Isolation and Partial Characterization of Host Cell Wall Surface Glycoproteins: Their Possible Involvement in Agglutination, Attachment and Appressorium Formation by Piptocephalis virginiana

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Abstract

Cell surface proteins obtained by alkaline extraction from isolated cell walls of Mortierella pusilla and M. candelabrum, host and nonhost, respectively, to the mycoparasite, Piptocephalis virginiana, were tested for their ability to agglutinate mycoparasite spores. The host cell wall protein extract had a high agglutinating activity (788 a.u. mg\(^{-1}\)) as compared with the nonhost extract (21 a.u. mg\(^{-1}\)). SDS-polyacrylamide gel electrophoresis of the cell wall proteins revealed four protein bands, a, b, c, and d (Mr 117, 100, 85 and 64 kd, respectively) at the host surface, but not at the nonhost surface, except for the faint band c. Deletion of proteins b or c from the host cell wall protein extract significantly reduced its agglutinating activity. Proteins b and c, obtained as purified preparations by a series of procedures, were shown to be two glycoproteins. Carbohydrate analysis by gas chromatography demonstrated that glucose and N-acetylglucosamine were the major carbohydrate components of the glycoproteins. It was further shown that the agglutinating activity of the pure preparation containing both b and c was 500-850 times that of the single glycoproteins, suggesting the involvement of both glycoproteins in agglutination. The results suggest that the glycoproteins b and c are the two subunits of agglutinin present at the host cell surface.

The two glycoproteins b and c purified from the host cell wall protein extract were further examined after various treatments for their possible role in agglutination, attachment and appressorium formation by the mycoparasite. Results obtained by agglutination and attachment tests showed: (1) the two glycoproteins are not only an agglutinin responsible for the mycoparasite spore agglutination, but may also serve as a receptor
for the specific recognition, attachment and appressorium formation by the mycoparasite; (2) treatment of the mycoparasite spores with various sugars revealed that arabinose, glucose and N-acetylglucosamine inhibited the agglutination and attachment activity of the glycoproteins, however, the relative percentage of appressorium formation was not affected by the above sugars; (3) the two glycoproteins are relatively stable with respect to their agglutinin and receptor functions. The present results suggest that the agglutination and attachment may be mediated directly by certain sugars present at the host and mycoparasite cell surfaces while the appressorium formation may be the response of complementary combinations of both sugar and protein, the two parts of the glycoproteins at the interacting surfaces of two fungi.
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The help of many members of the faculty and staff of the Department of Biological Sciences is acknowledged.
Dedication

I wish to dedicate this manuscript to my wife, Huajing, for her help and encouragement during this study and to my parents for their many years of dedication to my education.
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Introduction

Knowledge in specificity of interactions between fungal parasites and their hosts is fundamental to plant pathology. Although there has been considerable amount of research on this aspect, the details are still not completely clear. Investigation on this aspect may help us understand the nature and regulation of parasitism.

Mycoparasitism, the parasitism of one fungus on another, has been recognized by mycologists for about one hundred years. Mycoparasitic systems are ideal models for studies aimed at elucidation of complex problems of host-parasite interactions. They offer a unique combination of features and have numerous advantages over the higher plant-fungal pathogen systems, e.g. easily controlled cultural conditions, restricted host range, saving of time and space (Barnett and Binder 1973). In addition, the course of mycoparasitic infection has shown to parallel that of the plant-pathogen systems (Barnett and Binder 1973). Studies using fungal host-mycoparasite combinations may contribute to our understanding of specificity of interactions in other host-parasite systems.

*Piptocephalis virginiana*, a biotrophic haustorial mycoparasite used in this research, is a member of the order *Zoophagales* in the class *Zygomycetes*. The host range of this mycoparasite is restricted to members of the order *Mucorales*, which also includes some nonhosts (Berry and Barnett 1957; Barnett and Binder 1973). The infectious process on the hosts by *P. virginiana* is similar to that of some (other) plant pathogens, including recognition and attachment, appressorium formation, penetration, development and finally establishment of nutritional relationship (Manocha 1987). However, the mycoparasite does not show
any interaction whatsoever with the nonhost species. The mycoparasite's germ tube either crosses over or under, or sometimes continues to grow along side of the nonhost hypha without any indication of attachment and appressorium formation (Manocha and Golesorkhi 1981; Manocha 1986).

The mechanism of attachment of the mycoparasite to the host but not to the nonhost species has been under investigation in this laboratory for a long time, and a number of articles about this subject have been published. The gross cytological features and chemical compositions of cell walls did not reveal any differences between host and nonhost species (Letourneau et al. 1976; Manocha 1984). However, the attachment was found to be inhibited in the presence of specific sugars, chitobiose and chitotriose (Manocha 1985), suggesting the possible involvement of carbohydrate binding proteins in mycoparasitism. Both host and nonhost showed a positive binding reaction with fluorescent lectins specific for N-acetylglucosamine oligomer. The cell surface of the nonhost also revealed fucose, galactose and N-acetylgalactosamine residues at the lectin binding sites (Manocha 1985; Manocha et al. 1986). Further, SDS-polyacrylamide gel electrophoresis of cell wall extracts of host and nonhost species showed marked differences in their protein and glycoprotein profiles. Notably two bands of high molecular weight glycoproteins were observed in the extract of host species and not in the nonhost species (Manocha, 1984; Manocha 1985; Manocha et al. 1986).

It has been known that cell-cell interactions are usually mediated by specific ligands and receptors at the surfaces of the interacting partners (Callow 1977; Sequeira 1978; Longman and Carrow 1987). Various cell surface receptors identified during the last decade include carbohydrate binding proteins, i.e. agglutinins (Barak et al. 1986; Hohl and Balsiger
1988) and glycosidases (Shannone and Hankines 1981). Agglutinins have been shown to interact with glycoproteins or proteoglycans at the surfaces of the cells (Hamer et al. 1987; Bonfante-Fasolo 1988). A given cell expresses on its surface a set of specific glycoproteins or glycolipids which may be involved in recognition mechanism (Frazier and Glaser 1979; Daly 1984). A previous report from this laboratory showed that crude protein extract prepared from the cell walls of the host agglutinated cell wall fragments of the mycoparasite. However, the agglutinin contained in the crude extract has not been determined.

Obviously, further work is needed to isolate and characterize the two high molecular weight glycoproteins that are present only at the host surface and to elucidate the nature and role of the glycoproteins in the host-mycoparasite interactions.

For this study two species of the genus Mortierella, differing in their response to the mycoparasite P. virginiana, were selected. Mortierella canelabrum is not parasitized by the mycoparasite and served as a nonhost in the mycoparasite system; Mortierella pusilla is a susceptible host and supports good growth of the mycoparasite (Manocha 1984).
Review of Literature

Specificity of Attachment of Fungal Parasites to Their Hosts

The process of parasitism in host-fungal parasite systems usually consists of attachment and recognition, formation of an appressorium, penetration, development of a haustorium, formation of an extrahaustorial matrix, etc. Attachment of fungal parasites to their host cell surfaces is a prerequisite for further parasitic events. Attachment may involve parasite recognition of specific surface topography of the host or binding between complementary molecules on both host and parasite cell surfaces. As part of recognition, attachment may happen before, after, or together with the phenomenon of recognition between host and parasite.

There is a remarkable degree of specificity of attachment in host-fungal parasite interactions. That is, despite the presence of a diverse array of potential fungal pathogens in the environment, only a few organisms or plant species become attached to and subsequently infected by pathogens. Moreover, only certain biotypes or races of fungal pathogens are capable of attaching to and parasitizing certain strains or cultivars of a single organism or plant species.

Studies on the mechanism for the specificity of attachment of fungal parasites to their hosts, especially in mycoparasite systems, are still in their infancy. However, in recent years, remarkable progress in morphological, biochemical and physiological studies on this subject have been made toward a better understanding of the nature of this phenomenon. It is hoped that the genetics of the specificity of attachment and the determination of the molecular nature of binding sites will
eventually lead to the unravelling of the different mechanisms by which the fungi attach to their hosts.

I. Morphological Aspects

1. Specific host surface topography — a morphological explanation for the specificity of attachment

Cell surfaces provide the first point of interaction between a host and a fungal parasite. The cell surface could be an important centre of focus, as it is here where the attachment of fungal parasites and recognition occur. Apart from the biochemical events involved in the attachment, there has been considerable interest in the specific surface topography of the host which plays an important role in attachment and recognition in host-fungal parasite interactions.

Over the past several years, a number of researchers have published studies on germinating uredospores of rust fungi which suggest that germ tubes recognize and respond to specific surface topography of the hosts. Infection either fails or is erratic unless the correct information is present on the host surface to induce the attachment of the parasite to its host (Staples and Macko 1980). Furthermore, features of the host surface can guide the progress of germ tube differentiation after the fungal parasite attaches. When uredospores germinate on the surface of a host plant leaf, the first attachment stimuli for germ tube differentiation are the set of junction lines between cells of the epidermis, or the ridge, and indentations caused by the presence of the vascular elements (Dickinson 1949). These stimuli strongly orient germ tubes as they grow out over the surfaces of
the leaf. The perception by the germ tube of the ridge on the surface due to the underlying vascular system causes the germ tubes to travel at right angles to them. Such an orientation makes the search for the stomatal opening by the germ tube more efficient than a randomized growth pattern would have been (Lewis and Day 1972). Recently, the nature of the specific topographical signals of the host for growth orientation and infection structure formation were determined for the rust fungus *Uromyces appendiculatus* (Hoch et al. 1987). The differentiation signal was found to be a simple ridge, in this case one that was microfabricated on silicon wafers by using electron-beam lithography and had an optimum height of 0.5 μm. A similar ridge in the form of a stomatal lip was found associated with the stomatal guard cells of the bean (*Phaseolus vulgaris*) leaf. Ridge elevations greater than 1.0 μm or less than 0.25 μm did not serve as effective signals. The growth orientation signals for the germ tubes was found to be ridge spacings of 0.5 to 6.7 μm. The data suggest that the fungus is able to distinguish uniquely minute differences between host and nonhost leaf surface topographies, in order to attach to and subsequently infect the host plant.

Although the phenomenon of orientation has been discussed for both obligate (Dickinson 1949, 1977; Wynn 1976; Hoch et al. 1987) and facultative parasites (Preece et al. 1967), the fascinating mystery remains as to how the growing tip of the germ tube perceives the special topography of the junction to guide its direction of growth. It is reasonably certain that the germ tube can be attached to the host epidermis; however, surface contact is poor on nonhost species, and orientation is often confused (Wynn 1976). These observations suggest that in order for an effective stimulus, hyphal attachment with the host
surface must be specific and tight. In *Puccinia coronata*, the germ tube is bound to the host leaf specifically and apparently by a combination of fungal secretions (Onoe et al. 1972). This phenomenon was also observed in the interaction between a biotrophic haustorial mycoparasite, *Piptocephalis virginiana*, and its host (Manocha 1984).

2. Appressorium formation -- an attachment and recognition response in fungal parasites

There is considerable evidence that the formation of an appressorium by a fungal parasite on the host surface is the result of successful attachment and recognition between the two interacting species.

On an artificial smooth surface, the dikaryotic germ tubes of the bean rust fungus, *Uromyces appendiculatus*, may reach 1 mm in length, but they do not undergo nuclear division or form infection structures. On host leaves, however, germ tubes that attached to stomatal guard cells quickly underwent mitotic nuclear division and formed appressoria (Maheshwari et al. 1967; Staples et al. 1975; Wynn 1976). This suggests that the bean rust fungus has a contact-sensitive response to the stoma which is the fungal penetration site on a leaf (Epstein et al. 1985). Carver and Ingerson (1987) also found that conidia of *Erysiphe graminis* on barley (host) coleoptiles could germinate and form a normal appressorium. In a simulated air-borne state or on surfaces of glass and agar, multiple short germ tubes were formed, but almost no appressorium was produced. When suspended conidia with short germ tubes were transferred to coleoptiles, they produced a long second germ tube which differentiated an appressorium. Thus, stimuli causing the elongation of second germ tubes
and formation of appressoria are perceived through the short and long germ tubes attaching to the host coleoptile epidermis and that attachment plays an important role in differentiation of these structures.

