IMPROVED METHODS OF CANE SAMPLES STAINING DESTINED FOR MICROSCOPIC EXAMINATION AND IMAGING

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Abstract. In this study, staining methods were used to improve the effective visualization of grapevine cane sections under a microscope using 'ring light'. The stains applied to the sections included Aniline Blue, Safranin O, Toluidine Blue O, Bromophenol Blue, Basic Fuchsin, Methyl Green, Giemsa Stain, Carmine and Fast Green FCF. The natural color differences observed between the tissues were similar. Therefore, staining was proven to be the most useful tool to increase the contrast in grapevine cane tissues. Different stains reacted with different cell types and components and revealed their unique color properties. In addition to single use of stains, Carmine + Methyl Green, Fast Green FCF + Bromophenol Blue and Safranin O + Bromophenol Blue were utilized as double staining. Among these applications, staining with Aniline Blue, Bromophenol Blue, Fast Green FCF, Safranin O, and Toluidine Blue O showed effective results on cell and tissue levels. In addition, successful results were obtained from double stains. Double staining was recommended for examining grapevine cane sections under a microscope. 'Ring light' has been developed to improve the coloration and sharpness on the surface of the stained sections under the microscope and during photography. The ring light offers the opportunity to illuminate the entire sample by using white light, yellow light and both light intensities in different proportions.
Keywords: *sections, grapevine, stains, tissue, cane, stem*

Introduction

The development of technological methods has made it largely possible to visualize tissues and cell structures in plants (Žárský, and Cvrčková, 2014), but still dyeing of internal tissues proves to be valuable for quick and elaborate microscopic examination. Staining techniques were originally used to protect wooden materials. Later, they enabled scientists to gain information about plant anatomy. Their use in the identification of plant tissues began with Ehrlich in 1877, who used Safranin (Smith, 1915; Bracegirdle, 1986).

There are four groups of stains: positive stains, negative stains, nucleotide binding stains, and fluorescent proteins. Those that stains molecules by binding chemically in the sample are positive stains. As opposed, negative stains do not adhere to the sample. Stains that bind to genetic material of any type are called nucleotide binding stains. Fluorescent protein is a barrel-shaped molecule with a light-emitting chromophore at its center (Hulse, 2018).

Many studies have been conducted on staining mechanisms, theories and methods (Berlyn and Miksche, 1976; Horobin, 1982; Gahan, 1984; Ruzin, 1999; Horobin and Kiernan, 2002; Gökbayrak et al., 2023). The natural color differences in most plant stem tissues are minor. Therefore, too little concentration is available to show the surface and internal properties of such textures. The best way to increase the contrast of a sample in this way is through staining. In addition, with the appropriate selection of staining

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methods, a clear definition can be established by providing distinctive information on cell and tissue levels. The tools and chemicals used in preparing the samples, the methods used to soften and fix the hardwood tissue and the cleaning and drying of thin sections may affect the staining results.

The need to improve various methods for observing plant sections under a microscope is necessary. Staining techniques especially adapted to hard and woody stem anatomy, where natural color differences between tissues are minimal, should be developed. Since stains react with different tissue components to reveal unique color properties, different staining methods should be used to highlight them. In addition, tissues can be made translucent to highlight cell walls with their components, and compounds such as pigments and phenolics, which can obscure images, should be eliminated from the tissues. For this purpose, the success of some commercial chemicals, and reagents such as Aniline Blue, Safranin O, Toluidine Blue O, Bromophenol Blue, Basic Fuchsin, Methyl Green, Giemsa Stain, Carmine, Fast Green were used on grapevine cane sections to determine the best staining and imaging methods.

Materials and methods

Preparation of cross sections for staining

In the research, Cabernet Sauvignon (*Vitis vinifera* L.) grapevine cultivar was used as plant material. Considering the tissue profile structure and function of Cabernet Sauvignon cultivar, the natural color differences in the sections are very close to each other. Cane samples of the cultivar (approximately 1 cm in diameter and 6-8 cm long) were taken during the winter rest period and placed in PE bags at +1°C until used.

Staining of the cane sections included a series of processes: washing, and cleaning, fixing, and storing, softening, and sectioning of the cane parts. The cleaning and washing were carried out by depending on the tissue type and the dimension of the material to be examined. Following washing the samples under tap water, the cleaning process was performed with pure water. During the fixation and storage process, it is important to protect the tissue structure and cells of the samples from deterioration. Two different fixing and storage methods are generally used for this purpose (Gökbayrak, et al. 2024). The first is FAA (formaldehyde, alcohol, and acetic acid). The second is the Copenhagen mixture (ethanol, pure water, and glycerol). The samples were stored in 50 ml brown bottles with low light transmission, containing FAA solution (10:5:70, v/v). Softening the samples is important for woody sections. It is difficult to obtain good sections from overly softened or insufficiently softened samples. In the grapevine samples, the boiling method, also known as main softening, which has been proven effective in shrub and tree species, was used (Schubert et al. 1999).

