SOME OBSERVATIONS WITH LIGHT AND ELECTRON MICROSCOPE ON THE ENDOTROPHIC MYCORRHIZA OF ORCHIDS

P. J. NIEUWDORP

Laboratorium voor Microbiologie, Technische Hogeschool, Delft

SUMMARY

The endotrophic mycorrhiza of four heterotrophic and four autotrophic orchid species have been investigated by light and electron microscopy. Contrary to what has been found in some other plants, the cell wall of the cortex cells of the orchids lacks lignin which might preclude the invasion of fungi. Cellulose and pectin are attacked by the mycorrhizal fungi; these were all found to produce cellulase and pectinase. Within the cells of the plant, the hyphae of the fungus were found always to be enveloped within the invaginated plasmalemma which continues forming pectin and cellulose and, in the case of exodermal cells, may also form suberin. These pectin and cellulose layers are rather thin or even seem to be lacking in cells with young and active mycorrhizal fungi; on the other hand they are clearly visible in cells with decaying hyphae. It may be inferred that the active fungus digests pectin and cellulose about as fast as they are formed by the plant cell plasmalemma.

Decaying hyphae and the pectin and cellulose layer around them are usually found in the central part of the fungus-digesting cells and form a clot, which is also enveloped by a pectin-cellulose layer. During the process of digestion the plasmalemma shows pinocytosis.

INTRODUCTION

For their supply of nutrients many orchids depend to a greater or lesser degree on a mycorrhizal fungus, part of which lives within the plant's roots or in its rhizome. The other part lives in the soil where it feeds on decaying materials, which provide the fungus, and ultimately the orchid, with the nutrients needed (BURGEFF 1909, 1936; FUCHS 1924; HOLLAENDER 1932; HADLEY 1969; HADLEY & WILLIAMSON 1971).

The nutrients which the fungus passes on to the plant always comprise inorganic salts, but often also organic compounds. Some species of orchid lack chlorophyll altogether and are holosaprophytic, e.g. *Limodorum* and *Neottia* (*Neottia* has a little chlorophyll-*a* but cannot assimilate CO_2). Such plants appear in the flowering season and thereafter remain visible by their fruit-bearing stalks. Other species have a little chlorophyll enabling them to assimilate some CO_2, e.g. *Corallorhiza*. Most orchids, however, have green leaves, e.g. *Cymbidium*, *Epipactis* and *Goodyera*; some of these, e.g. *Cymbidium* and *Epipactis*, are also able to live – at least for some time – entirely heterotrophically. When aseptically raised plants of *Cymbidium* – some with mycorrhiza and the others without – are kept in the dark, those possessing a mycorrhiza may develop further (BURGEFF 1936). A few *Epipactis* plants were found to lack

chlorophyll and thus to live as holosaprophytes; however, these plants did not bear seeds (RENNER 1938; BURGEFF 1954).

Orchid seeds, reputedly very small, contain few reserves. After germination the first, often subterranean, state is a so-called protocorm which cannot develop into a plant unless it is invaded by a fungus. In due time the fungus is digested by the host. Likewise the full-grown orchid plants profit by the digestion of the mycorrhizal fungus. This has been the object of many investigations (for a review see BURGEFF 1959).

One finds the mycorrhizal fungus in the cortex of the roots and of the rhizome. In most species the fungus enters the roots through the root hairs. Usually the outer two layers of cortical cells are free of fungus, and so are the endodermal cells and the cells just outside the endodermis. Growth in the inner layers is assumed to be prevented by a protecting agent (GÄUMANN et al. 1950, 1959, 1960a, 1960b).

The investigation reported here aimed at explaining why and how the mycorrhizal fungus grows into the roots. The electron microscope enabled us to observe some details of the process of digestion of the fungus inside the cortical cells.

2. MATERIALS

2.1. Orchids

Heterotrophic species:

Neottia nidus-avis (L.) L. C. Richard collected in the Jura (France).

Limodorum abortivum (L.) Swartz collected near Le Rozier-Peyreleau (Tarn, France).

Corallorhiza trifida Châtel. collected in the Dolomites (Italy), from litter overgrown with moss in a spruce-fir wood.

Corallorhiza maculata Raf. collected by Professor Dr. B. J. D. Meeuse, near Seattle, Wash., under Douglas firs.

Autotrophic species:

Cymbidium hybr. (probably *lowianum* × *tracyanum*), gift of Mr. Maarse, Aalsmeer, the Netherlands.

Epipactis helleborine (L.) Crantz (= *E. latifolia*) collected in Meyendel, Wassenaar, the Netherlands, from dry sandy soil.

Goodyera repens (L.) R. Br. collected near the river Tarn, France.

Listera cordata (L.) R. Br. collected in the Jura (France), from litter overgrown with moss in a fir wood.

2.2. Fungi

Rhizoctonia spec. CBS 363.63, an isolate from *Cymbidium* roots, received from Centraalbureau voor Schimmelcultures, Baarn. Strains isolated from the orchids mentioned above. Three strains, which are probably identical, were isolated from the roots or the rhizome of each species. Identity of fungal strains isolated from different orchids cannot be excluded.

