Isolation and Characterization of a Chitinase-secreting Mutant of *Mortierella pusilla* with Altered Interaction with the Mycoparasite *Piptocephalis virginiana*

HANJE CHEN B.Sc., M. Sc. (Fuzhou, PRC)

Department of Biological Sciences

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Brock University
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Abstract

Mortierella pusilla is a susceptible host and supports good growth of the mycoparasite, Piptocephalis virginiana.

Uninucleate spores of M. pusilla were subjected to N-methyl-N'-nitro-nitrosoguanidine (MNNG). To attain a high mutation frequency , a 10-minute exposure to 10 mg/ml MNNG was used and lead to the survival of about 10 % of the spores. The exposed spores then were plated on chitin or milk plates. Approximately 30,000 colonies were examined after mutagenesis on the screening media. A strain, MUT23, with abnormal slow growth morphology was found to delay parasitism by P. virginiana. The particular morphology was not due to auxotrophy, because this strain displayed normal hyphae when glucose was used as the sole carbon source.

One interesting phenomenon was that MUT23 showed an extensive clearing zone around the colony on colloidal chitin agar after 20-25 d. On the same conditions, wild type strain did not show this phenotype. In addition, the MUT23 strain produced the same normal hypha as the wild type strain when it was grown on colloidal chitin agar. The MUT23 was also able to produce more spores on colloidal chitin agar than on malt-yeast extract and minimal media.

The parasite germ tubes formed appressoria at the point of contact on the cell surface of wild type and MUT23 grown for 6 days cell surface but not on the cel surface of MUT23 grown for 2 days. Thus, interaction between MUT23 strain and the mycoparasite was dependent on MUT23 age. The effect of MUT23 filtrate on germination of the parasite was tested. Lysis of germinated spores of the parasite were observed in concentrated MUT23 filtered solution.

MUT23 was compared to the wild type strain for their chitinase production in submerged culture. The chitinase isozymes of both wild type and MUT23 were shown by immunoblotting. Eight distinct chitinase molecules were detected. MUT23 showed markedly higher chitinase activity than the wild type cultured in chitin-containing medium. Maximum chitinase activities of MUT23 were 13.5 fold higher at 20 day of the culture then that of wild type.

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Dedication

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

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INTRODUCTION

It is commonly accepted that chitinases play essential roles in fungi. Chitinases are known to have diverse functions. First, chitinases have physiological roles in hyphal growth and morphogenesis. The regulation of apical growth, germination, and degration of septa for the mobilization of nuclei may all be dependent upon a delicate balance between chitinase and chitin synthetase (Bartnicki-Garcia, 1973). Pedraza-Reys and Lopez-Romero (1989) support the model proposed by Bartnicki-Garcia, showing that the parallel expression of chitinase and chitin synthetase activities during the growth cycle of filamentous cells of *Mucor rouxii*. Second, these enzymes perform a nutritional role in saprophytes such as *Mortierella* spp. and *Aspergillus* spp. (Gray and Baxby 1968; Reyes et al., 1988) and in pathogenesis of other fungi. Plant chitinase is induced in response to attack by fungal pathogens, through cell wall lysis (Schlumbaum et al., 1986; Roberts and Selitrennikoff, 1988), or release of fungal cell wall elicitors (Keen and Yoshikawa, 1983; Hadwiger et al., 1986). A large number of plants have been shown to have chitinase activity.

Chitin-containing fungi that have been studied so far produce chitinases (EC 3.2.1.1.4). Yet chitinases have been isolated and characterized only the limited number of fungi. Work by Manocha and Balasubramanian (1988) has shown that both chitinase and chitin synthetase occur in a membrane bound and partly zymogenic form. This chitinase acts in membrane fraction of *Choanephora cucurbitarum*. These observations support the idea that the lytic

cucurbitarum. These observations support the idea that the lytic enzymes are membrane bound zymogens activated by proteinases.

Chitinases are produced by *Trichoderma harzianum* when it is grown in the presence of chitin or isolated fungal cell walls (Elad et al., 1983; Ridout et al.,1988). A study by Srivastava et al. (1985) showed that the deuteromycete fungus *Aphanocladium album* produced high levels of extracellular chitinase when grown in minimal medium with crystalline chitin as sole carbon source. Chitinase-overproducing mutants of *A. album* were isolated and charactered by Vasseur et al.(1990).

In recent years several workers have suggested that chitinase-producing fungi such as species of *Trichoderma*, can be effective as biological control agents against fungal pathogens (Tokimoto, 1982; Chet, 1987). The main mechanism involved in the antagonism of *Trichoderma* spp. and pathogenic fungi appears to be the release of lytic enzymes, including chitinases.

REVIEW OF LITERATURE

Resistance and Susceptibility in Host-Parasite Interaction

Conventional genetic studies of both plants and pathogens have defined specific loci controlling many host-parasite interactions. These loci have been distinguished genetically with varying degrees of detail relative to modes of inheritance. Generally, three classes are recognized: oligogenic, polygenic and cytoplasmic (Day, 1974). The existence of heritable resistance is suggested by analysis of variation in progeny of crosses between resistant and susceptible parents or between generations of populations of plants selected on the basis of disease resistant phenotypes (Allard, 1960).

Specific interactions between plants and their fungal or bacterial pathogens basically have a pattern that operates in many plant-pathogen systems, wherein different host cultivars exhibit differential responses to distinct physiological races of the pathogen. These systems conform to the gene-for-gene hypothesis, first described by Flor (1946, 1947) for the interaction of flax with the rust fungus *Melampsora lini*. Such race-specific resistance is believed to function via the interaction of dominant avirulence gene-encoded functions in the pathogen with dominant disease resistance gene-encoded functions in the host. Furthermore, if the plant and its pathogen each contain a number of complementary resistance and avirulence genes, respectively, any gene combination determining incompatibility will be epistatic on

other complementary gene pairs conferring compatibility. This is illustrated in the so-called quadratic check.

The Quadratic Check

	Pathogen race			
Host cultivar	P1P1 P2P2	P1P1 p2p2	p1p1 P2P2	p1p1 p2p2
R1R1 R2R2	Res	Res	Res	Susc
R1R1 r2r2	Res	Res	Susc	Susc
r1r1 R2R2	Res	Susc	Res	Susc
r1r1 r2r2	Susc	Susc	Susc	Susc

a. pattern of interaction between a host and its pathogen where two resistance genes (R1 and R2) are complemented by two avirulence genes (P1 and P2). (Ellingboe, 1981). R=resistant; r=susceptible. P=avirulent; p=virulent.

b. Res, resistant (pathogen avirulence on the host cultivar); Susc, susceptible (pathogen virulence on the host cultivar).

The genetics of resistance of cultivated legumes to many of their fungal, bacterial, and viral pathogens has been reviewed (Meiners, 1981).

If fungal parasites have the ability to successfully parasitize host species, they are often able to avoid or inactivate the host defenses. More specialized parasites initially interact

without causing damage to the host, and for some time grow biotrophically within host tissue. In a compatible interaction, the fungal parasite either eludes or suppresses recognition and successful colonization ensues leading to parasitism. In an incompatible interaction, early molecular recognition events trigger the activation of host defense responses.

An example of this former type of parasite strategy is seen in the infection of fungus (*Choanephora cucurbitarum*) by the mycoparasite *Piptocephalis virginiana*, the cytology of which has been studied in detail (Manocha, 1988). Attachment of the germ tube of the mycoparasite to the host cell surface is a pre-requisite for parasitism. Manocha (1985) has developed a direct binding assay to quantify attachment of the parasite germ tubes to the isolated cell wall fragments of the host and nonhost. Within 2 hours of establishing contact with the host hyphal wall, the germ tubes of the parasite form the appressoria. After host penetration, intracellular infection vesicles-haustoria develop. The fine structure of haustoria of *Piptocephalis virginiana* has been elaborated by Manocha and Lee (1971). The cells of the host *Choanephora cucurbitarum* start to degenerate 6 hours after the development of the haustorium of the mycoparasite. When the mycoparasite has absorbed enough nutrients for the development of its sporangiospores the death of the host cell is apparently a normal result of the host-parasite interaction.

The gene-for-gene hypothesis does not imply a general molecular mechanism. There has been speculation that recognition of

host-parasite specificity at the functional level is the interaction of the products of an allele for resistance and the corresponding allele for avirulence (Ellingboe, 1981). The greatest impact of the gene-for-gene hypothesis is that it has encouraged study of resistance-virulence mechanisms with distinctly characterized single gene interactions. However, it must be remembered also that the gene-for-gene phenomenon has been extensively studied in only a few systems (Day et al., 1983). The focus has been on highly evolved biotrophic interaction, which corrected for those systems studied in detail, may not be representative of all host-parasite interactions.

During the past two decades a number of theories have been proposed to explain the molecular basis of host resistance and susceptibility (Ellingboe, 1981; Vanderplank, 1982). These proposals generally are founded on results from genetic (analysis) segregation ratios of disease phenotypes where either the host or the pathogen genotype is segregating and the alternate is fixed. Such studies have provided a genetic understanding of the number of loci involved in controlling disease and their allelic relationships. They also have exhibited dominance-recessiveness relationships, but have indicated little about the biochemical function of the gene products involved. In contrast, knowledge of the biochemical basis of interactive recognition and response phenomena in fungal parasites is expected to lead to new strategies for identification, incorporation, and maintenance of genetic resistance (Day et al., 1983; Vanderplank, 1982).

resistance (Day et al., 1983; Vanderplank, 1982).

The biochemical studies of disease control mechanisms have used genetically undefined systems, resulting in a large body of inconclusive evidence. Correlations have essentially not been made between heritable difference in disease phenotype and differences in pathogen or host metabolites possessing biological activity (e.g. pathotoxins). None of the resistance genes have been characterized functionally , nor are we aware of any resistance genes from plants which have been cloned. A primary benefit of having isolated genes is the potential that they offer for improving our understanding of the molecular mechanisms that control interactions between parasites and their hosts. One can imagine an exchange of molecular messages that may be simple undirectional or complex and multidirectional. The final outcome of the molecular communication is either compatibility leading to parasitic ingress, or incompatibility, where establishment of parasitism is avoided.

At the molecular level, defense response genes are activated for the production of antimicrobial barriers. A role in disease resistance has been proposed or might be suspected for a number of host hydrolases. An earlier review of this subject has appeared (Pegg, 1977). Chitinase has often been studied in plants as a secondary hydrolase involved in defense.

During the last decade, chitinases have been extensively studied due to their potentially wide range of applications. The enzyme could be applied directly in the biological control of

fungal pathogens, or indirectly, using the purified protein or the gene encoding the chitinase (Lund et al., 1989; Shapira et al., 1989).

Plants and Fungal Chitinases

Chitin, β 1, 4-polymer of N-acetylglucosamine (GlcNAC), is a major structural component of most fungal cell walls (Gooday and Trinci, 1980). All fungi that contain chitin also contain chitinase (poly-β-1.4-(2-acetamido-2-deoxy)-D-glucoside glycanohydrolases), (EC 3.2.1.14). Chitinase activity in fungi should have physiological implications in hyphal growth and morphogenesis. The ordered growth of the fungal wall at the hyphal apex is the result of a very delicate balance of lysis and synthesis of wall polymers. For the maintenance of this balance, Bartnicki-Garcia (1973) proposed that the gradient of wall synthesis in the apical dome must be accompanied by a parallel gradient of lytic activity. In this model, chitinase causes a limited degree of cell wall lysis so that the wall remains in a plastic and extensible condition. Pedraza-Reyes and Lopez-Romero (1989) showed parallel expression of chitinase and chitin synthase activities during the growth cycle of filamentous cells of *Mucor rouxii*.

