

Appressorium formation by AM fungi on isolated cell walls of carrot roots

SUMMARY

Cell walls were isolated and purified from Ri T-DNA-transformed carrot roots to determine whether arbuscular mycorrhizal (AM) fungi need a signal from the host root in order to form appressoria. Large cell-wall pieces were generated by the Parr nitrogen bomb technique. Purified cell walls were sterilized and inoculated with single spores of *Gigaspora gigantea* (Nicol. & Gerd.) Gerdemann & Trappe and *Gigaspora margarita* Becker & Hall using an *in vitro* culture technique. Appressoria only formed on epidermal cell walls isolated from host roots (*Daucus carota* L.) but did not form on epidermal cell walls isolated from non-host roots (*Beta vulgaris* L.). Although appressoria formed on host-cell walls, penetration hyphae did not fully develop. The results of this study indicate that appressorium formation by these AM fungi is a contact recognition event that does not require a signal secreted from the host root or the presence of intact host cells.

Key words: Cell walls (purified), appressorium formation, host, non-host.

INTRODUCTION

Appressorium formation is a decisive event in fungal recognition by and infection of a host (Staples & Macko, 1980). It is the first cell-to-cell recognition step during an AM fungus-plant-host interaction. Appressoria are morphologically identified as flattened, elliptical hyphal tips that form on the surface of host roots (Garriock, Peterson & Ackerley, 1989). Little is known about the phenomenon of binding or adhesion of AM fungal hyphae to host roots or of the nature of signals inducing appressorium formation (Giovannetti, Sbrana & Logi, 1994). It is not known whether secreted compounds or root exudates from the host act as signals for appressorium formation (Schreiner & Koide, 1993). We report results of experiments in which appressorium formation was studied using purified cell walls in the absence of root exudate, cytoplasmic components, or intact protoplasts.

MATERIALS AND METHODS

Root organ culture

Ri T-DNA-transformed carrot (*Daucus carota* L.) roots (host) and sugar beet (*Beta vulgaris* L.) roots

(non-host) established by Bécard & Fortin (1988), were routinely propagated at 24 °C on M medium in Petri dishes. Root explants of the host and non-host were standardized and prepared as described (Bécard & Piché, 1992). The gelling agent used was gellan gum (Phytigel®, Sigma) used at 0.2% (w/v) for maintaining root cultures and at 0.4% used for spore germination and the dual culture system (Bécard & Piché, 1992). Aseptic, germinated single spores were transferred to a Petri dish containing carrot roots to obtain infected root cultures (Bécard & Piché, 1992).

Spore inoculum

Azygospores of *Gigaspora gigantea* (Nicol. & Gerd.) Gerdemann & Trappe and *Gigaspora margarita* Becker & Hall (DAOM 194757) were produced in pot cultures in a greenhouse with *Paspalum notatum* Flugge as a host. Spores were collected, isolated and sterilized as described by Bécard & Fortin (1988). Spores were germinated on M medium in an incubator at 32 °C and 2% CO₂. Dual cultures were incubated at 24 °C and ambient CO₂.

Cell wall isolation procedures

To separate and recover carrot roots from the gellan gum, root cultures were shaken in 10 mM EDTA buffered at pH 7.5 with 25 mM TRIZA HCl-TRIZMA Base (Sigma, St. Louis, MO) for 5–10 min

at 120 r.p.m. in an orbital shaker (Nagahashi *et al.*, 1993). Roots were collected on a sintered glass filter, rinsed in distilled-deionized (DD) water, pulverized in liquid nitrogen, and suspended in homogenization medium (Nagahashi, Abney & Uknalis, 1994). The suspension was transferred to a Parr nitrogen bomb and placed under 12.4 MPa for 15 min at 4 °C (Nagahashi & Seibles, 1986). The contents were extruded from the bomb, and cell walls were trapped on cheesecloth. The crude cell-wall fraction was suspended in DD water and degassed for 5 min at 4 °C. The cell walls were purified by filter trapping and resuspending in DD water eight to 10 times. The large cell-wall pieces were collected on the filter while the cytoplasmic components readily passed through the trap. The purified cell walls were stirred in 10 mM CaCl₂ for 10 min at 4 °C to prevent autolysis, and the excess CaCl₂ was removed by washing. The cell walls were trapped on a sintered glass filter, suspended in absolute ethanol, sonicated to break up the cell-wall clumps, and stored at 4 °C overnight. The sterilized cell walls were then washed in sterile DD water and transferred to M medium with gellan which had been autoclaved and cooled to approx. 50 °C. The cell walls settled on the bottom of the Petri dishes as the gellan solidified.

