Isolation and partial characterization of a complementary protein from the mycoparasite, *Piptocephalis virginiana*, which specifically binds to two glycoproteins b and c of the host cell surface

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Abstract

Presence of surface glycoprotein in *Piptocephalis virginiana* that recognizes the host glycoproteins b and c, reported earlier from our laboratory, was detected by immunofluorescence microscopy. Germinated spores of *P.virginiana* treated with *Mortierella pusilla* cell wall protein extract, primary antibodies prepared against glycoproteins b and c and FITC-goat anti-rabbit IgG conjugate showed fluorescence. This indicated that on the surfaces of the biotrophic mycoparasite *P. virginiana* , there might be a complementary molecule which recognizes the glycoproteins b and c from *M. pusilla.* Immunobinding analysis identified a glycoprotein of M_r 100 kDa from the mycoparasite which binds with the host glycoproteins b and c, separately as well as collectively. Purification of this glycoprotein was achieved by (i) 60% ammonium sulfate precipitation, (ii) followed by heat treatment, and (iii) Sephadex G-100 gel filtration. The glycoprotein was isolated by preparative polyacrylamide gel electrophoresis by cutting and elution. The purity of the protein was ascertained by SDS-PAGE and Western blot analysis. Positive reaction to periodic acid-Schiff reagent revealed the glycoprotein nature of this 100 kDa protein. Mannose was identified as a major sugar component of this glycoprotein by using a Boehringer-Mannheim Glycan Differentiation Kit.

Electrophoretically purified glycoprotein was used to raise polyclonal antibody in rabbit. The specificity of the antibody was determined by dot-immunobinding test and western-blot analysis.

Immunofluorescence microscopy revealed surface localization of the protein on the germ tube of *Piptocephalis virginiana*. Fluorescence was also observed at the surface of the germinated spores and hyphae of the host, *M. pusilla* after treatment with complementary protein from *P. virginiana*, primary antibody prepared against the complementary protein and FITC-goat anti-rabbit IgG conjugate.

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Introduction

In the plant host-fungal parasite system and fungal host-fungal parasite or mycoparasitic system, the term "recognition" has become very popular in the literature of host-pathogen interactions. In the plant host-fungal parasite system, molecules responsible for recognition at the surface of the fungal parasite have been extensively studied as compared to the mycoparasitic system. Various types of molecules have been isolated from cell walls and culture filtrates of different parasitic fungi and from host plants (Callow, 1977; Manocha and Sahai, 1993). These molecules include elicitors, lectins and agglutinins, sugars and toxins. In necrotrophic mycoparasite systems, glycoproteins responsible for recognition have been isolated from the host and parasite (Inbar and Chet, 1994; Benyagoub et al. 1996).

The mechanism of recognition and attachment of the biotrophic mycoparasite, *Piptocephalis virginiana,* to the host but not to the nonhost fungal species has been under investigation for some time in this laboratory (Manocha and Chen, 1990; Manocha and Sahai, 1993). Most of the investigations concentrated on the isolation, purification and properties of agglutinin responsible for the specific attachment of the biotrophic mycoparasite to its host.

These studies (Manocha and Chen, 1991) have shown the presence of two high molecular weight glycoproteins that occurred in the cell wall of the host, but not the nonhost. Qualitative differences in protein profile, from NaOH extracts of isolated cell wall fragments of the host and nonhost fungi, were observed by SDS-PAGE. The bands a,

b, d were present in the host, but were absent in the nonhost. Only the band c was present in both host and nonhost. The molecular weights of proteins a, b, c, d are 117, 100, 85, and 64 kDa, respectively. Measurement of agglutinating activity with the mycoparasitic spores showed that the host cell wall extract had a higher agglutinating activity than the nonhost cell wall extract. To detect which protein was involved in agglutination, a host cell wall protein extract minus proteins a, b, c, or d was obtained by preparative PAGE. Deletion of protein a or d did not affect agglutinating activity. However, crude extract minus b or c showed significantly lower agglutination activity than the complex extract.

Further purification of b and c was confirmed by SDS-PAGE. Both b and c were proved to be glycoproteins. The agglutination activity of glycoproteins b and c increased with purification, 35 times more than the crude extract. However, purified preparation of b or c alone showed agglutination 510-850 times less than the pure preparation containing both glycoproteins. This indicated that both proteins b and c are required for agglutination of mycoparasitic spores. Pretreatment of *P. virginiana* germinated spores with b and c could also totally inhibit their attachment to host cell wall fragments. Chemical or enzymatic removal of host surface protein, including the two glycoproteins, resulted in a significant decrease in attachment by the mycoparasite. The major carbohydrate components of proteins b and c were N-acetylglucosamine, glucose, and arabinose. These three sugars also inhibited agglutination as well as attachment of *P. virginiana* germ tubes to host cell wall fragments.

Polyclonal antibodies prepared against the glycoproteins b and c were used to identify the exact location of the antigen at the host surface. Manocha and Su (1992) employed an indirect immunocytochemical technique using secondary antibodies labeled with either fluorescein isothiocyanate or gold particles. Immunofluorescence microscopy showed that antibodies bind to the hyphal cells of the host, *M. pusilla,* and exhibit strong but discontinuous fluorescence. Intense fluorescence was observed on the germinating spores and germ tubes. Antibodies conjugated with gold particles showed patches of gold particles over the cell wall of the host, confirming the discontinuous pattern observed by fluorescence microscopy.

It is hypothesized (Manocha and Chen, 1991) that the host cell surface glycoproteins b and c responsible for the agglutinating activity may also play a role in recognition and attachment of the mycoparasite to the host cell surface. It is likely that at the surface of the mycoparasite, there might be a specific molecule that is complementary to the glycoproteins on the host surface.

The objective of this project was (i) to ascertain the existence of the complementary molecule in *Piptocephalis virginiana* that binds specifically to the glycoproteins b and c at the host surface; (ii) to identify, isolate and characterize the complementary molecule.

<center>Review of Literature</center>

Molecular Recognition in Host-Parasite Interaction

As noted by Sequeira (1978, 1983), the term "recognition" has become very popular in the literature of host-pathogen interactions. Recognition is defined by Clarke & Knox (1978) as the "initial event in cell-cell communication that elicits a defined biochemical, physiological, or morphological response". Callow (1984) defined recognition as " an ability to discriminate materials present in the environment". 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).

Mutual molecular interactions between cell surfaces of pathogens and of host plants are suggested to play an important role in the control of pathogenesis and disease resistance. Specificity in plant disease may be considered to be a sequence of recognition. On the one hand, the plant host has to detect or recognize a potential pathogen as foreign or "non-self" then use this initial act of recognition to trigger induced resistance mechanisms. On the other hand, a potential pathogen may recognize surface features of a plant that transfer signals of its suitability for parasitism (Keen, 1982; Callow, 1988).

The studies on the specificity in host-parasite interaction of earlier years were concentrated only on nutrition and environmental

factors, host range, models of parasitism that may modify the degree of parasitism and disease. While in recent years, with the advantage of cytological and biochemical investigations, as well as modern techniques of molecular biology, the molecular recognition mechanisms which trigger and regulate plant-pathogen interactions have been understood more and more.

Undoubtedly the importance of molecular recognition between a plant and pathogen on cell surface is now universally accepted (Mazau et al. 1988). The intention of this review is to discuss my current understanding of the nature of some molecular signals and their receptors involved in recognition in both plant host-fungal pathogen systems and mycoparasitic systems.

Products of gene-for-gene system as receptors and signals

Flor's (1942; 1971) gene-for-gene hypothesis is perhaps this century's greatest contribution to plant pathology principle. According to his hypothesis, resistance or susceptibility in host species to distinct physiological races of a pathogen is determined by pairs of corresponding genes in the hosts 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, 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).

Proteins have two important characteristics: (i) They can store variation freely; indeed, most of the variation in the DNA of structural genes survives to be reflected in the amino acid residues of proteins. (ii) As in many antigen-antibody systems or in polymerization, proteins recognize one another precisely. These two attributes, variation storage and mutual recognition, are essential for a biochemical explanation of the gene-for-gene hypothesis; and a protein-for-protein hypothesis was proposed by Vanderplank (1978). The hypothesis states that in gene-for-gene disease the mutual recognition of host and pathogen is not by genes themselves but by their coded proteins (Vanderplank, 1982).

The high degree of specificity exhibited by gene-for-gene systems suggests that highly selective host receptor are capable of detecting specific features of parasite races. Callow (1987) 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.

In the past several years, a lot of fungal avirulence genes have been isolated and identified (Keller et al. 1989). Aimed at understanding the molecular basis for gene-for-gene interaction, a recent research (Tor et al. 1994) has showed map positions of three loci in *Arabidopsis thaliana* associated with isolate-specific recognition

of *Peronospora parasitica* (Downy Mildew). The proteins encoded by the 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 products of gene-for-gene system recognize each other as signals and receptors.

Elicitor and elicitor receptor

Another hypothesis based on the genetics of plant host-fungal parasite interaction is that avirulence gene products act indirectly. According to this model, the products of avirulence genes act as inducers of defense 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 or elicitins (Keen, 1975). Elicitors appear to be recognized by plant cells via interaction with specific receptors on plant plasma membranes (Yoshikawa, et al. 1993a). The elicitor-receptor interactions are presumed to generate signals that then activate nuclear genes involved in plant defense reactions, such as the biosynthesis of phytoalexins. The polysaccharides or glycoprotein molecules commonly found in fungal cell walls and peptides, oligosaccharides derived from fungal cell wall polymers such as β-glucan, chitin, chitosan, and lipid fractions have been shown to have elicitor activity (Keen, 1982, 1986; De Wit, 1988; Dixon, 1986; Yang et al. 1994; Zeng et al. 1993; Schaffrath et al. 1995). Even several plant lectins concanavalin A (ConA), *Phaseolus vulgaris* agglutinin (PHA), peanut agglutinin (PNA) and *Pisum sativum* (PSA) can act as elicitors (Toyoda et al. 1995).

A gene encoding a host-specific elicitor protein of *Phytophthora parasitica* has been studied by Kamoun et al. (1993). Based on elicitin amino acid sequences, elicitin-coding sequences from *P. parasitica* were amplified by the polymerase chain reaction. A genomic clone containing a complete elicitin gene, parA1, was isolated and sequenced. Bacterial expression of the cloned elicitin gene as a translational fusion protein containing glutathione S-transferase yielded a biologically active protein capable of inducing a hypersensitive response in tobacco.

The initial biochemical event of this model is thought to be the recognition of elicitor molecules by receptors on plant cells. Yoshikawa et al. (1983) obtained the first direct evidence for the suggestion that a phytoalexin elicitor is recognized by a specific receptor on soybean membranes. They employed a (^{14}C)-labeled fungal elicitor (mycolaminaran) and a direct membrane-binding assay. The binding was highly specific, as judged from results of studies with unlabeled ligands, no other carbohydrates competed for binding. The results indicated that the observed binding sites were specific receptors for mycolaminaran and suggested that they might be physiologically involved in the initiation of the production of phytoalexin. However, the biological significance of such a binding site remains unclear.

Recently, binding sites for β-glucan fragments, as well as hepta-β-glucopyranoside derived from the cell walls of *Phytophthoza megasperma* f. sp. *glycinea* with elevated elicitor activity, have been reported in soybean membranes (Cheong and Hahn, 1991; Cosio et al. 1988, 1990 a, b; Schmit and Ebel, 1987). These results suggest that β-glucan elicitors are recognized by soybean cells through receptor-like

binding sites. Yoshikawa and his co-researchers (1993b) recently demonstrated that soybean membranes do indeed contain a specific and discrete class of high-affinity binding sites for the glucanase-released elicitor. They further solubilized and isolated the binding molecules from soybean microsomal membranes and determined their partial amino acid sequence. They then attempted to clone the gene for the binding molecules (Yoshikawa et al. 1993a).

More recently, research on isolation, characterization, and biological function of two race-specific elicitors AVR4 and AVR9 of *Cladosporium fulvum* and cloning and regulation of their encoding genes has been done (De Wit et al. 1995).

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

The specificity of interactions between ligand and receptor on the cell surface may result in the specificity of plant-pathogen interaction. 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 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).

