Polychromatic Staining of Plant Cell Walls by Toluidine Blue O

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With 4 Figures

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The value of basic dyes as routine stains for the walls of plant tissues was recognized 34 years ago by Czaja (1930):

„Umgekehrt haben wir in den substantiven Farbstoffen ein sicheres Mittel in der Hand, die differente Struktur und Permeabilitätsverhältnisse verschiedener, sogar direkt benachbarter Zellen oder auch verschiedener Schichten einer und derselben Zellwand mit Leichtigkeit zu ermitteln." [2]

Although toluidine blue O is used routinely as a stain in animal cytology, and its metachromatic properties are widely known (Bergeron and Singer 1958), one finds but casual reference to the use of any of the thiazin dyes in plant histology. Indeed, Jensen (1962) recently remarked, "This stain (Azure B) has been little used by botanists, but shows great promise both as a stain for the nucleic acids and for lignin."

The purpose of this note is twofold: to draw attention to the wealth of structure revealed by toluidine blue O when it is used to stain fresh or fixed plant tissues and to outline very simple and rapid procedures for obtaining temporary or permanent mounts of stained sections. The methods are so simple that one may proceed from the intact plant via tissue sections (10–50 μ) cut free-hand with a razor blade to an examination of these sections in the microscope in a matter of 10 minutes. The staining procedure may also be applied to sections of fixed and embedded material. Magnifications of up to $\times$1000 may be usefully employed. The value of such a technique for teaching and research is evident.

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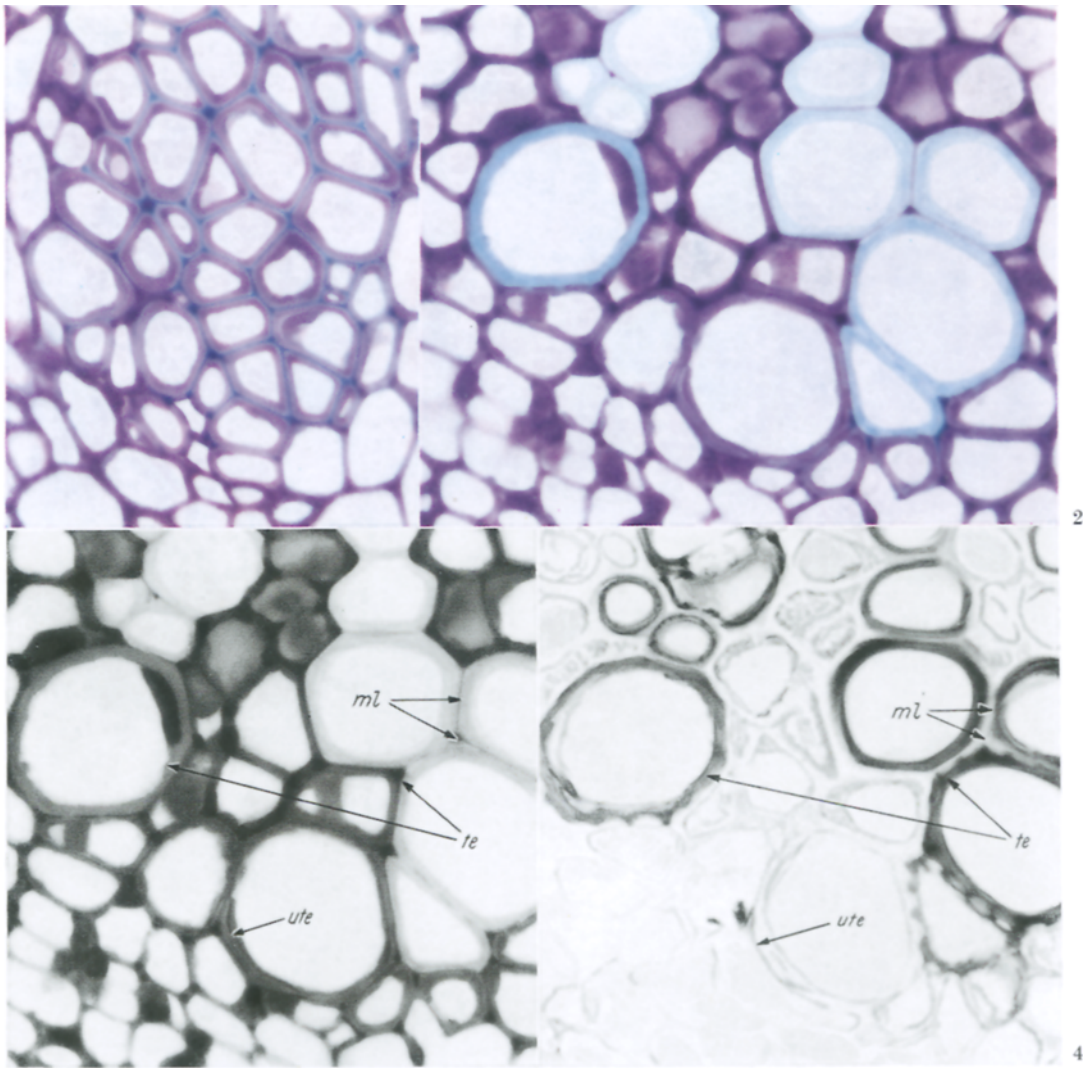
[2] Conversely, in the substantive dyes we have available a sure means of easily ascertaining the different structure and permeability relations of different cells, even of immediately adjacent cells, or even of different layers of one and the same cell wall.



