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Leveraging Micro-CT Scanning to Analyze Parasitic Plant-Host Interactions

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TITLE:

Leveraging Micro-CT Scanning to Analyze Parasitic Plant-Host Interactions

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Microtomography, haustorium, holoparasite, endoparasite, Cuscuta, mistletoe, cytochemistry

SUMMARY:

Micro-CT is a non-destructive tool that can analyze plant structures in three dimensions. The present protocol describes the sample preparation to leverage micro-CT to analyze parasitic plant structure and function. Different species are used to highlight the advantages of this method when coupled with specific preparations.

ABSTRACT:

Micro-CT scanning has become an established tool in investigating plant structure and function. Its non-destructive nature, combined with the possibility of three-dimensional visualization and virtual sectioning, has allowed novel and increasingly detailed analysis of complex plant organs. Interactions among plants, including between parasitic plants and their hosts, can also be explored. However, sample preparation before scanning becomes crucial due to the interaction between these plants, which often differ in tissue organization and composition. Furthermore, the broad diversity of parasitic flowering plants, ranging from the highly reduced vegetative body to trees, herbs, and shrubs, must be considered during the sampling, treatment, and preparation of parasite-host material. Here two different approaches are described for introducing contrast solutions into the parasite and/or host plants, focusing on analyzing the haustorium. This organ promotes connection and communication between the two plants. Following a simple approach, details of haustorium tissue and cellular organization can be explored three-dimensionally, as shown here for a euphytoid root parasite, parasitic vine, and mistletoe species. Selecting specific contrasting agents and application approaches also allow detailed observation of endoparasite spread within the host body and detection of direct vessel-to-vessel connection between parasite and host, as shown here for an obligate root parasite. Thus, the protocol discussed here can be applied to the broad diversity of parasitic flowering plants to advance the understanding of their development, structure, and functioning.

INTRODUCTION:

High-resolution x-ray microcomputed tomography (micro-CT) is an imaging method in which multiple radiographs (projections) of a sample are recorded from different viewing angles and later used to provide a virtual reconstruction of the sample[1]. This virtual object can then be analyzed, manipulated, and segmented, allowing non-destructive exploration in three dimensions[2]. Initially designed for medical analyses and later for industrial applications, micro-CT also offers the advantage of visualizing inner organs and tissues without the need for invasive procedures[3]. Like other forms of imaging, micro-CT works with a trade-off between the field of view and pixel size, which means that high-resolution imaging of large samples is nearly unattainable[4]. Advances in using high-energy X-ray sources (i.e., synchrotron) and secondary optical magnification are constantly being made, allowing the smallest resolution to reach under 100 nm[5,6]. Nevertheless, longer scanning times are necessary for large samples, increasing the chance of artifacts due to sample movement or deformation inside the scanner. Furthermore, micro-CT is generally limited by natural density variations within the sample and how the sample interacts with X-rays. While a higher X-ray dose is best for penetrating denser samples, it is less efficient in capturing variations in density within and between the sample and its surrounding medium[7]. On the other hand, a lower X-ray dose offers less penetration power and often requires longer scanning times but more sensitivity in density detection[7].

These restrictions have long hampered the use of microtomography for plant sciences, given that most plant tissues are composed of light (non-dense) tissue with low X-ray absorption[8]. The first applications of micro-CT were focused on mapping root networks within the soil matrix[9,10]. Later, plant structures with more significant differences in tissue density, such as wood, began to be explored. This has allowed investigations of xylem functionality[11,12], development of complex tissue organizations[13,14], and interactions among plants[15–17]. The analysis of soft and homogeneous tissue is becoming widespread due to contrast agents, which are now standard procedures in preparations for micro-CT scanning of plant samples. However, protocols for contrast introduction can have different results depending on sample volume, structural properties, and the type of solution used[8]. Ideally, the contrast agent should enhance distinction among different tissues, enable tissue/organ functionality evaluation, and/or provide biochemical information about a tissue[18]. Therefore, adequate sample treatment, preparation, and mounting before scanning become crucial for any micro-CT analysis.

Micro-CT of the parasitic plant haustorium

Parasitic flowering plants represent a distinct functional group of angiosperms characterized by an organ known as *haustorium*[19]. This multicellular organ, a developmental hybrid between a modified stem and a root, acts on the host's attachment, penetration, and contact by a parasite[20]. For this reason, the haustorium is considered to "embody the very idea of parasitism among plants"[21]. A detailed understanding of this organ's development, structure, and functioning is crucial for parasitic plant ecology, evolution, and management studies. Nevertheless, parasitic plants' overall complexity and highly modified structure and haustoria often hinder detailed analysis and comparison. Haustorium connections are also usually extensive and not homogenous tissue and cell distribution (**Figure 1**).In this context, while working with small tissue fragments allows easier manipulation and higher resolution, it can lead to erroneous conclusions

about the three-dimensional architecture of complex structures, such as the parasitic plant haustorium.

Although there is a vast literature on haustorium anatomy and ultrastructure for most parasitic plant species, the three-dimensional organization and the spatial relationship between parasite and host tissues remains poorly explored[17]. In a recent work by Masumoto et al.[22], over 300 serial semi-thin microtome sections were imaged and reconstructed into a three-dimensional virtual object representing the haustorium of two parasite species. This method's excellent level of detail provides unprecedented insights into the haustorium's cellular and anatomical 3-D structure. However, such a time-consuming technique would forbid a similar analysis in parasites with more extensive haustorium connections. The use of micro-CT emerges as an excellent tool for three-dimensional analysis of complex and often bulky haustoria of parasitic plants. Although not a substitute for detailed anatomical sectioning and complementary other forms of microscopy analyses[17,23], results obtained *via* micro-CT scanning, especially for large samples, can also serve as a guide to direct the sub-sampling of smaller segments, which can then be analyzed using other tools, such as confocal and electron microscopy, or re-analyzed with high-resolution micro-CT systems.

[Place **Figure 1** here]

As micro-CT becomes an increasingly popular technique in plant sciences, there are guides, protocols, and literature dealing with sample scanning, three-dimensional reconstruction, segmentation, and analysis[3,10,24]. Thus, these steps will not be discussed here. As with any imagining technique, appropriate treatment and mounting of samples are a fundamental, albeit often overlooked procedure. For this reason, this protocol focuses on the preparation of haustorium samples for micro-CT scanning. More specifically, this protocol describes two approaches for introducing contrast agents into haustorium samples to improve visualization of different tissues and cell types in the haustorium, to facilitate the detection of parasitic tissue within the host root/stem to analyze parasite-host vascular connections in three dimensions. The preparations described here can also be adapted to the analysis of other plant structures.

