Comparison of different bleaching agents for bryophyte spores – the genus *Riccia* as an example

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Abstract
We have tested various agents commonly used in microscopy for bleaching dark microscopic objects, using *Riccia* spores. Many *Riccia* species have very dark spores and therefore do not allow a clear view of all spore characteristics, like the wall, which is important for the determination. We have tested sodium hypochlorite, hydrogen peroxide and chloral hydrate. Sodium hypochlorite bleaches very strongly and makes the spore wall appear hyaline, but the ornamentation is no longer visible. Furthermore, many spores burst after this bleaching, probably because they are compressed by the cover glass. Hydrogen peroxide bleaches *Riccia* spores rather too little, and additionally causes unpleasant blistering. Chloral hydrate proved to be the most suitable for bleaching *Riccia* spores.

Zusammenfassung
Wir haben verschiedene in der Mikroskopie übliche Mittel zum Bleichen dunkler mikroskopischer Präparate an *Riccia*-Sporen getestet. Viele *Riccia*-Arten haben sehr dunkle Sporen und erlauben somit keinen klaren Blick aller Merkmale, wie die für die Bestimmung wichtige Beschaffenheit der Sporenwand. Wir haben Natriumhypochlorit, Wasserstoffperoxid und Chloralhydrat getestet. Natriumhypochlorit bleicht sehr stark und lässt die Sporenwand hyalin erscheinen, so dass die Ornamentierung nicht mehr sichtbar ist. Außerdem platzen nach dieser Bleichung viele Sporen, wahrscheinlich, weil sie vom Deckgläschen zusammengedrückt werden. Wasserstoffperoxid bleicht *Riccia*-Sporen eher zu wenig, und erzeugt zusätzlich unangenehme Blasenbildung. Chloralhydrat erwies sich für die Bleichung von *Riccia*-Sporen am geeignetsten.

Introduction

Spore characters are generally regarded as important diagnostic characteristics for bryophytes. For some genera, not only their size but also the condition of the spore surface are important determining characteristics. Among liverworts, the genera *Fossombronia* and *Riccia* have particularly ornamented spores. Here, the species differentiation is largely based on the ornamentation of the spores (PEROLD 1989, MEINUNGER & SCHRÖDER 2007).
Most *Riccia* species have large, but very dark spores (JOVET-AST 1986). The ornamentation, which is important for determination, is usually not visible in the transmitted light microscope. As generally in the light microscopy of plants (HAUS 2014), different bleaching agents are used to lighten up the objects.
We have applied the most common bleaching agents to spores of two *Riccia* species and compared their effect on the image shown in the light microscope. Furthermore, we investigated the question if the bleaching agent has an influence on the diagnostically important spore size. Often, distally sunken spores are seen, and the initial hypothesis was that the spores change its size under the influence of the bleaching agents.
We used the common species *Riccia glauca* and *Riccia sorocarpa* collected in autumn 2019 in the Styrian lowland as examples. Both have dark brown spores, which could hardly be photographed in natural pigmentation.

Material and Methods

From thalli of rich sporulating *Riccia glauca* and *Riccia sorocarpa* collected 2019 in the Styrian lowland (SE-Austria), we prepared five mature capsules each, placed them on a slide with a drop of water, opened the capsules, removed the remains of the capsule wall and mixed the spores. Then we divided the spore-water mixture into four drops of approximately equal size. To three of these

samples we added a bleaching agent commonly used in microscopy, the fourth sample served as a control.
As bleaching agent we used

- Sodium hypochlorite (commercial bleach Danklorix from Colgate-Palmolive, contains 2.8g sodium hypochlorite per 100g water, known as C in lichenology)
- Hydrogen peroxide (15% solution)
- Chloral hydrate (saturated aqueous solution)

MÜLLER (1906–1911) also recommends concentrated sulfuric acid, but we would rather not have such agents on our private working places.
Immediately after bleaching, we examined the samples in a light microscope. The samples were measured and photographed. Translucent light photos were taken with a Motic light microscope BA310 with Moticam 5.0 MP camera and supplied software. We applied focus stacking using the program Picolay (Freeware, www.picolay.de) to create meaningful images.

