A new and simple cryosectioning protocol for pollen analysis under light microscopy

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Conventional plant microtechniques typically involve fixation, dehydration, and embedding in acrylic or epoxy resins followed by room temperature microtomy. Some cryomethods have been used for pollen grain analysis under light microscopy. However, these protocols do not allow for obtaining sections with similar or better quality compared to resin embedding methods. In this paper, a simple cryoprotocol for use in pollen studies under light microscopy is presented. Aldehyde-fixed anthers were cryoprotected in an aqueous sucrose solution and frozen in liquid nitrogen. A simple and inexpensive water-based gelatin–sucrose solution was used to support samples for frozen sectioning. With this new method, 1 µm thick sections were cut in a cryostat at -30 °C. The pollen grain sections showed cell structures with excellent preservation. Furthermore, different sublayers of the intine can be visualised with unprecedented clarity. The pollen cryostat sections have a higher level of quality than those of the resin embedding protocols at the same thickness. The main advantage to the method is the absence of artefacts, such as shrinkage and lipid extraction, caused by solvent dehydration and resin inclusion. Since the sections remain hydrated and free of embedding resins, the stain action is faster and more effective.

Keywords: cryostat sectioning; fluorescence microscopy; intine; plant microtechnique; pollen wall

1. Introduction

A pollen grain is composed of the haploid male gametophyte of seed plants surrounded by a special cell wall, the sporoderm. Inside the sporoderm, a vegetative cell englobes the generative cell or two sperm cells [1]. After the opening of each microsporangium in the dehiscent anther, bicellular or tricellular pollen grains will await their arrival to the floral stigma. The sporoderm is a highly complex cell wall that is stratified into two distinct layers with different physical and chemical properties. The exine is the outermost stratum of the sporoderm with taxon-specific patterns of organisation (the pollen ornamentation), which is also found in fossil pollen. Exine structure has been used as a criterion of classification and taxonomic distinction [2]. The principal exine component is a high chemically stable substance called sporopollenin. Under the exine is the inner stratum, the intine, with a pectocellulosic composition [3]. The intine is thicker under the pollen apertures, from where the pollen tube emerges [4].

Palynological studies focus on the exine through the use of chemical treatment techniques, such as acetolysis [5], the standard method for pollen preparations for analysis under light microscopy or scanning electron microscopy [6]. This method usually does not involve sectioning of pollen grains, although optical sections of the transparent or translucent acetolysed pollen preparations can be obtained using the adjustable focus in bright field light microscopes. This procedure (the LO-analysis) allows for describing the fine structures of the exine, such as depressions, elevations, and ornamentations [2,7].

For the intine to be structurally and chemically characterised, the pollen grains need to be sectioned, generally using microtomy techniques. Pollen grains only can be sectioned using a matrix or embedding medium, since pollen grains are microscopic, and is not possible to make freehand pollen sections. This generally involves prior chemical fixation; dehydration; embedding in paraffin (for light microscopy), acrylic or epoxy resins (for light microscopy or transmission electron microscopy); and sectioning at room temperature [8]. The embedding media are not water miscible, and the use of intermediary solvents (the dehydration process) is necessary. So, many cellular components, such as structural or storage lipids, and cell wall proteins are usually extracted. In transmission electron microscopy preparations, the lipid extraction is minimised with the use of osmium tetroxide in a secondary fixation step [9]. Simultaneously, dehydration also causes morphological changes in the intine, since this layer is hydrophilic, with structural pectins as typical components of the layer [1]. Thus, the visual microscopy and chemical aspect of the intine are changed by dehydration followed by embedding.

Cryomicrotomy is presented as an alternative to histological specimen preparation in plants. Several publications have shown different procedures for obtaining cryosections of vegetative or reproductive organs in plants using a cryomicrotome or cryostat [10–12]. These methods have in common the use of cryoprotectants, such as dimethyl sulfoxide, glycerol or sucrose, that can minimise the effects of freezing, avoiding the formation of ice crystals. Although there are well-known commercial water-soluble embedding media based on polyvinyl alcohol and polyethylene glycol, such as the optimum cutting temperature medium (Tissue-Tek® O.C.T. Compound) [13], gelatin and glycerol have been used as sectioning embedding media of frozen plant materials, in pure or mixed formulations [10,14,15]. Both are inexpensive and easily prepared in the laboratory, and they are used as sectioning media that externally surround the frozen material, allowing for the obtainment of good sections.
The pollen grain sections obtained from the cryostat are important for cytological analyses of the intine components that normally would be extracted by conventional microtomy techniques at room temperature [15–17]. However, even though the cryosection protocols for pollen grains appear to be well established [18], there are few studies using cryotechniques for analyses of the cytology and pollen wall structure by light microscopy.

