Involvement of Fimbriae in Host-Mycoparasite recognition

by

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A Thesis
submitted to the Department of Biological Sciences
in partial fulfillment of the requirements
for the degree of
Master of Science

November 1991
Brock University
St. Catharines, Ontario

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Abstract

Extracellular, non-flagellar appendages, termed fimbriae are widespread among fungi. Fungal fimbriae range in diameter from 6-10 nm and exhibit lengths of up to 30 µm. Fungal fimbriae have been implicated in several functions: adhesion, conjugation and flocculation. A possible role of fimbriae in host-mycoparasite interactions was the focus of this study .

Using electron microscopy, fimbriae were observed on the surfaces of *Mortierella candelabrum, Mortierella pusilla* and *Phascolomyces articulosus* with diameter means of 9.1±0.4 nm, 9.4±0.5 nm and 8.6±0.6 nm, respectively, and lengths of up to 25 µm. Fimbriae were not observed on the surface of the mycoparasite, *Piptocephalis virginiana.*

Polyclonal antiserum (AU) prepared against the fimbrial protein of *Ustilago violacea* cross-reacted with 60 and 57 kDa *M. candelabrum* proteins. In addition, AU cross-reacted with 64 kDa proteins from both *M. pusilla* and *P. articulosus.* The proteins that cross-reacted with AU were electroeluted from polyacrylamide gels and were shown to subsequently form fibrils. The diameter means for the electroeluted fibrils were: for *M. candelabrum* 9.7±0.3 nm, *M. pusilla* 8.4±0.6 nm and *P articulosus* 9.2±0.5 nm.

Finally, to ascertain the role of fimbriae in host-mycoparasite interactions, AU was incubated with *P. virginiana* and *M. pusilla* (mycoparasite/susceptible host) and with *P. virginiana* and *P. articulosus* (mycoparasite/ resistant host). It was observed that AU

decreased significantly the level of contact between *P. virginiana* and *M. pusilla* and between *P. virginiana* and *P. articulosus* in comparison to preimmune serum treatments. Thus, it was proposed that fimbriae might play recognition and attachment roles in early events of mycoparasitism.

Acknowledgements

Sincer thanks to Dr. Alan Castle for his guidance and understanding. I would also like to extend my thanks to Dr. Manocha, my co-supervisor.

I wish to thank Dr. Carlone for lending us lab equipment. Many thanks to Rob Boulianne, Dr. Steve Lougheed, graduate students, and the biology department staff for their help. Special thanks go to members of the House of Masters for providing a much needed atmosphere.

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Introduction

Fungal extracellular non-flagellar appendages, termed fimbriae, were first described on the anther smut fungus *Ustilago violacea* (Poon and Day, 1974; Day and Poon, 1975). Polyclonal antiserum raised against *U. violacea* fimbrial protein was used to survey other fungi for the presence of fimbriae by agglutination and immunofluorescence techniques. Fimbriae were found to be widespread among fungi, and they are not restricted to specific taxonomic groups (Day *et al.,* 1986; Day and Gardiner, 1988; Gardiner *et al.,* 1981, 1982; Svircev *et al.,* 1986).

Fimbriae have been implicated in different functions: adhesion of *Candida albicans* to host tissue (Douglas *et al.,* 1981), flocculation of *Saccharomyces cerevisiae* (Day, Poon and Stewart, 1975) and conjugation in *U. violacea* (Day and Poon, 1975). A possible role of fimbriae in host-mycoparasite interaction, which is not yet determined, is under investigation in this study.

Mycoparasitism refers to a fungus parasitic on another fungus. The mycoparasite that was used in this study was *Piptocephalis virginiana* Leadbeater and Mercer. *P. virginiana* is a biotrophic haustorial mycoparasite with a host range limited to species in the Mucorales (Manocha, 1988). Moreover, all species of Mucorales are not equally susceptible. *P. virginiana* germ tubes are capable of directed growth towards and attachment to the hyphal surfaces of both susceptible (*Mortierella pusilla* Oudemen) and resistant hosts (*Phascolomyces articulosis* Boedijn ex Benny and Benjamin) but not to a non-host (*Mortierella candelabrum* V. Teigh and Le Monn).

Attachment is followed by the formation of an appressorium at the site of contact. A penetration peg develops from the appressorium to attempt penetration of the host hyphal wall. In the case of the susceptible host, penetration leads to the formation of a haustorium to draw nutrition from the host. In the resistant host, however, penetration is impeded by the thickening of the hyphal wall. Occasionally, penetration is successful and a haustorium is developed. When this occurs, a thick sheath forms around the haustorium thus preventing the establishment of a nutritional relationship with the host protoplast (Manocha, 1985,1988). It is obvious that these parasitic events require recognition between host and parasite at several levels: at the host plasma membrane, at the cell wall and in the cell wall vicinity.

This study was carried out to investigate the role of fimbriae in mycoparasitism and the specific aims were:

(1) determination of the presence of fimbriae on the above mentioned hosts, non-host and mycoparasite species.

(2) characterize partially the physical and chemical nature of fimbriae of these species.

(3) determine if the anti-fimbrial antiserum prepared against the fimbriae of *U. violacea* has an effect on the interactions between the hosts, *M. pusilla* and *P. articulosis,* and the mycoparasite, *P. virginiana.*

Literature Review

Bacterial Fimbriae

In 1949, Anderson and Houwink reported independently the presence of filamentous appendages on the surfaces of bacteria. These appendages were different from known flagella. Since then, these bacterial appendages have been referred to as threads, filaments, bristles, fuzz, fibrillae, cilia, colonization factor antigen, adhesins, pili and fimbriae by various workers (Paranchych and Frost, 1988). Fimbriae and pili, however, are the only terms that have received wide acceptance. Duguid and coworkers (1955) introduced the term fimbria (Latin for thread or fiber). Brinton (1959) argued that the term fimbriae is linguistically incorrect and introduced the term pilus (Latin for hair or hair-like structure). Since there is no general agreement on which name is appropriate, the term fimbriae will be used to refer to all bacterial non- flagellar surface filaments.

Fimbriae are ubiquitous among both gram-negative and gram-positive bacteria. Fimbriae are distributed over the whole cell, as in the enterobacteria, or in polar or bipolar arrangements as observed on various species of *Pseudomonas* (Elleman, 1988; Ottow, 1975). Several different types of fimbriae have been identified and serious attempts have been made to classify them. In 1965, Brinton classified fimbriae on the basis of their ultrastructure and biochemistry and distinguished six types of fimbriae (types I-V and F). At the same time, Duguid and coworkers (1966) devised a scheme to classify fimbriae on the basis of their agglutination properties, and they distinguished seven types of fimbriae (types 1-6 and F). However, as more types of fimbriae were described, it was difficult and inconsistent to classify them according to Brinton's and Duguid's schemes. Now only Duguid's type 1 classification is still in common use (Paranchych and Frost, 1988). More recently, another attempt to classify bacterial fimbriae based on serology of fimbrial antigens was made by Orskov and Orskov (1983). In this system only F designation (for fimbrial) would be used (e.g. F1-Fn). However, the scheme did not receive wide acceptance since it would lead to confusion between nonconjugative and conjugative fimbriae with designations FI-FV. Thus, it is not surprising that there is no general agreement on specific classification systems because of the bewildering array of fimbrial types exhibited on bacterial surfaces. Several different criteria are used to classify bacterial fimbriae. These include: morphology, function, and biochemical properties. However, the simplest way to classify bacterial fimbriae is that based on function using the biochemical properties and morphological characteristics to further divide into subpopulations. On the basis of function, bacterial fimbriae could be divided roughly into two broad groups: "conjugative" fimbriae and "non-conjugative" or "adhesive" fimbriae (Irvin, 1990; Parachych and Frost, 1988).

Conjugative Fimbriae

Conjugative fimbriae refer to bacterial non-flagellar surface filaments that are involved in the transfer of bacterial and viral nucleic acids, genetically determined by the fertility factor and may act as bacteriophage receptors (Ottow, 1975). To date, conjugative fimbriae have been identified only on gram-negative bacteria (Irvin, 1990). They are generally encoded by operons located on self transmissible plasmids that can pass a copy of their genetic material during conjugation (Paranchych and Frost, 1988; Irvin, 1990). Conjugative fimbriae can be subdivided further on the basis of their serology, phage sensitivity and morphology. Bradley (1980, 1983, 1984) classified conjugative fimbriae based on morphology into three classes: thin flexible with 6-7 nm diameter, thick flexible with 8-10 nm diameter and rigid with 8-11 nm diameter. These could be differentiated further into subclasses based on fimbriae associated structures.

Non-conjugative or adhesive fimbriae

Whereas conjugative fimbriae are described only for gram-negative bacteria and are encoded by plasmids with an extensive transfer operon, non-conjugative or adhesive fimbriae are present on both gram-negative and gram-positive bacteria and are encoded either chromosomally or on plasmids that have a more limited operon (Irvin, 1990). These fimbriae are implicated in promoting adherence to mammalian cells. Adhesive fimbriae of *E. coli* bind preferentially to intestinal or urinary epithelial cells (Gaastra and de Graaf, 1982; Klemm, 1985). In other bacteria such as *Pseudomonas aeruginosa, Neisseria gonorrhoeae, Moraxella bovis* and *Vibrio* *cholera,* fimbriae promote adherence to host mucosal surfaces (Paranchych and Frost, 1988).

Adhesive fimbriae can be classified into three classes based on morphology. The first class includes fimbriae such as type I, CFA/I, 987P, CS1, CS2 and Pap (pyelonephritis associated pili). These fimbriae have the appearance of thin, rigid rods with diameters of 7 nm (Gaastra and de Graaf, 1982). They are distributed all over the cell.

The second class includes MePhe or Type 4 fimbriae that are produced by *Neisseria sp., Moraxella sp. Ps. aeruginosa,* and *Bacteroides nodosus.* These fimbriae are thin and flexible of about 6 nm. They are characterized by the presence of a modified amino acid, N-methylphenylalanine (MePhe), at the N-terminal of the fimbrial subunit. There is an extensive homology at the N-terminal of the fimbrial subunits of these species (Paranchych and Frost, 1988).

Finally, the third class includes K88, K99, F41, and CS3 fimbriae which have an electron microscope appearance of very thin flexible threads of 2-5 nm in diameter they are arranged peritrichously and range from 100-1000 fimbriae per cell.

Structure of Fimbriae

Structural studies of bacterial fimbriae have been done only on a few types. Examples of these will be reviewed here to demonstrate differences and similarities between different types.

Escherichia coli type 1 fimbriae were the first to be examined by electron microscopy and X-ray fiber diffraction techniques (Brinton, 1965). These studies have indicated that the type 1 fimbriae are helical structures of identical subunits that are arranged in a manner of 3.125 subunits per turn of 2.3 nm pitch with an axial hole of approximately 2 nm.

Marvin and Folkhard (1986) have proposed a model for the structure of the F fimbriae. Using fiber diffraction studies, they concluded that F fimbriae are hollow fibers of 8 nm outer diameter with 2 nm axial hole. There is a five fold rotational symmetry in the fimbriae with 25 subunits per two turns of the helix with 16 nm pitch.

Fimbriae of the *Ps. aeruginosa* strains, PAK and PAO are examples of the NMePhe type. They have five repeating subunits, but there is no fivefold rotational symmetry. They are hollow helices with an outer diameter of 5.2 nm and a central channel of 1.2 nm diameter (Folkhard *et al.,* 1981; Watts *et al.,* 1983 a and b).

In contrast, the structure of *Bordetella pertussis* fimbriae was determined to be helical with 2.5 subunits per turn resulting in a pitch of 6.5 nm, and the fimbriae diameter is approximately 5.5 nm with no axial channel (Steven *et al.,* 1986). Thus, based on these studies, fimbriae are helical structures made up of subunits that are arranged in defined structures that may vary in the diameter, the pitch of the helix, or the presence of an axial channel depending on the fimbriae in question (Irvin, 1990).

