

Oxylipins in automictic yeast life cycles

By

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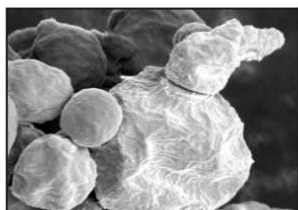
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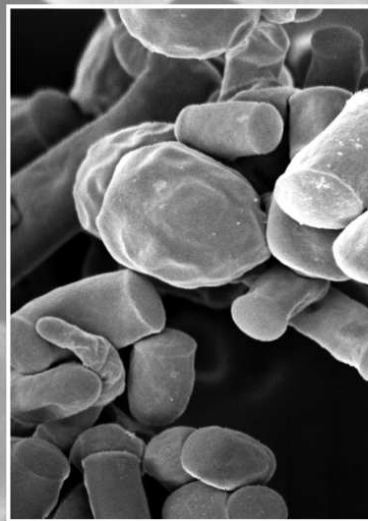
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This dissertation is dedicated to my family: my mother, M.M. Swart, father, P.H. Swart[†], brother, R.E. Swart, grandmother, M.M. Coetzer and my grandfather, C.J. Coetzer[†].



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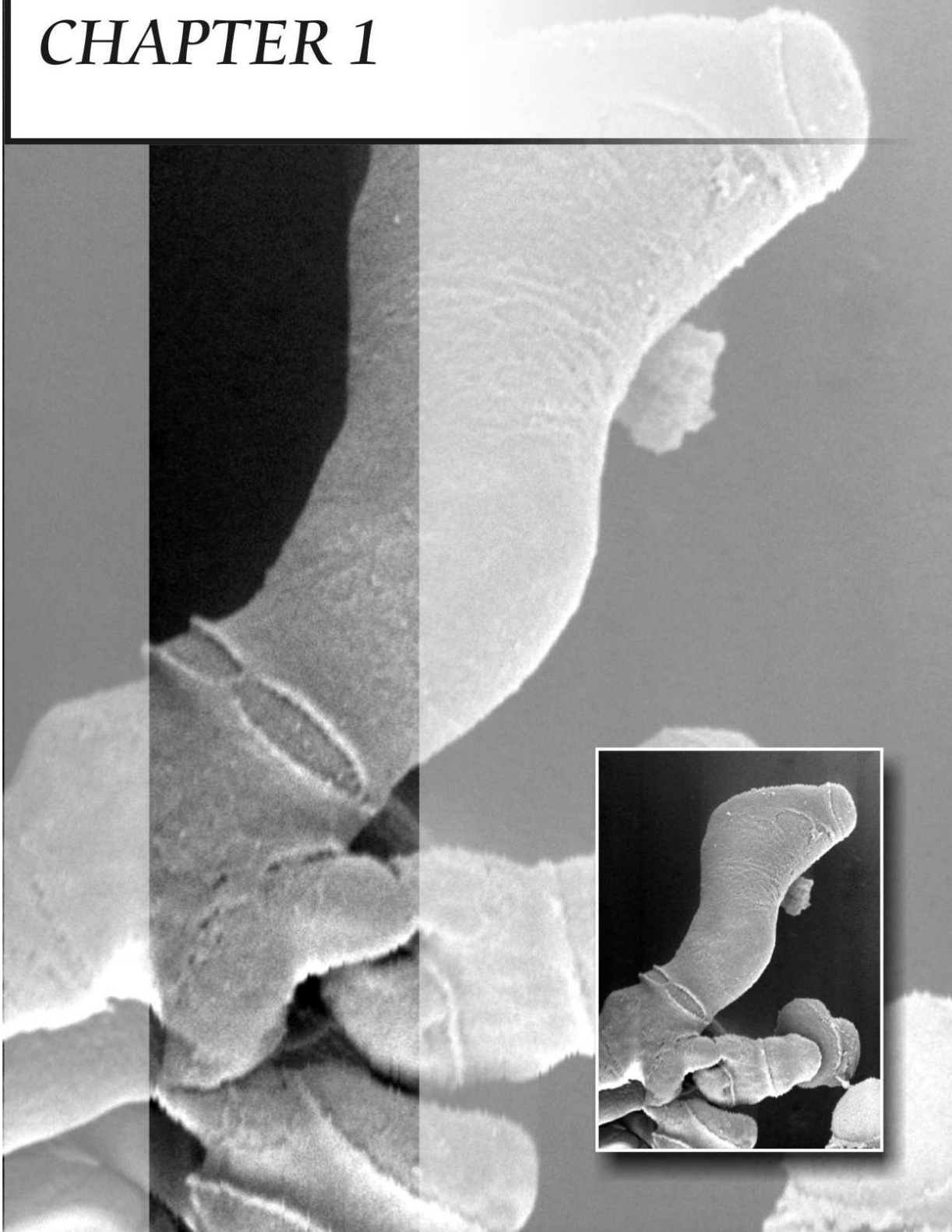
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CHAPTER 1



CHAPTER 1

Literature Review

1.1 Motivation

In the early 1990s, 3-hydroxy (OH) oxylipins were discovered in the yeast genus *Dipodascopsis* (Kock et al. 1991). Since then, various 3-OH oxylipins were uncovered in a variety of yeast species. These compounds were found to be associated specifically with the sexual structures, i.e. asci and ascospores (Kock et al. 2003), and were also discovered on the vegetative cell surfaces of certain yeasts, i.e. *Saccharomyces cerevisiae*, where they play a possible role in flocculation.

3-OH oxylipins are saturated and unsaturated oxidized fatty acids which are produced in the mitochondria via incomplete β -oxidation or fatty acid synthesis type II (Hiltunen et al. 2005; Kock et al. 2007). Literature suggests that these compounds play an important role in the sexual stage of yeast by assisting ascospore release from asci (Kock et al. 2004) by acting as a lubricant. Therefore it is not surprising that oxylipins accumulate in these sexual structures. Oxylipin accumulation in the automictic non-fermenting yeasts has been the main focus of studies performed so far (Kock et al. 2003, 2007; Leeuw et al. 2007).

When acetylsalicylic acid (ASA), a known mitochondrial (respiration) inhibitor, was added in increased concentrations to the yeast *Dipodascopsis uninucleata*, a dose dependent decrease in 3-OH oxylipin production as well as ascospore release was observed (Van Dyk et al. 1991; Botha et al. 1992). Ascospore release was the most sensitive part of the yeast life cycle towards ASA (Kock et al. 1999). Consequently it is

suggested that 3-OH oxylipins have a lubricating function in ascospore release from asci.

Since it is known that 3-OH oxylipins are produced in the mitochondria, we should also expect increased mitochondrial activity in asci. Is this true in both fermenting and non-fermenting automictic yeasts? What is the effect of ASA on growth and sexual reproduction of these yeasts? Consequently, this study aims (1) to map the distribution of 3-OH oxylipins and mitochondrial activity in the different reproductive phases of various fermenting and non-fermenting yeasts, (2) to assess the sensitivity of these yeasts to ASA, with regards to growth and sexual reproduction, (3) to determine if a link between ASA addition and oxygen deprivation (both mitochondrial inhibitors) exists and (4) to develop a biological assay that may find application in screening for effective anti-mitochondrial antifungals.

1.2 Automictic yeasts

1.2.1 Definition of a yeast

In short, yeasts are unicellular, ontogenic stages of the Ascomycetes and Basidiomycetes (Van der Walt 1987). Most yeasts are classified into three major groups namely, the Ascomycetes, Basidiomycetes and the anamorphic Deuteromycetes. According to Kurtzman and Fell (1998) yeasts are generally characterized by budding or fission as their primary means of vegetative reproduction

while the two teleomorphic taxa have sexual stages that are not enclosed within a fruiting body. The Deuteromycetes, however, is anamorphic and has no apparent sexual phase.

1.2.2 Automixis

Since this study emphasizes automixis in yeasts, it is important to first define this type of life cycle. Automixis or self-fertilization, is characterized by karyogamy occurring between two sister nuclei formed by the same cell. Therefore an alternation of generations in such an automictic cycle preserves the advantages of meiosis. Furthermore, the stability of an environmentally successful genome is guaranteed strictly through inbreeding. An automictic cycle therefore allows autodiploidization or self-fertilization, but excludes heterothallism (conjugation of mating types of different cells) (Van der Walt 1999).

In ascospore producing yeasts, the automictic cycle can be observed in both diplontic and haplontic species (Van der Walt 1999). In diplontic species, haploid ascospores are formed in unconjugated asci that conjugate after release to restore the diploid phase, while in haplontic species asci are formed that may consist of a mother cell and attached bud (mother-daughter cell conjugation). In haplontic species, asci can also be formed when two haploid vegetative cells conjugate to form a conjugated ascus.

In “anamorphic” species (budding, ascomycetous, diploid species that lack ascus stages) this automictic cycle is implicated in the formation of an “undifferentiated dangeardien”. This consists of a budding meioconidiophore structure that develops from a thick-walled chlamydospore or functional teliospore (Van der Walt and Johannsen 1974).

Automixis or autogamy occurring in uninucleate, budding yeasts, retains the advantages of meiosis by alternating the generations – karyogamy is therefore limited to two haploid sister nuclei derived from the same cell. The automictic cycle therefore excludes genetic exchange between different strains i.e. mating types of free-occurring cells (heterothallism). The automictic cycle is therefore characterized as (1) totally inbreeding, (2) renounces the evolutionary advantages of genetic exchange and recombination (heterothallism), and (3) confines further modification of an already environmentally successful genome, while (4) retaining the advantages of meiosis in eliminating deleterious mutations (Van der Walt 1999).

The automictic cycle differs from the amphimictic cycle considerably, where the latter (1) implicates karyogamy by conjugation of two free-occurring, vicinal or proximate haploid cells (mating types), (2) promotes genetic exchange and recombination, and (3) makes adaptation to new ecological niches possible. Amphimixis promotes diversity of the genome, while automixis is a sexual strategy where the stability and integrity of an already environmentally successful genome is ensured. Therefore it is suggested that the automictic cycle evolved from the amphimictic cycle (Van der Walt 1999). This

implies that species with exclusively automictic life cycles represent more recently evolved taxa. Table 1 and Figs 1-5 indicate various life cycle types. (A diplohaplontic homothallic life cycle is not indicated diagrammatically, but is a combination of a haplontic homothallic and diplontic homothallic life cycle.)

Since this study suggests a link between sexual reproduction in automictic yeasts, mitochondrial activity and 3-OH oxylipin accumulation/production, the next section will focus on mitochondrially produced oxylipins.

Table 1. Different sexual life cycles in yeasts with automixis in homothallic and amphimixis in heterothallic species.

Life cycle	Ascus type	Example
Haplontic homothallic	Mother-daughter cell conjugation	<i>Pichia farinosa</i>
Haplontic homothallic	Conjugated	<i>Zygosaccharomyces bailii</i>
Diplohaplontic homothallic	Conjugated/ Unconjugated	<i>Pichia angusta</i>
Haplontic heterothallic	Conjugated	<i>Pichia ohmeri</i>
Diplontic homothallic	Unconjugated	<i>Saccharomyces cerevisiae</i>
Cryptic*	Meioconidiophore	<i>Candida magnoliae</i>

* The cryptic cycle is also automictic.

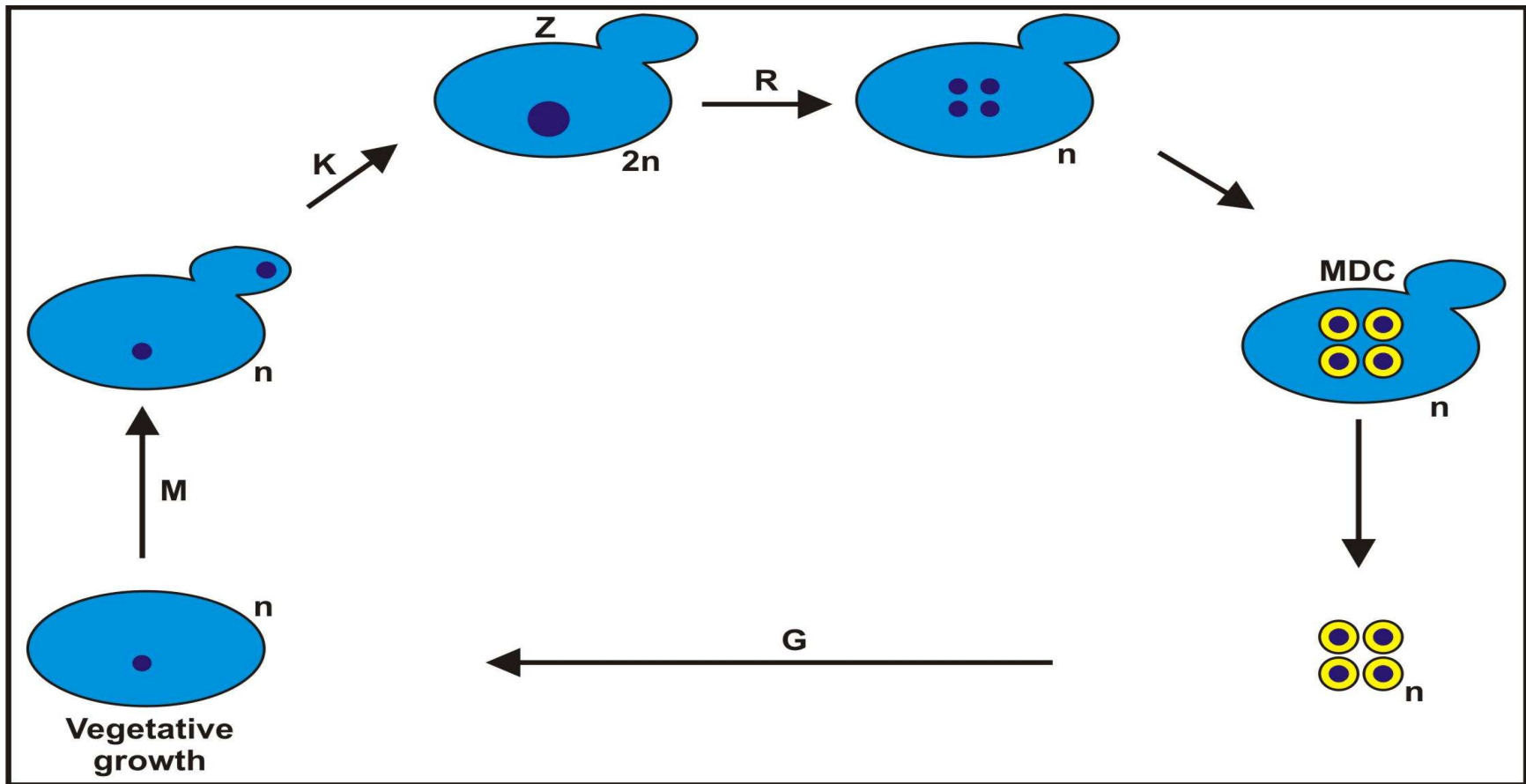


Fig. 1: Haplontic, homothallic life cycle in yeasts. In this sexual cycle a haploid (n) vegetative cell undergoes mitosis (M) to form 2 haploid nuclei. One of which enters a bud attached to the mother cell. This nucleus then migrates back into the mother cell where karyogamy (K) takes place to produce a diploid ($2n$) zygote (Z). Reduction division (R)/meiosis then occurs and four haploid nuclei are formed, which are enclosed within cell walls in a mother-daughter cell (MDC) conjugated ascus. These ascospores are then released and germinate (G) to return to the haploid vegetative stage (Table 1).

