THE ROLE OF POLYPHENOLS AND THE CELL WALL IN RELATION TO THE DESICCATION TOLERANCE OF THE RESURRECTION PLANT, *Myrothamnus flabellifolia* (Welw.)

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PREFACE

Associate Professor Wolf F. Brandt, Professor Jill M. Farrant and Associate Professor George G. Lindsey of the Department of Molecular and Cellular Biology and Doctor Neil Ravenscroft of the Department of Chemistry at the University of Cape Town supervised this thesis. Research undertaken in France was supervised by Professor Azeddine Driouich of the Centre Commun de Microscopie Electronique at the University of Rouen. It is hereby declared that this thesis, submitted for the degree of Doctor of Philosophy in Molecular and Cell Biology at the University of Cape Town, is the result of my own investigation, except where the work of others is acknowledged.

Signed by candidate

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LIST OF ABBREVIATIONS

% (w/v)	percentage gram(s) solute per 100 ml
% (w/w)	percentage gram(s) solute per 100 grams
µg	Microgram
µl	Microlitre
[M-H]	Molecular ion (deprotonated)
~	Approximately
°C	Degrees Celsius
^{13}C	Carbon-13
^{1}H	Proton
A_{260}	Absorbance reading at 260 nm
A_{280}	Absorbance reading at 280 nm
A_{600}	Absorbance reading at 600 nm
aa	Amino acid
AAPH	2,2- azobis(2-amidino-propane) dihydrochloride
AGPs	Arabinogalactan proteins
Ara	Arabinose
bar	one atmosphere pressure
bp	base pair (s)
BSA	Bovine serum albumin
C. plantagineum	*Craterostigma plantagineum*
C. wilmsii	*Craterostigma wilmsii*
Ca	Calcium
CCD	Charged coupled device
CDTA	Cyclohexane-1,2-diamine-tetra-carboxylic acid
cm	Centimetre
CO_2	Carbon dioxide
Cu^{2+}	Copper ions
Da	Dalton
dH_2O	distilled water
DHB	Dihydroxybenzoic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dw	Dry weight
ER	Endoplasmic recticulum
ESI	Electrospray ionisation
EtBr	Ethidium Bromide
eV	electron-Volt(s)
Fe	Iron
Fig.	Figure
FTIR	Fourier transform infra-red
Fuc	Fucose
g	gravitational acceleration (9.8 $m.s^{-2}$)
Gal	Galactose
GalA	Galacturonic acid
GC	Gas chromatography

GLC	Gas Liquid Chromatography
Glc	Glucose
GlcA	Glucuronic acid
h	hour(s)
H_2O	Water
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HF	Hydroflouric acid
HNO_3	Nitric acid
HPLC	High Performance Liquid Chromatography
hrs	hours
HSP	Heat shock protein
ICP MS	Inductively coupled plasma mass spectrometry
K	Potassium
kb	kilo base
kDa	kilo Dalton
KOH	Potassium hydroxide
kV	kilo-Volt(s)
LEA	Late embryogenesis abundant
m	Metre
M. flabellifolia	*Myrothamnus flabellifolia*
m/z	mass to charge ratio
mA	milli-Absorbance
MALDI-TOF	Matrix Assisted Laser Desorption/Ionisation Time-of-Flight
Man	Mannose
Mg	Magnesium
mg.g dw^{-1}	milligrams per gram dry weight
$MgCl_2$	Magnesium chloride
min	Minute(s)
ml	Millilitre
mm	Millilmetre
mM	milli-Molar
mm^2	Square millimetre(s)
M_r	Molecular mass
mRNA	Messenger RNA
MS	Mass Spectrometry
MW	Molecular weight
Na	Sodium
$NaBH_4$	Sodium borohydride
NaCl	Sodium chloride
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
nm	Nanometre(s)
nM	nano-Molar
NMR	Nuclear magnetic resonance
nt	nucleotide(s)
$O_2^{\cdot -}$	superoxide radical
PAGE	Polyacrylamide gel electrophoresis

PCR	Polymerase chain reaction
pH	Hydrogen ion concentration
PMSF	Phenylmethylsulphonyl fluoride
psbA	Photosynthetic subunit A
PVP	Poly[1-vinylpyrrolidone-2]
rbcL	rubisco large subunit
rbcS	rubisco small subunit
Rha	Rhamnose
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP	Reverse phase
rRNA	Ribosomal RNA
R_t	Retention time
RT-PCR	Reverse transcriptase polymerase chain reaction
RT	Room temperature
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RWC	Relative water content
S. cerevisiae	*Saccharomyces cerevisiae*
SD	Standard Deviation
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
smHSP	Small heat shock protein
Taq	*Thermus aquaticus*
TBS	Tris buffered saline
TEM	Transmission electron microscopy
TFA	Triflouroacetic acid
T_g	Glass transition temperature
T_m	Melting temperature
TOF	Time-of-Flight
Tris	2-Amino-2-(hydroxylmethyl)-1,3-propanediol
trnH	Histidine biosynthesis gene(s)
Tween 20	Polyoxyethylenesorbitan monolaurate
UV	Ultraviolet light
V	Volt(s)
X. humilis	*Xerophyta humilis*
X. viscosa	*Xerophyta viscosa*
Xyl	Xylose
ε	molar extinction coefficient
$\mu g.g\ dw^{-1}$	micrograms per gram dry weight
µM	micro-Molar
μm^2	micrometres square

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ABSTRACT

Resurrection plants are unique in that their vegetative tissue has the ability to survive reversible dehydration to an air-dry state. The widespread African resurrection plant *Myrothamnus flabellifolia* (Welw.), a woody shrub, is one of the largest of these plants. In addition to its resurrection ability it also is an important medicinal plant and is used by local tribes in the various regions where it grows to treat a variety of ailments. This thesis has investigated a number of morphological, ultratsructural and biochemical adaptations of the leaves of *M. flabellifolia* to desiccation and associated stresses. The main aim of this thesis was to ascertain the role of polyphenols and the cell wall of *M. flabellifolia* in relation to its desiccation tolerance. The first part of thesis investigated the anatomical and ultrastructural adaptations of the leaves before and after desiccation. This study illustrated that *M. flabellifolia* leaves utilise both constitutive protection (e.g. wax coated ribs and sclerenchyma support) as well as induced protective measures (e.g anthocyanin accumulation and tissue compaction) in response to desiccation. A further biochemical study revealed that dehydrating leaves accumulated saccharides and retained their photosynthesis associated proteins and pigments during desiccation while concomitantly decreasing their metal ion content. These studies suggested that *M. flabellifolia* prepares its leaves for controlled rehydration and the rapid resumption of photosynthesis upon favourable conditions. The main polyphenols isolated from the leaves of *M. flabellifolia* were characterised to be galloylquinic acids. These galloylquinic acids were shown to protect membrane systems against desiccation and free radical associated oxidation. These molecules were shown to accumulate in cell vacuoles to between 25 and 50 % of the leaf dry weight. These molecules also possess medicinal properties which may provide a rationale for the use of this plant in traditional medicine. A study of the genetic and galloylquinic acid compositional variation between populations of *M. flabellifolia* occurring in South Africa versus

those in Namibia revealed unique differences. These differences are suggestive of Namibian populations possessing the ability to remain in the quiescent state (desiccated) for significantly longer periods than those in South Africa. The final part of this thesis concentrated on the leaf cell wall of *M. flabellifolia* and its response to desiccation. A complete microscopic, biochemical and immunocytochemical analysis of the leaf cell wall was performed before and after desiccation. The microscopic study revealed that the bulk of the thin-walled mesophyll and epidermis cells folded their cell walls upon desiccation whilst the thick-walled vascular and sclerenchyma cells remained unfolded and likely supported the surrounding tissue during desiccation. The biochemical study revealed that the cell wall generally conformed to that found in other dicotyledonous plants except that a high constitutive concentration of arabinose polymers were noted in both hydrated and desiccated plants. Apart from some minor desiccation-induced wall remodelling the leaf cell wall remained largely unchanged upon desiccation. We propose that these constitutively present arabinose polymers, predominately associated with the pectin matrix, impart the necessary flexibility to the *M. flabellifolia* cell wall allowing it to withstand desiccation. It appears therefore that *M. flabellifolia* has evolved a unique combination of morphological, ultrastructural, chemical and biochemical adaptations in order to acquire tolerance to desiccation and its associated stresses.

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CHAPTER ONE

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Water is essential for life on this planet. It covers two thirds of the earth's surface and is a primary chemical constituent of our planet. It was in the depths of the primordial 'first' sea that it is believed that life evolved from simple chemical reaction cycles, chemical reactions that required an aqueous solvent system (Wächtershäuser, 1998). Water has been life's cradle by governing the physical and biochemical parameters that define life processes (Garrett and Grisham, 1995). Water is a biological solvent, it determines diffusion rates of chemical compounds in metabolism, being incompressible it provides structural support to cells and tissues, through lone pair interactions it forms a hydrogen bonded lattice through which it directs hydrophilic interactions with other molecules and indirectly controls hydrophobic interactions between molecules (Garrett and Grisham, 1995). The properties of water determine the biochemical processes of all known living organisms.

Green plants were amongst the first organisms to successfully invade the land (Niklas, 1997). Since then they have been 'waging a continuous war for water' (Niklas, 1997). Plants need water to maintain their physical and metabolic integrity as well as to facilitate reproduction (Campbell, 1993). The uneven distribution and limited availability of terrestrial water means that plants need to compete with each other as well as other organisms for this vital resource (Niklas, 1997). It has been estimated that approximately one third of the earths land surface is subjected to regular droughts and is defined as arid or semi-arid (Ramanathan, 1988). Many plants have adopted one of three strategies to survive water deficit stress (Bewley and Krochko, 1981). These strategies are evasion, avoidance or tolerance of water stress (Bewley and Krochko, 1981). Some plants

evade water stress by undergoing their growth and reproductive cycles when sufficient water is available (Bewley, 1979; Bewley and Krochko, 1981). Therophytes do not produce perennating vegetative buds and so survive the dry periods by producing desiccation tolerant (orthodox) seeds (Bewley, 1979). Geophytes evade water stress as dormant corms, rhizomes and bulbs buried beneath the soil (Bell and Woodcock, 1983). In contrast, succulents, such as the Cacti and Euphorbia, avoid water stress by increasing their capacity to store water in their leaves or stems and have concomitantly evolved mechanisms to prevent water loss from these storage organs such as thick cuticles (Niklas, 1997). These plants survive on their stored water reserves during periods of water scarcity (Niklas, 1997). Certain plants are able to tolerate water stress by dehydrating and rehydrating their cellular protoplasm without experiencing permanent damage and are termed desiccation tolerant (Bewley, 1979; Gaff, 1971; 1977). The common examples of plant tolerance to water deficit stress are orthodox seeds which are desiccation tolerant (Bewley, 1979). Yet examples of desiccation tolerance of vegetative tissue do occur in a number of lower plants and a few angiosperm families (Gaff, 1971; 1977). Many lower plants are capable of surviving desiccation including mosses such as *Tortula ruralis* (Alpert and Oliver, 2002) and lower tracheophytes such as *Selaginella dregii* (Goldsworthy, 1992). Many of the angiosperm species which are desiccation tolerant are unique to southern Africa (Gaff, 1971; 1977) (see Figure 1.1.). These desiccation tolerant species are commonly called 'resurrection plants' (Gaff, 1971; 1977).

Figure 1.1: South African resurrection plants: *Craterostigma wilmsii* in the desiccated (A) and hydrated (B) state; *Xerophyta humilis* in the desiccated (C) and hydrated (D) state; *Xerophyta viscosa* (E) and *Eragrostis nindensis* (F) in the hydrated state.

1.2 DESICCATION TOLERANCE IN PLANTS

Desiccation tolerance has been defined as the ability of an organism to revive following the loss of 80 – 95 % of cellular water (Bewley, 1979; Porembski and Barthlott, 2000). A more operational definition proposes that desiccation tolerance is the ability to dry to equilibrium with 'moderately dry' air, 'specified as 50 – 70 % relative humidity at 20-30°C', followed by the resumption of normal function upon rehydration (Alpert and Oliver, 2002). Effectively these organisms possess the capacity to suspend or reduce cellular metabolism when dehydrated and upon rehydration to resume their normal metabolism (Gaff, 1971; 1977).

Desiccation tolerance is common amongst lower plants such as mosses, ferns and their spores (Hoekstra et al., 2001). Most of the seeds of higher plants are orthodox and are therefore desiccation tolerant (Bewley, 1979; Hoekstra et al., 2001). In contrast to lower plants, vegetative desiccation tolerance in higher plants is rare (Oliver et al., 2000; Alpert and Oliver, 2002). Desiccation tolerance has evolved in only a few angiosperm families whilst no known gymnosperms display desiccation tolerance in their vegetative tissue (Alpert and Oliver, 2002; Hoekstra et al., 2001). Systematic and phylogenetic studies have indicated that desiccation tolerance has evolved independently on a number of occasions within the plant kingdom (Alpert and Oliver, 2002; Oliver et al., 2000). It has been suggested that desiccation tolerance is a 'universal potential' of all plant cells and tissues (Alpert and Oliver, 2002). This capability appears only to have been selected for in plants during the resting stages of their life cycle, i.e seeds, or in organisms that have not been able to evade or avoid desiccation, i.e. resurrection plants (Alpert and Oliver, 2002). Desiccation tolerance has been hypothesised to be the first widespread solution to land colonisation by aquatic green plants (Oliver et al., 2000). It has been proposed that evolution of early water transport and vascular tissue in certain plant lineages

resulted in a loss of tolerance to desiccation as the associated selection pressures diminished (Oliver et al., 2000). Spores, seeds and pollen separated from a constant water supply retained desiccation tolerance although this tolerance is believed to have been lost in the case of recalcitrant seed species (Bewley, 1979; Alpert and Oliver, 2002). It has been hypothesised that re-evolution of desiccation tolerance in adult plants of various lineages occurred as these vascular plants colonised new frontiers on land (Alpert and Oliver, 2002; Porembski and Barthlott, 2000).

Approximately 300 species of vascular plants are desiccation tolerant with the majority occurring in the ferns and monocotyledons (Porembski and Barthlott, 2000). In the monocotyledons the Velloziaceae is considered the largest resurrection plant family comprising over 200 desiccation tolerant species, an example being the resurrection plant *Xerophyta viscosa* (Kubitzki, 1998) (see Figure 1.1.). There are far fewer dicotyledonous resurrection plant taxa than there are monocotyledonous (Porembski and Barthlott, 2000). The Scrophulariaceae are among the more diverse dicotyledonous resurrection plant families (Porembski and Barthlott, 2000) with several members including the resurrection plant *Craterostigma plantagineum* which has been used as a model system for studying the molecular mechanisms of plant desiccation tolerance (Ingram and Bartels, 1996; Scott, 2000; Bartels and Salamini, 2001). Another member of the family, *Craterostigma wilmsii,* has been used as a model organism to study the physiological responses to desiccation by resurrection plants (Farrant, 2000; Farrant et al., 1999, 2003) (see Figure 1.1.). The small dicotyledonous family Myrothamnaceae comprises only two species, *Myrothamnus flabellifolia* and *Myrothamnus moschatus,* both of which are desiccation tolerant (Porembski and Barthlott, 2000) (see Figure 1.2.).

Many resurrection plants occur in southern Africa although species are also found in Australia, South America and southern Europe (Gaff and Churchill, 1976; Bewley and Krochko, 1981; Gaff, 1987). Over 90 % of resurrection plant species have been reported to occur on inselbergs

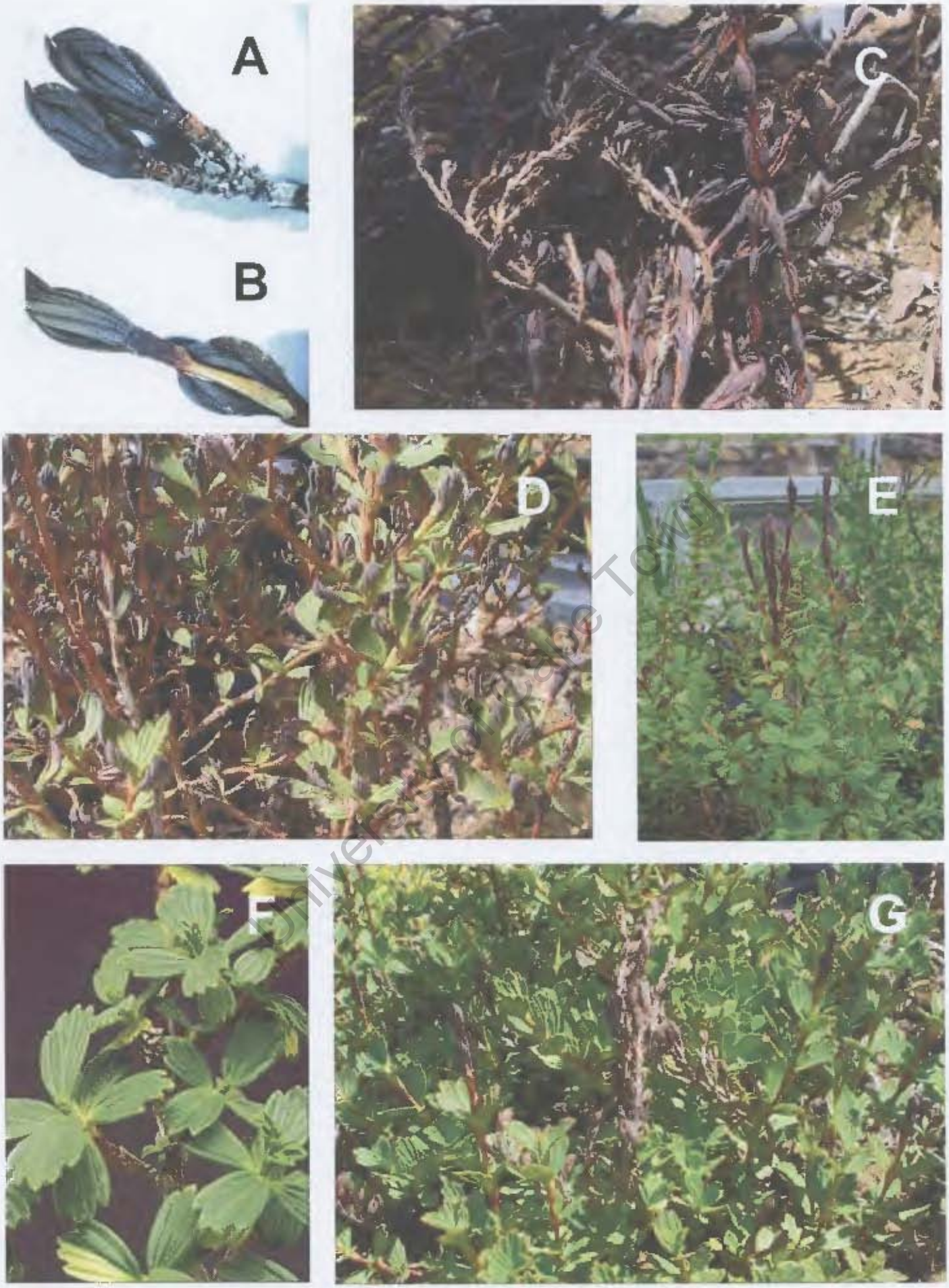


Figure 1.2: The African resurrection plant *Myrothamnus flabellifolia* (Welw.) in the desiccated (A, B, C), partially rehydrated (D) and hydrated (E, F, G) state.

