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Mycogone perniciosa, a pathogen of *Agaricus bisporus*

by

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DECLARATION

I, the undersigned, declare that the thesis, which I hereby submit for the degree of Master of Science to the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other institution.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
dH_2O	Distilled Water
ELISA	Enzyme-linked Immunosorbent Assay
EM	Electron Microscopy
GC-MS	Gas Chromatography Mass Spectometry
HPLC	High Performance Liquid Chromatography
ISEM	Immunosorbent Electron Microscopy
LM	Light Microscopy
LSD	Least Significant Difference
MA	Mushroom Agar
MDA	Mushroom Dextrose Agar
PAGE	Polyacrylamide Gel Electrophoresis
PDA	Potato Dextrose Agar
PVP	Poly(vinylpyrrolidine)
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
TLC	Thin Layer Chromatography
UV	Ultra Violet
WBD	Wet Bubble Disease

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(□ = mycelial plug; A= *Agaricus bisporus*; M= *Mycogone perniciosa*)

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Figure 5.4.

Thin layer chromatography (TLC) plates.

Plate A: TLC plate of hydrolysate of mycelial extracts of *Agaricus bisporus* and *Mycogone perniciosa* grown in paired culture on PDA in Petri plates migrated with dichloromethane: ethyl acetate at a ratio 1:1; lane1: *A. bisporus* in association with *M. perniciosa*; lane 2: *A. bisporus* control, lane 3: *M. perniciosa* in association with *A. bisporus*; lane 4: *M. perniciosa* control. *M. perniciosa* paired with *A. bisporus* in lane 3 gave darker bands with the same concentration hydrolysate compared to control in lane 4. *A. bisporus* paired with *M. perniciosa* (lane 1) possibly produced a compound not present in *A. bisporus* control (lane 2) indicated by arrow.

Plate B: TLC plate of hydrolysate of PDA media on which *A. bisporus* and *M. perniciosa* were grown in paired culture Petri plates after removal of the bulk of mycelia migrated with dichloromethane: ethyl acetate at a ratio 1:1; lane 1: *A. bisporus* in association with *M. perniciosa*; lane 2: *A. bisporus* control, lane 3: *M. perniciosa* in association with *A. bisporus*; lane 4: *M. perniciosa* control. The media where *M. perniciosa* was paired with *A. bisporus* (lane 3) had a higher content of all compounds compared to control (lane 4). In addition *M. perniciosa* paired with *A. bisporus* (lane 3) fluoresced under UV light (not shown).

Plate C: TLC plate of hydrolysate of mycelial extracts of *A. bisporus* and *M. perniciosa* grown in paired culture on PDA in Petri plates migrated with hexane: ethyl acetate at a ratio 5:1 lane 1: *M. perniciosa* in association with *A. bisporus*; lane 2: *M. perniciosa* control; lane 3: *A. bisporus* control; lane 4: *A. bisporus* in association with *M. perniciosa*. *A. bisporus* in association with *M. perniciosa* (lane 4) was the only

hydrolysate with compounds visible on the TLC plate, indicating that there is a difference between this sample and the *A. bisporus* control.

Plate D: TLC plate of hydrolysate of nutrient broth liquid media in which *A. bisporus* and *M. perniciosa* were grown either separately or together, samples were migrated with dichloromethane: ethyl acetate at a ratio 1:1; lane 1: *A. bisporus* control, lane 2: *A. bisporus* in the same liquid culture with *M. perniciosa*, lane 3: *M. perniciosa* control. The hydrolysates contained the same compounds in different concentrations, except for an uncharacterised compound in lane 2 (arrow) that was produced when *M. perniciosa* and *A. bisporus* grew in the same culture but was absent when both these fungi grew alone.

CHAPTER 1
GENERAL INTRODUCTION

Agaricus bisporus (Lange) Imbach is the most commonly cultivated mushroom in South Africa and many other places worldwide. Cultivating the edible mushroom, *A. bisporus* is a highly specialised process, aimed at producing the maximum yield of perfectly formed mushrooms (van Greuning, 1990). Success of production is dependant upon many interacting factors, of which diseases caused by fungal pathogens play a significant role.

Mycogone perniciosa (Magnus) Delacroix is the fungal pathogen causing wet bubble disease (WBD) in crops of commercial *A. bisporus* mushrooms worldwide (Fletcher *et al*., 1995). The disease is very contagious and results in severe crop loss (Umar *et al*., 2000). Mushrooms with wet bubble disease are very misshapen and not fit for sale (Dielemann-van Zaayen, 1976). The disease causes important economic losses in the South African mushroom industry and other mushroom industries across the globe.

The drastic changes in the shape of mushrooms infected with *M. perniciosa* including wet bubble, malformation, white, fluffy mycelial growth and amber droplets (Fletcher *et al*., 1994) have been investigated since the 1900s (Smith, 1924). It is known that *M. perniciosa* produces copious, flocculent mycelia on most substrata (Fletcher *et al*., 1994; Smith, 1924), while *A. bisporus* does not readily grow on agar media. However, the interaction between these fungi *in vitro* has not been described.

In this study different spore types of *M. perniciosa* were investigated, their morphology and development were studied using scanning electron and light microscopy. Interactions between *M. perniciosa* and *A. bisporus* grown in culture as well as in mushroom fruiting bodies, naturally infected with the pathogen, and infected under simulated commercial cultivation conditions were studied using light and electron microscopy in an effort to better understand WBD.

Growth of *M. perniciosa* and *A. bisporus* on agar media in paired, dual and half plate cultures on different media, was studied to get a better understanding of the effects these fungi have on each other *in vitro*. The enzymes secreted by *M. perniciosa* were investigated in order to achieve a better understanding of the pathogen's ability to enter and infect *A. bisporus*. Chemical components produced by *M. perniciosa* and *A. bisporus* when grown together were investigated using thin layer chromatography as an initial effort to elucidate the biochemical basis of the host-pathogen interaction in wet bubble disease.

The main aim of this study was to investigate interaction between *M. perniciosa* and *A. bisporus* in an effort to better understand the process of infection and subsequent manifestation of wet bubble disease.

The objectives were:

◊ Investigate the effect vegetative growth of *A. bisporus* has on *M. perniciosa*, and *vice versa*, on different agar media in paired, dual and half plate culture

◊ Examine the hyphal interactions between the host and the pathogen *in vitro* and *in vivo* using light and scanning electron microscopy

◊ Conduct enzyme assays to determine which enzymes *M. perniciosa* produces and whether it has the ability to degrade compounds present in *A. bisporus* cell walls

◊ Study the stimuli and volatiles involved in interactions between the host and pathogen in paired, dual and half plate culture and their effect on mushrooms cultivated under simulated commercial conditions.

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CHAPTER 2
LITERATURE REVIEW

2.1. INTRODUCTION

Agaricus bisporus (Lange) Imbach, the button mushroom is the most commonly cultivated mushroom worldwide (Sanchez, 2004). Cultivation is a highly specialised process, and commercial growing is done under strictly controlled environmental conditions (Eicker, 1990). The aim is always to produce a maximum yield of perfectly formed mushrooms (van Greuning, 1990). The success of mushroom production is dependant upon many interacting factors, of which disorders caused by pests and pathogens play a significant role (Muthumeenakshi and Mills, 1995). Fungi are probably the most important group of pathogens and their effect is most obvious on mushroom fruiting bodies (sporophores, basidiomes, hereafter referred to as mushrooms) rather than on mushroom mycelium (Fletcher *et al*., 1989). Farm hygiene, chemical and environmental control remains essential components of a disease control strategy (Muthumeenakshi and Mills, 1995). Changes in cultural practices within the mushroom industry often result in a shift in prevalence of pathogenic fungi. Production in different parts of the world is often threatened by common pathogens, which are rarely eradicated (Muthumeenakshi and Mills, 1995).

2.2. THE CULTIVATED MUSHROOM, *Agaricus bisporus*

2.2.1. HISTORY

The cultivated mushroom is classified by Alexopoulos and Mims (1996) as follows

Kingdom: Fungi
Phylum: Basidiomycota
Order: Agaricales
Family: Agaricaceae
Genus: *Agaricus*
Species: *bisporus*

The term mushroom was initially used to describe only the edible fungi originating from the order Agaricales, while poisonous varieties were referred to as toadstools (Eicker, 1990). *A. bisporus* was first cultivated in the seventeenth century when Louis XIV encouraged people to grow mushrooms in their outdoor gardens (Pacioni, 1985). The first book describing cultivation of mushrooms was written by De Tournefort in 1707, and in 1754 the first specialised building for mushroom growing was described (Eicker, 1990). Mrs. Robertson was the first person to grow *A. bisporus* in South Africa in 1940 (Eicker, 1990). Her operation was later bought by Monty Denny, who became one of the leading mushroom growers in South Africa. Today mushroom growing is a fully-fledged industry in South Africa (Mr. R. Richardson, personal communication, Highveld Mushrooms, South Africa) as well as other places across the globe (Pacioni, 1985).

2.2.2. CULTIVATION

Mushrooms are heterotrophic organisms grown on a highly complex compost substrate (Eicker, 1990). Raw materials that are used to make compost include wheat or barley straw and poultry manure. Nitrogenous substances and calcium sulphate are added to ensure a selective substrate for optimal development of the mushrooms (Pacioni, 1985; Eicker, 1990). Composting consists of two distinct phases i.e. phase I and phase II (Fletcher *et al*., 1989). The aim of phase I is to adjust the total nitrogen to between 1.8 and 2% and the moisture content to 75% (Eicker, 1990). Phase I composting lasts from 7 to 14 days, depending on the nature and characteristics of the material at the start (Wuest *et al*., 1980). There are two major purposes of phase II composting; the first is pasteurisation to kill insects, nematodes and other pests that may be present, the second is to lower the ammonia concentration formed during phase I to levels that are not inhibitory to mushroom spawn growth (Wuest *et al*., 1980). Due to advanced mechanisation on some South African farms the composting process is adjusted and controlled according to their cultural practices (Mr. R. Richardson, personal communication, Highveld Mushrooms, South Africa).

2.3. DISEASES OF THE CULTIVATED MUSHROOM

This work aims to provide the reader with a broad knowledge of the literature available on wet bubble disease. A brief overview of other common diseases of *A. bisporus* is provided merely as background and to acquint the reader with the problems faced by the mushroom industry. It is by no means a comprehensive study of all the literature available on diseases of the cultivated mushroom.

2.3.1. VIRAL DISEASES

Viruses as a cause of disease of mushrooms was first described in 1950 (Fletcher *et al*., 1989). To date more than five types of virus particles have been reported to infect commercial mushrooms (Howard *et al*., 1994) and are differentiated according to the size and shape of the particles (Fletcher *et al*., 1989). In many cases, viral diseases are so devastating that mushroom growers have to cease production temporarily in order to eradicate the problem (Howard *et al*., 1994).

Various symptoms are attributable to virus disease; the most diagnostic is reduced yields (Fletcher *et al*., 1989). Positive identification of mushroom viruses is available through comparative growth rates of mycelium on agar, electron microscopy (EM), immunosorbent electron microscopy (IEM or ISEM), polyacrylamide gel electrophoresis (PAGE), enzyme-linked immunosorbent assay (ELISA) (Howard *et al*., 1994) as well as molecular characterization including PCR based diagnostic techniques (Goodin *et al*., 1997). There are two possible means of viral disease transmission. The first is from mycelium to mycelium through hyphal fusion (anastomosis) and the second is by transfer in spores, which germinate and pass the virus to healthy mycelium, again by anastomosis (Fletcher *et al*., 1989). Control of viral diseases can be accomplished by a strict hygiene program, diseased mushrooms must not be allowed to open and release their spores (Howard *et al*., 1994).

Two viral diseases of importance are La France disease (Howard *et al*., 1994) and the serious disease phenomenon caused by the novel double-stranded RNA (dsRNA) virus, termed mushroom virus X (MVX) (Gaze *et al*., 2000). La France isometric virus (LIV) has 36-nm isometric particles that encapsidate nine dsRNAs, three of these have been completely sequenced (Goodin *et al*., 1997). The MVX disease is associated with 26 dsRNA elements, likely to represent a complex of more than one virus (Adie *et al*., 2004). Viral particles specifically associated with MVX are non-encapsidated (Grogan *et al*., 2003).

2.3.2. BACTERIAL DISEASES

The two most common bacterial diseases of *A. bisporus* are mummy disease and bacterial blotch (Doores *et al*., 1987; Eicker, 1990).

2.3.2.1. Bacterial blotch

Bacterial blotch (brown blotch) is caused by *Pseudomonas tolaasi* (Eicker, 1990; Munsch *et al*., 2000). *Pseudomonas tolaasi* is an anaerobic, Gram-negative, non-sporing rod. It is fluorescent, oxidase and arginine dihydrolase negative and does not grow below 4ºC (Howard *et al*., 1994). The most characteristic symptom of bacterial blotch is the occurrence of brown areas (blotches) on the surface of the cap (Fletcher *et al*., 1989). Symptoms occur more frequently on mushrooms that remain wet for a long time and in places where they touch one another (Howard *et al*., 1994).

Pseudomonas tolaasi is a natural inhabitant of the peat used for casing material (Howard *et al*., 1994). The pathogen appears to survive between crops on surfaces, in debris, on tools and on various structures (Fletcher *et al*., 1989). It can be spread through airborne dust particles (Eicker, 1990), sciarids and mites (Fletcher *et al*., 1989), contaminated harvesting equipment and the hands of pickers (Howard *et al*., 1994). Brown blotch is best managed by manipulating the growing environment (Howard *et al*., 1994). No free water should be left on the mushrooms, the relative humidity of the growing room should not be too high and mushrooms should dry

quickly after watering (Eicker, 1990). Adding sodium hypochlorite at a concentration of 150mg.dm^{-3} chlorine will keep the bacterial population levels low (Eicker, 1990). Much research has been done on control of the disease and although many control methods are known, none of them are completely successful and brown blotch disease remains a problem in the mushroom industry (Lee *et al*., 2002).

2.3.2.2 Mummy disease

Mummy disease is considered to be caused by a fluorescent pseudomonad, although no one has satisfied Koch's postulates (Howard *et al*., 1994). The symptoms of mummy disease are fairly distinctive (Fletcher *et al*., 1989). Affected mushrooms have curved stems and tilted caps (Howard *et al*., 1994). The base is covered by fluffy, white mycelium growth and there is a marked decrease in the mushroom crop (Eicker, 1990). Mummy disease is often observed in crops where the compost is exceptionally wet after pasteurisation (Howard *et al*., 1989). Control measures include digging a 20cm wide trench on either side of the affected area, thereby isolating the infected area (Fletcher *et al*., 1989). Following a strict hygiene regime on the farm proves to be an effective control measure (Eicker, 1990).

2.3.3. FUNGAL DISEASES

Fungi are the most important group of pathogens of the mushroom, occurring wherever the crop is grown (Fletcher *et al*., 1989). The management of fungal diseases of mushrooms poses problems, because both the host and pathogen are fungi (Bhatt and Singh, 2000). Fungal diseases include cobweb, green mould, mat and confetti, yellow mould, truffle, dry bubble, wet bubble, shaggy stipe, gill mildew and cap spotting.

2.3.3.1. Cobweb

Cobweb (soft mildew) is caused by *Cladobotryum dendroides* (Bull.) Gams (syn. *Dactylium dendroides* (Bull.) Fr.) (teleomorph *Hypomyces rosellus* (Alb. &

Schwein.) Tul.) (Howard *et al*., 1994; Grogan and Gaze, 2000). The disease is characterized by the growth of coarse mycelium covering affected mushrooms (Fletcher *et al*., 1989). Cobweb mycelium turns pink with ageing whilst affected mushrooms turn brown and rot (Howard *et al*., 1994). The pathogen is a soil-inhabiting fungus with two- or three-celled hyaline conidia, originating on erect, simple or branched conidiophores (Howard *et al*., 1994). The spores are dispersed by air, water splash, debris, infected casing material and pickers (Fletcher *et al*., 1989; Howard *et al*., 1994). Cobweb is controlled by managing hygiene and by the use of fungicides (Fletcher *et al*., 1989). Resistance of *C. dendroides* has led to research on the complex interactions between fungicide resistance, fungicide persistence in mushroom casing and on-farm disease management (Grogan and Gaze, 2000).

2.3.3.2. Green mould

Trichoderma diseases are characterized by the production of large quantities of dark green spores, hence the name green mould (Fletcher *et al*., 1989). *Trichoderma* species that are problematic in mushroom growing include *T. harzianum* Rifai (teleomorph *Hypocrea vinosa* Cook), *T. koningii* Oudem (teleomorph *H. ceramica* Ellis & Everh.) and *T. viride* (Pers.) Fr. (teleomorph *H. rufa* (Pers.) Fr.) (Howard *et al*., 1994). *A. bisporus* is highly susceptible to *T. harzianum* (Savoie and Mata, 2003). The vegetative mycelium of this fungus is septate, hyaline and initially grey-white, changing to grey-green when conidia develop (Howard *et al*., 1994). The growth requirements for the different species are not the same and the optimum growth temperature ranges from 22 to 26ºC (Fletcher *et al*., 1989). The fungus favours a pH below 6 and a carbon to nitrogen ratio (C:N) of 22:1 (Howard *et al*., 1994). *Trichoderma* spp. are found in soil and organic matter and dispersed by air currents, water, mites and mechanical means (Howard *et al*., 1994). The aim of control programmes is to eliminate inoculum by adopting suitable hygiene programmes (Fletcher *et al*., 1989). Correct compost preparation should reduce the C:N ratio to 15:1, thereby making conditions unfavourable for green mould (Howard *et al*., 1994).

2.3.3.3. Mat and confetti

Mat and confetti are diseases caused by *Chrysosporium luteum* (Constantin) Carmich. and *C. merdarium* (Ehrenb.) Carmich. respectively (Howard *et al*., 1994). Both diseases occur infrequently. Primordia do not form in the casing; consequently, mushrooms do not develop. Instead white mycelial mats of *Chrysosporium* are formed on top of the compost in the case of mat disease and interspersed throughout the compost in case of confetti (Howard *et al*., 1994). *Chrysosporium* spp. infest compost at spawning and infect after the casing is applied (Howard *et al*., 1994). The spores of these pathogens are readily air-borne (Howard *et al*., 1994). When present in compost, these diseases can only be managed through pasteurisation; otherwise management is based on reducing spore inoculum in the area surrounding the spawning operation (Howard *et al*., 1994).

2.3.3.4. *Sepedonium* yellow mould

Sepedonium yellow mould leads to poor yields (Wuest, 1982). The white mould turns dull yellow to tan with age (Howard *et al*., 1994). Spores of the pathogen can be spread to the compost by air currents prior to or during the filling operation, during the spawning operation or with unpasteurized compost sticking to wooden boards or trays (Wuest, 1982). Yellow mould can be prevented through careful attention to hygiene and by proper air filtration (Howard *et al*., 1994). Preventing spores from entering mushroom houses during spawning and spawn-running is also a means of control (Wuest, 1982).

2.3.3.5. Truffle

Truffle disease or false truffle is caused by *Diehliomyces microsporus* (Diehl & E.B. Lamb.) Gilkey (Wuest, 1982). Truffle is infrequently encountered in commercial mushroom production, but when present causes significant yield losses (Howard *et al*., 1994). The first symptom of false truffle is often the patchy appearance of the crop, with areas of the affected bed failing to produce mushrooms (Fletcher *et al*.,

1989). Mushrooms on the periphery of such areas experience premature opening of their veils and the stems are thicker than normal (Howard *et al*., 1994). The fungus grows concurrently with the vegetative mycelium of the host *A. bisporus*, forming ascocarps throughout the compost (Howard *et al*., 1994). Once the disease occurs, the only effective way to eliminate it from a farm is to sterilize the compost and woodwork at 75ºC for eight hours (Wuest, 1982).

2.3.3.6. Dry bubble

Dry bubble is one of the most significant fungal diseases. It is caused by the pathogen *Verticillium fungicola* var. *fungicola* (Preuss) Hassebr. previously known as *V. malthousei* (Wuest, 1982). Disease severity is not restricted to a specific time of the year, nor is it restricted to a specific type of casing material or mushroom strain (Wuest, 1982). The various common names for the disease (brown spot, fungus spot, verticillium spot, split stipe and dry bubble) are descriptive of symptoms resulting from infections at different stages of mushroom development (Howard *et al*., 1994).

Early infection of the developing mushroom primordium disrupts its growth, causing it to form a ball-like mass (dry bubble), 0.5 to 1.0 cm in diameter (Howard *et al*., 1994). When affected at a later stage, mushrooms are often imperfectly formed with partially differentiated caps, or with distorted stipes and tilted caps (Fletcher *et al*., 1989). When maturing mushroom caps are infected cinnamon-brown spots develop on the cap (Wuest, 1982). These necrotic lesions will eventually produce a greyish-white bloom of *Verticillium* spores (Howard *et al*., 1994).

Verticillium is carried onto the farm by infected casing soil and spread by spores in ventilation air, sciarid and phorid flies, dirty equipment, and hands and clothing of pickers (van Griensven, 1988). Hygiene is of utmost importance since the sticky spores cannot be removed by washing hands with hot, soapy water (Howard *et al*., 1994).

Dry bubble has been controlled through the use of chemicals, cultural practices and sanitation (Gea *et al*., 2005). One key to *Verticillium* control is knowledge that 10 to 14 days will pass from the time of infection before symptoms appear (Wuest, 1982). Cultural control is obtained by sanitizing all casing equipment and preventing insects from entering production rooms (Howard *et al*., 1994). Further spreading can be prevented by covering affected mushrooms with salt or pots. It is essential to push pots well into the casing to prevent the sideways drainage of water, which distributes spores (Fletcher *et al*., 1989). If the disease is severe the crop should be terminated by raising the compost temperature to 70ºC for 12 hours (Howard *et al*., 1994).

Chemical control can be used as a last resource to fight disease (Eicker, 1990). Almost all populations of *V. fungicola* are resistant to the benzimadazole fungicides, rendering this group of fungicides ineffective to use (Fletcher *et al*., 1989). Procloraz manganese gives good control of dry bubble and there are no records of fungicide resistance (Fletcher *et al*., 1989), however, there is *in vitro* evidence which points to a decrease in sensitivity to this fungicide (Gea *et al*., 2005). Dusting floors, bed surfaces and mushrooms with 15% zineb is also an effective means of control (Eicker, 1990).

2.3.3.7. Shaggy stipe, gill mildew and cap spotting

Shaggy stipe, gill mildew and cap spotting are less common fungal diseases (Howard *et al*., 1994). Shaggy stipe caused by *Mortierella bainieri* Constantin is characterised by the peeling of the stalk of affected mushrooms. The coarse grey-white mycelium of the pathogen can usually be seen growing over the affected mushroom tissue and also over the surrounding casing (Fletcher *et al*., 1989). *Cephalosporium* spp., the fungus responsible for gill mildew, causes stunting of gills with a growth of white mycelium on their surfaces (Howard *et al*., 1994; Fletcher *et al*., 1989). *Aphanocladium album* (Preuss) Gams and *Hormiactis alba* (Preuss) are fungi associated with cap spotting. Brown, circular and irregular spots about 10mm in diameter are symptoms of these two fungi, respectively. All these minor diseases can be readily controlled by spot-treatment with salt (Fletcher *et al*., 1989).

2.3.4. WEED MOULDS

There are several non-infectious fungal diseases of commercial mushrooms, all of which are usually referred to as indicator or weed moulds. Production can be significantly affected by their presence, but the mushroom itself is not generally infected (Howard *et al*., 1994).

2.3.4.1. Moulds chiefly in compost

2.3.4.1.1. Ink caps

Coprinus fimetarius Fr., *C. radiatus* (Bolton) Gray and other *Coprinus* spp. are the ink cap fungi, regarded as indicator moulds (Wuest, 1990). Ink caps appear if the compost contains too much nitrogen, this may be due to the use of too much chicken manure, too wet, too dry or too compacted compost and if the peak heating period is too short (van Griensven, 1988). *Coprinus* spp. are coprophillic (manure-loving) fungi (van Griensven, 1988) that can readily use free ammonia and grow in compost with an optimum pH of 8. Greater than 700 ppm of ammonia at spawning can stimulate ink cap production (Howard *et al*., 1994). Vegetative *Coprinus* spp. produce a luxurious growth of white, fine mycelium in or on the compost before or after spawning (Wuest, 1990).

Fruiting bodies of this mould generally develop after casing and before mushrooms are produced (Howard *et al*., 1994). Each pin develops into a mushroom with a narrow white stem and a scaly white to grey conical cap. The cap remains closed until the stem matures, at which time it opens umbrella-like releasing spores into the air. Once open the cap immediately starts to autodigest exuding the ink black liquid characteristic of this genus (Wuest, 1990).

Proper peak heating of good quality compost prevents the occurrence of ink caps, but if they do appear, they usually disappear by themselves, for example, when the

excess ammonia has disappeared from the compost. The mushroom mycelium then colonizes the previously infected area (van Griensven, 1988). The presence of a few ink caps however, has no effect on mushroom yield. It indicates that the compost has desirable nitrogen content, but that the ammonia has not completely dissipated before spawning (Howard *et al*., 1994). Where problems are severe a suitable hygiene program should be employed (Fletcher *et al*., 1989).

2.3.4.1.2. Olive-green mould

Olive green moulds are an indicator of poor-quality compost (van Griensven, 1988). The mould appears if phase II compost is improperly managed, especially where ventilation is inadequate (Wuest, 1990). *Chaetomium* is stimulated by oxygen concentrations lower than 16% combined with temperatures higher than 62ºC during phase II composting (van Griensven, 1988). These conditions permit the production of substances toxic to spawn growth, but favoured by *Chaetomium* (Wuest, 1982).

The mycelium in the compost is greyish-white and fine. Within 14 days of spawning, olive-green perithecia visible to the naked eye will appear on the straw (Howard *et al*., 1994). The heat tolerant spores of the fungus can survive 60ºC for six hours (Wuest, 1982) and can be carried along in air flows, clothes and other material (van Griensven, 1988). The olive-green mould fungus survives and thrives in conditions adverse for spawn growth (Howard *et al*., 1994) often occurring in places where compost is black and mushroom mycelium is not growing (van Griensven, 1988).

Once the compost is infested, it is not possible to control olive-green mould (Howard *et al*., 1994). Attention therefore has to be paid to prevention (van Griensven, 1988). Preparation of good compost during phase I, attention to filling trays, shelves or tunnels, and providing adequate aeration during phase II composting will prevent the occurrence of olive-green mould (Howard *et al*., 1994). Good hygiene must also be ensured, and cooking out at the end of the harvest is important (van Griensven, 1988).

2.3.4.1.3. *Penicillium* mould

Penicillium species are opportunistic fungi, which prefer simple carbohydrates but will also grow on cellulose, fats and lignin (Howard *et al*., 1994). This characteristic green mould is commonly seen growing on trays or the sideboards of shelves, or pieces of mushroom tissue left on the casing surface, and in grains of spawn. Apart from the appearance and some inconvenience, this mould has no measurable effect on crop yield or quality (Fletcher *et al*., 1989).

2.3.4.2. Moulds on compost and casing

2.3.4.2.1. Black whisker mould

This mould, *Doratomyces microsporus* (Sacc.) Morton, is so named because of the dark-grey to black whisker-like bristles produced on the surface of the casing (Fletcher *et al*., 1989). Black whisker mould in compost indicates an unbalanced nutritional base – specifically, the presence of certain carbohydrates – in the compost at spawning time. When *Chaetomium* green mould is present, whisker mould will often also be present, since both are cellulolytic fungi (Wuest, 1982).

Heavily infested compost will appear grey to black because of the high density of spores. When disturbed, the spores are released, resembling smoke. Human allergic responses to the spores have been reported (Howard *et al*., 1994). The fungus is thought to be antagonistic to mushroom mycelium and may affect yield, the full significance of this fungus however, is not understood (Fletcher *et al*., 1989).

2.3.4.2.2. Brown mould

Oedocephalum spp. in compost indicates that ammonia and amines were not completely eliminated during phase II, and are serving as food for this organism (Wuest, 1982). Brown mould is silver grey initially, but as the spores mature the

colour changes to a dark tan, beige or light brown (Howard *et al*., 1994). Conditions favouring *Oedocephalum* growth are not optimal for mushroom growth (Wuest, 1982) therefore techniques favouring the production of good compost will reduce the occurrence of this fungus (Howard *et al*., 1994).

2.3.4.2.3. Lipstick mould

The fungus *Sporendonema purpurascens* (Bonord.) Mason appears on the compost during spawn run or on the casing during production (Howard *et al*., 1994). The fungus produces a fine, white mycelial growth rather indiscernible from mushroom mycelium (Fletcher *et al*., 1989). The crystalline colonies may resemble frost on a windshield or small white cotton balls on straws or casing (Wuest, 1982). Its white colour changes gradually to bright pink and finally buff, with a powdery appearance as the spores are produced (Fletcher *et al*., 1989). Dissemination of this fungus is through spores and any means that transmit them can spread the organism (Howard *et al*., 1994). The presence of this mould in a mushroom crop causes great damage (van Griensven, 1988). Raising the temperature in peak-heat to 65ºC for four hours can eliminate lipstick mould, but this will increase the chances of other weed moulds developing (Fletcher *et al*., 1989). Formalin can be used to spot-treat small areas of lipstick mould (Howard *et al*., 1994) but strict attention to hygiene remains the most effective and safest approach to control (Fletcher *et al*., 1989).

2.3.4.2.4. Plaster mould

White plaster mould, *Scopulariopsis fimicola* (Costantin & Matr.) Vuill. and brown plaster moulds, *Papulaspora byssina* Hotson and *Scopulariopsis brevicaulis* (Sacc.) Bainier develop in mushroom compost when nitrogen sources from phase I are not completely utilised by the microbes during phase II and, in particular, when the nitrogen is not converted into microbial protein (Wuest, 1982). White plaster mould produces dense white patches of mycelium and spores on the casing surface and in the compost. In contrast with other weed moulds the mycelium of *S. fimicola* remains white (Fletcher *et al*., 1989).

Brown plaster mould characteristically produces large, dense, roughly circular patches of mycelium on the surface of the casing, initially whitish, but turning brown and powdery with age; it can also colonise the compost (Fletcher *et al*., 1989). Both plaster moulds grow well in compost with a pH of 8.0 or higher and reduce yields by competing with mushroom mycelium (Fletcher *et al*., 1989; Howard *et al*., 1994). It is not possible to control plaster moulds during growing, so great attention must be paid to prevention (van Griensven, 1988). Modification of composting practices to improve compost quality reduces the occurrence of plaster moulds (Wuest, 1982).