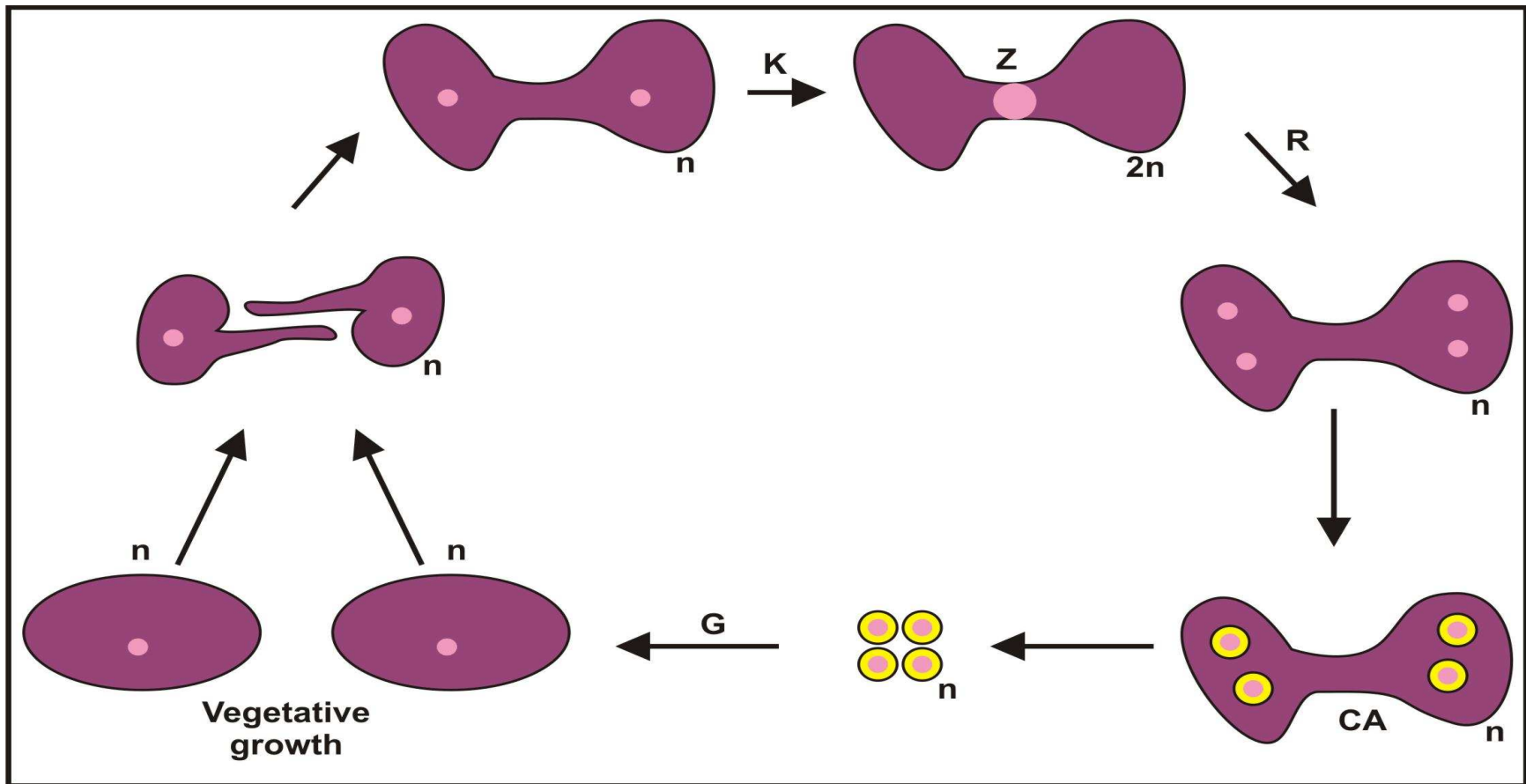


Fig. 2: Haplontic, homothallic life cycle in yeasts. During this sexual cycle two haploid (n) vegetative cells from the same mother cell conjugate and karyogamy (K) takes place to form a diploid ($2n$) zygote (Z). Reduction division (R)/meiosis then occurs to produce four haploid nuclei which are then enclosed within cell walls to form ascospores in a conjugated ascus (CA). After release these ascospores germinate (G) to return to the haploid vegetative stage (Table 1).

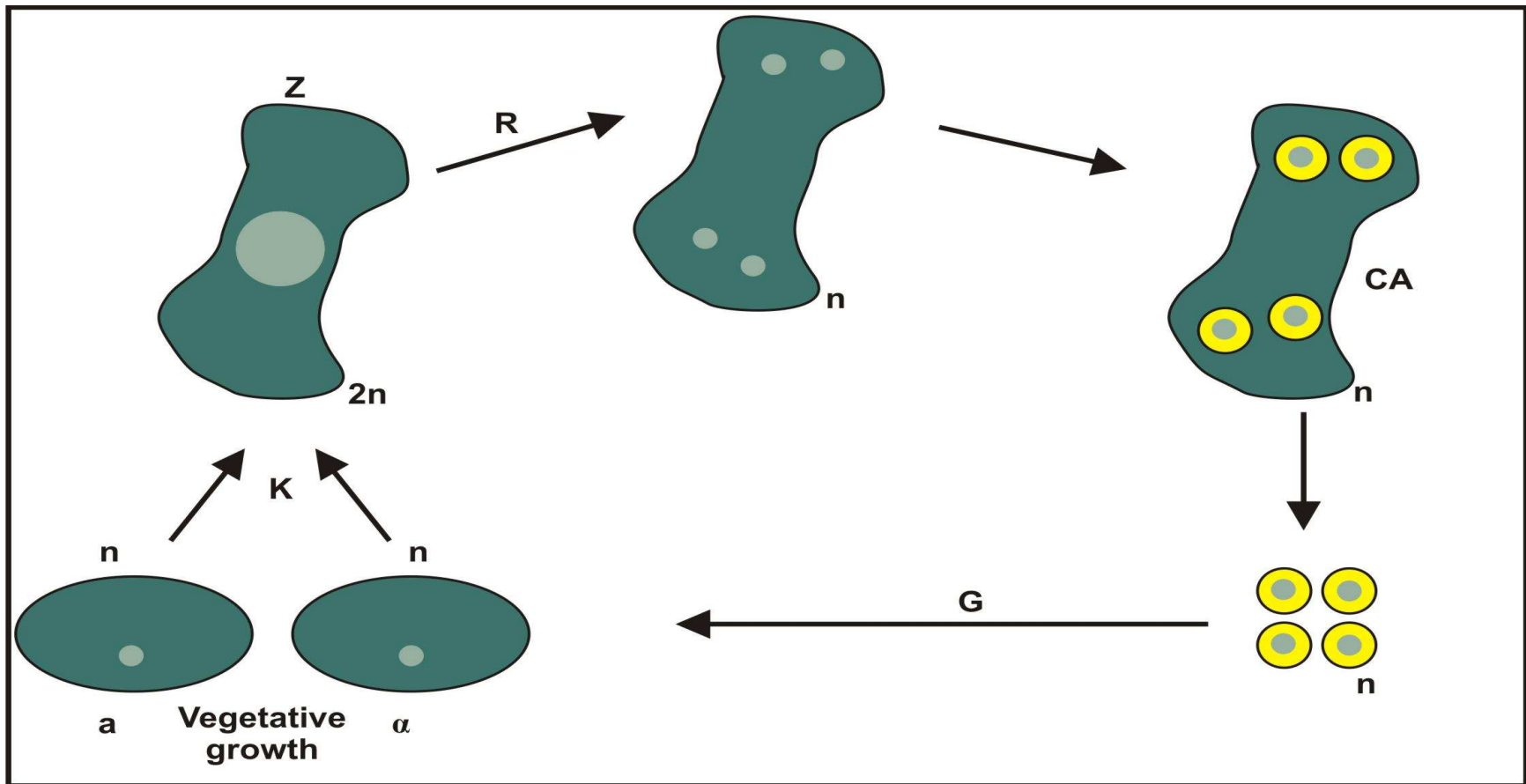


Fig. 3: Haplontic, heterothallic life cycle in yeasts. This life cycle requires two haploid (n) mating types, \underline{a} and $\underline{\alpha}$. These mating types conjugate and karyogamy (K) takes place to form a diploid ($2n$) zygote (Z). Reduction division (R)/meiosis then occurs to form four haploid nuclei (two \underline{a} mating types and two $\underline{\alpha}$ mating types). These nuclei are enclosed within cell walls in a conjugated ascus (CA) and after release these ascospores germinate (G) to return to the \underline{a} and $\underline{\alpha}$ mating types of the haploid vegetative stage (Table 1).

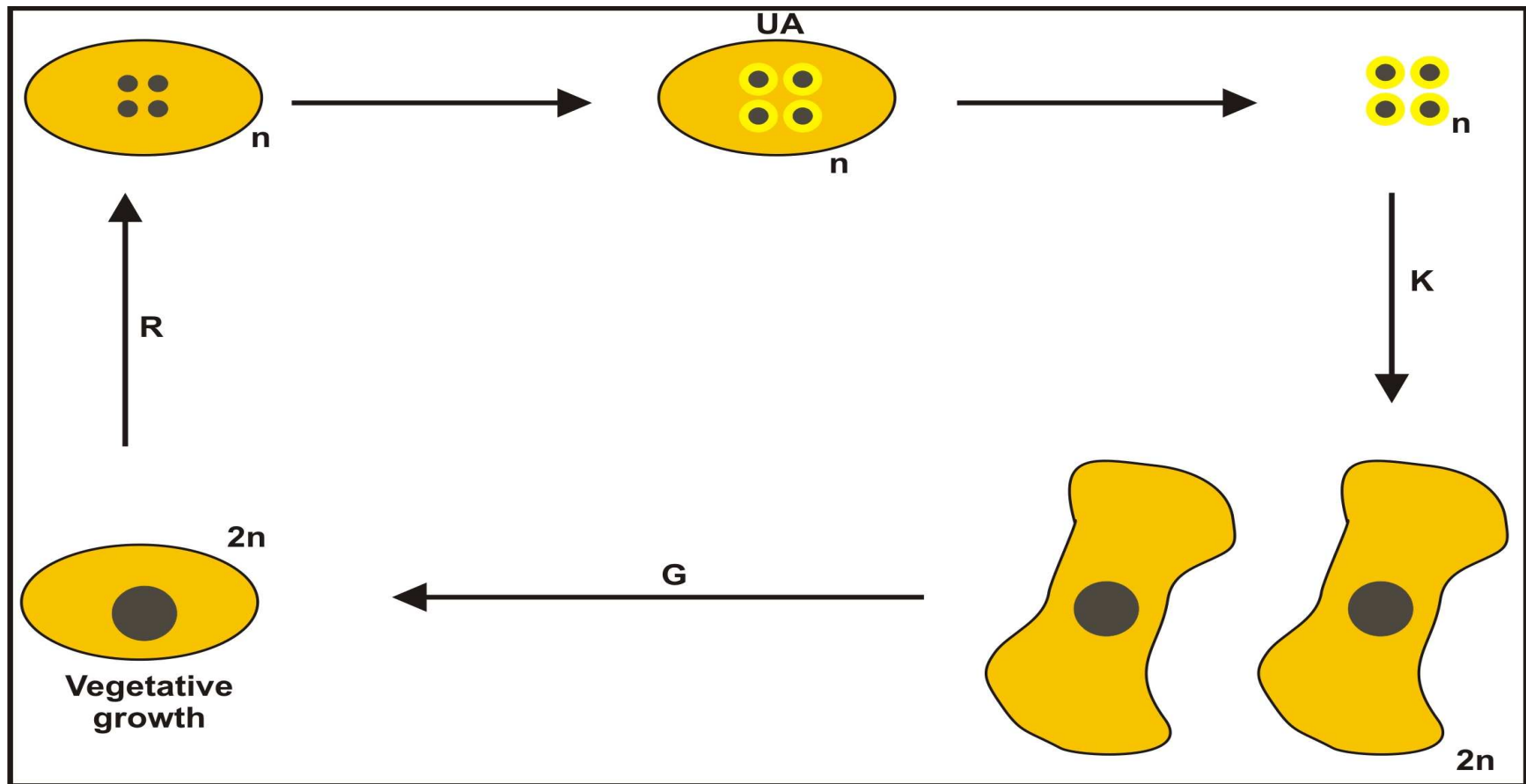


Fig. 4: Diplontic, homothallic life cycle in yeasts. During this life cycle a diploid ($2n$) vegetative cell directly undergoes reduction division (R)/meiosis to produce four haploid (n) nuclei which are enclosed within cell walls in an unconjugated ascus (UA). After release these haploid ascospores conjugate and karyogamy (K) takes place to return (G, germinate) to the diploid vegetative stage (Table 1).

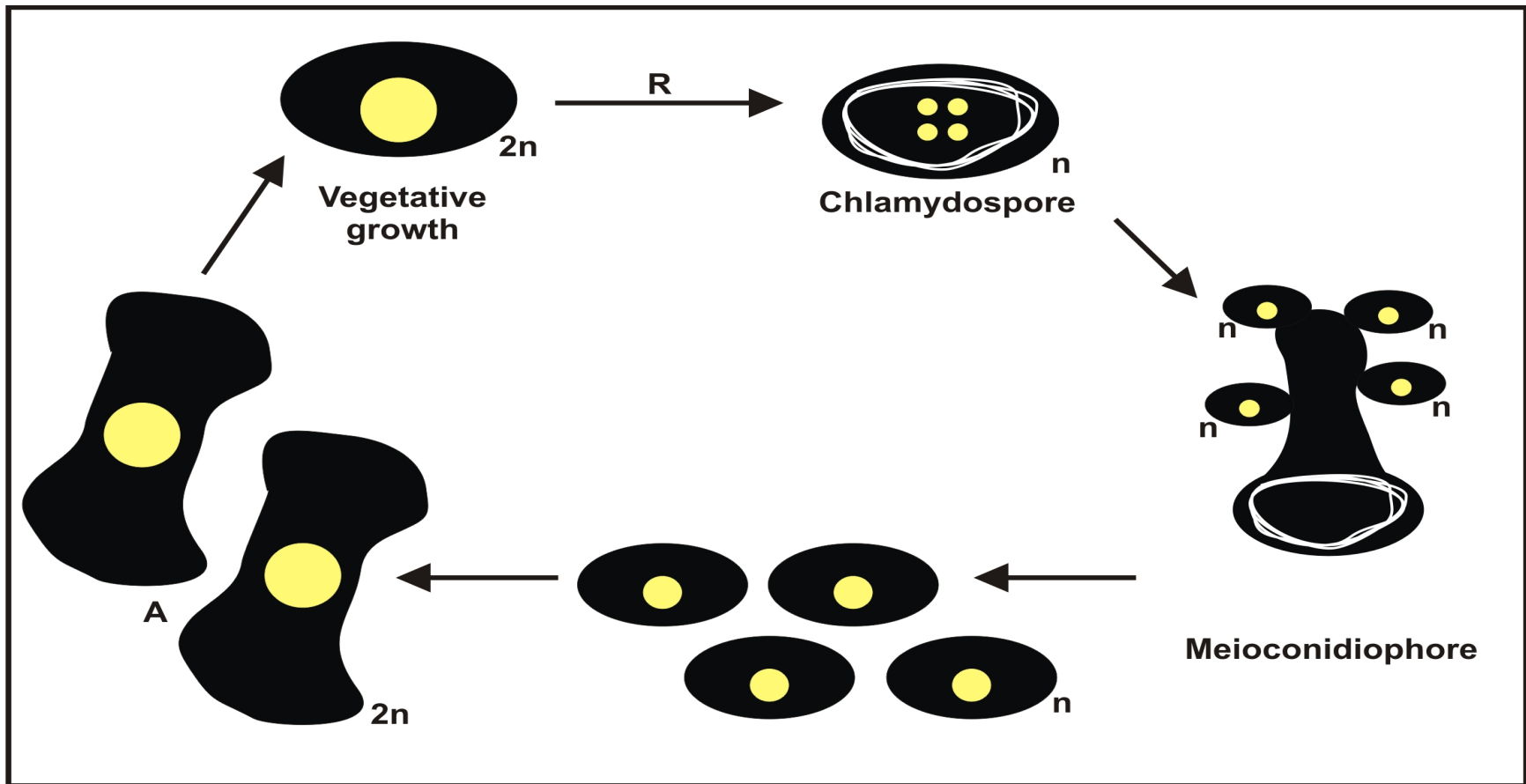


Fig. 5: Cryptic life cycle in yeasts. The cryptic cycle is expressed in “anamorphs”, which supposedly has no sexual cycle (*Candida magnoliae*). During this cycle a diploid ($2n$) vegetative cell undergoes reduction division (R)/meiosis to form four haploid (n) nuclei enclosed within a thick-walled chlamydospore. These nuclei are then enclosed within cell walls and externally released from the sexual structure (meioconidiophore). After release these meiospores conjugate and autodiploidization (A) takes place to return to the diploid vegetative stage (Table 1).

