Asian Journal of Mycology 4(2): 1–18 (2021) ISSN 2651-1339

www.asianjournalofmycology.org **Article**

Doi 10.5943/ajom/4/2/1

Methods for observing, culturing, and studying living ascospores

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Karakehian JM, Quijada L, Pfister DH, Tocci G, Miller AN 2021 – Methods for observing, culturing, and studying living ascospores. Asian Journal of Mycology 4(2), 1–18, Doi 10.5943/ajom/4/2/1

Abstract

Ascospore morphologies provide important characters with which to diagnose and describe taxa in *Ascomycota*. Ascospore features such as size, shape, color, septation, wall thickness, and guttulation, among others, are provided in identification manuals and descriptions of new species. Yet, by tradition, ascospores are usually described from dead fungarium material, and unfortunately, occasionally from immature or overmature ones. However, living, mature ascospores display a wealth of taxonomically informative morphological features that are lost or obscured when they die. Examples of the severe morphological changes that ascospores undergo when they die are provided here. Data from living ascospores may not be observed and recorded by mycologists because field and laboratory practices do not prioritize the study of freshly collected specimens. In this review, we discuss how to assess ascospore maturity and describe methods to produce an ascospore deposit for the purpose of obtaining living, mature ascospores. Ascospores are ejected from living, mature asci onto a cover glass or growth medium. The ascospores collected on these surfaces can be used in microscopy and culture studies. Notes on a method for isolating conidia on growth medium are also provided. This guide is aimed at those who have a basic understanding of ascomycetes, including the various types of ascomata and mechanisms of ascospore liberation. Methods given in this paper are primarily applied to ascomycete fungi that have active ascospore discharge. Some methods may be adapted for use with other groups that have passive discharge. Our purpose is to promote standardized, accurate, and thorough morphological characterization of living ascospores, as well as to encourage the routine employment of culture-based methods.

Key words – active spore discharge – fungal taxonomy – living state – spore maturity – spore print – vital taxonomy.

Introduction

Ascospore morphology provides important characters for delimiting taxa in many groups of *Ascomycota*. In this paper, we review and expand on methods to produce an ascospore deposit for study. Methods are adapted primarily from Ahmadjian (1963), Booth (1971), Baral (1992) and Kendrick et al. (1979). This guide is aimed at those who have a basic understanding of ascomycetes, including the various types of ascomata and ascospore liberation by active discharge

Submitted 2 February 2021, Accepted 4 May 2021, Published 6 August 2021
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(cfr. Ingold 1971, Trail & Seminara 2014). Ascospores are ejected from living, mature ascomata onto a cover glass or growth medium. The ascospores that are collected on these surfaces can be used in microscopy and culture studies. Our purpose is to instruct and provide advice to promote standardized, accurate, and thorough morphological characterization of living, mature ascospores, as well as to encourage the routine employment of culture-based methods. Notes on a method for isolating conidia on growth medium are also provided. We conclude this paper with remarks on its purpose – to promote repeatability, reliability, and thoroughness in morphological descriptions of ascomycete taxa. We advise that researchers become familiar with the literature on their fungi of interest and take into consideration relevant information regarding the biology of ascospore development and discharge while employing the methods outlined in this paper.

Mature ascospores

Mature ascospores must be studied to accurately characterize them. Mature ascospores are those that have been discharged from living asci into a moist environment and observed soon after (Baral 1992). A moist environment is important to keep the ascospores from drying out. A sealed Petri dish with dampened filter paper inside or the surface of agar growth media will do. Timely observations (within hours) are also important because deposited ascospores, though still living, may begin to display age-related morphological changes, including those that occur as the ascospores begin to germinate (Baral 1992, Baral et al. 2020).

Morphological data obtained from mature ascospores are important because they provide a standard by which data collected from other specimens can be compared. For example, studying mature ascospore morphology in specimens of a given species allows one to compare these observations among all the examined specimens. These features include size, shape, septation, contents (e.g., spore bodies, lipid guttules, nuclei), natural ascospore wall color, color reactions in iodine-based or other reagents, and spore wall thickness and layering, including ornamentation, appendages, or gelatinous sheaths.

However, it is important to discuss another criterion for assessing ascospore maturity. As mentioned above, mature, discharged ascospores may develop additional features over time, perhaps when passing into early stages of germination (Fig. 1). These morphological features may be observed not only in ascospores that have been discharged but also in those that remain within asci that for whatever reason have lost their turgor and thus their ability to discharge (Huhtinen 1990, Baral 1992). Baral has come to apply the term “overmature” to generally characterize the developmental stages of ascospores after they pass into the “first phase of germination” (cfr. Baral 1992 p. 375, Baral et al. 2020 p. 30). Overmature ascospores may also provide important taxonomic features, but attention must be paid to separate mature from overmature ascospores.

Briefly, some examples of morphological features that may be observed in overmature ascospores include “delayed” pigmentation in certain species of *Lambertella* (*Rutstroemiaceae*, *Helotiales*, *Leotiomycetes*) and *Velutarina rufoolivacea (Cenangiaceae*, *Helotiales*, *Leotiomycetes)*. Here, ascospores are hyaline when discharged but develop coloration over the course of several days to two weeks or longer (Fig. 1D1, D2), whereas in other *Lambertella* species and in *V. bertiscensis* the ascospores are ejected when brown (Dumont 1971, Baral 1992, Baral & Perić 2014). Dennis (1981) described the ascospores of *Phaeangellina empetri* as “long remaining hyaline then turning dark brown, becoming finally 1-septate, especially after expulsion from the ascus”. Dennis's statement hints that these brown spores may be overmature. Korf (1962) concluded that when dealing with *Hemiphacidiaceae* (= *Cenangiaceae*) and *Lambertella* species, the term maturity should be used for the stage when the spores are ejected and not for the stage when they achieved their final aspect prior to germination, which is how the term is often used. Korf (1962) noted that his definition would inevitably require experiments using living material to ascertain maturity. Indeed, judging whether ascospores are mature or overmature can only be done by studying living (not fungarium) material and producing an ascospore deposit is the primary approach toward making this assessment.

