

# **Oxylipins in the yeast genus *Ascoidea***

By

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# **CHAPTER 1**

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**Literature review**

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## 1.1 Motivation

Acetylsalicylic acid (ASA) sensitive 3-hydroxy (3-OH) oxylipins were uncovered in 1991 in the yeast *Dipodascopsis uninucleata* (Van Dyk et al. 1991). Since then, various similar oxylipins were found to be widely distributed in fungi (Pohl et al. 1998; Deva et al. 2000, 2001, 2003; Strauss et al. 2000, 2005; Sebolai et al. 2001, 2004, 2005, 2007; Kock et al. 2003, 2007; Noverr et al. 2003; Smith et al. 2003; Bareetseng et al. 2004, 2005; Ciccoli et al. 2005; Van Heerden et al. 2005, 2007; Leeuw et al. 2006, 2007). It is suggested in literature that these oxylipins are most probably produced through mitochondrial  $\beta$ -oxidation and/or mitochondrial fatty acids synthesis (FAS) type II in yeast (Ciccoli et al. 2005; Hiltunen et al. 2005).

By mapping the distribution of 3-OH oxylipins using immunofluorescence microscopy, it was reported that these compounds are mainly associated with the sexual stages of the non-fermenting yeasts *Ascoidea africana* (Bareetseng et al. 2005), *Dipodascopsis uninucleata* (Kock et al. 1998), *Dipodascus* (Van Heerden et al. 2005, 2007), *Eremothecium* (Bareetseng et al. 2004; Kock et al. 2004; Leeuw et al. 2006, 2007) and many other members of the family Lipomycetaceae (Smith et al. 2000a,b). Further studies showed that these compounds are also present on the surface of aggregating vegetative cells of *Saccharomyces cerevisiae* (Kock et al. 2000) and *Saccharomycopsis malanga* (Sebolai et al. 2001). Strikingly, when ASA, a known 3-OH oxylipin production and general mitochondrion inhibitor, was added to the yeasts *Dipodascopsis*, *Dipodascus* and *Eremothecium*, a dose dependant inhibition of the sexual stage and subsequently oxylipin production



were observed (Kock et al. 1999; Leeuw et al. 2007; Van Heerden et al. 2007).

Literature suggests that oxylipins may amongst others, act as lubricants during ascospore release from enclosed asci (Kock et al. 2004) where they are involved in assisting nano-scale gear-like (*Dipodascopsis uninucleata*, Kock et al. 1999); sliding (*Dipodascus albidus*, Van Heerden et al. 2005); drilling (*Eremothecium sinicaudum*, Baretseng et al. 2004) and piercing movements (*E. ashbyi*, Kock et al. 2004; *E. coryli*, Leeuw et al. 2006).

In *Ascoidea africana*, an oxylipin was found to be associated with hat-shaped ascospores carried inside ellipsoidal asci. The chemical structure of this compound was determined by gas chromatography – mass spectrometry (GC- MS) and found to be 3-OH 10:1 (Baretseng et al. 2005). In this study no function was proposed for this oxylipin.

Since only one species representing the genus *Ascoidea* was studied, it became the aim to further expand this study to also include *A. corymbosa* and *A. rubescens*. Consequently, the structures, distribution and possible function of 3-OH oxylipins in these species were assessed. In addition, the link between mitochondria and oxylipin accumulation in yeast sexual cells was investigated.

## **1.2 Yeast: definition and classification**

People have used yeast for fermentation and baking throughout history. Yeasts can be defined as those fungi that are mainly unicellular and whose vegetative growth predominantly results from budding or fission and which do not form their sexual states within or upon a fruiting body. Yeasts

are classified under the Ascomycetes i.e. when ascospores are produced within a naked ascus, the Basidiomycetes i.e. when basidiospores are formed outside a basidium or the Deuteromycetes i.e. when no sexual phase is observed (Kurtzman & Fell 1998; Barnett et al. 2000). Characteristics that are used to classify yeasts include morphology, sexual structures, biochemical, physiological properties (Yarrow 1998) and molecular methods such as nuclear DNA (nDNA) reassociation studies (Martini et al. 2003) and D1/D2 domain sequence of the 26S rDNA gene (Kurtzman & Fell 1998).

The ascomycetous and basidiomycetous yeasts are comprised of five orders, 90 genera and 678 species (Barnett et al. 2000). However, the latter authors do not recognize the genus *Ascoidea* in their classification monograph of the yeast. This taxon is recognized by Kurtzman & Fell (1998) as part of the ascomycetous yeasts and placed under the family Ascoideaceae. Their classification of the ascomycetous yeasts is as follows:

<b>Kingdom</b>	:	<b>Fungi</b>
<b>Phylum</b>	:	<b>Ascomycota</b>
Class	:	“Archiascomycetes”
Order	:	Schizosaccharomycetales
Family	:	Schizosaccharomycetaceae (1 genus)
Order	:	Taphrinales
Family	:	Taphrinaceae (2 genera)
Order	:	Protomycetales
Family	:	Protomycetaceae (2 genera)
Order	:	Pneumocystidales
Family	:	Pneumocystidaceae (1 genus)
Class	:	Euascomycetes (2 genera)
<b>Class</b>	:	<b>Hemiascomycetes</b>
Order	:	Saccharomycetales
<b>Family</b>	:	<b>Ascoideaceae (1 genus)</b>
		Cephaloascaceae (1 genus)
		Dipodascaceae (7 genera)
		Endomycetaceae (5 genera)
		Eremotheciaceae (2 genera)
		Lipomycetaceae (4 genera)
		Metschnikowiaceae (2 genera)
		Saccharomycetaceae (15 genera)

Saccharomycodaceae (4 genera)  
Saccharomycopsidaceae (2 genera)

### 1.3 *Ascoidea* Brefeld & Lindau

#### 1.3.1 Classification highlights

In 1891, Brefeld & Lindau were the first to isolate and describe the genus *Ascoidea* (member: *A. rubescens*) and to place it under the family Ascoideaceae (Endomycetales, Hemiascomycetidae). The yeast genus *Ascoidea* was constructed because it differed from all other ascomycetes by its possession of characteristic multispored asci which proliferate, the newer asci being formed through the collar-like wall remnants of older asci (Walker 1931; Batra & Francke-Grosmann 1961).

In 1898, Holtermann described a second species of *Ascoidea* i.e. *A. saprolegnioides* from materials collected from slime fluxes on various trees in Java. This species was later placed in synonymy with *A. rubescens* on the basis of morphological similarities (Walker 1931; Batra & Francke-Grosmann 1961). In 1900, Lindau also added two doubtful genera to the family Ascoideaceae i.e. *Oscarbrefeldia* Holtermann and *Conidiascus* Holtermann (Batra 1959).

In 1961, Batra and Francke-Grosmann isolated and described *A. hylecoeti* during studies of symbiotic relationships between ambrosia fungi and ambrosia beetles from oak wood infested with bark beetles (*Hylecoetus dermestoides*) in Sweden (Batra & Francke-Grosmann 1961).

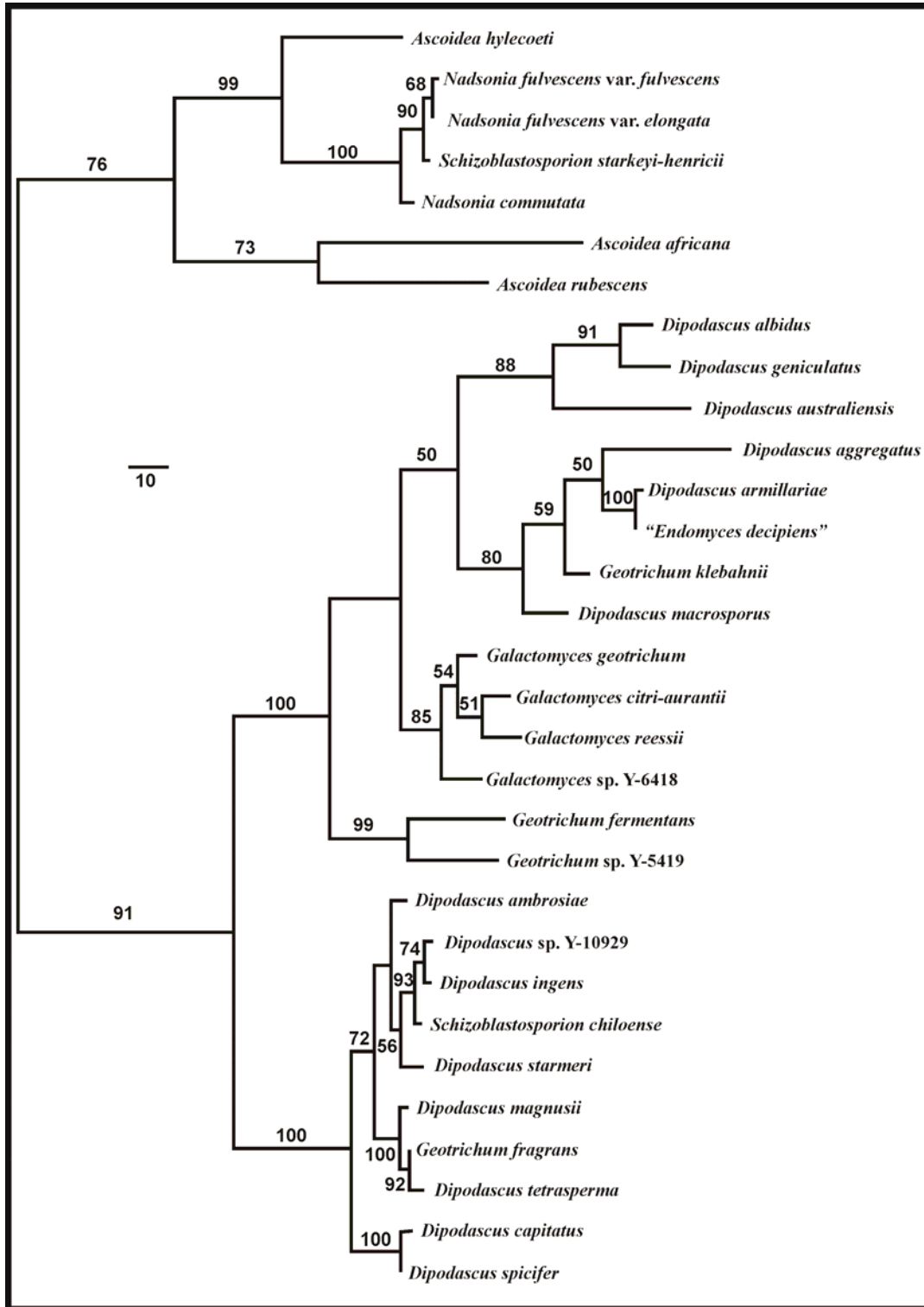
In 1963, Batra accepted five species in the genus *Ascoidea* i.e. *A. rubescens* Brefeld (type species), *A. hylecoeti* Batra & Francke-Grosmann, *A. asiatica* Batra & Francke-Grosmann, *A. africana* Batra & Francke-Grosmann

and *A. saprolegnioides* Holtermann. The first two species have multinucleate vegetative cells, many-spored asci and cucullate ascospores. *Ascoidea asiatica* has multinucleate vegetative cells, sixteen to thirty two-spored asci and ellipsoidal ascospores while *A. africana* has uninucleate vegetative cells, twenty four to sixty four-spored asci and ellipsoidal ascospores without any sheath. The fifth species, *A. saprolegnioides*, is known only from its original description and was reported to have many-spored asci and ellipsoidal ascospores (Batra & Francke-Grosmann 1961, 1964; Batra 1963).

In 1969, Wolf & Wolf included the following species: *A. rubescens*, *Spermophthora gossypii* and *Dipodascus albidus* under the family Ascoideaceae based on the observation that all have mycelium which are partially coenocytic and branched (Wolf & Wolf 1969). In 1973, Ainsworth classified the two genera *Ascoidea* and *Dipodascus* under the family Ascoideaceae, both with multispored asci, but divergent in the method of ascus formation (Ainsworth 1973).

On the basis of comparative morphological and physiological studies, Batra (1973) recognized the following taxa in the Ascoideales i.e. Ascoideaceae - *Ascoidea*; Nematosporaceae - *Nematospora*, *Ashbya*, *Metschnikowia*, *Eremothecium* and *Coccidiascus*.

Finally on the basis of D1/D2 domain sequence of the 26S rDNA gene a phylogenetic tree of *Ascoidea* was presented as part of the *Ascoidea* / *Nadsonia* / *Dipodascus* clade (Fig. 1).



**Fig. 1.** Phylogenetic tree of the *Ascoidea* / *Nadsonia* / *Dipodascus* - clade (Taken from Kurtzman & Robnett 1998).