Hyphal growth in plates containing purified cell walls

Plugs (Bécard & Piché, 1992) containing a single germinated spore of *G. margarita* or *G. gigantea* were transferred to the medium containing cell walls and incubated at 32 °C and 2% CO₂ for 7–10 d. As many as three spores were transferred to each plate containing cell walls. A minimum of 15 plates was analysed for the host and non-host interactions. Both fungal species produce a primary germ tube that exhibits negative geotropism. The plates were placed at a slight angle (30° from vertical) for the first night to allow the primary germ tube to grow towards the bottom of the Petri dish. The plates were then oriented vertically to maximize interactions with purified cell-wall fragments. The interactions between fungal hyphae and cell-wall fragments were observed with a Nikon Diaphot-TMD inverted microscope. Images were viewed with a JVC video monitor and stored using the NIH Image version 1.57 software. A Nikon 35 mm camera was used to take pictures of the video images.

Staining procedures

Colonized root cultures were dissolved as described above and cleared and stained with trypan blue (Phillips & Hayman, 1970). Plates containing purified cell walls and fungal hyphae were stained by covering the gellan with a thin layer of trypan blue,

lactic acid/glycerol solution for 1 h. The excess stain in the gellan medium was removed by covering the gel with water and changing the water several times daily for 5–7 d. This wash cycle leached the stain out of the gellan and reduced the blue background.

RESULTS

Although the interactions of the primary germ tube or lateral hyphae of AM fungi with different cell-wall types were not frequent, the only interactions which showed appressorium formation occurred with epidermal cell-wall surfaces of host roots (Fig. 1). The epidermal wall surface could be identified by its uniform curvature, small but long cellular structure without intercellular air spaces, and sometimes, the presence of root hairs. By contrast, vascular cell walls were easy to distinguish by the presence of ridges on xylem elements (Fig. 1*b*). Cortical cell walls (Fig. 1*b*) could be distinguished from epidermal cell walls by their amorphous clumps which originated from cortical cells with a larger diameter than epidermal cells. In some isolated cell-wall clumps, epidermal and cortical cell walls were still attached to each other.

Simple appressoria were formed on isolated epidermal cell walls of carrot roots by both *G. gigantea* (Fig. 1*a*) and *G. margarita* (Fig. 1*b*). The appressoria always formed on the walls or grooves between two adjacent epidermal cells. In a few interactions, root hairs were present, but it was difficult to obtain a micrograph with the root hairs and appressorium in the same plane of focus. These simple appressoria were identical to those formed on onion roots by *Glomus versiforme* (Garriock *et al.*, 1989). More complex appressoria with developing infection hyphae or penetration pegs were also observed for both species (Fig. 2*a, b*). Fully developed infection hyphae (non-septated and darkly stained) were not observed on isolated epidermal cell wall pieces. Penetration buds, like those shown in Figure 3*a*, only developed a short time before the infection hyphae became septated. Although it is not clearly shown in the micrographs, any infection hypha penetrating between two epidermal cell walls became septated and stopped growing.

Appressoria formed on intact carrot roots (Fig. 3*b*) were morphologically similar to those formed on isolated epidermal cell walls. The micrograph also shows the typical shape and curvature of intact epidermal cells which are identical to the isolated epidermal cell walls in Figures 1 and 2. The infection hyphae developed fully, penetrated the cortex of intact roots and arbuscule formation followed (micrographs not shown).