(Porembski and Barthlott, 2000). These are bare rock outcrops which experience extremes of irradiance, temperature and water availability (Kruger, 1998; Porembski and Barthlott, 2000). It is proposed that resurrection plants are not just passive survivors of harsh environments but are active colonisers of new land frontiers (Alpert and Oliver, 2002; Porembski and Barthlott, 2000). The first plants colonised the land from the sea, resurrection plants are believed to have gone one step further by colonising the rock from the soil (Alpert and Oliver, 2002).

1.3 PROTECTIVE MECHANISMS AGAINST DESICCATION

Desiccation causes considerable disruption of cellular structure and metabolic function in living cells and tissues (Crowe et al., 1997). Desiccation tolerant organisms have evolved mechanisms to repair damage upon rehydration, prevent damage using constitutively present mechanisms or achieve tolerance by using a combination of prevent and repair strategies (Alpert and Oliver, 2002; Oliver et al., 2000). Repair strategies are more prevalent in lower organisms and a well studied example of an organism using the repair strategy is the moss *Tortula ruralis* (Wood and Oliver, 1999; Alpert and Oliver, 2002). In contrast angiosperm resurrection plants primarily utilise a prevention of damage strategy by laying down protection during drying (Sherwin and Farrant, 1996; Farrant, 2000; Cooper and Farrant, 2002). This strategy requires the plants to be able to cope with desiccation induced mechanical and metabolic stress (Sherwin and Farrant, 1996; Farrant, 2000).

Mechanical stress can be defined as the tension that develops between and within the plasma membrane and cell wall due to a desiccation-induced loss of turgor pressure resulting in cell plasmolysis (Walters et al., 2002). To counter mechanical stress resurrection plants must be able to maintain the osmotic balance of the cell, prevent disruption of cellular integrity, i.e. membranes and cell walls, as well as stabilise cell macromolecules which are required for the resumption of normal cell function (Iljin, 1957; Vertucci and Farrant, 1995; Hoekstra et al., 2001; Walters et al., 2002). As a dehydrating cell loses turgor, tension develops between and within the collapsing plasma membrane and the rigid cell wall (Iljin 1957; Walters et al., 2002). This cytoplasmic shrinkage away from the cell wall places strain on plasmalemma-cell wall contacts and once plasma membrane rupture occurs cell death follows (Iljin, 1957; Walters et al., 2002). In addition cell wall rupture can occur in response to rapid drying should the tissue have insufficient

time to counter the stress or be too rigid as is the case for the leaf tissue of most desiccation sensitive species (Brett and Waldron, 1996; Farrant and Sherwin, 1998; Vander Willigen et al., 2003). Seeds fill their cell vacuoles with storage proteins and cytoplasm with lipid bodies and carbohydrates which are proposed to prevent mechanical damage (Farrant and Sherwin, 1998; Farrant et al., 1997). Certain resurrection plants, specifically *X. viscosa*, *X. humilis*, *Sporobolus stapfianus* and *Eragrostis nindensis*, adopt a similar vacuolar filling strategy (Farrant, 2002; Vicré et al., 2004a, Vander Willigen et al., 2004) (see Figure 1.1.). The opposite approach is to undergo extensive cell wall folding so that the plasma membrane remains appressed to the cell wall during dehydration. This strategy has been adopted by *C. plantagineum* and *C. wilmsii* (Jones and McQueen-Mason, 2004; Vicré et al., 2004b) (see Figure 1.1.). Concomitantly with desiccation-induced cell wall folding an accumulation of small vesicles has been observed to develop in the leaf mesophyll cells of certain resurrection plants (Farrant and Sherwin, 1998). These vesicles have been hypothesised to function as membrane reserves for rehydration and/or storage bodies containing osmoprotectants (Walters et al., 2002).

Loss of water from a cell results in an osmotic imbalance as cell organelles, membranes and macromolecules, dependent on water for their structure, begin to destabilise and denature (Crowe et al., 1997). In response to dehydration, desiccation tolerant organisms have been shown to accumulate compatible solutes (osmolytes) and water stress proteins which are believed to counter the destabilising and denaturing effects of desiccation on cell protoplasm (Crowe, 1992; Mtwisha et al., 2005).

Compatible solutes such as proline, glycine-betaine, glutamate, mannitol, cyclitols, trehalose, sucrose and oligosaccharides have all been shown to increase in response to drought or desiccation stress (Tymms and Gaff, 1979; Koster and Leopold, 1988; Crowe et al., 1993; Drennan et al., 1993; Horbowicz and Obendorf, 1994; Bohnert et al., 1995; Ingram and Bartels,

1996). Compatible solutes have been hypothesised to function in two ways to alleviate osmotic stress (Hoekstra et al., 2001). One is via a 'water replacement' mechanism (Crowe et al., 1993; Hartung et al., 1998). The polyhydroxy moieties of the solutes have been hypothesised to 'preferentially hydrate' the cell membranes and macromolecules replacing the lost water, thereby ensuring that intracellular stability is maintained (Hoekstra et al., 2001). This mechanism of 'preferential exclusion' and 'preferential hydration' has been proposed to function at high to moderate water contents during drought, freezing and general osmotic stress alleviation (Hoekstra et al., 2001). The concentration of accumulated compatible solutes is believed to be insufficient to account for protection at lower water contents (below 30 % H_2O w.dw^{-1}) as only in the case of saccharides is protection observed (Hoekstra et al., 2001). The second mechanism proposes that compatible solutes assist in the formation of cytoplasmic glasses in desiccated cells (Leopold, 1990; Ingram and Bartels, 1996; Buitink et al., 1998). A glass is an amorphous metastable state possessing both solid and liquid properties (Buitink et al., 1998; Hoekstra et al., 2001). Chemical reaction and molecular diffusion rates are much reduced in the glass state creating a condition of 'nearly suspended animation' (Hoekstra et al., 2001). Cytoplasmic glass formation (vitrification) can only occur at water contents below 10 % H_2O w.dw^{-1} governed by the Tg, the liquid-to-glass transition temperature (Hoekstra et al., 2001). Carbohydrates, such as sucrose and oligosaccharides, are particularly prone to glass formation and so are the main osmolytes implicated in cytoplasmic vitrification (Buitink et al., 1998; Hoekstra et al., 2001).

In addition to compatible solutes certain water stress proteins have been shown to accumulate in response to osmotic stresses (Mtwisha et al., 2005). The LEA (late embryogenesis abundant) proteins are small hydrophilic proteins which accumulate in seeds upon maturation and in vegetative plant tissue in response to light, heat, cold, osmotic and salt stress (Bartels et al., 1993; Bewley et al., 1983; Cai et al., 1995; Close, 1996; Imai et al., 1996; Ismail et al., 1999; Russouw et al., 1995; Russouw et al., 1997). LEA protein expression can also be hormonally induced with

abscisic acid (ABA) treatment (Bartels et al., 1993; Mundy and Chua, 1988). The function(s) of this diverse family of proteins is not certain although it has been suggested that they may function in 'water replacement' in an analagous manner to compatible solutes and as ion sequestration agents counteracting the increase in free metal ion concentration in dehydrating cells (Bray, 1993; Dure, 1993; Dure et al., 1989; Hoekstra et al., 2001; Wise and Tunnacliffe, 2004). Recently it has been shown that LEA proteins can prevent protein aggregation due to water loss (Goyal et al., 2005). In addition some small heat shock proteins (smHSP's) display LEA like properties and have been implicated in the desiccation stress response (Mtwisha et al., 1998). The 12 kDa heat shock protein, HSP 12, in the yeast *Saccharomyces cerevisiae* shows LEA like behaviour and has been shown to be able to stabilise artificial membranes against osmotic and desiccation stress (Mtwisha et al., 1998; Sales et al., 2000). These LEA and LEA-like proteins have more recently been suggested to play a role together with carbohydrates, in stable glass formation (Wolkers, 2000).

Metabolic stress, which can result from cell dehydration, can be defined as the disruption of cell metabolism and photosynthesis which can result in the production of reactive oxygen species (ROS) (Dalton, 1995; Walters et al., 2002). These oxygen radicals are dangerous because they can cause irreversible damage to cell macromolecules and structures (Bewley and Krotchko, 1981; Leopold, 1990; Winston, 1990; Walters et al., 2002). Metabolic stress control measures include the prevention of both free radical induced damage and the accumulation of potentially toxic metabolic intermediates (Seel et al., 1992 a,b; Smirnoff, 1993; Farrant, 2000). Two organelles, the mitochondria and the chloroplast, are normally considered the main sources of ROS in stressed plant cells (Dalton, 1995). In the mitochondria, uncoupling of the electron transport chain, due to membrane disorder and/or the denaturation of associated macromolecules, results in electron transfer to molecular oxygen forming the reactive superoxide radical (Winston, 1990). This electron transfer to oxygen is usually mediated by Ubiquinone (co-enzyme Q)

embedded in the mitochondrial membrane (Winston, 1990). The generated superoxide radical ($O_2^{\cdot}$-) is then able to react further with H_2O_2 and generate additional ROS in the form of singlet oxygen species and hydroxyl radicals (Cadenas, 1995).

In chloroplasts it is the thylakoid membranes, containing the light harvesting chlorophyll arrays, which function to convert light energy into electronic energy to drive carbohydrate synthesis from CO_2 and H_2O (Garrett and Grisham, 1995). The generation of large amounts of O_2 in close vicinity to the high energy electron pathways is a particularly risky albeit successful strategy to synthesise food (Dalton, 1995). Photoinhibition, the process by which molecular oxygen scavenges high energy electrons from the cascade pathways and generates ROS, is a problem faced by all plants on this planet (Dalton, 1995). This problem is compounded during the early stages of water stress when thylakoid membranes and associated macromolecules may denature. The resulting decompartmentation could result in an uncontrolled oxidative burst which may kill the plant (Leopold, 1990; Winston, 1990). Damage caused by ROS in cells include the oxidation of macromolecules, such as proteins, nucleic acids and polysaccharides, as well as lipid peroxidation which results in membrane damage and eventual degradation (Dhindsa and Matowe, 1981; Seel et al., 1992a; Smirnoff, 1993).

General countermeasures used by green plants against light induced ROS production include a combination of both chemical and enzymatic ROS scavenging systems (Dalton, 1995). The chemical systems include an array of antioxidants subdivided into the water-soluble reductants, such as ascorbate, catechol and glutathione, and the fat soluble reductants (vitamins) such as carotene and tocopherol (Cadenas, 1995). The enzymatic systems include enzymes such as glutathione reductase, catalase, superoxide dismutase and ascorbate peroxidase (Ahmad, 1995). These antioxidant enzymes link the chemical reductants to each other in redox cycles which generate reducing power from primary metabolism such as is the case in the Haliwell-Asada

antioxidant cycle (Asada, 1994; 1996). In addition, new chemical antioxidants are being investigated, such as polyphenols in plant and animal systems, for their role in oxidative stress tolerance (Larson, 1995). Green plants, dependent on light driven photosynthesis for carbon fixation, require an extensive system of interconnecting chemical and enzymatic antioxidant pathways to be able to rapidly respond to ROS formation before damage results. These antioxidant systems are further challenged during osmotic stress where uncoordinated metabolism and macromolecular denaturation can result in excess ROS production as well as limit the efficacy of the antioxidant response mounted by the plant. This oxidation problem is carried to the extreme in the case of resurrection plants where complete loss of protoplasmic water occurs.

Angiosperm resurrection plants have evolved two very different strategies to cope with light and oxidative stress (Farrant, 2000; Farrant et al., 2003).

The poikilochlorophyllous resurrection plants dismantle their thylakoid membranes and degrade their chlorophyll in a controlled manner thereby avoiding the generation of ROS from their photosynthetic complexes due to excess light (Farrant, 2000; Sherwin and Farrant, 1996; 1998; Farrant et al., 2003). Poikilochlorophytes include the *Xerophyta* species *X. viscosa* and *X. humilis* (Sherwin and Farrant, 1996) (see Figure 1.1.). Their photosynthetic complexes and chlorophyll pigments are then resynthesised upon rehydration (Dace et al., 1999; Sherwin and Farrant, 1996; 1998; Farrant et al., 2003).

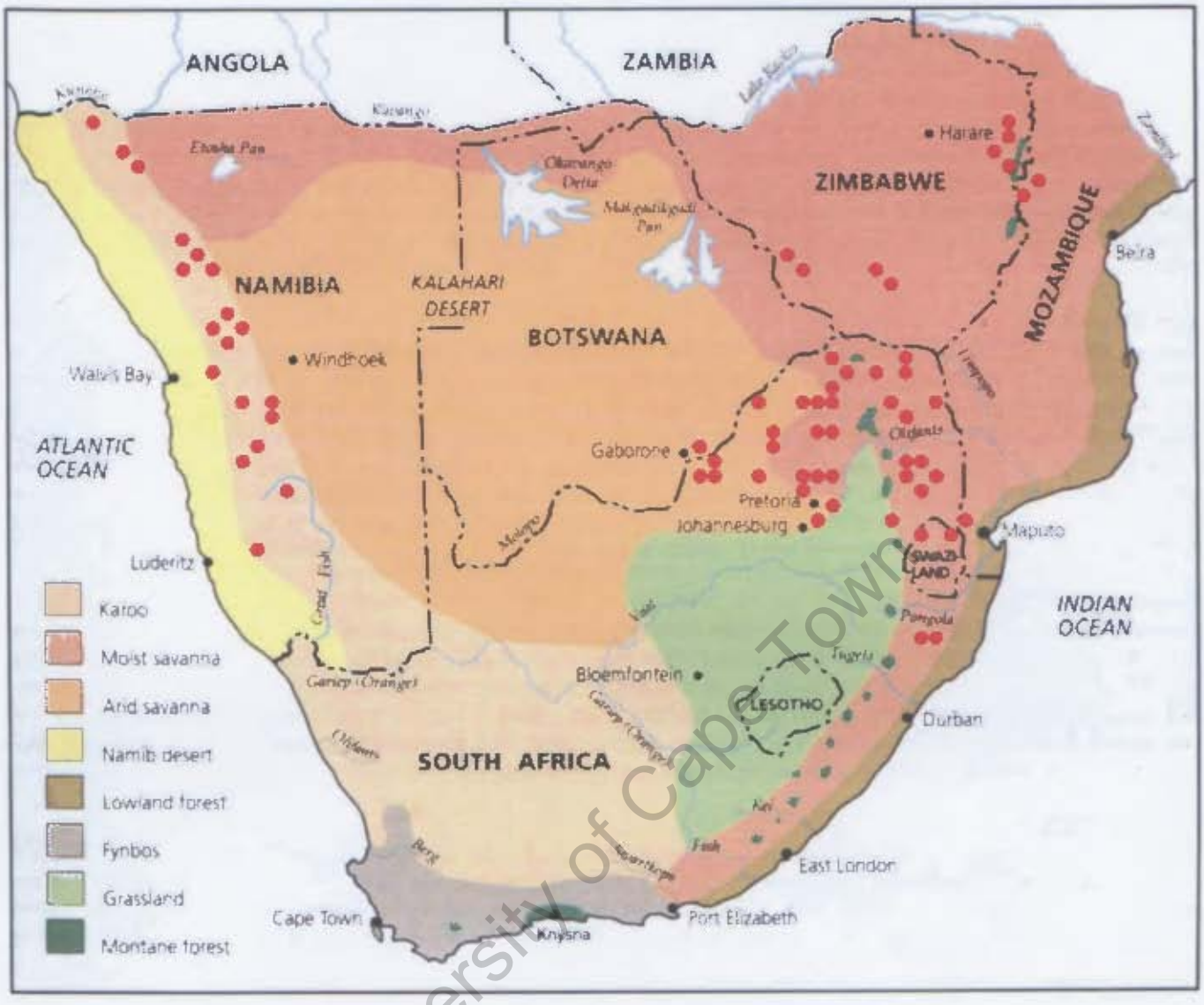
The homoiochlorophyllous resurrection plants, such as *Craterostigma wilmsii* and *Myrothamnus flabellifolia*, maintain their photosynthetic apparatus and chlorophyll intact during dehydration and rehydration (Sherwin and Farrant, 1996; 1998; Farrant et al., 2003) (see Figures 1.1. and 1.2.). Morphological adaptations such as leaf and cell wall folding results in a shielding of the photosystems from excess light upon and during desiccation as well as the subsequent

rehydration period (Farrant, 2000; Farrant et al., 2003). The advantage of this strategy is that a more rapid recovery of photosynthesis and growth is possible upon rehydration as compared to the poikilochlorophytes (Sherwin and Farrant, 1996; 1998; Farrant et al., 2003).

Resurrection plants have evolved unique and effective solutions to both the extreme stress of desiccation as well as its associated effects such as ROS production. Resurrection plants are unique and invaluable model systems for investigating the mechanisms of desiccation tolerance. In addition to desiccation tolerance resurrection plants display tolerance to other abiotic stresses such as hypersalinity and high temperature (Gaff, 1988; Gaff and Wood, 1989; Hartung et al., 1998). This is not surprising as acquiring tolerance to a specific stress often results in some tolerance of related stresses (Mowla at al., 2002) due to cross-talk in stress response pathways (Bartels and Salamini, 2001). The knowledge gained from investigating the mechanisms of desiccation tolerance in resurrection plants is therefore relevant to understanding related stresses such as water-deficit stress and so could be applied in improving the abiotic stress tolerance of economically important crop species.