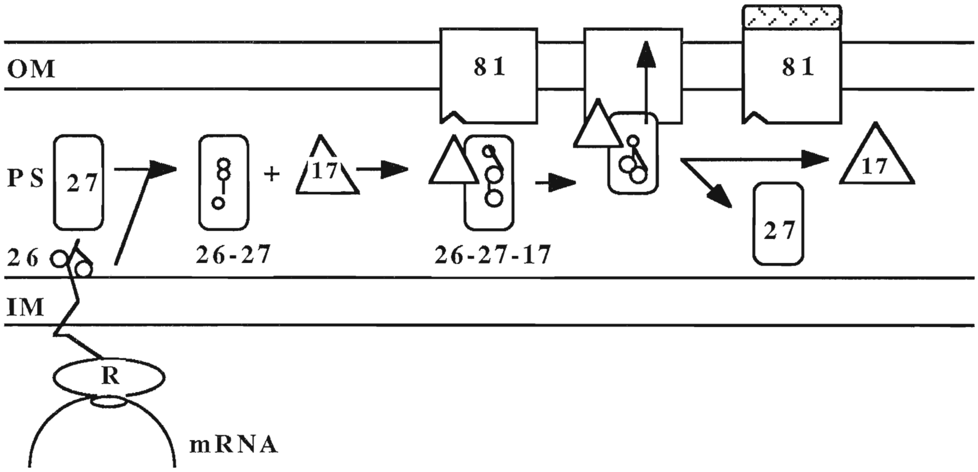
Assembly of Fimbrial Subunits

The assembly of fimbriae into morphologically defined structures is both highly specific and tightly regulated (Irvin, 1990). Bacterial fimbriae are assembled from a subunit precursor termed prepilin that is processed during protein export then stored in the outer membrane (Watts *et al.,* 1982). The fimbrial shaft is assembled from the pilin monomer pools. In some cases, dimers of pilins produce the final fimbria as in *N. gonorrhoeae* (Parge *et al.,* 1990). The final fimbria is the product of the assembly of a single type of pilin even though a bacterial cell may produce more than one type of fimbriae at a given time. Therefore, the processes that lead to the production of intact fimbriae are highly specific (Irvin, 1990).

Mooi and de Graaf (1985) have proposed a model for the export and assembly of the *E. coli* K88ab fimbriae, a serotype of the K88 fimbrial type (Figure 1). This model was based on the analysis of the derivatives of the recombinant plasmid pFM205 containing the six genes responsible for the synthesis, export and assembly of the K88ab (Mooi and de Graaf, 1985). The six genes encode six polypeptides: p17, p17.6, p81, p27, p26 and p27.5. All of these polypeptides have a signal peptide indicating that they are located in the periplasmic space or outer membrane (Mooi *et al.,* 1982, 1984). The major structural component of K88ab is p26. This protein accumulates transiently in the periplasmic space before the final assembly into intact fimbriae. As soon as it emerges from the inner membrane into the periplasmic space, p26 associates with p27 as a result of: 1) the high concentration of p27 in the periplasmic space

Figure1. A model for the transport and assembly of the K88ab fimbria

p26 is transported across the inner membrane (IM) into the periplasmic space (PS). p26 associates with p27 in PS. This association induces conformational change that is require for the transport of p26 across the outer membrane (OM). p17 binds to p26-27 complex inducing further conformational change that facilitates the binding to p81The association of p26-27-17with p81 induces the opening of the p81 channel through whichp26 is exported to the outer surface for the extension of the fimbria. The interaction with p81 brings about the dissociation of p17 and p27 from the complex. p17 and p27 are probably recycled in further transport events (Mooi and de Graaf, 1985).


OM
81
81
PS
27
+
17
17
27
26
26-27
26-27-17
IM
R
mRNA

(After Mooi and de Graaf, 1985)

and 2) the opposite net charges of these two proteins (p26 pI=4.2 and p27 pI≤9.3) (Mooi *et el,* 1983). This association of p26 with p27 induces conformational changes in p26 that are required to facilitate its transport across the outer membrane. Subsequently, p17 becomes associated with p26-p27 complex inducing a slight modification in p26. The association of these three proteins induces the opening of channels formed by the trans-membrane protein p81. The interaction with p81 results in the transfer of p26 across the outer membrane and the dissociation of p17 and p27. These proteins, p17 and p27, are probably recycled again in further transport events. At the cell surface, p26 folds in a manner that enables the binding of other subunits to form the fimbriae. The precise functions of p17.6 and p27.5 remain unresolved (Mooi and de Graaf, 1985). It must be noted that this model is based on gene mutation studies and is highly speculative.

Variability of Fimbrial Subunits

The expression of one type of fimbriae or more than one type simultaneously is a phenomenon known as phase variation. This process is characterized by switching between high and low levels of expression of fimbrial genes (Dorman and Higgins, 1987; Willems *et al.,* 1990). Since there may be more than one genetic copy of fimbrial genes, different serotypes could be produced. Therefore, through phase variation, a heterogeneous population of fimbriae is produced, and the best adapted variant for a certain environment can be selected (Willems *et al.,* 1990). It has been shown by

Silverblatt and Ofek (1979) that rapid switching between high and low levels of fimbriation is advantageous to the uropathogenic bacterium, *Proteus mirabilis*. Fimbriated bacteria are more virulent in the colonization phase of infection, whereas afimbriated cells are more virulent in the invasive phase (Silverblatt and Ofek, 1979).

The evolution of fimbrial phase variation by pathogenic bacteria is probably the result of selective pressure to elude host immune responses yet maintain the structural integrity and functionality of fimbriae (Irvin, 1990). Multiple genomic copies of the Pap fimbrial gene produce immunologically different fimbriae. In *E. coli*, the Pap genes are arranged in clusters that are found at a variety of locations. The different Pap gene clusters produce antigenically distinct fimbriae. Not all loci are expressed at once, and the expression of a certain fimbrial antigen depends on the individual gene cluster (Hull *et al.*, 1986; Rhen *et al.*, 1983). Even though the immunodominant region of the pilin proteins of these bacteria show considerable variation, immunologically conserved regions are retained as a consequence of functionality and/ or assembly of the intact fimbriae (Rothbard *et al.*, 1985). Similarly, *P. aeruginosa* pilin proteins vary considerably in one region of the protein, the immunodominant central region (Sastry *et al.*, 1985). The N-terminal of the protein is responsible for subunit assembly into polymers and therefore is highly conserved (Pasloske and Paranchych, 1988). The C-terminal of the pilin protein is semiconserved and harbours the epithelial cell-binding domain that facilitates the attachment of the bacteria to human buccal cells (Lee *et al.,* 1989).

The variation of the fimbrial structure using genetic manipulation is a strategy adopted by pathogenic bacteria to outcompete the host's immune responses against a significant part of the surface structure. These variations are to be accomodated within the framework of structure and functionality.

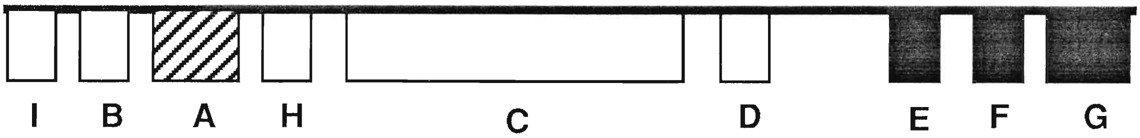
Organization and Expression of Fimbrial Genes

The events that lead to the production of the final intact fimbriae are under genetic regulation (Irvin, 1990). To appreciate the genetic complexity and organization of fimbrial genes, some examples of fimbrial gene systems will be examined in this section. The genes involved in the production of Type 1 fimbriae are mapped at 98 min of the *E. coli* linkage map (Swaney *et al.,* 1977; Freitag and Eisenstein, 1983). Klemm (1984) reported that *fim*A gene is responsible for the production of the structural component of Type 1 fimbriae. It encodes a polypeptide of 159 amino acids preceded by a signal peptide of 23 amino acids (Orndorff and Falkow, 1985). Other genes were found to be involved in the synthesis of Type 1 fimbriae: *fim*B, *fim*C, *fim*D, and *fim*E; their gene products are: (in molecular mass) 23,000, 26,000, 89,000 and 23,000 daltons, respectively (Klemm *et al.,* 1985). Type 1 fimbriae undergo phase variation between fimbriated and afimbriated forms at a rate of 10^{-3} to 10^{-4} per cell per generation (Brinton, 1959; Eisentein, 1981). This switch is regulated at the transcriptional level by an invertible promoter of

314 bp located upstream from *fim*A and is bounded by 9 bp inverted repeats (Abraham *et al.,* 1985). The switch is controlled by different *trans*-acting genes. *fim*B directs the switch in the "on" position, while *hyp*, equivalent to *fim*E, affects the level of transcription of *pil*A *(fim*A) in the "on" mode (Orndoff *et al.,* 1985). Two other genes, which map distally from *fim*A, encode integration host factor (IHF). It appears that IHF acts on the inverted repeats on either side of the invertible promotor mediating intramolecular recombination (Eisenstein *et al.,* 1987; Dorman *et al,* 1987). Thus in Type 1 fimbriae inversion controls phase variation of *fim*A, while other *trans*-acting elements modulate the level of expression (Paranchych and Frost, 1988).

B. pertussis, the causative agent of whooping cough, produces two serologically distinct fimbriae. Three genes have been identified: *fim*2, *fim*3, and *fim*X that code for serotype 2, serotype 3, and an unidentified product, respectively (Mooi *et al.,* 1987). These genes are regulated positively by the *bvg* locus which encodes three polypeptides involved in sensory transduction. Environmental factors such as temperature and concentrations of $MgSO_4$ affect the expression of virulence genes including the *fim* genes by acting on the *bvg* locus (Idigbe *et al.,* 1981; Arico *et al.,* 1989). A strain of *B. pertussis* can produce one type of fimbria, both types or none at all. This phase variation is controlled by insertion or deletion events within the promotor of the *fim* genes (Willems *et al.,* 1990). The *fim* promotor is characterized by the presence of a cytosine stretch (approximately 13-15 bp). Analysis of *fim*3 revealed that insertions and deletions within this stretch are responsible for the phase variation observed in *B. pertussis* (Willems *et al.,* 1990). Reiterated bases are hot spots for mutations due to transient misalignment during replication (Streisinger and Owen, 1985). The insertions and deletions affect phase variation, presumably, by affecting the distance between an activator binding protein site and a DNA sequence that contains TA sequences, termed the putative -10 box. The distance between these two sequences is probably important for the correct positioning of RNA polymerase (Willems *et al.,* 1990).

Uropathogenic *E. coli* strains usually produce Pap fimbriae (pyelonephritis associated pili) along with other virulence factors (Paranchych and Frost, 1988). The genes that encode Pap fimbriae are arranged in clusters, and more than one cluster can occur on each chromosome, even though not all loci are expressed at a given time (Hull *et al.,* 1986). Nine genes are found to be involved in fimbriae synthesis, expression of the adhesin and regulatory elements; these genes are: papIBAHCDEFG (Lindberg *et al.,* 1987):



PapA gene specifies the structural fimbrial subunit termed fimbrillin (16.5 kDa) (Baga *et al.,* 1984). The transcription of a papA is controlled positively by papB and papI. Transcription of papB proceeds through papA. The final transcript of papA is a 800 bp mRNA that is probably the processed papBA transcript (Baga *et al.,* 1985). PapC encodes a protein that forms the base of the fimbria

(Baga *et al.,* 1987). The other genes of the cluster, papEFG, are located on a separate operon. These genes are required for the production of the adhesin (Lindberg *et al.,* 1986). PapH seems to regulate the length of the fimbriae, since papH$^-$ mutants have longer fimbriae (Baga *et al.,* 1987). PapD gene encodes a periplasmic protein that is involved in the transport of the rest of the fimbrial-gene cluster products by forming transient complexes. The differences between pap gene clusters occur in papA and papG (the adhesin) which define the antigenic properties of the fimbriae (Lund *et al.,* 1985).

The synthesis, expression, and assembly of fimbriae is a highly specific, tightly regulated process. Therefore, the organization and expression of fimbrial genes are complex. Environmental factors play a role in the production of fimbriae by acting on elements controlling fimbrial gene transcription (e.g. the *bvg* locus in *B. pertussis).* Phase variation of fimbriae is controlled at the level of transcription and is modulated at the level of expression (Paranchych and Frost, 1988).

Fungal Fimbriae

The presence of fibrils on the surfaces of fungi was reported for several species: *Candida albicans* (Robin) Berkhout (Djaczenko and Cassone, 1971; Douglas *et al,* 1981), *Cryptococcus laurentii* (Kuff.) Skinner and *Rhodotorula glutinus* (Fres.) Harrison (Ruinen *et al,* 1968), *Psathyrrella coprophila* (Jurand and Kemp, 1972), and *Schizosaccharomyces pombe* Lindner (Calleja *et al,* 1977). These reports however did not provide descriptions of fimbriae and the correlation with bacterial fimbriae was only suggested in the case of *P. coprophila.* Thus, fibrils termed fungal fimbriae were first described by Poon and Day (1974) on the anther smut fungus, *Ustilago violacea* Fuckel. Since their discovery, fimbriae were found to be widespread among fungi and do not appear to be restricted to a certain taxonomic group (Gardiner *et al.,* 1981, 1982; Gardiner and Day, 1988).