2.3.4.3. Moulds chiefly in and on casing

2.3.4.3.1. Cinnamon brown mould

Cinnamon brown mould, *Chromelosporium fulvum* (Link) McGinty, perfect stage – *Peziza ostracoderma* Korf is found mainly on casing soil just after casing (van Griensven, 1988). It has been observed on the surface of the compost during spawn run (Howard *et al*., 1994). High air humidity in excess of 90 to 95% and a high temperature usually found in the first one and a half weeks after casing, are ideal for the development of this mould (van Griensven, 1988).

The fungus first appears as fine, white, aerial mycelium on the compost or casing (Howard *et al*., 1994). Within a few days spores form and the colour changes from white to light yellow or light golden brown (Wuest, 1982). The spores can be carried along in the air and infect new crops or cause secondary infections (van Griensven, 1988). Serious outbreaks on mushroom farms are usually indicative of poor hygiene or associated with over pasteurized casing material (Howard *et al*., 1994). Control is obtained by ensuring the relative humidity in the growing room does not exceed 95% (van Griensven, 1988). The casing material can also be treated with a 2% or less solution of commercial formalin (37% formaldehyde) (Howard *et al*., 1994).

2.4. THE WET BUBBLE PATHOGEN, *Mycogone perniciosa*

Mycogone perniciosa (Magnus) Delacroix causes wet bubble disease of *Agaricus bisporus* (Lange) Imbach. The disease is very contagious and results in severe crop loss (Umar *et al*., 2000).

2.4.1. HISTORY

Mycogone disease of mushrooms, also called wet bubble disease and La Molé, is caused by *M. perniciosa.* The disease has been known for many years, and is reported worldwide wherever *A. bisporus* is grown (Fletcher, *et al*., 1995). *M. perniciosa* causes distortion of affected mushrooms and the characteristic undifferentiated lumps of tissue which Smith (1924), called sclerodermoid mushrooms (Fig. 2.1; 2.2; 2.3 and 2.4). According to Smith (1924) the earliest scientific records of *Mycogone* disease of mushrooms are those of Magnus (1888) and Cooke (1889). Magnus was unable to identify the fungus, and although the ascus stage was not seen, he named the teleomorph stage *Hypomyces perniciosa* by analogy with *H. chryosospermus* (Eicker *et al*., 1989 and Smith, 1924). Cooke stated that the fungus he found resembled both *M. rosea* Link and *M. alba* Letell but differed in having amber-coloured chlamydospores (Smith, 1924). Constantin and Dufour (according to Smith, 1924) made an exhaustive study of the disease and after growing the fungus side by side with *M. rosea* decided that it was a distinct species and named it *M. perniciosa* (Magnus).

Figure 2.1. Mushroom infected with *Mycogone perniciosa* (left) compared to healthy *Agaricus bisporus* mushroom (Photo: Z. Pieterse).

Figure 2.2. Cross-section through mushroom infected with *Mycogone perniciosa* (left) compared to healthy mushroom (Photo: Z. Pieterse).

Figure 2.3. Mushroom infected with *Mycogone perniciosa* (Umar and van Griensven, 1999).

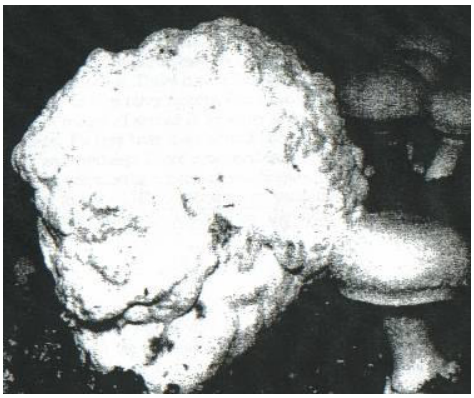

Figure 2.4. Mushroom infected with *Mycogone perniciosa* (left) compared to healthy *Agaricus bisporus* mushroom (Fletcher *et al*., 1994).

2.4.2. MORPHOLOGY

Mycogone perniciosa produces a copious flocculent mycelium on most substrata (Smith, 1924). In the early stages this mycelium is white, but changes to a light amber brown. Fletcher *et al*. (1995) found that colonies can have either an even or uneven edge and the amount of aerial mycelium varies from dense to sparse, while

the colour of colonies can be yellow-brown, dark brown, pale brown or white (Fig. 2.5 and 2.6).

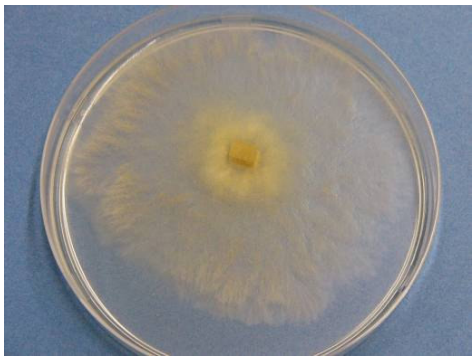

Figure 2.5. Yellow-brown *Mycogone perniciosa* mycelium on PDA (Photo: H. du Plooy).

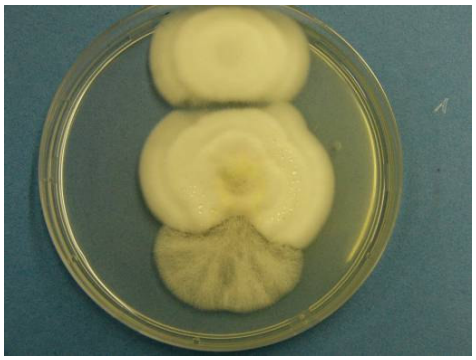

Figure 2.6. White *Mycogone perniciosa* mycelium on PDA (Photo: H du Plooy).

2.4.2.1. Spore types and properties

According to Smith (1924), Constantin and Dufour investigated the fungus in 1892 and found that it exhibited two forms of spores on the mushroom.

Gray and Morgan-Jones (1980) reported that *M. perniciosa* produces small thin-walled conidia (referred to as phialoconidia or phialospores) on *Verticillium*-like conidiophores (Fig. 2.7) together with much larger bicellular conidia (commonly referred to as either aleuriospores or chlamydospores, hereafter referred to as chlamydospores). Holland and Cooke (1991) described additional spore forms of *M. perniciosa*, including lateral smooth conidia (Fig. 2.8), intercalary chlamydospores (Fig. 2.9) and arthroconidia (Fig. 2.10). These spore types have not been reported since by any other investigators.

Figure 2.7. Light Microscopy photograph of the conidiophores of *Mycogone perniciosa* bearing phialoconidia (Hsu and Han, 1981).

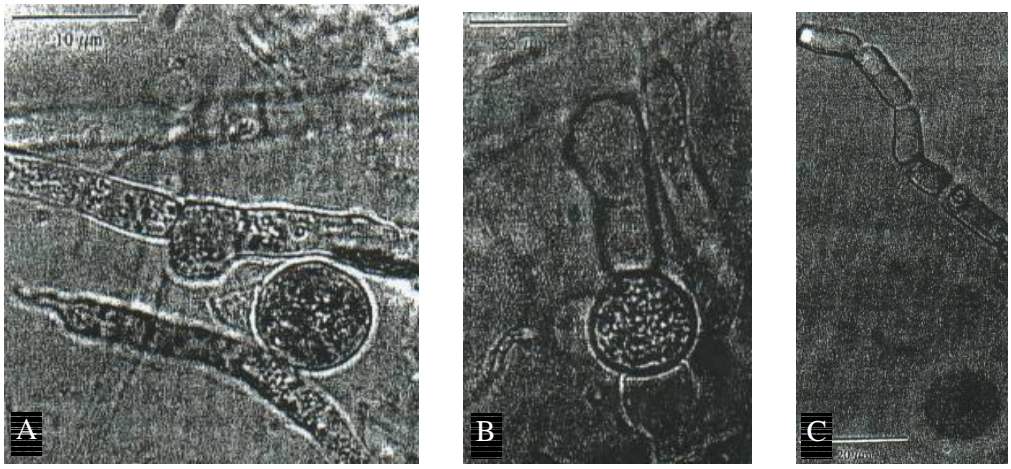

Figure 2.8 A) Lateral conidium of *Mycogone perniciosa*. B) Intercalary chlamydospore of *M. perniciosa*. C) Arthroconidia *M. perniciosa* (Holland and Cooke, 1991).

The phialoconidia are the first spores to appear on an infected mushroom and may be found at the edge of the *M. perniciosa* mycelium (Smith, 1924), these are also the first spores produced *in vitro* when nutrient levels are still high (Van Greuning, 2000). Conidia are produced by the formation of a constriction near the apex (Smith, 1924). The portion beyond the constriction enlarges until a long cylindrical spore with pointed ends is cut off. When the formation of the conidium is practically complete a median septum appears. The mature conidium is 1-septate with equal sized cells (Smith, 1924).

Phialospore size varies within the range 13.6-17.5 x 3.7-6.1µm (Fletcher *et al*., 1995). The upright verticillately branched conidiophores that bear the conidia (Smith, 1924), vary within the size range of 27.0-58.2 x 3.0-3.7µm (Fletcher *et al*., 1995). The phialospore and phialide measurements recorded by Brady and Gibson (1976) are larger.

Chlamydospores (Fig. 2.11 and 2.12) are formed later on short branches at the base of conidiophores and are much more resistant than thin-walled conidia (Smith, 1924). Formation of chlamydospores is the result of slight swellings of the hyphae followed by division and formation of one transverse septum. The upper cell swells and its wall thickens more quickly than the lower cell (Smith, 1924). Thus, a bicellular spore is formed, the upper cell of which is almost spherical with a thick stratified wall (Holland and Cooke, 1991).

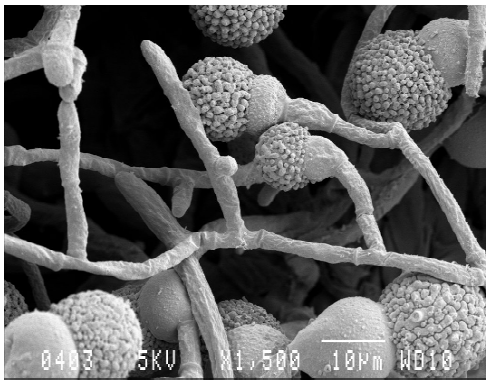

Figure 2.9. Scanning electron microscopy photograph of *Mycogone perniciosa* chlamydospores (Photo: Z. Pieterse).

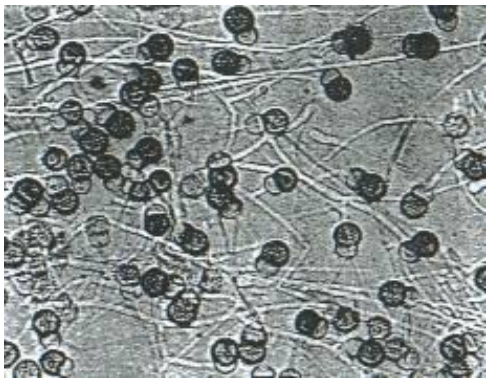

Figure 2.10. Light microscopy photo of *Mycogone perniciosa* chlamydospores (Hsu and Han, 1981).

The outer surface of the upper cell is slightly warted and the cytoplasm is dense and granular, containing several nuclei. The mature spore is light amber in colour. The lower cell appears to act only as a suspending cell for the upper thickened part of the spore as it is easily ruptured (Smith, 1924).

According to Brady and Gibson (1976), *M. perniciosa* can be distinguished from *M. rosae* by the paler colour of the chlamydospores and colony, and by the verticillium state, if present. Fletcher *et al*. (1995) was unable to observe these differences. Variations in colony morphology and physiology have been observed among isolates of *M. perniciosa* (Fletcher *et al*., 1995). Lapierre *et al*. (1971) found that some pathogenic strains of *M. perniciosa*, which were slow growing on agar, were highly pigmented and produced numerous chlamydospores.

Chlamydospore size varies within the range of 18.3-23.7 x 19.9-26.1µm for the upper cell and 10.4-14.5 x 12.5-17.3µm for the lower cell (Fletcher *et al*., 1995). Brady and Gibson (1976) also found the chlamydospore size fell within this range. According to Holland (1988), the dormant, thick-walled conidial cells of *M. perniciosa* are ethanol-soluble and wall-bound to some extent.

Phialospores germinate freely on a variety of substrata, while chlamydospores will germinate only when activated by uncharacterised factors emanating from vegetative mycelia of mushroom or mushroom tissues (Holland and Cooke, 1990). Fletcher and Ganney (1968) found that chlamydospores from an infected mushroom germinate through the basal cell but not through the resting cell.

2.4.2.2. Growth on various media

Sharma and Kumar (2000) investigated the growth of *M. perniciosa* in various liquid and on agar media. Maximal diametric growth (90mm) was recorded after 15 days on compost extract agar, followed by Czapek's Dox agar (82.1mm), dextrose nitrate agar (77.5mm), potato dextrose agar (PDA) (75.7mm), Martins rose bengal streptomycin agar (74.5mm), mushroom extract agar with sucrose (72.2mm), malt extract agar (71.9mm), Austhana and Hawker agar and Elliots agar (both 70.8mm), malt extract peptone dextrose agar (66.3mm), Waksman (65.0mm), mushroom extract agar without sucrose (63.8mm) and Sabourauds medium (45.7mm) (Sharma and Kumar, 2000).

Maximal conidia development (as x 10 spores per Petri plate) was recorded on malt extract peptone dextrose agar (8.64), followed by mushroom extract agar with sucrose (4.80), Austhana and Hawker agar (4.48), malt extract agar and Waksman (3.84), Sabourauds medium (3.52), mushroom extract agar without sucrose (3.20), potato dextrose agar (2.88), Martins rose bengal streptomycin agar and Elliots agar (1.92), compost extract agar (1.28), dextrose nitrate agar (0.96) and Czapek's Dox agar (0.64) (Sharma and Kumar, 2000).

The maximum growth, in liquid media, as measured by average mycelial dry weight (g) per Petri plate, was obtained in compost extract medium (0.77), followed by PDA (0.67), Czapek's Dox medium (0.38), Sabourauds medium (0.35), malt extract medium (0.33), Browns medium (0.24), mushroom extract medium with sucrose (0.22), malt extract peptone dextrose medium (0.21), Austhana and Hawker and Elliots media (both 0.17), Martins rose bengal streptomycin medium (0.12), mushroom extract medium without sucrose (0.09), dextrose nitrate medium (0.03) and Waksman medium (0.02) (Sharma and Kumar, 2000).

The optimum C/N ratio for *M. perniciosa* mycelial growth was found to be 1:5 and for sporulation 1:40 (Hsu and Han, 1981). Mycelial growth was best in liquid medium with mannose or glucose as carbon source compared to fructose, galactose, arabinose, xylose, lactose, trehalose and cellulose; and sodium nitrate and asparagine as nitrogen source was better in comparison to glutamine, cystine, lysine, ammonia, ammonium sulphate and calcium nitrate (Hsu and Han, 1981). Chlamydospore formation after 15 days of incubation was observed only in PDA, Elliots agar and malt extract dextrose agar. Sporulation of *M. perniciosa* was best in solid media containing glucose, mannose and starch as carbon source and urea, asparagine and glutamine as nitrogen source (Hsu and Han, 1981). When potassium and phosphate was lacking only a trace of growth occurred and sporulation was retarded (Hsu and Han, 1981).

These physiological studies indicate that compost extract agar supported the maximum growth of *M. perniciosa* in solid as well as liquid media, while maximal

spore formation was recorded in malt extract peptone dextrose agar (Sharma and Kumar, 2000).

2.4.2.3. Influence of temperature and pH

The optimum temperatures for mycelial growth and sporulation are 25ºC (Hsu and Han, 1981; Bech *et al*., 1989). This correlates with the findings of Smith (1924) and Lambert (1930). According to Lambert (1930), the fungus grew most rapidly when subjected to temperatures between 20ºC and 28ºC, which is above the normal temperature range for growing mushrooms. Smith (1924) found that light and darkness seemed to have little or no effect, but higher temperatures accelerated growth up to an optimum of 25ºC. At 15ºC growth was very slow (Lambert, 1930), while no mycelial growth was observed below 12ºC or above 32ºC (Hsu and Han, 1981). *M. perniciosa* chlamydospores can survive 105ºC for ten minutes in the dry state (Bech and Kovacs, 1981).

Radial mycelial growth of the pathogen in the pH range from 4-8 varied from 54.4 to 78.18mm, maximum being at pH 5.0 (Sharma and Kumar, 2000). This is slightly less than the optimum pH of 6.2 that was observed by Hsu and Han (1981). No growth of the pathogen was recorded at pH 3.5 and 9.0 (Sharma and Kumar, 2000).

2.4.2.4. Occurrence of virus-like particles

Albouy and Lapierre (1972) found that some pathogenic strains of *M. perniciosa*, which were slow growing on agar, were highly pigmented and produced numerous chlamydospores. Weakly pathogenic strains, produced more vegetative growth and little pigmentation. The slow growing forms were found to contain numerous virus-like particles of 39nm diameter (Fig. 2.13) (Fletcher *et al*., 1995). Atkey *et al*. (1976) also found 36nm diameter virus-like particles in slow growing isolates but all of their isolates were found to be highly pathogenic irrespective of the growth rate and particle content. It is not known with certainty whether mycoviral parasitism of *Mycogone* spp. is a widespread phenomenon (Umar *et al*., 2000). Virus-containing

strains of *M. perniciosa* grew slower and induced typical, pathological developmental errors in growing mushrooms (Fletcher *et al*., 1995). Mycoviruses may trigger the hyperplastic cellular changes observed in wet bubble disease by activating hyphal reserve cells to produce new hyphae (Umar *et al*., 2000).

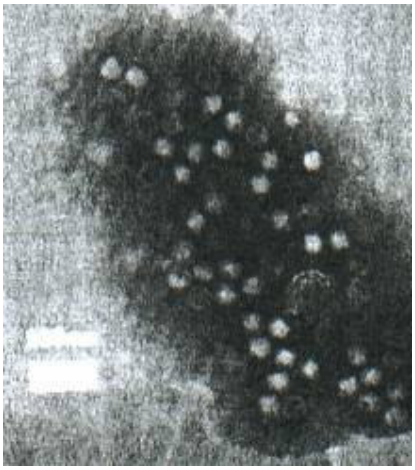

Figure 2.11. Virus-like particles from wet bubble diseased mushrooms infected with pathogenic strains of *Mycogone perniciosa* (Fletcher *et al*., 1995).

2.4.3. HOST RANGE

Mycogone perniciosa is not confined to cultivated mushrooms; there are early reports of *M. perniciosa* on *Amanita* and *Inocybe* (Holland and Cooke, 1990). According to Umar *et al*. (2000) this mycopathogen is not species specific, but the cultivated mushroom, *A. bisporus*, seems to be its most prevailing host world-wide (Brady and Gibson, 1976; Sohi and Upadhyay, 1987; Tu and Liao, 1989; Zhi *et al*., 1995). *Agaricus bisporus* may posses a type of 'germination inducing factor' that renders this species highly susceptible to interaction with *M. perniciosa* (Umar *et al*., 2000).

2.4.4. GEOGRAPHICAL DISTRIBUTION

Mycogone perniciosa is recorded worldwide wherever *A. bisporus* is grown (Fletcher *et al*., 1995).

2.4.5. DISEASE SYMPTOMS AND EPIDEMIOLOGY

Mushrooms with wet bubble disease are severely misshapen and not fit for sale (Dielemann-van Zaayen, 1976). *M. perniciosa* causes this distortion of mushrooms which Smith (1924) called sclerodermoid mushrooms (Fletcher *et al*., 1995). The sclerodermoid masses may be up to 10cm across (Fletcher *et al*., 1994) ranging in size from one half the size of a golf ball to as large as a grape fruit (Sharma and Kumar, 2000; Forer *et al*., 1974). Young mushroom pinheads infected with *M. perniciosa* develop amorphic shapes, which often do not even resemble mushrooms (Sharma and Kumar, 2000).

2.4.5.1. Symptoms of infection at different developmental stages of the mushroom

Mycogone perniciosa is a mycoparasite (Holland and Cooke, 1990) that may infect *A. bisporus* at various stages of its development. The result from infection at pinhead-stage is tumourous masses, later infections lead to malformation of the host (Hsu and Han, 1981), while even later infections cause the appearance of white, fluffy mycelial growth and less distortion (Fletcher *et al*., 1994). Cross and Jacobs (1969) found that most *M. perniciosa* infections occur at the pinhead stage. The pathogen can however, adhere to and penetrate the host during any stage of development (Umar *et al*., 2000). The base of the stipe, although symptomless, may be infected and up to 30% of apparently healthy mushrooms from an affected crop can be infected this way (Fletcher and Ganney, 1968)

2.4.5.2. Source and spread of the pathogen

The mycelium, phialoconidia and chlamydospores of *Mycogone* spp., are all proven to be infectious and are easily spread by vectors (Umar *et al*., 2000). The primary source of the pathogen is contaminated casing (Fletcher *et al*., 1994). When contaminated casing soil is the source of mushroom infection, symptoms develop within 10 to 14 days (Fletcher *et al*., 1994). The average interval between

inoculating casing and symptom appearance found by Hsu and Han (1981) was 13.74 days. The minimum time between inoculation of casing with *M. perniciosa* and symptom development however is eleven days (Fletcher and Ganney, 1968). The time symptoms take to develop appears to vary with the concentration of inoculum used and the quantity of mushrooms produced (Fletcher and Ganney, 1968). Neither compost (Hsu and Han, 1981) nor mushroom spawn seems to be the means of transmission for *M. perniciosa* (Gea *et al*., 1995). It is important to remove all the stipe bases from an infected crop in order to minimise build-up of disease (Fletcher and Ganney, 1968).

Waterborne spreading of the spores is the most important means of dispersal (Fletcher and Ganney, 1968). The dry spores of *M. perniciosa* can also be dispersed by wind (Cross and Jacobs, 1969). Spores may survive on the surfaces of buildings or may be carried in crop debris and, in this way, can contaminate crops. Pickers may spread the pathogen on their hands, on tools, boxes and clothing (Fletcher *et al*., 1994). In addition, the small conidia may be spread by sciarids (Dielemann-Van Zaayen, 1976).

2.4.5.3. Disease development

Mushroom fruiting bodies support all the nutrients needed by *M. perniciosa* for growth and sporulation (Hsu and Han, 1981). Fletcher and Ganney (1968) found that disease develops only if the pathogen inoculum is in close proximity to the developing mushroom. In contrast with these findings, Umar *et al*. (2000) found *M. perniciosa* able to affect the physiological state of the host cells and tissues from a distance they were unable to determine. There are many aspects of symptom and disease development, which could be accounted for by some form of stimulation provided by the mushroom mycelium (Fletcher and Ganney, 1968). Germination inducing factors are widespread within Agaricales but not confined to them (Holland and Cooke, 1990). All histological aspects observed during the pathogenesis of wet bubble disease indicate that host cells received very powerful and unusual stimuli to react upon (Umar *et al*., 2000).

2.4.5.4. Microscopical symptoms of the diseased host

A microscopical examination through the infected tissue revealed seriously disturbed histo-organogenesis (Umar and Van Griensven, 1999) showing three layers. The outer layer consists of hyphae and verticillated conidiophores bearing phialoconidia, the middle layer, chlamydospores, and the inner layer, a zone of brown mushroom tissue which has been destroyed (Hsu and Han, 1981). Pathological changes observed as a result of *M. perniciosa* include dedifferentiation, tissue necrosis and an excessive, nodular proliferation of hyphae (Umar *et al*., 2000).

In all perceivable lesions, one may regularly find atypical, swollen host cells and an increased number of hyphal reserve cells (Umar *et al*., 2000). Cytopathic changes are characterized by ribosomal condensation, rupture and perimitochondrial aggregations of ribosomes, hydropic degeneration of many segments, hypertrophy or occlusion of dolipore septa by coagulated materials, focal cell wall thickening or lysis (Umar *et al*., 2000). Hyphae of the mycopathogen coil around the host hyphae with firm adhesions but intrahyphal growth can be observed as well (Umar *et al*., 2000). Such intrahyphal growth is comparable to that of the dry bubble pathogen *Verticillium fungicola* var. *fungicola* (Dragt *et al*., 1996).

2.4.5.5. Symptoms in the latter stages of infection

After a few days the infected areas on the mushroom become creamish brown in colour due to the formation of dark coloured chlamydospores (Sharma and Kumar, 2000). Exudation of accumulated extracellular fluid results in a teardrop phenomenon (Umar *et al*., 2000). Clear brown coloured drops are exuded as a result of putrefying bacteria (Fig. 2.14). At this time the bubble is completely rotten and emits an unpleasant odour, which is characteristic of the disease (Sharma and Kumar, 2000). The wet decay and shape of affected mushrooms give the disease its common name, wet bubble disease (Fletcher *et al*., 1994).

Figure 2.12. Mushroom infected with *Mycogone perniciosa* showing amber droplets (arrows) (Photo: H. du Plooy).

2.4.5.6. Survival of the pathogen

Mycogone perniciosa sporulates heavily on *A. bisporus* mushrooms, producing small thin-walled phialospores together with large bicellular chlamydospores (Holland and Cooke, 1990). After secession the thin walled basal cell dies, leaving the thick-walled apical cell as the major survival propagule (Holland *et al*., 1985). The terminal chlamydospores can remain viable in stored casing for at least three years (Fletcher *et al*., 1994). Hsu and Han (1981) found that agar discs containing active mycelium and chlamydospores of *M. perniciosa* buried in casing soil and in autoclaved compost show that the fungus survives for 180 days and more than seven months, respectively. A very limited number (10 to 100) of chlamydospores were able to induce infection and manifest itself as a single nodule on the pilei of growing fruit bodies (Umar *et al*., 2000).

2.5. CONTROL

Since, chlamydospores have a thick, pigmented and heavily ornamented cell wall, known to be resistant to many physical and chemical factors, radical eradication of the mycopathogen may pose tremendous problems (Tu and Liao, 1989). These findings of Tu and Liao (1989) have proved to be right, because even today *M.*

perniciosa has not been eradicated and controlling the pathogen remains a difficult task.

The fungicide benomyl [methyl 1-(butylcarbamyoyl)-2-benzimidazolecarbamate] was released in Europe in 1969 and proved to be highly successful in controlling the major fungal pathogens of the commercially cultivated mushroom (Fletcher *et al*., 1975). Benomyl treatment applied to casing controlled wet bubble disease, whether the inoculum was introduced before or after casing (Nair and Baker, 1978). Benomyl treatment gave good control in the first flush and also controlled secondary spread (Fletcher *et al*., 1975). There was correlation between yield, disease incidence and rate of benomyl applied to the casing (Fletcher and Yarham, 1976). Widespread tolerance to this fungicide however appeared rapidly after the extensive use of benomyl on European mushroom farms (Eicker, 1987).

Thiabendazole was slightly less effective against bubble diseases, but differed from benomyl by the absence of detectable fungicidal residues in the mushrooms taken from any of the flushes (Fletcher, 1975). Thiabendazole proved to be successful in control of most fungal pathogens of cultivated mushrooms in South Africa (Eicker, 1984; 1990).

Prochloraz a member of the imidazole group of fungicides with the chemical name 1-{N-propyl-N-[2-(2,4,6 trichloro-phenoxy)ethyl]carbomyol}imidazole is also used to control wet bubble disease (Fletcher *et al*., 1983; Eicker, 1987). Prochloraz-manganese was found to be non-toxic to the cultivated mushroom and no evidence of any reduction in yield after split application of the fungicide could be found (Eicker, 1987).

Gandy (1979) performed experiments on biological control of *M. perniciosa* with *Acremonium strictum* Gams (Fig. 2.15), but no conclusions could be drawn from that study. The identity of the compound produced by *A. strictum* that caused abnormal mycelial growth of *M. perniciosa* was not determined.

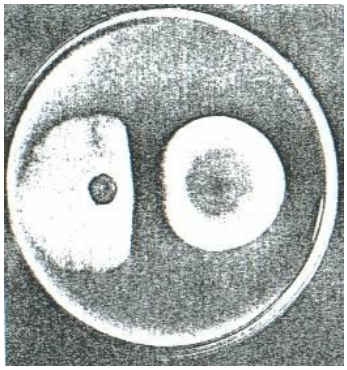

Figure 2.13. Inhibition of *Mycogone perniciosa* by *Acremonium strictum* (Gandy, 1979).

Control of wet bubble disease on commercial mushroom farms in South Africa and other places worldwide is currently obtained by applying salt to the infected areas as soon as they are spotted. Salting is followed by covering the infected area with a paper cup to prevent further spread (Mr. H. du Plooy, personal communication, Highveld Mushrooms, South Africa).

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CHAPTER 3

MICROSCOPICAL STUDY OF THE *IN VIVO* AND *IN VITRO* INTERACTION BETWEEN *Mycogone perniciosa* AND *Agaricus bisporus*

3.1. ABSTRACT

The macroscopical characteristics of wet bubble disease are well described, this work examined properties of the pathogen, *Mycogone perniciosa*, and its interaction with the host *Agaricus bisporus* using light and scanning electron microscopy. Samples from wet bubble diseased (WBD) mushrooms as well as from agar cultures where mycelia of *M. perniciosa* and *A. bisporus* were allowed to interact were investigated. *M. perniciosa* produced small, thin-walled phialoconidia, large, bicellular chlamydospores and intercalary propagules. *A. bisporus* spores were absent from WBD mushrooms, instead these mushrooms contained large amounts of *M. perniciosa* chlamydospores. Both phialoconidia and chlamydospores were present concurrently in agar cultures of *M. perniciosa* and in WBD mushrooms. No conclusive evidence of intrahyphal growth was found , but adhesion of both hyphae and phialospores of *M. perniciosa* to *A. bisporus* hyphae was observed. Coiling of *M. perniciosa* hyphae around *A. bisporus* hyphae and collapse of *A. bisporus* hyphae is an indication that *M. perniciosa* is a mycoparasite of *A. bisporus*.

3.2. INTRODUCTION

Mycogone perniciosa (Magnus) Delacroix produces two types of spores (Smith, 1924) small, thin-walled phialoconidia and larger, bicellular chlamydospores (Hsu and Han, 1981). Holland and Cooke (1991) also described additional spore forms: lateral smooth conidia, intercalary chlamydospores and arthroconidia, but their results have not been confirmed by other authors. In the first part of this chapter the different spore types of *M. perniciosa*, their morphology and development were studied with scanning electron and light microscopy.

