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Phenotyping xylem connections in grafted plants using X-ray micro-computed tomography

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Abstract

Plants are able to naturally graft or inosculate their trunks, branches and roots together, this mechanism is used by humans to graft together different genotypes for a range of purposes. Grafts are considered successful if functional vascular connections between the two genotypes occur. Various techniques can evaluate xylem connections across the graft interface. However, these methods are generally unable to assess the heterogeneity and three-dimensional (3D) structure of xylem vessel connections. Here we present the use of X-ray micro-computed tomography to characterize the 3D morphology of grafts of grapevine. We show that xylem vessels form between the two plants of natural root and human-made stem grafts. The main novelty of this methodology is that we were able to visualize the 3D network of functional xylem vessels connecting the scion and rootstock in human-made stem grafts thanks to the addition of a contrast agent to the roots and improved image analysis pipelines. In addition, we reveal the presence of extensive diagonal xylem connections between the main axial xylem vessels in 2-year old grapevine stems. In conclusion, we present a method that has the potential to provide new insights into the structure and function of xylem vessels in large tissue samples.

KEYWORDS
3D imaging, grafting, grapevine, micro-CT, scion, stock

1 | INTRODUCTION

Grafting is widely used in horticulture to provide desirable root traits to elite scion cultivars. Grafting makes use of the natural ability of plants to heal wounds and graft or inosculate their trunks, branches and roots together. The process of graft union formation involves an initial wound response, followed by callus formation, creation of a continuous cambium and the establishment of functional vascular connections between the two grafting partners (Loupit et al., 2023). In addition to the use of grafting stems and hypocotyls in

Abbreviations: 2D, two-dimensional; 3D, three-dimensional; micro-CT, X-ray micro-computed tomography.

Marilou Camboué and Anne Janoueix contributed equally to this study.

horticulture, natural grafts also form between different plants (Mudge et al., 2009). Natural root grafts can be beneficial for plant survival by improving anchorage, the acquisition of beneficial micro-organisms and allowing root systems to survive when the original shoot has died; however, they can also have negative consequences in terms of competition and pathogen transmission (Lev-Yadun and Sprugel, 2011).

Phenotyping the graft union in both human-made and natural grafts is challenging due to the size and heterogeneity of the tissues. Analyzing the graft union in one two-dimensional (2D) plane may not accurately capture the three-dimensional (3D) structure of the union (Loupit et al., 2023). Although histological studies (using light, confocal and electron microscopy) have provided detailed images of specific zones of the graft interface of a wide range of species, characterizing and quantifying the changes occurring at the graft interface is challenging. The use of 3D imaging techniques provides a comprehensive insight into the organization of various tissue types on a larger scale. Different techniques can be used to phenotype the 3D structure of large plant tissues, such as magnetic resonance imaging, which is generally based on the magnetic moment of protons and gives an indication of 3D structure largely based on the presence of water in different tissues. Magnetic resonance imaging has been previously used to study the graft interface of perennial grafts, but with limited resolution (Bahar et al., 2010; Korkutal et al., 2018). Another technique to study the 3D organization of tissues is X-ray micro-computed tomography (micro-CT), which provides information on tissue architecture in relation with tissue X-ray density (Brodersen, 2013). This technique is particularly useful to visualize empty xylem vessels and the formation of embolisms (which have a high contrast with the surrounding parenchyma tissues (Cochard et al., 2015; Kim and Lee, 2010; Nolf et al., 2017). X-ray micro-CT has also been previously used to study the graft interface (Milien et al., 2012), with a higher resolution than the aforementioned magnetic resonance imaging studies. However, the study of Milien et al. (2012) only identified a small number of empty xylem vessels connecting the scion and rootstock and did not comprehensively identify functional xylem vessels that span the graft interface.

In addition to understanding the 3D structure of the graft interface, it is also important to characterize functional vascular connections between the scion and rootstock. Fluorescent proteins, dyes and ^{14}C-labelled sucrose have been employed to characterize functional connections of the phloem and xylem at the graft interface in various species, but the morphological characterization was not done in 3D (Espen et al., 2005; Melnyk et al., 2015; Schoning and Kollmann, 1997). Recently, position electron tomography was used to track in 2D the movement of radiolabeled molecules between the scion and rootstock non-destructively in real time (Frank et al., 2022; Jahnke et al., 2009). As such, little is known about the 3D organization of functional xylem and phloem vessels at the graft interface, particularly in large perennial plant species.

X-ray micro-CT, coupled with functional xylem vessel labelling using contrast agents, has been employed to characterize xylem vessel connections in transverse sections of woody stems (i.e., in two dimensions (Pratt and Jacobsen, 2018; Pratt et al., 2020). The contrast agent used, iohexol, is a large, water-soluble, iodine-rich compound that can label actively transporting xylem vessels in woody species such as grapevine (Pratt and Jacobsen, 2018; Pratt et al., 2020). To identify conducting and non-conducting vessels, micro-CT images of stems with iohexol-labelled vessels were compared to micro-CT images of the same stem after drying the samples (Pratt and Jacobsen, 2018; Pratt et al., 2020). 3D analysis of these types of images has the potential to provide a more complete understanding of the spatial distribution of the functional xylem network in large plant samples.

