Intraspecies diversity of *Cryptococcus laurentii* (Kufferath) C.E. Skinner and *Cryptococcus podzolicus* (Bab'eva & Reshetova) originating from a single soil sample

by

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted to any university for a degree.

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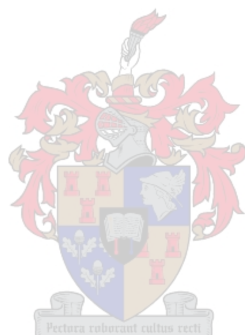

Summary

Intraspecific diversity among yeasts, including basidiomycetous yeasts has mostly been studied from a taxonomic point of view. The heterobasidiomycetous genus *Cryptococcus* is no exception and it was found to contain species that display heterogeneity both on a genetic and physiological level, i.e. diversity among strains originating from different geographical areas. It was stated that this diversity within yeast species is possibly caused by intrinsic attributes of the different habitats the strains of a particular species originate from. However, little is known about the diversity of a species within a specific habitat.

Thus, in this study intraspecific diversity among selected cryptoccoci isolated from a single soil sample originating from pristine Fynbos vegetation , was investigated. A total of 35 capsulated yeast strains, belonging to *Cryptococcus laurentii* and *Cryptococcus podzolicus* was isolated on a selective medium devoid of a nitrogen source. The identity of the isolates was determined using classical physiological taxonomic techniques, followed by sequence analysis of the D1/D2 region of the large-subunit within the ribosomal gene cluster. The isolates were all found to grow oligotrophically in biofilms. Carbon assimilation patterns, growth rates, optimum and maximum growth temperatures, differed among representatives of each of the yeast species. In addition, indications of intraspecific diversity were found upon phylogenic analysis of the D1/D2 region of the rDNA. Also, when binary interactions with two oligotrophic filamentous fungi (*Acremonium alternatum* and *Penicillium commune*) were studied, it was found that intraspecific differences occured. In the same yeast species some strains were found to be inhibitory to radial growth of a particular filamentous fungus , while others were found to stimulate the latter's radial growth. Preliminary investigations indicated that yeast proteases may be the cause of the antagonistic effects on the radial growth of the filamentous fungi.

Opsomming

Intra-spesifieke diversiteit tussen giste, insluitende basidiomisete giste is meestal vanuit 'n taksonomiese oogpunt bestudeer. Die heterobasidiomisete genus *Cryptococcus* is geen uitsondering en is gevind om spesies te besit wat heterogenesiteit op beide genetiese sowel fisiologiese vlakke openbaar, dit is diversiteit tussen stamme wat oorspronklik afkomstig is van verskillende geografiese areas. Die diversiteit binne-in gisspesies word moontlik veroorsaak deur intrinsieke eienskappe van verskillende habitatte waarin die stamme van 'n bepaalde spesies voorkom . Inteenstelling, baie min is egter bekend oor die diversiteit van 'n spesie in 'n spesifieke habitat.

Dus, in hierdie studie word intra-spesifieke diversiteit tussen geselekteerde cryptococci wat geïsoleer is vanuit 'n enkele grondmonster, afkomstig van 'n ongeskonde Fynbos veldtipe, bestudeer.'n Totaal van 35 gekapsuleerde gisstamme, wat aan *Cryptococcus laurentii* en *Cryptococcus podzolicus* behoort, is geïsoleer op 'n selektiewe medium sonder 'n stikstof bron. Die identiteit van die isolate was bepaal deur gebruik te maak van fisiologiese taksonomie tegnieke. Dit is opgevolg deur volgordebepaling van die D1/D2 streek van die groot subeeenheid binne die ribosomale geengroepering. Die isolate kon almal oligotrofies in biofilms groei. Koolstofassimilasie patrone, groeisnelhede, optimum en maksimum temperature, sowel die resultate van ribosomale geenvolgorde analise het verskil tussen verteenwoordigers van elke gisspesie. Daarby, is binêre interaksies met twee oligotrofiese filamentagtige fungi (*Acremonium alternatum* en *Penicillium commune*) bestudeer en is gevind dat intra-spesifieke verskille wel bestaan. In dieselfde gisspesie het sommige stamme 'n inhibitoriese effek op radiale groei gehad as 'n bepaalde filamentagtige fungus gebruik is, terwyl by ander 'n stimulerende effek op radiale groei van laasgenoemde waargeneem is. Voorlopige ondersoeke dui daarop dat gisproteases die moontlike oorsaak van die antagonistiese effekte op die radiale groei van die filamentagtige fungi teweegbring.

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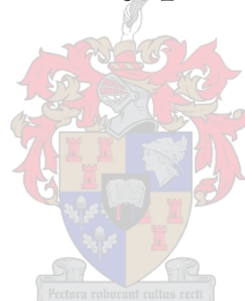
Dedicated to my parents.



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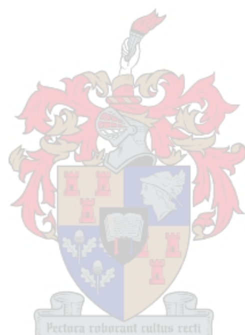
Preface

This thesis is presented as a compilation of five chapters. The Appendix consists of a number of compilations on compact disc (CD).



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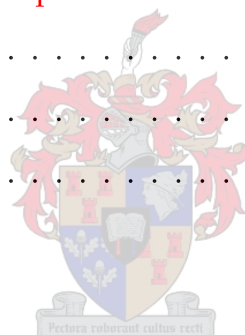

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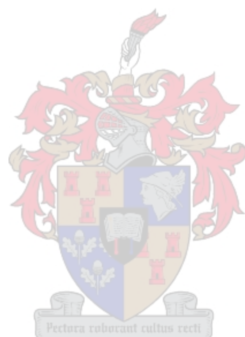

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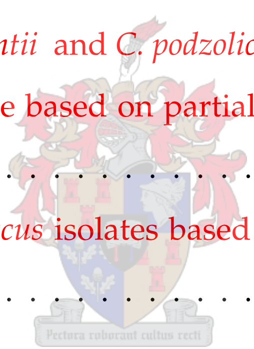

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Chapter 1

Introduction

1.1 General introduction and aims

Soil is regarded as the matrix that plants grow in, which contains mineral and organic matter (Jackson & Raw, 1966). It extends from the ground surface to the lower limit of the plants root system. The formation of soil is dependant on complex processes that include the chemical and physical weathering of parent rock providing the mineral substrate as well as the incorporation and decay of organic matter and the subsequent movement of soluble material in percolating or diffusible water. These processes are conditioned by the persistence of the ground surface and vegetation cover.

Thus, soil is a complex habitat that has a high solid/liquid ratio, making it unique among natural habitats (Stotsky, 1997). It is generally limited in nutrients, especially in carbon and nitrogen sources, and is constantly exposed to extreme environmental conditions (Gray & Williams, 1971; Poindexter, 1979). However, soil is able to sustain growth of a wide diversity of microbes and contains more genera and species of these organisms than most other habitats (Stotsky, 1997). This happens despite the fact that these microbes have to live in a varied and fluctuating environment. Not surprisingly, some species may be present in low numbers; this may be due to conditions that restrict them to discrete sites, in which nutritional and other physiochemical environmental factors are located that support growth of the particular species. Generally, it is difficult for exogenous (alien) microorgan-

isms to adapt and survive in soil (Stotsky, 1997). Most autochthonous (indigenous) soil microorganisms however, are either oligotrophic or zymogenous by nature.

The soil ecosystem harbours all major taxonomic groups of fungi including filamentous fungi and yeasts (Thorn, 1997). One of these is the genus *Cryptococcus* , which was found to be a dominant fungal group in some soils (Vishniac, 1995). The genus occurs over a wide geographical range and has been isolated from samples taken from animals, beverages, man, plants and soil (Fell & Statzell-Tallman 1998). Interestingly, intraspecific variation at the molecular and physiological levels was observed amongst *Cryptococcus* spp. isolated from different ecological niches (Sugita *et al.*, 2000; Sampaio & Fonseca, 1995). Some members of this genus are encapsulated, providing these yeasts with the ability to better survive in habitats diminished in nutrients, than non-encapsulated yeasts (Phaff *et al.*, 1987). It is also known that some cryptococci originating from soil, such as *Cryptococcus laurentii*, are able to grow oligotrophically (Kimura *et al.*, 1998). From the above, it may be assumed that cryptococci are adequately adapted for survival in soil environments.

Soil microbes may be considered aquatic organisms since their metabolism is dependent on a continual supply of available water (Stotsky, 1997). These microorganisms are essentially restricted to sites containing clay, as sand and silt particles do not retain water against their gravitational pull. The clay forms a coat around the sand and silt particles to produce a microaggregate ranging from 0.5 to 5 mm in diameter and which is stabilized by organic matter and inorganic materials. The water is retained within the aggregate. It is these aggregates that constitute the microhabitat wherein the microorganisms function.

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This soil microhabitat arrangement lends it to support the interactions amongst the microorganisms (Stotsky, 1997). These interactions can either be positive (commensalistic, mutualistic, synergistic) or negative (competitive, parasitic, predatory) (Gray & Williams, 1971). It is, however, wrong to assume that all microbes are in direct competition with each other, as negative interactions only occurs between those microbes competing for the same ecological niches (Lachance & Starmer, 1998) . Within the ecological niche, microbes will generally compete for nutrients, space, oxygen and other essential minerals. Such interactions in which one of the microbes is adversely affected, are termed antagonistic. These

interactions, whether antagonistic or not, may vary in their specificity. Also, an interaction between two soil isolates observed under laboratory conditions may never occur in soil, although it may highlight some aspects of the fundamental niche requirements of a particular microbe. An example of the latter approach is the data collected on carbon source assimilation among members of the yeast domain (Kurtzman & Fell, 1998).

1.2 Scope and outline of the thesis

With the above as background, the objectives of this study were: 1) To investigate the intraspecific diversity of selected *Cryptococcus* spp. present in a single soil sample originating from pristine Mountain Fynbos soil in the Western Cape region, South Africa. 2) To study the interactions of these cryptococci with filamentous fungi originating from the same soil sample.

Consequently, in Chapter 2 a review is presented on the biology of the genus *Cryptococcus* and yeast interactions. The results of a study on intraspecies diversity among isolates representing *Cryptococcus laurentii* and *Cryptococcus podzolicus*, all obtained from a single Mountain Fynbos soil sample, is presented in Chapter 3. In Chapter 4, the antagonistic effect of these isolates on filamentous fungi originating from the same soil sample, is reported on. In conclusion, an overview of the research, concluding remarks and opportunities for future research are presented in Chapter 5.

Chapter 2

Literature review

2.1 General features of the genus *Cryptococcus*

2.1.1 Taxonomic position

The generic name *Cryptococcus* was first applied by Kützing in 1833, when it was used in the taxonomy of the algal domain (Fell & Statzell-Tallman, 1998). In 1901, however, Vuillemin used the name to accommodate pathogenic yeasts when *Saccharomyces neoformans*, previously isolated from peach juice by Sanfelice, was assigned to the genus *Cryptococcus*. Although the two definitions of the genus caused confusion, the medical community embraced the nomenclature of the pathogen *Cryptococcus neoformans* (Fell & Statzell-Tallman, 1998). This species is known to occur in animals, humans and soil contaminated with pigeon droppings. It has also been isolated from various other sources such as eucalyptus trees and decaying wood forming hollows in living trees (Chakabarti *et al.*, 1997; Callejas *et al.*, 1998; Lazera *et al.*, 1998).

Although the name *Cryptococcus neoformans* was widely accepted, Skinner (1950) and Donk (1973) voiced their concern about the status of the genus *Cryptococcus* Kützing, favoring the emended genus by Vuillemin. In 1981, *Cryptococcus* Kützing of which *Cryptococcus mollis* was the type species at the time, was found to be heterogeneous after transmission electron microscopy was performed on the holotype species (Rodriques de Miranda & Batenburg-van der Vegte, 1981). Basidiomycetous elements were found to be present in

this sample. For this reason , it is not clear whether Kützing was referring to a yeast. Later, Fell *et al.* (1989) proposed to conserve the genus as *Cryptococcus* Vuillemin with *C. neoformans* CBS 132 as type species. This paved the way for the expansion of the genus during later years, when numerous anamorphic yeasts were classified in *Cryptococcus* Vuillemin.

According to the fourth edition of The Yeasts: A taxonomic study, the number of species belonging to the anamorphic genus *Cryptococcus* is 34 (Fell & Statzell-Tallman, 1998). These yeasts were classified as heterobasidiomycetous yeasts and grouped in the class Hymenomycetes. Based on sequence analyses of the small subunit rDNA, Swann *et. al* (1995b) recommended that this heterobasidiomycetous fungal class be subdivided into the subclasses Hymenomycetidae and Tremellomycetidae. The majority of members of the Tremellomycetidae produces a yeast phase, whereas an ontogenic yeast stage is not observed in the Hymenomycetidae.

Phylogenetic analyses of the hymenomycetous yeasts using sequence comparisons of the D1/D2 region of the large subunit (LSU) ribosomal DNA (Fig. 2.1) showed that *Cryptococcus* is indeed a polyphyletic genus (Fell *et al.*, 2000). Representatives of this genus were found to occur in more than one order *viz.* Cystofilobasidiales, Filobasidiales, Trichosporonales and the Tremellales (Fell & Kurtzman , 1990; Fell *et al.*, 2000).

The Tremellales contains mitosporic members of *Cryptococcus* (Fell *et al.*, 2000). However, the molecular systematics of this order has not yet been resolved and many of the clusters obtained during phylogenetic analyses lack statistical support (Fig. 2.1), which may be an indication of the heterogeneity of the order or of inadequate sampling of taxa. For example, *Cryptococcus cellulolyticus* and *Cryptococcus laurentii* in the Indecorata cluster lack statistical support. However, a strong statistical support exists for *Cryptoccocus skinneri* in the Foliacea cluster. The distributions of species within the Tremellales revealed that many *Cryptococcus* species appear to be related to *Bullera*, e.g. *Bullera pseudoalba/C. celluloyticus* and *Bullera armeniaca/Cryptococcus hungaricus*. Interestingly, the teleomorph of the pathogen *C. neoformans*, i.e. *Filobasidiella neoformans* grouped into the Tremellales; other representatives of *Cryptococcus* grouped in the order Filobasidium, which constitutes four clusters, although not all have bootstrap support. The order Trichosporonales has

bootstrap support of 89%. The recently described Cystofilobasidiales (Fell *et al.*, 1999) also contains *Cryptococcus* species.

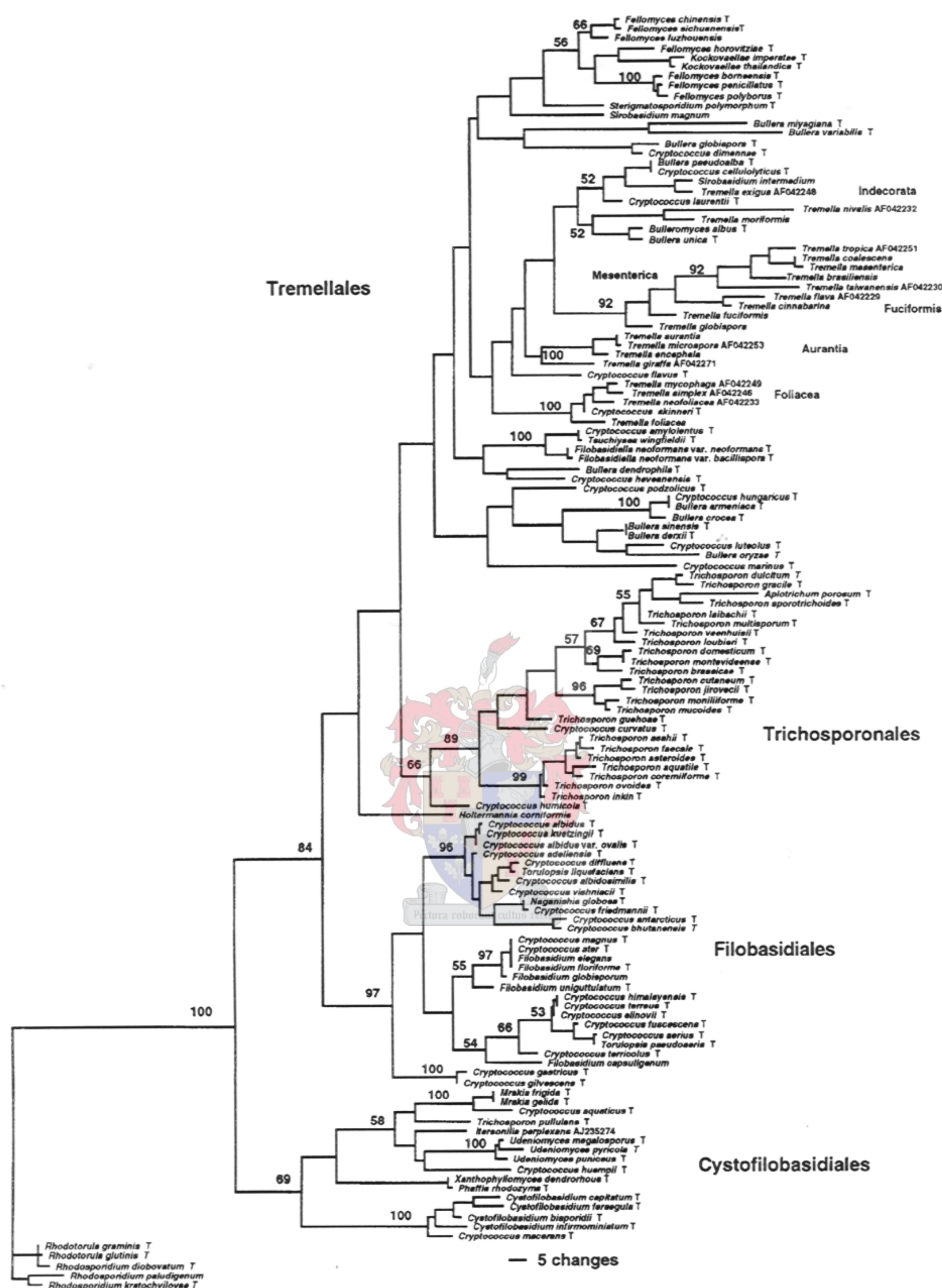

Fig. 2.1: Hymenomycetous yeasts: phylogenetic analysis of the D1/D2 region of the large subunit rDNA (one of 100 equally parsimony-uniformative trees). Numbers on the branches indicate bootstrap percentages from 100 full heuristic bootstrap replications, "T"indicates type strain. Reprinted from (Fell *et al.*, 2001).

2.1.2 Morphology

The genus *Cryptococcus* is characterized by spheroidal, ovoidal or elongated cells (Fell *et al.*, 1998). Members belonging to this genus reproduce by multilateral or polar budding and may develop pseudo- or true hyphae. Colony colour may be white or cream, while the colonies of some strains may develop a red or brown colour on solid media.

2.1.3 Physiology

Similar to the results obtained by phylogenetic analyses of ribosomal gene sequences (section 2.1.1), the cryptococci were found to be polyphyletic when grouped on the basis of the ability to synthesize starch and to utilize the carbon sources inositol and D-glucuronate (Fell *et al.*, 1998). However, none of the members of the genus exhibit the ability to ferment carbon sources.