The macroscopical characteristics of wet bubble diseased (WBD) mushrooms are well-documented (Smith, 1924; Holland and Cooke, 1991; Fletcher *et al*., 1994, Umar *et al*., 2000), but some microscopical properties are yet to be investigated. Furthermore the growth characteristics of *M. perniciosa in vitro* has been documented on various solid and liquid media (Sharma and Kumar, 2000) and illustrated with microscopy (Hsu and Han, 1981), but there is no literature on the microscopical characteristics of the pathogen in association with its host, *Agaricus bisporus* (Lange) Imbach, *in vitro*.

Infection of *A. bisporus* by *M. perniciosa* and subsequent development of disease has to take place due to interaction between the host and the pathogen, as in the case with other mycoparasitism reactions e.g. *Verticillium fungicola* var. *fungicola* (Preuss) Hassebr., causing dry bubble disease of *A. bisporus* (Dragt *et al*., 1996). Cooke and Rayner (1984) and Rayner and Boddy (1988) described three possible outcomes when two mycelial systems interact: (1) neutral intermingling of hyphae, (2) deadlock, in which neither individual mycelium enters territory occupied by the other and (3) invasion of domain, with one mycelium partially or completely replacing the other. It is not documented in which of these categories interaction of *M. perniciosa* and *A. bisporus* fits best.

The reasons for *M. perniciosa* infected mushroom tissue having seriously disturbed histo-organogenesis as examined microscopically by Umar and Van Griensven (1999) are not clearly documented. Interactions between *M. perniciosa* and *A. bisporus* grown in culture as well as in mushroom fruiting bodies, naturally infected with the pathogen, and infected under simulated commercial cultivation conditions were studied with light and scanning electron microscopy in an effort to better understand WBD.

3.3. MATERIALS AND METHODS

3.3.1. Samples

For *in vitro* studies, a mushroom infected with *M. perniciosa* was obtained from Dr. Petra Labuschagne (Plant Pathology Laboratories, University of Pretoria). The pathogen was isolated from this diseased mushroom and pure cultured on potato dextrose agar (PDA) (Merck, Johannesburg). A culture of the cultivated mushroom, *Agaricus bisporus*, was obtained from Dr. Martmari van Greuning (Sylvan Incorporated, South Africa). For the duration of experiments both fungi were maintained on PDA (Merck, Johannesburg).

Samples were taken from different parts of actively growing *M. perniciosa* cultures to examine spore morphology. Some agar cultures were kept for six months and studied for survival of the pathogen and its spores. These cultures were subsequently replated to fresh media and investigated for differences in growth compared to cultures obtained by isolation from diseased mushrooms.

For interaction studies, the pathogen and the host were grown together on potato dextrose agar (PDA), mushroom extract agar (MA) and mushroom extract dextrose agar (MDA) in 90mm Petri plates. Mushroom extract was prepared by blending 100g of randomly selected healthy mushrooms with 100ml distilled water (dH_2O), and filtering the resulting mixture through cheesecloth three times. Mushroom extract agar consisted of 10g mushroom extract and 15g agar-agar powder CP (Associated Chemical Enterprises, Johannesburg) in 1000ml dH_2O, while 10g dextrose (Protea Laboratory Services, Johannesburg) was added to produce MDA. Five millimetre diameter plugs from the edge of actively growing *A. bisporus* and *M. perniciosa* were inoculated opposite each other in 90mm Petri plates. Controls consisted of plates with plugs of either *M. perniciosa* or *A. bisporus* at both sides of the Petri plates. All Petri plates were incubated at 25ºC (the optimum growth temperature of the pathogen) for 20 days under alternating 12 hour light/dark

conditions. Samples were taken from the area where *M. perniciosa* mycelium came in contact with *A. bisporus* mycelium.

For *in vivo* studies mushrooms infected with *M. perniciosa* were obtained from Dr. Petra Labuschagne (Plant Pathology Laboratories, University of Pretoria) and Mr. H. du Plooy (Highveld Mushrooms, South Africa). The infected mushrooms were at various stages of development, from pinning stage to a stage of rotting due to bacterial infection. In addition, samples were taken from casing soil where possible interaction between mycelia of *A. bisporus* and *M. perniciosa* could occur at the time of pinning. Both casing material and mushroom tissue samples were investigated. Controls consisted of uncolonised casing soil and healthy agarics at different stages of development.

3.3.2. Scanning electron microscopy (SEM)

Samples (5mm diam.) were taken from culture plates and from *M. perniciosa*-infected mushrooms. The material was fixed in 2.5% glutaraldehyde solution buffered with 0.075M phosphate at pH 7.4 for two hours at 25ºC. The fixed samples were then rinsed three times for 10 min each in 0.075M phosphate buffer solution. Post-fixation was in 0.5% osmium tetroxide solution for two hours followed by three rinses in distilled water. Dehydration was done in a graded ethanol dilution series (30, 50, 70, 90, 100%), followed by immersion in 100% ethanol. The dehydrated material was critical-point dried with CO_2 in a Polaron Critical-point Dryer, mounted on SEM stubs using double-sided tape, sputter coated with gold and observed with a JEOL 840 SEM operated at 5kV.

3.3.3. Light microscopy (LM)

Five millimetre plugs were taken from the zone of intermingled *A. bisporus* and *M. perniciosa* hyphae on the Petri plates and from naturally infected mushrooms at different growth stages. The material was fixed in 2% glutaraldehyde solution buffered with 0.1M phosphate, pH 7 for one hour at 25ºC. The samples were then

washed with phosphate buffer solution and examined under 40X magnification with a Zeiss light microscope using lactofuchsin or toluene blue as staining solutions.

In addition various samples from wet bubble diseased mushrooms and mycelia from the zone of interaction were dissected into 1mm cubes or smaller sizes and fixed in a mixture of 4% formaldehyde and 1% gluteraldehyde solutions buffered with 0.1M phosphate buffer at pH 7.4, for one hour at room temperature (25ºC). These samples and freshly sampled diseased mushrooms and mycelial material were infused with mixtures of 2M sucrose and 20% poly(vinylpyrrolidone) (PVP; mol. Wt 10000; Sigma, USA). All samples were frozen at -28ºC for 24 hours and sectioned with a cryotome to approximately 40nm thickness. The ultrathin frozen sections were transfered from the knife edge to microscope slides by the use of distilled water (dH_2O) droplets. The samples were left in dH_2O for 24 hours to rinse out the sucrose and PVP, thereby allowing effective uptake of colouring fluid by the fungal samples. The samples were coloured with lactofuchsin or toluene blue and examined under 40X magnification with a Zeiss light microscope.

3.4. RESULTS

3.4.1. Spore morphology

Agaricus bisporus spores were produced in the gills of healthy mushroom fruiting bodies examined in this study (Fig. 3.1 A). *A. bisporus* spores were absent from mushrooms infected with *M. perniciosa*. Instead, WBD mushroom fruiting bodies contained large amounts of *M. perniciosa* chlamydospores (Fig. 3.1 B). Chlamydospores of *M. perniciosa* were found to be bicellular with basal cells ranging from 10 to 13µm in breadth and 13 to 17µm in length and the apical cell ranging from 18-22µm in breadth and 20-25µm in length (Figs 3.1 C and 3.1 D).

While all of the WBD mushrooms investigated in this study contained large amounts of *M. perniciosa* chlamydospores, only a few specimens produced phialoconidia, while other reproductive and survival structures were the exception rather than the

rule. *M. perniciosa* produced phialoconidia with and without septa (Fig. 3.1 E), on *Verticillium*-like conidiophores (Figs 3.1 F and 3.1 G).

Wet bubble diseased mushrooms as well as agar cultures of *M. perniciosa* produced both phialoconidia and chlamydospores simultaneously (Fig. 3.2 A). Mushrooms infected with *M. perniciosa* also produced chlamydospores that differed from the characteristics described in the literature. Some spores had verrucose basal cells (Fig. 3.2 B), while others seemed to have developed in the wrong sequence, with the verrucose apical cells being the first structure produced on a hypha (Fig. 3.2 C). Some chlamydospores were attached to swollen hyphal cells (Fig. 3.2 D). Wet bubble diseased mushrooms also produced abnormal propagules (Fig. 3.2 E) and intercalary swellings (Fig. 3.2 F).

3.4.1. Interaction

This investigation showed that the macroscopical differences exhibited by the reproductive stage of *A. bisporus* when infected with *M. perniciosa* are not distinct when *A. bisporus* is growing vegetatively on culture media (Fig. 3.3 A). No clearing zone of inhibition was observed. Hyphae of *A. bisporus* and *M. perniciosa* could be easily distinguished in agar plate cultures (Fig. 3.3 B) although their hyphae were in close proximity. *M. perniciosa* hyphae were approximately 2-3µm in diameter, while *A. bisporus* hyphae were 6-10µm in diameter. In experiments on agar media no intrahyphal growth was observed. The only apparent damage to host hyphae was hyphal collapse, where contact was made between the two fungi (Fig. 3.3 C).

Hyphae of *M. perniciosa* were found on the surface of infected mushrooms as well as in the interior of wet bubble diseased mushroom fruit bodies. Neither penetration structures, nor direct penetration were observed in any of the mushroom specimens studied. Similar to results from agar cultures, a collapse of *A. bisporus* hyphae was also observed when *M. perniciosa* came in contact with the mycelium of *A. bisporus* (Fig. 3.3 D).

In addition to causing hyphal collapse, the pathogen coiled around the hyphae of the host (Fig. 3.3 E). Scanning electron microscopy revealed adhesion of *M. perniciosa* hyphae (Fig. 3.3 D) and phialoconidia (Fig. 3.3 F) to hyphae of *A. bisporus*, but no penetration of the host by the pathogen was visible. Thinner hyphal-like structures were also found inside the larger *A. bisporus* hyphae in the scanning electron microscopy studies (Fig. 3.3 G) but light microscopy studies could not confirm these findings. We found *M. perniciosa*-infected fruit bodies contained large amounts of unidentified bacteria (Fig. 3.3 H).

Sections through wet bubble diseased mushrooms examined with light microscopy showed symptoms of cellular disruption (Fig. 3.4 A) compared to ultrathin cuts through healthy mushroom tissue (Fig. 3.4 B). Light microscopy also revealed several structures resembling hyphal reserve cells in samples from diseased tissue (Fig. 3.4 C). These structures were absent from healthy mushrooms (Fig. 3.4 D).

3.5. DISCUSSION

Agaricus bisporus basidiospores produced in the gills of healthy mushrooms were absent from mushrooms infected with *M. perniciosa*. We hypothesize that the undifferentiated nature of WBD mushrooms plays a role in the absence of *A. bisporus* spores. Infected mushrooms have no pileus area where young hymenial tissue can differentiate into lamellae. According to Umar and Van Griensven (1998) differentiation in the area of the universal veil is critical in histogenesis and subsequent development of basidiospores for sexual reproduction. Absence of reproductive organs in WBD *A. bisporus* thus lead to absence of basidiospores.

Mycogone perniciosa produced large, bicellular chlamydospores as has been found previously by various authors (Sharma and Kumar, 2000; Umar *et* al., 2000; Fletcher *et* al., 1994; Holland and Cooke, 1991; Eicker *et al*., 1989; Smith, 1924). Sizes of *M. perniciosa* chlamydospores investigated in this study were similar to those described in literature. Fletcher *et al*. (1995) found chlamydospore size varied within the range of 18.3-23.7 x 19.9-26.1µm for the upper cell and 10.4-14.5 x 12.5-17.3µm for the

lower cell. According to literature chlamydospores consist of a dark, spherical, thick-walled, verrucose apical cell and a smaller, subhyaline, thin-walled basal cell (Holland and Cooke, 1991). Agar cultures of *M. perniciosa* investigated in this study turned brown upon the production of chlamydospores around day 10 of incubation.

Cultures of *M. perniciosa* and WBD mushrooms produced phialoconidia in addition to chlamydospores. Smith (1924) found mature conidia have one septum dividing two equally sized cells. We found phialoconidia with similar characteristics as well as without septa. The spores without septa found in this study may still be immature, but considering their size (around 20µm in legth) it is more likely that they are mature phialoconidia without septa. Phialoconidia are short-lived and function exclusively as xenospores (Holland and Cooke, 1991).

Both phialoconidia and chlamydospores of *M. perniciosa* were simultaneously observed in agar cultures or WBD mushrooms. In addition to the two distinct major spore types described above, WBD mushrooms produced chlamydospores with anomalies as well as other reproductive/survival structures, similar to those described previously by Holland and Cooke (1991). These authors proposed two reasons for the production of these different spores. They hypothesized that these spores require less energy expenditure than production of phialoconidia and chlamydospores or were produced to increase the pathogen's chances of surviving in its current host and colonising new hosts. The latter hypothesis is more likely, since it has been found that phialoconidia are produced when ample nutrients are available (Van Greuning, 2000) at the edge of agar cultures (Smith, 1924). The high degree of pleomorphism exhibited by *M. perniciosa* confers flexibility of response to resource limitation and also, perhaps, contributes to genetic variation (Holland and Cooke, 1991).

The macroscopical symptoms seen in *A. bisporus* fruit bodies infected with *M. perniciosa* are not visible when *A. bisporus* is growing vegetatively on culture media. One possible explanation for this finding may be the different chemical composition and structure of fruit body and vegetative mycelial cells of *A. bisporus* (García-Mendoza *et al*., 1987).

Hyphae of *M. perniciosa* were found on the surface of infected mushrooms as well as in the interior of diseased mushroom fruit bodies. *M. perniciosa* hyphae were in close proximity to *A. bisporus* hyphae, characteristic of pathogenesis (North and Wuest, 1993). Neither penetration structures, nor direct penetration was observed in any of the mushroom specimens studied.

No intrahyphal growth was observed on agar media. The only apparent damage where contact was made between the two fungi and was accompanied by a collapse of the *A. bisporus* hyphae, similar to observations made for *Verticillium fungicola* infection of mushrooms (North and Wuest, 1993). No clearing zones of inhibition as observed for *V. fungicola* (Calonje *et al*., 2000) were observed for *M. perniciosa.*

In addition to hyphal collapse, *M. perniciosa* hyphae coiled around hyphae of *A. bisporus* in WBD mushrooms. Coiling is characteristic behaviour for a mycoparasite e.g. coiling of *Trichoderma harzianum* Rifai around various hosts' hyphae (Elad *et al*., 1983). Coils possibly play a role similar to appresoria in penetration of the host surface (Bayliss *et al*., 2001). *M. perniciosa* hyphae and conidia adhered to hyphae of *A. bisporus*, but no penetration of the host by the pathogen was visible. As was the case in the *in vitro* sudies, there was no conclusive evidence for intrahyphal growth *in vivo*.

Ultrathin cuts through WBD mushrooms illustrated cellular disintegration and increased amounts of hyphal reserve cells. Cellular disruption may play a role in WBD as it has been shown to play a major role in dry bubble disease of *A. bisporus* (Calonje *et al*., 2000; Dragt *et al*., 1996). Many cells of diseased mushrooms were collapsed, and less stainable than healthy tissue, probably due to the loss of cell wall material. Possible cellular disruption of *A. bisporus* cells by *M. perniciosa* was investigated with enzyme assays in Chapter 5, section 5.3.2.

In their inoculation studies, Umar *et al*. (2000) found that *M. perniciosa* infected fruit bodies were infected with various types of bacteria around day 6 of their

experiments. We also found *M. perniciosa*-infected fruit bodies contained large amounts of bacteria. Masaphy *et al*. (1987) found that rod like bacteria attach firmly to hyphae of *A. bisporus* during primordia development. These bacteria are possibly prevented to enter the cap-tissue in healthy *A. bisporus* by the protective barrier between the stipe and the cap formed by the HYPB protein, a hydrophobin produced by the highly expressed gene designated as *hypB*, which has been described by Umar and Van Griensven (1995). In WBD mushrooms this protective barrier is absent due to the lack of differentiation, the bacteria normally found as part of the microbiota of casing soil may therefore enter WBD mushrooms. According to Umar *et al*. (2000), this diffuse bacterial infection is the actual cause of death of the mushroom in most cases. The pathogen itself seemed not to be influenced by the changes of the host tissues (Umar *et al*., 2000).

From these microscopy studies we could conclude that the processes *M. perniciosa* employ to infect *A. bisporus* are complex making use of not only mechanical but also enzymatic means. Coiling, close proximity to host hyphae and host hyphal collapse, exhibited by *M. perniciosa* in this study is behaviour characteristic of mycoparasitsm, and raised questions on the methods *M. perniciosa* employs to infect *A. bisporus*. Future research should include transmission electron microscopy (TEM) studies to investigate possible penetration of *A. bisporus* by *M. perniciosa* and possible interhyphal growth of *M. perniciosa* inside *A. bisporus* hyphae.

3.6. REFERENCES

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Figure 3.1

A) *Agaricus bisporus* spores from the gills of a healthy mushroom. B) Masses of large, bicellular chlamydospores produced by *Mycogone perniciosa* on PDA in Petri plate culture. C) Scanning electron micrograph of chlamydospores (cs) of *M. perniciosa* produced in a mushroom infected with wet bubble disease. D) Light micrograph of a *M. perniciosa* chlamydospore from an infected mushroom, bc, basal cell; ap, apical cell. E) Light micrograph of non-septate (left) and 1-septate (right) phialoconidia of *M. perniciosa* (arrow indicates septum). F) Scanning electron micrograph from a wet bubble diseased mushroom illustrating *Verticillium*-like conidiophores (cp) bearing phialoconidia (pc) of *M. perniciosa*. G) Light micrograph of phialoconidia (pc) of *M. perniciosa* on conidiophores (cp).

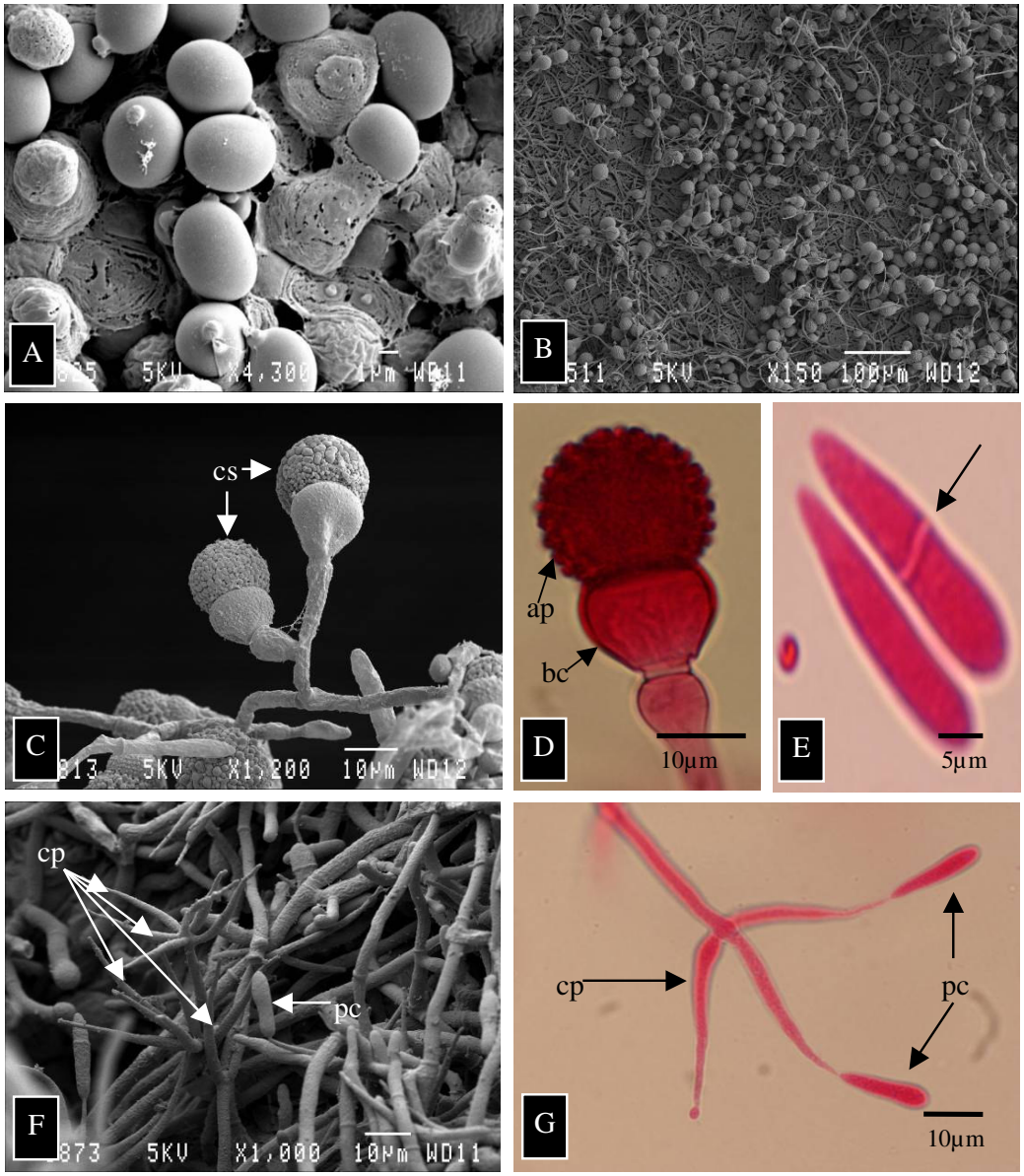

A
B
C
cs
D
ap
bc
10µm
E
5µm
F
cp
pc
G
cp
pc
10µm

Figure 3.2

A) Light micrograph of chlamydospores (cs) and phialoconidia (pc) of *Mycogone perniciosa* produced in same agar culture. B) Scanning electron micrograph of a *M. perniciosa* chlamydospore illustrating a verrucose basal cell (bs) and a verrucose apical cell (ap). C) Scanning electron micrograph of *M. perniciosa* chlamydospore (cs) with verrucose cell attached directly to hypha (h). D) Scanning electron micrograph of a *M. perniciosa* chlamydospore formed on a swollen hyphal cell (sh). E) Light micrograph of abnormal propagules produced by *M. perniciosa* similar to chlamydospores. F) Scanning electron micrograph of intercalary chlamydospore produced by *M. perniciosa* present in a wet bubble diseased mushroom.

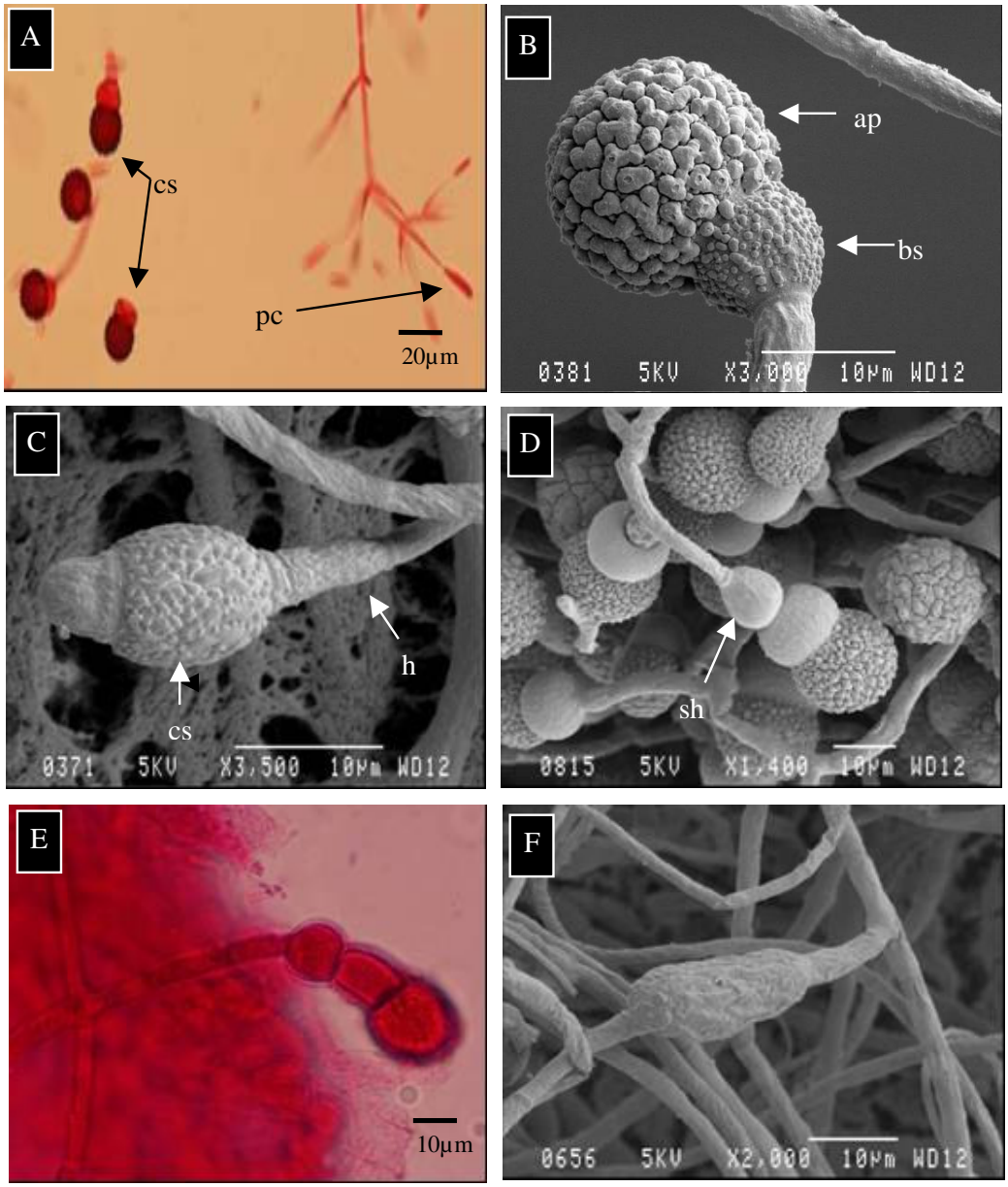

A
cs
pc
20µm
B
ap
bs
0381 5KV X3,000 10µm WD12
C
h
cs
0371 5KV X3,500 10µm WD12
D
sh
0815 5KV X1,400 10µm WD12
E
10µm
F
0656 5KV X2,000 10µm WD12

Figure 3.3

Scanning electron micrographs illustrating: A) Intermingled hyphae of *Mycogone perniciosa* (mh) growing over hyphae of *Agaricus bisporus* (ah) in a Petri plate culture on PDA. B) Thin hyphae of *M. perniciosa* (mh) in close proximity to *A. bisporus* hyphae (ah) in agar culture samples. C) Thin *M. perniciosa* hypha (mh) growing over a partially collapsed *A. bisporus* hypha (ah) on PDA. D) Section through a wet bubble diseased mushroom showing attachment of *M. perniciosa* hypha (mh) to an *A. bisporus* hypha (ah). E) *M. perniciosa* hypha (mh) coiled around an *A. bisporus* hypha (ah), sampled from the edge of a wet bubble diseased mushroom. F) Adhesion of *M. perniciosa* phialoconidium (pc) produced in a wet bubble diseased mushroom to an *A. bisporus* hypha (ah). G) Section through a wet bubble diseased mushroom illustrating possible growth of a thin *M. perniciosa* hypha (mh) inside a thicker *A. bisporus* hypha (ah). H) Wet bubble diseased mushroom infected with bacteria.

