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Cellular Interactions between *G. mosseae* and a Myc⁻ *dmi2* mutant in *Medicago truncatula*

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Abstract

Incompatibility towards *Glomus mosseae* (BEG 12) in a mycorrhiza-defective Myc⁻ *dmi2* mutant (TR26) in *Medicago truncatula* is associated with (i) abnormal plant wall reactions characterised by an autofluorescent apposition layer within cells adjacent to fungal hyphae, (ii) pectin-rich material deposited between plant and fungal walls and (iii) disorganisation of fungal hyphae in contact with root cells. No callose accumulation is observed. The mutation appears to affect fungal-root interactions in a different way than mutations reported in a pea Myc⁻ mutant.

Keywords: *M. truncatula*, mutant, *G. mosseae*, cellular incompatibility

1. Introduction

Medicago truncatula (Gaertn.) is a Mediterranean annual alfalfa, well adapted to semiarid conditions, with more than 100 described ecotypes (Armor,

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1965; Lesins and Lesins, 1979). It is used as a forage plant in Western Europe and the Mediterranean basin in rotation crop cultures (Crawford et al., 1989). *M. truncatula* is also a model plant for studying interactions between plant and symbiotic microorganisms as it nodulates with *Sinorhizobium meliloti* and forms a symbiotic association with arbuscular mycorrhizal fungi (AMF) (Barker et al., 1990; Harrison, 1997).

As for other legumes (Duc et al., 1989; Wegel et al., 1998), a *M. truncatula* mutant (TR26) defective for mycorrhizal and rhizobial symbiosis (Myc^-, Nod^-) has been obtained (Sagan et al., 1995). Genetic analysis has shown that mutation of only one gene, *dmi2*, is responsible for the double resistance to root micro-symbionts in this mutant (Sagan et al., 1998; Catoira et al., 2000). The cellular basis of resistance to AMF has previously been studied in another Myc^- mutant in pea using molecular cytology (Gollotte et al., 1993). Activation of wall defence reactions in epidermal root cells associated with this resistance was characterised by the accumulation of phenolic components, callose and PR1 protein in a wall apposition layer formed in cells in contact with the appressorium of *Glomus mosseae* (Gollotte et al., 1993). The purpose of the present work was to investigate whether a similar phenomenon is responsible for resistance to *G. mosseae* in the TR26 mutant of *M. truncatula* by studying the cellular interactions between the mycorrhizal fungus and mutant root.

2. Material and Methods

Medicago truncatula (Gaertn.) cv. Jemalong line J5 and the isogenic Myc^-, Nod^- mutant TR26 obtained by gamma-irradiation (Sagan et al., 1995) were inoculated with *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe (isolate BEG 12). Inoculum consisted of roots, spores, mycelium and soil from a pot culture of 3 month old mycorrhizal leek roots produced in the same clay loam soil. Surface-disinfected (6 min 98% sulphuric acid, 3 min 12° bleach), scarified seeds of *M. truncatula* were germinated on 0.7% water-agar *in vitro*. 3d after germination, seedlings were transplanted into pots containing 1/3 volume of the soil-based *G. mosseae* inoculum and 2/3 volume of calcined clay (Terra-green, Oil Dri Corporation, USA). The plants were grown under controlled conditions (20–24°C, 16 h photoperiod, 320 µmol photons $m^{-2}s^{-1}$, 70% relative humidity), and watered daily with osmosed water and once a week with a modified (Nx2, P/10) Long Ashton nutrient solution (Hewitt, 1966).

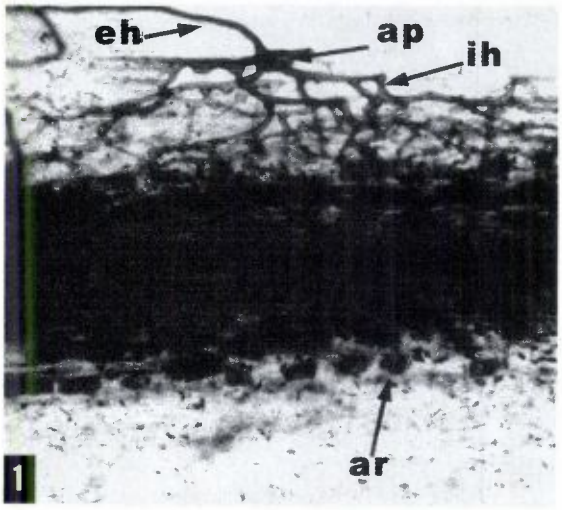
After 3 weeks' growth, plants were sampled and root systems carefully washed in water. Some roots were stained with 0.5% trypan blue (Phillips and Hayman, 1970) to monitor appressorium formation or fungal growth in TR26 or J5 roots. Other *G. mosseae*-inoculated roots were quickly dipped in a 0.5% trypan blue solution to localise external fungal structures, and 0.5 cm root pieces

