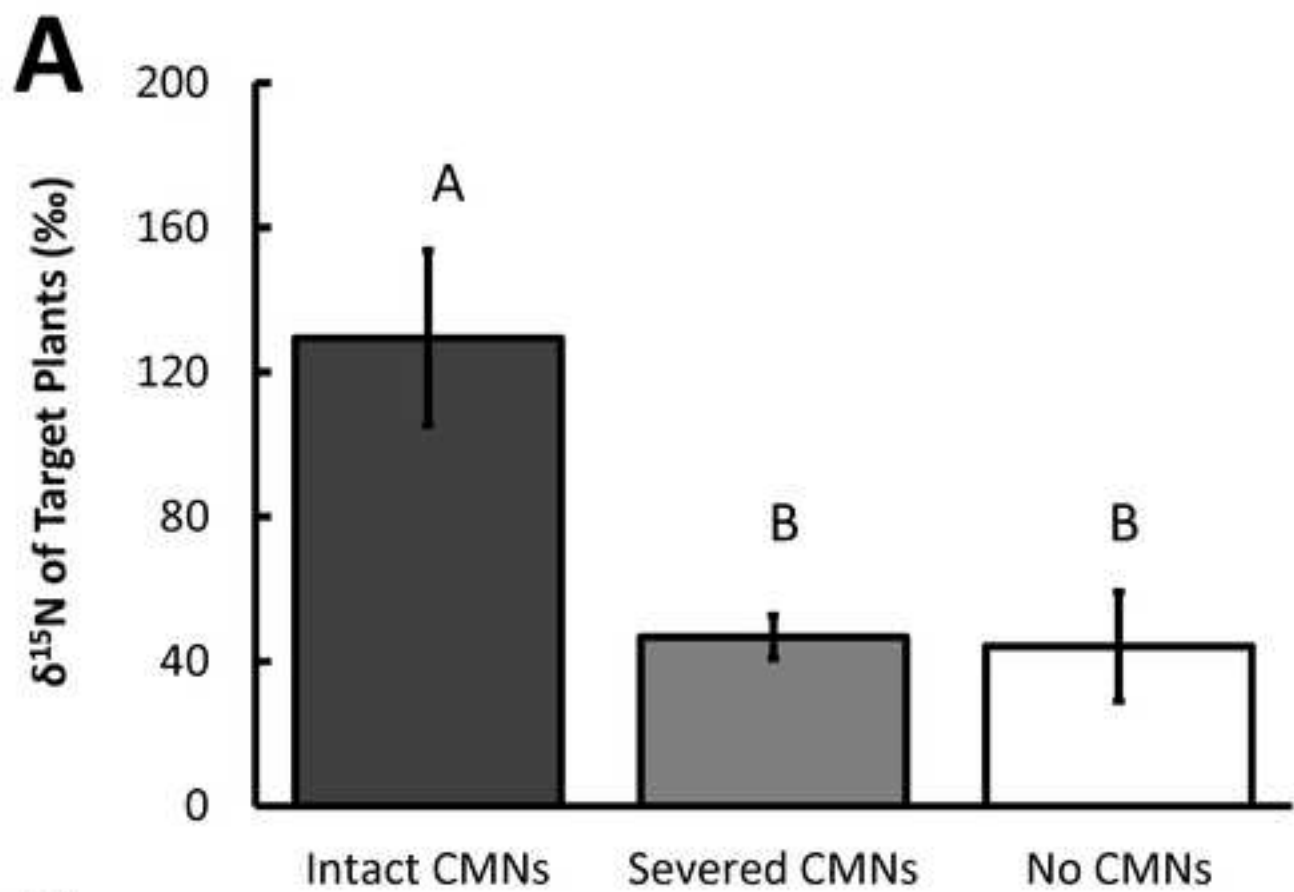