Andrew and Daleo (1988) found that the amount of soybean agglutinin (SBA) in seeds of resistant species to *Phytophthora megasperma* is 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 slides 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. 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. However, this factor 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. Also, another report (Furuichi et al. 1980) showed 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 of the hosts and their receptors

Hinch and Clarke (1980) found that adhesion of *Phytophora cinnamomi* zoospores to *Zea mays* roots depends on the integrity of the carbohydrate component of the secreted root surface slime. Adhesion is abolished by periodate oxidation of the root surface, enzymatic removal of terminal fucosyl residues of the slime and by interaction of the same residues with a fucose-binding lectin. The binding and encystment of zoospores of *Pythium aphanidermatum* to root surfaces of cress (*Lepidium satium*) was used as a quantitative model of fungal-plant recognition (Longman and Callow, 1987). Zoospore-root binding is abolished by trypsinization of zoospores or periodate oxidation of the root surface. Lectin and enzyme modification of the root surface mucilage polysaccharide reduced zoospore binding; treatments that block, or remove terminal fucosyl residues are especially effective. Incubation of zoospores with exogenous root mucilage polysaccharide also reduced zoospore binding but the effectiveness of the mucilage was reduced if pretreated with periodate or with exofucosidase. The results suggest that protein-containing receptors on the zoospore surface interact with fucose-containing ligands present in root surface mucilage to promote zoospore-binding and subsequent encystment. Whilst their interpretation of the data is consistent with a recognition based on the binding of root surface saccharide ligands by zoospore receptors, another study (Bacic, et al. 1985) has revealed saccharide receptors for certain lectins on the surfaces of *Phytophthora cinnamomi* zoospores. Fucose-specific receptors were also demonstrated on germinated cysts of other *Phytophthora* spp including *P. infestans* and *P. megasperma* (Hohl and Balsiger, 1988).

In order to understand the molecular basis of this interaction, it is necessary to isolate the fucose receptor and, if present, receptors for other glycosyl residues from the zoospore surface. For this purpose, Kelleher et al. (1990) have adapted a method whereby a defined ligand (e.g. polysaccharide) can be coupled to a matrix (e.g. fluorescent latex beads or carboxymethyl cellulose fibres). This method has successfully used to isolate receptors for glycosyl residues from the surfaces of lymphocytes, thymocytes and sponge cells (Coombe et al. 1987). Their results confirm the presence of a fucosyl-binding protein on the surface of zoospores, cysts and germinated cysts of *P. cinnamomi* and provides a means for isolation and subsequent characterization of this receptor.

The effects of several plant lectins on the production of a pea phytoalexin, pisatin, were examined (Toyoda et al. 1995). ConA, PHA, PNA and PSA each induced the production of pisatin in pea epicotyl tissues, demonstrating that plant lectins can act as elicitors. Their results indicate that the elicitor effect of ConA is attributable to its ability to bind to specific carbohydrate residues of pea cells.

Host-specific toxins and their receptors

Toxins with unusual characteristics are involved in some destructive diseases of plants. Certain parasitic fungi produce toxins of low molecular weight that selectively affect the host plant; nonhosts are tolerant. Selective toxins are produced by fungi that are specialized or restricted to certain plant cultivars and are toxic only to hosts of the fungus that produced the toxin. It was suggested that resistant cells may lack toxin receptor sites (Scheffer, 1984).

A number of fungal plant pathogens have been defined to produce host-specific toxins (HSTs) clearly responsible for the determination of host-specific pathogenicity in the species of *Alternaria, Helminthosporium,* and others (Nishimura et al. 1989; Scheffer, 1989; Xiao et al. 1991; Otani H. et al. 1995).

Specificity of the eyespot disease of sugar cane caused by *Helminthosporium* has been claimed to involve the selective binding of the toxin "helminthosporoside" to membranes of the susceptible host (Scheffer, 1989). 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 plasma membrane (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).

Other recognition molecules

In addition to the above mentioned recognition molecules, there are some others like small, cysteine-rich proteins, kinase-like protein, hydrophobic or hydrophilic surface structure, some cations like Ca^{2+} and Mg^{2+} ions, etc.

Some avirulence gene products, elicitins, and hydrophobins are small, cysteine-rich proteins (Templeton et al. 1994). The hydrophobins are between 90 and 150 amino acid in length, contain eight cysteine residues, and are strongly hydrophobic. The amino acid sequence homology between hydrophobins from different species is low, and alignments relies heavily on the pattern of cysteine residues in the protein. Another common feature is that the second and third cysteines form a doublet and are usually followed by an asparagine residue. The small, cysteine-rich proteins do not have known catalytic functions but tend to bind other molecules such as carbohydrates, proteins, and lipids. Many act as toxins or allergens and often bind to membrane-bound receptors. It is speculated (Templeton et al. 1994) that in fungi the recognition process might be mediated by an interaction between the cysteine-rich, hydrophobin-like domain of avirulence genes and similiar domains present on the receptor. The signal could then be passed on via a transmembrane region to a serine-threonine kinase domain that would be able to initiate intracellular responses.

The rice Xa21 gene, which confer resistance to *Xanthomonas oryzae pv. oryzae* race 6, was isolated by positional cloning (Song et al. 1995). Fifty transgenic rice plants carrying the cloned Xa21 gene display high levels of resistance to the pathogen. The sequence of the predicted protein, which carries both a leucine-rich repeat motif and a serine-threonine kinase-like domain, suggests a role in cell surface recognition of a pathogen ligand and subsequent activation of an intracellular defense response.

Colletotrichum trifolii causes anthracnose disease of alfalfa (*Medicago sativa*). Fungal perception and response to host signals are likely to be crucial in determining whether successful infection occurs. The research work done by Dickman et al. (1995) has shown that calmodulin, protein kinase C, and a novel protein kinase are probably the molecular signals during the early stages of alfalfa anthracnose.

Adhesion of a fungal pathogen on the surface of its host is often presumed to be a single, chemically mediated event associated with germ tube or appressorium formation. However, adhesion of ungerminated propagules may also occur, and evidence suggests that it is mediated by the release of adhesive materials directly from the propagule upon contact with a suitable substratum (Nicholson and Kunob, 1995). Fungi may require either a hydrophobic or a hydrophilic surface to initiate the infection process. The barley powdery mildew pathogen, *Erysiphe graminis*, requires a hydrophilic surface for appressorium formation, yet the barley leaf is extremely hydrophobic. The problem is resolved by the release of an exudate from conidia that makes the hydrophobic leaf surface hydrophilic. In contract, *Colletotrichum graminicola* requires a hydrophobic surface for the initiation of its infection process. Ungerminated conidia of this fungus release materials that allow for the rapid adhesion of conidia, which ensures that germination and appressorium formation occur, initiating the infection process.

The research work of early interactions of *Rhizobium leguminosarum by. phaseoli* and bean roots has been done by Lodeiro et al. (1995). Their results indicate that Ca^{2+} and Mg^{2+} ions are

required by *R. leguminosarum by. phaseoli* for the specificity, and those cations cause tighter binding of rhizobia to roots.

Molecular recognition in mycoparasitic systems

Mycoparasitism appears to be a complex process made up from several successive steps (Chet, 1987, 1990; Tunlid et al. 1992). It may be initiated by a remote sense of the host which stimulates directed growth of the parasite towards that host (Chet et al. 1981). Subsequently, contact is made between the fungal parasite and the host surface. This step provides the specific recognition event, which may be physical, as in thigmotropism, or chemical (chemotropism). The latter involves hydrophobic interactions or interactions between complementary molecules present on both the host and the parasite (e.g. lectin-carbohydrate interactions).

Compared with characterizations of plant host-fungal parasite interactions, only a limited amount of the research on recognition phenomena has concerned fungal host-fungal parasite or mycoparasitic interactions.

Based on the nature of parasitism, mycoparasites have been divided into two major groups, necrotrophs and biotrophs (Barnett and Binder, 1973).

(i) Necrotrophic mycoparasites

The principle work in the phenomena of recognition of necrotrophic mycoparasites has been carried out by Professor I. Chet and his coworkers in Israel, using *Trichoderma* species parasitic on *R. solani* and *S. rolfsii*. There is enough evidence to suggest the role of a

lectin or agglutinin type interaction facilitating recognition in these mycoparasite systems.

The initial study demonstrating the role of cell surface agglutinin in recognition of necrotrophic mycoparasite was performed by Elad et al. (1983). Their results along with the studies of Barak et al. (1986) clearly demonstrate the properties of an agglutinin on the cell surface of *R. solani* that interacts with L-fucosyl residues on the surface of *Trichoderma*. But there are no reports of further purification of agglutinin from *R. solani.* hyphae.

Culture filtrates and mycelial extracts of *S. rolfsii* demonstrated agglutinin activity (Barak et al. 1985). Their initial attempt to purify agglutinin from *S. rolfsii* yielded only small quantities of agglutinin due to high polysaccharide content in the crude agglutinin preparation. In a subsequent study (Barak and Chet, 1990), they purified an agglutinin with sugar binding specificity similar to that of the lectin concanavalin A (ConA), and also for D-mannose and D-glucose, a glycoprotein nature of this agglutinin was suggested. Polyclonal antiserum was prepared against the purified *S. rolfsii* agglutinin and indirect immunofluorescence studies were performed. Results indicated that the agglutinin is produced in restricted sites along the hyphae. In an attempt to test the role for lectins in the recognition , Inbar and Chet (1992) developed a biomimetic system based on the binding of lectins to the surface of nylon fibre.

A novel lectin was isolated and purified recently (Inbar and Chet, 1994) from the culture filtrate of *S. rolfsii* by anion-exchange chromatography using a DEAE-Sepharose column. SDS-PAGE analysis of the agglutinating fraction revealed a single band corresponding to

protein with molecular mass of 45 kDa. This lectin differs from that previously reported from *S. rolfsii*, which contained two major bands of 55 and 65 kDa. mono- or disaccharides inhibition tests and the treatments of various enzymes indicate that both protein and 1,3-β-glucan are necessary for agglutination. Using a biomimetic system revealed that the presence of the purified agglutinin on the surface of the fibres specifically induced mycoparasitic behavior in *Trichoderma harzianum*. They suggested that the recognition of *Trichoderma* to *S. rolfsii* cell wall surface is indeed mediated by a complexed agglutinating polymer which surrounds the host hyphae, thereby initiating a sequence of events which eventually lead to the destruction of the host (Inbar and Chet, 1994).

Except for the study of *Trichoderma* species parasitic on *R. solani* and *S. rolfsii*, interaction between the necrotrophic mycoparasite *Stachybotrys elegans* and its host *Rhizoctonia solani* was investigated by M. Benyagoub et al. (1996). By using various sugar specific lectins and anti-fimbriae antibodies, N-acetyl-D-glucosamine oligomers were revealed in the cell walls of both fungi. The absence of these sugar oligomers at the penetration sites of the host cell wall suggests that extracellular chitinases produced by *S. elegans* and may have a role in the recognition process. In *R. solani* cells, labeling studies revealed that papillae produced in response to attack by the mycoparasite contained N-acetyl-D-glucosamine and to a lesser extent either mannosyl or glucosyl sugar residues. Fimbrial proteins were localized within the extracellular matrix that surrounds *S. elegans* cells when they were confronted with its host. Their study has provided evidence that

surface molecules are implicated in the interaction between *S. elegans* and its host.

(ii) Biotrophic mycoparasites

The mechanism of recognition and attachment of the mycoparasite *P. virginiana* to the host but not to the nonhost fungal species has been investigated previously in this laboratory for some time (Manocha, 1985; Manocha and Chen, 1990).

Total protein of isolated cell wall fragments were not different in the host and nonhost fungi. However, qualitative differences in protein profiles, from NaOH extracts of isolated cell wall fragments of the host and nonhost fungi, were observed by SDS-PAGE. Three protein bands, a, b, and d, were observed in gels of the nonhost cell wall extract, but were absent in gels of the nonhost extract. A fourth band, c, could be seen on gels of both the host and nonhost extracts but was thin and faint in the latter. The apparent molecular masses of the proteins a, b, c and d were 117, 100, 85 and 64 kDa respectively (Manocha and Chen, 1991).

Measurement of agglutinating activity with the mycoparasitic spores showed that the host cell wall extract had a higher agglutinating activity than the nonhost cell wall extract, which had a very low titre. Deletion of proteins b or c from the crude protein extract of the host significantly reduced its agglutinating activity. Proteins b and c, purified by a series of procedures, were shown to be glycoproteins with glucose, N-acetylglucosamine and arabinose as major saccharides. The agglutinating activity of a mixture of pure proteins b and c was over 500 times that of either glycoprotein alone, suggesting an involvement of both glycoproteins in the agglutination

process. Further characterization showed that the two glycoproteins were heat-resistant with respect to their agglutinin function, which could be totally inhibited by three sugars: arabinose, glucose and N-acetylglucosamine. It is suggested (Manocha and Chen, 1991) that glycoproteins b and c are the two subunits of an agglutinin and may function as "receptors" to a complementary structure on the surface of the mycoparasite.