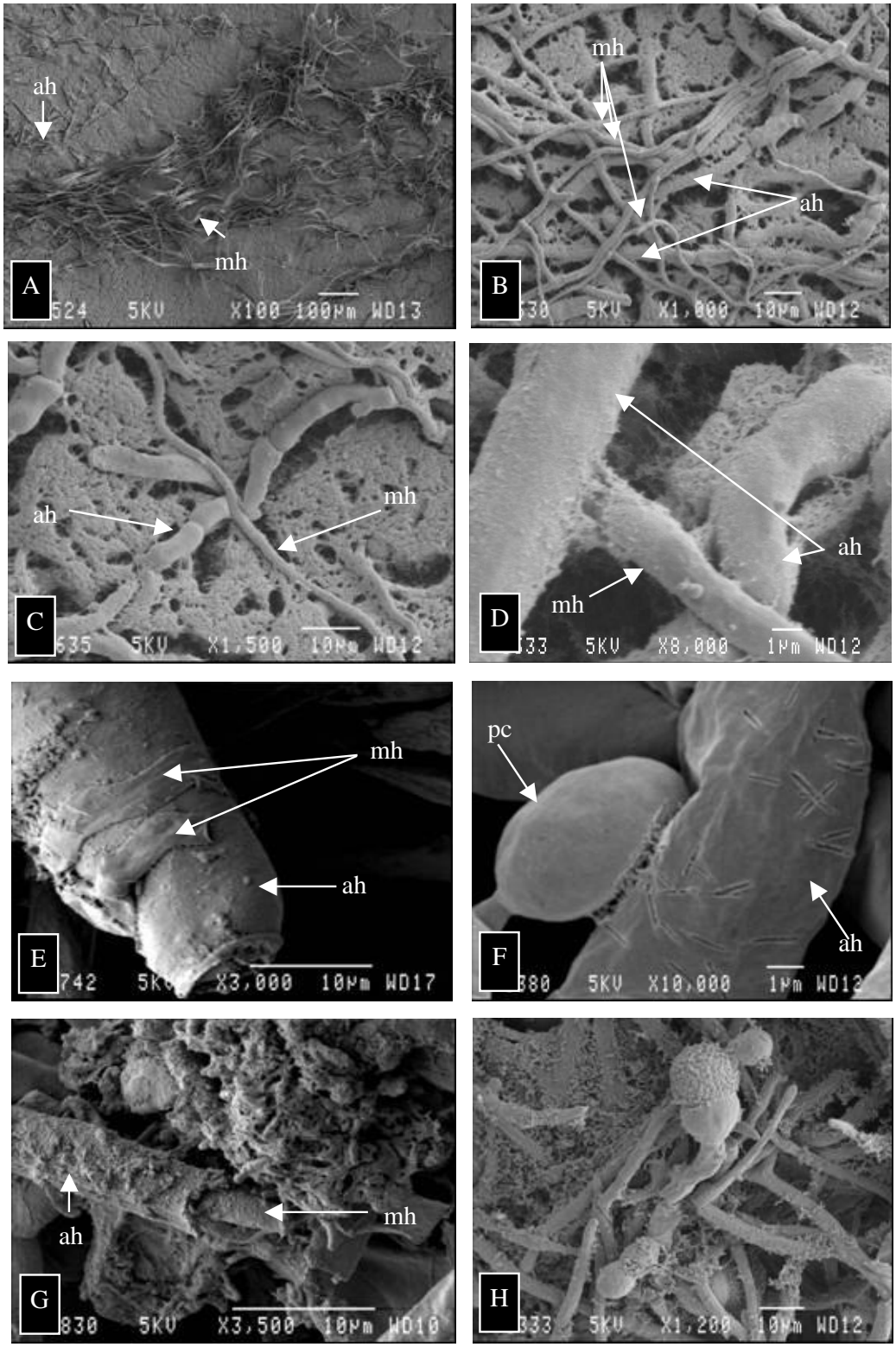
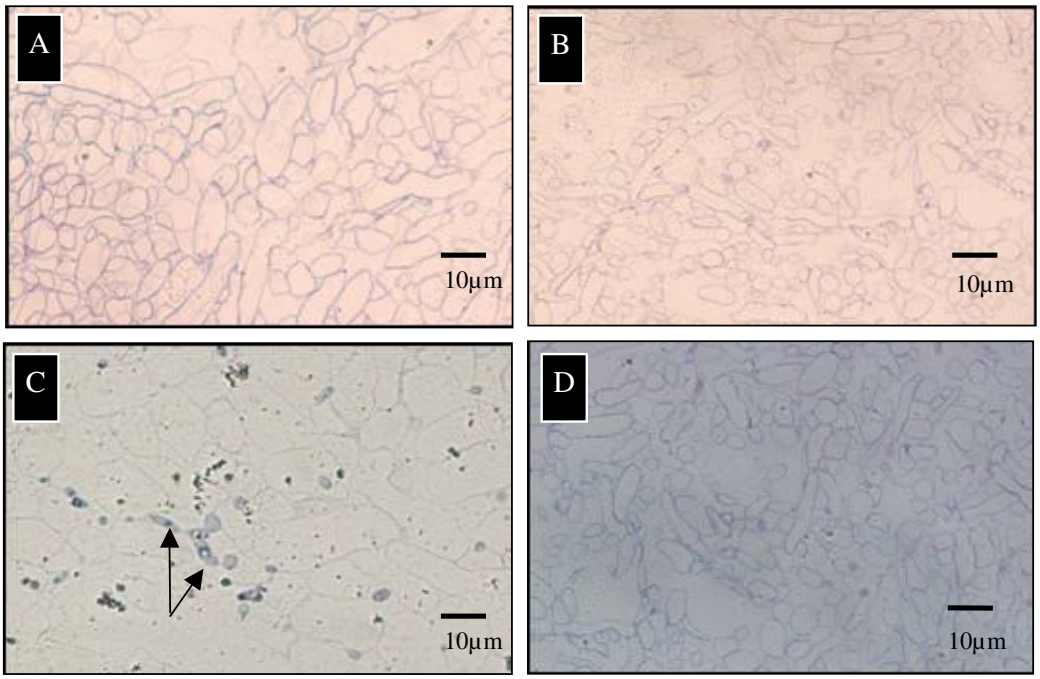

A
ah
mh
524 5KV X100 100µm WD13
B
mh
ah
30 5KV X1,000 10µm WD12
C
ah
mh
635 5KV X1,500 10µm WD12
D
ah
mh
33 5KV X8,000 1µm WD12
E
mh
ah
742 5KV X3,000 10µm WD17
F
pc
ah
80 5KV X10,000 1µm WD12
G
ah
mh
830 5KV X3,500 10µm WD10
H
33 5KV X1,200 10µm WD12

Figure 3.4

A) Light micrograph of an ultrathin cut through a wet bubble diseased mushroom stained with lactofuchsin illustrating disturbed cell structure. B) Light micrograph of an ultrathin cut through a healthy *Agaricus bisporus* mushroom stained with lactofuchsin. C) Light micrograph of an ultrathin cut through a mushroom infected with *Mycogone perniciosa* stained with toluene blue, illustrating hyphal reserve cells and possible appresoria (arrows). D) Light micrograph of an ultrathin cut through a healthy mushroom stained with toluene blue.


A
10µm
B
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C
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D
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CHAPTER 4

GROWTH OF *Mycogone perniciosa* AND *Agaricus bisporus* IN CULTURE MEDIA

4.1. ABSTRACT

Mycogone perniciosa grows on diverse agar media, producing mycelia abundantly, while *Agaricus bisporus* has difficulty growing vegetatively in agar culture. It is not known whether the dedifferentiation caused by *M. perniciosa* in fruit bodies of *A. bisporus* plays a role in the *in vitro* growth of both fungi. *M. perniciosa* was cultured on potato dextrose agar (PDA), mushroom agar (MA) and mushroom dextrose agar (MDA) in paired and dual cultures with vegetative mycelia of *Agaricus bisporus*, as well as in half plates, to view the interaction between the pathogen and the mushroom host *in vitro*. *M. perniciosa* grew significantly more in association with *A. bisporus* in paired and dual cultures and in half plates than *M. perniciosa* controls. The pathogen had increased growth even when it had no contact with the mycelia or growth media of *A. bisporus*. In contrast, *A. bisporus* inoculated alone as controls grew significantly more than in culture with *M. perniciosa*. Both fungi grew less on MA than on MDA and PDA. It is possible that the metabolites in the media of paired cultures, or volatiles in the case of dual and split cultures may influence the growth of both fungi *in vitro*.

4.2. INTRODUCTION

The pathogen causing wet bubble disease (WBD) of commercial *Agaricus bisporus* (Lange) Imbach mushrooms, *Mycogone perniciosa* (Magnus) Delacroix, produces copious flocculent mycelium on most substrata (Fletcher *et al*., 1994; Smith, 1924). The mycelia turned brown after 10 days growth on potato dextrose agar (PDA) due to the production of chlamydospores (Hsu an Han, 1981). Research indicates that *Mycogone* causes drastic changes in the reproductive growth of *A. bisporus* (Umar *et al*., 2000) while changes in vegetative growth have not been clearly documented. Fletcher and Ganney (1968) examined WBD mushrooms and found no evidence of

parasitism of the mushroom mycelium by *M. perniciosa* and suggested that many aspects of disease development were due to stimulation from the mushroom mycelium, while Umar *et al*. (2000) described *M. perniciosa* as a mycoparasite.

In other fungal-fungal interactions, hyphae of the different fungi interact before they come in contact e.g. the directed growth of hyphae of *Trichoderma hamatum* (Bonord.) Bainier to hyphae of *Rhizoctonia solani* Kühn prior to penetration (Chet *et al*., 1981). In all mycoparasitism reactions disease interaction between the mycoparasite and the host must take place before the development of external symptoms (Manocha *et al*., 1990). Different agar culture techniques are necessary to investigate interactions, because morphogenic stimulation may require direct contact in some cases, while in other cases, it may take place from a distance (Masaphy *et al*., 1987).

Sharma and Kumar (2000) investigated the growth of *M. perniciosa* on various agar media, but did not compare the pathogen's growth characteristics in the presence of *A. bisporus*. According to Sidirova and Velikanov (2000), *A. bisporus* exerts a significant influence on organisms in their natural environment, but the influence of *A. bisporus* on fungi in agar culture has not been investigated. To date there have not been any investigations on the influence of *M. perniciosa* on other organisms in agar culture. It has been found, however, that some species of *Mycogone* (*M. rosea* Link and *M. cervina* Link) produce secondary metabolites that affect the growth of other microorganisms in their natural environment (Wilhelm *et al*., 2004).

The purpose of this chapter was to study the interaction between *M. perniciosa* and the vegetative mycelial phase of *A. bisporus*, on different agar media *in vitro*. Although the parameters measured *in vivo* are very different from those measured *in vitro* (Fravel, 1988), *in vitro* interactions may provide knowledge on the effects that fungi exert on each other. This is the first investigation to study the effect that possible stimuli produced by *A. bisporus* may have on radial mycelial growth of *M. perniciosa* or *vice versa*.

4.3. MATERIALS AND METHODS

4.3.1. Cultures

A mushroom infected with *M. perniciosa* was obtained from Dr. Petra Labuschagne (Plant Pathology Laboratories, University of Pretoria). The pathogen was isolated from this diseased mushroom and pure cultured on potato dextrose agar (PDA) (Merck, Johannesburg). A culture of the cultivated mushroom, *Agaricus bisporus*, was obtained from Dr. Martmari van Greuning (Sylvan Incorporated, South Africa). For the duration of experiments both fungi were maintained on PDA and malt extract agar (MEA) (Merck, Johannesburg) at 25ºC in alternating 12 hour light/dark conditions.

4.3.2. Interaction

The pathogen and the host were grown together in paired culture on PDA, mushroom extract agar (MA) and mushroom extract dextrose agar (MDA) in 90mm Petri plates to view their interaction *in vitro*. Mushroom extract, MA and MDA was made as described in Chapter 3, section 3.3.1. Five millimetre diameter plugs from the edge of actively growing *A. bisporus* and *M. perniciosa* were inoculated opposite each other in 90mm Petri plates. Controls consisted of plates with plugs of either *M. perniciosa* or *A. bisporus* at both sides of the Petri plates (Fig. 4.1 A).

Five millimetre diameter plugs from actively growing *A. bisporus* and *M. perniciosa* cultures were also inoculated in the centre of the bottom part of 90mm PDA, MA and MDA Petri plates, to make dual cultures. After 48 hours the bottoms of Petri plates containing plugs of *A. bisporus* were joined with bottoms from Petri plates containing plugs of *M. perniciosa* by taping the respective bottom plates together with insulation tape (Fig. 4.1 B). In some cases plates containing *M. perniciosa* plugs were the bottoms of the dual culture and in others the *A. bisporus* plates were the bottom plates. Controls consisted of taped cultures with both top and bottom

plates of the dual culture containing *M. perniciosa* or *A. bisporus*, respectively. The lids of Petri plates were discarded.

In addition, the pathogen and the host were also grown together on PDA, in 90mm half plate Petri plates to examine the effect of volatiles on fungal growth and interaction *in vitro*. Five millimetre plugs from the edge of actively growing *A. bisporus* and *M. perniciosa* were inoculated in separate compartments of 90mm half plate Petri plates (Fig. 4.1 C). Controls consisted of half plates with plugs of either *M. perniciosa* or *A. bisporus* in both compartments of the half plate.

All Petri plates were incubated at 25ºC (the optimum growth temperature of the pathogen) for 20 days in alternating 12 hour light/dark conditions. Colony growth and radii were measured every five days. Each trial consisted of 10 replicates of 10 plates each and the experiment was repeated in triplicate. Two-way analysis of variance (ANOVA) was performed on the data of *in vitro* experiments and means were separated using the Student t-test ($P \leq 0.05$). The mean and least significant difference (LSD) for each replicate were determined.

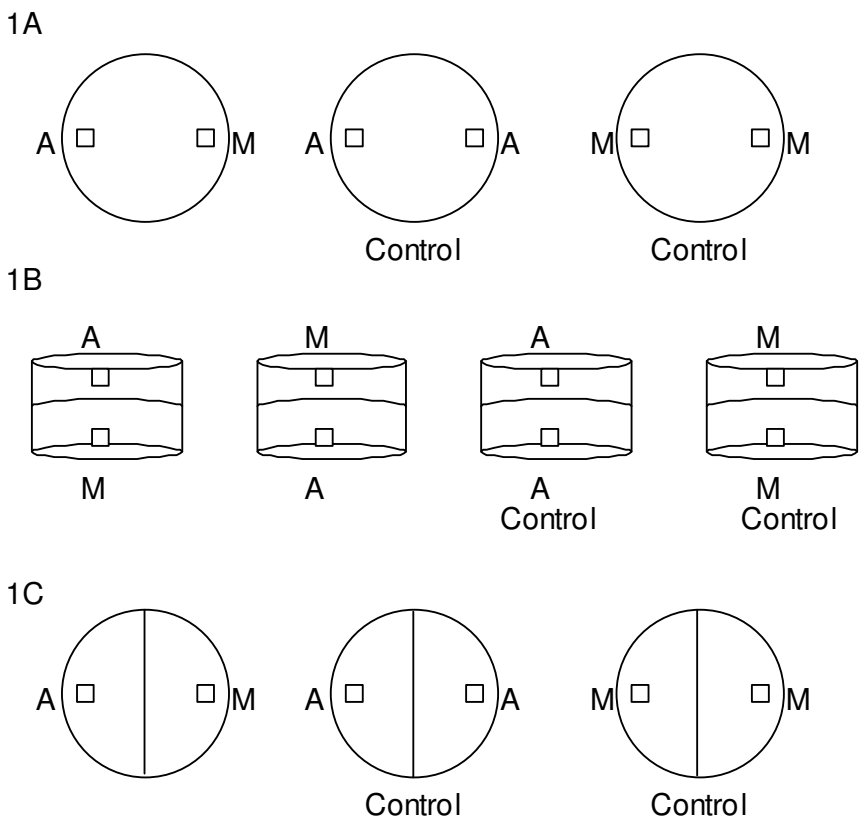

Figure 4.1

Diagrammatic illustration of *in vitro* screening assay of the host, *Agaricus bisporus* and pathogen, *Mycogone perniciosa*

1A) Paired culture of the pathogen and the host grown in the same Petri plate

1B) Dual culture of the pathogen and the host

1C) Half plate culture of the pathogen and the host grown in split plate

(□ = mycelial plug; A= *Agaricus bisporus*; M= *Mycogone perniciosa*)

4.4. RESULTS

4.4.1 *Agaricus bisporus* and *Mycogone perniciosa* grown on potato dextrose agar in paired and dual culture

Growth of *A. bisporus* on culture media was slow in comparison with *M. perniciosa*, with *A. bisporus* vegetative growth not exceeding radii of 20mm compared to more

than 30mm observed for *M. perniciosa* after 20 days growth on PDA (Fig. 4.3 A), MDA (Fig. 4.5 A) and MA (Fig. 4.7 A).

Agaricus bisporus inoculated on potato dextrose agar (PDA) in paired culture (in the same Petri plate: Fig. 4.1 A) with *M. perniciosa* started growing at the same rate as *A. bisporus* grown alone, with no significant difference in growth between paired cultures and the controls at 6 days (Fig. 4.2 A and 4.2 B). This changed as the growth period increased. After 10, 15 and 20 days, respectively the radial growth of the *A. bisporus* control significantly exceeded that measured for *A. bisporus* inoculated in the same plate with *M. perniciosa* (Fig. 4.3 A). *M. perniciosa* (on PDA) grew significantly more when inoculated in the same Petri plate with *A. bisporus* than when inoculated alone as control (Fig. 4.3 B).

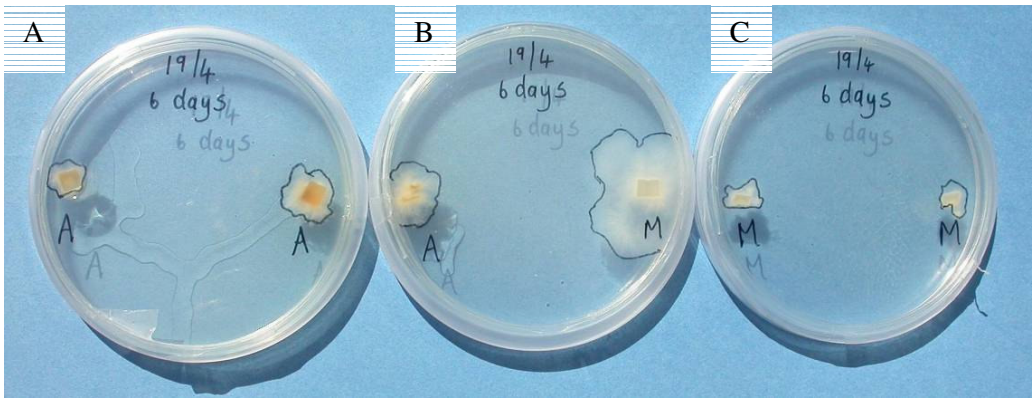

Figure 4.2 A) Growth of *Agaricus bisporus* control. B) Growth of *A. bisporus* in paired culture with *Mycogone perniciosa*. C) Growth of *M. perniciosa* control.

Agaricus bisporus growth in dual culture was inconsistent. Measurements were difficult to take because growth was very different when *A. bisporus* was growing in the bottom plate of a dual culture compared to when growing in the top plate of a dual culture, with growth in the top plate being much more dense and having inconsistent diameters. *A. bisporus* in the top part of a dual culture (Fig. 4.4 A) tend to grow more vigorously than *A. bisporus* growing in the bottom part (Fig. 4.4 B).

Results of growth of *M. perniciosa* in dual culture with *A. bisporus*, on PDA, were similar to results for *M. perniciosa* growth in paired cultures. Growth of *M. perniciosa* when growing in the bottom plate of a dual culture (Fig. 4.4 A) was similar to growth of *M. perniciosa* growing in the top plate of a dual culture (Fig. 4.4 B). *M. perniciosa*, in either top or bottom plate of a dual culture, grew significantly faster throughout the growth period when inoculated in dual culture with *A. bisporus* than when inoculated alone as control (Fig. 4.4).

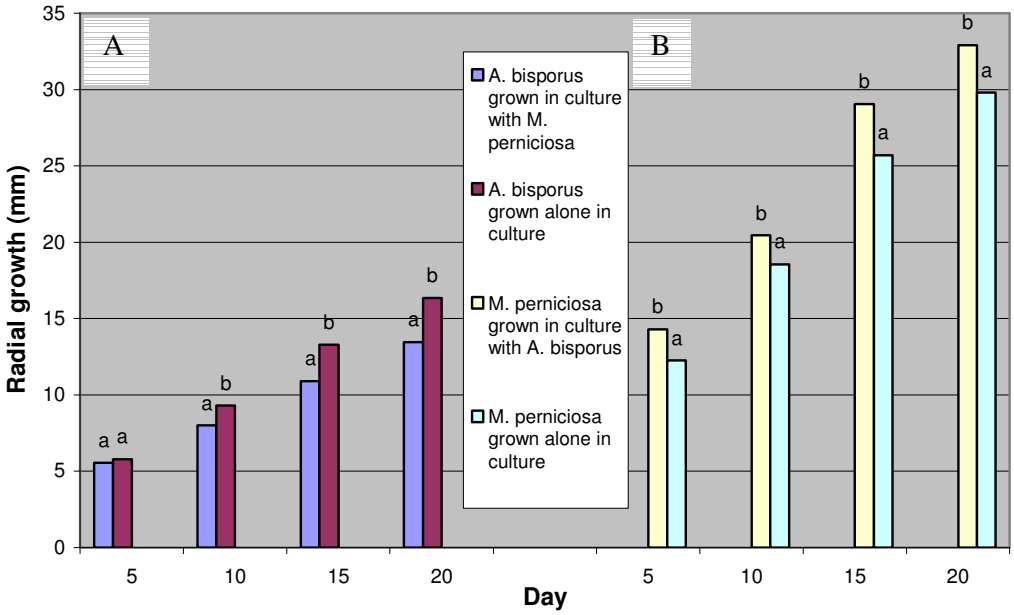

Figure 4.3 A) Growth of *Agaricus bisporus* in paired culture with *Mycogone perniciosa* compared to *A. bisporus* control, on PDA. B) Growth of *M. perniciosa* in paired culture compared to *M. perniciosa* control, on PDA. (a and b indicate significant differences within the day of measurement at $P \leq 0.05$).

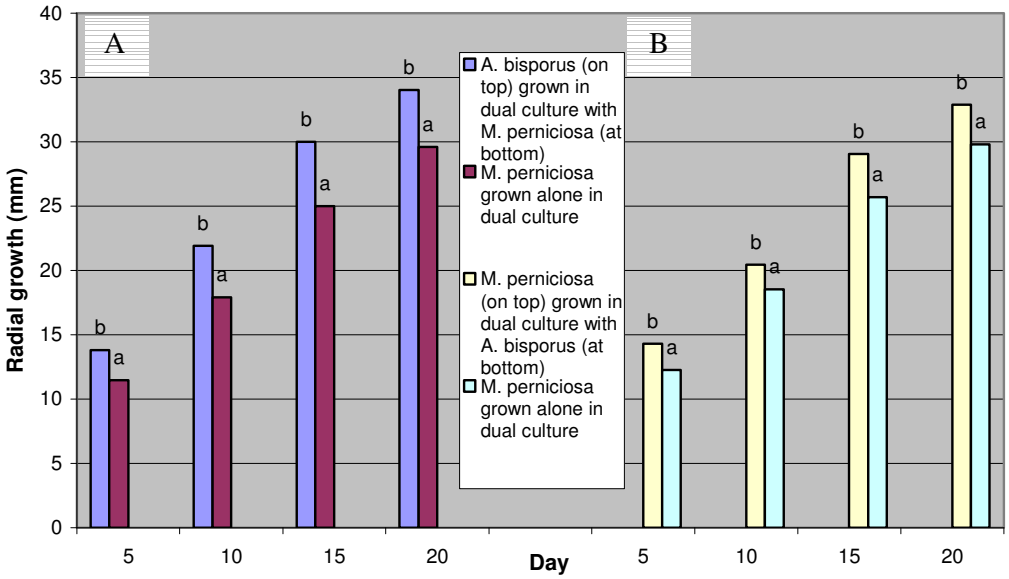

Figure 4.4 A) Growth of *Mycogone perniciosa* in the bottom plate of a dual culture with *Agaricus bisporus* in the top plate compared to growth of *M. perniciosa* grown alone as control on PDA. B) Growth of *M. perniciosa* in the top plate of a dual culture with *A. bisporus* in the bottom plate compared to *M. perniciosa* control on PDA (a and b indicate significant differences within the day of measurement at $P \leq 0.05$).

4.4.2. *Agaricus bisporus* and *Mycogone perniciosa* grown on mushroom extract dextrose agar in paired and dual culture

On mushroom dextrose agar (MDA) *A. bisporus* and *M. perniciosa* started with a slower growth rate (Fig. 4.5) than that recorded for paired cultures on PDA (Fig. 4.3). Growth rate of *A. bisporus* in paired culture on MDA remained slower than that observed on PDA for the duration of the experiment, with no culture exceeding growth of 28mm diameter in 20 days (Fig. 4.5 A).

Agaricus bisporus inoculated in the same Petri plate with *M. perniciosa* grew faster than *A. bisporus* inoculated alone during the entire growth period except on day 10 where there was no significant difference in growth (Fig. 4.5 A).

Measurement of *A. bisporus* growth in dual culture was difficult and growth was inconsistent for the same reasons as discussed for PDA (section 4.4.1). Growth of *M. perniciosa*, on MDA (Fig. 4.5 A), in paired culture started at a slower rate than that recorded for paired cultures on PDA (Fig. 4.3 A), but later growth increased to resemble a growth pattern similar to that of *M. perniciosa* in paired culture on PDA. *M. perniciosa* grew significantly more in association with *A. bisporus* in paired culture on MDA than when grown alone as control (Fig. 4.5 B).

Mycogone perniciosa grew more vigorous in dual culture on MDA with *A. bisporus* than when compared with *M. perniciosa* growing alone as control in both top (Fig. 4.6 A) and bottom plate (Fig. 4.6 B). Growth did not differ significantly between the treatment with *M. perniciosa* as top plate or as bottom plate. This trend was similar to that observed on PDA (Fig. 4.4). Growth of *M. perniciosa* in dual culture with *A. bisporus* was significantly more compared to growth measured for the *M. perniciosa* control on day 10 and 15. After day 15, *M. perniciosa* control grew just as well as *M. perniciosa* in dual culture with *A. bisporus* (Fig. 4.6).

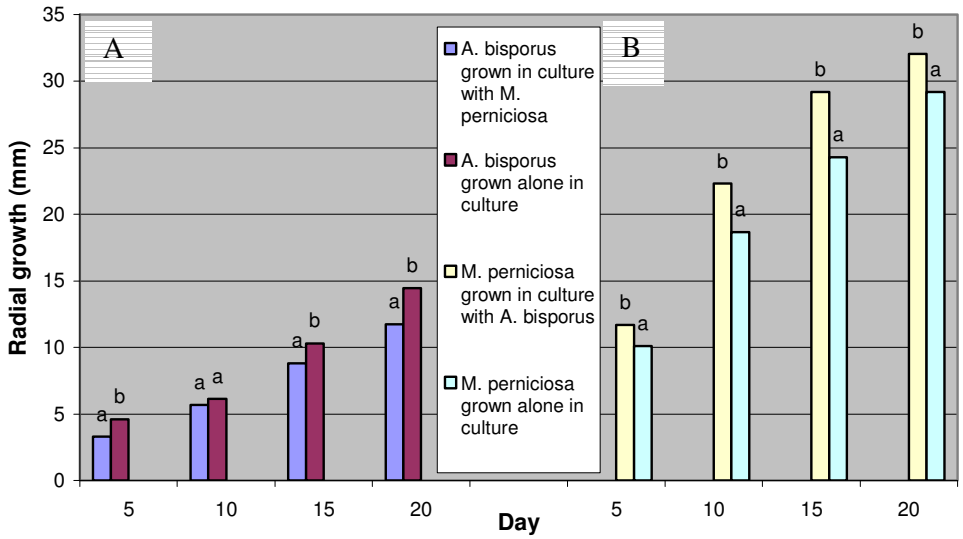

Figure 4.5 A) Growth of *Agaricus bisporus* in paired culture with *Mycogone perniciosa* compared to *A. bisporus* control, on MDA. B) Growth of *M. perniciosa* in

paired culture compared to *M. perniciosa* control, on MDA (a and b indicate significant differences within the day of measurement at $P \leq 0.05$).

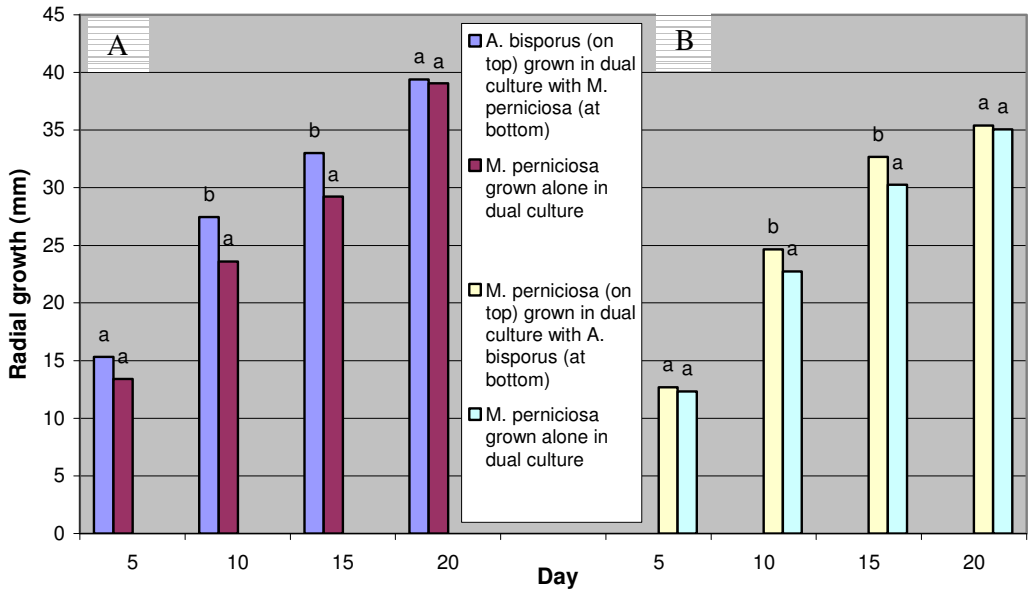

Figure 4.6 A) Growth of *Mycogone perniciosa* in the bottom plate of a dual culture with *Agaricus bisporus* in the top plate compared to growth of *M. perniciosa* grown alone as control on MDA. B) Growth of *M. perniciosa* in the top plate of a dual culture with *A. bisporus* in the bottom plate compared to *M. perniciosa* control on MDA (a and b indicate significant differences within the day of measurement at $P \leq 0.05$).

4.4.3. *Agaricus bisporus* and *Mycogone perniciosa* grown on mushroom extract agar in paired and dual culture

Paired cultures of *A. bisporus* and *M. perniciosa* on mushroom extract agar (MA) had a lower average growth for both fungi compared to growth of *M. perniciosa* and *A. bisporus* on the dextrose containing media i.e. PDA and MDA. The growth trend for paired cultures on MA was similar to that observed for PDA (section 4.4.1) and MDA (section 4.4.2)

At first (day 5), *A. bisporus* growth in the presence of *M. perniciosa* in paired culture was more than *A. bisporus* growing alone (Fig. 4.7 A). Radial growth measurements of *A. bisporus* growing in paired culture with *M. perniciosa* were not significantly different on day 10. On day 15 and 20 *A. bisporus* control grew significantly more than *A. bisporus* in paired culture with *M. perniciosa*. Findings on day 15 and 20 show a similar trend to that observed for PDA and MDA.

Growth of *M. perniciosa* in paired culture with *A. bisporus* on MA was significantly more than growth of the *M. perniciosa* control throughout the growth period (Fig. 4.7 B). Although the growth pattern was similar to that previously discussed for PDA and MDA, the average radial growth measured on this media was less than on PDA and MDA, for the duration of the experiment.

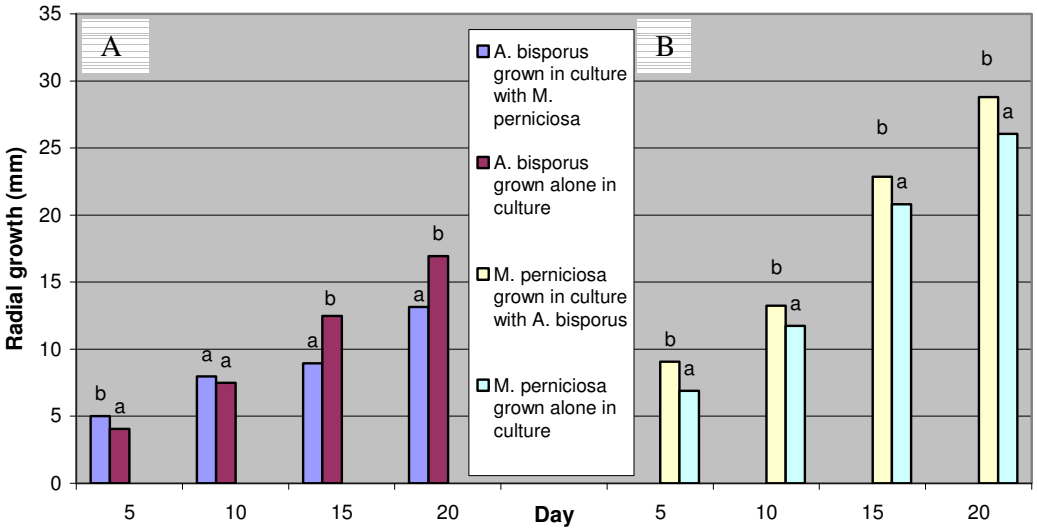

Figure 4.7 A) Growth of *Agaricus bisporus* in paired culture with *Mycogone perniciosa* compared to *A. bisporus* control, on MA. B) Growth of *M. perniciosa* in paired culture compared to *M. perniciosa* control, on MA (a and b indicate significant differences within the day of measurement at $P \leq 0.05$).

