CHAPTER 2-2b
SPHAGNUM STAINING

H. Rudi Zielman and Janice M. Glime

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CHAPTER 2-2b
SPHAGNUM STAINING

Figure 1. ***Sphagnum russowii***, a species for which pores are seen more easily when stained. Photo by Des Callaghan, with permission.

Following a discussion on Bryonet and the diminishing size of his trusty aniline pencil (Figure 2), Rudi Zielman set out to compare various stains used to make the pores of ***Sphagnum*** leaves and stems more visible. This subchapter is the result of that investigation. Another driver for this investigation is the toxicity of aniline. Furthermore, newer versions of this pencil simply didn't work – they didn't color wet leaves (Figure 3-Figure 4). And an aniline solution did not color the leaves easily. Then the leaves lost their color when they were placed in water.

Figure 2. Aniline blue pencil used to stain ***Sphagnum***. Photo by Rudi Zielman.

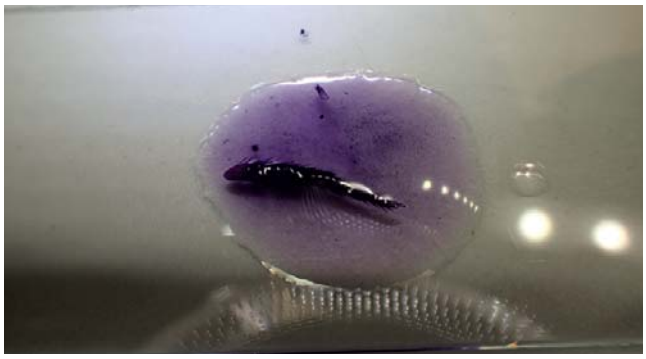

Figure 3. ***Sphagnum obtusum*** branch in water with aniline blue pencil scrapings. Photo by Rudi Zielman.

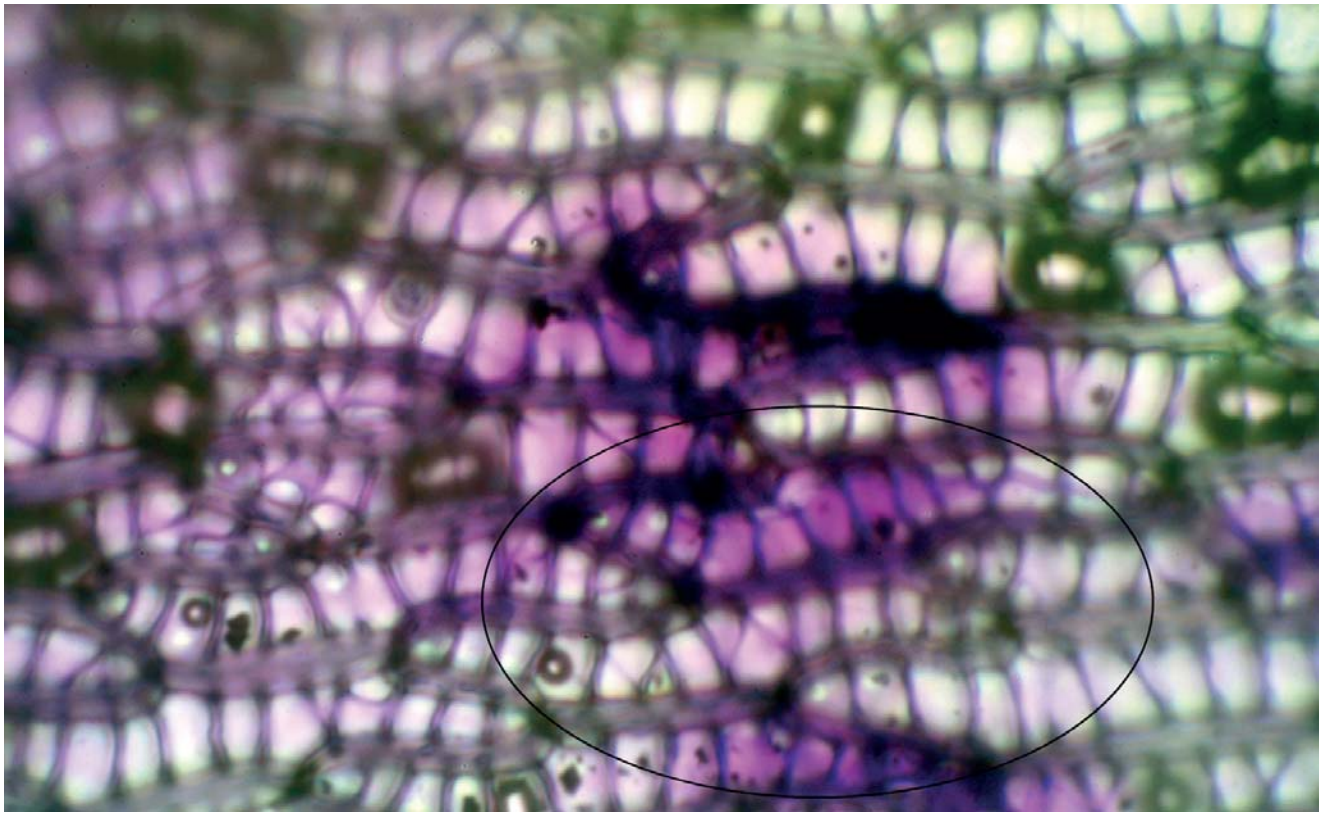

Figure 4. ***Sphagnum obtusum*** stained with an old aniline pencil. The branch was stained, leaves carefully removed, and placed on a slide in water. The pores became more visible, as seen in the area inside the ellipse. Photo by Rudi Zielman.

