MethodsX 13 (2024) 102999

Contents lists available at ScienceDirect

MethodsX

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

Protocol to analyse the structural composition by fluorescence microscopy and different conventional and fluorescence staining methods ☆

Agustín Maceda [a], Agustina Rosa Andrés-Hernández [b], Teresa Terrazas [a,*]

[a] *Universidad Nacional Autónoma de México, Instituto de Biología, Departamento de Botánica, Apdo. postal 70-367, 04510 Coyoacán, Cd. Mx., Mexico*

[b] *Benemérita Universidad Autónoma de Puebla, Blvd. Val-sequillo y Av. San Claudio, Edificio 112-A, Ciudad Universitaria, Col. Jardines de San Manuel, 72570 Puebla, Puebla, Mexico*

ARTICLE INFO

Method name:
Safranin-fast green conventional staining with fluorescence microscopy

Keywords:
Safranin-fast green staining
Congo red
Acridine orange
Epifluorescence
Intensity fluorescence
Imagej

ABSTRACT

The protocol shows the effectiveness of using safranin-fast green stain for fluorescence microscopy. This staining technique has been used in conventional microscopy to perform anatomical characterizations of plants. However, this protocol describes the procedure for using samples stained with safranin-fast green in conjunction with fluorescence microscopy. The strength of the protocol lies in the fact that the samples are permanent and allows for effective differentiation of lignified and cellulosic walls unlike conventional fluorescence microscopy stains such as Congo red-acridine orange, calcofluor, and autofluorescence. The protocol for making fluorescence intensity measurements is also standardized, allowing the data to be used for statistical analysis and inference about the chemical composition of plant cell walls.

Specifications table

Subject area:	Agricultural and Biological Sciences
More specific subject area:	Plant structural-anatomy
Name of your protocol:	Safranin-fast green conventional staining with fluorescence microscopy
Reagents/tools:	Synthetic resin (Entellan) that does not have autofluorescence. Leica tissue processors TP1020. Rotary or sliding microtomes Leica. Wide-field fluorescence microscope Zeiss Axio Imager Z2 with AxioCam MRc 5 (Zeiss) and metal-halide HXP 120, Zeiss. Triple filter set: 25, Zeiss, item number: 488,025–0000–000: excitation TBP 400 + 495 + 570 nm, beamsplitter TFT 410 + 505 + 585 nm, emission TBP 460 + 530 + 625.
Experimental design:	The plant parts are collected at random; the tissues are fixed and processed for sectioning and staining. The images are taken with similar equipment conditions; they are subsequently processed with ImageJ. Each image is divided into three-color channels and the intensity values are obtained and transformed into percentages for comparison between individuals or species stained with the same stain. For statistical analysis, at least 10 repetitions of the fluorescence intensity measurements are used.

(*continued on next page*)

☆ **Related research article:** Maceda, Agustín, Soto-Hernández, Marcos, Terrazas, Teresa, Cellulose in secondary xylem of Cactaceae: Crystalline composition and anatomical distribution, Polymers 14, 4840. https://doi.org/10.3390/polym14224840

* Corresponding author.
E-mail address: tterrazas@ib.unam.mx (T. Terrazas).

https://doi.org/10.1016/j.mex.2024.102999
Received 4 July 2024; Accepted 4 October 2024
Available online 10 October 2024

Trial registration:	N/A
Ethics:	N/A
Value of the Protocol:	• The common stain safranin – fast green can be used in fluorescence microscopy. • Micrographs with safranin – fast green favor the differentiation of lignified and cellulosic cell walls better than other specific fluorescence stains. • With the micrographs obtained, we can obtain fluorescence intensity data to compare between plant species.

Background

The characterization and analysis of lignocellulose in plants allows the identification of cellular structures, the composition of the walls, anatomical differences, and the possibility of use of plant species [1,2]. Fluorescence microscopy is a useful technique for the characterization of plant tissues. Most type of cells, protoplasm, and secondary metabolites in plant stem exhibit autofluorescence, such as lignin, suberin, cutin, chloroplasts and different secondary metabolites [3–8]. The cellulose of the primary walls of plant cells has little fluorescence emission, so staining methods such as calcofluor (Ca) or Congo red (Cr) were developed to emit fluorescence with greater intensity [9,10]. Lignin has autofluorescence because its structure has fluorophores [11]; however, by using dyes such as safranin and acridine orange, photobleaching can be reduced. Photobleaching is the photodestruction of the fluorophore: a high excitation intensity and a long time cause its destruction [12].

Safranin O alone was tested several times [13–16] or with another dyes as Ca [9] with microscopy fluorescence. Autofluorescence (Au), Congo red-acridine orange (CrA), and calcofluor (Ca) stains are typically used with semi-permanent mounting media and with fresh tissue or fixatives in FAA, so over time, the dyes can be diluted or leached to the background [17]. The safranin-fast green (SFg) stain becomes permanent because synthetic resin or Canada balsam are primarily used as a mounting medium. This stain is commonly used to make anatomical observations of plants [18–20]; however, it had been barely used in conjunction with fluorescence microscopy [21] until the protocol was tested for Cactaceae [22–24]. However, although the use of staining with fluorescence microscopy had been mentioned, the protocol had not been detailed so that it could be applied to other plant families. In addition, its potential for application to perform quantitative analyses through quantification of fluorescence intensity has not been explored. The fluorescence intensity is related to the intensity value of a pixel and the number of fluorophores present in the corresponding area of the specimen, so, with digital images, it is possible to perform spatial analysis and the presence of fluorophores [25].

Therefore, the aim of this protocol is to detail the processes of sample preparation, staining, photobleaching of unwanted fluorescence, image taking, standardization of image analysis with ImageJ, and obtaining fluorescence intensity data of the three RGB channels (red, green, and blue). The CrA, Ca and autofluorescence staining protocols were added to validate safranin-fast green staining.

Description of protocol

Fluorescence images are obtained after preparation of plant material, sectioning, staining, mounting, and imaging of the sample. The quality of the resulting images is defined by the clarity in the separation of structural components of plant stems based on the fluorescence emission of each structural compound.

The entire process from dehydrating the plant material to taking images with the microscope lasts nearly 84 consecutive hours. The dehydration process alone lasts 72 h and the staining process a maximum of 4 h. Waste dye, alcohols and paraffin are disposed of in appropriate containers to avoid contaminating the environment.

Collection, fixation, and preparation of plant material

The stems are collected and immediately fixed in a solution of formaldehyde (10%), glacial acetic acid (5%), alcohol (50%), and distilled water (35%) (FAA). This fixative is widely used in the preservation of plant material [26]. The samples are kept for a week and then changed to a 70% alcohol solution and can be stored for one year at room temperature. If the plant tissue has mucilage, the plant material must be kept for at least three months in 50% ethanol so that the mucilage separates and does not affect the process of paraffin infiltration into the plant tissue.

They are then stored in plastic cassettes and the samples are placed in the tissue processor. The equipment is programmed so that the samples go through different concentrations of ethanol (Table 1) and remain for four hours in each one. Then, put the samples in a solution of tertiary butyl alcohol (TBA, 67%) and mineral oil (33%) for 4 to 12 h (soft to hard tissues) and then to TBA (40%) and mineral oil (60%) for four hours. The samples are then passed through two consecutive occasions in liquid paraffin at a temperature of 70 ˚C for 4–12 h and finally the samples are removed from the tissue processor. In all solvents and paraffin, the samples are kept under constant agitation and vacuum so that they are infiltrated homogeneous with paraffin.

The paraffin-infiltrated samples are placed on an embedding stainless-steel base mold and positioned to make transverse, radial, and tangential anatomical sections. The trays are filled with liquid paraffin at 70 ˚C and allowed to cool until paraffin blocks are formed with the sample. Subsequently, the blocks are mounted on the rotary or sliding microtome.

The sections can vary between 5 and 20 μm, however, 10–12 μm is an optimal thickness. Once at least 10 sections in good condition are obtained, the slides are taken and a drop of Haupt adhesive is placed, which is made with 1 g of gelatin, 100 ml of water and three phenol crystals to prevent the appearance of fungi. The drop is spread on the slide and the sections are placed on

Table 1
Different concentrations of solvents used in the tissue processor.

