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Teaching techniques for mycology: 21. *Sclerotinia*, *Botrytis* and *Monilia* (Ascomycota, Leotiales, Sclerotiniaceae)

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Names of fungi

1. *Sclerotinia curreyana* (Berkeley ex Currey) P. Karsten = *Myriosclerotinia curreyana* (Berkeley ex Currey) Buchwald; conidial state *Myrioconium* Sydow
2. *Sclerotinia fuckeliana* (de Bary) Fuckel = *Botryotinia fuckeliana* (de Bary) Whetzel; conidial state *Botrytis cinerea* Persoon ex Fries
3. *Sclerotinia fructigena* (Persoon) Schröter = *Monilinia fructigena* Honey ex Whetzel; conidial state *Monilia fructigena* (Persoon) Eaton

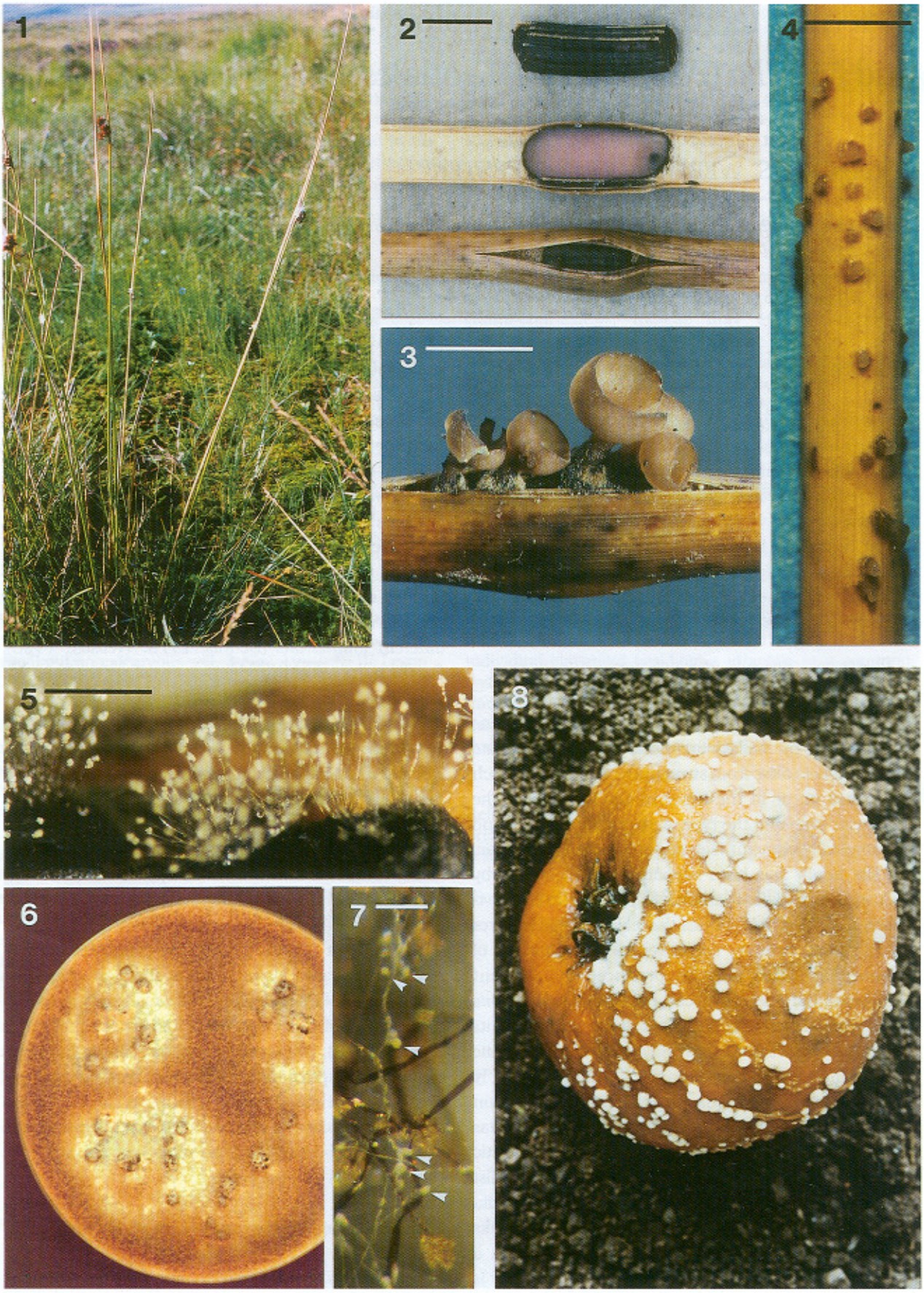
Introduction: Features of interest

Members of the Sclerotiniaceae are plant pathogens characterized by stalked apothecia with inoperculate asci. Apothecia arise from stromata, e.g. sclerotia which are formed on or in the tissue of the host plant. The apothecial state and the type of stroma are typical and taxonomically relevant features of the Sclerotiniaceae, and the family has been supported and delimited by DNA sequence data (Carbone & Kohn, 1993; Holst-Jensen *et al.*, 1997*a*,*b*). Whilst the teleomorphic features are therefore strongly conserved, a wide variety of anamorphic states may be produced by different members of the Sclerotiniaceae. For instance, *Sclerotinia sclerotiorum* (Libert) de Bary has only sclerotia as its anamorph whereas *S. curreyana* produces phialidic *Myrioconium*-type microconidia which probably function solely as spermatia, *i.e.* as agents of fertilization in sexual reproduction. In contrast, *S. fuckeliana* has *Botrytis cinerea* as its anamorph which produces blastic macroconidia in addition to phialidic spermatia, whilst *S. fructigena* has an anamorph with blastoconidia classified in the form-genus *Monilia* but lacks microconidia or spermatia. The occurrence of such a diversity of anamorphs within a monophyletic family is a striking example of the flexibility of asexual reproduction in the fungi. This uncertain character has nonetheless been used, along with the morphology of stromata, to separate from the formerly large genus *Sclerotinia* various genera such as *Botryotinia* for species with *Botrytis* anamorphs, *Monilinia* for forms with *Monilia* conidia, and *Myriosclerotinia* for those with *Myrioconium*-type spermatia (Whetzel, 1945, 1946; Buchwald, 1947). These segregations have been supported by Kohn (1979), although for practical reasons the generic name *Sclerotinia* continues to be used by some mycologists and plant pathologists in the original wider sense to embrace species with a range of different anamorphs (Dennis, 1978; Webster, 1980). For the purposes of introductory mycology teaching, we follow the latter approach.

