THE INFLUENCE OF MITOCHONDRIAL INHIBITORS ON FUNGAL LIFE CYCLES

By

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This thesis is lovingly dedicated to the late,

Simanga William Ncango (Grandfather, 2005)

Zoniselo Abraham Ncango (Uncle, 2006)

Hlekiwe Sarah Ncango (Aunt, 2010)

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CHAPTER 1

Literature review

1.1 Motivation

Research shows that non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, target yeast structures with elevated mitochondrial activity (Kock et al. 2007). Strikingly, this proposed mitochondrial inhibition function of aspirin has also been demonstrated in mammalian cells (Somasundaram et al. 2000; Norman et al. 2004). In 2007, the Kock group published the Aspirin Antifungal Hypothesis showing a clear link between 3-hydroxy (OH) oxylipin production, mitochondrial activity and acetylsalicylic acid (ASA, aspirin) sensitivity in respiring as well as fermenting yeasts.

In short, this hypothesis suggests among others that mitochondrial β-oxidation products such as 3-OH oxylipins are present in increased amounts in yeast sexual structures (asci) and in lesser amounts in vegetative asexual structures (hyphae and single cells). This implicates an increased mitochondrial activity in asci. Furthermore, according to the hypothesis, the development of the yeast sexual stage should therefore be more sensitive to mitochondrial inhibitors compared to the asexual stage. In addition, according to Leeuw and co-workers (2009) this phenomenon is not restricted to yeast asci, but is also found in dispersal structures such as sporangia in the mould *Mucor circinelloides*. Therefore the hypothesis can now be expanded to include structures (not only asci) where increased cell proliferation is observed. Here, elevated levels of mitochondrial activity are probably needed to meet the energy requirements to produce these structures characterized by an assembly of cells (ascospores and sporangiospores). This research may be of importance in the development of novel antifungals that target mitochondria.

In this study, the general validity of this hypothesis will be assessed by including more fungi with different cell assembly structures. Consequently, the following fungi will be studied i.e. an ascomycotan yeast plant pathogen characterized by naked asci but no yeast phase - *Eremothecium ashbyi* (Chapter 2), the anamorphic ascomycotan pathogenic mould – *Aspergillus fumigatus* (Chapter 3) as well as the pathogenic zygomycotan fungus – *Rhizopus oryzae* (Chapter 3).

1.2 Effects of mitochondrial inhibitors on fungal life cycles

Oxylipin studies mainly on yeasts have exposed the important role of mitochondria in fungal dispersal (Kock et al. 2003, 2007; Leeuw et al. 2009). Therefore, drugs that target mitochondrial function may find application in the control of human and plant fungal pathogens.

1.2.1 Discovery and structure of novel oxylipins in fungi

Oxylipins are defined as saturated and unsaturated oxidized fatty acids and are widely distributed in nature (Venter et al. 1997; Kock et al. 2003). Oxylipins such as prostaglandins (PGs) play an important role in mammalian cells where they induce labor and inhibit blood platelet aggregation (Samuelsson 1983; Needleman et al. 1986). A cheaper method of production of oxylipins such as PGs will have obvious advantages for the pharmaceutical industry. It was for this reason that the Kock group in South Africa (SA) and his international collaborators embarked on extensive bioprospecting studies to determine

whether yeasts can produce PGs. With radioimmunoassay (RIA), radio thin-layer chromatography (TLC) and gas chromatography – mass spectrometry (GC-MS), the presence of PGs was discovered in the yeast, *Dipodascopsis uninucleata* when a precursor of PGs, arachidonic acid (AA) was added. Interestingly, when NSAIDs, such as aspirin, were added they inhibited PG formation in this yeast (Kock et al. 1991). The discovery of PGs in yeasts, which was first reported by the Kock group in SA (Kock et al. 1991), was later confirmed by the Noverr group in the United States of America (USA) (Noverr et al. 2001, 2002). They demonstrated that pathogenic yeasts, *Cryptococcus neoformans* and *Candida albicans,* produce immunomodulatory PGs where they play a role as virulence factors.

In 1991, van Dyk and co-workers used a combination of techniques [TLC, ^{1}H two-dimensional (2D) correlation spectroscopy (COSY) nuclear magnetic resonance (NMR), electron impact - mass spectrometry (EI–MS), fast atom bombardment (FAB) and infrared spectroscopy analyses] to expose the chemical structure of a novel aspirin-sensitive oxylipin in *D. uninucleata.* They found this compound to be a 3-hydroxy 5Z,8Z,11Z,14Z-eicosatetraenoic acid (3-HETE, Fig. 1). Interestingly, Venter and co-workers revealed that *D. uninucleata* can produce a wide variety of novel 3-OH oxylipins (i.e. 3-OH 14:2, 3-OH 14:3, 3-OH 20:3, 3-OH 20:4, 3-OH 20:5) in the presence of different precursors (Venter et al. 1997).

To date, the presence of aspirin-sensitive 3-OH oxylipins has been reported to play key roles in the life cycle of non-related pathogenic and non-pathogenic fungi (Kock et al. 2003, 2007; Leeuw et al. 2009). The discovery of

aspirin-sensitive oxylipins in yeast was included in patents where aspirin and other NSAIDs where described as low cost, effective antifungals (Kock and Coetzee 1990; Davis et al. 2009). The dual function (i.e. anti-inflammatory as well as antifungal) of aspirin was hereby noted (Kock et al. 2007).

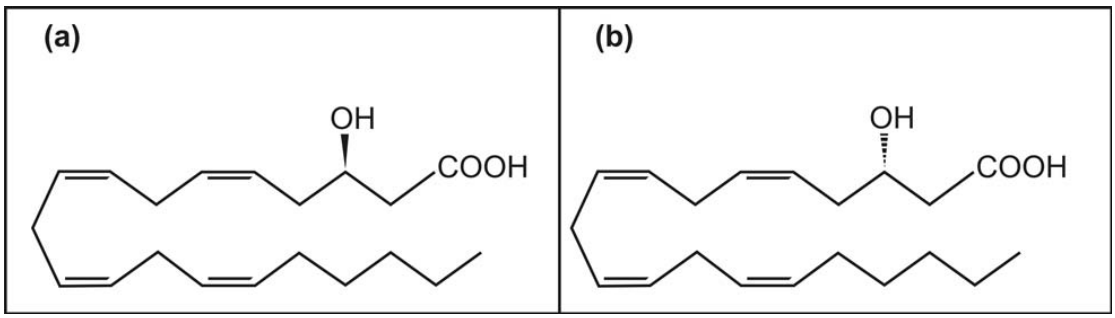

Fig. 1 The chemical structures of typical 3-hydroxy (OH) oxylipins. (a) *R*- and (b) *S*-3-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid (3-HETE). (Taken with permission from Kock et al. 2003).

1.2.2 Production of 3-OH oxylipins in fungi

Chemical and ultrascopic studies have suggested that 3-OH oxylipins are produced most probably in mitochondria of fungi (Venter et al. 1997; Sebolai et al. 2008). In 1997, Venter and co-workers reported that 3-OH oxylipins are produced via β-oxidation in the mitochondria of *D. uninucleata*. Here, various fatty acids (18:1, 18:2, 18:3, 20:0, 20:3, 20:4, 20:5) were fed to the yeast and GC-MS was used to analyze the extracted samples. Accumulation of 3-OH 14:2 (5Z,8Z) and 14:3 (5Z,8Z,11Z) were observed from the hydroxylation of 18:2 (9Z,12Z) and 20:3 (11Z,14Z,17Z) fatty acids which were shortened by four and six carbons respectively. Furthermore, 20:3 (5Z,8Z,11Z); 20:4 (5Z,8Z,11Z,14Z); 20:5 (5Z,8Z,11Z,14Z,17Z) fatty acids were 3-hydroxylated without chain length

alterations to 3-OH 20:3 (5Z,8Z,11Z), 20:4 (5Z,8Z,11Z,14Z) and 20:5 (5Z,8Z,11Z,14Z,17Z). It was demonstrated that 18:2 (9Z,12Z) and 20:3 (11Z,14Z,17Z) fatty acids were broken down by the β-oxidation degradation pathway i.e. reduction of 2 carbon for each cycle (Venter et al. 1997).

With ultrastructural studies, Sebolai and co-workers demonstrated in 2008, a 3-OH oxylipin production pathway in *Crypt. neoformans*. Transmission electron microscopy (TEM) showed that 3-OH oxylipins originate in the mitochondria and are then deposited inside the yeast cell wall, along capsule protuberances. Interestingly, aspirin, a known mitochondrial inhibitor, inhibited the mitochondrially produced 3-OH oxylipins. Furthermore, mitochondrial structural changes were observed which exposed the mitochondria as a target for aspirin action (Fig. 2). These studies strengthened the idea that a link between 3-OH oxylipins and mitochondria exists.

1.2.3 Distribution of 3-OH oxylipins in fungi

The first aspirin-sensitive 3-OH oxylipins were found in 1991 in *D. uninucleata* (van Dyk et al. 1991). Since then, there have been many reports on the presence of 3-OH oxylipins in pathogenic and non-pathogenic yeasts (Table 1). In 1998, Kock and co-workers, with the aid of immunofluorescence microscopy, studied antibodies directed against chemically synthesized 3-OH oxylipins (Bhatt et al. 1998; Groza et al. 2002, 2004). They found these antibodies to be specific for 3-OH oxylipins of different chain lengths and desaturation. In *D. uninucleata,* sexual cells (i.e. asci and ascospores) were

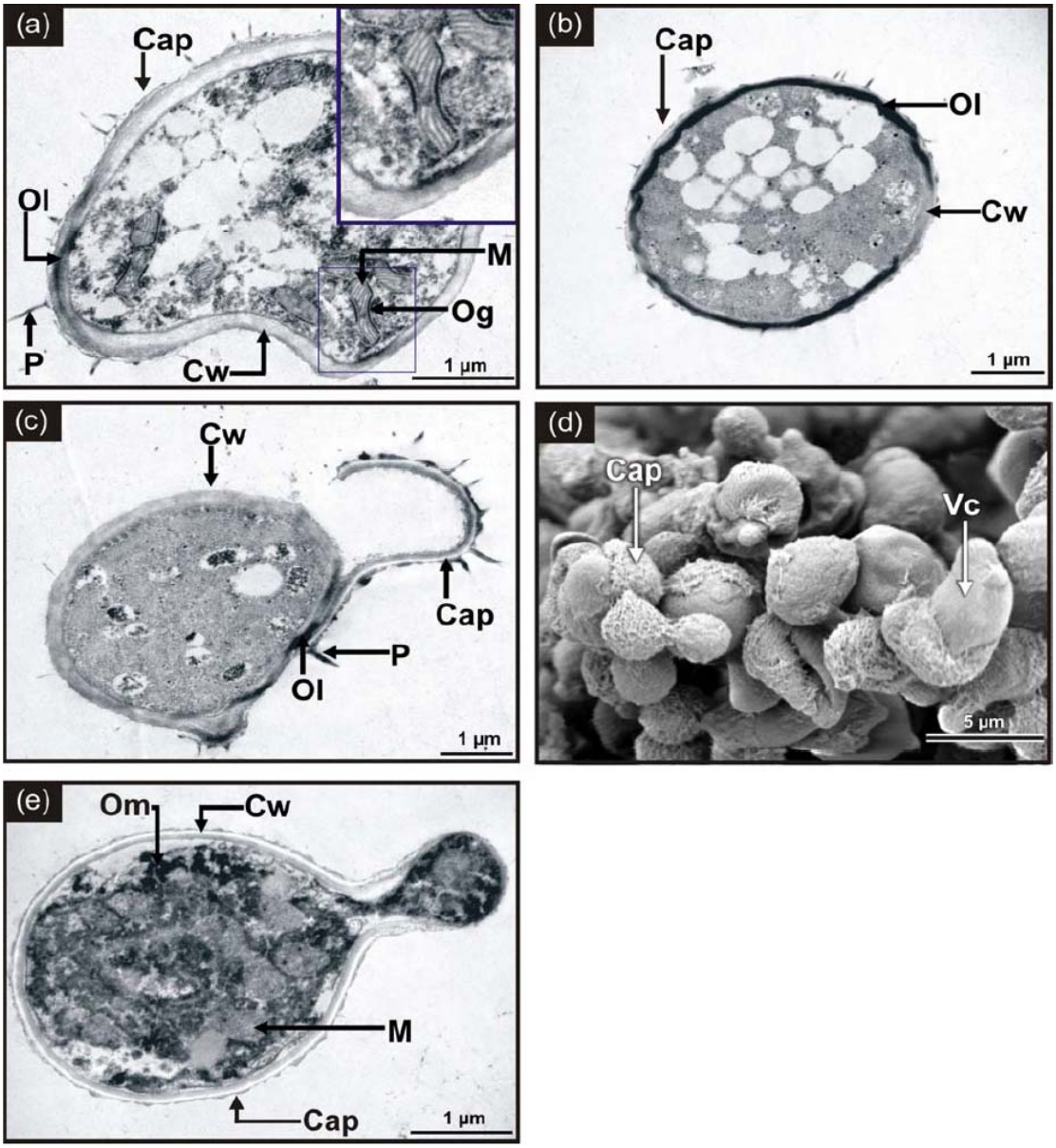

Fig. 2 Transmission electron micrographs depicting a possible migration route of osmiophilic material [containing 3-hydroxy (OH) oxylipins] from (i) mitochondria (a) (ii) to depositing of this material on the inside of the cell wall (b) and (iii) eventual excretion through the cell wall to the outside of capsule that becomes detached (c). Scanning electron micrograph depicting capsule detachment (d). In the presence of 5 mmol/L acetylsalicylic acid (ASA, aspirin) a mitochondrial ultrastructural change is observed while capsule detachment as well as osmiophilic material migration is inhibited (e). Cap = capsule, Cw = cell wall, M = mitochondrion, Og = osmiophilic globules, Ol = osmiophilic layer, Om = osmiophilic material, P = protuberance. Vc = vegetative cell. (Taken with permission from Sebolai et al. 2008)

observed to have higher affinity for the oxylipin antibodies compared to hyphae which had lower affinity. This demonstrated selective sensitivity of yeast sexual cells to aspirin-sensitive 3-OH oxylipin antibodies. In addition to immunofluorescence microscopy, the presence of 3-OH oxylipins in fungi was also confirmed by GC-MS.

The presence of aspirin-sensitive 3-OH oxylipins in yeasts seems to be a conserved character mainly associated with the sexual phase (Fig. 3) i.e. surface structures of the aggregating ascospores and asci (Kock et al. 1998; Leeuw et al. 2005, 2007; van Heerden et al. 2005, 2007; Ncango et al. 2006, 2008; Swart et al. 2008). Interestingly, in contrast to other yeasts, 3-OH oxylipin antibody fluorescence was associated with the ascus tip of *D. tóthii*, probably facilitating ascospore liberation (Smith et al. 2000a) while in *Ascoidea rubescens* (Fig. 3b), oxylipin deposition on the ascus tip is probably because of percurrent ascus formation and/or release of the oxylipin-containing slimy ascus-content (Ncango et al. 2008). With the aid of immunogold labelling TEM and immunofluorescence microscopy, 3-OH oxylipins were found also to be associated with the cell wall surface structures of the aggregating/flocculating vegetative cells of *Saccharomyces cerevisiae* and *Saccharomycopsis malanga* (Kock et al 2000; Sebolai et al. 2001; Strauss et al. 2005). Furthermore, these compounds were also reported in pathogenic yeasts and found to be associated with surfaces of pathogenic hyphae of *C. albicans* (Deva et al. 2000, 2001, 2003) and also in capsules, where they are released as hydrophobic droplets through protuberances into the extracellular environments by *Crypt. neoformans* (Sebolai et al. 2008).

Table 1. Distribution of 3-OH oxylipins in some yeasts.

