Response to editor and reviewers

I am thankful to the editor and the three anonymous reviewers for their suggestions and comments, which I firmly believe have helped me improve the manuscript. I hope to have addressed all concerns as indicated below. Requests are indicated in italics, and my answers are indicated in bold, green text.

Editorial comments

1. Please take this opportunity to thoroughly proofread the manuscript.

I apologize for the grammatical errors in the text. The manuscript was thoroughly proofread.

2. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors.

Numbering was adjusted following the JoVE Instructions for Authors.

3. Please ensure that all text in the protocol section is written in the imperative tense. Please include all safety procedures and use of hoods, etc.

4. The Protocol should contain only action items that direct the reader to do something.

5. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.

Thank you for your comments. The protocol section was revised, and verbs are now all in the imperative tense. Additional safety procedures were included where necessary. Only action items are included in the protocol, with a few, but important notes. Long paragraphs were eliminated.

6. For SI units, please use standard abbreviations when the unit is preceded by a numeral throughout the protocol.

7. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral throughout the protocol.

All SI and time units were revised and rewritten using the suggested abbreviations.

8. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

More steps were added to parts of the protocol to provide more detail.

9. Line 155: Is the whole plant submerged in the fixative? Does the volume of the fixative depend on the size of the plant? Please specify.

Yes, the whole sample is submerged, and the volume of fixative depends on sample size. This is now indicated in the text.

10. Line 185-188: What is the contrast solution used in this experiment? Please specify the conditions of the vacuum chamber.

Names of contrast solutions were added to this step of the protocol.

11. Line 212: Please specify the size of the tank used in this study.
The size of the tanks (small and large) was added to the text.

12. Line 160: How long is the sample submerged during the washing step?
A suggested duration of washing time was added to the text.

13. Line 266: Is the sample dried by placing it at room temperature?
This step of the protocol was explained in more detail, including timing and a better explanation of how to dry the samples.

14. Line 278-279: Please specify the micro-CT conditions/parameters used in this experiment.
A Table was added including information about the conditions and parameters used in all micro-CT experiments.

15. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video.
The main steps of the protocol, adding up to nearly 2.5 pages, were highlighted.

16. Please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations: a) Critical steps within the protocol. b) Any modifications and troubleshooting of the technique. c) Any limitations of the technique. d) The significance with respect to existing methods. e) Any future applications of the technique.
The Discussion has been thoroughly revised. These five points were taken into consideration and incorporated into the text, although not necessary in the order listed above.

17. Figure 2: Please include scale bars for all the images of the panel and define them in the Figure legends.
18. Figure 4/5: Please define the size of the sections in the figure legends to make the figure more informative to the readers. Include scale bars wherever possible.
Scale bars were added to all images in all panels. Definitions of the scale bars bar were added in the legend of each Figure.

19. Please ensure that the Table of Materials includes all the supplies used in the study. Please sort the table in alphabetical order.
Thank you for the reminder about pieces of software and other materials. The Table of Materials was revised and sorted in alphabetical order. All software used for controlling the micro-CT systems, as well as for image reconstruction and analyses were added. The vacuum chamber was also added to the Table.

Reviewer #1

1. In the introduction it is important to present more updated information about X-ray microscopy resolution.

Thank you for this suggestion. I was not aware that such fine resolution had been achieved while working with biological materials. I have updated the introduction to reflect this technical improvement.

2. Please give more technical details about the experimental conditions used to obtain the images (KV, current, etc).

A Table was added including information about the conditions and parameters used in all micro-CT experiments.

3. I would suggest trying to work with small tissue fragments processed as used for conventional scanning electron microscope.

In fact, working with small tissue fragments makes the process easier, not only in terms of scanning, but also for sample preparation and perfusion of contrast solutions. However, the complex organization of parasitic plant haustorium, as well as other plant structures that are often bulky (e.g., woodroses, long flowers, branch nodes, grafts, etc.) requires initial analyses that include the entire sample, or at least most of it. This rationale was reinforced in the introduction.

Reviewer #2

In the lines 135-139 the author discusses about the importance of fixation and at the same time present the possibility of fresh samples analysis. For this referee this situation could be ambiguous. The use or not of fresh samples could be deep discussed into introduction section or this information should be revised.

Thank you for pointing out this apparent ambiguity. I have revised the introduction and expanded on the explanation about the use of fresh samples, also mentioning the case of live plants.

For this referee, would be more interesting and easier for readers if at least the contrasting solutions were mentioned in the protocol section.

Names of the contrast solutions used in this study were added to the protocol.

The figure 2 could be improved. Please, if it is possible, show the surroundings more than what is shown in the figure 2d. Still in the figure 2, the docking of the plastic tube on the plant proximal end should be represented in the figure too.

Figure 2 was improved by adding an image of the docking of the plastic tube on the proximal end of the host branch (Figure 2b, detail). I was unable to produce a better image for the original (Figure 2d) in which the surroundings could be seen. I have, however, produced a schematic representation that should hopefully have the same effect.

In the lines 295-296 the author claimed that "XRM show the same level of detail as anatomical sections analyzed under a light microscope". For this referee, the comparison could not be done from this perspective. This sentence could be re-written.

Thank you for your perspective. The sentence was meant to indicate that XRM images are as useful as anatomical sections for the analysis of tissue organization and topology. The sentence was re-written to clarify this point.

In the line 324, the author mentioned untreated and treated samples. What are they? Does the author mention the contrasting solution or fresh/fixed samples? This should be clarified in the text. In the same paragraph, lines 328-329, it was compared the starch content analyzed in both figures 4 and 5. However, the author did not point out starch content in figure 5.

