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TECHNICAL ARTICLE: PART OF A SPECIAL ISSUE ON ROOT BIOLOGY

An easy method for cutting and fluorescent staining of thin roots

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• *Background and Aims* Cutting plant material is essential for observing internal structures and may be difficult for various reasons. Most fixation agents such as aldehydes, as well as embedding resins, do not allow subsequent use of fluorescent staining and make material too soft to make good-quality hand-sections. Moreover, cutting thin roots can be very difficult and time consuming. A new, fast and effective method to provide good-quality sections and fluorescent staining of fresh or fixed root samples, including those of very thin roots (such as *Arabidopsis* or *Noccaea*), is described here.
• *Methods* To overcome the above-mentioned difficulties the following procedure is proposed: fixation in methanol (when fresh material cannot be used) followed by *en bloc* staining with toluidine blue, embedding in 6 % agarose, preparation of free-hand sections of embedded material, staining with fluorescent dye, and observation in a microscope under UV light.
• *Key Results* Despite eventual slight deformation of primary cell walls (depending on the species and root developmental stage), this method allows effective observation of different structures such as ontogenetic changes of cells along the root axis, e.g. development of xylem elements, deposition of Casparian bands and suberin lamellae in endodermis or exodermis or peri-endodermal thickenings in *Noccaea* roots.
• *Conclusions* This method provides good-quality sections and allows relatively rapid detection of cell-wall modifications. Also important is the possibility of using this method for free-hand cutting of extremely thin roots such as those of *Arabidopsis*.

Key words: *Allium cepa*, *Arabidopsis thaliana*, *Brassica napus*, hand sections, endodermis, exodermis, fluorescence microscopy, *Noccaea caerulescens*, suberin lamellae, thin roots, *Triticum aestivum*, *Zea mays*.

INTRODUCTION

Cutting living plant tissues often gives high quality histological sections providing an adequate method for observation of their internal structure (e.g. Seago *et al.*, 2000; Lux *et al.*, 2004, 2005, 2011; Soukup *et al.*, 2006; Mesjasz-Przybyłowicz *et al.*, 2007; Zelko *et al.*, 2008; Vaculík *et al.*, 2009). However, in practice, a large number of samples often requires processing and, as fresh plant material cannot be preserved for long time, fixation and storage of the plant material is the solution often adopted before microscopic evaluation of the plant tissues. The usual consequence of current fixation procedures is an excessive softening or shrinking of the plant tissue, resulting in very deformed free-hand sections and, in case of aldehydes, also a chemical alteration of the cell walls resulting in strong autofluorescence under UV light which often does not allow fluorescent staining and observation of target objects. In previous work with maize (Redjala *et al.*, 2011), we successfully tested methanol fixation which preserves root elasticity and allows fluorescent detection of cell wall modification. In the case of thin roots, cutting is very difficult and often requires time-consuming embedding and use of various expensive devices such as (cryo-) microtomes or vibratomes. Moreover use of various embedding resins does not allow the penetration of many dyes, including those for the detection of lignin and suberin. Although the methods using whole-mount samples (Lux *et al.*, 2005) can be used, these methods have several limitations which often can be overcome only by making cross-sections.

The work presented here is aimed to improve existing methods for an effective detection of cell-wall modification also in thin roots. Free-hand cutting of these objects is very difficult. One aim was the use of appropriate fixation to store and process a huge number of samples and another one was to find an easy way of free-hand cutting and staining of the sections of thin roots.

MATERIALS AND METHODS

Plant material

Roots of various species were used for the study: *Arabidopsis thaliana* roots cultivated on agar plates; main and lateral roots

of *Noccaea caerulescens* cultivated in hydroponics and in aeroponics; roots of maize (*Zea mays*) grown in aeroponics, hydroponics and soil; main roots of Indian mustard (*Brassica juncea*) grown in hydroponics; seminal roots of barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) cultivated on filter paper; and onion (*Allium cepa*) adventitious roots grown in unaerated hydroponics. Before fixation the plant roots were carefully excavated (when soil grown) and washed with water.