Grapevine was chosen as the model grafted crop to study because grapevine is cultivated grafted in most viticultural regions of the world to provide resistance to Phylloxera (*Daktulosphaira vitifoliae*), an insect pest, originating from North America. Although Phylloxera attacks both the shoot and the root, insect feeding on the root system is what drives plant mortality. Towards the end of the 19th century, researchers realized that the roots of American *Vitis spp*. could provide a natural resistance to Phylloxera and the routine grafting of grapevines began (Pouget, 2015). Although most scion/rootstock combinations of grapevine graft easily, some combinations have poor grafting success rates and fragile graft unions (Loupit and Cookson, 2020). Being able to accurately phenotype the structure and function of graft unions in perennial crops would significantly advance our understanding of the processes underlying graft union formation. Furthermore, it would allow us to precisely phenotype mutants/transgenic lines of genes potentially involved in graft union development.

The objective of this work was to develop improved techniques for phenotyping xylem connections formed between the two different plants of natural root grafts and human-made stem grafts. We used micro-CT to visualize xylem vessels. Empty xylem vessels were easily identified in both types of graft, and functional xylem vessels were visualized in the human-made stem graft using a contrast agent. The novelty of this technique is that we can visualize functional xylem vessels in 3D in a large sample; previous studies have been of lower resolution in 2D and/or have not assessed whether xylem vessels are functional and transporting xylem sap.

2 | MATERIALS AND METHODS

2.1 | Grafted plant material

Natural root grafts were found during the excavation of the plants grown in an experimental setup similar to Tandonnet et al. (2018), in which a segregating population of *V. vinifera* cv. Cabernet-Sauvignon × *V. riparia* cv. Gloire de Montpellier was used as a rootstock and planted in rows 1 m apart with 33 cm between each plant in Bordeaux, France (44°47′ N, 0°34′ W; elevation, 22 m). The plants were excavated 5 years after planting, but we do not know when the

natural root graft formed. The natural root graft studied was air-dried and then used for micro-CT analysis described below.

The human-made stem grafts studied were *V. vinifera* cv. Tempranillo grafted onto the rootstock *V. berlandieri x V. rupestris* cv. 110 Richter; these grafts were made using an Omega grafting machine, grown in the Vitis Navarra nursery in Navarra, Spain, for one growing season and uprooted in the winter. The grafts studied were considered of good quality, with a mechanically resistant graft union and adequate scion and root growth during the first growing season (Carrere et al., 2022). The grafts were stored in plastic micro perforated bags, in a cold room (4°C), over the winter, planted in compost:sand-filled (50:50, V:V) pots, then transferred to a greenhouse and irrigated with tap water daily without fertilizer. When the plants had approximately 10 leaves (2 months after transferring the grafts to a greenhouse), one plant was used for micro-CT analysis and another was chosen for dye infiltration experiments. The graft interface was studied approximately 1 year after grafting.

2.2 | Tomography acquisitions and iohexol labelling

We used the EasyTom 150 (RX solutions) micro-CT facilities of the Institut des Sciences de l'Évolution de Montpellier, France. The natural root and human-made stem grafts were imaged with a spatial resolution of 14 and 29 µm voxel^{-1}, respectively. The scanned area was approximately 2 and 4 cm in length for the root and human-made stem grafts, respectively. Scans were done by rotating the sample 360° at <0.26° rotation steps. Each scan took around 5 min. The X-ray source was set at 60 kV and 301 µA. The distance between the X-ray source and the detector (SDD) was 475 mm for the root and 379 mm for the human-made stem graft. The distance between the source and the object (SOD) was 53 mm for root and 87 mm for human-made stem graft.

For the human-made stem graft, we first made a control acquisition without iohexol for each plant. Then, we cut the roots underwater and immersed the roots in an iohexol solution at 60 mg/mL for 24 h. Iohexol was selected as a contrast agent because it has previously been used successfully to characterize functional xylem vessels in perennial, woody plants (Pratt and Jacobsen, 2018; Pratt et al., 2020). The whole plants (with attached leaves) were then placed in a growth chamber so that the transpiration stream could label functional xylem vessels that cross the graft interface. A second acquisition was done after 24 h. For each plant, two acquisitions, at t_{0h} and at t_{24h}, were done.

2.3 | Image analysis

The analysis described below was conducted using Fiji/ImageJ (ImageJ 2.14.0/1.54 f; Java 1.8.0_322 [64 bit]) (Schindelin et al., 2012) and Imaris version 10 (Oxford Instruments) on desktop computer with an Intel® Xeon® Silver 4216 CPU with two processors with 2.10 GHz and 512 GB RAM, and the NVIDIA Quadro RTX 6000 4095MB graphic card.