The initial contact of a fungal parasite with the host surface may be a random event. A parasite may establish numerous contacts with both the host and nonhost, but it fails to attach to the surface of the nonhost. This can be proved by the results obtained in our laboratory. For this test, Manocha (1985) described a convenient method to quantify attachment of the mycoparasite, *Piptocephalis virginiana*, to the isolated cell wall fragments of the host by artificial inoculation and a washing-off procedure. The germ tubes of the mycoparasite did not form any appressorium on the surfaces of cell wall fragments of any of the nonhost species tested, indicating their failure to attach to them. Nineteen to 20 hr after inoculation of host cell wall fragments with parasite spores, however, many appressoria (53 - 69 %) were formed by the parasite germ tubes at the points of attachment, resembling those observed on intact hyphal walls (Manocha et al. 1986). Staples and Macko (1980) considered the formation of appressoria by fungi as a fungal response induced by the successful attachment and recognition between the fungal parasites and their hosts.

3. Evidence that attachment is required for induction of parasite infection structure differentiation and host resistance response initiation

Attachment and differential recognition of the outer and inner walls of epidermal cells of the host plant leaf by a crown rust fungus, *Puccinia*
coronata, could induce differential development of infection structures of the parasite (Mendgen 1982). At first, the uredospore germ tube did not form an appressorium with an infection peg until it attached to a stoma. Then, the peg pushed the guard cells apart, penetrated into the substomatal chamber and formed the substomatal vesicle. From this vesicle, infection hyphae emerged. When infection hypha attached to the inner wall of epidermal cells, a haustorium was differentiated from which an infection peg grew into a host cell and formed a haustorium. On the cuticular surface of the epidermis, however, no haustorial mother cells were seen. Thus, attachment of the parasite to different parts of the host appears to trigger different phases of infection structure development. This phenomenon was also seen in the bean rust fungus, *Uromyces phaseoli* (Mendgen 1978). The nature of this phenomenon has not been clarified.

An interesting experiment was performed by Hadwiger's group (Nichols et al. 1980) for investigation of the host resistance-inducing role of attachment in pea-*Fusarium solani* host-parasite interactions. Direct attachment between the host and parasite was found to be essential to initiate resistance responses in the host. If various synthetic membrane barriers were placed between the macroconidia and the plant cells, the normal induction of phytoalexin synthesis and yellow-green discoloration (typical of the hypersensitivity response) were suppressed or totally prevented. Further, the incompatible fungus grew uninhibited on the upper side of such a barrier even if the disease resistance responses had been previously induced in the host tissue on the barrier's opposite side (Hadwiger and Loschke 1981). Smith and Cruickshank (1987) have observed that the sustained attachment between endocarp tissue of pea
pods and conidia of *Monilinia fructicola* is required for the maximum outcome of the pisatin response.

II. Biochemical and Physiological Aspects

Considerable progress has also been made in biochemical and physiological studies in the specificity of attachment of fungal parasites to their hosts.

1. Involvement of cell wall proteins in attachment and recognition

Grambow and Riedel (1977) reported that two different protein fractions from the leaf surface of host plants could induce the differentiation of complete infection structures of *Puccinia graminis* at a high rate *in vitro* when they were supplied simultaneously to germinating uredospores at 22°C. It was suggested that *in vivo* the active factors were part of the biochemical environment prevailing at the stomata where the fungus attached and was induced to produce the appressorium. Similar fractions from nonhost plants were found to cause no effect on the fungal pathogen.

Evidence suggests that some proteins in the extracellular matrix of the germ tubes of some rust fungi are also involved in the attachment process. Epstein et al. (1985, 1987) reported that extracellular proteins consisting of six predominant peptides were required for the attachment of the bean rust fungus *Uromyces appendiculatus* to the leaf stomates of host plants. The amount of attachment could be reduced by pronase E, regardless of whether the enzyme was applied during growth or after attachment had
occurred, although higher concentrations were required after attachment had occurred. The data suggest that the extracellular proteins may bind the germ tube to an inductive surface and that binding may be necessary for induction of infection structures.

2. Agglutinin-carbohydrate and carbohydrate-carbohydrate interactions in attachment and recognition

Based on some preliminary results, Callow (1977) suggested that lectins and some carbohydrates of cell walls of some plants and fungi may play an important role in the specific attachment and recognition in host-pathogen interactions. There is now a very considerable body of information which strongly supports this suggestion, further indicating that in some host-fungal parasite interactions, binding of the cell wall components, agglutinins and carbohydrates, indeed directly mediates the specific attachment and recognition.

(i) Plant pathogens

Kojima and his co-workers isolated (Kojima and Uritani 1978) and purified (Kojima et al. 1982) a spore agglutinin factor from sweet potato root. The factor was a glycoprotein with a molecular weight of 1,600 kd and contained mainly galacturonic acid. It agglutinated differentially the germinated spores of several strains of *Ceratocystis fimbriata* in the presence of calcium ions at pH 6.5. It is suggested that the spore-agglutinating factors in host plants function as the determinants of
specificity in some host-fungal parasite interactions. The cell wall
fragments of the bean rust fungus, *Uromyces phaseoli*, can specifically
adhere to sections of host tissue but not at all to nonhost tissue (Mendgen
1978). In this case, the attachment appears to be achieved through
binding of complementary molecules on surfaces of the two interacting
species without any requirement of parasitic vitality.

Recently, evidence for the direct involvement in attachment and
recognition of specific sugar residues present on both host and fungal
parasite surfaces has been obtained by some workers. The attachment and
encystment of zoospores of *Pythium aphanidermatum* to root surfaces of
cress (*Lepidium sativum*) can be used as a quantitative model of fungal-
plant attachment and recognition (Longman and Callow 1987). Zoospore-
root attachment could be abolished by trypsinization of zoospores or
periodate oxidation of the root surface. Agglutinin and enzyme
modification of the root surface mucilage polysaccharide reduced zoospore
attachment; treatments that blocked, or removed terminal fucosyl residues
were especially effective. Incubation of zoospores with exogenous root
mucilage polysaccharide also reduced zoospore attachment but the
effectiveness of the mucilage was reduced if pretreated with periodate or
with exofucosidase. This suggests that protein-containing receptors on the
zoospore surface interact with fucose-containing ligands present on root
surface mucilage to promote zoospore attachment and subsequent
encystment.

Evidence obtained by Hamer et al. (1987) indicated that glucose
and/or mannose residues combined with the tip mucilage of spore of the
rice blast fungus, *Magnaporthe grisea*, was involved in the attachment of
the spore to its host rice leaf surface. Hohl and Balsiger (1988) have
demonstrated the presence of galactose and fucose binding sites on the outer surface of germinated cysts of the plant pathogens *Phytophthora megasperma* and *P. infestans*. They found that protoplasts of soybean hypocotyls had a glucosyl receptor exposed at their surface, probably one of the widely distributed \( \beta \)-lectins. In addition, a likely lectin-ligand type bond responsible, at least in part, for the attachment of the pathogen to the plasma membrane of the host was shown to occur between the terminal glucosyl residues present on the former and glycosyl receptors on the latter.

*Fusarium oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* f. sp. *lycopersici* are two morphologically identical plant pathogens with hosts restricted in the genus *Lycopersicon*, and they cause different and distinct symptoms. Most recently, Boyer and Charest (1989) observed, with three different lectins, quantitative differences in sugar residues on the surfaces of microconidia of the two fungal parasites. It is suggested that the surface sugars of the two fungi could be responsible for cell to cell attachment and recognition as well as specific interaction between the fungal isolates and their tomato plant hosts. Thus, the quantitative differences in surface sugar residues of the fungi may be the basis for their causing different and distinct symptoms in their hosts.

(ii) Mycoparasites — necrotrophs and biotrophs

The phenomenon of specificity of attachment in interfungal interaction has only recently received attention (Elad and Misaghi 1985; Baker 1987; Chet 1987). Agglutinin and some sugars are involved in attachment and recognition in mycoparasitism.
Interactions of the necrotrophic mycoparasite, *Trichoderma*, with its hosts was found to be specific and isolates of *Trichoderma* species differ from each other in their host specificity (Barak et al. 1985; Chet 1987). Most of the isolates examined can attack *Rhizoctonia solani*, but only a few are able to effectively parasitize *Sclerotium rolfsii*, *Pythium aphanidermatum*, or *Fusarium oxysporum*. Studies with the necrotrophic mycoparasite system indicate that attachment and recognition processes in the fungal host-mycoparasite interactions are mediated by agglutinin-carbohydrate binding on their cell surfaces. Thus, the host specificity of the mycoparasite isolates may be due to differences in their cell surface components.

In the study on *Trichoderma harzianum* parasitic on *R. solani*, a plant pathogen, Elad et al. (1982) demonstrated that the attachment of *Trichoderma* conidia with *R. solani* could be promoted by pretreatment of the conidia with trypsin. It is suggested that treatment with trypsin probably exposed receptors on the conidia which were responsible for attachment as was shown for *Fusarium* conidia (Kleinschuster and Baker 1974). It was also shown that the *Rhizoctonia* agglutinin activity could be inhibited by L-fucose and L-galactose. Furthermore, it was found that galactose and fucose residues were present on the mycoparasite surface and may serve as receptors to *Rhizoctonia* agglutinin (Elad et al. 1983; Barak et al. 1986). The fact that attachment was inhibited by preincubation of the fungus with specific carbohydrates allowed these researchers to conclude that an agglutinin on *R. solani* was involved in attachment and recognition, and these processes led to mycoparasitism. Since this agglutinin was found not to distinguish among biological variants
of the pathogen, *Trichoderma* species were able to attack different *R. solani* isolates.

Attachment and recognition between *Trichoderma* and another plant pathogen, *Sclerotium rolfsii*, may also depend on cell surface agglutinin-carbohydrate interaction. An agglutinin was isolated and partially purified from the extracts and culture filtrate of *S. rolfsii* (Barak et al. 1985). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) showed two protein bands of $M_r$ 60 kd and 50 kd after staining with Comassie brilliant blue. The agglutinin activity of extracts of the fungus was associated with the extracellular polysaccharides of *S. rolfsii*, and the activity was specifically inhibited by D-glucose, D-mannose and several of their derivatives. These researchers also showed that 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*, thus suggesting the role of *S. rolfsii* agglutinin in specific attachment and recognition in fungus-fungus interactions (Barak et al. 1985; Hertz and Chet 1986).

*Piptocephalis virginiana* with its host members restricted to the order *Mucorales* is an obligately biotrophic mycoparasite investigated in our laboratory. SDS-PAGE of alkali extracts from isolated cell wall fragments of the host, *Choanephora cucurbitarum*, *Mortierella isabellina* or *M. pussila*, and the nonhost, *Linderina pennispora*, *M. hygrophila*, or *M. candelabrum*, showed quantitative and qualitative differences after staining with Comassie brilliant blue R250 and periodic acid-Schiff's reagent. These differences were well marked in band patterns of glycoproteins. Two high molecular weight bands observed in the cell wall extracts of the hosts were absent in those of the nonhosts (Manocha 1984; Manocha 1985; Manocha et al. 1986). Attachment tests showed that by treatment of host cell wall
fragments with chitobiose or chitotriose and with acid or alkali which removed the surface proteins resulted in significant decreases of attachment and appressorium formation by the germ tubes, suggesting the possible involvement of glycoproteins in mycoparasitism (Manocha 1985). Further, agglutination tests demonstrated that the crude protein extract from the host cell walls were able to agglutinate the cell wall fragments of the mycoparasite (Manocha 1986). These results preliminarily verified the previous hypothesis proposed by Manocha (1987, 1988) stating that these two glycoproteins may play an important role in attachment and recognition in the fungal host-mycoparasite interaction. It was suggested that the two glycoproteins may function as "receptors" on the host surfaces which were attached and recognized by germ tubes of the mycoparasite, and this led to the further parasitic events (Manocha 1987). It is suggested that on the germ tube surface, there is a specific structure which is complementary to the host surface "receptor" and the interaction type is probably "agglutinin-carbohydrate". Recently, results obtained by a fluorescence-lectin labelling test with the mycoparasite germ tubes have shed some light on this supposition.

