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Full Length Research Paper

Variation in mitochondrial activity over the life cycle of *Nadsonia fulvescens*

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

[1]UNESCO-MIRCEN: Industrial Biotechnology, Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, P. O. Box 339, Bloemfontein, 9300, South Africa.
[2]Centre for Microscopy, University of the Free State, P. O. Box 339, Bloemfontein, 9300, South Africa.

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The yeast *Nadsonia fulvescens* is characterized by a unique life cycle. After conjugation between the parent cell and the first bud, the zygote moves into a second bud formed at the opposite end of the parent cell. This second bud is then delimited by a septum and becomes the ascus. Usually one, rarely two spherical, brownish, spiny to warty ascospores are formed within the ascus giving rise to brown coloured colonies. Strikingly, no increased mitochondrial activity was observed in the ascus when compared to the vegetative cells as previously reported for many yeast. In this study, the parent cell and attached first bud showed increased mitochondrial activity when compared to the ascus. When anti-mitochondrial compounds were added, the mitochondrial activity was inhibited in the parent cell and attached first bud followed by the formation of less asci with ascospores (many not fully developed and white coloured giving rise to white colonies). We conclude that sufficient mitochondrial activity in the parent cell and first bud is necessary to produce enough energy for the formation of a proper ascus with brown coloured ascospore (s).

Key words: Asci, ascospore, life cycle, mitochondria, mitochondrial inhibitors, *Nadsonia fulvescens*.

INTRODUCTION

Increased mitochondrial activity in sexual cells (asci), and not in asexual cells, seems to be a conserved characteristic in ascomycetous yeasts (Kock et al., 2007; Ncango et al., 2008). The only exception thus far noted was Zy*gosaccharomyces*. In this case both sexual and asexual reproductive structures are characterised by low mitochondrial activity. This is explained by the strong fermentative metabolism of this yeast probably yielding enough energy for ascus and ascospore formation (Swart et al., 2008).

In this study, variation in mitochondrial activity over the unique life cycle of *Nadsonia fulvescens* was investigated. This yeast performs heterogamic conjugation between the parent cell and the first bud, eventually producing a zygote in a second bud on the opposite side of the parent cell. The latter is then delimited by a septum and becomes the ascus containing ascospore(s) (Lodder and Kreger-van Rij, 1952; Kurtzman and Fell, 1998).

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

MATERIALS AND METHODS

Strain used and cultivation

In this study *N. fulvescens* UOFS Y-0705 was obtained from the yeast culture collection of the University of the Free State in Bloemfontein (South Africa). This yeast was grown on yeast-malt (YM) agar (Wickerham, 1951) at 22°C, for 6 – 8 days, until sporulation was observed (brown culture) using a light microscope (Axioplan, Zeiss, Göttingen, Germany) coupled to a Colourview Soft Digital Imaging System (Münster, Germany). The cells (vegetative and sexual) were then subjected to the following experimental methods:

Mapping of 3-OH oxylipins

The presence and distribution of 3-OH oxylipins in this yeast was mapped according to Kock et al. (1998) using sporulating yeast cells from the brown zone. In short, cells were treated with a primary antibody specific for 3-OH oxylipins, washed with

phosphate buffered saline (PBS) and further treated with a primary antibody-specific fluorescein isothiocyanate (FITC) conjugated secondary antibody (Sigma-Aldrich, USA). Cells were washed again with PBS to remove the unbound secondary antibodies and then fixed on a microscope slide and viewed using a Nikon TE 2000, confocal laser scanning microscope (Japan).

Mapping of mitochondria

Since 3-OH oxylipins in yeasts are produced by β-oxidation in mitochondria (Kock et al., 2007), it was decided to map the distribution of these organelles in normal and sporulating cells. Consequently, cells from the brown zone were treated with a primary monoclonal antibody (Genway Biotech Inc., San Diego, USA) specific for mitochondria (30 µl for 1 h in the dark at room temperature). Cells were then washed with PBS to remove unbound antibodies and further treated with a FITC conjugated secondary antibody (Sigma-Aldrich, USA), specific for the primary antibody, (30 µl for 1 h in the dark at room temperature). Cells were washed again with PBS to remove the unbound secondary antibodies. Staining was executed in 2 ml plastic tubes in order to maintain cell structure. After washing, the cells were fixed in Dabco (Sigma-Aldrich, USA) on a microscope slide and viewed using a Nikon TE 2000, confocal laser scanning microscope (Japan).