1.3 Oxylipins

1.3.1 Background

Oxylipins are widely distributed in nature i.e. in plants, animals and various microorganisms. These compounds are saturated and unsaturated oxidized fatty acids also including the eicosanoids (e.g. prostaglandins, thromboxanes, leukotrienes and lipoxygenase products). Many of these compounds are pharmacologically effective and have important biological activities (Samuelsson 1983; Needleman et al. 1986; Hinrichs et al. 1988; Spector et al. 1988; Van Dyk et al. 1994). This group also includes a large number of hydroxy oxylipins. Production of oxylipins occurs either mitochondrially or by lipoxygenase, dioxygenase or cytochrome P-450 mediated pathways, which have been reported in fungi (Shechter and Grossman 1983; Hamberg 1986; Hamberg et al. 1986; Mazur et al. 1991; Brodowski and Oliw 1992; Brodowski et al. 1992). Various hydroxyl groups can be attached at different carbon atoms e.g. 5, 7, 8, 9, 12, 13, 15 or 17 of the fatty acid molecule and are mostly produced from oleic or linoleic acid. In this study 3-OH oxylipins (with one hydroxyl group at carbon 3) were studied with regards to their distribution in non-fermenting and fermenting autotrophic yeasts (Fig. 6).

1.3.2 3-OH oxylipins

1.3.2.1 Chemical structure and production

Literature suggests that these 3-OH oxylipins are produced in the mitochondria via incomplete β -oxidation or fatty acid synthesis type II (Hiltunen et al. 2005; Kock et al.

2007). These compounds have a basic structure that consists of a hydrophilic carboxylate group (polar head) with a hydroxyl group at the carbon 3 position (Fig. 6) (Kock et al. 2003).

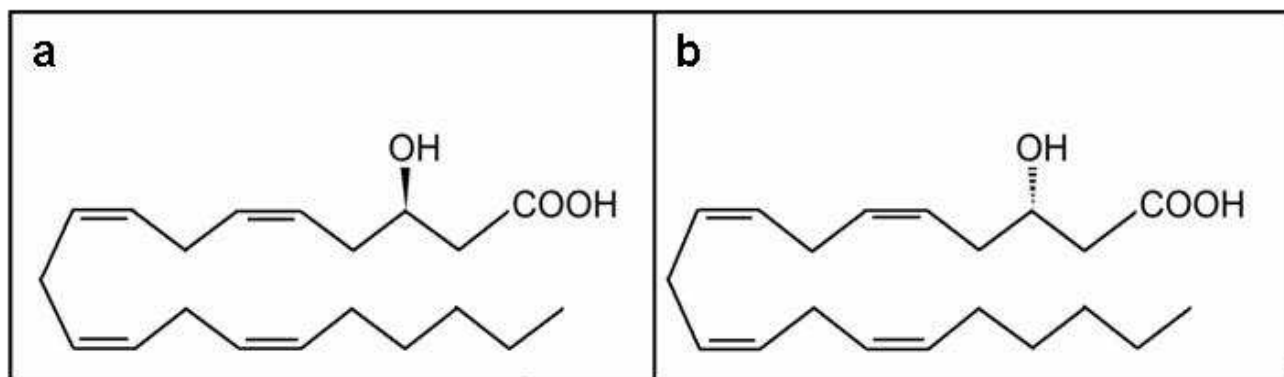


Fig. 6. The chemical structures of typical 3-hydroxy oxylipins. Two enantiomers are formed: *R*-3-hydroxy-5,8,11,14-eicosatetraenoic acid (3*R*-HETE) (a) and *S*-3-hydroxy-5,8,11,14-eicosatetraenoic acid (3*S*-HETE) (b). (Taken with permission from Kock et al. 2003).

1.3.2.2 Distribution

3-OH oxylipins were discovered in 1991 in the yeast *Dipodascopsis uninucleata* (Van Dyk et al. 1991), where the production of these compounds was also found to be ASA sensitive. Since then there has been ample reports on the distribution of 3-OH oxylipins in fungi (Kock et al. 1998, 2003, 2007; Pohl et al. 1998; Smith 2002; Van Heerden et al. 2005, 2007; Leeuw et al. 2006, 2007; Sebolai et al. 2007). Table 2 represents the latest information on the distribution of 3-OH oxylipins in fungi.

Table 2. Distribution patterns of 3-OH oxylipins in yeasts. (Taken with permission from Ncango 2007).

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
<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. 2000
<i>L. kockii</i>	3-OH metabolite	ascospores	Smith et al. 2000
<i>L. kononenkoae</i>	3-OH metabolite	ascospores	Smith et al. 2000
<i>L. starkeyi</i>	3-OH metabolite	ascospores	Smith et al. 2000
<i>L. yamadae</i>	3-OH metabolite	ascospores	Smith et al. 2000
<i>L. yarrowii</i>	3-OH metabolite	ascospores	Smith et al. 2000
<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

1.3.2.3 Function

Various bioprospecting studies imply that 3-OH oxylipins are associated with surface structures of aggregating ascospores of many yeasts. These include members of the non-fermenting genera *Ascoidea* (Bareetseng et al. 2005); *Dipodascus* (Van Heerden et al. 2005, 2007) and *Eremothecium* (Bareetseng et al. 2004; Kock et al. 2004; Leeuw et al. 2006, 2007). Literature suggests that these oxylipins play a possible role in the effective release of ascospores from enclosed asci by acting as a lubricant.

3-OH oxylipins were also found on surfaces of aggregating vegetative cells of *Saccharomyces cerevisiae* (Kock et al. 2000), *Saccharomycopsis malanga* (Sebolai et al. 2001) and various other yeasts (Table 2). Other functions for these compounds have also been reported where oxylipins act as an active substance in the lipopolysaccharide (LPS)-endotoxins of Gram-negative bacteria (Rietschel et al. 1994). It has also been reported that oxylipins exert an inflammatory response during *Candida* infection (Ciccoli et al. 2005).

1.3.2.4 ASA inhibition

A dose dependent inhibition of sexual cells (asci) development was observed when ASA (a known mitochondrial inhibitor) was added at different concentrations to the yeast *Dipodascopsis uninucleata* (Kock et al. 1999). This may be ascribed to the fact that these sexual cells possibly depend on mitochondria for normal development

(Marmioli et al. 1983; Codon et al. 1995). Since ascosporeogenesis requires active metabolism, sexual cells therefore need more energy for the development of large numbers of ascospores per ascus. It is expected that mitochondrial activity would increase in sexual cells when compared to the vegetative cells.

ASA contains a specific compound, salicylate, that is structurally similar to the acyl portions of the substrate and product of the 3-OH acyl-CoA dehydrogenase activity of the β -oxidation pathway (Glasgow et al. 1999), thereby inhibiting mitochondria and 3-OH oxylipin production. Inhibition by ASA may also be due to this compound uncoupling the oxidative phosphorylation and/or inhibiting electron transport (Somasundaram et al. 1997; Norman et al. 2004). This therefore explains why various yeasts have been reported to produce sexual cycles susceptible to ASA inhibition (Kock et al. 2003; Leeuw et al. 2007). These reports indicate a possible link between sexual reproduction, mitochondrial activity (needed for sexual reproduction) and 3-OH oxylipin accumulation/production (produced in mitochondria, needed for liberation of ascospores).

1.4 Purpose of research

With this information as background, it became the aim of this study to:

1. Map the distribution of 3-OH oxylipins and mitochondrial activity in autotrophic fermenting and non-fermenting yeasts.

2. Assess ASA sensitivity of these yeasts (sexual reproduction and growth).
3. Determine if a possible link between ASA addition and oxygen deprivation exists.
4. Develop a biological assay that may find application in screening for effective anti-mitochondrial antifungals.

Please note: The chapter to follow is presented in the format depicted by the journal of submission. As a result repetition of some information could not be avoided.

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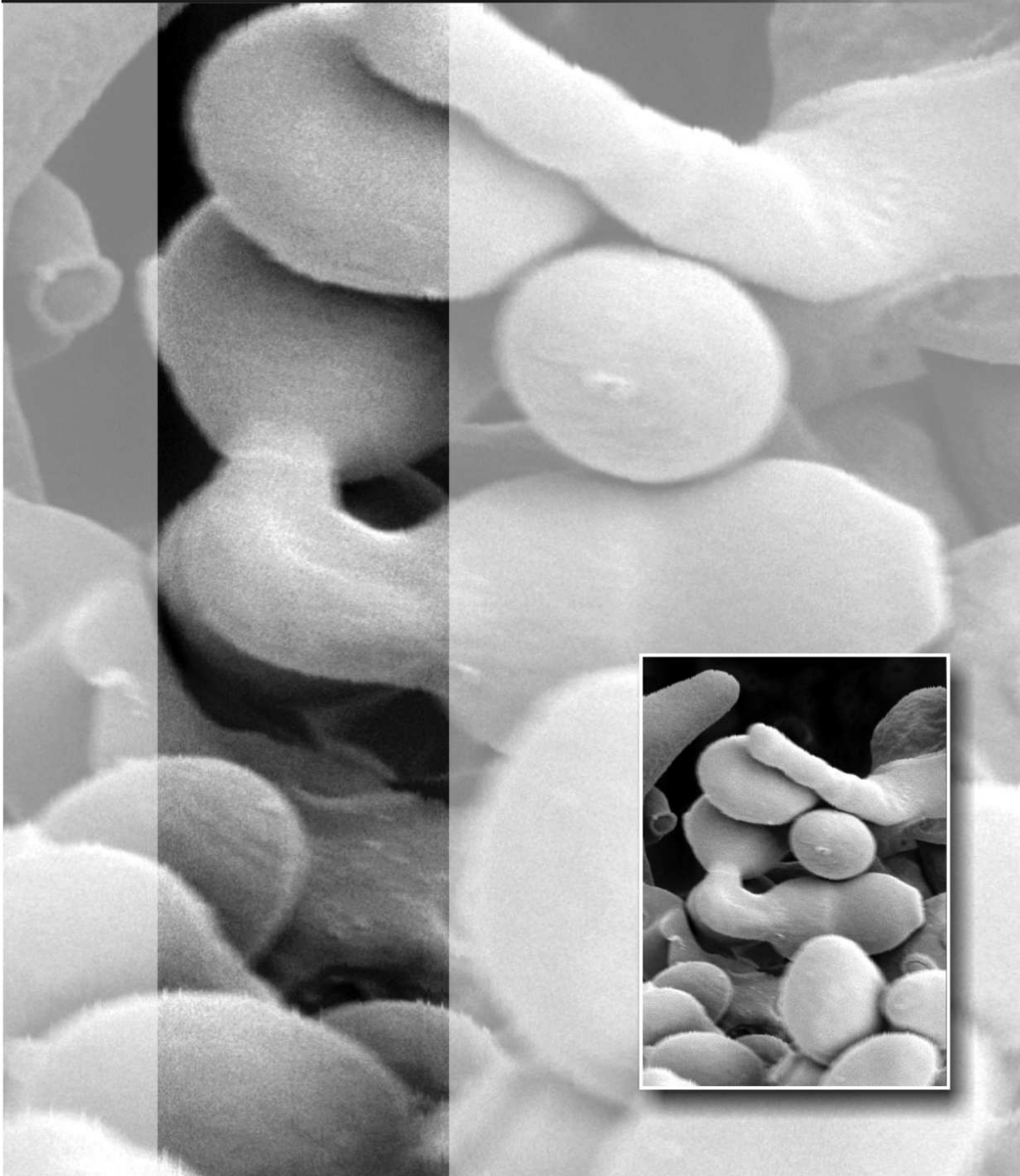
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CHAPTER 2



CHAPTER 2

**Oxylipin accumulation and acetylsalicylic acid sensitivity in
fermentative and non-fermentative yeasts**

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2.1 Abstract

3-Hydroxy (OH) oxylipins have been widely studied since their discovery in 1991 and were found to be distributed amongst various species in the fungal domain. Most studies, however, have been performed on non-fermenting yeasts, where 3-OH oxylipins were found to accumulate mainly in the sexual structures (asci). These studies also revealed that the sexual stages of non-fermenting yeasts are most susceptible to acetylsalicylic acid (ASA), a known mitochondrial (respiration and 3-OH oxylipin production) inhibitor. However, no information regarding oxylipin accumulation in asci or ASA-sensitivity of fermentative yeasts has so far been reported. Using confocal laser scanning microscopy in combination with an oxylipin probe for 3-OH oxylipins and coupled to a fluorescing secondary antibody, the accumulation of these oxylipins was discovered in the asci of the following fermentative yeasts i.e. *Pichia anomala*, *Pichia farinosa* and *Schizosaccharomyces octosporus*. Interestingly, no 3-OH oxylipin accumulation was observed in the asci of the fermenting yeast *Zygosaccharomyces bailii*. Using confocal laser scanning microscopy and a mitochondrial fluorescing probe (Rhodamine 123), an increase in mitochondrial activity was also observed in the asci of the fermenting yeasts tested, with the exception of *Z. bailii*. Furthermore during this study, links between yeast sexual reproduction, 3-OH oxylipin accumulation/production, mitochondrial activity and oxygen requirement were established. This study revealed that fermenting yeasts are more resistant to ASA than non-fermenting yeasts when grown in liquid media. This is probably due to the fact that these yeasts can use either aerobic respiration or a fermentative pathway for growth and reproduction. This

research prompted the development of a bio-assay that may find application in screening for effective antimitochondrial antifungals.

Keywords: Antifungals, Asci, Mitochondria, 3-Hydroxy oxylipins, Yeast.

2.2 Introduction

In the early 1990s, 3-hydroxy (OH) fatty acids and other oxylipins were discovered in the yeast genus *Dipodascopsis* (Kock et al. 1991; Van Dyk et al. 1991). Since then, a wide variety of 3-OH oxylipins were uncovered in various yeast species. These compounds were found to be associated specifically with the sexual structures i.e. asci and ascospores (Kock et al. 2003) and was also discovered on the vegetative cell surfaces of certain yeasts, i.e. *Saccharomyces cerevisiae*, where they play a possible role in flocculation.

3-OH oxylipins are saturated and unsaturated oxidized fatty acids which are produced in the mitochondria via incomplete β -oxidation or fatty acid synthesis type II (Hiltunen et al. 2005; Kock et al. 2007). Literature suggests that 3-OH oxylipins play an important role in the sexual stage of yeast by assisting ascospore release from asci (Kock et al. 2004). It is therefore not surprising that oxylipins accumulate in these structures. Studies done so far, however, focused mainly on oxylipin accumulation in non-fermenting yeasts (Kock et al. 2003, 2007; Leeuw et al. 2007).

When acetylsalicylic acid (ASA), a known mitochondrial (respiration) inhibitor, was added in increased concentrations to the yeast *Dipodascopsis uninucleata*, a dose dependent decrease in 3-OH oxylipin production as well as ascospore release was observed (Van Dyk et al. 1991; Botha et al. 1992). Here ascospore release was the most sensitive part of the yeast life cycle towards ASA (Kock et al. 1999). Consequently

it is suggested that 3-OH oxylipins, coating ascospores, may have a lubricating function in ascospore release from asci (Kock et al. 2003, 2007).

Since it is known that 3-OH oxylipins are produced in the mitochondria, we should also expect increased mitochondrial activity in asci. Is this true in both fermenting and non-fermenting yeasts? What is the effect of ASA on growth and sexual reproduction of these yeasts? Consequently, this study aims (1) to map the distribution of 3-OH oxylipins and mitochondrial activity in the different reproductive phases of various fermenting and non-fermenting yeasts, (2) to assess the sensitivity of these yeasts to ASA, with regards to growth and sexual reproduction, (3) to determine if a link between ASA addition and oxygen deprivation (both mitochondrial inhibitors) exists and (4) to develop a biological assay that may find application in screening for effective anti-mitochondrial antifungals.