2.4 | 3D reconstruction of a natural root graft

The aim was to reconstruct in 3D the xylem network of a natural root graft. The resulting raw stack had 1282 successive slices of 16-bit images (Figure 1a). These raw images were processed with Fiji software to subtract the background with a rolling ball radius of 50 pixels and the *sliding paraboloid* option. After this step, the background had a uniform intensity value (Figure 1b). Then, a threshold (from 687 to 65 535) was applied to all slices; this threshold was chosen to maximize contrast between empty xylem vessels and plant tissues. After this process, a binary stack of 8-bit images was produced so that black pixels represent the background and lumens of xylem vessels, and white pixels the root tissues (Figure 1c). Then, we selected the region of interest (ROI) (i.e., the area with the xylem vessels) using the free area selection tool in Fiji. The free area selection tool requires selecting a ROI on the first and last slice of the stack, and only one ROI per slice even when unconnected roots are present. We manually selected ROI in 29 slices of the stack in ROI manager. Then we applied interpolate ROI; this algorithm adds the ROI to all slices in the stack (as image contrast was higher in the 16-bit stack, the regions of interest were defined on the 16-bit stack, saved in the ROI manager and then applied to the 8-bit stack). The aim of this step was to select only xylem vessels so that automatic particle counting in Fiji (called 'Analyze Particle') can be used on each slice. This Plugin analyzed the particles in the segmented image and was used to set the minimum and maximum pixel area size to exclude any non-target objects or artefacts, we selected particles between 1 and 1.2034 mm². After this selection, a new stack was made. Then, the look-up table was inverted so that the background is black and the xylem vessels are white (Figure 1d). In the next step, we manually removed some artefacts of the stack (again, due to improved image contrast the 16-bit stack was used for this step, the regions were saved and applied to the new stack) (Figure 1e). We converted the stack of 2D images into 3D objects using the feature 3D manager (available in the plugin 3DSuite, V3.96). To do so, we selected the minimum volume option (1 voxel), the stack was then automatically converted to 32-bit as there were over 65 535 objects detected in the stack, and this was synchronized in the 3D viewer. After this step, a 3D segmentation was done so that objects are identified in 3D and defined by a grey-scale colour (Figure 1f). Thanks to the 3D segmentation, objects with a minimum volume of 0.05 mm^3 were imported into and visualized with the 3D manager (Figure 1f). In the example shown, 50 vessel objects (vessels and groups of vessels) were identified, and some were coloured and visualized in 3D (Figure 2); the image analysis pipeline is provided in Supporting Information S1: Figure 1A.

3D visualization of xylem vessels located around a natural root graft was selected (Figure 2), and quantitative information on the volume of objects of interest was obtained (Table 2). The vast majority of vessels

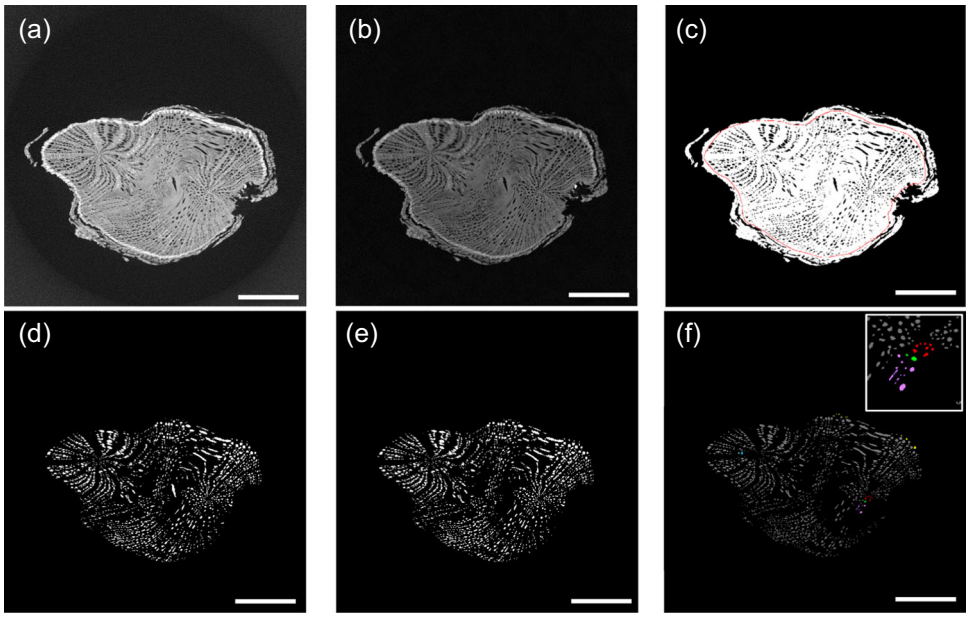

FIGURE 1 Image processing steps of a slice of a micro-CT scan of an air-dried natural root graft of grapevine: (a) raw image, (b) image after setting background, (c) binary image after the application of a threshold, red line shows the region of interest (ROI) defined by the ROI interpolation tool, (d) image after applying the Plugin analyze particles to select objects of less than 1.2 mm^2 in the ROI defined in image (c), (e) image after manual correction, and (f) image after selecting 3D objects >0.05 mm^3, insert of higher magnification of three objects coloured red, purple and green. Scale bar: 5 mm.