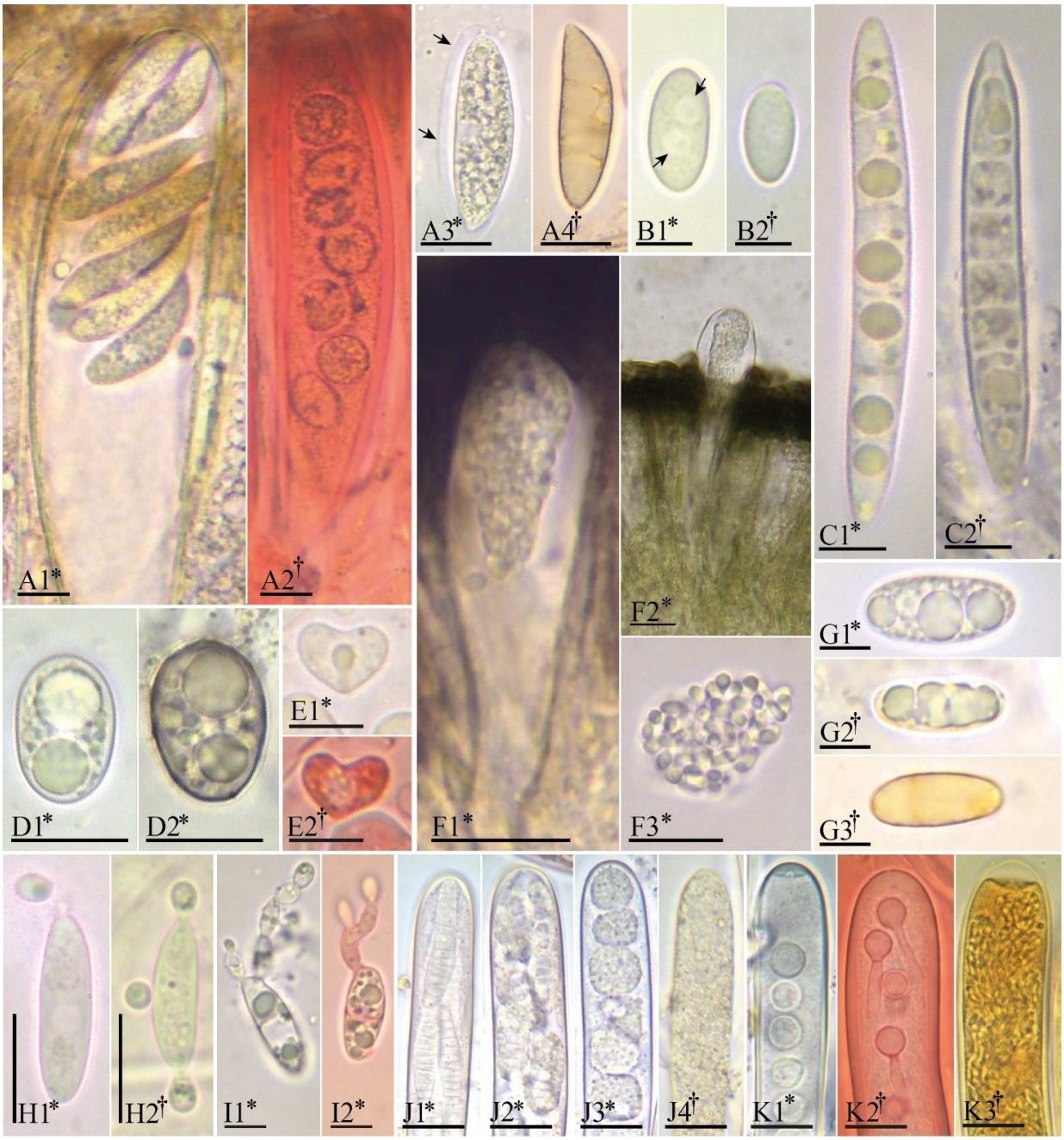

Fig. 1 – Comparison of ascospore morphologies in living and dead states, as well as in various stages of maturity. Also illustrated are examples of delayed pigmentation and development of ascoconidia within the living asci by germination of ascospores. Symbols in the figure panels (* = living, † = dead) and terminology used in the parenthetical comments below for guttulation type and other protoplasmic structures follows Baral (1992). KOH = Potassium hydroxide, CR = Congo red, MLZ = Melzer's iodine reagent. Scale bars: A, D, F, H, J, K = 10 µm; B, C, E, G, I = 5 µm. A1–A4 *Chloroscypha* sp. A1 mature ascus in water (ascospores multiguttulate). A2 immature ascus and ascospores in CR pretreated with KOH (note: change in ascus wall thickness). A3 mature ascospore in water (arrows indicate gelatinous sheath). A4 ascospore in MLZ (guttulation pattern optically obscured). B1–B2 *Ciboria acericola*. B1 mature ascospore in water (arrows indicate two nuclei). B2 ascospore in KOH (nuclei invisible). C1–C2 *Durella connivens*. C1 mature ascospore in water (oligoguttulate). C2 ascospore in KOH (note: plasma detachment, obscuration of lipid pattern). D1–D2 *Velutarina rufoolivacea* (in water, oligomultiguttulate). D1 mature, hyaline ascospore. D2 overmature, pale brown ascospore. E1–E2 *Orbilia corculispora*. E1 ascospore in water (spore body visible at the center). E2 ascospore in CR pretreated with KOH (refractivity of

spore body irreversibly destroyed). F1–F3 *Symbiotaphrina microtheca* (in water). F1 polysporous, nearly mature ascus with tip below the hymenial surface. F2 mature ascus protruding from the top of the hymenium (image courtesy of H-O Baral). F3 a discharged cluster of ascospores. G1–G3 *Discinella* sp. G1 ascospore in water with oligo multiguttulate lipid bodies. G2 ascospore in KOH (guttulation pattern obscured). G3 ascospore in MLZ (guttulation pattern optically obscured). H1–H2 *Rutstroemia* sp. H1 overmature ascospore in water with one nucleus, producing conidia at the pole. H2 overmature ascospore in KOH producing conidia laterally and at the poles, nucleus not clearly visible. I1–I2 *Ascocoryne sarcoides* (producing conidia holoblastically in chains). I1 overmature ascospore in water. I2 overmature ascospore in CR (note: cell wall staining). J1–J4 *Vexillomyces dacrymycetoideus* (J1-J3 illustrating the sequence of development). J1 immature ascus with primary ascospores in water. J2 as in J1 but with ascoconidia formed from the spore wall. J3 mature ascus with ascoconidia balls fully developed. J4 mature ascus filled with ascoconidia in MLZ, balls destroyed, and ascus evenly filled with ascoconidia. K1–K3 *Tympanis* sp. K1 immature ascus with primary spores in water. K2 immature ascus in CR with KOH pretreatment, with germ tubes emerging from primary spores. K3 mature ascus in MLZ, evenly filled with ascoconidia (as in J4), which arose from germ tubes that had formed ascoconidia balls (as in J3).

Notably, in certain species of *Vexillomyces*, *Myriodiscus*, and *Tympanis*, immature ascospores undergo radical morphological changes within the living ascus prior to being discharged. These species produce small and globose, or large and septate, "primary" ascospores that germinate or that continue to divide from the ascospore wall or germ tubes, producing very small "secondary" spores called ascoconidia. Clusters of ascoconidia are surrounded by a membrane and come to resemble balls. There may be four to eight ascoconidia balls per ascus. These are ultimately discharged from the ascus as independent units (Fig. 1J1–K3). However, at least in the cases of *Vexillomyces* and *Tympanis*, it is the primary ascospore morphology that is used to differentiate species (Fig. 1J1, K1) (Ouellette & Pirozynski 1974, Quijada et al. 2019).

Living ascospores

Many species of *Ascomycota* have been and continue to be characterized using morphological data obtained solely from dead ascospores (e.g., fungarium specimens) observed microscopically in crush mounts or sections. In contrast, when studied carefully, living, discharged ascospores represent an opportunity to obtain a wealth of taxonomically useful morphological data that can be compared to data obtained from dead state ascospores and given in a description (Baral 1992). This practice allows a researcher to link morphological data obtained from historical specimens (dead state) to observations made from living specimens to give a more complete picture of the ascospore morphology of the taxon of interest (Huhtinen 1990, Baral 1992, Perić 2019). Such an approach may inform species circumscriptions and higher-level taxonomies. For example, *Orbiliomycetes* is characterized by possessing distinctive and morphologically variable cytoplasmic structures within ascospores called "spore bodies" that are observable only in living ascospores when using standard light microscopy (Fig. 1E1–E2) (Baral et al. 2020).

The morphology of living ascospores changes drastically and irrevocably when they die (Figs 1, 2H–I) (see also Baral 1992: Figs 1–2, 5, 7–8, 12–13, 15–16, 21–22, 35 and Baral et al. 2020: Figs 3, 7, etc.). Mountants such as KOH or Melzer's iodine reagent kill the spores. Living, discharged ascospores are best observed in clean water (i.e., rain or tap water), a mountant that approximates natural conditions and that does not cause morphological changes due to osmotic stress or cell death, unlike distilled or deionized water (Baral 1992). This is the essence of the "vital taxonomy" approach. Baral (1992) reviewed the history of vital taxonomy methodologies and studied their effectiveness by observing and comparing fungal cells and tissues in both living and dead states. He thoroughly documented his observations with numerous high-quality drawings that dramatically illustrate the morphological changes that cells such as asci and ascospores undergo while transitioning from a living to a dead state. Recent literature that treats the impact of vital

taxonomy on taxonomic mycology may be found, e.g., in Kušan (2015), Perić & Baral (2019), and Baral et al. (2020).

