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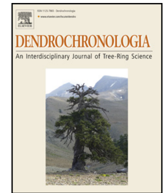

Applying methods of hard tissues preparation for wood anatomy: Imaging polished samples embedded in polymethylmethacrylate

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ABSTRACT

Cambial activity records short and long-term environmental signals in xylem anatomy, creating a permanent archive. Quantitative wood anatomy deciphers the relationship between cell structure and function in a spatiotemporal context. Obtaining high-resolution images of wood anatomical preparations is a critical stage in the process of decoding this information. Damage to cellular structures when sectioning by microtome is one of the main problems in the preparation of high-quality micro-sections. Cell damage leads to the occurrence of artifacts – most often related to broken cell walls – hindering the performance of image recognition programs, and increasing the time spent on the manual editing of images. In this work, we propose an alternative method to microtomy, based on embedding-polishing protocols established for hard tissue preparation. Wood samples are embedded in a transparent and non-reactive resin as polymethylmethacrylate (PMM) that is subsequently ground and polished. Being able to acquire images from the stained or unstained polished surfaces of the PMM-blocks and sections (thinner than 100 µm) by using a wide range of optical methods such as reflected polarizing microscopy, epifluorescence microscopy, bright-field microscopy with diffuse illumination and circularly polarizing microscopy. This embedding method improves the mechanical integrity and quality of wood anatomical preparations, eliminating the problem of broken cell walls. Furthermore, this technique allows the preparation and analysis of large tissue surfaces.

1. Introduction

Cambial activity records short- and long-term environmental signals in xylem anatomy, encoding information at inter- and intra-annual resolutions on the adaptation of plants to their environment (Arzac et al., 2018a, 2018b; Olano et al., 2013, 2012; Vaganov et al., 2011). Wood analysis using quantitative wood anatomy (QWA) techniques has significant potential to decrypt this information and reconstruct spatiotemporal environmental series based on the structural and functional characteristics of cells (Fonti et al., 2010).

Quantitative wood anatomy has been a research topic for a long time (see Speer, 2010); however its advance was hindered by the strenuous effort needed to obtain high sample sizes. The rapid evolution of digital imaging technology and image recognition programs (e.g., ROXAS, von Arx and Carrer, 2014) has changed this panorama and has accelerated the progress of QWA in recent times. The potential of QWA extends to the study of many parameters in the xylem, such as cell number (vessels and tracheids), cell size (e.g., lumen diameter and cell wall thickness), ray parenchyma features, and spatial arrangement of cells within the tree ring. Nevertheless, the accuracy and speed of QWA analysis are closely linked to the quality of wood anatomical preparations required for anatomical high-resolution images acquisition (von Arx et al., 2016).

Sectioning for wood anatomy analysis is commonly based on microtomy protocols (Gärtner and Schweingruber, 2013; Schweingruber and Poschlod, 2005), where the stability of the blade during cutting is crucial in order to obtain high-quality micro-sections of 10–15 µm thickness. However, when the blades cut structures with different density (as with lumen or wall dominated areas in conifers), it may occasionally fracture the cell wall (mainly in earlywood cells), creating artifacts that make difficult the automatic cell recognition, thereby increasing the time spent on manual editing. Different embedding techniques such as corn/rice starch (Schneider and Gärtner, 2013) or paraffin (Rossi et al., 2006) used before sectioning fills cell cavities and

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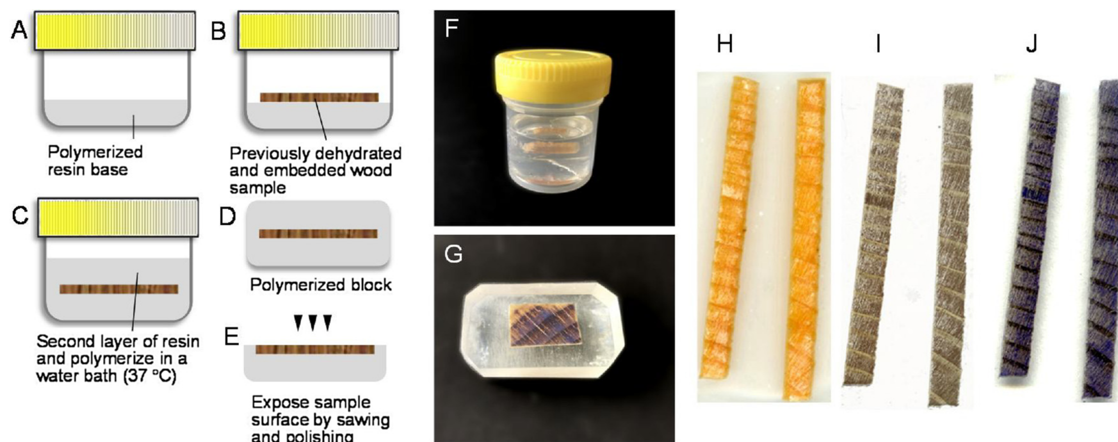