SPECIES	3-OH OXYLIPINS	ASSOCIATION	REFERENCE
Ascoidea africana	3-OH 10:1	Ascospores	Bareetseng et al. 2005
A. corymbosa	3-OH 17:0	Ascospores	Ncango et al. 2006
A. rubescens	3-OH 16:2	Ascospores	Ncango et al. 2008
Candida albicans	3,18 diHETE	Hyphal cells	Deva et al. 2000
Cryptococcus neoformans var. *neoformans*	3-OH 9:1	Vegetative cells	Sebolai et al. 2007
Dipodascopsis tothii	3-OH 14:2, 14:3, 20:3, 20:4, 20:5	Ascospores	Kock et al. 1997
D. uninucleata var. *uninucleata*	3-OH 14:2, 14:3, 20:3, 20:4, 20:5	Ascospores	Venter et al. 1997, Fox et al. 1997
Dipodascus albidus	3-OH metabolite*	Ascospores	Van Heerden et al. 2005
D. ambrosiae	3-OH metabolite*	Ascospores	Smith et al. 2003
D. geniculatus	3-OH metabolite*	Ascospores	Van Heerden et al. 2007
D. macrosporus	3-OH metabolite*	Ascospores	Smith et al. 2003
D. magnusii	3-OH metabolite*	Ascospores	Smith et al. 2003
D. spicifer	3-OH metabolite*	Ascospores	Smith et al. 2003
D. tetrasperma	3-OH metabolite*	Ascospores	Smith et al. 2003
Eremothecium ashbyi	3-OH 14:0	Ascospores	Kock et al. 2004
E. coryli	3-OH 9:1	Ascospores	Leeuw et al. 2005
E. cymbalariae	3-OH 13:1	Ascospores	Leeuw et al. 2007
E. gossypii	3-OH 10:1	Ascospores	Leeuw et al. 2007
E. sinecaudum	3-OH metabolite*	Ascospores	Bareetseng et al. 2004
Lipomyces doorenjongii	3-OH metabolite*	Ascospores	Smith et al. 2000b
L. kockii	3-OH metabolite*	Ascospores	Smith et al. 2000b
L. kononenkoae	3-OH metabolite*	Ascospores	Smith et al. 2000b
L. starkeyi	3-OH metabolite*	Ascospores	Smith et al. 2000b
L. yamadae	3-OH metabolite*	Ascospores	Smith et al. 2000b
L. yarrowii	3-OH metabolite*	Ascospores	Smith et al. 2000b
Nadsonia commutata	3-OH 9:1	Vegetative cells	Bareetseng 2004
N. fulvescens	3-OH metabolite*	Vegetative cells	Bareetseng 2004
Saccharomyces cerevisiae	3-OH 8:0, 10:0	Vegetative cells	Kock et al. 2000, Strauss et al. 2005
Saccharomycopsis capsularis	3-OH 9:1	Ascospores	Sebolai 2004
S. fermentans	3-OH metabolite*	Ascospores	Sebolai et al. 2005
S. javanensis	3-OH 9:1	Ascospores	Sebolai et al. 2005
S. malanga	3-OH 16:0	Vegetative cells	Sebolai et al. 2001
S. synnaedendra	3-OH 16:0, 17:0, 18:0, 19:0, 19:1, 20:0, 22:0	Vegetative cells	Sebolai et al. 2004
S. vini	3-OH 9:1, 10:1	Ascospores	Sebolai et al. 2005
Saturnispora saitoi	3-OH 9:1	Ascospores	Bareetseng et al. 2006
Schizosaccharomyces pombe	3-OH 11:0, 15:0	Vegetative cells	Strauss et al. 2006

*3-OH metabolite : 3-OH fatty acid structure not yet fully identified.

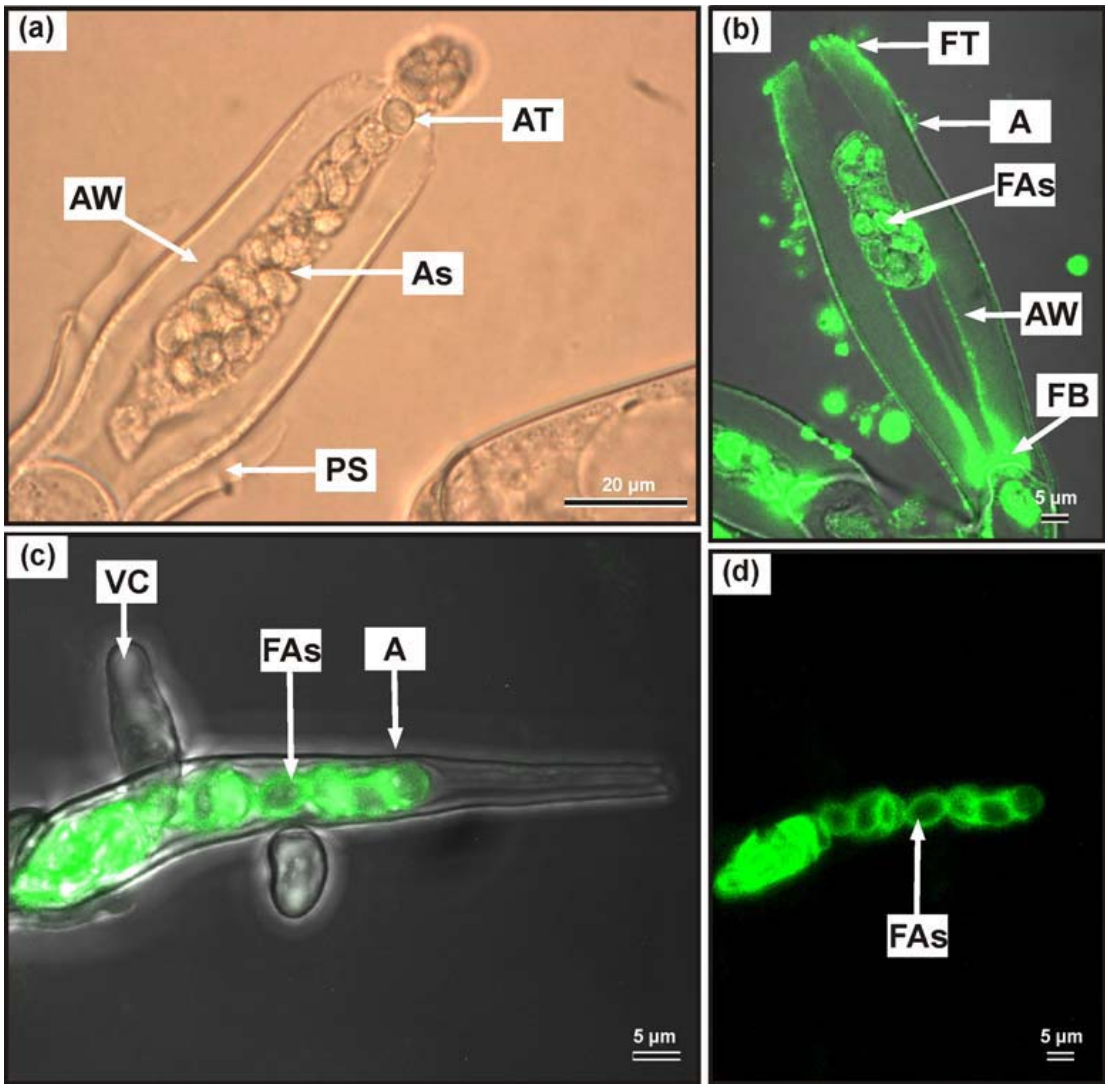

Fig. 3 Light and fluorescence micrographs of *Ascoidea africana* and *A. rubescens*. (a) Light micrograph of thick walled ascus of *A. rubescens* containing ascospores also showing percurrent succession, (b) immunofluorescence micrograph of partially filled mature ascus of *A. rubescens* in process of releasing spores from ascus tip, (c) immunofluorescence superimposed on corresponding light micrograph of ascus of *A. africana* and (d) only fluorescence micrograph of corresponding ascus in (c). A, ascus; As, ascospores; AT, ascus tip; AW, ascus wall; FAs, fluorescing ascospores; FT, fluorescing ascus tip; FB, fluorescing ascus base; PS, percurrent succession; VC, vegetative cells (Taken with permission from Ncango et al. 2008).

The presence of 3-OH oxylipins has not only been limited to yeasts. Using GC-MS and immunofluorescence microscopy, 3-OH oxylipins were also reported in *M. genevensis* and found to be mainly associated with sporangia and sporangiospores (Pohl et al. 1998). In 2000, Strauss and co-workers mapped the distribution of 3-OH oxylipins in various members of the order Mucorales. They found increased 3-OH oxylipin production in sporangia of *Absidia, Actinomucor, Cunninghamella, Mortierella* (subgenus *Micromucor*)*, Mucor* and *Rhizomucor*.

1.2.4 Functions of 3-OH oxylipins in fungi

Oxylipins have been implicated to have various biological functions in pathogenic and non-pathogenic fungi. They have been reported to play a role (i) in ascospore dispersal from enclosed asci in yeasts by acting as lubricants, (ii) in ascospore aggregation in yeast after dispersal, (iii) in yeast flocculation, (iv) as virulence factors, (v) in inflammation during infection as well as (vi) to act as antifungal agents against other fungi.

Aspirin-sensitive 3-OH oxylipins have been reported to be associated mainly with yeast sexual cells and this seems to be a conserved character (Kock et al. 2007). Here, they play a role as lubricants during ascospore release from enclosed asci, where they are involved in (i) drilling in the plant pathogen *E. sinecaudum* - using selectively oxylipin-coated tapered corkscrew ascospore tips (Bareetseng et al. 2004), (ii) piercing in the plant pathogen *E. cymbalariae* and *E. ashbyi* - through sharp oxylipin-coated ascospore tips (Kock et al. 2004; Leeuw et al. 2007), (iii) by cutting in *A. corymbosa* (Fig. 4) - through razor sharp selectively oxylipin-coated brims of hat-shaped ascospores (Ncango et al. 2006),

(iv) gear-like movement in *D. uninucleata* (Kock et al. 1999) and (v) sliding movement of sheathed ascospores in *Dipodascus* (van Heerden et al. 2007).

When yeast reach the sexual reproductive stage, ascospores coated with 3-OH oxylipins are liberated from the ascus tip followed by aggregation in clusters. In *D. uninucleata*, it was observed that ascospores are individually released through an opening formed at the apex during pressure build-up against the ascus wall. Before ascospore liberation, oil-like droplets are released first through the opened ascus tip. Liberated ascospores will then aggregate in these oil-droplets resulting in sticky aggretates (Kock et al. 1999). Similar results of ascospores forming a cluster after release were reported for *A. corymbosa* (Fig. 4b; Ncango et al. 2006), *A. rubescens* (Fig. 3a; Ncango et al. 2008) as well as in many other yeasts (Kock et al. 2003, 2007).

In 2000, Kock and co-workers implicated 3-OH oxylipins to play a role in flocculation in *S. cerevisiae*. Here, immunofluorescence microscopy revealed that these compounds were present on the cell wall surfaces and also in between cells of matured cells of *S. cerevisiae*. Similar observations were made with TEM where deposition of osmiophilic layers followed the same pattern as immunofluorescence microscopy results. Furthermore, detailed TEM studies showed that osmiophilic layers migrated from cells while protuberances were formed which crossed the cell wall to reach for other adjancent cells to attach to their cell wall. In addition, immunogold labelling TEM showed that osmiophilic layers contained 3-OH oxylipins. This implicated the role of 3-OH oxylipins in cell adherence/flocculation (Kock et al. 2000).

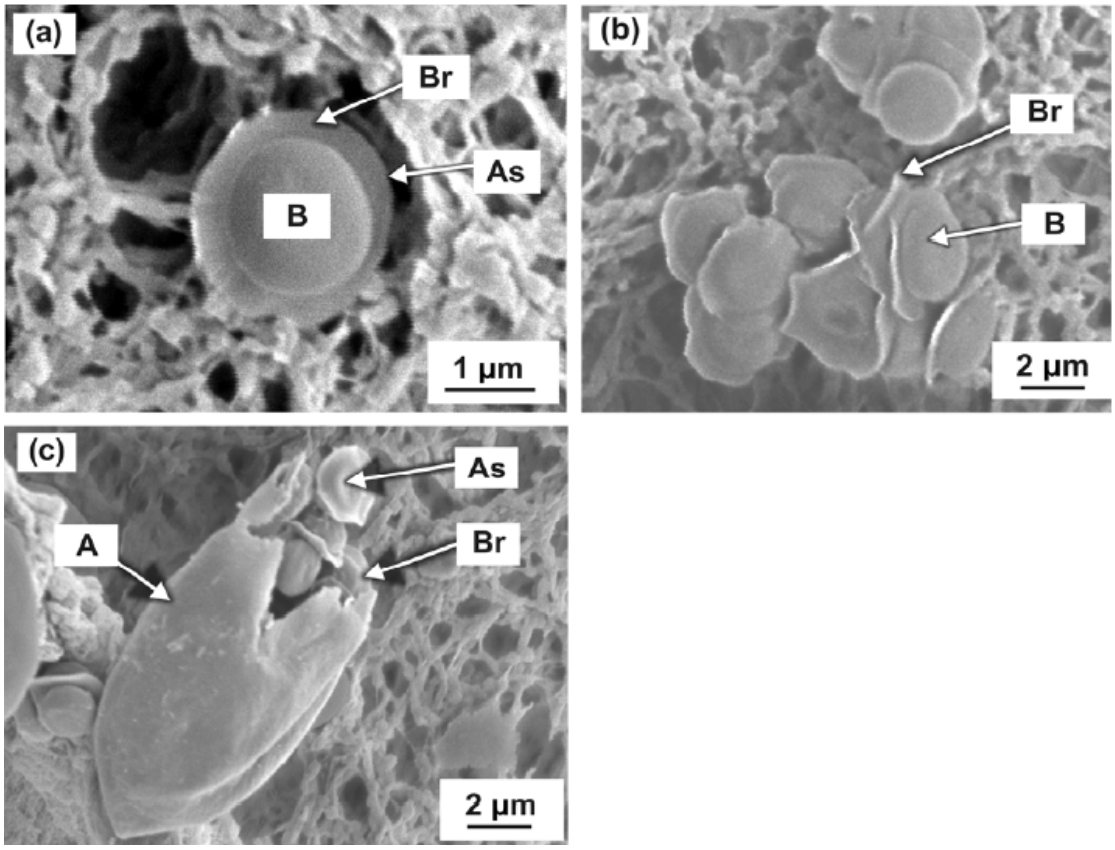

Fig. 4 Scanning electron micrographs of individually released ascospore (a) and aggregated released ascospores (b) in *Ascoidea corymbosa*. The release of ascospores from the ascus opening (tip) is shown in (c). A, ascus; As, ascospore; B, bowl; Br, brim (Taken with permission from Ncango et al. 2006).

In a study conducted by Sebolai and co-workers in 2007, 3-OH oxylipins were observed to accumulate in capsules of *Crypt. neoformans* where they are released as hydrophobic droplets through tubular protuberances. In literature it is known that the major virulence factor in *Crypt. neoformans* is the capsule (Yauch et al. 2006). Interestingly, when aspirin was added to cells of *Crypt. neoformans,* it inhibited capsule shedding as well as 3-OH oxylipin production (Sebolai et al. 2007).

A novel 3-OH oxylipin, 3,18 dihydroxy-5,8,11,14-eicosatetraenoic acid (3,18 diHETE) was identified in *C. albicans*, a causative agent of vulvovaginal candidiasis. Here it was shown that aspirin has a dual benefit in the treatment of this disease i.e. by inhibiting 3-OH oxylipin formation associated with the hyphal phase and also by inhibiting prostaglandin E_2 (PGE_2) formation in the infected host (Deva et al. 2000, 2001, 2003). These studies hinted at (i) a novel target for the control of *Candida* infections and (ii) the applicability of aspirin and other NSAIDs as strong antifungals in the control of yeast infections (Kock and Coetzee 1990).

3-OH oxylipins have also been reported in prokaryotes where they play a role in inflammation as well as to act as antifungals against other fungi (Rietschel et al. 1994; Sjogren et al. 2003). In Gram-negative bacteria such as *Escherichia coli*, 3-OH oxylipins are found as the active substances in the inflammatory-disease causing lipopolysaccharide (LPS)-endotoxin component (Rietschel et al. 1994). Furthermore, Sjogren and co-workers (2003) chemically characterized four different 3-OH oxylipins (i.e. 3-OH 10:0, 3-OH 11:0, 3-OH 12:0 and 3-OH 14:0) from a Gram-positive bacterium, *Lactobacillus plantarum*. These 3-OH oxylipins were reported to have strong antifungal activity against different moulds such as *A. fumigatus*, *A. nidulans*, *Penicillium commune* and *P. roqueforti* as well as against yeasts such as *Kluyveromyces marxianus, Pichia anomala* and *Rhodotorula mucilaginosa.*

1.2.5 Influence of mitochondrial inhibitors on fungal life cycles

Fungal structures such as asci and sporangia are characterized by increased mitochondrial activity. These structures have been shown to be more sensitive to mitochondrial inhibitors, such as aspirin, compared to asexual cells such as single cells and hyphae. The latter are characterized by decreased mitochondrial activity (Kock et al. 2007; Leeuw et al. 2009).

To investigate the role that mitochondrial activity plays in the life cycle of fungi, different serological techniques, enzymatic studies as well as inhibition studies were performed. The yeast, *D. uninucleata*, which is characterized by consecutive asexual and sexual stages in its life cycle, has been used as a model organism. In 1992, Botha and co-workers investigated the influence of mitochondrial inhibitors such as aspirin, on the life cycle of the aerobic respiring *D. uninucleata.* They reported the sexual stage of *D. uninucleata* to be more sensitive to mitochondrial inhibitors compared to the asexual stage. Furthermore, sexual phase development i.e. ascosporogenesis and ascospore liberation, was the most susceptible phase inhibited by lower concentration of aspirin (1 mM). In addition, TEM and 3-OH oxylipin inhibition studies on *D. uninucleata* revealed that 3-OH oxylipins associated with ascospore ornamentation i.e. nano-scale hooks, are sensitive to aspirin. In the absence of aspirin, ascospores formed nano-scale hooks that are perfectly interlocked compared to when aspirin was added. Here, the nano-scale hooks were not formed and ascospores were also not liberated (Kock et al. 1999). From these studies, it is clear that *D. uninucleata*, which is characterized by an aerobic respiring metabolism, probably

requires more energy for the production and dispersal of ascospores from enclosed asci compared to the hyphal stages (Leeuw et al. 2007). Similar inhibition results were obtained with indomethacin, also an NSAIDs (Botha et al. 1992). These studies on *D. uninucleata* exposed the strong antifungal action of mitochondrial inhibitors towards yeast sexual reproduction and dispersal.

