

# **Ascospore release and oxylipin production in the yeast**

## ***Dipodascopsis***

by

**Monique E. Goldblatt**

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Department of Microbial, Biochemical and Food Biotechnology

Faculty of Natural and Agricultural Sciences

University of the Free State

Bloemfontein

Republic of South Africa

**Supervisor:** Prof. J.L.F Kock

**Co-supervisors:** Prof. P.W.J van Wyk

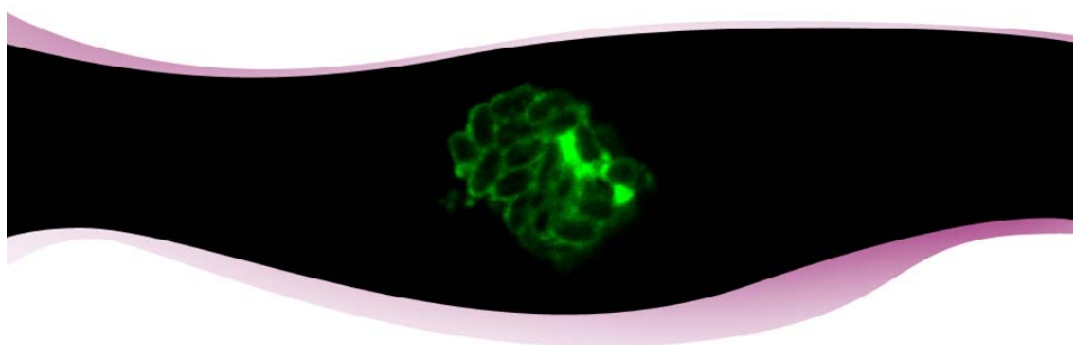
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- **Serijke Grobler**, for bringing the best out in me in all aspects of my life.





Two roads diverged in a yellow wood,  
And sorry I could not travel both  
And be one traveller, long I stood  
And looked down one as far as I could  
To where it bent in the undergrowth;

Then took the other, as just as fair,  
And having perhaps the better claim,  
Because it was grassy and wanted wear;  
Though as for that the passing there  
Had worn them really about the same,

And both that morning equally lay  
In leaves no step had trodden black.  
Oh, I kept the first for another day!  
Yet knowing how way leads on to way,  
I doubted if I should ever come back.

I shall be telling this with a sigh  
Somewhere ages and ages hence:  
Two roads diverged in a wood, and I--  
I took the one less travelled by,  
And that has made all the difference.

...Robert Frost

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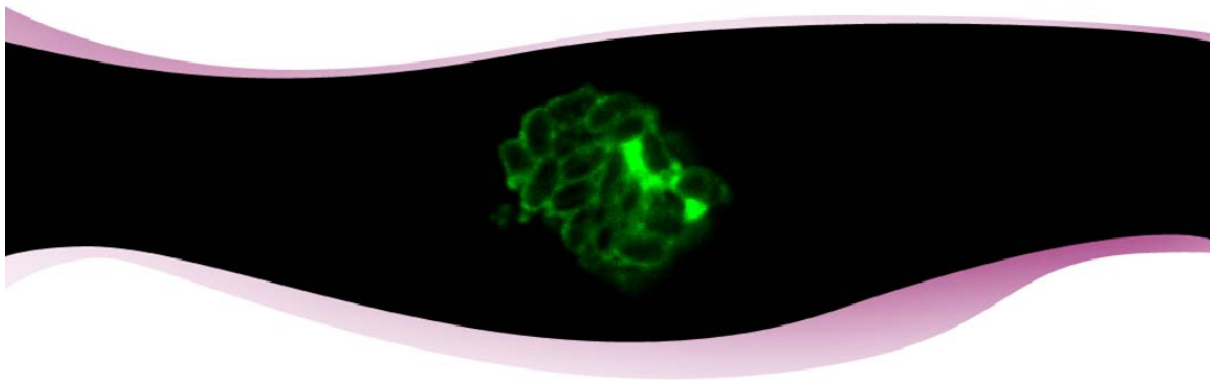
### Ascospore release and oxylipin production in *Dipodascopsis uninucleata*

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# Chapter 1



## Literature review

## 1.1. Motivation

The yeast genus *Dipodascopsis* was first described by Batra in 1973 (Cottrell and Kock 1989). Since then, this genus became an interesting model for performing novel ascospore release and oxylipin production experiments (Botha et al. 1992, 1993; Venter et al. 1997; Smith et al. 2000; Kock et al. 2007).

Synchronous life cycle studies were performed on a *D. uninucleata* var. *uninucleata* strain, isolated from fruit flies in Canada (Botha et al. 1992). They found that a large number of ascospores were released from asci (i.e. sexual stage) to restore the asexual stage. Interestingly, when this yeast was exposed to acetylsalicylic acid (ASA; a mitochondrial inhibitor) (Kock et al. 2007), the sexual stage was inhibited in a dose-dependent manner with ascospore release the most sensitive (Botha et al. 1992). Similarly, Van Dyk and co-workers (1991, 1993) found that 3-hydroxy (OH) oxylipins produced by this yeast, in asci and coating ascospores (Kock et al. 1998), are inhibited by ASA also in a dose-dependent manner. Therefore it is concluded that 3-OH oxylipins are involved in the release of the ascospores, probably having a lubricating function aiding their release (Kock et al. 1999). This was the first report on the function of 3-OH oxylipins in yeasts (Kurtzman et al. 1974; Van Dyk et al. 1994).

It was also reported that *D. uninucleata* var. *uninucleata* (Canadian strain) can serve as a source for the production of 3-OH oxylipins. When this strain was fed with exogenous arachidonic acid (AA), a precursor for prostaglandin formation in humans, a 3*R*-OH 5*Z*, 8*Z*, 11*Z*, 14*Z*-eicosatetraenoic acid (3-HETE) was found to be

produced (Van Dyk et al. 1991, 1993). A maximum 3-HETE yield of 1.9 % from AA was obtained (Fox et al. 1997).

Closer observation revealed that oxylipin-coated ascospores have a unique mode of release from asci (Kock et al. 2004). The ascospores are pushed through turgor pressure towards the narrow opening and then discharged (Kock et al. 2004). Upon individual release, the ascospores aggregate in ordered clusters outside the ascus (Kock et al. 1998, 1999). Transmission electron microscopy showed the ascospores to have nano-scale surface hooks which play a role in the ordered separation during release as well as the ordered aggregation after release by providing attachment sites for the oxylipin interspore matrix (Kock et al. 1999). Immunofluorescence microscopy performed, further confirmed the close association of 3-OH oxylipins with the sexual stage i.e. asci and ascospores (Kock et al. 1998).

The genus *Dipodascopsis* has been extensively studied with regards to reproductive cycles as well as the presence, distribution and function of 3-OH oxylipins. Most of this research has been carried out on the Canadian strain of *D. uninucleata* var. *uninucleata*, as well as *D. tóthii* (Kock et al. 2007). However, little is known concerning the South African strain of *D. uninucleata* var. *uninucleata* as well as *D. uninucleata* var. *wickerhamii*. Consequently, with this as background, it became the aim of this dissertation to assess and compare the morphologies, oxylipin production, mitochondrial activity as well as life cycles and ascospore release of *D. uninucleata* var. *uninucleata* (UOFS Y-2067, isolated from soil in South Africa) and *D. uninucleata* var. *wickerhamii* (UOFS Y-1129, isolated from a fruitfly).

## 1.2. Classification of *Dipodascopsis*

In 1890, Juel isolated *Dipodascus* from woods, defining it as a fungus which formed hyphae resulting in arthrospores (Cottrell and Kock 1989). Following this, Biggs isolated *D. uninucleatus* in 1937, and later Zsolt (1963) described *D. tóthii*. In 1973, Batra transferred these species to the new genus *Dipodascopsis*, essentially because of their distinctive septal ultrastructure, inability to produce arthroconidia and mode of ascus formation. He renamed *Dipodascus uninucleatus* as *Dipodascopsis uninucleata* and in 1978 renamed *Dipodascus tóthii* as *Dipodascopsis tóthii* (Kreger van Rij 1984). Finally in 1987, Kreger van Rij classified this genus under the Saccharomycetaceae together with *Lipomyces* (Cottrell and Kock 1989). *Dipodascopsis* as well as *Babjevia* were later placed under the family Lipomycetaceae together with *Lipomyces* (Van der Walt et al. 1987; Von Arx and Van der Walt 1987).

Within the Saccharomycetales Kudryavtsev, the yeast family Lipomycetaceae is regarded as one of the least evolved members (Jansen van Rensburg 1994). Four characteristics enable this family to be distinguished from other Saccharomycetaceae (Endomycetalean) families. These include the ability to produce viscous or slimy colonies on solid media as a result of extracellular amyloid material, the inability to ferment, the ability to utilize heterocyclic compounds, e.g. imidazole and thymine, as sole nitrogen source, as well as the production of sexually attached, multispored asci during reproduction (Van der Walt 1992; Jansen van Rensburg 1994).

This family is classified as follows (in red) in the current classification system of the ascomycetous yeasts proposed by Barnett and co-workers (2000):

**Kingdom Fungi**

**Phylum Ascomycota**

**Class** Archiascomycetes

**Class** Euascomycetes (1 genus)

**Class Hemiascomycetes**

**Order Saccharomycetales**

**Families**

- Candidaceae (11 genera)
- Dipodascaceae (7 genera)
- Eremotheciaceae (1 genus)
- Lipomycetaceae** (5 genera)
- Metschnikowiaceae (2 genera)
- Phaffomycetaceae (2 genera)
- Saccharomycetaceae (17 genera)
- Saccharomycodaceae (4 genera)
- Saccharomycopsidaceae (2 genera)
- Unclassified Saccharomycetales (3 genera)

The representatives of the genus *Dipodascopsis* are characterized by Smith and De Hoog (1998), as follows:

**Diagnosis:**

*“Colonies are whitish, moist, somewhat slimy; true hyphae are present; arthroconidia are absent; multilateral budding cells are sometimes present. Asci are acicular or cylindrical, and formed laterally on hyphae after fusion of gametangia. Asci have*

*persistant walls, and open by rupture at the apex. Ascospores are 32-128 per ascus, hyaline, ellipsoidal to reniform, and with a smooth wall and without a slime sheath. Fermentation is absent. Extracellular starch is produced. Diazonium Blue B reaction is negative. Coenzyme Q-9 system is present. Cells are multilamellar. Septa have narrow pores.”*

**Type Species:**

*Dipodascopsis uninucleata* Batra and P. Millner

**Species accepted:**

1. *Dipodascopsis tóthii* (Zsolt) Batra and P. Millner
2. *Dipodascopsis uninucleata* (Biggs) Batra and P. Millner
  - a. *Dipodascopsis uninucleata* (Biggs) Batra and P. Millner var. *uninucleata*
  - b. *Dipodascopsis uninucleata* var. *wickerhamii* Kreger van Rij

**Key to Species:**

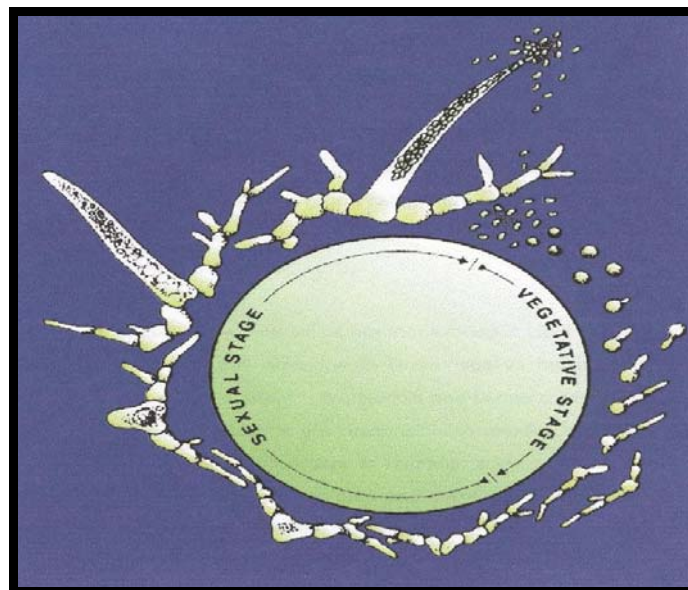
1.
  - a. Cellobiose and lactose assimilated ..... *D. tóthii*
  - b. Cellobiose and lactose are not assimilated → 2
- 2(1).
  - a. Sucrose and raffinose assimilated.....  
.....*D. uninucleata* var. *uninucleata*
  - b. Sucrose and raffinose are not assimilated.....  
.....*D. uninucleata* var. *wickerhamii*

**1.3. Morphology and sexual reproduction of *Dipodascopsis***

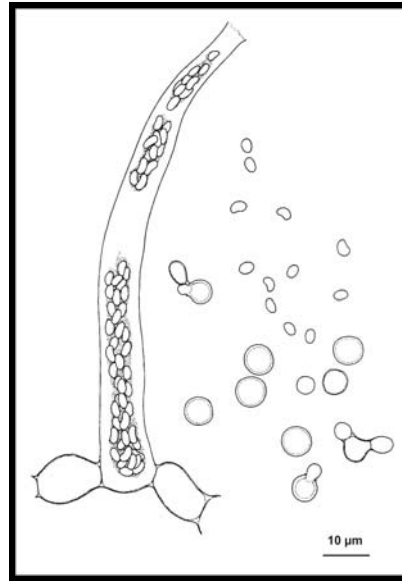
**1.3.1. *Dipodascopsis uninucleata* (Biggs) Batra and P. Millner. (Fig. 1 and 2)**

According to Smith and De Hoog (1998), this yeast species is homothallic and is characterized by a haplontic life cycle. *D. uninucleata* is the only yeast isolated to this date, known to depend on its sexual reproductive stage for growth (Smith 2002). Other known yeasts grow via asexual stages while their sexual stages are used

mainly for survival (Van Dyk et al. 1994). The haplontic life cycle is characterized by consecutive asexual (vegetative) and sexual reproductive stages (Botha et al. 1992; Fig. 1). During the asexual stage, ascospores swell and germinate to form germ tubes, which give rise to hyphae. The sexual stage then follows, firstly with plasmogamy of two gametangia (undifferentiated adjacent cells or hyphal tips which fuse), karyogamy (fusion of nuclei), meiosis and post meiotic mitosis resulting in the development of multi-spored asci. Asci are acicular and contain approx. (ca.) 30–120 ascospores. During ascospore release, asci open by rupturing at the apex and ascospores are pushed individually through the narrow ascus opening (Kock et al. 2004). Ascospores are ca. (0.8-1.2) x (2.0-2.8)  $\mu\text{m}$  in size, hyaline or subhyaline, ellipsoidal to bean-shaped and do not have a slime sheath (Smith and De Hoog 1998; Fig. 2).