Fig. 1. Scheme for the preparation of the PMM-block (A–C); PPM-block surfaced by sawing or grinding and (D and E); polymerized PMM-block with the infiltrated sample in a polypropylene container together with polyethylene screw cap (F); trimmed PMM-block with the sample surface exposed and polished (G); sections of PMM-block glued to an acrylic slide (3mm-thick) (H); ground & polished sections (< 100 µm thick), unstained (I) and stained (J). Tissues in figures H–J are 45 × 4 mm.

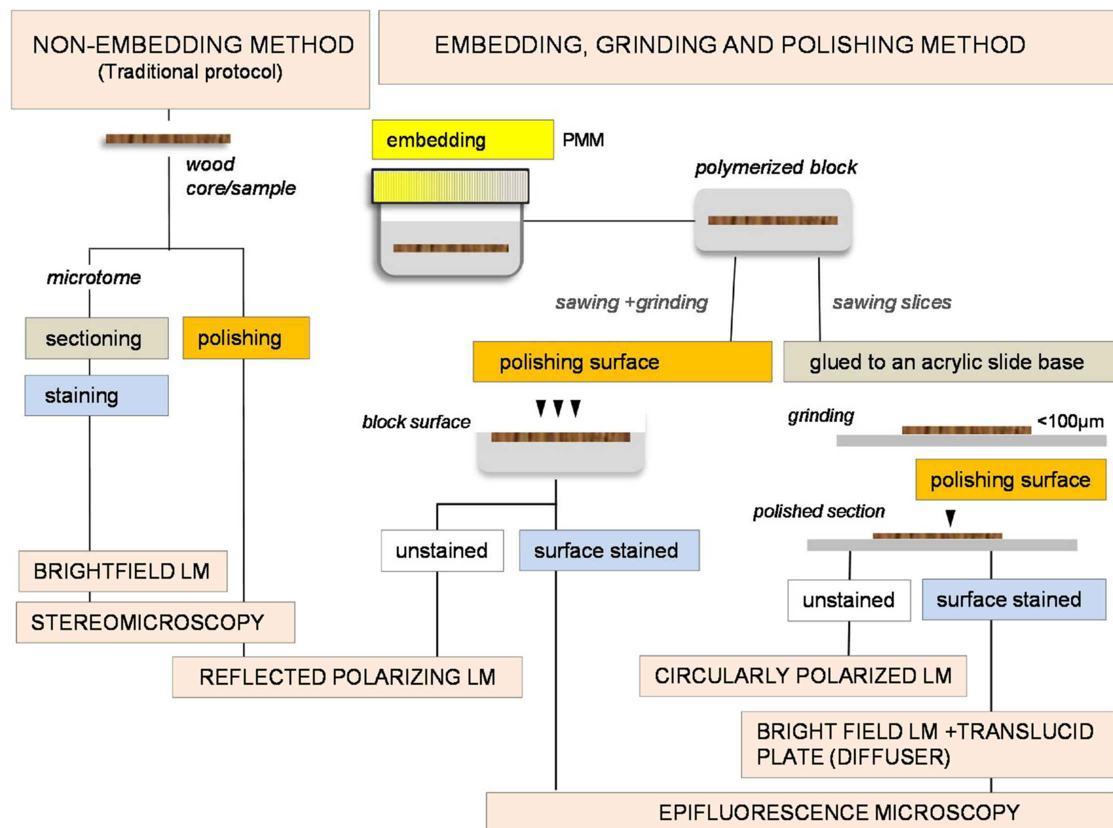

Fig. 2. Flowchart of preparation methods and optical microscopy strategies for imaging block surfaces and sections. The "non-embedding method" refers to the traditionally used protocol in the preparation of micro-sections for QWA, whereas the "embedding, grinding and polishing method" refers to the process described in detail this paper.

increases their resistance, improving cell structure stabilization and considerably decreasing the occurrence of these artifacts, leading to higher quality micro-sections. Although these methods help to improve the quality of micro-sections, the existence of differences between the density of the embedding substance and the wood material, the cutting speed and the clearance angle of the blade might still contribute to the occurrence of artifacts.

