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ORIGINAL ARTICLE

Rehydration of dried mushroom specimens with Aerosol® OT for scanning electron microscopy

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Abstract

Morphological, anatomical and ultrastructural characteristics are important for taxonomical and phylogenetic studies of fungi. For scanning electron microscopy (SEM), usually only dry voucher specimens are available. For dried plant material, Aerosol® OT (AOT) has been shown to be a suitable rehydration agent for SEM preparation. For swelling and stabilization of fungal cells, however, this simple method does not yield satisfactory results. Here, we show that a combination of AOT with ultrasonic bath and rehydration in a vacuum desiccator is a good method to distend fungal cells like basidiospores and pleuro- and cheilocystidia for SEM analysis. Tissues of several species of Agaricomycetes with diverse morphological structures were exposed to the treatment. Diverse concentrations of AOT as well as treatments in an ultrasonic bath and a vacuum desiccator were tested to optimize the surface reconstruction and to reduce preparation artefacts. The evaluated rehydration method is a cheap, quick and nontoxic method to prepare dried specimens of fungal cells for SEM analysis.

Keywords AOT · Agaricomycetes · Dried fungal specimen · Rehydration · SEM

Introduction

For scanning electron microscopy (SEM), diverse techniques for fixation and drying have been proposed to prepare fresh tissue. Fresh material of rare or new taxa, however, often is not available, and researchers have to use dried material from herbarium specimens (Erbar 1995; Neuhaus et al. 2017). Conventional dehydration inevitably causes distortion of cells. Therefore, structures of dried and sometimes brittle specimens differ significantly from structures of fresh material. Especially fungal cells suffer from desiccation.

The limited ability of fungal structures to absorb water after complete desiccation mostly leads to inacceptable results in SEM studies. Consequently, the preparation of fungal material is a challenge and varies depending on the analysed structures, such as surfaces (Williams and Veldkamp 1974; Ellis 1980) or subcellular organization (Müller et al. 2000). Nevertheless, SEM studies are important for detailed descriptions of known and new fungal taxa (Seifert and Rossman 2010; Adamčík et al. 2019).

The commercial product Aerosol® OT (AOT) is chemically sodium bis(2-ethylhexyl) sulfosuccinate (Caryl 1941). It is used as a wetting agent in medicine (Ayensu 1967) and is an important pharmaceutical compound with several applications (El-Laithy 2003; Sedgwick et al. 2011; Paré and Fedorak 2014). As a surfactant, AOT reduces the surface tension of water und facilitates the entry of liquids. It is soluble in water or oil depending on the concentration and temperature (Kunieda and Shinoda 1979). It forms reverse micelles in oil and has a strong solvent power (Shinoda and Kunieda 1987). Solution behaviour in non-polar solvents (De and Maitra 1995) and foaming behaviour of AOT solutions (Cheah and Cilliers 2005) have also been studied intensely.

AOT was first introduced by Pohl (1965) for wetting plant herbarium material as a solution of 1% AOT, 74% distilled

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water and 25% methanol. Only a few drops placed onto dried material show an immediate effect. In contrast to other methods, this preparation method needs no boiling of plant material and causes no discolouring of tissues (Pohl 1965). Ayensu (1967) proposed to use a 2.5 to 3.5% aqueous AOT solution and mentioned that it is chemically neutral and nontoxic. The protocol presented by Ayensu (1967) for light microscopy was applied to diverse types of dehydrated plant tissue (Tomasi and Rovasio 1997; Ronse De Craene et al. 2015; Vargas-Rodriguez et al. 2017). Peterson et al. (1978) modified the method by using a mixture (6:l) of 10% aqueous AOT and 95% acetone. This yielded good results for morphological analyses (Trovó and Stützel 2013; Thaowetsuwan et al. 2017).

In the context of SEM preparation, AOT was first mentioned as a swelling agent for plants by Erbar (1995) and was applied to bryophytes, hornworts and pteridophytes. Afterwards, diverse preparation techniques with AOT were reported as a surfactant for the rehydration of bryophytes and plant tissues (Hofmann et al. 1996; Frey et al. 2001). To our knowledge, AOT has not yet been used to rehydrate dried fungal material for SEM analyses.