At present the following species are recognized in genus *Ascoidea* (De Hoog 1998):

### **Type species**

*Ascoidea rubescens* Brefeld & Lindau

### **Species accepted**

*Ascoidea africana* Batra & Francke-Grosmann

*Ascoidea corymbosa* W. Gams & Grinbergs

*Ascoidea hylecoeti* Batra & Francke-Grosmann

*Ascoidea rubescens* Brefeld & Lindau

A morphological key to *Ascoidea* according to De Hoog (1998):

- |        |   |                            |
|--------|---|----------------------------|
| 1.     | a. Conidia 23-38 $\mu\text{m}$ long.....                | <b><i>A. rubescens</i></b> |
|        | b. Conidia less than 15 $\mu\text{m}$ long →            | 2                          |
| 2 (1). | a. Asci obclavate.....                                  | <b><i>A. hylecoeti</i></b> |
|        | b. Asci ellipsoidal to broadly clavate →                | 3                          |
| 3 (2). | a. Ascospores (2.5-4.0) X (3.5-5.5) $\mu\text{m}$ ..... | <b><i>A. africana</i></b>  |
|        | b. Ascospores (2.3-2.6) X (2.8-3.6) $\mu\text{m}$ ..... | <b><i>A. corymbosa</i></b> |

### **1.3.2 Diagnosis of the genus**

The present classification of yeasts, according to Kurtzman & Fell (1998) is based on morphology, sexual reproduction, fermentation, assimilation of carbon and nitrogen sources and other characteristics such as Co enzyme Q, Mol% G + C, and the Diazonium Blue B test. Consequently, the diagnosis of the genus *Ascoidea* Brefeld & Lindau according to Kurtzman & Fell (1998) is as follows:

*“Colonies are smooth, moist or dry mostly with an expanding, submerged mycelium. Species are often dimorphic, with colonies being restricted and yeast-like or expanding and hyphal. Budding cells and pseudomycelium are present or absent. Wide, true hyphae are present and form blastoconidia which are sessile or on denticles and occur singly or in short, branched chains. Asci are lateral or terminal on hyphae, ellipsoidal or acicular, with firm wall, and contain numerous ascospores which are liberated through a terminal opening; new asci are formed percurrently inside the remains of a previous ascus. Ascospores are ellipsoidal, with a unilateral, mucilaginous brim. Fermentation is absent. Urease is absent. Diazonium Blue B reaction is negative, rarely weak.”*

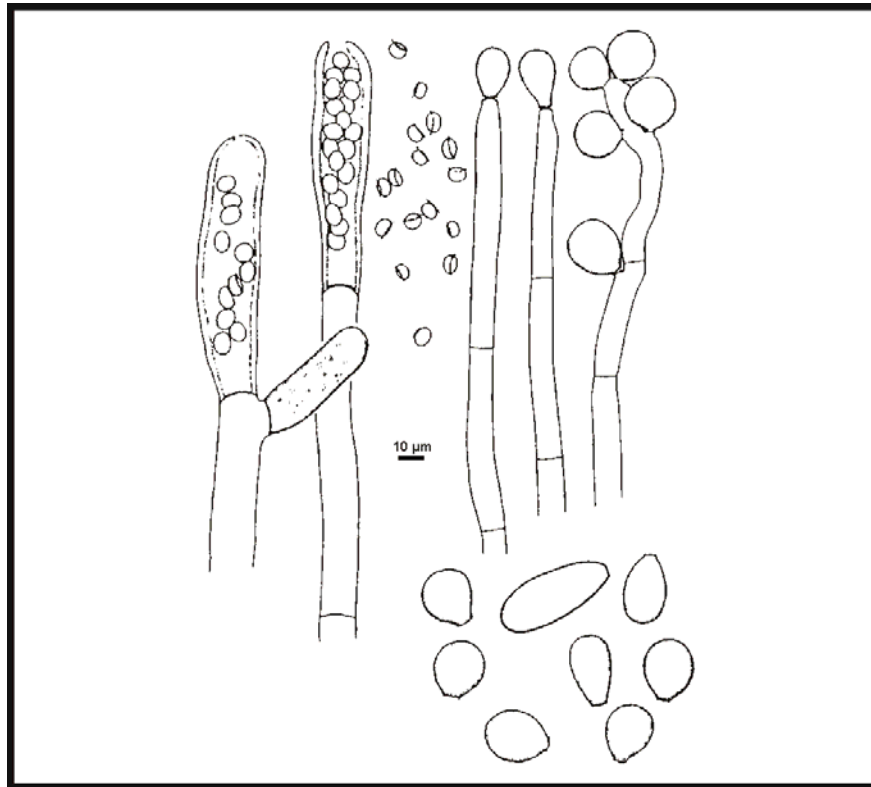
### **1.3.3 Sexual reproductive cycles**

Representatives of the Ascoideaceae have unique sexual reproductive cycles producing ellipsoidal ascospores with a unilateral, mucilaginous brim (De Hoog 1998). These phenotypic characteristics are of importance in the classification of this taxon as well as oxylipin function and are referred to in the chapters to follow.

#### ***Ascoidea rubescens* Brefeld & Lindau**

The sexual reproductive cycle of this yeast is characterized by the production of single asci which are formed terminally on hyphae or lateral branchlets and are clavate, (25-30) X (100-150)  $\mu\text{m}$ , with thick walls. They contain sixteen to one hundred-and-sixty ascospores which are liberated by

apical rupture of the ascus. Ascospores are ellipsoidal with a unilateral brim; (5.5-8) X (4-4.5)  $\mu\text{m}$  and cohere in slimy balls after liberation (Fig. 2).

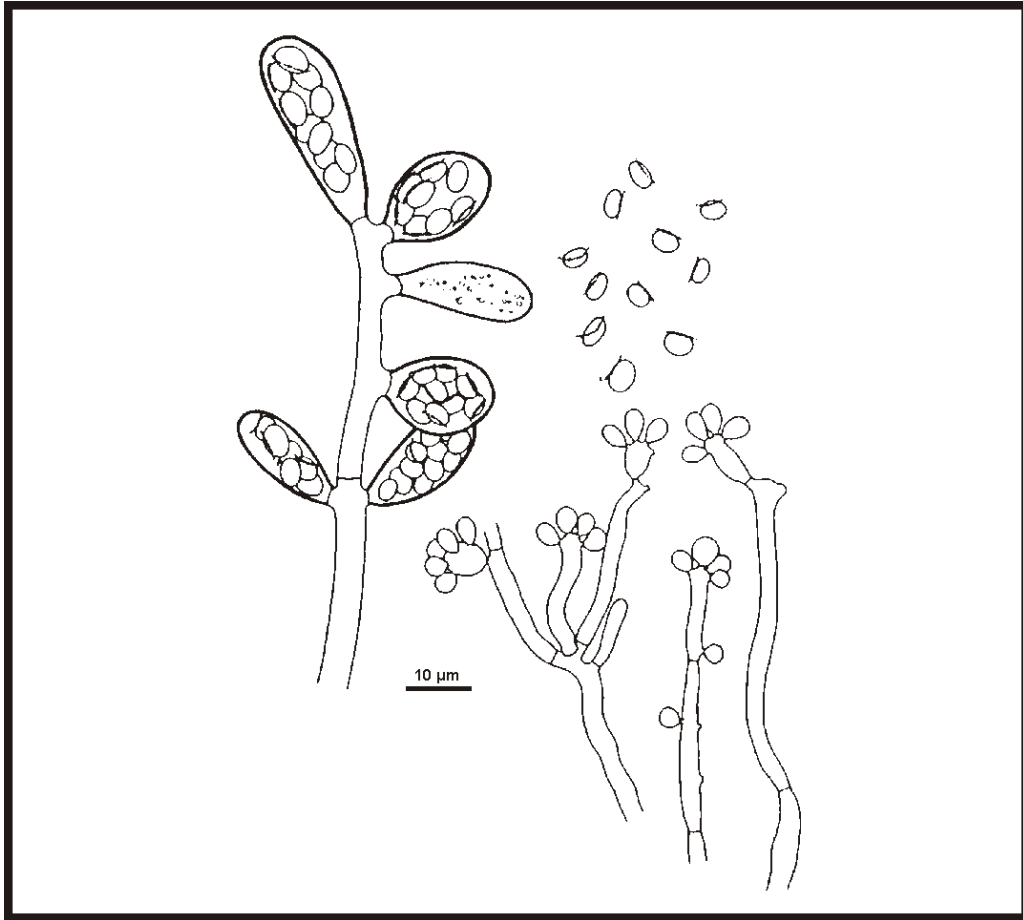


**Fig. 2.** Sexual reproduction and other morphological characteristics of *Ascoidea rubescens*. Asci, opening at the apex and liberating ascospores, true hyphae with sympodial conidia and liberated conidia with truncate bases are present (Taken from De Hoog 1998).

### ***Ascoidea africana* Batra & Francke-Grosmann**

Asci of this yeast are formed singly or in small whorls alongside hyphae, mostly inserted just below the distal septa, and are broadly ellipsoidal, (8-13) X (20-30)  $\mu\text{m}$ , with firm walls containing sixteen to seventy ascospores which are liberated by apical deterioration of the ascus. Ascospores are hat shaped, (2.5-4.0) X (3.5-5.5)  $\mu\text{m}$  cohering in slimy balls after liberation (Fig. 3).

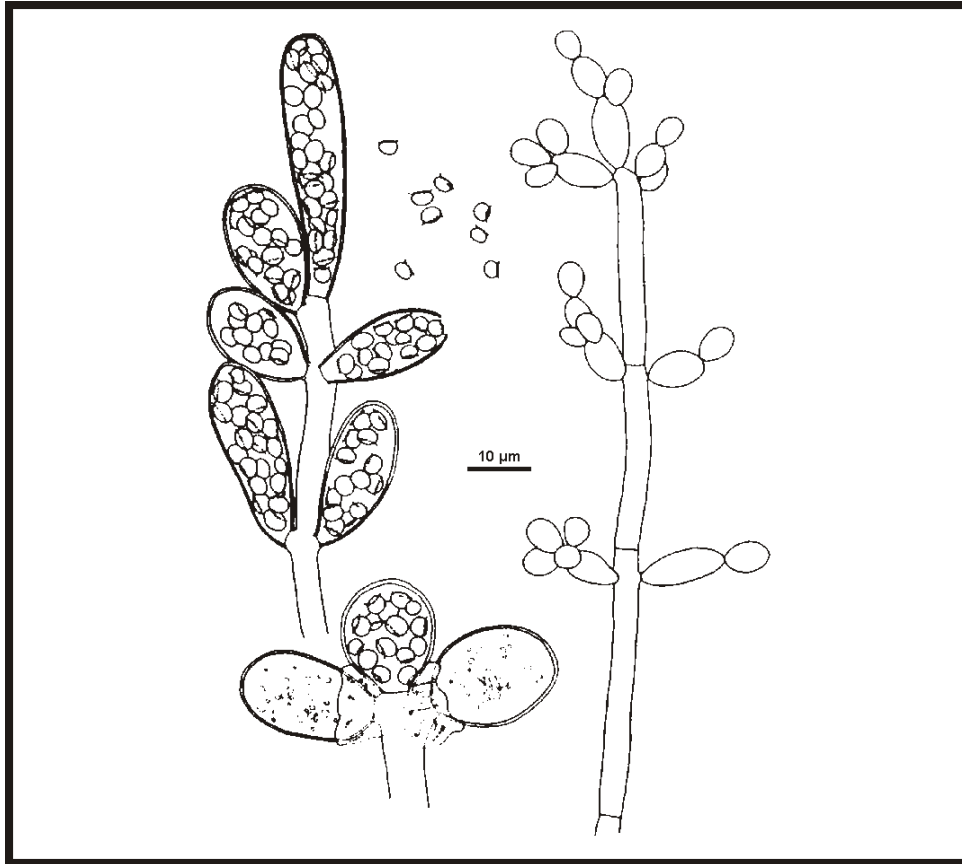




**Fig. 3.** Sexual reproduction and other morphological characteristics of *Ascoidea africana*. Asci, liberated ascospores and true hyphae with clusters of conidia are observed (Taken from De Hoog 1998).

### ***Ascoidea corymbosa* W. Gams & Grinbergs**

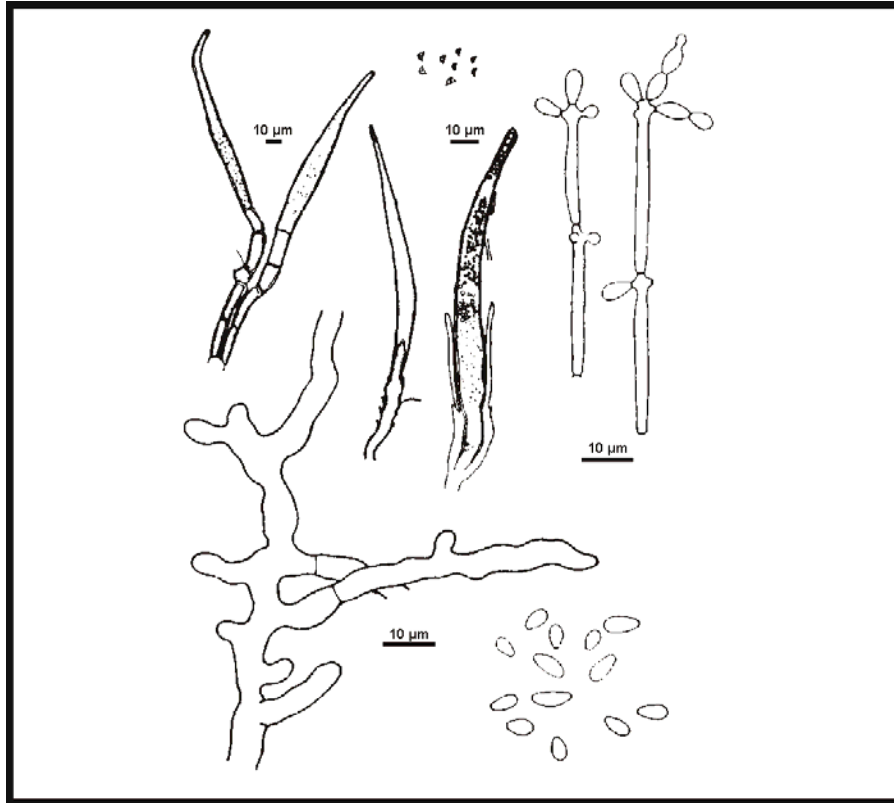
Asci of *A. corymbosa* are formed in small groups in distal portions of hyphae. They are mostly ellipsoidal; (10-14) X (20-40)  $\mu\text{m}$ , with firm walls and contain sixteen to forty ascospores which are liberated by apical deterioration of the ascus. Ascospores are hat-shaped, (2.3-2.6) x (2.8-3.6)  $\mu\text{m}$  in diameter, and cohere in slimy balls after liberation (Fig. 4).



**Fig. 4.** Sexual reproduction and other morphological characteristics of *Ascoidea corymbosa*. Asci deteriorate apically, with new asci arising through retracted remains of the previous ascus. Liberated ascospores and true hyphae with clusters of conidia are formed (Taken from De Hoog 1998).

#### ***Ascoidea hylecoeti* Batra & Francke-Grosmann**

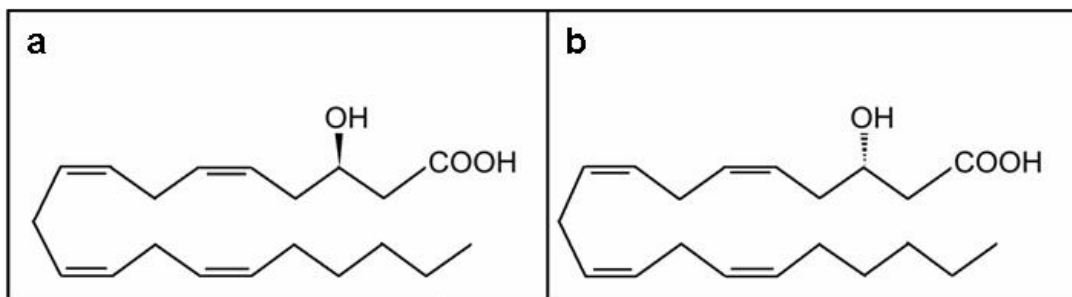
Asci of *A. hylecoeti* are borne terminally on erect hyphae later becoming lateral due to further growth of supporting hypha; asci are formed in percurrent succession and are obclavate, (15-24) X (160-400)  $\mu\text{m}$  and contain one hundred-and-fifty to four hundred ascospores. Asci are opened by terminal deterioration and ascospores are liberated. Ascospores are ellipsoidal; (2.5-3.2) X (1.5-2.0)  $\mu\text{m}$  and appear hat-shaped due to a unilateral mucilaginous brim (Fig. 5).