In this work, we propose an alternative to wood micro-sectioning based on the translation of techniques developed for hard tissue preparations to obtain a high-quality wood anatomical image. This technique is based upon the embedding of the wood sample in a transparent and non-reactive resin (polymethylmethacrylate, PMM), to then be ground and polished to a highly smooth surface (Bromage et al., 2018; Schenk et al., 1984) thereby allowing the observation by different illumination methods (e.g., reflected light, transmitted diffuse light and polarized light). Although the embedding of hard tissues has been used since the mid of the twentieth century (An et al., 2003; Schenk et al., 1984), as a technique for the study of different tissues (e.g., bones, teeth, fossils, seeds and paleobotanic material), it shares similar protocols with metallography techniques (Benedict, 2015; Jones and Rowe,

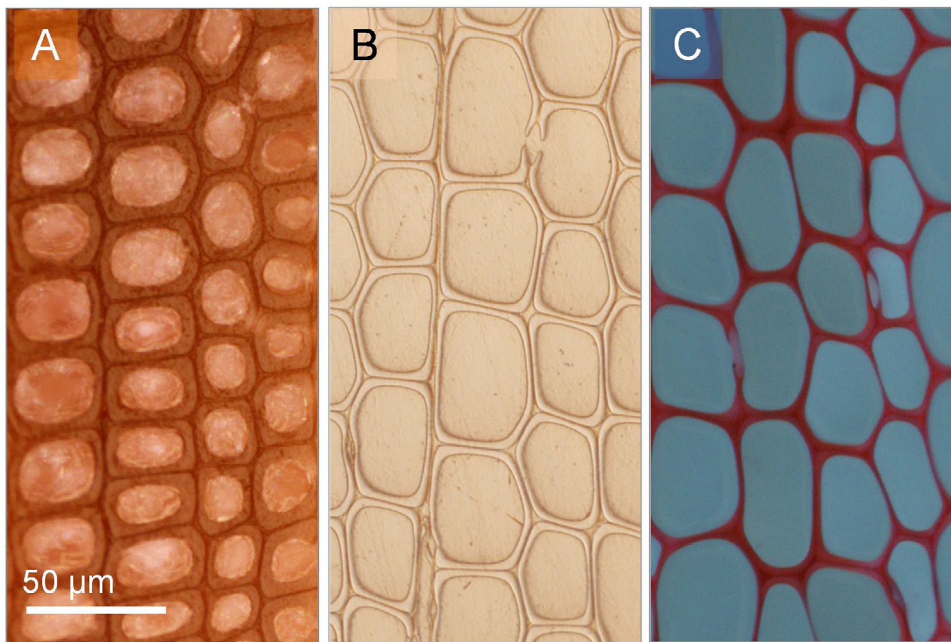

Fig. 3. Block surface imaging. *Pinus sylvestris* blocks polished surface photographed by reflected polarized light microscopy (A and B). Unstained and polished surfaces, non-embedded (A) and PMM-embedded (B) photographed by reflected polarizing microscopy; PMM-embedded and stained with safranin examined by epifluorescence microscopy using a Nikon cube filter V-2A (C).

