Oxylipin distribution in *Eremothecium*

by

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This dissertation is dedicated to the following people:

My mother (Nkotseng Leeuw)

My brother (Kabelo Leeuw)

My cousins (Bafokeng, Lebohang, Mami, Thabang and Rorisang)

Mr. Eugean Malebo

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SUMMARY

OPSOMMING

KEY WORDS

SLEUTELWOORDE

CHAPTER 1

Introduction

1.1 Motivation

In 1991, Kock and co-workers discovered acetylsalicylic acid (ASA)-sensitive oxylipins in yeasts (Kock et al. 1991; Noverr et al. 2003; Van Dyk et al. 1991). These compounds are oxidized saturated and unsaturated fatty acids and include the eicosanoids. Since 1991, various research groups showed the ubiquitous nature of oxylipins in fungi. In addition, their importance as target to control fungal infections as well as biofilm formation was recently highlighted (Alem and Douglas 2004; Deva et al. 2000, 2003; Noverr et al. 2003).

In 2004, Kock and co-workers exposed another function of these oxylipins. They found that these compounds may act as prehistoric lubricants during ascospore release from enclosed asci. In *Eremothecium ashbyi,* 3- hydroxy (OH) 14:0 was found to coat nano-scale fins protruding from the sides of sickle-shaped ascospores. It is suggested that these oxylipins increase the resistance of the fins to water movement and this enhances overall spore stability and boomerang speed. This is needed for the spiked spore-tip to gain enough momentum in order to pierce through the ascus-wall for dispersal purposes. In addition, Bareetseng et al. (2004) demonstrated the presence of oxylipins on the corkscrew part of ascospores of *Eremothecium sinecaudum* and suggested that these compounds act as lubricants that assist in ascospore release through asci walls by drilling movements under turgor pressure.

Since only a limited number of species representing *Eremothecium* (a genus producing curiously shaped ascospores) was thus far studied, it became the aim of this project to determine the distribution of 3-OH oxylipins in the remaining species of this

genus i.e. *Eremothecium coryli, Eremothecium cymbalariae* and *Eremothecium gossypii*. In addition, the possible functions of these oxylipins as well as ascospore shape and ornamentations will be assessed. The antifungal activity of ASA will also be investigated in this group of important plant pathogens.

1.2 Definition and classification of yeasts

Yeast is the informal name for single-celled members of the Ascomycetes, Basidiomycetes and imperfect fungi (also known as anamorphs) that tend to be unicellular for the greater part of their life cycle. One of the more prominent characteristics of yeasts is their ability to ferment sugars for the production of ethanol. However, according to literature many types of yeast known today cannot ferment. Yeasts are in general characterized by budding from a broad or narrow base or fission from a broad base as primary means of vegetative reproduction. In contrast to higher fungi, the sexual state of yeasts is not enclosed in fruiting bodies such as apothecia or cleistothecia; their asci are naked (Kurtzman and Fell 1998).

Characteristics that are used to classify yeasts include morphological (modes of reproduction, ascospore formation, shape and size) and physiological properties (ability to assimilate and ferment certain sugars and other carbon sources as well as to grow on different nitrogen sources) (Yarrow 1998). Recently, molecular techniques such as D1/D2 sequencing have been used to identify and classify yeasts (Kurtzman and Robnett 1998). On the basis of the Diazonium Blue B test, yeasts are divided into two main groups i.e. Ascomycetes (DBB negative) and Basidiomycetes (DBB positive). Classification on this level also depends on the mode of sexual reproduction (i.e. the

production of ascospores or basidiospores) which is highly conserved on higher taxon level.

These ascospores and basidiospores are produced by meiosis usually during adverse conditions and may have different shapes and nano-scale surface ornamentations. Some yeasts with ascomycetous or basidiomycetous affinity lack sexual stages, these are referred to as anamorphs. The yeasts representing the Ascomycetes, Basidiomycetes and their anamorphs at present comprise 90 genera and 678 species (Barnett et al. 2000).

The ascomycetous yeasts, of which *Eremothecium* is a member, are presently classified as follows (Barnett et al. 2000):

Kingdom: Fungi

Phylum: Ascomycota

Class: Hemiascomycetes

Order: Saccharomycetales

Families: Candidaceae (11 genera)

Dipodascaceae (7 genera)

Eremotheciaceae (1 genus)

Lipomycetaceae (5 genera)

Metschnikowiaceae (2 genera)

Phaffomycetaceae (2 genera)

Saccharomycetaceae (17 genera)

Saccharomycodaceae (4 genera)

Saccharomycopsidaceae (2 genera)

Unclassified Saccharomycetales (3 genera)

1.3 Classification of *Eremothecium* and related genera

The genera *Ashbya* (Guilliermond), *Coccidiascus* (Chatton), *Eremothecium* (Borzi), *Holleya* (Yamada), *Metschnikowia* (Kamienski) and *Nematospora* (Peglion) have been considered related on the basis of being characterized by needle or spindle-shaped ascospores. Also included in this complex was *Spermophthora* (Ashby and Nowell). Classification of these genera has been complicated by the perception that genera which commonly form budding yeast cells (*Holleya*, *Metschnikowia*, *Nematospora*) are phylogenetically separate from genera that do not normally form budding cells (*Ashbya*, *Eremothecium*) (Lodder 1970). The phylogeny of these yeasts will be discussed later under the sub-heading “Present classification”.

Classification of these yeasts began in 1931, when Stelling-Dekker brought together the genera *Coccidiascus, Metschnikowia* and *Nematospora* in the subfamily Nematosporoideae which was classified under the family Endomycetaceae belonging to the order Endomycetales (Batra 1973). At this stage, *Ashbya* and *Eremothecium* found no place in the yeast-like Hemiascomycetes. In 1950, Bessey proposed that the Hemiascomycetes represent the simplest forms of the Class Ascomycetes. He placed the genera *Ashbya* (Guilliermond), *Coccidiascus* (Chatton), *Eremothecium* (Borzi), *Metschnikowia* (Kamienski) and *Nematospora* (Peglion), in the Saccharomycetaceae (order Saccharomycetales).

In 1964, Gäumann proposed that the Hemiascomycetes should also include the most primitive Ascomycetes i.e. *Ashbya, Eremothecium* and *Nematospora* in the family Spermophthoraceae (Endomycetales) along with *Spermophthora* (Batra 1973). Also included in this order were the families Dipodascaceae, Endomycetaceae and Saccharomycetaceae. No mention was made of the genus *Coccidiascus.*

In 1973, on the basis of morphology and type of sexual reproduction, Batra placed *Spermophthora* as sole genus under the Spermophthoraceae while excluding genera with nematosporic ascospores i.e. *Ashbya* (Guilliermond), *Eremothecium* (Borzi), *Metschnikowia* (Kamienski), *Nematospora* (Peglion), and *Coccidiascus* (Chatton) (Kurtzman and Fell 1998). He classified these genera under the Nematosporaceae Novak and Zsolt which are characterized by yeast-like organisms mainly occurring on a wide variety of crop plants causing diseases such as "stigmatomycosis", "yeast spot", "eye spot", and "internal rot". They are associated with punctures made by insects having piercing-sucking mouthparts.

On the basis of comparative morphology and physiological studies, Batra (1973) classified the yeasts as follows:

Class: **Hemiascomycetes**

Subclass: **Hemiascomycetidae**

Order: **Spermophthorales** (Spermophthoraceae: *Spermophthora*)

Dipodascales (Dipodascaceae: *Dipodascus, Endomyces, Schizosaccharomyces*; Eremascaceae: *Eremascus*)

Cephaloascales (Cephaloascaceae: *Cephaloascus*)

Ascoideales (Ascoideaceae: *Ascoidea*; Nematosporaceae: *Nematospora, Ashbya, Metschnikowia, Eremothecium* and *Coccidiascus*; Saccharomycetaceae and most ascosporogenous yeasts).

He proposed the following diagnosis for the family Nematosporaceae Novak and Zsolt under which *Eremothecium* and *Metschnikowia* were classified:

"Thallus cellular or filamentous, uninucleate or multinucleate; asexual reproduction by blastosporic cells, thick walled, resting or nonresting chlamydospores also present; asci terminal or intercalary and arising from thallus or from proasci, one to many-spored, usually deliquescent in the middle; ascospores elongate, pointed at one or both ends, hyaline, with or without a flagellate appendage. Transmitted by hemipterous insects, parasitic on plants, on crustacea or saprophytes. Type genus: Nematospora Peglion nec. Nematospora Tassi. The genera of the family Nematosporaceae are distinguished on the basis of the shape of ascospores, the presence or the absence of proasci, and the behavior of the conjugant cell after caryogamy."

Batra (1973) proposed the following keys to the Nematosporaceae:

1. Ascospores needle-shaped……………………………………..…………….....2
1. Ascospores sickle-shaped or bent…………………………………..…*Eremothecium*
2. Thallus filamentous, coenocytic, sprout cells become absent or rare asci intercalary...……………………………………………………………………..…...*Ashbya*

2. Thallus cellular-colonial or occasionally filamentous and septate, sprout cells present, asci free-floating or terminal……………………………………………………..3

3. Ascospores with a flagellum-like cytoplasmic appendage and with two distinct uninucleate protoplasts, proasci thin-walled, non- refractive…………….*Nematospora*

3. Ascospores without an appendage and with one uninucleate protoplast, proasci thin or thick-walled, highly refractive ……………………………………..*Metschnikowia*

Based on phenotypic similarities, Batra (1973) and Von Arx et al. (1977) suggested that the genera *Ashbya, Eremothecium* and *Nematospora* may be congeneric and that *Spermophthora* is in fact similar to *Eremothecium ashbyi* (Kurtzman and Fell 1998).

Present classification

Kurtzman and Robnett (1994) investigated the extent of divergence in partial nucleotide sequences from large and small subunits of ribosomal RNAs from the type species of all culturable genera of ascomycetous yeast and yeastlike fungi. Results from this study demonstrated that the genera *Ashbya*, *Eremothecium*, *Holleya* and *Nematospora* represent closely related members of a subclade that is phylogenetically separate from the subclade that includes the genus *Metschnikowia*. Based on ribosomal DNA (rDNA) sequence divergence, Kurtzman (1995) found similar results and proposed the transfer of species of *Ashbya*, *Eremothecium*, *Holleya* and *Nematospora* to the genus *Eremothecium*. He pointed out that species that are to be placed in the genus *Eremothecium* are members of a monophyletic lineage and that this species show little interspecific divergence.

Kurtzman and Robnett (1998) were able to further verify their findings when they analyzed species of the ascomycetous yeasts and other anamorphic genera for extent of deviation in the variable D1/D2 domain of the large subunit (26S) rDNA. According to them, deviation in this domain is adequate to resolve individual species and with this they proved that species placed by Kurtzman in the genus *Eremothecium*, are closely related. Figure 1 shows a phylogenetic tree derived from maximum parsimony analysis representing the ascomycetous yeasts and yeastlike fungi indicating that the species belonging to the genus *Eremothecium* fall in the same clade. This implies that these species are closely related.

The current diagnoses for the genus *Eremothecium* (Eremotheciaceae) Borzi emend. Kurtzman is as follows:

"Budding cells are absent or present, and when present, budding is multilateral on a narrow base. Cells are globose, ovoidal, ellipsoidal or cylindrical. Enteroathric conidia are infrequently produced by one species. Pseudohyphae and true hyphae are generally present. Colonies are smooth or floccose and white, grayish or yellow in color. Asci, which become deliquescent, form 8-32 ascospores that are fusiform or acicular. Ascospores may have a central septum and those of some species have a tapered, terminal extension of the cell wall. Sugars fermented by some species. Nitrate is not assimilated. Coenzyme Q may have 5, 6, 7, 8 or 9 isoprene units in the side chain, some of which are in minor proportions. Diazonium blue B reaction is negative."

Type species:

Eremothecium cymbalariae Borzi

Species accepted:

1. *Eremothecium ashbyi* (Guilliermond ex Routien) Batra (1973)
2. *Eremothecium coryli* (Peglion) Kurtzman (1995)
3. *Eremothecium cymbalariae* Borzi (1888)
4. *Eremothecium gossypii* (Ashby and Nowell) Kurtzman (1995)
5. *Eremothecium sinecaudum* (Holley) Kurtzman (1995)

The latest key to *Eremothecium* (De Hoog et al. 1998):

1. *E. ashbyi*: Ascospores are curved and sickle-like in appearance

Ascospores are linear in appearance…………………………………………..……….2

2(1). *E. coryli*: Ascospores have a long whip-like terminal appendage.

Ascospores do not have whip-like appendages …………………………….……………3

3(2) *E. cymbalariae*: Ascospores are narrowly triangular in side view with one needle-like end.

E. gossypii: Ascospores are needle-shaped; length is greater than 20µm.

E. sinecaudum: Ascospores are needle-shaped; length is ca.10µm.

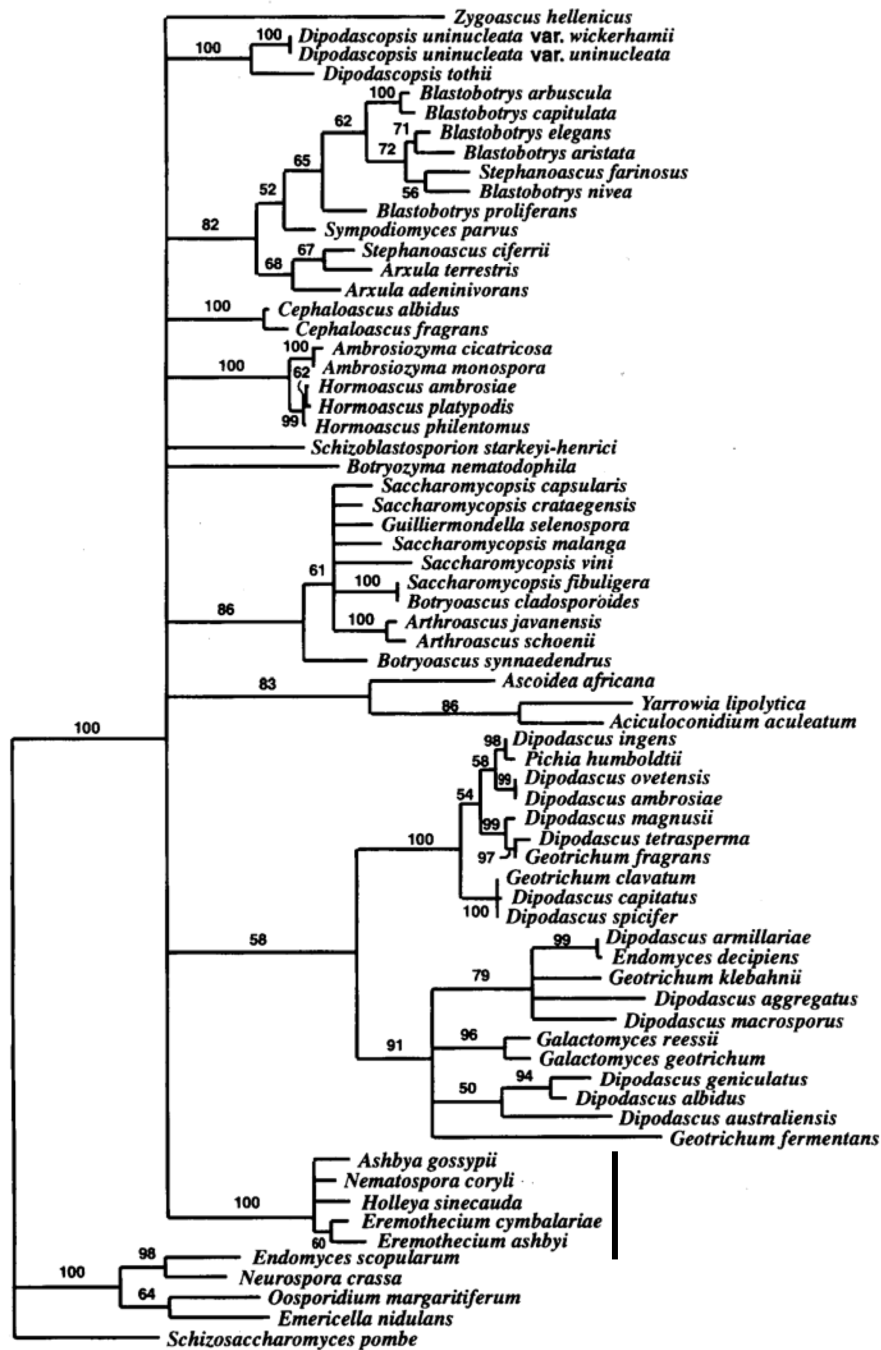

Figure 1: Phylogenetic tree derived from maximum parsimony analysis representing the ascomycetous yeasts and yeastlike fungi. The area marked with a bold vertical line represents the species of interest now classified under *Eremothecium* (Taken from Kurtzman and Robnett (1995)).

