OXYLIPIN PRODUCTION AND NOVEL ASCOSPORE RELEASE MECHANISMS IN THE YEAST *DIPODASCUS*

by

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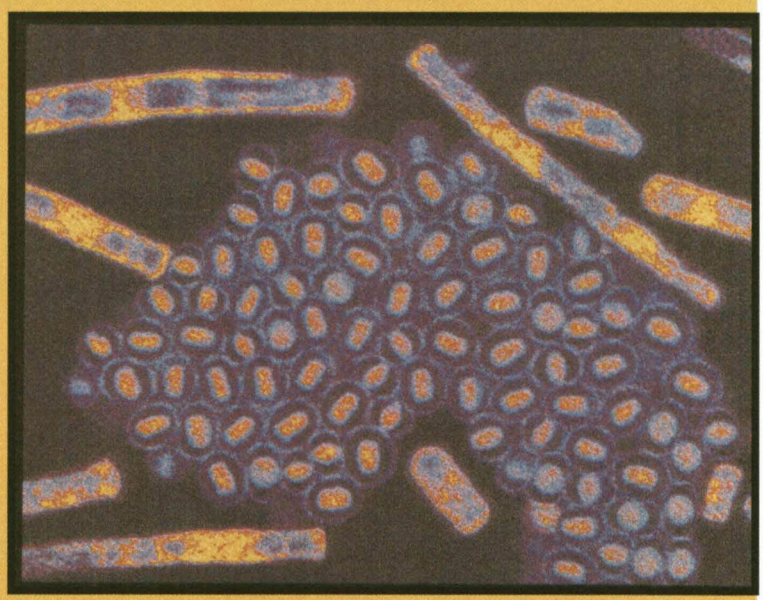

(Refer: p. 53, Fig. 2)

This thesis is lovingly dedicated to my parents, Hennie and Jeanette van Heerden

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CHAPTER 1

Introduction

1.1. Motivation

Some ascomycetous yeasts produce "lubricated" (oxylipin-coated), micron-scale sexual spores in a variety of shapes, sizes, colors and sometimes with nano-scale surface ornamentations (Yarrow, 1998; Kock *et al.*, 2003). In past literature, these oxylipin-coated ornamentations are only mentioned for use in classification and no thought was given to their possible purpose or function.

With the isolation and identification of a novel acetylsalicylic acid (ASA)-sensitive arachidonic acid metabolite, 3-hydroxy-5,8,11,14-eicosatetraenoic acid (3*R*-HETE), from *Dipodascopsis uninucleata* var. *uninucleata*, the first step towards a possible answer concerning the function of these structures was made (Van Dyk *et al.,* 1991). Here, this 3-hydroxy (3-OH) oxylipin is implicated as a prehistoric lubricant, facilitating ascospore water-driven movement and release from enclosed asci, probably for dispersal purposes. The practical application of this discovery was demonstrated when it was found that ASA inhibited the sexual cycle (both oxylipin production and ascospore release) of this yeast in a dose dependent manner. Consequently, the use of ASA and other non-steroidal anti-inflammatory drugs (NSAIDs) instead of expensive chemically produced antifungals, were suggested as an alternative method to combat fungal infections (Kock & Coetzee, 1990; Noverr *et al.,* 2003).

Since this discovery, researchers have demonstrated the widespread presence, distribution and possible function of ASA-sensitive oxylipins (i.e. prostaglandins and 3-OH oxylipins) in fungi (Kock *et al.*, 1991; 2003; 2004; Van Dyk *et al.,* 1991; Noverr *et al.*, 2003). Interestingly, research implicates oxylipins as new targets for controlling yeast infection. Alem & Douglas (2004; 2005) demonstrated that biofilm formation by the pathogenic yeast, *Candida albicans*, is enhanced by oxylipin production and can be decreased (uplifted) by the addition of physiological concentrations of ASA. In addition, Deva *et al.* (2000; 2001; 2003) demonstrated that ASA suppressed the pathogenic stage (hyphal formation) of *Candida albicans*. As a result, the use of ASA was proposed as an additional treatment for vulvovaginal candidiasis.

In 2003, Smith & co-workers revealed that 3-OH oxylipins are associated with the sheathed ascospores of some yeasts representing the genus *Dipodascus.* However, the distribution and function

of these oxylipins were not determined and the secret behind the fascinating release mechanics of oxylipin "lubricated" ascospores from bottle-shaped asci in *Dipodascus* still remains a mystery. With this information as background it became the aim of this study to map the distribution of 3-OH oxylipins in these yeasts and to expose the possible function of these compounds by using ASA inhibition studies.

1.2. Background

Yeasts are defined as unicellular, ontogenic stadia of true fungi that belong to the phylum Dikaryomycota and that literally means "foam" or "to rise" thus referring directly to the fermentation process (Phaff *et al.*, 1978; Kurtzman & Fell, 1998). They undergo vegetative reproduction by means of budding or fission and produce sexual stages that are not enclosed within a fruiting body. Ascomycetous yeasts are characterized by holoblastic budding and basidiomycetous yeasts by enteroblastic budding. Under adverse conditions, a wide variety of curiously shaped sexual spores (resembling needles, miniature corkscrews, hairy balls, hats, etc.) are produced, either through automixis or amphimixis, by some ascomycetous yeasts. The color of these ascospores can vary between colorless to yellow, amber, brown or reddish brown (Yarrow, 1998). Currently the ascomycetous yeasts comprise of 54 genera and 483 species (Barnett *et al.*, 2000).

1.2.1. Classification of the Dipodascaceae and related anamorphs

A schematic representation of the development of the classification of *Dipodascus* and its anamorph, *Geotrichum,* is shown in Fig. 1. The genus *Geotrichum* was first described by Link in 1809 as "white hyphomycetes that disarticulate into rectangular cells". Its teleomorph, *Dipodascus* was first isolated in 1890 by Juel from wood and trees (De Hoog *et al.,* 1986) where after it was defined as a fungus that formed hyphae resulting in arthrospores. In 1892, *D. albidus* was the first species to be described in the genus *Dipodascus* by de Lagerheim (De Hoog *et al.,* 1986).

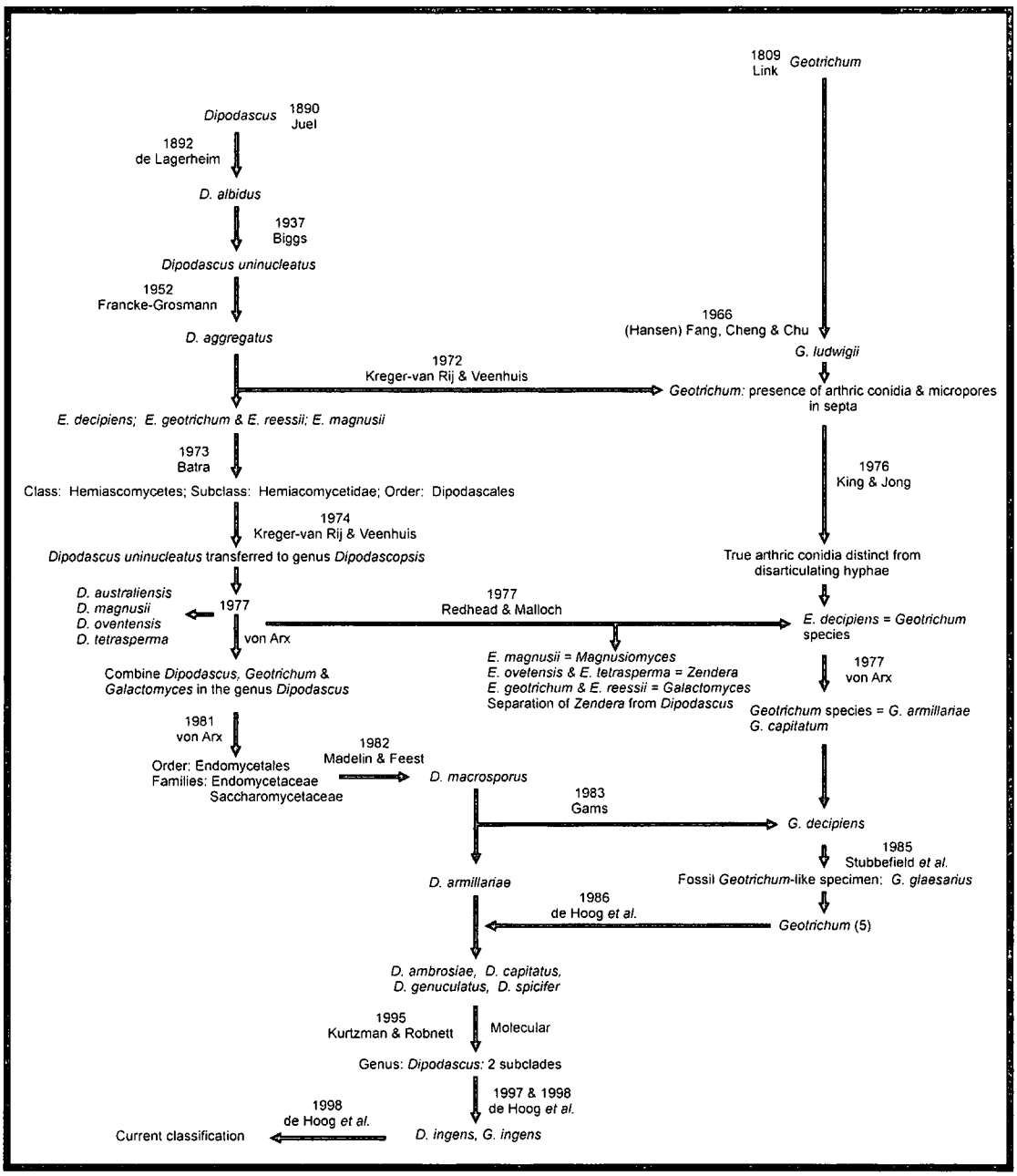

Fig. 1. A schematic representation of the historical development of the genus *Dipodascus* and its anamorphic state, *Geotrichum*.

In 1937, Biggs described *Dipodascus uninucleatus* and placed it in the genus *Dipodascus*, where its classification was based on the presence of multispored, elongated asci. In 1952, *Dipodascus aggregatus* was fully described by Francke-Grosmann and in 1966 *Geotrichum ludwigii* (Hansen) Fang, Cheng & Chu was introduced (De Hoog *et al.*, 1986). In 1972, Kreger-van Rij & Veenhuis divided the genus *Endomyces* into three groups based on the ultrastructure of the hyphae and the ascospores. These groups were *E. decipiens*, *E. geotrichum* together with *E. reessii* and *E. magnusii*. However, arthrospores but no ascospores were observed in *E. geotrichum*. Consequently, in the same year the genus *Geotrichum* was described by Kreger-van Rij & Veenhuis, based on the presence of arthric conidia (formed from fragmentation of pre-existing hyphae) and micropores in their septa. This discovery lead to the anamorphic genus *Geotrichum* being clearly distinguishable from the basidiomycetous genera *Trichosporon* and *Moniliella* as well as from the euascomycetous genera *Scytalidium* Pesante, *Rosulomyces* Marchand & Cabral, *Mauginiella* Cavara (simple septal pores with Woronin bodies) and *Arthrograhis* (Von Arx *et al.*, 1981).

Using morphological and physiological criteria, Batra (1973) classified the ascomycetous yeasts and especially the genus *Dipodascus*, as follows:

Class: **Hemiascomycetes**

Subclass: **Hemiascomycetidae**

Order: **Spermophthorales** Spermophthoraceae: *Spermophthora*

Dipodascales Dipodascaceae: *Dipodascus, Endomyces, Schizosaccharomyces*;

Eremascaceae: *Eremascus*

Cephaloascales Cephaloascaceae: *Cephaloascus*

Ascoideales Ascoideaceae: *Ascoidea*; Nematosporaceae: *Nematospora*, *Ashbya*, *Metschnikowia*, *Eremothecium, Coccidiascus*; Saccharomycetaceae & most ascosporogenous yeasts.

Using electron microscopy, Kreger-van Rij (1974) transfered *Dipodascus uninucleatus* to the genus *Dipodascopsis* based on asci that are laterally extended, tubular cells and colonies that are restricted, lobed, cerebriform and glassy. In addition, no anamorphic state is present, the septa have narrow, simple pores and the cell walls have a lamellar structure which is uncommon in fungi of the endomycetous yeasts. Consequently, this led to the conclusion that the genus *Dipodascopsis* is not related to *Dipodascus* (Curry, 1985) and it was placed together with *Lipomyces* Lodder & Kreger-van Rij, *Zygozyma* van der Walt *et al.* and *Waltomyces* Yamada & Nakase, in the family Lipomycetaceae Novák & Zsolt as redefined by Van der Walt & co-workers in 1986 (De Hoog *et al.*, 1986).

In 1976, King & Jong distinguished between true arthric conidia and disarticulating hyphae in the *Geotrichum* genus. In 1977, Redhead & Malloch observed the presence of two different yeast species on *Armillaria mellea*, one producing hat-shaped ascospores and the other, arthrospores (*Endomyces decipiens*). The latter was described as a *Geotrichum* species (Redhead & Malloch, 1977) and was later renamed *Geotrichum armillariae* by von Arx (1977). Redhead & Malloch (1977) also placed the other *Endomyces* species into the following genera:

1. *E. magnusii* Ludwig (1886)* under *Magnusiomyces*

2. *E. tetrasperma* Macy et Miller (1971)* and *E. ovetensis* Peláez et Ramirez (1956b)* under *Zendera*

3. *E. geotrichum* Butler et Peterson (1972)* and *E. reessii* van der Walt (1959c)* under *Galactomyces*

[*References obtainable from Redhead & Malloch (1977)]

At the same time the genus *Zendera* was separated from the genus *Dipodascus* while *D. australiensis* von Arx & Barker, *D. magnusii* (Ludwig) von Arx, *D. ovetensis* (Peláez & C. Ramírez) von Arx, *D. tetrasperma* (Macy & M.W. Miller) von Arx and *Geotrichum capitatum* (Diddens & Lodder) von Arx was fully described. Furthermore, *Geotrichum*, *Galactomyces* and *Dipodascus* were combined by von Arx in 1977 in the genus *Dipodascus*, based on asci that are formed after fusion of gametangial tips.

Von Arx (1981) divided the order Endomycetales into two families, the Endomycetaceae and the Saccharomycetaceae. In 1982, *D. macrosporus* Madelin & Feest was fully described (De Hoog *et al.*, 1986). A year later *D. armillariae* W. Gams and *Geotrichum decipiens* (L.R. Tulasne & C. Tulasne) W. Gams were described (De Hoog *et al.*, 1986). For the identification of *Geotrichum* species, van der Walt *et al.* (1983) & Gams (1984) proposed that the decisive tool should be the presence of micropores. However, to distinguish between *Geotrichum* and *Candida* proved to be a challenge. Here, the ability to produce arthric conidia was used as differentiation criterion.

During 1985, Stubbefield *et al.* described a fossil *Geotrichum*-like specimen resembling *Geotrichum candidum* on arachnoid remains in Oligocene amber from the Dominican Republic as *Geotrichum glaesarius*. In 1986, five new species of the genus *Geotrichum* as well as *D. ambrosiae, D. capitatus*, *D. geniculatus* and *D. spicifer* were introduced and fully described (De Hoog *et al.*, 1986). The description was based on morphology and the absence of budding cells. Von Arx & van der Walt (1987) accepted the proposal previously made by Redhead & Malloch (1977) that ascospore shape is of phylogenetic importance and suggested additional relationships between yeasts and euascomycetous families.

With the further development of the yeast classification system, more focus was placed on analysis using molecular sequences. Using D1/D2 sequencing, Kurtzman & Robnett (1995) divided *Dipodascus* into two subclades, one of which included species of *Galactomyces* and both with *Geotrichum* species (Fig. 2). The *D. ingens* clade is mainly characterized by species that produce only one to four ascospores (*D. ambrosiae, D. capitatus, D. ingens, D. magnusii, D. ovetensis, D. spicifer* and *D. tetrasperma*). The *D. albidus* clade is mainly characterized by species producing more than four ascospores (*D. aggregatus, D. albidus, D. armillariae, D. australiensis, D. geniculatus* and *D. macrosporus*) although the two *Galactomyces* species in this clade only produce one to two ascospores. This made any distinction between the two clades, based on ascospore numbers, ambiguous. Although *Endomyces* species produce hat-shaped ascospores, they are still closely related to *Dipodascus* because of their ability to produce arthrospores. In 1997, *D. ingens* Rodrigues

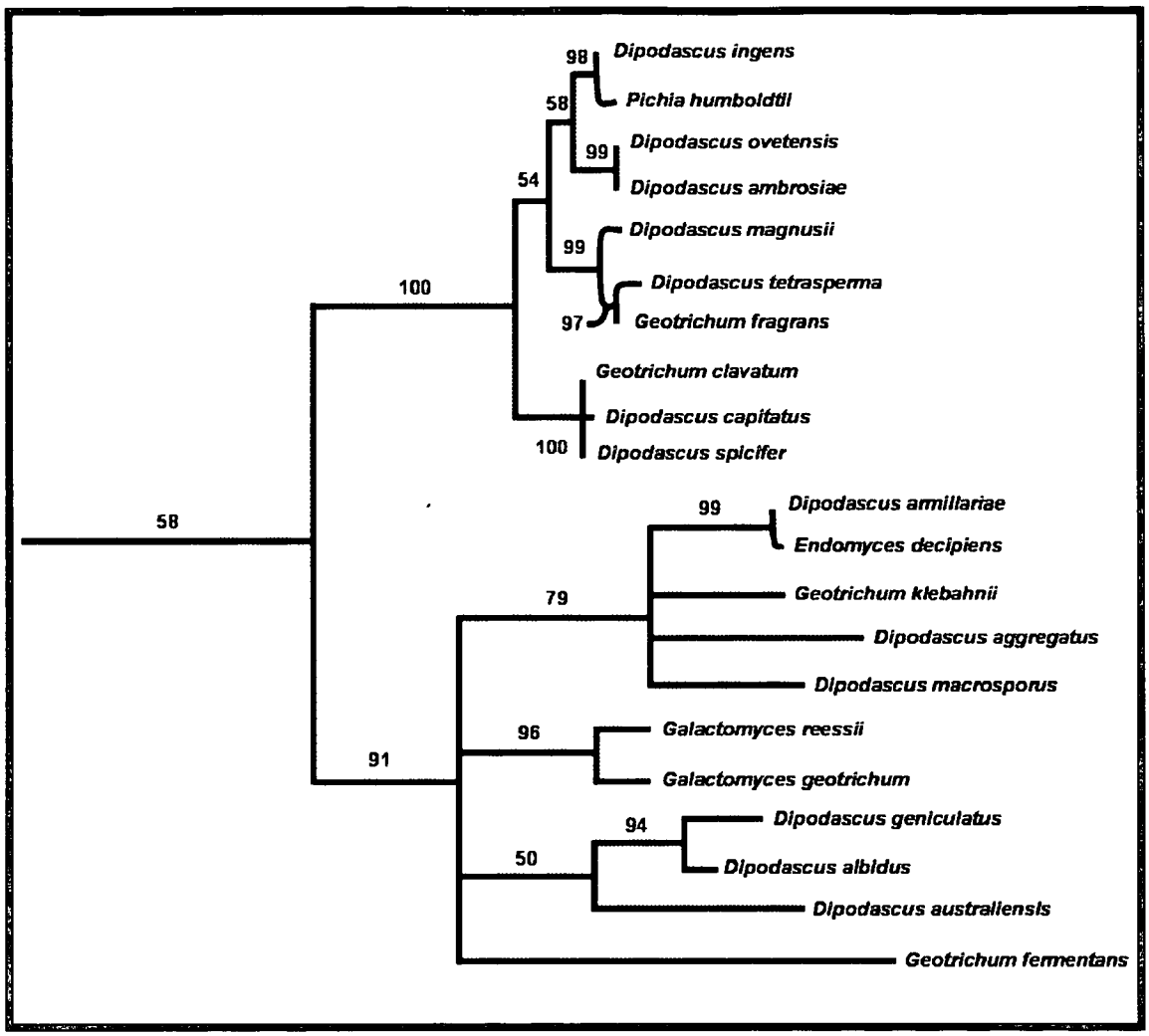

Fig. 2. A phylogenetic tree derived from maximum parsimony analysis indicating that species of *Dipodascus* are divided into two clades. Species in the *Dipodascus ingens* clade are characterized by asci containing 1-4 ascospores, whereas species in the *Dipodascus albidus* clade are characterized by asci containing more than 4 ascospores [Taken from Kurtzman & Robnett (1995)].

de Miranda ex de Hoog, M.Th. Smith & Guého and *Geotrichum ingens* (Van der Walt & Van Kerken) de Hoog, M.Th. Smith & Guého was fully described (De Hoog *et al.*, 1998). Based on their work in 1995, Kurtzman & Robnett evaluated D1/D2 sequencing further in 1998, resulting in the suggestion to move *Schizoblastosporion chiloense* into the genus *Geotrichum* since the data indicated that it is

phylogenetically close to *D. ingens*. The data also indicated the possibility of the following taxon pairs being conspecific: *D.* armillariae/"*E. decipiens*", *D.* ovetensis/*D. ambrosiae* and *D. spicifer*/*G. clavatum* (Fig. 3). The current classification of the genus *Dipodascus* as well as its anamorph, *Geotrichum* can be found in de Hoog *et al.* (1998).

