EVALUATION OF GROWTH MEDIA PARAMETERS FOR THE CULTIVATION OF SELECTED BIOLOGICAL CONTROL AGENTS

By

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Submitted in partial fulfilment
of the requirements for the degree of

MASTER OF SCIENCE

in the
Discipline of Plant Pathology
School of Applied Environmental Sciences
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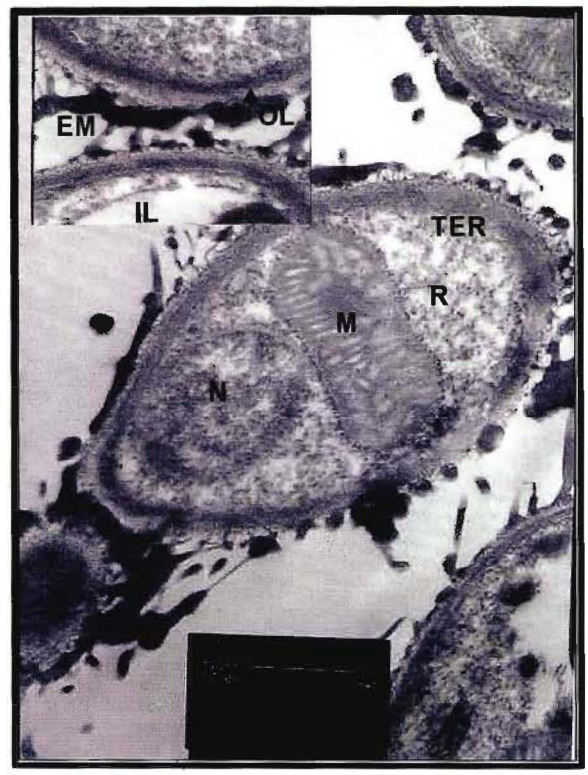
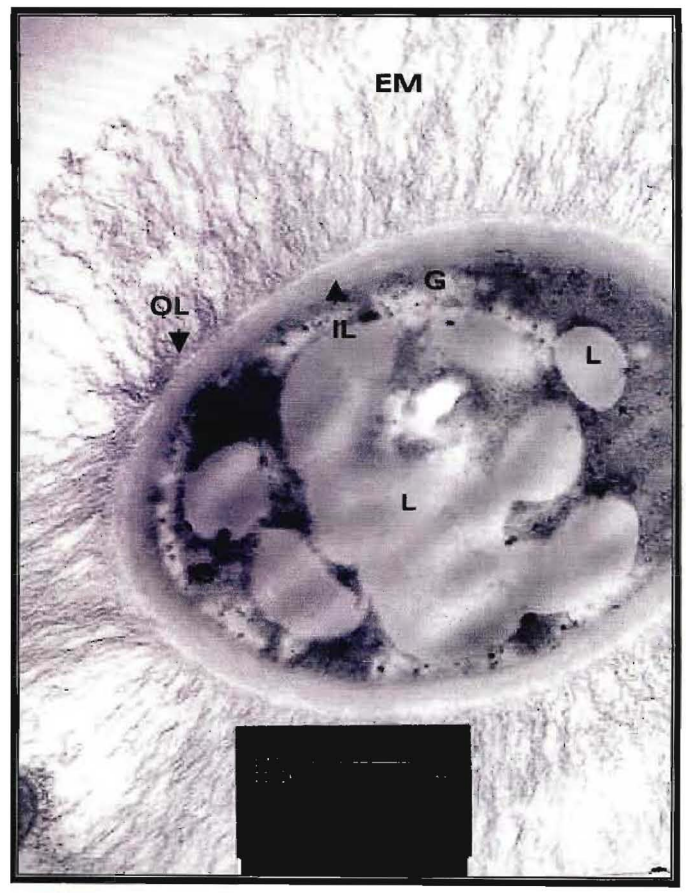
Republic of South Africa

December 2002

FRONTISPIECE



TEM micrograph of a *Colletotrichum gloeosporioides* C6 spore grown in a 5 g L^{-1} carbon concentration basal salts medium at a C:N ratio of 5:1



TEM micrograph of a *Colletotrichum gloeosporioides* C6 spore grown in a 20 g L^{-1} carbon concentration basal salts medium at a C:N ratio of 40:1

"Looking forward, it seems inevitable that the transition to biologically based practices will continue and thrive. A major driving force impacting agriculture is the exponential rate of population growth that will produce a population of over 6 billion people by 2000. The impact that this growth will have on entomology and agriculture is multifold".

Adapted from RL Metcalf, 1996.

ABSTRACT

Trichoderma harzianum kmd, *Gliocladium virens* MMI and *Colletotrichum gloeosporioides* C6 are potential biological control agents. *Trichoderma harzianum* kmd and *G. virens* MMI have been shown to have excellent growth stimulation and disease suppressive characteristics by the Biocontrol for Africa team of researchers at the University of Natal, Pietermaritzburg. *Colletotrichum gloeosporioides* C6 has been shown to have effective control of the invasive weed, *Hakea sericea* (Shrad.). The aim of this dissertation was to establish a method which was most effective for the mass production of the biological control agents (BCAs). Various parameters and the impact of carbon-to-nitrogen and total organic carbon (TOC) on the growth of the BCAs were investigated. Fingerprinting and detection of mutations between strains of *Colletotrichum gloeosporioides* C6 of different ages were attempted using AFLPs for patenting purposes.

Pine shavings and molasses were used in the semi-solid fermentation of *T. harzianum* kmd, *G.virens* MM1 and *C. gloeosporioides* C6. A 70% (v/v) ethanol soak was the most effective pretreatment in the removal of resin off the pine shavings as well as eradication of contamination. Parameters tested were pH, C:N ratios and TOCs. The optimal pH range for *T. harzianum* kmd and *C. gloeosporioides* C6 was between pH 6.5 and 7. The optimal pH for *G.virens* MM1 was pH6. Various C:N ratios and TOCs produced highly significant differences in spore yield and mycelial biomass ($P<0.0001$). The most prolific spore production of all three fungal cultures occurred at a C:N ratio of 31:1 and TOCs ranging between 10 g L^{-1} to 15 g L^{-1}. Chlamydospores of *T. harzianum* kmd were produced at high C:N ratios of 20:1 to 31:1. The growth of *C. gloeosporioides* C6 on a novel gel medium using solid-state fermentation was also investigated. Highest spore yield production was achieved at a C:N ratio of 5:1. The addition of basal salts to the gel medium enhanced spore production. The highest C:N ratio of 40:1 produced abundant mycelial growth. This was determined using Environmental Scanning Electron Microscopy. Scoring of the mycelial mats of the unamended gel medium resulted in the production of larger lipid bodies. The spore yield of *C. gloeosporioides* C6 was substantially decreased when grown on the novel gel as the mycelium integrated itself within the medium. It was concluded that semi-solid fermentation was a more suitable option for the production of *C. gloeosporioides* C6. Semi-solid state fermentation on pine shavings and molasses holds a lot of promise with regards up-scale of production of the BCAs.

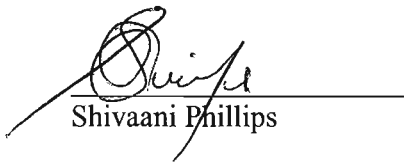
The effects of C:N ratios and carbon (C) concentration on growth, conidiation and germination of *C. gloeosporioides* C6 were investigated. Carbon concentration and C:N ratios produced highly significant differences on all parameters tested ($P<0.05$). Highest spore yields were obtained at C concentrations of 5 g L^{-1} and 10 g L^{-1}, at a C:N ratio of 15:1. Carbon concentration of 20 g L^{-1} and a C:N of 40:1 produced the heaviest mycelial biomass. Highest spore germination percentage occurred at a C concentration of 5 g L^{-1} and at a C:N ratio of 20:1. Specific spore yields (Ysp) and specific mycelial biomass (Yms) were indicators used to determine the most economical production of *C. gloeosporioides* C6. The optimum Ysp and Yms production occurred at a C concentration of 5 g L^{-1} and at a C:N of 15:1. Ysp was not affected by variation of C:N ratios at a C concentration of 10 g L^{-1}. The interactive relationship between C:N ratios and C concentration was significant in the production of Yms ($P=0.05$) and conidiation ($P=0.0001$).

The effect of C concentration and C:N ratios on the ultrastructure of *C. gloeosporioides* C6 was also investigated. Significant differences in the morphology of the spores at various C:N ratios and C concentrations were observed. Cell wall thickness increased with increasing C concentrations and reached a maximum thickness of 131.94 nm at a C concentration of 20 g L^{-1} and a C:N ratio of 40:1. Larger and fewer mitochondria was produced at the lower C concentrations and C:N ratios. Larger lipid bodies were observed at the higher C concentrations and C:N ratios. The nature of the extracellular matrix changed at various C concentrations and C:N ratios. A globular network was formed at a C concentration of 5 g L^{-1} and a fine fibrillar network was formed at a C concentration of 40 g L^{-1}. These criteria can be used to assess which spores would have the longest shelf life as well as best efficacy in the field.

Amplified fragment length polymorphism (AFLP) was used to detect mutations between the parent strain of *C. gloeosporioides* C6 and a culture that had been subcultured from the parent strain. The mutations affected both the nature and colour of the mycelial mats formed by the two cultures as well as the spore yield production. The AFLP technique was unsuccessful as various parameters need to be optimized, including the DNA isolation procedure. The work was discontinued due to financial and time constraints.

DECLARATION

I, Shivaani Phillips, hereby declare that the research reported in this thesis, except where otherwise indicted, is my own original research. This thesis has not been submitted for any degree or examination at any other university



Shivaani Phillips

ACKNOWLEDGEMENTS

I would like to extent my sincere gratitude to:

Prof. MD Laing, my supervisor, for all his guidance, patience and expertise throughout my research. His amazing wisdom in dealing with life's "daily battles" and "pick-me up" lectures have enabled me to face many a challenge.

Dr. Charles Hunter, my co-supervisor, for the colossal amount of work done in reviewing and editing my manuscripts and the ceaseless patience and wisdom he shared with me throughout my research.

Dr. Mike Morris, for all the time, advice and assistance he provided throughout this project.

Dr. Patricia May Caldwell, for the hours spent editing and proofreading my manuscripts.

Diane Fowlds, Ingrid Schlösser and Celeste Hunter in the Disciplines of Microbiology and Plant Pathology their technical assistance.

The National Research Foundation (NRF) and Innovation Fund for funding this project.

Kwasi Sackey Yobo, for all his assistance in the statistical analysis conducted in this dissertation.

The staff of the Centre for Electron Micoscopy, Pietermaritzburg, for their technical assistance in the EM studies performed. A special thanks to Mr. V H Bandu.

To my friends, the Phillips, Padayachee, Naicker and Chetty family for their support.

To my family, Vijaysagren, Premila, Rudheshan, Chrissun, Prenevin and Thashen, and Mr Kesavelu and Neela Naicker for being the inspiration and direction in my life. Without their encouragement and support, I would have been lost. Lastly, to my husband, Reynold Anthony Chetty, thank you having an inexhaustable amount of love and faith in me. You

give me the courage to face mammoth challenges. To my family and husband, thank you for all the sacrifices made so that that I may realize my dreams.

DEDICATION

To the Phillips family, Mr. and Mrs Kesavalu Naicker and
Reynold Anthony Chetty for their support, understanding and
spiritual encouragement
during my studies

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FOREWORD

The research conducted in this thesis was a result of a collaborative investigation driven by the need to effectively produce *Colletotrichum gloeosporioides* C6 to implement the biological control of the invasive weed, Silky Hakea (*Hakea sericea*). This research was also aimed at the production of two other biological control agents (BCAs), *Trichoderma harzianum* kmd and *Gliocladium virens* MM1 using cheap agro-industrial media. All laboratory and electron microscopy research was conducted at the University of Natal, Pietermaritzburg, South Africa. Research on the formulation of the products was partly done by Dr. M J Morris from Plant Health Products and field studies were conducted by the Plant Protection Research Institute in Stellenbosch, South Africa.

Our strategy was aimed at optimizing nutritional conditions and was based on developing a medium which maximized not only propagule yield but also propagule fitness as a biological control agent. The primary goals of this study were two-fold: to investigate semi-solid and solid- state fermentation as processes in the production of BCAs and to investigate the effect of carbon-to-nitrogen ratios and the total organic carbon on the morphology and physiology of *C. gloeosporioides* C6.

The approaches taken in order to achieve our aims were:

1. Chapter One presented a review of available literature on the steps in establishing a BCA and various fermentation methods, as well as growth substrates for the production of BCAs. Literature on the commercial application of various *Colletotrichum* spp. was also reviewed to ascertain the global use and manufacture of *C. gloeosporioides* C6 as a BCA for weed control.

2. Chapter Two evaluated the semi-solid fermentation of *C. gloeosporioides* C6, *T. harzianum* kmd and *G. virens* MM1 using pine shavings and molasses as a growth medium. Solid-state fermentation of *C. gloeosporioides* C6 was performed on a novel gel medium. The effects of parameters such as pH, C:N ratios and TOCs were investigated.

3. Chapter Three investigated the effects of C:N ratios and TOCs on the growth, conidiation and germination of *C. gloeosporioides* C6. Specific mycelial biomass and specific spore yield were also determined in order to assess the most economical production of the BCA.

4. Chapter Four assessed the impact of the C:N ratios and TOCs on the ultra-structure of *C. gloeosporioides* C6 spores.

6. Chapter Five is an overview of the experimental work conducted and also examines prospects for future research.

The information obtained from this research was used in up-scale of production of the aforementioned BCAs.

GENERAL INTRODUCTION

"Words are symbols with which we think. Therefore, our thoughts are profoundly influenced by how we define our words. The definitions that we give words constitute "mini-paradigms" which encapsulate ideas. These "mini-paradigms", if precisely defined can facilitate concise thinking. If poorly defined, our understanding becomes muddled"(Wilson, 1997).

Wilson (1997) defined biological control of plant diseases as:" *The control of a plant disease with a natural biological process or the product of a natural biological process".* This definition includes 'biological' chemicals 'delivered' by, and/or 'extracted' from living organisms as well as constitutive or elicited host resistance (Wilson, 1997). Biological control under this definition is clearly distinguishable from physical and synthetic chemical control of plant diseases.

Plant diseases have impacted on mankind since the advent of agriculture. The expansion and growth of the population has led to a need for more food resulting in increasing amounts of land being devoted to crop production (Lewis and Papavizas, 1991). Major outbreaks of plant disease have contributed to the current concepts concerning plant protection. The potato blight disaster in Ireland caused by the fungus *Phytophthora infestans* (Mont.) De Bary in the 1840s underpined the necessity for the development of effective control measures in order to render intensive agriculture sustainable (Deshpande, 1999). Additionally, approximately one million people suffer from the toxic effects of chemical pesticide residues annually and numerous deaths are attributed to poisoning from such compounds (Deshpande, 1999).

The introduction of a biocontrol agent (BCA) into the environment can be done naturally or by the manipulation of the environment, host or antagonist, or by the mass introduction of multiple antagonists (Baker and Cook, 1974; Cook and Baker, 1983). The different research strategies that may be adopted include inducing resistance to the host, phytoalexin production, modification of post harvest environments (viz., temperature, humidity, atmosphere), alteration of the host surface by changing plant nutrient status and direct or indirect use of foreign or natural antagonists (Korsten and

Jefferies, 2000).

Three biocontrol strategies are recognized.

- *Classical or inoculative biological control* involves the introduction of a self perpetuating or sustainable population of organisms into the environment that inflict a high level of biological control (Jackson, 1997). The use of a non-indigenous natural enemy/pathogen to control a non-indigenous pest species is defined as "classical biological control" (Korsten and Jefferies, 2000);
- *Inundative or augmentative biological control* involves the application of a natural antagonist to the environment to supplement the resident population (Anonymous, 2002). These resident microorganisms may be present either naturally or by previous introduction (Anonymous, 2002). Inundative application is used to instantly elevate the antagonistic population to a density that ensures rapid suppression of the target pathogen (Korsten and Jefferies, 2000). Some authors make the differentiation between inundative and augmentative biological control as the former aims to control the pests within the first generation of release; and,
- *Conservation biological control* involves the manipulation of agricultural practices to enhance the impact of beneficial insects. Examples of this practice include pest-specific insecticides and the timing of agricultural practices to minimize any negative effect on the beneficial species (Anonymous, 2002).

There are various types of BCAs used worldwide and include:

- Biopesticides such as entomopathogenic nematodes, baculoviruses, plant derived pesticides, insect pheromones and microbial pesticides. Biopesticides are used as alternatives to chemical pesticides and are often key components of integrated pest management (IPM) systems (Menn, 1996; Menn, 1997, Menn and Hall, 1999).
- Biofungicides such as strains of *Gliocladium* and *Trichoderma* are used to control plant diseases. The commercial development of biofungicides has made considerable progress due to the isolation and rapid characterization of new biofungicides. Molecular biology

techniques as well as new impressive fermentation technologies have significantly propelled the commercial development of cost effective biofungicides (Sundheim and Tronsmo, 1988). Biofungicides used for the control of seedling diseases caused by *Pythium*, *Fusarium*, *Rhizoctonia*, and *Verticillium* have received major attention as they are non-toxic and non-residue producing control agents (Papavizas, 1985). Research on the joint action or synergy of biofungicides could increase the utility of the biofungicides. An example of such an interaction is the joint action of a nonpathogenic strain of *Fusarium oxysporum* Schlechtendahl:Fries with a strain of *Pseudomonas fluorescens* Migula to control *Fusarium* wilts (Alabouvette, 1996).

- Bioinsecticides comprise a major portion of the biopesticides and are represented by a large selection of diverse microbials and natural products. The best known group of biopesticides are strains of *Bacillus thuringiensis* (Bts) which are produced by fermentation and produce insecticidal crystal proteins (Menn and Hall, 1999).
- Bioherbicides are used in the biological control of noxious weeds. Weeds pose a huge threat to the production of optimum yields as well as profits in the agricultural systems. Chemical herbicides have a negative effect on humans, non-target plants and the environment (Templeton *et al.*, 1984; Hoar *et al.*, 1986).

The general consensus among agricultural companies is that the future approach to the control of plant diseases is the use of biological fungicides. This is due to fewer regulatory requirements which in turn means faster registration (Froyd, 1997). It is surprising to note the keen interest in biologically based control shown by agricultural chemical companies. " Is it not a conflict of interest? Why would chemical companies show interest in biologically based products?" (Froyd, 1997). The answer lies in the fact that BCAs are much more eco-friendly and produce a safer environment for the human population. Increasing resistance of pests as well as fungi to chemical pesticides are spurring companies to investigate BCAs as an alternative control method. Chemical pesticides and BCAs are used for the same purpose, i.e., control of a pest which results in increases in crop yield. The difference in the two approaches of control is that the BCA must not only be effective in killing the target organism but must also multiply in the environment (Andrew, 1985).

There are many incentives for industry to opt for biological control agents as opposed to chemical control. The first is that the regulatory requirements in the United States are less for a BCA and this results in massive cost and time savings. The huge costs incurred in carcinogenic studies, plant and animal residues, and ecological fate and effects are not a requirement when a biological agent is being registered (Froyd, 1997). This also translates into rapid market entry which results in faster return on investments. Lastly, and most importantly, chemical companies are aware that public favour lies with the use of BCAs as opposed to chemical control. Companies such as Cyanamid, Ciba, DuPont, Monsanto, Sandoz and Zeneca are researching the development of genetically engineered agronomic and horticultural crops with resistance to diseases, insects, and chemical herbicides.

Increased demand for biological control agents have necessitated the provision of extensive funding for their production. The first major concern in commercial production systems is achieving optimal bulk growth of the BCA. The specific nutritional and environmental conditions of many BCAs make biomass production difficult to standardize (Bowers, 1982; Churchill, 1982; Lisansky, 1985). A major constraint in the BCA industry is the sterilization of potential substrates for this production. The mechanisms of infection and modes of action that biological control agents play in the environment have been widely researched but information on the commercial production and optimization of the growth of BCAs is severely lacking (Deshpande, 1999). The availability of this type of information is invariably hindered due to market competition and production processes, e.g. the patenting of the products. Another problem faced by the industry is genetic instability of established BCA products which undergo mutation as a result of long term storage and subculturing. Jutsum (1988) reported that only 5% of deliberate releases of BCAs had actually achieved their aim. This indicates that there is a serious gap between scientific observations in the laboratory and practicalities of mass production and field usage (Powell and Faull, 1989).

A major requirement of a BCA is that it must exhibit consistent disease control from the field to commercial level. The BCA must also provide the farmer with a similar success rate as that of a synthetic fungicide (Froyd, 1997). For successful *in vitro* production of biocontrol fungi, several

important factors should be taken into consideration. These include strain selection, strain stability, single spore isolation and media development (Powell and Faull, 1989). The optimum C/N ratio, O_2, temperature and pH also have to be ascertained. There are occasions when the optimum environmental conditions for growth do not coincide with those for optimal sporulation. It is therefore important that the conditions chosen should support the production of the product desired.

Certain environmental factors and laboratory practices have been shown to produce genetic variances in fungal cultures. Molecular markers are used to evaluate genetic diversity, characterize fungal pathogen populations and phylogenetic relationships between species. The use of molecular markers are also used in the fungal taxonomy to clarify genetic relationships of phytopathogenic fungal groups (Michelmore and Hulbert, 1987; Samuels and Siefert, 1995). This is important in fungal taxa such as *Colletotrichum* where species cannnot be clearly distinguished by their morphology (Majer *et al.*, 1996; O' Neill *et al.*, 1997). Amplified fragment length polymorphism (AFLP) is a molecular technique used to fingerprint DNA of any origin and complexity (Blears *et al.*, 1998). AFLPs can be used to map an entire genome and look for polymorphisms and its reproducibility.

The objectives of this study were:

- to ascertain the sterilization potential of agro-industrial by-products for use as substrates for the growth of selected BCAs;
- to evaluate molasses and pine shavings as substrates for bulk production of *Colletotrichum gloeosporioides* (Penz. and Sacc.), *Trichoderma harzianum* (Rafia) and *Gliocladium virens* (Miller, Giddens and Foster) by semi-solid fermentation;
- to optimize nutritional requirements for the production of *Colletotrichum gloeosporioides* C6 on a novel gel;
- to determine the effects of carbon-to-nitrogen ratio and carbon concentration on growth , conidiation and germiantion of *Colletotrichum gloeosporioides* C6.

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CHAPTER 1

LITERATURE REVIEW

A sharp increase in the research activity of biological control agents (BCAs) has been documented by Baker (1978) and is reported to have risen fourteen-fold from 1960 to 1981. The rate of publication of research papers has risen since then and is likely to be exceeded in subsequent years. Although the antagonistic activity of numerous strains of microbial strains have been documented in papers, biopesticides represent approximately 4.5% of all worldwide insecticide sales, with biofungicides an even smaller percentage compared to that of chemical control (Hall and Menn, 1999).

Numerous factors are responsible for the surge of results from the laboratory compared to the scarcity of commercially available products. These factors include: the cost of production of most BCAs and the limited market size; lack of protection of patents for the commercially produced BCA or active ingredients, and lack of sufficient knowledge on the ecology of the antagonist, target pathogen and associations with the microflora (Schisler and Slininger, 1997).

An important factor that is frequently overlooked in the commercialization of a BCA is the initial microbial selection strategies. These strategies play an important role in determining whether the commercialization of a BCA is feasible. A poorly conducted microbial selection selection strategy is an expensive mistake since it occurs at the beginning of a long arduous process towards the commercial development of the BCA (Slininger *et al.*, 1994).

1.1 STEPS IN ESTABLISHING A BIOCONTROL AGENT

1.1.1 Selection of strains

Earlier efforts in the selection of biological control agents have been done empirically, ranging from random selection from soil and plant surfaces to the observations of zones of inhibition on culture media. A number of these efforts have been unsuccessful (Lumsden and Lewis, 1989). Campbell (1986) suggested that emphasis should be placed on searching for an antagonist in environmental niches directly related to the incidence of the disease or lack thereof. An example of such a system is isolation of a potential BCA from disease suppressive soils. "Disease suppressive soils are defined as those soils in which disease development is suppressed when a pathogen is present or introduced in the presence of a susceptible host"(Cook, 1981, Scheidner, 1982). Another strategy is the isolation of potential antagonists from healthy plants in a heavily infested area (Lumsden and Lewis, 1989).

Choosing an appropriate pathosystem to search for a BCA may seem to be a common selection strategy. However, if presented with the opportunity, the choice of a pathosystem for biological control will have a great impact on developing a biological control agent (Schisler and Slininger, 1997). Characteristics that enhance the likelihood of developing a commercial microbial product for biological control include a pathogen which

- has exploitable weaknesses;
- operates in an environment favourable to introduced antagonists;
- has few or no alternative control options; and
- incites an economically important disease (Schisler and Slininger, 1997).

All selection approaches involve isolation of antagonistic fungi in pure culture followed by identification using biosystematic approaches (Lumsden and Lewis, 1989). Baiting techniques, using survival structures of the pathogen either caught by nylon or direct burial of sclerotia in soil, have resulted in the isolation of prospective antagonists (Lumsden and Lewis, 1989). This approach associates the potential antagonist with actual survival structures. Agar plate bioassays have also

been used to select for prospective antagonistic fungi in soil with antibiotic activity (Dennis and Webster, 1971). *In vitro* antagonism of 77 *Trichoderma* species, primarly *Trichoderma harzianum* (Rafai), were tested against six fungal plant pathogens and rated by Bell *et al.* (1982). The Bell rating system (1982) is a widely used *in vitro* screening method which assesses numerous isolates with varying degrees of antagonism against pathogens.

1.1.2 Isolation

Biological control agents should be isolated from areas of low disease incidence, based on the assumption that the search for BCAs would be more productive in areas where biological control is naturally occurring in the field (Baker and Cook, 1974). The following points summarize the microbial isolation methods that enhance the likelihood of discovering strains with high commercial development potential:

- isolating microbial strains in areas where incidence of disease is low or absent but expected;
- maximizing the number of microbial strains assayed for biological control efficacy;
- isolating microbial strains from appropriate plant parts;
- isolating microbial strains under appropriate environmental conditions; and
- avoidance of the use of highly selective isolation media (Schisler and Slininger, 1997).

The continuous search for new aggressive strains of biological control agents is an essential step in the development of effective BCAs. Askew and Laing (1994) evaluated distinct environmental sites as sources of aggressive strains of *Trichoderma* as well as 118 *Trichoderma* isolates for antagonistic action against *Rhizoctonia solani* Kuhn. This research was aimed at narrowing the search for potential BCAs. It was found that species of *Trichoderma* isolated from a pine bark source was more aggressive *in vitro* against *R.solani* than those isolated from various other sources i.e., soil, other bark in South Africa, contaminants and national and international sources (Askew and Laing, 1994). *Trichoderma* selective medium (TSM), used for the quantitative isolation of *Trichoderma* spp. was formulated by Elad *et al.* (1981) and developed later by Elad and Chet (1983) showing that the addition of benomyl to TSM was an effective medium for the isolation of *Fusarium* spp., while the

addition of captan selected for *Trichoderma* spp., specifically. Further investigations conducted by Askew and Laing (1993) proved that modifications to the TSM media by the replacement of fenaminosulf , which effectively controlled Oomycetes (Elad *et al.*, 1981), with propamocarb or metalaxyl to TSM was just as effective as suppressing Oomycetes as well as provided an excellent medium for the quantitative isolation of *Trichoderma* spp. (Askew and Laing, 1993).

1.1.3 Screening

Selection for a suitable biological control agent is difficult and tedious (Lumsden and Lewis, 1989). Ideally, assessment of biocontrol activity should be carried out in an environment similar to where the BCA is to be applied. It has been recognized that agar plate bioassays where potential antagonists are paired against the pathogens of interest, do not reflect a natural state and are very misleading. Isolates that show strong zones of inhibition against a target organism do not always perform when used *in vivo*. In addition, the BCAs may perform well in the field without demonstrating obvious interactions *in vitro* (Lumsden and Lewis, 1989). In some cases, indirect screening assays may provide useful information and give possible insights into mechanisms of action. These assays include studies on mycoparasitism, competition for space and nutrients, production of antibiotics, metabolites or lytic enzymes, induction of resistance, prevention of pathogen propagule germination and the ability of the BCA to proliferate in soil, colonize organic substrates, and affect pathogen survival (Papavizas and Lumsden, 1980; Cook and Baker, 1983).

1.1.4 Strain improvement

Typically, BCAs are naturally occurring antagonists. These organisms are readily obtainable and easily registered according to regulations formulated by the Environmental Protection Agency (EPA) (Betz *et al.*, 1987). Naturally occurring antagonists will continue to be a major source of potential BCAs. Genetic improvement may have a role to play in biological control. Conventional genetic crossings of strains as well as inducement of mutations by mutagenic agents are some of the methods that could be employed to improve potential biological control agents. However, more important

is the promise of molecular biology and genetic engineering for the creation of new and superior strains of BCAs (Lumsden and Lewis, 1989). Papavizas *et al.* (1982) induced mutations in *Trichoderma* spp. using ultra-violet light and then selected strains tolerant to methyl benzimidazole carbamate fungicides. Some mutants were found to be more effective than the wild strains in disease control. Mutation strains of *T. harzianum* have been selected for their ability to colonize the plant rhizosphere (Ahmad and Baker, 1987). Stasz *et al.* (1988) explored the possibility of gene transfer in *T. harzianum* .with protoplast fusion of strains requiring specific growth factors (auxotrophs) or those sensitive to specific antibiotics. Cells from two strains of *T. harzianum*, T12, which was resistant to *Pseudomonas* spp. bacteria and T95 which was rhizosphere competent, were fused. Of the resultant progeny 1-2 % showed increased biocontrol efficacy and rhizosphere competence. Strain T1295-22 showed effective control over a wide range of plant pathogenic fungi including *Pythium* spp., *R. solani*, *Fusarium* spp., *Botrytis cinerea* Pers. : Fr., *Sclerotium rolfsii* Sacc., and *Sclerotinia homoeocarpa* Lib. The new improved strain called T1295-22 also increased growth of the root tip in experiments with corn and cotton (Harman, 1991; Harman and Hayes, 1993).

1.2 FERMENTATION

The rapid expansion of fermentation biotechnology over the past 30 years has led to a greater awareness of the usefulness of filamentous fungi for the production of large amounts of acids, antibiotic, enzymes and fuels from inexpensive and/or waste ingredients (Smith *et al.*, 1980). Although biotechnology has been centered around food and energy production, fungal and bacterial biotechnology has moved to new areas such as production of biological insecticides and mycoherbicides (Churchill, 1982).

If widespread biological control of soilborne plant pathogens is to be achieved, it is necessary to mass produce promising antagonists rapidly and effectively in the form of spores, mycelia or mixtures (Papavizas *et al.*, 1984). For successful production of fungi various factors should be taken into consideration such as C:N ratios, O_2 requirements, as well as, temperature and pH optima. Optimal conditions for both vegetative growth and spore production should be ascertained as there are occasions when the conditions for required growth and sporulation do not coincide (Powell and

Faull, 1989). Problems associated with upscaling of production processes from shake flasks to small fermenters and then to large fermenters can be alleviated by judicious changes in the culture medium (Deshpande, 1999). During fermentation it is necessary to achieve fungal biomass with a desirable amount of living propagules, such as conidia and chlamydospores (Deshpande, 1999). Options for mass production of fungi include: surface cultivation on solid or semi-solid media; biphasic; liquid and submerged fermentation. Solid state and liquid fermentations are the most frequently used method of fermentation for production of fungi and will be discussed in detail.

1.2.1 Solid-state fermentation

Solid-state fermentation (SSF) is a process in which solid material is used as the substrate or the inert support for growing microorganisms (Sato and Sudo, 2000). Solid-state fermentations are distinguished from submerged cultures by the fact that microbial growth and product formation occur at or near the surface of solid materials (Mudgett, 1986). Substrates traditionally used include a variety of agricultural products, such as rice, soya beans, wheat (Fig. 1.1) and millet (Sato and Sudo, 2000). Non-traditional substrates which may be of interest in the industrial process include food-processing wastes and an abundant supply of agricultural and forest wastes (Mudgett, 1986). Solid-state fermentation may be regarded as gas-liquid-solid mixtures in which an aqueous phase is intimately associated with solid surfaces in various states of sorption and is in contact with a gas phase continuous with the external gas environment (Mudgett, 1986). Depending on moisture content, some water is tightly bound to solid surfaces, some is less bound and some exists in a free state in capillary regions of the solid. Heat derived from metabolism and growth of the microorganism raises the temperature of the solid substrate bed and may cause loss of moisture. The solid phase provides a rich and complex source of nutrients which may be complete or incomplete with regard to the organism cultured (Mudgett, 1986). Commonly, solid-state fermentations are performed on tray, static-bed, tunnel, drum, agitated-tank and rotary disc fermenters (Chisti, 1999).

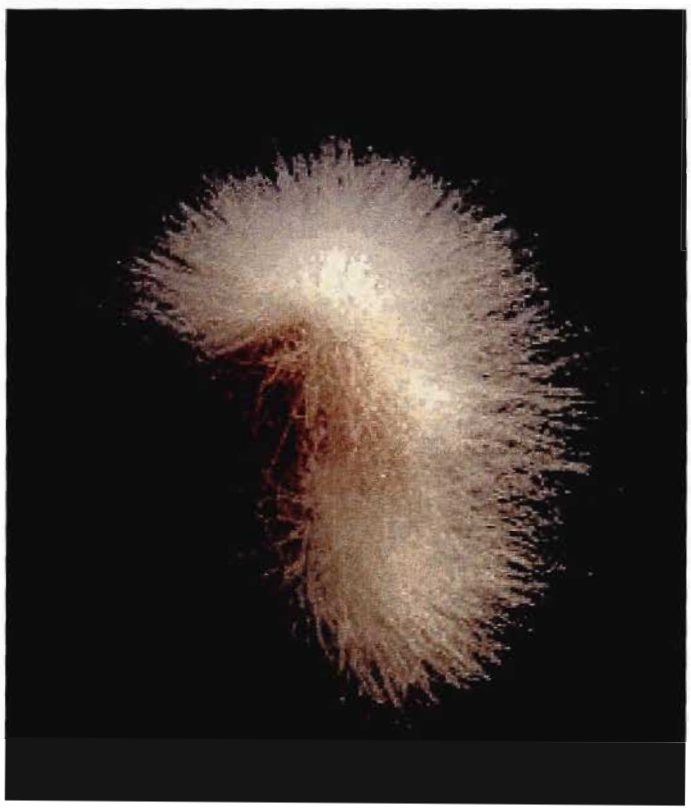

Figure 1.1. *Aspergillus oryzae* cultivated on a wheat grain (Anonymous, 2000)

1.2.1.1 Advantages and disadvantages

Solid-state medium production is the most expensive option as it requires specialist equipment for sterilization. The advantage of this system lies in its simplicity, and ease of harvesting of spores which can be achieved by vacuuming the spores off the surface of the culture, thereby, producing superior spore quality and larger quantity of spores (Powell and Faull, 1989).

Okara, a Japanese bean curd residue, is a by-product of the Tofu industry and is normally disposed of by incineration. Ohno *et al.* (1995) successfully used this waste product as a substrate for the cultivation of *Bacillus subtilis*. The major disadvantage in its utilization is that it is easily spoiled by microbial contaminants due to its abundance of nutrients and high water content. Dehydration of this residue as a preprocessing step to improve its quality before use has been investigated and has shown excellent potential (Ohno *et al.*, 1995). Wheat bran has also been commonly used as a solid-state substrate in both bacterial and fungal solid state fermentation (Ohno *et al.*, 1995).

Solid-state fermentation is often the first method evaluated because in nature most fungi form conidia on aerial hyphae (Jackson, 1997). Since fungal BCAs are typically grown on an agar plate, aerial conidia are usually the first propagules tested for host range and biocontrol efficacy (Jackson, 1997). Solid-state fermentation is advantageous since most fungi sporulate on a solid substrate, thereby facilitating simple culturing and harvesting. The conidia produced in an aerial environment tend to be more tolerant to desiccation and more stable as a dry preparation when compared to spores produced by submerged culture (Silman *et al.*, 1993). Unfortunately, the production of fungal spores using SSF suffers from numerous technical and economic constraints. Problems encountered in the scale-up process include substrate sterilization, gas exchange, temperature control, maintenance of pure culture, stability of culture and product recovery from the substrate. Fermentation time for sporulation on solid substrates generally requires weeks thereby increasing production costs. Deshpande (1999) suggested that a high inoculum can be used to decrease production time. However, it is possible that an organism may expend all the nutrients available for mycelial growth,

resulting in a decrease in conidiation. In general, solid-state substrate sporulation methods are too costly for commercial use, even when its advantages are taken into consideration (Jackson, 1997).

1.2.2 Semi-solid fermentation

Semi-solid fermentation involves the addition of solid granules to a culture medium, followed by fermentation with tumbling (Cannel and Moo-Young, 1980). This type of fermentation is plagued with sterility problems and requires specialist equipment. Semi-solid fermentation systems for commercial production of BCAs are generally not used in western industrialized countries (except in mushroom production) due to insufficient consumer demand for the products (Cannel and Moo-Young, 1980). Modification to SSF has been used successfully to produce large quantities of ascospores of the fungus *Talaromyces flavus* (Kloecker) Stolk and Samson (Fravel *et al.*, 1985) and for large scale production of the mycoparasite *Sporidesmium sclerotivorum* (Uecker, Ayers and Adams (Adams and Ayers, 1982). In the latter case, vermiculite moistened with a liquid medium was inoculated with the fungus in a large twin-shell blender, aseptically bagged, and incubated to allow the mycoparasite to grow and sporulate. Semi-solid fermentation technology is, therefore, useful for the production of fungi which either do not sporulate in liquid culture or do not survive the liquid fermentation process (Lisansky, 1985). Semi-solid fermentations on grains or other types of organic matter are usually allowed to progress until large amounts of conidia are evident. Semi-solid fermentation is appropriate for small-scale laboratory, greenhouse and field tests which require minimal facilities for implementation.

1.2.3 Bi-phasic fermentation

In bi-phasic fermentation, cultures are grown in a fermenter to the end of the log phase and then the mycelia are harvested, spread onto trays and allowed to sporulate. Spores can be harvested by vacuuming. The production method of choice however remains submerged fermentation. This method utilizes existing equipment, and has similar downstream processing requirements as for other fermented products (Powell and Faull, 1989).

