MUCORALEAN FUNGI PRESENT IN SOIL FROM ARID REGIONS IN SOUTH AFRICA

by

BUTI OSCAR SEABI

Submitted in fulfillment of the academic
requirements for the degree

MAGISTER SCIENTIAE

in the
Department of Microbiology and Biochemistry
Faculty of Natural Sciences
University of Orange Free State
Bloemfontein, South Africa

Supervisor: Dr. A. Botha
Co-supervisor: Prof. B.C. Viljoen

November 1999

This work is dedicated to my mother, Alina Moliehi Seabi, for believing in me, never losing hope when times were tough and giving me love. I love you.

PREFACE

The experimental work conducted and discussed in this thesis was carried out in the Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein, South Africa. The study was conducted during the period February 1998 to November 1999 under the supervision and co-supervision of Dr. A. Botha (University of Stellenbosch) and Prof. B. Viljoen (University of Free State) respectively.

The study represents original work undertaken by the author and has not been previously submitted for degree purposes to any other university. Appropriate acknowledgements in the text have been made where use of work conducted by others has been included.

Oscar. B. Seabi.

ACKNOWLEDGEMENTS

I would like to express and convey my sincere gratitude to all who assisted and contributed to the successful completion of this study. Included are the following:

Dr. Alfred Botha for his guidance, support, never ending patience and encouragement during the course of the study;

Prof. Benny C. Viljoen for being there for me when I had no one to turn to and also for a nice environment that you created for my work;

Mev. Yvonne Dessels, Department of Soil Science, Faculty of Agriculture, University of the Free State for all the chemical analysis of the soil samples that you did for us, thank you very much;

Zawadi Chipeta and Mzi Mkhize for the technical assistance, especially the computer, you are the best friends guys;

Tersia Strauss and Charlott Maree with the culture collection every time I needed mucoralean strains, thank you very much;

The academic and non-academic staff and students, Department of Microbiology and Biochemistry, UOFS, for having created an atmosphere where research was a joy;

The National Research Foundation (NFR) for the financial support of this study.

My wonderful parents, lovely family and supportive friends, who were by me every step of the way during this study; and

Sadly, to
The late Stephen Kabelo Matsôlo who passed away before this work could be finished. You were my inspiration and my mentor, R.I.P.

TABLE OF CONTENTS

CHAPTER 1

LITERATURE REVIEW

1. 1. Motivation

The Mucorales is a group of fungi which recently attracted substantial attention from biotechnologists, who see these fungi as potential sources for a number of products ranging from enzymes such as lipases, to high value fatty acids and chitin (Domsch *et al.*, 1980; Aggelis *et al.*, 1987; Hansson & Dostalek, 1988; Sajbidor *et al.*, 1988; Tsuchiura & Sakura, 1988; Eroshin *et al.*, 1996). Although the physiology directly related to these products, as well as the morphology of mucoralean fungi have been studied thoroughly (Hesseltine & Ellis, 1973; Domsch *et al.*, 1980; Ueng & Gong, 1982; Ratledge, 1989), much is still unknown about the natural habitat of these fungi. Therefore, to utilise the full potential of the Mucorales, it is essential that basic knowledge of the ecology and physiology of these fungi be obtained.

Mucoralean fungi are mostly saprophytes (Hesseltine & Ellis, 1973; Domsch *et al.*, 1980) that are usually associated with moist environments, such as leaf litter in forests, and are known to have a relative low tolerance to reduced water activity (Brown, 1976). However, these fungi have also been recorded in soil, debris and on plant roots from arid regions (Steiman *et al.*, 1995; Roux & Van Warmelo, 1997).

Mucoralean fungi are known as first colonisers of decaying organic material in soil, since these fungi are able to rapidly utilise the limited number of simple carbohydrates that are usually available, before other fungal groups take over the mineralisation of carbon (Hesseltine & Ellis, 1973). It is therefore not surprising that studies have indicated that mucoralean fungi are able to utilise organic nitrogen as well as ammonium salts (Inui *et al.*, 1965; Aggelis *et al.*, 1987; Sajbidor *et al.*, 1988; Tsuchiura & Sakura, 1988). Certain authors have found, however, that some mucoralean fungi are able to utilize nitrate (Hansson & Dostalek, 1988; Certik *et al.*, 1993). This characteristic is not essential for a primary colonizer of dead organic matter, since during the mineralisation of organic nitrogen, nitrification only occurs after ammonification (Sparling, 1998). The specific position of many mucoralean fungi in the biogeochemical nitrogen cycle, however, remains unknown since only

fragmentary information on the utilisation of different nitrogen containing compounds by these fungi, exists in literature.

With the above as background, the aim of this study was to investigate aspects of the physiology and geographical distribution of mucoralean fungi, which would give more insight into the ecological niche these fungi occupy in arid soil. The first was the ability to utilise a series of organic and inorganic nitrogen compounds, which would position these fungi in the biogeochemical cycle of nitrogen, while the second was the ability of the fungi to grow at a reduced water activity. In addition, to explore the ability of mucoralean spores to survive elevated temperatures in soil of arid regions, representatives of mucoralean taxa frequently encountered in soil were tested for survival in soil incubated at 55°C for 14 h.

1. 2. General characteristics of mucoralean fungi

1. 2. 1. Morphological features. To introduce the Mucorales, it was necessary to present a short discussion on the main morphological features, which characterise members of this fungal order. These fungi, which mostly produce velvet to cotton-like colonies on solid media, are characterised by the formation of coenocytic hyphae containing haploid nuclei (Benjamin, 1979). Sexual reproduction occurs when two, usually similar gametangia, conjugate to produce a zygospore. After meiosis these zygospores, which can survive prolonged periods of adverse conditions (Spotts & Servantes, 1986), give rise to haploid progenies. However, sexual reproduction infrequently occurs in mucoralean isolates (Benjamin, 1979). In contrast, asexual reproduction frequently occurs in nearly all mucoralean fungi (Benjamin, 1979). During this type of reproduction, spores are dispersed by means of splashing rain, air currents or insects (Domsch *et al.*, 1980). These spores, called sporangiospores, are either produced in many-spored sporangia, or in sporangiola which can contain one or several spores. In addition, to survive adverse conditions, thick-walled chlamydospores may also be formed in some hyphae. The morphology of these asexual reproductive structures, including the morphology and dimensions of the sporangia, sporangiola, sporangiophores, columellae and chlamydospores, are used to classify mucoralean fungi (Hesseltine & Ellis, 1973; Benjamin, 1979; Benny & Benjamin, 1991).

In order to give the reader background knowledge on the diversity within the Mucorales occurring commonly in soil, a number of taxa that are known to occur in this habitat (Domsch *et al.* 1980) and show distinct morphological features, are to be discussed below. The well known genus *Rhizopus* Ehrenb., a member of the family Absidiaceae (Benny & Benjamin, 1991), is characterised by forming dark unbranched sporangiophores on stolons opposite rhizoids. The sporangia, borne on the tips of sporangiophores are columellate and apophysate. (Hesseltine & Ellis, 1973; Alexopoulos & Mims, 1979; Schipper, 1984). *Absidia* Tiegh., also a genus within this family (Benny & Benjamin, 1991), may form branched sporangiophores which borne pyriform, apophysate sporangia containing columellae. The sporangiophores are formed on stolons, never opposite rhizoids (Hesseltine & Ellis, 1973). Members of the genus *Gongronella* Ribaldi, which is closely related to *Absidia*, form characteristic constricted zones between the sporangium and the apophysis.

Actinomucor Schost., a member of the family Mucoraceae (Benny & Benjamin, 1991), is another stoloniferous genus forming rhizoids, however, contrary to *Absidia*, *Gongronella* and *Rhizopus,* there is no apophysis present in *Actinomucor.* The sporangiophore bears a terminal sporangium and below this sporangium a whorl of short branches each terminating in a small sporangium (Hesseltine & Ellis, 1973; Alexopoulos & Mims, 1979). *Mucor* Fresen. on the other hand, produces multispored sporangia with columellae, whereas, no stolons and rhizoids are formed. Members of this genus also produce zygospores suspended between two oppositely aligned equal-sized suspensor cells (Hesseltine & Ellis, 1973; Alexopoulos & Mims, 1979; Domsch *et al.* 1980). Another member of this family (Benny & Benjamin, 1991), *Zygorrhynchus* Vuill., is homothallic and produces gamentangia and suspensor cells of unequal size (Alexopoulos & Mims, 1979).

A common soil borne genus, which occupies a rather distinct position within the Mucorales regarding morphology of colonies and sporangia, is *Mortierella* Coem. This genus is currently classified in the family Mortierellaceae (Benny & Benjamin, 1991) and is characterised by the formation of small fragile sporangia, which are always produced in low numbers on culture media. In contrast, chlamydospores are formed in abundance in the aerial hyphae of the cultures (Domsch *et al.* 1980). The sporangia that are produced within this genus mostly lack columellae. However, when these structures are formed, they are greatly reduced in size. Two

subgenera within *Mortierella* are currently recognised (Gams, 1977). The first is *Mortierella* subgenus *Micromucor*, which is characterised by velvety growth and mostly pigmented sporangia, which may contain small columellae. The other subgenus, *Mortierella* subgenus *Mortierella*, is characterised by white, arachnoid colonies often with lobed or rosette patterns and mostly with a garlic-like odour. The sporangia formed by members of this group may contain rudimentary columellae. During sexual production, zygospores are formed between two thong-like suspensor cells embedded in meshlike sterile hyphae (Hesseltine & Ellis, 1973; Gams, 1977). Due to the distinct characteristics of the genus *Mortierella* within the Mucorales, the taxonomy of the genus is likely to change in the near future (Streekstra, 1997). It is expected that the genus *Mortierella* will be elevated to the rank of a separate order within the Zygomycetes: namely the Mortierellales. In addition, the species currently classified in *Mortierella* subgenus *Micromucor* will be classified in a separate genus, *Umbelopsis* Amos *et* Barnett.

Cunninghamella Matr., a genus that is periodically isolated from soil, is classified in the family Cunninghamellaceae (Benny et al.,1992), characterised by the formation of branched sporophores terminating in swollen vesicles bearing pedicellate, unispored sporangiola (Benjamin, 1979; Alexopoulos & Mims, 1979). The sporangiola have no columellae and are usually spinose.

1. 2. 2. Physiological properties. Like all life forms, mucoralean fungi require macroelements and trace elements for growth. As typical chemoorganotrophic heterotrophs, these fungi utilise reduced organic molecules as carbon, energy and hydrogen sources. Generally, mucoralean fungi can easily be cultivated on complex agar media, such as malt extract agar, incubated at *circa* 25°C (Hesseltine & Ellis, 1973). Some species, however, like *Mortierella alpina* Peyronel is psychrotolerant and can grow at temperatures as low as 0°C (Domsch et al. 1980), while others such as *Rhizomucor tauricus* (Milko *et* Schkurenko) Schipper can grow up to 55°C (Schipper, 1978). In general, mucoralean fungi can grow at pH values between 4 and 8.

These fungi are known to aerobically utilise a variety of carbon sources (Table 1), such as hexoses, pentoses, di- and trisaccharides, polysaccharides as well as organic acids (Botha *et al.*, 1997; Botha & Du Preez, 1999). Certain carbohydrates can also be fermented (Table 2). Complex organic molecules are hydrolysed by

some mucoralean fungi (Domsch *et al.* 1980), for example, chitin can be utilised by representatives of *Absidia corymbifera* (Cohn) Sacc. *et* Trotter and *Mortierella* Coemans. Pectin was found to be utilised by *Absidia glauca* Hagem, *Absidia spinosa* Lendner, *Cunninghamella elegans* Lendner, *Mucor hiemalis* Wehmer, *Mucor piriformis* Fischer, *Mucor racemosus* Fres. and *Zygorrhynchus moelleri* Vuill. Hemicelluloses can be utilised by *M. racemosus* and *Z. moelleri*, while humic acids can be utilised by *Absidia cylindrospora* Hagem. and *M. plumbeus*.

Nitrogen sources that are known to be utilised by mucoralean fungi, include ammonium, nitrate, nitrite and organic nitrogen compounds such as amino acids (Inui *et al.*, 1965; Aggelis *et al.*, 1987; Aggelis *et al.*, 1988; Hansson & Dostalek, 1988; Du Preez *et al.*, 1997). However, a more detailed discussion of this will be presented later in this chapter.

Some mucoralean fungi require growth factors such as amino acids, vitamins, or siderophores. Although no ecological generalisations can be made, it is accepted that most saprophytic mucoralean fungi isolated from soil, such as representatives of *Mucor*, *Rhizopus* and *Zygorrhynchus* exhibit an absence of growth factor requirements (Jennings, 1995). It is known, however, that some mucoralean soil fungi require certain vitamins for growth (Domsch *et al.* 1980). *Absidia corymbifera* (Cohn) Sacc. *et* Trotter requires thiamine, while *Mortierella ramanniana* (Möller) Linnem. var. *ramanniana* requires thiamine or its thiazole moiety whereas *Mortierella vinacea* Dixon-Stewart requires thiazole for growth. The coprophilous mucoralean genus *Pilobolus* Tode, is known for its requirement for the siderophore, coprogen (Alexopoulus & Mims, 1979). This iron-binding ferrichrome normally occurs in dung of herbivores, which is the natural habitat of *Pilobolus*.

TABLE 1. Aerobic carbon source utilisation in synthetic liquid media (Botha *et al.*, 1997; Botha & Du Preez, 1999).

Carbon sources	*Mucor circinelloides* CBS 119.08	*Mucor circinelloides* CBS 108.16	*Mucor circinelloides* CBS 203.28	*Mucor rouxii* CBS 416.77	*Mucor flavus* CBS 234.35	*Mucor mucedo* CBS 109.16	*Rhizopus oryzae* CBS 112.07	*Thamnostylum piriforme* PPRI 5534	*Mortierella alpina* ATCC 3221
Pentoses									
D-arabinose	-	-	-	-	-	-	-	-	-
L-arabinose	+	+	+	+	+	+	+	+	-
D-ribose	+	+	+	+	-	+	+	-	+
D-xylose	+	+	+	+	+	+	+	+	-
L-xylose	+	+	+	+	-	-	+	-	-
Hexoses									
D-galactose	+	+	+	+	+	+	+	+	-
D-glucose	+	+	+	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+
L-sorbose	-	-	-	-	-	-	-	-	-
D-fucose	-	-	-	-	-	-	-	-	-
L-fucose	-	-	-	-	-	-	-	-	-
L-rhamnose	+	-	-	-	+	-	+	-	-
Disaccharides									
Cellobiose	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+
Melibiose	+	-	-	-	-	+	-	+	-
Sucrose	+	-	-	-	-	-	-	-	-
Trehalose	+	+	+	+	+	+	+	+	+
Trisaccharides									
Melezitose	+	+	+	+	+	+	-	+	-
Raffinose	-	-	-	-	+	-	-	-	-
Polysaccharides									
Insulin	+	+	+	+	+	+	-	+	+
Soluble starch	+	+	+	+	+	+	+	+	+
Glycoside									
Salicin	+	+	+	+	+	+	+	+	-

+ = growth occurred; - = no growth

Table 1 continues

Carbon sources	*Mucor circinelloides* CBS 119.08	*Mucor circinelloides* CBS 108.16	*Mucor circinelloides* CBS 203.28	*Mucor rouxii* CBS 416.77	*Mucor flavus* CBS 234.35	*Mucor mucedo* CBS 109.16	*Rhizopus oryzae* CBS 112.07	*Thamnostylum piriforme* PPRI 5534	*Mortierella alpina* ATCC 3221
Alcohols									
Erythritol	-	-	-	-	+	-	-	-	-
Ethanol	+	+	+	+	+	-	+	-	-
Galactitol	+	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	+	-	+	-	+
Inositol	-	-	-	-	+	-	n.d.	-	-
D-mannitol	+	+	+	+	+	+	+	-	-
Methanol	-	-	-	-	-	-	-	-	-
Ribitol	+	+	+	+	-	-	+	-	-
Sorbitol	+	+	+	+	+	+	+	+	-
Organic acids									
Acetic acid	+	+	+	+	+	+	+	+	-
Butanoic acid	+	+	+	+	-	-	n.d.	+	-
Citric acid	-	-	-	-	-	-	+	-	-
Formic acid	-	-	-	-	-	-	n.d.	-	-
Gluconic acid	+	+	+	+	+	-	+	-	-
Lactic acid	+	+	+	+	+	+	-	+	-
Succinic acid	+	+	+	+	+	+	+	+	-
Propionic acid	-	-	-	-	-	-	-	-	-

+ = growth occurred; - = no growth; n.d. = not determined
(Botha *et al.*, 1997; Botha & Du Preez, 1999)

TABLE 2. Carbohydrates fermented by *Mucor circinelloides f. circinelloides* CBS 108.16 (Botha & Du Preez, 1999).

Pentoses	
D-arabinose	-
L-arabinose	-
D-ribose	-
D-xylose	-
Hexoses	
D-galactose	+
D-glucose	+
Disaccharides	
Maltose	+
Sucrose	-
Trisaccharides	
Raffinose	-

+ Fermented

- not fermented

Water activity (a_w), defined as the ratio of the vapour pressure of the substrate to that of pure water (Brown, 1976; Bullerman, 1993), is also an important factor for growth of fungi, including the Mucorales. It is an indication of the amount of water not bound to the substrate, which is available for fungal growth and survival (Bullerman, 1993), and is related to the moisture content of the environment. Most fungi grow well over an a_w range of 0.72 – 0.94, while growth of more osmotolerant taxa (e.g. *Eurotium* Link:Fr) is inhibited at a_w values below 0.65 (Brown, 1976). However, it was found that representatives of *Absidia, Mucor* and *Rhizopus* could only grow above a_w values of 0.92 to 0.93 (Ottaviani 1993). As a group these fungi therefore seem to be less osmotolerant than most higher fungi. A comparison of the different taxa within the Mucorales with regard to osmotolerance, has thus far not been attempted. Studies are therefore needed to determine which mucoralean taxa are more osmotolerant and able to grow at reduced water activities.