A previous fluorescent lectin binding assay showed obvious differences in the sugar residues exposed at the host and nonhost cell wall surfaces. N-acetyl-D-glucosamine oligomers were shown to be present at both the host and nonhost surfaces; nevertheless, the nonhost species also had D-galactose and N-acetyl-D-galactosamine residues on their surfaces as specific lectin binding sites (Manocha 1985; Manocha et al. 1986). It was suggested that the two sugars at the nonhost cell surfaces may play a role in interfering with the attachment by the mycoparasite (Manocha 1987, 1988).
For ericoid mycorrhizae, some fungi related to *Hymenoscyphus ericae* species or *Clavaria* infect a limited number of plant species within the *Ericales* and are characterized by a strong host specificity (Bonfante-Fasolo and Gianinazzi-Pearson 1982). A role of cell surface structures in the specificity of interaction in the plant-fungal symbiont systems has been recently suggested (Gianinazzi-Pearson et al. 1986).

Some fungal symbionts possess extracellular material which is particularly abundant in the presence of the host plant. By using different fungal strains and computer-aided image analysis, a strong correlation between the ability of the fungus to infect a host plant and production of extracellular material was observed (Gianinazzi-Pearson et al. 1986). Correlations between the different surface organization of the fungal strains and their infectivity were also demonstrated (Bonfante-Fasolo 1988).

Cytochemical examination showed the presence of Thiery-reactive polysaccharides and soluble sugars in the extracellular material (Bonfante-Fasolo et al. 1987). In addition, the fungal strains with different infective capabilities showed a dissimilar distribution of their surface sugar residues both in pure culture and in the presence of the host plant (Bonfante-Fasolo and Perotto, 1986; Bonfante-Fasolo et al. 1987). Lectin labelling by Con A, specific for mannose and glucose residues, exhibited that the binding was scanty on the noninfective strains, abundant on the infective strains and occurred precisely to the extracellular material which was sensitive to mannosidase digestion. In the presence of the host plant, binding of Con A seemed to be significantly increased.
These results strongly suggest that the extracellular material rich in mannose residues is a key component in the specific host plant-fungal parasite attachment and in mycorrhizal establishment which only occurs in its presence (Bonfante-Fasolo 1988).

In the host plants, Calluna vulgaris and Vaccinium myrtillus, it was found that the root hair epidermal cell surfaces were covered by a mucilage layer. Tests by labelled lectins as probes for sugar residue localization showed that binding of wheat germ agglutinin (WGA), specific for N-acetylglucosamine (GlcNAc), was specifically localized on the root hair (Bonfante-Fasolo 1988).

Current research on the specificity of attachment and recognition between plants and ericoid fungi suggest that a cell surface carbohydrate-carbohydrate interaction is responsible for the specificity. It is possible that the specific attachment and recognition are brought about by the binding of GlcNAc residues at the plant root hair to the fungal extracellular material rich in mannose residues.

(iv) Fungal parasites of insects and nematodes

Evidence for the involvement of agglutinin present at the surface of fungal parasite of insects has been obtained by Ishikawa et al. (1979, 1981). They have isolated by treatment with β-glucanase, a lectin-binding hemagglutinin from both the cell wall and culture filtrate of Conidiobolus lamprauges. The purified agglutinin from both sources consisted of two glycoproteins, and the molecular weight of the major band of this agglutinin was approximately 86 kd as detected by SDS-PAGE. The cell wall agglutinin showed sugar specificity and immunological properties
similar to that purified from culture filtrates (Ishikawa et al. 1981, 1983). The activity of both cell wall and culture filtrate agglutinins was inhibited by N-acetylglucosamine oligomers and some other sugars (Ishikawa et al. 1979, 1981). These researchers suggest that the chitin-binding hemagglutinin plays an important role in the infection of insects by *C. lamprauges*, especially in the initial attachment of the fungus to the insect cuticle.

Nematodes have been observed to get attached and captured by a number of nematode-trapping fungi employing an adhesive present on specific capture organs (Barron 1977). However, the mechanism of attachment was not fully known until 1979. By using *Arthrobotrys oligospora*, one of the most common nematophagous fungi, Nordbring-Hertz and Mattiasson (1979) obtained indirect evidence for the presence of a lectin on traps of the fungi which bound to N-acetyl-galactosamine (GalNAc) on the surface of the nematode, *Panagrellus redivivus*. It was demonstrated that flooding of the fungal colony with 20 mM GalNAc solution resulted in total inhibition of capture. However, treatment of the nematodes with carbohydrates, including GalNAc, at 20 and 200 mM failed to inhibit capture. Thus, these results suggest that GalNAc residues located on the nematode surfaces are responsible for the attachment and subsequent capture. The fact that nematode-trapping ability could be suppressed by preincubating the fungus with GalNAc led to the suggestion that a lectin on the fungal surface is involved in the interaction. This was further proved by the work done by Borrebaeck et al. (1984) and Premachandran and Pramer (1984). They isolated and partially characterized the lectin from the fungal surfaces, and the molecular weight of the lectin was shown to be approximately 20 to 22 kd.
Evidence presented by Nordbring-Hertz and co-workers also indicated that the lectin of *Dactylaria candida* was specific for 2-deoxyglucose (Nordbring-Hertz et al. 1982). Rosenzweig and Ackroyd (1983), in a study of the attachment specificity of three fungi, found that the surface lectins of *Monacrosporium eudermatum*, *Monacrosporium rutgeriensis*, and *Arthrobotrys conoides* were specific for L-fucose, 2-deoxyglucose, and glucose-mannose, respectively. These results may possibly verify the hypothesis proposed by Nordbring-Hertz and Mattiasson (1979) that the attachment of nematode-trapping fungi to nematodes, despite the struggle of the nematodes, is due to a series of events beginning with an interaction between complementary molecules on the fungal and nematode surfaces.

The activity of lectins from *Arthrobotrys oligospora* could be inhibited by more than one sugar (Nordbring-Hertz and Mattiasson 1979). In addition, a study of cuticle surface carbohydrates of five different nematodes revealed the presence on all the nematodes of glucose-mannose and GalNAc residues (Rosenzweig et al. 1985). These results may explain the phenomenon that some nematode-trapping fungi were able to attach to and capture more than one species of nematodes, although not all nematodes are captured with the same degree of efficiency (Rosenzweig et al. 1985).

3. Quantitative and qualitative differences of agglutinins from different sources in relation to their role in host-fungal parasite interactions

Results obtained by Gibson et al. (1982) indicated that the amount of soybean agglutinin (SBA) detected by radioimmunoassay in seeds of
resistant cultivars to *Phytophthora megasperma* was approximately twice that of susceptible cultivars. At the same concentration, purified SBA from the resistant cultivar was more inhibitory to mycelial growth than that from the susceptible cultivar.

Apart from the quantitative and qualitative differences between agglutinins from susceptible and resistant hosts, the differences are also present among the agglutinins from different parts of a single plant host. *Phytophthora infestans* grew better on slices from the stem end rather than the rose end of potato tubes. Differences in electrophoretic properties as well as carbohydrate content were observed between the two preparations. 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 (Andreu and Daleo 1988). The results show a correlation between the agglutinating activity of the agglutinins and the ability of the parasite to attack its host. Thus, these results may in part explain the differential interactions, including the attachment, of the fungus with different zones of the tubers.

III. Genetic and Molecular Aspects: Future Perspectives

Genetic analysis of interactions between pathogen races and host varieties has shown that such interactions are controlled by gene-for-gene systems involving R genes for resistance in the host and complementary A genes for avirulence in the pathogen. A number of models have been proposed to explain recognition in plant host-fungal pathogen systems based on molecular complementary in primary gene products; i.e., the products of R gene recognize the products of A genes to initiate active
resistance (Ellingboe; Keen, 1982; Day, 1984). Most current models (Bailey, 1987; Callow, 1987) invoke two classes of resistance gene; i.e., recognition genes and response genes. Recognition genes coding for receptors located at the cell surface are involved in the signalling mechanisms leading to cascade of expression of the response gene which in turn leads to local defence reactions including hypersensitivity, phytoalexin accumulation, lignification and other wall modifications, accumulation of hydro-rich glycoproteins, release of hydrolytic enzymes, and the possibility of systemic responses, notably the induced systems of protease inhibitors (Callow et al. 1988). Validity of such models can only be tested by the isolation and cloning of genes encoding specificity determinants in the pathogen and recognition and response genes in the host. So far there is little or no information on plant host-fungal pathogen systems.

Current knowledge on the components of the complementary recognition system and on the molecular component of the surface receptors is dismal. Virtually nothing is known about the genetic details in specificity of attachment of fungal parasites to their hosts. However, the phenomenon of attachment specificity is suggested to be consistent with gene-for-gene hypothesis. We can imagine that in a fungal parasite there is a gene which encodes a surface protein or glycoprotein involved in attachment and recognized by the complementary gene product, glycoprotein or agglutinin, at the surface of the host. Until such receptors are isolated and fully characterized, the existence of receptors at the cell surface must remain hypothetical.

It is evident from the above discussion that future approaches should be directed to the molecular analysis of the components of cell surface receptors and the products of recognition gene. Current technologies can
advance our understanding of specificity of attachment and recognition in fungal parasitism. Monoclonal antibodies raised against cell surface components provide subtle and precise tools for probing and identifying antigens concerned with receptor function. Such an approach has been adopted for *phytophthora cinnamomi* by Hardham et al. (1986) to analyze zoospore recognition at the plant root surface. Recombinant DNA technologies offer an alternative approach to isolate and study genes and their products determining whether a factor for cell adhesion is being produced or altered during interaction.

IV. **Summary**

1. Attachment of fungal parasites to their hosts is one of the first events required for parasitism. Attachment usually takes place together with recognition which decides the outcome of the interaction between two interacting species.

2. Although the mechanism for the specificity of attachment of fungal parasites to their hosts has not been fully clarified, studies of the past twenty years in morphology, biochemistry and physiology have contributed to our knowledge on this subject. In some host-fungal parasite systems, the specificity is established by parasite recognition of specific surface topography and cell wall ultrastructure of the hosts. In most host-fungal parasite systems, the specificity is determined through binding of complementary macromolecules on the surfaces of both the host and the parasite. Those macromolecules are found to be proteins or glycoproteins (some are agglutinins) and carbohydrates. Pretreatments of the host and
parasite with some physical, chemical or biochemical agents can significantly or totally inhibit attachment. However, till now, the mechanism of interaction specificity in the mycoparasite system investigated in our laboratory is not clear. Undoubtedly, further work is needed to elucidate the nature and role of the two high molecular weight glycoproteins that are only present at the host surface.

3. The formation of appressoria by fungal parasites on surfaces of their hosts can be considered as a response of successful attachment and recognition. Attachment to different parts of a host by its parasite can induce differential development of infection structures of the pathogen, and direct attachment is essential for the initiation of host resistance responses. All of these interaction characteristics can be attributed to the specificity of attachment.

4. Till now, information on genetics of the specificity of attachment has been poor. However, genetic and molecular mechanisms of the specificity are suggested to be based on the gene-for-gene hypothesis. Obviously, further work along these lines, aided by monoclonal antibodies and recombinant DNA technology, is needed for complete explanations of the specificity of attachment of fungal parasites to their hosts.
Materials and Methods

Parasite inoculum and preparation of its ungerminated and germinated spores

Cultures of the biotrophic, haustorial mycoparasite, *Piptocephalis virginiana*, Leadbeater and Mercer, were maintained on its susceptible host, *Choanephora cucurbitarum* (Berk. & Rav.) Thaxter, in complete darkness, which is known to inhibit sporulation of the host while the parasite sporulates normally (Berry and Barnett, 1957). An axenic population of spores of the parasite was obtained by growing cultures in 9 cm Petri dishes at 23 ± 1°C for two weeks. The spores were harvested by adding 10 mL of sterile distilled water over the culture and gently shaking the culture dish for a few minutes. The resulting spore suspension was filtered through muslin cloth and immediately centrifuged at 1500 g for 10 min at 4°C. The spore pellet was then washed three times with sterile distilled water by centrifugation, and resuspended in 0.05 M phosphate buffer saline (PBS: 0.58% Na₂HPO₄, 0.13% NaH₂PO₄ and 0.85% NaCl), pH 7.4, for agglutination tests.

