Advanced imaging and quantification of the cambium and developing xylem in eucalypts using X-ray micro- and nano-computed tomography

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Summary – In recent years, the popularity of X-ray computed tomography (CT), as a non-destructive imaging technique, has continued to expand in various research domains. In wood research, X-ray CT has proven to be useful for three-dimensional (3D) structural studies investigating the complex tissues of trees. Wood formation (i.e., xylogenesis) initiates in the cambium and a narrow zone of subsequent differentiation, both of which play key roles in plant growth and development. However, the dynamics of xylogenesis in eucalypts remain relatively poorly understood, in large part due to challenges in sampling, imaging, and characterizing the cambium. Therefore, the aim of this study was to present a workflow to evaluate the feasibility of using X-ray CT to characterize and quantify the structural properties of the cambium in eucalypts. The growth responses of Corymbia hybrid seedlings, exposed to either irrigated or droughted conditions, was investigated by monitoring the structural development of the cambium. To track microstructural changes in the cambium, the same seedlings were imaged with X-ray micro-CT (μCT) one day before the treatments and again six days after the respective treatments. After the last X-ray μCT scan, X-ray nano-CT was also applied. Using image analysis techniques, the morphological characteristics of the cambium could be determined. X-ray μCT displayed a larger, thicker cambial zone in irrigated plants, while a much thinner cambium was visible in droughted seedlings. X-ray nano-CT revealed that droughted plants were associated with a significantly \( p \leq 0.05 \) smaller cambium volume containing smaller cells, compared to the cambium of irrigated plants. Light microscopy was used to validate the CT results and demonstrated no significant \( p > 0.05 \) difference in the cambium width and cell diameter obtained from the two respective CT techniques. The findings of this study proved X-ray CT to be a valuable tool for examining the effect of changing environmental conditions on the complex cambium structure of Corymbia hybrid seedlings.

Keywords – cambial activity, Corymbia hybrid, drought stress, image analysis, microtomy, non-destructive, wood formation, X-ray computed tomography.

Introduction

Trees comprise a large component of the biosphere and serve an exceptional role in biodiversity conservation (Barlow et al. 2007). On a global scale, trees play a fundamental part in eradicating excess CO₂ from the environment, consequently stabilizing climatic fluctuations (Shin et al. 2022).

Trees facilitate secondary growth through the secondary vascular system, which comprises the secondary phloem (bark), vascular cambium and secondary xylem (wood). Central to this dynamic developmental system is the cambium; a layer of meristematic tissue that regulates secondary growth by generating xylem and phloem, thereby
ensuring the perennial life of trees (Plomion et al. 2001). Understanding the cambium is a very enticing problem. An array of research on cambium structure and function has been published in the last few decades. For example, Risopatron et al. (2010) highlighted research that led to a clearer understanding of the molecular forces that affect the cambium structure and function. Prislan et al. (2013) reviewed the cellular and subcellular changes in the cambium, focusing on structural changes associated with seasonal activity. However, the criteria for describing cambium activity are not entirely explained, particularly when examining it at various resolutions (on cellular, subcellular, and ultracellular levels) (Prislan et al. 2013). In a recent review by Wang (2020), the regulation of cambium activity during secondary growth in the stem is reported. However, this tissue has yet to be fully exploited from the viewpoint of structural cell biology (Bossinger & Spokevicius 2018).

A fundamental cause of variability in wood properties is changes in the micro-environment experienced by the cambium and differentiating cells (Savidge 2000b). Wood properties are attributable to cambium cell division, expansion, and secondary wall production; since the cambium serves as an intercellular communication network that integrates growth and development throughout the whole plant (Savidge 2000a). However, there are many unanswered questions on the development phenomena of the cambium, and this study aims to shed some light on this aspect.

Generally, the cambium is a very difficult system to examine (Wang 2020) and few papers have been published on xylogenesis in angiosperms, since most existing studies are mainly focused on hydraulic properties and wood anatomy (Marchand et al. 2021). The reason for this scarcity is due to the technical difficulty in handling the cambium, which comprises very narrow, thin-walled, axially elongated cells that are crammed between considerably thicker-walled secondary vascular tissues. Furthermore, the cambium is easily damaged during sampling and very prone to tearing during excision, a phenomenon known as ‘slippage’ (Larson 1994).

Although a variety of two-dimensional (2D) imaging techniques, ranging from optical to electron microscopy, underpin the basic understanding of plant biological structure-function relationships, these techniques are constrained by a restricted field-of-view (transmission electron microscopy), optical transparency (essential for confocal) and these tools require very demanding sample preparation (e.g., freeze-fixation, embedding and subsequent sectioning) (Rawson et al. 2020). For cellular-level studies, light and electron microscopy are mostly employed (Koddenberg & Militz 2018). However, studying the spatial architecture of wood with microscopy is challenging, since it applies a 2D representation to a three-dimensional (3D) matter. Conventional methods for investigating xylogenesis is tedious and time-consuming and has undoubtedly been a bottleneck to progress in this research area. In particular, the need to work with a sample of tissue that must be killed for microscopy preparation is a major drawback.

In plant research, detailed 3D imaging is becoming increasingly available and is a powerful approach to understanding biological and mechanical functions. In particular, X-ray computed tomography (CT) appears to be a very good solution to answer these problems. This diagnostic 3D imaging technique exploits the penetrating power of X-rays and allows non-destructive multi-dimensional imaging of a large field-of-view, across a series of resolutions to attain qualitative and quantitative information on the internal structural properties of a sample (Rawson et al. 2020). When X-rays pass through a sample, they will be attenuated depending on the sample density, thickness and atomic number, and the energy of the X-rays (Koddenberg & Militz 2018). Thus, differences in X-ray attenuation create contrast in images, which then enables differentiation between low- and high-density areas in a sample. Essentially, during X-ray CT numerous X-ray projections are generated from various angles, while the sample is rotating. These 2D projection images are then mathematically reconstructed into a 3D volumetric model, which can be used to gain qualitative and quantitative information. After reconstruction of the dataset, virtual slices (like histology sections) can be extracted and visualized at any desired depth and orientation (Rawson et al. 2020). Moreover, segmentation can be performed to discern a specific region of interest (ROI) in 3D, allowing quantification of parameters such as volume, surface area, and porosity. Furthermore, X-ray CT requires no time-consuming sample preparation and enables excellent spatial resolution that resolves microstructures at scales down to the submicron range.
In the last decade, X-ray CT has been established in a variety of research domains to examine the 3D structure of plants. Several studies demonstrated that X-ray CT is especially useful to investigate xylem embolism (Suuronen et al. 2013; Cochard et al. 2014; Johnson et al. 2020), to perform anatomical analysis (Van den Bulcke et al. 2009; Brodersen, 2013), to examine wood microstructure and its properties (Lautner & Beckmann 2012; Yang et al. 2019), to study hydraulic function (Pratt & Jacobsen 2018) and to characterize and quantify plant tissues and structures (Mathers et al. 2018).

