

Immuno-cytochemical localization of glycoproteins  
involved in recognition and attachment in a mycoparasitism.

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## ABSTRACT

Polyclonal antibodies prepared against the two glycoproteins ( $M_r$  100 and 85 kDa) involved in recognition and attachment of the mycoparasite, *Piptocephalis virginiana*, to its hosts, *Mortierella pusilla* and *Phascolomyces articulatus*, susceptible and resistant, respectively, were employed to localize the antigens at their cell surfaces. Indirect immunocytochemical technique using secondary antibodies labelled with either FITC or gold particles as probes, were used. FITC-labelled antibodies revealed a discontinuous pattern of fluorescence on the hyphae of *Mortierella pusilla* and no fluorescence on the hyphae of *Phascolomyces articulatus*. Intensity of fluorescence was high in the germinating spores of both the fungi. Fluorescence could be observed on *P. articulatus* hyphae pretreated with a commercial proteinase. Fluorescence was not observed on either hyphae or germinating spores of the nonhost *Mortierella candelabrum* and the mycoparasite *P. virginiana*. Antibodies labelled with gold conjugate showed a different pattern of antigen localization on the hyphal walls of the susceptible and resistant hosts. Patches of gold particles were observed all over the whole cell wall of the susceptible host but only on the inner cell wall layer of the resistant host. Cell wall fragments of the susceptible host but not those of the resistant host, previously incubated with the antibodies inhibited attachment of the mycoparasite. Implications of preferential localization of the antigen in the resistant host and its absence in the nonhost are described.

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## INTRODUCTION

In the investigation of the specificity of host-parasite interaction, one crucial question is how the host and parasite recognize each other, that is, how a given parasite can distinguish its hosts from many potential species and parasitize on them and how a given host can discriminate number of diverse parasites around it. Considerable evidence has demonstrated that the specificity is the consequence of recognition and is mediated by surface-localized complementary molecules. In many cases, the interaction have been shown to be between lectins (or agglutinins) and carbohydrate-containing molecules (Kojima, 1982; Barak et al. 1985). By now, several models have been proposed to elucidate the mechanisms of specificity in host-parasite interactions (Callow, 1987a; De Wit et al., 1988a & 1988b; Keen, 1988; Heath, 1981b). However, none is universally accepted and the detail of recognition process is still not clear.

Recent study in biotrophic mycoparasitism performed in our laboratory has shown involvement of lectin-like glycoproteins in the attachment and recognition of the biotrophic, haustorial mycoparasite *Piptocephalis virginiana* Leadbeater and Mercer to its fungal hosts (Manocha and Chen, 1990 & 1991). It was reported that the germ tubes of *P. virginiana* attach to the cell surface of the host but not to that of a nonhost (Manocha, 1984 & 1985; Manocha et al., 1986). Agglutination tests demonstrate that the agglutinating ability of the host *Mortierella pusilla* cell wall protein extract to agglutinate the spores of

mycoparasite *P. virginiana* is 37 times as high as that of the nonhost *Mortierella candelabrum*. SDS-PAGE of alkali extract from isolated cell wall fragments of susceptible host *M. pusilla* showed four prominent bands of  $M_r$  117 (a), 100 (b), 85 (c) and 64 (d) kDa which are absent in nonhost *M. candelabrum* except for the faint band c (Manocha and Chen, 1991). The host cell protein extract minus protein a and d did not affect agglutinating activity, however, crude extract minus b or c showed a significantly lower agglutinating ability than the complete extract and was similar to that of the nonhost cell wall protein extract. The agglutination units of the single glycoprotein b and c were very low, about 510-850 times less than those of the pure preparation containing both glycoproteins b and c (Manocha and Chen, 1991). In light of such evidence, it was hypothesized that these two high molecular weight glycoproteins may serve as two subunits of receptors on the host cell surface which interact with carbohydrate moieties on the cell surface of mycoparasite thus leading to attachment and further parasitic events.

The possible role of the glycoproteins b and c ( $M_r$  100 and 85 kDa) in recognition between the host and the mycoparasite is based on the results of agglutination and attachment tests. This indirect evidence can not explain the detailed mechanism for recognition process. Furthermore, the fact that glycoproteins b and c are from cell wall protein extract of susceptible host does not mean they are present at the cell surface of the susceptible host, or they are only present in the susceptible host. To evaluate their role in recognition and attachment, elucidation of their exact subcellular localization in hosts (susceptible resistant), nonhost and mycoparasite is imperative. The objectives of

this study are to: (i) characterize antibodies produced against the purified glycoproteins b and c from cell wall of *M. pusilla*; (ii) investigate the localization of the two glycoproteins (Mr 100 and 85 kDa) in the cells of hosts (susceptible and resistant), nonhost and mycoparasite; (iii) determine the effect of antibodies on attachment and appressorium formation by mycoparasite on cell wall surface of susceptible and resistant host fungi. For this study two complementary techniques are used with antibody probes either labelled with fluorescein isothiocyanate (FITC) or complexed with colloidal gold particles.

## LITERATURE REVIEW

### RECOGNITION BETWEEN HOST AND FUNGAL PARASITES

A cell associates with another cell or its products in a special way. By doing so it acquires information that is conveyed through chemical or physical signals in the process of recognition. "Recognition" in this sense, means an ability to discriminate materials present in the environment (Callow, 1984). "Molecular recognition" is considered to be the process of binding of receptors to complementary ligands (Burke et al., 1980). "Cell recognition" implies a series of cellular and biochemical events triggered by molecular recognition (Callow, 1984).

Specificity in plant disease may be considered to be a consequence of recognition. On the one hand, a potential pathogen may recognize features of a plant which signal its suitability for parasitism. On the other hand, the host plant may be able to detect or recognize a potential fungal pathogen as foreign and use the initial act of recognition to trigger a range of induced resistance mechanisms (Callow, 1988; Keen, 1982). Earlier studies on the specificity in host-parasite interactions were limited to host range, models of parasitism, nutrition and environmental factors that may modify the degree of parasitism and disease. In recent years, cytological and biochemical investigations, as well as modern techniques of molecular biology have contributed to the unravelling of the mechanisms which regulate plant-pathogen interactions.

The importance of molecular recognition between a plant and pathogen on cell surface is now universally accepted (Mazau et al., 1988). It is the intention of this review to consider how specificity in host-parasite interaction, involving mutual recognition of host and parasite, may be mediated by surface-localized complementary molecules. Necessary attention will be paid to our current understanding of the nature of specificity in plant host-fungal pathogen systems and mycoparasitic systems.

### **MODELS FOR MECHANISM OF RECOGNITION AND ATTACHMENT IN PLANT HOST-FUNGAL PARASITE RELATIONSHIPS**

For the past decade or so, laboratories around the world have been attempting to explore fundamental aspects of host-parasite interaction in terms of models implicating molecular recognition as a critical early determinant. After arrival of the fungal parasite at the surface of a potential host, a cellular confrontation between the parasite and its host takes place. There is considerable evidence that recognition at the cellular and molecular levels is due to specific interactions between constitutively produced molecules at the surface of the fungal parasite and its host cells. Several models for mechanisms of recognition in host-fungal parasite systems have been proposed by some authors according to their experimental results. These models are discussed in detail as follow:

## MODELS BASED ON THE GENETICS OF PLANT HOST-FUNGAL PARASITE INTERACTION

Although much of resistance to natural disease in plants lies in static, constitutive and preformed defensive barriers, it is now clear that plants also possess effective, inducible resistant mechanisms which are considered to involve the synthesis of phytoalexins, hypersensitive cell death or the formation of a structure barrier. It seems that these mechanisms are accompanied by changes in gene expression, involving RNA and protein synthesis (Ryder et al., 1986; Hadwiger et al., 1986).

### "Gene-for-gene" hypothesis

The specificity underlying the determination of resistance or susceptibility operates in many cases at the level of different host species exhibiting differential responses to distinct physiological races of the pathogen (Lamb et al. 1989). Detailed genetic analysis of a relatively small number of plant-pathogen interactions has shown a "gene-for-gene" interaction. That is, resistance or susceptibility in host species to distinct physiological races of a pathogen is determined by pairs of corresponding genes in the host and pathogens (Ellingboe, 1981). In such "gene-for-gene" interactions, a resistance gene in a particular host species confers resistance against physiological races that express the matching avirulence gene. This leads to genetic incompatibility characterized by the hypersensitive response (HR), which involves rapid death of the first infected cell and elaboration of a number of inducible defenses. A dominant resistance gene (R) in a

particular host species would only confer resistance against a particular physiological race of the pathogen if that race expressed a complementary dominant avirulence gene (A) (Ellingboe, 1981). There are two major implications of this "gene-for gene" hypothesis: First, resistance will be functionally dominant and result from molecular recognition between the products of the complementary genes in host and pathogen; second, the plant and its pathogens may contain a number of resistance and avirulence genes, respectively, but any gene combination determining incompatibility will be epistatic on other gene pairs conferring compatibility. The combination R:A interaction has been called "stop signal" (Lamb et al., 1989) since it triggers incompatibility.

Gene-for-gene systems are of great value in designing and testing biochemical hypotheses which seek to explain the nature of specificity. The high degree of specificity exhibited by gene-for-gene systems suggests that highly selective host receptors are capable of detecting specific features of parasite races. By direct analogy with other examples of biological recognition, it is suggested that the products of host resistance genes are surface-localized lectins or lectin-like molecules which serve as receptors, to recognize specific parasite signals which are most likely cell-surface localized or secreted carbohydrate-containing molecules (Callow, 1987). The resulting molecular interaction serves as a trigger for the induction of mechanisms leading to resistance or susceptibility (Callow, 1987).

A major question is how the avirulence gene products confer the avirulent phenotype. In the last several years, a number of bacterial and fungal avirulence genes have been isolated and identified



(Keller et al., 1989). The proteins encoded by the bacterial and fungal avirulence genes sequenced to date are hydrophilic and contain no readily identifiable signal sequences for extracellular transport (Keen, 1988). Therefore, it is not clear how these proteins could interact directly with a plant recognition factor such as the products of disease-resistance gene.

#### Elicitor and elicitor receptor model

An alternative hypothesis is that avirulence gene products act indirectly. In the absence of information on the biochemical mode of action of the direct products of defined avirulence genes, as putative products of avirulence genes which act as inducers of defence responses, various molecules have been isolated from cell walls and culture filtrates of different parasitic fungi. These molecules have come to be known as elicitors (Keen, 1975). A number of reviews have considered how plants may use a variety of polysaccharides or glycoprotein molecules commonly found in fungal cell walls or secretions as signals for the elicitation of host responses such as phytoalexin synthesis (Keen, 1982 & 1986; De Wit, 1988b; Dixon, 1986). Peptides, glycoproteins, and oligosaccharides derived from fungal wall polymers such as  $\beta$ -glucan, chitin, chitosan, and lipid fractions also have elicitor activity.

Race-specific elicitors, which should induce a response only in hosts on which the pathogen race is avirulent, have been predicted to represent direct, or indirect, products of avirulence genes (Keen, 1986). However, the vast majority of elicitors are not race specific (Dixon,

1986). Only a few reports provide good evidence for the isolation of race-specific elicitors from pathogenic fungi. A partially purified galactose/mannose-rich glycoprotein from the a race of *Colletotrichum lindemuthianum* induces phytoalexin accumulation in a bean species resistant to the a race, but not in the susceptible species (Tepper and Anderson, 1986). A necrosis-inducing polypeptide isolated from intercellular spaces of tomato leaves infected with *Cladosporium fulvum* species exhibit appropriate species specificity on differential tomato species (De Wit et al., 1988a).

A functional receptor for a race-specific elicitor would be a candidate for the product (direct or indirect) of the complementary host resistance gene. The plasma membrane offers a logical site for the receipt and transduction of stimuli originating from pathogens. However, significant progress on characterization of binding sites has been restricted to recent studies on binding to soybean cell membranes of a purified glucan elicitor from the cell wall of *Phytophthora megasperma* (Schmidt and Ebel, 1987). Binding to cell membranes was competed by a range of unlabelled elicitors, but not by inactive, chemically modified oligosaccharides, consistent with a signal receptor function for this binding site. Until such receptors are isolated and fully characterized particularly with respect to the binding of pure, synthetic elicitors of known structure, the existence of the elicitor receptor on the cell surface must still be considered as hypothetical. By now definitive evidence is lacking that the R gene products exert their effect through some special surface recognition event localized at the host cell surface involving host receptors. It is currently an open question whether the avirulence gene products interact directly with disease resistance gene

products and how this interaction determines the outcome of infection.

### Resistance suppressors-alternative model

It is unlikely that each plant has a specific and different gene to control the recognition of the myriad of potential pathogens in a manner analogous to the gene-for-gene relationship (Callow, 1987; De Wit et al., 1988a). It seems more probable that each plant species has evolved the ability to detect the majority of pathogens through a limited range of common surface components and the resulting molecular interaction serves to trigger resistance mechanisms. Specificity at the species level may be explained by the ability of successful or compatible pathogens to suppress or divert this general or non-specific recognition so that the fungus is recognized as "self", resulting in disease (Heath 1981a & 1981b, 1982; Ouchi and Oku 1981, Komura and Kobayashi, 1990). In other words, non-specific elicitor and specific suppressors produced by virulent races only, would work in concert resulting in the observed specificity *in vivo*. Some plant pathogens have been shown to release suppressors which inhibit or delay active defence mechanisms of host plants. This delay provides the pathogen with sufficient time to enter host tissues and establish successful colonization.

Biochemical evidence for suppressors in the induction of basic compatibility at the species level has been reported. Prior inoculation of a plant with a compatible pathogen permits a susceptible reaction to an otherwise incompatible pathogen (Ouchi and Oku, 1981). Incorporation of resistant genes into a basic compatible host permits

the new host to recognize the pathogen as "non-self" (Bushnell and Rowell, 1981). Komura and Kobayashi (1990) reported that prior attack of coleoptile cells by the pathogen *Erysiphe graminis* induced accessibility in those cells to a challenge by the non-pathogen *Erysiphe pisi*. In contrast, if the nonpathogen was applied to the cell first, it induced inaccessibility to *E. graminis*, the normally pathogenic fungus. These results suggest that *E. graminis* might have the ability to suppress the resistance mechanisms of the host cells and conversely that *E. pisi* might have some factor that enhances resistance mechanisms of host cells. Preparations which inhibit or suppress the response have been reported (Heath, 1981b). But until the active components of the preparations are isolated and characterized, it is difficult to assess the precise significance of suppressors as the determinants of basic compatibility at the species level.