The grapevine cane samples were sectioned as follow (Hacke, 2015). Very thin cross-sections are necessary for successful microscopic examinations. In this study, semi-automatic microtome (Reichert Jung, Germany) with a classical sliding was used. The samples removed from the fixing solution for sectioning with a microtome were prepared depending on their diameter. They were basically cut into four equal slices. Small-bladed hand scissors (Felco, Switzerland) were used for slicing. Before starting the cross-sectioning, the outside of the samples was covered with tape with epoxy embedded material. This process prevented the tissues from breaking and intertwining with the blade. After adjusting the microtome blade, 80% ethanol was dropped on the

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surface of the samples. The breadth of the cross-sections taken was approximately 100 μm. Very sharp blades were used and changed frequently. The cross-sections were placed in a petri dish also containing 80% ethanol before moving to the staining procedure. They examined under the microscope (Olympus SZ61, Japan) and samples with cracks were not included in the staining test.

Staining

Three different application methods are generally used in staining the sections. The first is to prepare pipettes filled with prepared stain solutions and drop staining solutions directly onto the section. The second is to immerse sections in the solution in a petri. Third, sections are transferred from one solution to another (Gökbayrak et al, 2023). The list of commercial stains and some materials used in the research was given in *Table 1*. Once labeled, all solutions were stored appropriately to prevent deterioration.

Due to their interaction inside cell and tissues, when stains enter a cell and tissue, they might produce a characteristic pattern of staining different from the staining of an already fixed cell. To achieve desired effects, the stains are used in very dilute solutions and times. For this reason, our research focused on the application of different stains for different periods of time.

Researchers have proposed different methods for section staining (Lancelle et al. 1986; Fields et al., 1997; Anderson and Bancroft, 2002; Bond et al., 2008; Hacke, 2015; Hacke et al., 2015). In our study, methods developed by us were used to stain the sections. In addition, three different double staining methods were examined in our research: Carmine and methyl green; fast green FCF and bromophenol blue; safranin O and bromophenol blue.

Single stains

Aniline blue (AB)

This dye comes with different names, such as diphenylamine blue, China blue, or soluble blue. It has an aqua blue color. It is a water-soluble dye that develops a yellow green color after application (Anderson and Bancroft, 2002). It was used to distinguish rust fungi on pines (Jewell, 1958). For the application of AB stain, a 0.5% solution was prepared in a petri dish. The sections were immersed in the solution for 5-10 min. The surface of the sections, from which excess dye was removed using filter paper, were washed with pure water, and examined under a microscope.

Toluidine blue O (TB)

TB gives a multicolored sample due to its nature of a cationic, polychromatic dye and variously responds to different chemical components in plant cells. It reacts to carboxylated polysaccharides producing a purplish color, to lignin and tannins a greenish blue or bright blue, and to nucleic acids a purplish or greenish blue (Fields et al., 1997). TB stain was directly applied to the sections for 4 min. Excess dye was later removed using filter paper. The surface of the sections was washed with pure water. This process was repeated 3 times until no more dye was left on the edges. A drop of pure water was added to the sections and they were made ready for examination under a microscope.

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Table 1. *Stains and some chemicals and materials used in their preparation*

Stains	Manufacturer	Prepare solution with	Suggested laboratory equipment
Aniline Blue	Sigma-Aldrich	Distilled water	Erlenmeyer, beakers, slides, coverslips of various size
Basic Fuchsin	Sigma-Aldrich	Ethanol	Amber glass bottles with glass eye dropper for stain solution
Bromophenol Blue	Carlo-Erba	Distilled water	Fine camel hairbrush, nitrile gloves
Carmine	Merck	Distilled water	Absorbent filter paper (to remove excess stain or solvent)
Fast Green FCF	Sigma-Aldrich	Distilled water	Hand scissors, knives, chisel
Giemsa Stain	Sigma-Aldrich	Ethanol	Pipettes, angled dissecting needle, tweezers, razor blades, scalpels
Methyl Green	Sigma-Aldrich	Distilled water	Wash bottles of various size for alcohol solutions
Safranin O	Sigma-Aldrich	Ethanol	Microscope slides of standard size
Toluidine Blue O	Merck	Distilled water	Stopwatch to observe for staining time, electronic precision scale

Safranin O (S)

S is a routine dye used in anatomy studies. It dissolves in water or alcohol to produce a red color. It stains lignified cells and structures (Bond et al., 2008). S stain in aqueous solution was filtered twice to remove residues before use. Excess stain was removed from the sections by using ethanol. 1% S solution (0.7 g S in 100 mL of pure water or 1 g S in 100 mL of 90% ethanol) were used.

Bromophenol blue (BB)

BB changes color from yellow to purple. It is a regressive stain with a tendency to bind to cellulose and is soluble either in water or alcohol (Camerero et al., 2010). It was applied directly to the sections for 3 min. The excess stain was removed using a filter paper. The surface of the sections was washed 3 times with pure water. A drop of pure water was added to the sections before examination under a microscope.

Methyl green staining (MG)

MG is a basic stain that binds to nucleic acids. It stains DNA, but is not specific. When used alone in all cells, it stains almost everything, especially the pectocellulosic wall (Anderson and Bancroft, 2002). For the application of MG stain, a 0.2% solution was prepared in a petri. The sections were immersed in the prepared solution for 5 min. The surface of the sections, from which excess stain was removed using filter paper, was washed with pure water and examined under a microscope.

