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Full Length Research Paper

The influence of mitochondrial inhibitors on the life cycle of *Phytophthora*

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

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

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The anti-mitochondrial antifungal hypothesis implies a link between mitochondrial activity, fungal fruiting structures and susceptibility towards mitochondrial inhibitors. Here it is shown that the oomycete, *Phytophthora nicotianae* fits the hypothesis. Fruiting structures (zoosporangia) of this oomycete showed increased beta ( )-oxidation when probing levels of 3-hydroxy fatty acids (3-OH oxylipins) with specific polyclonal antibodies. In addition, increased mitochondrial activity was also observed in the zoosporangia when the mitochondrial transmembrane potential ($_m$) probe, Rhodamine 123 was added to the culture. This indicates increased mitochondrial activity in the zoosporangia when compared to the hyphae. When the anti-mitochondrial drug acetylsalicylic acid (ASA) was added to cultures of this oomycete, the zoosporangia were, as expected, most susceptible and were drastically inhibited in the presence of 1 mM of this compound. Similar ASA inhibition results were recorded for *P. citrophthora*. It is concluded that anti-mitochondrial compounds may find application in combating these devastating plant pathogens and that urgent further research is needed in this direction.

Key words: Acetylsalicylic acid, antifungal, anti-mitochondrial, asci, *Phytophthora*, plant pathogen.

INTRODUCTION

In 2007, Kock and co-workers proposed the Acetylsalicylic acid (ASA) Antifungal Hypothesis, stating that the sexual stages in yeast life cycles have increased mitochondrial activity when compared to asexual vegetative stages and that the former is most susceptible to mitochondrial inhibitors. Increased mitochondrial activity in sexual cells was observed in many yeasts using different fluorescing probes to determine transmembrane potential ($_m$), a polyclonal antibody to locate 3-hydroxy (OH) fatty acids [3-OH oxylipins from mitochondrial beta ( ) -oxidation], as well as a monoclonal antibody (specific for prohibitin) to locate mitochondria. This is to be expected since an increase in energy is probably needed to produce multiple ascospores inside the ascus. This phenomenon seems to be a conserved characteristic in most ascomycetous yeasts except in *Zygosaccharomyces* (Kock et al., 2007; Ncango et al., 2008; Swart et al., 2008, 2010a, b).

Based on the above mentioned studies, Kock and co-workers also investigated the possibility that this characteristic could be conserved amongst other fungi. They obtained positive results for fruiting structures (sporangia and phialide-conidia structures) for various non-yeast fungi and fungi-like organisms including *Aspergillus* , *Mucor* and *Rhizopus* . Consequently they decided to expand the hypothesis (Leeuw et al., 2009; Ncango et al., 2010).

In this study two pathogenic species of the oomycete genus, *Phytophthora* were investigated to assess if they also fit the hypothesis. This group includes the notorious destructive crop pathogens and is considered a distinct lineage of fungi-like eukaryotes that are related to organisms such as brown algae and diatoms. This group of pathogens has already caused worldwide crop losses of in access of $6.7 billion (Alexopoulos, 1962; Brasier,

*Corresponding author. E-mail: kockjl@ufs.ac.za. Tel: +27 51 401 2249. Fax: +27 401 9376.

1992; Judelson, 1997; Haverkort et al., 2008; Haas et al., 2009).

MATERIALS AND METHODS

Strain used and cultivation

Phytophthora species, including *P. nicotianae* and *P. citrophthora* were obtained from Dr. W.J. Botha, Agricultural Research Council – Plant Protection Research Institute (ARC-PPRI), Roodeplaat, South Africa. The organisms were first plated onto soft potato dextrose agar (PDA) plates (0.7% m/v agar). From there, plugs were taken and placed in 20 mL sterile soilwater (soil extract) . Round discs were cut from surface sterilised citrus leaves and placed in the soilwater to aid sporulation. The plates were then incubated at 21°C under near UV and white light to aid sporulation. Following this, the cells were subjected to the following procedures:

Morphology studies

Cells from both the species were scraped from the leaves and the PDA agar plugs and viewed with an Olympus YX70 light microscope (Tokyo, Japan) to determine the morphological characteristics of these oomycetes.

