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A NEW PROGRESSIVE POLYCHROME PROTOCOL FOR STAINING BRYOPHYTES

RICHARD H. ZANDER
Missouri Botanical Garden
P.O. Box 299
St. Louis, Missouri 63166
richard.zander@mobot.org

ABSTRACT

A general staining protocol for bryophytes is described, with exemplars of species of the family Pottiaceae (Bryophyta). The protocol assesses the color reaction to dilute potassium hydroxide, then to the addition of a mixture of dilute toluidine blue O and dilute ruthenium red. Colors are distinctive in various portions of plant anatomy. In addition to the preliminary KOH reactions of taxonomically important yellow or red anatomical structures, particularly reactive to TBO and RR are collenchyma in the distal laminal cells (green), laminal papillae (blue or pink), and leaf awns, axillary hairs, tomentum and paraphyses (deep pink). Mounting in glycerol further clarifies the distinctions between anatomical features. It is hypothesized that pectic mucilage is present in several plant organs, including tomentum and leaf awns, and may be important in water conservation.

Aqueous or vital staining of bryophytes for histochemical and taxonomic study has a long history in bryology, recently reviewed by Glime and Wagner (2013), including chromosome staining, histology, and clarification of pore type in sphagna. Additional discussion of the histochemical use of stains is recently reviewed by Konrat et al. (1999). Major works on aqueous stains for plants and animals in general include those of Gatenby and Beams (1950), Preece (1972), Ruzin (1999), and Yeung et al. (2015).

Potassium hydroxide

A 2% solution of potassium hydroxide (KOH) has been used in bryology, particularly in the study of the moss family Pottiaceae (Zander 1993: 3), for many years. It commonly demonstrates a yellow or red reaction in the distal lamina and other plant parts, these color reaction usually specific to genera. The colors last a fairly long time in glycerol mounts.

Toluidine blue O

Certain dyes are alone metachromatic (as discussed at length by Keating (2014), and these express different colors depending on the tissue stained. Toluidine blue O, a basic (acidophilic) thiazine dye, is one of these (see O'Brien et al. 1964; others are cresyl violet acetate, methyl violet and pseudoisocyanin). According to Keating (p. 88) staining with toluidine blue O (TBO) will express:

(1) pinkish purple with carboxylated polysaccharides (e.g. pectic acids);
(2) purplish or greenish blue with macromolecules with free phosphate groups (e.g. nucleic acids);
(3) green, greenish or bright blue with polyphenolics (lignin, tannins, while xylem walls are blue-green and phloem fibers bright blue);
(4) unstained are hydroxylated polysaccharides like starch and cellulose; and
(5) pinkish purple with polyuronides such as primary walls of epidermis or parenchyma.

Parker (1982) gave a shortened version: green or blue-green for xylem and sclerenchyma; red for phloem, red-purple for collenchyma and parenchyma.

TBO provides a pinkish purple color with carboxylated polysaccharides such as pectic acid; green, greenish blue or bright blue with polyphenolic substances such as lignin and tannins; and purplish or greenish blue with nucleic acids. Staining is more intense at low pHs (2.5–3.5) (Preece 1972: 266).

Collenchyma contains only primary cell wall tissue and is characterized by its uneven walls. It is, with sclerenchyma, a support tissue, but collenchyma provides a flexible support because it stretches. Angular collenchyma is common in moss leaves, most strongly expressed as cell wall trigones.

Ruthenium red

Ruthenium red is generally considered a specific stain for pectin mucilage but Luft (2005) found it only semispecific for pectin. The dye binds to polysaccharides that are highly substituted with acidic residues such as carboxyl groupings (Yamada 1970), for instance polyanions like pectic rhamnogalacturonans. Gums and mucilages are also stained, doubtless because they are composed in part with pectin (Engelbrecht et al. 2014). It is much used in histochemistry and electron microscopy (Fallel & Edmiston 2009). As the metachromatic TBO reacts to pectic substances with a pink-purple color that is different but not particularly distinctive, adding RR to the stain makes pectic-laced anatomical structures clearly stand out as bright pink or at least deep red-purple against TBO-stained green or blue non-pectic cells.

A progressive polychrome protocol

This staining schedule is termed "progressive" in that dilute KOH is the first test reagent. Adding RR and TBO changes the color responses to a significant extent. The greatest change is that bryophyte tissue of primary walls included collenchyma reacting yellow in KOH will respond green in TBO, particularly cell walls thickened with collenchyma. Thus, one notes the color in KOH, which is taxonomically significant, before adding TBO and RR for a final stain.

There are other polychrome stains, such as polychrome methylene blue, a mixture of methylene green, methylene azure, methylene violet, and methylene blue (Focosi 2015), or astra blue and basic fuchsin (Kraus et al. 1998), or safranin and alclan blue (Tolivia & Tolivia 2011), or even a combination of several dyes (Shellhorn & Hull 1961). These are generally used in medicine, not for bryophytes. The present protocol, as far as I can tell, has not been used previously on any organism.

METHODS

The images were captured with an Olympus CX41 microscope mounting a Moticam 2000 2.0 MP resolution camera, Motic Images Plus software (Motic China Group 2004) and Moticam Multi-focus software. They were cropped and slightly sharpened and color-balanced with Neopaint (Neosoft 2010) graphics software.

The mounting medium is Zander's (2014) polyvinyl alcohol-glycerol solution (PVOH-G). This is a mixture of polyvinyl alcohol-based clear glue (sold commercially by Elmer's and Colorations) and glycerol in a 7:3 proportion (emended from the 1:1 proportion of the 2014 article which has proved to solidify too slowly or sometimes not at all). This is essentially a glycerol mount stabilized by the polymer. Perhaps because the water-based glue greatly dilutes the glycerol, there is little osmotic effect that might shrivel the plant cells. The index of refraction in glycerol favors clarity of observation of small anatomical features over viewing in water mounts.

The staining reagents and dyes used for the progressive polychrome staining protocol (PPSP) are 2% solution of potassium hydroxide (KOH), 0.2% ruthenium red (RR) stain solution, and 0.2% toluidine blue O (TBO) stain solution.

