Advanced Journal of Microbiology Research Vol. 2016
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

Mitochondrion activity and dispersal of *Aspergillus fumigatus* and *Rhizopus oryzae*

Desmond M. Ncango[1], Chantel W. Swart[1], Carolina H. Pohl[1], Pieter W. J. Van Wyk[2] and Johan L. F. Kock[1]*

[1]UNESCO MIRCEN, Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, P.O. Box 339, Bloemfontein, 9301, South Africa.
[2]Centre for Microscopy, University of the Free State, P.O. Box 339, Bloemfontein, 9301, South Africa.

Accepted 23 March, 2016

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 concluded 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.

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.

MATERIALS AND METHODS

Strains used

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

*Corresponding author. E-mail: Kockjl@ufs.ac.za. Tel: +27 (51) 401 2249. Fax: +27 (51) 444 3219.

Africa.

Cultivation

All fungi were cultivated for 48 h on yeast- malt (YM) agar (Wickerham, 1951) at 25°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°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 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 ($_m$)

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 l 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 milliliters 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 l menadione (Fluka, 1 mmol/L in acetone) were added. Cells were incubated at 37°C 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 was 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°C for 48 h. Oxygen was removed with the use of

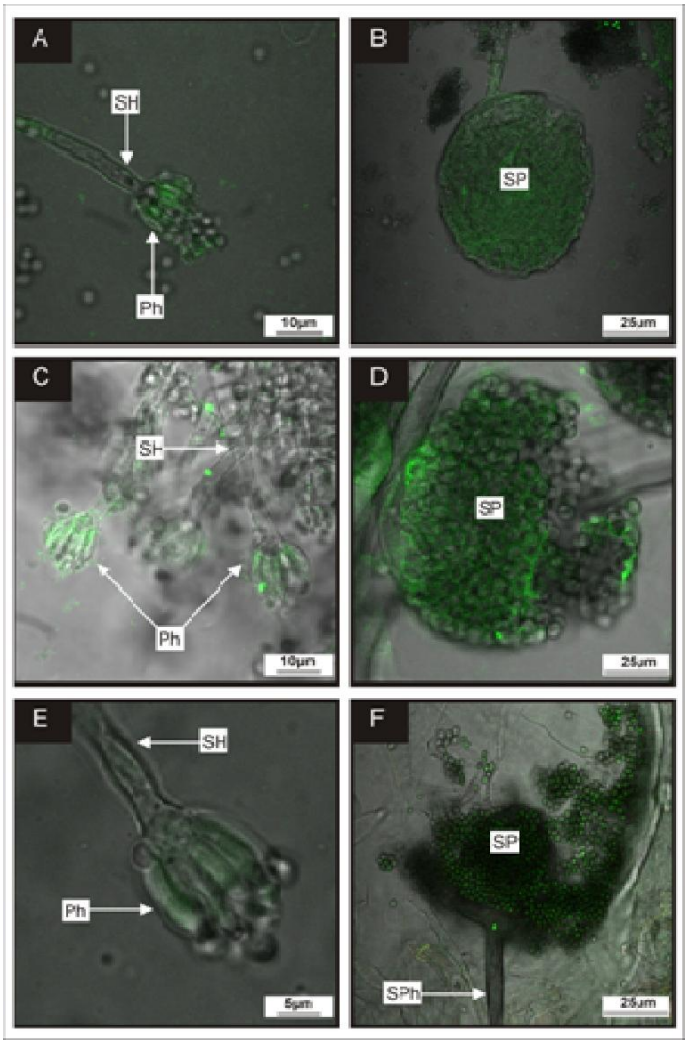

Figure 1. Confocal laser scanning micrographs of *A. fumigatus* (A, C and E) and *R. oryzae* (B, D and F). Cells stained with monoclonal antibodies specific for mitochondria (A and B). Cells treated with 3-hydroxy oxylipin antibodies and fluorescein secondary antibody (C and D). Cells stained with Rhodamine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

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.

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.

Table 1. Effects of different NSAIDs on the life cycle (hyphae and fruiting structure) of *A. fumigatus.*

Compounds tested	Zones		
	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 NSAIDs on the life cycle (i.e. hyphae and sporangia development) of *R. oryzae.*

Compounds tested	Zones		
	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.

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 -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; $_m$) was added (Figures 1E and F).