1.4 Pathogenicity

Most ascomycetous yeasts are not known to be plant pathogens but the genus *Eremothecium* (syn. *Ashbya*, *Holleya*, and *Nematospora*) is an exception. In a review by Batra (1973) providing the host range of the Eremotheciaceae, it was noted that infections are usually insect-vectored. Yeast cells or spores are introduced into various host plants during the feeding piercing-sucking action of insects and cause a variety of symptoms during their development in the host plant tissue. This was first noted by Ashby in 1926. He concluded that infection of plants is dependent on insect punctures and the infecting organisms (i.e. yeasts) are carried by the insects themselves. *Eremothecium ashbyi, E. cymbalariae* and *E. gossypii* commonly infect cotton (*Gossypium* spp.) as well as the fruits of *Citrus* species. *Eremothecium coryli* has a broader infection range than the preceding species, infecting cotton, citrus, hazelnuts and soybeans, whereas *E. sinecaudum* infects seeds of mustard (Kurtzman and Fell 1998). Infections caused by *E. sinecaudum* are restricted to seeds of oriental mustard [*Brassica jucea* (L) Coss] and yellow mustard (*B. hirta* Moench) (Holley et al. 1984).

Infections caused by these yeasts often take the form of surface lesions, especially on fruits, but in cotton bolls only the lint and seeds are affected and there is no external evidence of decay. Fibers from infected cotton bolls may show yellow discoloration (Phaff and Starmer 1987). It has been reported that these yeasts made it virtually impossible to grow cotton in certain parts of the world (Hopkins 1950). Even though these yeasts are pathogens, two species have been found to produce riboflavin. It has also been reported that some representative species produce oxylipin-coated ascospores (Bareetseng et al. 2004; Kock et al. 2004). Researchers have proposed

that, the water-driven drilling movement used by oxylipin-coated ascospores of *E. sinecaudum* for spore liberation, could also be used to induce plant infection (De Hoog et al. 1998). What remains to be observed now, is whether ascospores produced by other species of *Eremothecium* are also coated with 3-OH oxylipins.

1.5 Oxylipins

1.5.1 Definition

Oxylipins are saturated and unsaturated oxidized fatty acids and include the eicosanoids which are derived from arachidonic acid (AA). They exert potent biological effects and some have been found to play major roles in physiological processes such as aggregation of blood platelets and labour induction (Needleman 1986; Noverr et al. 2003; Samuelsson 1983). Also included are 3-OH oxylipins, which are believed to be synthesized via β-oxidation with the implication of inverse stereochemistry and the requirements of a 5*Z*, 8*Z*, diene system (Van Dyk et al. 1991; Venter et al. 1997). In this study emphasis will be placed on 3-OH oxylipins.

1.5.2 Distribution in yeasts

The discovery of 3-OH oxylipins was recorded in 1964 by Tulloch and Spencer when they reported the presence of 3-D-OH palmitic acid (16:0) and stearic acid (18:0) as part of the extracellular glycolipids of strains of *Rhodotorula glutinis* and *R. graminis.* This discovery was followed by another report by Stodola and co-workers (1967) who found 3-OH oxylipins in the same yeasts. In 1968, Vesonder and co-workers identified 3-OH 16:0 in the ascomycetous yeast *Saccharomycopsis malanga*. In these studies no mention was made of their metabolism, ASA sensitivity or possible function.

In an attempt to determine if yeasts are capable of producing expensive ASA-sensitive oxylipins such as prostaglandins, the Kock-group embarked on an extensive bioprospecting campaign. The motivation for this study was based on the fact that prostaglandins, synthesized chemically through expensive processes, are widely used/applied in medical practice to elicit various physiological actions (e.g. induce labour, inhibit platelet aggregation), therefore a biotechnological route to produce these compounds, using cheaper processes, was needed for obvious advantages (Dixon 1991). Strikingly, in the early 1990's the Kock group uncovered ASA-sensitive AA metabolites in the *Lipomycetaceae* family (*Dipodascopsis*, *Lipomyces*, *Zygozyma*, and *Myxozyma*), that were identified as prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and $PGF_{2\alpha}$-lactone (Kock et al. 1991). In addition, an aspirin-sensitive 3-OH oxylipin i.e. 3*R*-hydroxy 5Z,8Z,11Z,14Z eicosatetraenoic acid (3*R*-HETE) was found in the yeast *Dipodascopsis uninucleata* when the yeast was fed AA (Van Dyk et al. 1991). Consequently, 3*R*-HETE was found to affect signal transduction processes in human neutrophils and tumour cells in multiple ways thereby rendering a biotechnological value to this compound (Nigam et al. 1996).

In order to determine the functions of oxylipins in yeasts, the effect of different low concentrations of aspirin on the life cycle of *D. uninucleata* was determined (Botha et al. 1992). The most susceptible part of the life cycle was found to be the sexual stages (i.e. liberation of ascospores from the ascus) while the production of 3*R*-HETE was also inhibited. The distribution of 3*R*-HETE in *D. uninucleata* was mapped using antibodies against this compound (Kock et al. 1998). Consequently, immunofluorescence microscopy indicated that these oxylipins are associated with

aggregating ascospores and gametes. Using transmission electron microscopy these ascospores were found to be ornamented with 3-OH oxylipin coated hooked surface ridges that are linked in a gear-like fashion inside the ascus. These ornamentations are believed to be needed for effective individual release of these spores from asci (Kock et al. 2004).

Subsequent studies show that 3-OH oxylipins are produced by various yeasts and mucoralean fungi and were found to be associated with the surfaces of aggregating vegetative and sexual spores (Kock et al. 2003). The distribution of 3-OH oxylipins in ascomycetous yeasts is shown in Table 1. Interestingly, other oxylipins known as precocious sexual inducers or psi factors (a collection of hydroxylated oleic and linoleic acid derivatives) have been shown to play a key role during the sexual reproductive stage of *Aspergillus nidulans* (Tsitsigiannis et al. 2005). In *A. nidulans*, these oxylipins are involved in the switch between vegetative and sexual reproductive growth.

Oxylipins were also reported in the pathogenic yeast *Candida albicans* and were observed on the surfaces of infectious hyphae (Deva et al. 2000; 2001; 2003). They are believed to play a role in the morphogenesis and possibly pathogenicity of this yeast. Evidence for the role of 3-OH eicosanoids during candidiasis was provided by Ciccoli et al. (2005) when they showed that AA, released from infected host cells is converted by *C. albicans* to 3*R*-HETE, which in turn serves as a substrate for COX-2 in the host cells to produce pro-inflammatory 3-OH-PGE_2.

In other studies, Alem and Douglas (2004) established that biofilm formation by *C. albicans* could be inhibited by low concentrations of aspirin. However when PGE_2 was added in conjunction with aspirin, the inhibitory effect of aspirin was eradicated, indicating a possible role for prostaglandins in the regulation of biofilm formation. In 2005, Alem and Douglas also exposed the ability of both biofilms and planktonic (suspended) cells of *C. albicans* to produce extracellular prostaglandins. Their results suggest that these oxylipins might be important virulence factors in biofilm-associated infections.

In a review article in 2006, Erb-Downward and Huffnagle forecast that the next 10 years should not only witness an increase in what is known about nonmammalian oxylipins i.e. fungal oxylipins but should be the beginning of the practical applications of this information.

Table 1. Distribution of oxylipins in some ascomycetous yeasts

Genus	Type of 3-OH oxylipin	Associated structure	Reference
Ascoidea			
A. africana	3-OH 10:1	ascospores	Bareetseng et al. 2005
A. corymbosa	3-OH 17:0	ascospores	Ncango et al. 2006
Candida			
C. albicans	3,18 diHETE	hyphal cells	Deva et al. 2000
C. magnoliae	3-OH 17:1, 18:2	meiospores and meioconidiophore	Swart 2005 (Personal communication)
Dipodascopsis			
D. tóthii	3-OH 14:2, 14:3, 20:3, 20:5	ascospores	Kock et al. 1997
D. uninucleata var. uninucleata	3-OH 14:2, 14:3, 20:3, 20:5	ascospores	Fox et al. 1997 Venter et al. 1997

Genus	Type of 3-OH oxylipin	Associated structure	Reference
Dipodascus			
D. albidus	3-OH metabolite	ascospores	van Heerden et al. 2005
D. ambrosiae	3-OH metabolite	ascospores	Smith et al. 2003
D. geniculatus	3-OH metabolite	ascospores	van Heerden et al. 2006
D. macrosporus	3-OH metabolite	ascospores	Smith et al. 2003
D. magnusii	3-OH metabolite	ascospores	Smith et al. 2003
D. spicifer	3-OH metabolite	ascospores	Smith et al. 2003
D. tetrasperma	3-OH metabolite	ascospores	Smith et al. 2003
Eremothecium			
E. ashbyi	3-OH 14:0	ascospores	Kock et al. 2004
E. sinecaudum	3-OH metabolite	ascospores	Bareetseng et al. 2004
Lipomyces			
L. doorenjongii	3-OH metabolite	ascospores	Smith et al. 2000
L. kockii	3-OH metabolite	ascospores	Smith et al. 2000
L. kononenkoae	3-OH metabolite	ascospores	Smith et al. 2000
L. starkeyi	3-OH metabolite	ascospores	Smith et al. 2000
L. yamadae	3-OH metabolite	ascospores	Smith et al. 2000
L. yarrowii	3-OH metabolite	ascospores	Smith et al. 2000
Nadsonia			
N. commutata	3-OH 9:1	vegetative cells	Bareetseng 2004
N. fulvescens	3-OH metabolite	vegetative cells	Bareetseng 2004
Saccharomyces			
S. cerevisiae	3-OH 8:0, 10:0	vegetative cells	Kock et al. 2000
Saccharomycopsis			
S. capsularis	3-OH 9:1	ascospores	Sebolai 2004
S. fermentans	3-OH metabolite	ascospores	Sebolai et al. 2005
S. javanensis	3-OH 9:1	ascospores	Sebolai et al. 2005
S. malanga	3-OH 16:0	vegetative cells	Sebolai et al. 2001
S. synnaedendra	3-OH 16:0, 17:0, 18:0, 19:0, 19:1, 20:0, 22:0	vegetative cells	Sebolai et al. 2004
S. vini	3-OH 9:1, 10:1	ascospores	Sebolai et al. 2005
Saturnispora			
S. saitoi	3-OH 9:1	ascospores	Bareetseng et al. 2005

1.5.3 Distribution of 3-OH oxylipins in the genus *Eremothecium*

Recently the distribution of 3-OH oxylipins was mapped in the yeasts *E. ashbyi* and *E. sinecaudum*, using immunofluorescence confocal laser scanning microscopy (Bareetseng et al. 2004; Kock et al. 2004). In *E. ashbyi* (Figure 2a-d) these compounds

were found to be present as part of a V-shaped fin-like structure on sickle-shaped ascospores (Kock et al. 2004). It is suggested that these fins act as stabilizers and hydrofoils thereby assisting effective water-propelled boomerang movement. This is needed for the spiked spore-tip to gain enough momentum in order to pierce through the ascus wall for dispersal purposes. Furthermore gas chromatography-mass spectrometry (GC-MS) revealed the structure of this oxylipin as a saturated 3-OH 14:0.

In *E. sinecaudum* (Figure 3a-d) ascospores are acicular with a smooth surface at the blunt end and concentric ridges at the pointed end (similar to a tapered corkscrew). Here, only the tapered corkscrew end is coated with 3-OH oxylipins (Bareetseng et al. 2004). It was suggested that the oxylipin-lubricated corkscrew part with spiked tip plays a role in water-driven drilling through the ascus wall affecting ascospore release. Since no oxylipin studies have been performed on three species of *Eremothecium*, i.e. *E. coryli E. cymbalariae* and *E. gossypii* these will be attended to in this study.

1.6 Aim of study

The aforementioned literature review motivated the following aims:

1. The distribution mapping of 3-hydroxy oxylipins in *E. coryli, E. cymbalariae* and *E. gossypii*,
2. Determining the function of these oxylipins, especially during sexual reproduction in these yeasts,
3. Determining the effect of ASA a 3-hydroxy fatty acid production inhibitor on sexual reproduction in *Eremothecium,*
4. Determining the antifungal properties of ASA in *Eremothecium*.