To obtain adequately germinated spores for use in the agglutination and attachment tests, the concentration of the spores was adjusted to 10⁵ mL⁻¹ with malt-yeast extract medium and the spores were allowed to germinate for 20 h at 23 ± 1°C. The germinated spores (80-90% germination) were washed with sterile distilled water by centrifugation, and then resuspended in PBS.
Host and nonhost inocula

Cultures of Mortierella pusilla Oudeman and Mortierella candelabrum V, Teigè and Le Monn, host and nonhost to the mycoparasite P. virginiana, respectively, were selected and routinely grown at 23 ± 1°C on malt-yeast extract agar medium (Manocha et al 1986). For preparation of cell wall fragments, cultures were grown in a liquid medium of the same composition as above but minus agar, for 36 h and 5 d, respectively, in an Environmental incubator shaker (120 rpm) at 23 ± 1°C (Manocha 1984).

Isolation of cell wall and protein extraction

For preparation of host or nonhost cell walls, mycelium was collected on filter paper in a Buchner funnel, washed with distilled water until free of the medium. Washed mycelium was homogenized in a cold PBS containing 1 mM EDTA and 1 µM phenylmethylsulfonyl fluoride (5 mL. g⁻¹ wet weight of mycelium) for 2 min at 3400 rpm in a Sorvall Omni-Mixer with the cup immersed in ice. The resulting slurry poured into 70 mL homogenizing flasks containing c/a. one-third volume of 0.45-mm glass beads was further disrupted using a mechanical cell homogenizer, Braun model MSK, for 40s at 4°C. The homogenate was centrifuged at 1500 g for 3 min and the pellet was resuspended in PBS. An additional cell disruption for 2-3 min with a sonicator (model W375, Heat Systems - Ultrasonics, Inc.) released the cytoplasm and membranes completely from the cell walls. The isolated cell walls were cleaned by repeated suspension in cold PBS and centrifugation till the supernatant appeared clear. Cleaned preparations yielding 2 - 2.5% of the whole mycelial mass, were lyophilized and stored at -20°C until further use. Purity of isolated cell
walls free from cytoplasmic and membrane contamination was determined as described earlier (Manocha, 1984).

Cell wall proteins were extracted by suspending isolated cell walls (0.25 g) in 10 mL of ice-cold 0.1N NaOH and blended in a chilled Sorvall Omni-Mixer cup at full speed for 20 s. The suspension was stirred for 18-20 h in an ice bath. The extract was centrifuged at 8000 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 Lowry et al. (1951) using bovine serum albumin as standard.

Purification of glycoproteins from crude protein extract

The crude extract obtained from the isolated cell walls of *M. pusilla* was used for the purification of two glycoproteins. The crude protein was dissolved (5 mg mL⁻¹) in 0.1M PBS, pH 7.4, and the solution was adjusted to 30% 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 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⁻¹ was applied to a sephadex G-100 column (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 two glycoproteins emerged between 175 - 240 mL,
i.e. 110-175 mL 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 contained 10% (W/V) acrylamide, 0.27% (W/V) N,N'-methylenebisacrylamide, 0.038% (V/V), N,N,N',N'-tetramethylethylenediamine, 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 litre. Freeze dried sample obtained following Sephadex G100 chromatography was dissolved at a concentration of 8 mg mL⁻¹ 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. Electrophoresis was performed at 2.5 mA per tube until the marker dye, bromophenol blue, reached the end of the gel. Following electrophoresis, one gel tube was stained with periodic acid - Schiff reagent to reveal glycoprotein bands. A gel slice, corresponding to the glycoprotein region, was cut from the remaining 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 5000 g for 10 min, dialyzed against distilled water at 4°C for overnight and then lyophilized.

**SDS-polyacrylamide gel electrophoresis**

Cell wall 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. Sample of 300 μg of crude proteins or 25 μg of purified...
glycoproteins or 10 μg of individual glycoprotein (b or c) 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 until the samples penetrated the separation gel and then at 3.5 mA per gel. The running time was about 4 h. The gels were removed from the glass tubes 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, U.S.A.) or in 40% ethanol in aqueous 5% acetic acid for carbohydrate staining with periodic acid - Schiff's reagent (Segrest and Jackson 1972).

Measurement of agglutinating activity of purified fractions of host cell wall protein extract

For detection of agglutinating activity of purified fractions of crude proteins extracted from cell walls of the host, spores of the mycoparasite P. virginiana were used and a series of twofold dilutions of the test sample (50 μL) were prepared with 0.05 M PBS, pH 7.4.

The spores were harvested from cultures after 2 week's growth by washing off the agar surface with 10 mL of sterile distilled water. The resulting spore suspension was centrifuged at 1500 g for 10 min at 4 0C. The pellet was washed three times with cold PBS and resuspended in PBS to a concentration of 2 x 10^6 ml^{-1}. The spore suspensions were used for agglutination tests immediately after preparation. The test was performed on depression slides, and 50 μL of spore suspension was incubated with the same volume of the solutions of various purified protein fractions in a series of twofold dilutions in a humid chamber. The slides were shaken at
room temperature on a gyrotary shaker at 100 rpm for 30 min. Spore agglutination was examined under a light microscope.

The nonhost cell wall crude extract was also tested for their possible agglutinating activity. Control tests were performed with thyroglobulin and \( \gamma \)-globulin.

Agglutinating activity towards the mycoparasite spores was given as titre, defined as the reciprocal of the greatest dilution at which agglutination could still be detected in the test. In addition, one unit of agglutination (a.u.) was defined as the quantity of protein in a 50 \( \mu \)L sample which gave a titre of one in the test.

**Carbohydrate analyses of cell wall proteins and purified glycoproteins**

Sugars were identified as their alditol derivatives on the basis of their retention time comparative to the retention time of authentic standards (Sigma Chemical Co., St. Louis, MO, U.S.A.). Alditol acetate derivatives were prepared according to the method of Stadler (1976).

The cell wall proteins (10 mg of crude protein or 0.5 mg of purified glycoproteins) were hydrolysed overnight (14 h) in a sealed reaction tube in a final concentration of 1 mg mL\(^{-1}\) with 0.6 N HCl at 100 \(^\circ\)C (Dutton, 1973; Schranger and Oats, 1968). The suspensions were centrifuged at 15,000 g for 10 min and the supernatants were dried in a rotary evaporator. The residues were redissolved in 1 mL of a freshly prepared 0.2 M solution of sodium borohydride and stirred at room temperature for 2 h in a sealed reaction tube. The excess of sodium borohydride was then eliminated by addition of acetic acid and subsequently removed in the rotary evaporator as volatile trimethyl borate after the addition of methanol. The dried residue was acetylated in a mixture of dry pyridine
and acetic anhydride (0.3 mL each) at 105 °C for 20 min in a sealed tube. After cooling, but prior to the addition of distilled water, salts were pelleted by centrifugation (3500 g for 5 min) and discarded. The supernatant was freed from pyridine and acetic acid by repeated addition and evaporation of water (2 mL at each step). The dried residue was then extracted with chloroform (1 mL) and the extract was washed with water (2 mL). The chloroform phase was dried by a stream of nitrogen, redissolved in 20 µL of chloroform, and 1 µL of this solution was injected in the gas chromatograph.

A gas chromatograph (Hewlett Packard 5700 A) equipped with a flame ionization detector was used. The glass column (180 cm x 2 mm ID) was packed with 3% SP-2340 on 100/120 mesh Supelcoport and programmed at 180 °C - 240 °C (4 °C min⁻¹; 4 min initial hold) with a flow rate of 40 mL/min⁻¹ prepurified nitrogen carrier gas. The injector temperature was 200 °C and the detector temperature was 250 °C. The peak areas were ascertained using a Fisher Recordall 5000 series recorder fitted with an integrator.

Agglutination test with various treatments of cell wall proteins and purified glycoproteins

To examine the agglutinating activity of the cell surface proteins under different conditions, the host and nonhost cell wall proteins and the two purified glycoproteins from the host were treated with (i) heat at 95 °C for 10 min; (ii) 3% glutaraldehyde at 4 °C for 30 min; and with (iii) Pronase E (2.9 unit mL⁻¹, Sigma Chemical Co., St. Louis, Mo, USA) in 1 mL PBS at 37 °C for 8 h. In the latter treatment, the reaction mixture was then heated at 95 °C for 5 min to inactivate residual pronase. The reaction
solutions obtained after treatment with glutaraldehyde or pronase were
dialyzed overnight at 4°C against excess PBS with several changes.

The experimental procedures for agglutination test with ungerminated
spores (2 x 10^6 mL^{-1}) or germinated spores (4 x 10^5 mL^{-1}) were performed
according to those described above. For rationally and comprehensively
evaluating the results, spore agglutination was also judged as agglutination
degree and agglutination extent besides agglutination titre. Agglutination
degree was scaled as: -, no agglutination mass was found, or only 2-3
ungerminated or germinated spores contacted together; +, agglutination
mass consisted of 4-10 spores; ++, agglutination mass consisted of 11-20
spores; ++++, agglutination mass consisted of more than 20 spores. For each
recorded agglutination degree, the percentage of the corresponding
agglutination mass was at least 15% of the total agglutinating masses.
Agglutination extent was arbitrarily scaled from 0 (no agglutination) to 4
(100% agglutination). Each test was run in triplicate (this was the same
with other tests described below).

Attachment test with various treatments of cell walls

To test the attachment and appressorium formation by the
mycoparasite at the host and nonhost surfaces, isolated cell wall fragments
treated with physical, chemical and biochemical agents were used.

Physical treatment of the cell wall fragments was performed with
either heat at 95 °C for 15 min or lyophilization. For chemical treatment,
the cell wall fragments were treated with 0.1 N NaOH at 4 °C for 14 h or 3%
glutaraldehyde at 4°C for 1 h. In addition, biochemical treatment of the
isolated cell wall fragments (8 mg mL^{-1}) was carried out with Pronase E
(0.58 unit mL^{-1}) in PBS at 37 °C for 3 h. After chemical or biochemical
treatment, cell wall fragments were washed by centrifugation at 5000 g with excess sterile, cold distilled water for 5-6 times, and then resuspended in PBS.

Attachment of mycoparasite spores to the isolated cell wall fragment was studied by using a method described earlier (Manocha 1985). Isolated host and nonhost cell walls with or without treatment were suspended in PBS (1 mg mL⁻¹) and 0.1 mL of this suspension was spread over an area of 15 x 20 mm on a glass slide. The slides were air dried to fix the cell wall fragments. Suspension of germinated spores (0.25 mL) of the mycoparasite was spread over the fixed cell wall fragments. The slides were incubated for 2 h at room temperature, washed twice under a stream of slow running distilled water, and were stained with lactophenol-cotton blue. The germinated spores that attached to cell wall fragments and formed appressoria were counted from at least 20 randomly selected fields on each slide using the high power x40 objective of a light microscope.

