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Teaching Techniques for Mycology: 12. A demonstration of the teleomorph-anamorph connection using *Pleospora herbarum*

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Names of fungus

Pleospora herbarum (Pers.) Rabenh. ex Ces. & de Not.; *Stemphylium herbarum* Simmons

Introduction: features of interest

The life-cycles of many fungi include an asexual state, the anamorph, and a sexual state, the teleomorph. These together make up the holomorph (Hennebert & Weresub, 1977). Because these two states may bear little morphological resemblance to each other, it was not always appreciated that they were genetically identical, i.e. that they were merely different parts of the same fungus. Hence, anamorphs and teleomorphs were classified into different fungal groups (e.g. hyphomycetes and ascomycetes, respectively), and they were given separate generic and specific names. Some of the early mycologists such as the Tulasne brothers recognized pleomorphism in fungi by the frequent association of conidial states with certain ascomycetes. However, such conclusions based solely on associations could be misleading e.g. in cases where a mycoparasite was growing on a particular host fungus. Firmer evidence had to await the development of solid media and pure culture techniques, pioneered notably by the German mycologist, Oskar Brefeld. The connection between a teleomorph and anamorph of a fungus is made if it can be proven that one develops into the other in pure culture. It is customary to isolate sexual spores (ascospores or basidiospores) and show that cultures derived from them develop conidia. However, in some cases the reverse is also possible.

It is relatively simple to isolate clean cultures of ascomycetes with a violent ascus discharge mechanism by arranging for the fungus to shoot its ascospores onto an agar plate. Single germinated spores can then be transferred to fresh media where they may develop conidia. The technique is valuable because it can be used to demonstrate the range of asexual reproductive structures in different groups of fungi, and to clarify the taxonomic relationships between teleomorph genera and form-genera of the anamorphs.

Here we describe the isolation of ascospores of *Pleospora herbarum* (Figs 1 and 2) and the development of its conidial state (Figs 3 and 4). Wehmeyer (1961) regards *P. herbarum* as a species complex, and it is in this wide sense that we have used the name. Simmons (1969, 1985) has shown that there are several species of *Pleospora* similar to *P. herbarum* but with distinct *Stemphylium* anamorphs. For instance, *P. tarda* Simmons has *S. botryosum* Wallroth as its anamorph whilst *P. herbarum* has *S. herbarum*. In addition to being a suitable subject for teleomorph-anamorph studies, *P. herbarum* provides a good example of bitunicate (double-walled) asci characteristic of the Loculoascomycetes (Fig 1), and of the formation of poroconidia (Fig 4).

Source of material

Pleospora herbarum sensu lato is very common and widespread as a saprotroph or weak parasite on a wide range of herbaceous plants (Ellis & Ellis, 1997), especially on dead overwintering stems of maritime plants. The black, somewhat flattened pseudothecia are visible through the raised epidermis of the host plant. Plant stems with pseudothecia should be collected in autumn or winter. Dried material retaining viable pseudothecia can be stored at room temperature (r.t.) for over a year. We will provide our isolate of *P. herbarum* upon request to RWSW.

Maintenance of the fungus

Conidial cultures can be maintained on corn meal agar (CMA) slopes at 4°C with subculturing every 6 months, but they may lose the ability to produce pseudothecia which are often formed by fresh isolates on CMA or 0.2% malt extract agar (MEA). It is therefore preferable to freeze-dry conidia produced in culture from a recent ascospore isolate. Pseudothecia for practical classes may be produced by adding a few autoclaved plant stems (e.g. of legumes such as clover) to fresh isolates grown for 2 wk on CMA at r.t. Within a further 2 wk at r.t. in the light, pseudothecium initials will form on the stems. Maturation of pseudothecia can be induced by incubation for 3 wk at 8° in the dark, followed by 3 wk at 15° in the dark (Leach, 1971). Pseudothecia are ripe when discharged ascospores are found on the underside of the Petri dish lid within 24 h exposure to near-UV light or daylight (avoid direct sunshine). At this stage, the stems should be allowed to dry over 2-3 d. They can then be stored dry at r.t. or at 4°, ready for use in the next practical class.