Mitochondrion inhibition

When ASA (Tables 1 and 2), known to inhibit mitochon-

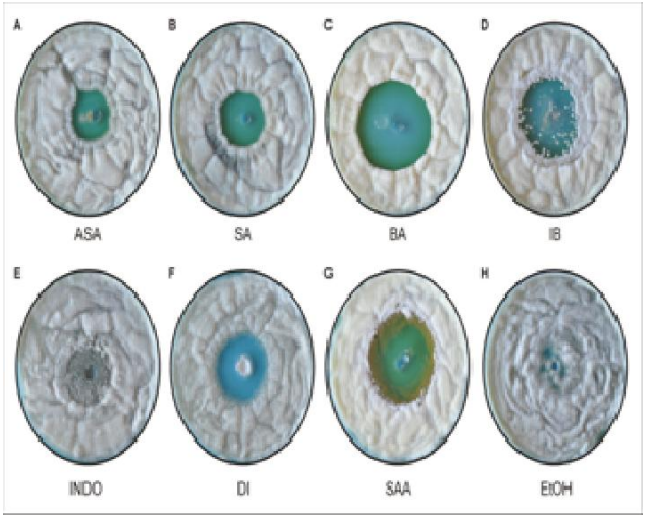

Figure 2. Bio-assays of *A. fumigatus* showing effects of different NSAIDs. (A) Acetylsalicylic acid (ASA), (B) Salicylic acid (SA), (C) Benzoic acid (BA), (D) Ibuprofen (IB), (E) Indomethacin (INDO), (F) Diflunisal (DI), (G) Salicylamide (SAA) and (H) Ethanol (EtOH) control.

dria (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, 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).

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 renders 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

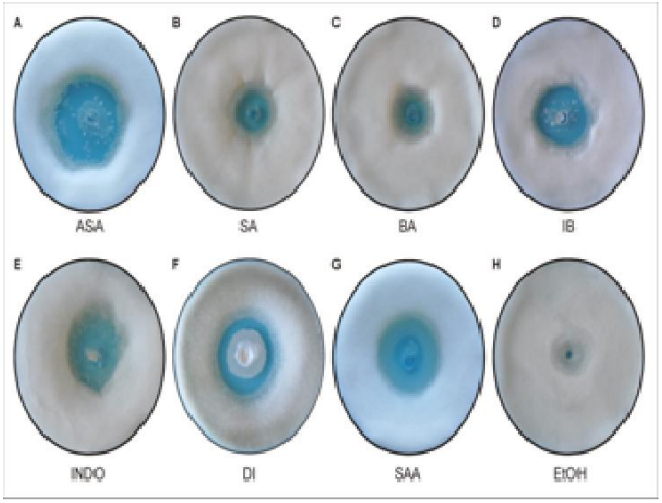

Figure 3. Bio-assays of *R. oryzae* showing effects of different NSAIDs. (A) Acetylsalicylic acid (ASA), (B) Salicylic acid (SA), (C) Benzoic acid (BA), (D) Ibuprofen (IB), (E) Indomethacin (INDO), (F) Diflunisal (DI), (G) Salicylamide (SAA) and (H) Ethanol (EtOH) control.

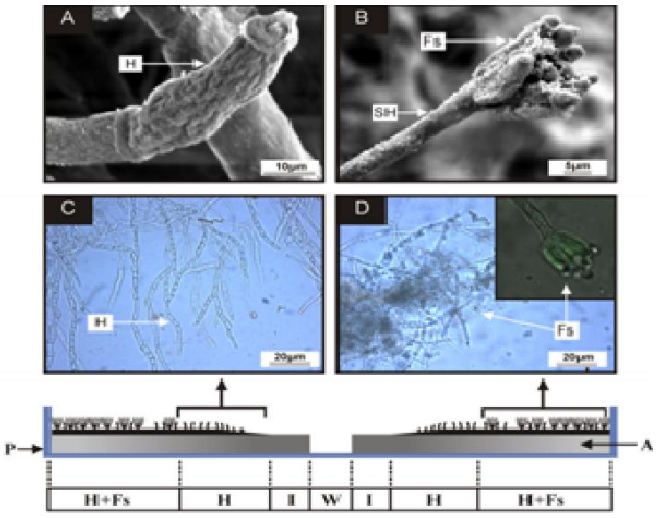

Figure 4. Detailed microscopic and ultrascopic analysis of *A. fumigatus* bio- assay plate (P) showing a well (W) and different growth zone as acetylsalicylic acid (ASA) concentration decreases across the agar (A) from the center to the periphery of the agar plate, that is Inhibition zone (I), Hyphal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

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 in the prevention of opportunistic fungal infections in humans who are immune compromised