Results

Control

The unbleached spores can be photographed by exposing them to strong light. In doing so, structures at the margins are overexposed, such as the hems of the *Riccia* spores. With Focus Stacking, the overexposed margins of the individual images create unattractive light effects that do not exist in reality (Fig. 1).

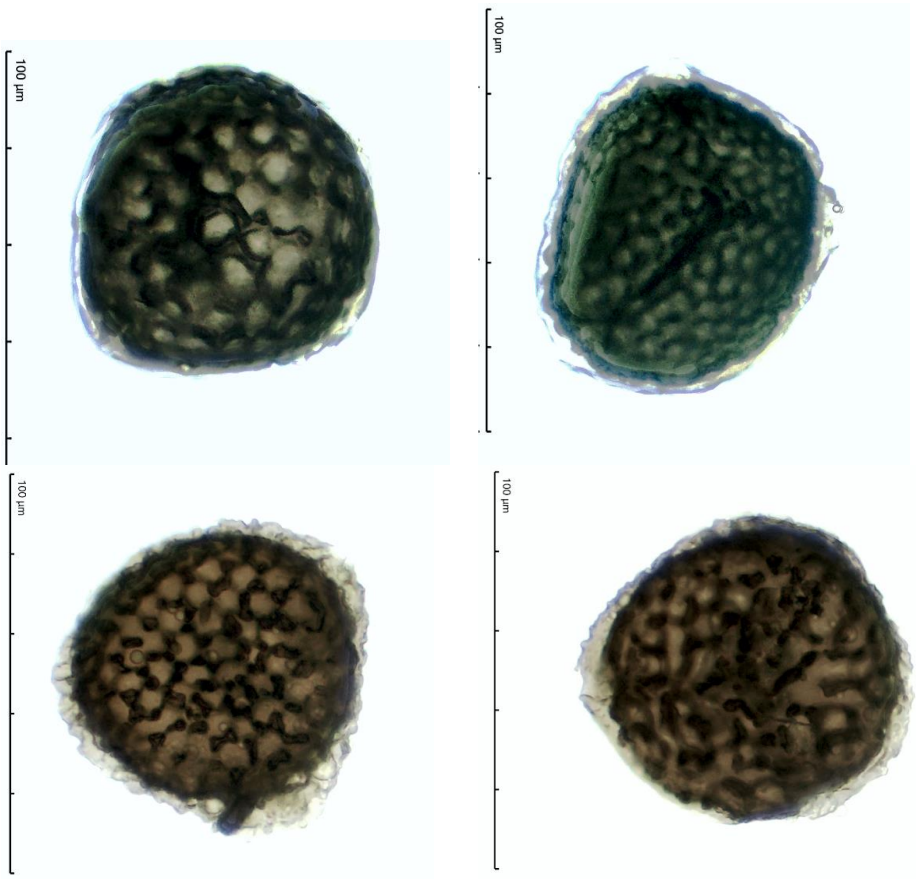

Fig. 1. Spores of *Riccia glauca* (up) and *Riccia sorocarpa* (down) distal (left) and proximal (right) side; for unbleached control.
Abb. 1. Sporen von *Riccia glauca* (oben) und *Riccia sorocarpa* (unten) distal (links) und proximal (rechts), welche als ungebleichte Kontrolle dienen.

Sodium hypochlorite

This bleaching agent seems to bee too drastic for *Riccia* spores. The spores become clearly transparent. The interesting surface becomes hyaline and is therefore not visible any more (Fig. 2). After half an hour the spores are completely hyaline and thus hardly visible.

