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Dear Editor,

We are pleased to submit for consideration our invited methods paper, “An optimized rhizobox protocol to visualize root growth and responsiveness to localized nutrients,” for publication in the Journal of Visualized Experiments. The enclosed protocol is accessible and versatile, and we feel it will be a valuable contribution to the field of root and rhizosphere research.

Interest in roots and rhizosphere has grown rapidly in recent years given increasing acknowledgement of their importance to plant nutrient uptake, biogeochemical cycling, and carbon sequestration. However, visualizing roots *in situ* poses a substantial challenge, especially when the goal is to study the development of a single root system over time. Destructive harvesting methods such as shovelomics eliminate the possibility of time-series observations on single experimental units, and more recent non-destructive imaging techniques such as magnetic resonance imaging and x-ray computed tomography require specialized, expensive equipment.

We present a protocol to construct rhizoboxes, clear rectangular units that allow visualization of roots in soil (or a soil-like substrate) over time. Our protocol focuses on the application of rhizoboxes to root plasticity research, in which root proliferation in nutrient-enriched patches is compared to a nutrient-free control patch. We also discuss the ways that other rhizosphere researchers have used rhizoboxes to address other research questions and outline potential future applications. This protocol is inexpensive and approachable in comparison to many published rhizobox experiments, including one recently published in JoVE (Bodner et al. 2017).

Given recent interest in roots and rhizosphere interactions and the diverse potential applications of rhizoboxes to answer these research questions, we feel this protocol is a unique and valuable contribution that is well-suited to publication in JoVE.

Thank you for your consideration.

Sincerely,

A handwritten signature in black ink, appearing to read 'AG', with several horizontal and diagonal strokes extending from the letters.

Dr. Amélie Gaudin

TITLE:

An Optimized Rhizobox Protocol to Visualize Root Growth and Responsiveness to Localized Nutrients

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KEYWORDS:

Isotope labeling, nitrogen, nutrient uptake, plasticity, proliferation, rhizobox, rhizosphere, root.

SUMMARY:

Visualizing and measuring root growth *in situ* is extremely challenging. We present a customizable rhizobox method to track root development and proliferation over time in response to nutrient enrichment. This method is used to analyze maize genotypic differences in root plasticity in response to an organic nitrogen source.

ABSTRACT:

Roots are notoriously difficult to study. Soil is both a visual and mechanical barrier, making it difficult to track roots *in situ* without destructive harvest or expensive equipment. We present a customizable and affordable rhizobox method that allows the non-destructive visualization of root growth over time and is particularly well-suited to studying root plasticity in response to localized resource patches. The method was validated by assessing maize genotypic variation in plasticity responses to patches containing ¹⁵N-labeled legume residue. Methods are described to obtain representative developmental measurements over time, measure root length density in resource-containing and control patches, calculate root growth rates, and determine ¹⁵N recovery by plant roots and shoots. Advantages, caveats, and potential future applications of the method are also discussed. Although care must be taken to ensure that experimental conditions do not bias root growth data, the rhizobox protocol presented here yields reliable results if carried out with sufficient attention to detail.

INTRODUCTION:

Although often overlooked compared to their aboveground counterparts, roots play a critical role in plant nutrient acquisition. Given the substantial carbon cost of root construction and maintenance, plants have evolved mechanisms to develop roots only where foraging is worth the investment. Root systems can thus efficiently and dynamically mine resource patches by proliferating in hotspots, upregulating rates of uptake, and rapidly translocating nutrients to the phloem for further transport¹. Plasticity responses can vary widely among plant species or genotypes^{2, 3} and depending on the chemical form of the nutrient involved^{4, 5}. Variation in root plasticity should be explored further, as understanding complex root responses to heterogeneous soil resources could inform breeding and management strategies to increase nutrient use efficiency in agriculture.

Despite its necessity and relevance for understanding plant systems, visualizing and quantifying root plasticity at relevant scales poses technical challenges. Excavating the root crown from the soil (“shovelomics”⁶) is a common method, but fine roots exploit small pores between soil aggregates, and excavation inevitably leads to some degree of loss of these fragile roots. Furthermore, destructive harvest makes it impossible to follow changes in one root system over time. *In situ* imaging methods such as X-ray computed tomography allow direct visualization of roots and soil resources at high spatial resolution⁷, but are expensive and require specialized equipment. Hydroponic experiments avoid constraints associated with extracting roots from soil, but root morphology and architecture differ in aqueous media as compared to the mechanical constraints and biophysical complexity of soils^{8, 9}. Finally, rhizosphere processes and functions cannot be integrated with developmental plasticity in these artificial media.

We present a protocol for the construction and use of rhizoboxes (narrow, clear-sided rectangular containers) as a low-cost, customizable method to characterize root growth in soil over time. Specially designed frames encourage roots to grow preferentially against the bottom panel due to gravitropism, increasing the accuracy of root length measurements. Rhizoboxes are commonly used to study root growth and rhizosphere interactions^{10–12}, but the method presented here offers an advantage in simplicity with its single-compartment design and inexpensive materials, and is designed to study root responses to localized nutrients. However, the method could also be adapted to study a range of other root and rhizosphere processes such as intra/interspecies competition, spatial distribution of chemical compounds, microbes, or enzyme activity. Here, we investigate genotypic differences among maize hybrids in response to patches of ¹⁵N-labeled legume residue and highlight representative results to validate the rhizobox method.

PROTOCOL:

1. Preparation of the Front and Back Panels, and Spacers

1.1. Prepare the front and back panels.

1.1.1. Cut two pieces of clear 0.635 cm thick acrylic to 40.5 cm wide by 61 cm long per box or purchase pre-cut pieces (see **Table of Materials**).

1.1.2. Using a drill bit designed for acrylic, drill holes 0.635 cm in diameter 1.3 cm from the side edges at 2.5, 19, 38, and 53.3 cm from the top. Drill holes 1.3 cm from the bottom edge at 2.5, 20.3, and 38 cm from the left side (**Figure 1**).

Note: It is most efficient to use a drill press for a stack of six to ten sheets at a time, but a hand drill can also be used.

[Insert **Figure 1** here.]

1.1.3. Remove any protective coverings from the acrylic and gently clean both panels before assembling the boxes.

1.2. Prepare the side and bottom spacers.

1.2.1. Cut three spacers per box from high-density polyethylene (HDPE) or purchase two pre-cut side spacers (0.635 cm thick, 2.5 cm wide, 57 cm long), and one pre-cut bottom spacer (0.635 cm thick, 2.5 cm wide, 40.5 cm long). See the **Table of Materials**.

1.2.2. Align the spacers between the front and back panels along the sides and bottom of the box. Using a hand drill or drill press, drill through the existing holes in the front and back again so that the holes pass through all three layers cleanly.

1.2.3. Hold the layers place using clamps or by installing a combination of bolts, nuts, and washers in each newly drilled hole (see step 3.1).

2. Installation of a Strip of Polyester Batting at the Bottom of the Box

Note: This will prevent soil and water from leaking through joints between spacers.

2.1. Cut polyester batting into 2.5 cm wide by 40.5 cm long strips (see the **Table of Materials**).

2.2. With the back panel lying flat and the spacers on top of it, lay the batting directly above the bottom spacer and hold it in place with the top panel (**Figure 2**).

[Insert **Figure 2** here.]

3. Assembly of the Rhizoboxes

3.1. Assemble the rhizoboxes using 20-thread screws (3.2 cm length by 0.635 cm diameter), washers (0.635 cm internal diameter), and hex nuts (sized to fit the screws, see **Table of Materials**).

3.2. Tighten each screw through a washer, front panel, spacer, back panel, washer, and hex nut. Make sure the screws are very tight; if the box is assembled loosely, soil will spill out through gaps between the panels and side spacers.

Note: Acrylic is easily scratched, and the scratches can interfere with root measurements, so handle the assembled boxes with care. Avoid stacking boxes unless protective material is placed between them.

3.3. Prepare two patch spacers (spacers that will be used to create the treatment and control patches) per box. Cut spacers from high-density polyethylene (HDPE) sheets or purchase them pre-cut (0.635 cm thick, 3.8 cm wide, 28 cm long; see **Table of Materials**). Drill one hole 0.635 cm in diameter in each spacer, 2 cm from the top along the mid-line (**Figure 3**).

[Insert **Figure 3** here.]

3.4. Secure a screw through each hole with a nut so that the spacer can be partially inserted into the rhizobox until the screw prevents it from going further (**Figure 3**).

Note: When the soil is wetted around the spacers and the spacers are removed, two empty spaces will remain that can be filled with the appropriate substrates for the nitrogen-containing treatment patch and control patch.

4. Building PVC Frames to Support the Rhizoboxes at an Angle

Note: When the box is placed at an angle, gravitropism will encourage the roots to grow against the back panel so that all roots are visible for tracing. The polyvinyl chloride (PVC) dimensions in **Figure 4** result in a frame that maintains the rhizobox at an approximately 35° angle to the bench.

4.1. Cut 13 pieces of 1.3 cm diameter PVC per box: 2× 44 cm lengths, 3× 42 cm lengths, 2× 36.3 cm lengths, 2× 25.4 cm lengths, and 4× 3.8 cm lengths (see **Table of Materials**).

Note: A chop saw is highly recommended for efficiency and even cuts.

4.2. Use 4× 2-way elbows, 2× 3-way elbows, and 4 T-joints (see **Table of Materials**) to assemble the box as shown in **Figure 4**.

Note: Frames should be stable without additional glue, but PVC glue can be used if necessary.

[Insert **Figure 4** here.]

5. Sewing Protective Cases to Reflect Light and Heat

Note: Roots avoid light, so these cases exclude light in order to make sure that observed root plasticity responses are driven by the nutrient source in the patches and not by light avoidance.

Light deprivation fabric also reduces the temperature inside the rhizoboxes, helping to avoid heat stress.

5.1. Cut the light deprivation fabric (specialized material that is white on one side and black on the other) into pieces approximately 95 cm wide and 69 cm long (see **Table of Materials**). One piece per box is required.

5.2. Fold each piece in half along the long edge to form a 47.5 cm × 69 cm sleeve. Using a sewing machine needle designed for denim, heavy-duty quilting thread, and a narrow seam, sew along the bottom and $\frac{3}{4}$ of the way up the side of each sleeve. Pin the top corners together with a safety pin.

6. Preparation of 1:1 (V/V) Soil : Sand Substrate to Fill the Rhizoboxes

6.1. Collect approximately 1000 cm³ of field soil (from the site of interest) per box. Dry the soil to constant weight in shallow trays at 60 °C.

Note: Soil for this experiment was collected immediately following harvest in an organically managed corn field from 0–10 cm depth.

6.2. Grind the soil with a mortar and pestle to pass through a 2 mm sieve. Measure the bulk density of the soil by weighing a known volume of soil.

6.3. Obtain sand (such as play sand, which can be purchased inexpensively from a hardware store; see **Table of Materials**) and measure the bulk density.

6.4. Measure out equal volumes sand and soil into a bucket and mix thoroughly. Use a funnel to fill the box slowly and evenly to 2.5 cm from the top, without shaking the box to cause the substrate to settle. Measure this volume of substrate; it should be approximately 1272 cm³.

6.5. Multiply the bulk density of sand by half this volume to obtain the mass of sand needed for each box. Do the same with the bulk density of soil to obtain the mass of soil needed for each box.

Note: For the field soil and sand used in this experiment, this was 976 g of sand and 774 g of soil, but these amounts will vary depending on the bulk density of the soil used.

6.6. Label one large zip-top plastic bag per rhizobox, weigh the appropriate masses of sand and soil into the bag, and homogenize thoroughly.

6.7. Analyze this 1:1 soil-and substrate for nutrient content and the natural abundance of ¹⁵N (δ¹⁵N).

7. Substrate Preparation for the Treatment and Control Patches

7.1. Label two small zip-top plastic bags per rhizobox, one for the treatment patch and one for the control patch. Weigh 30 g of the soil : sand substrate from each large bag (step 6.6) into the two corresponding small bags.