A feature of tremellaceous yeasts that seems to have taxonomic significance is the utilization of nitrate as sole nitrogen source (Sampaio & Fonseca, 1995). Numerical analysis of physiological traits in this genus showed that *Cryptococcus* species clustered into 12 groups (Sampaio & Fonseca, 1995). Cluster 1 to 4 included all nitrate-positive species, e.g. *C. aerius*, *C. albidus*, *C. antarticus*, *C. terreus* to mention a few, whereas cluster 5 to 12 comprised nitrate negative cryptococci, e g. *C. ater* and *C. magnus*. Strains of *C. laurentii* were found to cluster with *C. podzolicus*, which both test negative for nitrate utilization.

During studies on the utilization of 20 low molecular weight aromatic compounds by a wide diversity of basidiomycetous yeasts, it was found that *C. laurentii* and *C. podzolicus* are characterized by a poor ability to utilize monomeric aromatic compounds as sole carbon sources (Sampaio, 1999). Only a few of these compounds of which the degradation is catalyzed by oxidoreductases (Wackett & Hershberger, 2001) were assimilated (Sampaio, 1999). Three of the twelve strains representing *C. laurentii* were able to utilize gallic acid, the rest were unable to utilize any of the compounds. The four strains representing *C. podzolicus* were all able to utilize gallic acid and catechol, while protocatechuic acid, caffeic acid, gentisic acid and m-hydroxybenzoic acid were only utilized by some of the strains

representing this species. These variable assimilation patterns highlighted the heterogenous physiological nature of species belonging to the genus *Cryptococcus*.

Both *C. laurentii* and *C. podzolicus* are able to produce extracellular capsules (Fell & Statzell-Tallman, 1998). During experiments with a representative of *C. laurentii*, it was found that the quantity of capsule material produced is dependant on culture conditions such as the extent of aeration, nutrient concentrations, pH and temperature (Cadmus *et al.*, 1962). Later, it was found that this capsule material consists of heteropolysaccharides, which, in addition to smaller quantities of galactose, glucose, inositol and mannitol, contains mainly mannose and xylose (Martens & Frankenberger Jr, 1991).

2.1.4 Habitat

Studies of yeasts occurring in nature usually involve the distribution of the yeasts and not their specific ecological roles (Fell *et al.*, 2001). However, it was found that terrestrial habitats such as plants, animals or soil may support the growth of cryptococci the world over (Lachance & Starmer, 1998). These cosmopolitan species are highly heterogeneous in their nutritional abilities enabling them to aerobically utilize a wide diversity of organic compounds. The availability of these organic compounds in substrates may result in the establishment of species within a specific habitat (Phaff & Starmer, 1987).

Cryptococci that were isolated from plant sources, include *C. neoformans*, which in addition to its occurrence in clinical specimens has been isolated from eucalyptus trees and rotting wood (Ellis & Pfeiffer, 1990), *C. albidus* that was isolated from leaves and *Cryptococcus skinneri* that has been isolated from slime fluxes of trees (Fell & Statzell-Tallman, 1998). As part of a phylogenetic diverse yeast community cryptococci may sometimes also occur on the outer and inner surfaces of flowers (Spencer & Spencer, 1997) .The interior of a flower contains nectars, which act as carbon rich substrate that supports growth. The occurrence of yeasts in the nectar may also aid in the dispersion of these yeasts, especially in situations where the plant is pollinated by insects. Yeasts that are part of the natural microflora on fruits rarely become spoilage organisms unless damage occurs due to handling or attacks by insects, birds or animals. *C. albidus* and *C. laurentii*, isolated from strawber-

ries, may have originated from the soil, or possibly the flowers of these plants (Buhagiar & Barnett, 1971). Other habitats of cryptococci include insect frass, fermented beverages produced from fruit and a number of animals (Fell & Statzell-Tallman, 1998).

Cryptococci not only occur in nutrient rich environments, some species were repeatedly and exclusively isolated from soil, suggesting that these *Cryptococcus* species are specific to this habitat (Lachance & Starmer, 1998), although available nutrients in soil usually occur in low concentrations (Williams, 1985). Furthermore, studies conducted by Phaff and Starmer (1987) showed that yeast microflora of soils are dependant on the type of nutrients reaching them and their numbers increase by the addition of metabolizable substances. Soils from the Antarctic region contain basidiomyceteous yeasts, which revealed the presence of among others, *C. albidus* (Fonseca *et al.*,2000), which is classified in the order Filobasidiales (Fig. 2.1). The majority of members of this order are *Cryptococcus* species that occur in a variety of habitats, including the Antarctic (Fell *et al.*, 2000; Fonseca *et al.*,2000). Furthermore, in a study conducted in semi-arid prairie soils, the majority of yeasts isolated constituted cryptococci *viz*. *C. albidus*, *C. laurentii* and *C. terreus* (Spencer & Spencer, 1997). Another basidiomycetous yeast, *Candida podzolica* (syn. *Cryptococcus podzolicus*), is widely distributed in podzolic and sod-podzolic soils in Russia (Bab'eva & Reshetova, 1975).

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In was stated that yeasts have the ability to multiply asexually in favourable conditions within some soils and that this increase is followed by a decrease in cell numbers during unfavourable conditions (Lund, 1954). Furthermore, it was suggested that when this situation prevails, the yeast survives as a vegetative cell rather than a spore. However, more recently it was stated that the ability to sporulate may favour the survival of some soil yeast species (Phaff & Starmer, 1987). Also, evidence suggests that encapsulated soil yeasts such as *Cryptococcus* spp. survive better in poor habitats diminished in available nutrients and during periods of desiccation, than their non-capsulated counterparts (Golubev *et al.*, 1984).

2.1.5 Importance

Members of the genus *Cryptococcus* are of clinical, industrial and agricultural significance (Adhearn, 1998). One of the most important pathogenic yeasts for humans is *Cryptococcus neoformans*, usually causing infections of the lungs with mild symptoms. However, pneumococcal-type pneumonia may present in stressed patients. This species has a preference for the central nervous system and may also result in fatal meningoencephalitis, especially in the immunocompromised patient. This could perhaps be ascribed to the nervous tissue contains relatively high concentrations of thiamin, an essential growth factor for *C. neoformans* (Spencer & Spencer, 1997). Other medically important yeasts such as *Cryptococcus albidus*, *Cryptococcus ater*, *Cryptococcccus humicolus*, *Cryptococcus laurentii* and *Cryptoccocus uniguttulatus* have been implicated in diseases such as cryptococcal meningitis and extrapulmonary cryptococcosis.

However, *Cryptococcus* may not always be detrimental to mankind and some species are being utilized in the biocontrol of plant diseases. Strains representing *C. laurentii* were found to inhibit gray mold on apples caused by *Botrytis cinerea* (Roberts, 1990). An effective commercially available biocontrol agent, Yieldplus, was developed and registered in South Africa (Abadias *et al.*, 2003) and contains the yeast, *C. albidus*.

Enzymes produced by cryptococci are also being explored for possible use in biotechnology (Demain *et al.*, 1998). *C. albidus* has been shown to produce xylanases (Biely *et al.*, 1981), in another case the production of cellulases in *C. cellulolyticus* has been demonstrated (Nakase *et al.*, 1996). Amylolytic activity was found in *C. curvatus* (De Mot *et al.*, 1984). Various members of this genus such as *Cryptococcus diffluens*, *C. humicolus*, *C. laurentii* and *C. terreus* have the ability to assimilate a variety of aromatic compounds (Mills *et al.*, 1971; Middelhoven *et al.*, 1992; Sampaio, 1999). This indicates that these species have potential as agents to minimize the occurrence of pollutants in the environment.

2.2 Soil as nutrient limiting habitat

2.2.1 Nutrient status of soil

Environments such as soil are considered lacking sufficient carbon to sustain microbial growth (Gray & Williams, 1971; Poindexter, 1979; Williams, 1985). Organic matter entering the soil has already been partially exploited. Thus, the assimilable substrates in the soil have mostly been removed. This situation also applies where there is energy enrichment by energy-yielding substrata for example, rhizospheres. Investigations of the microbial growth in the rhizosphere showed that energy input from the roots to the soil was still insufficient to maintain active soil microbial communities (Barber & Lynch, 1979). In a study conducted by Ko and Lockwood (1967) it was found that water extracts from soils contained only 4.2μg.ml^{-1} carbohydrates and 1.9μ g.ml^{-1} amino acids. Low nutrient habitats such as soil may therefore be viewed as being in a state of oligotrophy (Williams, 1985). Oligotrophy was first described by environmental microbiologists in marine systems pertaining to growth rates of microorganisms in ecosystems containing only small amounts of low energy substrates. An oligotrophic habitat contains a low energy flux equivalent to less than 1 mg C.l^{-1} .d^{-1} (Poindexter, 1981). In order for microbial communities to survive and grow in soil several strategies need to be employed. Under these low nutrient conditions adaptations for survival include: anaplerotic CO_2 fixation, chemolithoautotrophy, nitrogen fixation, nutrient scavenging from the atmosphere and oligotrophic growth.

Fungi acquire energy for basic metabolic functions by oxidizing organic substrates in the soil to CO_2 (Gray & Williams, 1971). As a result of the low nutrient status fungi may either exist in most soils in a state of quiescence of spores or have established an endogenous metabolism. Other fungi such as *Acremonium* and *Penicillium* spp. are capable of oligotrophic growth under these conditions (Wainwright, 1993). With a sudden flux of utilizable carbon substrates the metabolism of the microbial community increases resulting in an increase of CO_2 levels. Therefore, soil may be considered as a low nutrient medium in which microbial growth, including fungal growth, responds to carbon influxes. However, it is not only available carbon which may act as a growth limiting factor in soil. Nitrogen

(N) may also be in short supply in some soils. An example of such soils is the soil from the mountainous regions in the Western Cape, South Africa (Specht, 1979). These soils are covered with a unique vegetation type called fynbos.

2.2.2 Fynbos soil

Fynbos is described as heathland, which comprises of an unique collection of hard leafed evergreen shrubs, found in the Western Cape region (Kruger, 1979; Specht, 1979). This type of vegetation is divided into arid, coastal and mountain fynbos. Fynbos soil types are mainly structure-less, low in organic matter content and acidic, with a low base saturation , giving it a low nutrient status (De Bano & Dunn, 1982). These acid soil types, with pH ranging from 3.5 to 5.5, are highly leached, mainly podzolics derived from quartzites, typical sand or sand loams (Kruger, 1979). Processes which form soil have a net tendency to differentiate the materials on which they act into horizons (Fig. 2.2). In typical podzol, a surface O-horizon is present, consisting of partially decomposed litter and other plant remains (Jackson & Raw, 1966). A thin A- horizon overlying a bleached E- or A2-horizon is present. It is from this layer that plant nutrients, iron and aluminium compounds are leached by humic acids percolating down from the organic layer where they are formed. Leached materials are then deposited in the B-horizon, which is usually more compact than the overlying E-horizon, with a brown, black or red colour. The parent rock, C-horizon, is typically very acid.

The physical and chemical characteristics of these fynbos soils were previously recorded by Fry (1987) in which mixed suite soils had a clay content less than 6% (m/m) whereas, the majority of quartzite soils had a clay content less than 6% (m/m). The mixed suite soils had organic contents (CT) values between 2 and 3.5% (m/m), whereas quartzite derived soils displayed CT values of approximately 1% (m/m). The quartzite-derived soils had N concentrations no higher than 0.06% (m/m), whereas the dark brown soils showed N concentrations no less than 0.1% (m/m). The C:N ratio between the quartzite (mean, 17 ± 1) and mixed suite soils (mean, 21 ± 2) showed no significant difference. Therefore, it is obvious that these soil types are nitrogen- limited. This phenomenon may be explained by

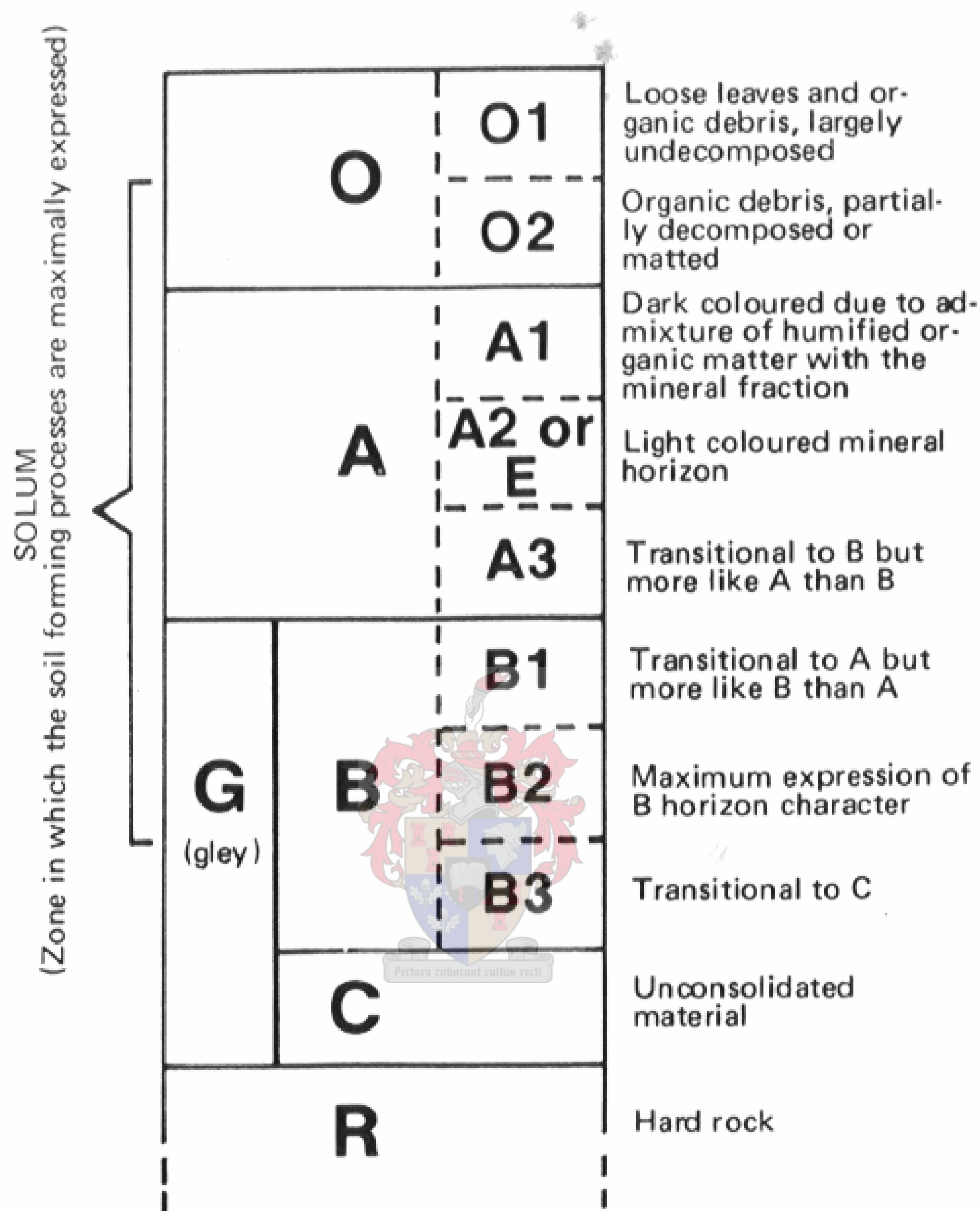

Fig. 2.2: A schematic representation of the soil horizons, brought about by soil processes acting on different materials such as rocks and various kinds of loose sediments (Macvicar *et al.*, 1977).

either particulate loss or volatilization as a result of fire (De Bano & Dunn, 1982) or even denitrification processes (Rundel *et al.*, 1983). Another possibility may be the low microbial activity and the presence of highly resistant organic compounds responsible for the slow rate of nitrogen mineralization (De Bano & Dunn, 1982).

2.3 Oligotrophy

2.3.1 General characteristics of oligotrophy

Our knowledge regarding microbial growth has mostly been obtained from studies using pure cultures growing on nutrient rich media (Wainwright *et al.*, 1991). Since the conditions in the laboratory are often created for optimum growth of single cultures, this situation rarely occurs in nature. The nutrient conditions in ecosystems are such that bioavailable energy is nearly always in short supply (Fry, 1990). In some cases, unrelated microbial community members may depend on each other for various inorganic and organic compounds to sustain continued growth (Barakah, 1992).

Although microbial nutritional requirements differ with respect to different environmental conditions, the environment must be able to supply elements essential for the synthesis of cell contents and maintenance (Wainwright *et al.*, 1991). These elements are the macroelements C, H, O, N, S, P, K, Ca and Mg and trace elements Mn, Zn, Mo, Co, Cu, Ni, V, B, Cl, Na, Se, Si and W. A species may be absent from an environment if one of these essential elements is lacking or is in short supply. Hence, microorganisms may grow and survive with difficulty in the presence of a diminished amount of essential substances (Veldkamp & Jannasch, 1972). The ability of microorganisms to grow and survive under these conditions may depend on adaptive survival strategies such as oligotrophy (Wainwright *et al.*,1991; Barakah, 1992).

Generally, oligotrophic microorganisms are those microbes that have the ability to grow in low concentrations of organic substrates and augment in a natural habitat where the nutrient flux is low. A universal definition for the term oligotrophy does not exist. Some authors such as Kuznetsov *et al.* (1979), defined oligotrophs as bacteria that are isolated on organic media containing between 1 and 15 mg carbon per liter ($C.l^{-1}$). A more stringent definition was proposed by Japanese researchers, who recommended that oligotrophs should be able to grow in the presence of 1 mg $C.l^{-1}$ (Ishida & Kadota, 1981), whilst Poindexter (1981) defined oligotrophs relative to the nutrient flux of the bacterial habitat. According to this author, the flux of an oligotrophic habitat should be between zero and a fraction of

a milligram of carbon per liter per day.

According to Kuznetsov *et al.* (1979), oligotrophs in water systems may be grouped according to their nutritional abilities. The first group is those microbes that can only grow at first cultivation on sterile water, but cannot be recultivated. The second group can be isolated on nutrient poor media, but not on rich media. However, the microorganisms can be recultivated on rich media after initial cultivation on a nutrient poor medium. The third group contains bacteria that can be isolated on special nutrient poor media or by using special isolation methods. These bacteria have peculiar morphological and physiological features. The last group includes bacteria that can only be detected using an electron microscope and are uncultivable. Some researchers regard the third group as the only true oligotrophic microbes (Kutznetsov *et al.*, 1979).

The majority of studies on oligotrophy were devoted to oligocarbotrophy (growth in a carbon deficient environment) of bacteria (Wainwright, 1993). However, oligotrophy may also be studied when microbes grow in low nitrogen, iron and phosphorus conditions. These conditions are known respectively as oligonitrotrophy, oligoferrotrophy or oligophosphotrophy.

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An oligotroph must be able to use a wide variety of growth substances and must possess enzymes to utilize a wide diversity of carbon sources (Wainwright, 1993). Furthermore, high affinity uptake systems among these microbes are likely, thus low saturation constants for growth (Ks) values when growing at a low substrate concentration are expected. This would result in low maintenance energies and increased survival. Oligotrophs also have the ability to scavenge nutrients from the air. However, the mechanisms involved in these processes have yet to be fully elucidated.