This part of the paragraph was indeed confusing. Thank you for your observation. "Untreated/treated" refer to samples before and after the application of the contrast solution. Species showed in figure 5 (opposite to the species in figure 4) show little or no starch reserves in their tissues, thus leading to different results obtained with the same method and solution. These points were clarified in the text.

In the legend of figure 4, in (a) the image should be cited as a light microscopic related. There are arrowheads in figure 4f-g, where these arrowheads are pointing?

The legend was improved to indicate that figure 4A refers to a micro section. To avoid confusion, arrowheads were substituted by outlines like that in figure 4E; in both cases, they indicate the cortical strand tissues of the parasite.

In the figure 5 some information are missing for this referee: In the line 374 the author compares sections of fresh material. Are all samples fresh? This information should be mentioned in the text.

This was an oversight; thank you for noticing it. The legend was improved to indicate that only figure 5A and B refer to fresh material.

Reviewer #3

The method of segmentation should also be very briefly described. Alternatively, if segmentation is not possible at the level of contrast achieved with the described protocols, and the benefits of the protocol are limited to interpreting the raw data without segmentation, the author should state clearly that this is the case.

Thank you for your suggestion. The method of segmentation is indeed powerful and useful for the analysis of micro-CT scans. Other methods, such as volumetrical measurements and the quantitative analysis of density are also valuable. However, the focus of this manuscript and protocol is on sample preparation, not on analytical methods. The methods discussed here are potentially useful for different forms of analysis, but my limited knowledge about segmentation and quantitative analyses have hindered the application of such methods in the context of this manuscript. I have, however, in this revised version, added a short paragraph about the use of such methods as future applications.

L24-25: Please reword this to "the possibilities of three-dimensional visualization and virtual sectioning have…."
Corrected, thank you.

L43: Please correct to "High resolution X-ray microcomputed tomography".
Corrected, thank you.

L52: The highest spatial resolution achievable by synchrotron and micro-focus laboratory X-ray sources now exceeds 1 micron. Please correct and cite for example (Langer et al. 2012; Walton et al. 2015; Busse et al. 2018).
Thank you for this suggestion. I was not aware that such fine resolution had been achieved while working with biological materials. I have updated the introduction to reflect this technical improvement.

L87: Here the author should make clear the disadvantages and trade-offs of using micro-CT compared to the serial sectioning approach of Matsumoto et al. (2020), i.e., a reduction in the structural detail available. L94: At the end of the sentence, please reference works on the subject of correlative microscopy/tomography e.g. (Clark et al. 2020; Calo et al. 2020). The author should also give examples for which "other tools" they mean and what their usefulness is.
This disadvantage was added to this paragraph indicating that, at least for large samples, micro-CT does not substitute anatomical sectioning. References of works comparing microtomography and microscopy were also cited.

L244: From the text, the reader assumes that the proximal end of the host stem/root must fit snugly within the tube, and samples of larger diameter will require larger tubing. However, this is not clearly stated. Please describe whether any specific method is required to connect the proximal end of the stem to the tubing.
Figure 2 was improved by adding an image of the docking of the plastic tube on the proximal end of the host branch (Figure 2b, detail). The panel now also includes a photo of tubes of various diameters and other items that can be used to produce a better fit between tubes and between tubes and samples in the apparatus.

L292: Please include the concentration of phosphotungstate used in the text and figure legends, in addition to the table of materials. Please repeat this for all contrast agents used.
The final concentration of all contrast solutions was added throughout the text and figure legends.

L295: I do not agree that the XRM images show the same level of detail as the histology images in Figure 3. Additionally, for every XRM image in the manuscript, please list the voxel size within the figure legends.

Thank you for your perspective. The sentence was meant to indicate that XRM images are as useful as anatomical sections for the analysis of tissue organization and topology. The sentence was re-written to clarify this point. Voxel size was also added the legend of Figure 3.

L302: It is difficult for the reader to appreciate how different the haustorium structure is from typical roots and stems when comparing two images taken using different modalities (Figs 3d-e).

Thank you for this observation. The phrase was ambiguous and is now corrected. Figures 3D and 3E show the same haustorium observed under a XRM and a microscope, respectively. The comparison with the typical organization of roots and stems, although interesting, does not make sense in the context of a methods paper focusing on microtomography. This part of the sentence was then removed.

L305: please include scale bars in all panels of Figures 3, 4, 5 and 6

Scale bars were added to all images in all panels. Definitions of the scale bars bar were added in the legend of each Figure.

L324: Please explain what the specific differences are between Figure 5 panels c and g which illustrate the importance of the staining. For example, which features are visible in the stained sample which are more difficult to discern in the unstained sample?

Thank you for this suggestion. The main differences have been added to the text and indicated in Figures 5C – F.

L333: Please include the final concentration of the lead nitrate solution used for staining in the text and Figure 6 legend.

The final concentration of all contrast solutions was added throughout the text and figure legends.

L377: Is Figure 6 panel d really a light microscopy image as described in the figure legend? It instead appears to be a volume rendering of the micro-CT data.

Yes, figure 6D is an anatomical section mounted onto a large slide and photographed under stereomicroscope. It was sectioned, stained, and mounted in the same way as the other sections. I chose not to detail these differences to not stray from the focus of this manuscript.

L414: Please cite (Busse et al. 2018), and briefly explain that they demonstrate how use of a Bromine-based staining agent is indeed effective for generating contrast.

Thank you for this suggestion. I unaware for the work by Busse et al. (2018). I have now cited it to corroborate the idea that bromine-base staining could be used in this case. I am also thankful for all other references indicated. Although I chose not to cite all of them, they are certainly important papers that might motivate me to do further work in this area.