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# Fine Structure of the Host-Fungus Interface in Orchid Mycorrhiza

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*Summary.* Electron microscopy of protocorms of *Dactylorhiza purpurella* infected with a symbiotic *Rhizoctonia* sp. showed that the intracellular hyphae examined did not penetrate the plasmalemma of the host cell. Walls of hyphae within cells bore many hemispherical protuberances over which the host plasmalemma was closely pressed. We estimate that these protuberances would increase the area of contact between hyphae and host plasmalemma by about 15%. They were not found on hyphae growing on agar. Except for these protuberances, and some vesicles or tubules which invaginated the fungus plasmalemma, no other structures were seen which could be suggested to be adaptations to transport across the living fungus-host interface.

## Introduction

Studies of orchid mycorrhiza show that the host controls the growth of the fungus through its cells and restricts it to certain regions of cortical tissue (Burgeff, 1959; Harley, 1969). Intracellular coils of branched anastomosing hyphae (pelotons) develop in such regions and are eventually lysed or digested. The living fungus may disappear from cells, although a second infection may take place. Infected host cells are apparently not damaged by the fungus. Hypertrophy of their nuclei is a characteristic feature of infection (Williamson, 1970) but nothing is known about other cytological features.

Germinated orchid seeds contain limited food reserves. Thus the translocation of carbohydrate from the substrate to the young protocorm of the orchid may be an important function of the endophytic fungus (Smith, 1967; Hadley, 1969). It is not known how nutrients are transferred from fungus to host, or whether the fungus obtains any nutrients from the host cell in return. Nor is it known whether the stimulation of growth of the orchid occurs before or after the fungus is lysed (Hadley and Williamson, 1971).

We have examined the fine structure of orchid mycorrhiza to see whether the interface between host and fungus shows any special features which might be related to transport across it or to any reaction between host and fungus. For this work we used protocorms infected

with pure cultures of a known symbiotic fungus under sterile conditions in the laboratory. Orchid roots infected in the field would have been unsuitable material because they may be infected with more than one fungus and because little is known about symbiosis in them.

## Materials and Methods

Seeds of *Dactylorhiza purpurella* (T. and T. A. Steph.) Sóo were surface sterilized and sown on Pfeffer agar containing 0.1% dextrose. Cultures were inoculated with an undescribed species of *Rhizoctonia* (Aberdeen University Botany Department isolate T) previously isolated from roots of *D. purpurella* and known to be symbiotic (Hadley, 1970). Single protocorms were observed and harvested about five days after infection. At this stage typical hyphal coils were present in many cortical cells.