Polyclonal antibodies against b and c were prepared and immuno-cytochemical localization of b and c was performed (Manocha and Su, 1992) using immunofluorescence and colloidal-gold immunocytochemistry techniques.

My current work has shown the existence of the complementary molecule on the surface of the mycoparasite *P. virginiana* by immunofluorescence microscopy after treating germinated spores of the mycoparasite, *P. virginiana* with the protein of the host, *M. pusilla*, primary antibody raised against the glycoproteins b and c of *M. pusilla* and secondary antibody. By a dot-immunobinding assay and an immunoblot analysis, one $M_r 100$ kDa protein was found complementary to b and c, further purification and characterization of this protein have been done through my research project.

In addition to studies of glycoproteins b and c and the purified 100 kDa glycoprotein complementary to b and c, the involvement of fimbriae in biotrophic mycoparasite and fungal host interaction has been studied (Rghei et al. 1992). Fimbriae were observed by electron microscopy on the surfaces of the host and non-host species but not the mycoparasite. Polyclonal antiserum prepared against the fimbrial protein of *Ustilago violacea* cross-reacted with 64kDa proteins from

both *M. pusilla* and *P. articulosus* and 60 and 57 kDa proteins from *M. candelabrum*. These proteins were electroeluted from polyacrylamide gels and were shown subsequently to form fibrils with the same diameter as the cell surface fimbriae. To ascertain the role of fimbriae in host-mycoparasite interaction, the antiserum was incubated with *P. virginiana* and *M. pusilla*, and with *P. virginiana* and *P. articulosus*. Contacts between mycoparasite and host were blocked significantly by the antiserum. It was proposed that the recognition of fimbriae by the mycoparasite leads to directed growth and contact between mycoparasite germ tubes and the hyphae of a potential host.

Comparing the recognition mechanisms of fungal parasite-plant host and mycoparasite-host interaction with that of animal cell-cell recognition, we find a major difference between plants, fungi and animals, namely, the presence of a cell wall which encases and protects the protoplast. Whether the complementary ligands and receptors are located on the cell membranes has not been defined (Ralton et al. 1987). We can presume that the fungal cell wall and plant cell wall may act as a primary target from which secondary signals are generated.

To reveal the nature of these complementary recognition molecules, it will be clear that in the future recombinant DNA technology enable us to investigate a number of problems which were hitherto difficult or even impossible to study.

Materials and Methods

Host cultures and cultural conditions

The susceptible host *Mortierella pusilla* Oudermans was
routinely grown on malt-yeast extract (MYE) solid medium consisting
of malt extract (20 g), yeast extract (2.5 g), and agar (20 g) in 1 L
distilled water at $23 \pm 1^{\circ}C$. The spores of the host were resuspended in
a liquid malt-yeast extract (MYE) without agar after adjusting their
concentration to $1x10^5$ cells/ml and germinated for 20-24 h at $23^{\circ}C$ on
a shaker incubator. The germinated spores were washed by
centrifugation with sterile distilled water, suspended in 0.01 M
phosphate buffered saline (PBS) at pH 6.8 and used for
immunoflurescence microscopy.

Parasite cultures and cultural conditions

Culture of biotrophic, haustorial mycoparasite, *Piptocephalis
virginiana* Leadbeater and Mercer was maintained on its susceptible
host, *Choanephora cucurbitarium* (Berk. and Rav.) Thaxter. Axenic
population of spores of mycoparasite were obtained by growing
cultures in 9 cm petri dishes at $23^{\circ}C$ 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 10^6 cells/ml with malt-yeast extract (MYE) medium containing 20 g of malt extract and 2 g of yeast extract and 10 ml glycerol in 1 L of distilled water. The spores were allowed to germinate for 20 h at 23 $^{\circ}$C. The germinated spores (80-90% germination) washed with sterile distilled water by centrifugation (x 1000g), were resuspended in 0.01 M PBS, pH 6.8. These germinated spores were used for immunofluorescence microscopy.

Preparation of cell wall fragments of the host

For isolation of cell wall fragments, culture of *M. pusilla* was grown in MYE liquid medium for 24 h in an environmental incubator shaker (120 rpm) at 23 $\pm$ 1 $^{\circ}$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 g 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 System-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°C until further use.

Crude extraction of *P. virginiana* proteins and *M. pusilla* cell wall proteins

P. virginiana proteins and *M. pusilla* cell wall proteins were extracted by suspending 0.25 g isolated *M. pusilla* cell wall fragments or *P. virginiana* germinated spores 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 x g for 10 min and the pellet was washed with ice-cold distilled water. The pooled supernatants were neutralized with 1N HCl at 0°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.

Immunofluorescence microscopy of *P. virginiana* with the treatment of *M. pusilla* cell wall proteins

An immunofluorescence technique with the treatment of *M. pusilla* cell wall proteins was used involving fluorescein isothiocyanate (FITC)-labeled 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 *P. virginiana* culture grown at 23±1°C for 24h, 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 *M. pusilla* protein by adding 10 µl of *M. pusilla* protein extract (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 2 h. The germinating spores were then 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 prepared against glycoproteins b and c of *M. pusilla* (Manocha and Su, 1992) 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 clean glass slide. A coverslip was placed on the drop of suspension and was sealed with fingernail polish. Wet mounts of samples labeled with fluorescent-antibody were viewed with a Wild-Leitz DIAPLAN fluorescence microscope fitted with an automatic camera.

Control tests were performed as follows: (i) omission of *M. pusilla* protein; (ii) omission of primary antibody against glycoproteins

b and c of *M. pusilla* ; (iii) omission of secondary antibody; and (iv) *P. virginiana* germinated spores were checked for autofluorescence.

SDS-polyacrylamide gel electrophoresis

Cell proteins or glycoproteins were dissolved in a solution of 2% sodium dodecyl sulfate (SDS) and 5% mercaptoethanol and heated at 95°C for 4 min. Samples of 300 µg of crude proteins or 10 µg of the purified glycoprotein were loaded on gels consisting of 10% acrylamide for the separation gel and 4% for the stacking gel. Electrophoresis was performed at 2.5 mA per gel for the tube gels and 60 V for the slab gel until the samples penetrated the separation gel and then at 3.5 mA per gel for the tube gels or 100 V for the slab gel. The running time was about 4 h for the tube gels and 2 h for the slab gel. The gels were removed from the glass tubes or plates and were fixed by shaking for 20 h in 100 ml of 40% methanol in 10% acetic acid for protein staining with Coomassie brilliant blue R250 (Sigma Chemical Co., St. Louis, MO, USA) or in 40% ethanol in aqueous 5% acetic acid for carbohydrate staining with period acid- Schiff reagent (Segrest and Jackson 1972).

Dot-immunobinding assay of *P. virginiana* proteins and *M. pusilla* cell wall proteins using prepared antibody against glycoproteins b and c of *M. pusilla*

Dot-immunobinding assays of cellular proteins were performed according to the methods outlined by Parent et al. (1985) and were modified by adding *M. pusilla* proteins before adding the primary antibody. Briefly, 2 µl samples of cell protein extracts from *M. pusilla* and *P. virginiana* (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 strips 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 strip was then transferred to *M. pusilla* protein extract(1mg/ml) diluted to 1:1,000 (20µl in 20 ml) in TBS containing 1% gelatin for 1 h. After washing 3x30 min in TTBS (20 mM Tris, 500 mM NaCl and 0.05% Tween-20, pH 7.5), the nitrocellulose strip 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 (2µl in 20 ml) in TBS containing 1% gelatin for 1 h. After washing 3x30 min in TTBS, the nitrocellulose strip was treated for 1 h with secondary antibody (2 µl/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-phosphatase (BCIP) (purchased from Promega corporation, Madison, WI, USA). 120 µl of 50 mg/ml NBT and 60 µl of 50 mg/ml BCIP were added to 20 ml of 0.1 M TBS, pH 9.5 (containing 1 mM $MgSO_4$). The strips were incubated in total darkness until the development of the color. Control tests were performed as follows: (I) omission of the *M. pusilla* proteins; (II) omission of the primary antibody against glycoproteins b and c of *M. pusilla* ; (III) omission of the secondary antibody.

Immunoblots of *P. virginiana* proteins with the treatment of *M. pusilla* cell wall 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. Omissions of the *M. pusilla* proteins, primary antibody against glycoproteins b and c of *M. pusilla* and secondary antibody respectively were performed as control experiments.

Purification of the glycoprotein from *P. virginiana* crude protein extract

The crude extract obtained from *P. virginiana* was used for the purification of the glycoprotein. The crude protein was dissolved (5 mg/ml) in 0.1M PBS, pH 7.4, and the solution was adjusted to 60% saturation with solid ammonium sulfate. The precipitate collected by centrifugation at 10,000 g for 15 min was redissolved in 20 ml of PBS containing 0.1 mM EDTA, pH 7.4. This suspension was heated in a water bath at 60°C for 5 min and, after cooling, the precipitate was removed by centrifugation.

Ten millilitre of the supernatant at the protein concentration of 4 mg/ml was applied to a Sephadex G-100 (100 x 2.5 cm) and eluted with 0.1 M PBS containing 0.1 mM EDTA, pH 7.4, at 4°C. The protein fraction containing the glycoprotein emerged between 60-100 ml (4

ml per tube) beyond the void volume. The eluate was dialyzed overnight against distilled water at 4°C and then lyophilized.

Preparative polyacrylamide gel electrophoresis

A modified method of Young and Kauss (1982) was used. Tube gels or slab gels contained 10% (W/V) acrylamide, 0.27% (W/V) N,N'-methylene-bisacrylamide, 0.038% (V/V) N,-N,-N',-N'-tetramethylenediamine, 0.07% (W/V) ammonium persulfate, and 38 mM Tris-HCl buffer, pH 8.8. The electrode buffer contained 2.4 g Tris and 22.5 g glycine per liter. Freeze dried sample obtained following Sephadex G-100 chromatography was dissolved at a concentration of 8 mg/ml in 50 mM Tris-HCl buffer, pH 6.8, containing 0.008% (W/V) bromophenol blue and 12% glycerol. Each tube gel was loaded with 100 μl (0.8 mg) of the sample solution or each lane of slab gel loaded with 30 μl sample solution. Electrophoresis was performed at 2.5 mA per tube or 100 V for the slab gel until the marker dye, bromophenol blue, reached the end of the gel. Following electrophoresis, a gel slice, corresponding to the glycoprotein region according to the prestained markers, was cut from the gels. Cut slices were homogenized in a Sorvall Omni mixer cup at full speed for 10 s, and eluted by stirring overnight with 20 ml of PBS at 4°C for 24 h. The eluate was collected by centrifugation at 5,000 g for 10 min, dialyzed against distilled water at 4°C for overnight and then lyophilized.

Identification of the sugar present on the glycoprotein

To determine if any carbohydrate could be identified on the *P. virginiana* protein, a Boehringer-Mannheim Glycan Differentiation Kit

was used. *P. virginiana* partially purified protein was separated by electrophoresis on 10% acrylamide mini-gels. Control glycoproteins, carboxypeptidase Y, fetuin, asialofetuin and transferrin provided with the kit, were separated along with the *P. virginiana* protein.

The proteins were then transferred from the gels onto a nitrocellulose membrane in a TansBlot Cell (Bio-Rad) by overnight electroblotting at a constant voltage (30 V) and an increase in voltage to 60 V applied for the last hour. The transfer buffer consisted of 0.30% w/v Tris; 1.44% w/v glycine; 20% v/v methanol. Membranes were then placed in blocking solution (1% w/v gelatin and 3% w/v Blocking Reagent (Boehringer-Mannheim) in TBS) for at least 30 min. The membranes are then washed three times in TBS (x 10 min) and once in Buffer 1 (TBS pH7.5 with 1 mM $MgCl_2$, 1mM $CaCl_2$).