Basic fuchsin staining (BF)

BF is a fluorescent stain consisting of rosaniline, magenta II, pararosaniline and fuschsine. The solution was prepared with 0.5 g BF in 50 ml ethanol (95%). The

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sections were immersed in the solution for 5 min. The surfaces of the sections from which excess stain was removed were washed with pure water and examined under a microscope.

Giemsa stain (GS)

GS is a mixture of methylene blue, eosin and azure. It is usually prepared from commercially available Giemsa powder. It is soluble in alcohol (Osipov and Andreyan, 2014). GS solution was prepared as 0.8 g GS in 100 ml ethanol (90%).

Fast green FCF (FG)

FG is a widely used to counterstain. Double filtered FG solution (0.5 g powdered FG in 100 ml of 90% alcohol) was used. Being a progressive stain, the section was immersed in the solution until the desired density was reached. Care is needed because an uneven staining may occur when sections are exposed to excess solution (Rauter and Zufa, 1972).

Carmine (C)

C is also called ‘cochineal’ stain because it is obtained from the cochineal bug. It is a bright red dye coming from red containing aluminum components. In our research, natural red and its special code name C.I. Serial 1% solution of 75470 was used.

Double stains

C and MG

After the sections were washed with pure water, they were kept in 70% ethanol for 3 min, then dipped in 0.1% C solution (containing 10% glycerol and 5% acetic acid) for 5 min. They were later rinsed three times in pure water to remove excess stain. Then, they were immersed in MG solution (0.2 g MG in 50 ml 95% ethanol) for 5 min. The samples dried at room temperature were examined under a microscope by adding a drop of pure water.

FG and BB

The sections were washed twice with pure water, kept in 50% ethanol for 5 min, then dipped in FG solution (0.5 g FG in 50 ml 95% ethanol) for 10 min. Then, it was immersed in 0.75% BB solution (containing 10% glycerol and 10% acetic acid) for 10 min and rinsed three times in pure water to remove excess stain. The samples dried at room temperature were examined under a microscope by adding a drop of pure water.

S and BB

The sections were washed 3 times with pure water, later kept in 1/1 ratio of 96% ethanol for 10 min, then in 1 g/L S solution for 20 min and rinsed 3 times in pure water to remove excess. After that, they were immersed in 0.75% BB solution (containing 10% glycerol and 10% acetic acid) for 25 min. Following the removal of excess stain with a filter paper, they were rinsed again three times in pure water. A drop of pure water was added to the sections before the examination.

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Imaging and photography of cross sections

LC20-Bundle LCmicro software program (Olympus Corp., Japan) was used to image and photograph the stained sections. The staining sections were examined under an Olympus SZX7 (Olympus Corp., Japan) stereomicroscope and an Olympus CX-41 (Olympus Corp., Japan) light microscope. The sections were imaged with a digital microscope camera (Olympus LC20) that can be connected to both microscopes, and the photographs were transferred to the computer. The properties of the images were checked in digital files. Imaging and photography under stereomicroscope were performed directly in petri dishes. To photograph the surface features of the examined samples more clearly, some sections were partially immersed in pure water. Since light microscope has a better resolving power than a stereomicroscope, thinner and smaller sections were examined under it.

In addition to providing appropriate lighting when examining sections, desired structures can be highlighted by creating shading. Different lighting should be provided on the sample when imaging and photographing the sections. For this purpose, we developed a 'ring light' that can be mounted on a microscope (*Fig 1*). The ring light offers a different illumination opportunity over the entire sample when viewing and photographing stained sections using a microscope. In addition, it provides illumination of the samples by using white light, yellow light and both light intensities in different proportions. The ring light technique can be used to increase the contrast in stained samples at the microscopic level. This technique was developed upon imaging-stained samples, typically using bright field or white and yellow illumination. Thus, the morphologies of certain cells and structures on contrasting colors can be easily seen and examined. The ring light model, unlike classical illumination, can be used to color the stained sample against a bright background. Since we have applied for a patent for the lighting model used, limited information on technical details are included.

Figure 1. *The 'ring light' model developed by us, providing yellow and white light illumination under the microscope*

Results and discussion

There are very few natural color differences in grapevine cane tissues (*Fig. 2*). Therefore, staining is the best way to increase the contrast of the tissues to illustrate surface and internal features. Toluidine blue O (*Fig. 3A*), safranin O (*Fig. 3B*), bromophenol blue (*Fig. 3C*), fast green FCF (*Fig. 3D*), aniline blue (*Fig. 3E*), methyl green (*Fig. 3F*) stains showed effective results in imaging cells and tissues under the microscope in cane.