1.4 THE RESURRECTION PLANT *Myrothamnus flabellifolia* Welw.

Myrothamnus flabellifolia (Welw.) is one of southern Africa's most well known and widely distributed resurrection plants (Weimarck, 1936; Van Wyk et al., 1997; Glen et al., 1999) (see Figures 1.2. and 1.3.). It is a woody shrub usually growing to a height of between 0.5 and 1.5 metres (Sherwin et al., 1998) and occurs on rock inselbergs distributed throughout the southern African region (Goldsworthy, 1992; Porembski and Barthlott, 2000). The plant is a geophyte and possesses an extensive root system which extends into the crevices and cracks of the sandstone and granite slopes upon which it grows (Child, 1960; Mauve, 1966; Glen et al., 1999). It has earned the English vernacular name 'resurrection plant' because of its remarkable capacity to dehydrate its vegetative tissue (e.g. leaves) to an air dry state during the dry season and upon summer rains to revive completely (Glen et al., 1999). During dehydration the plant curls both its leaves and stem segments and changes colour from green to dull-brown (Farrant et al., 1999; Glen et al., 1999) (see Figure 1.2.). When water is provided to the roots the plant revives in a dramatic manner by rehydrating its desiccated tissue resulting in a slow uncurling of both stems and leaves and a return to its original green colour (Glen et al., 1999; Farrant et al., 2003) (see Figure 1.2.). In addition to its resurrection abilities it is also an important medicinal plant in southern Africa well known in tribal African culture (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Van Wyk et al., 1997). The Zulu name the plant 'uvukwabafile' which literally means 'wakes from the dead'. It is this reviving ability seen as a symbol of hope which is believed to be passed on to the ill person during treatment (Hutchings, 1996; Van Wyk et al., 1997). It therefore occupies an important position in traditional African folklore and medicine. The plant was first recorded in the scientific literature by the well known explorer Friedrich Welwitsch in 1859 after whom the bizarre Namib desert plant *Welwitschia mirabilus* is named (Welwitsch, 1859, Welwitsch, 1869; Glen et al., 1999). Welwitsch named the plant *Myrothamnus* a contraction of the greek words *myron* which means aromatic and *thamnos* which



HABITAT MAP OF SOUTHERN AFRICA

Figure 1.3. Habitat map of southern Africa with the different vegetation biomes indicated as different colours (see key on left) and the distribution of *Myrothamnus flabellifolia* (Welw.) indicated by red dots (based on the distribution data reported in Weimarck, 1936; Lisowski et al., 1970; Puff, 1978a and Glen et al., 1999). (Map reproduced from Sinclair et al., 2002 with the kind permission of Struik Publishers, Cape Town)

means bush or shrub because of the characteristic 'balsamic' smell of plant's leaves (Welwitsch, 1869; Puff C, 1978a; Glen at al., 1999). His specific epithet *flabellifolia* means fan-like leaves which are characteristic of this plant (Puff C, 1978a; Glen et al., 1999) (see Figure 1.2.). One of the first articles to refer to the resurrection ability of *M. flabellifolia* was written by Weiss in 1906 and included photographic plates of desiccated and fully hydrated twigs (Weiss, 1906). In the article he remarked on the 'miraculous manner' with which the plant revived when supplied with water and that twigs that were detached and placed in water after four months were still able to recover to full vigour (Weiss, 1906). Thoday later in 1921 referred to *M. flabellifolia* as an example of an extreme xerophyte in his article on drought behaviour of Passerina species in the Cape (Thoday, 1926). Yet although the plant is well known in African tradition and in the early botanical literature it has only been in recent years that significant attempts have been made to fully characterize the plant in a scientific manner (Glen at al., 1999). The resurrection plant has been rather enigmatic in the past as its taxonomic position and evolution remained uncertain, no scientific characterisation of its medicinal usage and active principles had been undertaken while its amazing resurrection ability continued to be a great mystery.

Populations of *Myrothamnus flabellifolia* are found throughout the mountainous regions of central and southern Africa (Weimarck, 1936; Glen at al., 1999) (see Figure 1.3.). The African distribution of the plant is disjunct with populations occurring along the west coasts of Namibia and Angola separated by the Kalahari desert and low lying central regions from eastern populations which extend from South Africa to Kenya (Puff, 1978a; Glen et al., 1999). In the east, populations occur in northern South Africa as far south as northern Kwa-Zulu Natal, as well as in Botswana, Mozambique, Zimbabwe, Malawi, Tanzania and Kenya (Weimarck, 1936; Glen at al., 1999). Isolated populations have been reported in Zamibia and south eastern regions of the Congo (Lisowski et al., 1970). The populations in South Africa have been shown to correlate with

specific granitic and quartzitic geological sequences (Kruger, 1998) and in Namibia populations occur in mountainous regions such the Komas Hochland and the Spitzkoppe (Puff, 1978a). Supporting these observations is a recent study which has shown that rock inselbergs are centres of endemism for the majority of resurrection plants (Porembski and Barthlott, 2000). *M. flabellifolia* is commonly found growing high on mountain slopes and plateaus usually between 900 and 1200 metres above sea level (Puff, 1978a) although there have been reports of populations growing at altitudes of greater than 2000 metres above sea level (Schneider et al., 1999). They are considered to be the initial colonisers of the bare rock slopes and provide opportunities, possibly by improving local growing conditions, for other plant species such as grasses to become established (Child, 1960; Porembski and Barthlott, 2000). The plants occur singly or in colonies with extensive root systems that extend into the crevices of the rocky outcrops (Child, 1960; Goldsworthy, 1992). The older colonies generally consist of a number of individual plants and occur higher up on the mountain slopes whereas the younger plants occur singly and are found lower down on these same slopes (Child, 1960). Plants grow on exposed bare rock crevices where their root network extends into shallow hollows with soil depths of around 15 cm (Child, 1960). Their roots are able to intercept water draining into these hollows after rainfall thereby initiating rehydration but it has also been shown that plants died if transplanted into hollows that retained water for prolonged periods suggesting that *M. flabellifolia* is susceptible to flooding (Child, 1960). Erosion debris and mammal droppings were commonly observed (Child, 1960) around the root network of the plant colonies suggesting a possible means by which *M. flabellifolia* is able to obtain vital nutrients such as nitrogen. *M. flabellifolia* plants seem to be avoided by most herbivores and pests likely due to the high tannin content of both the hydrated and desiccated leaves which probably limits the plants' nutritional value. There is evidence of leaf bite marks on collected material (personal observation) and beetles have been observed chewing the leaves (Child, 1960). The species of beetle responsible has not been identified though beetle pupae have been found attached to plant stems (personal observation)

suggesting a more specific plant-insect interaction. The conditions on the exposed rock outcrops where the colonies are found are such that *M. flabellifolia* is exposed to considerable day/night temperature extremes, high light intensities in addition to an erratic water supply (Child, 1960; Goldsworthy, 1992; Kruger, 1998). These generally unfavourable conditions necessitate that the plant must exist in a desiccated quiescent state for extended periods during the dry winter months and then resurrect when the summer rains arrive allowing it to continue life processes such as growth and reproduction (Goldsworthy, 1992; Glen et al., 1999). These extended quiescent periods have been reported to be variable with different geographic regions (Puff, 1978a; Kruger et al., 2001). In northern areas of South Africa the plants have been reported to survive maximally nine months in the quiescent state (Kruger et al., 2001) whereas in Namibia it is believed that they survive for longer periods, perhaps for even years at a time without resurrecting (Puff, 1978a). Shorter periods between rainfall of only four months have also been reported (Puff, 1978a).

After rainfall the plant is able to undergo a rapid transformation from an air-dry state of suspended animation to a fully transpiring, rehydrated state (Child, 1960; Vieweg and Ziegler, 1969; Glen et al., 1999) (see Figure 1.2.). The absorption of water is reported to only occur via the roots as it has been shown that desiccated leaves are unable to absorb any moisture supplied to their external surfaces (Child, 1960). As *M. flabellifolia* possesses a woody stem (see Figure 1.2.) the ability to successfully refill its xylem conduits and to efficiently transport water to the leaves is a unique challenge encountered by this resurrection plant (Sherwin et al., 1998; Schneider et al., 2000; Zimmermann et al., 2001). The general stem anatomy of the plant reveals a relatively primitive vascular system with narrow xylem vessels and reticulate perforation plates (Tippo, 1938; Carlquist, 1976; Puff, 1978b; Sherwin et al., 1998). Unusual knob-like structures of unknown chemical composition were observed on the outer surface of the xylem vessels

(Sherwin et al., 1998). It is likely that these structures are lipophilic in nature as further studies have revealed novel lipid coatings of the vascular tissue (Wagner et al., 2000). Lipids were found lining all the xylem element inner walls and lipid inclusions were observed to occur within the intervessel pits and tracheid edges (Schneider et al., 1999; Wagner et al., 2000). Lipids were shown to be spatially distributed within the stem where two zones were observed (Shcneider et al., 2003). An inner zone of water non-dispersable lipids as well as an outer zone of water-dispersable lipids where water travels (Schneider et al., 2003). The lipids of the outer zone are believed to facilitate and enhance the advance of water in the xylem elements and pits (Schneider et al., 2003). The lipid distribution of the stem is also believed to be responsible for the distinct pattern of axial and radial water movement imaged by nuclear magnetic resonance spectroscopy of hydrating branches of *M. flabellifolia* (Schneider et al., 2000; 2003). The major physical factors believed to be involved in the refilling kinetics of *M. flabellifolia* branches include root pressure, capillary action and associated driving forces (Schneider et al., 2000; 2003). It was suggested that the plants unique internal cuticle is important for preventing complete water loss as well as maintaining anatomical and cellular separation during desiccation and subsequent rehydration (Schneider et al., 1999). *M. flabellifolia* has therefore evolved a remarkable hydraulic system designed to overcome the extreme challenges of desiccation and rehydration.

The most conspicuous change upon desiccation in *M. flabellifolia* occurs in the leaves which fold like a fan and close with their adaxial surfaces appressed against the stem (Sherwin and Farrant, 1996; Farrant et al., 2003) (see Figure 1.2.). Much of the recent research aimed at elucidating the mechanisms responsible for desiccation tolerance in this species has focussed on the leaves. The loss of protoplasmic water results in considerable anatomical and ultrastructural re-organisation of the leaf tissue in this species (Genkel and Pronina, 1969; Sherwin and Farrant, 1996). The leaves of *M. flabellifolia* have been reported to possess a high tannin content (Pizzi and Cameron,

1986; Koonjul et al., 1999). It was suggested that these tannins may be polymeric flavonoids which could facilitate the concertina like folding of the cell walls observed to occur in *M. flabellifolia* leaves (Pizzi and Cameron, 1986). However, this flavonoid-tannin hypothesis was based on very limited evidence (Pizzi and Cameron, 1986). The first ultrastructural study of *M. flabellifolia* was performed by Wellburn and Wellburn in 1976 which showed an unusual stacking of the thylakoid membranes of the chloroplasts in desiccated leaf tissue (Wellburn and Wellburn, 1976). The term 'staircase grana' was introduced by Wellburn and Wellburn to describe this unique arrangement (Wellburn and Wellburn, 1976). Generally, their attempts at fixation of desiccated leaf tissue were frustrated due to rehydration occurring upon addition of an aqueous aldehyde based fixative to desiccated samples (Wellburn and Wellburn, 1976). The 'staircase grana' or blistered arrangement of the thylakoid membranes of *M. flabellifolia* chloroplasts are believed to be important in the reduction of photo-oxidative stress during dehydration and rehydration (Sherwin and Farrant, 1996; Koonjul et al., 2000; Farrant et al., 2003). This is important as *M. flabellifolia* is homoiochlorophyllous (Sherwin and Farrant, 1996). Later studies of *M. flabellifolia* concentrated on analysing the chemical composition of its leaves with the aim of determining the substances responsible for desiccation tolerance. Suau et al. (1991) showed that arbutin and sucrose were present in desiccated leaves. These findings were disputed by Drennan et al. (1993) who concluded that sucrose and trehalose were the major saccharides present in the leaves with sucrose being induced upon desiccation. A further study of leaf chemistry by Bianchi et al. (1993) reported the presence of large amounts of glucopyranosyl-β-glycerol a known osmoprotectant which increased upon desiccation. A molecular study of the desiccation response of *M. flabellifolia* provides some support to these observations as partial putative carbohydrate biosynthesis and sugar transport mRNA transcripts were found to be upregulated upon desiccation (Koonjul, 1999). However, the levels of some of these saccharides are believed to be insufficient to provide protection to the leaves against desiccation as compared to the saccharide levels observed in other desiccation-sensitive plant species (Drennan et al.,

1993). Finally, a recent study has revealed that the ability of *M. flabellifolia* to revive from the desiccated state is dependent on the levels of antioxidants present in the leaves when rehydrated (Kranner et al., 2002). This highlights the important role the antioxidant biochemistry of resurrection plants has in their desiccation tolerance. Yet despite the recent advances made, the mechanisms which allow resurrection plants to survive desiccation are still enigmatic.

The general morphology of the plant reveals anatomical features suggestive of its unique ecology and reproductive biology (see Figures 1.2., 1.4., 1.5., 1.6. and 1.7.). The following anatomical description is based on previous characterisation by Mendes, (1978); Bywater, (1984); Goldsworthy (1992) and Glen et al. (1999). The leaves are wedge shaped with crenate-dentate leaf apices and are flabellate with 5-7 folds (Figure 1.2.). The leaf folds close parallel to the leaf face upon dehydration (Figure 1.2.). Leaf bases are entire and cuneate. Stipules are simple, connate and persistant with petioles at the leaf base. The plants are dioecious (Glen et al., 1999) with catkin-like inflorescences usually occurring on the shorter lateral branches (Figures 1.4. and 1.5.). Flowers are opposite, decussate and lack a perianth. Male flowers consist of 3-6 stamens with basally connate filaments (Figure 1.4.). The reddish anthers (in tetrads) dehisce longitudinally releasing yellow tricolpate pollen (Figure 1.4. and 1.6.). Female flowers are zygomorphic and are reduced to 3 carpels attached basally (Figure 1.4.). The female ovary is superior and consists of three chambers with each chamber bearing an outwardly curved style (Figures 1.4. and 1.7.). The papillose/spathulate stigmas are reddish-purple and are feather-like in appearance (Figure 1.4.). The pollination syndrome of *M. flabellifolia* is puzzling as its flowers possess both wind pollination and insect pollination features (Glen et al., 1999). It is likely that due to its unique habitat and limited growth period the plant utilises multiple mechanisms to achieve fertilisation. It has been reported that bees visit the male flowers for

Figure 1.4. Hydrated male (A, C, E) and female (B, D, E) flower clusters of *Myrothamnus flabellifolius* (Welw.). Immature male flowers (A). Mature male flowers (C, E) consist of reddish-purple anthers which dehisce longitudinally releasing yellow pollen (E). Female flowers (B, D, E) are zygomorphic, possess three carpels each and have pink (B) or reddish-purple (D, E) stigma's. | Scale bars: a, b, c, d, 10 mm; e, 2.5 mm; f, 5 mm. |

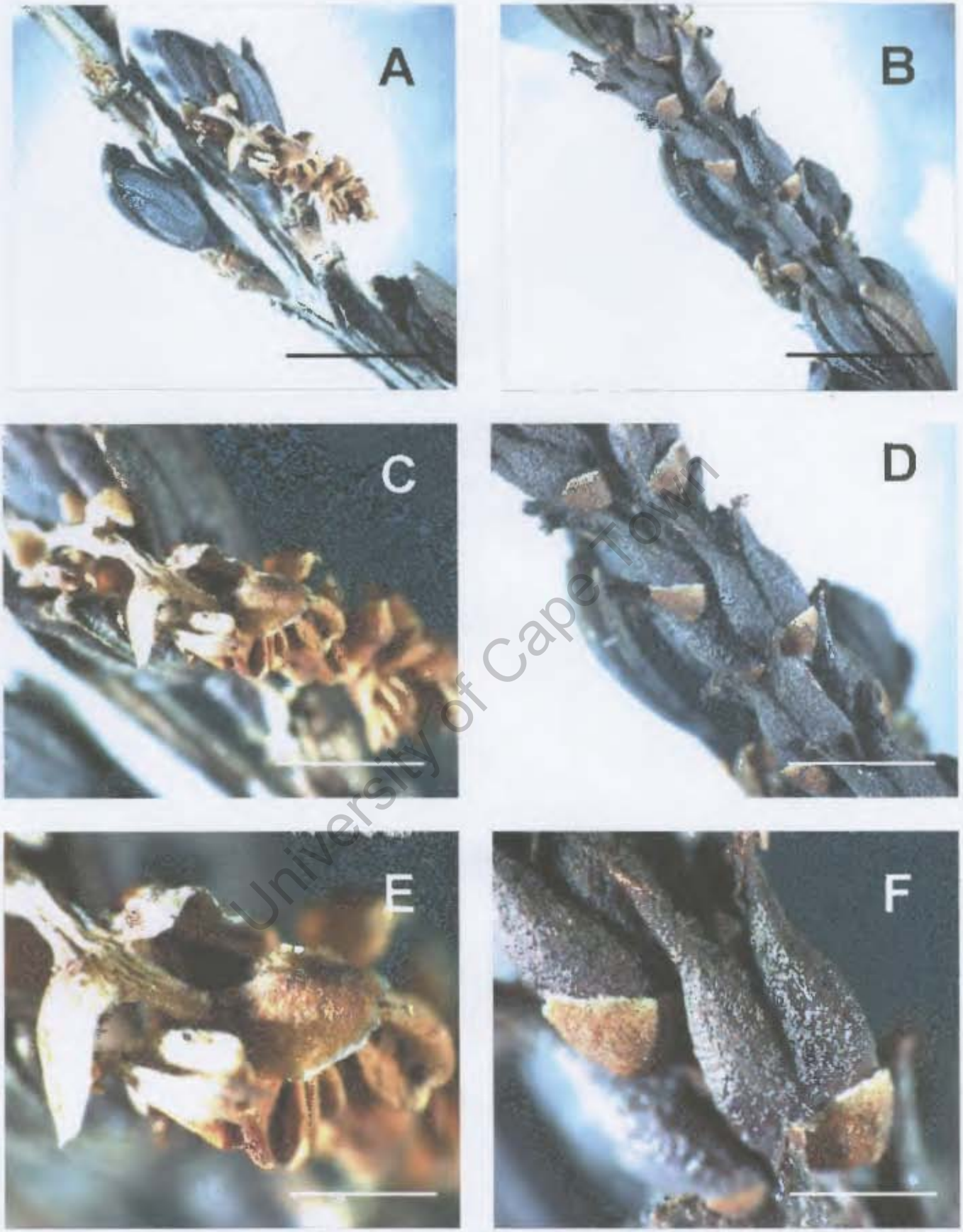


Figure 1.5. Desiccated male (A, C, E) and female (B, D, E) flowers of *Myrothamnus flabellifolius* (Welw.). [Scale bars: a, b, 10 mm; c, 2 mm; d, 5 mm; e, 0.5 mm; f, 2 mm.]

pollen (Child, 1960) and certainly the colourful zygomorphic female flowers (Figure 1.4.) point to an entomophilous pollination mechanism. The fruits are three-lobed coriaceous, dehiscent capsules which are slightly larger than the carpels at anthesis (Figure 1.7.). The remains of the style and stigma crown the capsules (Figure 1.7.). The 0.3-0.5 mm long seeds are ovoid possesing wrinkled seed coats and seem almost certainly wind dispersed (Figure 1.7.). The seeds have been reported to germinate after 10-15 days at 25°C (Goldsworthy, 1992).