	96% ETOH (%)	Distilled water (%)	TBA* (%)	Mineral oil (%)	Paraffin (%)
Station 1	50	40	10	–	
Station 2	50	30	20	–	
Station 3	50	15	35	–	
Station 4	50	–	50	–	
Station 5	25**	–	75	–	
Station 6	25**	–	75	–	
Station 7	50**	–	50	–	
Station 8	–	–	100	–	
Station 9	–	–	67	33	
Station 10	–	–	40	60	
Station 11	–	–	–	–	100
Station 12	–	–	–	–	100

*Tertiary butyl alcohol. ** Ethanol 100.%

top. Immediately, a drop of 2% formaldehyde is added and allowed to dry. Samples can be stored this way for a year or more without changes in the tissue.

Paraffin-free materials

If the plant has hard tissues, for example, many lignified fibers or parenchyma, after fixing the plant material with FAA, wash the sample with distilled water and a sliding microtome is used to make the sections between 10 and 25 μm thick. Sections can be stored in 50% ethanol until staining.

Deparaffinization and staining

When they are going to be dyed, they are placed in stainless steel basket and placed in the oven at a temperature of 60 °C for 15 min. Subsequently, they are removed and placed in 100 % xylol for 5 min. The basket is lifted and placed again in 100% xylol for 5 min. The samples are then hydrated using different concentrations of ethanol: 100%, 96%, 70%, 50%, and maintained for five minutes in each ethanol concentration, to carry out the different stains.

Fast green safranin staining

This process was based on [19]. Once the samples are in 50% ethanol, they are placed in 1% safranin in 50% ethanolic solution and 2 g of NaCl, the samples are kept in the dye for 1 h. Two washes are done with distilled water for 2 s each and the samples are dehydrated in different ethanol concentrations: 50, 70, 96, 96, and 100%, keeping the samples for two seconds at each concentration. Then it is dipped into the fast-green stain (at 5 % in absolute ethanol - clove oil- methyl cellosolve with similar proportion), held for three seconds, switched to 100% ethanol for one second, then changed to a new 100% ethanol solution for one second, after in xylol, and finally to histoclear for one second. The samples are changed once again to histoclear, held for 5 min and then put into new histoclear for 10 min. The samples are mounted with synthetic resin (Entellan), they are covered with a cover slip. To eliminate possible bubbles, so the samples remain flat are placed some nuts on top of the coverslip, the resin is allowed to dry for at least a week and the images are taken with the fluorescence microscope. Paraffin-free materials(safranin-fast green staining)

For material sectioned directly with the sliding microtome and stored in 50% ethanol. Sections are placed in a Petri dish and dehydrated in different ethanol concentrations: 50, 70 to 96%, then, use safranin in 96% ethanolic solution for 1 h. Give one wash with 96% ethanol followed by 1 or 2 washes of 100% and change to a fast-green stain, moving the petri dish for three seconds. Finally, wash the excess with 100% ethanol for one second, then change to a new 100% ethanol solution for one second, change to histoclear until mounting with synthetic resin (Entellan) and covered with a cover slip. For the different staining methods listed below, the process is similar for paraffin and non-paraffin plant materials with the difference that for non-paraffin materials, Petri dishes are used as described in this paragraph

Congo red-acridine orange stain

This method was based on [9,17]. Samples in 50% ethanol are changed to 25% ethanol, then to distilled water for 5 min in each step. Then they are put in the 0.01% acridine orange dye and left for 5 min. After, washes are made with different concentrations of ethanol that gradually increase: 25, 50, 70, and 96% each per min. Then the ethanol concentration decreases: 70, 50 and 25% with a duration of 5 min in each step. The samples are placed in the Congo red dye for 5 min and 5 washes are performed with distilled water for 2 min each. The samples are mounted with pure glycerin or with a 50% glycerin-phosphate buffer solution at a pH of 9. The edges of the coverslip are sealed with varnish to prevent the mounting medium from evaporating.

Calcofluor staining

This method was based on [9,17]. Samples in 50% ethanol were changed to 25% ethanol, then to distilled water for 5 min in each step. They are changed to the calcofluor dye for 5 min, then five washes are made in distilled water for 2 min each and they are mounted with pure glycerin or with a solution of phosphate buffer and 5 0 % glycerin at a pH 9. The edges of the coverslip are sealed with varnish to prevent the mounting medium from evaporating.

Autofluorescence

This method was based on [17]. One of the most practical methods for imaging with fluorescence microscopy is to use the autofluorescence of plants. To do this, the samples in 50% ethanol are changed to 25% ethanol, then distilled water for 5 min in each step. They are mounted with pure glycerin or with a 50% phosphate buffer solution and glycerin at a pH of 9. The edges of the coverslip are sealed with varnish to prevent the mounting medium from evaporating.

Imaging with fluorescence microscopy

The mounted slides were observed with the Zeiss Axio Imager Z2 epifluorescence microscope with AxioCam MRc 5 camera (Zeiss) and a metal halide HXP 120 fluorescent light source. For imaging, the samples in safranin-fast green had a different process to the other stains. This process was proposed by [22,27], but here the process is described step by step.

Safranin-green fast. The dried slides were cleaned with ethanol to eliminate any remaining stains. They are then placed in the epifluorescence microscope, where initial photos can be taken with a bright field and later taken with a wide field and fluorescence. For fluorescence, a photobleaching process is first performed to eliminate unwanted fluorescence from the adhesive and benzene crystals. The lamp power is increased to 90 %, and the sample is exposed with a triple band pass filter set (DAPI-FITC-TRITC) with an excitation at 405 + 495 + 575 nm and an emission filter TBP 460 + 530 + 625 nm (HE) (Fig. 1). Because it is a multiple filter, the samples are excited and the fluorescence emission is observed with three different lengths of light at the same time.

When the sample exposed to fluorescent light is immediately observed, a lot of unwanted fluorescence emission is observed (Fig. 1A), which decreases after 5 min but is still present since the cell lumen is seen with bluish tones (Fig. 1B). The optimal exposure time is 10 min with the 20x objective and we take the image after that (Fig. 1C). If the excitation with light is maintained, the sample begins to decrease the fluorescence emission after 15 min, mainly in the fluorescence emission of the cellulose (Fig. 1D). At 20 min the cellulose walls began to photobleaching (Fig. 1E) and after 25 min blue tones again predominated in the lumen and cell walls (Fig. 1F).

If the 40x objective is used to photobleach unwanted fluorescence from the sample, the time is cut in half. To take images, the lamp power is set to 50% and similar parameters are placed in the camera so that the results can be compared between the different species being analyzed.

The photos in Fig. 1 and subsequent ones were taken under the same conditions of white gain, contrast and brightness. Because a triple filter was used and the camera took color images, the photomicrographs were in color (RGB). The lamp power is maintained at 50% and the three excitation and emission filters (DAPI-FITC-TRITC) are used.

Other stains. With Congo red-acridine orange, calcofluor stains, and autofluorescence, imaging is done directly without the need to photobleach unwanted fluorescence. Imaging was performed under the same conditions as previously described.

Analysis of fluorescence intensity of cell walls

To analyze the fluorescence intensity, the process was based on [28] but with some modifications. Fluorescence images taken with the microscope are stored in ".tif" or ".jpg" format. Afterwards, the free software ImageJ 1.54f (Wayne Rasband and National Institutes of Health, USA) is opened (See supplementary files to see detailed and visually steps). Using the "Straight" button, a line is drawn on the image scale bar. Click on the "Analyze" button, "Set Scale" and in the box that opens, place the value of the scale bar in the "Known distance" space and click the "Ok" button. By doing this we can make measurements of cellular structures.

Subsequently, select the "Rectangle" button, and make a rectangle in the cell lumen of an empty cell or in some empty space. With the right mouse button, click on the box to display options and select "Add to ROI Manager", so that the rectangle area is saved in the ROI box. Click on the "Analyze" button, then on "Measure" and a box will open where the intensity value of the rectangle area will appear in the "Mean" column, write down the value, and save it (for example a value of 40).

Remove the ROI box by left-clicking anywhere else on the image. Click on "Process", "Math" and then on "Subtract", to open a box where we write a value between 5 and 10 units higher than the value that had been previously saved, for example 40, we add ten units and write the value of 50 and then click on "OK".