with fungal growth on their surface were selected under a binocular microscope. These were fixed in 2% glutaraldehyde in 0.1 M sodium-cacodylate buffer (pH 7.2), dehydrated through an ethanol series and embedded in LR White resin (Company Ltd., Reading, UK), as described previously (Gianinazzi and Gianinazzi-Pearson, 1992). Sections were cut with a Riechert ultramicrotome.

Light microscope observations were made on stained roots, and semithick sections (0.5 µm) were examined for autofluorescence (Fernandez and Heath, 1986) and after staining with toluidine blue (Feder and O'Brien, 1968) using a Leica Laborlux microscope with epifluorescence optics. Autofluorescence was assessed using a filter combination of 450–500 nm and 515–560 nm. Thin sections (90 nm) were collected on gold grids for analytical cytochemistry and immunolabelling. Polysaccharides and glycoproteins were located by the periodic acid-thiocarbohydrazide-silver proteinate reaction (PATAg; Thiéry, 1967). An indirect immunogold labelling technique (Gianinazzi and Gianinazzi-Pearson, 1992) was used to detect pectin and β-1,3 glucan-containing components in thin sections. Non-esterified pectins were located with a rat monoclonal antibody (JIM5; provided by K. Roberts, John Innes Institute, UK), diluted 1:50, and revealed using a gold labelled goat anti-rat secondary polyclonal antibody, diluted 1:20. β-1,3 glucans were detected using a mouse monoclonal antibody (Biosupplies Australia Pty Ltd., Australia) diluted 1:200, and revealed using a gold-labelled goat anti-mouse secondary polyclonal antibody, diluted 1:20. Gold labelled wheat germ agglutinin (WGA) (Sigma Chemical Company, USA), which binds to N-acetyl-glucosamine, was used diluted 1:50 to detect chitin as described by Lemoine et al. (1995). Sections were contrasted 10 min with 3% aqueous uranyl acetate before observation. Ultrathin sections were examined using a Hitachi (Tokyo, Japan) 600 electron microscope at 75kV.