Five species were used to illustrate better the convenience of the protocol described here. Each species represents one of the five functional groups of parasitic flowering plants, thus addressing specific points related to the functionality of each group. *Pyrularia pubera* (Santalaceae) was chosen to represent euphytoid parasites, which germinate in the ground and form multiple haustoria that connect the parasite to the roots of its hosts[25]. The haustoria created by these plants are often tenuous and easily torn apart from the host[26] (**Figure 1A**), thus requiring a more delicate handling process. Endoparasites are represented here by *Viscum minimum* (Viscaceae). Species in this functional group are only visible outside the body of their hosts for short periods (**Figure 1B**) and live most of their life cycles as significantly reduced and mycelial-like strands of cells embedded within host tissues[25]. A third functional group comprises parasitic vines, which germinate on the ground but form only rudimentary roots, relying on multiple haustoria that attach to the stems of host plants[25] (**Figure 1C**). Here, this functional group is represented by *Cuscuta americana* (Convolvulaceae). Contrary to parasitic vines, mistletoes germinate directly

upon the branches of their host plants and develop either multiple or solitary haustoria[25]. The species chosen to illustrate this functional group is *Struthanthus martianus* (Loranthaceae), which forms various connections with the host branch (**Figure 1D**). Analysis of solitary mistletoe haustoria using a combination of micro-CT and light microscopy can be found in Teixeira-Costa & Ceccantini[17]. Finally, obligate root parasites comprise species that germinate on the ground and penetrate the roots of host plants, upon which they are entirely dependent from the earliest growth stages[25]. These plants are represented here by *Scybalium fungiforme* (Balanophoraceae), which produce large tuber-like haustoria (**Figure 1E**).

All plant samples used in this protocol were fixed in a 70% formalin acetic acid alcohol (FAA 70). The fixation upon sampling is crucial for preserving plant tissues, especially if subsequent anatomical analyses are needed. In the case of parasitic plant haustorium, fixation is also essential, as this organ is often primarily composed of non-lignified parenchyma cells[20]. Detailed protocols for plant tissue fixation, including the preparation of fixative solutions, can be found elsewhere[27]. On the other hand, to a greater or lesser degree, fixatives can cause alterations of a sample's physical and chemical properties, rendering it unsuitable for specific biomechanical and histochemical analyses. Thus, fresh samples, i.e., non-fixated material collected immediately before preparation, can also be used with this protocol. Details on how to handle fresh samples and troubleshooting suggestions for fixated material are provided in the discussion section.

PROTOCOL:

1. Parasitic plant sample selection

1.1 Collect the entire parasitic plant haustorium, including the attached host stem/root and segments of both proximal and distal ends of the parasitized host organ; the ideal length of each segment is equivalent to double the diameter of the haustorium.

NOTE: For lateral haustoria, include part of the parasite mother stem/root from which the haustorium was formed (**Figure 1A,B,D**). For endoparasites, collect a segment of the host stem/root in which signs of the parasite are visible (**Figure 1B**). In the case of terminal connections, the whole plant should be collected (**Figure 1E**).

1.2 Submerge the whole sample in fixative solution (e.g., FAA) in a volumetric proportion of 1:10 (sample: fixative). Leave samples in fixative for at least 1 day, depending on the size of the sample[27].

NOTE: Samples can be stored in fixative before scanning or be transferred to a preserving solution (e.g., ethanol 70%). Fresh samples can also be used if the subsequent anatomical analysis is not warranted (see Discussion section). If working with fresh material, set up the apparatus for perfusion of the contrast solution, then collect the sample. The sample need not be allowed to dry out.

2. Application of contrasting solutions

2.1 Chose the application method to be used. Use the vacuum method (step 2.3) for small (**Figure 1A**) or non-woody (**Figure 1B**) samples. Use the perfusion method for most samples, provided it includes a segment of the host stem/root is (**Figure 1A,C-E**).

2.2 Wear rubber gloves and other appropriate personal protection (e.g., laboratory coat) equipment when manipulating the sample regardless of the approach chosen in the following steps.

CAUTION: All contrasting solutions include heavy metal salts in their composition, and therefore should not be handled without adequate personal protection equipment and under a fume hood.

2.3 For the vacuum method, follow the steps mentioned below.

2.3.1 Choose an appropriate vial and label it. The vial must be large enough to accommodate the sample and the contrasting solution, usually in a proportion of 1:10. Check instructions from the manufacturer to ensure that the vial can withstand low to moderate vacuum.

NOTE: Do not fill the vial to the brim, as the negative pressure (vacuum) can cause the liquid to spill.

2.3.2 Place the sample in the vial with the contrasting solution (1% iodine, 0.2% lead nitrate, or 3% phosphotungstate, see **Table of Materials**). Then place the vial in a vacuum chamber or desiccator connected to a vacuum pump. Remove the lid from the vial, then close the vacuum chamber or desiccator.

2.3.3 Check that there are no cracks on the vacuum chamber/desiccator and that the vacuum pump has enough oil.

2.3.4 Close the exhaustion valve of the pump to prevent air from scaping and open the exhaustion valve of the chamber/desiccator to force the air out.

2.3.5 Turn on the pump and wait until the pressure reaches approximately 20" Hg.

CAUTION: This process is usually fast, so do not leave the vacuum system unattended.

NOTE: While the metric system unit for pressure is Pascal (Pa), pressure gauges in most laboratory vacuum pumps display pressure in inches of mercury (" Hg), pound per square inch (psi), or bars. 20" Hg equals ca. 67.7 Pa.

2.3.6 Close the chamber/desiccator exhaust valve to prevent air from re-entering, then quickly turn off the pump.

2.3.7 Leave the sample under vacuum for at least 2 h; larger samples require longer for the contrasting solution to penetrate it.

2.3.8 After the desired period, remove the sample from the contrasting solution to prepare it for scanning.

2.3.9 Slowly open the chamber/desiccator exhaustion valve to allow air to enter it.

2.3.10 Wait for the pressure in the chamber/desiccator to be fully exhausted (i.e., pressure gauge reaches near 0), then carefully open it to retrieve the sample.

2.3.11 Discard the contrasting solution appropriately and keep the sample in preparation for scanning.

2.4 For the perfusion method, follow the steps mentioned below.

2.4.1 Select a supply tank for the contrasting solution according to the size of the sample. Use a 50 mL syringe (with no needle or plunger) for small samples (**Figure 2A**) or a 1 L intravenous medical kit for large samples (**Figure 2B**).

2.4.2 Connect one end of a transparent plastic tubing (see **Table of Materials**) to the supply tank, then connect the other end to a two-way or three-way valve. Connect a second tubing to a different outlet in the valve (**Figure 2A,B**).

2.4.3 Secure the supply tank at an elevated position without disassembling the apparatus set up in the previous step.

NOTE: The vertical distance between the tank and the sample will dictate the solution perfusion pressure **(Figure 2B)**. A distance of 20-50 cm is enough for small samples. For large samples, a distance of 1 m is more adequate.

2.4.4 Close the three-way (**Figure 2C**) or two-way (**Figure 2D**) valve to prevent liquid from exiting the tubing system, then pour the contrasting solution into the supply tank. If using an intravenous medical kit, fill the bag with the solution and close the valve before securing the apparatus at an elevated position.

2.4.5 Ensure that no large air bubbles are formed along with the tubing system (**Figure 2E**). If necessary, allow the contrast solution to flow out of the tubing until the bubble is removed. Close the valve again and leave the apparatus in place.

2.4.6 To prepare the sample for perfusion of the contrast solution, keep it submerged in liquid (water, ethanol, or fixative) and cut off the tip of the proximal end of the host stem/root in the sample (**Figure 2F**).

2.4.7 Remove the sample from the liquid in which it was stored and wrap it in a paraffin film to avoid desiccation. Keep the sample nearby and ready to be connected to the apparatus.