The image will subtract the fluorescence intensity, repeat the process using the "ROI manager" box, activate the box that was drawn in the cell lumen and perform the measurement again by clicking on "Analyze" and "Measure". If the value is above 1, the amount obtained is subtracted again plus 3 more units using the "Process", "Math" and "Subtract" procedures. The measurement of the lumen ROI box is repeated until a fluorescence intensity value of <1 is obtained. Fig. 2 shows the difference between the image obtained with the microscope (Fig. 2A) and the image from which the fluorescence of the cell lumen was subtracted (black areas, Fig. 2B).

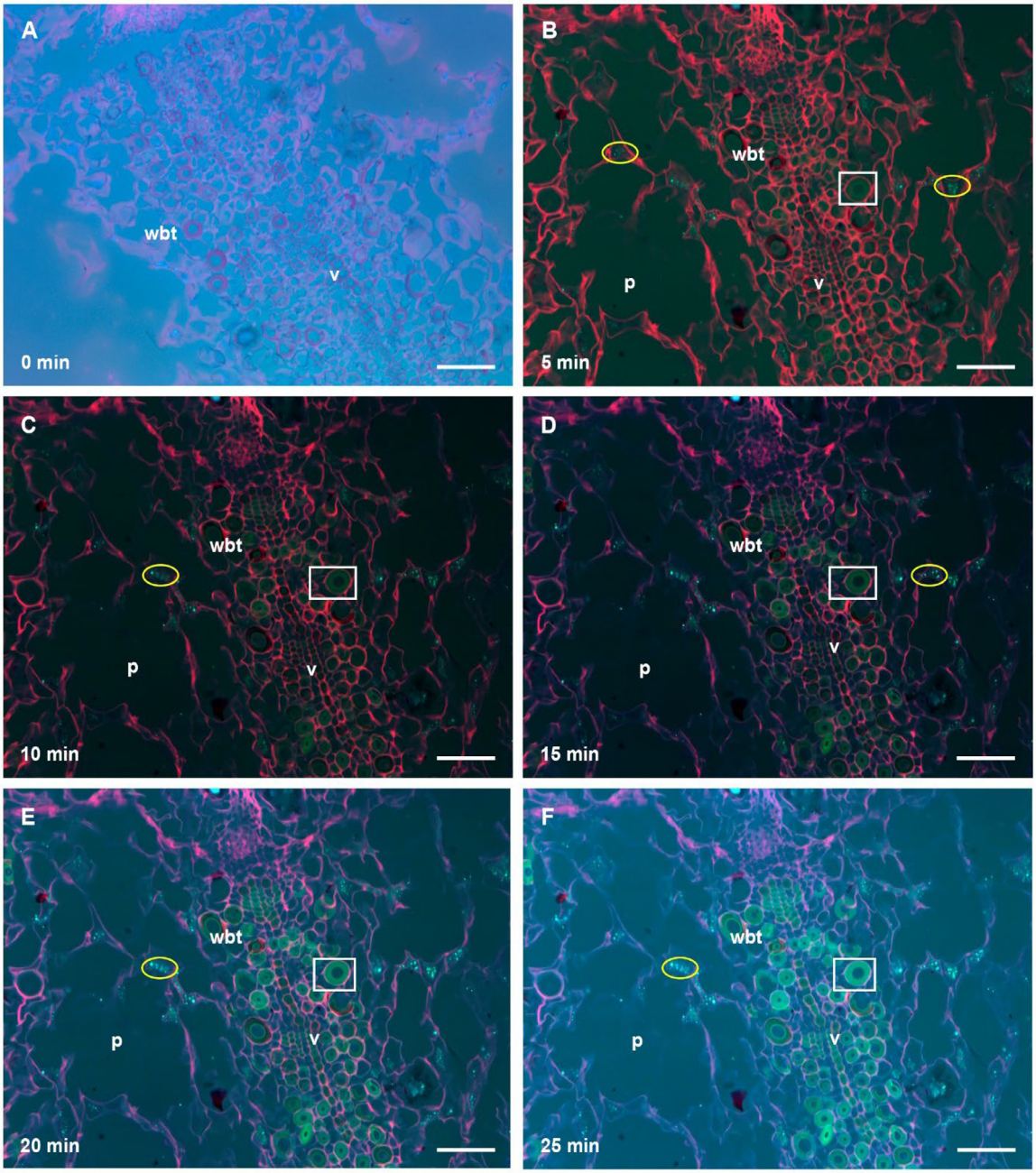

Fig. 1. Images taken with a triple band filter that excites and detects fluorescence in three different light lengths at the same time, so the resulting images are in color. Changes in fluorescence emission at different exposure times tested in *Pterocactus australis*. The shots were taken every 5 min from A to F. No changes were made to the brightness or contrast settings and the lamp power was maintained. p: parenchyma, v: vessel, wbt and white squares: wide-band tracheid, yellow circles: starch. Scale bar: 100 μm.

Next, remove the lumen box in the “ROI manager” by clicking on the “Delete” button and we will divide the image into the three channels red, green, and blue (RGB) (Fig. 3B-D). We click on the “Image” button, “Color”, and then on “Split Channels”. The image will be divided into the three channels. In the green channel, we approach the cell wall of a vessel or tracheid, with the “Square” button, we draw a square on the wall of the vessel and click on it with the right mouse button to click on “Add to Roi”. Manager". The process is repeated at least 10 times in 10 different vessels or cells to obtain 10 ROI frames or depending on the number of vessels in the microphotographed area, measurements of the available vessels are made as in Fig. 3A. Then, in the “ROI manager” select each frame of each cell and press the Ctrl + *M* keys on each selection to measure the fluorescence intensity of the 10 traced frames. For each measurement, the intensity values appear in the “Results” box.

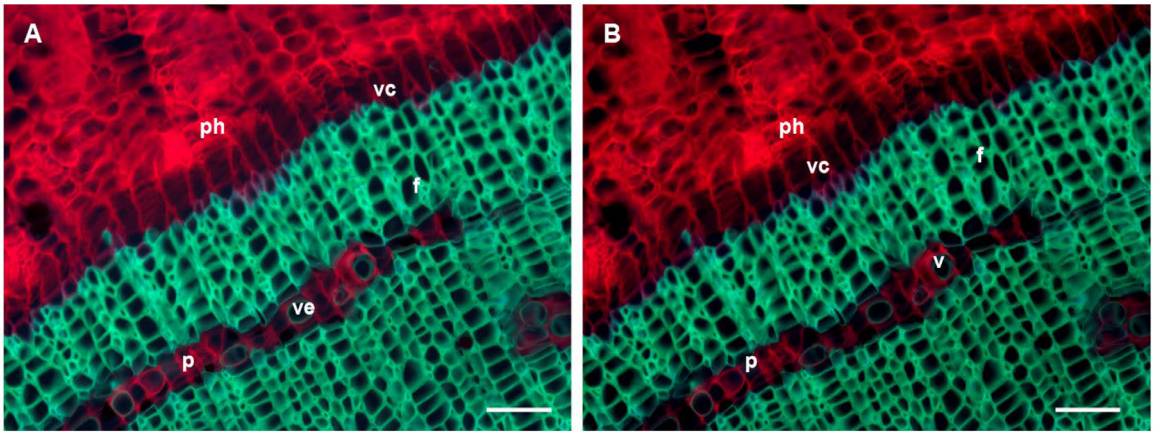

Fig. 2. Secondary xylem of *Thompsonella mixtecana*. Fluorescence subtraction of the background pixels using ImageJ. A. Image was obtained with the epifluorescence microscope. B. Image with fluorescence subtraction. f: fiber, p: parenchyma, ph: phloem, v: vessel, vc: vascular cambium. Scale bar: 100 μm.