More recently, X-ray micro-CT (µCT) was used to investigate whether embolism refilling is a routine mechanism of recovery from drought stress in *Eucalyptus* plants (Gauthey et al. 2022; Saunders & Drew 2022). Lehnebach et al. (2021) presented a new methodology to examine xylogenesis in trees using X-ray CT to observe cell wall density changes. High-resolution X-ray CT proved to be an effective avenue to study tree xylogenesis for a range of wood species and structures (Lehnebach et al. 2021). Additionally, Dhondt et al. (2010) recognized high-resolution X-ray CT as a preferred tool to investigate developmental plant biology.

Although X-ray CT has been applied to studies that examine the secondary vascular system of trees, the scope of this technique for studying the cambium, which plays a vital role in plant growth and development, remains to be fully exploited. To date, no study applied X-ray CT to characterize and quantify the cambium structure in living plants. To better understand cambium development in eucalypts, a non-destructive method for characterizing the developmental processes is potentially very useful. Therefore, the aim of this study was to investigate the feasibility of X-ray CT, as a non-destructive research tool, to assess the growth responses of eucalypts by monitoring the cambium and developing xylem tissue at a short time scale. In response to the opportunity to study cambial activity *in situ* in a living plant, this study utilized both X-ray micro- and nano-CT to visualize and quantify structural changes in the cambium in samples subjected to two contrasting environmental conditions (irrigated vs. droughted). Non-destructive X-ray µCT was performed on the same samples before and after the respective treatments to determine changes in the cambium at tissue level (i.e., volume and width), while X-ray nano-CT was used to determine cell level information (i.e., cell number and size) and then validated using a classical microtomy approach. We hypothesized that it is possible to visualize and quantify the morphology of the complex cambium tissue and cells with X-ray µCT- and nano-CT, respectively.

**Materials and methods**

**Plant material and experimental trial**

To determine the influence of environmental conditions on cambium development, six 3-month-old, fast-growing, highly responsive *Corymbia* hybrid (*C. torelliana × C. henryi*) seedlings, with similar pre-experimental conditions, for the purpose of uniformity, were randomly selected from the nursery at Stellenbosch University, Western Cape, South Africa. The seedlings were grown in plastic bags in a shade net nursery under continuous irrigation. A drip irrigation system supplied water to all the plants from the date of planting until the 24th of March 2021, when different treatments were applied. The stem diameter of the seedlings was monitored with a calliper at 10 cm above the base of the stem, from five days before the start of the experiment until the end of the trial. From the 24th until the 30th of March 2021, the seedlings were categorized into two groups, which represented the two respective treatments based on water availability: (1) an irrigation regime (irrigated seedlings) in which three seedlings remained in the shaded area in the nursery where they were irrigated to field capacity twice a day through micro-sprinklers and (2) a dry regime (droughted seedlings) in which three seedlings were exposed to drought conditions ("water-stress" treatment) as they were removed from the nursery and placed in direct sunlight, where they received no/limited water. Drought was interrupted only by rainfall, or by limited irrigation to avoid death.

To track microstructural changes in the cambium, the samples were subjected to lower-resolution scans using X-ray µCT and high-resolution scans using X-ray nano-CT. On the first and the last day of the trial, X-ray µCT was used
to non-destructively scan the same stem section. Due to high-resolution X-ray nano-CT being destructive, it was only performed on the last day of the experiment to obtain subcellular information. The same stem section scanned for X-ray μCT, was used to scan a sub-sample with X-ray nano-CT and the sample was stored for subsequent light microscopy analyses. X-ray CT image acquisition and data analysis were performed at the CT-scanner unit which forms part of the Central Analytical Facility of Stellenbosch University (Du Plessis et al. 2016).

X-ray μCT

*Image acquisition* For woody species, excised and dried samples are the simplest to scan and yield good-quality images (McElrone et al. 2013). With more rapid scan times and sample stabilisation, it is possible to scan living plants (McElrone et al. 2013). X-ray μCT was used to non-destructively explore stem sections of living seedlings to determine whether there is a difference in tissue microstructure and more specifically cambium development between seedlings subjected to different environmental conditions. Stem sections of small seedlings were scanned, because imaging of living plants is best performed on small plants, since the smaller the sample, the higher the resolution that can be obtained. Image acquisition required no sample preparation, besides sample mounting.

A challenging aspect of time-lapse imaging is detecting the same ROI for subsequent scans when removing the sample between scans. Thus, before each X-ray μCT scan, a piece of tape was placed on the stem, 10 cm from the soil surface to serve as a reference marker, indicating the area of interest, to allow the same section to be re-scanned at the same location. As suggested by Brodersen et al. (2010), the leaves of the seedlings were wrapped in paraffin film to prevent dehydration during image acquisition.

Minimizing sample movement is essential during a scan. Thus, the seedlings were mounted in a thin-walled polyvinyl chloride (PVC) cylinder to stabilize the sample and to reduce any vibrations of the upper foliage and stem movement that could cause the plant tissue to move and ultimately lead to image distortion (McElrone et al. 2013). The low density of the PVC cylinder makes it a suitable mounting material, as it can easily be distinguished from the subject of interest.

Setting up a scan relies on the optimization of the voxel size, noise level, number of projections, exposure time and radiation dose, to find the optimal configuration for imaging live plants, i.e., the shortest scan time that still results in adequate image quality (Suuronen et al. 2013). Generally, scan time increases as the resolution is increased. When imaging live plants, where biological processes occur on short time scales, selecting shorter scan times is desirable to reduce the potentially damaging effects of X-ray radiation (McElrone et al. 2013). Increased acquisition times can be accomplished by decreasing the number of projections captured during a 360° rotation or by decreasing the projection time, which will result in a decrease in X-ray dose (Rawson et al. 2020).

In this study real-time X-ray μCT scans of the intact seedlings were attained using a General Electric Phoenix V|Tome|X L240 (General Electric Sensing & Inspection Technologies, Wunstorf, Germany) laboratory-based imaging system, with a tungsten target X-ray tube operating at a source voltage of 90 kV and an electron current of 170 μA. A total of 1800 images were captured in one 360° rotation, resulting in a scan time of 30 minutes and resolution of 9.5 μm. Living samples or dose-sensitive samples are better suited for laboratory CT instead of synchrotron sources (Rawson et al. 2020). The mode of action of the cone-beam X-ray μCT is illustrated in Fig. 1.