Preparation of material

This experiment is ideal for courses in which two practicals are held in successive weeks. Once

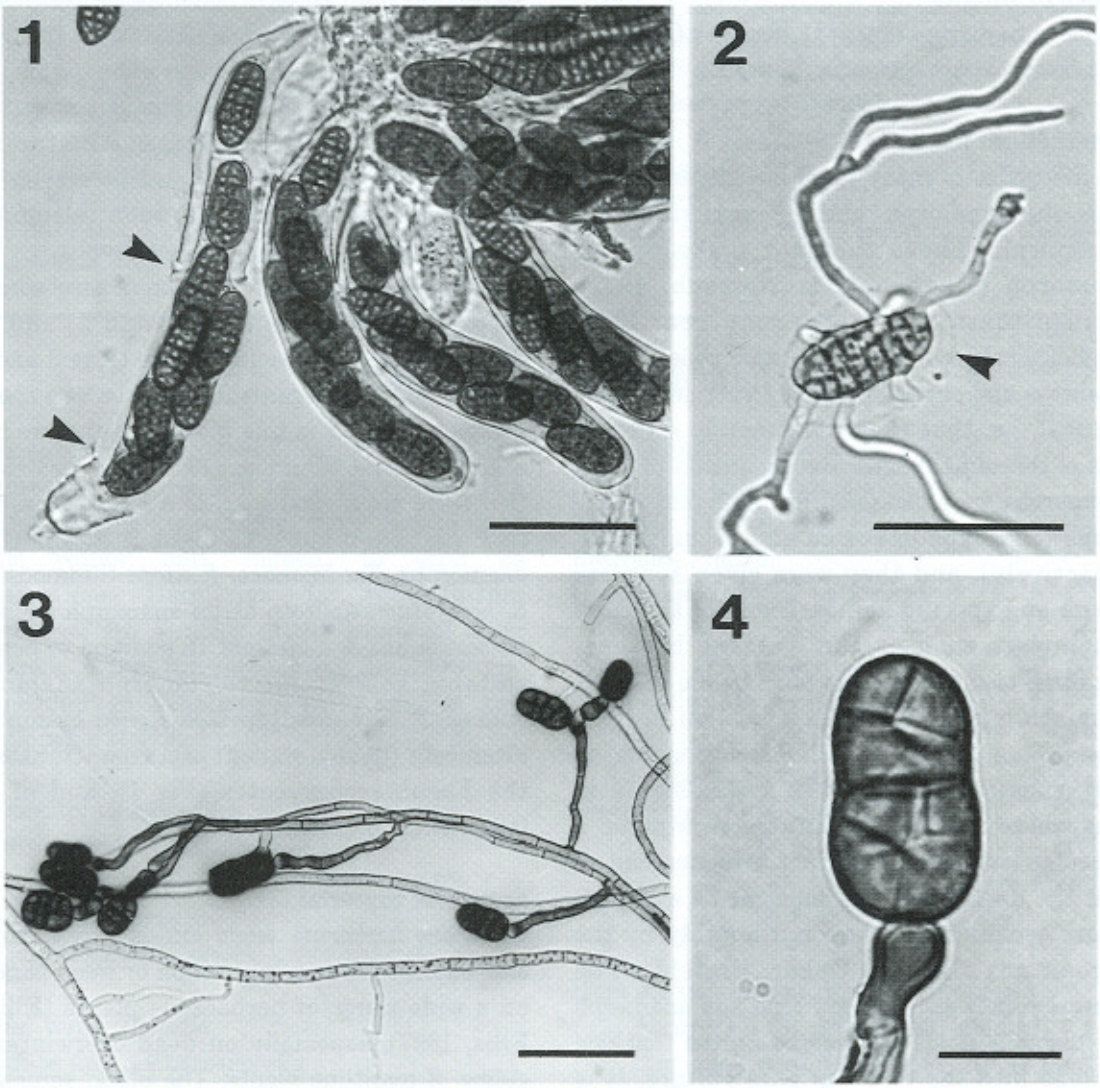

Fig 1 Squash preparation from a pseudothecium of *Pleospora herbarum*. The asci are bitunicate, and in one ascus the outer wall has broken (arrowheads) by stretching of the inner wall. Bar = 50 μm. Fig 2 An ascospore with its surrounding gelatinous epispore (arrowhead) 12 h after projection onto 0.2% MEA. Several cells of the ascospore have germinated, each producing one germ-tube. Bar = 50 μm. Fig 3 Conidiophore with conidia of the *Stemphylium herbarum* type in a 7 d old culture on CMA derived from a single ascospore. The conidiophore and conidia are pigmented whereas the vegetative hyphae on the agar surface are hyaline. Bar = 50 μm. Fig 4 Higher magnification of a *Stemphylium* conidiophore and conidium. The inner wall has ballooned out through a narrow pore in the swollen apex of the conidiophore to form a terminal conidium. The conidiophore may subsequently extend laterally to form a second conidium. Bar = 10 μm.

suitable pseudothecial material has been obtained, it involves little preparative work.

Day -1. Pour Petri dishes with a shallow layer of a clear agar medium such as CMA, MEA or potato dextrose agar. Host material with ripe pseudothecia, recognized by their papillate ostioles, should be selected under a dissection microscope and cut into short lengths (1 cm). About 24 h before the practical, place a length of suitable material on a piece of moistened filter paper (1 x 2 cm) near the edge of the lid of an inverted Petri dish and lower the base containing agar over it. Incubate in daylight, but avoid direct sunlight. The pseudothecia will revive, and ascospores will be shot off within a few hours. They are visible on the inverted agar surface under a dissection microscope. By rotating the base of the dish over the plant material, a succession of ascospore deposits can be obtained. Incubate at r.t. overnight.

