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EXPLANATION OF PLATE 16

Culicidospora gravida, strain CCM F 120.

Fig. 1. The basal cell of conidium axis and the apical cell of conidiophore at the beginning of proliferation.

Fig. 2. Later stage of the process shown in fig. 1.

Fig. 3. The proliferating conidiophore tip. Note the relatively narrow area of growth. Stained with aceto-nigrosin.

Fig. 4. Conidiophore with 'rings' as the result of repeated spore formation. Stained with aceto-nigrosin.

IMPROVED PROCEDURES FOR CLEARING ROOTS AND STAINING PARASITIC AND VESICULAR-ARBUSCULAR MYCORRHIZAL FUNGI FOR RAPID ASSESSMENT OF INFECTION

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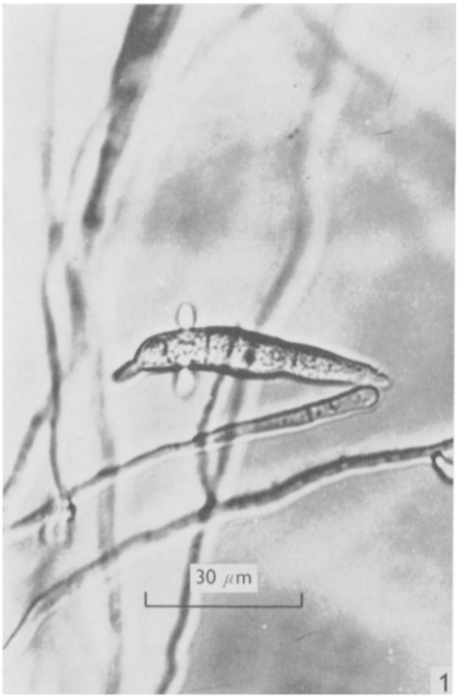
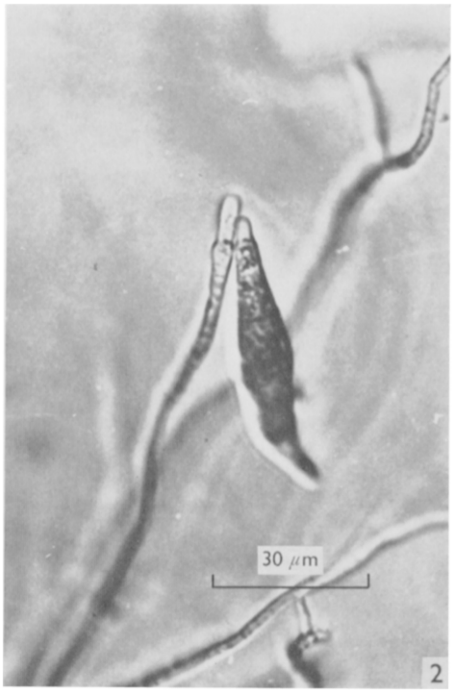
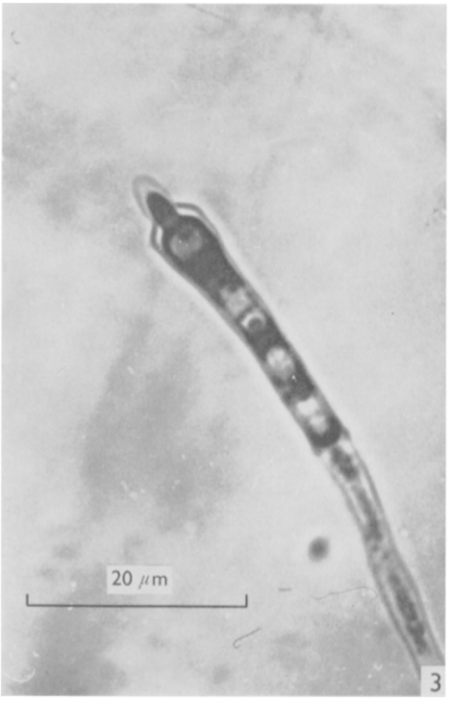
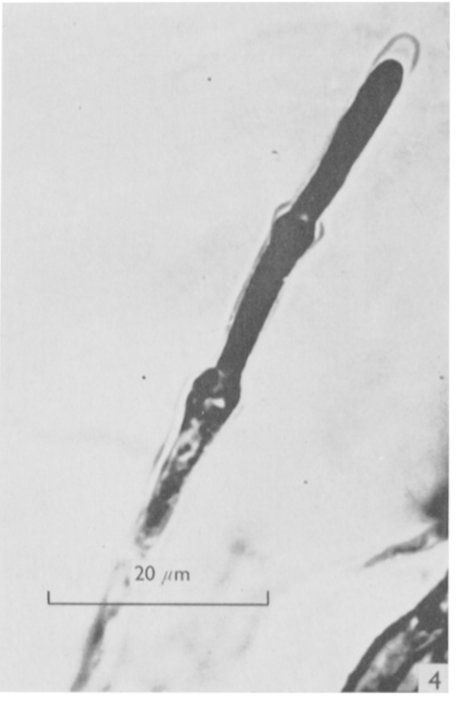
During studies on the effect of vesicular-arbuscular (VA) mycorrhiza, caused by species of *Endogone*, on plant growth, we needed to estimate the amount of mycorrhizal infection in the root cortex. Such infections in gramineous roots stain fairly clearly with standard procedures such as simmering in 0·1 % trypan blue in lactophenol (Hayman, 1970), or cotton blue and Sudan IV in lactophenol (Nicolson, 1959), and in some dicotyledonous roots by boiling for 10 min in 0·01 % acid fuchsin in saturated chloral hydrate (Gerdemann, 1955), or standing cold overnight in any of these stains. Infection in onion, our main test plant (Hayman & Mosse, 1970), is difficult to estimate using these methods because, although external hyphae and entry points are strongly stained, internal hyphae, arbuscules and vesicles are usually only well defined near the cut ends of the root segments. To improve stain penetration and clearing in whole mycorrhizal roots of onion and other host plants, and in roots infected by other fungi, we developed the following two procedures, which give deeply stained fungal structures which show distinctly against the outlines of the cells in the cortex of intact roots.