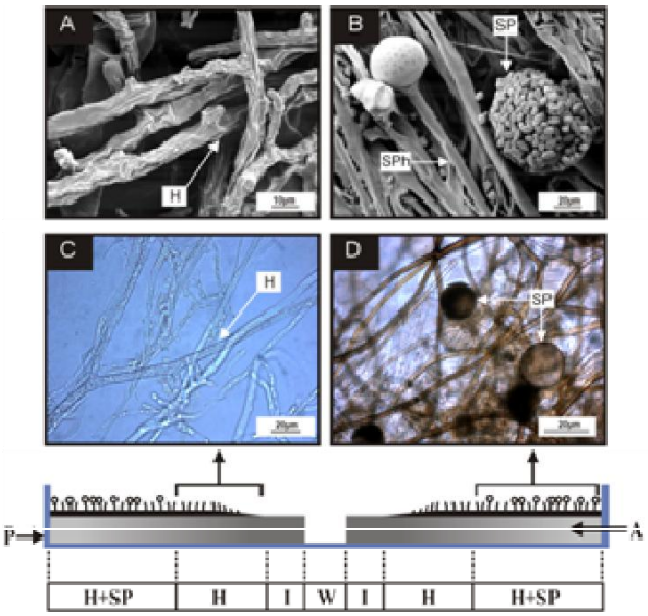

Figure 5. Detailed microscopic and ultrascopic analysis of *R. oryzae* bio-assay plate (P) showing a well (W) and different growth zones as acetylsalicylic acid (ASA) concentration decreases across the agar (A) from the center to the periphery of the agar plate, that is, Inhibition zone (I), Hyphal zone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

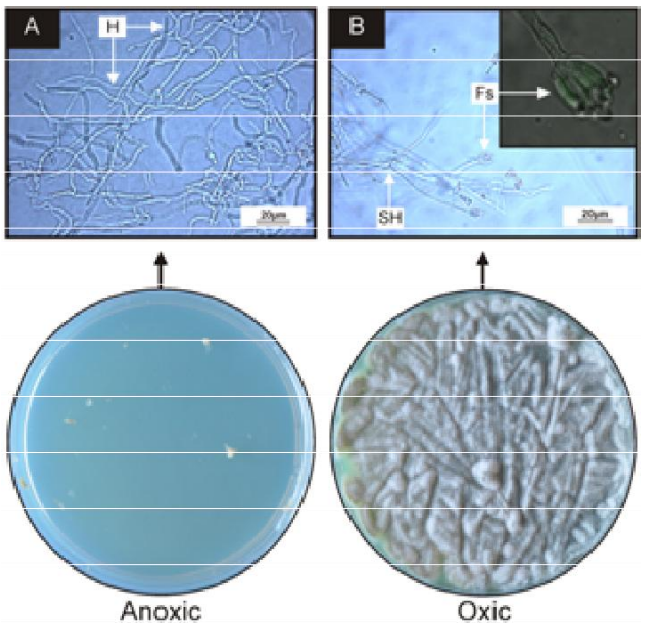

Figure 6. Different morphological forms when oxygen is limited in *A. fumigatus*. (A) Inhibition of spore-releasing-structures, that is, fruiting structure development under anoxic conditions and (B) fruiting structure development under oxic conditions. Fs, Fruiting structure; H, Hyphae; SH, Specialized Hyphae. (Davis et al., 2009).

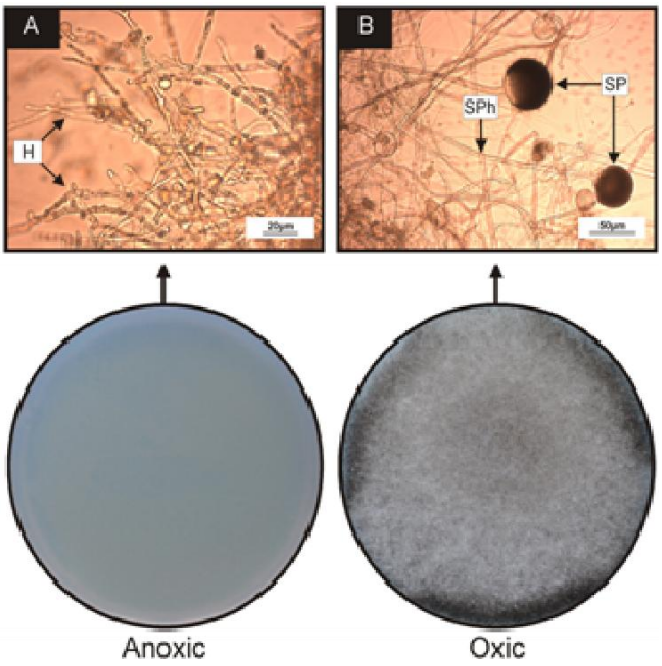

Figure 7. Different morphological forms when oxygen is limited in *R. ryzae.* (A) Inhibition of spore-releasing-structures, that is, absence of sporangia development under anoxic conditions and (B) sporangia development under oxic conditions. H, Hyphae; SPh, Sporangiophore; SP, Sporangia.

ACKNOWLEDGEMENTS

The authors wish to thank the National Research Foundation (NRF) and Lipid Biotech in South Africa for financial support.

REFERENCES

Bachmann SP, VandeWalle K, Ramage G, Patterson TF, Wickes BL, Graybill JR, López-Ribot JL (2002). *In vitro* activity of Caspofungin against *Candida albicans* biofilms. Antimicrobial Agents Chemother. 46: 3591-3596.