2.4.8 Carefully open the valve to allow the contrasting solution to flow slowly and fill the plastic tubing connected to the tank while holding the open end of the system at a slightly elevated position to prevent the contrasting solution from spilling. Again, ensure that no large air bubbles form along the tube.

2.4.9 Connect the proximal end of the host stem/root in the sample to the open end of the tubing system (**Figure 2B,** magnified region). Avoid introducing air bubbles into the system during this step. If necessary, disconnect the sample from the apparatus and remove air bubbles from the system by allowing the solution to flow.

2.4.10 While keeping the sample connected to the apparatus, place it inside a container to avoid leakage of the contrasting solution into the area where the experiment was set up. Use tubes of different diameters, plastic zip-ties, and valve adaptors (**Figure 2G**) to ensure all connections in the apparatus are well-fit, accommodate host branches of various sizes, and that the solution is not leaking out of the tubing system (**Figure 2B,** magnified region).

2.4.11 Let the solution perfuse the sample for at least 2 h, or until the solution accumulates inside the container.

2.4.12 Close the valve and carefully disconnect the sample from the apparatus. Drain the remainder of the solution into the container and dispose of it appropriately.

2.4.13 Remove the paraffin film from the sample in preparation for scanning.

3. Sample preparation and mounting

3.1 Wash the sample by submerging it in water for 2 min.

CAUTION: Do not wash samples in the sink, as all contrasting solutions include heavy metal salts in their composition. Consider the water used for washing as a diluted contrasting solution and dispose of it appropriately.

3.2 Place the sample in a paper towel at room temperature to allow excess water to evaporate for 2-5 min depending on the size of the sample. Alternatively, dry the sample slightly with the aid of a paper towel. Do not allow the sample to dry out completely.

3.3 Wrap the sample in a paraffin film by stretching it to a thin layer. Avoid folding the paraffin film on top of the sample.

3.4 Mount the wrapped sample onto a sample holder, keeping it stable and in position while moving it during the scanning. Use adhesive tape, low-density foam, pipette tips, and/or clear plastic containers to secure the sample in place.

3.5 Scan the sample and analyze the images following specific protocols and guidelines established for the micro-CT system available.

REPRESENTATIVE RESULTS:

The haustorium of parasitic plants is a complex organ comprising different tissues and cell types that intertwine and connect with the tissues of another plant, used as a host[20]. Micro-CT scanning can be leveraged to better understand this complex structure in a non-destructive and three-dimensional way when analyzing small (**Figure 1A-C**) and large (**Figure 1D,E**) haustoria. To do this, contrasting solutions can be applied into the parasite-host interface, thus making it possible to analyze samples that would otherwise have low and homogenous X-rays absorption. The two simple approaches described in this protocol rely on pressurizing the contrasting solution to accelerate penetration through the sample. As expected, the protocols described here work for different micro-CT systems. However, settings and parameters for the scan differ depending on the system and the analyzed samples (**Table 1**).

[Place **Table 1** here]

Following the first approach for contrast application (step 2.3), the vacuum was used to perfuse the haustorium of the euphytoid parasite *P. pubera* with a 3% phosphotungstate solution for two hours. The sample was then scanned in a 3D X-ray microscope (XRM) system (see **Table of Materials**), achieving high image resolution *via* secondary optical magnification[4]. XRM images were observed to be as effective as anatomical sections analyzed under a light microscope to analyze tissue organization and topology at the parasite-host interface (**Figure 3**). The use of phosphotungstate highlights the abundance of parenchyma tissue, which appears in a bright white tone due to the high absorption of the contrasting agent. Vessels appear in a dark gray tone due to low absorption of contrast. Based on this color difference, it is possible to observe the abundance of parenchyma surrounding the vascular core of the haustorium (**Figure 3**). The intricate vessels and thin parenchyma strands form the vascular core, especially in longitudinal sections (**Figures 3A-C**). The vascular core is easily observed in cross-sections as two vascular strands separated by a parenchyma core (**Figures 3D,E**).

[Place **Figure 3** here]

The same approach introduced contrast into the stem of a succulent host plant (*Euphorbia polygona*, Cactaceae) parasitized by *V. minimum*. Here, 1% iodine solution was chosen as a contrasting agent based on preliminary histochemical analysis, which showed that parenchyma cells of the endoparasite store carbohydrates in the form of starch (**Figure 4A**). At the same time, cells in the host cortex and parenchyma do not store detectable quantities of starch (**Figure 4B-D**). The sample was then scanned using an appropriate scanning system (see **Table of Materials**). The difference in iodine absorption allowed the detection of the intricate web of cortical strands

formed by the endoparasite within the host body (**Figure 4E**). These cortical strands were observed to concentrate around the host vascular center and eventually branch towards the periphery of the host stem when associated with the emersion of a parasite flower (**Figures 4F,G**) following this methodology.

[Place **Figure 4** here]

In the second approach described here (step 2.4), a supply tank containing the contrasting solution is elevated from the level in which the sample is placed, thus using gravity to drive the passage of the solution through the sample. Scanning was carried out using the appropriate scanning system (see **Table of Materials**). Results obtained for *C. americana* (**Figures 5A,B**) and *S. martianus* (**Figures 5C-G**) help illustrate the convenience of this approach for both small and large samples. Comparing samples scanned before (**Figure 5C,E**) and after (**Figure 5D,F**) perfusion of 1% iodine solution stress the importance of contrast application even in woody haustorium samples. It is noteworthy that in the associations between both *C. americana* and *S. martianus* (**Figure 5**) and their respective hosts, parasites have little to no starch content in their tissues. This explains the different results described for the endoparasite *V. minimum* (**Figure 4**), in which the same method and type of contrast solution were used.

[Place **Figure 5** here]

A final result obtained with the protocols described here is the possibility to detect vascular connections between parasites and host plants. Scanning was also carried out using a scanning system. This was achieved by perfusing a 0.2% lead nitrate solution through a segment of the host root around which the tuber of the obligate root parasite *S. fungiforme* had developed. Sequential projections show that vessels in the host root bifurcate into the parasite tuber (**Figure 6A-C**). Following the analysis of these results, the same sample was cut into a smaller sub-sample and prepared for anatomical study using light microscopy. Serial sectioning of this sub-sample shows that the xylem continuity between the two plants is formed by vessel-to-vessel connection via perforation plates (**Figure 6D-F**).

[Place **Figure 6** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Parasitic plants of different functional groups used in this protocol. Euphytoid parasite *Pyrularia pubera* (**A**), endoparasite *Viscum minimum* (**B**) with green fruits (dashed black circle), parasitic vine *Cuscuta americana* (**C**), mistletoe *Struthanthus martianus* (**D**), obligate root parasite *Scybalium fungiforme* (**E**). Segments of the host root (Hr) or stem (Hs) facilitate the application of contrast into the parasite haustorium (P). The presence of parasite mother root/stem (arrows) in the sample allows analysis of haustorium vessel organization. Rectangles indicate segments of the sample used for analysis. Scale bars = 2 cm.