Ultrasonic baths are used to rehydrate fruits and vegetables (Režek Jambrak et al. 2007; Ricce et al. 2016; Galvao et al. 2019). A vacuum desiccator helps to remove air from fungal tissues. To prepare dried fungal tissue for SEM, ultrasound and a treatment in a vacuum desiccator were applied to specimens in a solution with AOT.

Materials and methods

The following procedure is conceived for tissue samples with sizes of 5 to 10 mm in diameter. Tissue of dried fruiting bodies was cut under a Wild Heerbrugg M5 microscope with a razor blade. Parts of the fruiting bodies were selected for the analysis of species specific characteristics of basidiospores, basidia, pleuro- and cheilocystidia, pileipellis, dermato- and caulocystidia. Species studied are listed in Table 1.

The steps of the preparation were conducted in 10-mL vials. A 4-mL plastic transfer pipette was used to remove and refill the solutions without letting the samples run dry. The entire preparation was done at room temperature. The impact of AOT concentration, time of soaking, time of application of ultrasound and vacuum were tested. To compare the efficiency of the method, freshly collected material and a dried specimen were directly fixed, washed and dried as described in the following text.

Aerosol®-OT as swelling agent

Dried tissue samples were treated with AOT in bi-distilled water. The concentrations used were 1, 2, 3, 3.5, 5, 6 and 10%. Soaking times of 24 h and 60 h were tested.

Ultrasound treatment

In order to increase the effect of the surfactant, the vials containing AOT and the fungal tissues were placed in an ultrasonic bath (Bandelin Sonorex Digitec, DT 52 H, P: 240 W, f:

Table 1 Species used in this study, including collection year, voucher numbers and country. All the specimens are deposited at Herbarium Marburgense (MB)

Species	Collection year	Voucher nr.	Country
Entoloma conferendum (Britzelm.) Noordel.	2003	MB-011683	Germany
Entoloma undatum (Gillet) M.M. Moser	2011	MB-011682	Germany
Mycena aurantiomarginata (Fr.) Quél	2015	MB-002835	Austria
Mycena meliigena (Berk. & Cooke) Sacc.	2011	MB-010231	Germany
Mycena rosella (Fr.) P. Kumm	2009	MB-001252	Austria
Mycena cf. *abramsii* (Murrill) Murrill	2020	MB-006232	Germany
Russula velutipes Velen	2011	MB-000567	Austria
Russula ochroleuca Fr.	1992	MB-011681	Germany
Strobilurus esculentus (Wulf) Singer	2015	MB-002278	Germany
	2002	MB-002226	Germany
Strobilurus tenacellus (Pers.) Singer	2015	MB-308550	North Macedonia
	2016	MB-308572	North Macedonia
Strobilurus stephanocystis (Kühner & Romagn. ex Hora) Singer	2010	MB-010929	North Macedonia
	2015	MB-308568	North Macedonia