A trade-off was made between scan time and resolution, as fast scanning mitigates challenges of X-ray-induced damage that could arise with repetitive scanning. However, this gives rise to a potential loss of image quality. It should be noted that the impact of ionizing radiation is mainly manifested in tissues undergoing cell division. In the stem, the only dividing tissue is the cambium; other tissues largely contain cells that are either dead (xylem vessels and fibres), or alive but no longer dividing (phloem cells and xylem parenchyma) (Suuronen et al. 2013). Literature on the effect of X-rays on cells during X-ray CT is scarce and there is limited advice on specific exposure thresholds (Rawson et al. 2020). For living plants, a cumulative dose limit of 33 Gy has been suggested (Zappala et al. 2013). However, due to the small size of a desktop X-ray source, e.g., the samples are subjected to less than 1 Gy during a one-hour
X-ray micro-CT imaging of cambium

Fig. 1. Scheme of the image acquisition and reconstruction process during X-ray μCT. Following X-ray generation in the X-ray tube, X-ray photons penetrate and interact with the sample, resulting in X-ray attenuation. The attenuated radiation enters the detector and produces a 2D projection image. Through rotating the sample, a stack of projection images is captured and transformed into a 3D volumetric model during image reconstruction. SOD, source-to-object-distance; SDD, source-to-detector-distance.

scan, which is well below the observed threshold for acute radiation effects (Suuronen et al. 2013). Thus, in our study, the scan time of 30 min is below the upper limit for severe radiation effects. Furthermore, according to Rawson et al. (2020) cell damage is generally negligible for μCT systems, since after scanning at micron resolution, living cells remain viable.

Previous studies demonstrated that a typical X-ray CT experiment, with repeated sample scanning (employed at a low dosage), did not significantly impact plant development (Zappala et al. 2013). Provided that the right precautions are in place to limit dose effects, X-ray CT is suitable for the repeated scanning of live plants (Dutilleul et al. 2005). While high and repeated X-ray doses will undoubtedly damage living cells, we refer to Brodersen et al. (2010) who were able to observe refilling in grapevines that were repetitively scanned over a 24-h period. This indicates that X-ray radiation damage does not alter physiological processes within short time scales (hours to days) but may cause significant effects over longer time frames (weeks to months). Furthermore, evaluating the effect of X-ray exposure on living samples is very difficult because they have variable responses, which is reliant on the plant type, variety, and developmental stage (Zappala et al. 2013). This inconsistency in responses validates the need to include unscanned controls in CT experiments. Thus, during preliminary studies suitable scan parameters were tested and optimized to minimize radiation exposure to the seedlings. Unscanned controls were included as verification to ensure that the X-ray parameters and resulting dose are not significantly affecting the experimental treatment.
Image processing and analysis Image processing and analysis involved image reconstruction, characterization, and quantification. System-supplied Datos reconstruction software (Datos|x® 2.1, General Electric Sensing & Inspection Technologies) was used to reconstruct the 2D projection images into 3D volumes. Image analysis was performed with Volume Graphics VGStudio Max 3.4 software (Volume Graphics, Heidelberg, Germany). A flow diagram of the image processing and analysis procedure is displayed in Fig. 2. To obtain quantitative data from the X-ray μCT scans, the samples were divided into two respective ROI’s: the stem section and the extracted cambium. The greyscale images were filtered and then further processed to identify structural differences in selected ROIs in the seedlings subjected to the two respective treatments. Adaptive Gauss filtering was applied to improve image quality and remove noise.

X-ray CT enables one to move beyond the traditional 2D static micrograph and allows the examination of samples using virtual serial sections in any orientation. To visualize the samples in 3D, images were segmented using a variety of semi-automated and manual routines to separate the stem from the mounting material, remove background material, and extract the cambium from surrounding tissues (Fig. 2). The Region Growing tool and Drawing tool was used for these purposes. Once segmentation was completed, it was possible to quantify target plant structures e.g., changes in volume and dimensions of the stem and cambium.

Density differences between various ROIs can be detected as differences in grey values and therefore the cambium could easily be delineated and extracted. The cambial zone was visualized as a bright ring and quantified by manual segmentation using the Drawing tool (Fig. 2). The Wall Thickness Analysis tool was applied to determine the average, maximum and minimum thickness of the cambium.

The term “cambium” is used to describe the cambial initials and “cambial zone” usually refers to the region containing the initial and mother cells (Larson 1994). However, most published data do not differentiate between the two cell types, since the cambial initials and mother cells are only characterized cytologically by a small variation in length (Savidge 2000b). Thus, the terms “cambium” and “cambial zone” are often used to denote all undifferentiated cells that can divide (Prislan et al. 2013) and is therefore used interchangeably in this paper.

X-ray nano-CT

Image acquisition The General Electric Phoenix V|Tome|X L240 X-ray μCT system has a limited resolution (down to 5 μm voxel size), which is not sufficient for examining the cellular structure of plants. Thus, a more advanced approach using high-resolution X-ray nano-CT was applied. To determine cellular level information, nano-CT was performed on a small sub-section of the same stem samples used for X-ray μCT. Image acquisitions were conducted using the Phoenix Nanotom®S system (General Electric Sensing & Inspection Technologies).

A cylindrical tissue sample with a height of 1 cm was excised from the stem with a scalpel blade. The sample was further halved to obtain an even smaller sample and thus higher resolution. To prevent dehydration during image acquisition, the sample was wrapped in parafilm (McElrone et al. 2013). Subsequently, the excised samples were placed in polystyrene foam and fixed on a glass rod to facilitate mounting onto the rotation stage. An accelerating voltage of 80 kV and current of 180 μA was required to obtain a resolution of 1.25 μm. A total of 3200 images were captured in one 360° rotation, resulting in a scan time of 54 min. Although X-ray nano-CT was destructive (sub-sample had to be excised), it allowed quantification of microstructural parameters at a cellular level, enabling the characterization of cells. After the scans, the tissue samples were stored in Eppendorf tubes containing a formalin-acetic acid-alcohol (FAA) solution and placed in a refrigerator until light microscopy analysis.

Image processing and analysis Reconstruction, involving filtered back-projection algorithms, was performed using Datos reconstruction software (Datos|x® 2.1, General Electric Sensing & Inspection Technologies). Image visualization and quantitative analysis were performed using Volume Graphics VGStudio Max 3.4 software (Volume Graphics). A nano-CT image analysis protocol (see Fig. 3) was developed to segment the cell clusters of the cambium from the stem sample. Adaptive Gauss filtering was applied to remove noise and to allow better determination of the boundaries between cells.
Fig. 2. Flow diagram illustrating the consecutive steps involved in the stem and cambium analysis protocol. The 2D cross-sectional images obtained with X-ray μCT (9.5 μm) were filtered before further analyses and then processed in a separate workflow. In the 2D tomographs differences in grey level intensities indicate density variations (light grey, high density; dark grey, low density), while black areas represent air voids.
Due to the complexity of analysis and computation time of X-ray nano-CT, 3D volumes are reliant on the size of the analysed images. It is, therefore, more data efficient to follow a targeted trajectory tracking ROIs (Rawson et al. 2020) by selecting a sub-volume of the original image stack. This sub-volume should be small enough to be treated as a mathematical point of the continuum scale, i.e., large enough to provide a representation of the macroscopic properties of the sample. A representative ROI cube with a size of 0.5 mm × 0.5 mm × 0.5 mm was selected. This sub-volume was virtually cropped and extracted from the datasets for further analyses to be performed.