Welwitsch initially considered *M. flabellifolia* to belong in the family Saxifragaceae and that it was closely related to the genus *Myrica* because of certain similarities such as their shrub-like habit and aromatic leaves (Welwitsch, 1859; 1869). Sonder then re-aligned *Myrothamnus* with the genus *Cliffortia* also in the Saxifragaceae (Glen et al., 1999) as both plants possess superficial similarities such as small fibrous leaves although on closer inspection the leaves are quite dissimilar from each other (personal observation). *Cliffortia flabellifolia* has entered the literature which may explain the erroneous entries in *Flora zambesica* (Mendes, 1978) and *Flora of Tropical East Africa* (Bywater, 1974) stating that *M. flabellifolia* populations occur in the Cape province. *Cliffortia* species are present in the Cape flora (personal observation) but there have been no reports of *M. flabellifolia* populations occurring as far south as the Cape provinces (Glen et al., 1999). The family Myrothamnaceae consists of two species, both resurrection plant species, these being *M. flabellifolia* as well as *M. moschatus* its Madagascan cousin (Glen et al., 1999). Little is known of *M. moschatus* other than it resembles *M. flabellifolia* in morphology as no extensive scientific investigation of the plant has been performed (Glen et al., 1999). The taxonomic position of the Myrothamnaceae has been uncertain and anatomical studies of the stem and leaf structure suggested the placement of the plants in the Hamamelidaceae (Welwistch, 1869; Glen at al., 1999). This position has been appealing as many Hamamelidaceae species

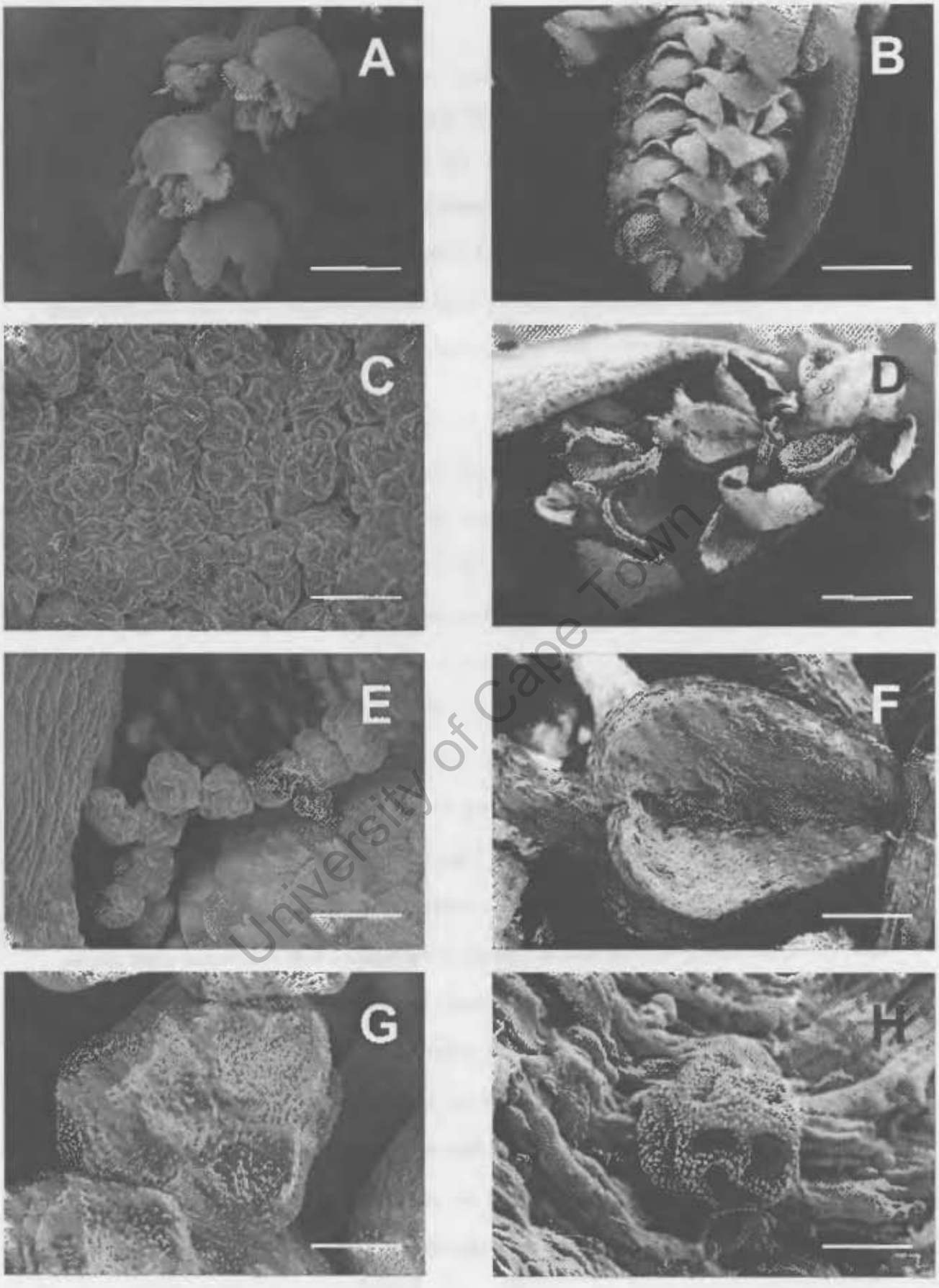


Figure 1.6. Scanning electron micrographs of flash frozen hydrated male flowers (A) and pollen grains (C, E, G) as well as desiccated male flowers (B, D), anther (F) and pollen grain (H) of *Myrothamnus flabellifolia*. [Scale bars: a, b, 1000 µm; c, 50 µm; d, 600 µm; e, 50 µm; f, 200 µm; g, 10 µm; h, 10 µm.]

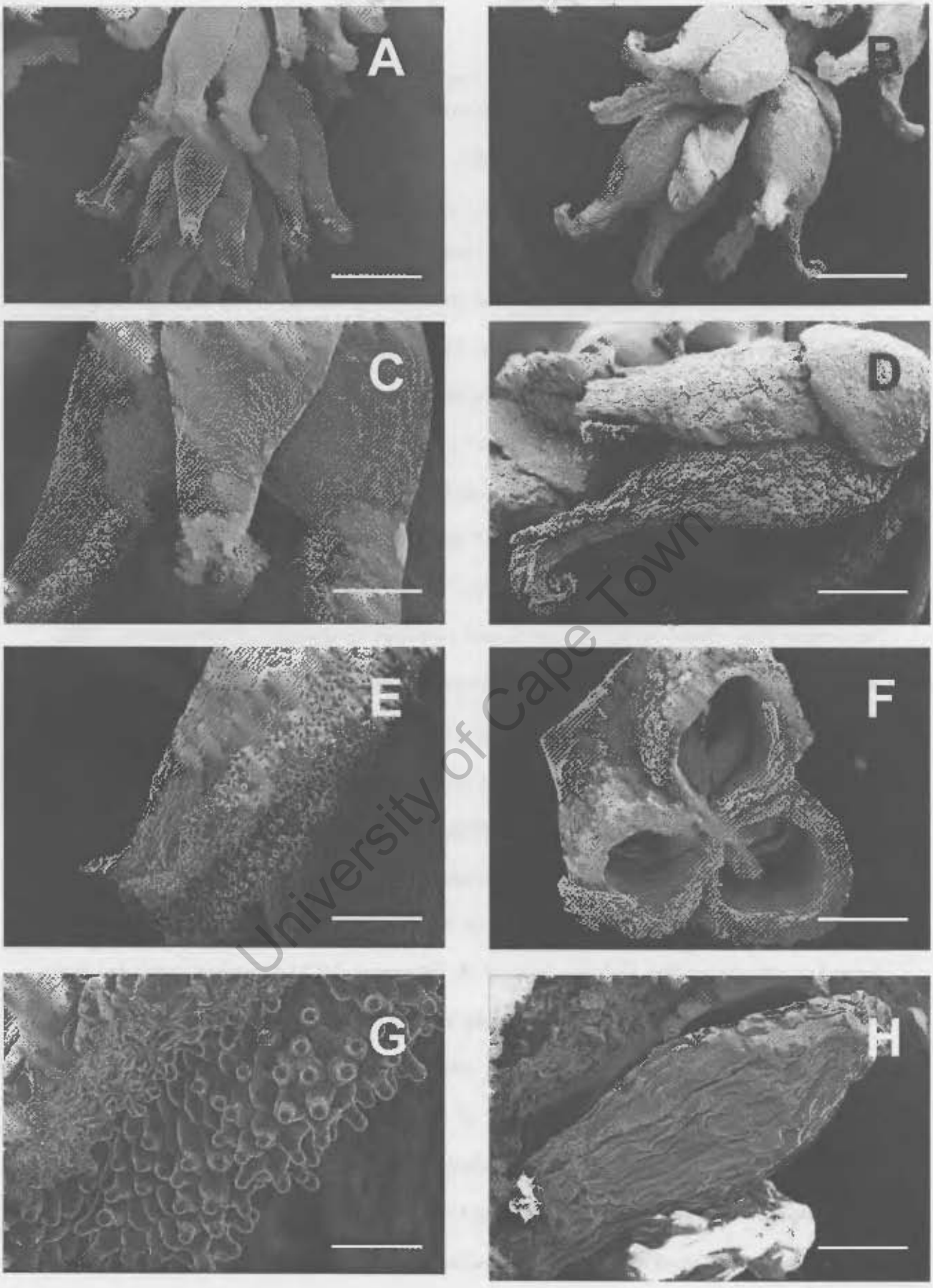


Figure 1.7. Scanning electron micrographs of flash frozen hydrated (A, C, E, G) female flowers and desiccated (B, D) female flowers, (F) seed pod and (H) seed of *Myrothamnus flabellifolia*. [Scale bars: a, 2000 µm; b, 1200 µm;.c, 400 µm; d, 600 µm; e, 300 µm; f, 400 µm; g, 100 µm; h, 50 µm.]

occur in southern Africa (Kubitzki, 1993). Carlquist even used *M. flabellifolia* in a comparative analysis aimed at determining the relationship of the Bruniaceae to the Geissolomataceae (Carlquist, 1990). A molecular analysis of the Hamamelidaceae by Qui et al. (1998) concluded that *M. flabellifolia* did not belong in the family Hamamelidaceae as previously thought (Kubitzki, 1993). Instead it was found to be more closely related to the angiosperm genus *Gunnera* (Qui et al. 1998). The *Gunnera* is one of the oldest genera of flowering plants consisting of approximately 30-40 species primarily distributed in the Southern Hemisphere (Wanntorp et al., 2001; 2003). The African species *Gunnera perpensa*, a large leaved aquatic plant, shares a similar geographical distribution to *M. flabellifolia* (Van Wyk et al., 1997). The *Gunnera-Myrothamnus* divergence has been dated at 120 million years ago from fossil evidence combined with molecular analysis (Wanntorp et al., 2001). The *Gunnera* distribution has been shown to have arisen from a combination of dispersal and vicariance mechanisms (Wanntorp et al., 2003) and it is possible that the distribution of the Myrothamnaceae has been similarly caused. At the population level, the first systematic and biogeographical study of *M. flabellifolia* was performed by Weimarck (Weimarck, 1936). The study proposed the presence of three subspecies *M. flabellifolia sensu stricta*, *M. flabellifolia elongata* and *M. flabellifolia robusta* based on a combination of morphological characters (Weimarck, 1936). The location of the subspecies did not correlate with geographical distribution of *M. flabellifolia* as the same subspecies were located on either side of the Kalahari desert divide (Weimarck, 1936). Weimarck concluded from the study that *M. flabellifolia* is a phenotypically very diverse species and that it was difficult to provide a definitive classification (Weimarck, 1936). Puff later reappraised Weimarck's initial classification and proposed the abandonment of subspecies *M. flabellifolia elongata* as being indistinguishable from subspecies *M. flabellifolia sensu stricta* (Puff, 1978a). A thorough systematic and biogeographical study, based on both molecular and morphological characters, of *M. flabellifolia* throughout its entire central southern African distribution as well its Madagascan cousin *M. moschatus* is needed. This is especially important as *M. flabellifolia* is an endangered

species in certain regions of its distribution due to excessive plant collecting practises (Glen et al., 1999).

M. flabellifolia is a well known and widely used medicinal plant by African tribes throughout its central southern African distribution (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Van Wyk et al., 1997). It has been given separate names by different tribes in the various regions where it is found (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996). Many of these names refer to its remarkable resurrection ability which is regarded as a powerful symbol of hope in African tradition (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996; Van Wyk et al., 1997). The plant is used as a psychological tool to treat severe depression in traditional African culture (Van Wyk et al., 1997) and in Zimbabwe detached twigs are carried as good luck charms (Watt and Breyer-Brandwijk, 1962). The Pedi, Shona and Vavhenda inhale smoke from burning leaves as a treatment for coughs and chest complaints (Watt and Breyer-Brandwijk, 1962; Van Wyk et al., 1997). While in Tanganyika the fruit is burnt and the smoke used for chest pains whilst in addition an aromatic salve is made by mixing ground fruit or leaves with butter (Watt and Breyer-Brandwijk, 1962). The Karanga reportedly chew the leaves as a treatment for scurvy, halitosis and Vincents gingivitus (Watt and Breyer-Brandwijk, 1962). A herbal tea is made from the leaves in Namibia, Botswana and some regions of central Africa as a tonic for the treatment of coughs, influenza and diseases of the breast (Watt and Breyer-Brandwijk, 1962; Van Wyk et al., 1997). Strong decoctions are taken for backache, kidney disorders, haemorrhoids and menstrual pains (Van Wyk et al., 1997). The Shona use the plant for the treatment of epilepsy, madness, coughs and abdominal pains whilst root decoctions are given for headaches and tropical ulcers (Watt and Breyer-Brandwijk, 1962). In addition, early European settlers in Zimbabwe have been reported to have used the plant for the treatment of headaches and for general pain alleviation (Watt and Breyer-Brandwijk, 1962). It has also been discovered that the Indian community of south

Tanganyika has been exporting the plant to India as a medicinal drug (Watt and Breyer-Brandwijk, 1962).

It is not surprising given the characteristic aromatic nature of the plant that the first chemical analyses of the plant focused on its essential oil components (Da Cunha and de Lurdes Rodriguez Roque, 1974; Gibbs, 1974). Essential oils are major odourant molecules in plants believed to play an important role in ecological interactions between the plant and its environment (Harborne, 1998). Da Cunha reported carvone and perillic acid as the major essential oil compounds in the leaves of *M. flabellifolia* (Da Cunha and de Lurdes Rodriguez Roque, 1974). In addition, Gibbs (1974) determined the presence of 1,8 cineole and diosphenol. A study by Chagonda et al. (1999) identified 43 compounds in the leaf essential oil of plants sourced from Zimbabwe. They reported trans-pinocarvenol, pinocarvone, alpha-pinene and beta-selinene as the major constituents (Chagonda et al. 1999). In order to clarify the conflicting data from the prior studies a re-analysis of essential oil composition using gas chromatography coupled mass spectrometry was performed by Viljoen et al. (2002). The study also examined the anti-microbial activity of *M. flabellifolia* essential oil against a range of pathogens (Viljoen et al. 2002). The study identified 85 compounds with pinocarvone and trans-pinocarvone as the major compounds in plants sourced from north eastern South Africa (Viljoen et al. 2002). They also demonstrated that the hydro-distilled essential oil had activity against most of the pathogens tested except *Salmonella typhimurium* and *Alternaria alternata* (Viljoen et al. 2002). It was proposed that these data suggested a possible rationale for the use of the plant in regard to particular traditional medicine applications (Viljoen et al. 2002). Furthermore as the results differed from previous essential oil studies of plants sourced from other locations in the region they purported that possible chemotypes exist within the *M. flabellifolia* distribution (Viljoen et al. 2002). Apart from the essential oil studies no major chemical study has been undertaken to characterise the main chemical constituents of the leaves.

