Lipid metabolism in *Mucor genevensis* and related species

by

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Vir my ouers,

baie dankie vir al julle liefde en ondersteuning

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Chapter 1

Introduction and literature review

1.1 Motivation

For as long as man can remember, mucoralean fungi have been used in the preparation of fermented foods, especially in the East (Wagenknecht *et al.* 1961; Sorenson & Hesseltine 1966; Hesseltine & Ellis 1973; VanDemark & Batzing 1987; Nahas 1988). More recently scientists realised the biotechnological potential of these fungi in the production of alcohol, certain enzymes and in the biotransformation of particular molecules (Hesseltine & Ellis 1973; Streekstra 1997).

A significant contribution to modern biotechnology was the utilisation of mucoralean fungi in the production of high value oils containing dietetically important long-chain polyunsaturated fatty acids (PUFAs) (Rattray 1984; Shimizu *et al.* 1988; Shimizu *et al.* 1989a; Shimizu *et al.* 1989b; Nakajima & Sano 1991; Kendrick & Ratledge 1992a; Shinmen *et al.* 1992; Bajpai & Bajpai 1993; Kennedy *et al.* 1993; Gill & Valivety 1997a). The production of γ-linolenic acid [18:3(ω6)], arachidonic acid [20:4(ω6)] and eicosapentaenoic acid [20:5(ω3)] by strains representing the genera *Mucor* and *Mortierella,* growing on substrates with carbohydrates as carbon sources, was intensively studied (Hansson & Dostalek 1988; Sajbidor *et al.* 1988; Tsuchiura & Sakura 1988; Shimizu *et al.* 1988; Sajbidor *et al.* 1990; Lindberg & Hansson 1991; Shinmen *et al.* 1992; Kock & Botha 1993; Roux *et al.* 1994; Du Preez *et al.* 1995; Kock & Botha 1995; Botha *et al.* 1997a; Botha *et al.* 1997b). Production of fungal oils rich in 18:3(ω6) commenced in Yorkshire, England (Ratledge 1994). The process, operated by J & E Sturge (Ltd.), used a strain of *Mucor circinelloides* grown in a 220 m^3 stirred tank fermenter. *Mortierella isabellina* is used in Japan for the production of 18:3(ω6) (Du Preez *et al.* 1995). Currently, *Mortierella alpina* is being intensively

studied as a potential commercial source of 20:4(ω6) (Streekstra 1997). Interestingly, Rhône-Poulenc, Gist Brocades, Suntory, Idemitsu, Martek Biosciences Corporation and Lion are also working towards the commercial production of lipids rich in 18:3(ω6), 20:4(ω6) and docosahexaenoic acid [22:6(ω3)] using the genera *Mucor* and *Mortierella* (Gill & Valivety 1997a; Certik & Shimizu 1999a; Certik & Shimizu 1999b). Martek Biosciences Corporation and Huntington Life Sciences also conducted toxicity studies on a fungal oil (ARASCO^{R}) enriched with 20:4(ω6) (Koskelo *et al.* 1997). The results obtained from these studies were very favourable regarding toxicity as well as psychological and behavioural consequences.

A recent development in the field of fungal lipid biotechnology is the inclusion of carbon sources other than carbohydrates in growth media. Acetic acid, present in industrial effluents, was used as carbon source for the production of 18:3(ω6) by a number of mucoralean fungi (Tsuchiura & Sakura 1988; Kock & Botha 1993; Du Preez *et al.* 1995; Kock & Botha 1995; Jeffery *et al.* 1999). Other alternative carbon sources, targeted for this purpose, are vegetable and fish oils, containing PUFAs (Shimizu *et al.* 1989a; Jacob & Krishnamurthy 1990; Aggelis *et al.* 1991a; Aggelis *et al.* 1991b; Nakajima & Sano 1991; Shinmen *et al.* 1992; Aggelis *et al.* 1995; Kendrick & Ratledge 1996; Aggelis & Sourdis 1997; Certik *et al.* 1997).

To fully understand the process whereby PUFAs are taken up by mucoralean fungi and used as carbon sources or as precursors for other, higher value PUFAs or oxidised fatty acids, fundamental research is necessary. Although the uptake and oxidation of PUFAs, such as 20:4(ω6), have been studied intensively in higher fungi (Heinz *et al.* 1970; Musallam & Radwan 1990; Tan & Ho 1991; Coetzee *et al.* 1992; Kock *et al.* 1992; Kock & Ratledge 1993; Botha *et al.* 1994; Kock *et al.* 1997; Venter *et al.*1997), relatively little knowledge is available on the incorporation and oxidation of exogenous PUFAs by mucoralean fungi (Akpinar *et al.* 1998).

Consequently, the aim of this study became:

1. To investigate the changes in endogenous lipids during growth and development of *Mucor genevensis*, a representative of the order Mucorales.
2. To investigate the uptake and incorporation of 20:4(ω6) into the various lipid fractions of *Mucor genevensis* and another mucoralean fungus, *Rhizopus oryzae*.
3. To investigate the formation of oxidised products, such as hydroxy fatty acids, from exogenous PUFAs by *Mucor genevensis* and other mucoralean fungi.
4. To determine the location and possible role of oxidised fatty acids in *Mucor genevensis*.

1.2 Lipid metabolism

In order to fully understand the results of the above mentioned study, an overview of the lipid metabolism in eukaryotes, especially in fungal cells, is necessary. We may start this overview by asking the question: What are lipids and long-chain fatty acids?

Lipids are structural, storage and regulatory molecules present in all living cells (Mathews & Van Holde 1990). These molecules are sparingly soluble in water, but readily soluble in chloroform, hexane and other organic solvents (Ratledge & Wilkinson 1988a). There are two classes of lipids: those that consist of isoprene units (i.e. carotenoids and steroids) and those that contain long-chain fatty acids.

A long-chain fatty acid consists of a carbon chain with a methyl group at the ω-end and a carboxylic acid group at the α-end (Ratledge & Wilkinson 1988b). A PUFA contains more than one double bond in its carbon chain (Schweizer 1989). Within cells, fatty acids are mainly found esterified to a glycerol molecule, as part of the glycolipids (Fig. 1), phospholipids (Fig. 2) and neutral lipids. The neutral lipids consist of monoacylglycerols, diacylglycerols (DAGs), triacylglycerols (TAGs) (Fig. 3) as well as free long-chain fatty acids (Ratledge 1994). All these molecules are synthesised in the cell and the next part of this overview deals with the synthesis and desaturation of fatty acids, the building blocks of these complex molecules.

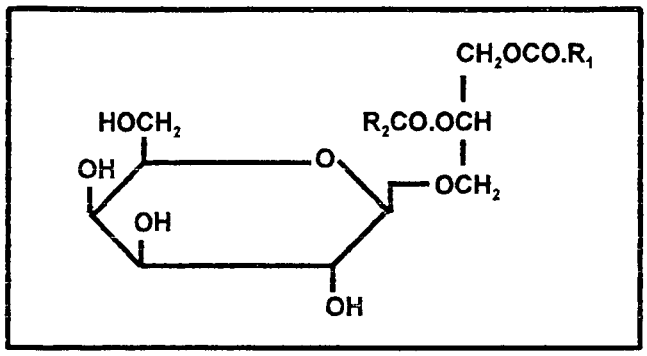
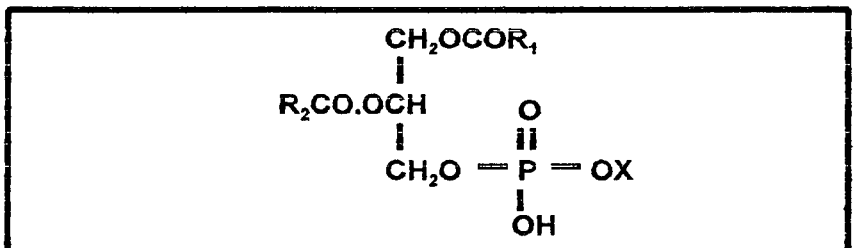


Fig. 1. A typical glycolipid (Ratledge & Wilkinson 1988b). R_1CO- and R_2CO- represent fatty acyl groups.



Phosphatidic acid:	X = H
Phosphatidylcholine:	X = $(CH_2)_2N(CH_3)_3$
Phosphatidylethanolamine:	X = $(CH_2)_2NH_2$
Phosphatidylinositol:	X = $(HCOH)_6$
Phosphatidylserine:	X = $CH_2CH(NH_2)CO_2H$

Fig. 2. The general structure and types of phospholipids found in fungi (Ratledge & Wilkinson 1988b). R_1CO- and R_2CO- represent fatty acyl groups; X- represents any of the indicated functional groups.

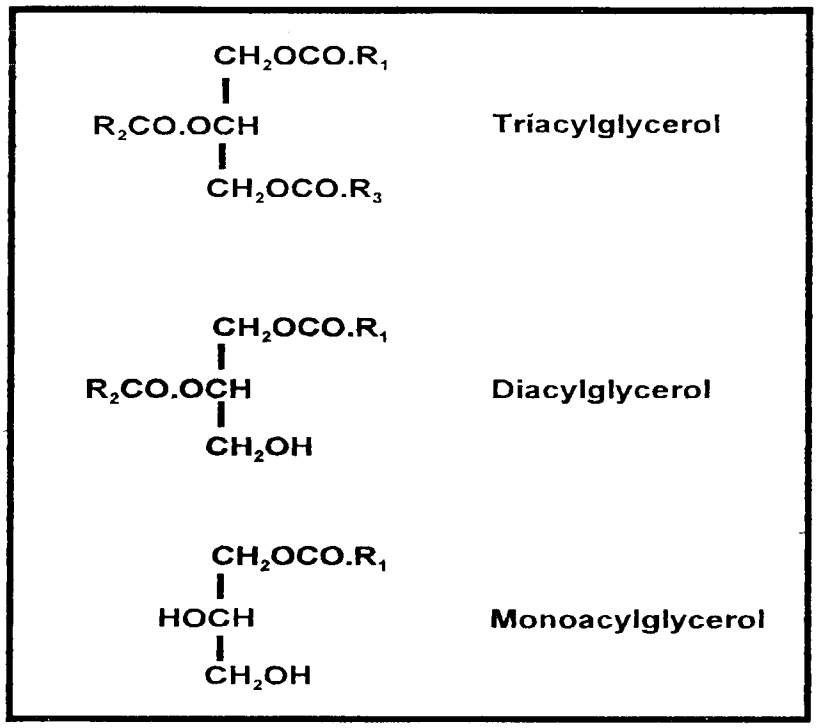


Fig. 3. Neutral lipids, represented by triacyl-, diacyl- and monoacylglycerol (Ratledge & Wilkinson 1988b). R_1CO-, R_2CO- and R_3CO- represent fatty acyl groups.

1.2.1 Fatty acid synthesis

The pathway for *ab initio* production of fatty acids has been elucidated by, among others, Ratledge (1994) and Certik and Shimizu (1999a, 1999b) (Fig. 4). Fatty acid synthesis is initiated when pyruvate is transported into the mitochondrion. There, pyruvate is transformed by pyruvate dehydrogenase to acetyl-CoA and by pyruvate carboxylase to oxaloacetate. Citrate synthetase, an enzyme in the citric acid cycle, then condenses acetyl-CoA and oxaloacetate to produce citrate (Aggelis 1996), which is the substrate for an aconitase enzyme, yielding isocitrate.

In oleaginous fungi, an isocitrate dehydrogenase, sensitive to adenosine monophosphate (AMP) levels, catalyses the production of α-keto-gluterate, which is

further metabolised in the citric acid cycle, yielding reduced coenzymes and ultimately adenosine triphosphate (ATP) (Mathews & Van Holde 1990). If the AMP concentration declines, due to a reduction in the levels of intracellular nitrogen (Botham & Ratledge 1979; Ratledge 1994), the activity of the AMP sensitive isocitrate dehydrogenase is reduced. As a result, the citrate concentration in the mitochondrion increases and malate (the precursor of oxaloacetate) is no longer produced from α-keto-gluterate in the citric acid cycle. Under these conditions, citrate is transported out of the mitochondrion into the cytoplasm (Aggelis 1996), where it is cleaved by ATP:citrate lyase, yielding acetyl-CoA and oxaloacetate (Botham & Ratledge 1979). The transformation of oxaloacetate to malate is catalysed by malate dehydrogenase (Ratledge 1989). In the cytoplasm, malate acts as a counter-ion for citrate transport out of the mitochondrion. In addition, the malic enzyme catalyses the formation of pyruvate and NADPH from malate and NADP.

The acetyl-CoA, produced by ATP:citrate lyase, is transformed to malonyl-CoA by the enzyme, acetyl-CoA carboxylase (Ratledge 1989). Malonyl-CoA and acetyl-CoA are substrates for the fatty acid synthetase complex, which catalyses the formation of acyl-CoA, utilising reducing power (NADPH) obtained from the action of the above mentioned malic enzyme.

Two acyl groups, originating from two acyl-CoA molecules, are esterified to a glycerol-3-phosphate molecule, resulting in the formation of phosphatidic acid. Phosphatidic acid may be transformed to different phospholipid molecules in the membrane or may be catalysed to a DAG by the action of phosphatase (Lösel 1988). This DAG may then act as a precursor for either phospholipid or TAG synthesis (Pieringer 1989; Mathews & Van Holde 1990). Furthermore, it is known that TAGs, through the action of lipases, may act as precursors for phospholipids via DAGs (Finnerty 1989).

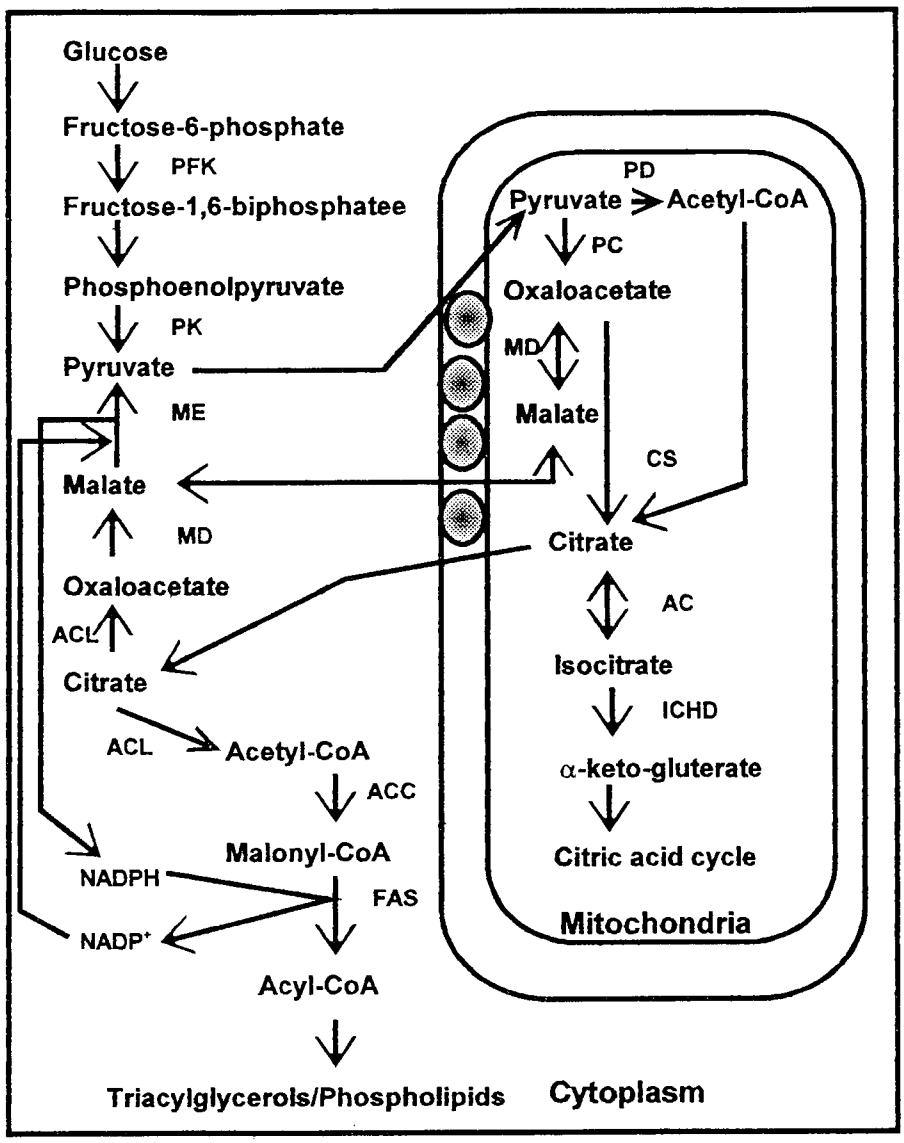


Fig. 4. Anabolic pathway for lipid production (Ratledge 1989).

AC = aconitase; ACC = acetyl-CoA-carboxylase; ACL = ATP:citrate lyase; CS = citrate synthetase; FAS = fatty acid synthetase complex; ICHD = isocitrate dehydrogenase; MD = malate dehydrogenase; ME= malic enzyme; PC = pyruvate carboxylase; PD = pyruvate dehydrogenase; PFK = phosphofructokinase; PK = pyruvate kinase

1.2.2 Fatty acid desaturation

The products of the fatty acid synthetase complex in fungi [i.e stearic acid (18:0) and palmitic acid (16:0)] (Schweizer 1989) are desaturated in the membranes (Kendrick & Ratledge 1992b; Certik & Shimizu 1999a; Certik & Shimizu 1999b) (Fig. 5) where they form part of phospholipid molecules, as explained under "1.2.1 Fatty acid synthesis". The desaturase enzymes, involved in these reactions, require molecular oxygen and NADPH (Kendrick & Ratledge 1992b).

Stearic acid is desaturated by Δ9 desaturase to produce oleic acid [18:1(ω9)], which may be used to produce the ω9 series of PUFAs, up to dihomo-γ-linolenic acid [20:3(ω9)] (Ratledge 1994). This pathway was discovered in a *Mortierella alpina* mutant, lacking Δ12 desaturase activity needed to transform 18:1(ω9) to linoleic acid [18:2(ω6)]. This mutant used Δ6 desaturase, usually involved in the formation of 18:3(ω6), to desaturate 18:1(ω9) to 18:2(ω9). Linoleic acid was elongated and further desaturated by Δ5 desaturase to yield 20:3(ω9). Alternatively, 18:1(ω9) may act as the precursor for the synthesis of 18:2(ω6) through the action of Δ12 desaturase. Linoleic acid may, in turn, act as precursor for the synthesis of the ω6-series of PUFAs, up to 20:4(ω6) or the ω3-series up to 20:5 (ω3) and 22:6(ω3). Since the enzymatic activity of all organisms vary depending on age and development, it stands to reason that changes in the lipid composition are also closely related to growth and development of organisms, including fungi. The next part of the overview deals with these changes that occur in fungal lipids.

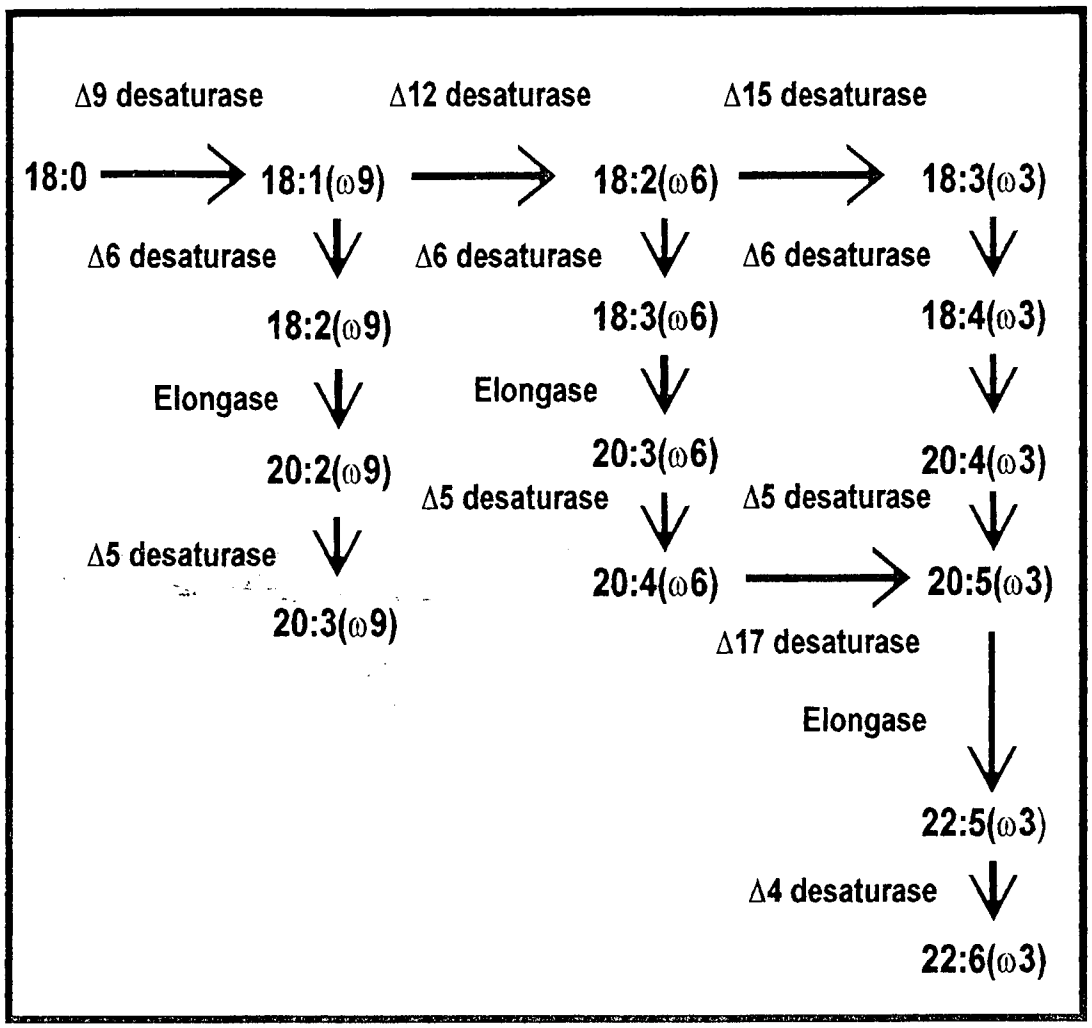


Fig. 5. Desaturation of fatty acids to produce the ω9-, ω6- and ω3-series of PUFAs (Ratledge 1994).

18:0 = stearic acid; 18:1(ω9) = oleic acid; 18:2(ω9) = 6,9-octadecadienoic acid; 20:2(ω9) = 8,11-eicosadienoic acid; 20:3(ω9) = 5,8,11-eicosatrienoic acid; 18:2(ω6) = linoleic acid; γ-linolenic acid; 20:3(ω6) = dihomo-γ-linolenic acid; 20:4(ω6) = arachidonic acid; 18:3(ω3) = α-linolenic acid; 20:4(ω3) = 8,11,14,17-eicosatetraenoic acid; 20:5(ω3) = 5,8,11,14,17-eicosapentaenoic acid; 22:5(ω3) = 7,10,13,16,19-docosapentaenoic acid; 22:6(ω3) = 4,7,10,13,16,19-docosahexaenoic acid

1.2.3 Lipid changes during growth and development

1.2.3.1 Changes in lipid content

Changes in lipid content during development and growth have been studied in a number of fungal strains. One of the most intensively studied of these fungi, is the protoctistan fungus, *Blastocladiella emersonii*.

The life-cycle of *B. emersonii* may be divided into four stages (Smith & Silverman 1973). The first stage is the free swimming zoospores, which germinate and produce vegetative cells that mature and sporulate. The lipid content of the zoospores was determined by Mills and Cantino (1974). They found that the ungerminated zoospores contained *circa* 11 % (w/w) lipids. This was comparable to the lipid content of *circa* 10 % (w/w) found in ungerminated zoospores of an *Achlya* species (Law & Burton 1976) and the *circa* 11 % (w/w) in the ungerminated ascospores of *Dipodascopsis tóthii* (Jansen van Vuuren *et al.* 1994).

Because the zoospores lack cell walls and must expend energy to maintain osmotic balance and motility (Suberkropp & Cantino 1973; Grant *et al.* 1988), the neutral lipids were the first and major lipid fraction utilised as energy source during swimming (Mills *et al.* 1974). It was found that the percentage neutral lipids decreased during the first five hours of swimming, after which the levels remained constant and the phospholipid fraction decreased as this fraction was used as energy source. The utilisation of phospholipids as energy source by zoospores was also observed for the protoctistan fungus, *Phytophthora capsici* (Gay *et al.* 1971).

Encystment of the zoospore heralds the start of the germination phase (Smith & Silverman 1973). Mills and co-workers (1974) reported that the glycolipid fraction decreased sharply during this stage. These authors suggested that this fraction may play an important role during encystment as it is a major constituent of the gamma particle, an organelle involved in cyst wall synthesis. During this phase, the neutral

lipids remained constant and the phospholipids increased.

Smith and Silverman (1973) reported a decrease in all three the lipid fractions as germination continued. This decrease of lipids during spore germination was also observed in an *Achlya* species, *Aspergillus nidulans*, *Dipodascopsis uninucleata*, *Stemphylium sarcinaeforme and Tilletia caries* (Weber & Hess 1974; Law & Burton 1976; Murray & Maxwell 1976; Weber & Trione 1980; Kock & Ratledge 1993). Shu and co-workers (1956), Murray and Maxwell (1976) as well as Weber and Trione (1980) suggested that the lipids were used as energy source during germination.

During the vegetative growth phase of *Blastocladiella emersonii*, phospholipids were formed as membranes were synthesised (Smith & Silverman 1973). This phenomenon was also demonstrated by authors working with *Achlya, Dipodascopsis tóthii, Dipodascopsis uninucleata, Phytophthora palmivora, Rhizopus arrhizus* and *Schizosaccharomyces pombe* (Weber & Hess 1974; Law & Burton 1976; Grant *et al.* 1988; Kock & Ratledge 1993; Jansen van Vuuren 1994; Jeffery *et al.* 1995). Before sporulation of *Blastocladiella emersonii*, the lipid composition of cells approached that of the spores i.e. a decrease in phospholipids and an increase in neutral and glycolipids (Smith & Silverman 1973).

1.2.3.2 Changes in cellular long-chain fatty acids

Changes in long-chain fatty acid composition during growth, were studied in strains representing *Acremonium persicinum, Agaricostilbum palmicolum, Candida albicans, Conidiobolus cornatus, Cunninghamella elegans, Debaryomyces vanrijiae, Dipodascopsis uninucleata, Endomyces fibuliger, Filobasidiella neoformans, Malassezia furfur, Metschnikowia reukaufii, Microdochium bolleyi, Microsporum canis, Penicillium camembertii, Penicillium paraherquei, Penicillium roqueforti, Phialophora verrucosa, Phytophthora palmivora, Rhizomucor pusillus, Rhodotorula glutinis, Rhodotorula gracilis, Saccharomyces cerevisiae, Saccharomycodes ludwigii, Sphacelotheca reiliana, Sporothrix schenckii, Tolyposporium ehrenbergii, Tremella*

aurantia and *Wangiella dermatitidis* (Farag *et al.* 1983; Viljoen *et al.* 1986; Smit *et al.* 1987; Grant *et al.* 1988; Kock 1988; Jacob & Krishnamurthy 1990; Kock & Ratledge 1993; Lomascolo *et al.* 1994; Van der Westhuizen *et al.* 1994; Stahl & Klug 1996).