**Fig. 5.** Sexual reproduction and other morphological characteristics of *Ascoidea hylecoeti*. Included are asci, in part being produced through remains of a previous ascus, ascospores, true non-sporulating hyphae as well as pseudomycelial budding states (Taken from De Hoog 1998).

#### 1.4 Oxylipins

Oxylipins can be defined as saturated or unsaturated oxidized fatty acids (Venter et al. 1997; Bhatt et al. 1998). The basic structure of 3-OH oxylipins comprises of a carboxyl group at one end of the carbon chain and a hydroxyl group at the carbon-3 position (Figure 6). The carbon chain can vary in length (number of hydrocarbons) and in the degree of desaturation (presence of double bonds). These compounds can also be present in two enantiomeric forms, i.e. 3*R* (Fig. 6a) and 3*S* (Fig. 6b) (Venter et al. 1997; Kock et al. 2003).



**Fig. 6.** The chemical structures of typical 3-hydroxy oxylipins. (a) *R*- and (b) *S*-3-hydroxy-5,8,11,14-eicosatetraenoic acid (3-HETE). (Taken with permission from Kock et al. 2003).

In 1991, Van Dyk and co-workers uncovered acetylsalicylic acid (ASA)-sensitive oxylipins in the yeast *Dipodascopsis uninucleata*. They also reported that this yeast is capable of transforming exogenously fed arachidonic acid (AA) to 3*R*-hydroxy 5,8,11,14- eicosatetraenoic acid (3*R*-HETE) (Kock et al. 1991; Van Dyk et al. 1991). Since then there has been many reports on 3-OH oxylipin distribution in fungi (Kock et al. 1998, 2003, 2007; Pohl et al. 1998; Van Heerden et al. 2005, 2007; Leeuw et al. 2006, 2007; Sebolai et al. 2007).

Following extensive bioprospecting studies, 3-OH oxylipins were found to be associated with the surface structures of aggregating ascospores and asci of many yeasts, including members of the non-fermenting *Ascoidea* (Baretseng et al. 2005); *Dipodascus* (Van Heerden et al. 2005, 2007) and *Eremothecium* (Kock et al. 2004; Baretseng et al. 2004; Leeuw et al. 2006, 2007). These compounds are also found on surfaces of aggregating vegetative cells of *Saccharomyces cerevisiae* (Kock et al. 2000), *Saccharomycopsis malanga* (Sebolai et al. 2001) and other yeasts (Table 1). Interestingly, it was also uncovered that these oxylipins are associated with

the zygomycotan fungi. In 1998, Pohl and co-workers found that *Mucor genevensis* was capable of biotransforming exogenous AA to 3-OH 5,8,-tetradecadienoic acid (3-OH 14:2) from linoleic acid (18:2) (Pohl et al. 1998). Further bioprospecting studies utilising immunofluorescence microscopy, showed that these oxylipins are specifically associated with the sporangia and columella of mucoralean fungi (Strauss et al. 2000).

3-OH oxylipins were also found to have various other functions. In 2004, Kock and co-workers proved that ascospore shape and oxylipin-coated surface ornamentations play a role in ascospore release from enclosed asci, where they are involved in assisting nano-scale gear-like (*Dipodascopsis uninucleata*, Kock et al. 1999); sliding (*Dipodascus albidus*, Van Heerden et al. 2005); drilling (*Eremothecium sinicaudum*, Bareetseng et al. 2004) and piercing movements (*E. ashbyi*, Kock et al. 2004; *E. coryli*, Leeuw et al. 2006). Furthermore, these oxylipins were found to be the active substance in LPS-endotoxins of Gram-negative bacteria (Rietschel et al. 1994), have an inflammatory function during *Candida* infection (Ciccoli et al. 2005) and show antifungal activity against certain fungi (Sjogren et al. 2004).

**Table 1.** Distribution patterns of 3-OH oxylipins in yeasts.

Species	Type of 3-OH Oxylipin	Association	Reference
<i>Ascoidea africana</i>	3-OH 10:1	ascospores	Bareetseng et al. 2005
<i>Candida albicans</i>	3, 18 diHETE	hyphal cells	Deva et al. 2000
<i>Cryptococcus neoformans</i> var. <i>neoformans</i>	3-OH 9 :1	vegetative cells	Sebolai et al. 2007
<i>Dipodascopsis tothii</i>	3-OH 14:2, 14:3, 20:3, 20:5	ascospores	Kock et al. 1997

**Table 1. Cont.**

<b>Species</b>	<b>Type of 3-OH Oxylipin</b>	<b>Association</b>	<b>Reference</b>
<i>D. uninucleata</i> var. <i>uninucleata</i>	3-OH 14:2, 14:3, 20:3, 20:5	ascospores	Venter et al. 1997, Fox et al. 1997
<i>Dipodascus albidus</i>	3-OH metabolite	ascospores	Van Heerden et al. 2005
<i>D. ambrosiae</i>	3-OH metabolite	ascospores	Smith et al. 2003
<i>D. geniculatus</i>	3-OH metabolite	ascospores	Van Heerden et al. 2007
<i>D. macrosporus</i>	3-OH metabolite	ascospores	Smith et al. 2003
<i>D. magnusii</i>	3-OH metabolite	ascospores	Smith et al. 2003
<i>D. spicifer</i>	3-OH metabolite	ascospores	Smith et al. 2003
<i>D. tetrasperma</i>	3-OH metabolite	ascospores	Smith et al. 2003
<i>Eremothecium ashbyi</i>	3-OH 14:0	ascospores	Kock et al. 2004
<i>E. coryli</i>	3-OH 9:1	ascospores	Leeuw et al. 2006
<i>E. cymbalariae</i>	3-OH 13:1	ascospores	Leeuw et al. 2007
<i>E. gossypii</i>	3-OH 10:1	ascospores	Leeuw et al. 2007
<i>E. sinecaudum</i>	3-OH metabolite	ascospores	Bareetseng et al. 2004
<i>Lipomyces doorenjongii</i>	3-OH metabolite	ascospores	Smith et al. 2000b
<i>L. kockii</i>	3-OH metabolite	ascospores	Smith et al. 2000b
<i>L. kononenkoeae</i>	3-OH metabolite	ascospores	Smith et al. 2000b
<i>L. starkeyi</i>	3-OH metabolite	ascospores	Smith et al. 2000b
<i>L. yamadae</i>	3-OH metabolite	ascospores	Smith et al. 2000b
<i>L. yarrowii</i>	3-OH metabolite	ascospores	Smith et al. 2000b
<i>Nadsonia commutata</i>	3-OH 9:1	vegetative cells	Bareetseng 2004
<i>N. fulvescens</i>	3-OH metabolite	vegetative cells	Bareetseng 2004
<i>Saccharomyces cerevisiae</i>	3-OH 8:0, 10:0	vegetative cells	Kock et al. 2000, Strauss et al. 2005
<i>Saccharomycopsis capsularis</i>	3-OH 9:1	ascospores	Sebolai 2004
<i>S. fermentans</i>	3-OH metabolite	ascospores	Sebolai et al. 2005
<i>S. javanensis</i>	3-OH 9:1	ascospores	Sebolai et al. 2005
<i>S. malanga</i>	3-OH 16:0	vegetative cells	Sebolai et al. 2001
<i>S. synnaedendra</i>	3-OH 16:0, 17:0, 18:0, 19:0, 19:1, 20:0, 22:0	vegetative cells	Sebolai et al. 2004
<i>S. vini</i>	3-OH 9:1, 10:1	ascospores	Sebolai et al. 2005
<i>Saturnispora saitoi</i>	3-OH 9:1	ascospores	Bareetseng et al. 2006
<i>Schizosaccharomyces pombe</i>	3-OH 11:0, 15:0	vegetative cells	Strauss et al. 2006

It has been reported in literature that 3-OH oxylipins are produced via  $\beta$ -oxidation most probably in mitochondria of yeasts and mammals (Venter et al. 1997; Glasgow et al. 1999; Ciccoli et al. 2005). When long chain fatty acids were fed to the yeast *Dipodascopsis uninucleata*, some were broken down by the  $\beta$ -oxidation degradation mode, pointing towards this metabolic route (Venter et al. 1997). In yeasts, these compounds are subsequently deposited on cell walls or ascospore surfaces (Kock et al. 2004). It is interesting to note that in yeasts such as *Saccharomyces cerevisiae*, no 3-OH oxylipin production, as observed above, could be found. This yeast could only produce 3-OH 8:0 *ab initio* (Strauss et al. 2005). It is possible that these oxylipins may be formed via a synthesis route such as fatty acid synthesis (FAS) type II, which has been demonstrated in mitochondria of *S. cerevisiae* and reported to be a conserved character throughout eukaryotes (Hiltunen et al. 2005).

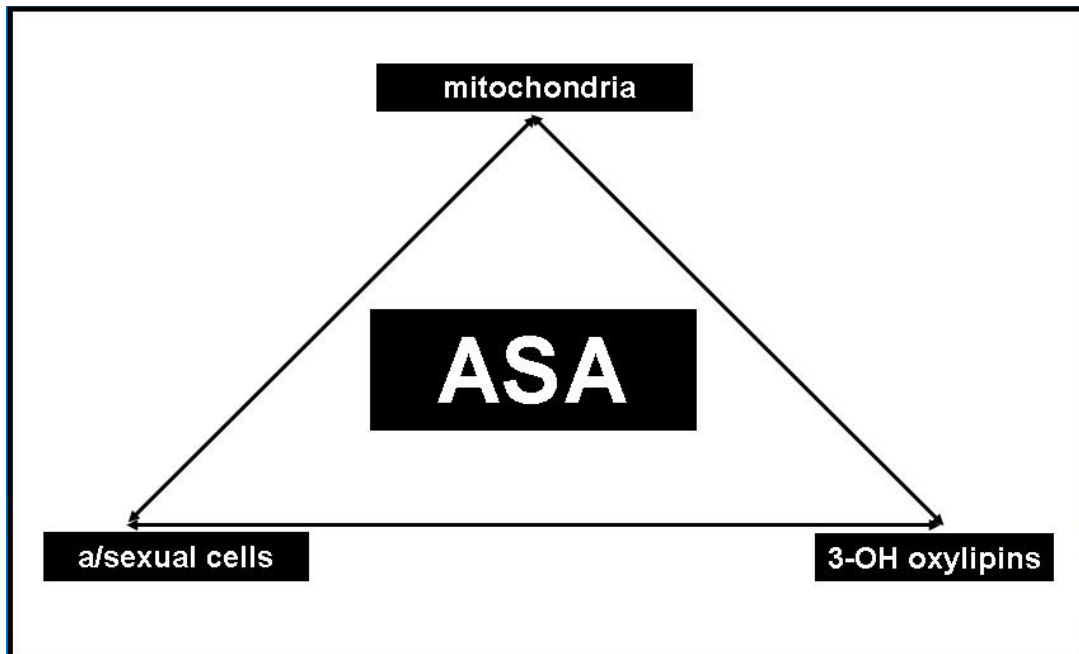
When ASA, a known mitochondrion inhibitor, was added to the yeast *Dipodascopsis uninucleata* at different concentrations, a dose dependant inhibition of sexual cell (asci) development was observed (Kock et al. 1999). This may be due to the fact that these sexual cells depend on mitochondria for normal development (Marmioli et al. 1983; Codon et al. 1995). These cells probably need more energy for the development of the many spores per sexual cell. ASA inhibits mitochondria and 3-OH oxylipin production by producing a compound, salicylate, that has structural similarities to the acyl-portions of the substrate and product of the 3-OH acyl-CoA dehydrogenase activity of the  $\beta$ -oxidation pathway (Glasgow et al. 1999). In addition, ASA may also inhibit mitochondrial activity by uncoupling oxidative phosphorylation and/or inhibit electron transport (Norman et al. 2004; Somasundaram et al.

1997). It is therefore not surprising that literature reports various yeast sexual cells to be more susceptible to ASA compared to vegetative cells (Kock et al. 2003; Leeuw et al. 2007).

A recent study by Strauss et al. (2007) provided evidence suggesting a link between oxylipin production, mitochondrial activity and flocculation in a flocculating strain of the biotechnologically important *Saccharomyces cerevisiae*. They reported that mitochondrial activity and oxylipin production were higher in the flocculent phase compared to the less flocculent phase. In addition, when ASA was added, oxylipin production, mitochondrial activity and flocculation were inhibited (Strauss et al. 2007). These results implicate a possible role for 3-OH oxylipins in flocculation. A final proof will be to produce pure 3-OH oxylipins (e.g. 3-OH 8:0) and add these to ASA-inhibited flocculating cells of *S. cerevisiae* and then determine if the ASA effect can be uplifted. It may of course also be possible that the uncoupling of oxidative phosphorylation and/or inhibition of the electron transport chain (Somasundaram et al. 1997; Norman et al. 2004) by ASA is responsible for the inhibition of flocculation.

This discussion suggests that 3-OH oxylipins accumulate in flocculating asexual and/or sexual cells that are characterized by elevated mitochondrial activity. These oxylipins as well as mitochondrial-linked cell function is inhibited by ASA (Fig. 7).





**Fig. 7.** Schematic representation showing the link between oxylipins, mitochondria, a/sexual cells and ASA inhibition.

### 1.5 Aims of the study

With this as background, the aim of this study became the following:

1. To investigate the distribution, function and chemical structures of 3-OH oxylipins in *Ascoidea* (**Chapters 2 and 3**).
2. To assess the link between yeast mitochondria, sexual cells, oxylipin production and ASA sensitivity in *Ascoidea* (**Chapter 3**).