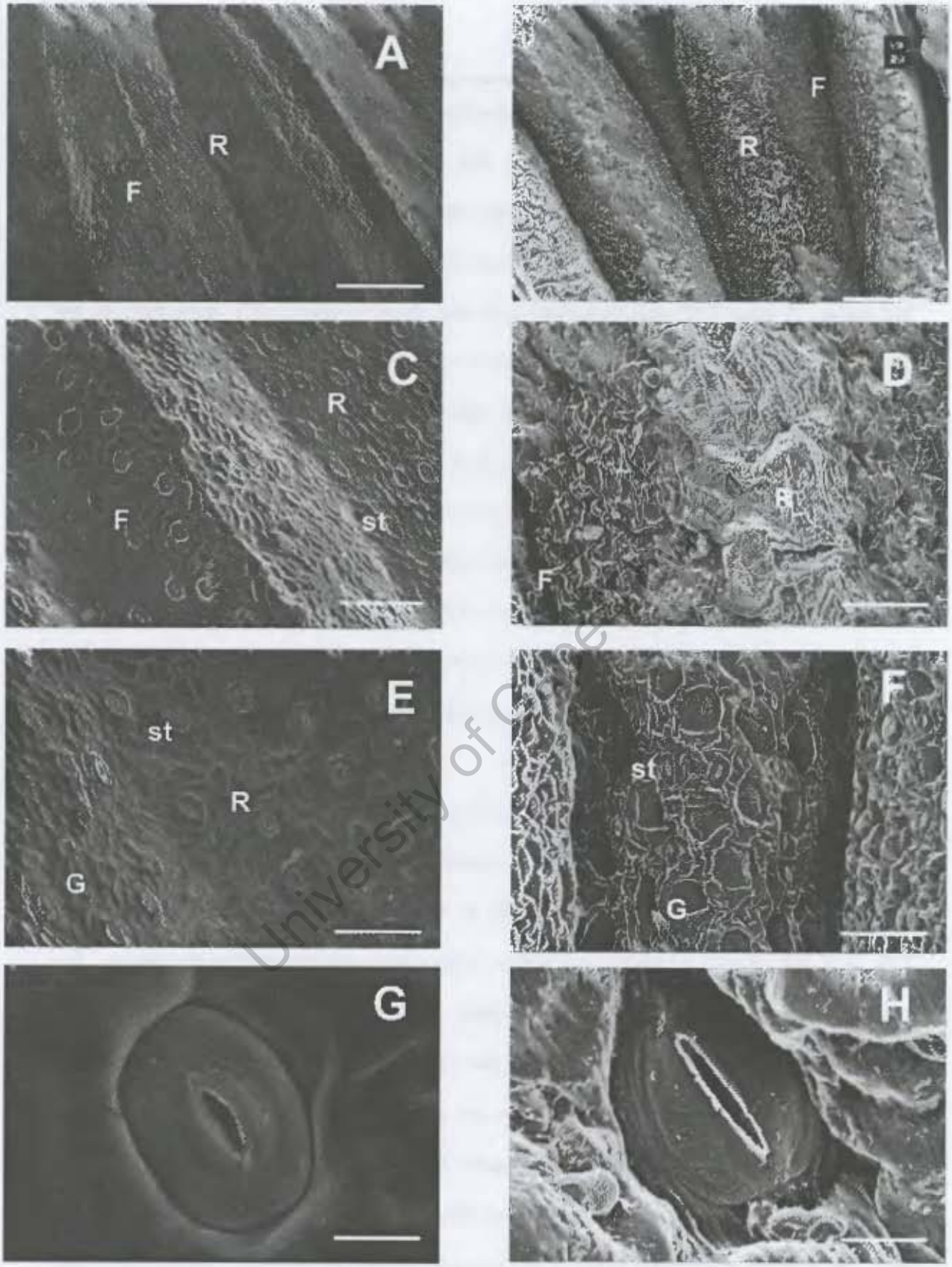


Figure 2.4. Scanning electron micrographs of adaxial leaf surfaces of hydrated (A, C, E, G) and desiccated (B, D, F, H) *M. flabellifolia* leaves. Key: F, furrows; R, ridges; st, stomata; G, gland cell. Scale bar: a, 150 μm; b, 300 μm; c, 80 μm; d, 80 μm; e, 60 μm; f, 60 μm; g, 8 μm; h, 6 μm.

surface resulted in the tissue between the cuticular ridges reducing in volume and the ridges closing together, thereby reducing the leaf face area (Figure 2.4B). The cuticular ridges underwent a considerable degree of folding upon desiccation and wax striations were visible on the ridges running parallel to the leaf longitudinal axis (Figure 2.4D). In contrast to the hydrated leaf surface (Figure 2.4E) the desiccated epidermal tissue (Figure 2.4F), particularly in the furrows, underwent extensive desiccation induced cell folding with only the stomata and gland cells remaining identifiable. The desiccated gland cells (Figure 2.4F) appeared enlarged compared with those on the hydrated leaves (Figure 2.4E) which we suggest is due to shrinkage of the surrounding epidermal tissue exposing more of the seemingly rigid gland cells. Desiccation resulted in considerable shrinkage of the stomatal guards cells leaving only an open stomatal pore and the associated wax lips clearly visible (Figure 2.4H) as compared to hydrated stomata (Figure 2.4G). The desiccation-induced folding of the epidermal tissue around the stomata resulted in the stomata appearing sunken beneath the epidermal folds in desiccated tissue (Figure 2.4H).

Further characterisation of the abaxial leaf surface of *M. flabellifolia* was also undertaken to determine the effect of desiccation on leaf morphology (Figures 2.5 and 2.6). The abaxial surfaces of flash frozen hydrated (Figures 2.5A, C, E and 2.6 A, C, E) and desiccated (Figures 2.5B, D, F and 2.6 B, D, F) leaves were viewed using a cryo-scanning electron microscope. The cuticular ridges present on the abaxial surface from hydrated leaves (Figure 2.5A) are considerably broader than those observed on the corresponding adaxial surfaces (Figure 2.4A). Leaf furrows consisting of endodermal tissue are also evident at the abaxial surface (Figure 2.5A). Higher magnification micrographs (Figures 2.5C and E) revealed that the broad abaxial ridges consisted of only endodermal and gland cells and were also covered in a thick layer of wax. The adjacent endodermal tissue in the furrows consisted of numerous stomata and gland cells interdispersed amongst endodermal cells (Figure 2.5E). Previous analysis by Goldsworthy (1992)

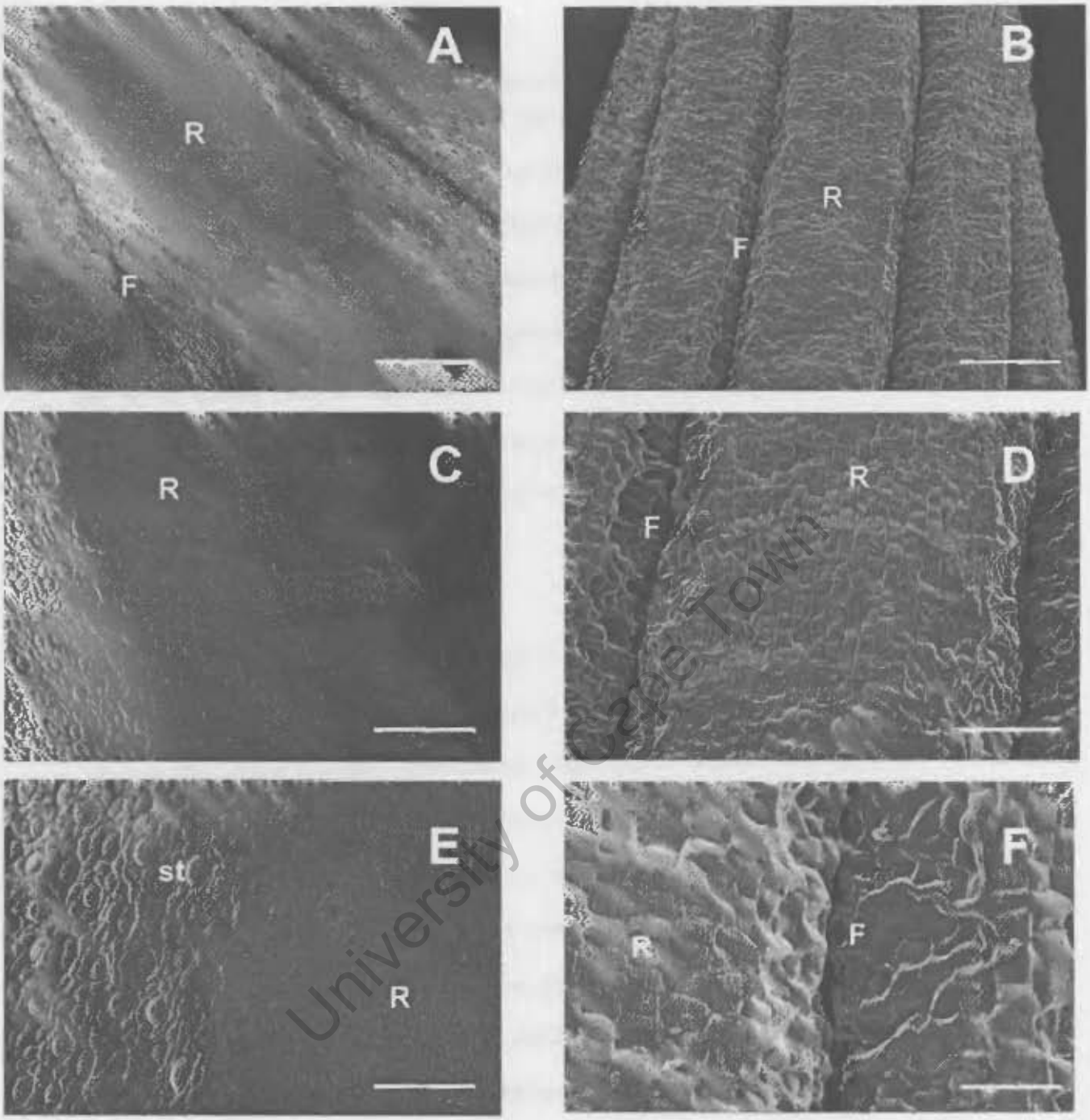


Figure 2.5. Scanning electron micrographs of abaxial leaf surfaces of hydrated (A, C, E) and desiccated (B, D, F) *M. flabellifolia* leaves. Key: F, furrow; R, ridge; st, stomata. Scale bars: a, 200 μm; b, 300 μm; c, 100 μm; d, 150 μm; e, 100 μm; f, 80 μm.

revealed a stomatal index of 9.0 ± 0.9 for the leaf furrows while no stomata were located on the cuticular ridges. Leaf trichomes and hairs were not present on the abaxial leaf surface. Similarly to that observed at the adaxial leaf surface, desiccation of the abaxial surface resulted in the tissue between the cuticular ridges reducing in volume and the ridges closing together, thereby reducing the leaf face area (Figure 2.5B). The cuticular ridges had undergone a limited degree of folding upon desiccation and wax striations were visible on the ridges running parallel to the leaf longitudinal axis (Figures 2.5D and F). The furrows of the abaxial surface (Figure 2.5E) appeared to have moved considerably closer together as compared to that observed on the desiccated adaxial leaf surface (Figure 2.4D and F).

Additional features of the abaxial leaf surface evident at higher magnifcations include the thick rib-like wax of the cuticular ridges (Figure 2.6A) and the presence of stomata and glands exclusively in the furrows (Figure 2.6B). Higher magnification images of glands cells revealed two distinct morphologies present (Figures 2.6C and D). Those gland cells found on the cuticular ridges (Figure 2.6C) are covered in a thick layer of wax in the form of parallel rods whilst those in the furrows have no such wax covering (Figure 2.6D). Stomata on the hydrated leaf abaxial surface, which were observed to be flush with the leaf surface, consisted of two crescent shaped guard cells forming the stomatal pore (Figure 2.6E). The lips of these pores appeared wax-like (Figure 2.6E). In contrast, desiccated stomata resembled those found on the adaxial leaf surface and had acquired a sunken appearance (Figure 2.4H). Furthermore, stomata located near the cuticular ridges were covered with wax-like material (Figure 2.6F).

A microscopic investigation of the leaf apices of *M. flabellifolia* was next undertaken in order to characterise the anatomical structure of the leaf apices as well as to assess the effect of desiccation on their morphology (Figure 2.7). Cryo-scanning electron microscopy was used to

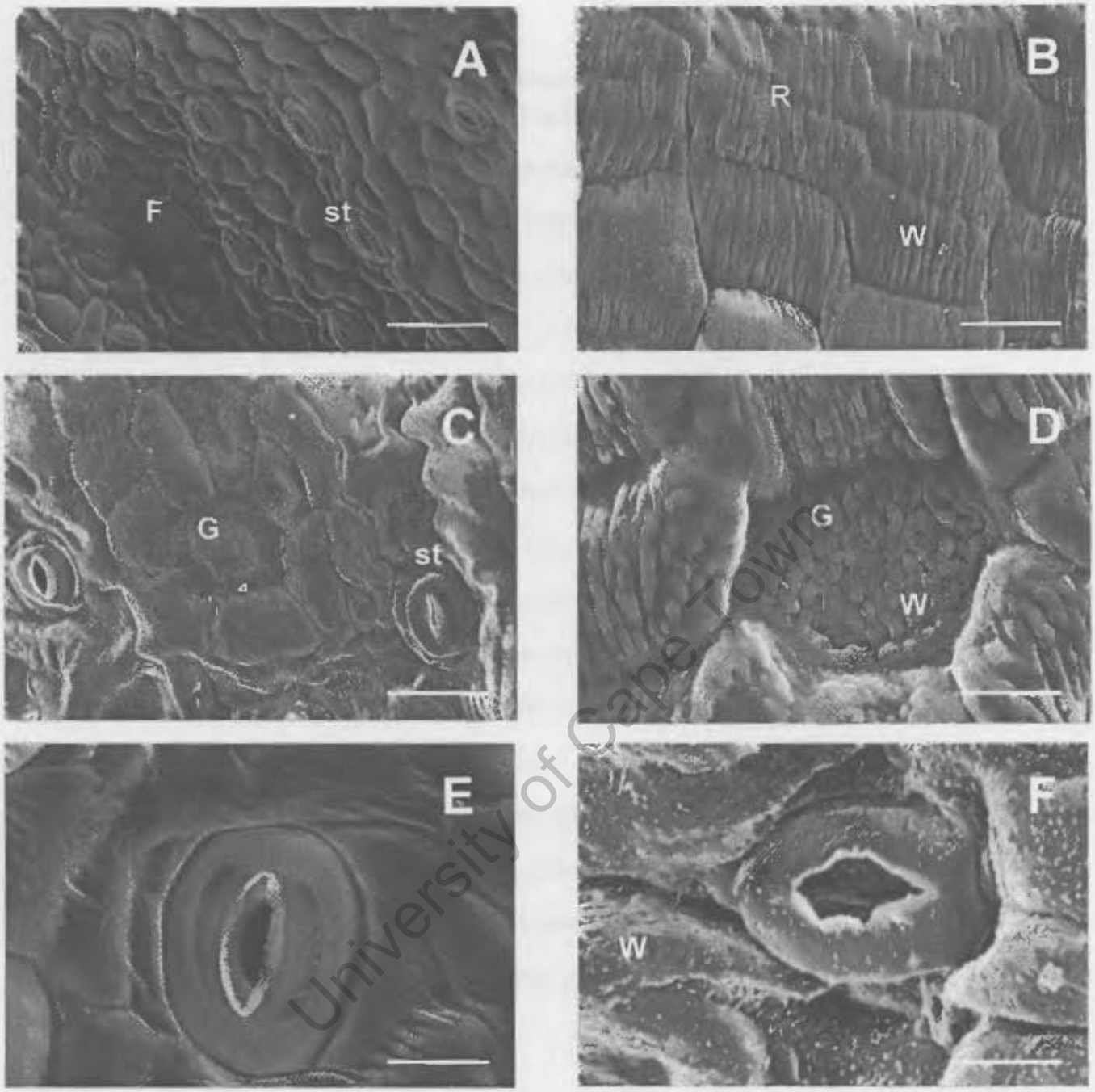


Figure 2.6. Higher magnification scanning electron micrographs of abaxial leaf surfaces of hydrated (A, C, E) and desiccated (B, D, F) *M. flabellifolia* leaves. Key: F, furrow; st, stomata; G, gland cell; R, ridge; W, wax. Scale bars: a, 40 μm; b, 20 μm; c, 20 μm; d, 10 μm; e, f, 5 μm.

characterise the leaf apices of hydrated (Figures 2.7A, C, E, G) and desiccated (Figures 2.7B, D, F, H) leaves of *M. flabellifolia*. General features of a hydrated leaf apex of *M. flabellifolia* viewed at the abaxial surface (Figure 2.7A) included the occurrence of numerous gland cells and stomata interdispersed between regular endodermal cells in a similar pattern to what was found on the general abaxial leaf surface (Figures 2.5A and C). A ridge, possibly an extension of the cuticular ridge characterised on the leaf abaxial surface (Figures 2.5A and C), was found to extend to the extreme apex of the abaxial leaf tip (Figure 2.7A). In contrast the view at the adaxial surface is quite different as there are no cuticular ridges visible but rather leaf furrows which extend to the leaf apices (Figure 2.7C). Higher magnification micrographs of the leaf apex viewed at the adaxial surface revealed numerous open stomata protruding above the level of the leaf surface (Figure 2.7F). These unusual raised stomata possessed fully open stomatal pores (Figure 2.7G) and resembled leaf hydathodes. The diameter across the width of these open stomatal pores (Figure 2.7G) appeared greater than those found in the other stomata described previously (Figures 2.4G,H and 2.6E,F) amorphous deposits of unknown composition were observed adjacent to many of these hydathode-like stomata. Elemental analysis of this material revealed that it consisted of a relatively high concentration (> 98 %) of calcium ions. These data suggest that these amorphous deposits may be calcium salts. It is possible that these are the remains of salt secretions from the hydathode-like stomata.

Desiccation caused similar effects at the abaxial surface near the leaf apex as described previously for the general abaxial leaf surface of desiccated leaves. Extensive cell wall folding of endodermal cells occurred whilst the cuticular ridge cells, gland cells and stomata remained less affected (Figure 2.7B). A cuticular ridge, consisting of wax striations running parallel to the leaf longitudinal axis, was observed to extend to the leaf apex at the abaxial surface of the desiccated leaf (Figure 2.7B). In contrast, inspection of the adaxial surface of desiccated leaves (Figure 2.7D) revealed that extensive folding of the epidermal tissue located between the ridges as well as

1.5 AIM OF THIS INVESTIGATION

Myrothamnus flabellifolia is clearly an important plant in the southern African region due to its wide use in African medicine and traditional culture. Furthermore, its remarkable resurrection ability has remained a mystery to Western science since its discovery by Welwitsch in 1858. This thesis aims to investigate the chemistry and biology of this interesting plant with a view to elucidating important aspects of its desiccation tolerance and to possibly provide further rationale for its medicinal applications.

The primary aim of this thesis can be divided into three parts. Firstly, to undertake a more thorough and systematic comparison of the morphological and ultrastructural changes associated with desiccation in the leaves of *M. flabellifolia* than that performed previously (Goldsworthy, 1992). In addition an ultrastructural study, a detailed biochemical (qualitative and quantitative) comparison of anthocyanin pigments, saccharides, metal ions and major proteins present in hydrated and desiccated *M. flabellifolia* leaves was undertaken. Secondly, to characterise the polyphenols of *M. flabellifolia* and investigate the role(s) they may play in abiotic stress tolerance and specifically desiccation tolerance in this species. Finally, to perform a complete ultrastructural, biochemical and immunocytochemical characterisation of the leaf cell wall and its response to desiccation in *M. flabellifolia.*

CHAPTER TWO

THE DESICCATION-INDUCED ULTRASTRUCTURAL AND BIOCHEMICAL CHANGES IN THE LEAVES OF THE RESURRECTION PLANT *Myrothamnus flabellifolia*

2.1 INTRODUCTION

The nature of plant cells poses particular challenges to investigators preparing plant material for microscopy (Robard, 1985; Hall and Hawes, 1991; Dashek, 2000). These challenges, not encountered with animal tissue, arise due to the diverse structural and chemical composition of plant tissues (Fahn, 1982; Hall and Hawes, 1991). As opposed to mammalian cells, plant cells possess unique components such as cell walls, plastids and vacuoles which complicate sample preparation (Hall and Hawes, 1991; Buchanan et al., 2002). The plant cell wall is composed of cellulose strands and hemicellulosic glycans embedded in a pectin-phenolic network (Brett and Waldron, 1996). This wall acts as a barrier to the penetration of fixative reagents and resins and, due to its toughness, impedes sample sectioning (Hall and Hawes, 1991). A further wall associated hydrophobic barrier, the plant cuticle, prevents the passage of aqueous solutions into tissue samples (Juniper and Jeffree, 1983). The plant vacuole, another plant specific component, is a dynamic storage component which sequesters a range of soluble and insoluble compounds, such as calcium oxalate crystals, polyphenols, toxic alkaloids and oligosaccharides (Fahn, 1982). The extreme diversity of chemicals stored coupled with the fact that individual plant species specialise in producing a single class of compound in abundance conspires to prevent the development of a universal plant fixative solution (Hayat, 1981; Fahn, 1982; Hall and Hawes, 1991). Plastids also store substances such as starch in granules and accessory pigments which inhibit fixative penetration (Hall and Hawes, 1991). Additional complications such as varying

osmolarity or the generation of gas bubbles because of intercellular air spaces can result in poor fixation (Hall and Hawes, 1991). Adequate knowledge of the specific plant tissue to be fixed and choice of a suitable fixative mixture is necessary to ensure satisfactory sample preparation for microscopy (Hayat, 1981; Hall and Hawes, 1991).