The goal of this work is to present a new and simple protocol for obtaining 1 µm thick cryosections of pollen grains using a cryostat. The procedure results in the production of sections with excellent cytological structure preservation using an inexpensive medium of sectioning that is easily prepared in the laboratory. Each step is detailed, from the chemical fixation to cryomicrotomy and cryosection staining, allowing to obtain bright field and fluorescence microscopy images with unprecedented quality of the cell appearance and sporoderm stratification, especially the intine, without the damage caused by dehydration with the use of solvents. The results obtained with this protocol are compared to conventional acrylic resin embedding and room temperature microtomy.

2. Materials and methods

Flower buds of *Bromelia antiacantha* Bertol. (Bromeliaceae) were collected from the *ex situ* plant collection (registration number CV1369) of the Plant Anatomy Laboratory (LAVeg) of the Department of Botany of the Federal University of Rio Grande do Sul (UFRGS). Under a stereomicroscope, several anthers were removed from dissected buds and immediately processed according to the protocols described below.

2.1 Chemical fixation in the microwave (MW)

The following steps were performed according to the procedures described by Russin and Trivett [19]. A PELCO BioWave® Pro MW processor, equipped with a PELCO Cold Pro® plate (that acts as a power transfer and dissipates the heat generated by the MW), and a PELCO TissueVac® vacuum chamber were used during this step. The MW maximum temperature was configured to 35 °C. Water (1 cm depth) and some ice cubes were added inside the vacuum chamber.

a) Intact anthers were immersed in 600 µL of a fixative solution containing 2.5% glutaraldehyde and 2% formaldehyde in a 0.1 M sodium phosphate buffer, pH 6.8 [20], in 1.5 mL polypropylene microcentrifuge tubes. The microcentrifuge tubes (with snap caps opened) were vertically positioned in a polytetrafluoroethylene (PTFE) tube holder, transferred to vacuum chamber and submitted to a vacuum at -20 inHg for 5 min (no MW power; 0 W). This step is important for removing stored air in the anther locular cavity.

b) The anthers were fixed in three consecutive MW cycles: 60 s at 750 W, 60 s at 0 W (no power) and 60 s at 750 W, at atmospheric pressure (no vacuum). The microcentrifuge tubes stayed closed during this step.

c) The anthers were washed in 600 µL of a 0.1 M sodium phosphate buffer, pH 6.8, for 2 min each at 150 W.

2.2 Cryosections in freezing microtome (cryostat)

An aqueous solution of 2% gelatin and 10% sucrose was prepared by dissolving 0.4 g gelatin and 2 g sucrose in a beaker containing 20 mL distilled water at 40 °C. For this purpose, a colourless and flavourless commercial (food grade) gelatin powder (Dr. Oetker 12 g sachet) was used for the medium preparation. The gelatin–sucrose solution was used only at room temperature (20–23 °C). When a larger stock solution was prepared (e.g., 100 mL for refrigerator storage), 0.05% sodium azide was added to avoid microbial contamination.

Clean and dry histological glass slides (treated in acidified ethanol for 1 h and rinsed in distilled water) were covered by a thin layer of Haupt’s adhesive solution [21]. Using a finger, a thin layer of the solution was spread onto the slide surface, which then dried for at least 30 min at room temperature prior to use.

Small aluminium moulds, with a volume of approximately 1 cm³, were previously handmade and prepared with aluminium foil (Fig. 1a) in a similar method to making paper folding moulds or boats used in the production of blocks in the paraffin tissue processing [22]. A solid 1 cm² square profile steel (4–5 cm in length) was used as an aid in shaping the mould.