**Combined agglutination-attachment test**

To study involvement of the two glycoproteins in attachment to host cell walls by the mycoparasite, the germinated spores of the mycoparasite were first incubated with the two purified glycoproteins (1 mg mL⁻¹) or the single glycoprotein b or c (0.5 mg mL⁻¹) as described in "Agglutination test" (the dilution titre of the glycoprotein samples was 1:256), and then incubated with the host cell wall fragments as described in "Attachment test". The control tests contained either no glycoproteins or thyroglobulin or γ-globulin at concentration of 1 mg mL⁻¹.
Treatment of mycoparasite spores with various sugars

To investigate involvement of carbohydrates in the agglutination and attachment, 0.5 mL of the ungerminated or germinated spore suspension were incubated at room temperature for 10 min with the same volume of various sugar solutions in PBS at the concentration of 200 mM. As control, PBS was used instead of sugar solution. Ungerminated and germinated spores were then washed twice with PBS by centrifugation, and adjusted to the original concentrations. The resulting spore suspensions were used for further tests as described under "Agglutination test" and "Attachment test".
Results

Isolation of protein from cell walls

The protein contents of isolated cell wall fragments as determined by the procedure of Lowry et al. (1951) were not significantly different in the two fungi, Mortierella pusilla and Mortierella candelabrum (Fig. 1), host and nonhost, respectively, to the mycoparasite, Piptocephalis virginiana. There was a slight increase in protein yield with culture age in M. pusilla (18.7 ± 1.6% in 5 d compared with 16.9 ± 0.8% in 36 h) whereas in M. candelabrum the protein yield as a function of age remained more or less the same (14.1 ± 0.9% in 5 d compared with 14.5 ± 1.4% in 36 h).

SDS-polyacrylamide gel electrophoresis of crude extracts from cell walls of host and nonhost

Protein profiles, from a NaOH extract of isolated cell wall fragments obtained by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue R250, showed quantitative and qualitative differences between M. pusilla and M. candelabrum (Fig. 2). Three protein bands marked as a, b, and d were observed on gels with the host cell wall extract, but were absent on gels prepared from the nonhost cell wall extract. Band c could be seen on gels with both the host and nonhost extracts; however, band c was thin and faint in the latter. The molecular weights of the four proteins, a, b, c and d were 117, 100.5, 85 and 64 kd, respectively.
Fig. 1. Protein yield from the cell walls of M. pusilla and M. candelabrum grown at 23 C on malt-yeast extract medium.
Abbreviations: Mp, Mortierella pusilla; Mc, Mortierella candelabrum; S, Standard; a, protein a; b, protein b; c, protein c; d, protein d.

Fig. 2  SDS-polyacrylamide gel electrophoresis of crude protein extracts of cell walls of Mortierella species. Gels were stained with Coomassie brilliant blue. The protein standards were purchased from Bio-Rad Laboratories (1414 Harbour Way South, Richmond, CA 94804, USA). The protein standards are as follows: rabbit muscle phosphorylase b, Mr 97 Kd; bovine serum albumin, Mr 66 Kd; hen egg white ovalbumin, Mr 43 Kd; bovine carbonic anhydrase, Mr 31 Kd; soybean trypsin inhibitor, Mr 22 Kd, and hen egg white lysozyme, Mr 14 Kd.
Agglutinating activity of host and nonhost cell wall extracts

Measurement of agglutinating activity with the mycoparasite spores showed that the host cell wall extract had a high agglutination titre (1:128) at the original concentration of 6.5 mg mL⁻¹. The nonhost cell wall extract at the original concentration of 7.5 mg mL⁻¹ also exhibited agglutinating activity; however, the titre level was very low (1:4, Table 1). The agglutination unit in the host group was about 37 times as high as that in the nonhost group (Table 1). In the control tests with thyroglobulin and γ-globulin at the concentration of 10 mg mL⁻¹, there was no spore agglutination observed.

Based on the results obtained by SDS-PAGE and the measurement of agglutinating activity, further investigation was focused on the four proteins which, except for the protein c, were present only at the cell wall surface of the host species. For detecting which protein was involved in the agglutination, the host cell wall protein extracts minus protein a, b, c, or d were obtained by preparative PAGE followed by gel cutting and eluting described under "Materials and Methods".

Measurement of agglutinating activity of the modified crude extracts showed that the deletion of protein a or d did not affect its agglutinating activity; however, crude extract minus protein b or c showed a significantly low agglutinating activity (37.7 and 23.9 a.u. mg⁻¹ protein) in comparison with that of the complete extract. The activity of the crude extract minus protein b or c was similar to that of the nonhost cell wall protein extract (Table 1).
Table 1. Agglutinating Activity of Crude Extracts from Cell Walls of *M. candelabrum* and *M. pusilla* Grown at 23 °C for 36 h

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein concentration (mg mL⁻¹)</th>
<th>Agglutination titre a</th>
<th>Agglutination unit (a.u. mg⁻¹ protein) b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>10.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>γ-Globulin</td>
<td>10.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>McCE c</td>
<td>7.5</td>
<td>4</td>
<td>21.3</td>
</tr>
<tr>
<td>MpCE d</td>
<td>6.5</td>
<td>128</td>
<td>787.7</td>
</tr>
<tr>
<td>MpCE minus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein a</td>
<td>6.2</td>
<td>128</td>
<td>825.8</td>
</tr>
<tr>
<td>MpCE minus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein b</td>
<td>8.5</td>
<td>8</td>
<td>37.7</td>
</tr>
<tr>
<td>MpCE minus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein c</td>
<td>6.7</td>
<td>4</td>
<td>23.9</td>
</tr>
<tr>
<td>MpCE minus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein d</td>
<td>6.8</td>
<td>128</td>
<td>752.9</td>
</tr>
</tbody>
</table>

a A series of dilutions was made in twofold. Agglutination titre was the reciprocal of the greatest dilution of extract in PBS at which agglutination could still be detected.

b a.u., agglutination unit, defined as the quantity of protein in a 50 μL aliquot that gave a titre of one in the assay.

c McCE: Crude Extract of *M. candelabrum*

d MpCE: Crude Extract of *M. pusilla*
Purification of two glycoproteins b and c from host cell wall extract

Table 2 provides a summary of purification of the two glycoproteins, b and c, from the crude protein extract obtained from isolated cell wall fragments of *M. pusilla*. The yield of the two glycoproteins after 36 h incubation period was 0.49%. The corresponding yield of the two glycoproteins from a 5 d old culture was 0.43%. Even though the amount of total protein in the cell wall of *M. pusilla* increased with age (Fig. 1), the content of the two glycoproteins decreased (the reduction percentage was 12.25%). In addition, the agglutinin activity of the two glycoproteins in the aged host was also decreased (the reduction was about 20%).

Purification of the two glycoproteins was confirmed by SDS-PAGE which revealed two distinct bands on gels after staining with Coomassie brilliant blue R250 (Fig. 3). By staining with periodic acid-Schiff's reagent, both proteins b and c were shown to be glycoproteins (Fig. 4).

Agglutination tests showed that the agglutinating activity of purified fractions was increased with purification procedures (Table 2). The agglutination unit of the pure preparation was about 35 times as high as that of the crude extract.

For detection of the agglutinating activity of the individual band, the single glycoproteins b and c were obtained by preparative PAGE and confirmed by SDS-PAGE (Fig. 5). Results in Table 3 show that the agglutination unit of the single glycoproteins b and c are very low and are about 510-850 times less than that of the pure preparation containing both glycoproteins.
Table 2. Purification of Two Proteins b and c from Cell Walls of *M. pusilla* Grown at 23°C for 36 h

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Protein yield (%)</th>
<th>Pv spore agglutination (a.u. μg^{-1} protein)</th>
<th>Agglutinin yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>36 h</td>
<td>5d</td>
<td>36 h</td>
<td>5d</td>
</tr>
<tr>
<td>Crude extract</td>
<td>I</td>
<td>1254.0</td>
<td>1588.5</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fractionation</td>
<td>II</td>
<td>368.4</td>
<td>475.8</td>
<td>29.4</td>
<td>30.0</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>III</td>
<td>164.3</td>
<td>204.4</td>
<td>13.1</td>
<td>12.9</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>IV</td>
<td>57.8</td>
<td>77.2</td>
<td>4.6</td>
<td>4.9</td>
</tr>
<tr>
<td>Preparative PAGE</td>
<td>V</td>
<td>6.1</td>
<td>6.8</td>
<td>0.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

** Product of total protein and agglutination unit in percentage terms relative to fraction I.
Table 3. Agglutinating Activity of Glycoproteins b and c Purified from Crude Cell Wall Protein Extract of *M. pusilla* Grown at 23°C for 36 h

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein Concentration (mg mL⁻¹)</th>
<th>Agglutination titre *</th>
<th>Agglutination unit (a.u. µg⁻¹ protein) **</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycoproteins b and c</td>
<td>0.8</td>
<td>512</td>
<td>25.20</td>
</tr>
<tr>
<td>Glycoprotein b</td>
<td>0.8</td>
<td>4</td>
<td>0.05</td>
</tr>
<tr>
<td>Glycoprotein c</td>
<td>0.8</td>
<td>2</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* A series of dilutions was made in twofold. Agglutination titre was the reciprocal of the greatest dilution of glycoproteins in PBS at which agglutination could still be detected.

** a.u., agglutination unit, defined as the quantity of protein in a 50 µL aliquot that gave a titre of one in the assay.
Fig. 3  Purified proteins b and c (left) isolated from the crude cell wall protein (right) of the host, *M. pusilla*. Gels were stained with Coomassie brilliant blue R250.
Fig. 4  Bands b and c stained with Coomassie brilliant blue R250 (right) for protein and with periodic acid-Schiff's reagent (left) for glycoprotein.
Fig. 5  Gels showing both the proteins b and c and the single proteins b or c. Gels were stained with Coomassie brilliant blue.
Carbohydrate analysis of cell wall proteins and two glycoproteins

Table 4 shows that the cell wall proteins of *M. pusilla* are more glycosylated than those of *M. candelabrum*. Percentages of three monosaccharides, i.e. arabinose, N-acetylglucosamine and glucose, in crude protein extracts are much higher in *M. pusilla* than in *M. candelabrum*. These are also reflected in Table 5. Calculation of the percentage of monosaccharides in the total carbohydrate shows that the above sugars are three major carbohydrate components contained in the host cell wall protein. However, the results show that the nonhost cell wall protein contains more N-acetylglalactosamine and galactose than the host.

The purified glycoprotein preparation contains no fucose. N-acetylglucosamine and glucose are the two major carbohydrate components of the two glycoproteins, b and c (Table 4 and 5). Although the percentage of total carbohydrate in the cell wall protein of the host increases with age, the corresponding percentage in the two glycoproteins decreases in 5 d culture, compared with that in the 36 h culture. Among those monosaccharides detected, more notable are the glucose levels in the glycoproteins obtained from 36 h and 5 d cultures. Results show that the content of glucose in the host crude protein extract slightly increases with age while it significantly decreases in the two glycoproteins (the reduction percentage is 60.04%). It seems that the quantitative reduction of glucose in the two glycoproteins obtained from the aged culture is the major factor responsible for the decrease in the level of total carbohydrate of the glycoproteins. This point is further elaborated in the ratio of three sugars, arabinose : N-acetylglucosamine : glucose in the two glycoproteins obtained from 36 h and 5 d cultures as shown in Table 6. The ratio of the two
Table 4. Percentage of Monosaccharides in Cell Wall Proteins of *Mortierella* Species and Two Purified Glycoproteins of *Mortierella pusilla* grown at 23°C for 36 h and 5 d

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Mc crude protein</th>
<th>Mp crude protein</th>
<th>Two glycoproteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>36 h 5 d</td>
<td>36 h 5 d</td>
<td>36 h 5 d</td>
</tr>
<tr>
<td>Fucose</td>
<td>0.68 ± 0.06 *</td>
<td>0.81 ± 0.07</td>
<td>1.48 ± 0.31</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.72 ± 0.08</td>
<td>0.44 ± 0.03</td>
<td>2.32 ± 0.54</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>0.55 ± 0.11</td>
<td>0.47 ± 0.10</td>
<td>2.70 ± 0.38</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>3.43 ± 0.57</td>
<td>1.45 ± 0.34</td>
<td>0.73 ± 0.06</td>
</tr>
<tr>
<td>Mannose</td>
<td>1.95 ± 0.65</td>
<td>1.72 ± 0.57</td>
<td>2.17 ± 0.85</td>
</tr>
<tr>
<td>Galactose</td>
<td>1.24 ± 0.22</td>
<td>1.09 ± 0.20</td>
<td>0.44 ± 0.25</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.26 ± 1.74</td>
<td>2.68 ± 0.37</td>
<td>5.39 ± 0.99</td>
</tr>
<tr>
<td>Unknown</td>
<td>3.02 ± 0.95</td>
<td>3.66 ± 0.71</td>
<td>3.64 ± 0.52</td>
</tr>
<tr>
<td>Sum</td>
<td>14.98 ± 1.66</td>
<td>12.32 ± 1.53</td>
<td>18.87 ± 1.23</td>
</tr>
</tbody>
</table>