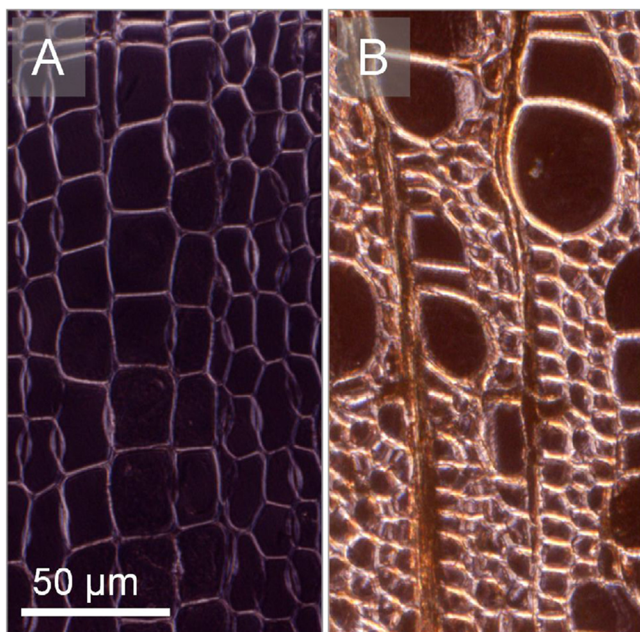

Fig. 4. Section imaging. Unstained polished thin-sections photographed under circularly polarized light. 10 µm thick *Pinus sylvestris* section (A) and 20 µm thick *Betula pendula* section (B).

1999). However, its application in wood anatomy is novel.

This new process outperforms conventional techniques by: (i) the replacement of microtome sectioning by the polishing of embedding tissue, facilitating the stabilization of cell walls inside the polymerized resin; (ii) the absence of cell wall breakage, avoiding the occurrence of the most common artifacts, which arise from the geometric distortion effect at the cutting edge by the microtome blade effective angle; and (iii) offering the ability to use the process with diverse kinds of wood materials of very different elastic and hardness properties, as well as varying sizes up to several centimeters long (20–25 cm, limited only by the size of the platen diameter of the grinder-polisher machine).

2. Wood material and proposed method

2.1. Wood material and sampling

Twenty dominant or codominant *Pinus sylvestris* L. and *Betula pendula* Roth trees were sampled during September 2016 in a forest-steppe zone in Southern Siberia near Shira (Khakassia Republic, Russia). This region is characterized by moderate continental climate conditions. Two cores per tree were taken at breast height from the stem with a 5 mm diameter increment borer, labeled and taken to the laboratory where they were air dried. After visual cross-dating, tree-ring width (RW) was measured to the nearest 0.001 mm using a LINTAB-5 sliding stage micrometer interfaced with the specialized package TSAP Win (RINNTECH, Heidelberg, Germany). Crossdating accuracy was checked using the software COFECHA (Grissino-Mayer, 2001). The average age of *P. sylvestris* and *B. pendula* trees was 125 ± 32 and 90 ± 19 (mean ± SD) years respectively, and the mean tree-ring width was 1.46 ± 0.4 mm (mean ± SD) for *P. sylvestris* and 1.19 ± 0.3 mm (mean ± SD) for *B. pendula*. Further, and in order to standardize the infiltration time for the protocol, *P. sylvestris* sections of 22 × 11 mm cut in progressive thickness (from 2 to 10 mm) were used.

2.2. Embedding grinding and polishing of blocks and sections

The embedding of the wood sample consists first in the dehydration of the sample with an acetone solution of increasing concentration in three stages: 75%, 95% & 100% for a duration of 60 min each stage, which would preferably take place at 4 °C. Then, the dehydrated wood sample is submerged in a mixture of methyl methacrylate (MMA) monomer (Sigma-Aldrich, Ref. M55909) and dibutyl phthalate (a plasticizer, 10:1 v/v; Sigma-Aldrich, Ref. 524980) catalyzed with benzoyl peroxide (2 g/100 ml MMA solution; Fluka, Ref. 33581). The duration of this embedding (infiltration) process will depend on the sample characteristics (i.e., thickness, density and porosity) and will ordinarily range from one-three days up to three weeks, being of shorter duration for samples taken from living trees (with empty tracheids/vessels) than with fossil wood with mineralized cells. During this process, samples should be subjected to continuous agitation at a speed of 80–100 rpm in an orbital shaker (Ovan, Spain) and should also be sheltered from intense light and high temperature to avoid the polymerization of PMM.