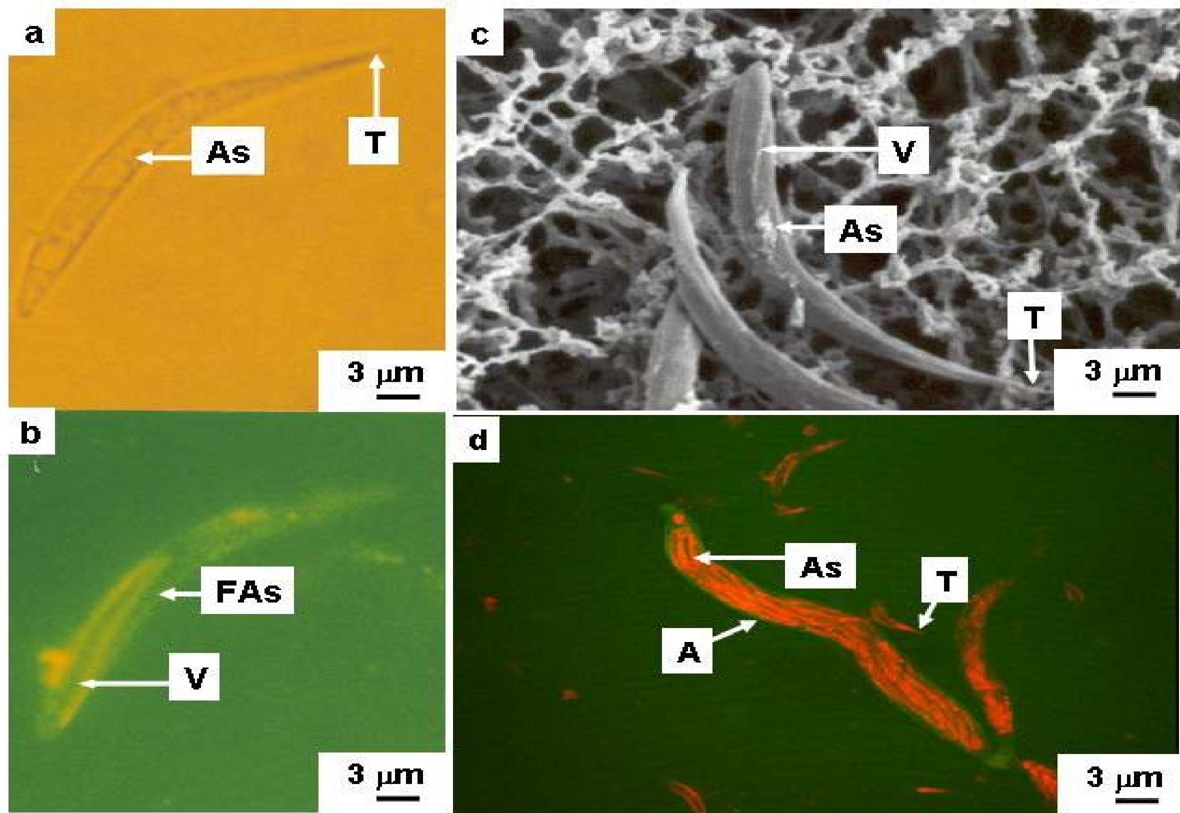

Figure 2: Different images of sickle-shaped ascospores produced by *Eremothecium ashbyi* UOFS –Y 630: (a) Light micrograph showing sickle-shaped ascospores (As) of *E. ashbyi* with ascospore tip (T). (b) Confocal laser scanning micrograph showing a fluorescing V-shaped (V) structure on the surfaces of these ascospores. (c) Scanning electron micrograph indicating fin-like (V) protuberances on the surfaces of these sickle-shaped ascospores. (d) Confocal laser scanning micrograph showing fluorescing ascospores (As) inside an ascus (A) when treated with orange G (Taken from Kock et al. (2004)).

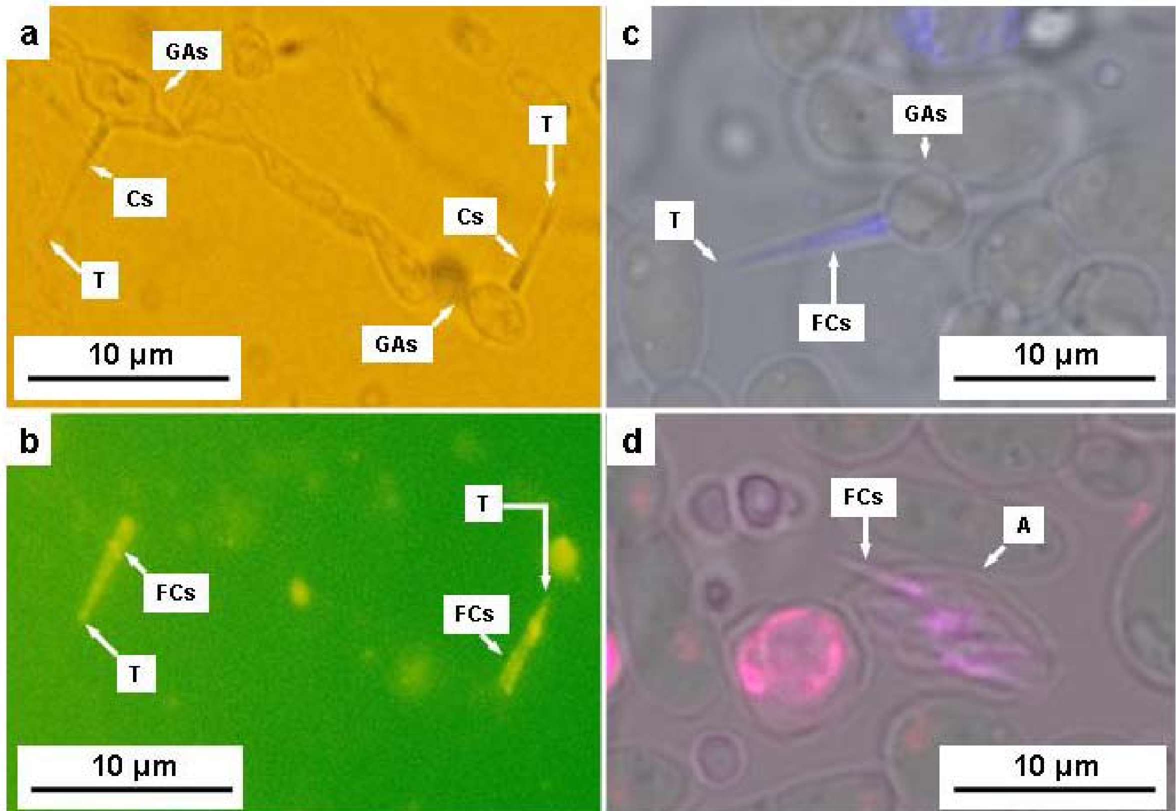

Figure 3: (a) Light micrograph of *Eremothecium sinecaudum* showing germinating ascospores (GAs) with sharp tips (T) that resembles a corkscrew (Cs). (b) Corresponding confocal laser scanning micrograph showing fluorescing of the corkscrew (FCs) part as well as the tip (T). (c - d) Fluorescing corkscrew part treated with Orange G as studied by confocal laser scanning microscopy (Taken from Bareetseng et al. (2004)).

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Please note: The chapters to follow are presented in format dictated by the journal of submission. As a result repetitions of some information could not be avoided.

CHAPTER 2

Oxylipin covered ascospores of *Eremothecium coryli*

The candidate performed preliminary studies on parts of chapter 2 during her B. Sc. Honours in 2004. After additional work in 2005, this study has been published in Antonie van Leeuwenhoek 89: 91-97 (2006) [Impact factor: 2.9] and also included with permission in this M. Sc. study. This is the independent work of the candidate.

Authors: Leeuw et al.

2.1 Abstract

Eremothecium coryli is known to produce intriguing spindle-shaped ascospores with long and thin whip-like appendages. Here, ultra structural studies using scanning electron microscopy, indicate that these appendages serve to coil around themselves and around ascospores causing spore aggregation. Furthermore, using immunofluoresence confocal laser scanning microscopy it was found that hydrophobic 3-hydroxy oxylipins cover the surfaces of these ascospores. Using gas chromatography-mass spectrometry, only the oxylipin 3-hydroxy 9:1 (a monounsaturated fatty acid containing a hydroxyl group on carbon 3) could be identified. Sequential digital imaging suggests that oxylipin-coated spindle-shaped ascospores are released from enclosed asci probably by protruding through an already disintegrating ascus wall.

2.2 Introduction

Ascospores often possess intriguing morphologies that have been used in the past to define genera of ascomycetous yeasts (Yarrow 1998). Although the unusual shapes and ornamentations of ascospores may be accidental, it is possible that in some cases, they offer a selective advantage. For example, it was found that *Dipodascopsis uninucleata* produces reniform to ellipsoidal spores with 3-hydroxy (OH) oxylipin-covered hooked surface ridges that are linked in gear-like fashion (Kock et al. 2004). These authors concluded that oxylipin-covered spore surface ornamentations are needed for effective individual discharge of spores through the narrow openings of bottle-shaped asci probably for dispersal purposes.

In addition, Kock and co-workers reported in 2004 that sickle-shaped ascospores of *Eremothecium ashbyi* contain nano-scale fin-like structures that are selectively covered with 3-OH oxylipins. It was suggested that these fins act as stabilizers and hydrofoils thereby assisting effective water-propelled boomerang movement. This is needed for the spiked spore-tip to gain enough momentum in order to pierce through the ascus-wall for dispersal purposes. Furthermore, *Eremothecium sinecaudum* produces needle-shaped ascospores of which a part simulates a tapered corkscrew. Strikingly, only the corkscrew part was found to be covered with 3-OH oxylipins (Bareetseng et al. 2004). It was suggested that the oxylipin-lubricated corkscrew part with spiky tip play a role in water-driven drilling through the ascus wall affecting ascospore release. This mechanism may also be used to induce plant infection (De Hoog et al. 1998).

Eremothecium coryli is known to produce intriguing spindle-shaped ascospores with long whip-like appendages (De Hoog et al. 1998). What are the functions of these structures? Do they contain 3-OH oxylipins? How are these ascospores released from asci? These questions will be addressed in this investigation.

2.3 Materials and methods

Strains used and cultivation

Eremothecium coryli UOFS Y-1155, obtained from the culture collection of the University of the Free State (UFS), Bloemfontein, South Africa was used throughout the study. *E. coryli* was cultivated on yeast-malt (YM) agar (Wickerham 1951) for 7 days at 25 °C to reach its sexual stage.

Microscopic studies

During the sexual stage, a light microscope (Axioplan Zeiss, West Germany) coupled to a Colorview Soft Digital Imaging System (Münster, Germany) was used for ascospore and sequential ascospore release studies.

Immunofluorescence microscopy

Synthesis of 3-hydroxy oxylipins and preparation of antibodies: R- and S- isomers of 3-OH eicosatetraenoic acid (3-HETE) were synthesized by Bhatt et al. (1998) and Groza et al. (2002). Antibodies against synthetic 3-HETE were raised in rabbits and characterized as described (Kock et al. 1998). Interestingly, antibodies were specific against all fatty acids carrying a C3-OH group and not only 3-HETE.

Microscopy: Immunofluorescence of yeast cells was performed as described (Kock et al. 1998) and includes treatment with primary antibody against 3-OH oxylipins as well as Fluorescein Isothiocyanate (FITC)-conjugated secondary antibody (Sigma, U.S.A.). In order to maintain cell structure, antibody, fluorescence and wash treatments were performed in 1 ml plastic tubes. Following adequate washing, the cells were fixed on a microscope slide and studied using a Nikon 2000 Confocal Laser Scanning Microscope (Japan).

Scanning electron microscopy (SEM)

Cells and spores were collected from agar plates and suspended in 3% sodium phosphate buffered (0.1 M, pH 7.0) glutardialdehyde and fixed for 3 h. The suspension was rinsed once by centrifugation with the same buffer to remove excess aldehyde fixative and then post-fixed for 1 h in 1% osmium tetroxide in similar buffer solution. The suspension was rinsed twice by centrifugation to remove excess osmium solution before dehydration commenced in an ethanol series (50%, 70%, 95% and two changes of 100%). The cell and ethanol suspension was centrifuged between each dehydration step. The cells and spore pellet was finally transferred to 5 µm critical point dryer baskets (Biorad, London, United Kingdom) for the critical point drying process. The dried pellet of cells and spores was dispersed over a thin layer of epoxy glue (Pratley, Gauteng, South Africa) on SEM stubs for mounting. The material was coated by 200 nm gold in a sputter coater (Biorad, London, United Kingdom) and examined with the scanning electron microscope (Jeol 6400 WINSEM, Jeol Japan, London, United Kingdom branch).

3-Hydroxy oxylipin extraction and derivatisation

Yeast cells in their sexual stage were suspended in 200 ml distilled water and the pH was dropped to below 4 using 3 % formic acid (Merck, Germany). Lipids were extracted by two volumes of ethyl acetate (200 ml; Merck, Germany) and the organic phase was evaporated using nitrogen gas (AFROX, South Africa). Lipid extracts were methylated using self-prepared diazomethane and silylated with *bis*-(trimethylsilyl) trifluoroacetamide (BSTFA – Merck, Germany) for 1 h respectively then dissolved in chloroform: hexane (4:1, v/v; Merck, Germany).

Gas chromatography-mass spectrometry (GC-MS)

The treated samples were injected into a Finnigan TraceGC Ultra gas chromatograph (GC; Finnigan, San José, Calif., USA) with a HP5 fused silica capillary column (60m long, 0.32 cm diam., 0.1 µm coating thickness) coupled to a Finnigan Trace DSQ mass spectrometer (MS). The carrier gas was helium at 1.0 ml min^{-1}. The initial oven temperature of 110 °C was maintained for 2 min, then increased to a final temperature of 280 °C at a rate of 5 °C min^{-1}. The GC-MS was auto-tuned for *m/z* of 50-502. Each sample (1 µl) was injected into the GC-MS at a split ratio of 1:20 at an inlet temperature of 250 °C (Venter et al. 1997).

2.4 Results and discussion

Light microscopy of ascospores (Figure 1a, b) confirmed their structure as described by De Hoog et al. (1998). Figure 1a shows lengthy spindle-shaped ascospores with whip-like appendages that are formed by *E. coryli* within oval-shaped unconjugated asci. When released, the whip-like appendages are intertwined (Figure 1b) thereby attaching

the ascospores in groups. Similar results were found when using SEM (Figure 2). These studies further illustrate that except for the appendages, the ascospores of this strain have no other ornamentations.

When 3-OH oxylipin-specific antibodies were added to the ascospores of *E. coryli*, the whole ascospore as well as whip-like appendage fluoresced as visualized by confocal laser scanning microscopy (Figure 3a, b). These results are at variance with those reported for *E. ashbyi*, where only parts (nano-scale fins) of the ascospore selectively fluoresced (Kock et al. 2004) or *E. sinecaudum* where only the corkscrew ascospore-part fluoresced thereby indicating the position of these oxylipins (Bareetseng et al. 2004). The side view of a cluster of ascospores of *E. coryli* shows as before intertwined whip-like appendages, this time also coiling around a germinating ascospore thereby illustrating the possible function of these ultra-thin strings (Figure 3b).

Following these observations, the identity of the 3-OH oxylipin was determined by GC-MS and found to be a 3-OH 9:1 (Figure 4 a,b). This oxylipin, which eluted at 11.66 min (Figure 4a) is characterized by a major ion (Figure 4b) at *m/z* 175 [$CH_3O^{\cdot}(CO)^{\cdot}CH_2{}^{\cdot}CHO^{\cdot}TMSi$], indicating an OH group at carbon 3. Moreover, this oxylipin is characterized by a peak at *m/z* 243 (M^+-15) indicating a M^+ (mother ion) of *m/z* 258 which is typical of a 3-OH 9:1 (Van Dyk et al. 1991).