* Figures indicated the quantity (mg) of various monosaccharides in 100 mg of cell wall proteins or glycoproteins. Values were reported as mean ± standard deviation (N=3).
Table 5. Percentages of Various Monosaccharides in Total Carbohydrate of Cell Wall Proteins of *Mortierella* Species and Two Purified Glycoproteins of *M. pusilla* Grown at 23 °C for 36 h and 5 d

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Mc crude protein</th>
<th>Mp Crude protein</th>
<th>Two glycoproteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>36 h 5d</td>
<td>36 h 5 d</td>
<td>36 h 5d</td>
</tr>
<tr>
<td>Fucose</td>
<td>4.59 ± 0.49 *</td>
<td>6.62 ± 0.43</td>
<td>7.78 ± 1.11</td>
</tr>
<tr>
<td>Arabinose</td>
<td>4.82 ± 0.74</td>
<td>3.62 ± 0.39</td>
<td>12.32 ± 2.86</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>3.70 ± 0.93</td>
<td>3.81 ± 0.42</td>
<td>14.25 ± 1.04</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>23.32 ± 6.02</td>
<td>12.15 ± 4.52</td>
<td>3.85 ± 0.05</td>
</tr>
<tr>
<td>Mannose</td>
<td>13.42 ± 5.55</td>
<td>13.77 ± 3.23</td>
<td>11.58 ± 4.94</td>
</tr>
<tr>
<td>Galactose</td>
<td>8.40 ± 1.97</td>
<td>8.81 ± 0.60</td>
<td>2.29 ± 1.16</td>
</tr>
<tr>
<td>Glucose</td>
<td>21.78 ± 5.21</td>
<td>21.75 ± 1.19</td>
<td>28.63 ± 5.38</td>
</tr>
<tr>
<td>Unknown</td>
<td>20.65 ± 7.34</td>
<td>29.47 ± 2.67</td>
<td>19.29 ± 2.59</td>
</tr>
</tbody>
</table>

* Figures indicated the quantity (mg) of various monosaccharides in 100 mg of total carbohydrate contained in cell wall proteins or glycoproteins. Values were reported as mean ± standard deviation (N=3).
Table 6. Ratio of Ara:GluNAc:Glu of Two Purified Glycoproteins from Cell Walls of *M. pusilla* Grown at 23°C for 36 h and 5 d

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age of culture</th>
<th>Ara:GluNAc:Glu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two Mp glycoproteins</td>
<td>36 h</td>
<td>1.0:6.7:8.6</td>
</tr>
<tr>
<td></td>
<td>5 d</td>
<td>1.0:6.8:3.1</td>
</tr>
</tbody>
</table>
sugars, arabinose and N-acetylglucosamine, remains almost the same with age while the ratio of glucose significantly decreases in the aged mycelium.

*Effects of various treatments of host and nonhost cell wall proteins on their agglutinating activity*

Host and nonhost cell wall surface proteins as well as the two glycoproteins with or without treatment (see Materials and Methods) were tested at their original concentrations up to 12.5 mg mL⁻¹ for their agglutinating activity, and the results are summarized in Table 7. All the treated or untreated cell wall proteins and glycoproteins were able to agglutinate both ungerminated and germinated spores (Figs. 6-13); however, there were large differences between the agglutination titres of the host and nonhost cell wall proteins with or without treatment (except for pronase treatment). The agglutination titres of the host cell wall protein and the two glycoproteins were about 30 and 100 times higher than those of the nonhost cell wall protein, respectively (Table 7). Treatment of the host cell wall protein with heat or 3% glutaraldehyde did not significantly affect its agglutinating activity. The results indicate that the contained glycoproteins which are responsible for the agglutination are relatively stable with respect to their agglutinating activity. This was further verified by heat treatment of the two glycoproteins followed by detection of their agglutinating activity (Table 7). Treatment of the host cell wall protein with Pronase E, a protein-degrading enzyme, could greatly inhibit the agglutination, as shown by the decreased agglutination titre, degree and extent (Table 7). In addition, there were also differences between agglutination degrees and extents of the host and nonhost cell
Table 7. Effects of Various Treatments of Cell Wall Extracts from Mortierella species (36 h) on Agglutination of Ungerminated and Germinated Spores from *P. virginia*.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Treatment</th>
<th>Protein concentration (mg mL⁻¹)</th>
<th>Agglutination titre *</th>
<th>Relative agglutination **</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. pusilla</em> crude extract</td>
<td>UH a</td>
<td>10.56</td>
<td>256</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>H b</td>
<td>10.58</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td>CT c</td>
<td>10.50</td>
<td>256</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>PT d</td>
<td>2.47</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>Two glycoproteins UH</td>
<td></td>
<td>1.78</td>
<td>1024</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>1.64</td>
<td>512</td>
<td>1024</td>
</tr>
<tr>
<td><em>M. candelabrum</em> crude extract</td>
<td>UH a</td>
<td>12.25</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>12.00</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>12.44</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>PT</td>
<td>2.65</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

* A series of dilutions was made in twofold. Agglutination titre was the reciprocal of the greatest dilution of cell wall proteins or glycoproteins in PBS at which agglutination could still be detected.

** Relative agglutination was expressed as agglutination degree (+++++) and agglutination extent (1-4). For details, see "Materials and Methods".

a UH = Unheated extract.

b H = Extract heated at 95°C for 10 min.

c CT = Extract treated with 3% glutaraldehyde at 4 °C for 30 min.

d PT = Pronase treatment of extract, see "Materials and Methods".
Fig. 6 Germinated spores without treatment (control); Fig. 7 Agglutination of germinated spores with the cell wall protein of the nonhost, *M. candelabrum*. The agglutinating degree of the most agglutinating masses was shown to be + and ++; Fig. 8 Agglutination of germinated spores with the cell wall protein of the host, *M. pusilla*. The agglutinating degree was shown to be +, ++ and +++; Fig. 9 Agglutination of germinated spores with the two glycoproteins, showing a +++ agglutinating mass.
Fig. 10 Ungerminated spores without treatment (control); Fig. 11 Agglutination of ungerminated spores with the heat-treated cell wall protein of the nonhost; Fig. 12 Agglutination of ungerminated spores with the 3% glutaraldehyde-treated cell wall protein of the host; Fig. 13 Agglutination of ungerminated spores with the heat-treated glycoproteins.
wall proteins (Table 7; Figs. 6-13). Agglutination tests with thyroglobulin, γ-globulin and fetuin were shown to be negative.

**Effects of various treatments of host and nonhost cell wall fragments on attachment and appressorium formation by the mycoparasite**

Results in Table 8 obtained by attachment test and microscopic examination showed that the mycoparasite germ tubes attached and formed appressoria at host but not at nonhost cell surfaces (Figs 14 and 15). Treatment of host cell wall fragments with alkaline solution or Pronase E could greatly inhibit the attachment by the mycoparasite, *P. virginiana*, in comparison with the untreated control. Treatments of host cell walls with other agents showed no obvious effects on the attachment. Whereas the relative percentages of appressorium formation in all other treatments were similar to that of the control (Table 8; Fig. 16), the percentage of appressorium formation was very low (8.1%) in heat-treatment (Fig. 17), as compared with that of the control (57.1%). The percentage of attachment in nonhost, including the control and treatments, was significantly low as compared with that of the corresponding host species (Table 8). Microscopic examination showed that numerous germ tubes could establish attachments and further form appressoria on the host cell walls, however, they either crossed over or under, or sometimes continued to grow along side of the nonhost cell walls without any indication of attachment and appressorium formation (Figs. 14 and 15). Only a few attachments, but definitely no appressorium formation were shown on nonhost cell walls with or without treatment.
Table 8. Effects of Various Treatments of Cell Wall Fragment of Host and Nonhost *Mortierella* Species on Attachment and Appressorium Formation by the Mycoparasite, *P. virginiana*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of attachment a</th>
<th>Relative Percentage of appressorium formation b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>M. pusilla</em> cell wall</td>
<td><em>M. candelabrum</em> cell wall</td>
</tr>
<tr>
<td>Control c</td>
<td>68.8 ± 12.7 *</td>
<td>3.2 ± 1.1</td>
</tr>
<tr>
<td>Heat d</td>
<td>63.5 ± 12.5</td>
<td>3.8 ± 1.8</td>
</tr>
<tr>
<td>Lyophilization</td>
<td>72.8 ± 15.1</td>
<td>2.2 ± 2.0</td>
</tr>
<tr>
<td>Extraction e</td>
<td>12.3 ± 7.2</td>
<td>0.7 ± 0.8</td>
</tr>
<tr>
<td>Glutaraldehyde f</td>
<td>59.0 ± 16.2</td>
<td>0.5 ± 0.7</td>
</tr>
<tr>
<td>Pronase E g</td>
<td>26.7 ± 7.7</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Values reported as mean ± standard deviation (N=3).

a Number of germinated spores that attached to cell wall fragments from a total of 100 germinated spores.

b Number of germinated spores that formed appressoria from a total of 100 germinated spores that attached to cell wall fragments.

c No treatment of cell wall fragments.

d Cell wall fragments heated at 95 °C for 15 min.

e Extraction of the cell wall fragments with 0.1 N NaOH at 4 °C for 14 h.

f Cell wall fragments were treated with 3% glutaraldehyde at 4 °C for 1 h.

g Treatment of the cell wall fragments (8 mg ml⁻¹) with Pronase E (0.58 unit ml⁻¹) in 0.05 M PBS (pH 7.4) at room temperature for 3 h.
Fig. 14 The mycoparasite germ tubes do not attach or form appressoria on cell walls of the nonhost, *M. candelabrum*; Fig. 15 The mycoparasite germ tubes attach and form prominent appressoria (arrow) at the points of contact with the cell walls of the host, *M. pusilla*; Fig. 16 Attachment and appressorium formation of the mycoparasite germ tubes on the host cell wall fragment pretreated with 3% glutaraldehyde; Fig. 17 The heat treatment of the host cell walls results in an inhibition of appressorium formation by the mycoparasite germ tubes, although their attachment is normal (arrow).
Involvement of glycoproteins b and c in attachment and appressorium formation

In the combined agglutination-attachment test, incubation of the two purified glycoproteins with the germinated spores and host cell wall fragments could totally inhibit the attachment and appressorium formation. The germinated spores were agglutinated and formed agglutinating masses near the host cell wall fragments, but no attachment and appressorium formation could be observed, even in those unagglutinated spores. It is interesting that in those spores preincubated with the single glycoprotein b or c, neither agglutination nor attachment could be seen under microscope. In the control tests with thyroglobulin or γ-globulin, no agglutinating mass could be seen and the attachment and appressorium formation were normal in comparison with those observed in the mycoparasite spores without any pretreatment.

Involvement of carbohydrates in agglutination and attachment

Incubation of the mycoparasite spores with various monosaccharides followed by incubation with the host cell wall protein or the two glycoproteins showed that three sugars, arabinose, glucose and N-acetylglucosamine, at the concentration of 100 mM could fully inhibit the agglutination of the mycoparasite spores (Table 9, Figs. 19 and 20). The weak agglutinating activity of the nonhost cell wall protein could also be inhibited by the three sugars (Fig. 18).