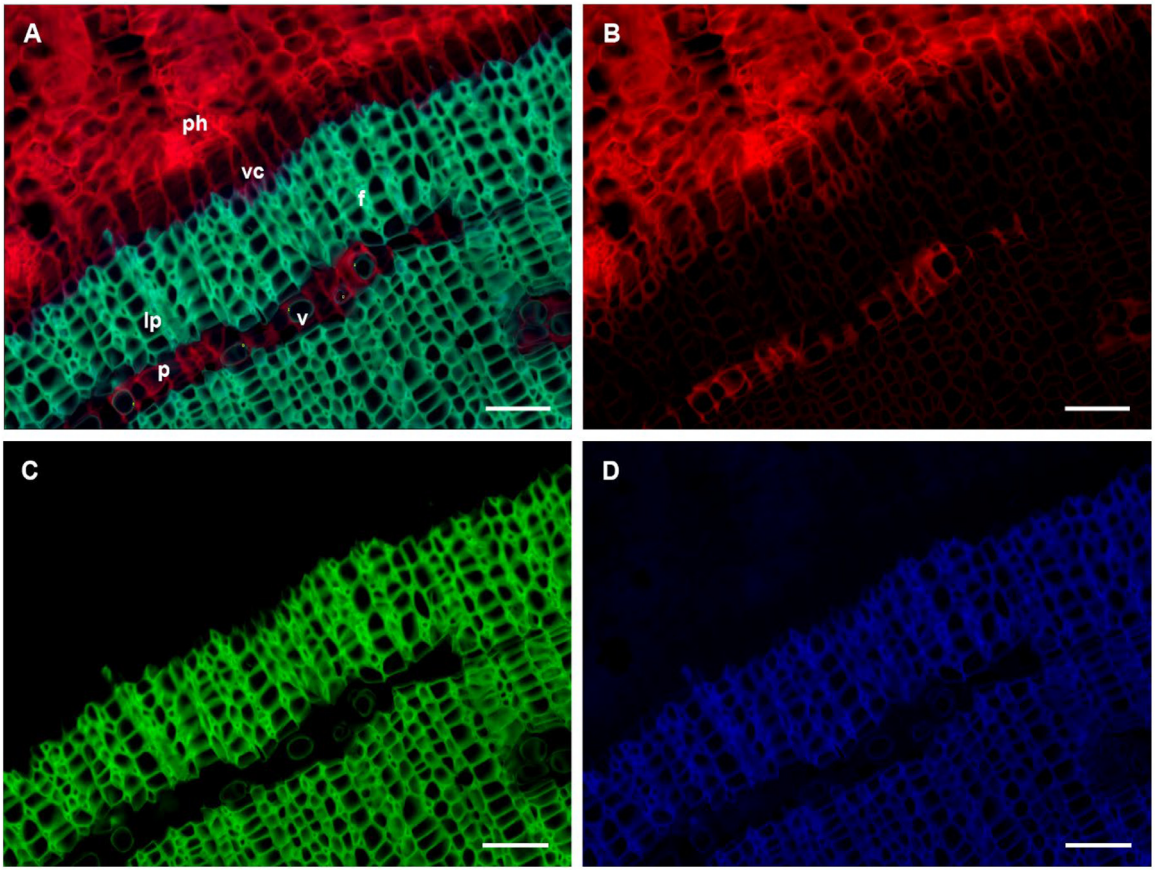

Fig. 3. Secondary xylem of *Thompsonella mixtecana*. Separation of the figure into the three channels red, green and blue. The color of each channel was added with ImageJ. A. Image with the channels joined and the 5 ROI frames. B. red channel, C. green channel. D. blue channel. f: fiber, lp: lignified parenchyma, p: parenchyma, ph: phloem, v: vessel, vc: vascular cambium. Scale bar: 100 μm.

Table 2
Example of the transformation of the values obtained in the intensity measurement into percentages.

	Vessel 1	Vessel 2	Vessel 3	Vessel 4	Vessel 5	Mean
Red Channel intensity	35.8	22.1	25.6	12.8	36.6	26.6
Green channel intensity	67	54.4	27.2	54.9	59.7	52.6
Blue channel intensity	61.8	51	39.6	56.8	53.7	52.6
Total intensity channels	164.6	127.5	92.4	124.5	150	131.8
Red channel%	21.7	17.3	27.7	10.3	24.4	**20.3**
Green channel%	40.7	42.7	29.4	44.1	39.8	**39.3**
Blue channel%	37.6	40	42.9	45.6	35.8	**40.4**
Total intensity%	100	100	100	100	100	100

	Parenchyma 1	Parenchyma 2	Parenchyma 3	Parenchyma 4	Parenchyma 5	
Red Channel intensity	11.3	17.0	16.5	10.7	15.5	14.2
Green channel intensity	128.5	121.2	107.8	114.1	106.3	115.6
Blue channel intensity	73.9	58.7	59.1	71.2	60.1	64.6
Total intensity channels	213.7	196.9	183.4	196.0	181.9	194.4
Red channel%	5.3	8.6	9.0	5.4	8.5	**7.4**
Green channel%	60.1	61.5	58.8	58.2	58.4	**59.4**
Blue channel%	34.6	29.8	32.2	36.3	33.1	**33.2**
Total intensity%	100	100	100	100	100	100

The fluorescence intensity measurement process is repeated for the red and blue channels. To compare the results of the three channels between species or treatments, the values of each channel are transformed into percentages using the following formula:$\%Fl = (VFl * 100\ \%) / Ti$. Where *VFl* is the value of the fluorescence intensity of channel X (red, green or blue), Ti is the value of the sum of the total intensity of the three channels (Table 2). The formula is applied to all three channels so they can be used to make comparative statistical analyzes to determine the variation in fluorescence emission of different cellular structures and between different species. For example, in the Table 2, the percentage of green channel intensity is greater in lignified parenchyma than in the lignified vessels and this is correlated with what we saw in the Fig. 3 between vessels and lignified parenchyma and fibers. What cannot be done is a comparison between dyes because each dye affects the fluorophores of lignin and cellulose differently.

Protocol validation

Comparison between stains

The images of the different staining methods can be seen in Fig. 4. *Thompsonella mixtecana* (Crassulaceae) was one of the species used to compare the different stains (Fig. 4A-D). Comparisons between the different types of staining were made with the same triple excitation and emission filter only to validate the methodology; however, each stain may present improvements depending on the filter used and even the microscope used (epifluorescence or confocal). In this work, the multiple filter allowed comparisons to be made quickly, while the image analysis was carried out with the ImageJ software.

Calcofluor (Ca) and autofluorescence (Au) present similar fluorescence emission tones in the parenchyma because bluish tones are observed for both cellulosic tissue and lignified tissue. In safranin-fast green (SFg) and Congo red-acridine orange (CrA) stains, the tones observed are red for parenchyma and bluish-green tones for lignified tissue. Empty spaces such as the cellular lumen are observed in black tones. Where there are secondary metabolites such as tannins, they are observed in orange tones.

Comparison of staining with *Yucca* species was also carried out (Fig. 4E-F) and the similarity in the fluorescence emission patterns was observed. With the Ca and Au stains, the tones of the lignified tissue are greenish-yellow, while the parenchyma has bluish tones in Ca staining (Fig. 4G). In the SFg and CrA stains, the lignified tissue has green to bluish tones while the cellulosic tissue has red tones.

Based on Fig. 4, it can be seen that the fluorescence emission with SFg is useful for the observation of the different lignified and cellulosic structures, in addition to the fact that the quality is similar or superior compared to the other stains that have been developed for its use with fluorescence microscopy.

Ca staining does not allow contrasts between cellulose and lignin, which makes the separation of the compounds difficult, although it is widely used for the identification of cellulose in plants [29–31].

Acridine orange dye can fluoresce green as a single molecule or red when it is a dimer. Additionally, it can stain non-lignified walls by intercalating or negatively binding with charged carbonyl functional groups [9]. Therefore, to avoid this double fluorescence emission, washes are carried out at a low concentration. However, there may be remnants in the stain [17,32]. In addition, Congo red stain some parts of the lignified cell walls [18], as can be seen in Fig. 4B, so care must be taken in interpreting the emission of fluorescence colors, a situation that does not occur with SFg staining.