### **MODELS BASED ON CELL SURFACE LIGAND AND RECEPTOR ACTIVITY**

The specificity of interactions between ligand and receptor on the cell surface may result in the specificity of plant-pathogen interactions. A few lines of evidence support such a notion.

#### **Lectins and agglutinins as the receptors on the surface of host cells**

To carry the requisite amount of specificity and variability it is quite likely that the elements involved are macromolecules. Many recognition phenomena are based upon carbohydrate binding by

protein or glycoprotein. Plant carbohydrate-binding proteins, known as lectins, have the ability to interact with different types of animal cells to produce various effects. The biological effect of the lectin is due to the binding of the lectin to carbohydrate-containing receptors localized at the cell surface (Callow, 1977). The binding of lectins to carbohydrate-containing molecules is both sufficiently variable and specific to permit discrimination against different pathogen races (Callow, 1987).

Quantitative and qualitative differences of agglutinins from host and nonhost plants were discovered. The amount of soybean agglutinin (SBA) in seeds of resistant species to *Phytophthora megasperma* was found to be about twice that of susceptible species. At the same concentration SBA from the resistant species was more inhibitory to mycelial growth than that from the susceptible species. Within different parts of a single plant host, the fungal pathogen *Phytophthora infestans* grew better on slices from the stem end rather than the rose end of potato tubers. Total haemagglutinating activity of the preparation from the stem end was 10 times higher and the specific activity was 30 times higher than that of the agglutinin from the rose end (Andrew and Daleo, 1988). These results show a correlation between the agglutinating activity of the agglutinin and the ability of the parasite to infect its host.

Many lectins or lectin-like agglutinin factors have been isolated from plants, but the majority of them are not race-specific. Kojima et al., (1982) reported that a lectin-like agglutination factor from sweet potato roots can agglutinate non-germinated spores of seven strains of *Ceratocystis fimbriata* including one parasitic on sweet potato. This

factor, however, also showed agglutinating activity with germinated spores of strains parasitic on hosts other than the sweet potato, while germinated spores of strains parasitic on sweet potato were not agglutinated. Furuichi et al. (1980) also reported that potato lectin is involved in binding the cell wall surface of *Phytophthora infestans* to cell-membrane. However, no race-specificity was found.

### Sugar residues and their receptors

The primary point of contact between the zoospores and the host is the root surface which is comprised of a polysaccharide-rich gel-like matrix, referred to as the root slime. This slime is secreted primarily by cells of the root cap (Rougier, 1981). The specific binding of zoospores of *Phytophthora cinnamomi* to maize roots (Hinch and Clarke, 1980) and *Pythium aphanidermatum* to cress roots (Longman and Callow, 1987) is inhibited if the root surface slime polysaccharide is oxidized with periodate. Mild trypsinization of *Pythium* zoospores, at concentrations which had no effect on motility, also inhibited the binding. Pretreatment of maize roots with the L-fucose-specific lectin from *Ulex europaeus*, or selective hydrolysis of terminal L-fucosyl-specific residues from the root surface slime, also inhibits the binding. Lectins with specificities for other sugar residues in slime polysaccharides, notably galactose, glucose and mannose, were relatively less effective in reducing spore binding. The zoospore attachment could be reduced by agglutinin and enzymic modification of the root surface mucilage polysaccharide, and by incubation of zoospores with exogenous root mucilage polysaccharide. These

discoveries indicate that the initial contact recognition between the zoospore and the host surface is specific and involves fucosyl residues on the root surface and a fucose-binding protein on the surface of the zoospore. Subsequent steps during the early stages of encystment involve release of a non-specific adhesive material from small peripheral vesicles of the zoospore (Gubler and Hardham, 1988 & 1989) which ensures continued attachment during germination and infection. The presence of cell surface receptors for fucosyl residues on *Phytophthora cinnamomi* has been verified by Kelleher et al. (1990).

#### Host-specific toxins and their receptors

At least 14 fungal plant pathogens have been defined to produce host-specific toxins (HTSs) clearly responsible for the determination of host-specific pathogenicity (Nishimura, 1987). Most of the reported HSTs are produced by species of *Alternaria* and *Helminthosporium* (Nishimura and Nakatsuka, 1989; Scheffer, 1989). It is now possible to explain host-parasite specificity in several diseases caused by necrotrophic pathogens in terms of host-toxins secreted by the fungus (Dunkle and Cantone, 1991). The pathogenic capacity of a number of fungi is strictly correlated with their ability to produce phytotoxin metabolites affecting only the host genotypes that are susceptible to the pathogen (Scheffer, 1984; Yoder, 1980). The most convincing demonstration of the molecular differences involved in this type of specificity has been obtained for the eyespot disease of sugar cane caused by *Helminthosporium sacchari*. Here, it is claimed that specificity involves the selective binding of the toxin

"Helminthosporoside" to membranes of the susceptible host. A binding protein of molecular weight 48 kDa has been isolated from membranes of the susceptible host and shown to consist of four subunits with at least two toxin-binding sites. The same protein has also been found in membranes of the resistant host but in a form which does not bind to toxin. Prior treatment of susceptible plant tissue with antiserum to the binding protein protects the tissue from the toxin. Sugar cane protoplasts from susceptible tissue were agglutinated by antiserum of the binding protein. These results indicate that the binding protein is localized in the plasm membrane (Scheffer, 1989). Other host-specific toxins may act in similar ways (Xiao and Tsuge, 1991).

### **SPECIFICITY OF HOST-PARASITE INTERACTIONS IN MYCOPARASITISM**

A great number of fungi have been observed growing on other fungi. In nature, most of these must be considered merely as fungal colonies until a nutritional relationship has been demonstrated. Mycoparasitism, a term now much in use, strictly means a fungus parasitic on another fungus. Based on the nature of parasitism, mycoparasites have been divided into two major groups (1) the necrotrophic mycoparasite which destroys the host cell and utilizes nutrients from the dying or dead host cell, (2) the biotrophic mycoparasite which is able to obtain nutrients from living cells and with no apparent harm to its host (Barnett and Binder, 1973). The phenomenon of specificity of attachment in fungus-fungus interactions has received attention only recently (Manocha and Chen, 1990).



Agglutinin and some sugar residues have been found to be involved in mediating attachment in mycoparasitism.

#### Specificity of attachment of mycoparasite to fungal host

In biotrophic mycoparasitism, the mycoparasite *Piptocephalis virginiana*, was reported to attach to the intact cell and cell wall fragments of the susceptible host fungal species, but not to the non-host fungal species (Manocha, 1985). Attachment of the germ tube of the mycoparasite to the host cell surface is a pre-requisite for parasitism. Failure to attach to the cell surface results in a non-host response (Manocha et al., 1986). The mechanism of recognition and attachment of *Piptocephalis virginiana* to the host but not to the non-host fungal species has been under investigation for some time, and a series of articles about this subject has been published (Manocha and Chen, 1990 & 1991). The cell walls of host and non-host fungi were thoroughly investigated. The gross cytological features and chemical composition of the cell walls did not reveal any difference between host and non-host species (Manocha, 1984). Manocha (1985) described a convenient method to quantify attachment of the parasite germ tubes to the isolated cytoplasm-free cell wall fragments of the host and non-host. The germ tubes of the mycoparasite attached irreversibly to the cell wall fragment of the susceptible host and formed a distinct appressorium at the points of contact similar to those observed on intact hyphal walls. This finding suggested that mycoparasite attachment and appressorium formation do not require a viable host. Germ tubes previously inactivated by exposure to 65°C for 15 minutes

or to 3% glutaraldehyde for 30 minutes did not attach to the host cell fragments. This suggested the requirement of vital activity of the germ tube in seeking out a specific binding site on host cell surface. The coiling of mycoparasites around the host hyphae as observed in a number of cases could be the result of such a search for binding sites.

Involvement of lectin and sugar in attachment of the mycoparasite to its fungal host.

In studying necrotrophic mycoparasitism between the yeast *P. guilliermondii* and a plant pathogen *Botrytis cinerea*, Wisniewski et al. (1991) found that the cell surfaces of both host and parasite play an important role in attachment of the yeast to the fungal pathogen. When trypsin or proteinase were present in the culture medium, the attachment was blocked. Pretreatment of *B. cinerea* with proteinase, trypsin, and 0.1 N HCl and NaOH inhibited attachment of *P. guilliermondii*. In general, the inhibition of attachment by protein degrading enzymes, NaOH and HCl indicates the involvement of protein or glycoprotein in recognition of carbohydrates at the cell surface (Manocha, 1990).

Nelson et al. (1986) showed that attachment of *Enterobacter cloacae* to hyphae of *Pythium ultimum* could be negated if sugars were applied. They concluded that a lectin-like binding was functioning in attachment. A lectin was also found to play a major role in the agglutination of spores of *Trichoderma harzianum* by culture filtrates of *Sclerotium rolfsii* and *Rhizoctonia solani*. This agglutination was blocked by both sugars and trypsin (Elad et al., 1983).

Attachment of *Trichoderma* conidia with *R. solani* can be promoted by pretreatment of the conidia with trypsin. Treatment with trypsin probably exposed receptors on the conidia which were responsible for attachment as was shown for *Fusarium* conidia (Kleinschnster and Baker, 1974). *Rhizoctonia* agglutinin activity could be inhibited by L-fucose and L-galactose. Glucose and fucose residues present on the mycoparasite surface may serve as receptors to *Rhizoctonia*. The fact that attachment was blocked by preincubation of fungus with specific carbohydrates indicates that an agglutinin on *R. solani* was involved in attachment and recognition.

In biotrophic mycoparasitism, much of the work has been carried out using a single mycoparasite species, *Piptocephalis virginiana* on *Choanephora cucurbotarum*. *P. virginiana*, with its host members restricted to the order *Mucorales*, is an obligate biotrophic mycoparasite and is being investigated currently in our laboratory (Manocha, 1987). Attachment of *Piptocephalis virginiana* to *Mortirella pusilla* is inhibited by the addition of sugar or by removal of binding sites with acid and alkali. Inhibition by sugar suggests that the attachment of *P. virginiana* to the host cell wall fragments is due to specific sugar residues at the host cell surface. Attachment is inhibited when parasite germ tubes are treated with wheat germ agglutinin before their application to the host cell wall fragments. This lectin specifically binds to the tip of the parasite germ tube (Manocha et al., 1990) .

Collectively, evidence indicates that a lectin or agglutinin may be involved in binding the fungal parasite to its host.

### Agglutinating activity of host and non-host cell proteins

In necrotrophic mycoparasitism, an agglutinin was isolated and purified from the extract and culture filtrate of *S. rolfsii* (Elad and Misaghi, 1985). The agglutinating activity of extract of the fungus was associated with the extracellular polysaccharide of *S. rolfsii* and the activity was specifically inhibited by D-glucose, D-mannose and several of their derivatives. The ability of different isolates of the mycoparasite *Trichoderma spp.*, to attack *S. rolfsii* was correlated with the agglutination of conidia of *Trichoderma* by *S. rolfsii* (Barak et al., 1985). This indicates that *S. rolfsii* agglutinin plays a role in specific attachment and recognition in fungus-fungus interaction.

In biotrophic mycoparasitisms, agglutinin tests demonstrate that crude protein extract from the host *M. pusilla* cell walls was able to agglutinate the mycoparasite spores and the agglutinin ability in the host cell wall extract was 37 times as high as that in the non-host *M. candelabrum* (Manocha and Chen, 1991). SDS-PAGE of alkali extracts from isolated cell wall fragments of host and non-host species showed quantitative and qualitative differences after staining with Coomassie brilliant blue and periodic acid-Schiff's reagents. Measurement of agglutinating activity with the mycoparasite spores showed that the host cell wall extract has a high agglutinating activity as compared with the non-host cell wall extract which has a very low activity. Four prominent bands of  $M_r$  117(a), 100(b), 85(c) and 64(d) kDa were observed in the cell wall extracts of host species, and all except for the band c, were absent in nonhost species (Manocha and Chen, 1991). The band c could be seen on gels of both the host and nonhost extracts but

was thin and faint in the latter. It is possible that the glycoproteins represented by these bands play a role in attachment and recognition of the mycoparasite. To determine which protein was involved in agglutination, the host cell protein extract minus one of these proteins was obtained by preparative PAGE followed by gel cutting and elution. Deletions of proteins a and d did not affect agglutinating activity. However, crude extract minus b or c showed a significantly lower agglutinating ability than the complete extract and was similar to that of the nonhost cell wall protein extract. The agglutination units of the single glycoprotein b and c were very low, about 510-850 times less than that of the pure preparation containing both glycoproteins b and c (Manocha and Chen, 1991). These results suggest that the two proteins b and c may be subunit of a receptor on the host cell. It was hypothesized that these two high molecular weight glycoproteins may serve as receptors on the host cell surface which interact with carbohydrate moieties on the cell surface of mycoparasite leading to attachment and further parasitic events.

## MATERIALS AND METHODS

### Parasite inoculum

Culture of biotrophic, haustorial mycoparasite, *Piptocephalis virginiana* Leadbeater and mercer was maintained on its susceptible host, *Choanephora cucurbitarum* (Berk. and Rav.) Thaxter. Axenic populations of spores of mycoparasite were obtained by growing cultures in 9 cm petri dishes at 23<sup>0</sup>C for two weeks in complete darkness which inhibits the sporulation of the host without interfering with the sporulation of the mycoparasite (Manocha 1985). The spores were harvested by adding sterile distilled water over the cultures and gently shaking the culture dishes for a few minutes. The resulting spore suspension was filtered through sterilized muslin cloth and was immediately washed twice by centrifugation. The spore pellet was washed three times with sterile distilled water by centrifugation (x 1000 g), then adjusted to a concentration of 10<sup>6</sup>/mL with malt-yeast extract (MYE) medium containing 5 gm of malt extract and 0.5 gm of yeast extract in 1 L of distilled water. The spores were allowed to germinate for 20 h at 23 <sup>0</sup>C. The germinated spores (80-90% germination), washed with sterile distilled water by centrifugation (x 1000g ), were resuspended in 0.01 M phosphate buffered saline (PBS), pH 6.8. These germinated spores were used for attachment tests and as well as immuno-fluorescence and immuno-cytochemistry.