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# CHAPTER 2

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## Oxylipin-coated hat-shaped ascospores of *Ascoidea corymbosa*

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The candidate performed preliminary studies during his B.Sc. Honours in 2005. After additional work during his M.Sc. study in 2006, this section was published in Canadian Journal of Microbiology **52**: 1046-1050 (2006). Consequently, this chapter is written in this journal's format and also included with permission in this study. Antibodies were obtained from Prof. S. Nigam, Free University of Berlin, Germany. The rest of work presented in this chapter has been performed by the candidate. **Note: Fig 1d was published as a cover picture in Canadian Journal of Microbiology: November 2006 issue.**



## 2.1 Abstract

We previously implicated 3-hydroxy oxylipins and ascospore structure in ascospore release from enclosed asci. Using confocal laser scanning microscopy on cells stained with fluorescein-coupled, 3-hydroxy oxylipin-specific antibodies, we found that oxylipins are specifically associated with ascospores and not the vegetative cells or ascus wall of *Ascoidea corymbosa*. Using gas chromatography - mass spectrometry the oxylipin 3-hydroxy 17:0 could be identified. Here, we visualize for the first time the forced release of oxylipin-coated, hat-shaped ascospores from terminally torn asci, probably through turgor pressure. We suggest that oxylipin-coated, razor sharp, hat-shaped ascospore brims may play a role in rupturing the ascus to affect release.

*Key words:* *Ascoidea corymbosa*, ascospore release, confocal laser scanning microscopy, gas chromatography-mass spectrometry, hat-shaped ascospores, 3-hydroxy oxylipins.

## 2.2 Introduction

In 1991, we discovered aspirin-sensitive 3-hydroxy oxylipins (3-OH oxylipins) in yeasts (Van Dyk et al. 1991). Since then, the ubiquitous nature of these compounds in yeasts has been reported (Deva et al. 2000, 2001, 2003; Kock et al. 2003; Bareetseng et al. 2004, 2005; Ciccoli et al. 2005; Strauss et al. 2005). It is suggested that these oxylipins, among others, may act as lubricants during ascospore release from enclosed asci (Kock et al. 2004). This research exposed a whole new world of ascospore movement in micron-space, which might find application in nano-, aero-, and hydro-technologies.

It is suggested that ascospores have developed lubricated nano-scale surface ornamentations necessary for smart release from enclosed asci. For example, 3*R*-OH 5*Z*,8*Z*,11*Z*,14*Z* eicosatetraenoic acid (3*R*-HETE) lubricates a complex gearbox-like system found within asci of the yeast *Dipodascopsis uninucleata*, where micron-scale ascospores with nano-scale ridged surfaces interact in gear-like fashion for unhindered water-propelled rotational release from a narrow opening (Kock et al. 1999). By upscaling the ascospore structure of this yeast, a novel generation of water-propelled pipe-cleaning devices was suggested (Kock et al. 2004). Strikingly, in *Eremothecium sinicaudum* 3-OH oxylipins are only observed on needle-shaped ascospores where they cover parts characterised by nano-scale surface ornamentations simulating a tapered corkscrew ending in a sharp spiky tip (Bareetseng et al. 2004). The authors suggested that the lubricated tapered corkscrew part as well as turgor pressure is responsible for drilling through the ascus wall to affect ascospore release. This is similar to the familiar lubricant assisted drilling into solid metal surfaces.

Recently, Bareetseng et al. (2005) demonstrated the presence of 3-OH oxylipins covering the surfaces of aggregated hat-shaped ascospores of *Ascoidea africana*. As an extension to these studies, we further map the distribution of 3-OH oxylipins in the related yeast *A. corymbosa* and show for the first time the forced release of oxylipin-covered hat-shaped ascospores through terminal ends of torn asci, probably by turgor pressure (Fisher et al. 2004).

## **2.3 Materials and methods**

### **2.3.1 Strain and cultivation**

*Ascoidea corymbosa* UOFS Y-0732, maintained in culture at the University of the Free State, was used in this study. The strain was cultivated on yeast malt agar medium (Wickerham 1951) at 22 °C until reaching its sexual reproductive stage. All experiments were performed in duplicate.

### **2.3.2 Microscopy**

#### **2.3.2.1 Light microscopy**

To investigate terminal ascospore release from asci, ascospores were stained according to Yarrow (1998) and studied using a Zeiss Axioskop light microscope (Zeiss, Gottingen, Germany).

#### **2.3.2.2 Scanning electron microscopy**

Scanning electron microscopy (SEM) was carried out as described by Van Wyk and Wingfield (1991). The yeast cells were chemically fixed overnight using 3% v/v (1.0 mol/L) of a sodium phosphate buffered glutaraldehyde (Sigma-Aldrich, St. Louis, Mo., USA) solution at pH 7.0 and a

similarly buffered solution (1% *m/v*) of osmium tetroxide (Sigma-Aldrich) for 4 h. After this, the material was dehydrated in a graded series of ethanol solution (30%, 50%, 70%, 90%, and 100% for 30 min per solution). The ethanol-dehydrated material was critical-point dried, mounted, and coated with gold to make it electrically conductive. This preparation was then examined using a Joel WINSEM (JSM 6400) SEM (Joel, Tokyo, Japan).

### **2.3.3 Immunofluorescence studies**

#### **2.3.3.1 *Synthesis of 3-OH oxylipins and preparation of antibodies***

The R and S isomers of 3*R*-HETE were synthesized according to Bhatt et al. (1998) and Groza et al. (2002). Antibodies against synthetic 3*R*-HETE were raised in rabbits and were characterized as described by Kock et al. (1998). Interestingly, antibodies were specific against all fatty acids (irrespective of chain length and desaturation) carrying a C<sub>3</sub>-OH group and not only 3*R*-HETE.

#### **2.3.3.2 *Microscopy***

Immunofluorescence of yeast cells was performed as previously described (Kock et al. 1998) and included treatment with primary antibody against 3-OH oxylipins as well as Fluorescein Isothiocyanate-conjugated secondary antibody (Sigma-Aldrich). Antibody, fluorescence, and wash treatments were performed in 1 mL plastic tubes to maintain cell structure. After adequate washing, the cells were fixed on a microscope slide using 1,4-diazabicyclo[2.2.2]octane (Sigma-Aldrich) and examined with a Nikon 2000 Confocal Laser Scanning Microscope (Nikon, Tokyo, Japan).

### 2.3.4 3-OH oxylipin extraction and derivatisation

Yeast cells in their sexual stage were suspended in distilled water (pH < 4). Lipids were extracted by two volumes of ethyl acetate (Merck, Haar, Germany), and the organic phase was evaporated with nitrogen gas (AFROX, Port Elizabeth, South Africa). Lipid extracts were methylated with diazomethane and silylated with bis(trimethylsilyl)trifluoroacetamide (Merck) for 1 h each and then dissolved in chloroform-hexane (4:1 v/v) (Merck).

### 2.3.5 Gas chromatography-mass spectrometry

The treated samples were injected into a Finnigan Trace GC Ultra gas chromatograph (Thermo Electron Corporation, San Jose, Calif., USA) with a HP5 (60m x 0.32 mm diameter) fused silica capillary column (0.1 µm coating thickness) coupled to a Finnigan Trace DSQ MS (Thermo Electron Corporation). The carrier gas was helium at 1.0 mL/min. 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. The gas chromatography – mass spectrometer (GC-MS) was auto-tuned for an  $m/z$  of 50-400. One micro-liter of the sample was injected into the GC-MS at a split ratio of 1:50 at an inlet temperature of 230 °C (Venter et al. 1997).

## 2.4 Results and discussion

Using light microscopy, it is clear that *A. corymbosa* produces ascospores within asci (De Hoog 1998) that are carried terminally on hyphae (Fig. 1a). Strikingly, these ascospores fluoresced selectively compared with the surrounding vegetative cells or the asci walls when antibodies against 3-



OH oxylipins with fluorescein isothiocyanate-conjugated secondary antibodies were added to the yeast culture (Fig. 1*b*). Here, mainly the outer part of the ascospore fluoresced strongly, resulting in a fluorescing circle surrounding each ascospore (Fig. 1*c*, 2*a*, and 2*b*). SEM studies of ascospore shape lead us to conclude that the fluorescing circles correspond to the surrounding nano-scale, razor-sharp brims (Fig. 2*b*). Why should only the brims be coated with oxylipins?

Ascospores are positioned in a curious pattern in the ascus, with hats lying on top of each other across the length of the ascus. This is implicated in Fig. 1*c*, which shows the fluorescing circles (brims) layered on top of each other across the ascus length. Is this ascospore orientation necessary so that the razor sharp brims can be pressed against the ascus tip (at end of the elongated ascus) via turgor pressure to affect rupturing? Are the oxylipin-coated brims also necessary for spores to effectively slide past each other as was reported in *Dipodascus* (Van Heerden et al. 2005)?

Microscopic studies showed that ascospores are forcibly released from the ascus tip, as evident from stained preparations (Fig. 1*d*) and SEM (Fig. 2*c*). We suggest that ascospores are probably liberated by apical rupturing of the ascus, which may be a result of ascospores being forced by turgor pressure through a disintegrating ascus tip with the brims cutting the ascus wall, probably for dispersal purposes. When released, the ascospores tend to aggregate in clusters (probably through entropic-based hydrophobic forces) probably for protection or for conjugation purposes (Fig. 1*d* and 2*b*). GC-MS analysis detected the presence of 3-OH 17:0 that presumably covers the

ascospore surfaces (Fig. 3). Analysis is underway to further characterize this oxylipin.

It is intriguing that ascospore surfaces presumably involved in ascus rupturing are covered with 3-OH oxylipins, such as 3-OH 17:0. It has been suggested that these oxylipins act as lubricants involved in nano-scale, gear-like (*D. uninucleata*, Kock et al. 1999), sliding (*Dipodascus*, Van Heerden et al. 2005), and drilling (*E. sinecaudum*, Bareetseng et al. 2004) mechanics, as well as boomerang movements, where they cover nano-scale hydrophobic fins of sickle-shaped ascospores of *E. ashbyi* (Kock et al. 2004). This study further extends these lubricant functions by demonstrating that the hypothesized cutting edges of hat-shaped ascospore brims in *A. corymbosa* are also selectively coated with 3-OH oxylipins, probably to assist in the cutting process.

The question that now arises concerns the lubricity properties of these oxylipins. How do they compare with other lubricants found in the market today? Well-known castor oil that contains mainly ricinoleic acid (12-OH 18:1) is essential for producing high quality lubricants for jet engines (Wood 2001), among others. What influence will the positional change of the hydroxyl group from position C12 to C3, the chain length, and desaturation have on the lubricating properties of these oxylipins? The only way to assess this is to produce significant amounts of 3-OH oxylipins with different chain lengths and desaturation probably through biotechnological and (or) existing chemical synthesis routes (Bhatt et al. 1998; Groza et al. 2002).

Research should now be directed towards capturing live images (Pringle et al. 2005) of forced hat-shaped ascospore release from asci of *A.*

*corymbosa* and determining the effect of oxylipin inhibitors and oxylipins on these mechanics.

## 2.5 Acknowledgements

The authors wish to thank the Volkswagen Foundation, Germany (1/74643) and the National Research Foundation in South Africa for financial support.

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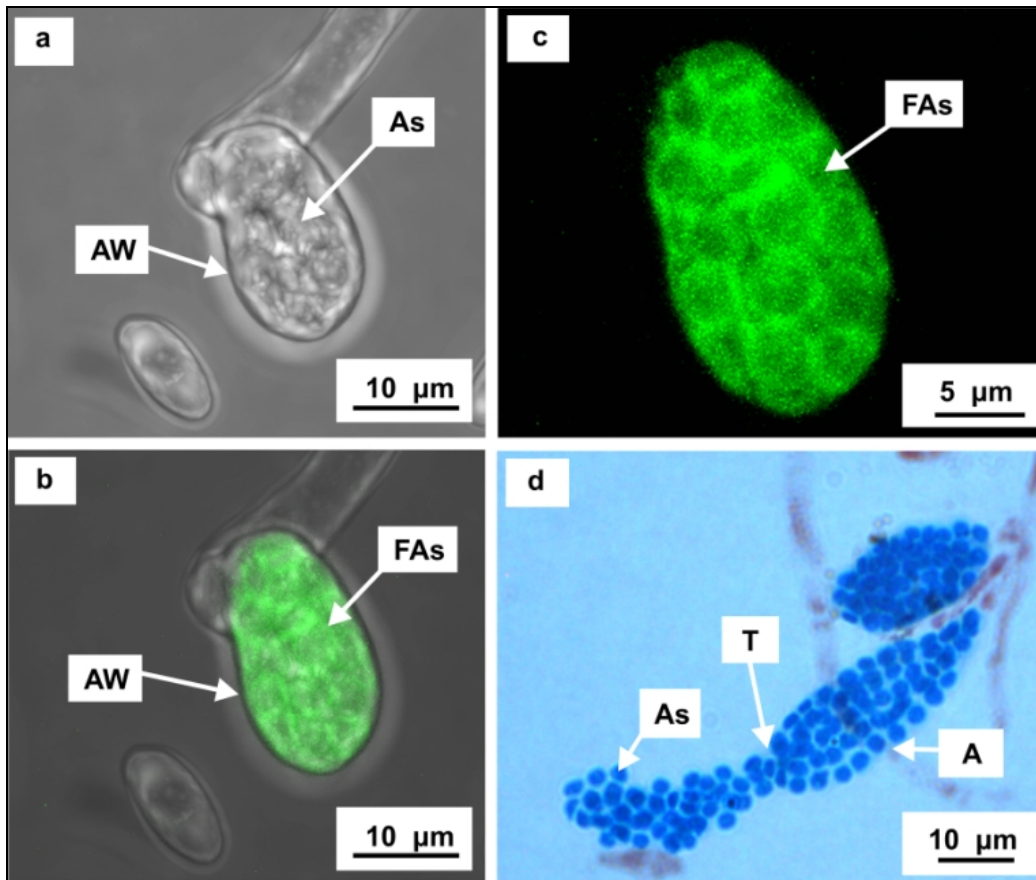
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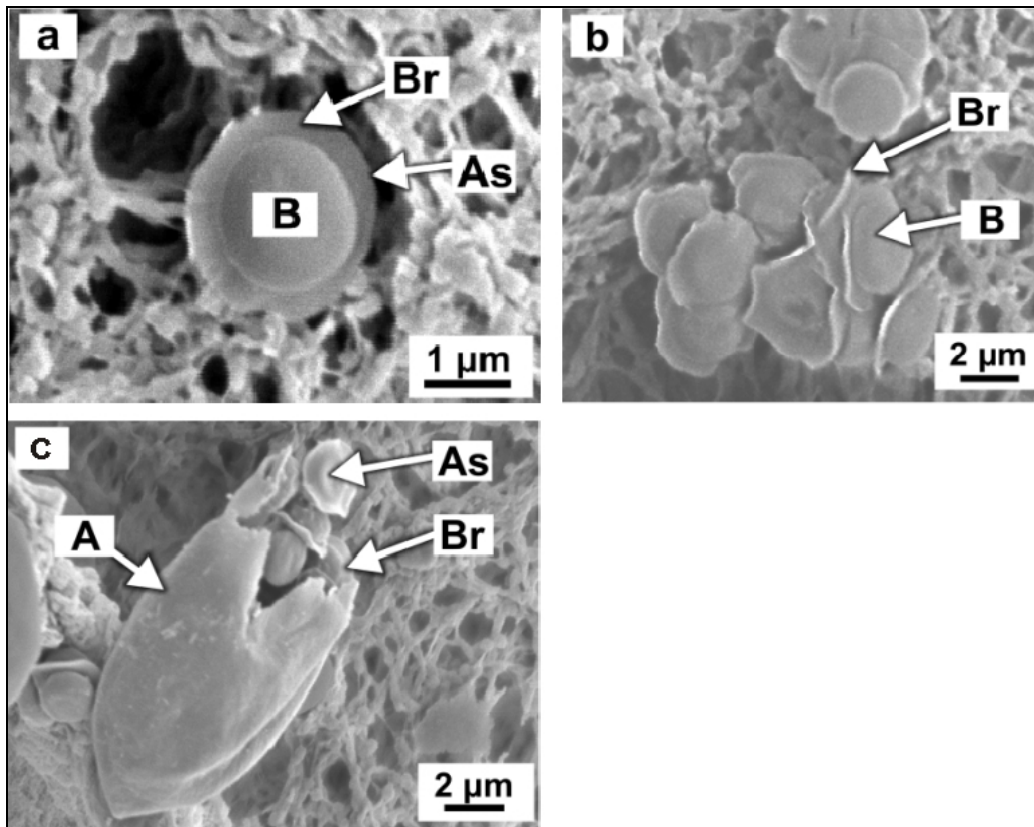
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## 2.7 Figures