For autofluorescence, although no marked differences were observed in the lignified tissue in *T. mixtecana* and *Yucca* sp., it is useful to identify fluorescent structures [9], differences in the composition, and concentration of lignin [33,34]. Therefore, by presenting a

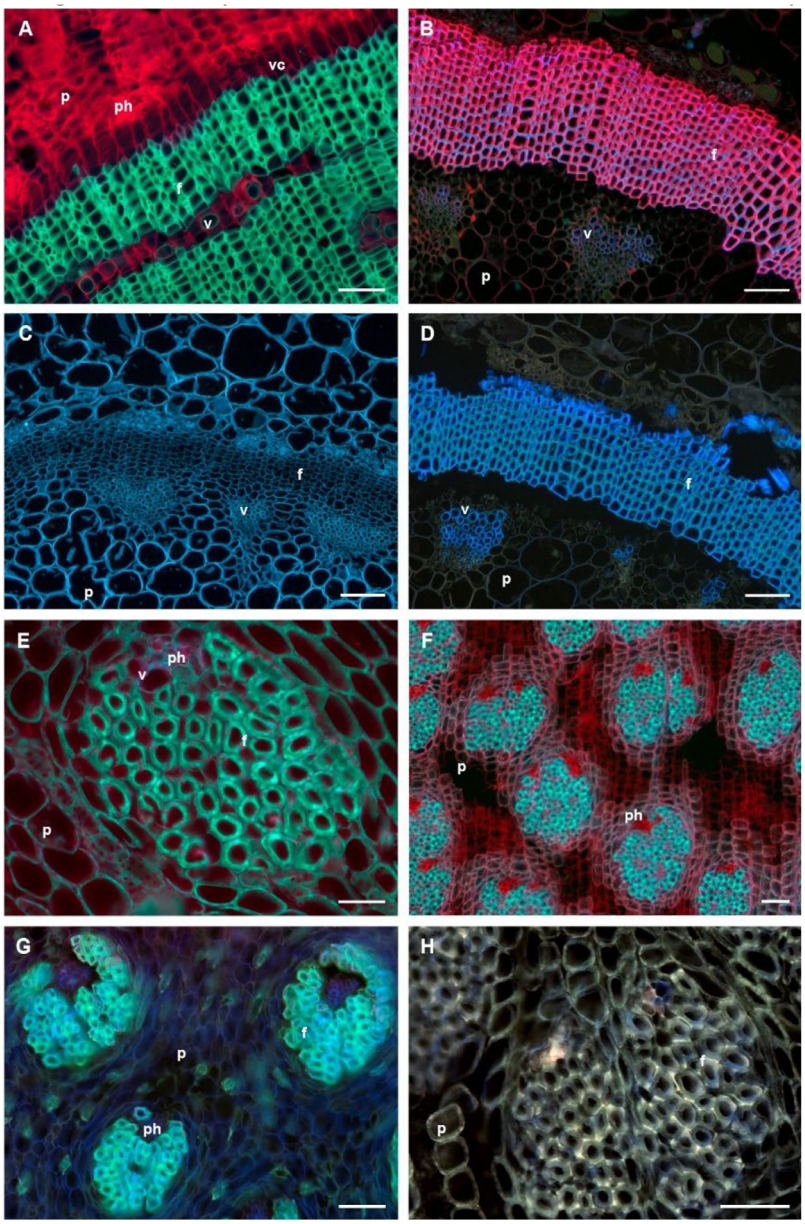

Fig. 4. Different stains and autofluorescence of *Thompsonella mixtecana* (A-D) and *Yucca periculosa* (E-H). A, E) Safranin-fast green. B, F) Congo red and acridine orange. C, G) Calcofluor. D, H) Autofluorescence. f: fibers, p: parenchyma, ph: phloem, v: vessel, vc: vascular cambium. Scale bar: A-B and G-H = 100 μm, E = 50 μm, F = 200 μm.

wide range of fluorescence emission, fluorescence lignin was used to characterize wood and delignification or degradation processes [34–39].

In Fig. 5, we can see that with SFg staining, transverse, longitudinal and radial sections can be observed. The fluorescence emission corresponds between the different sections. The primary walls will emit red tones corresponding to cellulose, while the secondary walls will emit blue, green or yellow tones, depending on whether fibers, vessels or lignified parenchyma are observed. In addition, details such as the presence of gums that fluoresce in red tones and secondary metabolites in green tones can be observed in the vessels lumina of *Cnidoscolus spinosus* Lundell (Fig. 5A, B).

In the bark of *Dendropanax arboreus* (L.) Decne. & Planch. the non-lignified parenchyma (red tones) and the lignified sclereids (green tones) could be observed as well as all xylem in green tones (Fig. 5C). In the xylem radial section of *D. arboreus,* the majority of cells had green tones due to the presence of lignin in the pits of vessels, fibers and parenchyma, the perforation plates, and in the primary walls of the fibers the presence of cellulose in red tones is observed. In the case of leaves, trichomes have fluorescence

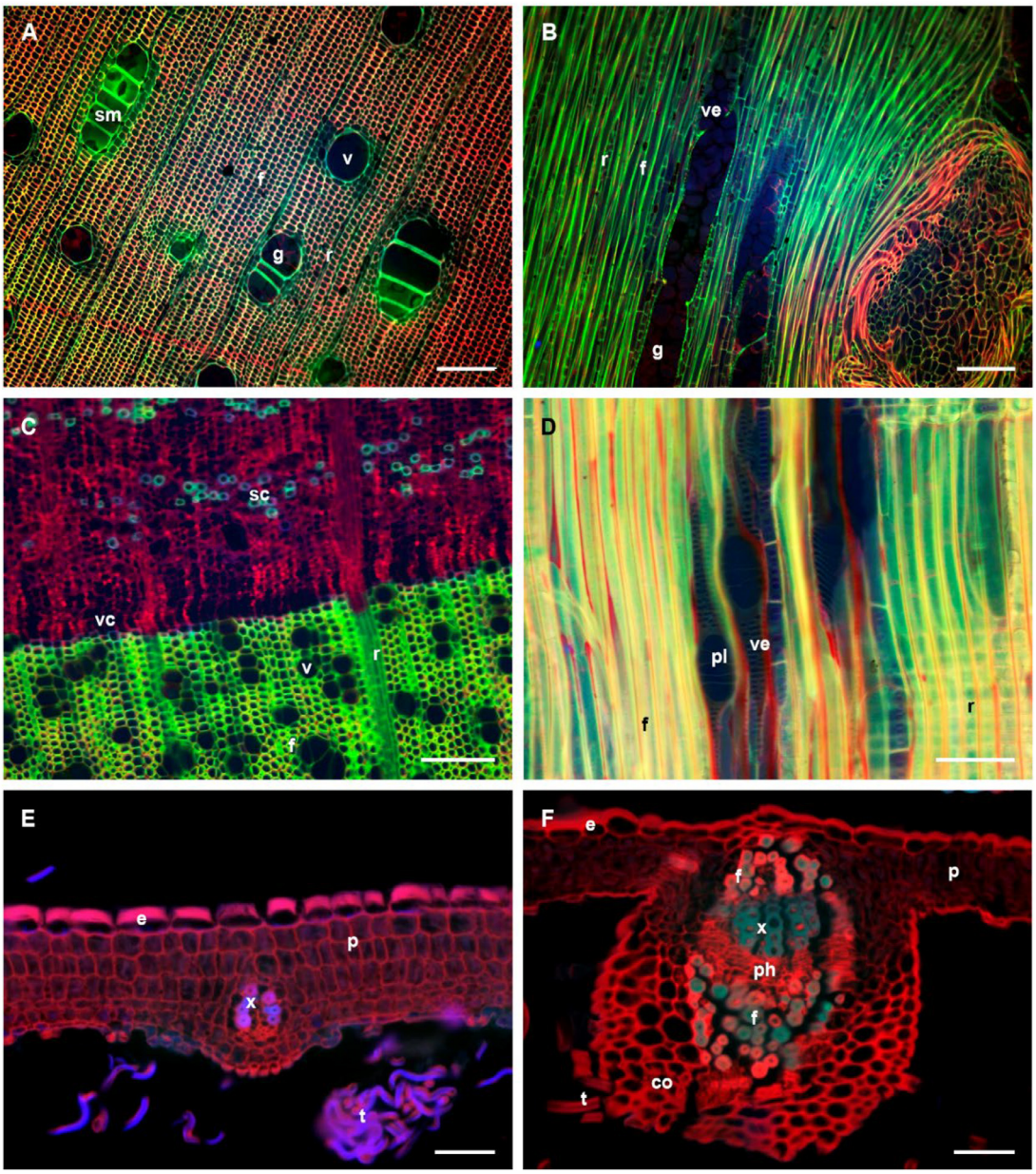

Fig. 5. Transverse, longitudinal and radial sections of stem (s) and leaves (l) of different plant species. A, B) *Cnidoscolus spinosus* (s). C, D) *Dendropanax arboreus* (s). E) *Nahuatlea hiriartiana* (l). F) *Tehuasca magna* (l). A, C, E, F) Transverse sections. B) Longitudinal tangential section. D) Longitudinal radial section. e: epidermis, co: collenchyma, f: fibers, g: gums, p: parenchyma, ph: phloem, pl: perforation plate, r: ray, sc: sclereids, sm: secondary metabolites, t: trichomes, v: vessel, ve: vessel elements, vc: vascular cambium, x: xylem. Scale bar: A-D = 100 μm. E-F = 50 μm.

emission in blue tones due to the presence of lignin similar to the vessels of the midvein (Fig. 5E). The non-lignified parenchyma is noticeable in the midvein, including the epidermis and collenchyma, compared to the presence of lignified tissue only in vascular bundle, vessels, and fibers (Fig. 5F).