CH, Van Wyk PWJ (2009). Development of a Yeast bio- assay to screen anti-mitochondrial drugs. Curr. Drug Disc. Technol., 6: 186-191.

Davis HJ, Sebolai OM, Kock JLF, Lotter AP (2009). Use of non-steroidal anti-inflammatory drugs in the treatment of opportunistic infections. Pat. Appl. No. 2009/06259.

Ghannoum MA, Rice LB (1999). Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clin. Microbiol. Rev., 12: 501-517.

Ikonen E, Fiedler K, Parton RG, Simons K (1995). Prohibitin, an antiproliferative protein, is localized to mitochondria. FEBS Lett. 358: 273-277.

Kamei K (2000). Animal models of Zygomycosis – *Absidia, Rhizopus, Rhizomucor* and *Cunninghamella*. Mycopathologia, 152: 5-13.

Kock JLF, Sebolai OM, Pohl CH, Van Wyk PWJ, Lodolo EJ (2007). Oxylipin studies expose aspirin as antifungal. FEMS Yeast Res., 7: 1207-1217.

Kock JLF, Swart CW, Ncango DM, Kock Jr JLF, Munnik IA, Maartens MMJ, Pohl

Kock JLF, Venter P, Linke D, Schewe T, Nigam S (1998). Biological dynamics and distribution of 3-hydroxy fatty acids in the yeast *Dipodascopsis uninucleata* as investigated by immunofluorescence microscopy. Evidence for a putative regulatory role in the sexual cycle. FEBS Lett., 427: 345-348.

Lal N, Kumar J, Erdahl WE, Pfeiffer DR, Gadd ME, Graff G, Yanni JM (2009). Differential effects of non-steroidal anti-inflammatory drugs on mitochondrial dysfunction during oxidative stress. Arch. Biochem. Biophys., 490: 1-8.

Leeuw NJ, Swart CW, Ncango DM, Kriel WM, Pohl CH, Van Wyk PWJ, Kock JLF (2009). Anti-inflammatory drugs selectively target sporangium development in *Mucor*. Can. J. Microbiol., 55(12): 1392-1396.

Mircus G, Hagag S, Levdansky E, Sharon H, Shadkchan Y, Shalit I, Osherov N (2009). Identification of novel cell wall destabilizing antifungal compounds using a conditional *Aspergillus nidulans* protein kinase C mutant. J. Antimicrobial. Chemother., 64: 755-763.

Moss BJ, Kim Y, Nandakumar MP, Marten MR (2008). Quantifying metabolic activity of filamentous fungi using a colorimetric XTT assay. Biotechnol. Prog., 24: 780-783.

Ncango DM, Swart CW, Goldblatt ME, Pohl CH, Van Wyk PWJ, Botes PJ, Kock JLF (2008). Oxylipin and mitochondrion probes to track yeast sexual cells. Can. J. Microbiol., 54(6): 450-455.

Norman C, Howell KA, Millar AH, Whelan JM, Day DA (2004). Salicylic acid is an uncoupler and inhibitor of mitochondrial electron transport. Plant Physiol., 134: 492-501.

Somasundaram S, Sigthorsson G, Simpson RJ, Watts J, Jacob M, Tavares IA, Rafi S, Roseth A, Foster R, Price AB, Wrigglesworth JM, Bjarnason I (2000). Uncoupling of intestinal mitochondrial oxidative phosphorylation and inhibition of cyclooxygenase are required for the development of NSAID-enteropathy in the rat. Alimentary Pharm. Therapeutics, 14: 639-650.

Strauss CJ, van Wyk PWJ, Lodolo EJ, Botes PJ, Pohl CH, Nigam S, Kock JLF (2007). Mitochondrial associated yeast flocculation – the effect of acetylsalicylic acid. J. Inst. Brew., 113(1): 42-47.

Trofa D, Agovino M, Stehr F, Schafer W, Rykunov D, Fiser A, Hamari Z, Nosanchuk JD, Gacser A (2009). Acetylsalicylic acid (aspirin) reduces damage to reconstituted human tissues infected with *Candida* species by inhibiting extracellular fungal lipases. Microbes Infections, 11: 1131-1139.

Van Wyk PWJ, Wingfield MJ (1991). Ascospore ultrastructure and development of *Ophiostoma cucullatum.* Mycologia, 83: 698-707.

Wickerham LJ (1951). Taxonomy of Yeasts. Technical Bulletin No. 1029, US Department of Agriculture, Washington D.C.

Wolfe MM, Lichtestein DR, Singh G (1999). Gastrointestinal toxicity of nonsteroidal antiinflammatory drugs. New Eng. J. Med., 340: 1888-1889.