3. METHODS

3.1. Isolation of the fungus

Mycorrhizal fungus-containing roots of orchids about to flower were thoroughly cleaned, superficially sterilized with alcohol, and flushed with sterile water. Longitudinal sections cut with a sterile knife were slipped under an agar layer in a Petri dish. The agar contained: K_2HPO_4, 0.3 g; KH_2PO_4, 0.7 g; $CaCl_2$, 0.1 g; NaCl, 0.1 g; $MgSO_4.7H_2O$, 0.3 g; $FeSO_4.7H_2O$, 0.05 g; $(NH_4)_2SO_4$, 0.5 g; agar, 15 g; starch, 3 g; H_2O, one litre. Incubation at 25 °C. After c. two weeks fungi grew into the agar; bacteria grew around the sections but did not spread as fast as the fungi. Thus, the fungi could be isolated and subcultured on malt agar. From each of the orchids used, 3 isolations were made.

3.2. Testing of isolates for capacity to act as mycorrhizal fungi

The isolates from *Cymbidium* were tested for the ability to act as mycorrhizal fungi. *Cymbidium* seeds were superficially sterilized with a c. 6.5% Ca hypochlorite solution for 10 min and after washing with sterile water placed on agar in an Erlenmeyer. The agar was prepared as follows (BURGEFF 1936): Solution A. $Ca(NO_3)_2$, 1 g; $(NH_4)_2SO_4$, 0.25 g; $MgSO_4.7H_2O$, 0.25 g; $FeSO_4.$ $7H_2O$, 0.02g; 500 ml H_2O. Solution B. K_2HPO_4, 0,25 g; KH_2PO_4, 0.25 g; 500 ml H_2O.

These solutions were mixed; 15 g agar and 20 g saccharose added; pH adjusted to 5,1 – 5,2; and sterilized. Incubation at 21 °C; illumination for 12 hr per day with TL-tubes 30 cm from the agar. From time to time sterile deionized water had to be added.

It took about 4 months to obtain dark-green protocorms with a diameter of a few mm (the diameter varied considerably). Some were transferred to culture tubes with the same medium, except that saccharose was replaced by 0.5% starch. A few weeks later the isolated fungus to be tested was inoculated on the agar near the protocorm. It soon appeared that the fungi developed too fast on this medium. Therefore another medium and another sequence of inoculation were tried. The sterile tubes now contained an agar with less nitrogen (BURGEFF 1936): Solution A. $CaCl_2$ sic., 0.1 g; $MgSO_4$, 0.3 g; NaCl, 0.1 g; $FeSO_4.7H_2O$, 0.01 g; agar, 15 g; H_2O, 500 ml. Solution B. KH_2PO_4, 1 g; starch, 5 g; Na nucleinate, 0.5 g; H_2O, 500 ml. These solutions were mixed and sterilized. The tubes were inoculated with the fungus, and after a fungal mat had developed, a protocorm was placed on the mat. The lower part of the culture tubes had to be screened from illumination as the mycorrhiza fails to develop in the light.

3.3. Electron microscopy

Longitudinal sections sufficiently thick to contain one layer of intact cells were fixed in 3–5% glutaraldehyde in veronal acetate buffer pH 6.8 at 4 °C for one night, washed in the buffer solution, then fixed again in buffered 1% OsO_4 at room temperature for a few hours. After washing and dehydration via alcohol and propylene these sections were embedded in Epon. Thin-sec-

tioning was followed by staining with Pb citrate (after REYNOLDS 1963) and U acetate, if necessary.

3.4. Test for cellulose

Light microscopic preparations were used for tests for the presence of cellulose, which has been reported by MAGNUS (1900) and BURGEFF (1959) to be a major component of the outer layer of the "clot", a layer which can only be a product of the plasmalemma of the plant cell. The preparations had to be treated for a long time with Eau de Javelle before being treated with zinc chloroiodine as otherwise the cellulose would not be stained.

3.5 Tests for production of cellulase and pectinase

The fungus to be tested was grown for 21 days at 27°C. One litre of medium contained: K_2HPO_4, 0.3 g; KH_2PO_4, 0.7 g; $CaCl_2$, 0.1 g; NaCl, 0.1 g; $MgSO_4.7H_2O$, 0.3 g; $FeSO_4.7H_2O$, 0.05 g; $(NH_4)_2SO_4$, 0.5 g; yeast water 1 ml, and either 1% carboxymethylcellulose (CMC) or 0.5% Na pectate.

At the end of this period the mycelium was spun down. The supernatant of the culture with CMC could be used directly for an estimation of the activity displayed by the cellulase during the growth period by comparing the viscosity of a blank (medium not inoculated) with that of the supernatant of the fungal culture. Viscosity was measured in an Oswald viscosimeter.

The supernatant of the culture with Na pectate could not be used in this way as Na pectate is hydrolysed by sterilization of the medium, so neither the blank nor the supernatant would show the viscosity of a Na pectate solution. Therefore 0.5% Na pectate was added to the supernatant, and some toluene to prevent growth of microorganisms. The mixture was incubated for 4 hrs at 27°C. Viscosity was measured before and after incubation.