TBO usually comes from the sales source in a 1% solution, which is far too concentrated as it quickly overstains and precipitates small, solid particles of TBO. Dilute to about 1:5 to 1:10, and continue diluting until the stain is just adequate (makes an olive leaf color) for the group you are working on. The same is true with RR, and a perfectly adequate solution is made by placing a fingernail-sized bit of the power in the bottom of a dropper bottle, and fill with water until you can just read print through the glass of the bulb-tipped dropper. Or simply follow the instructions by Glime and Wagner (2013: 2–2–18). Both TBO and RR are enhanced by buffering but this is not advised as KOH is a crucial ingredient in the protocol.

The protocol

1. Wet plant or plant portion in 2% KOH solution on a microscope slide. Detach leaves, make sections, and other dissections when necessary.
2. Daub off excess KOH solution by tilting the slide against absorbent tissue.
3. Add one or two drops of dilute RR solution, immediately add the same amount of dilute TBO solution. Put the RR on first as TBO has a tendency to shrivel leaves. Stir.
4. If the plant material does not turn weak olive color after a short wait, then heat gently with a butane lighter (carefully so as to warm whole slide or it will break) or better on a warm hot plate like a coffee warmer. The solution will deepen in color and the tissue should get darker, a deep green.
5. Daub off the excess.
6. Add three or four drops of glycerol or polyvinyl alcohol-glycerol. The toluidine blue O will regain a blue color in solution, and the glycerol may gain a blue cast. Stir, arrange, add cover slip.
8. One can examine the slide immediately. For best color development leave overnight or use mild heat. A small fan or mild heat also helps solidify the mount. Under-staining of the leaf cells may be due to over dilution of the TBO, in which case, daub off the RR before adding TBO. Over-staining can be reversed by addition of a tiny amount of KOH, but this may clear the stain completely.

One might mount a bit of the plant in KOH + PVOH on the left, and in the PPSP on the right to show the original plant with its reaction to KOH for comparison purposes. The turquoise stain may be more green than in color, but should not be mistaken for chlorophyllose tissue because it stains the leaf completely, including otherwise clear cells, also stains older leaves in which chlorophyll has degraded, and close study shows the interior cell walls green.

TBO plus KOH stains leaf cell walls green but tomentum has hyaline walls with only cell contents bluish. RR stains the tomentum (and hyaline leaf awns) pink. Leaving the RR or TBO for a longer time on the tissues will deepen each stain. After adding both RR and TBO, wait until the leaves turn from a yellow color in KOH to a weak olive; if red in KOH, wait until there is a color change, usually brownish. The RR pink may be hidden by the TBO blue if the latter is left on too long or is too concentrated. Either stain may over-stain the tissues, in which case, dilute the solution in the dropper bottle and try again with new plant material. Strong heat (to boiling) changes TBO from blue to green, and will help develop the turquoise color if the tissues seem resistant.

RESULTS

The color reactions

The resultant yellow, red, blue, and green colors on the slides are not all permanent. Pink and blue fade over a few days, but yellow and green are quite fast in the PVOH-glycerol mountant.

Green: in plants with KOH yellow-staining leaves, collenchyma; angular cells walls, trigones, porose cell walls (Figs. 1 and 4). May take some time (up to a few hours) to develop as a deep bluish green (Fig. 10).

Red-brown to orange: red brown for stain-resistant yellow-brown cell walls (Fig. 3), or becoming orange or blue for cell walls of originally KOH-red staining tissues (chlorophyllose cell contents remain green but walls are orange).

Blue: cellulose, antheridia (Fig. 6), thin cell walls, cancellinae, some papillae (Figs. 10 and 11).

Blue-violet: cellulose; parenchyma.

Pink, violet or red: gums, mucilage, pectin; rhizoids (Fig. 9) and paraphyses (Fig. 6), some papillae (Fig. 12).

Yellow to brown: stain resistant; structural tissues, some wall thickenings that are not angular.

Papillae of laminal cells are often stained blue-violet or sometimes pink against the background of green-stained cell walls. This differential staining is valuable in that discerning the type of papillae is a major taxonomic exercise. Papillae morphology is occasionally difficult to describe because papillae are often very small, reduced, of one or more salients, or massively thickened into a somewhat amorphic mass ("flower-shaped papillae"), or sometimes hollow and appearing C- or O-shaped in optical cross section.

Perigonial buds (Fig. 6) are quite responsive to this staining procedure with unburst antheridia light blue (probably from a concentration of nucleic acids) and paraphyses pink or even deep red.

Details of technique and problems

Instead of daubing off excess KOH, RR and TBO, use just a small amount of KOH, then add the RR, then the TBO over that, wait for a color change or heat moderately, then drop the PVOH-G mountant into the middle of the preparation, and move the leaves and other material into the clear mountant, then add a cover slip and daub off stain surrounding the cover slip. The mountant will cleanse the extra stain from the plant material as the material is swished through it.

Adding a drop of vinegar (5% acetic acid) to the KOH with create a lighter, more bluish stain with TBO (becoming blue-green with time), and not affect the RR; however, there is then less difference between the green collenchymatous tissue and blue-staining leaf marginal cells and laminal papillae. Also, adding vinegar to neutralize the KOH removes the KOH-stained structural tissue reaction, such as the spaced streaks of brownish cells in linear rows running up the basal cells of certain *Leptodontium* species.

RR premixed with KOH throws a precipitate, and becomes deep red, but may be used although RR in water is best. TBO premixed with KOH precipitates fully and should not be used. RR and TBO react with each other. Thus water solutions of dilute RR and TBO in separate individual bottles is best.