Mitochondrial activity studies - mitochondrial products (3-OH oxylipins)

To determine if 3-OH oxylipins are also present and where they are mainly located, cells of *P. nicotianae* were subjected to the Oxytrack (fluorescing probe to track oxylipins) system as described by Kock et al. (1998). In short, cells from *P. nicotianae* were treated with a primary antibody specific for 3-OH oxylipins, washed with phosphate buffered saline (PBS; Oxoid, Hampshire, England) and further treated with a primary antibody- specific fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Jackson Immunoresearch Laboratories, USA). Cells were washed again with PBS to remove the unbound secondary antibodies and then fixed on a microscope slide in Dabco (Sigma-Aldrich, USA) and viewed using a Nikon TE 2000, confocal laser scanning microscope (CLSM; Japan).

Mitochondrial activity studies - membrane potential ($_m$)

To determine the mitochondrial $_m$, cells of *P. nicotianae* were scraped from the citrus leaves and the PDA agar plugs and placed in a 2 mL plastic tube. Cells were then washed with PBS 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 (21°C). 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 Nikon TE 2000, CLSM.

Mitochondrial location

Since 3-OH oxylipins in yeasts are produced by -oxidation in mitochondria (Kock et al., 2007), it was decided to map the distribution of these organelles in *P. nicotianae* as well. Cells were treated with a primary monoclonal antibody (Genway Biotech Inc., San Diego, USA) specific for mitochondrial prohibitin (30 µL for 1 h in the dark at room temperature) and then washed with PBS to remove unbound antibodies before further treatment with a FITC-conjugated secondary antibody (Jackson Immunoresearch Laboratories, 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 performed in 2 mL plastic tubes in order to maintain cell structure. After washing, the cells were fixed in Dabco on a microscope slide and viewed using a Nikon TE 2000, CLSM.

Mitochondrial inhibition studies

The effects of ASA [a non-steroidal anti-inflammatory drug (NSAID) and anti-mitochondrial compound] on mitochondrial activity in cells of both *Phytophthora* species were assessed (Kock et al., 2007). The organisms were firstly plated onto PDA plates, from where plugs were taken and placed in 20 mL sterile soilwater. Round discs were cut from surface sterilised citrus leaves and placed in the soilwater to aid in sporulation. Before incubation, ASA (8% m/v ethanol; dissolved in 96% ethanol) (ethanol obtained from Merck, Gauteng, South Africa) was added to the plates to a final concentration of 0.1, 1, 2.5 and 5 mM respectively. In addition, ethanol alone was tested as control (the same volume used to dissolve 5 mM of ASA). The plates were then incubated at 21°C under near UV and white light to aid in sporulation. Samples were taken at random from the growth surrounding the plugs and leaves and viewed with an Olympus YX70 light microscope. The number of full and empty zoosporangia were then counted in four randomly selected microscopic fields (n = 4) for each of the experimental plates (containing 0, 0.1, 1, 2.5 and 5 mM ASA as well as the ethanol control) and the average and standard deviation calculated. Results were compared using the Student's *t*-test to determine if there is a significant difference between the results obtained. These experiments were done in triplicate.

RESULTS AND DISCUSSION

Morphology studies

In *P. nicotianae*, characteristic hyphae as well as zoosporangia with zoospores could be observed with light microscopy (Figure 1a). The exit pore, where zoospores are usually released from, is clearly visible. Similar results were obtained for *P. citrophthora*.

Mitochondrial activity studies - mitochondrial products (3-OH oxylipins)

Results obtained indicate an accumulation of 3-OH oxylipins situated in the exit pore of the zoosporangium (Figure 1b) from where the zoospores are released (indicated by the intense green fluorescence) in *P. nicotianae*. This accumulation was not observed in the surrounding vegetative hyphae. The zoosporangium is empty, indicating that the zoospores have already been liberated (Figure 1b). Literature suggests that 3-OH oxylipins may act as lubricants during ascospore release through narrow bottleneck ascus openings in yeast (Kock