Farag and co-workers (1983) studied the influence of culture age on cellular long-chain fatty acid composition in *Sphacelotheca reiliana* and *Tolyposporium ehrenbergii* in lipid free media, containing different carbon and nitrogen sources. These authors compared the relative percentages of fatty acids present in the total lipids of cultures, after one and two weeks of incubation at 30°C. The long-chain fatty acids studied, were 16:0, palmitoleic acid [16:1(ω7)], margaric acid (17:0), 18:0, 18:1(ω9), 18:2(ω6), α-linolenic acid [18:3(ω3)] and eicosenoic acid [20:1(ω9)]. Depending on the particular carbon and nitrogen sources in the medium, differences were observed between the long-chain fatty acid composition of one and two week old cultures of *Sphacelotheca reiliana* and *Tolyposporium ehrenbergii* (Farag *et al.* 1983).

From the results obtained by Farag and co-workers (1983), it was concluded that in order to compare the effect of culture age on cellular fatty acid composition of different fungal cultures, standardised culture conditions (e.g. carbon source and nitrogen source) should be used. The results obtained by other workers supported the conclusion that culture conditions have an impact on the fatty acid composition of fungal cultures (Lösel 1988).

Viljoen and co-workers (1986) determined the effect of culture age on strains representing *Debaryomyces vanrijiae, Endomyces fibuliger, Metschnikowia reukaufii* and *Saccharomycodes ludwigii* in a synthetic, liquid medium, containing glucose as carbon source, incubated at 30°C for 16 hours. A high degree of variation in the relative percentages of long-chain fatty acids was observed during the exponential growth phase and early stationary phase of all the strains. In cultures of *D. vanrijiae*, these phases were characterised by a sharp decrease in the percentage 18:2(ω6) and a sharp increase in the percentage 18:0. The percentage 16:0 in cultures of *D. vanrijiae* increased during exponential growth and early stationary phase, while the

percentages of both 16:1(w7) and 18:3(w3) decreased during these phases. However, the levels of all fatty acids were constant and reproducible during late stationary phase.

The levels of cellular fatty acids [i.e. 16:0, 16:1(ω7), 18:0, 18:1(ω9) and 18:2(ω6)] in cultures of *Endomyces fibuliger,* remained relatively constant during growth (Viljoen *et al.* 1986). The fatty acids of *Metschnikowia reukaufii* were also constant during growth, except for 18:1(ω9) and 18:2(ω6), which demonstrated increases during the exponential phase. Similarly, the levels of the cellular fatty acids of *Saccharomycodes ludwigii* cultures were relatively constant during growth, except for 16:1(ω7), which showed a decrease, followed by an increase to a level slightly lower than the original.

Smit and co-workers (1987) examined the relative percentages of the total cellular fatty acids present in growing and stationary phase cultures of the basidiomycetous yeasts, *Agaricostilbum palmicolum, Filobasidiella neoformans* and *Tremella aurantia,* cultivated at 22°C in a synthetic medium with glucose as carbon source. These yeasts contained mainly 16:0, 18:0, 18:1(ω9) and 18:2(ω6). *Agaricostilbum palmicolum* also contained small amounts of myristic acid (14:0) and 16:1(ω7). The percentages of these two fatty acids as well as that of 16:0, remained constant during the incubation period. During the growth phase the percentage 18:2(ω6) decreased, while the percentages 18:0 and 18:1(ω9) increased. During stationary phase the levels of the different fatty acids in *A. palmicolum* remained constant.

In addition to 16:0, 18:0, 18:1(ω9) and 18:2(ω6), cultures of *Filobasidiella neoformans* contained trace amounts of 14:0, which remained constant during the growth and stationary phases (Smit *et al.* 1987). Most of the changes in percentage long-chain fatty acids in this yeast, occurred during the growth phase, with 18:1(ω9) and 18:2(ω6) showing the most variation. During the initial growth phase, the percentage 18:1(ω9) decreased, while the percentage 18:2(ω6) increased. During mid-exponential growth, the percentage 18:1(ω6) showed a marked increase with a

concomitant decrease in the level of 18:2(ω6). During the whole of the growth phase, the percentage 18:0 showed a slight increase. As the cells entered stationary phase, the levels of the different fatty acids became remarkably constant.

Similar changes in cellular fatty acid composition to those observed in *Filobasidiella neoformans*, were observed in growing cultures of *Tremella aurantia* (Smit *et al.* 1987). In addition to 16:0, 18:0, 18:1(ω9) and 18:2(ω9), cultures of *T. aurantia* contained trace amounts of 14:0 and 16:1(ω7). The levels of 14:0, 16:1(ω7) and 18:0 remained constant throughout incubation, while the percentages of 18:1(ω9) and 18:2(ω6) showed the same pattern of increase and decrease as in *F. neoformans* during growth. The relative percentages of all the fatty acids remained unchanged during the stationary phase of the culture.

Using gas chromatography-mass spectrometry, Grant and co-workers (1988) examined the long-chain fatty acid content of zoospores and mycelium of the protoctistan fungus, *Phytophthera palmivora,* grown in complex media. They found that the main long-chain fatty acid present in zoospores, was 20:4(ω6) and that arachidic acid (20:0) and 20:1(ω9) were also present at lower concentrations. In addition, two unidentified PUFAs with 22 carbon atoms each, were detected in the zoospores. The levels of all these fatty acids decreased with 40 % during the 20 minutes of cyst formation and subsequent germination. The zoospores and cysts contained higher levels of 18:1(ω9) and 18:2(ω6) than the mycelium. Consequently, it was suggested that these PUFAs may have a specific, but as yet unelucidated function in zoospores.

Definite patterns of increase and decrease in the relative percentages of certain long-chain fatty acids were recorded during growth of the oleaginous yeast, *Rhodotorula gracilis,* in a complex medium (Jacob & Krishnamurthy 1990). During exponential growth, the percentage 16:0 showed a decrease, which was followed by an increase at the onset of the stationary phase. Similarly, during exponential growth, the relative percentage 18:1(ω9) decreased, followed by an increase at the onset of the

stationary phase. During exponential growth, the percentage 18:2(ω6) increased. However, the percentage of this fatty acid decreased at the onset of the stationary phase and remained at this level up to the end of the incubation period.

Kock and Ratledge (1993) studied changes in the relative percentage 16:0, 16:1(ω7), 18:0, 18:1(ω9), 18:2(ω6) and 18:3(ω3) during growth and development of *Dipodascopsis uninucleata,* incubated at 30°C in a complex medium. Growth of this fungus is characterised by consecutive sexual and asexual phases. The sexual phase encompasses the production of gametangia and subsequent conjugation as well as the formation of elongated asci, while ascospore germination and hyphal growth occur during the asexual phase. When the relative percentages of the fatty acids in the neutral, glyco- and phospholipid fractions of the culture were determined, the following results were obtained. The percentage 18:1(ω9) in all three lipid fractions decreased during germination of the ascospores and early vegetative growth. At the onset of the sexual phase and ascosporogenesis, the percentage 18:1(ω9) increased in all the lipid fractions. The percentage 18:2(ω6) and 18:3(ω3) in all three lipid fractions increased during germination of the ascospores. At the start of the sexual phase, a decrease in the percentages 18:2(ω6) and 18:3(ω3) occurred in all three lipid fractions.

In contrast with the above, no changes in the relative percentages of the cellular long-chain fatty acids {i.e. 16:0, 16:1(ω7), 18:0, 18:1(ω9), 18:2(ω6), 18:3(ω3), 18:3(ω6), 20:1(ω9) and eicosadienoic acid [20:2(ω6)]} were observed in cultures of *Penicillium camembertii* (Lomascolo *et al.* 1994). In this case, the fungus was cultivated at 15°C in a synthetic medium, devoid of fatty acids, containing glucose as carbon source. The only change observed during growth of *Penicillium roqueforti* in the same medium, was in the percentage of 18:1(ω9) relative to the other long-chain fatty acids. The percentage 18:1(ω9) in the neutral and polar lipids increased. The other long-chain fatty acids were not affected by culture age.

Stahl and Klug (1996) determined the changes in fatty acid composition of *Acremonium persicinum, Microdochium bolleyi* and *Penicillium paraherquei* after three, four and five days of growth on a complex medium at room temperature. The fatty acids present in *A. persicinum* and *M. bolleyi* were pentadecanoic acid (15:0), 16:0, heptadecenoic acid [17:1(ω7)], 18:0, 18:1(ω9) and 18:2(ω6). *Penicillium paraherquei* contained these fatty acids as well as 14:0, 16:1(ω7) and 17:0. After four days, trace amounts of lauric acid (12:0) could also be detected in *P. paraherquei*. The percentage 14:0 in this fungus remained constant during the six days of incubation. The percentage 16:1(ω7) in cultures of *P. paraherquei* showed a slight increase over the same incubation period. For all three fungi, it was observed that the level of 18:1(ω9) increased slightly with age and that the level of 18:2(ω6) decreased slightly.

From the results recorded in literature, it may be concluded that, depending on the fungal species and the culture conditions, the cellular long-chain fatty acid composition of a fungal culture goes through a number of changes during exponential growth. The fatty acid composition usually stabilised as the culture enters stationary growth phase. Consequently, the results of fatty acid analyses performed while cultures are in stationary growth phase, are more reproducible than results obtained from cultures in the exponential growth phase. This phenomenon may be explained by the fact that the neutral lipid content increases and the glyco- and phospholipids decrease as the cultures enter the stationary growth phase (Taylor & Parks 1979; Du Preez *et al.* 1995; Jeffery *et al.* 1995). It has also been determined that the long-chain fatty acid composition of the neutral lipids remained relatively stable during growth (Du Preez *et al.* 1995; Jeffery *et al.* 1995). It may be these long-chain fatty acids, present in such constant and high levels in the total lipids during the stationary growth phase, that are observed. In addition, the stable long-chain fatty acid composition may be attributed to a general decrease in cellular metabolism, including lipid synthesis and fatty acid desaturation during the stationary phase (Kock & Ratledge 1993).

It is important to note that, when studies are conducted on the cellular fatty acid composition of fungal cultures, the media should be devoid of fatty acids (Kendrick & Ratledge 1996; Certik *et al.* 1997). This is to ensure that exogenous fatty acids, which may be incorporated into the cellular lipids, do not distort the intrinsic fatty acid profiles of the cultures. The uptake and incorporation of exogenous fatty acids by fungi is the subject of the next part of this overview on fungal lipid metabolism.

1.2.4 Exogenous lipid utilisation

Kendrick and Ratledge (1996) reported that exogenous lipids in media repress fatty acid desaturation and elongation in filamentous fungi. This results in the phenomenon of cellular lipids mirroring the composition of the exogenous lipids (Ratledge 1989). However, there are some reports of fungi capable of utilising exogenous fatty acids as precursors for the synthesis of longer chain fatty acids with a higher degree of unsaturation (Shimizu *et al.* 1989a; Shimizu *et al.* 1989b; Shinmen *et al.* 1989; Shinmen *et al.* 1992; Aggelis *et al.* 1991b; Bajpai & Bajpai 1993; Aggelis *et al.* 1995; Certik *et al.* 1997).

1.2.4.1 Lipase production

The first step in lipid utilisation by microorganisms is the production of extracellular lipases (triacylglycerol hydrolases) which hydrolyse acylglycerol ester bonds, releasing the fatty acid moieties from the glycerol molecule (Akhtar *et al.* 1983; Ratledge 1984; Ratledge 1989; Aggelis *et al.* 1995; Certik *et al.* 1997). However, a number of factors must be borne in mind when fungi are cultivated in lipid rich media. In order for the fungal lipases to function adequately in liquid media, the lipids must be available to the lipase enzyme (Nahas 1988; Ratledge 1989). This may be accomplished by emulsifying the lipid substrate or by shaking the liquid culture during incubation. The aeration obtained through shaking also enhances lipase production. It should, however, be noted that shaking does have a denaturing effect on lipase, resulting in a lower than optimum enzyme activity. Another important factor

influencing lipase activity, is the pH of the medium (Ratledge 1989). Most lipase enzymes have a pH optimum between pH 6 and pH 8 and although there are reports of lipase activity at lower pH values, few if any are stable at pH 11 or above. The pH value of the medium should, therefore, be maintained near neutrality so that lipase activity and fatty acid uptake are at an optimum. Interestingly, residual, exogenous lipids are often modified due to the stereo- and typo-specificity of these lipolytic enzymes and the fatty acid specificity of the cellular membrane (Aggelis *et al.* 1995; Aggelis & Sourdis 1997).

1.2.4.2 Fatty acid uptake and accumulation

At high concentrations, free fatty acids in the medium are taken up by diffusion, however, at lower concentrations, the uptake of free fatty acids is achieved through facilitated diffusion. Since free fatty acids are toxic to cells, fatty acyl-CoA synthetase catalyses the formation of a thiol ester, acyl-CoA, using a free fatty acid and coenzyme A as substrates (Gunstone 1984; Ratledge 1984). The fatty acids are then available to be used in the production of lipid free biomass or lipid reserves (Aggelis *et al.* 1995; Certik *et al.* 1997).

Oleaginous microorganisms, cultured in media containing lipids as carbon source, accumulate reserve lipids by mechanisms differing from those encountered when carbohydrates are used as carbon source (Aggelis *et al.* 1995; Aggelis & Sourdis 1997). In the latter case, lipid accumulation only commences with the depletion of certain nutrients, such as nitrogen, from the medium (Botham & Ratledge 1979; Aggelis 1996). In contrast, it has been shown that members of the genus *Mucor* accumulate significant quantities of lipids, when grown on a medium containing lipids, regardless of the amount of nitrogen present (Aggelis *et al.* 1995).

When fungi are grown on lipids as carbon source, it is assumed that the accumulation of endogenous lipids is determined by the exogenous lipid concentration (Aggelis *et al.* 1991b; Aggelis *et al.* 1995). It was suggested that the

rate of 18:2(ω6) accumulation by fungi grown on vegetable oil is proportional to the initial concentration of this fatty acid in the medium. It was also suggested that the final 18:2(ω6) concentration in the total endogenous lipids is approximately 80 % of the initial concentration of 18:2(ω6) in the medium (Aggelis *et al.* 1991a). Certik and co-workers (1997) could only confirm this for a *Rhizopus* strain grown on sunflower oil.

When the exogenous lipid concentration in a medium, containing lipids as sole carbon source, reaches a critical point and the metabolic requirements of the culture can no longer be supported, the carbon pool is supplemented by biodegradation of endogenous lipid reserves (Aggelis *et al.* 1995). As the exogenous lipids are exhausted, any further metabolic activity becomes dependant on the degradation of these reserve lipids (Akhtar *et al.* 1983; Aggelis *et al.* 1995).

1.2.4.3 Fatty acid metabolism

Fatty acids, which were incorporated into the endogenous lipids of a fungus, may either be degraded by β-oxidation, yielding energy and acetyl-CoA, or may act as substrates for biotransformation processes (Aggelis *et al.* 1995). These processes may lead to changes in endogenous fatty acid concentrations and to the production of fatty acids which did not exist in the substrate (Aggelis *et al.* 1995; Aggelis & Sourdis 1997; Certik *et al.* 1997).

The enzymatic capabilities of the fungus are determining factors in the biotransformation processes and resulting products (Schweizer *et al.* 1978; Aggelis *et al.* 1995; Aggelis & Sourdis 1997). Members of the genus *Mortierella* are capable of incorporating fatty acids from cottonseed oil, linseed oil, olive oil, perilla oil and soy bean oil into their mycelia and biotransforming them to lipids containing 20:4(ω6) or 20:5(ω3) (Shimizu *et al.* 1989a; Shimizu *et al.* 1989b; Shinmen *et al.* 1989; Shinmen *et al.* 1992; Bajpai & Bajpai 1993). When *Mortierella alpina* 1S-4 was grown on linseed oil at a low temperature (12°C), this strain was able to produce 20:5(ω3) *via*

both the ω3 and ω6 pathways (Shimizu *et al.* 1989a; Shimizu *et al.* 1989b), but only through the ω3 pathway at 28°C (Shimizu *et al.* 1989b). A strain of *Mortierella elongata* produced 29.5 $mg.g^{-1}$ biomass 20:5(ω3) when grown at 15°C, in a medium containing linseed oil (Bajpai & Bajpai 1993). Certik and co-workers (1997) demonstrated that *Mortierella alpina*, grown on sunflower oil, produced 465 $mg.l^{-1}$ 20:4(ω6) and that *Mucor mucedo* and *Cunninghamella echinulata* were able to produce significant quantities of 18:3(ω6) on sunflower oil. It should, however, be noted that Certik and co-workers used a complex medium containing glucose. Therefore the *ab initio* production of fatty acids could not be ruled out (Kendrick & Ratledge 1996).

In addition to the above mentioned biotransformations, comprising elongation and desaturation reactions, other metabolic pathways are known which utilise PUFAs as direct precursors. The products of these pathways are oxygenated fatty acids which may play important physiological roles in the organisms.

1.2.5 Oxygenated fatty acid production

Oxygenated fatty acids, such as eicosanoids, are produced in eukaryotic cells from PUFAs in response to a stimulus, which may be of a thermal, chemical, mechanical, hormonal or enzymatic nature (Fig. 6) (Weksler *et al.* 1978; Grenier *et al.* 1981; Kirschenbaum *et al.* 1983; Ogburn & Brenner 1983; Smith 1989; Piomelli 1993; Lambert 1994). The result of the stimulus is the activation of lipase systems such as phospholipase A_2 and phospholipase C, which, through hydrolysis, release an eicosanoid precursor [e.g. 20:4(ω6)] from the cellular phospholipids (Dennis 1987; Smith 1989; Smith & Marnett 1991; Piomelli 1993; Lambert 1994; Gill & Valivety 1997a). Arachidonic acid or other PUFAs may undergo reesterification to produce phospholipids, diffuse out of the cell or may enter one of the pathways for eicosanoid synthesis (Penneys 1980; Piomelli 1993; Gill & Valivety 1997a). These pathways differ in the manner and position in which PUFAs are oxidised. A short review of the different oxidative pathways, each containing different enzyme systems, will be given

in the next part of the overview on fungal lipid metabolism.

1.2.5.1 Cyclo-oxygenase

Products of this pathway include prostaglandins and thromboxanes (Slater & McDonald-Gibson 1987). These molecules contain a cyclopentane ring and are considered to be derivatives of a theoretical molecule, prostanoic acid (Fig. 7) (Holland *et al.* 1988). Two prostaglandin endoperoxide H synthase enzymes (PGHSs) play important roles in the formation of the above mentioned molecules (Smith 1989; Smith & Marnett 1991; Lambert 1994; Smith *et al.* 1996). The first enzyme, PGHS-1 or cyclo-oxygenase-1 (COX-1), which is membrane-bound and particularly abundant in the endoplasmic reticulum, is a constitutive enzyme (Needleman 1986; Smith 1989; Lambert 1994; Morita *et al.* 1995; Smith *et al.* 1996). The second enzyme, PGHS-2 or COX-2, which is inducible (Smith *et al.* 1996) and more abundant in the outer nuclear membrane, has sofar only been demonstrated in mammalian cells (Morita *et al.* 1995; Smith *et al.* 1996). The reason for the existence of two similar enzymes is unclear (Morita *et al.* 1995; Smith *et al.* 1996). However, since both enzymes are often expressed in the same cell, it was suggested that the enzymes are part of separate prostaglandin synthesis systems, which distribute prostaglandins to both the nucleus and the extracellular domain. In both enzymes, two activities are associated with a single protein molecule (Miyamoto *et al.* 1976; Van der Ouderaa *et al.* 1977; Pagels *et al.* 1983; Needleman *et al.* 1986; Smith 1989; Lambert 1994; Smith *et al.* 1996). Firstly, the cyclo-oxygenase activity catalyses the formation of the unstable prostaglandin G_2 (Fig. 8) (Gunstone 1984; Smith *et al.* 1996). The second activity, the peroxidase, catalyses a two electron reduction of the 15-hydroperoxyl group of PGG_2 to produce prostaglandin H_2 (PGH_2). Then, depending on which metabolising enzyme predominates in the cell, PGH_2 is transformed to any of the biologically active prostaglandins, such as prostaglandin D_2, prostaglandin E_1, prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or prostaglandin I_2 (Gunstone 1984; Smith 1989; Lambert 1994). The prostaglandins (mainly PGHS-1 products) leave the cell possibly *via* carrier mediated transport (Smith 1986) and

interact with specific receptors on the membranes of the parent cell or neighbouring cells to elicit the desired biological response (Gorman & Marcus 1981; Ogburn & Brenner 1983; Samuelson *et al.* 1987; Smith 1989; Smith & Marnett 1991; Piomelli 1993; Lambert 1994; Smith *et al.* 1996).

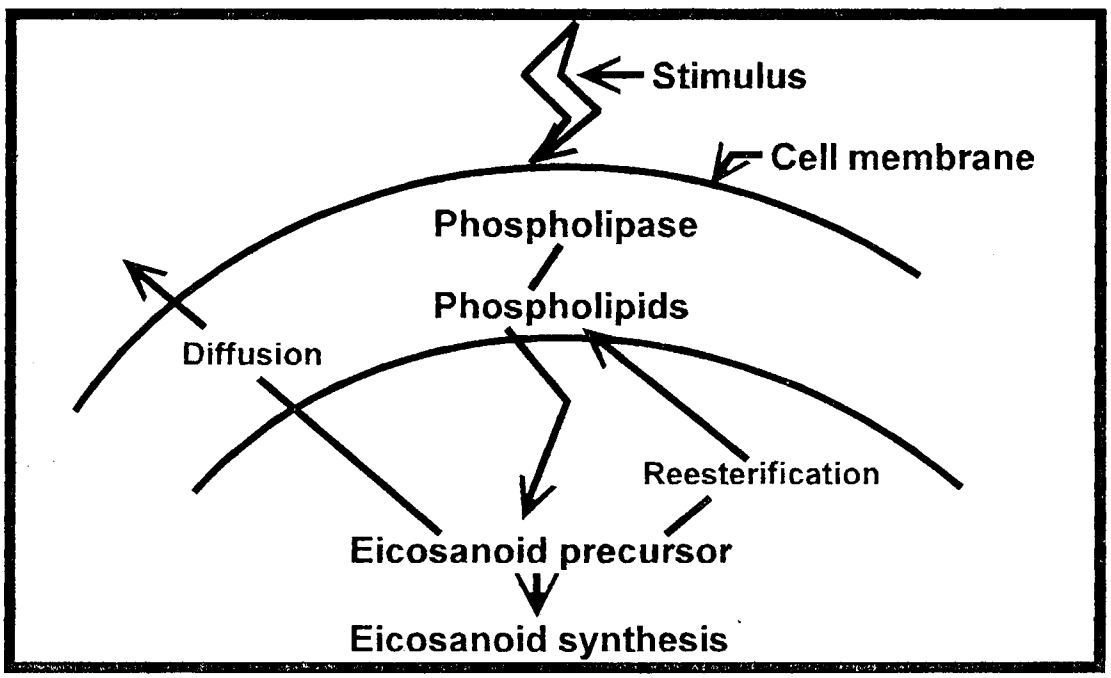


Fig. 6. Mobilisation of eicosanoid precursors.

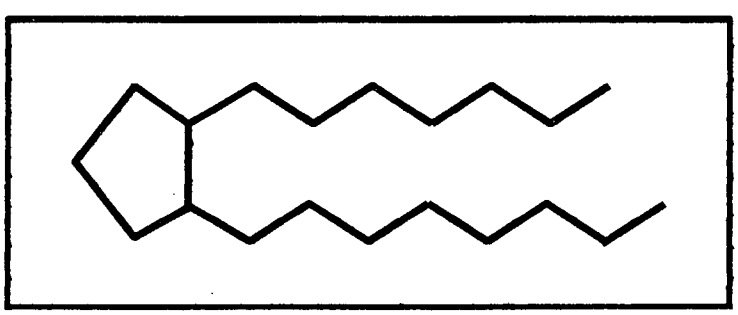


Fig. 7. Prostanoic acid (Holland *et al.* 1988).

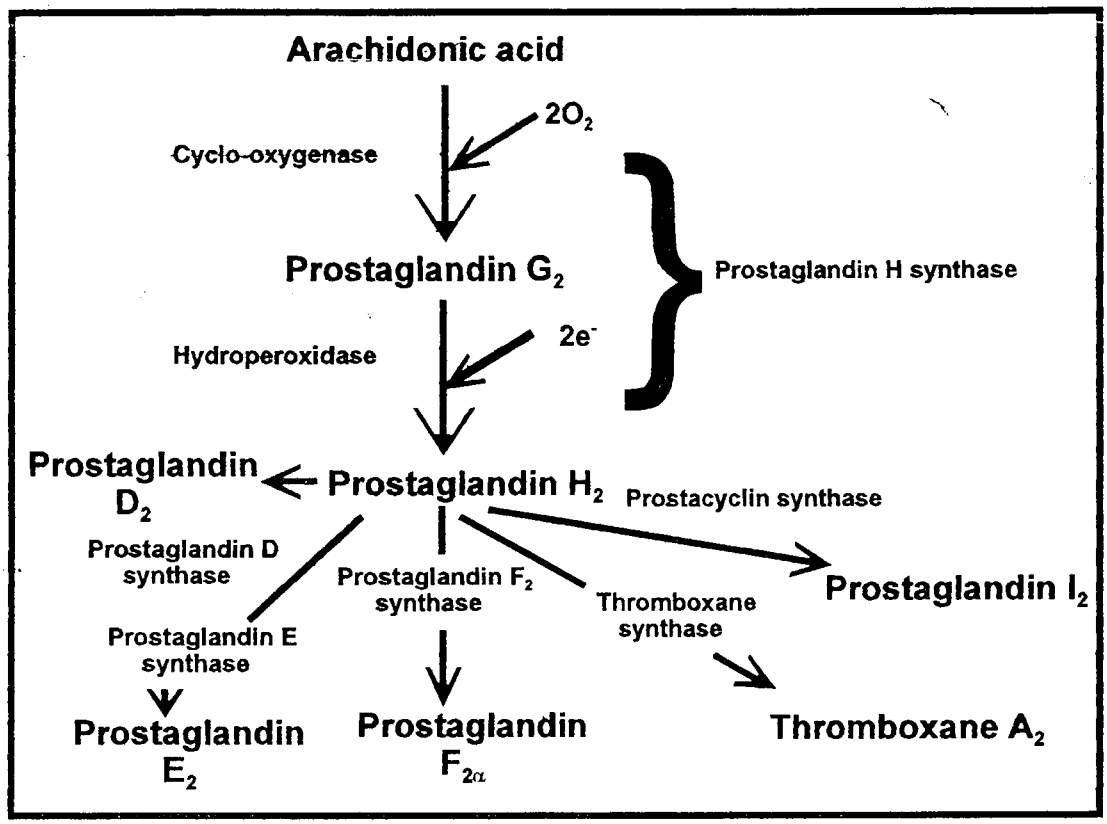


Fig. 8. Cyclo-oxygenase pathway (Smith & Marnett 1991).

1.2.5.2 Lipoxygenase

The most important difference between cyclo-oxygenase and lipoxygenase products is the absence of a cyclopentane ring in the products formed *via* the lipoxygenase pathway (Gorman & Marcus 1981). A range of lipoxygenases exists with different substrate specificities, stereo- and regiochemistry of hydroperoxide insertion, as well as different secondary lyase and oxidase activities (Gunstone 1984; Funabiki 1997; Gill & Valivety 1997b). However, the following may be considered typical reactions in the lipoxygenase pathway. Lipoxygenase introduces oxygen into a PUFA, such as 20:4(ω6), which is transformed to a hydroperoxy fatty acid (Fig. 9) (Vance & Vance 1985; Hamberg *et al.* 1986; Needleman *et al.* 1986; Slater & McDonald-Gibson 1987; Smith 1989; Funabiki 1997; Gill & Valivety 1997b). This molecule contains a pair of

cis/trans conjugated double bonds. The hydroperoxy fatty acid may then be transformed along one of three different metabolic pathways.