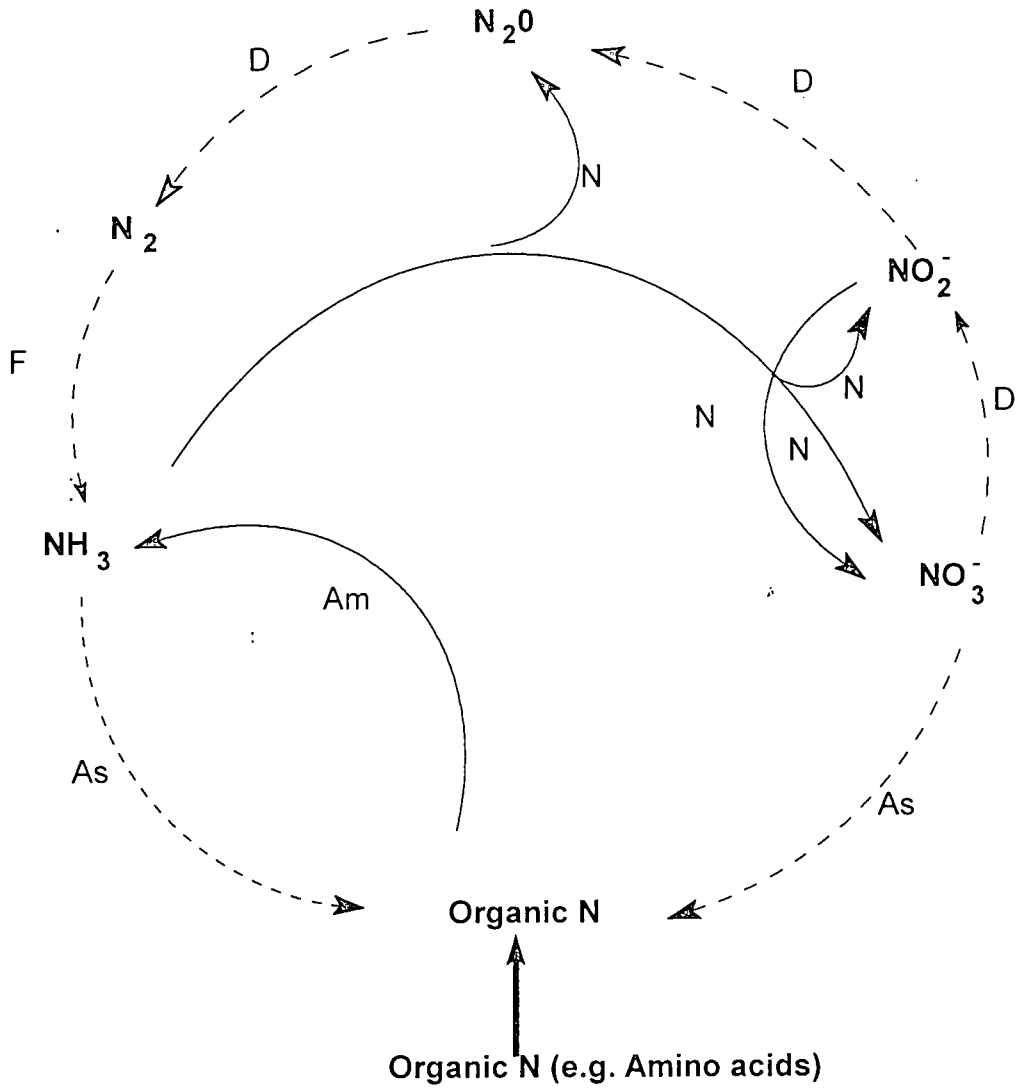
1. 3. Nitrogen Cycle

An essential process in the biosphere, in which the soil microbial community plays a pivotal role, is the biogeochemical cycling of nutrients. The nitrogen cycle (Fig. 1) forms part of this process and is the pathway for recycling nitrogen in the biosphere (Ferguson, 1987). It includes a variety of oxidation and reduction reactions that can be divided into dissimilatory and assimilatory reactions. Dissimilatory reactions are found principally amongst prokaryotes, while assimilatory reactions occur in both the eukaryotes and prokaryotes.

Atmospheric nitrogen (N_2) is reduced to ammonium during the process of nitrogen fixation (Fig. 1) occurring in some prokaryotes, like in members of the genera *Rhizobium*, *Azotobacter* or *Clostridium* (Brock *et al.*, 1994). Ammonium can be assimilated and incorporated into the cells as organic nitrogen compounds by bacteria and fungi. Ammonia or ammonium ions (Atlas & Bartha, 1981) can also be oxidised to nitrate or nitrite via the process of nitrification by nitrifying bacteria. Different microbial populations carry out the two steps of nitrification, that is the formation of nitrite followed by the formation of nitrate. These nitrifying bacteria are chemolithoautotrophs that utilise the energy derived from nitrification in order to assimilate CO_2. The most dominant bacterial genus in soil capable of oxidizing ammonium to nitrite is *Nitrosomonas*, while *Nitrobacter* is the dominant bacterial genus capable of oxidizing nitrite to nitrate (Atlas & Bartha, 1981). Nitrate is transformed back to nitrogen gas via a dissimilatory nitrate reduction pathway, called denitrification. *Pseudomonas, Thiobacillus* and other facultative aerobic prokaryotes are involved in the latter process (Atlas & Bartha, 1981). However, nitrate can also undergo assimilatory nitrate reduction to be incorporated as organic nitrogen in bacteria, fungi and plants. The assimilation of nitrogen containing compounds by mucoralean fungi, in relation to the biogeochemical cycling of nitrogen, has thus far not been studied.

1. 4. Nitrogen utilisation in fungi

1. 4. 1. Uptake of inorganic nitrogen. In order for a fungus to assimilate and utilise a particular nitrogen-containing compound, it is essential that the cell can



ure 1. Nitrogen cycle (Ferguson, 1987).
Am ammonification; As nitrogen assimilation; F nitrogen fixation; N nitrification; D denitrification

take up the compound. However, limited investigations have been conducted on the transport of inorganic nitrogen compounds across fungal cellular membranes (Jennings, 1995). An inducible nitrate transport system was uncovered in *Neurospora* Shear *et* B.O. Dodge. Nitrate and nitrite induced this transport system, but not ammonia or Casamino acids. It was found that the K_T for nitrate transport in this fungus is 0.25 mM and that nitrate and ammonia are non-competitive inhibitors.

Another ascomycetous fungus, *Candida utilis* (Henneberg) Lodder et Kreger-van Rij, was found to possess an electrogenic nitrate proton/symport system, which at pH 4-6 transports 2 protons for each nitrate ion. The charge balance is obtained by the expulsion of potassium from the cells (Jennings, 1995).

Nitrite transport has also been studied in *Neurospora*, (Jennings, 1995). Interestingly, the presence of ammonia, Casamino acids and nitrate had no effect on the transport of nitrite in this fungus, which has a K_T of 86 μM. In addition, it was found that nitrate cannot be taken up by *N. crassa* via the nitrite transport system.

Conclusive results on the transport of ammonia into fungal cells is also lacking in literature (Jennings, 1995). When *N. crassa* is presented with ammonia, the membrane potential is depolarised and the membrane interior becomes more positive. This depolarisation was found to be consistent with the transport of NH_4^+ across the cell membrane via an uniport transport system. Evidence indicates the presence of a single uniport transport system for ammonia, methylamine and ethylamine in *Penicillium chrysogenum* Thom. The K_T values for transport of these compounds are respectively *circa* 2.5×10^{-7} M, *circa* 1×10^{-5} M and *circa* 1×10^{-4}M.

The transport of ammonia into *Saccharomyces cerevisiae* Meyen *ex* E.C. Hansen also seems to be by a methylamine system (Jennings, 1995). This transport system, which shows biphasic Lineweaver - Burk kinetics, has two functions that can be lost separately by two genetically unlinked mutations. In *mep*-1 mutations, the yeast only has a high affinity ($K_T \approx 2.5 \times 10^{-4}$ M) low capacity system [$V_{max} \approx 20$ nmol (mg protein) $^{-1}$ min^{-1}] operating. In *mep*-2 mutations however, the yeast only has a low affinity ($K_T \approx 2.0 \times 10^{-3}$ M) high capacity [$V_{max} \approx 50$ nmol (mg protein) $^{-1}$ min^{-1}] operating. Interestingly, whilst double mutants grow very slow on 1×10^{-3} M ammonia, significant growth can still occur on 1×10^{-2} M of this compound, indicating the presence of a third transport system for ammonia in *S. cerevisiae*.

Except for the studies mentioned above, very little is known about the uptake of inorganic nitrogen compounds in the remainder of the fungal domain including the Mucorales.

1. 4. 2. Uptake of simple organic nitrogen compounds. The majority of studies on the uptake of simple organic compounds by fungi were conducted on yeasts, especially *S. cerevisiae* and other ascomycetous yeasts. Only limited studies on specific filamentous fungi were undertaken (Jennings, 1995; Walker, 1998). The kinds of simple organic compounds mostly studied were amino acids, purines and pyrimidines. To explain these uptake systems, it was decided to briefly review the transport systems of these compounds in a number of selected fungal taxa.

Saccharomyces cerevisiae. Two types of uptake systems exist for amino acids in *S. cerevisiae* (Walker, 1998). One is less specific and is involved in the uptake of all natural amino acids, including citruline. This is known as the so-called "general amino acid permease" or "GAP". The other system exhibits specificity for one or smaller groups of structurally related amino acids. Several of these more specific amino acid transport systems have been characterised in *S.cerevisiae* (Table 3). Amino acid transport, both general ("GAP") and specific transport, as represented in *Candida albicans* (Robin) Berkhout and *S.cerevisiae,* were found to be active and dependent on proton symport mechanisms. The transmembrane pH gradient therefore provides energy for the uptake of amino acids, while the secretion of K+ aided by an anti-port system balances the uptake of protons.

Two types of uptake systems have also been reported for urea in *S. cerevisiae* (Walker, 1998). The one is a high-affinity ($K_T \approx 14 \mu M$), nitrogen-repressible system that can actively concentrate urea 200-fold in the fungal cells (Table 3). The other is a constitutive, low-affinity ($K_T \approx 2.5$ mM) system that functions by facilitated diffusion when more than 0.5 mM urea is present in the medium.

TABLE 3. Specific amino acid and urea transport systems in *S. cerevisiae* (Jennings, 1995; Walker, 1998).

Amino acid	Affinity (K_T)	Remarks
L-Arginine	High, $K_T \approx 10$ μM Low, $K_T \approx 1.0$mM	System seems facilitates the uptake of all basic amino acids.
L-Lysine	High, $K_T \approx 25$ -78 μM Low, $K_T \approx 0.2$ mM	
L-Histidine	High, $K_T \approx 20$ μM Low, $K_T \approx 0.5$ mM	Histidine permease was the first of all the yeast permeases of which the molecular structure has been resolved. Diffusion may be involved.
L-Methionine	High, $K_T \approx 3$ -12 μM Low, $K_T \approx 0.6$ -0.8 mM	One high and two low affinity methionine Permeases have already been discovered
S-adenosyl-L-Methionine	High, $K_T \approx 1.6$ - 3.3 μM	
L-Cysteine	Low, $K_T \approx 0.25$ mM	There is doubt about the presence of this permease.
L-Serine	Low, $K_T \approx 0.58$ mM	The uptake of these amino acids is through the action of single, but not necessarily identical systems.
L-Threonine	Low, $K_T \approx 0.21$ mM	
L-Leucine	High, $K_T \approx 30$ μM Low, $K_T \approx 0.5$ -4.5 mM	The transport of leucine and other branched amino acids (isoleucine and valine) are mediated by a specific gene, BAP2, which in turn is regulated by the availability of leucine.
L-Glutamate	High, $K_T \approx 20$ μM Low, $K_T \approx 3.3$ mM	Three transport systems may exist, the other one is the "GAP"
L-Asparagine	Low, $K_T \approx 0.35$ mM	Also transports glutamine, histidine, threonine and tryptophan.
L-Proline	High, $K_T \approx 25$ μM	A low affinity system may also exist.
L-Alanine	High	"GAP" is responsible for high affinity uptake.
L-Glycine	Low	There is doubt about the role of this system.
Urea	High, $K_T \approx 14$ μM Low, $K_T \approx 2.5$ mM	Active transport involved Facilitated diffusion involved.

Purines and pyrimidines are actively taken up by *S. cerevisiae, Schizosaccharomyces pombe* Lindner and *Candida utilis* (Henneberg) Lodder *et* Kreger-van Rij (Walker, 1998). It was found that the transporting molecules undergo no chemical changes during this transport. Two systems were found in *S. cerevisiae*: The first is specific for adenine, cytosine, guanine and hypoxanthine. This system, of which the maximum activity is obtained during the exponential growth phase, is powered by a proton gradient and is inhibited by Na^+ or K^+. The

second system was found to have uracil as primary substrate. It appears to operate by facilitated diffusion, independent of the H^+ pump.

Achlya Nees. A number of amino acid transport systems have been reported for the oomycotan genus, *Achlya* (Jennings, 1995). Interestingly, it was found that methionine, inhibits in a non-competitive manner the transport of every other amino acid listed in Table 4. However, these other amino acids, do not inhibit transport of methionine.

TABLE 4. Amino acid transport systems in *Achlya* (Jennings, 1995).

System notation and amino acids it transports	Affinity (K_T)	Remarks
(i) L-Methionine	$K_T \approx 5.33$ μM $K_T \approx 0.20$ mM	Two saturable components exist for the transport of this amino acid
(ii) L-Cysteine	$K_T \approx 75.00$ μM	
(iii) L-Proline	$K_T \approx 0.15$ mM	
(iv) L-Serine L-Threonine	$K_T \approx 0.13$ mM $K_T \approx 25.00$ μM $K_T \approx 0.20$ mM	Two saturable components exist for the transport of threonine
(v) L-Aspartate L-Glutamate	$K_T \approx 4.00$ μM $K_T \approx 25.00$ μM	
(vi) L-Asparagine L-Glutamine	$K_T \approx 75.00$ μM $K_T \approx 0.15$ mM	
(vii)L-Alanine L-Glycine	$K_T \approx 0.17$ mM $K_T \approx 0.15$ mM	
(viii)L-Arginine L-Lysine L-Histidine	$K_T \approx 33.33$ μM $K_T \approx 8.33$ μM $K_T \approx 0.10$ mM $K_T \approx 0.17$ mM	Two saturable components exist for the transport of lysine
(ix) L-Phenylalanine L-Tyrosine L-Tryptophan L-Leucine L-Isoleucine L-Valine	 $K_T \approx 50.00$ μM $K_T \approx 33.33$ μM $K_T \approx 0.17$ mM $K_T \approx 0.11$ mM $K_T \approx 66.70$ μM $K_T \approx 83.00$ μM	

Neurospora crassa Shear & B.O. Dodge. The dikaryomycotan filamentous fungus *N. crassa* possess only five genetically and biochemically distinct transport systems for amino acids (Jennings, 1995). The five systems (Table 5) are: (I) for neutral and aromatic amino acids; (II) for general transport of neutral, basic and

acidic amino acids; (III) for basic amino acids; (IV) for acidic amino acids; and (V) for methionine. It was found that after germination of the conidia, 85 to 95 percent of the amino acid uptake, occurs via the general amino acid transport system (System II). Before germination, only 40% of amino acid uptake occur via this system. Results indicate that the uptake of amino acids with System II occurs via a proton symport system.

TABLE 5. Amino acid transport systems in *Neurospora crassa* (Jennings, 1995).

System notation and amino acids it transports	Affinity (K_T)	Remarks
(I) Phenylalanine Tyrosine Tryptophan Leucine Histidine Aspartate Glutamate	$K_T \approx 30.00$ μM $K_T \approx 50.00$ μM $K_T \approx 0.10$ mM $K_T \approx 0.65$ mM $K_T \approx 80.00$ μM	System also transports Val, Ala, Gly, Ser, Met; Asp and Glu transported optimally at acidic pH values.
(II) Tryptophan Methionine Phenylalanine Tyrosine Leucine Asparagine Aspartate Glutamate Citrulline Arginine Lysine Glycine Histidine	$K_T \approx 40.00$ μM $K_T \approx 3.00$ μM $K_T \approx 40.00$ μM (C); $K_T \approx 2.00$ μM (M) $K_T \approx 4.90$ μM $K_T \approx 20.00$ μM $K_T \approx 3.40$ mM (C); $K_T \approx 10.00$ μM (M) $K_T \approx 40.00$ μM $K_T \approx 3.20$ μM $K_T \approx 8.00$ μM $K_T \approx 1.20$ mM	Asp and Glu transported optimally at acidic pH values.
(III) Arginine Lysine Histidine Canavanine	$K_T \approx 2.00$ μM $K_T \approx 5.00$ μM $K_T \approx 3.50$ mM/1.6μM $K_T \approx 7.00$ μM	
(IV) Cysteic acid Aspartate Glutamate	$K_T \approx 7.00$ μM $K_T \approx 1.300$ μM $K_T \approx 1.60$ μM	Active under carbon, nitrogen or sulphur starvation conditions
(V) Methionine	$K_T \approx 2.30$ μM	Active under sulphur starvation conditions

Abbreviations: (C) values for conidial stage of development; (M) values for mycelium

An interesting phenomenon was uncovered in molecular studies on the *nit-2* gene of this fungus (Jennings, 1995). It was found that under nitrogen starvation, the presence of a functional *nit-2* gene product and the presence of certain amino acids lead to the production of an extracellular deaminase. The enzyme, which is L-stereospecific, converts the amino acid to its respective keto acid plus equimolar amounts of ammonia, which then act as nitrogen source. It was found that several neutral amino acids elicit the production of this enzyme, while arginine elicits enzyme production in mycelium in which the general amino acid transport system is non-functional.

1. 4. 3. Uptake of peptides. Studies on *C. albicans*, have shown that there are two peptide transport systems in this yeast (Jennings, 1995). One system is able to transport dipeptides with a reduced affinity for oligopeptides, while the other transports oligopeptides with a low affinity for dipeptides. Unlike in *C.albicans*, *S. cerevisiae* takes up di- and tripeptides via a single transport system. In both *S. cerevisiae* and *C. albicans,* the peptides are taken up by active transport systems (Walker, 1998). The peptides are then hydrolysed inside the cell.

Studies on peptide transport in *N. crassa* revealed that dipeptides do not support growth of the fungus, while tripeptides, tetrapeptides and pentapeptides are taken up and utilised (Jennings, 1995). The transport of peptides into mucoralean fungi is still unexplored.

1. 4. 4. Nitrogen metabolism in fungi. As depicted in Fig. 2, the catabolism of nitrogen containing compounds in fungi leads to the formation of two key compounds, ammonium and L-glutamate, via two separate pathways (Large, 1986; Jennings, 1995). These pathways are interlinked by basically two enzyme systems, NADP- dependent glutamate dehydrogenase, which converts ammonium to glutamate and NAD- dependent glutamate dehydrogenase, which converts glutamate to ammonium.