The first procedure was used successfully for non-pigmented roots such as those of onion. Root segments or whole roots, fresh or fixed in FAA (13 ml formalin, 5 ml glacial acetic acid, 200 ml 50 % ethanol), were heated at 90 °C for about 1 h in 10 % KOH. This removed the host cytoplasm and most of the nuclei, and the roots became very clear with









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the vascular cylinder distinctly visible. The roots were then rinsed in water and acidified with dilute HCl. They were stained by simmering for 5 min in 0·05 % trypan blue in lactophenol, and the excess stain removed in clear lactophenol. For roots thicker than 2 mm, or older roots of perennial plants, more than 1 h in 10 % KOH at 90° is recommended. Root segments were mounted on slides either temporarily in lactophenol or permanently in PVA (Polyvinyl alcohol resin)-lactophenol. Slight pressure on the coverslip flattened KOH-treated roots for observations within a limited range of focus. In mycorrhizal onion roots so treated the characteristic vesicles, arbuscules, longitudinally running internal hyphae and entry points of the endophyte stood out sharply against the outlines of the root cells (Pl. 17, figs. 1–6). Mycorrhizal infections also showed clearly in tomato, tobacco, sugar cane, bean, *Fuchsia* (Pl. 18, fig. 7), *Liquidambar styraciflua* and *Coprosma robusta* treated in this way, and in gramineous roots, e.g. *Nardus stricta* (Pl. 18, fig. 8). Other fungal infections showed up clearly in these roots. As the KOH treatment firstly removes the host cytoplasm and then the nuclei, the stain penetrates readily and there is no stained host cytoplasm to obscure the deep blue fungal tissues. With a dissecting microscope, many root systems were seen at a glance to be heavily mycorrhizal, usually around 80 % in inoculated onion plants grown in pots—only the root tips and a few rootlets were not mycorrhizal. Quantitative estimates of root infection were made on 1 cm segments, cut 3 and 4 cm below the onion bulb, by recording the number of segments with any infection and the amount of infection per unit length of root (Hayman & Mosse, 1970). At the stage when cytoplasm was removed but not the nuclei, nematodes present in the roots also stained clearly.