It is commonly accepted that chitinases play essential roles in the life cycle of fungi. The regulation of apical growth, wall softening during hyphal branching, germination, and degradation of septa for the mobilization of nuclei may all be dependent upon a delicate balance between wall synthesis and wall lysis

In plant, chitinases can be considered secondary proteins since they have no known role in the primary metabolism of the plant. However, with their established ability to attack important microbial structures, chitinase can also be considered defense weapons against microbial pathogens. Hence this enzyme has been studied in plants as a secondary hydrolase involved in defense. Its substrate, chitin, does not occur in higher plants but is present in the cell walls of many fungi. Therefore chitinase is considered as an enzyme directed against fungal pathogens. Plant chitinase was first discovered in bean seeds (Powning and Irzykiewcz, 1965).

Disease Resistance and Chitinase

Induction of chitinase and 1,3-β-D-glucanase activities appears to be closely related to induced defense in many plant species (Dixon et al., 1990, Mauch et al., 1988a). These enzymes have the potential to hydrolyze polymers of the fungal cell wall.

Purified chitinase is antifungal, particularly against species which have a high chitin content in the cell walls, such as *Trichoderma* spp. (Schlumbaum et al., 1986). Lysis of the fungus occured in tomato plants infected with *Verticilium albo-atrum* (Pegg and Vessey, 1973). Lysis of fungi was also observed in the mycorrhizal symbiosis of orchids with fungi and in other plant-fungus interactions (Pegg, 1977). β-1,3-glucanase can act as a cooperator to increase the antifungal activity of chitinase against a range of plant pathogenic fungi (Mauch et al., 1988b).

The cell walls of many Ascomycetes and Basidiomycetes contain a large proportion of chitin and of a β-1,3-glucan with some β-1,6-linkage in their cell walls (Wessels and Sietsma, 1981). It is no surprise that plant chitinase and β-1,3-glucanase have been found to digest a considerable part of the isolated cell walls of many fungi (Young and Pegg, 1982; Boller et al., 1983). Complete lysis of the mycelial cell wall is not obligated to stop a growing fungus. Growth of the hyphal tip appears to be a delicate balance of lytic and synthetic processes. The imbalance caused by the addition of exogenous hydrolase activity is often sufficient to break the tip open and kill the advancing hypha (Farkas, 1979). It is interesting that chitinase is particularly active on the chitin which is in the process of being formed (Molano et al., 1979).

Some chitinases can also exhibit lysozyme-like activity against bacteria, although a number of plant pathogenic bacteria appear to be resistant to this activity. In addition to pathogen attack, chitinase and glucanase can also be induced in many plants by treatment with elicitors or ethylene, although pathogen-induced ethylene production does not appear to be causally related to hydrolase induction (Mauch et al., 1984).

Is antifungal activity a general property of all kinds chitinases from different sources or unique to plant enzymes? Roberts and Selitrennikoff (1988) proved that plant and bacterial chitinases differ markedly in antifungal activity. This difference in biological activity correlates with differences in their

substrate specificity. Chitinases from the bacteria *Streptomyces griseus* and *Serratia marcescens* have been shown to lyse chitin by an exochitinolytic mechanism (Molano et al., 1977; Roberts and Cabib, 1982). These enzymes cleave the nonreducing end of chitin in the disaccharide diacetylchitobiose in a stepwise fashion. In contrast, endochitinases from wheat-germ (Molano et al., 1979) and bean leaf (Boller et al., 1983) degrade chitin to the N-acetyled derivatives of chitobiose, chitotriose and chitotetraose as reaction mixtures.

Cabib (1987) has reviewed a few reports about the purification of fungal chitinases. Chitinase preparations from *Saccharomyces cerevisiae* have been separated into several bands with chitinolytic activity after polyacrylamide gel electrophoresis (Gorrea et al., 1982). Pedraza-Reyes and Lopez-Romero (1989) purificated two forms of chitinase from mycelial cells of *Mucor rouxii*. These chitinases, with molecular masses of 30.7 (I) and 24.2 KDa (II), hydrolysed nascent chitin more effectively than preformed chitin. There are different chitinases in germinating cells of *Mucor rouxii* than in growing cells of the fungus. After being subjected to Bio-Gel P-100 column chromatography, the chitinases from aerobically germinating cells of *Mucor rouxii* showed two activity groups, which have molecular weights of 53 KDa and 28 KDa respectively. Further purification of these two chitinases from peaks A and B (53KDa and 28KDa) by ion exchange chromatography, resolved four and five species respectively (Pedraza-Reyes and Lopez-Romero, 1991). Hydrolysis of nascent chitin by these chitinases yield disaccharide

diacetylchitobiose as the main product of hydrolysis. These results indicate that *M. rouxii* chitinases show exochitinolytic activity.

Release of Elicitors of Pathogens by Chitinase

Cell wall components of pathogenic fungi act as elicitors of defense reactions in the plant. Pathogenic avirulence genes may somehow encode race-specific elicitors of defense responses. The avirulence genes have been cloned from plant pathogenic bacteria but not from fungi (Staskawicz et al., 1987; Kearney et al., 1988). In contrast, most of the biochemical studies on elicitors have involved fungal pathogens (reviewed by Dixon and Lamb, 1990). There is therefore no genetic confirmation that any of the molecules so far proposed to act as inducers of defense in host-parasite interaction are primary determinants of avirulence.

The recent elucidation of the primary structure of a highly potent heptaglucoside elicitor and of a number of inactive isomers (Sharp et al., 1984) suggests that relatively small, highly specific fungal wall fragments are natural elicitors. Fragments of such size might well be released from the cell surface of invading fungi by the hydrolases of the plant (Yoshikawa, 1983). It should be noted that reports of race specificity are few, the best example being for the elicitor of necrosis from the tomato pathogen *Cladosporium fulvum*, which is only effective on tomato cultivars which carry the A9 resistance gene (De Wit et al., 1985). The role of non-race-specific elicitors in plant-parasite interactions is still not clear. However, they are clearly potent inducers of

defense gene activation.

In support of this concept, a crude enzyme preparation from peas was found to release elicitor-active fragments from the cell walls of *Fusarium solani f. sp. phaseoli* (Hadwiger and Beckman, 1980). Moreover, β-1,3-glucanase has been purified from soybean and shown to release elicitor-active fragments from phytopathogen or a cell walls (Keen and Yoshikawa, 1983; Keen et al., 1983). On the other hand, plant glucanases may also inactivate elicitors.

In the case of chitinase, it is not known whether chitin fragments released from cell walls by purified chitinase have activity. In wheat leaves, chitin was found to act as an elicitor of the lignification response, and it was suggested that enzymatically generated chitin fragments were the natural elicitors in this system (Pearce and Ride, 1982).

The elicitor-receptor area is a complex one. Not only are a wide variety of different chemical structures from plant pathogens able to act as elicitors, but some pathogens also appear to produce molecules that act as suppressors of elicitation (Kessmann and Barz, 1986). The site at which such suppressors bind is not yet clear (Dixon, 1986). Hosts themselves also contain molecules with elicitor activity. The best defined are oligogalacturonides, which may be released from cell wall pectic polymers during infection, or perhaps by host processes in response to tissue damage (Lee and West, 1981; Davis and Hahlbrock, 1987). It is possible that other as yet unidentified plant components, possibly including secondary metabolites, may also have elicitor activity (Dixon et al., 1989).

Pectic polysacharides are believed to play an important role in the systemic induction of proteinase inhibitors in plants in response to wounding (Ryan, 1988).

The Purification and Genetic Encoding of Chitinase

Chitinase and glucanase have been purified from a range of plant species (Young and Pegg, 1981; Boller et al., 1983; Kurosak et al., 1987; Mauch et al., 1988a ; Metraux et al, 1988; Kragh et al., 1991). Ethylene-induced chitinase in bean leaves appears to be located primarily in the central vacuole of the cell, whereas glucanase is also found in the cell wall space (Mauch and Staehelin, 1989). This has introduced to the argument that vacuolar chitinase and glucanase represent a final line of defense in pathogen infection (Mauch and Staehelin, 1989).

cDNA sequences have been published for the chitinase from bean (Broglie et al., 1986; Hedrick et al., 1988), tobacco (Shinshi et al., 1987; Payne et al., 1990), and cucumber (Metraux et al., 1989).

In bean, there appears to be at least three chitinase genes, of which at least two are expressed on treatment of leaves with ethylene (Broglie et al., 1986). One bean chitinase cDNA encoded a polypeptide of Mr 35,400, with a 27-amino-acid-N-terminal leader sequence characteristic of other eukaryotic signal peptides. This signal peptide suggests that the chitinase is synthesized on

membrane-bound ribosomes.

Two wound-induced genes from potato, win-1 and win-2, encode proteins with 25-amino acid leader sequences followed by a region of extensive homology to chitinase and wheat germ agglutinin (Stanford et al., 1989). It is not known whether these proteins exhibit chitinase activity.

The mature basic chitinase from tobacco shows 73% homology, at the amino acid level, to the mature bean chitinase (Shinshi et al., 1987). Acidic tobacco chitinases are similarly homologous to their basic counterparts (Hooft Van Huijsduinen et al., 1987). In contrast, the cDNA sequence of an extracellular acidic chitinase encoded by a single gene in cucumber shows no homology to the bean or tobacco enzymes. The leader sequence of this chitinase is presumably involved in targeting to the extracellular space.

Chitinase produced by fungi has so far received little attention. Syivastava et al. (1985) published that the deuteromycete fungus *Aphanocladium album* produced high levels of extracellular chitinase when grown in minimal medium with crystalline chitin. Blaiseau et al. (1992) isolated recombinant clones from a cDNA library of an *A. album* chitinase-overproducing mutant strain. An isolated positive clone was used as a hybridization probe to isolated an 8 Kbp DNA fragment from a genomic library of the wild-type strain.

Changes in Gene Activation for Known Defense Response Proteins

cDNA and genomic clones have now been obtained for a number of plant defense response genes encoding enzymes of phytoalexin and lignin biosynthesis, hydroxyproline-rich glycoproteins, hydrolases, and pathology-related proteins. This diversity reflects the fact that different plant families use different biosynthetic pathways for the elaboration of defensive metabolites.

Increased mRNA levels for other defense response genes, including HRGPs (Hydroxyproline-rich glycoproteins) (Showalter et al., 1985; Corbin et al., 1987), chitinase (Hedrick et al., 1988), 1,3-β-D-glucanase (Edington and Dixon, 1990), and coniferyl alcohol dehydrogenase (CAD) (Walter et al., 1988), are likewise induced in bean cells in response to fungal elicitors, infection, and show differential timing in compatible and incompatible interactions. Particularly noteworthy is the very rapid induction of CAD in response to fungal elicitor (preceding the accumulation of phenylalanine ammonia-lyase and chalcone synthase mRNAs); since phenylalanine ammonia-lyase is the first enzyme in the synthesis of ligmin precursors, and CAD is the last, this may suggest some alternative role for phenylpropanoid alcohols in the very early stages of the defense response.

Infection-induced appearance of chitinase and/or glucanase transcripts has been measured in tobacco mosaic virus-infected tobacco (Hooft et al., 1987; Vogeli-Lange et al., 1988; Payne et al., 1990), ethylene-treated bean leaves (Vogeli et al., 1988), and

Pseudomonas syringae-infected bean leaves (Voisey and Slusarenko, 1989). The induction of the mRNAs encoding the two hydrolases appears to be closely coordinated (Vogeli et al., 1988). As well as being induced by ethylene, glucanase and chitinase mRNA expression, at least in tobacco cells, is controlled by levels of other growth regulators. Such control is to be expected in view of the roles of these enzymes in hormonally regulated developmental processes such as tissue-specific flavonoid accumulation.

