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An intact microtubule cytoskeleton is not necessary for interfacial matrix formation in orchid protocorm mycorrhizas

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Abstract This paper reports the changes that occur in the microtubule cytoskeleton of cells of orchid protocorms during infection by a compatible mycorrhizal fungus. In cells of protocorms uninfected by a mycorrhizal fungus, microtubules occurred in regular arrays. In contrast, the cells of orchid protocorms with established mycorrhizas appeared to contain irregularly arranged microtubules. Double labelling with anti-β-tubulin and rhodamine-labelled wheat-germ agglutinin demonstrated that these irregularly arranged microtubules occurred only inside fungal hyphae and that microtubules were absent from host cells containing mycorrhizal fungi. Microtubule depolymerisation was shown to occur at the early stages of fungal infection. There was neither loss of nor obvious organisational change in microtubules in cells adjacent to others containing fungal hyphae. Electron microscopy confirmed the presence of an interfacial matrix between the host plasma membrane and the hyphal wall. The loss of microtubules from cells infected by mycorrhizal fungi suggests that an intact host microtubule cytoskeleton is not necessary for the formation of the interfacial matrix in mycorrhizas of orchid protocorms.

Key words Interfacial matrix · Orchid mycorrhizas · Microtubules · Cell wall formation

Introduction

Mycorrhizas are mutually beneficial associations between many plants and soil-borne fungi. Several types of mycorrhiza are found, but all can be classified as either penetrating the host cell (endomycorrhizas) or enveloping host cells (ectomycorrhizas). Orchid mycorrhizas are endomycorrhizal associations which occur in either roots, stems or germinated seeds of members of the Orchidaceae. During formation of endomycorrhizas, fungi may form extensive coils or pelotons within the cortex. As infection occurs, the plasma membrane is invaginated and a new cell wall is formed in the host cell between the invaginated host plasma membrane and the hyphal wall. The formation of this new cell wall in mycorrhizas, called the encasement layer (Hadley 1975; Harley and Smith 1983) or the interfacial material or matrix (Harley and Smith 1983; Bonfante-Fasolo 1992; Bonfante-Fasolo and Scannerini 1992), is considered to be part of the expression of the compatibility between host and symbiont (Bonfante-Fasolo 1992).

Although recent research on arbuscular mycorrhizas has shown that the materials that make up the interfacial matrix are host derived (Bonfante-Fasolo and Scannerini 1992), both their organisation during the matrix formation and their origin in orchid mycorrhizas are unknown. It has been suggested that the compartment may form through stretching of the preexisting host cell wall (Bonfante-Fasolo and Scannerini 1992). Alternatively, the host reaction to invasion by compatible mycorrhizal fungi may be to initiate the formation of new cell wall material. Such a process would involve an array of cellular activities, including activation of cellulose formation and alterations in targeting pathways of polysaccharide and glycoprotein transport (Bonfante-Fasolo and Scannerini 1992).

Microtubules appear to play major roles in wall formation in plant cells. During interphase, microtubules direct the orientation of cellulose microfibrils at the plasma membrane (Gunning and Hardham 1982). The directed transport of Golgi vesicles containing wall precursors during formation of the cell plate appears to be along phragmoplast microtubules (Pickett-Heaps and Northcote 1966). In plant cells invaded by potentially pathogenic fungi, microtubules may be involved in the resistance response through the transport and deposi-

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tion of callose-containing wall vesicles (Kobayashi et al. 1994).

If the formation of the interfacial matrix in mycorrhizas involves changes in the pattern of host wall formation, then such changes might be reflected by alterations of microtubule organisation away from the host wall and towards the interfacial region. The aim of this present study was to examine this possibility. Our approach was to observe the organisation of the microtubule cytoskeleton within uninfected protocorms and to compare this with the organisation of the microtubule cytoskeleton within orchid protocorms infected by a mycorrhizal fungus.