7.2. Mix the substrate with a ^{15}N -labeled nitrogen source for the treatment patch. For this, weigh out 1 g of ^{15}N -labeled plant residue or other N-source (the amount can be adjusted as desired) into each treatment bag (small zip-top bag), and mix thoroughly.

Note: For this experiment, a mixture of ^{15}N -labeled clover and vetch residue was used. Clover and vetch seeds were planted in a 1:1 mix of vermiculite and sand, and grown under greenhouse conditions. Plants were watered daily with deionized water and twice weekly with 1/100 strength of Long-Ashton solution¹³ containing ^{15}N -labeled nitrogen sources. All aboveground biomass was harvested at four weeks after planting, dried, and ground to pass through a 2 mm sieve. If a different nutrient is chosen, particularly if that element is mobile in soil, pilot experiments to test for leaching are encouraged. Slow-release forms of nutrients could be used or a different rhizobox design could be chosen to restrict leaching (*e.g.*, by separate compartments¹⁰) if necessary.

8. Loading Rhizobox with Substrate, and Establishing Treatment and Control Patches

8.1. Weigh each empty rhizobox and record the weights for later use.

8.2. Insert two patch spacers (see step 3.2) into one rhizobox until the screw prevents them from going further. Mark the depth of the bottom edge with a light mark on the side of the rhizobox (**Figure 3**) and remove the spacers.

8.3. Using a funnel with a stem opening that is as narrow as the rhizobox opening, fill the rhizobox from the corresponding large bag of substrate to the marked depth. Move the funnel back and forth slowly and evenly so that the substrate fills uniformly and does not create preferential flow channels.

8.4. When the substrate level reaches the marked depth, put the spacers back in 5 cm from each side of the box. Continue filling the box until the substrate level is approximately 5 cm from the top of the box (there should be substrate remaining in the bag).

8.5. Wet thoroughly around each spacer.

Note: In this experiment, this was achieved by delivering 50 mL of water through drip emitters inserted between the outer edge of each spacer and the side of the rhizobox, and pouring 50 mL of water evenly between the two spacers. Slow irrigation is necessary for uniform wetting.

8.6. Remove the spacers while the soil is wet, leaving an empty cavity for the patches.

8.7. Tape a transparency film to the outside of each rhizobox (see **Table of Materials**). Mark one side as treatment and one as control, and fill the patches from the appropriate bags using the funnel. Trace the boundaries of each patch on the transparency using permanent marker.

8.8. Fill the rhizobox evenly with the remaining substrate. Trace the top of the substrate on the transparency.

8.9. Repeat for the remaining rhizoboxes. Save all the bags for harvest.

9. Even Watering to 60% Water-Holding Capacity

Note: This amount of soil moisture was found to prevent plants from experiencing drought stress while preventing the development of anoxic conditions or algal growth.

9.1. Measure the water-holding capacity (WHC) of the substrate¹⁴.

9.2. Calculate the ideal weight of each box; here defined as the sum of the weight of the empty rhizobox combined with the weight of the substrate at 60% water-holding capacity.

9.3. Multiply the WHC (grams of water / grams of dry substrate) by 0.6 to obtain the mass of water held in the substrate at 60% WHC. Add this mass to the mass of dry substrate and the mass of the ¹⁵N source.

9.4. Add the empty weight of each box to the number obtained above.

9.5. Weigh the boxes once they have been filled. Subtract the weight of each box (in g) at this point from its ideal weight (in g) calculated in step 9.2. Water with this volume (in mL) of de-ionized (DI) water slowly and evenly.

Note: This step may be done using drip irrigation or watering by hand. If watering by hand, allow the water to percolate completely before adding more to avoid heterogenous soil moisture conditions and preferential flow channels.

10. Seed Germination and Transplantation

10.1. If using unplanted controls, set those rhizoboxes aside.

10.2. Surface-sterilize maize seeds by stirring for 1 min in 5% NaOCl, then rinse thoroughly in DI water.

Note: In this experiment, seeds of six different maize genotypes were used in order to investigate genotypic differences in root plasticity.

10.3. Germinate sterilized seeds by placing them on a wet laboratory tissue (*e.g.*, Kimwipe) inside Petri dishes and covering with another moist tissue. There should not be any standing water. Place Petri dishes in a dark place for 48–72 h until the radicle just begins to emerge.

10.4. Use a narrow spatula to dig a hole to 2.5 cm depth at the center of each rhizobox. Transplant a germinated seed into the hole, ensuring that the radicle is oriented directly downwards.

Note: If the radicle is angled towards either patch, the comparison of root growth rates will be biased.

10.5. Trace the location of the seed on the transparency.

10.6. Cover the seed and water in with up to 50 mL of DI water.

11. Plants Growth

11.1. Grow plants for 25 days (or as long as desired), maintaining 60% WHC throughout the growing period. Monitor root growth by tracing the roots.

11.2. Weigh each box every 3–4 days and water until it is within 5 g of its ideal weight. Stop watering the rhizoboxes four days before harvest to facilitate separation of the panels. Remove weeds by hand frequently so that only plant roots of interest are present.

11.3. Trace visible roots every 3–4 days using a permanent marker with clearly distinguishable colors for each tracing day.

Note: Different diameter markers can be used for primary and lateral roots, if desired. It can be useful to define criteria for root tracing at the outset since a degree of subjectivity is involved, particularly if multiple researchers will be tracing roots or if roots of different orders or diameter are to be distinguished with different markers. In this experiment, the accuracy of tracing visible roots on only one side of the box was tested by tracing visible roots on both sides and comparing total root length measured on the scanned transparencies to total root length measured by washing and scanning roots. The correlation between traced and scanned root length was significant regardless of whether only the back transparency or both transparencies were used. It is therefore possible to just trace visible roots on the back panel.

12. Harvesting Shoots, and Obtaining Root and Soil Samples for Analysis

12.1. Lay the first rhizobox flat and remove all the screws.

12.2. Harvest the shoot samples. Clip shoots at the base, rinse off any soil with DI water, and dry at 60 °C. Grind shoots with a mortar and pestle to pass through a 2 mm sieve and weigh subsamples into tin capsules for isotope analysis (see section 14).

12.3. Using the transparency as a guide, cut around the treatment and control patches with a razor. Use a spoon or spatula to scoop the roots and the adhering rhizosphere soil into the respective treatment or control bag.

Note: While many methods exist to separate rhizosphere soil, the soil under the influence of plant roots¹⁵, and the rhizosphere can be considered a gradient rather than a strictly delineated zone¹⁶, this method follows the widely used definition of soil that adheres to plant roots after shaking¹⁷.

12.4. Scoop the remaining roots and soil into the third bag.

12.5. Pass the treatment, control, and bulk samples through a 2 mm sieve to separate roots from substrate, removing any visible roots or fragments >1 cm in length with fine tweezers. Keep these samples separate from one another for a total of three root and three substrate samples.

13. Validation of Tracings and Estimation of Relative Root Growth Rates

13.1. Scan treatment, control, and bulk samples and calculate the root length.

13.1.1. Working with one sample at a time, rinse roots carefully with DI water to remove any remaining substrate. Arrange samples in a clear tray so that the roots are not overlapping.

13.1.2. Scan samples using a scanner compatible with root analysis software (*e.g.*, WinRhizo). Ensure that the software is calibrated to reliably distinguish roots from the image background.

13.1.3. Use the software to measure total root length and root length in the diameter classes of interest (*e.g.*, <0.2 mm, 0.2–0.4 mm, 0.4–0.8 mm, 0.8–1.6 mm, >1.6 mm).

13.2. Calculate root length density (RLD) for treatment and control patches and for each rhizobox as a whole.

13.2.1. Calculate the volume of treatment and control patches by multiplying the area traced on each transparency (see step 8.1) by 0.635 cm, the depth of the box. Use those volumes to calculate root length density in treatment and control patches using the total root length in each patch (see step 13.1.3).

$$RLD_{patch} = \frac{\text{Total root length in patch (cm)}}{\text{Patch volume (cm}^3\text{)}}$$

13.2.2. Calculate the volume of substrate in each rhizobox by multiplying the area traced on the transparency (see step 8.1) by 0.635 cm. Calculate RLD as for the treatment and control patches.

$$RLD_{rhizobox} = \frac{\text{Total root length in rhizobox (cm)}}{\text{Substrate volume (cm}^3\text{)}}$$

393 13.3. Validate the root tracing method by comparing scanned root systems and traced images.

394
395 13.3.1. Scan each transparency and calculate total root length using the software. Save the
396 scanned image for growth rate calculations.

397
398 13.3.2. Sum the total root length measurements of treatment, control, and bulk samples for each
399 box (see step 13.1.3).

400
401 13.3.3. Test the scanned and traced measurements of total root length to see whether the
402 correlation is statistically significant.

403
404 Note: If so, the tracing method is validated, and relative growth rates can be calculated at each
405 time point. If not, only the scanned root system data provides an accurate indication of root
406 growth. This could be the case if tracing methodology was inconsistent or if roots were not
407 equally visible for all genotypes, for example.

408
409 13.4. If the tracing method was validated, calculate relative root growth rates for each rhizobox.

410
411 13.4.1. Use root analysis software calibrated to distinguish between the chosen tracing colors to
412 measure total root length in treatment, control, and bulk samples at each time point. Calculate
413 cumulative total root length at each time point.

414
415 13.4.2. Calculate relative root growth rates (RGR_{root}) for each rhizobox as well as for treatment
416 and control patches for each time interval t_1 - t_2 as follows.

$$RGR_{root\ t1-t2} = \frac{\ln(L_2) - \ln(L_1)}{t_2 - t_1}$$

418
419 Note: Here L_1 is total root length in the patch (cumulative sum from 11.3) at t_1 days after
420 transplanting (DAT) and L_2 is total root length in the patch at t_2 DAT.

421 422 **14. Analysis of ^{15}N partitioning among root, shoot, and treatment soil samples**

423
424 14.1. Dry roots at 60 °C, weigh biomass, and grind to pass through a 2 mm sieve.

425
426 14.2. Dry subsamples of treatment soil at 60 °C.

427
428 14.3. Package roots and treatment into tin capsules as with the shoots.

429
430 Note: Ideal sample weight per capsule should be calculated separately for shoots, roots, and soil
431 based on the estimated C/N ratio of the material to achieve the target amount of total N for
432 analysis. Contact the stable isotope facility where the samples are to be submitted for more
433 information. For this experiment, the sample preparation instructions and Sample Weight
434 Calculator provided by the UC Davis Stable Isotope Facility were followed¹⁸.

CAUTION: Take special care to mix samples evenly before packaging into capsules and prepare multiple capsules per sample. If samples are not evenly mixed, apparent recovery of ^{15}N can exceed the amount originally present.

14.4. Analyze total N, $\delta^{15}\text{N}$, and ^{15}N content of each shoot, root, and treatment soil sample.

Note: In this experiment, plant samples were analyzed via combustion with a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer at the UC Davis Stable Isotope Facility (UCD SIF). Soil samples were analyzed with an Elementar Vario EL Cube elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer at the UCD SIF.

14.5. Calculate the amount of ^{15}N obtained from the label in plant shoot and root samples.

14.5.1. First, calculate the amount of ^{15}N in excess of atmospheric ^{15}N in each pool, $^{15}P^*$:

$$^{15}P^* = ^{15}P - 0.3663$$

where ^{15}P is the ^{15}N content in atomic% of the pool of interest.

14.5.2. Second, calculate the amount of ^{15}N in excess of atmospheric ^{15}N in the label, $^{15}L^*$:

$$^{15}L^* = ^{15}L - 0.3663$$

where ^{15}L is the ^{15}N content in atomic% of the labeled N source.