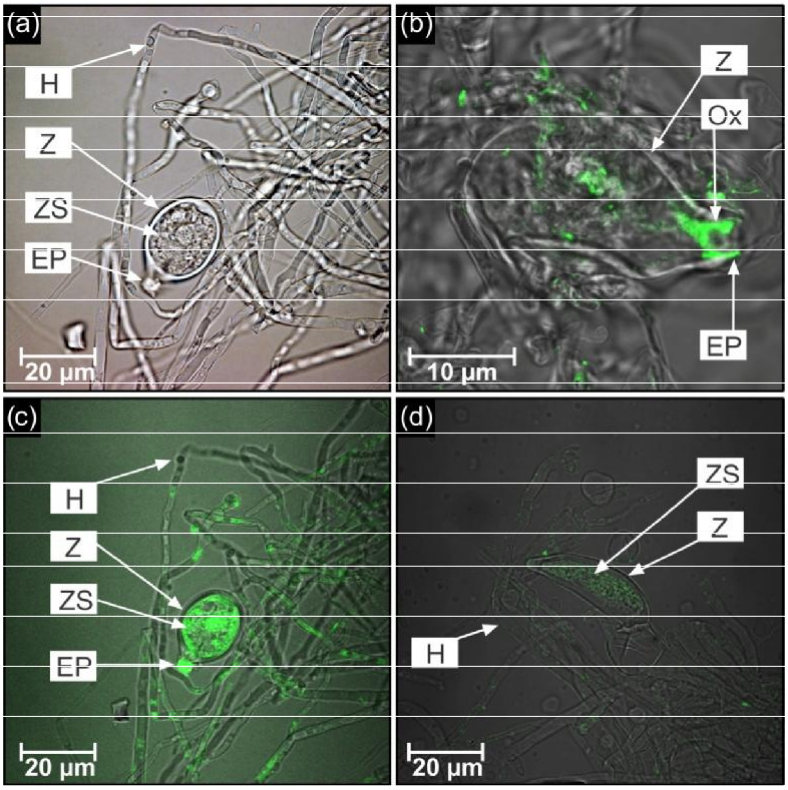

Figure 1. (a), A light micrograph indicating vegetative hyphae (H) and a zoosporangium (Z), containing zoospores (ZS) of *Phytophthora nicotianae*. The exit pore (EP) where the zoospores are released can also be observed. (b), An empty zoosporangium (Z) surrounded by vegetative hyphae. Here, 3-hydroxy (OH) oxylipins (Ox; indicated by the green fluorescence) can be observed covering the exit pore of the zoosporangium where the zoospores are released. (c), A confocal laser scanning micrograph indicating again the vegetative hyphae (H) and a zoosporangium (Z) with zoospores (ZS). Here we can see an increase in mitochondrial membrane potential ($_m$; indicated by the green fluorescence), as determined by Rhodamine 123 staining, associated with the zoospores inside the zoosporangium, including the exit pore (EP) and not the surrounding hyphae. (d), A confocal laser scanning micrograph indicating an increase in the number of mitochondria (determined using a monoclonal antibody specific for prohibitin in the mitochondria; indicated by the green fluorescence) in the zoosporangium (Z) containing zoospores (ZS). This increase could not be observed in the surrounding hyphae (H).

et al., 2007). A similar phenomenon may be observed here, where the 3-OH oxylipins situated in this bottleneck or exit pore of the zoosporangium, probably act as lubricants to ensure the effective release of zoospores into the environment. On the other hand, this may also be 3-OH oxylipins that were scraped off in the narrow exit pore opening during zoospore release.

Mitochondrial activity studies - membrane potential ($_m$)

In *P. nicotianae*, an increase in mitochondrial $_m$, indicated by the intense green fluorescence, was observed in the zoosporangium and not in the surrounding vegetative hyphae (Figure 1c). This indicates that an increase in mitochondrial activity is probably needed to produce multiple zoospores in the zoosporangium. Interestingly, the mitochondrial $_m$ was very intense at the exit pore – the position where 3-OH oxylipins accumulated. Here the same trend is observed as previously reported for yeasts (Kock et al., 2007), where there was an increase in $_m$ associated with the sexual phase.