Materials and methods

Culturing procedure

Seeds of *Microtis parviflora* R. Br. were surface sterilised for 15 min in a saturated solution of calcium hypochlorite (Aldrich Chemical Company) and, after rinsing 5 times in sterile distilled water, placed onto one-sixth strength nutrient dextrose yeast agar (NDY/6, Warcup 1959). After 14 days incubation at 21 °C, the seeds had germinated, as indicated by doubling of size, rupture of the seed coat and growth of epidermal hairs. At this stage, the germinated seeds or protocorms were placed in an approximately 0.5-cm-diameter ring using a sterile scalpel. A 0.1×0.1 cm diameter agar plug containing hyphae of *Epulorhiza repens* (Bernard) Moore was then placed into the centre of the ring of protocorms. Other plates were left uninoculated to act as controls. The plates were incubated under the same conditions until the edge of the fungal colony had reached the ring of protocorms, and the material fixed 2 or 5 days later.

Fixation and embedding for immunofluorescence microscopy

Protocorms were fixed for 1 h at room temperature in 4% paraformaldehyde in 50 mM 1,4-piperazine-diethanesulfonic acid (Pipes) buffer (pH 7.0). After fixation, protocorms were rinsed 3 times in Pipes buffer and then transferred with fine forceps to an agarose plug [Sigma; low temperature gelling, 1% (w/v) in distilled water]. The samples were passed through 0.5-h steps of 10, 25, 50, 75, 100% and 100% ethanol at 4 °C, then through 2-h steps of ethanol: butyl-methylmethacrylate (BMM; Baskin et al. 1992) 2:1, 1:1, 1:2 (v/v) at 4 °C and two infiltration periods of 2 h in 100% BMM; the plugs were then left in 100% BMM for 24 h. The samples were polymerised in BMM through which nitrogen gas had been bubbled for 20 min to remove oxygen, in inverted BEEM capsules (Probing & Structure, Thuringowa Central, Queensland, Australia), modified by removing the conical end and replacing it with a plastic cap from another capsule and then sealing this with Parafilm. The samples were polymerised under UV light at 4 °C for 6 h.

Processing for immunofluorescence microscopy

Sections of mycorrhizal and control samples (1, 4, 10 μm) were cut on a Reichert ultracut microtome. For bright-field microscopy, 4-μm sections of mycorrhizal and control protocorms were dried down onto glass slides and stained for 30 s in 0.05% toluidine blue. Sections for immunolabelling were dried down onto welled slides coated with poly-L-lysine [Sigma; 0.01% (w/v) in distilled H_2O] using a hot plate (30 s). These sections were incubated in 100% acetone for 5 min to remove the polymerised resin. Following this, the sections were rehydrated for 10 min in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 7 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and then blocked for 5 min in PBS containing 1% bovine serum albumin (BSA, Fraction V, Sigma). The sections were then incubated in β-tubulin-specific monoclonal antibodies (Amersham International, Sydney, Australia; 1:500 in PBS/1% BSA) for 2 h at room temperature, rinsed for 5 min in PBS and then incubated for 2 h at room temperature in fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse immunoglobulin [$F(ab')_2$ fragment; Silenus, Dandenong, Victoria, Australia; 1:60 in PBS/1% BSA]. Sections of mycorrhizal protocorms were then rinsed in PBS and incubated for 2 h in rhodamine-labelled wheat-germ agglutinin (WGA; 50 μg/ml in PBS/1% BSA), which specifically binds the *N*-acetylglucosamine polymers of the chitin of hyphal walls. Finally, all slides were rinsed for 5 min in PBS, then briefly in distilled water, and mounted in Mowiol (Osborn and Weber 1982) to which 2.5% 1,4-diazabicyclo (2,2,2) octane (Sigma) had been added to prevent fading. The sections were examined and photographed with a Zeiss Axiophot microscope equipped with epifluorescence optics (for FITC visualisation: excitation 450–490 nm, dichroic mirror 510 nm, barrier 520 nm; for rhodamine visualisation: excitation 546 nm, dichroic mirror 580 nm, barrier 590 nm).