Structure and Distribution of Fungal Fimbriae

Fimbriae appear as hair-like structures that emanate from the cell wall in individual fibers or multistranded cables. It was observed that on the surface of *U. violacea,* fimbriae sometimes appear as multistranded cables (Gardiner and Day, 1988). However, it is not known whether they exist normally in bundles or as individual fibres. The fimbriae of *U. violacea* appear to be helical assemblies as observed in negatively stained electron micrographs. In *U. violacea*, fimbriae have lengths of up to 20µm and a uniform diameter of 7 nm (Poon and Day, 1974; Gardiner and Day, 1988). High resolution studies on fungal fimbriae have not been reported yet, and therefore the presence of an axial channel and the fine structure of fimbriae remain unknown.

Fimbriae of *U. violacea* are distributed all over the cell wall, and they range from 0-200 fimbriae per cell. Log phase cells exhibit higher numbers of fimbriae than stationary phase cells which at times lack fimbriae (Poon and Day, 1974). Fimbriae are present on the surface of conjugating cells and their budding daughter cells, on the promycelium and on infection hyphae, but they are not present on the thick-walled teliospores (Poon and Day, 1974).

A survey of other taxonomic groups showed that fimbriae are extremely widespread in fungi (Gardiner and Day, 1988; Gardiner *et al.,* 1981, 1982). All isolates of *U. violacea* and 27 other species of the Ustilaginaceae were shown to have fimbriae resembling those of *U. violacea* (Gardiner *et al.,* 1981). Fungi from the Tilletiaceae species, closely related to Ustilaginaceae, that were examined possessed fimbriae with the exception of *Tilletia caries* which lacked fimbriae under all examination conditions (Gardiner *et al.,* 1981). In the heterobasidiomycetous yeasts, 26 out of 37 species that were tested had fimbriae. While fimbriae of basidiomyceteous yeasts are typically long (<20μm), most of the ascomycetous species tested, such as *Neurospora, Botrytis* and *Saccharomyces,* possess short fimbriae (0.5-1μm) that appear as fringes (Day *et al.,* 1975; Gardiner *et al.,* 1982). Fimbriae of up to 10 μm have been observed in deuteromycetes such as *Fusarium graminearum* and *Aspergillus niger*. The zygomyceteous fungi, *Mucor rouxii, Rhizopus stolonifer* and *Phycomyces blakesleeanus,* were all observed to have fimbriae. In the case of the latter species, fimbriae of up to 10 μm were reported (Gardiner and Day, 1988).

Environmental Stability

Fimbriae of *U. violacea* are helical assemblies of uniform diameter of 7 nm that reach lengths of up to 20μm (Gardiner *et al.,* 1981). Production of fimbriae is temperature dependent; fimbriae are produced at a temperature range of 10°C to 25°C but are absent from 25°C to 30°C ; even though cells continued to grow up to 30°C. In contrast, *U. maydis* and *U. bullata* cells are fimbriated better at 30°C than 22°C and *U. nigra* produced fimbriae only at 15°C (Day and Poon, 1975; Gardiner *et al.,* 1981). Therefore, the production of fimbriae is dependent on suitable temperature conditions.

Studies on the effects of monovalent and divalent cations showed that fimbriae were stable in concentrations of up to 10^{-1}M of lithium, sodium, potassium and ammonium salts. Similarly, fimbriae were found to be stable in concentrations of up to 5×10^{-2} M of divalent cations, such as calcium, magnesium, manganese and iron (Gardiner and Day, 1985).

Additionally, *U. violacea* cells retained visible fimbriae over a wide pH range of 3 to 9. These results show that fimbriae are very stable structures that can resist temperature, pH and ionic strength fluctuations (Gardiner and Day, 1985).

The chelators, EDTA and EGTA, at concentrations exceeding 5×10^{-2}M and 10^{-4} M, respectively, resulted in loss of fimbriae. This observation suggests that calcium plays an important role in the structure of fimbriae since EGTA chelates calcium several orders of magnitude more efficiently than it does magnesium. EDTA chelates them equally (Gardiner and Day, 1985).

Gardiner and Day (1985) also reported the effect of various chemical treatments on fimbriation of *U. violacea* sporidial cells. Fimbriae can resist various chemical treatments and were shown to be present under conditions such as 0.4M periodic acid, 10% formaldehyde, 30% hydrogen peroxide, 60% acetone, xylene and chloroform exposure. Although these treatments showed that fimbriae are stable structures, other chemical treatments resulted in the loss of fimbriation. Fimbriae were absent after treatment with 1M sodium chloride, 1% sodium dodecyl sulfate, 0.1N sodium hydroxide, 0.1N hydrochloric acid and 80-100% acetone (Gardiner and Day, 1985). From this study, it is clear, however, that fimbriae are very stable appendages and can withstand numerous harsh chemical treatments.

Subunit Structure

For experimental procedures, fimbriae can be isolated mechanically by: sonication, high speed agitation or sucrose gradient centrifugation which work best for unicellular yeast-like cells, or can be isolated thermally (15 min at > 45°C) which works well with filamentous fungi (Poon and Day, 1975; Gardiner and Day, 1985). Defimbriated *U. violacea* cells can regenerate fimbriae at a rate of 1-3μm/h in optimal growth conditions. Fimbrial regeneration does not proceed in the absence of continuous protein synthesis as it is inhibited by cycloheximide but not chloramphenicol (Poon and Day, 1975).

Enzymatic and inhibitor studies indicate that fimbriae are proteinaceous as they are digested by proteases such as papain, chymotrypsin or pronase but not by DNase, RNase, cellulase, α-amylase, chitinase, lysozyme, zymolase, β-glucurosidase, or lipase (Poon and Day, 1974; Day and Poon, 1975; Gardiner and Day, 1988).

The fimbrial subunits of *U. violacea, U. hordei, U. nigra, U. cynodontis,* and *Rhodotorula rubra* have molecular mass of 74 kDa (Gardiner *et al.,* 1979) and are substantially greater than the molecular masses of bacterial fimbrial subunits such as type 1 (17 kDa), K88ab (26 kDa) and Pap (16.5 kDa). However, size variation does exist as in the case of *Coprinus cinereus* and *Schizophyllum commune* whose fimbrial proteins are 37 and 51 kDa, respectively (Boulianne *et al.,* manuscript in preparation).

The fimbrial subunits of *U. violacea* are glycoproteins as indicated by periodic acid Schiff staining. Further, the 74 kDa fimbrial protein has at least six isoforms as shown on isoelectric focusing gels (Castle *et al.* submitted for publication). These isoforms of the protein may be the result of post-translational modifications such as phosphorylation, methylation, or glycosylation. Day and Cummins (1981) indicated that one of these isoproteins is phosphorylated. However, it cannot be ruled out conclusively that these isoforms are encoded by different genes.

Serological Studies

Polyclonal antisera raised against the fimbrial proteins of *U. violacea* (AU) and *R. rubra* (AR) proved useful in screening numerous species for fimbrial antigens (Gardiner *et al.,* 1981, 1982; Gardiner and Day, 1988). Using agglutination tests and immunofluorescence techniques, it has been shown that these antigens are widespread among fungi. (Gardiner *et al.,* 1981, 1982; Gardiner and Day, 1988; Gardiner, 1985).

Agglutination tests revealed that species of Ustilaginaceae agglutinated rapidly on treatment with AU but showed no response or agglutinated slowly with AR (Gardiner *et al.,* 1981, 1982). However, among other basidiomycetous yeasts, only 24% of species tested responded to AU; whereas 42% responded to AR. Most species of ascomycetous yeasts tested responded strongly to one or both antisera. Interestingly, ascomyceteous species that were examined produce short fimbriae (0.5-1μm) with the exception of *Hansenula wingei* and *Metschnikowia pulcherrima* which produce relatively long fimbriae, up to 4μm and 10μm, respectively. These two species did not agglutinate with either antisera (Gardiner *et al.,* 1982).

Filamentous fungi from all major divisions tested positive in immunofluorescence tests using AU (Gardiner and Day, 1988). Therefore, fungi share common antigens that are assembled into fibrils. These antigens appear to be highly conserved among fungi.

Based on observations from agglutination tests, electron microscopy and immunofluorescence, fungi tested with both antisera can be divided into five distinct groups:

Type 1) afimbriated; negative response with either antisera
Type 2) fimbriated; negative response with either antisera
Type 3) fimbriated; positive response to AU only
Type 4) fimbriated; positive response to AR only
Type 5) fimbriated; positive response to both antisera

It is of interest to note that AU did not agglutinate *R. rubra;* inversely, AR did not agglutinate *U. violacea* (Gardiner *et al.,* 1982). This observation suggests that there are at least two distinct antigens on the surface of fungi as seen in *U. violacea* and *R. rubra.* Using immunocyclochemical localization of antigen-binding sites with protein A-gold labeled antisera, Benhamou and coworkers (1986) showed that AU and AR bind to different locations of *Ascocalyx abietina* cells. AU cross-reacted with the fibrillar sheath surrounding the cells; whereas AR heavily labeled the cell wall and plasma membrane of these cells. It is apparent that *A. abietina* expresses both antigen types, whereas other fungi have only one of the antigens or neither antigen.

In order to study antigenic variation of fimbrial proteins, a wide variety of antibodies need to be employed especially monoclonal antibodies against various epitopes. This would give insight on the different regions of the subunit (i.e. conserved and variable regions) and the association of other fimbrial components such as adhesins as in the case of Pap fimbriae of *E. coli* that has an antigenically distinct low copy number adhesin (Lindberg *et al.*, 1987). Furthermore, species that responded negatively in agglutination or immunofluorescence tests may indeed have fimbriae that are antigenically different from either *R. rubra* or *U. violacea*. Since the fimbrial mutants of *U. violacea* obtained all revert back to fimbriated phase after a few subcultures and efforts to obtain stable mutants have not been successful (Gardiner, 1985), loss of fimbriation in fungi might be the result of phase variation as is observed in bacteria. Phase variation in bacteria is controlled at the gene level by mechanisms such as inversion or deletion and insertional events (Abraham *et al.*, 1985; Willems *et al.*, 1990). It is possible that such mechanisms are operative in fungi.

Function

The similarities between fungal fimbriae and those of bacteria suggest similar functions. The roles of bacterial fimbriae are mentioned earlier. Indeed fungal fimbriae might be multifunctional and adapted to the variation of environmental conditions and therefore serve different functions in different species.

Role of Fimbriae in Conjugation in *U. violacea*

Indirect evidence for the role of fimbriae in conjugation was derived from studies on *U. violacea* (Poon and Day, 1974). There is a strong correlation between the presence of fimbriae and the ability to complete conjugation. Conjugation proceeds at a temperature range of 2°C to 25°C, with an optimal range from 10°C to 22°C. Conjugation does not occur from temperatures between 26°C to 30°C even though cells continue to grow in this range. The ability to conjugate is correlated to the ability to regenerate fimbriae. *U. violacea* is able to regenerate fimbriae at a rate of 1-3µm per hour after mechanical defimbriation (Gardiner, 1985). This regeneration is temperature dependent. While fimbriae regenerate between temperatures of 5°C to 24°C, they do not regenerate at 26°C to 28°C (Day and Poon, 1975; Gardiner *et al.* 1981). Thus, the lack of fimbriae and the inability to complete conjugation at temperatures of 26°C to 28°C suggest that fimbriae play a role in conjugation. Furthermore, cells of compatible mating type in 'shift-down' temperature experiments (Day and Poon, 1974) were unable to conjugate at 27°C; however, when they were placed in 20°C subsequent to 27°C, conjugation proceeded normally. Defimbriated cells, treated as above (27°C then 20°C) in the presence of cycloheximide were unable to complete conjugation. Further, in the presence of pronase, which completely digests fimbriae, cells were unable to complete conjugation (Day and Poon, 1974).

Mating at distances of up to 5µm in water suspension have been reported (Day, 1976). During distant mating, the conjugation tube may not grow directly but could curve or turn in a sharp angle and may by-pass a compatible mating type. This led to the suggestion that the conjugation tube grows along the fimbrial connection made between two cells rather than a chemical diffusion gradient (Day, 1976). It has been postulated that fimbriae may provide chemical communication between mating cells in which inducer molecules transmitted along the fimbriae initiate the conjugation tube development by local activation of a wall-softening enzyme (Day, 1976).

Role of Fimbriae in Yeast Flocculation

The role of fimbriae in flocculation is also based upon correlations. A study by Day *et al* (1974) reported that flocculant strains of *Saccharomyces cerevisiae* and *S. carlsbergensis* were densely fimbriated. These fimbriae were short (0.5μm) with diameters of 5-7 nm. They also reported that flocculant strains were non-flocculant and afimbriated at log phase, but they gained the ability to flocculate and became densely fimbriated as they entered the stationary phase of the growth. Furthermore, pronase treatment, which completely digested fimbriae, destroyed the ability to flocculate. These correlations suggest that fimbriae may play an essential role in flocculation, however no mechanism for this role was proposed.