Images of stained examples in the moss family Pottiaceae

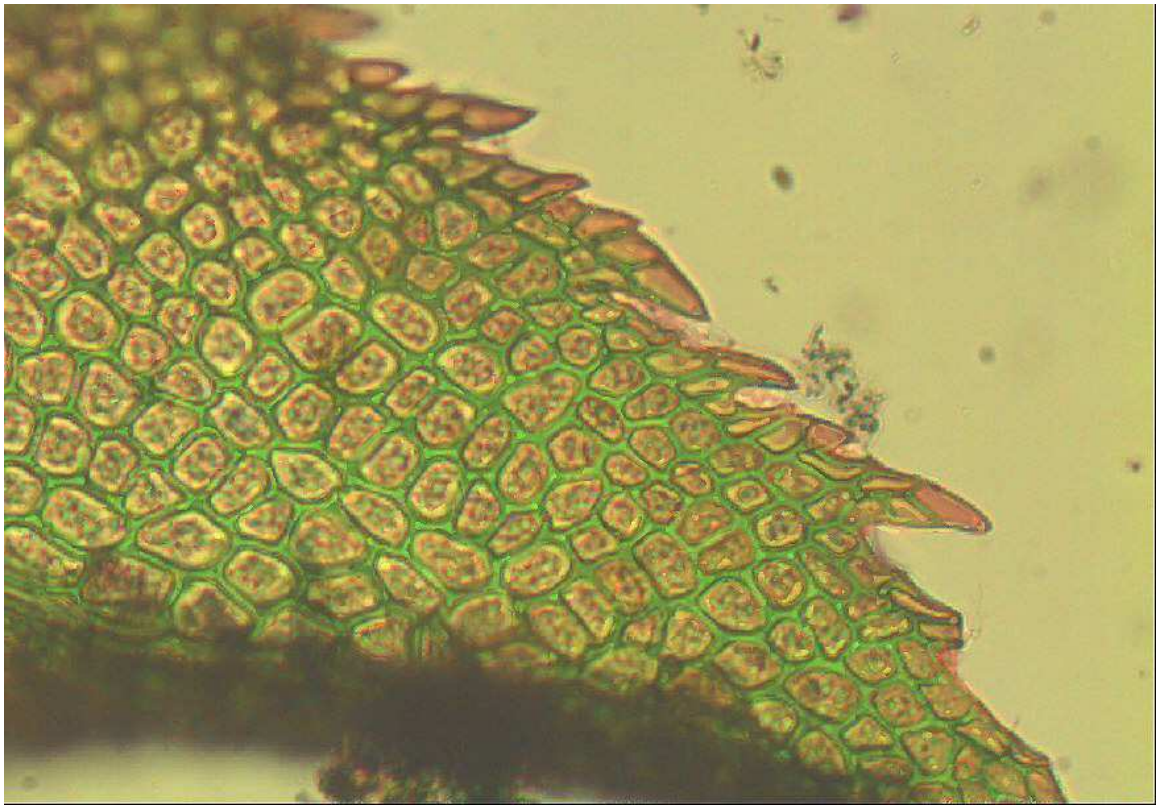

Figure 1. Leaf apex of *Leptodontium viticulosoides*. The collenchymatous internal cell walls are bright green, and the marginal teeth are reddish, indicating thinner cell walls.

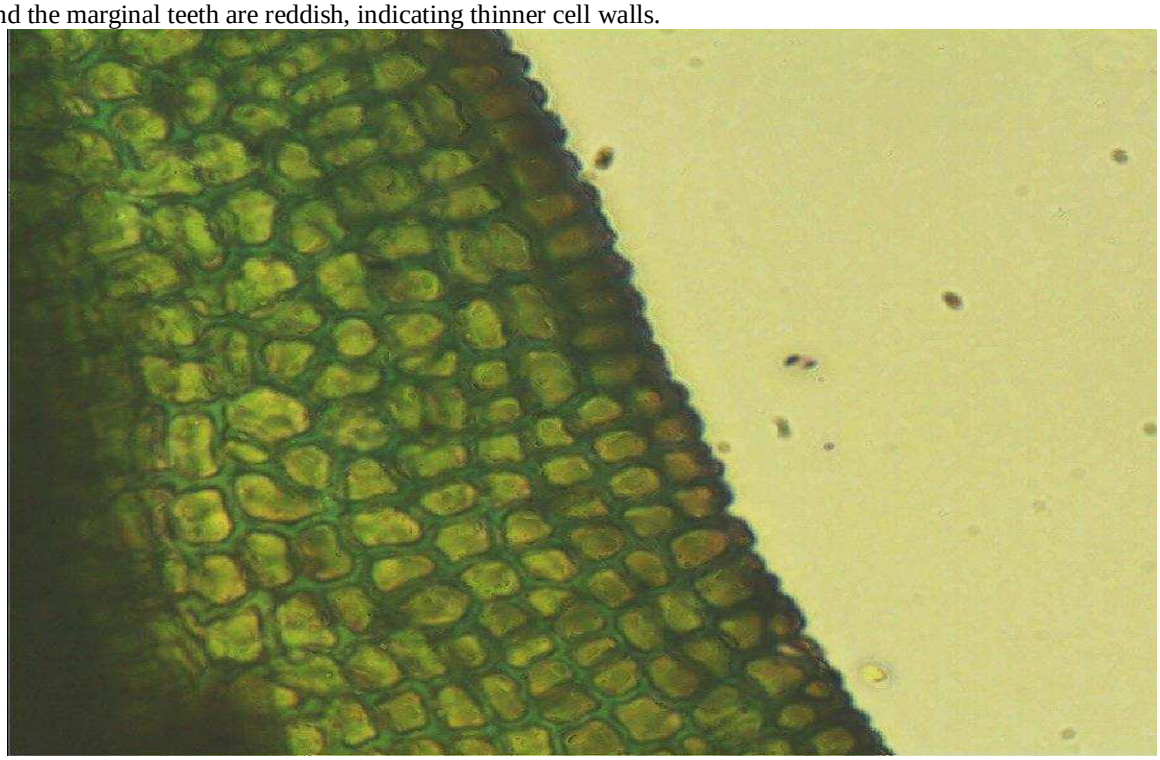

Figure 2. Margin of leaf of *Vinealobryum vineale*. Again the internal cell walls are green, while the cell contents are green from chlorophyll. Without the stain, the cell walls are reddish in this species.