Electron microscopy

To confirm the presence of a mycorrhizal interfacial matrix within protocorms infected by *E. repens,* protocorms were fixed for 1 h at room temperature in 1% glutaraldehyde in 100 mM Pipes buffer (pH 7.4) and then postfixed in 1% osmium tetroxide for 1 h. After rinsing several times in distilled water, the protocorms were transferred to agarose blocks (see above) before dehydration in a graded acetone series of 10, 30, 50, 70, and 100% acetone (30 min each). The samples were then passed through 2-h steps at room temperature of acetone: Spurr's resin in the ratios 2:1, 1:1, 1:2 (v/v). After two infiltration periods in 100% Spurr's resin of at least 2 h, the samples were polymerised overnight at 60 °C. Ultrathin sections (60 nm) were cut with a diamond knife on a Reichert Ultracut microtome, post-stained for 10 min in 2% aqueous uranyl acetate and for 5 min in Reynold's lead citrate (Reynolds 1963) and examined in a Phillips CM 12 electron microscope.

Results

In cells of protocorms uninfected by the mycorrhizal fungus (Fig. 1), microtubules existed in regular arrays

Fig. 1 *Microtis* protocorm uninfected by mycorrhizal fungi. A 4-μm section stained with 0.05% toluidine blue; *bar* 47 μm ▶

Fig. 2 *Microtis* protocorm with established mycorrhizas. A 4-μm section stained with 0.05% toluidine blue; *bar* 45 μm

Fig. 3 Regular arrays of microtubules in an uninfected *Microtis* protocorm. A 4-μm section labelled with anti-β-tubulin; *bar* 9.5 μm

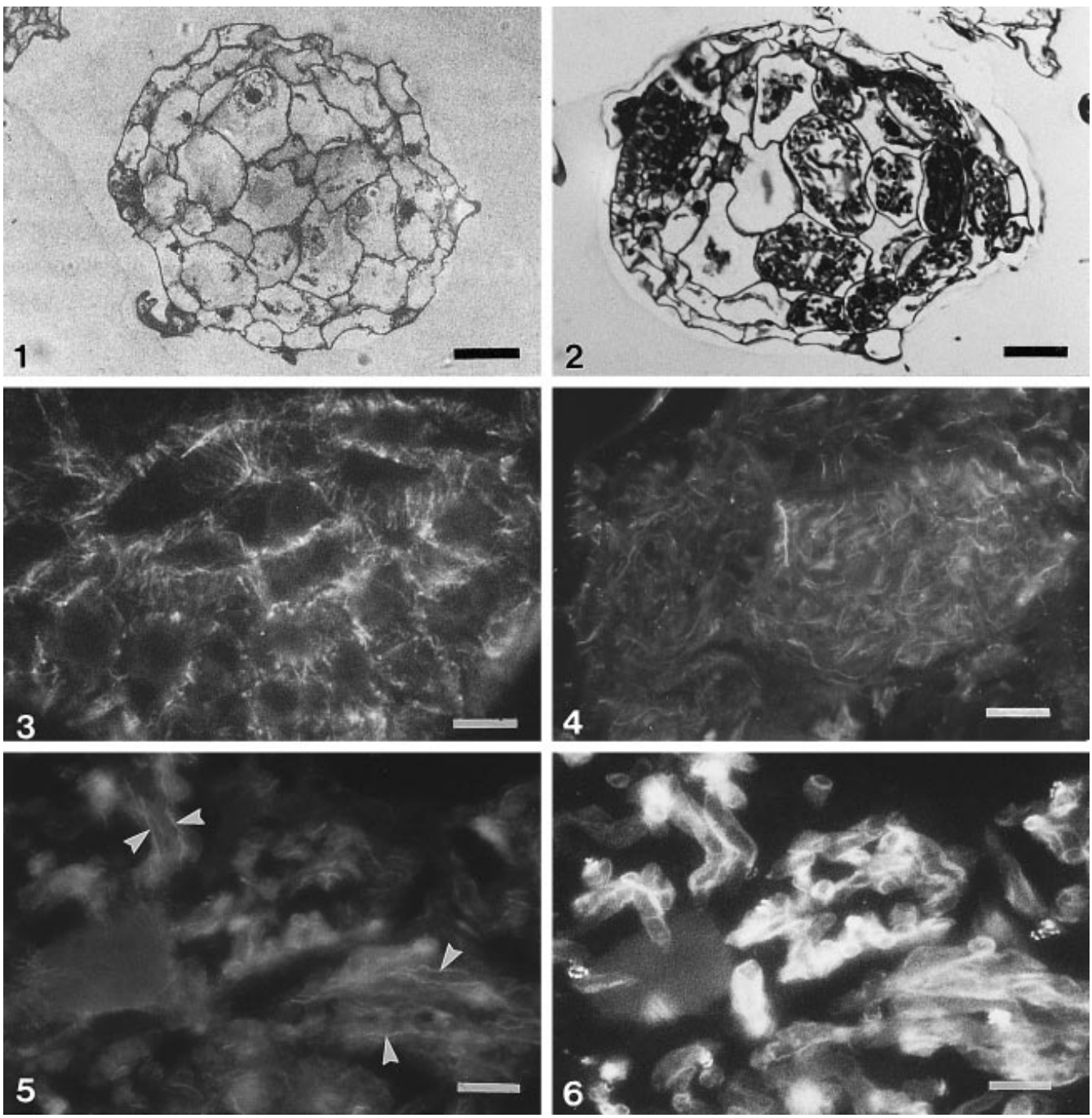
Fig. 4 Irregularly arranged microtubules within cells of a mycorrhizal *Microtis* protocorm. A 4-μm section labelled with anti-β-tubulin; *bar* 14 μm