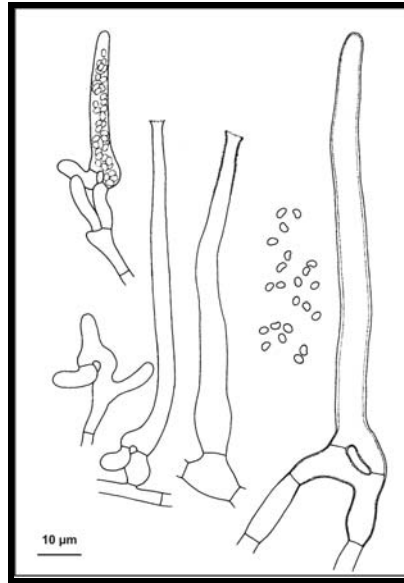
**Fig. 1. Life cycle of *Dipodascopsis*. Vegetative stage (11-13 h): Considerable swelling of the minute reniform ascospores, 15-20 h: Formation of germ tubes, 28-35 h: Number of swollen ascospores and cells with germ tubes decrease and filaments of cells form. Sexual stage (ca. 40 h): Ascosporangogenesis and ascospore dispersal from asci signal the end of the life cycle. Many ascospores are formed by post meiotic mitosis (reproduced from Venter 1999).**



**Fig. 2. *D. uninucleata* var. *uninucleata*. Ascus containing ascospores, liberated ascospores swell, become thick-walled prior to germination and occasionally form yeast-like cells (reproduced from Smith and De Hoog 1998).**

### **1.3.2. *Dipodascopsis tóthii* (Zsolt) Batra and P. Millner.**

According to literature this species is homothallic and is characterized by a haplontic life cycle (Smith and De Hoog 1998). During the asexual stage, ascospores swell and germinate to form germ tubes, which give rise to hyphae (similar to Fig. 1). The sexual stage consists of plasmogamy of two gametangia, karyogamy, meiosis and then post meiotic mitosis resulting in many ascospores (ca. 30-120) in acicular shaped sac-like asci. Here the ascospores are allowed to develop and then released through the narrow ruptured opening at the ascus tip (Fig. 3). During ascosporogenesis small bean-shaped ascospores with sheath-like appendages are released individually from the ascus tip, which then assemble in sheathed cluster balls (Smith et al. 2000).



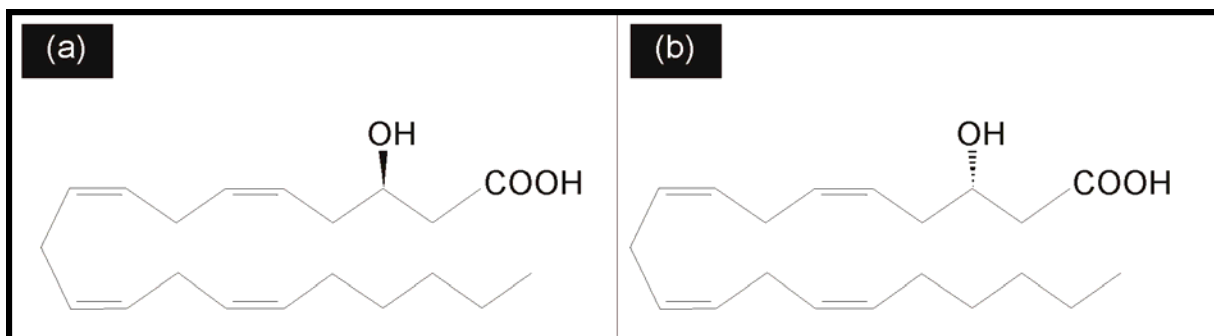
**Fig. 3. *D. tothii*. Ascii in various stages of maturation (reproduced from Smith and De Hoog 1998).**

#### **1.4. 3-Hydroxy oxylipins**

For the purpose of this study, emphasis will be placed on 3-OH oxylipin production and function in the yeast *Dipodascopsis*.

##### **1.4.1. Structure**

Oxylipins are defined as saturated and unsaturated hydroxy fatty acids (FAs; Bhatt et al. 1998) and are widely distributed amongst animals and plants where they exert a wide array of biological actions. 3-OH oxylipins have a basic structure comprising a hydrophilic carboxylate group (polar head) attached to one end of a hydrocarbon chain and a hydroxyl group at the C3 position (counted from the carboxylate group) (Finnerty 1989; Kock et al. 2007). These compounds can also be present in two enantiomeric forms i.e. *R* and *S* (Fig. 4).



**Fig. 4. The chemical structures of the enantiomers (a) *R*- and (b) *S*-3-hydroxy-5, 8, 11, 14-eicosatetraenoic acid (3-HETE; reproduced from Bhatt et al. 1998).**

#### 1.4.2. Metabolism

It has been proposed that these 3-OH oxylipins are produced through an incomplete  $\beta$ -oxidation pathway (Finnerty 1989). This is a catabolic pathway and can be divided into two phases (Fig. 5). In the first phase, a long chain fatty acid, which is suitable for catabolism, diffuses into the cell through the cytoplasmic membrane from the environment. The second phase then follows, starting with the formation of a fatty acyl CoA, which is catalysed by acyl CoA synthetase. This fatty acyl CoA is then transported into the mitochondria through a carnitine transport mechanism. Here, dehydrogenation of the fatty acyl CoA by removal of a pair of hydrogen atoms from the  $\alpha$ - and  $\beta$  carbon atoms, to yield an unsaturated acyl CoA, occurs. This compound is then enzymatically hydrated to form a racemic mixture of *L* and *D*  $\beta$ -OH-acyl-CoA (3-OH fatty acids). In fungal  $\beta$ -oxidation only the *L*-enantiomer of the 3-OH-acyl-CoA is further dehydrogenated via a  $\text{NAD}^+/\text{NADH}$  system to yield a  $\beta$ -ketoacyl-CoA. In the last step, the  $\beta$ -ketoacyl-CoA undergoes enzymatic cleavage to yield an acetyl CoA as well as an acyl CoA i.e. with two carbon atoms less than the original. The *D*-enantiomer may be produced in excess amounts and can either undergo epimerization to yield the *L*-enantiomer, which then proceeds through the

degradation process, or the excess may be secreted to the outside and accumulate in the ascus surrounding the ascospores as 3-OH oxylipins (Venter et al. 1997; Kock et al. 1998, 2007).

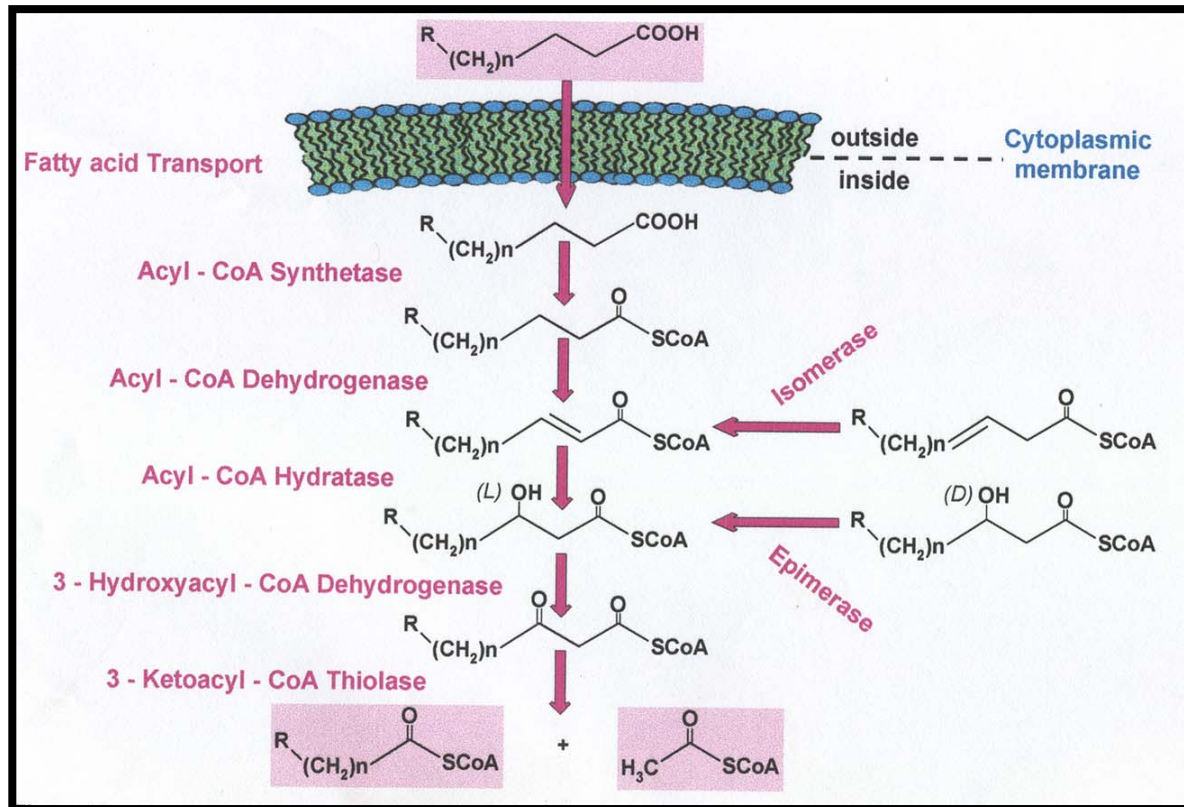


Fig. 5.  $\beta$ -oxidation of fatty acids (reproduced from Venter 1999).

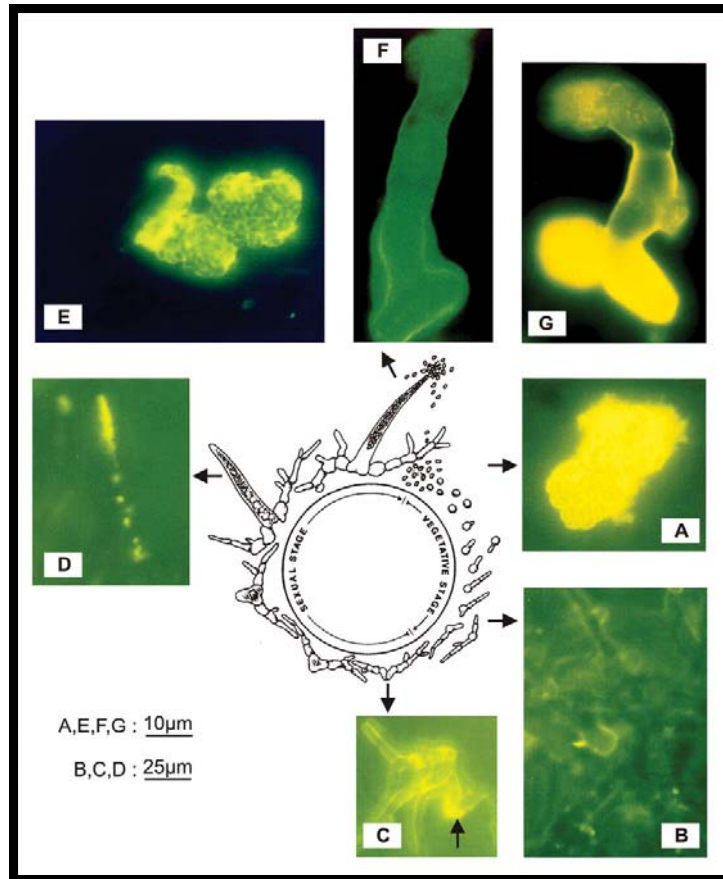
### 1.4.3. Distribution

Records on the presence of 3-OH oxylipins in fungi started as early as 1967 when Stodola and co-workers reported the presence of saturated 3(D)-OH-hexadecanoic (16:0) and -octadecanoic acid (18:0) as components of the extracellular glycolipids in strains of the yeasts *Rhodotorula graminis* and *R. glutinis*. In 1968, Vesonder and co-workers reported the formation of large quantities of saturated 3-OH 16:0 by *Saccharomycopsis malanga*. Further reports of the formation

of these 3-OH 16:0 by *S. malanga* followed in 1974 (Kurtzman et al. 1974). 3-OH hexanoic acid (6:0) and –octanoic acid (8:0) were also found to be present in the glycolipids of the smut fungi, *Ustilago nuda* and *U. zea* (Lösel 1988).

Unsaturated 3-OH oxylipins have also been found to be produced by fungi. In 1991, Van Dyk and co-workers reported the biotransformation of exogenously fed arachidonic acid (AA) to 3-HETE by the yeast *D. uninucleata* var. *uninucleata* (Canadian strain). In 1997, Kock and co-workers constructed a process by which approximately 4 % of the AA fed to the yeast was transformed to 3-HETE. Biotransformation of AA to oxylipins was also found to occur in *Babjevia anomala* and *D. tóthii* (Kock et al. 1991; Van Dyk et al. 1991; Coetzee et al. 1992). Fox and co-workers (1997) found yields of approximately 1.9 % of 3-HETE from AA using *D. uninucleata* in an optimized bioreactor process.

In order to map the distribution of 3-HETE in *D. uninucleata* var. *uninucleata* (Canadian strain), polyclonal antibodies specific for 3-OH oxylipins in general were developed (Kock et al. 1998). Kock and co-workers found that 3-OH oxylipins are formed during the sexual stage i.e. during gametangiogamy and ascosporeogenesis, and not during the asexual stage (Fig. 6). 3-OH oxylipins were found associated with the surfaces of adhering gametes (gametangiogamy; Fig. 6c), within the developing asci (Fig. 6d), as well as surrounding the aggregating liberated ascospores (Fig. 6e; Kock et al. 1998).



**Fig. 6.** The distribution of 3-HETE in the life cycle of *D. uninucleata* var. *uninucleata* (Canadian strain) through immunofluorescence mapping. **A,** Liberated ascospores. **B,** Hyphae. **C,** Gametangiogamy. **D,** Young ascus with ascospores. **E,** Liberated ascospores from ascus. **F,** Empty ascus protoplast: still with characteristic morphology. **G,** Deformed mature ascus protoplast containing ascospores mainly at base (reproduced from Kock et al. 1998).