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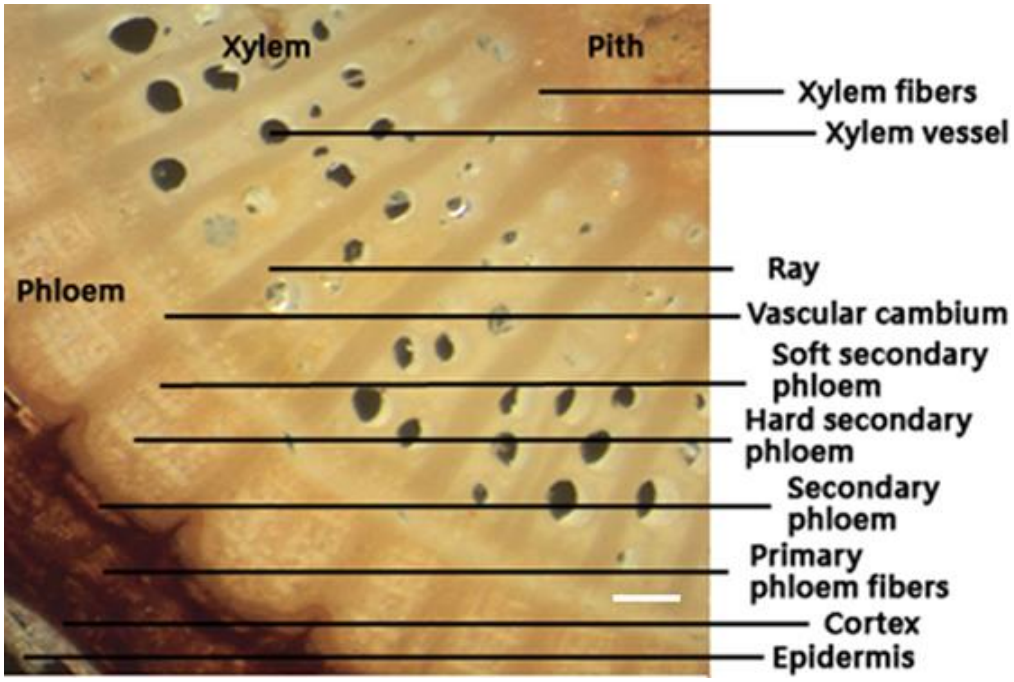

Figure 2. *Cell and tissues of grapevine cane sections, natural and unstained. Scale bar, 500 μm*

Different stainings allowed us to visualize the phloem ray parenchyma and the xylem ray parenchyma, as shown in *Figures 3, 4, 5* and *6*. Our findings regarding the stainings indicated a strong correlation between the substance concentrations in the tissues and the staining compounds. The correlation between these methods has been previously reported (Fanton et al., 2022).

TB and AB were one of the best stains on cane sections taken from thin canes (diameter smaller than 5 mm) (*Fig. 3A, E*). They reacted to different components of tissues and created color differences in the sections. It is known that TB reacts differently with different substances in a plant (Fields et al., 1997). Therefore, the colors appearing in TB staining in grapevine sections also provided information about the structure and walls of the cells. In lignified cane sections, cells with secondary walls generally appeared blue. Companion cells towards the pith were stained greenish purple. In the inner sections, reddish purple was more prominent.

S is easily soluble in water and alcohol and is used to stain lignified structures in the examination of branch anatomy due to its red color (Galigher and Kozloff, 1971; Ruzin, 1999). S created an effective red color on the examined sections. This effect increased even more as the waiting time in S solution increased. In S staining, it was necessary to remove excess stain from the sections. Otherwise, the very intense red color made it difficult to distinguish textures. For this purpose, sections with S staining should be rinsed with alcohol before being examined under the microscope. The waiting time for the sections in the solution could be reduced to prevent excessive S staining. Different S solutions applied to the sections (1% S solution, 0.7 g S in 100 mL pure water or 1 g S in 100 mL 90% ethanol) were effective and could easily be used as a double staining. However, it must be filtered for repeated applications. Otherwise, it would precipitate. In the grapevine sections examined, C did not give effective results when used alone. The use of a counterstain MG against C enabled discrimination between lignified tissues in cane sections (*Fig. 6*).