To demonstrate the possible function of the spindle-shaped ascospores with whip-like appendages, sequential live digital imaging was attempted using a light microscope coupled to a digital image analyzer. The sequential release of ascospores

from an ascus is presented in Figure 5a-c. Figure 5a shows spindle-shaped ascospores within an intact ascus. First the pointed ascospores protrude through a dissolving ascus wall – the latter probably through enzymatic action (Figure 5b). Finally the ascus wall almost completely disintegrates as the ascospores are liberated (Figure 5c). The whole process took about 12 h.

In conclusion, results obtained in this study suggest that the pointed ascospore shape of *E. coryli* may be involved in rupturing and therefore further assisting probably enzyme induced disintegration of the ascus wall while the ultra-thin whip-like appendages may be responsible for attaching these ascospores in clumps. This may be through entropic based hydrophobic forces affected by the 3-OH oxylipin covering the surfaces of these ascospores. Here, no forcible spore discharge mechanics could be observed as reported in *D. uninucleata* (Kock et al. 2004) and *Metschnikowia australis* (Lachance et al. 1976). The possible functions of ascospore shape and associated oxylipins in plant infection causing stigmatomycosis should now be addressed for *E. coryli* (De Hoog et al. 1998).

Research so far implicates 3-OH oxylipins to function as both lubricant and adherent that assist water-propelled spore movement and aggregation in polar medium respectively (Kock et al. 2003). The question that now arises concerns the lubricity properties of these oxylipins. How do they compare with normal lubricants in the market today? Castor oil, containing mainly ricinoleic acid (12-OH 18:1) is essential for producing high quality lubricants for, amongst others, jet engines (Wood 2001). What effects do (i) the shifting of the hydroxyl group from position C12 to C3, (ii) chain length

and (iii) desaturation have on the lubricating properties of these oxylipins? In order to assess these, significant amounts of 3-OH oxylipins with different chain lengths and desaturation will first have to be produced. This may be achieved by exploring biotechnological and/or existing chemical synthesis routes (Bhatt et al. 1998; Groza et al. 2002).

2.5 Acknowledgements

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2.7 Figures

Figure 1: Light micrograph of *Eremothecium coryli*, showing (a) spindle-shaped ascospores (As) in an ascus (A) as well as (b) released ascospores (As) held together by thin whip-like appendages (Ap).

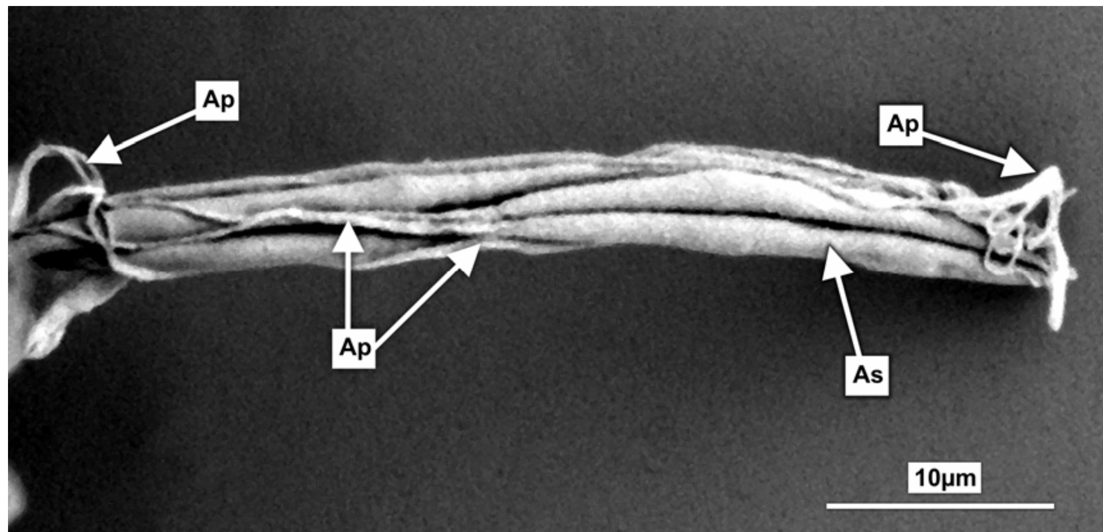

Figure 2: A scanning electron micrograph of ascospores (As) with appendages (Ap) coiling around each other and ascospores of *Eremothecium coryli*.

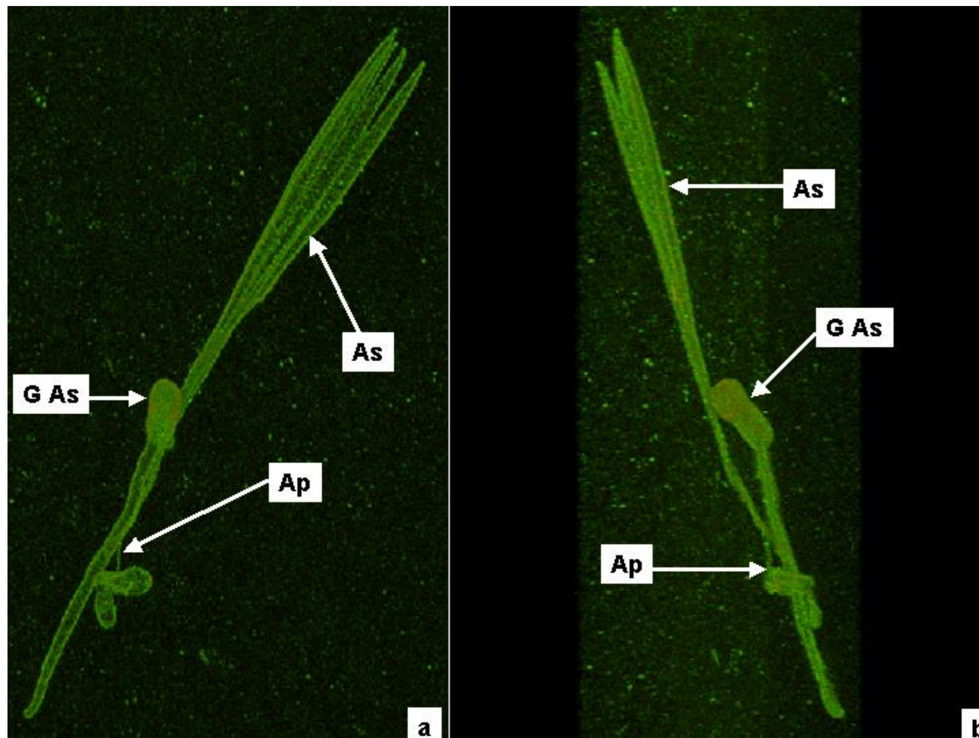

Figure 3: Immunofluorescence staining of ascospores (As) in *Eremothecium coryli*. (a) Indicates fluorescing ascospores (As) suggesting the presence of 3-hydroxy oxylipins. (b) Is a side-view of (a) showing appendages (Ap) coiling around a germinating ascospore (G As).

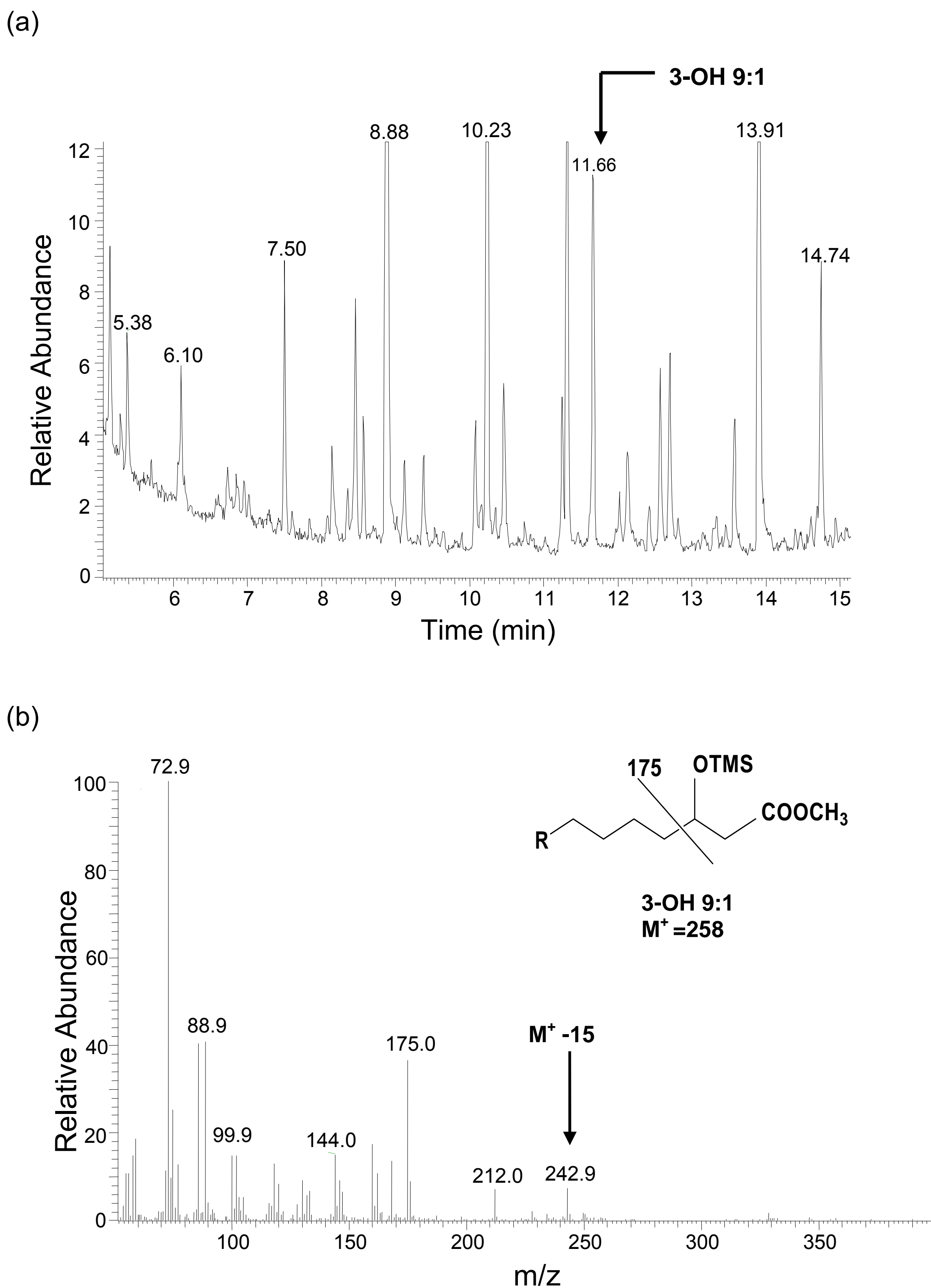

Figure 4: Total ion chromatogram (a) and mass spectrum (b) obtained from *Eremothecium coryli* during its sexual stage.

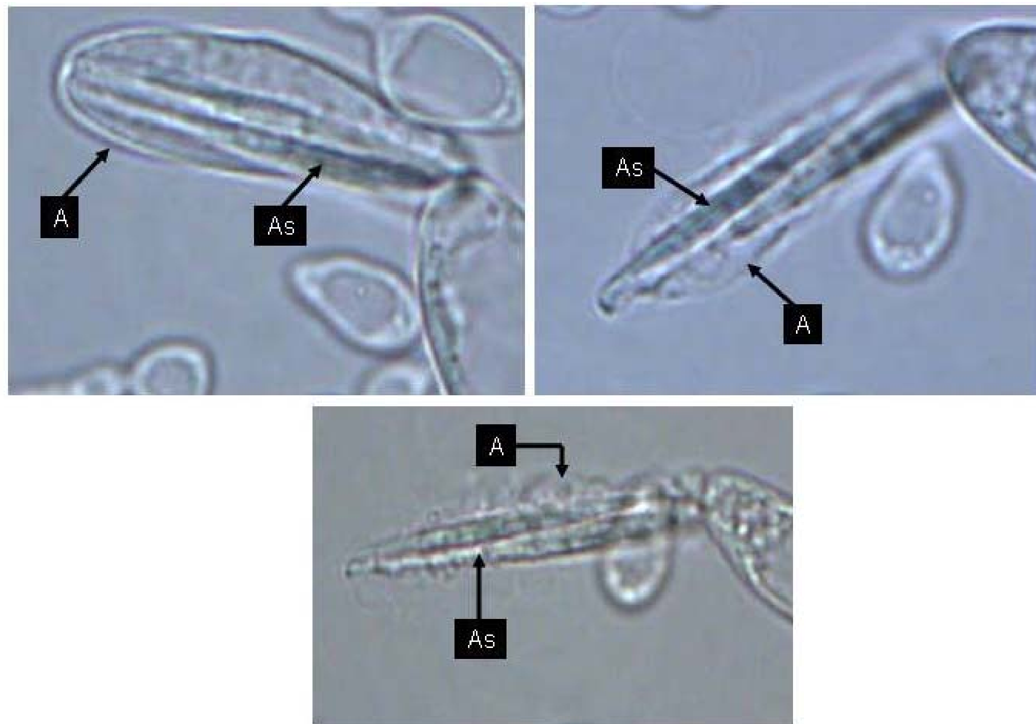

Figure 5: Sequential light micrographs of *E. coryli* over a 12 h period. (a) Shows an undamaged ascus wall (Aw) with spindle-shaped ascospores (As) inside an ascus. (b) Depicts ascospores protruding through a disintegrating ascus. (c) Indicates further disintegration of ascus wall with protruding spindle-shaped ascospores.

CHAPTER 3

Acetylsalicylic acid as antifungal in *Eremothecium* and other yeasts

This study was accepted for publication in Antonie van Leeuwenhoek (In Press; DOI 10.1007/s10482-006-9124-4) [Impact factor: 2.9]. Each experiment shown in table 1 was performed in duplicate by myself, further repetitions to prove proposed theory were also done by separate hands i.e. Miss C.W. Swart and Mr. M.D. Ncango.

Authors: Leeuw et al.