Figs. 1–4. All photomicrographs are of pea epicotyl, fixed in acrolein and embedded in polyester wax and sectioned at 8 μ.

Fig. 1. Differentiating fibers in the "caps" of the vascular bundles, stained with toluidine blue O. The lignified compound middle lamella (blue-green) is sharply differentiated from the as yet unlignified secondary wall (reddish purple). ×860.

Fig. 2. Vascular bundle stained with toluidine blue O. The legend is given in Fig. 3 in which part of the same field is reproduced. The lignified secondary walls of the tracheary elements (*te*) stain blue-green, which contrasts sharply with the reddish purple of the unlignified middle lamella (*ml*) separating two such lignified elements. The walls of unlignified tracheary elements (*ute*) and those of xylem parenchyma stain various shades and intensities of reddish purple. ×860.

Fig. 3. Part of the same field as Fig. 2, but photographed with light passing Corning yellow filter # 3484. ×860.

Fig. 4. The same section as shown in Fig. 2 and 3 after treatment with phloroglucinol-HCl to demonstrate the Wiesner-positive lignin. The stained section was photographed with the light passing a Corning green filter # 1010 to enhance contrast. There is an excellent correlation between walls or wall layers which are Wiesner positive, and the development of a blue-green to green color with toluidine blue O. ×860.

Materials and Methods

Staining solution: An aqueous solution containing 0.05% of toluidine blue O (C. I. #52040)[3] in 0.1 M phosphate buffer at pH 6.8.

Procedures: Fresh or fixed tissues may be used (but see comments under Fixatives for the limitations imposed by fixatives which contain heavy metals). The tissues may be sectioned by hand with a razor, or with a sliding microtome or cryostated microtome. Specimens embedded in polyester wax may be sectioned in the usual way (Sidman, Mottla, and Feder 1961). The list of materials which we have examined from hand-cut fresh material includes storage tissues of carrot and potato, epicotyl, stem, root and cotyledons of pea, petioles of celery, stem of *Elodea densa* (Planch.) and *Hippuris vulgaris* L., leaf of *Zea mays* L., stems of *Coleus* sp., *Thuja occidentalis* L. and *Pinus Strobus* L., coleoptiles of *Avena sativa* L. and *Zea mays* L., and rhizome of *Lycopodium* sp.