Role of Fimbriae in Adhesion and Pathogenesis

Evidence for the role of fungal fimbriae in adhesion comes primarily from studies with the deuteromycetous yeast *Candida albicans* (Critchley and Douglas, 1987; Houston and Douglas, 1989; Douglas, 1987). *C. albicans* adheres to human buccal, vaginal, uro-epithelial cells, corneocytes and mucosal surfaces (Douglas, 1987). This fungus produces a fibrillar matrix termed the extracellular polymeric material (EP) which appears to contain the adhesin (McCourtie and Douglas, 1985). These fibrils are likely to be fimbriae, however conclusive evidence have not yet been demonstrated. In media containing high concentrations of galactose, the production of fibrils is stimulated and this presumably enhances the adhesion of some *C. albicans* strains (McCourtie and Douglas, 1981, 1984). Critchley and Douglas (1987) reported that a mannoprotein contained in the EP is responsible for the attachment to buccal epithelial cells. Furthermore, they showed that the protein portion of the mannoprotein not the carbohydrate moiety is responsible for the adhesion since heat, dithiothreitol, or proteolytic enzymes inhibited the adhesion but not sodium periodate or α-mannosidase. Interestingly, the fibrillar layer was reported to increase virulence of *C. albicans* by enhancing resistance to intracellular killing after phagocytosis by neutrophils (Houston and Douglas, 1989).

C. albicans adhesion to host cells is most likely to be mediated by carbohydrate - lectin interactions as is the case in bacteria-host interactions (Ofek and Perry, 1985). The nature of the epithelial cell receptors for *C. albicans* was reported by Crichley and Douglas (1987) as glycosides, either glycoprotein or glycolipid. They showed that while adhesion of *C. albicans* strains GDH 2346, MRL 3153, GRI 681 or GRI 682 was inhibited by L-fucose, adhesion of strain GDH 2023 was inhibited by N-acetyl-D-glucosamine. They also showed that isolated EP from the strain GDH 2023 did not inhibit adhesion of the other strains. Furthermore, whereas the isolated EP of the GDH 2346 strain inhibited adhesion of strains MRL 3153, GRI 681 and GRI 682 by more than 50%, it inhibited adhesin of strain GDH 2023 by only 30%. It was suggested therefore that there are different types of adhesins in these strains mediating carbohydrate-lectin interactions. The protein portion of the mannoprotein is responsible for the adhesion to epithelial cell glycosides containing either L-fucose or *N*-acetyl-D-glucosamine (Critchley and Douglas, 1987).

Further support for notion that fungal fimbriae play a role in adhesion and pathogenesis comes from studies of immunocytochemical localization of fimbrial antigens on plant host surfaces using protein A-gold labelling (Svircev *et al.*, 1986). Antiserum raised against surface components of *Botrytis cinerea* and antiserum raised against fimbriae of *U. violacea* were used to screen for the presence of antigens in infected leaves of *Vicia faba.* Heavy gold labeling was detected on the surfaces and inside the plant cells of infected leaves but not on uninfected tissue. The presence of fimbrial antigens inside host cells suggests that fimbriae penetrate host cells and establishes contact between the host and pathogen. However it cannot be ruled out that these antigens are of dissociated fimbrial subunits, or they might be plant products antigenically similar to the fimbrial antigen produced in response to infection.

Similar studies were carried out on leaves of *Nicotiana tabacum* L. infected with *Peronospora hyoscyami* f.sp. *tabacina* and on leaves of *Erythronium americanum* Ker. infected with *Ustilago heufleri* (Day *et al.*, 1986). Results were similar to the previous study. Both findings suggest a possible role fungal fimbriae may play in host-pathogen interactions. Significance of this role has yet to be determined.

The role of fimbriae in mycoparasitism is the interest of this study. The term mycoparasitism refers to one fungus parasitic on another fungus. Although there are different types of mycoparasites, consideration will only be given to the biotrophic, haustorial mycoparasite. An example of this parasite is the zygomycete *Piptocephalis virginiana* with a host range is restricted to members of Mucorales. Not all members of the Mucorales are equally susceptible. While some species are susceptible hosts e.g. *Mortierella pusilla*, one species is completely resistant (*Phascolomyces articulosus*). *Mortierella candelabrum* is non-host and the mycoparasite does not seem to recognize it at all (Manocha, 1988).

The first step in the interaction between a mycoparasite and potential host is directed growth of the mycoparasite germ tube towards the host hypha. This directed growth is likely to be facilitated by physical or diffusible chemical gradient (Jeffries, 1985; Evans and cooke, 1982). The result of the directed growth is physical contact with the host hypha. At the site of contact, the germ tube of the mycoparasite attaches to the hyphal wall and develops an appressorium. From the appressorium a penetration peg is formed to attempt host penetration. In the susceptible host, a haustorium is formed after successful penetration. Through the haustorium the mycoparasite draws nutrition from the host cytoplasm. In the case of the resistant host, penetration is impeded by the thickening of the host cell wall. The thickening of the cell wall is apparently a defence mechanism mounted by the host in response to the attempted penetration. In some instances, the penetration of the mycoparasite is successful. This results in the formation of a haustorium. The resistant host responds by forming a thick sheath around the haustorium preventing the mycoparasite from establishing a nutritional relationship with the host (Manocha, 1988).

The mycoparasite does not appear to recognize the non-host even if the cell walls are in contact. Recognition is the initial event that determines the fate of parasitism. Positive recogition leads to subsequent parasitic events. Negative recognition means no parasitism. Recognition has been defined as an early specific event that triggers a rapid, overt response by the host that either facilitates or impedes the growth of the pathogen (Sequeira, 1978). Therefore for any parasitic event, there must be basic recognition between the host and mycoparasite. This recognition is facilitated by the interactions of complementary macromolecules present on the surfaces of the host and the pathogen.

Fimbriae of some species extend quite a distance away from the cell wall. these fibrils, therefore, may provide the recognition stimulus or receptor which leads to directed growth of the parasite towards the host. The focus of this study was (a) to investigate the presence of fimbriae in a mycoparasitic system that includes four zygomycetes: *P. virginiana, M. pusilla, P. articulosis,* and *M. cadelabrum,* (b) to partially characterize the physical dimentions of fimbriae and the chemical nature of the fimbrial subunits, and (c) to ascertain the role of fimbriae in the contact and attachment of the parasite to the host.

Materials and Methods

Stock Cultures and Growth Conditions

Cultures of *Mortierella candelabrum* V. Teigh and Le Monn, *Mortierella pusilla* Oudemen, and *Phascolomyces articulosus* Boedijn ex Benny and Benjamin were maintained at 22°C±1°C on media consisting of the following media (Manocha *et al.,* 1986):

Malt extract	20g
Yeast extract	2g
Agar	20g
Distilled water	1L

Cultures of *Piptocephalis virginiana* Leadbeater and Mercer were maintained by growing *P. virginiana* with its host *Choanephora cucurbitarum* (Berk and Rav.) Thaxter on media consisting of the following (Phipps and Barnett, 1975):

Malt extract	20g
Yeast extract	2g
Agar	20g
Thiamine	100µg
Biotin	5µg
$FeCl_2.4H_2O$	0.2mg
$ZnSO_4.7H_20$	0.4mg
$MnCl_2.4H_2O$	0.2mg
Distilled water	1L

Conidial suspensions of *P. virginiana* and *C. cucurbitarum* were grown at 22°C ± 1°C in total darkness to inhibit the sporulation of *C. cucurbitarum* while *P. virginiana* sporulated normally (Barnett and Lilly, 1955; Berry and Barnett, 1957).

P. virginiana germ tubes without the host were germinated in a medium consisting of the following (Balasubramanian and Manocha, 1986):

Malt extract	20g
Yeast extract	2g
Glycerol	10ml
Distilled water	1L
(pH6.8)	

Protein Isolation

M. candelabrum, M. pusilla and *P. articulosus* were grown in liquid medium (2% malt extract (W/V), 0.2% yeast extract (W/V)) for 1-3 days. *P. virginiana* was grown in liquid medium and harvested after 19-72 h. Cultures were filtered through cheese cloth and the mycelia were rinsed repeatedly with distilled water. Mycelia were then dipped in liquid nitrogen for five minutes to induce cell breakage. Proteins were isolated by grinding the frozen mycelia in mortar and pestel with two parts silica gel and one part cold TEPI (10mM Tris, 1mM ethylenediaminetetra-acetic acid (EDTA), 1μM phenylmethylsulfonyl fluoride, and 1mM iodoacetamide; pH 6.8). The slurry was centrifuged at 10,000xg for 10 min at 4°C. The supernatant was shaken vigorously with two parts cold n-butanol to remove lipids associated with the protein isolate. The mixture was then centrifuged at 1000xg for 10 min at 4°C; the bottom aqueous layer was collected carefully. Samples were placed in dialysis tubing and dialyzed against several changes of TE buffer (10mM Tris and 1mM EDTA; pH 7.5) for 2 h at 4°C. The samples were lyophilized and resuspended in minimal volumes of TEPI buffer. Protein concentrations of the samples were then determined using the Bio-Rad protein assay based on the Bradford (1976) assay.

Gel Electrophoresis

Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Discontinuous polyacrylamide gels consisting of 4% stacking and 11% separating gels were used (Laemlli, 1970) . The separating gel (0.37M Tris (pH 8.8) , 10.1% acrylamide, 0.99% N,N'-methylenebisacrylamide (BIS), 0.1% sodium dodecyl sulphate (SDS), 0.05% N,N, N', N'-tetramethylenediamine (TEMED), and 0.05% ammonium persulphate (APS)) was cast into a mini slab gel apparatus assembled with 0.75mm thick spacers. The separating gel was overlaid with water-saturated n-butanol. After the separating gel was polymerized, the n-butanol was washed off with distilled water. The stacking gel (0.125M Tris (pH 6.8), 3.9% acrylamide, 0.347% Bis, 0.1% SDS, 0.1% TEMED, and 0.05% APS) was then cast, and 10 well combs were used to produce the sample wells.

Protein samples were heated for 4 minutes at 95°C in SDS reducing buffer (0.05M Tris (pH6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.001% bromophenol blue). Samples were loaded into the wells with a Hamilton syringe. Electrophoresis was carried out buffer conditions of 0.007M Tris, 1.44% glycine and 0.1% SDS (pH8.3) at 200 V (constant voltage) until the tracking dye (bromophenol blue) reached the bottom edge of the separating gel.

Gel Staining Procedures

Coomassie Blue

Protein bands on the acrylamide gels were visualized using 0.1% Coomassie brilliant blue R-250, 40% methanol, and 10% acetic acid. Gels were left to stain for 1-2 hours. The excess stain was then removed by placing the gels in a destaining solution (40% methanol and 10% acetic acid).

Periodic acid-Schiff staining

Glycoproteins separated on polyacrylamide gels were visualized with the Segrest and Jackson (1972) method. Gels were fixed overnight in 40% ethanol and 5% glacial acetic acid. They were then treated with 0.7% periodic acid and 5% acetic acid for 2 h, followed by a treatment of 0.2% sodium metabisulfite and 5% acetic acid for 2 hours with one change of solution in the first half hour. Gels were then placed in a solution of Schiff reagent for 12-18 h. Schiff reagent was prepared by (0.5% basic fuchsin, 10% 1N HCl, 0.85% sodium metabisulfite and treated with HCl washed charcoal). The gels were subsequently treated with 0.2% sodium bisulfite, 40% ethanol and 5% acetic acid for 90 minutes at 55° C for an improved sensitivity of glycoprotein detection (Konat et al., 1984), followed by several changes of a destaining solution (40% ethanol and 5% acetic acid) until the excess background stain was removed.

Immunoblotting

Proteins on acrylamide gels were transferred to nitrocellulose membranes (BioRad) in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol (v/v), pH 8.3) (Towbin et al., 1979) in a Transblot cell (BioRad). An overnight transfer of proteins was carried out at room temperature at 30 V (constant voltage) with one change of voltage to 60 V in the last hour. Nitrocellulose membranes were washed with Tris Buffered Saline (TBS) (20 mM Tris and 500 mM sodium chloride (pH 7.5) for 10 minutes. The membranes were then placed in a blocking solution (3% gelatin in TBS) for at least 3 hours for subsequent immunoblotting. Membranes were washed in 2 changes of Tween-TBS (TTBS) (20mM Tris, 500 mM sodium chloride and 0.05% Tween-20) for 5 min each wash. The immunoblotting was carried out in an indirect immunoblotting system in which primary (1°) and secondary (2°) antibodies were employed for detection. The membranes were incubated with the 1°antibody buffer (anti-*Ustilago violacea* fimbriae antibody (AU) and 1% gelatin in TTBS) for 2 hours or in rabbit pre-immune serum (NS) buffer and 1% gelatin in TTBS for 2 h with gentle agitation. The membranes were then washed in TTBS for 2 h (4 X 30 min) followed by an incubation with the 2° antibody (goat-anti-rabit IgG (Fc) with an alkaline phosphatase conjugate (promega)) buffer (1/15000 2°antibody and 1% gelatin in TTBS) for 2 h. Membranes were then washed for 5 min in TTBS followed by three 10 min washes in TBS. Protein bands with bound antibodies were visualized with a color development buffer (0.1 M Tris (pH9.5) , 1mM $MgS0_4$, 0.03% nitroblue tetrazolium and 0.015% bicloro-indolyl-phosphate). The membranes were left in this buffer until a sufficient color had developed and were then washed with distilled water and stored dry.

