The role of endophytes in the metabolism of fluorinated compounds in the South African Dichapetalaceae

by

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Title of treatise: The role of endophytes in the metabolism of fluorinated compounds in the South African Dichapetalaceae

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Abstract

Dichapetalum cymosum (poison leaf) is a very common problem plant in southern Africa. Fluoroacetic acid, believed to be the poisonous entity in the plant, is produced by the plant, but the micro-organisms associated with this plant may also play a role in the production thereof. A previous study on *Burolderia cepacia*, an endophyte of *D. cymosum* showed active metabolism of fluoroacetate by this endophyte. The isolated endophytes from *D. cymosum* were studied to determine whether they synthesise any fluorinated compounds. It seemed from preliminary results that symbionts might play a role in the synthesis of the poisonous entities in *D. cymosum*, but further investigation is required.

The detection of glandular lesions on the abaxial side of the leaf led to closer examination and the cross sections revealed unusually deformed epidermis cells with adjacent cells containing vacuoles filled with phenolic-like crystals. Transmission electron microscopy (TEM) of the spongy parenchyma cells directly above the glandular lesions indicated the presence of clusters of small, virus-like particles (VLPs) in the chloroplasts. Observations by TEM showed that these VLPs have analogous structures to phytoferritin.

Tapura fischeri (leafberry tree) is a tree member of the same family, and it was found to also contain a fluorinated compound. Endophytes were also found in the plant and similar glandular lesions with analogous VLPs were observed at these sites. This

might indicate that endophytes have a share in the biosynthesis of the fluorinated compounds found in Dichapetalaceae. Numerous factors ought to be considered in order to fully understand the chemical ecology of the intricate system regarding the endophytes and the possible toxicity of the family Dichapetalaceae.

Keywords: Dichapetalaceae, *Dichapetalum cymosum*, *Tapura fischeri*, fluorinated compounds, endophytes, virus-like particles, transmission electron microscopy, phytoferritin.

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List of abbreviations used

CoA: co-enzyme A

D_2O: deuterium oxide

$_dH_2O$: distilled water

EDS: energy-dispersive X-ray spectroscopy

EELS: electron energy loss spectroscopy

GC: gas chromatography

HPF: high-pressure freezing

HPF-FS: high-pressure freezing and freeze substitution

MFA: monofluoroacetic acid / monofluoroacetate

NM: nutrient medium

NMA: *N*-methyl-L-alanine

NMR: nuclear magnetic resonance

NMS: *N*-methyl-L-serine

PFK: phosphofrucktokinase

SAM: *S*-(5′-adenosyl)-L-methionine

SEM: scanning electron microscopy / scanning electron microscope

SFM: soy flour mannitol

SIA: *Streptomyces*-isolation agar (medium)

sp.: species (singular)

spp.: species (plural)

TEM: transmission electron microscopy / transmission electron microsope

TLC: thin layer chromatography

TRIS: tris(hydroxymethyl)aminomethane

VLP: virus-like particle

WHO: World Health Organisation

Chapter 1 - Introduction

1.1.Background

Comprising of trees, shrubs, dwarf-shrubs, and lianas, the Dichapetalaceae forms part of the order Malpighiales and consists of three genera, mainly distributed throughout the tropics, although central Africa shows the centre of diversity. The two genera represented in southern Africa are that of *Dichapetalum* and *Tapura* with the latter having a rather restricted distribution. The genus *Dichapetalum* consists of at least 124 species with no less than 86 species occurring in the tropics of Africa, including Madagascar, with several species being poisonous to domestic animals (Breteler, 1988). Only *D. cymosum* (Hook.) Engl. is found in South Africa (Kellerman *et al.*, 1988)

Over the past century, *D. cymosum* was extensively studied in the attempt to find the toxic entity (Steyn, 1928; Marais, 1944), to understand the metabolism of monofluoroacetate (Eloff, 1968; Eloff & Von Sydow, 1971; Eloff & Grobbelaar, 1972; Meyer & Grobbelaar, 1989; Meyer, 1991), to determine if other toxic compounds are associated with the plant (Eloff & Grobbelaar, 1967; Eloff, 1968; Eloff & Grobbelaar, 1969; Eloff, 1972), and to identify endophytes associated with the plant (Meyer *et al.*, 1990). Not only was poison leaf investigated but other members of the family Dichapetalaceae as well (Eloff, 1972; Schwikkard *et al.*, 1998). Because of poison leaf being a common plant in southern Africa, and the fact that numerous operations to control or eradicate poison leaf have failed, implications such as livestock deaths brought about financial crises and economic repression to farmers (Kellerman *et al.*, 1996).

Fluoroacetic acid, which was identified as the poison in *D. cymosum* might, as speculated, be produced by the plant (Marais, 1944), but the microorganisms associated with this plant may play a role in the production thereof (Meyer *et al.*, 1990). Symbiotic relationships between *D. cymosum* and its endophytes are not clear and possible preventative measures against poisoning should be investigated as related to the production of the poisonous compounds. It may be found that fluoroacetic acid production corresponds to a defence response to pathogens or

parasites? Antidote studies might also convey potential solutions for preventing poisoning of livestock by fluoroacetic acid.

The genus *Tapura* is mainly restricted to the tropics of South America with only seven of the 28 species occurring in Africa. *T. fischeri* Engl. is the only member that is found in southern Africa (Breteler, 1988) and is known to be toxic, yet little is known about the biochemistry of this plant. This forest-tree member is not as common as the related *D. cymosum* and, hence, poisoning incidents are encountered very seldom (Schwikkard *et al.*, 1998). Even though it is related to *D. cymosum*, the overall anatomy, habitat preference, and distribution differs considerably and, thus, raises the question of similarity on physiological level.

1.2. *Dichapetalum cymosum*

D. cymosum (**Figure 1.1**), commonly known as poison leaf or "gifblaar", is a geophytic member of the Dichapetalaceae family (Klopper *et al.*, 2006). The genus comprises of 124 species occurring in the tropics of Africa, America, Madagascar, and South-East Asia (Moss, 1928; Wink & Van Wyk, 2008). Occurring mostly in the northern parts of the Republic of South Africa, Botswana, Namibia, and Zimbabwe (Kellerman *et al.*, 1988), *D. cymosum* prefer deep sandy soils where it forms dense stands (**Figure 1.2**), usually associated with indicator species such as *Ochna pulchra* and *Burkea africana* (Vahrmeijer, 1981; Kellerman *et al.*, 1988), which allows for the primary root system and enormous woody structure of the stem to grow underground (Nel, 1980). Apical parts of the branches protrude from the soil, giving the appearance of a pseudo-herbaceous plant, bearing alternating, oblong leaves, bright green above and below, with secondary veins forming loops near the margin (Nel, 1980). Young leaves tend to be hairy becoming more leathery and smooth with age (Nel, 1980). Small white flowers are borne in dense clusters which may, on rare occasions, develop into orange or yellow spherical fruits when ripe (Nel, 1980).

The caterpillar of the moth *Sindris albimaculatus* Reg. (**Figure 1.3**) feeds on the flowers, fruits and leaves of *D. cymosum*, which might explain why the fruits are

rarely found in nature (Meyer, 1991). Interestingly, this caterpillar not only defluorinates monofluoroacetate (MFA), but also tend to accumulate it as a form of defence (Meyer & O'Hagan, 1992).

Figure 1.1 *Dichapetalum cymosum* appears as a pseudo-herbaceous plant occurring on sandy plains and slopes as this specimen growing on the eastern aspect of the Bronberg range in Pretoria.

Figure 1.2 Dense stands of *D. cymosum* forming in sandy plains nearby Roodeplaat Dam in the Pretoria area. Note the extensive growth in the clearing in the foreground.

Figure 1.3 Caterpillar of the moth (inset) *S. albinaculatus* feeding on the abaxial side of an older leaf of *D. cymosum*.

Fluoroacetate poisoning, as a result of the consumption of *D. cymosum*, most frequently affects cattle in the spring and early summer (Kellerman *et al.,* 1996) because of the young, newly emerged shoots, immature seeds, and flowers which are particularly toxic (Steyn, 1927; Eloff, 1968; Eloff, 1972a; Tannock, 1975b; Meyer & Grobbelaar, 1989). Monofluoroacetate was first discovered in *D. cymosum* (Marais, 1944; Vickery & Vickery, 1972) and *D. toxicarium* (Marais, 1944), but has since been identified in more than forty tropical and sub-tropical plants (**Figure 1.4**) from Africa, Australia, and South America (Hall, 1972b). Monofluoroacetate is probably also found in other *Dichapetalum* species (**Table 1.1** (Thienpont & Vandervelden, 1961; Dalziell, 1937; Van der Walt & Steyn, 1946; Hall, 1972b)). In addition, MFA is known to be present in *Acacia georginae* (Australia (Oelrichs & McEwan, 1960)), *Cyamopsis tetragonolobus* (India (Vartiainen, 1984)), *Gastrolobium grandiflorum* (Australia (McEwan, 1964)), *G. parviflorum* (Australia (Alpin, 1967)), *Glycine max* (Lovelace *et al.,* 1968) and *Palicourea marcgravii* (South America (Oliverira, 1964)). Interesting to note is the fact that these plants contain the same poisonous entity but don't share any common characteristics or even the same distribution. These plants containing MFA are responsible for several cases of livestock poisoning when accidentally browsed (Vahrmeijer, 1981; Kellerman *et al.,* 1988) especially during unfavourable conditions when pastures become deprived because of drought, fires and overgrazing, or trampling (Botha & Penrith, 2008). *D. cymosum* also contains toxic amino acids such

as *N*-methyl-L-alanine (NMA (Eloff & Grobbelaar, 1967)) and *N*-methyl-L-serine (NMS (Eloff & Grobbelaar, 1969; Eloff, 1972a)).

Table 1.1 Fluorine content of the leaves from various *Dichapetalum* spp. (Adapted from Hall 1972b).

Species	Sample	Fluorine ($\mu g \cdot g^{-1}$ in air-dried tissue)	
		Total	Organic
Dichapetalum cymosum	1	40	13
	2	111	97
	3	23	8
	4	79	73
	5	12	1
D. guineese		9	< 1
D. mossambicense		11	2
D. stuhlmannii	1	134	90
	2	142	47
	3	148	10
	4	42	13
D. toxicarium	1	81	9
	2	145	29