Day 0. Students should remove the infected plant stem and investigate pseudothecia mounted in water on a microscope slide. They contain large, broad, bitunicate asci (Fig 1) interspersed by pseudoparaphyses. Ripe asci contain 8 yellowish-brown muriform ascospores, typically with 7 transverse and 1-2 longitudinal septa. Each ascospore is surrounded by a conspicuous transparent epispore (Fig 2). The ascospore deposits in the base of the Petri dish should be examined with a dissection microscope. Many of the spores will have germinated overnight (Fig 2). Incubate plates bearing ascospore deposits for 7 d at r.t. in natural daylight. For critical work avoiding the possibility of contamination by other fungi, a single germinated ascospore should be transferred with a flame-sterilized needle to a fresh agar plate and incubated as above.

Day 7. Students should examine the surface of their cultures under a dissection microscope, noting the dark sub-globose to oblong conidia borne on aerial conidiophores (Fig 3). Squares of agar with mycelium can be mounted in water under a coverslip to study formation and morphology of conidia. The conidia develop tretically (i.e. as poroconidia) by an extension of the inner wall of the conidiophore apex through a narrow canal (Fig 4; Carroll & Carroll, 1971).

Useful hints

The production of conidia is stimulated by near-ultraviolet light (400-500 nm). Good sporulation can be induced by subjecting cultures to periods of dark alternating with light from fluorescent lamps emitting near-UV light ("black light"; Leach, 1967).

When incubated in a moist chamber, host material infected by *P. herbarum* will also develop the *Stemphylium* conidial state. Individual conidia can be picked off (from the infected plant material or the ascospore isolates) and transferred to CMA plates with a flame-sterilized fine needle. The resulting single-conidium cultures can be compared with cultures derived from single ascospores. Because *P. herbarum* is homothallic, ripe pseudothecia can be produced on agar from single-conidium isolates. After incubation of the CMA plates with the single-spore isolates for 5 weeks at 20° in the light, asci containing ascospores will be visible, though discharge may not occur. In this way, the development of the teleomorphic from the anamorphic state can be demonstrated.

The technique described here can be adapted for use with many other subjects, e.g. *Leptosphaeria acuta* (Hoffm.) P. Karsten which forms its pseudothecia along with its pycnidial anamorph *Phoma acuta* Fuckel at the base of overwintered stems of stinging nettle (*Urtica dioica* L.).

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