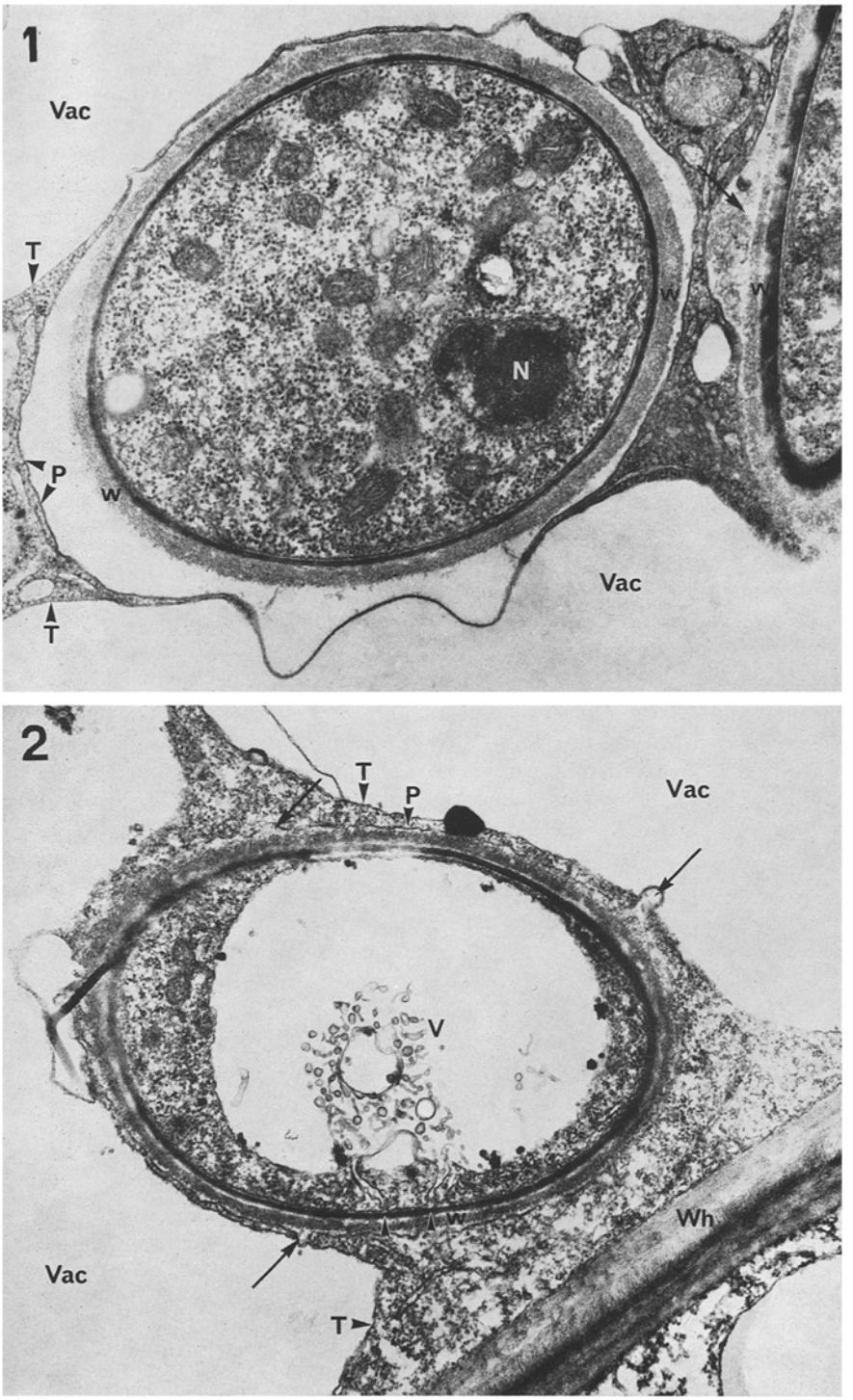
Protocorms were prepared for transmission electron microscopy in two ways; they were embedded and sectioned, or they were freeze-etched (Moor and Mühlethaler, 1963).

Specimens to be sectioned were fixed for two, or three, hours in 4% glutaraldehyde solution, washed, and post-fixed for thirty minutes in 2% $OsO_4$ solution. Fixatives and washing solution contained 0.01 M phosphate buffer at pH 6.8. Specimens were dehydrated in ethyl alcohol and passed through propylene oxide to Epikote 812 resin. Sections were stained with uranyl acetate followed by lead citrate (Venable and Coggeshall, 1965).

Specimens to be freeze-etched were either fixed for 15 minutes in 3% glutaraldehyde, then soaked in 20% glycerol solution for 90 minutes, mounted in depressions in gold discs and frozen in Freon 22 (chlorodifluoromethane) at about $-145°$C, or they were mounted on the discs and frozen in Freon 22 without pretreatment. They were stored under liquid nitrogen and then transfered to the specimen table of a Balzers BA 500R freeze-etching apparatus where they were fractured, etched for one minute at $-100°$C at a pressure of less than 0.7 mN $m^{-2}$, i.e. $5 \times 10^{-6}$ Torr, shadowed with a mixture of carbon and platinum and replicated with carbon (Moor, 1959). The replicas were cleaned by treating them with 2% cellulase solution, then with a solution containing 7% sodium hydroxide and 7% sodium hypochlorite, and then with 75% sulphuric acid. Replicas were examined, at 60 kV, in an AEI EM6B electron microscope. In this paper photographs

Fig. 1. Section through two hyphae with cytoplasm and vacuole of the host cell round them. Spaces appear between host cell plasmalemma and fungus wall. A wall protuberance, which may be the same as those on freeze-etched hyphae (see Fig. 6) is arrowed. ×25,000. Symbols used on figures: *Fw* fungus wall fractured obliquely, *if* inward exposed surface of fungus plasmalemma, *ih* inward exposed surface of host cell plasmalemma, *N* fungus nucleus, *of* outward exposed surface of fungus plasmalemma, *P* host cell plasmalemma in section, *T* host cell tonoplast in section, *Vac* host cell vacuole, *vh* inward exposed surface of host tonoplast, *w* fungus wall, *Wh* host cell wall, *X* ice crystals and eutectic mixtures

Fig. 2. Section through a hypha within a host cell to show invaginations of the fungus plasmalemma (arrowheads) and vesicles or tubules (*V*) in the fungus vacuole. Wall protuberances which may be the same as those on freeze-etched hyphae (see Fig. 6) are arrowed. ×25,000




Figs. 1 and 2

of replicas are printed so that deposited platinum appears dark. The direction along which the platinum was evaporated onto the replica is shown by an encircled arrow on each photograph.