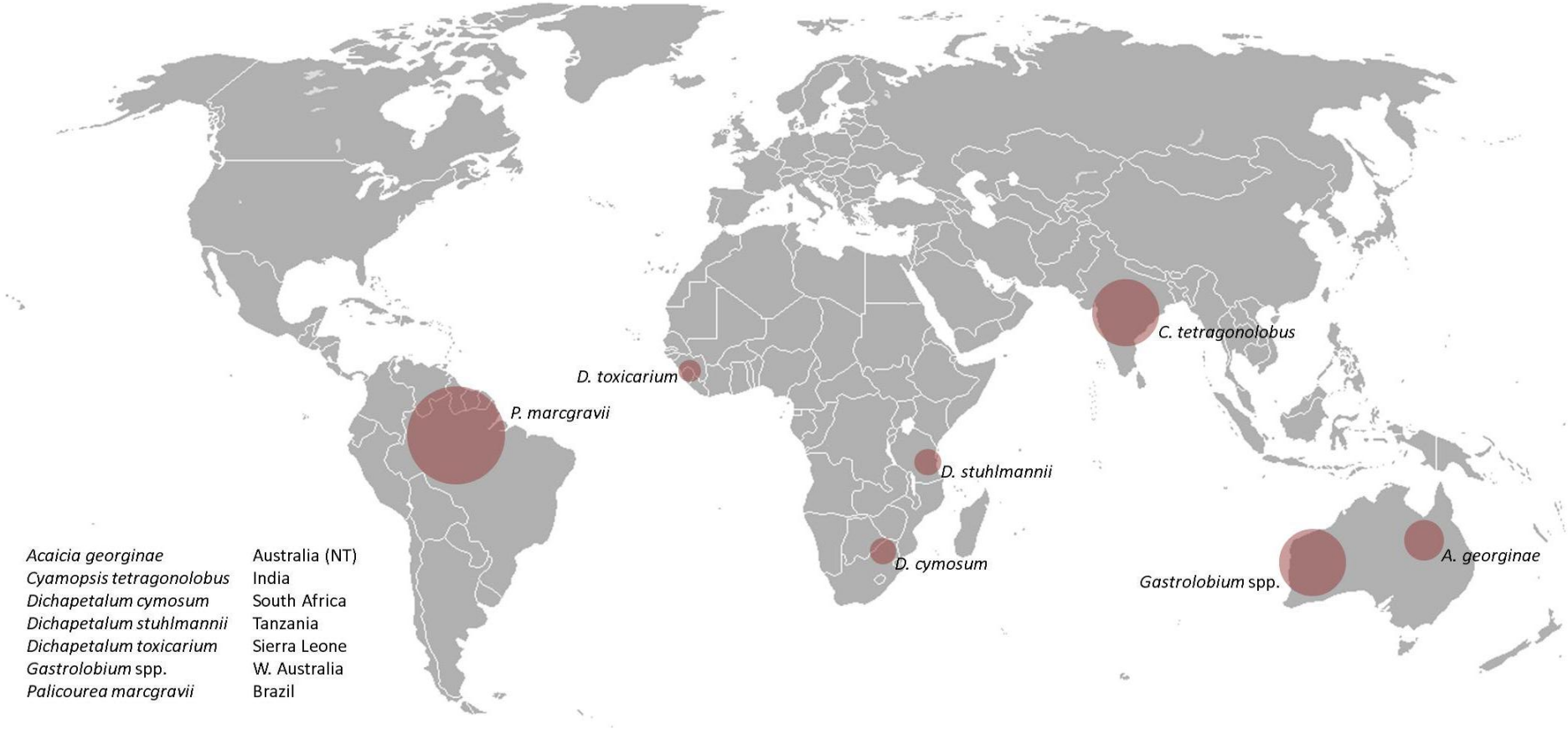

Figure 1.4 The worldwide distribution of plants containing organo-fluorine compounds, including MFA (adapted from Hall, 1972b).

Monofluoroacetate is highly toxic to vertebrates (Class 1a as recommended by the World Health Organisation (WHO)[1] (Wink & Van Wyk, 2008)), is also insecticidal (Notman, 1989; Booth & Wickstrom, 1999) and is commonly used to control pest species (Sherley, 2004). The lethal oral dose for cattle is 0.15 mg•(kg bodyweight)$^{-1}$ (Kellerman *et al.*, 1988), for humans 0.5 mg•(kg bodyweight)$^{-1}$ (Wink & Van Wyk, 2008) and even as little as 20 g of fresh leaves may kill a sheep (Wink & Van Wyk, 2008). Young leaves of the plant may accumulate up to 2500 µg•g^{-1} (dry weight) in early spring (Tannock, 1975a). Monofluoroacetic acid is relatively harmless when ingested, but it is, however, converted to monofluorocitric acid (**Figure 1.5**) in the body during the Krebs cycle (Peters, 1952). Fluorocitrate inhibits the enzyme aconitase (Morrison & Peters, 1954; Treble *et al.*, 1962; Fanshier *et al.*, 1964; Villafranca & Platus, 1973) and a mitochondrial acetate carrier (Wink & Van Wyk, 2008). A subsequent increase in the levels of citrate in the tissues and the blood leads to acidosis, a general metabolic imbalance. The citrate build-up also inhibits the enzyme phosphofructokinase (PFK) and, hence, inhibits glucose metabolism (Duin & Bernem, 1968). Due to the drastic reduction of cellular respiration within 24 hours of eating the plant, abrupt death usually occur as a result of acute heart failure provoked by the animal drinking water, exercising or due to anxiety (Kellerman *et al.*, 1996). The nervous system may also be affected (Chenoweth & Gilman, 1946; Sherley, 2004).

[1] 5mg or less/ kg bodyweight by oral administration to show LD_{50} in rats.

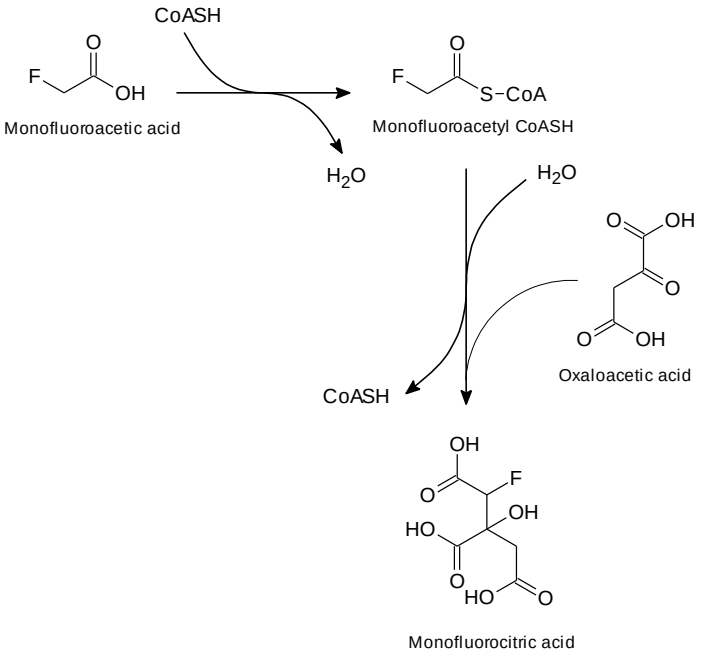

Figure 1.5 The conversion of monofluoroacetic acid to monofluorocitric acid in a 'suicide' reaction.

Peculiar to note is that *D. cymosum* itself does not succumb to the poisoning effect of MFA (Treble *et al.,* 1962). Eloff (1972b), in a tracer study, showed that *D. cymosum* possesses an active Krebs cycle and that the plant is capable of metabolising MFA (Eloff & Von Sydow, 1971). Monofluoroacetate, when added, stimulate respiration in the plant, whereas fluorocitrate indicated a strong inhibition of respiration. Following these findings, it was speculated that the tolerance of *D. cymosum* to MFA can be ascribed to the fact that the conversion of MFA to fluorocitrate does not take place within the plant and that the citrate synthetase possesses varying affinities for acetyl-CoA and its fluorinated derivative, fluoroacetyl-CoA (Eloff & Von Sydow, 1971). The presence of a possible fluoroacetyl-CoA hydrolase in the plant cell may serve as an additional explanation as to why the plant is not poisoned by MFA as this hydrolase could not use fluoroacetyl-CoA as substrate (Meyer, 1991).

A high degree of MFA degradation takes place in older leaves compared to that of the younger leaves (Meyer, 1991). The MFA concentration in young leaves tends to be relatively high (231.9 mg•(kg fresh weight)$^{-1}$) whereas the MFA concentration in

older leaves is much lower (97.0 mg•(kg fresh weight)$^{-1}$) and, hence, it is suggested that MFA is extensively metabolised by older leaves (Meyer & Grobbelaar, 1989). When leaves (both young and old) were supplied with fluoroacetate-2-^{14}C it was found that the older leaves contained more radioactive amino acids and carbohydrates than the young leaves (Eloff & Grobbelaar, 1972)

1.3. *Tapura fischeri*

Tapura fischeri Engl. is another member of the family Dichapetalaceae and is considered to be poisonous, yet little is known about this plant species and, therefore, little literature is available. Restricted mostly to the tropics, the genus *Tapura* consists of tree members unlike many *Dichapetalaceae* spp. which vary in size. *T. fischeri* Engl. (leaf berry tree) is a semi-deciduous to deciduous forest tree which grows in the understory or along forest edges (Friis & Vollesen, 1985) and is usually associated with *Terminalia* spp. restricted to the northern parts of KwaZulu-Natal (Torre, 1963). Although this plant species may not contain MFA, novel phaeophytins were discovered in the plant which may prove worth investigating in future (Schwikkard *et al*., 1998).

1.4. Endophytes of *D. cymosum*

Endophytes are considered to be micro-organisms that associate with a plant to form a symbiotic relationship without any sign of disease. This symbiotic relationship may vary and include neutralism, mutualism and commensalisms. The agronomic performance of a plant may be increased by the endophytic microbes which are sequestered to the protective and nutritious environment of the plant and in return the endophytic entity may convey continued protection of the plant in the form of a natural pesticide or similar poison (Bacon & Hinton, 2007).

Seemingly normal, field-grown *D. cymosum* plants were found to be infested with endophytic microbes (Grobbelaar & Meyer, 1989). It has not been shown that the

microbes are responsible for the synthesis of the MFA found in the plant, but relatively high concentrations of MFA were detected in the soils in which *D. cymosum* grows and it is speculated that soil microbes might be responsible for the synthesis of the fluoroacetate (Hall & Cain, 1972). The discovery of *Burholderia cepacia* (previously *Pseudomonas cepacia*) as a common endophyte of *D. cymosum* by Meyer *et al.* (1990) showed that this endophyte is capable of degrading MFA by cleaving the C-F bond (2.69mg of MFA per hour) after which the defluorinated acetate is then degraded by the Krebs cycle of the microbe to release CO_2 (23.59mg of MFA per hour).

Burkolderia cepacia was also found to contain the toxic amino acid *N*-methyl-L-serine (NMS), and that the aseptic callus of *D. cymosum* did not contain any of the toxic amino acids, including NMA, as with the *B. cepacia* infected plant material. The presence of NMA in the leaves (but not bacteria) suggested that the cooperation of plant and endophyte is required for the synthesis of NMA. It is, however, possible that the synthesis of NMS and NMA requires a high degree of differentiation (Meyer, 1991).

1.5.Aims and objectives

The main aim of this study was to determine what the relationship between endophytes and the South African members of the family Dichapetalaceae is

In order to gain a better perspective on the poisonous compounds and endophytes of *D. cymosum* and *T. fischeri* the following objectives were set:

- Determine whether the endophytes of *D. cymosum* produce poisonous organo-fluorine compounds;
- Determine whether *T. fischeri* produces organo-fluorine compounds;
- Ascertain whether there is a relationship between isolated endophytes and the production of organo-fluorine compounds;
- Establish the cause of abnormality in the epidermal cells on the abaxial side of most leaves and whether it relates to organo-fluorine compound synthesis; and
- Investigate what the unknown structures found in the chloroplasts of the affected areas are and whether they are related to the endophytes or any organo-fluorine compounds.

1.5.1. Hypothesis

Both the plant and the associated endophytic entity are required to produce the organo-fluorine compounds associated with *Dichapetalum cymosum* and *Tapura fischeri*.

1.6. Structure of the thesis

This Master's study aimed mainly to investigate the possibility of a symbiotic association between the members of the family Dichapetalaceae and their attendant endophytes. Rather than to answer numerous questions on the influences of poisonous compounds on the environment, this thesis aimed to help achieve the appreciative paradigm regarding the intricate system of symbiosis. It was, therefore, necessary to conduct various experiments in order to achieve the outcomes depicted in the following chapters. Opportunities for further research were also created as many topics remain unsolved.

- ***Chapter 1***

This chapter introduces the multi-disciplinary scope of the project by including initial studies and recent findings.

- ***Chapter 2***

This chapter investigates the isolation of endophytic symbionts from both South African members of the family Dichapetalaceae to determine whether any similarity could be observed in the assortment of endophytes in the plants. The metabolism of organo-fluorine compounds by the endophytes was the main focus of these studies with the synthesis of monofluoroacetate (MFA) being the principal priority.

- ***Chapter 3***

In this chapter it was attempted to determine the morphological properties of the glandular structures on the lamina and to characterise the virus-like particles (VLPs) which are sequestered to the chloroplasts in spongy parenchyma cells adjacent to the glandular epidermis cells.