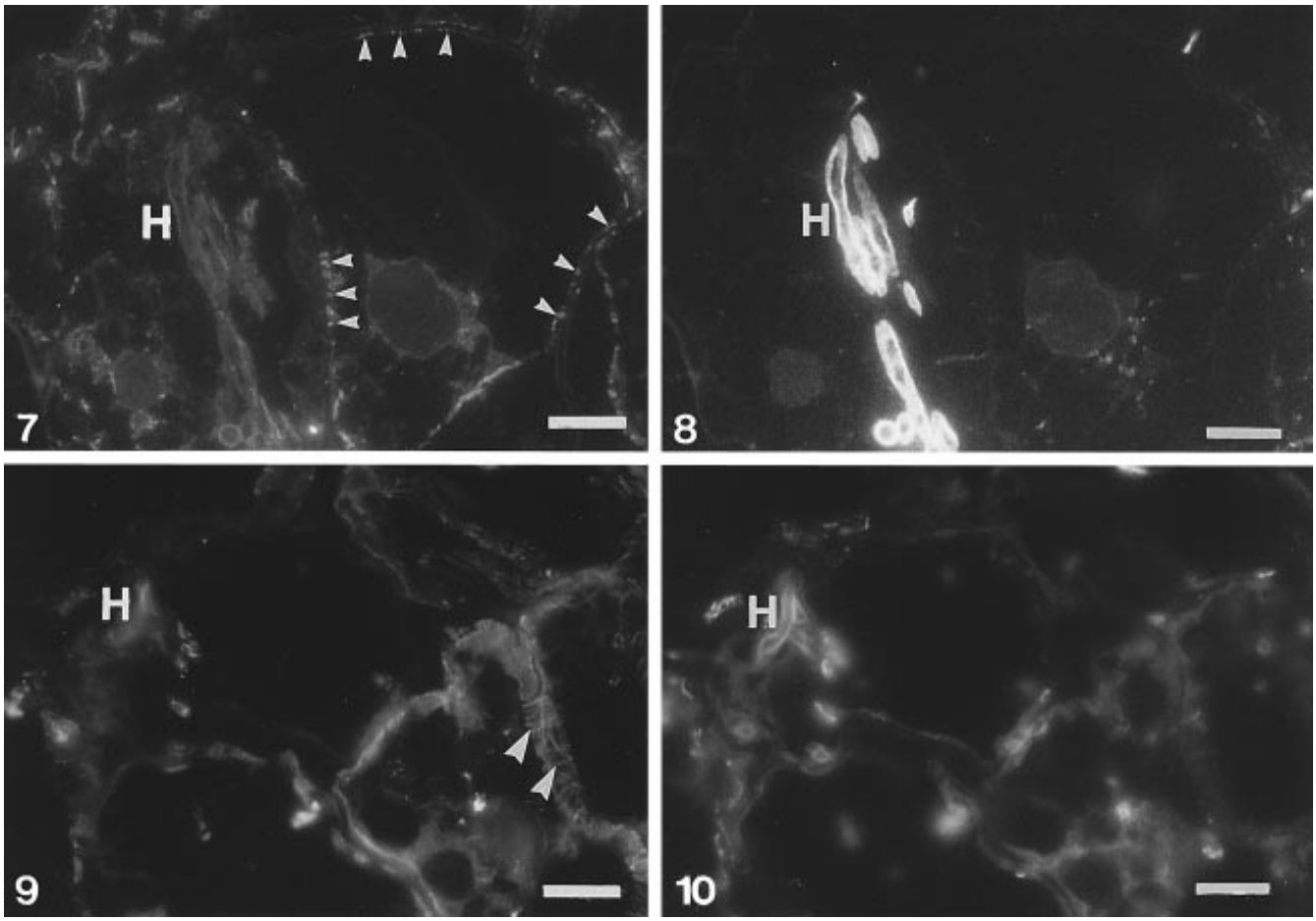
Figs. 5, 6 Irregularly arranged microtubules occurred only inside hyphae and not in the host cytoplasm. A 10 μm section double labelled with anti-β-tubulin and with WGA-rhodamine; *bar* 8.5 μm