Due to differences in tissue density, cambium cells could be segmented using thresholding techniques. To discriminate between different tissues, the cambium cells were segmented using the Surface Determination and Region Growing tool, which is grey value based. This approach compares a selected voxel with other voxels based on the similarity and spatial proximity of pixels inside a user-defined range (Koddenberg & Militz 2018). During thresholding, an ROI was created using the Region Growing algorithm. After selecting a starting point, the algorithm expands the selection. The expansion continues as long as new voxels are found which are connected to the growing selection and where the data value is within the specified tolerance relative to the starting point. The cambium ROI (a representative voxel situated in the bright cambial zone) was selected as the starting point and voxels were added to the growing region if they were connected to the region and their grey value did not deviate by more than half the tolerance from the average grey value of the voxels within the growing region. After thresholding for cambium cells, the respective ROI was extracted, and quantification was performed. The Wall Thickness Analysis algorithm was applied to obtain the thickness of the cambium cells. Additionally, the Foam Structure Analysis algorithm was performed on the cells to quantify the morphometric parameters which are described in Table 1.

**Light microscopy** For the preparation of high-quality slides, paraffin wax embedding was performed. The protocol used was adapted from a report on wood sample preparation for microscopy analysis (Prislan et al. 2022) and was
Table 1. Morphometric parameters obtained from X-ray nano-CT image acquisition, with the unit and description used to define the 3D microstructure of cambium cells.

<table>
<thead>
<tr>
<th>Morphometric parameter</th>
<th>Unit</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cell clusters in ROI</td>
<td>–</td>
<td>Number of individual segmented cell clusters in the defined ROI volume</td>
</tr>
<tr>
<td>Cell size</td>
<td>mm³</td>
<td>Volume of the cells</td>
</tr>
<tr>
<td>Cell surface area</td>
<td>mm²</td>
<td>Surface area of the cells</td>
</tr>
<tr>
<td>Cell diameter</td>
<td>μm</td>
<td>Width/diameter of cells</td>
</tr>
<tr>
<td>Sphericity (cells)</td>
<td>–</td>
<td>Ratio of the surface area of a sphere, with the same volume as the cell, to the surface area of the cell. A perfectly round shape has a sphericity of 1, while distorted structures have lower values.</td>
</tr>
</tbody>
</table>

optimized specifically for eucalypts by extending the time for automatic dehydration and infiltration in the tissue processor. Additionally, all the steps in the tissue processor were performed under vacuum to improve penetration. The same stem samples, previously used for nano-CT and which were stored in FAA, were trimmed with a razor blade to embedding size (<1 cm in height) and dehydrated with successive immersions in ethanol and xylene and embedded in paraffin wax. Once removed from the mould the outermost surface of the wax blocks was removed with a scalpel blade and trimmed using a rotary microtome at 8 μm to expose the sample surface. These trimmed sample blocks were placed into beakers with distilled water at 4°C for 3 weeks. This was done to soften the woody tissue, to ultimately produce higher quality slices. After 3 weeks, transverse sections were cut to a thickness of 6 μm. The sections were dewaxed and stained with safranin and astra-blue stain; staining lignin in red and cellulose/hemicellulose in blue, respectively. Dehydration was performed with a series of ethanol, after which DPX mounting medium and a coverslip were added to the glass slide. To investigate the cambial zone, transverse sections were examined using the Nikon Eclipse E400 Microscope (Nikon Instruments, Melville, NY, USA) and the Leitz Polarizing Light Microscope (Leitz, Wetzlar, Germany). The cambium width and cell diameters obtained from X-ray μCT and nano-CT, respectively were validated using this optimized paraffin wax embedding technique. For each sample, the cambium width and cambium cell diameters were measured at five random positions or five narrow rectangular cambium cells, respectively, using ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA).

Statistical analysis

One-way analysis of variance (ANOVA) was performed to analyze the statistical significance of the respective quantitative measurements with respect to the two treatments. All values are expressed as the mean ± standard deviation. Data analyses were performed using XLSTAT software. The level of confidence required for statistical significance was selected at p ≤ 0.05.

Results

Several studies investigated wood formation in trees (De Micco et al. 2019; Chen et al. 2022), but very little data are available, particularly on hardwoods like eucalypts, elucidating the dynamics of wood formation. Moreover, studies tracking microstructural changes within the same sample over time are, to our knowledge, completely absent. The development of a complete X-ray CT workflow from sample preparation, to optimizing scanning parameters and image acquisition, reconstruction, and analysis, allowed the estimation of relevant cambial parameters to track developmental changes. The following sections will demonstrate how X-ray μCT (Fig. 4a) and nano-CT (Fig. 4b) were utilized to explore the cambium in living plants and excised tissue, respectively. With X-ray CT the cambium was detected as a bright ring due to the high-density tissue that could be distinguished from other lower-density
tissues. Techniques capable of visualizing and quantifying 3D microstructures have been the answer to many research questions. Thus, our understanding of cambium development depends on insights into the 3D microstructure.

X-ray μCT

A representation of the grey level 2D projection images and 3D reconstructed volumes before and after the respective treatments are presented in Figs 5 and 6. Images of only one seedling for each treatment are displayed since similar trends were observed. Within the stem section, different components can be identified and characterized in 2D and 3D. Here the focus was to segment, characterize and quantify the cambium in terms of structural changes (i.e., volume and thickness) occurring as a result of either the irrigation or drought treatment.

In the irrigated sample, stem diameter and ROI volume increased after irrigation (Fig. 5). Cambium growth took place since there was an increase in cambium volume and average cambium thickness/width. In the droughted sample, by contrast, a decrease in ROI stem volume and diameter occurred (Fig. 6). Furthermore, a decrease in cambium ROI volume and average cambium thickness is visible from the 2D tomographs and 3D volume renderings (Fig. 6). From the X-ray μCT slice images in Figs 5 and 6, obtained at a resolution of 9.5 μm, it was not possible to display cells through this visualization process. Nevertheless, the tissue of interest, the cambium, was easily discernible.