Chemically fixed anthers (stored in phosphate buffer) were processed at room temperature (step a) and in a Leica CM1850 cryostat chamber (steps b–k) following the protocol detailed below. The cryostat chamber was configured at -30 °C.

a) Aldehyde-fixed anthers were transferred to a sucrose-based cryoprotectant treatment consisting of a series of increasing aqueous sucrose solutions (10, 20 and 30% w/v; 0.05% sodium azide added) for at least 1 h at each step (or until the anthers did not float in the solution). When in the 30% sucrose solution, the anthers remained in the solution for 12 h.

b) Using thin steel-tipped tweezers, the cryoprotected anthers were quickly removed from the sucrose solution and rapidly immersed in 1 cm deep liquid nitrogen for 20 s (stored in a small polystyrene vessel).

c) A frozen anther was transferred to the bottom of the aluminium mould (Fig. 1b) on the integrated cryostat Peltier element surface at -60 °C (5 min after the Peltier activation). Two drops of absolute ethanol were added between the Peltier plate and the aluminium mould (Fig. 1b, white asterisk) to provide good thermal conductivity.
d) The frozen anther was covered with 2 or 3 drops of the gelatin–sucrose solution at room temperature (using a glass or plastic pipette). The volume of the added solution was enough to cover the anther. Once frozen, the added gelatin–sucrose acquired a white appearance (Fig. 1c).

e) Additional gelatin–sucrose solution was added until completing the volume of the mould (approximately 1 cm³). The solution solidified completely after 5 min (Fig. 1d).

f) Using tweezers and gloved hands, the mould was disassembled, and the aluminium foil was removed from the frozen gelatin–sucrose block. For adhesion of the block to the cryostat specimen holder (specimen disc), the frozen block was positioned on the specimen holder (mounted on the integrated Peltier element), taking care that the top surface of the block (when in the mould) was maintained in contact with the holder surface (not the reverse). Sucrose–gelatin solution was added around the block for a perfect anchorage to the specimen holder (Fig. 1e).

g) One or 2 drops of the gelatin–sucrose solution were added to block upper surface (Fig. 1f). This procedure adjusted for possible imperfections, such as air gaps or bubbles between the frozen anther and the solidified gelatin–sucrose, before the initial microtome trimming.

h) The specimen holder with the block was inserted into the specimen head of the cryostat, with the necessary adjustments for correct block orientation. A precooled microtome blade (Duraedge® high-profile disposable microtome blade) was mounted in the knife holder (inserted 30 min before the sectioning), and the appropriate clearance angle (between 1 and 2°) was adjusted for and selected. Several sections (10–20 µm thickness) were obtained until a desired sectioning plane was acquired and to ensure the first sections of the anther were obtained (Fig. 1g).

i) The section thickness was decreased to 1 µm, and the sections were obtained with the aid of a previously cooled soft brush, which was used to guide and avoid the rolling of the cryosection on the collector plate surface (Fig. 1h). With this procedure, it was possible to obtain complete unfolded 1 µm thick sections (Fig. 1i). The use of the cryostat anti-roll glass plate was not necessary.

j) A previously prepared Haupt's adhesive-coated glass slide (maintained in a room at a temperature between 21 and 23 °C) was rapidly transferred to the inside of the cryostat chamber and held near the cryosection (5–10 mm distance) for 10 s (Fig. 1j). Gently, the slide was pressed against the cryosection, which rapidly adhered to it. It is important to use room temperature slides (no cold slides) since the temperature gradient allows quick adhesion and distention of the sections.

k) The mounted cryosections were removed from the cryostat chamber, stored in a horizontal position for 12 h at room temperature and transferred to a plastic storage box in a laboratory refrigerator (approximately 4 °C) until the staining protocols. Some slides were maintained in refrigerated conditions for 1 year before microscopy analysis.

2.3 Acrylic resin embedding and room temperature microtomy

Chemically fixed anthers were dehydrated in an ascending ethanol series (10, 30, 50, 70, 90 and 100% v/v) in a MW processor at 150 W for 1 min at each step. Subsequently, the anthers were transferred to a pre-infiltration solution containing a mixture (1:1 v/v) of absolute ethanol and 2-hydroxyethyl methacrylate resin (Technovit® 7100, Kulzer) for 2 h and pure resin for 12 h at room temperature [23]. The embedded anthers were polymerised at room temperature in 1 mL of pure resin in PTFE moulds with the addition of dimethyl sulfoxide (1:16 v/v) as a catalysing agent. Longitudinal sections 1 µm thick were obtained with a Leica RM2265 rotation microtome equipped with a high-profile disposable microtome blade and adhered to glass slides on a hot plate at 60 °C.