3.1 Abstract

Interesting distribution patterns of acetylsalicylic acid (ASA, aspirin) sensitive 3-hydroxy (OH) oxylipins were previously reported in some representatives of the yeast genus *Eremothecium* – an important group of plant pathogens. Using immunofluorescence microscopy and 3-OH oxylipin specific antibodies in this study, we were able to map the presence of these compounds also in other *Eremothecium* species. In *E. cymbalariae*, these oxylipins were found to cover mostly the spiky tips of narrowly triangular ascospores while in *E. gossypii*, oxylipins covered the whole spindle-shaped ascospore with terminal appendages. The presence of these oxylipins was confirmed by chemical analysis. When ASA, a 3-OH oxylipin inhibitor, was added to these yeasts in increasing concentrations, the sexual stage was found to be the most sensitive. Our results suggest that 3-OH oxylipins, produced by mitochondria through incomplete β-oxidation, are associated with the development of the sexual stages in both yeasts. Strikingly, preliminary studies on yeast growth suggest that yeasts, characterized by mainly an aerobic respiration rather than a fermentative pathway, are more sensitive to ASA than yeasts characterized by both pathways. These data further support the role of mitochondria in sexual as well as asexual reproduction of yeasts and its role to serve as target for ASA antifungal action.

3.2 Introduction

Eremothecium is regarded as a monophyletic group consisting of the previously described genera *Ashbya, Eremothecium, Holleya* and *Nematospora*. These yeasts are widely distributed and of economic importance since they are pathogens of a variety of plants such as cotton, citrus fruit and flax (De Hoog et al. 1998). In certain African, Caribbean and American countries they have been the most destructive pathogens of various crops and continue to cause extensive damage (Batra 1973). Studies exposing new perspectives on target sites that can assist in inhibiting dispersal and growth of these plant pathogens will therefore be of value.

3-Hydroxy (OH) oxylipins (oxygenated free fatty acids) in yeasts were reported by Kurtzman and co-workers in 1974. However, the presence of acetylsalicylic acid (ASA, aspirin) - sensitive oxylipins in yeasts, especially prostaglandins and 3-OH oxylipins, was discovered only in the early 1990's (Dixon 1991; Kock et al. 1991; Van Dyk et al. 1991). Consequently, the use of non-steroidal anti inflammatory drugs (NSAIDs) such as aspirin as antifungal that acts on these target sites, have been suggested (Kock and Coetzee 1990). These oxylipins were later found in various yeasts (Ciccoli et al. 2005; Deva et al. 2000, 2001, 2003; Kock et al. 2003; Noverr et al. 2003; Van Heerden et al. 2005) while subsequent studies further exposed these oxylipins as new target sites for developing novel antifungals (Alem and Douglas 2004, 2005; Erb-Downward and Huffnagle 2006; Noverr et al. 2003). Literature also suggests that 3-OH oxylipins are associated with the surfaces of ascospores where they probably assist in ascospore release from enclosed asci via for example turgor pressure (Fisher et al. 2004; Kock et al. 2004). Here, aspirin inhibits not only the production of these oxylipins,

but also yeast dispersal. It is suggested that smart mechanical movement of oxylipin-coated ascospores with different shapes, may find application in the field of engineering (Kock et al. 2006).

Since interesting spore mechanics as well as oxylipin distribution patterns and function have been suggested in some species of *Eremothecium*, it will be of interest to further assess this phenomenon in the remaining representatives. Consequently, in this study, the distribution of oxylipins in the yeasts *E. cymbalariae* and *E. gossypii* is mapped and characterized chemically. In addition, the influence of ASA, a known 3-OH oxylipin synthesis and β-oxidation inhibitor, on the life cycles and spore dispersal of both these yeasts, is assessed (Botha et al. 1992; Glasgow et al. 1999; Kock et al. 1999).

Literature suggests that ASA may act as antifungal that targets 3-OH oxylipin production and therefore also mitochondria (Ciccoli et al. 2005; Kock et al. 2003). Is it possible that yeasts which are dependable on mitochondrial activity for growth i.e. through aerobic respiration, are more susceptible to ASA compared to yeast capable of also utilizing the anaerobic fermentation pathway? This will also be assessed in this study.

3.3 Materials and methods

Strains used and cultivation

Eremothecium cymbalariae UOFS Y-2534 and *E. gossypii* UOFS Y-2535 are preserved in the culture collection of the University of the Free State (UFS), Bloemfontein, South Africa. These yeasts were used throughout the study. Both yeasts were cultivated on

yeast-malt (YM) agar (Wickerham 1951) for 4 days at 25 °C to reach their respective sexual stages. During this stage, a light microscope (Axioplan, Zeiss, Göttingen, Germany) coupled to a Colorview Soft Digital Imaging System (Münster, Germany) was used for ascospore observation.

Scanning electron microscopy (SEM; Leeuw et al. 2006)

Sporulating yeast cells were collected from agar plates and immediately fixed with 3% sodium phosphate buffered (0.1M, pH 7.0) glutardialdehyde (Merck, Darmstadt, Germany) for 3 h. The suspension was rinsed once by centrifugation with the same buffer to remove excess aldehyde fixative and then post-fixed with 1% aqueous osmium tetroxide (Merck, Darmstadt, Germany) in similar buffer solution. The suspension was rinsed twice by centrifugation to remove excess osmium solution and dehydrated by using a graded ethanol sequence (50%, 70%, 95%, 100% x 2 for 30 min per step). The cell and ethanol suspension was centrifuged between each dehydration step. The cells and spore pellet was finally transferred to 5 µm critical point dryer baskets (Biorad, London, UK) for the critical point drying process. The dried pellet of cells and ascospores was dispersed over a thin layer of epoxy glue (Prately, Gauteng, South Africa) on SEM stubs for mounting. The material was coated with 200 nm gold in a sputter coater (Biorad, London, UK) and viewed using a Joel 6400 WINSEM scanning electron microscope (SEM, Jeol, Tokyo, Japan) (Van Wyk and Wingfield 1991).

Immunofluorescence studies (Leeuw et al. 2006)

Synthesis of 3-hydroxy oxylipins and preparation of antibodies: R- and S-isomers of 3-OH eicosatetraenoic acid (3-HETE) were synthesized by Bhatt et al. (1998) and Groza et al. (2002). Antibodies against 3-OH oxylipins were raised in rabbits and characterized according to Kock et al. (1998). These oxylipins were found to be specific against all fatty acids carrying a C3-OH group and not only 3-HETE.

Immunofluorescence microscopy: Yeast cells during their sexual stage were treated with a primary antibody against 3-OH oxylipins (30 µl for 1 h at room temperature). Cells were then washed to remove unbound antibodies and further treated with a FITC conjugated secondary antibody (30 µl for 1 h in the dark at room temperature - Sigma, USA). The cells were again washed to remove unbound antibodies. These treatments were executed in 2 ml plastic tubes in order to maintain cell structure. After adequate washing the cells were fixed on a microscope slide and viewed using a Nikon TE 2000, confocal laser scanning microscope (Japan).

3-Hydroxy oxylipin extraction and derivatisation (Leeuw et al. 2006)

Yeast cells in their sexual stage were suspended in 200 ml distilled water and the pH was dropped to below 4 using 3% formic acid (Merck, Germany). Lipids were extracted by two volumes of ethyl acetate (400 ml; Merck, Germany) and the organic phase was evaporated using nitrogen gas (AFROX, South Africa). Lipid extracts were methylated using diazomethane for 2 h at -20°C and silylated with *bis*- (trimethylsilyl) trifluoroacetamide (Merck, Germany) for 40 min at room temperature then reconstituted in chloroform: hexane (4:1, v/v; Merck, Germany).

Gas chromatography-mass spectrometry (GC-MS; Leeuw et al. 2006)

Treated samples were injected into a Finnigan TraceGC Ultra gas chromatograph (GC; Finnigan, San José, Calif., USA) with a HP5 fused silica capillary column (60 m long, 0.32 cm diam., 0.1 µm coating thickness) coupled to a Finnigan Trace DSQ mass spectrometer (MS). The carrier gas was helium at 1.0 ml min^{-1}. The initial oven temperature of 110 °C was maintained for 2 min then increased to a final temperature of 280 °C at a rate of 5 °C min^{-1}. The GC-MS was auto-tuned for *m/z* of 50-502. Each sample (1 µl) was injected into the GC-MS at a split ratio of 1:20 at an inlet temperature of 230 °C (Venter et al*.* 1997).

ASA inhibition studies

Sexual cycle: Both yeast species were streaked out on YM agar (Wickerham 1951) and cultivated at 25 °C in Petri dishes until sporulation was observed. Cells were transferred from the Petri dishes into a 250 ml conical flask containing 50 ml of glucose-YM broth (10 g l^{-1} glucose, 5 g l^{-1} peptone, 3 g l^{-1} yeast extract, 3 g l^{-1} malt extract). Appropriate volumes were then transferred to several 500 ml conical flasks containing 100 ml of the same medium. ASA (Sigma, Steinheim, Germany) was first diluted in a minimal volume ethanol and added to each individual flask at the start of cultivation to reach a final concentration of 0 mM (control) 1 mM, 2 mM, 3 mM, 4 mM and 5 mM. These cultures were incubated at 25 °C for 48 h. Since ASA had to be dissolved in minimum amounts of 98% ethanol (Merck, Gauteng, South Africa), further control experiments containing similar amounts of ethanol without ASA were performed. Since it is not possible to quantify these asci with the aid of a counting chamber due to the extensive aggregation of hyphae and ascospores, the effect of different ASA concentrations on the sexual

cycle was determined for each yeast by counting at least 35 mature asci in four adjacent microscope fields of each culture using a Zeiss light microscope. In each case the percentage empty asci (indicating ascospore release) was calculated. This experiment was repeated in triplicate for each yeast species. Light micrographs were taken using a light microscope (Axioplan, Zeiss, Göttingen, Germany) coupled to a Colorview Soft Imaging System (Münster, Germany).

Asexual cycle: Yeasts in Table 1 were all subjected to fermentation tests on glucose (Radchem, Johannesburg, South Africa) at 30 ^{0}C as described, using Durham tubes to measure carbon dioxide release (Yarrow 1998). Here + indicates strong fermentation, with gas filling the insert tube within 4 days, +w indicate weak fermentation, with the inserted tube only partially filled after 4 days and -, no gas in inserted tube after 4 days of incubation.

In addition, these yeasts were cultivated aerobically in glucose containing liquid media in test tubes while agitating on a Rollordrum according to the assimilation tests in liquid medium protocol (Yarrow 1998). Each set consisted of eight test tubes each containing 6.7 g l^{-1} Yeast Nitrogen Base (Difco, Becton, Dickinson and Company, MD) and 2% (w/v) glucose medium. In addition, the following ASA concentrations dissolved in 98% ethanol (ETOH) were added. Tube 1: no ASA (control); Tube 2: 1 mM ASA (in 11.3 µl ETOH); Tube 3: 2 mM ASA (in 22.5 µl ETOH); Tube 4: 3 mM ASA (in 33.8 µl ETOH); Tube 5: 4 mM ASA (in 45 µl ETOH); Tube 6: 5 mM ASA (in 56.3 µl ETOH); Tube 7: 56.3 µl ETOH without ASA (ETOH control); Tube 8: no ASA, ETOH or inoculum (neg. control) – only medium. For *Eremothecium*, 0.1% yeast extract (Merck, Wadeville,

Gauteng, South Africa) was additionally added (De Hoog et al. 1998). In all cases, cells were cultivated at 28 ^{0}C for 4 days. Growth was measured after 4 days with a white card containing black lines as prescribed (Yarrow 1998). Here, +++ indicates good growth (no black lines visible), ++ indicates growth (black lines just visible) and + indicates weak or no growth (black lines similar to that of inoculated tube at start of growth).

3.4 Results and discussion

Eremothecium cymbalariae

According to literature, *E. cymbalariae* is characterized by asci that are formed on hyphal tips containing hyaline, narrowly triangular ascospores with needle-shaped ends (De Hoog et al. 1998). The strain used in this study showed similar ascospore and ascus structures (Figure 1a – d). In addition, interesting patterns of ascospore aggregation after release from asci could be observed using light microscopy. Sometimes the second longest side of one ascospore attaches to the longest side of the adjacent triangular ascospore with the sharp tips showing in the same direction thereby forming a circular structure (Figure 1a). Scanning electron microscopy (SEM) revealed smooth narrowly limoniform asci that are formed on hyphal tips (Figure 2a) as well as the presence of surface ornamentations on the narrowly triangular ascospores (Figure 2b). Some preparations also clearly demonstrate a possible piercing action of the razor sharp nano-scale ascospore tips through cellular material and ascus (Figures 1d, 2c).

Using 3-OH oxylipin- specific antibodies, we were able to map the presence of 3-OH oxylipins on the surfaces of these ascospores. Here, the ascospore tip showed the highest affinity for the antibody thereby implicating increased concentrations of these

compounds at this position of the ascospore (Figure 3a). Strikingly, this is similar to the results found in *E. sinecaudum* where only the tapered corkscrew end of the ascospore was covered with oxylipins (Bareetseng et al. 2004). Do these ascospore tips also function to pierce through the ascus wall/substrate for release or attachment purposes respectively?

It is interesting to note that the ascospores sometimes adhered at the oxylipin-covered ascospore tips (Figure 3a, b). This suggests an adhesion role for 3-OH oxylipins as suggested in literature for other yeasts through entropic based hydrophobic forces in polar medium (Kock et al. 2000). Does this surface oxylipin contribute to the interesting ascospore aggregation patterns (Figure 1a) observed in this yeast?

Using GC–MS, (Figure 3c) a peak could be observed at retention time 31.60 min that is characterized by a major ion at *m/z* 175 [$CH_3O^{\cdot}(CO)^{\cdot}CH_2^{\cdot}CHO^{\cdot}TMSI$] indicating an OH group at carbon 3. In this case the oxylipin, produced only in trace amounts, was identified as 3-OH 13:0 (Van Dyk et al. 1991). Studies are underway to confirm the structure of this oxylipin. Since only one 3-OH oxylipin type was observed, we conclude that this should be the same as that indicated with immunofluorescence and may therefore be associated with the spores.

When ASA, a known 3-OH oxylipin inhibitor, was added at different concentrations to *E. cymbalariae*, ascospore formation and release was visibly inhibited in a dose dependant manner (Figure 4a-d). This was also true for *E. sinecaudum* (Bareetseng et al. 2004). The addition of corresponding amounts of only ethanol yielded

similar results as the untreated control. In the absence of ASA, 8% of the observed mature asci contained well developed ascospores, while 92% were empty and had already released their ascospores. As the ASA concentration increased, ascospore release and growth decreased in a dose dependent manner i.e. 1 mM ASA (44% empty asci, hyphae well developed), 2 mM ASA (16% empty asci, some hyphae appear granular and dead), 3 mM ASA (no asci formed, many hyphae appear granular and dead), 4 mM ASA and 5 mM ASA (no growth). Similar patterns were observed when this experiment was repeated. In the presence of 2 mM ASA, some asci contained underdeveloped ascospores (Figure 4c).

Eremothecium gossypii

Light microscopy of ascospores confirmed their structure as described by De Hoog et al. (1998). This yeast is characterized by spindle-shaped ascospores inside clavate asci that separate at maturity to release their ascospores (Figure 5a). When liberated from asci, the ascospores unite in small groups by attachment of ascospore appendages (Figure 5b) in a similar way as reported for *E. coryli* (Leeuw et al. 2006). No empty asci could be observed since they disintegrate after ascospore release probably by enzymatic action, thereby releasing ascospore clumps that are similar in orientation than those found within enclosed asci. No active piercing ascospore release mechanisms could be observed.