Qualitative image analysis

The qualitative results illustrated structural changes before and after the respective treatments, and this was confirmed by the quantitative measurements presented in Table 2. For the quantitative measurements, the stem section and cambium ROI were characterized independently. No significant differences \((p > 0.05)\) were observed in the stem ROI volume for the respective irrigated and droughted samples. However, the irrigated samples displayed an increase of 1.2% in stem volume, while the droughted samples presented a decrease of 11.4% (Table 2). Porosity was expressed as the fraction of air volume over the total ROI volume and provided an estimation of the percentage air present in the stem. Both treatments exhibited a significant \((p \leq 0.05)\) increase in porosity, with this percentage increase being more than double in the droughted samples (Table 2). Although not significant \((p > 0.05)\), there was an increase of 0.7% in the average stem diameter in the irrigated samples, while the droughted samples displayed a decrease of 8.5% (Table 2).

The cambium ROI volume increased significantly \((p \leq 0.05)\) with 56.2%, while a significant \((p \leq 0.05)\) decrease of 42.3% was observed for the droughted samples (Table 2). This is related to the percentage that the cambium comprises...
Fig. 5. 2D tomographs and 3D volume renderings displaying the changes in the stem ROI and cambium ROI in a representative sample before and after the irrigation treatment.
Fig. 6. 2D tomographs and 3D volume renderings displaying the changes in the stem ROI and cambium ROI in a representative sample before and after the drought treatment.
Table 2. Microstructural parameters of the stem and cambium ROI before and after the respective irrigation and drought treatments, obtained with X-ray μCT.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Irrigated</th>
<th>Droughted</th>
<th>% Increase/decrease</th>
<th>% Increase/decrease</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Before (n = 3)</td>
<td>After (n = 3)</td>
<td></td>
<td>Before (n = 3)</td>
</tr>
<tr>
<td>Stem</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume (mm³)</td>
<td>104.76 ± 17.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>105.99 ± 17.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.2</td>
<td>138.57 ± 38.89&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Air volume (mm³)</td>
<td>1.59 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.22 ± 1.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>165.4</td>
<td>3.16 ± 2.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Porosity (%)</td>
<td>1.9 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.9 ± 1.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.4</td>
<td>2.9 ± 2.52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>5.53 ± 0.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.57 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.7</td>
<td>6.39 ± 0.96&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cambium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume (mm³)</td>
<td>5.96 ± 0.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.31 ± 1.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>56.2</td>
<td>6.13 ± 0.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>% of stem ROI</td>
<td>5.7 ± 0.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.8 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.1</td>
<td>4.4 ± 0.91&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Average thickness (μm)</td>
<td>108.40 ± 4.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>144.25 ± 12.89&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.1</td>
<td>110.92 ± 5.68&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maximum thickness (μm)</td>
<td>186.17 ± 16.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>244.15 ± 37.87&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.4</td>
<td>190.01 ± 9.72&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Minimum thickness (μm)</td>
<td>28.08 ± 16.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.35 ± 9.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.3</td>
<td>21.81 ± 8.69&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation of three replicates. Different letters in the same row indicate significant differences before and after the respective treatments (p ≤ 0.05).

of the stem ROI, increasing significantly (p ≤ 0.05) with 3.1% for irrigated plants and decreasing slightly with 1.5% for droughted plants (Table 2). The average cambium thickness also demonstrated the effect of water availability on the cambium structure, as irrigation imposed a significant (p ≤ 0.05) increase of 33.1% in cambium thickness, while drought resulted in a significant (p ≤ 0.05) decrease of 31.6%. After the treatments, the average cambium width was 144.3 μm and 75.8 μm for the irrigated and droughted samples, respectively (Table 2). Even though no significant (p > 0.05) differences were observed between the maximum and minimum cambium thicknesses for both treatments, the maximum thickness of the irrigated samples increased, while the inverse was true for the droughted samples. Similarly, there was an increase of 47.3% in the minimum cambium thickness for the irrigated samples, whereas a decrease of 56.3% occurred in the droughted samples (Table 2). It should be noted that in this study young seedlings were used, mainly due to height restrictions in the X-ray μCT system, and therefore results obtained will differ from older trees due to different age-related xylogenesiss patterns (Rodriguez-Zaccaro et al. 2019).

X-RAY NANO-CT

Qualitative image analysis X-ray nano-CT provided high-quality images (Fig. 7) of the cambial zone, allowing morphometric analysis of the cells. These observations provided a more detailed insight into changes arising on the structural level in the cambial cells in the irrigated and droughted samples (Fig. 7).

The cambium was manually segmented from the 2D tomographs, and individual cells were subsequently color-coded according to their volume, enabling quantification of cellular features (Fig. 7). The 2D tomographs and 3D volumes in Fig. 7 provide information on the shape and size of the cambium cells in a representative irrigated and droughted sample. From the 2D slice images and 3D volumes, it can be observed that the cambium is much thicker in the irrigated sample compared to the droughted sample. This is in alignment with the results obtained from X-ray μCT. Looking at the 2D and 3D representations of the average thickness of the cambium cells, it can be observed that in that specific irrigated and droughted sample the average thickness of the cambium cells was 13.0 and 9.7 μm, respectively (Fig. 7).
The irrigated sample comprised more and larger cambial cells, compared to the droughted sample (Fig. 7). The cambium is comprised of cells that are organized in radial files, which produce the secondary xylem towards the inside (top section on tomograph) of the stem and phloem towards the outside (bottom section on tomograph) (Fig. 7). Theoretically, each radial file comprises of one initial cell, which stays in the meristem, and xylem and phloem mother cells which are generated by the division of the cambial initials (Larson 1994).

**Quantitative image analysis** From Table 3 it can be observed that the irrigated and droughted samples differed significantly \((p \leq 0.05)\) with regards to the cambium ROI volume and % cambium, being higher in the irrigated samples. Although not significantly different \((p > 0.05)\), the number of cell clusters in the cambium ROI and the average cell size were larger in the irrigated samples (Table 3). Furthermore, the cell diameter was significantly \((p \leq 0.05)\) larger in the irrigated samples (Table 3). Cell shape was quantified by sphericity, indicating the roundness of cambial cells. Cells of the irrigated samples were significantly \((p \leq 0.05)\) more spherical than cells of the droughted samples (Table 3).
Table 3. Microstructural parameters of a sub-section of the cambium ROI after the respective irrigation and drought treatments, determined using X-ray nano-CT.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cambium ROI volume (mm³)</th>
<th>% Cambium</th>
<th>Number of cell clusters in ROI</th>
<th>Average cell size (× 10⁻⁶ mm³)</th>
<th>Average cell surface area (× 10⁻³ mm²)</th>
<th>Average cell diameter (μm)</th>
<th>Sphericity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irrigated</td>
<td>0.0114 ± 0.001 a</td>
<td>9.1 ± 0.98 a</td>
<td>2524 ± 1137 a</td>
<td>5.30 ± 1.79 a</td>
<td>2.75 ± 0.56 a</td>
<td>19.56 ± 2.52 a</td>
<td>0.55 ± 0.018 a</td>
</tr>
<tr>
<td>Droughted</td>
<td>0.0051 ± 0.001 b</td>
<td>4.1 ± 0.48 b</td>
<td>1817 ± 591 a</td>
<td>3.05 ± 0.79 a</td>
<td>1.77 ± 0.42 a</td>
<td>14.05 ± 1.08 b</td>
<td>0.47 ± 0.011 b</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation of three replicates. Different letters in the same column indicate significant differences between the respective treatments (p ≤ 0.05).