### Host, non-host and resistant host inoculum

Cultures of susceptible host *Mortierella pusilla* Oudermans, resistant host *Phascolomyces articulatus* Boedjin ex. Benny and

Benjamin and nonhost *Mortierella candelabrum* V. Teigh and Le Monn, were routinely grown on MYE solid medium consisting of malt extract (20 g), yeast extract (2.5 g), and agar (20 g) in 1 L distilled water at  $23 \pm 1^{\circ}\text{C}$ . The spores of the host, nonhost and resistant host were resuspended in a liquid MYE (after adjusting their concentration to  $1 \times 10^5$ ) and germinated for 20-24 h at  $23^{\circ}\text{C}$  on a shaker incubator. The germinated spores were washed by centrifugation with sterile distilled water, suspended in 0.01 M PBS at pH 6.8 and used for immuno-fluorescence assay and immuno-cytochemistry.

**Preparation of cell wall fragments of susceptible host, nonhost and resistant host.**

For isolation of cell wall fragments, cultures of *M. pusilla*, *M. candelabrum* and *P. articulatus* were grown in MYE liquid medium for 24 h in an environmental incubator shaker (120 rpm) at  $23 \pm 1^{\circ}\text{C}$  (Manocha 1984). Mycelium was collected on filter paper in a Buchner funnel and was washed with distilled water until free of the medium. Washed mycelium was homogenized in cold PBS (1 gm wet weight of mycelium/5mL PBS) for 3x30 sec at the highest speed in a Sorvall Omni-mixer with the cup immersed in ice. The resulting slurry was centrifuged at 1500 g for 5 min and the pellet was resuspended in PBS. Additional cell disruption for 6-10 min with a sonicator (model W375, Heat Systems-Ultrasonic, Inc) released cell cytoplasm and membranes completely from the cell walls. The isolated cell walls were cleaned by repeated suspension in cold PBS and centrifugation until the supernatant appeared clear. Purity of isolated cell walls free from cytoplasmic and membrane contamination was determined as

described earlier (Manocha, 1984). The cleaned preparations were lyophilized and stored at  $-20^{\circ}\text{C}$  until further use.

#### Isolation of cellular proteins

Cell wall proteins were extracted by suspending (0.25 gm) in 10 mL of ice-cold 0.1 N NaOH and blending in a chilled Sorvall Omni-Mixer cup at full speed for 20 sec. The suspension was stirred for 18-20 h in an ice bath. The extract was centrifuged at  $8000 \times g$  for 10 min and the pellet was washed with ice-cold distilled water. The pooled supernatants were neutralized with 1N HCl at  $0^{\circ}\text{C}$  and were dialyzed overnight against distilled water. The extract was either lyophilized or used immediately for further purification.

The amount of solubilized proteins in the cell wall samples was determined according to the procedure of Bradford (1976) using bovine serum albumin as standard

#### Purification of glycoproteins and preparation of antibodies

Two cell wall glycoproteins from *Mortierella pusilla* were purified by Mr Y. Chen. Details of this method are found in Manocha and Chen (1991).

Antibodies to the two purified glycoproteins were prepared by Dr. R. Balasubramanian in our laboratory following conventional techniques. The immunoglobulin fraction was separated from the whole serum according to the method described by Hurn and Chantler (1980). The volume of immunoglobulin solution was measured and the protein concentration was calculated by measuring the absorbance of a 1:25



dilution at a wavelength of 280 nm using a cuvet of 1 cm path length. The protein concentration was calculated as  $(OD_{280} \times 25)$  and was found to be 1.34 mg/ mL.

### Dot-immunobinding assay of cellular total proteins

Dot-immunobinding assays of cellular total proteins were performed according to the methods outlined by Parent et al. (1985). Briefly, 2  $\mu$ L samples of cell protein extracts from *M. pusilla*, *P. articulatus* and *M. candelabrum* (each containing 1 mg/mL in 0.01 M PBS, pH 7.2) were applied as dots on the nitrocellulose strips and dried at room temperature. Unoccupied binding sites on the nitrocellulose were blocked by incubating the strips in 3% gelatin in 20 mL TBS (20 mM Tris and 500 mM NaCl, pH 7.5) for 1 h. The nitrocellulose was then transferred to antibody (raised in rabbit against the 100 and 85 kDa glycoproteins of *M. pusilla* cell walls) diluted to 1:10,000 (2  $\mu$ L in 20 mL) in TTBS (20 mM Tris, 500 mM NaCl and 0.05% Tween-20, pH 7.5) containing 1% gelatin for 1 h. After washing 3x30 min TBS, the nitrocellulose was treated for 1 h with secondary antibody (2  $\mu$ L/20 mL) diluted to 1:10,000 in TBS containing 1% gelatin. The secondary antibody is the goat anti-rabbit IgG-alkaline phosphatase conjugate antibody (from Promega corporation, Madison, WI, USA). After washing with TTBS 2x20 min and then with TBS 1x10 min, membrane was thereafter stained for antigen detection with Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)(purchased from Promega corporation, Madison, WI, USA) which are substrates of alkaline phosphatase. 120  $\mu$ L of 50 mg/mL NBT and 60  $\mu$ L of 50 mg/mL BCIP were added to 20 mL of 0.1 M TBS, pH 9.5 (containing 1

mM MgSO<sub>4</sub>). The strips were incubated in this solution for 15 min. Control tests were performed as follows: (I) omission of the primary antibody step; (II) omission of the secondary antibody step.

### **SDS-polyacrylamide gel electrophoresis**

Cellular proteins were separated by SDS-polyacrylamide gel electrophoresis. Briefly, cellular proteins were dissolved in a solution of 2% sodium dodecyl sulfate (SDS) reducing buffer (0.05 M Tris pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.001% bromophenol blue) and heated at 95°C for 4 min. Cellular protein samples (20 µg for *M. pusilla* and *M. candelabrum*, 16 µg for *P. articulatus*) were loaded on gels consisting of 12% acrylamide for the separation gel and 4% for the stacking gel. Electrophoresis was run at 200 V (constant voltage).

### **Western immunoblots of cellular total proteins**

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes as described by Towbin et al. (1979). After transfer, Unoccupied binding sites on the nitrocellulose membranes were blocked by incubating the membranes in 3% gelatin in 20 mL TBS for 1 h. The procedure for detection of proteins was the same as used for the dot immuno-binding assay. Omission of the primary antibody was performed as a control experiment.

### **Immuno-fluorescence Microscopy**

An indirect immunofluorescence technique was used involving fluorescein isothiocyanate (FITC)-labelled secondary antibody, which was affinity pure, goat anti-rabbit IgG (Fc fragment specific). The FITC-

antibody conjugate was purchased from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA.

To 1 mL of cell suspension obtained from 250 mL liquid culture grown at  $23\pm 1$  °C for 24 h, 9 ml of 1% paraformaldehyde was added dropwise under constant stirring at 4°C. After fixation for 30 min, the germinating spores were washed (centrifuged at 125 x g for 5 min at 4°C) three times with 0.01 M PBS, pH 6.8. The cells were resuspended in 1:10 dilution of primary antibody by adding 10 µL of antibody (1 mg/ml in 0.01 M PBS, pH 6.8) to 90 µL of 0.01 M PBS, pH 6.8. The suspension was incubated at 4°C for 30-60 min. The hyphae were then washed with PBS for three times at 4°C (centrifuged 125 x g for 5 min each time) to remove unbound primary antibodies. After that, the hyphae were resuspended in a 1:10 dilution of secondary antibody-FITC conjugate and incubated for 1-2 h at 4°C in darkness. Hyphae were then washed three times in PBS by centrifugation and a drop of the suspension was placed on a glass slide. A coverslip was placed on the drop of suspension and was sealed with finger-nail polish. Wet mounts of samples labelled with fluorescent-antibody were examined under fluorescence microscopy immediately.

Control tests were performed as follows: (i) omission of primary antibody; (ii) treatment of primary antibody with purified glycoproteins from cell wall glycoprotein of *M. pusilla* in excess i.e. 10 µL 1 mg/mL primary antibody and 30 µL 1 mg/mL glycoproteins and 60 µL PBS, incubated for 2 h at 4 °C.

The cells of *P. articulosus* were treated with proteinase in order to expose the glycoprotein antigens inside the wall. A commercial proteinase, pronase E, obtained from Sigma Chemical Co., St. Louis, MO,

U.S.A was used in this study. A stock solution of 0.1 mM pronase E was prepared in phosphate buffer at pH 6.8. A suspension of 1 mL of germinated spores and 1 mL of the stock solution of pronase E were incubated at 4 °C for 30 min in darkness and then washed with PBS twice. The germinated spores were then resuspended to 1 mL in sterile distilled water and then processed as described above.

### Processing for Electron Microscopy

Mycelia of *M. pusilla*, *M. candelabrum*, *P. articulatus* and *P. virginiana*, grown in MYE liquid medium for 24 h at 23°C, were washed with sterile distilled water to remove any medium components from the culture by centrifugation at 125 x g for 5 min at 4°C. The mycelia were fixed with 3% glutaraldehyde in 0.1 M PBS, pH 6.8 at room temperature for 2 h, then washed with PBS and postfixed with 1% Osmium tetroxide in 0.1 M PBS for 1 h at 4°C. After washing three times with cold sterile distilled water to remove Osmium tetroxide, the fixed cells were dehydrated in graded concentrations of ethanol and propylene oxide as described below:

60% ethanol in water	15 min
75% ethanol in water	15 min
90% ethanol in water	15 min
100% ethanol	15 min
100% ethanol	15 min
100% ethanol : propylene oxide 1:1	15 min
propylene oxide	10 min
propylene Oxide : Spurr's medium 1:1	30 min

Dehydrated mycelia were transferred to fresh Spurr's medium and left overnight at room temperature. Mycelia were transferred into fresh Spurr's medium and were cured at 70 °C for 8 h. Ultrathin sections were made with an LKB ultramicrotome using glass knives. The sections were mounted onto uncoated 300 mesh Nickel grids.

### **Immuno-cytochemistry**

An indirect method for gold labelling was performed. The antibody which was raised in rabbit against the 100 and 85 kDa glycoproteins of *M. pusilla* cell walls as described above was used as the primary antibody. The secondary antibody was goat anti-rabbit IgG complexed with 10 nm colloidal gold particles. The goat anti-rabbit IgG-gold conjugate was purchased from Biocell Laboratories. Tris-HCl buffer (0.02 M, pH 7.2 and 8.2) containing 0.5 M NaCl and 1% (w/v) bovine serum albumin (Sigma Chemical) was used for all rinsing steps and for dilution of antibody. Sections were labelled by immersing grids in drops (10 µL) of antibody solution in petri dishes. The following protocol was used. Sections were treated for 2-3 min at 22 °C with 3%

Hydrogen Peroxide, rinsed with distilled water for 10 sec and incubated for 1 h at 22 °C in normal goat serum (Sigma Chemical) diluted 1:10 with pH 7.2 buffer. The sections were then treated with primary antibody (rabbit anti glycoprotein from *M. pusilla* cell wall) diluted 1:25 with pH 7.2 buffer for 2 h at 22°C. They were rinsed for 10 sec with pH 8.2 buffer and then treated with secondary antibody (goat anti-rabbit IgG) labelled with 10 nm colloidal gold particles (Sigma Chemical) diluted 1:10 with pH 8.2 buffer, for 30 min at 4 °C. The sections were rinsed with pH 7.2 buffer for 10 sec followed by a rinse with distilled water. They were stained for 5 min at 22°C with 2% uranyl acetate (in CO<sub>2</sub>-free distilled water), rinsed for 10 sec with CO<sub>2</sub>-free distilled water and then stained with Reynolds' lead citrate (Reynolds, 1963) for 10 min at 22 °C. The sections were rinsed for 10 sec with 0.02 N NaOH and for 10 sec with CO<sub>2</sub>-free distilled water, and they were examined with a Philips 300 TEM at 60 KV.

### **Immuno-cytochemical Controls.**

Specificity of labelling was assessed through different controls: (i) incubation of the section directly with secondary antibody-gold complex only, the primary antibody step being omitted; (ii) incubation with the primary antibody to which was previously added an excess of the purified glycoprotein from *M. pusilla* cell wall. (iii) the primary antibody was replaced by the pre-immune rabbit serum.

### **Inhibition of attachment by Ab-G**

Measurement of parasite attachment was tested according to the method outlined by Manocha et al. (1985). Isolated cell walls were suspended in sterile distilled water (1 mg/mL) and 0.1 mL of this

suspension was spread over an area of 15x20 mm on a glass slide. The slides were air dried to fix cell wall fragments at room temperature. Germinated spores (80-90%) of parasite were spread over the cell wall area. The glass slides were incubated for 2 h at room temperature, then washed twice (15 sec each washing) under a stream of slow running distilled water and were stained with Lactophenol-cotton blue. The germinated spores attached to the cell wall fragments were counted from 20 randomly selected fields on each slide using a dry high power (40x) objective. Each experiment was run in triplicate. The percentage of attachment and the relative percentage of appressorium formation were calculated.

*M. pusilla* cell wall fragments (air-fixed on the slides) were incubated with 0, 0.05, 0.15, 0.25, 0.35, 1.0 mg of antibodies per mL of 0.01 M PBS, pH 6.8 for 2 h at 4 °C. Cell wall fragments were also incubated with pretreated antibodies (mixed 100 µL 1 mg/mL antibodies with 200 µL 1 mg/mL purified glycoprotein from cell walls of *M. pusilla* and incubated for 2 h at 4 °C), and with normal rabbit serum (100 µg/300 µL of 0.01 M PBS, pH 6.8 ). Cell wall fragments of nonhost *M. candelabrum* were used as a control experiment.

For *P. articulatus*, the same protocol was used and the concentration of antibody used was also the same. Cell wall fragments of *M. pusilla* and *M. candelabrum* were used as control experiments.

The slides were washed three times (15 sec for each washing) under slowly running sterile distilled water to remove any unbound antibodies. 100 µL of 80-90% geminated spores suspension was spread over the slide as described before. The percentage of attachment and relative percentage of appressorium formation were calculated.

## RESULTS

### Specificity of antibodies

Specificity of antibodies raised in a rabbit against the two cell wall glycoproteins ( $M_r$  100 and 85 kDa) of *Mortierella pusilla* (Ab-G) was determined by (i) Dot-immunobinding assay and (ii) Western immunoblots.