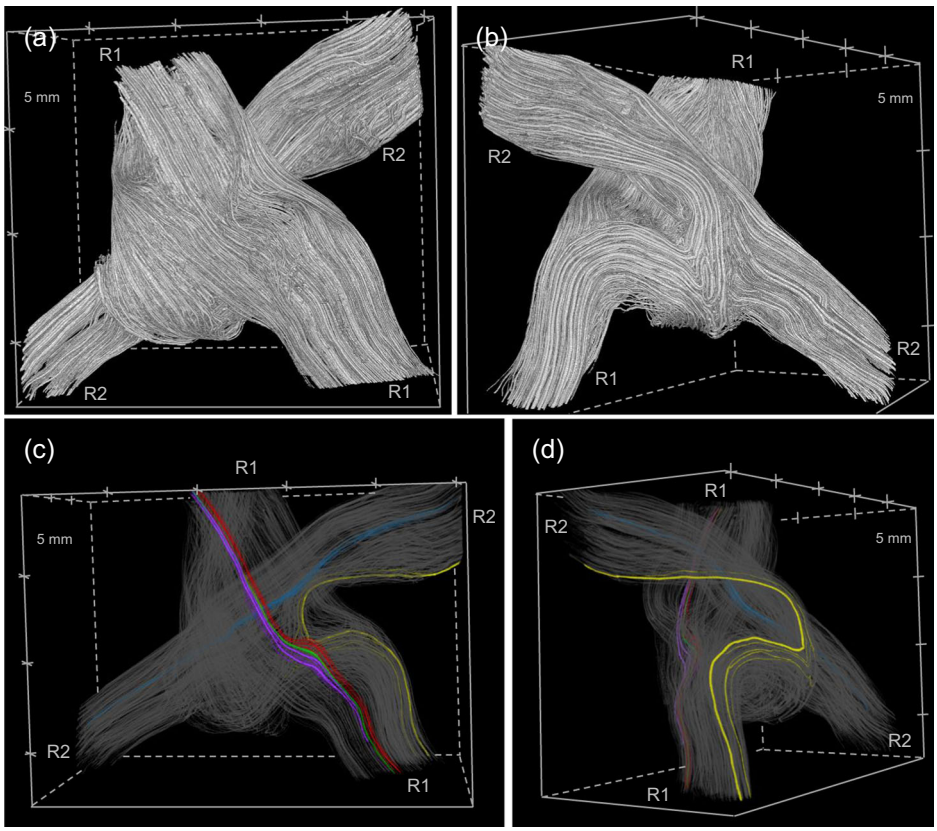

FIGURE 2 3D reconstruction of micro-CT images of the xylem vessels of an air-dried natural root graft of grapevine: (a, b) 3D representation of objects with a volume >0.05 mm^3, (c, d) 3D representation showing the six differentially coloured objects identified in Figure 1f. R1 and R2 correspond to root 1 and root 2. Scale bar: 5 mm.

appear interconnected at the resolution that we used. The grey object has a volume of 179.5 mm^3 and represents a large number of interconnected xylem vessels (Figure 2c,d). The coloured objects are individual or small groups of interconnected xylem vessels of the natural root graft: the yellow object appears to connect the two different roots (R1 and R2), whereas the purple, green and red objects are only in R1, and the blue object is only in R2 (Figure 2c,d). The different objects could be manually selected and coloured in the 3D reconstruction.

2.5 | 3D reconstruction of a human-made stem graft

The aim was to reconstruct in 3D the functional xylem vessel network connecting the scion and rootstock in human-made stem grafts of grapevine, 1 year after grafting, by labelling functional xylem vessels with the contrast agent iohexol. The 3D stacks collected at t_{0h} (Figure 3a) and t_{24h} (Figure 3b) were aligned using the FIJIYAMA plugin (Fernandez and Moisy, 2020) of Fiji/ImageJ (Schindelin et al., 2012), using default parameters (Figure 3c,d). Subtracting the t_{0h} image from the t_{24h} allowed us to select iohexol-containing xylem vessels (Figure 3e). Then iohexol-labelled xylem vessels were segmented using Waikato Environment for Knowledge Analysis/Weka segmentation (a trainable Fiji Plugin that employs machine-learning algorithms to generate pixel-based segmentations) (Figure 3f); manual cleaning to remove obvious artefacts followed this step. We then extracted the channel corresponding to iohexol-filled vessels from the segmented stack to create a new binary stack (Figure 3g). The organization of the functional xylem vessels at the graft interface of a human-made stem graft of grapevine was then visualized using the IMARIS software (Oxford Instruments) (Figure 3h–j) or the 3D viewer from Fiji.

We then used the same 3D manager plug-in that we used for the natural root graft, with the same settings. Only 14 objects with a volume >0.05 mm^3 were detected (Table 3), and just one (object 3) corresponded to xylem vessels, since all the vessels were highly interconnected and they could not be separated into individual objects. Selecting only object 3 allowed us to remove additional artefacts. If we compare an image of the stack at different steps of image processing, we can follow the removal of artefacts at each step (Supporting Information S1: Figure 2): for example, by comparing a slice after Weka segmentation (Supporting Information S1: Figure 2A), after manual cleaning (Supporting Information S1: Figure 2B), and after selecting the 3D object corresponding to xylem vessels (Supporting Information S1: Figure 2C), and the composite image of all three steps in 2D (Supporting Information S1: Figure 2D) and 3D (Supporting Information S1: Figure 2E). The image analysis pipeline is provided in Supporting Information S1: Figure 1B.