#### 1.4.4. 3-OH oxylipin function and ASA sensitivity in *Dipodascopsis*

In 1992, Botha and co-workers studied the effects of ASA on the reproductive cycle of *D. uninucleata* var. *uninucleata* (Canadian strain) and *D. tóthii*. At the start of growth, ASA was added to synchronous cultures, i.e. started from ascospores only. They found that in the absence of ASA, the life cycles were completed, which was indicated by a sharp rise in the released ascospores after ca. 40 h. In the presence of 1 mM ASA, the life cycles were severely inhibited. Inhibition was observed also in the transition of germinating ascospores to the hyphal stage resulting in a decrease

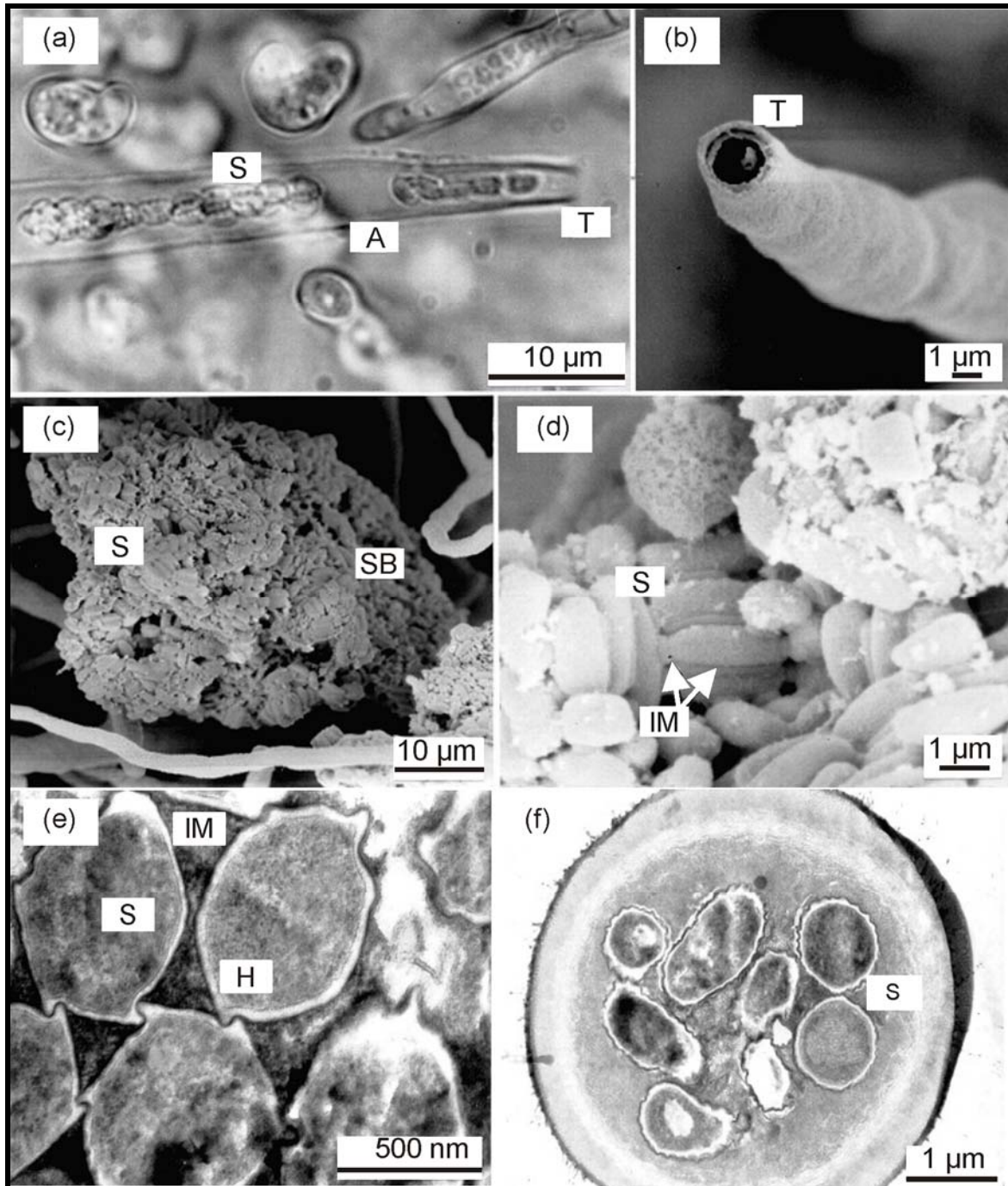
in the maximum number of hyphae formed. The type strain of *D. tóthii* was much more sensitive to 1 mM ASA in this regard. They further observed decreased amounts of asci produced in *D. uninucleata* var. *uninucleata* (Canadian strain) when compared to the controls. This is in contrast to *D. tóthii*, where no asci were produced in the presence of 1 mM ASA. However, the asci in *D. uninucleata* var. *uninucleata* (Canadian strain) were much less when compared to the controls and did not liberate ascospores even when cultivated for 60 h.

Van Dyk and co-workers (1991) studied the nature of this aspirin sensitive AA derived compound produced during the sexual reproductive phase. This compound was purified and identified as a 3-OH-all *cis*-5,8,11,14-eicosatetraenoic acid (3-HETE) – a novel eicosanoid. This yeast cannot produce 3-HETE *ab initio* since it can not produce AA. It was found that *D. uninucleata* produces 3-OH 14:2 *ab initio* from cellular linoleic acid (18:2; Venter et al. 1997). It was suggested by Mazur and co-workers (1991) that oxylipins exert a regulatory role during the sexual phase of fungi.

In 2002, Smith and co-workers observed the mechanism of spore release from asci. They found that ASA sensitive oxylipins and small nano-scale hooks located on the surface of the ascospores of *D. uninucleata* var. *uninucleata* (Canadian strain) are involved in the aggregation of the ascospores after they are released (Kock et al. 1999; Smith et al. 2000). It was found that in the absence of ASA, hundreds of ascospores initially packed in clusters inside a sac-like ascus, separate and were released individually from the tip of the ascus (Fig. 7a and 7b). The ascus opening is formed during turgor pressure build-up through which ascospores are released (Biggs 1937). 3-OH oxylipins act as a lubricant aiding in unimpeded ascospore

release through the narrow ascus opening (Kock et al. 2004). The ascospores then join in relatively large ascospore clusters in the culture medium (Fig. 7c). Before ascospore release, some 3-OH oxylipins are released from the ascus, and coalesce through entropic-based hydrophobic forces (Rudolph 1994) just outside the opened tip of the ascus. The ascospores are then released and are “attracted” within these oil droplets to form sticky aggregates. Ordered ascospore clusters, with a defined interspore matrix, were observed in this yeast (Fig. 7d).

Smith and co-workers (2000) observed the nano-scale surface hooks on the ascospores to be perfectly interlocked when the culture was grown in the absence of ASA (Fig. 7e). These hooks were found to play a role in ordered separation as well as ordered aggregation providing attachment sites for the interspore oil-like matrix. In the presence of ASA, it was found that oxylipin production (Van Dyk et al. 1991) as well as ascospore release was inhibited in *D. uninucleata*, in a dose-dependent manner. In the presence of 0.1 mM ASA, a small amount of ascospores were released, many ascospores contained no hooks, and some ascospores re-aggregated in a random pattern (Kock et al. 1999). In the presence of 1 mM ASA, no hooks were formed on the surface of the ascospores inside the ascus (Fig. 7f) and no ascospores were released (Kock et al. 1999).



**Fig. 7. Typical shaped asci of *D. uninucleata* var. *uninucleata* (Canadian strain) showing individual release of ascospores from the tip (a and b). After ascospore release, ascospores re-aggregate (c), in ordered clusters with distinct interspore matrix (d). Hooked surface ornamentations on ascospores in the absence of ASA (e), and the absence of hook formation on the surface of ascospores in the presence of 1 mM ASA (f). A = ascus, H = rib-like surface structures (hooks), IM = interspore matrix, S = ascospores, SB = ascospore bundle, T = ascus tip (reproduced from Smith et al. 2000).**

#### 1.4.5. Purpose of research

With this as background, it became the aim of this study to assess and compare the:

1. Morphologies,
2. Oxylin production,
3. Mitochondrial activity,
4. Life cycles,
5. Ascospore release, and
6. ASA sensitivity

of *D. uninucleata* var. *uninucleata* (UOFS Y-2067, isolated from soil in South Africa) and *D. uninucleata* var. *wickerhamii* (UOFS Y-1129, isolated from a fruitfy).

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

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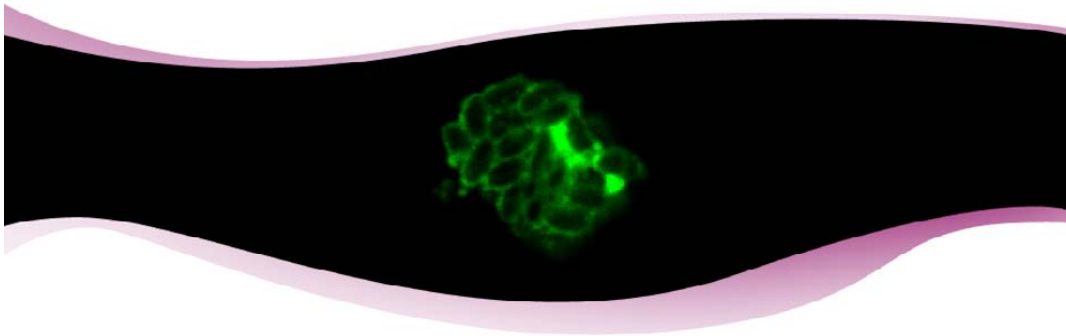
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# Chapter 2



## **Ascospore release and oxylipin production in *Dipodascopsis uninucleata***

The candidate performed preliminary studies during her B.Sc. Honours in 2005. After additional work during her M.Sc. study in 2006 and 2007, this section was prepared for publication in Canadian Journal of Microbiology. Consequently, this chapter is written in this journal's format as an article and also included with permission in this study. Antibodies were obtained from Prof. S. Nigam, Free University of Berlin, Germany. The work presented in this chapter has been performed by the candidate. Furthermore, parts of this chapter have been submitted to FEMS Yeast Research.

## 2.1. Abstract

*Dipodascopsis uninucleata* var. *uninucleata* is considered a source for the production of various bioactive 3-hydroxy (OH) oxylipins. In the search for other sources of similar oxylipins, as well as to map the distribution of 3-OH oxylipins in this species, a logical step was to also investigate the closely related species, *Dipodascopsis uninucleata* var. *wickerhamii*, for the production of these compounds. Consequently, using gas chromatography - mass spectrometry, electron microscopy and confocal laser scanning microscopy, the two varieties were compared regarding their morphologies, life cycles, oxylipin production, and mitochondrial activity. Similar morphologies and life cycles are reported while 3-OH oxylipin production is associated mainly with the sexual stage and concentrated in the ascus surrounding the ascospores. 3-OH oxylipins such as 3-OH 14:2 was found in *Dipodascopsis uninucleata* var. *uninucleata* (UOFS Y-2067) while a different 3-OH oxylipin was reported for *Dipodascopsis uninucleata* var. *wickerhamii*. Furthermore, increased mitochondrial activity was also observed for both varieties during the sexual stage and found to be concentrated in close vicinity of the ascospores. Since mitochondria produce 3-OH oxylipins, it is suggested that the increased activity during sexual development would be to aid in the production and release of the ascospores. In addition, acetylsalicylic acid was found to inhibit the production of 3-OH oxylipins in both varieties by probably decreasing mitochondrial activity resulting in the inhibition of ascospore release.

**Keywords:** *Dipodascopsis*; mitochondrion activity; rhodamine 123; 3-hydroxy oxylipins; yeast.

## 2.2. Introduction

The genus *Dipodascopsis* was first described by Batra in 1973 (Cottrell and Kock 1989). At present the genus consists of two species, namely *D. uninucleata* and *D. tóthii*, whereby *D. uninucleata* is designated as the type species (Cottrell and Kock 1989). *D. uninucleata* became an interesting model for performing novel ascospore release and oxylipin production experiments (Botha et al. 1992,1993; Venter et al. 1997; Smith et al. 2000; Kock et al. 2007).

In 1992, Botha and co-workers performed synchronous life cycle studies on a *D. uninucleata* var. *uninucleata* strain, isolated from a fruit fly in Canada. They found the life cycle to consist of consecutive asexual and sexual reproductive stages (Botha et al. 1992). In order to restore the asexual stage, a large number of ascospores were released from the sexual stage i.e. asci. It was also found that when the yeast was fed with acetylsalicylic acid (ASA; a mitochondrial inhibitor) the sexual stage was inhibited in a dose-dependent manner with ascospore release the most sensitive (Botha et al. 1992; Kock et al. 2007). Strikingly, it was found that 3-hydroxy (OH) oxylipins are inhibited by ASA also in a dose-dependent manner (Van Dyk et al. 1991, 1993). These compounds were found to coat the ascospores (Kock et al. 1998) and probably have a lubricating function during their release (Kock et al. 1999). It was also reported that *D. uninucleata* var. *uninucleata* UOFS Y-128T (Canadian strain) can serve as a source for the production of 3-OH oxylipins. A 3R-OH 5Z, 8Z, 11Z, 14Z-eicosatetraenoic acid (3-HETE) was found to be produced in *D. uninucleata* var. *uninucleata* (Canadian strain) in the presence of exogenous arachidonic acid (AA), a precursor for prostaglandin formation in humans (Van Dyk

et al. 1991, 1993). A maximum 3-HETE yield of 1.9 % from AA was obtained (Fox et al. 1997).

Closer observation revealed that oxylipin-coated ascospores have a unique mode of release from asci (Kock et al. 2004). The ascospores are pushed through turgor pressure towards the narrow opening and then discharged (Kock et al. 2004). Upon individual release, the ascospores aggregate in ordered clusters outside the ascus (Kock et al. 1998, 1999). Transmission electron microscopy performed showed the ascospores to have nano-scale surface hooks which play a role in the ordered separation during release as well as the ordered aggregation after release by providing attachment sites for the oxylipin interspore matrix (Kock et al. 1999). This development made it possible to test the biological activity of these compounds and to prepare specific antibodies used to map their distribution (Kock et al. 1998). Is it possible that other strains and varieties of *D. uninucleata* also produce these oxylipins and show similar ASA sensitivity?

Since all the experiments so far performed in this genus have been on *D. tóthii* and *D. uninucleata* var. *uninucleata* (Canadian strain), it became the aim of this study also to assess and compare the morphologies, oxylipin production, mitochondrial activity as well as life cycles and ascospore release of *D. uninucleata* var. *uninucleata* (UOFS Y-2067, isolated from soil in South Africa) and *D. uninucleata* var. *wickerhamii* (UOFS Y-1129, isolated from a fruitfly).

## **2.3. Materials and methods**

### **2.3.1. Strains used**

*D. uninucleata* var. *uninucleata* (UOFS Y-2067, isolated from soil in South Africa) and *D. uninucleata* var. *wickerhamii* (UOFS Y-1129, isolated from a fruit fly) are preserved in the culture collection at the University of the Free State (UFS), Bloemfontein, South Africa. These yeasts were used throughout the study. *D. uninucleata* var. *uninucleata* (UOFS Y-128T, isolated from a fruit fly in Canada) was used as a reference strain to compare phenotypic characteristics as published (Bareetseng et al. 2004; Botha et al. 1992; Kock et al. 1998; Kock et al. 2004; Smith 2002) with that found in the other two strains. Mitochondrial staining was also performed on this strain.