In filamentous fungi, enzymes involved in the regulation of various metabolic pathways are known to be inducible by their substrate (Finchman et al., 1979). Vasseur et al. (1990) demonstrated the induction of chitinase activity by using soluble GlcNAC, a capsule diffusion test (Pirt, 1971). It is a very convenient method that the rate of diffusion of the substrate can be modulated and controlled according to the concentration of the capsule, and prevents catabolite repression. (Vasseur et al., 1990). Blaiseau et al. (1992) demonstrated that the repressing effect of glucose on the expression of chitinase 1 mRNA is controlled by the nature of the carbon source in the culture medium. This inducer-repressor mechanism by which it regulates the expression of chitinolytic activity is somewhat unclear. Blaiseau et al. (1992) considered that a low constitutive production of chitinase in the culture filtrate leads to initiation of a soluble inducer-chitin oligomers which triggers the expression of chitinase 1 mRNA.

MATERIALS AND METHODS

Fungi and Culture Conditions

Cultures of *Mortierella pusilla* Oudermans and *Mortierella candelabrum (v*.. Teigh and Le Monn), host and nonhost to the mycoparasite *Piptocephalis virginiana (*Leadbeater and Mercer), respectively, were grown routinely on a semisolid medium consisting of malt extract (20 g) yeast extract (2 g), and agar (20 g) in 1 L of distilled water (pH 6.5) at 25$\pm$1°C (Manocha, 1985). The pH was adjusted to 6.5 with NaoH befor autoclaving. The mutant strain of *M. pusilla* was also maintained on the same medium.

Large numbers of spores of *M. pusilla* were obtained from 4-day-old cultures grown at 25°C in petri dishes containing the same medium. Spores were harvested in sterile distilled water, washed twice by low-speed centrifugation and then resuspended to obtain a final concentration of $1*10^6$ spores/ mL. These spores were then subjected to mutagenesis.

For isolation of cell proteins and preparation of cell wall fragments, cultures were grown in a liquid medium of the same composition as above but minus agar, at pH 6.5 for either 2 days or 6 days at room temperature on a gyrorotary shaker (G24 Environmental Incubator Shaker, New Brunswick Instruments) at 100 rpm (Manocha, 1984).

Nuclear Staining of Spores

Freshly harvested spores of *M. pusilla* suspended in sterile distilled water (approximately $1*10^5$ spores per mL) were used. Twenty μL (1 μg/mL) of acridine orange (Sigma Chemical Co., St. Louis, MO) solution were added to 50 μL of spore suspension. A drop of treated spore suspension was placed on a slide and examined immediately under fluorescent microscope. The number of nuclei per spore were counted.

Mutagenesis

An adaptation of the method of mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) described for *Phycomyces* sp. by Cerda-Olmedo (1974) was used. Accordingly , freshly harvested spores ($1*10^6$ spores/ mL) of *M. pusilla* washed with sterile distilled water were suspended in 0.1 M citrate-NaOH buffer (pH 5.5). The spore suspension was placed in a water bath at 40°C for 5 min, just before treatment. The spore suspension was then exposed to (a) different concentrations of MNNG for 2 min; (b) 10 μg/mL MNNG for 10 min at room temperature. The mutagen-treated spores were washed twice with sterile distilled water by low-speed centrifugation.

Isolation of the Mutant

Experiments were done to select different morphological or chitinase producing mutants. The exposed spores were plated on petri dishes containing milk and colloidal chitin minimal agar

(minimal medium: glucose, 20g; potassium nitrate, 5g; potassium dihydrogen orthophosphate, 1g; magnesium suphate, 0.5g; disodium hydrogen orthophosphate, 0.3g; agar, 20g; in 1 L distilled water). After incubation for 24 h at 25 $\pm$ 1°C, the culture plates were examined for morphological and chitinase mutants. A number of colonies showing a typical hyphal growth on chitin and milk plates were picked up, transferred to malt-yeast agar plates and incubated for 4 days at 25 $\pm$ 1°C. A total of 67 colonies were used for a parasitism test with the mycoparasite. Four colonies with the capability of postponement of parasitism of *P. virginiana* were selected, and maintained on malt-yeast agar slants.

Medium for Selecting Chitinase Mutant

Colloidal chitin served as a substrate for chitinase. The colloidal chitin (described below) plates were made by adding 1% colloidal chitin to a minimal medium.

Medium with milk was used as an indicator for protease activity. Sterile milk was prepared by the addition of 10 g of nonfat powder milk to 100 mL of water. The mixture was autoclaved for 10 min and immediately placed on ice to prevent clotting. The milk solution was heated to 37°C and added to minimal agar cooled to 55°C.

Preparation of Colloidal Chitin

A modification of the method described by Shimahara and Takiguchi (1988) was used. Thirty grams of chitin (practical grade obtained from crab shells, Sigma Chemical Co.) were mixed with 250 mL of 10 M HCl and then vigorously stirred overnight at 4°C, Eight liters of distilled water was then added. Within a few minutes, the chitin precipitated and the supernatant was decanted. The remaining mixture was filtered with suction through Whatman No. 2 filter paper. The residue was washed with distilled water until the washings became neutral. The colloidal chitin was then lyophilized.

Parasite Inoculum and Preparation of its Germinated Spores

Cultures of the biotrophic, haustorial mycoparasite, *Piptocephalis virginiana* were maintained on its susceptible host, *Choanephora cucarbitarum* (Berk & Rav.) Thaxter, and incubated at 23$\pm$ 1°C for a period of 2-3 days. An axenic population of spores of the parasite was obtained by incubating the cultures in complete darkness, which is known to inhibit sporulation of the host while the parasite sporulates normally (Berry and Barnett, 1957). Mycoparasite spores were harvested by adding 10 mL sterile distilled water over the culture and gently shaking the culture plates. The resulting spore suspension was filtered through muslin cloth and then centrifuged. The spore pellet was then washed three times with sterile distilled water by centrifugation.

To obtain adequately germinated spores for use in the attachment tests, the concentration of the spores was adjusted to $1*10^5$/mL with malt-yeast extract medium and the spores were allowed to germinate for 20 h at $23\pm1^{\circ}C$. The germinated spores (80%-90% germination) were washed with sterile distilled water by centrifugation and resuspended in 0.01 M phosphate buffer saline (PBS-contains: 1.42 g Na_2PO_4, 1.38 g NaH_2PO_4, 0.85%(w/v) NaCl in 1L H_2O adjusted to pH 6.8 with NaOH).

The spores of *P. virginiana* were allowed to germinate in nutrient broth solution (8.0g nutient broth, 2.5g yeast extract, 10 ml glyerol in 1000 ml distilled water, pH 6.5).

Isolation of Cell Wall Fragments

Purity of isolated cell walls free from cytoplasmic and membrane contamination was determined as described by Manocha (1984) . For preparation of cell wall fragments from wild and mutant strains, mycelium was collected on Whatman No. 1 filter paper in a Buchner funnel, washed with distilled water until free of the medium. Washed mycelium was homogenized in cold PBS containing 1 mM EDTA and 1 μM phenylmethylsulfonyl fluoride (5 mL/g wet weight of mycelium) for 2 min at 3400 rpm in a Sorvall Omni-Mixer with the cup immersed in ice. The homogenate was centrifuged at 1000g for 5 min and the pellet was resuspended in cold distilled water. Additional cell disruption for 2-3 min with sonicator (model W375, Heat System-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 centrifuged until the supernatant appeared clear. The cell wall fragments were then lyophilized and stored at -20^{0}C until further use.

Attachment Test with Wild Type and the Mutant

To test attachment and appressorium formation by germinated spores of the mycoparasite with wild type and mutant cell surfaces, isolated cell wall fragments of both were used. Measurement of parasite attachment was described by Manocha (1984). Wild and mutant cell wall fragments(1 mg/ml) were suspended in 0.01 M PBS 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 then 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 dry high power 40 X objective of a light microscope.

Protein Isolation

Mycelia of wild and mutant strains were harvested from liquid cultures by filtration through cheese cloth, followed by

three washings with distilled water. The samples were then frozen with liquid nitrogen. Proteins were extracted by grinding the frozen mycelia in a mortar and pestle with three parts sterilized sea sand and one part cold TEPI (10 mM Tris, 1 mM EDTA, 1 mM PMSF and 1 mM iodoacetamide; pH 6.8). The resulting slurry was centrifuged at 10,000 xg for 10 min at 4 ^{0}C and the supernatant was collected at 4 ^{0}C. The samples were mixed vigorously with equal volumes of cold n-butanol to remove any lipids from the sample . After centrifugation at 1,000 xg for 10 min at 4 ^{0}C the bottom layer saturated with n-butanol was collected carefully, avoiding the central lipid layer. The samples were dialyzed three times against TE (10 mM Tris and 1 mM EDTA, pH 7.5) at 4 ^{0}C for several hours. Freeze dried samples suspended in a minimal volume of sterile distilled water were stored at -20 ^{0}C.

Gel Electrophoresis

Cellular proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemlli (1970). Protein samples were dissolved in 2% sodium dodecyl sulfate reducing buffer (0.05 M Tris pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.001% bromophenol blue) and were heated at 95 ^{0}C for 4 min. Samples (20 μg) of crude protein were loaded on gels consisting of 10% acrylamide (separation) and 4% (stacking gel). The

electrophoresis buffer consisted of 0.007 M Tris, 1.44 % glycine and 0.1% SDS (pH 8.3). The voltage applied to the gels to resolve the samples was 200 V (constant voltage). The gels were visualized by staining with 0.1% Coomassie brilliant blue R-250 in 40% methanol, 10% acetic acid and destained in 40% methanol, 10% acetic acid. Gels were scanned with IBM 386 computer gel scanner(Imaging Research Inco., MCID OS/2). Low molecular mass markers were obtained from Bio-Rad.

Western Blots

The protocol for Western blotting was described by Towbin et al. (1979). Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes (Bio-Rad) overnight at 30 V. After transfer, the nitrocellulose membranes were washed with Tris buffered saline (TBS) containing 20 mM Tris and 500 mM sodium chloride (pH 7.5) for 10 min. The nitrocellulose membrane was then blocked with 3% gelatin in TBS for at least 3 h on a rocking platform. The nitrocellulose membranes were washed twice (5 min each wash) in Tween-TBS (TTBS) containing 20 mM Tris, 500 mM sodium chloride and 0.05% Tween-20.

The nitrocellulose membrane was then incubated with anti-chitinase antibody and 1% gelatin in TTBS for 2 h with gentle agitation. After incubation, the anti-chitinase antibody was removed and the nitrocellulose membrane was washed in TTBS for 2 h (4 x 30 min) before incubation with second antibody (goat anti-rabbit IgG) in 1% gelatin in TTBS for 2 h. The

nitrocellulose membrane was then washed for 5 min in TTBS followed by three 10 min washings in TBS. Protein bands with bound antibodies were visualized with a colour development buffer (0.1 M Tris pH 9.5, 1 mM MgSO4, 0.03% nitroblue tetrazolium and 0.015% biochloro-indolyl-phosphate). After development, the nitrocellulose membrane was washed with distilled water and allowed to dry for storage.

Fluorescent Lectin Binding Assay

Details of this method are described in Manocha (1985). Ten lectins were used for the direct FITC lectin binding assay. Germinated spores of both wild type and mutant were suspended in 0.01 M PBS buffer (pH 6.8) to a final concentration of $5x10^6$ spores/ml. Spore suspensions (200 μL) were placed in sterile microcentrifuge tubes and centrifuged at 1000 x g for 5 min. The supernatant was discarded. The pellets of spores were suspended in 50 μl of fluorescent lectin solution (1 mg/ml) at a final concentration of 125 μg/ml of PBS (pH 6.8) containing 1% thimerosal and 6% bovine serum albumin. These suspensions were incubated in the dark for 30 min at room temperature. The pellets were washed three times with 200 μL of PBS containing 1% thimerosal, and finally resuspended in 50 μL the same buffer. 20 μL of the suspension was pipetted onto a clean glass slide and observed with epi-fluorescence microscopy. The control consisted of incubation of 43 μL of 200 mM of the

appropriate inhibitory sugar hapten and 7 μL of the lectin incubate for 30 min prior to the addition to the germinated spore suspension. Germinated spores, without any treatment, served as autofluorescence controls.