There was no significant difference in growth of *M. perniciosa* in dual culture with *A. bisporus* compared to the control on MA (Fig. 4.8). There was no significant difference between growth of *M. perniciosa* as top or bottom plate.

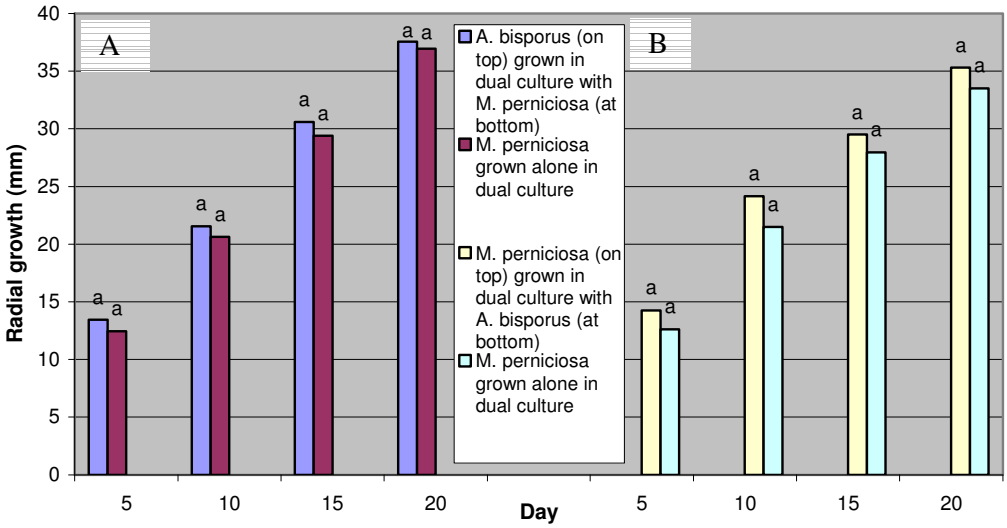

Figure 4.8 A) Growth of *Mycogone perniciosa* in the bottom plate of a dual culture with *Agaricus bisporus* in the top plate compared to growth of *M. perniciosa* grown alone as control on MA. B) Growth of *M. perniciosa* in the top plate of a dual culture with *A. bisporus* in the bottom plate compared to *M. perniciosa* control on MA (a and b indicate significant differences within the day of measurement at $P \leq 0.05$).

4.4.4. *Agaricus bisporus* and *Mycogone perniciosa* grown in half plate on potato dextrose agar

The *A. bisporus* control grew significantly more in half plate PDA Petri plates than when grown with *M. perniciosa*, while *M. perniciosa* grew more vigorously when grown in the same half plate Petri plate with *A. bisporus* than when grown alone (Fig. 4.9).

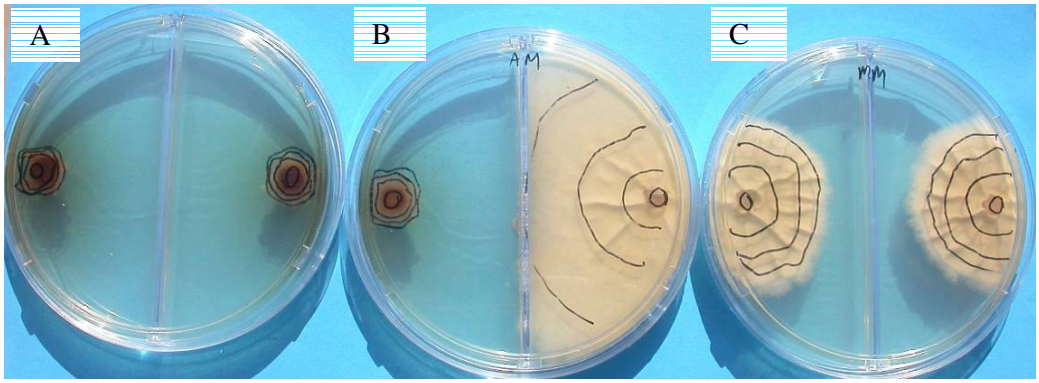

Figure 4.9 A) Growth of *Agaricus bisporus* control in half plate on PDA. B) Growth of *A. bisporus* (left side of half plate) in paired culture with *Mycogone perniciosa* (right side of half plate) on PDA. C) Growth of *M. perniciosa* control in half plate on PDA.

Agaricus bisporus growing alone in half plate as control grew significantly more at 5, 10 and 15 days compared to *A. bisporus* grown in the same half plate with *M. perniciosa* (Fig. 4.10 A).

Mycogone perniciosa grew significantly more in half plate containing *A. bisporus* mycelium in the other compartment than the control, with only *M. perniciosa* in both compartments of the half plate throughout the growth period (Fig. 4.9 B, C and 4.10 B). Radii and growth could not be recorded on day 20 as was previously done in this chapter for paired and dual cultures due to the sides and central separations acting as a physical barrier.

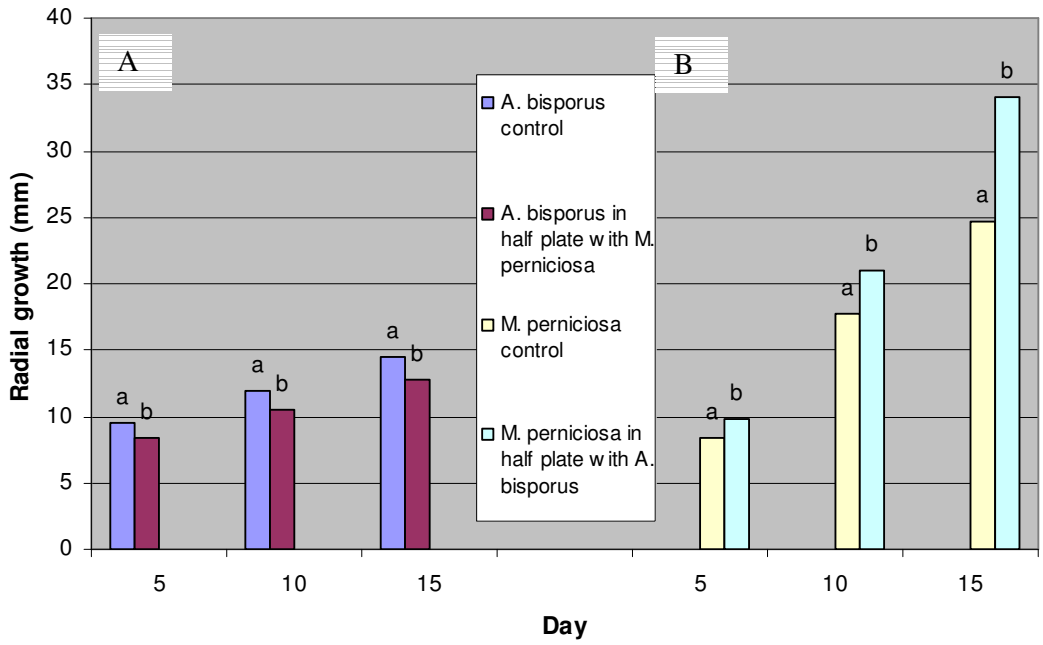

Figure 4.10 A) Growth of *Agaricus bisporus* control in half plate compared to *A. bisporus* grown in half plate with *Mycogone perniciosa* on PDA. B) Growth of *M. perniciosa* control in half plate compared to *M. perniciosa* grown in half plate with *A. bisporus* on PDA (a and b indicate significant differences within the day of measurement at $P \leq 0.05$).

4.5. DISCUSSION

Agaricus bisporus grew significantly more when growing alone in paired cultures on PDA, MDA and MA, in dual culture on the aforementioned media as well as in half plate on PDA. This may indicate that *M. perniciosa* produces an exudate into the media or a volatile that may inhibit *A. bisporus* growth. Sharma and Kumar (2000) found that, *M. perniciosa* had an average radial growth of 38mm in 15 days on PDA, whilst in these experiments *M. perniciosa* grew approximately 30mm in 15 days. Sharma and Kumar did not investigate growth of *M. perniciosa* on MDA, but rather on MA with sucrose as sugar source. The average radial growth was found to be 36mm compared to 33mm on MDA in this study. On MA *M. perniciosa* had radial

growth of 27mm in 15 days in this study, compared to 31mm measured by Sharma and Kumar (2004).

Growth of *M. perniciosa* in culture with *A. bisporus* was significantly more than the *M. perniciosa* controls in all experiments except in dual culture on MA. The reasons for increased growth of *M. perniciosa* in the presence of *A. bisporus* have not been investigated before and are not documented. According to Feovilova *et al*. (2004), *A. bisporus* vegetative growth changes the lipid and carbohydrate composition of agar media while Sidorova and Velikanov (2000) found that agaricoid fungi exert a significant influence on the microbiota formation under natural conditions in soil ecosystems. From our experiments it is evident that *A. bisporus* also influences the growth of *M. perniciosa* in agar culture. Increased *M. perniciosa* growth in split plate indicates that the stimulus received by *M. perniciosa* in the presence of *A. bisporus* is most likely volatile. Terekhova and Semenova (2005) have found that *A. bisporus* produces various, mostly uncharacterised volatile compounds.

According to Holland and Cooke (1991) *M. perniciosa* has the ability to move carbon compounds but not nitrogen compounds. From their experiments with *M. perniciosa* in half plate with one half having no sugar source and the other half being nutrient-rich it was found that *M. perniciosa* produced similar amounts of chlamydospores in both sides, while *M. perniciosa* produced no chlamydospores when a carbon source was absent from both sides of the half plate. They performed the same experiment with a nitrogen-source but *M. perniciosa* did not produce spores in the half plate without a nitrogen-source. In our experiments *M. perniciosa* grew significantly more when a carbon source was available and on MA when it was in contact with the same media as *A. bisporus* i.e. in paired culture, but not in dual culture.

M. perniciosa is a mycoparasite of *A. bisporus*, mycoparasitism in its simplest form is the acquisition of carbon from another fungus (Steyaert *et al*., 2004). It is hypothesized that *M. perniciosa* grown in paired culture could move carbon compounds present in *A. bisporus* hyphae even though there was no dextrose in the

MA media. By having the ability to mobilize the carbon from *A. bisporus*, *M. perniciosa* was still able to grow and be affected by the growth of *A. bisporus*. In dual cultures, however, the fungi were not in contact with the same media and no carbon translocation could take place, preventing increased growth of *M. perniciosa*.

We hypothesize that *M. perniciosa* may also produce compounds that affect *A. bisporus* growth and secondary metabolism. According to Terekhova and Semenova (2005) various micromycete organisms regulate the functional activity of basidiomycete mycelium. Some micromycetes regulate activity directly, but most micromycetes play an indirect role by controlling compounds in the environment (Terekhova and Semenova, 2005). While it is known that *M. perniciosa* has the ability to affect cells of reproductive *A. bisporus* from a distance (not measured) (Umar *et al*., 2000) our study has shown that *M. perniciosa* also has the ability to affect the vegetative growth of *A. bisporus*, *in vitro.*

These observations of *M. perniciosa* and *A. bisporus* growth *in vitro* raised questions of how the fungi interact and the effect that volatiles may have on the growth of either fungus. While it is known that results of interactions in agar culture and under natural conditions are not necessarily the same (Dowson *et al*., 1988) and nutrition, depth and age of agar, size of inoculum and temperature of incubation play a role in agar plate cultures (Fravel, 1988), these *in vitro* studies provide valuable information on the effects of *M. perniciosa* on *A. bisporus*, *in vitro* in agar plates.

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CHAPTER 5

ENZYME and VOLATILE PRODUCTION BY *Mycogone perniciosa*

5.1. ABSTRACT

To cause wet bubble disease, the pathogen, *Mycogone perniciosa* may need to enter *Agaricus bisporus* cells, possibly through production of enzymes. *M. perniciosa* influences growth of *A. bisporus* in agar culture and fruit body formation. Enzyme bioassays for *M. perniciosa* were done on agar media containing specific substrates. *M. perniciosa* produced chitinase, glucanase, cellulase, lipase, proteinase, and amylase, but not pectinase. The effects that volatile compounds produced by *M. perniciosa*, in agar culture, have on *A. bisporus* fruit bodies cultivated in simulated commercial conditions was investigated by attaching either closed or open Petri plates with *M. perniciosa* to pots with actively growing mushrooms at different growth stages. The differences in mycelial extracts of *M. perniciosa* grown in half plate with *A. bisporus*, and both fungi alone were determined with thin layer chromatography. Some pots containing closed-plates of *M. perniciosa* attached to them developed disease. In only one of these cases could it be identified as wet bubble disease. All pots containing open-cultures of *M. perniciosa* developed wet bubble disease. It was not possible to verify the volatile constituents from half plates of *M. perniciosa* and *A. bisporus*. *A. bisporus* grown with *M. perniciosa* in liquid culture produced an uncharacterised compound not present when either fungi grew alone. The identity of this compound was not determined.

5.2. INTRODUCTION

Umar *et al*. (2000) described *Mycogone perniciosa* (Magnus) Delacroix as a mycoparasite of *Agaricus bisporus* (Lange) Imbach. To cause infection *M. perniciosa* must have the ability to enter *A. bisporus* cells. The fungal cell wall is a rigid structure, which protects the fragile protoplast from damage and maintains the characteristic shape of the cell (García Mendoza *et al*., 2003). Fungal cell walls have a very complex chemical structure (Calonje *et al*., 2000). This structure is crucial in

fungal morphology and relations with other living creatures (García Mendoza *et al*., 2003), and contains glucans, glycans, chitin, proteins and lipids (Bartnicki-García, 1968). Mycoparasitism involves the degradation of a fungal cell wall by an antagonist via the production of lytic enzymes (Lima *et al*., 1993). Although it could not conclusively be determined in Chapter 3 that *M. perniciosa* does indeed infect and penetrate *A. bisporus* hyphe, hyphae and phialoconidia of *M. perniciosa* were shown to be firmly attached to *A. bisporus* hyphae. The present work was undertaken to investigate the enzymes produced by *M. perniciosa* in order to achieve a better understanding of the pathogen's ability to enter and infect *A. bisporus*.

It was found in Chapter 4 that *M. perniciosa* affects the growth of, and is in turn affected by *A. bisporus* in agar culture. *M. perniciosa* is said to also have the ability to affect the physiological state of the cells and tissues of *A. bisporus* from a distance (Umar *et al*., 2000). While it is well known that infection with *M. perniciosa* leads to an overwhelming cellular host reaction in *A. bisporus* with mushroom fruiting bodies perceivable as monstrosities (Umar *et al*., 2000), the mechanisms that enable the pathogen to cause infection are largely unknown. In this study the aim was to investigate the effects of chemical components produced by *M. perniciosa* in agar cultures on *A. bisporus* cultivated in simulated commercial conditions as an initial effort to elucidate the biochemical basis of the host-pathogen interaction in WBD.

5.3. MATERIALS AND METHODS

5.3.1. Cultures

A mushroom infected with *Mycogone perniciosa* was obtained from Dr. Petra Labuschagne (Plant Pathology Laboratories, University of Pretoria). The pathogen was isolated and pure cultured from this diseased mushroom as described in Chapter 3. A culture of the cultivated mushroom, *Agaricus bisporus*, was obtained from Dr. Martmari van Greuning (Sylvan Incorporated, South Africa). For the duration of experiments both fungi were maintained on PDA (Merck, Johannesburg) at 25ºC in alternating 12 hour light/dark conditions.

5.3.2. Enzymes

The production of extracellular chitinase, cellulase, lipase, protease, amylase and pectinase was investigated. Enzyme bioassays were carried out by inoculating fungal isolates onto agar media containing specific substrates. Inoculation was done by placing 5mm diam. mycelial plugs of actively growing *M. perniciosa* in the centre of Petri plates containing the required nutrients as discussed below. Four replicates per isolate were made for each test and all experiments were repeated.

5.3.2.1. Extracellular chitinase production

The production of chitinase was investigated using a solid agar medium modified from Lima *et al*. (1993). The agar medium contained ($g.l^{-1}$ of distilled water (dH_2O)): bacteriological peptone, 1.0; urea, 0.3; $(NH_4)_2SO_4$, 1.4; $MgSO_4.7H_2O$, 0.3; $CaCl_2.6H_2O$, 0.3; glucose, 1.0; colloidal chitin, 15.0 (wet weight) and agar (Merck), 20.0, adjusted to pH 6.0 (autoclaved for 15 min at 121ºC). Plates were inoculated as previously described and incubated at room temperature (20-25ºC) in the dark. Plates were examined every second day (for 10 days) for clearing of the opaque agar medium, which was indicative of chitinase activity (Lima *et al*., 1993).

5.3.2.2. Extracellular cellulase production

The production of extracellular cellulase was determined on agar medium supplemented with milled filter paper as an insoluble form of cellulose. The modified medium contained ($g.l^{-1}$ of dH_2O): bacteriological peptone, 1.0; NH_4NO_3, 1.0; $MgSO_4.7H_2O$, 0.2; KCl, 0.2; K_2HPO_4, 0.9; glucose, 1.0 and agar (Merck), 20.0, adjusted to pH 6.5. The medium was supplemented with $5g.l^{-1}$ milled filter paper and autoclaved for 15 min at 121ºC. Inoculated plates were incubated at room temperature (20-25ºC) in alternating 12 hour light/dark conditions and examined after 10 days. Plates were observed for zones of clearance indicating evidence of extracellular cellulase activity (Lima *et al*., 1993).

5.3.2.3. Extracellular lipase production

Lipolytic activity was determined using a solid agar medium. The basal medium contained ($g.l^{-1}$ of dH_2O): bacteriological peptone, 10.0; NaCl, 5.0; $CaCl_2.2H_2O$, 0.1 and agar (Merck), 20.0, adjusted to pH 6.0. Tween 20 (Sorbitan monlaurate) was used as lipid substrate and $10ml.l^{-1}$ was added to the basal medium before autoclaving at 121ºC for 15 min. Inoculated plates were incubated at room temperature (20-25ºC) in alternating 12 hour light/dark conditions and examined after 10 days. Lipolytic enzyme activity was determined by the observation of a visible precipitate resulting from the formation of crystals of calcium salt of the lauric acid liberated by the lipase enzyme (Cattelan *et al*., 1999).

5.3.2.4. Extracellular proteinase production

The production of extracellular proteinase was determined on agar medium supplemented with an 8% (w/v) solution of gelatine. The modified medium contained ($g.l^{-1}$ of dH_2O): bacteriological peptone, 1.0; NH_4NO_3, 1.0; $MgSO_4.7H_2O$, 0.2; KCl, 0.2; K_2HPO_4, 0.9; glucose, 1.0 and agar (Merck), 20.0, adjusted to pH 6.5. The medium was supplemented with $50ml.l^{-1}$ gelatine solution and autoclaved for 15 min at 121ºC. Inoculated plates were incubated at room temperature (20-25ºC) in alternating 12 hour light/dark conditions and examined after 10 days. Proteinase activity was detected by staining the plate with 0.1% (w/v) amido black in methanol-acetic acid-distilled water (30:10:60 v/v/v) for 15 min followed by destaining with methanol-acetic acid-dH_2O (30:10:60 v/v/v). The appearance of clear zones around growth was indicative of extracellular proteinase activity (Cattelan *et al*., 1999).

5.3.2.5. Extracellular amylase production

The ability of *M. perniciosa* to degrade starch was used as the criterion for determining amylase activity. The modified medium contained ($g.l^{-1}$ of dH_2O): bacteriological peptone, 1.0; NH_4NO_3, 1.0; $MgSO_4.7H_2O$, 0.2; KCl, 0.2; K_2HPO_4, 0.9; glucose, 1.0 and agar (Merck), 20.0, adjusted to pH 6.5. The medium was

supplemented with 2 $g.l^{-1}$ soluble starch and autoclaved for 15 min at 121ºC. Inoculated plates were incubated at room temperature (20-25º) in alternating 12 hour light/dark conditions and examined after 10 days. Plates were flooded with Lugols iodine solution and examined after three hours. Starch molecules were stained blue whereas zones of clearing were an indication of amylolytic activity (Cattelan *et al*., 1999).

5.3.2.6. Extracellular pectinase production

Pectolytic activity was determined on a modified M9 agar medium amended with 1.2$g.l^{-1}$ of yeast extract. The modified medium contained ($g.l^{-1}$ of dH_2O): Na_2HPO_4, 6.0; NH_4Cl, 1.0 NH_4NO_3, 1.0 $CaCl_2$, 0.2; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5; KH_2PO_4, 3.0; glucose, 1.0; yeast extract, 1.2 and agar (Merck) 20.0 (autoclaved for 15 min at 121ºC). Apple pectin (5$g.l^{-1}$) was added as a source of pectin was added to the media before autoclaving. Inoculated plates were incubated at room temperature (20-25ºC) in alternating 12 hour light/dark conditions and examined after 10 days. Plates were flooded with 2M HCl and fungal growth surrounded by clear halos were considered positive for pectinase activity (Cattelan *et al*., 1999).

5.3.3. Volatiles

To examine volatiles *in vivo, A. bisporus* was grown under simulated commercial conditions using Phase III compost that have been through spawn run and casing media obtained from Highveld Mushrooms (South Africa). Five hundred grams of Phase III compost was placed in 1L pots cleaned with a 1% solution of sodium hypochlorite. The compost was left to settle for one day. The next day 500g of pasteurized commercial casing media was placed on top of the compost layer in the pots. The pots were enclosed in large, black polythene bags. The polythene bags were opened and the developing primordia inside the pots sprayed with a fine mist of water once a day to create adequate moisture for mushroom growth. Six Petri plates with actively growing *M. perniciosa* sealed with insulation tape were attached to the sides of each pot with growing *A. bisporus* (Fig. 5.1 A and 5.1 B). There were three

treatments. Plates were added on day 0 (the day of casing), day 7 (the day of pinning) and day 10. For each treatment (day 0, 7 and 10) there were three control pots without plates. Plates were sealed with insulation tape whilst growing to ensure accumulation of volatiles. When attached to the pots inside the polythene bags small (5mm diameter) holes facing the developing primordia were aseptically made at the top of vertically attached Petri plates to allow volatiles to escape within the polythene bags (Fig. 5.1 A). Controls consisted of pots without Petri plates. This experiment was repeated in trial two.

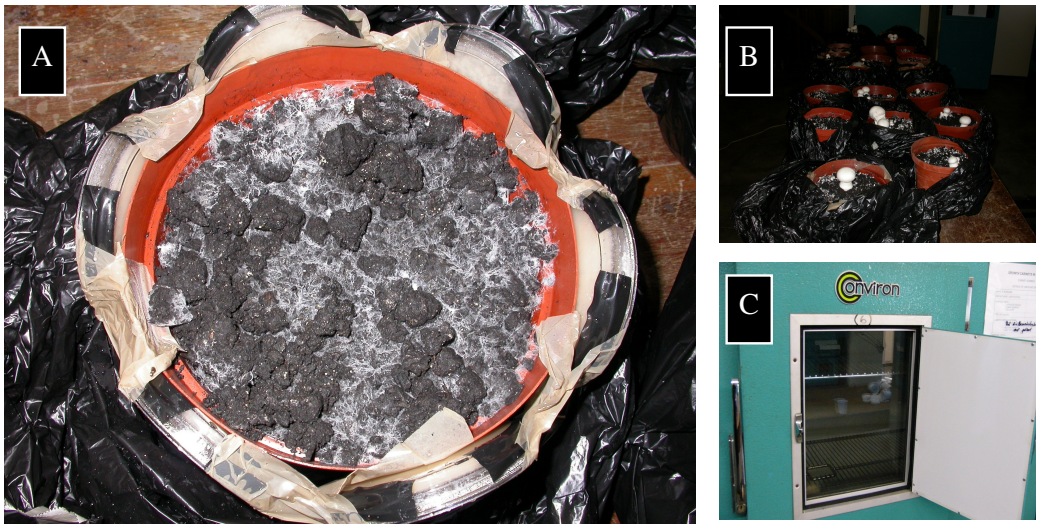

Figure 5.1 A) Pot (inside black polythene bag) with primordia of *Agaricus bisporus*, and closed Petri plates of *M. perniciosa* cultures attached to it (arrows). B) Pots with first flush of mushrooms inside black polythene bags. C) Growth chamber used to simulate commercial growth conditions used to cultivate *A. bisporus* mushrooms.

In addition six Petri plates, without their lids, with actively growing *M. perniciosa* were placed within polythene bags containing pots with growing *A. bisporus*. Therefore, the trial consisted of three treatments with three pots each of open and closed cultures. This trial was repeated. The open cultures were prevented from coming in direct contact with the developing mushroom by placing the Petri plates in the bottom of the polythene bags at the base of the pots.

Pots were placed in a randomised block design in a growing chamber at temperatures and humidity similar to commercial growing conditions (Fig. 5.1 C). Temperature and RH for spawn-run and pinning were 28ºC, 95% and 17ºC, 90%, respectively.

The effect of volatiles was assessed by comparing the pinning and subsequent fruit body development in controls, closed-plate and open-plate pot experiments. Disease in closed-plate and open-plate pots and absence of disease in control pots was an indication that volatiles produced by *M. perniciosa* may have had an effect on *A. bisporus* growth.

5.3.4. Thin layer chromatography

The hydrolysis products of agar cultures with *M. perniciosa*, *A. bisporus* and both fungi in paired culture were analysed using thin layer chromatography (TLC). In paired cultures the pathogen and the host were grown together on potato dextrose agar (PDA) in 90mm Petri plates as described in Chapter 3, section 3.3.1. Petri plates were incubated at 25ºC (the optimum growth temperature of the pathogen) in alternating 12 hour light/dark conditions up to the point where the hyphae of *M. perniciosa* and *A. bisporus* was in closest proximity without any contact.

The entire fungal colonies, with as little agar as possible were mashed with a mortar and pestle and the mycelial extracts were dissolved in 100ml of dichloromethane. The experiment was performed on ice to minimise the loss of volatiles. A 5ml aliquot of each hydrolysate was spotted onto a silica gel sheet using a microcapillary pipette. The spots were allowed to migrate to 1cm from the top of the TLC plate in hexane:ethyl acetate at a ratio of 5:1 and dichloromethane:ethyl acetate at a ratio of 1:1 respectively. After migration the TLC plates were examined under a UV light (254nm) and spots were circled with a lead pencil. Thin layer chromatography plates were subsequently stained for 5 min using vanillin (Sigma) to make compounds on the plates visible to the human eye.

In addition *M. perniciosa* and *A. bisporus* were grown alone and together in nutrient broth (Merck). Five millimetre plugs of actively growing cultures of both fungi were inoculated into 1L flasks containing 300ml nutrient broth each. *A. bisporus* was inoculated into the broth two days prior to inoculation of *M. perniciosa*, to compensate for its slower growth. The experimental flasks were inoculated with both *A. bisporus* and *M. perniciosa*. Controls consisted of flasks inoculated with either *M. perniciosa* or *A. bisporus*. Flasks, sealed with foil and parafilm, containing broth cultures were incubated at 25ºC in alternating 12 hour light/dark conditions for 14 days on a shaker at 75rpm.

The hydrolysis products of broth cultures were also examined using TLC. The mycelial mass and liquid broth were separated by running the broth culture through a separation funnel after the addition of 200ml hexane and subsequently 200ml dichloromethane. The resulting mycelial extract was dissolved in 200ml dichloromethane and 5ml aliquots of the resulting hydrolysates were spotted onto silica TLC plates. The liquid broth was vacuum-dried with a Bücchi Rotavapor R-200 and the sediment dissolved in 200ml dichloromethane. Five millilitres of the resulting hydrolysates were spotted onto silica TLC plates.

The spots on TLC plates were allowed to migrate up to 1cm from the top of the plate in a solution of dichloromethane:ethyl acetate at a ratio of 1:1. The plates were viewed and stained as previously described.

5.4. RESULTS AND DISCUSSION

5.4.1. Enzymes

Mycogone perniciosa produced chitinase and glucanase (Fig. 5.2 A), cellulase (Fig. 5.2 B), lipase (Fig. 5.2 C), proteinase (Fig. 5.2 D), and amylase (Fig. 5.2 E), but not pectinase (Fig. 5.2 F), under laboratory conditions.

In order for *M. perniciosa* to infect its host it needs to enter the host cells either through mechanical or enzymatic means. It is known that the fungal cell wall structure is crucial in fungal morphology and relations with other living creatures (García Mendoza *et al*., 2003), and contains glucans, glycans, chitin, proteins and lipids (Bartnicki-García, 1968). These compounds are also found in cell walls of *A. bisporus* (Manning and Wood, 1983; Wood *et al*., 1988).

Production of chitinase and glucanase by *M. perniciosa* could function in the degradation of *A. bisporus* cell wall components, since these hydrolytic enzymes have been shown to play a significant role in cell wall lysis (Lima *et al*., 1993). Calonje *et al*. (1997) noted an increase in production of glucanase by *Verticillium fungicola* var. *fungicola* (Preuss) Hassebr. (the pathogen causing dry bubble disease of *A.bisporus*) in the presence of *A. bisporus* cells. This current study suggests that glucanase and chitinase produced by *M. perniciosa* could also play a role in wet bubble disease of mushrooms.

The presence of protein in fungal cell walls is an accepted fact (Calonje *et al*., 2000). Proteinase produced by *M. perniciosa* could function in the degradation of the host's cell walls, since proteinase has been implicated previously in the degradation of *A. bisporus* cell walls in the interaction between the mushroom host and the mycoparasite *Trichoderma harzianum* Rifai (Geremia *et al*., 1993).

Both *M. perniciosa* and *A. bisporus* produce cellulase, with the latter producing a complete cellulase system enabling it to grow on crystalline cellulose as sole carbon source (Manning and Wood, 1983; Wood *et al*., 1988). *M. perniciosa* probably needs to produce cellulase as it also grows on the same compost substrate as the host, *A. bisporus*.