Glutamate is produced through the action of an amino transferase enzyme, catalysing the conversion of an amino acid and 2-keto glutarate to glutamate and an α-keto acid (Large, 1986). Ammonium can be produced as the end product of

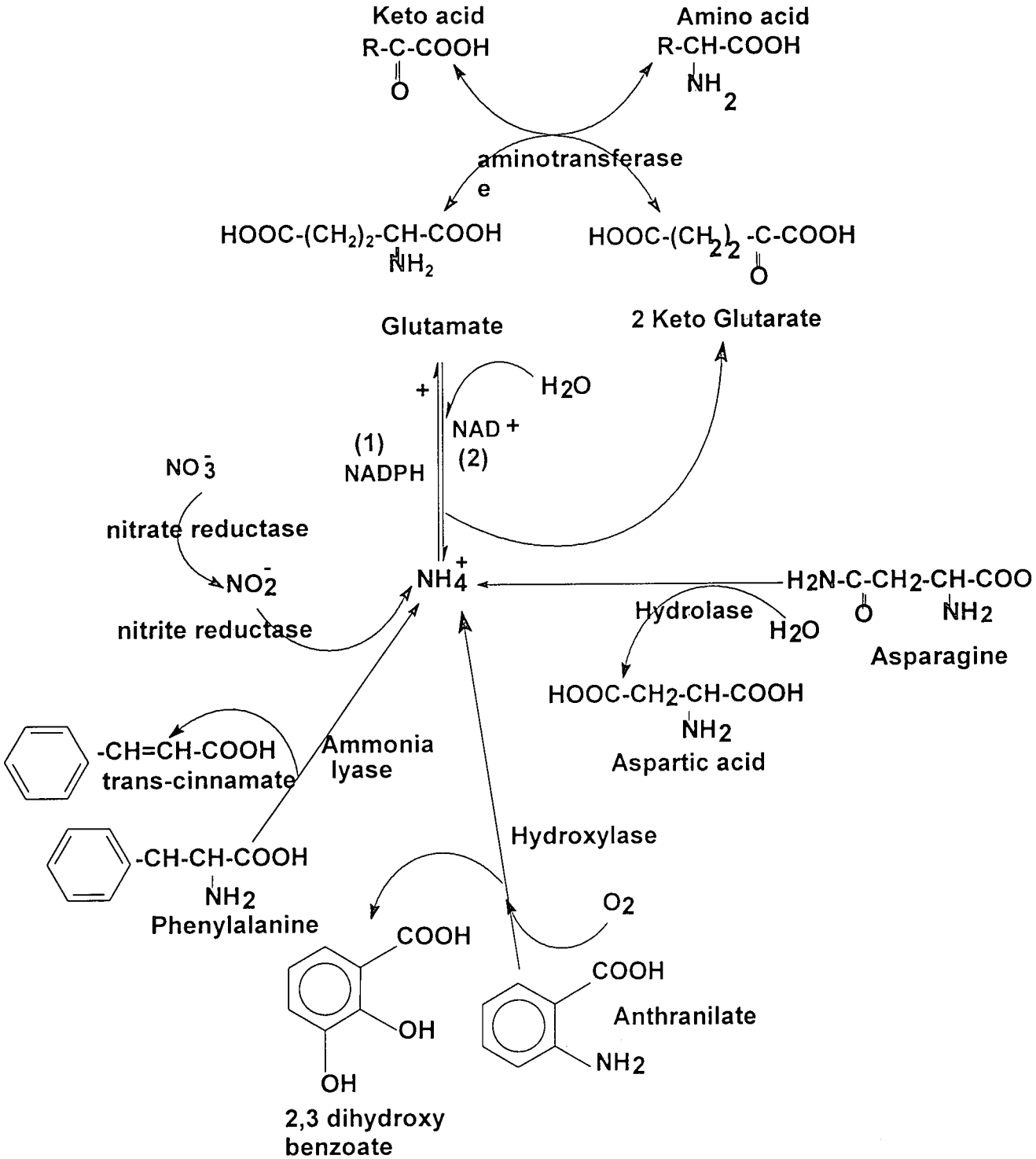


Figure 2. A simplified scheme depicting the catabolism of nitrogen containing compounds (Adapted from Large,1986). (1) NADP-dependant glutamate dehydrogenase
(2) NAD-dependant glutamate dehydrogenase

various catabolic pathways containing different enzymes. The enzyme ammonia lyase catalyses the formation of ammonium and trans-cinnamate from phenylalanine. On the other hand, hydroxylase catalyses the release of nitrogen from anthranilate during the degradation of the amino acid tryptophan. Another enzyme, catalyses the hydrolyses of amino acids like asparagine to produce ammonium and aspartic acid. Ammonium can now be incorporated into the cells by either glutamine synthetase / glutamate synthetase reactions or by direct amination of a α-keto-carboxylic acid to form an amino acid (Atlas & Bartha, 1981; Large, 1986).

Nitrate and nitrite are catalysed to ammonium by the actions of the enzymes nitrate and nitrite reductases (Jennings, 1995; Walker, 1998). However, it is known that not all fungi are capable of utilising both nitrate and nitrite. Extensive studies on nitrogen utilisation in the yeast domain have shown that certain yeast genera are unable to utilise nitrate (Large, 1986). This may be as a result of the absence of nitrate reductase in these yeasts, or it may be as a result of the absence of a transport system for nitrate.

1. 4. 5. Nitrogen utilisation in mucoralean fungi. Studies have been conducted on the ability of mucoralean fungi to utilise different nitrogen containing compounds. However, much of the emphasis was on the biotechnological production of high value fatty acids such as γ-linolenic acid (GLA) by this group of fungi. In these studies, ammonium sulfate [$(NH_4)_2SO_4$], peptone and sodium glutamate, included in synthetic liquid media, were used as nitrogen sources to study high value lipid production in the genera *Absidia*, *Mortierella*, *Mucor*, *Rhizopus* and *Zygorrhynchus*. These genera were found to be able to utilise the nitrogen sources mentioned above (Inui *et al.*, 1965; Aggelis *et al.*, 1987). However, it was found that sodium nitrite [$NaNO_2$] and sodium nitrate [$NaNO_3$] were unable to support growth of 447 strains of *Rhizopus* in synthetic liquid media (Inui *et al.*, 1965).

Members of the genera *Mortierella*, *Mucor* and *Rhizopus* were also found to be able to grow and produce GLA in complex liquid media containing either KNO_3, NH_4Cl, lysine, urea, malt extract (Hansson & Dostalek, 1988; Tsuchiura & Sakura, 1988; Sajbidor *et al.*, 1988; Certik *et al.*, 1993). All of these media were supplemented with $5g^{-1}$.l-yeast extract. However, it was also shown that representatives of the genus *Mortierella* can grow and produce high value lipids in

a medium containing $5g^{-1}$.l yeast extract as sole nitrogen source (Sajbidor *et al.*, 1990). Thus, it raised doubts about the previous results obtained on the ability of these fungi to utilise the tested nitrogen sources as sole sources of nitrogen, since yeast extract was also included in those experiments. The inclusion of yeast extract, which can serve as a nitrogen source on its own, makes it difficult to come to any definite conclusions concerning other nitrogen sources.

1. 5. Habitats of mucoralean fungi

As a result of their saprophytic nature, mucoralean fungi are often isolated from soil habitats. (Parkinson & Waid, 1960; Hesseltine & Ellis, 1973; Domsch *et al.*, 1980; Bokhary & Parvez, 1991; Brock *et al.*, 1994). In these habitats, the fungi may be associated with organic matter or the rhizosphere, which is a region in soil immediately adjacent to plant roots.

It was found that a number of mucoralean genera can often be isolated from the same soil sample (Hesseltine & Ellis, 1973; Domsch *et al.*, 1980; Botha *et al.*, 1997). The following genera occur commonly in soil habitats: *Absidia, Actinomucor, Cunninghamella, Mortierella, Mucor, Rhizomucor* and *Rhizopus*. Although the asexual reproductive structures of these fungi may differ significantly between taxa, within the Mucorales the asexual apparatus of these fungi are relatively simple. All these genera form sporangiophores, sporangia and/or sporangiola, but no elaborate clusters of sporangiola arranged along sporangiophores or sporangiophores with specialised means of dispersing their sporangia. Also, the nutritional needs of these fungi are usually simple and growth factors are mostly not needed (Hesseltine & Ellis, 1973; Domsch *et al.*, 1980).

In contrast, some of the mucoralean fungi that are commonly associated with dung, such as *Ellisomyces* Benny *et* Benjamin, *Thamnostylum* von Arx *et* Upadhyay, *Pirella* Bainier and *Radiomyces* Embreei all form complex clusters of sporangiola along sporangiophores (Benny & Benjamin, 1975; Benny & Benjamin, 1991; Benny & Schipper, 1992). Other coprophilous mucoralean genera, such as *Pilaira* v. Tiegheim and *Pilobolus* prefer moist environments with abundant growth factors (Webster, 1978; Domsch *et al.*, 1980; Kendrick, 1985). Both these fungi show specialised means of dispersing their mature sporangia to ensure that the

sporangiospores pass through the gut of herbivores in order to end up in fresh dung.

Some mucoralean fungi are known to be parasitic on insects, such as *Sporodiniella umbellata* Boedijn (Evans & Samson, 1977), while *Parasitella parasitica* (Bain.) Syd. is a facultative hyperparasite of other mucoralean fungi (Schipper, 1978; Domsch *et al.*, 1980). It has also been found that members of the genera *Absidia*, *Mucor* and *Rhizopus* cause mucormycosis in health compromised mammals (Hesseltine & Ellis, 1973). *Mucor amphibiorum* Schipper has been isolated from diseased frogs (Schipper, 1978). *Blakeslea trispora* Thaxter is a weak plant parasite that can be isolated from the leaves of higher plants in tropical regions (Zycha *et al.* 1969).

In general, mucoralean fungi are usually associated with moist environments that are rich in organic material. However, studies by some authors have shown that these fungi also occur in dry arid and semi-arid regions (Domsch *et al.*, 1980; Bokhary & Parvez, 1991; Steiman *et al.*, 1995; Guirand *et al.*, 1995; Roux & Warmelo, 1997).

1. 5. 1. Mucoralean fungi in soil of arid regions. During a survey on fungi present in desert soil of Northern Saudi Arabia, the microfungi associated with the ascocarps of truffles were studied (Bokhary & Parvez, 1991). The truffles investigated belonged to the genera *Tirmania* Chatin, *Terfezia* (Tul.) Tul. and *Phaeangium* (Sacc.) Sacc. In addition, fungi present in the rhizosphere of *Helianthemum lippi* as well as in non-rhizosphere soil were also surveyed. A total of 46 genera, the majority of which was dikaryomycotan fungi, were identified in these habitats. The most frequently encountered species were *Penicillium chrysogenum* Thom followed by *Aspergillus niger* van Tiegh. *Aspergillus* Micheli ex Link was found to be the most frequently isolated genus, represented by 13 species. It was then followed by *Penicillium* Link with 9 species, *Ulocladium* Preuss with 6 species, *Fusarium* Link with 5 species, *Alternaria* Nees with 4 species, *Cladosporium* Link and *Curvularia* Boedijn with 3 species each. The mucoralean fungi, which consisted of about 15% of the total number of isolates, were representatives of the genera *Absidia* v. Tieghem, *Circinella* v. Tieghem & le Monn., *Mucor*, *Rhizopus*, *Thamnidium* Link and *Zygorhynchus* Vuill.

In a survey of the mycobiota present in desert soil around the Dead Sea, samples from the top 100 mm of soil, were collected at 56 localities (Steiman *et al.*, 1995; Guirand *et al.*, 1995). A total of 23 fungal genera, most of which were dikaryomycotan fungi, were identified during the survey. As in the studies of Bokhary & Parvez (1991), *Aspergillus* was found to be the most frequently encountered genus, represented by 14 species (Steiman *et al.*, 1995). *Eurotium* Link, *Penicillium*, *Chaetomium* Kunze, *Microascus* Zukal and *Sporormiella* Ell. & Everh. were also encountered in substantial numbers. The most frequently encountered mucoralean fungal genus was *Rhizopus.* Other mucoralean fungi that were found belong to *Absidia*, *Cunninghamella*, *Mortierella* and *Mucor.*

Eicker *et al* (1982) did a survey of the microorganisms present in soil of the Giribes plains in northern Namibia. They found that *Aspergillus* and *Penicillium* species were generally the most numerous, while yeasts and dark coloured Dematiaceae also occurred frequently. The mucoralean fungi, which consisted of about 10% of the total number of isolates, comprised the genera *Absidia*, *Cunninghamella and Rhizopus*. Another study on fungi present in soil from an arid region in southern Africa, revealed that *Penicillium* and *Trichoderma* were the most frequently encountered fungi in a soil sample from Dry Sandy Highveld Grassland, taken near Bloemfontein in the Free State (Strauss, 1997). This author found that 12% of the total number of fungal isolates were mucoralean fungi belonging to the genera *Mortierella*, *Mucor* and *Rhizopus*. However, these results were obtained on a relatively non-selective medium, i.e. malt extract agar. When benomyl was included as selective agent in a series of enumeration media with different carbon sources, *Absidia*, *Cunninghamella* and *Gongronella* were also found in the soil sample.

In an extensive survey of the mycobiota associated with plants and their roots, as well as the leaf litter in a natural Karoo pasture near Middelburg (Eastern Cape Province), 135 fungal genera were identified (Roux & Warmelo, 1997). Hyphomycetes and Coelomycetes represented about 46% and 35% respectively of the identified taxa. The most prevalent hyphomycetous fungi were members of *Altenaria*, *Cladosporium* and *Fusarium*, while the most prevalent coelomycetous fungi belonged to the genera *Phoma* Sacc., *Ascochyta* Lib. and *Camarosporium* Schulz. Interestingly, there was a low incidence of *Aspergillus*, *Penicillium* and *Trichoderma*, which was contrary to the data obtained from other surveys in arid regions. The Mucorales, on the other hand, represented 4% of the total number of

all the isolates obtained during this survey. The mucoralean fungi encountered were *Actinomucor*, *Cunninghamella*, *Mortierella*, *Mucor*, *Rhizopus* and *Rhizomucor.*

Consequently, from the data obtained of surveys recorded in literature, it can be concluded that a survey conducted on soil from an arid region would probably reveal that *Aspergillus, Penicillium* or *Trichoderma* is the dominant dikaryomycotan genus. However, *Altenaria, Cladosporium* and *Fusarium* may also be present in significant numbers. Mucoralean fungi will be present and may constitute up to 12% of the total fungal isolates, when malt extract agar without selective agents, is used as enumeration medium. Furthermore, mucoralean genera that may be encountered in arid soil, would probably include *Absidia*, *Actinomucor*, *Cunninghamella*, *Gongronella*, *Mortierella*, *Mucor*, *Rhizopus* or *Zygorhynchus.*

1. 6. Aim

With the above as background, the aim of this study was to investigate aspects of the physiology and geographical distribution of mucoralean fungi, which would give more insight into the ecological niche these fungi occupy in arid soil. Consequently, selected mucoralean species occurring frequently in soil habitats, including strains from culture collections, as well as mucoralean isolates obtained from a soil sample from arid Upper Nama Karoo (Low & Rebelo 1996), were used to evaluate in vitro growth to determine nitrogen sources and a_w tolerances (Chapter 2). In chapter 3, the mucoralean fungal diversity of other arid regions in southern Africa, including a soil sample from Kimberley Thorn Bushveld, was compared to what is known on the diversity of these fungi in the Karoo soil. In addition, the experiments on nitrogen utilisation and a_w tolerances were repeated on the isolates of the Kimberley Thorn Bushveld soil sample. In this chapter again, the ability of mucoralean spores to survive elevated temperatures in soil of arid regions were also explored by testing mucoralean species occuring in soil for survival in soil incubated at 55 °C for 14hrs.

1. 7. References

Aggelis, G., Pina, M., Ratomaheninna, R., Arnaud, A., Graille, J., Galzy, P., Martin-Privat, P. and Perraud, J. P. (1987). Production d'huiles riches en acide gamma linolenique par diverses souches de Phycomycetes. *Oleagineux* **42,** 379 - 386.

Aggelis, G., Ratomaheninna, R., Arnaud, A., Galzy, P., Martin-Privat, P., Perraud, J. P., Pina, M. and Graille, J. (1988). Etude de l'influence des conditiones de culture sur la teneur en acide gamma linolenique de souches de *Mucor*. *Oleagineux* **43,** 311 – 317.

Alexopoulos, C. J. & Mims, C. W. (1979). Introductory mycology, 3rd Edition. John Wiley and Sons, New York.

Atlas, R. M. and Bartha, R. (1981). Microbial Ecology, In *Fundamentals and Applications*. Addison – Wesley Publishing Company.

Benny, G. L. and Benjamin, R. K. (1975). Observations on Thamnidiaceae (Mucorales). new taxa, new combinations, and notes on selected species. *Aliso* **8,** 301 – 351.

Benny, G. L. and Benjamin, R. K. (1991). The Radiomycetaceae (Mucorales; Zygomycetes). III. A new species of *Radiomyces*, and cladistic analysis and taxonomy of the family; with a discussion of evolutionary ordinal relationships in Zygomycotina. *Mycologia* **83,** 713 – 735.

Benny, G. L., Benjamin, R. K. and Kirk, P. M. (1992). A re-evaluation of Cunninghamellaceae (Mucorales). Sigmoideomycetaceae *fam. nov.* and *Reticulocephalis gen. nov.*; cladistic analysis and description of two new species. *Mycologia* **84,** 615 - 641.

Benny, G. L. and Schipper, M. A. A. (1992). Observations on Thamnidiaceae (Mucorales). IV. *Pirella*. *Mycologia* **84,** 52 – 63.

Benjamin, R. K. (1979). Zygomycetes and their spores, In *The whole fungus*, Vol 2, pp. 573 – 621. Edited by B. Ratledge. National Museum of natural sciences, Canada.

Bokhary, H. A. and Parvez, S. (1991). Soil mycology from wild desert truffle habitats in northern Saudi Arabia. *Arid Environments* **23,** 379 – 388.

Botha, A., Strauss, T., Kock, J. L. F., Pohl, C. H. and Coetzee, D. J. (1997). Carbon source utilisation and γ- linolenic acid production by mucoralean fungi. *System Appl Microbiol* **20,** 165 – 170.

Botha, A., Paul, I., Roux, C., Kock, J. L. F., Coetzee, D. J., Strauss, T. and Maree, C. (1999). Short communication: An isolation procedure for arachidonic acid producing *Mortierella* species. *Antonie van Leeuwenhoek* **75,** 253 - 256.