Wet mounts of the FITC labelled lectins were examined with 100X oil objective of a Zeiss D-7082 oborkochen epi-fluorescence microscope equipped with an HBO high-pressure mercury lamp, an excitation filter (450-490 nm) and a barrier filter (520 nm). The microscope was attached to a photomultiplier tube which was connected to an external DC voltage power supply and operated at 500 volts.

Preparation of Reacetylated Crystalline Chitin

The method described by Cabib (1988) was adapted. Chitosan (1 g) was ground in a mortar while adding slowly and in small portions 20 mL of 10% acetic acid. The solution was covered with a sheet of Parafilm and left overnight at room temperature. The next day, 90 mL of methanol was added slowly with stirring, and the solution was then filterted through five layers of cheese cloth. To the filtrate, 1.5 mL of acetic anhydride was added with constant stirring for 1-2 min. The gel was cut up into small pieces with a spatula and the fragments of the gel allowed to stand overnight. The liquid that oozed out was removed. After suspending in methanol, the suspension was homogenized with a motor-driven homogenizer for 1 min at maximum speed. Finally, the chitin was filtered with glass

funnel and washed extensively with 4 litres of distilled water. The reacetylated crystalline chitin was lyophilized.

Ammonium Sulfate Precipitation of Enzyme

150 ml cultures of different ages of the mutant and wild type were filtered through Whatman No.1 filter paper. To the filtrate, ammonium sulfate was added to give 70% saturation at 4 °C. After overnight incubation with gentle stirring, the precipitate was collected by centrifugation at 20,000 x g for 20 min at 4 °C and redissolved in 20 ml 40 mM phosphate/magnesium buffer (50 mM KH_2PO_4/NaOH, pH 5.5 containing 10 mM $MgCl_2$) and the pH raised to pH 8.4 with 0.1 N NaOH.

Chitinase Assay

1 ml of enzyme solution was mixed with 10 mg of regenerated chitin in 2 ml of 0.1 M sodium acetate buffer, pH 5.0, and incubated for 3 h at 37 ^{0}C with constant shaking. The reaction was stopped by boiling for 3 min. After centrifugation at 1000 x g for 10 min, 0.5 ml supernatant was mixed with 2 ml 0.05% potassium ferricyanide in 0.5 M sodium carbonate solution and the tubes were heated for 15 min in a boiling water bath. The absorbance at 420 nm was measured after cooling. Controls without enzyme or regenerated chitin were used (Ohtakara 1988).

A standard curve was constructed using N-acetylglucosamine and potassium ferricyanide reagent. The change in optical

density is directly proportional to the N-acetylglucosamine concentration (Imoto and Yagishita 1971).

One unit of chitinase activity was defined as the amount of the enzyme which produces 1 mmol of N-acetylglucosamine 3 hrs under the above conditions. Specific activity was expressed in units mg^{-1} protein.

Determination of Protein

Total protein of ammonium sulfate precipitation of wild type and MUT23 were determined according to the method of Bradford (1976) using BioRed dye. Bovine serum albumin (Freetou V) (Sigma Chemical Co., U. S. A.) was used as the standard.

RESULTS

Mutagenesis and Mutant Isolation

Mortierella pusilla is a susceptible host and supports good growth of the mycoparasite, *Piptocephalis virginiana*. When the wild type spores of *M. pusilla* were plated on malt-yeast extract agar medium, they germinated and developed a dense mass of hyphae within two days of incubation at 25°C. These cultures sporulated profusely within 4 days.

The proportion of uninucleate spores of *M. pusilla* was highest in 4 days old cultures grown on malt-yeast extract agar (Table 1). Uninucleate spores tended to be smaller and rounder than binucleate and multinucleate spores.

A mycelia-free 4 day spore suspension was subjected to N-methyl-N'-nitro-nitrosoguanidine (MNNG) or to UV light. Preliminary studies showed that *M. pusilla* was hypersensitive to UV light, hence the chemical method for obtaining mutants was preferred. Figure 1 shows the survival rate of wild-type spores exposed to various concentrations of MNNG for 10 min. Using 10 mg/mL of MNNG and different exposure times, a response curve of *M. pusilla* spores was also established (Figure 2).

Approximately 30,000 colonies were examined after mutagenesis at 10 μg/ml MNNG and 10 min exposure on the screening media, as described under Materials and Methods. A total of 67 colonies were used for a parasitism test by the mycoparasite. Four colonies were selected which showed postponement of the mycoparasitism.

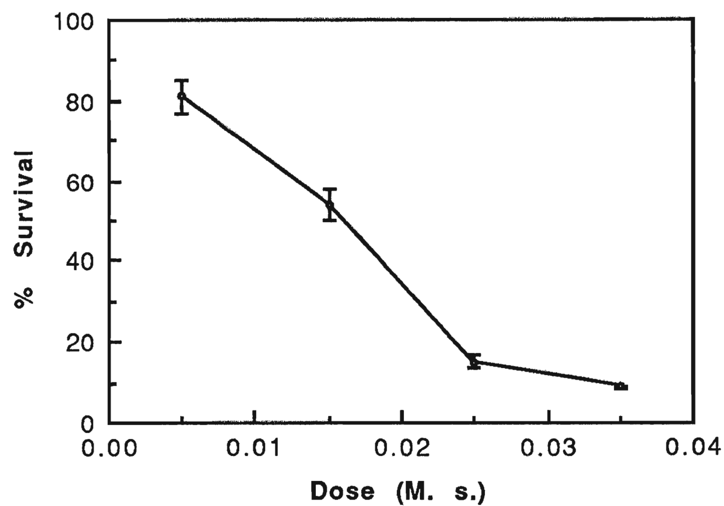


Figure 1. Survival rate of spores of *Mortierella pusilla* exposed for 2 min to different concentrations of N-methyl-N'-nitro-N-nitrosoguanidine. Data were average of three experiments. M.s-mole second/liter

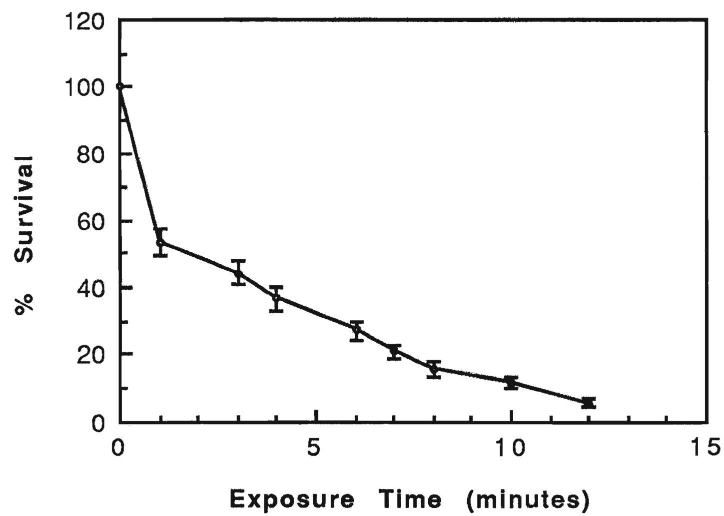


Figure 2. Survival rate of spores of *Mortierella pusilla* exposed to 10 μg/mL for different exposure time. Data were average of three experiments.

Table 1. Percentage of uni-, bi-, and multinucleate spores of *M. pusilla* grown for 4, 6, 8 days

Day	Number of spores	Per cent uninucleate	Percent binucleate	Percent multinucleate
4	487	70$\pm$4.9	9$\pm$0.5	18$\pm$0.8
6	462	64$\pm$1.4	11$\pm$0.3	25$\pm$1.4
8	437	68$\pm$2.4	8$\pm$0.5	24$\pm$1.8

Data were the average of three experiments.

Stability of the Mutant

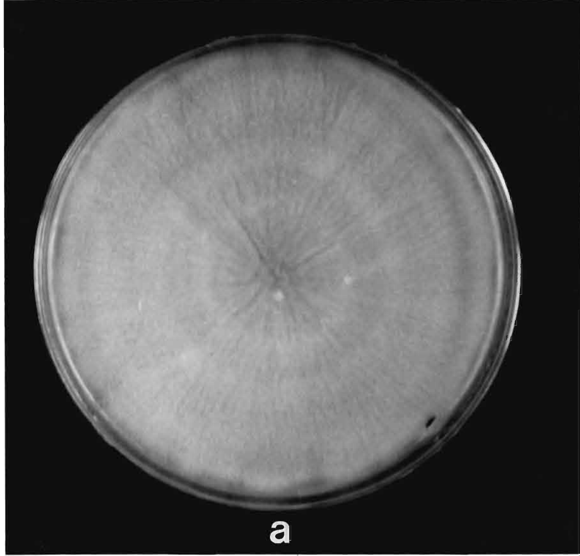
The stabilities of the mutants were tested by successive subcultures on malt-yeat extract medium. During this time, two mutants reverted back to their original phenotype. After four subcultures, two mutants were still able to delay parasitism by the mycoparasite, *P. virginiana* .

Both mutants had the same distinct morphology and were capable of delaying parasitism by *P. virginiana*, compared with the wild type strain under the same cultural conditions. This particular morphology (Figure 3) was not due to auxotrophy, because this strain displayed normal hyphal morphology when glucose was used as the sole carbon source (Figure 4) in a minimal medium. We studied a mutant designated MUT23.

Verification that MUT23 is a Strain of M. pusilla

Figure 5 shows protein profiles isolated from two day old cultures of *M. pusilla,* wild type, as well as MUT23 and *M. candelabrum*. Comparison of protein profiles of wild type and MUT23 with *M. candelabrum* showed marked differences, whereas profiles of wild type and MUT23 were very similar. Results of scans made of PAGE-GEL (MCID OS/2, Imaging Research Inc.) further confirmed the differences between MUT23 and *M. candelabrum*, and the similarity between MUT23 and wild type strain (Figure 6).