In 1998, Kock and co-workers mapped the life cycle of this yeast using polyclonal antibodies specific for 3-OH oxylipins as well immunofluorescence microscopy (Fig. 5a-g). Here, elevated mitochondrial activity expressed as 3-OH oxylipin production, was reported in the sexual stages compared to asexual stages. In the sexual stages, 3-OH oxylipins were found associated with tips of adhering gametes (Fig. 5c), young developing ascus (Fig. 5d) as well as liberated aggregating ascospores (Fig. 5e). This study linked mitochondrial activity expressed as 3-OH oxylipin production, with sexual cells of *D. uninucleata*. In addition to mapping, the presence of 3-OH oxylipins in *D. uninucleata* was confirmed with GC-MS. Here, lipids were extracted during sexual stage development and chemical structure analyzed with GC-MS. The presence of a major peak from the EI-MS at *m/z* 175 indicated a OH group at carbon 3 i.e. 3-OH oxylipin (Venter et al. 1997).

Increased mitochondrial activity, expressed as mitochondrial transmembrane potential ($\Delta\Psi m$) was also observed in the sexual stages of *D. uninucleata* compared to the asexual stage (Ncango et al. 2008). Here, rhodamine 123 (Rh123), a cationic lipophilic dye which selectively accumulates in the mitochondria with a high $\Delta\Psi m$ (Johnson et al. 1980; Ludovico et al. 2001),

was used. As expected, with the aid of confocal laser scanning microscopy (CLSM), the sexual stage i.e. asci, showed a higher affinity for Rh123 suggesting increased mitochondrial activity compared to the asexual stage i.e. hyphae which showed a low affinity. This study again linked mitochondrial activity expressed as $\Delta\Psi m$, with the sexual cells of *D. uninucleata*.

Interestingly, similar results were also reported in the life cycle of yeasts characterized by an aerobic respiring metabolism i.e. *Ascoidea* (Ncango 2007; Ncango et al. 2006, 2008), *Dipodascus* (van Heerden et al. 2005, 2007), *Eremothecium* (Leeuw et al. 2005, 2007) as well as *Lipomyces* (Swart 2007; Swart et al. 2008). Strikingly, the sexual stages of yeasts characterized by both aerobic respiring and fermenting metabolism i.e. *Kluyveromyces*, *Pichia*, *Schizosaccharomyces* as well as *Zygosaccharomyces*, were reported to be more resistant to aspirin (Leeuw et al. 2007; Swart 2007). Furthermore, elevated mitochondrial activity expressed as both 3-OH oxylipin production and $\Delta\Psi m$, was observed in the sexual stages compared to asexual stages of the tested aerobic respiring as well as fermenting yeasts, with the exception of *Zygosaccharomyces* (Swart et al. 2008). In *Zygosaccharomyces*, low mitochondrial activity was observed in both sexual and asexual cells. This may be ascribed to yeast depending more on the fermentative pathway for growth and sexual reproduction than aerobic respiration via mitochondria (Ludovico et al. 2001).

Increased mitochondrial activity in sexual cells is not only limited to yeasts as reported above but also to filamentous fungi. Recently, Leeuw and co-workers (2009) investigated the effects of mitochondrial inhibitors on sporangium

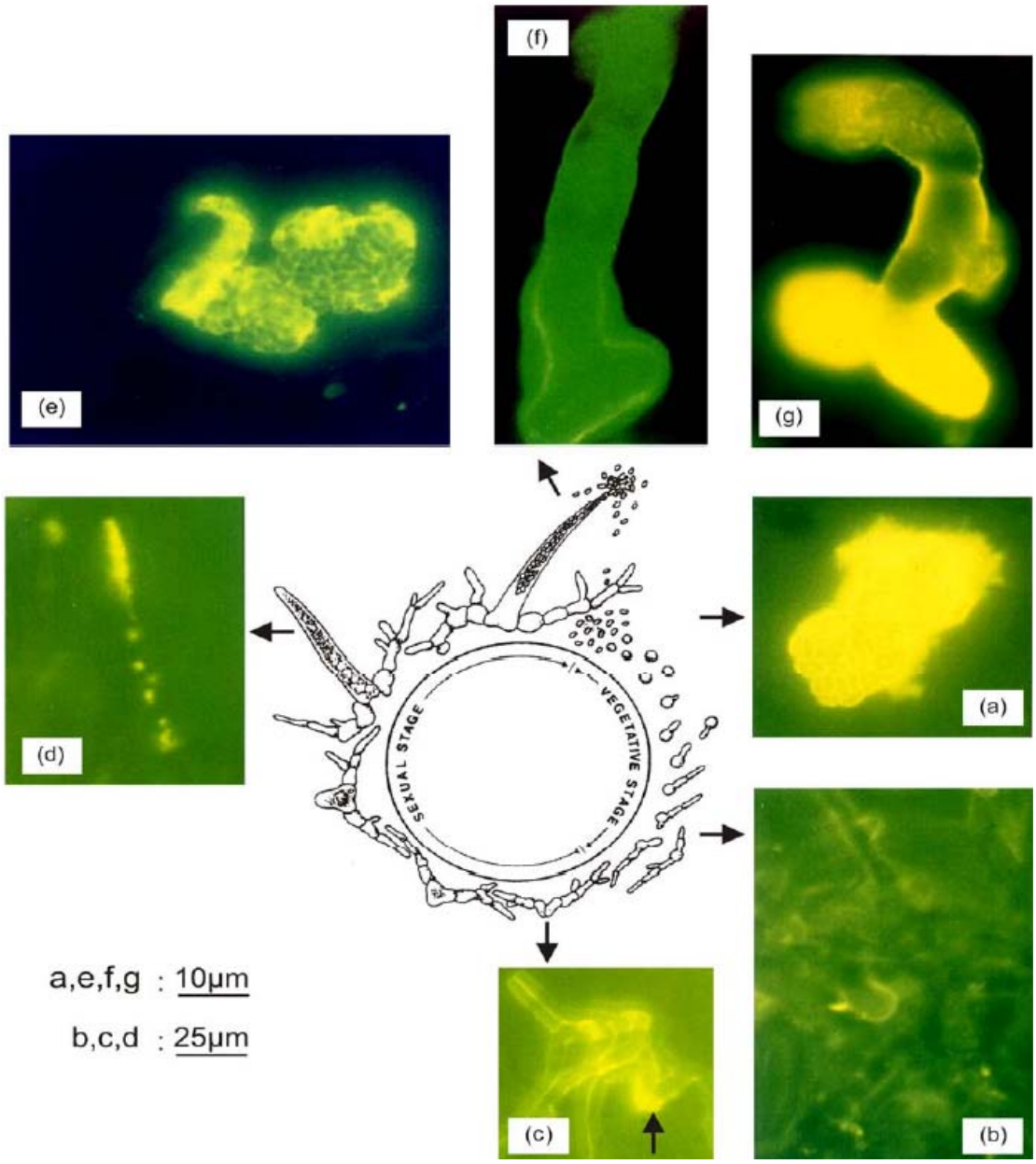

Fig. 5 The life cycle of *Dipodascopsis uninucleata* and distribution of 3-hydroxy (OH) oxylipins visualized through immunofluorescence mapping. (a) Liberated ascospores showing high affinity for oxylipin antibody. (b) Hyphae with low oxylipin antibody affinity. (c) Gametangiogamy with tip of adhering gametes showing high affinity for oxylipin antibody. (d) Young ascus with immature ascospores demonstrating high affinity for oxylipin antibody. (e) Liberated fluorescing ascospores from ascus. (f) Empty ascus: still with characteristic morphology. (g) Deformed mature ascus containing fluorescing ascospores mainly at base. Asexual vegetative stage (a, b). Sexual stage (c, d, e, f, g). (Taken with permission from Kock et al. 1998).

development of *M. circinelloides*. Here, increased mitochondrial activity expressed as $\Delta\Psi m$, was reported in the sporangium of *M. circinelloides* compared to hyphae. Consequently, in the same fungus, sporangium development was the most sensitive to mitochondrial inhibitors such as aspirin compared to hyphae. Furthermore, mitochondrial dehydrogenase activity in sporangium and hyphae of *M. circinelloides* was studied using the XTT reduction assay (Leeuw et al. 2009). Here, XTT (a tetrazolium salt) was cleaved by various mitochondrial dehydrogenase enzymes to produce a colored formazan, which indicates fungal metabolic activity. As expected, the sporangium of *M. circinelloides* contained increased mitochondrial dehydrogenase activity compared to hyphae. These findings are also corroborated by increased 3-OH oxylipin concentrations found in sporangia of *M. circinelloides* (Strauss et al. 2000). This study further supports the role that mitochondrial activity plays in the life cycle of fungi, especially in dispersal structures.

According to literature, 3-OH oxylipins are probably produced through β-oxidation in the mitochondria which will then be released and deposited onto spore surfaces of fungi (Kock et al. 2003, 2004, 2007). In addition, mitochondrial inhibitors such as aspirin have been reported to inhibit β-oxidation in the mitochondria and therefore 3-OH oxylipin production as well as sexual and/or asexual reproductive dispersal stages in fungi (Kock et al. 2003, 2007; Leeuw et al. 2007). According to Glasgow and co-workers (1999), mitochondrial β-oxidation is inhibited by the primary active metabolite of aspirin, salicylate, which has structural similarities to the acyl portions of the substrate and product of the 3-OH acyl-CoA dehydrogenase enzyme of the β-oxidation pathway. Another

possible mode of inhibition by aspirin is that it induces changes in mitochondrial energy production through uncoupling oxidative phosphorylation (Somasundaram et al. 2000; Norman et al. 2004). From this, it is clear that there is a link between 3-OH oxylipin production, mitochondria, sexual and/or asexual reproduction as well as mitochondrial inhibitors such as aspirin in fungi.

1.2.6 Aspirin Antifungal Hypothesis

Since mitochondrial dependence seems to be linked to aspirin sensitivity in fungi, we can conclude that fungal life cycles that are characterized by a mitochondrial-dependent aerobic respiring pathway will be more sensitive to mitochondrial inhibitors such as aspirin compared to fungal life cycles that can generate energy also through an anaerobic glycolytic fermentative pathway in which the mitochondria are less involved (Leeuw et al. 2007).

In 2007, Kock and co-workers reported that a clear link exists between 3-OH oxylipin production, mitochondrial activity and aspirin sensitivity in aerobic respiring as well as fermentative yeasts. These authors hypothesized (Fig. 6) that: (i) 3-OH oxylipins in yeasts are produced by mitochondria through β-oxidation, (ii) aspirin inhibits mitochondrial β-oxidation and 3-OH oxylipin production, (iii) yeast sexual stages, which are probably more dependent on mitochondrial activity are also characterized by higher 3-OH oxylipin production as well as $\Delta\Psi m$ compared to asexual stages, (iv) yeast sexual developmental stages as well as cell adherence/flocculation are more sensitive to aspirin than corresponding asexual growth stages and (v) mitochondrion-dependent sexual

yeast cells with an aerobic respiring metabolism are more sensitive to aspirin than those that can also produce energy through an alternative anaerobic glycolytic fermentative pathway where mitochondria are less involved in the energetic pathway. Can this yeast hypothesis (Fig. 6) be expanded to include other fungi?

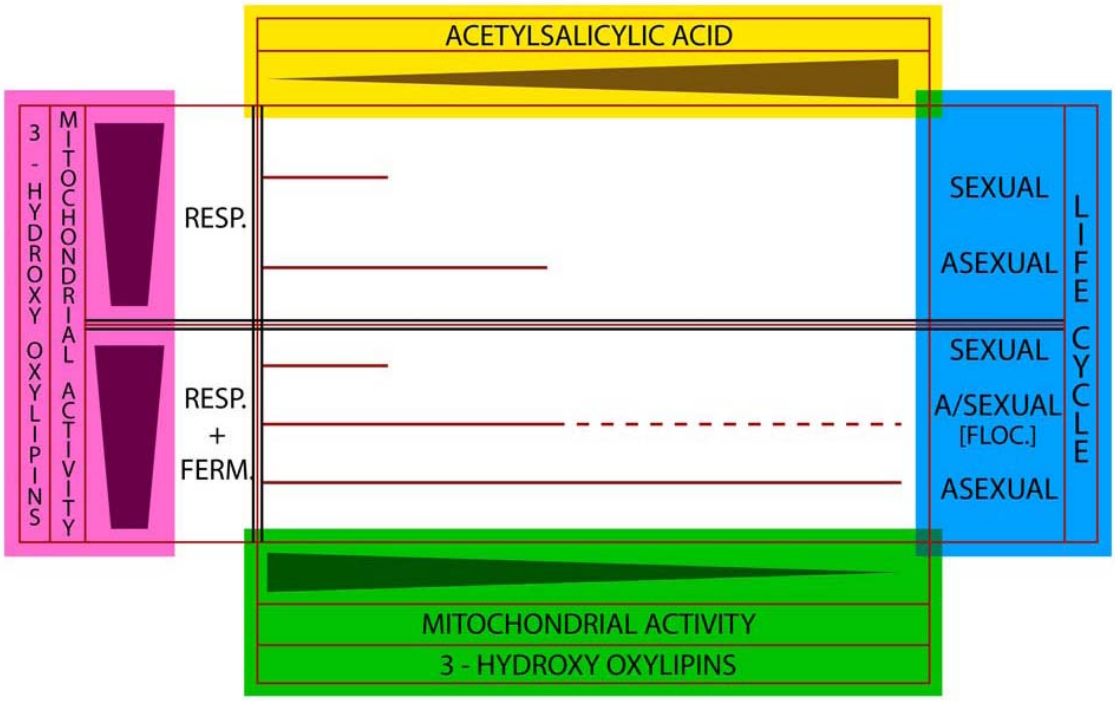

Fig. 6 A visual representation of a hypothesis suggesting a possible link between 3-hydroxy (OH) oxylipin production, mitochondrial activity, and aspirin sensitivity. *x*-axis, top: increase in aspirin concentration from left to right. *x*-axis, bottom: decrease in mitochondrial activity and 3-OH oxylipin levels from left to right. *y*-axis, left: decrease in mitochondrial activity and 3-OH oxylipin levels from sexual reproductive to asexual growth phases in both strict aerobic yeasts (RESP.) and yeasts with both aerobic and fermentative pathways (RESP. + FERM.). *y*-axis, right: different phases of yeast life cycles i.e. sexual, asexual as well as asexual/sexual flocculation (FLOC.). Middle block: response surface showing the relative sensitivities of different yeast phases towards increasing levels of aspirin (Taken with permission from Kock et al. 2007).

1.3 Purpose of the study

With the above information as background, the purpose of the study became to assess if this Aspirin Antifungal Hypothesis could be expanded to also include other fungal structures where increased mitochondrial activities are expected. Consequently, the life cycles of the ascomycotan yeast with naked asci (no yeast phases) – *E. ashbyi* (Chapter 2), anamorphic ascomycotan mould – *A. fumigatus* (Chapter 3) as well as zygomycotan fungus – *R. oryzae* (Chapter 3) were studied in this respect.

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CHAPTER 2

The effect of mitochondrial inhibitors on the yeast *Eremothecium ashbyi*

Parts of this study have been published in Current Drug Discovery Technologies **6:** 186-191 (2009) and written in this journal's format.

2.1 ABSTRACT

Previous studies show that acetylsalicylic acid (aspirin) at low concentrations affects yeast sexual structure development in a similar fashion than oxygen depletion. This is ascribed to its anti-mitochondrial action. In this study, we report the same for other anti-inflammatory (i.e. ibuprofen, indomethacin, salicylic acid, benzoic acid) as well as anticancer (Lonidamine) drugs, also known for inhibiting mitochondrial activity in mammalian cells. This is shown by a unique yeast bio-assay, with the mitochondrion-dependent sexual structure, riboflavin production, and hyphal morphology of the yeast *Eremothecium ashbyi* serving as indicators. These drugs affect this yeast in a similar way as found under oxygen limitation conditions by inhibiting sexual structure development (most sensitive), riboflavin production, and yielding characteristically wrinkled and granular hyphae, presenting a unique “anoxic” morphological pattern for this yeast. Only drugs associated with anti-mitochondrial activity presented such a pattern. This bio-assay may find application in the screening for novel drugs from various sources with anti-mitochondrial actions. In addition, anti-mitochondrial compounds may serve as antifungals to combat the dispersal of *E*. *ashbyi* that is notorious for causing diseases such as yeast-spot in soybeans.