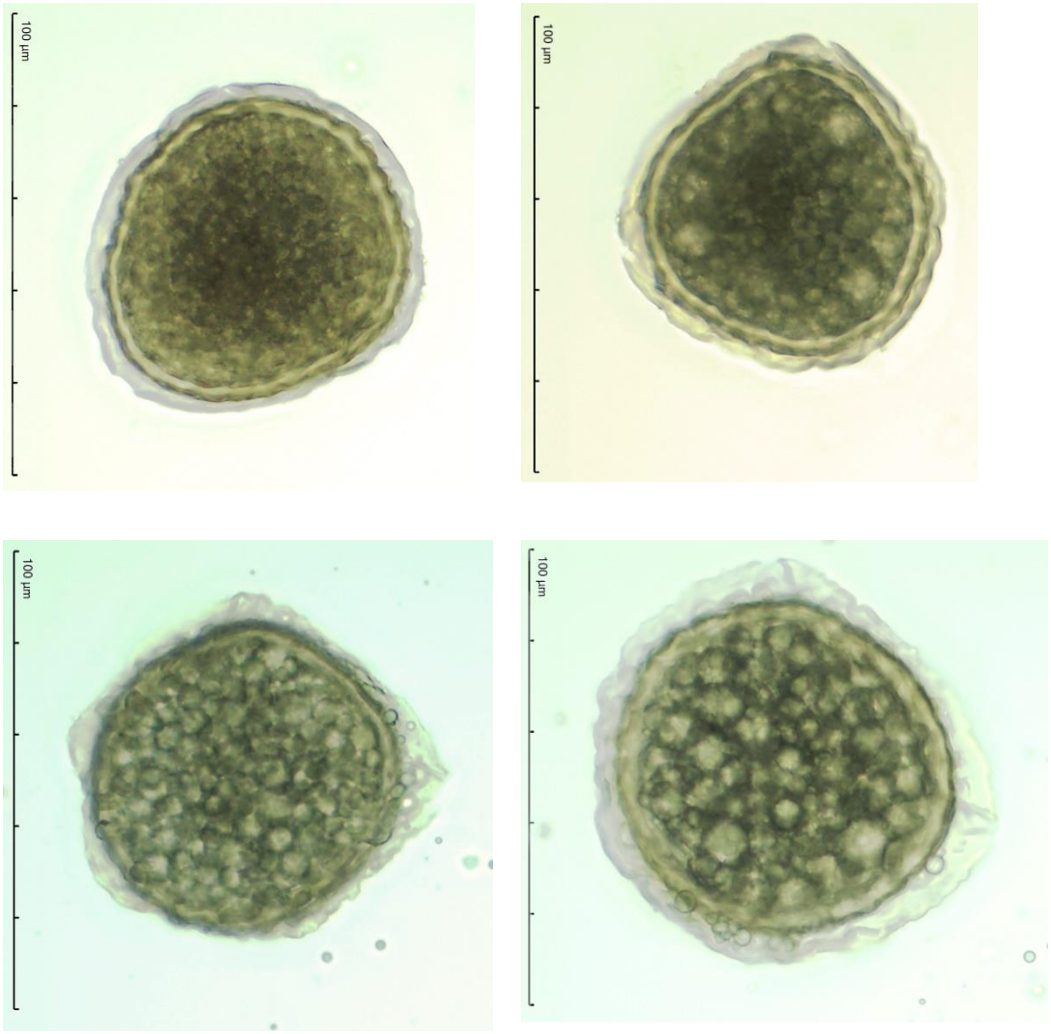

Fig. 2. Spores of *Riccia glauca* (up) and *Riccia sorocarpa* (down) distal (left) and proximal (right) side; bleached with sodium hypochlorite.
Abb. 2. Sporen von *Riccia glauca* (oben) und *Riccia sorocarpa* (unten) distal (links) und proximal (rechts) gebleicht mit Natriumhypochlorit.

Hydrogen peroxide

This product has a slight bleaching effect (Fig. 3). However, this effect is only weak, although the agent was used in a high concentration. Heating should increase the bleaching effect, but this would be an additional working step. A further disadvantage is that hydrogen peroxide releases gaseous oxygen in contact with numerous substances, including contact with the inside of the *Riccia* spores. The consequences are gas bubbles inside the spores (see Fig. 3, *Riccia sorocarpa*, proximal), and a strong bubble formation of the preparation, which makes photography more difficult (Fig. 4).