It is interesting that those three sugars, which could totally inhibit agglutination of the mycoparasite spores, also greatly suppressed attachment of the mycoparasite germ tubes to the host cell wall fragments (Table 10, Fig. 21), as compared with the control. However, these sugars
Table 9. Effects of Various Monosaccharides on Agglutination of Germinated Spores of *P. virginiana* by Cell Wall Extracts from *Mortierella* Species Grown at 23 °C for 36 h

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Concentration (mM)</th>
<th>Relative agglutination of <em>Piptocephalis</em> spores</th>
<th>Two glycoproteins</th>
<th>M. candelabrum crude extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>M. pusilla</em> crude extract</td>
<td>Two crude extract</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1:128)</td>
<td>(1:256)</td>
<td>(1:8)</td>
</tr>
<tr>
<td>Control</td>
<td>----</td>
<td>+--++, 3-4</td>
<td>+--++, 3-4</td>
<td>+--++, 2-3</td>
</tr>
<tr>
<td>L-Fucose</td>
<td>100</td>
<td>+, 3-4</td>
<td>+--++, 3-4</td>
<td>+++, 2-3</td>
</tr>
<tr>
<td>D-Arabinose</td>
<td>100</td>
<td>-, 0</td>
<td>-, 0</td>
<td>-, 0</td>
</tr>
<tr>
<td>N-Acetyl-D-glucosamine</td>
<td>100</td>
<td>-, 0</td>
<td>-, 0</td>
<td>-, 0</td>
</tr>
<tr>
<td>N-Acetyl-D-galactosamine</td>
<td>100</td>
<td>+--++, 3-4</td>
<td>+--++, 3-4</td>
<td>+--++, 3-4</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>100</td>
<td>+--++, 3-4</td>
<td>+--++, 3-4</td>
<td>+++, 2-3</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>100</td>
<td>+, 2-3</td>
<td>+--++, 3-4</td>
<td>+, 2-3</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>100</td>
<td>-, 0</td>
<td>-, 0</td>
<td>-, 0</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>100</td>
<td>+--++, 3-4</td>
<td>+--++, 3-4</td>
<td>+++, 2-3</td>
</tr>
</tbody>
</table>

* Relative agglutination was expressed as agglutination degree (+--++) and agglutination extent (1-4). For details, see "Materials and Methods".

* b Original protein concentration was 10.28 mg mL⁻¹.

* c Original protein concentration was 1.78 mg mL⁻¹.

* d Original protein concentration was 11.74 mg mL⁻¹.
Table 10. Effects of Various Monosaccharides on Attachment and Appressorium Formation by *P. virginiana* on Cell Wall Surface of *Mortierella* Species

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Concentration (mM)</th>
<th>Percentage of attachment <em>a</em></th>
<th>Relative percentage of appressorium formation <em>b</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>M. pusilla</em> cell wall</td>
<td><em>M. pusilla</em> cell wall</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>M. candelabrum</em> cell wall</td>
<td><em>M. candelabrum</em> cell wall</td>
</tr>
<tr>
<td>Control</td>
<td>----</td>
<td>62.7 ± 9.0 *</td>
<td>53.2 ± 12.3</td>
</tr>
<tr>
<td>L-Fucose</td>
<td>100</td>
<td>56.3 ±12.0</td>
<td>49.7 ± 15.0</td>
</tr>
<tr>
<td>D-Arabinose</td>
<td>100</td>
<td>22.3 ± 10.1</td>
<td>47.7 ± 6.2</td>
</tr>
<tr>
<td>N-Acetyl-D-glucosamine</td>
<td>100</td>
<td>9.2 ± 7.0</td>
<td>50.9 ± 15.3</td>
</tr>
<tr>
<td>N-Acety-D-galactosamine</td>
<td>100</td>
<td>67.5 ± 15.5</td>
<td>58.0 ± 5.7</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>100</td>
<td>59.6 ± 10.9</td>
<td>61.8 ± 11.3</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>100</td>
<td>64.8 ± 18.3</td>
<td>56.3 ± 6.5</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>100</td>
<td>6.3 ± 4.4</td>
<td>44.7 ± 5.7</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>100</td>
<td>60.5 ± 11.4</td>
<td>51.2 ± 13.2</td>
</tr>
</tbody>
</table>

* Values reported as mean ± standard deviation (N=3).

*a* Number of germinated spores that attached to cell wall fragments from a total of 100 germinated spores.

*b* Number of germinated spores that formed appressoria from a total of 100 germinated spores that attached to cell wall fragments.
Figs. 18-20  Inhibitory effect of sugars on agglutination by the cell wall proteins of the mycoparasite spores; Fig. 18 Ungerminated spores incubated with arabinose and the nonhost cell wall protein; Fig. 19 Germinated spores with glucose and the host cell wall protein; Fig. 20 Germinated spores with N-acetylglucosamine and the two glycoproteins; Fig. 21 Inhibition of the attachment and appressorium formation at the host cell surface by the parasite germ tubes previously treated with N-acetylglucosamine.
did not significantly affect the appressorium formation (Table 10). In nonhost, the attachment was not affected by those sugars and no appressorium formation could be seen.

Relationships between host age and parasitism as well as agglutinin activity

A comparison of effects of different host culture ages on the attachment, appressorium and agglutination was performed. Results (Table 11) showed that the percentages of attachment and appressorium formation in the aged host (5 d) decreased, as compared with those of the young host (36 h). Consistent with these observations was the finding that the two purified glycoproteins from the aged host culture had a lower agglutinating activity than those of the young host culture as shown by agglutination degree and extent (Table 11).
Table 11. Effects of Host Age on Parasitic Ability and Agglutination of Germinated Spores of *P. virginiana*

<table>
<thead>
<tr>
<th>Age of culture</th>
<th>Percentage of attachment (^a)</th>
<th>Relative percentage of appressorium formation (^b)</th>
<th>Relative agglutination (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>36 h</td>
<td>65.8</td>
<td>55.3</td>
<td>+++++, 3-4</td>
</tr>
<tr>
<td>5 d</td>
<td>41.7</td>
<td>48.7</td>
<td>+++, 2-3</td>
</tr>
</tbody>
</table>

\(^a\) Number of germinated spores that attached to cell wall fragments from a total of 100 germinated spores.

\(^b\) Number of germinated spores that formed appressoria from a total of 100 germinated spores that attached to cell wall fragments.

\(^c\) Relative agglutination was expressed as agglutination degree (+++++) and agglutination extent (1-4). For details, see "Materials and Methods". In the test, two glycoproteins purified from the crude cell wall extract of *M. pusilla* were used. The original concentration of the glycoproteins was 1 mg mL\(^{-1}\) and the dilution titre was 1:256 for both 36 h and 5 d old cultures.
Discussion

A key question in the research on host-parasite interactions is how a parasite can discriminate its host from nonhost and subsequently parasitize the former. In this aspect, cell surfaces provide the first point of interaction between a nonhost or host and a parasite. It is here that the fate of the nonhost- or the host-parasite interaction is determined. Regardless of the various factors which determine the nature of the interactions, cell surfaces could be the important centres where the phenomena of specificity and recognition take place (Manocha 1985). Attachment and appressorium formation by the parasite involve the host cell surface. Further, considerable evidence has demonstrated that the interaction specificity is mediated by surface-localized complementary molecular mechanisms and most of the interaction have been shown to be between agglutinin and carbohydrate which are involved as key specificity determinants in establishing host-parasite relationships (Kojima and Uritani 1978; Kojima et al. 1982; Furuichi et al. 1980; Barak et al. 1985).

In recent years, investigations on the mechanism of attachment of the mycoparasite, *Piptocephalis virginiana*, to the host but not to the nonhost species have been focused on the host and nonhost cell wall surfaces (Manocha 1984, 1985; Manocha et al. 1986). However, both gross cytological features and chemical composition of cell walls did not reveal any significant differences between host and nonhost species.

Present investigation has shown, by SDS-PAGE, that four high molecular weight bands marked as a, b, c, and d were observed on gels with the cell wall extract from the host, *M. pusilla*, but were absent (except for the band c) in gels prepared from the extract of the nonhost, *M.*
candelabrum (Fig. 2). Among the four bands, glycoprotein b and c seem to be two subunits of an agglutinin on the host cell wall surface. The evidence for this comes from the following observations. (I) Deletion of either glycoprotein b or c from the host cell wall crude extract resulted in significant decrease of agglutinating activity, in comparison with that of the complete extract (Table 1). The agglutination unit in the former was about 20-32 times less than that of the latter, and the decreased agglutinating activity was similar to that of the nonhost crude extract. (II) The agglutinating activity of single glycoprotein b or c was low. It was almost the same as that of the crude extract minus glycoprotein c or b; however, in the case of pure preparation containing both glycoproteins b and c, the agglutination unit was about 510-850 times more than those of the single glycoprotein b or c (Table 3). These results indicate that the agglutinin activity requires the presence of both the glycoproteins. (III) In the purification of the two glycoproteins, the agglutination unit of purified fractions increased with the purification procedures. The agglutination unit of the pure preparation was almost 35 times more than that of the crude extract (Table 2). (IV) Deletion of protein a or d from the crude extract did not affect its agglutinating activity (Table 1).

Moreover, observations in the agglutination test that the host cell wall protein and the two glycoproteins with or without treatment (except for pronase treatment) can strongly agglutinate the mycoparasite spores at high titres is consistent with the results obtained in the measurement of agglutinating activity of host cell wall crude extract and the purified glycoprotein fractions, further indicating that the two glycoproteins b and c are an agglutinin present at the host cell surface. The finding that the two cell wall glycoproteins are required for the interaction specificity is
partially in accord with previous results obtained in three separate investigations where glycoproteins extracted with 0.1 N NaOH from isolated cell walls of the hosts and nonhosts of the mycoparasite, *P. virginiana*, exhibited markedly different patterns after SDS-PAGE. Two distinct bands of high molecular weight glycoprotein, a and b (Manocha 1984) or a double peak b and c (Manocha 1985, 1986), similar to the band b and c in the present investigation, were observed in the extracts of the host species, but not in the nonhost species. From these studies, it seems that one band, i.e., band b, is common to all the host species investigated, and the glycoprotein represented by band b was suggested to be essential for the interaction specificity (Manocha 1985). However, based on the present results, it is demonstrated that two glycoproteins, i.e., b and c, are required for agglutination and are possibly involved in attachment of the mycoparasite.

The results of combined agglutination-attachment test showed that the two glycoproteins from the host could not only strongly agglutinate the mycoparasite spores but totally inhibit their attachment to the host cell walls fragments. Interestingly, although the single glycoprotein b or c only had a very weak agglutinating activity, they also could effectively hinder the attachment and appressorium formation. The inhibition of attachment may be due to a complete or a partial competitive occupation of the complementary binding sites at the germ tube surfaces by the two glycoproteins or the single glycoproteins which prevent their further binding with the identical receptor glycoproteins at the host cell wall fragment surface. The data presented here suggest that the two glycoproteins at the host cell surface may be involved in recognition and attachment by the mycoparasite. These findings also suggest that the two
glycoproteins b and c at the host surface may not only serve as agglutinin 
*in vitro* but also act as two subunits of the receptor *in vivo*. The dual 
function of certain host cell surface glycoproteins was also observed in 
other host-fungal parasite systems (Callow 1984; Ralton et al. 1986, 1987; 
Hohl and Balsiger 1988). The above results preliminarily verify the 
previous suggestion proposed by Manocha (1987) that the attachment by 
the mycoparasite may depend upon specific receptors on the host surface. 

The results obtained by attachments with or without treatments of the 
host cell walls also support the above suggestion. The inhibition of 
attachment of mycoparasite by alkaline and pronase treatments of the host 
cell walls may be caused by reducing or degrading the cell wall protein, 
including the two glycoproteins, which are probably involved in the 
make-up of the surface receptors. Suppression with pronase treatment of 
the agglutinating activity of the host cell wall protein is consistent with the 
above results. Since other physical and chemical treatments did not result 
in loss or degradation of the cell wall proteins including the two 
glycoproteins, the results of the agglutination and the attachment tests 
remained similar to those of the controls. These observations also indicate 
that the two glycoproteins are relatively stable with respect to their 
agglutinin and receptor functions.