Brock, T. D., Madigan, M. T., Martinko, J. M. and Parker, J. (1994). *Biology of microorganisms*, seventh edition, Prentice Hall, New Jersey.

Brown, A. D. (1976). Microbial water stress. *Bacteriol Rev* **40,** 803 – 846.

Bullerman, L. B. (1993). Fungi in Food – An Overview, In *Encyclopaedia of food science, food technology and nutrition,* Vol. 7, pp. 4327 – 4338. Edited by R. Macrae, R.K. Robinson & M.J. Sadler. Academic Press, London.

Certik, M., Sajbidor, J. and Stredanska, S. (1993). Effect of carbon and nitrogen sources on growth, lipid production and fatty acid composition of *Mucor mucedo*. *Microbios* **74,** 7 - 15.

Domsch, K. H., Gams, W. and Anderson, T-H. (1980). *Mortierella* Coemans 1863. In *Compendium of soil fungi*, Vol. 1, pp. 431 – 460. Edited by K.H. Domsch, W. Gams & T-H. Anderson. Academic Press, London.

Du Preez, J. C., Immelman, M., Kock, J. K. L. and Kilian, S. G. (1997). The effect of acetic acid concentration on the growth and production of γ- linolenic acid by *Mucor circinelloides* CBS 203.28 in fed batch culture. *World J Microbiol Biotechnol* **13,** 81 – 87.

Eicker, A., Theron, G. K. and Grobbelaar, N. (1982). 'n Mikrobiologiese studie van 'kaal kolle' in die Giribesvlakte van Kaokoland, S.W.A.-Namibië. *S Afr J Bot* **1,** 69 – 74.

Eroshin, V. K., Dedyukhina, E. G., Christyakova, T. I., Zhelifonova, V. P. and Bothast, R. J. (1996). Studies on arachidonic acid production by *Mortierella* fungi: A microbial method for selecting arachidonic acid producers. *Microbiol* **65(1),** 26 – 31.

Evans, H. C. and Samson, R. A. (1977). Sporodiniella umbellata, an entomogenous fungus of the Mucorales from cocoa farms in Ecuador. *Con J Bot* **55,** 2981 – 2984.

Ferguson, S. J. (1987). The redox reactions of the nitrogen and sulphur cycles. In *The nitrogen and sulphur cycles*, pp 1 – 98. Cambridge University Press, Cambridge.

Gams, W. (1977). A key to the species of *Mortierella*. *Persoonia* **9,** 381 - 391.

Guirand, P., Steiman, R., Seigle-Murandi, F. and Sage, L. (1995). Mycoflora of Soil Around the Dead Sea II – Deuteromycetes (except *Aspergillus* and *Penicillium*). *System Appl Microbiol* **18,** 318 – 322.

Hansson, L. and Dostalek, M. (1988). Effect of culture conditions on mycelial growth and production of linolenic acid by the fungus *Mortierella ramanniana*. *Appl Microbiol Biotechnol* **28,** 240 - 246.

Hesseltine, C. W. and Ellis, J. J. (1973). Mucorales. In *The fungi, an advanced treatise*, Ch 11. pp. 187 - 217. Edited by G.G. Ainsworth, F.K. Sparrow & A.S. Sussman. Academic Press, Inc., Orlando.

Inui, T., Takeda, Y. and Iizuka, H. (1965). Taxonomical studies on genus *Rhizopus*. *J Gen Appl Microbiol* **11 (supplement),** 1 – 20.

Jennings, D. H. (1995). The Physiology of Fungal Nutrition Cambridge University Press, Cambridge, United Kingdom.

Kendrick, B. (1985). *The fifth kingdom.* Mycologue Publications, Ontario.

Large, P. J. (1986). Degradation of Organic Nitrogen Compounds by Yeasts. *Yeast* **2,** 1 – 34.

Low, A. B. and Rebelo, A. G. (1996). Vegetation of South Africa, Lesotho and Swaziland. Department of Environmental Affairs and Tourism, Pretoria, South Africa.

Ottaviani, F. (1993). Moulds in food spoilage. In *Encyclopaedia of food science, food technology and nutrition*, Vol. 7, pp. 4338 – 4344. Edited by R. Macrae, R.K. Robinson & M.J. Sadler. Academic Press, London.

Parkinson, D. and Waid, J. S. (1960). *The ecology of soil fungi.* University Press, Liverpool.

Ratledge, C. (1989). Biotechnology of fats. In *Microbial lipids*, Vol. 2. pp. 567 – 668. Edited by C. Ratledge & S. G. Wilkinson. Academic Press, London.

Roux, C. and Van Warmelo, K. T. (1997). A survey of the mycobiota of a natural Karoo pasture. *Bothalia* **27,2,** 167 - 183.

Sajbidor, J., Certik, M. and Dorbronova, S. (1988). Influence of different carbon sources on growth, lipid content and fatty acid composition in four strains belonging to Mucorales. *Biotechnol Lett* **10,** 347 - 350.

Sajbidor, J., Dorbronova, S. and Certik, M. (1990). Arachidonic acid production by *Mortierella* sp. S-17: Influence of C:N ratio. *Biotechnol Lett* **12,** 455 - 456.

Schipper, M. A. A. (1978). On certain species of *Mucor* with a key to all accepted species. *Stud Mycol* **17,** 1 - 52.

Schipper, M. A. A. (1984). A revision of the genus *Rhizopus* 1. The *Rh. stolonifer*-group and *Rh. oryzae*. *Stud Mycol* **25,** 1 - 19.

Sparling, G. P. (1998). Soil microbial biomass, activity and nutrient cycling as indicators of soil health. In *Biological indicators of soil health*, pp. 97 – 119. Edited by C. E. Pankhurst, B.M. Doube & V.V.S.R. Gupta. CAB International, Oxon.

Spotts, R. A. and Cervantes, L. A. (1986). Populations of *Mucor piriformis* in soil of pear orchards in the Hood River valley of Oregon. *Plant disease* **70,** 935 - 937.

Steiman, R., Guiraud, P., Sage, L., Seigle-Murandi, F. and Lafond, J-L. (1995). Mycoflora of soil around the Dead Sea, I – Ascomycetes (including *Aspergillus* and *Penicillium*), Basidiomycetes, Zygomycetes. *System Appl Microbiol* **18,** 310 - 317.

Strauss, T. (1997). The isolation of gamma-linolenic acid producing mucoralean fungi. M.Sc. Thesis, Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein, South Africa.

Steekstra, H. (1997). On the safety of Mortierella alpina for the production of food ingredients, such as arachidonic acid. *Journal of Biotechnology* **56,** 153 – 165.

Tsuchiura, O. S. and Sakura, T. Y. (1988). Method for the preparation of a fungal body and a lipid rich in γ-linolenic acid therefrom. *U.S. Patent number* 4,783,408.

Ueng, P. P. and Gong, C. (1982). Ethanol production from pentoses and sugar-cane bagasse hemicellulose hydrolysate by *Mucor* and *Fusarium* species. *Enzyme Microb Technol* **4,** 169 -171.

Walker, G. M. (1998). *Yeast, Physiology and Biotechnology.* John Wiley and Sons, Inc., New York.

Webster, J. (1978). *Introduction to fungi.* Cambridge University Press, Cambridge.

Zycha, H., Siepman, R. and Linnemann, G. (1969). Mucorales, Eine beschreibung aller gattugen und arten dieser pilzgruppe. Verlag Von J. Cramer. Lehre.

CHAPTER 2

NITROGEN UTILISATION AND GROWTH AT REDUCED WATER ACTIVITY BY MUCORALEAN FUNGI PRESENT IN ARID SOIL.

(This chapter has been accepted for publication in the South African Journal of Botany)

2. 1. Introduction

Mucoralean fungi are mostly saprophytes associated with decaying plant material, dung or other organic debris in soil (Hesseltine & Ellis, 1973; Domsch *et al.*, 1980). These fungi are usually associated with moist environments, such as leaf litter in forests, and are known to be relatively intolerant to low a_w (Brown, 1976). Growth of *Absidia, Mucor* and *Rhizopus* occur above a_w values of 0.92 to 0.93 (Ottaviani, 1993). However, mucoralean fungi have also been recorded in soil, debris and on plant roots from arid regions (Steiman *et al.*, 1995; Roux & Van Warmelo, 1997).

Availability of carbon and nitrogen sources is known to play a major role in the composition and succession of microbial communities on decaying organic matter (Daeschel *et al.*, 1987; Hudson, 1992). Mucoralean fungi are the first to colonise decaying organic material in soil, since these fungi are able to rapidly utilise the limited number of simple carbohydrates that are usually available, before other fungal groups take over the mineralisation of carbon (Hesseltine & Ellis, 1973). It is therefore not surprising that studies have indicated that mucoralean fungi are able to utilise organic nitrogen as well as ammonium salts (Inui *et al.*, 1965; Aggelis *et al.*, 1987; Sajbidor *et al.*, 1988; Tsuchiura & Sakura, 1988). However, certain authors found that some mucoralean fungi are able to utilise nitrate (Hansson & Dostalek, 1988; Certik *et al.*, 1993). This characteristic is not essential for a primary coloniser of dead organic matter, since during the mineralisation of organic nitrogen, nitrification only occurs after ammonification (Sparling, 1998). The specific position of particular species of mucoralean fungi in the biogeochemical nitrogen cycle, however, is unknown since only fragmentary information on the utilisation of different nitrogen containing compounds by these fungi, exists in literature.

With the above as background, the aim of this study was to investigate the ecological niche of mucoralean fungi in arid soil, with specific reference to the position these fungi occupy in the biogeochemical cycle of nitrogen. Consequently, selected mucoralean taxa occurring frequently in soil habitats, including strains from culture collections, as well as isolates obtained from a soil sample from arid Upper Nama Karoo (Low & Rebelo, 1996), were used to evaluate in vitro growth to determine nitrogen sources and a_w tolerances.

2. 2. Materials and Methods

2. 2. 1. Strains used

The fungal strains and isolates used in this study are listed in Tables 1 and 3. The strains were obtained from the Centraalbureau voor Schimmelcultures (CBS), Netherlands, and the mucoralean culture collection of the University of the Orange Free State (MUFS), South Africa. Other strains were isolated from a soil sample originating from Upper Nama Karoo (Low & Rebelo, 1996).

2. 2. 2. Physiological properties

2. 2. 2. 1. Preparation of inocula. A sterile wet inoculating loop was used for each fungal strain to transfer sporangiospores and/or hyphal fragments from a two-week-old culture on 2 % (w/w) Difco malt extract agar (MEA) to 5 ml sterile distilled water. Forty microliters of the resulting suspension, containing *circa* 2 X 10^6 colony forming units per ml, was used to inoculate each defined medium, in Petri dishes.

2. 2. 2. 2. Nitrogen utilisation on a solid defined medium. A series of solid defined media was prepared (Van der Walt & Yarrow, 1984). Each medium, in Petri dishes, consisted of 11.7 $g.l^{-1}$ Bacto yeast carbon base, Difco (YCB) and a different nitrogen source (Table 1), giving a final nitrogen concentration of 0.1$g.l^{-1}$. The media all had a pH of *circa* 5.5 and were solidified with 2 % (w/w) washed purified agar. The washed agar was prepared by repetitively washing solidified agar blocks (Difco) in demineralised water (Van der Walt & Yarrow, 1984).

Table 1. The ability of strains representing different mucoralean genera to utilise the series of nitrogen containing compounds and to grow at 0.955 a_w.

Strains	* Nitrogen source utilisation					#Growth
	$NaNO_2$	KNO_3	NH_4Cl	Asn	Glu	0.955a_w
Actinomucor elegans (Eidam) C. R. Benj. & Hesselt. MUFS 022	+++	+++	++	++	+++	+
Actinomucor elegans (Eidam) C. R. Benj. & Hesselt. MUFS 221	+++	+++	++	++	+++	+
Actinomucor elegans (Eidam) C. R. Benj. & Hesselt. MUFS 229	+++	+++	+	+++	+++	+
Backusella lamprospora (Lendn.) Benny & R. K. Benj. MUFS 002	+++	+++	+++	+++	+++	++
Backusella lamprospora (Lendn.) Benny & R. K. Benj. MUFS 008	+++	+++	+++	+++	+++	++
Backusella lamprospora (Lendn.) Benny & R. K. Benj. MUFS 011	+++	+++	+++	+++	+++	++
Cunninghamella echinulata (Thaxt.) Thaxt. MUFS 001	-	0	0	++	+++	++
Cunninghamella echinulata (Thaxt.) Thaxt. MUFS 002	-	0	0	++	+++	++
Cunninghamella echinulata (Thaxt.) Thaxt. MUFS 003	-	0	0	+++	+++	++
Gongronella butleri (Lendn.) Peyronel & Dal Vesco MUFS 1	+++	+	++	++	++	0
Gongronella butleri (Lendn.) Peyronel & Dal Vesco MUFS 2	+++	+++	++	+++	+++	0
Mortierella amoeboidea W. Gams CBS 889.72T	-	0	++	+++	+++	0
Mortierella globulifera Rostrup CBS 417.64	-	-	+++	+++	+++	0
Mortierella turficola Y. Ling-Yong CBS 430.76	-	0	+++	+++	+++	0
Mucor azygosporus R. K. Benj. CBS 292.63T	-	-	++	+++	+++	+++

Table 1 continues

Strains	* Nitrogen source utilisation					#Growth
	$NaNO_2$	KNO_3	NH_4Cl	Asn	Glu	$0.955a_w$
Mucor circinelloides Tiegh. CBS 119.08	+++	+++	++	+++	+++	++
Mucor flavus Bainier CBS 234.35	+++	+++	+++	+++	+++	+
Mucor plumbeus Bonord. CBS 111.07	+++	+++	0	+++	+++	+++
Mucor racemosus Fres CBS 115.08	+++	+++	++	+++	+++	0
Rhizomucor pusillus (Lindt.) Schipper MUFS 001	-	0	+++	+++	+++	0
Rhizomucor pusillus (Lindt.) Schipper MUFS 005	-	0	+++	+++	+++	0
Rhizopus microsporus Tiegh. CBS 631.82	-	0	0	+++	+++	+
Rhizopus microsporus Tiegh. PPRI 5560	-	0	0	++	+++	++
Rhizopus oryzae Went & Prins. Geerl. CBS 112.07	-	+	+++	+++	+++	++
Rhizopus stolonifer (Ehrenb.: Fr.) Vuill.CBS 609.82	-	0	0	+++	+++	++
Rhizopus stolonifer (Ehrenb.: Fr.) Vuill.CBS 319.35	-	0	0	+++	+++	++
Thamnostylum piriforme (Bainier) Arx & H.P. Upadhyay MUFS 025	-	+++	+	++	++	0

* Nitrogen utilisation, measured by calculating the colony diameter obtained on the medium with the particular nitrogen source, as a percentage of the colony diameter on the medium which best supported radial growth of the particular fungal strain. Symbols: 0 = 0 %; + = 1 -33 %; ++ = 34 - 66 %; +++ = 67 - 100 %; - = toxic, since the colony density and diameter on the particular solid medium were less than that obtained on the medium devoid of a nitrogen source; Abbreviations: Asn = Asparagine; Glu = Sodium glutamate.

Growth at reduced a_w, measured by calculating the colony diameter on the medium with 0.955 a_w, as a percentage of the colony diameter obtained on a non water-stressed control. Symbols: 0 = 0 %; 1 -33 %; 34 - 66 %; 67 - 100 %.

A superficial well of 8.0 mm in diameter, was made in the centre of the medium in each Petri dish, using a hot glass rod. Each Petri dish was inoculated by pipetting 40 µl of the inoculum into the well and then incubated at 25°C in the dark. The diameter of the resulting colony was measured after five days of incubation. A control experiment was conducted in which the ability was tested to grow on a solid medium devoid of a nitrogen source. All the experiments were conducted in triplicate.

2. 2. 2. 3. Measurement of radial growth with different nitrogen sources. Radial growth, as an indication of the rate of utilisation of the nitrogen source, was measured. This was done by calculating the colony diameter obtained after five days on the medium containing the particular nitrogen source, as a percentage of the colony diameter obtained after the same period on the medium which best supported radial growth of the particular fungal strain (Tables 1 and 4).

2. 2. 2. 4. Growth on a medium with a reduced water activity. A solid medium was prepared in Petri dishes. This medium consisted of the following ($g.l^{-1}$): 10.0, glucose; 5.0, peptone; 1.0, KH_2PO_4; 0.5, $MgSO_4.7H_2O$; 220.0, glycerol; 15.0, agar and 0.1, chloramphenicol. The medium had a pH of *circa* 5.6 and a final a_w of 0.955.

A superficial well in the medium was prepared and inoculated as described above. The inoculated Petri dishes were incubated at 25°C in the dark. The diameter of the resulting colony was measured after five days of incubation. To evaluate growth against a non water-stressed control, the ability of each strain to spread on an identical medium, except for the absence of glycerol, was also recorded. All the experiments were conducted in triplicate.

2. 2. 2. 5. Measurement of radial growth on medium with low a_w. Growth was measured by calculating the colony diameter obtained after five days on the medium with 0.955 a_w, as a percentage of the colony diameter obtained after the same period on the control devoid of glycerol. (Tables 1 and 4)

2. 2. 3. Determination of fungal taxa in soil

2. 2. 3. 1. Taking of soil sample. The sampling site (10 m^2) is located at De Aar in the Northern Cape, South Africa, amongst Upper Nama Karoo (Low & Rebelo, 1996). The mean annual rainfall is 305 mm, while the mean annual temperature is 23.3 °C. The sample was taken in mid-summer. The surface litter was first scraped away to reduce contamination from this habitat. A soil sample of *circa* 2900 g, consisting of nine subsamples, was taken at random over the area of the site, each to a depth of 10 cm. The subsamples were thoroughly mixed in the laboratory to produce the sample, which was further processed.

2. 2. 3. 2. Chemical analyses of soil sample. The pH of the soil sample was determined according to the method of Spotts and Cervantes, (1986). The soil moisture content was determined according to the method of Eicker, (1970). The organic carbon content was determined using the Walkley-Black method as described by Barnard *et al.*, (1990). Nitrate and nitrite were determined by the Institute of Ground Water Studies at the UOFS, South Africa, using ion chromatography (Dionex 2000 I/SP and Dionex oinpac columns with carbonate/bicarbonate as elluent; AG4A – SC was used for the guard column and AS4A – SC for the separation column). The ammonium content was spectrophotometrically determined by the Institute of Ground Water Studies at the UOFS, South Africa, using the Nessler method (Hach). The characteristics of the soil at the sampling site are summarised in Table 2.