A wide range of oligotrophic microorganisms may be isolated from various natural environments containing low quantities of nutrients, such as coastal waters, oceans and soils (Barakah,1992). However, oligotrophic growth is primarily limited by the availability of dissolved assimilable organic substances (Upton & Nedwell, 1989). Thus, the concentration of dissolved assimilable organic substances serves as one of the most important ecological factors for the presence of oligotrophic microorganisms (Kutznetsov *et al.*, 1979). In

terrestrial habitats such as soil, which has a low nutrient status (section 2.2.1), a suitable environment is created to encourage oligotrophic growth among microorganisms (Williams, 1985).

In habitats such as soil, microzones are readily formed that favour microorganisms or induce stress (Nikitin & Chumakov, 1986). In these habitats, microbes are exposed to constant gradient changes of biologically active substances, extreme temperatures, ion concentrations, redox potential and pH. The ability of oligotrophic soil microorganisms to utilize a substrate may also be influenced by the presence of different amino acids or changes of inorganic salts (Hattori, 1984).

2.3.2 Oligotrophy among fungi

Research on fungal oligotrophy has been neglected since most of the literature on microbial oligotrophy relates to bacteria (Parkinson *et al.*, 1989). Increasing evidence, suggest that this type of growth is not restricted to prokaryotes only, but that oligotrophy does indeed exist in the fungal domain. It was shown that fungi readily are able to grow in an apparent absence of any nutrients or in conditions limited in essential nutrients (Wainwright, *et al.* 1991). In a study by Parkinson *et al.* (1989), in which fungal isolates were obtained from culture collections and soils, the isolation of oligotrophs was done on silica gel without a source of C or N, respectively. A range of fungal species was able to germinate and grow oligocarbotrophically on silica gel. The carbon source involved was believed to be obtained from the atmosphere. Oligotrophic fungal genera include *Aspergillus*, *Cladosporium, Fusarium, Gliocladium, Mucor* and *Penicillium* .

Under these low nutrient conditions, fungi usually produce less biomass than when grown in nutrient rich media (Wainwright, 1993). Hyphae displayed a tendency to form shallow grooves within the silica gel when observed with light microscope or scanning electron microscope. The low nutrient conditions cause the fungi to produce fine hyphae, known as gossamers. Gossamers create a mycelial mat, which provide a large surface area that increases nutrient scavenging abilities from the atmosphere and nutrient poor medium. Often no lysis or assimilation of pre-formed hyphae occurs when fungi grow

oligotrophically. A logical reason might be that fungi exploit exogenous nutrient sources when grown oligotrophically.

The physiology of fungi under low nutrient conditions is not that well understood (Wainwright, 1993). Similar adaptive strategies to those employed by bacteria under low nutrient conditions may also be present in fungi. Fungal oligotrophs may also have the ability to scavenge nutrients from the air when fungi grow oligocarbotrophically, since it was assumed that fungi were incapable of growing autotrophically to fix CO_2. It has been suggested that fungi may use this metabolic strategy when present in soil, scavenging carbon from soil water or the atmosphere (Barakah, 1992). Evidence was obtained that fungal species such as *Fusarium oxysporum* may be able to fix atmospheric CO_2 (Stover & Freiburg, 1958). Later, Parkinson *et al.* (1990), demonstrated that *Fusarium oxysporum* was able to assimilate $^{14}CO_2$ under oligotrophic conditions to obtain energy.

It has been suggested that it may be possible that oligotrophic soil fungi can utilize energy sources other than reduced carbon through a specialized metabolism (Jones *et al.*, 1991). A range of different fungi were found to play a role in soil nitrification (Lang & Jagnow, 1986). Certain soil fungi also obtain energy from the oxidation of reduced S, Mn or Fe (Wainwright & Kilham, 1980; Wainwright, 1988; Jones *et al.*, 1991). This suggests that soil fungi may avoid spore dormancy or fungistasis (Lockwood, 1977) when organic C is depleted. Little is known about the ecological importance of oligotrophic fungal taxa in soils (Jones *et al.*, 1991).

2.3.3 Oligotrophy among yeasts

Since yeasts co-exist with bacteria and filamentous fungi within their natural habitat, it may be presumed that oligotrophic yeasts occur among other oligotrophic microbes (Kimura *et al.*, 1998). However, oligotrophy among yeasts is not well documented. Soil cryptococci in Iceland were isolated using an enation medium, which is essentially a diluted semisynthetic medium (Vishniac, 2002). These icelandic soils consist of tephra, a poorly vegetated, dark and rusty-looking gravel. However, the tephra sample consisted mainly of fine sand. It can be assumed that the sample was low in nutrients. In a study

conducted by Kimura *et al.* (1998), several asporogenic yeasts were isolated, predominantly from soil. Most of the isolates were members belonging to the genus *Cryptococcus*, except for the red yeast *Rhodotorula glutinis*. These yeast isolates display low Km values for D-glucose, L-leucine and other L-amino acids when compared to eutrophic yeasts such as *Saccharomyces cerevisiae*. These Km values were similar to those of three oligotrophic soil bacteria. This similarity in Km values is an indication that efficient uptake systems are an important feature of oligotrophic yeasts. Under conditions of nitrogen limitation, uptake systems in oligotrophic yeasts showed a high tolerance for starvation conditions. Thus, the oligotrophic yeasts are able to respond to low nutrient conditions. These features may assist soil yeasts in their growth and survival under oligotrophic conditions.

2.4 Interactions between yeasts

2.4.1 Killer toxins

2.4.1.1 General description of killer toxins

To survive and grow in the environment, a microbe should not only be able to take up nutrients in the most efficient way, but should also be able to successfully interact with other living organisms in the same habitat (Atlas & Bartha, 1993). The various types of interactions that may exist among microbes include neutralism, commensalism, synergism, symbiosis, competition, amensalism, predation and parasitism. Numerous yeast species, belonging to *ca.* 20 genera, secrete extracellular proteinaceous toxins, also known as mycocins (Boekhout *et al.*, 1993). Although mycocins have an inhibitory or even lethal effect on sensitive yeasts strains, it is not toxic for the killer producing strain. The killer phenomenon is not unique to yeasts. Similar systems are known for bacteria, paramecia, slime molds and smut fungi (Konisky, 1982; Koltin, 1988; Quackenbush, 1988; Mizutani *et al.*, 1990).

Initially, killer phenomenon studies were limited to *S. cerevisiae* and dealt mainly with biochemical and molecular aspects. Cytoplasmic non-Mendelian genetic determinants

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were found to be involved in the killer activity (Herring & Bevan, 1977; Bussey *et al.*, 1982) and it was suggested that these may be double-stranded RNA (dsRNA) in association with virus-like particles (VLP's). Mycocinogenic strains were, however, later also reported among other yeast genera (Stumm *et al.*, 1977). The toxins produced by these yeasts have diverse modes of action (Young, 1987).

The genetic basis for killer phenotype expression has been widely studied among ascomycetous yeasts (Young, 1987). It was found that these toxins have molecular masses of 10-300kDa (Golubev, 1989). For a number of the toxins mycocidal activity was found to be related to their ability to compromise cytoplasmic membranes of sensitive strains (Bussey, 1981).

The killer phenomenon was also observed among basidiomycetous yeasts, since it was found that *Cryptococcus* and *Rhodotorula* species produce killer toxins (Golubev, 1989). Recently, strains of *Cryptococcus humicola* were found to produce low molecular mass killer toxins with a size of only 1kDa, also known as microcins, highlighting the diversity of molecules involved in this phenonmenon and showed its broad antifungal activity among both ascomycetous and basidiomycetous yeasts (Golubev & Shabalin, 1994). Results showed that mycocins of *Cryptococcus*, *Cystofilobasidium* and *Filobasidium* usually kill members of the order Tremellales including the Filobasidiaceae. None of the mycocinogenic basidiomycetous yeasts were found to be active against ascosporogenous yeasts, except *C. humicola*, which was able to kill the latter ascomycetes.

Yeast strains belonging to the same species or closely related species of the same genus were found to have identical broad host responses to mycocins (Golubev, 1998). However, sometimes these responses may differ as a result of the heterogeneity of some taxa . These different responses were attributed to the immunity of killer or neutral strains, or to modified cell wall components. Such different sensitivity patterns were frequently found to occur within anamorphic heterogenous genera such as *Cryptococcus* and *Rhodotorula*.

The killer phenomenon was also studied among natural yeast communities such as the killer yeasts associated with decaying cactus stems and fruits, as well as slime fluxes of trees (Starmer *et al.*, 1987). Interestingly, the fruit habitat seemed to favour the estab-

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lishment of killer yeasts, whereas the yeasts isolated from necrotic cactus tissue and slime fluxes of trees had a lower incidence of killer yeasts among them. During cross-reaction studies fewer killer-sensitive interactions were found to occur within the same habitat at a particular time and locality. Killer-sensitive strains reacted more often with yeasts from a different habitat. It was also found that killer sensitive strains were more widespread since a yeast community generally has only one killer species, while the rest of the community consisted of sensitive strains.

2.4.1.2 Non-*Saccharomyces* killer systems

Since its first discovery in *S. cerevisiae* by Markover and Bevan (1963), the killer phenomenon has been extensively studied in this species (Herring & Bevan, 1977; Bussey *et al.*, 1982). However, killer strains were found in non-*Saccharomyces* yeast genera such *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniospora*, *Kluyveromyces*, *Pichia*, *Sporidiobolus*, *Tilletiopsis* and *Zygosaccharomyces* (Golubev, 1998). Similar to *S. cerevisiae*, other killer yeasts may also belong to the killer classes K1 and K2, which kill each other, but show immunity to their own toxins (Marquina *et al.*, 2002). K1 killer activity remains stable within a narrow range of acidic pH, is unstable at temperatures above 25° C and is inactivated by agitation (Golubev, 1998). Studies conducted on a variety of toxins showed that some characteristics of the K1 killer toxin may be similar to other toxins. The toxins from all killer strains are protease sensitive, heat-labile macromolecules.

However, the genetic basis of killer characters for non-*Saccharomyces* yeasts may be quite different from that of the K1 system. In contrast to the cytoplasmic genetic determinants of the latter system, genes encoding killer toxins of basidiomycetous yeasts, belonging to the *Bullera* and *Cryptococcus* were found to be chromosomally inherited (Suzuki *et al.*, 1989).

2.4.1.3 Mode of action of killer toxins

Very few toxins examined for action on *S. cerevisiae* appear to act like K1 toxin in causing plasma membrane damage (Marquina *et al.*, 2002). Nevertheless, despite having a differ-

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ent protein structure, the K1 and K2 toxins exhibit similar modes of action. The first step in K1 toxin activity is binding to the yeast cell-wall (1→6)-β-D glucan receptor (Hutchins & Bussey, 1983). This step occurs rapidly and is energy independent. It is, however, pH-dependent and may therefore be responsible for the pH range of toxin activity. The next step in toxin activity is energy dependent whereby the toxin interacts with the plasma membrane receptor causing the membrane to become permeable for protons and potassium. This is probably due to the killer toxin acting as a K^+ ionophore or protonophore. Subsequently, the plasma membrane elicits leakage of higher molecular mass molecules such as ATP. It is still unclear whether the killer toxin inhibits some component of the proton pump or forms a transmembrane protein channel.

The K28 killer toxin differs notably in its mode of action from the K1 and K2 toxins. It binds to the mannoprotein part of the yeast cell wall (Tipper & Schmitt, 1991). Contrary to the K1 and K2, K28 show no ionophoric effects, but rather inhibits nuclear DNA synthesis. This is achieved by arresting the cell in the G1 phase of the cell cycle.

2.4.1.4 Potential applications of killer yeasts

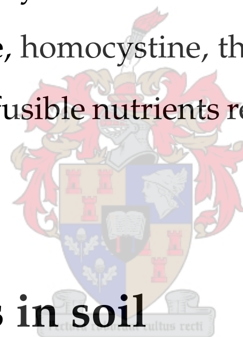
Killer yeasts may have applications in the brewing and wine industries since these yeasts may be able to compete efficiently with wild yeast strains (van Vuuren & Wingfield, 1986; Petering *et al.*, 1991). In wineries killer yeasts could be used as starter cultures during early stages of fermentation to prevent spoilage by undesirable strains. Other possible applications for killer yeasts relate to biocontrol in the agricultural sector as well as potential antifungal agents against wood-decay and plant pathogenic fungi (Walker *et al.*, 1995). As explained in section 2.4.1.1, the killer character is widely distributed among yeast genera with the toxins often being active against different yeasts outside the genus of the killer toxin producing strain.

2.4.2 Predation

It is generally accepted that destructive penetration of a fungus normally only occurs in filamentous fungi, a phenomenon ascribed to necrotrophic mycoparasitism (Barnett *et al.*,

1973). However, predatory yeasts also occur that attack yeasts and other fungi by production of small feeding appendages called haustoria (Lachance & Pang, 2000).

Yeast 'predation' was previously reported in *Arthroascus javenensis* and three other filamentous yeasts described by Kreger-van Rij and Veenhuis (1973). This phenomenon was interpreted as a variation by hyphal anastomosis of those yeasts that were capable of forming hyphae (Lachance & Pang, 1997). Later, Lachance and Pang (1997) demonstrated the occurrence of physical yeast predation among ascomycetous yeasts within the species *Saccharomycopsis* (syn. *Athroascus*) *javenensis*, *Saccharomycopsis synnaedendra*, *Saccharomycopsis selennospora*, *Saccharomycopsis fibuligera*, as well as *Candida* species. Organic sulphur, especially methionine, was identified as a major factor in growth of these specific microorganisms. This compound causes the predator to act as either an inhibitor for predation or a possible signal for prey. These yeasts also had the unique ability to utilize organic sulphur compounds such as cystine, homocystine, thioglycollate and thiosulphate as sole sulphur source at the expense of diffusible nutrients released by sulfate-assimilating yeasts (Lachance *et al.*, 2000).



2.5 Fungal interactions in soil

2.5.1 Antagonism

Soil microflora is a diverse community of constantly interacting beneficial and deleterious microorganisms that may influence root systems of higher plants (Elad, 1986). Interactions between these soil borne microbes or more specific, the phenomenon, antagonism may be ascribed to parasitism, antibiosis or competition. These antagonistic activities which are displayed especially by the beneficial microbes may be of use to reduce densities of soil borne plant pathogens. Various examples of such interactions are known. Mycoparasites *viz.*, *Pythium nunn* and *Trichoderma harzianum* are able to parasitize soil-borne plant pathogens such as *Rhizoctonia solani*, *Sclerotium rolfsii*, *Pythium* spp. and *Phytophthora* spp. These mycoparasites usually degrade the cell walls of their hosts by excreting superfluous enzymes such as β-1-3-glucanase and chitinase, upon which the host is penetrated by the

hyphae of the antagonistic fungus.

It was also suggested that such antagonistic interactions with disease-causing fungi may be as a result of competition for nutrient limiting factors such as carbon, nitrogen and iron in soil where both mycoparasites and potential plant pathogens are present in the same niche (Elad, 1986). The mechanism by which this nutrient-driven competition operates was suggested to be the suppressiveness of soils, which results in increased competition for carbon and energy sources by the larger total microbial activity in the soils (Baker & Cook, 1974). A pathogen may be present in these soils, but does not cause disease to a susceptible host (Whipps, 1997). A soil that is suppressive to one pathogen might not necessarily be suppressive to another pathogen. Suppressiveness was found to reduce the number of germinating chlamydospores of *Fusarium oxysporum*, thereby inhibiting wilt disease in the soil in question (Sivan & Chet, 1989b).

Microbes producing chelators of essential elements also affect competition among themselves by competing to bind these elements and transporting them into the cell (Elad, 1986). An example of chelated-mediated competition is provided by iron chelating compounds (siderophores), which provide their producers, with an effective means to compete with other microbes for a limited supply of iron (Elad, 1986; Chet, 2002).

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Antagonistic interactions among soil fungi may also occur by means of antibiosis that involves the production of a diffusible low molecular weight compound or antibiotic by a microorganism to inhibit the growth of another microorganism (Handelsman & Parke, 1989). This includes small toxic molecules, volatiles and lytic enzymes. Extensive reports exist for the production of inhibitory metabolites by fungal biocontrol agents (Wright, 1956; Ordentlich *et al.*, 1992). It must be noted, however, that although antibiosis reduces plant diseases, its impact in biocontrol is uncertain because other mechanisms, as mentioned above, may also be operating (Baker& Griffin, 1995).

In recent years the use of soil microorganisms as possible treatments against post-harvest diseases of fruit has been researched by a number of workers (Chand-Goyal & Spotts, 1997; Janisiewicz, 1987). The mechanistic action involved in such biocontrol appears to be competition for nutrients and space (Mari *et al.*, 1996b). Similarly, mechanisms

such as the production of anti-fungal metabolites (Janisiewicz *et al.*, 1991), direct parasitism and induced resistance sometimes associated with the reduction of pathogen enzyme activity (Zimad *et al.*, 1996) may also play a role.

2.5.2 Fungal Laccases

A fungal enzyme that has been demonstrated to play a pivotal role in the interactions of some fungi is laccase (EC.1.10.3.2) (Thurston,1994). Phenoloxidases such as laccases are extracellular glycoproteins (Yaropolov *et al.*, 1994) that have molecular masses of 60-80kDa and consist of 15-20% carbohydrates (Thurston, 1994). Laccases occur widely in fungi, especially in white rot fungi (Mayer & Staples, 2002) and are known to be multi-copper containing enzymes which reduce molecular oxygen to water by performing one electron oxidation of various aromatic substrates such as diphenols, methoxy-substituted monophenols and aromatic amines (Thurston, 1994). This oxidation of phenols and phenolic lignin substructures leads to the formation of radicals that result in polymerization, which may form an amorphous, insoluble melanin-like product (Fig. 2.3). This process initially involves a typically unstable product and may undergo various subsequent reactions. However, a second enzyme-catalyzed oxidation step may occur, also non-enzymatic reactions may result, such as hydration or disproportionation (undergoing both oxidation and reduction) leading eventually to polymerization.

2.5.2.1 Functions of laccase

The role of laccase in fungi has been well documented (Mayer & Staples, 2002). This enzyme plays a role in degradation of lignin and or detoxification of lignin products, pigmentation accumulation, sporulation (De Vries *et al.*, 1986) and plant pathogenesis (Thurston, 1994). Laccases have been implicated in the morphogenesis of rhizomorphs (Worrall *et al.*, 1986), which are unique fungal structures that are able to grow through soil containing no host material and hence facilitate colonization of new substrata (Rizzo & Blanchette, 1992).