Carbohydrate identification was carried out by incubating the membranes with the various digoxigenin-conjugated lectins provided in the Glycan Kit. *Galanthus nivalis* agglutinin (GNA) recognizes terminal linked mannose moieties (Shibuya et al. 1988); *Sambucus nigra* agglutinin (SNA) with specificity for sialic acid linked α(2-6) to galactose (Shibuya et al. 1987); *Maackia amurensis* agglutinin (MAA) which recognizes sialic acid linked α(2-3) galactose disaccharide (Wang and Cummings, 1988); Peanut agglutinin (PNA) recognizes core disaccharide galactose β(1-3)-N-acetylgalactosamine (Goldstein and Hayes, 1978) and *Datura stramonium* agglutinin (DSA) which recognizes galactose β(1,4)-N-acetylglucosamine (Crowley et al. 1984). The membranes were incubated for one hour with either 0.5 µg/ml GNA in Buffer 1; 1 µg/ml SNA in Buffer 1; 2.5 µg/ml MAA in Buffer 1; 2.5 µg/ml PNA in Buffer 1 or 1 µg/ml DSA in Buffer 1. The lectin

treated membranes were then washed three times in TBS (10 min) followed by a one hour incubation with anti-digoxigenin -Ap antibody (1 µg/ml in TBS). Anti-digoxigenin-Ap is conjugated with alkaline phosphatase (Ap) which can be visualized with Ap specific color reagents. A 5-10 min incubation with 4 nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in 0.1 M Tris-HCl pH9.5; 0.05 M $MgCl_2$; 0.1 M NaCl (Ap reagents) would visualize any glycoprotein bound by the lectin-antibody complex.

Preparation of antibody against the purified 100 kDa *P. virginiana* glycoprotein

Polyclonal antibody against the electrophoretically purified glycoprotein was raised in rabbit (New Zealand white rabbit): A total of 400 µg of glycoprotein was used for obtaining antibody. Totally 400 µg purified protein was dissolved into 500 µl 0.5M PBS, pH7.4, and injections were given 3 times, first 200 µg, second 100 µg, third 100 µg. The first injection was administered with an equal volume of Freund's complete adjuvant and the subsequent injections were given with incomplete adjuvant. All injections were administered into a rabbit by muscle. The rabbit was bled 21 days after the first injection. Subsequent bleedings were at 3-4 days intervals for a total of 3 bleedings. The blood samples were kept at room temperature for 1 h to allow clotting to occur, and then stored overnight at $4^{\circ}C$ before centrifugation at 2,500 g for 10 min at $4^{\circ}C$. The supernatant was used for the separation of the immunoglobulin fraction 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 then calculated by measuring the absorbance of a 1:25 dilution at a wavelength of 280 nm using a cuvete of 1 cm path length. The protein concentration was adjusted as (OD_{280} x 25) 1.0 mg/ml.

Dot-immunobinding assay of *P. virginiana* proteins and *M. pusilla* proteins using antibody raised against the purified *P. virginiana* glycoprotein

Dot-immunobinding assays of cellular proteins were performed according to the methods mentioned before. Samples of cell protein extracts from *M. pusilla*, *P. virginiana* and purified *P. virginiana* glycoprotein were treated with the primary antibody raised against the 100 kDa glycoprotein of *P. virginiana* and the secondary antibody (goat anti-rabbit IgG-alkaline phosphatase conjugate antibody, purchased from Promega corporation, Madison, WI, USA). Control test was performed as omission of the primary antibody.

Immunoblots of *P. virginiana* proteins and *M. pusilla* proteins using antibody raised against the purified *P. virginiana* glycoprotein

P. virginiana 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. Omissions of the primary antibody was performed as control experiment.

To investigate protein-protein interaction, immunoblot of *M. pusilla* cell wall proteins with the treatment of the purified *P. virginiana* glycoprotein was performed as before by using the purified glycoprotein as the first treatment. Purified *P. virginiana* glycoprotein was omitted in the control.

Immunofluorescence microscopy of *P. virginiana* and *M. pusilla* using antibody raised against the purified *P. virginiana* glycoprotein

The immunofluorescence technique was used as before except for using antibody against the purified *P. virginiana* glycoprotein as the first treatment to *P. virginiana* and *M. pusilla* germinated spores. The primary antibody was omitted in the control.

To investigate protein-protein interaction, another immunofluorescence technique was performed similar to the previous one by incubating *M. pusilla* germinated spores in *P. virginiana* protein suspension as the first treatment. *P. virginiana* protein was omitted in the control.

Results

Evidence of the presence of the complementary molecule on the surface of *P. virginiana*: Immunofluorescence microscopy of *P. virginiana* with the treatment of *M. pusilla* cell wall proteins

Figure 1 illustrates the fluorescence of the germinated spores of *P. virginiana* after treatment with *M. pusilla* cell wall protein extract, primary antibodies prepared against glycoproteins b and c of *M. pusilla* and FITC-goat anti-rabbit IgG conjugate. No fluorescence was observed in the controls: (i) minus *M. pusilla* cell wall protein extract (Figure 2); (ii) minus primary antibody (Figure 3); (iii) untreated *P. virginiana* germinated spores to check autofluorescence (Figure 4). The results indicated that on the surfaces of the biotrophic mycoparasite *P. virginiana*, there might be the complementary molecule existing which recognizes the b and c from *M. pusilla*. It might be either sugar residue or glycoprotein.

SDS-polyacrylamide gel electrophoresis of crude extracts of *P. virginiana*

The protein profile, from a NaOH extract of *P. virginiana* proteins, revealed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie brilliant blue R250 showed numerous distinct bands ranging from M_r 52-115 kDa (Figure 5).

Fig. 1. Fluorescence (A) and light (B) photomicrographs of germinated spores and hyphae of *P. virginiana* treated with *M. pusilla* cell wall protein, primary antibody raised against glycoproteins b and c of *M. pusilla* and FITC-goat anti-rabbit Ig-G conjugate (both primary and secondary antibodies diluted 1:10). Note the strong fluorescence on the surface of germinated spores and hyphae. X2,200
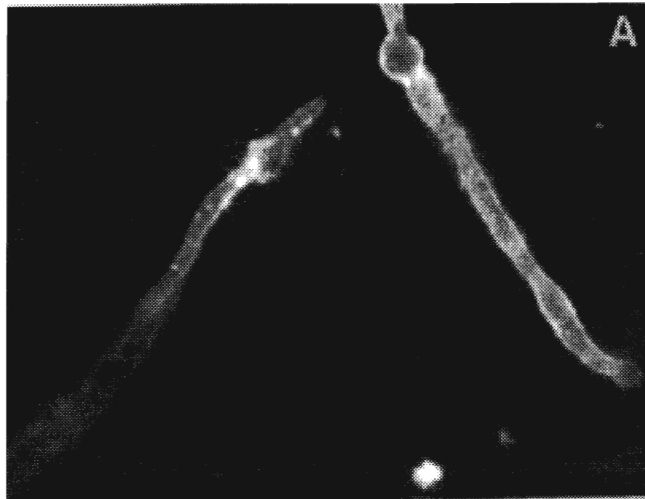
Fig. 2. Control Test: fluorescence (A) and light (B) photomicrographs of germinated spores and hyphae of *P. virginiana* treated with antibody prepared against glycoproteins b and c of *M. pusilla* and FITC-goat anti-rabbit Ig-G conjugate (both primary and secondary antibodies diluted 1:10). No fluorescence was observed. X2,200.
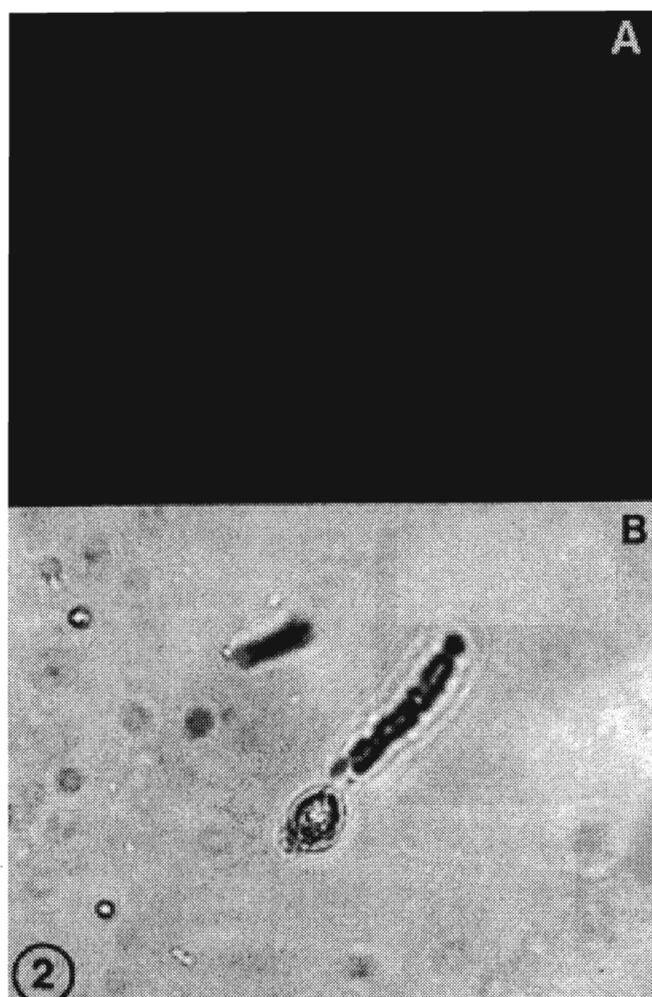
Fig. 3. Control Test: fluorescence (A) and light (B) photomicrographs of germinated spores and hyphae of *P. virginiana* treated with *M. pusilla* cell wall protein and FITC-goat anti-rabbit Ig-G conjugate (secondary antibody diluted 1:10). No fluorescence was observed. X2,200.
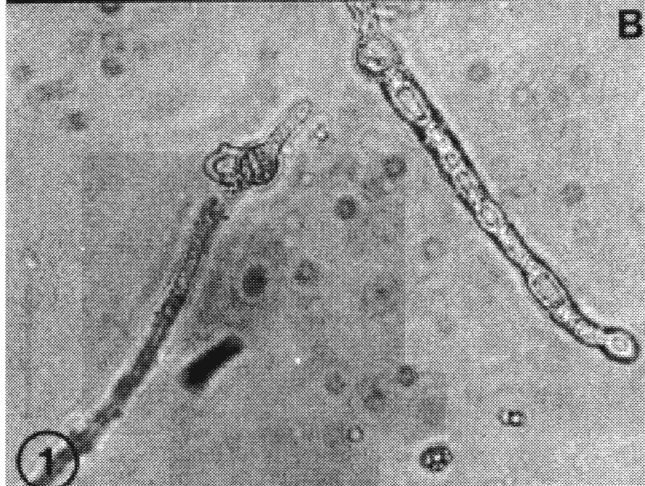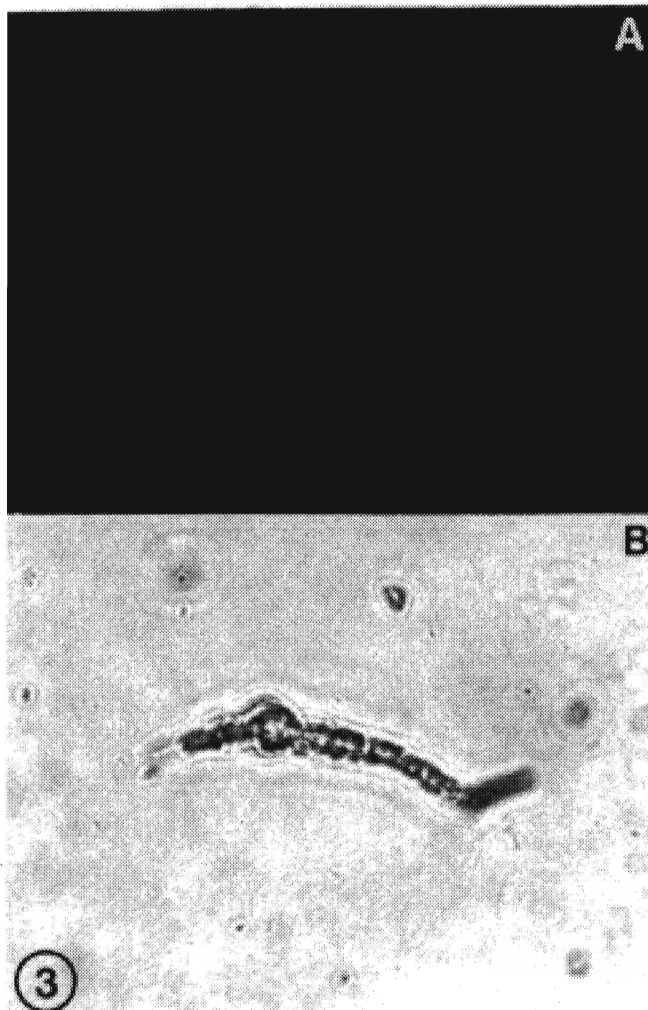
Fig. 4. Control Test: fluorescence (A) and light (B) photomicrographs of germinated spores and hyphae of *P. virginiana* with no treatment to check autofluorescence. No autofluorescence was observed. X2,200.