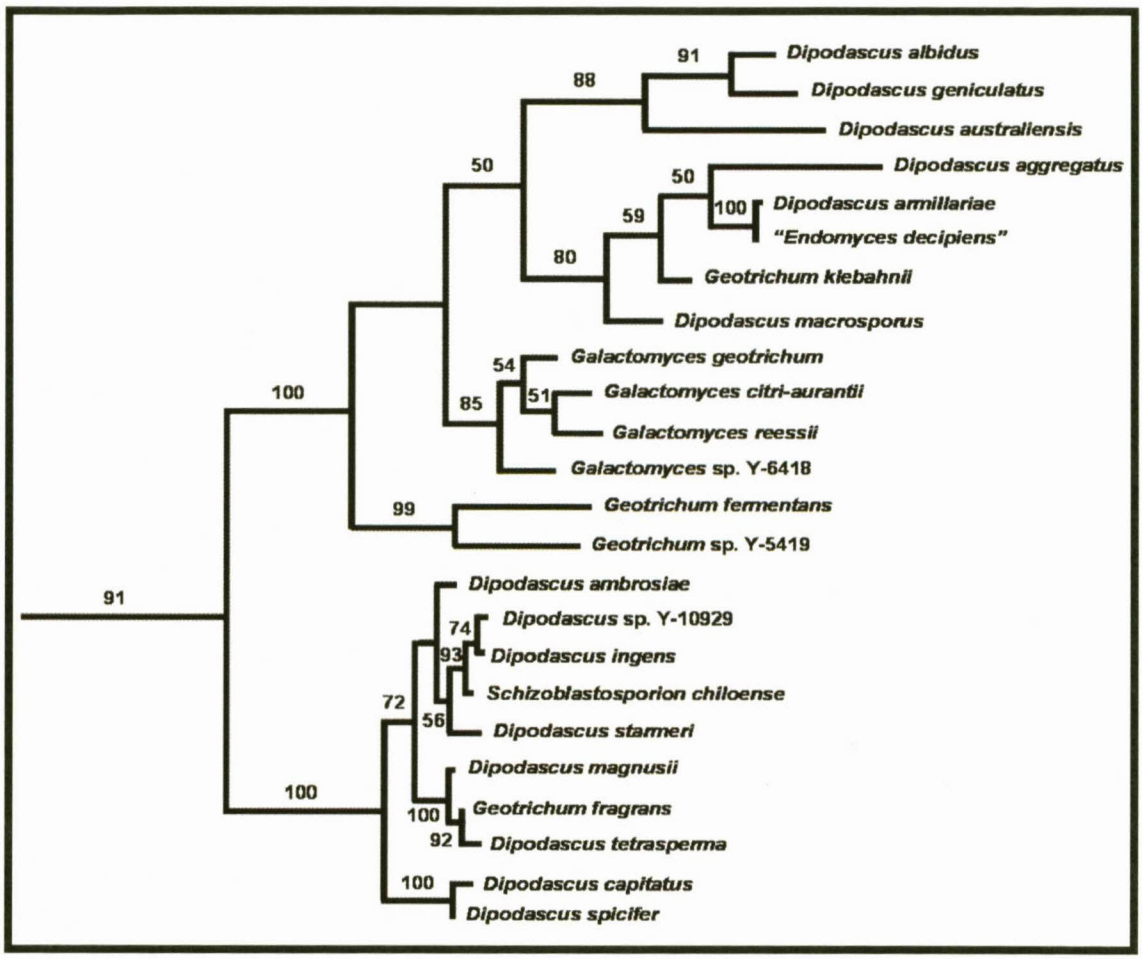

Fig. 3. A phylogenetic tree of the *Dipodascus* clade from maximum parsimony analysis indicating that *Schizoblastosporion chiloense* is closely related to *Dipodascus ingens* [Taken from Kurtzman & Robnett (1998)].

1.2.2. Species currently accepted (de Hoog *et al.*, 1998)

Type species:

Dipodascus albidus de Lagerheim

Species accepted:

1. *Dipodascus aggregatus* Francke-Grosmann (1952)
2. *Dipodascus albidus* de Lagerheim (1892)
3. *Dipodascus ambrosiae* de Hoog, M.Th. Smith & Guého (1986)
4. *Dipodascus armillariae* W. Gams (1983)
5. *Dipodascus australiensis* von Arx & Barker (1977)
6. *Dipodascus capitatus* de Hoog, M.Th. Smith & Guého (1986)
7. *Dipodascus geniculatus* de Hoog, M.Th. Smith & Guého (1986)
8. *Dipodascus ingens* Rodrigues de Miranda ex de Hoog, M.Th. Smith & Guého (1997)
9. *Dipodascus macrosporus* Madelin & Feest (1982)
10. *Dipodascus magnusii* (Ludwig) von Arx (1977)
11. *Dipodascus ovetensis* (Peláez & C. Ramírez) von Arx (1977)
12. *Dipodascus spicifer* de Hoog, M.Th. Smith & Guého (1986)
13. *Dipodascus tetrasperma* (Macy & M.W. Miller) von Arx (1977)

1.2.3. Present diagnosis (de Hoog *et al.*, 1998)

"Colonies are white or cream-colored, farinose or hairy, and usually dry; hyphae are hyaline, mostly disarticulating into rectangular arthroconidia (anamorph genus *Geotrichum*). Asci are acicular, cylindrical, ellipsoidal or subglobose, formed after fusion of gametangia located laterally on hyphae. Septa have micropores. Asci have persistent walls and open by rupture at the apex. Ascospores are 4-128 per ascus, hyaline, ellipsoidal, with smooth walls and surrounded by regular slime sheaths. Fermentation is mostly absent. Extracellular starch is not produced. Diazonium blue B reaction is negative".

Morphological key to species (de Hoog *et al.*, 1998):

1.	a- Asci acicular or long-cylindrical, with a narrow apex	2
	b- Asci usually globose or ellipsoidal; when cylindrical, with a broadly rounded apex	3
2(1).	a- Asci and ascospores cylindrical	*D. macrosporus*
	b- Asci subulate; ascospores ellipsoidal	*D. albidus*
3(1).	a- Asci 1-4 spored	7
	b- Asci containing more than 4 spores	4
4(3).	a- Asci cylindrical, up to 120 μm long, in rather dense groups, containing up to 30 ascospores; insect symbiont	*D. aggregatus*
	b- No combination of the above characters	5

5(4). a- Ascospores (2.8-3.2)x(3-4) μm; asci asymmetrically bipodal, somewhat tapering towards the tip *D. geniculatus*

b- Ascospores larger; asci cylindrical to ellipsoidal 6

6(5). a- Asci mostly in groups, broadly ellipsoidal, mostly present in culture; on rotting parts of tropical or subtropical succulents *D. australiensis*

b- Asci solitary, rather irregular in shape, not formed in culture; on carpophores of *Armillaria* in temperate zone *D. armillariae*

7(3). a- Asci borne on erect or suberect hyphae, anisogamous; ascospores (5.0-6.5)x(8.5-11.0) μm *D. magnusii*

b- Asci borne on undifferentiated hyphae, isogamous; ascospores smaller 8

8(7). a- Asci usually longer than wide 9

b- Asci appressed, usually shorter than wide; hyphae straight and stiff, 7-9 μm wide, with acuminate apices *D. tetrasperma*

9(8). a- Sympodial rachides abundant 12

b- Sympodial rachides absent or scarce 10

10(9). a- Initial growth with pseudomycelium 11

b- Initial growth with true hyphae *D. ambrosiae*

11(10). a- Thallus entirely pseudomycelial *D. ingens*

b- Thallus initially pseudomycelial, changing into

true hyphae *D. ovetensis*

12(9). a- Branching regular, often verticillate; rachides

straight; on warm-blooded animals *D. capitatus*

b- Branching rather irregular; rachides flexuose;

on rotting parts of tropical or subtropical succulents *D. spicifer*

1.2.4. Sexual reproductive cycles and ascospore morphology

In the genus *Dipodascus,* sexual spores (ascospores) are produced either through an automictic, homothallic sexual cycle or an amphimictic, heterothallic sexual cycle. During automixis (autogamy /selffertilization), inbreeding (genetic isolation) occurs although the advantages of meiosis are maintained (Van der Walt, 1999). This type of reproductive cycle is characteristic of various species such as *D. aggregatus, D. albidus, D. ambrosiae, D. australiensis, D. geniculatus, D. macrosporus, D. magnusii, D. ovetensis* and *D. spicifer* (De Hoog *et al.,* 1986) (Fig. 4).

During the haploid vegetative stage, the ascospores swell and germinate into hyphae. Some hyphae will break resulting in arthrospore formation. Mitosis also occurs during this stage. During the sexual stage, plasmogamy occurs and the two haploid nuclei will fuse through karyogamy to produce a diploid zygote. Meiosis or reduction division will follow to form four haploid nuclei. Post-meiotic mitosis will take place resulting in a mature ascus containing many ascospores, each surrounded by a

characteristic slime sheath. These ascospores will then be released from the ascus under adverse conditions after which it will swell again to restore the vegetative stage.

During amphimixis, different compatible mating types, a and α, are present. This type of life cycle promotes genetic exchange, recombination, genome diversity and also adaptation to new niches. Species that follow this type of life cycle is *D. capitatus* and *D. ingens*. The only species with an unknown ploidy is *D. armillariae* (De Hoog *et al.*, 1986; Van der Walt, 1999).

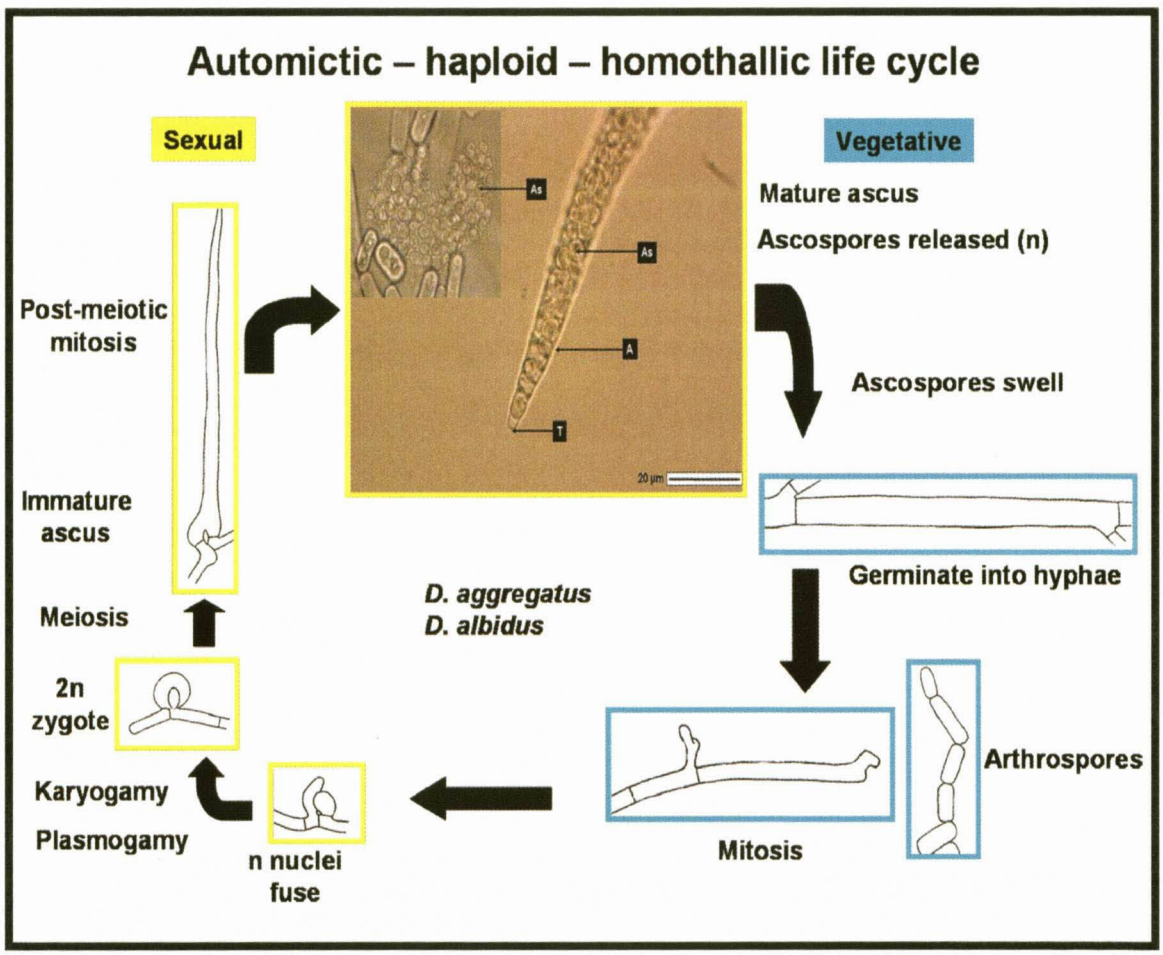

Fig. 4. An example of an automictic, haplontic, homothallic life cycle followed by some *Dipodascus* species.

1.2.5. Economic importance

Representatives of *Dipodascus* as well as its anamorph, *Geotrichum,* are found worldwide in soil, water, air, decaying leaves, rotting paper, textiles and sewage. They are usually involved in spoilage of food like bakery products, dairy products, juices, fruits and vegetables. They can also be found in indoor environments with some species producing strong odors. Other species are involved in gardening symbioses with arthropods (De Hoog *et al.*, 1986).

Dipodascus capitatus and *Geotrichum clavatum* are obligatory human pathogens and are usually associated with human lung disorders. They are frequently found in immunocompromised patients, especially patients with leukemia. *Dipodascus capitatus* again causes a disseminated disease in neutropenic patients. In addition, *D. armillariae* and *D. macrosporus* are mycoparasites restricted to certain fungi (De Hoog *et al.,* 1986; Ersoz *et al.*, 2004; Gadea *et al.*, 2004).

Geotrichum candidum (*Galactomyces geotrichum*) is a weak pathogen that can be found on plants, animals and humans. In humans it may cause geotrichosis (opportunistic bronchial, pulmonary and disseminated infections) as well as fungemia in immunocompromised hosts through inhalation or ingestion. It can also invade the internal organs and cause skin lesions, nail infections, black tongue and allergic reactions in patients with chronic urticaria. In animals it is known to cause skin diseases and play a role in abortions in cows due to fungal infection of the reproductive tract. It is also known as a spoilage organism in milk products and is present in polluted water. Fruit diseases are watery rot of tomato, rot of carrots and wet-stem of muskmelon. Although this yeast is mostly considered to be harmful, it plays an important role in the production of Nigerian fermented foods from watermelon seeds (De Hoog *et al.*, 1986).

1.3. Oxylipins in yeasts

1.3.1. Definition

Oxylipin is a general term used to describe oxygenated lipids that are widely distributed in nature. These compounds include the well-studied eicosanoids (e.g. prostaglandins, thromboxanes and

leukotrienes) and the hydroxy oxylipins with one or more hydroxyl groups at carbon 5, 7, 8, 9, 12, 13, 15 and 17. Eicosanoids, produced from a 20-carbon polyunsaturated fatty acid precursor via cyclooxygenases, play a vital role in cellular function and have potent biological activities (e.g. labour induction and platelet aggregation) (Samuelsson, 1983; Needleman *et al.,* 1986; Spector *et al.,* 1988; Van Dyk *et al.*, 1994). In contrast, most hydroxy oxylipins are produced by one of three pathways, either lipoxygenase, dioxygenase or cytochrome P-450 (Mazur *et al.,* 1991; Brodowski *et al.,* 1992). These oxylipins are widely distributed and can be found in plants, animals (Van Dyk *et al.,* 1991), algae (Gerwick, 1994; 1996) and in the fungal domain where it has been associated with vegetative growth and sexual reproduction (Herman & Herman, 1985; Kock *et al.,* 1998; 2000).