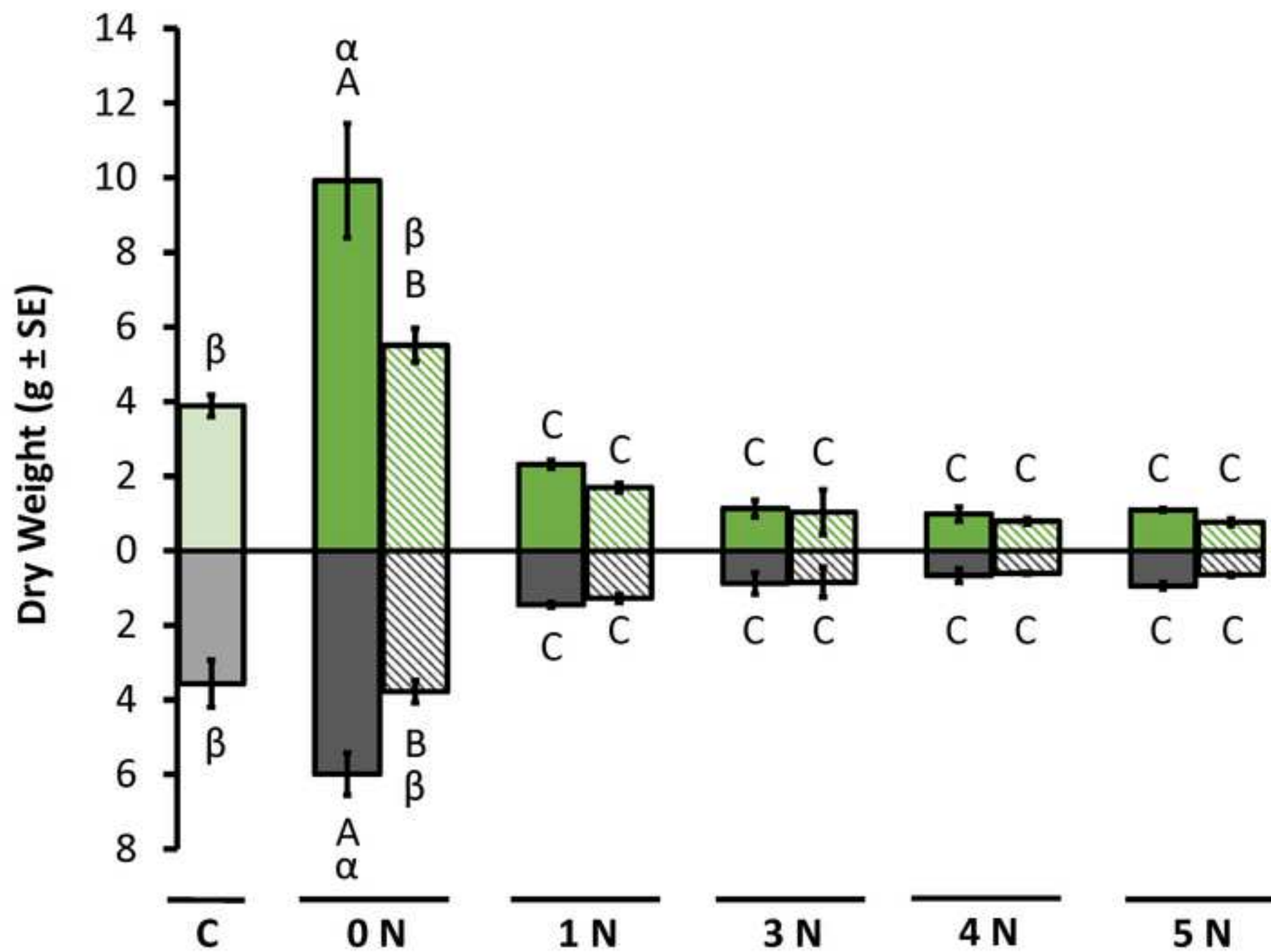