- ***Chapter 4***

The purification the fluorinated entity of *T. fischeri* and the attempt to elucidate the structure of this entity, are described in this chapter.

- ***Chapter 5***

This chapter is a discussion on all the results and includes the overall conclusions attained from the study. It also elaborates on future intended studies to be performed to achieve conclusive results.

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Chapter 2 – Endophytes from South African Dichapetalaceae

2.1.Introduction

Biosynthesis of fluorinated compounds is fascinating in the sense of prospects for biotransformation routes to organo-fluorine compounds by means of some enzymatic reactions. *Streptomyces* spp. were mostly of interest after the discovery of fluorinated products such as monofluoroacetate (MFA) and 4-fluorothreonine to be synthesised by *Streptomyces cattleya* (Sanada *et al.,* 1986). By utilising cell-free extracts of *S. cattleya* cultures, it was shown that the enzyme fluorinase is capable of synthesising MFA from free fluoride and the precursor *S*-(5′-adenosyl)-L-methionine (**Figure 2.1**). The detection of the presence of intermediates and the fluorinated products was conducted by utilising ^{19}F-NMR spectroscopy (Scaffrath *et al.,* 2002; Murphy *et al.,* 2003; Deng *et al.,* 2004, Cobb *et al.,* 2005).

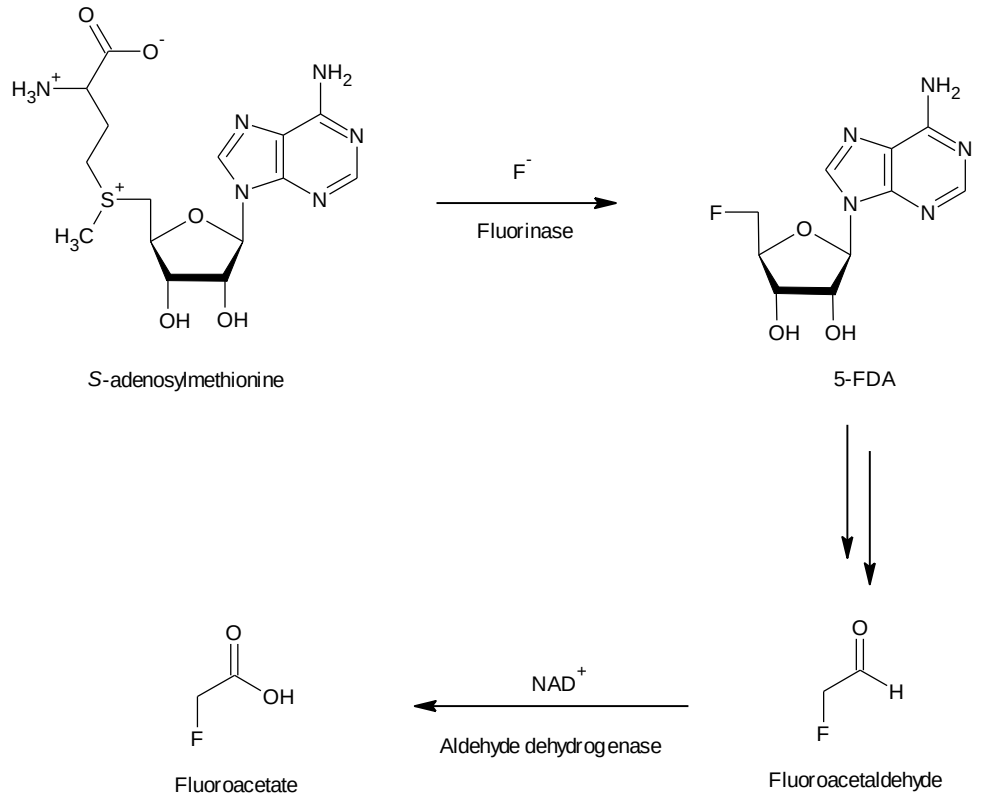

Figure 2.1 The biosynthesis of monofluoroacetate from the precursor *S*-(5′-adenosyl)-L-methionine (SAM) and ionic fluoride. 5-FDA, 5′-fluoro-5′-deoxyadenosine (Schaffrath *et al.*, 2002).

Hall (1972a) suggested the use of ^{19}F-NMR for the detection for fluoroacetic acid in plant material as it is easier than GC. The identity of MFA in *D. cymosum* and *D. toxicarium* extracts dissolved in D_2O has been established to be a triplet (J = 47 Hz) positioned at ca. -216 ppm in ^{19}F-NMR and this can also be confirmed on a ^{1}H-NMR spectrum by the presence of a doublet (J = 47 Hz) at δ 4.90 and shows a characteristic doublet FCH_2-signal at 82.1 ppm with ^{13}C-NMR (Kirms & Kirms, 2002). The limit of the detection of MFA is estimated to be ca. 4 $\mu g \bullet g^{-1}$ fluoroacetate in 1 g of plant material using a NMR spectrometer operating at 282 MHz. Another advantage is the abolishment of derivatisation which then allows for recovering of extracts after analyses in order to perform other analyses on the same sample (Baron *et al.*, 1987).

Since *B. cepacia* was revealed to be a common endophyte of *D. cymosum* by Meyer *et al.* (1990) and since this endophyte was shown to be capable of metabolising fluoroacetate, the centre of attention was on other unknown endophyte species possibly present in *D. cymosum*. Following the studies of O'Hagan *et al.* (2002) and Schaffrath *et al.* (2002) the emphasis was to isolate possible Actinomycete spp., especially *Streptomyces* spp., which might show similar activity than what was found with *S. cattleya*.

2.2. Methodology

Experiments pertaining to cell-free extracts were inspired by work done on *S. cattleya* by O'Hagan *et al.* (2002) and Schaffrath *et al.* (2002). It was decided to reproduce these experiments on some of the isolated endophytes resembling *Streptomyces* spp. in the attempt to replicate analogous enzymatic activity. Modifications were integrated into the protocol to provide for the shortage of precursors in the attempt to obtain cell-free extracts of the endophytes of interest without compromising results.

In later experimentations the cell-free extraction was omitted and, alternatively, whole-cell cultures were utilised to observe the fluorination capacity of the isolated endophytes.

The isolated endophytes from *T. fischeri* were studied as with the isolated endophytes from *D. cymosum* following the same protocol for culturing the endophytes in liquid broth.

2.2.1. Isolation of endophytes

Dichapetalum cymosum leaves and stems were collected at Faerie Glen Nature Reserve, Pretoria, South Africa (S 25° 46′ 15.34″; E 28° 17′ 33.29″), and at Kameelfontein near Pretoria, South Africa (S 25 35′ 54.14″; E 028° 24′ 31.01″). Plant collection took place in early spring to early summer and usually younger material was selected. *Tapura fischeri* leaves and stems were collected at the Onderstepoort campus of the University of Pretoria, Onderstepoort, South Africa (S 25° 39′ 2.00″; E 28° 10′ 54.56″). Collection took place from early spring to late summer and usually younger material was also selected.

The main intention was to determine the fluorinating capability of various bacterial endophytes isolated from inside tissue rather than including all of micro-organisms isolated from the exterior of plants. Various methods to isolate and culture *Streptomyces* spp. were also attempted as well as the preparation of spore cultures of the isolated *Streptomyces* spp.

The media used in this part of the study consisted of liquid and solid forms and of three different types. Firstly, a solid agar medium was used for the isolation, selection, and culture purification of endophytes, and secondly, a broth was used to culture these isolates. The three different types of media comprised of a nutrient medium (NM), soy flour mannitol (SFM) medium, and *Streptomyces*-isolation medium (SIA) which is adapted from the Kuster's agar medium (Balagurunathan & Subramanian, 1992). The NM contained nutrient agar or nutrient broth prepared according to the manufacturer's recommendations.

The SFM medium consisted of 20.0 g tryptone soy agar (Biolab, Merck, South Africa) and 20.0 g D(-)mannitol (B.D.H. Laboratory Chemicals Group, England) added to 1.0 ℓ $_{d}H_2O$. The isolated colonies were purified by streaking out the colonies on SFM

medium twice. The SIA consisted of 2.0 g of sodium caseinate (Sigma-Aldrich, New Zealand); 0.1 g anhydrous asparagine (Fluka Analytical, Sigma-Aldrich, Germany); 4.0 g sodium propionate (Fluka Analytical, Sigma-Aldrich, Germany); 0.5 g potasium dihydrogen phosphate (KH_2PO_4) (UnivAR, Saarchem, Merck, South Africa); 5.0 g glycerine (Promart Chemicals, India); anhydrous magnesium sulphate ($MgSO_4$) (UniLab, Saarchem, Merck, South Africa); 1.0 mg hydrous ferrous sulphate ($FeSO_4 \bullet 7H_2O$) (UnivAR, Saarchem, Merck, South Africa); and 15.0 g nutrient agar (Biolab, Merck, South Africa) added to 1.0 ℓ $_dH_2O$ in the order mentioned. The intention in the use of this medium was to utilise it to accelerate the growth of *Streptomyces* spp., resulting in the *Streptomyces* spp. colonies being a small fleck on the medium (Baskaran *et al.*, 2011). All media were supplemented with 10 mM sodium fluoride (NaF) (UnivAR, Merck, South Africa). All of these media were autoclaved for 20 minutes at 120°C and left to cool until ready to pour.

The different treatments of the leaves and stems included sterile material (in which case sterile material refers to the sterilisation of the leaf / stem surfaces) and non-sterile material. Sterilisation of material entailed the washing of the material in sterile $_dH_2O$ to remove soil particles and other debris, after which the material were rinsed vigorously of in a 3.5% (v/v) hypochlorite solution (Coss Ultra, G. Fox & Co., South Africa) for 30 seconds and thereafter in 70% (v/v) ethanol for one minute, and finally the leaves and stems were rinsed in sterile $_dH_2O$. Non-sterile control leaves and stems were rinsed in $_dH_2O$ to remove excess soil particles and debris, after which it was dried by dabbing on sterile paper towel (Webster *et al.*, 2003, Oyebanji *et al.*, 2009).

The positive control on non-sterilised material entailed that whole (uncut) material was pressed onto the NM agar and SFM agar plates as to observe the presence of non-endophytic microbes on the leaf surface (both adaxial and abaxial) and stem surface. The isolation of endophytes entailed the cutting of cross-sections through the centre of the sterile leaf after which the leaf sections were pressed onto the agar. Cross sections of the sterilised stems were also pressed onto the agar. Sterile material was also ground with a sterile pistil in an Eppendorf tube with 500 µℓ SFM broth

after which the homogenised liquid was spread onto NM agar and SFM agar. A negative control was included and consisted of nutrient agar with added SFM broth. Plates were incubated in the dark at 25°C for ca. 72 hours, or until growth was visible, after which they were observed under the stereomicroscope. Colonies were picked by using sterile pipette tips and streaked onto SFM agar plates for colony purification.

2.2.2. Preparation of spore suspensions

Isolated endophytes which proved to be spore-forming bacteria or actinomycetes (e.g. *Streptomyces* spp.) were included in a spore suspension preparation for future referencing. A fresh plate of pure spore-forming culture was selected to which ca. 9 mℓ sterile $_dH_2O$ was added. The surface was scraped with a sterile rigid inoculation loop without dislodging agar which would block the filtering system. The crude spore suspension was poured into a sterile container and agitated for 1 minute on a vortex. After the suspension was agitated, the crude suspension was filtered through a sterile non-absorbent cotton wool filter. Subsequently, the filtered suspension was centrifuged at 1,000×g for 10 minutes at 4°C to pellet the spores. The supernatant was decanted and the spores placed on ice for a few seconds. Spores were suspended in the residual supernatant by agitation on a vortex for a few seconds after which the spore suspension was placed on ice immediately. To avoid germination of the spores, 2 mℓ of a 20% (v/v) glycerol solution was added to the spore suspension and shortly agitated by vortex. The glycerol spore suspension was snap frozen by inserting the tube into liquid nitrogen and then stored at ca. -70°C until required.