Laccase has also been shown to be an important virulence factor in many diseases caused by fungi (Mayer & Staples, 2002). *Cryptococcus neoformans*, an encapsulated fungus

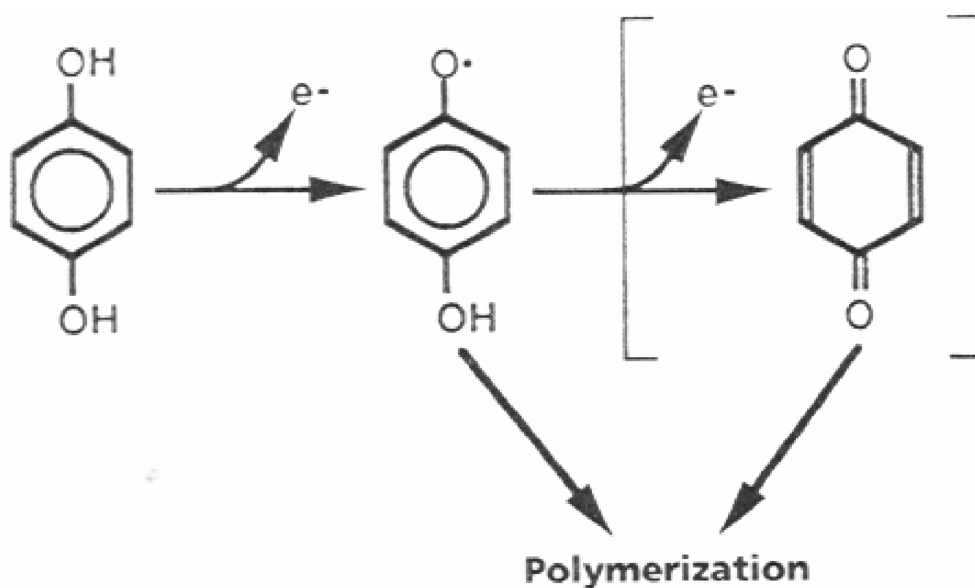

Fig. 2.3: Laccase catalysis reaction in which diphenol undergoes a one-electron oxidation to form an oxygen-centered free radical. This species can be converted to the quinone in the second enzyme-catalysed step or by spontaneous disportionation. Quinone and free radical products undergoes polymerization. Reprinted from (Thurston, 1994).

known to cause life-threatening infection in immunocompromised patients, was found to posses the laccase enzyme (Williamson, 1994). Zhu *et al.* (2001) showed that this laccase is a tightly associated cell wall enzyme that is readily available to interact with host immune cells. In the case of *C. neoformans*, laccase and its product melanin, are regarded as virulence factors, since melanin synthesis is dependent on a copper-dependent laccase. This was demonstrated in a study using knockout strains of *C. neoformans* (Williamson *et al.*, 1998). Melanization in *C. neoformans* occurs when exogenous or environmental catechols /aminophenols are oxidatively polymerized by laccase (Fig. 2.3). The cloning of the structural gene of laccase, *CNLAC1* and construction of *CNLAC1* knockout strains, also confirmed its role in the virulence of this yeast (Petter *et al.*, 2001; Williamson, 1994). A similar gene that showed homology with *CNLAC1* was also found in *C. podzolicus* (Petter *et al.*, 2001). In addition, laccase activity was detected among other clinical cryptococci *viz.*, *C. albidus*, *C. laurentii* and *C. curvatus* (Ikeda *et al.*, 2002).

2.6 Yeast-protozoan interactions

2.6.1 Development of virulence by *Cryptococcus neoformans*

Plants including trees may harbour *C. neoformans*, but it is also suggested that soil may act as natural habitat for *C. neoformans* (Kwon-Chung, 1998) . In nature certain factors play an important role in the protection of *C. neoformans* against fungal dessication and soil phagocytic predators such as amoeba. Although very little is known about the interactions of fungi with protozoa, it was found that *C. neoformans* was able to interact with soil amoebae (Land, 2002). This pathogenic yeast is known for causing life-threatening meningitis in immunocompromised patients with the capability to replicate inside macrophages. The cell capsule of *C. neoformans* is composed of polysaccharides, which protects the ingested yeast against attack by phagocystic cells. The polysaccharide capsule also promotes intracellular pathogenesis through the cytotoxic release of polysaccharide into macrophage vacuoles. Amoebae and macrophages have some common properties; both have phagocytose particles in their vacuoles and secrete enzymes such as lysozyme.

In a detailed study conducted by Steenbergen *et al.* (2001) the similarity of survival strategies for *C. neoformans* after ingestion by amoebae and macrophages, is hypothesized. The study revealed that when *C. neoformans* is phagocytosed by *Acanthamoeba castellani*, the yeast replicates within the amoeba leading to the death of the latter. It was proposed that melanin contributes to resistance against amoebae, because melanized *C. neoformans* cells without a capsule was more resistant to killing by amoebae than their non-melanized counterparts. This difference in the killing effect of the amoebae however, could not be observed when capsular *C. neoformans* strains were used as prey. This may have environmental significance since most environmental *C. neoformans* isolates have a small capsule which can be melanized. Similar results were observed in *C. neoformans* infected macrophages, with the formation of polysaccharide vesicles. Furthermore, when a phospholipase deficient *C. neoformans* mutant was ingested by *A. castellani*, the yeast displayed a decrease in virulence. The mutant, *plb* was unable to grow in *A. castellani* when a killing assay was performed. Interestingly, phospholipase is a known virulence factor for *C. neoformans*.

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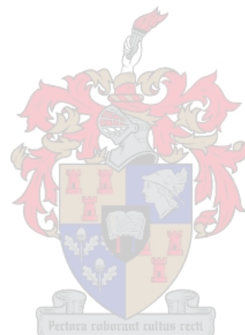
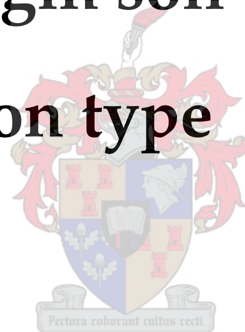
Thus, the development of virulence by *C. neoformans* is a consequence of adaptations that evolved for the protection against environmental predators such as amoebae and may give us insight into its broad host range.



Chapter 3

Intraspecies diversity of *Cryptococcus laurentii* and *Cryptococcus podzolicus* isolated from virgin soil covered with a pristine vegetation type



3.1 Introduction

The anamorphic capsulated yeast species *Cryptococcus laurentii* has been isolated in diverse habitats from various geographical areas, including beverages, diseased patients, plant material, seawater and soil (Fell & Statzell-Tallman, 1998). Intraspecific variation was observed with regards to the ability to assimilate certain carbon sources (Fell & Statzell-Tallman, 1998; Sampaio, 1999) and ribosomal gene sequence composition (Sugita *et al.*, 2000). Consequently, based on the results obtained with ribosomal gene analyses, strains of *C. laurentii* were recently reclassified into five distinct species (Takashima *et al.*, 2003). It can therefore be assumed that isolates of *C. laurentii* obtained prior to this reclassification may indeed belong to at least five different *Cryptococcus* species.

Capsulated yeasts, such as *Cryptococcus*, are known to survive better in habitats poor in available nutrients and during periods of desiccation than non-capsulated yeasts

(Phaff & Starmer, 1987). Recently, it was found that *C. laurentii* is able to grow under oligotrophic conditions (Kimura *et al.*, 1998), which frequently prevail in soil (Williams, 1985). A soil yeast that is physiologically related to some strains of *C. laurentii* (Sampaio & Fonseca, 1995), is *Cryptococcus podzolicus*. The intraspecies diversity of this yeast can be observed when isolates originating from different geographical areas that were deposited in the Centraalbureau voor Schimmelcultures (CBS) are compared (http://www.cbs.knaw.nl/databases/index.html).

As with many other taxonomic studies on fungi, observations on intraspecies diversity among isolates from different habitats and geographical areas may therefore provide us with a way to delimitate species. However, this approach will provide little information on the diversity needed within a species enabling it to sustain itself in a particular natural habitat. The aim of this study was therefore to obtain an indication of the natural intraspecies diversity of *C. laurentii* and *C. podzolicus* occurring in a single soil sample taken from virgin soil from a pristine area.

3.2 Materials and Methods

3.2.1 Yeast isolates used

A total of 35 yeast strains were used in this study, all isolated from fynbos soil.

3.2.2 Soil Sampling

The sampling site, which comprised an area of 10 m^2, is situated in the Jonkershoek valley (pristine Mountain Fynbos; S 33° 58′ 20″; E 18° 55′ 10″) near Stellenbosch, South Africa,within a cool temperate Mediterranean climatic region with a dry summer (Schulze, 1947). The mean annual temperature of this climatic region is 17°C (Fuggle & Ashton, 1979). The soil at the sampling site was classified as sandy loamy soil of the Oakleaf form derived from a mixture of granite and quartzite (Fry, 1987; Soil Classification Working Group 1991). Surface litter was removed to reduce contamination. In autumn (2000-04-15)

a soil sample of *ca.* 900 g consisting of nine sub-samples was taken at random over the area of the site, each to a depth of 5 cm. In the laboratory, the sub-samples were mixed to produce a composite sample (Table 3.1). Upon further mixing, aliquots of soil were extracted from the composite sample to be used as inocula for dilution plates.

Table 3.1: Characteristics of the soil at samping site

Mountain Fynbos Soil	
Physical characteristics[1]	
Stone % (Particle diameter > 2.0 mm)	23.4
Rough sand % (Particle diameter 0.5 - 2.0 mm)	16.6
Medium sand % (Particle diameter 0.2 - 0.5 mm)	28.0
Fine sand % (Particle diameter 0.02 - 0.2 mm)	32.9
Silt % (Particle diameter 0.002 - 0.02 mm)	16.4
Clay % (Particle diameter < 0.002 mm)	6.1
Soil moisture content (%) at 2000-04-15, 11:00[2]	11.75± 0.84
Mean monthly soil temperature up to a depth of 50 mm, for the month (April) the samples were taken in.[3]	16.94 ± 3.56
Chemical Characteristics	
Organic carbon[4] %	3.50
Total nitrogen [5] %	0.17
Ammonium (ppm)[6]	14.00
Nitrate and nitrite (ppm)[7]	0.40

[1]Determined by Bemlab CC using the hydrometer method (Van der Watt, 1966).

[2]The soil moisture content of the soil samples were determined in triplicate by drying the soil in an electric oven at 105°C for 12h (Eicker, 1970).

[3]Data provided for the top 5 cm of soil by a weather station situated in the Jonkershoek valley and owned by the Division for Water-, Environment and Forest Technology, CSIR, Stellenbosch.

[4]Determined by Bemlab CC using the Walkley-Black method (Nelson & Sommers, 1982).

[5]Determined by Bemlab CC through digestion in a LECO FP-528 nitrogen analyser.

[6]Determined in a 1M KCl extract by Bemlab CC (Bremner, 1965).

[7]Determined in a 1M KCl extract by Bemlab CC (Bremner, 1965).

Phosphorous (ppm)[8]	4.00
Copper (ppm)[9]	0.06
Zinc (ppm)[10]	0.50
Manganese (ppm)[11]	5.50
Boron (ppm)[12]	0.15
Exchangeable cations[13]	
Calcium (cmol/kg)	1.43
Magnesium (cmol/kg)	0.57
Potassium (cmol/kg)	0.31
Sodium (cmol/kg)	0.05
pH of a suspension containing 1 part soil and 2.5 parts 1M KCl. (2000-07-15)[14]	4.40
pH of a soil suspension in water prepared on the sampling date 2000-04-15 [15]	5.64 ± 0.37

[8]Determined by Bemlab CC.

[9]Determined in a di-ammonium EDTA extract by Bemlab CC according to the methods of Beyers and Coetzer(1971).

[10]Determined in a di-ammonium EDTA extract by Bemlab CC according to the methods of Beyers and Coetzer (1971).

[11]Determined in a di-ammonium EDTA extract by Bemlab CC according to the methods of Beyers and Coetzer (1971).

[12]Determined in a hot water extract by Bemlab CC according to the methods of the Fertilizer Society of South Africa (1974).

[13]Determined in a 1 M ammonium acetate extract by Bemlab CC according to the methods of Doll and Lucas (1973).

[14]Determined by Bemlab CC according to the method of McClean (1982).

[15]Determined in triplicate according to the method of Spotts and Cervantes (1986).

3.2.3 Yeast enumeration and isolation

A soil dilution series, ranging from 10^{-1} (using 1g soil in 9ml dH_2O to 10^{-5}) was prepared in triplicate and a 100μl aliquot of each dilution was spread in triplicate on plates, each consisting of a Petri dish (90 mm in diameter) containing an isolation medium (Table 3.2). This medium contained low concentrations of nitrogen thereby imitating the low nitrogen concentrations generally occurring in fynbos soils (Fry, 1987). After 7 days of incubation at 25°C, yeast colonies were enumerated. All the observed yeast colonies on the isolation plates were subsequently purified by successive inoculation and incubation on yeast malt extract (YM) agar (Wickerham, 1951) at 25°C. Each yeast strain was numbered according to the specific dilution in the series it originated from and the chronological order in which the associated colony was picked up for purification, e.g. ″1″ for the 10^{-1} dilution and ″a ″ for the first colony picked up at this dilution. Yeast cultures were subsequently maintained on YM slants at 25°C.

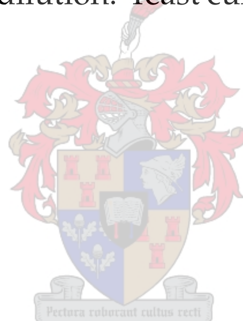


Table 3.2: The composition of isolation medium used to obtain the yeast isolates

Components per liter of distilled water			
Carbon source		**Vitamins**	
Glucose (g)	5.00	biotin (μg)	1
Mineral Salts			
$CaCl_2$ (g)	0.10	calcium pantothenate (μg)	200
KH_2PO_4 (g)	1.00	folic acid (μg)	1
$MgSO_4 \cdot 7H_2O$ (g)	0.50	inositol (μg)	1000
NaCl (g)	0.10	p-aminobenzoic (μg)	100
Trace elements		pyroxidine hydrochloride (μg)	200
$AlK(SO_2)_2 \cdot 12H_2O$ (μg)	10	riboflavin (μg)	100
$CuSO_4 \cdot 5H_2O$ (μg)	100	thiamine (μg)	500
$CoSO_4$ (μg)	100		
$FeCl_3 \cdot 6H_2O$ (μg)	200		
H_3BO_3 (μg)	500	**Anti-bacterial agent**	
KI (μg)	100	Chloramphenicol(g)	0.20
$MnSO_4 \cdot H_2O$ (μg)	40		
$Na_2MoO_4 \cdot H_2O$ (μg)	200	**Solidifying agent**	
$ZnSO_4 \cdot 7H_2O$ (μg)	400	Agar (g)	10.00
		Final pH	5.2

3.2.4 Yeast identification using physiological and morphological characteristics

All the isolates were identified using morphological and physiological criteria according to the keys and descriptions of Barnett *et al.* (2000), Payne *et al.* (1998) and Van der Walt & Yarrow (1984). Carbon source utilization was tested at 25°C on a Tissue Culture Rollor-drum rotating at 40 rph. Nitrogen source utilization was examined by the auxanographic method. The basidiomycetous nature of the isolates was confirmed by performing the Diazonium Blue B (DBB) colour test.

3.2.5 Molecular characterisation of yeast isolates

The identity of each isolate was confirmed by sequence analysis of the D1/D2 region of the rDNA. Yeast strains were grown for 24h in YPD broth (2% glucose, 2% peptone, 1% yeast-extract). Genomic DNA was extracted according to the method of Hoffman & Winston (1987). Using the polymerase chain reaction (PCR), the DNA was amplified with universal fungal primers ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and LR6 (5′-CGC CAG TTC TGC TTA CC-3′) in a Perkin-Elmer Thermal Cycler (Fell *et al.*, 2000). The PCR products were purified with Nucleospin (Separations) chromatography columns.

The 600-650bp D1/D2 region of the large subunit of ribosomal DNA (rDNA) was subsequently amplified by subjecting the above mentioned PCR products to another round of cycle sequencing (Fell *et al.*, 2000) with the forward primer F63 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and reverse primer LR3 (5′-GGT CCG TGT TTC AAG ACG G-3′) were used in the reactions. Sequences of the D1/D2 region of the rDNA from the strains were obtained using an ABI PRISM model 3100 genetic sequencer. The forward and reverse sequences were aligned with DNAMAN for WINDOWS Version 4.13 (Lynnon Biosoft). The yeast strains were identified by comparing the sequencing results with known sequences using the BLAST program (http://www.ncbi.nlm.nih.gov./BLAST).

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3.2.6 Molecular phylogenetic analysis

A molecular phylogenetic analysis of the D1/D2 region of the rDNA. was conducted (Appendix B, provided on compact disc). Phylogenetic relationships were inferred using distance analysis in PAUP* v.4.0b10 (Swofford 2001). Characters were treated as unweighted in the analysis and gaps were treated as missing data. A single tree for was obtained using neighbour-joining analysis with an uncorrected P-distance. *Trichosporon ovoides* CBS 7556 T was used as outgroup. Confidence levels were estimated with a Bootstrap analysis (1000 replicates) using the neighbour-joining option. Ambiguously aligned regions were coded and step matrices for assigning weight to the codes were computed using INAASE 2.3b (Lutzoni *et al.*, 2000). The weighted codes were included in the analysis, replacing the ambiguous aligned regions. Sequence data, other than yeast isolates, were obtained using the BLAST system (http://www.ncbi.nlm.gov/BLAST/) at the National Center for Biotechnology Information, Bethesda, Md.

3.2.7 Determination of growth rate in nutrient-rich medium

A pre-culture of each isolate was prepared by inoculating 50 ml YM broth (pH 5.5) contained in a 250 ml conical flask, with a loop-full of one-week-old cells from a YM slant. The pre-cultures were incubated at 30°C on a rotary shaker (100 rpm) until an optical density of 1 at 600 nm was obtained, using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech). Subsequently, these pre-cultured yeast cells of each isolate were used to inoculate 50 ml of YM broth contained in a 250 ml conical flask, resulting in an initial concentration of *ca.* $1{\times}10^6$ cells/ml in the culture. The culture was then incubated at 30°C on a rotary shaker (150 rpm). Growth was monitored by measuring the optical density of each flask at 640 nm using a Klett-Summerson colorimeter (red filter). The growth experiments were performed in triplicate (see Appendix B, table B.1 on compact disc).

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3.2.8 Determination of optimum growth temperature

A pre-inoculum of each isolate was prepared by inoculating 50 ml YM broth contained in a 250 ml conical flask, with a loop-full of one-week-old cells from a YM slant. The pre-inoculum was incubated at 30°C on a rotary shaker (150 rpm) until late logarithmic phase was reached. Subsequently, 40μl of the resulting cell suspension was used to inoculate each of a series of five 250 ml conical flasks, each containing 25 ml YM broth. The flasks were respectively incubated at 15°C, 20°C, 25°C, 30°C and 35°C. After 24h of incubation, growth was measured spectrophotometrically at 600nm using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech). All experiments were conducted in triplicate (see Appendix B, table B.2 on compact disc).

3.2.9 Indications for oligotrophic nature of yeasts by budding on silica gel

One-week-old yeast cells of isolates obtained from YM slants that were washed in physiological saline solution (PSS) were used. For each isolate, silica gel plates devoid of a carbon or nitrogen source were inoculated with *ca.* 1×10^4 cells per plate. A further set of inoculations on silica gel plates was performed. These plates contained 2.5mg/l sucrose with 0.067g/l Yeast Nitrogen Base (YNB), Difco or 5mg/l thymine with 0.117g/l Yeast Carbon Base (YCB), Difco. The plates were incubated at 25°C for three days after which microscopic analysis was done to confirm the presence of budding cells.