Fluorescence intensity analysis and bright field microscopy

With the triple band filter (DAPI-FITC-TRITC), fluorescence intensity measurements can be made for all the previously mentioned stains, by separating the three channels as seen in Fig. 3. In bright field microscopy, the intensity of safranin-stained tissue varies in relation to the amount of lignin present in the sample [40]. Therefore, different degrees of lignification can be observed in various structures and cell walls, similar to what was reported by [34] who, with autofluorescence, observed that there

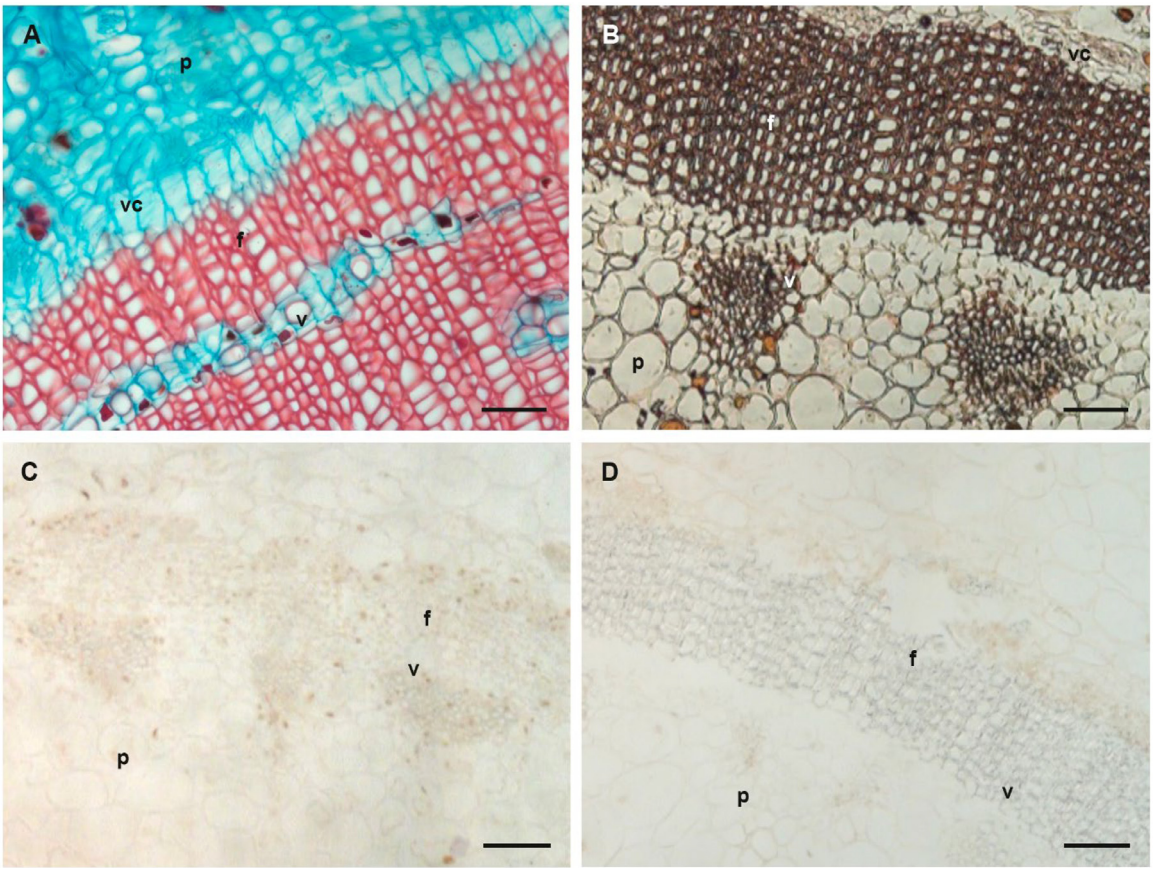

Fig. 6. Bright field micrographs of *Thompsonella mixtecana*. A) Safranin-green fast. B) Congo red and acridine orange. C) Calcofluor. D) Autofluorescence. f: fibers, p: parenchyma, r: ray, v: vessel, vc: vascular cambium. Scale bar: A = 100 μm, B-D = 200 μm.

was greater fluorescence intensity in the middle lamella and in the corners of the conifer cells due to a greater accumulation of lignin [34].

The fluorescence emission in the different channels varies in each stain, due to the influence of molecular environments as pH, temperature, concentration of dyes, and the interaction between fluorophores [9]. Therefore, the comparisons of intensity values must be made between the same stains and not between stains, since different values would be obtained [25,41].

The advantage between SFg staining with the two stains and autofluorescence is that, as it is permanent, observations can be made on samples that have been stored for decades, as long as synthetic resin (Entellan) has been used as a mounting medium and not Canada balsam because presents strong autofluorescence that affects the visibility of the sample with fluorescence microscopy [42]. Furthermore, samples stained with SFg can be used for both bright field microscopy and fluorescence microscopy, so they can have a dual purpose (Fig. 6A). Donaldson [34] mentioned that the use of glycerol and phosphated buffer (1:1 v/v) at pH 9 was the optimal mounting medium for lignin fluorescence. The Congo red – Acridine orange (Fig. 6B), calcofluor (Fig. 6C), and Autofluorescence (Fig. 6D) stains do not allow observations with bright field microscopy. In Fig. 4, the CrA, Ca, and Au stains had this mounting medium, and the images had a quality similar to that of the synthetic resin (Fig. 4A).

On the other hand, based on the fluorescence emission, assumptions can be made about the chemical composition of lignin [22], because by purifying and analysing lignin using Fourier transform infrared spectroscopy. It can be related to the differences in the fluorescence emission of the lignified walls. The light green to bluish tones with a wavelength of 420 nm to 510 nm, would correspond to the presence of syringyl type lignin (higher abundance of syringyl monomers) (Fig. 7A). The dark green to yellow tones with wavelengths from 510 nm to 610 nm would correspond to guaiacyl-type lignin (with a greater amount of guaiacyl monomers) (Fig. 7B). Red tones correspond to the presence of cellulose (Fig. 7A) and when cyan tones are observed within the parenchyma cells it is related to the presence of starches (Fig. 7C).

At this point, this protocol allows structural characterization of plants to be carried out at low cost compared, for example, to other specific staining methods that involve immunohistochemistry [43,44]. Then, to identify the presence of starches (Figure S1 A), secondary metabolites (Figure S1 B), suberin (in bark) (Figure S1 D), cutin (in seeds or leaves, Figure S1 C) and cellulose (Figure S1 in non-lignified parenchyma), is a quick way to carry out these characterizations.