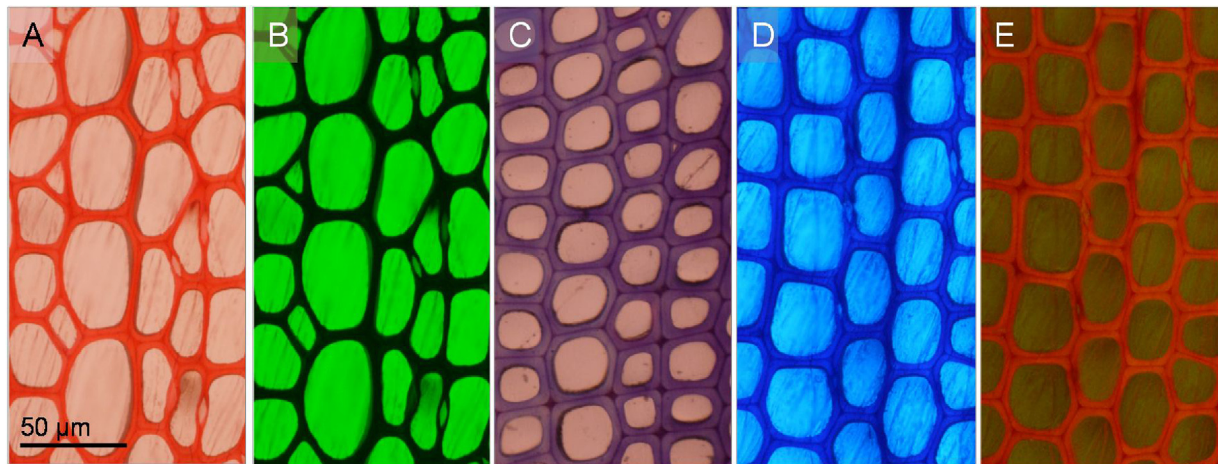

Fig. 5. *Pinus sylvestris* section imaging. Photographed by using bright-field microscopy with a diffuser (a translucent plate located just below the sample, A–C) and by epifluorescence microscopy (D and E). Polished section (30 µm thick) of pine which has been stained on surface with safranin (A); the same field as (A) but with the insertion of a 546 nm bandpass interference filter in the optical path to obtain a monochromatic image (B); polished section (50 µm thick) of pine stained on its surface with toluidine blue (C); polished section (30 µm thick) of pine surface stained with safranin and examined using a Nikon cube filter V-2A (D); the same field examined with a Nikon cube filter cube B-2A (E).

After embedding, a resin block containing the infiltrated wood sample is made (Fig. 1). First, a PMM base is created by pouring MMA (around 10–100 ml of MMA, depending on the sample size) at the bottom of a polypropylene container with polyethylene security screw cap. This layer of resin is polymerized by placing the capped container close — at a distance of 30 cm — to a black light source (Philips TL-D 36W BLB Blacklight Blue, Netherlands) for a period of one or two days until the resin hardens (Fig. 1A). The base must be prepared in advance, and for this purpose, surplus MMA left over from the wood embedding can be used. Once the base is polymerized, the infiltrated wood sample is placed upon it and covered by a second layer of MMA (providing a layer of 10 mm in thickness above the sample surface, Fig. 1B and C). The final polymerization of the PMM-block is then carried in a water bath at a temperature no higher than 37 °C for one to two days. Slow polymerization avoids the overheating of the resin caused by the exothermic reaction from the polymerizing agent that could lead to the production of bubbles or to resin deformation. The MMA solution can be pre-polymerized to a syrup consistency and stored in a flammable-safe refrigerator at −20 °C until use.

When the resulting PPM-block with the infiltrated sample is completely hardened by polymerization (Fig. 1D–G), it may be processed in two different ways: (i) surfaced of wood tissue on the PPM-block surface (Fig. 1G) or (ii) sectioning the PPM-block in thick sections by sawing (no more than 1 mm thick, Fig. 1H–J). Exposure of the PPM-block surface could be achieved by sawing the resin closely to the sample with the use of a slow speed precision saw (Isomet 100, Buehler; Lake Bluff, IL, USA), lubricated and refrigerated by water; or alternatively, by grinding the block surface with a grinder-polisher machine (Ecomet 250, Buehler). The sectioning of the surfaced PMM-block requires that the block be glued to an acrylic slide (3 mm thick) with cyanoacrylate glue (Loctite, Germany). Acrylic slides could be either “ready to use” microscope plastic slides (Exakt Technologies, Germany), or “cut to size” from acrylic sheets (Policril, IRPEN, Spain). To avoid the occurrence of air bubbles between the PMM-block and the acrylic slide, a weight or clamping device can be used for 24 h to ensure the release of air. Once the glue has hardened, the PMM-block is sawed using 0.5–0.9 mm diamond blades (IsometLC, Buehler), resulting in sections of 0.7–1 mm thick, that are then further thinned by grinding to result in a section of less than 100 µm thick. The thickness of the sections can be checked by a micrometer (considering the thickness of the slide and approximately 50 µm of the glue layer).