2.6 | Histological analysis

The human-made stem graft was analyzed by micro-CT was then placed in formal-acetic-alcohol/FAA prepared in the v/v ratio 4:50:5:41 of formaldehyde:ethanol:acetic acid:water to preserve the tissue. Afterwards, the sample went through a progressive rehydration in ethanol:-water (v:v) solutions at 50%, 40%, 30%, 20% and 10% for half a day. Once the samples were rehydrated, 40–60 µm thick sections were prepared on a slide microtome, the GSL1 (MIKROT L, Schenkung Dapples), which has been specifically designed to cut woody tissues (Gärtner et al., 2014) using MB35 premier microtome blades, 34°/80 mm, Thermo Scientific. Sections were then visualized under a bright field on an Axiozoom V16 macroscope, Zeiss, with the ZEN software, Zeiss.

2.7 | Dye infiltration

We used a dye infiltration system similar to the one described in Pouzoulet et al. (2019), which consists of a three-way valve, one connected to the water, one to the dye syringe and one to the sample (a graft). The sample was connected on one end to a tubing system filled with deionized water and deionized water was infiltrated with a 20 kPa pressure head for 5 min. Then, the water valve was closed and 5 mL of filtered Safranine O solution (1 mg mL^{-1} in deionized water) was added to the system with a syringe. The dye valve was then closed and the water valve reopened for a further 5 min to clean up excess dye. Longitudinal sections were then cut using the GSL1 microtome, with a thickness of approximately 30–40 µm and visualized on a LEICA® microscope DM750.

3 | RESULTS

3.1 | Micro-CT can characterize xylem vessel connections in air-dried natural root grafts

Natural root grafts were found upon excavating a high-density experimental vineyard; two natural root grafts were found among the 1137 plants studied (this is an underestimate of the number of natural root grafts present as the vineyard was excavated with a tractor and little attention was paid to the identification of natural root grafts). One of these natural root grafts was air-dried and used for micro-CT analysis (Figures 1 and 2). Imaging processing was used to isolate empty xylem vessels (Figure 1) and visualize the 3D xylem network (Figure 2, Supporting Information: Video 1 and 2). These images show that many xylem vessels connect the vascular systems of the two different roots of the naturally grafted plants (Figure 2, Supporting Information: Video 1 and 2). The volume of individual or small groups of xylem vessels was obtained using image analysis software (Tables 1 and 2).

3.2 | X-ray micro-CT can accurately image stem tissue structures

The comparison between micro-CT and microscopy images showed that micro-CT can be used to accurately visualize the tissues of a