Figure 3. Colonial morphology of wild-type (a) and MUT23 (b) malt-yeast extract medium. (10 day)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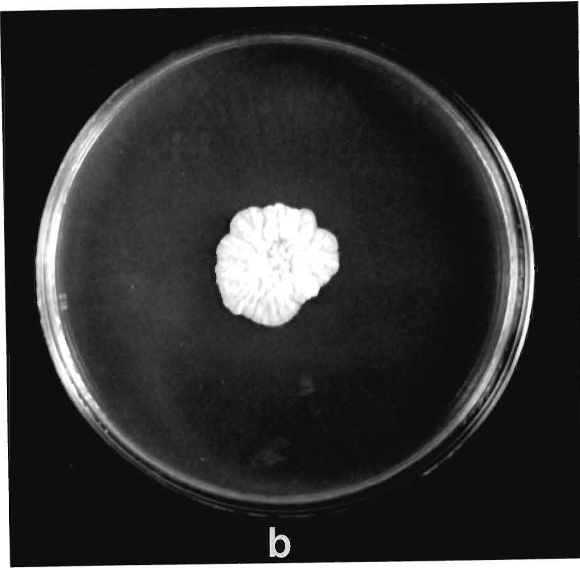
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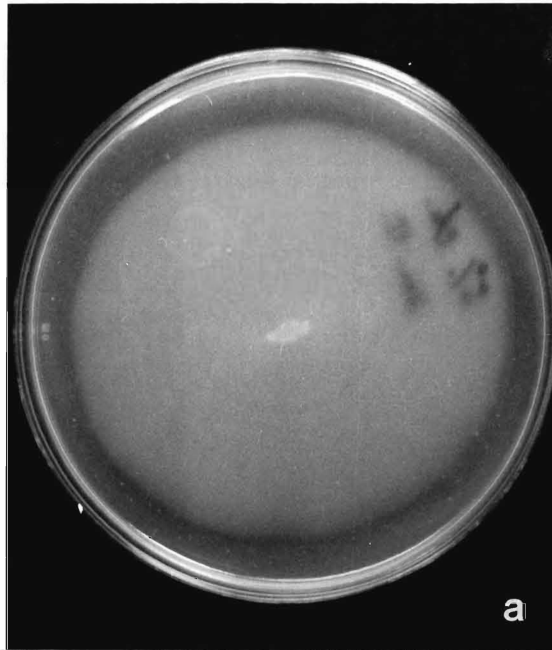
Figure 4. Colonial morphology of wild-type (a) and MUT23 (b) on minimal medium after 10 day growth.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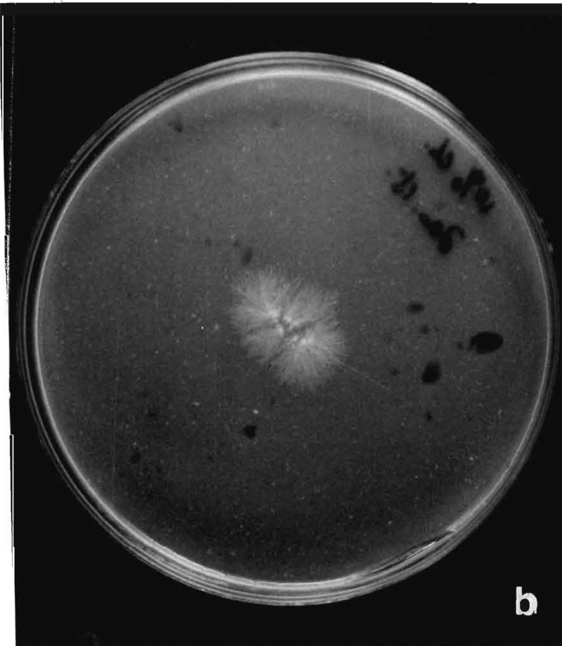
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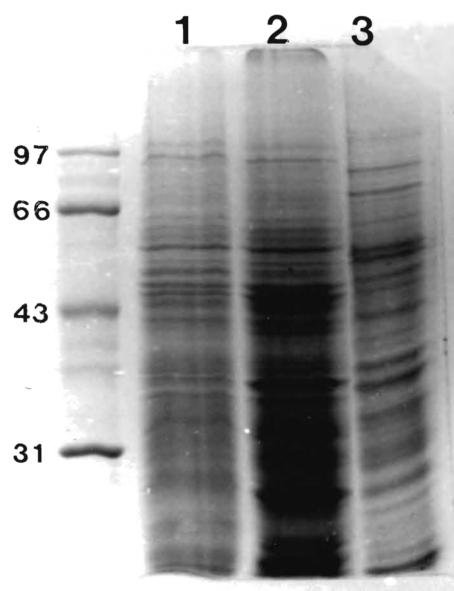
Figure 5. Patterns of protein bands of wild-type (lane-1), MUT23 (lane-2), and *M. candelabrum* (lane-3).The protein standards are as follows: rabbit muscle phosphorylase b, Mr 97 kDa: Bovine serum albumin, Mr 66 kDa: Hen egg white ovalbumin, Mr 43 kDa; Bovine carbonic anhydrase, Mr 31 kDa.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2
3
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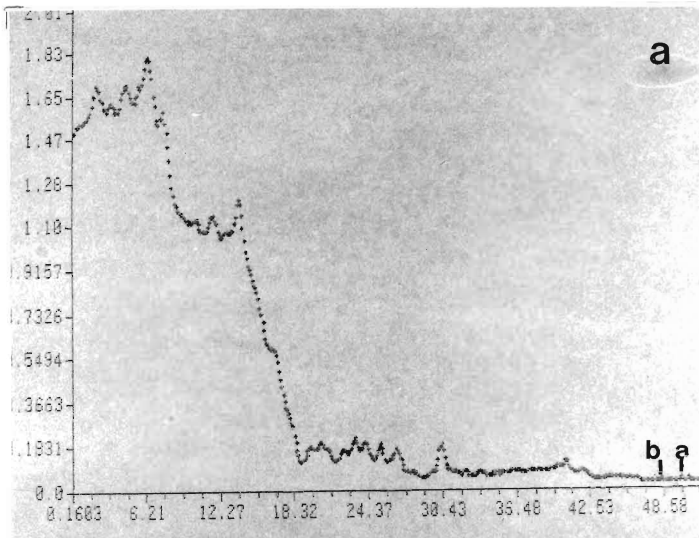
66
43
31

Figure 6. Graphs of PAGE-gels scanned with computer-386 Gel Scanner Image system (MCID OS/2, Imaging Research Inco.). (a) the graph of wild type; (b) the graph of 6 d MUT23; (c) the graph of 2 d MUT23; (d) the graph of *M.candelabrum*.Tick=1.513 mm.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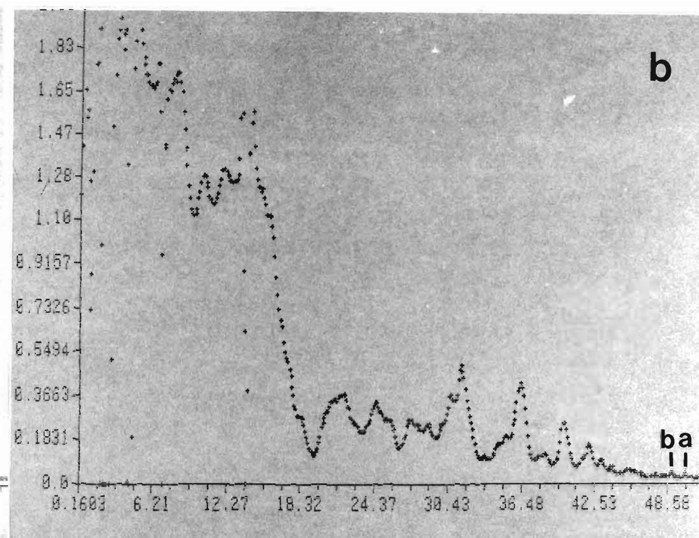
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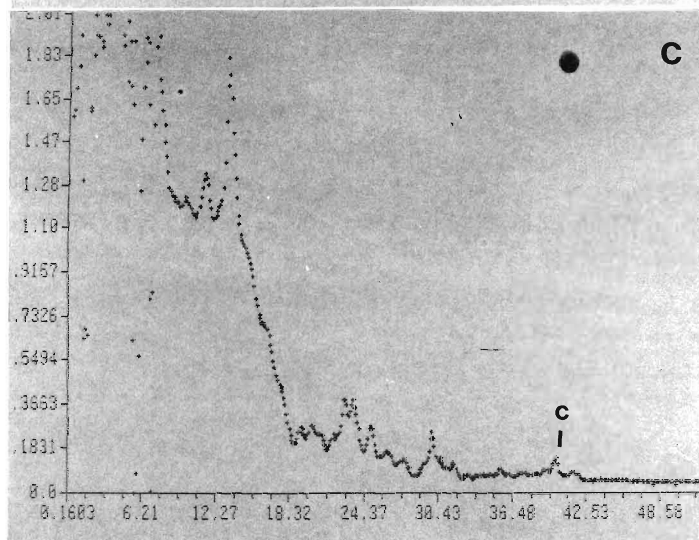
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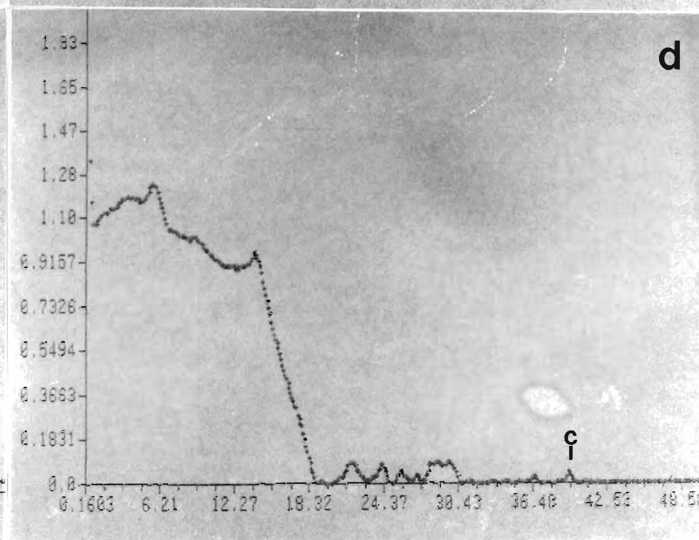
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42.53
48.58
c

The sporangia and spores of wild type and MUT23 strains were also observed by light microscopy (Figure 7). There were no apparent differences between these strains.

Growth and Morphology of Wild Type and MUT23 of *M. Pusilla*

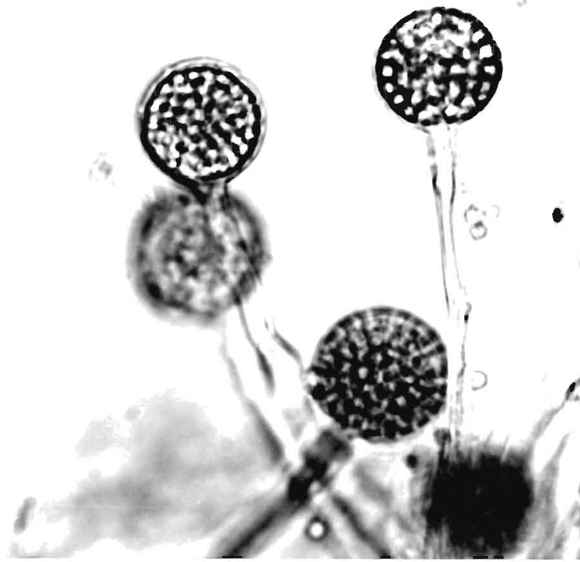
When MUT23 strain was grown on malt-yeast extract, which was used as the routine culture medium for wild type strain of *M. pusilla*, MUT23 strain unlike the parent strain, failed to spread but instead gave rise to tight colonies of intensely branched hyphae (see Figure 3).

The wild type strain showed different growth rates on different media such as malt-yeast extract and minimal medium (Table 2), as expressed in colony diameter. MUT23 strain, however, did not show any difference in its growth rate when grown on either malt-yeast extract or minimal medium (Table 2). Addition of ions (Fe^{2+}, Zn^{2+}, Mn^{2+}) and certain vitamins (Thiamine, Biotin) to the malt-yeast extract medium, resulted in increased growth rate of wild type strain, but there was no difference in the case of MUT23 strain (Table 3 and Table 4).

One interesting phenotype of MUT23 strain was an extensive clearing zone around the colony on colloidal chitin agar after 20-25 days (Figure 8). In addition, when MUT23 grew on colloidal chitin agar, its morphology reverted back to the normal wild type state. Maybe this will explain the high growth rate of MUT23 strain on the colloidal chitin medium (Table 5).

The MUT23 strain was also able to produce more spores on colloidal chitin medium than on malt-yeast extract and minimal media (Table 6).

Figure 7. The sporangia of wild-type (a) and MUT23(b) when grown on malt-yeast extract medium (mag. 1000).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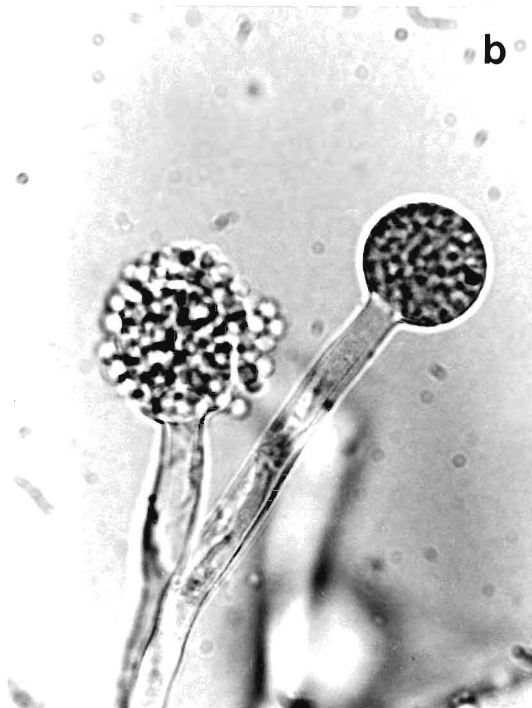
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Table 2. Colony diameter (mm) wild type and MUT23 grown on malt-yeast extract and minimal media for 5 days

Incubation time (d)	Malt-yeast		Minimal	
	WT.	MUT23	WT.	MUT23
1	5±0.2	1±0.1	4±0.3	0±0
2	22±1.4	5±0.3	12±0.5	4±0.1
3	27±1.4	8.5±0.2	18±0.4	7±0.3
4	35±1.2	11±0.5	21±1.5	13±0.5
5	45±2.6	14±0.8	28±1.4	15±0.7

0.1 ml spore suspension (10^5 spore ml^{-1}) was inoculated at center of agar dishes.