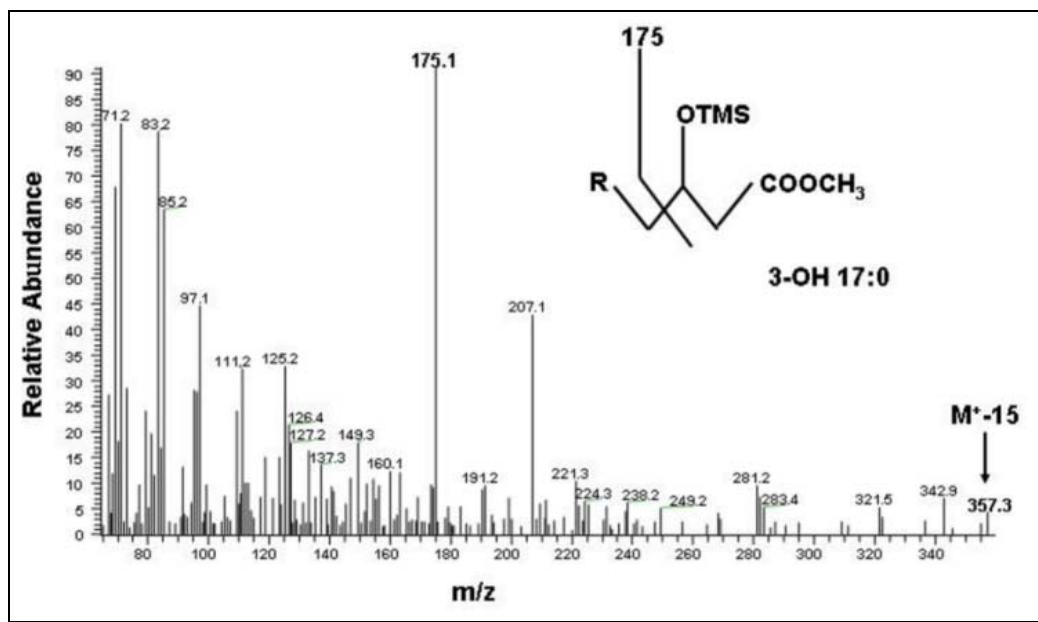


**Fig. 1.** Corresponding light micrograph (a), light combined with immunofluorescence micrograph (b), immunofluorescence micrograph showing in more detail selectively fluorescing brims surrounding ascospores in circles (c) (compare Fig. 2a), and light micrograph of stained ascospores (d) of *Ascoidea corymbosa*. A, ascus, As, ascospore, AW, ascus wall, Fas, fluorescing ascospores, T, ascus tip.



**Fig. 2.** Scanning electron micrographs of individually released ascospore (a) and aggregated released ascospores (b) in *Ascoidea corymbosa*. The release of ascospores from the ascus opening (tip) is shown in (c). A, ascus, As, ascospore, B, bowl, Br, brim.





**Fig. 3.** Mass spectrum obtained for *Ascoidea corymbosa* during its sexual stage.

# CHAPTER 3

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## **Increased mitochondrial activity uncovered in yeast sexual cells**

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This part of the study has been submitted for publication in FEMS Yeast Research (2007) and is written in this journal's format. Results shown in Figure 5 were obtained by Miss Chantel W. Swart and Miss Monique E. Goldblatt. Antibodies were obtained from Prof. S. Nigam, Free University of Berlin, Germany. The rest is the work of the candidate.

### 3.1 Abstract

Literature suggests a link between increased mitochondrial activity and yeast sexual cells (asci). In this study this association is demonstrated for the first time in fermentative and non-fermentative yeasts. Using selective fluorescence mitochondrial staining and confocal laser scanning microscopy, we provide evidence that mitochondrial function is higher in asci containing increased amounts of 3-hydroxy oxylipins compared to the corresponding asexual vegetative cells. This explains the accumulation of these oxylipins in asci, which is produced in mitochondria. Furthermore, when acetylsalicylic acid, a mitochondrial activity inhibitor, was added in increased concentrations to cultures of the non-fermenting yeast *Ascoidea* which include *A. africana*, *A. corymbosa* and *A. rubescens*, the sexual stage was found to be more sensitive. Ascospore liberation from asci was first inhibited followed by asci formation while some vegetative growth could still be observed. This work further demonstrates mitochondria as target site for aspirin antifungal action.

**Keywords** *Ascoidea*; ascospores; aspirin; mitochondrion activity; rhodamine 123; 3-hydroxy oxylipins; yeast.

### 3.2 Introduction

In 1992, Botha and co-workers reported that the life cycles of species of the non-fermenting yeast *Dipodascopsis* (*D. tothii* and *D. uninucleata*) are characterized by similar consecutive asexual and sexual reproductive stages. In the presence of different concentrations of the non-steroidal anti-inflammatory drugs (NSAIDs), acetylsalicylic acid (ASA, aspirin) and indomethacin, a dose dependent inhibition of the asexual stage was observed in both yeasts. Interestingly, the sexual stages of these yeasts were found to be more sensitive to NSAIDs (Botha *et al.*, 1992) with ascospore liberation in *D. uninucleata* the most sensitive stage towards aspirin (Kock *et al.*, 1999).

When aspirin was added to *D. uninucleata*, the production of 3-hydroxy (OH) oxylipins is inhibited in a dose dependent manner. It was therefore not surprising that later studies showed that these oxylipins are produced during the sexual cycle (Van Dyk *et al.*, 1991, 1993). This was visualized with immunofluorescence microscopy showing that the sexual stage i.e. ascospores are coated with 3-OH oxylipins (Kock *et al.*, 1998). So far, similar results concerning aspirin sensitivity and 3-OH oxylipin distribution were obtained in the non-fermenting yeasts *Dipodascus* and *Eremothecium* (Van Heerden *et al.*, 2005, 2007; Leeuw *et al.*, 2007).

The presence of 3-OH oxylipins was also reported in many yeasts (Kock *et al.*, 2003; Leeuw *et al.*, 2006), including some members of *Ascoidea* (Bareetseng *et al.*, 2005; Ncango *et al.*, 2006). Here increased amounts of oxylipins were associated with the ascospores of *A. africana* and *A. corymbosa*. Strikingly, the growth of these yeasts were found to be inhibited

by low concentrations of aspirin (Leeuw *et al.*, 2007), thereby implicating a similar phenomenon described for *Dipodascopsis*.

It is forecasted that increased amounts of 3-OH oxylipins, probably produced through mitochondrial incomplete  $\beta$ -oxidation and/or a mitochondrial fatty acid synthesis type II route such as FAS II (Ciccoli *et al.*, 2005; Hiltunen *et al.*, 2005), will be associated with the development of the sexual stages in all ascomycetous yeasts. Consequently, higher mitochondrial activities are expected in yeast sexual cells compared to asexual cells. In this study this hypothesis is tested in the yeast *Ascoidea* and other non-related yeasts such as the non-fermentative *Dipodascopsis uninucleata* and fermentative *Pichia anomala*. In addition, the effect of aspirin, a mitochondrion activity inhibitor, on the sexual and asexual stages of *Ascoidea*, is assessed.

### **3.3 Materials and methods**

#### **3.3.1 Strains used**

*Ascoidea africana* (UOFS Y-1217), *A. corymbosa* (UOFS Y-0732), *A. rubescens* (UOFS Y-0733), *Dipodascopsis uninucleata* (UOFS Y-2067) and *Pichia anomala* (UOFS Y-0157) maintained at the University of the Free State in South Africa, were used in this study.

#### **3.3.2 Cultivation and analysis**

##### **3.3.2.1 Cultivation**

All yeasts were cultivated on YM agar (Wickerham, 1951) in Petri dishes at 22 °C until sexual stage was reached. Cells of *Dipodascopsis uninucleata* were then resuspended in 100 mL YNB media (40 g L<sup>-1</sup> glucose and 6.7 g L<sup>-1</sup> Yeast

Nitrogen Base - Difco, Becton, Dickinson and Company, Sparks, MD) contained in 500 mL conical flasks. Cultures were cultivated at 25 °C on a rotary shaker (160 r.p.m.) for 48 h until the sexual stage was reached after which the cells were separated by centrifugation at 10000 r.p.m. Cells of above yeasts were subjected to light and electron microscopy, confocal laser scanning microscopy and gas chromatography- mass spectrometry (GC-MS) for morphological studies, oxylipin analysis as well as mitochondrion mapping as described below. All experiments were performed in duplicate.

### **3.3.2.2 Electron microscopy**

This was performed on *Ascoidea rubescens* (UOFS Y-0733). Scanning electron microscopy (SEM) was carried out as described by Van Wyk & Wingfield (1991). In short, the yeast cells (in sexual stage) were chemically fixed overnight using 3% (v/v) of a sodium phosphate buffered glutaraldehyde (Sigma-Aldrich, St. Louis, Mo., USA) solution at pH 7 and a similarly buffered osmium tetroxide (Sigma-Aldrich, USA) solution (1% m/v) for 4 h. Following this, the material was dehydrated in a graded series of ethanol (Merck, Gauteng, South Africa) at 30 %, 50 %, 70 %, 90 % and 100 % for 30 min per step. The ethanol-dehydrated material for SEM was critical point dried, mounted and coated with gold to make it electrically conductive. This preparation was then examined using a Shimadzu Superscan (SSX 550) SEM (Shimadzu, Tokyo, Japan).

### **3.3.2.3 Immunofluorescence studies**

This was performed on *Ascoidea africana* (UOFS Y-1217), *A. rubescens* (UOFS Y-0733) and *Pichia anomala* (UOFS Y-0157). Primary antibodies, previously prepared against synthetic 3R-HETE (Bhatt *et al.*, 1998) and

characterized as described (Kock *et al.*, 1998) were used in this study. These antibodies were found to be specific against all fatty acids (irrespective of chain length and desaturation) carrying a hydroxyl group on carbon 3.

A small amount of yeast cells was scraped from a Petri dish, transferred to a plastic tube and suspended in phosphate buffer solution (PBS; Oxoid, Hampshire, England). Cells were centrifuged for 10 min to remove debris and agar. The supernatant was disposed of with a Pasteur pipette. Thirty micro-liter of the primary antibody, which is specific to 3-OH oxylipins, was added to the cells and then incubated for 60 min in the dark. The unbound primary antibodies were washed off with PBS. Thirty micro-liter of the secondary antibody (fluorescein isothiocyanate – conjugated secondary antibody; Sigma-Aldrich, U.S.A.) was added to the tube and incubated for 60 min in the dark. Unbound FITC secondary antibody was washed off with PBS as described before. In order to maintain cell structure - antibody, fluorescence and wash treatment were performed in 2 mL plastic tubes. Cells were fixed on a microscope slide using 1,4-diazabicyclo [2.2.2] octane (Sigma-Aldrich, U.S.A.) and examined with a Nikon 2000 Confocal Laser Scanning Microscope (Nikon, Tokyo, Japan).

#### **3.3.2.4 Oxylipin analysis**

This was performed on *Ascoidea rubescens* (UOFS Y-0733). Yeast cells in their sexual stage were suspended in distilled water (pH < 4). Lipids were extracted by two volumes of ethyl acetate (Merck, Haar, Germany) and the organic phase evaporated with nitrogen gas (AFROX, Port Elizabeth, South Africa). Lipid extracts were methylated with diazomethane and silylated with

*bis*- (trimethylsilyl) trifluoroacetamide (BSTFA – Merck, Germany) for 1 h each and then dissolved in chloroform-hexane (4:1, v/v; Merck, Germany).

The treated samples were injected into a Finnigan Trace GC Ultra gas chromatograph (Thermo Electron Corporation, San Jose, California USA) with a HP5 (60 m x 0.32 mm diameter) fused silica capillary column (0.1 µm coating thickness) coupled to a Finnigan Trace DSQ MS (Thermo Electron Corporation, San Jose, California). The carrier gas was helium at 1.0 mLmin<sup>-1</sup>. 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<sup>-1</sup>. The gas chromatograph - mass spectrometer (GC-MS) was auto-tuned for an *m/z* of 70-355. One micro-liter of the sample was injected into the GC-MS at a split ratio of 1:50 at an inlet temperature of 230 °C (Venter *et al.*, 1997).

