Dendrochronologia 35 (2015) 87–90

Contents lists available at ScienceDirect

Dendrochronologia

journal homepage: www.elsevier.com/locate/dendro

TECHNICAL NOTE

Using steam to reduce artifacts in micro sections prepared with corn starch

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ARTICLE INFO

Article history:
Received 23 February 2015
Received in revised form 7 July 2015
Accepted 8 July 2015
Available online 23 July 2015

Keywords:
Wood anatomy
Corn starch
Microtome
Artifacts
Cell lumen
Micro section preparation
Non-Newtonian fluid

ABSTRACT

Preparation of micro sections to measure cell wall thickness and lumen diameter is a widely used method in the fields of dendroecology, dendroclimatology and tree physiology. Efficient sample preparation and image analysis are critical for studies with long time series and large sample sizes. Recently, there have been substantial improvements in micro section preparation techniques, including a corn starch-based non-Newtonian fluid treatment. This method reduces cell wall damage during cutting with a microtome, which in turn decreases artifacts during image analysis. Although this procedure does in fact improve sample quality, we found starch grains sometimes to be difficult to remove and to cause artifacts during image analysis. This technical note outlines a simple, fast and effective steam treatment that causes starch gelatinization and a reduction in the number of starch grain artifacts.

1. Introduction

Measuring cell wall thickness and lumen diameter in xylem cells requires the preparation of micro sections, typically 10–20 μm thick. Measurements of these microscopic structures are important in dendroecology, dendroclimatology, and tree physiology research (Fonti et al., 2010). In tree physiology, cell wall thickness and cell lumen diameter are closely correlated with cavitation resistance (Hacke et al., 2001), and can therefore be used to infer drought resistance of tree species or populations. In dendroecology, this information enables the assessment of tree species' abilities to acclimate to changing environmental conditions and their overall suitability to increased drought severity under climate change (Bryukhanova and Fonti, 2013). In dendroclimatology, time series of cell parameters can serve as proxies for climate reconstructions (Wimmer, 2002). The proportion of cell walls to cell lumen is closely related to wood density (Wassenberg et al., 2014), which is an important proxy for summer temperatures (Briffa et al., 2004).

Preparing micro sections from large stem diameters, representing long dendrochronological time series is a challenging and labor-intensive task, however. These obstacles have led to the development of alternative preparation techniques, including the use of high-precision diamond-fly-cutters for reflected light microscopy (Spiecker et al., 2000), but also to advances in microtome techniques. Sliding microtomes using common cutter blades have emerged as being capable of producing high-quality micro sections, while also being much easier to maintain than earlier alternatives (Gärtner et al., 2014; 2015b). These instruments are intended to develop long anatomical time series, with a single micro section covering multiple tree-rings (Gärtner et al., 2015a, b).

One remaining issue, however, is that secondary cell walls of tracheids are sensitive to pressure induced by the cutting blade, often causing them to be pushed into the cell lumen (Schneider and Gärtner, 2013). Such damage to the cell structure can create problems for automatic image analysis, which are time-consuming to manually remove. Until recently, this problem was addressed by embedding the sample in paraffin wax, for example (Feder and O'Brien, 1968). However, this procedure is time-consuming and the wax must be removed before staining (Schneider and Gärtner, 2013).

As an alternative method to reduce cutting artifacts in the development of long time series of anatomical properties in tree-rings, Schneider and Gärtner (2013) proposed using a non-Newtonian fluid to stabilize the cell structure. A fluid mixture of corn starch and water is applied to the surface of the sample prior to the microtome cut. The effectiveness comes from the fluid's non-Newtonian properties. While being applied with a brush, the mixture is liquid, and

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http://dx.doi.org/10.1016/j.dendro.2015.07.003
1125-7865/