The first is a two electron reduction of the hydroperoxy group to produce the corresponding hydroxy acid (Vance & Vance 1985). The second pathway includes a lipoxygenation at another position on the side chain, resulting in the formation of a dihydroxy fatty acid after reduction of the two hydroperoxy groups. In the third pathway, the hydroperoxy fatty acid is dehydrated to form an epoxy fatty acid, such as the unstable leukotriene A_4 (Samuelson *et al.* 1987), which may undergo additional transformations to more stable leukotrienes (Smith & Borgeat 1985; Needleman *et al.* 1986; Samuelson *et al.* 1987; Lambert 1994).

1.2.5.3 Cytochrome P-450

The mono-oxygenase or cytochrome P-450 pathway refers to a family of membrane-bound heme proteins which catalyse the mono-oxygenation of lipophilic substances and exhibit a wide range of substrate heterogenicity (Laniado-Schwartzman *et al.* 1988; Needleman *et al.* 1986; Porter & Coon 1991; Shimada *et al.* 1997). Some of these enzymes are responsible for the formation of mono-epoxides at each of the double bonds of unsaturated fatty acids. According to Pace-Asciak (1989), the enzymes appear to be site specific on the fatty acid. The presence of molecular oxygen and NADPH is also required (Lambert 1994). These epoxy fatty acids may be transformed to epoxy-prostaglandins.

1.2.5.4 β-oxidation

It is known that 3-hydroxy fatty acids are released during the course of β-oxidation (Ratledge 1989; Mathews & Van Holde 1990; Jin *et al.* 1992). When an unsaturated fatty acid enters β-oxidation, it is oxidised until the first double bond is in position five, counted from the CoA moiety. Enoyl-CoA-hydratase is the enzyme responsible for the transformation of trans-Δ^2-enoyl-CoA to 3-hydroxylacyl-CoA. This enzyme gives

a racemic mixture of L- and D-3-hydroxylacyl-CoA. Since only the L-isomer is the substrate for the next enzyme in β-oxidation, an epimerase transforms the D-isomer into the L-isomer. In organisms with an inefficient epimerase, the D-3-hydroxylacyl-CoA may accumulate and eventually leak out of the pathway. Venter and co-workers (1997) analysed the oxidation products of 20:4(ω6) in *Dipodascopsis uninucleata* and found that the D-enantiomer of 3-hydroxy-5,8,11,14-eicosatetraenoic acid was nearly exclusively produced from this PUFA. Thus, if β-oxidation were involved, the enoyl-CoA-hydratase in this yeast had a specificity different from that found in normal β-oxidation. Another possible explanation for the release of 3-hydroxy fatty acids during β-oxidation, is an increase in 3-hydroxylacyl-CoA concentration due to a limited rate of transformation of L-3-hydroxylacyl-CoA to 3-ketoacyl-CoA by 3-hydroxyacyl-CoA-dehydrogenase (Jin *et al.* 1992).

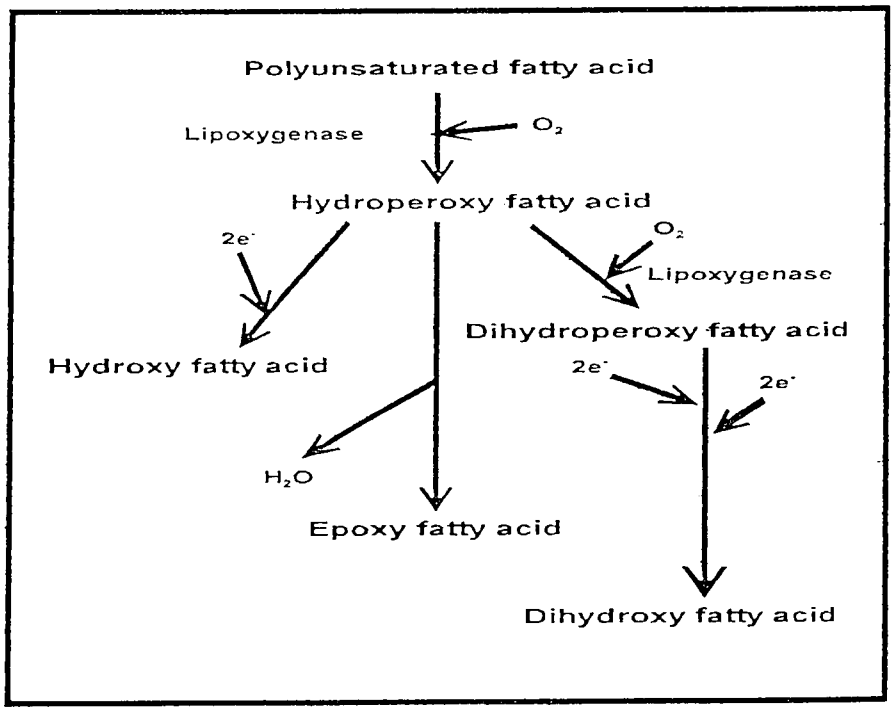


Fig. 9. Lipoxygenase pathway (Smith 1989).

1.2.6 Occurrence of oxygenated fatty acid producing enzymes and\or their products in the fungal domain

Extensive studies have been conducted on the occurrence of oxygenated fatty acids in the fungal domain. These reports on the presence of oxygenated fatty acids, either synthesised *ab initio* or produced as a result of biotransformation of certain exogenously added precursors as well as evidence for lipoxygenase and cyclo-oxygenase activity in different taxa, are listed in Tables 1 to 3.

As can be seen in Tables 1 to 3, oxygenated lipids occur in the protoctistan fungi, the Mucorales and both sub-divisions of the Dikaryomycota, suggesting that the production of oxygenated lipids is ubiquitous in the fungal domain. However, as a result of the different methodologies followed during experimentation, it is not possible to infer any taxonomic implications regarding the distribution of oxygenated lipids among the above mentioned members of the fungal domain. Certain authors have, however, highlighted the fact that certain plant pathogenic fungi contain 9,10-dihydroxy-18:0 and 9,10-epoxy-18:0 in their spores and that the sclerotia of members of the genus *Claviceps* contain 12-hydroxy-18:1(ω9) (Lösel 1988).

Table 1. Occurrence of oxygenated fatty acids in primitive protoctistan fungi.

Species	Oxygenated fatty acid producing enzymes or products	Exogenous precursors
Achlya ambisexualis	Cyclo-oxygenase product Lipoxygenase product	None 20:4(ω6)
Achlya caroliniana	Cyclo-oxygenase product	None
Lagenidium giganteum	Cyclo-oxygenase products Lipoxygenase products	None None
Leptomitus lacteus	9-hydroxy-20:4(ω6) 17-hydroxy-20:4(ω6) 18-hydroxy-20:4(ω6) 19-hydroxy-20:4(ω6)	20:4(ω6) 20:4(ω6) 20:4(ω6) 20:4(ω6)
Saprolegnia diclinia	11,12,15-trihydroxy-20:3(ω7) 11,14,15-trihydroxy-20:3(ω8) 13,14,15-trihydroxy-20:3(ω9)	20:4(ω6) 20:4(ω6) 20:4(ω6)
Saprolegnia ferax	Lipoxygenase products	None
Saprolegnia parasitica	9,10,13-trihydroxy-18:1(ω7) 9,12,13-trihydroxy-18:1(ω9) 11,12,15-trihydroxy-20:3(ω7) 11,14,15-trihydroxy-20:3(ω8) 13,14,15-trihydroxy-20:3(ω9) 15-hydroperoxy-20:4(ω7) 11,12-epoxy-15-hydroxy-20:3(ω7) 13,14-epoxy-15-hydroxy-20:3(ω9)	None None 20:4(ω6) 20:4(ω6) 20:4(ω6) 20:4(ω6) 20:4(ω6) 20:4(ω6)

Herman & Herman (1985); Hamberg (1986); Hamberg *et al.* (1986); Kerwin *et al.* (1986); Herman *et al.* (1989); Herman & Luchini (1989); Van Dyk *et al.* (1994); Akpinar *et al.* (1998)

Table 2. Occurrence of oxygenated fatty acid producing enzymes and their products in mucoralean fungi.

Species	Oxygenated fatty acid producing enzymes or products	Exogenous precursor
Cunninghamella elegans	PGE_2 $PGF_{2\alpha}$	None None
Mortierella alpina	PGE_2 $PGF_{2\alpha}$	None None
Mortierella isabellina	15-hydroxy-20:4(ω7) 11,12-dihydroxy-20:3(ω6) 13,14-dihydroxy-20:3(ω9) 11,12,17-trihydroxy-20:3(ω6) 11,12,18-trihydroxy-20:3(ω6) 11,12,19-trihydroxy-20:3(ω6) PGE_2 $PGF_{2\alpha}$	20:4(ω6) 20:4(ω6) 20:4(ω6) 20:4(ω6) 20:4(ω6) 20:4(ω6) None None
Mucor sp.	7-hydroxy-10:0 7-hydroxy-12:0 7-hydroxy-14:0	None None None
Mucor circinelloides	7-hydroxy-10:0 7-hydroxy-12:0 7-hydroxy-14:0	None None None
Mucor dimorphosporus	7-hydroxy-10:0 7-hydroxy-12:0 7-hydroxy-14:0	None None None
Mucor griseo-cyanus	7-hydroxy-10:0 7-hydroxy-12:0 7-hydroxy-14:0	None None None
Mucor hiemalis	7-hydroxy-10:0 7-hydroxy-12:0	None None
Mucor mucedo	7-hydroxy-10:0 7-hydroxy-12:0 7-hydroxy-14:0	None None None

Tahara *et al.* (1980); Akpinar *et al.* (1998); Lamacka & Sajbidor (1998)

Table 2. (Continued)

Species	Oxygenated fatty acid producing enzymes or products	Exogenous precursor
Mucor praini	7-hydroxy-10:0 7-hydroxy-12:0 7-hydroxy-14:0	None None None
Mucor pusillus	7-hydroxy-10:0 7-hydroxy-12:0 7-hydroxy-14:0	None None None
Rhizopus sp.	Lipoxygenase activity	None
Rhizopus arrhizus	Prostaglandin analogs	15-deoxy-prostanoids
Rhizopus stolonifer	Prostaglandin analogs	15-deoxy-prostanoids

Satoh *et al.* (1976); Tahara *et al.* (1980); Holland *et al.* (1988); Kock *et al.* (1992)

Table 3. Occurrence of oxygenated fatty acid producing enzymes and their products in dikaryomycotan fungi.

Species	Oxygenated fatty acid producing enzymes or products	Exogenous precursor
Ascomycetous fungi		
Aspergillus sp.	Lipoxygenase activity	None
Aspergillus ochraceus	Prostaglandin analogs	15-deoxy-prostanoids
Aspergillus sydowi	2-hydroxy-26:0	None
Emericella nidulans (Anamorph = *Aspergillus nidulans*)	5,8-dihydroxy-18:1(ω9) 5,8-dihydroxy-18:2(ω6) Precocious sexual inducers	None None None
Candida sp. (= *Torulopsis* sp.)	17-hydroxy-18:1(ω9) 15-hydroxy-18:2(ω6)	18:1(ω9) None
Candida apicola (= *Torulopsis apicola*)	15-hydroxy-16:0 16-hydroxy-16:0 16-hydroxy-17:0 17-hydroxy-17:0 17-hydroxy-18:0 17-hydroxy-18:1(ω9) 18-hydroxy-18:2(ω9) 18-hydroxy-19:0 19-hydroxy-20:0	None None 17:0, 19:0, 21:0 17:0, 19:0, 21:0 None None None 19:0, 21:0 C20 hydrocarbon
Candida bombicola (= *Torulopsis bombicola*)	17-hydroxy-18:0 17-hydroxy-18:1(ω9)	n-octadecane, alkanes, 18:1(ω9), soy bean oil None
Candida borgoriensis	13-hydroxy-22:0	Fatty acids
Candida mycoderma	15-hydroxy-18:2(ω6)	None
Candida rugosa	3-hydroxy-3:0 3-hydroxy-4:0	3:0 4:0

Stodola *et al.* (1967); Heinz *et al.* (1970); Fujii & Tonomura (1971); Domsch *et al.* (1980); Tahara *et al.* (1980); Satoh *et al.* (1976); Ito & Inoue (1982); Göbbert *et al.* (1984); Holland *et al.* (1988); Lösel (1988); Boulton (1989); Mazur *et al.* (1991); Van Dyk *et al.* (1994)

Table 3. (Continued)

Species	Oxygenated fatty acid producing enzymes or products	Exogenous precursor
Candida tropicalis	12-hydroxy-12:0	n-dodecane
Candida utilis	2-hydroxy-26:0	26:0
Cephalosporium sp.	2-hydroxy-4:0 3-hydroxy-4:0	? ?
Cephalosporium herbarum	2-hydroxy-4:0 3-hydroxy-4:0	? ?
Ceratocystis ulmi	Lipoxygenase products Methyl-jasmonate	None None
Claviceps fusiformis	12-hydroxy-18:1(ω9)	None
Claviceps gigantea	12-hydroxy-18:1(ω9) 9,10-dihydroxy-18:0	None None
Claviceps paspali	12-hydroxy-18:1(ω9) 9,10-dihydroxy-18:0	None None
Claviceps purpurea	12-hydroxy-18:1(ω9) 9,10-dihydroxy-18:0	None None
Claviceps sulcata	12-hydroxy-18:1(ω9) 9,10-dihydroxy-18:0 9,10-epoxy-18:0	None None None
Curvularia lunata	Prostaglandin analogs	15-deoxy-prostanoids
Dipodascopsis tóthii	13-hydroxy-18:2(ω7) 3-hydroxy-20:4(ω6) 9,10,13-trihydroxy-18:1(ω7) 9,12,13-trihydroxy-18:1(ω8) 9,10-epoxy-11-hydroxy-18:1(ω6) 12,13-epoxy-11-hydroxy-18:1(ω9) $PGF_{2\alpha}$	None 20:4(ω6) None None None None None

? = Lösel (1988) made no mention of exogenous precursors in her review.

Morris (1967); Stodola *et al.* (1967); Mantle *et al.* (1969); Fujii & Tonomura (1971); Kren *et al.* (1985); Holland *et al.* (1988); Lösel (1988); Boulton (1989); Ratledge (1989); Kock *et al.* (1991); Van Dyk *et al.* (1991a); Van Dyk *et al.* (1991b); Jensen *et al.* (1992); Kock *et al.* (1992); Van Dyk *et al.* (1994)

Table 3. (Continued)

Species	Oxygenated fatty acid producing enzymes or products	Exogenous precursor
Dipodascopsis uninucleata	3-hydroxy-14:2(ω6) 3-hydroxy-14:3(ω3) 3-hydroxy-20:3(ω3) 3-hydroxy-20:3(ω6) 3-hydroxy-20:3(ω9) 3-hydroxy-20:4(ω6) 3-hydroxy-20:5(ω3) 9-hydroxy-18:2(ω6) 13-hydroxy-18:2(ω7) 9,10,13-trihydroxy-18:1(ω7) 9,12,13-trihydroxy-18:1(ω8) 9,10-epoxy-11-hydroxy-18:1(ω6) 12,13-epoxy-11-hydroxy-18:1(ω9) $PGF_{2\alpha}$ α-pentanor-$PGF_{2\alpha}$-γ-lactone	18:2(ω6) 20:3(ω3) 20:3(ω3) 20:3(ω6) 20:3(ω9) 20:4(ω6) 20:5(ω3) 18:2(ω6) None None None None None None 20:4(ω6)
Fusarium sp.	2-hydroxy-4:0 3-hydroxy-4:0	? ?
Fusarium anguioides	Lipoxygenase activity	Soy bean oil
Fusarium caucasicum	Lipoxygenase activity	Soy bean oil
Fusarium culmorum	Lipoxygenase activity	Soy bean oil
Fusarium lini	Lipoxygenase activity	Soy bean oil
Fusarium oxysporum	9-hydroperoxy-18:2(ω6) 13-hydroperoxy-18:2(ω6) Lipoxygenase activity	18:2(ω6) 18:2(ω6) Soy bean oil
Fusarium solani	Lipoxygenase activity	Soy bean oil

? = Lösel (1988) made no mention of exogenous precursors in her review.

Satoh *et al.* (1976); Matsuda *et al.* (1978); Lösel (1988); Kock *et al.* (1991); Van Dyk *et al.* (1991a); Van Dyk *et al.* (1991b); Botha *et al.* (1992a); Botha *et al.* (1992b); Coetzee *et al.* (1992); Kock *et al.* (1997); Venter *et al.* (1997); Akpinar *et al.* (1998)

Table 3. (Continued)

Species	Oxygenated fatty acid producing enzymes or products	Exogenous precursor
Gaeumannomyces graminis	10-hydroxy-18:1(ω7)	18:1(ω7)
	8-hydroxy-18:1(ω9)	18:1(ω9), 12-hydroxy-18:1(ω9)
	17-hydroxy-18:1(ω9)	18:1(ω9)
	7,8-dihydroxy-18:1(ω9)	18:1(ω9), 12-hydroxy-18:1(ω9)
	7-hydroxy-18:1(ω9)	12-hydroxy-18:1(ω9)
	7,12-dihydroxy-18:1(ω9)	12-hydroxy-18:1(ω9)
	8,12-dihydroxy-18:1(ω9)	12-hydroxy-18:1(ω9)
	12,17-dihydroxy-18:1(ω9)	12-hydroxy-18:1(ω9)
	12,18-dihydroxy-18:1(ω9)	18:2(ω6)
	12,13-dihydroxy-18:1(ω9)	18:2(ω6)
	8-hydroperoxy-18:2(ω6)	18:2(ω6)
	8-hydroxy-18:2(ω6)	18:2(ω6)
	9-hydroxy-18:2(ω6)	18:2(ω6)
	10-hydroxy-18:2(ω6)	18:2(ω6)
	11-hydroxy-18:2(ω6)	18:2(ω6)
	13-hydroxy-18:2(ω7)	18:2(ω6)
	16-hydroxy-18:2(ω6)	18:2(ω6)
	17-hydroxy-18:2(ω6)	18:2(ω6)
	7,8-dihydroxy-18:2(ω6)	18:2(ω6)
	8,16-dihydroxy-18:2(ω6)	18:2(ω6)
	18,17-dihydroxy-18:2(ω6)	18:3(ω3)
	8-hydroxy-18:3(ω3)	18:3(ω3)
	17-hydroxy-18:3(ω3)	18:3(ω3)
	7,8-dihydroxy-18:3(ω3)	18:3(ω3)
	15,16-dihydroxy-18:2(ω6)	20:4(ω6)
	17-hydroxy-20:4(ω6)	20:4(ω6)
	18-hydroxy-20:4(ω6)	20:4(ω6)
	19-hydroxy-20:4(ω6)	20:4(ω6)
	17,18-dihydroxy-20:4(ω6)	20:4(ω6)
	19-hydroxy-20:5(ω3)	20:5(ω3)
Lasiodiplodia theobromae	Jasmonic acid	None
Lipomyces sp.	12,13-epoxy-18:1(ω9)	None
	$PGF_{2\alpha}$	None
Lipomyces anomalus	Cyclo-oxygenase activity	None
Lipomyces kononenkoae	$PGF_{2\alpha}$	None

Aldridge *et al.* (1971); Brodowski *et al.* (1992); Brodowski & Oliw (1992); Kock *et al.* (1991); Kock *et al.* (1992); Van Dyk *et al.* (1994)

Table 3. (Continued)

Species	Oxygenated fatty acid producing enzymes or products	Exogenous precursor
Lipomyces starkeyi	$PGF_{2\alpha}$	None
Lipomyces tetrasporus	$PGF_{2\alpha}$	None
Myxozyma geophila	$PGF_{2\alpha}$	None
Myxozyma lipomycoides	$PGF_{2\alpha}$	None
Myxozyma melibiosi	$PGF_{2\alpha}$	None
Myxozyma mucilagina	$PGF_{2\alpha}$	None
Neurospora crassa	2,3-dihydroxy-iso-5:0 2,3-dihydroxy-methyl-5:0	? ?
Penicillium sp.	Lipoxygenase activity	None
Penicillium frequentens	2-hydroxy-4:0 3-hydroxy-4:0	? ?
Rhodotorula sp.	8,9,13-trihydroxy-18:0 8,9-dihydroxy-13-oxo-18:0	None None
Rhodotorula glutinis	3-hydroxy-16:0 3-hydroxy-18:0	None None
Rhodotorula gracilis	3-hydroxy-16:0 3-hydroxy-18:0	None None
Rhodotorula graminis	3-hydroxy-16:0 3-hydroxy-18:0	None None
Saccharomyces cerevisiae	9-hydroperoxy-18:2(ω6) 13-hydroperoxy-18:2(ω6) $PGF_{2\alpha}$ Lipoxygenase activity	18:2(ω6) 18:2(ω6) None None
Saccharomycopsis malanga (= *Hansenula malanga*)	3-hydroxy-16:0 3-hydroxy-18:0	None None
Zygozyma oligophaga	$PGF_{2\alpha}$	None

? = Lösel (1988) made no mention of exogenous precursors in her review.

Stodola *et al.* (1967); Versonder *et al.* (1968); Kurtzman *et al.* (1974); Satoh *et al.* (1976); Schechter & Grossman (1983); Lösel (1988); Boulton (1989); Ratledge (1989); Kock *et al.* (1991); Kock *et al.* (1992); Van Dyk *et al.* (1994)

Table 3. (Continued)

Species	Oxygenated fatty acid producing enzymes or products	Exogenous precursor
Basidiomycetous fungi		
Cronartium fusiforme	9,10-epoxy-18:0	None
Cronartium ribicola	9,10-dihydroxy-18:0 9,10-epoxy-18:0	None None
Gymnosporangium claviceps	9,10-dihydroxy-18:0 9,10-epoxy-18:0	None None
Laetisera arvalis	8-hydroxy-18:2(ω6)	None
Melampsora lini	9,10-dihydroxy-18:0 9,10-epoxy-18:0	None None
Puccinia graminis	9,10-epoxy-18:0	None
Uromyces phaseoli	3-hydroxy-4:0	?
Ustilago nuda	2-hydroxy-16:0 3-hydroxy-6:0 3-hydroxy-8:0 2,15-dihydroxy-16:0	None None None None
Ustilago zea	2-hydroxy-16:0 3-hydroxy-6:0 3-hydroxy-8:0 2,15-dihydroxy-16:0 15,16-dihydroxy-16:0 2,15,16-trihydroxy-16:0	None None None None None None

? = Lösel (1988) made no mention of exogenous precursors in her review.

Stodola *et al.* (1967); Weete & Kelley (1977); Schechter & Grossman (1983); Bowers *et al.* (1986); Lösel (1988); Boulton (1989); Ratledge (1989); Kock *et al.* (1991); Van Dyk *et al.* (1994)

Although the role of oxygenated fatty acids in higher eukaryotes has been studied in much detail, relatively little is known about the role of these molecules in fungi. The next section will review the literature on the role of oxygenated fatty acids in the fungal domain.

1.2.7 Role of oxygenated fatty acids in the fungal domain

Authors studying the role of lipoxygenase and cyclo-oxygenase products in unrelated fungal taxa, are in agreement that oxygenated lipids play important roles in the growth, development and reproduction of fungi (Herman & Herman 1985; Kerwin *et al.* 1986; Herman *et al.* 1989; Herman & Luchini 1989; Kock *et al.* 1991; Mazur *et al.* 1991; Coetzee *et al.* 1992; Jensen *et al.* 1992; Van Dyk *et al.* 1994; Kock *et al.* 1998). Herman and Herman (1985) studied the effect of acetylsalicylic acid, a known inhibitor of cyclo-oxygenase, on growth and reproduction of the protoctistan fungi, *Achlya ambisexualis, Achlya caroliniana* and *Saprolegnia parasitica*. They observed abnormal hyphal branching, resulting in characteristic asterisk shaped colonies in the presence of acetylsalicylic acid. These colonies did not reproduce sexually and the authors suggested a possible role for prostaglandins or prostaglandin-like substances in Oomycete development. Similar results were obtained for an unrelated fungus, the yeast *Dipodascopsis uninucleata* (Coetzee *et al.* 1992). In the presence of acetylsalicylic acid, the sexual stage of the life-cycle of this yeast was disrupted. Kock and co-workers (1998) found that 3-hydroxy fatty acids are important regulators of sexual reproduction of *D. uninucleata* and that these molecules occur selectively in the gametangia, asci and between the ascospores of this yeast. Other oxygenated lipids, so-called precocious sexual inducers, are responsible for premature sexual sporulation in *Emericella nidulans* (Anamorph = *Aspergillus nidulans)* (Domsch *et al.* 1980; Mazur *et al.* 1991).

Herman and Luchini (1989) and Herman and co-workers (1989) found that lipoxygenase products are involved in vegetative growth of *Saprolegnia ferax, Saprolegnia parasitica* and *Achlya ambisexualis*. They observed a decrease in lipoxygenase activity prior to sexual reproduction of these fungi. In contrast, Kerwin and co-workers (1986) concluded that lipoxygenase products are necessary for oosporogenesis, including induction of antheridia, fusion of antheridia with oogonia, spore wall formation and oospore maturation, of the protoctistan fungus, *Lagenidium giganteum*. However, these authors also concluded that lipoxygenase products were

not involved in regulation of growth or asexual reproduction of this fungus. Jensen and co-workers (1992) examined the yeast/mycelium dimorphism of *Ceratocystis ulmi* and found that when lipoxygenase activity was inhibited, this fungus occurred in the yeast form.

Oxygenated lipids and 3-hydroxy fatty acids play important roles in host-pathogen interactions between fungi and plants (Van Dyk *et al.* 1994) and have been extracted from fungi and yeasts isolated from leaves (Stodola *et al.* 1967; Lösel 1988; Brodowsky *et al.* 1992). Jasmonic acid, a known regulator of plant growth, was detected in the plant pathogen, *Lasiodiplodia theobromae* (Aldridge *et al.* 1971) and methyl-jasmonate was detected in *Ceratocystis ulmi* (Jensen *et al.* 1992).

Certain fungi may also produce hydroxy fatty acids as antimicrobial agents, as in the case of *Laetisaria arvalis*, a soil-dwelling basidiomycetous fungus (Bowers *et al.* 1986). This fungus produces 8-hydroxy-18:2(ω6), which effectively inhibits the growth of fungi such as *Fusarium solani, Fusarium oxysporium, Mucor globosus, Mucor racemosus, Phoma batae, Phythophthora megasperma, Pythium ultimum, Rhizoctonia solani* and *Verticillium albo-atrum.* Brodowsky and co-workers (1992) indicated that 8-hydroxy-18:2(ω6) was also present in *Gaeumannomyces graminis* and suggested that it may serve a similar fungicidal purpose as in *L. arvalis.* Another hydroxy fatty acid, 3-hydroxy-16:0, with antibacterial action against *Vibrio tyrogenus*, was found in *Saccharomycopsis malanga.*

Certain hydroxy fatty acids are components of complex lipids such as the glycolipids (Stodola *et al.* 1967; Lösel 1988) and in the case of *Candida bombicola,* form part of the sophorolipids (Ito & Inoue 1982). As part of sophorolipids it was suggested that hydroxy fatty acids play a role in alkane utilisation.

It is important to note that, although the presence of cyclo-oxygenase and lipoxygenase products as well as other oxygenated fatty acids was revealed in mucoralean fungi (Tahara *et al.* 1980; Akpinar *et al.* 1998; Lamacka & Sajbidor

1998), the role of these oxygenated fatty acids in the Mucorales is still unknown. It may be speculated that, similar to their roles in unrelated fungi, these molecules are involved in the regulation of cellular processes, the interaction with other organisms or the formation of more complex lipid molecules.