To effectively document the morphology of living ascospores as well as to generally implement methods of vital taxonomy, it is important to start with undamaged, freshly collected specimens. Ideally, specimens should be mature and free of other fungi, soil, and minute animals such as insects and their eggs, larvae, and worms. The fresh specimen should be studied before drying a portion for the fungarium. Those working with lichens and certain desiccation-tolerant fungi may be able to store air-dried material for later examination. These may discharge ascospores up to a month or more after collection if the specimens are maintained in cool, dry conditions. Understanding the ecology of the fungi of interest will inform decisions regarding handling specimens to obtain living ascospores.

Crush mounts and sectioning are employed to observe ascospores in both fresh and dried material. Ascospores liberated in crush mounts and sections may be used for routine identification. However, care must be taken to be certain that one is observing fully mature ascospores, since immature ascospores may be liberated from broken asci. This is especially important when observations are being made for descriptions. Observations made from an ascospore deposit obviates this issue. Alternatively, one may study ascospores inside fully turgescent asci in sections or gently squashed mounts made in water and directly observe the ascospores that are discharged from them. This approach also enables unequivocal recognition of mature ascospores. For the recognition of full turgescence, see Baral (1992) pp. 339–341 Figs 1, 6–10, 20, 43.

Methods

To demonstrate these methods, we used a fresh collection of *Rhytidhysteron rufulum* (ILLS00121324). This species has desiccation-tolerant ascomata and produces relatively large ascospores that become dark brown while still inside the living asci. When discharged, these can be seen clearly against a white background using 3–5× magnification. To produce images for Fig. 2, we used a dissecting microscope and a macro lens with a digital camera. Because *R. rufulum* is a presumed saprobe, we expected the ascospores to germinate and produce hyphae on potato dextrose agar (PDA). All methods were executed at room temperature.

Methods are presented in sections A–C, with numbered steps. General guidance for the methods, as well as for microscopic observations, imaging, and documenting, is given in section D. Methods are written in a "how-to" style that includes advice and illustrative figures.

A. How to make an ascospore deposit on a cover glass

Purpose – To make morphological observations of living, mature ascospores, spore balls, ascospore germination, germ tube formation, hyphal growth, and differentiation.

Notes – For this method, tools and supplies should be clean, but it is generally unnecessary to sterilize them or use a sterile working environment like a biological safety cabinet or a laminar flow hood. Therefore, these methods may be executed in a field setting as conditions permit.

Materials and equipment

Petri dish, Parafilm (laboratory film), absorbent paper, tap water, cover glasses, pieces of cork (pith or eraser will also do), scalpel, forceps, fine point paintbrush (it may be necessary to carefully trim away excess bristles under a dissecting microscope), dissecting and compound microscopes.

Method

1. *Select the specimen*. Select fresh, mature specimens. Immature or decayed ascomata will not actively discharge ascospores. Senescent ascomata may discharge some ascospores, though inconsistently; it is likely that the ascospore deposit will fail. In some cases, active ascospore discharge can be seen with the unaided eye, appearing as a smoke-like cloud of ejected ascospores that arises from the hymenial surface of a fresh ascoma (Ingold 1971). This "puffing" may be

observed, for example, among *Peziza* species that produce vast numbers of ascospores that are discharged simultaneously from an apothecium. This phenomenon is not observed in species that produce few ascospores.

To assess whether ascospores are being actively discharged, make a section or crush mount in tap water. Under a compound microscope, scan the preparation for signs of discharge, such as groups of spores collected in the medium near open asci.

Dried ascomata may sometimes be mistaken as senescent or dead. Many living, desiccation-tolerant discomycetes have covering layers that close over the hymenium when dry or otherwise appear shriveled or hysteriform, but these open widely to expose the hymenium when rehydrated (Fig. 2 A–B). Some fleshy pyrenomycetes may appear collapsed when dry but balloon in humid conditions. Those with heavily carbonized outer tissues, such as *Rosellinia*, externally appear much the same wet or dry. In these cases, a few ascomata can be broken open with a probe under the dissecting scope to check for intact hymenia. Also, there may be a visible spattering of ascospores clustered around the ostioles and surrounding surfaces – an indication of recent ascospore discharge. Similarly, with hydrated ascomata of some groups of pyrenomycetes, ascospores may collect at the ostiole in masses, forming a spore-tendril (cirrus) or simply a globular mass (Ingold 1971). In these cases, the work of making an ascospore deposit is already done, and the spores may be transferred with a fine point paintbrush or needle. Care must be taken with such ascospores because it is unknown how long they have been there, and they might be overmature.

Certain long-lived, desiccation-tolerant ascomata, such as those of *Orbilia* spp. or lichens, may discharge only a few ascospores. If you suspect that the fungus that you are working with may not discharge ascospores copiously, then keep these ascomata air-dried until step 3.

There is always the possibility that parasitic fungi may be present on the specimen. These are a potential source of contamination because they may go undetected and possibly discharge their ascospores onto cover glasses or growth media along with the fungus of interest. For example, though perhaps uncommonly encountered except in certain groups of discomycetes, species of *Acremonium*, *Helicogonium* and *Gelatinopsis* are hymenium-inhabiting parasites. These could go undetected in the absence of a thorough micromorphological inspection of the portion of the apothecium that is to be used in the spore deposit (Baral 1999, Baral & Marson 2000, Baral 2019).

2. *Prepare the Petri dish*. Place a square of filter paper or other absorbent paper in the Petri dish and dampen it with tap water. The paper should be well moistened but not flooded. An ascoma will be placed on this paper, and the addition of too much water risks inundating it. Furthermore, bacteria and microalgae may be suspended in the excess water and may cling to ascospores as they are ejected. Contamination may result from these "stowaways" if the ascospore deposit is being made on growth media, as described in section B.

3. *Prepare the ascoma*. For large, fleshy apothecia, a portion bearing hymenium approximately 2 mm^2 is excised from freshly collected material using a clean scalpel, taking care to cut deep enough to include some fleshy tissue underlying the hymenium. For small ascomata (i.e., < 5 mm diam), a single ascoma or a cluster of a few ascomata may be used. Ascomata smaller than a few millimeters in diameter are best left on a small portion of the substratum for ease of handling. However, check in the vicinity of the ascomata of interest for the presence of extraneous ascomata of other species. Such unwanted fruit bodies can be scraped or cut away with a scalpel under the dissecting microscope. This is important to prevent ascospores of more than one fungus from being deposited on the same cover glass.

When the ascoma is prepared, use forceps to place it on the damp paper in the Petri dish. Take care to orient it with the hymenium facing upward. As discussed in step 2, the ascoma should become hydrated and very moist but not so moist that it is flooded – a particular problem with tiny apothecia. Furthermore, in apothecia, the surface tension from excess water on the hymenium may capture discharged ascospores. Take up any excess water with a small piece of absorbent paper.

4. *Place a cover glass over the ascoma*. Using forceps, a cover glass may be placed directly on the ascoma, but better results are obtained by placing the cover glass on supports that raise the cover glass above the ascoma. A few small pieces of cork, eraser or pith will do (Fig. 2 C). The

rationale is that water droplets will form by condensation on the undersurface of the cover glass. Later, when the cover glass is picked up to make a microscopy preparation, the condensation droplets and any ejected ascospores that are suspended in them may flow back onto the ascoma, damaging the ascospore deposit. Raising the cover glass above the surface of the ascoma reduces the likelihood of excess droplet formation.