2.4 Staining the cryosections and resin embedded sections

The sections obtained from frozen and resin embedded anthers were stained in 0.1% aqueous Toluidine Blue O (CI 52040), pH 4.4 [8], for 45 s and 2 min, respectively, followed by washing in distilled water. To avoid detachment of the cryosections during the washing step, the slides were gently submerged in water for 1 min. Unstained cryosections and Toluidine Blue-stained cryosections and resin embedded sections were covered with a glass cover slip in distilled water and immediately analysed under the microscope.

To carry out microscopy fluorescence analysis, cryosections were treated with a drop of the following solutions: 0.01% Calcofluor White M2R in distilled water for 1 min followed by washing in distilled water, for cellulose identification [24]; Nile Red (5 µg/mL in 75% glycerol) with the addition of 2 µL/mL of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) from a 1 mg/mL stock solution, for lipid and DNA detection [18,25]; and 0.01% Auramine O in 0.05 M Tris-HCl buffer for 1 min followed by washing in distilled water, for localisation of sporopollenin [26,27]. The slides were mounted in distilled water (with the exception of the Nile Red/DAPI staining, which was mounted directly in the staining mixture) with a cover slip and immediately analysed under the microscope.

Photomicrographs were obtained using a Leica DMR light microscope outfitted with bright field and fluorescence optics, equipped with a Zeiss Axiocam HRc digital camera. Calcofluor White and DAPI fluorescence images were obtained using a Leica DM A filter cube (PNS13804; BP 340-380 nm, DM 400 nm, LP 425 nm), while a Leica DM I3 filter cube (PNS13808; BP 450-490 nm, DM 510 nm, LP 515 nm) was used for Nile Red and Auramine fluorescence images. Nile Red and DAPI images were combined with Zerene Stacker 1.04 software.
Fig. 1  Sample preparation of frozen anthers for cryomicrotomy. (a) Handmade aluminium foil mould with a capacity of 1 cm$^3$. (b) The mould, inside the cryostat chamber, containing a frozen anther on the integrated Peltier element (quick freeze shelf) at -60 °C; the disk below the mould is the parking station. Note the liquid ethanol for best thermal conductivity (white asterisk). (c) The frozen anther covered by a drop of gelatin–sucrose solution (already frozen). (d) Mould completely filled with gelatin–sucrose solution. (e) Gelatin–sucrose frozen block (and the frozen sample) attached to specimen disk (cryostat chuck), after manual removing of the aluminium mould. (f) The block after the addition of one drop of the gelatin–sucrose solution for filling imperfections on the pre-cutting surface. (g) The specimen disk mounted on the specimen head, with the cutting surface after the trimming. (h) The use of a soft brush to guide and avoid the rolling of the sections. (i) One micrometre thick cryosection on the cryostat collector plate surface. (j) Cryosections collected by the slow approach of a histological glass slide previously treated with an adhesive solution.
3. Results and discussion

3.1 Freezing protocol and cryosectioning in the cryostat

Chen and Zhao [11] described a cryosectioning protocol using anthers transferred to a fixative–sucrose solution, with concentrations ranging from 4–10% sucrose (directly added to the fixative solution) in four plant species. However, the protocol of these authors allowed 10 µm sections, 10 times thicker than the one obtained with the method presented here. Furthermore, differently from the previous work, this new protocol used fixation that preceded cryoprotection. The goal was to allow for any fixed anther (even without the initial objective of cryosectioning) to be used with the new protocol. Thus, anthers already fixed (since they are not dehydrated) can be used without problem. Additionally, the MW fixation applied to the anthers is not a prerequisite, and conventional chemically fixed materials (without a MW processor) also can be used without problem or protocol adaptations.

Gelatin as sectioning media has been previously employed but in different formulations [10,16]. This is the first time that the use of a mixture of gelatin and sucrose allowed for obtaining 1 µm thick sections (Fig. 1i) with quality comparable to those of acrylic resin embedding methods. The medium is easy to prepare and is inexpensive. Different temperatures were tested, and -30 °C was the optimal cryostat chamber temperature that allowed for obtaining 1 µm thick sections. Higher temperatures (e.g., -20 °C) did not enable the same thickness in the sections, although 3–5 µm sections could be obtained without difficulty. The medium prepared with a food brand gelatin powder allowed very good sectioning properties, which eliminate the use of a laboratory grade reagent. It should be emphasised that 2 µm thickness gelatin-glycerol cryosections were obtained only after the use of “high-molecular-weight gelatin samples” [16].