1. For hand-cut sections of fresh material:

a) Cut sections with a razor blade into tap water and allow them to soak for at least 2–3 minutes.

b) Transfer selected sections (10–50 μ) to the staining solution. Immerse them for 1 minute.

c) Wash the stained sections for 1–2 minutes in tap water.

d) Examine the stained sections mounted in tap water under a coverslip, or photograph at magnifications up to $\times 250$ in the microscope.

e) For examination with high-dry and oil immersion objectives remove as much of the wash water as possible from around the section on the slide and mount it under a coverslip in a drop of the following liquid:

Cadmium iodide	2 g.
Potassium thiocyanate	4 g.
Fructose	10 g.
Water	4 ml.

This liquid is relatively viscous, has a refractive index of approximately 1.5, and sections mounted in it maintain their full color differentiation for at least 2 days. The liquid does not set and the mounts are temporary. The composition of this mountant is modified from that proposed by Spurr (1954).

2. For sections cut on a sliding or cryostated microtome:

i) Cut sections at 10–16 μ and allow them to dry on gelatin-coated slides in the usual way.

ii) Stain for 1 minute in the staining solution, wash for 1 minute in tap water, and mount and examine as for 1. d) or e) above.

[3] Available from National Aniline Division, Allied Chemical Corporation.

3. For sections embedded in polyester wax:

Sidman, Mottla, and Feder (1961) give instructions for preparing sections stained with toluidine blue O while still in the ribbon of wax. From such sections, permanent mounts can be obtained by dewaxing the sections in xylene and mounting in Diaphane.[4] This treatment results in some change from the colors developed by the same material stained while fresh. The color change can be minimized by a slight rehydration of the material before mounting. This is most simply done by breathing on the dried wax sections just before immersing them in xylene.

Results

Toluidine blue O resolves tissue sections into their component cell types by coloring various types of wall strikingly different colors (Figs. 1 and 2). In some instances the color resolution extends to different layers of the

Table 1.

Tissue Element	Color Developed by Toluidine Blue O
Tracheary elements (lignified walls)	Green, or bluish green
Lignified sclerenchyma	Blue-green, but occasionally green
Collenchyma	Reddish purple
Parenchyma	Reddish purple
Sieve tubes and companion cells	Red
Unlignified compound middle lamellae	Reddish purple or red
Callose, starch	Unstained

wall of one cell. For example, the lignified secondary wall of the tracheary elements of pea epicotyl are stained a bluish green while the compound middle lamella separating two such adjacent elements is stained a brilliant reddish purple (Fig. 2). The "caps" of the vascular bundles of the same tissue are composed of fibers in which lignification commences in the primary wall adjacent to the intercellular air spaces. A section of such young fibers stained with toluidine blue O shows the lignifiying primary wall stained a clear blue-green, while the middle lamella in the region adjacent to the air spaces, and the unlignified secondary walls, are stained intense reddish purple. As the lignification of the fibers proceeds, the blue-green staining progresses into the middle lamella (Fig. 1) and finally into the secondary wall. The colors developed typically by toluidine blue O are shown in Table 1. In all cases "lignified" means that the particular wall or wall-layer was positive in the Wiesner test (phloroglucinol-HCl, Johansen 1940).

Other dyestuffs: Several other members of the thiazin group of dyes were tested in preliminary experiments on fresh sections of pea stem. Of those tested (azure A, azure B, toluidine blue O, thionin, methylene blue, new methylene blue, and methylene violet) only toluidine blue O and

[4] The Will Corporation, Rochester 3, N. Y.

azure A were satisfactory in the range and stability of the colors developed. Thionin was also satisfactory, but the colors were shifted towards the red. Although we selected toluidine blue O for our further tests, azure A should be equally satisfactory and other members of this group of dyes might be useful in certain circumstances.

Effect of pH: 0.1 M phosphate buffer at pH 6.8 is recommended because it is easy to prepare. However, tests on sections of pea epicotyls cut on a cryostated microtome showed that the same colors were developed by toluidine blue O dissolved in water or in buffers in the pH range 3 to 9. At pH less than 4 the metachromatic red colors are steadily reduced in intensity, but lignified walls will stain either blue or green even at pH 1.0.