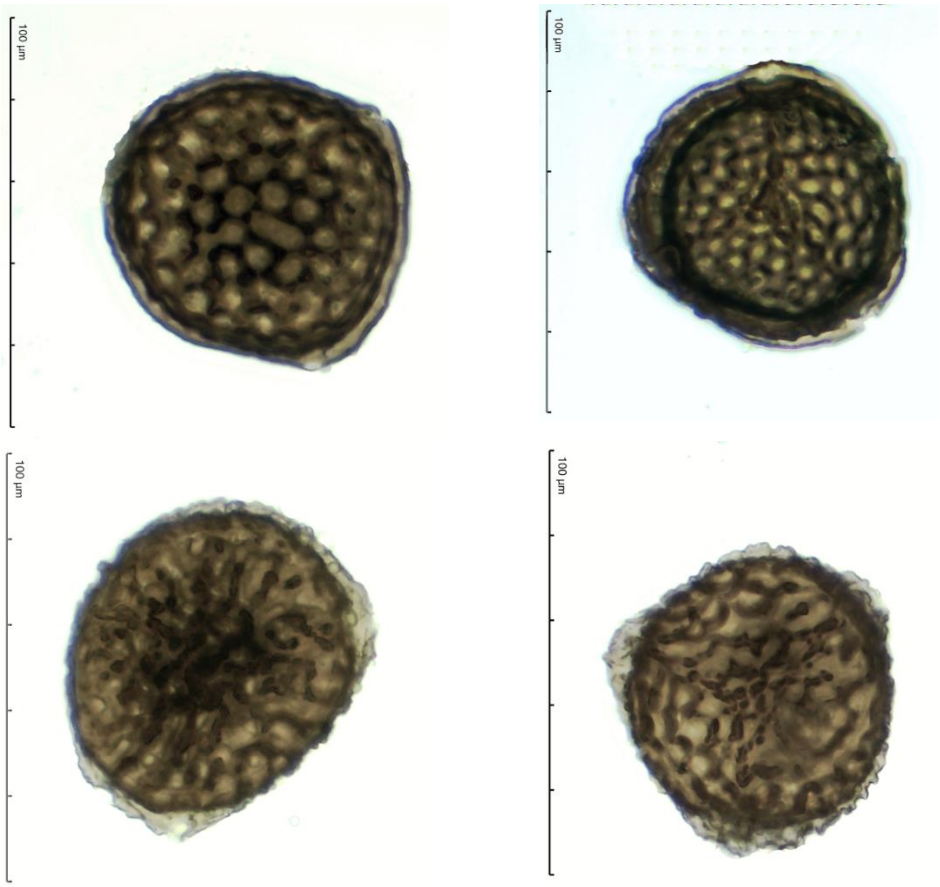

Fig. 3. Spores of *Riccia glauca* (up) and *Riccia sorocarpa* (down) distal (left) and proximal (right) side; Bleached with hydrogen peroxide.
Abb. 3. Sporen von *Riccia glauca* (oben) und *Riccia sorocarpa* (unten) distal (links) und proximal (rechts); gebleicht mit Wasserstoffperoxid.

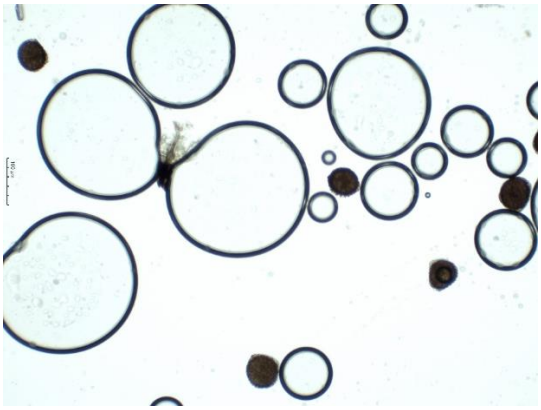

Fig. 4. Preparation of *Riccia sorocarpa* spores bleached with hydrogen peroxide with strong oxygen bubble formation.
Abb. 4. Zubereitung von mit Wasserstoffperoxid gebleichten *Riccia sorocarpa*-Sporen mit starker Sauerstoffblasenbildung.