1.2.4 Liquid Fermentation

Small-scale fermentations are undoubtedly the most frequent fermentations used in industry (Hilton, 1999). "Small-scale" can be interpreted as individual fermentations ranging from microliter volumes in microwell plates, up to a one liter range in flasks or carboys. In batch fermentations the sterilized media components are supplied at the beginning of the fermentation with no nutrients added after inoculation. Although batch fermentations are simple to set up and run, they are dynamic processes that never reach a steady state. They are, therefore, more complex than the more controlled processes such as stirred, monitored, and fed tank processes (Hilton, 1999). Often in liquid batch fermentations the critical parameter is gas exchange or balance between respiration rate and oxygen transfer.

Shaken Erlenmeyer flasks with cotton plug closures have been used in classical small-scale liquid fermentation systems (Fig.1. 2A). The basic shake flask system can be modified to increase mixing and mass transfer with the incorporation of indentations, baffles and "corners" (Fig.1.2B). Simple sparged and lightly stirred carboys (Fig.1.2C) are used for large volumes when shaking is impractical or would damage the microbe. Liquid fermentation of fungal BCAs usually involves production of a large number of conidia in a liquid, deep-tank fermentation system (Hayes, 1992).

The ideal starting point with liquid media design is to provide a balance of carbon, nitrogen, phosphorus, minerals, and essential vitamins. Medium composition can be divided into two extreme categories: nutrient-balanced media typically used for biomass and growth associated products; and unbalanced media that support only low growth rates but ongoing metabolism (Bul'lock, 1974). Usually, media composition lies somewhere between these two extremes. When this hybrid media composition is used, the balanced nutrients support rapid growth to an initial cell mass (a vegetative stage). The remaining nutrients are unbalanced and can trigger a secondary metabolic phase (Bushell and Fryday, 1983).

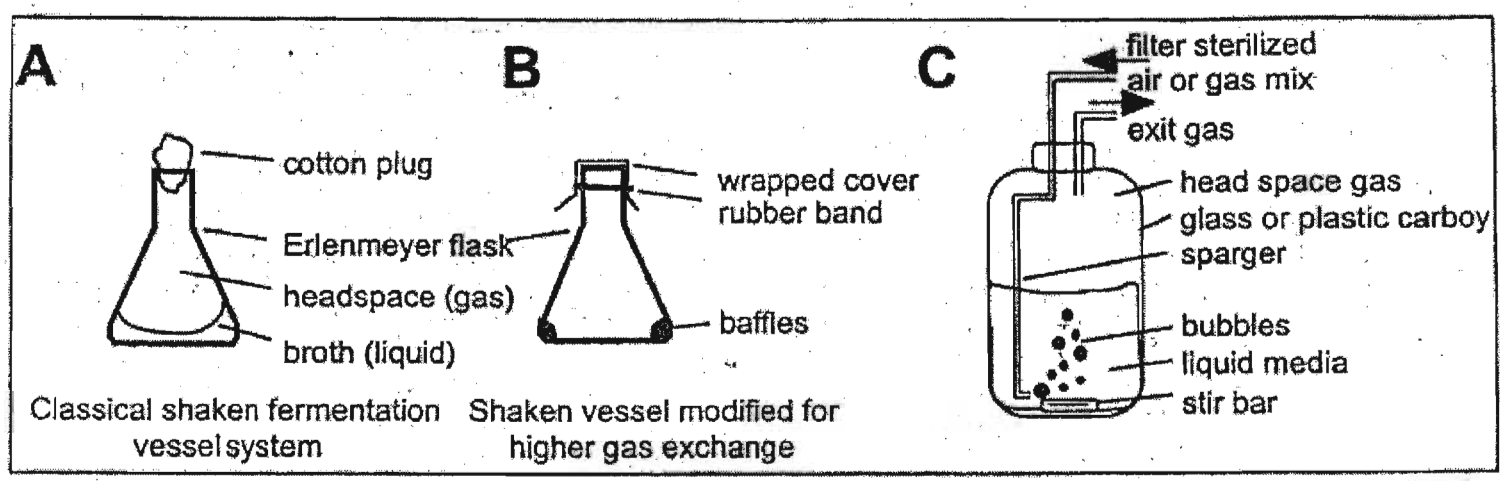

Figure 1.2. Diagrams illustrating common small-scale liquid fermentation systems (Hilton, 1999).

1.2.4.1 Advantages and disadvantages of liquid fermentation

Liquid fermentation has several advantages over other methods of fermentation. Process control is simplified and both the organism end products exhibiting biocontrol activity can be easily harvested. Various parameters such as pH, agitator speed, aeration rate, temperature, etc., can be controlled to optimize the timing for harvesting spores and to obtain suitable biomass for storage (Deshpande, 1999).

Papavizas *et al.* (1984) produced large batches of *Gliocladium virens* (Miller *et al.*) von Arx, *Trichoderma hamatum* (Bonorden) Bainier, *T. harzianum, T. viride (*Persoon:Fries*)* and *Talaromyces flavus* biomass using liquid fermentation in 20L vessels simulating industrial conditions. The substrates utilized were commercially available and inexpensive, i.e., molasses and brewers yeast. Contamination was not a serious problem when reasonable precautions were taken (Papavizas *et al.*, 1984). Several isolates of *Trichoderma* spp. have been shown to develop large quantities of biomass containing conidia and chlamydospores in both liquid and solid fermentation containing inexpensive media (Lewis and Papavizas, 1983). The problems encountered were related to filtering, drying, and the grinding of the biomass. This was due to lack of appropriated downstream processing facilities during experimentation. This problem would be overcome in industry with the availability of proper equipment.

Liquid culture fermentation is currently the most cost-effective method for the bulk production of BCAs (Jackson, 1997). Other than the technical constraints, the sporulation time of BCAs on solid substrates generally takes weeks rather than days elevating production costs. The extensive research conducted in the production methods of antibiotics and organic acids by submerged culture fermentation have provided a considerable amount of data and blueprints for optimizing the design and conditions for fermentation vessels and methods of production of BCAs (Jackson, 1997). The production methods used in the dairy industry has also provided numerous examples proving that living biomass can be efficiently produced from liquid culture fermentations and dried into a stable preparation. These commercial successes have enhanced the industries perception of liquid fermentation as a economically viable method for the mass production of BCAs. Presently, three-

quarter of the registered fungal BCAs for the commercial use in North America are produced using liquid fermentation, reinforcing the fact that liquid culture fermentation is most viable method for the bulk production of BCAs (Jackson, 1997).

1.2.5 Submerged fermentation

Submerged fermentations may use a dissolved substrate, e.g., a sugar solution, or a solid substrate, suspended in a large amount of water to form a slurry. Submerged fermentation are used for pickling vegetables, producing yoghurt, brewing beer and producing wine and soya sauce (Chisti, 1999). Industrial fermentations may be carried out either batchwise, as fed-batch operations, or as a continuous cultures. Batch and fed-batch fermentations are quite common, while continuous fermentations are relatively rare. Batch fermentations typically extend over 4-5 days, but some traditional food fermentations may last months. In fed-batch systems, sterile culture medium is added either continuously or periodically to the inoculated fermentation batch. The volume of the fermenting broth increases with each addition of the medium, and the fermenter is harvested after an appropriate batch time. The major types of submerged-culture bioreactors include bubble columns, stirred-tank, airlift, flautist-bed and trickle-bed fermenters (Chisti, 1999).

1.3 COMPONENTS OF INDUSTRIAL FERMENTATION MEDIA

Industrial media contains complex ingredients that are poorly defined. These ingredients often contain multiple nutrients that the organisms can utilize. For medium development, it is assumed that a given ingredient provides a single nutrient. For example, soya protein which contains amounts of metabolizable carbohydrates and minerals is used to primarily supply complex nitrogen (Dahod, 1999). An important factor that should be taken into consideration when choosing the components of industrial fermentation media is cost. A significant aspect of cutting costs can be attained by the substitution of high cost medium ingredients by complex regional agro-industrial residues and by-products (Alves *et al.*, 1997). The advantage is these ingredients are usually substances with low cost and high availability.

An example of the growth of a microorganism on alternative media is the production of entomopathogenic bacteria *Bacillus thuringiensis* Berlin var. *kurstaki.* Agro-industrial residues and by-products available in southeastern Brazil were used as ingredients for low-cost culture media for liquid fermentation. Medium containing cheese whey, soya bean milk and molasses (WSM) produced the highest spore yield. When all factors, namely, potencies, cost and yields of the final product, and lowest relative cost were taken into consideration, the BMM (Bombyx mori *pupae* and molasses) media was the best option (Alves *et al.*, 1997).

1.4 DESIGN AND PREPARATION OF FERMENTATION MEDIA

Criteria that need to be taken into consideration in the design and preparation of industrial fermentation media are listed as follows:

- **media supply** that is cost effectiveness (raw materials, transport, etc.); a consistent and reliable source of supply; has little nutritional variability and there should be alternative carbon and nitrogen sources;
- **media type** can either be liquid or solid, defined or complex, nutrient limited, balanced or unbalanced;
- **media properties** that have good buffering capacity, heat-liable components, control of redox potential and microbial stability in storage;
- **media treatment** that may be necessary are sterilization, pasteurization, pre-treatments and separate treatments for heat-liable components.

(Dahod, 1999)

1.5 OPTIMIZATION OF CULTURE PARAMETERS FOR THE PRODUCTION OF BCAs

Fungal propagules are the preferred biological control agent as compared to bacteria and can be produced by the methods of fermentation as discussed above. The medium used should be inexpensive, abundant and readily available with an appropriate nutrient balance (e.g., agricultural by-products). Suitable materials include molasses, corn steep liquor and sugar cane bagasse, to name a few. Cereal grains that are husked, cooked, crushed, or cracked have been extensively used (Deshpande, 1999).

Jackson (1997) implemented a strategy for optimizing nutritional conditions for the production of fungal biocontrol agents in submerged culture. It is based on the development of media which maximizes both propagule yield and fitness. Propagule fitness for use as a biopesticide is measured in terms of desiccation tolerance, stability as a dry preparation, and biocontrol efficacy. All these parameters are important if the biopesticide is to become a commercial reality (Jackson, 1997).

The initial approach to the optimization strategy is the formulation of a defined or semi-defined medium. The concentrations of the various nutritional components such as carbon concentration and carbon:nitrogen ratios are known, therefore, the impact of varying the various nutritional components of the media can be ascertained. The parameters tested are propagule formation, mycelial biomass and stability of the BCA as a dry preparation. The propagule of interest varies according to the BCA being evaluated and can be spores, sclerotia or mycelia. The first experiments are designed to assess which nutritional components result in significant biomass production. Generally, optimal spore yields are directly dependant on the endogenous nutrients accumulated by the fungus during the vegetative growth stage. Other nutritional factors that affect growth, propagule formation and efficacy are carbon sources, nitrogen sources, trace metals and vitamins. Once a suitable defined medium has been formulated, the various nutritional factors can be varied individually and the impact on spore yield and fitness can be determined. The optimized defined medium is then used as a reference to determine the optimal concentrations of the various nutritional components in the formulation of production media. The major advantage of having the nutritional framework is that

nutritional components of the defined medium are substituted with low-cost complex substrates in the formulation of the production media. This framework can also be used in the development of media for the production of other BCAs (Jackson, 1997).

Eyal *et al.*, (1997) optimized the large scale production of chlamydospores of *G. virens* strain GL-21 in submerged culture. Harvesting time plays an important role in the formation of chlamydospores. Transmission Electron Microscope (TEM) studies showed that spores matured during the stationary phase when the mycelium lysed completely and defined walls formed around each chlamydospore(Fig. 1.3). The viability of the spores was measured as a function of shelf life of the final formulated product.

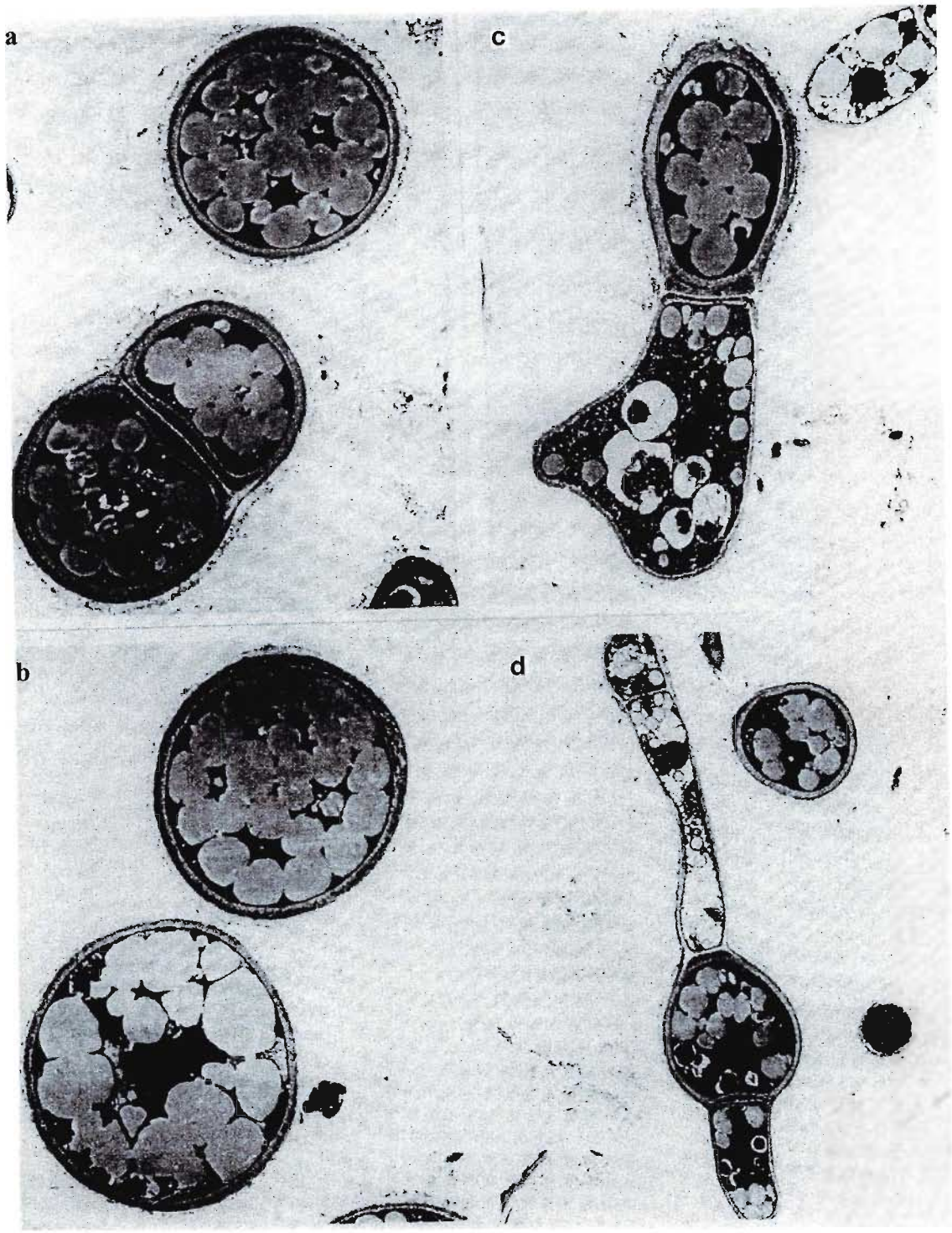

Fig. 1.3. TEM micrographs of *G. virens* (GL-21) showing morphological variability (a) and (b) represent mature spores harvested at stationary phase. (c) and (d) represent immature spores harvested at exponential phase (Eyal *et al.*, 1997).

1.5.1 Optimization strategies for the production of *Colletotrichum truncatum*

Colletotrichum truncatum (Schwein) has been isolated and patented as a fungal pathogen of *Sesbania exaltata*, a weed causing significant agronomic loses in the South-eastern United States in their rice, cotton and soyabeans industries (Bryson, 1987). Jackson (1997) found that *C. truncatum* sporulated poorly when grown in modified Richard's V-8 medium, a standard liquid medium, and hence an optimization program for spore production was established. Initial studies led to the development of a semi-defined basal medium supplemented with trace elements, vitamins and organic carbon and nitrogen sources which satisfied the requirements of *C. truncatum* NRRL 13737 in submerged culture (Jackson and Bothast, 1990). Various C:N ratios and overall carbon concentrations to determine their effect on conidia were assessed. Under the nutritional conditions tested the highest conidial concentrations were produced in media with carbon concentrations of 4.0 to 15.3 g L^{-1}. Higher carbon concentrations (20.4 to 40.8 g L^{-1}) inhibited sporulation and enhanced the formation of micro-sclerotium-like hyphal masses (Jackson and Bothast, 1990).

Jackson and Slininger (1993) conducted submerged culture experiments to determine the optimal nitrogen source for the rapid production of *C. truncatum* conidia. Conidial germination of inocula in submerged culture occurred most rapidly (>95 % in 6 hours) in media provided with a complete complement of amino acids. When ammonium sulfate, urea, or individual amino acids were provided as a sole nitrogen source, conidial germination was less that 20 % after a 6 hour incubation. Supplementation of media with methionine, lysine, tryptophan, isoleucine, leucine or cysteine as sole nitrogen sources severely inhibited *C. truncatum* conidial production.

1.5.2 Optimization strategies for growth of *Paecilomyces fumosoroseus* at various carbon concentrations

Recent interest in *Paecilomyces fumosoroseus* (Wize) A.H.S Brown and G. Smith as a bio-insecticide for the control of various soft-bodied insects, such as greenhouse and silver leaf whitefly, has risen from the ability of this fungus to develop epidemics within a host insect population (Jackson, 1997). The blastospores produced by this entomopathogens, has been shown to be highly infective structures but are intolerant to drying (Inch and Trinci, 1987). Primary goals in optimizing nutritional conditions for the production of *P. fumosoroseus* blastospores have focused on blastospore yield and on tolerance to desiccation. A synthetic medium has been developed, which supports good growth and sporulation (Jackson *et al.*, 1997). The basal medium supporting the growth of *C. truncatum* (Section 1.3.1) also supported the growth of *P. fumosoroseus*. However, an organic nitrogen source was required for adequate growth. Media which contained the high concentrations of glucose (> 20 g L^{-1}) and nitrogen resulted in highest concentration of blastospores (5.8×10^8 ml^{-1}) (Jackson *et al.*, 1997). Desiccation tolerance of blastospores was found to be affected by prevailing conditions. Media containing Casamino acids between 13-40 g L^{-1} and 1-3 g nitrogen L^{-1} achieved a 79 % survival rate of blastospores (Jackson *et al.*, 1997). These results demonstrate that desiccation tolerant blastospores can be produced under appropriate nutritional conditions, thereby alleviating a critical constraint associated with their commercial use.

1.5.3 Optimization strategies for growth of *Penicillium oxalicum*

Induction of submerged conidiation of *Penicillium oxalicum* (Currie and Thom.) has been examined using a range of synthetic and complex media supplemented with by-products of the brewery industry (Pascual *et al.*, 1997). Successful conidiation has been induced by growing *P. oxalicum* in a glucose/salts-based medium (C:N ratio 62.5) for 24 hours and then through the transference of the same mycelium to a new medium lacking a nitrogen source (Pascual *et al.*,1997). Levels of

sporulation have also been significantly increased by the addition of calcium or polyethylene glycol to the medium (Pascual *et al.*, 1997).

1.5.4 Optimization strategies for growth of *Fusarium oxysporum*

Conditions for optimizing spore production in 2.5 liter fermentors, especially chlamydospores, by host-specific mycoherbicide strains of *Fusarium oxysporum* (Schlechtendahl and Fries) were studied by Hebbar *et al.* (1997). Dissolved oxygen concentration and pH significantly affected growth characteristics of *F. oxysporum* strains. After 14 days of fermentation, microconidia and chlamydospores were produced with very little production of macroconidia. Total viable spore counts were significantly higher under high dissolved O_2, whereas chlamydospore counts were significantly higher under low dissolved O_2. The percentage of chlamydospores increased when the pH was increased (Hebbar *et al.*, 1997).

1.5.5 Medium optimization studies on *Trichoderma*

Saprophytic soil fungi such as species of *Trichoderma* can use a wide range of compounds as sole carbon and nitrogen sources (Papavizas, 1985). The carbon and energy requirements of *Trichoderma* can be satisfied by monosaccharides and disaccharides, complex polysaccharides, purines, pyrimidines, amino acids, condensed tannins, catechins, aldehydes and organic acids (Papavizas, 1985).

Ammonium appears to be the most readily utilised source of nitrogen used by *Trichoderma* spp. in buffered media. Other sources of nitrogen, such as amino acids, urea, nitrate and nitrite, also support abundant growth. The role of mineral salts, sulfur sources and vitamins as growth-affecting ingredients has not been extensively researched (Danielson and Davey, 1973).

There has been considerable interest in the effects of CO_2 on the *in vitro* growth of *Trichoderma* and the implications of such effects on the ecology of the antagonist in the soil. These effects on growth,

are variable and appear to depend on CO_2 concentration, medium pH and species aggregate tested (Danielson and Davey, 1973). Rapid growth has been found to occur at high CO_2 concentrations in alkaline media. This may explain why *Trichoderma*, a normally acidophilic soil inhabitant, thrives in very wet, slightly basic habitats (Papavizas, 1985). Since the effect of CO_2 is most pronounced at high pH values, it can be ascribed to the bicarbonate ion rather than CO_2 directly (Danielson and Davey, 1973).

1.6 *Colletotrichum* STRAINS AS MYCOHERBICIDES

Colletotrichum species are widespread and occupy specific ecological niches on plant surfaces (Korsten and Jeffries, 2000). They can exist as saprotrophic competitors in dead and dying organic plant material, or as necrotrophic or semi-biotrophic pathogens (Bailey and Jeger, 1992).

Mycoherbicides are fungal pathogens that are applied inundatively to specific weeds in the same manner as chemical herbicides (Templeton, 1992). This results in infection, disease development and eventual death of the weed host (Templeton, 1992). In contrast to classical control, mycoherbicides employ indigenous strains of fungi which are well adapted to prevailing conditions and consequently, are more predictable in the region where they are to be used (Templeton *et al.*, 1986). Furthermore, many of the constraints to classical biological control, such as spatial, temporal and environmental constraints to establishment, increase and spread, are alleviated or substantially reduced by a single, timely application of inoculum to each individual weed plant at a climatically suitable time (Templeton, 1992).

The bioherbicide tactic differs from classical biocontrol method of weed control where the dissemination and reproduction of the biocontrol agent is left largely up to nature. In the bioherbicide tactic, massive doses of inoculum are applied directly to all the targeted weeds. Thus the bioherbicide method requires large numbers of viable propagules and a satisfactory method of storing them so that their viability is maintained until they are required for application (Amsellem *et al.*, 1990).

1.6.1 Commercial development of inundative bioherbicide products and strains for weed control

Nineteen strains of *Colletotrichum* have been isolated exhibiting potential biocontrol activity (Templeton, 1992). *Colletotrichum gloeosporioides* f.sp. *aeschynomene* (Penz and Sacc.) (Collego) has been used commercially for many years and will be discussed in detail. In addition, four strains which have shown potential for commercialization prospects are summarized in Table 1.1.

1.6.1.1 *Collego (Colletotrichum gloeosporioides* f.sp. *aeschynomene)*

Collego, a dry formulation used as a selective post-emergent mycopesticide was marketed from 1982 to 1992 for the control of northern jointvetch (*Aeschynomene virginica* (L.) B.S.P) in rice (*Oryza sativa* L.) (Templeton, 1992).

Northern jointvetch is a leguminous annual that is commonly found in rice fields, where it can develop into dense colonies if not controlled. It competes with rice, interferes with harvest and reduces the value of rough and milled rice. The black kidney-shaped seeds are difficult to remove from harvested rice because they are nearly the same diameter as rice grains (Smith, 1986). The Collego pathogen infects a number of leguminous hosts but not as effectively as on northern jointvetch. In initial host range tests in the greenhouse with seedlings of 30 plant species and 46 cultivars of economic and native plants, only northern jointvetch was killed. A related species, Indian jointvetch (*A. Indica* L.), was slightly susceptible as small lesions formed but plants were not killed. Hence the strain was designated *C. gloeosporioides* f.sp. *aeschynomene* (Daniel *et al.*, 1973).
This disease caused by *C. gloeosporioides* f.sp. *aeschynomene* is an endemic one, occurring naturally throughout the rice-growing region in eastern Arkansas, United States (Smith *et al*, 1973). The lesions are black and elongate along the axis of the stem, tapered at the ends and speckled with pink and orange, non-setose pustular acervuli that at first are covered by host epidermis and later become erumpent. Lesions range in size from 1-4 cm long by 0.25-1.5 cm wide. The round pustules range from 0.1-0.5mm wide (Daniel *et al,* 1973).

Conidia are elliptical with rounded ends and range from 14-17 by 4-5.7μm in size. Conidia bud from the tips of short condiophores on a subepidermal stoma. Masses of conidia, in a water soluble matrix, are released by rupture of the covering epidermis (TeBeest *et al.*, 1978). The fungus is readily isolated from diseased tissue and can be grown on a number of common media such as potato dextrose agar, lima bean agar, oatmeal agar or Emerson's Ypss agar. The optimum temperature for linear growth and sporulation on lima bean agar ranges from 28 - 30°C. Optima for spore production and spore germination are similar to those of linear growth (Daniel *et al.*, 1973).

Liquid culture is the preferred method of spore production for field evaluation. The Collego fungus grew well in liquid culture and sporulated abundantly when aerated by continuous agitation or by bubbling air though the cultures along with stirring (Daniel *et al.*, 1973). High spore yields were also obtained in modified Richard's solution in three to five days, depending on inoculum volume. Preliminary field tests were conducted over three seasons in flooded rice paddies to obtain information on timing, rates, volume and methods of application, as well as water management and other crop practices (Smith, 1982; Smith, 1986). Control of northern jointvetch in the experimental plots were equivalent to, or superior to chemical herbicides. No damage to rice and neighbouring crops occurred as is frequently experienced with chemical control. The onset of mycoherbicide action developed more slowly than for chemical treatment. Spores used for field tests were packed as wet cakes or pastes. These formulations had to be air freighted to the test area and used promptly upon arrival to prevent spoilage (Templeton, 1992).

Dry spore formulations were developed and eventually became the commercial product of choice (Bowers, 1982; Bowers, 1986). Spore germination in formulation ranged from 35 - 80% depending on storage conditions and the spores had a self-life of at least 18 months. A spore re-hydrating agent was provided by the supplier to improve spore germination (Bowers, 1986).

Approximately 24710 hectares were treated annually with reliable and reproducible results. Since initial field plot studies were initiated in 1970, and during the nine years of commercial use no perturbation or crop damage were reported (Templeton, 1992). Approximately 2500 hectares were

treated annually. The sale of Collego was discontinued because of its low perceived market potential andthe difficulties of producing an economically viable product (Watson *et al.*, 2000).

1.6.1.2 Lubao (*Colletotrichum gloeosporioides* f. sp. *cuscutae*)

Lubao has been used in China since 1996 for the control of dodder species (*Cuscuta chinensis* Lam. and *C. australis* R. Br) parasitizing soyabeans (*Glycine max* (L.) Merr) (Watson *et al.*, 2000). Inoculum has been produced and distributed locally though small-scale rural factories. This fungus is currently being tested in Israel. Production of inoculum has been erratic, and cultures are easily lost unless they produce chlamydospore-like cells. An intensive host-range study was conducted in which dodder-parasitized crops were inoculated. None were infected, while dodder was completely destroyed by the fungus (Watson *et al.*, 2000).

1.6.1.3 Biomal (*Colletotrichum gloeosporioides f.sp. malvae*)

Biomal was registered in 1992 in Canada for post-emergence control of round-leaved mallow (*Malva pusilla* Sm.), lentil (*Lens esculenta* Moench), flax (*Linum usitatissimum* L.), and wheat (*Triticum aestivum* L.), but it was never marketed. The project was terminated because of high cost associated with inoculum production. The product was in the process of re-registration on the date of publication (Templeton and Heiny, 1989; Watson *et al.*, 2000).

Table 1.1 Commercially produced strains of *Colletotrichum gloeosporioides* used as mycoherbicides

Genus, Species, Pathovar	**Registered name**	**Target weed(s)**	**Biological control (%)**
Colletotrichum gloeosporioides f. sp. *cuscutae*	Lubao	Dodder (*Cuscuta chinensis* Lam. and *C. australis* R. Br) which parasitize soyabean (*Glycine max* (L.) Merr).	85
	Velgo	Velvet leaf (*Abutilon theophrasti*)	46
Colletotrichum gloeosporioides f. sp. *malvae*	BioMal	round-leaved mallow (*Malva pusilla* Sm.) which compete with lentil (*Lens esculenta* Moench), flax (*Linum usitatissimum* L.), and wheat (*Triticum aestivum* L.)	89-100

(Templeton, 1992 and Watson *et al.*, 2000).

1.7 *Trichoderma AND Gliocladium*: POTENTIAL FOR BIOCONTROL

Trichoderma and *Gliocladium* are widely distributed fungi and occur in nearly all soils and other natural habitats which contain organic matter (Danielson and Davey, 1973). *Trichoderma* appears to be a secondary colonizer, as its frequent isolation from well-decomposed organic matter indicates. *Trichoderma* is also commonly found on root surfaces of various plants, on decaying bark, especially when it is damaged by other fungi, or on sclerotia or other propagules of fungi (Danielson and Davey, 1973).

Bliss (1951) attributed the control of *Armillaria mellea* (Vahl:Fr.) Kummer in citrus following carbon disulfide fumigation to the action of indigenous *Trichoderma* that rapidly colonized and reproduced in the fumigated soil. Ohr *et al.* (1973) provided direct evidence indicating the involvement of indigenous *Trichoderma* in the biocontrol of *A. mellea* in methyl bromide fumigated soil. *Trichoderma* was found to be more resistant to methyl bromide than *A. mellea.*

The addition of sulfur to soil to maintain the pH below 3.9 has been shown to control root rot of pineapple in Australia (Cook and Baker, 1983). This control was attributed to a decrease in zoosporangium formation of the causal agent (*Phytophthora*) under acidic conditions, and to an increase of acidophilic *T. viride* numbers (Cook and Baker, 1983). The ability of *Trichoderma* and *Gliocladium* to act as mycoparasites of hyphae and the resting structures of plant pathogens has been demonstrated *in vitro* (Adams and Ayers, 1982), as well as in natural soil (Hubbard *et al.*, 1983). The ability of *Trichoderma* and *Gliocladium* to produce diffusible substances toxic to other fungi *in vitro* and in organic substances in soil also strengthens this hypothesis suggesting the importance of indigenous *Trichoderma* and *Gliocladium* as biocontrol agents (Papavizas, 1985).

Ahmed *et al.* (1999) studied the ability of *T. harzianum* to control the rotting of pepper (*Capsicum annuum*) caused by *Phytophthora capsici* (Leonian). Analysis of the fungal population showed that

T. harzianum consistently reduced the population of *P. capsici* over time and the associated reduction of root rot ranged between 24 and 76%.

A wheat-bran/peat mixture (1:1 v/v) was found to be most efficient of the raw material substrates tested for growing *T. harzianum* (T-315) (Sivan *et al.*, 1984). *Trichoderma harzianum* grown on a bran/peat mixture efficiently controlled damping-off caused by *Pythium aphanidermatum* (Edison) Fitz. in peas, cucumbers, tomatoes, peppers, and gypsophila (Sivan *et al.*, 1984). Disease reduction in tomatoes of up to 85% was achieved.

1.8 SCIENTIFIC AND COMMERCIAL REQUIREMENTS FOR BCAs

The following traits are desirable in BCA products: (Powell and Faull, 1989; Froyd, 1997).

- *a rapid mode of action* relative to that of the pest or host plant is critical. The possibility that a pathogen may outgrow the BCA and exploit new niches must be minimised. If the BCA is slow growing then the pathogen can use available substrate and this increases the chance of disease. Kelley (1976) reported that *T. harzianum*-impregnated clay granules provided nutrients for the growth of *Phytophthora cinnamomi* which worsened rather than controlled the disease;
- *persistence* in the target environment is important. Host plant vulnerability to some pests and disease has a "window" of time, before and after which susceptibility does not occur. In some cases the host is continuously susceptible to attack throughout its lifetime. Thus, it is important that the BCA should be present and active for extended periods in order to protect the plant during its vulnerable period. Ideally, the BCA should be able to establish itself and multiply in the environment;
- *tolerance* to prevailing environmental conditions is desirable. The environmental tolerance of the BCA to moisture, temperature, UV light, and pH should correlate to that of the targeted

niche. If they do not match that of the targeted niche, the formulation of the BCA can be designed in such a way so as to try and modify that niche;

- *a mechanism of action* that acts specifically against the targeted pest or pathogen at a desired application rate. Biocontrol agents release volatile antibiotic compounds which dissipate into the surrounding environment, or may require close physical contact between the BCA and the target, as in the case with hyperparasitism and hyphal interference (Lewis *et al.*, 1989).

Criteria that should be taken into consideration for commercialization are:

- *a viable market size* and customer demand for the BCA's. It is preferable that there is a broad spectrum activity against a number of targets;
- *high performance and consistency* of effect of the BCA must be equal to or better than competing chemical pesticides;
- *persistence of effect*: the BCA must persist long enough to offer protection to the plant against the target organism;
- *safety*: the product should not pose toxicological problems;
- *stability*: the product should have at least a two year shelf life over a range of temperatures, though this criterion could be modified;
- *indigenous microorganisms* : unless aimed at a very valuable market, BCAs should be indigenous microorganisms which are not genetically engineered;
- *saprotrophic microorganisms*: the BCA should preferably be a saprotroph;
- *minimal capital cost*;
- *minimal cost and maximal practicality of production*;
- *application*: current conventional application methods should be the first option and their application should not involve major changes in current agricultural practices. (Powell and Faull, 1989; Froyd, 1997)

1.9 FUTURE NEEDS AND DIRECTIONS

The next few years will see more applications of BCAs in agriculture, with particular emphasis on the use of Gram-positive bacteria. These organisms have superior capacities for survival in formulated products resulting in products with extended shelf-life and viability. These criteria are crucial in the development of commercially successful BCA (Wilson, 1997).

The goals of fermentation are low cost and high productivity. The first imperative step in the process is the production of top quality inoculum. Liquid culture fermentation is considered to be the most efficient and economical. However, certain filamentous fungi do not sporulate well in liquid culture (Daniel and Davey, 1973). Future alternatives to both solid and liquid fermentations are the development and conditioning of new strains of fungi to sporulate when submerged, development of media or media additives that enhance sporulation, and further research of physical treatments that stimulate sporulation (Templeton and Heiny, 1989).

The difficulty of formulating mycoherbicides into effective products lies in the fact that, as living organisms, their viability must be preserved throughout processing and storage. Additionally, the final packaged product must show efficacy in the field under natural environmental conditions. A viable concept that has not been sufficiently explored is the use of adjuvants that interact with the plant and/or microbial agent to promote the efficacy of the plant (Daigle and Connick, 1990). The fact that each disease and host interaction is unique enhances the formulator's challenge.

Another aspect which should be taken into consideration in the future development of formulations is the microbial interaction on plant surfaces. Researchers have proposed that an integrated biocontrol system by the addition of chemical and/or insects make some mycoherbicides more efficacious (Daigle and Connick, 1990). Technology for formulating and applying spores in the absence of dew has been developed and is continually being improved (Quimby *et al.*, 1988). The combination of more than one fungus in a formulation increases market potential substantially.

Boyette *et al.* (1979) mixed the Collego fungus with *C. gloeosporioides* f. sp. *jussiaea* and successfully controlled two unrelated weed species in one application in a rice field.

There are several stages in the development of successful BCAs that need further research. Greater research emphasis needs to be placed on exploration, both domestic and foreign, to find new strains of weed pathogens. More knowledge of potential biopesticides is required to make this biocontrol tactic more generally available. Candidates may be exotic or indigenous microorganisms or specific natural enemies (Templeton, 1990; Lewis and Papavizas, 1991). A greater number of investigations need to be employed concerning persistent problems related to dependability and reliability of microbial agents. Models need to be developed for analysis of epidemiological, economic and biological data to assess maximum benefits and how they might be introduced into integrated pest management systems (Lumsden and Lewis, 1989). More information on the formulation of microbial products is essential. More basic research on formulation is needed in the public domain for all categories of antagonists (Mathe *et al.*, 1999).

Most laboratory experiments are conducted on a small-scale level. One of the greatest challenges during scale-up is the maintenance of quality control. Contamination and mutation can occur at any stage of the scale-up process. The consequence of this is that the organisms applied in large-scale field trials or commercially are different from the organisms used in the research phase of the project. This problem has to be addressed in terms of maintaining sterility through out the process of scale-up (Mathe *et al.*, 1999).

New developments and prospects in biotechnology give cause for optimism. It can be expected that powerful new genetic engineering tools can be used to enhance existing biocontrol tactics, improve our ability to manipulate the agent practically based on an understanding of molecular interactions between the agent and target (Templeton, 1990). Genetic manipulation and hybridization can be used to induce new biotypes for improved performance, adaptation of microbial agents to new disease control requirements, and improved compatibility with chemical fungicides (Lumsden and Lewis, 1989).

The use of an integrated biological control strategies in which several tactics are employed to combat the same pathogen, is a promising approach to improve the level of disease control or the consistency of the biological treatment. The use of mixtures of organisms on the same plant organ will likely become more commonplace (Wilson, 1997).

Commercialization of a product may begin at several levels. The most effective system is to develop a close liaison between industry, public research institutions, extension or advisory services and eventually growers. This enables the formation of a network of experimentation to develop the most effective product to agriculture (Lumsden and Lewis, 1989). Foremost among these is the greater interaction and cooperation between industrial and non-industrial scientists. This interaction is imperative in the transference of basic scientific information into research and development activities (Lumsden and Lewis, 1989). These areas of communication at the various levels are severely lacking and need to be improved in future endeavors (Justum, 1988).

Integrated pest management (IPM) is most advanced in the entomopathological applications but advances are being made with the incorporation of biological control agents with other test management methods. One approach is the use of BCAs that are tolerant to fungicides or the incorporation of tolerance to fungicides of the BCA. The use of a fungicide resistant BCA can increase seed protection initially using the chemical component and the biological component becomes active later enhancing growth and as a disease control measure (Harman and Bjorkman, 1998). Most genera of insects and mites are prone to infection by fungi, and pathogenic strains of fungi are quite specific and may infect only a specific type of host. Therefore, fungi can be used to infect pests without killing beneficial predators and parasitoids (Gillespie and Moorhouse, 1989). An advantage of such a system is that fungi can be applied to provide prolonged pest control without polluting the environment. The use of two or more different types of biological control agents may be required to control situation where there is a variety of pests that need to be controlled. Extensive trials need to be conducted to establish the compatibility of the two or more biological control agents as well as timing releases as to avoid the two BCAs encountering each other if they are incompatible (van Driesche and Bellows, 1996).