Mitochondrial location

Since the results indicate an increase in mitochondrial activity i.e. increased $_m$ as well as increased

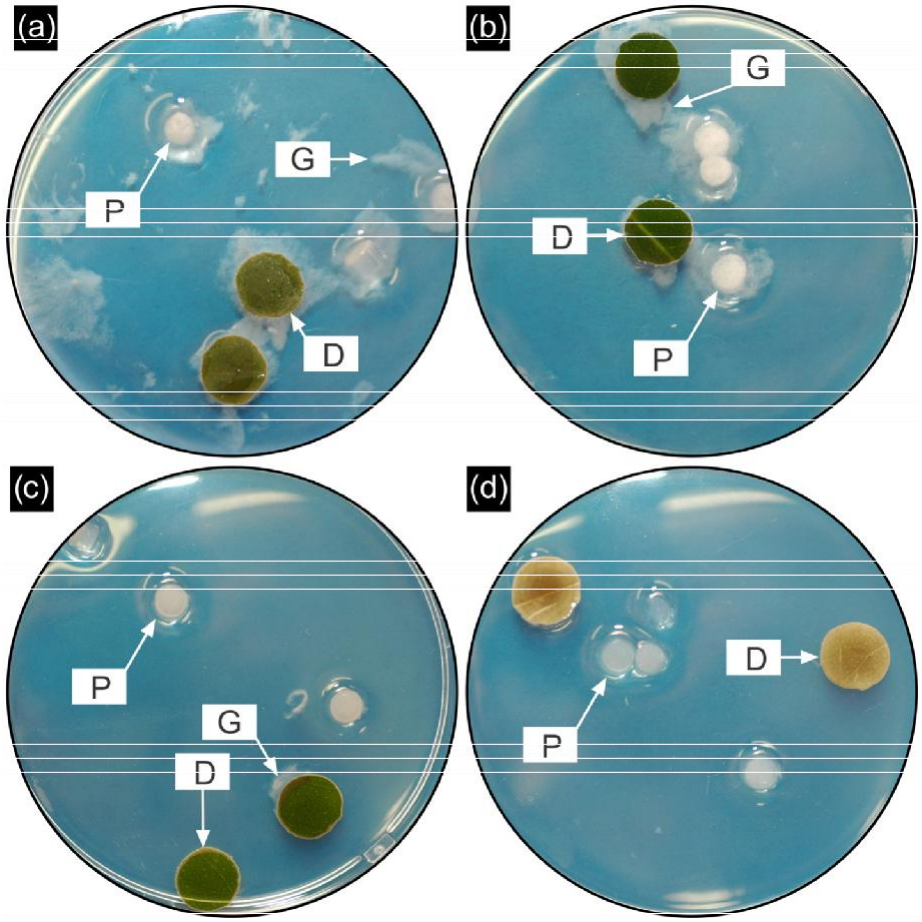

Figure 2. Photographs of plates containing *Phytophthora nicotianae* on soft potato dextrose agar (PDA) plugs and discs of surface sterilized citrus leaves. (a), Control plate containing only the organism on plugs (P) as well as citrus discs (D). Here good growth (G) could be observed around the plugs as well as discs. (b), Ethanol control plate containing the same amount of ethanol used to dissolve 5 mM of acetylsalicylic acid (ASA). Here good growth was also observed around the plugs as well as the discs. (c), A plate containing 1 mM of ASA. In this case a drastic decrease in growth could be observed, with only little growth around the disc. (d), A plate containing 5 mM of ASA. Here no growth could be observed around the plugs or discs, demonstrating the effect that ASA has on the growth of this organism.

-oxidation (3-OH oxylipin production) in the zoosporangium, the accumulation of mitochondria in this location was not surprising. According to CLSM results, mitochondria are situated mostly in the zoosporangium surrounding the zoospores of *P. nicotianae* as indicated by an increase in green fluorescence (Figure 1d). In the surrounding hyphae the mitochondria are less, as indicated by very little green fluorescence (Figure 1d). This is probably due to the fact that a higher number of mitochondria (to produce necessary energy) are needed for the production of multiple zoospores inside the zoosporangium. Similar results have been obtained with various other fungi and fungi-like organisms where the fruiting structures (i.e. sexual phase, phialide-conidia complex and sporangia) showed increased mitochondria populations (Leeuw et al., 2009; Ncango et al., 2010).