3. Results and Discussion

G. mosseae formed appressoria on the surface of roots of *M. truncatula* in both the wild type J5 genotype (Fig. 1) and the TR26 genotype mutated in the *dmi2* gene (Figs. 2 and 3). Appressorium formation and root colonisation patterns (Fig. 1) by *G. mosseae* were fairly invariable on wild type J5 roots, but two types of fungal behaviour were observed close to and on the root surface of the TR26 *dmi2* mutant. One resembled that described for *G. mosseae* on roots of a Myc⁻ pea genotype mutated in the *sym30* gene (Gollotte et al., 1993; Gianinazzi-Pearson, 1996) in that external hyphae ran over the root surface and formed thick, lens-shaped appressoria at epidermal cell junctions (Fig. 2). The second type of fungal development was seen as intense external hyphal ramification over the root surface, which led to the formation of many ill-defined appressoria (Fig. 3).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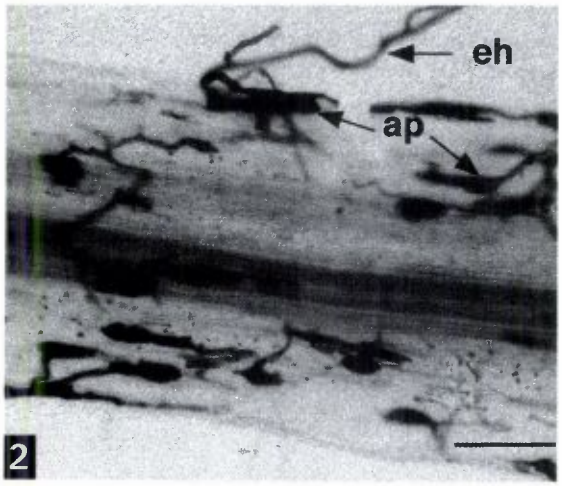
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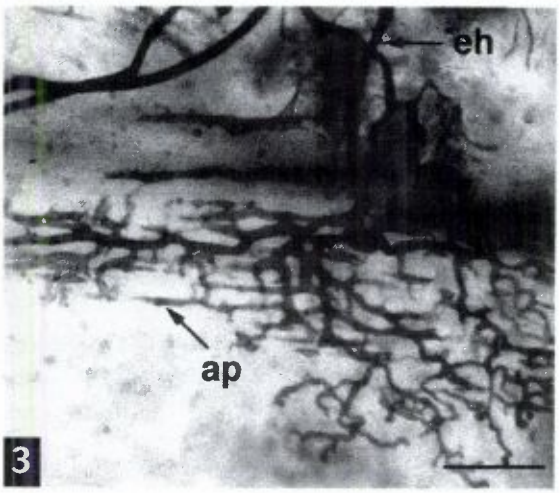
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Toluidine staining of semi thin sections through appressoria at the surface of TR26 mutant roots clearly revealed an accumulation of material at the junction between the plant epidermal cells and *G. mosseae* (Fig. 5). Intense staining with toluidine blue has been suggested to indicate the accumulation of phenolic components (Feder and O'Brien, 1968). This modification was consistently associated with appressoria formation on mutant TR26 roots, but was not observed when appressoria formed at the root surface of the wild type J5 genotype (Fig. 4). Blue light excitation, without toluidine blue staining, of the same region of mutant TR26 roots in contact with appressoria gave an intense bright-yellow autofluorescence (Fig. 7), again suggesting the accumulation of phenolic components (Fernandez and Heath, 1986) at the junction between the mutant epidermal cells and *G. mosseae*. No such autofluorescence was ever observed in the wild type genotype epidermal cells in contact with *G. mosseae* appressoria (Fig. 6). Cessation of fungal growth and accumulation of material at the junction between epidermal cells of TR26 roots was confirmed from observations of twenty different appressoria on mutant root sections.

Strong autofluorescence and toluidine blue staining were also observed in the cell wall thickenings of the Myc⁻, Nod⁻ pea *sym30* mutant, where the epidermis was in contact with *G. mosseae* appressoria (Gollotte et al., 1993). Here, it was proposed to reflect a wall reaction by the root cells to impeach fungal penetration into the root, so recalling plant defence responses involving phenolic accumulation in interactions between plants and pathogens (Graham and Graham, 1991).

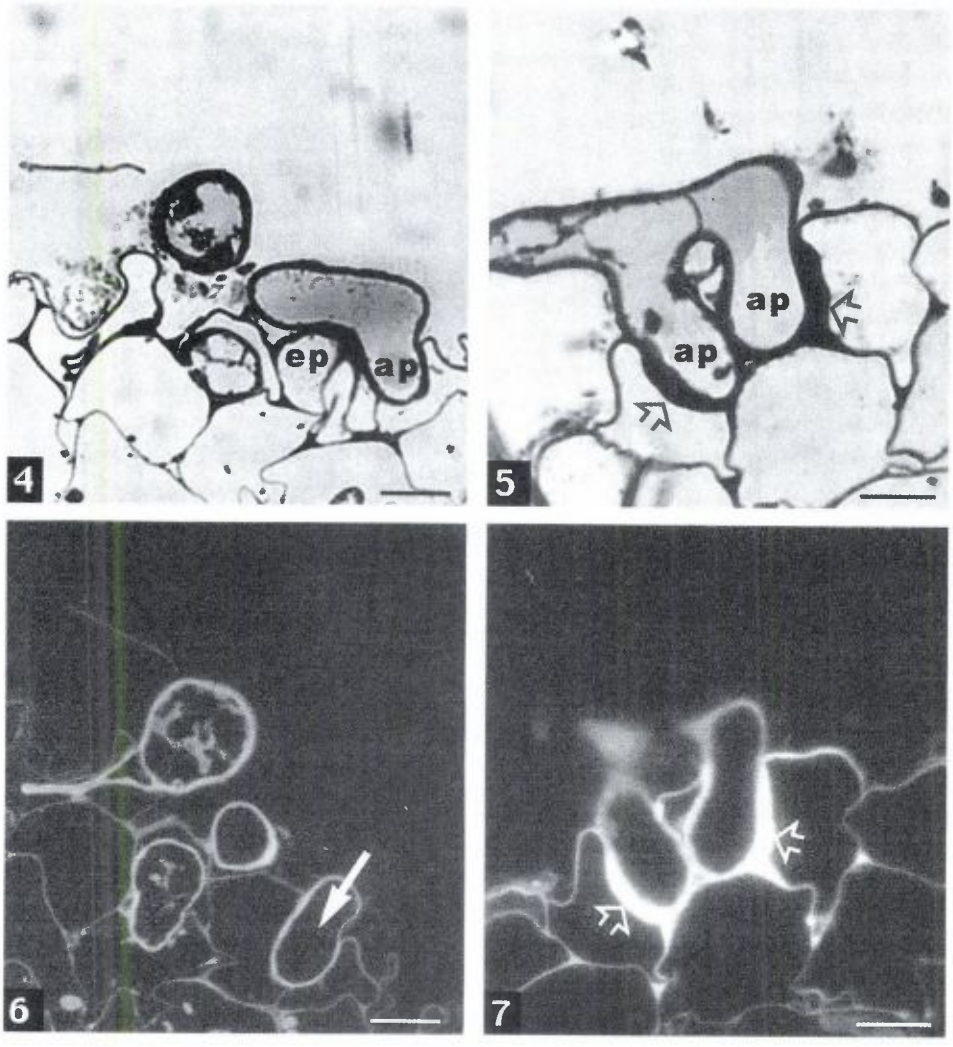
Ultrastructural observations of TR26 root sections showed that where *G. mosseae* penetrated between two epidermal cells, there was a thickening of the adjacent plant cell wall (Figs. 8 and 11) and/or a deposit between the fungus and root cell wall (Figs. 12 and 15), corresponding to the toluidine blue material.