Key words: Anticancer, antifungal, anti-inflammatory, anti-mitochondrion, bio-assay, yeast.

2.2 INTRODUCTION

Oxylipin studies show that the commonly used non-steroidal anti-inflammatory drug (NSAID), acetylsalicylic acid (aspirin) acts as a potent anti-mitochondrion antifungal thus exposing a dual action which may have therapeutic benefits in combating disease while decreasing the host inflammatory response [1, 2]. These studies hypothesize a link between aspirin sensitivity, mitochondrion function and sexual reproduction in strict respiring yeasts and yeasts that can also ferment [1]. Here an increase in aspirin concentration results in a decrease in mitochondrion function and consequent decrease in sexual reproduction followed by decreased asexual growth at higher aspirin concentrations. Similar results were obtained with oxygen depletion studies [1].

In this study a practical yeast bio-assay was constructed using *Eremothecium ashbyi* to evaluate the ability of various anti-inflammatory, antifungal and anticancer drugs to selectively target oxygen-dependent yeast sexual structure development. Here growth, ascus formation, ascospore release and mitochondrion activity of *E. ashbyi* over decreasing concentration gradients of various anti-inflammatory, antifungal and anticancer drugs were assessed. For the purpose of this study, any compound that inhibits mitochondrion activity in a direct or indirect manner will be referred to as an anti-mitochondrial. *E. ashbyi* is a plant pathogen widely responsible for yeast-spot disease as well as lesions on the surface of citrus fruits and cotton boll [3, 4, 5]. Uncovering novel effective anti-mitochondrial antifungals will therefore be of importance in combating this yeast.

2.3 EXPERIMENTAL SECTION

Strain Used and Cultivation

Eremothecium ashbyi UOFS Y-630 was used in the study and is preserved at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Bloemfontein, South Africa. The yeast was streaked out on yeast malt (YM) agar [6] and cultivated at 25^0C in Petri dishes until sporulation was observed.

Bio-Assay Preparation and Application

The bio-assay is based on the agar diffusion method where activity of a compound is measured along a concentration gradient across the agar plate (i.e. from position of compound addition) by observing the growth inhibition-zone (i) and changes in yeast reproductive structures from the asexual-zone c_1 to the sexual-zone b_2 (Fig. **1a**).

Cells were scraped from YM-agar grown cultures and suspended in sterilized dH_2O (3+ density according to Yarrow [7]) from where 0.2 ml were streaked out on YM–agar (0.5% agar m/v) to produce a uniform lawn completely covering the agar surface. A well of 0.5 cm in diameter and depth was constructed aseptically in the middle of the agar plate followed by the addition (46 µl) of the following anti-inflammatory compound solutions [8-10]: aspirin (Sigma, Steinheim, Germany), ibuprofen (Sigma-Aldrich, Steinheim, Germany), indomethacin (Sigma, Steinheim, Germany), salicylic acid (The British Drug Houses Ltd., Poole, England) and benzoic acid (The British Drug Houses Ltd.,

Poole, England) – compound concentration: 80 mg/ml 96% ethanol (Merck, Gauteng, South Africa). In addition, controls were constructed by the addition of similar amounts of only 96% ethanol to wells. Tests were also conducted with 40 µL of the anticancer drug Lonidamine (LND) [11] dissolved in DMSO (Merck, Germany; 0.5% m/v), obtained from Sigma-Aldrich, Steinheim, Germany (added to a similar well as described); aqueous solutions (10 µl) of HCl (1N; Merck, Gauteng, South Africa), formic acid (1N; Saarchem, Gauteng, South Africa) and NaOH (1N; Thomas Baker Chemicals, Mombai, India) as well as 10 µL aqueous ethidium bromide (ETB; 8% m/v; Sigma-Aldrich Ltd., St Louis, MO, USA) all added to filter paper discs (0.5 cm in diameter). ETest gradient strips (AB Biodisk, Dalvagen 10, 169 SG Solna, Sweden) containing amphotericin B (MIC reading scale: 0.002 to 32 µg/mL) and flucytosine (MIC reading scale: 0.002 to 32 µg/mL) were applied as non anti-mitochondrial antifungal drugs tests. Filter discs and ETest strips were placed in the centre of the bio-assay plates while all plates were incubated at 25^0C until the yellow sexual-zone b_2 (Fig. **1a**) could be observed (usually within 2 days) [3]. Since the bio-assay has been evaluated as a qualitative screen for compounds with specialized antifungal activity, no attempts were made at this stage to determine MICs (minimum inhibitory concentration).

For each anti-mitochondrial compound tested, four different areas (sampling points) for each zone (c_1, b_1, $b_{2;}$ Fig. **1a**) on the yeast lawn were aseptically sampled at random, suspended in a drop of dH_2O on a glass slide with cover slip and then subjected to light microscopy analysis. All asci (empty and filled with well developed ascospores) in *E. ashbyi* were counted in four

adjacent microscope fields as described by Ncango in 2007 [12]. The percentage empty asci relative to asci filled with well developed ascospores (indicating ascospore release), was calculated for each sample. These experiments were repeated at least in triplicate resulting in a total of 3 plates (lawns) tested, each containing four sampling points per zone, totaling 3 (repetitions) x 4 (sampling points) x 3 (zones) microscopic fields. Representing light micrographs of cells in the different zones studied, were taken using a light microscope (Axioplan, Zeiss, Göttingen, Germany) coupled to a Colorview Soft Imaging System (Münster, Germany).

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was carried out as described by Ncango *et al.* [13]. Aspirin treated bio-assays containing cells of yellow sexual-zone b_2 and white asexual-zone c_1 were chemically fixed overnight using 3% v/v of a sodium phosphate buffered glutardialdehyde (Sigma-Aldrich, St. Louis, Mo., USA) solution at pH 7.0 and a similarly buffered solution (1% m/v) of osmium tetroxide (Sigma-Aldrich, St. Louis, Mo., USA) 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 Jeol WINSEM (JSM 6400) SEM (Jeol, Tokyo, Japan).

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 as described for bio-assay preparation. A sterilized cover slip was placed on the plate to create an anoxic environment [7]. Cells were grown for two days until growth was observed. Cells from the plate and also beneath the cover slip were directly viewed under the light microscope for growth and sexual reproduction (Fig. **2d**; Table **2**).

Mitochondrion Distribution

A small amount of yeast cells (about 2-10 g/l according to wet biomass) in their sexual stages (yellow zone) was scraped from a Petri dish, transferred to a plastic tube and suspended in 2 ml Phosphate-buffered Saline (PBS; Oxoid, Hampshire, England). Cells were centrifuged for 10 min at 1232 *g* to remove debris and agar. The supernatant was disposed of with a Pasteur pipette. Thirty micro-liter of the monoclonal antibody (mAb; Geneway, San Diego, USA) specific for prohibitin localized in mitochondria [14] was added to the cells and then incubated for 60 min in the dark. The unbound mAb was washed off with PBS. Thirty micro-liter of the secondary antibody [fluorescein isothiocyanate (FITC) – 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. Appropriate controls were included as described by Kock *et al.* [15]. Cells were fixed on a microscope slide using 1,4-diazabicyclo [2.2.2] octane (Dabco; Sigma-

Aldrich, U.S.A.) and examined with a Nikon 2000 Confocal Laser Scanning Microscope (CLSM; Nikon, Tokyo, Japan).

Mitochondrion Mapping

This was performed according to Ncango *et al*. [16]. In short, sporulating cells were washed with PBS in a 2 ml plastic tube, to remove agar and debris, and treated with 31 µl Rhodamine 123 (Rh123; Molecular Probes, Invitrogen Detection Technologies, Eugene, Oregon, U.S.A.). Cells were treated for 1 h in the dark at room temperature after which cells were washed again with PBS to remove excess stain. These were fixed on microscope slides in Dabco (Sigma-Aldrich) and viewed with a CLSM. Rh123 is a cationic lipophilic mitochondrion stain used to map mitochondrion function ($\Delta\psi_m$) selectively. This is attributed to the highly specific attraction of this cationic fluorescing dye to the relative high negative electric potential across the mitochondrion membrane in living cells [11, 17, 18]. With this dye, a high $\Delta\psi_m$ is signified by a yellow-green fluorescence (collected at 450 nm), while a low $\Delta\psi_m$ is signified by a red fluorescence collected at 625 nm (Fig. **4c**).

Quantitative Measurement of Metabolic State

The XTT colorimetric assay was used to determine the activity of mitochondrion dehydrogenases, an indicator of metabolic activity [19 – 21], in cells of *E. ashbyi* (1.0 g/l) scraped from the c_1 and b_2 zones (Fig. **1a**) respectively. A hundred micro-litre of the standardised yeast suspension was transferred to wells of a 96-well flat bottom polystyrene microtiter plate (Corning Incorporated, NY, USA). Following this, 50 µl XTT [0.5 g XTT (Sigma Chemicals,

St. Louis, Mo.) in 1 L Ringer's lactate solution] and 4 µl menadione (Fluka, 1 mM in acetone) were added to each of these wells. Plates were incubated at 37ºC for 2 h in the dark, whereafter the formazan product in the supernatant was spectrophotometrically measured in terms of optical density at 492 nm using a Labsystems iEMS reader (Thermo BioAnalysis, Helsinki, Finland). Experiments were performed in at least triplicate.

2.4 RESULTS AND DISCUSSION

Extensive oxylipin studies expose the NSAID, aspirin as a potent anti-mitochondrion antifungal drug. This led to the Aspirin Antifungal Hypothesis forecasting the same in all yeasts and probably other fungi as well [1]. The challenge of this study became the construction of a practical and easy to use yeast bio-assay that will not only render this hypothesis a visual reality but may also be used as a first screen for potential anti-mitochondrion antifungal drugs from different sources.

In order to construct the bio-assay, the yeast *E*. *ashbyi* [4], which produces sexual reproductive structures that stains yellow due to associated riboflavin production [3], was chosen. This would make it easy to observe the results without resorting to tedious and time consuming microscopy.

When the NSAID, aspirin was applied to the bio-assay with *E. ashbyi* as indicator organism, four zones corresponding to the hypothesis [1] could be detected (Fig. **1a**). These include the no growth inhibition-zone (i), pale coloured

asexual-zone c_1, borderline-zone b_1 and yellow sexual-zone b_2. Detailed microscopic analysis indicated that an increase in ascospore release from asci in *E. ashbyi* occurred across these zones (Table **1**) i.e. over a decrease in aspirin concentration. This suggests ascospore release to be most susceptible to aspirin compared to ascus formation and asexual growth. At relatively high concentrations of aspirin (i.e. the pale coloured asexual-zone c_1), no mature asci were formed (Fig. **1a**) while at lower concentrations (yellow sexual-zone b_2; Fig. **1a**), relative high percentages of mature asci, many already empty due to ascospore release, were observed (similar to Fig. **2b**). Consequently, a large number of released sickle-shaped ascospores were also present (Fig. **2b**). Similar results were obtained for all anti-inflammatory compounds i.e. ibuprofen, indomethacin, salicylic acid, benzoic acid, LND as well as ETB (gave pink instead of pale colonies in asexual-zone) tested (Table **2**). It was recorded that ETB interferes with mitochondrial DNA and may cause the formation of respiration-deficient petite mutants at high frequency as resistance mechanism in *Candida glabrata* [22]. According to Table **2,** the Etest strips with amphotericin B and flucytosine did not preferentially inhibit the sexual phase. Furthermore, no reference to preferential anti-mitochondrial effects of amphotericin B and flucytosine could be obtained in literature.

On the basis of these results we conclude that the anti-inflammatory compounds selectively inhibit ascospore and ascus development in *E. ashbyi*, targeting ascospore release and therefore probably β-oxidation (i.e. necessary for spore release [1]) at low concentrations. These results are corroborated by

previous mammalian and plant studies that suggest that these anti-inflammatory compounds also have anti-mitochondrion activity [23-26].

In all cases the growth inhibition-zone (i) formed by the ethanol control was smaller than those obtained with the anti-inflammatory anti-mitochondrion compounds dissolved in ethanol thereby indicating the antifungal role of these compounds i.e. inhibiting both asexual and sexual reproductive phases (Fig. **1a** and **b**). Since no additional zones were observed i.e. no selective inhibition of sexual structure development or appearance of wrinkled granular hyphae, we conclude that the sterilant ethanol does not selectively target the sexual reproduction in this yeast and is therefore not anti-mitochondrial.

Oxygen limitation studies based on the Dalmau method [7] (Fig. **2d**) suggest a similar mechanism of inhibition compared to when anti-inflammatory, antifungal and anticancer drugs associated with anti-mitochondrial activity were added (Table **2**). In all cases the sexual phase development and riboflavin production were drastically inhibited while similar mainly granular and wrinkled hyphae developed (Fig. **2a, c** and **3a**) compared to the less granular more smooth walled hyphae and mature asci with sickle-shaped ascospores (Fig. **2b** and **3b**) found on the yeast lawn on the outside of the cover slide area shown in Fig. **(2d)**. Similar morphologies for hyphal and sexual cell structures were obtained from the yellow sexual-zone b_2 (Fig. **1a**).

When monoclonal antibodies (mAbs) specific for mitochondria [14] were added to sporulating cells of *E. ashbyi*, fluorescing V-shaped fins were observed

on the sickle-shaped ascospores inside the asci found in the yellow sexual b_2-zone (Fig. **4d**). This suggests that mitochondria are probably localized on the V-shaped fins where they release 3-OH oxylipins (Fig. **4a** and **b**). This is the first report showing mitochondria and oxylipins at the same structural position.

When Rh123, a mitochondrion transmembrane potential ($\Delta\psi_m$) probe [17, 27, 28] was added to the yeast (Fig. **4c**), the enlarged sexual cells (asci; dominant in zone b_2; Fig. **1a**) showed a much higher affinity for the stain compared to the hyphae (also dominant in zone c_1; Fig. **1a**). This suggests increased mitochondrion function in sexual cells. This is also highlighted by the fact that mitochondrially β-oxidation produced 3-hydroxy oxylipins (3-OH 14:0) accumulate in sexual cells where it is deposited on fin-like structures on both sides of the blunt ends of sickle-shaped ascospores necessary for boomerang movement to affect release through oxylipin lubricated piercing mechanics (Fig. **4a** and **b**) [29]. No such accumulation of Rh123 was evident in the inhibition white zone c_1 that did not contain any sexual structures (Fig. **1a**).

Also, changes in mitochondrion function in the c_1 and b_2 -zones (Fig. **1a**) were investigated using the XTT reduction assay. With this assay, XTT (a tetrazolium salt) is cleaved by various mitochondrial dehydrogenases to produce coloured formazans, which are indicators of fungal metabolic activity [30, 31]. As expected, we found that mitochondrion function in the sexual b_2-zone was significantly higher ($p < 0.001$) compared to their respective asexual zone c_1 (Table **1**; Fig. **1a**). Similar results were obtained for all anti-inflammatory compounds tested (results not shown).

When LND, an anticancer drug [11] was added to the yeast bio-assay (Table **2**), again similar hyphal morphological changes (Fig. **2c** and **3a**) occurred while the sexual stage (especially ascospore release) with associated riboflavin release was most susceptible to inhibition which suggests anti-mitochondrion activity. Strikingly, LND has been found to exert a direct effect on the mitochondrion permeability transition pore and induces a drop in mitochondrion transmembrane potential ($\Delta\psi_m$) in different mammalian cell lines. LND has been used successfully in combination chemotherapy phase II and III trials in patients with metastatic breast cancer and inoperable non-small-cell lung cancer [11].

In addition, tests were conducted with aqueous solutions of compounds on filter paper discs that do not generally target mitochondria i.e. HCl (1N), formic acid (1N) and NaOH (1N) (Table **2**). Only a small inhibition zone (i) and no selective inhibition of the sexual development and riboflavin production or change in hyphal morphology (similar to Fig. **2b** and **3b**) were observed when applying these solvents, thereby also ruling out a pH effect for the selective antifungal action of the anti-inflammatory drugs tested. Similar results were obtained when ETest strips containing amphotericin B and flucytosine were added to the bio-assay. No reports on selective anti-mitochondrial activity of these antifungals could be obtained in literature.

In this study, anti-inflammatory, antifungal and anticancer drugs that inhibit mitochondrial activity in mammalian cells were found to yield a unique growth pattern (Fig. **1a**) when added to the yeast *E. ashbyi.* As expected, this growth pattern also found under anoxic conditions, is characterized by a significant

decrease in mitochondrial metabolic activity as well as $\Delta\psi_m$. Since no such growth patterns could be found for other non anti-mitochondrial compounds tested, we conclude that these inhibitory patterns visualized as white zones may be used as indicators to identify anti-mitochondrials. This bio-assay should now be further tested with many other anti-mitochondrials and non anti-mitochondrials to evaluate the validity of this proposal (Refer to Supplementary Information). Can this yeast bio-assay be used as a first screen for anti-mitochondrial anti-inflammatory and antifungal drugs from various sources including plants and food with claims of medicinal value as well as traditional medicines? The antibacterial activity of these agents should also be determined especially since mitochondria are regarded as endosymbiotic derived Gram-negative bacteria [32]. In order to obtain high throughput screening, a possibility is to adapt the bio-assay to include various small wells on one plate testing various drugs at the same time. If water soluble, drug crystals can also be placed directly on agar plate to observe inhibition patterns.