4. RESULTS

4.1. Description of the symbiotic fungus-containing roots and rhizomes of the species studied

Heterotrophic species:
Neottia nidus-avis has short (2–8 cm) and comparatively thick roots. As in other orchids, the rhizodermis is composed of two layers, the outer of which is called "epidermis" and the inner either "hypodermis" or sometimes "exodermis". In *Neottia* the epidermis does not form root hairs through which the mycorrhizal fungus might enter the root; instead, the fungus invades the root from the rhizome, spreading from the base of the root to the tip. The connexion of the mycorrhizal part of the fungus with the soil is via the oldest part of the rhizome. The roots from this part are already decaying. The spreading of the fungus through the root takes place in the third layer of cortical cells, reckoned from the hypodermis; the cells of this layer are called "host cells". From the host cells the fungus invades other cortical cells and in these, rather than in the host cells, it ramifies and forms loops. Later on, the fungus is digested by the

orchid plant cells and for this reason these cortical cells are called "fungus-digesting cells". Before the invasion by the fungus the cortical cells contained much starch, but thereafter only the uninvaded cortical cells, especially the layer closest to the endodermis, are full of starch grains. In the end, the host cells also digest the fungus. In general, this description applies also to the other orchid species described in this paper.

Limodorum abortivum has longer roots. The epidermal cells may form root hairs through which the mycorrhizal fungus may enter the root. These epidermal cells are short-lived; the exodermal cells take over their function; they have also thick walls, but lack layers of suberin. A few layers of small cortical cells directly under the exodermis act as host cells; the fungus-digesting cells and the starch-containing cells are larger. The clamp-connexions on the fungal hyphae show that the fungus is a hymenomycete. As the fungus enters the roots through the root hairs, some parts of the roots may remain free of fungus; instead these parts contain starch.

Corallorhiza trifida from Europe and *C.maculata* from N. America have no roots, but only a rhizome bearing papillae which are outgrowths of the cortex. The epidermal cells of these papillae form hairs which function as root hairs, and which may also be invaded by mycorrhizal fungi. The fungus spreads through an outer small-celled cortical layer, as in *Limodorum*.

Autotrophic species:
Cymbidium hybr. roots, though growing in soil and not like those of epiphytic orchids in air, possess a velamen. The cell walls of the velamen cells lignify, whereafter the protoplasts in these cells die off and are replaced by air. The exodermal cells, directly under the velamen, now form suberin layers on the inside of the cell wall. It is clear that the mycorrhizal fungus can only enter the young tips of the roots.

Epipactis helleborine has long straight roots with root hairs which can be invaded by the fungus. Still, the greatest development of the mycorrhiza is found in darkly coloured parts of last year's roots.

Goodyera repens symbiosis with mycorrhiza has been studied by DOWNIE (1940, 1943) and by MOLLISON (1943). The fungus enters the plant through hairs on the rhizome from where it invades the roots.

Listera cordata has a long rhizome from which several stems rise The thin roots have an epidermis with root hairs and the fungus accordingly is not found throughout the root, but only in limited areas. Fungus-digesting cells occur in two cell layers.

4.2. Capacity of the isolated fungi to act as mycorrhizal fungus.

The results of experiments are presented in *table 1*.

Table 1 shows that protocorms could not develop on starch agar if no mycorrhizal fungus was present. All young plants had mycorrhiza; this proves that our isolates A, B and C may well be, and most probably are, mycorrhizal fungi.

Table 1. Development of protocorms of *Cymbidium* spec. which were sterile when they were placed on starch agar inoculated 5 days earlier with one of the new mycorrhizal fungus isolates (strain A, B or C), or with *Rhizoctonia* spec. CBS 363.63, or not inoculated with a fungus.

Strains	Number of experiments	After 3 months		After 6 months		After 12 months		Further development[1]
		Number of living protocorms	Number of living young plants	Number of living protocorms	Number of living young plants	Number of living protocorms	Number of living young plants	
A	20	20	0	15	3	11	4	1 well devel.
B	20	20	0	16	1	15	1	
C	20	19	0	16	1	13	2	
363.63	20	20	0	14	6	12	7	4 well devel.
No fungus	20	17	0	13	0	0	0	

[1]) Further development may have been hampered by deficiency of the medium.

Roots which had grown into the agar had root hairs all around, but those creeping over the agar had no root hairs where exposed to air.

4.3. Production of cellulase and pectinase

All of our isolates produced cellulase and pectinase, as did the symbiotic fungus *Rhizoctonia* spec. CBS 363.63. In the viscosity measurements with the latter fungus, the outflow of the supernatant of the CMC culture took 20.5 sec, that of the blank 7 min 14.5 sec. The outflow of the mixture of the supernatant of the Na pectate culture, and 0.5% Na pectate, took 30.6 min before, and 15.7 min after 4 hr incubation.

4.4 Penetration of the root cells by the mycorrhizal fungus

The hyphae of the mycorrhizal fungus penetrate the plant cell by perforating the cell wall (*Figs.2–4*) but they never enter the cytoplasm as the plasmalemma recedes before the growing hyphae *(figs.4–6)*. At the same time it proceeds with its normal activity: the synthesis of the cell wall components pectin and cellulose. Around young hyphae with an active protoplast, however, a cellulose layer is rarely found: the cellulase and pectinase of the fungus may have attacked the products of the plasmalemma enabling the fungus to digest them, and also enabling it to grow by forming loops (pelotons) filling quite a large part of the cell.