Figs. 1–3. Light microscope observation of trypan blue stained roots of the wild type (J5) and the Myc⁻ mutant (TR26) genotypes of *Medicago truncatula* colonised by *Glomus mosseae*.

Figure 1. An extraradical hyphae (eh) forms a typical appressorium (ap), intercellular hyphae (ih) and arbuscules (ar) in association with roots of the wild type J5. Bar = 50 µm.

Figure 2. Development of *G. mosseae* on roots of the Myc⁻ mutant TR26 where external hyphae (eh) run over the root surface and form thick appressoria at epidermal cell junctions. Bar = 50 µm.

Figure 3. Development of *G. mosseae* on roots of the Myc⁻ mutant TR26 where external hyphal (eh) ramify intensely over the root surface leading to the formation of many "ill-defined" appressoria. Bar = 50 µm.



Figs. 4–7. Semithick sections of *M. truncatula* roots colonised by *G. mosseae* and embedded in LR White resin, observed under normal light after staining with toluidine blue (Figs. 4 and 5), or under blue light without staining (Figs. 6 and 7).

Figure 4. Appressorium (ap) formed on the root of the wild type J5 genotype. No thickening of the epidermal (ep) cell wall occurs around the appressorium. Bar = 10 µm.

Figure 5. Appressoria (ap) formed on the root of the Myc⁻ mutant TR26. A strongly stained thickening develops in the epidermal cell wall (arrows) in contact with the appressorium. Bar = 10 µm.

Figure 6. A serial section of the same root region as in Fig. 4. No strong fluorescence is observed around the appressorium (arrow). Bar = 10 µm.

Figure 7. A serial section of the same root region as in Fig. 5. A strong autofluorescence is observed around the appressoria (arrows) corresponding to the wall thickening shown in Fig. 5. Bar = 10 µm.

The JIM5 pectin antibody labelled the internal thickening of the epidermal cell wall (Fig. 11), and also the extracellular deposits between the root and fungal walls, confirming their plant origin (Fig. 12). The thickened epidermal cell wall and the extracellular deposit between the root and fungal wall were not labelled with β-1,3 glucan antibodies (Figs. 15 and 16), indicating that there was no accumulation of callose in these structures.