Fixation and storage

Plant roots were submerged in undiluted methanol (≥99 %, for synthesis; Carl Roth, Germany) and stored at 4 °C. Samples stored for periods of several hours up to 1 year were used.

Pre-staining

To facilitate manipulation with very thin roots and for simplifying eventual subsequent Casparian band detection, we used pre-staining with toluidine blue. Roots from methanol were put into water for approx. 1 min and then into 0·25 % (w/v) solution of toluidine blue for 0·5–3 min depending on the root thickness. Thereafter the roots were washed in distilled water to remove excessive stain. In some cases the non-target roots (e.g. adventitious roots and lateral roots of various orders) were removed to make the observation easier.

Embedding

Pre-stained and rinsed roots were placed into a mould. A mould can be easily prepared from aluminium foil to the following dimensions: height about 10 mm, width about 10–15 mm (depending on the number of roots embedded, the width should not exceed the width of the microscope slide) and the length depending on the size of the sample. Up to three roots were placed into one mould. Embedding itself was in a 6 % solution of agarose (agarose NEEO ultra quality; Carl Roth, Germany) at the temperature near the point of solidification (approx. 45 °C). Since the solution was very viscous, it was necessary to prepare approximately double the volume of the mould. Before pouring the agarose solution, roots were carefully positioned to be parallel to the longitudinal axis of the mould and aligned according to their apices to simplify the measurements. Agarose solution was poured into the mould to cover the roots in a gel layer 7–10 mm thick and left to solidify at room temperature. After this step, the roots could be cut; or, if necessary, they could be stored (at 4 °C, in water) up to 3 d before cutting.

In certain species (e.g. *A. thaliana*) embedding may cause collapse of peripheral cell layers or even whole roots. To prevent this, it is recommended to make a two-step embedding. In the first step, the agarose solution is poured along the root which is hanging (e.g. held in tweezers), making a thin agarose layer around the root. After solidifying, this layer provides mechanical support which prevents the collapse. The second step consists in placing the agarose-covered root(s) into the mould and finishing the embedding as previously described. Another modification of the embedding procedure reducing cell-wall deformation is to use less-concentrated agarose (e.g. 4 %) in the first or both steps of the procedure.

Sectioning

After removing the mould and eventual excessive agarose around the roots, samples were cut using razor blades (sharp blades are especially important when making sections of thin roots). Roots were cut together with agarose, the embedding medium providing mechanical support. Agarose also helped for identification of root segments since it was possible to carve marks (e.g. letters, numbers) on its surface. Asymetrical trapezoids were the ideal shapes for the agarose sections, allowing us to identify samples easily in cases when several root samples were embedded and cut at the same time.

The cross-sections aimed for fluorescence microscopy do not have to be very thin. More important is the angle, which should be perpendicular to the root axis. Thickness of these sections can reach up to 1 mm and even more when an inverted microscope is used. However, sections about 0·5 mm thick were usually prepared. Sections were placed into a drop of water on the slide. To achieve higher quality sections, a vibratome can be used and/or the agarose surrounding the section can be manually removed on the microscope slide using a preparation needle.

This method also allows longitudinal sections, but this is not feasible in the case of thin roots.

Staining

To detect Casparian bands the method of Brundrett *et al.* (1988) was used after modification. The first step was staining by berberine 0·01 % (w/v) for 1 h. Usually, the aniline blue counterstaining was not necessary, since roots were pre-stained with toluidine blue. The next step was rinsing the sections in water and mounting them in a solution of $FeCl_3$ 0·1 g L^{-1} in 50 % (v/v) glycerol. When visualization is poor, this procedure can be modified using a more concentrated solution of berberine. When there is excessive staining of the agarose surrounding the section (which may interfere with the visualization of exodermal Casparian bands), the agarose can be removed manually.

To detect suberin lamellae, the procedure of Brundrett *et al.* (1991) was followed.