Bio-assay preparation

Cells were scraped from above YM-agar grown plates and suspended in sterile distilled water.200 µl of this suspension was then spread out on soft agar plates (containing 0.5% m/v agar) to form a homogenous lawn. A well was then constructed in the middle of the plate (0.5 cm in diameter and depth) and 46 µl of the mitochondrial inhibitors that is aspirin (ASA; Sigma, Steinheim, Germany), benzoic acid (The British Drug Houses Ltd., Poole, England), ibuprofen (Sigma-Aldrich, Steinheim, Germany) and salicylic acid (The British Drug Houses Ltd., Poole, England), all at a concentration of 8% m/v in ethanol (dissolved in 96% ethanol) were added to each well respectively. Similar experiments were performed where only 96% ethanol was added to the plates as a control to study the effect of ethanol. Fluconazole was tested using an E-test strip (Davies Diagnostics, South Africa) containing various concentrations of fluconazole (0.016 – 256 µg/ml). Plates were incubated at 22°C for 6 – 8 days and viewed for formation of inhibition zones as well as white (asexual) and brown (asexual and sexual) zones. All plates are referred to as bio-assay plates.

Identification and analysis of 3-OH oxylipins

In order to identify the 3-OH oxylipins observed microscopically and if anti-mitochondrial compounds inhibit these compounds, cells from the white and brown zones respectively were used for analysis. This experiment was performed as described by Van Heerden et al. (2005). In short, after scraping cells from respective zones, they were suspended in 100 ml dH_2O and the pH decreased to 3.8 with 3% formic acid (Merck, Darmstadt, Germany). Next, oxylipins were extracted and then dissolved in 2x volumes of ethyl acetate (Merck, Darmstadt, Germany). After the organic and water phases have separated, the organic phase was evaporated with N_2 gas (AFROX, Bloemfontein, South Africa). This was followed by derivatizing (methylating and silylating) extracts which were finally dissolved in 400 µl chloroform:hexane (4:1) (Merck, Darmstadt, Germany). All experiments were performed in at least duplicate. Derivatized samples from the white and brown zones respectively were injected into a Finnigan Trace GC Ultra gas chromatograph (Thermo Electron Corporation, San Jose, Calif., USA) with a HP5 (60 m x 0.32 mm diameter) fused silica capillary column (0.1 µm coating thickness) coupled to a Finnigan Trace DSQ MS (Thermo Electron Corporation, San Jose, Calif., USA). The carrier gas was helium at 1.0 ml/min. The initial oven temperature of 110°C was maintained for 2 min then increased to a final temperature of 280°C at a rate of 5°C/min. The gas chromatography – mass spectrometer (GC-MS) was auto-tuned for an *m/z* of 50 - 400. 1 µl of the sample was injected into the GC-MS at a split ratio of 1:50 at an inlet temperature of 230°C (Venter et al., 1997).

Transmission electron microscopy

Yeast material scraped from white and brown zones respectively were chemically fixed with 1.0 M (pH 7) sodium phosphate-buffered glutardialdehyde (3%) for 3 h and then for 1.5 h in similarly buffered osmium tetroxide (Van Wyk and Wingfield, 1991). These fixed cells were then embedded in epoxy resin and polymerized at 70°C for 8 h (Spurr, 1969). An LKB III Ultratome was used to cut 60 nm sections with glass knives. Uranyl acetate (Merck, Darmstadt, Germany) was used to stain these sections for 10 min, followed by lead citrate (Merck) (Reynolds, 1963) for 10 min. The preparation was viewed with a Philips 100 transmission electron microscope (Eindhoeven, The Netherlands).