Amylase was not found to be involved in the infection process of *A. bisporus* by *Trichoderma*, rather it aided in the survival of this mushroom pathogen in the field by mediating hydrolysis of starch (De Marco *et al*., 2003). This enzyme, also produced by *M. perniciosa* is probably also linked to saprophytic survival.

Production of pectic enzymes has been widely reported in host-pathogen interactions (Smith *et al*., 1986; De Lorenzo *et al*., 1997), but *M. perniciosa* did not produce pectinase. The pathogen does not need to produce this enzyme since pectin is mainly found in fruit and *M. perniciosa* is a pathogen exclusive of fungi.

Secretion of enzymes *in vitro* is a good indicator of the kind of enzymes that a pathogen can produce and the role they play in relation to disease (Calonje *et al*., 1997). Kuës and Liu (2000) found that considerable changes in enzyme production occur during fruiting of *A. bisporus* indicating that enzymes play an important role in fruit body development. The role of enzymes produced during fruit body development by *A. bisporus* itself is not yet understood, increasing the difficulty to determine the role of enzymes produced by the pathogen, *M. perniciosa*, and other mushroom pathogens.

5.4.2. Volatiles

From our *in vitro* studies discussed in Chapter 4, there is evidence that *M. perniciosa* produces volatiles that affect the growth of *A. bisporus* and *vice versa*. This section aimed to establish the effect of volatiles produced by *M. perniciosa* on growth and development of *A. bisporus* cultivated under simulated commercial conditions.

Control pots (from both trials) contained healthy *A. bisporus* fruiting bodies three days after pinning (Fig. 5.3 A) and these mushrooms increased in size (Figs. 5.3 B and 5.3 C) with no disease symptoms developing until the time of harvest 14 days after pinning (Fig. 5.3 D).

Pots with closed-plates of *M. perniciosa* attached to them developed disease (Fig. 5.3 E and 5.3 H). One of the three pots from day 0 had white, fluffy mycelial growth characteristic of wet bubble disease around day 5, with amber droplets, also a characteristic symptom (Smith, 1924) being exuded on day 6 (Fig. 5.3 F). A sample of this diseased mushroom viewed with light microscopy revealed the presence of *M. perniciosa* chlamydospores. No mushrooms in this pot developed into

undifferentiated fruiting bodies. No other mushrooms in any of the pots from day 0 in both trials developed into WBD mushrooms.

One of the three pots containing *M. perniciosa* closed-plates from day 7 of the first trial contained malformed mushrooms. The diseased mushrooms became covered with brownish coloured mycelia giving off a foul smell (Fig. 5.3 G). Amber droplets were found in the early stages of infection in this pot (day 8), but later the brown mycelia completely covered the mushrooms in this pot, extending into the casing soil. Samples taken from this pot could not be identified as *M. perniciosa*, but revealed that there was more than one fungus responsible for the dramatic colonisation and infection of these diseased mushrooms.

A pot from day 0 of the second trial also showed similar results to the diseased pot from day 0 in the first trial but, as was the case with the pot from day 7, the white, fluffy mycelial growth produced in this pot could not be unambiguously identified as *M. perniciosa.*

Pots containing open-cultures of *M. perniciosa* developed wet bubble disease without exception in both the first and second trials (Fig. 5.3 H). This may however be more likely due to spreading of spores than due to volatiles produced by *M. perniciosa*. It was difficult to design an experiment that facilitated the flushing of CO_2 while still enabling the accumulation of volatiles. Therefore, it is possible that the volatiles were not in high enough quantities to affect mushrooms in these trials.

Lesions of WBD mushrooms formed during infections from open-plate cultures contained amber-brown extracellular liquid. This extracellular fluid was abundant with bacteria (Fig. 5.3 F) which, according to Umar *et al*. (2000), was the actual cause of death of the WBD mushroom in most cases.

In the second trial one of the three pots from the control from day 0 also produced one wet bubble diseased mushroom. This may be due to *M. perniciosa* being resident in the casing soil used for the experiment or contamination from the air

during CO_2 flushing. However, considering the large amount of healthy mushrooms that developed it probably did not interfere with the experimental design.

From the above results, it appears that cultures of *M. perniciosa* being in close proximity to growing *A. bisporus* fruiting bodies do have an effect, although not directly linked to only WBD. Pots containing closed-plates of *M. perniciosa* were more susceptible to disease, compared to control pots, and developed disease on three occasions. In only one of these cases could the identity of the pathogen causing disease be identified as *M. perniciosa.* The only way that *M. perniciosa* could have affected growing *A. bisporus* mushrooms in this pot was through volatiles, or by contamination of the casing medium. The role of volatiles in disease development is uncertain and difficult to determine.

5.4.3. Thin layer chromatography

It was not possible to verify the volatile constituents that differed in half plate and dual cultures of *M. perniciosa* and *A. bisporus*, due to the vast rate of evaporation of volatiles and the lack of specialised equipment to analyse them. The concentration of volatiles produced in agar plate cultures were not sufficient to quantify on TLC. In addition, most of the volatile produced was lost due to a lack of equipment necessary to perform this type of TLC. Keeping the samples on ice for the duration of the experiment proved to be of value in keeping the little available volatiles from evaporating completely.

Hydrolysates of the mycelial mass of the host, pathogen and both fungi cultured together showed differences on TLC (Fig. 5.4 A. and 5.4 C). *A. bisporus* grown in culture with *M. perniciosa* produced a compound that was absent from cultures with *A. bisporus* growing alone (Fig. 5.4 A). This compound was the only compound that differed from the hydrolysate of the control and therefore may play a role in the signals received by *M. perniciosa* to increase its growth in the presence of *A. bisporus*. There was a difference in the concentration of compounds visible on a TLC plate with *M. perniciosa* in association with *A. bisporus* showing a higher

concentration of all compounds than the control (Fig. 5.4 A). *M. perniciosa* controls also fluoresced more than dual cultures.

Broth cultures were also used to determine whether clearer results could be obtained. If one looks at the volatile terpenoid constituents identified from fungi in the past, mainly abundant ones like drimenol from *Gloeophyllum odoratum* (Wulfen) Imazeki (Kahlos et al., 1994) have been identified. It has to be noted that these compounds were isolated from liquid cultures and that their occurrence in nature has not been confirmed.

Agaricus bisporus and *M. perniciosa* growing together in liquid culture produced a compound not produced when either of them was growing alone in nutrient broth (Fig. 5.4 D). It has to be noted that production of volatiles is time and temperature dependant (Wuest and Forer, 1975). Therefore the compounds produced by *A. bisporus* and *M. perniciosa* may only be produced when conditions are favourable and adequate recognition between the fungi has occurred.

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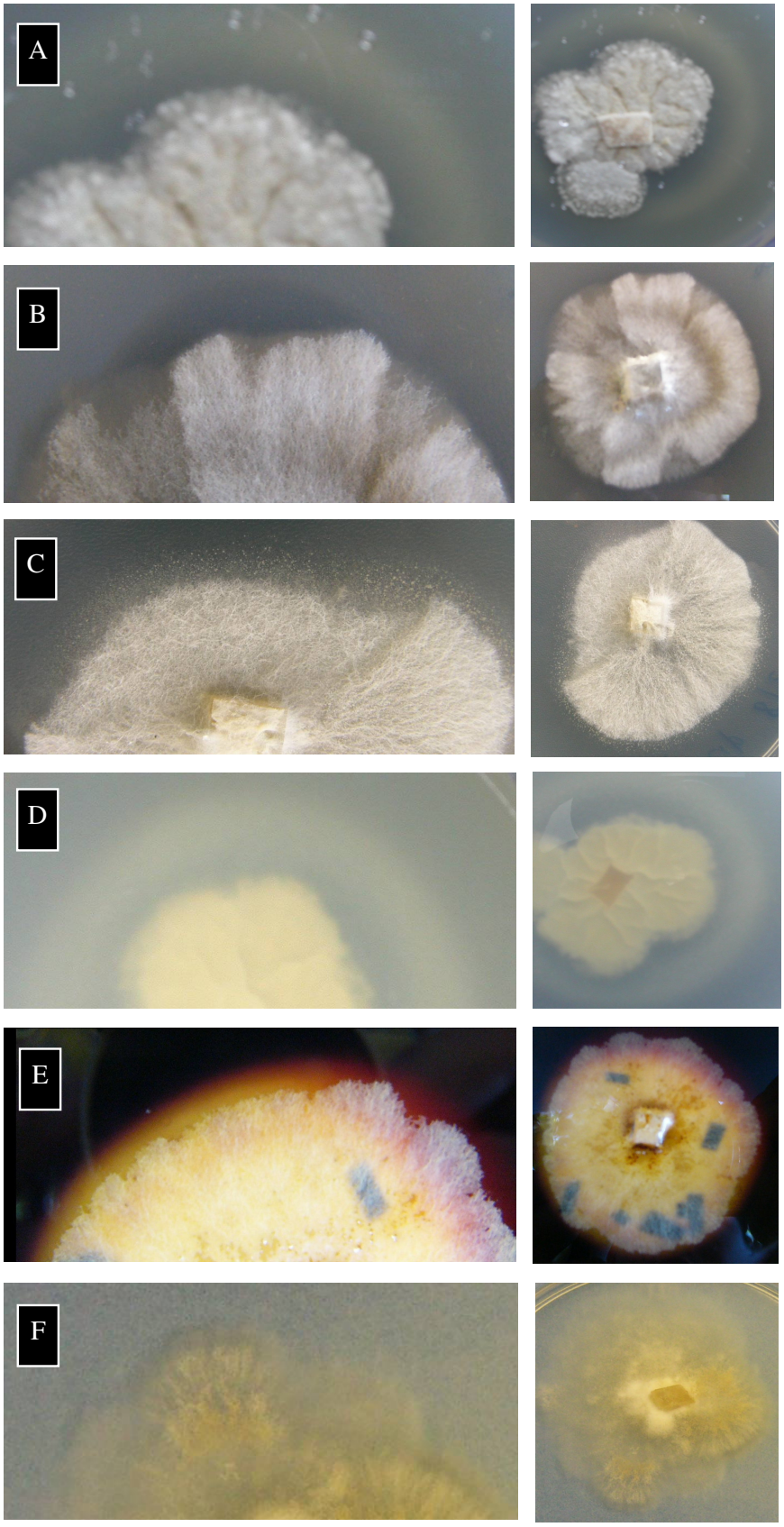
Figure 5.2.

Production of enzymes by *Mycogone perniciosa* in agar culture. A) Chitinase indicated by clearing of the opaque medium. B) Cellulase indicated by a zone of clearance surrounding the colony. C) Lipase indicated by the formation of salt crystal on the medium. D) Proteinase indicated by the appearance of clear zones around the colony. E) Amylase indicated by the clearing zones surrounding the colony F) *M. perniciosa* did not produce pectinase.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E
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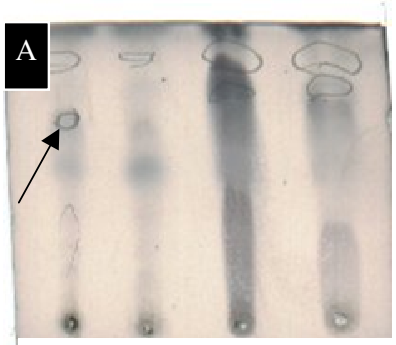
Figure 5.3

A) Developing mushrooms from trial two. B) Healthy developing *Agaricus bisporus* mushrooms on day 5 in a pot from the first trial. C) Healthy *A. bisporus* mushrooms on day 10 in a pot from the second trial. D) Healthy *A. bisporus* mushrooms on day 14 in a pot from the first trial. E) A pot with mushrooms developing with Petri plates containing *M. perniciosa* attached to it from the first trial. F) Mushrooms inside a pot from the first trial illustrating white, fluffy mycelial growth of *Mycogone perniciosa* and amber droplets (arrow). G) Diseased *A. bisporus* mushrooms completely covered in brown mycelial growth. H) Mushroom infected with *M. perniciosa* illustrating the accumulation of amber fluid in a wound.


A
B
C
D
E
F
G
H

Figure 5.4 Thin layer chromatography (TLC) plates.

Plate A: TLC plate of hydrolysate of mycelial extracts of *Agaricus bisporus* and *Mycogone perniciosa* grown in paired culture on PDA in Petri plates migrated with dichloromethane: ethyl acetate at a ratio 1:1; lane1 (A+M): *A. bisporus* in association with *M. perniciosa*; lane 2 (A): *A. bisporus* control, lane 3 (M+A): *M. perniciosa* in association with *A. bisporus*; lane 4 (M): *M. perniciosa* control. *M. perniciosa* paired with *A. bisporus* in lane 3 gave darker bands with the same concentration hydrolysate compared to control in lane 4. *A. bisporus* paired with *M. perniciosa* (lane 1) possibly produced a compound not present in *A. bisporus* control (lane 2) indicated by arrow. Plate B: TLC plate of hydrolysate of PDA media on which *A. bisporus* and *M. perniciosa* were grown in paired culture Petri plates after removal of the bulk of mycelia migrated with dichloromethane: ethyl acetate at a ratio 1:1; lane 1 (A+M): *A. bisporus* in association with *M. perniciosa*; lane 2 (A): *A. bisporus* control, lane 3 (M+A): *M. perniciosa* in association with *A. bisporus*; lane 4 (M): *M. perniciosa* control. The media where *M. perniciosa* was paired with *A. bisporus* (lane 3) had a higher content of all compounds compared to control (lane 4). In addition *M. perniciosa* paired with *A. bisporus* (lane 3) fluoresced under UV light (not shown). Plate C: TLC plate of hydrolysate of mycelial extracts of *A. bisporus* and *M. perniciosa* grown in paired culture on PDA in Petri plates migrated with hexane: ethyl acetate at a ratio 5:1 lane 1 (M+A): *M. perniciosa* in association with *A. bisporus*; lane 2 (M): *M. perniciosa* control; lane 3 (A): *A. bisporus* control; lane 4 (A+M): *A. bisporus* in association with *M. perniciosa*. *A. bisporus* in association with *M. perniciosa* (lane 4) was the only hydrolysate with compounds visible on the TLC plate, indicating that there is a difference between this sample and the *A. bisporus* control. Plate D: TLC plate of hydrolysate of nutrient broth liquid media in which *A. bisporus* and *M. perniciosa* were grown either separately or together, samples were migrated with dichloromethane: ethyl acetate at a ratio 1:1; lane 1 (A): *A. bisporus* control, lane 2 (A+M): *A. bisporus* in the same liquid culture with *M. perniciosa*, lane 3 (M): *M. perniciosa* control. The hydrolysates contained the same compounds in different concentrations, except for an uncharacterised compound in lane 2 (arrow) that was produced when *M. perniciosa* and *A. bisporus* grew in the same culture but was absent when both these fungi grew alone.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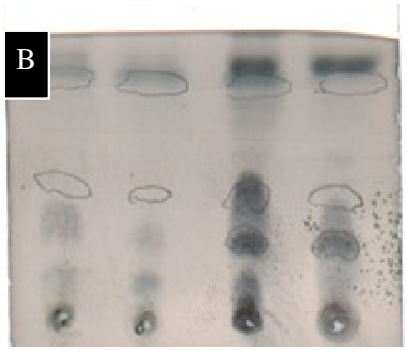
lane 1
A+M
lane 2
A
lane 3
M+A
lane 4
M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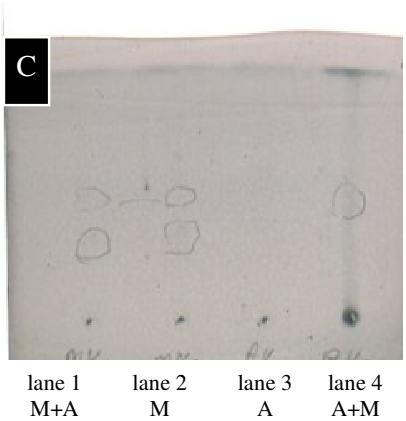
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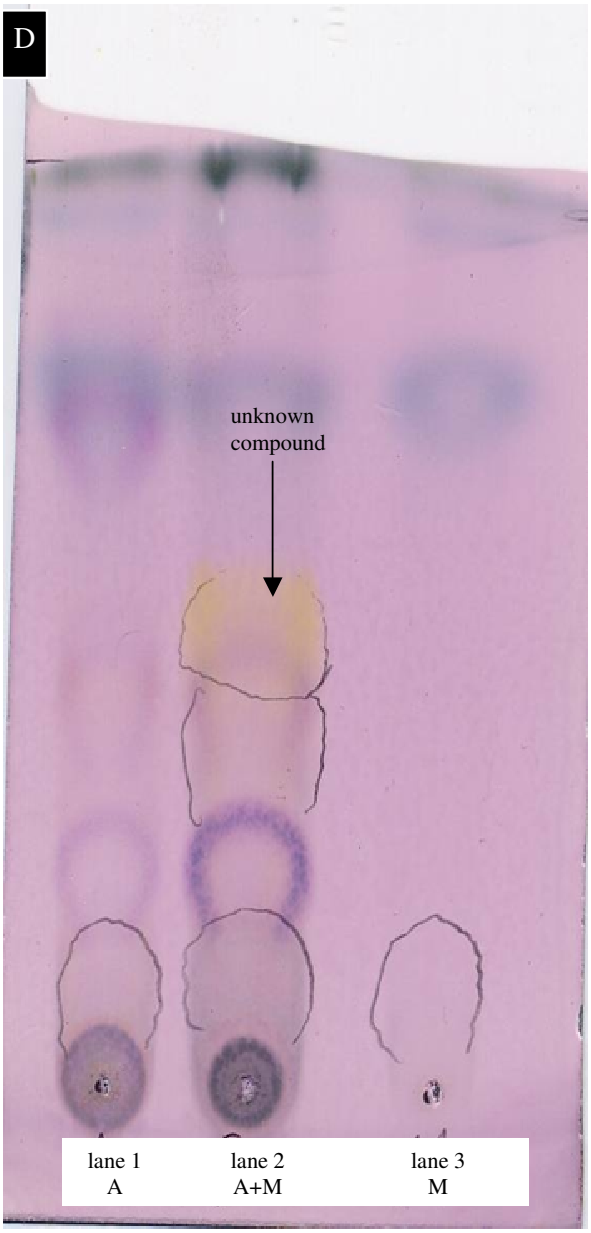
A+M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CHAPTER 6
GENERAL DISCUSSION

A major problem in mushroom cultivation is the poor understanding of fruit body development of *Agaricus bisporus* (Lange) Imbach (De Groot *et al*., 1998; Umar and Van Griensven, 1999). *Mycogone perniciosa* (Magnus) Delacroix is a fungal pathogen that affects fruit body development and manifests in wet bubble disease (WBD). Infected mushrooms are malformed and not fit for sale (Dielemann-van Zaayen, 1976), resulting in economic losses for the mushroom industry. Not a great deal of research has been done on the disease, in particular reference to the interaction between *M. perniciosa* and *A. bisporus* and the mechanisms involved.

Smith (1924) was the first to describe spore morphology of the pathogen. Spore morphology investigated using light and scanning electron microscopy in this study, revealed similar characteristics as found by other authors (Smith, 1924; Fletcher *et al*., 1994) however there were a number of anomalies, some which have been described before by Holland and Cooke (1991).

Umar *et al*. (2000) described intrahyphal growth of *M. perniciosa*, characterising the pathogen as a mycoparasite. Further investigation was required to further elucidate the various aspects of infection leading to WBD. In contrast to Umar *et al*. (2000) we found no conclusive evidence of intrahyphal growth in both WBD mushrooms or *in vitro* cultures. However adherence and coiling of *M. perniciosa* hyphae to *A. bisporus* hyphae, hyphal collapse of host hyphae and increased amounts of abnormal hyphal cells were found.

Due to evidence of cellular disruption and the close proximity of hyphae of the pathogen to those of the host, it was decided to investigate enzyme production by *M. perniciosa*, in an effort to determine whether the pathogen have the ability to degrade *A. bisporus* cell walls. *M. perniciosa* produced proteinase, cellulase, chitinase and lipase, all of which are cell-wall degrading enzymes that could play a role in cell wall collapse and penetration of the host. Considering host invasion is an essential

prerequisites for successful mycoparasitism, host cell wall degradation is conceivably one of the most harmful events associated with the antagonistic process (Hajloui *et al*., 1994).

To date the effect mycelial growth of *M. perniciosa* and *A. bisporus* have on each other has not been investigated *in vitro*. *A. bisporus* grew significantly more in the absence of *M. perniciosa*, while *M. perniciosa* had increased growth in the presence of *A. bisporus* in paired, dual and split culture, indicating that *A. bisporus* produced a compound, most likely volatile that stimulated *M. perniciosa*. *M. perniciosa* also affected growth of *A. bisporus* cultivated under simulated commercial conditions, possibly due to production of volatile compounds that affected *A. bisporus* growth and secondary metabolism. According to Terekhova and Semenova (2005), various micromycete organisms regulate the functional activity of basidiomycete mycelium, some directly but most by controlling compounds in the environment.

It was not possible to verify the volatile constituents produced by *M. perniciosa in vitro*, due to the low quantities of volatile being produced in agar media. Hydrolysates of *A. bisporus* and *M. perniciosa* growing together in liquid culture revealed a compound using thin layer chromatography. This coumpound was not produced when either of the fungi was grown alone in nutrient broth. Whether the compound was produced by *A. bisporus* or *M. perniciosa* could not be determined.

From this study we can conclude that hyphae of *M. perniciosa* are closely associated with hyphae of *A. bisporus* in WBD mushrooms as well as in culture. *M. perniciosa* is capable of producing enzymes required to penetrate the host and furthermore it is most likely that a volatile is involved in the host-pathogen interaction.

This study was the first to investigate the *in vitro* interaction between *A. bisporus* and *M. perniciosa*, to examine the compounds they produce in each other's presence and their effect. This study offers some insight into the host-pathogen interaction in wet bubble disease and provides numerous questions to be answered. Future research should include transmission electron microscopy (TEM) studies to investigate

possible penetration of *A. bisporus* by *M. perniciosa* and possible interhyphal growth of *M. perniciosa* inside *A. bisporus* hyphae. The possible volatiles and other compounds produced by *M. perniciosa* and *A. bisporus* when grown together needs to be further investigated by means of high performance liquid chromatohraphy (HPLC) as well as gas chromatography mass spectometry (GC-MS).

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CHAPTER 7

SUMMARY

Mycogone perniciosa, a pathogen of _Agaricus bisporus_

by

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Degree: Maters of Science (MSc)

Agaricus bisporus (Lange) Imbach, the most commonly cultivated mushroom worldwide, can be infected by *Mycogone perniciosa* (Magnus) Delacroix, the fungal pathogen causing wet bubble disease. This contagious disease, known since 1888, results in malformed mushrooms, crop loss and subsequent economic losses. This work examined the interaction between *M. perniciosa* and *A. bisporus* using light and scanning electron microscopy, growing the fungi together in agar plate culture on different media, enzyme assays and determining volatiles produced and their effect on mushrooms cultivated under simulated commercial conditions in an effort to better understand the process of infection and subsequent disease manifestation. No conclusive evidence of intrahyphal growth was found but *M. perniciosa* adhered to, coiled around and caused hyphal collapse of *A. bisporus* hyphae indicating that it is possibly an infective mycoparasite. *M. perniciosa* grew significantly more in the presence of *A. bisporus* in agar culture, while *A. bisporus* grew less than when cultured alone. The fungi influenced each other's growth when they had no contact with the same media, indicating that a volatile compound may be produced when they grow together. Petri plates with cultures of *M. perniciosa*, were attached to pots with developing mushrooms. These mushrooms developed disease, possibly due to *in vitro* volatile production by the pathogen. It was not possible to verify the volatile constituents produced by *M. perniciosa*, but hydrolysates from liquid cultures of *A. bisporus* grown with *M. perniciosa* examined with thin layer chromatography revealed a compound that was not present when either fungi grew alone. The identity of this compound was not determined. This study was the first to investigate

whether the dedifferentiation caused by *M. perniciosa* in fruit bodies of *A. bisporus* plays a role in the *in vitro* growth of both fungi and also the first attempt at elucidating the biochemical basis of the host-pathogen interaction in wet bubble disease.

Keywords: *Mycogone perniciosa*, wet bubble disease, *Agaricus bisporus*, mushroom, interaction, mycoparasite, volatile

APPENDIX

Raw data: *Agaricus bisporus* and *Mycogone perniciosa* grown in paired culture on potato dextrose agar (PDA), mushroom extract dextrose agar (MDA) and mushroom extract agar (MA).

Raw data: *Agaricus bisporus* and *Mycogone perniciosa* grown in dual culture on potato dextrose agar (PDA), mushroom extract dextrose agar (MDA) and mushroom extract agar (MA).

Raw data: *Agaricus bisporus* and *Mycogone perniciosa* grown in half plate culture on potato dextrose agar (PDA).

Raw data: Statistical analysis.

PAIRED CULTURES REPLICATE 1

5 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control		10 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control	
PDA							**PDA**						
1	11.5	4.0	7.0	9.0	4.0	3.5	1	16.0	6.0	13.0	18.0	5.0	5.0
2	11.0	4.0	10.0	10.0	4.0	4.0	2	15.5	5.0	16.0	18.0	5.5	5.5
3	11.5	4.0	11.0	9.5	3.5	4.5	3	15.0	6.0	17.0	16.5	6.0	5.5
4	12.0	4.0	12.0	9.0	3.0	4.0	4	20.0	5.5	20.0	16.0	5.5	5.5
5	10.0	4.0	10.5	9.0	4.5	3.5	5	15.0	6.0	15.5	16.0	5.5	6.0
6	11.5	4.0	12.0	10.0	3.5	3.5	6	19.0	6.0	18.0	17.0	5.0	5.0
7	11.0	4.0	12.0	9.0	4.0	4.5	7	19.0	5.0	17.0	17.0	5.0	6.5
8	11.0	4.0	11.0	9.0	3.5	4.0	8	15.5	5.0	16.0	17.0	6.0	5.0
9	11.0	4.0	12.0	10.0	4.0	4.0	9	19.0	6.0	18.0	17.0	5.0	6.0
10	11.5	4.0	11.5	11.0	3.5	4.0	10	19.0	5.5	17.0	18.0	6.5	5.5
MDA							**MDA**						
1	12.0	3.5	10.0	6.5	4.5	6.0	1	21.0	5.5	17.5	17.5	6.5	9.0
2	10.0	3.5	10.0	6.5	4.0	5.0	2	19.5	5.5	21.5	14.0	6.0	8.5
3	13.0	3.5	10.5	8.5	4.0	4.5	3	23.0	6.5	19.0	14.5	6.0	8.5
4	12.5	3.0	12.0	9.0	4.0	5.5	4	25.0	6.5	19.5	17.0	5.5	8.0
5	10.0	3.0	7.5	8.5	4.0	4.5	5	21.0	5.5	15.0	17.0	5.5	8.5
6	12.0	3.5	10.0	8.0	5.0	6.5	6	22.0	3.5	16.0	16.0	7.0	12.0
7	10.0	3.5	10.0	8.0	4.5	5.5	7	20.0	3.5	23.0	16.0	6.5	9.5
8	13.0	3.5	9.5	7.5	4.0	5.5	8	23.0	3.5	16.5	15.0	6.0	9.5
9	12.5	3.0	11.5	7.5	5.5	6.5	9	22.5	3.0	20.5	15.0	12.0	10.5
10	10.0	3.0	14.0	8.0	6.5	6.0	10	21.0	3.0	23.0	13.5	10.5	10.5
MA							**MA**						
1	9.5	3.5	5.5	8.0	4.0	4.5	1	17.5	6.0	15.0	18.0	7.5	6.5
2	9.0	3.5	7.0	7.5	4.0	4.5	2	16.0	7.0	17.0	17.0	6.0	8.0
3	8.0	4.0	5.0	8.0	4.0	4.5	3	18.0	6.5	15.0	18.0	5.0	8.0
4	9.0	3.5	8.0	9.0	4.0	5.0	4	19.0	7.0	16.0	17.0	8.0	7.0
5	9.5	4.0	6.5	8.0	4.0	5.0	5	18.0	7.0	16.5	17.0	7.5	8.0
6	8.0	3.5	8.5	9.0	4.0	5.5	6	15.0	6.5	17.0	18.0	9.5	8.5
7	9.0	3.0	7.0	8.0	4.0	6.0	7	16.0	6.5	18.0	18.0	7.5	8.5
8	9.0	3.5	5.0	5.0	4.0	5.5	8	18.0	6.5	10.0	11.0	7.0	9.0
9	9.5	3.5	8.0	8.0	4.0	6.0	9	18.0	7.0	18.0	19.0	8.5	9.0
10	9.5	3.5	8.0	8.0	4.0	5.0	10	18.0	6.5	19.0	18.0	7.5	8.5