2.3 Materials and Methods

2.3.1 Strains used and cultivation

The following yeasts were used in this study: *Galactomyces reessii* (UOFS Y-1120 T), *Lipomyces starkeyi* (UOFS Y-1999 T), *Pichia anomala* (UOFS Y-0157), *Pichia farinosa* (UOFS Y-0872), *Schizosaccharomyces octosporus* (UOFS Y-1715) and *Zygosaccharomyces bailii* (UOFS Y-1865). These strains are preserved in the culture collection of the University of the Free State (UFS), Bloemfontein, South Africa. The

various yeasts were grown on yeast-malt (YM) agar (Wickerham 1951) at 25 °C, ranging from 3 days to 14 days, until sporulation was observed using a light microscope (Axioplan, Zeiss, Göttingen, Germany) coupled to a Colourview Soft Digital Imaging System (Münster, Germany). The cells were then subjected to the following experimental methods:

2.3.2 Ultrastructure

2.3.2.1 Scanning electron microscopy (SEM): Sporulating yeasts cells were collected from agar plates and fixed immediately with 3 % sodium buffered (0.1 M, pH 7.0) glutardialdehyde (Merck, Darmstadt, Germany) for a period of 3 h (Van Wyk and Wingfield 1991). The suspension was then rinsed once by centrifugation with the same buffer to remove excess aldehyde fixative. Post-fixation was performed with 1 % aqueous osmium tetroxide (Merck, Darmstadt, Germany) in a similar buffer solution. The suspension was rinsed twice by centrifugation to remove excess osmium tetroxide and then dehydrated by using a graded ethanol sequence (50 %, 70 %, 95 % and 100 % x2 for 30 min per step). Centrifugation took place between each dehydration step. Drying was performed by using a critical point dryer. The specimens were mounted on stubs and coated with gold to make them electron conductive. These were then viewed with a scanning electron microscope (Jeol 6400 WINSEM, Jeol, Tokyo, Japan).

2.3.2.2 Transmission electron microscopy (TEM): This was performed on sporulating *P. anomala* and *Z. bailii* cells. This material was chemically fixed with 1.0 M (pH 7)

sodium phosphate-buffered glutardialdehyde (3 %) for 3 h and then for 1.5 h in similarly buffered osmium tetroxide (Van Wyk and Wingfield 1991). These fixed cells were then embedded in epoxy resin and polymerized at 70 °C for 8 h (Spurr 1969). An LKB III Ultratome was used to cut 60 nm sections with glass knives. Uranyl acetate (Merck, Darmstadt, Germany) was used to stain these sections for 10 min, followed by lead citrate (Merck) (Reynolds 1963) for 10 min. The preparation was viewed with a Philips 100 transmission electron microscope (Eindhoven, The Netherlands).

2.3.3 3-OH oxylipin mapping and mitochondrial activity

2.3.3.1 Immunofluorescence microscopy

3-OH oxylipin staining: This was performed according to Kock et al. (1998). Sporulating yeasts cells were treated with a primary antibody specific for 3-OH oxylipins (30 µl for 1 h in the dark at room temperature). Cells were then washed with phosphate buffered saline (PBS) to remove unbound antibodies and further treated with a fluorescein isothiocyanate (FITC) conjugated secondary antibody (Sigma-Aldrich, U.S.A.), specific for the primary antibody, (30 µl for 1 h in the dark at room temperature). The cells were then washed again with PBS to remove the unbound secondary antibodies. Staining was executed in 2 ml plastic tubes in order to maintain cell structure. After washing, the cells were fixed in Dabco (Sigma-Aldrich) on a microscope slide and viewed using a Nikon TE 2000, confocal laser scanning microscope (Japan).

Mitochondrial staining: Sporulating cells were washed with PBS in a 2 ml plastic tube to get rid of agar and debris and then treated with Rhodamine 123 (31 µl per sample), a mitochondrial stain (Molecular Probes, Invitrogen Detection Technologies, Eugene, Oregon, U.S.A.), for 1 h in the dark at room temperature. Cells were washed again with PBS to remove excess stain and fixed on microscope slides in Dabco (Sigma-Aldrich). Cells were then viewed with a confocal laser scanning microscope (Nikon TE 2000, Japan).

2.3.4 Ascospore staining

Ascospores and vegetative cells were selectively stained with malachite green and safranin (Yarrow 1998).

2.3.5 Oxygen inhibition studies

Cells were scraped from YM agar plates and suspended in sterilized distilled water. A homogenous lawn was then spread out onto YM agar plates containing 1.6 % (m/v) agar. Two sterilized cover slips were placed on the plate to create an anoxic environment (Yarrow 1998). Cells were grown for 14 days until growth was observed. Cells from the plate and also beneath the cover slip (if growth occurred) were scraped off and viewed under the light microscope for growth and sporulation (Table 1).

2.3.6 ASA inhibition studies

Inhibition of growth and sexual phases on solid media: Sporulating cells were scraped from plates and suspended in sterilized distilled water. This suspension was spread out on soft YM agar plates containing 0.5 % (m/v) agar to form a homogenous lawn. Each plate contained one of the following concentrations of ASA (Sigma, Steinheim, Germany) i.e. 5 mM and 10 mM. A control containing 0 mM of ASA was also inoculated. Ethanol (EtOH) controls containing equivalent amounts of ethanol (Merck, Gauteng, South Africa) as used in the 5 mM and 10 mM ASA plates were also inoculated. These plates were prepared by first dissolving the ASA in a minimum volume of ethanol and then mixed with the YM-media to reach the appropriate concentrations. Liquid media were not used since no sporulation could be induced in some yeasts studied. These plates were then incubated at 25 ° C for 14 days until a homogenous lawn could be observed on the surface of the agar plate. Solid YM agar plates containing 1.6 % (m/v) agar could not be used since ASA (dissolved in ethanol) was unable to dissolve in this medium. Cells on four different areas of the plate were scraped of and placed in a drop of distilled water (dH₂O) on a microscope slide and viewed with the light microscope to determine growth and sporulation (Table 3).

Inhibition of growth in liquid media: Yeasts in Table 2 were cultivated in glucose containing liquid medium in test tubes, while agitating on a Rollordrum according to assimilation tests in liquid medium protocol (Yarrow 1998). The liquid medium contained 6.7 g.L⁻¹ Yeast Nitrogen Base (Difco, Becton, Dickinson and Company, MD) and 2 %

(w/v) glucose. Each set (for each organism) contained 8 tubes with different ASA concentrations dissolved in 98 % ethanol (Table 2). Tube 1: no ASA (control), tube 2: 1 mM ASA (dissolved in 11.3 µl EtOH), tube 3: 2 mM ASA (22.5 µl EtOH), tube 4: 3 mM ASA (dissolved in 33.8 µl EtOH), tube 5: 4 mM ASA (dissolved in 45 µl EtOH), tube 6: 5 mM ASA (dissolved in 56.3 µl EtOH), tube 7: 56.3 µl EtOH (ethanol control), tube 8: no inoculum (negative control), only medium and ASA (dissolved in ethanol). Cells were cultivated at 25 °C for 4 days. After this period growth was measured as prescribed (Yarrow 1998). Here +++ (no black lines visible) indicates good growth, ++ (black lines just visible) indicates growth and + (black lines similar to that of inoculated tube at start of growth) indicates no or weak growth (Leeuw et al. 2007).

2.3.7 Biological assay

Cells of *L. starkeyi* were scraped from YM plates and spread out on soft agar plates (0.5 % m/v agar) containing different amounts of ASA dissolved in ethanol (Table 4). Ethanol controls were also included where equal amounts of ethanol only (same volume added together with dissolved ASA) were added to the cultures. Cells were grown at 25 °C for 14 days until sporulation occurred. The plates were studied for sporulation using light microscopy and visually inspected for brown coloration of cultures due to the formation of amber-colored ascospores.

2.4 Results and Discussion

2.4.1 Morphology, oxylipin- and mitochondrial mapping

Morphology: Electron- as well as light micrographs of selectively stained yeast cells are shown in Figs 1-6. This is in accordance to that reported in literature (Kurtzman and Fell 1998). In Fig. 1(a) thick walled asci as well as characteristic arthrospores and hyphae of *G. reessii* is shown. From the light micrograph it is clear that each sub-spherical ascus contains only one broadly ellipsoidal ascospore which stained blue with malachite green/safranin (Fig. 1b). Scanning electron microscopy (Fig. 1c) confirms these structures, indicating arthrospores, hyphae and sub-spherical asci.

In addition, characteristic sac-like to elongated asci (with several ascospores) can be observed in Fig. 2(a) for *L. starkeyi*. Interestingly, this micrograph shows ascospore release from asci (Fig. 2a – inserts), probably in single file, as reported previously for *Dipodascopsis* (Kock et al. 1999). These ascospores stained typically blue with malachite green/safranin staining (Fig. 2b). A typical round ascus is observed in Fig. 2(c) using scanning electron microscopy.

P. anomala produces characteristic hat-shaped ascospores (Fig. 3a). Two ascospores are observed in each ascus. These ascospores also selectively stained blue during ascospore staining (Fig. 3b). A scanning electron micrograph of a typical hat-shaped ascospore, showing well demarcated brims is shown in Fig. 3(c). The bowl and brim of

these ascospores are also clearly visible in the transmission electron micrograph (Fig. 3d).

Fig. 4(a) shows a light micrograph of a persistent ascus formed by mother-daughter cell conjugation in *P. farinosa* (Yarrow 1998). Here the attached daughter cell (bud) is clearly indicated. The same is observed when the cells were stained with malachite green/safranin (Fig. 4b). In addition, such an ascus can be observed using scanning electron microscopy (Fig. 4c).

Furthermore, an elongated ascus of *S. octosporus* is shown in Fig. 5(a). In this case malachite green/safranin staining could not be performed, since this organism has a very thick ascus wall that is not easily penetrated by the respective dyes. Scanning electron microscopy depicts a typical elongated ascus (Fig. 5b).

Interestingly shaped asci (dumbbell-shaped) with a clear conjugation tube are visible in *Z. bailii* (Fig. 6a). Selective ascospore staining confirmed these results (Fig. 6b). The same dumbbell-shaped ascus was observed using scanning- and transmission electron microscopy (Fig. 6c, d).

It is interesting to note that according to literature (Kock et al. 2007) many yeasts accumulate oxylipins in their sexual structures (i.e. within asci and surrounding ascospores). These oxidized lipids assist in effective ascospore release from enclosed asci. For example, it is suggested that 3-OH oxylipins play a role in ascospore release

by lubricating ascospore surface parts to affect (1) drilling in the plant pathogen *Eremothecium sinicaudum* - using selectively oxylipin-coated tapered corkscrew ascospore tips (Bareetseng et al. 2004), (2) piercing in the plant pathogen *Eremothecium cymbalariae* - through sharp oxylipin-coated ascospore tips (Leeuw et al. 2007), (3) piercing probably through boomerang movement in the plant pathogen *Eremothecium ashbyii* (Kock et al. 2004) - using sharp ascospore tips which are stabilized by oxylipin-coated nano-scale V-shaped fins on sickle-shaped spores, and (4) by cutting in *Ascoidea corymbosa* (Ncango et al. 2006) - through razor sharp selectively oxylipin-coated brims of hat shaped ascospores. Consequently, it would be of interest to determine if 3-OH oxylipins also accumulate in the sexual structures, surrounding ascospores of the strains studied.

3-OH oxylipin- and mitochondrial mapping: According to literature, 3-OH oxylipins are produced in the mitochondria via incomplete β -oxidation or fatty acid synthesis type II (Hiltunen et al. 2005; Kock et al. 2007). Studies performed so far indicate that oxylipins accumulate in the sexual structures of yeasts to assist in ascospore release (Kock et al. 2004). Therefore we expect an increase in mitochondrial activity in the sexual structures associated with this accumulation of oxylipins. Interestingly, an increase in mitochondrial activity was observed in the sexual structures of all the studied fermenting and non-fermenting yeasts that also show oxylipin accumulation in these structures. Strikingly, no 3-OH oxylipin accumulation or, as expected, no increase in mitochondrial activity was observed in *Z. bailii*, which probably depends more on

fermentation for growth and reproduction, than mitochondrial respiration (Ludovico et al. 2001).

3-OH oxylipins were mapped using a primary antibody specific for these components and a fluorescing secondary antibody (showing green fluorescence at 520 nm) specific for the primary antibody. Using Rhodamine 123, a cationic lipophilic mitochondrial stain, mitochondrial activity could be mapped. Rhodamine 123 stains mitochondria selectively. This is attributed to the highly specific attraction of this cationic fluorescing dye to the relative high negative electric potential across the mitochondrial membrane in living cells (Johnson et al. 1980). With this dye, a high mitochondrial activity is signified by a yellow-green fluorescence (collected at 450 nm), while a low mitochondrial activity is signified by a red fluorescence collected at 625 nm (Fig. 7).

Using these probes, 3-OH oxylipins are observed surrounding the single ascospores of *G. reessii* (Fig. 7a). Here the oxylipins are situated between the ascospore and the ascus wall. The surrounding arthrospores did not show a high affinity for the antibody used, thus confirming the accumulation of oxylipins in the sexual structures. Corresponding to this accumulation of 3-OH oxylipins, an increase in mitochondria is observed in the ascus of *G. reessii* (green fluorescence). The red fluorescence indicates a low mitochondrial activity in the surrounding vegetative hyphal cells and arthrospores (Fig. 7b).

The characteristic sac-like to elongated asci of *L. starkeyi* also contain increased amounts of oxylipins (green fluorescence) that surround the ascospores not yet

released (Fig. 7c). Again an increase in mitochondrial activity was observed in these asci, associated especially with the ascospores (Fig. 7d). In both oxylipin- and mitochondrial mapping, the attached and surrounding vegetative cells (not shown in Fig. 7d) showed a low affinity for the respective dyes used, thus indicating low mitochondrial activity and no accumulation of oxylipins in these cells. Fig. 7(e) depicts oxylipin-coated hat-shaped ascospores characteristic of *P. anomala*. This is in accordance with the increased mitochondrial activity found in asci of this yeast upon Rhodamine 123 staining (yellow-green fluorescence) (Fig. 7f). As expected no oxylipin accumulation occurred in the vegetative cells. Here, only red fluorescence indicating low mitochondrial activity was observed.

A typical “mother-daughter cell conjugation” – sexuality (adelphogamy) is observed in *P. farinosa* (Fig. 7g). Here oxylipins are again concentrated in the ascus (surrounding the ascospores) and also the attached daughter cell (green fluorescence), while the surrounding vegetative cells did not show this accumulation. Fig 7(h) again indicates “mother-daughter cell conjugation” - sexuality, yet only the ascus, and not the attached daughter cell or the surrounding vegetative cells, show high mitochondrial activity.

The sexual structures of *S. octosporus* (i.e. ascus and ascospores) and not the surrounding vegetative cells showed a high affinity for the 3-OH oxylipin specific antibody, again indicating accumulation of these compounds in asci (Fig. 7i). In Fig 7(j) a high mitochondrial activity can be observed in the elongated ascus of this yeast

(green fluorescence), while only low mitochondrial activity was observed in the surrounding vegetative cells.

Strikingly, *Z. bailii* deviates from the above trend as can be observed in Fig. 7(k). Here no accumulation of oxylipins in the dumbbell-shaped ascus occurred - the vegetative cells and the sexual structures show relatively equal low affinity for the oxylipin-antibody. A similar trend can be observed in Fig. 7(l) where no increased mitochondrial activity could be observed in the ascus. This may be due to the fact that this yeast depends more on a fermentative pathway for growth and sexual reproduction than aerobic respiration via mitochondria (Ludovico et al. 2001). These experiments were repeated and yielded similar results.

2.4.2 Oxygen- and acetylsalicylic acid inhibition studies

Oxygen inhibition studies: Since increased mitochondrial activity was found to be correlated with sexual reproductive structures of most yeasts studied (except *Z. bailii*), it was attempted to perform preliminary experiments to assess the possible role of mitochondria in these structures. Consequently these cells were cultivated on YM-agar medium on petri dishes where part of the cultures was covered with sterile cover slips, thereby establishing an anoxic environment (Table 1). This is based on the Dalmau Plate Technique described by Yarrow (1998).