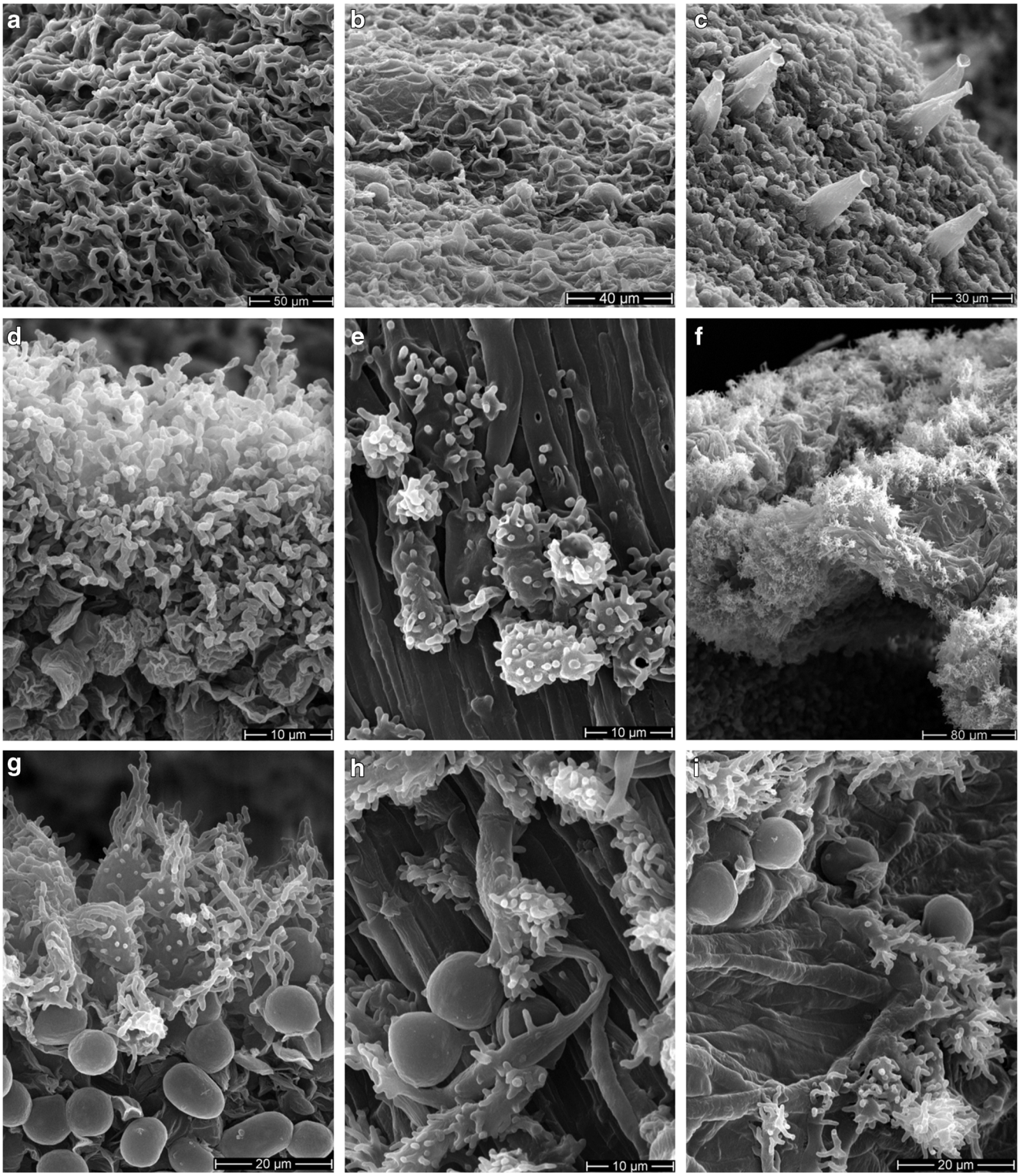
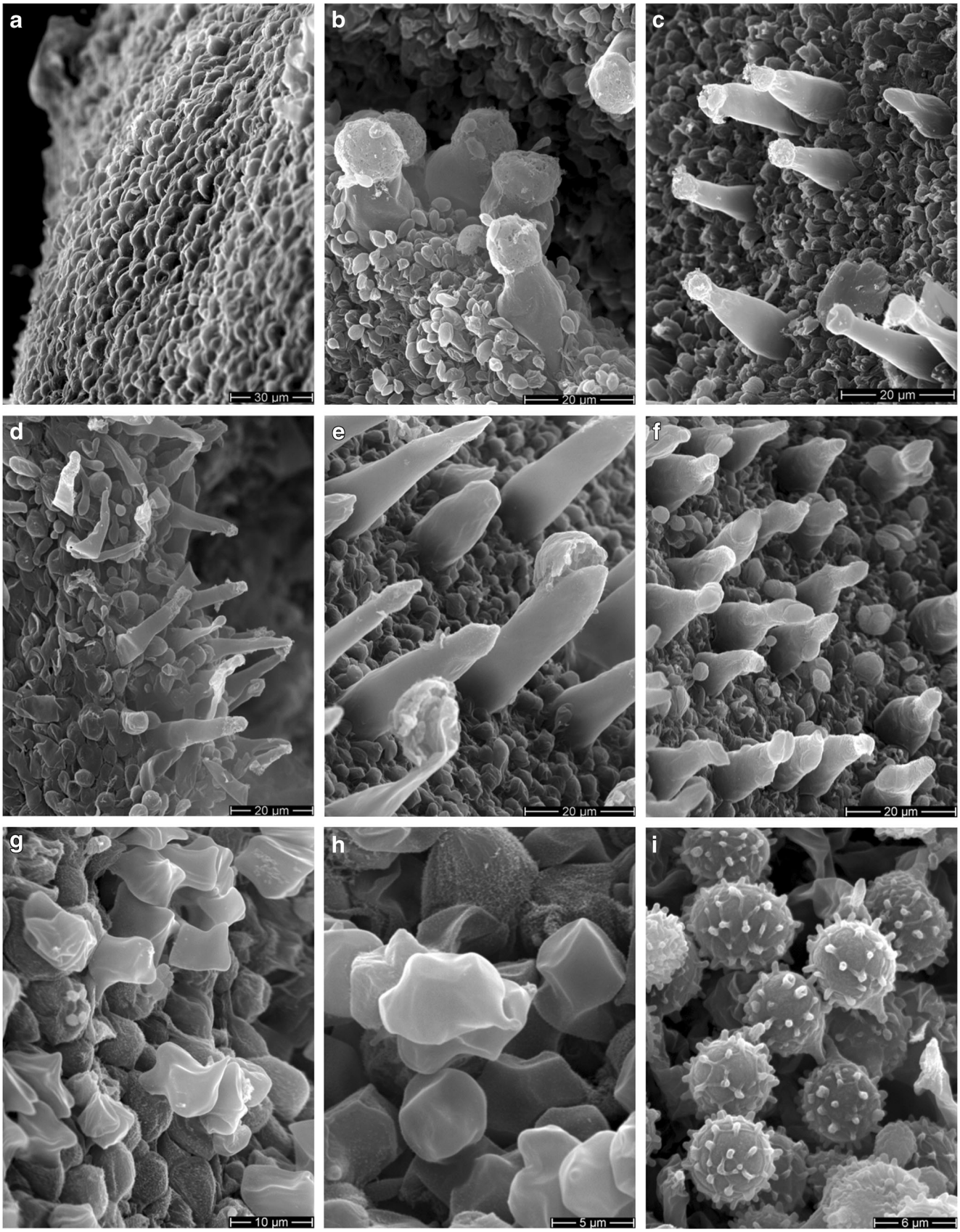