SEM revealed that the surface of the ascospore-part carrying the appendage is smooth whereas that of the other (blunted) end is ornamented with concentric ridges (Figure 6a). Here, appendages seem to be coiling around each other and around

ascospores probably for aggregation purposes (Figure 6b) as also reported in *E. coryli* (Leeuw et al. 2006).

When 3-OH oxylipin specific antibodies were added to the ascospores of *E. gossypii*, the entire ascospore as well as the terminal appendage fluoresced as visualized by confocal laser scanning microscopy (Figure 7a). These results are similar to that found in *E. coryli* (Leeuw et al. 2006). The structure of the 3-OH oxylipin, present in only trace amounts, was determined by GC-MS and found to be a 3-OH decenoic acid (3-OH 10:1) that eluted at 25.25 min (Figure 7c). The mass spectrum of this compound contained a major peak of *m/z* 175$[CH_3O^{\cdot}(CO)^{\cdot}CH_2^{\cdot}CHO^{\cdot}TMSi]$ which is characteristic for a 3-OH oxylipin (Van Dyk et al. 1991). Studies are underway to confirm the structure of this oxylipin. Since only one 3-OH oxylipin type was observed, we conclude that this should be the same as that indicated with immunofluorescence and may therefore be associated with the spores. Interestingly, a 3-OH oxylipin was also characterized in *E. coryli* using immunofluorescence as well as chemical analysis (Leeuw et al. 2006).

When ASA, a known 3-OH oxylipin inhibitor was added to *E. gossypii*, again the sexual stage was found to be more sensitive towards ASA than the vegetative hyphal stage. The effect of ASA on the sexual cycle was however difficult to assess, since mature asci disintegrated upon ascospore release. It was therefore not possible to count mature empty asci. In the presence of 3 mM ASA, some asci contained underdeveloped ascospores (not shown) while no asci could be observed in the presence of 4 and 5 mM ASA (Figure 8). Here, hyphal growth could still be observed

although to a limited extend – many became granular and appeared dead with increased concentrations of ASA (Figure 8). A similar pattern was observed when these experiments were repeated. *Eremothecium cymbalariae* was found to be more sensitive to ASA in this respect (Figure 4c) since no asci were formed by this yeast at 3 mM ASA and spores were underdeveloped already at 2 mM ASA. Since ASA inhibits reproduction in both yeasts, further studies should be performed to assess the antifungal ability of this and other NSAIDs on these yeasts when infecting plant crops.

It is interesting to note that in *E. coryli* and *E. gossypii*, characterized by needle-shaped ascospores with whip-like appendages, the whole of the spore was covered with 3-OH oxylipins. This is probably necessary for attachment in polar medium such as water for protection purposes. In *E. cymbalariae* and *E. sinecaudum*, only the sharp tips of the ascospores were selectively covered with these oxylipins. Is this necessary to assist in piercing and drilling movements respectively in order to be released from birth sacs (asci) and for attachments to host substrates? This study further demonstrates the ubiquitous nature of 3-OH oxylipins associated with ascospore surfaces of yeasts.

According to our results, the addition of 3-OH oxylipin inhibitors such as ASA at low concentrations mainly inhibited the sexual cycle such as ascospore development in the yeasts studied while these yeasts carried on to reproduce mainly asexually, although to a limited extend. These results point towards a selective association of these oxylipins with sexual reproductive stages. This is supported by recent genetic evidence that oxylipin production appears to play a role in fungal life cycle control particularly in sexual and asexual development through oxylipins called “psi factors”

[precocious sexual inducers i.e. secreted mixtures of mainly C8 - hydroxylated oleic- (18:1) and linoleic acid (18:2)]. These compounds, probably produced by fatty acid oxygenases (PpoA, PpoB and PpoC), have been shown to alter the ratio of asexual to sexual sporulation in the filamentous fungus *Aspergillus nidulans* (Tsitsigiannis et al. 2005). It is interesting to note that the gene *ssp1* has been identified previously to be involved in the production of fungal oxylipins in *Ustilago maydis* (Huber et al. 2002). Here, the Ssp1 protein (similar to linoleate diol synthase, a fatty acid dioxygenase) is localized on lipid bodies in germinating teliospores, probably suggesting a role in the mobilization of storage lipids during spore germination. Consequently, these studies suggest that oxylipins of different structures (hydroxyl groups on different carbons), chain lengths and origin are associated with sexual/asexual reproduction modes in fungi. It is interesting to note that lipid globules present in the asci of the yeast *Dipodascopsis uninucleata* also contain 3-OH oxylipins as demonstrated through immunogold labeling (Smith et al. 2000). This has most probably been produced in mitochondria and then deposited in the lipid globules.

It is suggested that 3-OH oxylipins are produced through incomplete β-oxidation in the mitochondria which is then released and deposited on, amongst others, ascospore surfaces (Kock et al. 2004). It is also reported that ASA inhibits β-oxidation in mitochondria and therefore 3-OH oxylipin synthesis (Deva et al. 2001; Glasgow et al. 1999). According to Glasgow and co-workers (1999), salicylate, the primary metabolite of aspirin inhibits β-oxidation of medium and long-chain fatty acids in rodent liver mitochondria but not peroxisomes. This may be ascribed to aspirin metabolites having structural similarities to the acyl-portions of the substrate and product of the 3-

hydroxyacyl-CoA dehydrogenase activity of the β-oxidation pathway. It has also been reported that aspirin induces changes in mitochondrial energy production through the uncoupling of oxidative phosphorilation (or inhibition of electron transport) in the rat intestine (Somasundaram et al. 1997). Interestingly, ascosporogenesis in *Saccharomyces cerevisiae* only occurs in the presence of metabolic active mitochondria (Codon et al. 1995; Marmiroli et al. 1983). Consequently, any decrease in mitochondrial respiration caused for instance by antibiotics which inhibit mitochondrial protein synthesis would therefore negatively affect the sexual cycle. It is important to assess if this is also true for yeasts representing *Eremothecium*.

Our results so far suggest that ASA also inhibits the asexual reproductive cycle (growth) of yeasts. Yeasts with both a fermentative as well as aerobic respiration metabolism were found to be more resistant in this respect to ASA compared to yeasts characterized by mainly an aerobic respiration pathway (fermentation - ; Table 1). In most cases, yeast strains that show fermentation, could also grow well in the presence of 5 mM ASA. However exceptions were observed in the diverse sample of yeasts tested. A weak fermenting *E. coryli* and strong fermenting *Schiz. octosporus* strain were more susceptible to ASA compared to the other strong fermenters. Those yeasts showing no CO_2 liberation i.e. no or limited fermentation, could scarcely or not grow in the presence of 5 mM ASA. Is it possible that ASA selectively inhibits mitochondrial respiration while fermentation is less affected thereby rendering enough energy for growth? It is important to further extend this database and also include amongst others respiratory deficient yeasts to further assess the general validity of this phenomenon. The affect of ASA on the growth of petit–phenotype–positive *versus* petit-phenotype-

negative yeasts should be further addressed (Moller et al. 2001). This will assess the use of low-cost NSAIDs such as aspirin as antifungals in animal and crop disease.

Before any final conclusions regarding the involvement of mitochondrially produced 3-OH oxylipins in the development of sexual stages and liberation of ascospores can be drawn, it is necessary to perform genetic studies. If genes responsible for mitochondrial development are inactivated, will the sexual cycle, spore release and 3-OH oxylipin production also be affected? The use of petit-mutants by inducing for example deletions in yeast mitochondrial DNA, would be a place to start such research (Moller et al. 2001).

According to literature, mitochondria evolved from Gram-negative bacteria such as the rickettsias, through endosymbiosis many millions of years ago (Gray et al. 2001). These bacteria also produce 3-OH oxylipins as part of their extracellular lipopolysaccharide (LPS) layer. This intriguing fact raises several important questions. Is it possible that this characteristic was maintained over these years? Can 3-OH oxylipins, necessary for bacterial endotoxicity also be inhibited by ASA (Kock et al. 2005)? It is interesting to note that Ciccoli and co-workers suggested in 2005 that 3-OH oxylipins, produced by *Candida albicans* upon infection from released host arachidonic acid, might be excreted in the host where in turn it is transformed via cyclooxygenase to inflammatory 3-OH prostaglandins. Can the type of 3-OH oxylipins produced by these bacteria today as well as those of yeasts, assists in elucidating the origin of yeast mitochondria? What is the conserved status of 3-OH oxylipin production in mitochondria

and their release in yeasts? How do yeasts manage to selectively deposit these oxylipins on nano-scale structures present on surfaces of ascospores?

3.5 Acknowledgements

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3.7 Tables

Table 1. The influence of acetylsalicylic acid on growth (after 4 days of cultivation) of some fermentative and non- fermentative yeasts.

Yeast	Cult. No.	Growth ASA concentration range (mM) 0	1	2	3	4	5	ETOH	-*	Ferment
A. africana	UOFS Y-1217	+++	++	++	+	+	+	+++	-	-
A. corymbosa	UOFS Y-732	+++	++	++	+	+	+	+++	-	-
A. rubescens	UOFS Y-733	++	+	+	+	+	+	++	-	-
E. ashbyi	UOFS Y-1122	+++	++	++	++	+	+	+++	-	-
E. coryli	UOFS Y-1155	+++	++	++	++	++	++	+++	-	+(w)
E. cymbalariae	UOFS Y-2534	+++	+	+	+	+	+	+++	-	-
E. gossypii	UOFS Y-2535	+++	+	+	+	+	+	+++	-	-
E. sinecaudum	UOFS Y-2358	+++	++	++	++	+	+	+++	-	-
Crypt. neoformans	UOFS Y-1378	+++	+++	++	++	+	+	+++	-	-
D. albidus	UOFS Y-1145	+++	+++	++	+	+	+	+++	-	-
Dip. uninucleata	UOFS Y-128	+++	+	+	+	+	+	+++	-	-
R. glutinus	UOFS Y-519	+++	+++	+	+	+	+	+++	-	-
L. starkeyi	UOFS Y-1999	+++	++	++	+	+	+	+++	-	-
C. magnoliae	UOFS Y-1039	+++	+++	+++	+++	+++	+++	+++	-	+
C. tropicalis	UOFS Y-210	+++	+++	+++	+++	+++	+++	+++	-	+
K. marxianus	UOFS Y-988	+++	+++	+++	+++	+++	+++	+++	-	+
P. anomala	UOFS Y-157	+++	+++	+++	+++	+++	+++	+++	-	+
S. cerevisiae	UOFS Y-1529	+++	+++	+++	+++	+++	+++	+++	-	+
Schiz. octosporus	UOFS Y-1715	+++	+++	+++	+++	++	++	+++	-	+
Schiz. pombe	UOFS Y-2174	+++	+++	+++	+++	+++	+++	+++	-	+
Z. baillii	UOFS Y-1865	+++	+++	+++	+++	+++	+++	+++	-	+

-* = negative control; 0-5 = ASA concentration in mM; ETOH = 0 mM ASA control + 56.3 µl ethanol; - = fermentation negative; + = fermentation positive; w = weak; +++: good growth; ++: growth; + weak or no growth. *A.* = *Ascoidea*; *C.* = *Candida*; *Crypt.* = *Cryptococcus*; *Dip.* = *Dipodascopsis*; *D.* = *Dipodascus*; *E.* = *Eremothecium*; *K.* = *Kluyveromyces*; *L.* = *Lipomyces*; *P.* = *Pichia*; *R.* = *Rhodotorula*; *S.* = *Saccharomyces*; *Schiz.* = *Schizosaccharomyces*; *Z.* = *Zygosaccharomyces*.

3.8 Figures

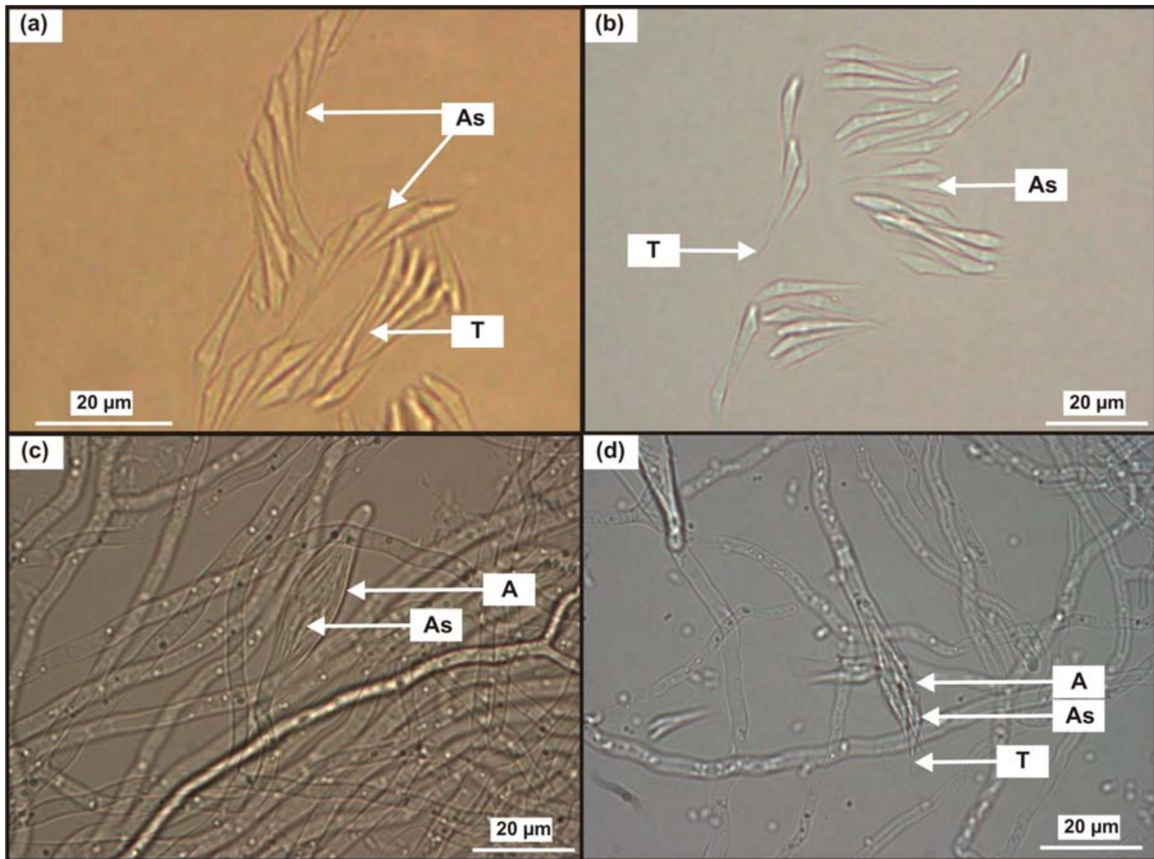

Figure 1: Light micrograph of *Eremothecium cymbalariae* showing ascospores (As) as well as sharp ascospore tips (T) in (a, b). Light micrograph of an ascus (A) with ascospores (As) inside (c) and light micrograph showing ascospore release, sharp tip first from asci (d).