According to Table 1, all yeasts could grow and form asci under oxic conditions. The non-fermenting yeasts (*G. reessii* and *L. starkeyi*) could not grow or produce asci under anoxic conditions. This could be expected since these yeasts only depend on aerobic mitochondrial respiration and cannot grow fermentative (fermentation negative) under anoxic conditions. Here, increased mitochondrial respiration is probably needed for producing enough energy for the development of the sexual reproductive phase (asci and ascospores). The fermenting (fermentation positive) yeasts (*P. anomala*, *P. farinosa*, *S. octosporus* and *Z. bailii*) could grow under anoxic conditions, yet no sexual reproduction occurred except in *Z. bailii* where abundant asci could be observed. This suggests that these fermenters can also use the fermentative pathway for growth. Yet, *Z. bailii* could also reproduce sexually under anoxic conditions, indicating that this yeast probably obtains enough energy through fermentative metabolism to maintain this reproductive phase. This is in accordance with the lack of mitochondrial activity and consequent lack in 3-OH oxylipin increase in the asci of *Z. bailii*. This experiment was repeated with similar results.

Acetylsalicylic acid inhibition studies: Mitochondrial activity is not only inhibited by an oxygen shortage, but also by non-steroidal anti-inflammatory drugs (NSAIDs) such as ASA (Kock et al. 2007). Strikingly, results in Table 2 show that the non-fermenting yeasts (*G. reessii* and *L. starkeyi*) which are solely dependent on mitochondrial respiration for energy are more sensitive to growth inhibition by ASA. Here good growth occurred in the absence of ASA and also in the presence of 1 mM ASA. Yet, growth was inhibited already at 2 mM and no or weak growth occurred at 3 mM to 5 mM ASA.

In contrast, those yeasts that can produce energy through both respiration as well as fermentation were, as expected, more resistant towards the mitochondrial inhibitor, ASA. Here good growth occurred in the absence as well as the presence of 1 mM to 5 mM of ASA. An ethanol control was used in all cases, where the same amount of ethanol used to dissolve 5 mM of ASA was added to these cultures. Table 2 clearly indicates that the ethanol had no effect on growth (good growth occurred in all cases).

Literature suggests that sexual reproduction in yeasts is more sensitive to ASA compared to vegetative growth (Ncango et al. 2006; Kock et al. 2007; Leeuw et al. 2007; Van Heerden et al. 2007). Table 3 indicates the effect of different concentrations of ASA on the growth and sexual reproductive cycle of the various yeasts studied. Here, cells were cultivated on agar plates containing 0.5 % (m/v) agar and different concentrations of ASA. In this case, liquid media (Table 2) were not used since, with the exception of *Z. bailii*, the yeasts did not sporulate probably due to low oxygen content. Interestingly, *Z. bailii* was the only yeast studied that produced asci at all ASA concentrations when cultivated in liquid medium (Table 2). From Table 3 it appears that the growth of the non-fermenting yeasts *G. reessii* and *L. starkeyi* are more resistant to ASA when grown on agar plates instead of liquid media in test tubes (Table 2, Leeuw et al. 2007). Here growth occurred at 5 mM and 10 mM ASA in both yeasts (Table 3), while no growth was observed in liquid medium already at 3 mM ASA (Table 2). This may be due to the poor solubility of ASA in agar medium, thereby influencing its availability to cultures. Is it possible that ASA is more inhibitory to growth of obligate

respiring yeasts when oxygen availability is restricted as is probably the case in these slow rotating test tubes containing liquid medium (Table 2)?

Strikingly, *G. reessii* could also produce asci when grown on plates with high ASA concentration (5 mM, Table 3). This is in accordance with studies of another representative of the *Dipodascaceae* i.e. *Dipodascus geniculatus* (Barnett et al. 2000). In this case the latter could also produce asci at 5 mM ASA, although no ascospores were liberated (Van Heerden et al. 2007). In addition, no malformed asci were observed at 5mM ASA compared to that found in *D. geniculatus* (Van Heerden et al. 2007). *L. starkeyi*, however could not produce asci at 5 mM or 10 mM ASA. As expected all the fermenting yeasts studied could grow and produce asci at 5 mM ASA. Growth also occurred at 10 mM ASA but no asci could be observed in fermenting as well as non-fermenting yeasts.

2.4.3 Biological assay

It is of importance to develop new, effective antifungals and antibacterial compounds, especially since multi drug-resistant bacteria and fungi, including yeasts, are responsible for many mortalities worldwide (Annane et al. 2005). The development of a rapid, safe assaying system using eukaryotic yeasts with their prokaryotically derived mitochondria as target, is a strategy that may assist in exposing novel antimitochondrial/antibacterial compounds that can be used to eventually combat yeast and Gram-negative bacterial infections. This is especially true since Gram-negative bacteria are considered ancestors of mitochondria (Gray et al. 2001).

In this proposed assay, *L. starkeyi* was used since it produces within 14 days ample distinguishable amber/brown colored ascospores that form a clearly visual brown coloration of the culture when cultivated on YM agar in petri dishes (Fig. 8). It has been reported previously (Kock et al. 2007) that asci formation in non-fermenting yeasts are dependent on increased mitochondrial activity for development – probably since especially ascospore development requires more energy. Literature suggests that the development of the sexual stage may therefore be more sensitive towards antimitochondrial compounds where more energy (mitochondrial activity) is required. Consequently, any change in coloration upon addition of potential antifungal agents, may implicate antimitochondrial activity especially if vegetative growth still occurs. This means that the sexual cycle which is mostly dependent on mitochondrial activity, is more susceptible to an antimitochondrial antifungal. This research should now be followed up to further assess specificity of this proposed antimitochondrial biological assay.

Fig. 8 (a-k) shows cells of *L. starkeyi* in a homogenous lawn on agar plates containing 0.5 % (m/v) agar (Table 4). Fig. 8(a) depicts a control with no ASA added. Here the culture turned brown, indicating that ample sporulation has occurred (i.e. formation of many amber/brown colored ascospores). After the addition of 1 mM ASA (added before cultivation, Fig. 8b) an effect on the sexual phase of this yeast could already be observed, since the brown color (indicating sporulation) was starting to fade. With the addition of 2 mM ASA (added before cultivation, Fig. 8d) the sexual phase was severely inhibited with only a slight brown color formation. From 3 mM ASA to 5 mM ASA (added

before cultivation) the sexual stage is completely inhibited since no brown color could be observed (culture creamy), therefore no sporulation occurred (Fig. 8(f) – 3 mM ASA; Fig. 8(h) – 4 mM ASA; Fig. 8(j) – 5 mM ASA). This was also confirmed with light microscopy. For each concentration of ASA dissolved in ethanol an equivalent ethanol control is shown in Fig. 8(c) – equivalent amount of ethanol used to dissolve 1 mM ASA; Fig. 8(e) – equivalent amount of ethanol used to dissolve 2 mM ASA; Fig. 8(g) – equivalent amount of ethanol used to dissolve 3 mM ASA; Fig. 8(i) – equivalent amount of ethanol used to dissolve 4 mM of ASA; Fig. 8(k) – equivalent amount of ethanol used to dissolve 5 mM ASA. Since sporulation occurs abundantly on the ethanol controls (indicated by the intense brown coloration) it can be concluded that the ethanol has no or a limited effect on sporulation. This experiment yielded similar results when repeated.

If this assay is specific for mitochondria, new antimitochondrial compounds may be easily and rapidly identified visually without using microscopy. This makes such a biological assay suitable for a first phase in high throughput screening of antimitochondrial, antifungals. Consequently, these compounds that mainly inhibit the sexual stage, may now be further studied for their antifungal and antimitochondrial activities e.g. inhibition of mitochondrial β -oxidation, FAS II, etc.

2.5 Conclusions

In this study, links between mitochondrial activity, 3-OH oxylipin production, sexual and vegetative (growth) reproduction, oxygen requirement and ASA sensitivity were

established in both fermentative and non-fermentative yeasts (Fig. 9). In general, an increase in mitochondrial activity in the sexual cells (asci) coincided with an increase in 3-OH oxylipin production in asci of both the non-fermentative (Fig. 9a) and fermentative (Fig. 9b) yeasts studied, with the exception of *Z. bailii* (Fig. 9c). This is to be expected since 3-OH oxylipins are produced through β -oxidation and/or the fatty acid synthesis type II (FAS II) pathways in mitochondria (Fig. 9d, Hiltunen et al. 2005; Kock et al. 2007). Vegetative growth of non-fermentative yeasts was also more sensitive towards oxygen depletion compared to fermentative yeasts (Table 1) probably since the former are more dependent on mitochondria (require oxygen for respiration) rather than fermentative metabolism for energy production (Fig. 9a). Linked to this, growth of non-fermentative yeasts in liquid media was more sensitive towards ASA (a mitochondrial inhibitor) compared to the fermentative yeasts again probably due to the same reason as mentioned before (Fig. 9a, b, c, Table 2).

However, growth of *G. reessii* and *L. starkeyi* appeared to be less resistant to ASA when cultivated in liquid (Table 2) compared to when grown on solid medium (Table 3). This may be ascribed to the poor solubility of ASA in solid medium and therefore decreased availability for uptake.

Strikingly, in asci of *Z. bailii* no increase in mitochondrial activity and as expected, no increase in 3-OH oxylipin accumulation was observed (Fig. 9c). In addition, the formation of asci in this yeast was not affected by oxygen depletion which suggests that this phase requires less oxygen for development compared to the other fermentative

yeasts. This may be ascribed to the fact that this yeast depends more on fermentative than respiratory metabolic pathways for energy production, needed for the development of the sexual stage (Fig. 9c).

This research prompted the development of a biological assay (Fig. 8), where yeasts, producing amber colored ascospores (*L. starkeyi*) may be used as a first visual screen for compounds with antimitochondrial activity. These selected compounds may then be subjected to more specific antimitochondrial biochemical tests. Yeast capable of energy production through a fermentative pathway (i.e. *Z. bailii*, Fig. 9c) may be included as control to assess if the selected inhibitory compounds also inhibit other processes (e.g. meiosis), during sexual reproduction. This bio-assay should also be tested with known antimitochondrial agents such as streptomycin. This antibiotic specifically interferes with 70S-ribosome function (also found in bacteria and mitochondria) thereby also inhibiting protein synthesis in mitochondria (Chang and Flaks 1972).

This bio-assay is in the process of being patented. The biological assay should also be expanded to include the agar diffusion method (for only water-soluble compounds) where known amounts of the compounds to be tested are added to for instance filter paper discs, which are then placed on the agar surface (Johnson and Case 1995). The effect of different concentrations of the compound found in the agar diffusion gradient on sporulation (brown color) can then be visually assessed. Will it be possible to select antimitochondrial compounds with selective inhibitory effects on mitochondrial function such as β -oxidation, FAS II, etc.? Will it be possible to select antimitochondrial

compounds that will also inhibit for example the production of mycolic acid (Rietschel et al. 1994; Takayama et al. 2005), a disease-causing bacterial 3-OH oxylipin, produced by the prokaryotic pathogen *Mycobacterium tuberculosis* (Korf et al. 2005)?

2.6 Acknowledgements

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2.7 References

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2.8 Figures

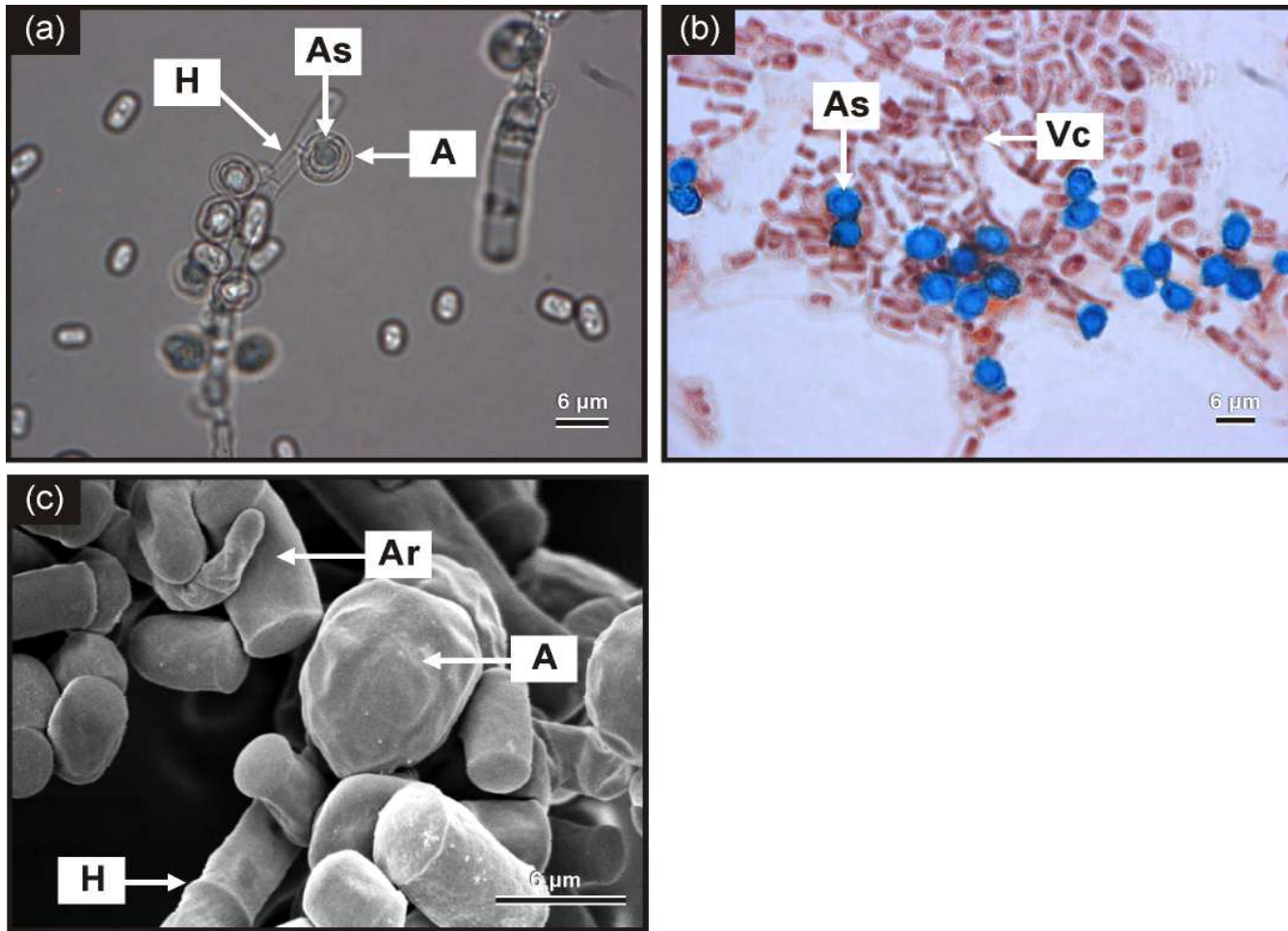


Fig. 1: Light micrograph (a) showing the hyphae (H), with ascospores (As) and an ascus (A) of *Galactomyces reessii*. In (b), ascospores (As) stained blue and vegetative cells (Vc) red with malachite green/safranin staining. Scanning electron micrograph (c) shows the ultrastructure of the ascus (A), hyphae (H) and arthrospores (Ar).