Unit of diameter was mm.

Data were the average of three experiments.

Table 3. Colony diameter (mm) of wild type and MUT23 grown on malt-yeast agar supplemented with ions (Fe^{2+}, Zn^{2+}, Mn^{2+})

Incubation time (d)	Ions		Control	
	WT.	MUT23	WT.	MUT23
1	5±0	3±0	5±0.2	1±0
2	19±1.2	5±0.3	12±1.2	5±0.4
3	27±1.7	7±0.7	21±0.8	8.5±0.9
4	45±2.4	12±1.6	34±2.8	11±1.3
5	60±2.9	14±1.4	44±2.2	13±1.8

0.1 ml spore suspension (10^5 spore ml^{-1}) were inoculated at center of agar dishes.

The concentrations of ions were Fe^{2+}-0.2mg l^{-1}, Zn^{2}-0.4mg l^{-1}, Mn^{2+}-0.2mg l^{-1}.

Data were average of three experiments.

Table 4. Colony diameter (mm) of wild type and MUT23 grown on malt-yeast agar supplemented with vitamins (Thiamine, and Biotin)

Incubation time	Thiamine		Biotin		Thiamine + Biotin	
(d)	WT.	MUT23	WT.	MUT23	WT.	MUT23
1	6±0.4	2±0	5±0.2	4±0.1	5±0.1	3±0
2	23±1.4	5±0.5	19±1.7	7±0.4	18±0.4	6±0.2
3	26±1.7	7±0.8	25±1.6	8±0.6	26±1.7	7±0.1
4	46±2.9	10±1.4	44±2.0	12±1.6	44±2.1	12±0.3
5	68±3.3	12±1.1	61±3.2	15±1.8	60±3.4	15±1.22

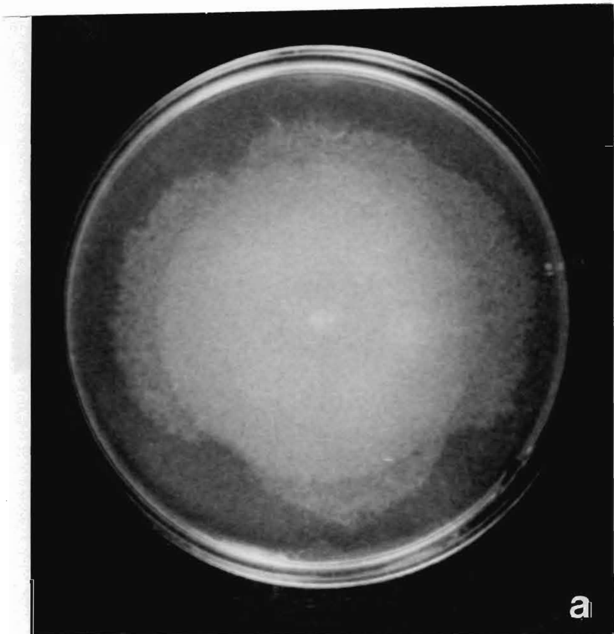
0.1 ml spore suspension (10^5 spore ml^{-1}) were inoculated at center of agar dishes.

The concentrations of vitamines were thiamine-100μg l^{-1}, and biotin-5μg l^{-1}.

Data were the average of three experiments.

For Control, see Table 3.

Figure 8. Colonies of wild-type (a) and MUT23(b) on colloidal chitin medium. MUT23 was 20 days. Wild type was 10 days.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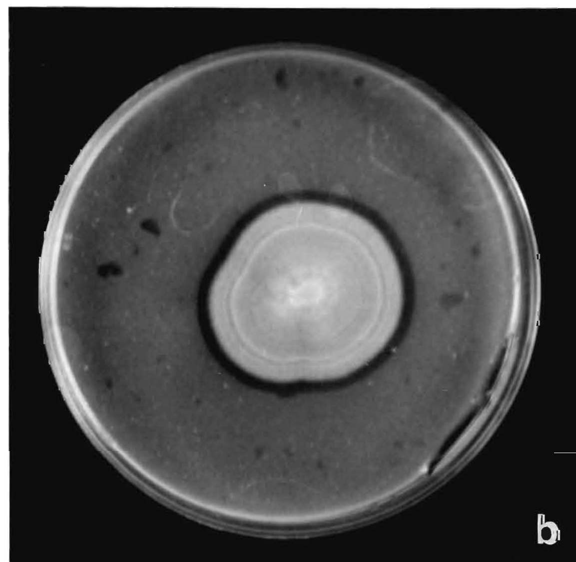
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Table 5. Colony diameter (mm) of wild type and MUT23 grown on media with or without 0.01% (w/v) Colloidal chitin (C.chitin)

Time	Malt-yeast				Minimal			
	-C.chitin		+C.chitin		-C.chitin		+C.chitin	
(d)	WT.	MUT23	WT.	MUT23	WT.	MUT23	WT.	MUT23
1	7±0	2±0	6±0	3±0	4±0	1±0	3±0	2±0
2	18±0.2	6±0.6	20±0	6±0	12±0.2	4±0	9±0.1	5±0
3	22±0.2	9+0.2	28±0.9	10±0.3	18±0.6	7±0.3	15±0.4	8±0.2
4	30±0.7	12±0.5	35±1.4	17±0.4	25±0.7	11±0.5	20±0.7	16±0.6
5	40±1.6	16±0.7	44±1.2	26±0.8	30±0.6	14±0.3	26±0.5	24±0.5

0.1 ml spore suspension (10^5 spore ml^{-1}) were inoculated at center of agar dishes.

Unit of diameter was mm.

Data were average of three experiments.

Table 6. Comparison of spore production (spores ml^{-1}) of wild type and MUT23 grown on different media

Medium	Wild type		MUT23	
	10 days	15 days	10 days	15 days
Malt-yeast	$1.6x10^6$	$2.7x10^7$	$3.8x10^4$	$8.5x10^5$
Minimal	$2.5x10^6$	$2.5x10^6$	$4.8x10^4$	$5.7x10^5$
0.01% C.chitin*	$1.6x10^6$	$2.7x10^6$	$7.2x10^5$	$2.9x10^6$
0.05% C.chitin*	$1.2x10^6$	$3.7x10^6$	$8.5x10^5$	$8.9x10^6$

*Minimal medium supplemented with 0.01 % and 0.05 % colloidal chitin.

An agar plug 2.25 mm in media was suspended in 1 ml d.d. H_2O. The suspension was shaked for several minutes. The suspension was counted with a hemcytometer.

Data were the average of three experiments.

Fluorescent Lectin Binding Assay

Table 7 summarizes the results of lectin binding assay recorded as absence (-) or presence (+, ++ with increasing intensity) of fluorescent reaction with wild type and MUT23 strains. Both strains showed a positive reaction to wheat germ agglutinin (WGA) and *Phytolacca americana* lectin agglutinin (PAA), both specific for N-acetylglucosamine oligomer, indicative of binding with the sugar residues at the surface of the germinating spores (Figure 9). *Lotus tetragonolobus* lectin (LtA) specific for L-fucose showed binding with both strains but gorse lectin (UeA), also specific for L-fucose, failed to show any binding with either of the strains. Castor bean lectin (RcAII) specific for D-galactose reacted positively only with MUT23 strain, whereas the peanut lectin failed to bind with any of the two strains. No binding was observed with the Con A and pea lectin (PsA), both specific for D-mannose. The soybean lectin (SBA) and Castor bean lectin (RcAI) also showed negative reactions with both strains.

The sugar specificity of lectin binding at the surface of MUT23 germ tubes was similar to that of the wild strain. However, the binding of castor bean lectin (RcAII) was positive with MUT23 but not with wild type strain. Manocha et al. (1986) have shown that castor bean lectin RcAII reacts positively with the nonhost, *M. candelabrum*.

Table 7. Summary of direct FITC labelled lectin binding assay of wild type and the mutant (MUT23)

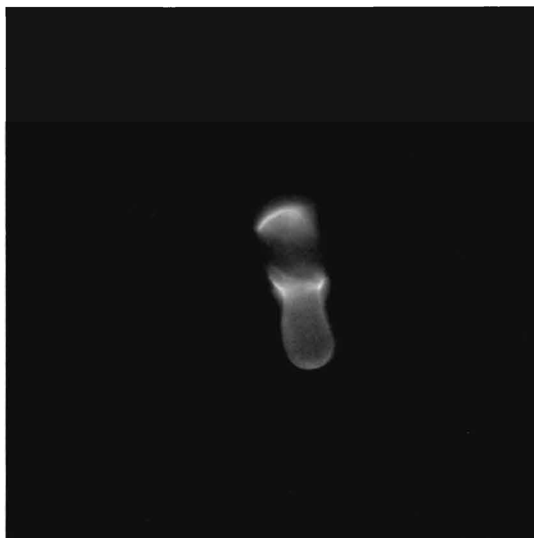
Fluorescence lectin	Sugar-binding specificity	WT.	MUt23
WGA	N-acetylglucosamine oligomers	++	++
PaA	N-acetylglucosamine oligomers	++	++
ConA	D-mannose	-	-
PsA	D-mannose	-	-
PNA	D-galactose	-	-
RCA2	D-galactose	-	+
RCA1	N-acetyl-D-galactosamine	-	-
SBA	N-acetyl-D-galactosamine	-	-
LTA	L-fucose	+	+
UeA	L-fucose	-	-

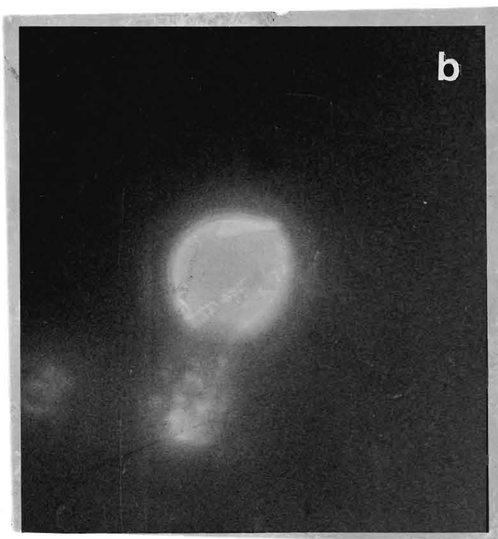
Fluorescence reactions recorded: -, no fluorescence: + to ++, increasingly intense FITC green fluorescence.

Data were from three experiments.

Figure 9. Both germinating spores of wild type (a) and MUT23 (b) showed positive reaction to wheat germ aglutinin

(a)





Interaction with the Mycoparasite

There were marked differences in the mycoparasite's interaction with the wild type and MUT23 strains (Table 8). The parasite germ tube formed appressoria at the point of contact on the cell surface of the wild type strain. However, in the case of MUT23 strain the mycoparasite formed appressoria at the point of contact on the cell surface of the 6 day culture but not on the 2 day culture. Thus, the interaction between MUT23 strain and the mycoparasite was age dependent. The mycoparasite, *P. virginiana* was not able to parasitize young 2 day old cultures of MUT23. MUT23 strain was capable of delaying parasitism by the mycoparasite.