The dyes used are all dyes with a ring structure; especially with toluidine blue it is emphasized that it should be the toluidine blue-O, *i.e*. the methyl and amine groups in the ortho position. This is also true with safranin, and apparently is the case in all the stains described here and currently available for staining the moss. Methylene blue and safranin are sold at (web) stores that also sell microscopy equipment.

Toluidine blue is currently the most difficult to obtain; when Zielman collected all the materials about 8 years ago he was able to collect the needed materials from a web shop and a university lab; gentian violet was also a problem at that time, now to a lesser extent; one needs a doctor's prescription to get it from a pharmacist. It is used in the treatment of **thrush** (a fungal infection) in infants, and can apparently also be found at shops that focus on supplies for breastfeeding. An advantage is that all these dyes are, in contrast to aniline, non-toxic. A 10 ml ready-to-use solution costs approximately 15-20 euros. Methylene blue, toluidine blue-O, and gentian violet are also available as a powder (quite difficult); to use them one places a few grains (forceps tip) on a slide and dissolves this in water or ethanol. You can also use the powder to prepare a "stock solution" (additional recipes on the internet; several are listed here, but unfortunately no URL's or author names were available), but then some stirring and filtering facilities are required. For the staining effects it does not matter whether you use the solution or the powder, but the solution works more easily.

A word about safety: methylene blue is the most annoying of these four dyes tested here. It is non-toxic, but it can cause eye and skin irritations. All solutions contain alcohol and are therefore slightly irritating. Spilled dyes can be easily removed with a tissue and some methylated alcohol.

Methods

The stains used are:

No colorant (stain) applied
Methylene Blue
Toluidine Blue (actually tolonium chloride)
Gentian Violet (also called crystal violet or methylrosanaline)
Safranin

Methylene Blue from Powder

Prepare a saturated solution of methylene blue by adding 1.5 g powdered methylene blue to 100 mL 95% ethyl alcohol. Slowly add the alcohol to dissolve the powder. Add 30 mL saturated alcoholic solution of methylene blue to 100 mL distilled water and 0.1 mL 10% potassium hydroxide. Always make these in a 1% ETOH solution, a saturated solution in water.

Toluidine Blue-O from Powder

Dissolve the toluidine blue powder in distilled water (0.1 g of toluidine blue in 100 ml of distilled water). Check the *p*H of the solution, it is very important. The stock solution should be *p*H 2.3 (and less than 2.5), achieved with 5 ml 1% sodium chloride in 45 ml; mix well. The working solution should be *p*H 2.0-2.5. Make this solution fresh and discard after use.

Alternatively, mix powder to dissolve and adjust *p*H to 2.0~2.5 using glacial acetic acid or HCl.

Gentian Violet Powder

Dissolve 2 g of gentian violet powder dye in 20 ml of 95% ethanol (Histanol 95) and mix with 80 ml of 1% aqueous solution of ammonium oxalate.

Safranin-O

Mix 10 ml of basic solution with 90 ml of distilled/demineralized water.

Applying Stain

For each stain, a dry branch or stem piece is quickly dipped in a few drops of the stain, stirred and slightly pressed to make sure the stain is distributed everywhere. If you dip too briefly, the leaf parts (often the proximal half) may not be properly stained because the stain solution could not reach them. After dipping, the branches or stem pieces are rinsed in demineralized water. Do this carefully; ***Sphagnum*** mosses very easily lose leaves or become damaged. Just dip in water, replace drops, re-dip, until the water no longer colors. After that, the material is mounted on the slide.

Microscopic images in this subchapter were taken with a Leica DM E microscope with 40 X achromatic objective and trinocular head with a Leica 1 X photo lens on which a Nikon D5300 camera body was attached. The diaphragm opening of the microscope is equal for all photos; the exposure intensity is not. Because a microscopic image has no depth of field, stacking is needed. First focusing is done slightly above the leaf blade or section and then the fine adjustment knob is used manually through small steps to change the focal plane through the cell wall, going deeper and deeper. The recordings are then stacked with CombineZM and reworked (color levels automatically balanced, stack edges clipped) with GIMP 2.10. (It is also possible to have a camera that does automatic stacking and combining the images.) The resulting photos are composed of a variable number of individual photos, depending on visual evaluation (or the number provided by an automatic camera).

Results

In the overviews below we show a few species in which pores are important to observe. For each species in the images shown, the different stains were applied to adjacent branches of the same stem just below the capitulum. The order is always no coloring, methylene blue, toluidine blue-O, gentian violet, safranin. This sequence shows a fairly even gradient in the colors seen, from blue through purples to orange-red.