The lipid metabolism as well as the distribution and role of certain lipid classes in the fungal domain have been reviewed. The next section will deal with the general biology of the oleaginous mucoralean fungi.

1.3 The order Mucorales

1.3.1 General characteristics

A typical mucoralean fungus is characterised by a coenocytic, eucarpic thallus which forms an extensive mycelium containing haploid nuclei (Fig. 10) (Hesseltine & Ellis 1973; Benjamin 1979). Asexual reproduction occurs by means of sporangiospores formed in a mitosporangium. A zygospore is formed during sexual reproduction, as a result of the conjugation of similar gametangia.

The classification of mucoralean taxa is largely based on the mode of asexual reproduction (Hesseltine & Ellis 1973). According to Hawksworth and co-workers (1995), the order Mucorales is comprised of 13 families, each characterised by a unique set of asexual and sexual reproductive structures (Table 4). In order to give the reader a brief overview of the diversity within this order, some distinguishing characters of taxa within the Mucorales will be discussed.

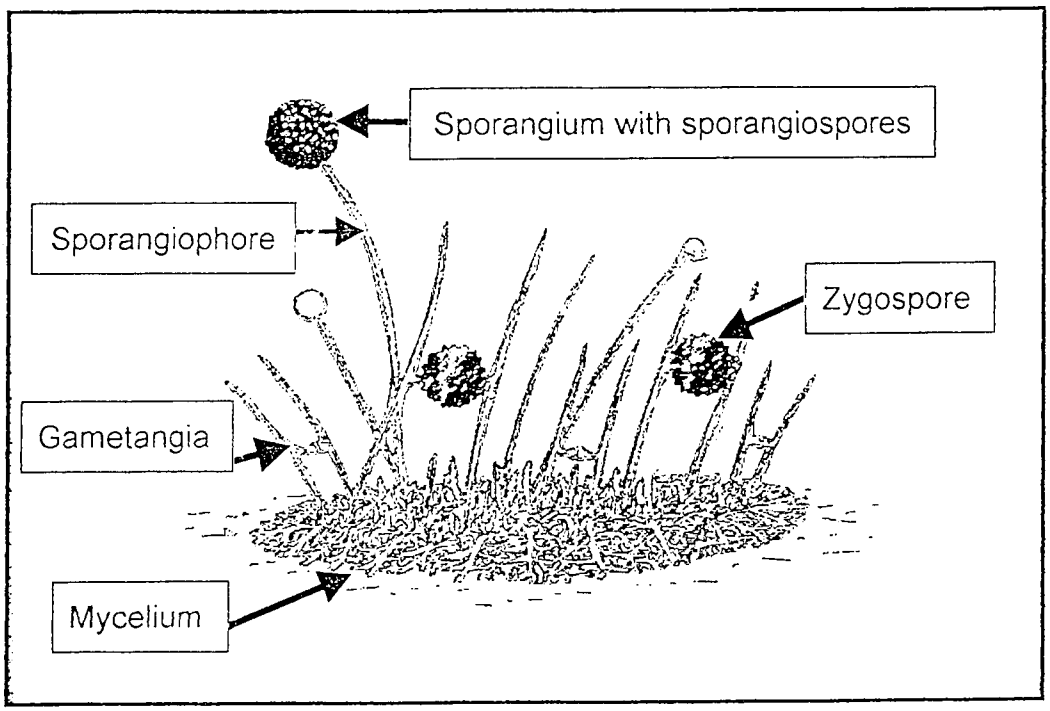


Fig. 10. *Mucor genevensis* Lendner, a representative of the order Mucorales, with mycelium, sporangiophores, sporangia and zygospores.

Table 4. Families of the Mucorales (Hawksworth *et al.* 1995).

Family	Characteristics and numbers of genera and species within each family
Chaetocladiaceae	A. Fisch., 2 gen., 7 spp., sporangiospores terminating in sterile spines; pedicelate sporangiola unispored, formed on fertile vesicles; zygospores rough-walled, suspensors opposed
Choanephoraceae	J. Schröt., 3 gen. (+ syn.), 5 spp., sporangia and sporangiola borne on seperate and distinct sporangiophores; zygospores striate, borne on tong-like suspensors
Cunninghamellaceae	Naumov ex R.K. Benj., 1 gen. (+ 3 syn.), 7 spp., sporangiospores appendaged; zygospores warty, borne on opposed suspensors
Gilbertellaceae	Benny, 1 gen., 1 sp., sporangia columellate, sporangiospores appendaged; zygospore rough-walled, suspensors opposed
Mortierellaceae	A. Fisch., Mucorales. 7 gen. (+ 7 syn.), 106 spp., sporangia and sporangiola typically with columella absent or rudimentary; zygospores smooth or angular, borne on opposed suspensors
Mucoraceae	Dumort., 20 gen. (+ 27 syn.), 122 spp., sporangia columellate, specialised sporangiola absent; zygospores smooth to warty, borne on opposed, tong-like or apposed, naked or appendaged suspensors; polyphyletic
Mycotyphaceae	Benny and R.K. Benj., 2 gen. (+ 1 syn.), 6 spp., sporangiola borne on dehiscent pedicels

gen. = genus / genera; sp. / spp. = species; syn. = synonym / synonyms

Table 4. (Continued)

Family	Characteristics and numbers of genera and species within each family
Phycomycetaceae	Arx, 1 gen., 3 spp., sporangiophores large, unbranched; zygospores with coiled, tong-like suspensors bearing branched appendages
Pilobolaceae	Corda, 3 gen. (+ 3 syn.), 13 spp., sporangia columellate, specialised liberation mechanism present; zygospores smooth, borne on tong-like suspensors
Radiomycetaceae	Hesselt. and J.J. Ellis, 2 gen. (+ 1 syn.), 4 spp., sporangiola borne on complex ampullae, simple or branched, often stoloniferous sporangiophores, sporangia absent; zygospores smooth, borne on apposed, appendaged suspensors
Saksenaeaceae	Hesselt. and J.J. Ellis, 1 gen., 1 sp., sporangia lageniform and columellate; zygospores unknown
Syncephalastraceae	Naumov ex R.K. Benj., 1 gen., 2 spp., merosporangia present; zygospores warty, borne on opposed suspensors
Thamnidiaceae	Fitzp., 12 gen. (+ 9 syn.), 22 spp., sporangia (diffluent and columellate) and sporangiola (few to one spored, persistent walled and columellate) borne on the same or separate, morphologically identical sporangiophores; or sporangiola only present; zygospores warty, borne on opposed suspensors, polyphyletic

gen. = genus / genera; sp. / spp. = species; syn. = synonym / synonyms

The genus *Absidia,* of the family Mucoraceae, is characterised by a sporangiophore, which terminates in a swollen section, called an apophyses, which supports the sporangium (Hesseltine & Ellis 1964). Stolons and rhizoids are produced by members of this genus.

Benny and Benjamin (1993) described members of the genus *Chaetocladium,* of the Chaetocladiaceae, as having pedicelate, unispored sporangiola on small, fertile vesicles. Members of this genus have vertically or dichotomously branched fertile hyphae, often bearing sterile spines.

The genus *Choanephora,* of the Choanephoraceae, is characterised by large, columellate, multispored sporangia with persistent walls which split in half to release sporangiospores (Hesseltine & Ellis 1973). These sporangiospores are dark and possess stif hair-like structures at both poles. Members of this genus also form dark unispored sporangiola.

According to Benjamin and co-workers (1992), the genus *Cunninghamella,* of the Cunninghamellaceae, is characterised by unispored sporangiola, borne on swollen, round vesicles at the tip of a sporangiophore. No multispored sporangia occur, but the presence of chlamydospores was reported (Hesseltine & Ellis 1973). Members of the genus *Gilbertella,* of the Gilbertellaceae, produce sporangia similar to those of members of the *Choanehora* but no sporangiola are formed (Benny 1991).

The genus *Mortierella*, of the Mortierellaceae, is composed of species which produce uni- or multi-spored sporangia, with no or only vestigial columellae (Hesseltine & Ellis 1973). Although sporangia are scarce, chlamydospores with spiny and rough walls are abundant. Certain members of this genus are known to produce zygospores with tong-like suspensor cells. The zygospores are inwebbed in sterile hyphae.

Members of *Mucor*, of the Mucoraceae, have columellate, multispored sporangia (Hesseltine & Ellis 1973). Rhizoids and stolons are reduced or absent and zygospores are suspended between opposite aligned suspensor cells.

Sporangiophores of members of the genus *Mycotypha*, of the family Mycotyphaceae, end in elongated vesicles, covered with sporangiola (Alexopoulos & Mims 1979). The genus *Phycomyces,* of the family Phycomycetaceae, is characterised by slender, unbranched sporangiophores with single, dark, multispored sporangia at the tips (Alexopoulos & Mims 1979). The zygospores have tong-like suspensor cells and sterile spines which develop from one of the suspensor cells.

The genus *Pilobolus,* of the family Pilobolaceae, is characterised by persistent walled, multispored sporangia and large, elongate sporangiophores, which are often phototrophic (Hesseltine & Ellis 1973; Benjamin 1979). No unispored sporangia are formed in this genus (Benjamin 1979), but zygospores, formed between tong-like suspensors, are known to be produced (Hesseltine & Ellis 1973).

Members of *Radiomyces,* of the Radiomycetaceae, have unique sporangiolum-bearing structures, consisting of once-septate stalks ending in secondary vesicles and arising terminally from the enlarged apex of a sporangiophore (Hesseltine & Ellis 1973; Benjamin 1979; Benny & Benjamin 1991). One or many uni- or multispored sporangiola are borne per vesicle.

The sporangia of members of the genus *Saksenaea,* of the Saksenaeaceae, are unlike any other in the order Mucorales (Hesseltine & Ellis 1973). They are elongate and lageniform with distinct columellae. No zygospores are known in this genus.

Another member of the Mucorales, is the genus *Syncephalastrum*, of the family Syncephalastraceae. This genus is characterised by distinctly shaped sporangia, called merosporangia (Hesseltine & Ellis 1973; Benjamin 1979). These cylindrical sporangia each contain a chain-like series of sporangiospores and are borne deciduously on the swollen ends of sporangiophores. Sporangiophores are branched and may be modified into stolon-like structures with rhizoids. Dark, warty zygospores are produced between opposed suspensors.

The members of the genus *Thamnidia*, of the Thamnidiaceae, produce small, few-spored sporangiola which may have columellae and persistent walls (Hesseltine &

Ellis 1973; Benny & Benjamin 1975; Benjamin 1979). Warty zygospores are borne on opposed suspensors.

1.3.1.1 Mucor genevensis Lendner

Although zygospores are known to occur in mucoralean cultures, these sexual reproductive structures are not always produced (Hesseltine & Ellis 1973). This may be as a result of the presence of only one mating type, as in the case of heterothallic fungi. It may also be that the particular environmental conditions do not favour zygospore formation.

Homothallism is not common in the Mucorales (Hesseltine & Ellis 1973, Schipper 1978). However, *Mucor genevensis* Lendner is a homothallic species, which, apart from producing asexual reproductive structures, also readily produces zygospores in the same culture (Fig.10) (Schipper 1973). Thus, *M. genevensis* is one of a few mucoralean fungi of which the biology of both the asexual and sexual reproductive structures may be studied in a single strain. This species produces pale grey colonies on beerwort agar at 20°C (Schipper 1973). On this medium, the height of the colonies may be up to 5 mm. The sporangiophores are sympodically branched and may be up to 10 μm in diameter. The glistening yellow-brown sporangia are up to 70 μm in diameter and have deliquescent walls and piriform-ellipsoidal columellae. The ellipsoidal sporangiospores may vary in size, but the dimensions of these structures are usually 4 - 8.1 x 2.4 - 4 μm. Dark brown, globose zygospores with diameters of up to 80 μm are formed between two similar suspensors. These zygospores have stellate spines up to 4 μm in length.

On beerwort agar, growth and sexual reproduction of *M. genevensis* occur between 5°C and 25°C (Schipper 1973). Hardly any growth occurs at 30°C, while no growth occurs at 37°C.

It was observed by Schipper (1973) that the substrate hyphae of *M. genevensis* are often swollen and filled with yellow droplets. These droplets in the fungal cytoplasm are lipid globules, consisting of neutral lipids (Ratledge & Wilkinson 1988a; Immelman

1993). *Mucor genevensis* occurs in habitats typical of mucoralean fungi, such as in soil, dung and on fruit (CBS, List of cultures 1994). However, as discussed in the next section, other habitats also exist for the Mucorales.

1.3.2 Habitat

Mucoralean fungi are capable of the rapid utilisation of simple carbohydrate molecules, including pentoses, hexoses, disaccharides, trisaccharides, polysaccharides, glucosides, alcohols and organic acids (Botha *et al.* 1997a; Botha *et al.* 1997b; Pohl *et al.* 1997; Botha & Du Preez 1999) and are considered to be the first colonisers of dead or decaying plant material (Alexander 1961). A good example of this is the role of mucoralean fungi in fruit decay (Dennis & Blijham 1980; Spotts & Cervantes 1986; Botha & Du Preez 1999). *Mucor* and *Rhizopus* species were found to rapidly colonise picked pears, strawberries and tomatoes during cold storage and are always present in stored grains as well as on unspoiled food such as spices, flour, nuts, dried fruit, fresh vegetables, milk products, dog food and meats (Hesseltine & Ellis 1973; Botha & Du Preez 1999).

Although these fungi are mostly encountered when isolating from soil, air, dung or decaying plant material, a few species have been found to be parasitic on animals (Hesseltine & Ellis 1973). *Absidia corymbifera* and *Absidia ramosa* were found to be common fungal pathogens of domestic animals (Hesseltine & Ellis 1973) and *Mortierella wolfii*, the only member of this genus that can grow at 35°C, is a well known pathogen of cattle (Streekstra 1997). *Sporodinella umbellata* is an insect parasite (Evans & Samson 1977), while *Parasitella parasitica* is a parasite of other fungi (Schipper 1978). Members of the genus *Mucor* are considered to be opportunistic pathogens of humans with diabetes, leukemia or an immune deficiency, causing mucormycosis (Botha & Du Preez 1999).

Although the genus *Mucor* is accepted to be non-toxic to humans, studies have indicated that mycotoxins may be present in extracts from certain *Mucor* species (Botha & Du Preez 1999). Such an extract from *Mucor mucedo* was found to be mildly toxic to brine shrimp, but highly toxic to chicken embryos. Extracts from *Mucor*

indicus and *Mucor circinelloides* were toxic to ducklings.

An economically important habitat for certain *Rhizopus* species are sunflowers (*Helianthus anuus*) to which they are pathogenic, causing head rot. The species mostly responsible for this disease are *Rhizopus arrhizus*, *Rhizopus stolonifer* and *Rhizopus oryzae* (Weiss 1983; Kolte 1985; Berglund 1994). The economic importance of this disease is that it reduces seed and oil yield (Kolte 1985) and oil from infested seeds has an increased amount of free fatty acids: from 0.8 % (w/w) in oil from healthy seeds to 19.4 % (w/w) in oil from infected seeds (Weiss 1983; Kolte 1985). The ability of these fungi to colonise and grow on oil-rich sunflower seeds is not surprising when taking into account that *Rhizopus* species (e.g. *R. delemar, R. japonicus, R. niveous, R. oligosporus, R. oryzae*) are known producers of significant quantities of lipases (Wagenknecht *et al.* 1961; Iwai & Tsujisaka 1974a; Iwai & Tsujisaka 1974b; Aisaka & Tereda 1981; Nahas 1988; Espinoza *et al.* 1990; Hayes & Gulari 1992; Cruz *et al.* 1993; Talaro & Talaro 1993; Chen *et al.* 1995; Christen *et al.* 1995).

1.4 Purpose of this study

With the above as background, the purpose of this study is to examine lipid metabolism in representatives of the Mucorales. This includes the turnover of lipids during growth and development (Chapter 2), the uptake and incorporation of 20:4(ω6) (Chapter 3), the hydroxylation of exogenous long-chain PUFAs (Chapter 4) and the localisation of these hydroxylated fatty acids (Chapter 5) in a selected member of the order Mucorales.

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Chapter 2

Lipid turnover during growth and development of *Mucor genevensis*

2.1 Introduction

Applied and fundamental research on fungal lipids have been conducted in various laboratories the world over. Consequently, several workers have studied changes in lipid content and composition during growth and development of phylogenetically unrelated fungal taxa (Gay *et al.* 1971; Smith & Silverman 1973; Suberkropp & Cantino 1973; Mills & Cantino 1974; Mills *et al.* 1974; Weber & Hess 1974; Law & Burton 1976; Murray & Maxwell 1976; Farag *et al.* 1983; Grant *et al.* 1988; Jacob & Krishnamurthy 1990; Kock & Ratledge 1993; Lomascolo *et al.* 1994; Van der Westhuizen *et al.* 1994; Stahl & Klug 1996). Unfortunately, no general conclusions regarding changes in lipid and fatty acid composition during growth and development of fungi could be drawn from these studies, since the culture conditions were not standardised (Lösel 1988). Furthermore, these analyses were performed on fungi grown in liquid media and many filamentous fungi do not produce all their developmental structures, such as conidia or sporangia, in submerged cultures (Lösel 1988). In addition, lipid changes during the development of different stages in the life-cycle of mucoralean fungi, grown on solid substrates, received very little attention.

Mucor genevensis is a homothallic mucoralean fungus, capable of producing sexual and asexual reproductive structures on solid media. In order to fully appreciate the lipid turnover in such a fungus, it is necessary to examine the life-cycle of *M. genevensis* (Fig. 1). Asexual development is characterised by germination of sporangiospores to form an extensive mycelium. After *circa* two days of incubation

on a solid synthetic medium containing glucose, Yeast Nitrogen Base (YNB, Difco) and vitamins (Van der Walt & Yarrow 1984) at 20°C, sporangiophores develop from the substrate mycelium. In order to complete asexual reproduction, columellate sporangia, containing sporangiospores, are produced. Upon maturation of sporangia, the spores are released. After another ten days of incubation, sexual reproductive structures may be observed in the culture. During sexual reproduction, gametangia grow toward each other, fuse and produce dark, thick walled zygospores (Hesseltine & Ellis 1973). Although zygospores may in turn germinate and give rise to mycelium, in nature they occur mainly as dormant structures which may survive prolonged periods of adverse conditions (Michailides & Spotts 1988). It is important to note that the different stages in the life-cycle may all be present at the same time on solid media, although specific stages may dominate at certain times. Thus, with the above as background, the aim of this study was to investigate the endogenous lipid turnover during the life-cycle of *Mucor genevensis* on a solid synthetic medium.

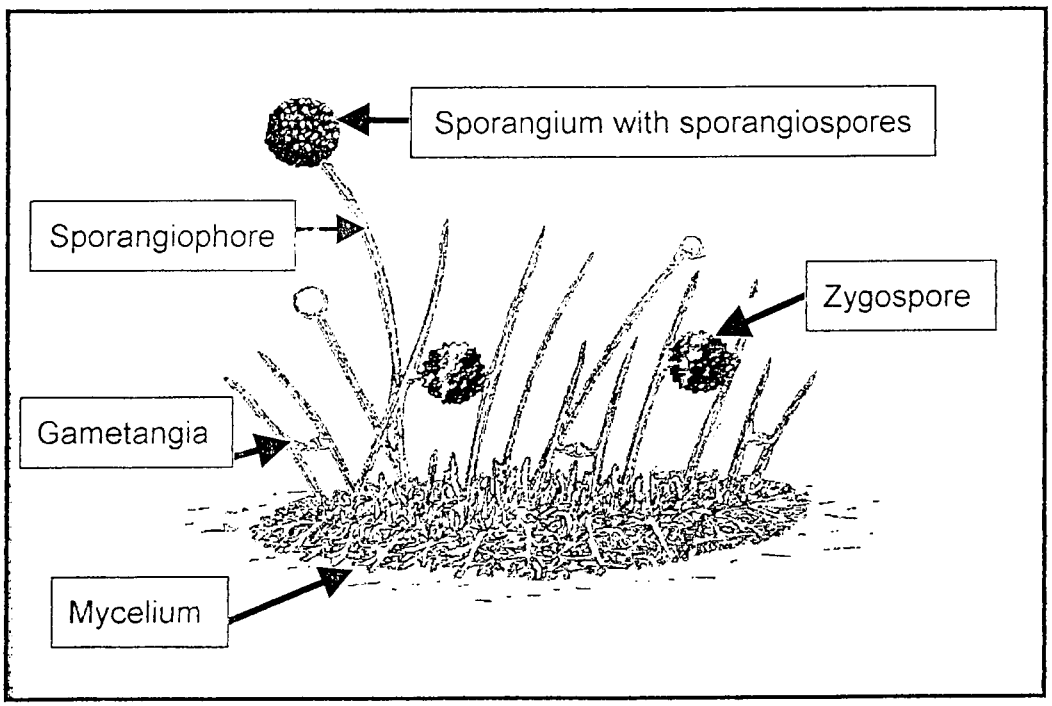

Fig. 1. Different developmental stages present during the life-cycle of mucoralean fungi.

2.2 Materials and methods

2.2.1 Strain

Mucor genevensis (MUFS 038), a homothallic species, isolated from soil in the Knysna forest in South Africa, was used in this study. The strain is held in the Mucoralean Culture Collection at the Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein, South Africa.

2.2.2 Cultivation and harvesting

A spore suspension of the fungus was prepared from seven day old cultures grown on a solid synthetic medium, containing 10 $g.l^{-1}$ glucose, 6.7 $g.l^{-1}$ YNB and 20 $ml.l^{-1}$ vitamin solution (Van der Walt & Yarrow 1984). This served as inoculum for 63 Petri dishes containing the same synthetic medium. Cultures were incubated at 20°C. Biomass was periodically obtained by harvesting nine cultures simultaneously at two day intervals. The Petri dishes with growth were frozen overnight and the biomass scraped of and freeze dried. The rest of the original spore suspension was filtrated and the sporangiospores frozen, freeze dried and weighed. To harvest the zygospores, the biomass of 20 Petri dishes, containing 12 day old colonies, was flash-frozen with liquid nitrogen, broken and suspended in distilled water. The suspension was repeatedly centrifuged at 500 x *g* for ten seconds and the supernatant (containing sporangiospores and hyphal pieces) discarded. The pellet was examined microscopically to verify that it only contained zygospores. The zygospores were also freeze dried and weighed.

2.2.3 Lipid extraction and fatty acid analyses

Lipids were extracted from the freeze dried biomass, zygospores and sporangiospores using chloroform:methanol (2:1 v/v) (Kendrick & Raltedge 1992) and

washed with distilled water (Folch *et al.* 1957). The organic solvents were evaporated under vacuum, the lipids dissolved in a minimal volume of diethyl ether and transferred to preweighed vials. Prior to determination of lipid weights, samples were dried to constant weight in a vacuum oven over P_2O_5 at 50°C. The extracted lipids were dissolved in chloroform and applied to a column (140 x 20 mm) of clean, activated silicic acid. Neutral, glyco- and phospholipids were eluted by the application of organic solvents as described by Kendrick and Ratledge (1992). The samples were dissolved in chloroform and methylated with trimethyl sulphonium hydroxide (TMSH) (Butte 1983). The fatty acid methyl esters were analysed using a Varian 3300 gas chromatograph equipped with a polar Supelcowax 10 glass capillary column (0.75 mm x 30 m) with N_2 (5 $ml.min^{-1}$) as carrier gas (Kock 1988). Peaks were identified by reference to authentic standards.

2.2.4 Statistical analyses

In all cases Student-T tests were performed to determine the significance of the differences between data averages. Only significant differences (p value < 0.5) are discussed (Schefler 1979).

2.3 Results and discussion

Periodic microscopic examination of the fungal cultures, grown on the solid synthetic medium revealed that, within two days of inoculation, the sporangiospores had germinated and had given rise to mycelium. After six days of incubation, sporogenesis had occurred and mature sporangiospores were released by sporangia formed on the mycelium. After 12 days, zygospores were observed in the mycelium. The changes in lipid content during the 12 day incubation period are depicted in Figure 2. Interestingly, a statistically significant decrease in lipid content was observed after six days of incubation. This decrease coincided with the maturation and release of sporangiospores. Since the sporangiospores contributed less than 0.1% (w/w) towards the total biomass on day six [0.035 (± 0.001) g sporangiospores

obtained from 40.5 (± 1.67) g biomass], the decrease in total lipids could not be ascribed to the release and loss of sporangiospores. Therefore, the results indicate that lipids were utilised during sporogenesis. This finding is in contrast with results regarding lipid accumulation during asexual sporogenesis of *Achlya ambisexualis* (Law & Burton 1976). However, Kock and Ratledge (1993) examined the changes in lipid composition during the life-cycle of *Dipodascopsis uninucleata* and recorded a decrease in the lipid content of the biomass from 7.2 $mg.g^{-1}$ biomass (during vegetative growth) to 3.4 $mg.g^{-1}$ biomass (during plasmogamy and the early stages of sporogenesis). Furthermore, it was found that reserve materials such as lipid droplets and glycogen, were accumulated during early stages of ascosporogenesis in *Saccharomyces cerevisiae*, but diminished during later stages of sporogenesis (Kane & Roth 1974; Aon *et al.* 1996).

A decrease in the neutral, glyco- and phospholipids after six days of incubation, indicated that all three fractions were involved in the process(es) of lipid utilisation (Fig. 3). The increase in lipids in the culture upon further incubation, may be due to continued production of cellular lipids. A second decrease in lipids could be observed after another six days (Fig. 2) which coincided with the production of zygospores in the culture. Similar to the findings after six days of incubation, all three lipid fractions had decreased by day 12. (Fig. 3).

It is known that acetate is an important precursor for the biosynthetic reactions that occur during sporogenesis of *Saccharomyces cerevisiae* (Esposito *et al.* 1969). It may, therefore, be possible that through β-oxidation of fatty acids (Mathews & Van Holde 1990), the observed lipid utilisation during sporogenesis of *M. genevensis* provides the necessary procursor (i.e. acetyl-CoA) for biosynthetic reactions as well as energy in the form of adenosine triphosphate (ATP). This may, however, not be the only possible explanation for the observed decrease in lipids. Cellular lipids may also be metabolised to oxygenated lipids, such as hydroxy fatty acids. Oxygenated fatty acids, such as prostaglandins and 3-hydroxy fatty acids, have been implicated as regulatory molecules during various developmental stages in the life-cycles of a

number of unrelated fungi and as such may be ubiquitous regulatory molecules in the fungal domain (Herman & Herman 1985; Kerwin *et al.* 1986; Herman *et al.* 1989; Herman & Luchini 1989; Kock *et al.* 1991; Mazur *et al.* 1991; Coetzee *et al.* 1992; Jensen *et al.* 1992; Van Dyk *et al.* 1994; Kock *et al.* 1998).

Fatty acid analyses of the cellular lipid fractions gave the results depicted in Figures 4 to 6. In all three fractions the major fatty acids were palmitic acid (16:0) and oleic acid [18:1(ω9)]. The other fatty acids were palmitoleic acid [16:1(ω7)], stearic acid (18:0), linoleic acid [18:2(ω6)], α-linolenic acid [18:3(ω3)] and γ-linolenic acid [18:3(ω6)]. This fatty acid composition is typical of a fungus of the Mucorales (Van der Westhuizen *et al.* 1994).

Similar to the results of others, recorded in literature (Jeffery *et al.* 1995), the percentage fatty acids in the neutral lipids remained relatively constant during growth (Fig. 4). The same phenomenon was observed for the percentage fatty acids in the glycolipids (Fig. 5).