Unlike the commercial O.C.T. medium, it is not necessary to use an anti-roll glass plate to prevent folding of the sections from the gelatin–sucrose block, even with 1 µm sections. The sections obtained with the frozen gelatin–sucrose media are easily guided by using a soft brush only (Fig. 1h). In addition, the sections are not sticky, and they are easily captured (Fig. 1j); when outside of the chamber, the mixture of gelatin and sucrose thaws smoothly and uniformly. It is very important that a temperature gradient exists between the cryosections and the glass slide. This is a fundamental factor to be considered, as the use of very cold or very hot glass slides will not produce good results. When the slides are very cold (e.g., kept inside the cryostat) the desired distention of the sections does not occur nor their proper adhesion to the slide surface. When very hot (without several seconds in the cryostat chamber), excessively rapid thawing causes damage to the pollen grains, which is displayed as a separation of the vegetative cell and the sporoderm. It is recommended to test this several different times (in seconds), as the gradient between the room and the cryostat chamber (at -30 °C) will influence the quality of the collected sections.

3.2 Pollen cryosections: quality and staining proprieties

The sections obtained from the acrylic resin embedded anthers (Fig. 2a) showed good cytological preservation of the male gametophyte. The intine layer regularly appears stained with a purple colour when stained with Toluidine Blue (2 min staining), with the exception of the innermost layer, which remains unstained (Fig. 2a, asterisk). The 1 µm cryosection preparations have similar aspects of the vegetative nucleus and cytoplasm, but the sections obtained from the cryostat shows outstanding cytological quality (Figs. 2c–d). The vegetative nucleus has a shape and size similar to those of resin embedded sections. Several starch grains can be identified in the vegetative cell.

The main difference between resin and frozen sections can be seen in the sporoderm, especially the intine. While the water in the embedded resin sections allowed a limited hydration of the acrylic resin (and the intine), the absence of an insoluble embedding medium (such as the acrylic resin) allowed a larger hydration of the intine under the pollen apertures, leading to identification of sublayers in this polysaccharide wall. In the *B. antiacantha* pollen grains, used as model plant in this work, four intine sublayers (Fig. 2c) have been identified, in an unprecedented result. Recent papers published about Bromeliaceae pollen of other genera, using conventional acrylic resin embedding methods, do not show any additional intine stratification [28,29], similar to the resin embedding sections of *B. antiacantha* pollen obtained here. More than two intine sublayers are considered an uncommon character in monocotyledons [30]. Based on the results found here, this could be a mistake interpreted as derived from a ‘methodological artefact’ that interferes with the correct structural characterisation of the intine, including Bromeliaceae pollen grains. The cryosections mounted in water showed a progressive increase in the intine thickness due to water absorption just a few minutes after the slide preparation, when the intine thickness stabilised until a maximum was reached. Therefore, different from other pollen preparations, intine thickness of the hydrated pollen cryosections could not be used in comparative and quantitative analyses, since the time lapse after the mounting of the slide and the microscopy analysis can interfere with the measurements.

Some prepared slides of cryosections of *B. antiacantha* pollen were stored dry in a refrigerator at 4 °C for 1 year. Although the recommendation that cryosections should be stored in an ultra-freezer at temperatures below -80 °C exists [31], the unstained water-mounted slides, analysed under bright field microscopy, showed excellent structural quality and no signs of fungal or bacterial contamination (Fig. 2b). The absence of biological contamination can be explained by the use of the Haupt’s adhesive, a mixture of gelatin, glycerin, and phenol (2 g/100 mL distilled water) [21]. Phenol is well known for its antimicrobial, disinfecting and antiseptic activity [32]. Thus, these properties make the Haupt’s
adhesive the correct choice when good adhesion and conservation of the cryosections over a long period (such as 1 year or more) are required, without the expected damage caused by microorganisms during storage at low (not extremely low) temperature.

The staining time of the pollen cryosections was shorter than that necessary for acrylic embedded anthers with equal thickness. Only 45 s were required to obtain an optimum staining of the 1 µm cryosections (Figs. 2c–d). Longer times (such as 1 min) with the Toluidine Blue stain resulted in excessive staining of the cytoplasm and sporoderm, leading to masking of the intine sublayer. For acrylic embedded sections, 2 min of staining are necessary. This is easily explained by the absence of an embedding medium. After their melting onto the slides, the cryosections are comparable to handmade sections, since no tissue penetration occurs with the sectioning medium (gelatin–sucrose). Epoxy resins can be progressively dissolved from slide preparations with the use of sodium ethoxide (a saturated solution of sodium hydroxide in absolute ethanol), but acrylic resins cannot be removed without a destructive tissue method [33]. Therefore, we can see a clear advantage of the cryotechniques with the conventional room temperature procedures.