Electroelution of Proteins

Protein samples were separated by SDS-PAGE followed by a quick 10 min Coomassie Brilliant Blue R-250 staining. The gels were then destained until protein bands were apparent. Target bands were excised for subsequent electroelution according to Hanaoka et al. (1979). Glass tubes of 105 mm in length and 9 mm diameter were constructed. A notch 30 mm from bottom was indented to keep the gel from sliding. Parafilm were placed on the bottom, and 50% sucrose was pipetted into each tube. The gel solution (6% acrylamide, 0.16% BIS, 6M urea, 0.03% APS, 0.1% TEMED, 1mM dithiothreitol (DTT) and 40 mM glycine; pH 10.4) was pipetted on top of the 50% sucrose solution. The gels were allowed to polymerize for two hours. The parafilm and the sucrose solution were then removed. Electrophoresis buffer (40mM glycine and 1mM DTT; pH 10.4) was pipetted to replace the sucrose solution and act as a reservoir for the eluted protein. Dialysis membranes were affixed onto the bottom of each tube. A vertical disc gel apparatus was then assembled with the polymerized gels. These gels were pre-run at 2 mA/tube for 1 hour. Protein bands that had been previously cut from the poyacrylamide gel were placed inside the tubes and the gels were run again at 2mA/tube overnight at 4°C. After electrophoresis, the buffer trapped between the gel and the dialysis membrane was collected and dialyzed against several changes of TE buffer (10 mM Tris and 1mM EDTA). Dialyzed protein samples were lyophilized and resuspended in a minimal volume of distilled water.

Electron Microscopy

Grid Preparation

Microscope slides cleaned with 70% ethanol were dipped half way in a solution of 0.25% formvar in ethylene dichloride. The slides were then allowed to air dry. The edges and the bottom ends of the dried formvar were cut with a razor blade. The slides were then dipped in a container of distilled water producing formvar films floating on the surface of the water. Electron microscope specimen supports (Gilder G300 or G400 copper grids) were dropped on the surface of the formvar film. Sheets of stiff file card paper were used to recover the grid formvar films trapping the grids between the paper and formvar film. These were allowed to air dry and were then carbon reinforced in a vacuum evaporator (Varian).

Shadow Casting

M. candelabrum, M. pusilla and *P. articulosus* mycelia (24-72 h) were harvested by centrifugation in a clinical centrifuge at setting 7 for 5 min. They were resuspended in 15% acetone and were mixed vigorously. They were left in the solution for 10 min prior to centrifugation in clinical centrifuge at setting 7 for 10 min. The samples were washed twice with distilled water. The samples were then left the stand in distilled water for 2 hours. A drop of sample was placed on each formvar coated and carbon reinforced grid. Samples were left to settle on the grids for 5 min. Excess fluid was removed with filter paper. The grids were then placed on a grid holder which was in turn placed in a vacuum evaporator (Varian).

Gold palladium oxide was evaporated onto the grids at an angle of 19-21°. These samples were then viewed with a Philips 300 electron microscope.

Negative Staining

Acetone treated mycelia of *M. candelabrum, M. pusilla, P. articulosis*, electroeluted proteins from these three species and the germ tubes of *P. virginiana* germinated for 19-72 h were each mounted on a separate carbon reinforced, formvar coated copper grid. The grids were dried with filter paper. A drop of either 1% uranyl acetate or 3% ammonium molybdate was placed on the sample grid for 5-10 min. The excess stain was removed with filter paper. Air dried grids were viewed with a Philips 300 transmission electron microscope.

Incubation of parasite/host and non-host with antisera

Conidial spores of *M. candelabrum, M. pusilla, P. articulosis,* and *P. virginiana* were obtained from culture plates by addition of sterile distilled water followed by gentle scraping of the cultures and finally filtered through cheese cloth. Both the mycoparasite and one of each host were mixed in a total volume of 1 mL at a spore concentration of 10^7 spores mL^{-1} for *P. virginiana* and 10^4 spores mL^{-1} for each of host. AU was added at the following concentrations: 0.045, 0.087 and 0.125 μg protein/μL to each of the mycoparasite-host mixture. In control experiments, preimmune serum was added in the same concentrations. The mycoparasite-host-antiserum mixtures were mixed well and left to stand for 1 h at room temperature. Then one drop of each were spread over semi-solid MY medium overlaid with dialysis membrane. The plates were incubated for 19-24 h at 22±1°C. The dialysis membrane with the germinated spores was placed on a microscope slide and were stained with cotton blue (1% methylene blue in lactophenol) before examination. Using light microscopy (Leitz, Diaplan) at least 300 spores of the mycoparasite were counted in their relation to host or non-host hyphae: contact, non-contact, and appressorium formation. Germinated spores of *P.virginiana* observed to have no apparent physical contact with host hyphae were scored as non-contact events. Germ tubes of *P. virginiana* in contact along any part of the host hyphae were placed in the contact category. Germ tubes which showed contact and appressorium formation were considered separately as attachments. In control experiments, a 2:1 ratio of fimbrial protein from *U. violacea* and AU antiserum was incubated for 1 h at room temperature then overnight at 4°C. This pre-absorbed antiserum was then used in attachment experiments as outlined above.

Results

Electron microscopy on whole hyphal mounts

Fimbriae were observed on the surfaces of the hyphae of *M. candelabrum, M. pusilla, and P. articulosus* (Fig. 2-7). They were distributed non-uniformly along the cell wall and did not seem to form cables. For all three species, fimbriae were estimated to be up to 25 µm in length as determined from electron micrographs. Diameters of the fimbriae did not vary significantly between the three species. *M. candelabrum* fimbriae had a mean diameter of 9.1±0.4 nm, *M. pusilla* fimbriae were 9.4±0.5 nm and *P. articulosus* fimbriae were 8.6±0.6 nm. These measurements were determined from electron micrograph negatives of the negatively stained samples. Fimbriae have not yet been observed on *P. virginiana* asexual spores or on 19-72 h germ tubes grown in liquid or on semi-solid medium. It should be noted that germ tubes of *P. virginiana* are capable of contacting and forming appressoria on host species on semi-solid medium.

Immunoblot analysis

Proteins isolated from *M. candelabrum, M. pusilla, P. articulosus,* and *P. virginiana* were separated by SDS-PAGE (Fig. 8) and were transferred to nitrocellulose membranes for immunoblot identification of fimbrial protein. Polyclonal antiserum raised

Figure 2. Shadow cast preparation of *M. candelabrum* hyphae

An electron micrograph of *M. candelabrum* hyphae shadow casted with gold palladium oxide at an angle of 20°. Magnification, 100,000X.

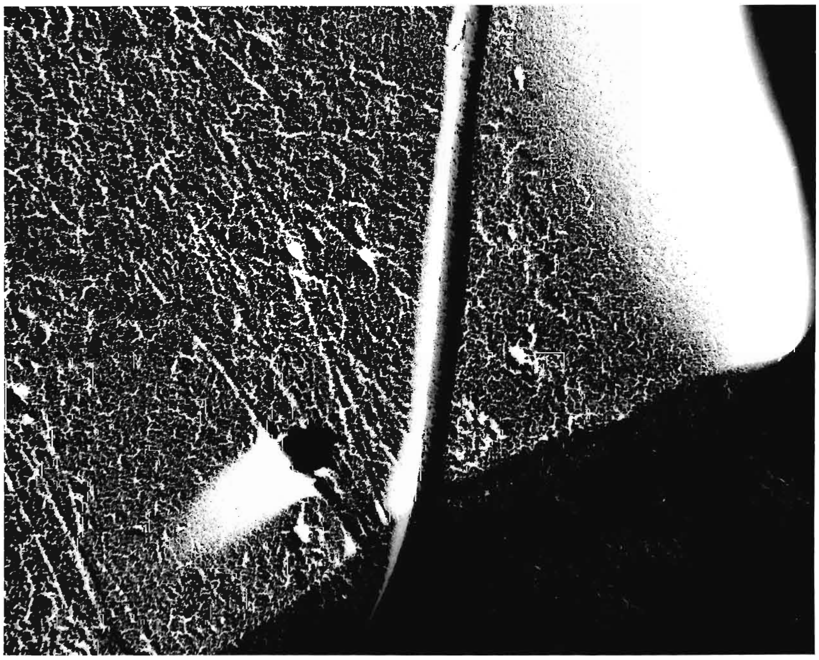


Figure 3. Shadow cast preparation of *M. pusilla* hyphae

An electron micrograph of *M. pusilla* hyphae shadow casted with gold palladium oxide at an angle of 20°. Magnification, 100,000X.

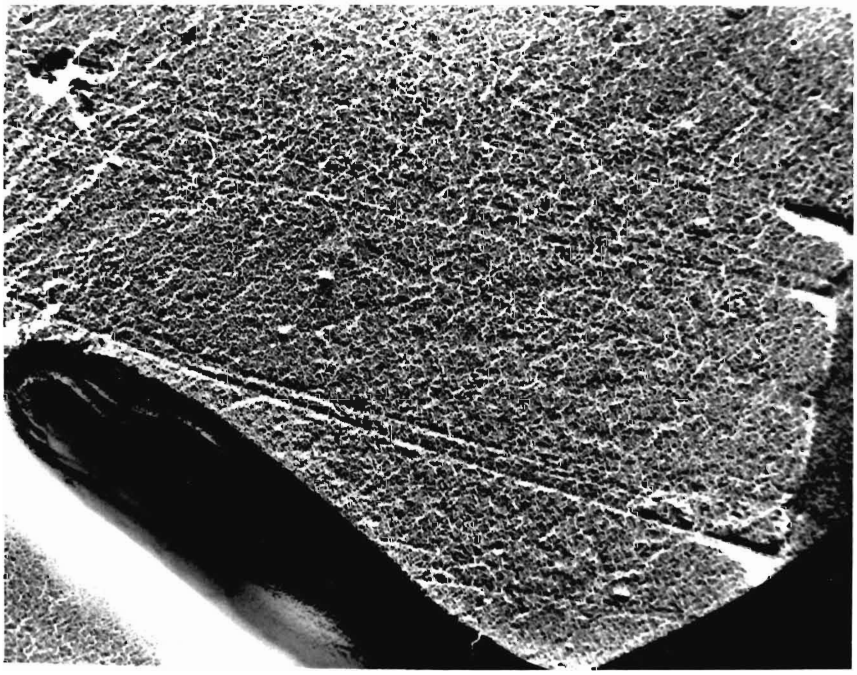


Figure 4. Shadow cast preparation of *P. articulosus* hyphae

An electron micrograph of *P. articulosus* hyphae shadow casted with gold palladium oxide at an angle of 20°. Magnification, 100,000X.

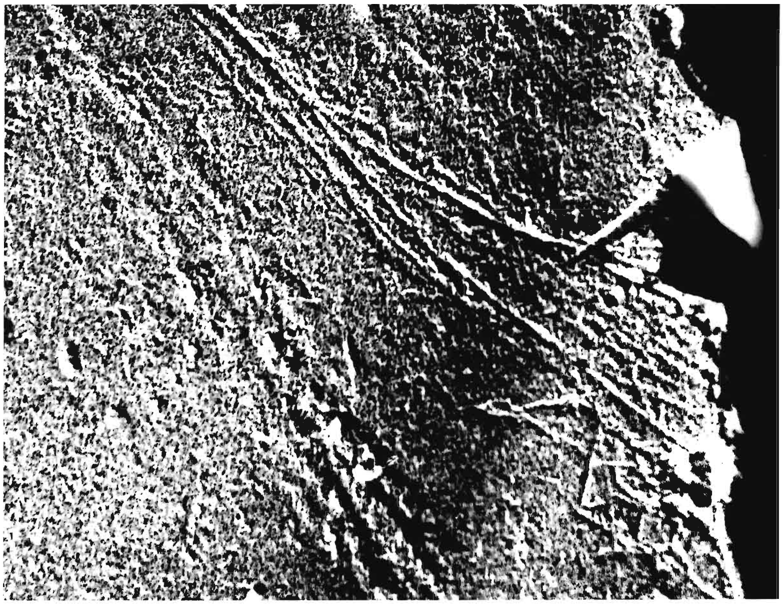


Figure 5. Negative stain preparation of *M. candelabrum* hyphae

Hyphae of *M. candelabrum* negatively stained with 1% uranyl acetate. The mean diameter of these fibrils was calculated to be 9.1±0.4 nm. Magnification, 100,000X.