As was found in the glycolipid and neutral lipid fractions, the percentages 16:0, 16:1(ω7), 18:0, 18:2(ω6), 18:3(ω6) and 18:3(ω3) in the phospholipids, remained relatively stable over the incubation period. Changes did, however, occur in the percentage 18:1(ω9) in the phospholipids. During the first six days of incubation, no significant change could be observed in the percentage of this fatty acid, but after six days of incubation, the percentage increased from 26.00 (± 4.31) % to 44.65 (± 3.89) % after eight days of incubation. This was followed by a decrease to 24.14 (± 0.74) % after 12 days of incubation (Fig. 6).

It is important to note that the lipid and fatty acid compositions depicted in Figures 2 to 6 represent that of the total culture. The only developmental stages of which the lipid composition could be exclusively determined, were the sporangiospores, the mycelium containing no sporangia and the zygospores harvested from the mycelium. Figure 7 illustrates the percentages of the neutral, glyco- and phospholipids in the

lipids of these three stages. No significant differences could be detected in the percentage neutral lipids between the sporangiospores, mycelium and zygospores. The neutral lipid fraction of these developmental stages was the largest lipid fraction and ranged from *circa* 44 % (w/w) to *circa* 55 % (w/w). The mycelium contained a smaller percentage glycolipids [*circa* 27 % (w/w)] than the zygospores [*circa* 39 % (w/w)]. The percentage phospholipids of the sporangiospores was *circa* 22 % (w/w) and that of the mycelium was similar at *circa* 18 %.(w/w). The zygospores contained less phospholipids [*circa* 11 % (w/w)].

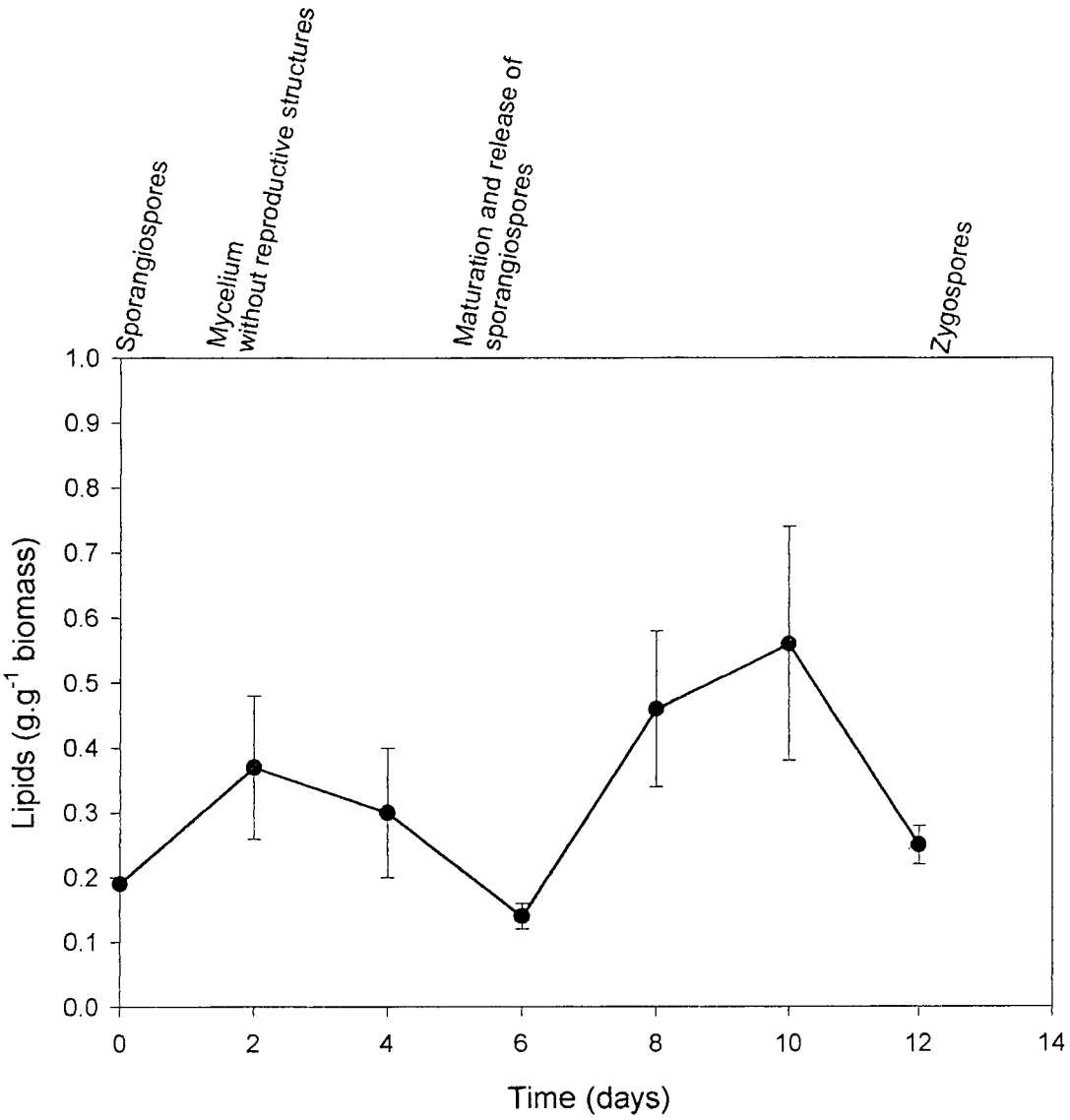

Fig. 2. Changes in the amount of lipids in the biomass of *M. genevensis* during the incubation period. Each point is the mean of three repetitions. The standard deviations are indicated by the bars and the major developmental stages present during a specific day are indicated across the top.

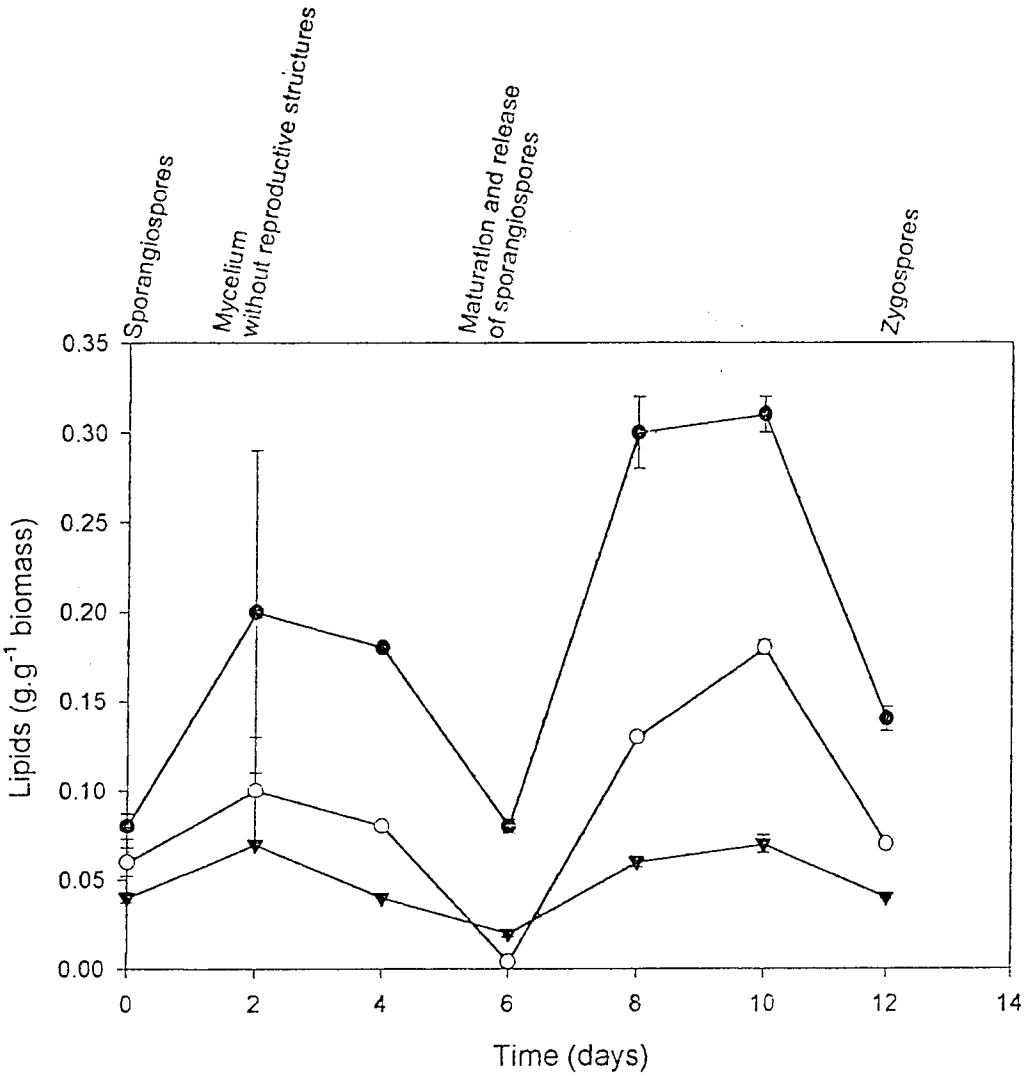

Fig. 3. Changes in the amount of lipids in the different lipid fractions of *M. genevensis* during the incubation period. Each point is the mean of three repetitions. The standard deviations are indicated by the bars and the major developmental stages present during a specific day are indicated across the top.

● = Neutral lipids; ○ = Glycolipids; ▼ = Phospholipids

The long-chain fatty acid composition of the different lipid fractions of the developmental stages are depicted in Figures 8 to 10. As was found when the fatty acids of the whole culture were analysed, the major fatty acids of the developmental stages were 16:0 and 18:1(ω9). When comparing the percentages of the PUFAs in the neutral lipids of the developmental stages, it was observed that the neutral lipids of the zygospores contained a lower percentage 18:3(ω6) than the sporangiospores. However, no significant differences could be detected between the percentages of PUFAs in the mycelium and the sporangiospores (Fig. 8). This is in accordance with results obtained by Gordon and co-workers (1971) who found that the sporangiospores of *M. genevensis* had a lipid composition similar to that of the actively growing mycelium. Although 18:3(ω3) could be detected in the neutral lipids of the sporangiospores and mycelium, this PUFA could not be detected in the neutral lipids of the zygospores.

When comparing the fatty acid composition of glycolipids (Fig. 9), it was found that all three the developmental stages had a similar fatty acid composition regarding 16:0, 16:1(ω7), 18:0, 18:1(ω9) and 18:2(ω6). However, the percentage 18:3(ω6) in the zygospores was lower than the percentage 18:3(ω6) in the glycolipids of the sporangiospores and mycelium. No 18:3(ω3) could be detected in the glycolipids of the zygospores.

The phospholipids of the zygospores contained a higher percentage of the unsaturated fatty acid, 18:0 than the mycelium and the sporangiospores. No 18:3(ω3) could be detected in the phospholipids of the zygospores (Fig. 10).

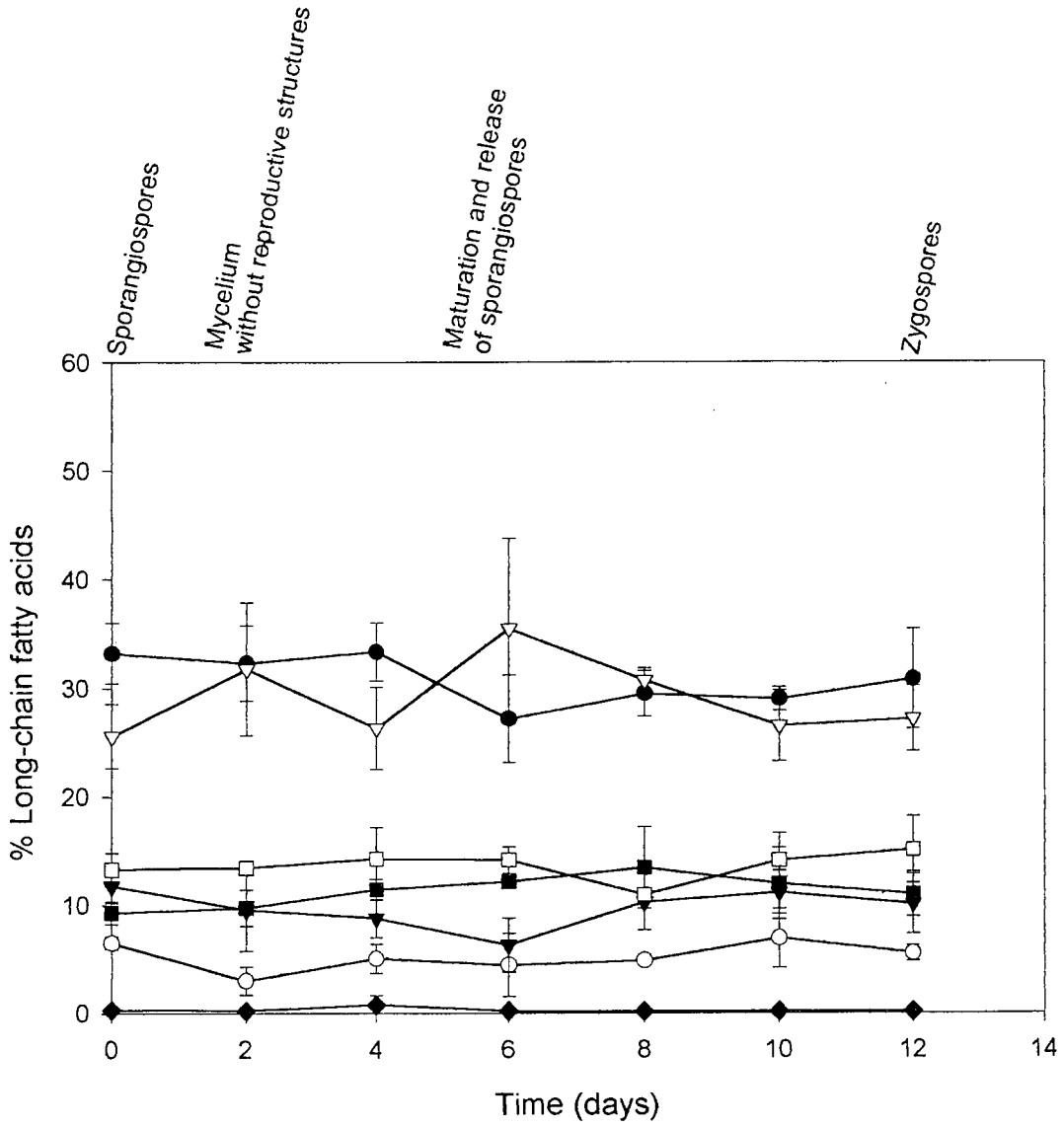

Fig. 4. Changes in percentage long-chain fatty acids in the neutral lipids of *M. genevensis* during the incubation period. Each point is the mean of three repetitions. The standard deviations are indicated by the bars and the major developmental stages present during a specific day are indicated across the top.

● = 16:0; ○ = 16:1(ω7); ▼ = 18:0; ▽ = 18:1(ω9); ■ = 18:2(ω6); ❐ = 18:3(ω6); ◆ = 18:3(ω3)

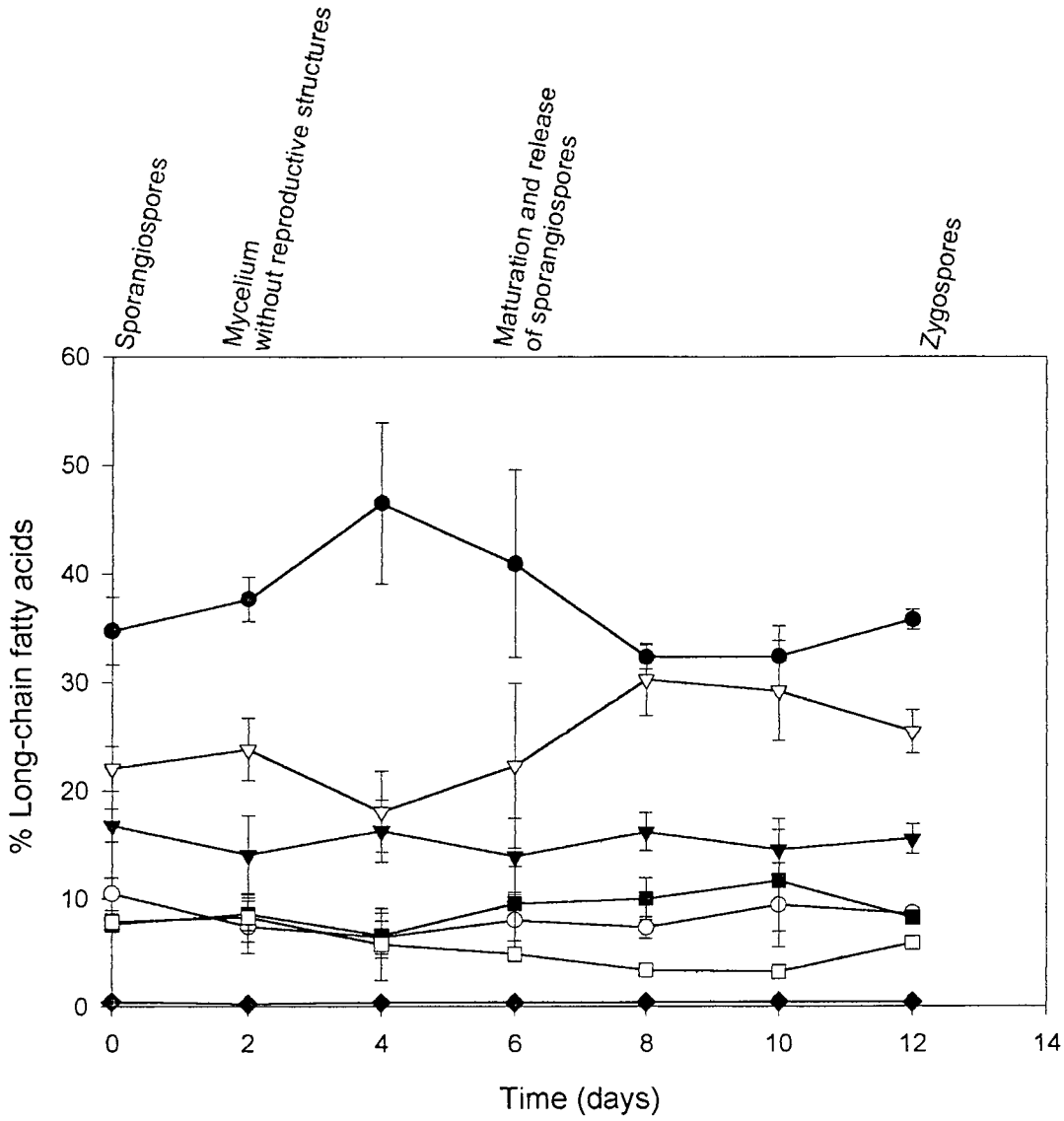

Fig. 5. Changes in percentage long-chain fatty acids in the glycolipids of *M. genevensis* during the incubation period. Each point is the mean of three repetitions. The standard deviations are indicated by the bars and the major developmental stages present during a specific day are indicated across the top.

● = 16:0; ○ = 16:1(ω7); ▼ = 18:0; ▽ = 18:1(ω9); ■ = 18:2(ω6); ❐ = 18:3(ω6); ◆ = 18:3(ω3)

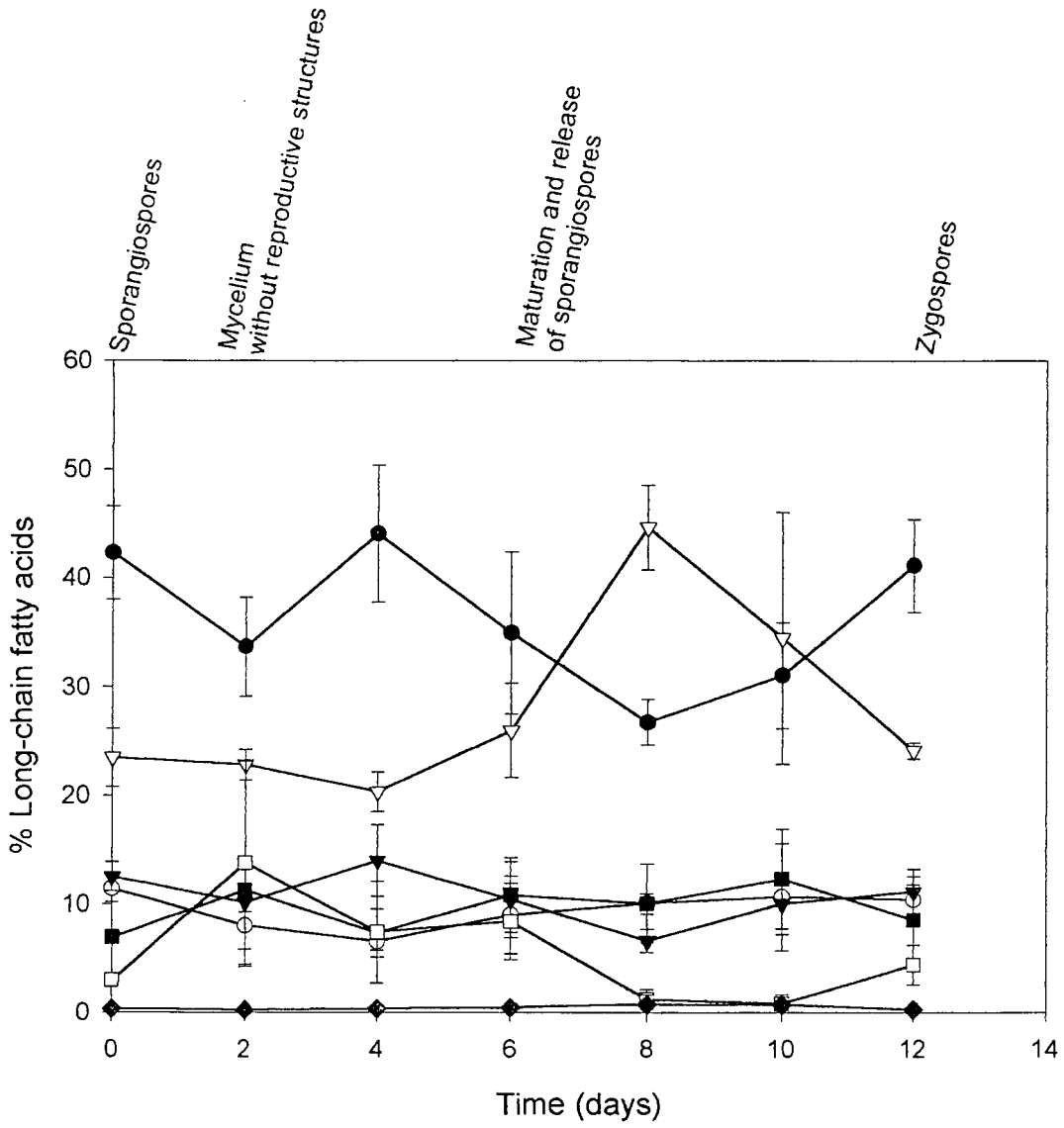

Fig. 6. Changes in percentage long-chain fatty acids in the phospholipids of *M. genevensis* during the incubation period. Each point is the mean of three repetitions. The standard deviations are indicated by the bars and the major developmental stages present during a specific day are indicated across the top.

● = 16:0; ○ = 16:1(ω7); ▼ = 18:0; ▽ = 18:1(ω9); ■ = 18:2(ω6); ❐ = 18:3(ω6); ◆ = 18:3(ω3)

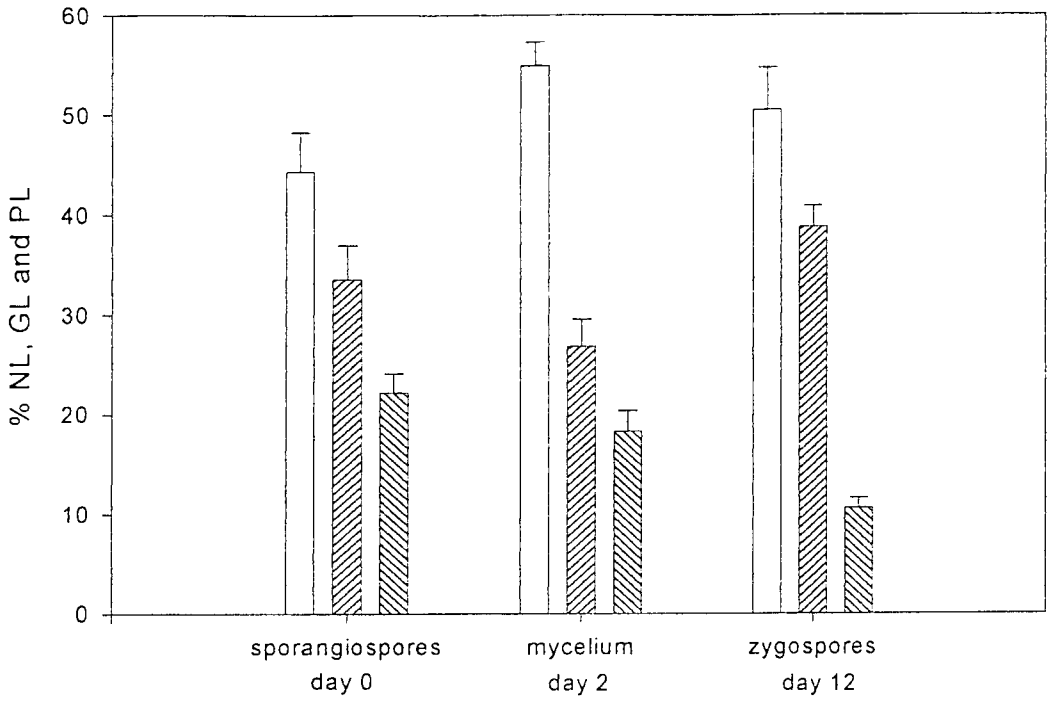

Fig. 7. Percentage neutral, glyco- and phospholipids in the sporangiospores, mycelium and zygospores of *M. genevensis*. Each point is the mean of three repetitions. The standard deviations are indicated by the bars.

= Neutral lipids; = Glycolipids; = Phospholipids

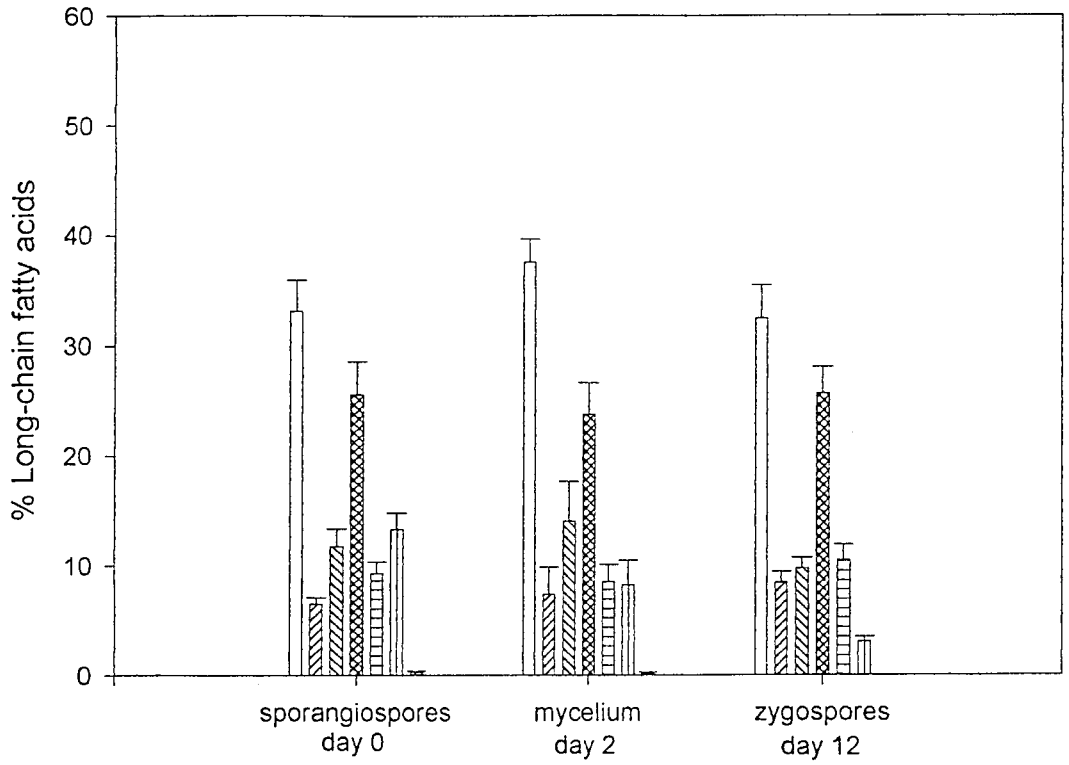

Fig. 8. Percentage long-chain fatty acids in the neutral lipids of the sporangiospores, mycelium and zygospores of *M. genevensis*. Each point is the mean of three repetitions. The standard deviations are indicated by the bars.