Sphagnum divinum (Figure 5-Figure 15)

First of all, ***Sphagnum divinum***, where the width of pores in the proximal part of branch leaves and the thickness of the wall between **chlorocytes** (cells with chloroplasts) and **hyalocytes** (colorless cells) are important to observe. What you see in these images of ***Sphagnum divinum*** is that the pores in the hyalocytes are clearly visible and are less than half the width of the cell. The leaf cross section is less clear. This is caused by the sigmoid cell pattern; the wall between hyalocytes and chlorocytes is visible through many sections behind one another, and thus is often blurred in stained leaf transections, so it is recommended to inspect these in unstained condition.

Figure 5. ***Sphagnum divinum***, Ireland, a segregant from ***Sphagnum magellanicum*** that can be identified more easily when stained. Photo by David Long, with permission.

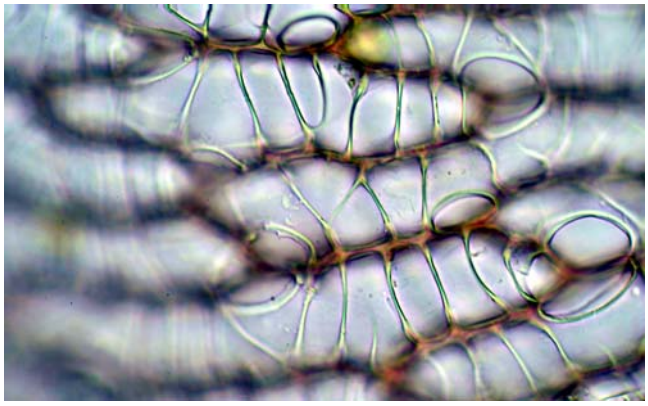

Figure 6. ***Sphagnum divinum*** leaf cells with no staining. Photo by Rudi Zielman.

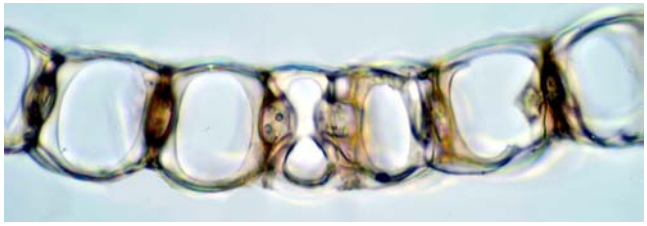

Figure 7. ***Sphagnum divinum*** leaf cross section with no staining. Photo by Rudi Zielman.

Figure 8. ***Sphagnum divinum*** leaf cells stained with methylene blue. Photo by Rudi Zielman.

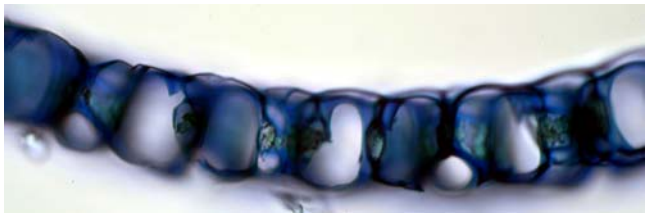

Figure 9. ***Sphagnum divinum*** leaf cross section, stained with methylene blue. Photo by Rudi Zielman.

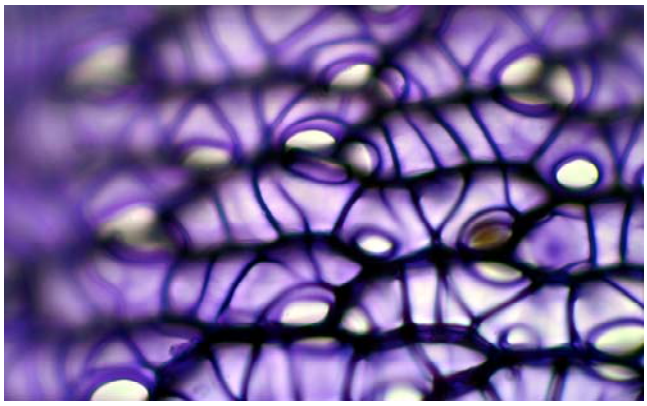

Figure 10. ***Sphagnum divinum*** leaf cells stained with toluidine blue-O. Photo by Rudi Zielman.

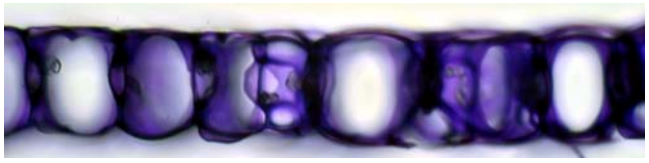

Figure 11. ***Sphagnum divinum*** leaf cross section, stained with toluidine blue-O. Photo by Rudi Zielman.