2. 2. 3. 3. Isolation of fungi. By using the soil plate technique of Warcup, (1950), two selective media (containing benomyl and starch or sucrose as carbon source), as well as a relative non-selective medium, was used to isolate mucoralean fungi from the soil sample. The first selective medium contained (g. l^{-1}); starch, 10.00; NH_4Cl, 1.00; KH_2PO_4, 1.00; $MgSO_4.7H_2O$, 0.50; yeast extract (Difco), 0.50; and chloramphenicol, 0.20. It also contained the following mineral salts (mg.l^{-1}): $FeSO_4.7H_2O$, 10.00; $ZnSO_4.7H_2O$, 10.00; $MnSO_4.H_2O$, 0.80 and $CuSO_4.5H_2O$, 0.05. The medium had a pH of 5.5 and was solidified with 16 g. l^{-1} agar (Biolab); 0.02 g.l^{-1} benomyl (Methyl 1-(butylcarbamoyl)-2-benzimidazole-carbamate, Aldrich catalog no. 38,158-6) was added before autoclaving. The

Table 2. Characteristics of the soil at the sampling site.

CHARACTERISTICS	
Chemical properties	
pH of soil	7.25 ± 0.05
Soil moisture content	6.66 % ± 0.22 %
Organic carbon	0.72 %
Total nitrogen	0.20 %
Nitrite	1.10 ppm ± 0.10 ppm
Nitrate	18.60 ppm ± 2.40 ppm
Ammonium	2.80 ppm ± 0.40 ppm
Physical properties	
Clay	30 %
Silt	21 %
Sand	48 %

second selective medium was similar to the first, except that it contained 10 $g.l^{-1}$ sucrose as carbon source instead of starch. The relatively non-selective medium (MYPps) (Carreiro & Koske, 1992), contained ($g.l^{-1}$): Malt extract (Difco), 7.00; peptone (Oxoid), 1.00; yeast extract (Difco), 0.50; penicillin G, 0.50; streptomycin sulphate, 0.50; and agar (Biolab), 16.00.

For each medium, soil plates were prepared by transferring 0.001 g of soil from the sample to each of 10 sterile Petri dishes. Eight ml of cooled molten isolation medium (45°C) was then added to each Petri dish. The plates were incubated at 25°C in the dark and examined for growth of colonies.

Mycelium from each colony on the isolation plates, was transferred to plates with fresh 2% (w/w) MEA and incubated at 25°C. Further purification was obtained by successive subculturing of each isolate on 2% (w/w) MEA. The number of fungi in the soil sample that grew on the isolation plates were calculated as colony forming units per gram of soil ($CFU.g^{-1}$).

2. 2. 3. 4. Identification of fungi. Isolates belonging to the higher fungi were identified up to genus level using the keys of Baxter *et al.*, (1994), Ellis, (1976) and Kreger-van Rij, (1984). The mucoralean fungi were identified using the keys and descriptions given by Gams, (1977), Hesseltine & Ellis, (1973), Lunn & Shipton, (1983) and Schipper, (1976, 1978 and 1984).

2. 3. Results

2. 3. 1. Utilisation of nitrogen sources by species and strains of the selected genera

The ability of species and strains of the selected fungal genera to utilise the series of nitrogen containing compounds as sole nitrogen source is given in Table 1. All the strains were able to utilise the amino acids, asparagine and glutamate. However, only 70% of the strains were able to utilise ammonium chloride, while 52% of the strains were able to grow on nitrate as sole nitrogen source. The latter included strains of *Actinomucor*, *Backusella*, *Gongronella*, *Mucor*, *Thamnostylum*, and *Rhizopus oryzae*. The growth results obtained when nitrite was added indicated that the concentrations used in this study were toxic to species of *Cunninghamella*, *Mortierella*, *Rhizomucor*, *Rhizopus* and *Thamnostylum*.

2. 3. 2. Growth at 0.955 a_w by species and strains of the selected genera

Species and strains of the genera *Actinomucor*, *Backusella*, *Cunninghamella* and *Rhizopus* were able to grow at 0.955 a_w (Table 1). *Mucor racemosus* was the only strain representative of the genus *Mucor* that was unable to grow at 0.955 a_w. Representative strains examined of *Gongronella*, *Mortierella*, *Rhizomucor* and *Thamnostylum* were unable to grow at this a_w value.

2. 3. 3. Fungi present in the soil sample

The fungi in the soil sample, which were enumerated using the three isolation media, are listed in Table 3. Strains of 13 genera of higher fungi were found to

Table 3. Fungi occurring in the soil sample.

	MYPps (CFU/g soil)	Med. A (CFU/g soil)	Med. B (CFU/g soil)
Mucorales			
Actinomucor			
Elegans (Eidam)			
C.R. Benj. Hesselt.	240	760	760
Mortierella isabellina			
Oudem.	180	-	-
Mucor circinelloides			
Tiegh.	-	320	40
Rhizopus oryzae			
Went & Prins.Geerl.	860	80	180
TOTAL	1280	1160	980
Genera of other fungi			
Acremonium	-	40	-
Aspergillus	480	-	60
Botryotrichum	360	-	
Cladosporium	60	-	-
Curvularia	120	120	300
Fusarium	20	-	-
Geotrichum	200	-	-
Paecilomyces	20	-	-
Penicillium	120	20	-
Phaeoisaria	60	-	-
Polyscytalum	-	-	20
Rhizoctonia	40	-	40
Trichoderma	700	20	-
TOTAL	2180	200	420

CFU: Colony forming units; MYPps: Relative non-selective medium (Carreiro & Koske, 1992); Med. A: Isolation medium A containing starch as carbon source and 20 ppm benomyl; Med. B: Isolation medium B containing sucrose as carbon source and 20 ppm benomyl;

occur in the soil sample. The most frequently occurring of these were *Trichoderma* and *Aspergillus*. Only four species of mucoralean fungi could be detected in the soil sample i.e. *Actinomucor elegans* and *Rhizopus oryzae,* which were the most numerous, and *Mortierella isabellina* and *Mucor circinelloides.*

2. 3. 4. Utilisation of nitrogen sources by mucoralean isolates

The ability of mucoralean isolates from the soil sample to utilise the series of nitrogen containing compounds as sole nitrogen source is given in Table 4. All the isolates were able to utilise the amino acids, asparagine and glutamate, as well as ammonium chloride. The isolates of *Mortierella isabellina* were unable to utilise nitrate as sole nitrogen source and at the concentration of nitrite used, this compound was toxic to the isolates of *Mortierella isabellina* and *Rhizopus oryzae*.

2. 3. 5. Growth at 0.955 a_w by mucoralean isolates

The isolates of *Actinomucor elegans, Mucor circinelloides*, and *Rhizopus oryzae* were able to grow at 0.955 a_w (Table 4). Similar to the *Mortierella* strains from culture collections (Table 1), the isolates of *Mortierella isabellina* were unable to grow at 0.955 a_w (Table 4).

2. 4. Discussion

2. 4. 1. Nitrogen utilisation within the Mucorales

A number of workers testing for the utilisation of defined nitrogen sources by mucoralean fungi, also included yeast extract in the culture media (Hansson & Dostalek, 1988; Certik *et al.*, 1993). However, yeast extract was found by Saijbidor *et al.*, (1990) to be able to act as nitrogen source on its own. This complicates the interpretation of results. Therefore, it was decided during the present study to evaluate for nitrogen utilisation using defined media. In order to determine the nitrogen requirements of hyphal cultures on solid substrates, rather than those of submerged cultures in liquid substrates, the media in the present study were solidified with washed agar. It is obvious from the results obtainable from literature as well as the results depicted in Tables 1

Table 4. The ability of mucoralean isolates from the arid soil sample to utilise the series of nitrogen containing compounds and to grow at 0.955 a_w.

Strains	* Nitrogen source utilisation					#Growth
	$NaNO_2$	KNO_3	NH_4Cl	Asn	Glu	0.955a_w
Actinomucor elegans A19	+++	+++	+++	++	+++	++
Actinomucor elegans B28	+++	+++	+	+++	+++	++
Actinomucor elegans B65	+++	+++	++	+++	+++	++
Mortierella isabellina 15	-	0	++	+++	+++	0
Mortierella isabellina 35	-	0	+++	+++	+++	0
Mortierella isabellina 40	-	0	++	+++	+++	0
Mucor circinelloides A46	+++	+++	+++	++	+++	++
Mucor circinelloides A66	+++	+++	+++	++	+++	+
Mucor circinelloides B8	+++	+++	+++	++	+++	+
Rhizopus oryzae 54	-	++	+++	+++	+++	+
Rhizopus oryzae 79	-	+	+++	+++	+++	++
Rhizopus oryzae 82	-	++	+++	+++	+++	+

* Nitrogen utilisation, measured by calculating the colony diameter obtained on the medium with the particular nitrogen source, as a percentage of the colony diameter on the medium which best supported radial growth of the particular fungal strain. Symbols: 0 = 0 %; + = 1 -33 %; ++ = 34 - 66 %; +++ = 67 - 100 %; - = toxic, since the colony density and diameter on the particular solid medium were less than that obtained on the medium devoid of a nitrogen source; Abbreviations: Asn = Asparagine; Glu = Sodium glutamate.

Growth at reduced a_w, measured by calculating the colony diameter on the medium with 0.955 a_w, as a percentage of the colony diameter obtained on a non water-stressed control. Symbols: 0 = 0 %; 1 -33 %; 34 - 66 %; 67 - 100 %.

and 4, that within the Mucorales, differences exist regarding the ability to utilise oxidised inorganic nitrogen.

Inui *et al.*, (1965) found that *Rhizopus* species are unable to utilise 21.0 $g.l^{-1}$ sodium nitrite (3.5 $g.l^{-1}$ nitrogen) in a defined liquid medium contained in stationary test tubes. The results obtained in the present study are in agreement with these findings. However, it was found that the strains and isolates representing *Rhizopus oryzae* (Tables 1 and 4), were able to utilise nitrate when grown on the defined solid media. Interestingly, when the *Rhizopus* strains listed

in Table 1 were tested for growth and nitrate utilisation in defined liquid media with identical compositions except for the absence of agar, none of the *Rhizopus* strains could grow on nitrate (results not shown). The discrepancy with the results of Inui *et al.*, (1965) can therefore be ascribed to the use of different culture conditions. The latter authors used stationary liquid media containing 3.5 $g.l^{-1}$ nitrogen, while solid media containing only 0.1 $g.l^{-1}$ nitrogen were used in the present study. The results of the present study indicate that *R. oryzae* is less specialised in its nitrogen source requirements than the other *Rhizopus* species tested in this study. The ability to grow on ammonium may be as a result of the presence of a transport mechanism for this compound (Jennings, 1995), which is absent in other members of this genus. It may also be that spores of *R. oryzae* are more resistant towards changes in pH upon germination in the ammonium containing medium, than the spores of other species in this genus (Jennings, 1995).

Generally, the *Mucor* species tested in this study were able to utilise inorganic nitrogen compounds (Table 1). However, *M. azygosporus*, a species that can readily be distinguished from the other *Mucor* species by the formation of reddish-brown thick-walled azygospores, did not utilise nitrate nor nitrite, since these compounds were toxic to the fungus at the specific concentrations used in this study (Table 1). Another *Mucor* species, *M. plumbeus,* did not utilise ammonium, which is unusual since most fungi able to utilise nitrate are also able to utilise ammonium (Jennings, 1995). The inability to grow on ammonium might be as a result of the absence of a transport mechanism for this compound, or it may be as a result of a too low pH upon utilisation of ammonium during the early stages of culture.

A low concentration of nitrogen containing compounds (0.1 $g.l^{-1}$ nitrogen) was used in the present study in order to minimise potential toxic effects. Despite this low concentration however, sodium nitrite at a concentration of 0.65 $g.l^{-1}$, was found to be toxic for the representatives of *Cunninghamella echinulata*, *Mortierella spp.*, *Mucor azygosporus*, *Rhizomucor pusillus*, *Rhizopus spp.* and *Thamnostylum piriforme* (Tables 1 and 4).

The ability of the mucoralean fungi to utilise the amino acids presented to them in this study, is in agreement with their role as initial colonisers of organic matter in soil. However, the ability to utilise nitrate, would also place certain mucoralean fungi among the succession of microbes that are involved in the mineralisation of organic matter. According to the results of this study such mucoralean fungi may include the genera *Actinomucor, Backusella, Gongronella*, *Mucor, Rhizopus oryzae* and *Thamnostylum*. Interestingly, it is known that *Mucor* and *Rhizopus* may colonise dead leaves after a few months, when the leaf litter has been incorporated in the upper layers of the soil (Gray & Williams, 1979).

2. 4. 2. The ability of mucoralean fungi to grow at 0.955 a_w

From the results depicted in Tables 1 and 4, it can be seen that within the Mucorales, differences exist regarding the ability to grow at 0.955 a_w. Strains of *Gongronella butleri, Mortierella spp.*, *Mucor racemosus, Rhizomucor pusillus* and *Thamnostylum piriforme* were unable to grow at this a_w value and hence were less osmotolerant than the strains of *Actinomucor elegans, Backusella lamprospora, Cunninghamella echinulata, Rhizopus spp.* and most of the *Mucor* strains.

Comparing the results on the ability to grow at 0.955 a_w, it can be concluded that tolerance towards low water activity may vary within a species. This is obvious when comparing the data on *A. elegans*, *M. circinelloides*, *R. microsporus* and *R. oryzae* (Tables 1 and 4). However, the difference in radial growth on the medium with low a_w, between the culture collection strains of *A. elegans* (Table 1) and the isolates of this species (Table 4), may also reflect a lack of selection pressure during the period of storage in the culture collection.

It must be noted however that, although the mucoralean fungi mentioned above showed osmotolerance compared to the other mucoralean fungi, the Mucorales are known to be significantly less osmotolerant than higher fungi like *Aspergillus* and *Penicillium*, which may have a growth limiting a_w value as low as 0.65 (Brown, 1976).

2. 4. 3. Fungi present in the soil sample

The occurrence of *Aspergillus* and *Penicillium* in the soil sample is not surprising, since it is known that members of these genera frequently occur in soil from arid regions (Griffen, 1972; Eicker *et al.*, 1982; Allsopp *et al.*, 1987). Similarly, *Cladosporium*, *Fusarium* and *Paecilomyces* are known to occur commonly in soil (Gilman, 1959; Waid, 1960; Eicker, 1970; Eicker, 1974; Carreiro & Koske, 1992). Isolates representing *Acremonium, Botryotrichum, Curvularia* and *Geotrichum*, have also been obtained from southern African soils (Eicker, 1969; Eicker *et al.*, 1982; Strauss, 1997).

The overall number of higher fungal genera recorded in this study is significantly less than the 135 genera recorded by Roux and Van Warmelo, (1997) during an extensive survey of fungi associated with plants, litter and soil in a natural Karoo pasture at Middelburg in the Eastern Cape Province, South Africa. However, it must be borne in mind that in the present study, the fungal populations of one soil sample without litter, was investigated with methods which were selective for mucoralean fungi (Strauss, 1997).

In their survey of fungi associated with soil, plants and litter in the Karoo, Roux and Van Warmelo, (1997) found six mucoralean taxa, i.e. *Actinomucor elegans*, *Cunninghamella echinulata, Mortierella, Mucor, Rhizopus stolonifer* and *Rhizomucor.* When strains of these taxa were tested for utilisation of the series of nitrogen containing compounds during the present study (Table 1 and 4), only representatives of *Actinomucor elegans* and *Mucor* were able to utilise oxidised nitrogen compounds. The others were only able to utilise organic nitrogen compounds, which may explain their role as initial colonisers of organic matter.

In the present study, isolates of four mucoralean fungi were isolated from the soil sample, i.e. *Actinomucor elegans*, *Mortierella isabellina*, *Mucor circinelloides* and *Rhizopus oryzae* (Table 3). Isolates of *Mortierella isabellina* occurred less frequently on the isolation media than the other mucoralean isolates and were unable to utilise oxidised nitrogen containing compounds (Table 4). Members of *Mortierella isabellina*, are known to occur on organic material, including the rhizospheres of various plant species (Domsch *et al.*, 1980, Allsopp *et al.*, 1987).

The soil sample analysed in the present study contained only 180 CFU.g^{-1} of *Mortierella* on MEA. This is significantly less than what was found during identical studies on soil from Alti Mountain Grassland conducted in mid-winter (3630 CFU.g^{-1}), and on Dry Sandy Highveld Grassland conducted in mid-summer (8600 CFU.g^{-1}) (Strauss, 1997; Botha *et al.*, 1999). This phenomenon may be ascribed to the preference members of this genus seems to have for moist environments (Domsch *et al.*, 1980). The moisture content of the soil samples taken from Alti Mountain Grassland and Dry Sandy Highveld Grassland, were respectively *circa* 29 % (w/w) and *circa* 13% (w/w) at the time of sampling (Strauss, 1997; Botha *et al.*, 1999). These figures are significantly more than the moisture content of *circa* 7 % (w/w) of the soil sample from Upper Nama Karoo, which was analysed during the present study (Table 2).

Interestingly, it was found that the *Mortierella* species that was isolated in the present study, *M. isabellina,* can be isolated amongst the heat-resistant fungi in soil, after heating soil at 70°C for 30 min. (Domsch *et al.*, 1980). Similarly, the major mucoralean species obtained on the MEA plates in the present study, *R. oryzae*, was found to survive dry heat at 80 °C for 72 h on cured tobacco leaves (Domsch *et al.*, 1980). Heat-resistance may favour the survival of these species in hot arid regions such as the Karoo.

Oligotrophic growth is a prerequisite for fungal growth in many soils (Wainwright, 1993). It was found that *Actinomucor*, *Rhizopus stolonifer* as well as *Mucor rouxii* are capable of oligotrophic growth on silica gel medium with no added nutrients. According to literature, the concentration of soluble inorganic nitrogen (i.e. ammonium, nitrate and nitrite) in the soil sample (Table 2) would be adequate to serve as nitrogen source for oligotrophic growth by these fungi (Wainwright, 1993). This growth however, would be subjected to a number of chemical and physical factors (Jennings, 1995) including the growth limiting a_w for each fungus (Tables 1 & 4; Brown, 1976).