14.5.3. Third, calculate the amount of total N in each pool, N_p :

$$N_p = m_p * \%_p$$

where m_p is the mass of the pool (e.g., total dry shoot or root biomass) and $\%_p$ is the percent of N of that pool.

14.5.4. Finally, use the results of 14.5.1–14.5.3 in the Ndff equation¹⁹ to calculate the amount of N obtained from the label, N_l :

$$N_l = N_p \frac{^{15}P^*}{^{15}L^*}$$

Note: The Ndff equation is used to determine the amount of N from a labeled source that is recovered by plants. It assumes that no isotopic discrimination occurs during N uptake by the plant and is generally valid for N sources enriched ~1–10%¹⁹.

REPRESENTATIVE RESULTS:

Roots grew preferentially against the back of the box, as anticipated. Total traced root length on the back of the box ranged from 400 to 1956 cm, as compared to 93–758 cm on the front of the box. Pairwise Pearson correlation coefficients were calculated between scanned root length and traced root length on the front of the box, back of the box, and the sum of front and back was used to determine whether tracing accurately reflected total root length ($n = 23$, as the plant in one box died during the experiment). Scanned total root length was significantly correlated with traced root length on the back of the box (**Figure 5A**, $p = 0.0059$), front of the box (**Figure 5B**, $p = 0.022$), and sum of back and front (**Figure 5C**, $p = 0.0036$). Tracing only the back of the box is thus validated as giving a representative measure of root growth while halving the time required to trace roots. It should be noted, however, that tracing captures only 21.6–54.6% of total root length. While roots do grow preferentially against the surface of the rhizobox, fine lateral roots in particular may not be visible for tracing. Tracing is well-suited to relative comparisons of root length over time, especially early in development, but harvesting and scanning root systems is preferable if the goal is to accurately quantify total root length.

[Insert **Figure 5** here.]

Root growth rates over time were similar among boxes, as shown by consistent slopes when plotting the natural log of total root length against time (**Figure 6**). While slight variability is to be expected, consistent growth rates indicate that experimental conditions were uniform for all boxes. Dramatically different slopes would indicate that plants were growing at different rates, suggesting the need to check for differences in variables such as temperature or moisture.

[Insert **Figure 6** here.]

Roots of all maize genotypes proliferated in patches containing ^{15}N -labeled cover crop residue. Two-way ANOVA with patch type and genotype as fixed factors ($n = 23$) revealed that root length density was higher in treatment than control patches using scanned root data (**Figure 7a**, $p = 0.013$) as well as traced root data (**Figure 7b**, $p = 0.005$). RLD was not significantly different among genotypes in either case.

[Insert **Figure 7** here.]

Root diameter can be used to make inferences about root function and turnover. Fine roots are more likely to be lateral roots that rapidly develop and proliferate in response to resource hotspots, while larger coarse roots are more likely to be long-lived, slow-to-respond axial roots. Scanned root systems were analyzed for the proportion of roots in each diameter class: <0.2 mm, 0.2 – 0.4 mm, 0.4 – 0.8 mm, 0.8 – 1.6 mm, and >1.6 mm, and each size class was tested for genotypic differences. Genotypes with more fine roots in treatment patches might indicate a more effective proliferation response. One-way ANOVA with genotype as a fixed factor ($n = 23$) revealed that genotypes did not differ in root length in each size class for the root system overall (**Figure 8a**), treatment patches (**Figure 8b**), or control patches (**Figure 8c**). A majority of the roots were fine roots (<0.2 mm).

[Insert **Figure 8** here.]

Label N was higher in shoot than root samples across genotypes according to two-way ANOVA with sample type and genotype as fixed factors ($n = 23$, **Figure 9**), showing that 77–81% of N taken up from the treatment patch was translocated from roots to shoots during the experiment. One-way ANOVA ($n = 23$) showed that $\delta^{15}\text{N}$ of root and shoot samples did not vary by genotype.

[Insert **Figure 9** here.]

FIGURE AND TABLE LEGENDS:

Figure 1: Layout of drilled holes. Holes are drilled 1.3 cm from the side edges at 2.5, 19, 38, and 53.3 cm from the top, and 1.3 cm from the bottom edge at 2.5, 20.3, and 38 cm from the left margin.

Figure 2: Assembled rhizobox with batting. A narrow strip of batting at the bottom of the rhizobox prevents soil and sand from leaking out.

Figure 3: Patch spacers. Screws inserted through the center of HDPE strips keep them from falling into the box. The rhizobox is filled with soil around the spacers, the soil is wetted, and the spacers are removed in order to leave empty treatment and control patches.

Figure 4: Frame to support rhizoboxes. The lightweight frame is constructed from PVC cut to the specified lengths and connected using the joint types indicated.

Figure 5: Correlations between traced and scanned root length data. **A)** Traced root length was significantly correlated with scanned root length when only the front of the box was traced. **B)** Tracing roots on the back of the box, where the majority of roots were visible, improved the R^2 value of the regression against scanned root length over tracing the front of the box; the correlation was again significant. **C)** The most accurate method is to trace roots on both sides of the box, as shown by the highest R^2 value of the three methods and the significant correlation with scanned root length.

Figure 6: Root growth rates over time. Similar slopes of root length vs. time among rhizoboxes indicate that roots grew at equal rates. Non-uniform slopes could indicate that experimental conditions vary among rhizoboxes.

Figure 7: Root length density by genotype and root data type. **a)** Scanned root data showed that all genotypes (**A-F**) proliferated in the treatment (T) patch, and genotypic differences were not significant. **b)** Harvested and scanned root data confirmed the significant effect of legume residue, but not genotype (**A-F**), on root length density in patches. Error bars represent standard error. C: control; T: treatment.

Figure 8: Proportions of roots in different diameter classes by genotype and location. a) In each rhizobox (excluding treatment and control patches), the majority of roots were fine (<0.2mm in diameter). Genotypes did not differ in the proportions of roots in each diameter class. b) In treatment patches, root length per size class likewise decreased with increasing diameter across genotypes. c) Control patches were characterized by the same patterns. Error bars represent standard error.

Figure 9: Nitrogen obtained from legume residue in roots and shoots at harvest. All genotypes were equally effective at taking up N from the patch containing ¹⁵N-labeled legume residue. The majority of N taken up from the patch was translocated from roots to shoots. Error bars represent standard error.

DISCUSSION:

The rhizoboxes described in this protocol can be used to answer varied questions in root and rhizosphere science, and have found diverse uses elsewhere^{10, 20–25}. Other researchers have captured time-lapse images of rhizoboxes^{21, 25, 26}, some using automated systems^{22, 27}. These approaches may be used for quantitative analyses of root length and architecture not possible with tracing methods. Rhizoboxes have also been used to visualize microbial communities with techniques such as fluorescent *in situ* hybridization (FISH) and micro-autoradiography (MAR)^{21,22}, or to capture spatially explicit patterns of water and nutrient resources with RGB imaging²⁴ or extracellular enzyme activity with zymography^{11, 30}. The rhizoboxes presented here are unique from previous designs in that they are relatively large, making it possible to study species with extensive root systems; have a simple single-compartment design; use readily available, inexpensive materials; and are specially designed to study localized patches. The versatility of this rhizobox protocol could allow it to be customized for a range of other applications in root plasticity and rhizosphere interactions. Other nutrients could replace nitrogen in the patches. Immobile nutrients such as phosphorus would subject to lesser leaching, likely making them a good fit for this approach. The rhizoboxes are also well-suited to comparisons of bulk and rhizosphere soil, as the zone of root influence (a long-standing definition for the rhizosphere¹⁵) can be more clearly delineated than in pot studies and separated from bulk soil with a razor at harvest. Adapting this method to study rhizosphere processes opens up a broad new range of ways to extend the protocol, including study of both abiotic and biotic interactions²³.

The rhizobox method presented here is well-suited to measuring relative differences among genotypes or species in root growth early in development, characterizing relationships among root traits, and exploring the effects of soil characteristics on root growth. Certain steps of the protocol are particularly critical because they affect factors with disproportionate influence on root growth: soil moisture, bulk density, and slope. Watering evenly and equally across boxes is critical given the influence of soil moisture on root growth patterns^{1, 31}. Pilot experiments showed that water delivered through drip emitters became evenly distributed after 24–48 h due to capillary action; however, variability among emitters in the volume of water delivered during a given irrigation period was too high to recommend this method using our arrangement. Hand watering slowly and evenly was the best of the techniques tested, but other irrigation methods are certainly possible. Watering to a previously calculated weight ensures that all boxes maintain

the same soil moisture content, preventing variability in root growth due to water stress³². The weight of empty rhizoboxes can vary significantly, so calculating ideal weights for individual boxes is important.

As with moisture, establishing even bulk density of the substrate throughout the rhizobox is critical to measure root proliferation responses. Roots grow more extensively in less-dense soil³³ and compaction may create channels for preferential water flow, further affecting resource distribution and root growth patterns³⁴. Drying and sieving field soil, thoroughly mixing sand and soil, and moving the funnel back and forth slowly and evenly help create a homogeneous matrix for root growth.

A number of components within this experiment will depend on the research questions of interest as well as the soil type and plant species utilized within the study. The ratio of sand to soil may need to be adjusted for soils of different texture, to ensure that the substrate wets evenly without clumping. Pilot experiments showed that a 1:1 (v/v) soil : sand mixture was superior to 1:2 or 1:3 mixtures for the soil used in this experiment, a very fine sandy loam. The dimensions of the rhizoboxes and duration of the experiment can be adjusted depending on the research question, root traits, and plant species of interest. Maize is a relatively fast growing plant species; we therefore selected larger rhizobox dimensions compared to other rhizobox studies^{20, 35} to provide a sufficient volume of soil that allows the experiment to continue for the optimal duration as plants become increasingly rootbound over time. Finally, the fitting of statistical models to visible and traced root growth must be determined for each study and species of interest, perhaps with pilot experiments. Visible root growth may follow a saturating curve rather than a linear model^{25, 36}, and the slope of the regression of visible on harvested roots varies by plant species²¹.

Roots have an inherent tendency to pursue gravity and avoid light³⁷, but care must be taken to ensure that gravitropism and light avoidance do not bias root growth data. If greenhouse benches are tilted slightly, for example, roots will grow down the slope rather than responding to nutrient patches. Similarly, light gradients in the greenhouse could cause shifts in shoot growth and roots to grow preferentially on one side if the rhizoboxes are not kept completely dark. The light deprivation cases presented in the protocol are an effective means of reducing incident light, but other methods such as wrapping the rhizoboxes in aluminum foil may also work.

The non-destructive rhizobox method presented here facilitates the tracking of roots *in situ* over time³⁸ and can be implemented by a graduate student with a basic working knowledge of power tools. As such, it offers advantages over destructive harvesting methods such as shovelomics⁶, which are better suited to studying mature root architecture but do not allow repeated measurements of the same root system over time, and MRI or X-ray computed tomography^{39, 40}, which allow high-quality imaging of root-substrate interactions but requires expensive equipment. However, it is not without limitations. Construction of the rhizoboxes can be time-consuming, and ensuring that factors such as moisture, bulk density, slope, and light are as uniform as possible, as described above, is non-trivial. Nonetheless, given sufficient time and

attention to detail, the rhizoboxes deliver reliable results and can be reused for many experiments.