Figure 2: Perfusion approach for contrast application. Small (**A**) and large (**B**) versions of the perfusion apparatus include a supply tank (st) and two plastic tubes (t1 and t2) connected by a valve (va). The proximal end of the host stem (H) bearing a parasite (P) connected to it *via* the haustorium (ha) is connected to the open end of the system (B, expanded). A three-way (**C**) or a two-way (**D**) valve is used to help prevent the formation of air bubbles inside the tubing system, which block the passage of contrasting solution (**E**). The tip of the proximal end of the host stem (**H**) is cut underwater to allow passage of the contrast solution (**F**). Zip-ties, valve adaptors, and tubing of different diameters can secure tighter connections and avoid leakage in the system (**G**). Figures 2B, D and F were created with BioRender. Scale bars = 2 cm.

Figure 3: Euphytoid parasite haustorium. Longitudinal (**A-C**) and transversal (**D-E**) sections through the haustorium were observed under a light microscope (**A,E**) and *via* 3D X-ray microscopy following application of 3% phosphotungstate solution using vacuum (**B-D**). Red outlines indicate parenchyma tissue (par), and blue outlines indicate the vascular core (vc). Hx: host xylem. Hb: host bark. Scale bars = 500 µm (A, E) and 2.5 cm (B-D; voxel size = 2.8382 µm).

Figure 4: Endoparasite tissue network. Anatomical (**A**) and macro (**B-D**) sections show reaction with 1% iodine solution indicating that starch (stained in black) is present in the parenchyma (par) associated with the vessels (ve) of the parasite. Because starch is absent in the host cortex (Hc) and xylem (Hx), 1% iodine solutions were selectively used to enhance the contrast of parasite cortical strand tissues (purple outlines, **E-G**), seen against the host background (**H**). The presence of a flower (Pf) confirms that the stained tissue is part of the endoparasite structure (**A,F,G**). Scale bars = 0.25 cm.

Figure 5: Parasitic vine and mistletoe haustorium. Longitudinal sections through the haustorium of a parasitic vine (**A,B**) and a mistletoe (**C-G**). Comparison between scan and macro section of fresh material shows that the intricate haustorium structure is well captured in micro-CT scans (**A,B**). Comparison between fixated samples before (**C,E**) and after (**D,F**) the perfusion of 1% iodine solution show that contrast is enhanced even in lignified samples, facilitating the observation of parasites (P) structures such as vessels in the epicortical root (pink arrowheads), vascular strands (blue arrowheads), and sinker (yellow arrowhead). Vessels and annual rings of the host xylem (Hx) and starch in the host bark (Hb) are also more easily observed. Scale bars = 1 cm (A-C) and 500 µm (D-F).

Figure 6: Obligate root parasite haustorium. Longitudinal sections through the haustorium as observed *via* micro-CT scanning (**A-C**) and anatomical sections observed under the light microscope (**D-F**). The accumulation of 0.2% lead nitrate solution within the vascular system of the host root (Hr, pink outline) allows observation of host vessels branching within the parasite tube (Pt) and the detection of direct vascular connection between the two plants (dashed white outline). Scale bars = 1 cm (A-C) and 500 µm (D-F).

Table 1: Settings and parameters for the scan of the analyzed samples.

DISCUSSION:

The use of heavy metal solutions to improve plant tissue contrast has become a crucial step in sample preparation for micro-CT analysis. Several compounds commonly available in plant micro-morphology laboratories have been tested by Staedler et al., who recommend using phosphotungstate as the most effective agent in penetrating samples and increasing contrast index[8]. Results obtained here in the analysis of the haustorium of *P. pubera* corroborate this recommendation. In terms of contrast application, Steadler et al. describe that the contrast solution was passively infiltrated through the analyzed material (flowers and flower buds ranging from 1 mm to 10 mm) during 1 to 8 days[8]. Other authors have used the same protocol of passive infiltration over extended periods (1-4 weeks) when analyzing larger samples, such as the 30 cm wide flowers of endoparasitic Rafflesiaceae species[28]. While this process has proven successful, results obtained with the protocol described here show that the infiltration process can be significantly accelerated under vacuum, thus improving the previously established method. A vacuum chamber or pump forces air to escape from the plant tissues, thus allowing easier infiltration of the contrast solution. Vacuum can be applied even to fragile structures, as shown here for the haustorium of *P. pubera* and the soft tissues of the succulent host plant *E. polygona* parasitized by *V. minimum*. Moreover, the use of vacuum pumps is a common procedure for the infiltration of fixatives or embedding substances in many laboratories of plant micro-morphology, thus making this protocol more accessible.

The same approach was used here with a contrasting agent that selectively infiltrates samples if specific storage compounds are present. When applied through the vacuum or passive infiltration, selective staining might provide poor contrast enhancement to an entire sample. On the other hand, it is beneficial for highlighting specific plant structures or tissues. As reported here, when combined with previous anatomical or histochemical analysis, the use of selective contrast agents such as iodine can aid in the differentiation of parasitic plant tissue within the host's body provided that either parasite or host (but not both) show abundant starch reserves. Selectively increasing X-ray absorption of parasite tissues is shown to be especially useful for exploring, for the first time, the complex three-dimensional network formed by an endoparasitic plant such as *V. minimum* within the stem of its host plant. Moreover, it is noteworthy that endoparasitic plants and certain fungi show an interesting evolutionary convergence, both growing "incognito" inside their hosts for much of their life cycle, emerging only during brief periods[29]. Thus, a potential new application of the protocol described here would be to use phloxine B, a stain containing bromine[30], to detect endophytic fungi within plant tissues. Eosin, another bromine-based staining solution only seldomly used for plant tissues, has been shown to enhance contrast in mouse kidney samples analyzed using a specific micro-CT system[31].

Another approach described in this protocol is the perfusion of contrasting agents through the vascular tissue of the host stem or root. This approach, which differs significantly from previously reported methods, is based on the work of Sperry et al., who perfused wood samples with safranin dye to analyze hydraulic conductivity[32]. As discussed by the authors and highlighted here, a critical step in the protocol is setting up the stain-perfusing apparatus to avoid introducing air bubbles into the system[32]. Three-way valves can temporarily diverge the solution flux and eliminate bubbles from the liquid comlumn[32]. Nevertheless, emboli can be present inside the vessels of the host plant, caused either artificially during sampling or naturally due to the

generally high transpiration rates of parasitic plants[33,34]. This can severely decrease perfusion efficiency, leading to contrast not being improved in the sample. It is essential to apply low to moderate vacuum when fixating the specimen after sampling to prevent this issue. Complementarily, the fixated specimen can be flushed under high pressure using the fixative solution in which the sample is stored. Flushing can help restore conductivity in the sample by removing air bubbles[32], thus clearing the path for the perfusion of the contrast solution. If working with fresh material, air bubbles into the plant can be avoided by submerging the specimen in water immediately after sampling, cutting it again underwater, and smoothing the end surfaces with sharp blades[35].