5. *Replace the Petri dish lid.* Wrap the closed Petri dish with laboratory film, taking care not to disturb the orientation of the ascoma or the cover glass. Sealing the Petri dish stills the air within and allows humidity to build up. After sealing, use a dissecting microscope to check that the ascoma has not shifted. This is often a concern with tiny ascomata. Carefully set the Petri dish aside where it will not be disturbed.

6. *Routinely check the progress of the ascospore deposit*. Progress can be checked by carefully examining the cover glass in the Petri dish under a dissecting microscope. The Petri dish lid does not necessarily need to be removed. Using medium to high magnification and strong illumination, focus down on the cover glass. Ejected ascospores are seen as small, gem-like, shining bodies suspended in droplets of condensation and ascoplasm (Fig. 2D–F). Generally, an ascospore deposit takes an hour or two, but leaving the Petri dish set up for longer periods is sometimes necessary. However, it is important to make observations as soon as sufficient ascospores have accumulated on the cover glass. Over time, ascospores may germinate (sometimes this may occur within 4–6 hours after deposition on the cover glass!). Germination may lead to drastic changes in ascospore morphology. For example, ascospores may swell considerably and develop septa. In some groups of *Ascomycota*, ascospores give rise directly to conidia. Observation of germination or conidiation events present an excellent opportunity to document development – these are valuable biological and morphological data that may also have taxonomic value.

A recalcitrant ascoma that will not produce an ascospore deposit after being left for a long period can be a challenge. Placing the Petri dish in direct sunlight for a few hours may induce ascospore discharge. Sometimes, peeling away the laboratory film to lower the humidity in the Petri dish, or raising and lowering the Petri dish lid to produce changes in air pressure, may also stimulate discharge. If these approaches fail, it may be that the ascoma is immature or senescent. To check this, make a longitudinal hand section of the ascoma, mount this in water, and observe the preparation using a compound microscope. If turgid asci are present, it may be that the ascoma is not quite mature. In this case, it may be possible to "incubate" the ascoma, or another portion of the specimen, in a moist chamber and try the process again in a few days. It must be emphasized not to put all of the specimen in the moist chamber in case other fungi, such as fast-growing hyphomycetes, overgrow it. If this happens the entire specimen may be ruined. In some desiccation-tolerant, nonlichenized discomycetes we have observed in air-dried collections that active discharge fails after remaining dry for roughly two months, but other species can discharge ascospores after even longer periods. A telltale sign that asci may fail to discharge is that the hymenial surface of these desiccation-tolerant discomycete species does not swell very much when hydrated and that water droplets form on it, as though the water was simply filtering up through the hymenium. In a senescent ascoma, few asci will be observed, and those that are present may not be turgid.

Even in cases where active discharge has failed, seemingly mature ascospores within asci may remain viable for many months. One must resort to a crush mount in tap water with these non-discharging collections. Because mature, immature, and overmature ascospores may be intermixed in crush mounts, care must be taken to distinguish among them. This may be difficult to do in practice and requires a judgement on each ascospore that is observed. Unfortunately, species have been described using characters observed from immature ascospores (e.g., *Cookeina garethjonesii* in Ekanayaka et al. 2016). Generally, mature ascospores display an outer wall with good optical contrast. The outline of these ascospores appears clear and sharp in tap water mounts. Similarly, guttules, when present, are clearly delimited from the surrounding cytoplasm and may be organized in certain regions of the ascospore and in particular numbers (e.g., Fig. 1A3, B1, C1, D2, E1, G1, H1, I1–I2). When observing a group of ascospores, the arrangement and number of guttules in each

ascospore is somewhat stereotypical. For example, a group of ascospores in a water mount may show typical clusters of a few guttules that are organized near the ascospore poles. In contrast, immature ascospores display optically low contrast outer walls and protoplasmic bodies that are not clearly delineated (see Baral 1992 pp. 357–359 Figs 15–16, pp. 375–378 Figs 42–43). Guttules may be numerous, small, and scattered haphazardly within the immature ascospore. There may be no detectable pattern to the number and placement of guttules when observing a group of such ascospores. Careful observations of mature ascospores gathered by an ascospore deposit observed in a tap water mount will provide a standard with which to compare ascospores observed in crush mounts prepared from other specimens of the same fungus. In this way, a researcher may gain experience identifying "good looking" ascospores to describe (Baral 1992).

7. *Observe the ascospore deposit*. When you estimate that at least 10–30, or more, ascospores have accumulated on the cover glass, the Petri dish can be unwrapped, and the cover glass carefully lifted with forceps. This is placed ascospore side down on a microscope slide. Usually, the condensation and ascoplasm that the ascospores are suspended in provide sufficient mounting media, but a very small amount of tap water can be added to the edge of the cover glass if necessary. The preparation is ready for microscopic examination and imaging (Fig. 2G–I). It is important to document the morphology of the ascospores soon after the deposit is made in order to ensure that they are mature.

A word of caution regarding the amount of tap water to use in a microscope slide preparation is helpful here. In the microscope's field of view, if there is too much water in the preparation, the ascospores will appear to be rapidly floating toward the edges of the cover glass where they will escape into the excess water at the edge. This can be a particularly frustrating problem when examining small ascospores. Large ascospores or those with gelatinous sheaths tend to remain in place in moderate amounts of water. If floating ascospores are observed put the slide aside for a few minutes until they settle and some of the water evaporates. Wicking the water away risks inadvertently drawing up some ascospores. Stable ascospores are necessary to produce clear images and to make accurate measurements. To settle floating ascospores Baral (1992) suggests using a viscous solution of albumin as a mountant. On the other hand, having too little water in preparation will introduce compression of the spores and air bubbles (Fig. 2G). This is a minor problem solved by the addition of a tiny amount of tap water to the edge of the cover glass. This should be done before evaporation begins drawing even more air under the cover glass. In the microscope's field of view, expanding air pockets under the cover glass causes ascospores to be swept forward along the leading edge of an air bubble. Compression by the coverslip results in distorted ascospore shapes and exaggerated measurements.

Issues with floating ascospores and rapidly evaporating tap water mounts can be addressed by coating the edges of the cover glass with a very thin layer of petroleum jelly before the preparation is made. This is done by applying an extremely thin layer of petroleum jelly on a microscope slide. Then, while carefully holding the edges of the cover glass (with the ascospore deposit on it), very gently scrape each edge of the cover glass at a slight angle against the microscope slide like a squeegee, taking care to pick up less than a hair's thickness of petroleum jelly. The cover glass is then placed in a very small droplet of water, with the ascospore/petroleum jelly-treated side down. A preparation made in this way is temporarily stabilized.

B. How to make an ascospore deposit onto growth media

Purpose − To establish multi- or single-ascospore cultures for study, allowing observation of ascospore germination and asexual morphs that might develop, and to cultivate hyphae for DNA extraction.

Notes − This method follows the procedures outlined in section A with a few modifications. To make the ascospore deposit on growth media, it is generally unnecessary to use sterile tools (though these should be clean) or to work in a sterile environment. We have noted where sterile procedures are required. This method may be executed in the field. Growth media might include water agar, potato dextrose agar, or malt extract agar. These are transparent and allow ascospore

deposits to be observed using the methods described below under *Helpful tip* and in *Additional information…* (sections b–c).