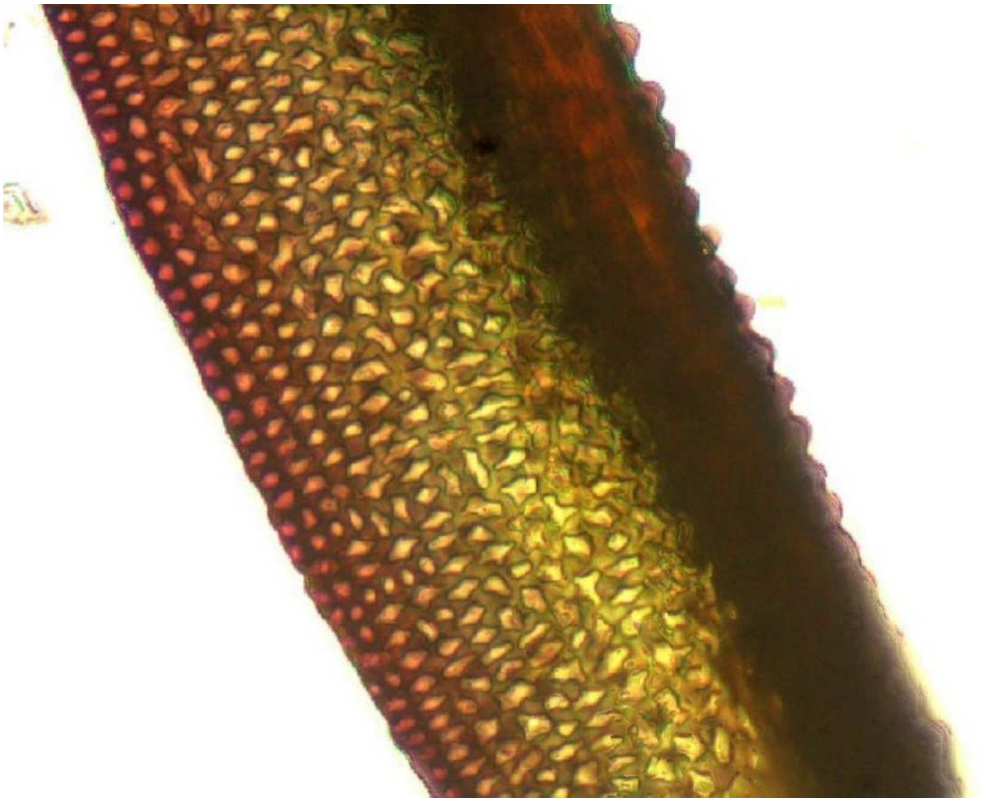

Figure 3. Portion of the upper leaf of *Geheebia gigantea*. The thick angular collenchyma is in the form of bulging trigones, which are yellow to greenish yellow, retaining the KOH reaction. The reddish brown coloration of the unstained or KOH soaked plants is typically somewhat unreactive, in this and other resistant species, to the TBO and RR.

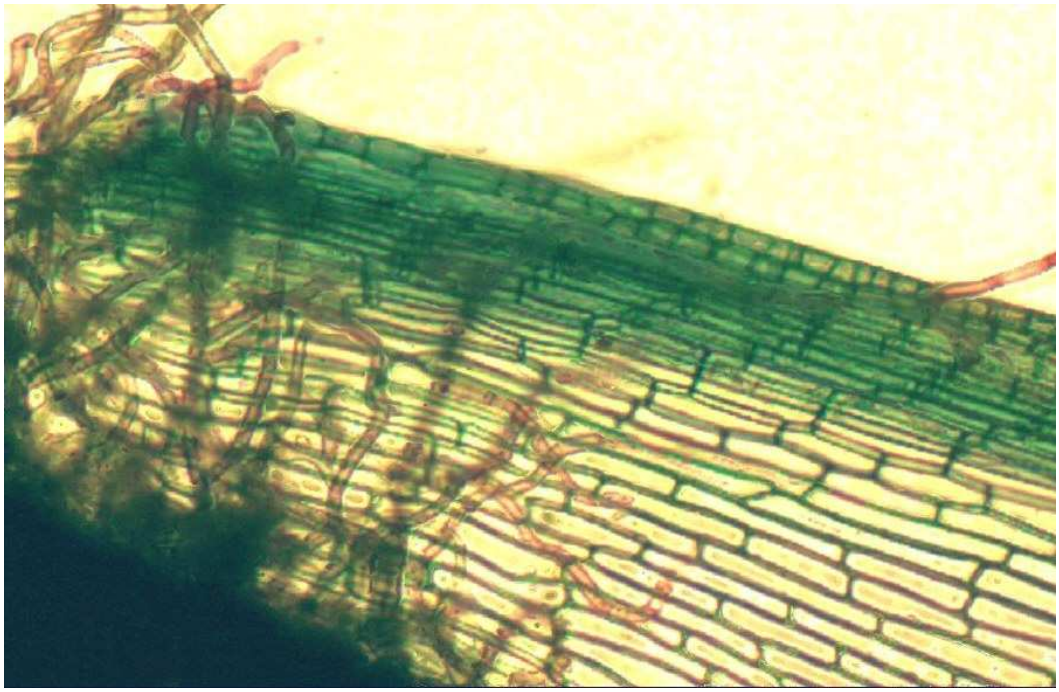

Figure 4. Basal cells of *Syntrichia princeps.* Although this species is russet red in nature, the protocol results in deep green marginal cells and green enlarged mid basal cells. The rhizoids are pink.