2. 5. Concluding Remarks

Although more factors play a role in growth and survival of fungi in soil, the results showed the interplay between a_w and nitrogen utilisation, which provided some pointers to the likely successions of mucoralean fungi in soil. Although *Mortierella* species were able to utilise amino acids, they were unable to utilise inorganic oxidised nitrogen compounds. In addition, they were unable to grow at 0.955 a_w. This restricts the potential nitrogen sources of these fungi to organic sources containing an abundance of available water. However, it was found that isolates of *A. elegans*, *M. circinelloides* and *R. oryzae* obtained from the Karoo soil, were more osmotolerant and able to utilise organic nitrogen sources as well as inorganic oxidised nitrogen produced during nitrification.

In the next chapter, the mucoralean fungal diversity of other arid regions, including a soil sample form Kimberley Thorn Bushveld, will be compared to the diversity of these fungi in the Karoo soil. In addition, isolates from Kimberley Thorn Bushveld will be tested on nitrogen utilisation as well as on a_w tolerances. The ability of mucoralean soil fungi to survive elevated soil temperatures in arid regions will also be explored.

6. References

Aggelis, G., Pina, M., Ratomaheninna, R., Arnaud, A., Graille, J., Galzy, P., Martin-Privat, P. and Perraud, J. P. (1987). Production d'huiles riches en acide gamma linolenique par diverses souches de Phycomycetes. *Oleagineux* **42,** 379 - 386.

Allsopp, N., Olivier, D. L. and Mitchell, D. T. (1987). Fungal populations associated with root systems of proteaceous seedlings at a lowland fynbos site in South Africa. *S Afr J Bot* **53,** 365 – 369.

Barnard, R. O., Buys, A. J., Coetzee, J. G. K., Du Preez, C. C., Meyer, J. H., Van Der Merwe, A. J., Van Vuuren, J. A. J. and Volschenk, J. E. (1990). Handbook of standard soil testing methods for advisory purposes. Soil Sci. Soc. South Africa, P.O. Box 30030, Sunnyside, Pretoria.

Baxter, A. P., Rong, I. H., Roux, C., Schutte, L. and Van Der Linde, E. (1994). Elementary keys to common fungi in South Africa. Plant Protection Research Institute, Pretoria, South Africa.

Botha, A., Paul, I., Roux, C., Kock, J. L. F., Coetzee, D. J., Strauss, T. and Maree, C. (1999). Short communication: An isolation procedure for arachidonic acid producing *Mortierella* species. *Antonie van Leeuwenhoek* **75,** 253 - 256.

Brown, A. D. (1976). Microbial water stress. *Bacteriol Rev* **40,** 803 – 846.

Carreiro, M. M. and Koske, R. E. (1992). Room temperature isolations can bias against selection of low temperature microfungi in temperate forest soils. *Mycologia* **84,** 886 - 900.

Certik, M., Sajbidor, J. and Stredanska, S. (1993). Effect of carbon and nitrogen sources on growth, lipid production and fatty acid composition of *Mucor mucedo*. *Microbios* **74,** 7 - 15.

Daeschel, M. A., Andersson, R. E. and Fleming, H. P. (1987). Microbial ecology of fermenting plant materials. *FEMS Microbiol Rev* **46,** 357 - 367.

Domsch, K. H., Gams, W. and Anderson, T-H. (1980). *Mortierella* Coemans 1863. In *Compendium of soil fungi,* Vol. 1, pp. 431 – 460. Edited by K.H. Domsch, W. Gams & T-H. Anderson. Academic Press, London.

Eicker, A. (1969). Microfungi from surface soil of forest communities in Zululand. *Trans Br Mycol Soc* **53,** 381 – 392.

Eicker, A. (1970). Ecological observations on soil fungi. *S Afr J Sci* **66,** 327 - 334.

Eicker, A. (1974). The mycoflora of an alkaline soil of the Open – Savannah of the Transvaal. *Trans Br Mycol Soc* **63,** 281 – 288.

Eicker, A., Theron, G. K. and Grobbelaar, N. (1982). 'n Mikrobiologiese studie van 'kaal kolle' in die Giribesvlakte van Kaokoland, S.W.A.-Namibië. *S Afr J Bot* **1,** 69 – 74.

Ellis, M. B. (1976). More dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, England.

Gams, W. (1977). A key to the species of *Mortierella. Persoonia* **9,** 381 - 391.

Gilman, J. C. (1959). A manual of soil fungi. The Iowa State Press, Iowa.

Gray, T. R. G. and Williams, S. T. (1979). Soil microflora and the decomposition of dead organic matter. In *Soil micro-organisms,* pp. 75 – 89. Edited by T.R.G. Gray & S. T. Williams. Longman, London.

Griffen, D. M. (1972). Ecology of soil fungi. Chapman and Hall, London.

Hansson, L. and Dostalek, M. (1988). Effect of culture conditions on mycelial growth and production of linolenic acid by the fungus *Mortierella ramanniana*. *Appl Microbiol Biotechnol* **28,** 240 - 246.

Hesseltine, C. W. and Ellis, J. J. (1973). Mucorales. In *The fungi, an advanced treatise,* Ch 11. pp. 187 - 217. Edited by G.G. Ainsworth, F.K. Sparrow & A.S. Sussman. Academic Press. Inc., Orlando.

Hudson, H. J. (1992). Fungal Biology. Cambridge University Press, Cambridge.

Inui, T., Takeda, Y. and Ilzuka, H. (1965). Taxonomical studies on genus *Rhizopus*. *J Gen Appl Microbiol* **11 (supplement),** 1 – 20.

Jennings, D. H. (1995). The physiology of fungal nutrition. Cambridge University Press, Cambridge.

Kreger-Van Rij, N. J. W. (1984). The yeasts - a taxonomic study. Elsevier Science Publishers B.V., Amsterdam, The Netherlands.

Low, A. B. and Rebelo, A. G. (1996). Vegetation of South Africa, Lesotho and Swaziland. Department of Environmental Affairs and Tourism, Pretoria, South Africa.

Lunn, J. A. and Shipton, W. A. (1983). Re-evaluation of taxonomic criteria in *Cunninghamella*. *Trans Br Mycol Soc* **81,** 303 - 312.

Ottaviani, F. (1993). Moulds in food spoilage. In *Encyclopaedia of food science, food technology and nutrition,* Vol. 7, pp. 4338 – 4344. Edited by R. Macrae, R.K. Robinson & M.J. Sadler. Academic Press, London.

Roux, C. and Van Warmelo, K. T. (1997). A survey of the mycobiota of a natural Karoo pasture. *Bothalia* **27,2,** 167 - 183.

Sajbidor, J., Certik, M. and Dorbronova, S. (1988). Influence of different carbon sources on growth, lipid content and fatty acid composition in four strains belonging to Mucorales. *Biotechnol Lett* **10,** 347 - 350.

Sajbidor, J., Dorbronova, S. and Certik, M. (1990). Arachidonic acid production by Mortierella sp. S-17: Influence of C:N ratio. *Biotechnol Lett* **12,** 455 - 456.

Schipper, M. A. A. (1976). On *Mucor circinelloides*, *Mucor racemosus* and related species. *Stud Mycol* **12,** 1 - 40.

Schipper, M. A. A. (1978). On certain species of *Mucor* with a key to all accepted species. *Stud Mycol* **17,** 1 - 52.

Schipper, M. A. A. (1984). A revision of the genus *Rhizopus* 1. The *Rh. stolonifer*-group and *Rh. oryzae*. *Stud Mycol* **25,** 1 - 19.

Sparling, G. P. (1998). Soil microbial biomass, activity and nutrient cycling as indicators of soil health. In *Biological indicators of soil health,* pp. 97 – 119. Edited by C.E. Pankhurst, B.M. Doube & V.V.S.R. Gupta. CAB International, Oxon.

Spotts, R. A. and Cervantes, L. A. (1986). Populations of *Mucor piriformis* in soil of pear orchards in the Hood River valley of Oregon. *Plant disease* **70,** 935 - 937.

Steiman, R., Guiraud, P., Sage, L., Seigle-Murandi, F. and Lafond, J-L. (1995). Mycoflora of soil around the Dead Sea, I – Ascomycetes (including *Aspergillus* and *Penicillium*), Basidiomycetes, Zygomycetes. *System Appl Microbiol* **18,** 310 - 317.

Strauss, T. (1997). The isolation of gamma-linolenic acid producing mucoralean fungi. M.Sc. Thesis, Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein, South Africa.

Tsuchiura, O. S. and Sakura, T. Y. (1988). Method for the preparation of a fungal body and a lipid rich in γ-linolenic acid therefrom. *U.S. Patent number* 4,783,408.

Van Der Walt, J. P. and Yarrow, D. (1984). Methods for the isolation, maintenance, classification and identification of yeasts. In *The yeasts, taxonomic study,* pp. 45 – 104. Edited by N.J.W. Kreger-van Rij. Elsevier Science Publishers B.V., Amsterdam.

Waid, J. S. (1960). The growth of fungi in soil. In *The ecology of soil fungi,* pp. 55 – 75. Edited by D. Parkinson & J.S. Waid. University Press, Liverpool.

Wainwright, M. (1993). Oligotrophic growth of fungi – stress or natural state? pp. 127 – 144. Edited by D.H. Jennings. Marcel Dekker, Inc., New York.

Warcup, J. H. (1950). The soil-plate method for isolation of fungi from soil. *Nature* **166,** 117 - 118.

CHAPTER 3

A STUDY ON THE DIVERSITY AND PHYSIOLOGY OF MUCORALEAN FUNGI PRESENT IN THE KAROO AND OTHER ARID REGIONS.

3. 1. Introduction

It is known that mucoralean fungi may occur in soil habitats of arid regions (Steiman *et al.*, 1995). In Chapter 2 mucoralean fungi belonging to *Actinomucor* Schostak., *Mortierella* Coem., *Mucor* Fresen and *Rhizopus* Ehrenb were found in a soil sample from Upper Nama Karoo (Low & Rebelo, 1996). These genera were also obtained when the mycobiota of a natural Karoo pasture was surveyed by other authors (Roux & Van Warmelo, 1997).

Growth and survival of these fungi depend on a number of factors, such as the ability to utilise available nutrients, for example carbon and nitrogen sources, as well as the ability to grow and survive at a particular pH, a_w and temperature (Domsch *et al.*, 1980; Jennings, 1995). In Chapter 2 some of these factors have been explored for a number of mucoralean strains and species. Nine mucoralean fungal genera including 18 species were examined for the ability to utilise a series of nitrogen containing compounds and to grow at an a_w of 0.955 on solid media. The nitrogen concentration in the media was 0.1 $g.l^{-1}$ and the series of nitrogen containing compounds were ammonium chloride, asparagine, sodium glutamate, sodium nitrite and potassium nitrate. The genera were *Actinomucor*, *Backusella* Hesselt. & J.J. Ellis, *Cunninghamella* Matr., *Gongronella* Ribaldi, *Mortierella*, *Mucor*, *Rhizomucor* Lucet & Costantin., *Rhizopus* and *Thamnostylum* Arx & H. P. Upadhyay. Culture collection strains, as well as soil isolates from the arid Upper Nama Karoo soil sample, were tested. All the species and strains tested in Chapter 2 were able to utilise asparagine and glutamate. Strains belonging to *Cunninghamella*, *Mucor racemosus* Fresen., *Rhizopus microsporus* Tiegh. and *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill. were unable to utilise ammonium chloride. Strains of *Cunninghamella,*

Mortierella, Rhizomucor, Rhizopus microsporus and *Rhizopus stolonifer* were unable to grow on nitrate as sole nitrogen source. Nitrite was found to be toxic to species belonging to *Cunninghamella, Mortierella, Rhizomucor, Rhizopus* and *Thamnostylum*. Members of *Gongronella, Mortierella, Mucor racemosus, Rhizomucor* and *Thamnostylum* were unable to grow at an a_w of 0.955.

Interestingly, it was found in Chapter 2 that the only *Mortierella* species that could be detected in the Upper Nama Karoo soil sample, *M. isabellina,* is known to be heat-resistant (Domsch *et al.*, 1980). Similarly, the major mucoralean species obtained on the malt extract agar isolation plates, *R. oryzae*, is also known to be heat-resistant. It was subsequently speculated that heat-resistance may favour the survival of these species in hot arid regions such as the Karoo.

With the above as background, the aim of this study was firstly to get an indication whether the mucoralean diversity of the Karoo, as observed in Chapter 2 and in the records of Roux and Van Warmelo, (1997), differs from data on mucoralean diversity from other arid regions. The latter included data from literature and what could be found in a soil sample taken from Kimberley Thorn Bushveld (Low & Rebelo, 1996). Secondly, the aim was to test the isolates obtained from the Kimberley Thorn Bushveld soil sample in order to further explore the ability of mucoralean fungi to utilise the above mentioned series of nitrogen sources and to grow at an a_w of 0.955. In addition, selected mucoralean taxa occurring frequently in soil habitats, including strains from culture collections, as well as isolates obtained from the soil sample of Kimberley Thorn Bushveld, were tested for the ability to survive elevated temperatures in soil.

3. 2. Materials and Methods

3. 2. 1. Strains used

The fungal strains and isolates used in this study are listed in Tables 3 and 4. The strains were obtained from the Centraalbureau voor Schimmelcultures (CBS), The Netherlands, and the mucoralean culture collection of the University of the Orange

Free State (MUFS), South Africa, as well as from the National collection of Fungi (PPRI) in Pretoria, South Africa. Other strains were isolated from a soil sample originating from Kimberley Thorn Bushveld (Low & Rebelo, 1996).

3. 2. 2. Determination of fungal taxa in soil

3. 2. 2. 1. Taking of soil sample. The sampling site (10 m^2) is located at Taung in the North West Province, South Africa, amongst Kimberley Thorn Bushveld (Low & Rebelo, 1996). The mean annual rainfall is 455 mm while the mean annual temperature is 25.3 °C. The sample was taken in late summer. The surface litter was first scraped away to reduce contamination from this habitat. A soil sample of *circa* 2000 g, consisting of nine subsamples, was taken at random over the area of the site, each to a depth of 10 cm. The subsamples were thoroughly mixed in the laboratory to produce the sample, which was further processed.

3. 2. 2. 2. Chemical analyses of soil sample. The pH of the soil sample was determined according to the method of Spotts and Cervantes, (1986). The soil moisture content was determined according to the method of Eicker, (1970). The organic carbon content was determined using the Walkley-Black method as described by Barnard *et al.*, (1990). Nitrate and nitrite were determined by the Department of Soil Science at the UOFS, South Africa, using the standard soil testing methods as compiled by the Non-affiliated soil analysis work committee (1990). The ammonium content was also determined using the same methods by the Soil Science department of UOFS. The characteristics of the soil at the sampling site are summarised in Table 1.

Table 1. Chemical properties of the soil at sampling site.

CHARACTERISTICS	
pH of soil	6.06 ± 0.05
Soil moisture content	6.10 % ± 0.25 %
Organic carbon	6647.00 ppm
Total nitrogen	745.50 ppm
Nitrite & Nitrate	8.63 ppm
Ammonium	9.33 ppm

3. 2. 2. 3. Enumeration and isolation of fungi. The soil plate technique of Warcup, (1950) was used in combination with two selective media (containing benomyl and starch or sucrose as carbon source), as well as a relative non-selective medium, to isolate mucoralean fungi from the soil sample. The first selective medium contained ($g.l^{-1}$); starch, 10.00; NH_4Cl, 1.00; KH_2PO_4, 1.00; $MgSO_4.7H_2O$, 0.50; yeast extract (Difco), 0.50; and chloramphenicol, 0.20. It also contained the following mineral salts ($mg.l^{-1}$): $FeSO_4.7H_2O$, 10.00; $ZnSO_4.7H_2O$, 10.00; $MnSO_4.H_2O$, 0.80 and $CuSO_4.5H_2O$, 0.05. The medium had a pH of 5.5 and was solidified with 16 g/l agar (Biolab); 0.02 $g.l^{-1}$ benomyl (Methyl 1-(butylcarbamoyl)-2-benzimidazole-carbamate, Aldrich catalog no. 38,158-6) was added before autoclaving. The second selective medium was similar to the first, except that it contained 10 $g.l^{-1}$ sucrose as carbon source instead of starch. The relatively non-selective medium (MYPps) (Carreiro & Koske, 1992), contained ($g.l^{-1}$): Malt extract (Difco), 7.00; peptone (Oxoid), 1.00; yeast extract (Difco), 0.50; penicillin G, 0.50; streptomycin sulphate, 0.50; and agar (Biolab), 16.00.

For each medium, soil plates were prepared by transferring 0.001 g of soil from the sample to each of 10 sterile Petri dishes. Eight ml of cooled molten isolation medium (45°C) was then added to each Petri dish. The plates were incubated at 25°C in the dark and examined for growth of colonies.

Mycelium from each colony on the isolation plates, was transferred to plates with fresh 2% (w/w) MEA and incubated at 25°C. Further purification was obtained by successive subculturing of each isolate on 2% (w/w) MEA. The number of fungi in the soil sample that grew on the isolation plates were calculated as colony forming units per gram of soil ($CFU.g^{-1}$).

3. 2. 2. 4. Identification of fungi. Isolates belonging to the higher fungi were identified up to genus level using the keys of Ellis, (1976), Barron, (1983), Kreger-van Rij, (1984) and Baxter *et al.*, (1994). The mucoralean fungi were identified using the keys and descriptions given by Hesseltine & Ellis, (1973), Domsch *et al.*, (1980) and Schipper, (1984).

3. 2. 3. Physiological properties

3. 2. 3. 1. Preparation of inocula. A sterile wet inoculating loop was used for each fungal strain to transfer sporangiospores and/or hyphal fragments from a two-week-old culture on 2 % (w/w) Difco malt extract agar (MEA) to 5 ml sterile distilled water. Forty microliters of the resulting suspension, containing *circa* 2 X 10^6 colony forming units per ml, was used to inoculate each medium, in Petri dishes.

3. 2. 3. 2. Nitrogen utilisation on a solid defined medium. A series of solid defined media were prepared (Van der Walt & Yarrow, 1984). Each medium, in Petri dishes, consisted of 11.7 $g.l^{-1}$ Bacto yeast carbon base, Difco (YCB) and a different nitrogen source (Table 3), giving a final nitrogen concentration of 0.1 $g.l^{-1}$. All the media had pH values of *circa* 5.5 and were solidified with 2 % (w/w) washed purified agar. The washed agar was prepared by repetitively washing solidified agar blocks (Difco) in demineralised water (Van der Walt & Yarrow, 1984).

A superficial well of 8.0 mm in diameter was made in the center of the medium in each Petri dish, using a hot glass rod. Each Petri dish was inoculated by pipetting 40 µl of the inoculum into the well and then incubated at 25°C in the dark. The diameter of the resulting colony was measured after five days of incubation. A

control experiment was conducted in which the ability was tested to grow on a solid medium devoid of a nitrogen source. All the experiments were conducted in triplicate.