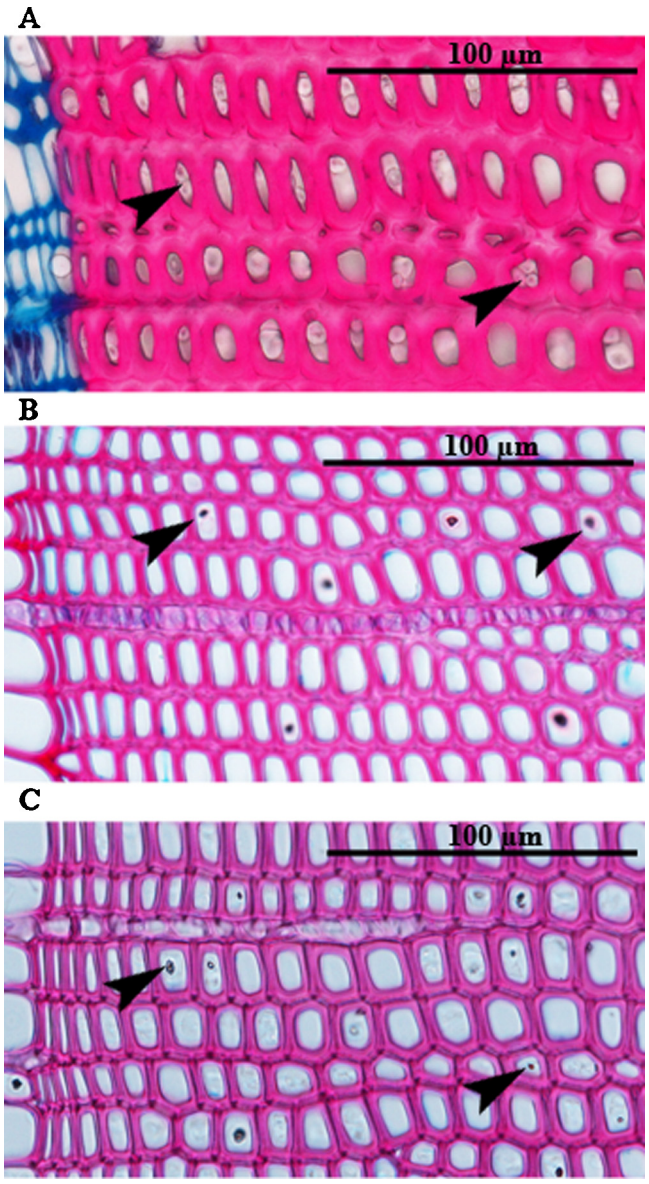

Fig. 1. Three examples of micro sections of stained xylem cells made from lodgepole pine samples (*Pinus contorta* Douglas ex Loudon). Two black arrows per panel highlight examples of starch grains that remained after rinsing, staining and dehydration. The center of the grains appears darker, especially in panels B and C. The starch grains sometimes absorbed Safranin during the staining stage, as seen in Panel B.

flows into the empty cell lumen. Under pressure, the starch grains form a solid structure, stabilizing the cell walls. As a result, artifacts due to deformed cell walls can be mitigated. We applied this method to lodgepole pine (*Pinus contorta* Douglas ex Loudon) samples. The corn starch mixture worked as expected and did indeed minimize the aforementioned artifacts. We found the corn starch solution efficient, easy to use, and to improve accuracy of the image analysis.

However, during our sample preparation, we sometimes found it difficult to completely wash the corn starch grains out of the cells. We found that the starch grains were more easily removed from the larger earlywood cells, leading to good contrast between cell walls and cell lumen, but the removal could be more problematic in the narrower latewood cells. Here, the grains were held in place by friction and adhesion, and even long and intensive rinsing could not always overcome these forces. Examples of remaining starch grains are shown in Fig. 1 and become especially visible under higher magnification. The dark, crystalline centers of the grains are especially visible. Sometimes, it appears that the grains also take up Safranin stain, which could lead to poorer contrast in the image analysis stage. We found these grains to interfere with the automatic detection of WinCELL Version 2013a (Regent Instruments Canada Inc., 2013), despite trying to eliminate the starch by setting appropriate filters. Often, the grains had to be removed by manual image manipulation. Although more sophisticated tools for image manipulation may better filter the remaining starch grains, we found their complete removal, without altering other structures in the image, to be laborious.

Starch gelatinization by inducing heat is a common procedure in the food industry (Bauer and Knorr, 2005). By adding water and applying heat, the intermolecular bonds of the starch are broken and more water molecules can link to hydrogen bonding sites. Therefore, the starch grains change their crystalline structures to a gelatinous, viscous solution (Zobel, 1984). This reaction may be useful when starch grains need to be completely removed from the micro sections. In this technical note, we describe how a steaming procedure can be used to dissolve and wash out the starch before staining. This presents an additional step to the methodology proposed by Schneider and Gärtner (2013). This procedure could be useful, where starch grains remain abundant after rinsing and interfere with image analysis, and where image processing to remove starch grains may cause other inaccuracies.

2. Materials and methods

Stem disks of lodgepole pine were collected in a 40-year-old provenance trial located in the vicinity of Kamloops, British Columbia, Canada, at an elevation of approximately 1400 m a.s.l. (Illingworth, 1978). The air-dry disks were already sanded to allow for measuring of tree-ring width and latewood proportions. In preparation of the wood anatomical measurements, 1 cm wide, diagonal cross-sections were marked on the surface of the disks. Subsequently, the cross sections were sawed from the disks using a standard circular saw. The resulting samples were then trimmed to a thickness of 1.5 cm with a small circular saw. The saw cuts were also made perpendicular to the fibers, to ensure a vertical orientation of the tracheids (Gärtner and Schweingruber, 2013). These samples were split at the pith, yielding two pieces per disk, between 4 cm and 7 cm long.

Prior to cutting micro sections with the microtome, the corn starch solution was prepared as a mixture of corn starch, water and glycerol in the ratio of 10 g:8 ml:7 g (Schneider and Gärtner, 2013). Following this step, the sample was placed in the object holder of a GSL1-microtome (Gärtner et al., 2014), was softened by applying water with a brush, and then transverse micro sections were cut. The first few cuts were needed to remove the top layer of fibers damaged by the saw. We then changed the blade (A170, NT-Cutter, Japan) and applied the corn starch solution with a brush to the surface of the sample (Schneider and Gärtner, 2013; Gärtner et al., 2015b). We aimed to produce sections of a thickness of approximately 10–20 µm, which were subsequently placed on a glass slide. To wash out the starch grains, the section was rinsed for 4 min by pumping water through the sample with a pipette (Gärtner and Schweingruber, 2013) and subsequently stained as described below.

Our simple laboratory set-up for steaming the micro sections is illustrated in Fig. 2. A lab grade, 2.5 l stainless steel container is placed on a hot plate and is filled with about 500 ml of distilled water. A metal rack is placed in the bottom of the container so that the top sits above water level. It is important that the glass slide remains well above water to prevent the sample from being washed off the slide and damaged by the boiling water. A lid is