In this study, emphasis is placed on 3-hydroxy (OH) oxylipins (Fig. 5) produced in the mitochondria of fungi through incomplete β-oxidation (Deva *et al.,* 2000; 2001; 2003; Ciccoli *et al.,* 2005). These compounds are characterized by a hydroxyl group at the C3 position (counted from the carboxylate group), while the carbon chain can vary in length and degree of desaturation (Van Dyk *et al.,* 1991). These compounds were found to be ubiquitous in yeasts (Van Dyk *et al.,* 1991; Smith, 2002). 3-Hydroxy oxylipins can be present in two enantiomeric forms, i.e. 3R and 3S. Incomplete β-oxidation can be divided into two phases (Fig. 6). First, a long chain fatty acid suitable for catabolism enters the cell from the environment. Secondly, this fatty acid is converted via acyl CoA synthetase to a fatty acyl CoA molecule, which then enters the mitochondria. Next, this molecule is dehydrogenated by an acyl CoA dehydrogenase enzyme to yield a Δ2 unsaturated acyl CoA molecule. This acyl CoA molecule is then enzymatically hydrated to form a racemic mixture of *D* and *L* 3-OH fatty acids (FAs). In fungal β-oxidation, only the *L*-enantiomers are further dehydrogenated and cleaved to form fatty

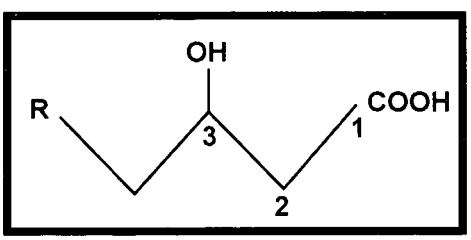

Fig. 5. The structure of a 3-hydroxy oxylipin [Taken from Venter *et al.* (1997)].

acids with two less carbons and an acyl CoA molecule. Some of the *D*-enantiomers undergo epimerization to yield *L*-enantiomers which then proceed through the normal system. It is suggested that the rest of the *D*-enantiomers (3*R*-form) is released from the mitochondria and deposited on fungal cell surfaces (Venter *et al.*, 1997; Kock *et al.*, 2003). It is generally believed that the mitochondria (where β-oxidation takes place), evolved from Gram-negative bacteria, i.e. rickettsias (Gray *et al.*, 2001) through endosimbiosis many millions of years ago. Strikingly, these bacteria also produce 3-OH oxylipins as part of their lipopolysaccharide layers (Armano *et al.*, 1998).

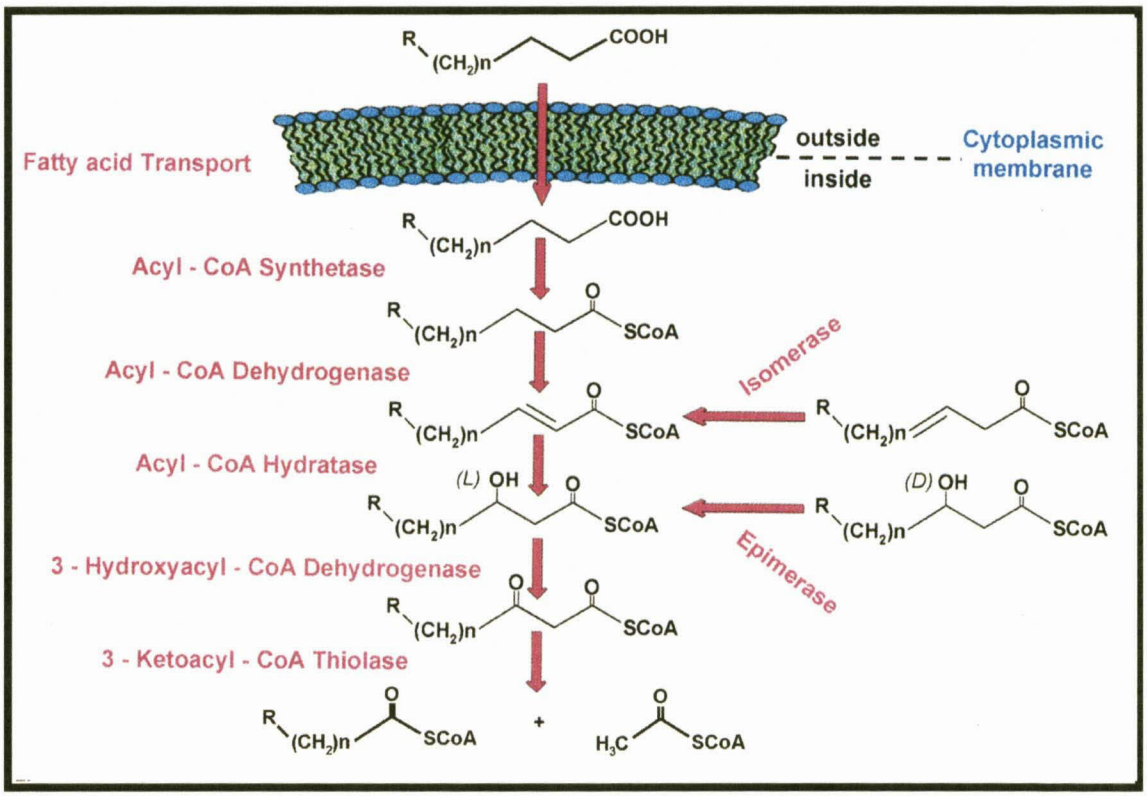

Fig. 6. β-oxidation in fungi [Taken from Finnerty (1989)].

1.3.2. Acetylsalicylic acid (ASA)-sensitive oxylipin distribution in fungi, especially yeasts: A historical review

The first evidence for the presence and production of 3-OH oxylipins in fungi has been documented as far back as 1967 by various researchers (Stodola *et al.*, 1967; Vesonder *et al.*, 1968;

Kurtzman *et al.,* 1974; Lösel, 1988). In the late 1980's Kock & co-workers embarked on an extensive study to determine whether yeasts can also produce ASA-sensitive oxylipins (i.e. eicosanoids such as prostaglandins). Eicosanoids have a number of medical uses such as labour induction and the control of inflammation and platelet aggregation (Kock *et al.*, 1991). Unfortunately these compounds, when produced synthetically, are very expensive due to their complex chemical structure. However, if these compounds could be biotechnologically produced (e.g. by yeasts), it may have a major impact on reducing the production costs, therefore making them more readily available for application (Dixon, 1991).

In 1991, the first step towards this goal was achieved with the combined use of radio TLC and H^1 2DCOSY NMR, gas chromatography-mass spectrometry (EI and FAB) and IR spectroscopy analysis. Strikingly, a 3-hydroxy-5,8,11,14-eicosatetraenoic acid (3*R*-HETE) was found, amongst others, to be produced from arachidonic acid (AA) during the sexual stage of the yeast *Dipodascopsis uninucleata* var. *uninucleata* (Van Dyk *et al.*, 1991). This compound however, does not have the cyclopentane ring that is characteristic of the cyclooxygenase formed prostaglandins (Coetzee *et al.,* 1992). It also displayed different chromatographic properties than that of the usual cyclooxygenase products. Studies indicated that the sexual stage of this yeast's life cycle as well as 3*R*-HETE production, were inhibited by ASA in a dose dependant manner (Van Dyk *et al.,* 1991; Botha *et al.,* 1992), implicating a role of this oxylipin in sexual reproduction.

Acetylsalicylic acid has various medical uses such as relieving mild to moderate pain, pyrexia, prophylaxis of platelet aggregation, treatment of rheumatic fever and treatment of acute and chronic inflammatory disorders (Gibbon *et al.,* 2003). These actions may be ascribed to the fact that ASA is a potent inhibitor of cyclooxygenase, leading to the subsequent reduction in prostaglandin synthesis. Since it has also been discovered that ASA inhibits yeast growth and sexual reproduction, the possibility exists that this NSAID can also be used as an antifungal. As a result a patent was registered based on the possible application of NSAIDs (e.g. ASA and indomethacin) to combat fungal infections (Kock & Coetzee, 1990).

In 1996, the biological effects of 3*R*-HETE in mammalian cells were explored. This lead to the recognition of the biotechnological importance and value of this compound (Nigam *et al.,* 1996). It was discovered that it affects signal transduction in human tumor cells and neutrophils, activates the phospholipase-D pathway to increase the formation of diacylglycerol via phosphatidic acid metabolism and causes aggregation of rabbit platelets (Kock *et al.,* 1994; Nigam *et al.,* 1996). Consequently, research was aimed at the production of sufficient quantities of 3*R*-HETE for further testing in mammals. The production of 3*R*-HETE from AA fed to *D. uninucleata* var. *uninucleata* and a close relative, *D. tóthii,* was evaluated. The presence of 3*R*-HETE was reported in both yeasts, but *D. uninucleata* var. *uninucleata* produced more 3*R*-HETE, resulting in it remaining the yeast of choice for 3*R*-HETE production (Kock *et al.*, 1997). The exploration of the metabolism of 3*R*-HETE, to arrive at a possible pathway for oxylipin production in yeasts, indicated that *D. uninucleata* var. *uninucleata* could produce a variety of 3-OH oxylipins (i.e. 3-OH 20:3; 3-OH 20:5; 3-OH 14:3), when fed with different precursors (Venter *et al.,* 1997; Fox *et al.,* 1997). This made it possible to produce 3-OH oxylipins of different chain lengths and desaturation.

3-Hydroxy-5,8,11,14-eicosatetraenoic acid was chemically synthesized for the first time in 1998 (Bhatt *et al.*, 1998; Groza *et al.,* 2002). This was achieved by coupling a chiral aldehyde with a Wittig salt, derived respectively from 2-deoxy-D-ribose and AA (Bhatt *et al.*, 1998). In order to produce polyclonal antibodies against 3*R*-HETE, the carboxyl group of 3*R*-HETE was attached to the amino groups of bovine serum albumin. Next, it was emulsified in an equal volume of Freund's complete adjuvant (first injection) and later in incomplete adjuvant. The emulsion was injected into a white, female Nieu-Zealand rabbit, every second week for three months. Blood was collected from the carotid artery and after centrifugation, the sera were purified by Biogenes, Berlin, and the antibody characterized by determining its titer, sensitivity and specificity (Kock *et al.,* 1998). Cross-reactions occurred with 3-OH oxylipins of different chain lengths and desaturation, indicating the high specificity of the antibodies for 3-OH oxylipins in general. In combination with secondary FITC- coupled antibodies, this assisted in the successful mapping of 3-OH oxylipins over the life cycle of *D. uninucleata* var. *uninucleata*.

In 1998, immunofluorescence microscopy of *D. uninucleata* var. *uninucleata* cultures, indicated that 3-OH oxylipins were only associated with structures present during the sexual stage (Fig. 7). The liberated ascospores (Fig. 7 A;E), tips of adhering gametes (Fig. 7C) and ascospores in young asci (Fig. 7D) show a high oxylipin-antibody affinity. In contrast, the hyphae (Fig. 7B) show a low oxylipin-antibody affinity. Since this yeast has characteristically thick cells walls which could have prevented the antibody from entering the cell, the process was repeated with protoplasts which showed that the empty ascus protoplast (Fig. 7F) had a low affinity for the oxylipin-antibody. In addition, ascospores in an ascus protoplast fluoresced intensely (Fig. 7G) (Kock *et al.*, 1998).

Using transmission electron microscopy (TEM) and oxylipin inhibition studies in *D. uninucleata* var. *uninucleata* (Fig. 8), it was concluded that the 3-OH oxylipins are not only associated with the ascospores, but more specifically with nano-scale surface ornamentations i.e. interlocked hooked ridges on the ascospores. Interestingly, the ascospores are connected by these interlocked hooked ridges (surface hooks) (Fig. 8C) that in combination with 3-OH oxylipins may play a role in ensuring ordered ascospore liberation from enclosed asci as well as aggregation after release. This is probably due to entropic based hydrophobic forces (Rudolph, 1994). Interestingly, the presence of 1mM ASA not only inhibited the production of 3-OH oxylipins, but also resulted in the malformation of the surface hooked ridges (Fig. 8B). In addition, ASA also caused the ascus tip to be partly closed resulting in impaired ascospore release (Fig. 8A) (Kock *et al.*, 1999). These findings were the first report and the start of various studies on the mechanics of ascospore release and the probable function of these 3-OH oxylipins in yeasts. Interestingly, in contrast to *D. uninucleata* var. *uninucleata,* only the ascus tip of *D. tóthii* contained 3-OH oxylipins with a small amount being present within the ascospore clusters (Smith *et al.,* 2000). Some species in the Lipomycetaceae (such as *Lipomyces kockii, L. starkeyi, Zygozyma oligophaga*) tested positive for the presence of 3-OH oxylipins. Immunofluorescence indicated that these compounds were also associated with the sexual spores (ascospores) (Smith *et al.,* 2000).

The presence of 3-OH oxylipins was also found in the Mucorales. Gas chromatography-mass spectrometry revealed that *Mucor genevensis* could transform exogenously fed AA to 3-OH 5Z, 8Z-tetradecadienoic acid (3-HTDE). This was thought to be achieved by the retroconversion of AA to

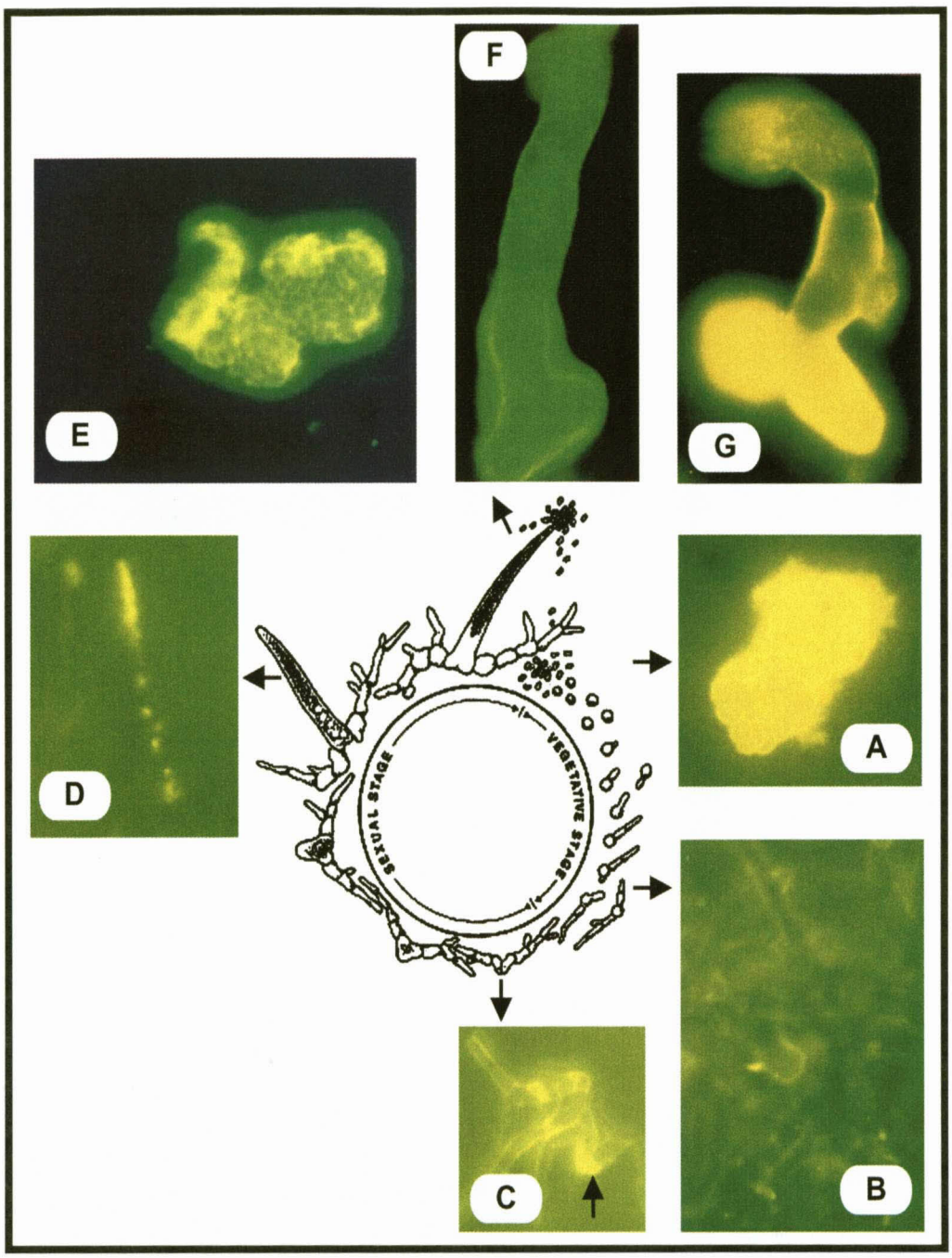

Fig. 7. The life cycle and distribution of 3*R*-HETE in *Dipodascopsis uninucleata* var. *uninucleata* visualized through immunofluorescence. (A) Liberated ascospores (10 mm = 10 µm cell size). (B) Hyphae with cell wall (10 mm = 25 µm cell size). (C) Gametangiogamy (10 mm = 25 µm cell size). (D) Young ascus with cell wall (10 mm = 25 µm cell size). (E) Liberated ascospores from ascus (10 mm = 10 µm cell size). (F) Empty ascus protoplast-still with characteristic morphology (10 mm = 10 µm cell size). (G) Deformed mature ascus protoplast (10 mm = 10 µm cell size) [Taken from Kock *et al.* (1998)].

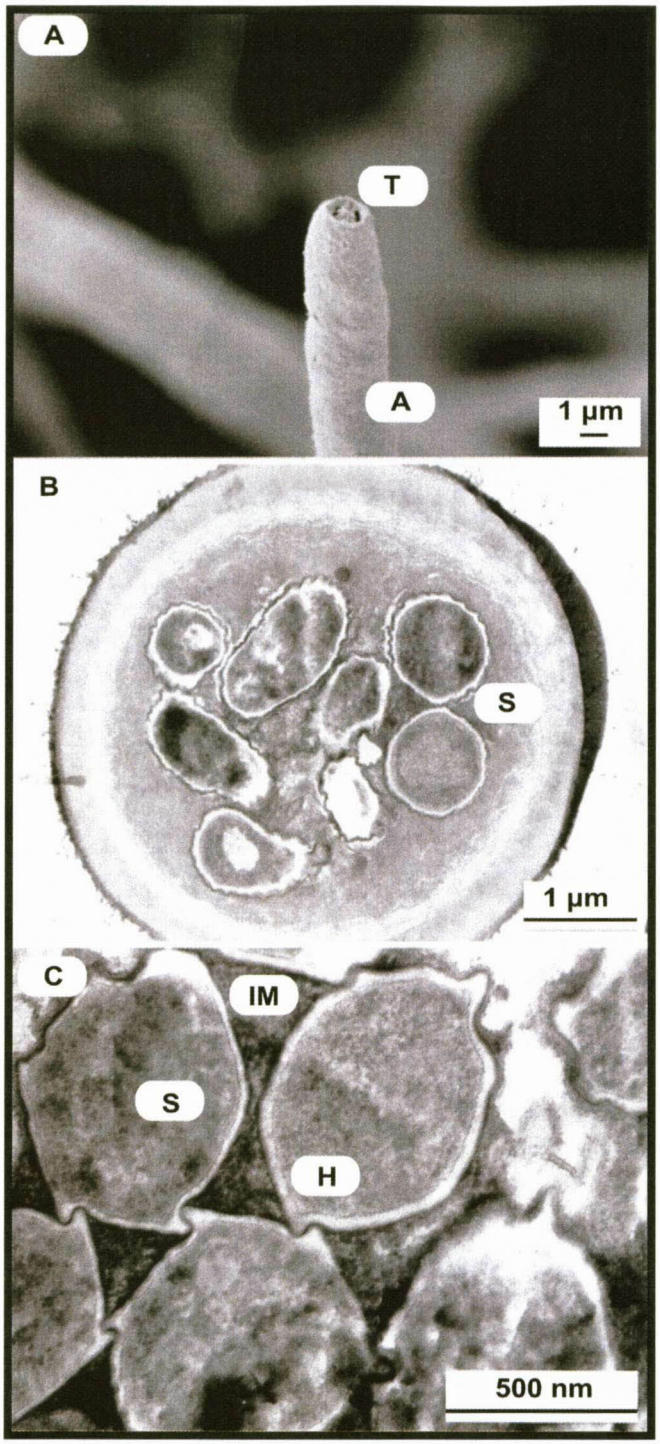

Fig. 8. Representative photomicrographs illustrating the effect of non-steroidal anti-inflammatory drugs (NSAIDs) i.e. 1 mM acetylsalicylic acid (ASA) on ascospore release and ultrastructure. **(A)** Upper part of mature ascus (A) with partly closed tip (T). **(B)** Ascospores (S) without defined hooks inside an ascus. **(C)** Hooked (H) mature ascospores inside an ascus in the absence of ASA. Interspore matrix (IM). [Taken from Kock *et al.* (1999)].

linoleic acid followed by the production of 3-HTDE (Pohl *et al.,* 1998). Later studies indicated that the columellae, sporangia and aggregating sporangiospores are the structures associated with the 3-OH oxylipins (Strauss *et al.,* 2000). Interestingly, with the aid of immunofluorescence and gas chromatography-mass spectrometry, a 3-OH 9:1 was found to be present on the sub-sporangial vesicle and between aggregating sporangiospores of *Pilobolus* (Kock *et al.,* 2001).