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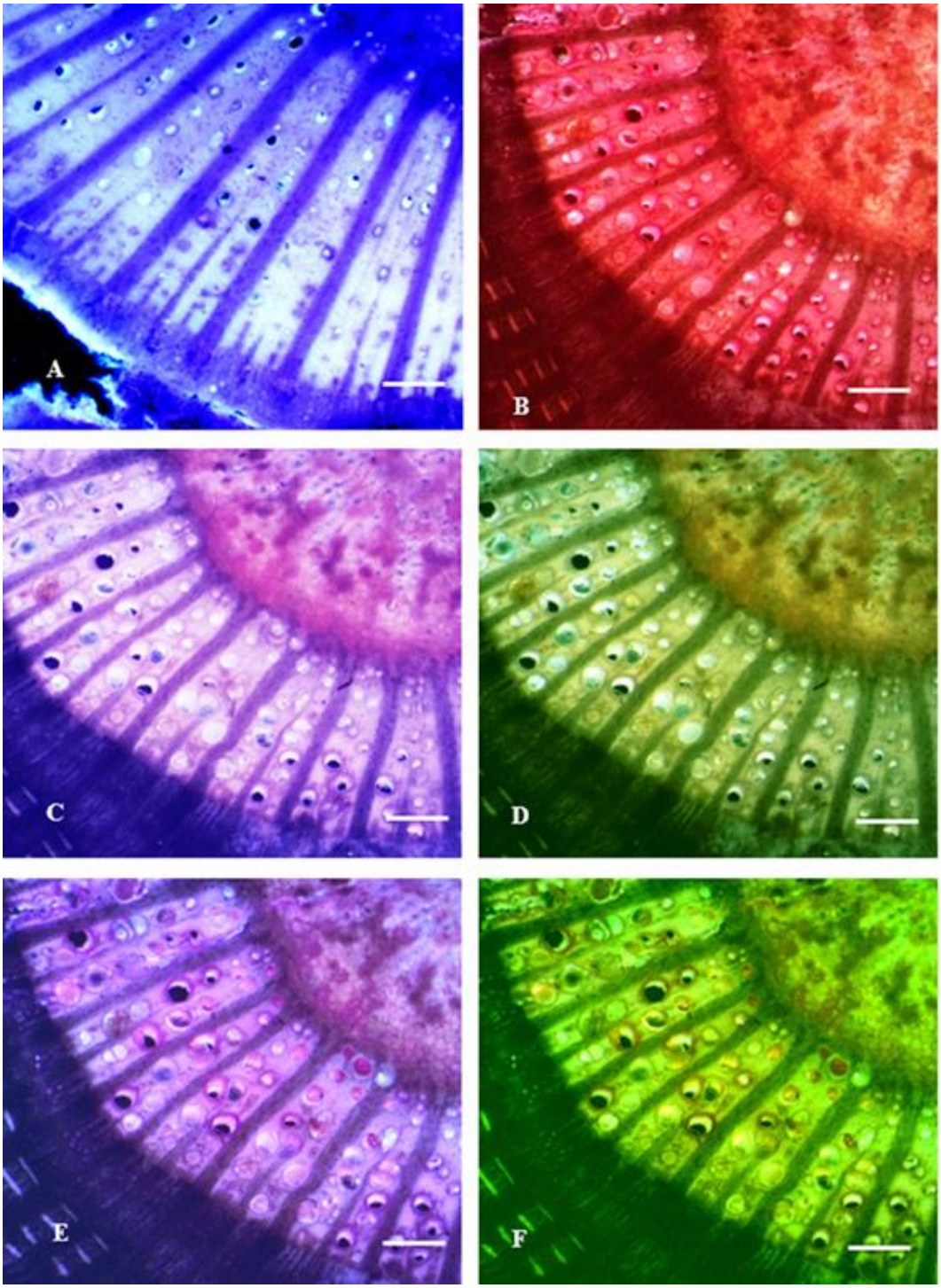

Figure 3. *Grapevine cane tissues stained with (A) toluidine blue O (TB), (B) safranin O (S), C) bromophenol blue (BB), (D) fast green FCF (FG), (E) aniline blue (AB), (F) methyl green (MG). Scale bar, 500 μm*

Our research shows that BB staining (*Fig. 3C*) may be among the counterstains that should be used. Studies show that AB and FG are the most used counterstains (Micco and Aronne, 2007). While S stains lignin regardless of the presence or absence of cellulose, AB has been determined to stain cellulose only in the absence of lignin

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(Schwarze, 2007). BB is an effective stain with affinity for cellulose. As a counterstain against S, it can be used to identify early stages of cane in grapevine cuttings. While S stains lignin, BB stains only cellulose and the distinction between tissues can be revealed (*Fig. 4*).