#### (i) Dot-immunobinding assay of total cellular proteins

Antigen-antibody interaction was determined by a recently introduced dot-immunobinding procedure. The results are shown in Figure 1. Ab-G cross-reacted strongly with the protein extracts from *M. pusilla* and *P. articulatus* cells, but did not react with the protein extract from *M. candelabrum* cells. The total absence of a color reaction with all control tests demonstrated the specificity of the antigen-antibody reaction. These results indicate that the antibody prepared against the two glycoproteins ( $M_r$  100 and 85 kDa) is specific for the proteins of *M. pusilla* and *P. articulatus*. In order to show which proteins in *P. articulatus* and *M. pusilla* cross-react with the antibody, western immunoblots were performed.

#### (ii) Western immunoblots of total cellular proteins

Western immunoblots (Fig. 2) show that Ab-G specifically react with two protein bands with molecular masses in the range of 100 and 85 kDa in both *M. pusilla* and *P. articulatus*.



Fig 1. Dot-immunobinding detection of antigen-antibody reaction.

- Row. 1. Reactions obtained after treatment of total proteins with Ab-G and goat anti-rabbit IgG alkaline phosphase conjugate antibody. In Mp, Pa, Mc, total proteins of *M. Pusilla*, *P. articulatus* and *M. candelabrum* were used as antigen respectively. A significant colored reaction occurred for Mp and Pa
- Row. 2. Reactions obtained after treatment of the same antigen directly with goat anti-rabbit IgG alkaline phosphase conjugate antibody. No response can be detected.
- Row. 3. Reactions obtained after treatment of the same antigen only with Ab-G. Results are negative indicating the absence of nonspecific binding of the secondary antibody.

**Mp Pa Mc**


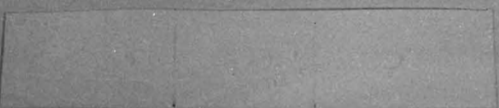

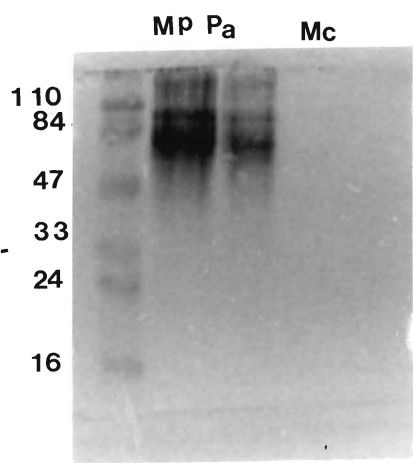
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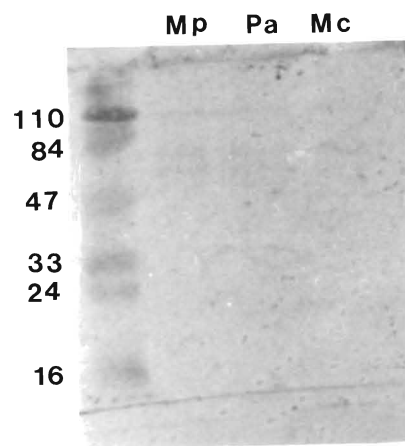
Fig 2. Immunoblot analysis of total proteins of *M. pusilla*, *P. articulatus* and *M. candelabrum*

- ( A ) An immunoblot of total proteins of *M. pusilla*, *P. articulatus* and *M. candelabrum* incubated with Ab-G. Ab-G cross-reacted with proteins of *M. pusilla* and *P. articulatus* (both at about  $M_r$ 100 and 85 kDa)
- (B) An immunoblot of total proteins from *M. pusilla*, *P. articulatus* and *M. candelabrum* incubated with normal rabbit serum. No cross-reactivity was observed.

The marker lane was that of low molecule range prestained protein standards (purchased from Bio-Rad) as follows:  
Phosphorylase B (rabbit muscle), 100 kDa; Bovine serum albumin, 84 kDa; Ovalbumin (hen egg white), 47 kDa; Carbonic anhydrase (bovine), 33 kDa; Soybean trypsin inhibitor, 24 kDa; Lysozymes (hen egg white), 16 kDa.



A



B

No protein from *M. candelabrum* was observed to react with the antibody. An immuno-blot of total protein of *M. pusilla*, *P. articulatus* and *M. candelabrum* incubated with normal rabbit serum did not show cross-reactivity (Fig. 2B).

### **Localization of two glycoprotein antigens on host, nonhost and mycoparasite**

Localization of cell wall glycoproteins ( $M_r$  100 and 85 kDa) was determined by using the complementary techniques of (A) Immuno-fluorescence microscopy and (B) Immuno-cytochemistry with gold labelling.

#### **(A) Immuno-fluorescence Microscopy**

Intact hyphal cells and germinated spores were investigated with the use of indirect immuno-fluorescence techniques. Both primary and secondary antibodies were usually diluted to 1:10.

Table 1 summarizes the binding affinity of secondary antibody-FITC conjugate for the surfaces of the mycoparasite and its hosts (susceptible and resistant) and nonhost fungi, which were previously treated with primary antibodies. The results are based on five replicate experiments. The secondary antibody-FITC conjugate did not bind to the surfaces of the mycoparasite, nonhost and the hyphae of the resistant host. Intense fluorescence, on the other hand, was observed on the surfaces of the hyphae and germinated spores of the susceptible host, *M. pusilla*, and on the surface of the germinated spore

of the resistant host, *P. articulatus*.

### **Immunofluorescence labelling pattern over *M. pusilla*.**

Figures 3, 4 A and B illustrate that germinated spores and hyphae of *M. pusilla* that were pretreated with primary antibodies could bind FITC-secondary antibody conjugate at their cell surface. The fluorescence distribution on cell surface is discontinuous. The fluorescence is absent in some areas of cell surface and very strong in other areas. The morphological comparison as observed by phase contrast microscopy and fluorescence microscopy showed no difference between the areas of fluorescence and non-fluorescence on the cell surface. A difference in fluorescence intensity can also be observed. The germinated spores always showed the most intense fluorescence (Fig. 3). The binding of the secondary antibody-FITC conjugate to the cell surface of *M. pusilla* was completely eliminated by preincubation of the primary antibody with the  $M_r$  100 and 85 kDa glycoproteins (Fig. 5) and by the omission of the primary antibody (Fig. 6). When normal rabbit serum was used instead of Ab-G, only slight autofluorescence was observed on the surface of hyphae and germinated spores.

Table 1. Comparison of intensity of binding of fluorescent antibody on germinated spores and hyphae of hosts *M. pusilla* and *P. articulatus* and non-host *M. candelabrum* and the mycoparasite *P. virginiana*

Fungal species	Hyphae (or germ tube)	Germinated spores
<i>Mortierella pusilla</i>	+++/**	+++
<i>Phascolomyces articulatus</i>	-	+++
<i>Mortierella candelabrum</i>	+/-	-
<i>Piptocephalis virginiana</i>	-	-

Intensity of fluorescent labelling is shown as follows: +++, strong, ++, moderate; +, weak; -, unlabelled. Variable reaction in labelling intensity is indicated.

Fig. 3. Light and fluorescence photomicrographs of germinated spores and hyphae of *M. pusilla* which were treated with Ab-G and FITC-goat anti-rabbit IgG conjugates (both primary and secondary antibody diluted 1:10). Note the strong fluorescence on the surface of germinated spores as compared to the hyphae. x 1,092.





3

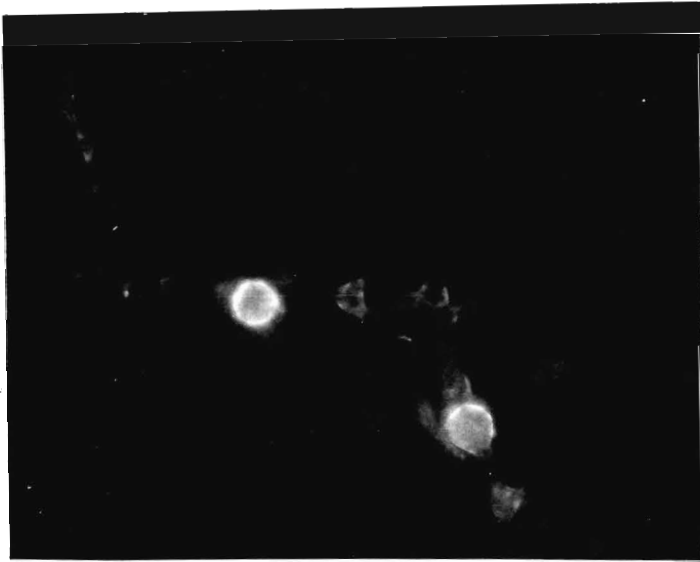


Fig. 4. Light and fluorescence photomicrographs of hyphae of *M. pusilla* treated with Ab-G and FITC-goat anti-rabbit IgG conjugates (both primary and secondary antibody diluted 1:10). Note the patchy and discontinuous fluorescence pattern on the hyphae. The variation in the intensity of fluorescence can not be attributed to focusing of the hyphae in the microscope. A and B: x 2,730.

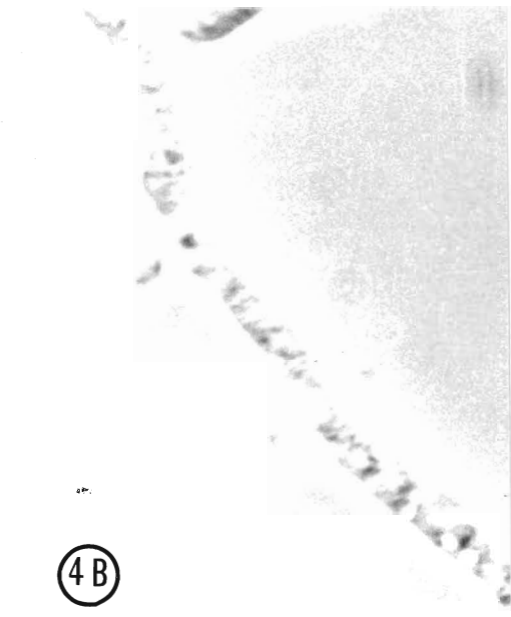
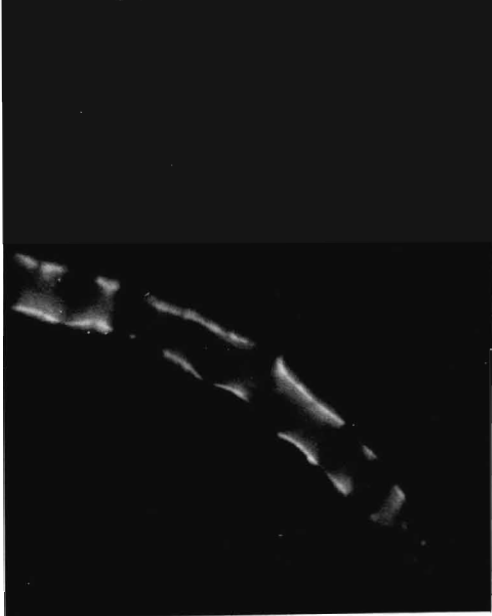
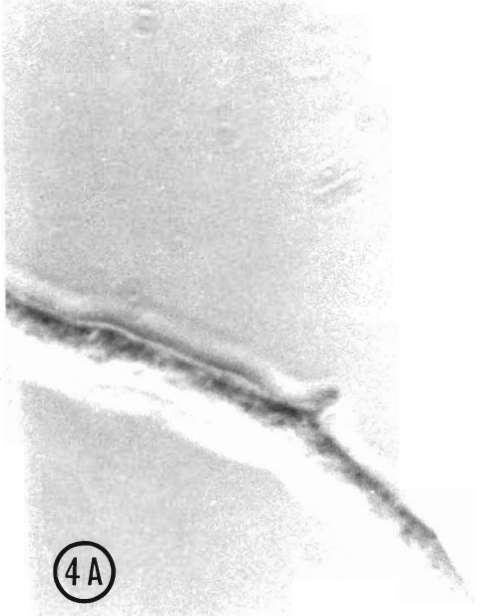
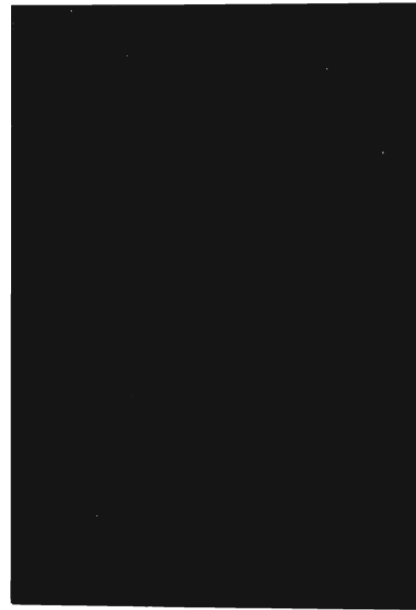
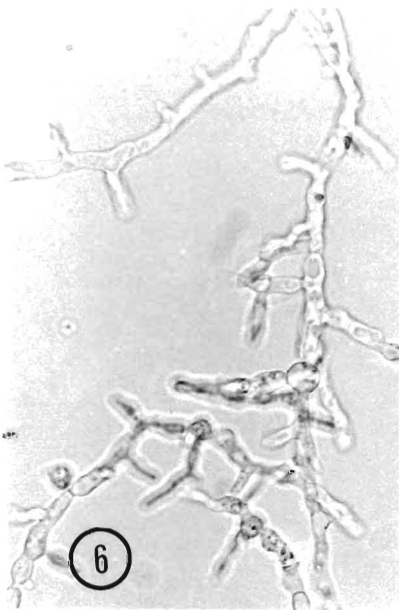
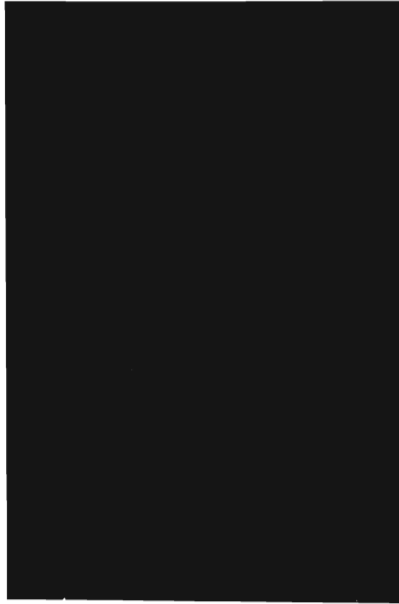
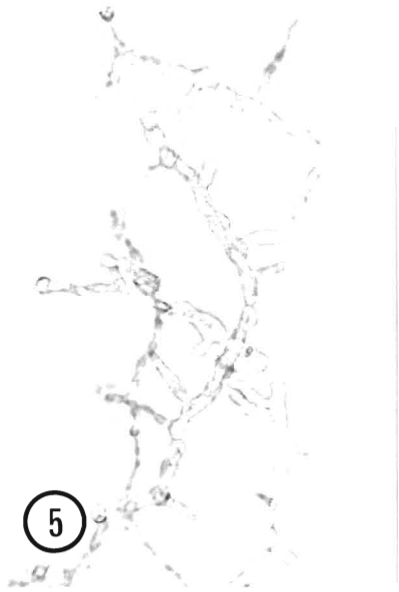


Fig. 5. Control Experiment. Light and fluorescence photomicrographs of hyphae and germinated spores of *M. pusilla* treated with Ab-G and FITC-goat anti-rabbit IgG conjugates (both primary and secondary antibody diluted 1:10 ). Ab-G was previously adsorbed with purified glycoproteins from cell wall of *M. pusilla*. Only weak autofluorescence can be observed. x 1,092.