= 16:0; = 16:1(ω7); = 18:0; = 18:1(ω9);

= 18:2(ω6); = 18:3(ω6); = 18:3(ω3)

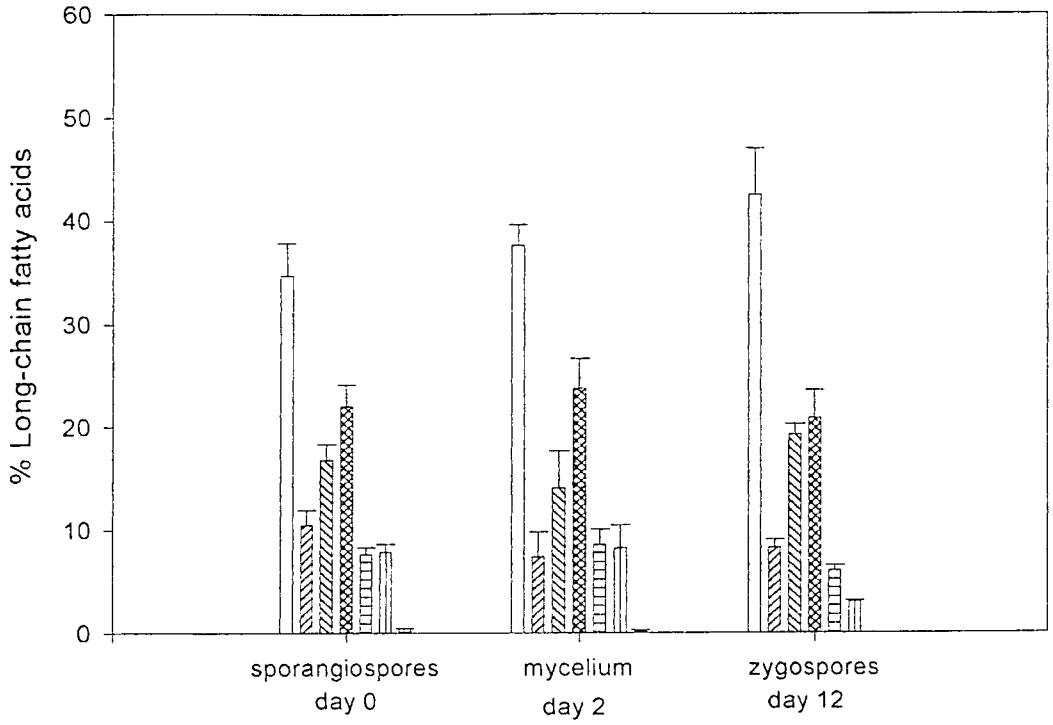

Fig. 9. Percentage long-chain fatty acids in the glycolipids of the sporangiospores, mycelium and zygospores of *M. genevensis*. Each point is the mean of three repetitions. The standard deviations are indicated by the bars.

= 16:0; = 16:1(ω7); = 18:0; = 18:1(ω9);

= 18:2(ω6); = 18:3(ω6); = 18:3(ω3)

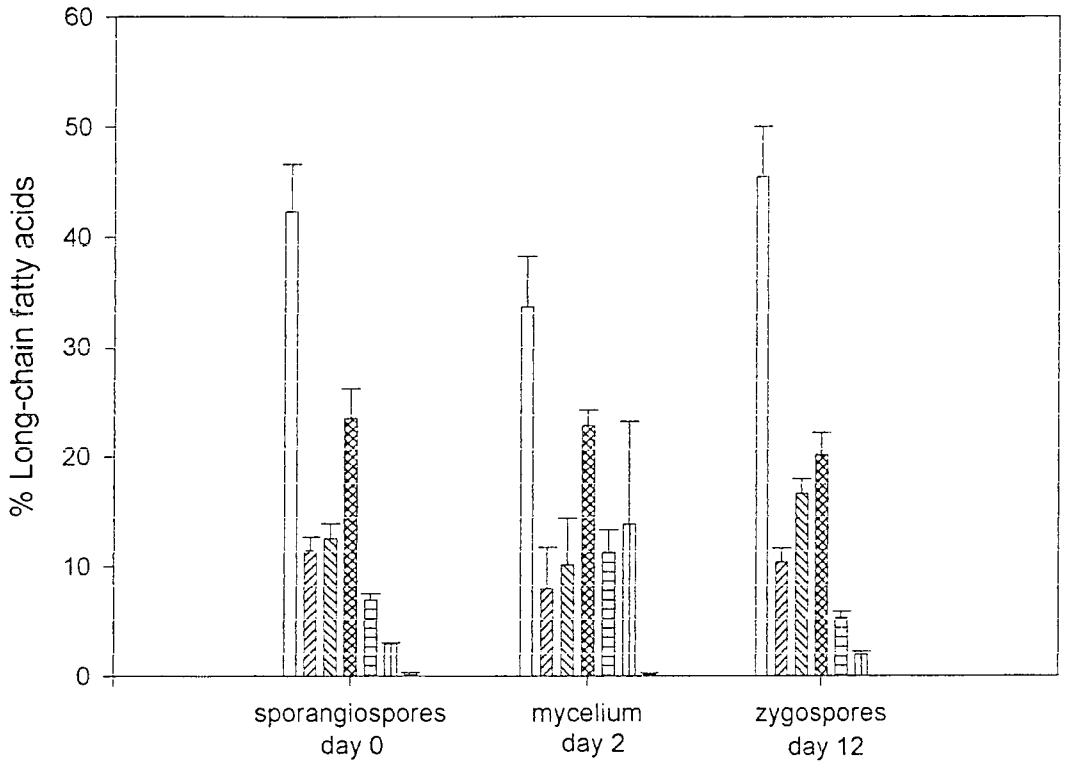

Fig. 10. Percentage long-chain fatty acids in the phospholipids of the sporangiospores, mycelium and zygospores of *M. genevensis*. Each point is the mean of three repetitions. The standard deviations are indicated by the bars.

= 16:0, = 16:1(ω7); = 18:0, = 18:1(ω9);

= 18:2(ω6), = 18:3(ω6), = 18:3(ω3)

2.4 Conclusions

Through all the growth and developmental stages of the life-cycle of *Mucor genevensis* MUFS 038, the lipid and fatty acid composition remained similar to that of a typical mucoralean fungus. Decreases in all three lipid fractions were observed during sporangiosporogenesis as well as zygosporogenesis. These decreases may be as a result of the production of precursors for structural molecules and/or ATP necessary for sporogenesis and/or as a result of the production of oxygenated fatty acids, involved in the regulation of sporogenesis.

The lipid composition of the sporangiospores and mycelium regarding neutral, glyco- and phospholipids was comparable. Similarly, the fatty acid compositions of the lipid fractions of the sporangiospores and mycelium were alike. This is not surprising since mucoralean sporangiospores act mainly as dispersal and to a lesser extent as survival structures (Parkinson & Waid 1960; Ingold & Zoberi 1963; Dennis & Blijham 1980). Therefore the lipid composition of these structures would be expected to be similar to that of actively growing mycelium. In contrast, the percentages of phospholipids and PUFAs in the zygospores were significantly less than that of the sporangiospores. This correlates with the fact that zygospores are dormant structures designed to survive prolonged periods of adverse conditions (Michailides & Spotts 1988). The presence of fewer membranes with a lower degree of fluidity in these structures, is, therefore, understandable (Kock & Ratledge 1993).

Having studied the changes in endogenous lipids during growth and development of the various reproductive stages in *M. genevensis*, the aim of the next chapter was to investigate the uptake and incorporation of exogenous PUFAs such as 20:4(ω6), into the biomass of *M. genevensis* and another mucoralean fungus, *Rhizopus oryzae.*

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Chapter 3

Arachidonic acid uptake and incorporation by *Mucor genevensis* and *Rhizopus oryzae*

3.1 Introduction

In a search for novel biologically active compounds among fungi, it was recently discovered that, when challenged with exogenous polyunsaturated fatty acids (PUFAs), the yeast, *Dipodascopsis uninucleata,* can produce a series of long-chain hydroxy fatty acids (Kock *et al.* 1997; Venter *et al.* 1997). One of these products, 3-hydroxy-5,8,11,14-eicosatetraenoic acid, has already been shown to be biologically active. These experiments were, however, preceded by studies on the uptake and turnover of PUFAs such as arachidonic acid [20:4(ω6)] in the lipids of *D. uninucleata* (Kock & Ratledge 1993). These experiments were conducted in order to obtain basic knowledge regarding the initial steps of 20:4(ω6) catabolism in the yeast, with the ultimate aim of manipulating the yeast to produce significant quantities of certain long-chain hydroxy fatty acids.

A group of fungi, known for the utilisation of exogenous lipids, including long-chain fatty acids, is the mucoralean fungi (Weiss 1983; Kolte 1985; Berglund 1994). Members of the order Mucorales demonstrated the ability to further elongate and desaturate fatty acids taken up from the medium (Jeffery *et al.* 1997) and this ability has been exploited in the fungal biotransformation of vegetable oil, containing PUFAs, to higher value oils (Certik *et al.* 1997). However, unlike the studies on *Dipodascopsis uninucleata*, the uptake and incorporation of PUFAs into the cellular lipids, as well as the subsequent oxidative degradation of these fatty acids, have not been studied in detail in mucoralean fungi.

The aim of this study was therefore, to investigate the uptake and incorporation of 20:4(ω6) into the various lipid fractions of two mucoralean fungi, *Mucor genevensis* and *Rhizopus oryzae*. In addition, the effect of exogenous 20:4(ω6) on the endogenous long-chain PUFAs of the neutral, glyco- and phospholipids was documented.

3.2 Materials and methods

3.2.1 Strains

Mucor genevensis (MUFS 038), isolated from soil in the Knysna forest in South Africa (held in the Mucoralean Culture Collection at the Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein, South Africa), and *Rhizopus oryzae* (PPRI 5172) (held in the National Culture Collection, Pretoria, South Africa) were used in this study.

3.2.2 Analyses of cellular lipids and exogenous 20:4(ω6)

3.2.2.1 Cultivation

Eighty four cultures of each fungal strain were prepared by inoculation of yeast-malt-extract gelatine (YMG) in Petri dishes. The YMG consisted of 10 $g.l^{-1}$ glucose, 3 $g.l^{-1}$ yeast extract, 3 $g.l^{-1}$ malt extract and 5 $g.l^{-1}$ peptone. Gelatine (100 $g.l^{-1}$) was used as solidifying agent. Cultures were incubated at 20°C for seven days. During this period the sexual stage (i.e. zygospores) of *M. genevensis* became visible. The colonies of both strains were harvested by melting the gelatine in a water bath (30°C), followed by filtration. For each strain, six colonies were transferred to each of seven Roux flasks, containing 375 ml physiological salt solution [0.85 % NaCl, (w/v)] (PSS). The biomass was challenged with 20:4(ω6) by adding it in ethanolic solution (1 g fatty acid in 100 ml ethanol) (Kock *et al.* 1997) to each flask, resulting in a final 20:4(ω6)

concentration of 10 $mg.l^{-1}$. The flasks were incubated at 25°C for six hours (Kock *et al.* 1997). The biomass was harvested by filtration at one hour intervals, washed and freeze dried.

3.2.2.2 Lipid extraction and fatty acid analyses

Lipids were extracted from the freeze dried biomass using chloroform:methanol (2:1 v/v) (Kendrick & Raltedge 1992) and washed with distilled water (Folch *et al.* 1957). The organic solvents were evaporated under vacuum. The lipids were dissolved in a minimal volume of diethyl ether and transferred to preweighed vials. Prior to determination of lipid weights, samples were dried to constant weight in a vacuum oven over P_2O_5 at 50°C. The extracted lipids were dissolved in chloroform and applied to a column (140 x 20 mm) of clean, activated silicic acid. Neutral, glyco- and phospholipids were eluted by the application of organic solvents, as described by Kendrick and Ratledge (1992). An internal standard [lauric acid (12:0)] was included before the samples were dissolved in chloroform and methylated with trimethyl sulphonium hydroxide (TMSH) (Butte 1983). The fatty acid methyl esters were analysed using a Varian 3300 gas chromatograph equipped with a polar Supelcowax 10 glass capillary column (0.75 mm x 30 m) with N_2 (5 $ml.min^{-1}$) as carrier gas (Kock 1988). Peaks were identified by reference to authentic standards.

Exogenous lipids in the PSS were extracted with ethyl acetate (Nigam 1987), which was subsequently evaporated under vacuum. The lipids were dissolved in a minimal volume of diethyl ether and transferred to preweighed vials. Prior to determination of lipid weights, samples were dried to constant weight in a vacuum oven over P_2O_5 at 50°C. Lauric acid was included as internal standard before the samples were dissolved in chloroform and methylated with TMSH. The fatty acid methyl esters were analysed as described above.

3.2.3 Determination of the percentage free 20:4(ω6) in the cellular neutral lipids and medium

3.2.3.1 Cultivation

Seven day old cultures of the two fungal strains growing at 20°C on YMG, were harvested as described in the section "3.2.2 Analyses of cellular lipids and exogenous 20:4(ω6)". For each strain, six colonies were transferred to each of two Roux flasks, containing 375 ml PSS. The biomass was challenged with 20:4(ω6) by adding it in ethanolic solution (1 g fatty acid in 100 ml ethanol) (Kock *et al.* 1997) resulting in a final 20:4(ω6) concentration of 10 $mg.l^{-1}$. The flasks were incubated at 25°C (Kock *et al.* 1997). The biomass was harvested by filtration after two and six hours and freeze dried.

3.2.3.2 Lipid extraction and fatty acid analyses

The neutral lipids of both fungal strains were extracted using hexane (Lomascolo *et al.* 1994). The resulting organic phase was divided into two aliquots, 12:0 was added as internal standard to each and the samples were dried under a stream of N_2. The contents of one aliquot was dissolved in chloroform and methylated with TMSH (Butte 1983). The contents of the other aliquot was dissolved in methanol and methylated with diazomethane (Kock *et al.* 1997). The fatty acid methyl esters were analysed using a Varian 3300 gas chromatograph equipped with a polar Supelcowax 10 glass capillary column (0.75 mm x 30 m) with N_2 (5 $ml.min^{-1}$) as carrier gas (Kock 1988). Peaks were identified by reference to authentic standards.

Exogenous lipids in the PSS were extracted using ethyl acetate (Nigam 1987). In each case, the extract was divided into two aliquots, 12:0 was added as internal standard to each and the samples were dried under a stream of N_2. The contents of one aliquot was dissolved in chloroform and methylated with TMSH. The contents of the other aliquot was dissolved in methanol and methylated with diazomethane. The

fatty acid methyl esters were analysed as above.

3.2.4 Statistical analyses

In all cases Student-T tests were performed to determine the significance of the differences between data averages. Only significant differences (p value < 0.5) are discussed (Schefler 1979).

3.3 Results and discussion

3.3.1 Arachidonic acid uptake by biomass

The concentration of the exogenous 20:4(ω6) in the cultures of both *M. genevensis* and *R. oryzae* decreased significantly during the first hour of incubation (Fig. 1 & Fig. 2). A similar phenomenon was observed by Kock and Ratledge (1993) when 20:4(ω6) was fed to cultures of *Dipodascopsis uninucleata.* These authors found that more than 90 % of the 20:4(ω6) was taken up by this fungus within the first hour of incubation.

In the present study, this decrease in exogenous 20:4(ω6) was not accompanied by a similar increase in this PUFA in the neutral, glyco- or phospholipids of the two fungi after one hour of incubation. This overall decrease in 20:4(ω6) in the cultures may be ascribed to the catabolism of the PUFA, since no such decrease in 20:4(ω6) in the cultures (including biomass and medium) of both strains could be detected when this PUFA was fed to autoclaved biomass.

The largest lipid fraction in both fungi was the neutral lipids which, throughout the six hour incubation period, comprised 54.60 (± 6.27) % (w/w) and 68.21 (± 2.64) % (w/w) of the crude lipid extract of *M. genevensis* and *R. oryzae,* respectively. After one hour of incubation, 20:4(ω6) was taken up and detected in the neutral lipids of *M.*

genevensis as well as in the neutral lipids of *R. oryzae* (Fig. 1 & Fig. 2). When the concentration of 20:4(ω6) (w/w) in the cellular neutral lipids of *M. genevensis* was calculated, a significant increase in the concentration of 20:4(ω6) was detected over the incubation period (Table 1). Similarly, a significant increase could be observed in the 20:4(ω6) concentration in the neutral lipids of *R. oryzae* (Table 2).

The glycolipids of *M. genevensis* comprised 24.59 (± 5.09) % (w/w) of the total crude lipid extract throughout the incubation period, while the phospholipids comprised 20.57 (± 0.94) % (w/w). No significant increase in the concentration of 20:4(ω6) associated with the glyco- and phospholipids of *M. genevensis,* could be noted between the time it was first detected (i.e. two hours) and after six hours of incubation (Table 1).

The glycolipids of *R. oryzae* comprised 16.29 (± 1.45) % (w/w) of the crude lipid extract throughout the incubation period, while the phospholipids of this fungus during the incubation period, comprised 15.28 (± 1.51) % (w/w). Arachidonic acid was detected in the glyco- and phospholipids of *R. oryzae* after only one hour of incubation (Table 2). The concentration of 20:4(ω6) in the glyco- and phospholipids of *R. oryzae* increased significantly during the six hour incubation period (Table 2).

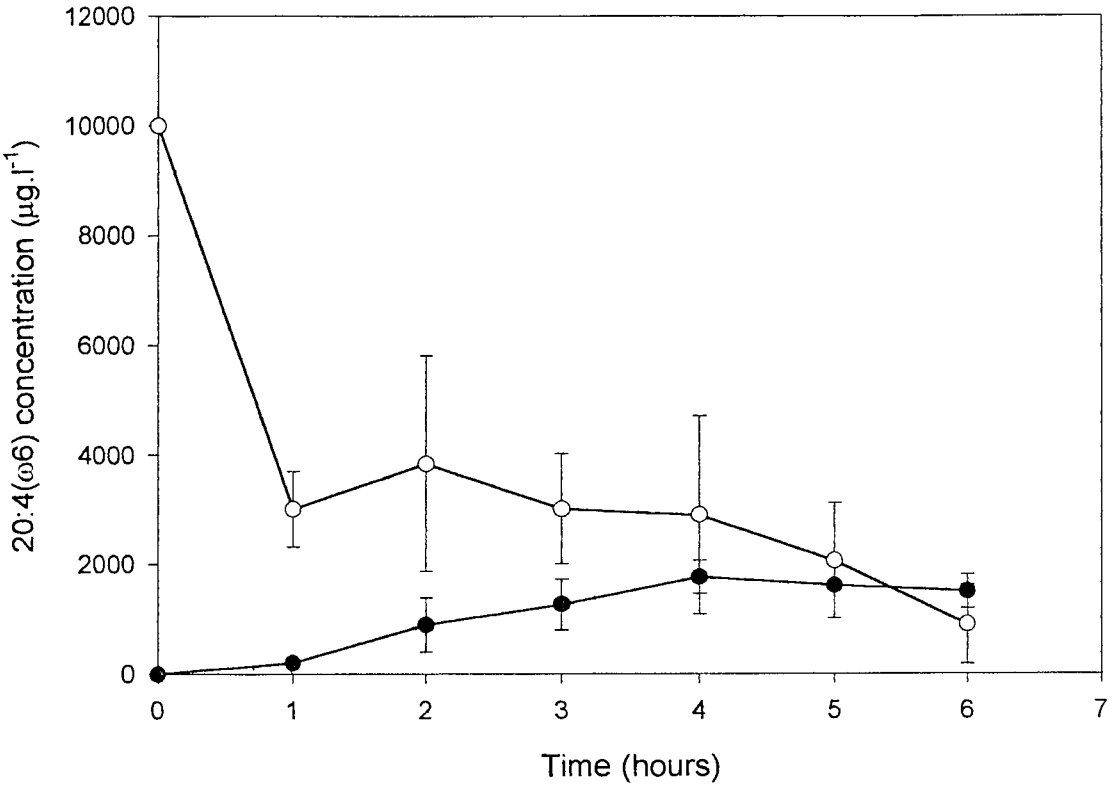

Fig. 1. Volumetric concentration of 20:4(ω6) present in the medium and in the cellular neutral lipids of *M. genevensis*. Each point represents the mean of three repetitions. The standard deviations are indicated by the bars.

● = 20:4(ω6) concentration in the cellular neutral lipids; ○ = 20:4(ω6) concentration in the medium

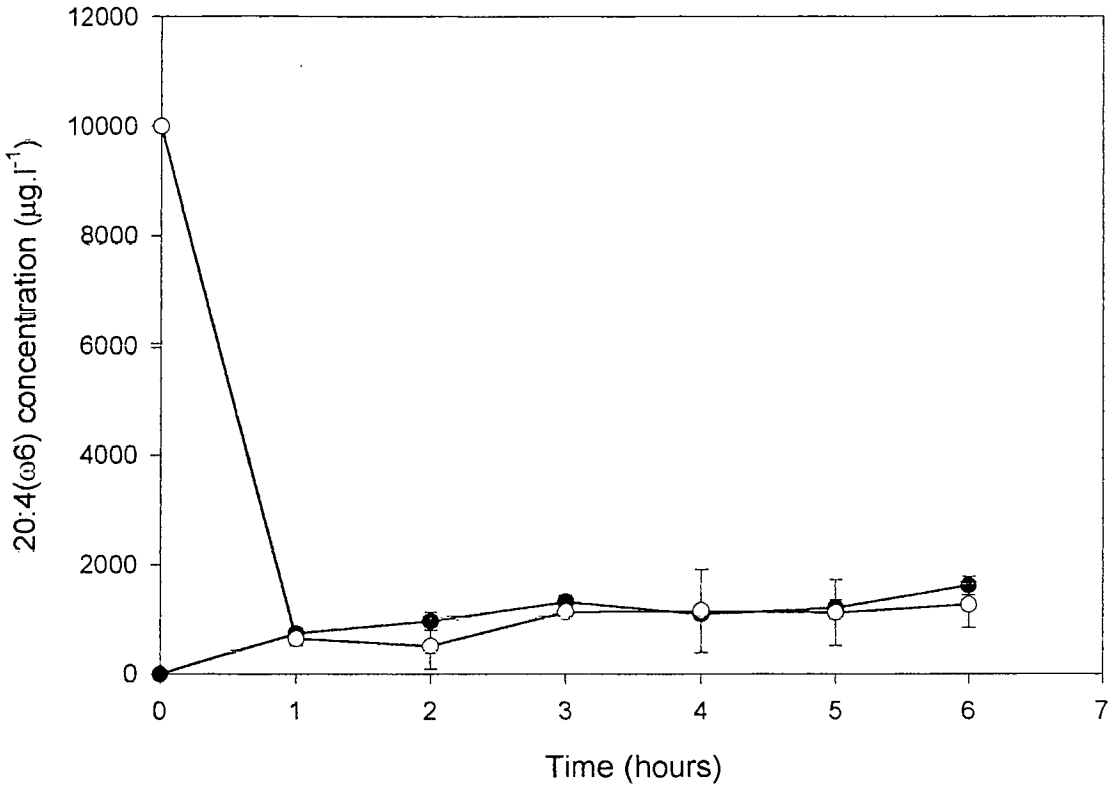

Fig. 2. Volumetric concentration of 20:4(ω6) present in the medium and in the cellular neutral lipids of *R. oryzae*. Each point represents the mean of three repetitions. The standard deviations are indicated by the bars.

● = 20:4(ω6) concentration in the cellular neutral lipids; ○ = 20:4(ω6) concentration in the medium

Table 1. Concentration of 20:4(ω6) in the lipid fractions of *M. genevensis* upon initial detection and after six hours of incubation.

Lipid fraction	20:4(ω6) μg.g^{-1} biomass upon initial detection		20:4(ω6) μg.g^{-1} biomass after six hours of incubation
Neutral lipids	1 hour	86.63 (20.01)	931.10 (155.05)
Glycolipids	2 hours	100.20 (27.00)	106.51 (6.49)
Phospholipids	2 hours	46.12 (14.02)	71.58 (3.78)

Values represent the mean of three repetitions. The standard deviations are indicated in brackets.

Table 2. Concentration of 20:4(ω6) in the lipid fractions of *R. oryzae* upon initial detection and after six hours of incubation.

Lipid fraction	20:4(ω6) μg.g^{-1} biomass upon initial detection		20:4(ω6) μg.g^{-1} biomass after six hours of incubation
Neutral lipids	1 hour	4.23 (1.32)	118.74 (16.20)
Glycolipids	1 hour	1.05 (0.22)	10.94 (0.03)
Phospholipids	1 hour	20.46 (5.68)	109.80 (0.15)

Values represent the mean of three repetitions. The standard deviations are indicated in brackets.

From the results depicted in Table1 and Table 2, it was evident that more 20:4(ω6) was incorporated into the lipid fractions of *M. genevensis* after six hours of incubation, than into the lipid fractions of *R. oryzae*. However, more 20:4(ω6) was incorporated into the membranes of *R. oryzae*, than into the membranes of *M. genevensis* (Table 1 & Table 2). More 20:4(ω6) was incorporated into the glycolipids of *M. genevensis*, than into the glycolipids of *R. oryzae*. Similarly, after six hours, more 20:4(ω6) was incorporated into the neutral lipids of *M. genevensis*, than into the neutral lipids of *R. oryzae*. It must be borne in mind that the neutral lipids are not only comprised of acylglycerols, but also contain free fatty acids (Ratledge 1994).

3.3.2 The percentage free 20:4(ω6) in the cellular neutral lipids and medium

The percentage free 20:4(ω6) in the cellular neutral lipids of *M. genevensis* and *R. oryzae* are depicted in Figure 3. After two hours of incubation 100 % of the 20:4(ω6) in the neutral lipids of *M. genevensis* was still in the free form. After 6 hours of incubation, no significant change could be detected in this percentage.

After two hours of incubation, *circa* 65 % of the 20:4(ω6) in the cellular neutral lipids of *R. oryzae* was in the free form (Fig. 3). This percentage decreased to *circa* 48 % after six hours of incubation. These results indicate that *R. oryzae* has a greater ability than *M. genevensis*, to reduce the amount of free 20:4(ω6) in its cells. All the 20:4(ω6) in the medium was in the free form.