When the fungus gets older the reverse process sets in: the plant now attacks the fungus. Pinocytosis is seen along the plasmalemma, both where it envelopes the hypha *(fig. 8)* and where it lines the cortical cell wall *(fig. 9)*. The fungal protoplast disappears *(figs.7, 8, 10)* and now the hyphae are seen to become enveloped by a cellulose slime layer which is continuous with the original plant

cell wall, and sometimes also by a layer of suberin which may be recognized by its lamellar structure (*Cymbidium, fig. 5*). Gradually the hyphae become more and more flattened, the contents disappear (especially in *Epipactis* and *Good-yera* the protoplast was found to have disappeared completely) and only cell walls are left. The fungus forms glycogen and lipid as a reserve material *(fig. 1)*. Both are transportable in the protoplast. A so-called clot *(fig. 2)* is formed out of the cell walls of several disintegrated hyphae and (all around the latter) a common cellulose envelope *(figs. 13, 14, 15)*. The cell walls of the hyphae may be rather thick *(fig. 11*; still thicker in *Listera*) and of a loose fibrillar structure (reserve material?).The digestion by the plant cell of the hyphal walls is a slow process *(figs. 10, 12, 16*; in the latter figure the hyphal wall is seen to disintegra-te).

DISCUSSION

The cell wall of the cortical cells of the orchids examined was found not to contain lignin; neither is lignin formed by these cells if a fungus has invaded neighbouring cells or intracellular spaces. Many other plants make lignin under these conditions, see GRIFFITHS & ISAAC 1966. Therefore, a fungus which pro-duces cellulase and pectinase can perforate the cell wall. The fact that all of our fungal isolates produce both these enzymes shows that these isolates may be mycorrhizal fungi. More evidence for their actually being mycorrhizal fungi was provided by our finding that three of our strains, brought together with the plant roots, proved capable of forming a mycorrhiza.

The cortical cells of the orchids, when invaded by the mycorrhizal fungus, do not die; the hyphae are enveloped by the plasmalemma. The same reaction has been found in the root-nodule cells of the soy bean when they are invaded by symbiotic bacteria (GOODCHILD & BERGERSEN 1966).

We have not found any indication as to why the fungus at a certain moment loses its vitality and is digested by the plant cell. DÖR & KOLLMANN (1969) described a well developed endoplasmatic reticulum in the cortical cells which might produce fungus- digesting enzymes, but we have not been able to corro-borate this finding.

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Abbreviations used in the figures.