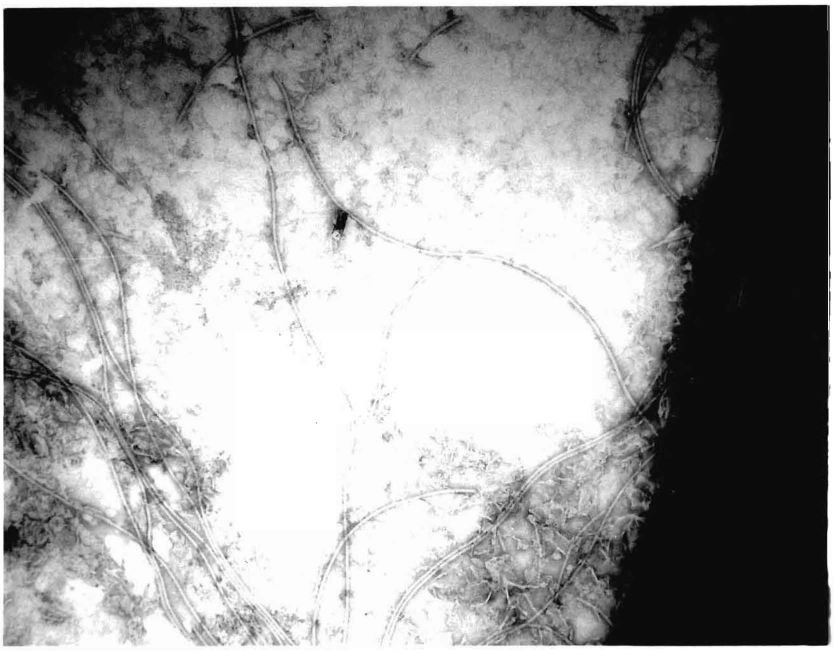


Figure 6. Negative stain preparation of *M. pusilla* hyphae

Hyphae of *M. pusilla* negatively stained with 1% uranyl acetate. The mean diameter of these fibrils was calculated to be 9.4±0.5 nm. Magnification, 100,000X.

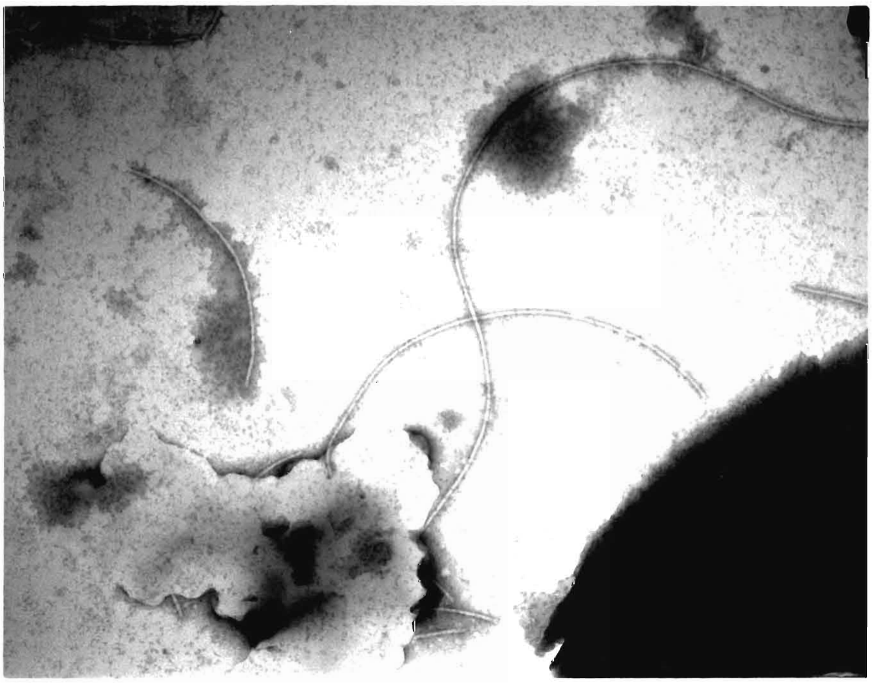


Figure 7. Negative stain preparation of *P. articulosus* hyphae

Hyphae of *P. articulosus* negatively stained with 1% uranyl acetate. The mean diameter of these fibrils was calculated to be 8.6±0.6 nm. Magnification, 100,000X.

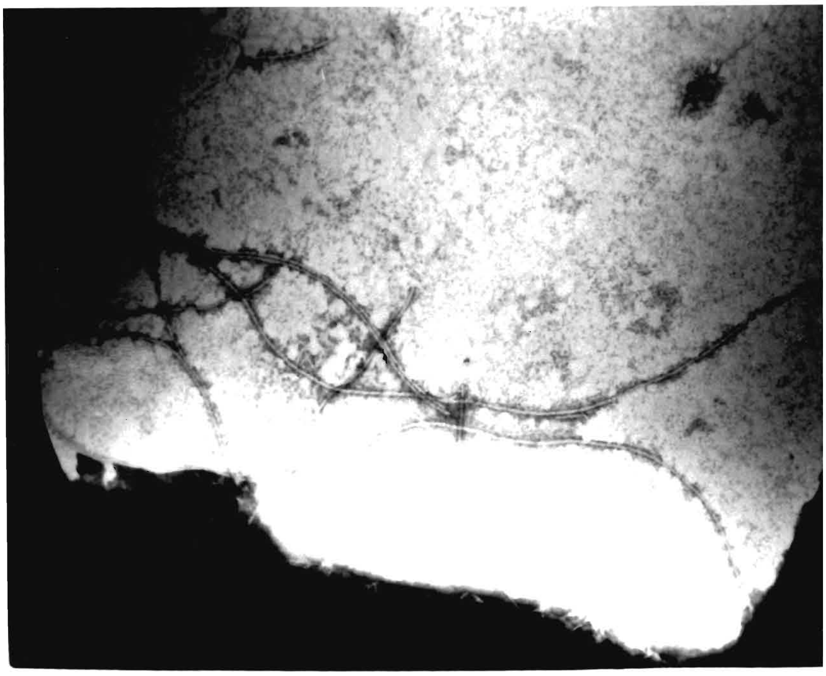
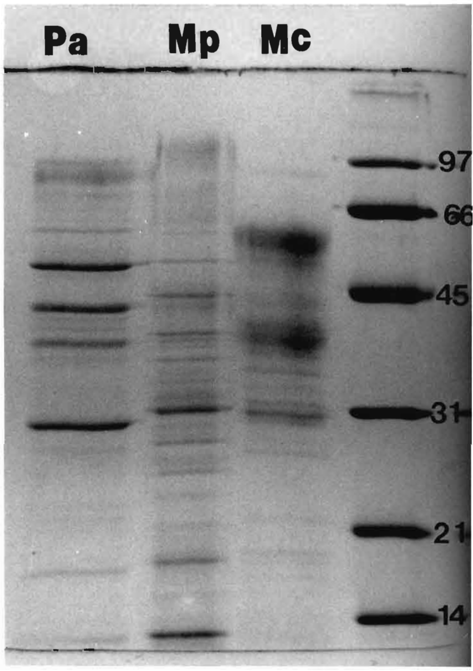


Figure 8. SDS/PAGE separation of proteins of *M. candelabrum, M. pusilla* and *P. articulosus.*

Total proteins of *M. candelabrum* (Mc), *M. pusilla* (Mp) and *P. articulosus* (Pa) separated with SDS/PAGE. Marker lane was that of low molecular range protein standards (Bio-Rad®).


Pa
Mp
Mc
97
66
45
31
21
14

against fimbriae of *U. violacea* (AU) cross-reacted with proteins from all four species. Two *M. candelabrum* proteins with molecular masses of 60 and 57 kDa were visualized. In contrast, the presumptive fimbrial proteins of both the susceptible and resistant hosts, *M. pusilla* and *P. articulosus*, had masses of 64 kDa (Fig. 9). Even though fimbriae had not been observed on *P. virginiana,* AU cross-reacted with two *P. virginiana* proteins with molecular masses of 94 and 91 kDa (Fig.10).

Electron microscopy of electroeluted proteins

In order to determine whether AU was cross-reacting with fimbrial protein subunits, the proteins recognized by AU were electroeluted from the gel matrix and the SDS was removed. Fibrils were formed by the electroeluted proteins of *M. pusilla*, *M. candelabrum* and *P. articulosus* (Fig. 11-13). The reformed fibrils diameters were approximately as the fimbriae observed on the hyphal surfaces, a diameter mean of 9.7±0.3 nm, 8.4±0.6 nm and 9.2±0.5 nm for *M. candelaburm, M. pusilla,* and *P. articulosus,* respectively.

Effect of AU on host-mycoparasite interactions

To determine if fimbriae are required for events in mycoparasitism, spores of the mycoparasite, *P. virginiana,* and the susceptible host, *M. pusilla,* or *P. virginiana* and the resistant host, *P. articulosus*, were incubated with AU in varying concentrations. Incubation with AU resulted in an inhibition of contact between parasite and host. Once contact was achieved, however, appressorium formation was apparently normal (Fig. 14 and15). Using the nonparametric Mann-Whitney test, the inhibition of contact in the presence of AU was significantly different ($U_{0.10(2)3,3}$) than either that of pre-immune serum or AU pre-incubated with purified *Ustilago violacea* fimbrial protein.

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Figure 9. Immunoblots analysis of proteins of *M. candelabrum*, *M. pusilla* and *P. articulosus*

(A) an immunoblot of total proteins of *M. candelabrum* (Mc), *M. pusilla* (Mp) and *P. articulosus* (Pa) incubated with AU. AU cross-reacted with two *M. candelabrum* proteins (57 and 60 Kd) and one protein of each of *M. pusilla* and *P. articulosus* (both at 64 Kd). (B) an immunoblot of total proteins of *M. candelabrum* (Mc), *M. pusilla* (Mp) and *P. articulosus* (Pa) incubated with preimmune serum. No cross-reactivity observed. The marker lane was that of low molecular range protein standards (Bio-Rad®).

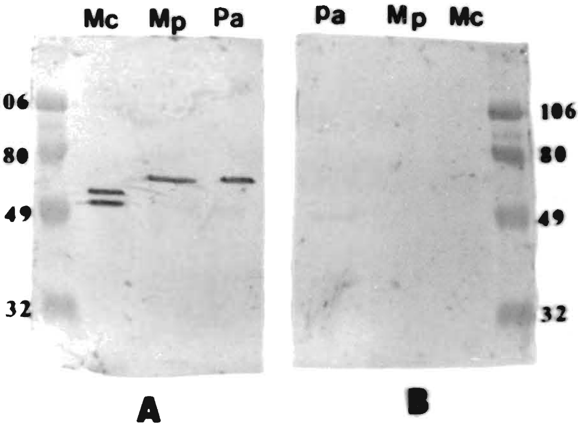

Mc
Mp
Pa
Pa
Mp
Mc
106
80
49
32
106
80
49
32
A
B

Figure 10. Immunoblot analysis of proteins of *P. virginiana*.

AU cross-reacted with two *P. virginiana* (Pv) proteins (94 and 91 Kd). *U. violacea* (Us) proteins gave a positive reaction at ≈ 78 Kd. Marker lane was that of high molecular range protein standards (Bio-Rad®).

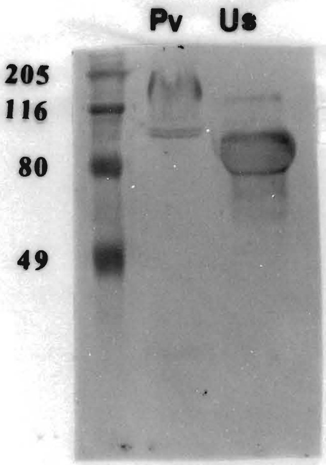

Pv
Us
205
116
80
49

Figure 11. Negative stain preparation of electroeluted *M. candelabrum* proteins

An electron micrograph of *M. candelabrum* electroeluted proteins negatively stained with 1% uranyl acetate. The mean diameter was calculated to be 9.7±0.3 nm. Magnification, 100,000X.