Figure 6



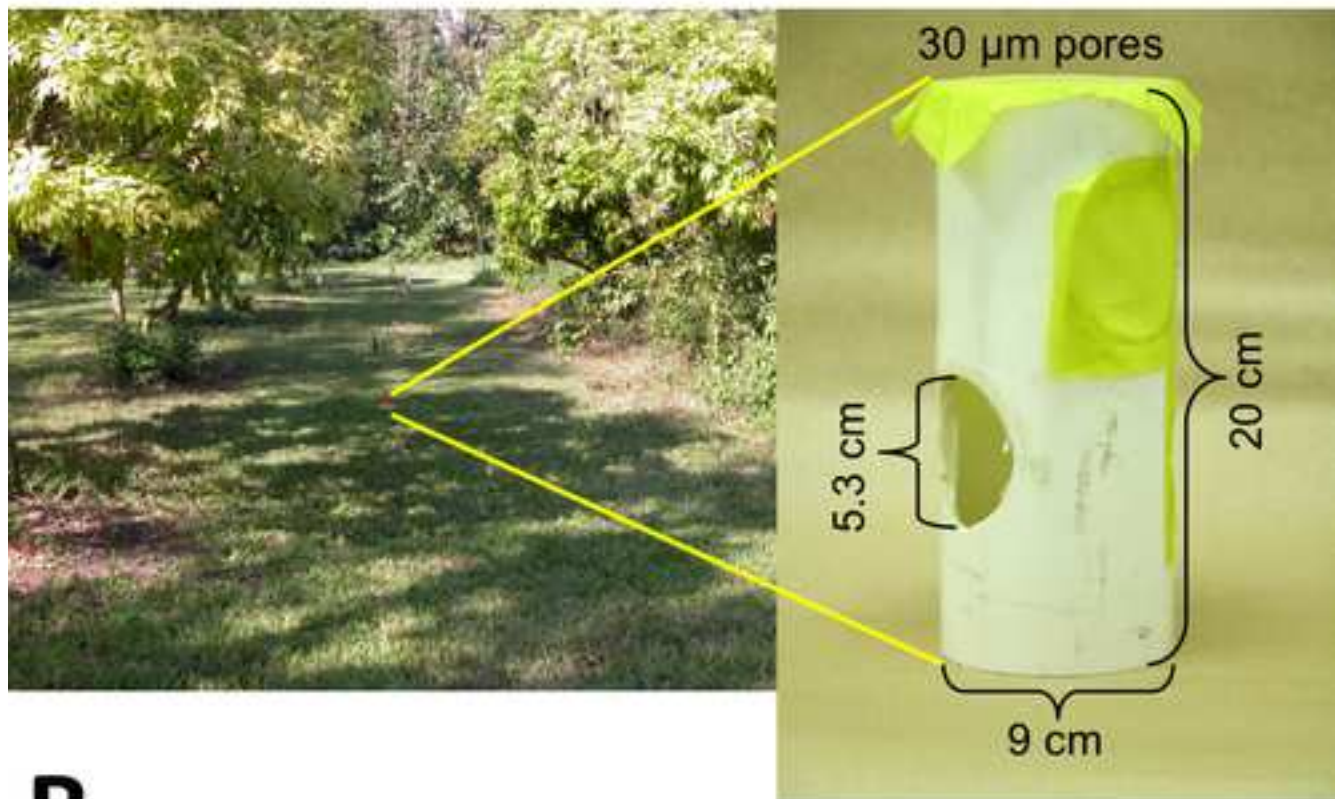
A**B**

Table 1. List of commercial materials, suppliers, part descriptions, and part numbers