The second procedure was developed for pigmented roots. These were heated at 90° in 10 % KOH for at least 2 h, washed with fresh KOH, and immersed in an alkaline solution of hydrogen peroxide (approx. 10 vol) at 20° until bleached (10 min–1 h). They were then rinsed thoroughly in water to remove all the H_2O_2, acidified in dilute HCl and stained as already described. The time of treatment in alkaline H_2O_2 depended on the colour intensity of the root which in turn depended on its size, age and the plant species. This method also gives better contrast of fungal infections in slightly pigmented roots, such as older roots of bean (Pl. 18, fig. 11), cotton and *Liquidambar styraciflua*, than does the KOH treatment alone. With very dark roots, such as those of Sitka spruce (Pl. 18, figs. 9, 10), complete clearing and bleaching by this method is essential for root-infecting fungi to be seen, otherwise such fungi can be detected only by isolation methods. Root pigments can also be decolorized by treating with sodium hypochlorite (Bevege, 1968) or nitric acid. Nitric acid gave fairly satisfactory results but only after several hours. After bleaching with hypochlorite to the same extent as with peroxide, the fungal structures in the pigmented roots we tested stained poorly.

Our method for clearing non-pigmented roots is simpler and faster than the procedure involving KOH required for cereal embryos (Popp, 1958). For pigmented roots, alkaline hydrogen peroxide was the best bleaching

agent and made observations of stained fungal infections easy where previously they had been impossible. The methods greatly increase both the accuracy and the speed of estimating fungal infection in roots. Whole root systems can be scanned under a dissecting microscope, and fungus structures can be observed in situ at high magnifications for comparing qualitative differences (see Pl. 17, figs. 3, 6) without laborious sectioning. These methods are especially valuable for root endophytes such as *Endogone* spp. which can neither be cultured nor estimated by plating.

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EXPLANATION OF PLATES 17 AND 18

PLATE 17

Intact roots of onion infected with vesicular-arbuscular mycorrhizal fungus, cleared and stained with KOH-trypan blue treatment. The roots in figs. 1–5 were inoculated with the yellow vacuolate spore type of *Endogone* and the one in fig. 6 with the honey-coloured sessile spore type (see Mosse & Bowen, 1968).

Fig. 1. Root with external hyphae and several entry points but little internal infection. Without clearing with KOH such roots appear heavily infected as judged by the amount of external hyphae and entry points present. × 30.

Fig. 2. Entry point with appressorium (*ap*) and longitudinally running internal hyphae with arbuscules (*ar*) in the root cortex. × 105.

Fig. 3. Vesicles (*v*) and longitudinally running hyphae in root cortex. × 105.

Fig 4. Arbuscules (*ar*) and longitudinally running hyphae in root cortex. × 105.