### **2.3.2. Cultivation for morphological, oxylipin and mitochondrial analysis**

Yeast strains of *D. uninucleata* (UOFS Y-128T, UOFS Y-2067, UOFS Y-1129) were cultivated on yeast malt (YM) agar medium (Wickerham 1951) at 25 °C until the sexual stage was reached (about 48 h to 52 h). In all cases, a light microscope (Axioplan, Zeiss, Göttingen, Germany) was used to follow growth and ascospore formation. Yeast cells were scraped from agar plates and resuspended in 50 mL YNB liquid media [40 g/L glucose and 6.7 g/L Yeast Nitrogen Base (YNB – Difco, Becton, Dickinson and Company, Sparks, MD)]. One milliliter was then transferred to 100 mL YNB medium and allowed to grow at 25 °C on a rotary shaker (160 r.p.m) for about 48 h to 52 h until the sexual stage was reached. Cells obtained in this way were subjected to malachite green/safranin-, Orange G- and Nile red staining as well

as scanning electron microscopy, oxylipin mapping, mitochondrial mapping and oxylipin analysis.

### **2.3.3. Morphology, oxylipin- and mitochondrial- mapping**

***Ascospore staining:*** Cells were fixed on microscopic slides and then heat treated with malachite green (Merck, Darmstadt, Germany) for 5 min to ensure successful penetration into the ascospores. The cells were then washed with dH<sub>2</sub>O, counter stained with safranin (Merck, Darmstadt, Germany) for 10 sec and finally washed with dH<sub>2</sub>O before being viewed under a light microscope (Yarrow 1998) coupled to a Colorview Soft Digital Imaging System (Münster, Germany).

***Orange G/Nile red staining:*** A drop of 1 % Orange G/Nile red (Molecular Probes, Eugene, OR, USA) was added to cells on a microscope slide for 5 min at room temperature to allow the stain to penetrate the cells. The stained cells were then examined using a Nikon TE 2000 Confocal Laser Scanning Microscope (CLSM; Nikon, Tokyo, Japan).

***Oxylipin mapping:*** (1) *Antibody preparation and characterization:* Primary antibodies used for immunofluorescence CLSM were raised in female New Zealand white rabbits and characterized according to its titer, sensitivity and specificity (Kock et al. 1998). The antibodies were obtained from Prof. S. Nigam (Free University, Berlin, Germany).

(2) *Immunofluorescence protocol:* A small amount of sporulating culture was transferred to an Eppendorf tube. In order to get rid of debris and agar, the cells

were centrifuged for 10 min at 5 880 g, using a Hermle Z 160 M centrifuge (Lasec, Cape Town, South Africa) and the supernatant discarded using a Pasteur pipette. Thirty microliters of the primary antibody, specific to 3-hydroxy (OH) oxylipins, was added to the cells and then incubated in the dark for 60 min to allow sufficient binding to the oxylipins. The unbound primary antibodies were washed off with phosphate buffer saline (PBS; Oxiod, Hampshire, England). Thirty microliters of the secondary antibody (FITC anti-rabbit IgG; Sigma-Aldrich, Steinheim, USA) was added to the tube and then incubated in the dark for another 60 min. The unbound FITC anti-rabbit IgG were washed off with PBS. In order to maintain cell-antibody structure and fluorescence, wash treatments were performed in 2 mL Eppendorf tubes. Fluorescing cells submerged in 1,4-diazabicyclo [2.2.2] octane (Sigma-Aldrich, Steinheim, U.S.A.) were prepared on microscope slides and then examined using a Nikon TE 2000 CLSM.

***Mitochondrial activity mapping:*** Two milliliters of culture in sexual stage were transferred to a 2 mL Eppendorf tube. The cells were centrifuged for 10 min at 5 880 g, using a Hermle Z 160 M centrifuge (Lasec, Cape Town, South Africa), to form a pellet containing the yeast cells. The supernatant was removed with a Pasteur pipette. The cells were then suspended in PBS and centrifuged for 5 min at 5 880 g to get rid of debris and agar. The supernatant was removed with a Pasteur pipette. Four microliters of a 31  $\mu$ L 160 nM Rhodamine 123 (Rh123) probe suspension in PBS (Rh123 from Molecular probes, Invitrogen Detection Technologies, Eugene, Oregon, U.S.A.), an effective mitochondrion  $\Delta\Psi_m$  probe (Johnson et al. 1980; Ludovico et al. 2001), were added to the cells and then incubated in the dark for 60 min. The unbound Rh123 was washed off with PBS.

Stained cells submerged in 1,4-diazabicyclo [2.2.2] octane were examined using a Nikon TE 2000 CLSM. In order to obtain confocal images, the samples were excited with a 488 nm argon-ion laser. Sexual stage (green fluorescence) images i.e. asci were obtained through a 540 nm long-pass dichroic filter and a 550 nm barrier filter, while vegetative stage (red fluorescence) images i.e. hyphae were obtained through a 625 nm long-pass filter. In order to observe more distinctly the abundant sporulation observed relative to vegetative cells, the staining can be optimized by either adding less biomass to the 160 nM Rh123-probe suspension or by increasing the probe concentration.

**Scanning electron microscopy (SEM):** Preparation of sporulating cells for SEM was carried out as described by Van Wyk and Wingfield (1991). Cells were obtained from the YNB liquid medium and then chemically fixed using 3 % (1.0 M) of a sodium phosphate buffered gluteraldehyde (Sigma-Aldrich, Steinheim, USA) solution at pH 7 for 3 h and a similarly buffered osmium tetroxide (Sigma-Aldrich, Steinheim, USA) solution (1 %) for 1 h. Following this, the cells were dehydrated in a graded series of ethanol 30 %, 50 %, 75 %, 90 % and 100 % for 15 min per step. The ethanol dehydrated material for SEM was critical point dried, mounted and coated with gold to make it electrically conductive. Scanning electron micrographs were then taken using a Joel WINSEM JSM 6400 (Japan) and a Shimadzu SSX 550 (Japan) SEM.

#### **2.3.4. Oxylipin analysis**

The pH of the liquid culture (100 mL YNB medium) was lowered to below 4 using 30 % (m/m) formic acid (Merck, Darmstadt, Germany). Oxylipins were

extracted using 100 mL of ethyl acetate (Merck, Darmstadt, Germany). The organic and water phases were allowed to separate followed by the removal of the organic phase. This procedure was repeated twice. The ethyl acetate was allowed to evaporate under a stream of nitrogen (AFROX, Bloemfontein, South Africa) and the extract then stored under nitrogen in a freezer overnight. Extracts were methylated by dissolving the sample in 500  $\mu$ L methanol and 2 mL of diazomethane (Sigma-Aldrich, Steinheim, USA) in a vial that was closed with fat-free cotton wool. The solution was then left at -20 °C for 2 h and dried under a stream of nitrogen. Next, the extracts were silylated by adding 100  $\mu$ L dry pyridine (Sigma-Aldrich, Steinheim, USA) and 100  $\mu$ L *bis*- (trimethylsilyl) trifluoroacetamide (BSTFA; Sigma-Aldrich, Steinheim, USA), left for 40 min and dried under a stream of nitrogen. The samples were finally dissolved in 400  $\mu$ L chloroform: hexane (4:1, v/v; Merck, Darmstadt, Germany) mixture and 1  $\mu$ L of the sample was injected into the gas chromatogram - mass spectrometer (Venter et al. 1997).

***Gas chromatography – mass spectrometry (GC-MS):*** A Finnigan Trace Ultra gas chromatogram (San Jose, California) which is equipped with a HP-5-60 m fused silica capillary column (0.23  $\mu$ m i.d. and 0.1  $\mu$ m, coating thickness) coupled to a Finnigan Trace DSQ MS (San Jose, California) was used to record the electron impact mass spectra of 3-OH oxylipins. Helium was used as a carrier gas at a constant flow of 1 mL/min. The MS was auto-tuned to *m/z* 69-512. The temperature was programmed to rise at 5 °C per min and held for 2 min from an initial temperature of 110 °C to a final temperature of 230 °C. A sample volume of 1  $\mu$ L was injected at a split ratio of 1:50.

### 2.3.5. Life cycle studies

**Cultivation:** Yeast strains of *D. uninucleata* (UOFS Y-2067, UOFS Y-1129) were cultivated as described before. Spore suspensions of the yeasts were obtained by filtering 48 h to 52 h old liquid cultures (40 g/L glucose and 6.7 g/L YNB) through sterile glasswool (Botha et al. 1992). The filtrate was centrifuged (18 879 g; 40 min) and the resulting pellet, which consisted mainly of ascospores, was resuspended in an appropriate volume of liquid media (40 g/L glucose and 6.7 g/L YNB). The cultures used in this study were initiated by inoculating 2 mL of these ascospore suspensions in 100 mL YNB liquid media in 500 mL conical flasks, yielding a final spore concentration of between  $3.88 \times 10^6$  and  $4.6 \times 10^6$  spores/mL for *D. uninucleata* var. *uninucleata* and between  $0.25 \times 10^6$  and  $0.33 \times 10^6$  spores/mL for *D. uninucleata* var. *wickerhamii*. The cultures were then grown at 25 °C on a rotary shaker (160 r.p.m). Growth and ascospore development were monitored by periodically withdrawing from each culture (0.1 mL sample) and counting the cells with a hemocytometer under a light microscope coupled to a Colorview Soft Digital Imaging System. This experiment was performed in triplicate for *D. uninucleata* var. *uninucleata* (South African strain) and *D. uninucleata* var. *wickerhamii* (only one strain available).

**Acetylsalicylic acid (ASA) inhibition studies:** ASA (Sigma-Aldrich, Steinheim, Germany) was first diluted in a minimal volume ethanol and added to cultures as prepared above (at time 0 h) to reach a final concentration of 0 mM (control) and 1 mM respectively. These cultures were grown at 25 °C on a rotary shaker (160 r.p.m). Growth and ascospore development was monitored by periodically withdrawing from each culture and analyzing with a hemocytometer

under a light microscope coupled to a Colorview Soft Digital Imaging System. This experiment was repeated with the addition of only ethanol in similar amounts to cultures at time 0 h as in corresponding ASA experiments. These experiments were performed in triplicate for *D. uninucleata* var. *uninucleata* (South African strain) and *D. uninucleata* var. *wickerhamii*.

## 2.4. Results and discussion

### 2.4.1. Morphology

*D. uninucleata* forms colonies that are whitish, moist and somewhat slimy. True hyphae are formed and the asci are acicular, ca. 100-130  $\mu\text{m}$  long, have persistent walls, and open by rupture at the apex. Ascospores are ca. 32-128 per ascus, hyaline or subhyaline, ellipsoidal to reniform, ca. (0.8-1.2) x (2.0-2.8)  $\mu\text{m}$ , and without a slime sheath (Smith and De Hoog 1998). Although clear differences between the two species of *Dipodascopsis* exist i.e. *D. tóthii* and *D. uninucleata*, information concerning the differences in the size and shape as well as the sexual reproduction and ascospore release mechanisms between the two varieties of *D. uninucleata* are poorly reported. It therefore became an aim of this study to assess if differences exist.

#### ***D. uninucleata* var. *uninucleata* (UOFS Y-2067; South African strain)**

Using light microscopy (Fig. 1a), an acicular ascus was observed which formed laterally on hyphae after fusion of gametangia. This ascus is filled with ca. 30-120 hyaline or subhyaline, ellipsoidal to reniform, ascospores (Smith and De Hoog 1998).

These ascospores are formed through meiosis and then post meiotic mitosis (Botha et al. 1992). From Fig. 1(b) it is clear that the ascospores stained typically blue when using malachite green/safranin staining (Yarrow 1998) while vegetative cells and the ascus wall stained red. This is in accordance with literature (Yarrow 1998). In Fig. 1(b) ascospores (blue), lined-up for individual release through a close fitting open ascus tip, was observed. From this figure it is clear that ascospores (similar to that reported for the Canadian strain of *D. uninucleata* var. *uninucleata*; Kock et al. 1999) are released individually through the narrowing ascus neck.

A 3D-image of a mature ascus filled with ascospores was obtained with the aid of CLSM on cells stained with Orange G (Fig. 1c). Orange G is an azo-dye that accumulates on the surface of ascospores (attaches to lipophilic surfaces), fluorescing pink/red when combining blue and red emission of the stain with 454/543/633 nm laser excitation. The fluorescing ascospores in pink can be observed as well as the thick ascus cell wall in blue. When the asci open by rupturing at the apex, the ascospores are released individually after which they tend to aggregate in clusters as shown in Fig. 1(d).

The various morphological phases of the sexual stage could clearly be observed when using SEM. A typical ascus, formed laterally on hyphae after fusion of gametangia (Fig. 2a), showing a broad base and a narrow tip, is visible. In Fig. 2(b), an opened ascus tip of ca. 1  $\mu\text{m}$  in diameter, which is the same size as the ascospore diameter, was observed. Once the ascospores have been released from the ascus tip they aggregate into slimy balls or ascospore clusters (Fig. 2c). These ascospore clusters contain ascospores forming ordered clusters with the interspore

matrix linking the ascospores (Fig. 2d). Similar results were reported for *D. uninucleata* var. *uninucleata* (Canadian strain; Kock et al. 1999) although this strain i.e. *D. uninucleata* var. *uninucleata* (South African strain) produced less distinct interspore matrices. In Fig. 2(e, f) single ascospores with rib-like surface structures could be observed. Results reported for *D. uninucleata* var. *uninucleata* (Canadian strain) showed that the ascospores are connected through surface hooks and that these hooks together with the presence of oxylipins may contribute to the aggregation phenomenon as well as the ascospore cluster ordering found once the ascospores are released from the asci (Kock et al. 1999). These rib-like surface structures (Fig. 2e and 2f) could indeed be the surface hooks found by Kock and co-workers (1999).

The size of the ascospores was measured in all the experiments performed in this section. It was found that the size was similar to that of the Canadian strain, being ca. (0.8-1.2) x (2.0-2.8)  $\mu\text{m}$  (Smith and De Hoog 1998).

#### ***D. uninucleata* var. *wickerhamii* (UOFS Y-1129)**

Using light microscopy (Fig. 3a), a mature acicular ascus, showing a broad base and narrow tip, was observed. The ascus clearly shows the alignment of the well developed hyaline to subhyaline, ellipsoidal to reniform, ascospores to be released individually through the ruptured ascus tip. From Fig. 3(b) it is clear that the ascospores stained typically blue when using malachite green/safranin staining (Yarrow 1998) while the vegetative cells and ascus wall stained red. This is in accordance with literature (Yarrow 1998).

In an Orange G stained preparation, viewed under the CLSM, it was found that once the ascospores have been released from the mature ascus, the ascospores again aggregate into clusters (Fig. 3c). In Fig. 3(d), a mature ascus with developing ascospores in pink (Nile red staining) as well as an empty ascus that has already released its ascospores was observed. Nile red is a lipophilic stain that accumulates on ascospore surfaces thereby fluorescing pink/red ascribed to blue/red combined emission.