By contrast, appressoria were not formed on intact sugar beet roots (micrographs not shown). Similarly, appressoria were not formed on isolated epidermal cell walls from the non-host. The interactions were

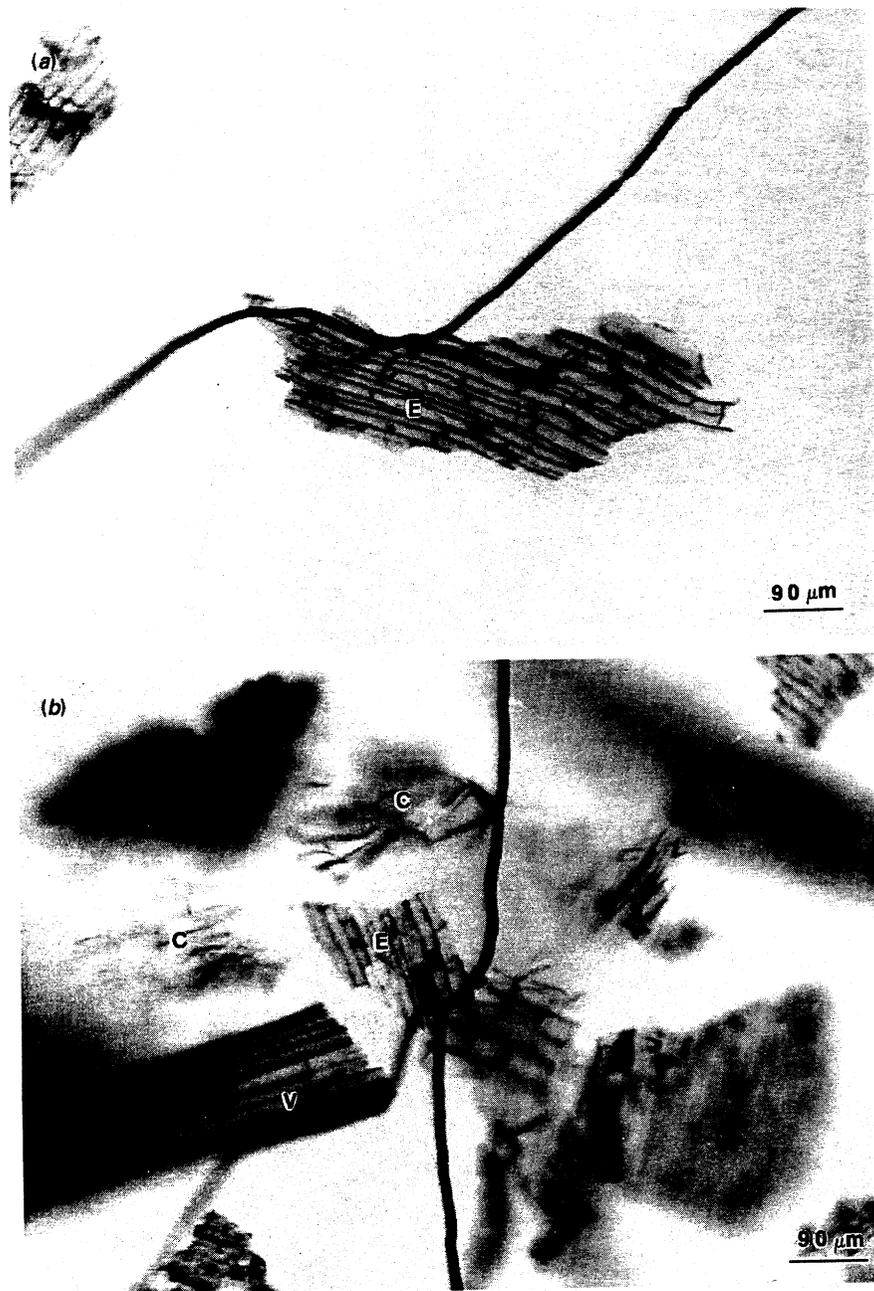


Figure 1. (a) Light micrograph of a simple appressorium formed by *Gigaspora gigantea* on isolated cell walls of Ri T-DNA transformed carrot roots. E, epidermal cell walls. (b) Light micrograph of a simple appressorium formed by *G. margarita* on isolated cell walls of Ri T-DNA transformed carrot roots. C, cortical cell walls; V, vascular cell walls.

limited to hyphal diversion or simple branch formation as observed for cortical cell walls and vascular cell walls of carrot roots.