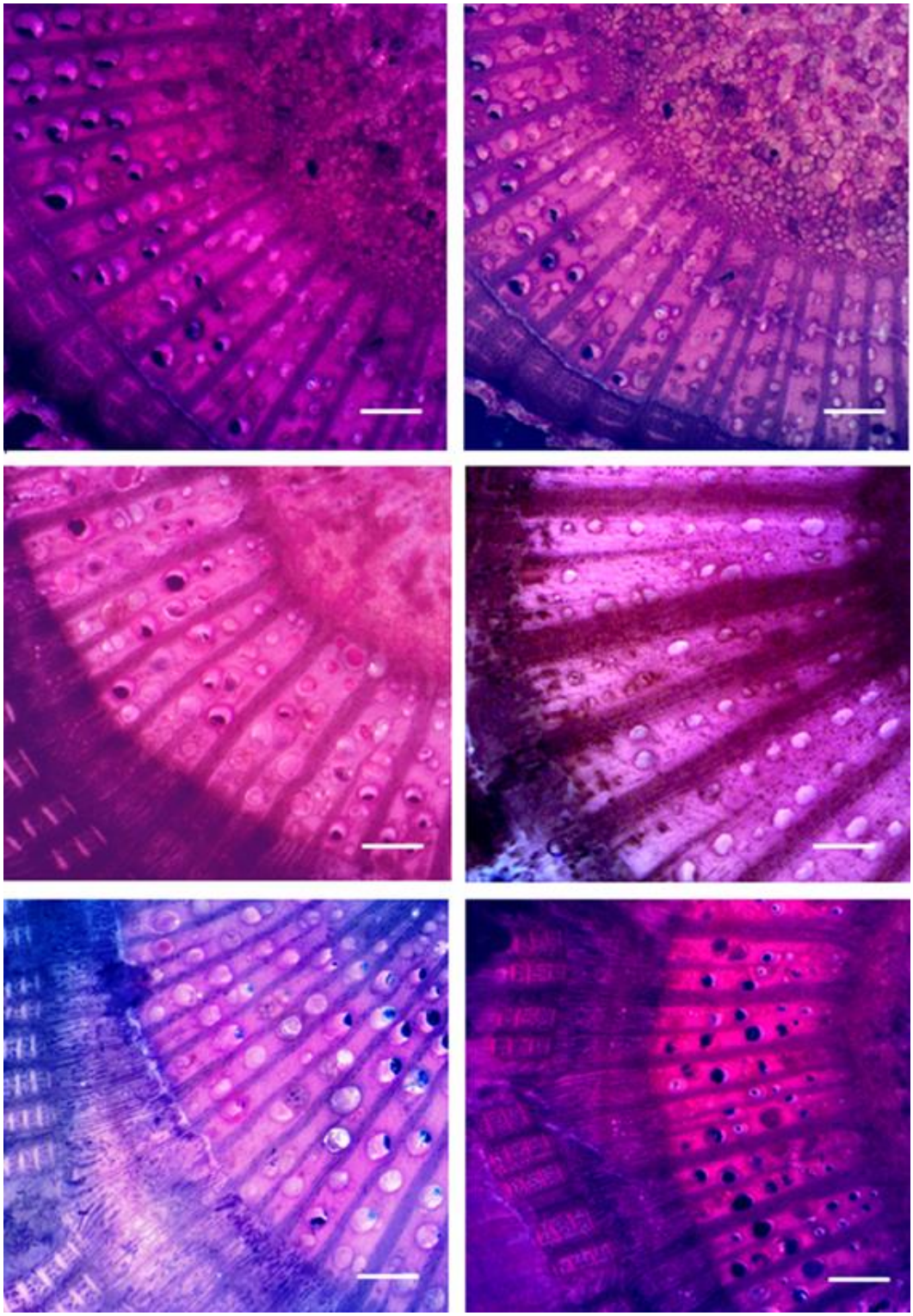

Figure 4. *Tissue levels of cane in cross sections taken from grapevine cuttings, double staining safranin O and bromophenol blue. Scale bar, 500 μm*

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The double staining technique was used to reveal cell wall lignification in the rings of poplar (*Populus tremuloides* Michx.) trees (Sutton and Tardif, 2005). Studies conducted with samples with woody structure showed that using water in the solution softens the tissues (Srebotnik and Messner, 1994), while using alcohol hardens the tissues (Vazquez-Cooz and Meyer, 2002). In the stainings we used, aqueous and alcoholic solutions we used helped visualizing the differences in the tissues. The grapevine cane cross section was well observed with the lignified walls being colored red and the cellulose rich layer being colored blue (*Fig. 4*).

A 0.75% BB (10% glycerol and 10% acetic acid) solution was used in our double staining applications. There are minor differences in the preparation or application of counterstains such as BB solution (Schweingruber et al. 2007). It is also recommended to heat the sections at 60°C for 10 s after dropping AB solution on them (Chaffey, 2002). Double staining processes are difficult and takes a long time. Simultaneous staining of the sections can be performed to facilitate these processes by combining both S and BB, and BB and FG solutions in equal proportions, with a staining time of 3-5 min. Some researchers adopted this approach. For example, a mixture of S and AB (40 mg S and 150 mg AB, 100 mL of pure water and 2 ml of acetic acid) with a staining time of 10 min by mixing 1% S and 1% AB solutions in equal proportions was suggested by Werf et al. (2007). It has been reported that Basacryl Brilliant Rot BG (10 mg Basacryl Brillant Rot BG (C.I.16)) can be used instead of S, which is used in simultaneous dyeing with AB, and unlike S, it does not form a deposit (Rapp and Behrmann, 1998). FG and MG are other stains with good results when used as a counterstain.

Our study shows that FG was more effective when used in double staining rather than alone (*Fig. 5*). During FG staining, dipping the sections into the staining solution until the desired density was reached was more effective than just dropping on the sections. Therefore, immersion in a weak FG solution (1%) is generally recommended (Wegner et al., 2014).

Results of double staining with carmine and methyl green indicated tissues and metabolically active cells in the cross sections of grapevine cane (*Fig. 6*). C and MG-stained sections was visualized with compound microscopy and then red, pink and green stained tissues were identified in the xylem and phloem ray parenchyma via thresholding under ring light (*Fig. 6*). In contrast, C and MG staining was evident in the xylem ray parenchyma appearing as grey and green pixels because of the absence of carmine granules in a representative image from the cross sections.

Very intense color formations occurred on the surfaces of the sections in BF, GS and C staining solutions. This situation, which we think was due to overstaining, resulted in the inability to distinguish the tissues. The stains in question should be used in lower amounts or as counterstains. FG staining leaked from the section surfaces, causing uneven staining. FG can be used in double staining processes of cane sections, when it was prepared using both alcoholic and aqueous solutions. Textures could be clearly distinguished in the sections especially in FG solutions taken from thicker and lignified cane cuttings. AB is another staining that can be used on cane sections. It stands out especially with its sharp tone stainings. It is stated that its first use was in the identification of tree fungi (Jewell, 1958). In a study on the shaping of wood tissues in fir (*Abies balsamea* (L.) Mill.), more than ten staining solutions were tested, and very good results were reported using AB (Kutscha and Gray, 1972).