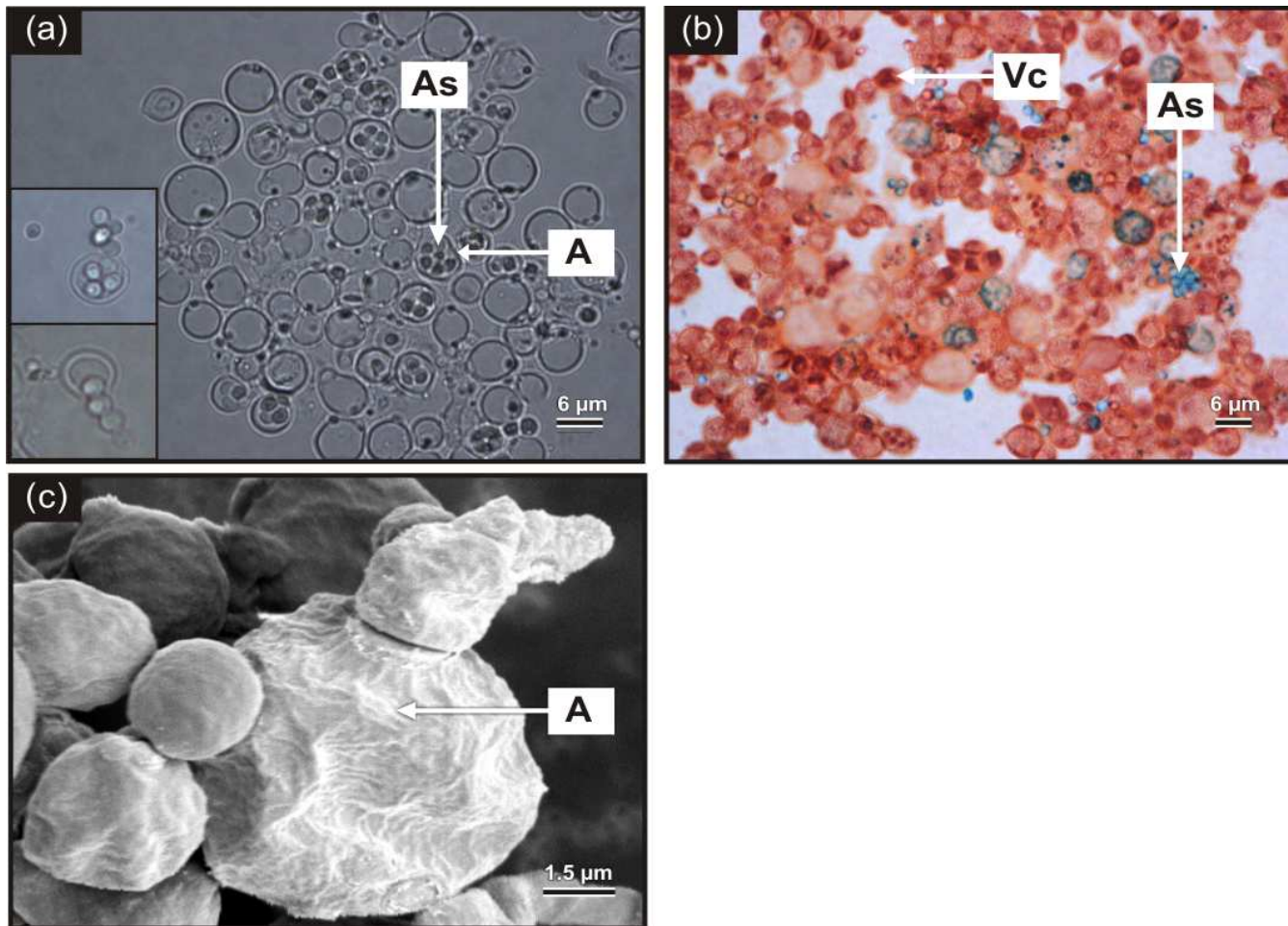


Fig. 2: Light micrograph (a) showing the ascus (A) with ascospores (As) of *Lipomyces starkeyi*. The insert in (a) shows the release of ascospores in single file from asci. Ascospore staining is indicated in (b) where the ascospores (As) stained blue and vegetative cells (Vc) red with malachite green/safranin staining. Scanning electron micrograph (c) shows the ultrastructure of the ascus (A).

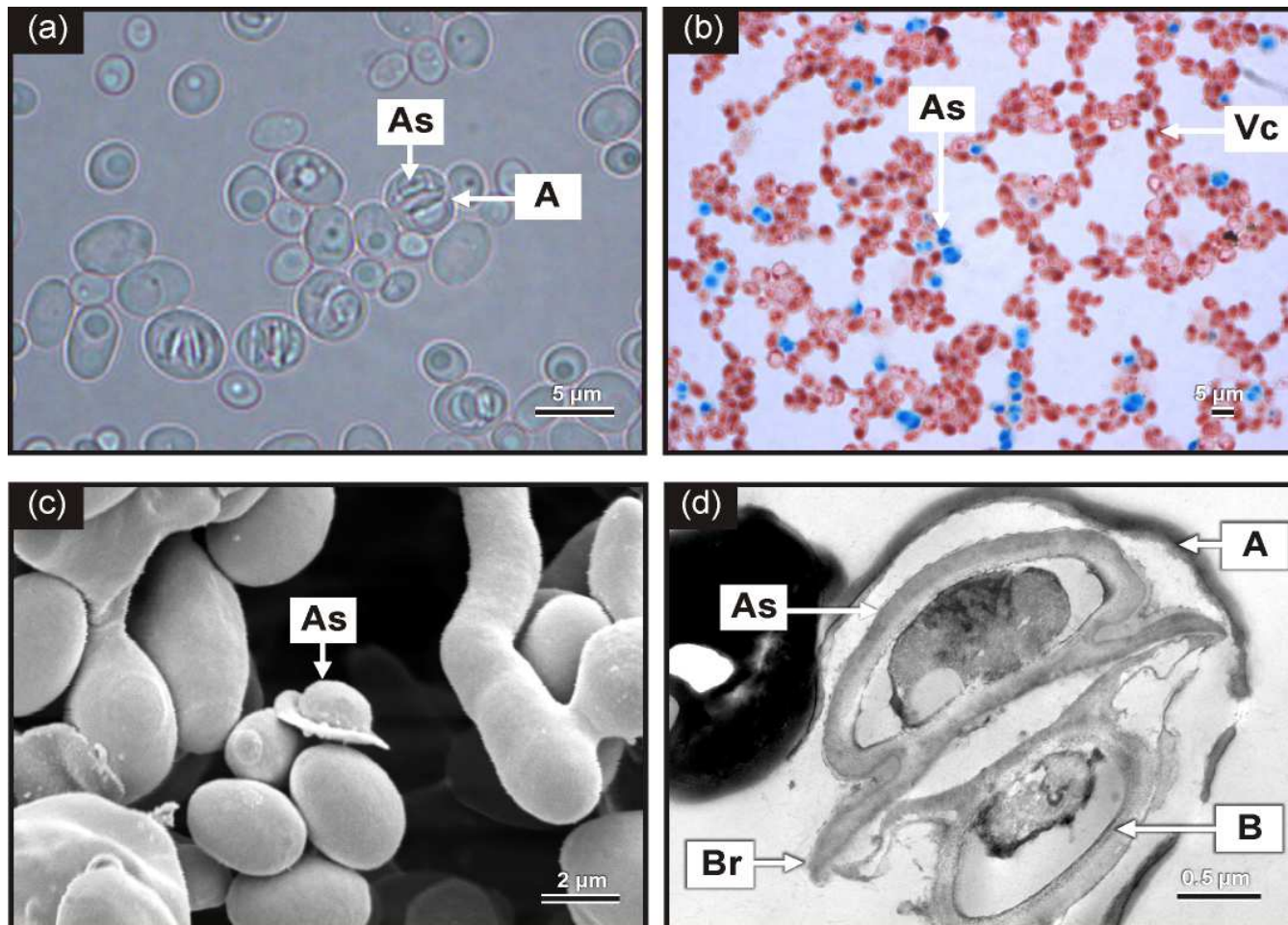


Fig. 3: Light micrograph (a) depicts hat-shaped ascospores (As) of *Pichia anomala* in asci (A). Ascospore staining is indicated in (b) where the ascospores (As) stained blue and the vegetative cells (Vc) red with malachite green/safranin staining. Scanning electron micrograph (c) shows the 3D-ultrastructure of a hat-shaped ascospore (As) while transmission electron microscopy (d) clearly shows the boile (B) and brim (Br) of the hat-shaped ascospores (As) in the ascus (A).

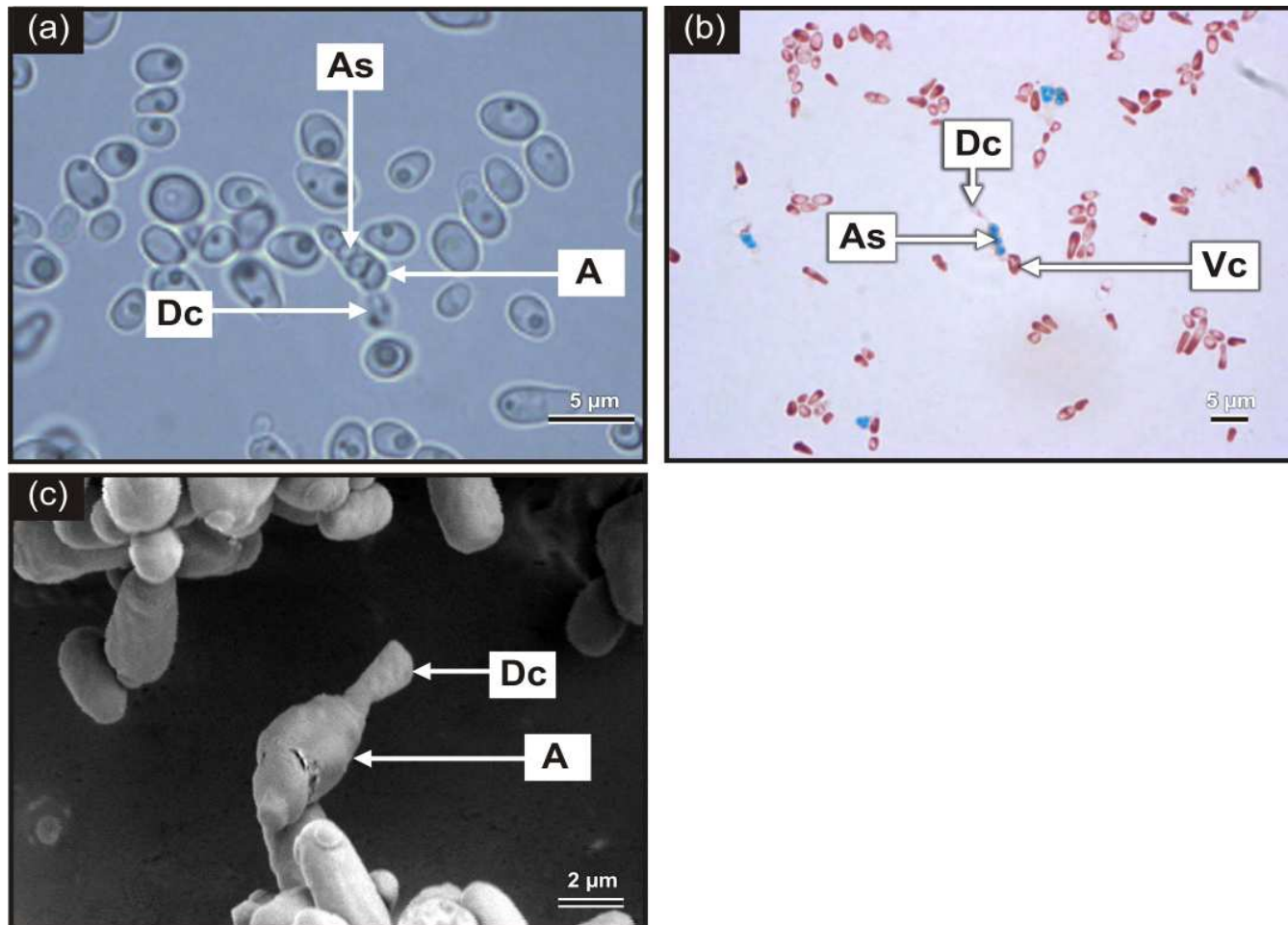


Fig. 4: Light micrograph (a) clearly indicates mother-daughter cell conjugation (adelphogamy) typical of *Pichia farinosa*. Here the daughter cell (Dc) is attached to the mother cell (ascus; A) containing several ascospores (As). Ascospore staining is indicated in (b) where the ascospores (As) stained blue and vegetative cells (Vc) red with malachite green/safranin. Scanning electron micrograph (c) shows the ultrastructure of mother-daughter cell conjugation. A, ascus; Dc, daughter cell.

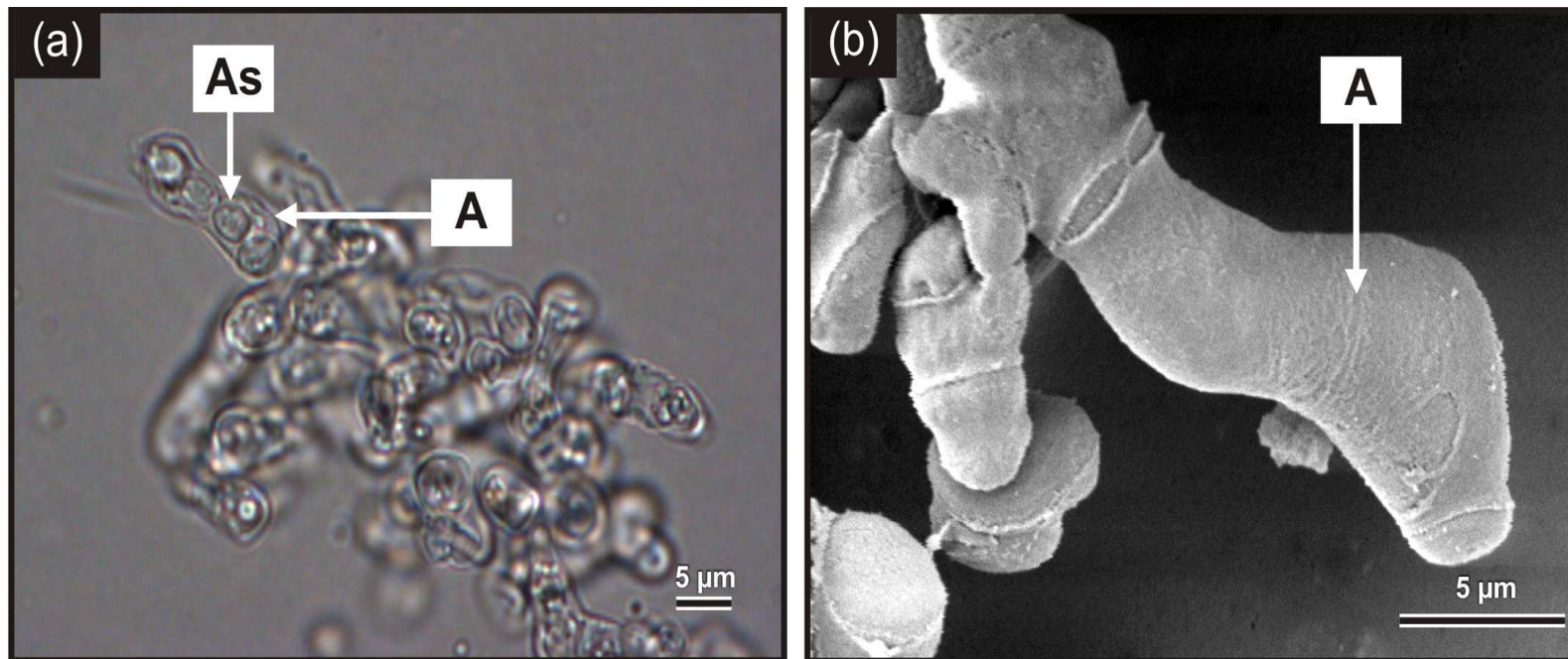


Fig. 5: Light micrograph (a) depicts an elongated ascus (A) with ascospores (As) of *Schizosaccharomyces octosporus*. Scanning electron micrograph (b) shows the ultrastructure of the ascus (A).