Fig. 1 **a–c** *Strobilurus esculentus*. **a** Pileipellis without AOT treatment. **b** Pileipellis with 1% AOT only. **c** Hymenium with pleurocystidia, 3% AOT, without further treatment. **d–i** *Mycena meliigena.* **d** Edge of the lamellae with cheilocystidia and basidiospores, 3.5% AOT, without further treatment. **e** Caulocystidia in detail, 6% AOT, 3 min ultrasonic bath, 24 h incubated in a vacuum desiccator. **f** Overview of the edge of the cap, 10% AOT, without further treatment. **g** Cheilocystidia and basidiospores in detail, 5% AOT, 1 min ultrasonic bath, 24 h incubated in a vacuum desiccator. **h** Caulocystidia and basidiospores, 3% AOT, 1 min ultrasonic bath, 24 h incubated in a vacuum desiccator. **i** Terminal hyphae of the pileipellis and basidiospores, 5% AOT, 1 min ultrasonic bath, 24 h incubated in a vacuum desiccator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◀ **Fig. 2** **a–b** *Strobilurus stephanocystis*. **a** Overview of the pileipellis, 3.5% AOT, 1 min ultrasonic bath, 24 h incubated in a vacuum desiccator. **b** Pleurocystidia and basidiospores, 3.5% AOT, 1 min ultrasonic bath, 24 h incubated in a vacuum desiccator. **c** *Strobilurus esculentus*, hymenium with 3.5% AOT, 1 min ultrasonic bath, 24 h incubated in a vacuum desiccator. **d–e** *Strobilurus tenacellus*, dermatocystidia, 3.5% AOT, 1 min ultrasonic bath, 24 h incubated in a vacuum desiccator. **e** Pleurocystidia with exudate crystals, 3.5% AOT, 1 min ultrasonic bath, 24 h incubated in a vacuum desiccator. **f** *Mycena rosella*, pleurocystidia with exudate and basidiospores, 3.5% AOT, 1 min ultrasonic bath, 24 h incubated in a vacuum desiccator. **g** *Entoloma conferendum*, hymenium with basidiospores, 3.5 % AOT, 1 min ultrasonic bath, 24 h incubated in a vacuum desiccator. **h** *Entoloma undatum*, hymenium with spores, 3.5% AOT, 1 min ultrasonic bath, 24 h incubated in a vacuum desiccator. **i** *Russula ochroleuca*, hymenium with basidiospores in detail, 3% AOT, 24 h incubated in a vacuum desiccator