Recently, *E. ashbyi* has been reported to cause soybean yeast-spot disease when transmitted by the stink bug *Riptortus clavatus* after feeding on infected soybean [5]. It will be interesting to assess the antifungal properties of the anti-mitochondrial compounds tested (Table **2**) in this study on infected soybean seed especially the sexual cycle which are responsible for dispersal via many ascospores produced in asci [4].

2.5 ACKNOWLEDGEMENTS

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2.7 TABLES

Table 1. Percentage ascospore release and mitochondrion function over decreased aspirin concentration-gradient [from (i) to b_2 in *Eremothecium ashbyi* UOFS Y-630].

Zone	% Ascospore Release (± SD)*	Mitochondrion Function (± SD)**
(i)	No growth	
c_1	No asci	1.5 ± 0.3
b_1	30.0 ± 1.4	
b_2	62.0 ± 2.8	2.6 ± 0.6

*% Ascospore release: [Empty asci / (Full + Empty asci) x 100].

**XTT colorimetric assay [19-21, 31].

Table 2. Compounds tested with yeast bio-assay for specialized antifungal activity.

Compound tested	Selective inhibition of sexual cells
Anti-inflammatory/anti-mitochondrial	
Aspirin	+
Ibuprofen	+
Indomethacin	+
Benzoic acid	+
Salicylic acid	+
Anticancer/ anti-mitochondrial (loss of $\Delta\psi_m$)	
Lonidamine (LND)	+
Antifungal/non anti-mitochondrial	
Amphotericin B	-
Flucytosine	-
Others	
HCl (aq)	-
Formic acid (aq)	-
NaOH (aq)	-
Ethanol (sterilant)	-
ETB (mutant inducer/anti-mitochondrial)	+
Anoxic conditions (anti-mitochondrial)	+

ETB, ethidium bromide. All compounds inhibited asexual growth at higher concentrations i.e. at start of concentration gradient.

2.8 FIGURES

Fig. (1). The Antifungal Bio-Assay. (**a**) Growth response over decreasing aspirin concentration-gradient in *Eremothecium ashbyi* UOFS Y-630 is indicated by four different zones i.e. from growth inhibition-zone (i) to pale coloured asexual-zone c_1 to transition-zone b_1 to yellow sexual-zone b_2. (**b**) Ethanol control with only a small growth inhibition-zone (i) in *Eremothecium ashbyi* UOFS Y-630.

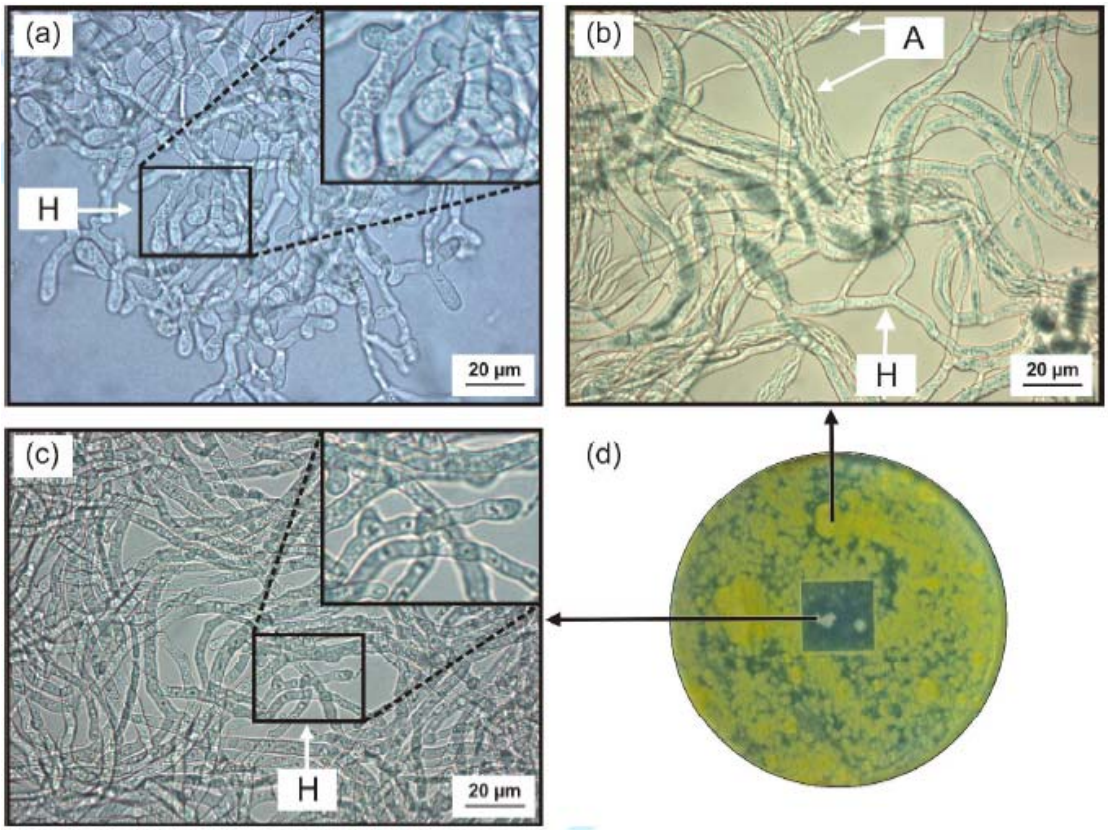

Fig. (2). Light micrographs demonstrating the similar effects of oxygen limitation and aspirin on the sexual stage of *Eremothecium ashbyi* UOFS Y-630. (**a**) Inhibition of sexual reproduction in the c_1-zone (Fig. **1a**) – mainly wrinkled and granular hyphae (H) visible. (**b**) Asci (A) with ascospores and more smooth hyphae obtained from the yellow lawn outside cover slide area (**d**). Similar results were obtained from the yellow b_2-zone (Fig. **1a**). (**c**) Mainly wrinkled hyphae (similar to (**a**)), from underneath the cover slide (*i.e.* anoxic pattern). (**d**) Dalmau plate method with cover slide (covering pale asexual cells with similar morphology as c_1-zone – Fig. **1a**) in middle of yellow lawn (latter has similar morphology as b_2-zone – Fig. **1a**).

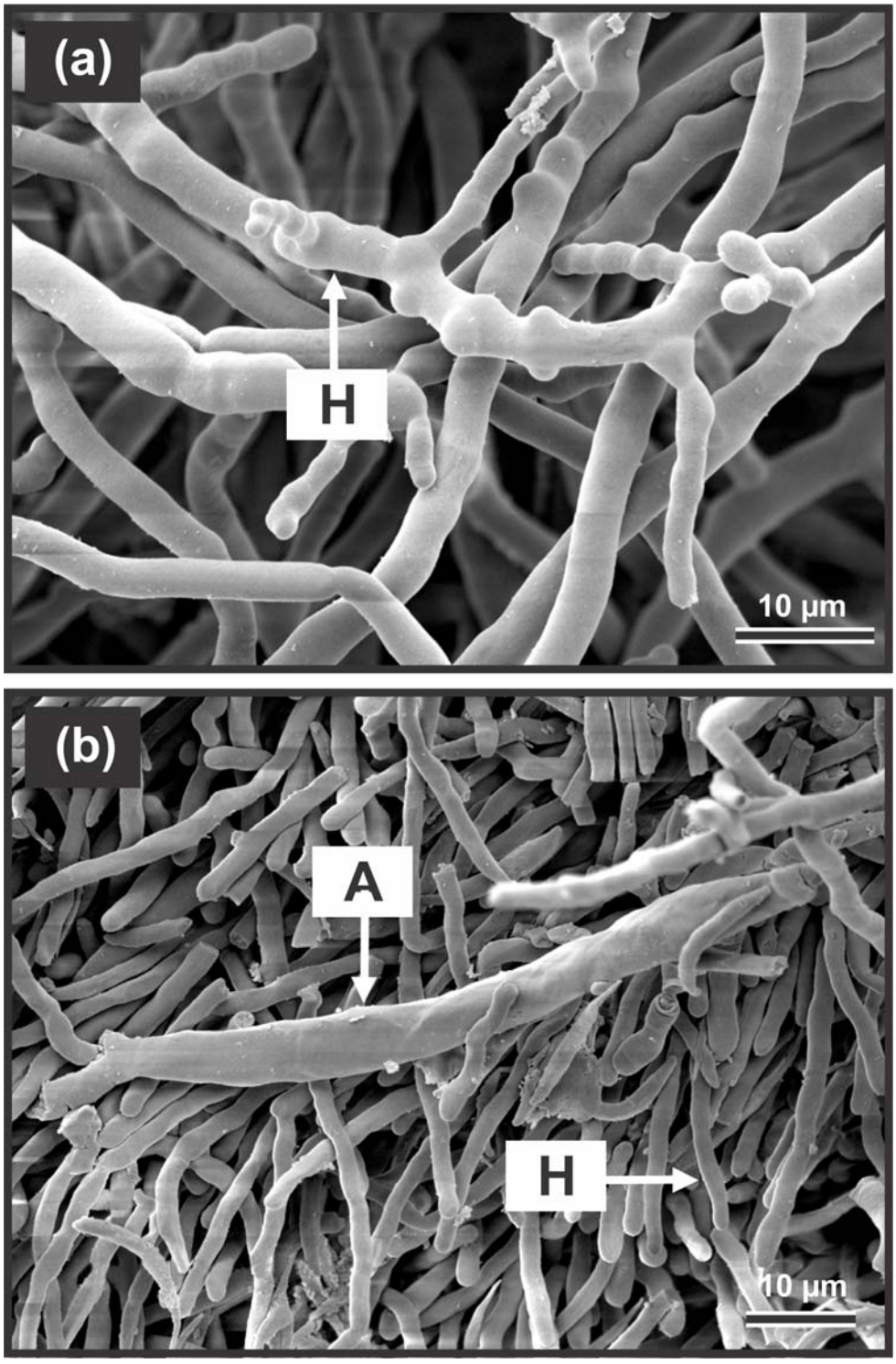

Fig. (3). Scanning electron micrographs of *Eremothecium ashbyi* UOFS Y-630 scraped from white asexual-zone c_1 showing only wrinkled and granular hyphae (**a**) and yellow sexual-zone b_2 showing matured full asci and well developed hyphae (**b**). A, asci; H, hyphae.

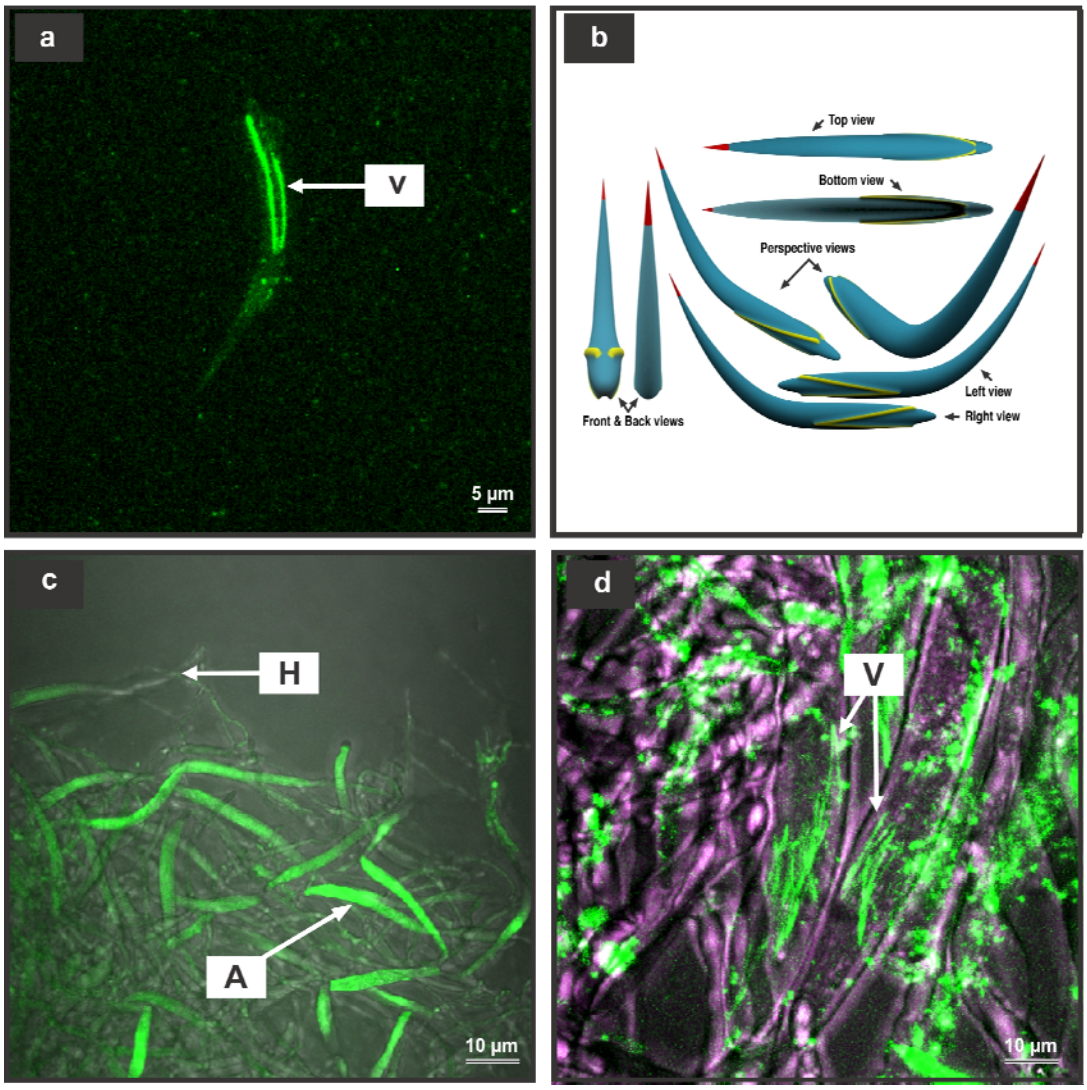

Fig. (4). Fluorescence confocal laser scanning micrographs of *Eremothecium ashbyi* UOFS Y-630 scraped from yellow sexual-zone b_2 (Fig. **1a**). (**a**) Stained with 3-hydroxy (OH) oxylipin antibodies (Abs) coupled to fluorescing secondary Abs. (**b**) 3-D simulated sickle-shaped ascospores indicating morphology and position of 3-hydroxy oxylipins and mitochondrial activity on fin-like structures on both sides of ascospores (in yellow). Figure **4a** and **b** taken with permission from Kock *et al.* [29]. (**c**) Stained with Rhodamine 123. (**d**) Stained with monoclonal antibodies (mAbs) specific for mitochondria [14]. A, ascus with ascospores; H, hyphae; V, fluorescing V-shaped fins.

2.9 SUPPLEMENTARY INFORMATION

To evaluate the efficacy of the bio-assay (Fig. **1a**) as a first screen for anti-mitochondrial antifungal drugs, different compounds were tested (Table **1S**). Compounds known in literature to have anti-mitochondrial properties [1, 10, 24, 25, 33, 34, 35, 36, 37, 38, 39, 40], all selectively inhibited the sexual phase development i.e. yellow sexual-zone b_2. Other compounds tested that did not selectively inhibit the sexual phase development have also not been reported in literature to have anti-mitochondrial properties. These should now be further researched for possible effects on mitochondria and the database expanded by testing more antifungals.

It is interesting to note that all the anti-inflammatory compounds tested, except for Fenbufen, Indoprofen, Mefenamic acid and Tenoxicam as well as the antimalarials Chloroquine and Quinine, showed anti-mitochondrial activity. All of the antifungals but only Pepperbark Tincture, of all the Traditional Medicines tested, inhibited mitochondrial activity. The known anti-mitochondrial compounds Antimycin A [39] and Ethionine [40] as well as the plant extracts Capsaicin [37] and Curcumin [38] also demonstrated anti-mitochondrial activities. Strikingly, all compounds tested (Table **1S**) inhibited asexual growth at higher concentrations i.e. at start of concentration gradient.

Table 1S. Compounds tested with yeast bio-assay for specialized antifungal activity.

