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.*

1. *The highlighting must include all relevant details that are required to perform the step. 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 included in the highlighting.  
   2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.  
   3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.  
   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. *• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."*

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 bedevilling 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 fine 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 form 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. 1 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 15N abundance in the soil (δ15N) at this stage? This should be clarified.*

Yes, the reference is to the natural 15N 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 our 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.