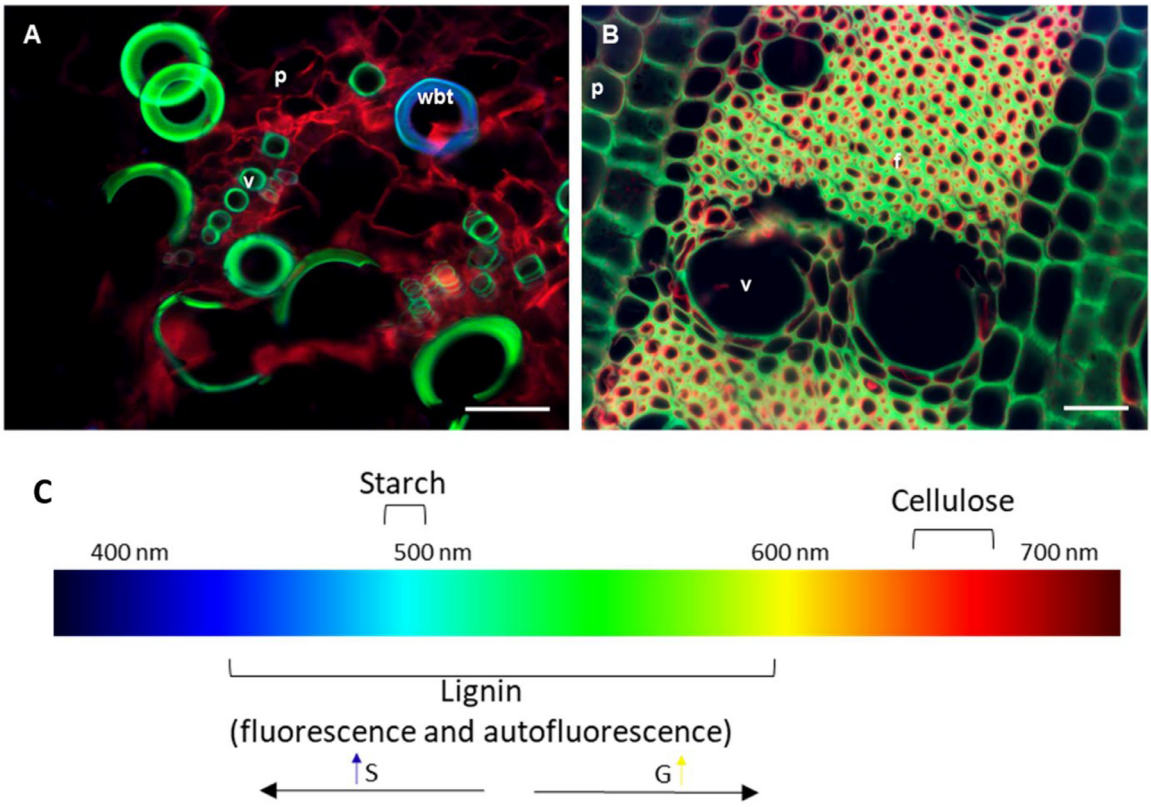

Fig. 7. Relationship between fluorescence emission tones with the structural composition of the observed wall. A) Wide-band tracheids of *Opuntia streptacantha* with different fluorescence emission. B) Similarity in fluorescence emission between vessels and parenchyma but different from fibers of *Dendropanax arboreus*. C) Scale of the wavelength of light and the structures that emit fluorescence. f: fibers, p: parenchyma, v: vessel, wbt: wide-band tracheids. Scale bar: 50 μm.

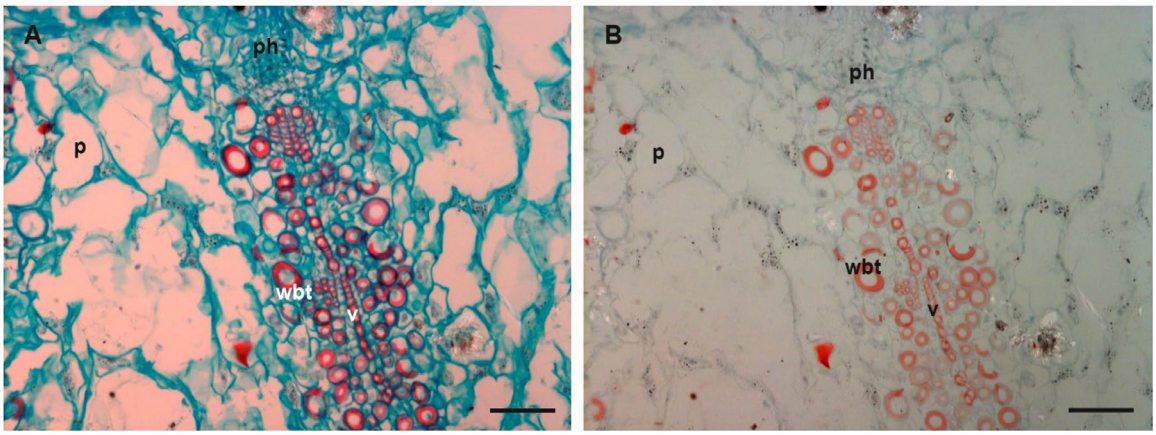

Fig. 8. Loss of SFg staining when taking images with bright field microscopy in *Pterocactus australis*. A. Before photobleaching. B. After photobleaching of the sample. p: parenchyma, v: vessel, wbt: wide-band tracheids. Scale bar: 100 μm.

Limitations

The main limitation of this method is the time it takes to photobleach unwanted fluorescence. Because, for each area to be taken photos, it takes 10 min to expose the sample to fluorescent light, while in both staining and autofluorescence, images are taken immediately. Furthermore, once the sample is photobleached, the stain is lost to be able to take images with bright field microscopy, mainly the fast green stain is the one that is lost (before photobleaching Fig. 8A, after photobleaching Fig. 8B), so images must first

be taken in the field. clear and later with fluorescence. Also, the drying time of the resin is at least a week, while for the other stains and autofluorescence, the mounting medium allows photos to be taken immediately after an hour of drying of the nail varnish.

CRediT author statement

Agustín Maceda and Teresa Terrazas: Conceptualization, development, writing of the original preparation of the manuscript. **Rosa Andrés:** validation of the protocol and review of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

The funding was provided by the DGAPA-UNAM postdoctoral fellowship to AM (CJ IC/CTIC I5OO7I2O2I) and by the DGAPA-PAPIIT: UNAM grants IN209012 and IN210115 to TT.

Authors thank Jerónimo Reyes for providing us with plant material of Crassulaceae, Roberto Kiesling for collecting with us South American cacti, Pedro Mercado Ruaro, Morpho-Anatomy and Cytogenetics Laboratory (LANABIO, UNAM) for allowing us to use the fluorescence microscope, Alicia Rojas-Leal for lab work and Julio César Montero-Rojas for artwork. The positive comments of both reviewers are appreciated.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2024.102999.