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CHAPTER 2

Evaluation of methods of semi-solid and solid-state fermentation for the cultivation of *Colletotrichum gloeosporioides* C6, *Trichoderma harzianum* kmd and *Gliocladium virens* MM1.

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The potential for large-scale production of fungal biocontrol agents exists within the agricultural industry, either as fungal cells themselves or cell-free fungal components. A major consideration in the production of biological control agents is the cost and availability of raw materials. The aim of this study was to investigate the semi-solid fermentation of three potential biological control agents, *Colletotrichum gloeosporioides* C6, *Trichoderma harzianum* kmd and *Gliocladium virens* MM1 on pine shavings and molasses and the solid-state fermentation of *C. gloeosporioides* C6 on a novel gel medium. Parameters investigated for semi-solid state fermentation were pH, carbon-to-nitrogen (C:N) ratios and optimal total available organic carbon (TOC), with the primary aim of producing biomass, spores and chlamydospores. The initial focus of the research was the effective sterilization and removal of resin from pine shavings. It was concluded that the most efficient method was a 30 minute 70% (v/v) ethanol treatment. The optimal pH range for both *C. gloeosporioides C6* and *T. harzianum* kmd was found between pH 6.5-7 and for *G. virens* MM1, it was pH 6. Highly significant differences ($P<0.0001$) were recorded at various C:N ratio treatment, with the highest spore yield for all three fungal cultures occurring at a C:N ratio of 31:1. TOC treatments produced highly significant differences ($P<0.0001$) with the optimal TOC ranging between 10g L^{-1} to 15g L^{-1} for all three fungal cultures. The distribution of chlamydospores produced by *T. harzianum* kmd on the pine shavings was sparse and produced at high C:N ratios. It was concluded that the growth of biological control agents on pine shavings and molasses

holds promise with regard to large-scale production. Best spore yield production of *C. gloeosporioides* C6 on the novel gel was achieved at a C:N ratio of 5:1 and was enhanced by the addition of a basal salts to the medium. A C:N ratio of 40:1 produced abundant mycelial mass. Larger lipid bodies were produced when the mycelial mat of the unamended gel was scored. A higher spore yield of *C. gloeosporioides* C6 was achieved when grown on the pine shavings and molasses medium than on the gel medium. It was concluded that semi-solid fermentation was a better option for the large scale production of *C. gloeosporioides* C6 than solid fermentation.

2.1 INTRODUCTION

In recent times solid-state fermentation (SSF) has been employed in the production of microbial biomass for use as mycopesticides (Stowell, 1991). It is essential that the biomass be stable, remain viable, have a long term shelf life and still retain high efficacy in the field (Papavizas *et al.*, 1984). Solid-state fermentation processes involves the growth of microorganisms on moist solid substrates or inert supports in the absence of free-flowing water (Mudgett, 1986; Losane *et al.*, 1992; Ooijkaas *et al.*, 2000; Sato and Sudo, 2000). Two types of SSF systems may be used depending on the nature of the solid phase used. The most commonly used system involves cultivation of microorganisms on support medium comprised entirely of natural materials. The second involves cultivation on an inert support impregnated with a nutrient medium (Barrios-Gonzales and Mejia, 1996).

Natural materials which are used as both a support and nutrient source are typically starch- or (ligno-)-cellulose-based and include agricultural products such as grains and grain by-products, cassava, potato, beans, and sugar beet pulp (Pandey, 1992). In instances where inert matrices are used, substrates such as hemp, perlite, polyurethane foam (PUF), sugarcane bagasse and vermiculite have been used (Oriol *et al.*, 1988).

Chemically defined media are rarely used in industry since complex media usually give higher fermentation yields at lower cost. Complex media containing ingredients such as by-products from the brewery, meat and corn-milling industries are commonly used (Dahod, 1999). However, information obtained using chemically defined media is useful in optimising nutrient requirement for a particular organism. Typical components of chemically defined media include carbon, nitrogen, phosphorus, sulfur and trace metals. The limiting nutrient is usually carbon since carbon depletion results in cessation of growth, whereas the depletion of other nutrients results in abnormal growth (Miller and Churchill, 1986; Walker, 1999).

The pH of chemically defined media is often overlooked as a growth parameter. Complex media have natural peptides, amino acids and other organic compounds that provide pH buffering capacity. In contrast, defined media need the addition of an external acid or base to control the pH during the fermentation process (Minoda, 1986).

An important factor that should be taken into consideration when choosing the components of industrial fermentation media is cost. Cost cutting can be achieved by substituting expensive ingredients with complex regional agro-industrial residues and by-products (Alves *et al.*, 1997). The advantage is that such ingredients are usually readily available at low cost.

There are four basic nutrient classes in industrial fermentation media. These are carbon, nitrogen, minerals and materials used for special purposes. Glucose is the most commonly used carbon source in fermentation in the laboratory but not widely used in industrial fermentation media (Dahod, 1999). Alternative carbon sources commonly used in fermentation media include maltose, sucrose, fructose, xylose and lactose (Walker, 1999). More complex glucose based carbohydrates such as dextrins have been also used (Churchill, 1982). Major factors that influence the choice of an appropriate carbon source include availability, cost and ability of the microorganism to metabolize the carbon source.

Nitrogen plays a critical role in the anabolic biosynthesis of proteins and nucleic acids. Microorganisms that are non-diazotrophic require an exogenous supply of either organic or inorganic nitrogen (Walker, 1999). Inorganic sources of nitrogen include ammonia, ammonium salts and nitrate which is reduced to ammonia by many bacteria, fungi and yeasts. Urea has also been used as an inexpensive nitrogen source in industrial fermentation media (Walker, 1999). The range of potential organic nitrogen sources of nitrogen is large, the main constraints being availability and cost. Examples include grain, bean commodity products, cottonseed flour, corn steep liquor, peanut meal, barley malt meal, corn gluten meals, linseed meal, rice meal, wheat meal, meat and fish waste products (Dahod, 1999). The composition of amino acids in complex nitrogen is not an important factor, but the overall level of protein is a particularly important constituent of the media (Miller and Churchill, 1986).

Minerals serve many purposes in fermentation media. These roles include ionic strength balancing agents, precursors for secondary metabolite syntheses, as buffering agents, pH control agents, and reactants that remove specific inhibiting nutrients from the medium (Dahod, 1999).

The primary aim of the experimental work in this chapter was the cultivation of *Colletotrichum gloeosporioides* C6, *Trichoderma harzianum* kmd and *Gliocladium virens* MM1 on pine shavings supplemented with molasses to obtain maximum spore yield. Various sterilization methods were evaluated and total organic carbon concentrations and C:N ratios tested. The growth of mycoherbicide *C. gleoesporioides* C6 on a gel medium was also investigated. Total organic carbon and C:N ratios were the parameters tested in order to obtain maximum spore yield.

2.2 MATERIALS AND METHODS

PART A

Evaluation of pine shavings and molasses as a support matrix for the growth of *C. gloeosporioides* C6, *T. harzianum* kmd and *G. virens* MM1.

Cultures

Stock cultures of *Colletotrichum gloeosporioides* (Penz and Sacc.) C6, *Trichoderma harzianum* (Rafai) kmd and *Gliocladium virens* (Miller, Giddens and Foster) MM1 were obtained from Plant Health Products[1]. Cultures were stored on potato dextrose agar (PDA) slants or on wheat at 4EC, under mineral oil. Agar plugs (± 1 cm^2) of stock culture were transferred to PDA and V-8 plates and incubated for 7-10 days at 20EC and used as inoculum for pine shaving and molasses experiments for all fungal isolates.

Media Composition

Pine shavings and molasses were tested as potential growth substrates. Pine shavings placed in Petri dishes were supplemented with a commercially available molasses/ protein feed source (Voermolas[2]). Subsequent to sterilization media were then inoculated with a plug of fungi; and incubated at 20EC for 7 days.

Sterilization

Several methods of bulk media sterilization were evaluated. These include autoclaving at 121°C for various time intervals; tyndillization (autoclaving at 121°C for 30 minutes and then again 16 hours after first autoclave treatment); steaming for 1, 2 and 4 hours, and pressure cooking for 0.5, 1 and 2 hours, respectively.

Several treatments were also evaluated to assess the efficacy of sterilizing pine shavings as well as removing resin from the substrate. Three treatments were employed, namely: autoclaving at 121°C for 15, 30, 40, 50, and 60 minutes; steaming for 30, 60, and 120

[1] Plant Health Products, PO Box 207, Nottingham Road, 3280, KZN, South Africa.

minutes; and soaking in 70% (v/v) ethanol for 10, 15, 20, 30, 40, 50, 60, and 120 minutes, respectively.

Further to the sterilization step, pine shavings were then boiled for 30 minutes in 250 ml molasses, placed in a Petri dish and then inoculated with a plug of fungal culture and incubated at 20°C for 7 days. Untreated pine shavings were used as negative controls.

A contamination problem was experienced in the initial experimental procedure. To determine if pine shavings or molasses were the source of the problem, the following treatments were performed: pine shavings were sterilized with 70 % (v/v) EtOH for 30 minutes; sterilized pine shavings and untreated pine shavings were added to sterile nutrient broth. To investigate if molasses was the source of contamination, the following experiments were conducted: pine shavings were treated with 70 % (v/v) EtOH and added to molasses; treated pine shavings were added to autoclaved molasses; treated pine shavings were added to molasses and boiled for 30 minutes, and molasses without pine shavings.

pH Optimization

pH ranges of 4 - 7.5 were obtained by the addition of 1N NaOH or 1N HCl to pine shavings and molasses media after the boiling stage.

Total Organic Carbon Analysis

Media was made up to evaluate the effect of total organic carbon (TOC) concentration on biomass and spore production. The Walkey-Black oxidation procedure (Walkey, 1947), (Appendix 2.1) was used to determine the initial TOC of the molasses. The TOC of the concentrated sample was found to be 284.03 g L^{-1} (Appendix 2.2). The desired TOCs for pine shavings and molasses media were obtained by making up a range of water:molasses dilutions (Appendix 2.3). The TOCs tested were 5 g L^{-1}, 13 g L^{-1}, 15 g L^{-1}, 16 g L^{-1}, 17 g L^{-1} and 21 g L^{-1}.

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[2] Voermolas, Tongaat Foods (Pty) Ltd, P.O. Box 13, Maidstone, 4380, KZN, South Africa.

C:N Ratios

The C:N ratio of the Voermolas molasses formulation was determined using the Walkey-Black oxidation procedure. A detailed composition of the Voermolas according to the supplier is given in Appendix 2.4. The C:N ratio was determined to be 31:1 (Appendix 2.2). A range of C:N ratios from 5:1 (Appendix 2.5) to 31:1 were then made up by the adding urea to the medium (Appendix 2.6) and then tested. Laboratory analysis of the cane molasses was done by Cedara Fertilizer Advisory Services[3]. Total nitrogen was determined by the Kjeldahl (TKN) method (Farina, 1981; Perstorp, 1993; Matejovis, 1996). The C:N ratio was found to be 49.03 g L^{-1} (Appendix 2.7). However, Voermolas (C:N ratio 31:1) was the substrate of choice in experiments because of its availability and low cost.

Evaluation

Effectiveness of media sterilization and resin removal were assessed by rating fungal growth on agar plates. Fungal growth was rated visually on a scale from 1 to 5. The rating scale used was as follows: 5 = dense mycelial mat covering entire plate; 4 = dense mycelial covering ¾ of plate; 3 = dense mycelial mat covering ½ of plate; 2 = sparse mycelial mat covering ½ - ¾ of plate; 1 = sparse mycelial mat covering less than ½ of plate; 0 = no growth. The optimum C:N and TOC were determined by spore count using a heamocytometer. Dissected samples of colonized pine shaving were viewed under a light microscope to assess chlamydospore formation of *T. harzianum* kmd. Environmental Scanning Electron Microscope (ESEM) was also undertaken to assess growth of fungal isolates being evaluated.

PART B

Evaluation of semi solid gel formulation for the cultivation of *C. gloeosporioides* C6

[3] Cedara Agricultural Development Institute, Private Bag X9059, Pietermaritzburg, 3200, South Africa

Culture

For the inoculation of gel media, plugs of *C. gloeosporioides* C6 were grown in 50 ml of Modified Richard's liquid medium (Yu *et al.*, 1998) in 250ml Erlenmeyer flasks. The medium comprised sucrose, 10 g L^{-1}; KNO_3, 10 g L^{-1}; KH_2PO_4, 5 g L^{-1}; $MgSO_4.7H_2O$, 2.5 g L^{-1}; $FeCl_3.6H_2O$, 0.02 g L^{-1}; V-8 juice, 150 ml L^{-1} and was made to a volume of 1 liter with distilled water in 250 ml Erlenmeyer flasks. Cultures were incubated for 5 days on a rotary shaker at 150 rpm at room temperature. An atomizer was used to spray the spores (1x 10^5 ml) obtained from these cultures onto the gel.

Media Composition

A novel gel medium was formulated to support growth of *C. gloeosporioides* C6. The basic gel medium was composed of cornflour[4], 74.8 g L^{-1}; soya bean flour[4], 15 g L^{-1} and glucose[5], 30 g L^{-1}. A basal salts medium used to supplement the gel contained KH_2PO_4, 5.0 g L^{-1}; $MgSO_4.7H_2O$, 0.5 g L^{-1}; $CaCl_2$, 0.5 g L^{-1}; $FeCl_3.6H_2O$, 0.05 g L^{-1}; and $CuSO_4$, 0.05 g L^{-1}.

Amendments were made to the gel medium to evaluate growth and spore production of the test fungal isolates. These included: basic gel plus basal salts; basic gel with 5 g L^{-1}, 10 g L^{-1} and 15 g L^{-1} soya protein; basic gel with 5 g L^{-1}, 10 g L^{-1} and 15 g L^{-1} soya protein plus basal salts; basic gel with 2 g L^{-1}, 5 g L^{-1}, 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} commercial glucose; basic gel with 2g L^{-1}, 5 g L^{-1}, 10 g L^{-1}, 20 g L^{-1}, and 30 g L^{-1} commercial glucose plus basal salts.

Sterilization

Each gel medium was sterilized by autoclaving at 121°C for 15 minutes.

Scoring Of Mycelium

An investigation to observe the effect of physically damaging the mycelial mat on spore formation was conducted. A scalpel blade was used to score the surface of mycelial mats

[4] Attwell's Wholesome Foods, P.O.Box 2313, Hillcrest, 3650

[5]. Alpha Glucose Powder, Allied Drug Co. (Pty) Ltd, 27 Hannah Road, Durban, 4001, South Africa

that had formed on the gel media. Differential interference contrast (DIC) was used to assess the effect of mycelial damage on spore morphology using the Axiophot light microscope (Zeiss, Germany).

pH Determination

The pH of the gel was determined to be pH 6.5.

Total Organic Carbon Analysis

The Walkey-Black oxidation procedure was used to determine the total organic carbon of the constituents of the corn and soy bean flour used in the gel formulation (Appendix 2.8). The TOC of the medium was determined to be 52.92 g L^{-1}(Appendix 2.9).

C:N Ratios

To obtain C:N ratios of 5:1; 10:1; 20:1; 30:1 and 40:1, the gel formulation was amended with 44.53g; 20.72g; 8.34 g ; 4g and 2.15g $(NH_4)_2SO_4$, respectively (Appendix 2.9). Nitrogen analysis was done by the Department of Animal and Poultry Science[6], at the University of Natal, on soy bean flour (35.35 % w/w) and cornflour (0.001% w/w) using the Dumas (1831) combustion method (LECO FP 2000).

Evaluation

Growth of fungi on gel media was evaluated using ESEM. Spore morphology of spores from different treatments were viewed using an the Axiophot light microscope (Ziess, West Germany). Effect of physically damaging the mycelial mat and varying carbon concentration on the unstained spores was assessed using differential interference contrast (DIC) microscopy (Axiophot, Zeiss, West Germany).

Statistical Analysis

A general linear model was used to run Analysis of Variance (ANOVA) on the results for both Part A and Part B using Statistical Analysis System (SAS, 1987).

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[6] Department of Animal and Poultry Science, University of Natal, Private Bag x 01, Scottsville, 3209, KZN, South Africa.

2.3 RESULTS

PART A

A 45 and 60 minute autoclave period at 121°C produced prolific fungal growth for all fungal strains tested on the inoculated pine shaving medium, and a 30 minute treatment was sufficient for the growth of *C. gloeosporioides* C6 (Fig. 2.1). Steam pretreatment for 60 and 120 minutes produced similar results, however, 30 minutes was sufficient for *C. gloeosporioides* C6 (Fig. 2.2). Contamination was minimal for both treatments. Where pine shavings were immersed in ethanol (70% v/v), a soaking period of 20 minutes was the shortest treatment that produced sufficient growth of all three fungi (Fig. 2.3). However, a 30 minute treatment was the most effective sterilization procedure, eradicating bacterial and fungal contamination. Inoculated shavings which had not been pretreated did not support fungal growth and high levels of bacterial contamination were apparent. Contamination was absent in experiments were pine shavings were treated and molasses autoclaved. The highest level of bacterial contamination was found in untreated molasses.

Optimal pH for *T. harzianum* kmd and *C. gloeosporioides* C6 ranged from 6.5-7, whereas, G. *virens* MM1grew best at pH 6 (Fig. 2.4).

For the range of C:N ratios evaluated it was found that there were no significant difference in spore yields of *T. harzianum* kmd from C:N ratios 5:1–15:1 (Fig. 2.5 and Table 2.1). Highest spore yield was obtained at a C:N ratio of 30:1. No significant differences in spore yield for *G. virens* MM1 and *C. gloeosporioides* C6 were observed between C:N ratios 5:1-15:1. Highest spore yields for *G. virens* MM1 were obtained between C:N ratios of 20:1–30:1. Highest spore yield for *C. gloeosporioides* C6 was obtained at a C:N ratio of 30:1 (Table 2.1).

Optimum total organic concentrations for the maximum spore yields of all three fungi ranged between 16 – 17 g L^{-1} (Fig. 2.6 and Table 2.2). Spore yields of all three fungi

drastically decreased when the TOC exceeded 17 g L^{-1}. There were no significant differences in spore yields of all three fungi from 5–15 g L^{-1} (Fig. 2.6 and Table. 2.2).

An example of the rating scale used in experiments testing pH is shown in Fig. 2.7. Comparisons of *C. gloeosporioides* C6 inoculated onto a plate of the molasses and pine shavings media with an uninoculated control are shown in Fig. 2.8. Inoculated plates that were considered to support optimal growth of *G. virens* MM1 and *T. harzianum* kmd are shown in Fig. 2.9 and Fig. 2.10, respectively.

ESEM micrographs of *T. harzianum* kmd, *G. virens* MM1 and *C. gloeosporioides* C6 grown on molasses and pine shavings medium are shown in Fig. 2.11 A-D.

PART B

Mycelial growth and conidia formation of *C. gloeosporioides* C6 were compared when grown on a plain gel medium to that supplemented with basal salts (Fig. 2.12 – Fig. 2.13). Gel supplemented with basal salts produced more conidia and a denser mycelial mat.

The effects of scoring mycelial mats of *C. gloeosporioides* C6 on spore formation are shown in Fig. 14 A-D. Conidia produced from undamaged mycelial mats grown on a plain gel medium and basal salts supplemented gel are shown in Fig. 14 A– B. Conidia produced on the undamaged mycelial mat grown on a plain gel produced more lipid droplets (L) than undamaged spores cultivated on gel supplemented with basal salts (Fig. 2.14 B). Large lipid droplets (L) were observed in spores produced on the scored mycelial mats produced on unamended gel medium (Fig. 2.14 C). The damaged mycelial mat of *C. gloeosporioides* C6 grown in the supplemented gel gave rise to spores which contained numerous smaller lipid droplets (L) (Fig. 2.14 D).

Spore counts for *C. gloeosporioides* C6 were not obtained since the mycelial mass entwined its hyphae into the gel medium. ESEM was therefore used to assess the best

C:N ratio and what effect basal salts had on fungal growth of the fungus. At a C:N ratio of 5:1 few condia and a sparse hyphal network were observed (Fig. 2.15). The addition of basal salts into the gel medium resulted in a denser mycelial mass with a larger number of conidia (C) on thicker condiophores (Cp) (Fig. 2.16). Conidia collapsed as the sample was not critical point dried.

A C:N ratio of 40:1 in an unamended gel medium gave rise to a dense hyphal mass but with fewer spores (Fig. 2.17). The gel medium amended with basal salts produced a denser mycelial mass than the unamended gel media at lower C:N ratios (Fig. 2.18). Visible are conidia (C) on conidiophores (Cp). More conidia were observed at the lower C:N ratio (5:1) on the gel amended with basal salts.

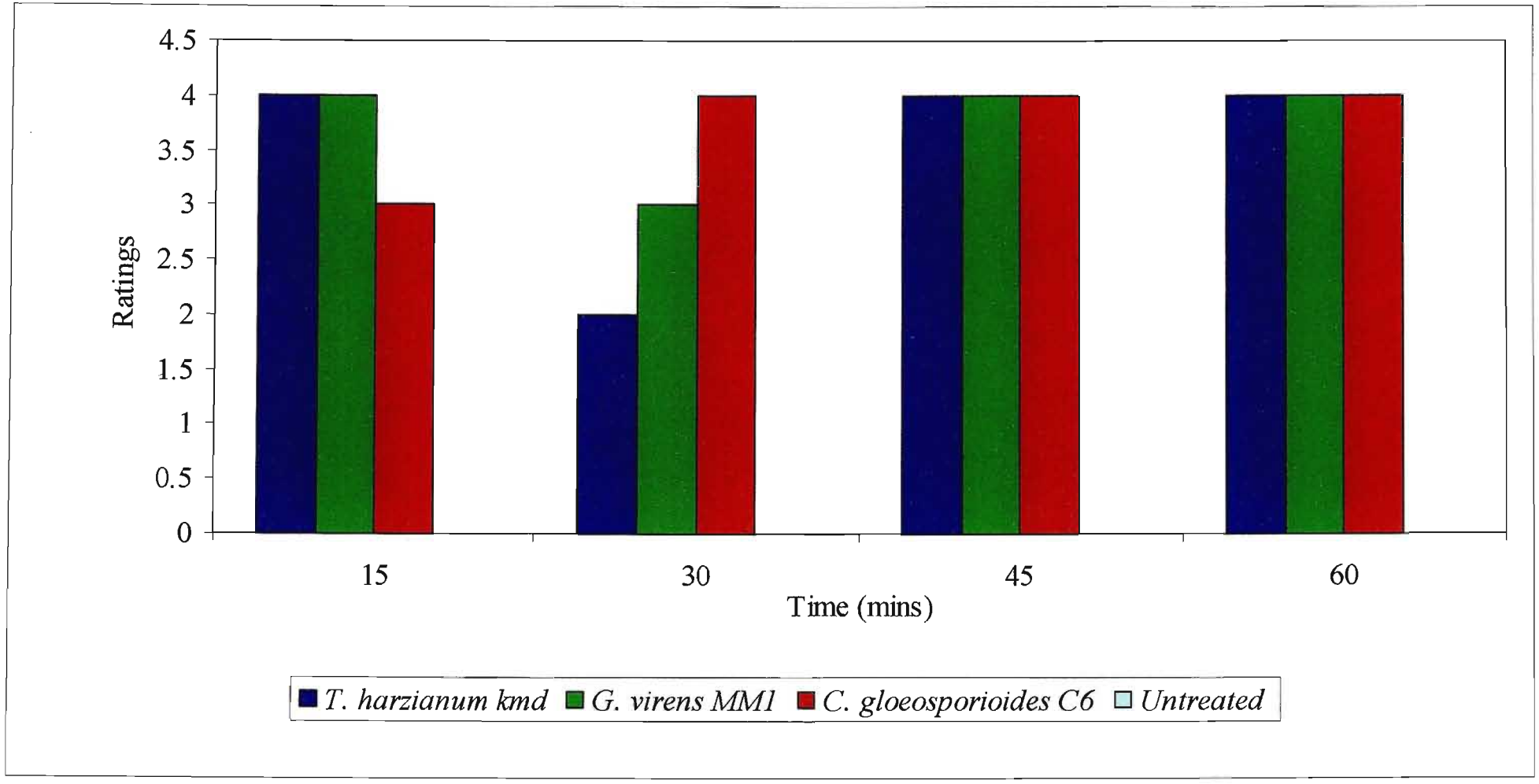


Fig. 2.1. Growth ratings of fungi cultivated on molasses and pine shavings media autoclaved at 121°C over a range of time intervals. There were three reps per treatment and these were visually evaluated. The results between the reps were constant.

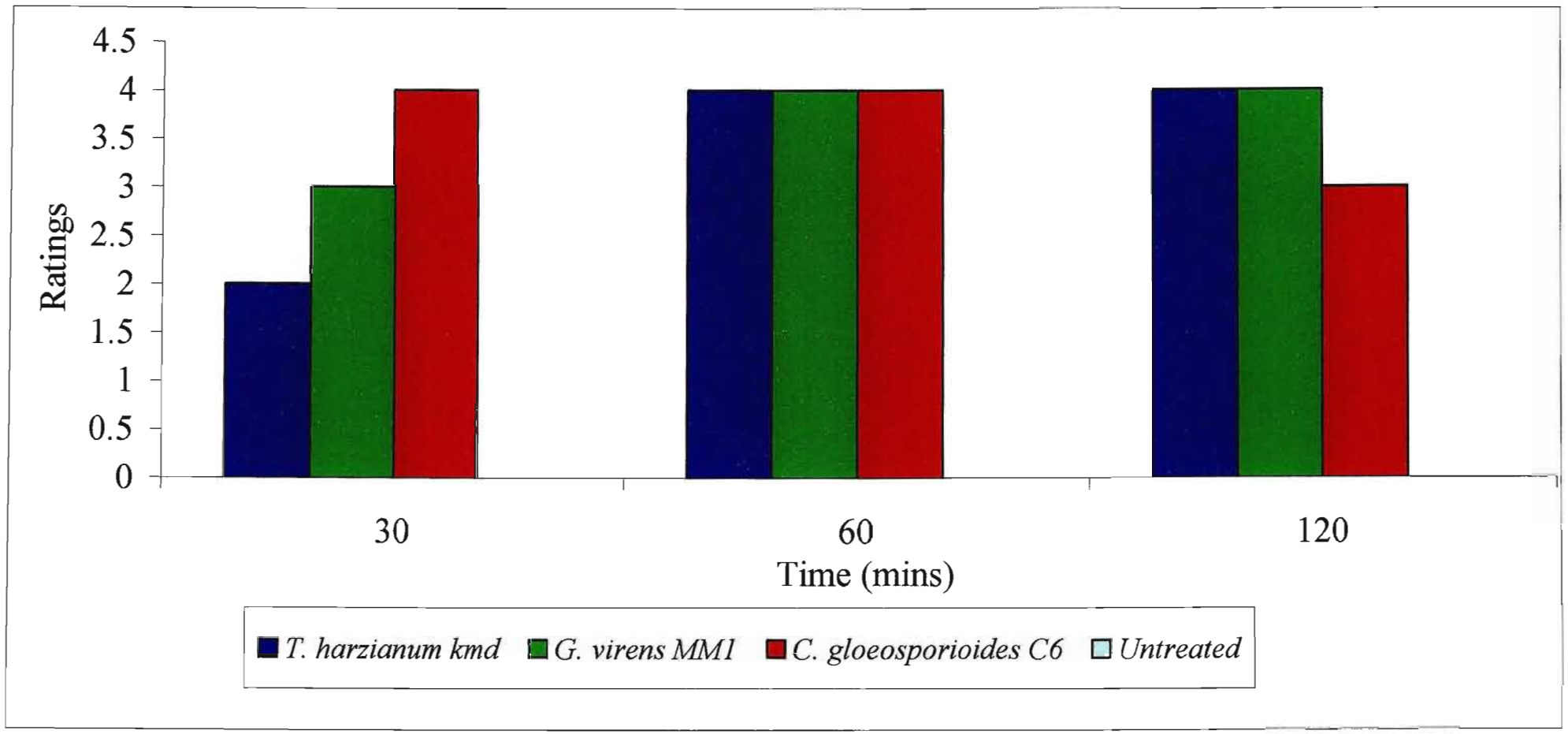


Fig. 2.2. Growth ratings of fungi cultivated on molasses and pine shavings media autoclaved at 121°C over a range of time intervals. There were three reps per treatment and these were visually evaluated. The results between the three reps were constant.

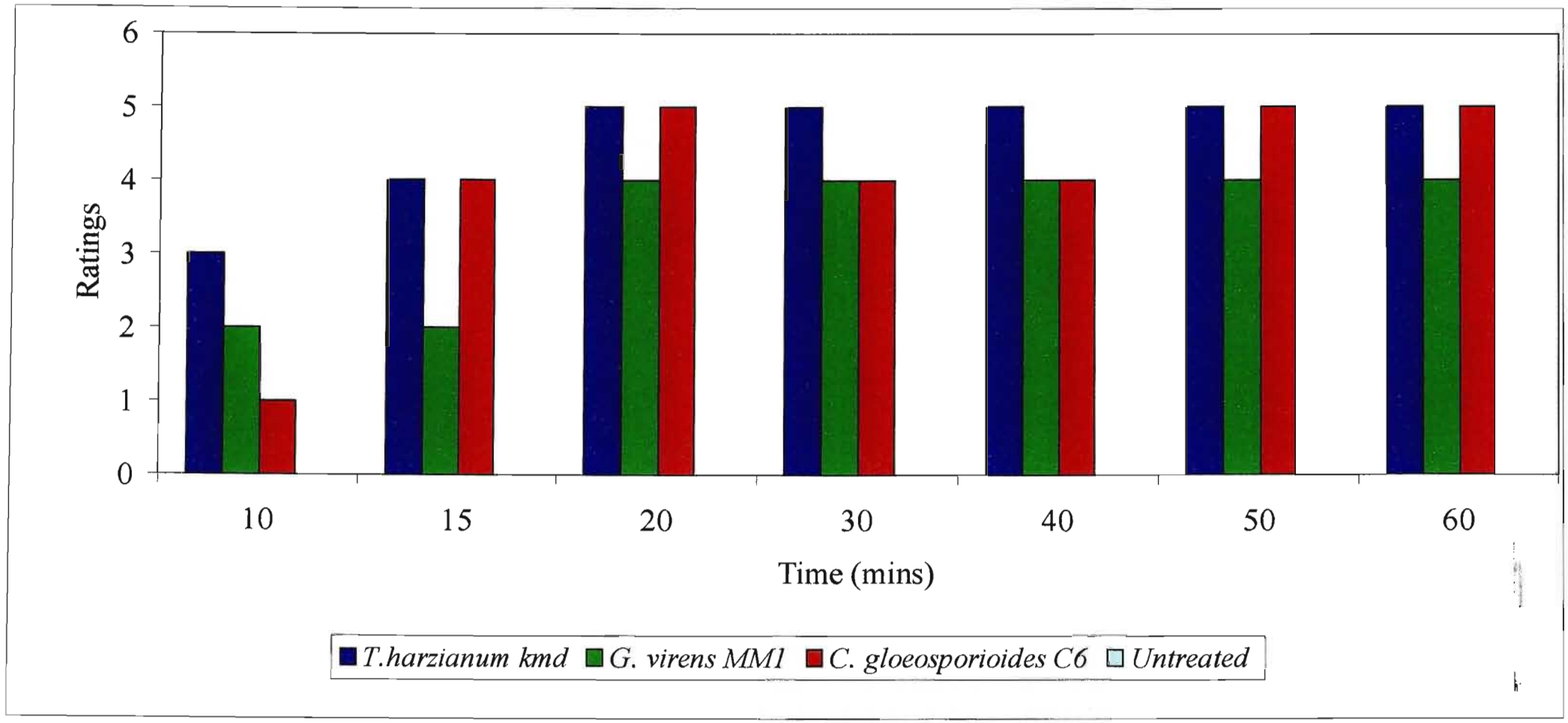


Fig. 2.3. Growth ratings of fungi cultivated on pine shavings treated with 70% (v/v) ethanol and supplemented with molasses. There were three reps per treatment and these were visually evaluated. The results between the three reps were constant.

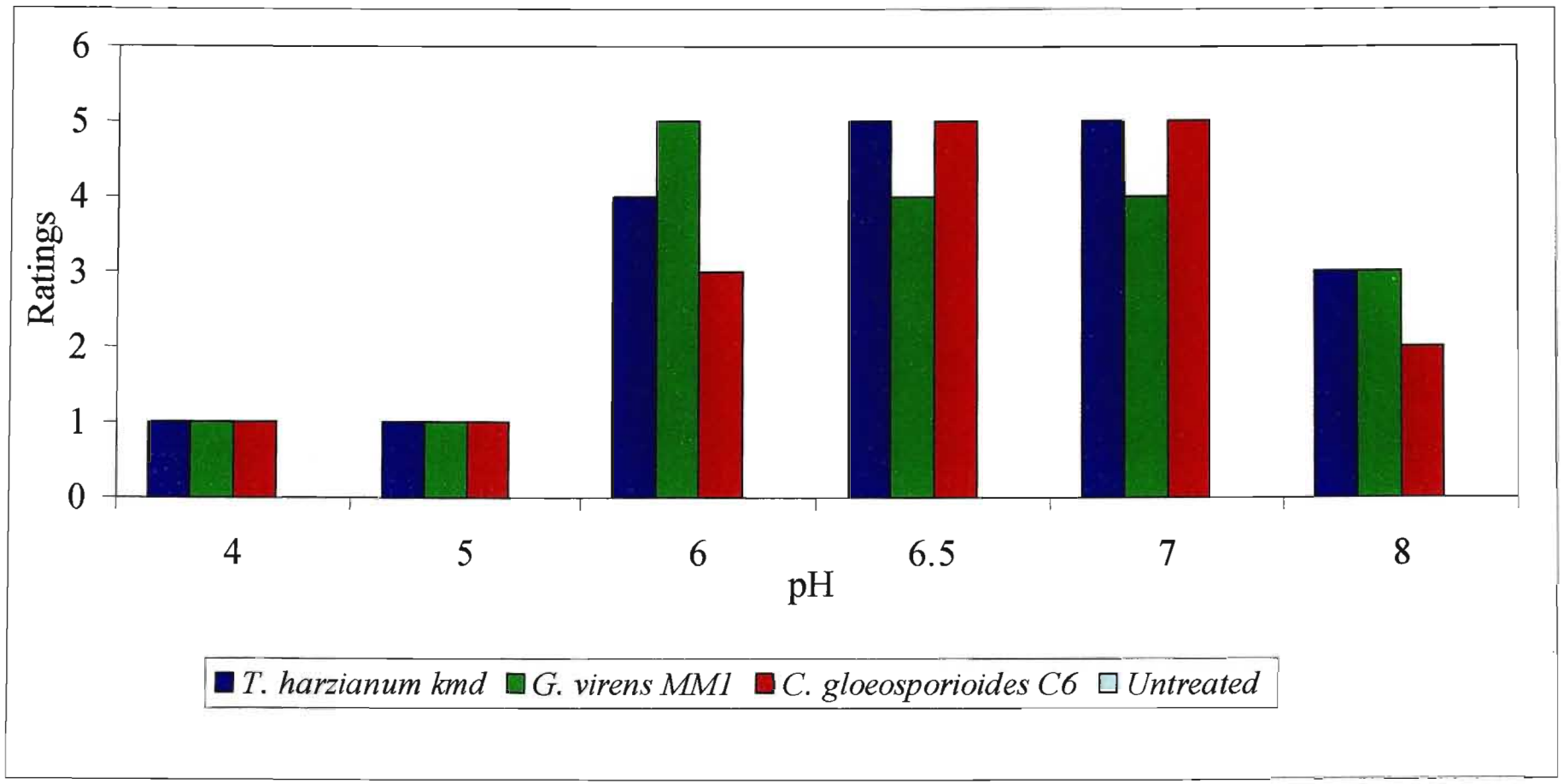


Fig. 2.4. Growth ratings of fungi cultivated on sterile pine shavings supplemented with molasses at various pH units. There were three reps per treatment and these were visually evaluated. The results between the three reps were constant.

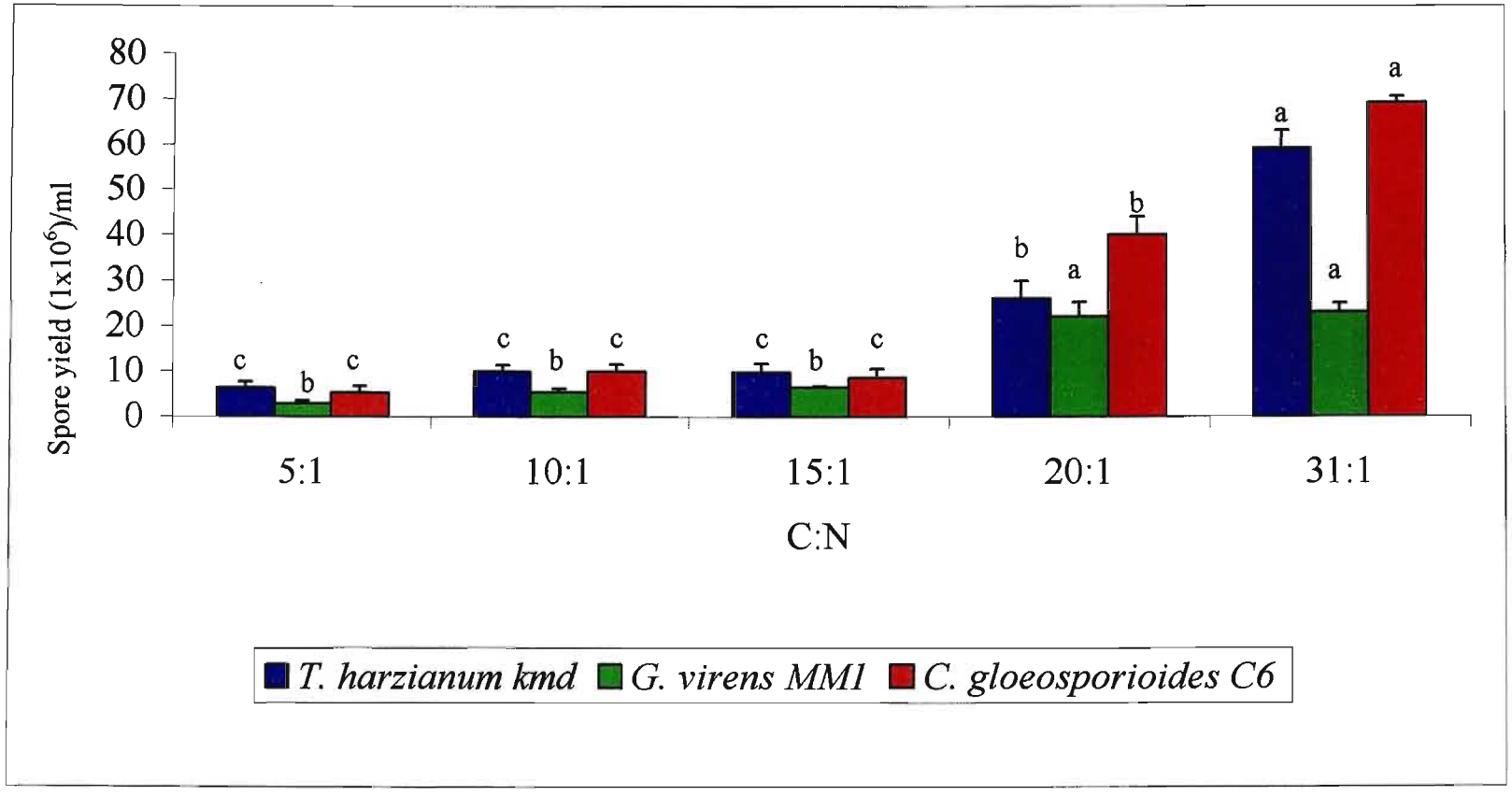


Fig. 2.5. Spore yields of fungi cultivated on pine shavings supplemented with molasses at various carbon-to-nitrogen ratios. Each C:N ratio treatment with a different letter represents a significantly different mean spore concentration for each fungi ($P < 0.05$).