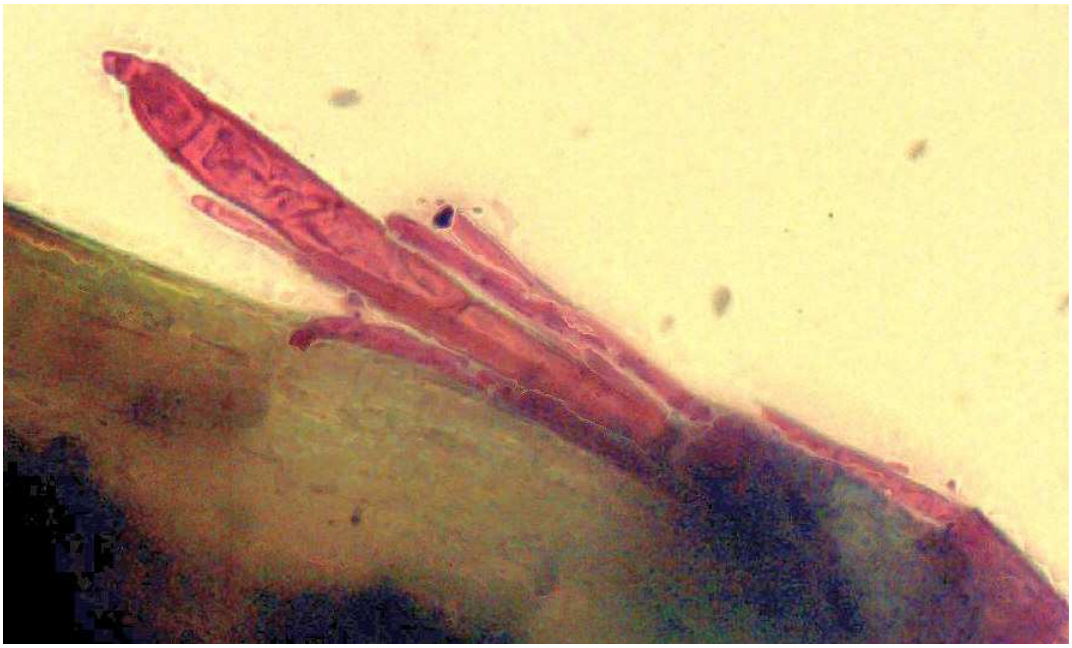

Figure 5. Axillary hairs of *Hyophila involuta.* The larger structure is probably the immature basal stalk of angular gemmae often produced in this species. Both hairs and gemmae stalks stain strongly for pectic mucilage.

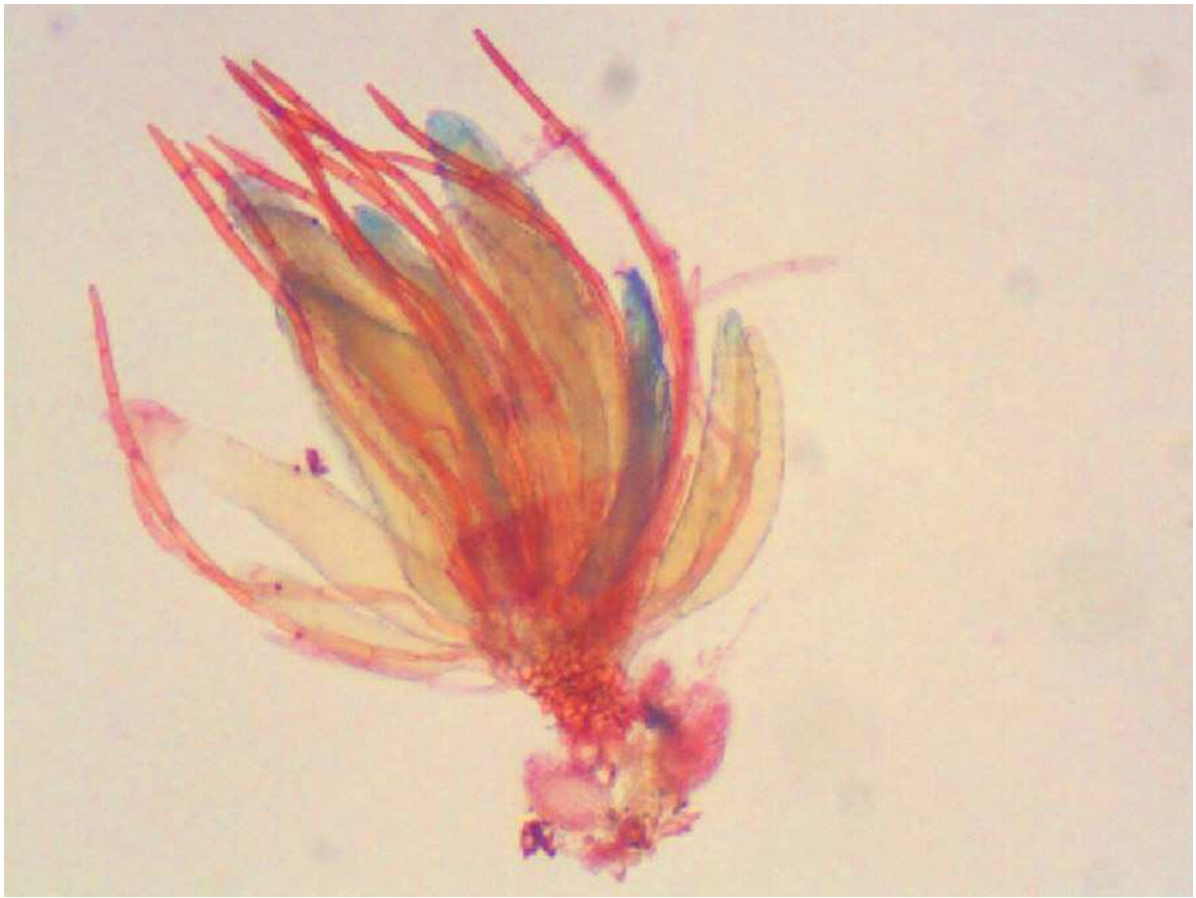

Figure 6. Androecium of *Trachyodontium zanderi.* The paraphyses stain a deep pink while the unburst antheridia are blue.