In addition to illustrating taxonomic problems caused by anamorph flexibility, the Sclerotiniaceae also provide suitable material for plant pathology courses, not least because of their exceptionally wide host range and economic importance. Thus, *S. fructigena* causes the common brown rot of apples (Fig 8) and other fruits, and can be used to demonstrate Koch's postulates (see Pitt *et al.*, 1998). *Sclerotinia laxa* Aderhold & Ruhland is another pathogen of fruit trees, causing blossom wilt, spur blight and brown rot (Moore, 1959). The conidial state of *S. fuckeliana*, *Botrytis cinerea*, is one of the most ubiquitous necrotrophic plant pathogens and causes grey mould on a wide range of fruits, vegetables and ornamental plants (Moore, 1959). *Sclerotinia sclerotiorum* infects the stems of numerous agriculturally important crops by means of ascospores released from apothecia produced on overwintered sclerotia (Parry, 1990). Wild plants are also hosts to members of the Sclerotiniaceae, e.g. soft rush (*Juncus effusus*) to *S. curreyana*.

Source, storage and preparation of *S. curreyana*

Infected culms of *Juncus effusus* are easily recognized in summer by their white, bleached appearance which contrasts with the dark green colour of uninfected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culms (Fig 1). A difference in colour persists throughout the winter so that the bleached culms can be distinguished from the straw-coloured dead uninfected culms. From late September onwards, infection can also be confirmed by drawing the base of a bleached culm through finger and thumb when the firm sclerotia embedded in the pith resist crushing (Fig 2). The interior of sclerotia is pink, explaining why this state was earlier known as *Sclerotium roseum* (John Ramsbottom, personal communication). The sclerotia survive the winter and in early spring develop one or several apothecia which mature by April or May (Fig 3) in a wet environment. Infected culms often break at the position of a sclerotium.

Spring preceding the practical class. Short lengths of *Juncus* containing overwintered sclerotia should be collected in early to late March, depending on latitude and altitude. Air-dried material will remain viable for up to 12 months when stored at 4 °C or -20°C. Development of apothecia can be induced by thoroughly wetting sclerotia and incubating them for a few days on wet filter paper or *Sphagnum* moss at room temperature (r.t.) in the light.

Day -21. If the spermatial (*Myrioconium*) state is required in culture for the practical class, a few sclerotia should be incubated on wet filter paper at r.t.