3-Hydroxy oxylipins have also been implicated in yeast infection. *Candida albicans* is a dimorphic yeast and depending on environmental status, can either grow as blastospores or switch to a filamentous form (Deva *et al.*, 2000; 2001; 2003). The pathogenic filamentous form often involves the formation of biofilms on tissue (vulvovaginal and oral candidiasis) or on implanted devices (i.e. catheters). However, management and treatment of infections are very difficult due to the drug resistance of biofilms (Alem & Douglas, 2004; 2005). It was illustrated that 3-OH oxylipins are associated with the surface of the filamentous structures of *Candida albicans* and could possibly play a role in the morphogenesis and pathogenicity of this yeast (Deva *et al.*, 2000; 2001; 2003). In 2005, the role that 3-OH eicosanoids plays in candidiasis was demonstrated. Upon infection, *C. albicans* causes release of AA form the infected host tissue which is then in turn converted by *C. albicans* to 3-OH AA. This compound then serves as a substrate for the cyclooxygenase-2 enzyme (COX-2) in the host tissue to produce pro-inflammatory 3-OH-PGE_2 (Ciccoli *et al.,* 2005). Interestingly, with the addition of ASA, up to 95% of infectious biofilms formed by *C. albicans* were inhibited *in vitro* (Alem & Douglas, 2004). According to Ciccoli *et al.* (2005), in order to control this infection, ASA is added to inhibit β-oxidation in the pathogen and to target the COX-2 enzyme in the host cell. In addition, the mechanism behind the inhibition of β-oxidation by ASA metabolites was studied in skin fibroblasts in Reye's syndrome and control patients. Results indicated that the ASA-sensitive β-oxidation reaction is the conversion of 3-hydroxyacyl CoA to 3-ketoacyl CoA by 3-hyroxyacyl-CoA dehydrogenase (Glasgow *et al.,* 1999). Since ASA inhibits both 3*R*-HETE and COX-2-produced 3-OH prostaglandins, research suggests new targets for the control of yeast infection.

It was revealed that 3-OH oxylipins can also be associated with vegetative cells of other yeasts. During the growth cycle of the brewing yeast, *Saccharomyces cerevisiae,* 3-OH 8:0 and 3-OH 10:0 are produced in association with "sticky" ornamentations on the surfaces of flocculating cells (Kock *et al.,*

2000). This suggests a possible involvement of these compounds in cell flocculation. At the start of flocculation, wrinkled cell surfaces produce these protuberances or "sticky" ornamentations. Transmission electron microscopic studies revealed that they consist of osmiophilic layers that migrate through the cell walls in a "ghost-like" fashion, without visually damaging the cell wall structure (Kock *et al.,* 2000). This seems to be a prerequisite for flocculation (cell adherence) since it causes the binding of these osmiophilic layers to the cell walls of adjacent cells. In addition, further studies revealed a link between flocculation and 3-OH 8:0 produced in strains of *Sacch. cerevisiae*. With the addition of 1 mM ASA, the production of 3-OH 8:0 was totally inhibited and a 30% reduction in flocculation was observed. These findings could assist to partially control yeast flocculation and help reduce costs involved with centrifugation during the brewing process (Strauss *et al.,* 2005). Studies on *Saccharomycopsis malanga* revealed the presence of 3-OH 16:0 which formed thread-like micelles (Sebolai *et al.,* 2001). Micellar threads, characterized by an osmiophilic-hydrophilic outer layer and hydrophobic inner layer, were found to link aggregating vegetative cells of this yeast. This further illustrated the adhesive role that 3-OH oxylipins play in yeasts when present in a polar medium. In addition, a whole cascade of even and uneven carbon numbered as well as saturated and unsaturated 3-OH oxylipins was discovered in *Saccharomycopsis synnaedendra* (Sebolai *et al.,* 2004).

In 2004, it was reported that some ascomycetous yeasts produce ascospores (resembling needles, corkscrews, walnuts, hairy or warty balls and hats) with curiously shaped nano-scale ornamentations that was found to be coated with 3-OH oxylipins (Kock *et al.*, 2004). Interestingly, hydroxy oxylipins are today used in high-quality motor oils and lubricants (Johnson, 1999). Could these compounds have a similar function on the surface ornamentations of ascospores? Consequently, this research may find application in nano-, aero- and hydrotechnologies. Furthermore, these studies indicate that amongst teleomorphic fungi, 3-OH oxylipins are highly conserved and may have some taxonomic value due to its potential to be used as taxonomic markers for yeast identification (Kock *et al.,* 2003).

Interestingly, oxylipins have been mentioned in the switch between sexual and asexual reproductive growth and dimorphism in yeasts (Kock *et al.,* 2003) and filamentous fungi (Noverr *et al.,* 2003). In 2005, the first genetic evidence for prostaglandin production by fungi was provided. Three

dioxygenase-encoding genes (*ppoA, ppoB* and *ppoC*), produced by *Aspergillus nidulans*, was discovered. The genes are involved in prostaglandin production, virulence and integration of the sexual and asexual development of this filamentous fungus (Tsitsigiannis *et al.*, 2005). These studies indicate the possible role of oxylipins as regulators in sexual and asexual spore formation in *Aspergillus nidulans* (Tsitsigiannis *et al.*, 2005).

1.4. Purpose of research

In 2003, Smith & co-workers found that novel 3-OH oxylipins are associated with the sheathed ascospores of some species representing the genus *Dipodascus*. However, these compounds have not yet been studied in detail in this genus. In addition, no thought was given to the mystery behind the release mechanics of sheathed ascospores from enclosed bottle-shaped asci and the role of these 3-OH oxylipins during dispersal. With this information as background, it thus became the aim of this study to:

1. determine 3-OH oxylipin structure, distribution and function in *D. albidus* and *D. geniculatus* and to
2. reveal the secrets behind the release mechanics of sheathed ascospores from bottle-shaped asci in these two species.

Please note: The chapters to follow are presented in the format required by the journal of submission. As a result repetition of some information could not be avoided.

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CHAPTER 2

Ascospore release from bottle-shaped asci in *Dipodascus albidus*

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The candidate performed preliminary studies during her B.Sc. Honours in 2004. After additional work during her M.Sc. study in 2005, this section was published and also included with permission in this study. Part of the work (Fig. 2.) is presented on the cover page of all 2006 FEMS Yeast Research issues. All the work presented was performed by the candidate.

Abstract

Yeasts utilize different mechanisms to release ascospores of different lengths from bottle-shaped asci. Using electron microscopy, confocal laser scanning microscopy, gas chromatography-mass spectrometry and digital live imaging, the individual release of oval ascospores from tight-fitting narrow bottle-necks, is reported in the yeast *Dipodascus albidus*. These ascospores are surrounded by compressible, oxylipin-coated sheaths enabling ascospores to slide past each other when forced by turgor pressure and by possible sheath contractions towards the narrowing ascus-neck. In this paper, the release mechanisms of ascospores of various lengths from bottle-shaped asci and produced by different yeasts are compared. We suggest that different release mechanisms, utilizing compressible sheaths or geared-alignment, have possibly evolved to compensate for variation in ascospore length. Alternatively, sheaths and ridges might be two evolutionary solutions to the same biomechanical problem, i.e. to release ascospores irrespective of length from bottle-shaped asci.

2.1. Introduction

In 1991, we discovered the first aspirin-sensitive oxylipins in yeasts [1,2]. Since then studies by various research groups have demonstrated the ubiquitous nature of these compounds in yeasts and their importance as target to control fungal infections [1,3-6]. We recently exposed another feature of fungal oxylipins [7]. In some yeasts oxylipins, such as 3-hydroxy oxylipins, were found to act as lubricants during ascospore release from enclosed asci [7]. This research opened new views on ascospore movement in micron-space, which may find application in nano-, aero- and hydro-technologies [7].

Microscopic studies revealed that representatives of the yeast genus *Dipodascopsis* and some *Dipodascus* species produce bottle-shaped asci with a broad base and narrow neck, containing ascospores of various shapes (round, oval, or elongated) with surface ornamentations (compressible sheaths or surface ridges linked in gear-like manner) [7,8]. Each yeast species produces only one kind of ascospore structure. These morphological differences may influence the type of release mechanism used by a particular species to force ascospores, probably by turgor pressure, through tight-fitting ascus openings without blocking the ascus tip [7-9]. This is in accordance with the literature where it

has been reported that many ascomycetous fungi release their ascospores forcibly from asci through osmotic or turgor pressure [10].

In *Dipodascus aggregatus,* round to oval-shaped ascospores are enveloped in oxylipin-coated compressible sheaths [7]. These sheaths enable ascospores to slide past each other when reaching the narrowing ascus neck. However, more elongated ellipsoidal to reniform ascospores of *Dipodascopsis uninucleata* var. *uninucleata* are released differently [7-9]. Here, the elongated ascospores remain aligned within the bottle-shaped ascus before release. Otherwise, we believe they might turn sideways, thereby blocking the ascus-neck and eventually inhibiting individual ascospore release. These ascospores do not contain sheaths, but are linked by means of interlocked ridges on the surfaces of neighboring ascospores, thereby keeping them aligned while being pushed towards the ascus-tip. It is proposed that 3-hydroxy oxylipins also assist in this release mechanism by acting as a lubricant between ascospores [7-9,11].

This study explores the secret behind the release mechanism of oval-shaped ascospores from bottle-shaped asci in the yeast *Dipodascus albidus*. These findings are compared with possible mechanics involved in effective release of ascospores of different lengths from similarly shaped asci.

2.2. Materials and methods

2.2.1. Strains and cultivation

Dipodascus albidus UOFS Y-1445T, *Dipodascus aggregatus* UOFS Y-1358 and *Dipodascopsis uninucleata* var. *uninucleata* UOFS Y-128 were used in this study.

These strains are held at the University of the Free State, Bloemfontein, South Africa. The yeasts were streaked on yeast malt agar [12] and cultivated at room temperature for 2-10 days until sporulation was observed. All experiments were performed at least in duplicate.

2.2.2. Asci and ascospore measurements

The dimensions (diameter and length) of one hundred ascospores within various asci of *Dipodascus albidus, Dipodascus aggregatus* and *Dipodascopsis uninucleata* var. *uninucleata* were measured using a micrometer fitted to a light microscope. Subsequently, the ratios of ascospore

diameter:length were calculated. Since the release mechanisms of *Dipodascus aggregatus* and *Dipodascopsis uninucleata* var. *uninucleata* have been studied previously [7], only the ascospore release mechanism of *Dipodascus albidus* was further investigated.

2.2.3. Ascospore release studies in Dipodascus albidus

To illustrate release of individual ascospores from asci tips, asci with ascospores (unstained and stained according to [13]) were studied by light microscopy. Photographs and a movie showing active ascospore release were taken using an Axioplan light microscope (Zeiss, Göttingen, Germany) coupled to a Colorview Soft Imaging System (Münster, Germany).

2.2.4. Immunofluorescence microscopy of Dipodascus albidus

Antibodies against chemically synthesized 3*R*-hydroxy-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid [14] were raised in rabbits and then characterized by determining their sensitivity, titer and specificity [11].

Immunofluorescence microscopy was performed as described [11]. Cells (5 mgl^{-1}) were suspended in 100 ml phosphate buffer solution and centrifuged for 10 min to remove debris and agar. Next, 30 µl of primary antibody was added to the cells and incubated for 60 min to allow sufficient binding to the oxylipins. After washing with phosphate buffer solution, fluorescein isothiocyanate (FITC) secondary antibodies (Sigma, St. Louis, MO, USA) were added and incubated in the dark for 30 min to allow sufficient binding to the primary antibody. To ensure that aggregated ascospore structure was maintained, antibody, fluorescence and wash treatments were performed in 2-ml plastic tubes. The cells were fixed on a microscope slide and photographed using a Nikon 2000 Confocal Laser Scanning Microscope (Nikon, Tokyo, Japan).

2.2.5. Orange-G staining

Cells from a 10-day-old culture of *Dipodascus albidus* were stained with 1% Orange G (Molecular Probes, Eugene, OR, USA) for 5 min at room temperature. After staining, cells were rinsed twice with distilled water. Micrographs were obtained using a Nikon 2000 Confocal Laser Scanning Microscope.

2.2.6. Electron microscopy

Cells from a 10-day-old culture of *Dipodascus albidus* were chemically fixed, using 3% glutaraldehyde (Merck, Darmstadt, Germany) and 1% osmium tetroxide (Merck) [15]. These cells were dehydrated by a graded ethanol series, followed by drying using a critical-point dryer. The specimen was made electron-conductive by mounting the sample on a stub and coating it with gold. Scanning electron micrographs were taken with a Jeol 6400 WINSEM (Jeol, Tokyo, Japan).

2.2.7. 3-Hydroxy oxylipin extraction and derivatisation

Cells of *Dipodascus albidus* were harvested and suspended in 100 ml distilled water, after which the pH was decreased to <4 using 3% formic acid (Merck). Oxylipins were extracted and dissolved using 200 ml ethyl acetate (Merck). The organic and water phases were allowed to separate, after which the organic phase was evaporated using N_2 gas (AFROX, Bloemfontein, South-Africa). The sample was methylated and silylated using diazomethane [Prepared from Diazald (Aldrich, Schnelldorf, Germany)] and bis-(trimethylsilyl) trifluoroacetamide (Merck). Finally, the sample was dissolved in 400 µl chloroform:hexane (4:1) (Merck, Germany) and injected into the gas chromatograph–mass spectrometer for chemical analysis [16].

2.2.8. Gas chromatography–mass spectrometry

A Finnigan Trace Ultra gas chromatograph (San José, CA, USA) with an HP-5-60 m fused silica capillary column (0.23 µm i.d. and 0.1 µm coating thickness), equipped with a Finnigan Trace DSQ MS-MS was used. Helium was used as a carrier gas at a constant flow of 1 ml min^{-1}. The initial oven temperature of 110 °C was held for 2 min before it was increased with 5 °C min^{-1} to a final temperature of 280 °C. The mass spectrometer was auto-tuned to *m/z* 50-500. A sample volume of 1 µl was injected at an inlet temperature of 230 °C at a split ratio of 1:50.

2.3. Results

2.3.1. Ascus and ascospore morphology of Dipodascus albidus

Using light microscopy on unstained and stained ascospores [13] it was observed that the ascus of *Dipodascus albidus* is typically bottle-shaped with a narrow neck at the tip and a broader bottom

base. Asci usually contain more than a hundred ascospores, which are forced through the ascus tip. Upon individual release, the ascospores aggregate in clusters outside the ascus (Fig. 1). Similar ascus-shapes were reported previously for *Dipodascus aggregatus* [17].

The ascospore shape and surface structure were investigated using confocal laser scanning microscopy on Orange-G-stained cells (Fig. 2). By measuring the dimensions of ascospores, it was found that ascospores of *Dipodascus albidus* are oval to ellipsoidal (ascospore diameter:length = 1:1.5–2.0), and each ascospore was enveloped in a sheath of approximately 1 µm in diameter. These structures were confirmed using scanning electron microscopy (Fig. 3). However, the sheaths formed ridges due to dehydration during preparation for electron microscopy studies, implicating the presence of water in these compressible enveloping matrices.

2.3.2. Oxylipin distribution in Dipodascus albidus

Using 3-hydroxy oxylipin-specific antibodies coupled to a fluorescing compound (FITC anti-IgG), it was possible to map the distribution of 3-hydroxy oxylipins through confocal laser scanning microscopy. In Fig. 4, aggregated ascospores (cross-section view) are observed in red (auto-fluorescence), while oxylipins are shown by fluorescent green, indicating the presence of the oxylipins on the surface of the compressible sheaths. Only the outer surface fluoresced, since oxylipin antibodies could not penetrate the aggregating ascospore mass.

The presence of 3-hydroxy oxylipins, as observed with confocal laser scanning microscopy, was confirmed by gas chromatography–mass spectrometry analysis. The ion chromatogram indicated that the 3-hydroxy oxylipin eluted at around 9 min (Fig. 5). The mass spectrum showed a characteristic peak of *m/z* 175 indicating the presence of a hydroxyl group on carbon-3 (Fig. 6). However, a chemical structure could not be fitted to the mass spectrum. A similar unique spectrum has been reported in other *Dipodascus* species [18] and could probably be a conserved characteristic of this genus. Is it possible that this compound acts as a lubricant assisting the release of ascospores from asci, as was observed in *Dipodascopsis uninucleata* [7-9]? Future oxylipin inhibition studies should clarify this.

2.3.3. Ascospore release mechanism in Dipodascus albidus

By using digital live imaging, the ascospore release mechanism in *Dipodascus albidus* was assessed (Movie 1, Supplementary data). Here, ascospore traffic, moving in single file, is illustrated. The characteristic compressible sheath is clearly visible as a light halo surrounding each ascospore. It is suggested that ascospores, while being pushed by turgor pressure [10], slide past each other using their compressible sheaths to effect unhindered individual release from the narrow ascus-tip. In addition to osmotic pressure pushing these ascospores, it is also possible that the sheaths are capable of exerting pressure against the inner surface of the ascus wall that might well assist in pushing the spores towards the ascus bottle-neck. This might be achieved by the repetitive swelling and contraction of the sheaths through hydration and dehydration, respectively. Our scanning electron microscopy observation on sheath dehydration demonstrates that this structure may act as a gel that expands upon hydration (Fig. 3). In other words, the sheath swells as water enters its matrix, and contracts as it dehydrates.