DISCUSSION

Vascular tissue was not separated from cortical tissue during the cell-wall isolation procedure used in this study (Nagahashi *et al.*, 1994). This provided the opportunity to determine whether AM fungal hyphae would interact with cortical cell walls or vascular cell walls as well as with epidermal cell walls. Appressoria were not formed on cortical cell walls or

vascular cell walls by either AM fungal species (data not shown). If the primary germ tube ran into a cortical cell-wall fragment, it usually formed a single branch or worked its way around the wall fragment and continued on its negative geotropic course. It is interesting that these fungi did not form appressoria on cortical cell walls, which must also be penetrated for the eventual formation of arbuscules. It is possible that a host-derived signal for penetration of cortical cell walls was absent or that penetration requires the presence of intact cortical cells.

No appressoria formed on isolated vascular cell-wall pieces. In some instances, the germ tube grew

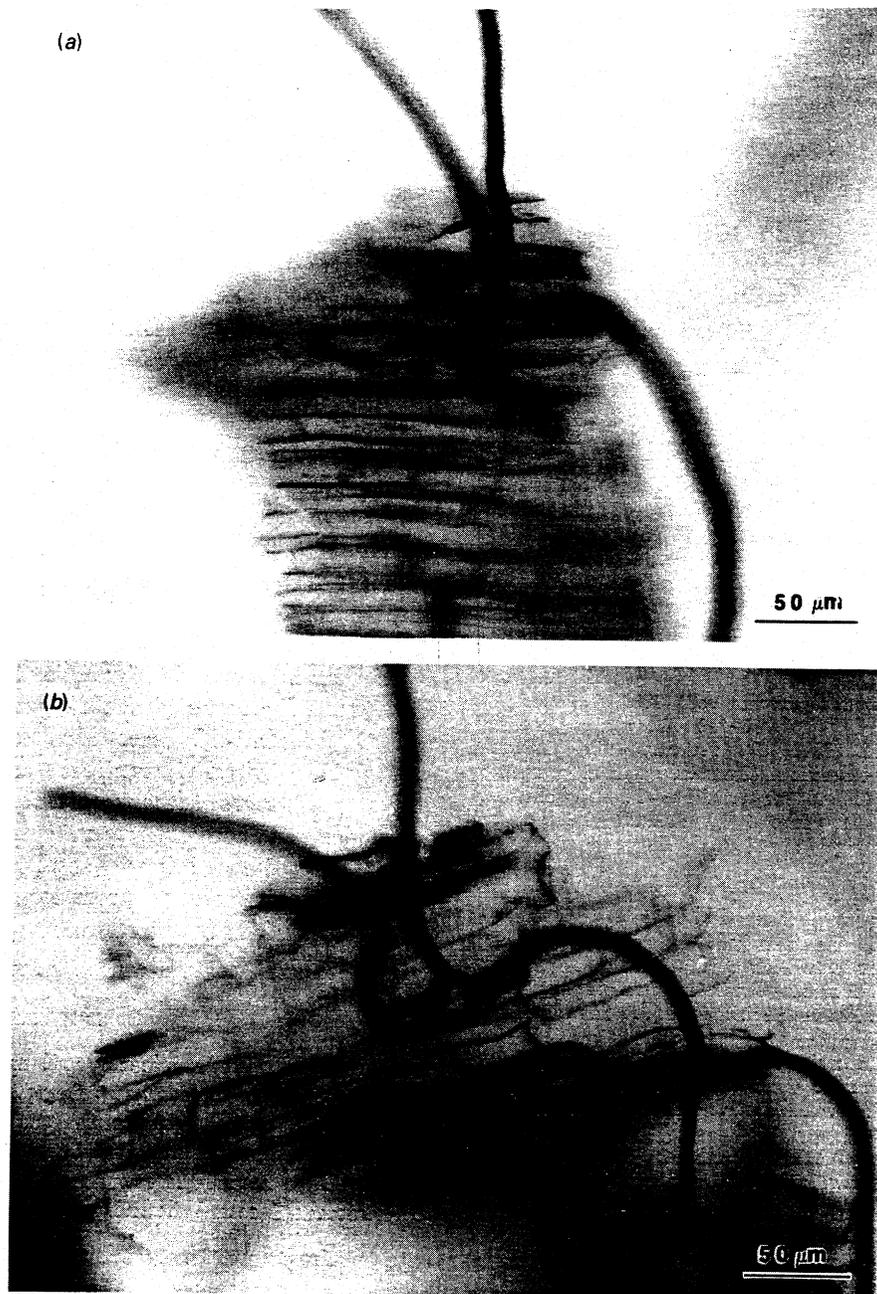


Figure 2. (a) Light micrograph of a complex appressorium formed by *Gigaspora gigantea* on isolated carrot cell walls of Ri T-DNA transformed carrot roots. (b) Light micrograph of a complex appressorium formed by *G. margarita* on isolated carrot cell walls of Ri T-DNA transformed carrot roots.

right through the hole in a xylem element or diverted around a clump of vascular cell walls, suggesting that if a breach occurred in the endodermis of an intact root, appressorium would not be likely to form on vascular-tissue cell-walls since they do not provide an appropriate surface.

Many interactions with epidermal cell walls were similar to those with cortical or vascular cell walls, where hyphal diversion occurred or single branches formed but no appressoria developed. A single hyphal branch formed when the hyphal path was impeded by a cell-wall fragment as when the hyphal tip came up against the side or bottom of the Petri dish. The limited appressorial formation with a

mixture of cell walls from all regions of the root was consistent with the fact that only specific epidermal regions of the intact root are infected. If the binding sites or adhesion sites for AM fungi are restricted to certain areas of the growing root, then the number of interactions observed with isolated wall pieces is a function of the number of appropriate pieces a fungal hypha can encounter, the orientation of the pieces, and the size of the pieces. The success of this study can be attributed to the use of concentrated cell-wall fragments and more importantly, the size of the cell-wall fragments used in the experiments. The isolation and purification procedure used in this study specifically allows for the recovery of very