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3.2.10 Confirmation of the oligotrophic nature of yeasts in continuous flow cells

Continuous-flow cells were used to confirm the oligotrophic nature of the yeast isolates. An inoculum of each yeast isolate was obtained from a one-week-old liquid culture in 50 ml YM broth (pH 5.5), contained in a 250 ml conical flask and incubated on a rotary shaker (100 rpm) at 30°C.

Flow cells were constructed as previously described by Wolfaardt *et al.* 1994. The flow

cells were surface sterilized with a 2% hypochlorite solution and irrigated with a low nutrient medium at a flow rate of 0.1 mm/s using a Watson Marlow 205S peristaltic pump. The medium contained 25 mg/l sucrose and 67 mg/l YNB. Each flow cell was inoculated by adding a single 0.2 ml pulse of a yeast culture, with the pump turned off, for 2 h before flow was resumed.

After 1 week of incubation at 22°C, the yeasts in the biofilm were examined for the presence of budding and viability using epi-fluorescence microscopy. Yeasts were visualized after staining with the fluorescent probes FUN-1^{TM} and/or Calcofluor White M2R^{TM} (both from Molecular Probes). FUN-1^{TM} is a yeast viability stain, marking intravacuolar structures fluorescent red in metabolically active cells. Calcofluor is an ultraviolet-excitable dye that labels yeast cell walls fluorescent blue, regardless of the cell's metabolic state. Both dyes were diluted according to the manufacturer's product information sheet. A volume of 100 μl of FUN-1^{TM} was injected into an individual channel of the flow cell at a concentration of 40 μM, with the peristaltic pump turned off for 3h before flow was resumed for 1h before microscopic examination of the channel. One hundred μl of a 25μM solution of Calcofluor White M2R^{TM} was injected in a similar way into the same channel, either after microscopic examination of FUN-1^{TM} stained yeasts, or before staining with FUN-1^{TM}. In every case, flow cells could be examined directly after staining, but better results were obtained when the stained flow channels were rinsed by resuming flow for a limited time. Microscopic examination was carried out with a Nikon Eclipse E400 epifluorescence microscope, using a filter set with excitation of 430 nm and emission 520nm / a multipass filter set appropriate for viewing DAPI. Images were captured with a Nikon Coolpix 990 digital camera.

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3.2.11 Response of isolates to phenotypic criteria

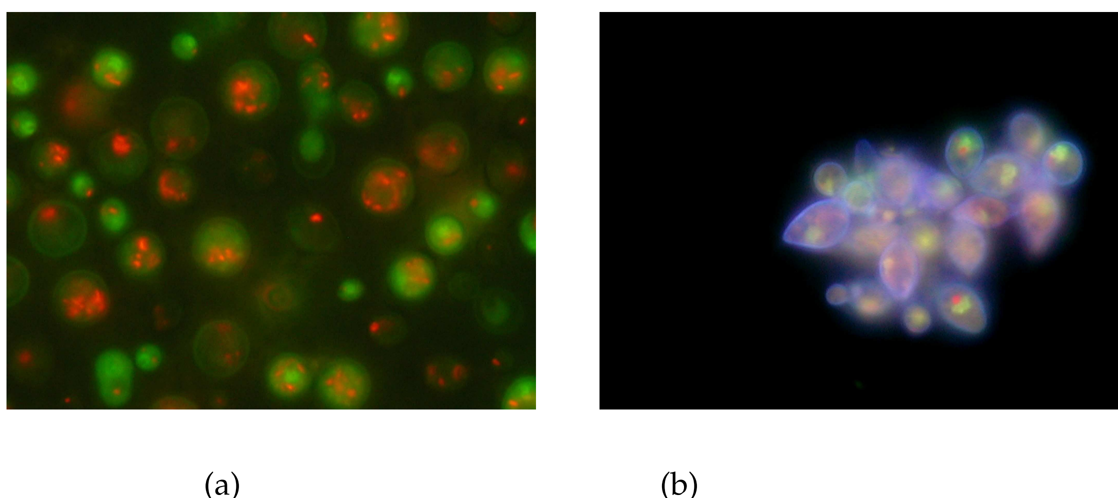
Based on tests that were performed on the isolates, a phenogram was constructed with the use of the computer software programme Statistica for Windows Version 6 (StatSoft). Cluster analysis was done on 60 phenotypic characteristics (Appendix A, tables A.1, A.2, A.3 and A.4) using complete linkage method.

3.3 Results and Discussion

The soil sample yielded only two yeast species able to grow on the isolation medium (Table 3.2), i.e. *Cryptococcus laurentii* (Kufferath) C.E. Skinner and *Cryptococcus podzolicus* (Bab'eva & Reshetova). Isolates representing the latter species amounted to ca. 900 cells/g soil, while only ca. 300 cells/g soil was found representing *C. laurentii*. These values were calculated by dividing the number of colonies representing each species by the dilution factor of the soil dilution. It must however be noted that these numbers only represent a single point in time and that seasonal changes in soil yeast numbers are known to occur (Slávikovά & Vadkertiovά, 2000). Consequently, to obtain a true indication of the numbers of these yeasts in the soil their numbers at the sampling site should be monitored over different seasons.

All the isolates were found to be oligotrophic, since all were able to bud on the silica gel devoid of added carbon and nitrogen sources. In addition, viable yeasts cells showing active budding within a biofilm were detected in flow cells after one week of irrigation with a medium containing only 10 mg/l carbon (fig. 3.1 a and b). Similar results were obtained by others when it was found that *C. laurentii* is able to produce extracellular polymeric substances (EPS) and form biofilms when cultivated in flow cells under oligotrophic conditions (Joubert *et al.*, 2003). The formation of biofilms is a mechanism which enables microbes to sequester and concentrate nutrients while growing in low nutrient environments (Decho, 1990). It is therefore tempting to speculate that these yeast species are able to survive and grow in low nutrient soils by forming biofilms on soil granules. Low nutrient conditions, especially low nitrogen concentrations, often occur in fynbos soils (De Bano & Dunn, 1982; Fry 1987). However, both *C. laurentii* and *C. podzolicus* seem to be well adapted for growth in these soils, for not only were the isolates able to grow oligotrophically but these two species were the only yeasts growing on the isolation plates devoid of an added nitrogen source.

All the isolates were tentatively identified, using morphological and physiological criteria, according to the keys and descriptions of Barnett *et al.* (2000) and Kurtzman *et al.* (1998). In malt extract, the isolates representing *C. laurentii* formed spheroidal to ovoidal



(a) (b)

Fig. 3.1: Active budding of (a) *C. laurentii* cells in a biofilm within a continuous flow cell irrigated at 22°C for 1 week with a medium containing 25mg/l sucrose and 67mg/l YNB. The biofilm was subsequently stained with FUN-1 and observed using epifluorescence microscopy. Similar observations were made for (b) *C. podzolicus* cells when stained with Calcofluor White M2R and observed using epifluorescence microscopy.

cells, while the isolates of *C. podzolicus* tended to be polymorphic with lateral or polar appendages. The identity of each isolate was confirmed by sequence analysis of the D1/D2 region of the rDNA. Bearing in mind that some representatives of the species *C. laurentii* have recently been renamed (Takashima *et al.*, 2003), phylogenetic analyses of our isolates and related species resulted in a similar tree as obtained by Takashima *et al.*, (2003). This phylogenetic analyses of the D1/D2 region confirmed that the isolates were closely related to the type strains representing each of the two species, since in both cases the isolates grouped into well supported clades together with their respective type strains. However, some variation was noted among isolates representing *C. laurentii* and especially among the *C. podzolicus* isolates (Fig.3.2). In the clade representing the latter species, subgroups with bootstrap values as high as 100% were noted. This phenomenon may be indicative of intraspecific variation.

It is generally accepted that the D1/D2 domain of 26S rDNA and ITS1/ITS2 sequences are useful in the delimitation of basidiomycetous yeasts species. However by studying selected yeast taxa, evidence is increasingly being obtained showing that in some cases the 26S rDNA, as well as the more variable ITS regions may exhibit very little intra and even

interspecies variation (Lopandic *et al.*, 2005). An example is biologically distinct species like *Trichosporon montevideense* and *Trichosporon domesticum* which cannot be distinguished by ITS sequences (Sugita *et al.*, 1999). Lopandic *et al.* (2005) emphasized that ribosomal DNA-based phylogeny should be supported by fingerprinting of the whole genome. Two methods, random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) and amplified fragment length polymorphism (AFLP) were successfully applied in the typing of both prokaryotic and eucaryotic genomes (Lopandic *et al.*, 2005).

Investigations into several *Fellomyces* strains using both methods, RAPD-PCR and AFLP showed that these techniques are more discriminative than ITS sequences, although the latter may play a valuable role in investigations of phylogenetic relationships (Lopandic *et al.*, 2005). The AFLP technique showed seven times more DNA loci amplified fragments than RAPD-PCR. Another advantage of AFLP is that it has a higher reproducibility due to the high stringency of the PCR conditions. Both methods suffer from the problem that co-migrating fragments may not be homologous. However, using a sequencer in conjunction with a highly resolving polyacrilamide capillary array, may overcome the problem of co-migrating fragments. It was stated that the AFLP technique can serve as a useful taxonomic tool for strain and species delineation, although DNA-DNA hybridization is necessary to evaluate its full discrimination potential.

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In this study, intraspecific differences regarding physiological characteristics, were found among isolates representing both *C. laurentii* and *C. podzolicus*. The number of carbon sources that can be utilized, the maximum growth temperature and the growth rate in a nutrient rich medium differed within the species (Table 3.2). Cluster profiles of phenotypic criteria revealed that both *C. laurentii* and *C. podzolicus* are physiologically diverse (Fig. 3.3) and representatives of these two species may even group together. In addition, physiological tests that according to literature (Kurtzman & Fell, 1998; Takashima *et al.*, 2003) may be used in the differentiation of *C. laurentii* and *C. podzolicus* could not be used to differentiate the isolates representing the two species. These physiological tests include testing for the ability to assimilate L-sorbose, ribitol, erythritol, and testing for the ability to grow at 35°C, as well as to grow in a medium without vitamins (Appendix A). This dis-

crepancy should be further investigated in future, since the similar physiological profiles of some isolates representing the two different species may be a result of a shared natural niche in this soil.

Table 3.3: Characteristics of the isolates obtained from the soil sample

Isolate no.	Species	No. of C sources utilized [1]	μ max [2]	Max temp of growth °C [3]	Opt temp of growth °C
1a	*Cryptococcus laurentii*	30	0.23	37	25/30
1e	*C. laurentii*	30	0.25	37	25/30
1f	*C. laurentii*	30	0.25	37	20/25/30
1g	*C. laurentii*	30	0.27	37	20/25/30
1h	*C. laurentii*	30	0.25	37	20/25/30
1I	*C. laurentii*	30	0.19	37	20/25/30
2c	*C. laurentii*	28	0.08	30	20/25/30
3m	*C. laurentii*	29	0.16	30	25
3n	*C. laurentii*	30	0.11	30	25
1b	*C. podzolicus*	29	0.10	30	25
1c	*C. podzolicus*	28	0.13	30	25
1d	*C. podzolicus*	28	0.09	35	25
1j	*C. podzolicus*	29	0.12	35	20/25/30

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[1] Number of the following compounds the isolate was able to utilize as sole carbon source, as determined according to the methods described by Van der Walt and Yarrow (1984): D-glucose, galactose, L-sorbose, sucrose, maltose, trehalose, lactose, melibiose, raffinose, melezitose, Soluble starch, inulin, D-xylose, L-arabinose, D-ribose, L-rhamnose, D-arabinose, erythritol, ribitol, D-mannitol, inositol, xylitol, methanol, ethanol, Propane-1,2-diol, Butane-1,2-diol, glycerol, galactitol, Sorbitol, Succinic acid, Citric acid, DL-lactic acid, D-galactonate ,glucoronic acid, 2-ketogluconate, arbutin, salicin.

[2] Maximum growth rate calculated using the equation μ max= lnx_t -ln x_0 / t_t - t_0 , where x_0 is A_{640} at the start of logarithmic growth, ln x_t is A_{640} at mid-logarithmic growth, t_t is time at mid-logarithmic growth and t_0 is time at the start of logarithmic growth.

[3] Maximum temperature at which growth occurred when growth was tested, according to the methods of Van der Walt and Yarrow (1984), at 25°C, 30°C, 35°C, 37°C, 40°C and 42°C.

2a	*C. podzolicus*	29	0.14	35	20/25
2b	*C. podzolicus*	29	0.12	30	20/25
2d	*C. podzolicus*	30	0.13	30	20/25/30
2e	*C. podzolicus*	28	0.16	30	25
2f	*C. podzolicus*	30	0.11	30	25
2g	*C. podzolicus*	30	0.16	30	25
3a	*C. podzolicus*	30	0.09	30	25
3b	*C. podzolicus*	28	0.13	35	30
3c	*C. podzolicus*	29	0.10	35	25
3d	*C. podzolicus*	30	0.10	35	30
3e	*C. podzolicus*	30	0.09	35	30
3f	*C. podzolicus*	28	0.12	35	25
3g	*C. podzolicus*	28	0.11	30	25/30
3h	*C. podzolicus*	28	0.12	35	25/30
3i	*C. podzolicus*	28	0.15	35	25/30
3j	*C. podzolicus*	29	0.11	30	25/30
3k	*C. podzolicus*	29	0.12	35	25/30
3l	*C. podzolicus*	29	0.11	35	25/30
3o	*C. podzolicus*	29	0.10	35	25/30
3p	*C. podzolicus*	31	0.13	30	25/30
4a	*C. podzolicus*	28	0.14	37	25
5a	*C. podzolicus*	28	0.12	35	25/30

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3.3.1 Conclusions

The results of the present study highlighted intraspecies heterogeneity of isolates representing *C. laurentii* and *C. podzolicus* originating from fynbos soil. Although indications of intraspecific genetic heterogeneity were uncovered during sequence analyses of the D1/D2 domain of 26S rDNA, more discriminative typing techniques such as RAPD-PCR and AFLP should be used in future to study intraspecific genetic differences among these isolates.

While the phylogenetic analysis of the D1/D2 domain of 26S rDNA showed that the isolates belonged either to *C. laurentii* or to *C. podzolicus*, the isolates showed notable intraspecies heterogeneity regarding carbon assimilation profiles, maximum growth temperature and growth rate in a nutrient rich medium. The extent of this physiological heterogeneity was such that the species could not be differentiated on the basis of the physiology of the isolates.

The question thus arises whether similar heterogeneity exists among isolates representing each species regarding their interactions with other microbes originating from the same habitat. Consequently, in chapter 4 the interactions of these cryptococci with filamentous fungi originating from the same soil sample are discussed.

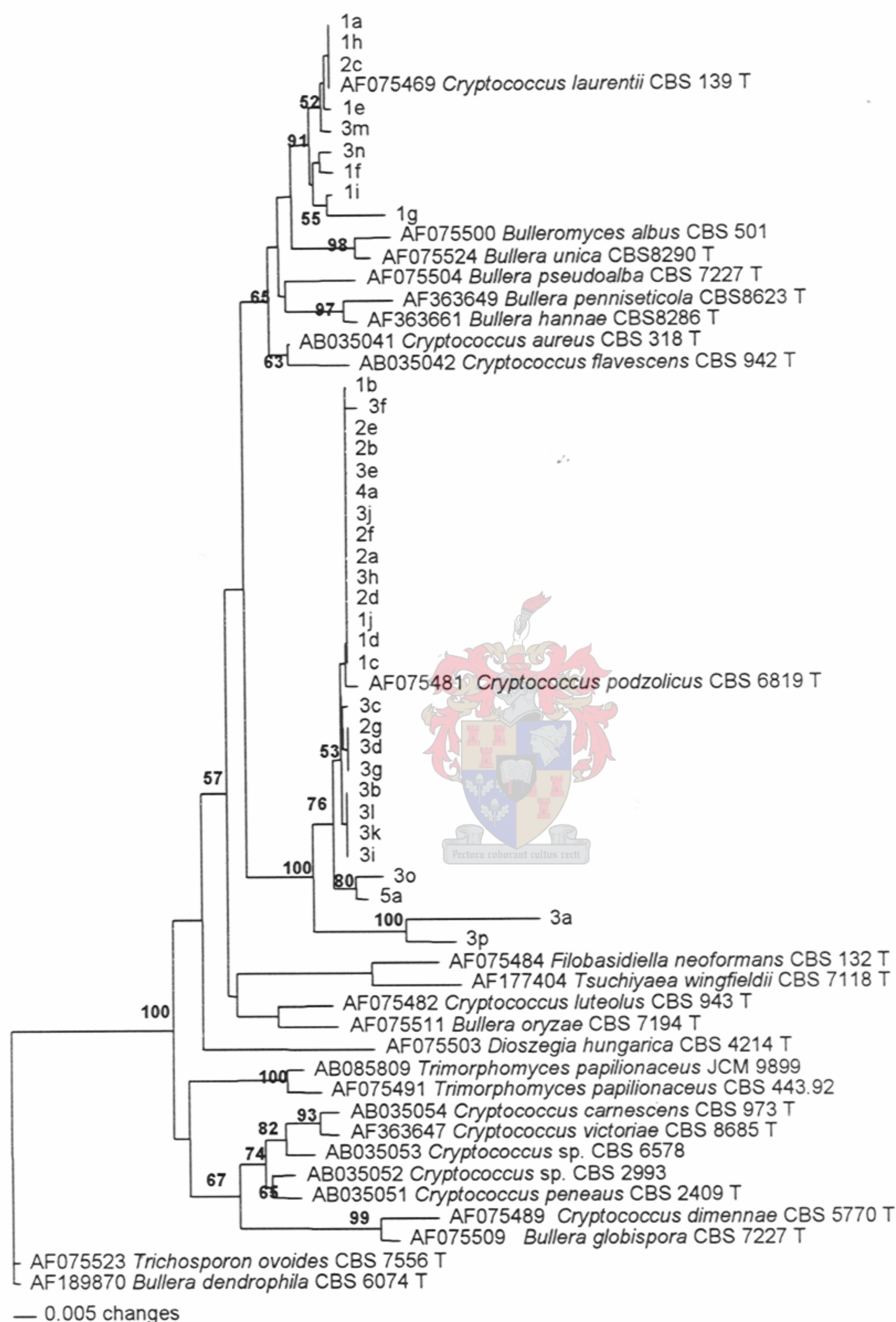

Fig. 3.2: Molecular phylogenetic tree based on partial sequences of D1/D2 domain of rDNA. The tree was constructed with the neighbour-joining method. Numerals represent confidence levels of 1000 replicate bootstrap samplings (frequencies less than 50% are not indicated). "T" indicates type strains. Accession numbers of the reference sequences, that were obtained from the Genbank database at the NCBI server, are provided in front of each strain designation.