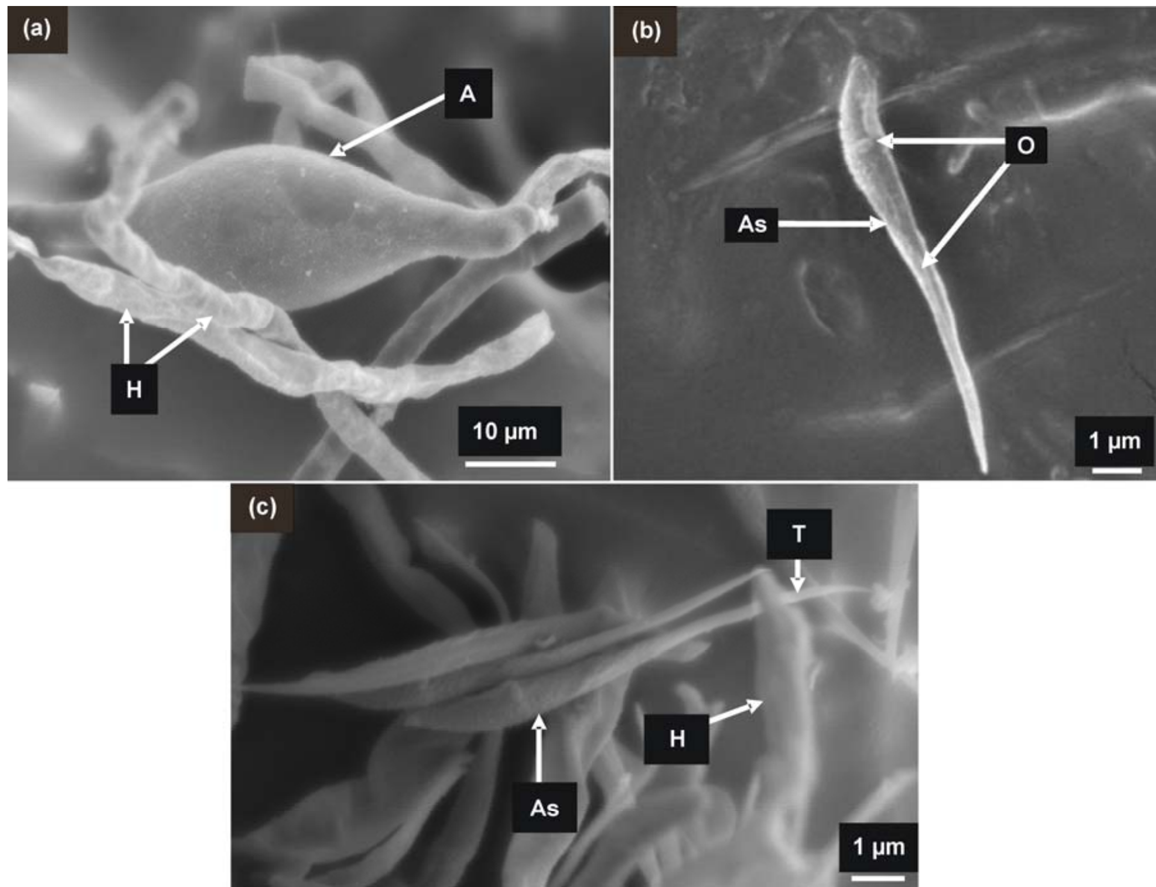

Figure 2: Scanning electron micrograph showing hyphae (H) surrounding a limoniform ascus (A) of *Eremothecium cymbalariae* (a). An ascospore (As) of *E. cymbalariae* (b) with surface ornamentations (O). Scanning electron micrograph (c) showing sharp ascospore (As) tip (T) piercing through cellular hyphal material (H).

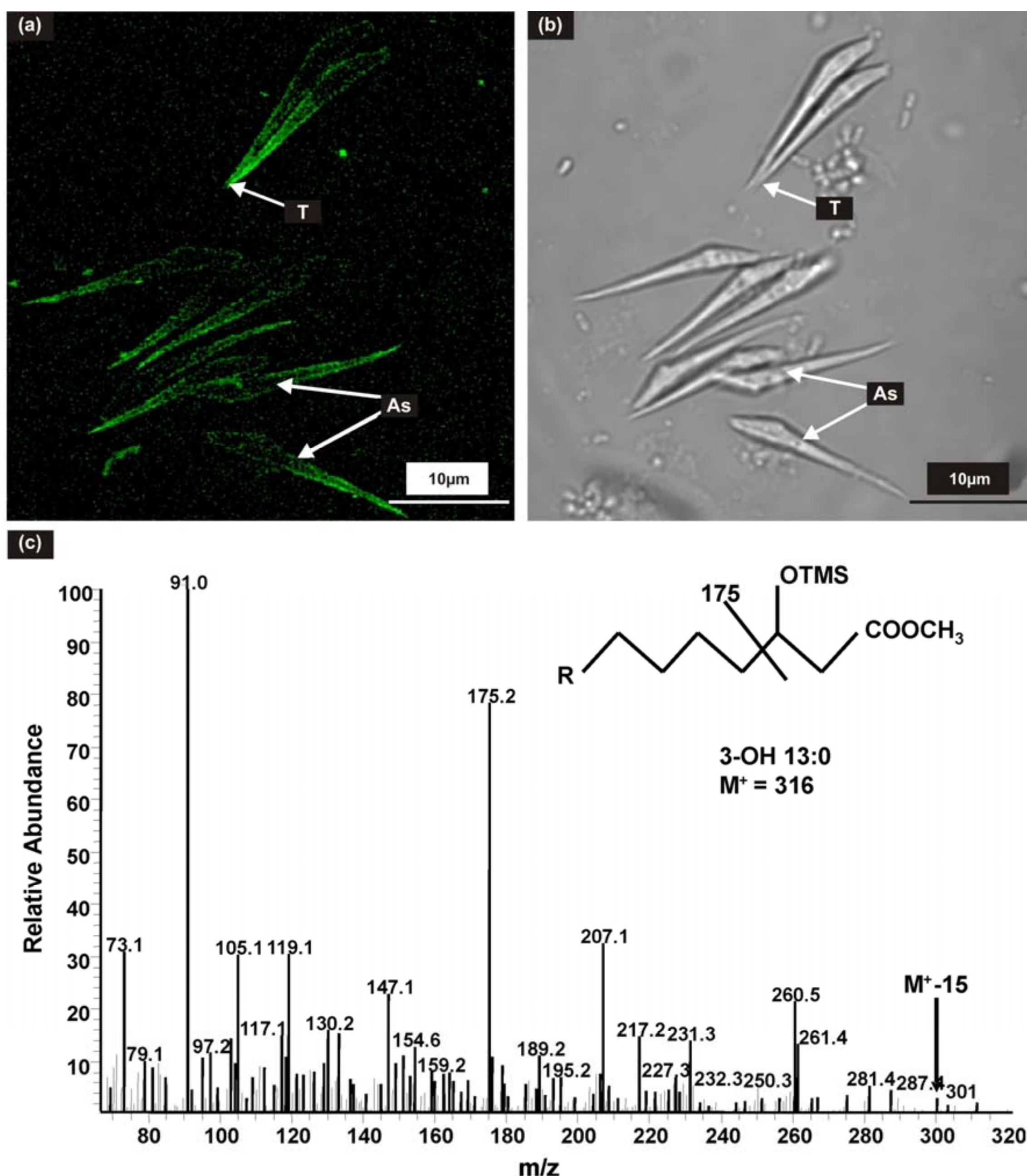

Figure 3: Oxylipin specific immunofluorescence micrograph (a) with corresponding light micrograph (b) of ascospores of *Eremothecium cymbalariae*. Fluorescing ascospores (As) suggest the presence of 3-hydroxy oxylipins mainly at tip (T) of ascospore. A mass spectrum of an oxylipin is shown in (c) obtained from *E. cymbalariae*.

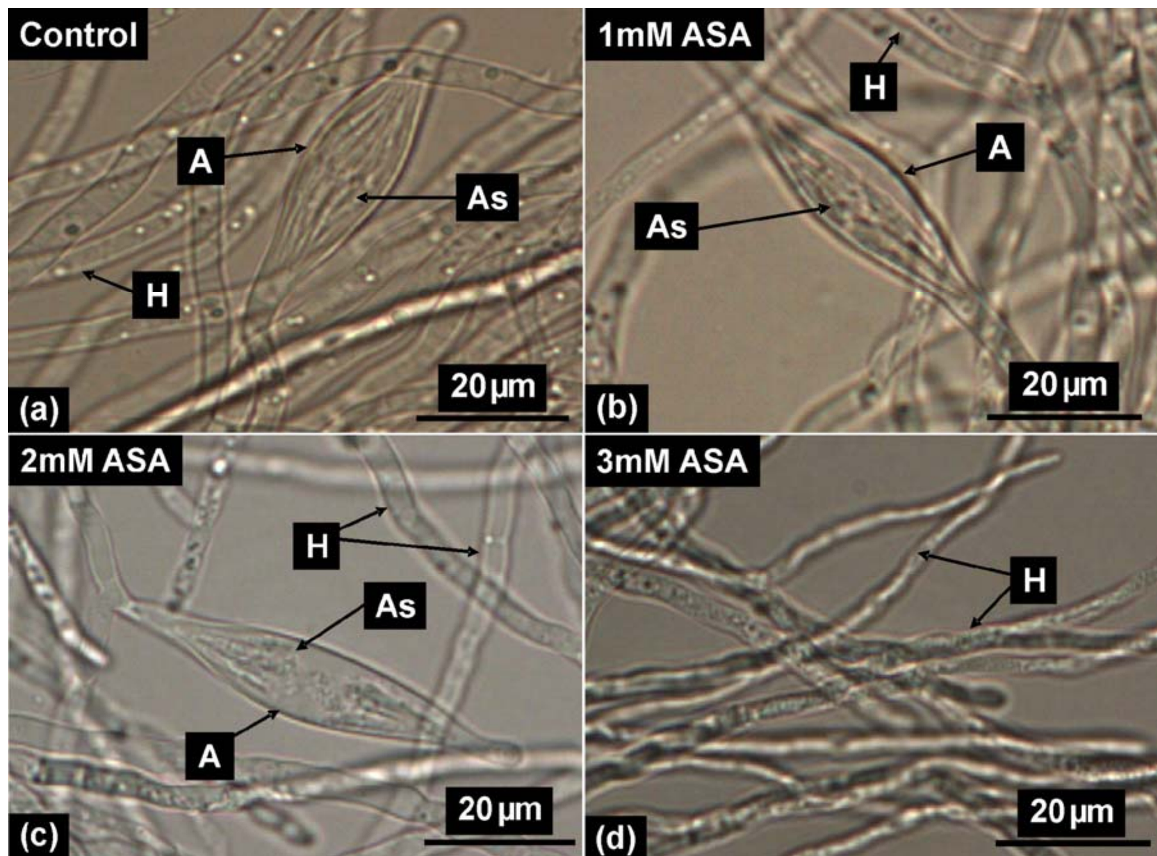

Figure 4: Light micrographs demonstrating the dose dependant effect of acetylsalicylic acid (ASA) on ascus and ascospore development in *Eremothecium cymbalariae*. Light micrograph of an ascus (A) with ascospores (As) as well as hyphae (H) in the absence of ASA (a). Ascus (A) with ascospores (As) as well as hyphae (H) in the presence of 1mM ASA (b). Light micrograph showing hyphae (H) as well as an ascus (A) with ascospores (As) that are not fully developed in the presence of 2 mM ASA (c) and (d) showing the absence of a sexual stage, only hyphae (H), in the presence of 3mM ASA.

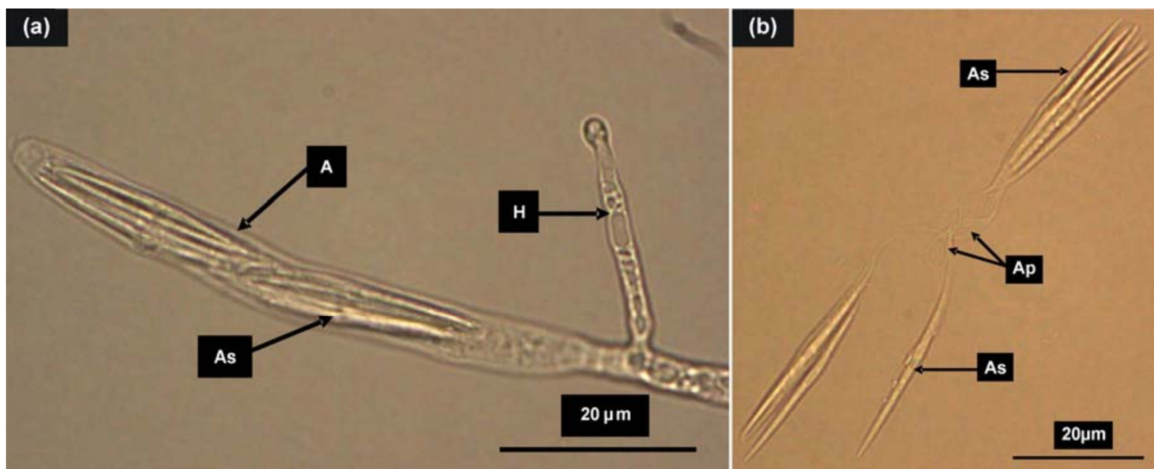

Figure 5: Light micrograph (a) of an ascus (A) with ascospores (As) and hyphae (H) of *Eremothecium gossypii*. Ascospores of *E. gossypii* with terminal appendages (Ap) coiling around each other to keep these ascospores in small groups, are shown in (b).