A

B

4
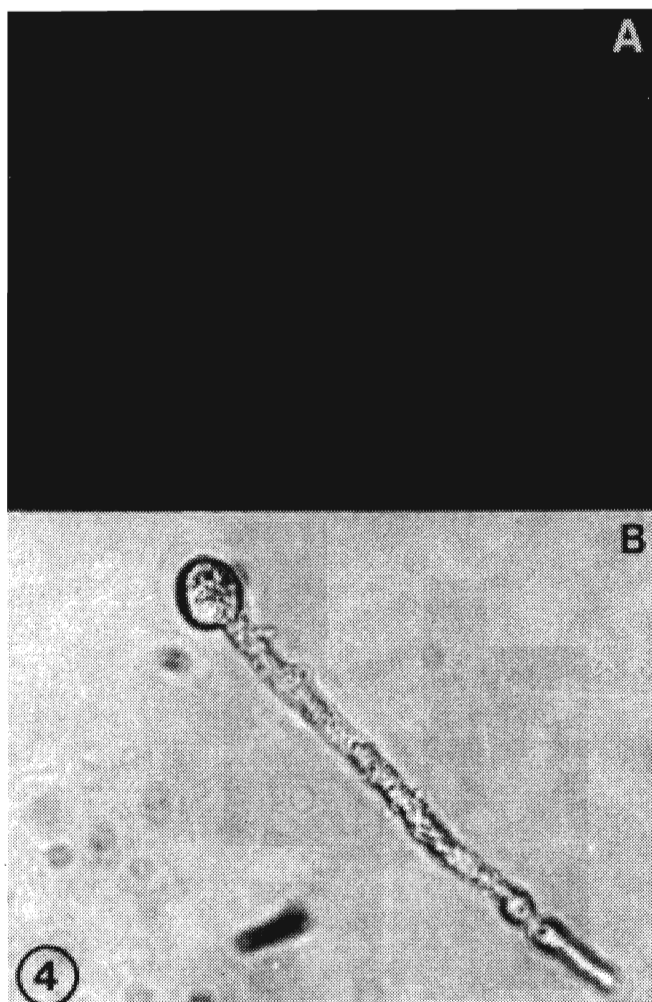
Fig. 5. SDS-polyacrylamide gel electrophoresis of protein extracts of *P. virginiana*. Gel was stained with Coomassie brilliant blue. From left to right: prestained protein standards; *P. virginiana* crude protein extract. The prestained protein standards purchased from Bio-Rad are: Phosphorylase B, M_r 112 kDa; Bovine serum albumin, M_r 84 kDa; Ovalbumin, M_r 52 kDa; Carbonic anhydrase, M_r 35 kDa; Soybean trypsin inhibitor, M_r 29 kDa; Lysozyme, M_r 21 kDa. The arrow points to a band of M_r 100 kDa.

kDa
112
84
52
35
29
21

5
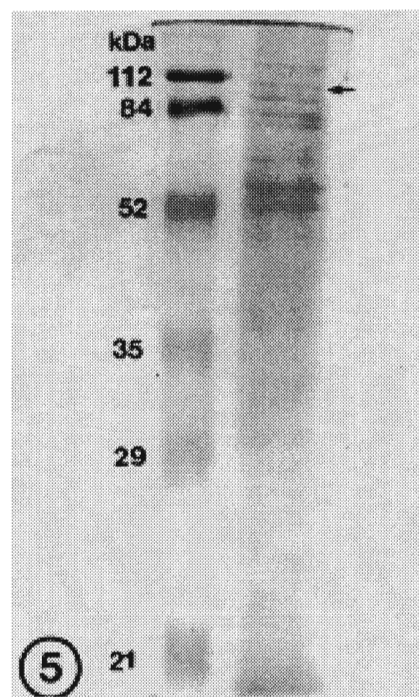
Dot-immunobinding tests of *P. virginiana* and *M. pusilla* protein extracts using antibodies prepared against glycoproteins b and c of *M. pusilla*.

The results from dot-immunobinding tests showed (Figure 6) that on the test membrane, after treatments of antibodies raised against glycoproteins b and c of *M. pusilla* and goat anti-rabbit IgG alkaline phosphase conjugate antibody, only *M. pusilla* sample had color reaction (Row 2), which confirmed the specificity of the antibodies against b and c previously reported (Manocha and Su, 1992). Both *P. virginiana* and *M. pusilla* protein have color reactions (Row 1) if the membrane was treated with *M. pusilla* cell wall protein, and minus primary (Row 3) and secondary antibody (Row 4), no reaction in both cases. This means in *P. virginiana* protein extract, there might exist the complementary protein which reacts especially with *M. pusilla.* protein or b and c.

Identification of the complementary molecule in *P. virginiana* protein extract.

The results of a immunoblots with *P. virginiana* proteins separated by SDS-PAGE on gels and transferred onto the nitrocellulose membrane, treated with *M. pusilla* cell wall protein extract and primary antibody raised against glycoproteins b and c of *M. pusilla* showed a strong reaction in a protein band of M_r 100 kDa (Figure 7A). The corresponding band is marked by an arrow in Figure 5. In the control test, omission of *M. pusilla* protein treatment gave no color reaction (Figure 7B). This indicates that this M_r 100 kDa

Fig. 6. Dot-immunobinding detection of antigen-antibody reaction. Total protein of *P. virginiana* (A), *P. virginiana* protein after ammonium sulfate fractionation (B), and total protein of *M. pusilla* (C) spotted on nitrocellulose membranes were treated with *M. pusilla* cell wall protein, primary antibody prepared against glycoproteins b and c of *M. pusilla* and secondary (goat anti-rabbit IgG alkaline phosphase conjugate) antibody (Row 1). Control tests were: (i) omission of *M. pusilla* cell wall protein (Row 2); (ii) omission of primary antibody (Row 3); (iii) omission of secondary antibody (Row 4).

Fig. 7. Immunoblot analysis of total proteins of *P. virginiana* incubated with *M. pusilla* cell wall protein, primary antibody prepared against glycoproteins b and c of *M. pusilla* and secondary antibody goat anti-rabbit IgG alkaline phosphase conjugate (A); the control (B) is the omission of *M. pusilla* cell wall protein. The protein band of M_r 100 kDa showed a colored reaction. The prestained protein standards are the same as those in Figure 5.
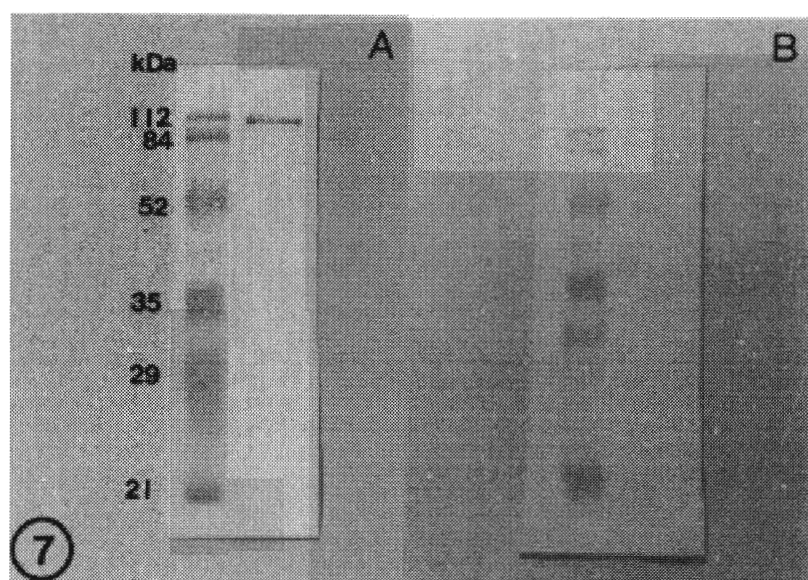
protein band is the complementary molecule which binds with the proteins b and c of the host.

Purification of the complementary protein from *P. virginiana* crude protein extract

Purification of this glycoprotein was achieved by (i) 60% ammonium sulfate precipitation, (ii) followed by heat treatment, and (iii) Sephadex G-100 gel filtration and (iv) preparative polyacrylamide gel electrophoresis by cutting and elution. Figure 8 shows the fractionation by a Sephadex G-100 column. The yield of this protein was 0.3% measured by Bradford method (1976) and the protein appeared between 60 and100 ml beyond the void volume.

Purification of this protein was confirmed by SDS-PAGE which revealed one distinct band on the gel after staining with Coomassie brilliant blue R250 (Figure 9). By staining with periodic acid-Schiff reagent, this 100 kDa protein was shown to be a glycoprotein (Figure 10). Western blot was performed with the purified protein (Figure 11), which showed the same color reaction with crude extract (Figure 7).

Carbohydrate analysis of the glycoprotein

Results of the identification of sugar present on this glycoprotein by using a Boehringer-Mannheim Glycan Differentiation Kit with various digoxigenin-conjugated lectins: *Galanthus nivalis* agglutinin (GNA) (Figure 12); *Sambucus nigra* agglutinin (SNA) (Figure 13); *Maackia amurensis* agglutinin (MAA) (Figure 14); Peanut agglutinin (PNA) (Figure 15); and *Datura stramonium* agglutinin (DSA) (Figure
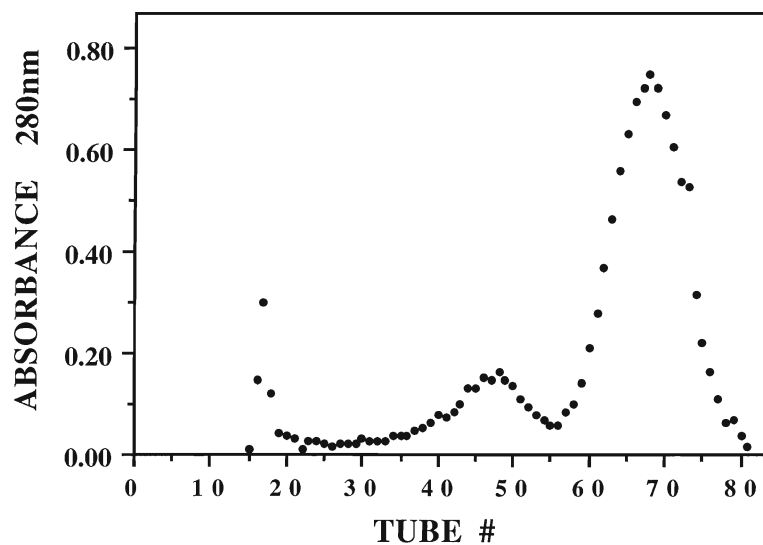
Fig. 8. Fractionation by a Sephadex G-100 column of the mycoparasite P. virginiana cell wall protein containing the complementary glycoprotein.
The fractionation range is 40,000-150,000.
The 100 kDa protein appeared in the first fraction.

45

Fig. 9. SDS-polyacrylamide gel electrophoresis of the purified protein of *P. virginiana*. Gel stained with Coomassie brilliant blue shows one band of M_r 100 kDa (arrow). The protein standards purchased from Bio-Rad are: Myosin, M_r 200 kDa; β-galctosidase, M_r 116 kDa; Phosphorylase B, M_r 97 kDa; Serum albumin, M_r 66 kDa; Ovalbumin, M_r 45 kDa.