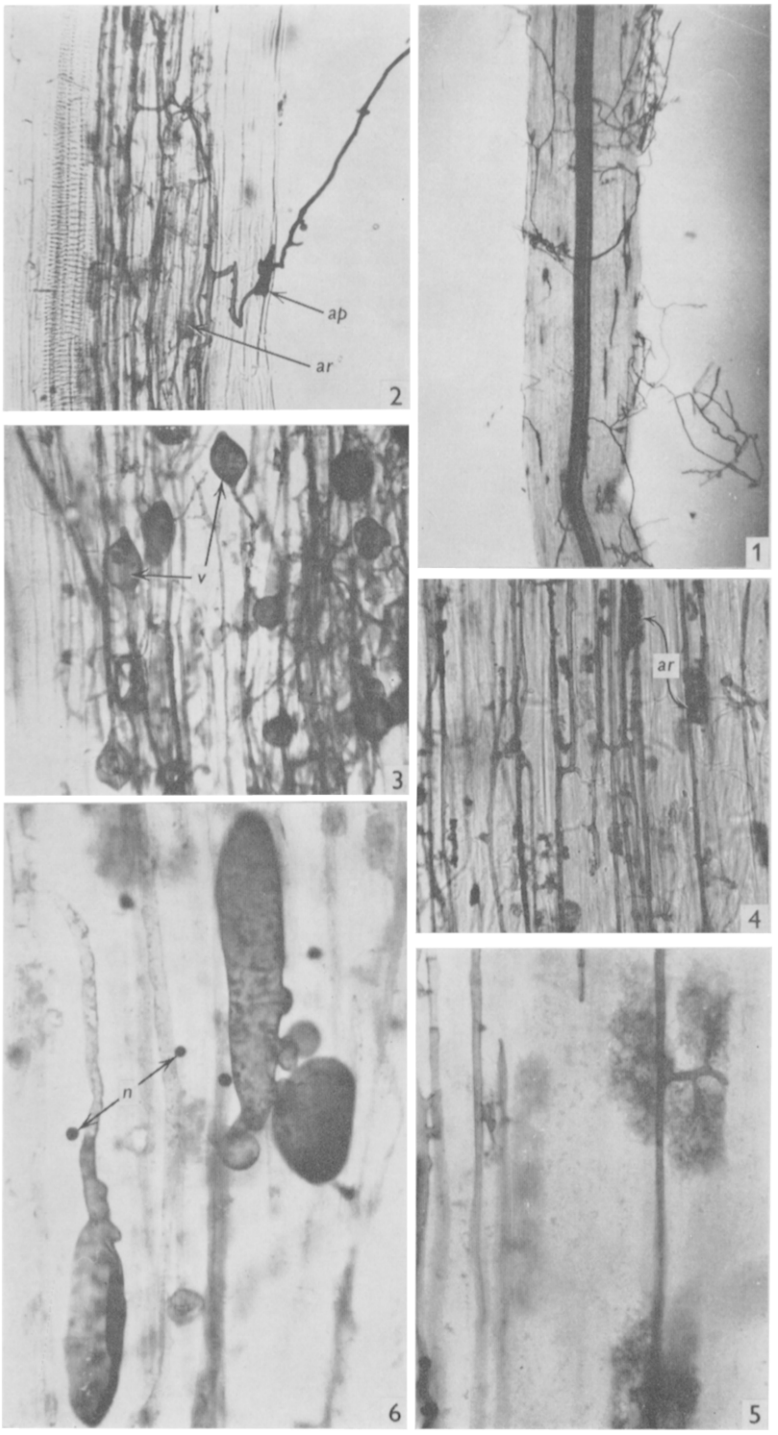
Fig. 5. Arbuscules branching off along the longitudinally running hyphae (the one on the left is at a lower plane of focus). × 320.

Fig. 6. Vesicles and longitudinally running hyphae in root cortex. The black dots (*n*) are root nuclei. Note the irregular shape and 'budding' in these vesicles produced by the honey-coloured sessile spore type compared to the lemon-shaped ones in fig. 3. × 320.

PLATE 18

Fig. 7. *Fuchsia* root inoculated with yellow vacuolate *Endogone* spore type; the dense areas are arbuscular infections. × 30.

Fig. 8. Root of *Nardus stricta* inoculated with yellow vacuolate *Endogone* spore type. Most of the cortex contains VA mycorrhiza, and attached to the roots are external hyphae, some subtending vesicles (*v*). × 30.



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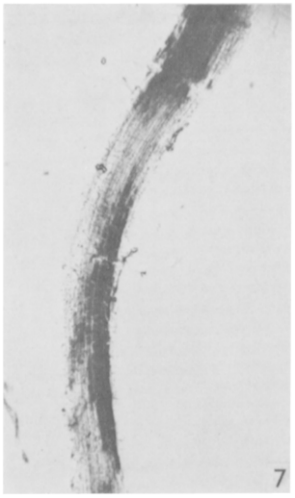

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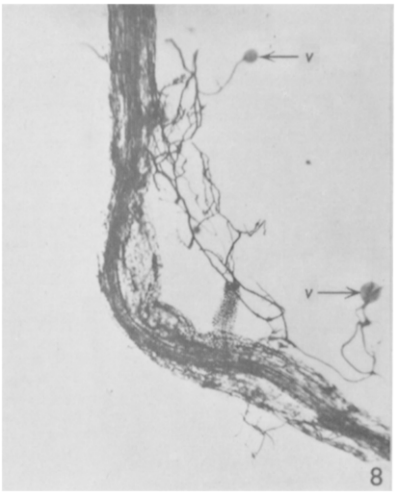