Determination of mitochondrial membrane potential

In order to assess the influence of anti-mitochondrial compounds on mitochondrial activity, yeast cells were collected from the white and brown zones respectively and then washed with PBS in a 2 ml plastic tube to get rid of agar and debris. Next, cells were treated with Rhodamine 123 (Rh123; 31 µl per sample), a mitochondrial stain (Molecular Probes, Invitrogen Detection Technologies, Eugene, Oregon, USA), for 1 h in the dark at room temperature. Cells were washed again with PBS to remove excess stain and fixed on microscope slides in Dabco (Sigma-Aldrich, USA). Finally, cells were viewed with a confocal laser scanning microscope (Nikon TE 2000, Japan) and the relative intensity of the fluorescence of the cells (parent cell and first bud before ascospore formation) from the different zones, determined.

Quantitative measurement of metabolic state

Cells (equal weight) of *N. fulvescens* were scraped from the bio-assay plates (white and brown zones respectively) and suspended in sterile PBS solution.100 µl of the cell suspension was added to a 96-well flat bottom polystyrene microtiter plate (Corning Incorporated, NY, USA). 50 µl of menadione (Fluka, USA; 1 mM in acetone) was added to 2.5 ml XTT [0.5 g XTT (Sigma Chemicals, St. Louis, Mo., USA) in 1 l Ringer's lactate solution] and transferred to the cell suspension. The mixture was incubated in the dark for 3 h at 37°C. After incubation the formazan product was spectrophotometrically measured in terms of optical density at 492 nm using a Labsystems iEMS reader (Thermo BioAnalysis, Helsinki, Finland).

Oxygen inhibition studies

Mitochondrial activity in cells of *N. fulvescens* was inhibited by limiting oxygen availability. Cells were scraped from YM-agar grown plates and suspended in sterilized distilled water. A homogenous

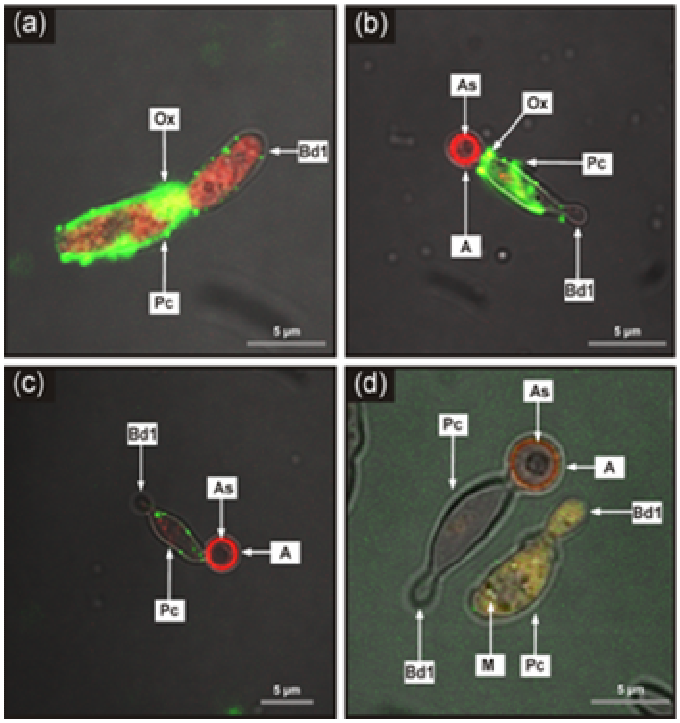

Figure 1. Confocal laser scanning micrographs of cells in different developmental stages towards ascus (A) formation. Cells were probed with fluorescing antibodies specific for oxylipins (Ox) and mitochondria (M). Oxylipins are observed in the parent cell (Pc) with first attached bud (Bd 1) (a) and parent cell attached to both first bud and ascus (b). Oxylipins are depleted in parent cell after further development (c). Monoclonal antibody probes indicate a higher mitochondrion concentration in the parent cell and first bud compared to mature ascus, attached to empty parent cell with attached first bud (d).

lawn was then spread out onto YM agar plates containing 1.6% (m/v) agar. Plates were placed in an anoxic jar. An Anaerocult A System (Merck, Darmstadt, Germany) was used to create an anoxic environment within the anoxic jar. Anaerotest Test Strips (Merck, Darmstadt, Germany) were placed in the jar, confirming the anoxic atmosphere. The jar was incubated for 6 - 8 days at 22°C. As the control, the corresponding agar plates were placed next to the jar in the incubator (oxic conditions) and incubated for the same period.