PAIRED CULTURES REPLICATE 2

5 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control		10 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control	
PDA							**PDA**						
1	8.5	5.5	7.5	8.0	5.5	5.0	1	18.5	7.5	18.0	17.5	9.5	9.0
2	7.0	5.5	6.5	7.5	6.0	5.0	2	17.5	7.5	19.0	15.0	10.0	8.5
3	8.0	6.0	8.0	4.5	6.0	6.5	3	17.5	9.0	15.0	14.5	10.0	10.0
4	8.5	5.0	7.5	8.5	5.0	6.0	4	17.0	7.5	17.5	16.0	9.0	9.5
5	8.5	6.0	5.0	8.0	6.5	6.0	5	18.5	8.0	17.0	18.5	9.0	9.0
6	8.0	5.5	8.5	8.0	7.5	7.0	6	17.5	8.5	16.5	18.0	11.5	12.0
7	9.0	5.5	7.5	8.5	5.5	6.5	7	17.5	8.0	18.0	18.5	8.5	9.5
8	9.0	5.5	7.0	8.0	7.0	5.5	8	18.0	7.5	16.0	18.0	11.0	9.0
9	7.0	6.0	7.5	8.0	5.0	5.5	9	16.0	8.5	16.0	18.0	8.0	10.0
10	7.0	5.0	7.0	8.0	4.0	4.0	10	16.0	8.0	16.0	17.0	6.5	8.5
MDA							**MDA**						
1	8.0	5.0	6.5	7.0	4.5	5.0	1	15.5	7.5	15.5	15.0	7.5	8.0
2	7.0	6.0	8.5	8.0	4.5	4.5	2	16.0	11.0	13.5	15.0	8.0	9.0
3	8.0	5.5	7.5	7.0	5.0	4.5	3	15.0	8.5	15.0	15.0	8.5	9.0
4	7.5	5.5	8.0	7.5	4.5	4.0	4	17.0	9.0	15.0	14.0	8.0	8.5
5	8.0	5.0	8.0	8.0	5.0	4.0	5	16.0	8.5	16.5	14.5	8.5	10.0
6	8.0	5.5	7.0	7.0	5.0	5.0	6	15.5	9.0	14.0	15.5	8.5	8.5
7	8.0	5.5	7.5	7.0	5.0	5.0	7	14.5	9.5	16.0	15.0	11.5	9.0
8	7.5	5.0	7.0	7.0	5.5	5.5	8	15.0	9.5	15.0	15.5	10.0	9.5
9	8.0	5.5	7.5	8.0	5.0	4.5	9	15.5	9.5	15.0	15.5	9.0	8.0
10	7.5	5.5	8.5	8.0	4.5	5.0	10	15.0	9.0	14.5	15.0	8.0	8.5
MA							**MA**						
1	7.5	5.0	7.0	6.5	4.0	4.0	1	12.0	8.0	11.5	13.0	7.5	7.5
2	6.5	4.5	6.5	7.0	4.0	4.0	2	11.5	8.5	12.0	14.0	8.5	6.0
3	5.5	5.5	8.0	4.5	4.0	4.0	3	12.5	8.5	14.0	10.0	8.0	5.0
4	7.0	5.5	6.5	6.5	4.0	4.0	4	14.0	8.5	12.5	11.0	6.5	8.0
5	7.5	5.0	6.5	7.0	4.0	4.0	5	14.5	8.5	12.0	12.0	7.5	7.5
6	9.0	5.5	7.5	7.0	4.0	4.0	6	13.5	9.0	12.5	13.5	8.5	9.5
7	8.5	7.0	5.5	6.5	4.0	4.0	7	13.0	11.5	10.5	11.5	8.5	7.5
8	6.5	4.5	7.5	7.0	4.0	4.0	8	12.0	7.0	11.5	12.5	8.0	7.0
9	7.5	5.5	6.0	7.5	4.0	4.0	9	12.5	8.5	15.0	12.0	8.5	8.5
10	7.5	5.0	5.5	8.0	4.0	4.0	10	12.5	8.5	10.5	14.5	8.0	7.5

PAIRED CULTURES REPLICATE 3

5 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control		10 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control	
PDA							**PDA**						
1	11.0	5.5	10.0	11.5	4.0	5.0	1	18.0	6.0	11.5	15.0	5.0	5.5
2	11.0	4.5	10.0	11.0	4.0	4.0	2	14.5	4.5	13.5	15.5	4.5	5.0
3	16.0	5.0	9.0	10.0	4.5	5.0	3	22.0	5.5	12.0	13.5	5.0	5.5
4	14.0	4.5	9.0	9.0	4.0	4.5	4	18.0	5.0	12.5	10.0	4.0	5.5
5	10.0	4.5	11.0	12.0	5.0	4.5	5	15.5	5.0	14.5	15.5	5.5	5.5
6	10.0	6.0	9.5	10.0	5.0	4.0	6	14.0	7.0	11.5	19.0	5.0	5.0
7	13.0	4.5	7.5	8.0	4.5	4.0	7	18.0	4.5	11.0	12.0	5.0	5.5
8	11.0	5.0	8.0	9.5	4.5	4.0	8	19.0	5.5	12.0	14.5	5.5	5.0
9	14.0	5.0	11.5	12.0	4.5	4.5	9	19.5	5.5	13.0	16.5	5.0	5.5
10	11.0	5.5	9.5	10.0	4.0	4.0	10	17.0	6.0	12.0	15.5	4.5	4.5
MDA							**MDA**						
1	12.0	3.5	10.0	10.0	4.5	4.0	1	21.0	5.5	15.5	16.0	6.5	5.5
2	10.0	3.5	10.0	10.0	4.0	5.0	2	18.5	5.5	21.5	21.0	6.0	7.0
3	13.0	3.5	10.5	9.5	4.0	4.5	3	23.0	6.5	19.0	16.5	6.0	6.5
4	12.5	3.0	12.0	11.5	4.0	4.0	4	26.0	6.5	19.5	20.5	5.5	6.0
5	10.0	3.0	9.5	12.0	4.0	4.0	5	21.0	5.5	15.0	21.0	6.0	6.0
6	12.0	3.5	10.5	12.0	4.0	4.0	6	22.0	5.5	16.5	18.0	6.0	6.5
7	10.0	3.5	11.0	12.0	4.0	4.0	7	20.0	5.5	18.0	18.0	6.0	6.0
8	13.0	3.5	10.0	11.5	4.0	4.0	8	23.0	5.5	17.0	17.0	6.0	6.0
9	12.5	3.0	10.5	10.0	4.5	4.5	9	22.5	5.0	17.0	16.5	6.0	6.5
10	10.0	3.0	10.0	11.0	4.0	4.5	10	20.0	5.0	17.0	17.5	6.0	6.0
MA							**MA**						
1	9.5	3.5	5.5	8.0	4.0	4.0	1	17.5	6.0	15.0	18.0	7.5	7.5
2	9.0	3.5	5.0	7.5	4.0	4.5	2	16.0	7.0	17.0	17.0	6.0	8.5
3	8.0	4.0	5.0	8.0	4.0	5.0	3	18.0	6.5	12.0	18.0	5.0	8.0
4	9.0	3.5	8.0	9.0	4.0	4.5	4	19.0	7.0	16.0	17.0	8.0	6.5
5	9.5	4.0	4.0	8.0	4.0	5.0	5	18.0	7.0	14.5	17.0	7.5	7.5
6	8.0	3.5	8.5	9.0	4.0	6.0	6	15.0	6.5	17.0	18.0	9.5	8.5
7	9.0	3.0	7.0	8.0	4.0	6.0	7	16.0	6.5	18.0	18.0	7.5	8.5
8	9.0	3.5	5.0	5.0	4.0	4.0	8	18.0	6.5	12.0	11.0	7.0	8.0
9	9.5	3.5	8.0	8.0	4.0	5.5	9	18.0	7.0	18.0	19.0	8.5	8.5
10	9.5	3.5	8.0	8.0	4.0	5.5	10	18.0	6.5	19.0	18.0	7.5	8.0

PAIRED CULTURES REPLICATE 1

15 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control		20 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control	
PDA							**PDA**						
1	21.0	8.0	24.0	21.0	8.0	8.5	1	24.5	10.0	26.0	23.5	11.0	11.5
2	20.0	7.0	23.0	19.5	8.0	7.5	2	25.0	8.0	25.5	21.0	12.0	12.0
3	20.0	7.0	21.0	18.0	8.0	8.5	3	22.0	9.0	23.0	20.5	11.0	11.0
4	26.0	7.5	20.0	22.0	8.0	8.5	4	29.0	9.5	21.5	25.0	12.0	12.5
5	19.0	8.0	22.0	20.0	8.0	8.0	5	22.0	10.0	25.0	22.0	12.0	11.5
6	23.0	8.0	20.0	23.0	8.0	8.0	6	27.0	10.0	22.5	25.5	12.0	13.0
7	24.0	6.5	21.0	22.0	7.5	8.0	7	28.0	8.0	24.0	24.0	11.5	12.0
8	22.0	7.0	20.0	20.0	8.5	8.0	8	25.0	9.0	22.5	23.0	12.5	12.5
9	24.0	8.0	22.0	24.0	7.5	8.0	9	28.0	10.0	25.0	26.0	11.0	11.5
10	23.0	8.0	24.0	23.0	8.5	7.5	10	26.0	10.0	25.5	24.0	13.0	11.0
MDA							**MDA**						
1	27.0	8.5	28.0	24.0	10.5	13.0	1	30.0	11.0	27.5	30.0	15.0	18.0
2	24.5	9.0	31.5	24.0	10.0	12.5	2	27.0	10.0	30.0	30.0	14.0	15.0
3	27.0	11.0	27.0	27.0	9.5	13.0	3	29.0	13.0	33.0	30.0	13.5	16.0
4	32.0	9.0	31.0	23.0	10.0	10.5	4	33.0	12.0	30.0	30.0	14.0	17.5
5	31.0	11.0	23.0	23.5	9.5	12.0	5	33.0	12.0	29.0	30.0	13.0	15.0
6	32.0	7.0	27.0	23.0	11.0	17.5	6	34.0	10.0	30.0	30.0	16.0	20.5
7	28.0	8.0	33.0	25.0	10.5	17.5	7	30.0	12.5	30.0	30.0	14.0	21.0
8	30.0	8.5	26.0	26.0	10.5	18.0	8	33.0	12.0	28.0	30.0	15.0	23.0
9	31.5	8.0	29.0	24.0	17.5	15.5	9	33.5	12.0	31.5	30.0	22.0	20.0
10	29.0	8.0	28.0	23.5	15.0	17.5	10	31.0	13.0	30.0	30.0	19.0	20.5
MA							**MA**						
1	26.0	8.5	20.5	25.0	11.0	9.0	1	30.0	14.0	22.0	28.0	14.0	10.0
2	24.0	9.0	21.0	25.0	12.0	11.0	2	28.0	13.5	23.0	27.0	16.0	12.5
3	26.0	9.0	20.0	24.0	11.5	11.0	3	31.0	12.0	20.0	28.0	17.0	12.5
4	27.0	9.5	21.0	21.5	12.0	10.0	4	32.0	14.0	23.0	24.0	15.5	12.5
5	25.0	10.0	20.5	24.0	12.5	10.5	5	30.0	14.0	22.0	27.0	17.0	13.5
6	23.0	8.5	21.0	24.0	14.5	11.0	6	30.0	12.0	23.0	28.0	20.0	14.0
7	26.0	8.5	21.0	20.0	12.0	12.5	7	28.0	12.0	24.0	24.0	17.0	14.0
8	26.0	9.0	12.0	16.0	12.5	13.0	8	31.0	13.0	16.0	19.0	17.0	14.5
9	27.0	9.0	21.0	22.0	14.0	11.0	9	33.0	14.0	24.0	25.0	18.5	14.5
10	26.0	8.5	22.0	21.0	13.0	11.5	10	30.0	13.0	26.0	24.0	17.5	13.5

PAIRED CULTURES REPLICATE 2

15 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control		20 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control	
PDA							**PDA**						
1	29.0	10.5	25.0	26.5	13.5	14.0	1	30.0	11.5	30.0	30.0	16.5	17.0
2	21.0	10.5	26.0	24.5	14.5	12.0	2	30.0	13.0	25.0	30.0	18.0	15.5
3	24.5	13.5	21.5	25.5	14.5	13.0	3	30.0	16.5	27.0	26.0	18.0	17.5
4	28.0	10.5	25.0	23.5	12.0	14.5	4	28.0	13.5	30.0	29.0	15.0	16.0
5	30.0	9.0	25.0	27.5	13.5	14.5	5	30.0	12.5	30.0	30.0	16.0	18.5
6	28.5	11.5	24.0	25.0	16.0	16.0	6	30.0	13.5	30.0	29.0	18.0	19.5
7	28.0	11.0	26.5	24.0	12.0	13.0	7	30.0	14.0	30.0	30.0	15.0	17.5
8	26.0	12.0	24.0	25.5	14.5	13.5	8	30.0	14.0	29.0	29.0	18.5	16.0
9	26.0	10.0	26.0	26.0	11.5	14.0	9	30.0	13.0	28.0	30.0	13.5	17.5
10	24.0	10.5	26.5	27.5	11.0	13.5	10	30.0	13.0	28.5	30.0	15.0	19.0
MDA							**MDA**						
1	25.0	13.5	25.5	22.0	10.5	12.5	1	30.0	17.0	30.0	28.0	14.5	16.0
2	24.0	16.5	23.5	23.5	12.5	14.0	2	30.0	18.5	30.0	29.0	14.5	17.0
3	24.5	14.0	23.0	24.0	14.0	14.0	3	30.0	18.0	30.0	28.0	17.0	17.0
4	25.0	13.5	24.5	25.0	13.0	12.5	4	30.0	16.5	30.0	30.0	15.0	15.5
5	23.0	13.5	24.5	24.0	13.0	13.0	5	30.0	17.0	30.0	29.0	15.5	18.0
6	26.0	12.0	23.0	23.0	13.5	12.5	6	30.0	16.5	30.0	26.0	15.5	16.5
7	24.0	16.5	28.0	23.5	19.0	13.5	7	30.0	20.0	30.0	27.0	23.0	16.5
8	25.0	14.5	22.0	25.0	15.0	14.5	8	30.0	18.0	30.0	30.0	16.5	17.5
9	23.5	13.0	23.0	24.0	15.0	14.5	9	30.0	16.0	30.0	28.0	18.0	18.0
10	23.0	13.0	23.0	24.0	14.0	14.5	10	30.0	16.5	30.0	28.0	17.0	16.0
MA							**MA**						
1	22.0	11.0	20.0	20.0	11.0	12.0	1	29.0	13.5	23.0	27.0	14.0	17.0
2	21.5	12.5	21.0	20.0	12.0	11.0	2	28.0	14.5	24.5	29.0	16.0	15.0
3	21.0	12.5	25.0	21.0	11.5	11.0	3	30.0	14.0	27.0	24.0	17.0	15.5
4	24.0	11.5	22.5	20.0	12.0	13.0	4	29.0	13.5	25.0	28.0	15.5	16.0
5	21.0	11.0	24.0	22.0	12.5	11.5	5	25.0	13.5	26.0	27.0	17.0	15.0
6	25.5	13.0	22.0	23.0	14.5	12.5	6	29.5	15.5	30.0	23.0	20.0	16.5
7	21.5	16.0	17.5	22.0	12.0	11.5	7	28.0	18.5	26.0	30.0	17.0	16.0
8	20.0	10.0	22.0	20.5	12.5	11.0	8	30.0	12.5	27.0	29.0	17.0	16.0
9	21.5	12.0	21.5	21.0	14.0	13.0	9	30.0	14.0	26.5	30.0	18.5	17.5
10	21.5	12.0	22.0	23.0	13.0	12.5	10	28.0	14.5	27.0	28.0	17.5	16.5

PAIRED CULTURES REPLICATE 3

15 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control		20 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control	
PDA							**PDA**						
1	25.5	6.5	13.5	21.5	5.0	6.0	1	35.0	8.0	18.0	27.5	6.0	7.0
2	22.5	6.0	19.0	20.0	5.0	6.0	2	29.0	8.0	23.0	22.0	5.5	6.5
3	26.5	6.0	18.0	20.5	5.5	6.0	3	30.0	7.0	25.0	25.0	6.5	6.5
4	25.0	5.0	16.0	16.0	5.0	5.5	4	32.0	8.0	24.0	25.0	6.0	6.0
5	22.5	6.0	18.0	24.5	6.0	6.5	5	30.5	7.0	22.0	27.5	6.5	7.0
6	22.0	8.0	14.5	22.5	5.0	5.5	6	27.5	9.0	20.5	28.0	6.0	6.0
7	24.5	6.0	15.0	20.0	5.5	6.5	7	30.0	8.5	21.0	27.5	6.0	6.5
8	25.0	6.5	17.0	17.5	6.0	6.0	8	31.0	7.0	20.0	21.0	7.0	6.5
9	26.0	5.5	16.5	21.0	6.0	6.5	9	33.0	8.0	18.0	28.0	6.5	7.5
10	25.5	8.0	15.0	21.0	5.0	5.5	10	33.0	10.0	17.0	24.0	6.0	6.5
MDA							**MDA**						
1	27.0	8.5	26.0	23.0	10.5	9.5	1	30.0	11.0	27.5	32.5	15.0	13.0
2	24.5	9.0	27.5	28.0	10.0	11.0	2	27.0	10.0	31.0	33.0	14.0	16.0
3	27.0	11.0	27.0	24.0	9.5	10.5	3	29.0	13.0	30.0	28.0	13.5	14.0
4	32.0	9.0	31.0	28.0	10.0	10.5	4	33.0	12.0	34.0	28.0	14.0	15.0
5	31.0	11.0	23.0	28.0	10.5	9.5	5	33.0	12.0	27.0	32.0	14.0	13.0
6	32.0	9.0	26.0	28.0	10.0	10.5	6	32.0	13.5	28.0	30.0	14.0	15.5
7	28.0	8.5	28.0	26.0	10.0	10.5	7	30.0	13.0	30.0	28.0	14.5	14.0
8	31.0	9.5	25.5	25.0	10.5	10.0	8	33.0	12.5	28.0	28.0	14.5	14.0
9	28.5	8.0	26.0	24.5	10.0	10.0	9	32.5	11.5	29.0	27.5	14.0	14.0
10	26.0	9.0	28.0	25.0	10.0	10.0	10	30.0	12.0	29.0	28.0	14.0	14.0
MA							**MA**						
1	26.0	8.5	20.5	25.0	11.0	10.5	1	30.0	14.0	22.0	28.0	14.0	13.0
2	24.0	9.0	21.0	25.0	12.0	11.5	2	28.0	13.5	23.0	27.0	16.0	14.0
3	26.0	9.0	17.0	26.0	11.5	11.5	3	31.0	12.0	20.0	30.0	17.0	14.5
4	27.0	9.5	21.0	21.5	12.0	9.0	4	32.0	14.0	23.0	24.0	15.5	11.0
5	25.0	10.0	17.5	24.0	12.5	9.5	5	30.0	14.0	20.5	27.0	17.0	11.5
6	23.0	8.5	21.0	24.0	14.5	10.5	6	30.0	12.0	25.0	28.0	20.0	13.0
7	26.0	8.5	21.0	20.0	12.0	11.0	7	28.0	12.0	24.0	24.0	17.0	13.5
8	26.0	9.0	17.0	17.0	12.5	10.5	8	31.0	13.0	20.0	23.0	17.0	13.0
9	27.0	9.0	21.0	22.0	14.0	11.5	9	33.0	14.0	24.0	25.0	18.5	13.5
10	26.0	8.5	22.0	21.0	13.0	11.5	10	30.0	13.0	26.0	24.0	17.5	13.0

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DUAL CULTURES REPLICATE 1

5 days	*M. perniciosa* top *A. bisporus* bottom		*M. perniciosa* bottom *A. bisporus* top		*M. perniciosa* control		*A. bisporus* control		10 days	*M. perniciosa* top *A. bisporus* bottom		*M. perniciosa* bottom *A. bisporus* top		*M. perniciosa* control		*A. bisporus* control		15 days
PDA									**PDA**									**PDA**
1	14.0	5.5	13.0	6.0	9.5	10.0	8.5	8.0	1	23.0	6.0	22.0	7.0	15.0	15.0	10.0	9.0	1
2	12.0	6.5	15.0	6.0	10.0	11.0	7.0	7.5	2	18.5	7.0	23.0	7.0	14.0	14.5	9.0	8.5	2
3	12.5	8.0	11.0	5.0	12.0	12.5	6.0	7.0	3	23.5	10.0	19.0	6.0	16.5	17.0	6.5	8.0	3
4	12.5	7.5	17.0	7.5	16.0	13.0	8.5	8.0	4	20.5	8.5	26.0	9.5	22.0	17.0	9.0	8.5	4
5	12.0	5.5	14.0	7.0	13.0	13.0	7.0	7.5	5	19.0	6.0	23.0	8.0	17.0	17.0	7.0	8.5	5
6	11.0	10.5	15.0	4.5	11.0	11.0	7.0	6.5	6	20.0	11.0	24.5	5.5	16.0	18.0	8.0	8.0	6
7	11.0	6.0	12.0	6.5	11.5	11.0	7.0	7.0	7	20.0	7.5	19.0	7.5	13.0	15.0	7.5	8.0	7
8	13.0	8.0	11.0	4.5	8.0	11.0	5.5	6.5	8	17.0	9.0	20.0	5.0	13.5	14.0	6.5	7.5	8
9	11.0	8.5	16.0	7.5	11.0	11.0	6.0	7.0	9	21.0	10.0	24.0	8.5	15.0	14.0	8.0	8.0	9
10	11.0	8.0	14.0	8.0	13.0	11.5	6.0	6.0	10	21.0	9.5	21.0	9.0	17.0	15.0	6.5	7.5	10
MDA									**MDA**									**MDA**
1	12.0	3.5	11.5	3.0	10.5	11.0	4.0	4.0	1	25.0	8.5	23.0	7.0	23.0	18.0	7.5	8.5	1
2	12.0	3.5	13.0	4.5	12.5	12.0	4.0	3.5	2	26.0	8.5	25.0	8.5	21.5	20.0	6.0	7.0	2
3	14.0	3.0	13.0	3.0	11.5	11.0	4.0	4.5	3	25.0	9.0	25.0	8.0	20.5	19.5	5.0	8.0	3
4	13.0	3.5	11.0	3.5	13.0	12.5	4.0	4.0	4	25.0	8.0	23.0	8.5	24.0	20.5	8.0	8.0	4
5	14.0	4.0	11.0	3.0	14.0	13.0	4.0	4.0	5	25.0	10.0	23.5	8.0	23.0	22.0	7.5	7.0	5
6	12.0	3.5	13.5	5.0	11.0	11.5	4.0	4.0	6	24.0	8.0	22.0	9.0	21.5	20.5	9.5	8.0	6
7	12.5	3.0	13.5	5.0	12.0	12.5	4.0	4.5	7	25.0	9.0	25.0	9.0	25.0	22.5	7.5	8.5	7
8	13.0	3.5	12.5	4.0	13.0	13.0	4.0	3.5	8	26.0	8.5	21.5	8.5	23.0	23.0	7.0	7.5	8
9	12.0	4.0	12.0	5.0	13.5	13.5	4.0	4.0	9	22.0	9.0	23.0	9.0	24.0	23.0	8.5	8.0	9
10	12.5	4.0	11.5	4.0	12.5	12.5	4.0	4.0	10	23.5	9.0	22.0	8.0	22.0	22.0	7.5	8.0	10
MA									**MA**									**MA**
1	12.0	3.5	13.0	4.5	10.0	11.0	3.5	3.0	1	21.0	5.5	24.0	6.0	17.0	17.0	5.0	5.0	1
2	10.0	3.5	11.0	4.0	10.0	12.0	3.0	3.0	2	18.5	5.5	21.0	6.0	18.0	19.0	5.0	5.0	2
3	13.0	3.5	11.5	3.5	10.5	9.0	3.5	3.5	3	23.0	6.5	21.5	5.5	19.0	15.0	6.0	5.5	3
4	12.5	3.0	12.0	3.0	12.0	10.0	3.0	3.0	4	24.0	6.5	21.0	6.0	19.5	18.0	4.5	5.0	4
5	10.0	3.0	10.5	4.0	7.5	10.5	3.0	3.0	5	21.0	5.5	21.0	6.0	15.0	18.0	5.0	5.0	5
6	12.0	3.5	12.0	3.0	10.0	10.5	3.5	3.0	6	21.5	3.5	23.0	5.5	17.5	18.5	5.0	5.0	6
7	10.0	3.5	11.5	4.0	10.0	11.0	3.5	3.0	7	19.0	3.5	22.0	6.0	18.0	20.0	5.0	5.0	7
8	13.0	3.5	13.0	4.0	9.5	10.5	2.5	3.5	8	22.0	3.5	22.0	6.0	16.5	19.5	5.0	5.5	8
9	12.5	3.0	11.0	3.5	11.5	10.0	3.5	3.5	9	20.5	3.0	20.5	6.0	20.5	19.0	5.5	6.0	9
10	10.0	3.0	12.0	4.0	14.0	10.5	2.5	3.0	10	18.5	3.0	22.0	6.5	21.5	19.5	5.5	5.0	10

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DUAL CULTURES REPLICATE 2

5 days	*M. perniciosa* top *A. bisporus* bottom		*M. perniciosa* bottom *A. bisporus* top		*M. perniciosa* control		*A. bisporus* control		10 days	*M. perniciosa* top *A. bisporus* bottom		*M. perniciosa* bottom *A. bisporus* top		*M. perniciosa* control		*A. bisporus* control		15 days
PDA									**PDA**									**PDA**
1	11.0	7.5	13.0	5.5	12.5	13.0	10.0	9.0	1	17.5	9.0	21.0	6.5	18.5	20.0	13.5	13.0	1
2	13.0	7.0	12.0	4.5	14.0	9.0	10.5	9.5	2	22.5	9.0	21.0	5.5	20.0	16.0	13.0	12.0	2
3	12.0	6.0	12.0	6.0	11.5	9.0	10.0	12.0	3	21.0	7.0	21.5	7.5	18.0	18.0	13.5	16.5	3
4	16.0	6.5	12.0	6.5	12.0	12.0	9.5	12.5	4	24.0	8.0	19.0	6.5	18.5	21.0	12.0	17.0	4
5	14.0	5.0	18.0	6.0	12.0	12.0	9.5	9.0	5	21.5	6.0	26.0	6.5	22.0	19.5	13.5	11.5	5
6	16.0	5.0	12.0	4.0	12.0	12.5	10.5	8.5	6	23.0	5.0	23.0	4.0	19.0	17.5	15.0	12.5	6
7	14.0	4.5	14.0	4.5	14.0	10.5	8.5	10.0	7	20.5	5.0	24.0	4.5	22.0	14.0	12.5	14.5	7
8	14.0	4.0	13.0	4.5	17.0	14.5	9.5	6.5	8	21.0	4.5	17.5	4.5	25.0	21.5	13.5	11.5	8
9	14.0	4.5	15.5	5.0	14.0	12.5	8.0	9.0	9	22.0	4.5	25.5	5.5	18.0	16.0	11.5	12.5	9
10	15.0	4.0	14.5	5.0	13.0	11.5	9.0	9.5	10	23.0	4.5	23.5	5.0	18.0	18.5	13.0	12.0	10
MDA									**MDA**									**MDA**
1	16.0	9.0	17.0	9.5	15.0	14.5	7.5	9.5	1	28.0	12.0	28.0	14.0	23.0	21.0	11.0	12.0	1
2	20.0	9.0	18.0	8.5	16.0	13.0	7.5	8.5	2	29.0	13.0	30.0	11.0	25.0	24.5	10.5	12.0	2
3	23.0	9.0	15.0	8.5	13.0	14.0	8.5	9.5	3	36.0	12.0	27.0	11.5	22.0	24.0	11.0	12.0	3
4	22.0	11.0	14.5	7.5	12.5	12.0	9.5	8.5	4	34.0	14.0	24.5	12.0	20.5	20.0	12.0	11.5	4
5	18.0	12.0	20.0	7.0	18.0	14.0	8.5	8.0	5	34.0	15.0	30.0	10.0	25.0	22.5	12.0	11.0	5
6	15.0	9.0	17.5	7.5	15.5	12.0	8	8.0	6	30.5	12.5	22.5	11.0	18.5	19.0	13	12.0	6
7	18.0	8.0	15.0	6.5	13.0	13.0	8	8.0	7	31.5	12.5	23.5	10.0	19.5	20.0	12	13.5	7
8	17.5	8.0	15.0	7.0	13.0	10.5	7.5	8.0	8	33.0	12.0	27.0	10.0	22.0	19.0	11	12.0	8
9	18.0	8.5	15.5	7.0	13.5	11.0	7.5	8.0	9	32.0	12.5	28.5	10.0	24.5	20.0	10.5	12.0	9
10	15.5	7.0	16.0	9.0	14.0	15.0	7	7.0	10	30.5	10.5	30.5	13.5	22.5	25.5	11.5	10.5	10
MA									**MA**									**MA**
1	12.0	9.5	15.5	7.5	15.0	12.5	7.0	7.5	1	24.0	13.0	26.0	11.0	22.5	21.5	10.0	11.0	1
2	14.5	10.0	14.0	5.5	11.5	14.0	8.0	6.5	2	24.0	12.5	24.5	9.0	19.5	22.0	11.5	10.0	2
3	13.0	9.0	13.0	7.0	13.0	14.5	8.5	7.5	3	20.0	12.0	24.0	9.0	21.0	20.5	9.5	11.0	3
4	16.0	9.0	12.5	6.5	11.0	12.5	7.0	7.0	4	26.5	11.5	22.0	10.0	22.0	20.0	12.0	10.0	4
5	11.5	9.0	13.0	6.5	13.5	12.0	7.0	7.5	5	19.5	13.0	22.5	9.5	23.0	20.0	10.0	10.0	5
6	12.5	8.5	13.0	8.0	15.5	11.0	7.0	6.0	6	23.0	11.5	23.5	13.0	26.0	18.5	10.0	9.5	6
7	15.0	8.0	12.5	6.5	12.0	15.0	7.5	8.0	7	24.0	11.0	23.0	10.5	22.5	24.0	10.5	11.0	7
8	15.0	8.5	15.5	7.0	11.5	11.0	6.5	7.0	8	27.5	11.5	25.0	11.0	16.5	19.0	10.5	9.5	8
9	18.0	9.0	12.5	7.5	12.0	10.0	5.5	6.5	9	28.0	11.5	22.0	11.5	22.0	18.0	8.5	9.5	9
10	15.0	7.0	10.5	7.0	11.0	11.0	6.0	6.0	10	25.0	10.0	21.0	10.0	20.0	20.0	9.0	10.0	10