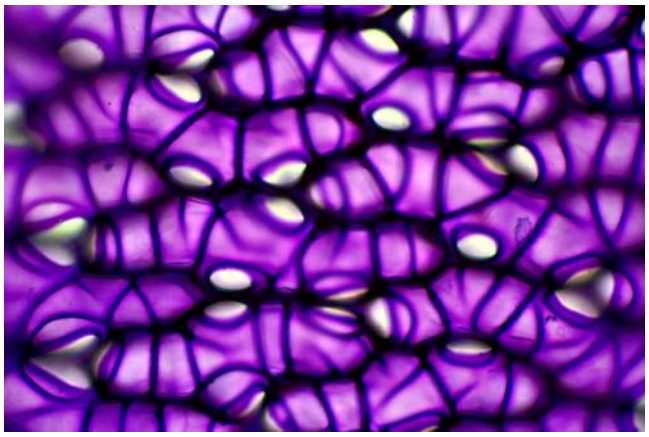

Figure 12. ***Sphagnum divinum*** leaf cells stained with gentian violet. Photo by Rudi Zielman.

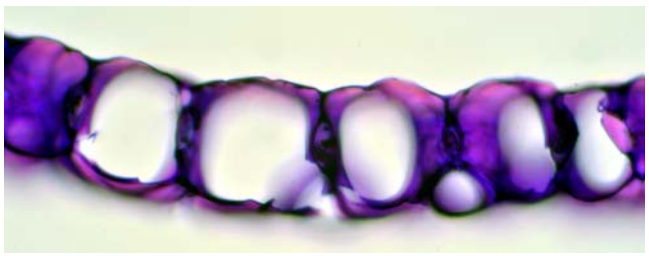

Figure 13. ***Sphagnum divinum*** leaf cross section, stained with gentian violet. Photo by Rudi Zielman.

Figure 14. ***Sphagnum divinum*** leaf stained with safranin. Photo by Rudi Zielman.

Figure 15. ***Sphagnum divinum*** leaf cross section, stained with safranin. Photo by Rudi Zielman.

Sphagnum obtusum (Figure 4, Figure 16-Figure 26)

A true challenge with staining lies in making visible the very small and very unclear pores of ***Sphagnum obtusum***. The cell wall thinnings that matter most are primarily located proximally in the leaf at the lateral sides; this zone is therefore always pictured.

Figure 16. ***Sphagnum obtusum***, a species with faint pores that require staining for observation. Photo by Michael Lüth, with permission.

It should be clear that all stains enhance the visibility of the structures in the branch leaf cells of ***Sphagnum obtusum***, while without such staining the faint pores remain invisible. But again, the stained cross-sections of the branch leaves are more difficult to interpret than the unstained ones.

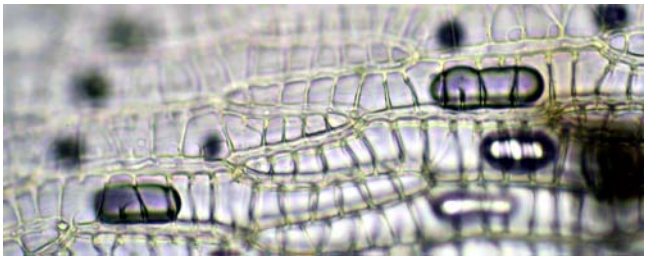

Figure 17. ***Sphagnum obtusum*** leaf cells, with no staining. Photo by Rudi Zielman.

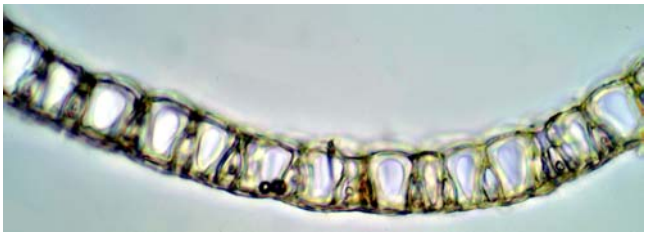

Figure 18. ***Sphagnum obtusum*** leaf cross section, with no staining. Photo by Rudi Zielman.

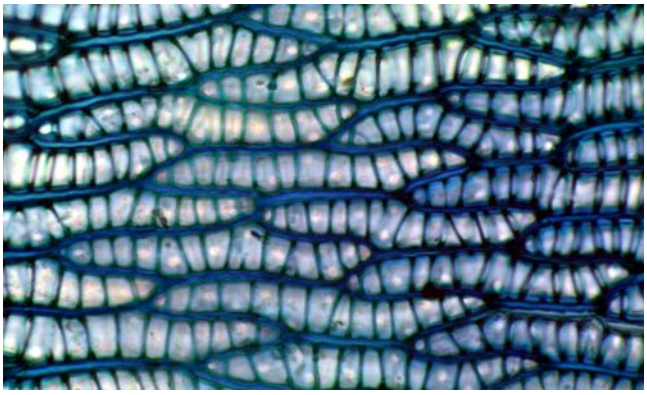

Figure 19. ***Sphagnum obtusum*** leaf cells, stained with methylene blue. Photo by Rudi Zielman.

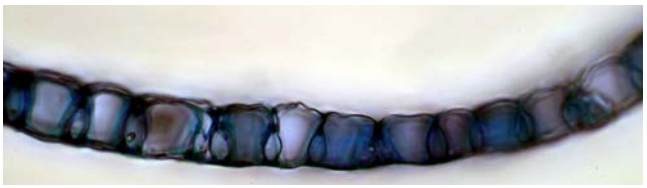

Figure 20. ***Sphagnum obtusum*** leaf cross section, stained with methylene blue. Photo by Rudi Zielman.