LIGHT MICROSCOPY

The reliability of the developed X-ray CT protocol was evaluated by comparing the results with those of a parallel study of the same stem tissue by analysing serial sections of paraffin wax-embedded samples. In the brightfield cross-sections in Fig. 8, different cell types can be distinguished; however, the focus was only on the cambial zone. The individual cells of the generative cambium tissue are bordered by and interconnected with neighbouring cells at various developmental phases (Chaffey 1999). Cambium cells are combined into a circumferential zone, which is sandwiched between two distinctive tissues of contrasting anatomy, the xylem, and phloem (Savidge 2000a). Even though the cambial tissue is easily damaged during sampling and sectioning, reasonable images of the cambial zone have been obtained by conventional light microscopy (Fig. 8).

Cambial cells were characterized as radially flattened cells with small diameters and thin, non-lignified cell walls that stain blue with astrablue (De Luis et al. 2011). The onset of cell wall lignification could be detected as the red stain by safranin, which gradually replaced the blue stain (Fig. 8). From the brightfield micrographs in Fig. 8 it can be observed that cells on the xylem section of the cambium in the irrigated sample comprised of more cellulose since it stained more blue, in contrast to the droughted plants which stained more red.

The cambium width is much wider and contains more cell layers in the irrigated sample, compared to the droughted sample (Fig. 8). Regarding the morphology of the cambial cells, it can be observed that cells in the irrigated sample appear larger and more turgid compared to cells in the droughted sample (Fig. 8).

From the polarized light images, the same conclusion can be made, with the irrigated sample displaying a wider cambial zone. Under polarized light, the deposition of secondary wall layers was detected for both treatments, as the cell walls displayed birefringence (De Luis et al. 2011).

Light microscopy was used as a means of validating the cambium thickness (Table 4) and cambium cell diameter (Table 5) results obtained with X-ray µCT and nano-CT, respectively. In Table 4 the X-ray µCT cambium width results were compared with the microscopy data and no significant (p > 0.05) difference was detected. Table 5 presents the X-ray nano-CT cambium cell diameter measurements and here also no significant (p > 0.05) differences were detected when compared with microscopy. Polarizing light microscopy was only included in the X-ray µCT comparison since individual cambial cells cannot be observed under polarizing light and were therefore not included in the X-ray nano-CT comparison.

STEM DIAMETER

Figure 9 presents the before and after stem diameters for the respective irrigation and drought treatments. As expected, although not significant (p > 0.05), there was an increase of 2.5% in diameter for the irrigated samples and a decrease of 8.2% for the droughted samples (Fig. 9).
Fig. 8. Brightfield and polarizing light micrographs of transverse sections of the cambium with the adjacent xylem and phloem of a representative irrigated and droughted *Corymbia* sample, magnified with a 20× (25× for the polarizing microscope) and 40× objective lens. The red arrows indicate the cambial zone. CZ, cambial zone; PH, phloem; X, xylem. Scale bar = 80 μm.
Table 4. Comparison of the cambium ROI width as measured using X-ray μCT, brightfield microscopy, and polarizing microscopy techniques, respectively in irrigated and droughted Corymbia stem sections.

<table>
<thead>
<tr>
<th></th>
<th>X-ray μCT (μm) (3D)</th>
<th>Brightfield microscopy (μm) (2D)</th>
<th>Polarizing microscopy (μm) (2D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irrigated</td>
<td>144.25 ± 12.89³</td>
<td>134.43 ± 5.04³</td>
<td>155.32 ± 12.83³</td>
</tr>
<tr>
<td>Droughted</td>
<td>75.84 ± 16.29³</td>
<td>71.55 ± 6.02³</td>
<td>77.33 ± 8.76³</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation of three replicates for the X-ray μCT technique and five random point measurements per micrograph for three replicates for microscopy. Different letters in the same row indicate significant differences between the respective techniques ($p \leq 0.05$).

Table 5. Comparison of the cambium cell diameter as measured using X-ray nano-CT and brightfield microscopy, respectively in irrigated and droughted Corymbia stem sections.

<table>
<thead>
<tr>
<th></th>
<th>X-ray nano-CT (μm) (3D)</th>
<th>Brightfield microscopy (μm) (2D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irrigated</td>
<td>19.56 ± 2.52³</td>
<td>20.72 ± 0.99³</td>
</tr>
<tr>
<td>Droughted</td>
<td>14.05 ± 1.08³</td>
<td>15.65 ± 2.45³</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation of three replicates for the X-ray μCT technique and five random cell measurements per micrograph for three replicates for microscopy. Different letters in the same row indicate significant differences between the respective techniques ($p \leq 0.05$).

Fig. 9. Average stem diameter before and after the respective irrigation and drought treatments. Values are the means of three replicates. Different letters for the specific treatment indicate significant differences ($p \leq 0.05$).

Discussion

X-RAY μCT

*Qualitative image analysis* Due to differences in X-ray attenuation between different plant tissues, good image contrast could be achieved without the use of contrast solutions. Furthermore, air-filled vessels (observed as black)
were also easily discernible from other surrounding water-filled tissues (observed as greyscale). This is in alignment with Koddenberg & Militz (2018) who reported that in angiosperm datasets with a resolution of >3 μm, larger structures and vessels can be identified and visualized adequately. Smaller structures such as the fibres and axial parenchyma cells are usually difficult to visualize, as they tend to fade into the background noise at lower resolution (Koddenberg & Militz 2018). Even though X-ray μCT was non-destructive and provided a larger field-of-view, it is necessary to obtain more structural detail through high-resolution X-ray nano-CT.

Although the damaging effects of X-ray radiation on the long-term metabolic activity of plants are poorly researched, Earles et al. (2018) noted varying responses that are dependent on the species, tissue type, and radiation exposure time. These effects are particularly important when repeated scans are performed. When scanning living plants, shorter scan times are preferable, to decrease the potential harmful effects of radiation. However, shorter scan times again result in a potential loss of image quality. Provided that X-ray damage can be averted, the non-invasive nature of X-ray imaging presents the prospect to track tissues and cells over time in response to external changes (Rawson et al. 2020). Thus, a series of scans can be performed to reflect changes over time, termed time-lapse CT (Rawson et al. 2020). In our time-lapse study, we observed no adverse X-ray radiation effects at the specific CT scan parameters applied.