Any fixation technique seeks to preserve biological systems in their natural state and make them amenable to microscopic investigation (Hall and Hawes, 1991). Plant microtechniques have progressed with instrumentation advances and the development of novel methodologies (Dashek, 2000). Fixation techniques can be divided into two main methods, those that use chemical solutions to preserve cellular structure and those that use low temperature (physical) treatments to freeze cell constituents in their *in vivo* state (Hall and Hawes, 1991; Dashek, 2000).

Resurrection plants pose unique difficulties to both chemical and physical fixation techniques due to the extreme variation in hydration of their leaf tissues (Vicré, 2001; Wesley-Smith, 2001). Chemical fixation of desiccated leaf tissue in aqueous buffers results in some reydration occurring (Wellburn and Wellburn, 1976). Use of non-aqeous fixatives also results in rehydration occurring albeit at a slower rate than that observed with aqueous solutions (Opik, 1980; 1985; Goldsworthy and Drennan, 1991). Artifacts are also present in chemically fixed desiccated resurrection plant tissue such as plasmolytic separation of the expanding cell wall from the compacted cytoplasm (Opik, 1980; Thomson and Platt, 1997; Wesley-Smith, 2001). Aldehydes present in fixative solutions preferentially cross-link cytosolic proteins as opposed to wall carbohydrates and so the cell wall, remaining largely unfixed, swells in the aqueous solution (Hayat, 1981; Hall and Hawes, 1991). Desiccated leaf tissue is also not suitable for fixation via rapid freezing techniques as the lack of sufficient tissue water prevents the adequate immobilisation of cell structures (Hall and Hawes, 1991; Vicré, 2001; Wesley-Smith, 2001). Problems are then encountered if pre-fixative steps are taken such as adding a cryo-protectant,

usually a sucrose solution which facilitates rehydration, or in post-fixative processing should freeze substitution be employed (Vicré, 2001; Wesley-Smith, 2001). Substitution of the frozen water in a desiccated sample is usually carried out with a suitable solvent, however many solvents unfortunately are also able to extract cell constituents and thereby damage cell structures (Hall and Hawes, 1991; Dashek, 2000). Complications with freezing methods in hydrated samples include the formation of ice crystals, large water vacuoles which do not freeze and the creation of freezing gradients in the specimen since samples can usually only be frozen to a depth of 10-20 μm from the surface (Hall and Hawes, 1991; Dashek, 2000). Other fixation techniques, such as acrolein vapour phase fixation, have also proven unsuitable for resurrection plant tissue (Goldsworthy and Drennan, 1991; Vicré, 2001). A combination of both chemical and physical fixation methods is generally advised in order to obtain complementary information on the sample under study thereby providing a clearer overview of the cellular processes or structures present (Hall and Hawes, 1991; Dashek, 2000).

In this study we use both chemical and physical fixation techniques to investigate the anatomical adaptations and ultrastructural changes associated with desiccation in the leaves of *M. flabellifolia*. We used both low temperature scanning electron microscopy to systematically compare hydrated and desiccated leaves with respect to their surface and internal ultrastructure. In addition to the microscopic analysis we performed a qualitative and quantitative biochemical analysis of components such as pigments, saccharides, proteins and metal ions present in both hydrated and desiccated *M. flabellifolia* leaf tissue. Our analysis revealed a number of unique anatomical adaptations to desiccation-associated stresses, such as thick wax covered leaf ribs and an internal wax cuticle, present in this species. Furthermore, we showed that leaf tissue underwent considerable shrinkage and collapse but was supported by a framework of vascular and sclerenchymous tissue. Our biochemical analysis showed *M. flabellifolia* increases the anthocyanin pigment (cyanidin 3-glucoside) concentration as well as the saccharide content of its

leaves in response to desiccation. We further confirmed that this species utilises a strategy of maintaining its photosynthetic (chlorophyll and rubsico) and translation (ribosomal proteins) machinery intact during the desiccation period in preparation for rehydration. In summary, we suggest that the leaves of *M. flabellifolia* possess a number of both constitutively present and desiccation-induced adaptations which significantly contribute to their desiccation tolerance.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

M. flabellifolia plants, collected from the Buffelskloof Nature Reserve and the Magaliesberg Mountains, Mpumalanga Province, South Africa, were maintained in a glasshouse at the Department of Botany, University of Cape Town. Desiccation of whole hydrated (~ 90 % RWC) plants was performed by withholding water and allowing the plants to dry naturally under ambient environmental conditions until the plants reached an air-dry state (~ 12 % RWC). Desiccated leaves were sampled for analysis at least 6 months after drying. Water content was determined gravimetrically by oven drying at 70°C for 48 h and RWC was calculated as described previously (Cooper and Farrant, 2002).

2.2.2 Light microscopy

Whole leaves and leaf segments were viewed and photographed using a WILD W400 photomakroskop microscope fitted with an AxioCam digital camera and images were captured using Zeiss software. Leaf segments (1 - 2 mm^2) were excised from the mid-blade of hydrated or desiccated leaves and fixed overnight at 4 °C with 2.5 % glutaraldehyde in 0.1 M phosphate buffer pH 7.4 supplemented with 0.5 % caffeine. Fixed samples were dehydrated in ethanol and embedded in epoxy resin (Spurr, 1969). Semi-thin sections (0.25 µm) were cut using a Reichert Ultracut-S ultramicrotome. Sections were then transferred to glass slides and stained with a 1 % w/v solution of toluidine blue. The sections were then rinsed with dH_2O and viewed with a Leitz Diaplan optical microscope fitted with an AxioCam digital camera. Images were captured using Zeiss software.

2.2.3 Scanning electron micoscopy

Scanning electron microscopy was performed using a fully analytical Leica Stereoscan 440 digital scanning electron microscope equipped with a Fisons LT7400 Cryo Transfer System. Leaves from hydrated and desiccated plants were flash frozen using liquid nitrogen and viewed directly or after freeze-fracturing. In addition leaf segments (1 - 2 mm^2) were excised from the mid-blade of hydrated or desiccated leaves and fixed overnight at 4 °C with 2.5 % glutaraldehyde in 0.1 M phosphate buffer pH 7.4 supplemented with 0.5 % caffeine. Fixed samples were dehydrated in ethanol and embedded in epoxy resin (Spurr, 1969). Resin blocks were polished with 0.5 µm alumina slurry and viewed in the SEM using a 4 quadrant backscatter detector. For elemental analysis certain specimens were coated with gold-palladium or carbon prior to viewing.

2.2.4 Anthocyanin, chlorophyll and carotenoid analysis

Anthocyanins were extracted from lyophilised leaf material from hydrated and desiccated leaves using 0.1 % (v/v) aqueous HCl (Harborne, 1998). The extract was loaded onto a C18 Sep-pak cartridge (Phenomenex) (Skrede et al., 2000), eluted with 0.1 % HCl (v/v) in methanol and the eluate dried using a Speed-Vac. The material present was dissolved in the minimum volume of and applied to a Strata SCX cartridge (Phenomenex) pre-equilibrated with successive solutions of 0.1 % (v/v) aqueous NaOH and 0.1 % (v/v) aqueous HCl. Purified anthocyanins were eluted with 0.1 % HCl (v/v) in methanol. Purity and characterisation of the main anthocyanin constituent(s) were achieved using a combination of HPLC and MALDI-TOF MS (Skrede et al., 2000; Sugui et al., 1998). Sugar analysis of the purified anthocyanins was performed after hydrolysis with 2 M HCl for 2 h at 80 °C. The liberated monosaccharide(s) were analysed using DIONEX HPLC (Salvador et al., 2000). Anthocyanins were quantitated at 520 nm using cyanidin 3-glucoside as

the reference compound (Harborne 1998; Rivas-Gonzalo, 2003). Chlorophyll and carotenoids were extracted from leaf material using 80 % acetone (Lichtenthaler, 1987; Harborne, 1998). The chlorophyll and carotenoid contents were determined (Lichtenthaler, 1987) from the absorbance of the extracts at 470, 644.8 and 661.6 nm.

2.2.5 Qualitative and quantitative analysis of protein content

Total protein was extracted from lyophilised leaf material using a modified acetone precipitation method (Harborne, 1998). Briefly, leaf material was ground to a fine powder using a mortar and pestle before being extracted at -20 °C with 0.1125 M Tris-HCl pH 8.8, 0.1 mM PMSF, 10 mM NaCl, 1 % SDS, 0.1 % ascorbic acid, 5 % insoluble PVP and 8 % glycerol (Koonjul, 1999). Extracted proteins were precipitated with 4 volumes of ice-cold acetone and collected by centrifugation. The protein pellet was subjected to repeated washing with ice-cold acteone before being dried under vacuum and weighed. The lyophilate was dissolved in 4 M urea 2 % SDS and electrophoresed on a SDS-PAGE gel (Laemmli, 1970). After staining the gel with Coomassie Brilliant Blue R250, major protein bands were excised and analysed using MALDI-TOF mass spectrometry after tryptic digestion (Wilm et al., 1996). Protein was quantitated using the Bradford method (Bradford, 1976).

2.2.6 Sugar and metal ion analysis

Sugars were extracted from lyophilised leaf material with 70 % aqueous methanol and analysed using a DIONEX HPLC system (Harborne, 1998; Salvador et al., 2000). Saccharides were identified by comparison with the retention time of commercial standards and quantitated relative to mannitol, used as the internal standard.

The metal ion content of lyophilised leaf material was determined using a modified protocol according to le Roex et al. (2001). The samples were digested using concentrated HF and HNO_3 in Teflon beakers and analysed using a Perkin Elmer/Sciex Elan 6000 ICP-MS. Calibration was achieved using a set of standards made from artificial standard solutions. Internal standardisation against rhodium was used to correct for instrument drift. The analytical procedure involved 300 individual intensity measurements with dwell times of 35 ms at each of the analyte masses.

2.3 RESULTS

2.3.1 The effects of desiccation on the general anatomy and morphology of *M. flabellifolia* leaves

The shrinkage, folding and colour change of the leaves of *Myrothamnus flabellifolia* that occurs upon dehydration constitute the most conspicous desiccation-induced changes that occur in this resurrection plant species. The general appearance of both hydrated (Figures 2.1A and B) and desiccated (Figures 2.1C and D) adaxial and abaxial leaf surfaces were assessed using a dissecting light microscope. The hydrated adaxial leaf surface (Figure 2.1A) was green and reflective consisting of alternating lighter ridges and darker furrows. This arrangement of the ridges and furrows caused the leaf to resemble an open fan (*flabellifolia* = fan-like). The hydrated abaxial leaf surface (Figure 2.1B) viewed under the same conditions revealed a green reflective surface with alternating ridges and narrow furrows. However the abaxial ridges appeared markedly broader in width than those found on the adaxial surface (Figure 2.1A). In contrast the desiccated adaxial leaf surface (Figure 2.1C) was found to consist of a reflective light green-yellow surface with red pigmented regions found to occur along the leaf ridges. The ridges of the adaxial surface (Figure 2.1C) in the desiccated leaf are closed together and so the effective leaf surface area is reduced as compared to the hydrated leaf. The appearance of the abaxial leaf surface after desiccation (Figure 2.1D) is also considerably altered in comparison to that observed in hydrated tissue (Figure 2.1B). The broad ridges visible, in the hydrated leaf, are closed completely together in desiccated tissue with the leaf furrows no longer visible (Figure 2.1B). The abaxial ridge surfaces (Figure 2.1D) are dark reddish-brown in colour, appear pitted and uneven as well as being highly reflective. A transverse section through a hydrated leaf (Figure 2.1E) revealed a uniformly green cross section with no visibly distinct tissue layers. In addition a wax-

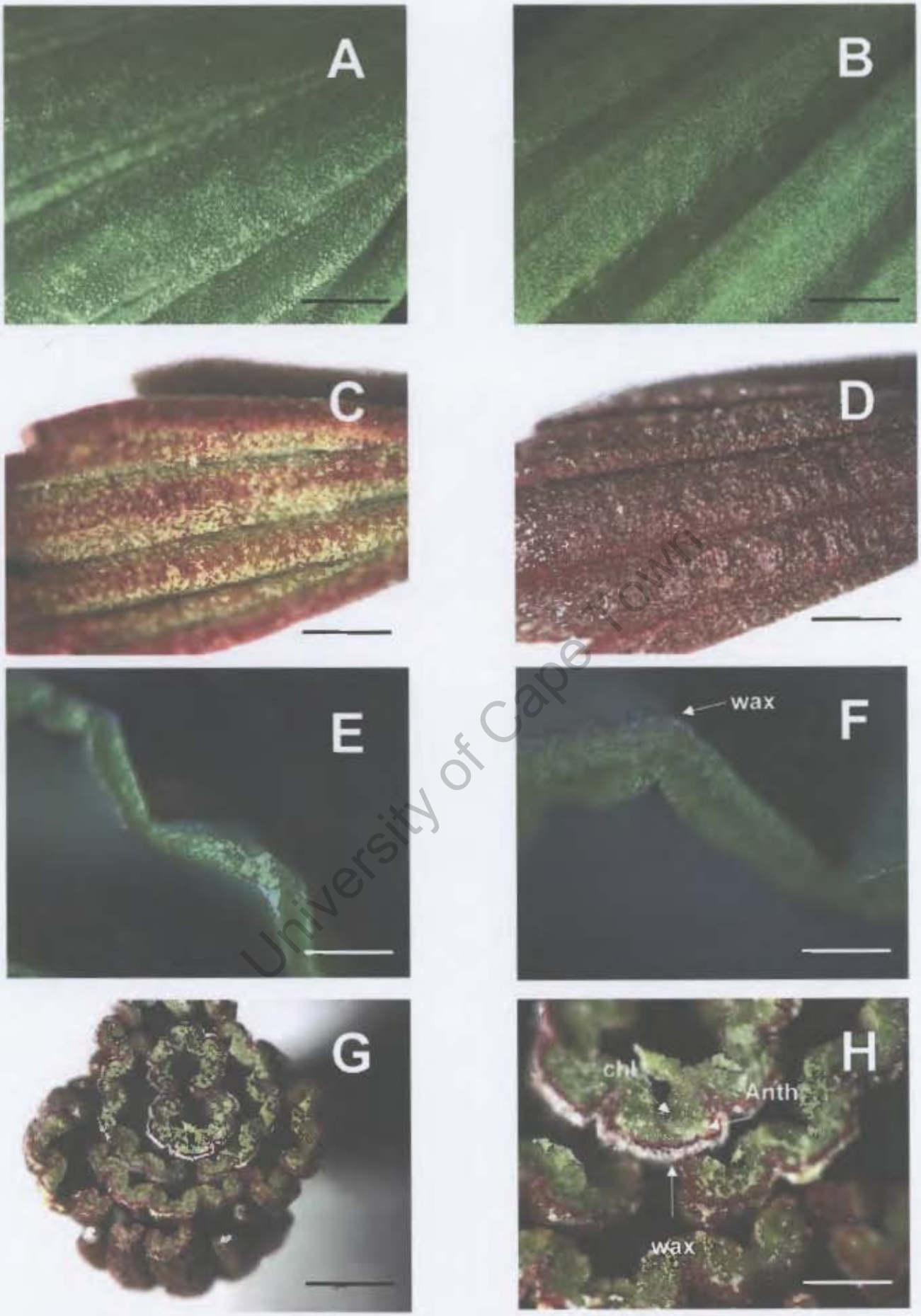


Figure 2.1. Light micrographs of surface and transverse sections of hydrated and desiccated *M. flabellifolia* leaves. A. Adaxial surface of hydrated leaf; B. Abaxial surface of hydrated leaf; C. Adaxial surface of desiccated leaf; D. Abaxial surface of desiccated leaf; E, F. Transverse sections of hydrated leaves; G, H. Transverse sections of desiccated leaves. Key: chl, chlorophyll; Anth, anthocyanin; Wax, wax covering. Scale bars: a,b, 1 mm; c,d, 0.5 mm; e, 3 mm; f, 1 mm; g, 3 mm; h, 1 mm.

like coating is observable at the abaxial surface (Figure 2.1F). In contrast the corresponding transverse section through desiccated leaves (Figure 2.1G) revealed that the leaves have become considerably folded with two differently coloured tissue layers being found. The inner 'palisade' mesophyll layer, adjacent to the adaxial surface, is green which is indicative of chlorophyll being present whilst the outer 'spongy' mesophyll tissue, adjacent to the abaxial surface, is dark red in colour (Figure 2.1H). This dark red pigment is likely to be anthocyanins and we suggest that these molecules are responsible for the dark colouration of the abaxial desiccated leaf surface (Figure 2.1D) previously described. As was found in hydrated leaves the abaxial surface (Figure 2.1H) appears to be coated with a layer of wax.

We next investigated the general leaf anatomy of *M. flabellifolia* in order to determine the anatomical features that may be contributing to desiccation tolerance in this species. Resin embedded tissue blocks of hydrated (Figures 2.2A and B) and desiccated (Figures 2.2C and D) leaf specimens were sectioned, stained and viewed using a light microscope. General features of *M. flabellifolia* hydrated leaf anatomy (Figures 2.2A and B) include small cell size (~20 μm in diameter), primitive vasculature (tracheids) and the abundance of darkly stained cells throughout the leaf tissue. The epidermis (Figure 2.2B) is uniseriate and epidermal cells appear devoid of darkly stained material. Large round cells (Figure 2.2B) are observed adjacent to the epidermis in the mesophyll and are spaced at regular intervals along the epidermal mesophyll leaf border. The inner mesophyll layer cells (Figures 2.2 A-D) contain an abundance of darkly stained material. This darkly stained material present in the majority of the leaf mesophyll cells is likely phenolic in nature and resemble phenolic containing cells found in other plant species (Schneider, 1976; Mueller and Greenwood, 1978; Hayat, 1981). In addition, crystalline deposits were also visible interdispersed amongst the spongy and palisade mesophyll cells of leaf (Figures 2.2A and B).