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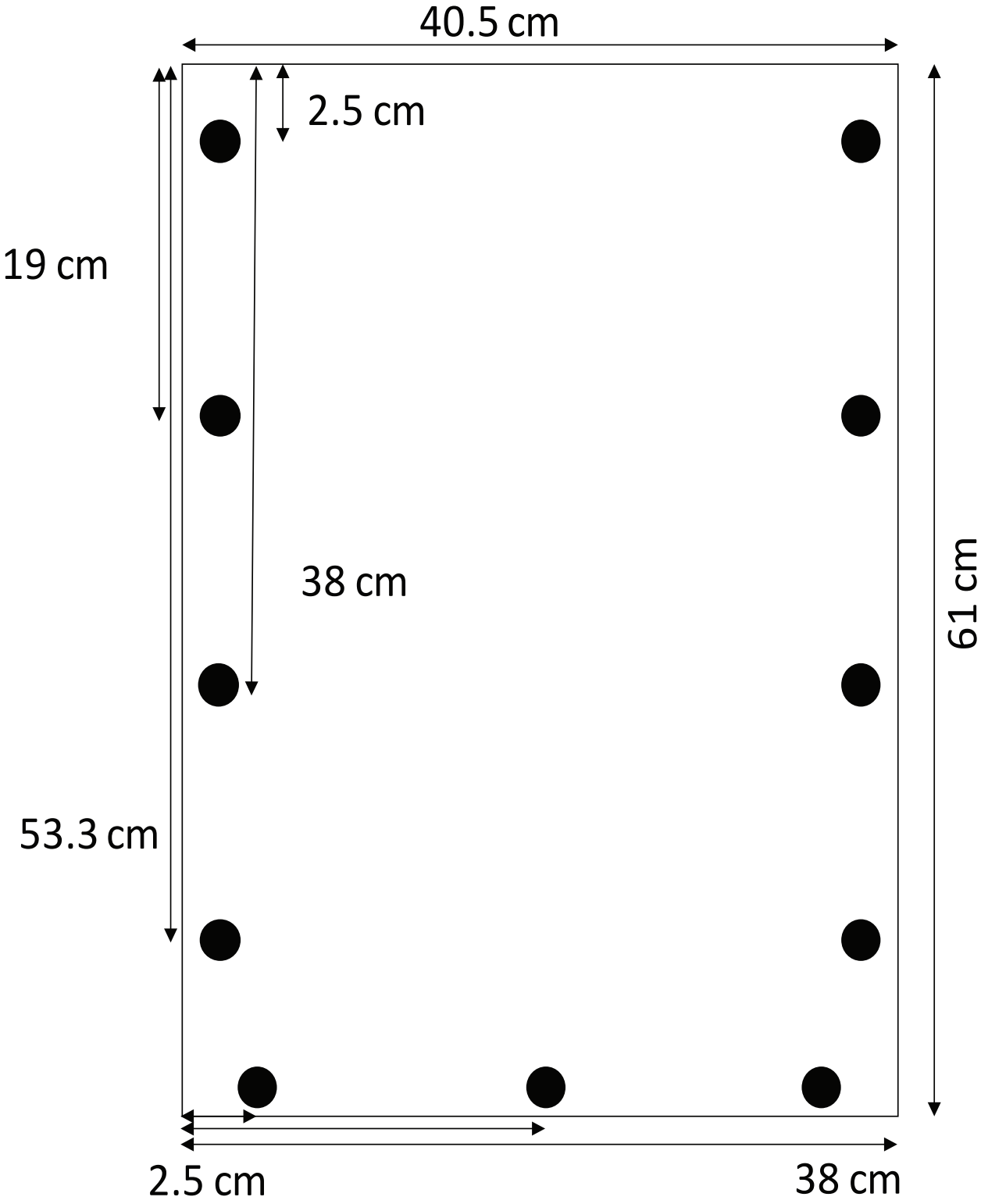
The authors have nothing to disclose.

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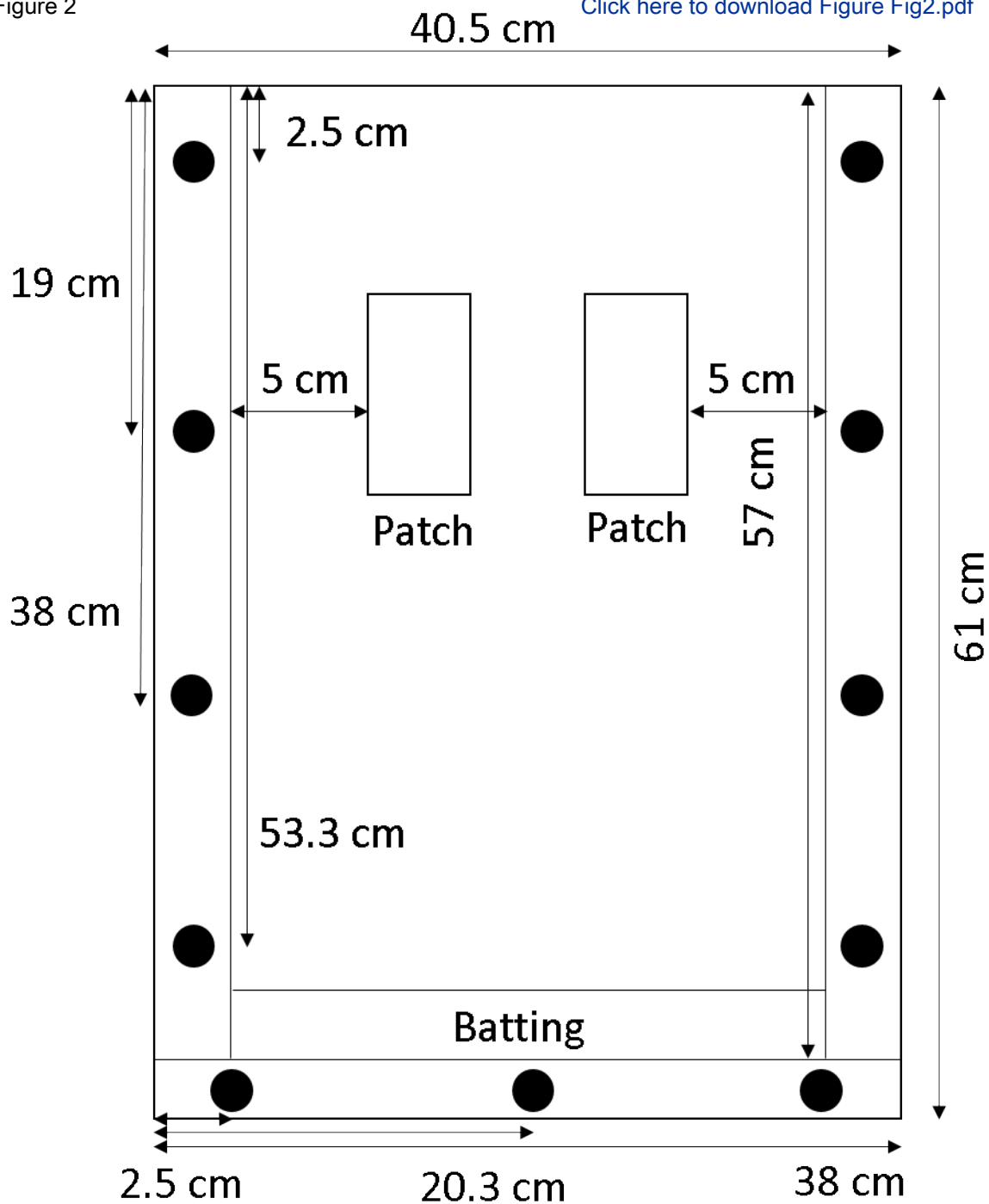