Fig. 6. Control Experiment. Light and fluorescence photomicrographs of hyphae and germinated spores of *M. pusilla* which were treated with FITC-goat anti-rabbit IgG conjugates (diluted 1:10) only. Note the very weak autofluorescence on hyphae. x 1,092

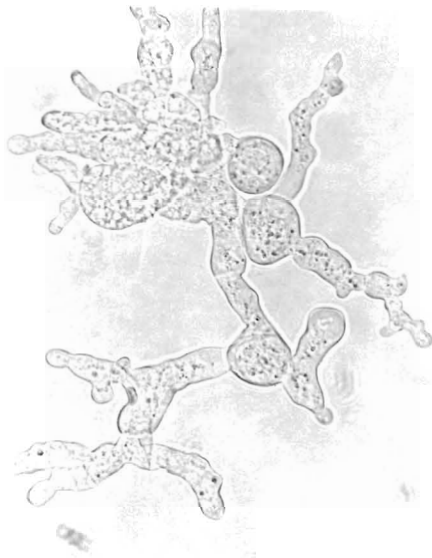


### **Immunofluorescence labelling pattern over *P. articulosus*.**

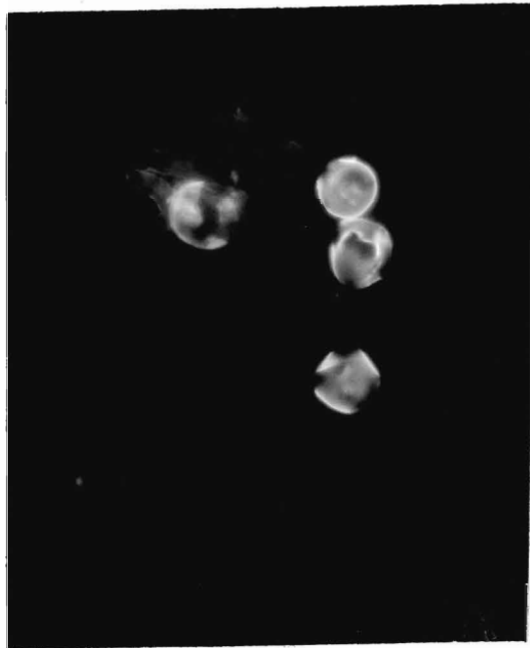
Different fluorescence patterns were observed on the surface of germinated spores and hyphae of *P. articulosus*. It is clear that the binding of secondary antibody conjugate was restricted to the surface of germinated spores (Figs. 7 A & B) previously treated with the primary antibody. There was only weak fluorescence at the surface of the hyphal cells, probably the auto-fluorescence. The fluorescence present on the surface of germinated spores of *P. articulosus* was as intense as on that of *M. pusilla*. Higher concentrations of primary antibody and/or secondary antibody-FITC conjugate did not extend the fluorescence from surface of germinated spores to that of the hyphal cells and the intensity of fluorescence on the surface of germinating spores remained constant. The fluorescence was reduced to the level of auto-fluorescence by preincubation of the primary antibody with purified glycoproteins from cell wall of *M. pusilla* (Fig. 8) and the omission of primary antibody (Fig. 9). Auto-fluorescence was observed on the surface of both hyphal cells and germinated spores.

However, the pretreatment of hyphal cells of *P. articulosus* with pronase E (0.05 mM in 0.02 M PBS at pH 6.8 for 30 min) followed by treatment with primary and secondary antibody increased the fluorescence (Fig. 10). This fluorescence on hyphae can not be attributed to auto-fluorescence. Higher concentrations of pronase E can remove the fluorescence on both germinated spores and hyphae.

Fig. 7. Light and fluorescence photomicrographs of germinated spores and hyphae of *P. articulatus* treated with Ab-G and FITC-goat anti-rabbit IgG conjugates (both primary and secondary antibody diluted 1:10). Note the strong fluorescence on the germinated spores and the absence of fluorescence on the hyphae. A and B: x 1,092.



7A



7B

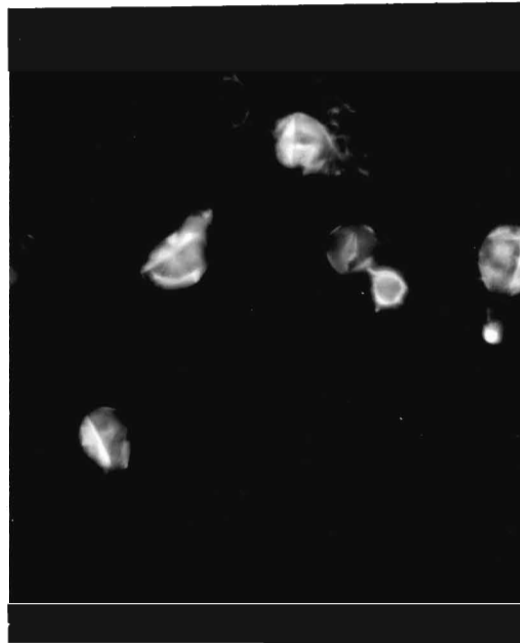


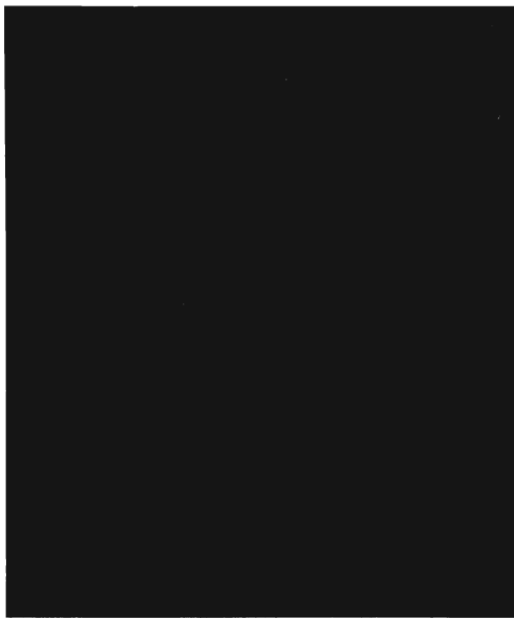


Fig. 8. Control Experiment. Light and fluorescence photomicrographs of germinated spores and hyphae of *P. articulosus* treated with Ab-G and FITC-goat anti-rabbit IgG conjugates (both primary and secondary antibody diluted 1:10, secondary antibody diluted 1:10). Ab-G was previously adsorbed with purified glycoproteins from the wall of *M. pusilla*. Only auto-fluorescence can be observed. x 1,092.

Fig. 9. Control Experiment. Light and fluorescence photomicrographs of germinated spores and hyphae of *P. articulosus* treated only with the FITC-goat anti-rabbit IgG conjugate (diluted 1:10 ). Note the very weak auto-fluorescence. x 1,092



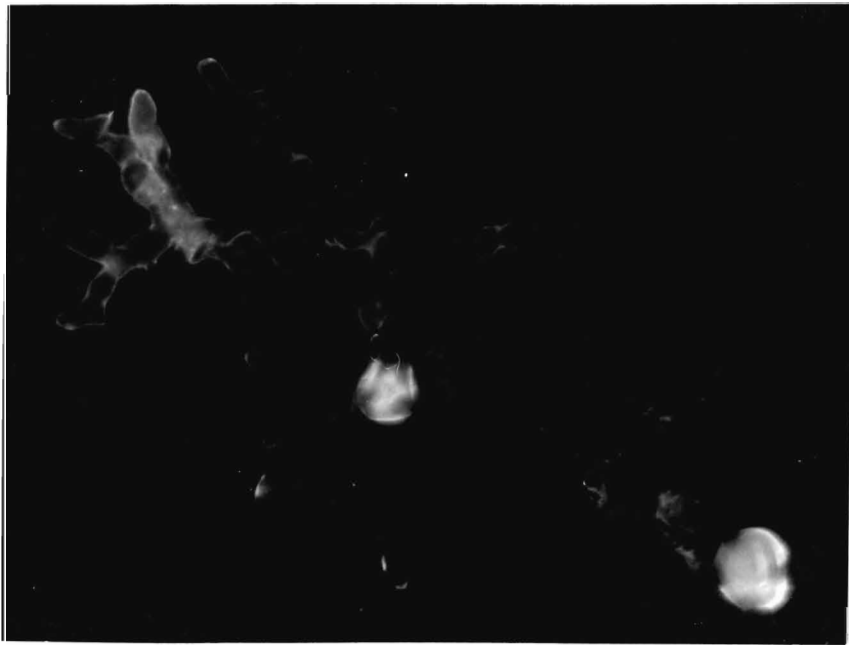
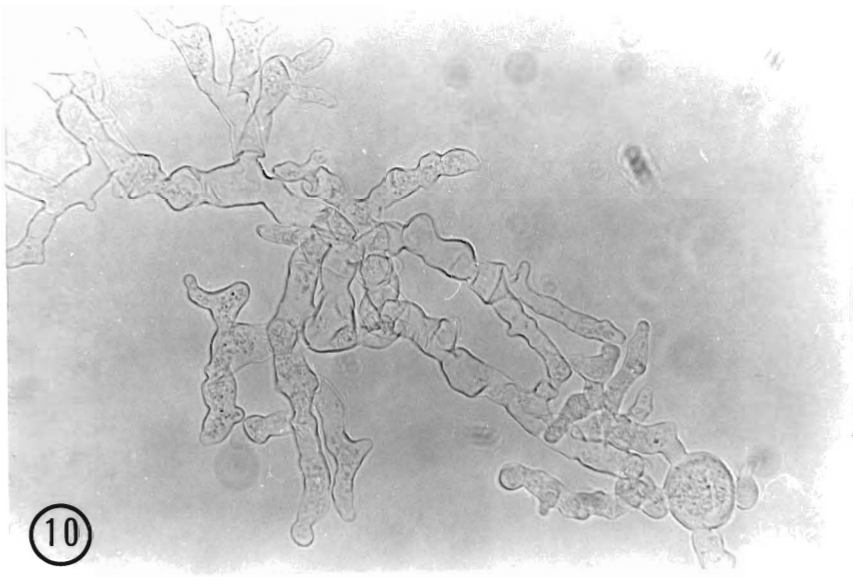
8



9



Fig. 10. Light and fluorescence photomicrographs of germinated spores and hyphae of *P. articulosus* treated with Ab-G and FITC-goat anti-rabbit IgG conjugates (both Primary and secondary antibody diluted 1:10). Germinated spores and hyphae were previously incubated with pronase E (0,05 mM in 0,02 M PBS at pH 6,8) for 30 min. Note evenly distributed fluorescence on the hyphae. x 1,092.



### **Immuno-fluorescence labelling pattern over *M. candelabrum*.**

Some fluorescence spots were observed on the hyphae of *M. candelabrum* treated with the primary and secondary antibodies (Fig. 11). Higher concentrations of primary antibody and secondary antibody or prolongation of incubation time did not extend the fluorescence. The fluorescence spots could be removed by omission of the primary antibody (Fig. 12). Auto-fluorescence was also observed.

### **Immunofluorescence labelling pattern over *P. virginiana*.**

No fluorescence was observed on the surface of germinated *P. virginiana* spores or germ tubes treated with both primary and secondary antibodies (Fig. 13).

### **(B) Immuno-cytochemistry**

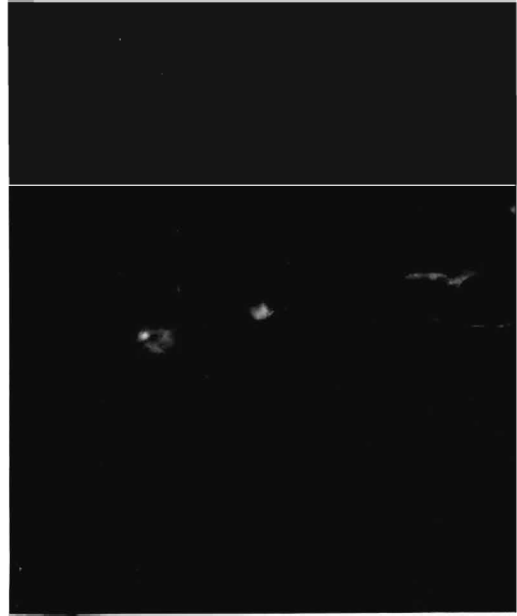
An indirect method of immuno-gold labelling was used as described under Materials and Methods. The best results were obtained when thin sections were treated for 2 h at 22 °C with Ab-G diluted 1:25, followed by 1 h incubation at 4 °C with goat anti-rabbit IgG-gold complex diluted 1:10 in PBS. As for any cytochemical experiment, the success of the indirect immuno-gold method is entirely dependent on the maintenance of receptor sites and on the good preservation of relevant structures. In this experiment, the double fixation (Glutaraldehyde and OsO<sub>4</sub>) of the hyphal cells did not alter the glycoprotein antigenicity and gave a satisfactory preservation

Fig. 11. Light and fluorescence photomicrographs of hyphae of *M. candelabrum* treated with Ab-G and FITC-goat anti-rabbit IgG conjugates (both primary and secondary antibody diluted 1:10). Note the fluorescence spots on hyphae. x 1,092.

Fig. 12. Light and fluorescence photomicrographs of hyphae of *M. candelabrum* treated with FITC-goat anti-rabbit IgG conjugates (diluted 1:10). Note the faint fluorescence on germinated spores and hyphae. x 1,092.