To ascertain the peculiar capability of delayed interaction between the mycoparasite and MUT23 strain, the effect of the culture filtrate of MUT23 strain on the germination of the mycoparasite spore was tested. In the concentrated culture filtrate solution (see Materials & Methods), the germination of mycoparasite spores was inhibited. In contrast, the germination of the mycoparasite spores was stimulated by the culture filtrate of wild type strain, compared with malt-yeast extract used as control (Table 9).

In addition, the germinated spores of the mycoparasite were lysed, after 10 hours following inoculation in concentrated culture filtrate of MUT23 strain. Figure 10 shows the results of lysis of the mycoparasite spores at different time intervals. No lysis was observed in the concentrated culture filtrate of the wild type strain and unconcentrated culture filtrate of MUT23 strain (Table 10).

Table 8. Per cent attachment of *Piptocephalis virginiana* to wild-type and MUT23

Strain (time)	Number of spores	Per cent contacted	Per cent appressoria
WT (2 days)	274 ± 11	68.4 ± 8	49.1 ± 5
MUT23(2 days)	275 ± 14	5 ± 0	0.0 ± 0
WT (6 days)	352 ± 19	64.3 ± 7	58.2 ± 5
MUT23 (6 days)	234 ± 7	64.5 ± 7	40.3 ± 6

% contacted-apparent contacts between the parasite germ tubes and host hyphae.

% appressoria-development of appressoria and penetrations of both cell surfaces by the parasite germining tubes.

Data were the average of three experiments.

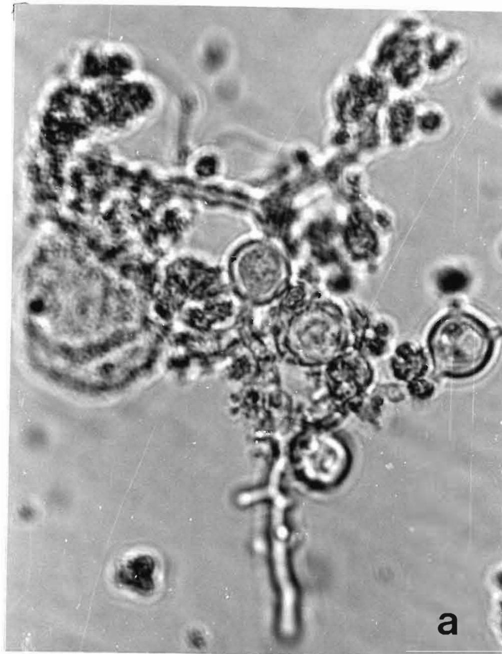
Table 9. Per cent spore germination of *Piptocephalis virginiana* in wild type and the mutant (MUT23) filtrates

Strain	Concentrated Filtrate			Filtrate
	10 X	5 X	2.5 X	
MUT23	11.3 $\pm$0.7	12.5 $\pm$0.9	18.2 $\pm$0.2	31.7 $\pm$0.4
WT.	92.3 $\pm$0.8	90.7 $\pm$0.5	89.4 $\pm$0.4	92.8 $\pm$0.5
Control*	---	---	---	48.5 $\pm$0.7

* Malt-yeast extract filtrate. Data were the average of three experiments.

The two days filtrate of wild type and mutant was dried by lyophilization and prepared in several concentrated filtrate solutions.

Figure 10. Lysis of the germinated spores of the mycoparasite, *Piptocephalis virginiana*, incubate in concentrated filtrate of MUT23. (a) incubated 10 h. (b) incubated 20 h. (c) incubated 30 h (mag. 40X).


a

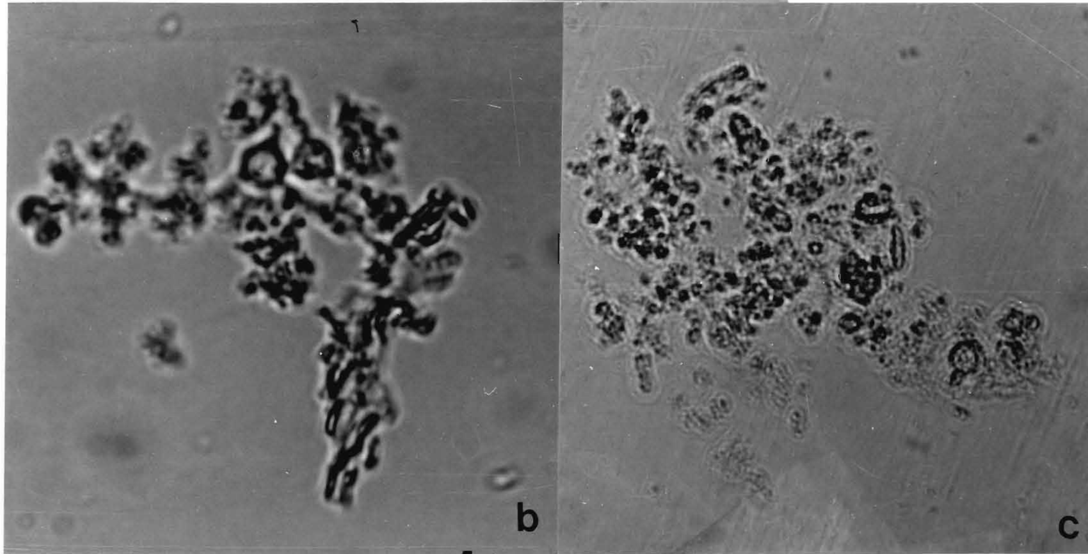

b
c

Table 10. Per cent lysis of germinated spores of Piptocephals virginiana in wild type and mutant filtrate

Strain	Concentrated Filtrate			Filtrate
	10 X	5 X	2.5 X	
MUT23	69.3±5	42.1±5	18.7±4	0.0
WT.	0.0	0.0	0.0	0.0
control*	---	---	---	0.0

*Malt-yeast extract filtrate.

Data were the average of three experiments.

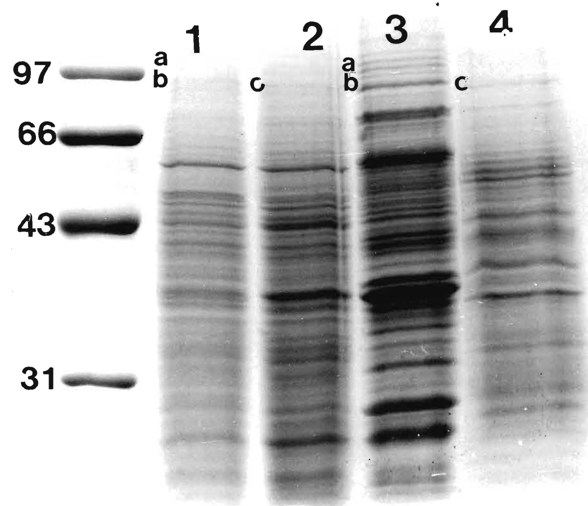
The lysis of *P. virginiana* of the culture filtrate appeared to be heat-labile. Lysis was also observed when the spores and germinated spores were incubated for 5 h on the clear zone area (formed by mutant's growth on a medium containing colloidal chitin).

SDS-PAGE Gel Electrophoresis Analysis

Figure 11 shows patterns of protein bands of wild type and MUT23 (2 and 6 days) strains, and nonhost *M. candelabrum*. Quantitative and qualitative differences were noticed among them. Two protein bands marked as **a** and **b** were observed in wild type (lane-1) and 6 d MUT23 (lane-3). Band **c** could be seen in 2 d MUT23 (lane-2) and *M. candelabrum* (lane-4). The molecular mass of the three proteins **a**, **b**, and **c** were approximately 105, 90, and 85 KDa, respectively. This was proven to be the case as reported earlier by Manocha and Chen (1990). Two prominent bands of electrophoretic mobility were observed in wild type and 6 d MUT23, but not in the nonhost, *M. candelabrum* and 2 d MUT23.

Gel scans (MCID OS/2, 386, Imaging Research Inc.) showed two prominent peaks in both figures of wild type (Figure 6 a) and 6 d MUT23 (Figure 6 b). These peaks were at 50.09 and 48.58 mm. Peak C presented at 41.02 in another two figures, *M. candelabrum* (Figure 6 c)and 2 d MUT23 (Figure 6 d).

Figure 11. SDS-Polyacrylamide gel electrophoresis of proteins of mycelial extract of *Mortierella pusilla* (lane-1); MUT23, 2 d (lane-2); MUT23, 6 d (lane-3); and *M. candelabrum* (lane-4), grown in malt-yeast extract. Gels were stained with Coomassie brilliant blue. The protein standards were purchased from Bio-Rad. The protein standards are as follows: Rabbit muscle phosphorylase b, Mr 97 KDa: Bovine serum albumin, Mr 66 KDa: Hen egg white ovalbumin, Mr 43 KDa; Bovine carbonic anhydrase, Mr 31 KDa.


1
2
3
4
97
66
43
31
a
b
c
a
b
c

Western Blotting

In Western blot analysis (Figure 12), eight distinct chitinase molecules were detected. These chitinases showed the following distinct sizes, Mr 96 KDa; 88 KDa; 61 KDa; 42 KDa; 38 KDa; 33 KDa; 28 KDa; 25 KDa. The chitinase of Mr 42 KDa only showed in the supernatant of the colloidal chitin containing culture.

Recently, nine chitinase species have been identified in germinating cells of *Mucor rouxii* by Pedraza-Reyes and Lopez-Romero (1991). In tobacco plant, *Nicotiana tabaccum L.*, eight chitinase species were detected by polyacrylamide gel electrophoresis (Pan et al.,1992). Six chitinases of a bacterium have also been demonstrated in the culture supernatant of *Bacillus circulans* (Takeshi et al., 1990). These finds strongly suggest a complex chitinolytic system and the association of chitinase isozymes with systemic protection.

Chitinase Production

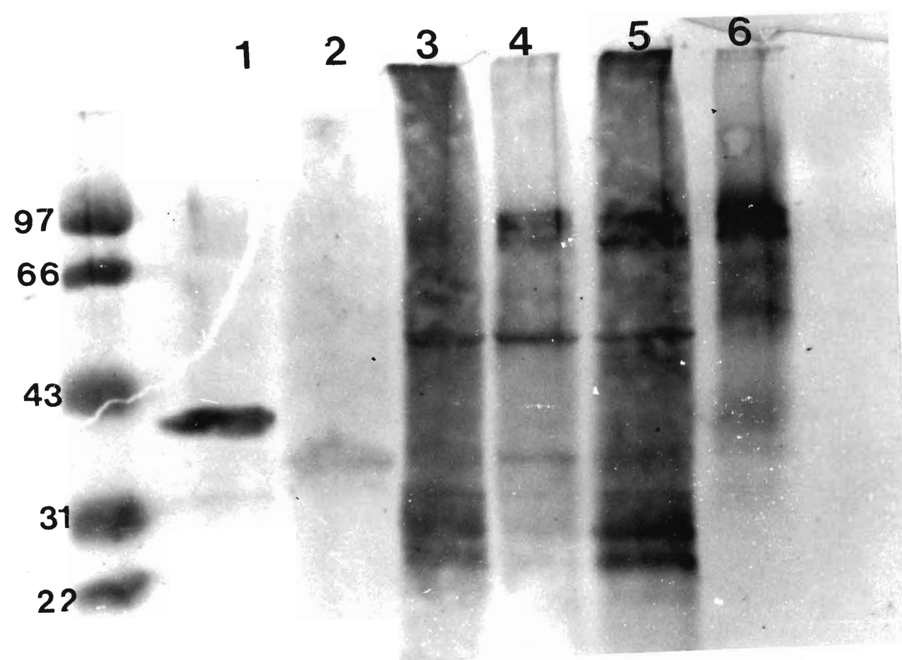
MUT23 showed markedly higher chitinase activity than the wild type strain grown in both, malt-yeast extract and 1% colloidal chitin-containing medium (Tables 11 and 12).