Following this approach, 1% iodine solution was perfused through the haustorium of *C. americana* and *S. martianus* to enhance the contrast of the host-parasite interface as a whole. Results obtained for these species show that the perfusion protocol described here works well for both fresh and fixated samples. That introduction of contrast solutions improves visualization even in lignified samples. These observations agree with results reported elsewhere[17]. The same approach of introducing contrast *via* perfusion was also used to apply a 0.2% lead nitrate solution to visualize specific aspects of haustorium structure. In contact with carbon dioxide, lead nitrate reacts to form a highly insoluble precipitate composed of lead carbonate crystals, which efficiently absorb X-rays[8,36]. Once perfused in the sample vascular tissue, this precipitate clogs up vascular pit connections, allowing the solution to flow only through direct, vessel-to-vessel connections *via* perforation plates. Following this approach, the presence of direct parasite-host xylem connections *via* perforation plates is shown here for *S. fungiforme* and confirmed *via* detailed anatomical analysis. These results highlight a further application of this approach to test haustorium functionality. Given that haustorium initiation might not culminate in developing a fully functional organ due to either parasite-host incompatibility or host resistance against the parasite, the approach described here can be used to test whether vascular connections are formed between the two plants leading to the occurrence of effective parasitism. Considering the value of using micro-CT scanning to investigate xylem embolisms more broadly[12,37], the protocol presented here can also improve the visualization and quantitative analysis of xylem embolism in other non-parasitic plants species.

In conclusion, this protocol approaches one of the critical advances expected in plant microtomography, which is applying contrasting agents to help differentiate structures with low X-ray absorpion[24]. As shown here, contrast solutions can significantly improve the visualization of haustorium structures at the interface between parasitic plants and hosts. Different compounds can be chosen according to their specificity in reacting with reserve compounds, such as starch, which is often differently distributed among parasite and host tissues. The approach to contrast application into haustorium samples can also be chosen to investigate specific features of the parasite-host interface, such as direct vessel-to-vessel connection. Furthermore, this protocol may also be applied to non-parasitic species, potentially improving the detection of endophytic fungi and quantification of xylem embolism. In any application, the protocol described here to improve microtomography analysis can be combined with other tools, such as optical projection tomography and automated virtual segmentation, to provide new and exciting insights into the three-dimensional development of the connection between these plants and

their hosts[38,39]. Furthermore, this protocol can also be applied to non-parasitic species, potentially improving the detection of endophytic fungi and quantification of xylem embolism.

ACKNOWLEDGMENTS:

I would like to thank Dr. Simone Gomes Ferreira (Microtomography Laboratory, University of Sao Paulo, Brazil) and Dr. Greg Lin (Center for Nanoscale Systems, Harvard University, USA) for their indispensable help and user training for different microtomography systems and data analysis software. I also thank the staff at the EEB Greenhouse at the University of Connecticut (USA), especially Clinton Morse and Matthew Opel for providing the specimens of *Viscum minimum*. Dr. John Wenzel provided the opportunity and great help for the sampling of *Pyrularia pubera*. The funding was provided by the Harvard University Herbaria.

DISCLOSURES:

The author has nothing to disclose.

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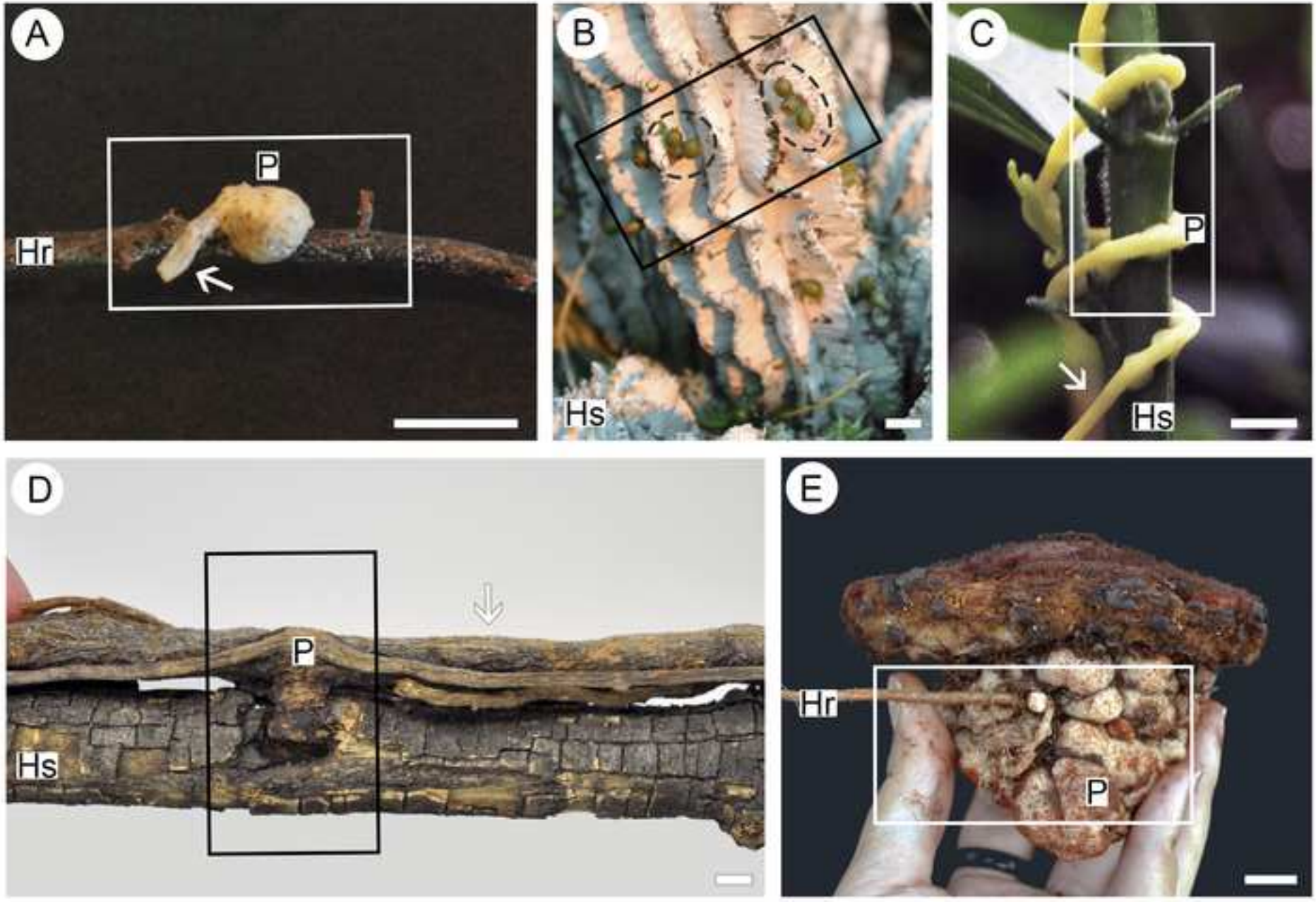