35 kHz) with water. Samples were treated for 1 min or 3 min. As control, samples were observed without ultrasonic treatment.

Vacuum treatment

To make the AOT solution penetrate into the fungal tissue and to cast out air trapped between the hyphae, vials with samples in the AOT solution were placed in a vacuum desiccator for 24 h. Vacuum was created with the help of a water jet pump.

Further preparation steps

Afterwards, the AOT solution was removed by three washing steps with bi-distilled water (10 min each) followed by three washing steps with phosphate-buffered saline (PBS, pH between 5.8 and 7.4, 10 min each). Fungal cells were fixed with glutaraldehyde (4% dissolved in PBS) overnight. The fixative was removed by one washing step with 100% PBS and 5 washing steps with bi-distilled water (10 min each). In order to exchange water against acetone, a graded series of 10%, 20%, 30%, 50%, 70%, 95%, 2 × 100% (10 min each) was applied. The samples were critical point dried (Polaron E3000), mounted on stubs and sputter coated with gold (Balzers Union, Lichtenstein) for 2.5 min at 120 V. Specimens were analysed with a Hitachi S-530 scanning electron microscope equipped with Diss5 (point electronic, Halle, Germany) at 20 kV.

Results

Mainly specimens of *Strobilurus* and *Mycena* species were used to test the effects of diverse implementations of the rehydration method to prepare fungal specimens for scanning electron microscopy (SEM). *Strobilurus* spp. have a hymeniderm consisting of pear-shaped, thin-walled cells and dermatocystidia which are thin-walled and fusiform. Pleuro- and cheilocystidia with species specific morphology are found on the hymenophores (Rexer and Kost 1989a, 1989b; Qin et al. 2018). Most species of *Mycena* have an epicutis of thin radiating hyphae with wart- or fingerlike projections (diverticulate hyphae). Hyphae of the same type were also found on the surface of the stipe, often terminating in characteristic cells. On the hymenophore, cheilocystidia are always present but pleurocystida are only found in some species (Rexer 1994; Aronsen and Læssøe 2016).

In the *Strobilurus* specimen prepared without AOT treatment (control), the cells of the hymeniderm were deformed, with sunken apexes and lateral walls seen as rings as seen by SEM (Fig. 1a). In a specimen of *S. esculentus* treated with 1% AOT without ultrasound and without vacuum treatment, single cells are slightly inflated (Fig. 1b), but the overall result is similar to the result of the control (Fig. 1a). An increase of the AOT concentration to 3% led to a minor improvement, as seen for the hymenium of *S. esculentus* (Fig. 1c). Although the solubility of AOT decreases strongly with concentrations larger than 6%, solutions with up to 10% AOT were tested. With 10% AOT, the diverticulate hyphae of the epicutis of *Mycena meliigena* and their terminal cells were insufficiently rehydrated and showed wrinkled surfaces (Fig. 1f). Concentrations of AOT between 3 and 6% (Fig. 1 c, d) led to better shapes of the cells as compared to the control, but the results were not satisfactory. Exposure times of up to 60 h in AOT were tested, but the quality of the result did not increase compared to 24 h of soaking.