11



12

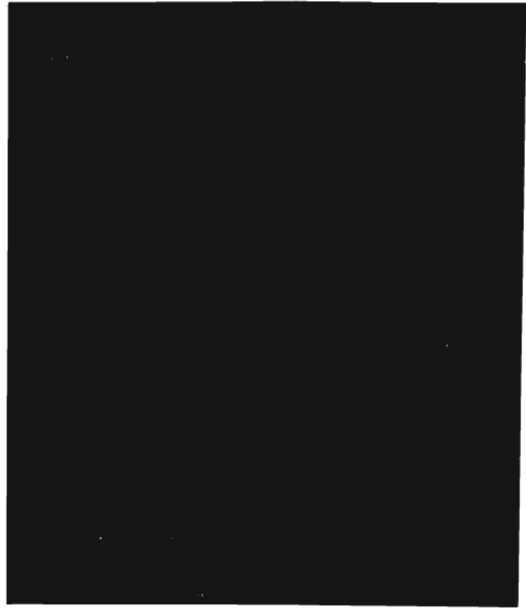


Fig. 13. Light and fluorescence photomicrographs of germinated spores of mycoparasite, *P. virginiana* treated with Ab-G and FITC-goat anti-rabbit IgG conjugates (both primary and secondary antibody diluted to 1:10). No fluorescence can be observed. x 2,730





13



of cell's fine structure. The best results yielding low nonspecific background staining were achieved when relatively highly diluted antibody was used. Table 2 summarizes the distribution of  $M_r$  100 and 85 kDa glycoproteins in the main cell substructures of mycoparasite and its hosts (susceptible and resistant), and nonhost species.

**(i) Immunogold labelling pattern over *M. pusilla*.**

Figures 14 and 15 illustrate the gold labelling of *M. pusilla* cells after incubation with the antibody raised against *M. pusilla* cell wall glycoproteins and the secondary antibody-gold conjugate. A qualitative evaluation of the labelling revealed that the gold particles were unevenly distributed over the whole cell walls (Fig.14). Most of the gold particles were found in groups over the cell wall and cytoplasmic membranes (Figs. 14 and 15 A-D). Labelling over the plasma membranes was as intense as over the cell walls (Figs. 15 A-D). All other cytoplasmic organelles were free of labelling. Interestingly, these results seem consistent with the result of earlier experiments with FITC-secondary antibody conjugates which showed the discontinuous distribution pattern of fluorescence on the cell surface. The multilayered structure of the cell wall could be identified in some micrographs, but the preferential distribution over various layers was not observed.

The specificity of this immuno-cytochemical labelling was verified by several controls. When sections were treated with secondary antibody-gold conjugate only (primary antibodies omitted),

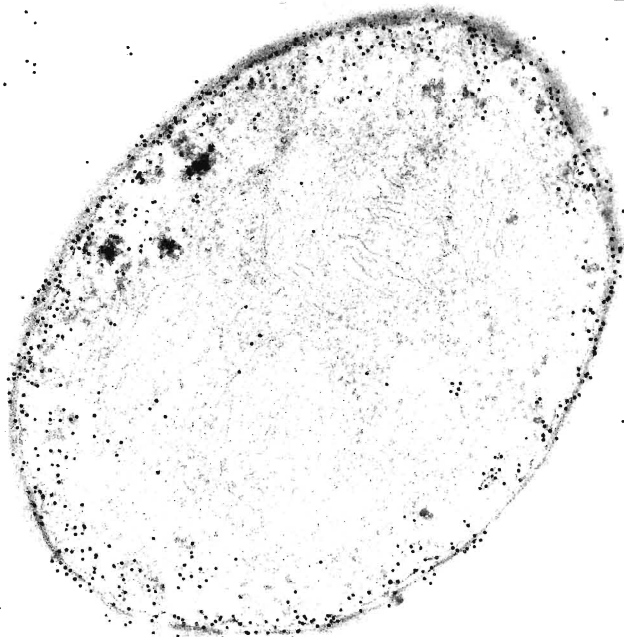
Table 2. Immuno-gold localization of M<sub>r</sub>100 and 85 kDa glycoproteins in the subcellular structures of mycoparasite *P. virginiana* and its hosts *M. pusilla* and *P. articulatus* and nonhost *M. candelabrum*

Fungal Species	cell wall		cell membrane	Cytoplasm		Cell organelles
	Inner	Outer		Close to plasm membrane	other area	
<i>M. pusilla</i>	+	+	+	+	-	-
<i>P. articulatus</i>	+	-	+	+	-	-
<i>M. candelabrum</i>	-	-	-	-	-	-
<i>P. virginiana</i>	-	-	-	-	-	-

The intensity of immunogold labelling is shown as follows: +, labelled, - , unlabelled.

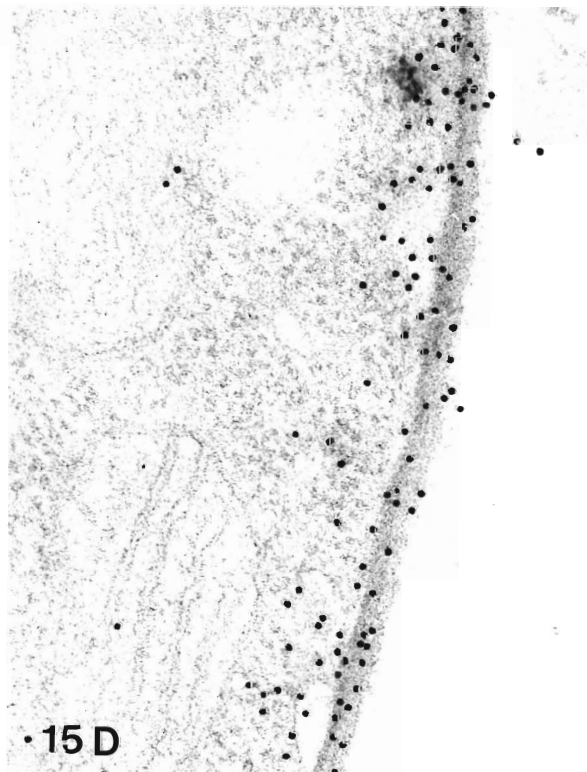
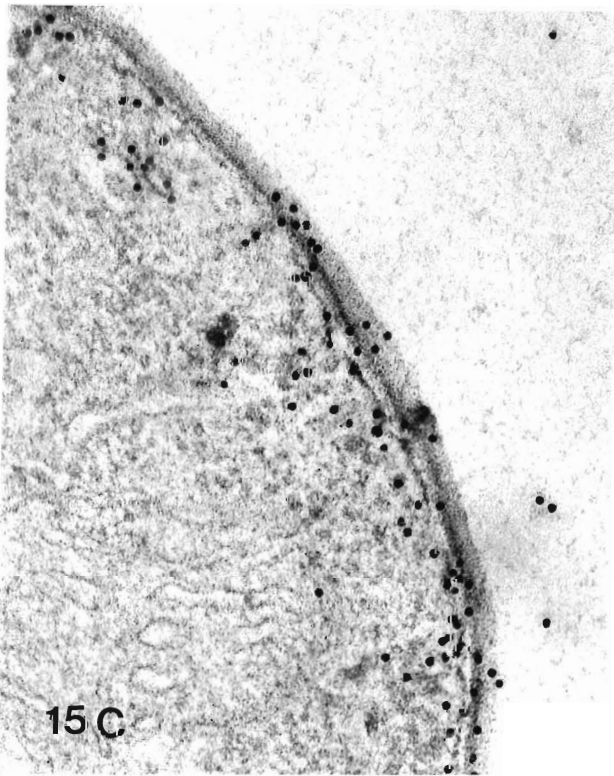
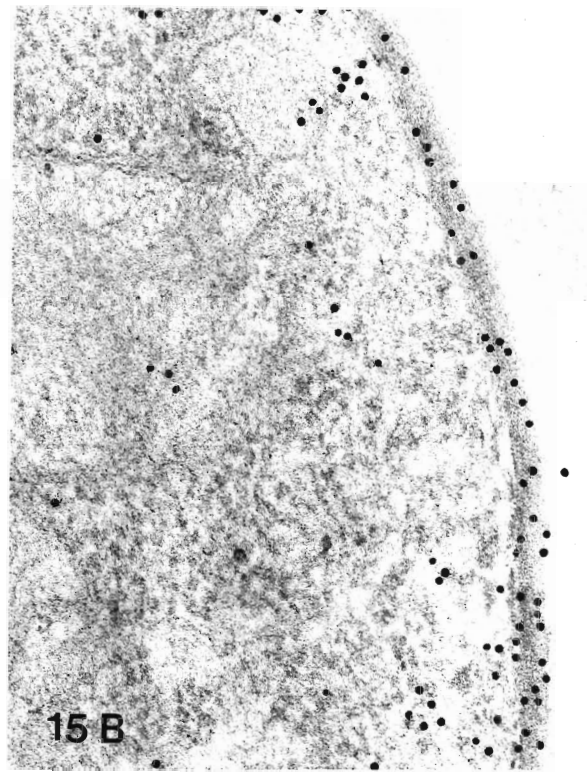
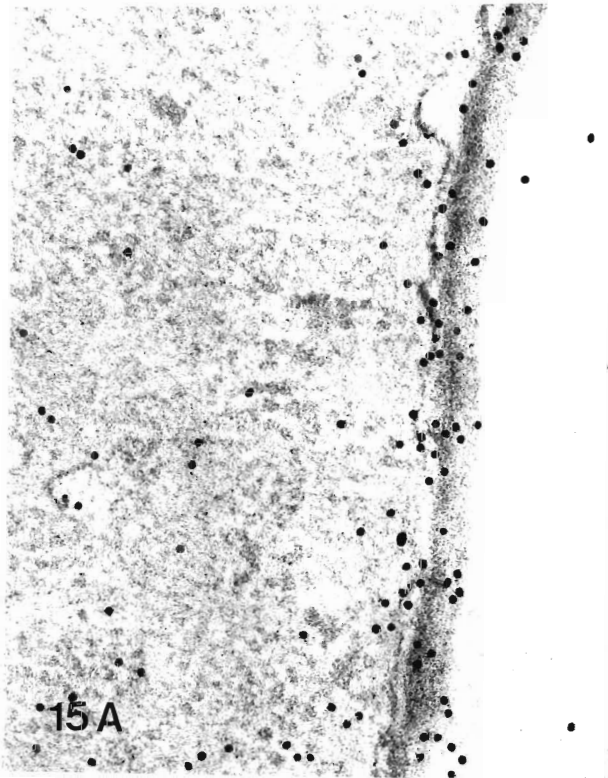
The results are based on five replicative experiments.

Fig. 14. Electron micrograph of a thin section of *M. pusilla* hyphae treated with Ab-G and goat anti-rabbit IgG-gold complex (Ab-G diluted 1:25 and goat anti-rabbit IgG-gold complex diluted 1:10). Note that gold particles are predominantly localized over the cell wall, plasma membrane and associated areas. Cytoplasm and all organelles are not labelled. x 58,000.



14

Fig. 15. A-D. Electron micrographs of thin sections of *M. pusilla* hyphae treated with Ab-G and goat anti-rabbit IgG-gold complex (Ab-G diluted 1:25 and goat anti-rabbit Ig-G gold complex diluted 1:10). Portions of fungal cells showing gold particles localized over the whole cell wall and are present in groups. The plasma membrane and adjacent cytoplasm are also labelled. A, x110,880; B, x 85,086; C, x 110,880; D, x 82,940.



gold deposition did not occur (Fig.16). This indicates the absence of nonspecific interactions between the secondary antibody-gold complex and the cell sections. When sections were incubated with primary antibodies, previously adsorbed with an excess of purified glycoproteins from *M. pusilla* cell walls, only a few dispersed particles could be observed (Fig.17).

### (ii) Immuno-gold labelling pattern over *P. articulatus*

Ultrastructure studies showed that the cell wall of *P. articulatus* is much thicker than that of *M. pusilla* (Fig 18 A & B, 19, 20). Figures 18 A & B illustrate the labelling of *P. articulatus* cells after incubation with Ab-G and Goat anti-rabbit IgG-gold conjugate. A qualitative evaluation of the labelling revealed that the gold particles were evenly and predominantly localized in the inner layer of the thick cell walls, and cell membrane (Fig. 18 A & B). Cytoplasmic organelles and cytoplasm were free of any significant labelling. A higher concentration of primary and/or secondary antibodies leads to nonspecific gold labelling over the whole cell. Gold labelling could be blocked by omission of the primary antibody (Fig. 19) or preincubation of the primary antibody with purified glycoproteins from the cell walls of *M. pusilla* (Fig. 20).

### (iii) Immuno-gold labeling pattern over *M. candelabrum*

Thin sections of *M. candelabrum*, processed in the same way as



Fig. 16. Control Experiment. Electron micrograph of a thin section of *M. pusilla* hyphae treated with goat anti-rabbit IgG-gold complex (diluted 1:10) and Ab-G (diluted 1:25) which was previously incubated with purified glycoproteins from cell wall of *M. pusilla*. Very few gold particles attributable to background can be observed. x 47,190.

Fig.17. Control Experiment. Electron micrograph of a thin section of *M. pusilla* hyphae treated with goat anti-rabbit IgG-gold complex (diluted 1:10) only. A few gold particles probably representing background are noticeable. x 47.190.