Table 2.1. Mean spore yield (SY) of *Trichoderma harzianum* kmd, *Gliocladium virens* MM1 and *Colletotrichum gloeosporioides* C6, respectively, at various carbon-to-nitrogen ratios

C:N ratio	Mean SY (10^6/ml) of *T. harzianum* kmd	R	Mean SY (10^6/ml) of *G. virens* MM1	R	Mean SY (10^6/ml) of *C. gloeosporioides* C6	R
5:1	6.32c	3	2.90b	2	5.34c	3
10:1	9.94c	3	5.38b	2	9.87c	3
15:1	9.82c	3	6.46b	2	8.63c	3
20:1	26.00b	2	22.00a	1	40.00b	2
31:1	59.00a	1	23.00a	1	69.00a	1
Effects	**P-level**		**P-level**		**P-level**	
C:N	<.0001 * * *		<.0001 * * *		<.0001 * * *	
	C.V.		**C.V.**		**C.V.**	
	21.42		24.49		15.27	
	MSE		**MSE**		**MSE**	
	4.76		2.93		4.06	

1. **Significance** — **Description**
   - * * * — Very Highly significant
   - * * — Highly significant
   - * — Significant
2. Means with the same letter are not significantly different ($P < 0.05$) according to Student, Newman and Keuls comparison test.
3. R = ranking
4. C:N = carbon-to-nitrogen

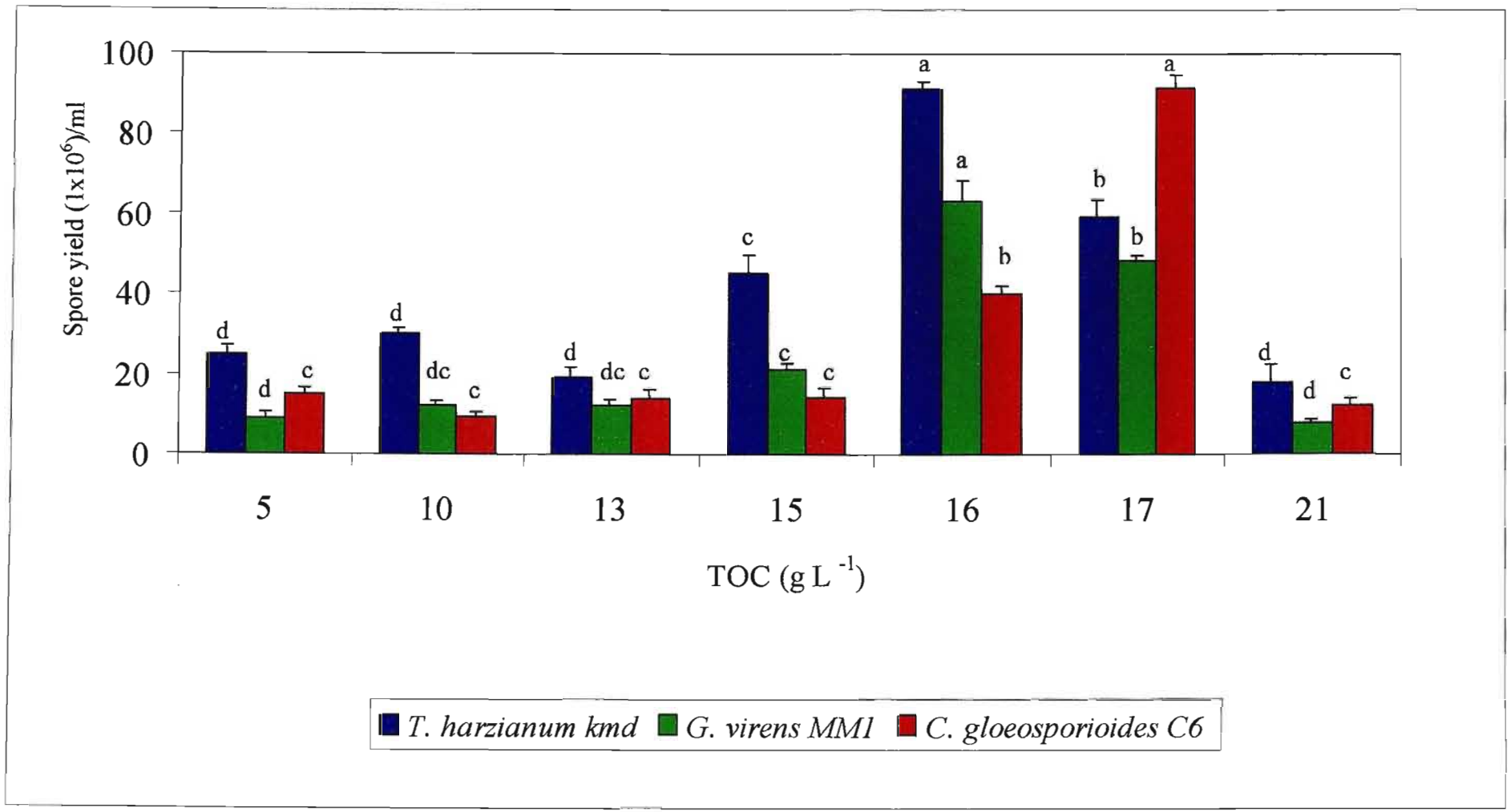


Fig. 2.6. Spore yields of fungi cultivated on pine shavings supplemented with molasses at various total organic carbon concentrations. Each TOC treatment with a different letter represents a significantly different mean spore concentration for each fungi ($P < 0.05$).

Table 2.2. Mean spore yield (SY) of *Trichoderma harzianum* kmd, *Gliocladium virens* MM1 and *Colletotrichum gloeosporioides* C6, respectively, at various total organic carbon concentrations

TOC	Mean SY (10^6/ml) of *T. harzianum* kmd	R	Mean SY (10^6/ml) of *G. virens* MM1	R	Mean SY (10^6/ml) of *C. gloeosporioides* C6	R
5 g L^{-1}	24.50d	4	9.00d	4	15.00c	3
10 g L^{-1}	30.00d	4	12.00dc	3	9.50c	3
13 g L^{-1}	19.00d	4	12.00dc	3	13.85c	3
15 g L^{-1}	45.00c	3	21.00c	3	14.00c	3
16 g L^{-1}	91.00a	1	63.00a	1	40.00b	2
17 g L^{-1}	59.00b	2	48.00b	2	91.00a	1
21 g L-^{1}	18.00d	4	7.74d	4	12.00c	3
Effects	**P-level**		**P-level**		**P-level**	
TOC	<.0001 * * *		<.0001 * * *		<.0001 * * *	
	C.V.		**C.V.**		**C.V.**	
	13.21		17.29		14.5	
	MSE		**MSE**		**MSE**	
	5.4		4.26		4.04	

1. **Significance** — **Description**

* * *	Very Highly significant
* *	Highly significant
*	Significant

2. Means with the same letter are not significantly different ($P < 0.05$) according to Student, Newman and Keuls comparison test.
3. R = ranking
4. TOC = total organic carbon

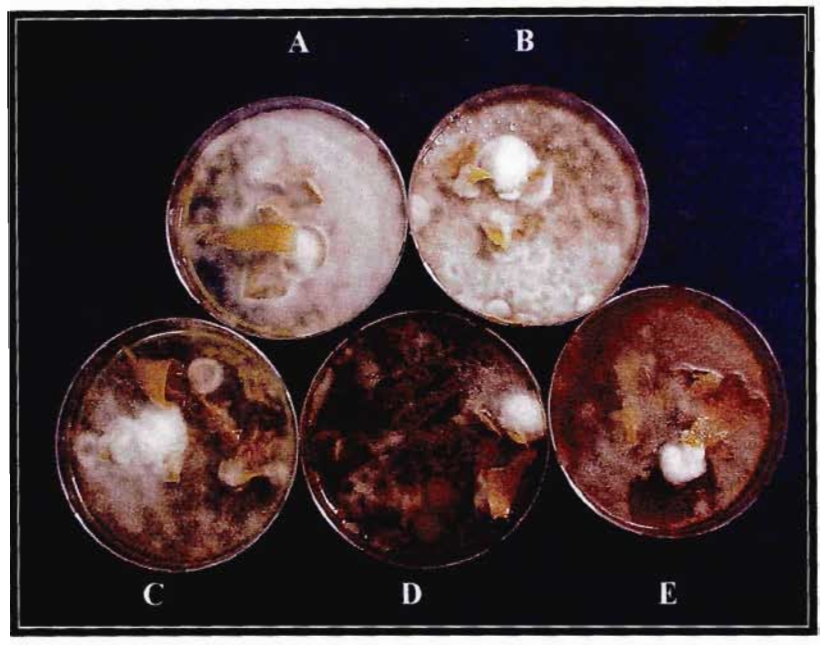


Fig. 2.7. Growth of ` *Colletotrichum gloeosporioides* C6 at TOC of 17 g L^{-1} and C:N ratio of 31:1. Plate A has a pH of 7; B, pH 6.5.; C, pH 6; D, pH 5 and E, pH 4. The visual rating scale used in all experiments are as follows: A = 5; B = 5; C = 4 ; D = 1 and E = 1.

Fig. 2.8. Comparison between control plate (molasses and pine shavings) and inoculated plate of *Colletotrichum gloeosporioides* C6 grown in media at pH 6.5 with a TOC of 17 g L^{-1} and a C:N ratio of 31:1. The visual ratings for the control = 0 and plate A = 5.

Fig. 2.9. *Gliocladium virens* strain MM1 grown on Voermolas amended pine shavings after four days. The medium was adjusted to pH 6.5 with 1N NaOH. The TOC of the medium was 16.08 g L^{-1} carbon. The C:N ratio of the unamended Voermolas was 31:1.

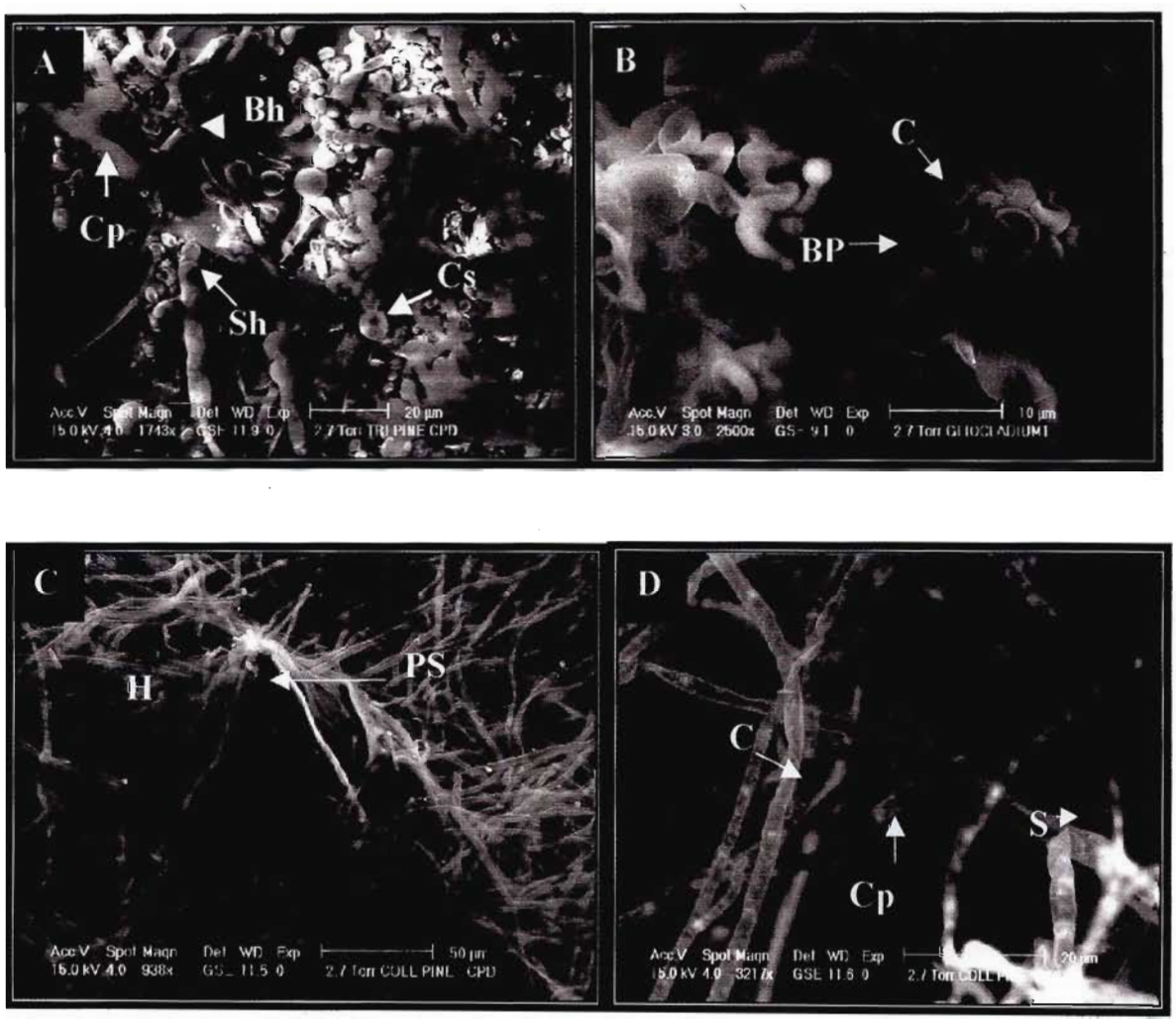
Fig. 2.10. *Trichoderma harzianum* strain kmd grown on Voermolas amended pine shavings after four days. The medium was adjusted to pH 6.5 by the addition of 1N NaOH. A TOC of 16.08 g L^{-1} carbon was used. The C:N of Voermolas was 31:1.

Fig. 2.11 A. ESEM micrograph of a critical point dried sample of *Trichoderma harzianum* kmd grown on Voermolas and pine shavings. Branched hyphae (Bh) and septate hyphae (Sh) are visible. Visible are large hyaline, ovoid chlamydospores (Cs). Chlamydospores are situated on the terminal ends condiophores (Cp).

B. ESEM micrograph of *Gliocladium virens* MM1 grown on Voermolas and pine shavings. Conidia (C) of *Gliocladium virens* MM1 are gathered at tips of branched phialides (BP) in a tight, ball-shaped cluster. Condia appear to have collapsed as a result of the sample being untreated before viewing.

C. ESEM micrograph of *Colletotrichum gloeosporioides* C6 grown on Voermolas and pine shavings (PS). Visible is a protruding piece of pine shaving covered by mass of septate hyaline hyphae (H).

D. ESEM micrograph of *Colletotrichum gloeosporioides* C6 illustrating single celled, ovoid and oblong conidia (C). Conidia are attached to simple septate (S) condiophores (Cp).


A
Bh
Cp
Cs
Sh
B
C
BP
C
PS
H
D
C
S
Cp

PART B

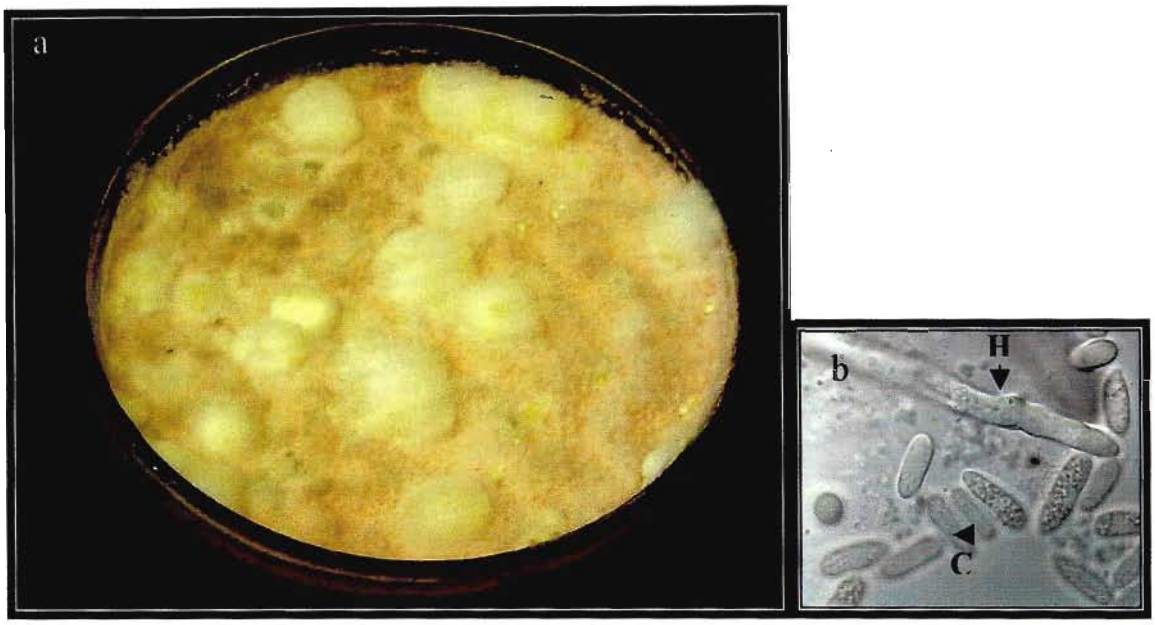


Fig. 2.12a. *Colletotrichum gloeosporioides* C6 grown on a plain gel medium. b. Morphology of *C. gloeosporioides* C6 observed with bright field microscope (Mag. X100). b. Conidia (C) and hyphae (H) (Mag. X100)

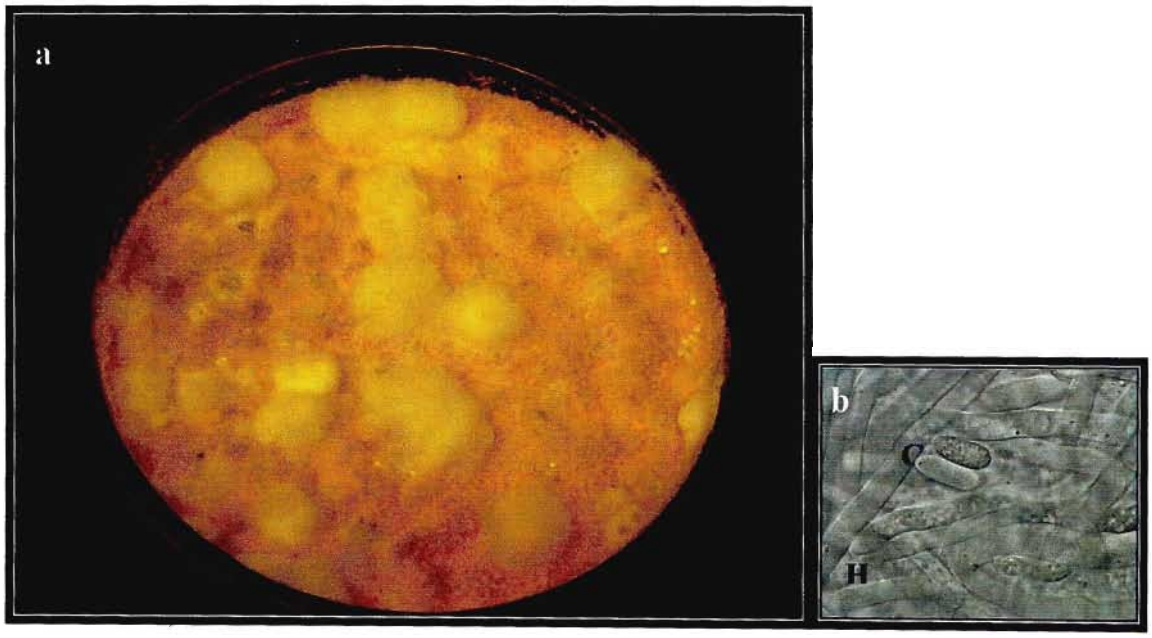


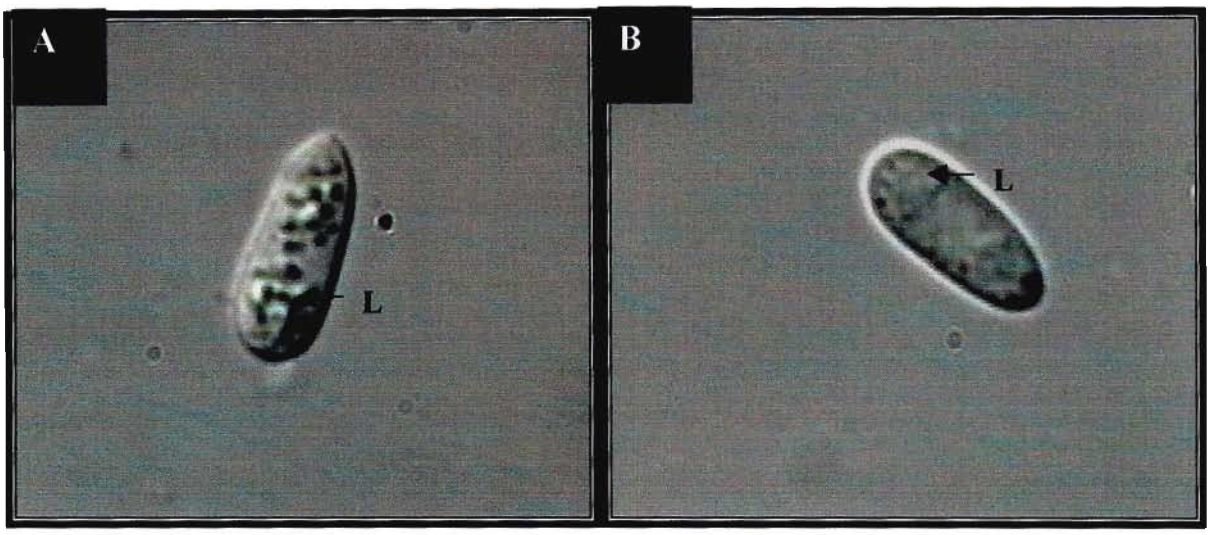
Fig. 2.13a. *Colletotrichum gloeosporioides* C6 grown on a gel medium supplemented with basal salts. b. Conidia (C) and a mass of hyphae (H) are visible using bright field microscopy (Mag X100)

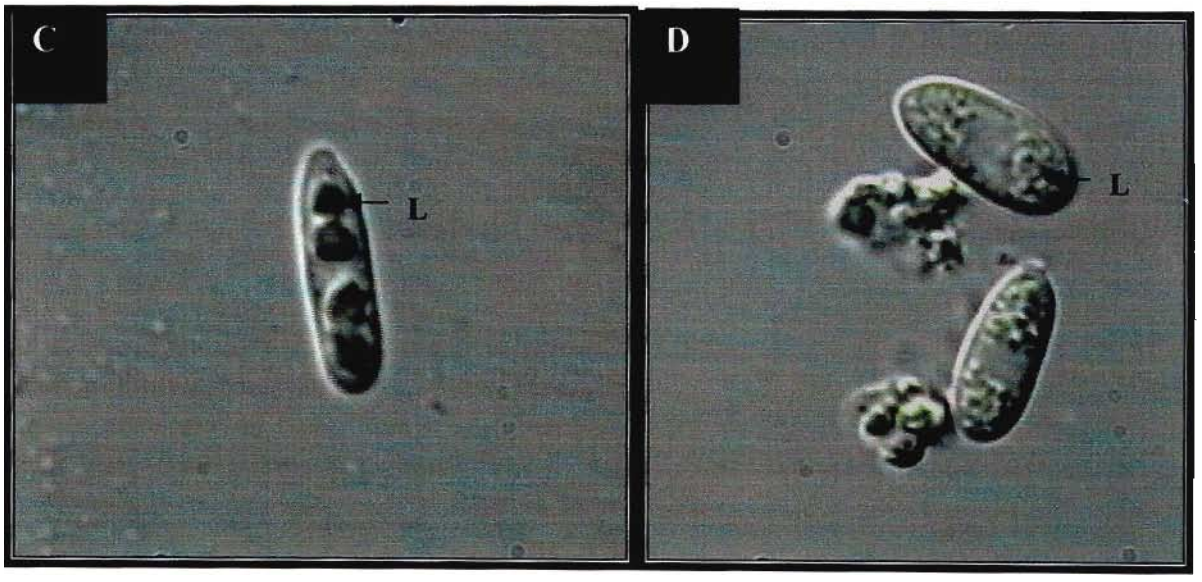
Fig. 2. 14 A. *Colletotrichum gloeosporioides* C6 spores viewed with a bright field microscope exhibiting lipid droplets (L) (Ker-Chung Kuo, 1999) (Mag. X100). Spores were grown on unamended gel medium. The mycelial mat was undamaged.

B. Bright field microscopy view of spores of *Colletotrichum gloeosporioides* C6 grown on gel media supplemented with basal salts (Mag. X100). No prominent lipid droplets were observed. The mycelial mat was undamaged.

C. Bright field microscopy of *Colletotrichum gloeosporioides* C6 spores grown on unamended gel (Mag. X100). The mycelial mat of the fungus was damaged using a scalpel blade. Large lipid bodies (L) inclusions were present in the spores.

D. Bright field microscopy of *Colletotrichum gloeosporioides* C6 spores (Mag. X100) grown on scored mycelial gel medium supplemented with basal salts. Small lipids droplets were observed (L).


A
L
B
L


C
L
D
L

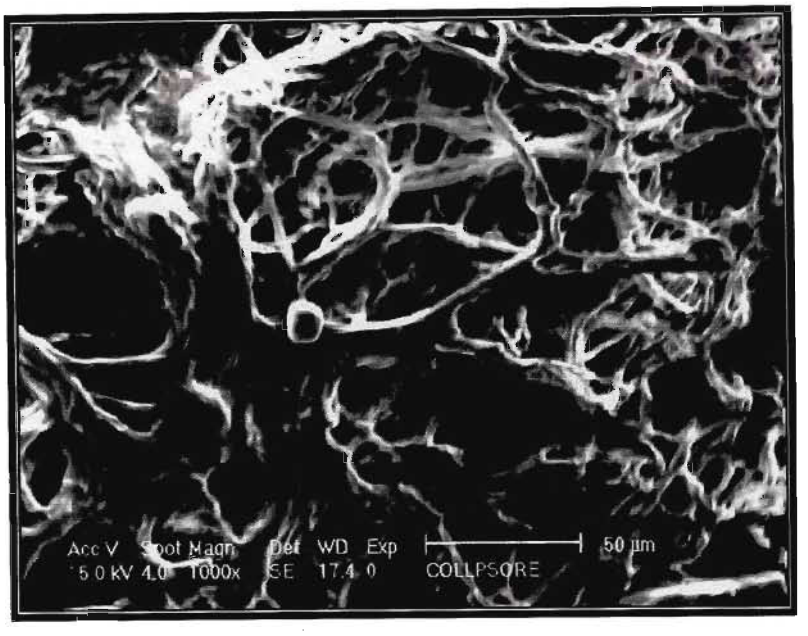


Fig. 2.15. ESEM plate of *Colletotrichum gloeosporioides* C6 grown on plain gel medium at a C:N ratio of 5:1. Visible is a dense hyphal mass that has integrated into the gel. Few spores visible in the mycelial mass.

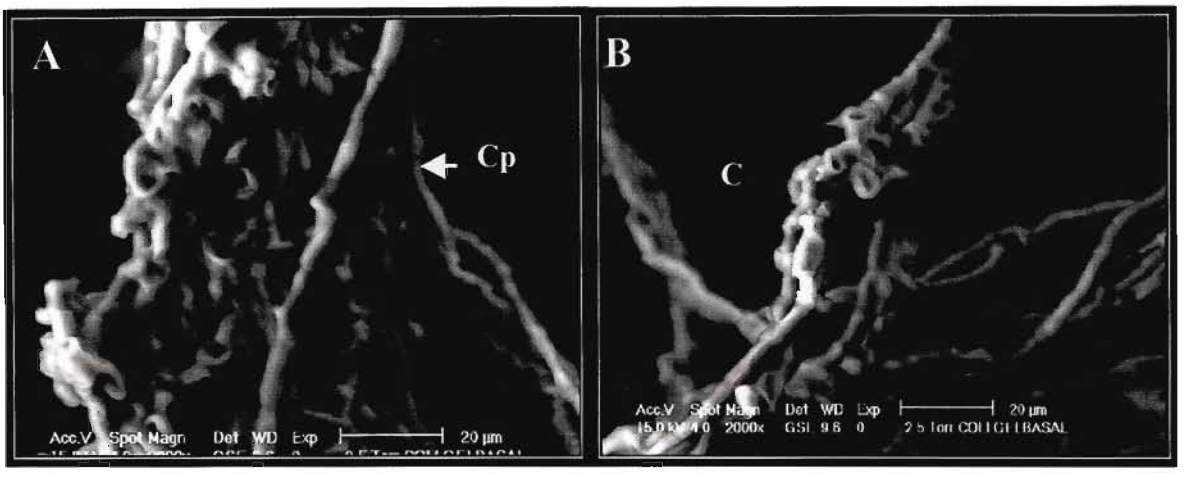


Fig. 2.16 A and B. ESEM of *Colletotrichum gloeosporioides* C6 spores produced on a gel medium amended with basal salts. Conidia (C) produced on condiophores (Cp) are visible.

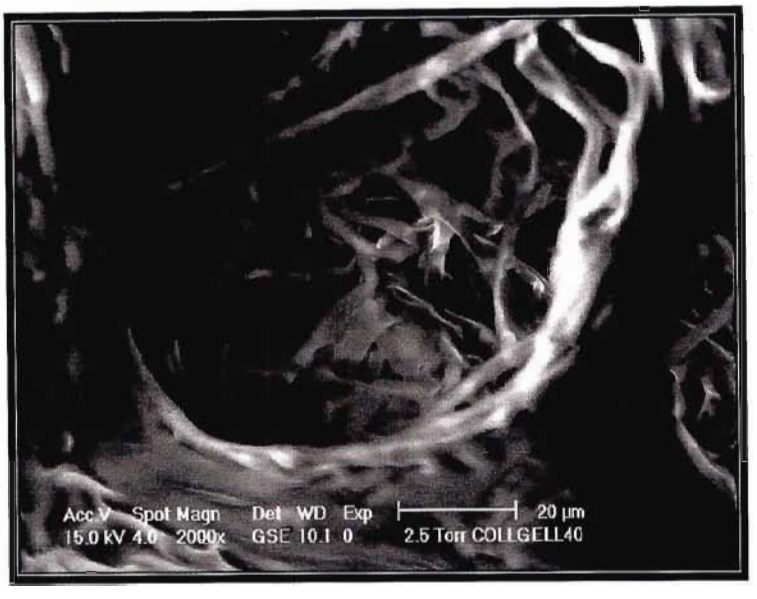


Fig. 2.17. ESEM micrograph illustrating hyphal mass of *Colletotrichum gloeosporioides* C6 when grown on a plain gel at a C:N ratio of 40:1.

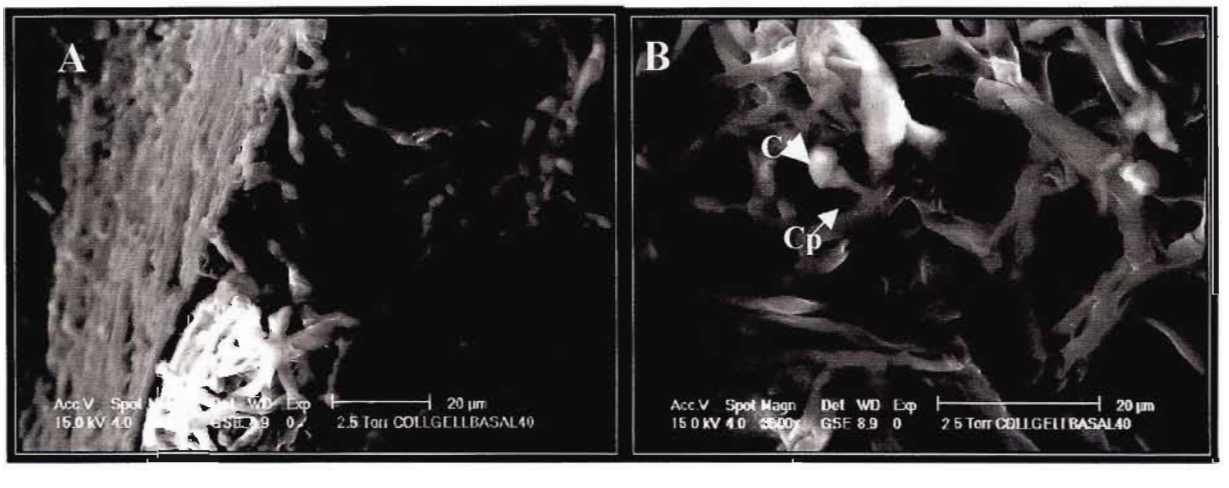


Fig. 2.18 A and B. ESEM micrograph of *Colletotrichum gloeosporioides* C6 grown on gel amended with basal salts. The C:N of the gel was at 40:1. A condium (C) produced on a condiophores (Cp) is visible.

2.4 DISCUSSION

An inert system was chosen as potential growth media over natural substrates, as a major disadvantage of using natural substrates in SSF is that carbon constitutes part of the structure of the material. During microbial growth, the solid medium is degraded, resulting a change in the geometric and physical characteristics of the medium, as well as changes in the C:N ratios and total organic carbon concentration in the medium. For instance, oats shrink due to the degradation of starch and the evaporation of water. Consequently, there is a reduction in mass and heat transfer (Webber *et al.*, 1999). This inherent disadvantage can be overcome by using an inert system such as pine shavings since the physical structure remains largely intact for the duration of the process. Inert systems also facilitate improved control of heat and mass transfer, enhance product recovery, introduce fewer impurities and allow for the reuse of the support material (Larroche and Gros, 1997). For example, the spores of *Penicillium roquefortii*, which are formed inside buckwheat grains can only be harvested by the disruption of the grain. In contrast, spores produced on pozolano particles can be extracted without destruction of the inert material, allowing the particles to be reused (Larroche and Gros, 1997). Another advantage of an inert SSF system is that it allows for a relatively simple scale up procedure as all concentrations of the nutrients in the production media are known. Hence, in the work presented in this dissertation, pine shavings were used as an inert support system in conjunction with molasses as a growth substrate in the semi-solid fermentation of the selected BCAs.

Media sterilization is one of the biggest problems facing the large scale cultivation of BCAs (Churchill, 1982). A 20 minute period was the shortest time for any treatment to both eradicate bacterial and fungal contamination as well as support fungal growth of all three isolates. Ethanol (70% v/v) was chosen as the preferred pretreatment method since it was effective at removing resin and exhibits both bactericidal as well as fungicidal activity and at 70% (w/w) ethanol proteins are more readily denatured in the presence of water than in absolute alcohol (Sykes, 1965). Ethanol is a very effective sterilant due to its capacity to denature proteins (Sykes, 1965).

All methods of sterilization investigated were also found to be effective in the removal of resin, evident by the growth of the three fungal cultures (Fig. 2.1 – Fig. 2.3). The 70% (v/v) ethanol soak was selected as the method that was the most effective at removing resin as well as acting as a sterilant (Fig. 2.3). It was imperative that resin be removed from the pine shavings since it has been shown that pine timber contains resin acids and tannins which exhibit antimicrobial action (Eberhardt and Young, 1994 ; Eberhardt *et al.,* 1994). Studies by Celimene *et al.* (1994) demonstrated that compounds isolated from pine resin exhibited antifungal activity and were capable of inhibiting both white rot and brown rot fungal growth. The results obtained supported this finding where no growth was observed on the untreated pine shavings (Fig. 2.1 – Fig. 2.3).

A variability of growth was found at different time intervals, with each sterilization treatment. Sterilization of the media can be a cause of variability. Autoclaving resulted in failure to kill spores (especially *Bacillus* spores, when present in high numbers), or resulted in inconsistency due to the difference in chemical reactions triggered by the heat in the autoclave (Hilton, 1999). One of the disadvantages of heat treatment to sterilize growth media is the loss of available carbon and the build-up of potentially toxic or inhibitory compounds (Walker, 1999). For example, the excessive heating of molasses may generate undesirable caramelization products, following the Maillard reaction (Walker, 1999). The Maillard reaction occurs between reducing sugars and amino compounds to produce growth inhibiting amino sugars (Dahod, 1999). To prevent this reaction, molasses was first boiled and urea was added once the medium cooled to 40EC.

It was also observed that the consistency between batches of the gel medium differed even though the heat sterilization treatment remained constant. Inconsistent results were produced each time experiments were replicated as the constituents of the media changed after each heat sterilization procedure. Heat sterilization of a mixture of ingredients in water has a profound effect on the media. For instance, insoluble particles such as grain flour and meals are partially solubilized. Macromolecules such as proteins and starch maybe partly denatured. The inorganic compounds of the medium react among

themselves and with organic components, which result in the formation of new compounds (Dahod, 1999).

Optimal pH for fungal growth on molasses amended pine shavings was between pH 6.5-7 (Fig 2.4), and pH 6.5 for the gel medium (Fig. 2.12 and Fig. 2.13). It should be noted that studies on the effects of hydrogen ions on the growth of fungi suffer many inadequacies. The pH of the culture medium is often drastically changed during growth of the fungus due to the differential uptake of cations and anions from the medium and the secretion of protons (H^+) during the transportation of substrates across the cell membrane (Griffin, 1994). The pH of molasses and pine shavings media was not adjusted over the growth period as buffers have a very limited effective range of approximately pK ± 0.5 pH unit. Another factor taken into consideration was that the concentration of buffer sufficient to be effective may have been inhibitory to fungal growth and many fungi grow well when the initial pH of the media is adjusted to a range of 4-7 (Griffin, 1994). The extracellular pH typically has limited influence on the cytoplasmic pH. pH effects are therefore indirect, either on the cell surface or on extracellular components. The pH of the medium during sterilization is also very important. pH effects both the rate of reactions and equilibrium composition. Manipulation of sterilization pH can increase or decrease protein solubilization from media containing insoluble sources such as grain flour and meals (Dahod, 1999).