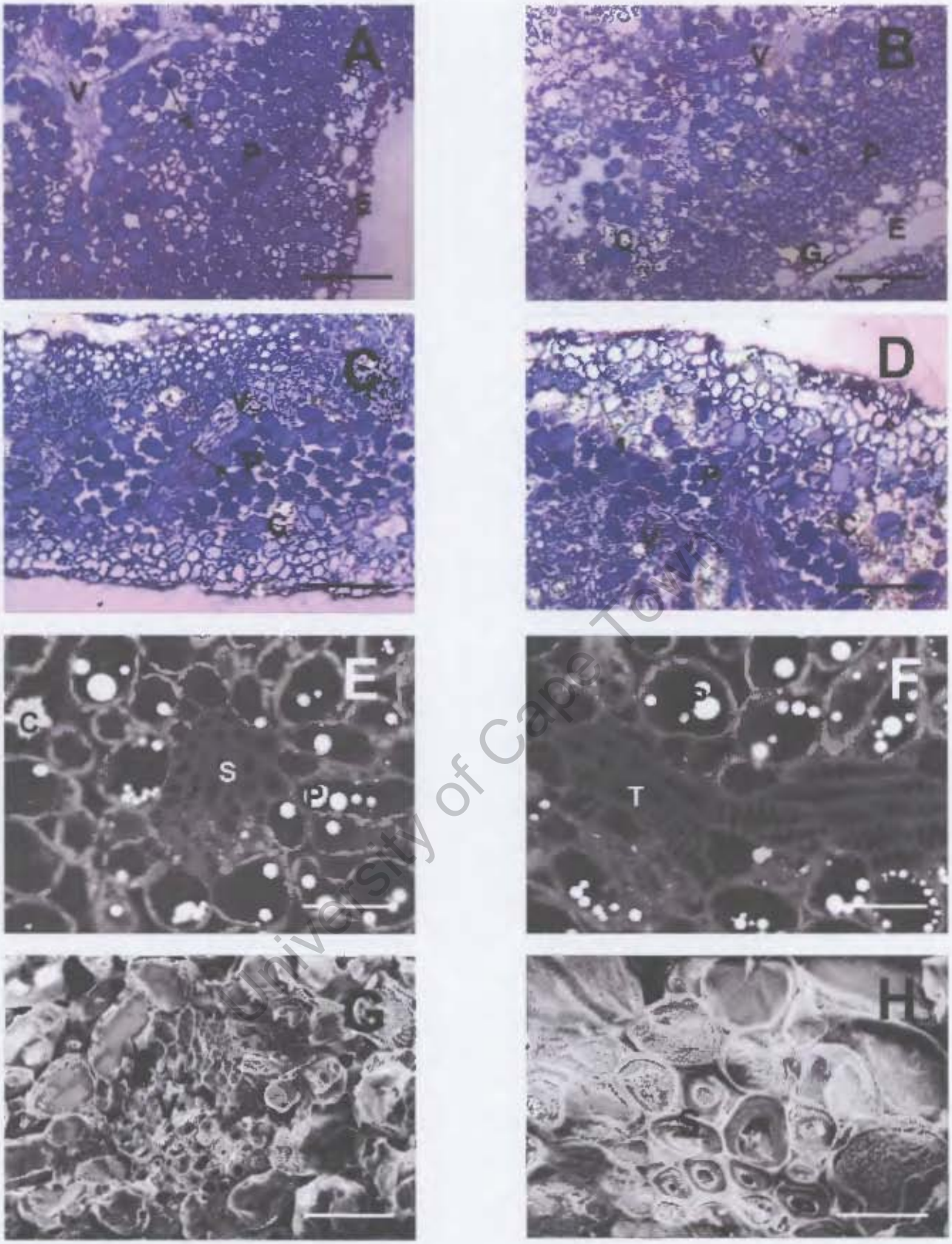


Figure 2.2. Light microscope (A-D) and scanning electron microscope (E-H) images of fixed and resin infiltrated leaf tissue from hydrated and desiccated *M. flabellifolia* plants. A, B. Light micrographs of transverse sections through hydrated leaves; C, D. Light micrographs of transverse sections through desiccated leaves; E, SEM image (using backscatter detector) of transverse section through a hydrated leaf; F, SEM image (using backscatter detector) of transverse section through a desiccated leaf; G, H; SEM micrographs of freeze-fractured surfaces of hydrated leaves showing sclerenchymous and vascular tissue. Key: V, vacuole; P, polyphenol body; E, epidermis; G, gland cell; C, calcium oxalate crystal; S, sclerenchyma tissue; T, tracheids, arrow, darkly stained material. Scale bars: a-d, 180 μm; e, 30 μm; f, 20 μm; h, 8 μm.

The anatomical features found in hydrated leaves such as small mesophyll cells, uniseriate epidermis, tracheids and calcium oxalate crystals were also found in desiccated leaves (Figures 2.2C and D). Desiccated leaf tissue was fixed in an aqueous aldehyde based fixative and viewed using light microscopy. Transverse sections of desiccated specimens (Figures 2.2C and D) revealed cells with markedly less regular spherical shapes than those present in hydrated tissue (Figures 2.2A and B). In addition, some of the cell contents display irregular morphology and many cells show partial cell wall folding (Figures 2.2C and D). As rehydration and swelling of leaf cells is known to occur during aqueous fixation of desiccated resurrection plant tissue (Wellburn and Wellburn, 1976; Vicré, 2001), we believe that the general cellular anatomy of desiccated leaves is likely to have a considerably more compressed and compacted appearance *in vivo* than is evident in our micrographs (Figures 2.2C and D).

Osmium tetroxide is a heavy metal stain commonly used in electron microscopy (Hayat, 1981; Hall and Hawes, 1991). It is known to fix cellular structures such as membrane lipids, unsaturated macromolecules and phenolics (Hayat, 1981; Hall and Hawes, 1991). We post-fixed certain aldehyde fixed specimens with osmium tetroxide with the view to ascertaining the cellular location of various cytoplasmic, organellar and vacuolar substances in hydrated (Figure 2.2E) and desiccated (Figure 2.2F) leaf samples. Resin embedded specimen blocks were polished using alumina and imaged in the scanning electron microscope using a 4 quadrant backscatter detector. This detector allows imaging of regions of high atomic number elements (osmium rich) in specimens which appear as lighter areas in the micrographs contrasting with the darker low atomic number elements of the background (Figures 2.2E and F). The brightest regions were found to occur as generally spherical intra-vacuolar deposits in the bulk of the mesophyll cells (Figures 2.2E and F). These deposits are likely phenolic compounds which have become oxidised and cross-linked with osmium tetroxide in the cell vacuole. In addition, analysis of the elemental composition of these spherical metal-rich deposits revealed a high relative concentration (> 95 %)

of osmium metal present. This provides support for the previous assertion that the bulk of the leaf mesophyll cells are filled with phenolic material. Less bright regions, as compared with the regions corresponding to intra-vacuolar deposits, occurred along the periphery of the majority of the mesophyll cells adjacent to the cell walls, co-localising with the cell cytoplasm and structures resembling chloroplasts (Figures 2.2E and F). Additional features were also evident such as vascular tissue supported by thick walled sclerenchyma cells (Figure 2.2E) and xylem tracheids (Figure 2.2F). Irregular crystalline deposits, corresponding to those observed in the light micrographs (Figures 2.2A – D), were observed as bright regions in the micrographs (Figure 2.2E). Elemental analysis of these irregular deposits revealed a high relative concentration (> 98 %) of calcium ions present which suggested that these irregular structures are likely calcium oxalate crystal deposits (Franceschi and Horner, 1980; Webb, 1999). These crystal deposits had been reported previously by Grundell (1933) to be present in *M. flabellifolia* leaf tissue. No marked difference in the arrangement of osmium rich (phenolic) bodies between hydrated (Figure 2.2E) and desiccated (Figure 2.2F) leaves was observed.

Further characterisation of leaf vascular tissue was performed by imaging freeze-fractured surfaces of frozen hydrated leaves using cryo-scanning electron microscopy (Figures 2.2G and H). The scanning electron micrographs revealed small sclerenchyma and vascular cells surrounded by larger turgid mesophyll cells (Figure 2.2G). The cell walls of these sclerenchyma and vascular cells are considerably thickened as compared to the adjacent mesophyll cell walls (Figure 2.2H). The sclerenchyma tissue which occurs in the leaf as vascular strands (Grundell, 1933) likely acts to reinforce the general leaf shape and we suggest is responsible for the the fan-like ridges described previously.

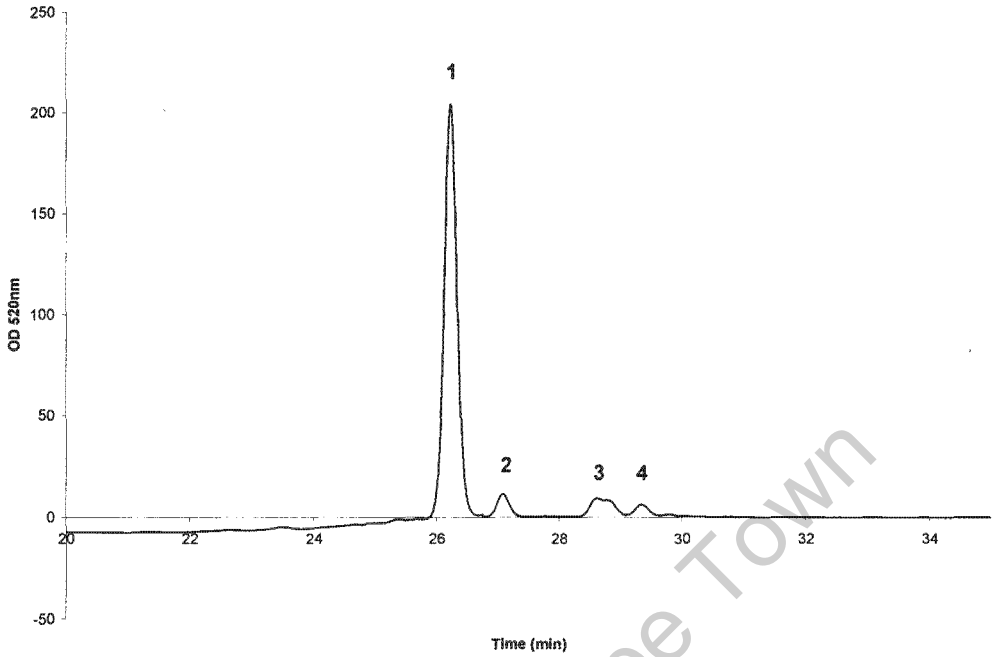
2.3.2 Pigments present in *M. flabellifolia* leaves and their change upon desiccation

The presence of red leaf pigment(s), tentatively identified as anthocyanin(s), in *M. flabellifolia* was shown to correlate with desiccation, as no pigment(s) was visible in hydrated leaves (Figures 2.1A-H). Thus, a study was performed to compare anthocyanin levels in hydrated and desiccated leaves. Anthocyanin pigments were extracted from both desiccated and hydrated leaves and assayed at 529 nm (Harborne, 1998; Rivas-Gonzalo, 2003). Determination of anthocyanin levels in hydrated and desiccated leaf tissue confirmed the visual observation of desiccation-correlated accumulation of anthocyanin in *M. flabellifolia* as an approximate two fold increase in the anthocyanin concentration was measured in desiccated leaves as compared to hydrated leaves (Table 2.1). Furthermore, analysis of chlorophyll and carotenoid pigment concentrations in hydrated and desiccated leaves were also performed. No difference between chlorophyll and carotenoid levels in hydrated and desiccated leaves was found (Table 2.1). As these data suggest a possible role for the leaf anthocyanin pigments in desiccation protection we undertook to purify and identify the main anthocyanin(s) present in desiccated leaves using HPLC and Mass Spectrometry (Figure 2.3). Anthocyanin pigments were extracted and sequentially enriched using a Sep-pak cartridge purification protocol (see methods). The enriched fraction was shown to be composed of one main compound as evidenced by the presence of a single predominant peak in the HPLC chromatograph (Figure 2.3A). Additional peaks were present in markedly lower abundance (Figure 2.3A) confirming the main detectable compound to be present at > 90 % concentration. The main peak was purified by HPLC and subjected to MALDI TOF MS which displayed a single peak in the mass spectrum at *m/z* 451 (data not shown). Fragmentation of this peak resulted in an additional ion occurring in the mass spectrum at *m/z* 289 suggesting the presence of cyanidin as the parent anthocyanidin (data not shown). The identical fragment ion at *m/z* 289 corresponding to the cyanidin nucleus is also present in the unhydrolysed mass spectrum (data not shown). This was

Table 2.1. Content of various pigments in the leaves of desiccated and hydrated *M. flabellifolia* plants. Data represents the means and standard deviations of the mean for two independent experiments with at least two replicates per experiment.

Compound	Unit of Measure	Hydrated	Desiccated
Anthocyanins	$\mu g.g.dw^{-1}$	11.95 ± 1.91	32.26 ± 1.03
Chlorophyll a	$mg.g.dw^{-1}$	2.74 ± 0.40	2.28 ± 0.47
Chlorophyll b	$mg.g.dw^{-1}$	1.58 ± 0.19	1.26 ± 0.24
Chlorophyll a + b	$mg.g.dw^{-1}$	4.05 ± 0.36	3.52 ± 0.72
Carotenoids	$mg.g.dw^{-1}$	0.56 ± 0.06	0.61 ± 0.12

A



B

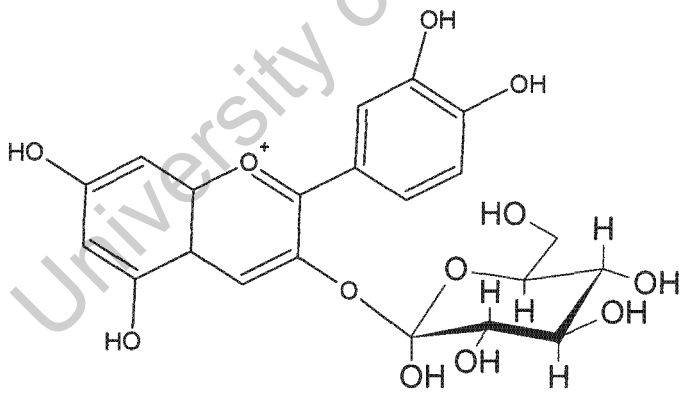


Figure 2.3. HPLC analysis of the anthocyanin composition of *M. flabellifolia* leaves. (A) Anthocyanins eluted from a C18 column (see methods) monitored at 520 nm; Peak 1 corresponds to (B) cyanidin 3-glucoside isolated and identified (see text), peaks 2-4 are unidentified derivatives of cyanidin (likely glycosides). Ordinate scale denotes milli-Absorbance units.

further confirmed by hydrolysis with TFA which resulted in a single peak eluting later on the HPLC and showing a *m/z* of 289 (data not shown). The attached molecule, which corresponds to a mass difference of *m/z* 162, was shown to be glucose by hydrolysis of the anthocyanin extract and subsequent saccharide analysis of the soluble hydrosylate using DIONEX HPLC (data not shown). We therefore propose that the main leaf anthocyanin is cyanidin 3-glucoside (Figure 2.3B) (Harborne, 1998). Cyanidin 3-glucoside is reported to be the most common anthocyanin being present in the leaves of most angiosperms (Harborne, 1998).

2.3.3 The surface and internal ultrastructure of *M. flabellifolia* leaves before and after desiccation

Further characterisation of the adaxial leaf surface of *M. flabellifolia* was undertaken to determine the effect of desiccation on leaf morphology (Figure 2.4). The adaxial surfaces of flash frozen hydrated (Figures 2.4A, C, E, G) and desiccated (Figures 2.4B, D, F, H) leaves were viewed using a cryo-scanning electron microscope. The adaxial surface of a hydrated leaf (Figure 2.4A) consists of cuticular ridges that run parallel to the leaf longitudinal axis and result in the appearance of alternating light (ridge) and dark (furrow) regions across the leaf surface. This arrangement causes the leaf to appear fan-like. Higher magnification images of the adaxial hydrated leaf surface (Figures 2.4C and E) show that the ridges appear to be covered in a layer of wax. Both the epidermal tissue of the ridge surfaces as well as the tissue between the ridges in the furrows were found to consist of numerous stomata, gland cells and epidermal cells (Figures 2.4C and E). The stomata, which were observed to be flush with the leaf surface, consisted of two crescent shaped guard cells forming the stomatal pore (Figure 2.4G). The lips of these pores appeared wax-like (Figure 2.4G). Previous analysis by Goldsworthy (1992) revealed stomatal indices of 6.2 ± 0.2 for the leaf furrows and 1.3 ± 0.6 for the cuticular ridges. Leaf trichomes and hairs were not present on the adaxial leaf surface. Desiccation of the adaxial leaf