Material	Supplier	Part
Commercial tubular seedlings container (called 'containers' in the manuscript)	Stuewe and Sons, Inc	Ray Leach Cone-tainer TM
Course glass beads	Industrial Supply, Inc.	12/20 sieve
Course silica sand	Florida Silica Sand	6/20 50lb bags
Fine glass beads	Black Beauty	Black Beauty FINE Crushed Glass Abrasive (50 lbs)
Hydrophobic membrane	Gore-tex	None
Large commercial tubular seedling containers	Stuewe and Sons, Inc.	Deepot TM
Medium silica sand	Florida Silica Sand	30/65 50 lb bags
Nylon mesh	Tube Lite Company, Inc.	Silk screen
Soil and foliar nutrient analysis facility	Kansas State University Soil Testing Lab	None
Stable isotope core facility	University of Miami	None

part numbers used in the protocol

Part No.
RLC3U
Size #1
None
BB-Glass-Fine
None
D16L
None
LE7-380-34d PW YEL 60/62 SEFAR LE PECAP POLYESTER
None
None

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Investigation of plant interactions across common mycorrhizal networks using rotated cores
Author(s):	Joanna Weremijewicz and David P. Janos

Item 1: The Author elects to have the Materials be made available (as described at <http://www.jove.com/publish>) via:

☒ Standard Access

☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: **"Agreement"** means this Article and Video License Agreement; **"Article"** means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; **"Author"** means the author who is a signatory to this Agreement; **"Collective Work"** means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; **"CRC License"** means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; **"Derivative Work"** means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; **"Institution"** means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; **"JoVE"** means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; **"Materials"** means the Article and / or the Video; **"Parties"** means the Author and JoVE; **"Video"** means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion

of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum

rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to


the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Joanna Weremijewicz	
Department:	Department of Biology	
Institution:	North Central College	
Title:	Assistant Professor	
Signature:		Date: October 30, 2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Dear Dr. Weremijewicz,

Your manuscript, JoVE59338 "Investigation of plant interactions across common mycorrhizal networks using rotated cores," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 x 1080 pixels or 300 dpi.

Your revision is due by **Dec 19, 2018**.

To submit a revision, go to the [JoVE submission site](#) and log in as an author. You will find your submission under the heading "Submission Needing Revision".

Best,

Bing Wu, Ph.D.

Review Editor

[JoVE](#)

Follow us: [Facebook](#) | [Twitter](#) | [LinkedIn](#)

[About JoVE](#)

We would like to thank the editor for the advice and suggestions. We have attached a Word document of the revised manuscript that uses the "track changes" feature in Microsoft word to identify all edits.

We have checked each figure, and confirmed that they exceed 1920 x 1080 pixels, which should ensure high quality publication.

Please see our responses to all suggestions (shown in blue, below).

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

[We have proofread the manuscript for spelling and grammar.](#)

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account.

The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Explicit copyright permission has been granted by John Wiley and Sons and uploaded to the account. Each re-used figure has been appropriately cited in its legend.

3. Figure 5A: Please indicate the unit of delta 15N in the figure.

The unit of delta 15N has been added to 5A.

4. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

All sentences with the phrase "can be" have been changed to the imperative tense or moved to be "Notes" in the manuscript, and all discussion about the protocol is in the discussion.

5. Please describe Figure 6 and Figure 7 in the Representative Results.

Figures 6 and 7 are now described in the Representative Results sections in lines 377 – 390.

6. Please include single-line spaces between all paragraphs, headings, steps, etc.

The entire document has been modified to include only single-line spaces between paragraphs, heading, steps, etc.

7. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted 2.75 pages of protocol including headings and spacing.

8. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

All steps that have been highlighted are complete sentences and are in the imperative tense.

9. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Done.

10. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

We have sorted all materials in alphabetical order.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript describes a method to modify and arrange core tubes for plant growth, in order to make experiments on the effect of common mycelium network (CMN) on plant physiology and growth. The method allow the generation of an intact CMN, a challenged CMN (by rotating the cores) and absence of CMN (by using closed, unmodified cores), simultaneously in microcosm- or mesocosm-experiments.

Major Concerns:

I have no major concerns.