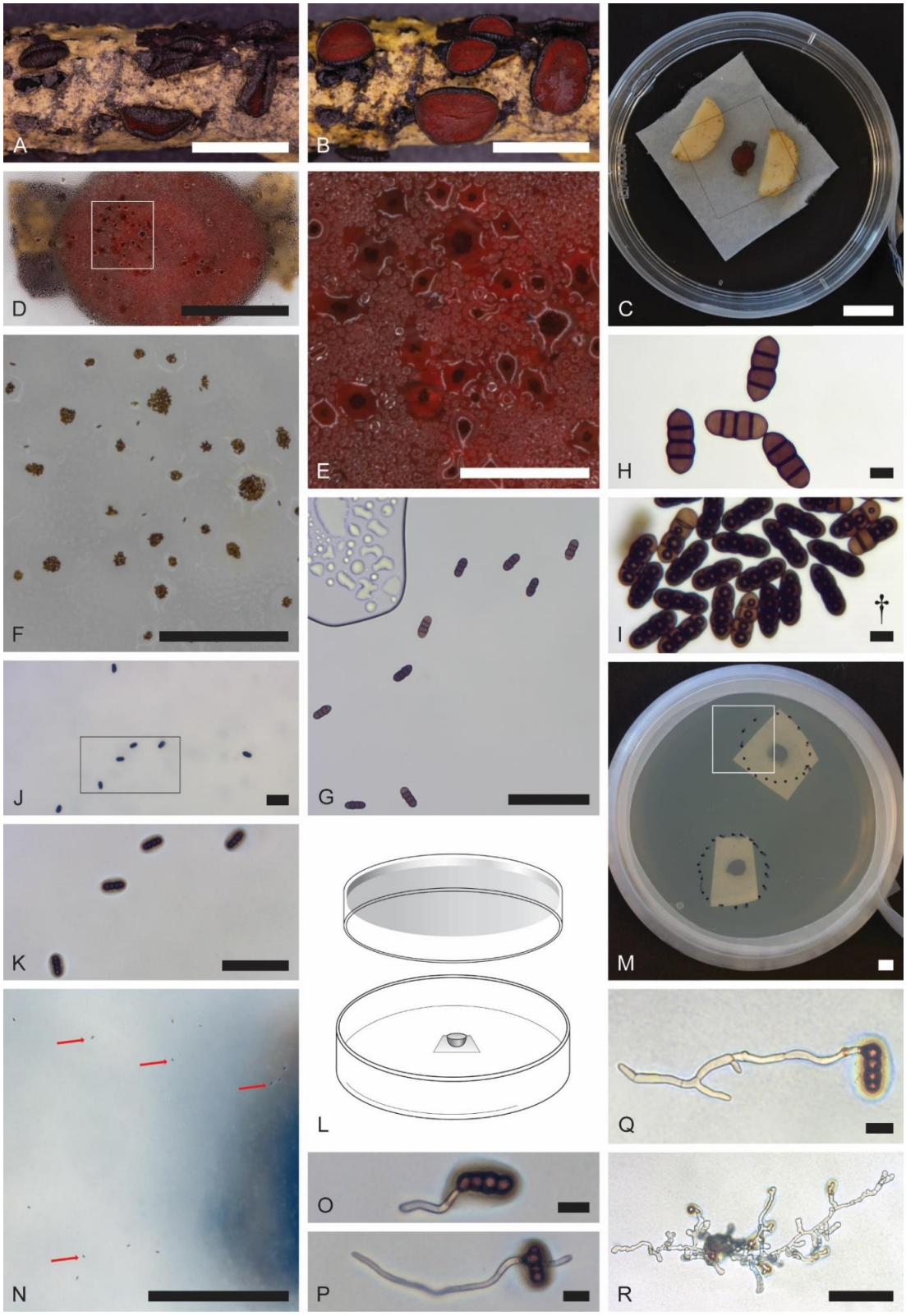

Fig. 2 – The process of making an ascospore deposit onto a cover glass or growth medium using *Rhytidhysteron rufulum* (ILLS00121324) as a model, with observations of living, discharged and germinating ascospores. All images are of living subjects except for I († = dead). Scale bars: A–B = 5 mm, C = 10 mm, D = 2 mm, E–F = 500 µm, G = 100 µm, H–I = 10 µm, J–K = 100 µm, M = 3 mm, N = 1.5 mm, O–Q = 20 µm, R = 100 µm. A–B habit of fresh apothecia on a twig. A air-dried.

B same as A but rehydrated with tap water. C–I making an ascospore deposit onto a cover glass using an apothecium (described in section A), with features of discharged ascospores. C top view of the setup for producing an ascospore deposit onto a cover glass using a piece of dampened filter paper, hydrated apothecium, two pieces of pith and a cover glass (edges lined using a black, indelible-ink marker for this photograph) in a 60 mm Petri dish sealed with laboratory film. The apothecial surface faces the cover glass but does not touch it. D discharged ascospores on the undersurface of the cover glass in C after 12 hours. E ascospores suspended in condensation and ascoplasm (detail of region bounded by the white square in D). F same as E, but with the apothecium removed. G discharged ascospores shown in D–F mounted in tap water and observed using a compound microscope at 400× magnification. H same as G, but at 1000× magnification. I the same preparation as shown in D–H, but with all ascospores treated by the addition of Melzer's iodine reagent, at 1000× magnification (note de Bary bubble formation; mean shrinkage of ascospores is 14.3% in length and 20.8% in width (30 ascospores measured in both living and dead states). J–R making an ascospore deposit onto growth medium using apothecia (described in section B), with features of discharged and germinating ascospores. J discharged ascospores on PDA, observed using a compound microscope at 40× magnification. K same ascospores as J, but at 100× magnification (detail of region bounded by the black square in J). L schematic diagram of setup to produce an ascospore deposit on PDA (modified from Ahmadjian, 1963). M setup for producing an ascospore deposit onto PDA using pieces of dampened filter paper and hydrated apothecia in a 60 mm Petri dish sealed with laboratory film. The apothecial surfaces face the growth medium. The dotted circles roughly demarcate where ascospores should land. N ascospores on the surface of PDA observed using a dissecting microscope at high magnification (detail of region bounded by the white square in M, red arrows point to some discharged ascospores resting on the surface of the growth medium, the dark, out-of-focus region at the right is the apothecial surface). O–R germinating ascospores and growing hyphae on PDA, observed using a compound microscope at 100× magnification.

Materials and equipment

Petri dishes with transparent agar growth media (antibiotics optional), laboratory film, absorbent paper, tap water, scalpel, forceps, fine point paintbrush, dissecting and compound microscopes.

In our experience, a general use, antibiotic supplemented growth medium for wood decay ascomycetes can be made using chloramphenicol and streptomycin sulfate. These inhibit protein synthesis in Gram+ and Gram− bacteria by binding the 50S and 30S ribosomal subunits, respectively. We provide the following protocol for making 1 L of agar growth medium supplemented with these two antibiotics.

Chloramphenicol. Dissolve 600 mg chloramphenicol in 10 mL 95–100% ETOH to make the stock solution. Make 1 mL aliquots in microcentrifuge tubes and store these at −20 °C.

Streptomycin sulfate. Dissolve 600 mg streptomycin sulfate in 10 mL molecular grade water then filter sterilize the solution into a sterile tube using a syringe and a 0.20 µm filter. Aliquot and store as above.

Add 1 mL of chloramphenicol stock solution and 1 mL of streptomycin sulfate stock solution to a flask of 998 mL autoclaved, warm, molten, agar growth medium and swirl gently to mix. This media will contain 0.06 mg/mL of each antibiotic.

Concentrations of antibiotics may be adjusted depending on the application and the number of bacteria that are expected to be present. Familiarity with the literature on growth media and antibiotic supplements specific to the fungus of interest will help produce successful results.