Fig. 2 Resin embedded sections and cryosections of bicellular pollen grains of *Bromelia anticantha* under a bright field light microscope. All the preparations contain 1 µm sections mounted in distilled water. (a) Acrylic resin embedded pollen grain stained for 2 min with Toluidine Blue. The resin background has diagonal and parallel lines produced by nicks in the knife blade. The inner layer of the intine (in) is indicated by an asterisk. (b) Unstained 1 µm cryosection. This prepared glass slide was kept dry in a refrigerator for about 1 year. Note the exceptional section cleanliness and no evidence of bacterial or fungal contamination. The vegetative nucleus (vn) can be easily identified as well as the intine (in) and exine (ex). (c–d) Toluidine Blue-stained cryosections. (c) Pollen grain with evident four-layered intine (1–4 layers), under the pollen aperture. Differences between the pollen size and shape from this image and the pollen grain sectioned in (a) are due to small differences in the developmental stages. (d) Pollen grain showing excellent cytological preservation. The vegetative cell (vc) has no shrinkage, and the generative cell (gc) and the vegetative nucleus (vn), with an amoeboid shape in this stage, show excellent aspects. Abbreviations: ex, exine; gc, generative cell; in, intine; s, starch grains; vc, vegetative cell; vn, vegetative nucleus. Scale bars: 10 µm.
The same fast staining was observed when stains were used to display nuclear DNA, cellulose and lipids in the pollen cryosections (Figs. 3a–d) under fluorescence microscopy. Only 1 min of staining was necessary to obtain strong fluorescence of the exine (Fig. 3a) and the inner cellulosic layer of the intine (Fig. 3b). The DNA of the generative nucleus (Fig. 3c–d) and lipids in the cytoplasmic oleosomes (Fig. 3d) were visualised immediately after applying a coverslip and also showed strong fluorescence. Moreover, the melted gelatin–sucrose medium around the pollen grains showed extremely low autofluorescence and created a perfectly black background, without the typical background fluorescence usually present in sections of acrylic resins generally due to staining retention, eliminating the need for image post-processing.

Although the described protocol presented here was used in pollen grain applications, the same methodology (with some possible adaptations) could be employed to obtain cryosections of simple or complex tissues of different vegetative or reproductive plant organs. The collenchyma is a tissue of difficult structural preservation when prepared by conventional methods at room temperature, involving dehydration and paraffin or even hydrophilic resin embedding because their cells have a thickened and hydrated pectocellulosic wall [34], which is similar in many aspects to a thickened intine under the pollen grain apertures. Due to these cell wall properties, freehand sections of fresh plant organs are most suitable for the study of the collenchyma. However, it is very difficult to obtain freehand sections of 1–5 µm thickness.

Fig. 3 Cryosections of pollen grains of *Bromelia antiacantha* via fluorescence microscopy. (a) Auramine O staining with a strong green fluorescence of the exine. (b) Calcofluor White fluorescence of the inner cellulosic sublayer of the intine (sublayer 4; see Fig. 2c); (c) DAPI reaction showing different fluorescence between the generative and vegetative nuclei. The autofluorescence in the vegetative cytoplasm is an aldehyde fixation artefact. (d) Combined Nile Red/DAPI staining for lipids and DNA. Lipid droplets in the vegetative cell cytoplasm show a golden yellow colour; the vegetative nucleus is pale green and the generative nucleus blue. The exine acquires an orange fluorescence colour (image software stacking; see Materials and Methods, section 2.4). Abbreviations: ex, exine; gn, generative nucleus; in, intine; ol, oleosomes; vn, vegetative nucleus. Scale bars: 10 µm.
Thus, the method described here would be employed in preparation and analysis of other plant tissues, which could reveal details not typically seen with the room temperature methods. Specifically, for pollen grain studies, I expect the new protocol to expand our view of the sporoderm layers and their functional and chemical proprieties.

Acknowledgements I am very grateful to Dr. Jorge E. A. Mariath, head of the Plant Anatomy Laboratory (LAVeg) of the Department of Botany, UFRGS, and the lab staff for their technical support.

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