The precipitated culture filtrate of 2 d and 4 d of MUT23 showed much higher chitinase activity than that of wild type cultured in malt-yeast extract. After 6 days, the chitinase production of MUT23 decreased under similar conditions.

Again, MUT23 showed higher chitinase activity than the wild type cultured in chitin-containing medium. Maximum chitinase

activities of MUT23 were 13.5 fold higher at 20 d of the culture then that of wild type.

Figure 12. Western blotting: Lane-1, protein from culture filtrate of MUT23 grown in colloidal chitin medium; Lane-2, protein from mycelial extract of MUT23 grown in colloidal chitin medium; Lane-3, proteins from 2 d mycelial extract of MUT23 grown in malt-yeast extract medium; Lane-4, proteins from 6 d mycelial extract MUT23 grown in malt-yeast extract medium; Lane-5, proteins from wild type grown in malt-yeast; Lane-6, proteins from *M.candelabrum* grown in malt-yeast extract medium.


1
2
3
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5
6
97
66
43
31
22

Table 11. Production of chitinase by wild type and MUT23 grown in malt-yeast extract

Incubation time (d)	Wild type			MUT23		
	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1}protein)	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1} protein)
2	18.2	1002	55.1	12.6	1067	84.7
4	33.4	1740	52.1	13.8	1197	86.7
6	35.3	1772	50.2	26.3	1604	61.0
8	24.7	1189	48.1	12.9	579	44.9

Data were the average of three experiments. Standard deviations were omitted for clarity.

Table 12. Production of chitinase by wild type and MUT23 grown in 1% colloidal chitin medium

Incubation time (d)	Wild type			MUT23		
	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1} protein)	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1} protein)
5	2.7	136	50.7	8.2	652.2	79.5
10	2.3	131	57.1	8.6	922.4	107
15	1.9	104	54.0	4.0	1545.6	301
20	1.9	96.8	50.9	4.0	2753.7	687
30	1.2	53.0	44.0	2.6	1416.8	554

Data were the average of three experiments. Standard deviations were omitted dor clarity.

DISCUSSION

This is the first report of chitinase-secreting morphological mutant in a filamentous fungus. Vasseur et al.(1990) reported that they obtained two chitinase-overproducing mutants from the fungus *Aphanocladium album* by UV irradiation. The deuteromyceteous fungus *Aphanocladium album* produced high levels of extracellular chitinase when grown in minimal medium with crystalline chitin as sole carbon source (Syivastava et al., 1985). The fungus *Mortierella pusilla* does not process this metabolic system to produce more extracellular chitinase when grown in the chitin medium. After two consecutive steps of selection, a stable physiological and chitinase-secreting mutant was isolated. As a host of the mycoparasite, the mutant is a significant tool for understanding the mechanisms of host-parasite interaction.

Formation of chitinase in micro-organisms is thought to be controlled by a repressor-inducer system in which chitin or products of degradation serve as an inducer (Monreal and Reese, 1969). There are few reports on the regulation of chitinase activity in filamentous fungi. St Leger et al. (1986), using slow-feeding with sugar or alanine in a carbon-deficient medium, demonstrated that the chitinase activity in the entomopathogenic fungus *Metarhizium anisopliae* was regulated by products of chitin i.e. N-acetylglucosamine. Smith and Grula (1983) reported that the sterilization of

chitin by autoclaving or boiling causes release of GlcNAc, glucosamine or chitobiose as the inducer for chitinase synthesis.

Vasseur et al.(1990) reported the isolation of a mutant of *Aphanocladium album* with a high level of constitutive chitinase activity, which allowed an early degradation of chitin and early induction of chitinase when compared to the wild-type. Ulhoa and Peberdy (1991) showed in *Trichoderma harzianum* that high chitinase activity was found only in cultures supplied with chitin but not with other polymers such as cellulase and chitosan. They considered that the inducers of chitinase in *Trichoderma harzianum* were probably soluble oligomers derived from the chitin preparation or by the action of constitutive chitinase. In my experiments, high chitinase activity was found only in the mutant growing on chitin-containing medium but not in the wild type under the same conditions. Chitinase activity of the mutant strain was also repressed by addition of glucose, suggesting that catabolic repression may be involved in the regulation of chitinase synthesis. Poor metabolization of GlcNAc in the mutant strain is perhaps due to the presence of readily metabolizable compounds. This phemomenon has been called the glucose effect (Epps and Gale, 1942) or catabolic repression (Neidhardt, 1960, Holzer, 1976).

The basic tenet of Bartnicki-Garcia's (1973) model of apical growth in fungi is the existence of a delicate balance

and the lysis of the microfibrils of the cell wall. This model requires all chitinous fungi to possess chitinase and for the latter's activity to be displayed during growth.

Manocha and Balasubramanian (1988) demonstrated that the chitinase was a membrane-bound enzyme in the mucoraceous species, *Choanephora cucurbitarum* and *Phascolomyces articulosus*. Similar results were obtained by Dickinson et al. (1991) who detected the enzyme in *Candida albicans*. Gooday and Trinci (1980) have proposed that microsomal chitinase and chitin synthase may be regulated in consort to play a morphogenetic role during hyphal growth in *Mucor mucedo*. There are some accounts of chitinase activities that may play a role in regulating chitin deposition in cells, such as a periplasmic chitinase activity from *Saccharomyces cerevisiae* (Elango et al., 1982) and a wall-bound chitinase activity from *Aspergillus nidulans* (Polachek and Rosenberger, 1978). Meanwhile, the inhibitors of chitin synthase from *Mucor rouxii* (Lopez-Romero et al., 1982), *Neurospora crassa* (Zarain-Herzberg and Arroyo-Begovich, 1983) and *Choanephora cucurbitarum* (Manocha and Balasubramanian, 1988) have proved to be chitinases. This coexistence of chitinase with chitin synthase might represent a lytic and synthetic complex, as is required for branch and septum formation and for hyphal apical extension. The steady-state condition for wall addition, wall expansion, and wall rigidification were visualized for hyphal tip growth. The morphogenetic role of chitinases could be

tip growth. The morphogenetic role of chitinases could be cross-linking of chitin to other wall components through glycosidic linkages. Covalent linkages between chitin and glucans have been described for a variety of fungal walls by Wessels and Sietsma (1981). In our case, possibly the general component of wall expansion in the mutant resulted from a low rate of cross-linking and rigidification. The wall addition is less polarized than the hyphal growth in the wild type because more secreting of chitinase in MUT23. Under the light microscope, the mutant exhibited shorter branches. This result suggests that the large chitinase production in the mutant must have associated with morphological change in the filamentous fungus. The present study confirms the results of earlier investigations showing that chitinase plays a morphogenetic role.

Lim et al. (1991) found that the anti-fungal mechanism of the bacterium (*Pseudomonas stutzeri* YPL-1) may involve a lytic enzyme rather than a toxic substance. They showed that chitinase-defective mutant, *P. stutzeri* YPL-M122, did not inhibit fungal growth at all. The chitinase-overproducing mutants, YPL-M26 (UV) and YPL-M178 (MNNG), displayed larger inhibition of mycelial growth of *Fussarium solani* after mutagensis with UV and MNNG. These results led to the conclusion that the antifungal mechanism of *P. stutzeri* is due to chitinase. Several studies have shown that efficient parasitic biocontrol agents excrete extracellular lytic

enzymes that are capable of degrading chitin and laminarin (Horikoshi et al., 1959, Elad et al., 1982, and Ordentlich et al., 1988). Furthermore, the antifungal activity of crude chitinase in mycelial growth was much higher than that of crude laminarinase. In our experiment, MUT23 released much more extracellular chitinase than the wild type, which is a key enzyme in the lysis of mycoparasite. Vogelsang and Barz (1990) have identified the function of chitinase in higher plants against microbial pathogens. They demonstrated that the cells of the resistant line contained a five times higher levels of chitinase activity in comparison to the susceptibility cell culture. After a short log-phase, an appreciable amount of hydrolase activity is released into the medium by the resistant cell culture. This coordinated pattern of hydrolase induction has also been found in various plants (Vogeli-Lange et al., 1988, Mauch et al., 1988). This induction kinetic is considered to be an important prerequisite for an effective inhibition of fungal growth in infected tissues since chitinase and β-1, 3-glucanase act synergistically in the degradation of fungal cell walls (Mauch et al., 1988). Vogelsan and Barz (1990) also assumed that β-1,3-glucanase may be involved in different reactions of cellular metabolism, because of the differences in β-1,3-glucanase and chitinase activated plant cell suspension cultures derived from the resistant and the susceptible chickpea cultivars. Their results illustrated that it seems

results illustrated that it seems likely that β-1,3-glucanases and chitinases play a major role in recognition and defense of the invading pathogen.

Pedraza-Reyes and Lopez-Romero (1990) showed the complex chitinolytic system in germinating cells of *Mucor rouxii*. Eight chitinase species were identified in their work by molecular exclusion and ion exchange chromatography. *Bacillus circulans* WL-12 also secretes a variety of chitinases into culture medium (Watanabe et al., 1990). They detected six distinct chitinase molecules in the culture supernatant, when the chitinases of the bacterium were induced with chitin. Nine chitinases species were shown in our western blotting trails.

An interesting result was that the induction of secreted chitinase of the mutant by the colloidal chitin was different from the constitutive chitinases of the mutant and the wild type when grown in malt-yeast extract medium. Furthermore, the result showed that two distinct chitinase molecular forms could be detected in the culture medium and in the cells, when the mutant strain grew in colloidal chitin containing medium. This simultaneous presence of chitinase isoforms occurs also in green plants (Roby and Esquerre-Tugaye, 1987; Majeau et al., 1990). This diversity of chitinase species could be the result of a simultaneous expression of more than one chitinase gene, or post-translational modification of one primary translation product. Multiple genes could contribute to the diversity of chitinases produced. Miyashita et al.(1991)

described the cloning of genes from *Streptomyces lividans* that specified the production of four chitinases. Initially, we tried to isolate chitinase-negative mutants of *M. pusilla*. However, chitinase-negative mutants could not be isolated. The multiple genes for chitinase in fungi probably result in the failure to isolate chitinase-negative mutants.

Watanabe et al. (1990) demonstrated that chitinase A1 was the key enzyme in chitinase system of *Bacillus circulans* WL-12. Chitinase A1 showed strong affinity to insoluble substrate chitin and degraded colloidal chitin directly into $(GlcNAc)_2$. The different chitinases might have slightly different preference for substrates and functional differences. Daugrois et al. (1990) showed that the induced β-1,3-glucanase activity was a basic enzyme which differs both from the acidic constitutive enzyme in control seedlings and from an acidic enzyme secreted by the bean plant, *Phaseolus vulgaris*. The extracellular chitinase from *Streptomyces sp.* s-84 were two distinct chitinases (A and B). The chitinase A hydrolyzed 4-MU-disaccharide more rapidly than 4-MU-trisaccharide. Chitinase B had the reverse effects (Ueno et al., 1990). Nevertheless, a definite assignment of the individual chitinases to somewhat different functions requires a separate investigation with the highly purified enzymes.

SUMMARY

1. This is the first report of a physiological and chitinase-secreting mutant in a filamentous fungus, *Mortierella pusilla*.

2. The present study confirms the results of earlier investigations showing that chitinase plays a morphogenesis role. The large chitinase production in the mutant was associated with morphological change.

This morphological change was observed on inhibition of growth of the mutant on artificial media by inhibiting its own cell wall synthesis. This inhibition was relieved by the addition of colloidal chitin which probably competed for chitinase binding sites.

3. In the interaction between host and mycoparasite, the extracellular chitinase of host is involved in defense reaction against mycoparasite. The extracellular lytic enzyme was capable of degrading the cell wall of the mycoparasite.

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