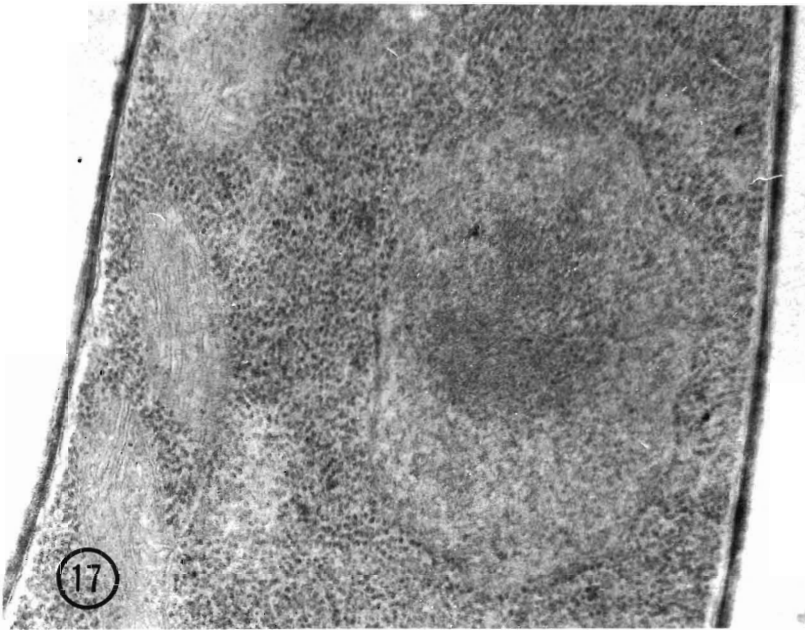
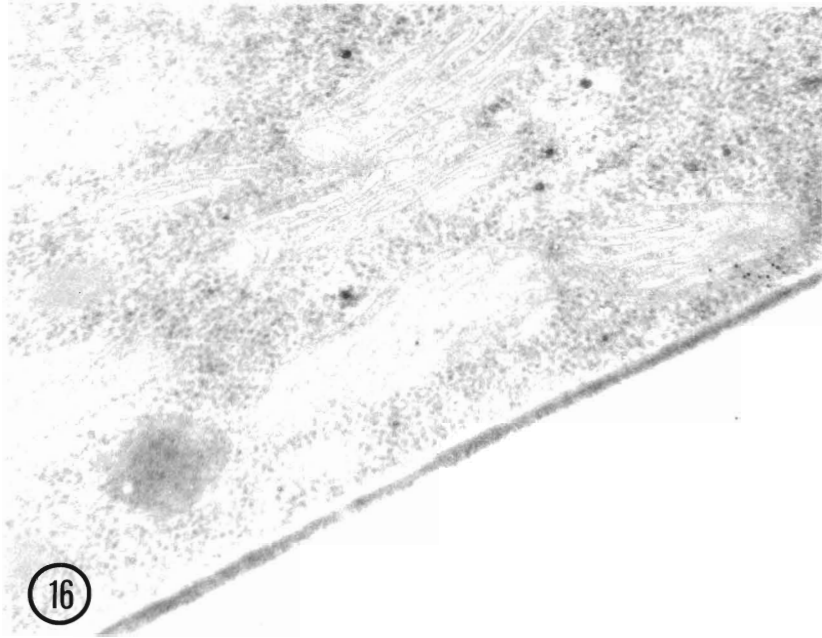
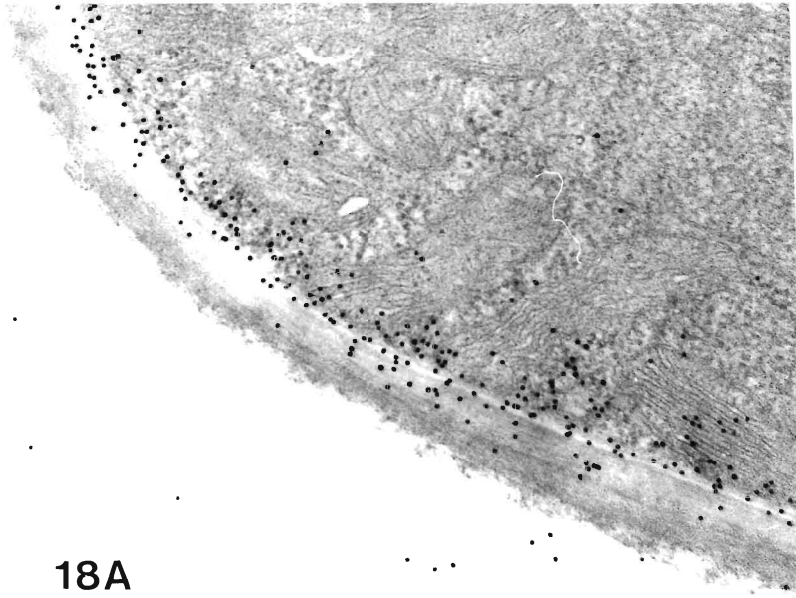


Fig.18. A & B. Electron micrographs of thin sections of *P. articulatus* treated with Ab-G and goat anti-rabbit IgG-gold complex (primary antibody diluted 1:25, secondary antibody diluted 1:10). The labelling is restricted over the inner layer of the cell wall, plasma membrane and its associated area. The cytoplasm lacks gold particles but has numerous densely stained organelles. A, x 60,984; B, x 60,984.



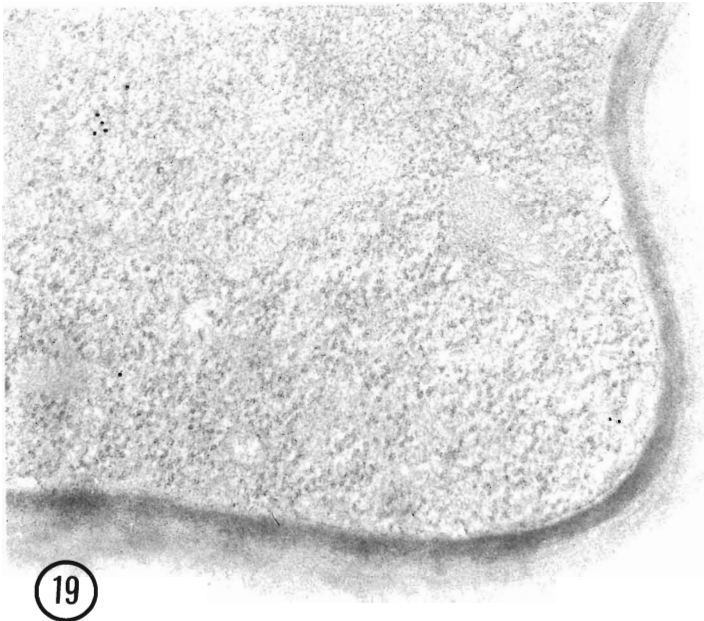
18A



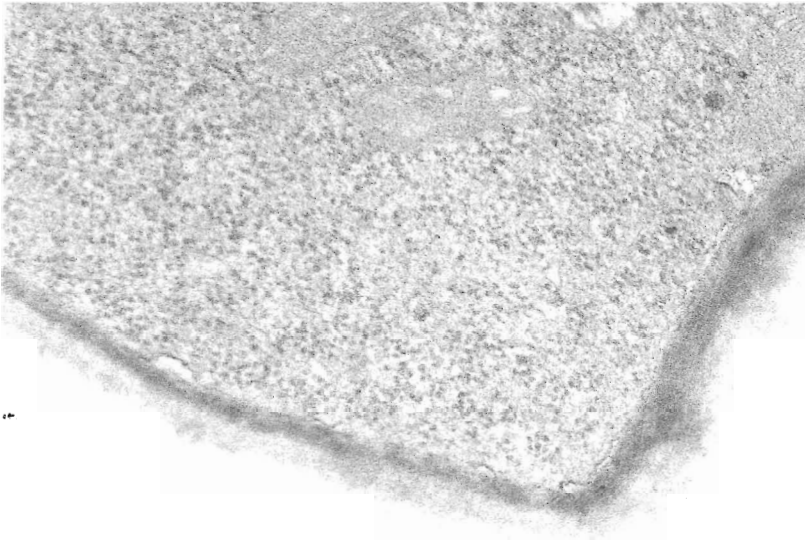
18B

Fig.19. Control Experiment. Electron micrograph of a thin section of *P. articulatus* treated with goat anti-rabbit IgG-gold complex (diluted 1:10) and Ab-G (diluted 1:25) which was previously adsorbed with purified glycoprotein from the cell wall of *M. pusilla*. Only a few gold particles are observed scattered all over the cell section. x 47,190.

Fig. 20. Control Experiment. Electron micrograph of a thin section of *P. articulatus* treated with goat anti-rabbit IgG-gold complex (diluted 1:10) only. A few gold particles are noticeable. x 47,190.



19



20

the sections of the host cells, showed only a few gold particles dispersed randomly over the whole cell (Fig. 21). When the primary antibody was omitted, a few gold particles could still be observed over the sections (Fig.22).

#### (iv) Immuno-gold labelling pattern over *P.virginiana*

Thin sections of *P. virginiana* treated with both primary and secondary antibodies showed a low level of nonspecific binding of gold particles (Fig. 23) which are scattered randomly over both cell and clear resin. When the primary antibody was omitted, a few gold particles were noticeable (Fig. 24).

#### Attachment inhibition test

In order to test the possibility that the antibody binding may inhibit the attachment of the mycoparasite to the host cell surface, cell wall fragments of *M. pusilla* and *P. articulatus* previously treated with the antibody were used. Attachment was measured as described under Materials and Methods.

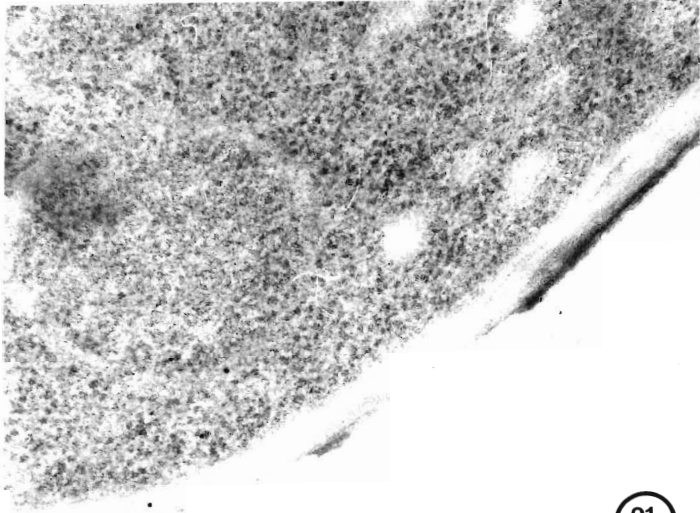
#### Effects of Ab-G on attachment and appressorium formation by the mycoparasite *P. virginiana* on the cell wall surface of *M. Pusilla*

The results are summarized in Table 3. Between the control groups, *M. pusilla* and *M. candelabrum*, the differences of attachment and appressorium formation were significant. The degree of attachment

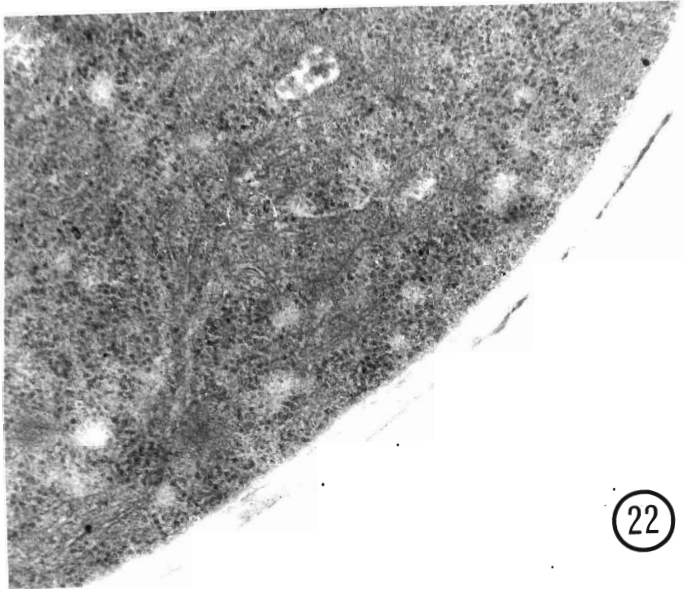
Fig. 21. Electron micrograph of a thin sections of *M. candelabrum* hyphae treated with Ab-G and goat anti-rabbit IgG-gold complex (primary antibody diluted 1:25, secondary antibody diluted 1:10). Only a few gold particles are noticeable probably representing background. x 61,908.

Fig. 22. Control Experiment. Electron micrograph of a section of *M. candelabrum* hyphae treated with goat anti-rabbit IgG-gold complex (diluted 1:10) only. A few gold particles are noticeable. x 45,276.





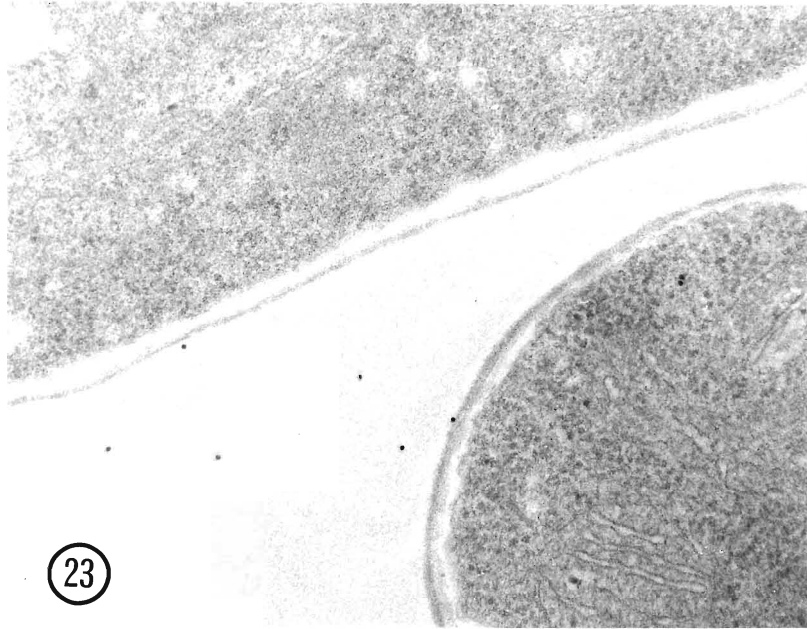
21



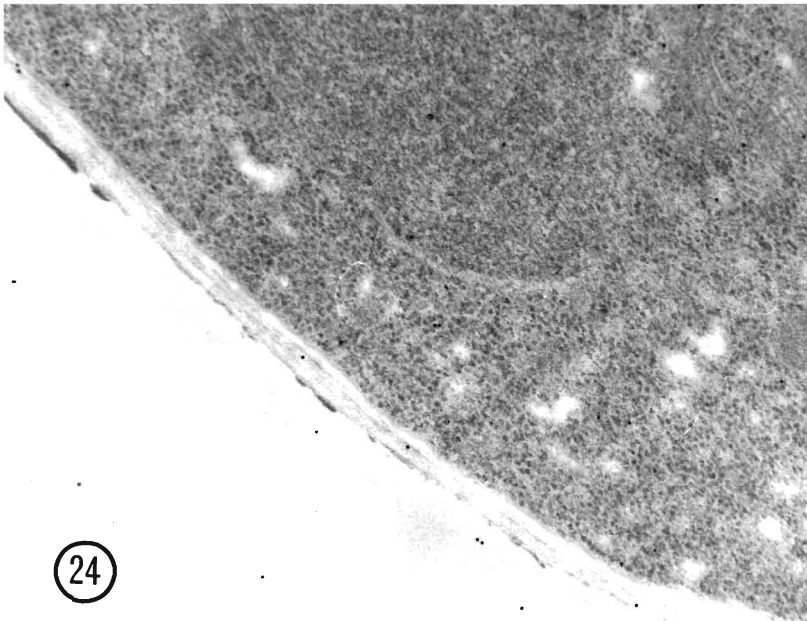
22

Fig. 23. Electron micrograph of a thin section of *P. virginiana* treated with Ab-G and goat anti-rabbit IgG-gold complex (primary antibody diluted 1:25, secondary antibody diluted 1:10). Only a few gold particles are noticeable probably representing background. x 60,984.