SEM was performed to observe the various morphological phases of the sexual stage in detail. In Fig. 4(a), a typical ascus with a broad base and a narrow tip was observed. Interestingly, an ascospore bundle which formed from individually released ascospores from a mature ruptured ascus and aggregated at the ascus tip, was also observed (Fig. 4a). In Fig. 4(b) an undeveloped ascus is shown, with the ascus tip still closed and the ascospores not ready to be released. Once the ascospores have been released they aggregate into irregular ascospore clusters (Fig. 4d), leaving the ascus tip ruptured (Fig. 4c). These ascospore clusters (Fig. 4d) do not seem to have the same distinct ordered clustering as observed in *D. uninucleata* var. *uninucleata* (Canadian strain; Kock et al. 1999).

Ascospores of *D. uninucleata* var. *wickerhamii* were found to be ca. (0.5-1) x (1-2.2)  $\mu\text{m}$  which is much smaller when compared to *D. uninucleata* var. *uninucleata* (South African- and Canadian- strains), measuring ca. (0.8-1.2) x (2.0-2.8)  $\mu\text{m}$ .

#### **2.4.2. Oxylipin and mitochondrial mapping**

For the mapping of 3-OH oxylipins, an antibody specific to these oxylipins and coupled to a fluorescing secondary antibody, were used (from now on referred to as OXYTRACK). OXYTRACK is specific for 3-OH oxylipins, irrespective of their side chain length or desaturation level (Kock et al. 1998). GC-MS was then followed to confirm the presence as well as the possible structures of the 3-OH oxylipins (Venter et al. 1997; Kock et al. 2003).

Since we know that the production of 3-OH oxylipins occurs within the mitochondria through an incomplete  $\beta$ -oxidation pathway and/or a mitochondrial fatty acid synthesis type II route such as FAS II (Ciccoli et al. 2005; Hiltunen et al. 2005), the next step was to perform mitochondrial mapping. Mitochondrial activity within the cells can be determined through the addition of Rhodamine 123 (Rh123; from now on referred to as MITOTRACK). This is a cationic lipophilic dye which accumulates specifically within the mitochondria (Chen 1988). The interaction of the mitochondria with the MITOTRACK is dependent on the high membrane potential ( $\Delta\Psi_m$ ) which is maintained by functional mitochondria (Johnson et al. 1980, 1981).

#### ***D. uninucleata* var. *uninucleata* vs. *D. uninucleata* var. *wickerhamii***

The ellipsoidal to reniform ascospores are clearly visible for both varieties when ascospores were selectively stained with the OXYTRACK antibodies and viewed with a CLSM (Fig. 5a and 5c). These results also confirm that these oxylipins are mainly associated with the ascospores. Similar results were reported for *D. uninucleata* var. *uninucleata* (Canadian strain; Kock et al. 1998) whereby oxylipins were associated with the sexual stage and less with the vegetative stage (Fig. 5e).

MITOTRACK results viewed under CLSM showed the highest mitochondrial activity associated with the sexual stages i.e. asci and surrounding the liberated ascospores of both *D. uninucleata* var. *uninucleata* strains as well as *D. uninucleata* var. *wickerhamii* (Fig. 5b, 5d and 5f). A higher mitochondrial activity is expected in the sexual cells since a large amount of energy is required during the formation and maturation of the ascospores while 3-OH oxylipin accumulation is also observed (Kock et al. 2007).

It also became an objective of this study to chemically characterize the structure of 3-OH oxylipins in both varieties, using GC-MS. A major peak at  $m/z$  of 175 was observed in both varieties (Fig. 6a and 6b), indicating the presence of a hydroxyl group on the 3rd carbon as counted from the carboxyl group. A mother ion of 326 was observed for *D. uninucleata* var. *uninucleata* (South African strain; Fig. 6a). This value was found to be typical for the chemical structure of a 3-OH 14:2 oxylipin. A similar spectrum and hence 3-OH oxylipin was reported for *D. uninucleata* var. *uninucleata* (Canadian strain; Venter 1999). For *D. uninucleata* var. *wickerhamii*, no diagnostic mother ion could be found and hence a chemical structure could not be fitted to this mass spectrum (Fig. 6b). It is concluded that the two varieties of *D. uninucleata* produce different types of 3-OH oxylipins.

#### **2.4.3. Life cycle studies**

The life cycle of *Dipodascopsis* is considered to be homothallic and haplontic, and consists of alternating asexual and sexual reproductive stages (Biggs 1937; Zsolt 1963; Botha et al. 1993). In 1992 Botha and co-workers performed life cycle studies on synchronous cultures of *D. uninucleata* var. *uninucleata* (Canadian strain)

as well as *D. tóthii*. At the start of growth (ca. first 20 h), ascospores of both species at first increased in size (swell) and then started to form germ tubes. From ca. 28 h to 35 h germ tubes gave rise to hyphae which reached a maximum number in this period. At the same time, the number of swollen ascospores and germ tubes decreased. After ca. 35 h the asci were found to reach a maximum number and after ca. 40 h, the life cycle was completed, as indicated by the sharp rise in the number of released ascospores. In this study the life cycles of *D. uninucleata* var. *uninucleata* (South African strain) and *D. uninucleata* var. *wickerhamii* were assessed and compared.

***D. uninucleata* var. *uninucleata* (South African strain; Fig. 7)**

At the start of growth (ca. first 28 h), the minute reniform ascospores of *D. uninucleata* var. *uninucleata* first also increased in size and then started to germinate forming germ tubes (Fig. 7a). The germ tubes then gave rise to hyphae which continued to form and increase in number throughout the life cycle (ca. 0 h to 50 h). At the same time, the number of swollen and germinating ascospores decreased (Fig. 7a). Individual hyphae were observed up to ca. 50 h only, after this hyphae aggregated in bundles and could not be counted by hemocytometer. The sexual stage was entered (from ca. 28 h), indicated by the rise in the number of primordial asci (ca. 28 h to 50 h). At first, primordial asci formed through plasmogamy (fusion of cell cytoplasm) of gametangia, followed by karyogamy (fusion of two haploid nuclei; Yarrow 1998). After meiosis, post meiotic mitosis occurred resulting in multi-spored asci. This is indicated by mature asci which started to increase after ca. 28 h and showed a maximum at ca. 55 h. After ca. 55 h, the life cycle was completed, as indicated by the sharp rise in the number of released ascospores (Fig. 7a).

Primordial as well as mature asci were observed up to ca. 55 h only, after this the asci became too large and bundled to be counted by hemocytometer. Similar patterns were observed when performed in triplicate. Only general trends regarding the different cell types during the life cycle are reported. No accurate quantitative values for the transitions (e.g. concomitant increases and decreases of different cell types) could be obtained probably due to inaccuracies inherent to the counting chamber.

#### ***D. uninucleata* var. *wickerhamii* (Fig. 8)**

At the start of growth (ca. first 20 h), the minute reniform ascospores of *D. uninucleata* var. *wickerhamii* first also increased in size and then started to germinate forming germ tubes (Fig. 8a). The germ tubes then gave rise to hyphae which continued to form and increase in number throughout the life cycle (ca. 0 h to 49 h). At the same time, the number of swollen and germinating ascospores decreased in number (Fig. 8a). Individual hyphae were observed up to ca. 49 h only, after this hyphae aggregated in bundles that could not be counted by hemocytometer. The sexual stage was entered (after ca. 20 h), indicated by the rise in the number of primordial asci (ca. 20 h to 49 h). Mature asci started to increase after ca. 20 h and showed a maximum at ca. 45 h to 49 h. After about 49 h, the life cycle was completed, as indicated by the sharp rise in the number of released ascospores (Fig. 8a). Similar patterns were observed when performed in triplicate. Only general trends regarding the different cell types during the life cycle are reported. No accurate quantitative values for the transitions (e.g. concomitant increases and decreases of different cell types) could be obtained probably due to inaccuracies inherent to the counting chamber.

#### **2.4.4. Effect of ASA on life cycles**

In 1992, Botha and co-workers determined the sensitivity of the life cycle of *D. uninucleata* var. *uninucleata* (Canadian strain) towards NSAIDs when adding ASA in increased amounts. They found that when 1 mM ASA was added to the yeast, the life cycle was severely inhibited. They found the inhibition to occur during the transition of the germinating ascospores to the hyphal stage resulting in a decrease in the number of hyphae. They further observed decreased numbers of asci when compared to the controls. Under these conditions, this yeast did not liberate ascospores even after 60 h. When ASA was added in increased amounts (0 mM to 1 mM) the most susceptible stage was found to be ascospore release. This is probably due to the inhibition of mitochondrially produced 3-OH oxylipins that serve as lubricant during ascospore release through the narrow ascus opening (Kock et al. 1999).

#### ***D. uninucleata* var. *uninucleata* (South African strain; Fig. 7)**

When synchronous cultures were grown in media containing 1 mM ASA, it was observed that the vegetative stage proceeded similarly when compared to the control cultures – both cultures contained similar ascospore numbers at the start of growth (Fig. 7a and 7b). In the ASA treated culture (Fig. 7b), the minute reniform ascospores increased in size and then germinated forming germ tubes (ca. first 26 h). The germ tubes gave rise to hyphae which continued to form and increase in number throughout the life cycle. The maximum number of hyphae formed after ca. 49 h were however half of that formed when compared to the control (Fig. 7a and 7b). Individual hyphae were observed up to ca. 49 h only, after this hyphae aggregated in bundles that could not be counted by hemocytometer in both ASA

treated and control cultures. The sexual stage was entered only after *ca.* 25 h (ASA treated culture), indicated by the rise in primordial asci (*ca.* 25 h to 42 h). Although no mature asci could be observed during growth of ASA treated culture, some ascospore release was observed. After about 55 h, the life cycle (ASA treated culture) was completed, as indicated by a rise in the number of released ascospores (Fig. 7b). When compared to the control life cycle (no ASA added), it could be observed that the ASA treated life cycle was completed at about the same time although the number of ascospores released after 69 h was *ca.* 2.8 times less (Fig. 7c). This is in contrast when compared to *D. uninucleata* var. *uninucleata* (Canadian strain) where no ascospores were released even after 60 h of cultivation and the life cycle therefore not completed (Botha et al. 1992).

After 60 h of growth in the presence of 1 mM ASA (Fig. 7b), light microscopy (Fig. 9a) as well as SEM (Fig. 9b) indicate that the sexual stage was inhibited. Here, only primordial asci with immature ascospores as well as closed asci with mature ascospores could be observed indicating that the ascospore development and release was inhibited. Similar results compared to Fig. 7(a) were obtained when equivalent amounts of ethanol used to dissolve 1 mM ASA were added to the growth medium and the life cycle transitions studied. This shows that ethanol had no or little effect on the life cycle. Only general trends regarding the different cell types during the life cycle are reported. No accurate quantitative values for the transitions (e.g. concomitant increases and decreases of different cell types) could be obtained probably due to inaccuracies inherent to the counting chamber.

#### ***D. uninucleata* var. *wickerhamii* (Fig. 8)**

When synchronous cultures were grown in media containing 1 mM ASA, it was observed that the vegetative stage proceeded similarly when compared to the control cultures – both cultures contained similar ascospore numbers at the start of growth (Fig. 8a and 8b). In the ASA treated culture, the minute reniform ascospores increased in size and then germinated forming germ tubes. The germ tubes branched into hyphae which continued to form and increase in number (up to ca. 45 h). The maximum number of hyphae formed after 45 h (ASA treated culture) were similar when compared to the control after 49 h (Fig. 8a and 8b). Individual hyphae were observed up to ca. 49 h only, after this hyphae aggregated in bundles that could not be counted by hemocytometer in both ASA treated and control cultures. The sexual stage was entered only after ca. 21 h (ASA treated culture), indicated by the rise in primordial asci (ca. 21 h to 49 h). Mature asci were formed after ca. 21 h and reached a maximum after ca. 49 h. After ca. 45 h, the life cycle (ASA treated culture) was completed, as indicated by the rise in the number of released ascospores (Fig. 8b). When compared to the control life cycle (no ASA added), it could be observed that the ASA treated life cycle was completed at about the same time although the number of ascospores released after 49 h was again ca. 2.8 times less (Fig. 8c). This is similar when compared to *D. uninucleata* var. *uninucleata* (South African strain), but also in contrast when compared to *D. uninucleata* var. *uninucleata* (Canadian strain) where no ascospores were released in the presence of ASA even after 60 h of cultivation and the life cycle therefore not completed (Botha et al. 1992).

After 60 h of growth in the presence of 1 mM ASA, light microscopy (Fig. 9c) as well as SEM (Fig. 9d), indicate that the sexual stage was inhibited. Here, primordial asci with immature ascospores, closed asci with ascospores, as well as a small amount of released ascospores could be observed. Similar results compared to Fig. 8(a) were obtained when equivalent amounts of ethanol used to dissolve 1 mM ASA were added to the growth medium and the life cycle transitions studied. This shows that ethanol had no or little effect on the life cycle. Only general trends regarding the different cell types during the life cycle are reported. No accurate quantitative values for the transitions (e.g. concomitant increases and decreases of different cell types) could be obtained probably due to inaccuracies inherent to the counting chamber.

## 2.5. Conclusions

According to D1/D2 analysis (Kurtzman and Robnett 1998) and morphological studies using light microscopy (Smith and De Hoog 1998) the two varieties i.e. *D. uninucleata* var. *uninucleata* and *D. uninucleata* var. *wickerhamii* are similar. However, according to literature (Smith and De Hoog 1998) the two varieties differ in their abilities to assimilate certain carbon sources where *D. uninucleata* var. *uninucleata* is capable of assimilating sucrose, raffinose and inulin while, *D. uninucleata* var. *wickerhamii* is capable of assimilating L-rhamnose.

During this study it was found that variation in ascospore size and cluster-morphology exists between *D. uninucleata* var. *uninucleata* (South African strain) and *D. uninucleata* var. *wickerhamii*. In *D. uninucleata* var. *uninucleata* it was

observed that ascospores formed ordered clusters with a distinct interspore matrix after being released individually from the narrow ascus opening (Fig. 2d). This corresponds to results obtained for *D. uninucleata* var. *uninucleata* (Canadian strain), where ascospores formed ordered clusters and were surrounded by a distinct interspore matrix (Kock et al. 1999). It was however observed in *D. uninucleata* var. *uninucleata* (South African strain) that the interspore matrix surrounding the ascospores was less distinct than *D. uninucleata* var. *uninucleata* (Canadian strain). In *D. uninucleata* var. *wickerhamii* smaller and less ordered clustering with less defined interspore matrix was observed when compared to *D. uninucleata* var. *uninucleata* (South African- and Canadian- strains; Fig. 4d). This corresponds to results found by Baretseng and co-workers (2004) for *D. uninucleata* var. *wickerhamii*.