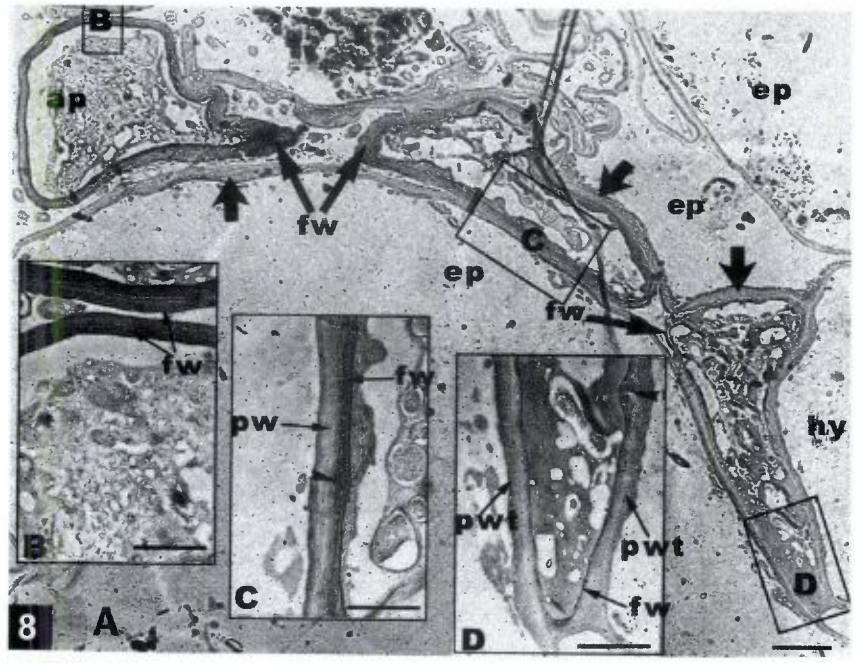
The lack of callose, but the presence of phenolics in the wall modifications produced in the TR26 *dmi2* mutant in reaction to the symbiotic fungus suggest a weak defence reaction, as compared to that observed in fungal pathogen interactions with resistant plants (Collinge et al., 1994) or with the Myc⁻ pea mutant in contact with *G. mosseae* (Gollotte et al., 1993). Indeed, in the pea mutant, callose was found in the thickening of the cell wall in contact with the appressoria, as is often the case of apposition wall layers layed down in response to pathogen invasion.

In most of the sections of TR26 mutant roots observed, the appressorial hyphae appeared to have disorganised or electron-dense cell contents (Figs. 8, 11, and 15), or were empty, although some hyphae at a distance from the root had active cell content (not shown). The appressoria and entry point hyphae observed on J5 roots were more frequently characterised by normal cell contents, with large vacuoles and scattered cytoplasm (not shown).

These observations suggest that appressoria on mutant TR26 roots were mainly inactive, as compared to those on J5 roots. Tisserant et al. (1993), have previously reported that a lower proportion of metabolically active appressoria or entry point hyphae of *G. intraradices* developed on roots of a Myc⁻ pea mutant than on wild-type pea roots.

Extraradical hyphae of *G. mosseae* frequently had thick, well-stratified walls which were labelled for β-1,3 glucans in the presence of both J5 (Fig. 13) and TR26 (Fig. 15) roots. It is well known that the appressoria cell walls of AM fungi develop into thickened, multilayered structures and that they become progressively thinner as hyphae penetrate root cells and develop into the cortical parenchyma (Gianinazzi-Pearson et al., 1981; Jacquelinet-Jeanmougin et al., 1987). This also occurred in the case of *G. mosseae* in the roots of the J5 genotype of *M. truncatula* (Figs. 9 and 10). However, *G. mosseae* appressoria did not develop thickened walls in contact with epidermal or hypodermal cells of the mutant TR26 root. Here, the fungal wall remained considerably thinner than that of the extraradical hyphae (Figs. 8 C and D, Fig. 15)