Day -14. Mature apothecia shooting off ascospores should have developed. Invert a thinly-poured tap-water agar (TWA) Petri dish base over ripe apothecial material. When ascospores have been deposited on the agar surface, place a lid on the TWA plate and incubate at r.t. under a fluorescent tube. The ascospores germinate into short hyphae which produce phialides and spermatia.

Day -7. Revive sclerotia in order to produce apothecial material for the practical class.

Day 0. Present material to the students who should make a squash preparation of the hymenium from mature apothecia in water to note the inoperculate asci each containing 8 curved ascospores (Fig 9). The spermatial state is best seen by mounting squares of TWA in water or lactic acid on a microscope slide (Fig 10).

Useful hints. The spermatial state of *S. curreyana* can also be observed on the upper part of bleached culms of *Juncus effusus* in August and September as minute brownish lesions termed spermodochidia (Whetzel, 1946; Webster, 1980). Within a few hours of wetting infected material, a hyaline mass of phialide clusters and spermatia will ooze out of irregular cracks (Fig 4). Such material can be collected with a fine needle and should be washed in 70% ethanol to remove some of the spermatia prior to mounting in lactic acid (Fig 11). Infected culms may be stored dry at 4°C or -20°C and rehydrated when needed.

Source, storage and preparation of *Botrytis cinerea*

In summer and autumn, *B. cinerea* can be found causing grey mould on a wide range of wild and cultivated plants. In winter and early spring, overwintered black sclerotia are seen especially on dead stems of umbellifers such as hemlock (*Conium maculatum*) and hogweed (*Heracleum sphondylium*), as well as many other herbaceous plants. Sclerotia on host stems collected in March remain viable for many months if stored dry at 4°C or -20°C. After incubation for 1-2 d in a Petri dish with moist filter paper, these sclerotia germinate to form prominent dark conidiophores with numerous macroconidia (Fig 5). The fungus is easily isolated from macroconidia produced either from sclerotia or taken directly from grey mould lesions. In order to prevent the loss of macroconidium production in laboratory cultures of *B. cinerea*, potato dextrose agar (PDA) plates should be inoculated by spreading macroconidia over the entire agar surface, followed by incubation for 4-5 d at r.t. in the dark, a brief (5 min) exposure to near-UV light which stimulates asexual reproduction (Tan & Epton, 1973), and a further 2-3 wks at r.t. in the dark (Weber & Pitt, 1997). The fungus can be maintained on PDA slopes at 4°C with subculturing every 12 months, or for several decades as a lyophilized vacuum-sealed spore suspension stored at r.t. (Smith & Onions, 1983).

Day -42. Revive *B. cinerea* by transferring inoculum from a storage culture onto a fresh PDA plate. Incubate at r.t.

Day -39. Inoculate a number of yeast extract-sucrose (YES) agar plates (20 g sucrose, 4 g yeast extract, 1 g

Figs 1-4 *Sclerotinia curreyana*. Fig 1 The host, *Juncus effusus*. Infected culms are recognisable by their bleached appearance. Fig 2 Fresh sclerotia collected in early March. Top, sclerotium dissected out of an infected culm. Centre, culm sliced open to reveal the sclerotium which has a pink interior. Bottom, sclerotium inside a culm. The sclerotium has swollen and is ready to germinate. Bar = 5 mm. Fig 3 Germinated sclerotium with several apothecia. Bar = 5 mm. Fig 4 The *Myrioconium* spermatial state on a bleached culm collected in August and incubated in a damp-chamber overnight. **Figs 5-7** *Botrytis cinerea*. Fig 5 Macroconidiophores obtained by incubating a stem of *Conium* with overwintered sclerotia on moist filter paper for 2 d. Bar = 0.5 mm. Fig 6 YES agar plate inoculated on day -39 as described in the text. The mycelium in marginal areas has produced abundant greyish-green macroconidia whereas the centres of inoculum are producing sclerotia. Microconidia are formed in the transition zone between these two areas. Fig 7 Binocular microscopy view of the transition zone. Among the collapsed dark macroconidiophores, minute droplets of microconidia (arrowheads) have been produced. Bar = 100 µm. **Fig 8** *Monilia fructigena*. Brown-rotted apple with whitish conidial pustules. The concentric zonation of the pustules is related to diurnal illumination.