46
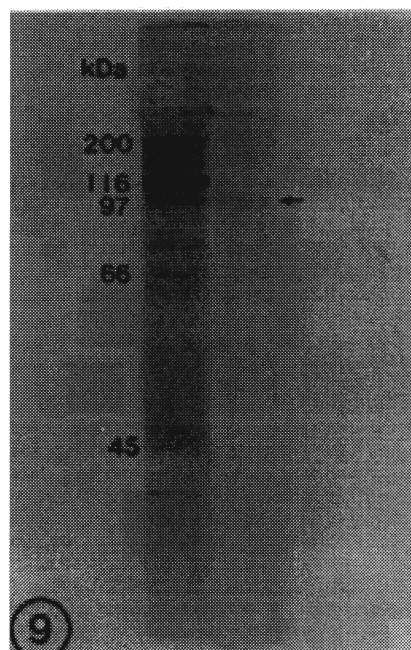
Fig. 10. SDS-polyacrylamide gel electrophoresis of the purified protein of *P. virginiana*. Gel was stained with periodic acid-Schiff reagent. The 100 kDa purified protein (arrow) showed pink color reaction of a glycoprotein. The prestained protein standards are the same as those in Figure 5.

kDa
112
84
52
35
29

10
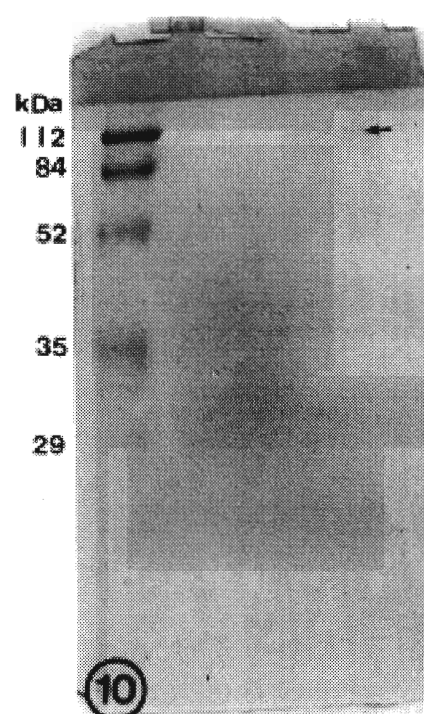
Fig. 11. Immunoblot analysis of purified glycoprotein of *P. virginiana* incubated with *M. pusilla* cell wall protein, primary antibody prepared against glycoproteins b and c of *M. pusilla* and secondary antibody goat anti-rabbit IgG alkaline phosphase conjugate. The purified protein showed colored reaction at the band of M_r 100 kDa. The prestained protein standards are the same as those in Figure 5.

kDa

112
84
52
35
29
21

11

Figure 12. Analysis of sugars on the 100 kDa glycoprotein from *P. virginiana*. lane 1, prestained SDS-PAGE standard protein; lane 2, partially purified *P. virginiana* protein; lane 3, asialofetuin; lane 4, carboxypeptidase Y; lane 5, transferrin and lane 6, fetuin after separated on 12% SDS-PAGE gels and were transferred to nitrocellulose membranes. Proteins were visualized by reaction with the lectin *Galanthus nivalis* agglutinin (GNA). The prestained protein standards are the same as those in Figure 5. Positive reactions were observed with *P. virginiana* protein (Lane 2) and the control glycoprotein carboxypeptidase Y (Lane 4).

Figure 13. Analysis of sugars on the 100 kDa glycoprotein from *P. virginiana*. lane 1, prestained SDS-PAGE standard protein; lane 2, partially purified *P. virginiana* protein; lane 4, carboxypeptidase Y; lane 5, transferrin; lane 6, fetuin and lane 7, asialofetuin after separated on 12% SDS-PAGE gels and were transferred to nitrocellulose membranes. Proteins were visualized by reaction with the lectin *Sambucus nigra* agglutinin (SNA). The prestained protein standards are the same as those in Figure 5. Positive reactions were observed only with the control glycoproteins transferrin (Lane 5) and fetuin (Lane 6).

Figure 14. Analysis of sugars on the 100 kDa glycoprotein from *P. virginiana*. lane 1, prestained SDS-PAGE standard protein; lane 2, partially purified *P. virginiana* protein; lane 4, carboxypeptidase Y; lane 5, transferrin; lane 6, fetuin and lane 7, asialofetuin after separated on 12% SDS-PAGE gels and were transferred to nitrocellulose membranes. Proteins were visualized by reaction with the lectin *Maackia amurensis* agglutinin (MAA). The prestained protein standards are the same as those in Figure 5. Positive reaction was observed only with the control glycoprotein fetuin (Lane 6).
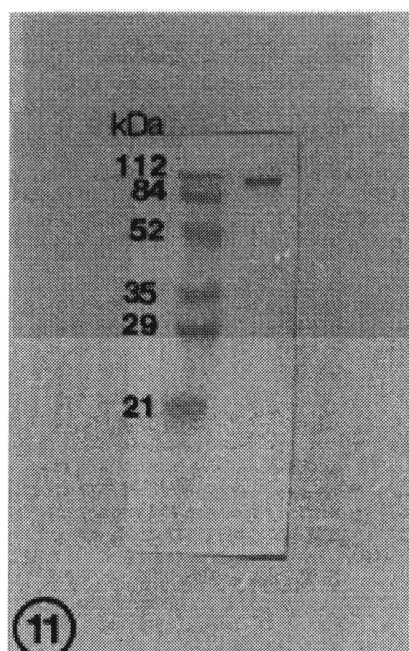
Figure 15. Analysis of sugars on the 100 kDa glycoprotein from *P. virginiana*. lane 1, prestained SDS-PAGE standard protein; lane 2, partially purified *P. virginiana* protein; lane 4, carboxypeptidase Y; lane 5, transferrin; lane 6, fetuin and lane 7, asialofetuin after separated on 12% SDS-PAGE gels and were transferred to nitrocellulose membranes. Proteins were visualized by reaction with the lectin Peanut agglutinin (PNA). The prestained protein standards are the same as those in Figure 5. Positive reaction was observed only with the control glycoprotein asialofetuin (Lane 7).
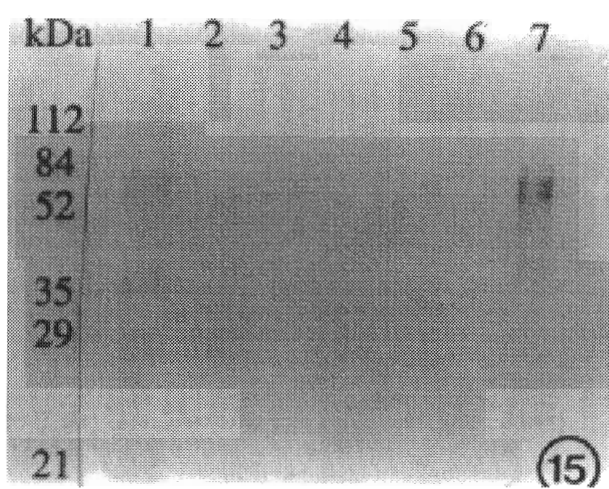
16) showed that only treatment with GNA showed the color reaction, which proved the presence of mannose in this 100 kDa glycoprotein.

Specificity of the antibody raised against purified complementary glycoprotein

Specificity of the antibody was determined by (i) a dot-immunobinding assay and (ii) a immunoblot analysis. Results of the dot-immunobinding test showed that the antibody raised against purified complementary glycoprotein reacted strongly with the purified protein and the crude protein extract of *P. virginiana*. No color reaction developed with *M. pusilla* protein sample (Figure 17). Control tests with the omission of primary and secondary antibody did not show any reaction.

Immunoblot analysis showed that *P. virginiana* protein extract contained a band of M_r 100 kDa protein which reacted positively with the antibody (Figure 18A). In the control test with the omission of primary antibody, there was no color reaction (Figure 18B).

Localization of complementary glycoprotein on the surface of *P. virginiana* germinated spores: Immunofluorescence microscopy

The results of the Immunofluorescence microscopy showed that the germinated spores of *P. virginiana* treated with primary antibody raised against the complementary protein, and FITC-goat anti-rabbit IgG conjugate exhibited fluorescence. In the early stages the strongest fluorescence was shown on the tips of the germ tubes (Figure 19A, B). When the tubes grew longer, strong fluorescence was observed in the
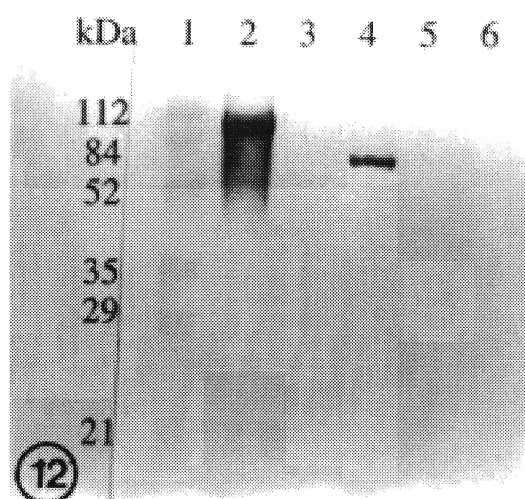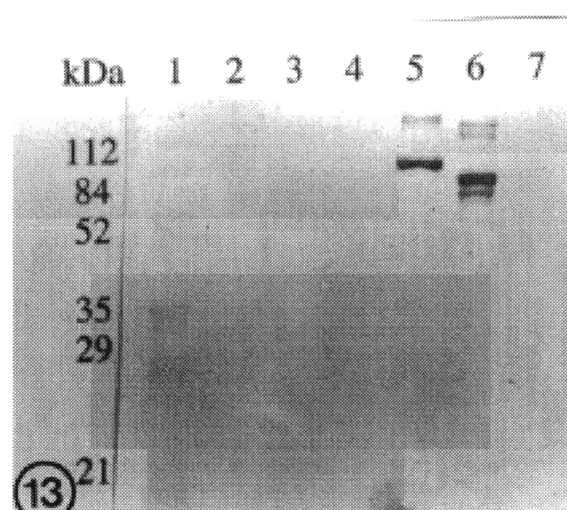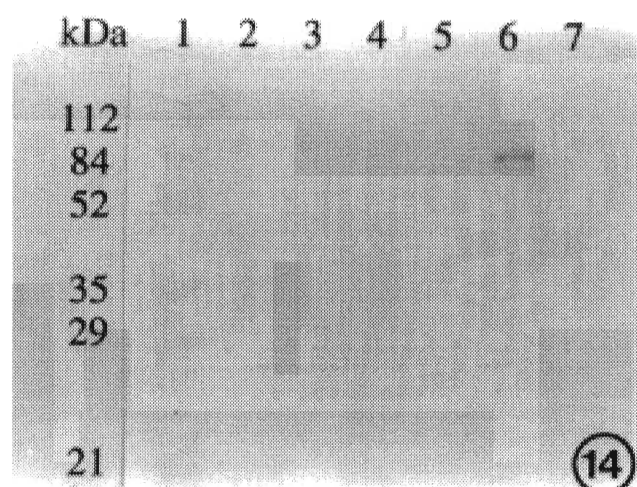
53