Quantitative image analysis  The decrease in stem diameter of the droughted samples was expected as drought stress usually causes a shrinkage in stem diameter (Drew et al. 2009). It is evident that the cambium was more affected by the respective treatments than the stem ROI, demonstrating that cambial growth performance is highly dependent on water availability. As expected, under continuous irrigation, cambium activity and cell differentiation were retained, in contrast to the droughted samples where cambial activity decreased. Thus, the highly dynamic process of wood formation is regulated by physiological and environmental influences (Güney et al. 2015). The increase in stem diameter and cambium thickness in the irrigated samples is in alignment with Patel et al. (2014), who reported that radial growth is positively related to cambial zone width. Our results are also in agreement with a previous study reporting that drought stress results in a decrease in cambium thickness and consequently delays xylem and phloem formation (Qaderi et al. 2019).

Pumijumnong et al. (2021) evaluated the climatic effects on the cambial activity of pine trees to determine the relationships between climatic variables. This study noted that monthly rainfall, relative humidity, soil moisture and monthly mean temperature were all positively correlated to cambial zone width. In another study, the effect of different irrigation regimes on the cambial activity and wood formation in pine trees was established (De Luis et al. 2011). It was found that dry conditions limit cambial activity, and that water availability is the main driving force in promoting cambial activity and primary and secondary growth in woody plants (De Luis et al. 2011).

One can assume that the droughted samples in this study endured quiescence or environmental dormancy, which is controlled by environmental conditions (in this case water availability) and involves structural changes in the cambium (Prislan et al. 2013). Alternatively, during drought, cell production might have occurred at a very slow rate or only on the phloem side and not on the xylem side. Cambium activity and the rate of xylem differentiation is mainly affected by water availability (Patel et al. 2014). This was also confirmed by Wimmer et al. (2002) who reported that most cambial activity occurs when water availability to the cambium is at a maximum.

X-ray nano-CT

Qualitative image analysis  With the introduction of sub-micron resolution systems, plant tissues can now be imaged with cellular resolution in a laboratory environment (Dhondt et al. 2010). Trees comprise complex heterogenous tissues with structural components that are three-dimensionally organized, and these structural attributes can be investigated at various levels (Koddenberg & Militz 2018). Therefore, a multiscale CT imaging approach was developed. In contrast to X-ray μCT, which provided a wider context to the arrangement of the cambium within the whole stem section, X-ray nano-CT (at 1.25 μm resolution) allowed the identification and segmentation of individual...
X-ray micro-CT imaging of cambium

cell clusters. Nano-CT provided a unique view of the cells as it bridges the gap between the abilities of light and electron microscopy techniques (Rawson et al. 2020).

Quantitative image analysis Kitin et al. (2000) reported that details of the 3D cambium structure can assist with forecasting the growth and development patterns of vascular tissues, which can then aid in the characterization of features describing wood structure and quality. A deeper understanding of the mechanisms that underlie cambium development requires quantitative data on the 3D morphology. Earlier studies on the shape, dimensions and arrangement of cambial cells were indirectly characterized based on the structure of cambial derivatives and the assumption that the architecture of secondary vascular tissues reflects that of the cambium (Cumbie 1967). However, the present study provides an understanding of the morphological and developmental changes of the cambial cells by performing 3D numerical analyses through semi-automatic procedures to quantify cell characteristics, such as volume, surface area, diameter, and sphericity.

As expected, irrigation induced cambial activity and cell differentiation (Balducci et al. 2013). Likewise, De Luis et al. (2011) reported a higher number of cambial cells in samples that were under irrigation. Balducci et al. (2013) explained that during water stress, the cambium reduces cell division to save energy to maintain minimum metabolism and defense. Rossi et al. (2009) evaluated the effect of a 20-day-long dry period on cambial growth and reported that cell diameter reduced significantly ($p \leq 0.05$) from 18–22 μm to 13–15 μm. These values are in line with the results reported in this study. The reduction in cell diameter in the droughted plants indicates drought stress, which results in a decline in xylem water potential, ultimately creating an unfavourable environment for cell enlargement as a loss of turgor inhibits cell expansion during water stress (Rossi et al. 2009).

Wu et al. (2016) examined cytological changes in the cambium and found a significantly positive correlation between cambium zone width and cambium cell numbers. Similarly in our study, we found that irrigated samples, with a larger cambium width, also comprised of more cambium cells. Water conditions are the key factor controlling cell size (Abe & Nakai 1999). During water deficit, cell expansion is physically constrained by the loss of cell turgor (Abe & Nakai 1999). Larson (1994) suggested that a decrease in tree water potential, causes a decrease in the frequency of cell division, which is followed by a decrease in cell diameter. This indicates that during drought, cell expansion is constrained by the decline in hydrostatic pressure.

Moreover, Savidge (2000a) reported that a decrease in cambial cell division (cell number) and a reduction in cell diameter usually occur in response to drought. Desoto et al. (2011) also reported that cells formed under drought conditions were smaller. Abe & Nakai (1999) examined the influence of the water status within a tree on the anatomical features of cambial cells and found that trees irrigated daily had more cell layers with larger diameters in the cambium, compared to trees watered every three days.

Water availability plays a vital role cambium development. This corroborates with previous studies reporting water availability to be a vital factor influencing cambial activity and wood formation (Camarero et al. 2010). Similarly, Balducci et al. (2013) stated that the cambium can exhibit sensitivity to water deficit by a decline in cell division and differentiation rate or the formation of smaller cells.

The significantly ($p \leq 0.05$) rounder cells observed in the irrigated samples can be attributed to the turgid versus flaccid state of the cells. Cells in the irrigated samples are in a more turgid state as they are swollen from water uptake and thus appear more spherical, while cells in droughted samples tend to shrink and become more flaccid due to turgor loss. Umami et al. (2021) reported that the cell walls of Eucalyptus become more elastic under drought stress and consequently more elastic cell walls allow for more turgor loss.

Cambial activity is very sensitive to the plant’s water status and drought inhibits or delays cambium cell division by reducing the turgor pressure of cambial cells and by interfering with metabolism and cell enlargement; directly leading to reduced plant growth (Rossi et al. 2009; Patel et al. 2014). A drought-induced growth reduction can also occur indirectly through an effect on photosynthesis and due to a decrease in auxin and carbohydrate synthesis, along with a slower translocation of assimilates to the cambium (Wimmer et al. 2002). However, usually in short-
term variations in water status, growth reduction is the result of a direct influence, because the rate of polar auxin transport is not quick enough to account for rapid reactivation of the cambium (Wimmer et al. 2002). Thus, the differential behaviour of cambial cells can be explained by shifts in the micro-environment.