Therefore, we applied ultrasound and vacuum treatments to the specimens in AOT solutions. This procedure significantly enhanced the rehydration effect of AOT. Fragments of specimens of *S. esculentus* were put in 3.5% aqueous solution of AOT and were first treated for 1 min with ultrasound and then incubated for 24 h in a vacuum desiccator. As a result, the more or less capitate, thin-walled apices of the pleurocystidia of *S. esculentus* were not sunken (as seen in Fig. 1c) but re-inflated as observed in fresh material (Fig. 2c). The amorphous or crystalline substances which are located at the tips of pleurocystidia of *Strobilurus* spp. were mostly washed away during the preparation so that the surface of the cell wall was visible (Fig. 2e).

The application of ultrasound for 3 min to material of *M. meliigena* caused artefacts. Hyphae of the stipitipellis and their terminal cells showed holes of variable sizes (Fig. 1e). Such holes were never seen in specimens treated with ultrasound for only one minute (Fig. 2d). Treatments with 3% AOT (Fig. 1h) or 5% AOT (Fig. 1i) resulted in minor differences. However, hyphae deeper in the tissue were better rehydrated with 3% AOT.

The effects of the optimum treatment parameters resulting from these observations were tested on further species as illustrated in Fig. 2. *Strobilurus stephanocystis* presented

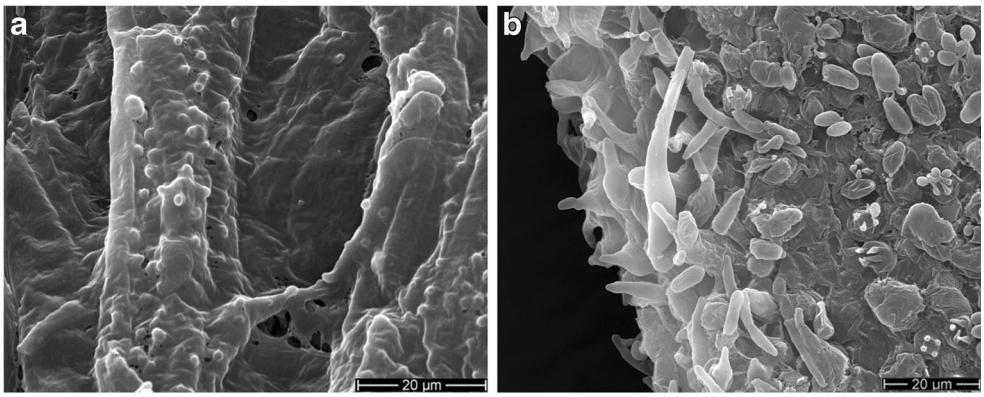

Fig. 3 **a–b** *Mycena* cf. *abramsii*. Fresh material directly fixed in 4% glutaraldehyde. **a** Overview of the pileipellis. **b** Edge of the lamellae with cheilocystidia and hymenium with basidia and basidiospores

perfectly convex cells of the pileipellis (Fig. 2a) and characteristic pleurocystidia (Fig. 2b). In this case, the amorphic exudate at the tips of the cystidia was not washed off during preparation. In *M. rosella* as well as *S. tenacellus*, thin-walled and fusiform pleurocystidia are formed. In both species, most pleurocystidia were inflated as seen by SEM. However, in each specimen a few collapsed cells were obvious (Fig. 2e, f).

The basidiospores of species in various genera like *Entoloma*, *Mycena*, *Russula* and *Strobilurus*, presented their typical shapes (Fig. 1g–i, Fig. 2b, g–i). *E. conferendum* has cruciform-stellate basidiospores which were almost completely rehydrated (Fig. 2g). The hexagonal to octagonal basidiospores with rounded angles of *E. undatum* (Noordeloos 1987), however, showed slightly wrinkled surfaces (Fig. 2h). The broadly ellipsoid basidiospores of *R. ochroleuca* (Romagnesi 1996) were completely inflated, and their warts and delicate line connections were perfectly visible (Fig. 2i).