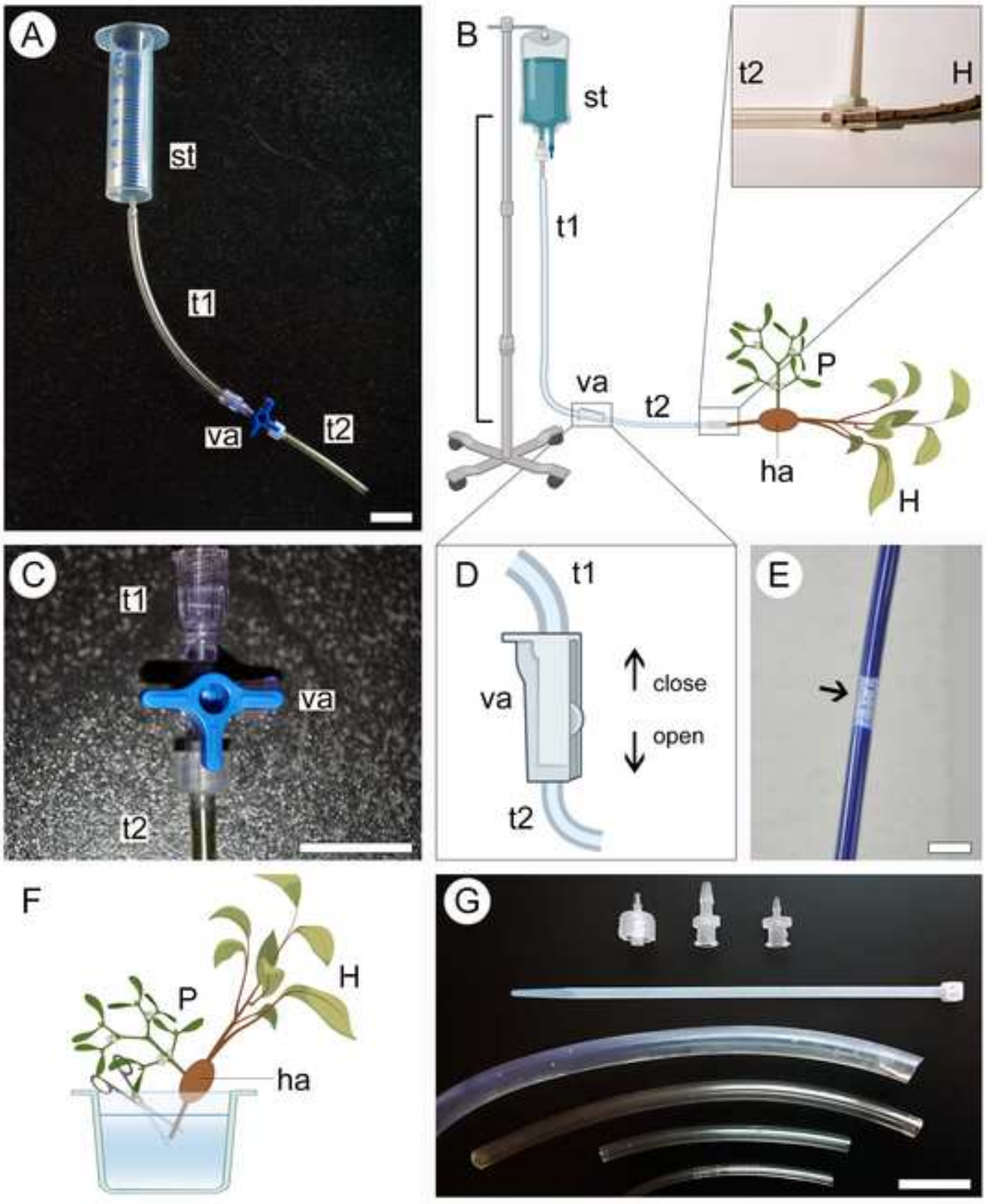

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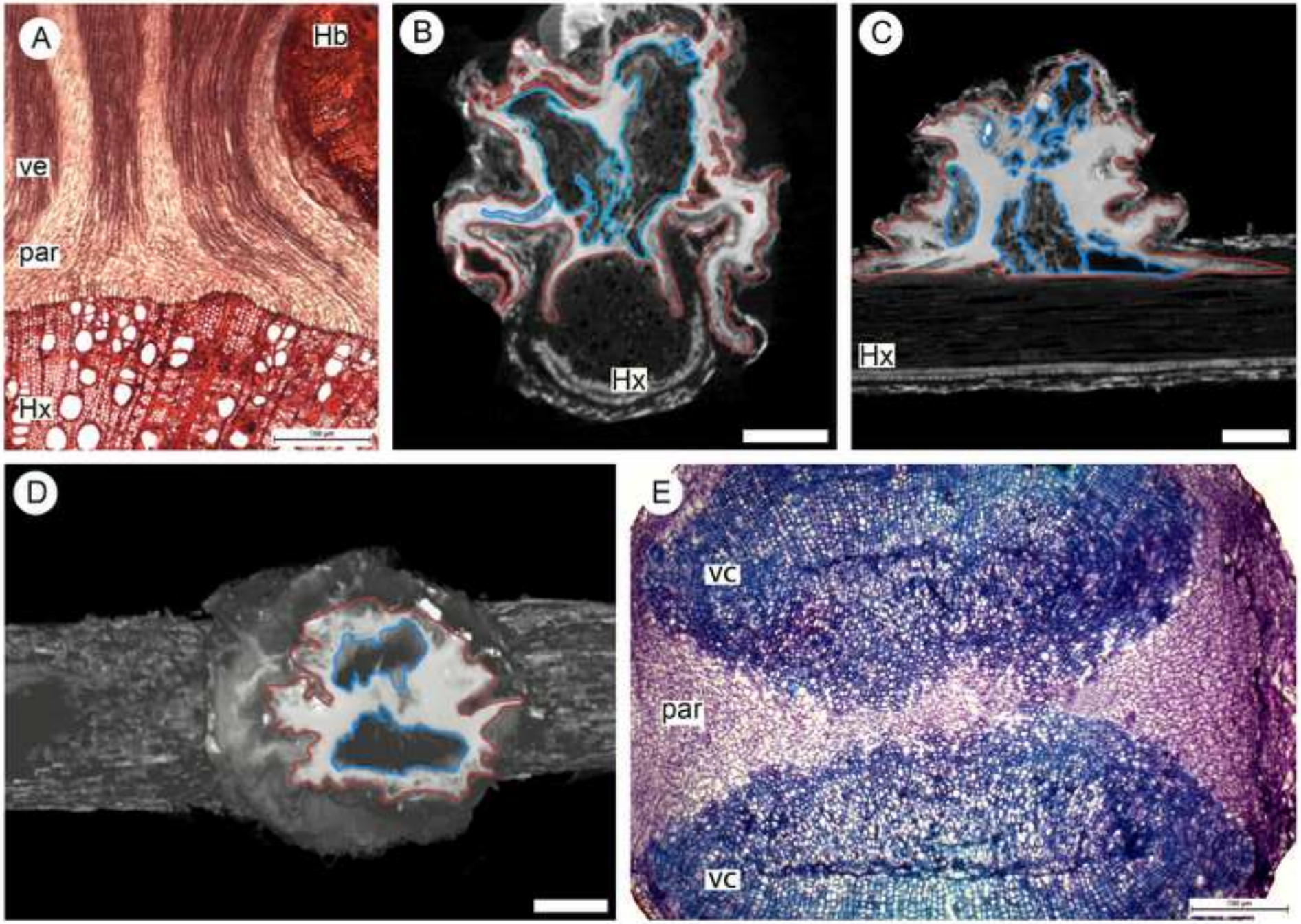