References

[1] C. Plomion, G. Leprovost, A. Stokes, Wood formation in trees, Plant Physiol. 127 (2001) 1513–1523, doi:10.1104/pp.010816.
[2] Y. Uraki, K. Koda, Utilization of wood cell wall components, J. Wood Sci. 61 (2015) 447–454, doi:10.1007/s10086-015-1492-9.
[3] L. Donaldson, Autofluorescence in plants, Molecules. 25 (2020) 2393, doi:10.3390/molecules25102393.
[4] L.A. Donaldson, K. Radotic, Fluorescence lifetime imaging of lignin autofluorescence in normal and compression wood, J. Microsc. 251 (2013) 178–187, doi:10.1111/jmi.12059.
[5] L. Donaldson, A. Vaidya, Visualising recalcitrance by colocalisation of cellulase, lignin and cellulose in pretreated pine biomass using fluorescence microscopy, Sci. Rep. 7 (2017) 44386, doi:10.1038/srep44386.
[6] N. Bergau, A.N. Santos, A. Henning, G.U. Balcke, A. Tissier, Autofluorescence as a signal to sort developing glandular trichomes by flow cytometry, Front. Plant Sci. 7 (2016) 949, doi:10.3389/fpls.2016.00949.
[7] L. Donaldson, N. Williams, Imaging and spectroscopy of natural fluorophores in pine needles, Plants 7 (2018) 1–16, doi:10.3390/plants7010010.
[8] R. Jupa, V. Didi, J. Hejátko, V. Gloser, An improved method for the visualization of conductive vessels in arabidopsis thaliana inflorescence stems, front, Plant Sci. 6 (2015) 211, doi:10.3389/fpls.2015.00211.
[9] P. Kitin, S. Nakaba, C.G. Hunt, S. Lim, R. Funada, Direct fluorescence imaging of lignocellulosic and suberized cell walls in roots and stems, AoB Plants. 12 (2020) 1–19, doi:10.1093/aobpla/plaa032.
[10] P. Kitin, J.C. Hermanson, H. Abe, S. Nakaba, R. Funada, Light microscopy of wood using sanded surface instead of slides, IAWa J. 37 (2021) 1–14, doi:10.1163/22941932-BJA10061.
[11] K. Radotić, A. Kalauzi, D. Djikanović, M. Jeremić, R.M. Leblanc, Z.G. Cerović, Component analysis of the fluorescence spectra of a lignin model compound, J. Photochem. Photobiol. B Biol. 83 (2006) 1–10, doi:10.1016/j.jphotobiol.2005.12.001.
[12] G. Paës, Fluorescent probes for exploring plant cell wall deconstruction: a review, Molecules. 19 (2014) 9380–9402, doi:10.3390/molecules19079380.
[13] J. Bond, L. Donaldson, S. Hill, K. Hitchcock, Safranine fluorescent staining of wood cell walls, Biotech. Histochem. 83 (2008) 161–171, doi:10.1080/10520290802373354.
[14] F. Baldacci-Cresp, C. Spriet, L. Twyffels, A. Blervacq, G. Neutelings, M. Baucher, S. Hawkins, A rapid and quantitative safranin-based fluorescent microscopy method to evaluate cell wall lignification, Plant J. 102 (2020) 1074–1089, doi:10.1111/tpj.14675.
[15] Q. Liu, X. Li, J. Fettke, Starch granules in arabidopsis thaliana mesophyll and guard cells show similar morphology but differences in size and number, Int. J. Mol. Sci. 22 (2021) 5666, doi:10.3390/ijms22115666.
[16] B.G. Ferreira, R. Falcioni, L.M. Guedes, S.C. Avritzer, W.C. Antunes, L.A. Souza, R.M.S. Isaias, Preventing false negatives for histochemical detection of phenolics and lignins in peg-embedded plant tissues, J. Histochem. Cytochem. 65 (2017) 105–116, doi:10.1369/0022155416677035.
[17] S. Nakaba, P. Kitin, Y. Yamagishi, S. Begum, K. Kudo, W.D. Nugroho, R. Funada, Three-dimensional imaging of cambium and secondary xylem cells by confocal laser scanning microscopy, Plant Microtech. Protoc. (2015) 431–465, doi:10.1007/978-3-319-19944-3_24/COVER.
[18] M.A. Hubbe, R.P. Chandra, D. Dogu, S. van Velzen, Analytical staining of cellulosic materials: a review, Bioresources 14 (2019) 7387–7464.
[19] S. Loza-Cornejo, T. Terrazas, Anatomía del tallo y de la raíz de dos especies de wilcoxia britton & rose (cactaceae) del noreste de México, Bot. Sci. 59 (1996) 13–23, doi:10.17129/botsci.1502.
[20] S. Loza-Cornejo, T. Terrazas, Epidermal and hypodermal characteristics in North American Cactoideae (Cactaceae), J. Plant Res. 116 (2003) 27–35, doi:10.1007/s10265-002-0066-2.
[21] A. Dié, P. Kitin, F.N. Kouamé, J. Van den Bulcke, J. Van Acker, H. Beeckman, Fluctuations of cambial activity in relation to precipitation result in annual rings and intra-annual growth zones of xylem and phloem in teak (Tectona grandis) in Ivory Coast, Ann. Bot. 110 (2012) 861–873, doi:10.1093/aob/mcs145.
[22] A. Maceda, J. Reyes-Rivera, M. Soto-Hernández, T. Terrazas, Distribution and chemical composition of lignin in secondary xylem of Cactaceae, Chem. Biodivers. 18 (2021) e2100431, doi:10.1002/CBDV.202100431.

[23] A. Maceda, M. Soto-Hernández, T. Terrazas, Cellulose in secondary xylem of cactaceae: crystalline composition and anatomical distribution, Polymers. (Basel) 14 (2022) 4840, doi:10.3390/POLYM14224840.
[24] A. Maceda, M. Soto-Hernández, C.B. Peña-Valdivia, C. Trejo, T. Terrazas, Differences in the structural chemical composition of the primary xylem of cactaceae: a topochemical perspective, Front. Plant Sci. 10 (2019), doi:10.3389/fpls.2019.01497.
[25] J.C. Waters, Accuracy and precision in quantitative fluorescence microscopy, J. Cell Biol. 185 (2009) 1135–1148, doi:10.1083/jcb.200903097.
[26] N. Feder, T.P. O'Brien, Plant microtechnique: some principles and new methods, Am. J. Bot. 55 (1968) 123–142, doi:10.1002/j.1537-2197.1968.tb06952.x.
[27] A. Maceda, M. Soto-Hernández, C.B. Peña-Valdivia, C. Trejo, T. Terrazas, Differences in the structural chemical composition of the primary xylem of cactaceae: a topochemical perspective, Front. Plant Sci. 10 (2019) 1497, doi:10.3389/fpls.2019.01497.
[28] S. Fontenete, D. Carvalho, A. Lourenço, N. Guimarães, P. Madureira, C. Figueiredo, N.F. Azevedo, FISHji: new ImageJ macros for the quantification of fluorescence in epifluorescence images, Biochem. Eng. J. 112 (2016) 61–69, doi:10.1016/j.bej.2016.04.001.
[29] J. Hughes, M.E. McCully, The Use of an Optical brightener in the study of plant structure, Stain. Technol. 50 (1975) 319–329, doi:10.3109/10520297509117082.
[30] D.W. Galbraith, Microfluorimetric quantitation of cellulose biosynthesis by plant protoplasts using Calcofluor White, Physiol. Plant. 53 (1981) 111–116, doi:10.1111/j.1399-3054.1981.tb04119.x.
[31] J. Flores-Félix, E. Menéndez, M. Marcos-García, L. Celador-Lera, R. Rivas, Calcofluor white, an alternative to propidium iodide for plant tissues staining in studies of root colonization by fluorescent-tagged rhizobia, J. Adv. Biol. Biotechnol. 2 (2015) 65–70, doi:10.9734/JABB/2015/12444.
[32] C.J. Houtman, P. Kitin, J.C.D. Houtman, K.E. Hammel, C.G. Hunt, Acridine orange indicates early oxidation of wood cell walls by fungi, PLoS. One 11 (2016) e0159715, doi:10.1371/journal.pone.0159715.
[33] B. Albinsson, S. Li, K. Lundquist, R. Stomberg, The origin of lignin fluorescence, J. Mol. Struct. 508 (1999) 19–27, doi:10.1016/S0022-2860(98)00913-2.
[34] L. Donaldson, Softwood and hardwood lignin fluorescence spectra of wood cell walls in different mounting media, IAWa J. 34 (2013) 3–19, doi:10.1163/22941932-00000002.
[35] L.A. Donaldson, A.P. Singh, A. Yoshinaga, K. Takabe, Lignin distribution in mild compression wood of *Pinus radiata*, Can. J. Bot. 77 (1999) 41–50, doi:10.1139/b98-190.
[36] L. Donaldson, K. Radotić, A. Kalauzi, D. Djikanović, M. Jeremić, Quantification of compression wood severity in tracheids of Pinus radiata D. Don using confocal fluorescence imaging and spectral deconvolution, J. Struct. Biol. 169 (2010) 106–115, doi:10.1016/j.jsb.2009.09.006.
[37] V. De Micco, G. Aronne, Combined histochemistry and autofluorescence for identifying lignin distribution in cell walls, Biotech. Histochem. 82 (2007) 209–216, doi:10.1080/10520290701713981.
[38] V. De Micco, G. Aronne, Anatomy and lignin characterisation of twigs in the chaparral shrub rhamnus californica, IAWa J. 33 (2012) 151–162, doi:10.1163/22941932-90000086.
[39] V. De Micco, K. Ruel, J.-P. Joseleau, J. Grima-Pettenati, G. Aronne, Xylem anatomy and cell wall ultrastructure of nicotiana tabacum after lignin genetic modification through transcriptional activator EgMYB2, IAWa J. 33 (2012) 269–286, doi:10.1163/22941932-90000093.
[40] J. Bond, L. Donaldson, S. Hill, K. Hitchcock, Safranine fluorescent staining of wood cell walls, Biotech. Histochem. 83 (2008) 161–171, doi:10.1080/10520290802373354.
[41] J. Pawley, The 39 Steps: a cautionary tale of quantitative 3-d fluorescence microscopy, Biotechniques 28 (2000) 884–887, doi:10.2144/00285bt01.
[42] S. Ravikumar, R. Surekha, R. Thavarajah, Mounting media: an overview, J. Dr. NTR Univ. Health Sci. 3 (2014) S1–S8.
[43] Y. Verhertbruggen, J.L. Walker, F. Guillon, H.V. Scheller, A comparative study of sample preparation for staining and immunodetection of plant cell walls by light microscopy, Front. Plant Sci. 8 (2017), doi:10.3389/fpls.2017.01505.
[44] R. Ursache, T.G. Andersen, P. Marhavý, N. Geldner, A protocol for combining fluorescent proteins with histological stains for diverse cell wall components, Plant J. 93 (2018) 399–412, doi:10.1111/TPJ.13784.