Teaching techniques for mycology: 14. Mycorrhizal infection of orchid seedlings in the laboratory

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Names of fungi

Rhizoctonia cerealis van der Hoeven = *Ceratorhiza* cerealis (van der Hoeven) Moore. Teleomorphic state, *Ceratobasidium cereale* Murray & Burpee:

Rhizoctonia solani Kühn = *Moniliopsis solani* (Kühn) Moore. Teleomorphic state, *Thanatephorus cucumeris* (Frank) Donk

Introduction: Features of interest

The term mycorrhiza ('fungus-root') denotes the close association between the roots of a terrestrial plant and one or more species of fungi. Most plants are capable of forming mycorrhiza, especially in nutrient-poor soils, whereby the fungal mycelium scavenges mineral nutrients from the soil and makes them available to the plant, which in turn provides the fungus with carbohydrates (Smith & Read, 1997). Orchids are unusual because in all natural situations they are dependent on mycorrhizal fungi throughout their life-cycle, from the establishment of seedlings which develop from tiny seeds devoid of storage tissue, through an often prolonged non-photosynthetic phase of growth in the soil, until the emergence of a shoot and the eventual production of flowers (Smith & Read, 1997). Thus, at least in its initial stages, the orchid seedling relies on its mycorrhizal partner for the supply of carbon as well as minerals, and there is no evidence of any translocation of photosynthetic product to the fungus even after green shoots have formed (Alexander & Hadley, 1985). Orchids therefore parasitize their fungal partner which, paradoxically, is often itself a serious pathogen of other plants. Species of *Rhizoctonia sensu lato*, which can kill a wide range of terrestrial plants and degrade their remains, are particularly common as mycorrhizal associates in orchids (Sneh *et al.*, 1991; Masuhara *et al.*, 1993).

We use the generic name *Rhizoctonia* in its widest sense as circumscribed in 1815 by DeCandolle. This denotes fungi capable of infecting living plants and growing as a mycelium which may produce sclerotia different from those of *Sclerotium* (Andersen & Stalpers, 1994). *Rhizoctonia sensu lato* has recently been divided into four genera, based on septal pore ultrastructure and other characteristics, and those genera correlate to different basidiomycete teleomorphic states (Moore, 1987; Andersen & Stalpers, 1994). However, the teleomorphs are rare and the anamorphic distinctions cannot be made with the light microscope. Further, a comparison of Moore (1987) and Smith & Read (1997) reveals that species forming orchid mycorrhiza arise from at least three of the four new genera, *i.e. Ceratorhiza, Epulorhiza* and *Moniliopsis*. For these reasons, we use *Rhizoctonia sensu lato* in the context of the present paper.

On mineral salts agar, the microscopic seeds of most native orchid species such as those of the heath spotted orchid (Fig 3) germinate by limited cell division to form a protocorm, a swollen storage structure which may extend a few epidermal hairs (Fig 4). In the absence of simple sugars such as glucose, further growth and differentiation of the protocorm (Fig 5) take place only after infection of the seedling by a suitable mycorrhizal fungus capable of degrading complex plant polymers such as cellulose (Smith, 1966; Hadley, 1969). Degradation of cellulose and translocation of sugars to the orchid seedlings can be demonstrated by the split-plate technique in which the mycorrhizal fungus crosses a physical barrier between the

food base (tissue paper) and the seedlings (Fig 7). After prolonged incubation of infected seedlings in the light, a green shoot will grow (Fig 6).

The infection process of orchid protocorms by hyphae of *Rhizoctonia* is readily demonstrated under laboratory conditions (Williamson & Hadley, 1970). Penetration occurs immediately upon contact (Hadley & Williamson, 1971), often through the tips of epidermal hairs (Fig 8), followed by the formation of hyphal coils (pelotons) in the outer cortical cells of the protocorm (Figs 2 and 9). Each peloton has a limited life-span which may be as short as 24 h (Hadley & Williamson, 1971) and is terminated when the peloton is degraded by the plant cell in which it is contained. Degradation seems to be particularly active in cells of the inner cortex (Fig 2), suggesting that the orchid is capable of restricting and regulating colonization by *Rhizoctonia*. Nevertheless, orchid mycorrhiza is an unstable association in which only a proportion of protocorms shows balanced mycorrhizal infection whilst other seedlings may resist penetration altogether or succumb to aggressive, soft-rotting infections of the same fungus (Hadley, 1970; Beyrle *et al.*, 1995). Heterogeneous developmental stages may be observed even in material raised under controlled laboratory conditions (*e.g.* Figs 6 and 7).