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Additionally, microLC imaging and ring light will inspire future studies in imaging studies to quantify and assess the structural dynamics of plants. Although individual phloem cells did not appear well in microLC images under ring light, especially in the C and BF staining treatments, the starch dense xylem ray parenchyma and rays appeared in bright colors from all three double staining treatments; where pixel brightness is a function of staining, illumination and molecular density.

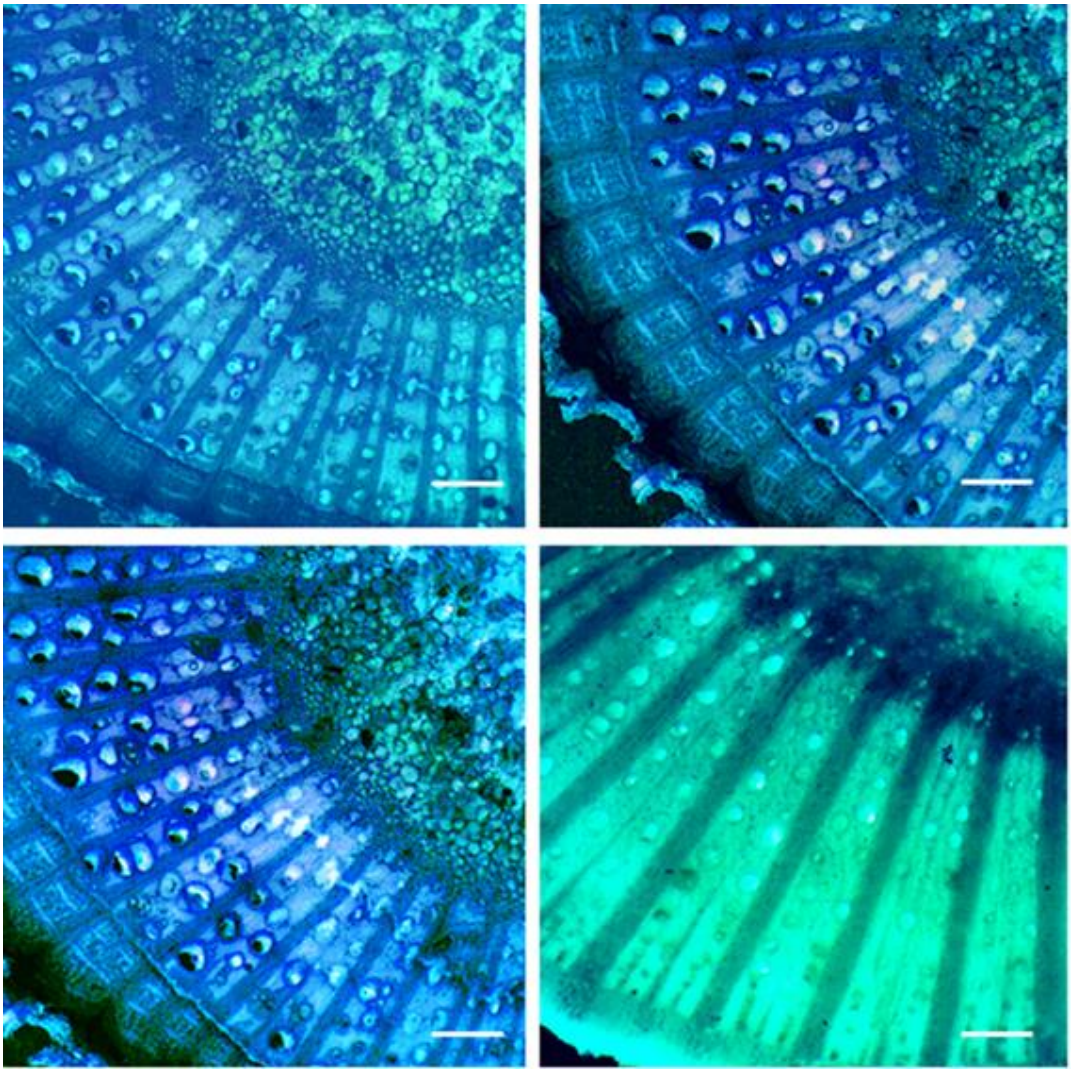

Figure 5. *Tissue levels of cane in cross sections taken from grapevine cuttings, double staining fast green FCF and bromophenol blue. Scale bar, 500 µm*

In studies using different stains, double stainings containing 1% S and AB were recommended to successfully stain cell walls and fibers (Schweingruber, 1990; Ruzin, 1999; Horobin and Kiernan, 2002). Successful results in our study were obtained from the three different double staining methods. All double staining methods are recommended for examining grapevine cane sections under a microscope. Researchers suggested that S and FG are quite satisfactory in defining branch structure and AB in visualizing cell structures (Gartner and Schweingruber, 2013). In addition, the combined use of MG, C and BB, in other words triple staining, could be studied in order

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to sharply stain cell structures, condensations and cell walls. The ring light developed by us prove to better visualize and photograph under the microscope. The ring light provides the desired illumination on the cross-sectional surfaces by using white light, yellow light and both light intensities in different proportions on the entire sample.