Figure 16. Analysis of sugars on the 100 kDa glycoprotein
from *P. virginiana.* lane 1, prestained SDS-PAGE standard
protein; lane 2, partially purified *P. virginiana* protein;
lane 4, carboxypeptidase Y; lane 5, transferrin; lane 6,
fetuin and lane 7, asialofetuin after separated on 12%
SDS-PAGE gels and were transferred to nitrocellulose
membranes. Proteins were visualized by reaction with
the lectin *Datura stramonium* agglutinin (DSA). The
prestained protein standards are the same as those in
Figure 5. Positive reactions were observed only with the
control glycoproteins transferrin (Lane 5), fetuin (Lane 6)
and asialofetuin (Lane 7).
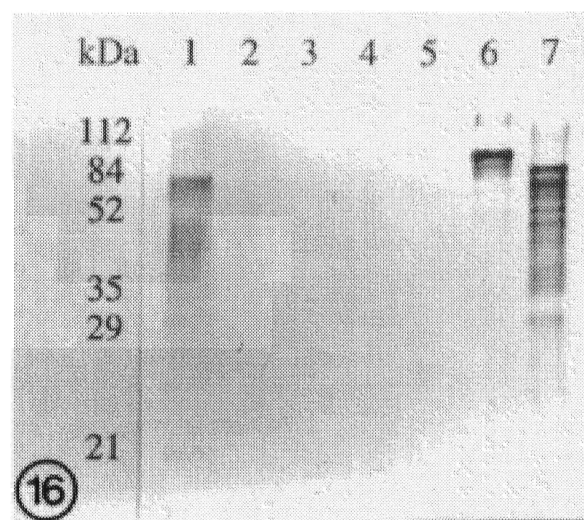
Fig. 17. Dot-immunobinding detection of antigen-antibody reaction. Total protein of *P. virginiana* (A), purified protein of *P. virginiana* (B) and total protein of *M. pusilla* (C) were spotted on nitrocellulose membranes. Samples were treated with primary antibody against purified glycoprotein of *P. virginiana* and secondary (goat anti-rabbit IgG alkaline phosphase conjugate) antibody (top row). Control (bottom row) was the omission of primary antibody.
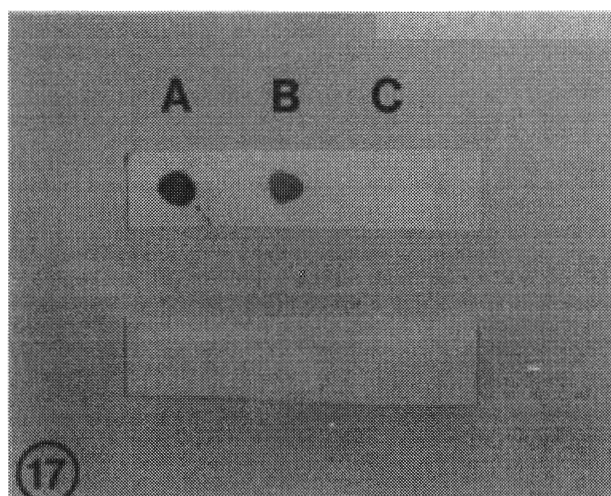
Fig. 18. Immunoblot analysis of total proteins of
P. virginiana incubated with primary antibody
raised against the complementary protein and goat
anti-rabbit IgG alkaline phosphase conjugate
antibody. The band at about M_r 100 kDa showed a
colored reaction (A). No color reaction was observed
in the control (B) with omission of the primary
antibody. The prestained protein standards are the
same as those in Figure 5.
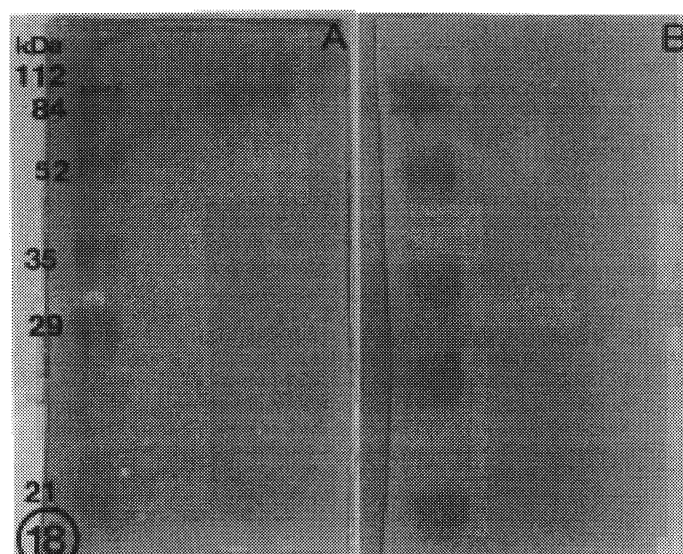
Fig. 19. Fluorescence photomicrographs of germinated spores of *P. virginiana* treated with primary antibody raised against the complementary protein and FITC-goat anti-rabbit Ig-G conjugate (both primary and secondary antibody diluted 1:10). Note the strong fluorescence on the tips of the germ tubes (A, B) and in the spores and along the whole length of the hypha (C, D). A, B, X2,200; C, D, X1,920.
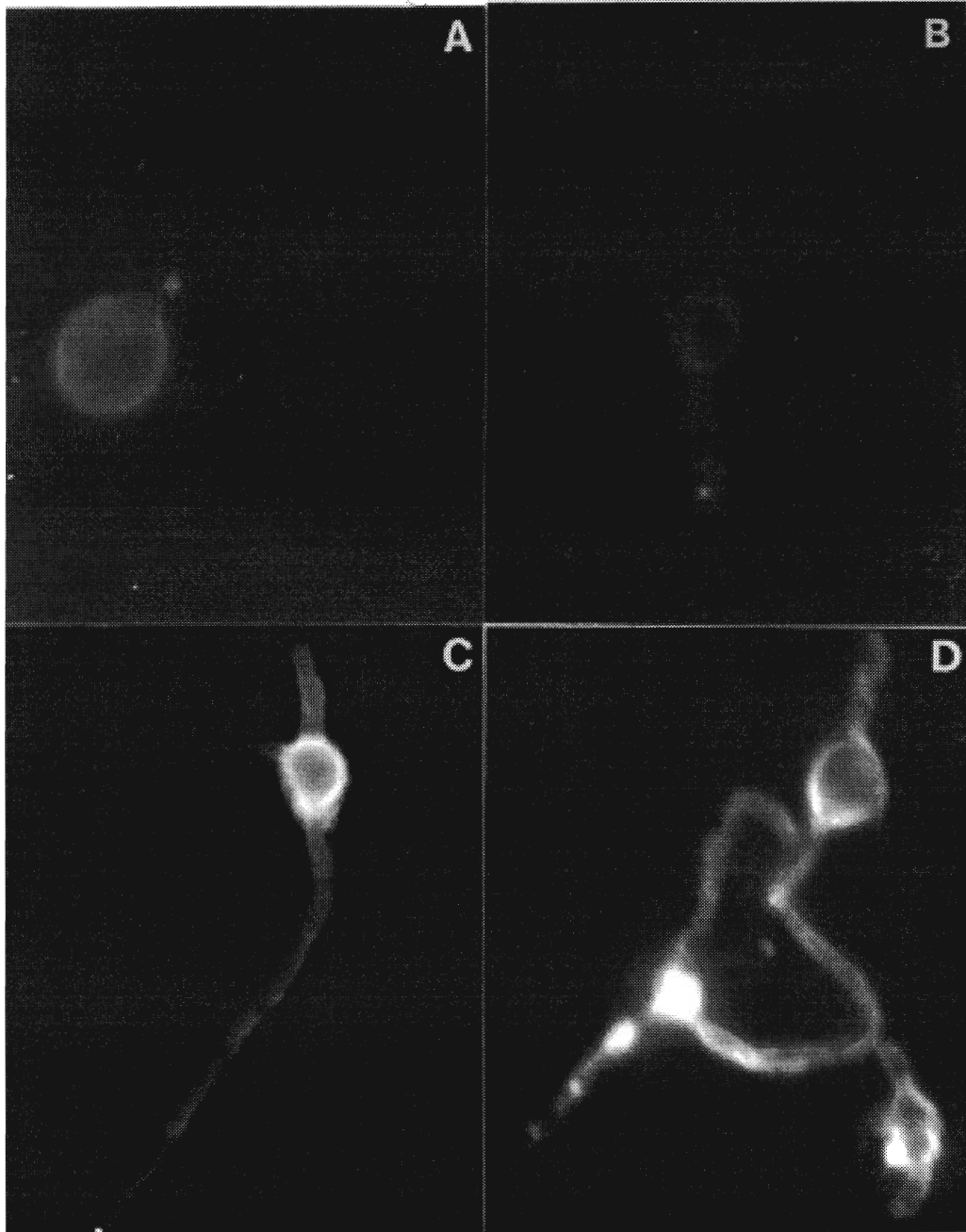
spores, and fluorescence was also observed along the whole length of the hypha (Figure 19C, D). With the same treatments as above, the germinated spores of *M. pusilla* did not show any fluorescence. However, when the germinated spores and hyphae of *M. pusilla* were treated with *P. virginiana* protein extract, primary antibody raised against the complementary protein of *P. virginiana* and FITC-goat anti-rabbit IgG conjugate, they exhibited fluorescence (Figure 20).

Determination of the specificity of complementary protein of *P. virginiana* for either b or c or both glycoproteins of *M. pusilla*

To ascertain whether the antibody raised against the complementary protein of the mycoparasite binds specifically with the b or c or both the glycoproteins of *M. pusilla,* immunoblot analysis was performed. The result in Figure 21A showed that both bands b and c areas reacted with the 100 kDa complementary protein. Omission of the *P. virginiana* protein treatment showed a negative result (Figure 21B).

Fig. 20. Fluorescence photomicrographs of germinated spore and a hypha of *M. pusilla* treated with *P. virginiana* protein extract, primary antibody raised against the complementary protein and FITC-goat anti-rabbit Ig-G conjugates (both primary and secondary antibody diluted 1:10). Note the strong fluorescence on the surface of germinated spore and hypha. X1,920.
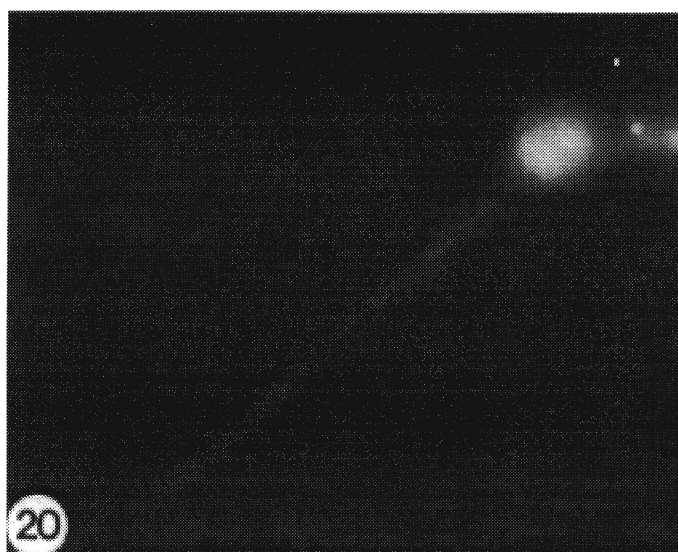
Fig. 21. Immunoblot analysis of total proteins of *M. pusilla* incubated with the purified protein from *P. virginiana*, primary antibody raised against the complementary protein and goat anti-rabbit IgG alkaline phosphase conjugate (A); the control test (B) is omission of purified *P. virginiana* protein. Two bands around b and c areas showed colored reaction. The prestained protein standards are the same as those in Figure 5.
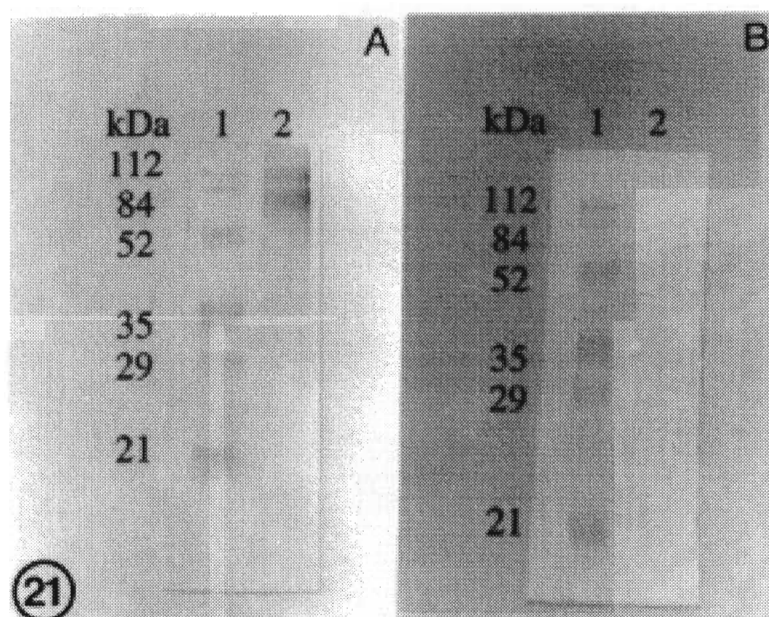
Discussion

Based on the results of the present and a previous study by Manocha and Chen (1991), a simplified recognition model between mycoparasite *P. virginiana* and it host *M. pusilla* has been suggested (Figure 22). This model suggests that two glycoproteins, b and c, from the host *M. pusilla* are the two subunits of an agglutinin and may function as receptor to a complementary molecule on the surface of the mycoparasite, *P. virginiana*. The complementary molecule has been the subject of the present study. In biotrophic mycoparasite systems this is the only system in which both host and parasite surface proteins have been isolated, purified and investigated for their role in recognition and attachment.