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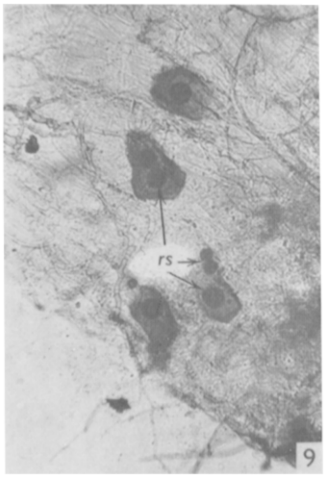

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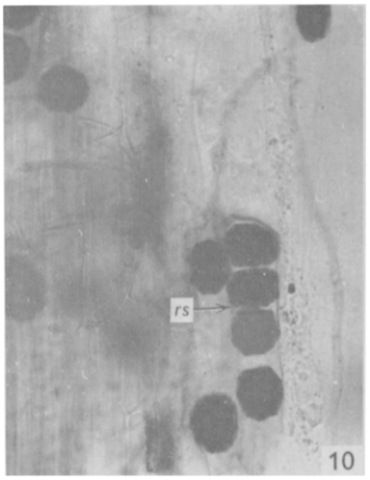

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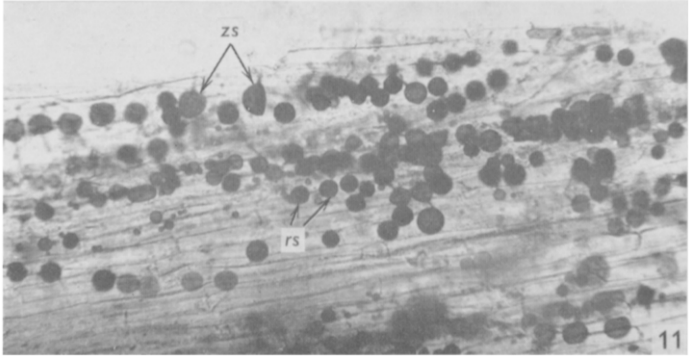

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Figs. 9, 10. Sitka spruce roots, cleared and stained with KOH-H_2O_2-trypan blue treatment, containing septate hyphae of unidentified fungus and resting spores (*rs*) of *Olpidium* sp. Fig. 9 × 105, fig. 10 × 320.

Fig. 11. Bean root cleared and stained with KOH-H_2O_2-trypan blue treatment, containing resting spores (*rs*) and empty zoosporangia (*zs*) of *Olpidium* sp. × 105.

A SELECTIVE MEDIUM FOR THE ISOLATION OF *POLYPORUS SCHWEINITZII*

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Studies of butt rot caused by *Polyporus schweinitzii* Fr. in conifer crops in Britain have failed to demonstrate that infection spreads directly by vegetative means from tree to tree (e.g. via root contacts, etc.). For some time it has seemed possible that mycelium and/or chlamydospores produced on a transient mycelial stage occurred in the soil and might act as the source of root infection. Alternatively, basidiospores produced in the autumn might remain viable in soil for a considerable period, and may be carried passively down to the surface of deeper roots where infection usually starts.

To facilitate examination of litter and soil (and wood colonized by many other organisms) for the presence of *P. schweinitzii*, an attempt was made to develop a selective medium for the growth of the fungus. Russell (1956) reported that malt agar containing 60 ppm *o*-phenyl-phenol was useful for the isolation of basidiomycetes from contaminated wood pulp, and Kuhlman & Hendrix (1962) described a medium incorporating 190 ppm PCNB and 100 ppm streptomycin for the isolation of *Fomes annosus* from wood. Neither of these media, with or without modifications, proved effective for the isolation of *P. schweinitzii* from soil.

The initial range of substances screened was largely arbitrary but included a number of antibiotics known to inhibit the growth of mould fungi and bacteria, as well as other materials used in timber preservation. In all tests, solutions of the candidate substances (over a range of concentrations) were introduced by gentle agitation into Petri dishes containing 15 ml of cooled (*c.* 45 °C) 2·5 % agar (5 % malt). The plates were inoculated with small plugs of 5 % malt agar supporting active growth of *P. schweinitzii*. The rate and type of subsequent growth were compared with those of similar inoculations on to normal malt agar. All plates were incubated at 23°. Only those substances which caused no or little suppression of growth of *P. schweinitzii* at reasonably high concentrations, with active suppression of common mould fungi, were selected for further tests.

Some of the materials showed high toxicity towards *P. schweinitzii*

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