2.2.3. Preparation of cell-free extracts

Cell-free extracts were prepared for both the isolated endophytes of *D. cymosum* and *T. fischeri*. Cultures isolated from the leaves and stems were inoculated in 10 mℓ of SFM broth with added 10 mM NaF. These broth cultures were incubated in a dark

incubator at 27°C for 72 hours after which the crude cultures were submitted to ultra-sonication of 25 pulses at a percentage duty cycle of 40%. Each cycle of 25 pulses was repeated fifteen times, or until a foam appeared, indicating the denaturing of proteins, with a 1 minute interval between each cycle to allow for the suspension to cool down. The crude suspensions were concentrated to ca. 10% of the original volume (ca. 1 mℓ). The crude suspensions were then analysed by ^{19}F-NMR. This was performed by adding 700 µℓ of the crude suspension to 300 µℓ of D_2O (Sigma-Aldrich, USA), after which the sample was submitted to 40,000 scans on a 200 MHz Varian NMR spectrometer. This extraction method proved ineffective as the broth impeded the concentration process by vacuum evaporation.

Alternatively, isolated endophytes were inoculated in 10 mℓ of SFM broth with 10 mM NaF added and incubated in a dark incubator at 27°C for 72 hours. The broth was filtered by vacuum through a sterile Buchi filter fitted with a sterile Whatmann No.1 filter paper. The wet cells (mass ranging from 1-2 g)[2], on the sterile filter paper, were transferred to a sterile container, suitable for ultra-sonication, containing tris(hydroxymethyl) aminomethane (TRIS) buffer at pH7.7. This buffer suspension was ultra-sonicated with 25 pulses at a percentage duty cycle of 40% with each cycle of 25 pulses repeated fifteen times, or until a foam appeared indicating the denaturing of proteins, with a 1 minute interval between each cycle to allow for the suspension to cool down. The sonicated suspension was then enriched with 10 mM NaF and 0.4 mM SAM. This enriched suspension was incubated in a dark incubator at 27°C for 25 hours after which the suspension was centrifuged at 15,000×g for 10 minutes and the supernatant decanted and submitted for analysis by ^{19}F-NMR. This was performed by adding 700 µℓ of the crude suspension to 300 µℓ of D_2O, after which the sample was submitted to 40,000 scans on a 200 MHz NMR spectrometer.

[2] The wet mass of the cells was not valued in this study as it only addressed the qualitative capability of endophytes to fluorinate.

2.2.4. Preparation of endophyte broth cultures

The capability of endophytes, isolated from both *D. cymosum*, to synthesise organo-fluorine compounds in broth was studied by providing the necessary precursors along with the supply of nutrition to the isolated endophytes. Cultures isolated from the leaves and stems were inoculated in 50 mℓ SFM broth with added 10 mM NaF and 4 mM SAM or NMA. Each experiment was conducted in replicates of two with one experiment containing SAM and another NMA. This was also compared to observe the difference between the effectiveness of the endophyte's fluorination capability when provided with different precursors. A control group of endophytes were cultured in the same medium, but without any precursor (SAM or NMA). These broth cultures were incubated in a dark incubator at 27°C for 72 hours and shaken at 110 rpm. The broth was filtered by vacuum through a sterile Buchi filter fitted with a sterile Whatmann No.1 filter paper after which the broth was submitted to vacuum evaporation until completely dry. The dried broth was dissolved in 700 µℓ D_2O and submitted for ^{19}F-NMR at 40,000 scans.

2.3.Results and discussion

2.3.1. Isolation of endophytes

The type of isolated endophytes from *D. cymosum* and *T. fischeri* appeared to be consistent throughout the various experiments respectively. No apparent *Streptomyces* spp. were isolated as of yet, but actinomycetes were isolated nonetheless. The actinomycetes were not verified or identified and visual validation was the main basis to select the endophytes of interest. The SFM medium proved to be the most efficient when isolating endophytes as well as culture purification (**Figure 2.2**).

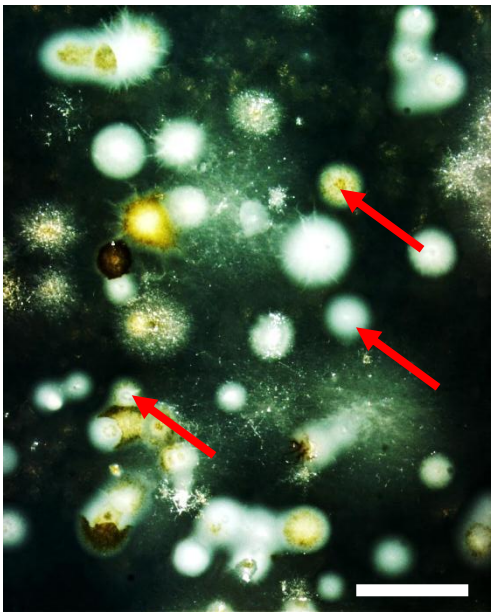

Figure 2.2 SIA plate indicating the diversity of endophytes isolated from *D. cymosum* leaves with the red arrows indicating the colonies which were picked for further investigation. Sclae bar = 5 mm.

2.3.2. Preparation of spore suspensions

The spore suspensions proved to be still viable after six months after preparation with no contamination. This method proved to be invaluable in the sustaining and maintenance of specific spore-forming cultures when applied correctly. This will prove useful in future studies when certain cultures need to be preserved for prospect studies without the organism losing its viability and its ability to synthesise metabolites of interest.

2.3.3. Preparation of cell-free extracts

Despite the modifications to the protocol, the experiments showed some fluorinase biosynthetic activity, but this appeared to be far less than that of the whole-cell broth experiments. This might be ascribed to the protease activity present when cells are lysed or because of the denaturing of the important enzymes during ultra-sonication.

Cell-free extracts of the isolated endophytes of *D. cymosum* showed fluorination capabilities (**Figure 2.3**) in this experiment, but that of *T. fischeri* failed to show any fluorination capabilities. The presence of an organo-fluorine compound with a shift of ca. -131 ppm was observed with ^{19}F-NMR. The concentration of the organo-fluorine compound appeared to be very little and, therefore, it was decided to consider another method to determine the fluorinating capabilities of the isolated endophytes. There was no clear distinction between the fluorination capabilities when isolates from leaves and stems were compared.

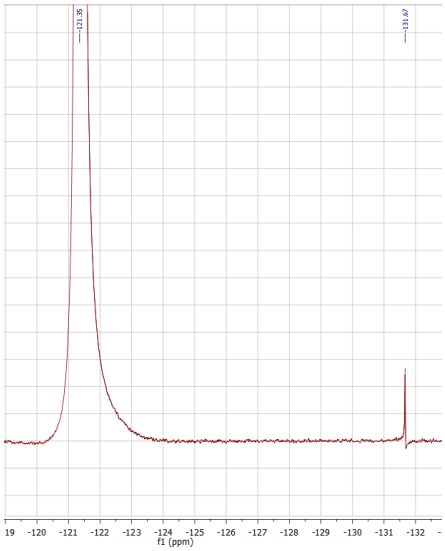

Figure 2.3 The organo-fluorine compound synthesised by a *D. cymosum* isolated endophyte extract is visible on the NMR spectrum at -131.67 ppm. Free fluorine is visible at -121.35 ppm.

2.3.4. Preparation of endophyte broth cultures

Five of the six endophytes isolated from the leaves and one of the two endophytes isolated from the stem of *D. cymosum* and none of the five endophytes isolated from the leaves of *T. fischeri* showed the synthesis of an organo-fluorine compound with a shift of ca. -133 ppm with ^{19}F-NMR (**Figure 2.4**). Although not MFA, this organo-fluorine molecule is due for further investigation nonetheless. Characterisation of the organo-fluorine molecules is an essential to establish the toxicity and the distinctive properties which might lead to the identification of this molecule as a

precursor. Ideally, this experimental setup would be suitable for the study of the synthesis of organo-fluorine compounds in *Streptomyces* spp. as it can be conducted on large scale and requires little input (Tamura *et al.*, 2003).

No distinction could be made between the fluorinating capabilities of endophytes isolated from leaves and endophytes isolated from stems. What appeared to be significant was the fact that the control groups (cultures not receiving precursors SAM or NMA) synthesised significantly less of the organo-fluorine product as compared to those cultures receiving possible precursors. Isolates cultured to obtain second generation progeny proved to synthesise considerably less or no organo-fluorine compounds, indicating that the inheritance of the capability to fluorinate is unstable. This indicates the presence of an enzyme similar or identical to the fluorinase described by Deng *et al.* (2006) which then might explain the volatile behaviour of the activity by the fluorinase homolog.

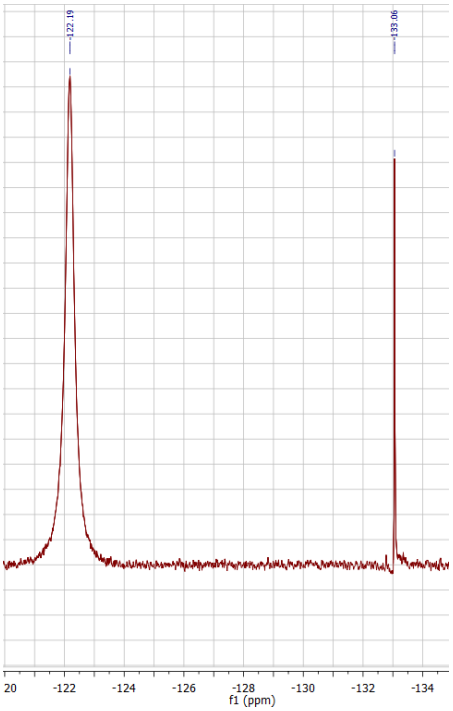

Figure 2.4 Organo-fluorine synthesis by cultured *T. fischeri* endophyte broth with the added precursor SAM. The organo-fluorine compound is visible at -133.06 ppm and free fluorine is visible at -122.19 ppm.

2.4.Conclusions

By utilising various techniques, the endophytes of *D. cymosum* and *T. fischeri* were isolated and, subsequently, their capacity to synthesise fluorinated compounds was determined. The organo-fluorine compound found in the endophyte culture medium might prove to be a trifluorinated compound or a difluorinated compound containing a double bond between the fluorinated carbon and the neighbouring carbon (represented as a singlet on a ^{19}F-NMR spectrum). It is possible that the endophytes of *D. cymosum* and *T. fischeri* synthesise the same fluorinated compound, but because of pH fluctuations in the different samples the chemical shifts of the free fluorine vary (**Figures 2.3 and 2.4**). This also indicate a homologous enzyme in both plant species' endophytes.

Furthermore, this study speculated on the ecological importance of the endophytic entities associated with the members of the family Dichapetalaceae. Evidently, it is clear that it is possible that endophytes might play a role in the production of organo-fluorine compounds, such as the case of *Streptomyces cattleya* producing MFA in culture (Schaffrath *et al.*, 2002). It has, however, not been shown to be an endophyte of *D. cymosum*. Other endophytes have shown to convey toxicity to plants which are present as antifeedants such as pyrrolizidine alkaloids (Johnson, 1985), host protecting compounds such as iron-chelating siderophores (Bacon, 1995; Selosse *et al.*, 2004), pesticides such as lolitrem B (Jensen, 2005), spermicides (Looper *et al.*, 2008), and poisonous alkaloids such as swainsonine (Ralphs *et al.*, 2008).

It might also be that endophytes elicit a response in the plant inducing the production of MFA as a defence mechanism against unwanted microbes.

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Chapter 3 – Morphology of glandular structures of the Dichapetalaceae

3.1.Introduction

A previous study by Van der Merwe and Du Plessis (2006) described the microscopic morphology of *D. cymosum* leaves with standard staining and microscopy methods. The morphological descriptions comprise of the typical features of the epidermal as well as mesophyll cells when haematoxylin and eosin staining (H&E staining) were performed. In their study the leaves of various plant species from the same habitat were studied because of the macroscopic similarities that these plants share with *D. cymosum,* but there was no report of any unusual occurrence pertaining to the features of the leaves.