In necrotrophic system, glycoproteins responsible for recognition have been isolated from the host. The principle work in the phenomena of recognition of necrotrophic mycoparasites has been carried out using *Trichoderma* species parasitic on *R. solani* and *S. rolfsii*. The initial study performed by Elad et al. (1983) along with the studies of Barak et al. (1986) clearly demonstrated the properties of an agglutinin on the cell surface of *R. solani* that interacts with L-fucosyl residues on the surface of *Trichoderma*. Barak and Chet (1990) purified an agglutinin from *S. rolfsii* with sugar binding specificity similar to that of the lectin concanavalin A (ConA) i.e. for D-mannose and D-glucose. This agglutinin was found to be a glycoprotein. Inbar and Chet (1994) isolated and purified another agglutinin from the culture filtrate of *S. rolfsii* . Mono- or disaccharides inhibition tests and

parasite

b

c
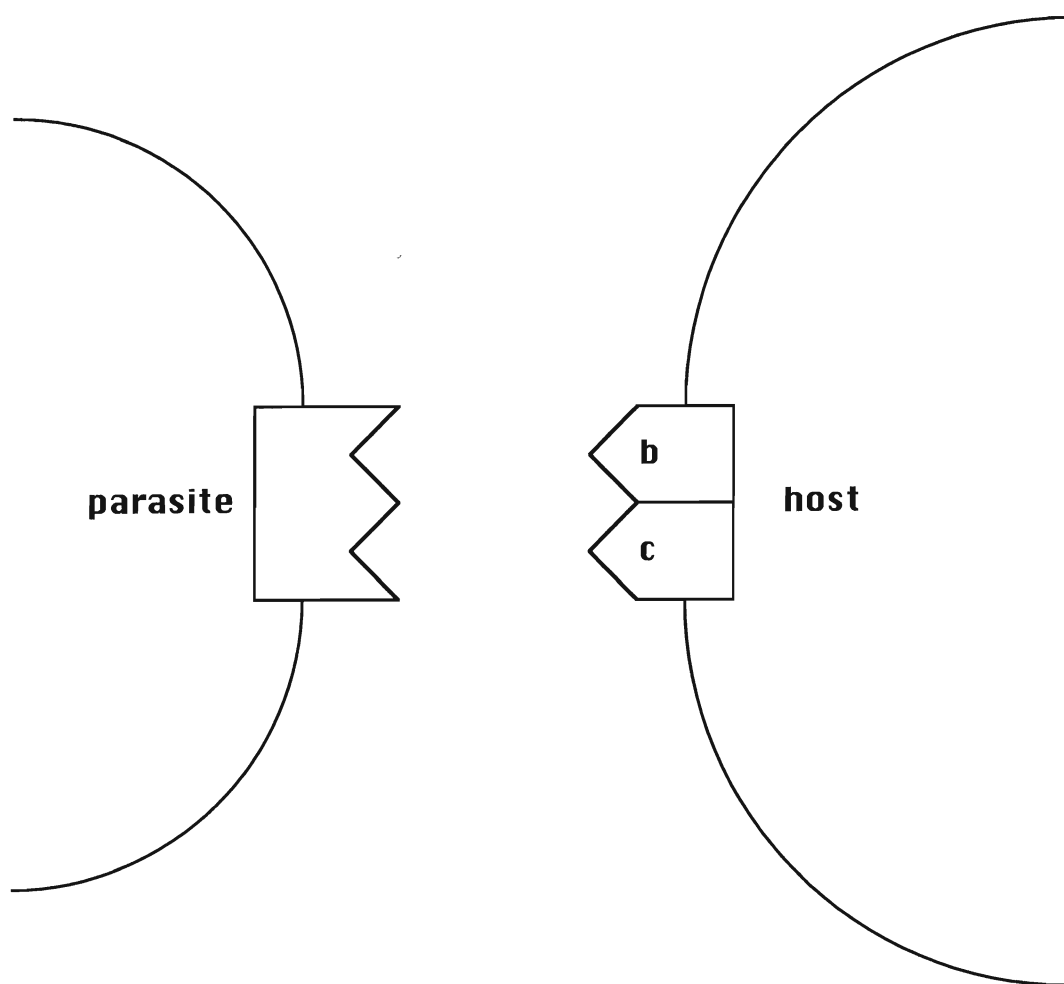
host

Figure 22. Recognition model for the mycoparasite-host
interaction between Piptocephalis virginiana
and Mortierella pusilla

the treatments of various enzymes indicated that both protein and 1,3-β-glucan are necessary for agglutination. Immunofluorescence studies of the biomimetic system based on the binding of lectin to the surface of nylon fibre (Inbar and Chet, 1992, 1994) was developed to test the role for lectin in the recognition. The biomimetic system revealed that the presence of the purified agglutinin on the surface of the fibres specifically induced mycoparasitic behavior in *Trichoderma harzianum*. They suggested that the recognition of *Trichoderma* to *S. rolfsii* cell wall surface is indeed mediated by a complexed agglutinating polymer which surrounds the host hyphae, thereby initiating a sequence of events which eventually lead to the destruction of the host. From the above results, there is enough evidence to suggest the role of a lectin or agglutinin type interaction facilitating recognition in these mycoparasite systems.

In another necrotrophic mycoparasitic system, cell surface protein responsible for recognition was investigated. Interaction between the necrotrophic mycoparasite *Stachybotrys elegans* and its host *Rhizoctonia solani* was investigated by Benyagoub et al. (1996). Fimbrial protein was localized within the extracellular matrix that surrounded *S. elegans* cells when confronted with its host. Their study has proved to be the first report on the presence of fimbrial proteins in a fungal mycoparasite and provided evidence that these proteins play a role in cell recognition and adhesion. Their study can be compared with an earlier report from this laboratory on the presence of fimbrial proteins on the surface of fungal host which play a role in biotrophic mycoparasite and fungal host interaction(Rghei et al. 1992). Fimbriae were observed by electron microscopy on the surfaces of the

host and non-host species but not the mycoparasite. Polyclonal antiserum prepared against the fimbrial protein of *Ustilago violacea* cross-reacted with 64kDa proteins from both *M. pusilla* and *P. articulosus* and 60 and 57 kDa proteins from *M. candelabrum*. These proteins were electroeluted from polyacrylamide gels and were shown subsequently to form fibrils with the same diameter as the cell surface fimbriae. To ascertain the role of fimbriae in host-mycoparasite interaction, the antiserum was incubated with *P. virginiana* and *M. pusilla*, and with *P. virginiana* and *P. articulosus*. Contacts between mycoparasite and host were blocked significantly by the antiserum. It was proposed that the recognition of fimbriae by the mycoparasite leads to directed growth and contact between mycoparasite germ tubes and the hyphae of a potential host.

In the plant host-fungal parasite system, proteins responsible for recognition at the surface of fungal parasite have been extensively studied as compared to the mycoparasitic system. Various types of 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). Elicitors appear to be recognized by plant cells via interaction with specific receptors on plant plasma membranes (Yoshikawa et al. 1993a). Some glycoprotein molecules and peptides commonly found in fungal cell walls have been shown to have elicitor activity.

Lectins and agglutinins at the surface of host cells have been found in both plants and fungi. The biological effect of the lectin is due to the binding of lectin to carbohydrate-containing receptors (carbohydrate or glycoprotein) localized at the parasite cell surface

(Callow, 1977; Manocha and Sahai, 1993). Some sugar residues at the plant host surface and their receptors on the fungal parasite play a role in recognition. Longman and Callow (1987) suggested that protein-containing receptors on the zoospore surface interact with fucose-containing ligands present in root surface mucilage to promote zoospore-binding and subsequent encystment. Some avirulence gene products, elicitins and hydrophobins are small, cysteine-rich proteins (Templeton, et al. 1994). It is speculated that in fungi the recognition process might be mediated by an interaction between the cysteine-rich, hydrophobin-like domain of avirulence genes and similar domains present on the receptor.

My study has provided a protein-protein interaction model. Proteins can store variation freely; indeed, most of the variation in the DNA of structural genes survives to be reflected in the amino acid residues of proteins. Proteins recognize one another precisely, as in many antigen-antibody systems or in polymerization. These two attributes, variation storage and mutual recognition, are essential for a biochemical explanation of the gene-for-gene hypothesis: and a protein-for-protein hypothesis as proposed by Vanderplank (1978). The hypothesis states that in gene-for-gene disease the mutual recognition of host and pathogen is not by genes themselves but by their coded proteins (Vanderplank, 1982).

To investigate protein-protein interaction, the immunofluorescence microscopy of the germinated spores of *P. virginiana* pretreated with *M. pusilla* cell wall protein extract, primary antibody against b and c from *M. pusilla* and FITC-goat anti-rabbit IgG conjugate have given us confidence of the previous

hypothesis from this lab of the existence of the complementary molecule on the surface of *P. virginiana*, while dot-immunobinding tests and immunoblots clearly indicated that a protein of about M_r 100 kDa from *P. virginiana* is complementary to the glycoproteins b and c of *M. pusilla*.

Actually, the existence of the recognition protein molecule at the surface of the mycoparasite *P. virginiana* was indicated by a previous study (Manocha, 1985). Germ tubes of *P. virginiana* previously inactivated by exposure to 65°C for 15 min or to 3% glutaraldehyde for 30 min did not attach to the host cell wall fragments, which suggested that attachment depends on the vital activity of the mycoparasitic germ tubes to seek out complementary receptor sites at the host cell surface.

The purification of the 100 kDa protein from *P. virginiana* crude protein extract by preparative polyacrylamide gel electrophoresis has proved to be successful. This technique was used in a previous study for the isolation and purification of glycoproteins b and c from *M. pusilla* (Manocha and Chen, 1991). Both the purified 100 kDa protein and b and c from *M. pusilla* are NaOH extract. The b and c are from cell wall, and I tried my best to break the cell walls of *P. virginiana* spores which are difficult to break. Fungal cell walls generally are constructed with an inner part containing either chitin or cellulose embedded in a matrix, and an outer alkali-soluble layer. Differences which might be species-specific are found in the alkali-soluble glycoproteins (Wessels and Sietsma, 1981). It is suggested that the fungal cell wall may act as a molecular sieve for extracellular signals originating from the host, or

it may act as a primary target from which secondary signals are generated(Ralton, et al, 1987).

The results of immunofluorescence microscopy, dot-immunobinding tests and immunoblots using previously prepared antibody against b and c for the investigation of protein-protein interaction are based on the good purity of previously prepared antibody against b and c. They confirmed the suggestion 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* (Manocha and Su, 1993). It means that after binding to complementary molecule from parasite in the recognition sites, two glycoproteins b and c have other binding sites for the polyclonal antibodies which secondary antibody can attach to. The results from immunofluorescence microscopy and immunoblots using antibody against purified glycoprotein for the investigation of protein-protein interaction also can draw similar conclusion, which is that the antigenic determinants of the glycoprotein which are recognized by antibodies are not necessarily the receptor sites recognized by the glycoproteins b and c of the host *M. pusilla.*

In my work, I used two sets of markers, one is prestained marker for immuno-binding tests to localize the protein bands, another is high-range SDS-PAGE markers for the determination of molecular weight of the protein bands. That's why serum albumin sometimes 84kDa and 66kDa, phosphorylase b 97 and 112kDa, ovalbumin 45 and 52 kDa.

The carbohydrate analysis of the 100 kDa protein has proved the presence of mannose in this 100 kDa glycoprotein. There might be other carbohydrate components existing, but due to the number of

lectins we used, only one carbohydrate component was found. This can be compared with the result of carbohydrate analysis of b and c which contain glucose, N-acetyl-glucosamine and arabinose as the major carbohydrate components (Manocha and Chen, 1991). These three sugars inhibited agglutination as well as attachment of *P. virginiana* germ tubes to host cell wall fragments but had no obvious effect on appressorium formation. On the other hand, appressorium formation was inhibited by heat treatment of host wall fragments, which still permitted attachment. It was suggested then that the factor(s) responsible for attachment were probably not involved in appressorium formation.

The immunoblot analysis by treating *M. pusilla* cell wall protein with purified *P. virginiana* complementary glycoprotein, newly prepared antibody and FITC-goat anti-rabbit IgG conjugate showed that the complementary glycoprotein binds to both proteins b and c. This result lent support to an earlier observation that both b and c proteins are required for the agglutination of mycoparasite spores (Manocha and Chen, 1991). In addition to the two proteins b and c, we can see some minor bands around b and c had color reaction too. They might be due to non-specific binding or color overdevelopment. These minor bands' function remains unknown though they are unlikely to serve any role in recognition and attachment.

It should be noted that glycoproteins like b and c from the host and the complementary glycoprotein from the mycoparasite are not the only proteins involved in the interaction between host and parasite. Fimbriae have been shown to play a role in fungal host-mycoparasite interaction as well. The interaction between the fungal host and the mycoparasite appears to occur at several levels: (1)

initially through fimbriae, resulting in directed growth and contact (Rghei et al. 1992); (2) through attachment of the mycoparasite to the host cell surface mediated by the host cell wall glycoproteins which are complementary to the purified 100 kDa glycoprotein from the parasite; (3) during appressorium formation (Manocha and Chen, 1991); and (4) at the host plasmalemma as indicated by the resistant host response to the mycoparasite e.g. formation of a papilla which inhibits penetrations by the mycoparasite (Manocha et al. 1990).

The present study provides a convincing evidence for the binding between the cell surface glycoproteins, b and c, of the host and the complementary glycoprotein of the mycoparasite. However, it is not clear what role the sugars of these glycoproteins play in host-parasite interaction. Does the complementary glycoprotein of the mycoparasite bind to the proteins b and c or to their sugar moieties, glucose and N-acetylglucosamine? Manocha et al. (1990) did show that pretreatment of the mycoparasite with glucose and N-acetylglucosamine inhibited its attachment to the host cell surface whereas mannose, the sugar in the complementary protein of the parasite, had no effect on the attachment.

Further investigations are needed to clarify whether the sugar moieties of the glycoproteins are involved in the interaction between the biotrophic mycoparasite, *Piptocephalis virginiana*, and its host, *Moetierella pusilla*. Other molecules involved in the recognition signal transduction should be determined too.

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