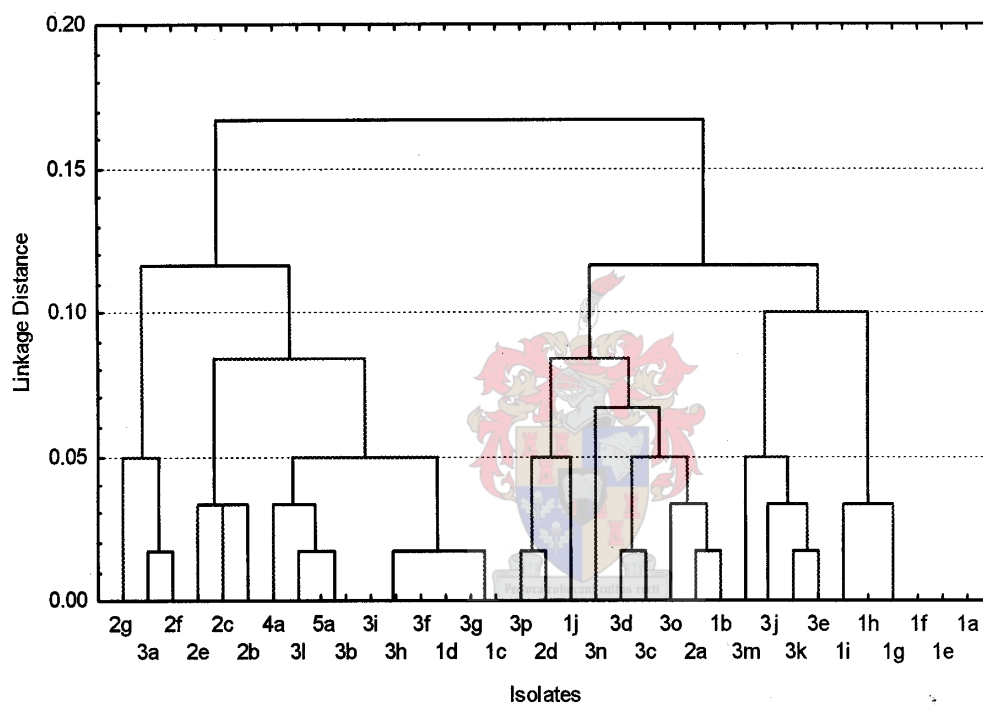

Fig. 3.3: Phenogram of 35 *Cryptococcus* isolates based on their responses to 60 physiological tests (see Appendix A: Tables A.1, A.2, A.3 and A.4). Percentage disagreement cluster method with complete linkage was used.

Chapter 4

Antagonism of isolates representing *Cryptococcus laurentii* and *Cryptococcus podzolicus* towards oligotrophic filamentous soil fungi

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4.1 Introduction

It is known that basidiomycetous soil yeasts belonging to the genus *Cryptococcus* may exert cytocidal and cytostatic effects on other eukaryotes. *C. neoformans* is a well-known human pathogen that was also found to kill amoebae that may occur in the natural habitat of this yeast (Steenbergen *et al.*, 2001). Factors that may affect the virulence of *C. neoformans* were found to be the ability of the yeast to produce extra- cellular polysaccharides, melanin-like pigments and laccases (Petter *et al.*, 2001). These abilities were also discovered in related *Cryptococcus* species, including autochthonous soil yeasts such as *C. laurentii* and *C. podzolicus*. (Petter *et al.*, 2001; Ikeda *et al.*, 2002).

The antagonistic effect of *Cryptococcus* is, however, not restricted to the protozoa and animalia. Strains representing *C. laurentii* were found to inhibit growth of fungal post harvest pathogens on wounded fruit (Roberts, 1990; Chand-Goyal & Spotts, 1997). Although

the exact mode of action of this antagonistic effect of yeasts is still unclear, it has been suggested that nutrient competition, killer activity or the production of glucanases may play a role (Robert 1990; Fredlund *et al.*, 2002; Masih & Paul, 2002).

Most of the above mentioned interactions of *Cryptococcus* were studied in nutrient rich environments, although *C. laurentii* commonly occurs in a nutrient poor environment such as soil. It was also found that this yeast is able to grow oligotrophically (Kimura *et al.*, 1998. In Chapter 3 it was demonstrated that although isolates of both *C. laurentii* and *C. podzolicus* show intraspecific variation regarding a number of physiological characteristics, they are all able to grow oligotrophically. Therefore, the aim of this study was to investigate the antagonistic effect of these isolates, representing *C. laurentii* and *C. podzolicus*, related oligotrophic yeasts towards oligotrophic soil fungi originating from the same soil sample.

4.2 Materials and methods

4.2.1 Isolates

The same isolates representing *C. laurentii* and *C. podzolicus*, that were isolated and identified as explained in Chapter 3, were used in this study. The yeast type strains, *Cryptococcus laurentii* CBS 139 and *Cryptococcus neoformans* var. *neoformans* CBS 132T were obtained from the culture collection of the University of the Free State, South Africa. Despite two efforts of the curator of this culture collection to obtain viable cultures representing the type strain of *C. podzolicus* from the Centraalbureau voor Schimmelcultures, the cultures could not be revived. Consequently, the latter type strain was omitted from this study.

Two filamentous fungal species *viz.*strains, representing the species, *Acremonium alternatum* and *Penicillium commune*, were also isolated from the same soil sample as the yeasts mentioned in Chapter 3. For this purpose silica gel plates devoid of carbon and nitrogen sources were used (Mirocha & De Vay, 1971). The oligotrophic nature of these filamentous fungi were confirmed by their ability to keep on growing, even when repetitively subcultured on these silica gel plates.

4.2.2 Screening for antagonistic activity among yeast isolates

To investigate the relative antagonism of the yeast isolates towards the two filamentous fungi, a method was developed in which a culture of each yeast isolate was prepared by inoculating 50 ml YM broth (pH 5.5) contained in a 250 ml conical flask, with a loop-full of one-week-old cells from a YM slant. The cultures were subsequently incubated overnight at 30°C on a rotary shaker (100 rpm) until stationary cell growth was reached. The cells were harvested using centrifugation ($13800 \times g$ for 15 min), washed three times with sterile physiological saline solution (PSS), and diluted with PSS resulting in a final cell suspension of *ca.* 1×10^6 cells/ ml, as determined using a haemacytometer.

Triplicate spread plates on malt extract (ME) agar and water agar were prepared with the cell suspension of each yeast strain. Each plate (diameter of 85 mm) received 1×10^5 washed yeast cells. After the plates were allowed to dry for 5h under sterile conditions, a well of 8 mm in diameter was made in the center of each plate and it was inoculated with *ca.* 3×10^4 filamentous fungal spores in PSS. The latter spore suspension was obtained by transferring spores from a two-week-old culture of *A. alternatum* to sterile PSS with an inoculation loop. Using a haemacytometer to determine spore numbers, the suspension was then appropriately diluted with sterile PSS.

The inoculated plates were incubated at 25°C and 30°C for one week and the colony diameter of the filamentous fungus in the center of each plate was recorded. This experiment was repeated with the *P. commune* strain (see Appendix A and B, Tables A.5 and B.3).

4.2.3 Statistical analysis

The data obtained when the colony diameters were measured, were used to analyze differences in growth with regard to temperature, medium, fungus and yeast isolate by four-way analysis of variance (ANOVA). Subsequent interactions, which proved to be significant were investigated by Bonferroni multiple comparison procedures. A significance level of α=0.05 was used. All statistical analyses were performed using Statistica for Windows

Version 6 (StatSoft).

4.2.4 Endoglucanase screening

The yeast isolates were screened for the production of extracellular endoglucanase enzymes according to the methods described by Ruijsenaars and Hartmans (2001). For each strain an inoculum of 1×10^4 cells were transferred to the center of the plate containing 0.15% (m/v) ostazin brilliant blue cellulose dissolved in 0.05M sodium acetate buffer, pH5. After an incubation period of two days at 25°C, the plates were observed for clear zones. The plates were also used to screen the culture filtrate of the selected yeast strains cultivated for two days in malt extract (ME) broth (Table 4.3).

4.2.5 Killer activity

The killer phenomenon was investigated according to the protocol of Golubev *et al.* (2003). The medium used essentially contained 5% (m/v) glycerol, 0.5%(m/v) glucose, 0.25%(m/v) peptone, 0.2%(m/v) yeast extract and 2%(m/v) agar with 0.05M citrate-phosphate buffer (pH4.5). Strains that were examined for killer activity against the yeast isolates, were *C. laurentii* (1a and 1f), *C. podzolicus* (3f), also part of the yeast isolate set and *C. laurentii* CBS139T.

4.2.6 Laccase activity among yeast isolates

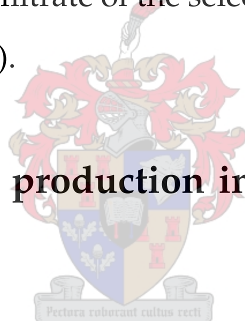
A plate assay for the presence of laccase activity was conducted on all the yeast isolates according to the methods of Kwon-Chung *et al.* (1982). For each strain an inoculum of 1×10^4 cells was transferred to the center of an agar plate containing dihydroxyphenyl-alanine (DOPA) in the medium. This differential medium was prepared by first autoclaving (121°C for 15 min) 800 ml of a basal medium (pH 5.5) consisting of 0.5g glucose, 10mg thiamine hydrochloride, 20μg biotin, 4g potassium dihydrogen phosphate, 2.5g magnesium sulfate heptahydrate and 25g agar. After the solution was allowed to cool, but before it solidified, 200ml filter sterilized solution (pH 5.5) containing 0.04g DOPA, 1g asparagine, 1g L-glutamine and 1g glycine was added to the basal medium. The pH of the DOPA-containing

solution adjusted to pH5.5 with 1M potassium hydrogen phosphate.

After inoculation and an incubation period of two days at 25°C, the plates were examined for dark brown to black zones. The plates were also used to screen the culture filtrate of the selected yeast strains cultivated for three days at 25°C in ME broth (Table 4.3).

4.2.7 Protease screening

All yeast isolates were screened for proteolytic activity on a modified gelatin agar as described by Wade *et al.* (2003). The medium (pH5.9)consisted of 30g gelatin, 20g glucose, 20g peptone, 10g yeast extract and 15g agar in 1000ml distilled water. For each strain, protease plates were inoculated with 1×10^4 cells and incubated at 25°C. After incubation at 25°C for three days, the plates were examined for the presence of clear zones. The plates were also used to screen the culture filtrate of the selected yeast strains cultivated for three days at 25°C in ME broth (Table 4.3).



4.2.8 Screening for enzyme production in the presence of *P. commune* culture filtrates

Selected yeast isolates, representing *C. laurentii* and *C. podzolicus* were screened for the induction/enhancement of enzyme production in the presence of the filamentous fungus *P. commune*, when grown in (1.5%m/v) malt extract (ME) broth at 25°C. Each yeast isolate was cultivated in freshly prepared ME broth, contained in conical flasks at 25°C until an OD_{600} of *ca.* 0.6 was reached. The cells were harvested using centrifugation ($13800 \times g$ for 5 min.) and $20\ \mu$l of the supernatant was spotted on the various screening media (sections 4.2.3, 4.2.4 and 4.2.6.) and observed for typical reactions. An inoculum of 1×10^6 cells was transferred to the filter-sterilized *P. commune* culture filtrate, obtained by growing the culture for 1 week in ME broth at 25°C. The yeast growing in the *P. commune* culture filtrate was incubated at 25°C until the same OD_{600} was reached as when the yeast was first harvested growing in fresh ME broth. The culture filtrate was harvested using centrifugation and 20μl quantities were tested on the screening media (sections 4.2.3, 4.2.4

and 4.2.6) (see Appendix A, Table A.6).

4.3 Results and Discussion

When the antagonistic effect of the *C. laurentii* isolates on the radial growth of *A. alternatum* was measured, it was found that not only did the effect of the isolates differed from one another, but the effect of a single isolate also differed on different media and at different temperatures (Figs.4.1 A & B, 4.3 A & B). In contrast to the results obtained with other conditions, a number of *C. laurentii* strains had a positive effect on the colony diameter of *A. alternatum* on water agar at 30°C (Figs. 4.1 A & B). Similar results were obtained when the radial growth of *P. commune* was measured on different media and at different temperatures (Figs.4.2 A & B). The isolates representing *C. podzolicus* also differed regarding their antagonistic effect on the radial growth of the two filamentous fungi (Figs. 4.3 A & B, 4.4 A & B). Compared to the isolates of *C. laurentii*, the *C. podzolicus* isolates seem to act antagonistically against *A. alternatum*, even at 30°C on the water agar. However, at 30°C this antagonistic effect was not as obvious against *P. commune* (Figs. 4.2 B & 4.4 B).

To obtain an indication of which factors are involved in the antagonistic effects of the yeasts on growth of other fungi, the yeast strains were screened for a number of characteristics known to be involved in fungal antagonism. It has been shown that wood decay fungi appear to be susceptible to killer yeast strains (Walker *et al.*, 1995). Since it is known that some cryptococci may exhibit killer activity (Golubev, 1998; Golubev & Kuznetsova, 1989), it was decided to screen for killer activity among the yeast isolates. None of the yeast isolates that were screened showed this activity (Table 4.1). The only killer activity observed was that of the type strain *C.laurentii* CBS139T. This is not surprising, since it is known that yeast strains from the same habitat do not exert a killer effect among themselves (Starmer *et al.*, 1987).

Pure cultures of the yeasts were subsequently screened for the presence of enzymes such as endoglucanases, laccases and proteases, which all have been implicated in antagonistic interactions in the fungal domain (Sietsma *et al.*, 1995; Score *et al.*, 1997; Schmidt *et*

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al., 2001). Endoglucanases are known to hydrolyze endoglucans, which are cell wall components involved in the construction of plasma membranes (Sietsma *et al.*, 1995). Laccases are known virulence factors which may disrupt cell walls (Thurston, 1994) and proteases may hydrolyze essential proteins in and around the cell (Horton *et al.*, 1999). Of the three types of enzymes screened for in pure cultures, proteases were the only enzymes detected (Table 4.2).

Since these results were obtained for pure cultures, it was decided to screen for the induction of these enzymes in culture filtrates from *P. commune*. Surprisingly, no induction of laccase or endoglucanase was detected. While evidence for a laccase gene in *C. podzolicus* does exist (Petter *et al.*, 2001), the results on the screening for the endoglucanase are in accordance with literature. It is known for some time that soil yeasts do not have the ability to degrade cellulose and related polymers (do Carmo-Sousa, 1969). Similar to the results obtained when pure yeast cultures were screened (Table 4.2), proteases were also detected when yeast isolates were grown in the culture filtrate of *P. commune* (Table 4.3). Interestingly, compared to the controls where each yeast strain was cultivated in fresh ME both, more protease activity was detected in yeast cultures grown on the filtrate of *P. commune* (Table 4.3). This indicates that yeast proteases are stimulated in the presence of this filamentous fungus and may play a role in the antagonistic effect of the yeast.

However, the technique used in this study to detect protease activity may be perceived as crude, especially when compared to modern quantitative assays based on 2-D electrophoresis and/or gel filtration (Motizuki, *et al.*, 1986). Nevertheless the method did indicate that proteases may be responsible for the antagonistic effect of the cryptococci and that the role of these enzymes should be investigated in future studies.

In future, other assays for the presence of laccases (e.g. the method of Petter *et al.* ,2001) should also be conducted, especially since the method used in the present study was not aimed at detecting cell-wall bound laccases, but to reveal laccases that are released into the medium, such as the laccases of *Trichoderma reesei*. The latter species is known for its antagonistic effects on a number of fungi (Score, *et al.* 1997)

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Table 4.1: Killing patterns of mycocins produced by *C. laurentii* CBS139T, *C. laurentii* (1a), *C. laurentii* (1f), *C. podzolicus* (3f)

Isolate	CBS 139T	1a	1f	3f
1a	+	-	-	-
1b	+	-	-	-
1c	+	-	-	-
1d	+	-	-	-
1e	+	-	-	-
1f	+	-	-	-
1g	+	-	-	-
1h	+	-	-	-
1i	+	-	-	-
1j	+	-	-	-
2a	+	-	-	-
2b	+	-	-	-
2c	+	-	-	-
2d	+	-	-	-
2e	+	-	-	-
2f	+	-	-	-
2g	+	-	-	-
3a	+	-	-	-
3b	+	-	-	-
3c	+	-	-	-
3d	+	-	-	-
3e	+	-	-	-
3f	+	-	-	-
3g	+	-	-	-

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3h	+	-	-	-
3i	+	-	-	-
3j	+	-	-	-
3k	+	-	-	-
3l	+	-	-	-
3m	+	-	-	-
3n	+	-	-	-
3o	+	-	-	-
3p	+	-	-	-
4a	+	-	-	-
5a	+	-	-	-
CBS 139T		-	-	-

Table 4.2: The production of enzymes screened by pure cultures of the yeast isolates on differential media

Isolate	Glucanases[1]	Laccases[2]	Proteases[3]
1a	-	-	+
1c	-	-	+
1d	-	-	+
1e	-	-	+
1f	-	-	+
1g	-	-	+
1h	-	-	+
1i	-	-	+
1j	-	-	+
2a	-	-	+

[1] A purified enzyme of *Trichoderma reesei* was included as positive control.
[2] *C. neoformans* CBS 132T was included as positive control.
[3] *C. laurentii* CBS 139T was included as positive control.

2b	-	-	+
2c	-	-	+
2d	-	-	+
2e	-	-	+
2f	-	-	+
2g	-	-	+
3a	-	-	+
3b	-	-	+
3c	-	-	+
3d	-	-	+
3e	-	-	+
3f	-	-	+
3g	-	-	+
3h	-	-	+
3i	-	-	+
3j	-	-	+
3k	-	-	+
3l	-	-	+
3m	-	-	+
3n	-	-	+
3o	-	-	+
3p	-	-	+
4a	-	-	+
5a	-	-	+

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Table 4.3: The ratio of the zone diameter on differential plates for protease to the OD_{600}, obtained with yeast cultures grown in fresh ME broth and growing in the culture fluid of *P. commune*

Yeast isolate	Only filamentous fungus[4]	Only yeast	Filamentous fungus and Yeast
1a	0	14.4	16.3
1b	0	14.1	14.6
1e	0	17.1	18.5
1f	0	18.2	18.8
1g	0	14.4	15.3
1h	0	16.7	16.2
1i	0	14.5	19
3e	0	13.9	16.5
3f	0	12.5	13.7
3g	0	10.8	10.9
4a	0	15.6	17.1
5a	0	17.04	18.66
control[5]	0	17.5	18.1

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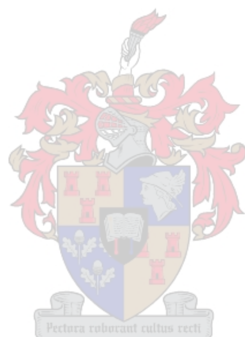
[4]Supernatant of *P. commune* was spotted on protease differential medium.
[5]*C. laurentii*CBS139T was included as positive control.

4.3.1 Conclusions

This study showed that intraspecific diversity regarding yeast-filamentous fungal interactions occurred among isolates representing *C. laurentii* and *C. podzolicus* originating from a single soil sample. Subsequent screening of the yeast isolates revealed that a yeast protease may be involved with the antagonistic effect of the yeast towards the filamentous fungi. According to our knowledge these findings are the first indications of proteases as factor playing a role in the antagonistic actions of *C. laurentii* and *C. podzolicus*. To confirm the role of these hydrolytic enzymes in the antagonism of *C. laurentii* and *C. podzolicus*, detailed molecular analyses should be conducted on the production of proteases by these cryptococci.