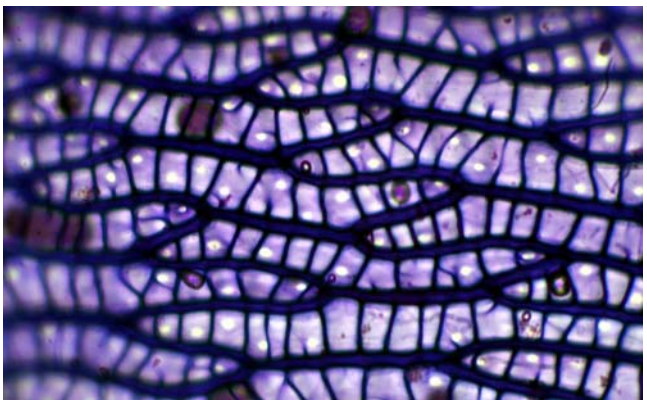

Figure 21. ***Sphagnum obtusum*** leaf ells, stained with toluidine blue-O. Photo by Rudi Zielman.

Figure 22. ***Sphagnum obtusum*** leaf cross section, stained with toluidine blue-O. Photo by Rudi Zielman.

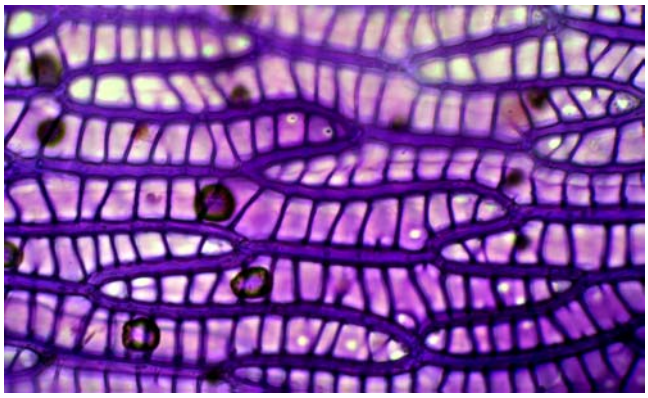

Figure 23. ***Sphagnum obtusum*** leaf cells, stained with gentian violet. Photo by Rudi Zielman.

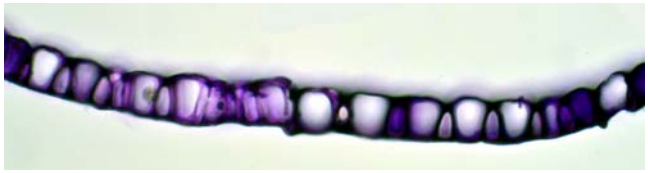

Figure 24. ***Sphagnum obtusum*** leaf cross section, stained with gentian violet. Photo by Rudi Zielman.

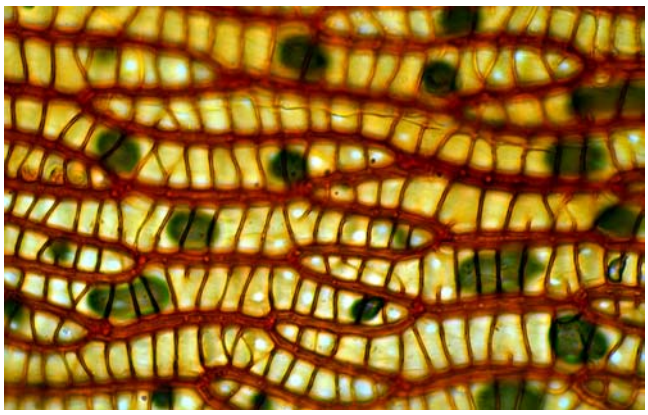

Figure 25. ***Sphagnum obtusum*** leaf cells, stained with safranin. Photo by Rudi Zielman.

Figure 26. ***Sphagnum obtusum*** leaf cross section, stained with safranin. Photo by Rudi Zielman.

Sphagnum russowii (Figure 27-Figure 37)

In ***Sphagnum russowii***, the **pseudopores** (thin spots in the cell wall) of the stem epidermis are of importance. The easiest way to prepare them is by holding a piece of stem with forceps and then cut the whole stem diagonally with a razor blade; sometimes it even works to get rid of the tissue below that epidermis completely (*e.g.* in the gentian violet preparation in Figure 35). Hölzer (2010) also mentions the large pores on the ventral side of branch leaves as characteristic (Figure 28); figs 30, 32, 34, 36 show the same pore structure.

Figure 27. ***Sphagnum russowii***, a species with pores that are more easily seen with staining. Photo by Hermann Schachner, through Creative Commons.

Figure 28 is the non-stained version of ***Sphagnum russowii*** leaf pores; this image comes close to what we see through the microscope. In all pictures of the stem epidermis (Figure 31, Figure 33, Figure 35, Figure 37), except the unstained (Figure 29), the faint pores are clearly visible. Also the large pores on the ventral side in the branch leaves are easily recognizable. Please realize that the white holes are a view where a pore on the ventral and dorsal side are aligned with each other!