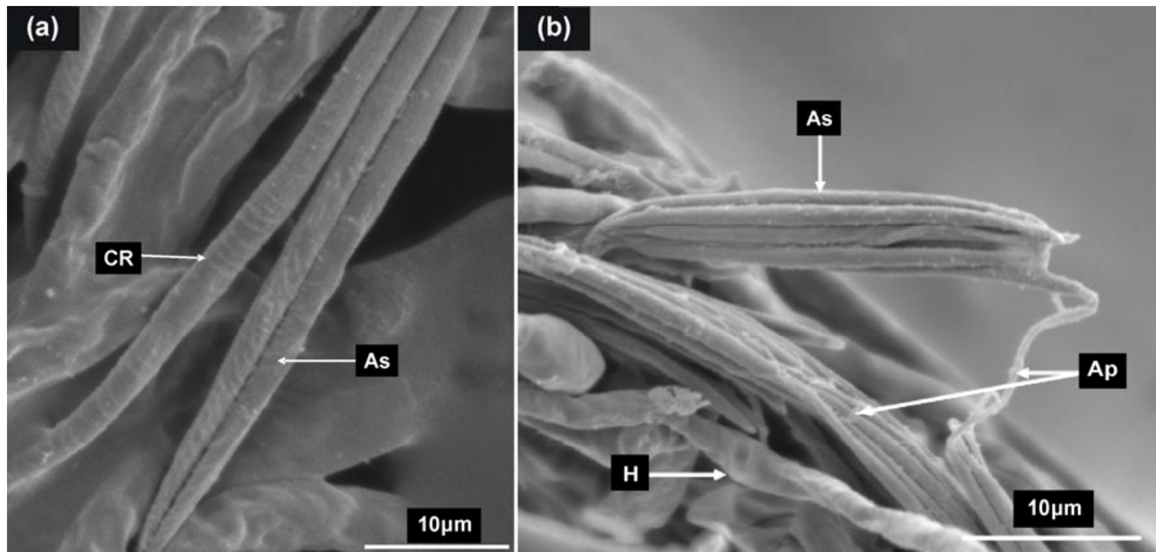

Figure 6: Scanning electron micrograph (a) of *Eremothecium gossypii* showing the presence of concentric ridges (CR) on surfaces of parts of these ascospores (As). An SEM (b) demonstrating the presence of intertwining terminal appendages (Ap). As, ascospores; H, hyphae.

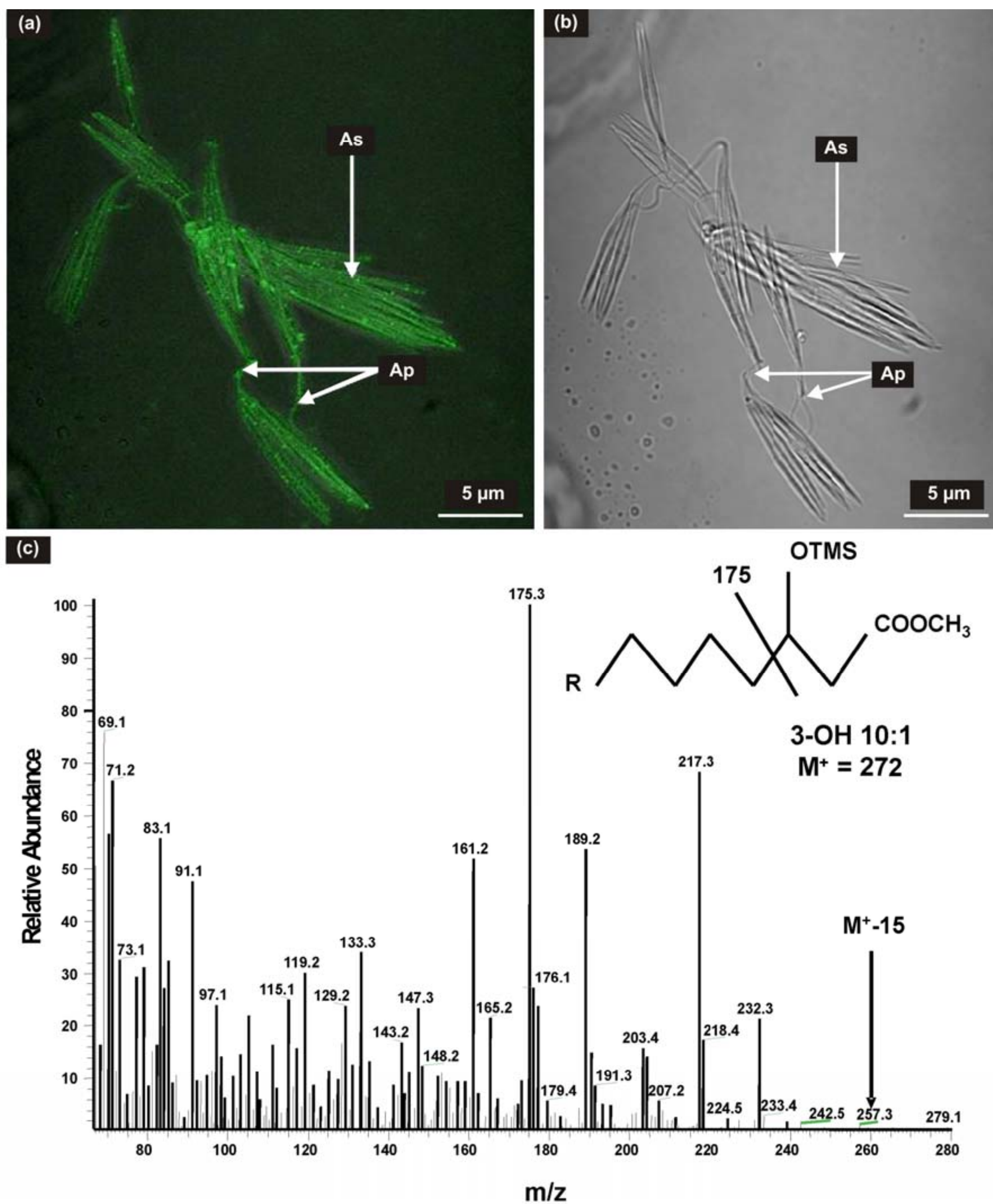

Figure 7: Immunofluorescence (a) with corresponding light (b) micrographs indicating the presence of 3-OH oxylipins on the entire surface of ascospores (As) as well as the terminal appendages (Ap) in *Eremothecium gossypii*. A mass spectrum of an oxylipin (c) obtained from *E. gossypii*.

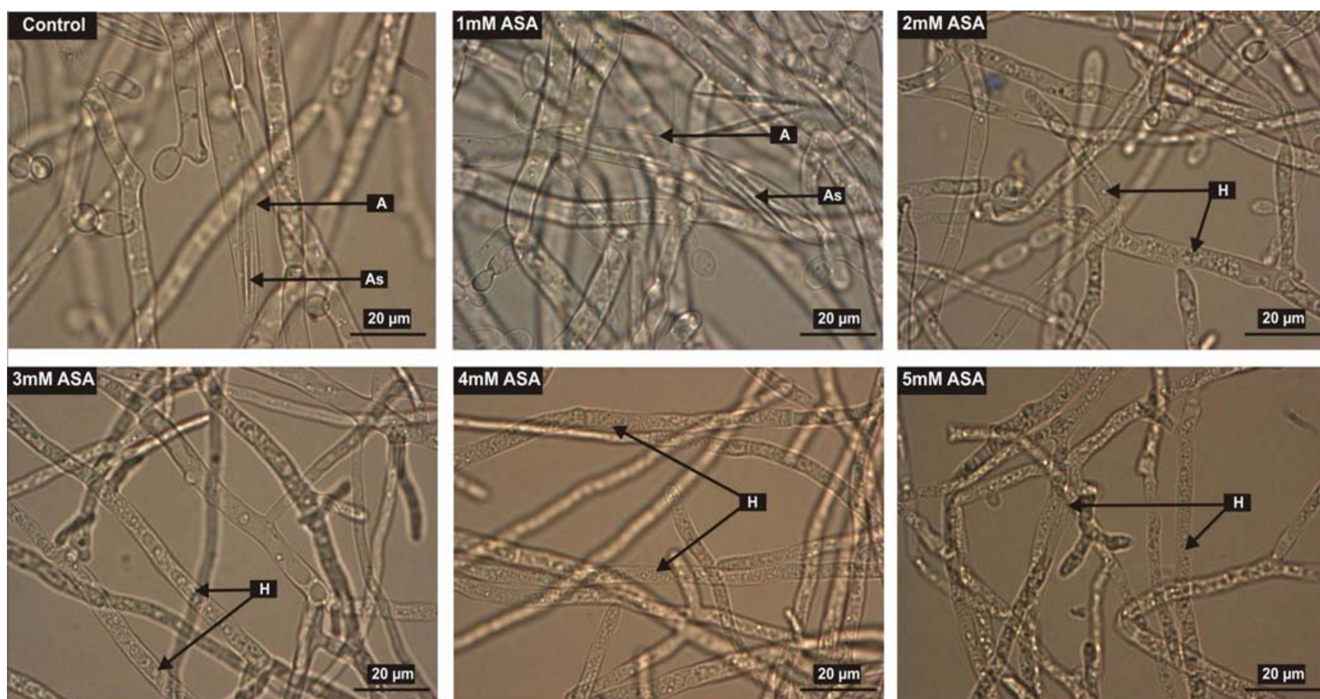

Figure 8: Light micrographs showing the effect of different concentrations of acetylsalicylic acid (ASA) on cultures of *Eremothecium gossypii*. A, ascus; As, ascospores; H, hyphae.

Summary

In the early 1990's, Kock and co-workers discovered acetylsalicylic acid (ASA)-sensitive oxylipins in yeasts. It was also reported that the site of production of these compounds may serve as important targets to control fungal infections. In 2004, researchers exposed another function for these oxylipins – they may act as lubricants during spore release from enclosed asci. Since oxylipin production in only a limited number of species representing *Eremothecium* was thus far studied, it became the aim of this project to further extend this study and to determine the type and distribution of 3-hydroxy (OH) oxylipins in the remaining species i.e. *Eremothecium coryli*, *E. cymbalariae* and *E. gossypii.* In addition, the possible functions of these oxylipins as well as ascospore shape and ornamentations were assessed. Finally, the antifungal activity of ASA was also investigated in this group of important plant pathogens as well as other yeasts. *Eremothecium coryli* is known to produce intriguing spindle-shaped ascospores with long and thin whip-like appendages. In this study, ultra structural studies using scanning electron microscopy, indicate that these appendages serve to coil around themselves and around ascospores causing spore aggregation. Furthermore, using immunofluoresence confocal laser scanning microscopy it was found that hydrophobic 3-OH oxylipins cover the surfaces of these ascospores. Using gas chromatography-mass spectrometry, only the oxylipin 3-OH 9:1 (a monounsaturated fatty acid consisting of a hydroxyl group on carbon 3) could be identified. Sequential digital imaging suggests that oxylipin-coated spindle-shaped ascospores are released from enclosed asci probably by protruding through an already disintegrating ascus wall. Using immunofluorescence microscopy and 3-OH oxylipin specific antibodies, it was possible to map the presence of these compounds also in other *Eremothecium* species. In *E. cymbalariae*, these oxylipins were found to cover

mostly the spiky tips of narrowly triangular ascospores while in *E. gossypii*, oxylipins covered the whole spindle-shaped ascospore with terminal appendages. The presence of these oxylipins was confirmed by chemical analysis. When ASA, a 3-OH oxylipin inhibitor, was added to these yeasts in increasing concentrations, the sexual stage was found to be the most sensitive. Results suggest that 3-OH oxylipins, produced by mitochondria through incomplete β-oxidation, are associated with the development of the sexual stages in both yeasts. Strikingly, preliminary studies on yeast growth suggest that yeasts, characterized by mainly an aerobic respiration rather than a fermentative pathway, are more sensitive to ASA than yeasts characterized by both pathways. These data further support the role of mitochondria in sexual as well as asexual reproduction of yeasts and its role to serve as target for ASA antifungal action.

Opsomming

Kock en medewerkers het gedurende die vroeë 1990's asetielsalisiensuur (ASS)-sensitiewe oksielipiene in giste ontdek. Dit is ook gerapporteer dat die setel van produksie van hierdie verbindings as belangrike teiken vir die beheer van fungale infeksies mag dien. In 2004 het navorsers nog 'n funksie van hierdie oksielipiene blootgelê – hulle mag as smeermiddels gedurende die vrystelling van spore dien vanuit geslote aski. Aangesien oksielipienproduksie sover slegs in 'n paar spesies van die genus *Eremothecium* bestudeer is, het dit die doel van hierdie projek geword om die studie uit te brei en om die tipe en verspreiding van 3-hidroksie (OH)-oksielipiene in die oorblywende spesies d.i. *Eremothecium coryli*, *E. cymbalariae* en *E. gossypii,* te bepaal. Verder is die moontlike funksies van die oksielipiene asook die askospoorvorm en ornamentasie ondersoek. Laastens is die antifungale aktiwiteit van ASS in hierdie groep belangrike plantpatogene asook in ander giste bestudeer. Dit is bekend dat *Eremothecium coryli* fassinerende naaldvormige askospore met lang, sweepagtige aanhangsels produseer. In hierdie studie is d.m.v. skandeerelektronmikroskopie van die ultrastruktuur, aangetoon dat die aanhangsels om mekaar en om askospore draai, om spooraggregasie tot gevolg te hê. Verder is d.m.v. immunofluoreserende konfokale laserskandeermikroskopie gevind dat hidrofobiese 3-OH-oksielipiene die oppervlaktes van die askospore bedek. Met behulp van gaschromatografie-massaspektrometrie kon slegs die oksielipien, 3-OH 9:1 ('n mono-onversadigde vetsuur met 'n hidroksielgroep op koolstof 3) geïdentifiseer word. Opeenvolgende digitale uitbeelding dui dat oksielipienbedekte, naaldvormige askospore deur vooraf disintegrerende askuswande vrygestel word, moontlik d.m.v. turgordruk. Met behulp van immunofluoresensie-mikroskopie en 3-OH-oksielipienspesifieke teenliggaampies was dit moontlik om die teenwoordigheid van hierdie verbindings ook in ander *Eremothecium* spesies te karteer.

In *E. cymbalariae*, is gevind dat die oksielipiene die skerp punte van die smal driehoekige askospore bedek, terwyl in die geval van *E. gossypii*, die hele naaldvormige askospoor en terminale aanhangsel met 3-OH-oksielipiene bedek is. Die teenwoordigheid van hierdie oksielipiene is deur chemiese analise bevestig. Indien ASS, ‘n 3-OH-oksielipieninhibeerder, in toenemende konsentrasies by die gis gevoeg word, is gevind dat die seksuele stadium die sensitiefste is. Resultate dui daarop dat 3-OH-oksielipiene, wat deur die mitochondria deur onvolledige β-oksidasie gevorm word, met die ontwikkeling van die geslagtelike fase in albei van laasgenoemde giste geassosieer is. Voorlopige studies dui daarop dat giste, gekarakteriseer deur hoofsaakllik aerobiese respirasie eerder as fermentasie, meer sensitief is vir ASS as giste wat deur beide metaboliese weë gekenmerk word. Hierdie data ondersteun die rol van mitochondria in seksuele en aseksuele voortplanting verder, asook die rol as teiken vir ASS se antifungale werking.

Key words: Acetylsalicylic acid, Antifungal, Ascospores, Aspirin, Confocal laser scanning microscopy, *Eremothecium coryli*, *Eremothecium cymbalariae*, *Eremothecium gossypii*, Mitochondria, 3-Hydroxy oxylipins

Sleutelwoorde: Asetielsalisiensuur, Antifungale, Askospore, Aspirien, Konfokale laserskandeermikroskopie, *Eremothecium coryli*, *Eremothecium cymbalariae*, *Eremothecium gossypii*, Mitochondria, 3-Hidroksie oksielipiene