All three fungi have had prolific growth when grown on the molasses supplemented pine media (Fig. 2.11 A-D). One of the major requirements for successful application of a BCA is the production of fungal propagules that are resistant to desiccation and adverse conditions in the field. *Trichoderma harzianum* kmd formed chlamydospores on extensively branched hyphae (Fig. 2.11 A). The spores formed had thick cell walls and were produced in media with the higher C:N ratios of 31:1. Chlamydospores formation is favourable since they are resting spores with resistant cell walls that accumulate fatty acids (Ingold, 1984). Advantages of fatty acids accumulation in spores are discussed in Chapter Four. Essentially, chlamydospores formation leads to a more resistant product suitable for application even under adverse field conditions.

The primary goal of the results obtained from the experiments conducted on the novel gel was to study the effect of TOC and C:N ratios and the use of various carbon and nitrogen sources on the sporulation of *C. gloeosporioides* C6. The basal salts served as a source of basic macro- and micro-elements that were lacking in the gel medium. The addition of basal salts to the gel medium resulted in higher spore production as well as a denser mycelial mat when compared to the growth of *C. gloeosporioides* C6 on the plain gel (Fig. 2.12 and Fig. 2.13). This is in accordance with the Klebs's (1899) principles that states " inorganic salts may have specific effects on the reproduction as well as on the overall vigor of the fungus".

Another interesting feature of the spores produced in these experiments was the effect of damage of the mycelial mat of the fungus. This was done both to the supplemented and plain gels. Spores cultivated on plain gel produced numerous larger lipid bodies than those on supplemented gel (Fig. 2.14 A and B). However, damaging the mycelial mat appeared to produce more lipid bodies which were larger then those found in the undamaged mycelial mat (Fig. 2.14 C and D). The production of lipid bodies in a biological control agent is a desirable quality, because besides acting as a storage body for fungi, lipids prevent spore desiccation (Deacon, 1980).

A spore yield count could not be obtained from the fungi grown on the gel medium as the fungi integrated its structures into the gel. ESEM studies was used to establish which medium produced more abundant growth. The micrographs show that plates supplemented with basal salts resulted in higher spore yield production (Fig. 2.15 and Fig. 2.16). This was, however, the case at only the lower C:N ratio of 5:1. The higher C:N ratio of 40:1 resulted in more abundant production of mycelial biomass (Fig. 2.17 and Fig. 2.18).

Basal salts supplemented the gel with macroelements phosphorus, magnesium, sulphur, potassium and microelements calcium and iron. Phosphorus and sulphur, along with carbon and nitrogen, play an important role in the structure of the fungal cell and hypha.

Magnesium is required as an activator of a number of key enzymes and plays a significant role in maintaining the internal osmotic potential of the fungal spore and potassium is required to maintain the appropriate ionic environment for enzyme functioning (Boddy *et al.*, 1989; Jennings and Lysek, 1996). The role of calcium in fungi is still unclear as to whether or not calcium is essential for growth. However, it has been shown that the addition of calcium to media has a variety of effects on different fungi including, growth and morphogenesis. Insoluble calcium carbonate is added to prevent the fermentation pH from dropping below pH 6.0. As the pH drops the calcium carbonate dissolves thereby raising the pH (Dahod, 1999). Iron is thought to play a role in the activation of enzymes (Griffin, 1994; Jennings and Lysek, 1996).

Glucose was chosen as a constituent of the gel media as it is the most widely utilizable carbon source when developing media for routine and rapid growth (Griffin, 1994). Also, the transport and enzyme systems for glucose are already present in fungi and no adaptation is required for growth to begin (Griffin, 1994; Jennings, 1995; Jennings and Lysek, 1996). Glucose is commonly used as a sole carbon source in the laboratory but is not generally freely available in industrial fermentation media (Walker 1999). Glucose has been found to exhibit a repressive effect on the assimilation of other sugars by microorganisms. Cornflour, also used as carbon source in the gel medium, formed an opalescent paste when mixed with water forming a solid matrix on which the fungus was cultivated. Cellulose, the insoluable polymer of linked molecules of glucose is cross-linked, forming thick, complex fibres. *Trichoderma*, *Gliocladium* and *Colletotrichum* are capable of breaking the bonds connecting the glucose units making cornflour a rich source of energy directly available for respiration or fermentation. Soy bean flour, used as a source of nitrogen also contained minerals (± 6%) which enhanced the growth of *C. gloeosporioides C6* (Dix and Webster, 1995; Jennings and Lysek, 1996; Stamets, 2000).

It was deduced that although *T. harzianum* kmd, *G. virens* MM1 and *C. gloeosporioides* C6 grew over a wide range of C:N ratios and TOCs, higher C:N ratios and TOCs are required for maximum spore yield and chlamydospore production of *T. harzianum* kmd. All three fungal cultures required a shorter time period to produce prolific growth on the

pine shavings and molasses media than on the gel medium. This is an important consideration as a longer growth time elevates production costs. The gel was dried at 20°C in a dehumidifier to final moisture content of 7% and harvested by vacuuming spores off the gel. Experiments by Cecila van Roy (prs. comm.) showed that the spore count of *C. gloeosporioides* C6 was $1X10^9$ g L^{-1} when 2 g of the formulation was added to a liter of distilled water, with a spore viability of 88-90%. The spore yield obtained when *C. gloeosporioides* C6 was grown on the pine shavings and molasses medium at the best C:N ratio of 31:1 was 6.9×10^{10} L^{-1} The spore yield of the *C. gloeosporioides* C6 grown on the gel medium could have been substantially reduced as only surface spores were harvested and majority of the spores produced were integrated in the gel medium as shown by the ESEM micrographs (Fig. 2.16). Therefore, it can be concluded that production of *C. gloeosporioides* C6 spores using semi-solid fermentation is a better option than solid fermentation as spores are easier to harvest and a much higher spore yield is obtained during the fermentation process. The spore content of the commercial formulation of *G. virens* MM1 and *T. harzianum* kmd in formulation is 1×10^9 g L^{-1} when 1 g of formulation is added to a liter of distilled water (Plant Health Products). The spore yields of *T. harzianum* kmd (5.9×10^{10} L^{-1}) and *G. virens* MM1 (2.3×10^{10} L^{-1}) produced in the pine shavings and molasses media is sufficient to be effectively formulated in kaolin. However, viability tests on spores produced on the pine shavings and gel formulation need further investigation and efficacy tests on all spores need to be conducted.

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2.6 APPENDIX A

Appendix 2.1. Organic carbon by the Walkey-Black oxidation procedure

Reagents

- Potassium dichromate: 1 N ($K_2Cr_2O_7$)
- Ferrous ammonium sulphate: approximately 0.5 N
- Ferroin indicator (use freshly made indicator)
- NaF
- H_3PO_4 (85 %)
- H_2SO_4 (100%)

Procedure

Carry out analysis in duplicate. Grind the air-dry sample using a mortar and pestle (porcelain) to pass through a 0.5 mm sieve and transfer 0.5 g to a 500ml Erlenmeyer flask. Add 10ml of the $K_2Cr_2O_7$ solution and mix by swirling. Carefully add 20ml c. H_2SO_4 and mix gently for 1 min by slowly rotating the flask.
CAUTION : ACID FUMES ARE LIBERATED WHEN H_2SO_4 IS ADDED, PERFORM THIS STEP IN A FUME HOOD WITH THE EXTRACTOR FAN RUNNING. Allow mixture to stand for 20 min.
Run a blank, omitting the sample being tested i.e. cornflour, soya bean flour and glucose, respectively and treat in exactly the same way above. After 20 min dilute the mixture by the addition of 170ml deionized water, add 10ml 85 % H_3PO_4, 0.2 g NaF and 5 drops of ferrion indicator. Swirl the flask to mix the contents and then titrate the blank first in order to standardize the ferrous ammonium sulphate (FAS) solution. This also enables one to recognize the titration end-point more easily.
Initially the blank is golden brown. With the addition of FAS the solution darkens and turns a dull green in colour. With further addition of FAS the solution turns a brighter green and then to a dark greenish blue. At this point add the ferrous ammonium sulphate drop by drop until the one-drop end point is reached when the blank turns a dark brownish black. NOTE THE AMOUNT OF FAS USED.

Calculation

If we use NaOH solution of known concentration (C_{NaOH} me/l) in a burette and titrate this against an known volume (V_{HCl} ml) of HCl of unknown concentration in a flask

until equivalence or end-point is reached (indicator), and then read from the burette then volume of NaOH used, we can calculate the concentration of the HCl by the following relationship:

$$V_{HCl} \times C_{HCl} = V_{NaOH} \times C_{NaOH}$$

Where C is the me/l (= mmol/l for a univalent salt or mmol/2 for a divalent salt), therefore :

$$C_{HCl} = V_{NaOH} \times C_{NaOH} / V_{HCl}$$

As applied to the Walkey-Black procedure:

The organic matter has been oxidized by some of the 10ml $K_2Cr_2O_7$ added. The amount that is left is titrated with the FAS of known charge concentration and can be found to be equal to:

$V_{FAS} \times C_{FAS} / C_{K2Cr2O7} = y$ ml

The volume (= x) of $K_2Cr_2O_7$ used by the organic matter is calculated as $x = (10 - y)$

To find % organic carbon : We know that 1 meq $K_2Cr_2O_7$ reacts with 1 meq C.

Convert this to a mass of C..... 1 meq C = ¼ mmol C (C^{4+} charge)

= 12/4 mg C (at wt. C = 12)

x mℓ of $K_2Cr_2O_7$ have been consumed = x meq $K_2Cr_2O_7$ (1N solution = 1 eq/ l

= 1 meq/ mℓ

Therefore mg C oxidized = X x 12/4 x 1.33

Where 1.33 is a correction factor as the method only recovers a proportion of the soil carbon.

Therefore, % organic C in sample = X x 12/4 x 1.33 x 100/ mass of sample (mg)

Appendix 2.2. **Calculation of the TOC and C:N ratio of Voermolas**

The sample tested using the Walkey-Black oxidation method was a 70:3 (water:molasses) ratio mixture. The total organic carbon concentration (TOC) was found to be 11 674 mg L^{-1}. The protein concentration of the sample was 56.5 g kg^{-1} (as per supplier; Tongaat Foods[1]).

The C:N ratio was determined as follows:

Nitrogen concentration = protein / 6.25

$= 56.5 \text{ g kg}^{-1} / 6.25$

$= 9.04 \text{ g kg}^{-1}$

Nitrogen concentration in the sample tested (70:3)

$= 9.04 \text{ g kg}^{-1} * 3/73$

$= 0.372 \text{ g kg}^{-1}$

$= 372 \text{ mg kg}^{-1}$

Therefore the C:N ratio is:

	C	:	N
	11674	:	372
±	31	:	1

In a water:molasses ratio of 70:3 the TOC was 11.674 g kg^{-1} and the C:N ratio was 31:1

Total organic carbon in the concentrated sample = 11674 mg kg^{-1}* 73/3

$= 284028.42 \text{ mg l}^{-1}$

The TOC in a 160:3 (water: molasses) ratio was calculated as follows

$= 3/163 * 284028.42 \text{ mg l}^{-1}$

$= 52275.17 \text{ mg l}^{-1}$

$= 5.2 \text{ g l}^{-1}$

TOCs for all dilutions were calculated as shown above.

Appendix 2.3 **Total organic carbon in each water: molasses mixture**

Table A 2.1. Total organic carbon in each water: molasses mixture

Ratio of water: molasses	**Total organic carbon (g L^{-1})**
160:3	5.22
80:3	10.27
70:3	11.67
60:3	13.53
70:4	15.35
50:3	16.08
60:4	17.75
50:4	21.04

Appendix 2.4. **General analysis of Voermolas**

Table A 2.2. General analysis of macro- and micro-nutrients found in Voermolas (Tongaat Foods)

Ingredient	Quantity (g kg^{-1})
Protein	56.5
Calcium	9.2
Phosphorus	1.1
Moisture	300

Appendix 2.5. **Amount of urea added to Voermolas to obtain desired C:N ratios**

Molecular weight of urea (NH_2CONH_2) = 60

% nitrogen in NH_2CONH_2 = 28/60 * 100 = 47% i.e. 47g in 100g

% carbon in NH_2CONH_2 = 12/60 * 100 = 20% i.e. 20g in 100g

In 4.6g NH_2CONH_2

Amount of nitrogen = 4.6g * 47 / 100 = 2.16 g L^{-1}

Amount of carbon = 4.6g * 20 /100 = 0.92 g L^{-1}

Carbon-to nitrogen of amended media = 12.594 : 2.532

= 4.97 : 1

± 5 : 1

All other desired C:N ratios were calculated as stipulated above.

Appendix 2.6 **Amounts of urea added to obtain desired C:N ratios**

Table A 2.3. Quantity of urea added to medium to obtain C:N ratios

C:N ratio	Amount of urea (g) added
5:1	4.6
10:1	1.75
20:1	0.45
31:1	Unamended

Appendix 2.7 **General analysis of nutrients in Voermolas**

Table A 2.4. General analysis of macro- and micro nutrients found in molasses

Constituent	Data on 100 % DM Basis
Carbon	30.0
Protein	3.88
Nitrogen	0.62
C:N Ratio	49.03
	ppm
C	0.3
Mg	0.72
K	4.67
Na	0.15
$K/Ca^{+}Mg$	1.12
P	0.1
Cu	6
Mn	182
Fe	178
Moisture content (%)	
Moisture content of molasses	22.41

Appendix 2.8. **Calculation of carbon in the cornflour and soy bean flour used in gel formulation.**

Table A 2.5. Quantity of ferrous ammonium sulphate (FAS) used to determine the percentage total organic carbon of cornflour and soy bean flour

Samples	Amount FAS used (ml)
Blank	19.5
Cornflour (Rep 1)	16.8
Cornflour (Rep 2)	17.3
Soy bean flour (Rep 1)	17.2
Soy bean flour (Rep 2)	17

Calculation :-

V_{FAS} x C_{FAS} / C $_{K2Cr2O7}$ = Y ml

Y ml is the amount of $K_2Cr_2O_7$ not used in initial reaction i.e.,

BLANK

C1V1 = C2V2 ($K_2Cr_2O_7$)

C1 = 10ml * 1N ($K_2Cr_2O_7$)/ 19.5 ml

= 0.513

CORNFLOUR

Y ml = 17.05 ml * 0.513 / 1N

= 8.88ml

The volume (= x) of $K_2Cr_2O_7$ used by the organic matter is calculated as x = (10 – Y) i.e.,

X ml = 10 – 8.88 ml

= 1.12ml

Therefore, % organic C in sample = X x 12/4 x 1.33 x 100/mass of sample (mg)

= 1.12 x 3 x 1.33 x 100/10

= 44.88 %

SOY BEAN FLOUR

The equations stipulated above were used to calculate the Y, X values and % organic carbon.

Y ml = 17.1 * 0.513 / 1

= 8.77 ml

X ml = 10 – 8.77 ml

= 1.23 ml

% organic carbon = 1.23 x 3 x 1.33 x 100/10

= 48.99 %

CARBON IN GLUCOSE

Molecular weight of glucose = 180

Carbon = 6 * 12

= 72

% carbon in glucose = 72 / 180 * 100

= 40 %

Appendix 2.9. **The quantity of $(NH_4)_2SO_4$ added to the gel formulation to obtain the desired C:N ratios**

% nitrogen in soya bean flour = 35.35 / 6.25 = 5.66

in 15 g L^{-1} there is = 5.66/100 * 15 g L^{-1}

= 0.85 g L^{-1}

The initial C:N ratio of the gel was 52.92: 0.85 = 62:1

Molecular weight of $(NH_4)_2SO_4$ = 132.13

Nitrogen in $(NH_4)_2SO_4$ = 14.007 * 2 = 28.014

% nitrogen in $(NH_4)_2SO_4$ = m / M

= 28.014 / 132.13 * 100

= 21.20 %

For a 30:1 C:N ratio you need 1.764g nitrogen

Therefore you need to add 1.764g – 0.85 = 0.914g

21.2/100 * X = 0.914

X = 4.35g

The 5:1; 10:1 and 20:1 C:N ratios were calculated as stipulated above.

CHAPTER 3

Effect of carbon concentration and C:N ratios on growth, conidiation and germination of *Colletotrichum gloeosporioides* C6

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Colletotrichum gloeosporioides (Penz. and Sacc.) C6 is a mycoherbicide used in the biological control of the invasive weed Silky Hakea (*Hakea sericea* Shrad.). The effect of carbon (C) concentration and carbon-to-nitrogen (C:N) ratio on growth, conidiation and spore germination of *C. gloeosporioides* C6 was investigated. For all parameters tested, significant differences were observed ($P < 0.05$). Highest spore yields were obtained at C concentrations of 5 g L^{-1} and 10 g L^{-1} at a C:N ratio of 15:1. Highest mycelial dry biomass was obtained at a C concentration of 20 g L^{-1} and at a C:N of 40:1. The highest spore germination percentage occurred at 5 g L^{-1} C and at a C:N ratio of 20:1. Optimum specific spore yield and specific mycelial dry weight were obtained at a C concentration of 5 g L^{-1} and at a C:N ratio of 15:1 and 40:1, respectively. Specific spore yield was not significantly affected at a C concentration of 10 g L^{-1} when C:N ratios were varied. The interactive relationship of C concentration and C:N ratios was significant, affecting the specific mycelial dry biomass (P=0.05) and conidiation (P=0.0001).

3.1 INTRODUCTION

Colletotrichum gloeosporioides is a fungus used in the 'bioherbicide' approach to weed control. The strategy applies the inundative application of fungal or bacterial propagules to selectively infect and kill a target weed (Templeton *et al.*, 1979). The use of fungal pathogens to control weeds that are not effectively managed by chemical herbicides is becoming a practical option (Templeton, 1982).

A number of *Colletotrichum* species have shown potential for use as mycoherbicides. Strains of *C. malvarum* (Braun and Casp.) and *C. coccodes* (Wallr.) which show biological control

against prickly sida (*Sida spinosa* L.) and velvet leaf (*Abutilon theophrasti* Medic.), respectively, have been identified (Kirkpatrick *et al.*, 1982; Wymore, 1988). Strains of *C. gloeosporioides* which specifically infect the noxious weeds *Clidemia hirta* (L.) D. Don. and *Malva pussilla* Smith were discovered by Trujillo *et al.* (1986) and Mortensen (1988), respectively.

Hemp sesbania (*Sesbania exaltata* Raf.) is a competitive weed which is responsible for considerable losses in the cotton, soyabean and rice industries in the southern United States. Boyette (1988) discovered a strain of *C. truncatum* NRRL 13737 (Schwen.) which specifically kills *S. exaltata*. The major constraint to the development of this mycoherbicide was that it was not amenable to traditional liquid fermentation technology.

Jackson and Bothast (1990) investigated the effect of C concentration and carbon-to-nitrogen ratios on submerged culture conidiation of *Colletotrichum truncatum* NRRL 13737. This study revealed that the C concentration and C:N ratios had a profound effect on production of conidia. The investigation used media with a C:N ratio of 15:1 and was measured in cultures with a C concentration between 5.1 and 40.8 g L^{-1}. Conidiation in 5-day old cultures was highest with a C concentration of 10.2 g L^{-1}. However, the highest levels of conidiation in the 6- and 7-day old cultures were found with concentrations of 5.1 to 15.3 g L^{-1}. Jackson and Bothast (1990) showed that an understanding of how that nutritional environment influences conidiation must be ascertained, in order to predict what type of low cost complex media are suitable for conidiation.

The invasive weed targeted in this study was the Silky Hakea (*H. sericea*). *Hakea sericea* grows as a shrub producing distinctive cream flowers (Fig. 3.1 A and B). Seeds are formed annually in woody fire resistant follicles (Fig 3.1 C). Seeds accumulate within the follicles until up to 75 million seeds per hectare are formed (Fig. 3.1). The seeds are released when the plant dies and germinate to produce monospecific stands. The biological control of Silky Hakea fall into two catogeries: seed reducing insects and fungi that damage the vegetative growth (Anonymous, 2001).

The biological control agent, *C. gloeosporioides* C6, the hakea gummosis fungus, is different from many other biocontrol agents used in South Africa since it was not intentionally

introduced into South Africa but probably transferred from indigenous species of protea onto Silky Hakea (Anonymous, 2001).

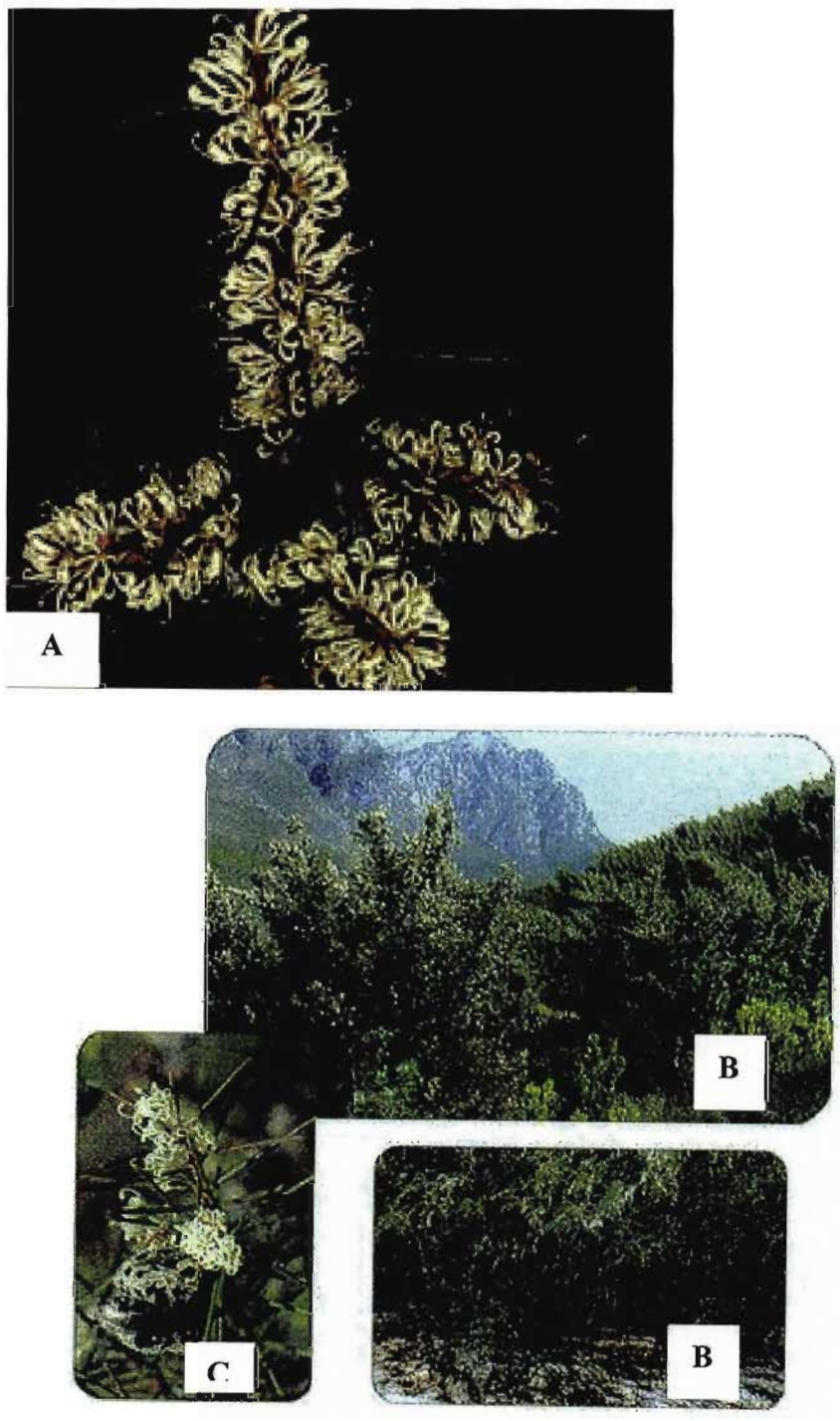

Fig 3.1. Silky Hakea (*Hakea sericea*), a shrub bearing small cream flowers (A) with prickly branches (B), up to 5m high, young stem covered in short silky hair, and distinctive fruits (C) (Anonymous, 2002).

Colletotrichum gloeosporioides C6 affects both mature plants as well as seedlings. Growth tips of seedlings are affected and die. The infection then spreads down the stem until the whole plant dies. A colourless gum oozes from lesions produced by the fungus when mature plants are infected (Fig. 3.2). The fungus kills the bark around the stem. If the base of the stem is infected the fungus may girdle the stem, resulting in the death of the tree (Anonymous, 2001). *Colletotrichum gloeosporioides* C6 spores are distributed aerially using a helicopter (Fig. 3.4) and brown patches are symptomatic of infected shrubs (Fig. 3.3)

Although there have been many promising strains of fungi that exhibit biological control activity, only three fungal bioherbicides are registered in Northern America (Templeton, 1982; Mortensen, 1988). A major constraint in the commercial production of bioherbicides is the availability of low cost methods for the mass production of viable propagules (Templeton *et al.*, 1979).

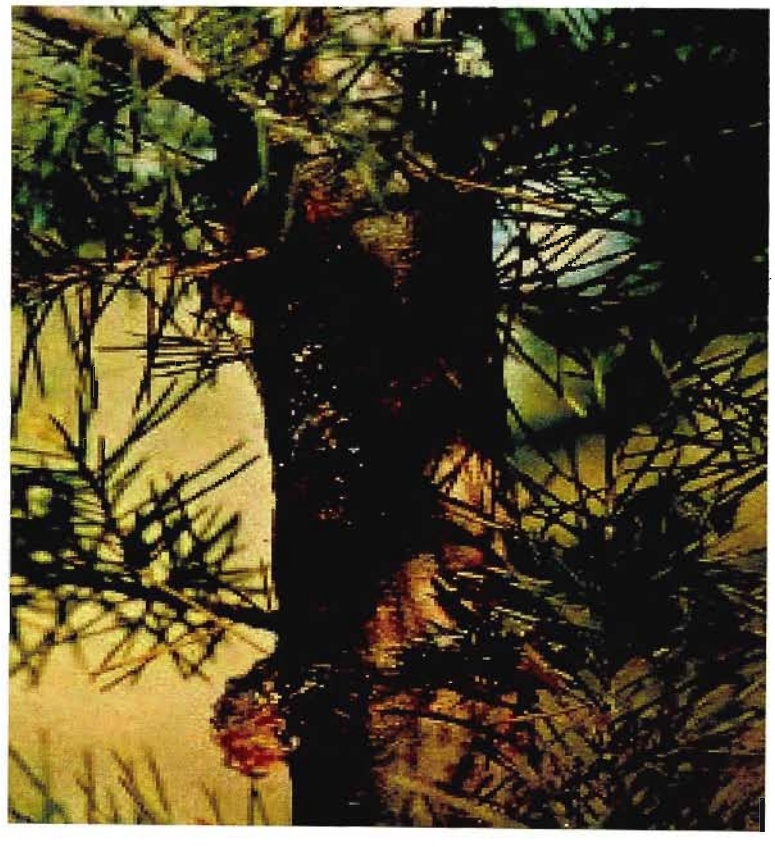

Fig 3.2. Gum exudate oozing from hakea attacked by the gummosis fungi, *Colletotrichum gloeosporioides* (Anonymous, 2001).

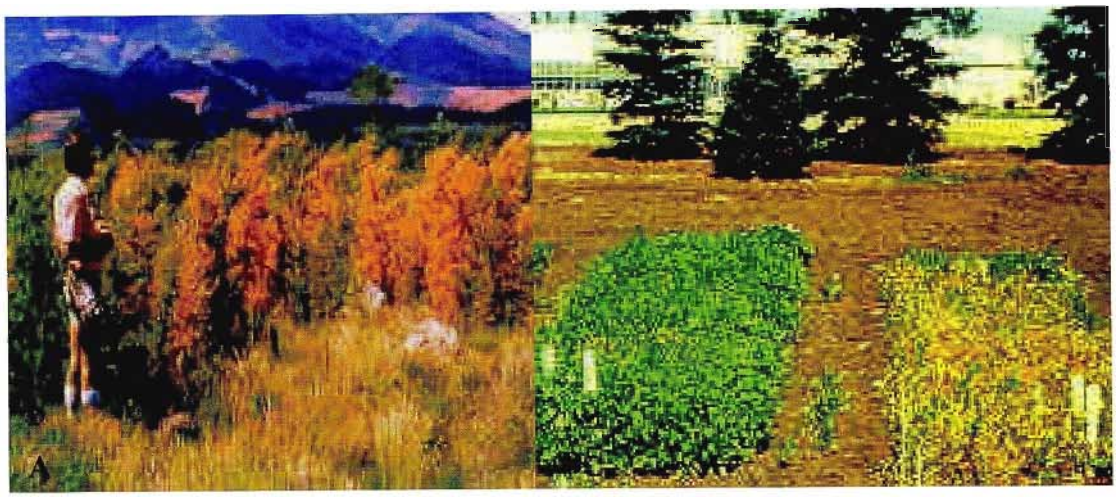

Fig. 3.3. A and B. Hakea plants killed by *Colletotrichum gloeosporioides*. Brown plants indicate gummosis (Anonymous, 2001).

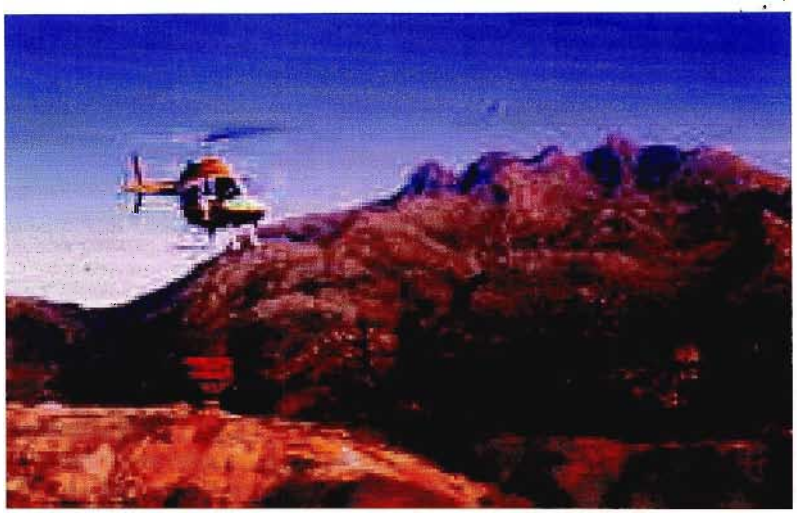

Fig. 3.4. Aerial distribution of *Colletotrichum gloeosporioides* spores on Silky hakea (*Hakea sericea*) plants using a helicopter (Anonymous, 2001).

Production costs of biological control agents are lowered by the development of media and environmental conditions which reduce fermentation times and increase yields of conidia. This is an imperative step in the formulation of biological control agents as commercial viability of a product is dependent on an economical production method (Jackson and Slininger, 1993).

This investigation has focused on using submerged culture conditions for the production of high concentrations of *C. gloeosporioides* C6 conidia. Previous research showed that the nutritional environment significantly impacts conidia yield and efficacy (Jackson and Bothast, 1990 ; Schilser *et al.*, 1991 ; Jackson and Schilser, 1992 ; Jackson and Slininger, 1993). Jackson *et al.* (1989) showed that the vitamin enriched basal salts medium used in this study supported growth of other fungi. Using this medium, evaluations were made on the suitability of various C:N ratios and C concentrations to *C. gloeosporioide* C6 and the effect on growth, conidiation and germination of *C. gloeosporioides* C6.

3.2 MATERIALS AND METHODS

Cultures

Stock cultures of *C. gloeosporioides* C6 were obtained from Plant Health Products[1]. The cultures were stored on potato dextrose agar (PDA) slants or on wheat grain at 4°C, under mineral oil. Small plugs of the stock culture were transferred to PDA plates and incubated for 7-10 days at 20°C. Small plugs of *C. gloeosporioides* C6 were inoculated into 50 ml Modified Richard's liquid medium in 250 ml Erlenmeyer flasks. The medium was composed of sucrose, 10 g L^{-1}; KNO_3, 10 g L^{-1}; KH_2PO_4, 5.0 g L^{-1}; $MgSO_4.7H_2O$, 2.5 g L^{-1}; $FeCl_3.6H_2O$, 0.02 g L^{-1}; V-8 juice, 150 ml L^{-1}, and made up with distilled water to a volume of 1 L. Cultures were kept at room temperature for five days and shaken at 150 rpm on a rotary shaker. Spores were harvested from these cultures and used as initial inoculum for the C:N and C concentration experiments.

Media

The defined basal salts medium used for the C concentration and C:N ratio experiments comprised KH_2PO_4, 5.0 g L^{-1}; $MgSO_4.7H_2O$, 0.5 g L^{-1}; $CaCl_2$, 0.5 g L^{-1}; $FeCl_3.6H_2O$, 0.05 g

[1] Plant Health Products, P.O. Box 207, Nottingham Road, KwaZulu Natal, 3802, South Africa

L^{-1}; and $CuSO_4$, 0.05 g L^{-1}. Stock solutions of sucrose (10%, w/v), and KNO_3 (10%, w/v) were added to the basal salts medium to obtain the desired C concentrations and C:N ratios. Soy Protein supplied by ICN Biochemicals [2] was supplemented to the medium as a nitrogen (N) source.

In each treatment, supplemented N was composed of 50% N from KNO_3 and 50% from soy protein (Yu *et al.*, 1998). Molecular C concentration and C:N ratios were calculated based on the concentration of C and N of sucrose (42% C); KNO_3 (14% nitrogen) and soy protein. The soy protein provided by ICN Biochemicals contained 92% protein, approximately 52% elemental C and 16% N.

Carbon concentration and C:N ratios

The C concentrations tested were 5 g L^{-1}, 10 g L^{-1} and 20 g L^{-1}. The C:N ratios tested at each C concentration were 5:1, 7.5:1, 10:1, 15:1, 20:1, 30:1, 40:1. Experiments were conducted in triplicate and a total of 63 flasks per experiment were used. Table 3.1 outlines the amounts of sucrose, KNO_3 and soy protein used at a C concentration of 5 g L^{-1} to obtain the various C:N ratios. For the C concentrations of 10 g L^{-1} and 20 g L^{-1}, the amounts of sucrose, KNO_3 and soy protein were two-fold and four-fold, respectively (Yu *et al.*, 1988). The pH of the media was adjusted to pH 5.5 by the addition of 1 N NaOH or 1 N HCl.

Table 3.1. Concentration of sucrose, soy protein and KNO_3 in media with 5 g L^{-1} total organic carbon at various C:N ratios (Yu *et al.*, 1998).

C:N Ratio	Sucrose (g L^{-1})	Soy Protein (g L^{-1})	KNO_3 (g L^{-1})
5:1	7.95	3.40	3.61
7.5:1	9.26	2.26	2.41
10:1	9.92	1.70	1.81
15:1	10.57	1.13	1.20
20:1	10.9	0.85	0.90
30:1	11.23	0.57	0.60
40:1	11.39	0.43	0.45

[2] ICN Biochemicals, Inc., 1263 South Chillicothe Road, Aurora, Ohio, 44202, USA.

Growth and sporulation

Experiments were conducted in 250 ml Erlenmeyer flask with 50 ml of medium. The flasks were inoculated with spores adjusted to a concentration of 2 x 10^5 spores ml^{-1} using a counting chamber. The cultures were incubated at room temperature (± 21-23°C) on a orbital shaker at 125 rpm for 7 days.

The medium was then filtered through two layers of sterilized cheesecloth to separate the spores from the mycelium. The cheesecloth was soaked in water for 20 minutes and autoclaved at 121°C for 15 minutes. Spore suspensions were centrifuged at 41 400 x g (18 500 rpm) for 10 minutes using a JA-20 Beckman rotor. The pellet was retained and washed with distilled water, centrifuged under the same conditions and resuspended in distilled water to the original volume.

Evaluation

Spore concentrations were determined microscopically using a counting chamber. The mycelial biomass was determined by drying the wet mycelial biomass at 60°C for 24 hours and the dry mass was weighed. Germination tests were conducted by spraying conidia on PDA coated slides. These slides were placed in petri dishes with moist filter paper. Spores were allowed to germinate at room temperature and were checked periodically every 6 hours until 100 % germination was achieved in any one treatment. The spores were stained with fast green (0.2 % w/v). The germinating spores were counted under the Axiophot light microscope (Zeiss, Germany) at 100X magnification.

The specific spore yield (Ysp) and specific mycelial biomass (Yms) was calculated as follows:

Specific spore yield (Ysp):

$$Ysp\ (spores\ g^{-1}) = Spore\ concentration\ (spores\ L^{-1}) \div initial\ carbon\ concentration\ (g\ L^{-1})$$

Specific mycelial biomass yields (Yms)

$$Yms\ (g\ g\ L^{-1}) = mycelial\ dry\ weight\ (g\ L^{-1}) \div initial\ carbon\ concentration\ (g\ L^{-1})$$

Analysis

A randomized block design with three replicates per treatment was used in all experiments. All the experiments were repeated three times and the data was pooled and analyzed using the Statistical Analysis System program. A general linear model (GLM) was used to run an Analysis of Variance (ANOVA) on the results using the computer statistical package, (SAS, 1987).

3.3 RESULTS

For each of the effects of C concentrations investigated varying C:N ratios were found to have a profound effect on spore yields, mycelial biomass and spore germination, Ysp and Yms. The effects on spore yield, dry weight of mycelial biomass and spore germination percentage are presented in Table 3.2 and Figures 3.5 to 3.11. The influence of C:N ratios and overall C concentration on Ysp and Yms is presented in Table 3.3 and Figures 3.12 and 3.13, respectively.

Sporulation

No clear trend in spore yield was discernable. Highest spore yields, in excess of 80 x 10^6 ml^{-1}, within all the C:N ratio groupings and C concentrations, were produced at C concentrations of 5 g L^{-1} and 10 g L^{-1} at C:N ratios of 7.5:1, 15:1 and 40:1 (Table 3.2 and Fig. 3.5). No significant differences ($P < 0.05$) in spore yields for a C concentration of 5 g L^{-1} at C:N ratios of 5:1, 10:1 and 20:1 were observed. At a C concentration of 10 g L^{-1}, significantly increased spore yields were achieved at C:N ratios of 15:1 to 20:1 (Table. 3.2). At a C concentration of 20 g L^{-1}, no highly significant increases in spore yield were achieved over the range of C:N ratios tested. The two lowest spore yields within all C:N groupings and C concentrations were produced at a C concentration of 20 g L^{-1} and C:N ratios 30:1 and 40:1 (Table 3.2 and Fig. 3.5). Carbon concentration, C:N ratios and the interactive effect of C concentration and C:N ratios were highly significant on spore yield ($P < 0.05$) (Table 3.2).