Chloral hydrate

Chloral hydrate bleaches the spores to a medium degree, so that the ornamentation of the surface is well visible without being disturbed by too much transparency (Fig. 5). In the microscope, the exposure can be adjusted so that no overexposed edges are created during focus stacking.

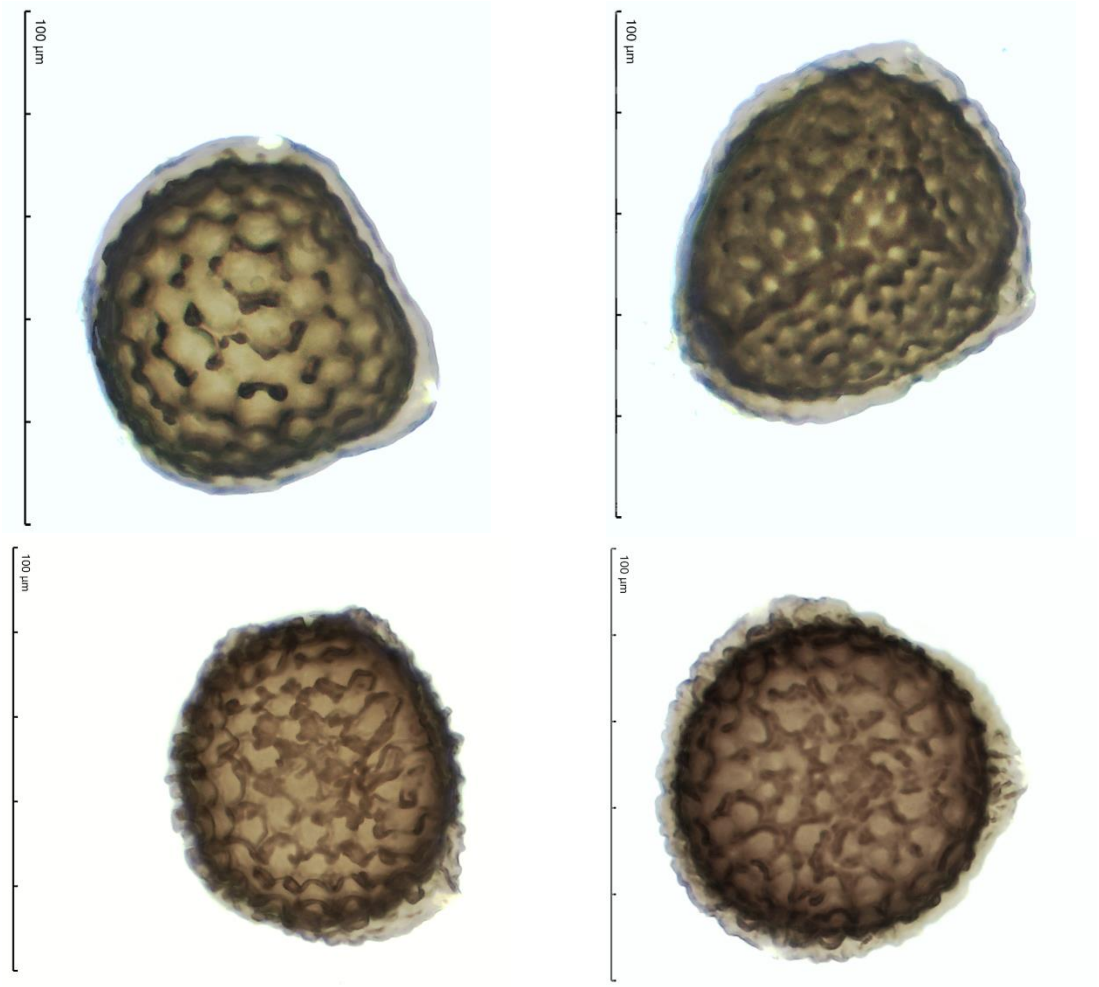

Fig. 5. Spores of *Riccia glauca* (up) and *Riccia sorocarpa* (down) distal (left) and proximal (right) side; bleached with choral hydrate.
Abb. 5. Sporen von *Riccia glauca* (oben) und *Riccia sorocarpa* (unten) distal (links) und proximal (rechts); mit Choralhydrat gebleicht.