Figure 3

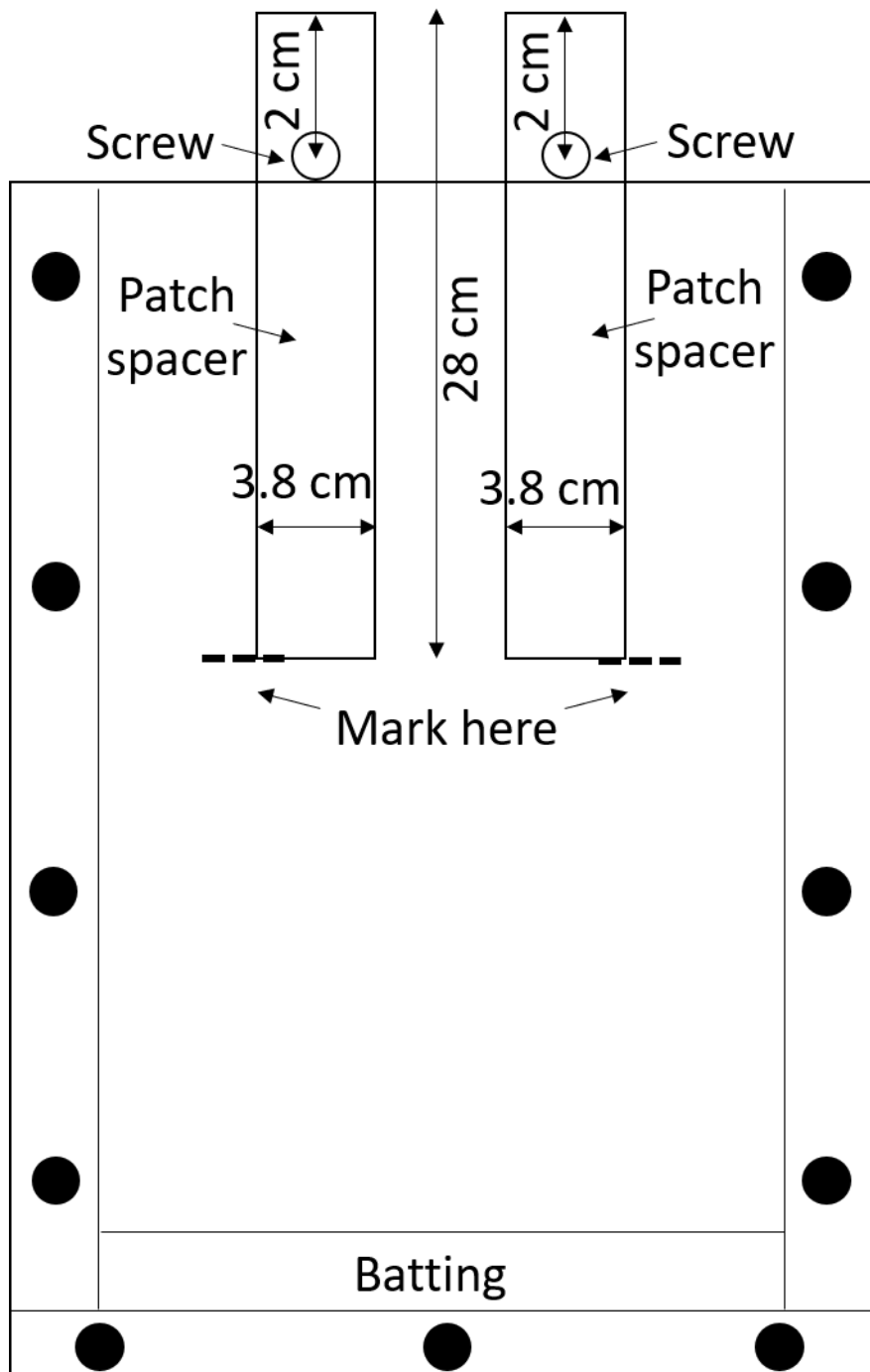
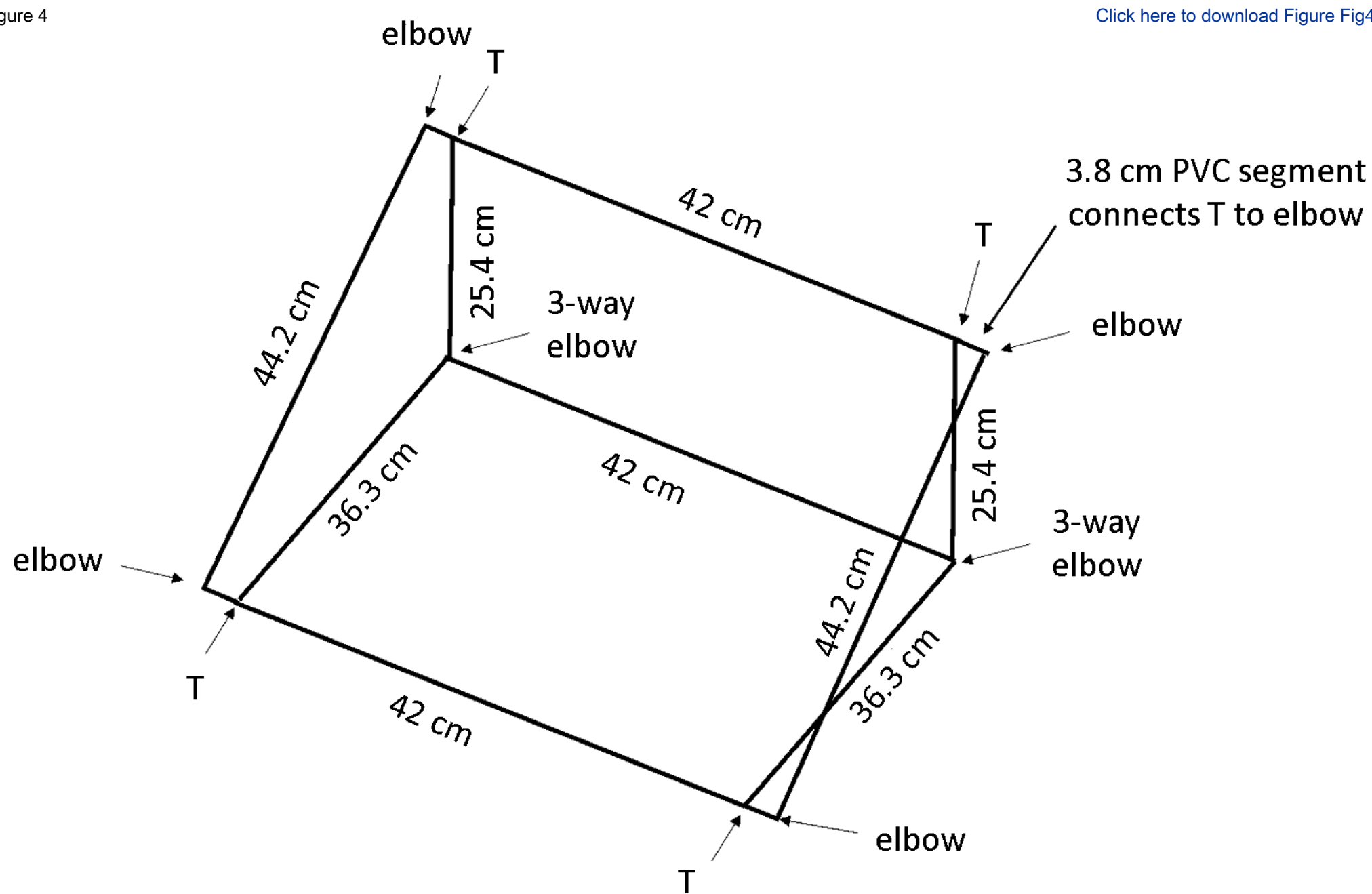
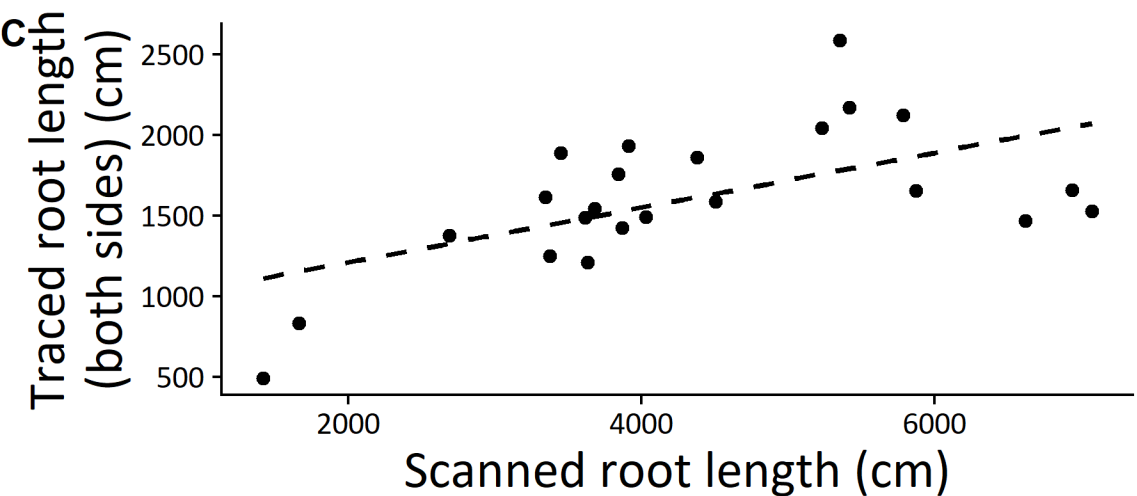
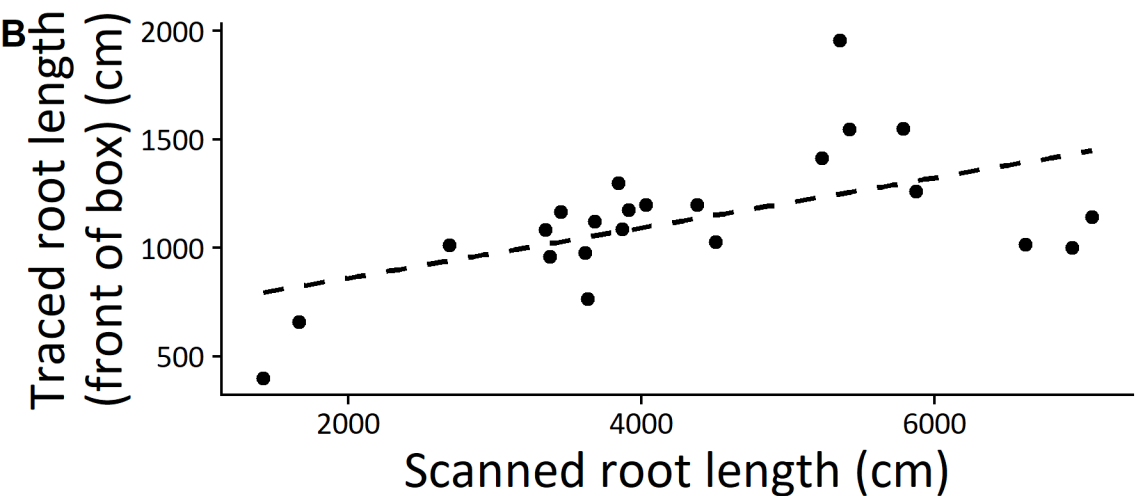
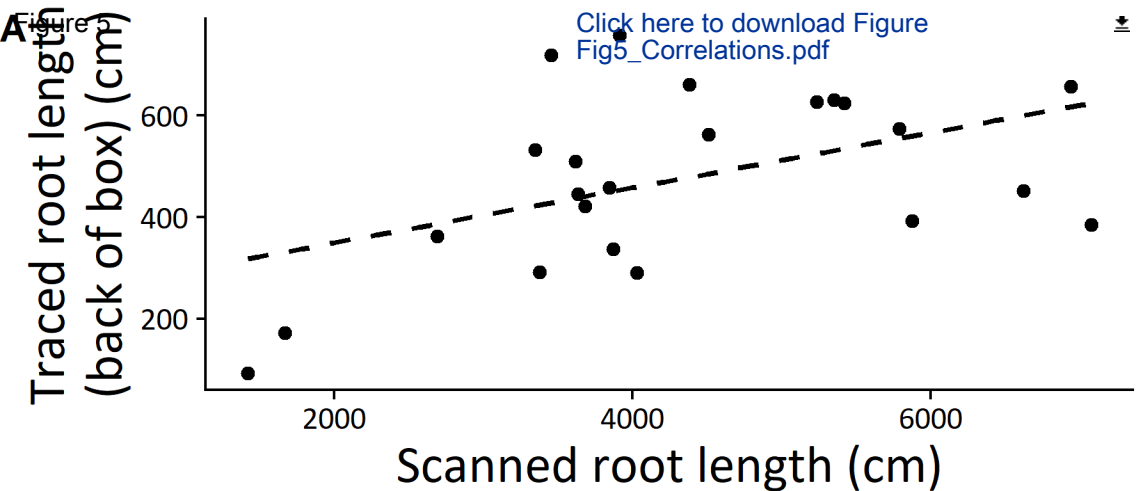