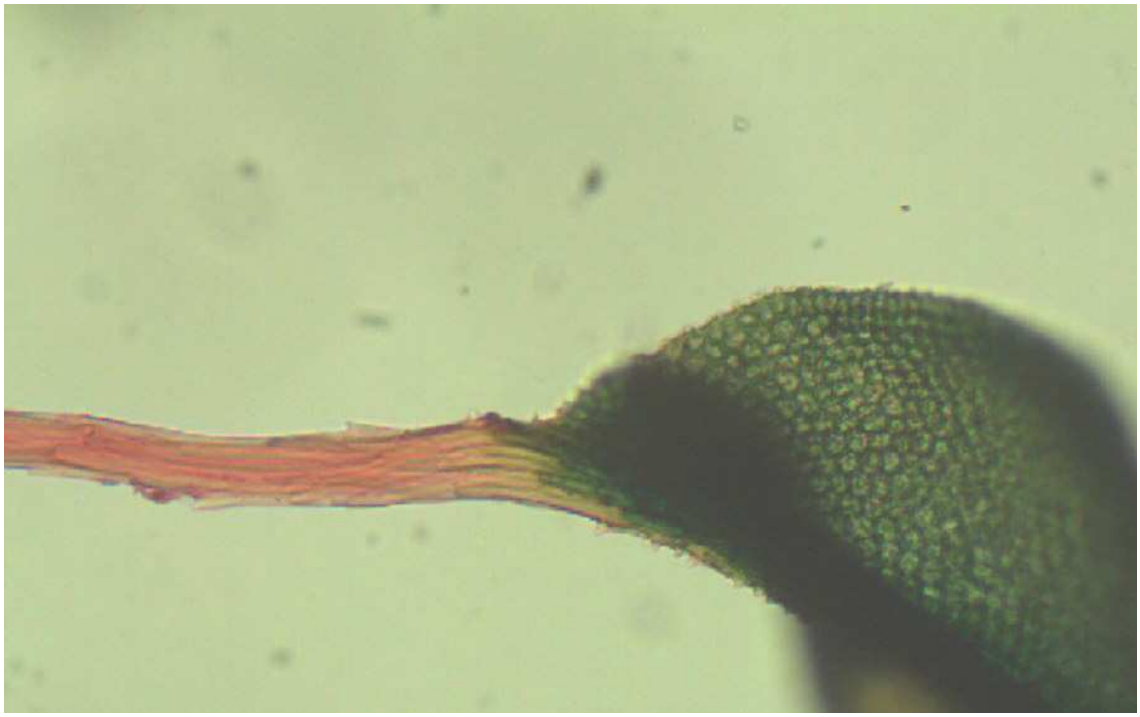

Figure 7. Apex of *Syntrichia princeps.* The leaf awn stains pink against the deep green of the distal lamina.