As final step prior to staining and imaging, either the PPM-block or the section surfaces must be ground to remove the marks of the saw applying silicon carbide (SIC) abrasive discs (CarbiMet SIC grinding discs, Buehler) in successively finer steps (P240, P400, P800, P1000, P2500 grit) with a grinder-polishing machine at 60–250 rpm (the finer the grit, slower the rotation speed). After using P2500 grit in the final stage of grinding, the sample might then be surfaced by polishing using 1 µm alumina paste (Micropolish II, Buehler) or 1 µm diamond suspensions (MetaDi, Buehler), with the final polishing step performed by hand using polishing cloth (Microcloth, Buehler) or cotton. This step must be repeated until the xylem structures are clearly visible under microscopic magnification.

2.3. *Staining and microscopy techniques*

Both the PPM-block surface and the sections allow surface staining (Bromage et al., 2018; Schenk et al., 1984) following different techniques, including the traditional protocols employed in wood anatomy (e.g., Safranin and Alcian blue solution; Schweingruber and Poschlod, 2005). As the tissues embedded in epoxy or acrylic plastics are usually poorly stained, it is advisable to heat the staining solution and adjust the timing of the staining process with reference to the sample, to improve the quality of the staining. In contrast to non-embedded sections, the staining solutions merely penetrate 5–10 µm below the surface, creating the effect of an optical section (Bromage et al., 2018; Schenk et al., 1984).

The choice between PPM-block surface or sections will depend on the microscopy technique to be used (see flowchart for the range of options, Fig. 2) and the specific research objectives. Thus, polished blocks can be imaged by a high resolution-scanner or observed under reflected light microscopy and after staining a thin superficial layer (of a few microns in depth) with a fluorochrome (e.g., safranin O, E_{max} 563 nm), by epifluorescence microscopy (using a Nikon cube filter V-2A). While polished section (less than 100 µm thick) glued to an acrylic base slide (Fig. 1H–J), can also be observed unstained under circularly polarizing microscopy or by bright-field microscopy with a diffuser (a 546 nm bandpass interference filter in the optical path to obtain a monochromatic image may be employed; Edmund Optics Ltd, UK). Alternatively, imaging can take place by way of epifluorescence microscopy after staining, using either a Nikon cube filter V-2A or B-2A (see Fig. 2 for further details). Images illustrating the different optical methods in this work were captured with a Nikon DMX1200F camera mounted on a Nikon Optiphot-2 light microscope.

3. Results and discussion

The application of methods from hard tissues in wood anatomy has produced high-quality anatomical preparations, from both the PMM-block surface and the block sections, without the occurrence of the most common artifacts often encountered when sectioning with microtomes. In our general use of this method, we have observed that the preparation of wood cores is technically more accessible than in mineralized tissues, with shorter infiltration times and absence of surface cracks while sectioning. It has been shown that the embedding of wood samples with PMM results in the protection of the fragile structures within the wood sample during sawing, grinding and polishing, thereby providing stability to edges of materials, avoiding the fragmentation of the sample. This has led to an increase in the quality of the images captured.

A good infiltration of the sample is critical to obtain high-quality preparations. During infiltration, the wood sample floats on the MMA solution to then become wholly submerged as a result of the wood pores filling with resin (e.g., a 45 × 4 × 2 mm wood sample would be wholly submerged after one day of infiltration). Even so, it is recommended to leave the sample in the solution several more days (1–3 weeks) so that the MMA also penetrates the micropores of the cell walls. However, the work may be organized in such a way so as to permit the simultaneous performance of different tasks, speeding the timing of the whole method. For example, while some samples are being infiltrated, other (previously infiltrated and polymerized) could be ground and polished or imaged.

When comparing images of unstained block surfaces by using reflected polarized microscopy prior to and after embedding (both ground and polished), the improvement in the image quality of embedded sample becomes evident (Fig. 3A and B), highlighting the definition of cell structures after embedding (Fig. 3B). Additionally, the block surface can be stained with a fluorochrome such as safranin (widely used in traditional staining protocols for wood anatomy imaging, and resulting in lignified cells becoming red), to then be observed by epifluorescence microscopy (Fig. 3C). However, the quality of epifluorescence imaging highly depends on the cell walls affinity to the fluorochrome and the optic quality of the microscope.