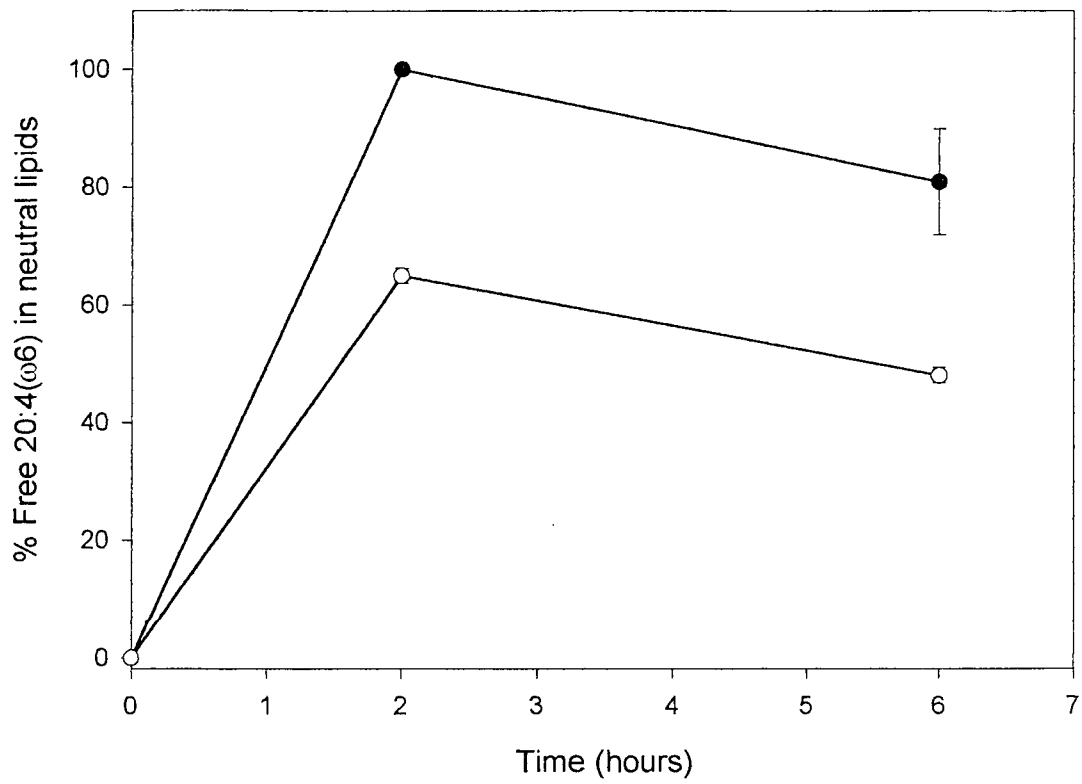

Fig. 3. Percentage free 20:4(ω6) in the cellular neutral lipids of *M. genevensis* and *R. oryzae*. Free 20:4(ω6) was determined as a percentage of the total 20:4(ω6) in the cellular neutral lipids. Each point represents the mean of three repetitions. The standard deviations are indicated by the bars.

● = % free 20:4(ω6) in the cellular neutral lipids of *M. genevensis*; ○ = % free 20:4(ω6) in the cellular neutral lipids of *R. oryzae*

3.3.3 Effect of exogenous 20:4(ω6) on the fatty acid profiles of the lipid fractions

The fatty acid compositions of the neutral, glyco- and phospholipid fractions of *M. genevensis* and *R. oryzae,* at the start and after six hours of incubation, are depicted in Tables 3 to 8.

3.3.3.1 Cellular neutral lipid fatty acid composition

After six hours of incubation in the presence of exogenous 20:4(ω6), the only fatty acids in the cellular neutral lipids of *M. genevensis* that showed significant changes in concentration, were oleic acid [18:1(ω9)] and linoleic acid [18:2(ω6)]. Both these fatty acids increased in concentration (Table 3). When *R. oryzae* was incubated under the same conditions, palmitic acid (16:0) was the only fatty acid in the neutral lipids which exhibited a significant change in concentration (Table 4). This fatty acid decreased in concentration during incubation. Although no α-linolenic acid [18:3(ω3)] could be detected in the neutral lipids of these fungi at the start of incubation, this PUFA was detected in the neutral lipids of both fungi after six hours. The reason for the presence of 18:3(ω3) in the cellular neutral lipids, when 20:4(ω6) is fed to the biomass, is unclear and should be investigated further in future studies. The different responses of the neutral lipid fatty acid concentrations during incubation, are indicative of the differences that exist between *M. genevensis* and *R. oryzae* regarding lipid metabolism.

Table 3. Fatty acid concentration ($\mu g.g^{-1}$ biomass) in the neutral lipids of *M. genevensis* at the start of incubation and after six hours of incubation.

Fatty acids	T0	T6
16:0	29380.23 (879.33)	30280.04 (349.29)
16:1(ω7)	6525.97 (501.33)	5721.48 (132.83)
18:0	6080.82 (1056.02)	5460.74 (0.01)
18:1(ω9)	21518.79 (422.67)	23771.43 (354.21)
18:2(ω6)	12615.69 (258.13)	14345.51 (98.40)
18:3(ω6)	13060.85 (2048.01)	16421.57 (206.62)
18:3(ω3)	0.00 (0.00)	339.45 (14.76)
20:4(ω6)	0.00 (0.00)	931.10 (155.05)

Values are the mean of three repetitions. The standard deviations are indicated in brackets.

T0 = Fatty acid concentration at the start of the incubation period; T6 = Fatty acid concentration after six hours of incubation; 16:0 = palmitic acid; 16:1(ω7) = palmitoleic acid; 18:0 = stearic acid; 18:1(ω9) = oleic acid; 18:2(ω6) = linoleic acid; 18:3(ω6) = γ-linolenic acid; 18:3(ω3) = α-linolenic acid; 20:4(ω6) = arachidonic acid

Table 4. Fatty acid concentration ($\mu g.g^{-1}$ biomass) in the neutral lipids of *R. oryzae* at the start of incubation and after six hours of incubation.

Fatty acids	T0	T6
16:0	5864.21 (532.16)	4081.66 (116.31)
16:1(ω7)	596.28 (55.36)	438.26 (54.32)
18:0	2532.48 (550.16)	1551.61 (569.62)
18:1(ω9)	11340.58 (1284.36)	10642.51 (6257.81)
18:2(ω6)	2763.44 (225.31)	2072.45 (214.06)
18:3(ω6)	1800.04 (251.49)	1223.58 (62.76)
18:3(ω3)	0.00 (0.00)	20.70 (2.18)
20:4(ω6)	0.00 (0.00)	118.74 (16.20)

Values are the mean of three repetitions. The standard deviations are indicated in brackets.

T0 = Fatty acid concentration at the start of the incubation period; T6 = Fatty acid concentration after six hours of incubation; 16:0 = palmitic acid; 16:1(ω7) = palmitoleic acid; 18:0 = stearic acid; 18:1(ω9) = oleic acid; 18:2(ω6) = linoleic acid; 18:3(ω6) = γ-linolenic acid; 18:3(ω3) = α-linolenic acid; 20:4(ω6) = arachidonic acid

3.3.3.2 Glycolipid fatty acid composition

The fatty acid concentrations in the glycolipids of *M. genevensis* and *R. oryzae,* at the start and after six hours of incubation, are depicted in Table 5 and Table 6. No significant changes occurred in the concentrations of the fatty acids in the glycolipids of *M. genevensis* (Table 5). In contrast, increases were detected in the concentrations of 16:0, palmitoleic acid [16:1(ω7)] and stearic acid (18:0) in the glycolipids of *R. oryzae.* The concentrations of 18:1(ω9), 18:2(ω6) and γ-linolenic acid [18:3(ω6)] in the glycolipids of this fungus did not change significantly (Table 6). Interestingly, no 18:3(ω3) could be detected in the glycolipids of either fungus (Tables 5 and Table 6).

Table 5. Fatty acid concentration ($\mu g.g^{-1}$ biomass) in the glycolipids of *M. genevensis* at the start of incubation and after six hours of incubation.

Fatty acids	T0	T6
16:0	887.03 (259.34)	845.16 (201.49)
16:1(ω7)	201.11 (33.24)	167.14 (40.78)
18:0	249.09 (20.38)	315.36 (83.33)
18:1(ω9)	646.85 (150.39)	648.06 (195.50)
18:2(ω6)	371.54 (100.87)	250.71 (65.55)
18:3(ω6)	262.20 (50.49)	239.65 (64.21)
20:4(ω6)	0.00 (0.00)	106.51 (6.49)

Values are the mean of three repetitions. The standard deviations are indicated in brackets.

T0 = Fatty acid concentration at the start of the incubation period; T6 = Fatty acid concentration after six hours of incubation; 16:0 = palmitic acid; 16:1(ω7) = palmitoleic acid; 18:0 = stearic acid; 18:1(ω9) = oleic acid; 18:2(ω6) = linoleic acid; 18:3(ω6) = γ-linolenic acid; 20:4(ω6) = arachidonic acid

Table 6. Fatty acid concentration ($\mu g.g^{-1}$ biomass) in the glycolipids of *R. oryzae* at the start of incubation and after six hours of incubation.

Fatty acids	T0	T6
16:0	271.38 (35.21)	1193.55 (261.53)
16:1(ω7)	52.11 (23.65)	173.18 (8.71)
18:0	111.28 (1.02)	564.88 (126.28)
18:1(ω9)	545.12 (55.06)	1447.93 (625.56)
18:2(ω6)	224.54 (41.38)	409.89 (135.77)
18:3(ω6)	163.25 (35.00)	98.61 (65.87)
20:4(ω6)	0.00 (0.00)	10.94 (0.03)

Values are the mean of three repetitions. The standard deviations are indicated in brackets.

T0 = Fatty acid concentration at the start of the incubation period; T6 = Fatty acid concentration after six hours of incubation; 16:0 = palmitic acid; 16:1(ω7) = palmitoleic acid; 18:0 = stearic acid; 18:1(ω9) = oleic acid; 18:2(ω6) = linoleic acid; 18:3(ω6) = γ-linolenic acid; 20:4(ω6) = arachidonic acid

3.3.3.3 Phospholipid fatty acid composition

No significant differences were observed in the concentrations of the 16 carbon fatty acids, 18:0, 18:1(ω9) and 18:2(ω6) in the phospholipids of *M. genevensis,* challenged with 20:4(ω6) (Table 7). However, the concentration of 18:3(ω6) in the phospholipids of this fungus decreased. The only significant change in fatty acid concentration in the phospholipids of *R. oryzae* was an increase in concentration of 18:0 (Table 8). No 18:3(ω3) could be detected in the phospholipids of either fungus (Table 7 and Table 8).

Table 7. Fatty acid concentration ($\mu g.g^{-1}$ biomass) in the phospholipids of *M. genevensis* at the start of incubation and after six hours of incubation.

Fatty acids	T0	T6
16:0	182.25 (50.24)	109.37 (42.68)
16:1(ω7)	45.29 (5.01)	32.96 (16.27)
18:0	34.48 (1.58)	26.16 (6.03)
18:1(ω9)	167.12 (10.29)	126.98 (74.21)
18:2(ω6)	120.79 (20.18)	53.27 (28.41)
18:3(ω6)	97.05 (5.99)	37.72 (19.42)
20:4(ω6)	0.00 (0.00)	71.58 (3.78)

Values are the mean of three repetitions. The standard deviations are indicated in brackets.

T0 = Fatty acid concentration at the start of the incubation period; T6 = Fatty acid concentration after six hours of incubation; 16:0 = palmitic acid; 16:1(ω7) = palmitoleic acid; 18:0 = stearic acid; 18:1(ω9) = oleic acid; 18:2(ω6) = linoleic acid; 18:3(ω6) = γ-linolenic acid; 20:4(ω6) = arachidonic acid

Table 8. Fatty acid concentration ($\mu g.g^{-1}$ biomass) in the phospholipids of *R. oryzae* at the start of incubation and after six hours of incubation.

Fatty acids	T0	T6
16:0	490.25 (125.01)	803.57 (76.03)
16:1(ω7)	60.10 (10.03)	145.92 (35.97)
18:0	92.21 (21.36)	219.08 (0.82)
18:1(ω9)	524.39 (165.55)	894.51 (14.91)
18:2(ω6)	514.11 (115.35)	557.42 (157.27)
18:3(ω6)	320.18 (90.47)	246.57 (28.31)
20:4(ω6)	0.00 (0.00)	109.80 (0.15)

Values are the mean of three repetitions. The standard deviations are indicated in brackets.

T0 = Fatty acid concentration at the start of the incubation period; T6 = Fatty acid concentration after six hours of incubation; 16:0 = palmitic acid; 16:1(ω7) = palmitoleic acid; 18:0 = stearic acid; 18:1(ω9) = oleic acid; 18:2(ω6) = linoleic acid; 18:3(ω6) = γ-linolenic acid; 20:4(ω6) = arachidonic acid

3.4 Conclusions

The exogenous 20:4(ω6) was rapidly taken up by the biomass of *M. genevensis* and *R. oryzae*. The overall concentration of 20:4(ω6) in the cultures of both fungi rapidly decreased due to catabolism of this PUFA. In addition, 20:4(ω6) was incorporated into the neutral, glyco- and phospholipids of the biomass of both fungi. The results further indicate that *R. oryzae* has a greater ability to reduce the amount of free 20:4(ω6) in its cells, than *M. genevensis*.

During incubation with exogenous 20:4(ω6), different changes in the concentrations of 16 and 18 carbon fatty acids were observed for *M. genevensis* and *R. oryzae*, indicating differences in the lipid metabolism of these two fungi. For *M. genevensis*, the only significant changes in fatty acid concentration in the neutral lipids, were increases in the concentrations of 18:1(ω9) and 18:2(ω6). The increase in 18:1(ω9) suggests a high activity of the Δ9 desaturase enzyme. The increase in 18:2(ω6) in the neutral lipids of this fungus, may be as a result of the non-utilisation of this PUFA in the presence of an exogenous carbon source in the form of 20:4(ω6). It may also be true that the concentration of this PUFA increased due to the catabolism of 20:4(ω6). In the presence of 20:4(ω6), no significant changes in the concentrations of the glycolipid fatty acids of *M. genevensis* could be observed. However, in the phospholipids of this fungus, a decrease in the concentration of 18:3(ω6) was noted. A possible explanation for this decrease may be that, in order to maintain the correct membrane fluidity, 18:3(ω6) is released from the membranes upon incorporation of 20:4(ω6) into these structures.

For *R. oryzae* the only significant change in the neutral lipid fatty acid concentration, was a decrease in the concentration of 16:0. This may indicate that the effectiveness of the fatty acid synthetase enzyme system is reduced during incubation. However, when the changes in fatty acid concentration of the glycolipids of *R. oryzae* were studied, an alternative explanation became evident. Since an increase in the

concentration of 16:0 in the glycolipids was observed, this fatty acid may have been transferred from the neutral lipids to the glycolipids. Other glycolipid fatty acids that may have been replenished from the neutral lipids, are 16:1(ω7) and 18:0.

In the phospholipids of *R. oryzae* the only significant change in fatty acid concentration, was observed for 18:0. The concentration of this fatty acid increased in the phospholipids over the incubation period. This may again be as a result of the incorporation of 20:4(ω6) into the membranes. The increased concentration of 18:0 may serve as a mechanism for maintaining the correct membrane fluidity.

This study provided indications of the fate of 20:4(ω6) after uptake and incorporation of this PUFA into the lipid fractions of *M. genevensis* and *R. oryzae*. Since 20:4(ω6) was catabolised by these fungi, the aim of the next chapter is to determine some of the oxidation products of exogenous PUFAs produced by these fungi.

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Chapter 4

Biotransformation of polyunsaturated fatty acids to 3-hydroxy-5,8-tetradecadienoic acid by selected members of the order Mucorales

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4.1 Introduction

Oxygenated derivatives of fatty acids are abundant not only in mammalian cells, but also in algae (Gerwick 1996) and fungi (Herman & Herman 1985), where they are implicated in the regulation of vegetative growth and sexual reproduction of protoctistan and dikaryomycotan fungi (Mazur *et al.* 1991; Van Dyk *et al.* 1994). It has been reported that the yeast, *Dipodascopsis uninucleata,* is capable of biotransforming certain polyunsaturated fatty acids (PUFAs) to a novel group of 3-hydroxy fatty acids (3-OH-FAs) (Van Dyk *et al.* 1991; Venter *et al.* 1997). Thus, exogenous arachidonic acid [20:4(ω6)] was transformed to a novel eicosanoid, 3-hydroxy-(5*Z*,8*Z*,11*Z*,14*Z*)-eicosatetraenoic acid (3-HETE) (Van Dyk *et al.* 1991), whereas 3-hydroxy-(5*Z*,8*Z*)-tetradecadienoic acid (3-HTDE) and 3-hydroxy-(5*Z*,8*Z*,11*Z*)-tetradecatrienoic acid were formed from linoleic acid [18:2(ω9)] and α-linolenic acid [18:3(ω3)], respectively (Venter *et al.* 1997). In contrast, oleic acid [18:1(ω9)], linoelaidic acid [(9*Z*,12*E*)-18:2(ω6)], and γ-linolenic acid [18:3(ω6)] were apparently completely metabolised without detectable formation of 3-OH-FAs. From

these observations it was concluded that the putative fungal mono-oxygenase requires a (5*Z*,8*Z*)-dienoic fatty acid as substrate, either directly or formed by preceding partial β-oxidation (Venter *et al.* 1997). However, Akpinar and co-workers (1998) obtained results regarding the biotransformation of PUFAs by *D. uninucleata*, indicating that a (5*Z*,8*Z*)-dienoic fatty acid is not the only substrate that can be hydroxylated.

A recent immunofluorescence microscopic study using an antibody which exhibited group-specificity towards 3-OH-FAs, revealed that 3-OH-FAs occurred selectively in the gametangia, asci and between released ascospores of *D. uninucleata,* even in the absence of exogenous PUFAs (Kock *et al.* 1998). This location, the abundance of 18:2(ω6) in fungal lipids as well as the inhibitory effects of acetylsalicylic acid on both 3-OH-FA formation and the sexual reproductive phase of the yeast, suggest a pivotal role for 3-OH-FAs in fungal growth and as regulators of sexual reproduction (Kock *et al.* 1998). In order to determine whether these 3-OH-FAs are present in other fungi and could therefore conceivably be of general physiological importance in the fungal domain, this study was extended to the order Mucorales. Consequently, strains belonging to the genera *Rhizopus, Thamnostylum* and *Mucor,* including the homothallic fungus, *Mucor genevensis,* were screened for the biotransformation of exogenous PUFAs to 3-OH-FAs.

4.2 Materials and methods

4.2.1 Strains

The following representatives of the order Mucorales were screened for the production of 3-OH-FAs when fed with exogenous PUFAs: *Mucor circinelloides* f. *circinelloides* CBS 107.16, *Mucor flavus* CBS 234.35, *Mucor mucedo* CBS 109.16, *Mucor plumbeus* CBS 111.07 (held at the Centraal Bureau voor Schimmelcultures, Delft, The Netherlands), *Mucor genevensis* MUFS 038, *Thamnostylum piriforme*

MUFS 025 (held at the Mucoralean Culture Collection at the Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein, South Africa) and *Rhizopus oryzae* PPRI 5172 (held at the National Collection of Fungi, Pretoria, South Africa).

4.2.2 Polyunsaturated fatty acids

The following PUFAs were used in this study: 18:2(ω6), 18:3(ω6), dihomo-γ-linolenic acid [20:3(ω6)]; 20:4(ω6) and eicosapentaenoic acid [20:5(ω3)].

4.2.3 Cultivation and biotransformation

For each PUFA tested for biotransformation, 12 cultures of each fungal strain were prepared by inoculation of yeast-malt-extract gelatine (YMG), in Petri dishes. The YMG consisted of 10 $g.l^{-1}$ glucose, 3 $g.l^{-1}$ yeast extract, 3 $g.l^{-1}$ malt extract and 5 $g.l^{-1}$ peptone. Gelatine (100 $g.l^{-1}$) was used as solidifying agent. The cultures were incubated at 20°C for seven days. The colonies were harvested by melting the gelatine in a water bath (30°C), followed by filtration. The biotransformation medium was prepared for each strain, by placing six colonies in a Roux flask containing 375 ml physiological salt solution [0.85 % NaCl (w/v)] (PSS). The biotransformation was started by the addition of an ethanolic solution (1 g in 100 ml ethanol) of one of the PUFAs listed above, giving a final fatty acid concentration of 10 $mg.l^{-1}$. An equal amount of ethanol was added to a second Roux flask containing six colonies, which served as solvent control. The flasks were incubated at 25°C for six hours.

4.2.4 Oxygenated lipid extraction from biomass and aqueous phase and analyses

Polar lipids were extracted from the biomass with 80 % (v/v) aqueous ethanol (Van Dyk *et al.* 1991) and partially purified using a pre-conditioned Sep-Pak C_{18} cartridge

(Millipore, USA) (Nigam 1987). The hydroxy fatty acids were eluted with 5 ml distilled ethyl acetate and purified using silica mini-columns (Van Dyk *et al.* 1991). The fatty acids, including hydroxy fatty acids, in the aqueous phase of the biotransformation medium, were extracted with ethyl acetate, and the hydroxy fatty acids were purified as described above.

The purified hydroxy fatty acids were methylated with diazomethane, dissolved in 50 µl dry pyridine and silylated with 50 µl O-*bis*(trimethylsilyl)trifluoroacetamine, as described by Kock and co-workers (1997). The samples were dissolved in 50 µl chloroform:hexane (4:1) and analysed on a Hewlett Packard 5890 gas chromatograph equipped with a 30 m fused silica capillary column coupled to a Hewlett Packard 5972 mass selective detector. The carrier gas was helium at 154 psi head pressure. The initial column temperature was 140°C and it was increased by 5°C.min^{-1} to a final temperature of 300°C.

4.3 Results and discussion

Most of the strains screened could not biotransform the exogenous PUFAs to detectable 3-OH-FAs. The exceptions were *Mucor genevensis* MUFS 038 and *Mucor circinelloides* f. *circinelloides* CBS 107.16.

Significant amounts of 3-hydroxy-5,8-tetradecadienoic acid (3-HTDE) could be detected in the aqueous phase of the biotransformation medium of *Mucor genevensis* fed with exogenous 20:4(ω6) as well as in the aqueous phase of the biotransformation medium of *Mucor circinelloides* f. *circinelloides* fed with exogenous 20:3(ω6). The electron impact mass spectrum of the methylated and silylated fatty acids from both these organisms, had a M^+ of 326 mass units (Fig. 1 & Fig. 2). The peak at m/z 175 [$CH_3O(CO)CH_2$·CHO·TMSi] is indicative of 3-OH-FAs (Van Dyk *et al.* 1991; Venter *et al.* 1997). Another characteristic fragment is one with a m/z of 311 [M^+ - 15 (CH_3)]. In addition to its identical mass spectrum, this metabolite co-

chromatographed with 3-HTDE produced by *Dipodascopsis uninucleata* when fed with 18:2(ω6) (Venter *et al.* 1997). Concomitant with the formation of 3-HTDE by *M. genevensis* and *M. circinelloides* f. *circinelloides*, the partial ion current chromatograms also revealed marked peaks of free 18:2(ω6) (Fig. 1a & Fig. 2a). This observation may indicate a conversion of exogenous 20:4(ω6) and 20:3(ω6) to 18:2(ω6). In contrast with *D. uninucleata* no 3-HETE could be detected in either the aqueous phase or biomass when *M. genevensis* and *M. circinelloides* f. *circinelloides* was fed with 20:4(ω6). In spite of the fact that 3-HTDE is expected to be formed from 18:2(ω6), this metabolite could not be detected when 18:2(ω6) was added to the biotransformation media of *M. genevensis* and *M. circinelloides* f. *circinelloides*. This discrepancy may suggest different metabolic routes for endogenous and exogenous 18:2(ω6) in these two *Mucor* species.

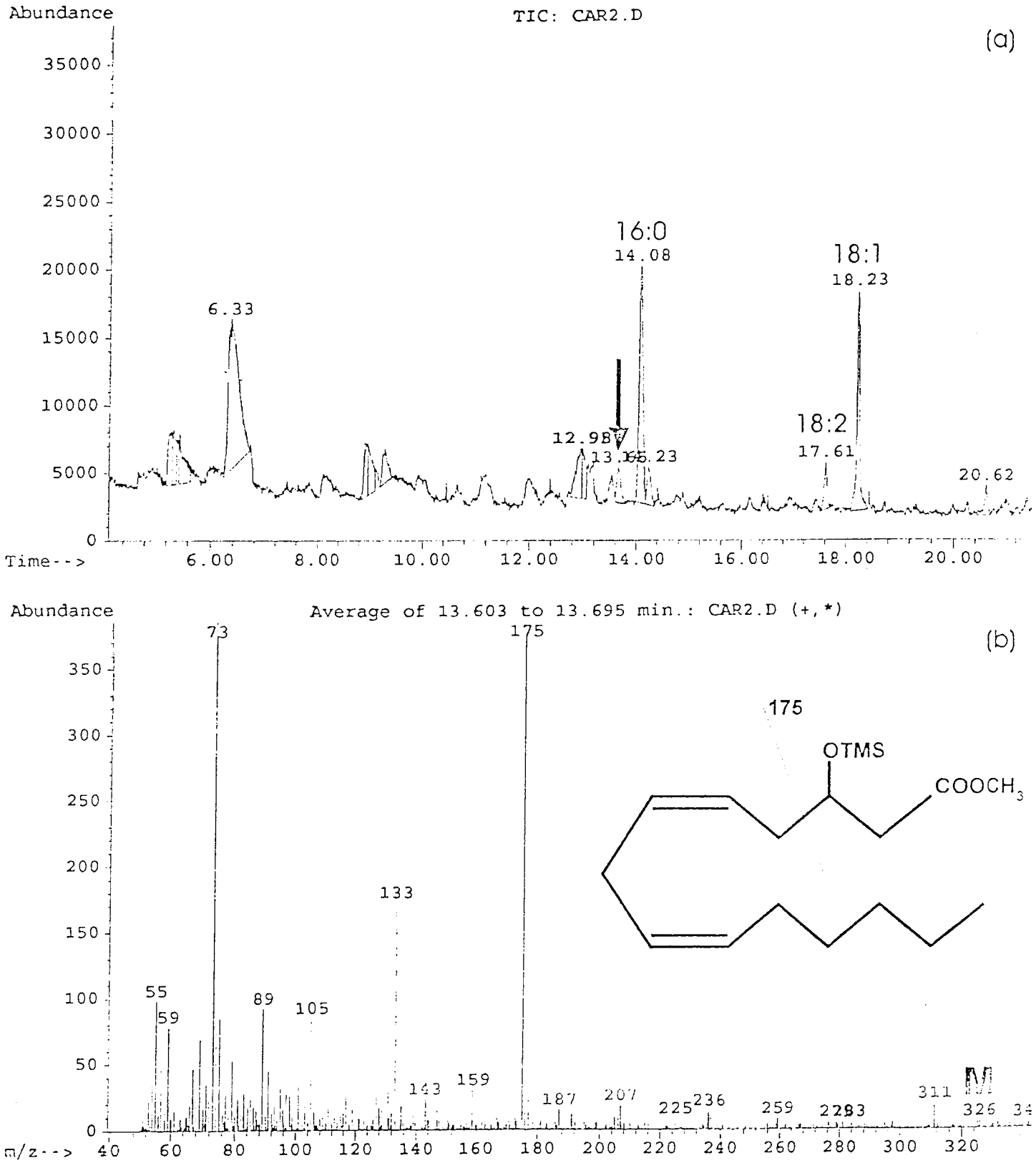

Fig. 1. (a) Partial ion current chromatogram of the methylated and trimethylsilylated extract from the aqueous phase of the biotransformation medium of *M. genevensis*.

(b) Electron impact mass spectrum of the compound representing the peak indicated by the arrow in (a). The chemical structure of this compound, which is methylated and trimethylsilylated 3-hydroxy-5,8-tetradecadienoic acid, is also shown.