3. 2. 3. 3. Measurement of radial growth with different nitrogen sources. Radial growth, as an indication of the rate of utilisation of the nitrogen source, was measured. This was done by calculating the colony diameter obtained after five days on the medium containing the particular nitrogen source, as a percentage of the colony diameter obtained after the same period on the medium which best supported radial growth of the particular fungal strain (Table 3).

3. 2. 3. 4. Growth on a medium with a reduced water activity. A solid medium was prepared in Petri dishes. This medium consisted of the following ($g.l^{-1}$): 10.0, glucose; 5.0, peptone; 1.0, KH_2PO_4; 0.5, $MgSO_4.7H_2O$; 220.0, glycerol; 15.0, agar and 0.1, chloramphenicol. The medium had a pH of *circa* 5.6 and a final a_w of 0.955.

A superficial well in the medium was prepared and inoculated as described above. The inoculated Petri dishes were incubated at 25°C in the dark. The diameter of the resulting colony was measured after five days of incubation. To evaluate growth against a non water-stressed control, the ability of each strain to spread on an identical medium, except for the absence of glycerol, was also recorded. All the experiments were conducted in triplicate.

3. 2. 3. 5. Measurement of radial growth on medium with low a_w. Growth was measured by calculating the colony diameter obtained after five days on the medium with 0.955 a_w, as a percentage of the colony diameter obtained after the same period on the control devoid of glycerol (Table 3).

3. 2. 3. 6. Survival of mucoralean fungi in soil at elevated temperatures. Culture collection strains and isolates were allowed to grow and sporulate abundantly by cultivating them for 2 weeks on MEA at 25°C. Two loopfulls of fungal growth from each strain or isolate, were subsequently used to inoculate each of three test tubes, each containing 3 g of sterile, dried soil. The soil which was taken from the soil

sample, was previously autoclaved (15 min, 121°C), dried in an oven (100°C, 24 h) and allowed to cool down to room temperature. After inoculation, the three test tubes containing soil were incubated for 48 h at 20°C. Subsequently, two of the test tubes of each strain or isolate, were subjected to higher temperatures for 14 h by incubating one at 45°C and the other at 55°C. The third inoculated test tube remained at 20°C and served as a control to determine if the fungal spores were still viable after incubation at lower temperatures in the autoclaved soil. After incubation of the inoculated soil at the different temperatures, the viability of the fungal spores in the soil from each tube was determined, by preparing soil plates with MEA and incubating it at 20°C for 1 week.

3. 3. Results

3. 3. 1. Fungi present in the soil sample

The fungi in the soil sample from Kimberley Thorn Bushveld, which were enumerated using the three isolation media, are listed in Table 2. Strains of ten genera of higher fungi were found to occur in the soil sample. As was found in the soil sample from Upper Nama Karoo (Chapter 2), the most frequently occurring of these were *Aspergillus* and *Trichoderma*. Only two species of mucoralean fungi could be detected in the soil sample i,e. *Gongronella butleri* (Lendner) Peyronel & Dal Vesco and *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill.

Table 2. Fungi isolated from the soil sample.

	MYPps (CFU/g soil)	Med. A (CFU/g soil)	Med. B (CFU/g soil)
Mucorales			
Gongronella butleri (Lendner) Peyronel & Dal Vesco.	520	1360	1600
Rhizopus stolonifer (Ehrenb.:Fr.) Vuill.	320	1020	740
TOTAL	840	2380	2340
Genera of other fungi			
Acremonium	120	-	-
Aspergillus	740	220	220
Cladobotryum	40	-	-
Curvularia	20	-	-
Dreschlera	-	20	20
Fusarium	540	-	-
Paecilomyces	20	-	-
Penicillium	500	-	-
Sporobolomyces	100	-	-
Trichoderma	700	-	-
TOTAL	2660	240	240

CFU: Colony forming units; MYPps: Relative non-selective medium (Carreiro & Koske, 1992); Med. A: Isolation medium A containing starch as carbon source and 20 ppm benomyl; Med. B: Isolation medium B containing sucrose as carbon source and 20 ppm benomyl;

3. 3. 2. Utilisation of nitrogen sources by mucoralean isolates

The ability of mucoralean isolates from the soil sample to utilise the series of nitrogen containing compounds as sole nitrogen source is given in Table 3. As was found for the isolates from the Upper Nama Karoo soil sample (Chapter 2), all the isolates were able to utilise the amino acids, asparagine and glutamate, as well as ammonium chloride (Table 3). The isolates of *Gongronella butleri* were all able to utilise nitrate and nitrite. However, the isolates of *Rhizopus stolonifer* were unable to utilise nitrate. In addition, nitrite at the concentration used in this study was found to be toxic to the isolates of *Rhizopus stolonifer*.

3. 3. 3. Growth at 0.955 a_w by mucoralean isolates

The isolates of *Gongronella butleri* and *Rhizopus stolonifer* were able to grow at 0.955 a_w (Table 3). However, growth of the *Rhizopus stolonifer* isolates, was less susceptible to this low a_w value than the isolates of *Gongronella butleri*.

3. 3. 4. Survival of mucoralean fungi in soil at elevated temperatures.

The ability of mucoralean isolates from the soil sample to survive in soil incubated for 14h at 45°C or 55°C is listed in Table 4. All the strains and isolates were able to survive in the autoclaved soil incubated at 20°C. However, strains of *Mortierella amoeboidea*, *Mortierella globulifera*, *Mortierella turficola* and *Rhizopus microsporus* were unable to survive in the soil incubated for 14 h at 45°C or 55°C.

Table 3. The ability of mucoralean isolates from the arid soil sample to utilise the series of nitrogen containing compounds and to grow at 0.955 a_w.

Strains	* Nitrogen source utilisation					#Growth
	$NaNO_2$	KNO_3	NH_4Cl	Asn	Glu	0.955a_w
Gongronella butleri A35	+++	+++	+++	+++	+++	++
Gongronella butleri A36	+++	+++	+++	+++	+++	++
Gongronella butleri A88	+++	+++	++	+++	+++	++
Gongronella butleri B24	+++	+++	+++	+++	+++	++
Gongronella butleri B77	+++	+++	+++	+++	+++	++
Gongronella butleri B99	+++	+++	+++	+++	+++	++
Rhizopus stolonifer A4	-	0	++	+++	+++	+++
Rhizopus stolonifer A83	-	0	++	+++	+++	+++
Rhizopus stolonifer A100	-	0	++	+++	+++	+++
Rhizopus stolonifer B2	-	0	++	+++	+++	+++
Rhizopus stolonifer B21	-	0	++	+++	+++	+++
Rhizopus stolonifer B25	-	0	++	+++	+++	+++

* Nitrogen utilisation, measured by calculating the colony diameter obtained on the medium with the particular nitrogen source, as a percentage of the colony diameter on the medium which best supported radial growth of the particular fungal strain. Symbols: 0 = 0 %; + = 1 -33 %; ++ = 34 - 66 %; +++ = 67 - 100 %; - = toxic, since the colony density and diameter on the particular solid medium were less than that obtained on the medium devoid of a nitrogen source; Abbreviations: Asn = Asparagine; Glu = Sodium glutamate.

Growth at reduced a_w, measured by calculating the colony diameter on the medium with 0.955 a_w, as a percentage of the colony diameter obtained on a non water-stressed control. Symbols: 0 = 0 %; 1 -33 %; 34 - 66 %; 67 - 100 %.

Table 4. The ability of strains representing different mucoralean genera to survive in soil incubated at 45°C and 55°C for 14h.

Strains	20°C control	45°C	55°C
Actinomucor elegans (Eidam) C. R. Benj. & Hesselt. MUFS 022	+	+	+
Actinomucor elegans (Eidam) C. R. Benj. & Hesselt. MUFS 221	+	+	+
Actinomucor elegans (Eidam) C. R. Benj. & Hesselt. MUFS 229	+	+	+
Backusella lamprospora (Lendn.) Benny & R. K. Benj. MUFS 002	+	+	+
Backusella lamprospora (Lendn.) Benny & R. K. Benj. MUFS 008	+	+	+
Backusella lamprospora (Lendn.) Benny & R. K. Benj. MUFS 011	+	+	+
Cunninghamella echinulata (Thaxt.) Thaxt. MUFS 001	+	+	+
Cunninghamella echinulata (Thaxt.) Thaxt. MUFS 002	+	+	+
Cunninghamella echinulata (Thaxt.) Thaxt. MUFS 003	+	+	+
Gongronella butleri (Lendn.) Peyronel & Dal Vesco MUFS 1	+	+	+
Gongronella butleri (Lendn.) Peyronel & Dal Vesco MUFS 2	+	+	+
Gongronella butleri (Lendn.) Peyronel & Dal Vesco A35	+	+	+
Mortierella amoeboidea W. Gams CBS 889.72T	+	-	-

Table 4 continues

Strains	20 °C	45 °C	55 °C
Mortierella globulifera Rostrup CBS 417.64	+	-	-
Mortierella isabellina Oudem. CBS 208.32	+	+	+
Mortierella turficola Y. Ling-Yong CBS 430.76	+	-	-
Mucor azygosporus R. K. Benj. CBS 292.63T	+	+	+
Mucor circinelloides Tiegh. CBS 119.08	+	+	+
Mucor flavus Bainier CBS 234.35	+	-	-
Mucor plumbeus Bonord. CBS 111.07	+	+	+
Mucor racemosus Fres CBS 115.08	+	+	+
Rhizomucor pusillus (Lindt.) Schipper MUFS 001	+	+	+
Rhizomucor pusillus (Lindt.) Schipper MUFS 005	+	+	+
Rhizopus microsporus Tiegh. CBS 631.82	+	-	-
Rhizopus microsporus Tiegh. PPRI 5560	+	-	-
Rhizopus oryzae Went & Prins. Geerl. CBS 112.07	+	+	+
Rhizopus stolonifer (Ehrenb.: Fr.) Vuill.CBS 609.82	+	+	+
Rhizopus stolonifer (Ehrenb.: Fr.) Vuill.CBS 319.35	+	+	+

Table 4 continues

Strains	20 °C	45 °C	55 °C
Rhizopus stolonifer (Ehrenb.: Fr.) Vuill. B25	+	+	+
Thamnostylum piriforme (Bainier) Arx & H.P. Upadhyay MUFS 025	+	+	+

3. 4. Discussion

3. 4. 1. Fungi present in the soil sample

The occurrence of *Aspergillus* and *Trichoderma* in the soil sample (Table 2) is not surprising, since other authors have frequently found these genera in soil from arid regions (Griffen, 1972; Eicker *et al.*, 1982; Allsopp *et al.*, 1987). *Aspergillus* and *Trichoderma* were also the most dominant of the higher fungi in the soil sample from Upper Nama Karoo (Chapter 2). Similarly, *Acremonium*, *Curvularia Fusarium*, *Paecilomyces* and *Penicilium* (Table 2) were also found in the Upper Nama Karoo soil sample (Chapter 2), as well as in studies conducted by others on soil of the arid Giribes plains (Eicker *et al.*, 1982) and the soil of Dry Sandy Highveld Grassland (Strauss, 1997). Isolates representing *Cladobotryum* and *Dreschlera* have also been obtained from soil habitats (Domsch *et al.*, 1980; Barron, 1983), while *Sporobolomyces* is basidiomycetous yeast normally isolated from dead or decaying leaves (Kurtzman & Fell, 1998). It is possible that this yeast in the soil sample originated from the leaf litter that covered the soil before sampling.

The overall number of fungal genera recorded in this study (12 genera), corresponded with the 17 genera found in the soil sample of Upper Nama Karoo (Chapter 2). However, as pointed out in Chapter 2, these numbers are significantly less than the 135 genera recorded by Roux and Van Warmelo, (1997) during an extensive survey of fungi associated with plants, litter and soil in a natural Karoo

pasture. These differences are attributed to the sampling of (Chapter 2 and 3) only the fungal populations present in two soil samples without litter. In addition, methods that were selective for mucoralean fungi were applied in these studies. (Strauss, 1997).

3. 4. 2. Mucoralean fungi in soil of Karoo and other arid regions in southern Africa.

In an extensive survey conducted over five years on fungi associated with plants, litter and soil in a natural Karoo pasture near Middelburg in the Eastern Cape Province, South Africa, Roux and Van Warmelo, (1997) found six mucoralean taxa, i.e. *Actinomucor elegans*, *Cunninghamella echinulata, Mortierella, Mucor, Rhizopus stolonifer* and *Rhizomucor.* The presence of the genera *Actinomucor*, *Mortierella*, *Mucor* and *Rhizopus* in Karoo soil was subsequently confirmed in this M.Sc study when the mucoralean fungal taxa present in a soil sample from Upper Nama Karoo was investigated (Chapter 2). The apparent absence of *Cunninghamella* and *Rhizomucor* could have been because only the fungal populations in one soil sample without litter or plant material was investigated (Chapter 2). Nevertheless, although more studies are necessary, the data showed that Karoo soil may contain mucoralean fungi belonging to the genera *Actinomucor*, *Cunninghamella, Mortierella, Mucor, Rhizopus* and *Rhizomucor.*

In studies on the mycobiota of soil from the arid Giribes plains, Dry Sandy Highveld Grassland and Kimberley Thorn Bushveld (this study), it was found that in addition to *Cunninghamella*, *Mucor*, *Mortierella* and *Rhizopus,* the genera *Absidia*, *Gongronella* or *Zygorhynchus* may also occur in arid soil from other regions in southern Africa (Eicker *et al.*, 1982; Strauss, 1997). However, *Actinomucor* and *Rhizomucor* occurred in the Karoo soil samples, but up until the present, could not be detected in surveys of other arid regions in southern Africa.

It can be concluded from the data obtained in literature (Eicker *et al.*, 1982; Strauss, 1997; See also Chapter 1, "Habitats of mucoralean fungi"), that the following

mucoralean genera may occur in arid soil; *Absidia*, *Actinomucor, Cunninghamella, Gongronella, Mortierella, Mucor, Rhizopus, Rhizomucor* or *Zygorhynchus.*

3. 4. 3. Selected physiological characteristics of mucoralean fungi in arid soil.

3. 4. 3. 1. Oligotrophic growth. The ability to grow in the presence of limited nutrients is a prerequisite for fungal growth in many soils (Wainwright, 1993). It was found that *Actinomucor*, *Rhizopus stolonifer* as well as *Mucor rouxii* are capable of oligotrophic growth on silica gel medium with no added nutrients. According to literature, the concentration of soluble inorganic nitrogen (i.e. ammonium, nitrate and nitrite) in the soil samples analysed in this M.Sc. study (Chapter 2, Table 2; Chapter, Table1) would be adequate to serve as nitrogen source for oligotrophic growth by these fungi (Wainwright, 1993). This growth however, would be subjected to a number of chemical and physical factors (Jennings, 1995) including the growth limiting a_w for each fungus and the ability to utilise the particular nitrogen source (Brown, 1976; Chapter 2, Tables 1 & 4; Chapter 3, Table 3).

3. 4. 3. 2. The utilisation of nitrogen sources. In this study, 12 mucoralean fungal isolates obtained from Kimberley Thorn Bushveld and representing two species, i.e. *G. butleri* and *R. stolonifer*, were examined for the ability to utilise a series of nitrogen containing compounds (Chapter 3; Table 3). The nitrogen concentration in the media was 0.1 $g.l^{-1}$ and the series of nitrogen containing compounds were ammonium chloride, asparagine, sodium glutamate, sodium nitrite and potassium nitrate. Similar to what was found in Chapter 2 (Tables 1 & 4), all the isolates tested were able to utilise asparagine and glutamate (Chapter 3, Table 3). The results (Chapter 3, Table 3) also confirmed the findings on the utilization of nitrite and nitrate by representatives of *G. butleri* and *R. stolonifer* (Chapter 2, Tables 1 & 4). However, in contrast to the findings on the utilisation of ammonium chloride by *R. stolonifer* obtained in Chapter 2, the isolates of this species from Kimberley Thorn Bushveld, were able to utilise ammonium chloride (Chapter 3, Table 3). This may be as a result of variation in the presence of a transport mechanism for ammonium in this species (Jennings, 1995). It may also be that variation exists within the species

R. stolonifer regarding resistance of spores towards changes in pH upon germination in the ammonium-containing medium.

Taking into account the results obtained in this M.Sc. study (Chapter 2 & 3) on the ability of strains and species of nine mucoralean genera including 18 species to utilise the above mentioned series of nitrogen sources in a solid synthetic medium, the following conclusions can be drawn. The amino acids asparagine and sodium glutamate were utilised by all the strains and species tested (Chapter 2, Tables 1 & 4; Chapter 3 Table 3). However, ammonium chloride was not utilised by representatives of a number of taxa. These include *Cunninghamella, Mortierella, Mucor plumbeus*, *Rhizopus microsporus* and *Rhizopus stolonifer.* Whether this inability is a result of intolerance towards pH changes in an ammonium rich medium, or as a result of the absence of a transport system (Jennings, 1995), is unknown and should be the subject of further study. At the specific concentration used (0.1 $g.l^{-1}$ nitrogen), nitrate was not utilised by representatives of *Cunninghamella, Mortierella, Rhizomucor*, *Rhizopus microsporus* and *Rhizopus stolonifer* (Chapter 2, Tables 1 & 4; Chapter 3 Table 3). A discrepancy regarding nitrate utilisation within the genus *Rhizopus* was also noted that contradicts the findings of Inui *et al.*, (1965). In contrast to the findings of Inui *et al.*, (1965), it was found that *Rhizopus oryzae* is able to utilise nitrate in a solid synthetic medium. As already suggested in Chapter 2, this discrepancy could be as a result of different culture conditions that were used. At the concentration used in this study, nitrite was found to be toxic to representatives of *Cunninghamella, Mortierella*, *Mucor azygosporus, Rhizomucor, Rhizopus* and *Thamnostylum.* As a result of this perceived inhibitory effect, questions arise about the ability of these fungi to utilise much lower concentrations of nitrite and nitrate during oligotrophic growth. In order to understand growth of mucoralean fungi in soil habitats with low nutrient concentrations, this phenomenon should be studied further in future.

3. 4. 3. 3. The ability of mucoralean fungi to grow at 0.955 a_w. From the results depicted in this M. Sc. study (Chapter 2, Tables 1 & 4), it can be seen that within the *Mucorales*, differences exist regarding the ability to grow at 0.955 a_w. Strains of *Gongronella butleri, Mortierella spp., Mucor racemosus, Rhizomucor pusillus* and

Thamnostylum piriforme were unable to grow at this a_w value and hence were less osmotolerant than the strains of *Actinomucor elegans, Backusella lamprospora, Cunninghamella echinulata, Rhizopus spp.* and most of the *Mucor* strains.