RESULTS AND DISCUSSION

Immunofluorescence studies (Figure 1) show that mitochondrially produced 3-OH oxylipins are associated mainly with the parent cell (Figure 1a) and decrease in concentration when the ascus is formed (Figure 1b, c). Strikingly, in Figure 1(b) the ascus contained significant lower levels of oxylipins when compared to the parent cell. Depletion of oxylipins is, as expected, in accordance with the loss of mitochondria (Figure 1d) from the parent cell when asci are formed. Using GC-MS (Figure 2), the fluorescing antibodies depicted the presence of only one

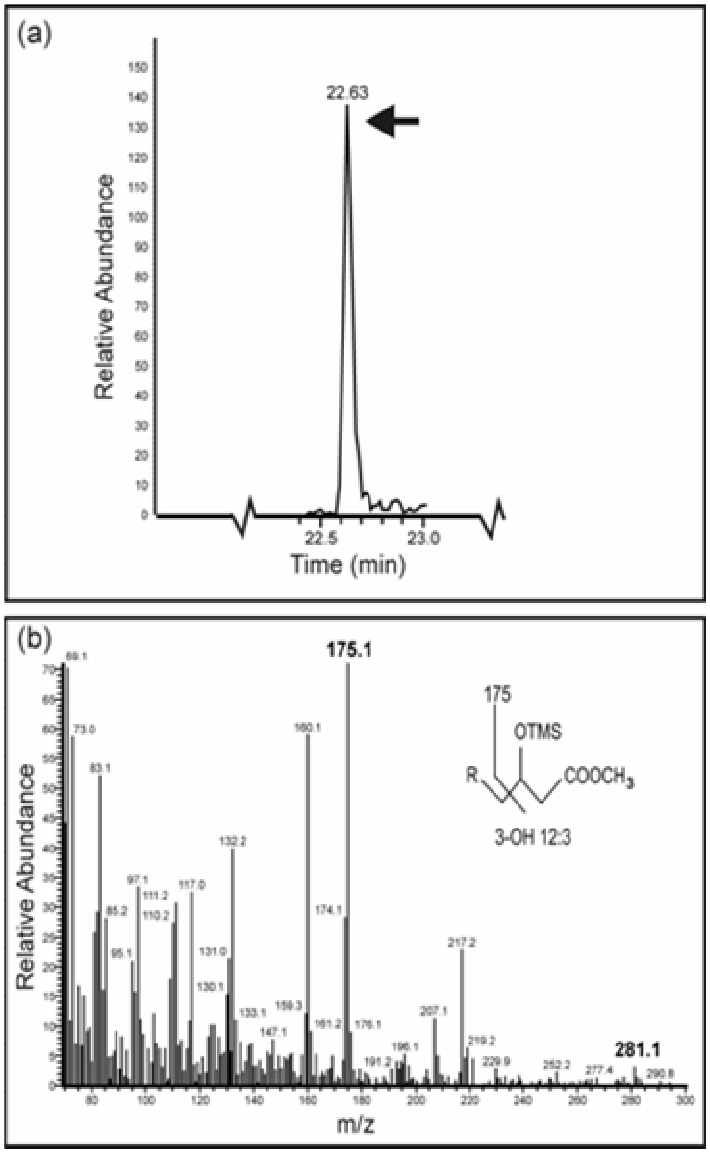

Figure 2. Gas chromatography – mass spectrometry profiles showing the total ion chromatogram (a) and mass spectrum (b) of 3-OH 12:3 found in the yeast *Nadsonia fulvescens*.

type of 3-OH oxylipin (3-OH 12:3; retention time = 22.63 min; Figure 2a) produced by *N. fulvescens*. The base peak at *m/z* = 175 (Figure 2b) depicts a hydroxyl group at carbon 3 as counted from the carboxyl group (Kock et al., 2007). These results are contrary to the Aspirin Antifungal Hypothesis proposed by Kock and co-workers in 2007. According to the hypothesis, asci should contain increased amounts of one or more 3-OH oxylipins which is an indication of increased mitochondrial activity probably necessary for ascospore formation. What role does mitochondria therefore play in the life cycle of *N. fulvescens*?