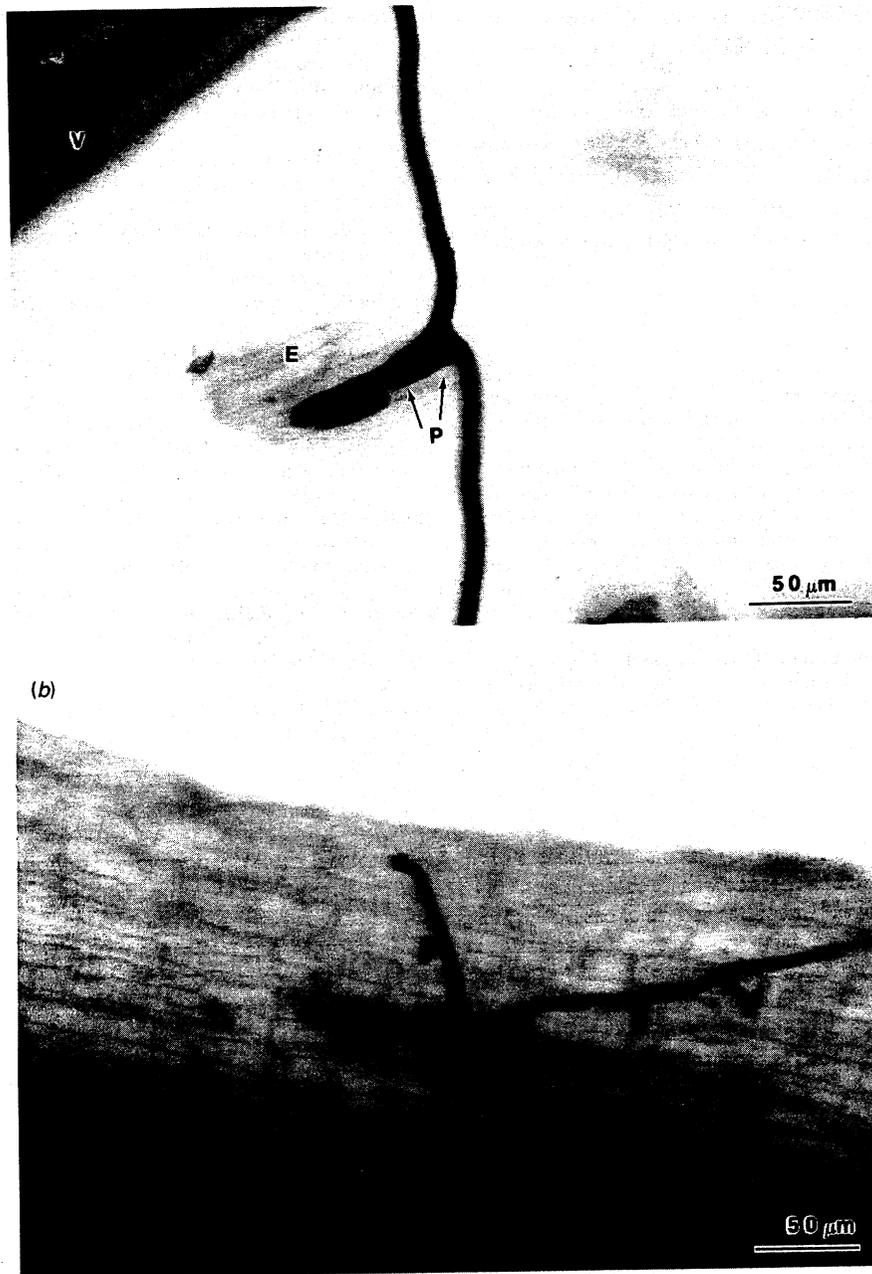


Figure 3. (a) Light micrograph of an appressorium formed by *Gigaspora margarita* on isolated carrot cell walls. Penetration buds (P) are developing from the appressorium. (b) Light micrograph of an appressorium formed by *G. margarita* on the surface of an Ri T-DNA transformed carrot root *in vitro*.

large cell-wall fragments. Cell-wall ghosts (complete removal of the protoplasts). 3–5 cells long are apparent in electron micrographs shown by Nagahashi *et al.* (1994) and in light micrographs shown here (Figs 1–3). When the Parr bomb is used, cracks through the wall are formed and the protoplasm is extruded when the pressure is released. The topology of the epidermal cell-wall surface is preserved so that contact by a fungal hypha occurs as if contact were made with an intact root. Pertinent to our results is the report that germ tubes from a fungal pathogen can detect the difference between the surface topography of a host and a non-host (Manocha & Chen, 1990). Appressoria were formed on isolated host-cell walls.

The observation that appressoria formed in the absence of the protoplast or root exudates was surprising. It is known that the first signal detected by AM fungi is present in the root exudate and that signal promotes rapid, three-dimensional branching of hyphal tips (Giovannetti *et al.*, 1993; Nagahashi, Douds & Abney, 1995) as they grow near the surface of host roots. This signal is not induced by the fungus but it is a constitutive component of the host exudate, as indicated by previous results with the *in vitro* assay (Nagahashi *et al.*, 1995). Since purified cell walls do not stimulate rapid branching of the fungus, the branching signal must either be secreted from intact cells or, possibly, be covalently bound to the cell wall and released during secondary root

formation. The branching signal apparently increases the chances of hyphal tip contact recognition with the host root.

Although the actual mechanism for recognition between a mycorrhizal fungus and host-root surface has not been elucidated, the results reported here show that neither intact host cells nor a signal in the exudate of host roots is necessary for appressorium formation.

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