Despite the possible limitation in the use of surface staining directly upon PMM-block surfaces, a direct examination with more sophisticated imaging methods (e.g., using confocal laser microscopy) could be employed. On the other hand, work with an unstained PMM-block surface might be helpful in studies other than wood anatomy, and could be applicable to other fields such as dendroarchaeology, improving the handling of delicate wood samples as subfossil wood, accelerating the crossdating and tree-ring width measurements with a reduced risk of damage to the sample.

Although the results of PMM-block surface imaging are promising, we think that images from block sections (of less than 100 μm thick) resulted in higher contrast (Figs. 4 and 5). The contrast of tracheids and vessels in unstained block sections of *P. sylvestris* (Fig. 4A) and vessels in *B. pendula* (Fig. 4B), was enhanced by the use of circularly polarizing microscopy. Moreover, after surface staining, sections can be observed either by bright-field microscopy with a diffuser (Fig. 5A–C) or by epifluorescence microscopy (Fig. 5D and E). The combination of surface staining and these microscopy methods, produce images in which only the superficially stained layer is visible, thereby creating an effect of optical sectioning (Bromage et al., 2018; Schenk et al., 1984).

As a consequence of the high structural stability conferred by the embedding, the resulting images are of high-resolution and excluded from the occurrence of mechanical artifacts, showing a complete absence of broken structures, facilitating the subsequent automatic image analysis. Even though we were able to produce high-quality images for PMM-block surface and sections, whether one method is to be preferred over the other will depend on the specific research objectives: thus block sections offer highest contrast images, whereas block surfaces would permit a shorter time for preparation.

The significant advantages of this procedure in comparison to existing methods include: (i) avoidance of tissue damage by microtome blade, and mechanical fracture, which are primary source of artifacts in wood anatomy sectioning; (ii) it does not necessarily involve the preparation of classical micro-sections, allowing the use of epi-illumination microscopy of the polished block surface; while still maintaining the possibility of using staining techniques (e.g. safranin, Astra blue, toluidine blue, etc.); (iii) preparation of PMM-block sections are able to be observed by bright-field microscopy, polarization microscopy, or epifluorescence microscopy. After images are captured, it is possible to follow the standard protocol for quantitative wood anatomy analysis (e.g., von Arx et al., 2016). On the other hand, the main disadvantages of the proposed method when compared to existing protocols are: (i) the time required for the wood infiltration, and (ii) serial sectioning (as used with microtomy) is not possible, since each block section required to be at least a 1 mm thick (usually two 1 mm thick sections can be obtained from a 5 mm wood core). Nevertheless, these inconveniences are more than compensated by the high quality of the obtained images, the ability to prepare larger surfaces, and the possibility of indefinite storage of the blocks and sections.

4. Conclusions

There are several methods to produce wood sections for xylem anatomy studies; however, most of them produce artifacts in the cell structures that present difficulties achieving the wholly automatic analysis of samples. The method we are proposing in this paper has produced high-quality anatomical preparations with an absence of artifacts in the cellular structure. High-resolution images can be obtained directly from polished resin blocks by using epi-illumination of a large tissue surface, without the need for micro-section preparation. Although, the resin block can also be sectioned to be analyzed with different microscopy techniques (e.g., circularly polarized light) increasing the contrast of cellular structures. Additionally, this method might be used with different wood material (e.g., core sections, whole cores, stem sections, sub-fossil and fossil wood) with a sample size only limited by the size of the platen diameter of the grinder-polisher machine (usually of 203–254 mm).

Besides the excellent results obtained by using this method for wood anatomy preparations, we continue working on the improvement of the technique so that it may be employed with different species and sample dimensions in order to provide more detailed information (e.g., duration of wood infiltration process for different species). However, we consider that this protocol could be a breakthrough for the studies on quantitative wood anatomy, reducing the time required for image analysis. We also think that polished PPM-block surfaces may be potentially useful in fields like dendroarchaeology. Finally, we would like to encourage other existing wood anatomy laboratories (according to each of their specific research objectives) to try variations of the presented parameters of the method described, in an attempt to optimize its efficiencies.

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