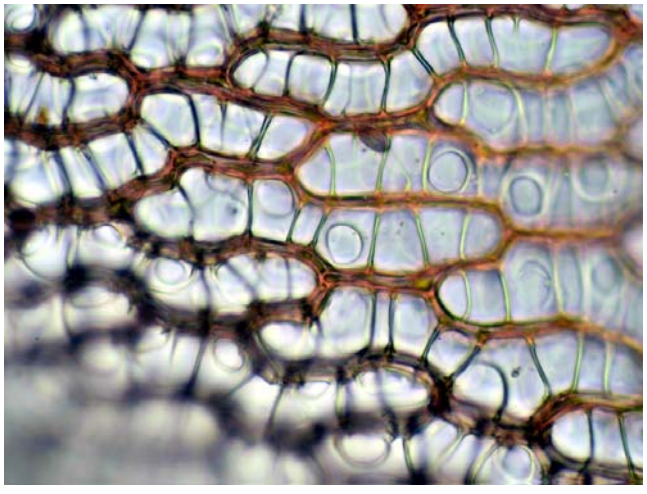

Figure 28. ***Sphagnum russowii*** leaf cells showing pores with no stain. Photo by Rudi Zielman.

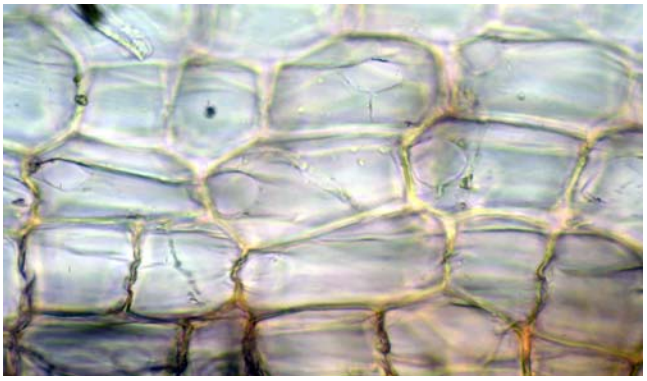

Figure 29. ***Sphagnum russowii*** stem epidermis, with no stain. Photo by Rudi Zielman.

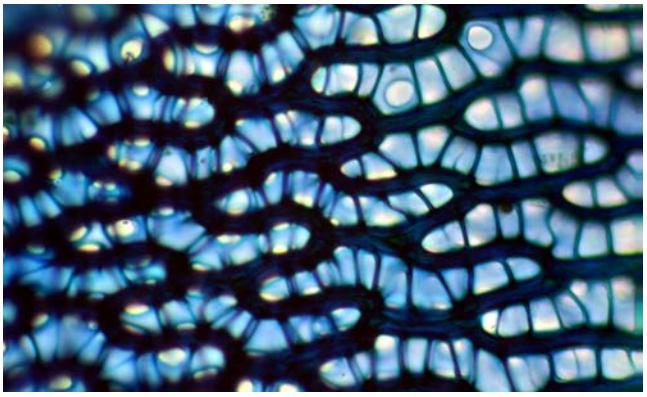

Figure 30. ***Sphagnum russowii*** leaf cells, stained with methylene blue. Photo by Rudi Zielman.

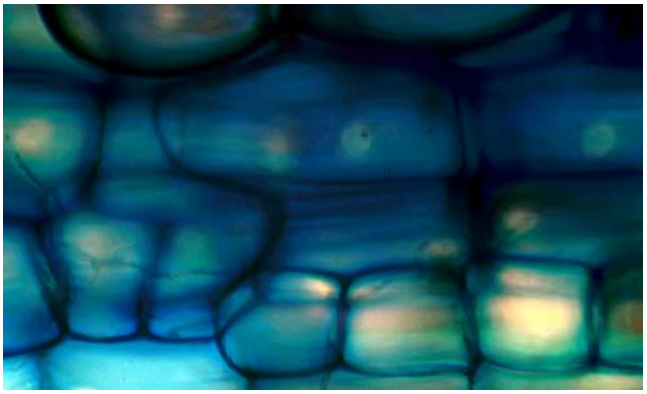

Figure 31. ***Sphagnum russowii*** stem epidermis, stained with methylene blue. Photo by Rudi Zielman.

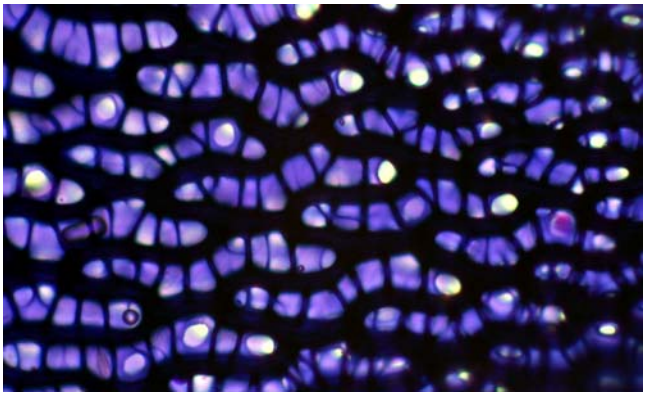

Figure 32. ***Sphagnum russowii*** leaf cells, stained with toluidine blue-O. Photo by Rudi Zielman.