Fig. 5 Microtubules (*arrows*) stained with anti-β-tubulin

Fig. 6 Hyphae stained with WGA-rhodamine

(Fig. 3) similar to those recorded for other interphase plant cells (Roberts et al. 1985; Wick 1985; Ishida and Katsumi 1991). In orchid protocorms with established mycorrhizas (Fig. 2), the pattern of microtubule organisation was quite different from uninfected protocorms, with irregularly arranged microtubules within cells containing fungal coils (Fig. 4). Double labelling with anti-β-tubulin and WGA-rhodamine demonstrated that these irregularly arranged microtubules occurred only inside hyphae and that microtubules were absent from the cytoplasm of host cells containing mycorrhizas (Figs. 5, 6). There was no loss of microtubules from cells adjacent to established mycorrhizas (Figs. 7, 8). Microtubule depolymerisation in host cells occurred during an early stage of fungal invasion (Figs. 9, 10). In neighbouring cells at this stage, there was neither a loss of nor an obvious organisational change in microtubules (Figs. 9, 10). Electron microscopy confirmed the presence of an interfacial matrix between host plasma membrane and the fungal cell wall (Fig. 11). This layer was less electron dense than the hyphal wall (Figs. 11, 12) and was similar in appearance to the host cell wall (Fig. 11), as reported previously for orchid protocorm mycorrhizas (Uetake et al. 1992).





Figs. 7, 8 There was no loss of microtubules from cells adjacent to established mycorrhizas. A 1-μm section double-labelled with anti-β-tubulin and with WGA-rhodamine; *bar* 12.5 μm

Fig. 7 Microtubules (*arrows*) stained with anti-β-tubulin (*H* mycorrhizae)

Fig. 8 Mycorrhizae (*H*) stained with WGA-rhodamine

Figs. 9, 10 Microtubules were absent from cells which were in the early stages of infection by fungal hyphae. In neighbouring cells at this stage, there was neither a loss of nor an obvious organisational change in microtubules. A 4-μm section double-labelled with anti-β-tubulin and WGA-rhodamine; *bar* 10 μm