Effects of acetylsalicylic acid on growth

Macroscopically, growth was visible around the plugs and leaves in the control (Figure 2a) and ethanol control plates (Figure 2b). However, a drastic decrease in growth was observed in the presence of 1 mM ASA (Figure 2c) while in the presence of 5 mM ASA (Figure 2d), no growth could be observed. These inhibitory effects can be attributed to the presence of ASA since in the ethanol control (ethanol concentration same as with 5 mM ASA culture) good growth was also observed. These experiments were performed in triplicate and reproducible observations were recorded. Biomass measurement was not possible in these experiments due to the extremely small amount of growth and difficulty to separate these from agar plugs and leave discs.

Table 1. The influence of acetylsalicylic acid (ASA) concentration on zoosporangium formation by *Phytophthora nicotianae*.

ASA concentration (mM)	Number of zoosporangia				Average	SD
	Field #1	Field #2	Field #3	Field #4		
0	72	65	80	67	71	6.7
0.1	15	4	3	2	6	6.1
1	0	0	0	0	-	-
2.5	0	0	0	0	-	-
5	0	0	0	0	-	-
Ethanol control	65	69	64	70	67	2.9

SD = standard deviation; Fields #1 - #4 = Microscopic fields selected at random around discs and plugs.

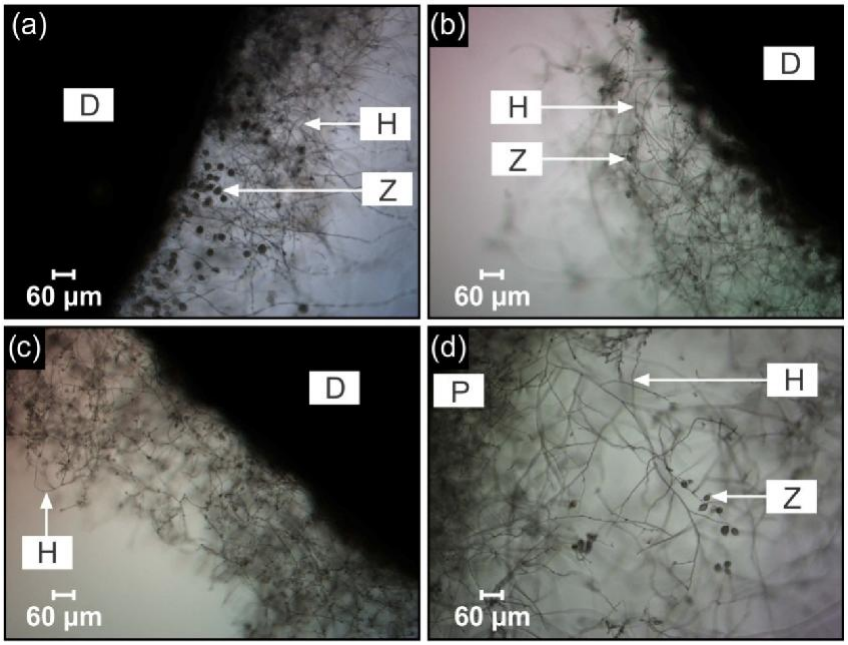

Figure 3. (a), Light micrograph of growth of *Phytophthora nicotianae* obtained from the control plate that contains no acetylsalicylic acid (ASA). Here, good hyphal (H) growth as well as a vast number of full and empty zoosporangia (Z) could be observed. (b), A drastic decrease in zoosporangia in the plate containing 0.1 mM of ASA could be observed in this light micrograph, yet good hyphal growth was evident. (c), Light micrograph of growth obtained from the plate containing 1 mM of ASA. In this case impaired hyphal growth, with the hyphae being misformed and showing unusual nodules, could be observed. In this plate no zoosporangia could be observed, indicating that the fruiting structure has increased susceptibility towards ASA when compared to hyphae. (d), Light micrograph of growth obtained from the plate containing only ethanol. Here, hyphal growth and zoosporangium production were similar to the control, indicating that ethanol did not influence the results obtained. These results are similar to that shown in Table 1. D = leave discs; P = plugs.