5 μm thick sections of material embedded as for electron microscopy were stained with toluidine blue and examined under a light microscope.

A Cambridge Instrument Co. Ltd. "Stereoscan" scanning electron microscope was used to examine some hyphae grown on potato dextrose agar. Clumps of fresh hyphae were glued to specimen support-stubs with colloidal-silver paint. A thin layer of carbon, followed by one of gold-palladium alloy, was then evaporated onto them under high vacuum. The specimens were rotated about two axes during the evaporation to obtain a uniform coating.

## Observations

Young intracellular hyphae could be distinguished from old ones under the light microscope because their contents stained more densely with toluidine blue. Also, they were circular or elliptical in section whereas older hyphae were more angular and often empty. Clumps of material which contained the remains of lysed hyphae also stained densely.

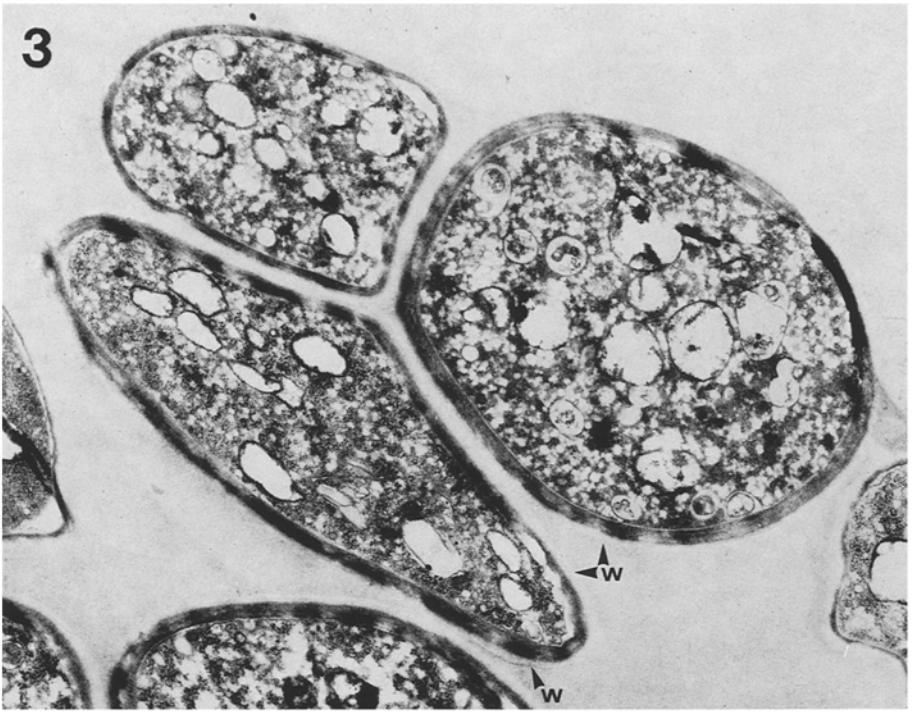
Under the electron microscope the young intracellular hyphae could be seen to contain dense cytoplasm with many ribosomes and mitochondria. A nucleus and a central vacuole were seen in some sections (Figs. 1 and 2). The plasmalemma of hyphae often appeared as a unit membrane; the tonoplast did so less often. The plasmalemma sometimes invaginated to form tubes into the cytoplasm. In one section it appeared continuous with membranes which surrounded channels right through the cytoplasm to the vacuole, where many vesicles or tubules appeared (Fig. 2, *V*).