Compounds Tested	Selective Inhibition of Sexual Cells
Anti-inflammatory (8% m/v)	
Acemetacin*	+
Diclofenac*	+
Diflunisal*	+
Etodolac	+
Fenbufen	-
Fenoprofen*	+
Flurbiprofen*	+
Flufenamic acid*	+
Indoprofen	-
Ketorolac	+
Meclofenamic acid*	+
Mefenamic acid	-
Methyl salicylate*	+
Nabumetone	+
Naproxen*	+
Piroxicam	+
Phenylbutazone	+
Salicylamide*	+
Sulfinpyrazone	+
Sulindac	+
Tenoxicam	-
Tolfenamic acid*	+
Tolmetin	+

Antimalaria (8% m/v)	
Artermisinin*	+
Chloroquine	-
Quinine	-
Thapsigargin	+
Antifungals (ETest gradient strips)	
Caspofungin* (MIC reading scale: 0.002 to 32 µg/mL)	+
Fluconazole* (MIC reading scale: 0.016 to 256 µg/mL)	+
Itraconazole* (MIC reading scale: 0.002 to 32 µg/mL)	+
Ketoconazole* (MIC reading scale: 0.002 to 32 µg/mL)	+
Posaconazole* (MIC reading scale: 0.002 to 32 µg/mL)	+
Voriconazole* (MIC reading scale: 0.002 to 32 µg/mL)	+
Traditional medicines	
African ginger (*Siphonochillus aethlopicus*)	-
African potato tincture (*Hemerocallidea*)	-
Natural antibiotic (*Pelargonium sidoides*)	-
Sutherlandia (*Sutherlandia frutescens*)	-
Vuka (African *Helichrysum*)	-
Pepperbark tincture (*Warburgia salutaris*)	+
Warburgia (*Warburgia salutaris*)	-
Plant extracts (8% m/v)	
Capsaicin*	+
Curcumin*	+
Others (8% m/v)	
DMSO (Solvent)	-
Antimycin A*	+
Ethionine*	+

DMSO, Dimethyl Sulfoxide; MIC, Minimum Inhibitory Concentration; *, anti-mitochondrial [1, 10, 24, 25, 33, 34, 35, 36, 37, 38, 39, 40]; +, zone present; -, zone absent. All compounds inhibited asexual growth at higher concentrations i.e. at start of concentration gradient.

CHAPTER 3

The effect of mitochondrial inhibitors on *Aspergillus* and *Rhizopus*

This part of the study has been published in African Journal of Microbiology Research **4(9):** 830-835 (2010) and written in this journal's format.

3.1 Abstract

We investigated the effects of anti-inflammatory and anti-mitochondrial compounds on spore dispersal in the pathogens *Aspergillus fumigatus* and *Rhizopus oryzae*. When acetylsalicylic acid (ASA) and other non-steroidal anti-inflammatory drugs (NSAIDs) were added to bio-assays of *A. fumigatus* and *R. oryzae*, spore-releasing structures were targeted first at lower concentrations. Similar results were obtained when oxygen was limited. These spore-releasing structures contained increased levels of mitochondrion activity compared to hyphae. We conclude that increased mitochondrion activity is necessary for dispersal of *A. fumigatus* and *R. oryzae*.

Key words*: Aspergillus fumigatus*, bio-assay, mitochondrion activity, non-steroidal anti-inflammatory drugs, *Rhizopus oryzae*, spore-releasing structure.

3.2 Introduction

An antifungal yeast hypothesis that links acetylsalicylic acid (ASA) sensitivity, mitochondrion function and sexual reproduction in strict respiring and non-respiring yeasts was published in 2007 (Kock et al., 2007). Based on this hypothesis, Kock and co-workers (2009) developed a yeast bio-assay to screen for anti-mitochondrial drugs.

Here, the ascomycotan yeast, *Eremothecium ashbyi* was used as an indicator organism. When anti-mitochondrial drugs, such as ASA were added to cultures of this yeast, the most susceptible stage was the formation of asci and concomitant riboflavin production. Riboflavin production is indicated by yeast colonies turning yellow. Consequently, de-colorization of yeast colonies was used as indicator in this bioassay to detect anti-mitochondrial activity.

Based on this work, Leeuw et al. (2009) developed a similar bio-assay using the zygomycotan fungus, *Mucor circinelloides* as indicator organism. In this study, sporangium development was reported to be most sensitive to anti-mitochondrial drugs. As expected, these authors showed that young sporangia contain increased mitochondrion activity when compared to hyphae and that ASA selectively inhibits structures with increased mitochondrion activity. Mitochondria are probably necessary to produce sufficient energy for development of these multi-celled spore-releasing-structures.

In this study the human pathogens *Aspergillus fumigatus* and *Rhizopus oryzae* were used to further assess the conserved status of this phenomenon.

Here, mitochondrion activity and the susceptibility of spore-releasing-structures towards anti-mitochondrials compared to hyphae are reported. This may be of importance to combat pathogenic fungal dispersal.

3.3 Materials and Methods

Strains used

Aspergillus fumigatus UOFS Y-2808 and *Rhizopus oryzae* UOFS Y-2807 were used in the study and are preserved at the UNESCO Mircen Culture Collection, Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Bloemfontein, South Africa.

Cultivation

All fungi were cultivated for 48 h on yeast-malt (YM) agar (Wickerham, 1951) at 25 ^{0}C in Petri dishes until spore-releasing-structures in *A. fumigatus* and *R. oryzae* were observed.

Bio-assay preparation

Each fungus was separately suspended in sterilized distilled water (dH_20) and 0.2 ml streaked out on YM (0.5 % m/v agar). This produced a homogenous lawn across the surface of the agar (Kock et al., 2009). Next, a well (0.5 cm in diameter and depth) was constructed at the center of the Petri dish and 46 µl of the following compounds added, that is, 2 g in 25 ml 96 % ethanol (Merck, Gauteng, South Africa); ASA (aspirin: Sigma, Steinheim, Germany), ibuprofen (Sigma-Aldrich, Steinheim, Germany), indomethacin (Sigma, Steinheim,

Germany), salicylic acid (The British Drug Houses Ltd., Poole, England), diflunisal (Sigma-Aldrich, Steinheim, Germany), salicylamide (Sigma-Aldrich, Steinheim, Germany) and benzoic acid (The British Drug Houses Ltd., Poole, England). Ethanol (96 %) was also added alone to the wells as a control. All plates where incubated at 25 ^{0}C until different textured growth zones where observed (usually after 48 h). A light microscope (Axioplan, Zeiss, Göttingen, Germany) coupled to a Colorview Soft Imaging System (Münster, Germany) was used to study each zone.

Mitochondrion distribution

Fungal cells (about 2-10 g/l according to wet biomass) were scraped from previous 48 h old YM plates and suspended in 2 ml Phosphate-buffered Saline (PBS; Oxoid, Hampshire, England). Next, cells were centrifuged for 10 min at 1232 *g* to remove debri and agar while the supernatant was removed. Thirty micro-liter of the monoclonal antibody (mAb; Geneway, San Diego, USA) specific for prohibitin localized in mitochondria (Ikonen et al., 1995) was added and the cell suspension then incubated for 60 min in the dark. The unbound mAb were washed off with PBS. Thirty micro-liter of the secondary antibody [fluorescein isothiocyanate (FITC) – conjugated secondary antibody; Sigma-Aldrich, U.S.A.] was added 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. Appropriate controls were included as described by Kock et al. (1998). Cells were fixed on a microscope slide using 1,4-diazabicyclo [2.2.2] octane (Dabco; Sigma-Aldrich, U.S.A.) and examined with a Nikon 2000

Confocal Laser Scanning Microscopy (CLSM; Nikon, Tokyo, Japan) as described by Kock et al. (2009).

Mitochondrion activity mapping

Oxidation products

This was performed on *A. fumigatus* and *R. oryzae* according to Kock et al. (1998). Cells (2-10 g/l according to wet biomass) of both fungi were removed from previously described YM plates and transferred to plastic tubes and suspended in 2 ml PBS. Cells were then centrifuged for 10 min to remove debris while the supernatant was removed by Pasteur pipette. Thirty micro-liter of the 3-hydroxy (OH) oxylipin specific primary antibody was added to the cells and then incubated for 60 min in the dark. The unbound primary antibodies were removed with PBS. Thirty micro-liter of the secondary antibody was added to the treated cells and again incubated for 60 min in the dark. Unbound FITC secondary antibody was removed with PBS as described before. To maintain cell structure, the antibody, fluorescence and wash treatments were performed in 2 ml plastic tubes. Appropriate controls were included (Kock et al., 1998). Cells were fixed on a microscope slide using Dabco and examined with a Nikon 2000 CLSM.

Transmembrane potential ($\Delta\psi_m$)

This was performed according to Ncango et al. (2008). In short, fungal cells of both fungi were scraped from YM plates. To remove agar and debris, fungal cells were washed separately with PBS in 2 ml plastic tubes and then treated with 31 µl Rhodamine 123 (Rh 123; Molecular Probes, Invitrogen Detection Technologies, Eugene, Oregon, U.S.A.). Cells were treated for 1 h in the dark at

room temperature after which cells were washed again with PBS to remove excess stain. These were fixed on microscope slides in Dabco and viewed with a Nikon 2000 CLSM.

Scanning electron microscopy

Scanning electron microscopy (SEM) was carried out according to Van Wyk and Wingfield (1991). Cells of *A. fumigatus* and *R. oryzae* were fixed using 3 % v/v of a sodium phosphate buffered glutardialdehyde (Sigma-Aldrich, St. Louis, Mo., U.S.A.) solution at pH 7.0 and a similarly buffered solution (1 % m/v) of osmium tetroxide (Sigma-Aldrich, St. Louis, Mo., U.S.A.) for 1 h. After this, the material was dehydrated in a graded series of ethanol solution. Next, 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 Jeol WINSEM (JSM 6400) SEM (Jeol, Tokyo, Japan).

Quantitative measurement of metabolic state

The XTT colorimetric assay was used to determine the activity of mitochondrion dehydrogenases, an indicator of metabolic activity (Bachmann et al., 2002; Strauss et al., 2007; Moss et al., 2008). Here cells of *A. fumigatus* and *R. oryzae* were scraped off from different textured zones on agar diffusion plates (bio-assay). Five milli-liters of PBS were used to suspend 1 g of cells from each respective zone. Following this, 2.5 ml XTT [2.5 g XTT (Sigma Chemicals, St. Louis, Mo., U.S.A.) in 1 L Ringer's lactate solution] and 400 µl menadione (Fluka, 1 mmol/L in acetone) were added. Cells were incubated at 37 ºC for 3 h in the dark. A 96-well, flat bottom polystyrene microtiter plate (Corning Incorporated,

NY, U.S.A.) was used and 150 µl of the formazan product transferred to each well and the formazan product in the supernatant spectrophotometrically measured in terms of optical density at 492 nm using a Labsystems iEMS reader (Thermo BioAnalysis, Helsinki, Finland). This was repeated on cells grown under oxygen limitation and normal oxic conditions as described.

Oxygen inhibition studies

Cells of both fungi were spread out onto YM agar plate to form a homogenous lawn as described for bio-assay preparation and placed in an Oxoid anaerobic jar (Oxoid, Cambridge, U.K.), cultivated at 25 ^{0}C for 48 h. Oxygen was removed with the use of Anaerocult A System (Merck, Darmstadt, Germany). To monitor the anaerobic atmosphere in the sealed jar, an Anaerotest Test-strip (Merck, Darmstadt, Germany) was used. Control cultures were placed in normal atmosphere. Cells from YM agar plates were examined with the light microscope. All experiments were performed in at least triplicate.

3.4 Results

In this study, different tests were performed and they showed the dependence of spore dispersal on mitochondrion activity when mitochondrion activity was inhibited, so was the formation of multiple spores.

Mitochondrion distribution and activity

When monoclonal antibodies (mAb) against mitochondria (Ikonen et al., 1995) were added to 48 h old cells of *A. fumigatus* and *R. oryzae*, we observed

increased amounts of mitochondria in phialides (Figure 1A) and sporangia (Figure 1B), respectively, when compared to hyphae (results not shown). A similar trend was found when polyclonal antibodies (pAb) against mitochondrial β-oxidation products (3-OH oxylipins) were added (Figure 1C and D). This indicates increased mitochondrion activity in these spore-releasing-structures. These results were corroborated by increased fluorescence in phialides and sporangia respectively when Rh 123, a stain that tracks mitochondrion activity (transmembrane potential; $\Delta\psi_m$) was added (Figure 1E and F).

Mitochondrion inhibition

When ASA (Tables 1 and 2), known to inhibit mitochondria (Somasundaram et al., 2000; Norman et al., 2004; Lal et al., 2009) was applied to the bio-assays of *A. fumigatus* and *R. oryzae*, three zones were observed (Figures 2-5), that is, inhibition zone (I), hyphal zone (H; fruiting structures/sporangia absent) and hyphal and fruiting structure/sporangia zone (H+Fs/SP). When ethanol (EtOH) was tested as a control, smaller zones of inhibition were observed and fruiting structure/sporangia development were not selectively inhibited (Figures 2H and 3H; Tables 1 and 2).

Detailed microscopic and ultrastructural studies of *A. fumigatus* across the ASA gradient showed that, at higher concentrations that is, H zone (Figures 4A and C), spore-releasing-structures that is, fruiting structure development was selectively inhibited. Here, hyphal cell walls appeared wrinkled compared to lower ASA concentrations that is, H+Fs zone (Figure 4B and D) where well developed smooth hyphal cell walls were observed. Benzoic acid produced the largest inhibition zone while indomethacin produced a hyphal zone without an

inhibition zone (Figure 2C and E). Interestingly, salicylamide produced a yellow colored hyphal zone while small petit colonies were formed in the inhibition zone where ibuprofen was applied (Figure 2). All NSAIDs tested, except for indomethacin produced the three zone pattern (Table 1).

In *R. oryzae*, spore-releasing-structures, that is, sporangium development was again inhibited at higher concentration of ASA, that is, H zone (Figure 5A and C) while hyphae had a wrinkled appearance. This is in contrast to lower mitochondrion inhibitor concentrations, that is, H+SP zone (Figure 5B and D) where sporangium development was not inhibited and well developed smooth walled hyphae were observed. Here ASA produced the largest inhibition zone while crystals were formed in the inhibition zones produced by ASA, ibuprofen, indomethacin and diflunisal (Figure 3A, D, E and F). Similar results were obtained for the other NSAIDs tested (Table 2).

XTT reduction assay studies performed on different growth zones of *A. fumigatus* treated with ASA showed significantly higher ($p < 0.001$) mitochondrion activity in the H+Fs zone (0.4 ± 0.01 measured at 492 nm) compared to the H-zone (0.3 ± 0.01 measured at 492 nm). Similar results were obtained for *R. oryzae* (H+SP zone: 0.4 ± 0.03 and H zone: 0.2 ± 0.01; all measured at 492 nm). When oxygen was limited, similar inhibitory effects were observed for both fungi at increased ASA concentrations (compare Figures 6A, 7A with Figures 4A, C, 5A and C).

3.5 Discussion

Previously we showed that spore-release-structures such as yeast asci and sporangia with increased mitochondrion activity are more sensitive to mitochondrial inhibitors compared to vegetative cells and hyphae (Kock et al., 2007; Leeuw et al., 2009). This may be of value in combating fungi that depend mainly on these structures for dispersal.

It was found that the pathogenic fungi, *A. fumigatus* and *R. oryzae* are also dependent on increased mitochondrion activity to effect spore-release-structure development. It is concluded that commonly used NSAIDs also target the development of these structures probably by decreasing energy production necessary for normal development and spore dispersal. This provides a dual function to these compounds, that is, anti-inflammatory as well as antifungal.

The incidence of human fungal infection, particularly those caused by *Aspergillus* spp. and *Rhizopus* spp., has continued to increase in immuno-compromised individuals (Kamei, 2000; Mircus et al., 2009). However, antifungal agents used for treatment of invasive aspergillosis and zygomycosis respectively, are limited. Resistance to currently available drugs is more prevalent. Furthermore, drug toxicity is also a problem because the same cellular machinery is operative in both fungi and mammalian cells (Ghannoum and Rice, 1999). The need to develop novel drugs and also novel fungal-specific target sites has therefore become a priority (Kock et al., 2007; Mircus et al., 2009; Trofa et al., 2009). This study suggests that NSAIDs target spore dispersal that is associated with increased mitochondrion activity.

In future, more NSAIDs and other anti-mitochondrial compounds should be screened with these bio-assays and the corresponding minimum inhibitory concentrations (MICs) determined and evaluated for possible *in vivo* application in combating fungal pathogens. However, the prolonged use of NSAIDs should be cautioned since some may results in gastrotoxicity (Wolfe et al., 1999). Recently, a patent has been registered by the Kock-group describing the daily continuous use of non-toxic, low dose aspirin and diclofenac in the prevention of opportunistic fungal infections in humans who are immune compromised (Davis et al., 2009).

3.6 Acknowledgements

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3.8 Tables

Table 1. Effects of different non-steroidal anti-inflammatory drugs (NSAIDs) on the life cycle (hyphae and fruiting structure) of *Aspergillus fumigatus.*

	Zones		
Compounds tested	**I**	**H**	**H+Fs**
Acetylsalicylic acid (ASA)	√	√	√
Salicylic acid (SA)	√	√	√
Benzoic acid (BA)	√	√	√
Ibuprofen (IB)	√	√	√
Indomethacin (INDO)	**X**	√	√
Diflunisal (DI)	√	√	√
Salicylamide (SAA)	√	√	√
Ethanol (EtOH)	√	**X**	√

I, Inhibition zone; H, Hyphal zone; H+Fs, Hyphae + Fruiting structure; √, zone present; X, zone absent.