As previously reported for *G. mosseae* colonizing Myc⁺ pea on roots (Lemoine et al., 1995), the walls of extraradical hyphae and of the appressoria in contact with the root surface of the J5 genotype of *M. truncatula* contained β-1,3 glucans (Figs. 13 and 14). In contrast, no β-1,3 glucans were detected in the fungal wall of the appressoria developing on mutant TR26 roots, although the thick-walled extraradical hyphae were immunolabelled (Fig. 14). In addition, the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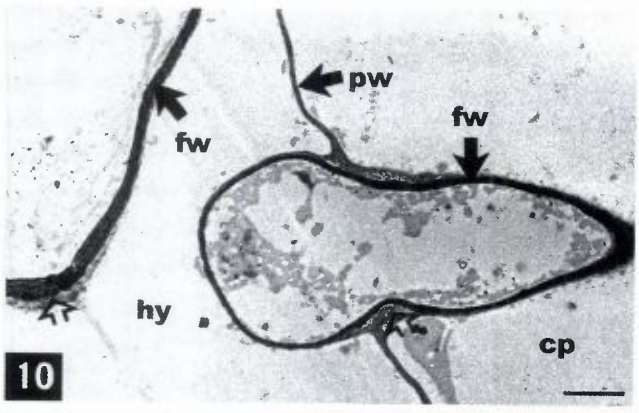
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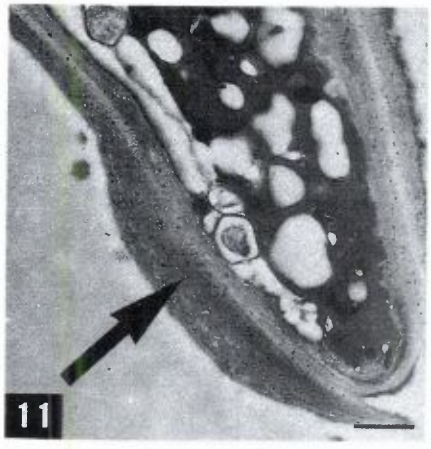
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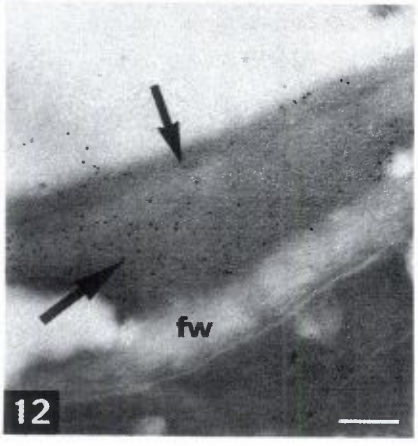
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surface of the wall of *G. mosseae* was irregular in contact with epidermal cells of TR26 roots (Figs. 15 and 16) suggesting some sort of lytic action. However, cell wall labelling with WGA was not different between extraradical and appressorial hyphae in association with either J5 or TR26 roots (not shown), suggesting no alteration in their chitin composition.

These different observations suggest that maturation of the hyphal wall of *G. mosseae* is somehow impeded in contact with roots of the Myc⁻ *dmi2 M. truncatula* mutant, so that there is not only a lack of thickening of the fungal wall in contact with roots, compared to wild type J5 roots, but also an alteration in the composition of the fungal wall.

In conclusion, the cellular bases of the Myc⁻ phenotype in the *dmi2 M. truncatula* mutant appears to differ from that of the Myc⁻ *sym30* pea mutant. The lack of penetration of *G. mosseae* into the roots of this Myc⁻ mutant of *M. truncatula* does not seem to be due to an important cell wall defence reaction as

Figs. 8–12. Thin sections of *M. truncatula* roots colonised by *G. mosseae* and treated with uranyl acetate (Fig. 9), the PATAg reaction (Figs. 8 and 10) or immunogold-labelled pectin antibodies (Figs. 11 and 12).