### **3.3.3 Mitochondrion function mapping**

This was performed on *Ascoidea africana* (UOFS Y-1217), *A. corymbosa* (UOFS Y-0732), *A. rubescens* (UOFS Y-0733), *Dipodascopsis uninucleata* (UOFS Y-2067) and *Pichia anomala* (UOFS Y-0157). A small amount of yeast cells in their sexual stage was transferred to a plastic tube and suspended in PBS. Cells were centrifuged for 5 min to get rid of debris and agar. The supernatant was disposed of with a Pasteur pipette. A 31 micro-liter suspension of rhodamine 123 in PBS (160 nM; Molecular Probes, Invitrogen Detection Technologies, Eugene, Oregon, U.S.A.), an effective mitochondrion tracker (Johnson *et al.*, 1980), was added to the cells and then incubated for 60 min in the dark. The unbound rhodamine 123 was washed off with PBS. Staining and washing treatment were performed in 2 mL plastic tubes. Cells



were fixed on a microscope slide using 1,4-diazabicyclo [2.2.2] octane and examined with a Nikon 2000 Confocal Laser Scanning Microscope.

### **3.3.4 ASA inhibition studies**

This was performed on *Ascoidea africana* (UOFS Y-1217), *A. corymbosa* (UOFS Y-0732) and *A. rubescens* (UOFS Y-0733). Yeasts were streaked on YM agar (Wickerham, 1951) and cultivated at 22 °C in Petri dishes. Cells were then spread out (to form a homogenous lawn) on soft YM agar plates containing only 0.5 % (m/v) agar. Each plate contained one of the following concentrations of aspirin i.e. 0 mM, 1 mM and 5 mM (Sigma, Steinheim, Germany). To achieve this, aspirin was first diluted in a minimum volume ethanol and then mixed with YM-agar media to reach the latter concentrations. Liquid media could not be used since no sexual cycle could be induced in such a medium. Yeasts on media containing low concentrations of agar were then incubated at 22 °C for 10 days until a homogenous lawn of cells could be observed on the surface of the medium. Solid YM-agar medium containing 1.6 % (m/v) agar could not be used since aspirin (in ethanol) was not able to dissolve in this medium. Since aspirin had to be dissolved in minimum amounts of 98 % ethanol, further control experiments containing similar amounts of ethanol without aspirin were performed. Next, four different areas on the yeast lawn were aseptically sampled at random and each area suspended in a drop of dH<sub>2</sub>O on a glass slide with cover slide and then subjected to light microscopy analysis. Here, all asci (at least 35) in four adjacent microscope fields were counted. This was done 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. In each case, the percentage empty asci (indicating ascospore release), was calculated. This experiment was repeated in triplicate for each yeast species resulting in a total of 3 plates (lawns) per organism tested, each containing four sampling points totaling 3 X 4 microscopic fields per yeast. Light micrographs of cells of each species studied were taken using a light microscope (Axioplan, Zeiss, Göttingen, Germany) coupled to a Colorview Soft Imaging System (Münster, Germany).

### **3.4 Results and discussion**

#### **3.4.1 Ultrastructure, oxylipin production and mitochondrial activity**

Scanning electron and light microscopy of *Ascoidea rubescens* revealed large clavate asci (Fig. 1a, and 2a). Here, ascospores are forcibly released in slimy balls (Fig. 1b) probably through turgor pressure (Fisher *et al.*, 2004) leaving a gaping ascus tip after release (Fig. 1c). This is in accordance with de Hoog (1998).

When cells of *A. rubescens* were treated with antibodies specific against 3-OH oxylipins and visualized with FITC-conjugated secondary antibody and immunofluorescence confocal laser scanning microscopy, ascospore material inside asci and especially the base of the spore discharging asci, fluoresced strongly (Fig. 2b). This may be attributed to the accumulation of these oxylipins at the ascus base when spores are forced through the narrow aerially orientated ascus tip. Interestingly the outside wall at the tips of these asci also showed increased fluorescence (Fig. 2b) which may be from oxylipins deposited on the outside of the ascus wall during

percurrent ascus formation (Fig. 2a) and/or during the release of the oxylipin-containing slimy ascus-content (Fig. 1b). A similar pattern was also observed in *Dipodascopsis tothii* where increased fluorescence was associated on the ascus tip after ascospore liberation (Smith *et al.*, 2000). In *A. africana*, fluorescence was mainly associated with the ascospores (Fig. 2c,d) while the ascus base as well as outside ascus tip did not fluoresce. As found in previous studies on *Ascoidea* (Bareetseng *et al.*, 2005; Ncango *et al.*, 2006), the vegetative cells of *A. africana* and *A. rubescens* showed only a very low affinity for this oxylipin antibody.

To confirm the presence of 3-OH oxylipins, lipids were extracted from cells during sexual reproduction stage and their chemical structures analyzed by GC-MS. The electron impact mass spectrum showed a major peak at  $m/z$  175 [ $\text{CH}_3\text{O}(\text{CO})\text{CH}_2\text{CHO}\text{TMSi}$ ], characteristic of a 3-OH oxylipin (Fig. 3). Further analysis indicates an  $M^+ - 15$  peak of 339 implying a mother ion ( $M^+$ ) of 354 (Van Dyk *et al.*, 1991). Hence, this oxylipin was identified as a 3-OH 16:2. Further chemical studies, including NMR and advanced GC-MS protocols (Ciccoli *et al.*, 2005) should now be used to confirm this structure.

Since 3-OH oxylipins are suggested to be produced by mitochondria (Ciccoli *et al.*, 2005) and are mainly associated with asci of *Ascoidea* (Bareetseng *et al.*, 2005; Ncango *et al.*, 2006), we reason that these sexual cells should therefore have higher mitochondrial function and activity compared to vegetative cells. This in fact is exactly what we found for *Ascoidea africana*, *A. corymbosa*, *A. rubescens*, *Dipodascopsis uninucleata* and *Pichia anomala*. When rhodamine 123 (an effective mitochondrion tracker, Johnson *et al.*, 1980) was added to cells of the five yeasts, the asci

showed preferential affinity for this stain implicating higher mitochondrion function in these sexual cells (Fig. 4a-e; Fig 5 a,b). The highly selective staining of mitochondria by rhodamine 123 is attributed to the selective attraction of this cationic fluorescing dye to the relative high negative electric potential across the mitochondrial membrane in living cells (Johnson *et al.*, 1980). Fluorescence studies show that 3-OH oxylipins are mainly associated with ascospores of *P. anomala* (producing hat-shaped ascospores) and not vegetative cells (Fig. 5b-insert, bottom left hand corner). This phenomenon has also been reported for *Dipodascopsis uninucleata*, producing reniform ascospores (Kock *et al.*, 1998). This links mitochondrial function and activity with 3-OH oxylipin accumulation in non-related yeast sexual cells.

#### **3.4.2 Aspirin inhibition studies**

To investigate the importance of high mitochondrial function and activity in asci, a known mitochondrion and 3-OH oxylipin production inhibitor, aspirin (Glasgow *et al.*, 1999; Leeuw *et al.*, 2007; Van Heerden *et al.*, 2007), was added at different concentrations to each of the selected species of the genus *Ascoidea*. The sexual stages of all three species were inhibited in a dose dependant manner (Figs 6, 7, and 8) while corresponding amounts of ethanol used to dissolve aspirin at different concentrations (Figs 6c, 7d, and 8d), yielded similar results to the control (Figs 6a, 7a, and 8a). These results are quantitatively expressed in Table 1, calculated according to Leeuw *et al.*, (2007) and Van Heerden *et al.*, (2007). In the absence of aspirin (control) all species showed a similar percentage (*A. africana* = 42 $\pm$ 3.7%; *A. corymbosa* = 41 $\pm$ 4.4%; *A. rubescens* = 48 $\pm$ 6.5%) of ascospore release, calculated as

percentage empty asci of total number of mature asci (with and without spores). In the presence of 1mM aspirin, a significant decrease ( $P < 0.001$ ) in ascospore release occurred in *A. africana* ( $26 \pm 4.1\%$ ) and *A. corymbosa* ( $28 \pm 3.2\%$ ) while no asci were formed in *A. rubescens* (most sensitive towards aspirin). In the presence of 5mM aspirin no mature asci were visible in all three species tested. In all cases, some hyphal growth could still be detected (Figs 6, 7, and 8). Similar dose dependant aspirin inhibition patterns were reported for *Dipodascus* (Van Heerden *et al.*, 2007) and *Eremothecium* (Leeuw *et al.*, 2007).

### 3.4.3 Conclusions

Literature suggests that 3-OH oxylipins are involved in the sexual cycle of yeasts i.e. ascospore release in *Dipodascopsis* (Kock *et al.*, 1999) as well as flocculation in *Saccharomyces cerevisiae* (Kock *et al.*, 2000; Strauss *et al.*, 2005). This is based on the fact that aspirin inhibits oxylipin synthesis and ascospore release as well as flocculation in these yeasts. One should realize that these events may be independent from each other and that changes in function may be ascribed to mitochondrial inhibition in general. A way to prove the function of these oxylipins is to add them together with low concentrations of aspirin (i.e. mitochondria only partially inhibited) and determine if aspirin inhibition can be uplifted. For this purpose, 3-OH oxylipins with different chain lengths and desaturation may be produced via biotechnological processes (Fox *et al.*, 1997) or chemical synthesis (Bhatt *et al.*, 1998; Groza *et al.*, 2002, 2004).

Ciccoli *et al.*, (2005) suggest that 3-OH oxylipins are produced via incomplete  $\beta$ -oxidation in mitochondria. This is mainly based on results by Venter *et al.*, (1997) that reported the production of 3-OH oxylipins from exogenously fed long chain fatty acids through different cycles of  $\beta$ -oxidation by the sexual stage of the yeast *D. uninucleata*. The conserved status of this oxylipin metabolic route in yeasts, is yet to be demonstrated. Especially since a fatty acid synthesis type II (FAS II) metabolic route, similar to that found in bacteria has been demonstrated in the mitochondria of *Saccharomyces cerevisiae* (Hiltunen *et al.*, 2005). Furthermore, when *Saccharomyces cerevisiae* was fed with arachidonic acid (a 20:4 fatty acid), no 3-OH 20:4 or other 20:4 derived 3-OH oxylipins could be observed (Strauss *et al.*, 2005). Only 3-OH 8:0 was produced *ab initio*. This suggests another mitochondrial route for the synthesis of this oxylipin. It is therefore possible that yeast 3-OH oxylipins that accumulate in sexual cells may also be produced by a mitochondrial FAS II. Both metabolic routes are presumed to be present in 3-OH oxylipin-producing Gram-negative bacteria (Hiltunen *et al.*, 2005) some of which is regarded as the ancestors of mitochondria (Gray *et al.*, 2001)

This is the first report directly linking mitochondrial function and activity with sexual reproduction in various yeasts. Previously, only the importance of the presence of mitochondria during ascospore development in *Saccharomyces cerevisiae* was reported using a mitochondrial protein synthesis inhibitor (Marmioli *et al.*, 1983; Codon *et al.*, 1995). We conclude that high mitochondrial function and activity is necessary for the production of sufficient energy through aerobic respiration to sustain a high production and assembly throughput during the formation of numerous ascospores within a

single enlarged ascus cell. Lower mitochondrial activity is needed in the presumably relatively less active vegetative cells. Is this a conserved phenomenon in all yeasts?

In future, studies of the function of oxylipins in *Ascoidea* and other yeasts should be assessed by adding 3-OH oxylipins to partially aspirin-inhibited cultures. Are these oxidised fatty acids also involved in forced spore release (Pringle *et al.*, 2005)? Furthermore, the applicability of rhodamine 123 as a rapid differential stain to detect fungal sexual cells, should now be investigated. This work further demonstrates mitochondria as target site for aspirin antifungal action and may have value in constructing biological assays for rapidly identifying other novel antibiotics and chemotherapeutic compounds with potent anti-mitochondrial activity (Leeuw *et al.*, 2007).

### **3.5 Acknowledgements**

The authors wish to thank the National Research Foundation (NRF) in South Africa for financial support and Professor S. Nigam for providing the antibodies.

### **3.6 References**

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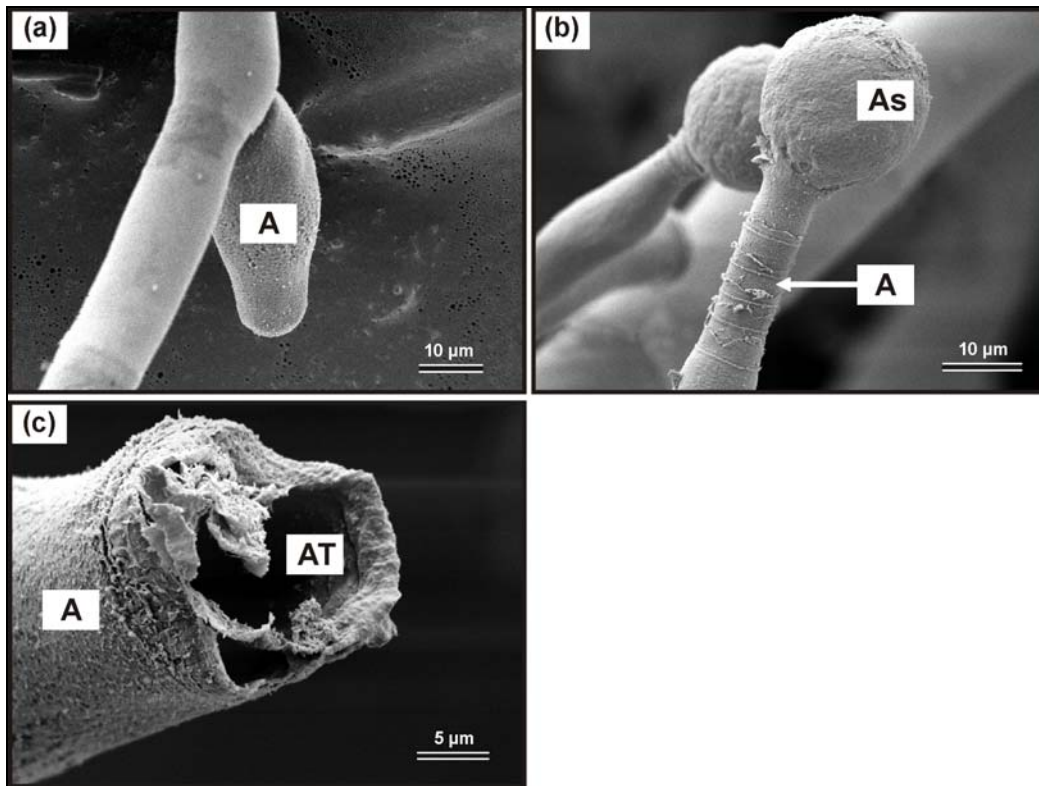
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**3.7 Table 1.** Effect of acetylsalicylic acid (ASA, aspirin) on ascospore release in the yeast genus *Ascoidea*.