To address this question, the influence of different

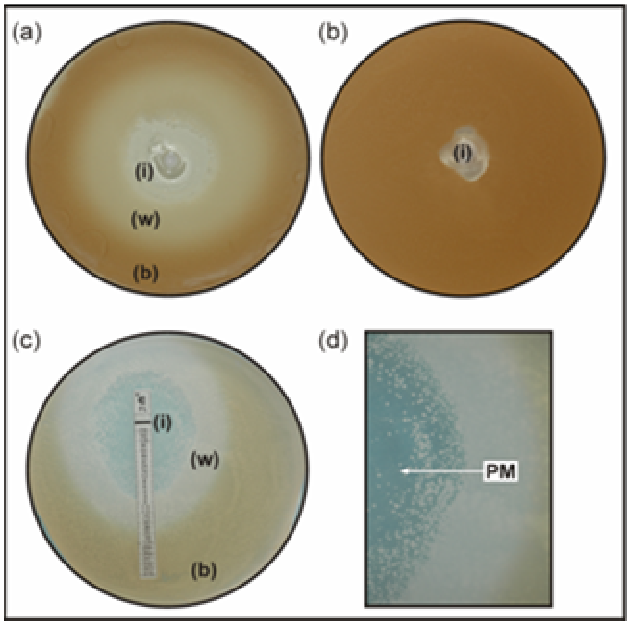

Figure 3. Photograph of a bio-assay containing the anti-mitochondrial drug acetylsalicylic acid (a) indicating the inhibition zone (*i*), the white zone (*w*) as well as a brown zone (*b*). Ethanol control is shown in (b) containing only a small inhibition zone (*i*). Fluconazole E-test strips were also tested using *Nadsonia fulvescens* as indicator yeast (c) showing an inhibition zone (*i*), white zone (*w*) and brown zone (*b*). Petite mutants (PM) are formed in the inhibition zone (d).

mitochondrial inhibitors dissolved in ethanol was tested on the life cycle of *N. fulvescens* using a bio-assay based on the agar diffusion test method. Similar results were obtained compared to that found for ASA (Figure 3a and b). At relative high concentrations (that is close to origin of well) ASA, benzoic acid, ibuprofen and salicylic acid, inhibited growth followed by a white zone where selective inhibition of the sexual stage was observed (Figure 3a). In this zone, underdeveloped cream coloured ascospores were observed microscopically. When ethanol alone (control) was added, only a small inhibition zone and brown zone were visible and no white zone (that is no selective inhibition of the sexual phase) (Figure 3b). Finally a brown zone containing normal cells with mature asci and amber coloured ascospores was observed microscopically on the periphery of the bio-assay plate after the addition of the different mitochondrial inhibitors. Microscopy studies of the brown zones observed in both cases (that is in the presence of anti-mitochondrials and ethanol alone) were similar to the brown coloured cultures observed on similar agar plates without addition of anti-mitochondrials or ethanol. Similar results were obtained when E-test strips containing the antifungal fluconazole was added to the bio-assay (Figure *3*c). It is interesting to note that small white “petit” colonies with underdeveloped ascospores developed in the inhibition zone (Figure 3d). Is this a resistance mechanism developed by this fermentative yeast to overcome the anti-mitochondrial function (Kontoyiannis, 2000) of this antifungal? The respiration function of these resistant colonies and possible mutations should now be further studied.

After observing cell cultures from the different zones macroscopically and microscopically, the next step was to study their ultrastructure (Figures 4a - d). Cells from the brown zone showed normal development with the formation of young primordial smooth ascospores and lipid globules between parent cell and second bud that eventually give rise to mature asci containing usually one ascospore with hair-like protruberances (Kurtzman and Fell, 1998), (Figures 4a and b). Cells from the white zone showed asci with underdeveloped small ascospores and no hair-like outgrowths or lipid globules as described above (Figures 4c and d). Some asci were empty while others contained ring-like structures.