Orchids and their mycorrhizal fungi therefore provide a valuable system to explore the diffuse boundaries between the nutritional concepts of saprotrophy, mutualistic symbiosis and parasitism (*e.g.* Lewis, 1973). Additionally, the split-plate technique is suitable for demonstrating the translocation of nutrients through fungal mycelia, and for illustrating the invasion of plant tissues by hyphae.

Source of material

Common plant-pathogenic *Rhizoctonia* spp. often form satisfactory orchid mycorrhizas under experimental conditions (Downie, 1957; Hadley, 1970), thus obviating the need to obtain isolates from roots of mature orchids. We use *R. solani* which causes stem canker and black scurf of potato (*Solanum tuberosum*) and is easily isolated from black sclerotia on the surface of potato tubers, and *R. cerealis* which causes sharp eyespot on the stem base of cereals and grasses. We will provide cultures of our isolates upon request.

Dactylorhiza maculata ssp. *ericetorum* (Fig 1), the heath spotted orchid common on acid peat soils throughout the British Isles (Stace, 1991), is particularly suitable for this experiment because its seeds germinate easily and the seedlings are readily infected by *Rhizoctonia* spp. Other native orchid species are also amenable to this technique (Hadley, 1970; Smreciu & Currah, 1989). In order to obtain seeds, it is convenient to locate the plants at their time of flowering (May) and to return to collect a few capsules when the seeds are ripe (July to August). Seeds are released by incubating the capsules on a Petri dish base overnight at r.t. under gently drying conditions. Seeds should be stored dry at 4°C in darkness and will remain viable for several months.

Maintenance of fungi

Rhizoctonia spp. are easily maintained on slopes of 2% malt extract agar (MEA) with added cellulose (*e.g.* sterilized tissue paper or filter paper) incubated at 4°, with subculturing every 6 months.

Preparation of material

Divided two-compartment plastic Petri dishes (9 cm diam.) are available from most laboratory suppliers (*e.g.* Bibby Sterilin Ltd., Stone, Staffs.). Once the split-plates have been prepared, little further work is required. Several students may share one plate.

Day -90. Prepare Pfeffer's agar with the following ingredients (per litre dist. water); 290 mg $Ca(NO_3)_2 \cdot 4H_2O$, 50 mg KH_2PO_4, 50 mg $MgSO_4 \cdot 7H_2O$, 50 mg KNO_3, 25 mg KCl, 1 g glucose, 1 ml of a trace element supplement (*e.g.* 2.2 g $ZnSO_4$, 1.1 g H_3BO_4, 0.5 g $MnCl_2 \cdot 4H_2O$, 0.5 g $FeSO_4 \cdot 7H_2O$, 0.17 g $CoCl_2 \cdot 6H_2O$, 0.16 g $CuSO_4 \cdot 5H_2O$, 0.15 g $Na_2MoO_4 \cdot 2H_2O$ and 5 g Na_4EDTA in 100 ml dist. water) and 15 g agar. Autoclave (20 min at 115°) and allow to cool to 48°. Meanwhile, surface-sterilize orchid seeds by immersion in 5% (v/v) Domestos (Lever, U.K.; active ingredient, sodium hypochlorite) with 0.1% Teepol or another detergent for 10 min at r.t. with occasional shaking, followed by centrifugation (approx. 4,000 rpm for 3 min) and two washes in sterile water. The seeds will strongly adhere to surfaces and can thus be transferred in a drop of dist. water to