The experiments conducted in this part of the study of the dissertation attempted to observe the plant's microscopic features in the vicinity where glandular structures have been seen on the leaves. During this part of the project many findings led to tentative speculations in order to infer possible elucidations for the inexplicable results obtained.

3.2. Methodology

The leaves of *D. cymosum* were collected at Kameelfontein near Pretoria, South Africa (S 25 35′ 54.14″; E 028° 24′ 31.01″), whereas the leaves for *T. fischeri* were collected at the Onderstepoort campus of the University of Pretoria, Onderstepoort, South Africa (S 25° 39′ 2.00″; E 28° 10′ 54.56″). Light and electron microscopy was implemented to study the morphology of the leaves and the unknown glandular structures on the abaxial sides of mostly the young leaves.

3.2.1. Sample preparation

Plant samples (with and without glandular structures) were excised from the leaves by using a sterile blade and by cutting the sample (ca. 1 mm^2) in a fixation mixture of gluteraldehyde (5%) and formaldehyde (5%) in 0.2 M phosphate buffer (KH_2PO_4 and K_2HPO_4) at pH 7.2. This fixation mixture also served as the fixation mixture in which

the plant samples were incubated for 3 hours at 23°C to ensure the infiltration of the mixture as to eliminate any air from the samples. Subsequently, the samples were infiltrated with a contrasting mixture of 5% osmiumtetraoxide (OsO_4) in phosphate buffer (for transmission electron microscopy (TEM) purposes) for 24 hours at 23°C. The samples were removed from the osmium mixture and then rinsed with an increasing concentration of ethanol starting at an initial concentration of 30% (v/v) ethanol after which the rinse steps that followed were increased incrementally by 30 % until the samples were finally rinsed in absolute ethanol. These steps were required to remove excess osmium from the sample to ensure contrast and to preserve the samples. The samples were stored in absolute ethanol to preserve the samples until they could be imbedded.

The plant samples were then infiltrated with a 50% (v/v) Quetol 651 epoxy resin mixture in ethanol for 3 hours at 60°C with agitation every hour to ensure uniform infiltration of the resin. Subsequently, the samples were imbedded in Quetol 651 epoxy resin for 48 hours at 60°C. Transverse sections of 1.5 µm were made by means of ultramicrotomy by using a glass knife. These sections were stained with toluidine blue O and observed under the light microscope.

Since MFA is water soluble, it was considered that chemical fixation might have a detrimental effect on the chemical composition of the cells found in the sample. This is especially true when samples are due for electron microscopic analyses such as energy dispersive X-ray spectroscopy (EDS) or electron energy loss spectroscopy (EELS). It was, therefore, decided that high-pressure freezing and freeze substitution (HPF-FS) would be incorporated in later studies rather than chemical substitution. The samples prepared for electron microscopic analyses were also used for light microscopy.

Plant samples, both for electron microscopy and for microscopic analyses, were immersed in hexadecene after which the glandular structures were punched from the leaves under hexadecene by means of a punch with a diameter of 1.2 mm. The punched samples were placed in a syringe filled with hexadecene and a vacuum was created to displace the air present in the samples with hexadecene. This is necessary

to preserve the integrity of the cells during HPF-FS and to prevent the sample from being compressed excessively.

Samples were placed in solid sample holders coated with egg ovalbumin to prevent the sample from sticking to the sample holder after HPF-FS. The samples were then submitted for high-pressure freezing (HPF) after which the samples were placed in either one of three different solutions composed of 1% OsO_4, 1% OsO_4 and 1% uranylacetate, or 1% uranylacetate respectively. These solutions served as pre-contrasting solutions to ensure better contrasting for TEM. These samples were then submitted to freeze substitution for 72 hours after which the samples were removed from the solutions and rinsed with absolute acetone to remove excessive contrasting solution. The rinsing step was repeated three times with each rinse lasting for 10 minutes after which the samples were imbedded as follows: 1% OsO_4 and 1% OsO_4 with 1% uranylacetate contrasted samples were imbedded in Quetol 651 epoxy resin whereas the 1% uranylacetate contrasted samples were imbedded in LR White resin (linear polymerisation resin). These samples were infiltrated and polymerised for 48 hours at 60°C after which transverse sections of 1.5 µm thick through the samples were made by means of ultramicrotomy by using a glass knife. These sections were stained with toluidine blue O and observed under the light microscope.

Both leaves possessing glandular structures and those without glandular structures (control leaves) were incorporated in these studies. Only the glandular structures found on the lamina were studied and the extrafloral nectaries found on the base of the leaves were disregarded for this study. It would, however, be necessary to include these extrafloral nectaries in future studies to serve as reference point for the glandular structures on the lamina.

Preparation for the transverse sections for TEM was performed by means of ultramicrotomy by using a glass knife to ensure a clear polished surface. The transverse sections were cut by means of ultramicrotomy by using a diamond knife. These transverse sections varied between 1.2-1.5 µm (gold to purple colour on the reflective spectrum) in the attempt to prepare samples which are appropriate for tomography analyses.

Sections from samples imbedded in the epoxy resin were picked up on 200 mesh grids after which half the number of grids was submitted to post-contrasting by adding lead acetate. Sections from samples imbedded in the LR White resin were picked up on 300 mesh carbon-coated grids to prevent the unstable resin from deteriorating too rapidly during TEM examination. All grids were then examined by TEM.

The leaves used for scanning electron microscopy (SEM) were collected together with the leaves used for light microscopy and TEM. The leaves were cut into smaller pieces and mounted on a specimen holder by adhesive after which it was sputtered with gold. Low voltage (5 kV) SEM was used to examine the samples.

3.2.2. Quantification of glandular structures

To determine the abundance of the glandular structures found on *D. cymosum* leaves, a simple quantification was performed with all the leaves collected. Both the occurrence of glandular structures on the leaf and the number of leaves housing any form of glandular structures were quantified. Counting of the glandular structures was performed using a stereomicroscope or handheld lens when in the field.

3.3. Results and discussion

In this study, peculiar glandular structures on the abaxial side of the lamina were observed (**Figure 3.1**) which appeared to excrete a watery fluid. At first it was thought to be bacterial nodules as are found with numerous *Pavetta* spp. (Boodle, 1923). The number of glandular structures found on each leaf and the number of leaves housing these glandular structures varies tremendously but, from this part of the study, it appears to be a relatively common occurrence.

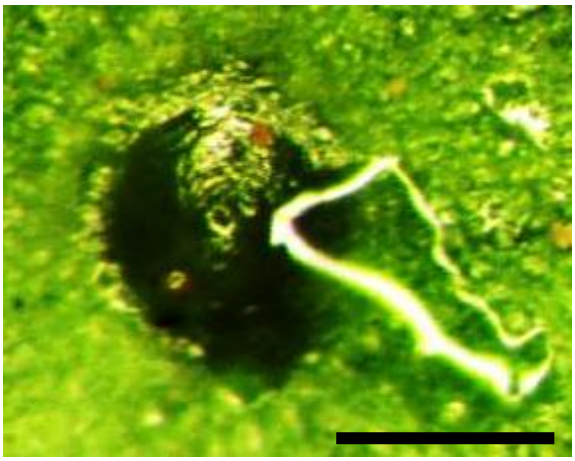

Figure 3.1 Glandular structure found on most leaves on the abaxial side. 'Fresh' marks are commonly found on younger leaves and are mostly associated with the leaf base but, however, are found spread all over the leaf lamina. Scale bar = 1 mm.

Light microscopy revealed the altered morphology, and most probably altered physiology, of the abaxial epidermal cells with the possibility that these structures might either be due to glandular development, or invasive infection. These interesting results led to the further examination of the glandular structures by utilising TEM examination. The results obtained by SEM also added to the intriguing results regarding the formation and purpose of these glandular structures.

3.3.1. Light Microscopy

Observed under low magnification (10×), it was clear that the glandular structures were due to abnormal elongated epidermal cells (**Figure 3.2**). When viewed under higher magnification (40×), the morphology of these abnormal cells showed palisade cell-like characteristics with an increased volume of the cytosol and the presence of polyphenol crystals in the vacuoles of adjacent cells (**Figure 3.3**). These characteristics may indicate the presence of glandular tissue, but confirmation is required. The presence of polyphenol crystals may also be indicative of invasive agents and that the leaf is in attempt to isolate the infected cells. Further study is required to establish the cause of this phenomenon.

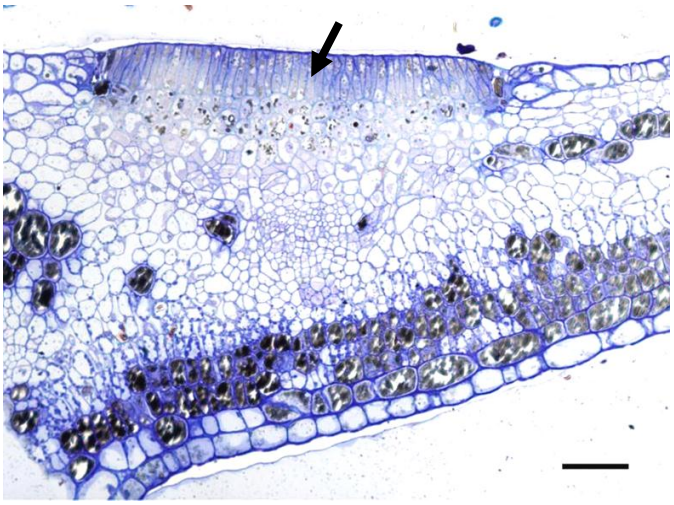

Figure 3.2 Abnormal, elongated epidermal cells (arrow), which are responsible for the appearance of the glandular structures, clearly show the altered morphology of the cells. Scale bar = 10 µm.

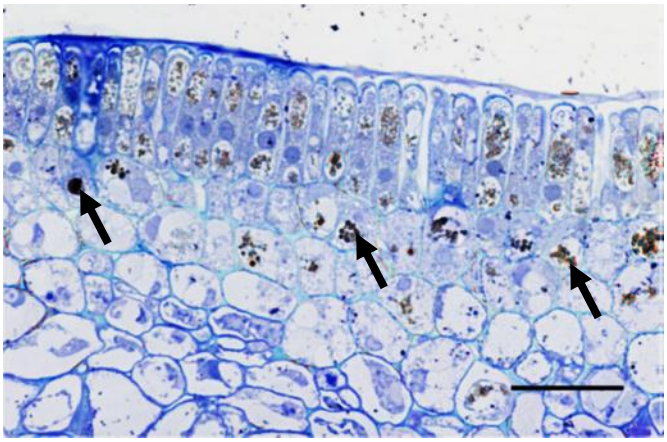

Figure 3.3 The occurrence of an increased volume of cytosol in the abnormal epidermis cells as well as the presence of polyphenol crystals in adjacent cells' vacuoles (arrows). Scale bar = 5 µm.

After imbedding the samples in LR White resin, it was attempted to determine the quality of staining and contrasting under light microscopy. The difference in staining and contrasting, compared to the results from the initial studies, was insignificant and, therefore, no improvement could be detected.

It was interesting to note that tissue damage was present in some of the samples (**Figure 3.4**). At first it was speculated that the HPF-FS might have caused the damage but this was ruled out by the fact that most samples contained this damage and all control samples showed no HPF-FS damage. The damage seems as though it might have been caused by a piercing action, but verification is required by observing the suspected parties (e.g. insects). A second explanation could be that a type of scale insect (tobacco whitefly (*Bemisia tabaci*) or greenhouse whitefly (*Trialeurodes vaporariorum*)), observed during SEM studies, could be the cause. These are thought to be a type of whitefly which is known to transmit plant viruses by means of their stiletto mouth parts (Duffus, 1987, Wisler *et al.*, 1998). This speculation, however, needs confirmation.