Minor Concerns:

Consider to replace the term "to severe" with the term "to challenge", which is more conventionally used in plant microbiology works (for example, at lines 38-40 "CMNs are left either intact between interacting individuals, CHALLENGED by rotation of containers, or prevented from forming by a solid barrier.").

We thank Reviewer #1 for the suggestion. After careful consideration, we have decided that the term "to challenge" may be potentially misleading to our audience because of its generality. "To challenge" may mean that the common mycorrhizal networks were treated with fungicide, or other stressors, while "to sever" explicitly states the action of the treatment. Another potential issue with "to challenge" is that it may mislead readers into assuming that the common mycorrhizal network was the focus of our work, because in the literature the individuals that are challenged with a fungal pathogen for example, are the subjects of the work. In our work, although the common mycorrhizal networks are severed, we investigated the effects of severing CMNs on plant nutrition and interactions.

Reviewer #2:

Manuscript Summary:

The manuscript describes a method to investigate the influence of common mycorrhizal networks on plant growth and plant interactions. The authors propose a relative simple method using rotated cores to disrupt mycorrhizal networks within the context given soil conditions. Plants growing with intact mycorrhizal networks, with disturbed networks by rotating cores or in conditions with completely prevented networks were compared. Rotation of the containers was sufficient to disrupt the mycorrhizal networks thus preventing plant interactions. The presented method is useful to investigate the functioning of plant - mycorrhizal networks in a given ecosystem by mimicking natural conditions. The authors combined this method with ¹⁵N tracer measurements between interacting plants and plant growth. The protocol describes in detail fabrication of the tools and the experimental setup. Representative results for a prairie grass demonstrate the efficient application of the described method. Figures illustrate the method and the results. The manuscript is well written and presents not only the method but also the scientific contexte and results of one example.

Minor Concerns:

(1) The two results illustrated by Figures 6 and 7 could have been mentioned also within the results part.

We thank Reviewer #2 for the suggestion, and have cited Figures 6 and 7 in the representative results in lines 377 – 390.

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Oct 31, 2018

This Agreement between Joanna Weremijewicz ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	4455481342323
License date	Oct 24, 2018
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	New Phytologist
Licensed Content Title	Common mycorrhizal networks amplify competition by preferential mineral nutrient allocation to large host plants
Licensed Content Author	Joanna Weremijewicz, Leonel da Silveira Lobo O'Reilly Sternberg, David P. Janos
Licensed Content Date	Jun 6, 2016
Licensed Content Volume	212
Licensed Content Issue	2
Licensed Content Pages	11
Type of use	Journal/Magazine
Requestor type	Author of this Wiley article
Is the reuse sponsored by or associated with a pharmaceutical or medical products company?	no
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Title of new article	he investigation of plant interactions across common mycorrhizal networks using rotated cores
Publication the new article is in	JoVE
Publisher of new article	MyJoVE Corp
Author of new article	Joanna Weremijewicz
Expected publication date of new article	Mar 2019
Estimated size of new article (pages)	5
Requestor Location	Joanna Weremijewicz 872 Quin Ct. Unit 101 NAPERVILLE, IL 60563 United States Attn: Joanna Weremijewicz
Publisher Tax ID	EU826007151

Total

0.00 USD

[Terms and Conditions](#)**TERMS AND CONDITIONS**

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your RightsLink account (these are available at any time at <http://myaccount.copyright.com>).