Next we determined the mitochondrial activity in parent cells as well as first bud before ascospore formation by staining cells with Rh123, a cationic lipophilic dye that assesses transmembrane potential ($\Delta\psi_m$). This was performed on equal amounts of cells from both the white and brown zones respectively (Johnson et al., 1980; Swart et al., 2008). Cells from the brown zone showed significant ($p < 0.001$) increased fluorescence (relative intensity = 4050 +/- 85; n = 10) compared to similar cells in the white zone (relative intensity = 2810 +/- 752; n = 10) as collected at 450 nm. This indicates increased mitochondrial activity in these cells present in the brown zone which is probably needed for proper ascospore formation. Likewise, no evidence of mitochondrially produced 3-OH fatty acids were found in cells in the white zone using GC-MS thereby further showing a decreased mitochondrial activity (that is β-oxidation) in this zone.

Mitochondrial activity by measuring mitochondrial dehydrogenase activity (Kuhn et al., 2003) in cells present in the white and brown zones, respectively, was also determined using the XTT-assay. These zones were obtained by the addition of the various anti-mitochondrial drugs to the bio-assay as previously described. Results indicate that anti-mitochondrial drugs inhibit mitochondrial activity (measured at 492 nm) significantly ($p < 0.001$, n = 8) that is ASA: White zone 1.4 +/- 0.24, brown zone 2.42 +/- 0.08; Benzoic acid: White zone 0.88 +/- 0.08, brown zone 2.58 +/- 0.12; Ibuprofen: White zone 0.99 +/- 0.33, brown zone 2.39 +/- 0.35; Salicylic acid: white zone 0.80 +/-0.12, brown zone 2.17 +/- 0.14.

Finally, the effect of anti-mitochondrial compounds was compared to the effect of oxygen limitation conditions on sporulation. As expected, anoxic conditions, which are known to also inhibit mitochondria, yielded white colonies (inhibit sporulation) compared to brown colonies (with normal sporulation) observed under oxic conditions (results not shown).

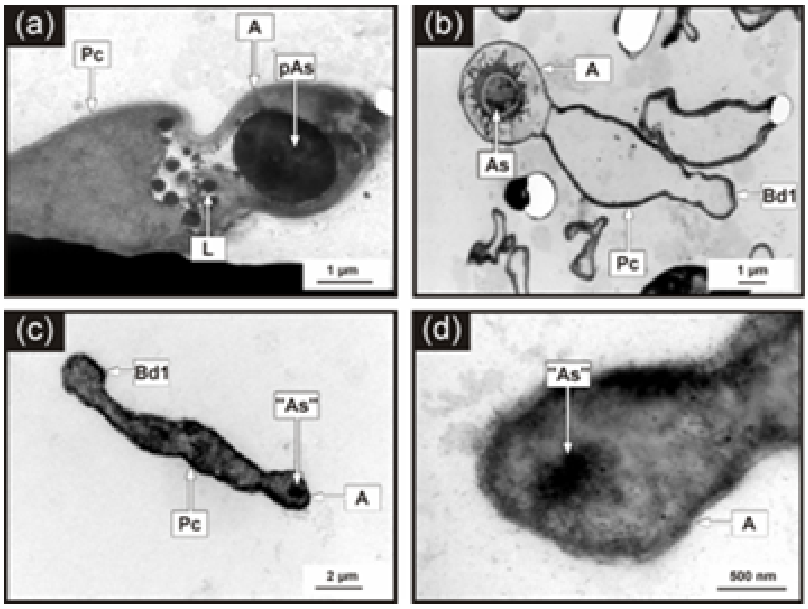

Figure 4. Transmission electron micrographs showing cells obtained from the brown as well as white zones. Cells obtained from the brown zone (a, b) indicate lipid (L) globules concentrated around the neck of the second bud cell (A) where a primordial ascospore (pAs) is being produced. A mature ascus (A) with mature ascospores (As) could also be observed in the cells obtained from the brown zone. Cells obtained from the white zone (c, d) only show small, immature ascospores ("As"). Bd 1, first attached bud; Pc, parent cell.