Spore size

The spore size shows no significant change due to bleaching. The spores tend to become slightly larger rather than shrinking (Fig. 6). In the case of sodium hypochlorite this can be lead to many burst spores.

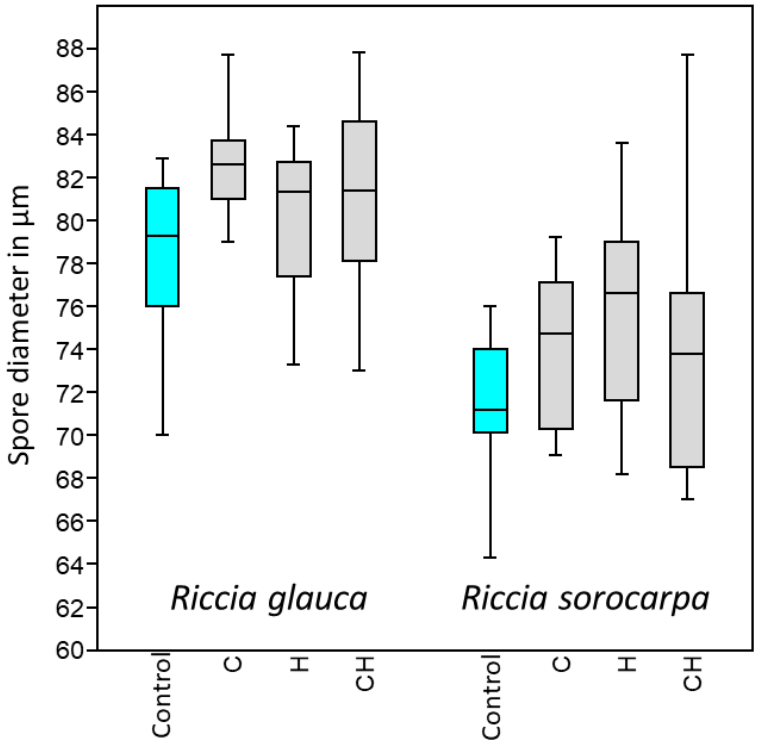

Fig. 6. Spore size of *Riccia glauca* (left) and *Riccia sorocarpa* (right) after bleaching compared with an unbleached control. C = Natriumhypochlorit, H = Hydrogen peroxide, CH = Chloralhydrate. All differences not significant (Kruskal-Wallis-test, Bonferroni corrected, n = 10).
Abb. 6. Sporengröße von *Riccia glauca* (links) und *Riccia sorocarpa* (rechts) nach dem Bleichen im Vergleich zu einer ungebleichten Kontrolle. C = Natriumhypochlorit, H = Wasserstoffperoxid, CH = Chloralhydrat. Alle Unterschiede nicht signifikant (Kruskal-Wallis-Test, Bonferroni korrigiert, n = 10).

Discussion

In our test chloral hydrate appears to be the most suitable bleaching agent. Chloral hydrate as a component of Hoyer's medium used for embedding spores for microscopic permanent preparations (Anderson 1954). For other, deep blackish spores like those of *Riccia ciliata*, the bleaching effect of Chloral hydrate is too weak. Sodium hypochlorite bleaches too strong and hydrogen peroxide rather too little. Both agents also have unpleasant side effects. Under influence of sodium hypochlorite many spores burst, while hydrogen peroxide develops gaseous oxygen inside and outside the spores, which leads to strong bubble formation under the cover slide.
In tendency, the spores tend to become larger after bleaching, probably because the spore wall weakened by bleaching and the spores are compressed under the cover glass. This would also explain why many spores even burst under the strongest bleaching agent sodium hypochlorite.
In summary, chloral hydrate is recommended as a bleaching agent for moderate dark *Riccia* spores. Light brown spores as in *Riccia bifurca*, with a sufficient transparency, should not be bleached. Additional, spore measurements are better done before bleaching. Although no significant changes in spore size could detected after bleaching, the spores showed a slight enlargement.

Literature

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