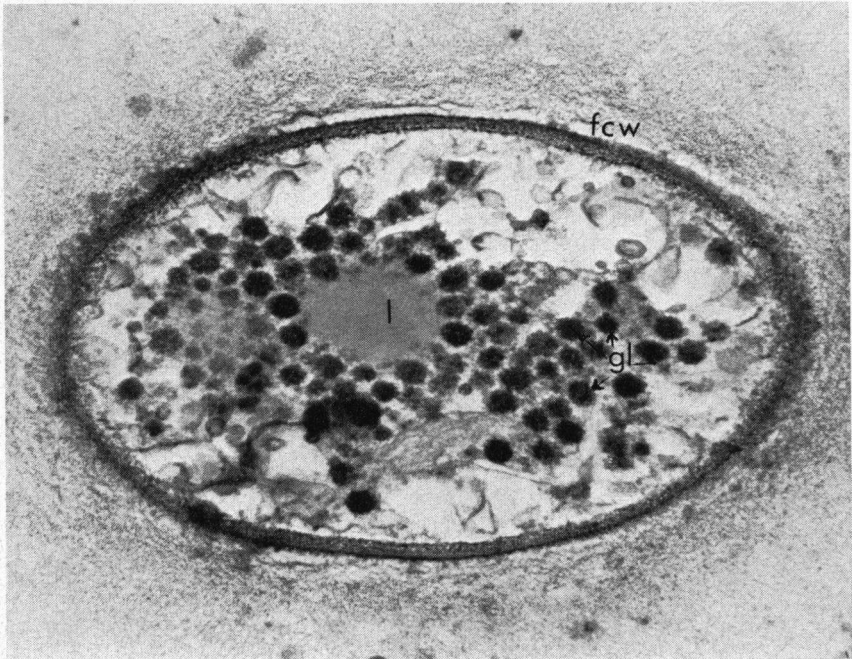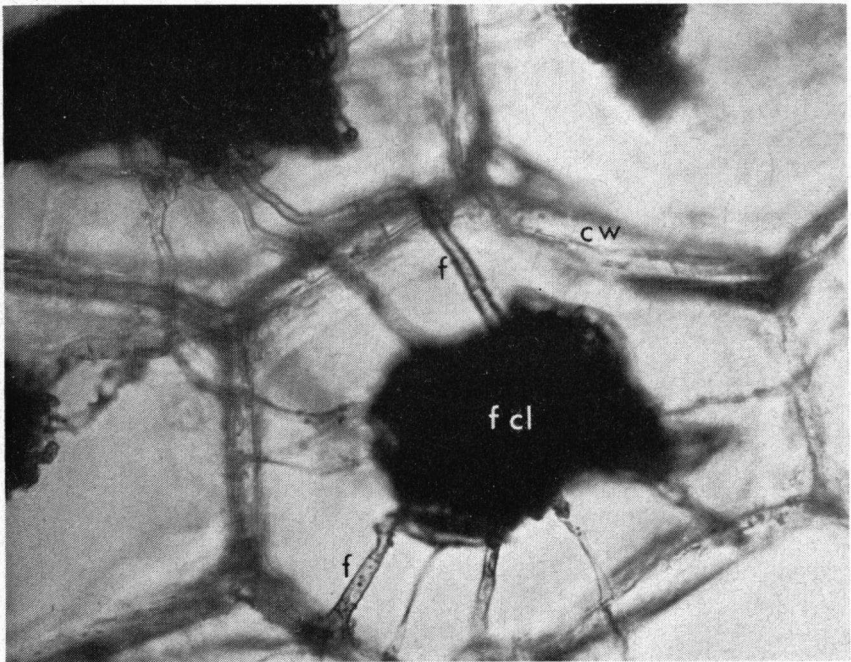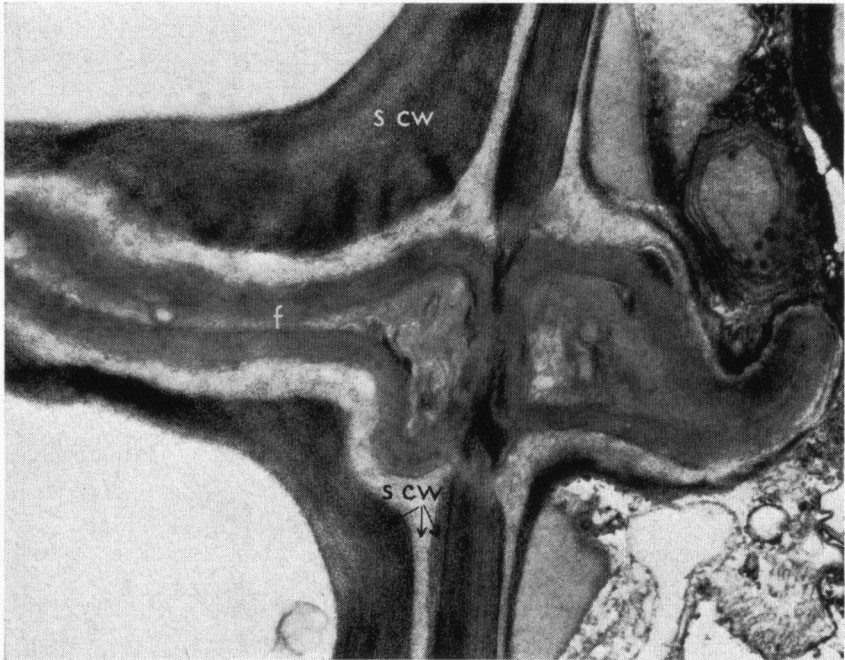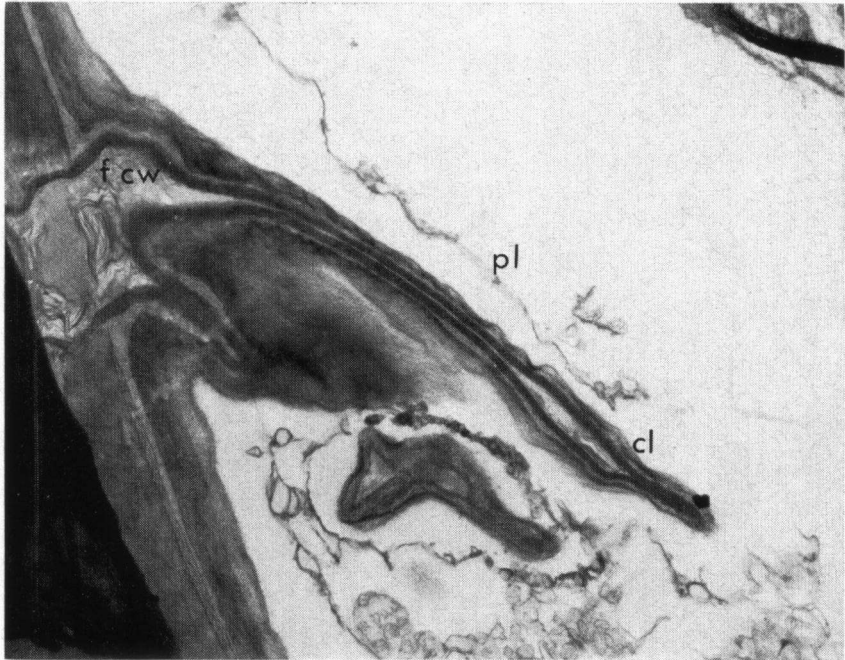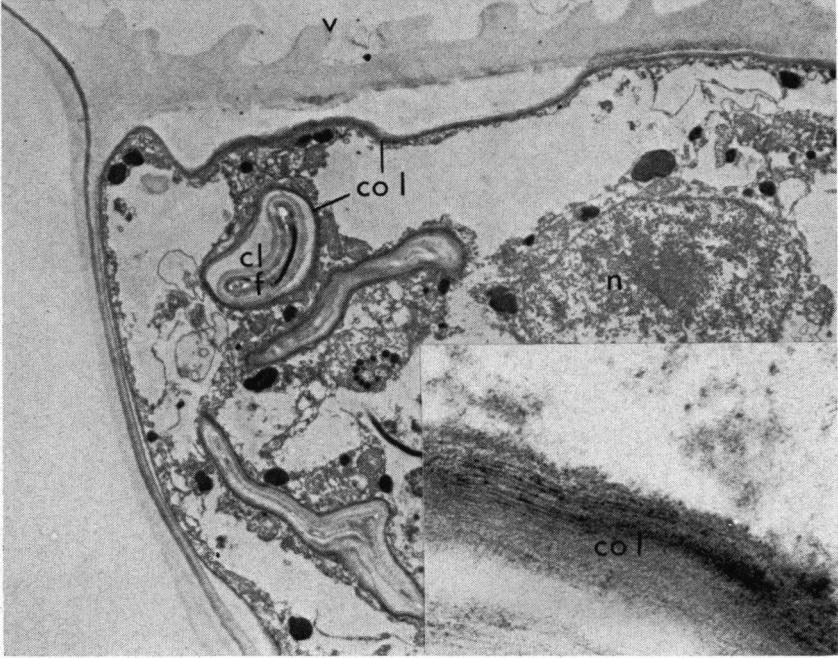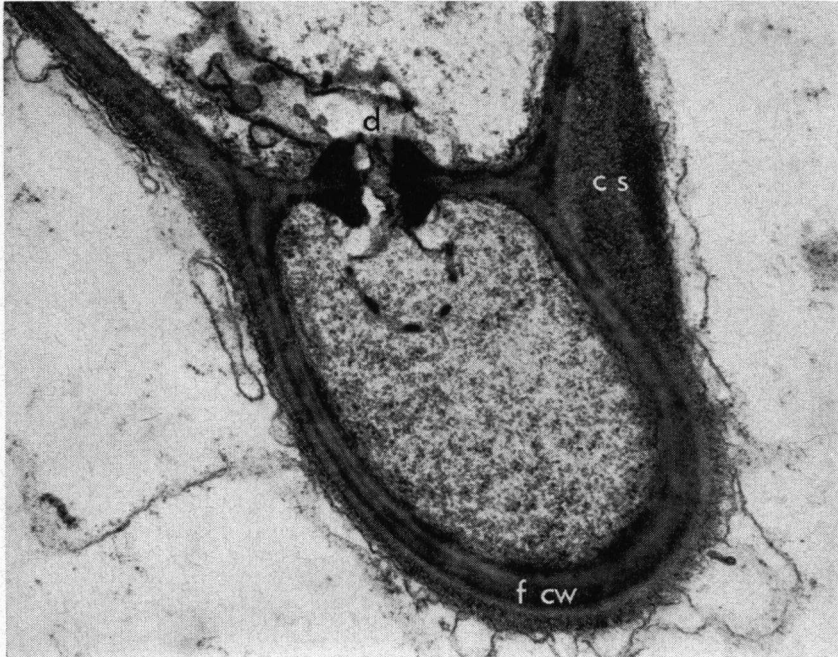|			
c l	cellulose layer	i	intercellular space
co l	cork layer	l	lipid
c s	cellulose slime	n	nucleus
c w	cell wall (of cortex cell)	pl	plasmalemma
d	dolichopore	p r	protoplasma remains
f	fungus	p v	pinocyt vesicle
f cl	fungus clot	s cw	secondary cell wall
f c w	fungus cell wall	t	tonoplast
f c w r	fungus cell wall remains	v	velamen
gl	glycogen		