Figure 4





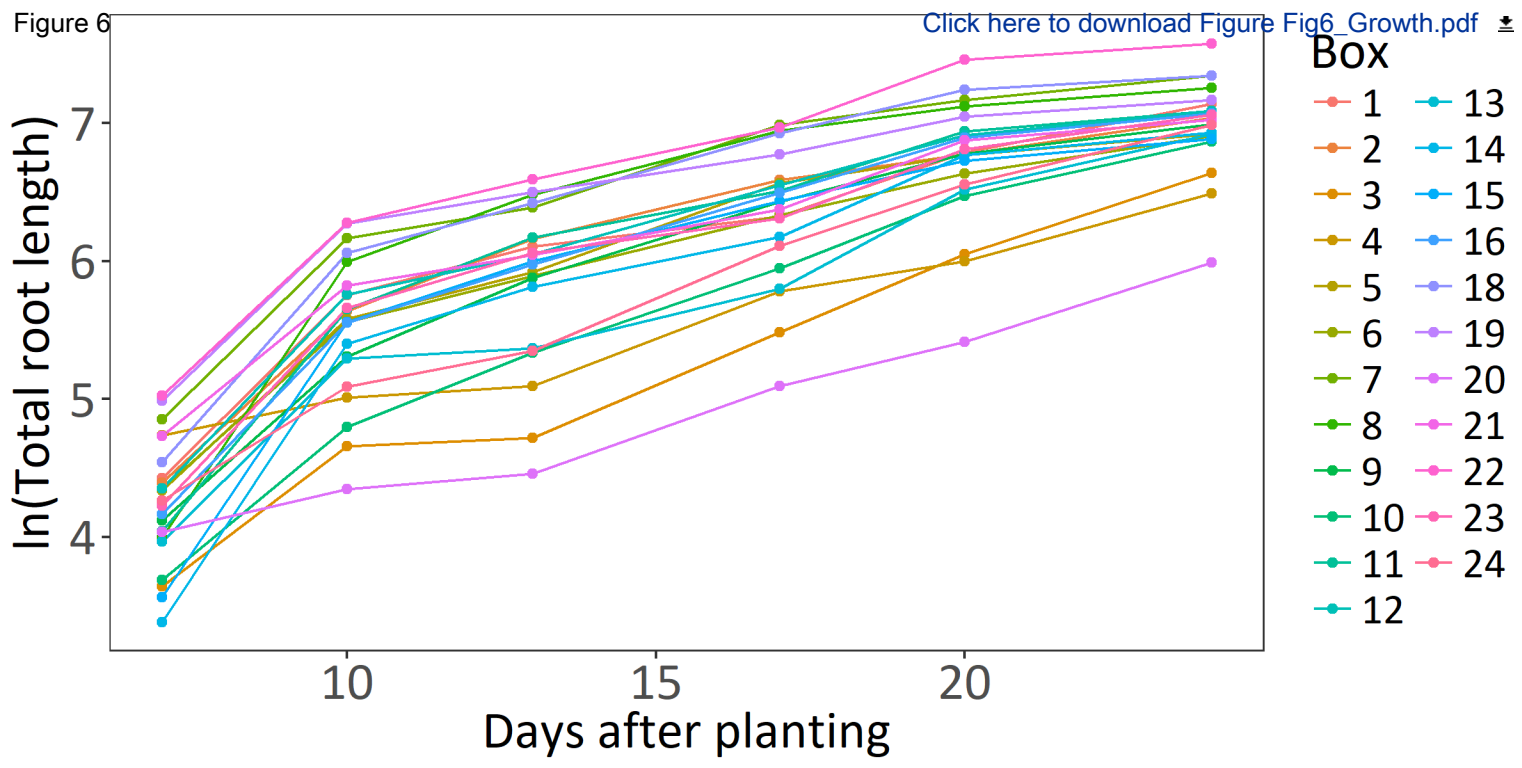
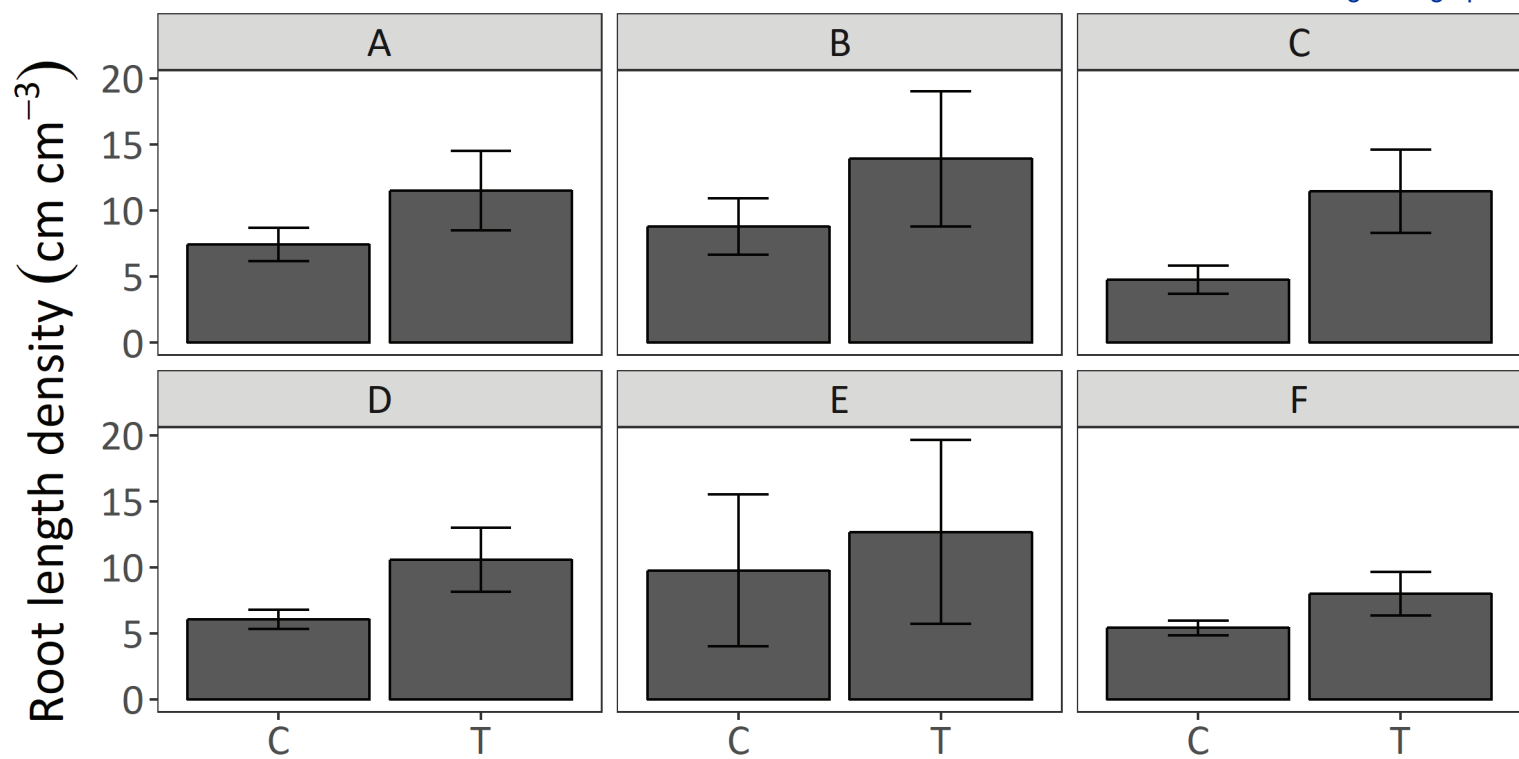


Figure 7

a.



b.

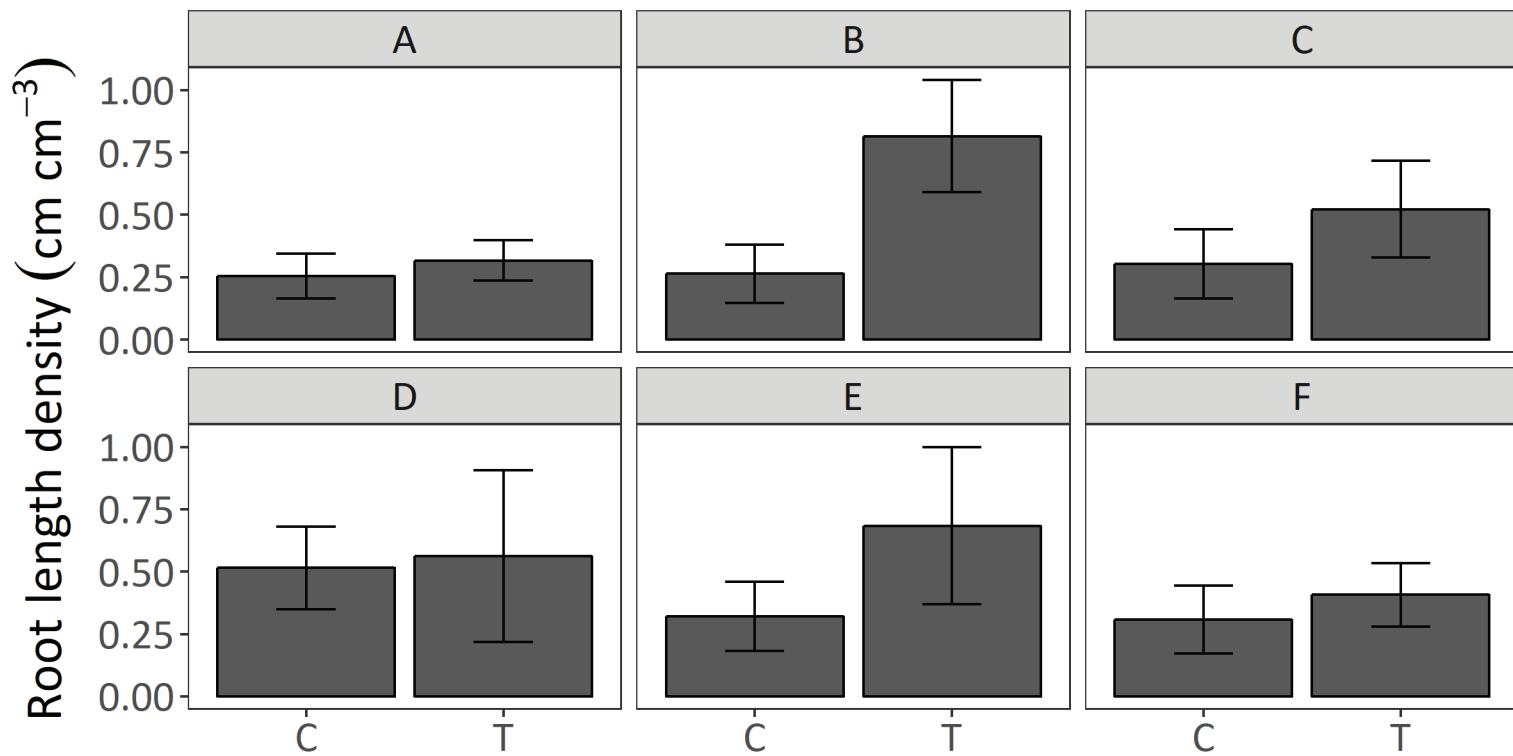
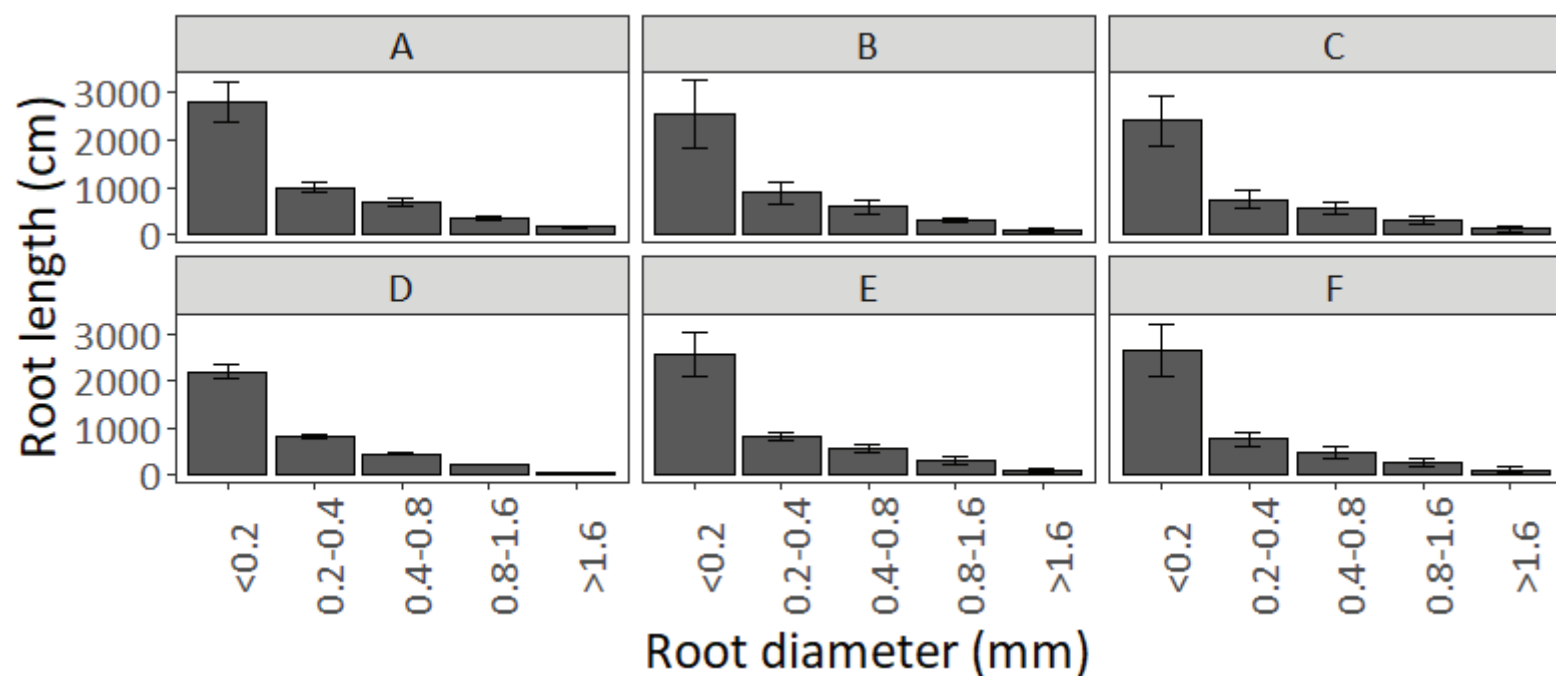