According to GC-MS, *D. uninucleata* var. *uninucleata* (South African strain) produces a 3-OH 14:2 oxylipin. This specific 3-OH oxylipin was also found to be produced by *D. uninucleata* var. *uninucleata* (Canadian strain; Venter et al. 1997). In *D. uninucleata* var. *wickerhamii* a characteristic 175 peak was observed indicating the presence of a 3-OH oxylipin. However, a structure could not be fitted to this 3-OH oxylipin. The latter differed also from the two strains of *D. uninucleata* var. *uninucleata* (South African- and Canadian- strains). Consequently, with the small number of strains available in this species (i.e. two varieties) oxylipin structure can be used as an identification tool.

During oxylipin production and distribution studies it was found that 3-OH oxylipins were associated with the sexual stage and not the vegetative stage in both *D.*

*uninucleata* var. *uninucleata* (South African strain) as well as *D. uninucleata* var. *wickerhamii*. The 3-OH oxylipins observed were associated with ascospores inside the asci as well as ascospores after release. This corresponds to results found for *D. uninucleata* var. *uninucleata* (Canadian strain; Kock et al. 1998).

As expected, increased mitochondrial activity, in both varieties, was observed in asci i.e. surrounding the ascospores, during mitochondrial tracking. Less activity was found associated with the vegetative stage. Since mitochondria produce 3-OH oxylipins possibly through  $\beta$ -oxidation and/or FAS II (Ciccoli et al. 2005; Hiltunen et al. 2005), it is suggested that the increased activity during sexual development is necessary for ascospore development as well as their effective release (Kock et al. 2007).

During life cycle studies, both strains followed similar asexual as well as sexual developmental stages. During hyphal formation and growth it was observed that *D. uninucleata* var. *uninucleata* (South African strain) produced approximately half the number of hyphae (after ca. 40 h) in the presence of 1 mM ASA than in the control culture. This could indicate possible effects of ASA on the vegetative stage. This can not be explained at present.

In both varieties the life cycle was completed at ca. the same time (45 h to 55 h) with a rise in ascospore release. In the presence of 1 mM ASA, ascospore release was found to be strongly inhibited (ca. three times) when compared to the controls (Fig. 7c and 8c), although, small amounts of ascospores were released in the two varieties in the presence of ASA (1 mM). No ascospores were released in *D.*

*uninucleata* var. *uninucleata* (Canadian strain), which suggests that *D. uninucleata* var. *uninucleata* (South African strain) and *D. uninucleata* var. *wickerhamii* are more resistant to ASA addition in this respect.

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## 2.8. Figures

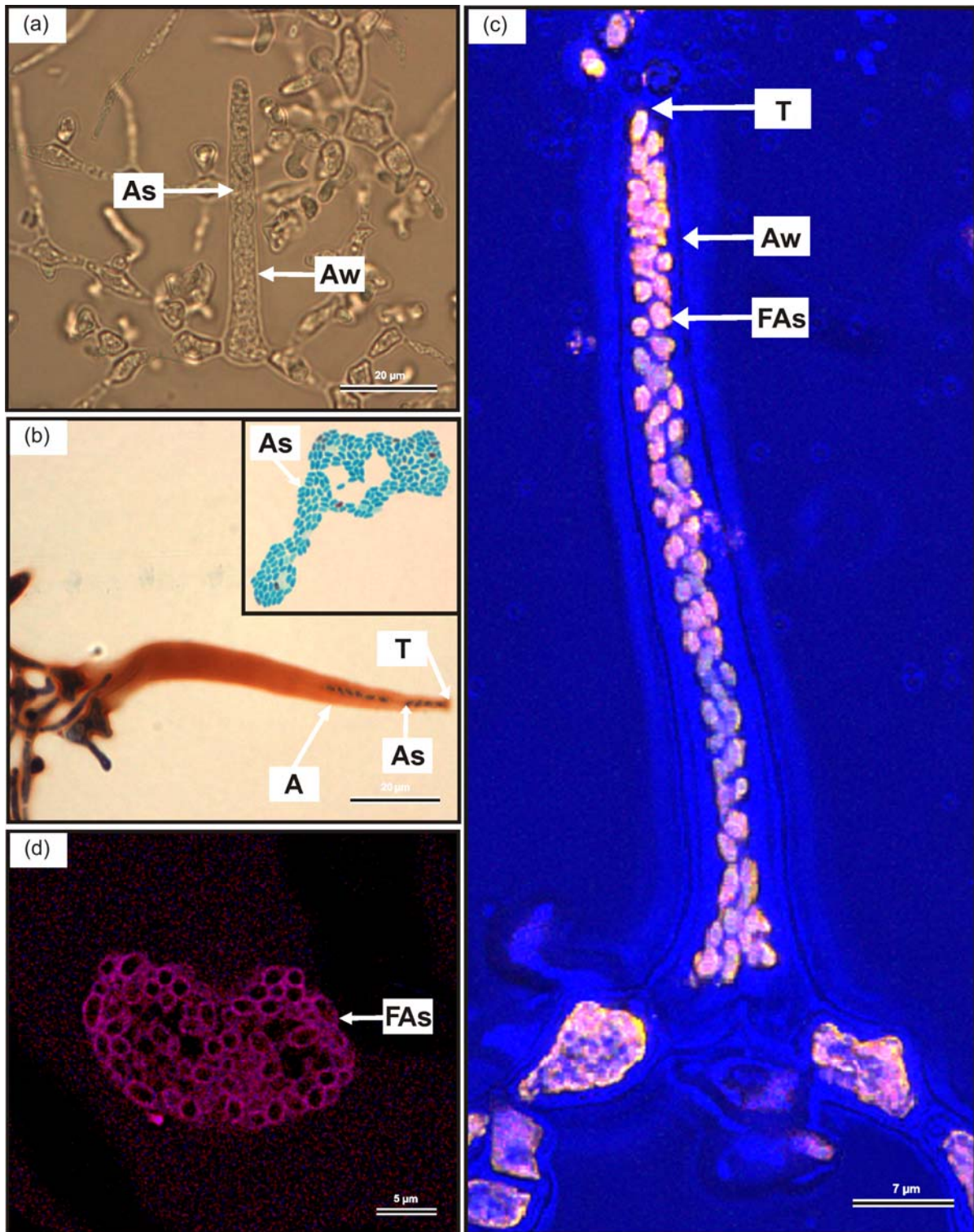
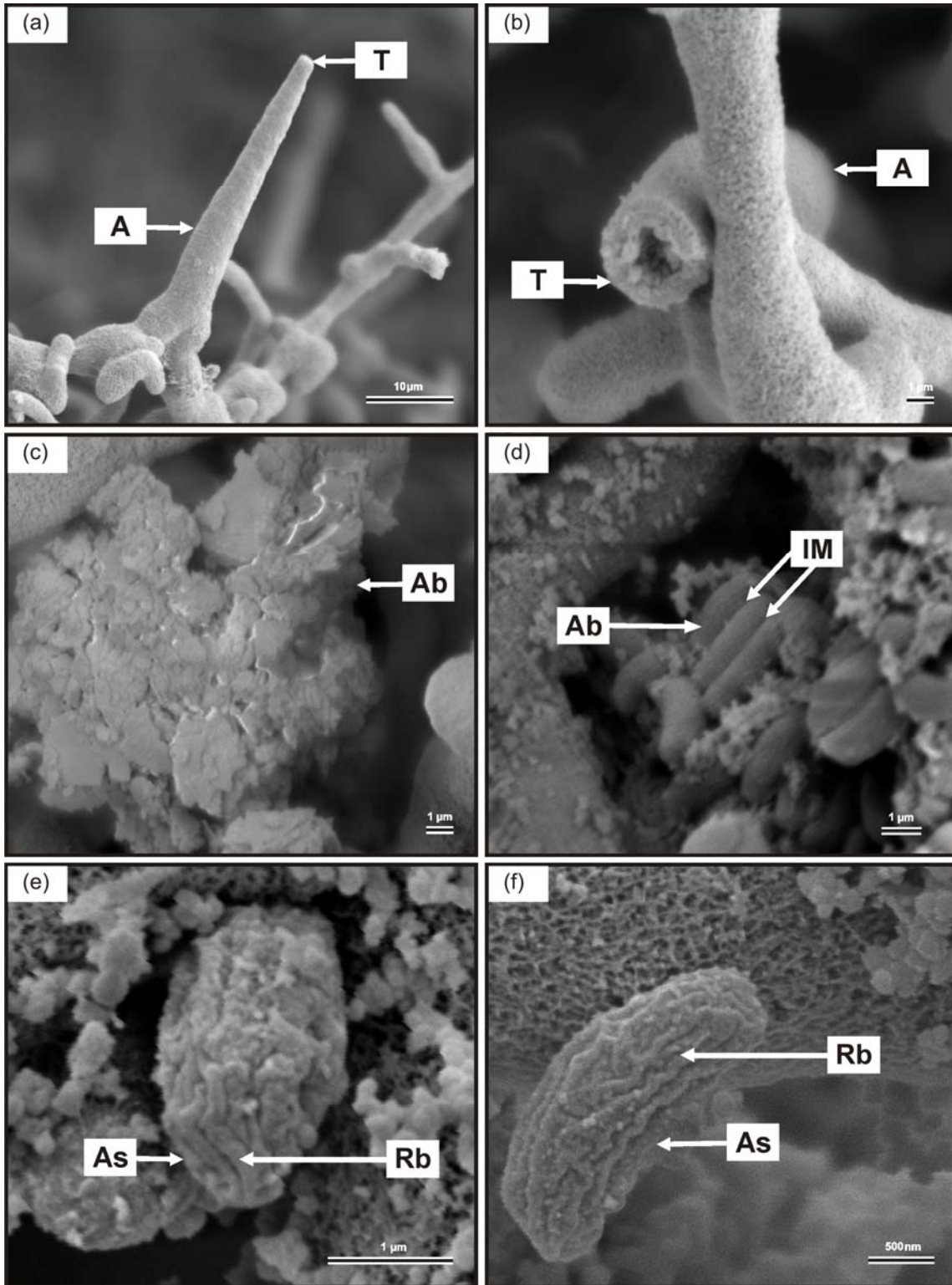
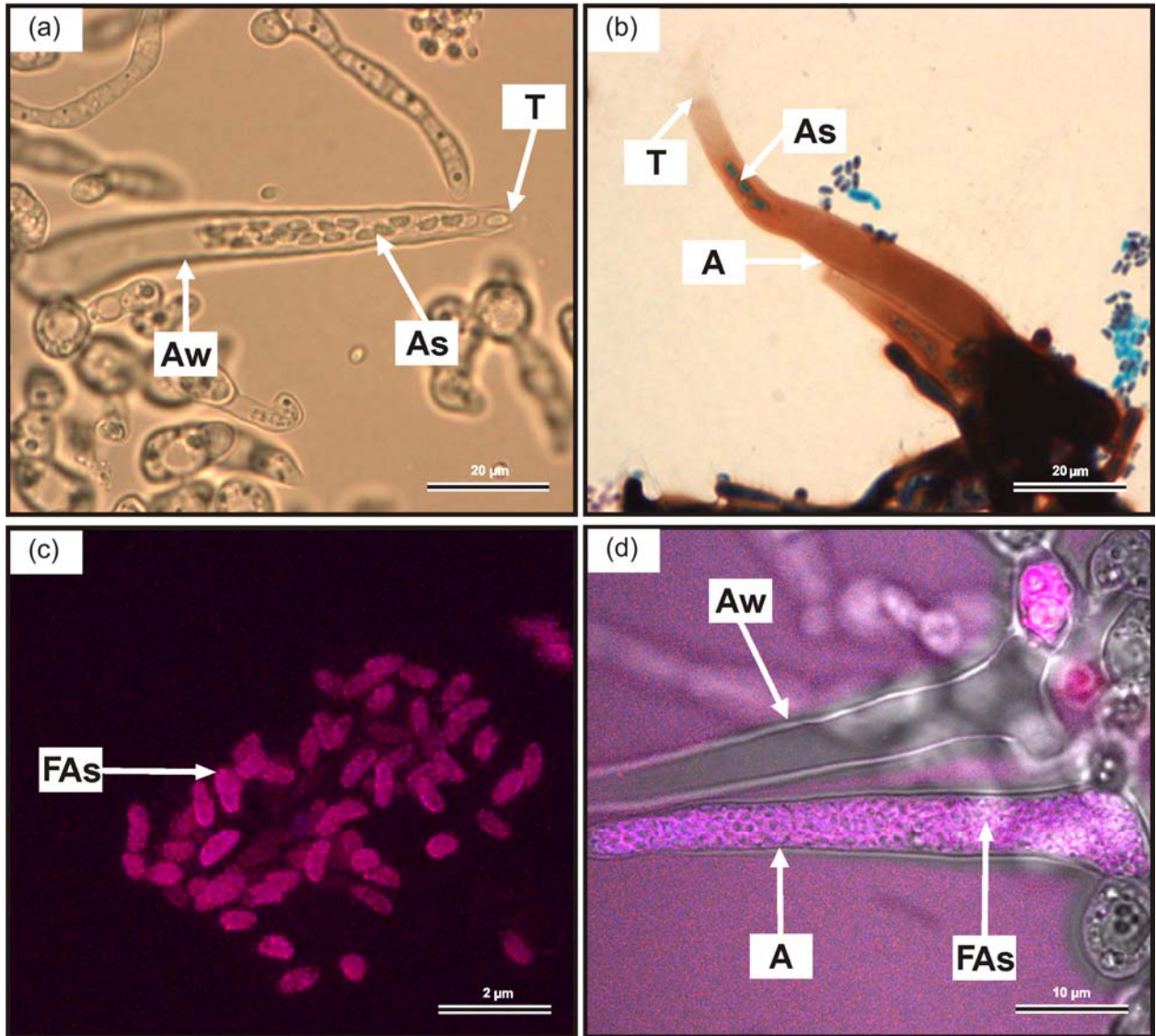