Table 2. Effects of different non-steroidal anti-inflammatory drugs (NSAIDs) on the life cycle (hyphae and sporangia development) of *Rhizopus oryzae.*

	Zones		
Compounds tested	**I**	**H**	**H+SP**
Acetylsalicylic acid (ASA)	√	√	√
Salicylic acid (SA)	√	√	√
Benzoic acid (BA)	√	√	√
Ibuprofen (IB)	√	√	√
Indomethacin (INDO)	√	√	√
Diflunisal (DI)	√	√	√
Salicylamide (SAA)	√	√	√
Ethanol (EtOH)	√	**X**	√

I, Inhibition zone; H, Hyphal zone; H+SP, Hyphae + Sporangia; √, zone present; X, zone absent.

3.9 Figures

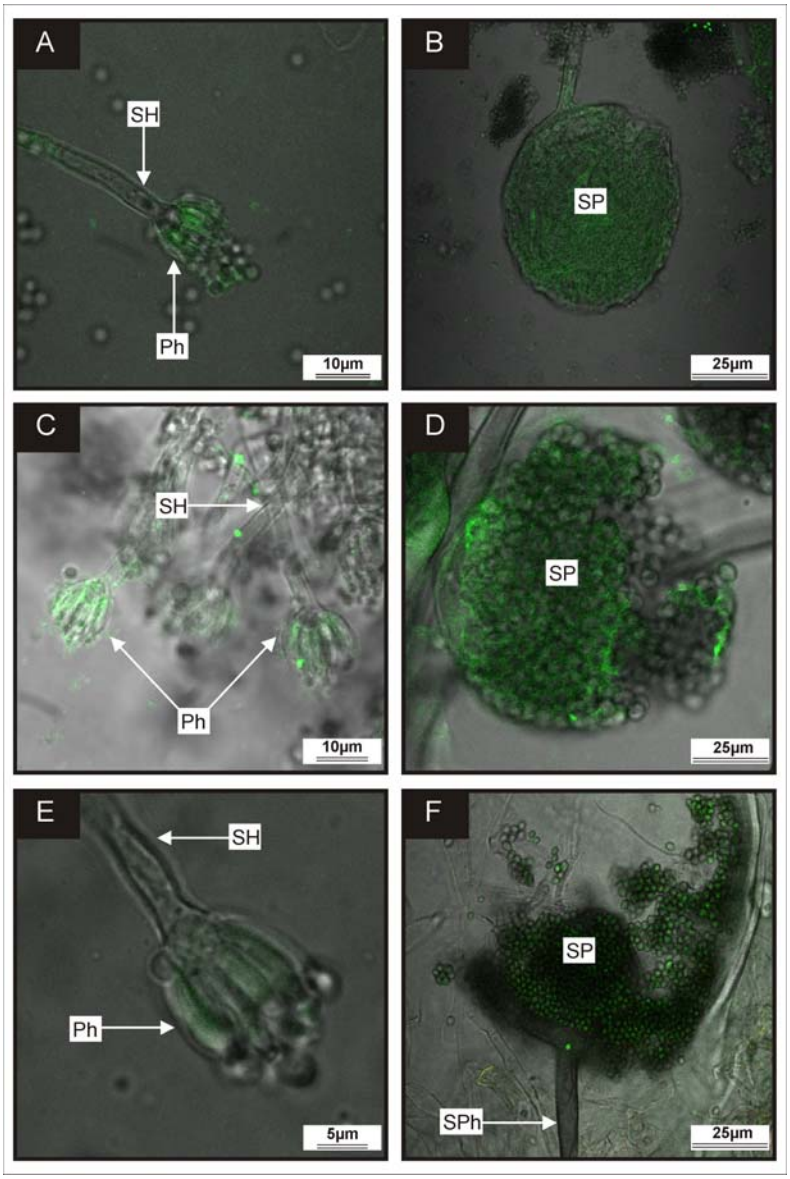

Figure 1. Confocal laser scanning micrographs of *Aspergillus fumigatus* (A, C and E) and *Rhizopus oryzae* (B, D and F). Cells stained with monoclonal antibodies specific for mitochondria (A and B). Cells treated with 3-hydroxy oxylipin antibodies and fluorescein secondary antibody (C and D). Cells stained with Rhodamine 123 (E and F). Ph, phialides; SH, specialized hyphae; SP, Sporangia; SPh, Sporangiophore.

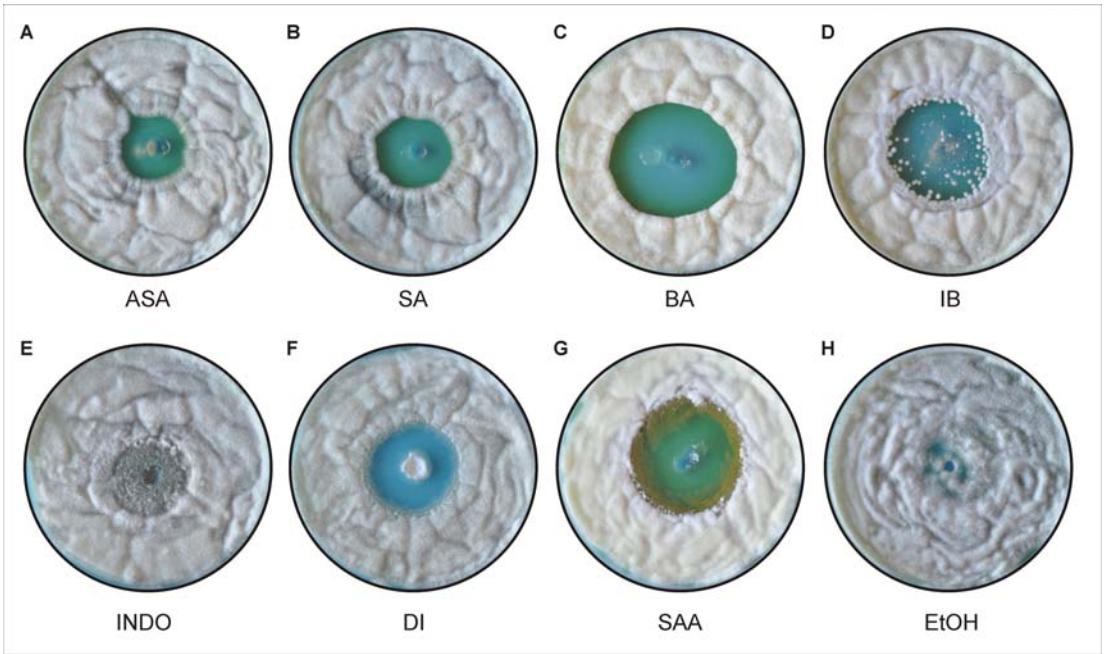

Figure 2. Bio-assays of *Aspergillus fumigatus* showing effects of different non-steroidal anti-inflammatory drugs (NSAIDs). (A) Acetylsalicylic acid (ASA), (B) Salicylic acid (SA), (C) Benzoic acid (BA), (D) Ibuprofen (IB), (E) Indomethacin (INDO), (F) Diflunisal (DI), (G) Salicylamide (SAA) and (H) Ethanol (EtOH) control.

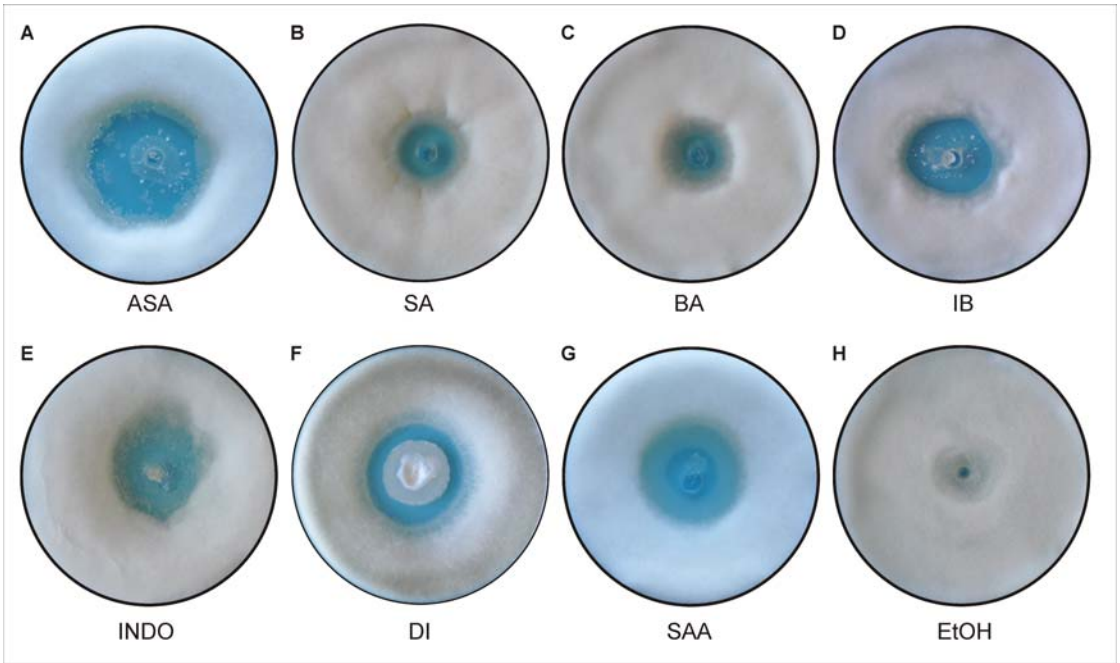

Figure 3. Bio-assays of *Rhizopus oryzae* showing effects of different non-steroidal anti-inflammatory drugs (NSAIDs). (A) Acetylsalicylic acid (ASA), (B) Salicylic acid (SA), (C) Benzoic acid (BA), (D) Ibuprofen (IB), (E) Indomethacin (INDO), (F) Diflunisal (DI), (G) Salicylamide (SAA) and (H) Ethanol (EtOH) control.

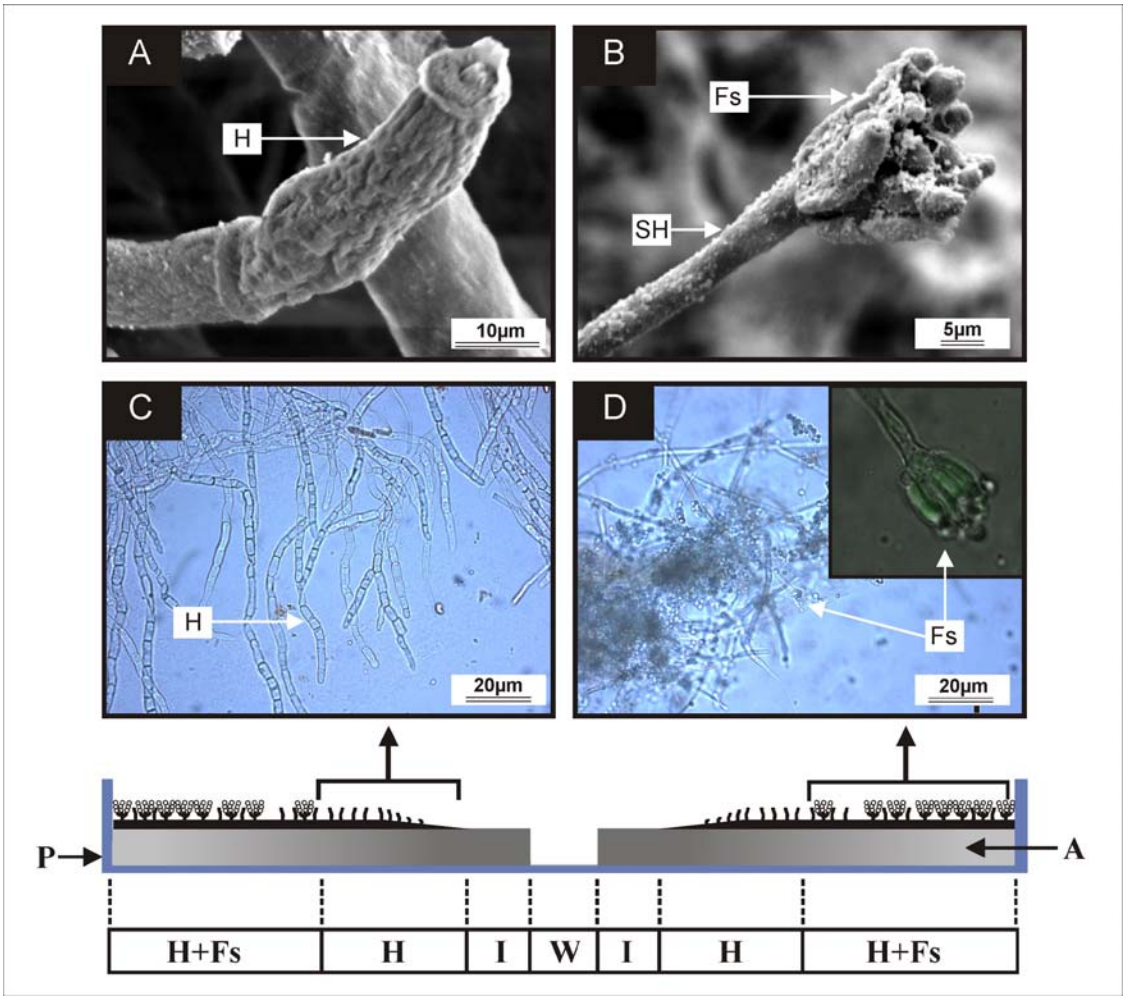

Figure 4. Detailed microscopic and ultrascopic analysis of *Aspergillus fumigatus* bio-assay plate (P) showing a well (W) and different growth zone as acetylsalicylic acid (ASA) concentration decreases across the agar (A) from the center to the periphery of the agar plate, that is, Inhibition zone (I), Hyphal zone only (H; A, C) and Hyphae & Fruiting structure (H+Fs; B, D).

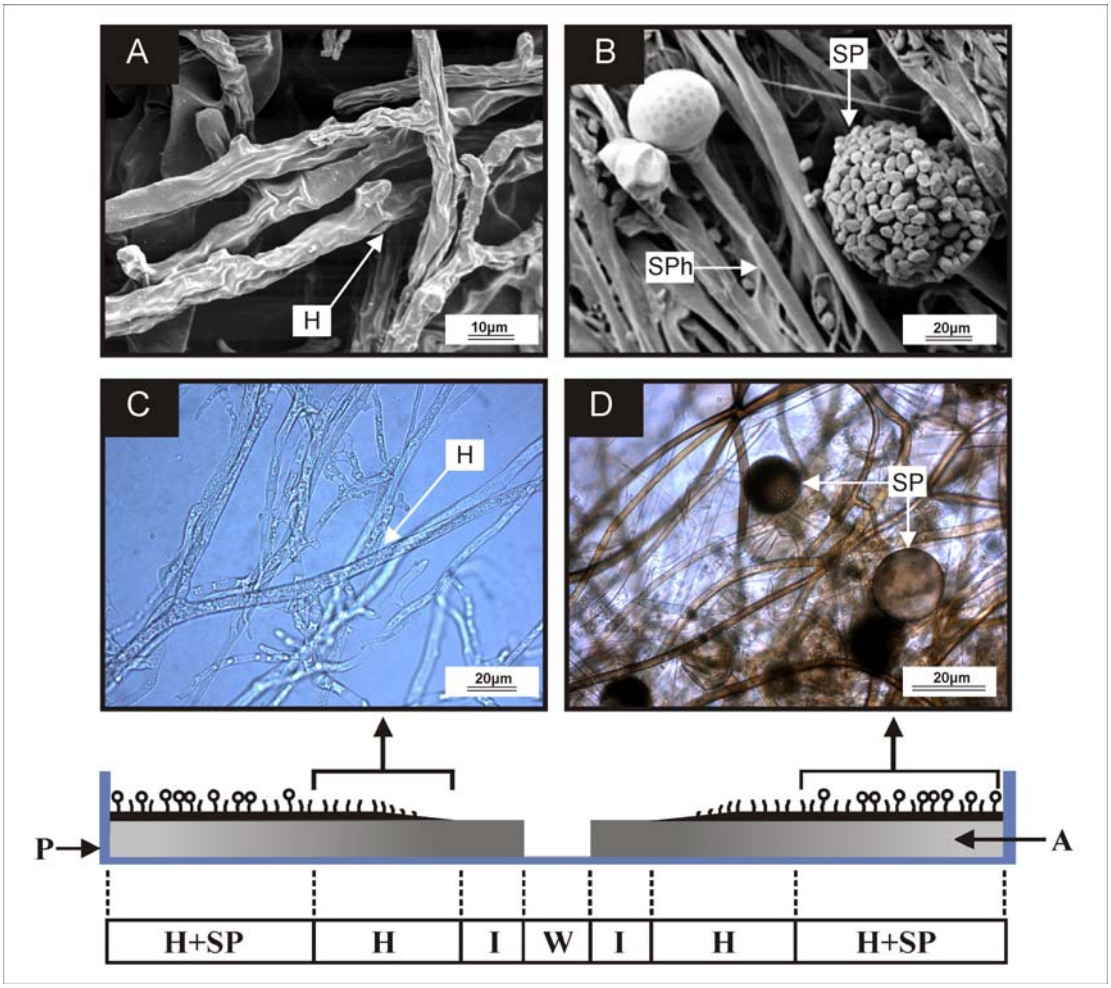

Fig. 5 Detailed microscopic and ultrascopic analysis of *Rhizopus oryzae* bio-assay plate (P) showing a well (W) and different growth zones as acetylsalicylic acid (ASA) concentration decreases across the agar (A) from the center to the periphery of the agar plate, that is, Inhibition zone (I), Hyphal zone only (H; A, C) and Hyphae & Sporangia zone (H+SP; B, D).