Effects of Fixatives: Air-dried sections of pea stems were fixed for 10 minutes, 1 hour or overnight in the following fixatives: 10% acrolein, 10% formalin, 5% glutaraldehyde, FAA, Bouin's, CRAF, Zenker's, and 1% osmium tetroxide. The fixed sections were washed for 1 hour in distilled water, stained and examined in tap water. The colors developed by sections fixed in acrolein, formalin, glutaraldehyde, FAA, and Bouin's were indistinguishable from those seen in sections soaked in water for the same period. The same was true of sections fixed for 10 minutes in Zenker's, CRAF, or 1% osmium tetroxide; however, after fixation for 1 hour or overnight in these fixatives a pronounced blue shift in the characteristic colors developed, and they began to fade within a few minutes of staining. This change could be prevented in material fixed in CRAF if the stained sections were mounted in the liquid mountant described above.

Discussion

The fact that toluidine blue O would stain plant cell walls polychromatically has been known for many years (Czaja 1934), but this dye does not seem to have gained the widespread use it deserves in plant cytology. Shortly after we began to use toluidine blue O for the study of fresh plant tissues, our attention was drawn to the extensive unpublished work of E. Rasch and H. H. Swift who have attempted to analyze the histochemical basis of the polychromatic staining developed by cell walls of different types in plant tissue embedded in paraffin. These workers are the first in recent years to recognize the value of polychromatic staining with toluidine blue O for the investigation of plant cell walls, and we are pleased to thank them for allowing us to see a preliminary description of their results, which are in good agreement with ours.

In the range of herbaceous materials examined here, there is an excellent but not invariable correlation between the color developed by toluidine blue O and the presence of lignin. All walls which give the Wiesner reaction (phloroglucinol-HCl) stain distinctly green, or blue-green. In the differentiating cortical fibers of pea epicotyl, it is very clear that the intensely blue-green, compound middle-lamella of these cells (Fig. 1) is the only part of the wall which gives the Wiesner reaction. Conversely, some of the

tracheary elements in the xylem of this tissue have Wiesner-positive secondary walls; the compound middle-lamella is negative (Fig. 4). When stained with toluidine blue O, the secondary wall stained green, the compound middle-lamella a distinct reddish purple (Fig. 2). However, in spite of these results, it is not certain that the green color necessarily indicates the presence of lignin. Sections of pea epicotyl were "delignified" for various periods of time by treatment with 2% sodium chlorite and ammoniacial 70% alcohol (Barghoorn 1948). After treatment for 1 hour, the sections appeared to be delignified as judged by the Wiesner test, but the colors developed by toluidine blue O were identical to those of controls. Prolonged delignification (overnight) did destroy all green colors when the delignified sections were stained with toluidine blue O. Those areas which previously stained green now stained a deep blue. However, different results were obtained with *Lycopodium* rhizome, in which some of the heavily lignified walls are Wiesner positive and also stain intensely green with toluidine blue O. In this tissue treatment of sections with sodium chlorite for 8 hours abolishes the Wiesner reaction but even treatment for two days has no effect on the green staining of cell walls with toluidine blue O.

A sample of Brauns' isolated native spruce lignin (kindly given to us by Dr. I. A. Pearl of the Institute of Paper Chemistry) does stain an intense bluish-green. The refractory nature of native lignin *in situ* raises doubts about the validity of any histochemical procedure that claims to identify lignin. Until more is known of the chemistry underlying the Wiesner reaction and the blue-green colors developed by toluidine blue O in lignified walls, it is impossible to decide with certainty which is more reliable for the identification of lignin.

It is disappointing not to be able to attach histochemical significance in some simple way to the polychromatic staining observed. Nonetheless, the convenience of the sectioning, staining and mounting procedures and the quality of the polychromatic staining achieved lead us to believe that the method may find widespread application in the teaching of plant anatomy and in research into plant histology.

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Summary

1. The polychromatic staining of plant cell walls by toluidine blue O is described and illustrated.

2. The effects of various common fixatives and the effects of the pH of the staining solution are evaluated.

3. Simple and rapid procedures are described for preparing stained temporary mounts of fresh material, or permanent mounts of embedded and sectioned material.

4. The relationship between the polychromatic staining observed and the lignification of the walls is discussed.

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