Terms and Conditions

- The materials you have requested permission to reproduce or reuse (the "Wiley Materials") are protected by copyright.
- You are hereby granted a personal, non-exclusive, non-sub licensable (on a stand-alone basis), non-transferable, worldwide, limited license to reproduce the Wiley Materials for the purpose specified in the licensing process. This license, **and any CONTENT (PDF or image file) purchased as part of your order**, is for a one-time use only and limited to any maximum distribution number specified in the license. The first instance of republication or reuse granted by this license must be completed within two years of the date of the grant of this license (although copies prepared before the end date may be distributed thereafter). The Wiley Materials shall not be used in any other manner or for any other purpose, beyond what is granted in the license. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Wiley Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Wiley Material. Any third party content is expressly excluded from this permission.
- With respect to the Wiley Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Wiley Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Wiley Materials without the prior permission of the respective copyright owner. **For STM Signatory Publishers clearing permission under the terms of the [STM Permissions Guidelines](#) only, the terms of the license are extended to include subsequent editions and for editions in other languages, provided such editions are for the work as a whole in situ and does not involve the separate exploitation of the permitted figures or extracts**, You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Wiley Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Wiley Materials on a stand-alone basis, or any of the rights granted to you hereunder to any other person.
- The Wiley Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc, the Wiley Companies, or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Wiley Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Wiley Materials or any of the intellectual property rights therein. You shall have

no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto

- NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU.
- WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.
- You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.
- IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.
- Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.
- The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.
- This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.

- Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.
- These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.
- In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.
- WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
- This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.
- This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

WILEY OPEN ACCESS TERMS AND CONDITIONS

Wiley Publishes Open Access Articles in fully Open Access Journals and in Subscription journals offering Online Open. Although most of the fully Open Access journals publish open access articles under the terms of the Creative Commons Attribution (CC BY) License only, the subscription journals and a few of the Open Access Journals offer a choice of Creative Commons Licenses. The license type is clearly identified on the article.

The Creative Commons Attribution License

The [Creative Commons Attribution License \(CC-BY\)](#) allows users to copy, distribute and transmit an article, adapt the article and make commercial use of the article. The CC-BY license permits commercial and non-

Creative Commons Attribution Non-Commercial License

The [Creative Commons Attribution Non-Commercial \(CC-BY-NC\) License](#) permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.(see below)

Creative Commons Attribution-Non-Commercial-NoDerivs License

The [Creative Commons Attribution Non-Commercial-NoDerivs License](#) (CC-BY-NC-ND) permits use, distribution and reproduction in any medium, provided the original work is properly cited, is not used for commercial purposes and no modifications or adaptations are made. (see below)

Use by commercial "for-profit" organizations

Use of Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee.

Further details can be found on Wiley Online Library

<http://olabout.wiley.com/WileyCDA/Section/id-410895.html>

Other Terms and Conditions:

v1.10 Last updated September 2015

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

COMMENTS/NOTES

When drilling rotatable cores, rigid large seedling containers (such as polyvinyl chloride pipe) can accommodate several large holes but must be drilled carefully with a central drill bit protruding from the hole saw.

Commercial silk screen cloth can be obtained in a range of micron openings and is relatively inexpensive.

Glass beads used as blast media (such as to remove rust or paint) also can be mixed in a large volume in a cement mixer to fill the interstitial space with a nutrient poor substrate through which hyphae can grow.

When clipping grasses, it is important to clip below the meristem to prevent re-growth.

When removing plants from containers, it is important not to pull or weed the seedlings, because root systems become intertwined and can damage the plant you wish to leave in the container.

Rotating once a week can be sufficient to produce a treatment effect while rotating more than two or three times a week may be too disruptive and is unnecessary.

Only a small amount of ^{15}N is needed to detect the molecule in plant tissue. For the weekly fertilization of nitrogen, 0.5 % ^{15}N enriched KNO_3 and NH_4Cl together with non-enriched KNO_3 and NH_4Cl is detectable in plant tissue after 12 weeks of weekly fertilization¹⁰.

It is important to monitor growth weekly because if large plants that have grown rapidly in one treatment become root-bound and greatly slow or cease growth, treatment differences may 'disappear' becoming statistically undetectable as plants in other treatments continue to grow.

At harvest, a balance that transmits data to a computer is helpful for ensuring data accuracy for the numerous plants that may be involved.

Macronutrients may be of greatest interest in nutrient analyses.