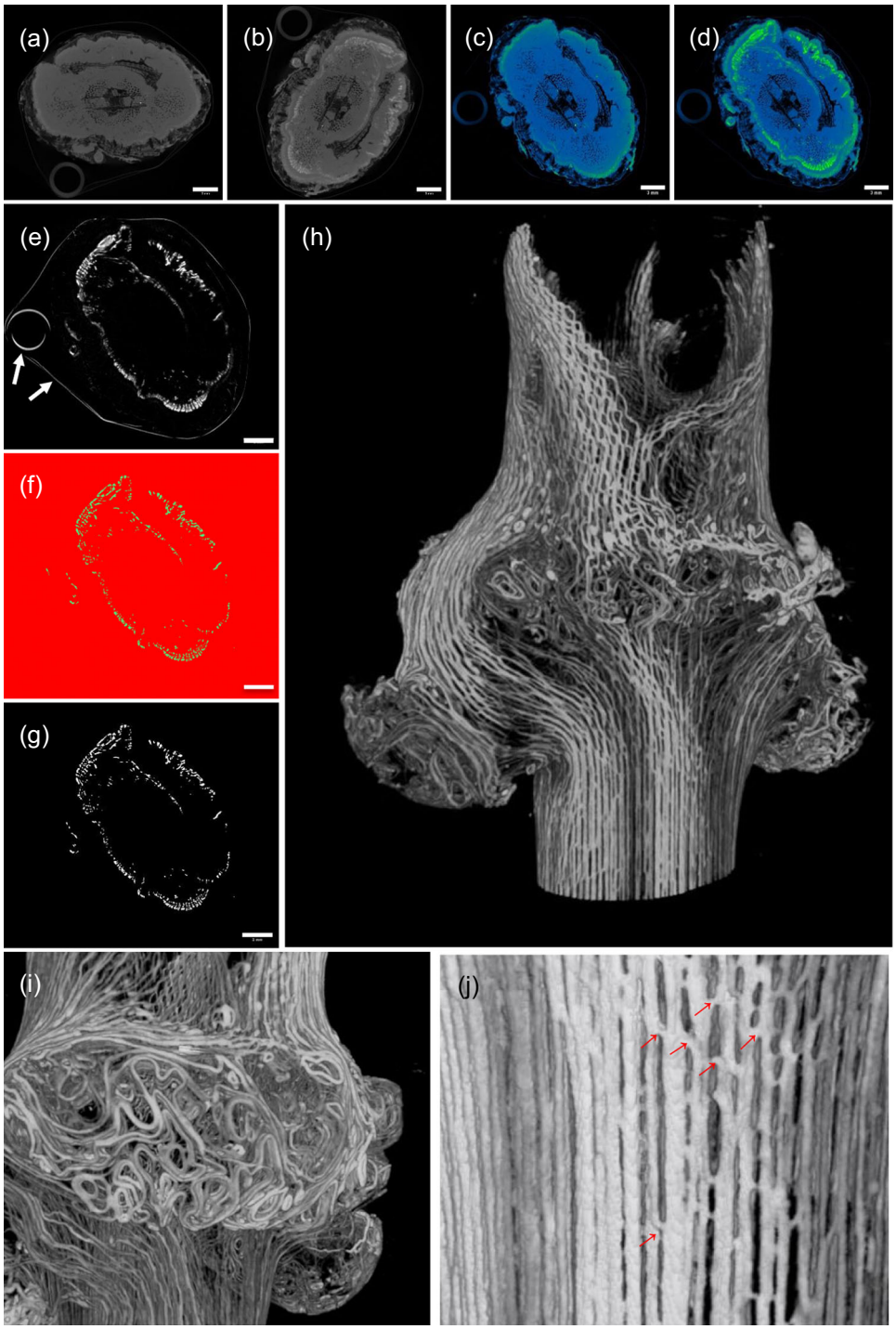

FIGURE 3 Image processing steps to visualize in 3D the functional xylem vessels connecting the scion and rootstock a human-made stem graft of grapevine. (a) raw image before and (b) after iohexol-labelling. (c) False colour image of (a) (LUT: green fire blue); (d) false colour image of (b) after alignment with image (a) using FIJIYAMA. (e) Subtraction of image (c) from image (d), artefacts removed from (e to f) indicated by arrows; (f) image (e) after Weka segmentation and region of interest selection; (g) binary image after selection of iohexol-labelled vessels. (h–j) Images of 3D reconstruction of functional xylem vessels using IMARIS. To improve image contrast in (h–j), the segmentation of (g) is shown with the grayscale of (d). Red arrows in (i) indicate some of the diagonal xylem connections between the main axial xylem vessels of the rootstock. Scale bar: 5 mm. [Color figure can be viewed at wileyonlinelibrary.com]

grapevine stem visible under light microscopy, in particular empty xylem vessels (Supporting Information S1: Figure 3). Furthermore, micro-CT scans of the graft interface of grapevine before and 24 h after applying iohexol to the roots showed that the contrast agent ascends to the scion and labels the functional xylem vessels spanning the graft interface (Figure 3, Supporting Information: Video 3, Supporting Information S1: Figures 3 and 4). There is also good agreement between the micro-CT images of iohexol-labelled xylem

TABLE 1 Distribution of object volumes in the 3D reconstruction of a root graft.

Volume (mm^3)	>150	150–1	1–0.1	0.1–0.01	0.01–0.001	<0.001
Number of objects	1	0	20	188	1670	117 921

TABLE 2 Quantification of the volume of the objects identified in Figure 2c,d.

Colour of object	Red	Green	Purple	Yellow	Blue	Grey
Volume (mm^3)	0.912	0.39	0.758	0.865	0.627	179.569

TABLE 3 Distribution of object volumes in a 3D reconstruction of a micro-CT scan of an artificial stem graft after image processing.

Volume (mm^3)	<150	150–1	1–0.1	0.1–0.01	0.01–0.001	<0.001
Number of objects	1	1	3	83	749	9410

vessels and the xylem vessels visible in microscopy images, particularly in the newly formed callus tissues (Supporting Information S1: Figure 4). Different objects were identified after image processing and their size could be measured (Table 3). This analysis shows that at the graft interface, some xylem vessels connect the scion and rootstock.

3.3 | Iohexol-labelling reveals that thin diagonal vessels connect the main axial vessels in woody stems of grapevine

The 3D xylem network of a human-made stem graft of grapevine revealed the presence of many thin diagonal xylem vessels connecting the main axial xylem vessels of the stem (in the areas not damaged by the grafting process) (Figure 3j); these vessels appear to be arranged in an organized manner and a relatively uniform angle. We used microscopy to confirm that these diagonal vessels were not artefacts (Figure 4) and found that the diameter of the diagonal vessels was significantly smaller than the main axial vessels (Supporting Information S1: Figure 5). Dye infiltration of Safranin O showed that these small-diameter vessels are able to transport liquid (Figure 4) and that the xylem vessel network is highly interconnected. Owing to the extremely high degree of xylem vessel interconnectivity, it was impossible to isolate individual xylem vessels that span the graft interface and characterize their sizes/volumes.