To conclude, this study shows evidence that sufficient mitochondrial activity associated with the parent cell and first bud before sporulation is needed for normal ascospore formation. In *N. fulvescens*, mitochondria seem not to be involved inside the ascus in ascospore formation. This is in contrast to the many other yeasts studied in this respect (Kock et al., 2007). The use of *N. fulvescens* as indicator to select compounds with anti-mitochondrial antifungal properties should now be assessed and compared to the *Eremothecium* bio-assay protocol (Kock et al., 2009).

N. fulvescens is regarded as a yeast with both respiring and fermentative capability (showing the Pasteur Effect). According to literature, the asexual and sexual reproductive phases of yeasts with and without the "Pasteur Effect", are both inhibited by anti-mitochondrial drugs (Kock et al., 2007). Here, yeasts that can respire and ferment were more resistant (regarding growth and ascus formation) to anti-mitochondrial compounds compared to strict respiring yeasts. This may be ascribed to the production of sufficient energy needed for asexual as well as sexual growth through an alternative anaerobic glycolytic fermentative pathway where mitochondria are less involved (Kock et al., 2007). It is interesting to note that the respiring and fermentative *Zygosaccharomyces baillii* may produce mature asci even in the presence of relative high concentrations of anti-mitochondrials and under anoxic conditions (Swart et al., 2008). In future the influence of anti-mitochondrial drugs on mutants of *Nadsonia* which are strict aerobic should be investigated.

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REFERENCES

Johnson LV, Walsh ML, Chen LB (1980). Localization of mitochondria in living cells with Rhodamine 123. PNAS 77: 990-994.

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 reproductive cycle. FEBS Lett. 472: 345-348.

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 JLF Jr., Munnik IA, Maartens MMJ, Pohl CH, Van Wyk PWJ (2009). Development of a yeast bio-assay to screen anti-mitochondrial drugs. Curr. Drug Disc. Technol. 6: 186-191.

Kontoyiannis DP (2000). Modulation of fluconazole sensitivity by the interaction of mitochondria and Erg3p in *Saccharomyces cerevisiae*. J. Antimicrob. Chemother. 46: 191-197.

Kuhn DM, Balkis M, Chandra J, Mukherjee PK, Ghannoum MA (2003). Uses and limitations of the XTT assay in studies of *Candida* growth

and metabolism. J. Clin. Microbiol., 41(1): 506-508.
Kurtzman CP, Fell JW (1998). Definition, classification and nomenclature of the yeast. In Kurtzman and Fell (eds) The yeasts – a taxonomic study, 4th ed, Elservier Science Publication BV, Amsterdam, The Netherlands.
Lodder J, Kreger-van Rij NJW (1952). The yeast – a taxonomic study. North-Holland Publishing Co., Amsterdam, The Netherlands.
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.
Reynolds ES (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell. Biol. 17: 208-212.
Spurr AR (1969). A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26: 1-43.
Swart CW, Van Wyk PWJ, Pohl CH, Kock JLF (2008). Variation in yeast mitochondrial activity associated with asci. Can. J. Microbiol. 54 (7): 532 - 536.
Van Heerden A, Kock JLF, Botes PJ, Pohl CH, Strauss CJ, Van Wyk PWJ, Nigam S (2005). Ascospore release from bottle-shaped asci in *Dipodascus albidus*. FEMS Yeast Res. 5: 1185-1190.
Van Wyk PWJ, Wingfield MJ (1991). Ascospore ultrastructure and development of *Ophiostoma cucullatum*. Mycologia 83: 698-707.
Venter P, Kock JLF, Sravan KG, Botha A, Coetzee DJ, Botes PJ, Bhatt RK, Falck JR, Schewe T, Nigam S (1997). Production of 3-hydroxy-polyenoic fatty acids by the yeast *Dipodascopsis uninucleata*. Lipids 32: 1277-1283.
Wickerham LJ (1951). Taxonomy of yeasts. Technical Bulletin No. 1029. US Department of Agriculture. Washington D.C., USA.