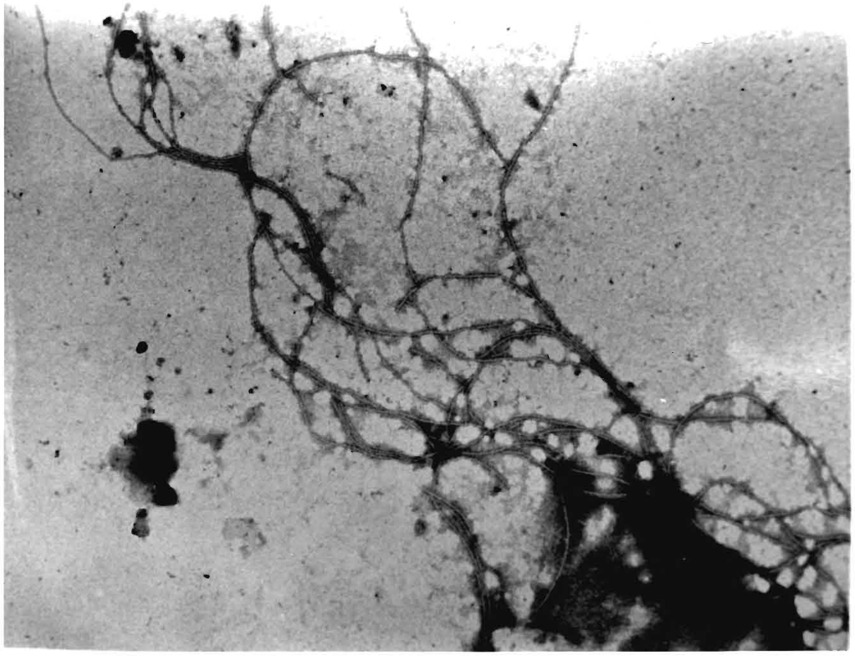


Figure 12. Negative stain preparation of electroeluted *M. pusilla* proteins

An electron micrograph of *M. pusilla* electroeluted proteins negatively stained with 1% uranyl acetate. The mean diameter was calculated to be 8.4±0.6 nm. Magnification, 100,000X.

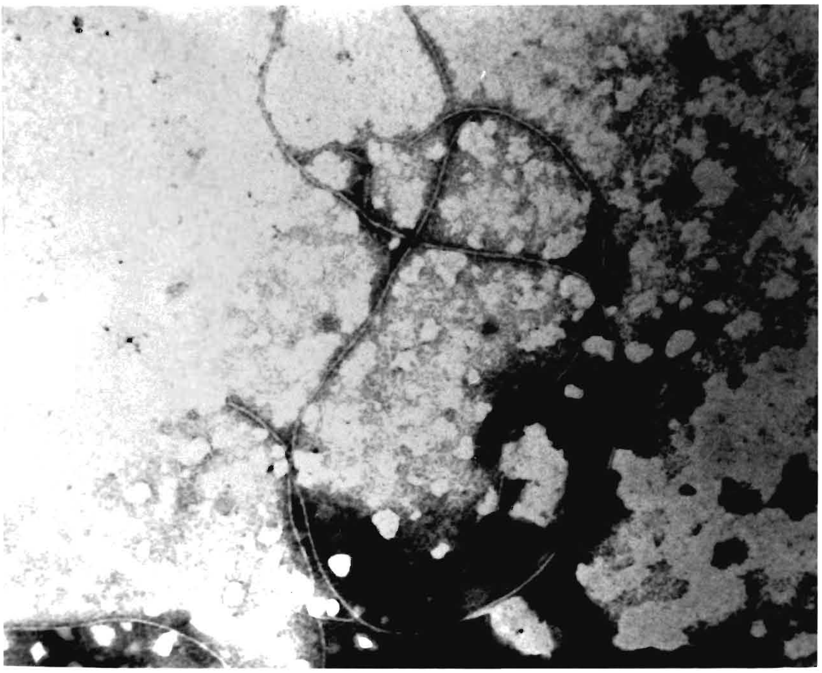


Figure 13. Negative stain preparation of electroeluted *P. articulosus* proteins

An electron micrograph of *P. articulosus* electroeluted proteins negatively stained with 1% uranyl acetate. The mean diameter was calculated to be 9.2±0.5 nm. Magnification, 100,000X.

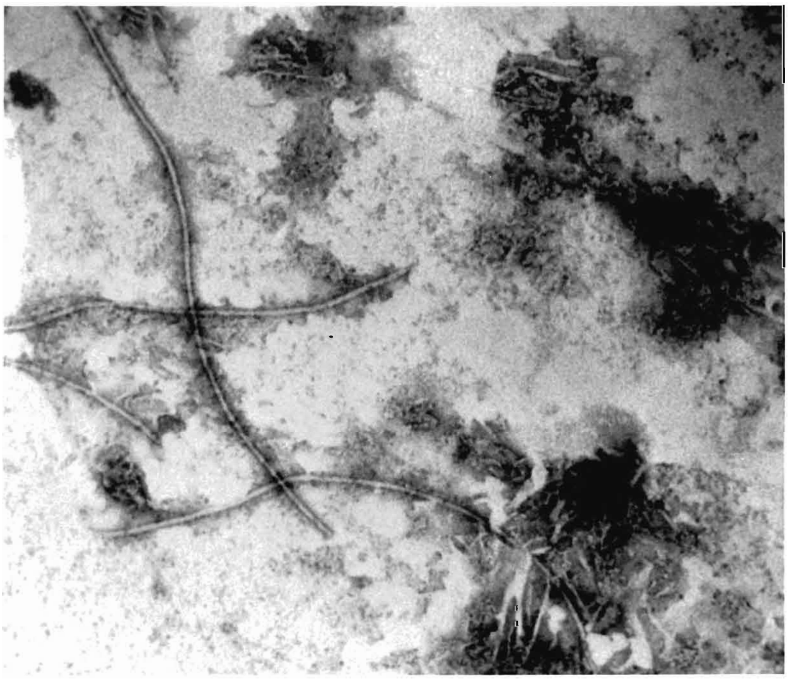


Figure 14 Inhibition of *P. virginiana* attchment to *M. pusilla* by anti-fimbrial protein antiserum

The level of contact between *P. virginiana* and *M. pusilla* decreased as the concentration of AU increased, in contrast with incubations with preimmune serum only. The observed decrease in samples incubated with AU was significantly different from those incubated with preimmune serume at all concentrations ($U_{0.10(2)3,3}$). Furthermore, levels of contact between the host and mycoparasite incubated with AU were significantly different from those incubated with AU preabsorbed with isolated *U. violacea* fimbrial protein at all concentrations ($U_{0.10(2)3,3}$). The decreased appressorium formation observed, however, was not due to AU inhibition but due to decreased levels of contact as appressorium formation per contact remained constant. (NS= preimmune serum; AU+FP= AU preabsorbed with isolated *U. violacea* fimbrial protein; AU= antisera against *U. violacea* fimbrial protein).

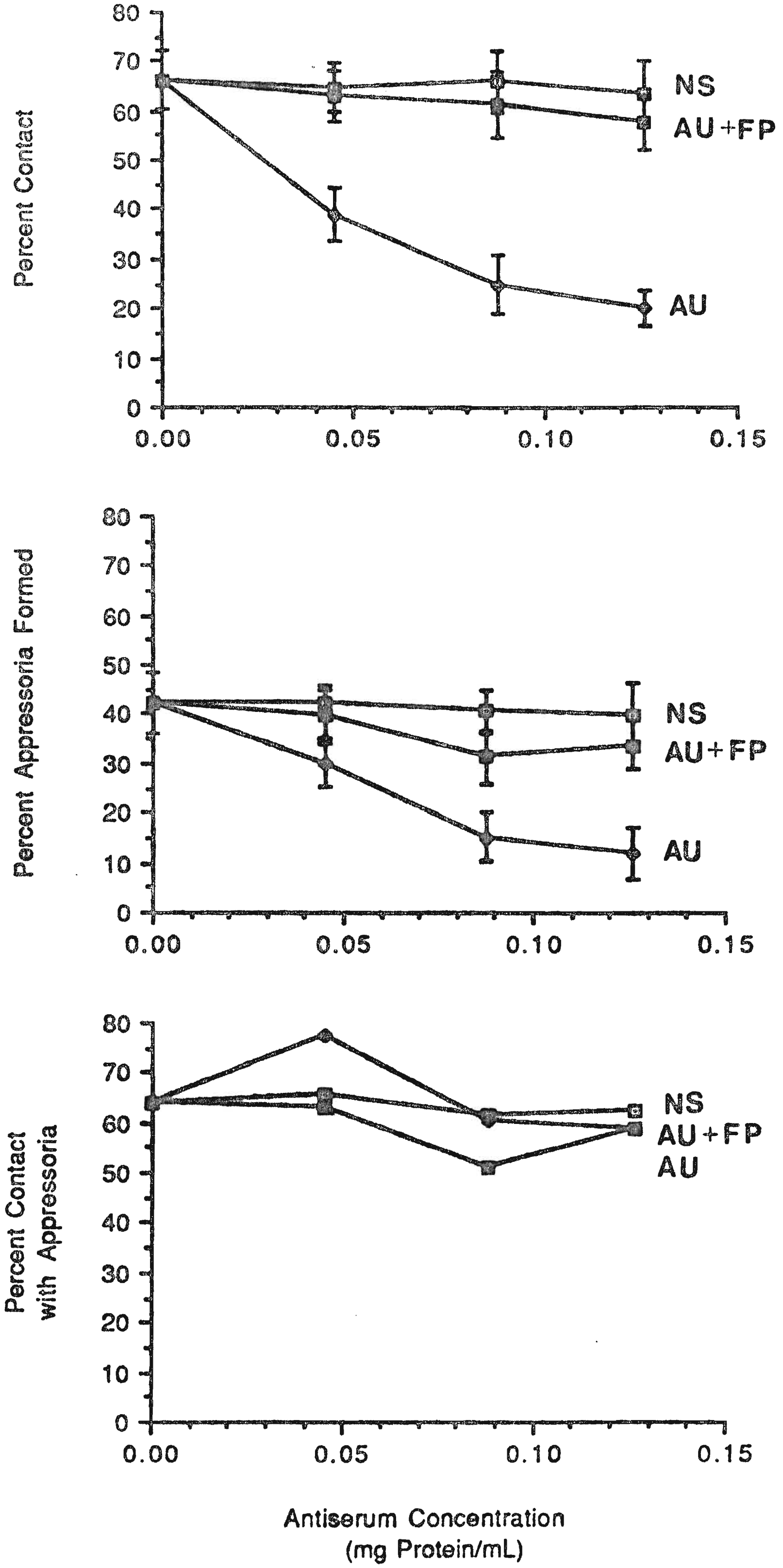
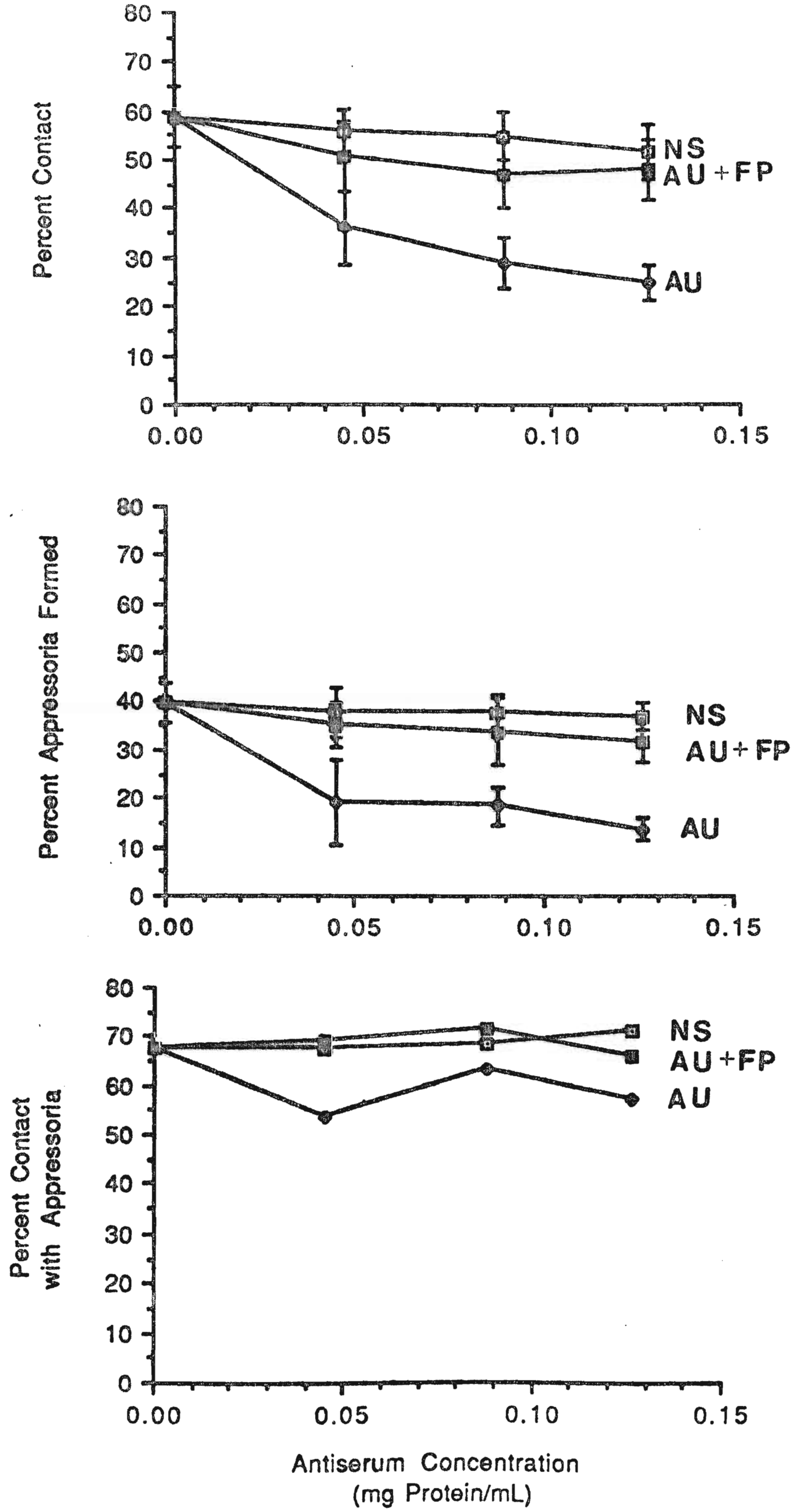