Specific spore yield

No significant effect on Ysp of C concentrations at C:N ratio groupings of 5:1 and 20:1 was observed (Table 3.3 and Fig. 3.6). There were no differences of Ysp at a C:N ratio of 7.5:1 at C concentrations of 10 g L^{-1} and 20 g L^{-1}; C:N ratio of 30:1 at C concentration 10 g L^{-1} and C:N ratio of 40:1 at C concentration 20 g L^{-1} (Fig. 3.12). The general trend was the highest Ysp was produced at the lowest C concentration of 5 g L^{-1}, which had the most significant difference within the C:N ratio grouping and the different C concentrations. The C concentration ($P = 0.0001$) and C:N ($P = 0.016$) affected Ysp yields and the interaction of C concentration and C:N ratios was very highly significant on Ysp yields ($P = 0.0005$).

Mycelial biomass

For most C:N ratios the general trend observed was for mycelial dry biomass to increase with an increasing C concentration (Fig. 3.7 and Fig. 3.9), with the exception of C:N ratio of 30:1. An increase was observed in wet mycelial biomass as C concentration increased from 5 g L^{-1} and 10 g L^{-1} to 20 g L^{-1} (Fig. 3.10-3.12). Highest mycelial dry weight of 0.34 g was formed at the highest C concentration, i.e., 20 g L^{-1} at the highest C:N ratio of 40:1 (Table 3.2 and Fig. 3.7). For each C concentration tested there were no highly significant differences found at C:N grouping evaluated (Fig. 3.7). The effect of C concentration and C:N ratios on mycelial biomass were very highly significant ($P = 0.0001$) (Table 3.2). Interactive effect of C concentration and C:N ratios did not have a significant effect on mycelial biomass formation ($P= 0.0659$) (Table 3.2).

Specific mycelial biomass (Yms)

Highest Yms was formed at a C concentration of 10 g L^{-1} with a C:N ratio of 40:1 (Table 3.3 and Fig. 3.8). The general trend was that as the C:N ratio increased with C concentration the Yms increased. The lowest Yms values were formed at C concentration of 20 g L^{-1} at all C:N groupings except at C:N ratios of 7.5:1 and 10:1. Carbon concentration ($P = 0.0002$) and C:N ($P = 0.0001$) had a very highly significant effect on Yms (Table 3.3). The interaction effect of C concentration and C:N ratios was significant ($P = 0.0313$).

Spore germination

The highest percentage germination coincided with the lowest C concentration of 5 g L^{-1}, within all C:N ratio groupings. Spore germination appears to favour low C concentrations, i.e., 5 g L^{-1} at higher C:N ratios from 20:1 to 40:1 (Table 3.2). The higher C concentration of 20 g L^{-1} resulted in significantly ($P< 0.05$) lower percentage spore germination (Fig 3.13). the effect of C concentration, C:N ratios and the interactive effect of C concentration and C:N ratios were very highly significant on spore germination ($P =0.0001$) (Table 3.2).

Table 3.2. Means of spore yield (SY), mycelial yield (MY) and percentage germination of *Colletotrichum gloeosporioides* C6 cultured over a range of C concentrations and C:N ratios

TOC	C:N	(SY) (10^6/ml)	R	(MY) (g)	R	% Germination	R
5 g L^{-1}	5.1	27.64cdef	14	0.05d	13	17.00def	15
5 g L^{-1}	7.5:1	106.37a	2	0.08cd	11	27.50cde	11
5 g L^{-1}	10:1	51.50bcdef	10	0.06d	12	70.00b	6
5 g L^{-1}	15:1	89.67ab	3	0.10cd	10	35.00cd	9
5 g L^{-1}	20:1	43.97bcdef	13	0.11cd	9	97.50a	2
5 g L^{-1}	30:1	78.49abc	6	0.12cd	8	97.50a	2
5 g L^{-1}	40:1	88.36ab	4	0.12cd	8	98.00a	1
10 g L^{-1}	5.1	25.61cdef	17	0.12cd	8	2.00f	19
10 g L^{-1}	7.5:1	24.56cdef	18	0.11cd	9	7.00ef	16
10 g L^{-1}	10:1	26.77cdef	16	0.08cd	11	29.50cde	10
10 g L^{-1}	15:1	112.00a	1	0.14bcd	7	40.50cd	8
10 g L^{-1}	20:1	81.56ab	5	0.12cd	8	80.50ab	4
10 g L^{-1}	30:1	26.99cdef	15	0.22abc	4	88.50ab	3
10 g L^{-1}	40:1	74.67abcd	7	0.27ab	2	71.50b	5
20 g L^{-1}	5.1	47.70bcdef	11	0.18bcd	6	1.00f	20
20 g L^{-1}	7.5:1	57.20bcdef	9	0.27ab	2	2.50f	18
20 g L^{-1}	10:1	46.26bcdef	12	0.19bcd	5	3.50f	17
20 g L^{-1}	15:1	21.65def	19	0.23abc	3	20.50def	14
20 g L^{-1}	20:1	72.06abcde	8	0.18bcd	6	45.50c	7
20 g L^{-1}	30:1	16.02f	21	0.18bcd	6	23.00cdef	12
20 g L^{-1}	40:1	18.32ef	20	0.34a	1	22.00cdef	13

Effects	**P-level**	**P-level**	**P-level**
C concentration	0.0001***	0.0001***	0.0001***
C:N	0.0004***	0.0003***	0.0001***
C concentration*C:N	0.0001***	0.0659	0.0001***
	C.V .	**C.V.**	**C.V.**
	26.85	26.72	17.38
	MSE	**MSE**	**MSE**
	14.5	0.04	7.28

1. **Significance**

	Description
* * *	Very Highly significant
* *	Highly significant
*	Significant

2. Means with the same letter are not significantly different ($P < 0.05$) according to Student, Newman and Keuls comparison test.
3. R= ranking
4. C = carbon
5. C:N = carbon-to-nitrogen ratio

Table 3.3. The effect of culturing *Colletotrichum gloeosporioides* C6 at various C concentrations and C:N ratios on specific spore yield (Ysp) and specific mycelials biomass (Yms)

TOC	C:N	(Ysp) (10^6/ml)	R	(Yms) (g)	R
5 g L^{-1}	5:1	5.5280de	10	0.0100de	14
10 g L^{-1}	5:1	2.5610de	15	0.0115cde	10
20 g L^{-1}	5:1	2.3850de	17	0.0090de	16
5 g L^{-1}	7.5:1	21.2740a	1	0.0160abcde	7
10 g L^{-1}	7.5:1	2.4560de	16	0.0105cde	13
20 g L^{-1}	7.5:1	2.8600de	12	0.0135bcde	8
5 g L^{-1}	10:1	10.3000cd	6	0.0110cde	12
10 g L^{-1}	10:1	2.6770de	14	0.0075e	18
20 g L^{-1}	10:1	2.3130de	18	0.0093de	15
5 g L^{-1}	15:1	17.9340ab	2	0.0190abcde	5
10 g L^{-1}	15:1	11.2000bcd	5	0.0135bcde	8
20 g L^{-1}	15:1	1.0830e	19	0.0113cde	11
5 g L^{-1}	20:1	8.7950de	7	0.0220abcd	4
10 g L^{-1}	20:1	8.1560de	8	0.0120bcde	9
20 g L^{-1}	20:1	3.6030de	11	0.0088de	17
5 g L^{-1}	30:1	15.6980abc	4	0.0240ab	2
10 g L^{-1}	30:1	2.6990de	13	0.0220abcd	4
20 g L^{-1}	30:1	0.8010e	21	0.0088de	17
5 g L^{-1}	40:1	17.6720ab	3	0.0230abc	3
10 g L^{-1}	40:1	7.4670ed	9	0.0270a	1
20 g L^{-1}	40:1	0.916e	20	0.0170abcde	6

Effects	P- level	P-level
TOC	0.0001* * *	0.0002 * * *
C:N	0.0016* * *	0.0001* * *
Carbon concentration*C:N	0.0005* * *	0.0313* *
	% C.V	**% C.V**
	34.39	23.54
	MSE	**MSE**
	2.43	0.003

1. **Significance** — **Description**
   - * * * — Very Highly significant
   - * * — Highly significant
   - * — Significant
2. Means with the same letter are not significantly different (P < 0.05) according to Student, Newman and Keuls comparison test.
3. R = Ranking
4. C=carbon
5. C:N= carbon-to-nitrogen

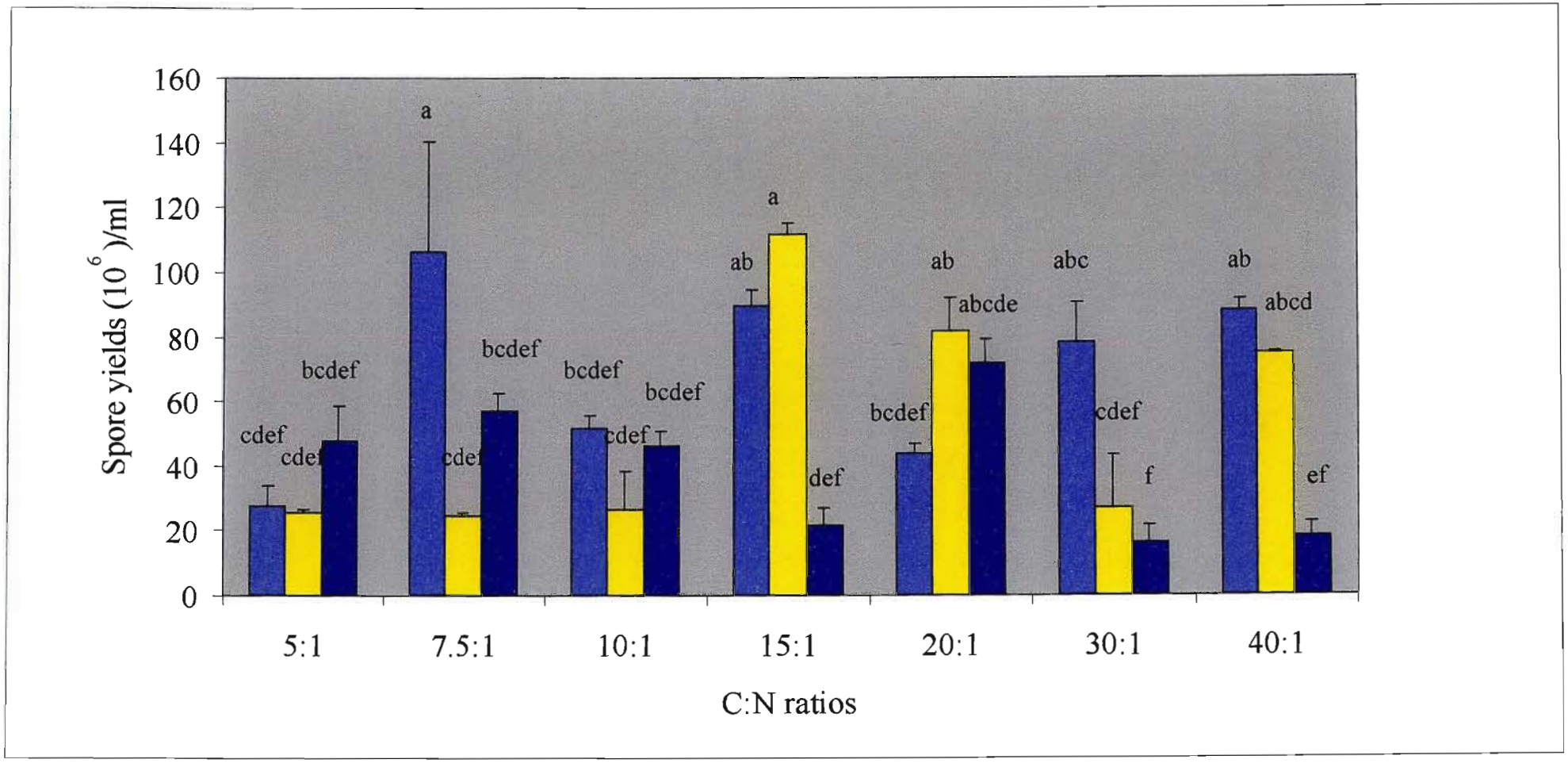

Fig. 3.5. Influence of carbon concentrations on conidiation of *Colletotrichum gloeosporioides* C6 grown at various C:N ratios. Within each C:N ratio grouping, mean spore concentrations with different letters are significantly different ($P < 0.05$).

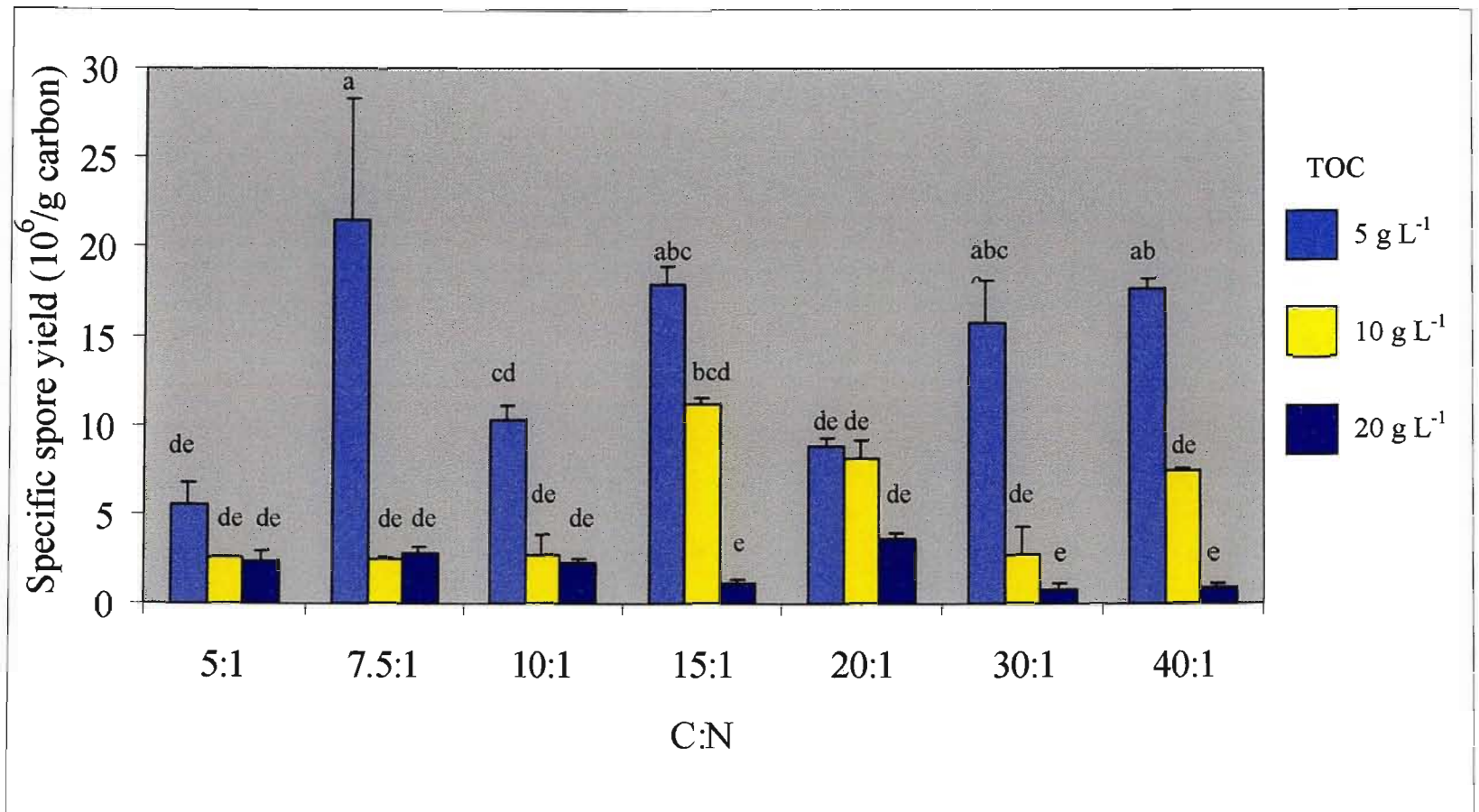

Fig. 3.6. Effect of C:N ratios and carbon concentration on the specific spore yield (Ysp) of *Colletotrichum gloeosporioides* C6. The mean Ysp within each C:N ratio grouping with the same letters are not significantly different.

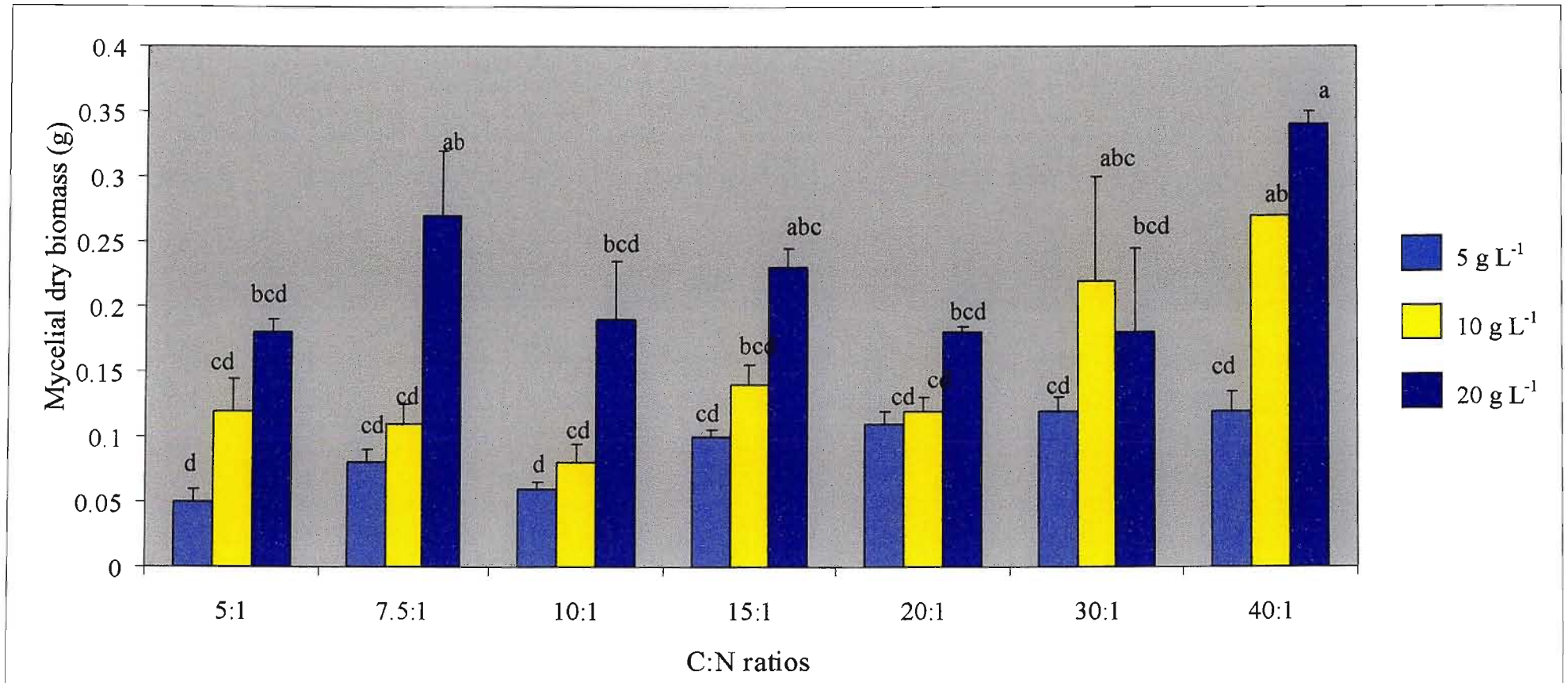

Fig. 3.7. Influence of carbon concentrations on dry mycelial biomass of *Colletotrichum gloeosporioides* C6 grown at various C:N ratios. Within each C:N ratio grouping, mean mycelial biomass with different letters are significantly different ($P < 0.05$).

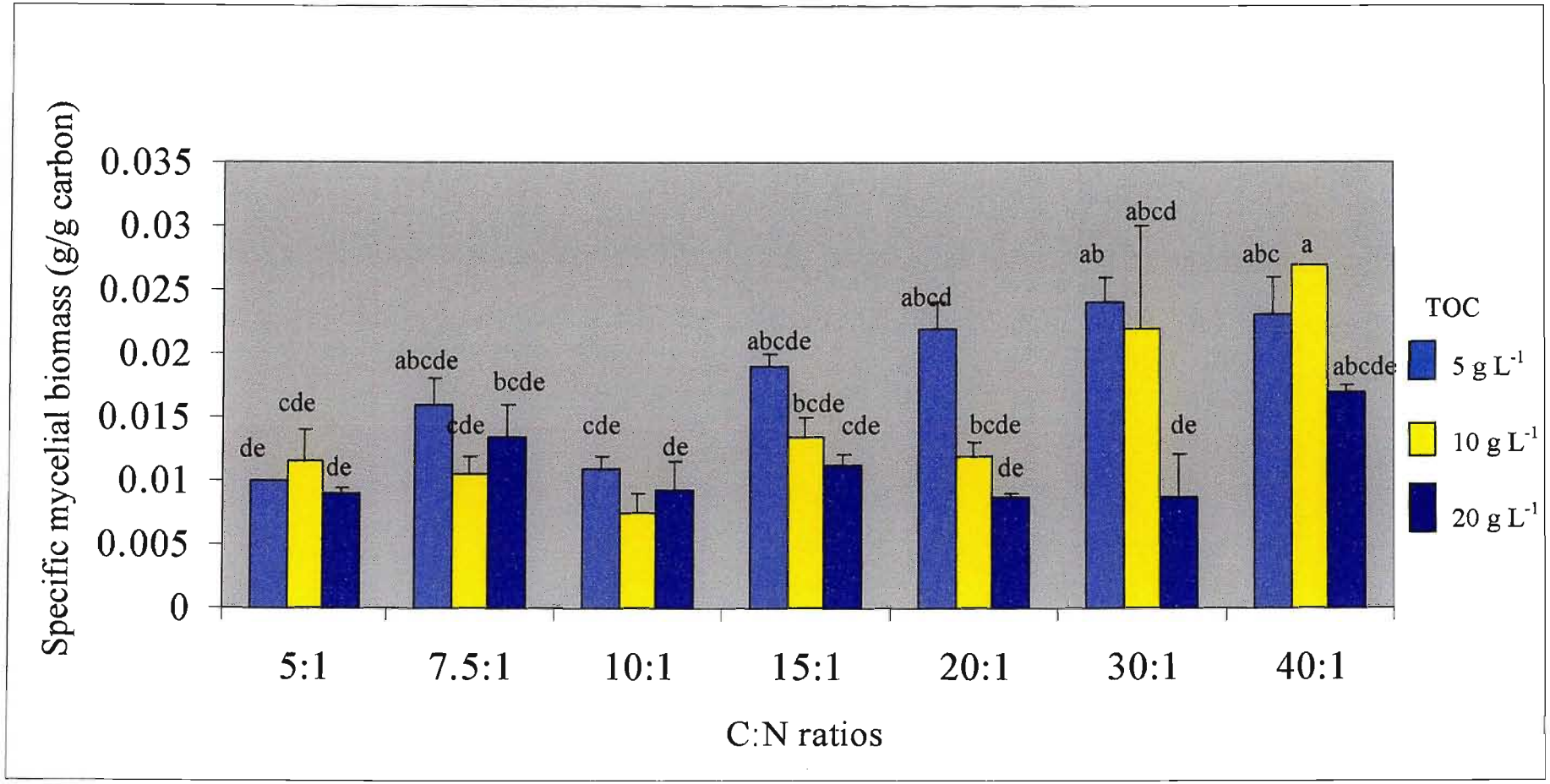

Fig. 3.8. Effect of C:N ratios and carbon concentration on the specific mycelial biomass (Yms) of *Colletotrichum gloeosporioides* C6. The mean Yms within each C:N ratio grouping with the same letters are not significantly different.

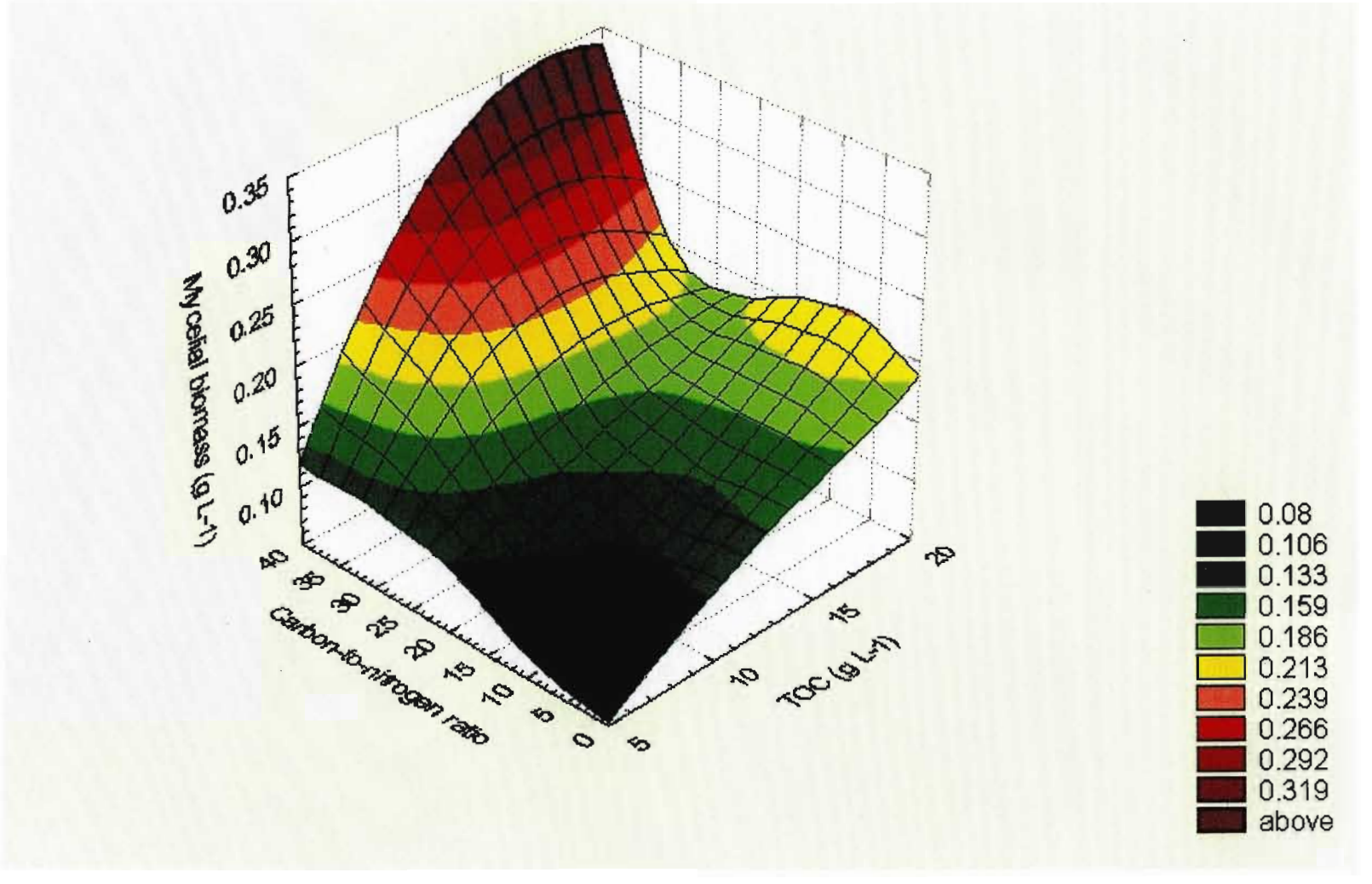

Fig. 3.9. The interactive effect of carbon-to-nitrogen and total organic carbon (TOC) on mycelial biomass.

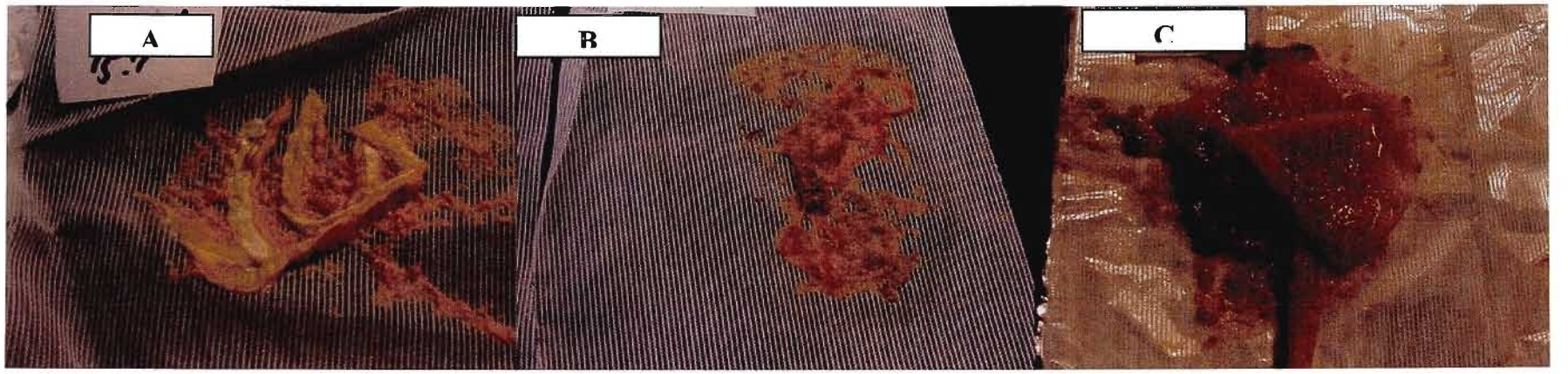

Fig. 3.10. Wet mycelial biomass of *Colletotrichum gloeosporioides* C6 at a C:N ratio of 15:1. A shows mycelial biomass at 5 g L^{-1} carbon; B shows mycelial biomass formed at carbon concentration of 10 g L^{-1}; and C shows the mycelial biomass at 20 g L^{-1} carbon.

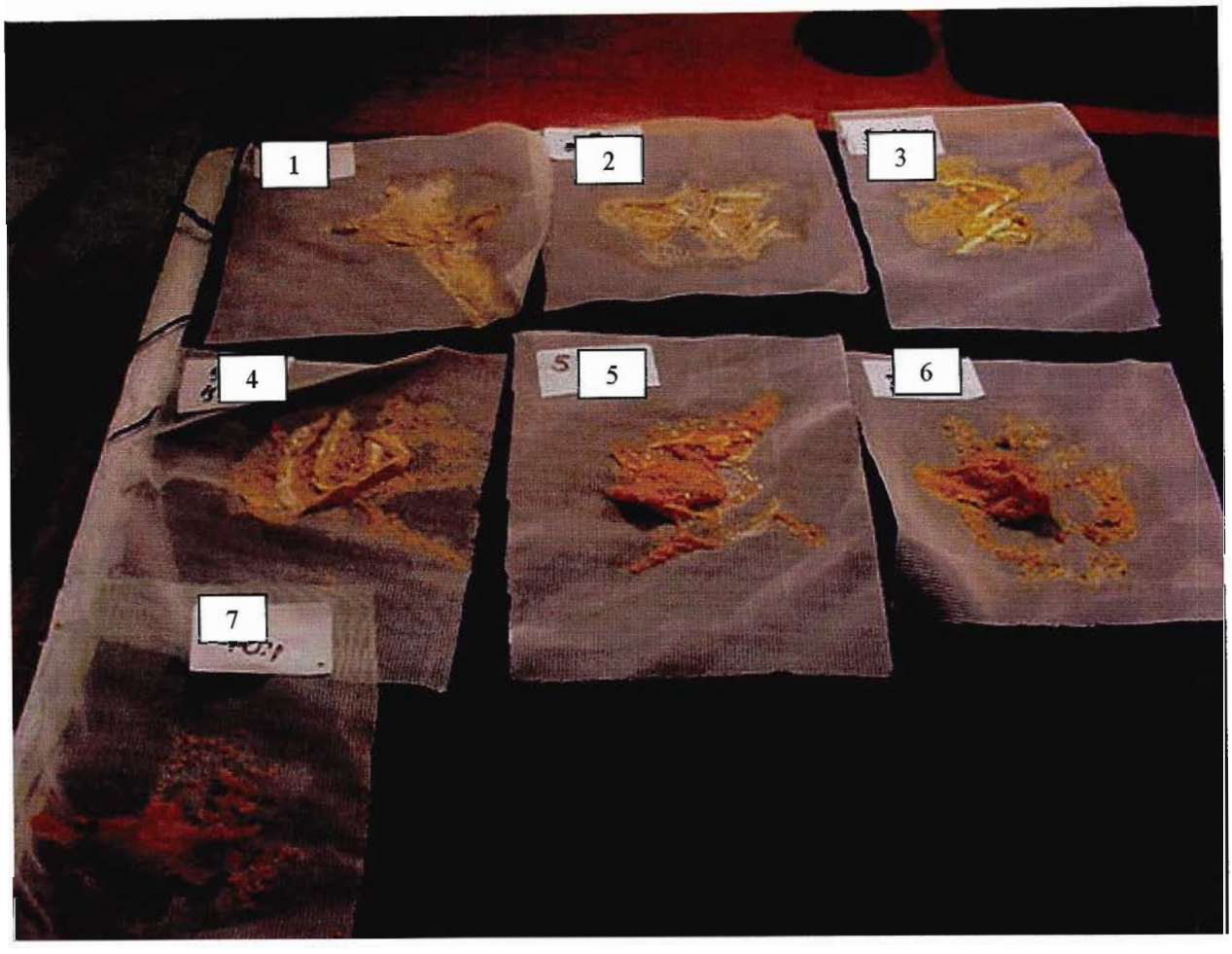

Fig. 3.11. Wet mycelial biomass of *Colletotrichum gloeosporioides* C6 at a carbon concentration of 5 g L^{-1}. The C:N ratios range from 1 (5:1) ; 2 (7.5:1) ; 3 (10:1) ; 4 (15:1) ; 5 (20:1) ; 6 (30:1) ; 7 (40:1). The illustration shows an increase in wet mycelial biomass as C:N ratios increases at C:N 7.5:1 and 15:1 to 40:1. The lowest biomass was formed at C:N ratios of 5:1 and 7.5:1. However, statistically there were no difference in dry mycelial biomass.

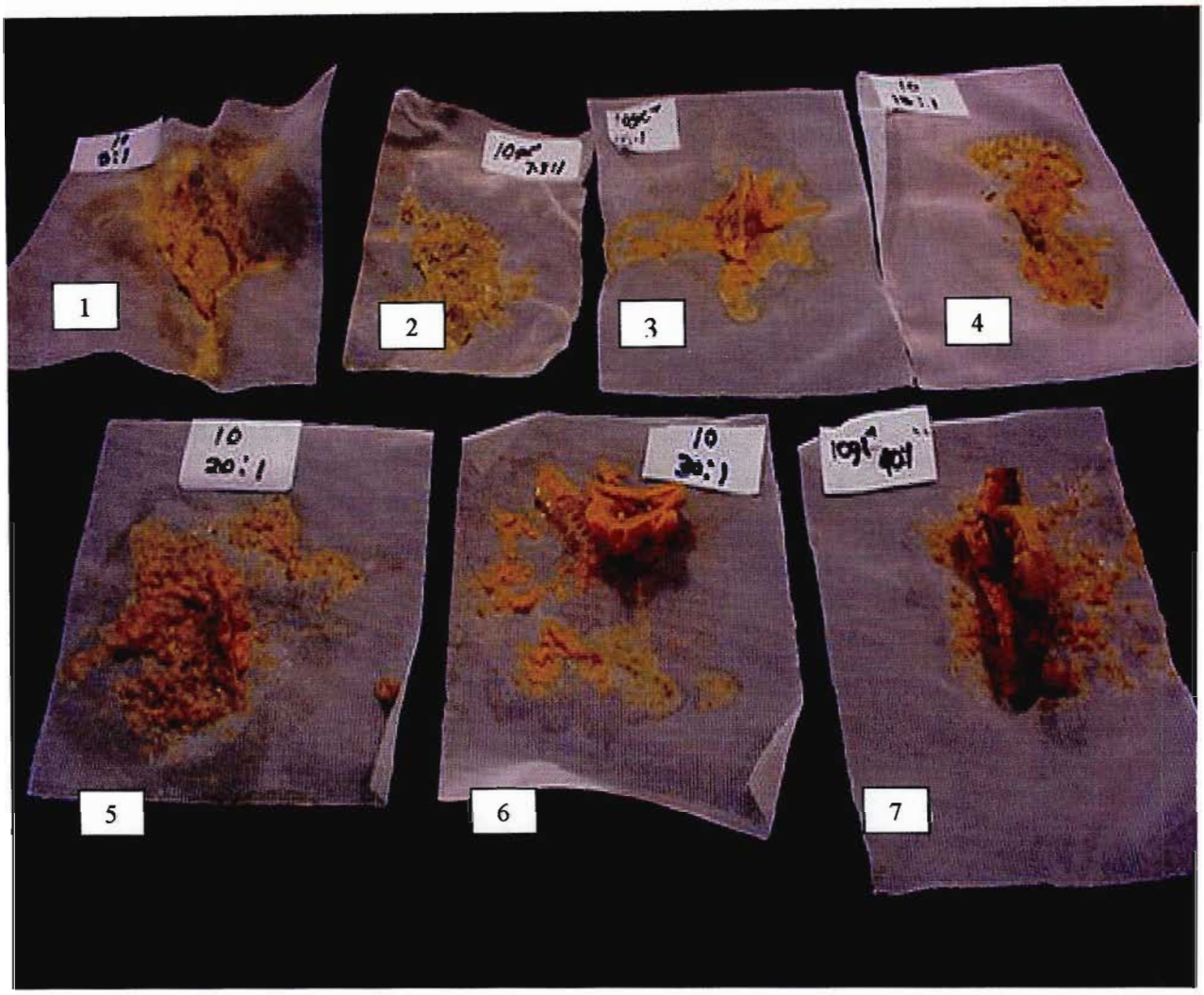

Fig. 3.12. Wet mycelial biomass of *Colletotrichum gloeosporioides* C6 at a carbon concentration of 10 g L^{-1}. The C:N ratios range from 1 (5:1) ; 2 (7.5:1) ; 3 (10:1) ; 4 (15:1) ; (20:1) ; 6 (30:1) ; 7 (40:1). There was a significant increase in mycelial biomass observed at C:N ratios of 30:1 to 40:1.