Helpful tip

Using a compound microscope to observe ascospores deposited on growth medium. Lower the compound microscope stage as far as possible and place the Petri dish on it with the lid side down so that the surface of the growth medium is facing down. The slide holder may be removed if

it is in the way, or, alternatively, put a large, 71 × 51 mm microscope slide (or two standard 75 × 25 mm slides) on the stage as one would place a slide in the holder. Place the Petri dish on this. This allows you to use the stage controls to move the Petri dish, otherwise, you can do this by hand. Using the objective with the lowest magnification, bring the Petri dish on the microscope stage up close to the objective lens. Then, looking through the eyepieces, slowly lower the stage so that you are focusing up through the lid (you may see condensation droplets on the inner surface of the lid) and airspace within the Petri dish. Stop when you have focused on the surface of the growth medium. Carefully move the Petri dish around and scan for ascospores if they are not already in the field of view. It helps to adjust the condenser diaphragm to produce an image with maximum contrast. If you have trouble finding the surface, focus to a level somewhere in the growth medium. Using the fine focus knob, slowly bring the stage up while watching any particles that are suspended in the growth medium pass into and out of focus. When you no longer see any particles, you know that you have focused past the surface of the medium and need to backtrack. When you find the ascospores (Fig. 2 J), an objective of higher magnification can be swung into place (Fig. 2 K). Most standard objectives higher than 10× magnification are too long to swing into place without hitting the Petri dish and do not have the focal length necessary to focus on the ascospores. However, a 20 or 40× objective may be employed by opening the Petri dish and placing it right side up on the stage. Swing the objective into place and slowly raise the stage until the ascospores come into focus, taking care to not plunge the objective into the medium. Petri dishes opened in this way become exposed to airborne contaminants and should be quarantined for a period before being returned to an incubator or container housing other cultures.

Method

1. *Select the specimen*. As described above in section A1.

2. *Prepare the Petri dish*. Using alcohol-soluble markers, write your initials, date, collection number, media type, multi-ascospore culture, and any other information around the outer rim of the Petri dish lid.

3. *Prepare the specimen*. As described above in section A3. It is very important to carefully check for mites and other phoretic vectors of contamination.

4. *Place the specimen in the Petri dish*. A Petri dish is inverted so that the surface of the lid is on the work surface and the surface of the growth medium faces down. Place the ascoma on a dampened piece of absorbent paper, as described above in section A3. Using forceps, place this on the inner surface of the lid so that the ascoma faces directly upward, towards the medium (Fig. 2L). It is advisable to position the ascoma off center so that the dish can be rotated to allow several deposits on the surface of the growth medium. In most cases, the discharge event is sufficient to carry the spores to the surface of the medium without elevating the ascoma.

5. C*lose the Petri dish*. Close the Petri dish and check the setup under the dissecting microscope to make sure that the ascoma is properly oriented to eject ascospores upward onto the growth medium. If not, open the dish slightly and adjust the ascoma with a probe. Wrap the Petri dish in laboratory film, as described above in section A5. Looking down onto the surface of the dish part of the Petri dish, mark where the ascospores are expected to be deposited on the surface of the growth medium by drawing a circle on the dish surface with an alcohol soluble marker around the area above the ascoma (Fig. 2M). This helps to locate the ascospores for observations later, as described above in *Helpful tip*, and the markings can be wiped away easily if you wish to photograph the culture later. Place this Petri dish setup aside in a location where it will not be disturbed.

6. *Check the progress of the ascospore deposit*. The progress of the ascospore deposit is checked hourly by focusing slowly down to the surface of the medium through the bottom of the dish using a dissecting microscope set to high magnification, as described above in section A6. It should be checked more frequently if ascomata are copiously discharging ascospores. In the field of view, ascospores appear as shining, gem-like bodies on the surface of the growth medium. Sometimes it may take patience and careful observation to find very small, hyaline ascospores.

Adjusting the direction and intensity of the incidental illumination might help, as well as using transmitted light if the dissecting scope is equipped with this. It may also be helpful to use transmitted light in a compound microscope rather than a dissecting microscope (refer to *Helpful tip*, above for how to set this up). In this case, it is necessary to assure that there is proper lighting by unwrapping the Petri dish and rotating the lid so that the dampened paper and ascoma do not block the path of the transmitted light.

After ascospores have been deposited (Fig. 2N), a fresh region of the growth medium may be exposed to another ascospore deposit by unwrapping the Petri dish and rotating the lid a few degrees. Mark the new location of the ascoma by drawing another circle on the surface of the dish using an alcohol-soluble marker. Reseal the Petri dish with laboratory film. By frequently rotating the Petri dish lid, dense deposits of ascospores are avoided. Multiple ascospore deposits that are spread over the surface of the growth medium facilitate maintenance of the culture by allowing easy removal of contaminants without losing the entire culture. It also facilitates isolating single ascospores (described below under *Obtaining single ascospore cultures*). Refer to section A6 above for suggestions and troubleshooting on checking the development of the ascospore deposit. When there are sufficient deposited ascospores on the growth medium, unwrap the Petri dish and open it enough to admit forceps to remove the paper and ascoma. Close and reseal the Petri dish with laboratory film.

If ascospores have not been released, you may excise a portion of the hymenium using a sterile tool, then prod this fragment across the surface of antibiotic supplemented growth medium in a fresh Petri plate. Lift the lid only enough to admit the tool. Free ascospores or those within asci may germinate. When germination is observed, portions of the medium supporting this growth may be transferred to fresh media (antibiotic supplemented) following the tip given below under *Obtaining single ascospore cultures*.

7. *Maintenance and monitoring*. Depending on ambient conditions (temperature), Petri dishes may be stored inverted (with the growth medium facing down) in order to try to prevent condensation from forming on the inner surface of the lid. Condensation can make observations difficult and facilitate the spread of contaminants when condensation droplets coalesce and begin splashing around inside the Petri dish when it is handled. As a standard, a Petri dish may be stored in an incubator set at room temperature with a 12-hour light/dark cycle. Duplicate Petri dishes may be stored at various temperature and light/dark regimens. Some fungi may produce conidia in light whereas this is not required for other fungi. Again, referencing the literature may inform the parameters that you set for your culture experiments.

Ascospore deposits should be checked daily for 7 days or longer to monitor germination, hyphal growth, and contamination. Hold the Petri dishes up to a light to check for any conspicuous contaminants that may be growing on the surface of the growth medium. These may be bacterial or fungal. Bacterial contaminants may appear as round, smooth, and glossy colonies with sharply defined margins. If you know the growth characteristics of your fungus of interest from literature or experience, then it should be straight-forward to identify any fungal contaminants. For example, a fast-growing fungus that produces a thin, web-like mycelium in a culture that is expected to produce a slow-growing, tough, hump-shaped mycelium is probably a contaminant and should be immediately excised. Fungal contaminants are often species of *Aspergillus*, *Penicillium*, or *Trichoderma*. After making this gross examination, observe the progress of the ascospore deposits using a compound microscope, following the procedure outlined above in *Helpful tip*.

Contaminants may be carefully removed from a culture by excising a block of growth medium with the contaminant and discarding it. Alternatively, the fungus of interest can be excised and transferred to fresh media in a new Petri dish. This may be a more convenient approach if the contaminant is heavily sporulating with fragile masses of conidia. Use sterile tools and conditions (such as a biological safety cabinet or laminar flow hood) to avoid further contamination. If these are not available, open the Petri dish in a room that has relatively still airflow and work quickly on a sterilized surface to cut out the contaminant.

Additional information, tips, and suggestions (a–c)

a. *Ascospore germination*. Ascospore germination may be rapid, occurring within 6–12 hours (Fig. 2 O–R) or sooner, but some ascospores may take up to 48 hours to germinate. Some ascospores may lyse and never produce a germ tube. If ascospores germinate, the majority of the ascospores in an ascospore deposit will germinate at roughly the same time. If only one or a handful out of dozens of ascospores germinate, then one should be suspicious that these arose from contaminant ascospores. There is also the phenomenon of crowded ascospore deposits inhibiting germination – dense deposits often have fewer germlings. Ascospores that look viable but that do not germinate within 36 hours may not germinate at all, or they may require stimulation. Reviews of methods to stimulate germination are given in Sussman & Halvorson (1966), Booth (1971), and Sussman (1976). Some ascospores have never been observed to germinate in culture.

b. *Observing ascospore germination and hyphal growth at magnifications greater than 10×*. When observing ascospores on growth medium in a Petri dish using a standard compound microscope, we usually cannot use objectives greater than 10× (refer to *Helpful tip*, above). If you want to use objectives at 40× or greater, it is necessary to make a microscope slide preparation with germinating ascospores, mounting medium, and a cover glass.