Percent Contact
80
70
60
50
40
30
20
10
0
0.00
0.05
0.10
0.15
NS
AU+FP
AU
Percent Appressoria Formed
80
70
60
50
40
30
20
10
0
0.00
0.05
0.10
0.15
NS
AU+FP
AU
Percent Contact with Appressoria
80
70
60
50
40
30
20
10
0
0.00
0.05
0.10
0.15
NS
AU+FP
AU
Antiserum Concentration (mg Protein/mL)

Figure 15 Inhibition of *P. virginiana* attachment to *P. articulosus* by anti-fimbrial protein antiserum

The level of contact of *P. virginiana* and *P. articulosus* decreased as the concentration of AU increased, in contrast with incubations with preimmune serum only. The observed decrease in samples incubated with AU was significantly different from those incubated with preimmune serume at all concentrations ($U_{0.10(2)3,3}$). Furthermore, levels of contact between the host and mycoparasite incubated with AU were significantly different from those incubated with AU preabsorbed with isolated *U. violacea* fimbrial protein at all concentrations ($U_{0.10(2)3,3}$). The decreased appressorium formation observed, however, was not due to AU inhibition but due to decreased levels of contact as appressorium formation per contact remained constant. (NS= preimmune serum; AU+FP= AU preabsorbed with isolated *U. violacea* fimbrial protein; AU= antisera against *U. violacea* fimbrial protein).


Percent Contact
80
70
60
50
40
30
20
10
0
0.00
0.05
0.10
0.15
NS
AU + FP
AU
Percent Appressoria Formed
80
70
60
50
40
30
20
10
0
0.00
0.05
0.10
0.15
NS
AU + FP
AU
Percent Contact with Appressoria
80
70
60
50
40
30
20
10
0
0.00
0.05
0.10
0.15
NS
AU + FP
AU
Antiserum Concentration
(mg Protein/mL)

Discussion

The presence of fimbriae on the zygomycetes *Mortierella candelabrum, Mortierella pusilla* and *Phascolomyces articulosis* adds to an increasing list of fungi possessing non-flagellar cell surface filaments. Previous work has demonstrated that fimbriae are widespread in the kingdom fungi (Day *et al.,* 1986; Day and Gardiner, 1988; Gardiner *et al.,* 1982; Svircev *et al.,* 1986). Based on electron microscope observations, the morphological characteristics of fungal fimbriae that have been previously reported are similar to those reported here. The length of fimbriae reported showed considerable variation from as short as 0.5-1 µm in some ascomycetes (e.g. *Arthroascus javanensis* and *Saccharomyces cerevisiae)* (Poon and Day, 1974; 1975; Gardiner, 1985) and up to 20 µm in length in *U. violacea. Phycomyces blakesleeanus*, a zygomycete, had fimbriae up to 10 µm in length with a diameter of 7.5 nm (Gardiner, 1985). The diameters of fimbriae on *M. candelabrum, M. pusilla* and *P. articulosis* are comparable to fimbrial diameters reported for all fungal species (Gardiner, 1985; Gardiner *et al,* 1981, 1982; Gardiner and Day, 1988). Although fungal fimbriae vary in diameter (6-10 nm), they do not show as much variation as their bacterial counterparts where diameters range from 2 to 11 nm (Paranchych and Frost, 1988).

Fimbriae were not detected in some basidiomycetous and ascomycetous fungi that were examined by electron microscopy, agglutination or immunofluorescence techniques (Gardiner, 1985). Since fimbrial production is dependent on suitable growth conditions such temperature, the lack of fimbriae might be linked directly to unsuitable growth conditions. Although *P. virginiana* was tested for the presence of fimbriae under the same conditions that are required for parasitism (i.e. 22°C and pH 6.8 on semisolid medium) fimbriae were still not observed. Thus it appears that fimbriae are indeed widespread but not universal in distribution.

Fimbriae of *M. candelabrum, M. pusilla,* and *P. articulosus* cross-react with the polyclonal antiserum AU yielding different molecular size protein bands. Variation in molecular sizes was reported in other fungi as well. The fimbrial subunit of *U. violacea* is 74 kDa protein and that of *Coprinus cinereus* is 37 kDa protein (Gardiner, 1985, Boulianne *et al*, in prep.). In contrast, bacterial fimbrial proteins show much less size variation. The molecular sizes of fimbrial proteins of *Escherichia coli, Serratia marcescens, Salmonella, typhimurium* and *Klebsiella pneumoniae* are 17, 19, 21 and 19.5 kDa, respectively (Salit and Gotschlich, 1977; Korhonen *et al.*, 1980; Fader *et al.*, 1982; Kohno *et al.*, 1984). However, there is a considerable variation in amino acid composition among bacterial fimbrial proteins. Within the fimbrial proteins there are variable and conserved regions. *Pseudomonas aeruginosa* pilin proteins vary considerably in one region of the protein, the immunodominant central region (Sastry *et al.*, 1985). The N-terminal of the protein is responsible for subunit assembly into polymers and therefore is highly conserved (Pasloske and Paranchych, 1988). The C-terminal of the pilin protein is semiconserved and harbours the epithelial cell-binding domain that facilitates the attachment of the bacteria to human buccal cells (Lee *et al.*, 1989). Even though the immunodominant region of the pilin proteins of these bacteria show considerable variation, immunologically conserved regions are retained as a consequence of functionality and/ or assembly of the intact fimbriae (Rothbard *et al.,* 1985).

Differences in molecular size in fungal fimbriae observed may be attributed to different functions. While functionality may be the driving force of molecular size variation, it must be stressed that the fimbrial monomer is under assembly constraints. Even though fungal fimbrial proteins vary greatly in size from species to species, they are antigenically related. This antigenic relatedness is not likely due to conservation of protein polymerization sites since the antiserum against fimbrial protein recognizes intact fibrils (Gardiner, 1985; Gardiner and Day, 1988). Since fimbrial monomers are antigenically conserved proteins, they, therefore, likely play an important role in the life cycle of fungi. Some functions had already been attributed to fimbriae: conjugation in *U. violacea* (Day and Poon, 1975), flocculation of *S. cerevisiae* (Day, Poon, and Stewart, 1975), and adhesion of *C. albicans* to buccal epithelial cells (Douglas *et al.,* 1981).

In the case of conjugation in *U. violacea* it was hypothesized that growth of the conjugation tube along the fimbriae provides a gradient by which the conjugation tube of a mating tube grows directly towards a compatible mating type (Day and Poon, 1975). The role of fimbriae in mycoparasitism is of interest since there is an analogous directed growth of the parasite germ tube towards the host hyphae over short distances. The directed growth is likely to be promoted by certain factor(s), physical or diffusible chemical stimulus produced by the host (Jeffries, 1985; Manocha, 1988). Directed growth of *P. virginiana* towards the hyphae of *Choanephora cucurbitarum*, a susceptible host, has been previously reported (Berry and Barnett, 1957). Jeffries and Young (1978) studied the host range of *Piptocephalis unispora*. The host range was found to be limited to certain members of the Mucorales. *P. unispora* germ tubes showed directed growth towards both susceptible and resistant hosts, including *Phacolomyces articulosus,* but did not show positive growth towards non-hosts (Jeffries and Young, 1978). This phenomenon is not universal. For example, *Piptocephalis fimbriata* germ tubes grow directly towards the hyphae of the host *Mortierella vinacea* but do not grow positively towards the hyphae of *Circinella mucoroides,* another host (Evans and Cooke, 1982). It has been proposed that the directed growth of *P. fimbriata* is promoted by high molecular weight, non-volatile, heat labile, proteinaceous or protein-associated diffusible factors released from *M. vinacea* but lacking from *C. mucoroides* (Evans and Cooke, 1982). This latter example makes it difficult to make conclusions regarding directed growth and led Evans and coworkers (1978) to suggest that recognition of a susceptible host before contact does not always occur.

Most haustorial mycoparasites have a host range limited to members of the Mucorales, and even within this order members are not equally susceptible (Jeffries, 1985; Manocha, 1988). It is not really known whether host discrimination is a consequence of metabolic biochemical differences or differences in recognition processes (Jeffries, 1985). Results presented in this thesis showed that the non-host *M. candelabrum* had fimbrial protein monomers with molecular masses different from fimbrial proteins of either host. It is tempting to speculate that it might be a basis for a differential recognition phenomenon. However, preliminary results have shown that *P. virginiana* exhibits growth towards both hosts and non-host (Manocha, 1988). Therefore, fimbriae are not likely involved in host/non-host differentiation.

The directed growth of the mycoparasite germ tube leads to contact and attachment to the host hyphae. At the site of contact, the germ tube forms an appressorium followed by a penetration peg. In a susceptible host a successful penetration results in the formation of a haustorium drawing nutrition from the host. In the the case of the resistant host, penetration is usually impeded by thickening of the hyphal wall. However, sometimes penetration is successful and results in haustorium formation. In this instance, a thick sheath is formed around the haustorium preventing the mycoparasite from establishing nutritional relationship with the host (Manocha, 1988).

In order to ascertain the role fimbriae may play in mycoparasitism, AU was used to block fimbriae. The effect of AU on events which occur very early in parasitism were examined. The decreased level of contact observed between *P. virginiana* and its hosts *(M. pusilla* and *P. articulosis*) when incubated with AU gives evidence that host fimbriae are recognized by the mycoparasite. It is likely that the recognition of the host fimbriae by the mycoparasite leads to directed growth towards the host hypha. The inhibition of contact between the mycoparasite and host fimbriae results in inhibition in subsequent parasitic events. Appressorium formation decreases when the level of contact decreases. However, percent appressorium formation remains constant per percent contact. This implies that AU had no effect on appressorium formation and fimbriae do not play a role in appressorium formation. These results suggest that host fimbriae may provide an initial point of contact between host and mycoparasite establishing recognition and a directed growth gradient. The recognition between the host and mycoparasite provides the initial events that sets the stage for subsequent parasitic events. Based on results of this study and earlier observations (Manocha and Chen, 1991) a model can be proposed in which the susceptible host fimbriae promotes recognition resulting in the directed growth of the mycoparasite towards the host. Once, in contact with the hyphal wall, the mycoparasite's adhesion is mediated by two host cell wall glycoproteins. These glycoproteins, rich in glucose and N-acetylglucosamine, may serve as a receptor for the mycoparasite's attachment to the host (Manocha and Chen, 1991). Subsequently, mycoparasite germ tube forms appressorium in preparation for a penetration attempt.

Further support for notion that fungal fimbriae play a role in a host-parasite interactions comes from studies of immunocytochemical localization of fimbrial antigens on plant host surfaces using protein A-gold labelling (Svircev *et al.,* 1986). Two antisera raised against surface components of *B. cinerea* and the other against fimbriae of *U. violacea* were used to screen for the presence of Fimbrial antigens on infected leaves of *Vicia faba.* Heavy gold labeling was detected on the surfaces of leaves and inside the plant cells of infected leaves but not on uninfected tissue. Similar findings were obtained from studies on *Nicotiana tabacum* L. infected with *Peronospora hyoscyami* f.sp. *tabacina* and *Erythronium americanum* Ker. infected with *Ustilago heufleri* (Day *et al.*, 1986). The presence of fimbrial antigens inside host cells suggested that fimbriae penetrate host cells establishing contact between the host and pathogen.

In summary, the occurance of fimbriae in the host and non-host species in this study supports the observation of the widespread distribution of fimbriae in fungi. Furthermore, the inhibition of contact between the mycoparasite and its hosts gives strong evidence fimbriae play a role in host mycoparasite interactions. Fimbriae promote recognition between the host and mycoparasite establishing the initial event that is followed by other parasitic events.

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