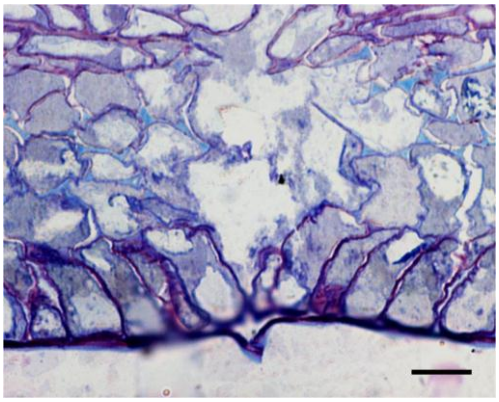

Figure 3.4 Tissue damage found in nearly all samples with damage mostly found near the centre of the glandular structure. Scale bar = 1 µm.

3.3.2. Transmission electron microscopy

Tedious searching for any indications that might explain the cause of the glandular structures led to interesting results. The spongy parenchyma cells adjacent to the abnormal epidermis cells contained in their chloroplasts a particular arrangement of what appeared as virus-like particles (VLPs) (**Figure 3.5**). After close examination it was suggested that these VLPs were too small to be viruses but it might be that they might have their origin from viruses. It is, nonetheless, not unfeasible for these VLPs to be viruses and all possibilities are considered, including the fact that they might be

protein bodies with viral origin. Analogous to these discovered VLPs are phytoferritin which is responsible for binding iron and share similarity in their arrangement within chloroplasts (Hyde *et al.*, 1963, Maramorosh & Hirumi, 1973).

Numerous formations were observed but all of the arrangements pertained to a circular or arched type arrangement (**Figure 3.6**) with what seemed to be a core which is globular in character (**Figure 3.7**). At higher magnification it is clear that these VLPs appear to have a similar morphology to viruses but the diameter of these particles range between 7-8 nm (**Figure 3.8**) and viruses between 20-400 nm (Melnick, 1983; Ghedin & Claverie, 2005). Phytoferritin was identified previously as a crystalline agglomerate of electron-opaque granules with similar dimensions arranged in slightly curved arrays occurring in deteriorated chloroplasts of mesophyll cells (Maramorosh & Hirumi, 1973) and even in cambial tissue (Robards & Humpherson, 1967). Electron-translucent areas around the opaque granules (**Figure 3.8**) are characteristic proteinaceous shells found in ferretin of both animals and plants (Robards & Humpherson, 1967). Therefore, the speculation was that the observed VLPs might prove to be phytoferritin.

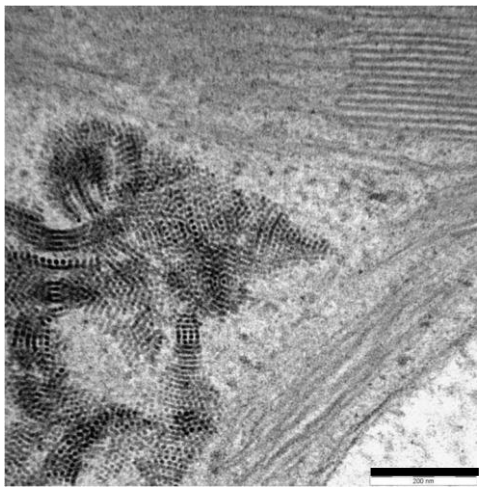

Figure 3.5 Virus-like particles found in the chloroplasts of the spongy parenchyma cells adjacent to the abnormal epidermal cells of the glandular structures. Scale bar = 200 nm.

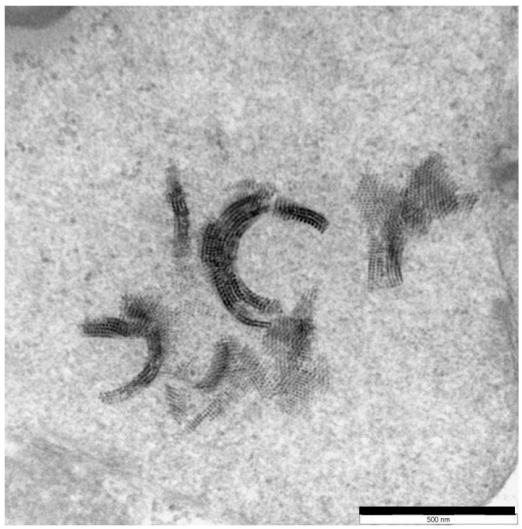

Figure 3.6 Virus-like particles were mostly arranged in a circular or arched formation. Scale bar = 500 nm.

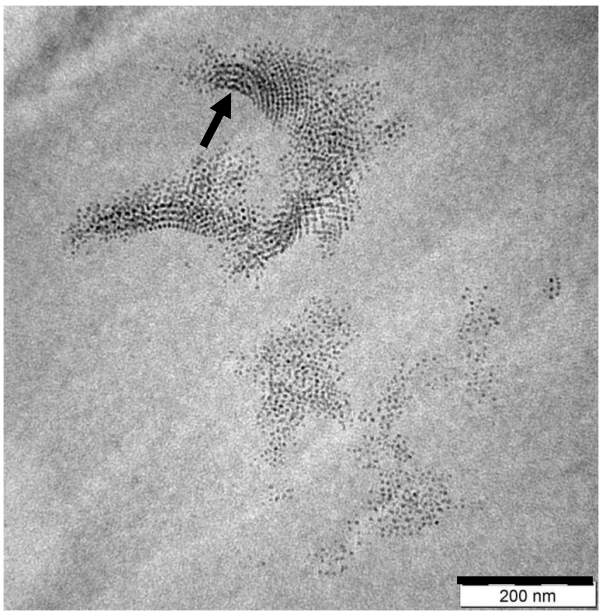

Figure 3.7 Virus-like particles showing the possibility of a globular core (arrow). Scale bar = 200 nm.

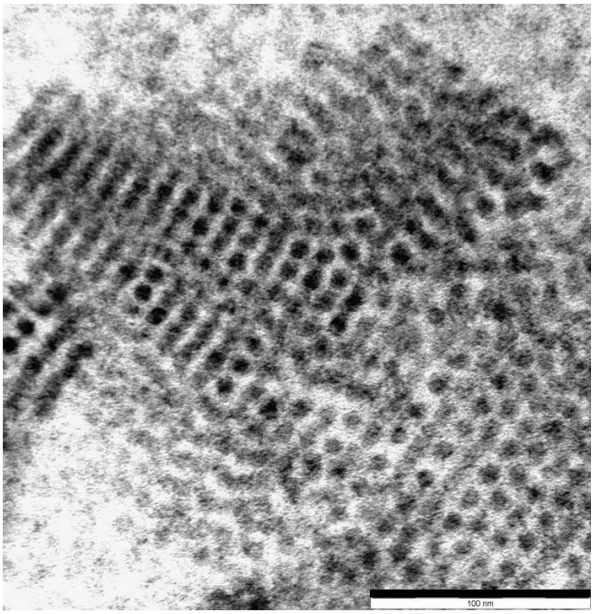

Figure 3.8 Higher magnification showed the morphology of these VLPs to correspond with that of virus capsid proteins and also closely resemble the structure of phytoferritin with clear presence of an electron-translucent area around the electron-opaque particles indicative of a proteinaceous shell. Scale bar = 100 nm.

Further investigation by performing reconstruction of the sample by means of tomography (Appendix A) showed that the VLP arrays appear as organised sheets of rod-like subunits with an aspect ratio of 4:1 bearing cubic symmetry. However, the curvature of the arrays could also be indicative of the VLPs interaction with nucleic acid, thus, leading to the curvature observed in the array. This might lead to the speculation that these VLPs are of viral origin. By utilising EELS, it can possibly be established whether iron is associated with these VLPs in order to confirm whether this might be phytoferritin. This technique may also lead to the answer whether these structures possess the ability to sequester MFA to a specific location to avoid poisoning of the plant. Because of the botanical iron (Fe) biomineral content of the phytoferritin in the form of magnetite (Fe_3O_4) and haematite (Fe_2O_3) (Gajdarziska-Josifovska & Owen, 2002), it may act as an electrostatic mechanism to non-covalently bind to negatively charged salts such as MFA to prevent it from entering other compartments of the cell such as mitochondria. This sequestering mechanism,

however, will still allow the plant to remain poisonous to any animal that consume the leaves as the MFA will be released as soon as the phytoferritin is degraded.

3.3.3. Scanning electron microscopy

To study the anatomy of the glandular structures, low voltage (5 kV) scanning electron microscopy (SEM) was employed. The glandular structures on the lamina showed some resemblance to that of the extrafloral nectaries found on the basal end of the leaves (**Figure 3.9**). This gave rise to various questions which pertain to the cause of the glandular structures on the lamina. During the study it was observed that a type of whitefly nymph was often present on the abaxial side of the leaf (**Figure 3.10**). This finding further substantiates the observation of tissue damage observed with light microscopy where the damage appeared as a piercing to the centre of the glandular structure. Another observation was the presence of some kind of 'footprint' composed of membranous residue that is supposedly left by these whitefly nymphs (**Figure 3.11**). It was also noted that a specific fungal spore with a remarkable structure (**Figure 3.12**) predominated the leaf surface and this may prove worth investigating.

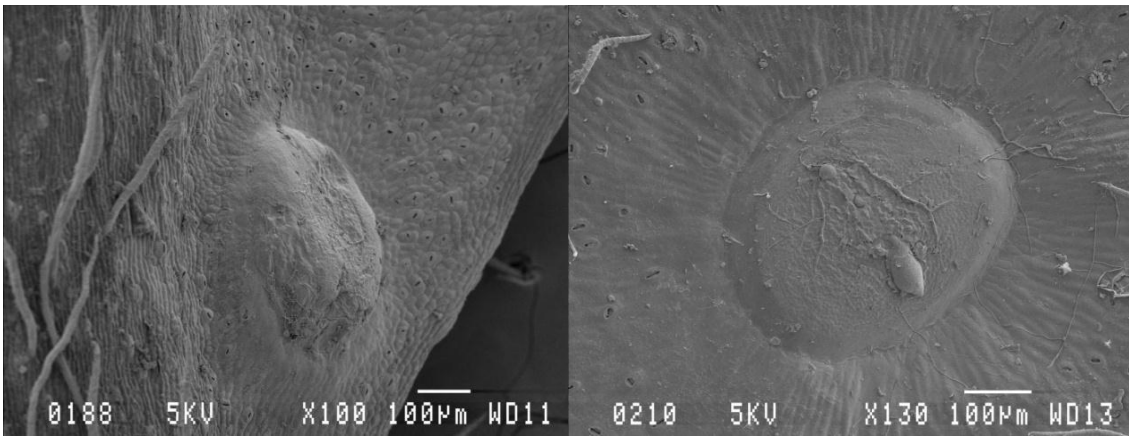

Figure 3.9 A comparison between the extrafloral nectaries (left) and the glandular structures on the lamina (right). Scale bars = 100 µm.

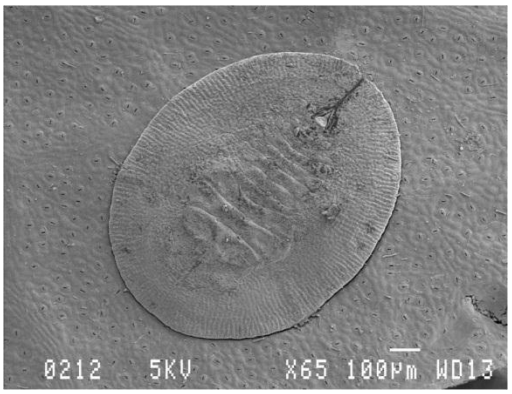

Figure 3.10 A whitefly nymph found on the abaxial side of the leaf of *D. cymosum*. Scale bar = 100 µm.