One approach to this is to remove a small (~2 mm) block of growth medium that supports a few germinating ascospores. This is done in sterile conditions following the instructions given in *Obtaining single ascospore cultures* below. Place this block in a drop of water on a microscope slide, ascospore side down, and apply a cover glass. Put this preparation on a heating block at low temperature (or wave it carefully over an alcohol lamp) to warm the agar until it melts, and the cover glass becomes flat against the microscope slide. Alternatively, a very small agar block may simply be placed in the water droplet on a slide. This is covered with a cover glass that is gently tapped to spread the agar square against the slide. Wick away any excess water with absorbent paper placed at the edge of the cover glass. These procedures are rapid, but the manipulations involved risk killing cells and disturbing the pattern of hyphal growth.

To address these challenges, one approach is simply to produce ascospore deposits on cover glasses (section A) but preserve these in a sealed Petri dish with damp absorbent paper to maintain humidity (moist chamber). Monitor the cover glasses regularly with a compound microscope to see if the ascospores germinate in the condensation droplets that they are suspended in (section A6). Because the cover glasses are rigid, the germinating ascospores are supported to a degree when the cover glass is manipulated to make a microscope slide preparation. The risk of cell damage or disturbance comes when the cover glass is placed in a droplet of mountant on a microscope slide.

A modification of this cover glass approach is to dip sterile cover glasses in sterile, melted growth medium. This supplies some nutrients and water, as well as a thin matrix that is supported by the rigid cover glass for hyphae to penetrate and grow through. This keeps hyphae in place when the microscope slide preparation is made and minimizes cell death and disturbance of hyphal growth patterns. Cover glasses that are dipped in growth medium are placed on agar media in a Petri dish, which serves as a moist chamber. They can be arranged in a circle so that after an ascospore deposit has been made on one cover glass, the next one can be rotated into place over the ascoma. Because a cover glass can be picked every *X* hours or days, mounted, and observations made, a sequence of germination through hyphal growth, including any hyphal differentiation that might develop, can be documented. Lethal mountants such as cotton blue/lactophenol may be used in a preparation because a different cover glass will be picked later.

Another, perhaps more straightforward technique is the modified slide culture method. Once the ascospores are deposited on the growth medium, a block supporting a few of them is cut out, placed on a slide, and covered with a cover glass. Germination and subsequent hyphal growth can be observed over several days using the same slide culture but tap water can only be used as a mountant to keep the fungus alive. The slide is maintained in a moist chamber during the period of study.

c. *Obtaining single ascospore cultures*. Generally, single ascospore cultures are unnecessary for routine morphological and physiological characterization (Kendrick et al. 1979) or DNA

extraction for Sanger sequencing. If single ascospore cultures are desired (e.g., for mating studies or genome sequencing), these can be made by transferring a germinating ascospore from an ascospore deposit to fresh growth medium in a separate Petri dish. To do this, ascospores must first be chosen and marked for transfer. Invert a Petri dish that contains the ascospore deposit so that the surface of the dish part (the bottom part) faces up and the surface of the growth medium faces down. Place it on the stage of a dissecting microscope (if the ascospores are large or dark enough to be clearly observed) or on the stage of a compound microscope following the directions given in *Helpful tip* above. Focus to the surface of the growth medium where the germinating ascospores lay and identify single ascospores that are isolated from each other by about 5 mm distance. Use a fine point colored marker to place a dot on the dish surface to mark their location. This may necessitate maneuvering the tip of the marker in between the objective and surface of the dish while looking through the eyepieces of the compound microscope. Then, in sterile conditions, open the Petri dish and with the surface of the growth medium facing you, find the colored dot(s) and use a sterile scalpel or similar tool to carefully cut out a 2 mm square block of medium over the dot. Carefully transfer this to the surface of fresh growth medium in a separate Petri dish, preferably with the ascospore-bearing side facing up (illustrated in Ahmadjian (1963), Kendrick et al. (1979)). After you have transferred blocks of media bearing germinating ascospores to different Petri dishes, check each one under the compound scope (as described above in *Helpful tip*) to ensure that only one ascospore was transferred. Mark the transfers where you can clearly see only one ascospore and monitor the growth of these until the colonies are well established. Be sure to mark the rim of each Petri dish lid to indicate that the culture is derived from a single ascospore. It is worth repeating that the selection of transparent growth media such as PDA or MEA agar will facilitate visualizing germinating ascospores with transmitted light. Growth media that is not so nutrient rich can also delay fast growing contaminants.

C. How to isolate conidia from natural substrates

In some groups of fungi, a suspected or known anamorphic state may co-occur with ascomata, and it may be desirable to isolate these in culture for further study. Kendrick et al. (1979) provided instructions for making conidial cultures by streaking a mass of conidia across the surface of the growth medium using a sterile probe. Similarly, a whole conidioma may be prodded across the surface of the growth medium with a sterile probe. This simultaneously produces a conidial streak and, ideally, cleanses the surface of the conidioma of fungal or bacterial contaminants so that the end of the streak should be contaminant-free. Finally, large conidia may be picked up and transferred using a very fine point paintbrush or a heat sterilized, steel or tungsten (Brady 1965) pin. This is first sterilized in 70% ethanol, 10% bleach, and sterile water for approximately 60 seconds per treatment. When applying conidia or a conidioma to the surface of a sterile growth medium outside of a biological safety cabinet or laminar flow hood, lift one side of the Petri dish up only as much as is necessary to admit the tip of the sterile probe or paintbrush. Growth medium supplemented with antibiotics (discussed above in section B, Materials and equipment) may be used as well.

When conidia germinate, blocks of growth medium bearing one to a few conidia from the end of a streak should be transferred to fresh growth medium following the instructions given above for *Obtaining single ascospore cultures*. Once this is done, it is important to monitor for contaminants and observe hyphal growth under the compound microscope until colonies are well established, as outlined above in section B7.

D. General suggestions and guidance

Voucher the ascoma (-ta) used in these studies and preserve ascospore deposits. An ascoma used to produce ascospore deposits should be dried and kept in a small paper packet. The same ascoma can be used for both making an ascospore deposit on a cover glass and on a growth medium (sections A and B). The packet should be labeled with details about what purposes the

ascoma was used for and it should be kept with the specimen. The ascoma can then be reexamined later should any questions arise regarding the source of the ascospore deposits.