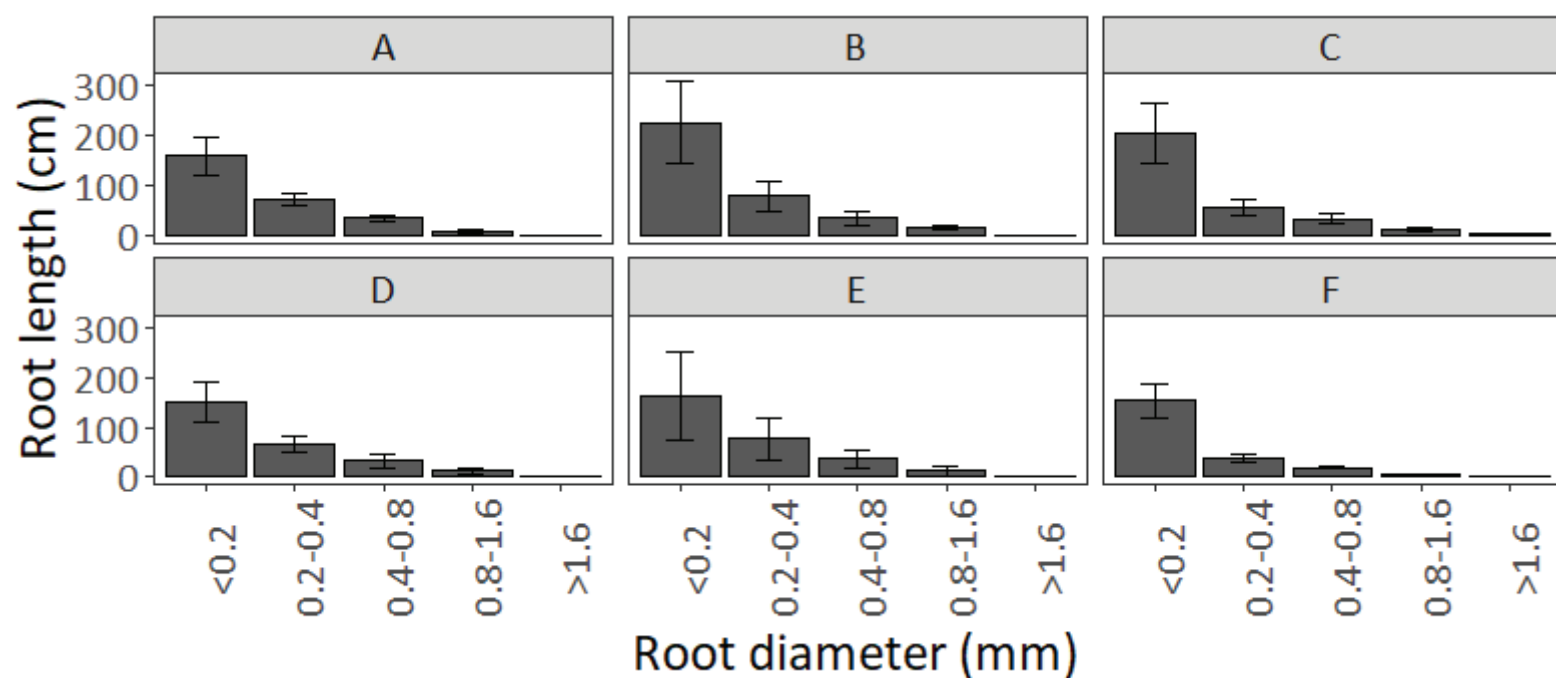
Figure 8

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a.



b.



c.

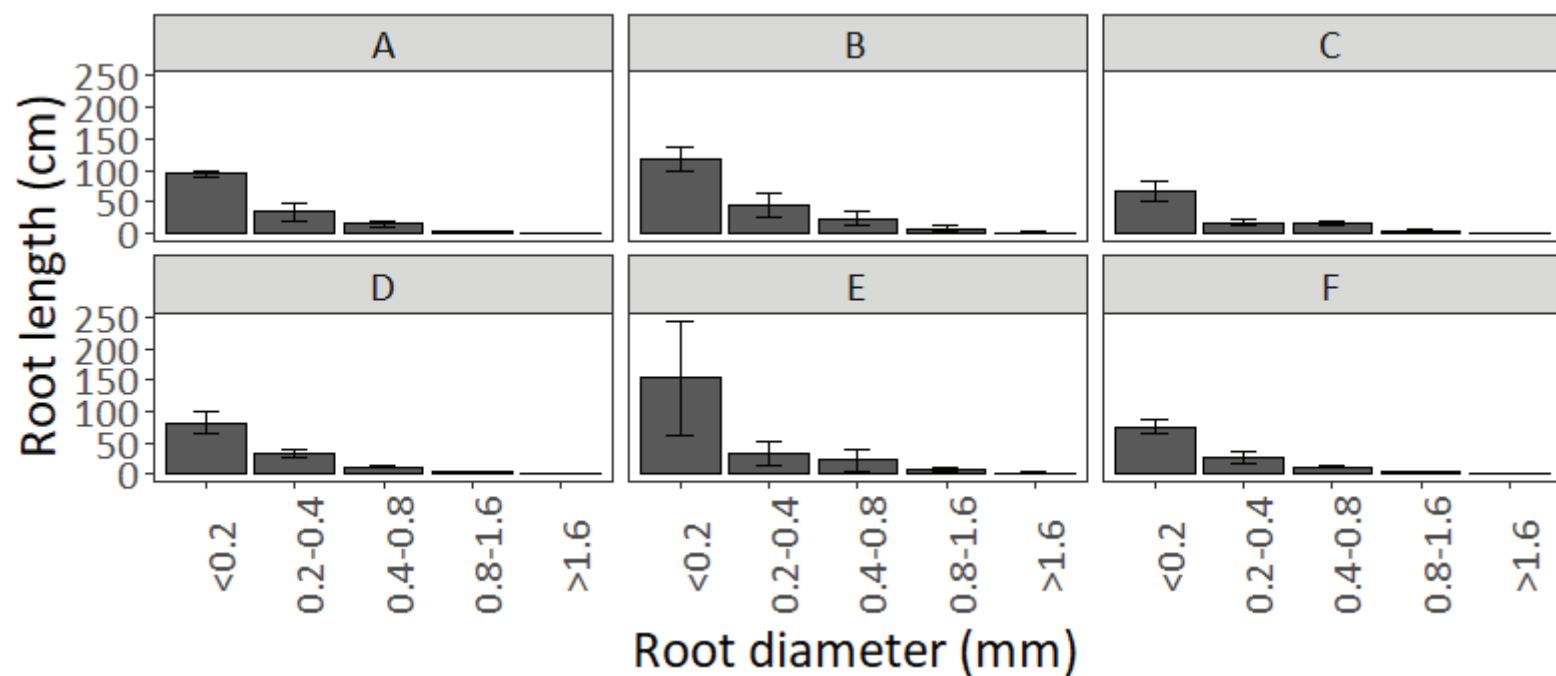
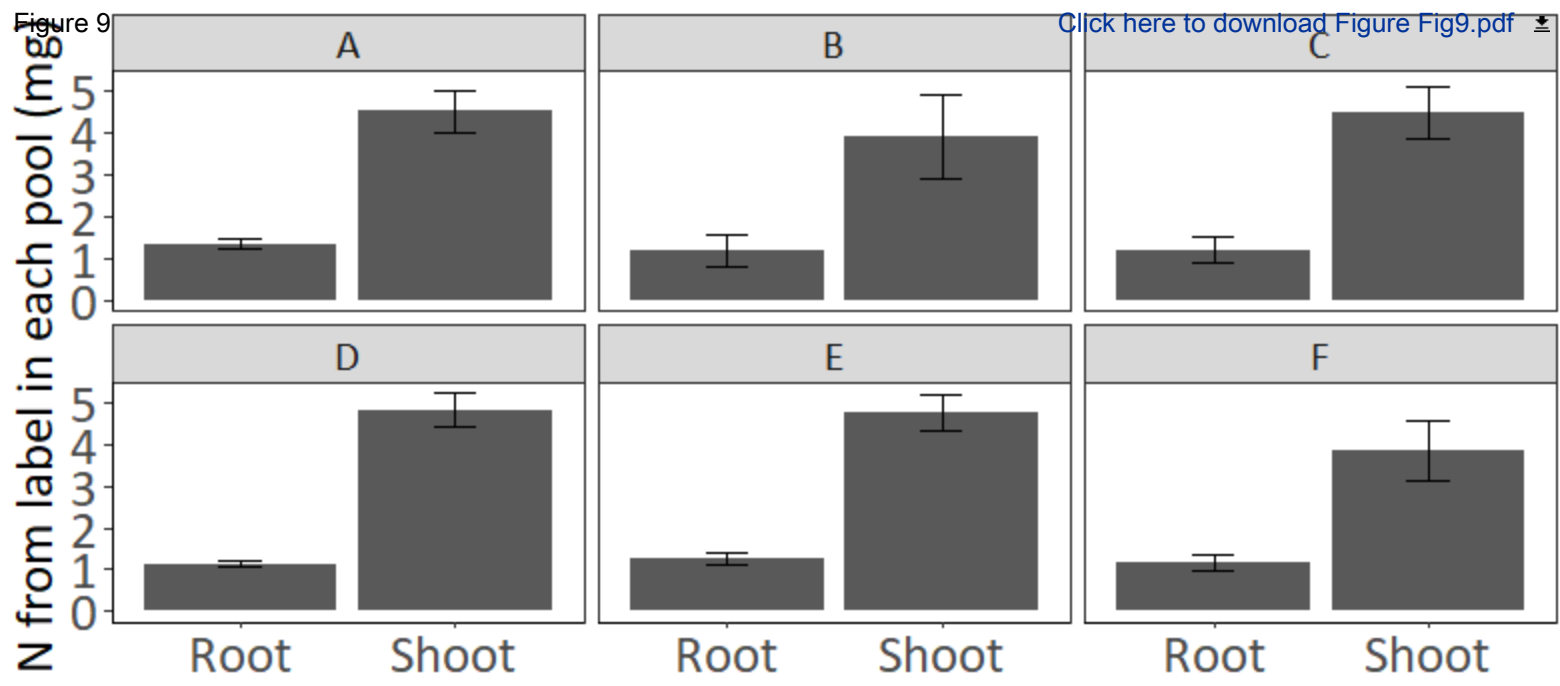


Figure 9

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.27 cm diameter PVC pipe	JM Eagle	530048	305 cm per box, cut into lengths as specified in the protocol
PVC side elbows	Lasco	315498	2 per box
PVC 90-degree elbows	Charlotte	PVC 02300 0600	4 per box
PVC T joints	Charlotte	PVC 02402 0600	4 per box
Extruded acrylic panes	TAP Plastics	N/A	2 per box, 0.64 cm thick x 40.5 cm wide x 61 cm long
HDPE spacers (sides)	TAP Plastics	N/A	2 per box, 0.64 cm thick x 2.5 cm wide x 57 cm long
HDPE spacers (bottom)	TAP Plastics	N/A	1 per box, 0.64 cm thick x 2.5 cm wide x 40.5 cm long
HDPE spacers (patch)	TAP Plastics	N/A	2 per box, 0.64 cm thick x 3.8 cm wide x 28 cm long
Polyester batting	Fairfield	#A-X90	2.5 cm x 40.5 cm strip per box
20-thread screws	N/A	N/A	3.2 cm long, 0.64 cm diameter
Washers	N/A	N/A	0.64 cm internal diameter
Hex nuts	N/A	N/A	sized to fit the screws
Light deprivation fabric	Americover, Inc.	Bold 8WB26.5	1 piece 95 cm wide and 69 cm long per box
Sand	Quikrete	No. 1113	
Field soil	N/A	N/A	
Transparencies for tracing	FXN	FXNT1319100S	One per side of the box to be traced

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Responses to Editorial and Reviewer Comments

We would like to thank the editor and reviewers, whose insightful comments identified areas where the manuscript could be improved. We have conducted a thorough revision in accordance with their suggestions. Major points include adding a new figure (Figure 3) to clarify a section of possible confusion and emphasizing the unique strengths of this protocol in comparison to other rhizobox designs. Please see below for our responses to individual comments.

Editorial Comments:

• *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.*

The manuscript has been proofread thoroughly.