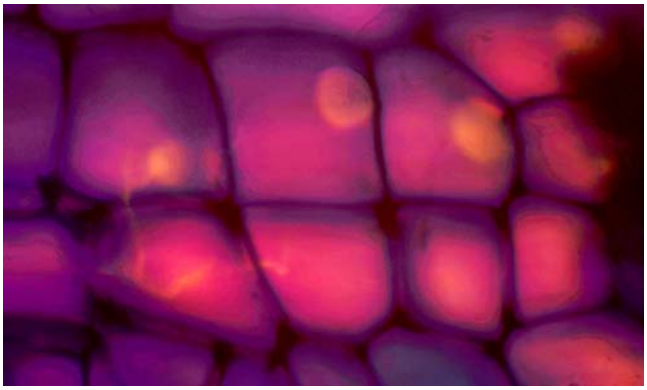

Figure 33. ***Sphagnum russowii*** stem epidermis, stained with toluidine blue-O. Photo by Rudi Zielman.

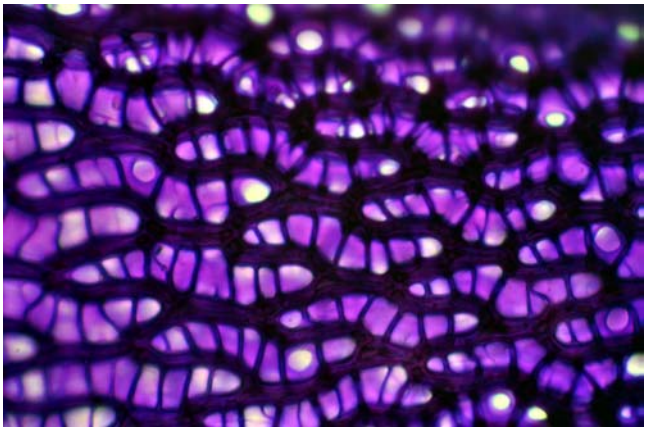

Figure 34. ***Sphagnum russowii*** leaf cells, stained with gentian violet. Photo by Rudi Zielman.

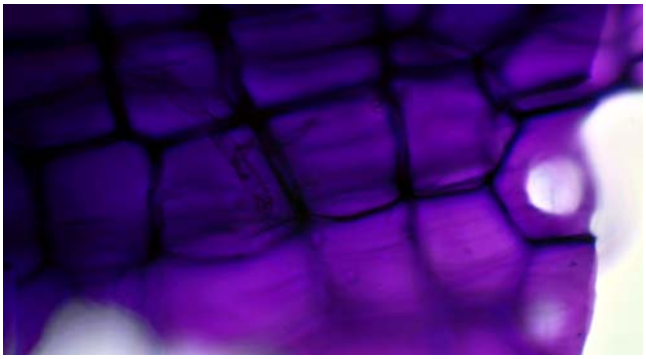

Figure 35. ***Sphagnum russowii*** stem epidermis, stained with gentian violet. Photo by Rudi Zielman.

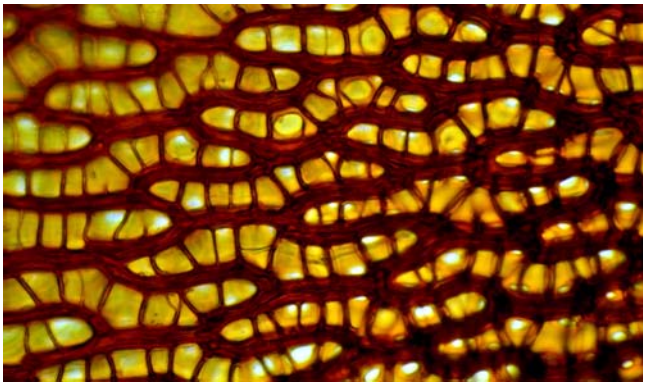

Figure 36. ***Sphagnum russowii*** leaf cells, stained with safranin. Photo by Rudi Zielman.

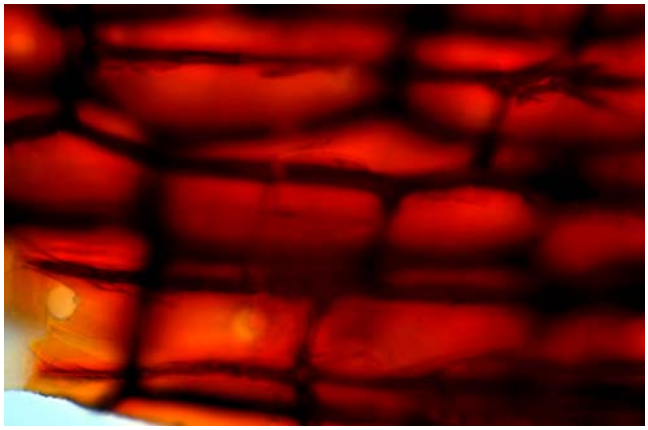

Figure 37. ***Sphagnum russowii*** stem epidermis, stained with safranin. Photo by Rudi Zielman.