These results were compared to structures observed on directly fixed fresh material. Immediately after being collected, fresh material of *Mycena* cf. *abramsii* (Rexer 1994; Aronsen and Læssøe 2016) was fixed in glutaraldehyde. The hyphae of the epicutis were densely covered with warts or short outgrowths and slightly embedded in a matrix. These hyphae were well preserved (Fig. 3a), but the matrix masked the morphology of the hyphae to some extent. The cells of the hymenium, however, were less well preserved. Many basidiospores and all the basidia were shrunken or collapsed, only the characteristic cheilocystidia were well preserved (Fig. 3b). These results were similar to results obtained from rehydrated specimens treated with AOT only (Fig. 1g–i).

Discussion

For analyses of cellular structures of dried specimens of fungi by scanning electron microscopy (SEM), an improved method for rehydration of the material was needed. For higher plants and bryophytes, the use of Aerosol® OT (AOT) yielded good results for light microscopy (Pohl 1965; Ayensu 1967; Peterson et al. 1978; Thaowetsuwan et al. 2017) and SEM (Erbar 1995; Frey et al. 2001). We adapted the application of AOT to the preparation of fragments of fruiting bodies of Agaricomycetes. AOT in concentrations of 3 to 5% increased rehydration, but the results were not satisfactory. The application of ultrasound enhanced water uptake of fungal tissues, but prolonged treatments caused damage to the cells. The method was further improved by putting the vials containing the fungal specimens in AOT solution in a vacuum desiccator. The vacuum helps to remove air from between hyphae in the fungal tissue. The combination of these treatments led to good results. This might be explained by hydrophobic surface properties of fungal structures and cell wall compounds that are not soluble in water (Rillig 2005; Chau et al. 2012; Ruiz-Herrera and Ortiz-Castellanos 2019).

With this method, even delicate fungal structures presented shapes similar to cells in fresh material. Ornamented basidiospores, cystidia or other terminal cells with outgrowths were similar to cells in fresh material, although they originated from dried specimens. Only basidia could not be completely reinflated. As in the fixed fresh material, basidia were also badly preserved; it becomes evident that any biological material can only reflect the cell condition at the time of fixation. If a fruiting body has already suffered from drying prior to collecting and fixation, it cannot be reconstructed. It might be helpful to apply the rehydration technique presented here to fresh material as well.

Osmium tetroxide (OsO_4) is a well-established post fixation agent for SEM preparation (Hayat 1986). We did not use it, because it is toxic. The rehydration method presented here does not imply the application of toxic reagents and it is cheap.

Based on our results, we recommend to follow this protocol for the preparation of fungal tissue for SEM:

1. Put fragments of fungal tissue in 3.5% AOT in bi-distilled water
2. 1 min treatment with ultrasound
3. Storage in a vacuum desiccator for 24 hours
4. 3 × washing with bi-distilled water (10 min each)

5. 3 × washing with phosphate-buffered solution (10 min each)
6. Fixation in 4% glutaraldehyde in phosphate-buffered solution (overnight)
7. 1 × washing with phosphate-buffered solution (10 min)
8. 5 × washing with bi-distilled water (10 min each)
9. Acetone series for dehydration (10%, 20%, 30%, 50%, 70%, 95%, 2 × 100%, 10 min each)
10. Critical point drying
11. Sputter-coating with gold

The fungal fragments must not desiccate during this preparation, as this might cause artefacts.

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Availability of data and materials Additional information on the origin of the specimens used are available via GBIF. All other data generated or analysed during this study are included in this published article.

Code availability Not applicable

Author contributions All authors contributed to the study conception and design, material preparation and data collection. The first draft of the manuscript was written by Koch, JA, Fischer, A, and all authors commented on previous versions of the manuscript. Rexer, K-H contributed supervision, reviewing and editing of the final version. All authors read and approved the final manuscript.

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Declarations

Ethics approval Not applicable

Consent to participate Not applicable

Consent for publication All authors agreed with the content of the work and all gave explicit consent to submit the manuscript to *Mycological Progress*.

Conflict of interest The authors declare that they have no conflict of interest.

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