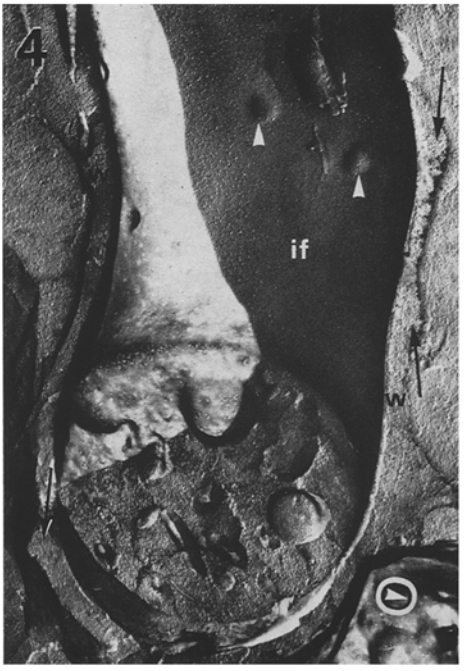
Young hyphae were surrounded by the equally dense cytoplasm of their host cells and often this host cytoplasm appeared as a very thin layer between host plasmalemma and host tonoplast (Figs. 1 and 2). Thus the vacuole of the host cell was close to the outer surface of hyphae in many places. In sections there was sometimes a space between the plasmalemma of the host cell and the surface of the fungal wall (Fig. 1), but such spaces were not seen in freeze-etched material;

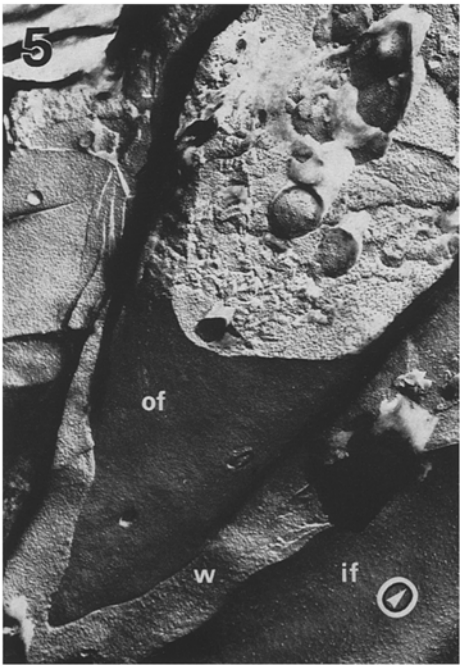
Fig. 3. Section through hyphae grown on potato dextrose agar. The outer layer of their walls has not stained. ×16,000

Fig. 4. Freeze-etched hypha within a host cell. Protuberances of the fungus wall are arrowed. Inward bulges of the fungus plasmalemma appear at arrowheads. ×14,000

Fig. 5. Freeze-etched hyphae within a host cell to show outward (*of*) and inward (*if*) exposed faces of the fungus plasmalemma. ×14,000