LIST OF FIGURES

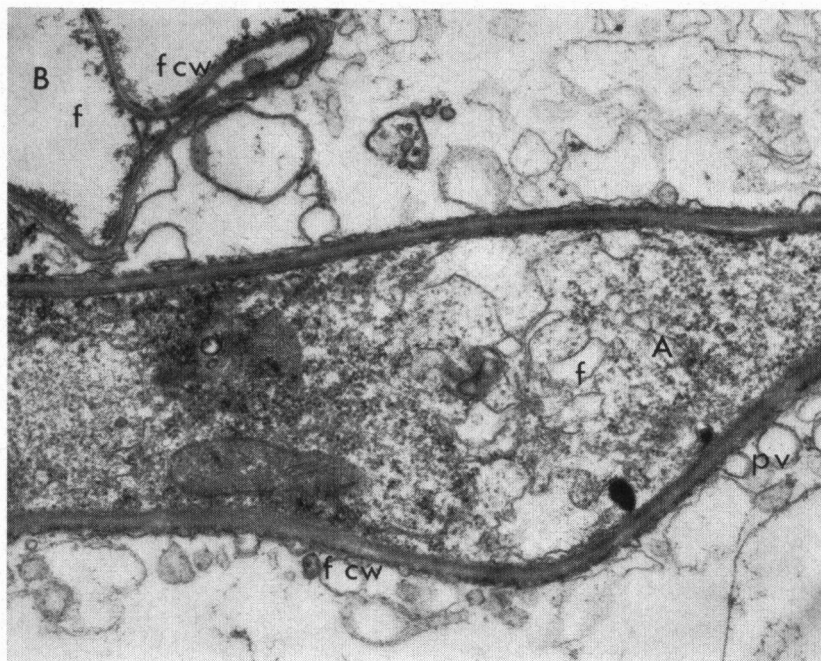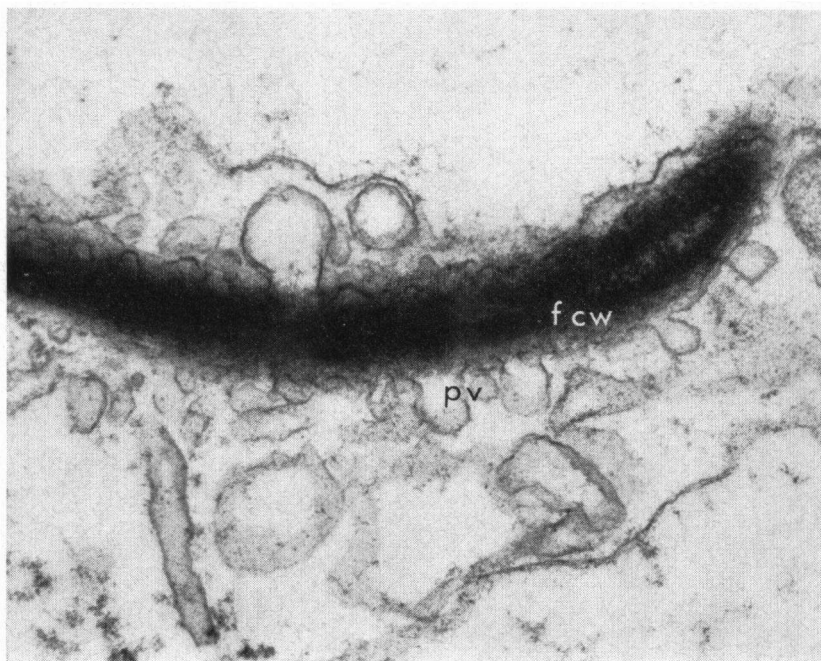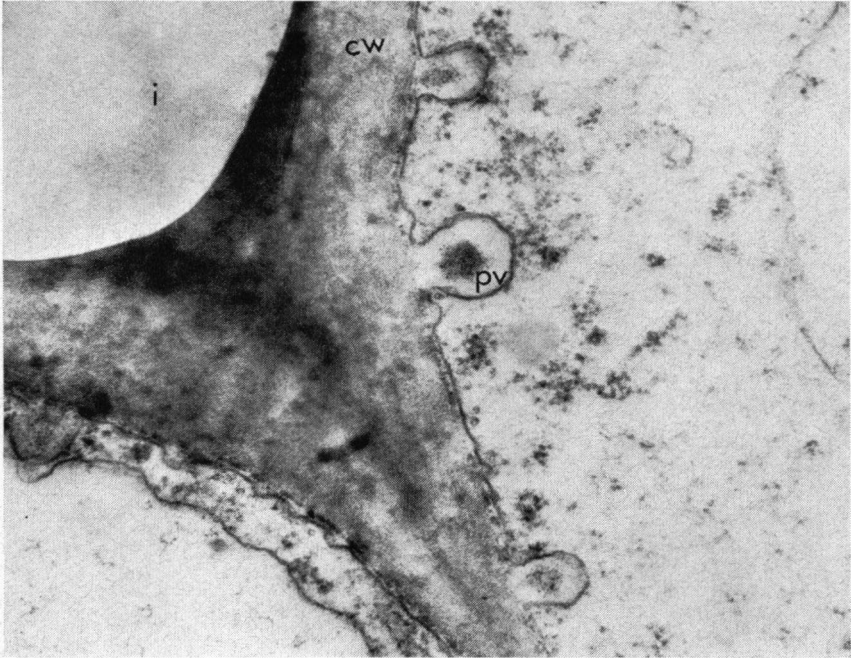Fig. 1

Fig. 2

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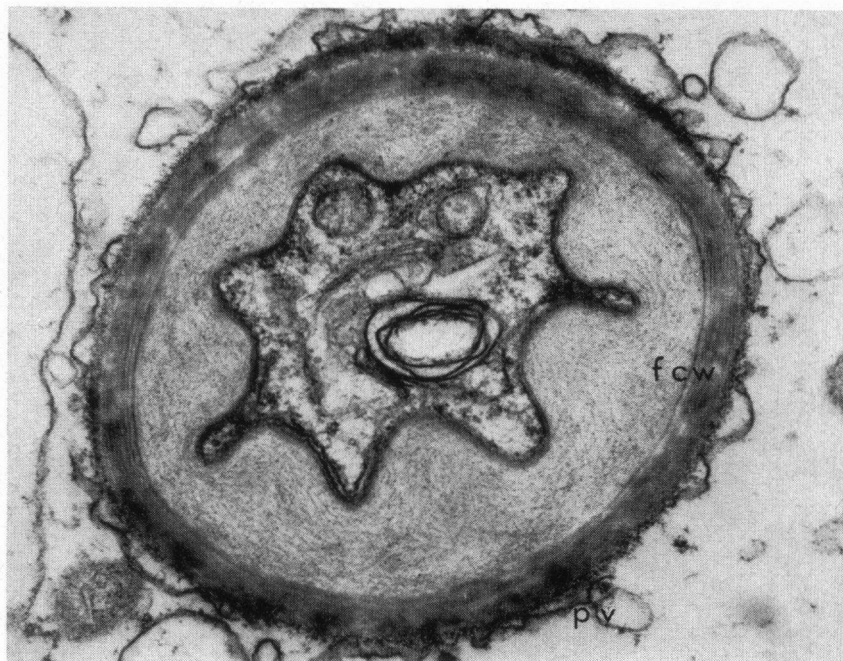Fig. 4