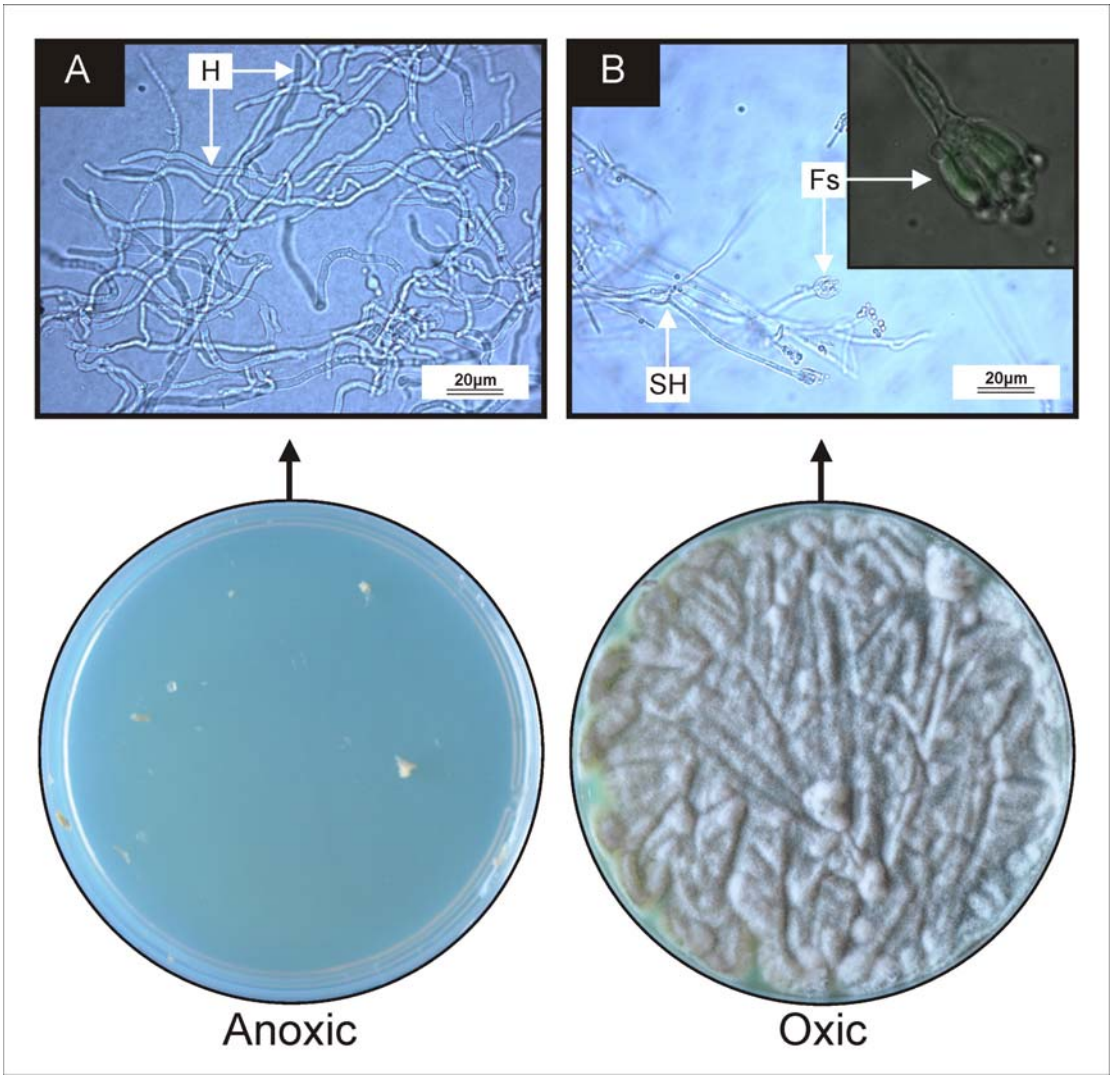

Fig. 6 Different morphological forms when oxygen is limited in *Aspergillus fumigatus.* (A) Inhibition of spore-releasing-structures, that is, fruiting structure development under anoxic conditions and (B) fruiting structure development under oxic conditions. Fs, Fruiting structure; H, Hyphae; SH, Specialized Hyphae.

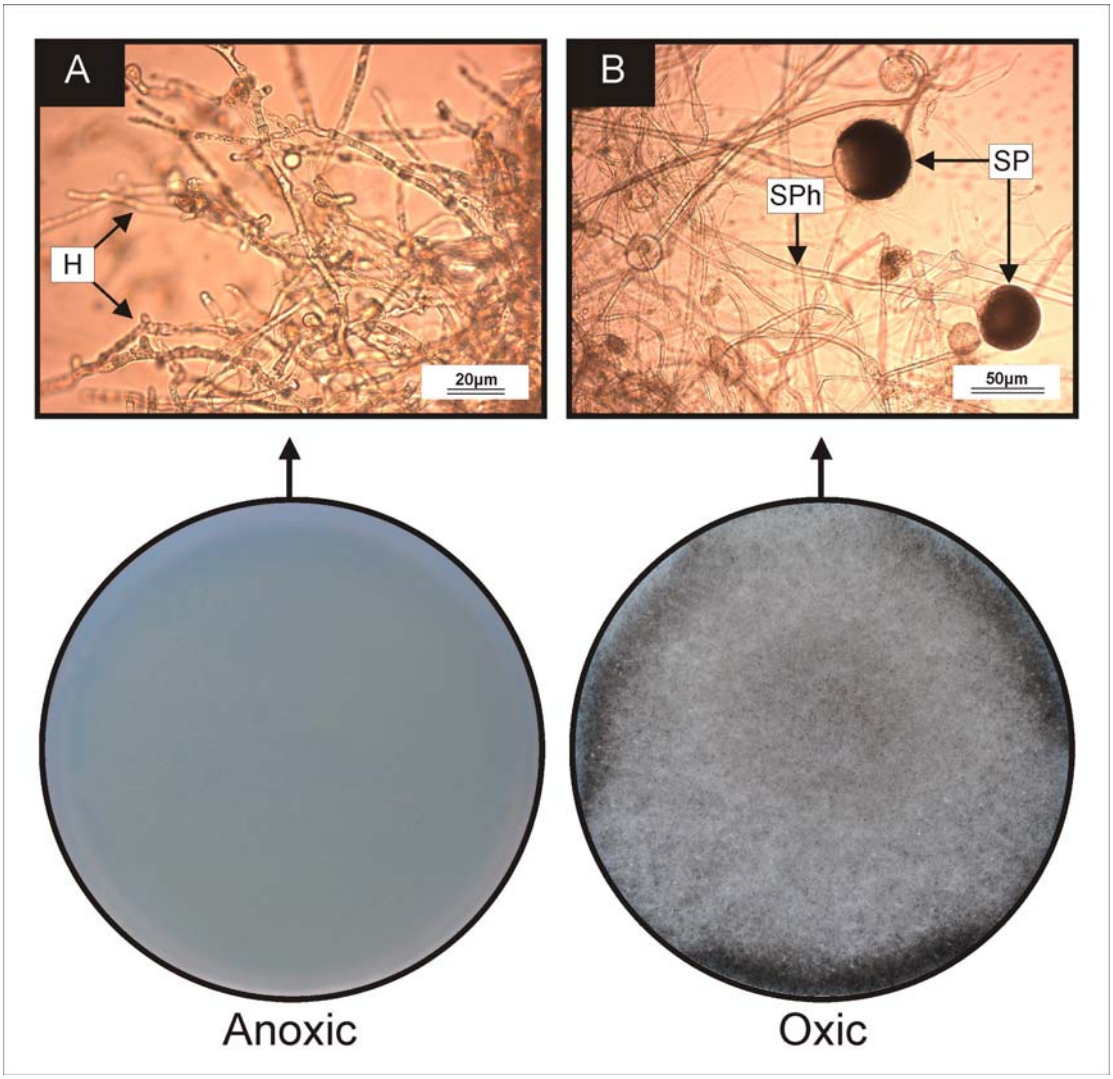

Fig. 7 Different morphological forms when oxygen is limited in *Rhizopus oryzae.* (A) Inhibition of spore-releasing-structures, that is, absence of sporangia development under anoxic conditions and (B) sporangia development under oxic conditions. H, Hyphae; SPh, Sporangiophore; SP, Sporangia.

SUMMARY

In 2007, the Kock group published the Aspirin Antifungal Hypothesis showing a clear link between oxylipin production, mitochondrial activity and acetylsalicylic acid (ASA, aspirin) sensitivity in respiring as well as non-respiring yeasts. This hypothesis suggests that mitochondrial inhibitors such as ASA selectively inhibits parts of yeast life cycles, especially the sexual stage. According to the hypothesis, mitochondrial β-oxidation products such as 3-OH oxylipins are present in elevated amounts in yeast sexual structures (asci) and lesser amounts in vegetative asexual structures (hyphae and single cells). This suggests increased mitochondrial activity in asci. Consequently, ascomycetous yeast sexual structures should be more sensitive to mitochondrial inhibitors compared to vegetative asexual structures. The purpose of the study became to assess if the Aspirin Antifungal Hypothesis could be expanded to also include other mitochondrial inhibiting drugs as well as other structures present in fungal life cycles where increased mitochondrial activities are expected. In this study, the anti-inflammatory drugs ASA, ibuprofen, indomethacin, salicylic acid and benzoic acid as well as anticancer drugs such as Lonidamine, also known for inhibiting mitochondrial activity in mammalian cells, were found to be antifungal and specifically target the sexual stage of yeast. This is shown by a unique yeast bio-assay, with the mitochondrion-dependent sexual dispersal structure producing many ascospores, riboflavin production, and hyphal morphology of the notorious yeast plant pathogen *Eremothecium ashbyi* serving as indicators. These drugs affect this yeast in a similar way as found under oxygen limitation conditions by inhibiting sexual structure development (most sensitive), riboflavin production, and yielding characteristically wrinkled and granular hyphae, presenting a unique “anoxic” morphological pattern. Only drugs associated with mitochondrial

inhibiting activity presented such a pattern. This bio-assay may find application in the preliminary screening for novel drugs from various sources with possible mitochondrial inhibiting actions. In another part of the study, the effects of anti-mitochondrial compounds on asexual fungal spore dispersal structures in the pathogens *Aspergillus fumigatus* and *Rhizopus oryzae* were investigated. When anti-mitochondrial ASA and other anti-mitochondrial non-steroidal anti-inflammatory drugs (NSAIDs) were added to *A. fumigatus* and *R. oryzae*, asexual fungal spore-releasing structures were targeted first at lower concentrations. Similar results were obtained when oxygen was limited. These asexual fungal spore-releasing structures contained increased levels of mitochondrial activity compared to hyphae. Increased mitochondrial activity may be necessary for the formation of asexual fungal spore dispersal structures of these fungi. Consequently, mitochondrial inhibitors may serve as effective antifungals to combat asexual fungal spore dispersal of these pathogenic fungi. In this study, the Aspirin Antifungal Hypothesis is expanded to also include various anti-inflammatory compounds, anticancer drugs, plant extracts, traditional medicines and others – many showing anti-mitochondrial activity. These compounds should be further investigated to determine their minimum inhibitory concentrations (MICs) and application to combat plant and human fungal pathogens. In this study, the hypothesis is also expanded to include asexual fungal dispersal structures with increased mitochondrial activity.

Key words: Antifungal, *Aspergillus fumigatus,* Aspirin Antifungal Hypothesis, *Eremothecium ashbyi*, fungal life cycles, mitochondrial activity, mitochondrial inhibitors, non-steroidal anti-inflammatory drugs, *Rhizopus oryzae*, spore-releasing structure.

OPSOMMING

Die Kock-groep het in 2007 die Aspirien Antifungale Hipotese gepubliseer en daarmee ’n duidelike verband tussen oksilipienproduksie, mitochondriale aktiwiteit en sensitiwiteit vir asetielsalisielsuur (ASS, aspirien) in respirerende en nie-respirerende giste aangetoon. Die hipotese stel dit dat mitochondriale inhibeerders soos ASS dele van gislewensiklusse, veral die geslagtelike fase, selektief inhibeer. Volgens die hipotese kom mitochondriale β-oksidasieprodukte, soos 3-OH oksilipiene, teen verhoogde vlakke in giste se geslagtelike strukture (askusse) en teen verlaagde vlakke in vegetatiewe ongeslagtelike strukture (hifes en enkel selle) voor. Dit stel voor dat daar verhoogde mitochondriale aktiwiteit in askusse is. Dus behoort askomisete giste se geslagtelike strukture meer sensitief te wees vir mitochondriale inhibeerders in vergeleke met vegetatiewe ongeslagtelike strukture. Die doel van die studie was om te bepaal of die Aspirien Antifungale Hipotese uitgebrei kan word om ook ander mitochondriale inhibeerders asook ander strukture in fungale lewensiklusse waar verhoogde mitochondriale aktiwiteit verwag word, in te sluit. Hierdie studie het gevind dat die anti-inflammatoriese middels ASS, ibuprofen, indometasien, salisiensuur en bensoësuur asook antikankermiddels soos Lonidamien wat ook mitochondriale aktiwiteit in soogdierselle inhibeer, antifungaal is en spesifiek die geslagtelike fase van giste teiken. Dit is aangetoon deur ’n unieke gisgebaseerde biotoets met die mitochondrionafhanklike geslagtelike verspreidingstrukture wat baie askospore vorm, riboflavienproduksie en hife morfologie van die berugte plant patogeniese gis, *Eremothecium ashbyi,* as aanduiders. Hierdie middels beïnvloed die gis op soortgelyke wyse as wat onder suurstofbeperkende toestande gevind is, deur die ontwikkeling van geslagtelike strukture (mees sensitief) en riboflavienproduksie te inhibeer, en om kenmerkende gekreukelde

en granulêre hifes te veroorsaak wat ’n unieke “anoksiese” morfologiese patroon daarstel. Slegs middels geassosieer met inhibisie van mitochondriale aktiwiteit het so ’n patroon veroorsaak. Hierdie biotoets mag toepassing vind in die soektog na nuwe middels met moontlike anti-mitochondriale aktiwiteit vanuit verskeie bronne. In ’n ander deel van die studie is die effek van anti-mitochondriale middels op ongeslagtelike spoorverspreidingstrukture van die patogene *Aspergillus fumigatus* en *Rhizopus oryzae* ondersoek. Toe anti-mitochondriale ASS en ander anti-mitochondriale nie-steroïed anti-inflammatoriese middels (NSAIMs) by *A. fumigatus* en *R. oryzae* gevoeg is, is ongeslagtelike spoorverspreidingstrukture eerste teen laer konsentrasies geteiken. Soortgelyke resultate is verkry onder suurstofbeperking. Hierdie ongeslagtelike spoorverspreidingstrukture bevat verhoogde vlakke van mitochondriale aktiwiteit in vergeleke met die hifes. Mitochondriale aktiwiteit mag nodig wees vir die vorming van ongeslagtelike spoorverspreidingstrukture van hierdie fungi. Gevolglik mag mitochondriale inhibeerders dien as effektiewe antifungale om ongeslagtelike spoorverspreiding van hierdie patogene fungi te bekamp. In hierdie studie is die Aspirien Antifungale Hipotese uitgebrei om ook verskeie anti-inflammatoriese verbindings, antikankermiddels, plantekstrakte, tradisionele medisyne en ander – baie met anti-mitochondriale aktiwiteit – in te sluit. Hierdie verbindings behoort verder ondersoek te word om hulle minimum inhibitoriese konsentrasies (MIKs) en hul toepassing om plant- en menslike patogene fungi te beveg, te bepaal. In hierdie studie is die hipotese ook uitgebrei om ongeslagtelike spoorverspreidingstrukture met verhoogde mitochondriale aktiwiteit in te sluit.

Sleutelwoorde: Antifungaal, *Aspergillus fumigatus,* Aspirien Antifungale Hipotese, *Eremothecium ashbyi*, fungale lewensiklusse, mitochondriale aktiwiteit, mitochondriale inhibeerderss, nie-steroïed anti-inflammatoriese middels, *Rhizopus oryzae*, spoorverspreidingstruktuur.