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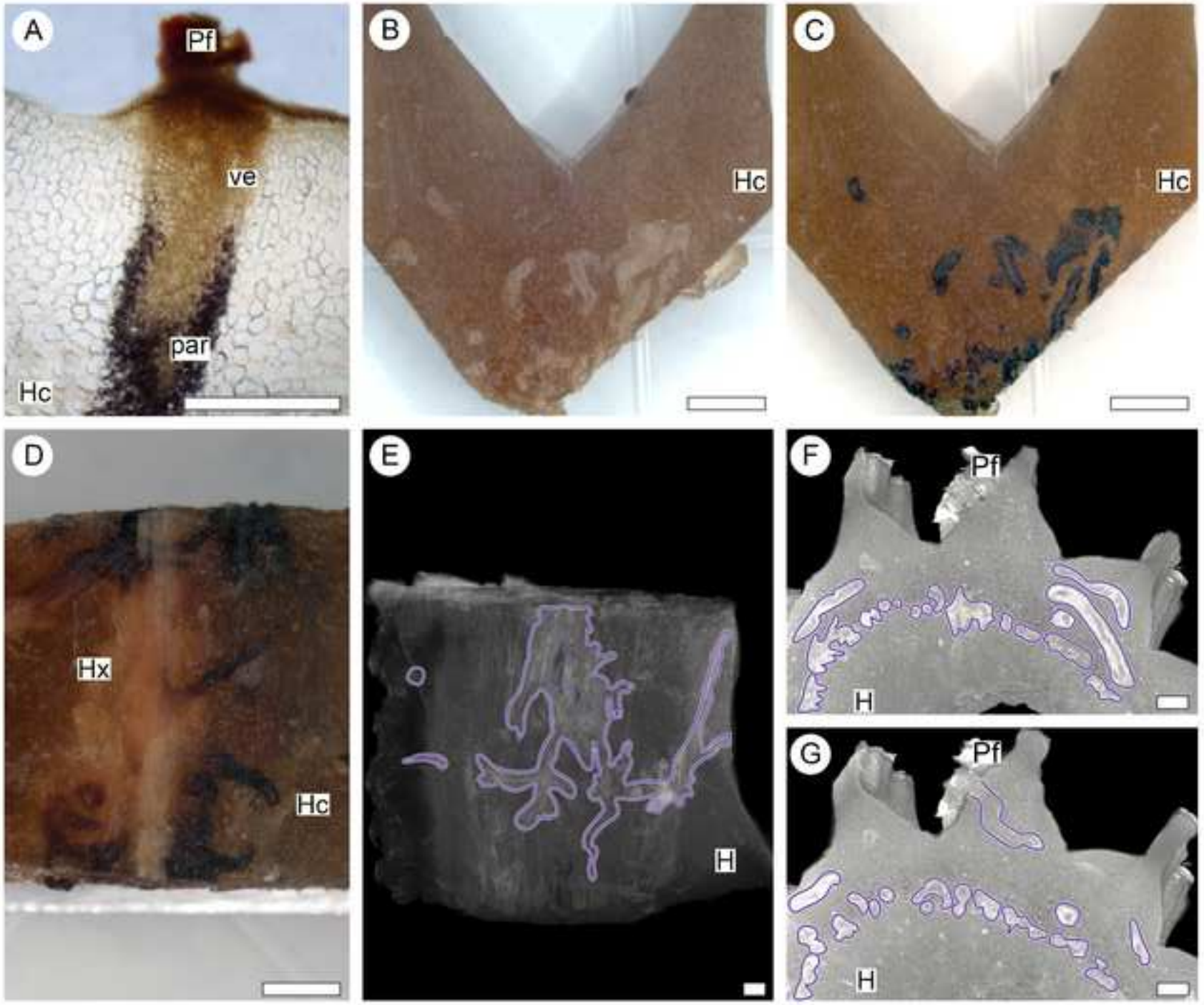