Figs. 3–5

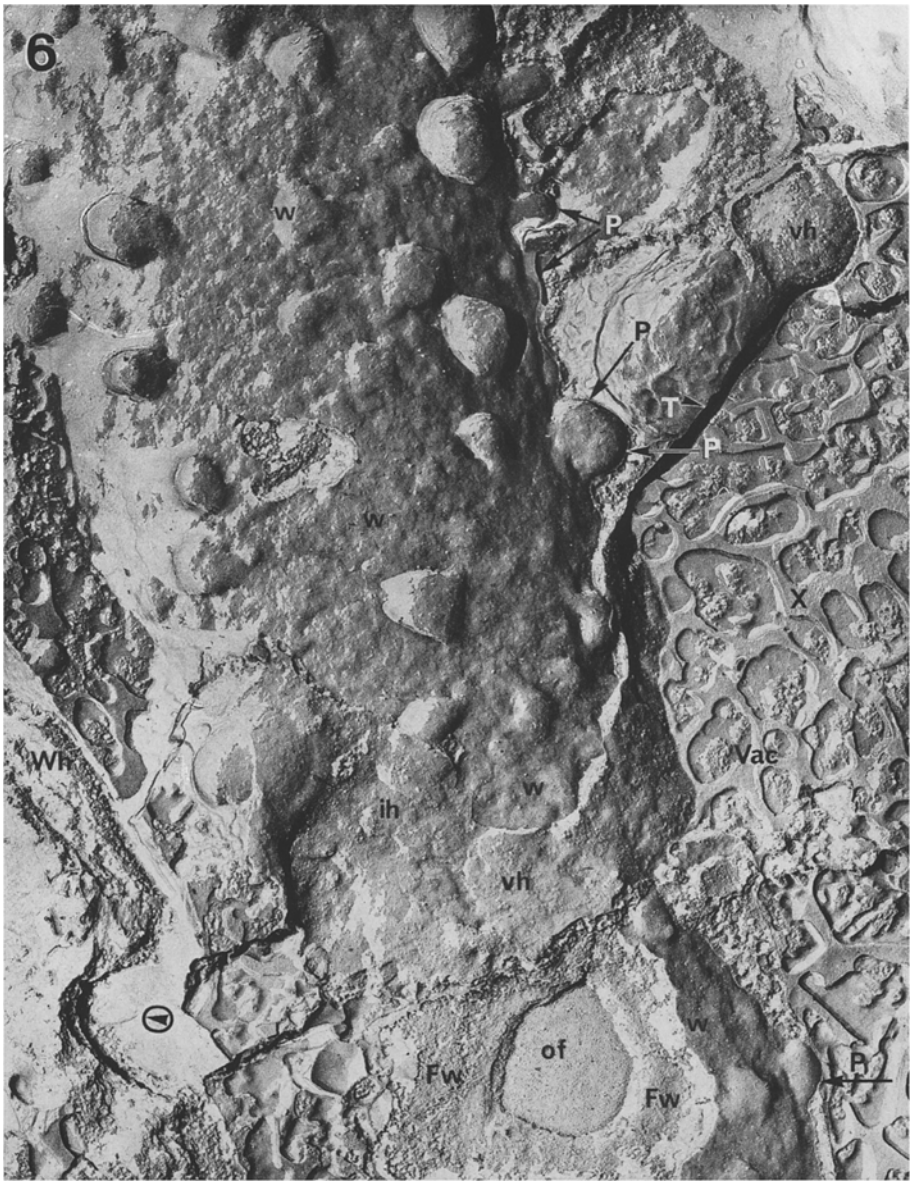



Fig. 6. A freeze-etched hypha within a host cell. The outside of the fungus wall bears protuberances. These are closely enveloped with host cell plasmalemma (*P*) at arrows. Part of the fungus wall has fractured obliquely at Fw to expose an inner layer of wall and part of the fungus plasmalemma (*of*). ×32.000

some spaces may have formed during the fixing and embedding process, or some may have contained material which did not stain to become electron-dense.

The hyphae seen in healthy host cells appeared to be outside the host cell plasmalemma and were never seen in direct contact with the cytoplasm.

In sections the walls of hyphae within host cells appeared to contain two layers, the inner layer stained more densely than the outer one (Figs. 1 and 2). The outer layer was usually the thicker, and it appeared granular.

The walls of hyphae grown on potato dextrose agar also showed two layers; the outer held little stain and could only be seen clearly as a gap between adpressed hyphae (Fig. 3). Some freeze-etched walls of hyphae within host cells appeared to contain two layers since a discontinuity could be seen at the middle of the wall where it fractured (Fig. 6, *Fw*). Others appeared to be of uniform texture throughout (Fig. 4, *w*).

The outside of the walls of intracellular hyphae seen by freeze-etching bore numerous hemispherical protuberances (Fig. 6). The plasmalemma of the host cell can be seen to be closely pressed over the surfaces of these (Fig. 6, *P*). We calculate that protuberances of the size and distribution of those shown in Fig. 6 would increase the surface area of the hyphal wall by about 15%. In cross fractures through frozen walls the protuberances appear continuous with the wall, not merely attached to it (Fig. 4, arrows). Some outward projections from the walls of hyphae seen in sections (Figs. 1 and 2, arrows) might be the same as the hemispherical protuberances seen in freeze-etched material. No protuberances were seen on sectioned or on freeze-etched hyphae of the same fungus grown on potato dextrose agar. The outer surfaces of many hyphae grown on agar were examined in a scanning electron microscope but none were seen to have protuberances.

Shallow inward bulges appear on the inside of the fungus plasmalemma where this was exposed by a suitable fracture (Fig. 4, arrowheads). But these bulges, which invaginate the plasmalemma, were too few in number to regularly correspond to protuberances on the outside of the wall. Furthermore, the bulges on the inside did not appear opposite protuberances on the outside when the walls appeared in cross-fracture (Fig. 4).

An inward surface of the fungus plasmalemma appears where the hyphal contents were fractured from it during the freeze-etching process (Figs. 4 and 5, *if*), and an outward surface appears where the hyphal wall was fractured from it (Figs. 5 and 6, *of*). Both kinds of surface bore particles. Similarly, exposed inward and outward surfaces of host cell plasma-

lemma also bore particles. But such surfaces may not be true inner or outer surfaces of membranes and the particles may be within, and not on, membranes (Branton, 1966). The several surfaces exposed bore different quantities of particles, but the significance of this variation is obscure; we do not know the range of variation over each membrane.