Photography

Sections were observed using the microscopes associated with digital cameras (Optiphot 2 Nikon and a D1 digital camera Nikon; inverted microscope DMI3000 B, Leica and digital camera DFC 295, Leica; Axioskop 2 plus, Zeiss and digital camera DP72, Olympus). Utraviolet illumination was used for fluorescence microscopy, with excitation filter TBP 400 nm + 495 nm + 570 nm, beam splitter TFT 410 nm + 505 nm + 585 nm, and emission filter TBP 460 nm + 530 nm + 610 nm.

RESULTS

Cross-sections of material prepared by the new method were relatively easy to make, since thickness is not a limiting factor when epifluorescence is used for viewing. However,

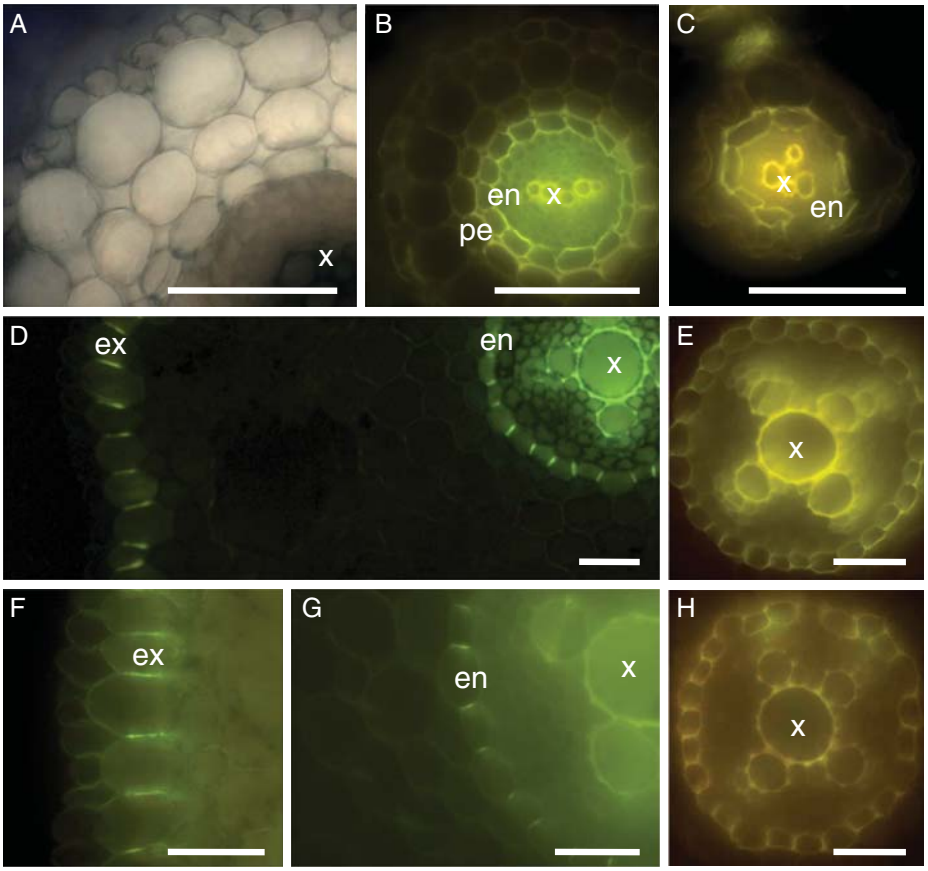

FIG. 1. Free-hand cross sections obtained from agarose embedded roots of *Brassica juncea* (A), *Noccaea caerulescens* (B), *Arabidopsis thaliana* (C) and *Allium cepa* (D–H). Samples of fixed (A–E) and fresh roots (F–H) were pre-stained with toluidine blue, cut and viewed with bright-field (A) and fluorescence microscopy (B–H). Visualization of Casparian bands (D, F, G) was obtained with berberine-aniline blue staining and visualization of suberin lamellae (B, C, E, H) with Fluorol yellow 088. Fixation did not influence fluorescent staining and provided good-quality sections suitable for observation of endodermis (B–H), exodermis (D, F), peri-endodermal thickenings (B) and xylem elements. Abbreviations: en, endodermis; ex, exodermis; pe, peri-endodermal thickenings; x, xylem. Scale bars = 50 μm.