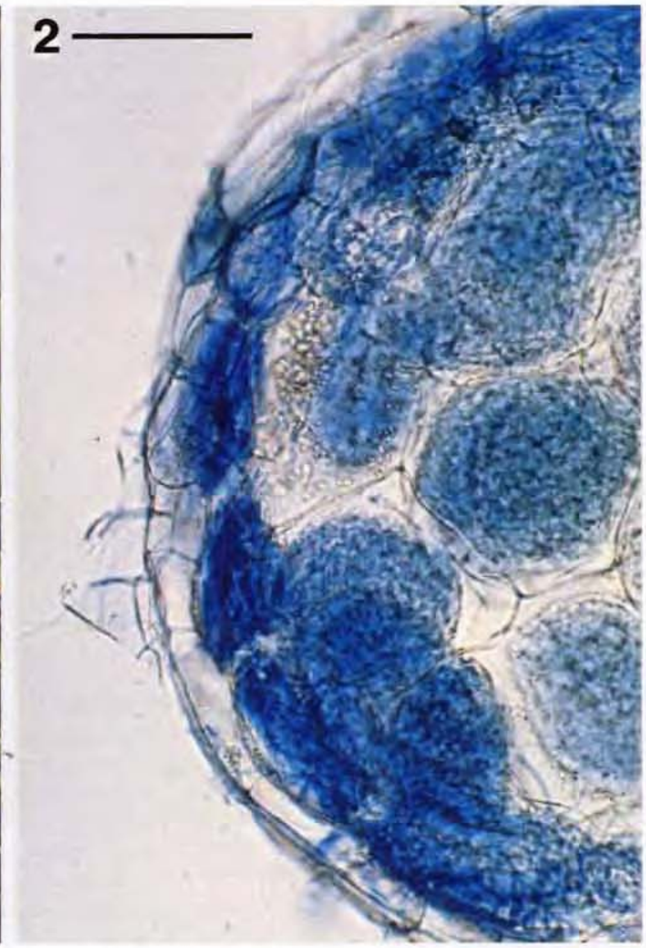

Fig 1 Flowering shoot of *Dactylorhiza maculata* ssp. *ericetorum*, the common heath spotted orchid. Fig 2 Section of an infected protocorm mounted in Cotton Blue/lactic acid (day -21). Note the hyphal coils (pelotons) in cells of the outer cortex of the protocorm and their disintegration in cells of the inner cortex. Bar, 100 μm.

the bottom of one side of a split Petri dish with a flame-sterilized needle or loop. Pour Pfeffer's agar onto the drop of seed suspension, distribute seeds with a flame-sterilized needle if necessary, and pour the remaining agar into the empty compartment of the split-dish. Incubate at r.t. in the dark.

Day -67. Subculture *Rhizoctonia* onto MEA plates and incubate at 20° in darkness.

Day -60. Many of the orchid seeds will have germinated by swelling and bursting of the testa, producing a few epidermal hairs (Fig 4). Place a piece of sterile tissue paper (approx. 2 x 5 cm) in the opposite half of the split Petri dish and inoculate this side of most of the plates with mycelium of *Rhizoctonia* from the MEA cultures set up on day -67, keeping a few control plates without the fungus. Incubate at r.t. in darkness.

Day -21. Mycelium of *Rhizoctonia* should have grown from the tissue paper food base across the partition into the compartment containing orchid seedlings which display a substantial increase in the size of the protocorms relative to uninfected controls, accompanied by the differentiation of a plumule (Fig 5). At this stage, plates may be transferred to daylight illumination at r.t.

Day 0. Infected protocorms in daylight should begin to produce chlorophyll in the developing shoot (Fig 6). Present material to the students to note the differences in size between infected seedlings and controls without the fungus, and also the differences between adjacent infected seedlings, some of which will have been parasitized by *Rhizoctonia.* In order to investigate penetration of orchids by hyphae of *Rhizoctonia,* the largest seedlings should be removed, sandwiched between pith or carrot slices, sectioned with a razor blade, and mounted in 0.1% Cotton Blue or acid Fuchsin in lactic acid. Alternatively, smaller infected protocorms may be squashed directly.