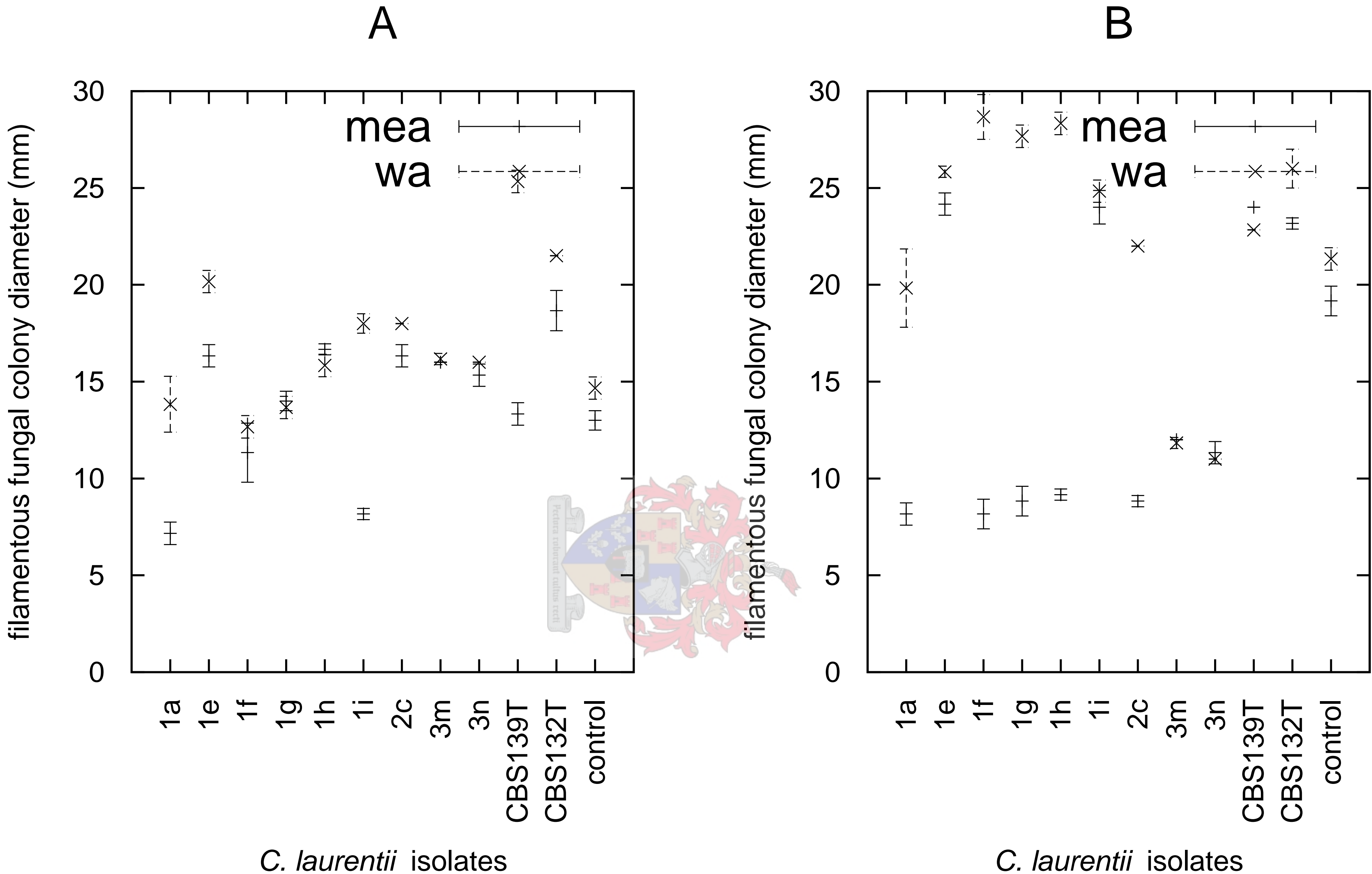


Fig. 4.1: The interspecific interactions of *C. laurentii* isolates with the filamentous fungus, *A. alternatum* on malt extract agar (MEA) and water agar (WA) at (A) 25°C and (B) 30°C, respectively. For reference purposes , *A. alternatum* was also respectively challenged with the type strains *C. laurentii* CBS139T and *C. neoformans CBS* 132T. Included in the experiment was a control comprising only of *A. alternatum* in the absence of a yeast

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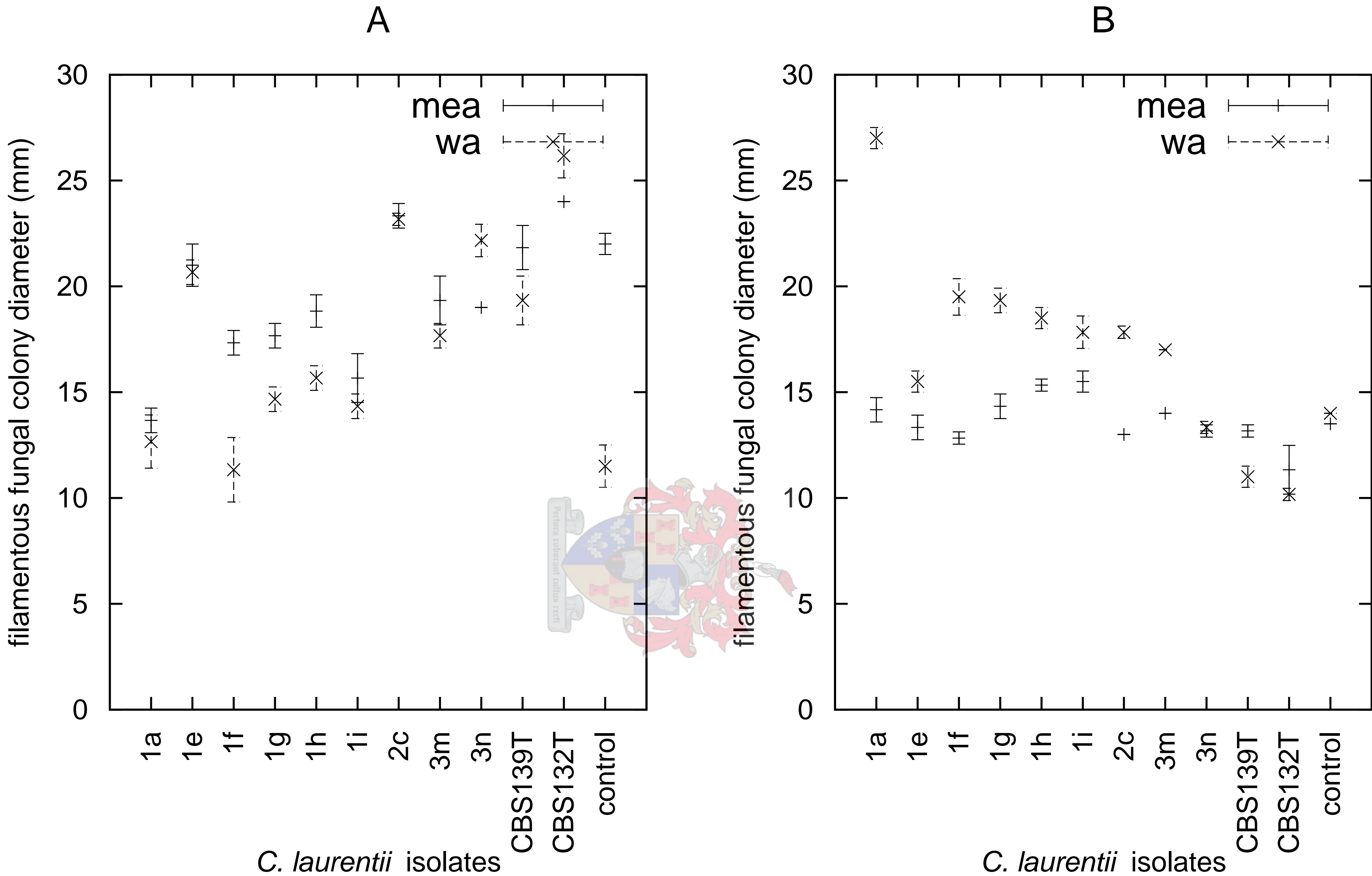

Fig. 4.2: The interspecific interactions of *C. laurentii* isolates with the filamentous fungus, *P. commune* on malt extract agar (MEA) and water agar (WA) at (A) 25°C and (B) 30°C, respectively. For reference purposes , *P. commune* was also respectively challenged with the type strains *C. laurentii* CBS139T and *C. neoformans CBS* 132T. Included in the experiment was a control comprising only of *P. commune* in the absence of a yeast

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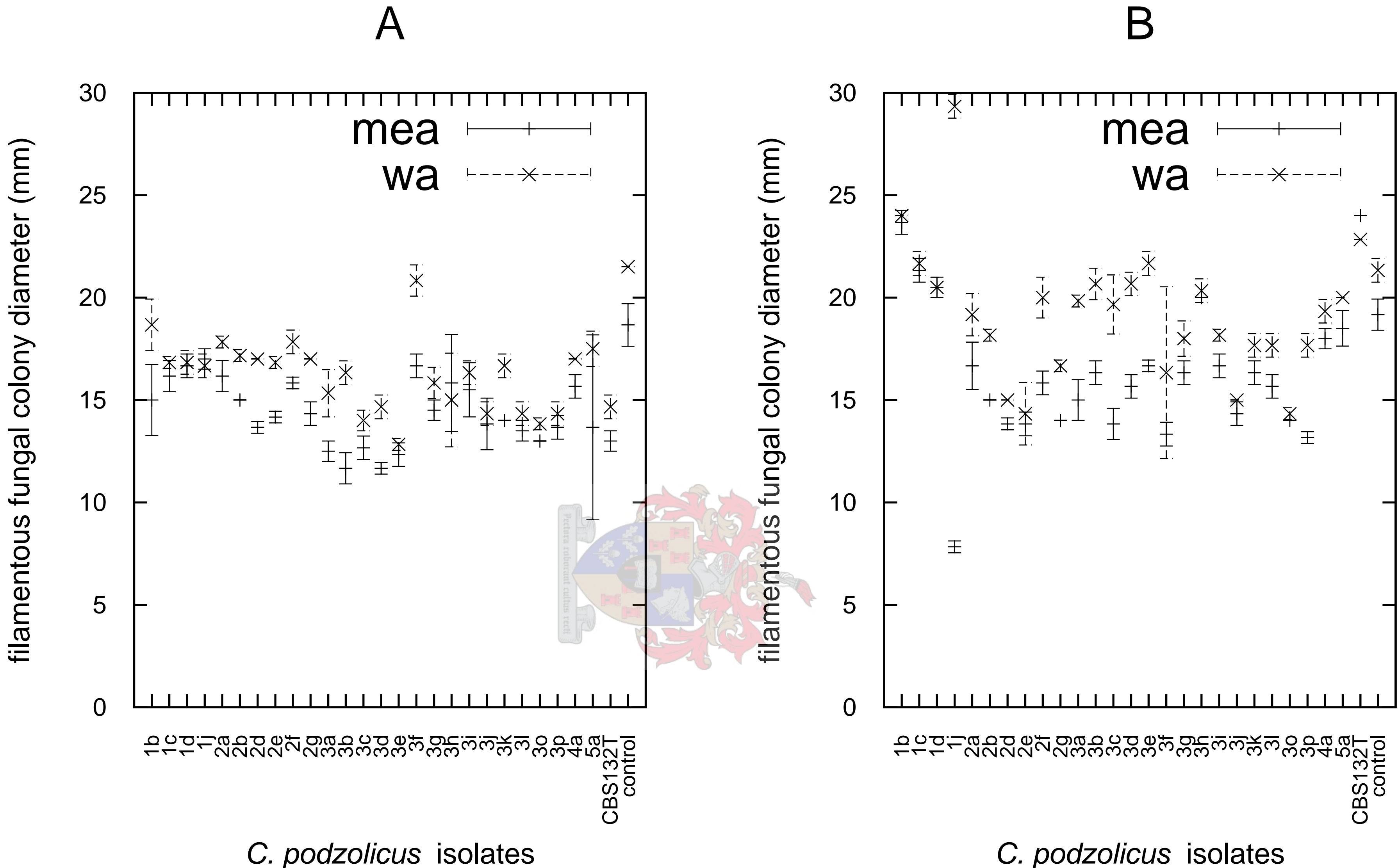

Fig. 4.3: The interspecific interactions of *C. podzolicus* isolates with the filamentous fungus,isolates with the filamentous fungus, *A. alternatum* on malt extract agar (MEA) and water agar (WA) at (A) 25°C and (B) 30°C, respectively. For reference purposes , *A. alternatum* was also challenged with the type strain *C. neoformans CBS* 132T. Included in the experiment was a control comprising only of *A. alternatum* in the absence of a yeast

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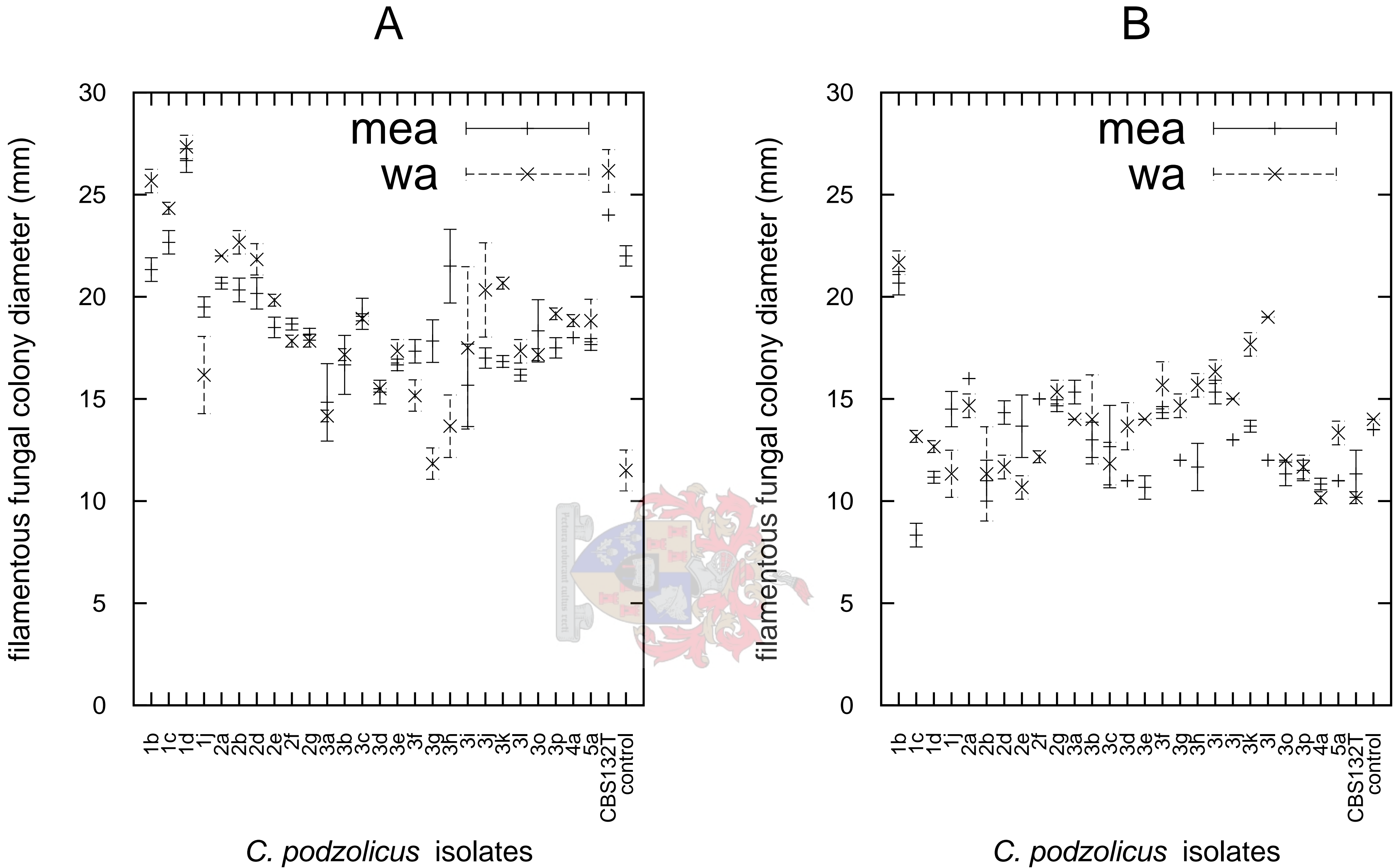


Fig. 4.4: The interspecific interactions of *C. podzolicus* isolates with the filamentous fungus, *P. commune* on malt extract agar (MEA) and water agar (WA) at (A) 25°C and (B) 30°C, respectively. For reference purposes , *P. commune* was also challenged with the type strain *C. neoformans CBS* 132T. Included in the experiment was a control comprising only of *P. commune* in the absence of a yeast

.

Chapter 5

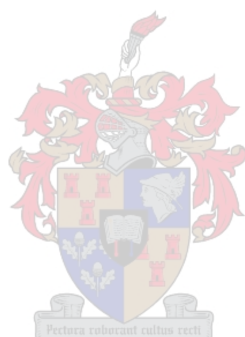
General Conclusions

The study highlighted the intraspecific diversity seen among various *C. laurentii* and *C. podzolicus* strains within a single soil sample originating from a pristine habitat. It was found that intraspecific variation exists regarding the ability to assimilate carbon sources, cardinal temperatures, growth rates and interactions with filamentous fungi. In addition, indications of intraspecific diversity were found upon phylogenic analysis of the D1/D2 region of the rDNA. However, this method is not the most appropriate method for studying intraspecific diversity and it was suggested that future studies should include techniques such as random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) and amplified fragment length polymorphism (AFLP). Nevertheless, the study provided a glimpse of the variation needed to sustain these species in a particular soil habitat in the absence of anthropogenic activities. The origin of this variation and the possible involvement of a cryptic sexual stage should also be investigated in future. Interestingly, Kurtzman *et al.* (1973) did observe mating between isolates representing *C. laurentii*. However, to confirm the formation of sexual stages among the yeast isolates from fynbos soil, mating studies should be conducted under a wide diversity of environmental conditions.

Furthermore, the results obtained in this study, showed that filamentous soil fungi may be antagonistically affected by strains representing *C. laurentii* and *C. podzolicus*. Preliminary indications show that proteases may play a role in these interactions but these findings need to be validated by employing more accurate assays. This phenomenon should

be the subject of further studies on the interactions of these yeasts.

To conclude, the results of this study on the intraspecific diversity of cryptococci in their natural environment have implications on how the biodiversity within soil samples are perceived. In a single soil sample numerous individual strains, differing in their characteristics, may be found that represent a single species. This phenomenon must be borne in mind when soil samples are investigated during bioprospecting or analysed to obtain representatives of a particular habitat during ecological surveys.