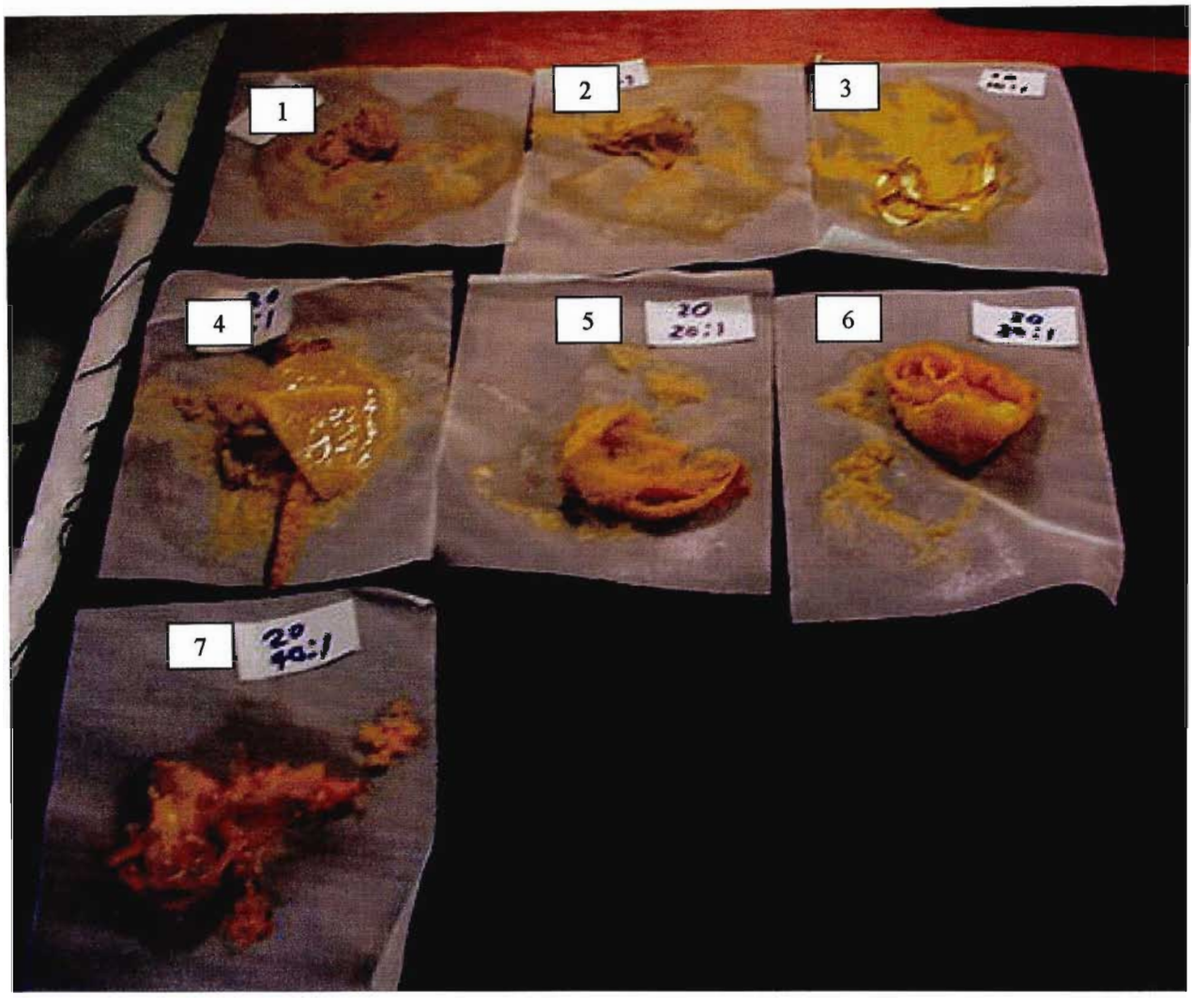

Fig. 3.13. Wet mycelial biomass of *Colletotrichum gloeosporioides* C6 at a carbon concentration of 20 g L^{-1}. The C:N ratios range from 1 (5:1); 2 (7.5:1); 3 (10:1); 4 (15:1); 5 (20:1); 6 (30:1); 7 (40:1). The highest mycelial biomass was formed at C:N of 40:1. There were no significant differences at C:N ratios 5:1, 10:1, 20:1 and 30:1.

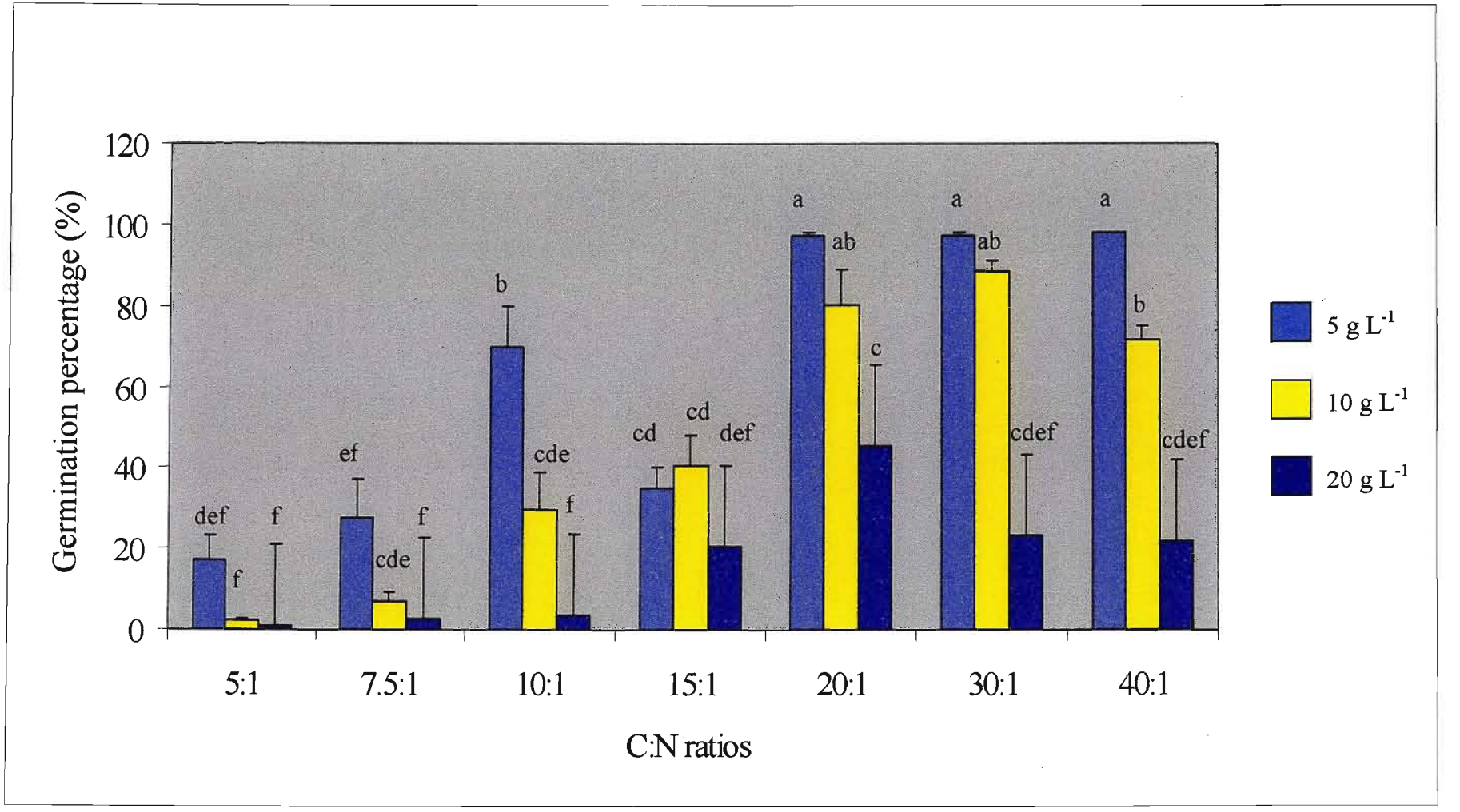

Fig. 3.14. Influence of carbon concentrations on germination pecentage of *Colletotrichum gloeosporioides* C6 grown at various C:N ratios. Within each C:N ratio grouping, mean germination percentage with different letters are significantly different ($P < 0.05$).

3.4 DISCUSSION

Carbon concentration and carbon-to-nitrogen (C:N) ratio had a dramatic effect on the submerged fermentation of *C. gloeosporioides* C6. Previous studies have shown that *Colletotrichum* spp. sporulate well in media containing soy protein. However, soy protein cannot be used as a sole nitrogen source as it is has poor solubility in water (Yu *et al.*, 1997; Yu *et al.*, 1998). In this study, KNO_3 and soy protein were used to supply equal amounts of molecular nitrogen to the defined basal salts medium.

The effect of C concentration on the sporulation of *C. gloeosporioides* C6 varied within the different C:N ratios tested (Table 3.2 and Fig. 3.5). Jackson *et al.* (1995) and Schisler *et al.* (1995) reported that the C:N ratio on the sporulation medium affected not only the spore yield but the ultimate effectiveness of the potential biological control agent (BCA). The rate at which biomass of the effective propagule, in this case, the conidia, is formed affects cost of production as well as chance of contamination and viability (Lisansky, 1985). It was concluded that *C. gloeosporioides* C6 was very sensitive to C:N ratios at both low and high C concentrations. There was no significant difference in the highest spore yields formed at 5 g L^{-1} at a C:N ratio of 7.5: 1 and the number of spores formed at 10 g L^{-1} at a C:N ratio of 15:1. The effect of C:N ratios within a constant C concentration of 5 g L^{-1} yielded variable significant differences. It can therefore be concluded that at a low C concentration, *C. gloeosporioides* C6 is very sensitive to changing C:N ratios. The effect of C:N ratios from 5:1 to 10:1 on *C. gloeosporioides* C6 at a constant C concentration of 10 g L^{-1} did not yield any significant differences. However, as C:N ratios increased at the same constant C concentration, there was a highly significant increase in spore yields. The effect of C:N ratios from 5:1 to 10:1, within a constant C concentration of 20 g L^{-1}, had no effect on the sporulation. As the C:N ratios increased from 15:1 to 40:1 there was a highly significant decrease in conidiation. It was therefore concluded that at a high C concentration, *C. gloeosporioides* C6 is highly sensitive to high C:N ratios. This indicated that both C concentration and C:N ratios had a dramatic effect on the conversion of raw materials to spores by *C. gloeosporioides* C6. Yu *et al.* (1998) showed that spores produced with optimal C:N ratios had the highest germination rates, incited more severe disease and resulted in more shoot dry weight reduction in velvetleaf.

The effect of C concentration within a C:N grouping had significant effect on mycelial biomass production (Table 3.2 and Fig. 3.7). The C concentration had a more profound effect on mycelial biomass formation than C:N ratios (Table 3.2). It was observed that an increase in hyphal melanization correlated with a significant decrease in conidiation. The mycelium of *C. gloeosporioides* C6 has not been proven to be invasive therefore the production of mycelial biomass is unfavourable. It was observed that as the C concentration with a fixed C:N ratio increased from 5 to 20 g L^{-1} carbon, there was a significant increase in mycelial biomass (Fig 3.11-3.13). However, at a constant C concentration of 5 g L^{-1} there were no highly significant differences in biomass (Table 3.2 and Fig. 3.9). It was evident that C:N ratios do not influence mycelial biomass at low C concentrations. A similar trend was observed at a constant C concentration of 10 g L^{-1} for C:N ratios of 5:1, 10:1 and 20:1 (Fig. 3.7). Significant effects of C:N on mycelial biomass at a C concentration of 10 g L^{-1} was observed at C:N ratios 15:1, 30:1 and 40:1. The highest C concentration of 20 g L^{-1} produced the largest mycelial biomass (Fig. 3.7). With a constant C concentration of 20 g L^{-1}, the C:N ratios had a varied effect, with a high mycelial biomass being produced at a C:N ratio of 7.5:1 but the highest at 40:1.

The results suggested that C concentration plays an important developmental role in the conidiation and mycelial development of *C. gloeosporioides*. However, although it was shown that higher C concentration resulted in a decrease in conidiation and an increase in mycelial biomass, analogous studies conducted by Jackson and Bothast (1990) on *C. gloeosporioides* f. sp. *aeschynomene* resulted in an increase in conidiation as the C concentration was increased, without the formation of the mycelial biomass. These results suggest that the regulation of differentiation by C concentration is not universal in *Colletotrichum* species.

A fast germinating spore is an essential feature of a commercial biological control agent (BCA). Fungal propagules must be hydrated to germinate and infect their hosts. A BCA that needs prolonged contact with free moisture is less likely to be competent in the field and has a higher risk to the adversities of nature (Templeton and Heiny, 1989; Charudattan, 1989). Therefore, a rapidly germinating spore has an added advantage in the field where free water is a limiting factor in the BCA efficacy.

The results showed that both C:N ratios and C concentrations have profound effects on germination rates. Germination significantly decreased at a fixed C:N as C concentration increased. The spores produced at lower C concentration and higher C:N ratios (5 g L^{-1}, C:N 15:1 to 40:1) germinated the fastest, i.e., spores took the shortest time to reach a 100 % germination. The highest germination percentage correlated with the lowest C concentration and higher C:N ratios from 20:1 to 40:1 (Table 3.2 and Fig. 3.10). As the C concentration increased within a C:N ratio, spore germination significantly decreased, except at C:N ratio of 15:1 where there were no significant differences at C concentrations of 5 g L^{-1} and 10 g L^{-1}. In ultrastructure studies (Chapter 4), it was observed that at the highest C concentration and C:N ratios, an extensive spore matrix was formed. The spore matrix has been shown to decrease germination. Yu *et al.* (1998) showed that at high C concentrations and high C:N ratios, nitrogen became a growth limiting factor when *C. coccodes* was produced. In this nitrogen limited environment, the extra C was converted to spore matrix when *C. coccodes* changed its metabolic pathway. These results coincide with the germination and ultrastructure studies performed on *C. gloeosporioides* C6.

One of the major constraints in commercial production of BCAs is the need to produce large numbers of viable spores at low cost. The cost of the production of a BCA directly determines its market potential and desirability. The specific spore yield (Table 3.3 and Fig. 3.12) presents the most economically viable option for the production of *C. gloeosporioides* C6 spores. Statistically, the best media was shown to have a C concentration of 5 g L^{-1} at C:N ratio of 7.5:1. These conditions coincides with highest spore yield produced at a C concentration of 5 g L^{-1} and C:N ratios 7.5:1 as well.

The highest specific mycelial biomass (Yms) was formed at C concentration of 10 g L^{-1} with a C:N ratio of 40:1 (Table 3.3 and Fig. 3.13). The highest Yms overall was formed at the lowest C concentration of 5 g L^{-1} and the Yms increased as the C:N ratio increased. It should be noted that at the C concentration of 5 g L^{-1} high Yms and Ysp values were obtained at higher C:N ratios (Fig. 3.6 and 3.8) The ideal would be the production of high Ysp and lower Yms. This could be achieved with a C concentration of 5 g L^{-1} with a C:N ratio of 7.5:1 and 15:1.

Efficiency should be taken into account when trying to optimize conditions for the mass production of any BCA. This prompted the question " Are media that produce the highest spore yield producing the most effective spores?" A study conducted by Jackson (1997) to evaluate the traits of conidia produced by *C. truncatum* in different nutritional environments (C:N ratios 10:1, 30:1 and 80:1) addressed this question. Media with a C:N ratio of 30:1 produced the highest spore yield. However, media with a lower C:N of 10:1 produced more effective spores. Compositional analyses showed that at lower C concentrations, conidia contain more protein and less lipids. The appearance of lipids at higher C:N ratios and C concentrations was also observed in studies conducted on the ultrastructure of the spores produced in the growth conditions defined in this chapters. This will be discussed more fully in Chapter Four. The excess C at higher C concentrations were converted to lipids (Jackson, 1997). Further green-house trials need to be conducted to determine the efficacy of the conidia obtained from each C:N and C concentration grouping. It was concluded that spore yield should not be the sole criterion used in media optimization as the media that produced that highest spore yield does not necessarily produce the most effective spores in the field

Taking into account all parameters, it was concluded that the best option for the commercial production of *C. gloeosporioides* C6 is at a C concentration of 5 g L-1 and at a C:N ratio of 20:1. The ideal option for highest spore yield is at a C:N ratio of 7.5:1. However, a low germination percentage was recorded at this C:N ratio and this would decrease the efficacy of the BCA in the field.

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CHAPTER 4

Effect of carbon concentration and carbon-to-nitrogen ratio on the ultrastructure of *Colletotrichum gloeosporioides* C6

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The effect of carbon (C) concentrations and the carbon-to-nitrogen (C:N) ratio of a selected medium on the ultrastructure of *Colletotrichum gloeosporioides* C6 (Penz. and Sacc.) spores was investigated. *Colletotrichum gloeosporioides* C6 is used in the biological control of the invasive weed Silky Hakea (*Hakea sericea* Schrad.). The morphological characteristics of the spore are significantly affected by C concentrations and C:N ratios of the media and this in turn affects the efficacy of the *C. gloeosporioides* C6 spores. Cell wall thickness increased with increasing C concentrations and C:N ratios reaching a maximum thickness at a C concentration of 20 g L^{-1} and a C:N ratio of 40:1. Fewer but larger mitochondria were observed at lower C concentrations and C:N ratios. Numerous and larger lipid bodies were formed at higher carbon concentrations and C:N ratios. The extracellular matrix appeared globular at a C concentration of 5 g L^{-1} whereas at 40 g L^{-1} C a fine fibrillar network was present. The extracellular matrix was sparse at a TOC of 10 g L^{-1}. These criteria can be used to optimize large-scale and economic production of *C. gloeosporioides* C6.

4.1 INTRODUCTION

Use of plant pathogens to economically achieve control of weeds exemplifies a welcomed effort to integrate plant pathology, weed science and plant physiology in their broadest sense (Charudattan and Walker, 1982). Conventional biological control involves the manipulation of agricultural practices to intensify the impact of natural enemies already present on the weed. Bioherbicides by definition include plant pathogens and phytotoxins, derived from pathogens or

other microorganisms, that may be useful for weed control (Hoagland, 1989). Biological control with plant pathogens is an effective, safe, selective and practical means of weed control (Charudattan, 1989).

Biological control agent (BCA) inocula should ideally, be cultivated on, and efficiently recovered from inexpensive media, be effective in the field and retain viability after long storage periods (Charudattan, 1989; Agosin *et al.*, 1997). In order to effectively control weeds with BCAs factors such as the economic mass production of infectious inocula should be thoroughly investigated.

Colletotrichum gloeosporioides C6 has been effectively used to control of Silky Hakea (*Hakea sericea* Schrad.) an invasive weed originating from New South Wales and Victoria, Australia (Anonymous, 2002). The effects of C concentration and C:N ratios on the growth, conidiation and germination of the *C. gloeosporioides* C6 spores were conducted (Chapter 3). The quality and the quantity of spores can be manipulated by the nutritional environment (Agosin *et al.*, 1997). Investigations by Jackson (1997) on *Colletotrichum truncatum* showed that conidia generated at optimum growth conditions for biomass production did not necessarily produce spores with the best efficacy. The optimum growth production parameters were found to be at C concentration of 5 g L^{-1} and at a C:N ratio of 20:1. Various morphological factors such as extracellular matrices, lipid bodies and cell wall structure have a profound effect on spore efficacy and viability (Nicholson and Moraes, 1980; Leite and Nicholson, 1992; Jones *et al.*, 1995; Mims *et al.*, 1995; Mims *et al.,* 1988; Agosin *et al.*, 1997).

A variety of microscopical techniques and molecular probes have been used to study the ultrastructure and composition of fungal spores. In this study, we intended to document the morphological changes of *C. gloeosporioides* C6 under different C concentrations and carbon-to-nitrogen (C:N) ratios using the Transmission Electron Microscope (TEM).

In addition correlations were made from findings in Chapter 3 regarding germination rates and morphological differences in spores.

4.2 MATERIALS AND METHODS

Colletotrichum gloeosporioides C6 spores produced as specified in Chapter Three were harvested for the ultrastructure experiments. The C concentration and C:N ratios of the defined basal salts medium were adjusted as tabulated in Table 4.1

Table 4.1. Concentration of sucrose, soy protein and KNO_3 in media with 5 g L^{-1} total organic carbon at various C:N ratios (Yu *et al.*, 1998)

C:N Ratio	Sucrose (g L^{-1})	Soy Protein (g L^{-1})	KNO_3 (g L^{-1})
5:1	7.95	3.40	3.61
7.5:1	9.26	2.26	2.41
10:1	9.92	1.70	1.81
15:1	10.57	1.13	1.20
20:1	10.9	0.85	0.90
30:1	11.23	0.57	0.60
40:1	11.39	0.43	0.45

Spores were separated from the mycelium by filtering the medium through two layers of sterilized cheesecloth. Spore suspensions were centrifuged at 41 400 x g (18 500 rpm) for 10 minutes using a JA-20 Beckman rotor. The pellet was retained and washed with distilled water, centrifuged under the same conditions and resuspended in distilled water to the original volume.

Transmission electron microscope (TEM)

Spores were fixed in 3% gluteraldehyde in 0.05M sodium cacodylate buffer (pH 7.2) for a minimum of 8 hours and washed twice in that buffer for 2 x 30 minutes. The spores were then post-fixed for 3 hours in 2% OsO4 in 0.05M buffer at room temperature. The sample was then washed twice in 0.05M buffer for 2 x 30 minutes. Samples were dehydrated in graded alcohol series (10%- 70%), for 10 minutes per solution. Spores were then embedded in Epon-Araldite resin. Serial sections were cut with a diamond knife and collected on copper slot grids. Sections

on copper grids were poststained with uranyl acetate and lead citrate. Specimens were viewed with a Jeol 100 CX transmission electron microscope.

4.3 RESULTS

Varying C concentrations and C:N ratios of the growth media were found to have a profound effect on the ultrastructure of the resulting *C. gloeosporioides* C6 spores. As C concentrations and C:N ratios were increased, corresponding increases in cell wall width were apparent (Table 4.2). All conidia were coated with varying amounts of extracellular matrix (EM). All conidia examined were uninucleate. Lipid bodies were more abundant in spores produced at higher C:N ratios and C concentrations. In some cases membranes of these bodies were smooth and in other cases were angular or irregular in appearance. Other organelles present in *C. gloeosporioides* C6 conidia were multivesicular bodies with electron dense contents thought to be autophagic vacuoles, strands of rough tubular endoplasmic reticulum (TER) and microtubules (MT).

At a C concentration of 5 g L^{-1} and C:N ratio of 5:1, spores with large conspicuous nucleus and mitochondria (M) surrounded by ribosomes (R) were apparent (Fig. 4.1). Conidia were surrounded by a globular copious layer of extracellular matrix (EM). Examination of the cell walls at higher magnification (x 74 K) revealed a thin inner and outer layer surrounded by the EM. This treatment produced conidia with the thinnest cell walls measuring an average of 42.75 nm (Table 4.2 and Fig. 4.2). All subsequent cell measurements were taken as shown in Fig. 4.2.

Large lipid (L) bodies dominated the cytoplasmic volume of spores grown at a C concentration of 5 g L^{-1} and a C:N ratio of 40:1 (Fig. 4.3). A number of small mitochondria, two autophagic vacuoles (AV) and glycogen deposits were also visible. Autophagic vacuoles are large digestive vacuoles which contain digestive enzymes and in which cellular organelles are digested (Jennings and Lysek, 1996). The insert clearly shows the surface of the conidium covered by a fine mucilaginous layer of extracellular matrix and a thicker cell wall than that produced at a lower C:N treatment of 5:1. The average cell wall thickness was measured to be 88.40 nm (Table 4.2).

Transmission electron micrographs of spores produced in a basal salts medium at a C concentration of 10 g L^{-1} and C:N ratio of 5:1 indicated numerous mitochondria, vacuoles and a single large nucleus (Fig. 4.4). Visible in the inner and outer cell walls are plasmalemma plates (PP) dislodged from the plasma membrane during sectioning (Fig. 4.4, inset). Conidia produced at this treatment show thin, sparse extracellular matrix barely visible at high magnification (x 74 K). Cell wall thickness was measured as 166.92 nm (Table 4.2).

The prominent characteristic of conidia produced at a C concentration of 10 g L^{-1}, C:N ratio of 40:1, was the presence of large lipid bodies (Fig. 4.5). These lipid bodies appear as electron-transparent spaces in the spore cytoplasm. A number of small mitochondria are distributed in the cytoplasmic region. The cell wall thickness was measured as 188.95 nm (Table 4.2).

Conidia grown in a basal salt treatment of 20 g L^{-1} C concentration and a C:N ratio of 5:1 produced numerous small lipid bodies. The inner and outer cell wall are only visible at much higher magnification (x 74 K) as illustrated in the inset. A cross wall or septum (S) is visible running down the length of the spore (Fig. 4.6). An interesting feature of this TEM micrograph is the arrangement of the numerous lipid bodies and the large nucleus on either side of the septum. A number of small mitochondria on either side of the septum was observed. The thickest cell wall thickness measuring 287.63 nm was formed at this treatment (Table 4.2).

Two types of spores were produced at a C concentration of 20 g L^{-1}, C:N ratio of 40:1. The first type (Fig. 4.7) exhibited an extensive fibrillar extracellular matrix and a number of large lipid bodies. Glygcogen deposits were also present but no mitochondria and nulceus were observed. Numerous large mitochondria and a single nucleus dominated the cytoplasmic region in the second type of conidia formed (Fig. 4.8). The extracellular matrix of this conidia was not like the extensive network found in the type one but formed a sparse layer around the spore coat. The cell wall thickness measured at this treatment was 131.94 nm (Table 4.2 and Fig. 4.9).

Table 4.2. Cell wall thickness of *Colletotrichum gloeosporioides* C6 at various carbon concentrations and C:N ratios

Treatments (Carbon; C:N)	5 g L^{-1}, 5:1	5 g L^{-1}, 40:1	10 g L^{-1}, 5:1	10 g L^{-1}, 40:1	20 g L^{-1}, 5:1	20 g L^{-1}, 40:1
Cell wall thickness (nm)	40.00	109.09	193.75	195.22	271.63	129.41
	42.50	85.45	156.40	176.64	295.89	136.12
	52.50	83.64	189.58	198.01	295.89	134.12
	37.50	123.64	152.94	187.92	287.67	127.06
	45.00	74.03	15625		282.19	132.98
	35.00	80.70	152.60		288.65	
	45.00	62.27			286.43	
	37.50					
	45.00					
	47.50					
Average cell wall thickness (nm)	42.75	88.40	166.92	188.95	287.63	131.94

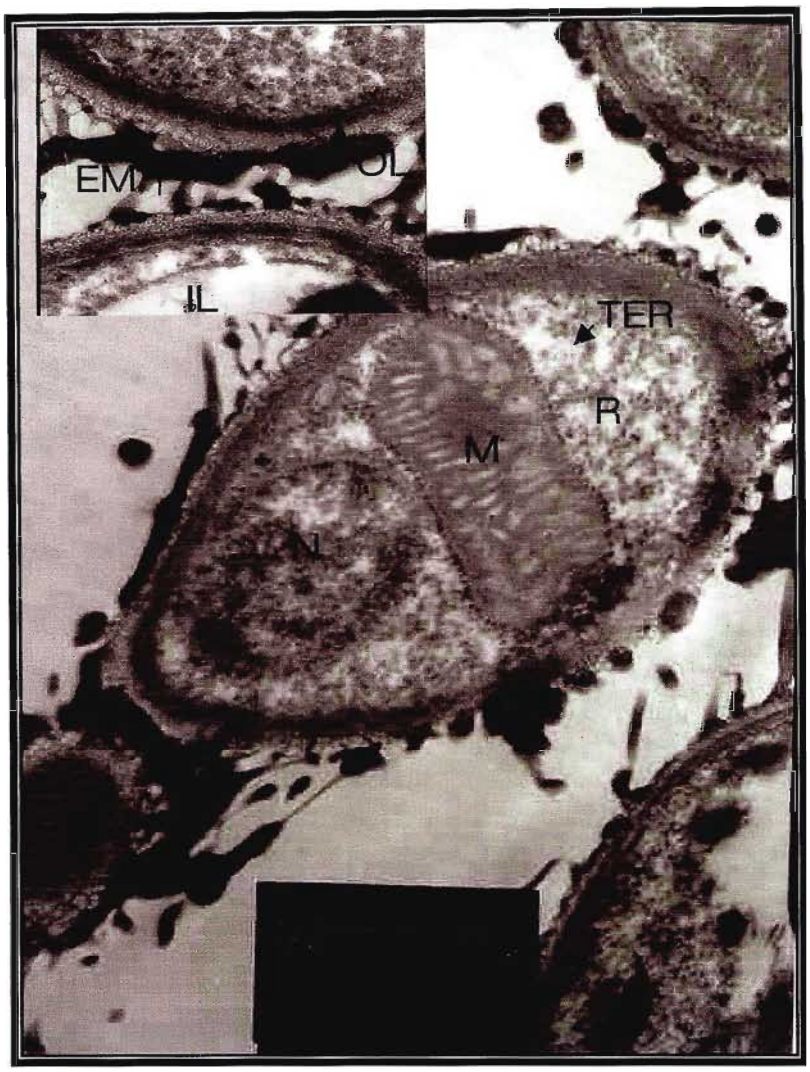

Fig. 4.1. TEM micrograph of *Colletotrichum gloeosporioides C6* spore grown in a 5 g L^{-1} carbon concentration basal salts medium with a C:N ratio of 5:1. Visible are, a single large mitochondrion (M) surrounded by ribosomes (R), a single nucleus (N) and tubular ER (TER). The inner (IL) and outer (OL) layers of the conidium wall are more clearly defined in the inset. The extracellular mucilage (EM) forms a globular layer around the conidium.

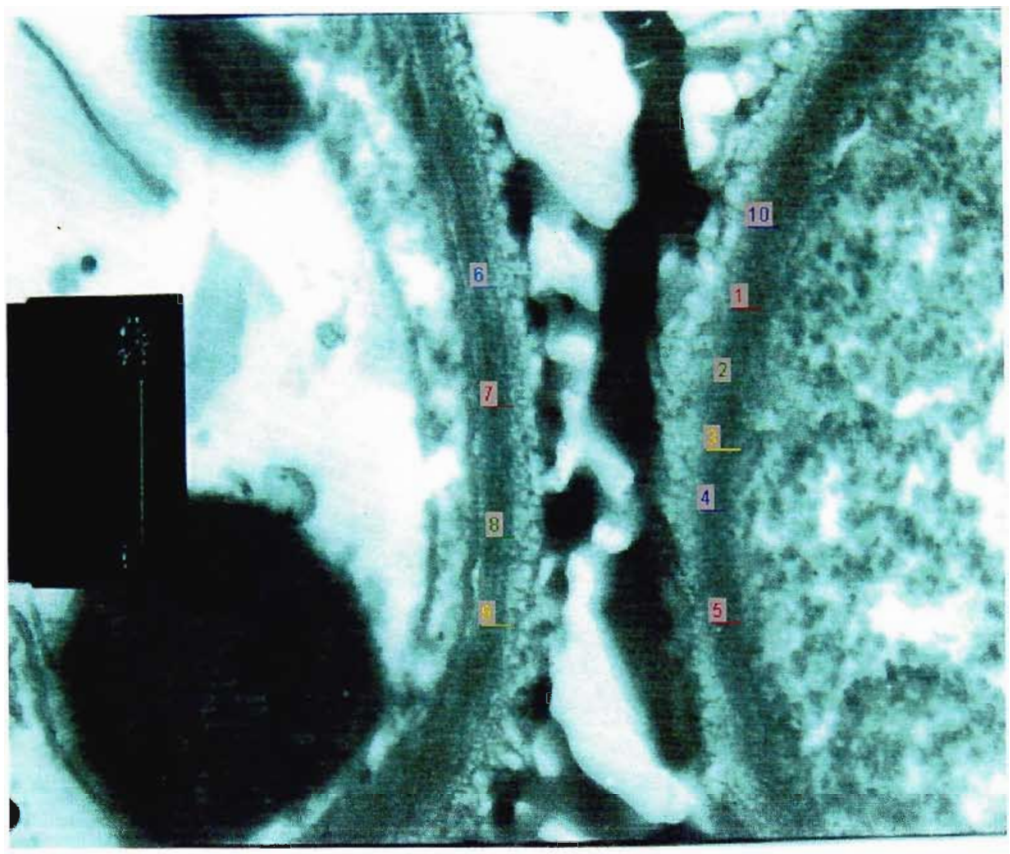

Fig. 4.2. Cell wall of conidium produced at carbon concentration of 5 g L^{-1} at a C:N ratio of 5:1. The numeric markings indicate various measurements of the cell wall thickness. The average cell wall thickness was calculated as 42.75 nm.

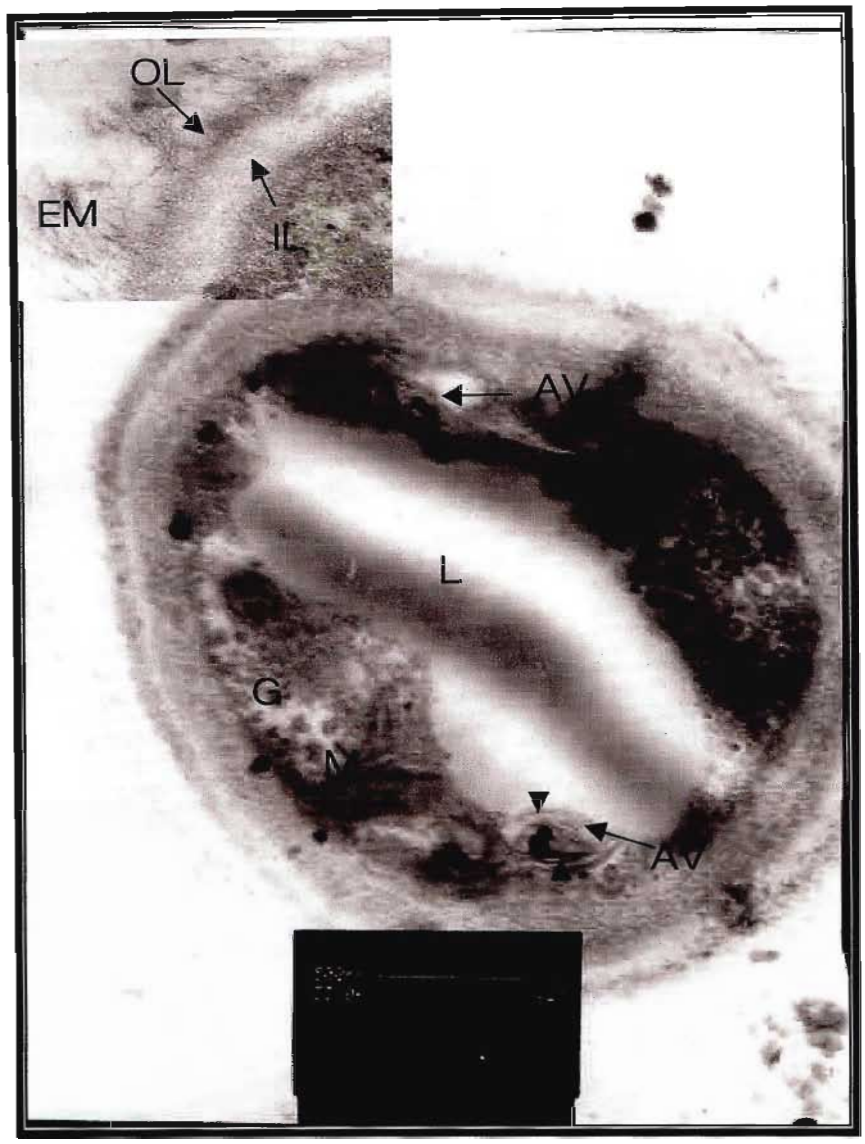

Fig. 4.3. Ultrastructure of a *Colletotrichum gloeosporioides* C6 spore grown in basal salts medium with a carbon concentration of 5 g L^{-1} and a C:N ratio of 40:1. Visible are small mitochondria (M), and what appears to be autophagic vacuoles enclosed by double membranes (AV). Lead citrate was used to stain the large lipid droplet visible. The inset shows the inner (IL) and outer (OL) layers of the conidium wall. The extracellular mucilage (EM) forms a fine fibrillar network. The fine granular electron dense bodies are possibly glycogen.

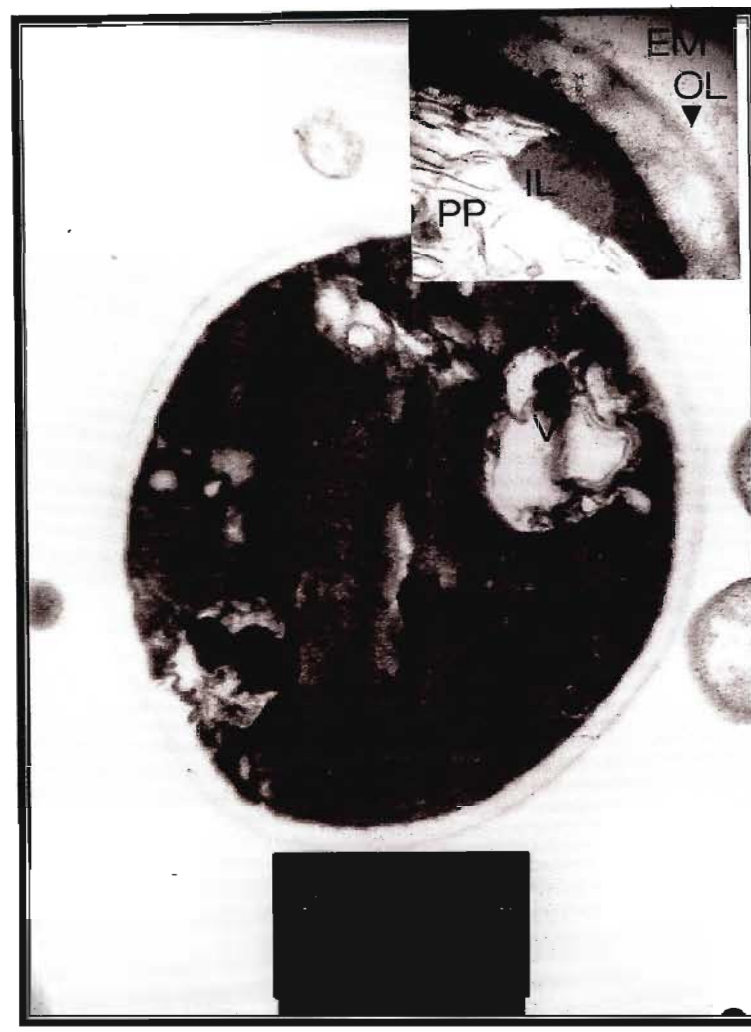

Fig. 4.4. TEM micrograph of *Colletotrichum gloeosporioides* C6 spore grown in a 10 g L^{-1} carbon concentration basal salts medium with a C:N ratio of 5:1. Visible are numerous small mitochondria (M) and a single large nucleus (N). The inset shows plasmalemma plates (PP), the inner (IL) and outer (OL) layers of the conidium wall. Extracellular mucilage (EM) is absent.