Microscope slide preparations made from ascospore deposits on cover glasses can be saved for future study. If necessary, carefully clean the microscope slide around the cover glass with ethanol and tack down two corners of the cover glass using a sealant such as nail polish or Paraloid B-72. Make sure that there is still adequate mountant in the preparation – a dehydrated mount may draw the sealant under the cover glass and damage parts of the ascospore deposit and make rehydration difficult. We note that nail polish, though inexpensive and readily available, is not stable for long term preservation (Alten 1998). Paraloid B-72 (CAMEO n.d.) is fast-setting and is archival quality. Ascospores in tap water mounts may be preserved by applying a very small drop of glycerol to the edge of the cover glass. The preparation is set aside overnight. As the water mount evaporates, the viscous glycerol will be drawn under the cover glass. Future workers may observe these directly or easily rehydrate the glycerol with water. Because the cover glass has been tacked down, immersion oil or excess glycerol may be cleaned off with ethanol and lens paper without the risk of disturbing the cover glass and damaging the ascospore deposit. Label the slide clearly, using a scribing/etching pen with a tungsten-carbide tip, a paper label with a pencil or archival pen, or by using the standard museum object practice of layering Paraloid B-72 on the clean slide and writing on it with permanent ink, as outlined by Sullivan & Cumberland (1993). The label should provide the name of the fungus (or field determination), the collection number, the date, your initials, and importantly, the mountant used. Place the slide in a cardboard or plastic slide case or wrap it in tissue paper so that it is not damaged. Keep this with the specimen. Generally, slides prepared in KOH or Melzer's iodine reagent are not preserved. For very rare or valuable specimens, a more durable mount can be made by the double-cover glass mounting method given by Volkmann-Kohlmeyer & Kohlmeyer (1996). In this method, two cover glasses of different sizes are used. A subject is placed on the large cover glass on a microscope slide. A mountant is added and a smaller cover glass is placed on the specimen. The edge of the small cover glass is sealed with nail polish or similar material. This can be examined under the compound microscope. The sandwiched cover glasses are then picked up and inverted so that the larger glass is on top. This is then placed in a drop of balsam or Permount on a microscope slide.

It is important that dried fungal specimens, notes, microscope slides, dried cultures, and other materials should be assembled into a packet and be deposited in a recognized institutional fungarium (see Index Herbariorum: http://sweetgum.nybg.org/science/ih/). Living cultures should be deposited in a recognized institutional culture collection (see World Federation for Culture Collections: http://www.wfcc.info/index.php/home/). These procedures are vital to ensure reproducibility and further studies of your materials by future workers (this includes yourself!).

Bacterial, algal, or fungal contaminants. In our experience, the ascospore deposit method described here results in few instances of contamination. Contamination usually involves bacteria or algae being carried on discharged ascospores. Fungal contaminants are often due to the presence of other fungi on the same piece of the substratum. More often contamination occurs from airborne spores gaining entry when the Petri dishes are opened. When the colony of a contaminating organism is small, a block of growth medium supporting it can simply be excised using sterile tools in a biological safety cabinet or laminar flow hood and disposed of. In cases where there is pervasive bacterial contamination, any tiny amount of uncontaminated mycelium may be transferred to a fresh growth medium with antibiotics. Another approach is to cut a flap in the growth medium of a fresh Petri plate and lift the edge to transfer a small portion of the fungus under it. The hyphae will tend to outgrow the bacteria as they move through the medium. For additional recommendations on dealing with contaminated cultures, including mite infestations, see Jewson & Tattersfield (1922), Ko et al. (2001), and Perkins (n.d.).

Use vital stains for microscopy. Vital stains are those that do not kill cells immediately. Vital stains that are routinely used include diluted Lugol's iodine solution and basic dyes such as aqueous brilliant cresyl blue and 1% aqueous Congo red (Fig. 1I2). It should be noted that these are lethal to cells after prolonged exposure. Cells are killed almost instantly when mounted in solutions

with a very high or low pH, or that exhibit other lethal properties. Melzer's iodine reagent, cotton blue in lactophenol, glycerol buffer, Hoyer's fluid, lactic acid, and 2–10% KOH are all immediately lethal (e.g., Figs 1A2, A4, B2, C2, E2, G2–G3, H2, J4, K2–K3, 2 I) (Baral 1992).

Killing ascospores. Ascospore deposits offer an excellent opportunity to characterize the morphology of a sample of mature, discharged ascospores in both living and dead states. After ascospores have been extensively documented in the living state they should be killed and documented again. This is beneficial because many fungi have been described from rehydrated fungarium material in the dead state and redescriptions of type material can only be done based on the dead fungus. Data obtained from dead state ascospores can be compared with such descriptions in the literature or identification keys based on explicitly dead elements. It may also be compared with data obtained from living ascospores (an example is to compare the percent shrinkage in ascospore size between living and dead states, provided in the legend for Fig. 2 under I). Results may be highly informative and provide useful taxonomic features. However, these data should never be intermixed, that is, data obtained from living ascospores should always be analyzed and reported separately from data obtained from dead ascospores. Ascospores may be killed by applying a small drop of a lethal reagent such as KOH or Melzer's iodine reagent to the edge of the cover glass (Baral 1992). This can be done while the slide is positioned on the microscope stage with a few subject ascospores in the field of view. As the reagent is slowly drawn under the cover glass, diffusing into the water mountant on the slide, you may observe morphological changes in the ascospores in real time. For example, you may observe ascospore shrinkage and shape changes, as well as the fusion of lipid guttules into a single mass. After the ascospores in a deposit are killed in this way, document dead-state ascospore morphology.

Differential Interference Contrast (DIC) microscopy. Certain features of hyaline, living ascospores are sometimes difficult to visualize because they are low contrast. DIC optics are helpful to clearly image low contrast structures such as gelatinous sheaths and hyaline appendages. It also clearly distinguishes crystalline inclusions in ascomatal and/or host tissues. If DIC microscopy is available, it is good practice to use it to document the gelatinous structures of living, mature ascospores. However, researchers should question whether DIC microscopy is appropriate to image *all* cellular and tissue structures. Often, the flattened image produced by transmitted light microscopy clearly and adequately presents ascospore guttulation and nuclei, as well as the cellular structure in ascomatal sections (i.e., in order to discern tissue types like *textura globulosa, t. angularis, t. prismatica*, etc.). In these cases, it is important to observe the 2-dimensional *pattern* of these structures that are observed in the image. DIC produces a certain 3-dimensional appearance of ascospores or tissues that may obscure these patterns and make their interpretation less immediately clear to the eye. For instance, Baral et al. (2020) noted a loss of resolution in that DIC microscopy utterly obscures the attachment point of spore bodies, the taxonomically important organelle in *Orbiliomycetes*, to the inner ascospore wall.

Clearly describe methods in figure legends. Provide clear information in your published figure legends on mountants or stains used, and whether the ascospores are living or dead. A convention is to type in the text of the legend, or in the image panel itself, an asterisk (*) for living; a dagger (†) to indicate dead cells (Figs 1 and 2). These notations may also be used in descriptions (Baral 1992).

Document everything in images and words. Conidia may swell considerably during germination. Sometimes, ascospores in culture may simply lyse, or produce a network of short hyphae before growth stalls. Hyphae may display distinctive branching patterns or produce distinctive structures such as appressoria or chlamydospores. Some groups of fungi grow rapidly and quickly produce conidia, but other groups grow slowly and form a tough, humped, or cerebriform mycelium. Asexual states may be present or lacking. All these conditions provide valuable information that should be well-documented and included with the specimen as notes, even if not published. Critical cultures should be deposited in publicly accessible culture repositories.

Concluding remarks

The methods employed and the care taken in standardizing microscopic examination is critical to producing morphological work that is dependable and repeatable. Toward this end, researchers should understand how the methods that they employ will affect cell morphology. These methods must then be clearly and thoroughly communicated in the text and figure legends of manuscripts. In this way, any artifacts that may have been introduced may be detected and discussed. This review has focused primarily on ascospore and conidium morphology, though these principles extend to the morphological analysis of other fungal cells and tissues, such as asci and paraphyses (Baral 1992). The morphology and biometry of living ascospores provide critical data for species delimitation in *Ascomycota*. Images of freshly collected specimens and taxonomically important cells and tissues in both living and dead states are vital to fully characterize the morphology of a subject. The methods outlined here are intended to aid those working in ascomycete systematics to study these fungi effectively.

Acknowledgements

We wish to thank our anonymous reviewers, as well as Rosanne Healy and Daniel Raudabaugh for their advice and input. Special thanks to Hans-Otto Baral for his comments and helpful advice. Luis Quijada was supported through the Farlow Fellowship offered by the Department of Organismic and Evolutionary Biology at Harvard University and the Harvard University Herbaria.

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