4 | DISCUSSION

4.1 | Micro-CT can be used to visualize the organization of xylem vessels in natural root grafts of grapevine

Micro-CT has been widely used to study xylem vessels in air-dried plant tissues, for example, in olive (Walker et al., 2023), grapevine (Pratt and Jacobsen, 2018; Pratt et al., 2020), and beech (Schreel et al., 2022) shoots. In general, these studies have only presented 2D slices taken from the 3D stacks produced by the tomography equipment. In some cases, the 3D organization of xylem vessels in air-dried samples has been studied in exceptional detail thanks to synchrotron CT, which can achieve excellent resolution (around 4.5 µm) (Brodersen et al., 2011; Brodersen, Choat, et al., 2013; Lee et al., 2013; McElrone et al., 2021). Similarly, xylem vessels in small sections of stem (approximately 1–2 mm) have been analyzed in 3D with a bench top micro-CT scanner with a resolution of 10 µm (Steppe et al., 2004). Usually, high-resolution studies of xylem vessel anatomy characterize only a limited number of vessels over a small area and do not provide insights into xylem vessel connectivity in large tissues. In this study, we characterized xylem vessels in an air-dried natural root graft of grapevine. We showed that these roots were truly grafted together, i.e., they had vascular connections between both genotypes of the grafted plant. Natural root grafts in forest ecosystems are widely reported in the literature in terms of their consequences on pathogen propagation or stress resistance (Lev-Yadun and Sprugel, 2011), and dye/^{32}P injection experiments have shown that these root grafts have functional xylem connections (Bormann, 1966). Natural root grafts of tree species are usually assessed in 2D images, which can show the development of a callus and the fusion of xylem and phloem layers to form a common growth ring spanning both genotypes (Fraser et al., 2005; Gaspard and DesRochers, 2020; Tarroux and DesRochers, 2010). The method that we present here allows the 3D characterization of vascular connections in natural root grafts of a perennial species for the first time.

4.2 | Iohexol can be used to visualize xylem connections in human-made stem grafts of grapevine

We have previously used micro-CT to study the graft interface of living samples of grapevine (Milien et al., 2012); however, major