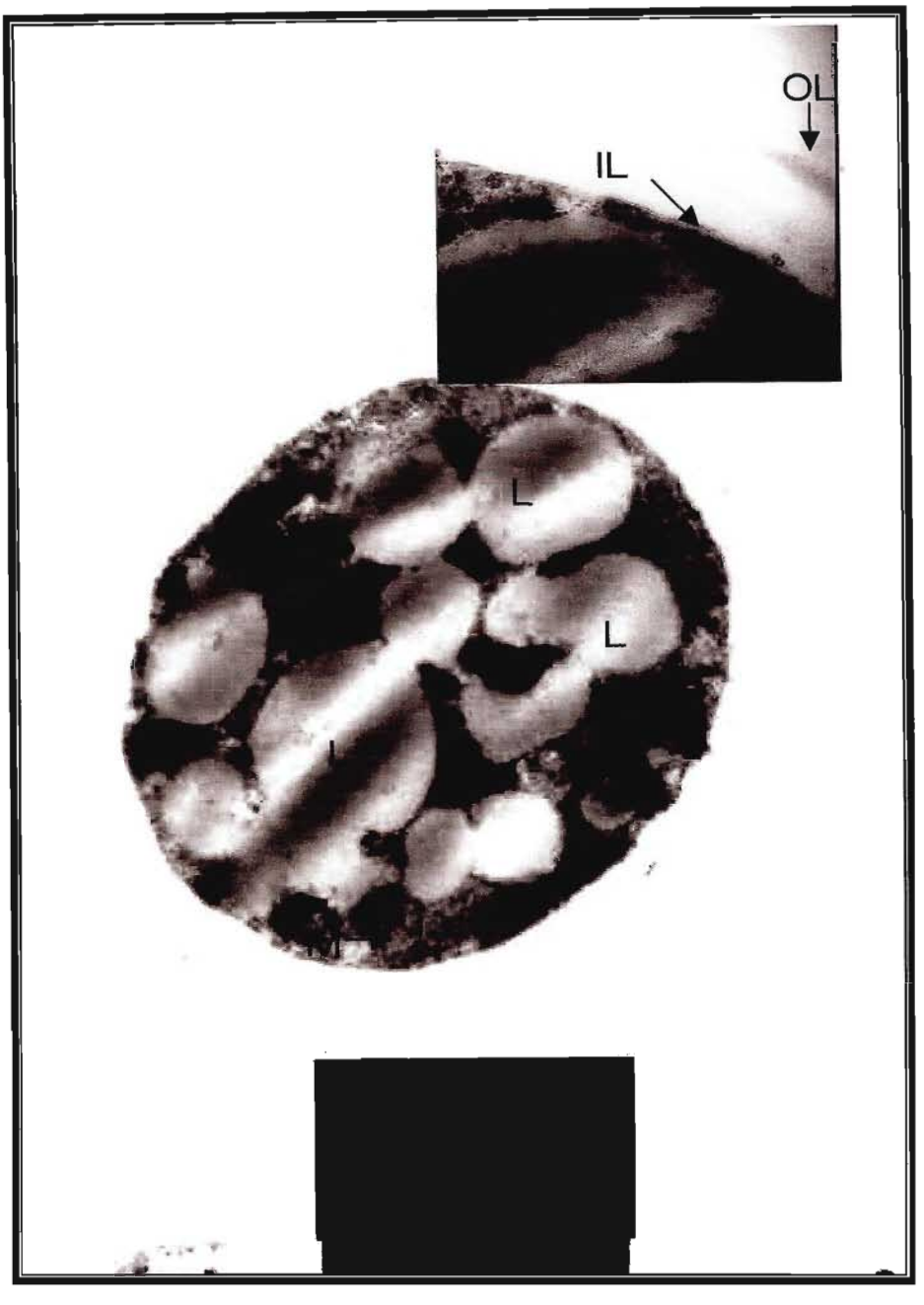

Fig. 4.5. Transmission Electron Micrograph of *Colletotrichum gloeosporioides* C6 spore treated with 10 g L^{-1} carbon basal salts medium with a C:N ratio of 40:1. Visible within the conidium are numerous small mitochondria (M) and the electron-transparent remains of extracted lipid bodies (L). Treatment with osmium tetroxide provides the contrasting of lipids relative to other organelles in the micrograph. The inner (IL) and outer (OL) layers of the conidium wall are more clearly illustrated in the inset. Extracellular mucilage (EM) is absent.

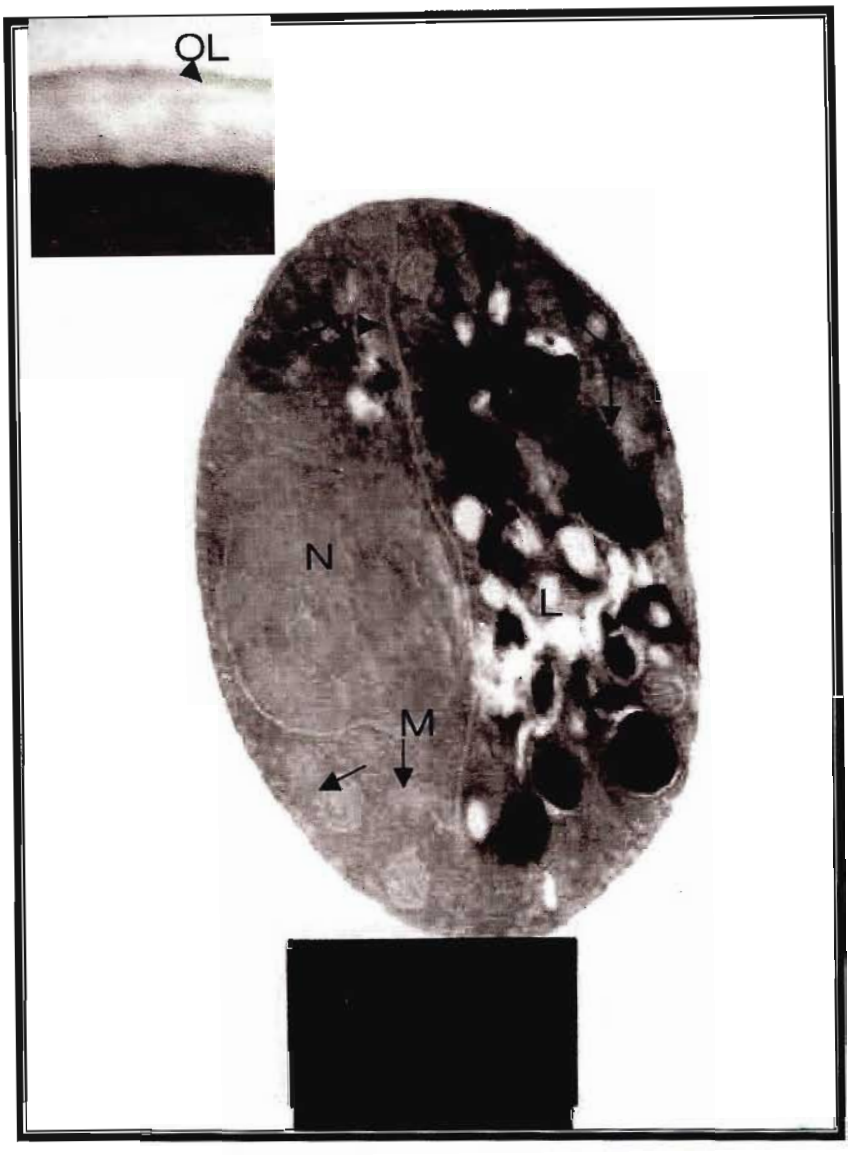

Fig. 4.6. TEM micrograph of *Colletotrichum gloeosporioides* C6 spore grown in a 20 g L^{-1} carbon basal salts medium with a C:N ratio of 5:1. Visible are numerous small mitochondria (M), a large single nucleus (N) and what appears to be a septum (S). The lipid (L) bodies are large and the electron-transparent regions are lipids that have been leached from the specimen. The inner (IL) and outer (OL) layer of the cell walls are very faint but more clearly visible in the inset.

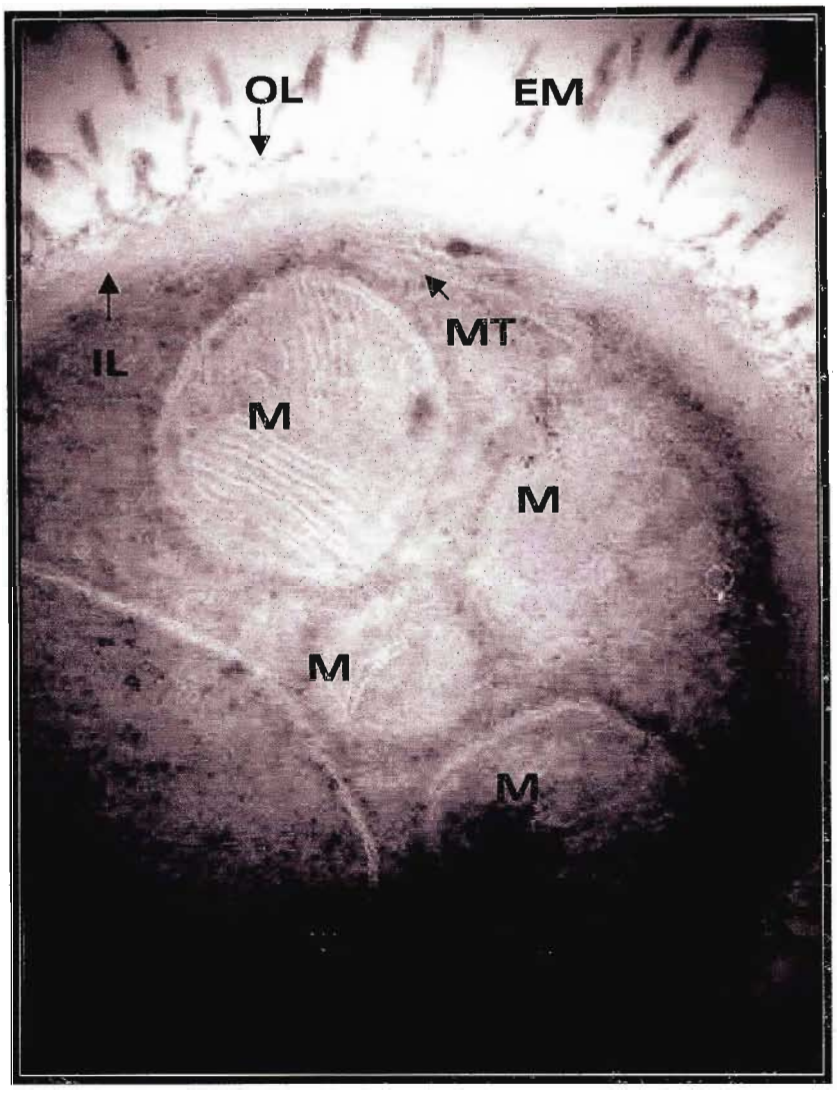

Fig. 4.7. TEM micrograph of *Colletotrichum gloeosporioides* C6 spore grown in a 20 g L^{-1} carbon basal salts medium with a C:N ratio of 40:1. Visible are numerous large mitochondria (M). Clearly defined are the inner (IL) and outer (OL) layers of the conidium wall. Extracellular mucilage forms a vast fibrillar network. Lipid (L) bodies are absent. A large nucleus (N) and microtubules (MT) are present.

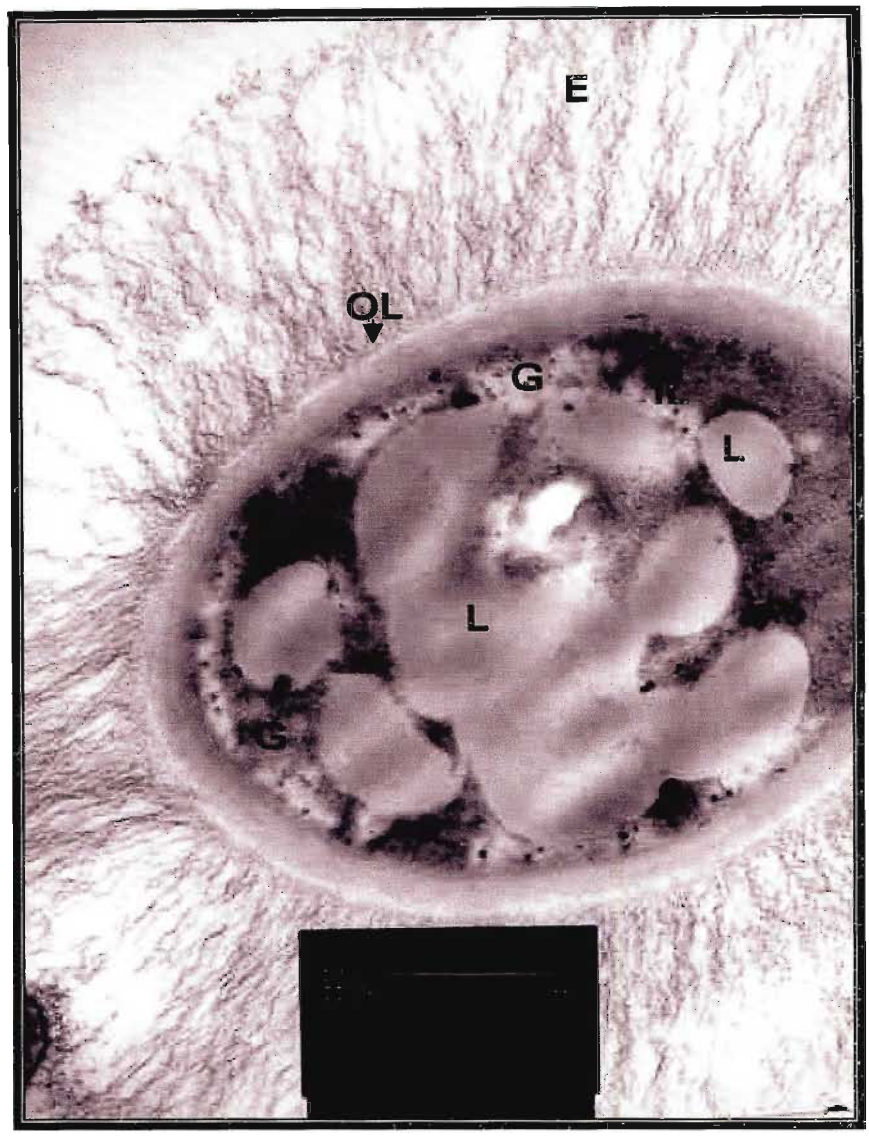

Fig. 4.8. TEM micrograph of *Colletotrichum gloeosporioides* C6 spore grown in a 20 g L^{-1} carbon basal salts medium with a C:N ratio of 40:1. The inner (IL) and outer (OL) layers of the conidium wall are cleary visible with a vast fibrillar network. Lipid (L) bodies are large and have been leached from the specimen. Small deposits, most likely to be glycogen are also present.

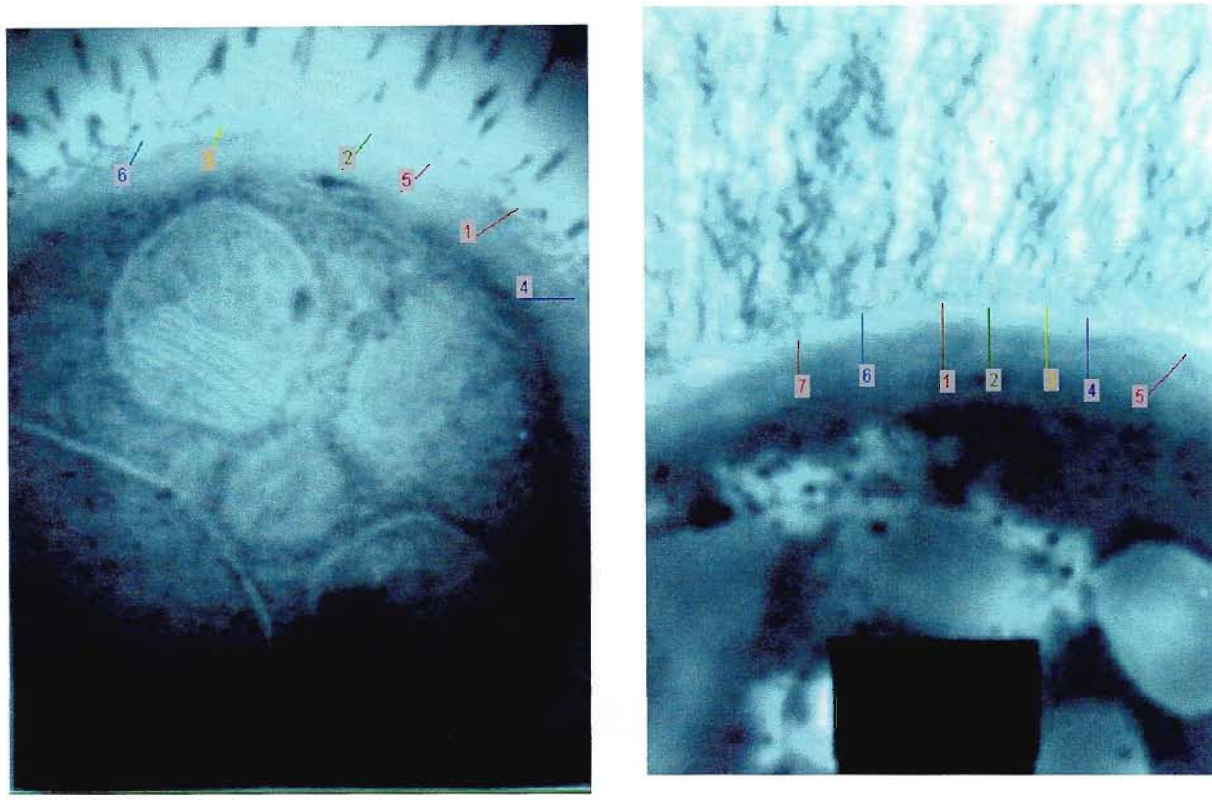

Fig. 4.9. Cell wall of conidia produced at carbon concentration of 20 g L^{-1}, C:N ratio of 40:1. The numeric markings are various measurements of the cell wall thickness. The average cell wall thickness was calculated to be 131.94 nm.

4.4 DISCUSSION

Research of biological control agents has focused on optimizing the production of propagules that are suitable for field application (Lewis and Papavizas, 1991). The ultrastructure of spores is a key factor contributing to the viability and effectivity of the BCA.

The cell wall thickness in spores used in BCAs is a very important feature, as the cell wall in the absence of the extracellular matrix is the spore's first barrier against adverse conditions and increases the shelf life of the BCA (Agosin *et al.*, 1997). As the carbon concentration was increased from 5 g L^{-1} (Fig. 4.2) to 20 g L^{-1} (Fig. 4.6), there was a significant increase in cell wall thickness from 42.75 nm to 287.63 nm (85.14 % increase). The decrease of the cell wall thickness at a C concentration of 20 g L^{-1} and C:N ratio of 40:1 was unexpected (Fig. 4.13).

The extracellular matrices play a pivotal role in spore differentiation and development on host surfaces. Evidence of the importance of extracellular matrix in the adhesion of fungal spores to the host has been reported by Nicholson and Epstein (1991) and Jones (1994). Adhesion is essential for initial spore attachment anchorage of the appressorium during mechanical penetration of the host cuticle (Hamer *et al.*, 1988; Howard *et al.*, 1991). Additionally, the extracellular matrix enables spores to survive extended periods of desiccation (Nicholson and Moraes, 1980) as well as the toxic effects of polyphenolic compounds released by plant tissue as a response to infection (Nicholson and Moraes, 1980; Nicholson *et al.*, 1986; Nicholson *et al.*, 1989). Another important feature of the extracellular matrix is its ability to act as a barrier to diffusion thereby reducing the loss of endogenous nutrients and maintenance of extracellular pH and ion balances in an aqueous medium (Moloshok *et al.*, 1993).

The extracellular matrix of some fungi contains a diverse number of degradation enzymes. Previous studies have shown that the spore matrix contains invertase (Bergstrom and Nicholson 1977; Bergstrom, 1978) and a non-specific hydrolase (Anthenill, 1978; Anthenill and Nicholson, 1978). Invertase plays a role in the acquisition of a C source for the germinating spore (Bergstrom, 1978) and hydrolase in the degradation of the leaf cuticle (Anthenill and Nicholson, 1978). The most extensive extracellular matrix formed a fibrillar network at the highest TOC of 20 g L^{-1} and C:N of 40:1, forming a large barrier around the spore (Fig. 4.7 and Fig. 4.8).

Lipid body formation was affected by the C concentration and C:N ratio. Lipid bodies found in the cytoplasm are made up of glycerolipids (Griffin, 1994). Glycerolipids are divided into two groups: the acylgycerols and phosphoglycerides. Acylgylcerols occur in fungi as the major constituents of oil droplets suspended in the mycelial and spore cytoplasm. They are also minor constituents of membranes and cell walls (Griffin, 1994).

The production of lipid bodies in conidia of biological control agents are of considerable importance. They help prevent desiccation of the spore and may contribute to the surface properties (Deacon, 1980). Rate of spore germination of a BCA is also a desirable feature of BCA spores. Faster germinating spores have an added edge in the field where free moisture is limited. During germination there has to be a supply of energy and metabolic precursors for growth. Glycogen, trehalose and lipids are common as reserve sources responsible for that energy supply (Jennings and Lysek, 1996). Lipids can constitute between 1-13 % of the dry weight of a fungal spore (Hoch, 1986). In electron microscopy studies, lipid droplets can be seen to disappear on germination. This evidence of lipids as a reserve source for germination are confirmed by studies which show that the respiratory quotient during germination of fungal spores can be correlated to the rate of lipid utilization. This theory correlates with the empirical results obtained in Chapter Three. It was noted that the lowest germination rate was found at the highest C:N ratio of 40:1 and at higher C concentrations, and the most abundant lipid bodies were observed at these conditions. Lipids are also more suited as storage bodies for fungi as they are lighter and contain more energy than polysaccharides or proteins (Jennings and Lysek, 1996). The acylgylcerols are particularly suited for their primary function, i.e., storage material having the highest caloric content of any fungus constituent (Griffin, 1994). Lipid bodies were most apparent when a C:N ratio of 40:1 was used, for the entire range of C concentrations tested (Fig. 4.3; Fig. 4.5; Fig. 4.7 and Fig. 4.8).

Mitochondria (M) were prominent in the treatment of 20 g L^{-1} C concentration and C:N ratio of 40:1 (Fig. 4.11- Fig. 4.12). Mitochondria are the energy producing organelles in spores (Griffin, 1994). The presence of mitochondria plays an integral role in the glyoxylate cycle. The relevance of the glyoxylate cycle to fungi lies in the fact that it plays a major role in the

conversion of stored lipids, especially fatty acids, to carbohydrates. Germinating fungal spores require a constant supply of sugar for new cell wall synthesis (Anonymous, 2002).

The lowest percentage germination was attained at the highest carbon concentration of 20 g L^{-1}. This correlates with the empirical studies conducted in Chapter Three. The most extensive extracellular matrices was formed at a carbon concentration of 20 g L^{-1} and it has been shown by Yu *et al.* (1998) that spore matrices inhibit germination.

Highest spore yield and specific spore yield, lowest mycelial biomass production and fastest percentage germination were the criteria used in Chapter Three to discern the optimum parameters to produce C. *gloeosporioides* C6 spores. The best option was deduced to be a carbon concentration of 5 g L^{-1} with a C:N ratio of 20:1. However, when taking into account favourable criteria of ultrastructure studies such as thick cell walls, production of lipid bodies and presence of extracellular matices, the ideal option is a media with a higher carbon concentration of 20 g L^{-1} with a C:N ratio of 5:1.

A medium with a higher carbon concentration and C:N ratio produces spores that would have a longer shelf life, would be more resistance to ultraviolet light and have a greater chance of survival in adverse conditions. However, media with a lower carbon concentration produce faster germinating spores that might have a greater efficacy in the field, especially where water is a limiting factor. Definite conclusions about the best option for the production of *C. gloeosporioides* C6 cannot be drawn without conducting pathogenicity tests. Pathogenicity tests were not within the scope of this investigation but is an area that requires further research before the optimum parameters for the commercial production of *C. gloeosporioides* C6 can be determined.

In conclusion, this study indicates that spore ultrastructure of *C. gloeosporioides* C6 can be manipulated by C concentration and C:N ratio of the production media. The control of C concentration and C:N ratios are fundamental in obtaining the most effective spores of *C. gloeosporioides* C6 for the biological control of *Hakea sericea*.

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CHAPTER 5

GENERAL OVERVIEW

5.1 INTRODUCTION

Colletotrichum gloeosporioides C6 fungal pathogenic spores used as contact biological control agents (BCAs) are highly effective in the control of weeds. The ability of the spores to effectively infect and kill their host has increased the demand of mycoherbicides. Special consideration needs to be given with regards to the mass production of the fungal pathogens as spore yield and spore quality are important factors when media optimization procedures are being assessed. Other than achieving high spore biomass, spores should be tolerant to desiccation, retain viability, even as a dry preparation and exhibit good biocontrol efficacy in the field (Jackson, 1997). Nutritional factors can be manipulated during media optimization to influence the quality of fungal spores, ensuring that endogenous reserves such as lipids are readily formed enhancing fungal spores quality. Yu *et al.* (1998) demonstrated the impact of nutrition on spore "fitness" of *Colletotrichum coccodes* (Wallr.) for use as a BCA. An understanding of how the nutritional environment influences sporulation is needed in order to predict what types of low-cost complex media can be used for the production of the BCA (Jackson and Bothast, 1990). It has been established that nutritional factors, in particular C:N ratios and TOC, influence both the vigor and viability of spores. Optimum conditions need to be determined for sporulation and enhancing the pathogenicity and viability of the spores produced. The main aim of the small-scale production of the BCAs in this thesis was the verification of laboratory procedures and their modification to permit the scale-up of the production to commercial scale.

5.2 SOLID-STATE vs SEMI-SOLID STATE FERMENTATION

It was established that semi-solid state fermentation was the better option for the production of *C. gloeosporioides* C6 than solid-state fermentation. Semi-solid state

fermentation produced prolific growth of *T. harzianum* kmd and *G. virens* MM1. The results indicated that :

- An inert support medium was more effectively sterilized and utilized than an organic support medium
- Pine shavings and molasses media produced prolific growth of *T. harzianum* kmd, *C. gloeosporioides* C6 and *G. virens* MM1.
- Semi-solid fermentation produced a higher spore yield and spores were easier to harvest
- Parameters such as TOC and C:N were easily adjusted in the pine shavings and molasses medium and sterilization procedures had fewer effects on the constituents of the medium than the organic gel medium.
- *C. gloeosporioides* C6 integrated its mycelium and spores within the gel matrix decreasing spore yield substantially
- Addition of basal salts media enhanced spore production

Literature pertaining to the use of solid-state fermentation (SSF) for the production of BCAs is very sparse (Cannel and Moo-Young, 1980). Reasons for this include, the need for a pre-processing step (viz., tyndallization experiments in Chapter Two), specialized equipment for sterilization and increased problems with contamination when a solid substance is used as growth media (Silman *et al.*, 1993). Contamination was one of the contributing factors that resulted in oats and sorghum being discarded as growth media in the solid-state fermentation experiments conducted in this dissertation. The formulation and harvesting of *C. gloeosporioides* C6 spores also posed a problem. Vacuuming of spores off the solid media is the most common method employed in harvesting the BCA spores. However, this was not an effective method in this study as majority of the spores had integrated themselves in the solid gel medium. A factor that should be taken into consideration in the SSF production of mould or filamentous fungi is that these fungi are adapted to penetrate solid substances thereby posing problem during the harvesting of spores. Fungi also produce extra-cellular hydrolases that break down biopolymers in solid substrates, thereby changing the media constituent concentrations. This problem can be overcome by the use of inert substrates to provide surface area for growth.

However, the ability of the filamentous fungi such as *Aspergillus oryzae* Cohn. to penetrate solid substrates is an advantage in the food industry in the production of soya sauce. It is also important to note that BCAs are living entities are therefore susceptible to all environmental and nutritional parameters that control their existence and activities (Nakas and Hagedorn, 1990). Physical and chemical factors are the primary factors that affect the efficacy of a BCA. A nutrition-related phenomenon in fungal BCAs is the effect of the substrate on the production of secondary metabolites (Nakas and Hagedorn, 1990). The substrate on which the fungus was cultivated affects not only the antibiotic production of a fungus but also the production of phytotoxic compounds (Howell and Stipanovic, 1984). Semi-solid fermentation, which was the preferred method of fermentation for production of *C. gloeosporioides* C6, is useful for the production of fungi that do not sporulate in liquid culture and do not survive in the liquid fermentation process (Lisansky, 1985).

5.3 CARBON-TO-NITROGEN RATIO AND TOTAL ORGANIC CARBON

From the experiments conducted (Chapter Three and Four) it is evident that C:N ratios and TOC have a profound effect on the morphology and physiology of *C. gloeosporioides* C6. It was established :

- an increase in TOC (5 g L^{-1} to 20 g L^{-1}) and C:N ratios (5:1 to 40:1) resulted in a decrease in spore yield and increase in mycelial biomass
- highest spore germination occurred at the lowest TOC of 5 g L^{-1}
- specific spore (Ysp) and mycelial (Yms) yield are important parameters in the economical production of *C. gloeosporioides* C6
- Best results for the formation of Ysp and Yms were at the lowest TOC of 5 g L^{-1}

ESEM and TEM are useful tools to study the morphology and ultrastructure of fungi. ESEM was used to:

- assess colonization of the *T. harzianum* kmd, *C. gloeosporioides* C6 and *G. virens* MM1 on the pine shavings and molasses medium.
- Formation of fungal structures such as *T. harzianum* kmd chlamydospores were observed.
- Assess growth of *C. gloeosporioides* C6 on the amended and unamended gel medium.

TEM studies showed that :

- higher C:N ratios and TOCs increased cell wall thickness
- higher C:N ratios and TOCs increased the lipid content of the spores
- the nature of the extracellular matrix changed at the various C:N ratios and TOCs

Electron microscopy can be used in initial laboratory experiments to assess the effects of various cheap industrial media on the morphology and physiology of the fungal BCA. It is a useful tool in predicating shelf life of fungal spores as well as spore fitness by observing the morphological changes occurring when parameters such as C:N ratios and TOCs are tested. This technique can be used to reduce the time taken to test or shelf life and endurance of the spore to adverse conditions in the field.

5.4.1 FORMULATION

The novel gel formulation and harvesting of spores (Chapter Two) produced a low spore yield as surface spores were vacuumed and used for efficacy tests. The field trials of *C. gloeosporioides* C6 on *Hakea sericea* (Shrad) are currently being conducted in Stellenbosch by the Plant Protection Research Institute (PPRI). The efficacy of a fungal BCA is affected by the extent of spread and the effective adhesion of the spore to the target plant. Close contact of the spore to the plant surface is imperative for effective action. Therefore, the sticker used in the formulation of the BCA is an important component. Another factor that should be thoroughly investigated is the compatibility of

the surfactant and the fungal culture as surfactants may reduce spore attachment to hosts and spore viability (Connick *et al.*, 1990).

Various approaches can be considered for formulation and this is dependant on the application of the spore. For mycoherbicides intended for application to the soil, alginate granules are the best option. These provide extended time for spore production after application but seldom redistribute the spores to aerial parts of the plant (Connick *et al.*, 1990). This approach is effective when weeds are short (less than 2-4 cm) or when the spores are effectively in contact with the target weed, in or on the soil (Storey *et al.*, 1990).

Fungal spores such as *C. gloeosporioides* C6, *T. harzianum* kmd and *G. virens* MM1 germinate rapidly in water, therefore, liquid formulations are not suitable. A dust or wettable-powder formulation such as kaolin may be used. The spores may be applied as conidia that germinate and infect the target weed after exposure to high humidity or free water such as dew. The timing for the application of the spores is crucial as natural free water on the target surface may be present for a short period of time (Connick *et al.*, 1991). Therefore, formulations should contain substances that retain water around the fungal spore. Invert emulsions of water in oil are ideal in arid conditions where the relative humidity is less than 35% (Bateman, 1992). The constraint in the commercial use of this system is the large quantities of liquid that is required to obtain droplet sizes needed for adequate water retention (Egley *et al.*, 1993). Inclusion of sucrose or soy flour to formulations may increase the efficacy of the BCA as the nutritional material supports fungal growth prior to invasion of the target plant (Walker, 1981).

5.5 FUTURE RESEARCH

Based on the work conducted in this thesis, a forecast of what is needed in order to complete the research is as follows:

- ***Mechanisms of control by the BCAs***

Numerous studies have been published of potential BCAs that have been successfully mass produced and tested under greenhouse conditions and produce promising results. However, a large percentage of these BCAs fail to exhibit effective control in the field when exposed to variable environmental conditions. The sparse knowledge of the scientist in understanding the biocontrol mechanism used by the fungal antagonist and the effects of the biotic and abiotic environment on the BCA contributes largely to the failure of the BCA in the field. Studies need to be conducted on the mechanisms of biocontrol of all three fungal cultures to ensure that the product being mass produced is effective in the field and can be exploited to its full potential (Nakas and Hagedorn, 1990).

- ***Formulation of BCAs***

Further research needs to be conducted in the formulation of the fungal cultures of *T. harzianum* kmd, *C. gloeosporioides* C6 and *G. virens* MM1 grown on the pine shavings and molasses medium. One of the salient traits of BCAs that distinguish them from chemical control agents is that they are living entities (Nakas and Hagedorn, 1990). Formulation of BCAs is often the most difficult hurdle to overcome in the commercialization of a product. Spores need to be formulated in a suitable medium that acts as a carrier. The formulated product should to facilitate easy application of the BCA, heighten chances of survival in adverse conditions in the field and optimize conditions providing extended shelf life.

- ***Shelf life trials***

Shelf life trials need to be conducted on all three fungal spore cultures to determine the best formulation method which allowed the BCA to retain its biological activity during production, formulation, packaging, distribution and storage, until application.

- ***Application methods of the BCAs***

Depending on the type of formulation, various application methods may be employed to ensure the best delivery of the BCAs. These include soil treatments granules, powders and drench and spray applications.

- ***Efficacy of spores***

The strains of *T. harzianum* kmd and *G.virens* MM1 investigated were found to be effective in the control of various pathogens such as *Pythium* spp. and *Rhizoctonia solani* Kuhn by colleagues whom conducted greenhouse trials. The spores used in these trials were produced using laboratory media, hence, further trials also need to be undertaken in order to establish the efficacy of the fungal spores grown on the pine shavings and molasses medium. Trials with *C. gloeosporioides* C6 spores are currently being undertaken by the PPRI.

- ***Synergy***

Trials investigating the joint action of the BCAs need to be conducted to determine the synergistic effect of the two BCAs. The efficacy of the BCAs may be synergized by combining them with the application of chemical herbicides, thereby reducing the rates of chemical herbicides needed (Zidack and Quimby, 1999). This phenomenon was demonstrated in the effective control of *Fusarium* wilts by combining a non-pathogenic strain of *Fusarium oxysporum* (Schlechtendahl and Fries) and a strain of *Pseudomonas fluorescens* (Alabouvette, 1996).

- ***Fingerprinting***

Future research in this area includes optimizing DNA extraction procedures and PCR reactions. An interesting observation was made when *C. gloeosporioides* C6 cultures of different ages were cultivated on PDA plates and sporulated in Modified Richards Medium (Yu *et al.,* 1998). The original master culture of *C. gloeosporioides* C6 which had been maintained in long term storage formed a black mycelial mat and produced a higher spore yield. The culture of *C. gloeosporioides* C6 used in the experiments in this dissertation (which had been obtained from the master culture and sub-cultured three times) produced a white to orange fluffy mycelial mass and lower spore yields. Studies conducted by Kubo *et al.* (1982) showed that albino mutants produced by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG) and ultraviolet radiation formed orange mycelium and formed appressoria that had a lower penetration rate on nitrocellulose membranes and a decrease in lateral germination. Various experiments have shown that the dark-brown pigmentation found in fungi display the typical characteristics of melanin (Campbell *et al.*, 1968, Bull, 1970; Bell *et al.*, 1976). Generally, pigmented, thick walled appressoria endure adverse conditions and have cell walls that possess melanin which increase the structural rigidity (Bloomfield and Alexander, 1967; Kuo and Alexander, 1967).

AFLP procedures were conducted to establish the banding patterns of the the parent and mutated *C. gloeosporioides* C6 DNA samples. The results were however not documented as the AFLP procedure was not successful. PCR reactions resulted in no product. A subsequent extraction with phenol/chloroform followed by an ethanol ammonium acetate precipitation and wash in 70% (v/v) ethanol was conducted to clean up DNA. However, still no restriction product was obtained. The control experiment with DNA supplied by Gifco restricted and produced banding patterns. Hence it was deduced that possible PCR inhibitors existed in the DNA sample. It was possible that the initial DNA extraction procedure resulted in fungal DNA product that contained a high Cesium chloride salt content which inhibited the restriction endonuclease digestion as well as ligation of the adapters. It was also hypothesised that the T4 DNA ligase concentration was too low in the enzyme master mix and resulted in insufficient enzyme

activity and the glycerol concentration of the *Eco*RI enzyme inhibited the ligation reaction (Alton van der Westhuyzen, Technical support specialist, Applied Biosystems, pers comm.) Research was discontinued due to time as well as financial constraints.

The results expected were the formation of common bands between the two *C. gloeosporioides* C6 DNA samples, as well as differences in banding patterns due to the polymorphims caused by subculturing which influenced the change in the morphology of the mycelial mat and decreased spore yield. It is expected that the banding patterns of the cultures of different ages would produce different banding patterns. It is of paramount importance that the master culture as well as culture in use be finger-printed as these products need to be patented for use as a biological control agent.

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