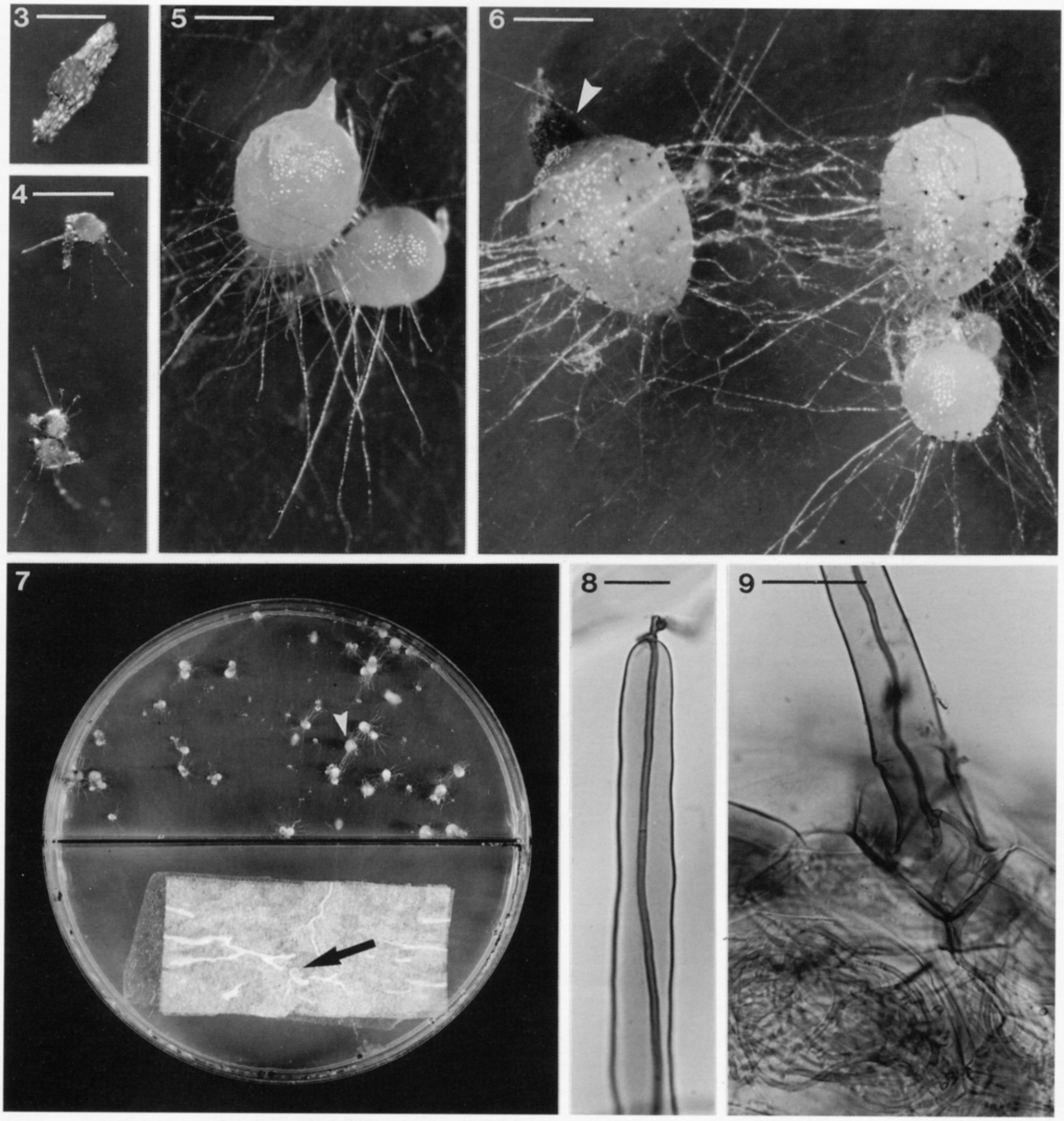

Fig 3 An ungerminated seed enclosed in its testa. Bar, 0.5 mm. Fig 4 Germinated but uninfected protocorms on a control plate (day -21). The seeds have increased in size, rupturing their testae, and have produced a few epidermal hairs. No further growth was observed. Bar, 1 mm. Fig 5 Germinated seedlings infected with *R. cerealis*, showing a great increase in size as well as differentiation and the establishment of polarity (day -21). Same scale as Fig 4. Fig 6 Well-developed protocorms, one of which has developed a green shoot tip (arrowhead) after exposure to daylight, photographed on day 0. Same scale as Fig 4. Fig 7 The split-plate photographed on day 0. Mycelium of *R. cerealis* has grown from its inoculation point on the tissue paper (arrow) across the partitioning wall, infecting germinated seedlings of *D. maculata* ssp. *ericetorum*. The seedling represented in Fig 6 is indicated by an arrowhead. Fig 8 Penetration of an epidermal hair by a hypha of *R. cerealis*. From an infected seedling (day -21) sectioned and mounted in acid Fuchsin/lactic acid. Bar, 25 μm. Fig 9 Penetration of *R. cerealis* hyphae through an epidermal hair into the root cortex of a seedling of *D. maculata* ssp. *ericetorum*. Material as Fig 8, but mounted in Cotton Blue/lactic acid. Bar, 50 μm

Useful hints

For critical research work, species of *Rhizoctonia* can be isolated from several common orchid species by surface-sterilizing root segments (about 1 cm long) in 5% (v/v) Domestos for 5-10 min, followed by several washes in sterile water and incubation on tap-water agar for several days at room temperature. Clean subcultures of growing hyphae should then be made on MEA. Alternatively, individual pelotons (see Fig 2) can be removed by dissection from infected roots, and transferred directly to MEA (Smith & Read, 1997).

Acknowledgements

We thank Mr P. M. Booth, a BMS Associate, for his support which enabled us to complete this paper.

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