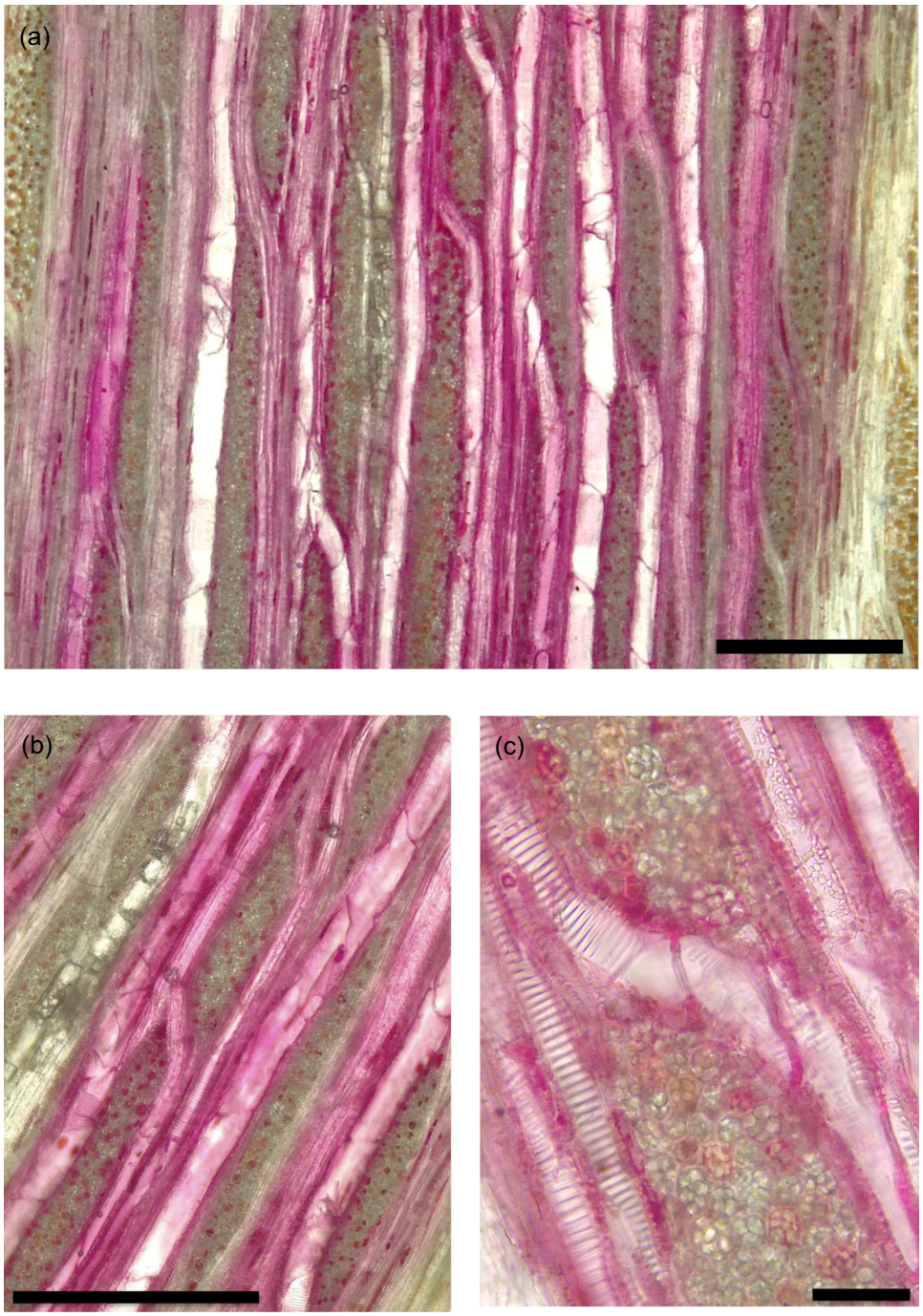

FIGURE 4 Images of Safranin O infiltrated xylem vessels of a woody grapevine stem showing the presence of many small diameter diagonal xylem vessels. Scale bars: (a, b) 0.5 mm and (c) 0.05 mm. [Color figure can be viewed at wileyonlinelibrary.com]

improvement described in the current study is that we have a higher contrast between the xylem vessels and surrounding tissues because functional xylem vessels were labelled with high X-ray dense iohexol. In addition, we used a higher resolution for the acquisition and improved image processing in comparison to Milien et al. (2012). Iohexol has previously been used to study functional xylem connections in grapevine and other perennial plants, but the xylem connections have rarely been visualized in three dimensions in large tissue samples. The 3D visualization presented here shows how the xylem vessels connections are heterogeneously distributed around the circumference of the graft. While some regions have relatively straight and direct xylem connections, others present only few direct connections between the scion and rootstock. The most callus tissue was produced around the lowest part of the omega cut which, to a certain extent, seems to impede xylem vessel connections between the scion and rootstock. The large and heterogeneous nature of the graft interface makes it difficult to characterize graft union formation; however, the use of iohexol and micro-CT can further our understanding

of tissue organization and function. This technique may be more easily applied to herbaceous annual grafts, these species may have fewer intervessel connections so that individual xylem vessels could be readily segmented and characterized in 3D.

The most advanced technique for analyzing the 3D xylem networks is the Tomography-derived Automated Network Analysis of Xylem add-on in the Avizo software (Brodersen et al., 2011; Lee et al., 2013); in this case, small samples were scanned using a synchrotron CT facility with very high resolution. Although the Tomography-derived Automated Network Analysis of Xylem method provides exceptional detail, high-resolution CT facilities are not widely available. The method that we describe in this manuscript is based on the use of a benchtop micro-CT scanner, which is much more accessible to researchers.

4.3 | 3D visualization of iohexol-labelled xylem vessels reveals that thin diagonal vessels connect the main axial vessels in grapevine woody tissues in the spring

High-resolution synchrotron CT has shown that xylem vessels of grapevine are radially connected by vessel rays, thin radial xylem vessels are aligned so that they connect large diameter vessels together (Brodersen, Choat, et al., 2013), and intervessel pits formed between adjacent vessels (Brodersen et al., 2011). These measurements were made in mature stem internodes of current year stems. Our 3D visualization of iohexol-labelled xylem vessels showed that there is considerable radial transport through thin diagonal vessels between the main axial xylem vessels of 2-year-old woody stems of grapevine (in areas far from the graft interface in both the scion and rootstock). These thin diagonal vessels could connect large-diameter axial vessels together through intervessel pits. To the best of our knowledge, this is the first time that these thin, diagonal vessels have been observed in wood tissues of grapevine. Iohexol-labelling and dye infiltration experiments showed that these vessels are functional. However, it has previously been shown that grapevine trunks have extensive lateral xylem connections in the autumn despite being functionally sectored (McElrone et al., 2021). it is possible that these thin, diagonal vessels observed in the spring mature into the vessels observed in the study of McElrone et al. (2021). The presence of such a dense network of diagonal vessels connecting the main axial vessels in grapevine in the spring could have consequences for embolism repair and pathogen movement (Chatelet et al., 2006) in the perennial tissues of grapevine during the winter months.

5 | CONCLUSION

In this study, we visualized xylem connections across the graft interface of both air-dried natural root grafts and iohexol-labelled vessels in living plant material in 3D. The major novelty of this work is the 3D assessment of functional xylem vessels connecting the scion and rootstock, as previous studies have been limited to characterizing the presence of vessels (which are not necessarily functional) or to characterizing xylem vessel function in 2D. In addition, this method was able to reveal that there are extensive diagonal xylem connections in 2-year-old woody tissues of grapevine. These diagonal vessels make the quantitative analysis of xylem vessel connections between the scion and rootstock very difficult, as all vessels appear to be connected with each other. The method that we describe here is an important advance in our ability to phenotype the graft interface of both natural and human-made grafts and could be used to visualize xylem connections in a wide range of plant species using relatively easily accessible equipment. The next step for this type of study is to quantify the number of xylem vessels formed across the graft interface and link these measurements to proxies frequently used to define grafting success such as break tests, hydraulic conductivity measurements, and wilting assays.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Recherche Data Gouv, doi. org/10.57745/NKQ2ZY.

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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