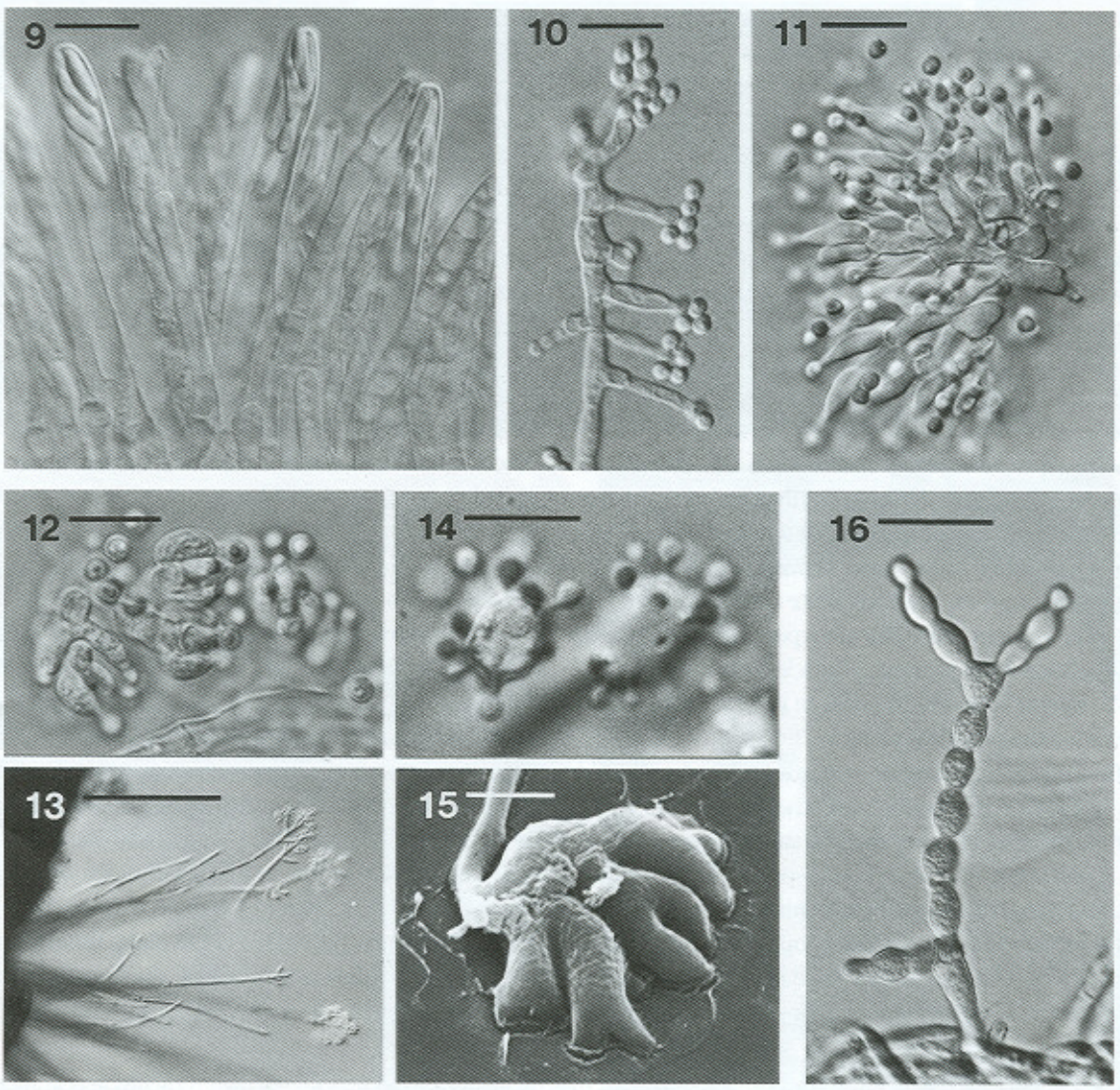
KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ and 15-20 g agar per litre tap water) with 3 mycelial plugs from the PDA plate. Incubate at r.t. in the dark.

Day -35. Mycelia of *B. cinerea* will have covered most of the YES agar surface. Expose these plates to near-UV light for 5 min. This will induce macroconidium formation only in the young, competent hyphae at the mycelial margins. Incubate at 18°C in the dark until the day of the practical.

Day -5. Remove macroconidia from one of the YES agar plates and streak out onto PDA plates. Incubate at r.t. in the dark.

Day -1. Expose the PDA plates inoculated on day -5 to near-UV light for 5 min. Return to r.t. in the dark.