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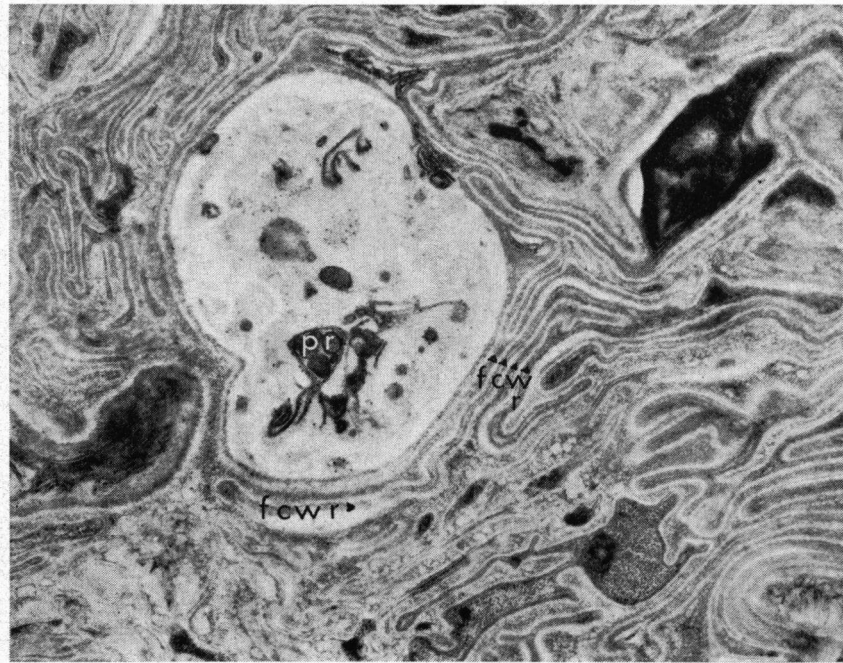Fig. 6

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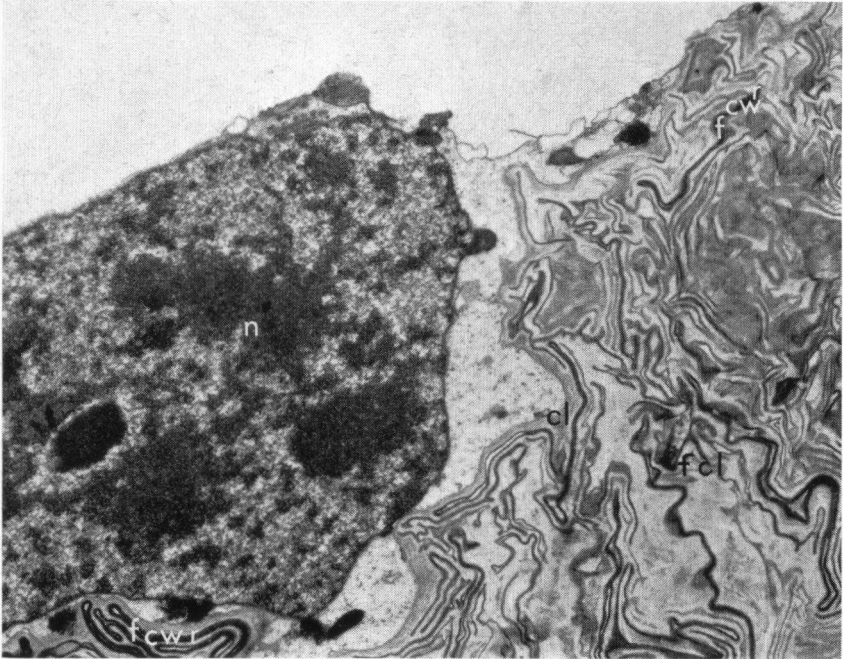Fig. 14

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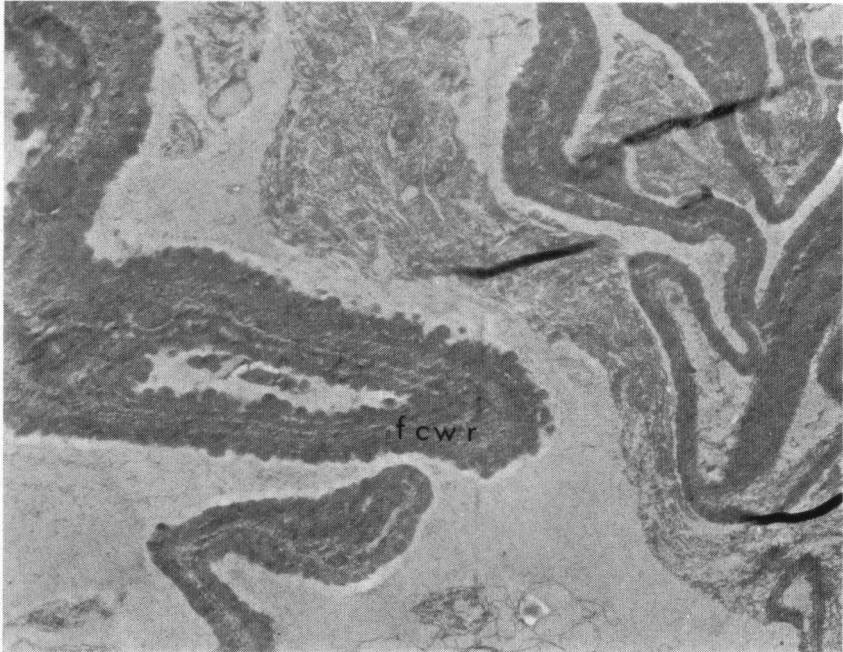

Fig. 16