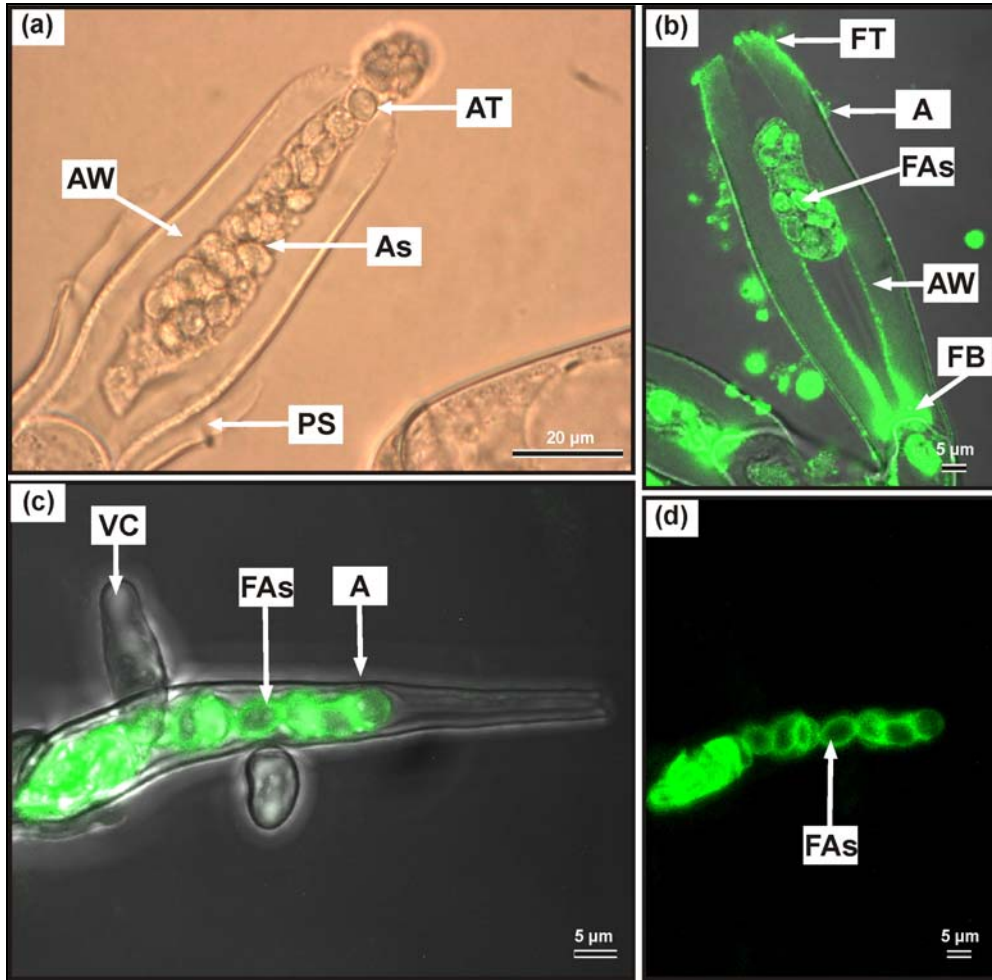
STRAINS	% ASCOSPORE RELEASE ( $\pm$ SD)		
	0 mM ASA	1 mM ASA	5 mM ASA
<i>A. africana</i> (UOFS Y-1217)	42 $\pm$ 3.7	26 $\pm$ 4.1	No asci
<i>A. corymbosa</i> (UOFS Y-0732)	41 $\pm$ 4.4	28 $\pm$ 3.2	No asci
<i>A. rubescens</i> (UOFS Y-0733)	48 $\pm$ 6.5	No asci	No asci

% Ascospore release = [Empty asci / (Full + Empty asci)] x 100; SD, standard deviation.

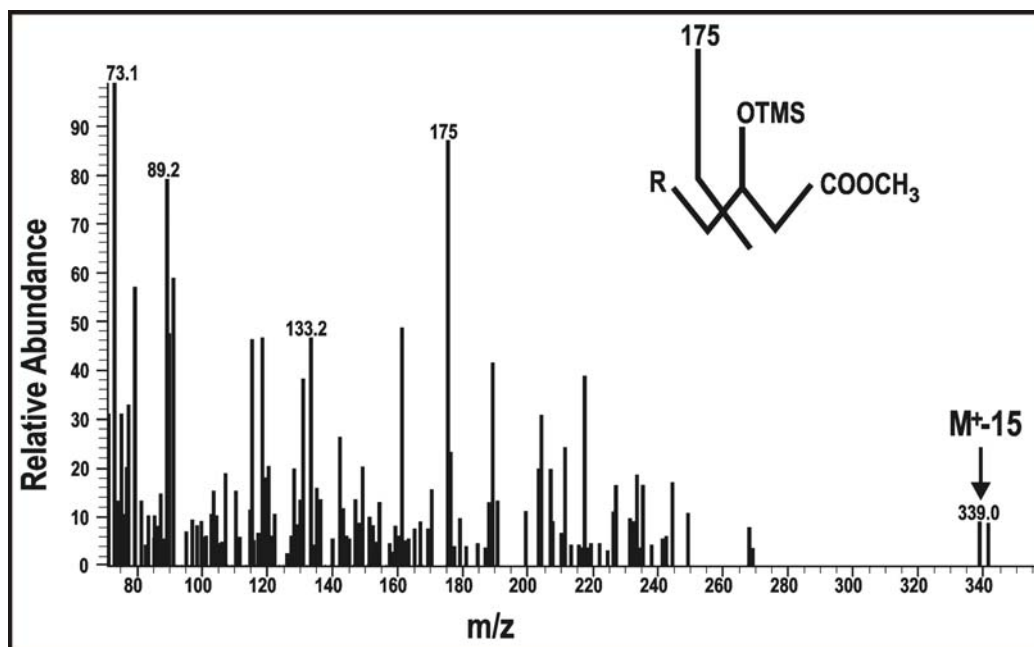
## 3.8 Figures



**Fig. 1.** Scanning electron micrograph of a premature ascus (a), ascospore liberation from a clavate ascus tip (b) and opened ascus tip after spore liberation (c). A , ascus; As, ascospore; AT, ascus tip.

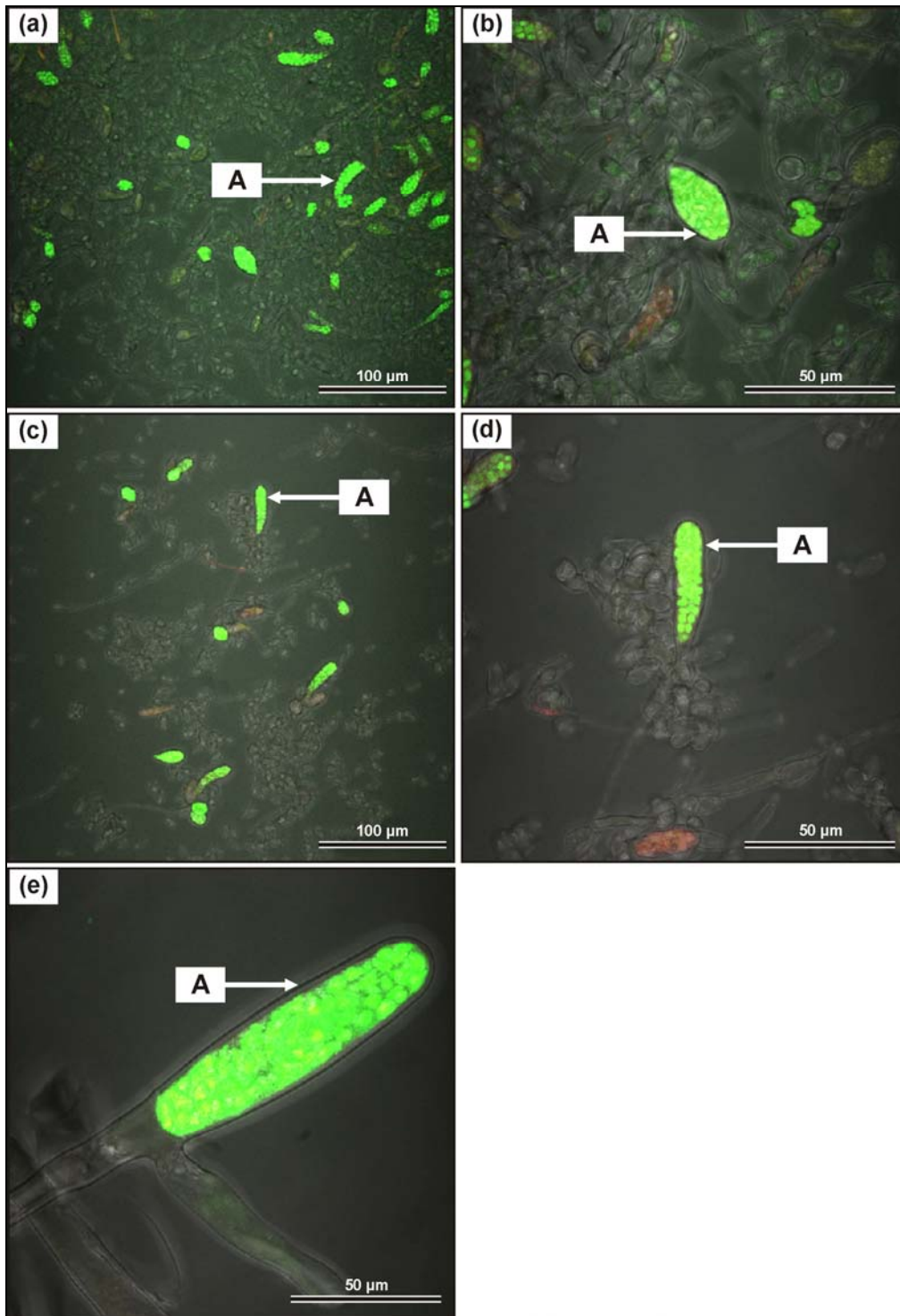


**Fig. 2.** Light and fluorescence micrographs of *Ascoidea africana* and *A. rubescens*. (a) Light micrograph of thick walled ascus of *A. rubescens* containing ascospores also showing percurrent succession, (b) immunofluorescence micrograph of partially filled mature ascus of *A. rubescens* in process of releasing spores from ascus tip, (c) immunofluorescence superimposed on corresponding light micrograph of ascus of *Ascoidea africana* and (d) only fluorescence micrograph of corresponding ascus in (c). A, ascus; As, ascospores; FAs, fluorescing ascospores; FT, fluorescing ascus tip; AW, ascus wall; FB, fluorescing ascus base; PS, percurrent succession; VC, vegetative cells.