Fig. 9 Microtubules (*arrows*) stained with anti-β-tubulin (*H* hyphae)

Fig. 10 Hyphae (*H*) stained with WGA-rhodamine

Discussion

In this paper we describe the invasion of orchid protocorm cells by a compatible mycorrhizal fungus. Using anti-β-tubulin to localise microtubules and WGA-rhodamine to locate fungal hyphae by chitin detection, we show that microtubules are absent from host cells containing the fungus. The validity of anti-β-tubulin labelling of microtubules in plant material embedded in BMM has been well established (Gubler 1989; Baskin et al. 1992; Kobayashi et al. 1994). WGA has been shown to have a high specificity for *N*-acetylglucosamine polymers (Allen et al. 1973) and has been used to detect chitin/fungal hyphae within infected plant cells at the ultrastructural level (Bonfante-Fasolo et al. 1990; Bonfante-Fasolo and Scannerini 1992) and using fluorescence microscopy (Bonfante-Fasolo et al. 1990), including research where the plant material was embedded in BMM (Kobayashi et al. 1994). The microtubules in uninfected protocorm cells displayed a morphology that has been extremely well characterised for interphase higher plant cells (Roberts et al. 1985; Wick 1985; Ishida and Katsumi 1991), and thus any change in microtubule organisation of infected protocorms can be considered to be related specifically to the presence of fungal hyphae within cells.

Since an intact microtubule cytoskeleton was not present in protocorm cells recently infected by the mycorrhizal fungus, it appears that microtubules do not play an important role in the formation of the interfacial matrix. This finding suggests that interfacial matrix formation does not involve activation of the synthesis of new wall materials by the host. Instead, invasion of protocorm cells by fungal hyphae may lead to interfacial matrix formation, at least partly through stretching of the preexisting host wall, as has been suggested for arbuscular mycorrhizas (Bonfante-Fasolo and Scannerini 1992).