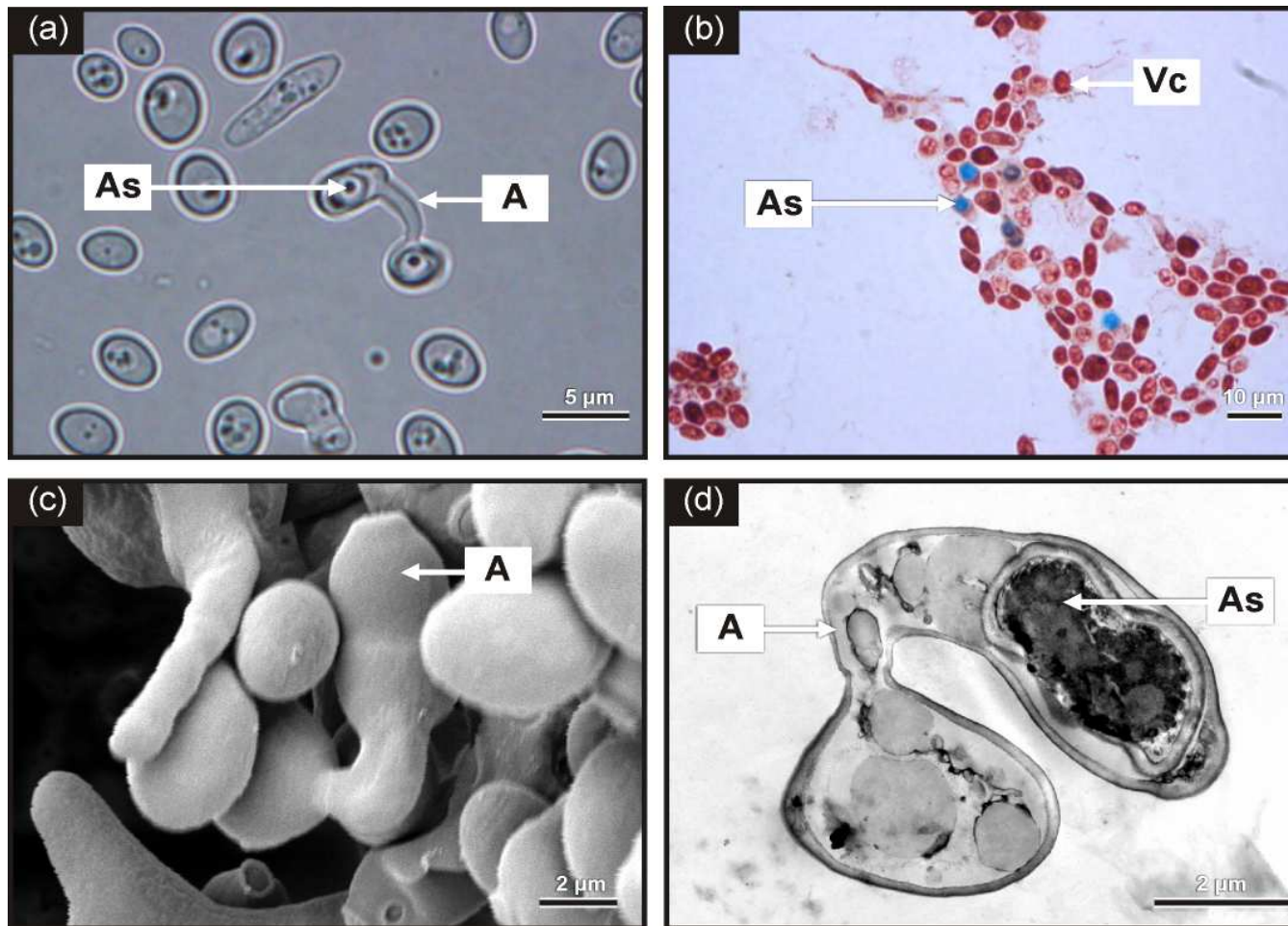


Fig. 6: Light micrograph (a) clearly indicates the interesting dumbbell-shaped ascus (A) of *Zygosaccharomyces bailii* with ascospores (As). Ascospores (As) stained blue and vegetative cells (Vc) red with malachite green/safranin (b). Scanning electron micrograph (c) shows the ultrastructure of a dumbbell-shaped ascus (A) while a transmission electron micrograph (d) depicts a cross section through a dumbbell-shaped ascus showing one ascospore (As).

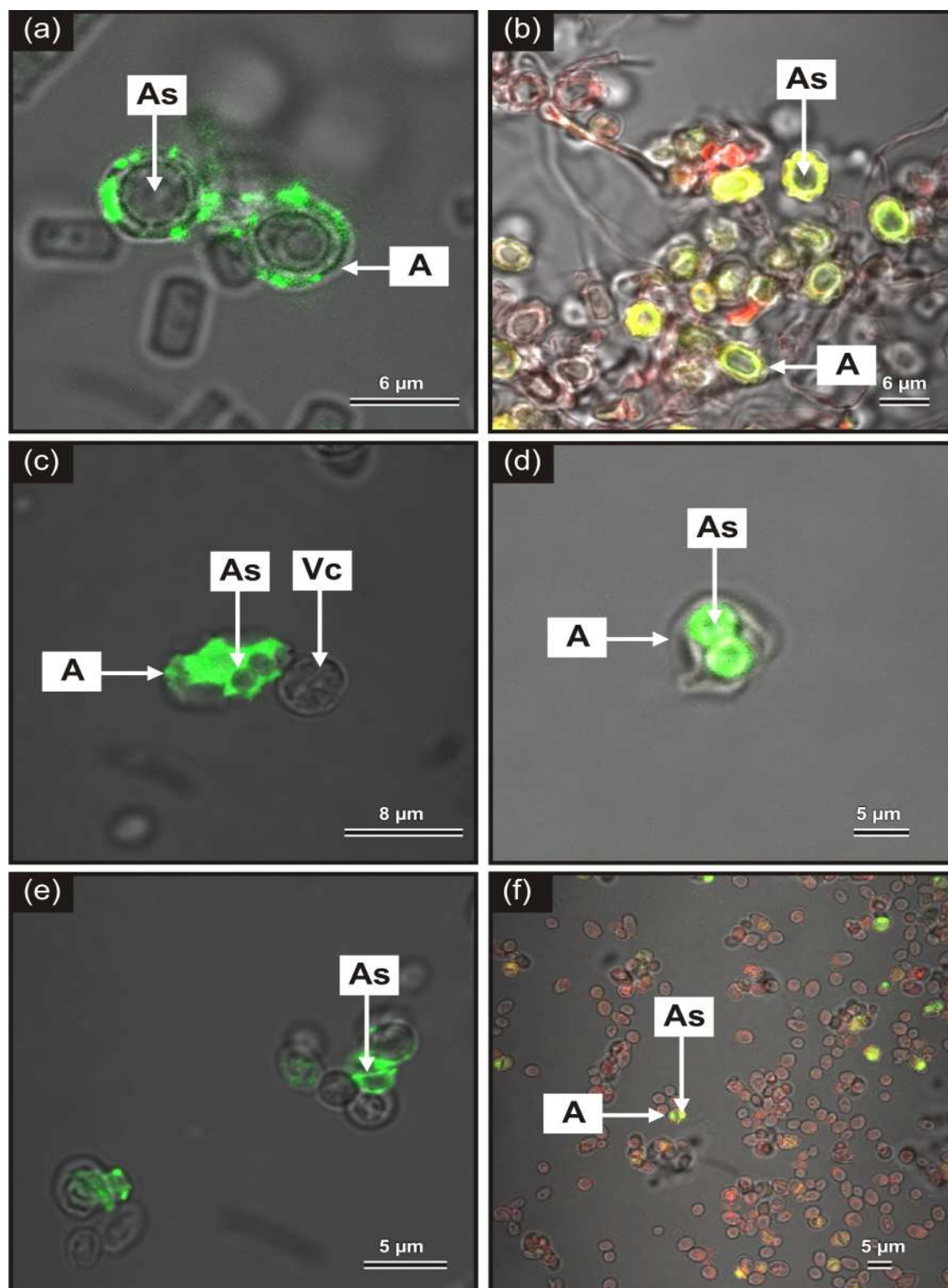


Fig. 7.

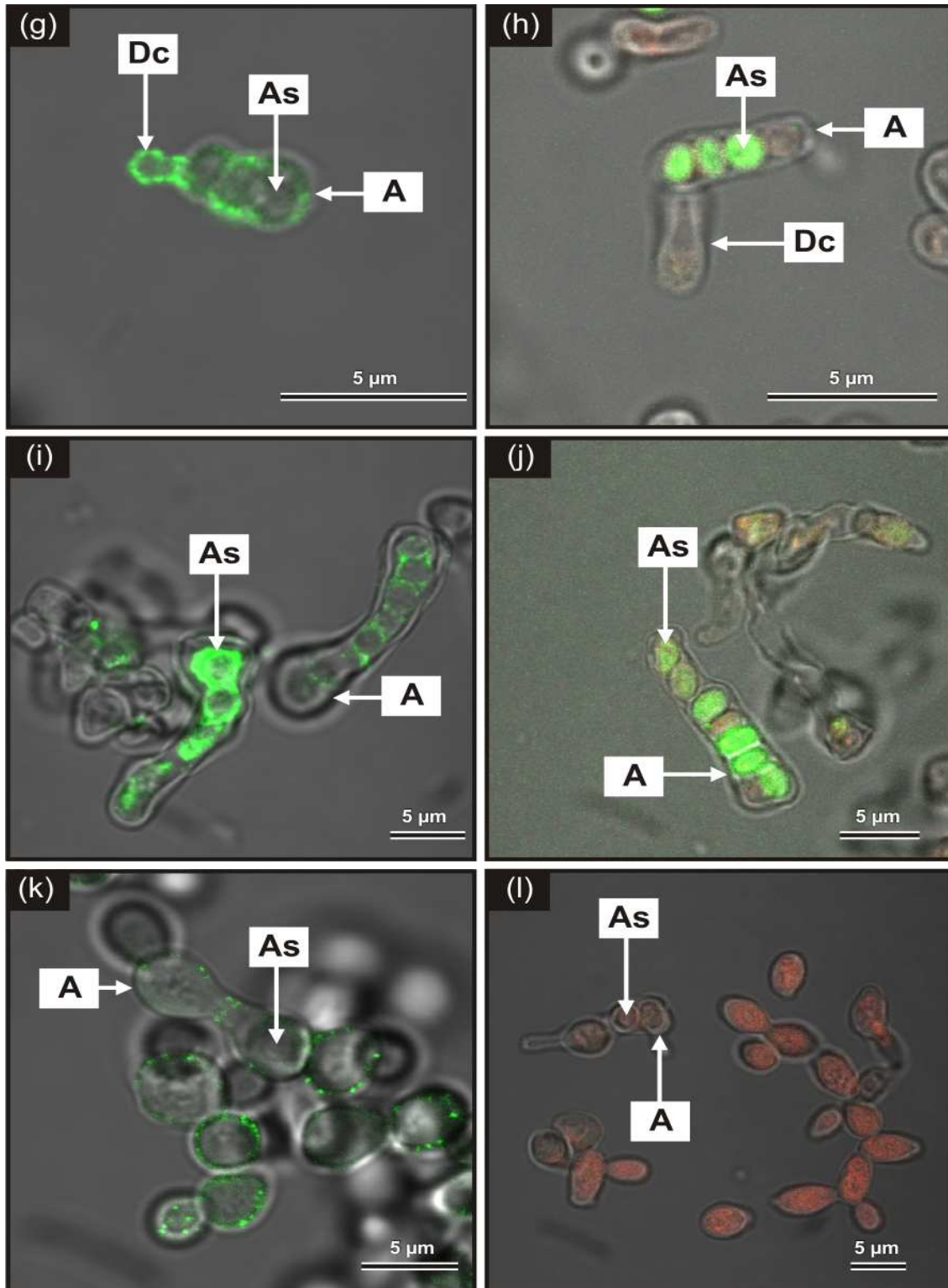


Fig. 7. (Cont.).

Fig. 7: Confocal laser scanning micrographs of different cultures treated with a primary antibody specific for 3-OH oxylipins and a secondary FITC-coupled antibody, specific for the primary antibody. The green fluorescence indicates the accumulation of 3-OH oxylipins in the sexual structures. Fig 7(a, c, e, g, i) shows that the 3-OH oxylipins (seen as the green fluorescence) are associated only with the sexual structures and not the vegetative cells of each yeast respectively. Fig 7(k), however, shows no accumulation of 3-OH oxylipins in the sexual structures. The oxylipins are relatively equally distributed between the sexual structures and the vegetative cells. (*Galactomyces reessii* (a), *Lipomyces starkeyi* (c), *Pichia anomala* (e), *Pichia farinosa* (g), *Schizosaccharomyces octosporus* (i) and *Zygosaccharomyces bailii* (k)). Confocal laser scanning micrographs of the different yeasts stained with Rhodamine 123 (a cationic, lipophilic dye, selectively accumulating in the active mitochondria) are depicted in Fig. 7 (b, d, f, h, j, l). The green fluorescence indicates a higher mitochondrial activity than reddish stained cells. Fig. 7(b, d, f, h, j) clearly indicates a higher mitochondrial activity in the sexual structures compared to vegetative cells. In *Zygosaccharomyces bailii* (Fig. 7l) no increased mitochondrial activity was found in the sexual structures. Mitochondria are relatively equally distributed between the sexual structures and the vegetative cells. (*Galactomyces reessii* (b), *Lipomyces starkeyi* (d), *Pichia anomala* (f), *Pichia farinosa* (h), *Schizosaccharomyces octosporus* (j) and *Zygosaccharomyces bailii* (l)). A, ascus; As, Ascospores; Dc, Daughter cell; Vc, Vegetative cell.

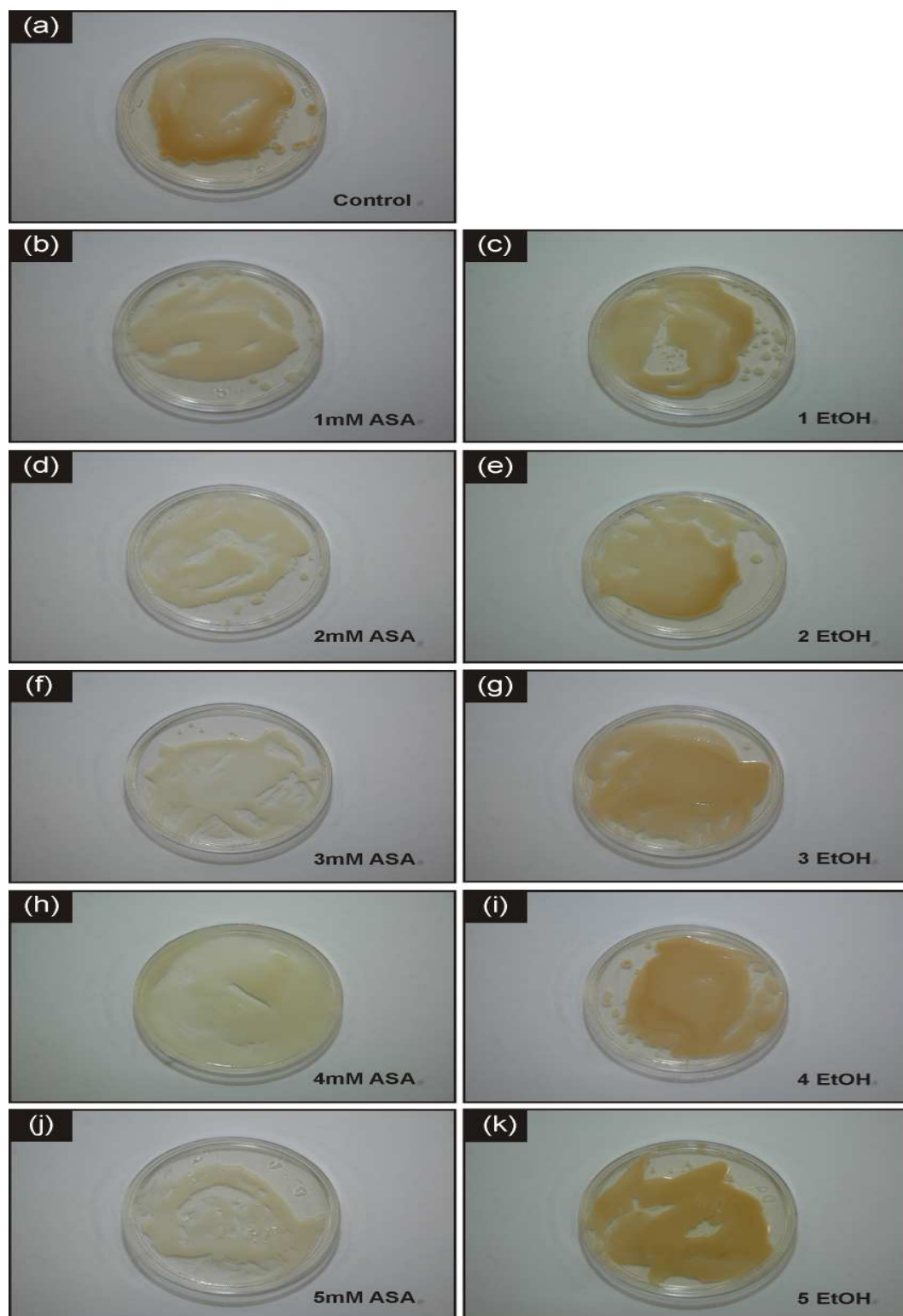


Fig. 8.