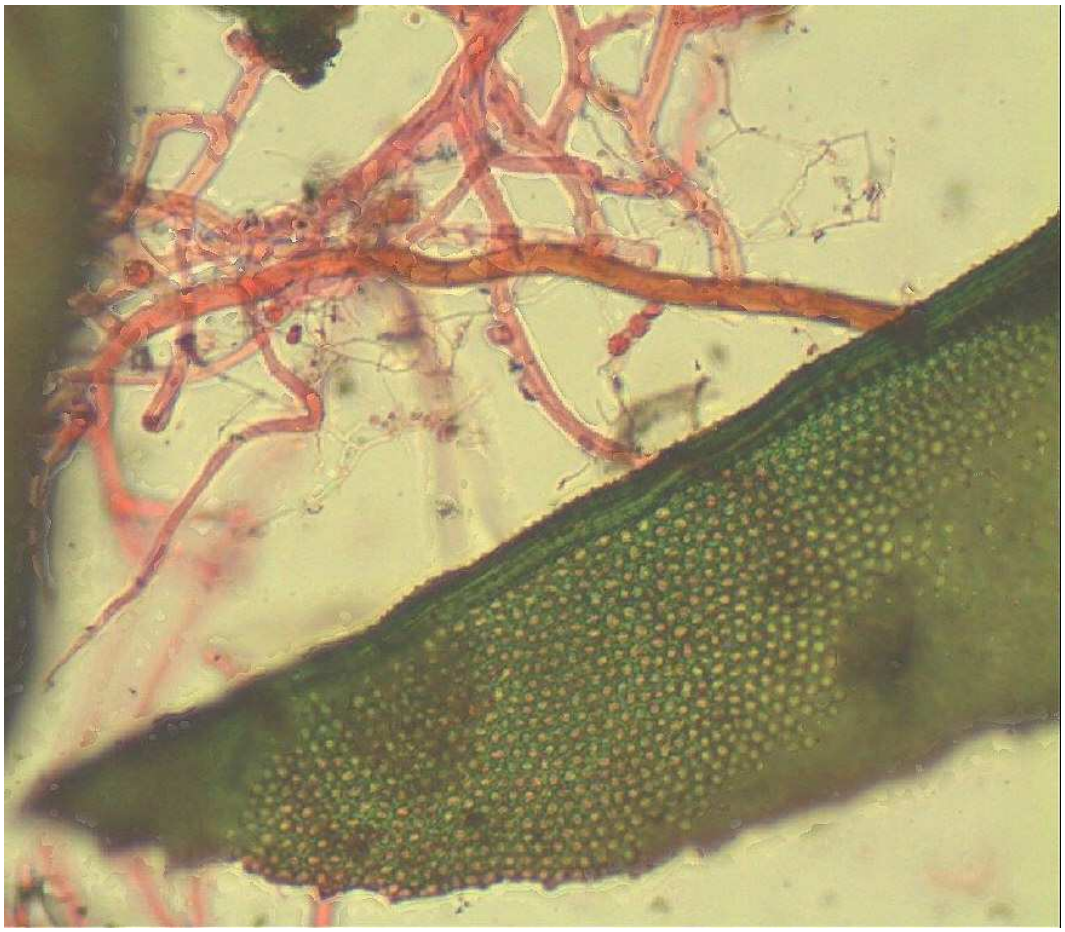

Figure 8. Tomentum and leaf tip of *Leptodontium viticulosoides.*

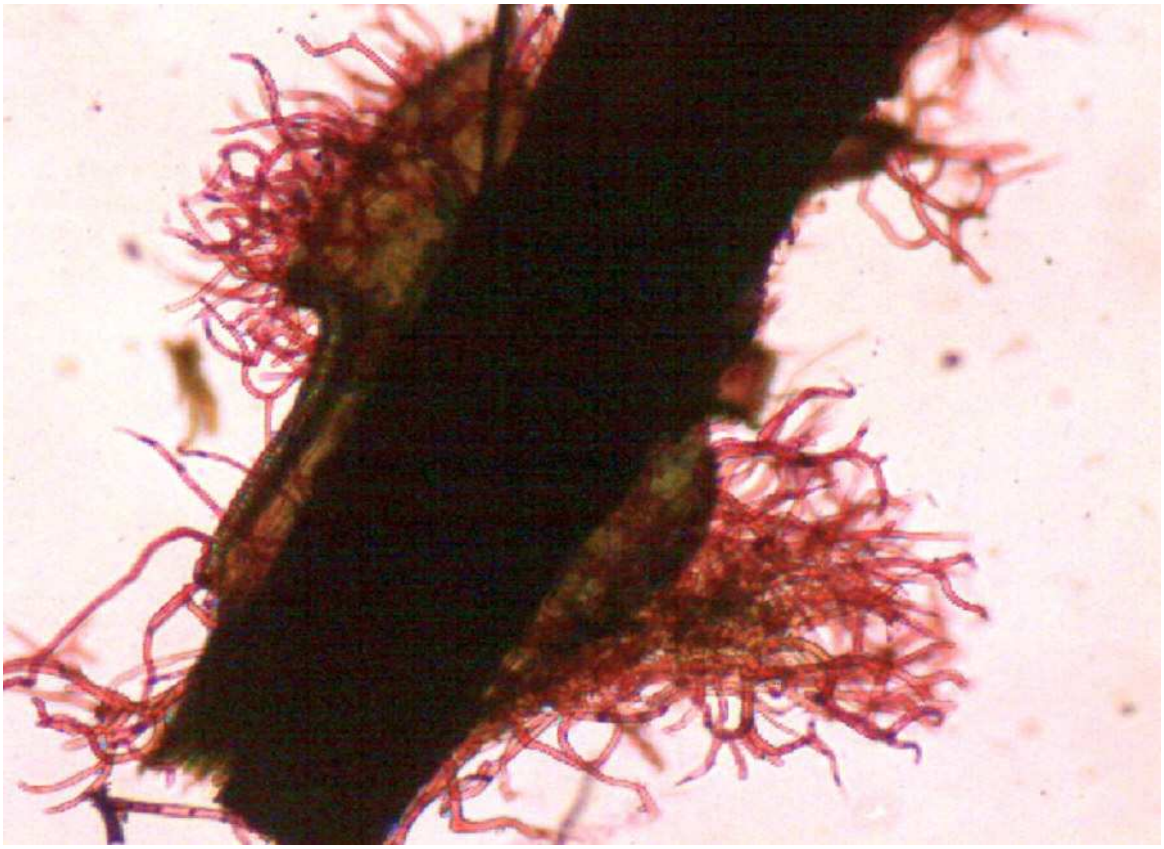

Figure 9. Tomentum of *Leptodontium tricolor.* The pink stain implies the present of pectic mucilage, which may be involved in water conservation.

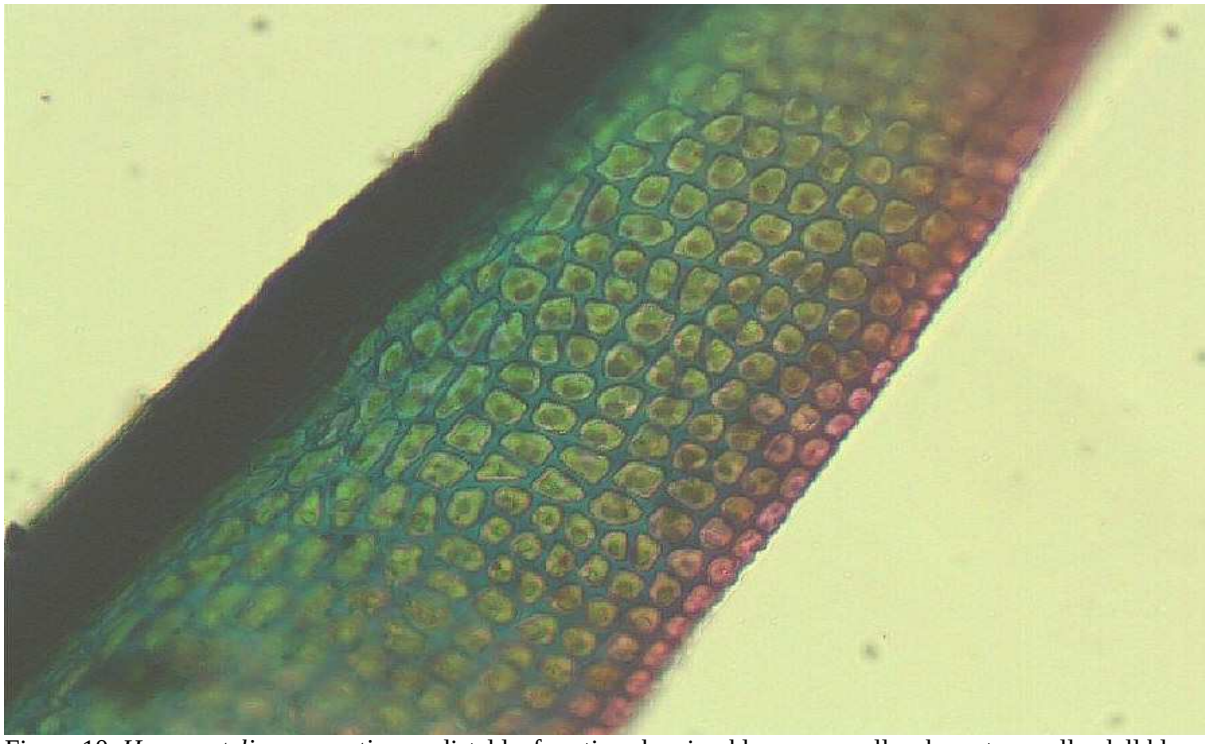

Figure 10. *Hymenostylium aurantiacum* distal leaf portion showing blue-green collenchymatous cells, dull blue large, simple papillae, and pink marginal cells that have thin cell walls.