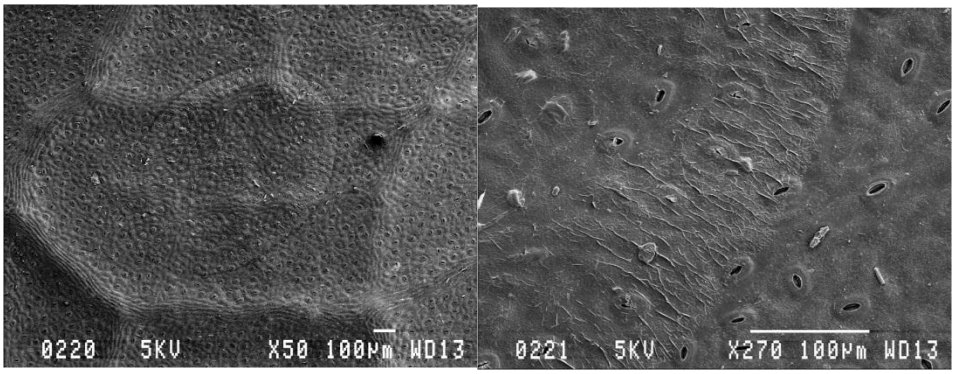

Figure 3.11 A 'footprint' (left) of the same size and shape as the whitefly nymph shown in **Figure 3.10**. Note the membranous characteristics of the 'footprint'. Scale bars = 100 µm.

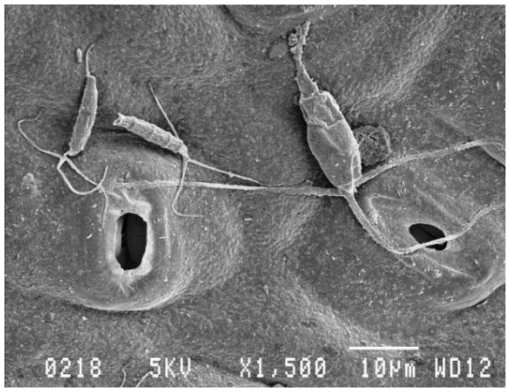

Figure 3.12 Fungal spores predominating the abaxial leaf surface. Scale bar = 10 µm.

3.3.4. Quantification of glandular structures

It was considered that the occurrence of the glandular structures on the leaves was affected by the change of seasons and, therefore, only leaves collected in spring and summer were surveyed to omit any variation. The number of leaves containing glandular structures varied and ranged from 28% to 97% of leaves showing the presence of glandular structures, but the average number of leaves containing glandular structures was 58% of all leaves collected (**Table 3.1**). The number of glandular structures per leaf varied tremendously and ranged from one to 15 glands per leaf, but the average was four glandular structures per leaf. This also correlates with the abundance of the whitefly nymphs during these months and may prove to be the cause for these glandular structures. This postulate arises after it was observed that the leaves collected at Faerie Glen Nature Reserve showed fewer of the glandular structures than what was found on the leaves collected at Kameelfontein. This correlates to the abundance of whiteflies in the respective areas where fewer whiteflies have been observed in Faerie Glen Nature Reserve. It is also known that whiteflies transmit virus diseases (Duffus, 1987) which, in turn, may lead to the formation of phytoferritin (Craig & Williamson, 1969; Allen, 1972; Goodman *et al.*, 1977).

Table 3.1 The quantification of glandular structures found on the leaves. Collections 1, 3-5 were at Kameelfontein and Collection 2 was at Faerie Glen Nature Reserve.

	Collection 1 03/2009	**Collection 2** 07/2010	**Collection 3** 09/2010	**Collection 4** 10/2010	**Collection 5** 02/2011	**Total**
Leaves with glandular structures	80	94	82	124	299	679
Total number of leaves	196	342	132	200	306	1176
% of glandular structures on a leaf	40.82	27.49	62.12	62	97.71	58.03
Average number of glandular structures per leaf (standard deviation)	2.46 (1.47)	2.76 (2.08)	7.46 (3.40)	2.51 (1.24)	1.71 (0.89)	3.38 (1.82)

3.4. Conclusions

At this stage it is not clear what the characteristics of the virus-like particles are and it is suggested that further studies are conducted to establish the properties of these particles. It is, however, clear that these VLPs share some similarities with phytoferritin agglomerates, and this indicates that the whitefly nymphs might be responsible for the formation of the glandular structures. It can be considered that whiteflies may transmit certain elicitors such as viruses and, in the event of infecting the plant, causes a response to limit the spread of the infection (Craig & Williamson, 1969). The formation of these phytoferritin agglomerates may also be ascribed to the high iron content of the soil (Gajdarziska-Josifovska & Owen, 2002) and that the plants are remediating the soil and sequestering the organic iron in mature plastids in a non-toxic form (Robards & Humpherson, 1967).

The relationship of the glandular structures on the lamina and the extrafloral nectaries needs to be established and verified. A definite explanation as to how and when the glandular structures are formed is still due but will expectantly be cleared by future examination. Another intriguing quest is to determine the correlation between the virus-like particles and the MFA synthesis within the plant, by endophytes, or perhaps both.

3.5. References

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Chapter 4 – Presence of fluorinated compounds in *Tapura fischeri*

4.1. Introduction

Little is known about this genus of the family Dichapetalaceae in terms of its phytochemistry but, however, it is known that some members are poisonous (Quignard *et al.*, 2004). Restricted mostly to the tropics, the genus *Tapura* consists of tree members unlike many *Dichapetalaceae* spp. which vary in size. *T. fischeri* Engl. (leaf berry tree) is a semi-deciduous to deciduous forest tree which grows in the understory or along forest edges (Friis & Vollesen, 1985) and is usually associated with *Terminalia* spp. restricted to the northern parts of KwaZulu-Natal (Torre, 1963). Although this plant species may not contain MFA, novel phaeophytins were discovered in the plant which may prove worth investigating in future (Schwikkard *et al.*, 1998).

Other members, especially *T. amazonica* Poepp., are known to be poisonous and have been recorded to be used for such purposes. *T. amazonica* (Quignard *et al.*, 2004) and *T. guianensis* (Fanshawe, 1953) are used as fish poisons by natives in America and, therefore, it can be suspected that *T. fischeri* may be poisonous as well. This being said, *T. fischeri* (**Figure 4.**1) is referred to by some as 'gifboom' but it is not sure what the poisonous entity is. It is important to identify and characterise the unknown compound(s) which might be responsible for the plant's toxicity.

The possible presence of a fluorinated compound in *T. fischeri* could prove to be a remarkable occurrence which may substantiate the fact that organo-fluorine compounds are commonly found amongst the members of the family Dichapetalaceae. This chapter, therefore, investigates the presence of organo-fluorine compound(s) and elaborates on the chemistry of *T. fischeri*.

Figure 4.1 The leaves and flowers of the *T. fischeri* specimen at Onderstepoort. Scale bar = 5 cm.

4.2. Methodology

4.2.1. Fluorinated compound analysis

Leaves of *T. fischeri* were collected during the late morning at the Onderstepoort campus of the University of Pretoria, Onderstepoort, South Africa (S 25° 39′ 2.00″; E 28° 10′ 54.56″). The fresh mass of the leaves was 500 g which were homogenised in a total volume of 2 ℓ absolute methanol for 5 minutes. The homogenate was shaken for 24 hours on a shaker to allow for proper extraction after which the homogenate was left to stand for 30 minutes to allow for the sediment to precipitate. The extract was filtered through a Buchi filter with a Whatmann No. 3 filter paper after which the sediment was rinsed with 1 ℓ absolute methanol and filtered. The remainder of the material was extracted with 1 ℓ distilled water for 24 hours and the filtration step was repeated as for methanol. After collecting all filtrate, the methanol-rich filtrate was added to the water-rich filtrate and then partitioned with ca. 500 mℓ absolute chloroform by shaking in a separating funnel. After separating the parts, the water/methanol part was dried by means of rotavaporation yielding an extract mass of 74.6 g.

Since fluorinated compounds react strongly with silica (Ou & Janzen, 1997), it was decided to utilise the separating capabilities of Sephadex and reverse-phase (C_{18}) silica. A gel preparation of ca. 250 g Sephadex LH20 powder was mixed with a 5% (v/v) water in methanol solution. A 500 mℓ column was filled to the top and, after settling of the Sephadex, the dried water/methanol sample was dissolved in a 5% (v/v) water in methanol solution and loaded onto the column. The eluting solvent consisted of the 5% (v/v) water in methanol solution from fractions 1-60 after which the running solvent was changed to absolute methanol from fractions 71-73. The column was washed with an additional 500 mℓ absolute methanol.

Fractions were collected at intervals of ca. 5.0 mℓ accompanied by the detection of compounds by means of thin layer chromatography (TLC) and UV detection (254 nm and 340 nm). This was performed by spotting the collected fraction onto the TLC plate and developing the plate in a running solution composed of 30 ethyl acetate: 5 formic acid: 5 acetic acid: 5 water. Once developed, the TLC plate was viewed under short and long wave length UV light to observe any fluorescing and absorbing compounds. The plate was subsequently dipped in vanillin (25g vanillin, 95 mℓ ethanol, 5 mℓ H_2SO_4) and heated to expose the reactive compounds present.

All fractions were dried by means of rotavaporation after which the fractions were combined into "superfractions" as shown in **Table 4.1**. Dried "superfractions" were dissolved in 1 mℓ D_2O and submitted to 40,000 scans on a Varian 200 MHz NMR machine for ^{19}F-NMR analysis.

Table 4.1 Fractions combined into superfractions based on similar composition of compounds as visualised by TLC.

Fractions combined	Superfractions
1-5	C1
6-12	C2
13-29	C3
30-36	C4
37-46	C5
47-72	C6
73-wash	C7

4.3.Results and discussion

Fractions were analysed as they were collected from the column. Thin layer chromatography was utilised to establish the presence of compounds by vanillin staining (**Figure 4.2**). The NMR results showed that fractions 30-46 contained a fluorinated compound but with traces of other compounds. It is, therefore, necessary to perform subsequent purification in future as the TLC indicated the presence of other compounds in the same fractions as the fluorinated compound. These steps, such as reversed phase HPLC, will ensure the recovery of the pure fluorinated compound (**Chapter 5**).

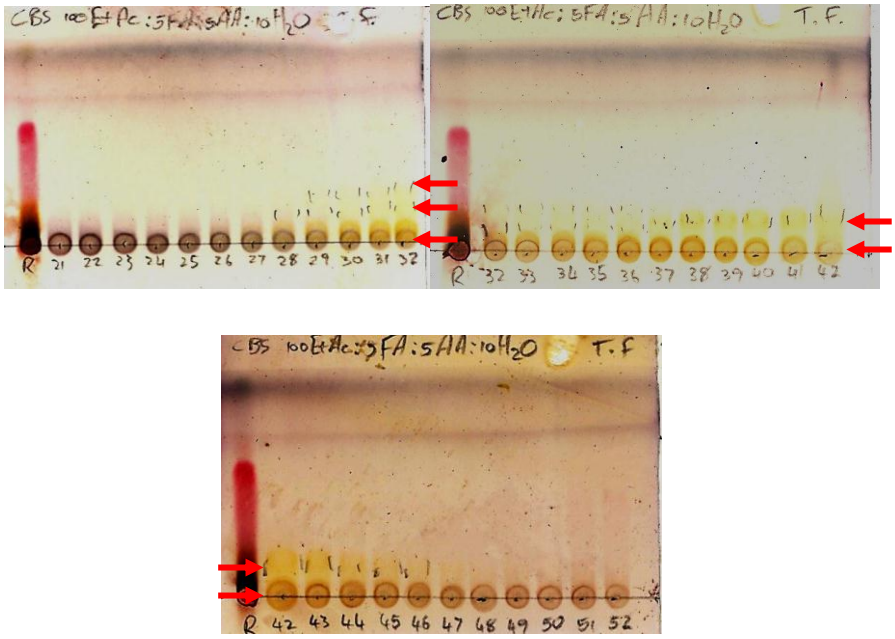

Figure 4.2 The composition of the fractions collected for the Sephadex column chromatography with a running solvent of 5% (v/v) H_2O in methanol. Fractions 30-46 showed the presence of a fluorinated compound after conducting ^{19}F-NMR, but at least one other compound was present (arrows) in each fraction. R = crude extract.