**LIGHT MICROSCOPY**

Sectioning of the cambium has not been widely applied by other researchers, due to the technical difficulties that arise because of the thin walls of cambial cells (Kitin et al. 2000). Lignified cells (detected by red stain) are presumed to be non-living, as the presence of lignin typically indicates that a cell has reached the end of its developmental phase and “died” to produce functional xylem components such as vessels or fibres. In contrast, blue-stained cells are typically living cells. Those on the xylem side of the cambium represent xylem mother cells, which are still in the process of differentiating into mature xylem elements. The differentiation process, from the un lignified xylem mother cells to the mature xylem with lignin-impregnated walls, was accelerated in the droughted samples since they stained more red. Furthermore, the narrow cambial zone in the droughted samples is indicative of low cambial activity, while a thicker cambial zone suggests an active and differentiating cambium.

Cambial cells in the droughted sample were more distorted, implying that these cells could not sustain their turgidity. This phenomenon can be described as follow. Before a drought event, cambial cells expand by absorbing water. During water deficit, there is a reduction in the pressure potential of the apoplastic water around the expanded cells, since it becomes lower than the osmotic potential of the expanded cells (Abe & Nakai 1999). Consequently, the cells lose their turgor, even though they developed secondary walls. Thus, cell expansion is directly constrained by the decrease in pressure potential, and subsequently, cell production is restricted by physiological factors, such as the decrease in cambial cell activity (Abe & Nakai 1999).

From the polarized images, a wide cambial zone is evident of cambial activity as cell division is taking place. Thus, trees under irrigated growth conditions can invest in a higher number of cambial cells, which in return will result in higher growth rates (Güney et al. 2015).

The benchmarking of X-ray CT with classical microtomy proved that X-ray CT produces accurate estimates of cambial dynamics in samples subjected to contrasting water availabilities. The drawbacks of the paraffin wax procedure include the fact that fixation, dehydration, wax embedding, microtome slicing, and the preparation of numerous slides are time-consuming and laborious. The consecutive sections are collected manually, and artifacts can occur as the sections are easily damaged with the microtome (Kitin et al. 1999). In contrast, X-ray-CT provides a quick and powerful method for 3D visualization of the cambium to examine the developmental changes in cambial cells, which, until now, have been challenging to study using other techniques. However, depending on the information required, the image analysis process may be lengthy.

**STEM DIAMETER**

A tree’s water status is reflected by changes in stem diameter and has frequently been shown to limit radial stem growth (Abe & Nakai 1999). Due to the low water availability in the droughted samples, growth ceased. Güney et al. (2015) confirmed that termination of growth is mainly influenced by restricted water availability. During drought stress, plants tend to close their stomata to reduce water loss, and this results in a decrease in photosynthesis and eventually limited plant growth (Umami et al. 2021). Our results agree with earlier studies demonstrating that stem radial growth is dependent on short-term water availability (Camarero et al. 2010). Water deficit influences the whole plant physiology and reduces or prevents cell metabolism, thereby indirectly constraining plant growth (Desoto et al. 2011).

Fluctuation in stem diameter is mainly triggered by variations in the water status of the cambium cells and reflects the discrepancies in turgidity of cells in the cambial zone (Klepper et al. 1971). Abe & Nakai (1999) demonstrated that cells in and around the cambial zone easily lose their turgor if the tree is water stressed. The decrease in stem
diameter of the droughted samples can thus be attributed to the decrease in xylem water potential during drought, which result in the movement of apoplastic water around the living cells to the xylem, leading to a decrease in the cambium water content (Abe & Nakai 1999). This radial water movement results in a decrease in stem diameter.

Lateral thickening of the stem is the result of cell division and expansion in the cambial zone which continuously produces xylem and phloem (Wang 2020). Stem radial growth is restricted by water deficit (drought stress), through changes in tissue development (cell division and expansion) (Camarero et al. 2010). Drought stress causes the cessation of cambial activity and therefore no stem growth (Drew et al. 2009). Hence, stem growth results from the activity in the cambium. The cambium ensures the production and renewal of functional vascular elements through cell divisions which result in an increase in stem diameter (Catesson 1994).

Conclusions

Understanding the process of cambium development is highly complex and studying the 3D morphology of this tissue is extremely challenging due to its localization, since it is embedded between layers of other cell types. Therefore, the need to go beyond the general destructive microscopic investigation. It was demonstrated how X-ray μCT in combination with nano-CT can be used to provide new insights and explore the cambium at unprecedented resolution in 3D in living samples and excised tissue, respectively. X-ray CT proved to be useful for the visualization and quantification of the soft and fragile meristematic cells of the cambium and allowed investigation beyond the traditional static, 2D light micrographs.

Researchers should, nevertheless, be aware that X-ray CT also poses some challenges; due to the cost of X-ray CT, it is not feasible for large-scale investigations and is generally only used for laboratory-based proof-of-concept studies making use of a small number of samples. Image analysis can also be very time-consuming, depending on the size of the dataset and the type of segmentation; especially when manual intervention is required to delineate ROIs. There are also sample size limitations, which are dependent on the type of instrument. While X-ray CT provides clear advantages over destructive techniques, previous studies raised concerns about the damage caused to plants when exposed to high X-ray doses (Petruzzellis et al. 2018). Currently, there is no recommended X-ray limit for time-lapse studies, and experimental trials should be conducted to identify X-ray settings that impose minimal dosages while producing acceptable scan quality (Rawson et al. 2020). With time-lapse studies of living plants, X-ray exposure is a concern, and caution is thus advised, and unexposed controls are recommended (Rawson et al. 2020). Depending on the instrument type, scan settings, and type of plant, X-ray radiation may have adverse effects on the sample. However, in our study, we observed no X-ray radiation effects (such as growth impairment) at the specific levels applied.

With this new X-ray CT approach, it was possible to monitor cambium development non-destructively over time at the same location in the tree stem before and after the respective treatments. Non-destructive methods, such as X-ray CT, for 3D microstructural investigation of the cambium, will result in increased knowledge of the developmental dynamics of this tissue, offering a key framework for the examination of the cellular processes underpinning these events. Since X-ray CT is a growing technology in the field of plant and wood sciences, future technological advancements in either scanning instruments, increased computer capacity, or protocol optimization, will lead to potential further developments in xylogenesis investigations. The CT approach developed in this study provides a promising avenue to explore future plant climate growth relationships covering a variety of species, climatic conditions, and wood structures. Moreover, detailed 3D renderings of plant structures are suitable for building morphological models within dynamic modelling approaches (Dhondt et al. 2010) and providing 3D data for mathematical simulations (Koddenberg & Militz 2018). It is anticipated that X-ray CT will play a key role in future discoveries in plant biology, especially when combined with other high-resolution systems, for instance, laser capture microdissection (LCM), or other advanced visualization tools such as combined magnetic resonance imaging (MRI) and positron emission tomography (PET) (Jahnke et al. 2009).
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References


X-ray micro-CT imaging of cambium


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