also using bright-field microscopy, good-quality pictures can be obtained (Fig. 1A). Time needed to obtain the data about the development of a particular root structure (e.g. apoplastic barrier) was notably shorter when processing embedded roots compared with non-embedded ones. The time-saving is more notable in the case of thin roots, e.g. with a diameter smaller than 150 μm (Fig. 1A–C). Methanol fixation did not influence fluorescent staining of root samples (Fig. 1B–E) when compared with fresh roots (Fig. 1F–H). In some cases we observed deformation of cell shape (Fig. 1C), which was more frequent in fixed samples compared with fresh ones. No deformations were observed in xylem elements. To obtain optimal staining of various root sections (e.g. from different plant species, age or way of cultivation) the staining the procedure was adjusted for each case by changing the time of staining or eventually diluting the fluorescent dyes.

DISCUSSION

This fast and low-cost method was designed for examining the development of root barriers in fixed root samples. It provides good-quality sections and is preferable in cases when the method for clearing tissues for observing whole-mount samples in not useful (e.g. for observing xylem element development or when lignified or suberized cell layer/s such as exodermis or the peri-endodermal layer, which may be hiding deeper localized tissues, are present). This method can be a cheaper alternative to optical sectioning using laser scanning confocal microscopy and, since it enables cutting more roots at the same time, it can be considered as a relatively rapid method.

Besides the already mentioned advantages of this simple fixation, there is also a possibility of combining it with a clearing and staining method that allows observation of whole-mount samples (Lux *et al.*, 2005) as well as lignin detection using phloroglucinol. For the fixation of samples, 70 % ethanol can also be used (Meyer *et al.*, 2009); however, in the case of soft and thin roots, better results were obtained with methanol fixation which preserves the cell wall elasticity better than ethanol. Neinhuis and Edelmann (1996) used methanol to fix plant samples for scanning electron microscopy. These authors reported that methanol instantly fixes the elastically extended cell walls. Owing to this instant fixation, shrinking of the specimens is prevented, resulting in an improved preservation of cell dimensions comparable to *in vivo* conditions.

Methanol is widely used in immunohisto- and immunocytochemistry since it causes both fixation of the cell and permeabilization of the cell membranes. Methanol fixation is also used to measure viscoelastic properties of root cell walls (Tanimoto *et al.*, 2000). Methanol is a non-chemical fixative which dehydrates the cells and causes partial lipid solubilization. Protein denaturation (and eventually precipitation) is without covalent modifications; however, it affects their antigenicity as discussed by Neunhaus *et al.* (1998).

Embedding in agarose is widely used in the preparation of vibratome sections, e.g. in neurobiology (Sallee and Russell, 1993), but has great potential to be used in plant biology as well. Agarose as an embedding medium acts only as a mechanical support for both free-hand and vibratome cutting and does not penetrate the tissues. As a consequence, it only minimally affects the staining of the cell walls. This method could be used also for cutting above-ground tissues such as stems, leaves, etc.

To lower the costs it is possible to use agar diluted in water (6 % w/v). Disadvantages compared with the use of agarose are the opacity, lower strength and lower mechanical flexibility. To obtain stronger and less opaque agar we dilute it in water (in the microwave) and let it solidify at room temperature. The agar is again melted in the microwave and only then used for embedding.

Disadvantages and limitations of this method are particularly seen in the collapsing of the peripheral root layers or in collapsing of the apical part of the thin roots. However, these deformations usually do not impede the detection of the apoplastic barriers or other cell-wall modifications, which is the main goal of the method.

To obtain higher quality sections (e.g. for photography), the use of fresh plant material is preferable (skipping the fixation and following all the other steps of the presented procedure). The use of a vibratome or/and manual removal of agarose which is around the root section can also improve the quality of the section, by reducing eventual deformations of the peripheral cells layers. The modification of the embedding procedure (using less concentrated solution of agarose and/or the two step procedure) can help prevent this problem as well.

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