Differences in tolerances towards low water activity may exist within a species. This is obvious when comparing the data on *A. elegans*, *G. butleri*, *M. circinelloides*, *R. microsporus* and *R. oryzae* (Chapter 2, Tables 1 & 4; Chapter 3 Table 3). These differences may indicate differences in growth patterns of the various fungi in soil after rainfall. It also seems that fungi that are intolerant towards low water activity may also occur in arid soil, e.g. *M. isabellina* (Chapter 2, Tables 4). Therefore, the presence of these fungi in the arid soil does not solely depend on the ability to grow at low a_w values. Other factors should also be investigated. One such factor is the ability to survive in the hot upper soil layer of the Karoo.

3. 4. 3. 4. Survival of mucoralean fungi in soil at elevated temperatures. Two of the mucoralean species isolated from the Upper Nama Karoo soil sample (Chapter 2), *M. isabellina* and *R. oryzae*, are known to be heat-resistant and can respectively survive temperatures of 70°C for 30 min, and 80 °C for 72 h (Domsch *et al.*, 1980). No data on heat resistance of the other mucoralean fungi were obtained from literature, since no systematic screening for survival of mucoralean taxa in soil at elevated temperatures has been attempted. Consequently, mucoralean taxa occurring frequently in soil habitats, including strains from culture collections, as well as isolates obtained from the soil sample of Kimberley Thorn Bushveld, were tested for the ability to survive at 45°C and 55°C for 14h in soil (Chapter 3; Table 4). Representatives of the following taxa were able to survive these conditions; *Actinomucor elegans*, *Backusella lamprospora, Cunninghamella echinulata, Gongronella butleri, Mucor azygosporus*, *Mucor circinelloides, Mucor plumbeus, Mucor racemosus, Rhizomucor pusillus*, *Rhizopus oryzae, Rhizopus stolonifer* and *Thamnostylum piriforme*. The only representative of *Mortierella* able to survive, was *Mortierella isabellina*, a member of *Mortieralla* subgenus *Micromucor* (Gams, 1977). Although more elaborate screenings at different temperatures and incubation times should be conducted, the results indicate that these species would be able to survive elevated temperatures in soil of hot arid regions such as the Karoo.

Interestingly, *Mortierella amoeboidea, Mortierella globulifera* and *Mortierella turficola,* all members *of Mortierella* subgenus *Mortierella* (Gams, 1977), did not survive at 45°C and 55°C for 14h in soil (Chapter 3; Table 4). Representatives of this subgenus are known to frequently occur in cold wet soil (Carreiro *et al.*, 1992; Botha *et al.*, 1999). In contrast, the numbers of these fungi in arid soil seem to be low, since none of these species were detected in neither the Upper Nama Karoo soil sample (Chapter 2; Table 3) nor the Kimberley Thorn Bushveld soil sample (Chapter 3; Table 2). The other mucoralean species that were not detected in these soil samples and failed to survive the elevated soil temperatures, were *Mucor flavus* and *Rhizopus microsporus* (Chapter 3; Table 4). Normally these species occur in habitats that are characterised by moderate temperatures (CBS, List of cultures 1994). Strains of *Mucor flavus* have frequently been isolated from dung and forest soil, while *Rhizopus microsporus* has been isolated from forest soil, stored grains and other plant material, as well as from animals (Domsch *et al.*, 1980).

3. 5. Concluding Remarks

The following species of the Mucorales may be encountered in the arid soil of the Karoo; *Actinomucor elegans*, *Cunninghamella echinulata, Mortierella isabellina, Mucor circinelloides, Rhizomucor* species, *Rhizopus oryzae and Rhizopus stolonifer.* Future surveys would reveal if genera like *Absidia, Gongronella* and *Zygorrhynchus*, which have been isolated from other arid regions, also occur in Karoo soil.

Representatives of mucoralean taxa occurring in arid soil were able to utilise organic as well as inorganic oxidised nitrogen sources. However, at the concentration tested in this study (0.1 $g.l^{-1}$ nitrogen), nitrite was found to be toxic to representatives of *Cunninghamella, Mortierella, Rhizomucor* and *Rhizopus.* Nitrate could not be utilised by *Cunninghamella, Mortierella, Rhizomucor* and *Rhizopus stolonifer.* Whether this inability to utilise inorganic nitrogen sources would prevail during oligotrophic growth in soil, remains a question to be addressed by future research.

Representatives of the above mucoralean taxa occurring in arid soil were able to survive 55°C for 14 h in soil. Although more elaborate screenings for survival at

elevated temperatures in soil must be done, the results indicate that spores of these fungi are able to survive in the hot top layer of soil in arid regions. When the conditions change to favour growth, the spores would germinate and growth would occur until the limits of growth for the particular species have been reached again.

3. 6. References

Allsopp, N., Olivier, D. L. and Mitchell, D. T. (1987). Fungal populations associated with root systems of proteaceous seedlings at a lowland fynbos site in South Africa. *S Afr J Bot* **53,** 365 – 369.

Barnard, R. O., Buys, A. J., Coetzee, J. G. K., Du Preez, C. C., Meyer, J. H., Van Der Merwe, A. J., Van Vuuren, J. A. J. and Volschenk, J. E. (1990). Handbook of standard soil testing methods for advisory purposes. Soil Sci. Soc. South Africa, P.O. Box 30030, Sunnyside, Pretoria.

Barron, G. L. (1983). The Genera of hyphomycetes from soil. Robert E. Krieger Publishing Co., Inc. Florida, U. S. A.

Baxter, A. P., Rong, I. H., Roux, C., Schutte, L. and Van Der Linde, E. (1994). Elementary keys to common fungi in South Africa. Plant Protection Research Institute, Pretoria, South Africa.

Botha, A., Paul, I., Roux, C., Kock, J. L. F., Coetzee, D. J., Strauss, T. and Maree, C. (1999). Short communication: An isolation procedure for arachidonic acid producing *Mortierella* species. *Antonie van Leeuwenhoek* (Accepted for publication).

Brown, A. D. (1976). Microbial water stress. *Bacteriol Rev* **40,** 803 – 846.

Carreiro, M. M. and Koske, R. E. (1992). Room temperature isolations can bias against selection of low temperature microfungi in temperate forest soils. *Mycologia* **84,** 886 - 900.

CBS, List of Cultures. (1994). Fungi and yeasts 33rd edition, Centraalbureau voor Schimmelcultures, Baarn/ Delft, The Netherlands.

Domsch, K. H., Gams, W. and Anderson, T-H. (1980). *Mortierella* Coemans 1863. In *Compendium of soil fungi,* Vol. 1, pp. 431 – 460. Edited by K.H. Domsch, W. Gams & T-H. Anderson. Academic Press, London.

Eicker, A. (1970). Ecological observations on soil fungi. *S Afr J Sci* **66,** 327 - 334.

Eicker, A., Theron, G. K. and Grobbelaar, N. (1982). 'n Mikrobiologiese studie van 'kaal kolle' in die Giribesvlakte van Kaokoland, S.W.A.-Namibië. *S Afr J Bot* **1,** 69 – 74.

Ellis, M. B. (1976). More dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, England.

Gams, W. (1977). A key to the species of *Mortierella*. *Persoonia* **9,** 381 - 391.

Griffen, D. M. (1972). Ecology of soil fungi. Chapman and Hall, London.

Hesseltine, C. W. and Ellis, J. J. (1973). Mucorales. In *The fungi, an advanced treatise,* Ch 11. pp. 187 - 217. Edited by G.G. Ainsworth, F.K. Sparrow & A.S. Sussman. Academic Press. Inc., Orlando.

Inui, T., Takeda, Y. and Ilzuka, H. (1965). Taxonomical studies on genus *Rhizopus*. *J Gen Appl Microbiol* **11 (supplement),** 1 – 20.

Jennings, D. H. (1995). The physiology of fungal nutrition. Cambridge University Press, Cambridge.

Kreger-Van Rij, N. J. W. (1984). The yeasts - a taxonomic study. Elsevier Science Publishers B.V., Amsterdam, The Netherlands.

Kurtzman, C. P. and Fell, J. W. (1998). The Yeasts, a taxonomic study. Fourth revised and enlarged edition. Elsevier Science Publishers B. V., Amsterdam, The Netherlands.

Low, A. B. and Rebelo, A. G. (1996). Vegetation of South Africa, Lesotho and Swaziland. Department of Environmental Affairs and Tourism, Pretoria, South Africa.

Roux, C. and Van Warmelo, K. T. (1997). A survey of the mycobiota of a natural Karoo pasture. *Bothalia* **27,2,** 167 - 183.

Schipper, M. A. A. (1984). A revision of the genus *Rhizopus* 1. The *Rh. stolonifer*-group and *Rh. oryzae*. *Stud Mycol* **25,** 1 - 19.

Spotts, R. A. and Cervantes, L. A. (1986). Populations of *Mucor piriformis* in soil of pear orchards in the Hood River valley of Oregon. *Plant disease* **70,** 935 - 937.

Steiman, R., Guiraud, P., Sage, L., Seigle-Murandi, F. and Lafond, J-L. (1995). Mycoflora of soil around the Dead Sea, I – Ascomycetes (including *Aspergillus* and *Penicillium*), Basidiomycetes, Zygomycetes. *System Appl Microbiol* **18,** 310 - 317.

Strauss, T. (1997). The isolation of gamma-linolenic acid producing mucoralean fungi. M.Sc. Thesis, Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein, South Africa.

Van Der Walt, J. P. and Yarrow, D. (1984). Methods for the isolation, maintenance, classification and identification of yeasts. In *The yeasts, taxonomic study,* pp. 45 – 104. Edited by N.J.W. Kreger-van Rij. Elsevier Science Publishers B.V., Amsterdam.

Wainwright, M. (1993). Oligotrophic growth of fungi – stress or natural state? pp. 127 – 144. Edited by D.H. Jennings. Marcel Dekker, Inc., New York.

Warcup, J. H. (1950). The soil-plate method for isolation of fungi from soil. *Nature* **166,** 117 - 118.

SUMMARY

The aim of the first part of the study was to investigate the ecological niche of mucoralean fungi in arid soil, with specific reference to the position these fungi occupy in the biogeochemical cycle of nitrogen. Consequently, selected mucoralean taxa occurring frequently in soil habitats, including strains from culture collections, as well as isolates obtained from a soil sample from arid Upper Nama Karoo, were used to evaluate *in vitro* growth to determine nitrogen sources and a_w tolerances. Nine mucoralean fungal genera including 18 species were examined for the ability to utilise a series of nitrogen containing compounds and to grow at an a_w of 0.955 on solid media. The nitrogen concentration in the media was 0.1 $g.l^{-1}$ and the series of nitrogen containing compounds were ammonium chloride, asparagine, sodium glutamate, sodium nitrite and potassium nitrate. The genera were *Actinomucor* Schostak., *Backusella* Hesselt. & J.J. Ellis, *Cunninghamella* Matr., *Gongronella* Ribaldi, *Mortierella* Coem., *Mucor* Fresen., *Rhizomucor* Lucet & Costantin., *Rhizopus* Ehrenb. and *Thamnostylum* Arx & H. P. Upadhyay. Thirty-nine fungal strains obtained from culture collections (CBS, MUFS and PPRI), as well as 12 soil isolates from the Karoo, were tested. All the species and strains tested in this study were able to utilise asparagine and glutamate. Strains belonging to *Cunninghamella*, *Mucor racemosus* Fresen., *Rhizopus microsporus* Tiegh. and *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill. were unable to utilise ammonium chloride. Strains of *Cunninghamella, Mortierella, Rhizomucor, Rhizopus microsporus* and *Rhizopus stolonifer* were unable to grow on nitrate as sole nitrogen source. Nitrite was found to be toxic to species belonging to *Cunninghamella, Mortierella*, *Rhizomucor*, *Rhizopus* and *Thamnostylum*. Members of *Gongronella*, *Mortierella*, *Mucor racemosus, Rhizomucor* and *Thamnostylum* were unable to grow at an a_w of 0.955. The aim of the second part of the study was firstly to get an indication whether the mucoralean diversity of the Karoo, as observed in the first part of the study and in the records obtainable from literature, differs from data on mucoralean diversity from other arid regions. The latter included data from literature and what could be found in a soil sample taken from Kimberley Thorn Bushveld. Secondly, the aim was to test the isolates obtained from the Kimberley Thorn Bushveld soil sample in order to further explore the ability of mucoralean fungi to utilise the above mentioned series

of nitrogen sources and to grow at an a_w of 0.955. In addition, selected mucoralean taxa occurring frequently in soil habitats were tested for the ability to survive elevated temperatures in soil. It was found that the following species of the Mucorales may be encountered in the arid soil of the Karoo; *Actinomucor elegans*, *Cunninghamella echinulata, Mortierella isabellina, Mucor circinelloides, Rhizomucor* species, *Rhizopus oryzae* Went. Prins. Geerl. and *Rhizopus stolonifer.* Future surveys would reveal if genera like *Absidia, Gongronella* and *Zygorrhynchus*, which have been isolated from arid regions, also occur in Karoo soil. Representatives of mucoralean taxa occurring in arid Karoo soil were able to utilise organic as well as inorganic oxidised nitrogen sources. However, at the concentration tested in this study, nitrite was found to be toxic to representatives of *Cunninghamella, Mortierella, Rhizomucor* and *Rhizopus.* Nitrate could not be utilised by *Cunninghamella, Mortierella, Rhizomucor* and *Rhizopus stolonifer.* Whether this inability to utilise inorganic nitrogen sources would prevail during oligotrophic growth in soil, remains a question to be addressed by future research. Representatives of the above mucoralean taxa occurring in arid soil were able to survive 55°C for 14 h in soil.

OPSOMMING

Die doel van die eerste gedeelte van hierdie studie was om die ekologiese nis van mucoraliese fungi in droë grond te bestudeer, met spesifieke verwysing na die posisie van hierdie fungi in die biogeochemiese siklus van stikstof. Uitgesoekte mucoraliese taksa wat gereeld in grondhabitatte voorkom, insluitend stamme vanuit kultuurversamelings en isolate verkry uit `n grondmonster van die droë Boonste Nama-Karoo, is gevolglik gebruik om *in vitro* groei vas te stel, ten einde stikstofbronne en $a_w$ toleransies te bepaal. Nege mucoraliese fungusgenera, wat 18 species insluit, is ondersoek vir hul vermoë om `n reeks stikstofbevattende verbindings te benut en by `n a_w van 0.955 op soliede media te groei. Die stikstofkonsentrasie in die media was 0.1 $g.l^{-1}$ en die reeks stikstofbevattende verbindings was ammoniumchloried, asparagien, natriumglutamaat, natriumnitriet en kaliumnitraat. Die genera was *Actinomucor* Schostak., *Backusella* Hesselt. & J.J. Ellis, *Cunninghamella* Matr., *Gongronella* Ribaldi, *Mortierella* Coem., *Mucor* Fresen., *Rhizomucor* Lucet & Costantin., *Rhizopus* Ehrenb. en *Thamnostylum* Arx & H. P. Upadhyay. Nege-en-dertig fungusstamme wat uit kultuurversamelings (CBS, MUFS and PPRI) verkry is, asook 12 grondisolate uit die Karoo, is getoets. Al die species en stamme wat in hierdie studie getoets is, kon asparagien en glutamaat benut. Stamme wat tot *Cunninghamella, Mucor racemosus* Fresen., *Rhizopus microsporus* Tiegh. en *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill. behoort het, was nie in staat om ammoniumchloried te benut nie. Stamme van *Cunninghamella, Mortierella, Rhizomucor, Rhizopus microsporus* en *Rhizopus stolonifer* kon nie op nitraat as enigste stikstofbron groei nie. Nitriet is gevind om toksies te wees vir species wat behoort tot *Cunninghamella, Mortierella*, *Rhizomucor*, *Rhizopus* en *Thamnostylum*. Lede van *Gongronella*, *Mortierella*, *Mucor racemosus, Rhizomucor* en *Thamnostylum* kon nie by `n $a_w$ van 0.955 groei nie. Die doel van die tweede deel van die studie was eerstens om `n aanduiding te kry of die mucoraliese diversiteit van die Karoo, soos waargeneem in die eerste gedeelte van die studie en ook uit verslae wat uit die literatuur beskikbaar is, verskil van data oor mucoraliese diversiteit in ander droë streke. Laasgenoemde sluit data in uit die literatuur en wat gevind kon word in `n grondmonster wat geneem is in Kimberley Doring-Bosveld. Die doel was tweedens om die isolate wat uit die Kimberley Doring-Bosveld monster

verkry is, te toets ten einde die vermoë van mucoralies fungi om die bogenoemde reeks stikstofbronne te benut en by `n a_w van 0.955 te groei verder te ondersoek. Mucoraliese taksa wat dikwels in grondhabitatte voorkom, is verder getoets vir hul vermoë om verhoogde grondtemperature te oorleef. Dit is gevind dat die volgende Mucorales-species in die droë grond van die Karoo aangetref mag word; *Actinomucor elegans*, *Cunninghamella echinulata, Mortierella isabellina, Mucor circinelloides, Rhizomucor* species, *Rhizopus oryzae* (Went. Prins. Geerl.) en *Rhizopus stolonifer.* Toekomstige opnames sal onthul of genera soos *Absidia, Gongronella* en *Zygorrhynchus,* wat ook uit droë streke geïsoleer is, ook in Karoo-grond voorkom. Verteenwoordigers van mucoraliese taksa wat in droë Karoo-grond voorkom, was in staat om organiese asook anorganiese geoksideerde stikstofbronne te benut. Teen die konsentrasie wat in hierdie studie getoets is, is nitriet egter gevind om toksies te wees vir verteenwoordigers van *Cunninghamella, Mortierella, Rhizomucor* en *Rhizopus.* Nitraat kon nie deur *Cunninghamella, Mortierella, Rhizomucor* en *Rhizopus stolonifer* benut word nie. Of hierdie onvermoë om anorganiese stikstofbronne te benut standhou tydens oligotrofiese groei in grond, is `n vraag wat deur toekomstige navorsing aangespreek moet word. Verteenwoordigers van bogemelde mucoraliese taksa wat in droë grond voorkom, was in staat om vir 14 h in grond met `n temperatuur van 55 °C te oorleef.

Keywords: Mucorales; soil; arid regions; Karoo; nitrogen utilisation; fungi; water activity; Kimberley Thorn Bushveld.