Day 0. Present the PDA and YES agar plates to the students. The latter will show all three asexual reproductive structures found in *B. cinerea* (Fig 6). Dark greyish-green macroconidiophores are abundant at the margins of the colonies whereas large black sclerotia are situated mainly in the central whitish areas. Using a binocular microscope, hyaline drops of sticky microconidia (Fig 7) may be detected at the border between the sclerotial and macroconidial regions (Fig 6). These should be mounted on a microscope slide in water



Figs 9-11 *Sclerotinia curreyana*. Fig 9 Squash preparation from the hymenium of a mature apothecium. Several ripe asci, each containing 8 curved ascospores, are visible. Bar = 10 µm. Fig 10 The *Myrioconium* state formed from germinated ascospores on TWA. Spermatia are produced from phialides. Bar = 10 µm. Fig 11 The *Myrioconium* state obtained from infected culms shown in Fig 4. The phialides are arranged in tight clusters. Bar = 10 µm. **Figs 12-15** *Botrytis cinerea*. Fig 12 Microconidia obtained from the droplets shown in Fig 7. The phialides are stout and tightly clustered. Bar = 10 µm. Fig 13 Macroconidiophores arising from a rehydrated sclerotium as shown in Fig 5. The tips of maturing conidiophores bear grape-like clusters of conidia. Bar = 250 µm. Fig 14 Details of the swollen conidiogenous cells at the tip of a macroconidiophore. Conidia are produced polyblastically at numerous points on the surface of the swollen cells. Bar = 10 µm. Fig 15 Scanning electron micrograph of a sclerotium initial produced on a coverslip. Bar = 10 µm. **Fig 16** *Monilia fructigena*. Conidiophore producing branching chains of blastoconidia. Bar = 20 µm.

or lactic acid in order to see the minute spherical conidia produced from phialides (Fig 12). For fresh macroconidial material, the PDA plates inoculated on day -5 should be used. The dark conidiophores terminate in thin-walled conidiogenous cells which give rise to grape-like clusters of unicellular egg-shaped macroconidia (Fig 13). Conidiogenesis is polyblastic, whereby conidia are produced synchronously at numerous points distributed over the surface of a conidiogenous cell (Fig 14; see also Tribe & Weber, 2001).

Useful hints. Macroconidiogenesis of *B. cinerea* is most readily seen in young conidiophores washed in 70% ethanol and then mounted in lactic acid. If the developmental sequence of sclerotium formation by repeated branching of a hyphal tip (Townsend & Willetts, 1954; Moore, 1995) is to be demonstrated, the Riddell slide technique is useful (Weber & Pitt, 2000). If plugs of PDA are inoculated 5 d before the practical class and incubated at r.t. in the dark, sclerotium initials of various sizes will be seen on the glass coverslip (Fig 15).

Source, storage and preparation of *Monilia fructigena*

Apples showing symptoms of brown rot (Fig 8) can be found on the tree, in storage or on the ground where they overwinter in a dried, mummified state, providing a source of inoculum in the following spring as conidia or, rarely, ascospores (Webster, 1980). The mummified fruit is an example of a pseudosclerotial stroma which incorporates host tissue (Whetzel, 1945). Concentric rings of buff-coloured pustules appear on infected fruits in response to light which stimulates conidium formation. These pustules are sporodochia bearing macroconidia which develop holoblastically at the tips of branching chains (Fig 16). Mummified fruits can be induced to form fresh conidia by incubation for a few days in a moist chamber in the light. The fungus is easily isolated by streaking out macroconidia onto the surface of a PDA plate where they germinate within a few hours. Alternatively, inoculum can be obtained from the brown margin of a developing lesion on an apple fruit by swabbing the surface with 70% ethanol, cutting out a small piece of infected tissue, and placing it on PDA. Hyphae will emerge from the inoculum within 2 d. Transfers of bacterium-free hyphal tips should be made onto fresh plates of YES agar which is particularly suitable for sporulation. It should be noted that brown rot of apples can be caused also by other fungi such as *Penicillium expansum* Link ex Gray which forms synnemata with greenish-blue conidia, so that care is needed in selecting the material to be used. *Monilia fructigena* can be maintained on YES agar slopes at 4° with subculturing every 12 months.

Day -14. Revive *M. fructigena* by placing inoculum on a YES agar plate.

Day -7. Inoculate several YES agar plates and place these at r.t. under fluorescent light on a light-dark cycle, or expose to near-UV light for 10 minutes each day.

Day 0. Present material to the students who should make a squash preparation of conidial chains in lactic acid (Fig 16).

Useful hints. The Riddell slide technique (Weber & Pitt, 2000) provides good material of *M. fructigena*.

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