Mitochondrial inhibition studies

Previous studies indicated that sites with increased mitochondrial activity (fruiting structures such as sexual stages, phialide-conidia-complex and sporangia) are more susceptible to mitochondrial inhibitors such as ASA. It was confirmed in this study that fruiting structures i.e. zoosporangia of *Phytophthora* are also more susceptible to ASA at low concentrations, than hyphal growth (Table 1). A significant difference was observed between the number of zoosporangia in the control plates (0 mM ASA; Figure 3a) and the plate containing 0.1 mM ASA ($P < 0.001$) although good growth could be observed in both cases (Figure 3b). This indicates that the fruiting structure or zoosporangium is more sensitive towards this antifungal at low concentrations, than the vegetative

hyphae. There was no significant difference between the number of zoosporangia and growth in the control (0 mM ASA) and the ethanol control (contains the same amount of ethanol needed to dissolve 5 mM of ASA) indicating that ethanol does not have an inhibitory effect on zoosporangium formation. No zoosporangia were observed in the plates containing 1 to 5 mM ASA. The plate containing 1 mM of ASA had impaired hyphal growth, with the hyphae being misformed and showing unusual nodules. No growth could be observed in the presence of 2.5 and 5 mM ASA. This pattern is also demonstrated in Figures 3a to 3d. Similar results were obtained with *P. citrophthora*.

Conclusions

The genus *Phytophthora* is a very large group of fungi-like organisms, including aquatic, amphibious and terrestrial species. The oomycetes consist of organisms that reproduce asexually by means of biflagellate zoospores. These spores are contained in zoosporangia of various types. Sexual reproduction is rare and almost always heterogametangic. Members of *Phytophthora* bear their zoosporangia directly on the somatic hyphae and the presence of zoosporangia of different ages can be observed on the same sporangiophore at any given time (Alexopoulos, 1962; Brasier, 1992; Judelson, 1997; Schumann and D'Arcy, 2000). It is important to note that zoospores (asexual reproduction) are mainly responsible for infecting plants. Consequently, the release of zoospores from the zoosporangium could be referred to as one of the main infectious stages.

Results from this study implicate novel targets by which *Phytophthora* infections may be inhibited. These findings necessitate urgent investigations into more oomycete representatives and assessing various anti-mitochondrials including NSAIDs as anti- oomycetous compounds. Who knows, these may prove in future to be effective in field applications. Detailed biochemical studies should now be performed on mitochondria to identify the type of mitochondrial inhibition exerted by different anti-mitochondrial drugs.

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REFERENCES

Alexopoulos CJ (1962). Introductory Mycology. Second Edition. John Wiley and Sons, Inc., USA, Toppan Company, LTD., Japan.

Brasier CM (1992). Evolutionary biology of *Phytophthora*: Genetic system, sexuality and the generation of variation. Ann. Rev. Phytopath., 30: 153 – 171.

Haas BJ, Kamoun S, Zody MC (2009). Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. Nature, 461(17): 393 – 398.

Haverkort AJ, Boonekamp PM, Hutten R, Jacobsen E, Lotz LAP, Kessel GJT, Visser RGF, Van der Vossen EAG (2008). Societal costs of late blight in potato and prospects of durable resistance through cisgenic modification. Potato Res., 51: 47 – 57.

Judelson HS (1997). The genetics and biology of *Phytophthora infestans*: Modern approaches to a historical challenge. Fungal Genet. Biol., 22: 65 – 76.

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.

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

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.

Ncango DM, Swart CW, Pohl CH, Van Wyk PWJ, Kock JLF (2010). Mitochondrion activity and dispersal of *Aspergillus fumigatus* and *Rhizopus oryzae*. Afr. J. Microbiol. Res., 4(9): 830 – 835.

Schumann GL, D'Arcy CJ (2000). Late blight of potato and tomato. The Plant Health Instructor. DOI: 10.1094/PHI-I-2000-0724-01.

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.

Swart CW, Van Wyk PWJ, Pohl CH, Kock JLF (2010a). Variation in mitochondrial activity over the life cycle of *Nadsonia fulvescens*. Afr. J. Microbiol. Res., 4(16): 1727 – 1732.

Swart CW, Swart HC, Coetsee E, Pohl CH, Van Wyk PWJ, Kock JLF (2010b). 3-D architecture and elemental composition of fluconazole treated yeast asci. Sci. Res. Essays, 5(22): 3411 – 3417.