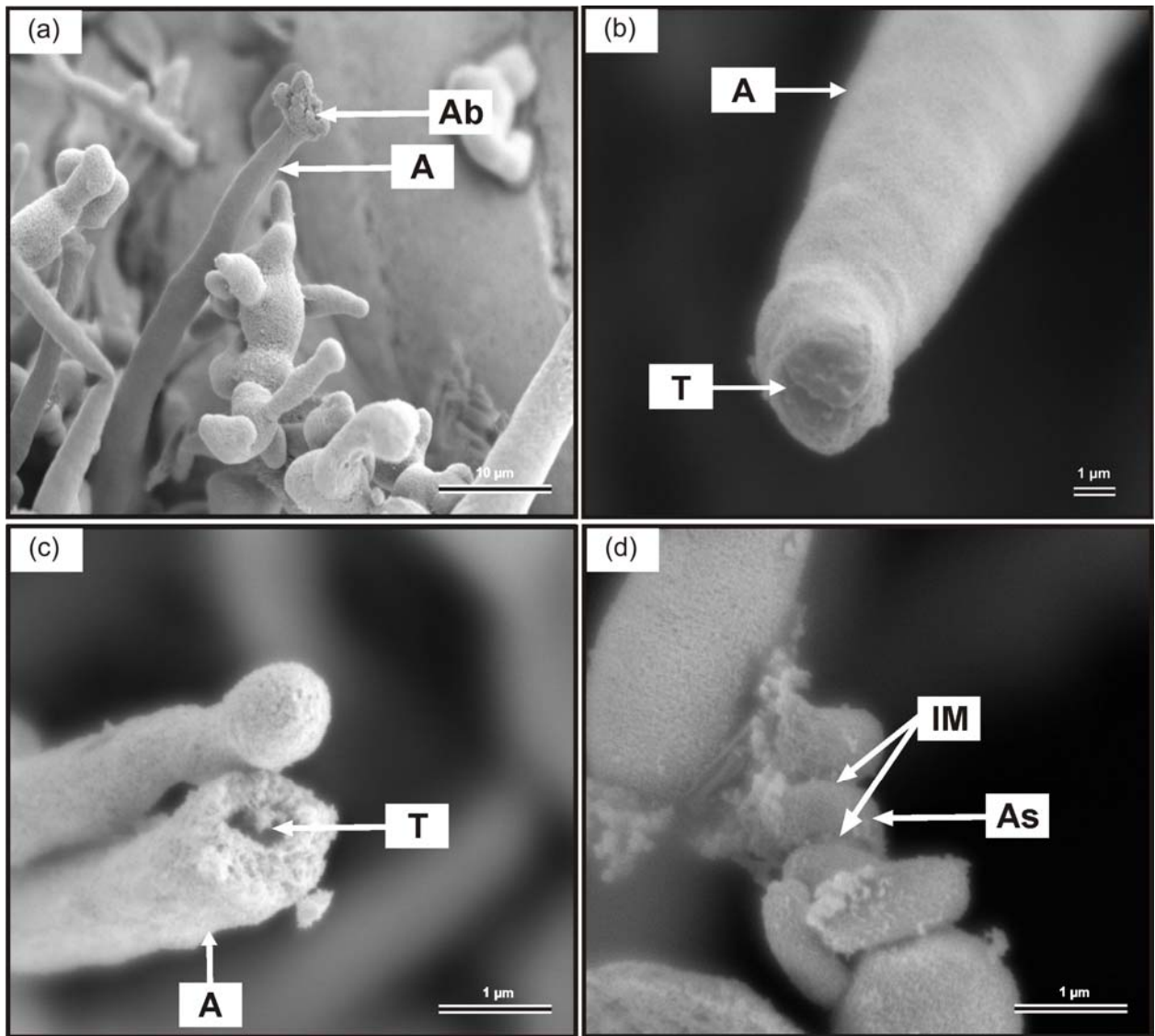
Fig. 1. Light micrographs of an ascus (a), of a malachite green/safranin stained ascus with released ascospores - insert (b), as well as confocal laser scanning micrographs of an Orange G stained ascus (c) and an ascospore cluster (d) showing the reniform shape of the ascospores of *D. uninucleata* var. *uninucleata* (South African strain). A = ascus, As = ascospores, Aw = ascus wall, FAs = fluorescing ascospores, T = ascus tip.



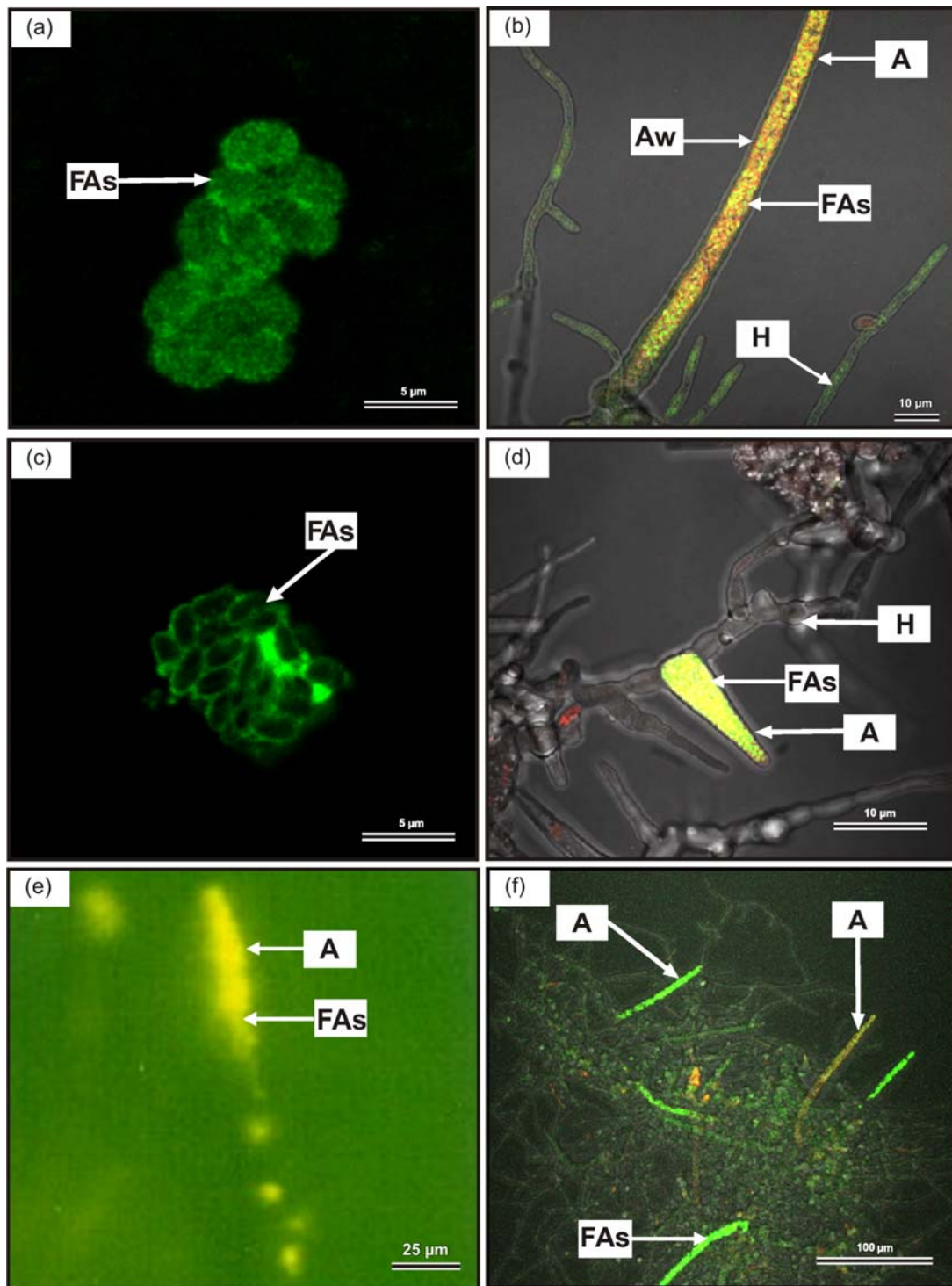
**Fig. 2. Scanning electron micrographs of an ascus with a closed tip (a), of an ascus with an open tip (b), of an ascospore bundle with the surrounding interspore matrix (c), of an ascospore bundle showing ordered clustering of the ascospores (d) and of a single ascospore with surface rib-like structures (e, f) of *D. uninucleata* var. *uninucleata* (South African strain). A = ascus, Ab = ascospore bundle, As = ascospore, IM = interspore matrix, RB = rib-like surface structures (hooks), T = ascus tip.**



**Fig. 3.** Light micrographs of an ascus showing individual release of the ascospores from the tip (a), of a stained ascus with ascospores (b), as well as confocal laser scanning micrographs of Orange G stained ascospores (c) and Nile red stained ascospores within an ascus (d) of *D. uninucleata* var. *wickerhamii*. A = ascus, As = ascospores, Aw = ascus wall, FAs = fluorescing ascospores, T = ascus tip.



**Fig. 4. Scanning electron micrographs of an ascus, with a released ascospore bundle at the tip (a), of still a closed ascus tip (b), of an opened ascus tip (c), and an ascospore bundle showing the irregular clustering and interspore matrix (d) of *D. uninucleata* var. *wickerhamii*. A = ascus, Ab = ascospore bundle, As = ascospores, IM = interspore matrix, T = ascus tip.**



**Fig. 5. Confocal laser scanning micrographs of OXYTRACK antibodies (green fluorescence) associated with the ascospores of *D. uninucleata* var. *uninucleata* (South African strain; a), of *D. uninucleata* var. *wickerhamii* (c) and of *D. uninucleata* var. *uninucleata* (Canadian strain; e), as well as MITOTRACK showing highest mitochondrial activity associated with the sexual stages i.e. asci surrounding the ascospores of *D. uninucleata* var. *uninucleata* (South African strain; b), of *D. uninucleata* var. *wickerhamii* (d) and of *D. uninucleata* var. *uninucleata* (Canadian strain; f). A = ascus, FAs = fluorescing ascospores, H = hyphae.**

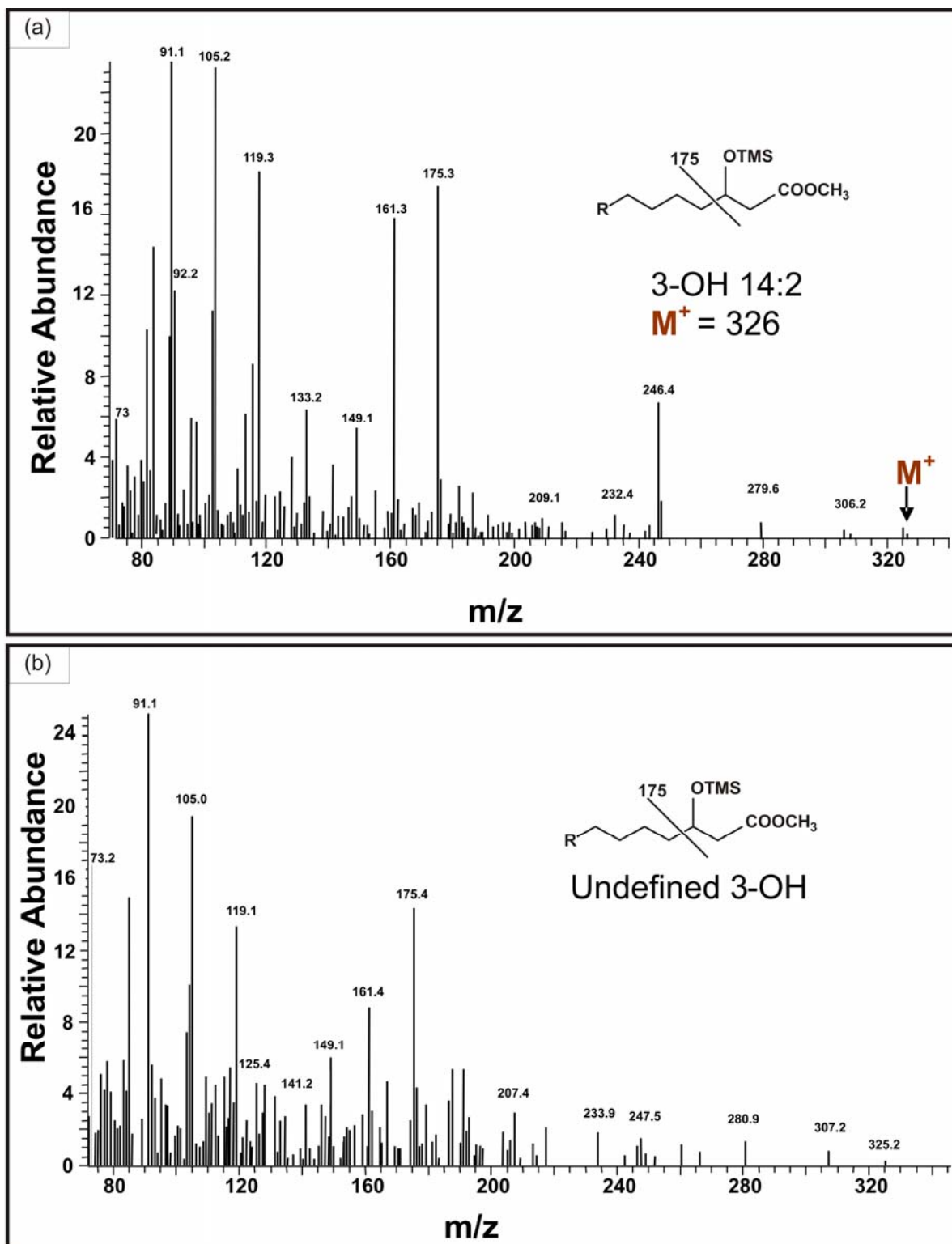


Fig. 6. Mass spectrum of methyl-trimethylsilylated 3-OH 14:2 obtained from *D. uninucleata* var. *uninucleata* (South African strain; a) and of an undefined methyl-trimethylsilylated 3-OH oxylipin from *D. uninucleata* var. *wickerhamii* (b). M<sup>+</sup> = mother ion, TMS = trimethylsilyl.