After analysing the superfractions by ^{19}F-NMR, it was confirmed that "superfractions" C4 and C5 contained a fluorinated compound of interest and these

"superfractions" was preserved for future study (**Figure 4.2**). It is speculated that the fluorinated compound may be a trifluorinated compound due to the singlet with a chemical shift of ca. -76.1 ppm which is similar to that of trifluoroacetic acid (-76.55 ppm) or other difluorinated carbons with double-bonded neighboring carbons (ca. -50 to -120 ppm) (Dolbier Jr., 2009), but elucidation of the structure will be performed once the compound is purified.

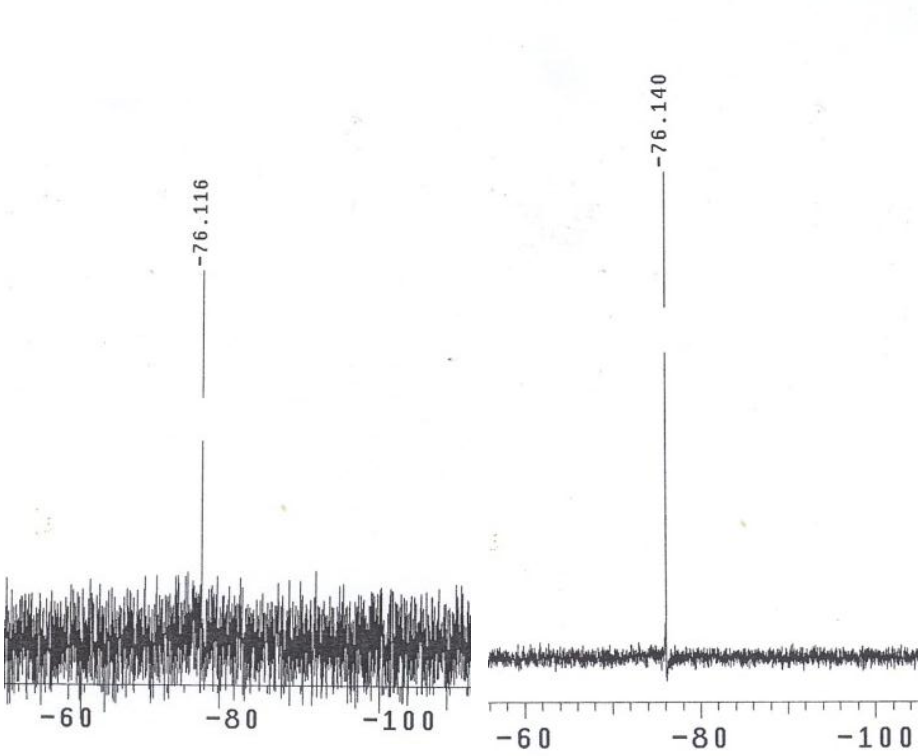

Figure 4.3 The ^{19}F-NMR results for superfraction C4 (left) and C5 (right) with C5 showing a larger quantity of the fluorinated compound.

No pure fractions could be obtained and the purification of the fluorinated compound is continued by utilising chromatographic techniques. Toxicity tests could also be conducted on the isolated compound to establish the toxicity of the fluorinated compound.

4.4.Conclusions

It was shown that *T. fischeri* contains a fluorinated compound which needs to be identified and the structure elucidated but it is required to first purify the compound of interest. This shows that fluorinated compounds occur in both of the members of South African Dichapetalaceae and might prove to be a shared characteristic among the family. Toxicity studies should be done in future to establish the toxicity of the organo-fluorine compound in the attempt to verify the plant's toxicity and confirm the native reference as being poisonous.

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Chapter 5 – General conclusions and future prospects

5.1.Conclusions

The relationship between endophytes and *Dichapetalum cymosum* remains unclear, but some understanding was gained regarding the metabolism of monofluoroacetic acid (MFA). Through this study perspective was gained on the poisonous compounds of *D. cymosum* as the following objectives were met:

- *D. cymosum* is not the only member of the family Dichapetalaceae that produces an organo-fluorine compound as the presence of a fluorinated compound in *Tapura fischeri* was shown. The presence of fluorinated compounds might prove to be a characteristic of the family Dichapetalaceae as it was shown in these two members;

- The endophytes isolated from *D. cymosum* needs to be identified but various endophytes showed the capability to synthesise a fluorinated compound and this requires further investigation to produce a definite conclusion on the relationship between isolated endophytic microbes and the production of fluoroacetate. Thus far it appears as though the endophytes might be responsible for providing the plant with a precursor to produce fluoroacetate;

- The cause of abnormality in epidermal cells on the abaxial side of most leaves are still unclear but the presence of virus-like particles should be investigated extensively as it might prove to be a physiological response to invading bodies or it might be the site of MFA production; and

- The relationship between these virus-like particles and the production and sequestering of MFA has not been established, but it is speculated that these particles might have a role in the synthesis or sequestering thereof. If these VLPs prove to be phytoferritin, further studies are required as to the reason why these particles form and whether they relate to infectious bodies.

Overall, it can be concluded that biological systems composed of symbiotic associations are complex and requires an appreciable extent of study to comprehend the full scope. This biological system shows just that and, therefore, further research is required. But the speculation is that the endophytic entities play a role in the

production of protective compounds such as the organo-fluorine compounds found in the members of Dichapetalaceae.

5.2.Future prospects

5.2.1. Molecular studies on endophytes

Further isolation of endophytes is required to establish the consistency of their occurrence in *D. cymosum*. Culturing of the endophytes isolated from *D. cymosum* ought to be performed to establish and confirm the presence of the biosynthetic pathway by which MFA might be synthesised. Concurrently to the cell-free extract experiments, genetic molecular techniques should be implemented to identify gene sequences related to the fluorinase gene.

5.2.2. Nuclear magnetic resonance (NMR) spectroscopy to determine the presence of organo-fluorine compounds

Nuclear magnetic resonance (NMR) spectroscopy will prove useful in the determination of the presence as well as the concentration of fluorinated substances. The level of organo-fluorine compounds can be determined by means of ^{19}F-NMR spectroscopy by adding internal standards which serve as references and aids in the comparison of shift values of detected fluorinated compounds. Isolated compounds can be further assessed by determining the concentrations and comparing the significance of the toxic level at such concentration to known LD_{50} values. Structure determination may be conducted in the event of presentation of an unknown fluorinated compound within the plant. Subsequently, ^{13}C-NMR, ^{1}H-NMR, and 2D-NMR spectroscopy could be performed to determine the structure of the isolated organo-fluorine compound. This, however, will be conducted after fractionation by means of HPLC to obtain pure compounds. Nuclear magnetic resonance spectroscopy may be used to establish the purity of fractions after fractionation. Similar steps will be followed for *T. fischeri* to elucidate the structure and properties

of the fluorinated compound. This method is ideal for rapid detection of certain compounds of interest given that the concentrations are adequate and the chemical shift of the compound is known.

After purification of the fluorinated compound, various chromatography techniques ought to be performed in order to isolate the pure fluorinated compound from contaminants. The structure of the fluorinated compound can then be elucidated by nuclear magnetic resonance (NMR) in conjunction with other techniques such as mass spectroscopy.

5.2.3. Analytical microscopy on the anatomy of Dichapetalaceae

Interesting results obtained by microscopy might inspire future initiatives to identify the possible cause of the glandular structures. Light microscopy will be incorporated to study the variation and extent of the glandular structures over time. The tissue damage observed in the samples ought to be verified by observing other samples and by studying the suspected insect.

Transmission electron microscopy (TEM) will prove useful for future studies especially due to the applications possible with this technique. Energy dispersive X-ray spectroscopy (EDS) will prove useful to determine the correlation between the structures and fluorinated compounds whereas tomography will aid in elucidating the spatial arrangement of the virus-like particles. In conjunction with scanning electron microscopy (SEM), TEM will assist in comparing the morphology of the glandular structures and the extrafloral nectaries. It is necessary to determine whether the virus-like particles are present in the extrafloral nectaries as this will clarify the ambiguity regarding the cause of these glandular structures. Examination of the insects will also be of significance as it can be determined whether these virus-like particles might be transmitted to the plant by these insects. Treatment of glandular tissue with ribonuclease and deoxyribonuclease will show if the VLPs are of viral origin (Robards & Robinson, 1968). If the VLPs are of viral origin, the

nucleases will lead to the disruption of the array leaving a disordered aggregate of proteins.

Recent applications with SEM, such as tomography with ultramicrotomy combined with surface scanning, can be used to observe the glandular structures. The observation of the scale insects might indicate some role in the development of the glandular structures and SEM will aid in quantifying the number of occurrences of these scale insects. Scanning electron microscopy will prove useful in studying the habit and occurrence of the fungal spores found on the leaves. It could be determined whether this fungus has any role in the production of MFA of the development of the glandular structures or both.

Microscopic studies to verify the presence of virus-like particles in *T. fischeri* will prove interesting and, therefore, might be included in the microscopic section of future studies.

5.2.4. Experiments on glandular structures

Statistical analyses regarding the abundance of the glandular structure in different plant communities from different areas is an important aspect and will be included in future studies. The verification of the difference in concentrations between glandular structures and lesion-free tissue will be a major aspect of future studies that will yield clarification regarding the function and origin of the glandular structures.

5.2.5. High Performance Liquid Chromatography (HPLC) to fractionate and analyse extracts

The fluoroacetate content of *D. cymosum* has been determined by various analytical methods, but they proved to be inadequate and more sensitive methods had to be developed (Vartiainen & Kauranen, 1984). Several chromatographic methods (Tannock, 1975a; Tannock, 1975b; Vartiainen & Kauranen, 1984; Vickery *et al.*, 1973)

and a ^{19}F-NMR spectroscopic method (Baron *et al*., 1987) have been used for the determining of fluoroacetate in plant extracts. A high-performance liquid chromatography (HPLC) method was first incorporated in a study to determine the fluoroacetate concentration of canine gastric content (Ray *et al*., 1981) and further developed by Meyer and Grobbelaar (1989) to determine the fluoroacetate content of *D. cymosum*. This method was developed to provide a direct, fast, sensitive, and specific method to quantify the fluoroacetate content of different parts of *D. cymosum*.

High performance liquid chromatography will provide a means to fractionate extracts to obtain a pure compound for further experimentation. This method is far more accurate to recover fractions and perform the process in a shorter time span. This, however, depends on the nature of the compounds, solvents, and the concentrations thereof. Bioassays may be utilised as to determine which fractions contain poisonous compounds.

5.3.Final remarks

Many findings emerged from the study of *Dichapetalum cymosum* throughout the past century, yet many a question arose from uncertain and unexplainable events which lead to the basis of this study. In an attempt to resolve at least some of these questions, or some new ones, methods and techniques proposed by research will be exploited as to verify, or even further develop, such methods and to promote our understanding for *D. cymosum*, and other members of Dichapetalaceae, and their associated aspects.

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Chapter 6 – Acknowledgements

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- God for granting me the opportunity to acknowledge and truly appreciate the blessings He has bestowed on me – may each frail word be praise to Your splendour.

Job 12:12-13 (KJV)

With the ancient is wisdom; and in length of days understanding. With him is wisdom and strength, he hath counsel and understanding.

Chapter 7 – Apendix: Tomography reconstructions

Tomography reconstructions

See attached CD[3]:

- Video 1: Tomography video constructed to determine the three dimensional orientation of the virus-like particles.
- Video 2: The representation of the virus-like particles in a spacial orientation after the reconstruction of tomography data.

[3] to be viewed with VLC media player run by Windows 7 or another compatible operating system