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Appendix A

A.1 Compilations in printed format:

A.1.1 Table A.1: Carbon source assimilation data of yeast isolates

A.1.2 Table A.2: Assimilation of various carbon containing compounds by the isolates

A.1.3 Table A.3: Additional physiological tests performed on the isolates

A.1.4 Table A.4: Nitrogen source assimilation by yeast isolates

A.1.5 Table A.5: The intraspecific interactions of yeast isolates with the filamentous fungi

A.1.6 Table A.6: Cell densities and corresponding protease screening of culture fluids of selected yeast isolates in the absence and presence of filamentous fungi

Table A.1: The assimilation of various carbohydrates by the isolates

Yeast isolate[1]	control[2]	D-glucose	galactose	L-sorbose	sucrose	maltose	trehalose	lactose	melibiose	raffinose	melezitose	Soluble starch	inulin	D-xylose	L-arabinose	D-ribose	L-rhamnose	D-arabinose
1a	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
1e	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
1f	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
1f	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
1g	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
1h	-	+	+	-	+	+	+	+	+	+	+	+	-	+	-	-	+	+
1i	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+
2c	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
3m	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
3n	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
1b	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
1c	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
1d	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
1j	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
2a	-	+	+	-	+	+	+	+	+	+	+	+	-	+	-	-	+	+
2b	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
2d	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
2e	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
2f	-	+	+	-	+	+	+	+	+	+	+	+	-	+	-	-	+	+
2g	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
3a	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
3b	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
3c	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+

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Table A.1: continued

Yeast isolate	control	D-glucose	galactose	L-sorbose	sucrose	maltose	trehalose	lactose	melibiose	raffinose	melezitose	Soluble starch	inulin	D-xylose	L-arabinose	D-ribose	L-rhamnose	D-arabinose
3d	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
3e	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
3f	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
3g	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
3h	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
3i	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
3j	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
3k	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
3l	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
3o	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
3p	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
4a	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+
5a	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+

[1]Assimilation of carbon source by each yeast isolate is denoted as either + / - or W indicating growth, no growth or weak growth, respectively.

[2]Control included contained only yeast nitrogen base (YNB).

Table A.2: The assimilation by yeast isolates of various carbon containing compounds

Yeast isolate[1]	erythritol	ribitol	D-mannitol	inositol	xylitol	methanol	ethanol	propane-1,2-diol	butane-1,2-diol	glycerol	galactitol	sorbitol	succinic acid	citric acid	DL-lactic acid	D-galactonate	glucoronic acid	2-ketogluconate	arbutin	salicin
1a	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
1e	+	+	+	+	+	-	W	-	-	+	-	+	+	+	+	+	+	+	+	+
1f	+	+	+	+	+	-	W	-	-	+	-	+	+	+	+	+	+	+	+	+
1g	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
1h	-	+	+	+	+	-	-	-	-	W	-	+	-	+	+	+	+	+	+	+
1i	+	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
2c	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
3m	+	-	+	+	+	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+
3n	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
1b	-	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
1c	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
1d	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
1j	+	+	+	+	+	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+
2a	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
2b	-	+	+	+	+	-	-	-	-	+	+	+	W	+	+	+	+	+	+	+
2d	-	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
2e	+	+	+	+	+	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+
2f	-	+	+	+	+	-	W	-	-	+	+	+	+	+	+	+	+	+	+	+
2g	-	+	+	+	+	-	W	-	-	+	-	+	+	+	+	+	+	+	+	+
3a	-	+	+	+	+	-	W	-	-	W	-	+	+	+	+	+	+	+	+	+
3b	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
3c	-	+	+	+	+	-	-	-	-	W	+	+	+	+	+	+	+	+	+	+

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Table A.2: continued

Yeast isolate	erythritol	ribitol	D-mannitol	inositol	xylitol	methanol	ethanol	propane-1,2-diol	butane-1,2-diol	glycerol	galactitol	sorbitol	succinic acid	citric acid	DL-lactic acid	D-galactonate	glucoronic acid	2-ketogluconate	arbutin	salicin
3d	-	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
3e	+	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
3f	+	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
3g	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
3h	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
3i	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
3j	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
3k	+	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
3l	+	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
3m	+	-	+	+	+	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+
3n	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
3o	+	+	+	+	+	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+
3p	-	+-	+	+	+	-	W	-	-	+	+	+	+	+	+	+	+	+	+	+
4a	+	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
5a	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+

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[1]Assimilation of carbon source by each yeast isolate is denoted as either +/ - or W indicating growth, no growth or weak growth, respectively. Control included, contained only YNB.

Table A.3: The results of additional physiological tests performed on the isolates

Yeast isolate[1]	Growth at 25°C	Growth at 30°C	Growth at 35°C	Growth at 37°C	Growth at 40°C	Growth at 42°C	0.01% cycloheximide	0.1% cycloheximide	W/o vitamins	Starch formation	Acetic acid production	Urea hydrolysis	Diazonium Blue B reaction
1a	+	+	+	W	-	-	+	-	-	+	-	+	+
1e	+	+	+	+	-	-	+	-	-	-	-	+	+
1f	+	+	+	+	-	-	+	-	-	+	-	+	+
1g	+	+	+	+	-	-	+	-	-	+	-	+	+
1h	+	+	+	+	-	-	+	-	-	+	-	+	+
1i	+	+	+	+	-	-	+	-	-	+	-	+	+
2c	+	+	-	-	-	-	+	-	-	-	-	+	+
3m	+	+	+	-	-	-	+	-	-	-	-	+	+
3n	+	+	-	-	-	-	-	-	-	+	-	-	+
1b	+	+	-	-	-	-	+	-	-	+	-	-	+
1c	+	+	-	-	-	-	+	-	-	+	-	-	+
1d	+	+	W	-	-	-	+	-	-	-	-	-	+
1j	+	+	W	-	-	-	+	-	-	+	-	-	+
2a	+	+	W	-	-	-	+	-	-	+	-	-	+
2b	+	+	-	-	-	-	+	-	-	+	-	+	+
2d	+	+	-	-	-	-	+	-	-	-	-	-	+
2e	+	+	-	-	-	-	+	-	-	+	-	+	+
2f	+	+	-	-	-	-	+	-	-	-	-	-	+
2g	+	+	-	-	-	-	+	-	-	-	-	-	+
3a	+	+	-	-	-	-	+	-	-	-	-	-	+

Table A.3: continued

Yeast isolate	Growth at 25°C	Growth at 30°C	Growth at 35°C	Growth at 37°C	Growth at 40°C	Growth at 42°C	0.01% cycloheximide	0.1% cycloheximide	W/o vitamins	Starch formation	Acetic acid production	Urea hydrolysis	Diazonium Blue B reaction
3b	+	+	-	-	-	-	+	-	-	-	-	-	+
3c	+	+	W	-	-	-	+	-	-	-	-	-	+
3d	+	+	W	-	-	-	+	-	-	-	-	-	+
3e	+	+	W	-	-	-	+	-	-	-	-	-	+
3f	+	+	W	-	-	-	+	-	-	-	-	-	+
3g	+	+	W	-	-	-	+	-	-	-	-	-	+
3h	+	+	-	-	-	-	+	-	-	-	-	-	+
3i	+	+	W	-	-	-	+	-	-	-	-	-	+
3j	+	+	W	-	-	-	+	-	-	-	-	-	+
3k	+	+	-	-	-	-	-	-	-	-	-	-	+
3l	+	+	W	-	-	-	-	-	-	-	-	-	+
3o	+	+	W	-	-	-	+	-	-	+	-	-	+
3p	+	+	-	-	-	-	+	-	-	+	-	-	+
4a	+	+	W	W	-	-	+	-	-	-	-	-	+
5a	+	+	W	-	-	-	+	-	-	-	-	-	+

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[1]Each yeast isolate is denoted by either + / - or W indicating growth, no growth or weak growth, respectively. Control included, being yeast nitrogen base.

Table A.4: The physiological evaluation of nitrogen assimilation on various nitrogen compounds by yeast isolates

Yeast isolate[1]	Ammonium sulphate[2]	Cadavarine	Creatine	Creatinine	D-glucosamine	Imidazole	L-lysine	Potassium nitrate	Sodium nitrite	Thymine
1a	+	-	-	+	-	-	+	-	-	-
1e	+	-	-	+	-	-	+	-	-	-
1f	+	-	-	+	-	-	+	-	-	-
1g	+	-	-	+	-	-	+	-	-	-
1h	+	-	-	+	-	-	+	-	-	-
1i	+	-	-	+	-	-	+	-	-	-
2c	+	-	-	-	-	-	+	-	-	-
3m	+	-	-	+	-	-	+	-	-	-
3n	+	-	-	+	-	-	+	-	-	-
1b	+	-	+	+	-	-	+	-	-	-
1c	+	-	-	+	-	-	+	-	-	-
1d	+	-	-	+	-	-	+	-	-	-
1j	+	-	-	-	-	-	+	-	-	-
2a	+	-	-	+	-	-	+	-	-	-
2b	+	-	-	+	-	-	+	-	-	-
2d	+	-	-	-	-	-	+	-	-	-
2e	+	-	-	-	-	-	+	-	-	-
2f	+	-	-	-	-	-	+	-	-	-
2g	+	-	-	+	-	-	+	-	+	-
3a	+	-	-	+	-	-	+	-	-	-
3b	+	-	-	-	-	-	+	-	-	-

Table A.4: continued

Yeast isolate	Ammonium sulphate	Cadavarine	Creatine	Creatinine	D-glucosamine	Imidazole	L-lysine	Potassium nitrate	Sodium nitrite	Thymine
3c	+	-	-	+	-	-	+	-	-	-
3d	+	-	-	+	-	-	+	-	-	-
3e	+	-	-	+	-	-	+	-	-	-
3f	+	-	-	+	-	-	+	-	-	-
3g	+	-	-	+	-	-	+	-	-	-
3h	+	-	-	+	-	-	+	-	-	-
3i	+	-	-	+	-	-	+	-	-	-
3j	+	-	-	+	-	-	+	-	-	-
3k	+	-	-	+	-	-	+	-	-	-
3l	+	-	-	-	-	-	+	-	-	-
3o	+	-	-	-	-	-	+	-	-	-
3p	+	-	-	-	-	-	+	-	-	-
4a	+	-	-	-	-	-	+	-	-	-
5a	+	-	-	-	-	-	+	-	-	-

[1] +/- denotes growth or no growth on auxanogram.

[2] Control compound included in tests is ammonium sulphate.

Table A.5: The intraspecific interactions of yeast isolates with the filamentous fungi, *A. alternatum* and *P. commune* on MEA and WA at 25°C and 30°C.

Yeast isolate	Filamentous fungi	MEA[1]	WA[2]	MEA[3]	WA[4]
1a	*A. alternatum*	7.2	13.8	8.2	19.8
1e	*A. alternatum*	16.3	20.2	24.2	25.8
1f	*A. alternatum*	11.3	12.7	8.2	28.7
1g	*A. alternatum*	14.0	13.7	8.8	27.7
1h	*A. alternatum*	16.7	15.8	9.2	28.3
1i	*A. alternatum*	8.2	18.0	24.0	24.8
2c	*A. alternatum*	16.3	18.0	8.8	22.0
3m	*A. alternatum*	16.0	16.2	12.0	11.8
3n	*A. alternatum*	15.3	16.0	11.3	11.0
1b	*A. alternatum*	15.0	18.7	23.7	24.0
1c	*A. alternatum*	16.2	16.8	21.3	21.7
1d	*A. alternatum*	16.7	16.8	20.5	20.5
1j	*A. alternatum*	17.0	16.7	7.8	29.3
2a	*A. alternatum*	16.2	17.8	16.7	19.2
2b	*A. alternatum*	15.0	17.2	15.0	18.2
2d	*A. alternatum*	13.7	17.0	13.8	15.0
2e	*A. alternatum*	14.2	16.8	13.8	14.3
2f	*A. alternatum*	15.8	17.8	15.8	20.0
2g	*A. alternatum*	14.3	17.0	14.0	16.7
3a	*A. alternatum*	12.5	15.3	15.0	19.8
3b	*A. alternatum*	11.7	16.3	16.3	20.7
3c	*A. alternatum*	12.7	14.0	13.8	19.7

Table A.5: continued

Yeast isolate	Filamentous fungi	MEA	WA	MEA	WA
3d	*A. alternatum*	11.7	14.7	15.7	20.7
3e	*A. alternatum*	12.3	12.8	16.7	21.7
3f	*A. alternatum*	16.7	20.8	13.3	16.3
3g	*A. alternatum*	14.5	15.8	16.3	18.0
3h	*A. alternatum*	15.8	15.0	20.0	20.3
3i	*A. alternatum*	15.5	16.3	16.7	18.2
3j	*A. alternatum*	13.8	14.3	14.3	15.0
3k	*A. alternatum*	14.0	16.7	16.3	17.7
3l	*A. alternatum*	13.5	14.3	15.7	17.7
3o	*A. alternatum*	13.0	13.8	14.0	14.3
3p	*A. alternatum*	13.7	14.3	13.2	17.7
4a	*A. alternatum*	15.7	17.0	18.0	19.3
5a	*A. alternatum*	13.7	17.5	18.5	20.0
CBS132T	*A. alternatum*	18.7	21.5	19.2	21.3
CBS139T	*A. alternatum*	13.3	25.3	23.2	26.0
control[5]	*A. alternatum*	15.7	17.0	24.2	22.8
1a	*P. commune*	13.7	12.7	14.2	27.0
1e	*P. commune*	21.0	20.7	13.3	15.5
1f	*P. commune*	17.3	11.3	12.8	19.5
1g	*P. commune*	17.7	14.7	14.3	19.3
1h	*P. commune*	18.8	15.7	15.3	18.5
1i	*P. commune*	15.7	14.3	15.5	17.8
2c	*P. commune*	23.3	23.2	13.0	17.8
3m	*P. commune*	19.3	17.7	14.0	17.0
3n	*P. commune*	19.0	22.2	13.2	13.3

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Table A.5: continued

Yeast isolate	Filamentous fungi	MEA	WA	MEA	WA
1b	*P. commune*	21.3	25.7	20.7	21.7
1c	*P. commune*	22.7	24.3	8.3	13.2
1d	*P. commune*	26.7	27.3	11.2	12.7
1j	*P. commune*	19.5	16.2	14.5	11.3
2a	*P. commune*	20.7	22.0	16.0	14.7
2b	*P. commune*	20.3	22.7	11.0	11.3
2d	*P. commune*	20.2	21.8	14.3	11.7
2e	*P. commune*	18.5	19.8	13.7	10.7
2f	*P. commune*	18.7	17.8	15.0	12.2
2g	*P. commune*	18.2	17.8	14.7	15.3
3a	*P. commune*	14.8	14.2	15.3	14.0
3b	*P. commune*	16.7	17.2	13.0	14.0
3c	*P. commune*	19.2	18.9	12.7	11.8
3d	*P. commune*	15.3	15.5	11.0	13.7
3e	*P. commune*	16.7	17.3	10.7	14.0
3f	*P. commune*	17.3	15.2	14.3	15.7
3g	*P. commune*	17.8	11.8	12.0	14.7
3h	*P. commune*	21.5	13.7	11.7	15.7
3i	*P. commune*	15.7	17.5	15.3	16.3
3j	*P. commune*	17.0	20.3	13.0	15.0
3k	*P. commune*	16.8	20.7	13.7	17.7
3l	*P. commune*	16.2	17.3	12.0	19.0
3o	*P. commune*	18.3	17.2	11.3	12.0
3p	*P. commune*	17.5	19.2	11.5	11.7
4a	*P. commune*	18.0	18.8	10.8	10.2

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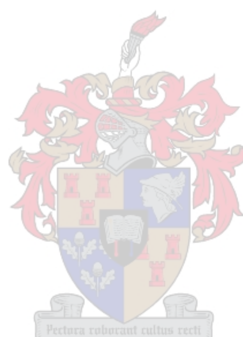
Table A.5: continued

Yeast isolate	Filamentous fungi	MEA	WA	MEA	WA
5a	*P. commune*	17.7	18.8	11.0	13.3
CBS132T	*P. commune*	24.0	26.2	11.3	10.2
CBS139T	*P. commune*	21.8	19.3	13.2	11.0
control[6]	*P. commune*	23.1	21.1	13.4	13.9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[1]denotes average growth of filamentous fungus on malt extract agar at 25°C, each value is the mean of three repetitions, see Appendix B, table B.3.

[2]denotes average growth of filamentous fungus on water agar at 25°C, each value is the mean of three repetitions, see Appendix B, table B.3.

[3]denotes average growth of filamentous fungus on malt extract agar at 30°C, each value is the mean of three repetitions, see Appendix B, table B.3.

[4]denotes average growth of filamentous fungus on water agar at 30°C, each value is the mean of three repetitions, see Appendix B, table B.3.

[5]denotes only average fungal growth of *A. alternatum* on either MEA or WA, see Appendix B, table B.3.

[6]denotes only average fungal growth of *P. commune* on either MEA or WA, see Appendix B, table B.3.

[7]

[8]

Table A.6: Cell densities and corresponding culture fluids of only yeast and combination of yeast and fungal culture fluids on protease plates

Isolate	OD 600nm [a]	Repeat 1	Repeat 2	Repeat 3	ave.	stdev.	OD 600nm [b]	Repeat 1	Repeat 2	Repeat 3	ave.	stdev.
1a	0.62	10	10.5	10	10.2	0.3	0.645	11	10.5	10	10.5	0.5
1b	0.65	9	9	9.5	9.2	0.3	0.64	9	9.5	9.5	9.3	0.3
1e	0.642	11	11	11	11.0	0.0	0.647	12	12	12	12.0	0.0
1f	0.633	11	11.5	12	11.5	0.5	0.656	12	12.5	12.5	12.3	0.3
1g	0.637	9.5	9.5	8.5	9.2	0.6	0.643	10	10	9.5	9.8	0.3
1h	0.639	10.5	11	10.5	10.7	0.3	0.648	10.5	10.5	10.5	10.5	0.0
1i	0.643	11.5	12	12	11.8	0.3	0.642	12	12	12.5	12.2	0.3
3e	0.673	10	9	9	9.3	0.6	0.656	11	11	10.5	10.8	0.3
3f	0.651	7.5	8.5	8.5	8.2	0.6	0.645	9	8.5	9	8.8	0.3
3g	0.647	7	7	7	7.0	0.0	0.643	7	7	7	7.0	0.0
4a	0.64	10	10	10	10.0	0.0	0.623	10	11	11	10.7	0.6
5a	0.655	11	11	11.5	11.2	0.3	0.643	12	12	12	12.0	0.0
control [c]	0.648	10	12	12	11.3	1.2	0.6	12	12	11	11.7	0.6

[a] Yeast only.

[b] Yeast and fungal culture fluid.

[c] *C. laurentii* was included as positive control.

Appendix B

B.1 Compilations provided on compact disc

B.1.1 Table B.1: Growth curves for yeast isolates

B.1.2 Table B.2: Optimum temperature data for yeast isolates

B.1.3 Table B.3: Raw data of intraspecific interactions of yeast isolates with the filamentous fungi

B.1.4 Yeast isolate sequences of forward reactions of the large subunit rDNA

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B.1.5 Compact disc-Appendix B