Fig. 8: Photographs of agar plates with different concentrations of acetylsalicylic acid (ASA) showing a homogenous lawn of *Lipomyces starkeyi* cells. (a), a control with brown lawn – brown color indicates ample sporulation due to the presence of many amber ascospores. Different ASA concentrations are added to these plates: (b) – 1mM ASA, (d) – 2mM ASA, (f) – 3mM ASA, (h) – 4mM ASA, (j) – 5mM ASA. Ethanol (EtOH) controls were also included: (c) – equivalent amount of ethanol used to dissolve 1mM ASA, (e) – equivalent amount of ethanol used to dissolve 2mM ASA, (g) – equivalent amount of ethanol used to dissolve 3mM ASA, (i) – equivalent amount of ethanol used to dissolve 4mM ASA, (k) – equivalent amount of ethanol used to dissolve 5mM ASA.

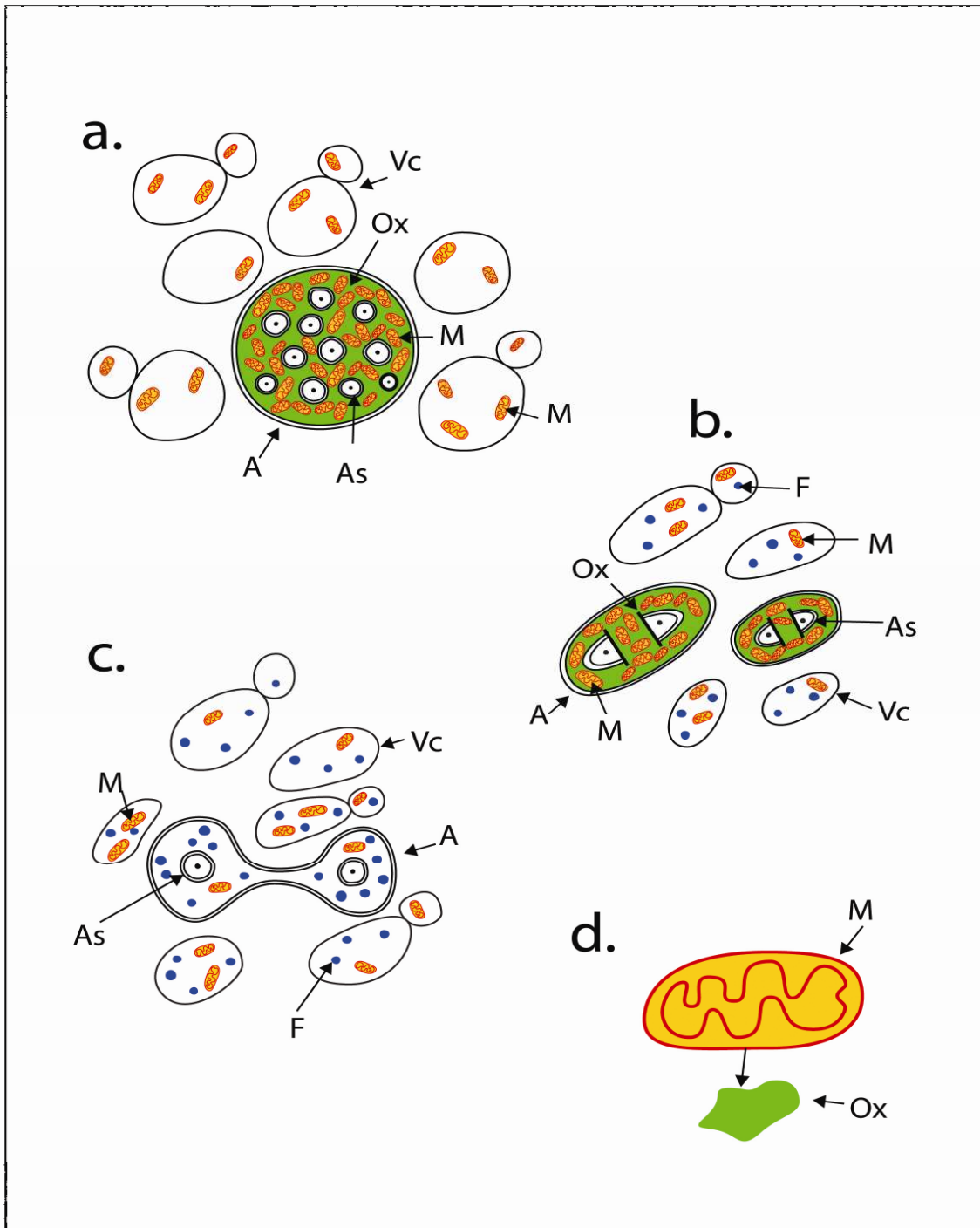


Fig. 9.

Fig. 9: A line diagram summarizing results of vegetative (Vc) and sexual (A, asci) cells (with ascospores, As) with high and low levels of mitochondrial activity (M, red), concomitant oxylipin (Ox) excretion (green) as well as ability to ferment (F, blue dots). (a), yeasts (e.g. *Lipomyces starkeyi*) that generate energy only through mitochondrial aerobic respiration. (b) and (c), yeasts (e.g. *Pichia anomala* and *Zygosaccharomyces bailii*) that generate energy through both mitochondrial aerobic respiration as well as fermentative metabolism. (d), active mitochondrion (M) excreting 3-hydroxy oxylipins (Ox).

2.9 Tables

Table 1. Growth and asci formation by fermenting and non-fermenting yeasts under oxic and anoxic conditions.

Yeast	Growth		Asci formation		Ferm.
	Oxic	Anoxic	Oxic	Anoxic	
<u>Non-fermentative</u>					
<i>G. reessii</i>	+	-	+	-	-
<i>L. starkeyi</i>	+	-	+	-	-
<u>Fermentative</u>					
<i>P. anomala</i>	+	+	+	-	+
<i>P. farinosa</i>	+	+	+	-	+
<i>S. octosporus</i>	+	+	+	-	+
<i>Z. bailii</i>	+	+	+	+	+

- = no growth/asci formation/fermentation; + = growth/asci formation/fermentation; oxic = culture without cover slip; anoxic = culture with cover slip; Ferm. = Fermentation G. = *Galactomyces*; L. = *Lipomyces*; P. = *Pichia*; S. = *Schizosaccharomyces*; Z. = *Zygosaccharomyces*.

Table 2. Growth inhibition of fermenting and non-fermenting yeasts by acetylsalicylic acid (ASA) when cultivated in liquid media.

Yeast	Growth							Ferm.	
	ASA concentration range (mM)								
	0	1	2	3	4	5	EtOH	-*	
<u>Non-fermentative</u>									
<i>G. reessii</i>	+++	+++	++	+	+	+	+++	-	-
<i>L. starkeyi</i>	+++	+++	++	+	+	+	+++	-	-
<u>Fermentative</u>									
<i>P. anomala</i>	+++	+++	+++	+++	+++	+++	+++	-	+
<i>P. farinosa</i>	+++	+++	+++	+++	+++	+++	+++	-	+
<i>S. octosporus</i>	+++	+++	+++	+++	+++	+++	+++	-	+
<i>Z. bailii</i> **	+++	+++	+++	+++	+++	+++	+++	-	+

+++ = good growth = ++, growth; + = no or weak growth; EtOH = ethanol control; Ferm. = Fermentation; - = no fermentation; -* = negative control (containing only media and ASA (dissolved in ethanol)); *G.* = *Galactomyces*; *L.* = *Lipomyces*; *P.* = *Pichia*; *S.* = *Schizosaccharomyces*; *Z.* = *Zygosaccharomyces*.

** *Z. bailii* was the only yeast in this study that could produce asci at all ASA concentrations.

Table 3. Asci formation and growth of fermenting and non-fermenting yeasts on solid media in the presence of 0 mM, 5 mM and 10 mM acetylsalicylic acid (ASA).

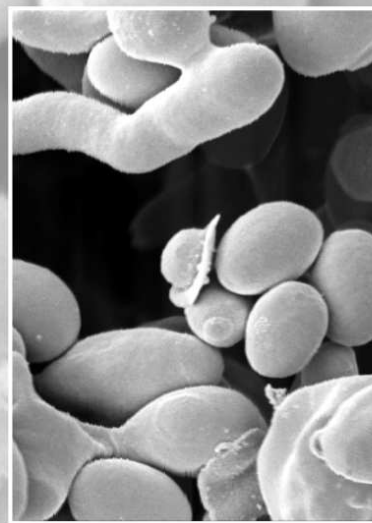
Yeast	ASA concentration range (mM)						EtOH	Ferm.
	0		5		10			
	Asci	Growth	Asci	Growth	Asci	Growth		
<u>Non-fermentative</u>								
<i>G. reessii</i>	+	+	+	+	-	+	+	-
<i>L. starkeyi</i>	+	+	-	+	-	+	+	-
<u>Fermentative</u>								
<i>P. anomala</i>	+	+	+	+	-	+	+	+
<i>P. farinosa</i>	+	+	+	+	-	+	+	+
<i>S. octosporus</i>	+	+	+	+	-	+	+	+
<i>Z. bailii</i>	+	+	+	+	-	+	+	+

- = no growth/asci formation/fermentation; + = good growth/asci formation/fermentation; EtOH = ethanol control; Ferm. = Fermentation; *G.* = *Galactomyces*; *L.* = *Lipomyces*; *P.* = *Pichia*; *S.* = *Schizosaccharomyces*; *Z.* = *Zygosaccharomyces*.

Table 4. Acetylsalicylic acid (ASA) concentrations used in the biological assay (see Fig. 8).

Plate	Contents
(a)	Positive control (no ASA)
(b)	1 mM ASA
(c)	Equivalent amount of EtOH used to dissolve 1 mM ASA
(d)	2 mM ASA
(e)	Equivalent amount of EtOH used to dissolve 2 mM ASA
(f)	3 mM ASA
(g)	Equivalent amount of EtOH used to dissolve 3 mM ASA
(h)	4 mM ASA
(i)	Equivalent amount of EtOH used to dissolve 4 mM ASA
(j)	5 mM ASA
(k)	Equivalent amount of EtOH used to dissolve 5 mM ASA

SUMMARY *OPSOMMING*



Summary

3-OH oxylipins are saturated and unsaturated oxidized fatty acids which are produced in mitochondria via incomplete β -oxidation or fatty acid synthesis type II. These compounds possibly play an important role in the sexual cycle of yeasts by assisting with ascospore liberation. Literature suggests that 3-OH oxylipins act as a lubricant during ascospore liberation, thereby ensuring efficient release of ascospores.

Since the discovery of oxylipins i.e. 3-hydroxy (OH) oxylipins in the early 1990's, these compounds were found to be distributed in various species of the fungal domain. Studies performed thus far, however, focused mainly on non-fermenting yeast species such as *Ascoidea*, *Dipodascopsis* and *Eremothecium*.

According to various studies performed so far, 3-OH oxylipins were found to accumulate specifically in the sexual structures (asci and surrounding ascospores) of various non-fermenting yeasts. These studies also revealed that the sexual stages of non-fermenting yeasts are most susceptible to acetylsalicylic acid (ASA), a known mitochondrial (respiration and 3-OH oxylipin production) inhibitor.

No information regarding oxylipin accumulation in asci or ASA-sensitivity of fermentative yeasts, however, has so far been reported. Using confocal laser scanning microscopy in combination with an oxylipin probe for 3-OH oxylipins and coupled to a fluorescing secondary antibody, the accumulation of these oxylipins was discovered in the asci of

the following fermentative yeasts i.e. *Pichia anomala*, *Pichia farinosa* and *Schizosaccharomyces octosporus*. Interestingly, no 3-OH oxylipin accumulation was observed in the asci of the fermenting yeast *Zygosaccharomyces bailii*. This could be ascribed to the fact that this yeast depends more on a fermentative pathway for growth and sexual reproduction, than on mitochondrial respiration.

Since 3-OH oxylipins are produced in the mitochondria, it is expected that there should be an increase in mitochondrial activity associated with these sexual structures. Using confocal laser scanning microscopy and a mitochondrial fluorescing probe (Rhodamine 123), an increase in mitochondrial activity was also observed in the asci of the fermenting yeasts tested, again with the exception of *Z. bailii*.

Furthermore, during this study links between yeast sexual reproduction, 3-OH oxylipin accumulation/production, mitochondrial activity and oxygen requirement were established. This study revealed that fermenting yeasts are more resistant to ASA than non-fermenting yeasts when grown in liquid media. This is probably due to the fact that these yeasts can use either aerobic respiration or a fermentative pathway for growth and reproduction. This research prompted the development of a bio-assay that may find application in screening for effective antimitochondrial antifungals.

Keywords: Acetylsalicylic acid, ascospore release, ascus, confocal laser scanning microscopy, fermentation, mitochondrial activity, mitochondrial respiration, rhodamine 123, 3-hydroxy oxylipins, yeast.

Opsomming

3-OH oksilipiene is versadigde en onversadigde geoksideerde vetsure wat in die mitochondria geproduseer word via β -oksidasie of vetsuur sintese tipe II. Hierdie komponente speel moontlik 'n belangrike rol in die seksuele fase van giste deur te help met die vrystelling van askospore. Literatuur stel voor dat 3-OH oksilipiene dien as 'n smeermiddel tydens askosporovrystelling en verseker dus effektiewe verspreiding.

Sedert die ontdekking van oksilipiene nl. 3-hidroksie (OH) oksilipiene in die vroeë 1990's, is hierdie verbindings in verskeie fungus spesies gevind. Verskeie studies alreeds uitgevoer, fokus meestal op nie-fermenterende gisspesies soos byvoorbeeld *Ascoidea*, *Dipodascopsis* en *Eremothecium*.

Na aanleiding van verskeie studies is gevind dat 3-OH oksilipiene in spesifiek die seksuele strukture (askus en askospore) van verskeie nie-fermenterende giste akkumuleer. Hierdie studies het ook onthul dat die seksuele fase van nie-fermenterende giste meer vatbaar is vir asetielsalisiensuur (ASA), 'n bekende mitochondriale (respirasie en 3-OH oksilipien produksie) inhibeerder.

Geen inligting rakende oksilipienakkumulasie in aski of ASA-sensitiwiteit in fermenterende giste is tot dusver beskikbaar nie. Deur die gebruik van konfokale laser skanderingsmikroskopie in kombinasie met 'n oksilipienmerker vir 3-OH oksilipiene (gebind aan 'n fluoresserende sekondêre teenliggaam) is die akkumulasie van hierdie

oksilipiene ontdek in die aski van die volgende fermenterende giste: *Pichia anomala*, *Pichia farinosa* en *Schizosaccharomyces octosporus*. Geen 3-OH oksilipien akkumulasie is egter in die aski van die fermenterende *Zygosaccharomyces bailii* gevind nie. Dit kan toegeskryf word aan die feit dat hierdie organisme moontlik meer op fermentasie staatmaak vir groei en reproduksie as mitochondriale respirasie.

Aangesien 3-OH oksilipiene in die mitochondria geproduseer word, verwag ons 'n toename in mitochondriale aktiwiteit in die seksuele strukture. Deur die gebruik van konfokale laser skanderingsmikroskopie en 'n fluoresserende merker (Rhodamine 123) is 'n toename in mitochondriale aktiwiteit in die aski van al die bogenoemde giste behalwe weereens *Z. bailii* waargeneem.

Tydens hierdie studie is daar 'n verband tussen seksuele reproduksie van giste, 3-OH oksilipien akkumulasie/produksie, mitochondriale aktiwiteit en suurstof benodigdheid gevind. Ook het hierdie studie onthul dat fermenterende giste meer weerstandbiedend is teen ASA as nie-fermenterende giste wanneer dit in vloeibare media gekweek word. Dit kan moontlik toegeskryf word daaraan dat fermenterende giste aerobiese respirasie of fermentasie kan gebruik vir groei en voortplanting. Hierdie navorsing het die weg gebaan vir die ontwikkeling van 'n bio-essaëring wat gebruik kan word vir die vinnige uitkenning van nuwe effektiewe antimitochondriale antifungale middels.

Sleutelwoorde: Asetielsalisiensuur, askosporovrystelling, askus, 3-hidroksie oksilipiene, fermentasie, gis, konfokale laser skanderingsmikroskopie, mitochondriale aktiwiteit, mitochondriale respirasie, rhodamien 123.