Judgment Call

The staining of ***Sphagnum*** helps in making pores visible, as unstained gaps in stained walls, but is not always necessary. As an example, one can make a good judgment on ***Sphagnum divinum*** (Figure 5-Figure 15) and ***Sphagnum centrale*** (Figure 38-Figure 41) without staining. Differentiating these species depends on the thickening of the cell walls of chlorocysts as seen in section, most obvious on the adaxial (= ventral) leaf side. Staining can help in assessing this wall. In general, however, we recommend the location of chlorocytes to be assessed by unstained cross-sections.

Figure 38. ***Sphagnum centrale***. Photo by Hermann Schachner, through Creative Commons.

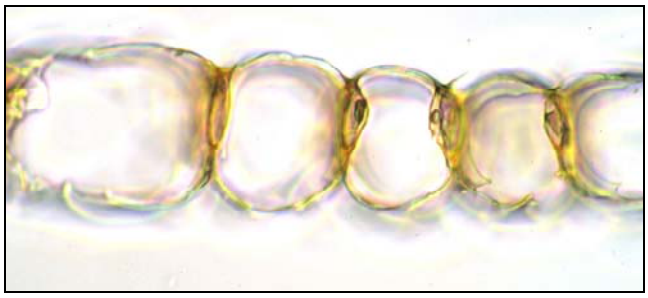

Figure 39. ***Sphagnum centrale*** unstained leaf cross section showing the almost hidden chlorocytes and thicker walls on the adaxial side of the hyalocytes. Photo by Rudi Zielman.

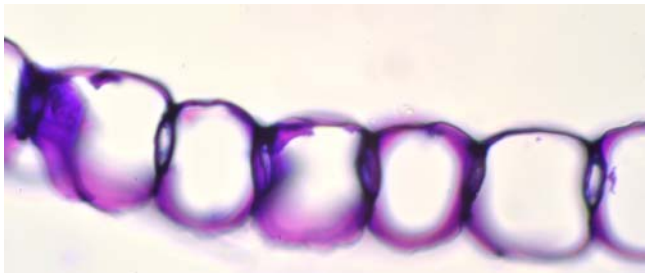

Figure 40. ***Sphagnum centrale*** leaf cross section with gentian violet stain. Photo by Rudi Zielman.

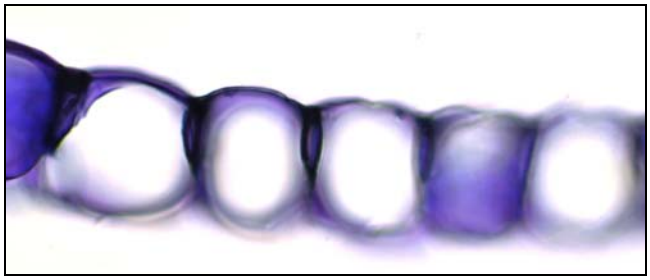

Figure 41. ***Sphagnum centrale*** leaf cross section with toluidine stain, giving a slightly better view of the thicker adaxial walls of the hyaline cells than in the gentian violet stain. Photo by Rudi Zielman.

If a decision has to be made as to whether faint pores or pseudopores are at hand, staining must be used. The dye which is used is less important as long as it is a cationic dye as already noted by Daniels and Eddy (1990). If I (Zielman) had read this in 2012 more carefully… For the rest it is merely a matter of taste; Adam Hölzer (2010) only wants to use gentian violet; Lisa op den Kamp (Bryonet, October 2012) has a strong preference for safranin; this is also the stain that was used by Laine *et al*. (2009), whereas in Australia all staining of botanical tissue is standardly done with toluidine blue (Rod Seppelt, pers. comm.). In general methylene blue, that used to be widely used, is considered a staining which is uncomfortably harsh and dark. Because of this I am, for the ease of use and availability, using more and more safranin, after my first bottle of gentian violet was empty. But after completing the work for this article, I tend to use toluidine blue, although it is tricky to obtain, or gentian violet. All in all it remains a tad a matter of personal taste, so not a firm conclusion. But I am going to use stains more often again, for an easier assessment.

Summary

Sphagnum pores are usually difficult or impossible to distinguish in unstained material. Some stains in use in the last century are toxic. And some current ones are difficult to obtain. Among the ones tested here, safranin and methylene blue are both safe and available from internet sources, gentian violet or toluidine blue might be preferred if obtainable. Some of the staining solutions can be made from powders, but it is easier to just buy the solutions ready-made.

Acknowledgments

This chapter is based on a publication in Dutch (Zielman 2020). We all owe Rudi Zielman a vote of thanks for documenting the differences among the available stains.

Literature Cited

Daniels, R. E. and Eddy, A. 1990. Handbook of European *Sphagna*. HMSO, London.

Hölzer, A. 2010. Die Torfmoose Südwestdeutschlands und der Nachbargebiete. Weisdorn, Jena.

Laine, J., Harju, P., Timonen, T., Laine, A., Tuittila, E.-S., Minkkinen, K., and Vasander, H. 2009. The Intricate Beauty of *Sphagnum* Mosses. Univ. of Helsinki. Dept. Forest Ecology Publ. 39.

O'Brien, T. P., Feder, N., McCully, M. E. 1964. Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 59: 368-373.

Zielman, H. R. 2020. Hoogveenveenmos in Nederland is *Sphagnum divinum* Hassel & Flatberg. Buxbaumiella 119: 27-34.