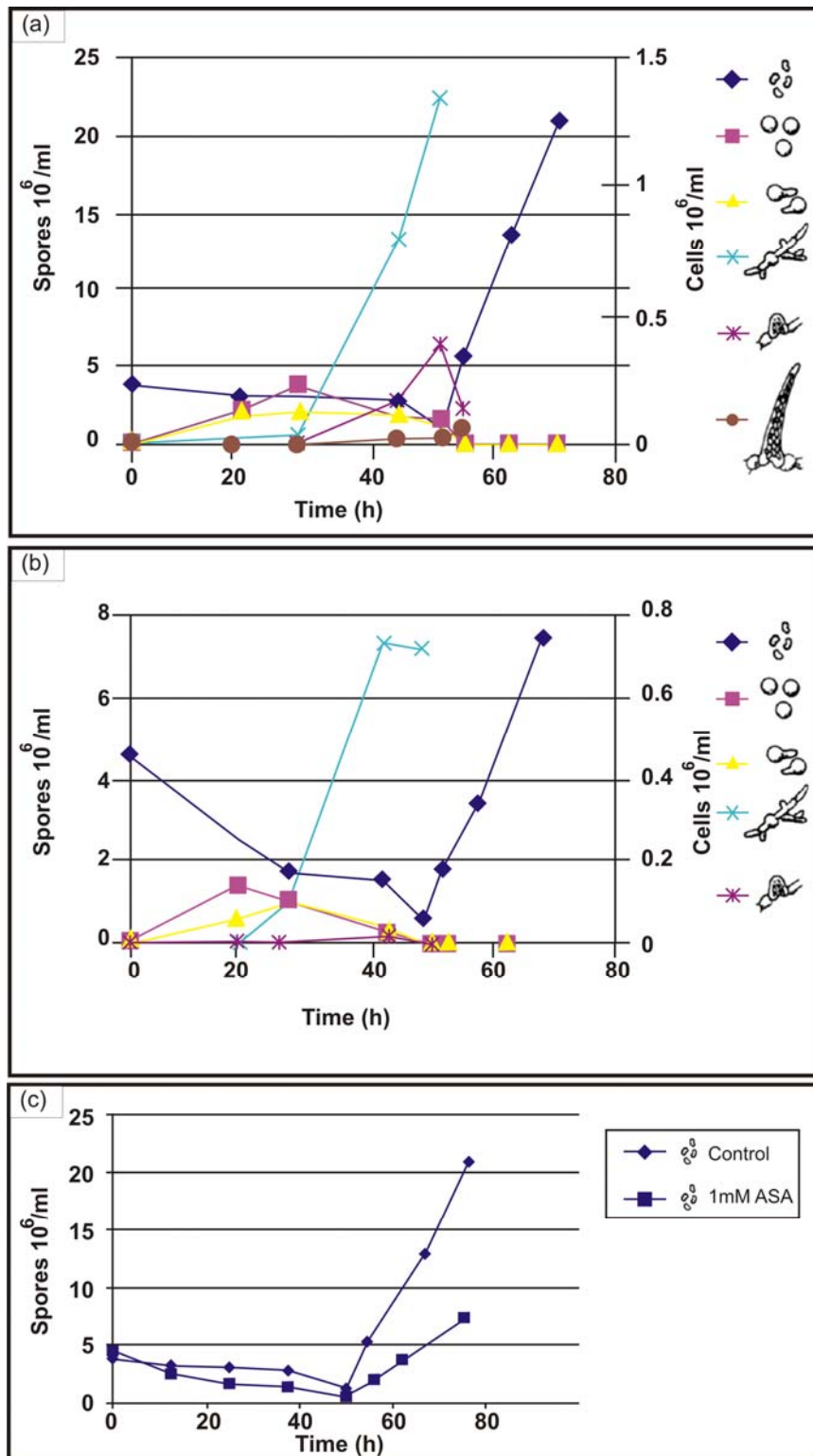


Fig. 7. Graphs of the life cycle of *D. uninucleata* var. *uninucleata* (South African strain) showing the development of the various stages from ascospores alone in the absence (a) and presence (b) of 1 mM ASA. In (c), ascospore release in the absence and presence of 1 mM ASA is shown. The key to the consecutive stages is given on the right hand side of the graph. From top to bottom: reniform ascospores; swollen ascospores; germinating ascospores; hyphae; primordial asci; mature asci.

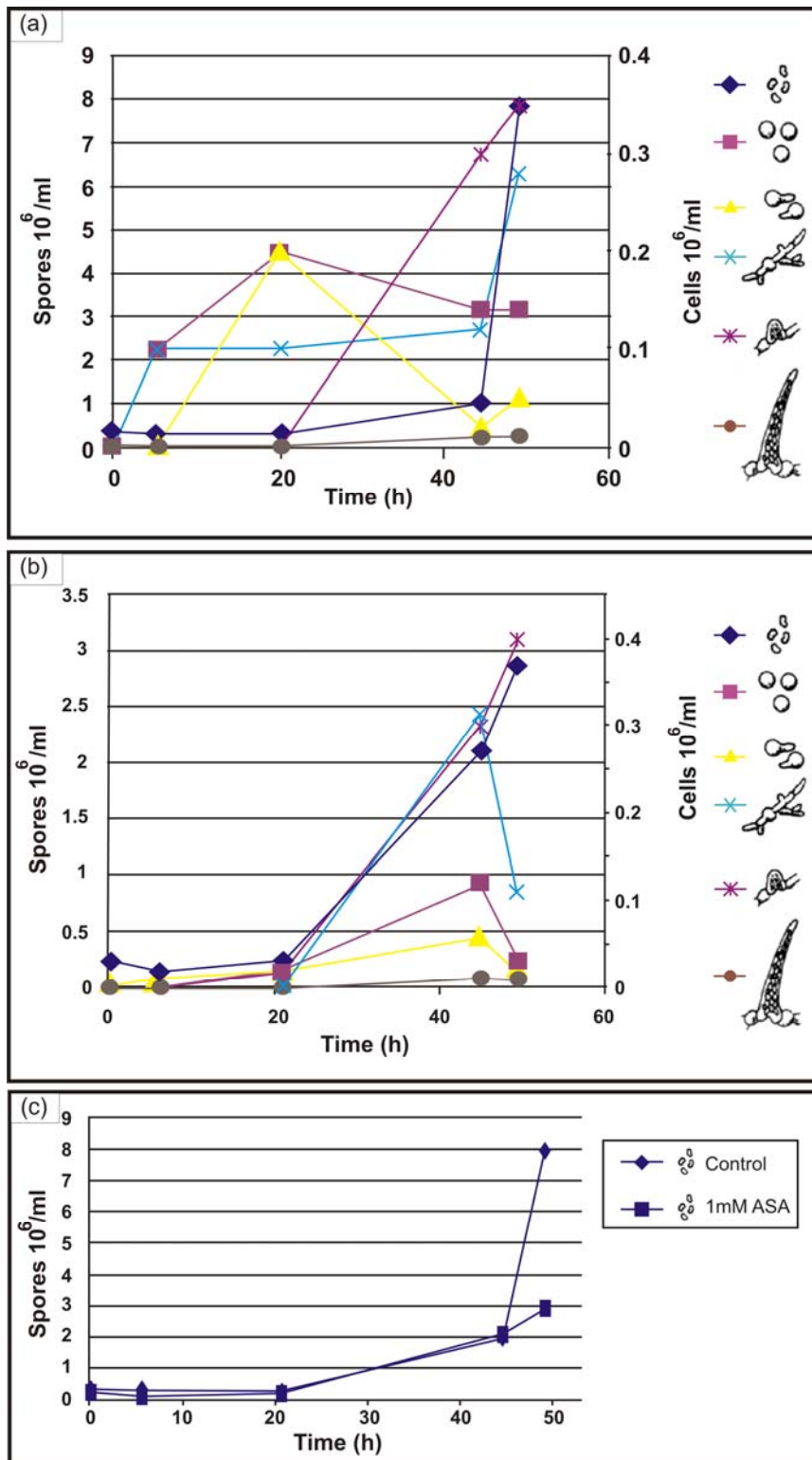
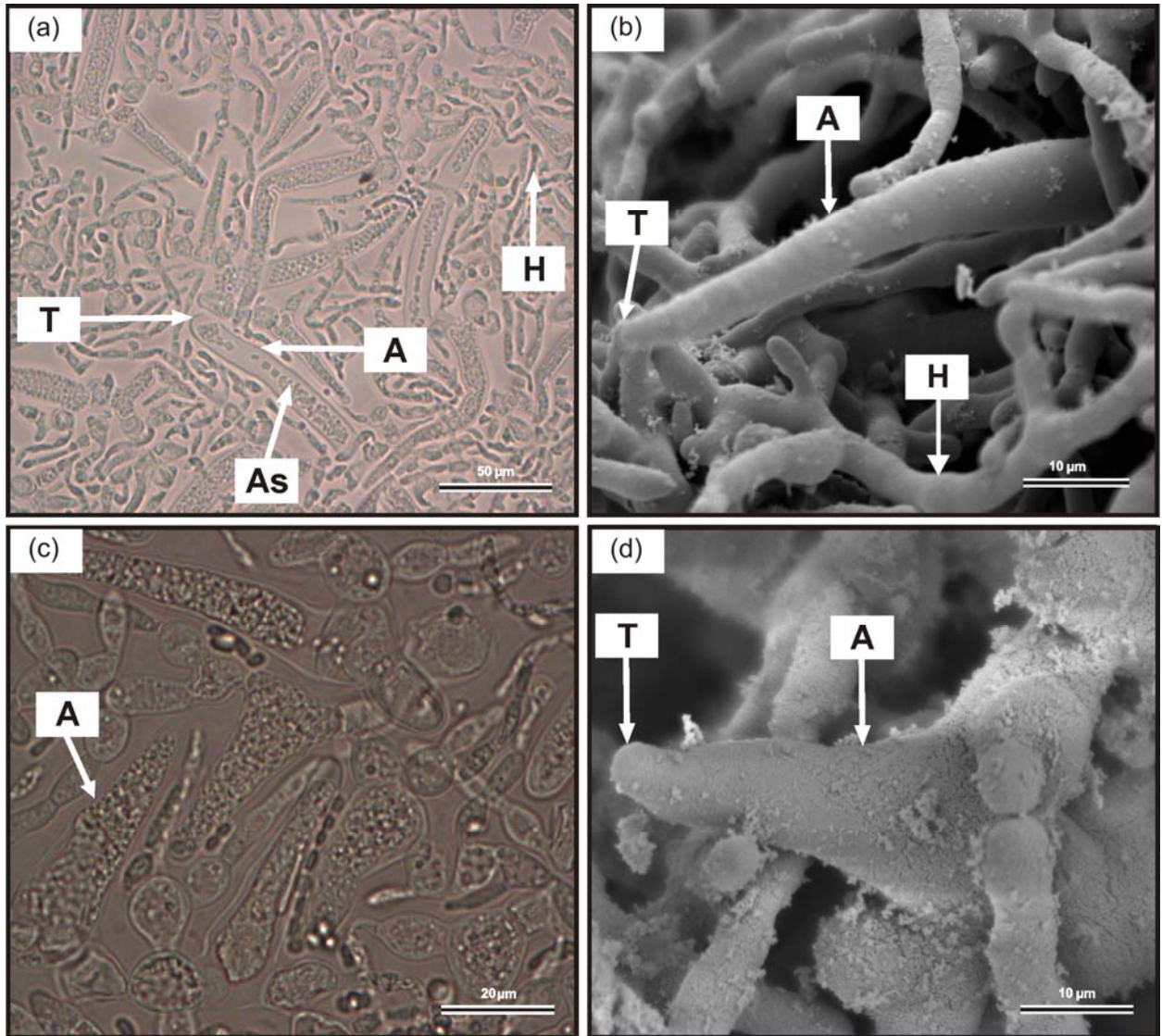


Fig. 8. Graphs of the life cycle of *D. uninucleata* var. *wickerhamii* showing the development of the various stages from ascospores alone in the absence (a) and presence (b) of 1 mM ASA. In (c), ascospore release in the absence and presence of 1 mM ASA is shown. The key to the consecutive stages is given on the right hand side of the graph. From top to bottom: reniform ascospores; swollen ascospores; germinating ascospores; hyphae; primordial asci; mature asci.



**Fig. 9.** Light and scanning electron micrographs showing the effect of 1 mM ASA on the sexual stage i.e. asci and ascospore release in *D. uninucleata* var. *uninucleata* (South African strain; a, b) and for *D. uninucleata* var. *wickerhamii* (c, d). A = ascus, As = ascospores, H = hyphae, T = ascus tip.

## Summary

The genus *Dipodascopsis* was extensively studied with regards to reproductive cycles as well as the presence, distribution and function of 3-OH oxylipins. Most of this research was carried out on *D. uninucleata* var. *uninucleata* (Canadian strain) as well as *D. tóthii*. However, little is known concerning *D. uninucleata* var. *uninucleata* isolated from South African soil, as well as *D. uninucleata* var. *wickerhamii*. Consequently, using gas chromatography-mass spectrometry, electron microscopy and confocal laser scanning microscopy, the two varieties were compared regarding their morphologies, oxylipin production, mitochondrial activity as well as life cycles and ascospore release. According to literature, the two varieties differ only in their ability to assimilate certain carbon sources. During this study, differences in ascospore size as well as differences in ascospore clustering, after release, was observed. Furthermore, differences in the type of 3-OH oxylipin produced by the two varieties, also existed. 3-OH oxylipin production was found to be associated mainly with the sexual stage and concentrated in the ascus surrounding the ascospores, in both varieties. Furthermore, increased mitochondrial activity was also observed during the sexual stage and found to be concentrated in close vicinity of the ascospores. Since mitochondria produce 3-OH oxylipins, it is suggested that the increased activity during sexual development would be to aid in the production and release of the ascospores, as well as the accumulation of these 3-OH oxylipins. In addition, acetylsalicylic acid was found to inhibit the production of 3-OH oxylipins by probably decreasing mitochondrial activity resulting in the inhibition of ascospore release.

**Keywords:** *Dipodascopsis*; mitochondrion activity; rhodamine 123; 3-hydroxy oxylipins; yeast.

## Opsomming

Die genus *Dipodascopsis* is goed bestudeer m.b.t. lewensiklusse asook die teenwoordigheid, verspreiding en funksie van 3-OH oksilipiene. Meeste van hierdie navorsing is uitgevoer op *D. uninucleata* var. *uninucleata* (Kanadese stam) en *D. tóthii*. Min is egter bekend aangaande *D. uninucleata* var. *uninucleata* geïsoleer vanuit Suid-Afrikaanse grond, asook van *D. uninucleata* var. *wickerhamii*. Gevolglik is die morfologie, oksilipienproduksie, mitochondriale aktiwiteit, lewensiklusse en askosporvrystelling van hierdie variëteite m.b.v. gaschromatografie-massaspektrometrie, elektronmikroskopie en konfokale laserskandeermikroskopie vergelyk. Volgens literatuur verskil die twee variëteite slegs in hul vermoë om sekere koolstofbronne te assimileer. Gedurende hierdie studie is verskille in askosporgrootte en -bondelvorming na vrystelling opgemerk. Verder bestaan daar verskille in die tipe 3-OH oksilipien wat deur die twee variëteite geproduseer word. 3-OH oksilipienproduksie in albei variëteite is meestal geassosieer met die geslagtelike fase en in die askus om die askospore gekonsentreer. Verder is verhoogde mitochondriale aktiwiteit ook opgemerk in die geslagtelike fase en is dit gekonsentreerd naby die askospore. Aangesien mitochondria 3-OH oksilipiene produseer, word dit voorgestel dat die verhoogde aktiwiteit gedurende geslagtelike ontwikkeling sal plaasvind om te help met die produksie en vrystelling van die askospore, asook die opeenhoping van hierdie 3-OH oksilipiene Asetielsalisielsuur

is ook gevind om die produksie van 3-OH oksilipiëne te inhibeer, moontlik deur mitochondriale aktiwiteit te verminder wat tot die inhibisie van askosporvrystelling lei.

**Sleutelwoorde:** *Dipodascopsis*; mitochondrionaktiwiteit; rhodamien 123; 3-hidroksieoksilipiëne, gis.