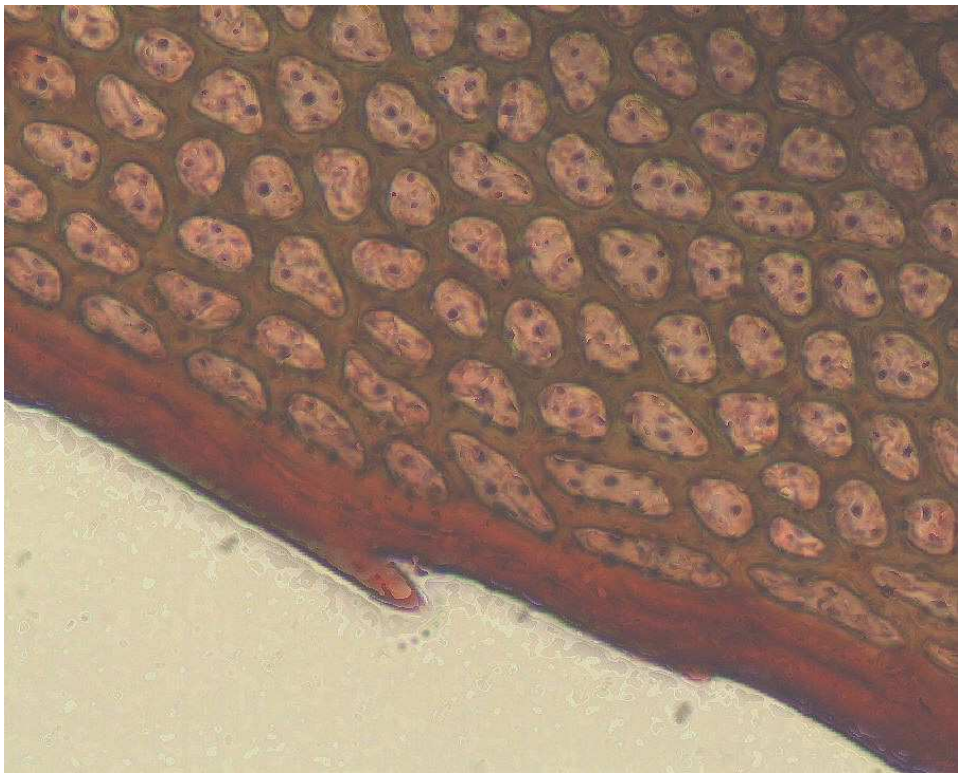

Figure 11. *Trachyodonium zanderi* showing blue, scattered, small, simple papillae over pink cell lumens and green(ish) collenchymatous internal cell walls.

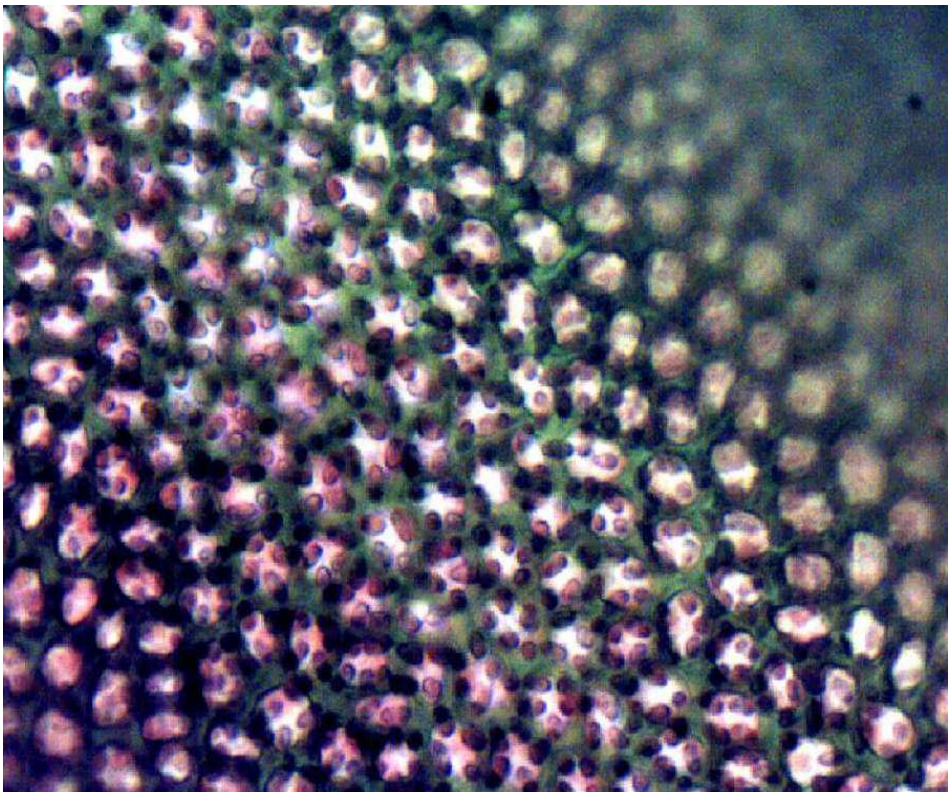

Figure 12. *Leptodontium viticulosoides* showing blue or violet bifid papillae and green collenchymatous internal cell walls.

DISCUSSION

The progressive polychrome stain protocol was revealing or at least potentially valuable in three ways. (1) Plant parts of intergrading morphology may be clearly distinguished on a chemical basis (e.g., laminal papillae morphology). (2) Both axillary hairs and perigonial paraphyses are apparently both essentially "mucilage hairs" and may have a lubricant function. Because tomentum and leaf costal awns also stain pink, a further selective function is possible, perhaps these last two act as water sponges. The habitat of tomentose or awned species is commonly arid or misty mountainous areas, often with tree bark substrates. (3) There are taxonomic differences in color reaction to TBO and RR in addition to that of KOH, but it is somewhat too early to declare these as indicators for chemical taxonomy. This is certainly so as multiple tests are necessary to ensure identification of cell constituents, as these stains are not perfectly selective.

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