Although alkaline extraction or pronase treatment of the host cell wall 
resulted in an inhibited attachment which led to a decrease in the total 
number of appressoria formed by the mycoparasite germ tubes, still the 
relative percentages of appressorium formation remained similar to that of 
the control. These results indicate that the appressorium formation by the 
mycoparasite was not affected by these treatments of the host cell walls. 
However, with heat treatment the results were different. There was
normal attachment but inhibited appressorium formation (lower relative percentage of appressorium formation). This phenomenon could be explained based on the following assumptions: (1) the alkaline extraction and pronase treatment did not remove or degrade all the host cell surface receptors, and the remaining receptors might have functioned normally in the processes of parasitism, i.e. attachment and appressorium formation; (2) the heat treatment, on the other hand, denatured the receptors but did not cause the loss of the sugars from the receptor glycoproteins. This investigation (see Table 10 and Fig. 21) and others (Hamer et al. 1987; Longman and Callow 1987; Bonfante-fasolo 1988) have shown that attachment, in fact, is mediated directly by certain sugars present at either or both interacting species surfaces; (3) the appressorium formation may be the response of the complementary combinations of both sugar and protein, the two parts of the glycoproteins at the host and mycoparasite surfaces. Recognition is possibly required at each stage of parasitic process, including appressorium formation. The research work carried out by Mendgen (1982) on *Puccinia coronata* has provided preliminary evidence for the latter.

Previous studies, using fluorescent lectin binding assay technique, have shown obvious differences in the sugar residues exposed at the host and nonhost cell surfaces. N-acetylglucosamine oligomers were shown to be present at both the host and nonhost surfaces; nevertheless, the nonhost species also had galactose and N-acetylgalactosamine residues on their surfaces as specific lectin binding sites (Manocha 1985; Manocha et al. 1986). In the present study, it was revealed by gas chromatography that the host and nonhost cell wall extracts contained the same sugars, including the three sugars mentioned above, although the extract from the
nonhost did contain higher levels of galactose and N-acetylgalactosamine than that from the host. The results of sugar analysis of the glycoproteins clearly indicated that the two sugars, glucose and N-acetylglucosamine, are the major carbohydrate component of the two glycoproteins.

Pretreatment of the germinated spores of the mycoparasite with various sugars before incubation with the host cell wall fragments or the cell wall protein has revealed that the three exogenous sugars, arabinose, glucose and N-acetylglucosamine, could significantly inhibit attachment of the germ tubes to the host cell walls and completely block the agglutinin activity of the two glycoproteins. This suggests that the three sugars present in the two glycoproteins, may be involved in agglutination, attachment and recognition. The result that N-acetylglucosamine could inhibit the attachment supports the previous finding that the attachment was blocked by the addition of chitobiose and chitotriose (Manocha 1985). Sugar analysis by gas chromatography has revealed that glucose and N-acetylglucosamine are major carbohydrate components of the two glycoproteins. It seems that the abundant quantities of the two sugars together with another sugar, arabinose, contained in the two glycoproteins are a material basis for the specific agglutinin and receptor functions of the two glycoproteins.

This study may also provide some possible explanations for well founded observations that resistance of the mucoraceous hosts to the mycoparasite increases with age (England 1969; Manocha and Campbell 1983). According to the present results, this phenomenon may be evoked by the quantitative changes in the cell surface components of the aged host cell: (1) decrease in the quantity of the two glycoproteins (Table 2), and (2) decrease in glucose content in the two glycoproteins (Table 4). Loss or
degradation of the two glycoproteins at the host cell surface, by an unknown mechanism, must result in the decrease of attachment locations required for parasitism. As mentioned above, numerous evidence has demonstrated that certain carbohydrates present at host cell surfaces are involved in attachment and recognition. Thus, the significant reduction of glucose in the two glycoproteins may also influence, at least in part, the host-mycoparasite cell surface complementary interactions in attachment and recognition processes. It seems that in the mature host the reduction in the quantity of glucose in the two glycoproteins is responsible for the weakened agglutinating activity, while the lower percentages of attachment and appressorium formation are caused by decreased contents of both the glycoproteins and glucose contained therein.

On SDS gels with the nonhost cell wall crude extract, a weak band corresponding to band c of the host was observed (Fig. 2). Measurement of agglutinating activity showed that the nonhost crude extract had a very weak agglutinating activity, which was similar to that of the host crude extract minus glycoprotein b. Furthermore, the low agglutinating activity and percentage of attachment in nonhost observed in agglutination and attachment tests were also reported in previous studies (Manocha 1985; Manocha et al 1986). These phenomena may be attributed to the low amount of protein c contained in the nonhost cell surface protein, as revealed by SDS-gel electrophoresis. The fact that the three sugars, arabinose, glucose and N-acetylglucosamine, which can inhibit agglutinating activity of the two host glycoproteins also suppress the activity of the nonhost cell surface protein may support the above suggestion. It is possible that the lack of the protein b at the nonhost cell wall surface may be responsible for the failure of the mycoparasitic germ
tube to successfully attach to it and to form appressorium on the surface even with the negligible attachment.

Based on the present results, it has become increasingly clear that further work on the host-parasite interaction specificity is needed. Further analyses of the two glycoproteins will enable the investigator to build on the trends which are established in this study. The eventual accumulation of the data in all aspects about the the glycoproteins will possibly lead to a complete elucidation of roles of them in the determination of host range and in the host-parasite interaction specificity in the mycoparasite system. Cloning and analysis of the glycoprotein-specific genes in the host and nonhost may provide some genetic information on the interaction specificity. In addition, numerous investigations have shown that the specificity of interaction between host and fungal parasite is achieved by the specific binding of complementary molecules at both the interacting species, and evidence for the involvement of specific components at fungal parasite surface in host-parasite interaction has been obtained (Barak et al 1986; Hamer et al. 1987; Boyer and Charest 1989). It is conceivable that on the germ tube surface of the mycoparasite, *Piptocephalis virginiana*, there may also be a specific structure which is complementary to the host surface glycoproteins. Obviously, further work is also needed to conduct toward the specific components at the mycoparasite surface which are involved in agglutination, attachment and appressorium formation.
Summary

1. Glycoproteins b and c present at the host but not the nonhost cell wall surface may serve as both the two subunits of agglutinin *in vitro* and receptor *in vivo*. The two glycoproteins are relatively stable with respect to their agglutinin and receptor functions.

2. Arabinose, glucose and N-acetylglucosamine present in the two glycoproteins are involved in the specific host attachment and recognition by the mycoparasite.

3. Agglutination and attachment are mediated directly by certain sugars present at the host and mycoparasite cell surfaces while the appressorium formation is the response of complementary combinations of both sugar and protein, the two parts of glycoproteins at the interacting surfaces of the two fungi.

4. Based on the present results, previous observations that resistance of the hosts to the mycoparasite increases with age can be explained by decreased quantities of the two glycoproteins and glucose contained therein in the mature host.

5. The low agglutinating activity and percentage of attachment in nonhost may be attributed to the low amount of protein c present at the nonhost surface and it seems that the lack of the protein b at the nonhost cell surface is responsible for the failure of the mycoparasite to successfully
attach to the surface and to form appressorium on it even with the negligible attachment.

6. On the mycoparasite germ tube surface, there may be a specific structure which is complementary to the host surface receptor and the interaction may be between agglutinin and carbohydrate.
Literature Cited


*roseum Avenaceum* and *Fusarium solani*. Phytopathology, 64: 394-399.


Appendix I

Ammonium sulfate fractionation of the host cell wall protein extract

1. Adjust the pH of 50 mL of saturated (NH₄)₂SO₄ to about pH 7.4 by addition of 2N NaOH. This is done just prior to precipitation of the host cell wall protein.

2. With constant stirring, slowly add (dropwise) to a 35-mL protein sample a total of 15 mL of saturated ammonium sulphate solution (pH 7.4), thus effecting 30% saturation. During the first stage, do not proceed with the addition of the salt solution until all precipitate from the precious addition has dissolved. Eventually the precipitate persists. Continue adding the salt solution slowly.

3. Upon completion of the addition of the ammonium sulfate solution, continue stirring the suspension for an additional 2 h.

4. Centrifuge the suspension at room temperature for 15 min at 10,000 g.

5. Dissolve the precipitate in 0.1 M PBS containing 0.1 mM EDTA, pH 7.4.

6. Purify the fraction containing the two proteins b and c by a second precipitation (repeat steps 1 through 5).

7. Remove the ammonium sulfate from the precipitate by dialyzing against 0.1 M PBS containing 0.1 mM EDTA, pH 7.4, for 24 h at 4 °C.

8. After dialysis is complete, remove the solution from the tubing.
Appendix II

Fractionation of the host cell wall protein extract by a Sephadex G-100 column

Forty micrograms of the crude protein extracted from the host cell walls were dissolved in 10 ml of 0.1 M PBS containing 0.1 mM EDTA, pH 7.4, and loaded onto a Sephadex G-100 column (100 x 2.5 cm). The protein was eluted with the above PBS solution at 4°C. The eluted solution was collected in 5 mL fractions and the contained protein was detected using a spectrophotometer. The fractions containing the two proteins b and c were confirmed by SDS-PAGE followed by staining with Coomassie brilliant blue R250.

The data from this test revealed that the fractions containing the two proteins emerged between 175-240 mL, i.e. 110-175 mL beyond the void volume (65 mL). After 240 mL of PBS solution passed through the column, only trace amounts of the two proteins were detected.

The data also showed that 35-38% of the protein which was loaded into the column was recovered within the first protein peak covering 65 mL of elutant containing the two proteins (Fig. 22). The agglutinin yield after purification with Sephadex G-100 column was about 87% of the agglutinin contained in the protein obtained after heat treatment. The calculated efficiency of the column in each instance is incorporated into the data which are presented in Table 2.
Fig. 22  Fractionation by a Sephadex G-100 column of the host cell wall protein containing the two glycoproteins

O.D. Value (280 nm)

Elution Volume (mL)
Appendix III. Data on agglutination test: Degree of agglutination of germinated spores from P. virginiana by cell wall proteins under various treatments from Mortierella species grown at 23 C for 36 h.

+, agglutinating masses consisting of 4-10 Piptocephalis spores; ++, agglutinating masses consisting of 11-20 spores; ++++, agglutinating masses consisting of more than 20 spores.

Abbreviations: MpCE(UH), unheated crude extract of Mortierella pusilla; MpCE(H), heated crude extract of *M. pusilla*; MpCE(CT), chemical-treated crude extract of *M. pusilla*; MpCE(PT), pronase-treated crude extract of *M. pusilla*; Gps(UH), unheated glycoproteins of *M. pusilla*; Gps(H), heated glycoproteins of *M. pusilla*; McCE(UH), unheated crude extract of *M. candelabrum*; McCE(H), heated crude extract of *M. candelabrum*; McCE(CT), chemical-treated crude extract of *M. candelabrum*; McCE(PT), pronase-treated crude extract of *M. candelabrum*. 
Appendix IV. Data on Agglutination test: Effect of various sugars on degree of agglutination of germinated spores from *P. virginiana* by cell wall protein extract of *M. pusilla* grown at 23 °C for 36 h. The agglutination was fully inhibited by the addition of arabinose, N-acetylglucosamine or glucose.

**Abbreviations:** Con, Control; Fuc, Fucose; Ara, Arabinose; NAcGlu, N-Acetylglucosamine; Man, Mannose; Gal, Galactose; Glu, Glucose; Xyl, Xylose.
Appendix V. Data on agglutination test: Effect of various sugars on degree of agglutination of germinated spores from P. virginiana by two glucoproteins b and c purified from the cell wall protein extract of M. pusilla grown at 23 C for 36 h.

The agglutination was fully inhibited by the addition of arabinose, N-acetylglucosamine or glucose.
Appendix VI: Data on agglutination test: Effect of various sugars on degree of agglutination of germinated spores from P. virginiana by cell wall protein extract of M. candelabrum grown at 23 ºC for 36 h. The agglutination was fully inhibited by the addition of arabinose, N-acetylglucosamine or glucose.