• **Protocol Detail:** *Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) are mentioned in your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.*

We have reviewed the protocol and feel that it contains sufficient detail.

• **Protocol Highlight:** *After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.*

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- 4) *Notes cannot be filmed and should be excluded from highlighting.*
- 5) *Please bear in mind that software steps without a graphical user interface/calculations/ command line scripting cannot be filmed.*

The highlighted section of the protocol has been reviewed to meet the above criteria.

• **Results:** *Please mention the statistical tests performed and report sample sizes.*

This section has been revised to include the statistical tests and report a sample size in each case.

• **Discussion:** *JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail: modifications and troubleshooting, limitations of the technique, significance with respect to existing methods, future applications and critical steps within the protocol.*

These sections are all included in the discussion, which is focused on the methodology as specified above.

• *Please define all abbreviations at first use.*

All abbreviations are now defined at first use.

• *Please use standard abbreviations and symbols for SI Units such as μL , mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.*

Abbreviations, symbols, and units are formatted as indicated.

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All figures are original.

Reviewer #1:

Manuscript Summary:

Manuscript is very well written and the information presented here is of great interest for studying root biology, plant-soil interface studies and many other aspects of rhizosphere biology.

Major Concerns:

Figure 1 is missing.

Thank you for catching this. Figure 1 has been added.

Neither in introduction, nor in discussion section you deliberated why this rhizobox design is better than other designs (see Spohn et al 2013, Wenzel et al 2001, Vollsnes et al 2010).

A brief discussion of the major advantages (simple one-compartment design, ease of construction and low-cost materials, and specifically well-suited to studying localized nutrients) has been added to the introduction (lines 70-73) and the discussion (lines 552-555).

Minor Concerns:

Line 162, 4.3 : Please mention how much weight of soil per box did you use for each of your boxes.

The weights of soil and sand have been added (lines 184-185), with the caveat that they will vary for soil or sand with a different bulk density.

Lines 186-190, 5.2.1.1 : Please include, how often plants were watered ? At what stage of plant growth harvesting was performed ? Which plant parts did you use as N source for your experiment ?

Plants were watered daily with deionized water and twice weekly with Long-Ashton solution. Harvest occurred at four weeks after planting and all aboveground biomass was ground and used as an N source. This information has been added to section 7.2.1.

Lines 322-325, 10.5: Mention the criterion used to differentiate between rhizosphere and bulk soil?

This information has been added to section 12.3, where the separation of rhizosphere soil is first mentioned (lines 322-325).

Reviewer #2:

Manuscript Summary:

I am a strong advocate for affordable plant phenotyping protocols that provide solutions to some of the phenotyping bottlenecks currently bedeviling plant breeders and rhizosphere scientists. Phenotyping root systems is definitely among the most difficult areas of the plant phenotyping discipline. Thus, relevant attempts to provide solutions are very welcome. Consequently, this paper is very relevant to the audience JOVE. The paper is largely well structured and the description of the rhizobox system is appropriate. The title and abstract are equally appropriate.

Major Concerns:

Perhaps, my concern about the paper is that the system described can hardly be used for high throughput screening. Given the enormous amount of data and time normally required for breeding for root traits, high throughput root screening platforms and image analyses routines that seek to address most of the root phenotyping limitations are becoming increasingly important. But I wonder if 'tracing on rhizoboxes' could be used for the currently required level of throughput in screening of root systems. This protocol could be stronger if automated imaging, tracing and extraction of root system features had been developed in addition to the building of the rhizoboxes.

Thank you for your feedback. The tracing method described here is indeed time-intensive and thus not particularly well-suited to high-throughput screening. However, as other methods incorporating automated imaging have already been developed and published in JOVE (see Bodner et al. 2017, cited in this manuscript), this protocol was developed to provide an alternative for researchers who require a lower-cost approach with less specialized equipment.

Minor Concerns:

This protocol is developed around the 'visualization of root growth and responsiveness to localized nutrients' but I was not convinced that patches described in the protocol could efficiently prevent the leaching of localised nutrients to other parts of the bulk soil, especially when dealing with nitrogen. I also think that the section on 'control patches' could be described more clearly. I think a graduate student trying to duplicate this protocol would struggle to follow the section. For the steps listed in that section to lead to the described outcome, the section has to be supported with illustrations and the write up made clearer.

Leaching was likely minimal with the organic nitrogen source used in this experiment, as evidenced by the root proliferation response seen in the patches, which would not have occurred if the nitrogen source had already leached away, and based on estimates of mineralization rates from the literature. Soil nitrogen measured outside the patches was not higher after the experiment than at the beginning, further supporting the idea that leaching was minimal. However, leaching could indeed occur with a soluble form of nitrogen such as nitrate or other mobile elements. If researchers choose to modify the protocol presented here to use a different, more mobile nutrient source, we would encourage them to do a pilot experiment and test for leaching; the nutrient source may need to be modified (e.g. slow-release fertilizers), or the patches could be enclosed in some sort of barrier. This suggestion has been added to section 7.2.1.

The sections on patches and spacers have been revised (see specific responses to comments below) to make this section clearer, and an additional figure has been added to support that section (see Figure 3).

Specifically, the following minor issues should be addressed:

L81: Could this and similar ones in 1.2, 1.3 etc. be made sub-headings?

The protocol steps have been renumbered and additional sub-headings have been added in multiple places to make the flow as clear as possible.

L81: It would be ideal if for any material that the authors describe, they also give the catalogue number and other details of what they used in their lab (or refer to a Table of Materials where these has been provided), to further assist readers in the sourcing of their materials.

Thank you for the suggestion. We have added references to the included Table of Materials where necessary.

L84-87: Does the size of these holes matter in the design? If so, what is the size or diameter of these holes?

The holes should be the same diameter as the screws, washers, and hex nuts. Section 1.1.1 now specifies that the holes should be 0.635 cm in diameter.

L87: I am struggling to find Figure 1. Has been provided? The page for Figure 1 is blank.

Thank you for catching this. Figure 1 has been added.

L92: Define this on first mention. High-density polyethylene

This has now been defined at first mention in section 1.2.

L103-104: Idem.

This should be made a sub-title.

As with the first minor comment, subheadings have been added throughout and sections have been re-numbered.

Because I asked these the following questions before I realized the details have been provided:

A bit more detail is required here. Installed where and how? In-between the plate and the bottom spacers? Or installed over the layers? Why is this not needed at the side of the box? Couldn't water and soil also leak from the sides?

As noted, these details are provided elsewhere. The batting is not needed on the sides because there are no joints between spacers on the sides; leakage only occurs when there is a slight gap between the side and bottom spacers.

L111-114: Create sub-heading e.g.: Assembling of Rhizobox 1.4. I Follow with the protocol

Revised as suggested.

L114: 'if the box is assembled loosely, soil will spill out the sides'; that brings back the question of, why there isn't batting at the sides?

The wording has been revised to clarify that soil would spill out any gaps between the front and back panels and the spacer. Since the batting is the same width as the spacer, it would not

prevent this even if installed along the sides; the batting only prevents leakage through the bottom gap.

L120-125: 'patch spacers'; Sub-heading - But what are patch spacers? Explain this clearly. This section lacks clarity. It has no figure reference and it uses unfamiliar jargons. Please be very explicit what you are describing and what is its purpose. Use figure to further elaborate.

Additional wording has been added to section 3.2 to clarify what the patch spacers are upon first mention. A new figure has been added to show how the holes are drilled in the patch spacers and how a screw keeps them from falling into the rhizoboxes.

L121: How big is the hole?

0.635 cm in diameter; this information has been added to this section.

L135-136: Are these elbows and Ts built of purchased? In any circumstance, more details should be provided.

A reference to the Table of Materials has been added and more details are available in the table.

L140: Yes, this how all section should be sub-headed.

L143: One each of black and white fabric?

Light deprivation fabric is black on one side and white on the other, so only one piece is required. This section has been rewritten to clarify that point, and details about the fabric are available in the Table of Materials.

L145-148: A figure/sketch could help elaborate the description. This section could go further to tell the reader why the protective cases are needed and what it would be used for. Currently it is not so clear why these fabrics have been sewed.

Additional rationale for why light and heat should be minimized has been added to section 5.

L154-156: 'Soil for this experiment was collected immediately following harvest in an organically managed corn field from 0-10 cm depth'. Yes. This satisfies the point I made earlier. After giving the instruction, it is prudent to tell the reader what material you used and where it was sourced.

L161-162: What did you use and what was your source?

A reference to the Table of Materials has been added to section 6.3, and more details about the sand used in this experiment are available there.

L177: Are the authors referring to natural ^{15}N abundance in the soil ($\delta^{15}\text{N}$) at this stage? This should be clarified.

Yes, the reference is to the natural ^{15}N abundance, and the wording has been changed to clarify this.

L186: This is why the 'patch' needed clarification previously. Until now, readers would have struggled to know what the patch was and its purpose.

Thank you for this observation. We hope that the revised wording earlier in the manuscript now makes this clear.

L195: Is this the ziptop plastic bag? If so, indicate this on first mention of the ziptop plastic bag. And indicate which of the bags, small for the patches or large ziptop bag for the rhizobox.

Yes, this section refers to the small ziptop plastic bag that was designated as the treatment bag in 7.1. The wording has been revised to clarify this point.

L198: Filling' rhizobox with patches? How? Is that what the authors literally mean?

This header has been revised to read “Fill rhizoboxes with substrate and establish treatment and control patches,” to make it clear that the box is filled with substrate, then the nitrogen-containing and control patches are put in place.

L200: Rhizobox?

Revised as suggested.

L202: The utility of these patch spacers should have been clarified in 1.5.

Additional wording has been added to section 3.3 in the revised version to clarify the purpose of the patch spacers.

L202: Which one is the 'first rhizobox'? Until now readers would not know rhizoboxes have been numbered.

This phrase was not meant to indicate that the rhizoboxes were numbered, but rather to indicate that the scientist following the protocol should start by filling one rhizobox and then repeat the procedure for all the other rhizoboxes. It has been changed to “one rhizobox”.

L203-204: It is difficult to picture this. Issues about these patch spacers should be described very well for a novice reader to picture and duplicate if required

A new figure (Figure 3) has been added to make this section clearer, and the markings referred to in this specific comment are shown on the figure to clarify.

L207-230: Whilst I admit that this protocol would be accompanied by a video, I still think this section could be elaborated further with sketches/figures. It is difficult to follow or picture the descriptions being given. There are 'spacers', 'patches' etc. Which is which? Which one is a permanent part of the rhizobox? Which one is just needed to aid filling of the rhizobox with substrate? Which one is needed to create space between successive rhizoboxes? Which one is required to join two plates etc?

We agree that the video protocol will be the best way to eliminate confusion about these terms! We feel that the additional figure and the revised wording (see responses to previous comments above) have made this section easier to follow.

L239-258: If all these are the processes to follow to "water boxes evenly to 60% water-holding capacity", then point 7 (L235) must clearly be written as a sub-heading.

Yes, this sub-heading is marked in bold to show that it is a sub-heading.

L395-397: For a graduate student, trying to adapt this protocol for his/her work, this information would not provide the needed help. Explain further. If possible illustrate the point with an example calculation; etc.

Because target sample weights and preparation instructions may vary by laboratory, we revised this section to include the sample preparation requirements used in this experiment and provided a citation. The citation contains all necessary information and a sample weight calculator for readers to use, should they want to submit samples to the same laboratory.

L435: Provide some details on the Ndiff equation here in addition to the reference.

Additional information about this approach and its assumptions has been added to the end of this section (lines 435-437). The reader is referred to the Barraclough et al. citation for more extensive treatment of the subject.

L464: I am just noticing that multiple genotypes were used. This did not come out previously in the methodology

The use of different genotypes was discussed in the abstract and introduction (as well as in the results and figures). An additional sentence has been added to section 10.2 of the protocol to clarify that six genotypes were used in this experiment.