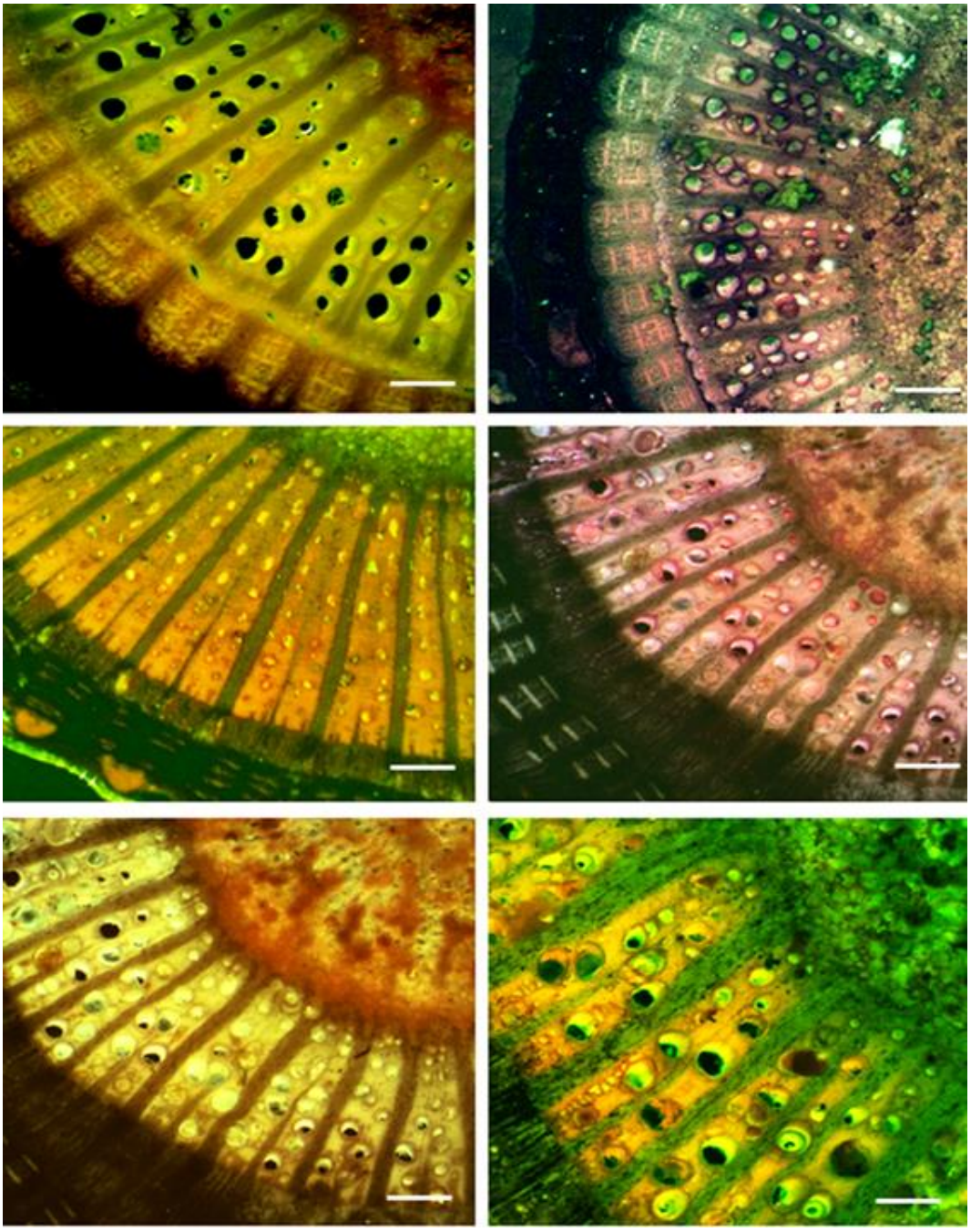

Figure 6. *Tissue levels of cane in cross sections taken from grapevine cuttings, double staining carmine and methyl green. Scale bar, 500 µm*

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Conclusions

As a result, staining applications, such as staining with Aniline Blue, Bromophenol Blue, Fast Green FCF, Safranin O, and Toluidine Blue O showed effective results at the cell and tissue levels. Among the double staining methods used in our research, Carmine + Methyl Green, Fast Green FCF + Bromophenol Blue and Safranin O + Bromophenol Blue were very effective in staining the tissues clearly. Therefore, all three double staining methods are recommended for examining grapevine cane cuttings under a microscope. Another important feature of this study is that the 'ring light' method was applied for the first time. The 'ring light' technique developed by us has increased the success in imaging and photographing samples under the microscope by increasing the coloration and sharpness on the surface of the stained sections. The ring light technique offers the opportunity to illuminate the entire sample using white light, yellow light and both light intensities in different proportions. Therefore, it is thought that it will be very useful in future studies to better visualize tissues and cells not only in grapevine but also in sections of other plant species.

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