2.4. Discussion

From the literature [7] and results reported here, it is clear that yeasts with round and oval to elongated ascospores (ascospore dimensions for *Dipodascus aggregatus*, 1:1-1.3; *Dipodascus albidus*, 1:1.5-2.0; *Dipodascopsis uninucleata* var. *uninucleata*, 1:2.5-) have different mechanisms for release from similar bottle-shaped asci (Fig. 7). Based on studies of the yeasts up to now, sheaths are used to assist in ascospore release when ascospores are round to oval in shape (ascospore dimensions: 1:1-2.0). Here, sheaths that envelope ascospores (about 1 μm in diameter, SE < 5%) assist in their release by allowing ascospores to slide past each other when reaching the bottle-neck (Fig. 7; 1(a)-(d), 2(a)-(c), 3(a)-(c)). If this ratio increases to 1:2.5 and more (Fig. 7; 4(a)-(d)), it appears that these ascospores, when sheathed, would probably not be released since they may turn sideways inside the ascus, thereby blocking their release. To evade this problem, elongated ascospores might have developed ridged surfaces that are interlocked in gear-like fashion [7-9], affecting alignment with the ascus-tip for unhindered individual release (Fig. 7; 5(a)-(d)).

The following questions can now be asked: (i) Have different mechanics of ascospore release evolved to compensate for variation in ascospore length? (ii) Is there a "cut-off" point where an evolutionary "decision" was made to utilize either sheaths or alignment? One should, however, bear in mind that sheaths and ridges may represent two evolutionary solutions to the same biomechanical problem and, therefore, may not be related to spore length. In this case it may be that sheathed lubricated spores, irrespective of their length, may slide easily through the bottle-neck by taking the path of least mechanical resistance, independent of their orientation within the broad base of the ascus. More insight may be obtained when appropriate mathematical and mechanical modeling, simulating these ascospores, their movement and ascus dimensions, are attempted for a large spectrum of yeasts characterized by bottle-shaped asci containing a large variation in ascospore lengths.

Future research should address this issue. The importance of oxylipins and the various ascospore shapes and ornamentations found in yeasts [7,13] will become clearer as investigations progress into this field of study, unlocking the secrets behind these fascinating release mechanisms. Consequently, the present results urge comprehensive comparative studies on mechanisms of ascospore release in fungi.

2.5. Acknowledgements

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2.6. Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version at doi:10.1016/j.femsyr.2005.04.010. or on the CD provided at the back of this dissertation.

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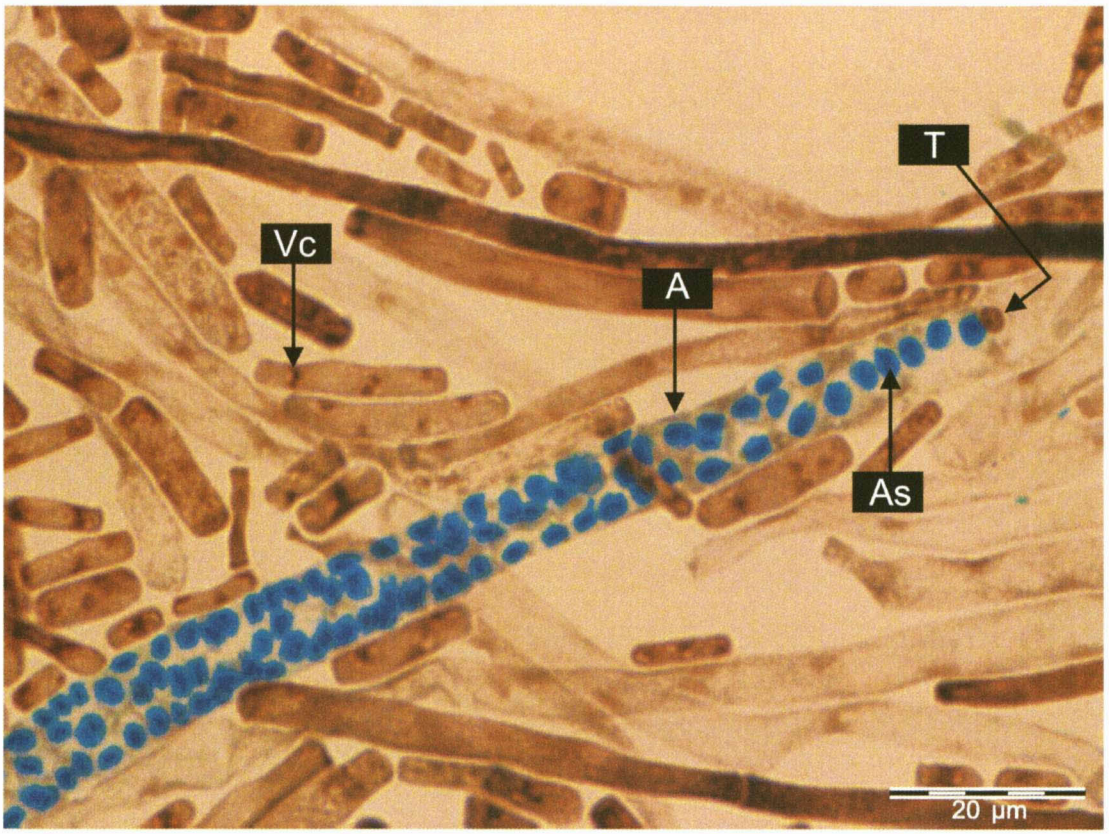

Fig. 1. A light micrograph of *Dipodascus albidus* showing stained ascospores within a bottle-shaped ascus. The vegetative cells (Vc) stained red while the ascospores (As) stained blue inside the ascus (A). Some of the ascospores are ready to be released individually from the narrow bottle-neck tip (T).

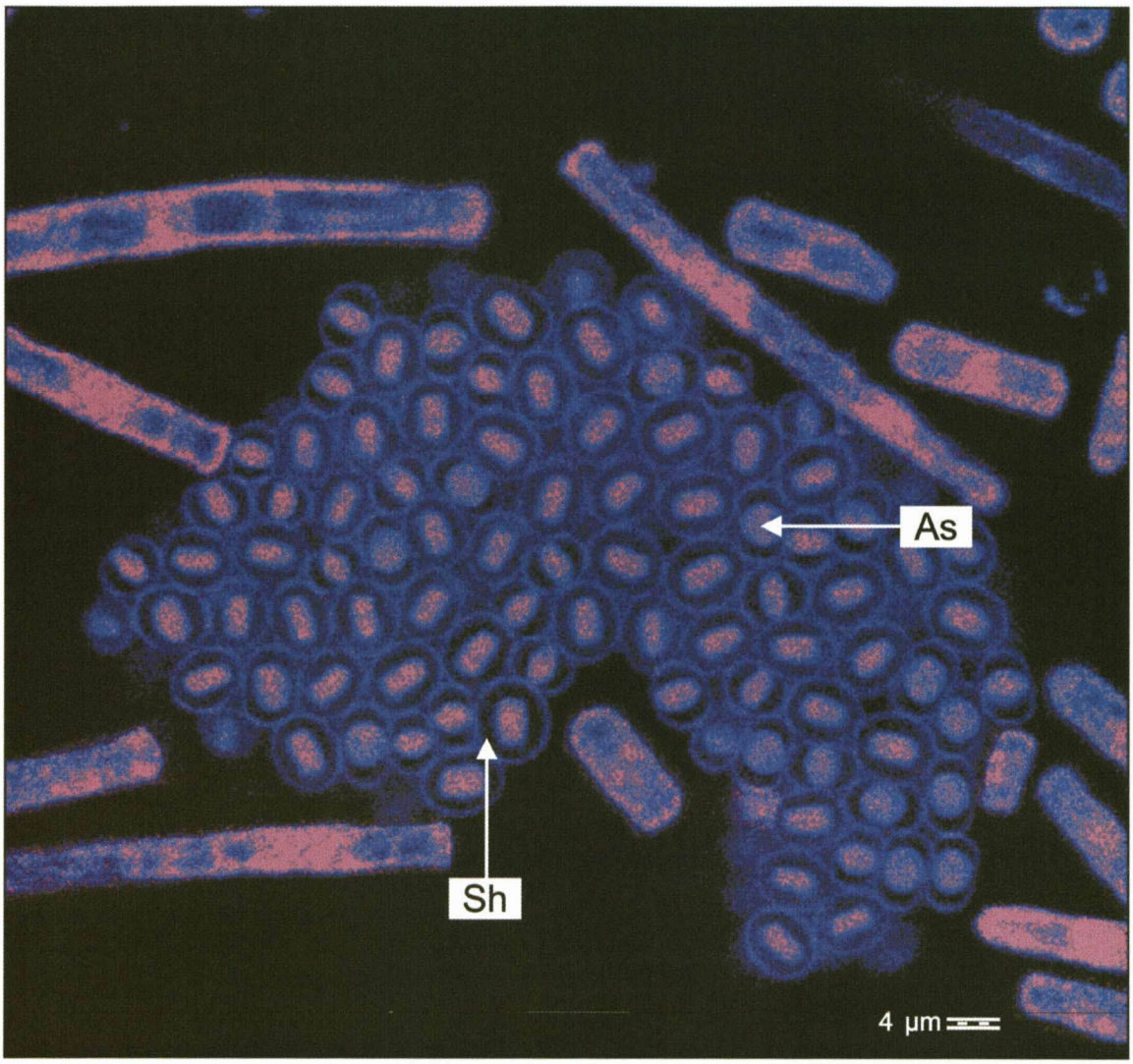

Fig. 2. A confocal laser scanning micrograph of Orange-G-stained cells of *Dipodascus albidus*. Ascospores (As) stained pink-blue while the compressible sheaths (Sh) remained black.

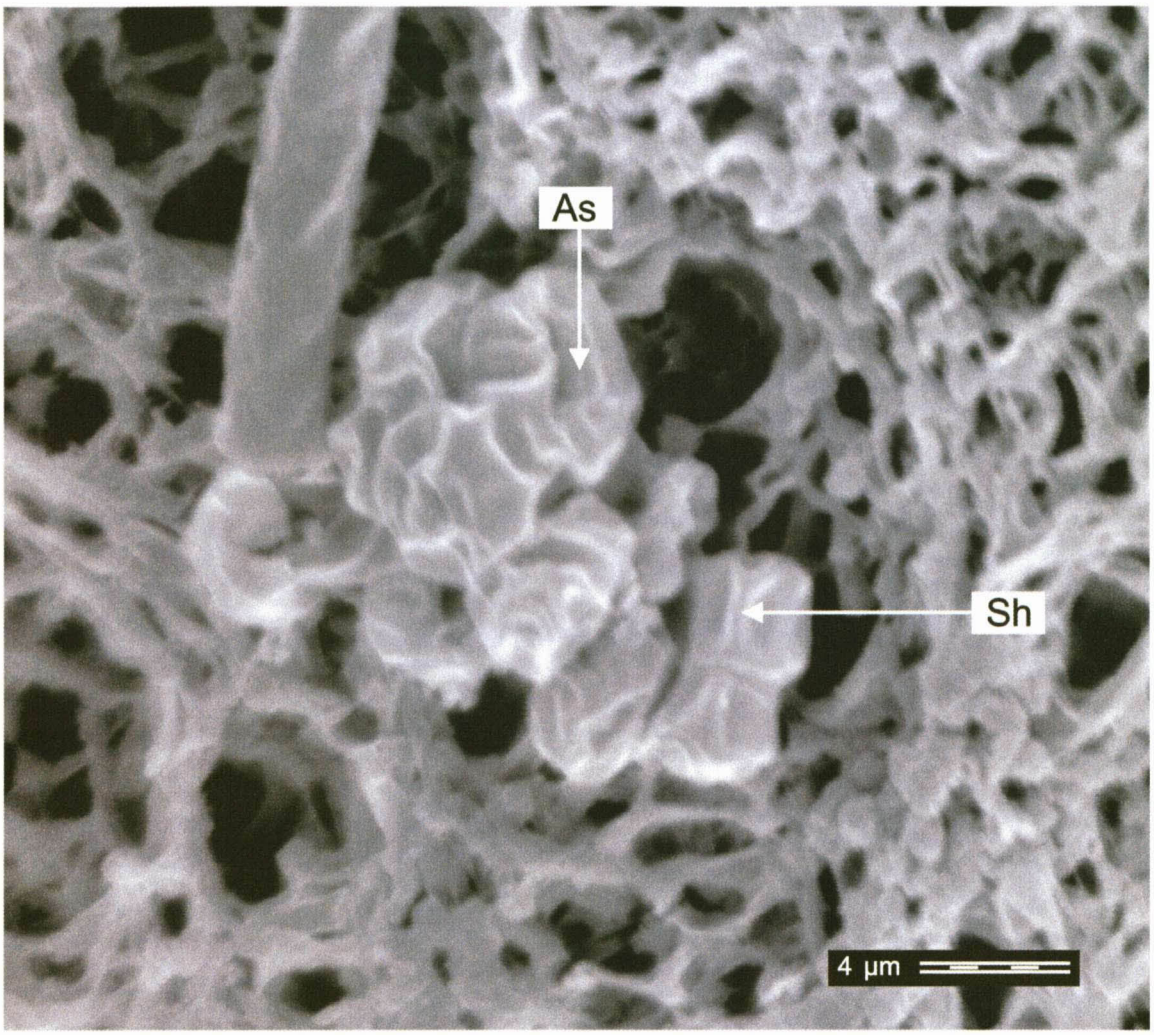

Fig. 3. A scanning electron micrograph of *Dipodascus albidus* revealing ascospores (As) with their associated, in this case dehydrated-ridged sheaths (Sh).

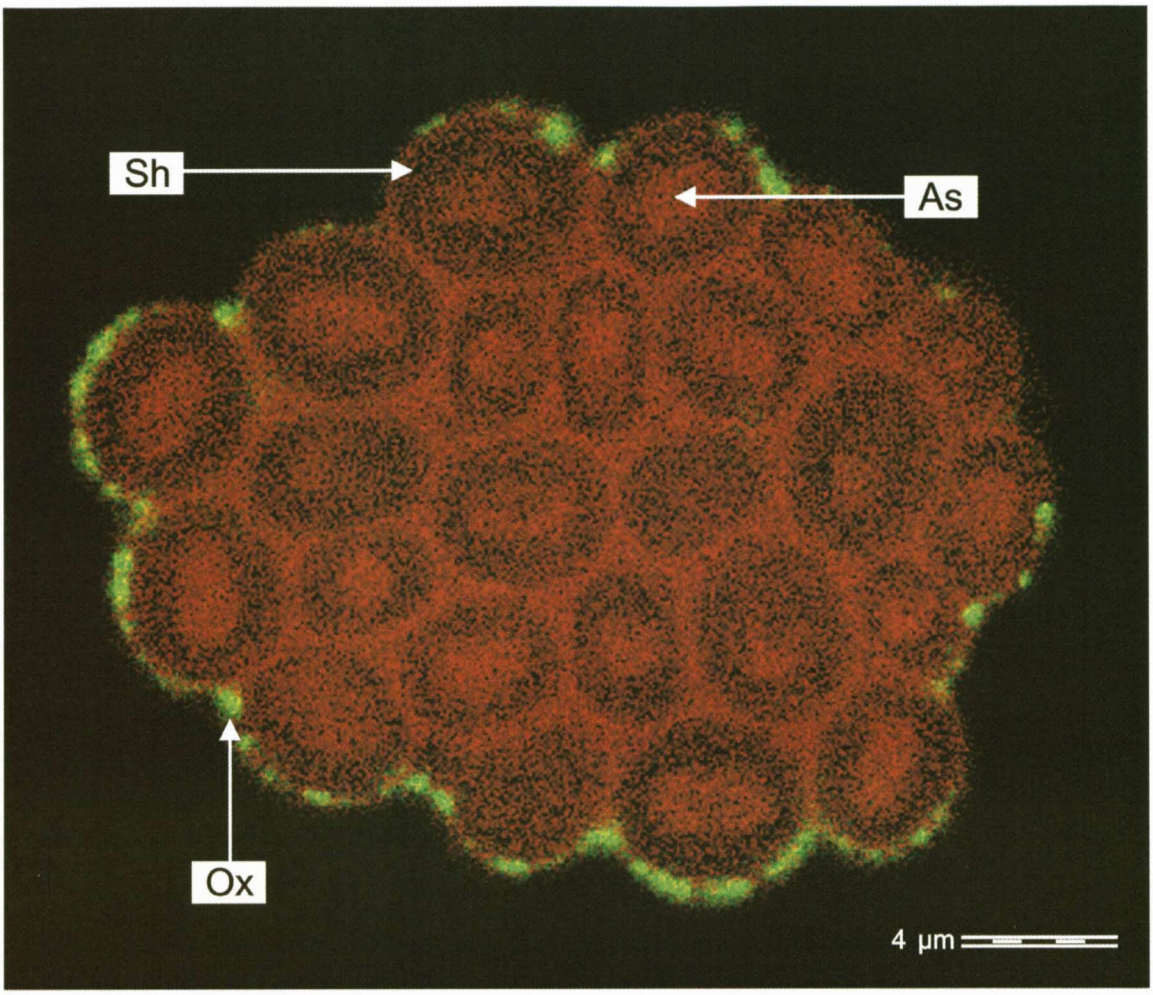

Fig. 4. Confocal laser scanning micrograph (cross-sectional view) of aggregated released ascospores (As, in red) and characteristic sheath (Sh, in darker black red) of *Dipodascus albidus*. 3-Hydroxy oxylipins (Ox) are shown as fluorescent green on the outside surface of the aggregating compressible sheaths.

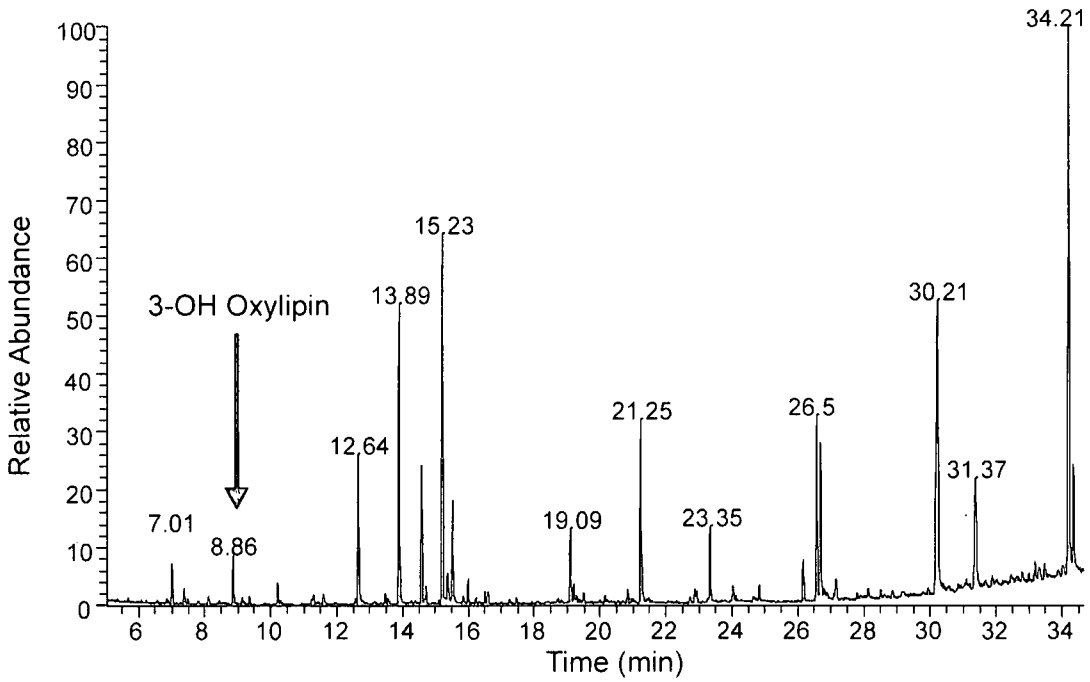

Fig. 5. A total ion chromatogram obtained through gas chromatography-mass spectrometry analysis of the derivatized lipid extract of *Dipodascus albidus*, showing a 3-hydroxy oxylipin eluting at around 9 min.