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DUAL CULTURES REPLICATE 3

5 days	*M. perniciosa* top *A. bisporus* bottom		*M. perniciosa* bottom *A. bisporus* top		*M. perniciosa* control		*A. bisporus* control		10 days	*M. perniciosa* top *A. bisporus* bottom		*M. perniciosa* bottom *A. bisporus* top		*M. perniciosa* control		*A. bisporus* control		15 days
PDA									**PDA**									**PDA**
1	12.0	6.0	12.5	7.5	10.5	13.0	5.0	5.5	1	18.5	12.5	22.0	10.5	18.5	18.5	5.5	6.0	1
2	16.0	8.5	12.0	7.5	13.5	10.5	5.5	6.0	2	23.0	12.5	17.5	11.5	17.5	15.5	6.0	7.0	2
3	15.0	8.5	13.0	6.5	13.0	15.5	5.0	5.0	3	22.0	12.5	23.0	11.5	19.5	20.5	6.0	5.5	3
4	15.5	8.5	12.5	9.0	14.0	15.5	5.0	5.5	4	20.5	12.5	20.5	13.0	19.0	20.5	6.0	6.5	4
5	14.5	7.0	15.5	7.0	13.0	11.0	5.5	6.0	5	20.0	10.5	17.0	12.0	18.0	16.5	6.0	6.5	5
6	15.0	9.0	14.0	11.0	14.5	11.5	5.0	5.5	6	20.5	12.5	19.5	14.0	20.0	19.5	7.0	9.0	6
7	13.0	7.5	14.0	10.0	14.0	14.5	6.5	5.5	7	21.0	13.0	21.0	14.0	19.0	19.5	9.5	9.0	7
8	12.0	8.0	14.0	9.0	12.0	13.0	5.5	5.5	8	19.0	12.0	22.0	12.0	18.0	18.5	8.5	10.0	8
9	11.0	8.5	15.0	9.5	14.0	12.0	5.0	6.5	9	19.0	11.0	22.0	12.5	19.0	19.5	6.0	7.5	9
10	13.0	8.5	15.0	9.0	13.5	15.0	5.0	6.0	10	16.0	12.0	18.5	13.0	18.5	19.0	6.5	8.5	10
MDA									**MDA**									**MDA**
1	18.0	8.5	15.5	8.5	11.5	16.5	7.0	7.0	1	32.0	12.5	28.0	11.0	24.5	23.5	11.0	12.0	1
2	15.5	7.0	17.0	7.5	13.0	16.0	6.5	6.5	2	30.5	10.5	29.5	11.0	27.5	27.0	10.0	10.5	2
3	15.0	8.0	15.0	8.5	12.0	14.5	8.0	8.0	3	32.0	11.5	27.0	13.5	26.0	26.0	11.5	12.0	3
4	17.5	8.0	19.0	8.5	11.5	12.0	7.5	8.0	4	32.5	11.5	29.0	11.5	21.5	21.0	11.5	12.0	4
5	17.0	7.5	15.0	6.5	11.5	14.0	7.5	7.0	5	34.0	11.5	24.5	11.5	25.0	22.0	11.0	10.0	5
6	18.5	8.0	14.0	7.5	14.0	13.5	7.5	6.5	6	31.0	14.0	28.0	10.5	20.0	21.0	11.0	10.5	6
7	13.0	7.0	11.0	7.5	17.0	12.5	9.0	8.0	7	27.0	11.5	26.0	11.0	20.5	21.0	13.0	12.0	7
8	13.5	8.5	16.0	8.5	15.0	16.0	8.0	8.0	8	29.0	14.5	29.0	11.0	23.5	28.0	11.0	11.0	8
9	17.0	7.0	14.5	8.0	15.0	13.5	8.0	9.0	9	29.0	10.0	26.0	10.0	27.0	20.5	12.0	12.0	9
10	12.0	9.0	16.5	8.0	13.5	13.5	7.5	8.0	10	27.0	12.5	27.5	11.0	26.0	26.0	11.0	11.5	10
MA									**MA**									**MA**
1	13.0	8.5	14.5	8.0	12.0	13.5	6.5	6.5	1	20.0	12.0	21.5	12.0	19.0	19.0	10.0	10.0	1
2	15.0	7.0	13.0	7.5	13.0	11.0	6.0	6.5	2	25.0	10.5	20.5	11.5	22.0	17.0	10.0	10.5	2
3	14.5	7.5	10.5	7.0	12.0	10.0	6.5	7.0	3	22.0	11.0	20.0	9.0	16.5	20.0	9.5	10.0	3
4	12.0	7.0	14.0	7.0	13.5	13.0	7.0	6.5	4	20.0	10.0	19.5	11.0	22.0	25.0	10.0	9.0	4
5	14.0	6.0	11.0	7.0	16.0	10.0	7.5	7.5	5	20.5	9.5	21.0	10.0	21.0	23.0	11.0	10.5	5
6	13.0	7.0	14.5	7.5	14.5	14.0	7.0	8.0	6	20.5	11.0	25.0	11.0	22.0	21.0	10.5	11.0	6
7	13.0	7.0	14.0	7.0	13.5	14.0	8.0	7.5	7	24.0	10.5	24.0	11.0	19.5	20.5	11.5	10.0	7
8	14.0	6.0	16.5	7.0	10.0	12.5	6.0	7.5	8	23.5	11.0	19.0	10.5	22.5	20.0	10.0	10.5	8
9	13.0	7.0	11.5	7.5	12.5	13.0	7.0	6.0	9	24.0	10.5	20.0	11.0	22.5	21.0	9.0	10.0	9
10	13.5	7.0	14.0	6.5	12.5	13.5	7.5	7.0	10	22.5	10.0	25.0	9.5	20.0	20.0	10.0	10.0	10

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DUAL CULTURES REPLICATE 1

M. perniciosa top *A. bisporus* bottom		*M. perniciosa* bottom *A. bisporus* top		*M. perniciosa* control		*A. bisporus* control		20 days	*M. perniciosa* top *A. bisporus* bottom		*M. perniciosa* bottom *A. bisporus* top		*M. perniciosa* control		*A. bisporus* control	
								PDA								
30.0	7.0	26.0	7.5	16.0	19.0	11.0	10.5	1	36.0	8.0	29.0	8.0	18.0	23.0	12.0	11.5
26.0	7.0	28.0	7.5	20.0	18.0	9.5	9.5	2	30.0	8.0	30.0	8.0	22.0	22.0	11.5	10.0
35.0	10.0	21.0	6.5	22.0	19.0	7.0	9.0	3	39.0	11.0	29.0	7.0	25.0	23.0	7.5	10.0
27.0	9.0	29.0	9.5	25.0	19.5	10.0	9.0	4	34.0	9.0	32.0	10.0	27.0	24.0	12.0	9.5
28.5	7.0	28.0	8.5	20.0	19.0	7.5	9.5	5	33.0	10.0	28.0	9.0	24.0	23.5	9.5	10.5
30.0	11.5	30.0	6.0	20.0	20.0	9.0	9.0	6	30.0	12.5	32.0	6.5	23.0	24.0	11.0	9.5
27.0	7.5	28.0	7.5	17.0	18.0	8.5	8.5	7	31.0	8.0	34.0	8.0	21.0	21.5	9.0	9.0
26.5	10.0	29.0	6.0	20.0	17.0	7.0	8.0	8	32.0	11.0	35.0	6.5	22.0	21.0	7.5	8.5
30.0	11.0	27.0	9.5	18.0	17.5	8.5	8.5	9	34.0	12.0	28.0	10.0	19.0	21.5	9.5	9.5
29.0	10.0	28.5	9.5	20.0	19.0	7.0	8.0	10	32.0	9.0	32.5	9.5	23.0	22.5	7.5	8.5
								MDA								
32.0	14.0	34.0	12.0	28.5	28.0	11.0	12.5	1	35.0	19.0	38.0	17.0	32.5	35.0	14.0	15.5
33.5	13.5	35.0	14.0	31.5	29.0	12.0	11.5	2	34.0	19.0	39.5	18.0	33.5	37.0	16.0	15.0
33.0	14.5	34.0	13.0	30.5	28.5	11.5	12.0	3	36.0	19.5	36.0	16.5	35.0	35.5	17.0	15.0
33.5	13.5	36.0	14.0	30.5	31.0	12.0	12.5	4	36.0	19.5	41.0	20.0	37.0	36.5	15.5	15.5
31.0	16.0	32.0	12.5	28.0	33.0	12.5	11.5	5	35.5	22.0	36.0	15.5	34.0	40.0	17.0	16.0
32.0	15.0	32.0	13.5	30.0	32.5	14.5	12.0	6	35.0	19.0	35.5	19.0	33.0	38.0	20.0	15.5
33.0	16.0	33.0	15.0	32.5	31.5	12.0	12.0	7	36.0	20.0	35.0	20.0	38.0	34.5	17.0	16.0
33.5	14.0	30.5	14.0	33.0	33.5	12.5	12.5	8	36.0	17.5	36.5	17.0	37.0	36.0	17.0	17.5
31.5	14.5	29.0	14.5	28.5	32.0	14.0	12.0	9	34.0	18.0	34.0	18.0	36.5	36.0	18.5	16.5
33.5	15.0	31.0	12.0	29.5	30.5	13.0	12.5	10	36.5	19.0	35.0	17.0	34.0	34.0	17.5	16.0
								MA								
27.0	8.5	33.5	8.5	24.0	22.0	7.5	8.0	1	30.0	11.0	37.0	11.0	34.5	28.0	8.5	9.5
26.5	9.0	31.5	8.5	26.0	24.0	9.0	8.0	2	27.0	10.0	33.0	12.0	34.0	30.0	10.5	9.0
27.0	11.0	30.0	8.0	27.0	21.0	8.0	8.5	3	29.0	13.0	34.0	12.0	33.0	27.5	10.0	10.0
32.0	9.0	31.0	7.0	25.5	25.0	7.0	7.5	4	33.0	12.0	35.0	10.0	33.0	30.0	9.5	9.0
31.0	11.0	31.5	8.5	23.5	24.5	7.0	8.0	5	33.0	12.0	35.0	11.0	30.5	28.5	8.5	9.0
29.0	7.0	30.5	8.0	26.0	25.0	7.5	8.0	6	32.0	13.5	34.5	11.0	30.0	29.0	9.5	10.0
26.0	8.0	33.0	8.0	29.0	27.0	8.0	8.0	7	30.0	13.0	37.0	11.0	34.0	32.0	9.5	9.5
30.0	8.5	31.5	7.5	24.0	26.0	7.5	8.5	8	33.0	12.5	35.0	10.5	30.0	30.5	9.0	10.5
29.0	8.0	32.0	8.5	29.0	25.5	8.0	8.0	9	32.5	11.5	36.0	12.0	35.5	29.0	11.0	9.0
27.5	8.0	33.5	8.0	28.0	26.0	9.0	8.0	10	31.0	11.0	35.5	11.5	35.0	32.0	11.5	9.5

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DUAL CULTURES REPLICATE 2

M. perniciosa top *A. bisporus* bottom		*M. perniciosa* bottom *A. bisporus* top		*M. perniciosa* control		*A. bisporus* control		20 days	*M. perniciosa* top *A. bisporus* bottom		*M. perniciosa* bottom *A. bisporus* top		*M. perniciosa* control		*A. bisporus* control	
								PDA								
27.0	9.0	26.0	7.0	26.5	24.0	16.5	16.5	1	33.0	10.5	28.0	7.5	33.0	28.0	19.0	18.0
30.0	9.0	25.5	6.5	27.0	20.0	17.5	16.5	2	37.0	10.0	27.0	6.5	35.0	27.0	21.5	19.5
32.0	7.0	30.0	7.5	24.0	23.0	19.0	20.0	3	36.0	8.0	32.0	7.5	33.0	28.0	21.5	23.0
34.0	8.0	27.0	7.0	26.0	24.0	17.0	20.0	4	38.0	8.5	29.0	7.0	36.0	30.0	19.0	22.0
32.0	6.0	34.0	6.5	31.0	24.0	17.5	14.5	5	40.0	7.0	39.0	7.5	37.0	29.0	20.0	17.5
29.0	5.5	33.0	4.5	23.5	26.0	20.0	18.0	6	35.0	6.0	37.0	5.0	30.0	30.5	24.5	22.5
29.0	5.5	32.0	4.5	28.0	25.5	18.5	20.0	7	37.0	6.0	37.0	4.5	32.0	28.5	23.0	24.0
31.0	5.5	28.5	4.5	30.0	30.0	17.0	16.0	8	39.5	6.0	32.0	4.5	35.0	35.0	20.5	19.5
26.0	5.0	34.0	7.0	27.0	24.5	16.0	17.5	9	31.0	5.0	40.0	8.5	29.0	30.0	19.0	20.5
30.0	4.5	33.0	5.0	25.0	29.0	17.5	15.5	10	38.0	5.5	35.5	5.0	31.0	34.0	22.0	19.0
								MDA								
35.0	18.0	40.0	17.0	28.0	27.0	17.5	18.0	1	40.0	20.0	40.0	19.0	40.0	38.0	22.0	24.0
36.0	20.0	39.0	13.0	30.0	26.0	16.5	18.0	2	40.0	23.0	40.0	16.5	40.0	36.0	21.0	21.5
40.0	19.0	37.0	15.0	26.0	33.0	17.0	18.0	3	40.0	21.0	40.0	17.5	40.0	40.0	22.0	22.0
40.0	22.0	38.0	14.0	24.5	27.0	18.5	18.0	4	40.0	25.0	40.0	17.0	40.0	37.0	22.5	22.5
35.0	22.0	40.0	13.5	31.0	28.5	18.5	16.5	5	40.0	26.0	40.0	17.5	38.0	40.0	22.5	21.0
36.0	15.5	36.5	14.0	22.5	25.0	15.0	14.0	6	40.0	18.5	40.0	17.0	35.0	35.0	17.0	15.5
36.0	15.0	34.0	13.0	24.5	27.0	14.0	15.0	7	40.0	18.0	40.0	16.0	35.0	36.0	15.5	18.0
38.0	15.5	37.0	12.0	27.5	26.5	13.0	16.0	8	40.0	19.5	40.0	16.0	40.0	39.0	16.0	18.5
37.0	16.0	31.0	14.0	28.5	24.5	14.0	14.0	9	40.0	19.5	39.0	17.0	35.0	35.0	17.0	18.0
35.5	14.0	33.5	15.5	27.5	30.5	14.5	14.0	10	40.0	18.0	36.0	18.5	33.0	38.0	18.5	18.5
								MA								
30.5	18.5	30.0	14.0	27.5	28.5	12.0	15.0	1	35.0	23.5	32.5	19.0	33.0	31.0	19.5	22.5
29.0	16.0	28.0	13.0	22.5	29.0	15.0	14.5	2	33.5	19.0	32.0	17.0	31.5	33.0	22.0	21.5
26.0	16.0	28.5	14.0	28.0	28.0	12.5	14.5	3	36.0	19.0	31.5	18.5	32.0	33.0	19.0	19.5
31.0	16.0	28.0	13.5	27.0	28.0	13.0	13.0	4	37.0	18.0	37.5	19.0	34.0	33.0	20.0	19.0
26.5	17.5	26.5	14.0	29.5	26.5	13.0	13.5	5	37.0	20.0	30.5	19.0	32.5	30.0	20.0	20.5
29.0	14.5	31.0	16.0	32.0	25.0	14.0	13.5	6	34.0	18.0	37.0	21.5	38.0	35.0	18.0	18.0
31.0	14.5	25.0	16.5	29.0	30.0	14.0	16.0	7	36.0	17.5	39.0	22.5	34.0	34.0	19.0	22.0
32.0	16.5	35.0	15.5	24.0	29.0	13.5	15.5	8	35.5	19.0	37.5	20.5	31.5	33.0	18.5	21.0
32.0	15.5	29.0	16.5	30.5	26.0	12.0	13.0	9	32.5	19.0	36.0	22.5	34.5	33.0	17.5	19.0
28.0	14.0	29.5	15.5	29.5	28.0	12.0	15.0	10	36.5	17.5	38.0	20.0	34.0	36.0	19.0	21.0

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DUAL CULTURES REPLICATE 3

M. perniciosa top *A. bisporus* bottom		*M. perniciosa* bottom *A. bisporus* top		*M. perniciosa* control		*A. bisporus* control		20 days	*M. perniciosa* top *A. bisporus* bottom		*M. perniciosa* bottom *A. bisporus* top		*M. perniciosa* control		*A. bisporus* control	
								PDA								
26.5	15.0	30.0	12.5	23.0	23.5	7.0	7.0	1	29.5	17.0	32.5	15.5	28.0	27.5	8.0	8.5
28.0	17.5	23.5	15.0	26.5	25.0	6.5	7.0	2	31.0	19.5	27.0	17.0	31.0	28.0	7.5	8.0
32.0	16.0	32.0	13.5	24.0	25.0	7.0	6.5	3	36.0	19.0	33.5	16.5	28.0	30.0	8.0	7.0
28.5	16.5	28.5	16.5	26.0	26.0	6.5	7.5	4	34.0	18.5	31.0	21.0	31.0	30.5	7.5	8.5
28.0	14.0	26.0	16.0	25.5	29.0	7.0	7.0	5	31.5	16.0	28.0	20.0	27.5	32.0	8.0	8.5
29.0	16.5	31.5	18.0	33.0	25.0	8.0	11.0	6	35.5	19.0	35.5	21.0	37.0	29.0	9.5	11.5
32.0	17.0	29.0	17.0	26.0	29.0	11.0	9.5	7	34.5	19.0	33.0	18.0	29.0	33.0	12.5	11.0
28.0	17.0	28.5	15.0	26.0	26.0	9.0	12.0	8	36.0	20.0	31.0	18.0	30.0	29.5	10.0	12.5
26.0	14.0	31.5	17.0	25.0	26.0	7.0	9.0	9	31.0	16.5	36.0	18.0	29.0	30.0	8.5	10.0
23.0	15.0	28.5	15.0	23.5	24.0	7.5	9.5	10	30.0	18.0	33.5	17.0	28.5	28.5	9.0	10.5
								MDA								
37.0	16.0	34.5	13.0	31.5	30.5	14.0	13.5	1	40.0	18.5	38.0	19.0	40.0	39.0	17.5	17.0
35.5	14.0	33.0	13.0	32.5	30.0	12.5	12.5	2	40.0	16.0	39.0	16.0	40.0	40.0	15.0	15.0
36.0	15.5	30.5	17.0	32.0	32.0	13.5	15.0	3	40.0	18.0	40.0	21.0	40.0	40.0	15.5	18.0
38.5	15.0	34.0	14.0	27.0	29.5	13.0	15.0	4	40.0	17.0	39.0	16.0	38.0	37.5	16.0	18.0
40.0	15.0	29.0	14.0	31.0	29.5	13.0	12.5	5	40.0	17.0	40.0	16.5	40.0	40.0	16.0	15.0
37.0	16.0	35.0	14.0	26.0	27.0	13.0	14.0	6	39.0	18.0	39.0	17.0	35.0	39.0	16.0	17.5
34.0	15.0	32.0	14.5	26.0	27.0	17.0	15.0	7	40.0	17.0	39.0	16.5	39.0	40.0	20.0	17.5
33.0	18.5	34.0	14.5	27.5	30.0	13.0	14.0	8	37.0	21.5	40.0	17.0	40.0	39.0	16.0	16.0
36.0	13.5	33.0	14.0	30.0	25.0	16.0	14.0	9	40.0	15.5	40.0	16.0	40.0	40.0	20.0	17.5
34.0	18.0	35.0	13.0	32.0	32.0	14.0	14.5	10	38.0	21.5	40.0	16.0	40.0	36.0	16.5	17.5
								MA								
33.0	20.5	27.0	14.5	27.0	25.5	14.0	13.5	1	40.0	25.5	32.5	21.0	32.5	35.0	20.0	20.0
33.0	15.5	33.0	16.0	31.0	23.0	13.5	14.0	2	40.0	21.0	38.0	21.5	38.0	37.0	19.5	19.0
32.5	14.5	31.0	14.5	26.5	27.0	13.0	13.5	3	39.0	19.0	36.0	20.5	36.0	38.0	19.0	20.0
26.0	14.5	29.0	14.5	31.0	28.5	13.5	13.0	4	37.0	19.5	38.0	21.0	38.0	34.0	19.5	19.0
27.5	13.0	31.0	13.5	31.5	30.5	14.5	13.5	5	35.0	18.5	40.0	20.0	40.0	39.5	21.0	20.0
27.0	14.5	32.5	16.5	32.5	28.5	14.5	15.0	6	36.0	19.0	39.5	21.5	39.5	35.5	20.0	22.0
30.5	14.0	30.0	16.0	25.5	28.0	16.0	14.5	7	37.0	20.0	37.0	21.5	31.0	34.0	22.5	20.5
27.0	15.0	28.5	15.0	30.0	28.0	14.0	15.0	8	36.0	20.0	38.0	20.5	38.0	31.5	20.0	19.0
30.0	16.5	32.0	15.0	30.0	33.0	14.5	14.0	9	37.0	24.5	37.5	19.5	37.5	39.0	20.5	19.5
30.0	17.5	32.0	14.0	29.0	30.0	15.0	14.5	10	35.5	20.5	39.0	20.0	39.0	39.5	20.5	20.0

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HALF PLATE CULTURES REPLICATE 1

5 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control		10 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control	
PDA							**PDA**						
1	9.0	8.0	10.0	8.0	8.0	8.0	1	23.0	10.0	20.0	22.0	12.0	12.5
2	10.0	8.0	6.0	8.0	8.0	7.0	2	23.0	11.0	22.0	20.0	11.0	13.0
3	10.0	8.0	9.5	8.0	8.0	8.0	3	24.0	11.0	19.0	19.0	13.0	12.5
4	10.0	8.0	9.0	9.5	7.0	8.0	4	22.0	9.0	19.0	22.0	11.0	12.0
5	14.0	8.0	8.0	9.0	8.0	8.0	5	30.0	10.0	22.0	24.0	12.0	12.0
6	9.0	7.0	9.0	6.5	8.0	8.0	6	21.0	9.0	18.0	21.0	12.0	12.0
7	11.0	7.0	9.0	7.0	8.0	8.0	7	20.0	10.0	15.0	18.0	13.0	14.0
8	9.0	7.0	7.0	9.0	8.0	7.5	8	26.0	11.0	17.0	20.0	12.0	13.5
9	10.0	7.0	8.5	9.5	7.0	8.0	9	24.0	12.0	20.0	18.0	11.0	12.0
10	11.0	8.0	9.0	8.5	8.0	7.5	10	23.0	11.0	18.5	19.0	12.0	12.0

HALF PLATE CULTURES REPLICATE 2

5 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control		10 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control	
PDA							**PDA**						
1	8.0	9.0	8.0	9.0	8.0	8.0	1	18.5	11.0	18.0	17.5	11.0	11.0
2	9.0	10.0	6.0	8.0	7.0	7.5	2	17.5	10.0	16.0	17.5	12.0	11.0
3	8.0	10.0	7.0	8.0	8.0	8.0	3	17.5	9.0	14.0	16.5	13.0	11.0
4	11.0	10.0	9.0	9.5	7.5	8.0	4	17.0	11.0	18.5	16.5	11.0	12.0
5	8.0	14.0	8.0	9.0	8.0	8.0	5	18.5	10.0	17.0	18.0	12.0	11.0
6	9.0	9.0	9.0	6.5	8.0	8.0	6	17.5	9.0	16.5	18.0	13.0	13.0
7	9.0	11.0	9.0	7.0	8.0	7.5	7	17.5	10.0	18.5	15.5	13.0	13.0
8	9.0	9.0	8.0	8.0	7.5	8.0	8	18.0	12.0	17.0	19.0	12.0	11.0
9	9.0	10.0	7.0	9.0	8.0	7.0	9	16.0	11.0	17.5	19.0	11.0	12.0
10	9.5	11.0	8.5	9.0	7.5	8.0	10	16.0	10.0	18.0	18.5	12.0	11.0

HALF PLATE CULTURES REPLICATE 3

5 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control		10 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control	
PDA							**PDA**						
1	10.0	9.0	8.0	8.0	9.0	10.0	1	22.0	12.0	13.0	14.0	12.0	14.0
2	10.0	10.0	8.5	7.0	10.0	9.0	2	19.0	12.0	14.5	14.0	13.0	12.0
3	9.0	8.0	9.0	8.0	9.0	8.0	3	23.0	9.0	14.0	15.0	10.0	9.0
4	8.0	9.0	7.5	7.0	9.0	9.0	4	16.0	10.0	15.0	15.5	12.0	11.0
5	9.0	9.0	8.0	7.5	10.0	10.0	5	19.0	11.0	14.0	14.0	12.0	12.0
6	9.0	9.0	8.0	8.0	9.0	10.0	6	21.0	12.0	17.0	20.0	12.0	12.0
7	10.0	9.5	7.5	7.5	10.0	11.0	7	19.0	12.0	14.5	16.5	13.0	14.0
8	9.0	6.0	8.0	8.0	10.0	10.0	8	19.0	9.0	12.5	14.0	12.0	13.0
9	9.0	8.0	8.0	7.5	9.0	9.0	9	20.0	9.0	14.5	17.5	14.0	11.0
10	9.0	6.0	8.5	8.0	9.0	9.0	10	20.0	9.0	17.5	18.0	12.0	11.0

HALF PLATE CULTURES REPLICATE 1

15 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control	
PDA						
1	33.0	12.0	30.0	30.0	14.0	15.0
2	34.0	13.0	31.0	30.0	15.0	13.0
3	34.0	13.0	27.0	25.0	13.0	16.0
4	33.0	12.0	25.0	30.0	16.0	14.0
5	35.0	13.0	32.0	32.0	14.0	15.0
6	35.0	12.0	31.0	28.0	14.0	16.0
7	34.0	12.0	27.0	28.0	16.0	13.0
8	33.0	11.0	26.0	29.0	13.0	15.0
9	34.0	12.0	29.0	28.0	16.0	14.0
10	34.0	12.0	27.5	28.5	15.5	15.5

HALF PLATE CULTURES REPLICATE 2

15 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control	
PDA						
1	32.5	13.0	25.0	26.0	14.0	13.5
2	34.0	12.0	26.0	26.0	15.0	15.0
3	33.0	12.0	25.0	22.0	13.0	13.0
4	32.5	13.0	24.0	22.5	16.0	14.5
5	34.0	13.0	22.0	26.5	14.0	14.5
6	35.0	12.0	24.0	23.0	14.0	16.0
7	34.0	12.0	27.0	24.5	16.0	13.0
8	32.0	11.0	25.0	25.5	13.0	13.5
9	32.5	11.0	26.0	26.0	16.0	14.0
10	34.0	11.0	26.5	25.5	15.5	13.5

HALF PLATE CULTURES REPLICATE 3

15 days	*M. perniciosa* (left) with *A. bisporus* (right)		*M. perniciosa* control		*A. bisporus* control	
PDA						
1	30.0	15.0	19.5	19.5	16.0	17.0
2	25.0	14.0	19.0	20.5	14.0	13.0
3	30.0	11.0	20.0	24.0	13.0	12.0
4	25.0	13.0	20.0	22.5	13.0	14.0
5	30.0	14.0	18.5	20.5	14.0	15.0
6	30.0	14.0	26.5	29.5	15.0	14.0
7	29.0	14.0	23.5	28.5	13.0	16.0
8	28.0	13.0	17.0	19.0	14.0	16.0
9	30.0	10.0	21.0	24.0	16.0	13.0
10	30.0	9.0	26.5	29.5	15.0	14.0

Potato dextrose agar dual culture: *Mycogone perniciosa* (top plate)							Potato dextrose agar dual culture: *Mycogone perniciosa* (bottom plate)						
	F-value	P-value	SE for diff	LSD (by t)	Set B (1)	Set A (2)		F-value	P-value	SE for diff	LSD (by t)	Set B (1)	Set A (2)
5 days	15.3000	0.0036	0.5241	1.1860	14.3000	12.2500	5 days	15.7700	0.0033	0.5918	1.3390	13.8000	11.4500
	F-value	P-value	SE for diff	LSD (by t)	Set B (1)	Set A (2)		F-value	P-value	SE for diff	LSD (by t)	Set B (1)	Set A (2)
10 days	15.5500	0.0034	0.4819	1.0900	20.4500	18.5500	10 days	36.4600	0.0000	0.6625	1.4990	21.9000	17.9000
	F-value	P-value	SE for diff	LSD (by t)	Set B (1)	Set A (2)		F-value	P-value	SE for diff	LSD (by t)	Set B (1)	Set A (2)
15 days	10.5700	0.0100	1.0300	2.3310	29.0500	25.7000	15 days	17.3700	0.0024	1.2000	2.7140	30.0000	25.0000
	F-value	P-value	SE for diff	LSD (by t)	Set B (1)	Set A (2)		F-value	P-value	SE for diff	LSD (by t)	Set B (1)	Set A (2)
20 days	15.6100	0.0033	0.7846	1.7750	32.9000	29.8000	20 days	12.3100	0.0663	1.2680	2.8690	34.0500	29.6000
Mushroom dextrose agar dual culture: *Mycogone perniciosa* (top plate)							Mushroom dextrose agar dual culture: *Mycogone perniciosa* (bottom plate)						
	F-value	P-value	SE for diff	LSD (by t)	Set A (1)	Set A (2)		F-value	P-value	SE for diff	LSD (by t)	Set A (1)	Set A (2)
5 days	1.0000	0.3434	0.3500	0.7918	12.7000	12.3500	5 days	2.9070	0.1224	1.1440	2.5870	15.3500	13.4000
	F-value	P-value	SE for diff	LSD (by t)	Set B (1)	Set A (2)		F-value	P-value	SE for diff	LSD (by t)	Set B (1)	Set A (2)
10 days	9.3090	0.0138	0.6227	1.4090	24.6500	22.7500	10 days	23.3900	0.0000	0.7960	1.8010	27.4500	23.6000
	F-value	P-value	SE for diff	LSD (by t)	Set B (1)	Set A (2)		F-value	P-value	SE for diff	LSD (by t)	Set B (1)	Set A (2)
15 days	41.8100	0.0000	0.3712	0.8397	32.6500	30.2500	15 days	14.8700	0.0039	0.9725	2.2000	33.0000	29.2500
	F-value	P-value	SE for diff	LSD (by t)	Set A (1)	Set A (2)		F-value	P-value	SE for diff	LSD (by t)	Set A (1)	Set A (2)
20 days	0.3554	0.5658	0.5871	1.3280	35.4000	35.0500	20 days	0.5244	0.4874	0.4833	1.0930	39.4000	39.0500
Mushroom agar dual culture: *Mycogone perniciosa* (top plate)							Mushroom agar dual culture: *Mycogone perniciosa* (bottom plate)						
	F-value	P-value	SE for diff	LSD (by t)	Set A (1)	Set A (2)		F-value	P-value	SE for diff	LSD (by t)	Set A (1)	Set A (2)
5 days	2.4020	0.1556	1.0650	2.4090	14.2500	12.6000	5 days	5.0000	0.0522	0.4472	1.0120	13.4500	12.4500
	F-value	P-value	SE for diff	LSD (by t)	Set A (1)	Set A (2)		F-value	P-value	SE for diff	LSD (by t)	Set A (1)	Set A (2)
10 days	3.5300	0.0930	1.4100	3.1900	24.1500	21.5000	10 days	0.7297	0.4151	1.0540	2.3830	21.5500	20.6500
	F-value	P-value	SE for diff	LSD (by t)	Set A (1)	Set A (2)		F-value	P-value	SE for diff	LSD (by t)	Set A (1)	Set A (2)
15 days	1.5700	0.2418	1.2370	2.7980	29.5000	27.9500	15 days	2.6130	0.1405	0.7424	1.6790	30.6000	29.4000
	F-value	P-value	SE for diff	LSD (by t)	Set A (1)	Set A (2)		F-value	P-value	SE for diff	LSD (by t)	Set A (1)	Set A (2)
20 days	4.3460	0.0668	0.8635	1.9530	35.3000	33.5000	20 days	0.3488	0.5693	1.0160	2.2980	37.5500	36.9500