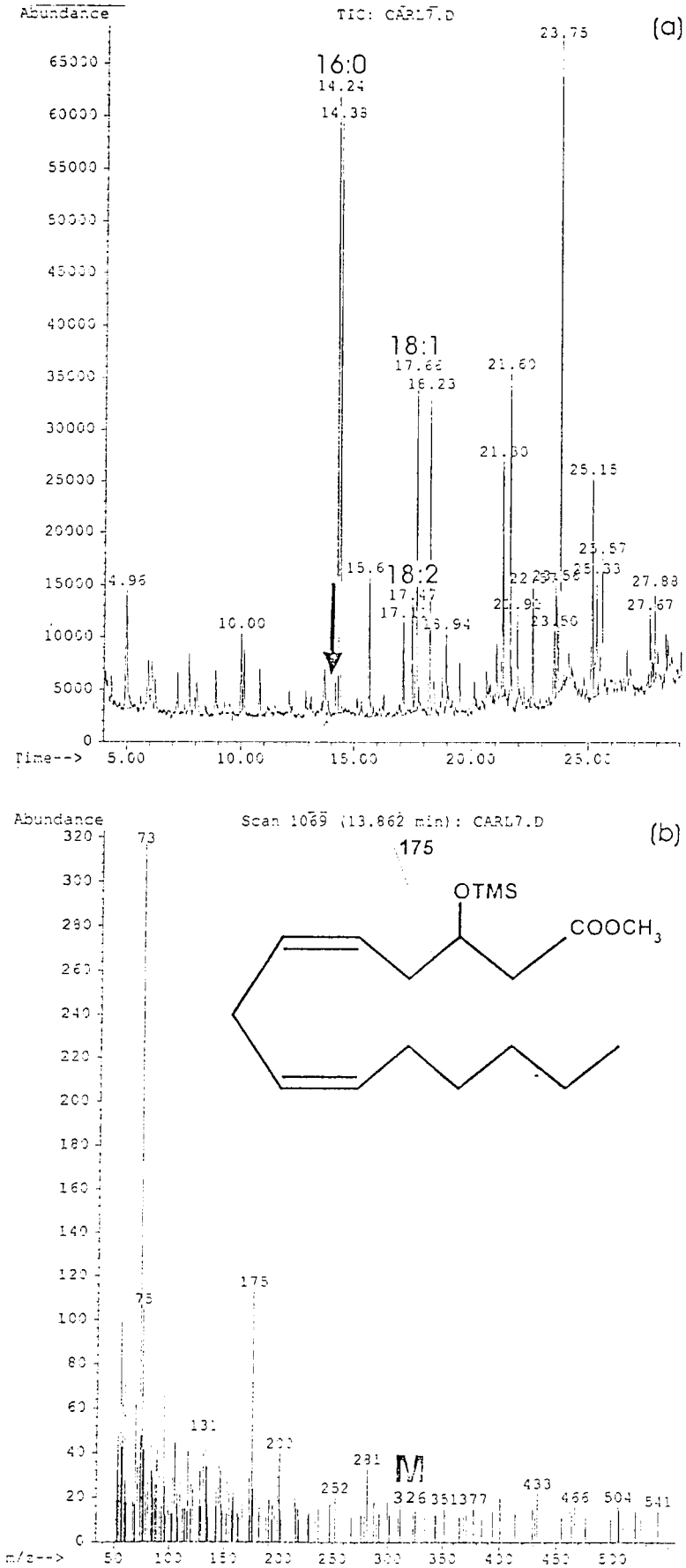

Fig. 2. (a) Partial ion current chromatogram of the methylated and trimethylsilylated extract from the aqueous phase of the biotransformation medium of *M. circinelloides* f. *circinelloides*.

(b) Electron impact mass spectrum of the compound representing the peak indicated by the arrow in (a). The chemical structure of this compound, which is methylated and trimethylsilylated 3-hydroxy-5,8-tetradecadienoic acid, is also shown.

4.4 Conclusions

The formation of 3-HTDE by *Mucor genevensis* and *Mucor circinelloides* f. *circinelloides* demonstrates that the production of 3-hydroxy fatty acids from PUFAs are not restricted to *Dipodascopsis uninucleata*, even though the metabolic routes in these species appear to differ. Taxonomically, *Mucor* and *Dipodascopsis* are not closely related, therefore, the formation of this 18:2(ω6) derived hydroxy fatty acid may be a general phenomenon of fungal lipid metabolism, although it could not be detected in most of the mucoralean strains screened in this study. A possible explanation may be that the metabolites are produced at such low concentrations, that detection is impossible. It may also be that the utilisation and further metabolism of the 3-OH-FAs occur rapidly in these strains.

The detection of 3-HTDE in the aqueous phase but not in the biomass, suggests that this hydroxy fatty acid is not a structural component of the fungal membrane lipids. It is, however, also conceivable that 3-HTDE is formed only at a defined physiological stage which does not significantly contribute to the final biomass and thus escapes analytical detection. The same reason may explain the absence of 3-HTDE in the absence of exogenous polyunsaturated fatty acids, in as much as free 18:2(ω6), the putative precursor of 3-HTDE, was also not detected under these conditions.

Esterified 18:2(ω6) is abundant in *M. circinelloides* f. *circinelloides* and *M. genevensis*. Gordon and co-workers (1971) observed that the level of this fatty acid in *M. genevensis* grown in liquid culture, decreased significantly during the transition from exponential growth to stationary phase whereas the 18:1(ω9) level remained constant, suggesting a growth-regulatory role for 18:2(ω6). When the lipid composition of *M. genevensis* was monitored during growth on solid media (Chapter 2), it could be seen that a rapid decline in esterified fatty acids [including 18:2(ω6)] occurred during sporogenesis. This also indicates a specific role for endogenous fatty acids during this part of the life-cycle.

It is tempting to speculate that the formation of 3-HTDE from endogenous 18:2(ω6) must be preceded by the liberation of the fatty acid from fungal lipids *via* a signal transduction process. Feeding of free 20:4(ω6) to *M. genevensis* or free 20:3(ω6) to *M. circinelloides* f. *circinelloides* may obviate this route. The lack of 3-HTDE formation upon feeding of free 18:2(ω6) to *M. circinelloides* f. *circinelloides* and *M. genevensis*, contrasts with earlier observations with *D. uninucleata* (Venter *et al.* 1997) and it may be hypothesised that exogenous 18:2(ω6) is preferably metabolised by *M. circinelloides* f. *circinelloides* and *M. genevensis via* peroxisomal and/or mitochondrial β-oxidation, whereas the reactions involved in the biotransformation of 20:3(ω6) or 20:4(ω6) to 3-HTDE are localised in another cell compartment.

The observation that exogenous 20:4(ω6) or 20:3(ω6) is transformed to 3-HTDE, may be explained by an effective preceding retroconversion (biohydrogenation) of these PUFAs to 18:2(ω6). Although such a retroconversion has been described in essential fatty acid deficient rats, fed with labelled 20:4(ω6) (Hansen *et al.* 1986) and in bacteria (Verhulst *et al.* 1986; Ratledge 1989), it is the first report of such a pathway in fungi. An alternative explanation for the results could, however, be a remodelling of the fungal membrane lipids in such a way that 20:4(ω6) or 20:3(ω6) is incorporated into the lipids resulting in the liberation of endogenous 18:2(ω6). Further studies are needed to discriminate between these conceivable mechanisms. Regardless of the nature of the mechanism involved, the PUFA-mediated supply of free 18:2(ω6) might be energetically advantageous to the fungus, since it allows a rapid switching to the sexual phase growth regulator, 3-HTDE (Kock *et al.* 1998), instead of steering the *ab initio* synthesis of 18:2(ω6) from glucose or other non-fatty acid carbon source and subsequent transformation to 3-HTDE.

From the results of this study it may be concluded that *M. genevensis* and *M. circinelloides* f. *circinelloides* do posses the necessary enzyme systems to produce 3-OH-FAs. In the next chapter immunofluorescence microscopy will be used to map the location of 3-OH-FAs in the different morphological structures of *M. genevensis*.

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Chapter 5

Localisation of 3-hydroxy fatty acids in *Mucor genevensis*

5.1 Introduction

It is known that, when the yeast, *Dipodascopsis uninucleata*, is challenged with certain exogenous polyunsaturated fatty acids (PUFAs) during sexual reproduction, it is capable of transforming these PUFAs to 3-hydroxy fatty acids (3-OH-FAs) (Van Dyk *et al.* 1991; Kock *et al.* 1997; Venter *et al.* 1997). Through immunofluorescence microscopy it was determined that endogenous 3-OH-FAs in *D. uninucleata* occur selectively in reproductive structures, such as gametangia, asci and between released ascospores, implicating these 3-OH-FAs in sexual reproduction of this yeast (Kock *et al.* 1998). In addition, evidence for the regulatory role of other oxygenated fatty acids (oxylipins) during reproduction of fungi, was obtained through work of Herman and Herman (1985) on the effect of cyclo-oxygenase inhibitors (acetylsalicylic acid and indomethacin) on colony growth, development and reproduction of certain lower fungi. These authors studied *Achlya ambisexualis, Achlya caroliniana* and *Saprolegnia parasitica*. They reported that cultures grown in the presence of the above-mentioned cyclo-oxygenase inhibitors, did not reproduce sexually. Kerwin and co-workers (1986) reported the production of prostaglandins by *Lagenidium giganteum*. These eicosanoids were observed to play a regulatory role in the induction and maturation of oospores. Herman and Luchini (1989) and Herman and co-workers (1989) studied lipoxygenase activity in several *Saprolegnia* species as well as in *Achlya ambisexualis* and found a decrease in lipoxygenase activity prior to sexual reproduction. Important roles during sexual reproduction in fungi such as *Emericella nidulans* (Anamorph = *Aspergillus nidulans*) have also been ascribed to

other oxylipins, namely, the so-called precocious sexual inducers (Mazur *et al.* 1991).

As was determined in the previous chapter, *Mucor genevensis* is capable of the biotransformation of exogenous arachidonic acid [20:4(ω6)] to 3-hydroxy-5,8-tetradecadienoic acid. In order to determine whether endogenous 3-OH-FAs are present in *M. genevensis* and whether these hydroxylated fatty acids may also be implicated in the development of reproductive structures in this fungus, the location of 3-OH-FAs in fixed reproductive structures was mapped by immunofluorescence microscopy.

5.2 Materials and methods

5.2.1 Strain

Mucor genevensis (MUFS 038), a homothallic species, isolated from soil in the Knysna forest in South Africa, was used in this study. The strain is held in the Mucoralean Culture Collection at the Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein, South Africa.

5.2.2 Cultivation

The fungal strain was inoculated on to yeast-malt-extract gelatine (YMG) in a Petri dish. The YMG consisted of 10 $g.l^{-1}$ glucose, 3 $g.l^{-1}$ yeast extract, 3 $g.l^{-1}$ malt extract and 5 $g.l^{-1}$ peptone. Gelatine (100 $g.l^{-1}$) was added as solidifying agent. Cultures were incubated at 20°C for seven days until zygospores were observed.

5.2.3 Detection of 3-hydroxy fatty acids by immunofluorescence microscopy

The antibody used in this study was obtained from prof. S. Nigam, who prepared and characterised it as discussed below.

5.2.3.1 Preparation of antibody

Synthetic 3*R*-hydroxy-5,8,11,14-eicosatetraenoic acid (3*R*-HETE), prepared by Bhatt and co-workers (1998) was used to raise antibodies in rabbits (Kock *et al.* 1998). The carboxyl group of 3*R*-HETE was conjugated to the amino groups of bovine serum albumin (BSA). The conjugate, containing 1 mg protein, was emulsified in an equal volume of Freunds complete adjuvant (for the first injection) or incomplete adjuvant (for later injections) (Kock *et al.* 1998). The emulsion was injected subcutaneously into several sites on the back of a female New Zealand white rabbit every second week, for a total of seven times over a period of three months. The whole blood was collected from the carotid artery, left at room temperature for two hours and centrifuged at 1200 x *g* at 4°C for 20 minutes. The sera were affinity purified by Biogenes in Berlin, Germany.

5.2.3.2 Characterisation of antibody

Characterisation was accomplished by determining the titer, sensitivity and specificity of the antibody preparation (Kock *et al.* 1998). The titer of the antibody gave a binding of approximately 30 % labelled 3*R*-HETE at a dilution of 1:100 in the absence of unlabelled 3*R*-HETE. The sensitivity (minimum detectable amount) of 3*R*-HETE was determined by 10 % displacement of radioactivity by unlabelled 3*R*-HETE from the zero point (maximum binding of labeled 3*R*-HETE). It was found to be 30 pmol. The specificity of the antibody, expressed in terms of cross-reactions with structurally related compounds, was found to be high for 3-OH-FAs.

5.2.3.3 Immunofluorescence microscopy

A loop full of biomass, obtained from the fungal culture, was washed and suspended in BSA-PBS buffer. The primary antibody, raised against 3-OH-FAs [(30µl; 1:10 dilution (v/v)] was added and the suspension was incubated at room temperature for 30 minutes. After washing in BSA-PBS buffer, the affinity purified fluorescein isothiocyanate anti-rabbit IgG (Sigma, Germany) was added [(30µl; 1:10 dilution (v/v)] and again incubated for 30 minutes at room temperature. Following adequate washing, microscope slides were prepared and photographed using Kodak Gold Ultra 200 ASA film on a Zeiss Axioskop standard microscope equipped for epifluorescence (500 W high pressure mercury lamp). The stained biomass was compared to appropriate controls.

5.3 Results and discussion

According to Schipper (1973), a zygospore with opposite aligned suspensor cells is typical of the genus *Mucor*. In the first micrograph (Fig. 1) such a zygospore and suspensor cells can be seen. Interestingly, an obvious difference in fluorescence could be observed between the zygospore (non-fluorescing) and the fluorescing suspensor cells, indicating the presence of 3-OH-FAs in the suspensor cells.

Figure 2 is the micrograph of asexual reproductive structures, i.e. sporangium, columella and sporangiospores. The sporangium and columella are clearly fluorescing while the sporangiophore and hyphae are not flourescent. Fluorescence may also be seen between the sporangiospores in the sporangium, again indicating the presence of 3-OH-FAs around the spores.

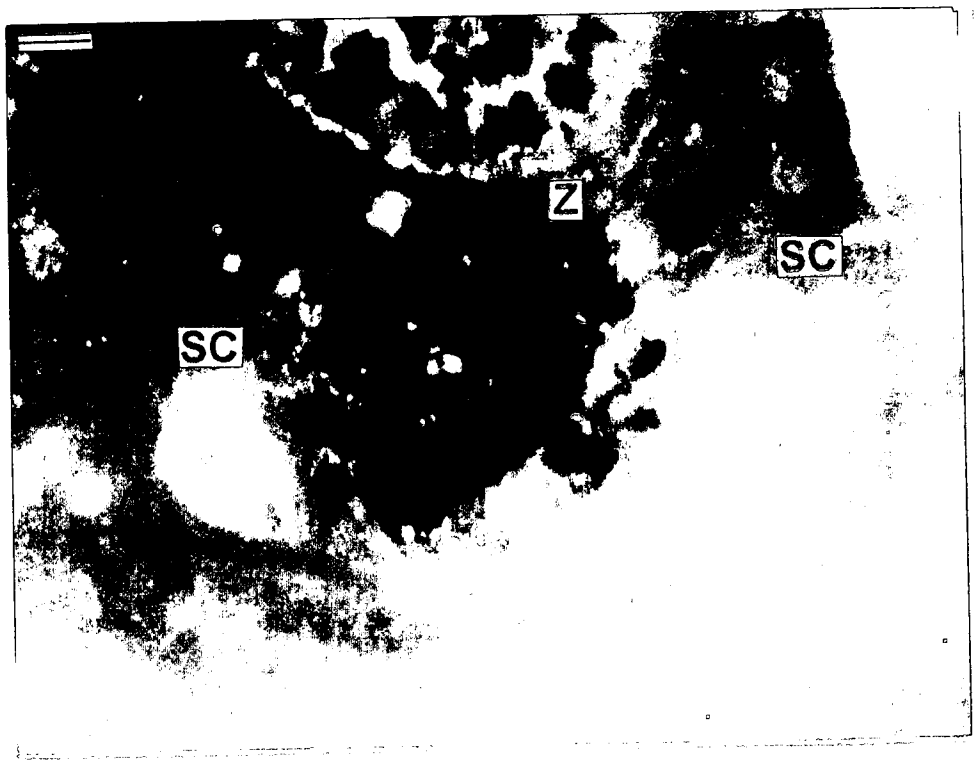

Fig. 1. Micrograph of the zygospore (non-fluorescing) with two attached suspensor cells (fluorescing). The bar represents 10 μm.

Z = zygospore; SC = suspensor cells

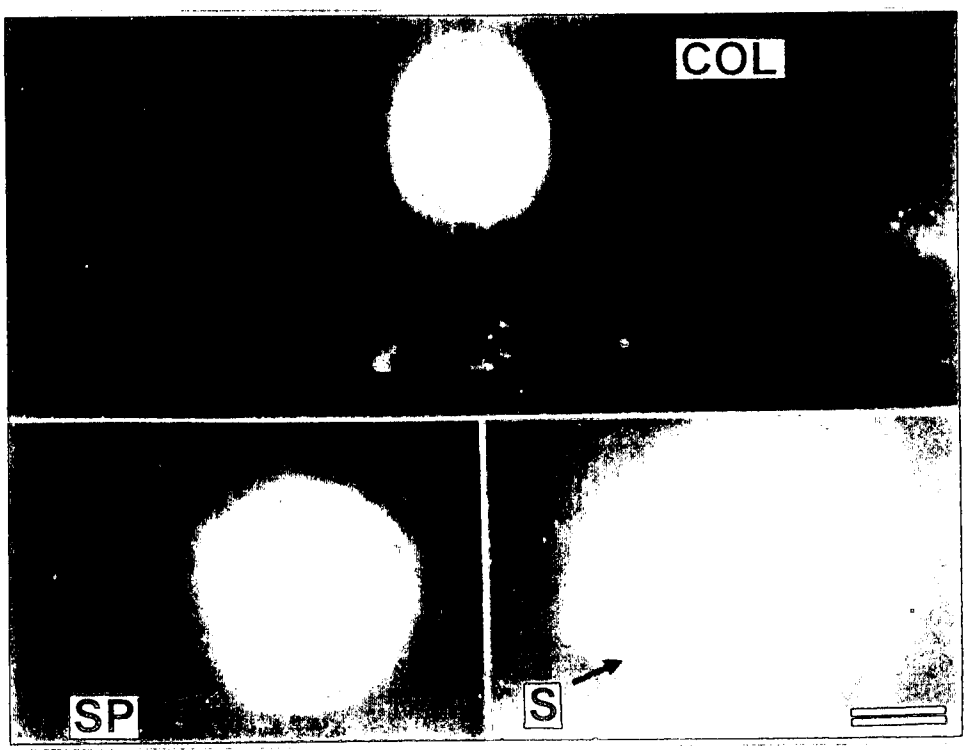

Fig. 2. Micrograph of different morphological structures of *Mucor genevensis*. The bar represents 20 μm.

COL = columella; SP = sporangium; S = sporangiospores.

5.4 Conclusions

From the results it may be concluded that spore bearing structures had a higher affinity for the antiboby, specific for 3-OH-FAs, than the somatic hyphae. Immunofluorescence microscopy not only indicated the presence of these hydroxylated fatty acids in structures associated with the formation of sexual spores, as was found in *D. uninucleata* (Kock *et al.* 1998), but it also indicated that 3-OH-FAs are located in the asexual spore bearing structures of *M. genevensis*. These results indicate that at least some of the esterified fatty acids in the lipids, which deminished during sporogenesis of *M. genevensis* (Chapter 2), were transformed to hydroxylated fatty acids during the formation of spores. It may also be speculated that the enzyme systems responsible for the biotransformation of exogenous arachidonic acid to 3-hydroxy-5,8-tetradecadienoic acid (Chapter 4) are located in the spore bearing structures. This is the first report on the location of 3-OH-FAs in mucoralean fungi and it paves the way for further studies on the specific role these compounds play during sporogenesis of these fungi.

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Summary

In this study the lipid metabolism of *Mucor genevensis* and related mucoralean fungi have been explored. Consequently, endogenous lipid turnover during growth and development of the different asexual and sexual reproductive stages, was studied in *Mucor genevensis* MUFS 038. Lipid analyses were periodically performed on cultures growing on solid synthetic media. It was found that during all developmental stages, the lipid composition remained similar to that of a typical mucoralean fungus. However, zygospores were found to have fewer membranes (i.e. less phospholipids) with a lower degree of fluidity (i.e. less polyunsaturated fatty acids - PUFAs) than sporangiospores and mycelium. Decreases in neutral, glyco- and phospholipids were observed during sporogenesis. The aim of the next chapter was to investigate the uptake and incorporation of arachidonic acid [20:4(ω6)] into the various lipid fractions of *Mucor genevensis* MUFS 038 and *Rhizopus oryzae* PPRI 5172. The exogenous 20:4(ω6) was rapidly taken up by the fungi. The overall concentration of 20:4(ω6) in both fungi decreased due to catabolism of 20:4(ω6). The PUFA was incorporated into the neutral, glyco- and phospholipids of both fungi. It was found that *Rhizopus oryzae* has a greater ability to reduce the amount of cellular free 20:4(ω6) than *M. genevensis*. During incubation with 20:4(ω6), different changes in the concentrations of 16 and 18 carbon fatty acids were observed for the two fungi, indicating differences in lipid metabolism of these two fungi. In the next chapter, mucoralean fungi were screened for oxidation products of exogenous PUFAs. Lipid extracts from biomass and aqueous phases of cultures of the following strains were analysed using gas chromatography-mass spectrometry: *Mucor circinelloides* f. *circinelloides* CBS 107.16, *Mucor flavus* CBS 234.35, *Mucor genevensis* MUFS 038, *Mucor mucedo* CBS 109.16, *Mucor plumbeus* CBS 111.07, *Rhizopus oryzae* PPRI 5172 and *Thamnostylum piriforme* MUFS 025. The following PUFAs were fed to the fungal cultures: linoleic acid, γ-linolenic acid, dihomo-γ-linolenic acid [20:3(ω6)], 20:4(ω6) and eicosapentaenoic acid. It was demonstrated that *M. genevensis* and *M. circinelloides*

are able to produce 3-hydroxy-5,8-tetradecadienoic acid (3-HTDE) from 20:4(ω6) and 20:3(ω6), respectively. No other 3-hydroxy fatty acids (3-OH-FAs) were detected in any other strains. It was suggested that the 3-OH-FAs were produced at such low concentrations that detection was impossible in most of the strains. Another reason for the apparent absence of 3-OH-FAs may be that these compounds are rapidly utilised or further metabolised by the strains. Detection of 3-HTDE in the aqueous phase only, suggested that it was not a structural component of membranes, or that 3-HTDE was formed at a physiological stage which did not contribute to the final biomass. Subsequently, immunofluorescence microscopy was used in the localisation of endogenous 3-OH-FAs in *M. genevensis* MUFS 038. It was found that 3-OH-FAs are associated with spore bearing structures. It was speculated that some of the lipids, which diminish during sporogenesis of *M. genevensis*, were transformed to hydroxylated fatty acids during sporogenesis.

Opsomming

In hierdie studie is die lipiedmetabolisme van *Mucor genevensis* en verwante spesies ondersoek. Gevolglik is die endogene lipiedomset gedurende groei en ontwikkeling van die verskillende ongeslagtelike en geslagtelike voortplantingsfases van *Mucor genevensis* MUFS 038 bestudeer. Lipiedanalises is met tussenpose op kulture wat op soliede sintetiese media gekweek is, uitgevoer. Dit is gevind dat, gedurende al die ontwikkelingsfases, die lipiedsamestelling vergelykbaar was met die van 'n tipiese mucoraliese fungus. Zigospore het egter minder membrane (d.i. minder fosfolipiede) met 'n laer graad van vloeibaarheid (d.i. minder poli-onversadigde vetsure - POVs) gehad as sporangiospore en miselium. Afnames in neutrale, gliko- en fosfolipiede is waargeneem tydens sporogenese. Die doel van die volgende hoofstuk was om die opname en inkorporasie van aragidonsuur [20:4(ω6)] in die verskillende lipiedfraksies van *Mucor genevensis* MUFS 038 en *Rhizopus oryzae* PPRI 5172, te bestudeer. Die eksogene 20:4(ω6) is vinnig deur die fungi opgeneem. Die algehele konsentrasie 20:4(ω6) in beide fungi het afgeneem a.g.v. die katabolisme van 20:4(ω6). Die POV is geïnkorporeer in die neutrale, gliko- en fosfolipiede van beide fungi. Dit is gevind dat *Rhizopus oryzae* 'n groter vermoë as *M. genevensis* het om die hoeveelheid vrye 20:4(ω6) in die selle te verminder. Tydens inkubasie met 20:4(ω6), is verskillende veranderinge in die konsentrasies van 16- en 18-koolstof vetsure vir die twee fungi waargeneem. Dit dui op verskille tussen die lipiedmetabolismes van die twee fungi. In die volgende hoofstuk is mucoraliese fungi ondersoek vir die vorming van oksidasieprodukte vanaf eksogene POVs. Lipiedekstrakte van biomassa en vloeistoffases van kulture van die volgende stamme is geanaliseer d.m.v. gaschromatografie-massa spektrometrie: *Mucor circinelloides* f. *circinelloides* CBS 107.16, *Mucor flavus* CBS 234.35, *Mucor genevensis* MUFS 038, *Mucor mucedo* CBS 109.16, *Mucor plumbeus* CBS 111.07, *Rhizopus oryzae* PPRI 5172 en *Thamnostylum piriforme* MUFS 025. Die volgende POVs is vir die fungikulture gevoer: linoleiensuur, γ-linoleensuur, dihomo-γ-linoleensuur [20:3(ω6)], 20:4(ω6) en

eikosapentanoësuur. Dit is gedemonstreer dat *M. genevensis* en *M. circinelloides* 3-hidroksie-5,8-tetradekadienoësuur (3-HTDE) vanaf 20:4(ω6) en 20:3(ω6) onderskeidelik, kan produseer. Geen ander 3-hidroksievetsure (3-OH-VSs) is in die ander stamme waargeneem nie. Dit word voorgestel dat die 3-OH-VSs in die meeste stamme teen te lae konsentrasies geproduseer is en dus nie waargeneem kon word nie. 'n Ander rede vir die skynbare afwesigheid van 3-OH-VSs kan wees dat hierdie verbindings vinnig deur hierdie stamme verbruik of verder gemetaboliseer word. Deteksie van 3-OH-VSs slegs in die vloeistoffase, het daarop gedui dat dit nie strukturele komponente van membrane was nie, of dat 3-HTDE gevorm is tydens 'n fisiologiese fase wat nie tot die finale biomassa bygedra het nie. Hierna is immunofluoressensiemikroskopie gebruik om die ligging van endogene 3-OH-VSs in *M. genevensis* MUFS 038 te bepaal. Dit is gevind dat 3-OH-VSs geassosieer word met spoordraende strukture. Dit is gespekuleer dat sommige lipiede, wat verminder tydens sporogenese van *M. genevensis,* getransformeer is na gehidroksileerde vetsure tydens sporogenese.

Key words / Sleutelwoorde

- lipid metabolism / lipiedmetabolisme
- Mucorales
- *Mucor genevensis*
- *Rhizopus oryzae*
- arachidonic acid / aragidonsuur
- 3-hydroxy fatty acids / 3-hidroksievetsure
- 3-hydroxy-5,8-tetradecadienoic acid / 3-hidroksie-5,8-tetradekadienoësuur
- immunofluorescence microscopy / immunofluoressensie-mikroskopie

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