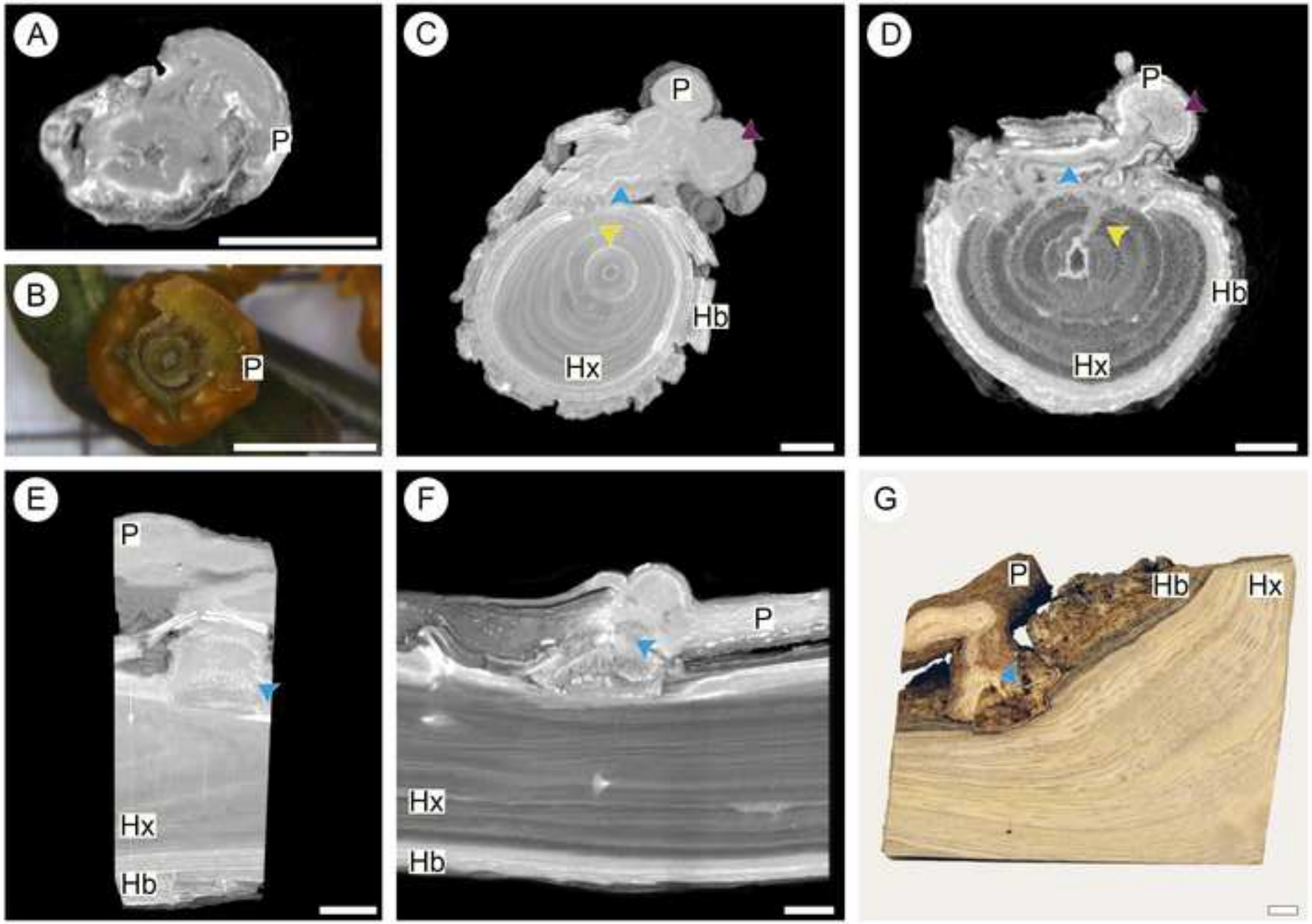

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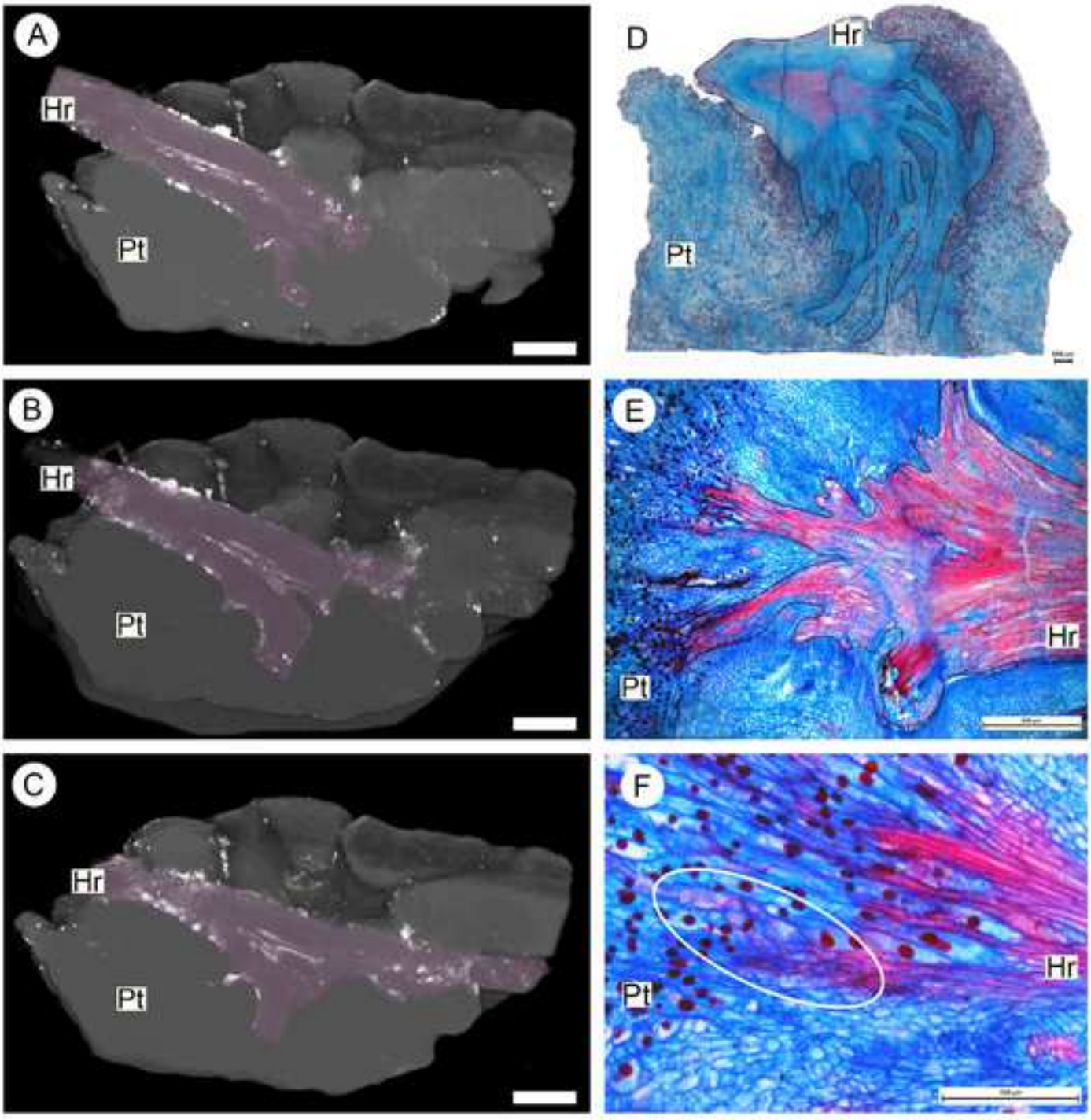

Table 1

Functional group	Euphytoid parasite	Endoparasite	Parasitic vine
Species (Family)	*Pyrularia pubera*	*Viscum minimum*	*Cuscuta americana*
Family	Santalaceae	Viscaceae	Convolvulaceae
Contrast solution	3% phosphotungstate	1% iodine	1% iodine
Application method	vacuum	vacuum	perfusion
Micro-CT system	Zeiss Versa 620	Nikon X-Tek HMXST225	Bruker Skyscan 1176
Projections	4500	3140	610
Frames	1	1	3 (averaged)
Exposure (ms)	5000	1000	680
Filter (mm)	none	Al 0.25	Al 1.0
Voltage (kV)	60	85	35
Current (µA)	108	80	375

Mistletoe (before/after contrast)	Obligate root parasite
Struthanthus martianus Loranthaceae	*Scybalium fungiforme* Balanophoraceae
1% iodine perfusion Bruker Skyscan 1176 1200 / 2400 3 / 3 (averaged) 1600 / 830 Al 1.0 / Al 1.0 90 / 45 180 / 390	0.2% lead nitrate perfusion Bruker Skyscan 1176 5300 3 (averaged) 750 none 40 600

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.

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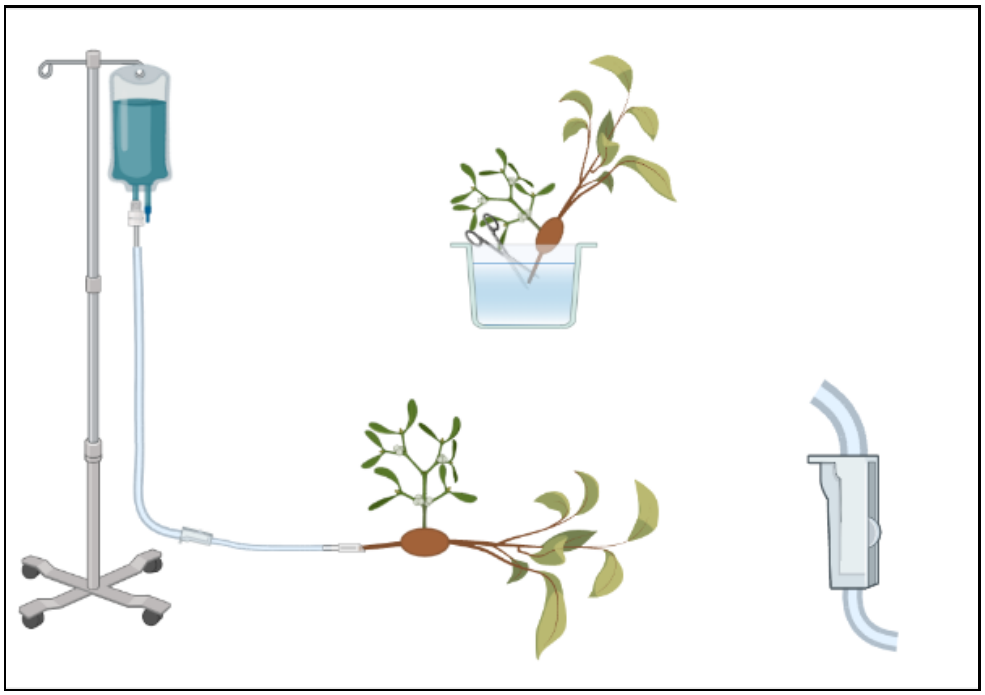

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