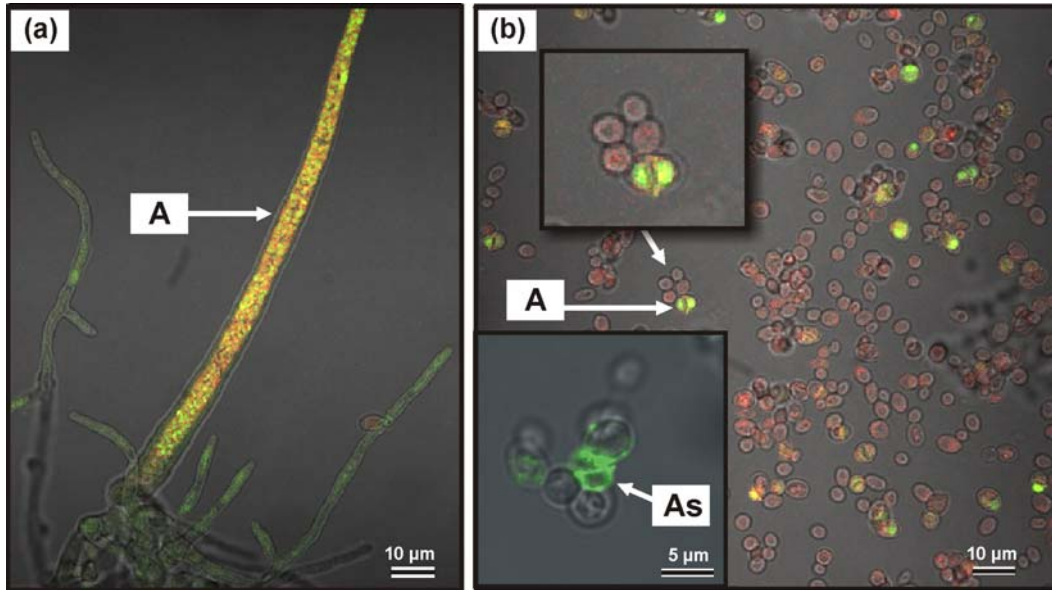


**Fig. 3.** Electron impact mass spectrum of a 3-OH oxylipin produced by *Ascoidea rubescens* during its sexual stage.

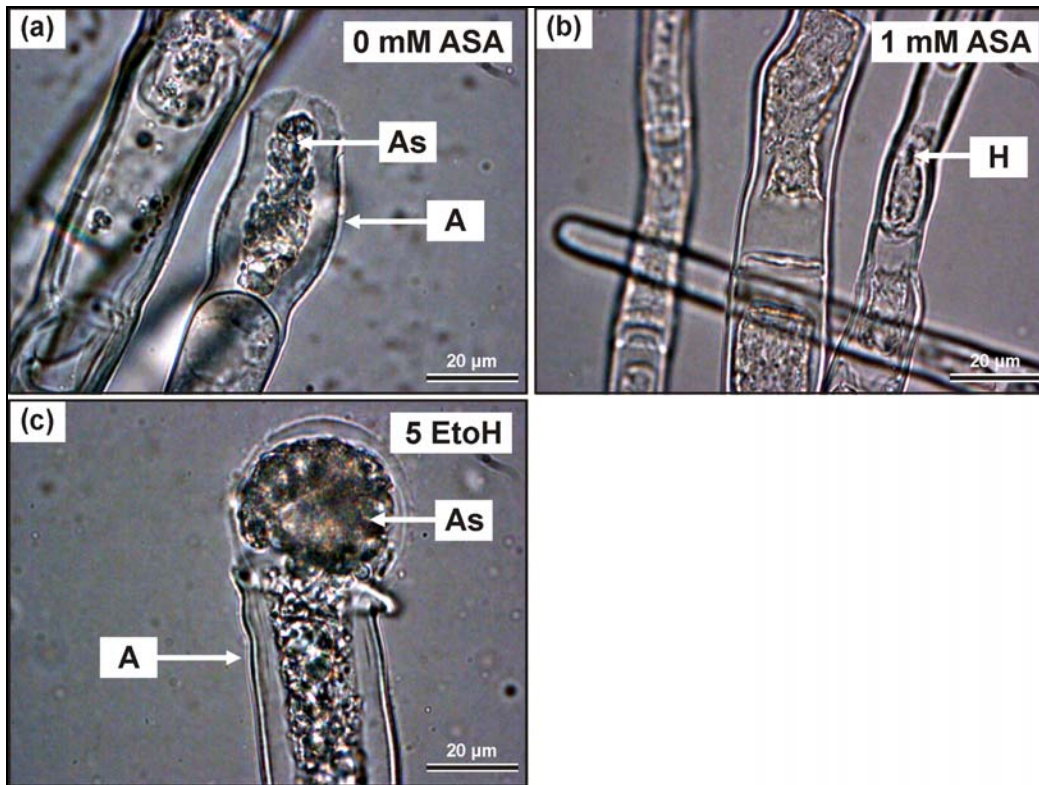




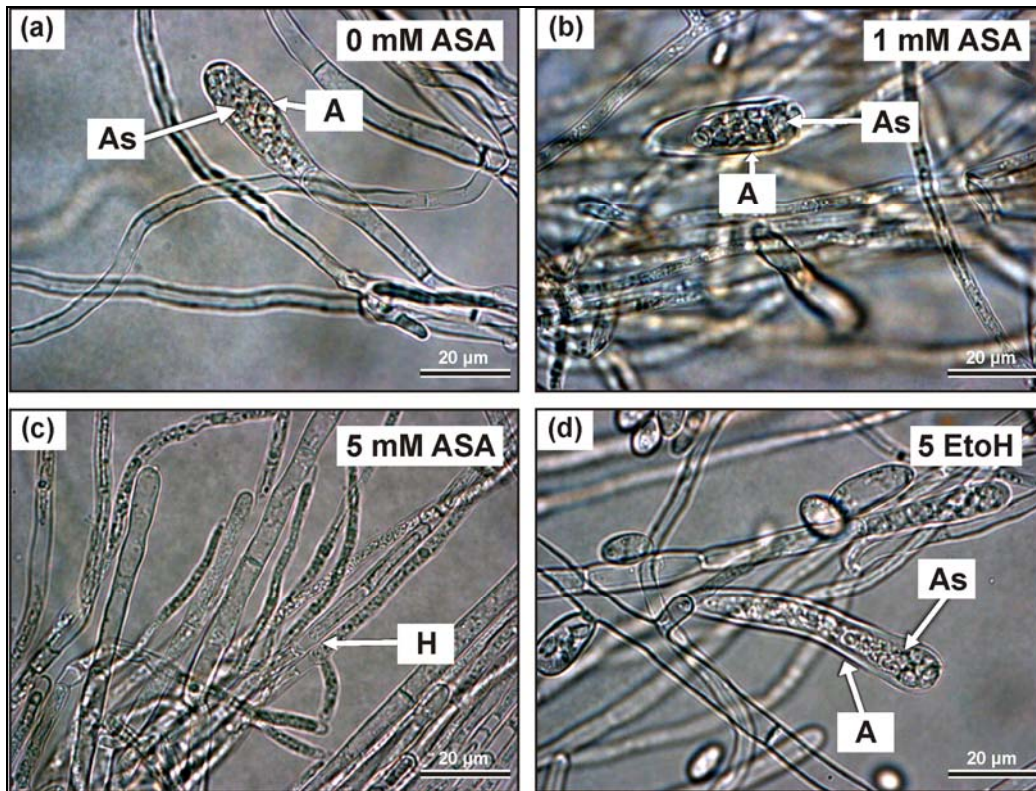
**Fig. 4.** Confocal fluorescence micrographs of cells stained with rhodamine 123 thereby mapping mitochondrial function. *Ascoidea africana* (a,b), *A. corymbosa* (c,d) and *A. rubscens* (e). A, ascus with ascospores.



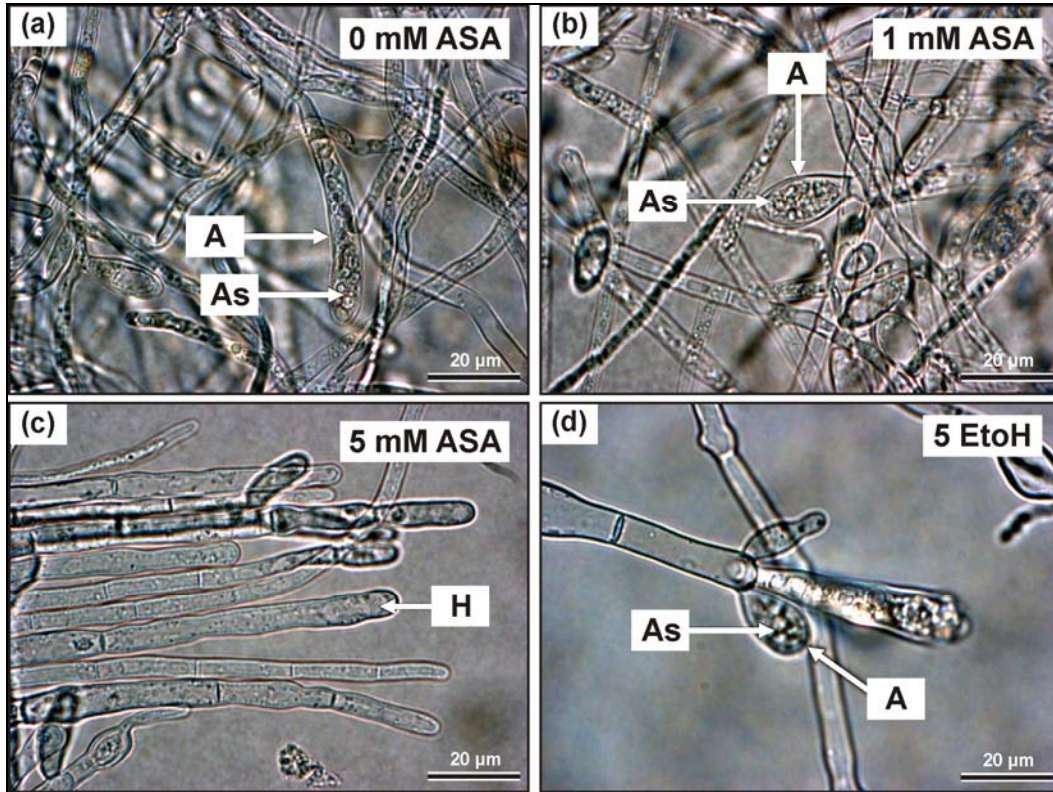
**Fig. 5.** Fluorescing micrographs depicting mitochondrial staining with rhodamine 123 in (a) *Dipodascopsis uninucleata* and (b) *Pichia anomala*. The insert (bottom left hand corner) in (b) depicts fluorescing hat shaped ascospores when treated with 3- hydroxy oxylin antibodies and fluorescein secondary antibody. The insert (top left hand side, b) depicts an enlargement of rhodamine 123- fluorescing ascus with two hat shaped ascospores. Many of these selectively fluorescing asci are visible in main micrograph (b).



**Fig. 6.** Light micrographs demonstrating the dose dependant effect of different concentrations of aspirin (ASA) on the sexual stage of *Ascoidea rubescens* (a, b). Only ethanol without ASA added to culture (c). A, ascus; As, ascospores; H, Hyphae; 5 EtoH, same amount of only ethanol added to culture as ethanol added together with ASA to yield 5 mM ASA concentration.



**Fig. 7.** Light micrographs demonstrating the dose dependant effect of different concentrations of aspirin (ASA) on the sexual stage of *Ascoidea africana* (a-c). Only ethanol without ASA added to culture (d). A, ascus; As, ascospores; H, Hyphae; 5 EtoH, same amount of only ethanol added to culture as ethanol added together with ASA to yield 5 mM ASA concentration.



**Fig. 8.** Light micrographs demonstrating the dose dependant effect of different concentrations of aspirin (ASA) on the sexual stage of *Ascoidea rubescens* (a-c). Only ethanol without ASA added to culture (d). A, ascus; As, ascospores; H, Hyphae; 5 EtoH, same amount of only ethanol added to culture as ethanol added together with ASA to yield 5 mM ASA concentration.

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**SUMMARY**

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Acetylsalicylic acid (ASA)-sensitive 3-hydroxy (3-OH) oxylipins were uncovered in 1991 in the yeast *Dipodascus uninucleata*. Since then, various similar oxylipins were found to be widely distributed in fungi. Interestingly, 3-OH oxylipins were reported to play a role in ascospore release from enclosed asci, where they are involved in assisting nano-scale gear-like (*D. uninucleata*); sliding (*Dipodascus*); drilling (*Eremothecium sinicaudum*) and piercing movements (*E. ashbyi* and *E. coryli*). In *Ascoidea africana*, a 3-OH 10:1 oxylipin was found to be associated with hat-shaped ascospores carried inside ellipsoidal asci. However, in this study no function was proposed for this oxylipin. Since only one species representing the genus *Ascoidea* was studied, it became the aim to further expand this study to also include *A. corymbosa* and *A. rubescens*. Using confocal laser scanning microscopy (CLSM) on cells stained with fluorescein-coupled 3-OH oxylipin specific antibodies, this study suggests that oxylipins are specifically associated with ascospores and not vegetative cells of *A. corymbosa* and *A. rubescens*. Using gas chromatography - mass spectrometry (GC-MS) the oxylipin, 3-OH 17:0, was identified in *A. corymbosa*. Here, oxylipin-coated razor sharp ascospore brims may play a role in rupturing the ascus to affect forced release of hat-shaped ascospores. Literature suggests that 3-OH oxylipins are produced by  $\beta$ -oxidation or fatty acid synthesis in mitochondria of yeasts. Since these oxylipins accumulate in sexual cells (asci), increased mitochondrial activity is therefore expected in these structures. Strikingly, this assumption is supported in this study. Using selective fluorescence mitochondrial staining and CLSM, evidence is provided that mitochondrial function is much higher in asci containing increased amounts of 3-OH

oxylipins compared to the corresponding asexual vegetative cells. Furthermore, when ASA, a mitochondrial inhibitor, was added in increased concentrations to cultures of *Ascoidea*, the sexual stage was found to be more sensitive. Ascospore liberation from asci was first inhibited followed by asci formation while some vegetative growth could still be observed.

**Keywords:** Acetylsalicylic acid, *Ascoidea*, ascospore release, confocal laser scanning microscopy, gas chromatography - mass spectrometry, mitochondrial activity, rhodamine 123, 3-hydroxy oxylipins, yeast.



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**OPSOMMING**

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Asetielsalisiensuur (ASA) sensitiewe 3-hidroksie (3-OH) oksilipiëne is in 1991 ontdek in die gis *Dipodascopsis uninucleata*. Sedertdien is verskeie soortgelyke oksilipiëne gevind in 'n groot verskeidenheid fungi. Daar is bevind dat 3-OH oksilipiëne 'n rol speel in askosporvrystelling vanuit 'n verseëelde askus, waar hierdie oksilipiëne betrokke is by verskillende nano-skaal ratagtige askosporbewegings (*D. uninucleata*), askospor glybewegings (*Dipodascus*), boor (*Eremothecium sinicaudum*) en prikbewegings (*E. ashbyi* en *E. coryli*). 'n Oksilipiën is ontdek in *Ascoidea africana* wat spesifiek geassosieer is met die hoedvormige askospore wat in 'n ellipsoïedale askus gedra word. Die chemiese struktuur van hierdie komponent is 3-OH 10:1. In hierdie studie is geen funksie voorgestel vir dié oksilipiën nie. Aangesien slegs een spesies van die genus *Ascoidea* ondersoek is, het dit die doel van hierdie studie geword om ook *A. corymbosa* en *A. rubescens* te ondersoek. Deur die gebruik van konfokale laserskanderingsmikroskopie op selle wat gekleur is met 'n fluoresien-gekoppelde 3-OH spesifieke teenliggaam, was 3-OH oksilipiëne duidelik om die askospore sigbaar, maar nie om die vegetatiewe selle of die askuswande van *A. corymbosa* en *A. rubescens* nie. Deur die gebruik van gaschromatografie-massaspektrometrie is die oksilipiën 3-OH 17:0 ontdek. Hier word voorgestel dat die vlymskerp askosporrante, wat bedek is met 3-OH oksilipiëne, 'n rol speel in die oopbreek van die askus tydens die geforseerde vrystelling van hoedvormige askospore uit die askus. Literatuur stel voor dat 3-OH oksilipiëne in die mitochondria van giste deur  $\beta$ -oksidase of vetsuursintese geproduseer word. Aangesien hierdie oksilipiëne in geslagtelike selle (askus) akkumuleer, word verhoogde mitochondriale aktiwiteit in hierdie strukture verwag. Hierdie bewering word in hierdie studie

ondersteun. Deur die gebruik van 'n selektiewe fluoresserende mitochondriale kleurstof en konfokale laserskanderingsmikroskopie, is bevind dat daar wel 'n verhoogde mitochondriale funksie in die askus, met verhoogde hoeveelhede 3-OH oksilipiene in vergelyking met die ooreenstemmende vegetatiewe selle, is. Verder is ook bevind dat wanneer ASA, 'n mitochondriale inhibitor, by die selle van *Ascoidea* in verhogende konsentrasies bygevoeg word, die geslagtelike fase meer sensitief was. Askosporvrystelling vanuit die askus word eerstens geïnhibeer, dan opgevolg deur die inhibisie van askusvorming. Daar kan egter nog in sommige gevalle vegetatiewe groei waargeneem word.

**Sleutelwoorde:** Asetielsalisiensuur, *Ascoidea*, askosporvrystelling, konfokale laser skandering mikroskopie, gas chromatografie-massa spektrometrie, mitochondriale aktiwiteit, rhodamien 123, 3-hidroksie oksilipiene, gis.