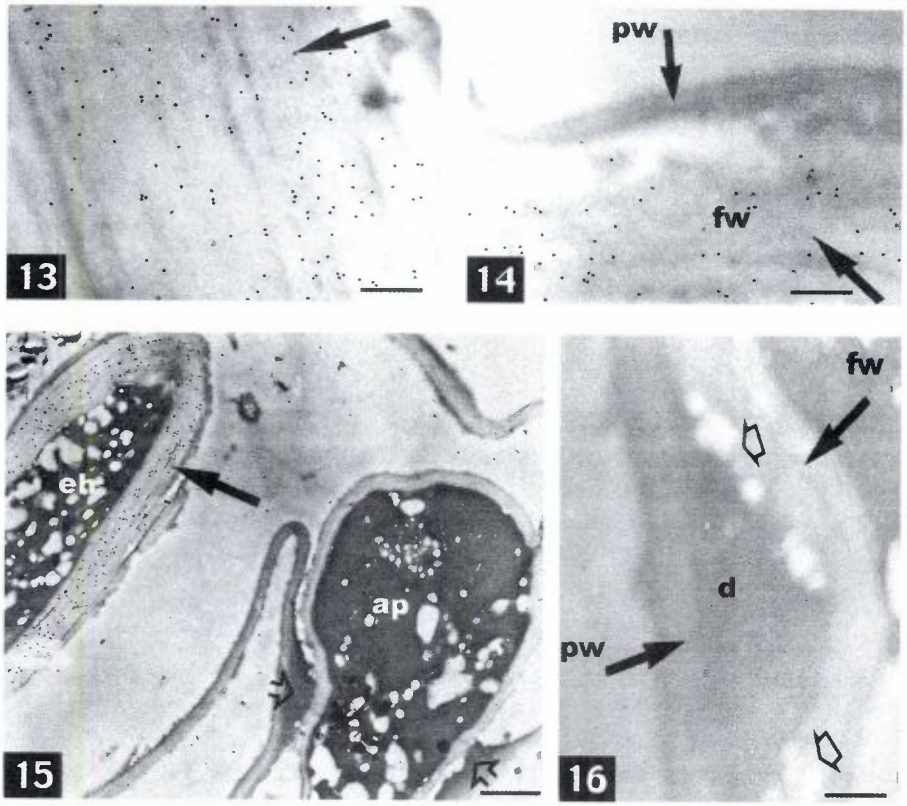
Figure 8. A. An appressorium develops on a Myc⁻ mutant TR26 root, between the epidermal cells (ep) up to a hypodermal cell (hy). The fungal wall (fw) becomes thinner in contact with the plant cells, whilst the plant cell wall thicker (short arrows) in contact with the fungus. Bar = 2 µm. B. Detail of the fungal wall of an extraradical hyphae running over the root surface. The fungal wall (fw) is well stratified and strongly stained with PATAg. Bar = 1 µm. C. Detail of the fungal wall in contact with an epidermal cell. The fungal wall (fw) is thin, and some dense material (arrow)is deposited between the fungal wall and the plant wall (pw). Bar = 1 µm. D. Detail of a hyphae at the epidermis/hypodermis junction. The fungal wall is extremely thin (fw) and we there is a thickening of the plant cell wall (pwt). Bar = 1 µm.

Figure 9. An appressorium (ap) formed on the root of the wild type J5 genotype has a thick and stratified wall. Bar = 1 µm.

Figure 10. Intracellular hyphae formed in hypodermal (hy) and cortical parenchyma (cp) cells of the genotype J5. The fungal wall (fw) thins out in the hypodermal (hy) and the cortical parenchyma. A light deposit of wall material is present around the hyphae (open arrows). The plant (fw) and the fungal (fw) cell walls are stained by PATAg reaction. Bar = 2 µm.

Figure 11. The wall thickening of epidermal cells of the TR26 mutant in contact with an appressorium (ap) contains immuno-labelled pectins (arrows) and suggests its plant origin. Bar = 1 µm.

Figure 12. The material deposited against the fungal wall (fw) on the root of the TR26 mutant (see also Fig. 15) is also immuno-labelled with pectin antibodies (arrow) which suggest its plant origin. Bar = 0.25 µm.



Figs. 13–16. Immunogold labelling of β-1,3 glucans on thin sections of *M. truncatula* roots colonised by *G. mosseae*.

Figure 13. The wall of an extraradical hypha close to a root of the J5 genotype is immuno-labelled (arrow). Bar = 0.25 µm.

Figure 14. The wall of an appressorium in contact with an epidermal cell of the J5 genotype contains β-1,3 glucans. The plant cell wall (pw) is not labelled. Bar = 0.25 µm.

Figure 15. Appressorium (ap) and external hyphae (eh) associated with TR26 roots. The external hyphae has a thick stratified cell wall which is richly immuno-labelled for β-1,3 glucans (plain arrow). There is no labelling over the thinner wall of the appressorium; a deposit is present between the fungal wall and the epidermal (ep) cell walls (open arrows). Bar = 1 µm.

Figure 16. Detail of the deposit (d) between the fungal (fw) and the plant cell walls in Fig. 15 showing that it is not labelled by β-1,3 glucans antibodies and indicating the absence of callose. This deposit is in close contact with the fungal wall which appears altered in some places (arrows). Bar = 0.25 µm.

in the case of the Myc⁻ pea mutant. There is, however, a reinforcement of the plant cell wall and this, together with a more direct action of the plant in

disorganising fungal cell contents and inhibiting wall maturation, may be sufficient to impede fungal progress into the root.

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