Masses of residual intracellular material, which were recognizable as digested or lysed hyphae under the light microscope were seen under the electron microscope to consist of completely collapsed and disorganized remnants of hyphal walls together with other unidentifiable material similar to that described by Dörr and Kollmann (1969). Host endoplasmic reticulum with densely stained globules appeared near these structures.

## Discussion

If the transfer of metabolites is a biotrophic transport process (i.e. between living organisms) then it must occur across the hyphal wall, the host plasmalemma, and perhaps other layers. The hyphae in orchid protocorms may be compared to the intracellular haustoria of obligate parasites (see Bracker, 1968) except that transfer of metabolites is principally from fungus to host. The extensive proliferation and anastomosis of hyphae to form pelotons increases their surface area within cells and, since the host plasmalemma invaginates round and closely envelops the invading hyphae, there is a large area of contact between host plasmalemma and fungus. This may facilitate transport between them.

We find only two obvious special features which might be concerned with the transfer of materials between living fungus and host cell. First, invaginations of the fungus plasmalemma occurred and in one section they appeared to be connected to complexes of vesicles in the central vacuole of a hypha (Fig. 2). But such invaginations and vesicles may be caused by fixatives. Second, protuberances occur on the outside of the walls of intracellular hyphae. These protuberances were clearly seen in preparations which had merely been frozen and freeze-etched. Therefore we think they must occur normally. They did not appear so clearly in embedded and sectioned material; they may consist of material which does not take up heavy metal stain strongly. These protuberances increase the surface area of fungus wall in contact with host cell plasmalemma. They did not appear on hyphae grown on agar in the absence of the host.

Gunning and Pate (1969) have shown that where there would otherwise be a small area of contact between two parts of transport pathways in plants then specialised cells ("transfer cells") occur which have ingrowths of wall. These ingrowths increase the area of plasma-

lemma in contact with the wall and may serve to enhance transport across it. Outward protuberances on the walls of hyphae in orchid cells might serve to enhance transport between fungus and host in the same manner.

Whether or not materials move biotrophically from living fungus to host the intracellular hyphae may eventually disorganize and collapse within the host cell. Materials from them may then be assimilated necrotrophically in the host cytoplasm and it may be that such assimilation is the principal means of nutrition of the protocorm.

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## References

Bracker, C. E.: Ultrastructure of the haustorial apparatus of *Erysiphe graminis* and its relationship to the epidermal cell of barley. Phytopathology **58**, 12–30 (1968).

Branton, D.: Fracture faces of frozen membranes. Proc. nat. Acad. Sci. (Wash.) **55**, 1048–1056 (1966).

Burgeff, H.: Mycorrhiza of orchids. The orchids; a scientific survey, p. 361–395, ed. C. Withner. New York: Ronald Press 1959.

Dörr, I., Kollmann, R.: Fine structure of mycorrhiza in *Neottia nidus-avis* (L.) L. C. Rich. (Orchidaceae). Planta (Berl.) **89**, 372–375 (1969).

Gunning, B. E. S., Pate, J. S.: "Transfer cells". Plant cells with wall ingrowths, specialised in relation to short distance transport of solutes—their occurrence, structure, and development. Protoplasma **68**, 107–133 (1969).

Hadley, G.: Cellulose as a carbon source for orchid mycorrhiza. New Phytol. **68**, 933–939 (1969).

— Non-specificity of symbiotic infection in orchid mycorrhiza. New Phytol. **69**, 1015–1023 (1970).

— Williamson, B.: Analysis of the post-infection growth stimulus in orchid mycorrhiza. New Phytol. **70**, 445–456 (1971).

Harley, J. L.: The biology of mycorrhiza, 2nd ed. London: Leonard Hill 1969.

Moor, H.: Platin-Kohle-Abdruck-Technik angewandt auf den Feinbau der Milchröhren. J. Ultrastruct. Res. **2**, 392–422 (1959).

— Mühlethaler, K.: Fine structure in frozen-etched yeast cells. J. Cell Biol. **17**, 609–629 (1963).

Smith, S. E.: Carbohydrate translocation in orchid mycorrhizas. New Phytol. **66**, 371–378 (1967).

Venable, J. H., Coggeshall, R.: A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. **25**, 407–408 (1965).

Williamson, B.: Induced DNA synthesis in orchid mycorrhiza. Planta (Berl.) **92**, 347–354 (1970).

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