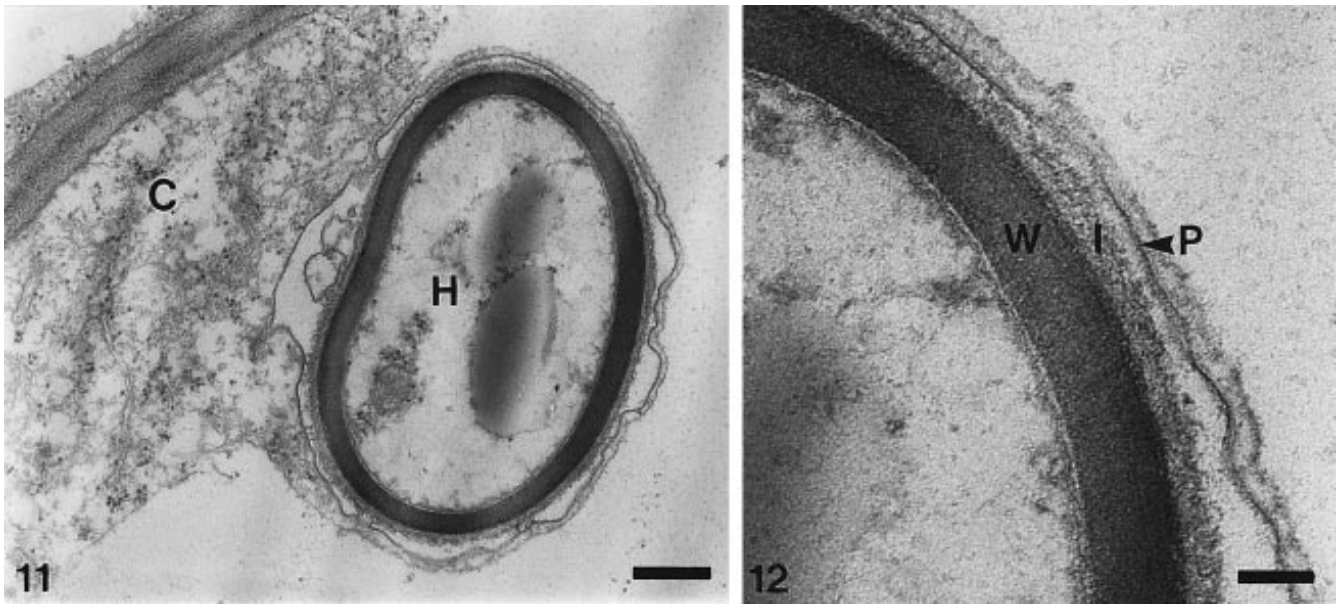


Fig. 11 Electron micrograph of hypha (*H*) in protocorm cell (*C*) showing the presence of an interfacial matrix between the hyphal wall and the host plasma membrane. Note the similarity in appearance between the interfacial matrix and the host cell wall; *bar* 0.3 μm

Fig. 12 Enlargement of Fig. 11 showing details of the interfacial matrix (*I*) between the hyphal wall (*W*) and host plasma membrane (*P*). Note that the interfacial matrix is less electron dense than the hyphal wall; *bar* 0.1 μm

Microtubule depolymerisation has been reported in other plant-fungal associations (Gross et al. 1993; Timonen et al. 1993; Kobayashi et al. 1994). In ectomycorrhizas, the presence of invading hypha in the root cortex appears to be related to a complete loss of microtubules from adjacent cells (Timonen et al. 1993). In plant-pathogen interactions, the presence of fungal hyphae within host cells may cause either a total (Kobayashi et al. 1994) or a local depolymerisation of microtubules (Gross et al. 1993). Microtubule depolymerisation may be important in the resistance response to pathogens through decreasing the viscosity of the host cytoplasm and thus allowing more efficient movement of organelles to the site of invasion (Gross et al. 1993). In mycorrhizas, reduced cytoplasmic viscosity would enhance penetration of fungal hyphae through the cell and ultimately lead to an increase in the efficiency of the mycorrhizal association with the plant. Although the invasion of protocorm cells by fungal hyphae appeared to be related to microtubule depolymerisation, the precise molecular mechanism behind this remains the subject of conjecture. Raised levels of Ca^{2+} can cause microtubule depolymerisation (Schliwa 1976), and it is possible that fungal infection of protocorm cells triggers the release of Ca^{2+} into the cytoplasm from previously stored compartments. It has also been speculated that fungi invading plant tissues release substances which disrupt the cytoskeleton (Palevitz 1980). Such substances released by the invading hyphae may have caused breakdown of the microtubule cytoskeleton in the infected protocorm cells. Although the cause of the depolymerisation of microtubules in this system is unknown, the intact microtubule cytoskeletons in uninfected cells adjacent to infected cells demonstrate that the mechanism is localised to the immediate vicinity of the infected cell and does not spread to adjacent cells before fungal infection. In a recent study of interactions between flax and rust fungi, Kobayashi et al. (1994) demonstrated that microtubules in cells adjacent to cells in which microtubule depolymerisation occurred became concentrated along the shared walls. Such changes may be related to the deposition of callose to prevent the spread of fungal infection, the converse of mycorrhizal infection where further fungal colonisation may be advantageous. These observations indicate that some characteristics of plant-pathogen interactions are found in orchid mycorrhizas, but that mycorrhiza formation induces some different responses in the host that can be explained in terms of the beneficial interaction that ensues.

Actin is the other major component of both the plant and fungal cytoskeleton which is known to play an important role in wall formation (Heath 1990; Steer 1990). Actin microfilaments in plants direct the transport of cell wall materials from the site of formation to final destinations in a number of systems (reviewed in Williamson 1993). During the infection of plant cells by *Phytophthora infestans,* localised depolymerisation of microtubules was shown to occur while the actin cytoskeleton remained intact (Gross et al. 1993). This suggests an important role for the actin cytoskeleton in the resistance response, including transport of wall materials to the site of fungal invasion. A follow-up study is necessary to assess the importance of actin microfilaments in the formation of the interfacial matrix in the protocorm system.

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