Fig. 24. Control Experiment. Electron micrograph of a thin section of *P. virginiana* treated with goat anti-rabbit IgG-gold complex (diluted 1:10) only. A few gold particles are noticeable. x 43,428.



23



24

and appressorium formation were significant. The degree of attachment and appressorium formation of the mycoparasite to the host cell surface was much higher than for the nonhost. These results are consistent with the previous work reported from this laboratory (Manocha, 1985). With an increase in the concentration of antibody, the percentage of attachment decreased from 58% to about 19%, corresponding to an inhibition of attachment of about 66%. At higher concentrations there was no further decrease in attachment and the percentage of attachment remained relatively stable. When the cell wall fragments were incubated with antibody previously adsorbed with an excess of a purified glycoprotein preparation from *M. pusilla* cell walls or the normal rabbit serum, the percentage of attachment remained high compared to the controls.

The percentage of appressorium formation (number of appressoria/number of attachments) seems relatively constant. This suggests that once the mycoparasite has attached to the host cell surface, further development proceeds as normal. These results suggest that factors responsible for attachment are not involved in appressorium formation.

Effects of Ab-G on attachment and appressorium formation by the mycoparasite *P. virginiana* on the cell wall surface of the resistant host *P. articulatus*.

Mycoparasite *P. virginiana* has been reported to attach to the intact hyphae of the resistant host *P. articulatus* and form an appressorium. In the present study, *P. virginiana* is found to attach to

the isolated cell wall fragments and form an appressorium. The degree of attachment and appressorium formation of the mycoparasite to the host *P.articulosus* cell surface was a little lower than for the susceptible host *M. pusilla*. Treatment of cell wall fragments with Ab-G seems to have no effect on the degree of attachment and appressorium formation of the mycoparasite. With increasing concentrations of antibodies, the percentage of attachment remains relatively constant (Table 4).

Table 3. Effect of Ab-G on attachment and appressorium formation by mycoparasite *P. virginiana* on cell wall surface of susceptible host *M. pusilla*

Concentration of antibody (mg/mL)	Percentage of attachment	Inhibition %	Relative percentage of appressorium formation
0.00	58.33 ± 1.29	0.00	80.54 ± 2.30
0.05	40.56 ± 2.28	30.46	75.86 ± 1.36
0.15	30.92 ± 0.76	46.99	81.23 ± 2.83
0.25	21.84 ± 1.30	62.56	78.76 ± 1.93
0.35	19.71 ± 1.71	66.21	79.74 ± 2.11
1.00	20.21 ± 2.22	65.35	76.87 ± 3.03
Mc	2.52 ± 0.75		0.00
Mp(PRE)a	57.25 ± 3.35	1.85	79.37 ± 2.33
Mp(NS)b	58.03 ± 2.38	0.05	79.50 ± 2.67

Values reported as confidence limits (95%)

- a. Cell wall fragments of *M. pusilla* were treated with Ab-G previously adsorbed with antigen (100 µg Ab-G incubated with 200 µg purified glycoprotein).
- b. Cell wall fragments of *M. pusilla* were treated with normal rabbit serum instead of Ab-G at a concentration of 0.35 mg/mL.

Mc = *M. candelabrum* (nonhost)

Table 4. Effect of Ab-G on attachment and appressorium formation by mycoparasite *P. virginiana* on cell wall surface of resistant host *P. articulatus*.

Concentration of antibody (mg/mL)	Percentage of attachment	Inhibition %	Relative percentage of appressorium formation
0.00	50.14 ± 3.01	0.00	74.42 ± 2.43
0.05	49.64 ± 2.61	0.99	76.32 ± 2.63
0.15	50.16 ± 2.63	-0.03	74.96 ± 3.41
0.25	48.07 ± 3.42	4.12	75.71 ± 2.79
0.35	49.02 ± 2.03	2.23	76.28 ± 3.15
1.00	49.43 ± 3.01	2.01	75.21 ± 3.11
Mc	1.94 ± 0.75		0.00
Mp	58.63 ± 1.70		79.37 ± 2.33

Values reported as confidence limits (95%)

Mc = *M. candelabrum* (nonhost)

Mp = *M. pusilla* (susceptible host)

## DISCUSSION

The Dot-immunobinding assay shows that the antibody produced against the two glycoproteins from *Mortierella pusilla* cell wall (Ab-G) cross-react with the protein extract of two hosts, *Mortierella pusilla* and *P. articulosus*, which are susceptible and resistant, respectively. The antibody did not cross-react with the protein extract of the non-host, *M. candelabrum*. Western immunoblots reveal that the antibody can recognize two glycoproteins from both *M. pusilla* and *P. articulosus* with molecular masses of about 100 and 85 kDa. The results from the antibody specificity test, which indicate that Ab-G can cross-react with only two glycoproteins of *M. pusilla* and *P. articulosus*, excludes the possibility of its cross-reacting with other proteins in the cell wall or in the whole cell. As a result, it is possible to use these antibodies to localize their corresponding antigens in the two host fungi, *M. pusilla* and *P. articulosus*.

The two glycoproteins of *M. pusilla* and *P. articulosus* are very similar, if not identical, in structure. Although the homology in the amino acid sequences and in the nucleotide sequence of their corresponding genes and cDNAs is unknown, they must share some epitopes. Interestingly, in a recent study on fungal fimbrial proteins of *M. pusilla*, *P. articulosus* and *M. candelabrum* (Reghei, 1992), it was reported that the antibody against fimbrial proteins of *Ustilago violacea* can also cross-react with one protein from each of *M. pusilla* and *P. articulosus* at the same molecular weight ( $M_r$  64 kDa) and with two other different proteins of *M. candelabrum* ( $M_r$  60 and 57 kDa).



These results support an earlier study (Manocha et al, 1990), suggesting that the surface features of *M. pusilla* and *P. articulatus* are similar in some aspects and are different from those of *M. candelabrum*.

The present study demonstrates that the precise subcellular localization of the glycoprotein antigens can be obtained with antibody complexed with colloidal gold. The absence of significant labelling with different control tests indicates clearly that the resultant staining depends on antibody-antigen binding. In contrast to the most previous studies in animal tissue, the present result revealed that the double fixation with Glutaraldehyde and Osmium Tetroxide and Spurr's embedding of fungal tissues did not impede accessibility of the antibody to their corresponding antigens. In the present study, even if some of the antigen sites could be masked, labelling was intense and well localized enough over definite structures to make the study meaningful. So far, it seems that surface glycoproteins of fungi are less affected by aldehyde fixation, osmication and Spurr's embedding. The high quality of the labelling obtained and good preservation of the underlying cellular fine structure confirmed that the immuno-gold cytochemical technique was a precise, reliable, immuno-staining procedure.

The host fungi, *M. pusilla* and *P. articulatus*, and the non-host fungus, *M. candelabrum* are taxonomically close. They are from the same order, Mucorales. Both fluorescence microscopy with fluorescent antibody and immuno-cytochemistry with colloidal gold labelled antibody show strong and specific labelling of antibody on *M. pusilla* and *P. articulatus*, but not on *M. candelabrum*. These results indicate

that the two glycoprotein antigens ( $M_r$  100 and 85 kDa) are not necessarily present in taxonomically close species and suggest their special role. The previous study on SDS/PAGE of cell wall proteins from host and non-host species also showed that several high molecular weight glycoproteins are present in hosts, but not in the non-host (Manocha and Chen, 1991).

In the two host species *M. pusilla* (susceptible) and *P. articulatus* (resistant), the results from immuno-fluorescence and immuno-cytochemistry are consistent. In *M. pusilla*, the fluorescent antibody labelling revealed fluorescence on the cell surface of hyphae and germinating spores. Correspondingly, there is the immuno-gold labelling on the *M. pusilla* cell wall and the plasma membrane. In *P. articulatus*, the fluorescent antibody labelling is present only on germinated spores, and not on hyphae. Treatment of *P. articulatus* hyphae with pronase E can unmask the antigen sites in the inner layer of the cell wall. It seems that exposure of the antigen sites to the outside is necessary for the fluorescent antibody to bind. The immuno-gold labelling is observed only over the inner layer of cell wall and plasma membrane of the hyphae.

The main difference between the results from two host species is the distribution pattern of both fluorescence and gold particles. Fluorescence is discontinuously distributed on both hyphae and germinated spores of *M. pusilla*. Fluorescence was only observed on the germinated spores of *P. articulatus*. Evenly distributed fluorescence can be observed on the hyphae treated with pronase E. The gold particles are present over the whole cell wall of *M. pusilla* and seem to appear in groups. In *P. articulatus*, the even distribution of gold

particles was observed in the inner layer. The different distribution pattern may suggest the different role the glycoproteins may play in the two host species.

Glycoproteins b (100 kDa) and c (85 kDa) may associate with each other structurally. The two glycoprotein antigens occupy the same subcellular location in *P. articulosus* (the inner layer of the cell wall). Immuno-gold particles are present in groups in the cell wall of *M. pusilla* hyphae. The possibility that each group may represent a receptor site consisting of both glycoproteins can not be excluded. Unfortunately, the limitation of polyclonal antibodies prevents further detailed analysis. The resolution, therefore, will depend on the use of monoclonal antibodies specifically directed against the well-defined epitopes of the two glycoproteins to determine if these would result in the same labelling pattern. The possible structural association of the two glycoproteins may suggest a functional relationship. If this observation is verified by the use of monoclonal antibodies, it will provide strong evidence to support the hypothesis that the two glycoproteins may be the two subunits of a receptor.

Another very important result in the present study is that, in spite of different distribution patterns of the two glycoprotein antigens in the cell wall of the hyphae, the two glycoproteins are present on the surface of germinated spores of both *M. pusilla* (susceptible) and *P. articulosus* (resistant), as revealed by the use of FITC-labelled antibody. The fluorescence intensity gradually decreased from the germinated spores to the hyphae. This finding suggests that the synthesis of these two glycoproteins probably takes place in germinating spores. In fact, it is possible that the germinated spores

are the source of the two glycoproteins where they are synthesized. After synthesis, they are transported to the surface of hyphae. As such, this study may provide a possible explanation for the well-known observation that resistance of the mucoraceous hosts to the mycoparasite increases with aging (England, 1969; Manocha and Campbell, 1983). According to the present results, the synthesis of two glycoproteins is restricted to the germinating spores, hence, the ability of the culture to synthesize these glycoproteins is limited and the quantity of the two glycoproteins on the surface of the host fungus will decrease proportionately with age as more and more hyphae are produced.

The attachment of the mycoparasite *P. virginiana* can be partially inhibited by the binding of antibody to the cell wall fragments of *M. pusilla*. However, it is possible that the antigenic determinant of the two glycoproteins which are recognized by antibodies are not necessarily the receptor sites recognized by the complementary molecules of the mycoparasite *P. virginiana*. There are at least two possible explanations. First, the antigenic determinants and the receptor sites may share some components. So, the antibody competes for the common components and as a result, preincubation of antibody with cell wall surfaces will partially inhibit the binding of the complementary molecules from the surface of *P. virginiana* to these sites. Second, the antigenic determinant and receptor site may not share any common component, but they might occupy two close sites in the same molecule. The binding of the antibody to its antigenic determinant will occupy some limited space which is necessary for interaction between the receptor site and the complementary molecule

at the surface of *P. virginiana*, thus inhibiting the attachment of mycoparasite to the surface of its host. Both of these hypotheses can explain why the percentage of attachment rate remains constant when the concentration of antibody reaches a certain level. Alternatively, some of the receptor sites may remain masked and are unmasked by the parasitic attack. From the above analysis, it is clear that the two glycoproteins are involved in the recognition process *in vivo*, because the binding of the antibody to these glycoproteins can successfully interfere with the recognition and attachment process.

Failure of the antibody to inhibit the attachment of the mycoparasite *P. virginiana* to its resistant host *P. articulatus* is probably due to the preferential localization of antigenic determinants in the inner layer of the thick wall. The surface sugar residues involved in attachment (Manocha and Chen, 1990) remain virtually unaffected. It is possible that a different recognition mechanism may be involved in the resistant host. It is tempting to speculate that in order to unmask these deep-seated glycoproteins, the mycoparasite deploys hydrolytic enzymes including proteinases which indirectly stimulate a membrane bound, zymogenic chitin synthase at the infection sites leading to the deposition of papillae or wall appositions. The supporting evidence for these events including the enzymatic penetration and role of wall apposition in the resistance of *P. articulatus*, has been described by Manocha (1991). Unmasking of glycoprotein antigen by proteinase is reported here.

Other authors (Mellon and Helgeson 1982) insist that the specificity of the lectin or agglutinin on the cell surface of host, resistant host and nonhost is necessary for them to be specifically

distinguished by the parasite and therefore, the compatibility or incompatibility could be determined. The present study provides the evidence that these lectins or agglutinins do not have to be different enough to be recognized by their parasites. The quantitative difference and spatial distribution may be the two important factors. The binding of complementary molecules on the surface of host and mycoparasite is the nature of recognition process. The appropriate combination of many events in space and time may determine whether an interaction will be compatible or incompatible (Benhamou, 1991).

Finally, the use of immuno-cytochemistry in conjunction with immuno-fluorescence may prove to be very useful for investigations of various proteins and glycoproteins in host-parasite interactions. The potential value of immunogold-cytochemistry techniques in elucidating the wall topochemistry has been stated (Benhamou, 1987; Chamberland et al., 1985) and is confirmed by the work presented here. The high specificity of gold-conjugated monoclonal antibody will undoubtedly prove to be a powerful probe for studies of cell wall protein and glycoprotein and protein in molecular plant biology and plant pathology.

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