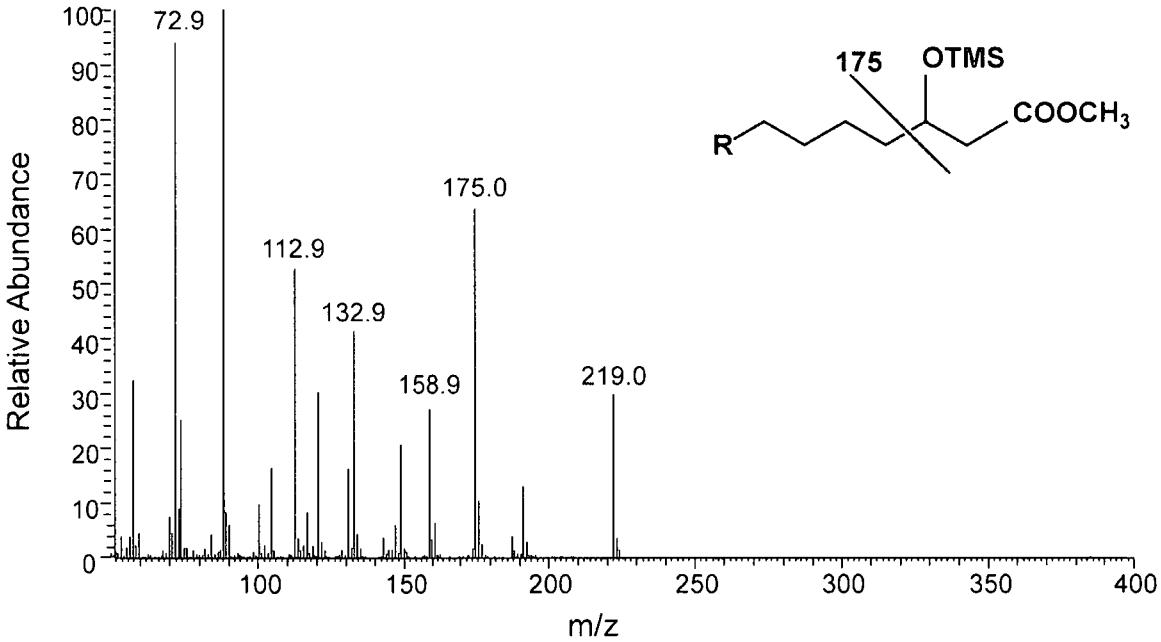

Fig. 6. The mass spectrum obtained through gas chromatography-mass spectrometry analysis of the 3-hydroxy oxylipin in *Dipodascus albidus,* indicating a characteristic peak of *m/z* 175. This is indicative of a 3-hydroxyl group on carbon-3 of the fatty acid.

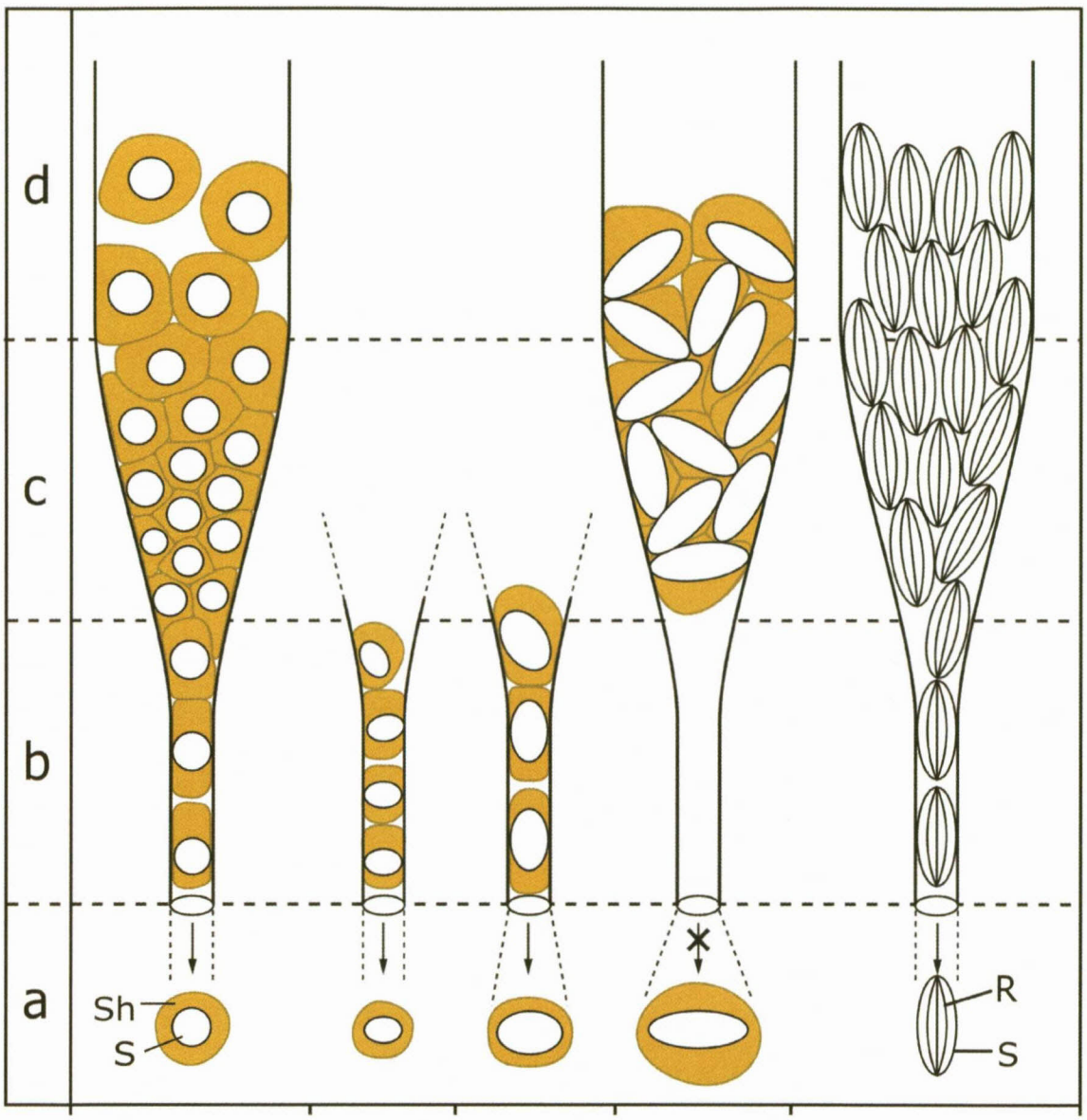

Fig. 7. A diagrammatic representation of ascospore-traffic (release) through bottle-shaped asci (row (b), bottle-neck; row (c), funnel; row (d), broad base) as observed by micorscopy. Round to oval-shaped ascospores (S, diameter:length = 1:1, 1:1.3 and 1:1.5-2.0) as observed for *Dipodascus aggregatus* (columns 1 and 2) and *Dipodascus albidus* (column 3) are enveloped in compressible sheaths (Sh, approximately 1 µm thick), enabling spores to slide past each other for unhindered individual release. Elongated ascospores, as observed for *Dipodascopsis uninucleata* var. *uninucleata* (column 5) are naked and aligned via surface ridges (R) in gear-like fashion for unhindered release [7]. We suggest, as one possible model (see text), that if elongated ascospores were sheathed (column 4) they would not be remain aligned and might easily turn sideways, thereby blocking ascospore release.

CHAPTER 3

The release of elongated, sheathed ascospores from bottle-shaped asci in *Dipodascus geniculatus*

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All the work presented was performed by the candidate.

Abstract

Yeasts use different mechanics to release ascospores of different lengths from bottle-shaped asci. Round to oval-shaped ascospores are enveloped in oxylipin-coated compressible sheaths enabling ascospores to slide past each other when reaching the narrowing ascus neck. However, more elongated ascospores do not contain sheaths, but are linked by means of oxylipin-coated interlocked hooked ridges on the surfaces of neighbouring ascospores, thereby keeping them aligned while being pushed towards the ascus-tip by turgor pressure. In this study, we uncovered elongated, oxylipin-coated sheathed ascospores in *Dipodascus geniculatus* that are released effectively from bottle-shaped asci without alignment. This is possible because the ascus neck and opening have the same diameter as the length of the ascospore, thus allowing the ascospores to turn sideways without blocking the ascus upon release. We found that increased concentrations of acetylsalicylic acid, inhibit both ascospore release and 3-hydroxy oxylipin production in this yeast thereby implicating this oxylipin in sexual reproduction.

3.1. Introduction

The presence of 3-hydroxy (3-OH) oxylipins in yeasts and their role in infections is well established (Van Dyk *et al.*, 1991; Kock *et al.*, 1998; Kock *et al.*, 1999; Deva *et al.*, 2000; Deva *et al.*, 2001; Kock *et al.*, 2003; Noverr *et al.*, 2003; Smith *et al.*, 2003; Deva *et al.*, 2004; Kock *et al.*, 2004; Ciccoli *et al.*, 2005; Van Heerden *et al.*, 2005). It was reported that these intermediates and products of incomplete β-oxidation are necessary for ascospore release and dispersal from asci (Kock *et al.*, 2004) by acting as lubricants. Strikingly, when acetylsalicylic acid (ASA) was added to these yeasts, 3-OH oxylipin production was inhibited which in turn impaired ascospore release (Kock *et al.*, 1999; Kock *et al.*, 2003; Kock *et al.*, 2004). This research opened new perspectives on ascospore movement which may find application in nano-, aero- and hydro- technologies (Kock *et al.*, 2004).

From literature, it is reported that yeasts with round and oval to elongated ascospores (ascospore dimensions as ratio of diameter:length = 1:1-1.3 for *D. aggregatus*; 1:1.5-2.0 for *D. albidus*;

1:2.5 and more for *Dipodascopsis uninucleata* var. *uninucleata*) have different release mechanisms from similar bottle-shaped asci (i.e. with a broad bottom part and a narrow bottle neck, releasing one ascospore at a time) (Van Heerden *et al.*, 2005). It was demonstrated that sheaths are used to assist in ascospore release when ascospores are round to oval in shape (ascospore dimensions: 1:1-2.0). These studies concluded that compressible sheaths of 1 µm in diameter envelop ascospores and assist in their release by allowing ascospores to slide past each other when reaching the bottle-neck. It is possible that, in combination with turgor pressure, forces generated by sheath expansion through hydration, drive the ascospores from the asci. If this ratio increases to 1:2.5 and more, these ascospores, when sheathed, would probably turn sideways inside the ascus thereby blocking their release. To solve this problem, elongated ascospores have developed hooked ridged-surfaces along spore length that are interlocked in gear-like fashion (Kock *et al.*, 1999; Kock *et al.*, 2004), affecting alignment with the ascus-tip for unhindered individual release under turgor pressure.

These studies prompted the following questions. Is it possible that different mechanisms of ascospore release evolved to compensate for variation in ascospore length, i.e. to ensure that ascospores, when too long, will not turn sideways thereby blocking the ascus? Is there a "cut off" point where sheaths or alignment are utilized? Do these different mechanics represent two evolutionary solutions to the same biomechanical problem and, may therefore be unrelated to ascospore length?

In this study, ascospore release in a strain of *D. geniculatus* was studied with these questions in mind. Strikingly, ascospores produced by this strain are sheathed (coated with a thin layer of oxylipins), elongated (ascospore dimensions: 1:2.0–2.7) and not aligned. How does this yeast manage to release the elongated oxylipin coated ascospores without blocking the bottle-shaped ascus? We also found that increased concentrations of ASA inhibit both ascospore release and 3-OH oxylipin production thereby implicating this oxylipin in sexual reproduction e.g. spore release.

3.2. Materials and methods

3.2.1. Strain used and cultivation

Dipodascus geniculatus UOFS Y-1144 was used in the study and is preserved at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Bloemfontein, South Africa. The yeast was streaked out on yeast malt agar (Wickerham, 1951) and cultivated at 25 ^{0}C in Petri dishes until sporulation was observed. Cells were then scraped off and subjected to the experiments to follow as described below.

3.2.2. *Ascospore measurements*

The dimensions (diameter and length) of one hundred ascospores within various asci of *Dipodascus geniculatus* were measured using a micrometer fitted to a light microscope. Subsequently, the ratios of diameter:length of the ascospores measured, were calculated.

3.2.3. *Ascospore release studies*

To illustrate the release of individual ascospores from asci tips, asci with ascospores were stained using malachite green and safranin (Yarrow, 1998) and Nile Red (Kimura *et al.*, 2004), respectively. Cells stained with malachite green and safranin (Merck, Gauteng, South Africa) were viewed with a Zeiss light microscope (Axioplan, Zeiss, Göttingen, Germany) coupled to a Colorview Soft Imaging System (Münster, Germany). In addition, cells stained with 10 % Nile Red (Sigma, Gauteng, South Africa) were viewed with a Nikon 2000 Confocal Laser Scanning Microscope (Nikon, Tokyo, Japan).

3.2.4. *Immunofluorescence microscopy*

Antibodies against chemically synthesized 3*R*-OH 5Z, 8Z, 11Z, 14Z eicosatetraenoic acid (Bhatt *et al.*, 1998; Groza *et al.*, 2002) were raised in rabbits and then characterized by determining their sensitivity, titer and specificity (Kock *et al.*, 1998). Immunofluorescence microscopy was performed as described previously (Kock *et al.*, 1998; Van Heerden *et al.*, 2005).

3.2.5. *Electron microscopy*

The protocol as described by van Heerden *et al.* (2005) was followed. Sporulating cells were chemically fixed, using 3% glutardialdehyde (Merck, Darmstadt, Germany) and 1% osmium tetroxide (Merck, Darmstadt, Germany) (Van Wyk & Wingfield, 1991). These cells were then dehydrated by a graded ethanol series, followed by drying using a critical point dryer. The specimens were mounted on stubs and coated with gold to make them electron conductive. Scanning electron micrographs were taken with a Jeol 6400 WINSEM (Jeol, Tokyo, Japan).

3.2.6. *3-OH oxylipin extraction and derivatisation*

These were performed as decribed by van Heerden *et al.* (2005). Cells were harvested and suspended in 100 ml dH_2O, after which the pH was decreased to 3.8 using 3 % formic acid (Merck, Darmstadt, Germany). Oxylipins were extracted and dissolved using 2 volumes of ethyl acetate (Merck, Darmstadt, Germany). The organic and water phases were allowed to separate, after which the organic phase was evaporated using N_2 gas (AFROX, Bloemfontein, South Africa). Extracts were derivatised (methylated and silylated), dissolved in 400 µl chloroform:hexane (4:1) (Merck, Darmstadt, Germany) and injected into the gas chromatograph – mass spectrometer (GC-MS) for chemical analysis (Venter *et al.*, 1997). All experiments were performed in at least duplicate.

3.2.7. *Gas chromatography – mass spectrometry*

A Finnigan Trace Ultra gas chromatograph (San Jose, CA, USA) with an HP-5-60 m fused silica capillary column (0.23 µm i.d. and 0.1 µm coating thickness), equipped with a Finnigan Trace DSQ MS-MS was used as described (Van Heerden *et al.*, 2005). Helium was used as a carrier gas at a constant flow of 1 mL min^{-1}. The initial oven temperature of 110 °C was held for 2 min before it was increased with 5 °C min^{-1} to a final temperature of 280 °C. The mass spectrometer was auto-tuned to

m/z 50-500. A sample volume of 1 μL was injected at an inlet temperature of 230 °C at a split ratio of 1:50.

3.2.8. Acetylsalicylic acid (ASA) inhibition studies

The yeast was streaked out on yeast malt agar (Wickerham, 1951) and cultivated at 25 ^{0}C in Petri dishes until sporulation was observed. Cells were then transferred into 250 mL conical flasks containing 50 mL of glucose-YM broth (10 g L^{-1} glucose, 5 g L^{-1} peptone, 3 g L^{-1} yeast extract, 3 g L^{-1} malt extract) and incubated at 25 ^{0}C while shaking (160 rpm) until late exponential phase was reached. Appropriate volumes were then transferred to several 500 mL conical flasks containing 20 mL of the same medium. ASA (Sigma, Steinheim, Germany) was first dissolved in a minimal volume ethanol and added to each individual flask at the start of cultivation to reach a final concentration of 0 mM (control), 1 mM, 2 mM, 3 mM, 4 mM and 5 mM, respectively. These cultures were incubated at 25 ^{0}C for 36 h. Since ASA had to be dissolved in minimum amounts of 98 % ethanol (Merck, Gauteng, South Africa), further control experiments containing similar amounts of ethanol without ASA were performed. Since it is not possible to quantify these asci with the aid of a counting chamber due to the extensive aggregation of hyphae and ascospores, the effect of different ASA concentrations on the sexual cycle was determined by counting up to 94 mature asci in four adjacent microscope fields of culture using a Zeiss light microscope. In each case the percentage empty asci (indicating ascospore release) were calculated. This experiment was repeated in triplicate. In addition, scanning electron micrographs of the asci were obtained using a Jeol 6400 WINSEM scanning electron microscope (Jeol, Tokyo, Japan). 3-OH oxylipins were extracted from the respective yeast cultures, derivatised, and analysed by gas chromatography – mass spectrometry as described (2.6 and 2.7). This experiment was performed in duplicate.

3.3. Results

3.3.1. Morphology

Using light microscopy to measure the shape and size of ascospores, we found these ascospores to be elongated (ascospore dimensions = 1:2.0-2.7) with each ascospore enveloped in a sheath of about 0.5 μm in diameter. Microscopy of unstained, as well as malachite green/safranin stained cells (Fig. 1) in their sexual cycle showed typical bottle shaped asci, each characterized by a broad base that tapers to form a thin ascus neck at the tip. These structures contained around 50 elongated, sheathed ascospores that are forced through the ascus tip upon release probably by turgor pressure in combination with sheath expansion through hydration. When released, these ascospores tend to aggregate. The asci are similar in shape to those reported for *Dipodascopsis uninucleata*, *Dipodascus aggregatus* and *Dipodascus albidus* (De Hoog *et al.*, 1998; Kock *et al.*, 2004; Van Heerden *et al.*, 2005).

Ascospores were further investigated using scanning electron microscopy (SEM) (Fig. 2). Similar results to that reported for *Dipodascus albidus* were obtained (Van Heerden *et al.*, 2005). The sheaths of aggregating ascospores again shrank, forming pronounced ridges due to dehydration during sample preparation. This implicates the presence of water in a gel-like structure present in these compressible sheaths.

3.3.2. Oxylipins

When 3-OH oxylipin-specific antibodies coupled to a fluorescing compound (FITC anti-IgG; Sigma, St. Louis, MO, USA) were added to the cells in their sexual cycle, it was possible to map the distribution of 3-OH oxylipins through confocal laser scanning microscopy. Our results suggest that, as reported in *D. albidus* (Van Heerden *et al.*, 2005), 3-OH oxylipins are mainly associated as a thin layer coating the surfaces (sheaths) of the ascospore clusters. This may be ascribed to the fact that these

antibodies could not penetrate the aggregating clusters. Subsequently, the presence of 3-OH oxylipins was confirmed using gas chromatography - mass spectrometry. The mass spectrum of the oxylipin showed a characteristic peak of *m/z* 175 which is characteristic of a hydroxyl group on carbon 3 (Fig. 3). The complete chemical structure however could not be fitted to the mass spectrum. The same mass spectrum, implicating the presence of the same 3-OH oxylipin structure, was found to be present in other *Dipodascus species* (Smith *et al.*, 2003; Van Heerden *et al.*, 2005).

3.3.3. Acetylsalicylic acid (ASA) inhibition studies

When ASA, a known 3-OH oxylipin production inhibitor, dissolved in minimum amounts of ethanol, was added at different concentrations to the yeast culture medium, ascospore release and oxylipin production were impaired with an increase in ASA concentration (Table 1, Fig. 3). The addition of corresponding amounts of only ethanol yielded similar results as the untreated control. In the absence of ASA, 76 % of the observed matured asci contained well developed ascospores while 24 % were empty and had already released their ascospores. The effect of increasing concentrations of ASA on ascospore release became pronounced after the addition of 3 mM or more ASA (Table 1). At 5 mM ASA, no empty asci could be observed. Interestingly, high concentrations of ASA also inhibited 3-OH oxylipin production. At 1 mM ASA, 3-OH oxylipins were still detectable, but at 5 mM ASA, no 3-OH oxylipins could be detected (Table 1; Fig. 3). Since 3-OH oxylipins were present in only minute amounts, accurate quantification was not possible. In the presence of 5 mM ASA, some asci (<1% of asci observed) were malformed compared to the untreated control, whereas in the presence of 1 – 4 mM ASA visibly normal asci developed from two gametangia that were filled with ascospores (Fig. 4 a,b).

3.3.4. Ascospore release

After staining sporulating cells (grown on solid medium) with Nile Red and examining these with confocal laser scanning microscopy (CLSM), the secret behind the release of sheathed elongated

ascospores from bottle-shaped asci was exposed (Fig. 5). From the micrograph it is clear that the elongated ascospores are not aligned as was observed in *Dipodascopsis uninucleata* (Kock *et al.*, 1999), but turned sideways in the ascus neck before release from the ascus tip. Here, to ensure ascospore release from bottle-shaped asci, the ascospore length is similar to the diameter of the ascus neck and opening (about 3 μm).

3.4. Discussion

Literature suggests that fungi use different mechanics to release and launch spores into the environment (Fischer *et al.*, 2004; Pringle *et al.*, 2005). In *Dipodascus* and *Dipodascopsis*, different release mechanisms utilizing compressible sheaths or geared-alignment respectively, have possibly evolved to compensate for variation in ascospore length (Fig. 6).

Using light microscopy and CLSM, the individual release of ascospores from necks of bottle-shaped asci has been studied in *Dipodascus geniculatus*. Here, ascospores are surrounded by compressible sheaths enabling ascospores to slide past each other when forced by probably turgor pressure in combination with sheath expansion towards the ascus neck. Strikingly, these ascospores are elongated (ascospore dimensions = 1: 2.0-2.7) and of similar dimensions as the aligned geared ascospores found in *Dipodascopsis uninucleata*. How is it then possible for these spores to be released without alignment inside the ascus? If these elongated ascospores turn sideways inside the ascus upon release, they will block the ascus bottle-neck and subsequent ascospore release (Fig. 6, column 4).

The solution to this puzzle was found when cells of *Dipodascus geniculatus* in their sexual cycle were stained with Nile Red and examined with CLSM. Strikingly, the diameter of the ascus bottle-neck was similar to the ascospore length and not ascospore diameter as found in *Dipodascopsis uninucleata*, *Dipodascus aggregatus* and *Dipodascus albidus* (Kock *et al.*, 1999; Van Heerden *et al.*, 2005). Therefore, when these elongated ascospores turn sideways, the ascus should not be blocked (Fig. 6). We conclude that alignment of elongated ascospores is not necessary if the ascus bottle-neck

is wide enough to allow elongated ascospores to turn sideways without blocking ascospore release. All that is needed are compressible, lubricated sheaths to affect unhindered release.

As reported previously for *D. albidus* (Van Heerden *et al.*, 2005), thin layers of oxylipins were found to coat the surfaces of sheathed ascospores of *D. geniculatus*. These probably act as lubricants during spore release (Kock *et al.*, 2004). Furthermore, the addition of a 3-OH oxylipin production inhibitor (ASA), mainly inhibited the sexual cycle such as ascospore release as well as 3-OH oxylipin production (Fig. 3), thereby confirming the association of these oxylipins with sexual reproductive structures (e.g. on ascospore surface as observed with immuno CLSM). Results on ascospore release are similar to those found for *D. albidus, Dipodascopsis uninucleata* var. *uninucleata* as well as *Dipodascopsis tothii* (Botha *et al.*, 1992; Botha *et al.*, 1993; Van Heerden *et al.*, 2005).

It was however not possible to follow the inhibitory effect of ASA through immunofluorescence, since ASA at neutral pH (i.e. when inside the cell), cross-reacts with the 3-OH oxylipin primary antibody that was found to be specific for the first 3 carbons of 3-OH oxylipins (irrespective of length of side chain or degree of desaturation) (Kock *et al.*, 1998). This may be ascribed to structural similarities (i.e. first 3 carbons) between the ASA metabolite (i.e. salicylate that spontaneously forms at neutral pH) and 3-OH oxylipins (Glasgow *et al.*, 1999). It has been suggested that 3-OH oxylipins are probably produced in the mitochondria via incomplete β-oxidation (Deva *et al.*, 2000; Deva *et al.*, 2001; Deva *et al.*, 2004; Ciccoli *et al.*, 2005). It was also reported that ASA inhibits β-oxidation (Glasgow *et al.*, 1999; Deva *et al.*, 2001). Strikingly, ascosporogenesis in *Saccharomyces cerevisiae* occurs only in the presence of mitochondrial respiration (Marmiroli *et al.*, 1983). Consequently, it is now important to investigate if this observation is also true in *D. geniculatus* and other yeasts. It is generally believed that mitochondria most probably evolved from Gram-negative bacteria, i.e. rickettsias (Gray *et al.*, 2001), through endosymbiosis many millions of years ago. Strikingly, these bacteria also produce 3-OH oxylipins as part of their lipopolysaccharide layers (Amano *et al.*, 1998). The influence of different growth conditions on oxylipin production, variation in oxylipin structure and mode of ascospore release in different strains of the same species should be investigated to assess the conserved status of these phenotypic characteristics.

This study as well as others suggests that 3-OH oxylipins, usually present in minute amounts, may play a role as lubricant to facilitate ascospore release from enclosed asci. It is interesting to note that the oxylipin 12-OH oleic acid (12-OH 18:1) from castor bean oil is used today for the production of high quality lubricants for, amongst others, jet engines (Wood, 2001). What effect does the shifting of the hydroxyl group from carbon 12 to carbon 3 have on the lubricating properties of these oxylipins? The application of these lubricants produced by yeasts in everyday mechanics should also be assessed and compared to those available on the market. The feasibility of producing these incomplete β-oxidation intermediates/products with different levels of unsaturation in sufficient quantities through biotechnological (Venter *et al.*, 1997) and chemical processes (Bhatt *et al.*, 1998; Groza *et al.*, 2002), should now be investigated.

From literature (Van Heerden *et al.*, 2005) and our results, we hypothesize that different mechanisms of ascospore release from bottle-shaped asci evolved to compensate for variation in ascospore length ensuring that elongated spores will not turn sideways thereby blocking the ascus (Fig. 6). This is only true if the ascospore diameter is equal to the diameter of the ascus opening. If the ascus opening is equal to the length of the spore, then gear-like alignment is not necessary and sheaths may be used. Elongated sheathed ascospores may also be aligned for effective forced release if the ascus is narrow and tube-like thereby aligning these ascospores throughout the ascus in single-file as observed in *Dipodascus macrosporus* (Fig. 6) (De Hoog *et al.*, 1998).

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Table 1. The effects of increased concentrations of acetylsalicylic acid (ASA) on ascospore release and 3-OH oxylipin production.

ASA (mM)	Ascospore release (+/-SD)	3-OH oxylipins
0	23.7 +/- 1.5	+
1	25.0 +/- 1.0	+
2	26.4 +/- 2.3	+
3	21.3 +/- 3.8	t
4	12.7 +/- 3.8	t
5	0.0 +/- 0.0	-

Ascospore release = % asci without ascospores (empty); SD = standard deviation; t = trace amounts; + = presence; - = absence.

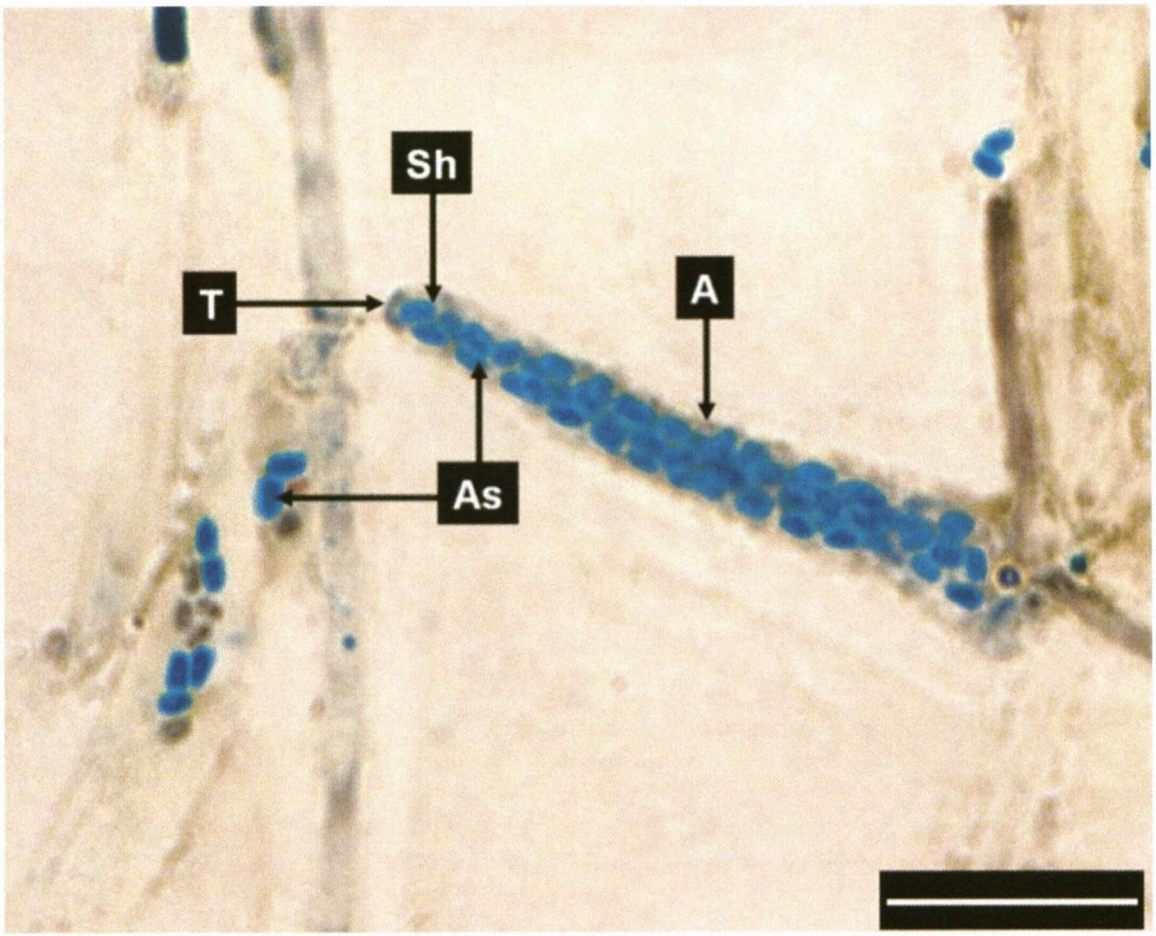

Fig. 1. A light micrograph of *Dipodascus geniculatus* showing stained elongated ascospores within a bottle-shaped ascus (A) with ascus tip (T). The ascospores (As) stained dark blue inside the ascus with the surrounding sheaths (Sh) in a lighter blue. Some of the blue stained ascospores are present as aggregates outside the ascus. Bar = 20 μm.

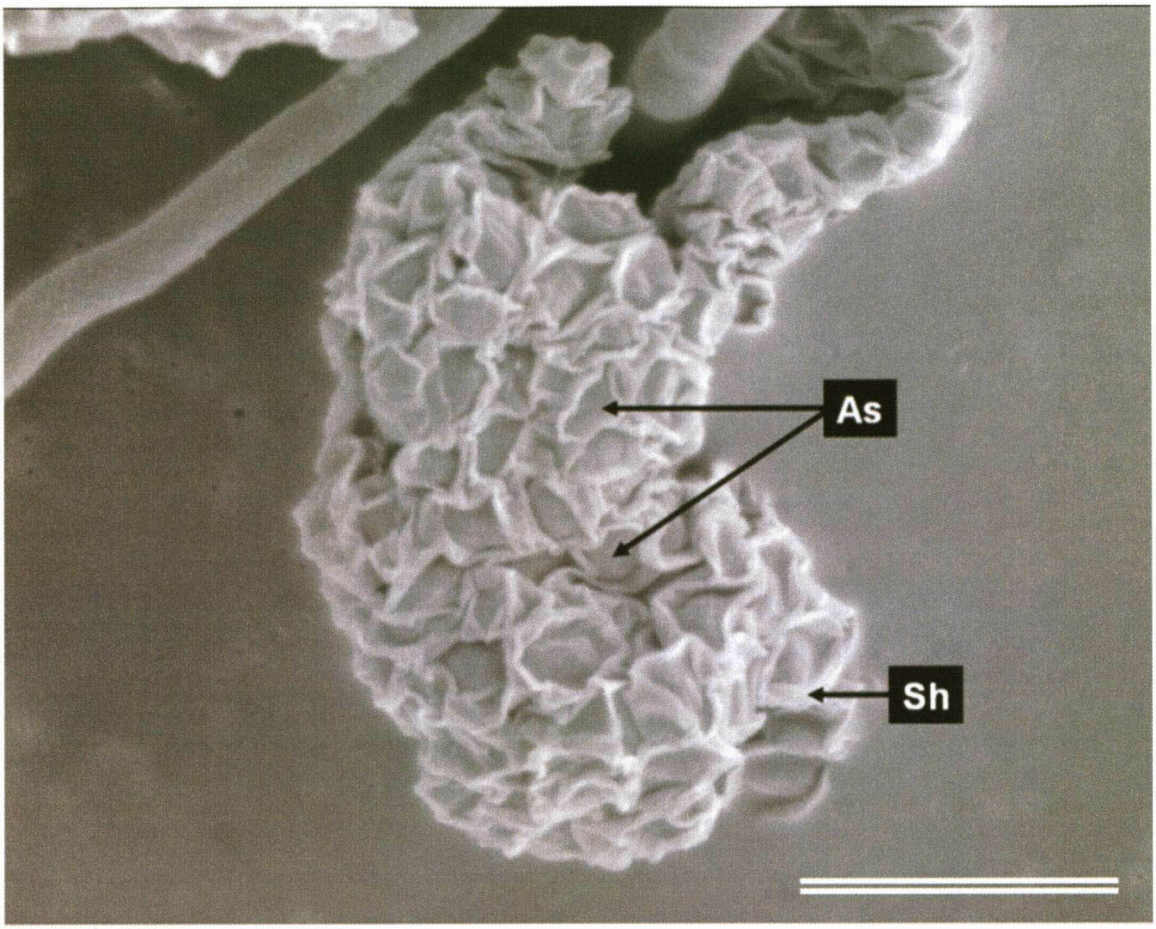

Fig. 2. A scanning electron micrograph of *Dipodascus geniculatus* revealing ascospores (As) with their associated dehydrated ridged and wrinkled sheaths (Sh). Bar = 10 μm.

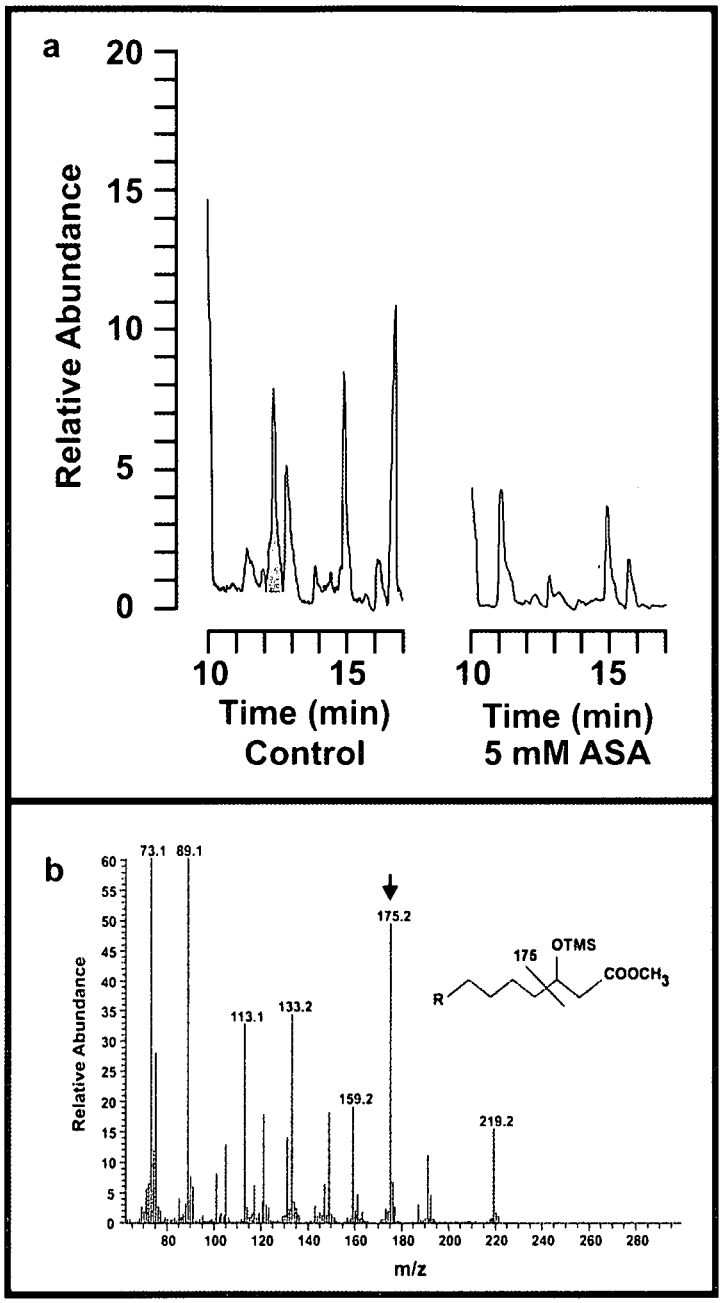

Fig. 3. The effect of acetylsalicylic acid (ASA) on 3-hydroxy (OH) oxylipin production in *Dipodascus geniculatus*. Total ion chromatograms of control (no ASA added) and in the presence of 5 mM ASA (a). Shaded peak indicates 3-OH oxylipin in (a). The mass spectrum (b) of the shaded peak in (a) indicating a characteristic peak of *m/z* 175. This is indicative of a 3-hydroxyl group on carbon 3 of the fatty acid.

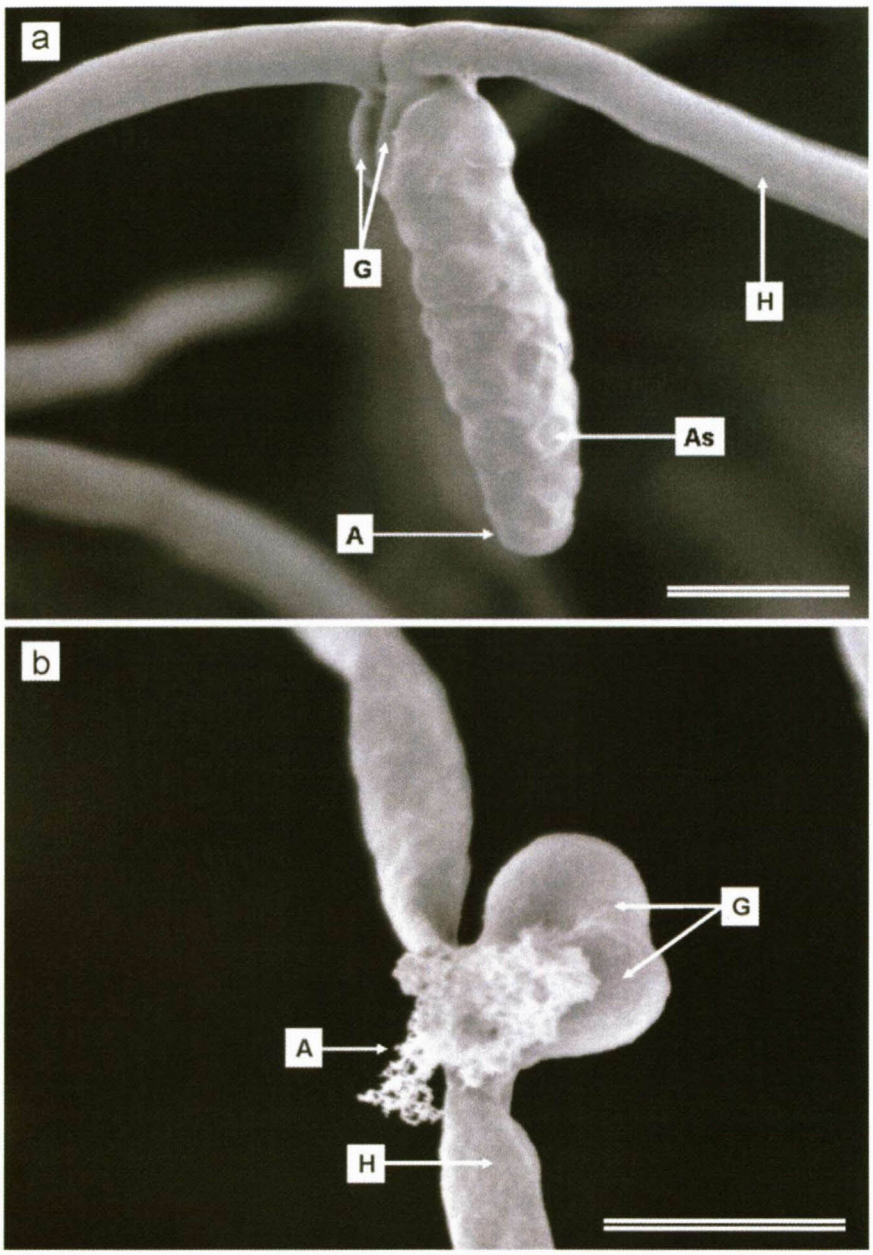

Fig. 4. A scanning electron micrograph of an ascus (A) with gametangia (G) produced by *Dipodascus geniculatus* when cultivated in the absence (a) and presence of 5 mM acetylsalicylic acid (b). A malformed ascus is visible in (b). As = ascospores inside ascus; H = hyphae. Bar = 10 μm.

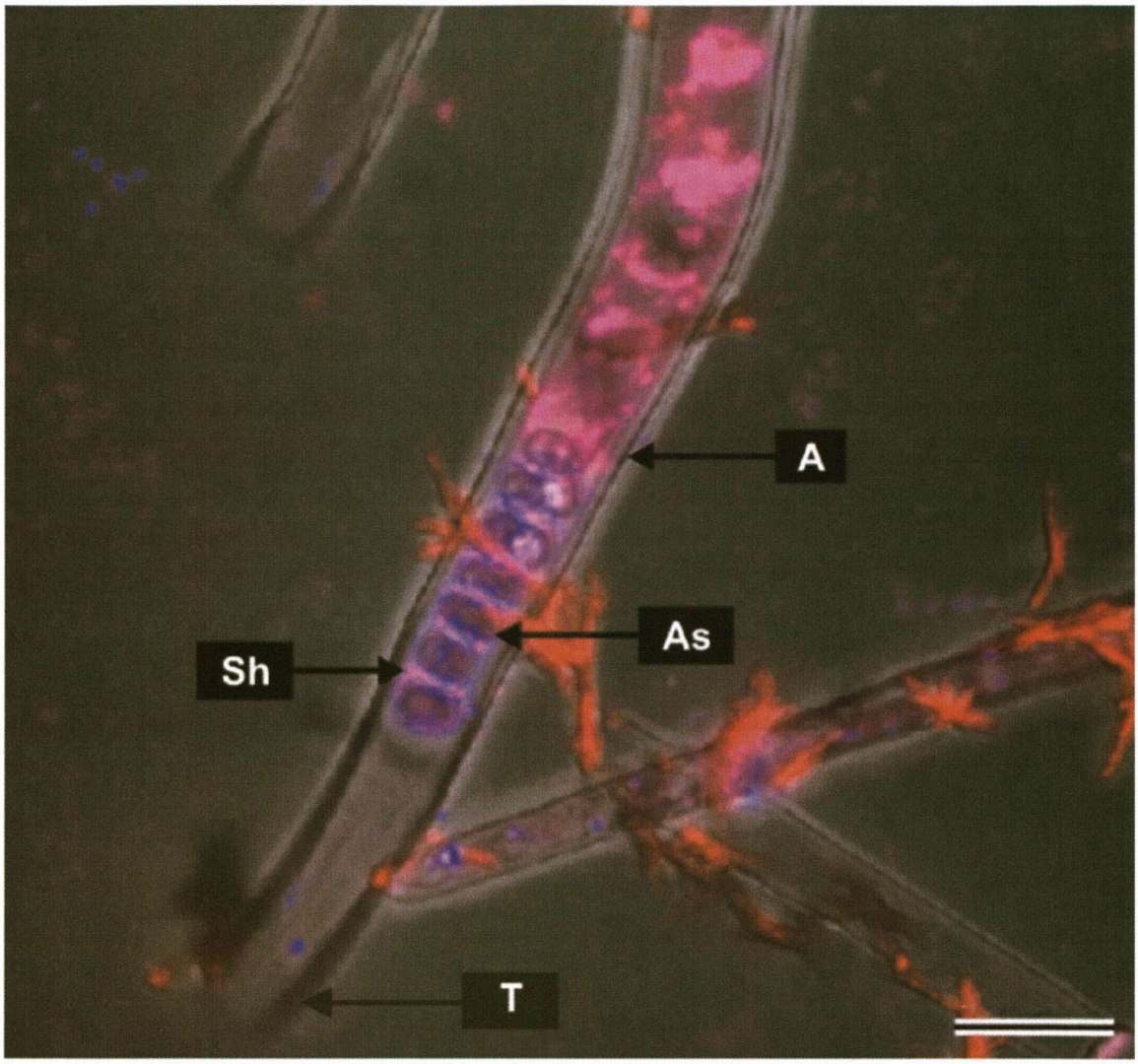

Fig. 5. A confocal laser scanning micrograph of Nile Red stained ascospores turned sideways in the ascus neck of *Dipodascus geniculatus.* Ascospores (As) within the ascus (A) stained purple-blue, while the compressible sheaths (Sh) stained light pink. T = tip. Bar = 10 μm.

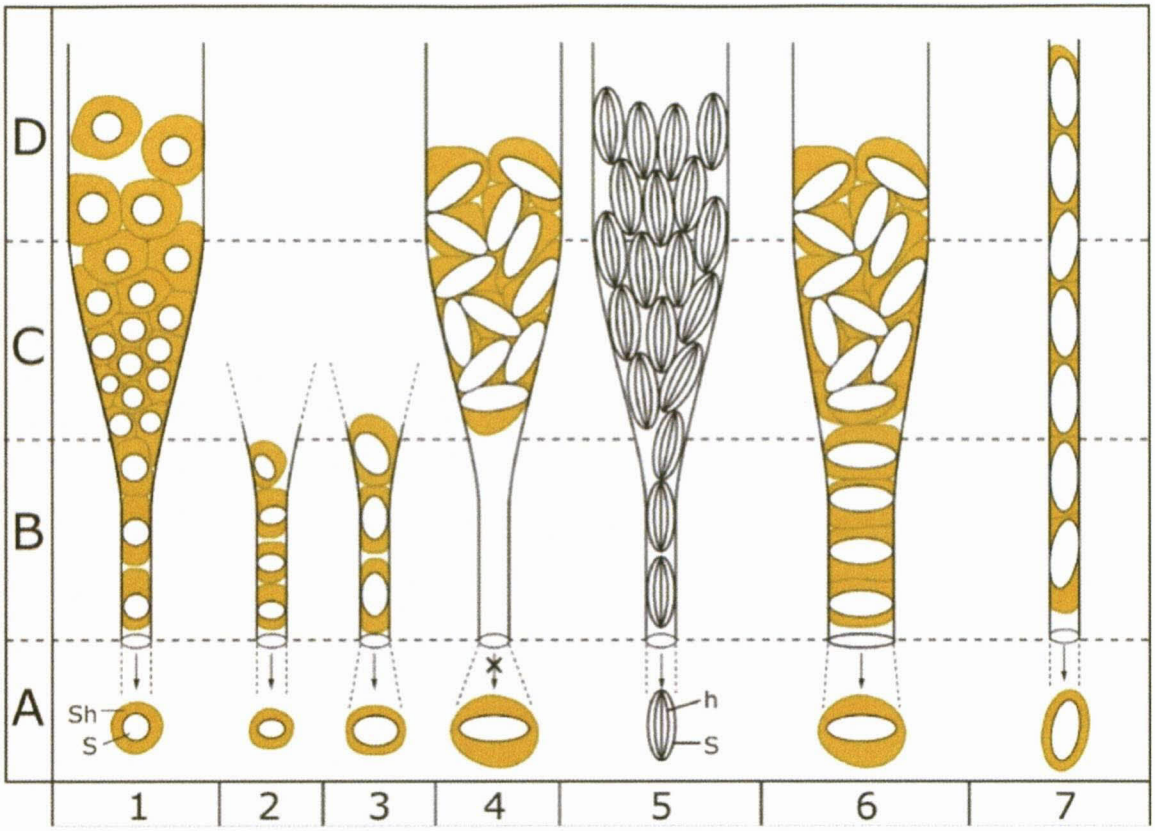

Fig. 6. A diagrammatic representation of ascospore-traffic (release) through bottle-shaped asci (row B, bottle-neck; row C, funnel; row D, broad base) as observed by light microscopy. Round to oval-shaped ascospores (S, diameter:length = 1:1, 1:1.3 and 1:1.5-2.0) as observed for *Dipodascus aggregatus* (columns 1 and 2) and *Dipodascus albidus* (column 3), respectively are enveloped by compressible sheaths (Sh, approximately 1 µm thick), enabling spores to slide past each other for unhindered individual release. Elongated ascospores (s), as observed for *Dipodascopsis uninucleata* var. *uninucleata* (column 5) are naked and aligned via surface hooked ridges (h) in gear-like fashion for unhindered release. Elongated ascospores, as observed for *Dipodascus geniculatus* (column 6) are enveloped in compressible sheaths. Here, the ascus dimensions changed by having the same bottle-neck diameter as the length of the ascospores, ensuring unhindered ascospore release. Elongated ascospores, as observed in *Dipodascus macrosporus* (column 7) are also enveloped in sheaths but are aligned inside the thin tube-like ascus for unhindered ascospore release. Columns 1-5 were taken from Van Heerden *et al.* (2005).

SUMMARY

Certain ascomycetous yeasts produce "lubricated" 3-hydroxy (3-OH) oxylipin-coated, micron-scale sexual spores in a variety of shapes, sizes, colors and sometimes with nano-scale surface ornamentations. In past literature, these oxylipin-coated ornamentations are only mentioned for use in classification and no thought was given to their possible purpose or function. These 3-OH oxylipins were found to be associated with the sheathed ascospores of some species representing the genus *Dipodascus*. However, these compounds have not yet been studied in detail in this genus. In addition, no thought was given to the mystery behind the release mechanics of sheathed ascospores from enclosing bottle-shaped asci and the role of these 3-OH oxylipins during dispersal. Therefore, it became the aim in this study to determine 3-OH oxylipin structure, distribution and function in *D. albidus* UOFS Y-1445T and *D. geniculatus* UOFS Y-1144 and to reveal the secrets behind the release mechanics of sheathed ascospores from bottle-shaped asci in these two species. Using electron microscopy, confocal laser scanning microscopy, gas chromatography-mass spectrometry (GC-MS) and digital live imaging, the release mechanisms of ascospores of various lengths from bottle-shaped asci, and produced by different yeasts, are compared in this study. It was found that *Dipodascus albidus* produces oval ascospores that are surrounded by compressible, oxylipin-coated sheaths enabling ascospores to slide past each other when forced by turgor pressure and by possible sheath contractions towards the narrowing ascus-neck. Usually elongated ascospores, as found in *Dipodascopsis uninucleata* var. *uninucleata,* are linked by means of oxylipin-coated interlocked hooked ridges on the surfaces of neighbouring ascospores, thereby keeping them aligned while being pushed towards the ascus-tip by turgor pressure. Interestingly, it was uncovered in this study that *Dipodascus geniculatus* produce elongated, oxylipin-coated sheathed ascospores that are effectively released from bottle-shaped asci without alignment. This is possible because the ascus neck and opening have the same diameter as the length of the ascospore, thus allowing the ascospores to turn sideways without blocking the ascus upon release. Interestingly, it was found that increased concentrations of acetylsalicylic acid (ASA), inhibit both ascospore release and 3-OH oxylipin production in these yeasts,

thereby implicating this oxylipin in sexual reproduction. Using GC-MS analysis, the oxylipin was characterized as a 3-OH metabolite. The same mass spectrum, implicating the presence of the same 3-OH oxylipin structure, was found to be present in other *Dipodascus species*. This implicates that this 3-OH metabolite might be conserved on genus level.

OPSOMMING

Sommige askomisete giste produseer mikron-skaal geslagtelike spore in `n verskeidenheid van vorms, grootes, kleure en somtyds met nano-skaal oppervlak ornamentasies wat met "smerende" 3-hidroksie-(3-OH)-oksielipiene bedek is. In literatuur word daar slegs melding gemaak van hierdie oksielipien-bedekte ornamentasies vir klassifikasiedoeleindes maar geen aandag word aan hul moontlike doel of funksie geskenk nie. Die 3-OH-oksielipiene word met die skedes, wat die askospore in sommige spesies van die genus *Dipodascus* omring, geassosieer. Hierdie komponente is egter nog nie in besonderheid in hierdie genus bestudeer nie. Geen aandag is ook aan die misterie van die vrylatingsmeganika van skede-omringde askospore vanuit geslote bottelvormige aski geskenk of die rol wat 3-OH-oksielipiene tydens verspreiding speel nie. Die doelstellings van hierdie studie is dus om die 3-OH oksielipienstruktuur, verspreiding en funksie in *D. albidus* UOFS Y- 1445T en *D. geniculatus* UOFS Y-1144 te bepaal en om die geheime agter die vrylatingsmeganika van skede-omringde askospore vanuit bottelvormige aski in the twee genoemde spesies te verklaar. In hierdie studie word m.b.v. elektron-mikroskopie, konfokalelaserskandeermikroskopie, gaschromatografie-massaspektrometrie (GC-MS) en digitale uitbeelding, die vrystellingsmeganismes van askospore, geproduseer deur verskillende giste, van verskillende lengtes vanuit bottelvormige aski vergelyk. Daar is bevind dat *Dipodascus albidus* ovaalvormige askospore, omring deur saampersbare, oksielipien-bedekte skedes, produseer. Deur turgordruk en vergroting van die skede, word hierdie askospore na die vernoude bottelnek gedruk deurdat die oksielipien-bedekte skedes die askospore verby mekaar laat skuur. Gewoonlik word verlengde askospore, soos in *Dipodascopsis uninucleata* var. *uninucleata* gevind, aan mekaar verbind deur oppervlakhoeke wat soos ratte aan mekaar gekoppel is, om te verseker dat die askospore in gelid bly soos dit na die askuspunt gedruk word. Interessant genoeg, is in hierdie studie ontdek dat *Dipodascus geniculatus* verlengde askospore met oksielipien-bedekte skedes produseer wat effektief vanuit bottlevorminge aski vrygestel word sonder dat hulle in gelid geplaas word. Dit is moontlik deurdat die askusnek en askusopening dieselfde deursnit as die lengte van die askospoor het. Dus kan die askospore skuins draai tydens vrystelling sonder om die askus te

blokkeer. Met die byvoeging van `n 3-OH-oksielipieninhibeerder, asetielsalisiensuur (ASS), is hoofsaaklik die geslagtelike fase, meer spesifiek, askospoorvrystelling geïnhibeer, asook 3-OH-oksielipienproduksie. Dit bevestig dat die oksielipiene met die geslagtelike voorplantingstrukture geassosieer word. Met die gebruik van GC-MS, is bepaal dat die oksielipien `n 3-OH metaboliet is. Dieselfde massaspektrum, wat op die teenwoordigheid van dieselfde 3-OH oksielipien struktuur dui, is ook in ander *Dipodascus* spesies gevind. Dit impliseer dat hierdie 3-OH metaboliet dalk op genus vlak gekonserveerd is.

Keywords

Alignment, Ascospore, Compressible sheaths, *Dipodascus albidus, Dipodascus geniculatus*, Elongated ascospores, 3-Hydroxy oxylipin, Release mechanism, Yeast

Sleutelwoorde

Askospore, *Dipodascus albidus, Dipodascus geniculatus,* Gis, 3-Hidroksie-oksielipien, In gelid plaas, Saampersbare skede, Verlengde askospore, Vrylatingsmeganisme