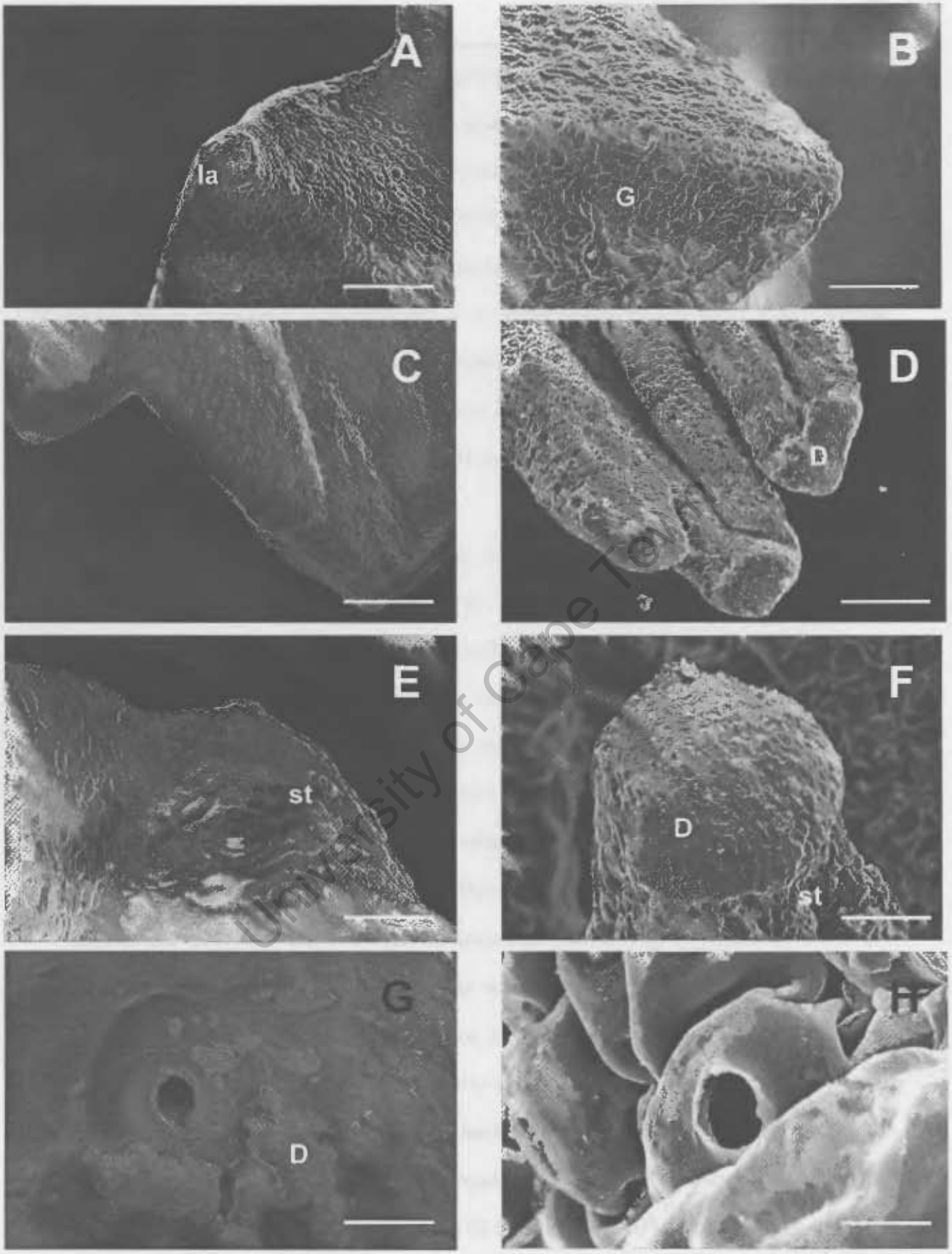


Figure 2.7. Scanning electron micrographs of the abaxial and adaxial apices of hydrated (A, C, E, G) and desiccated (B, D, F, H) *M. flabellifolia* leaves. A, B Abaxial surfaces; C, D. Adaxial surfaces; E, F. Adaxial surfaces; G, H. Adaxial surface hydathode-like stomata. Key: la, leaf apex; G, gland cell; st, stomata; D, deposit (calcium salts) a, 120 μm; b. 120 μm; c, d, 300 μm; e, 80 μm; f, 80 μm; g, 12 μm ; h, 8 μm.

epidermal cells had occurred resulting in the formation of distinct furrows which extended to the leaf apices. Furthermore, it was noted that large, amorphous deposits were located at the leaf apices on the adaxial surfaces of many desiccated leaves (Figures 2.7D and F). These deposits resembled desiccated liquid droplets (Figure 2.7F). Elemental analysis of these deposits revealed that they consisted of a relatively high concentration (> 98 %) of calcium ions. Hydathode-like stomata (Figure 2.7H) similar to those observed on hydrated leaves were found on the adaxial surfaces. The cells surrounding these stomata had become extensively folded due to desiccation (Figure 2.7H). These hydathode-like stomata occurred adjacent to many of the calcium rich deposits which further supports the previous assertion that these stomata secrete calcium salts.

We next determined the effects of desiccation on the internal ultrastructure of the leaf of *M. flabellifolia* (Figure 2.8). To investigate this, leaves from hydrated (Figures 2.8A, C, E) and desiccated (Figures 2.8B, D, F) plants were subjected to cryo-scanning electron microscopy after freeze fracture. Cross sections from hydrated leaves (Figure 2.8A) revealed the standard epidermal, mesophyll and endodermal cell layers found in other plant species (Fahn, 1982). The epidermal and endodermal cell layers (Figure 2.8A) consist of a single layer of cells (uniseriate) with occasional larger cells interspersed at regular intervals along the leaf margin. We propose that these larger cells correspond to the gland cells previously noted at the leaf margins (Figure 2.2 A) and cell surface (Figure 2.4 F). The mesophyll layer is further subdivided into palisade mesophyll (Figure 2.8A) adjacent to the adaxial surface, usually composed of 1-2 cell layers, as well as the spongy mesophyll (Figures 2.8A , C and E) adjacent to the abaxial surface generally composed of 4-5 cell layers. In contrast, a cross section through a desiccated leaf (Figure 2.8B) of *M. flabellifolia* revealed none of the clearly defined cell layers that were observed in the hydrated leaf section. A considerable amount of shrinkage and folding of epidermal, endodermal and mesophyll cells had occurred (Figures 2.8B and D) resulting in a distorted arrangement of the leaf cell layers. The thick outer cuticle (Figure 2.8B) on both the epidermis and endodermis

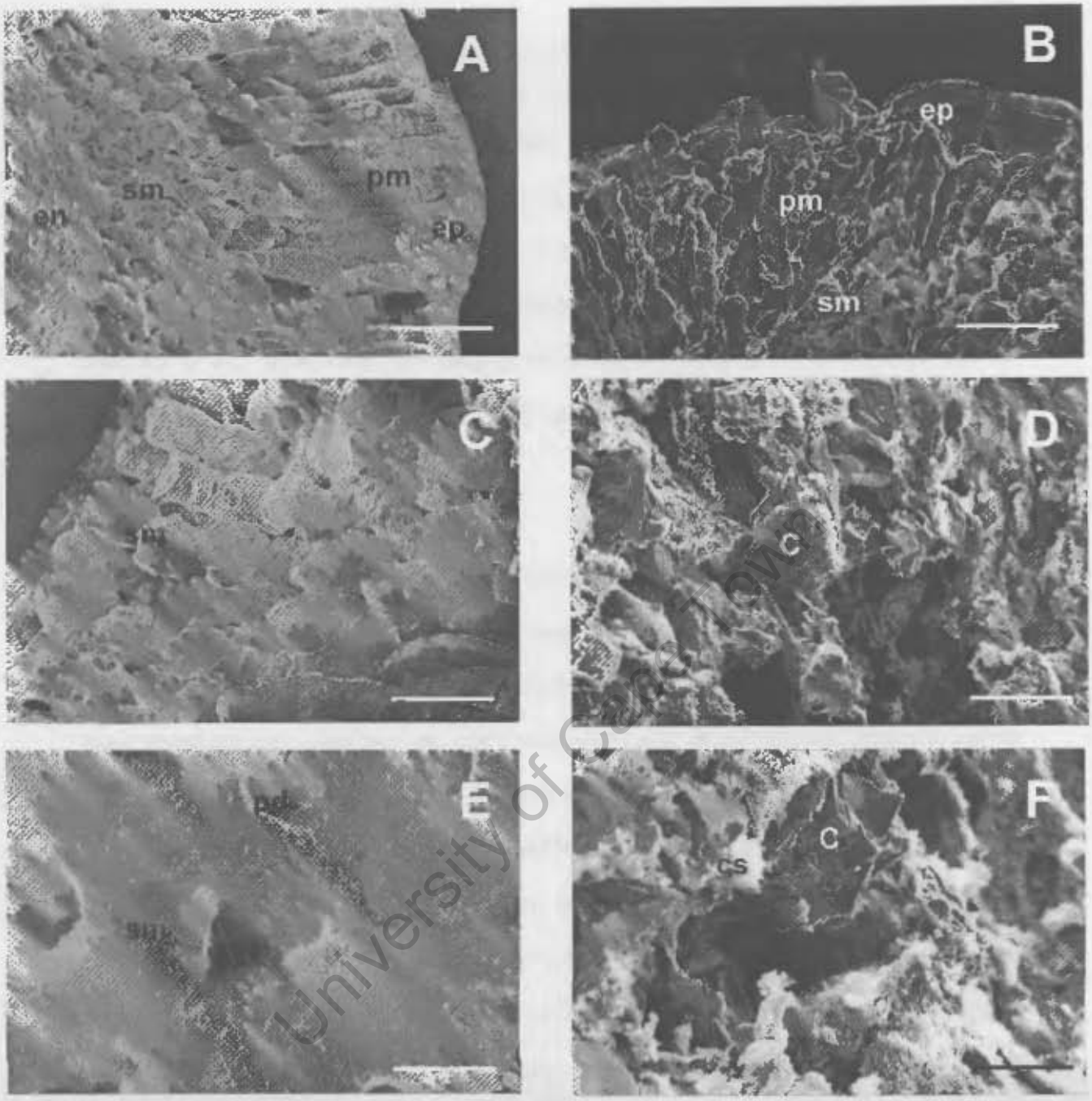


Figure 2.8. Scanning electron micrographs freeze-fracture transverse surfaces of hydrated (A, C, E) and desiccated (B, D, F) *M. flabellifolia* leaves. Key: en, endodermis; sm, spongy mesophyll; pm, palisade mesophyll; ep, epidermis; pd, plasmodesmata; c, calcium oxalate crystal; cs, cytosolic salts. Scale bars: a, 80 μm; b, 20 μm; c, 30 μm; d, 20 μm; e, 10 μm; f, 10 μm.

underwent a limited degree of folding thus likely providing the epidermal and endodermal cells with more support against shrinkage (Figure 2.8B). These cells also appeared generally more recognisable than the mesophyll cells (Figure 2.8B). Occasional amorphous deposits were observed near folded cells (Figures 2.8B and F) which we suggest are crystallised salts and cytosolic constituents resulting from the desiccation of the cell cytoplasm. As previously noted vascular and sclerenchyma tissue did not shrink (Figures 2.2 E-H) likely due to the thick reinforced nature of their cell walls (Brett and Waldron, 1996) which presumably prevented folding.

Higher magnification images of the cell surface of hydrated mesophyll cells (Figure 2.8E) revealed an unusual filamentous network (Figure 2.9A) as well an arrangement of wax beads (Figure 2.9C) covering the surface. This filamentous network was observed predominantly on the surface of spongy mesophyll cells. The filamentous fibres and wax beads were extremely small and were found to range from ~ 100 -200 nm in diameter (Figure 2.9A). At similar magnification the same filamentous network observed in hydrated tissue (Figures 2.9A and C) was found to occur in desiccated cells (Figures 2.9B and D) at cell junctions primarily in the spongy mesophyll region of the leaf. In addition, calcium oxalate crystals in the form of 'druses' (Franceschi and Horner, 1980; Webb, 1999) were observed in cross-sections of both hydrated (Figure 2.9E) and desiccated (Figure 2.9F) leaves.

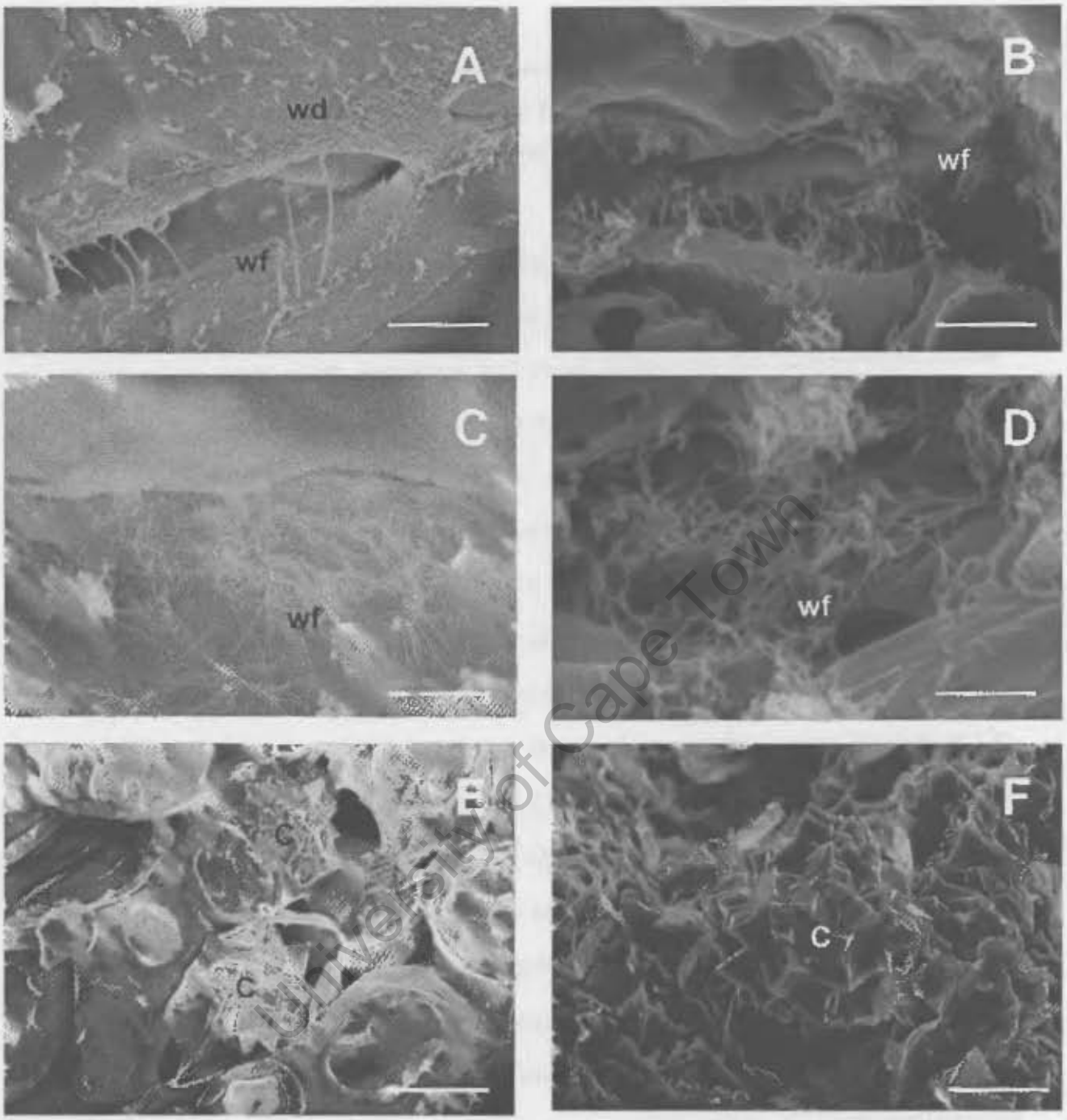


Figure 2.9. Scanning electron micrographs freeze-fracture transverse surfaces of hydrated (A, C, E) and desiccated (B, D, F) *M. flabellifolia* leaves. Key: wf, wax filaments; wd, wax droplets; C, calcium oxalate crystal. Scale bars: a, 3 μm; b, 2 μm; c, 2 μm; d, 1 μm; e, 20 μm; f, 10 μm.

2.3.2 Analysis of the protein, saccharide and metal ion content of hydrated and desiccated *M. flabellifolia* leaves

The presence of large amounts of amorphous deposits in desiccated leaf transverse sections seems to suggest that cytosolic constituents, such as proteins, salts and saccharides, may have precipitated/crystallised out of solution during leaf desiccation. It has been proposed that important changes in the leaf cell protein composition as well as the ratio of soluble sugars to cations occurs prior to desiccation in desiccation tolerant species (Schwab and Gaff, 1986; Kuang et al., 1995; Mtwisha et al., 2005). We therefore undertook a study to ascertain if any major qualitative or quantitative changes in these components occurred in the leaves upon desiccation (Figure 2.10, Tables 2.2 and 2.3). Total proteins were extracted from hydrated and desiccated leaves in an aqueous buffer and then precipitated using ice cold acetone (see methods). The yields of total leaf protein from hydrated and desiccated leaves as determined using the Bradford assay (Bradford, 1976) were not significantly different from each other and amounted to approximately 2.5 % of the leaf dry weight. Electrophoresis of the leaf protein extracts from hydrated desiccated material yielded no significant overall differences in protein composition between hydrated and desiccated samples (Figure 2.10). Peptide mass fingerprint analysis was performed on the major protein bands (Figure 2.10) present at masses ranging from approximately 10 kDa to greater than 50 kDa (Figure 2.10, Table 2.2). The proteins were tentatively identified as a either Chloroplast ribosomal proteins (A, B and D) or Rubisco protein fragments (C and E) (Figure 2.10, Table 2.2). Protein bands C and E (Figure 2.10) almost certainly correspond to the large (rbcL) and small (rbcS) subunits of Rubsico.

Analysis of the soluble saccharide composition of the leaves of *M. flabellifolia* was next performed using HPLC. The main saccharides present in the leaves of *M. flabellifolia* were fructose, glucose, sucrose, trehalose, raffinose and stachyose (Table 2.3). The concentration of

fructose and stachyose were found to be elevated in hydrated as opposed to desiccated leaves (Table 2.3). In contrast, the levels of sucrose, trehalose and raffinose were found to be higher in the desiccated relative to the hydrated leaves (Table 2.3).

In order to ascertain if a similar desiccation-correlated trend (Schwab and Gaff, 1986; Goldsworthy, 1992) of saccharide accumulation and cation decrease occurred in the leaves of *M. flabellifolia*, the metal ion content and concentrations in hydrated and desiccated leaves were determined. The common leaf cations (K, Ca, Na, Mg and Fe) (Ferry and Ward, 1959) were assayed using ICP-MS (Table 2.3). The only significant differences in cation content observed were elevated levels of potassium and sodium in hydrated samples relative to desiccated material (Table 2.3). A similar trend, although not significant, was observed in the calcium levels between the two states (Table 2.3).

Table 2.2. Swiss-Prot Proteomic analysis of excised bands from SDS PAGE gels of total leaf protein extracts from hydrated and desiccated leaves of *M. flabellifolia* (see Fig. 2.10). The best scores for each band analysed are displayed.

Band	Accession number	Description and Source Organism	peptide matches	Score	Mw
A (40kDa)	P30056	Chloroplast 30S Ribosomal Protein S4 *Epifagus virginiana*	4	0.50	undefined
	Q9GFL3	Chloroplast 30S Ribosomal Protein S7 *Ginkgo biloba*	4	0.50	undefined
B (35kDa)	Q9T029	40S Ribosomal protein S25-2 *Arabidopsis thaliana*	4	0.40	35088 Da
	Q9M314	Chloroplast 30S Ribosomal Protein S15 *Spinacia oleracea*	4	0.40	31163 Da
C (30kDa)	P28436	RuBisCO large chain fragment *Oxalis dillenii*	8	0.73	undefined
	P28423	RuBisCO large chain fragment *Hamamelis mollis*	8	0.73	undefined
D (20kDa)	Q8S0J7	Probable membrane associated 30 kDa chloroplast precursor *Arabidopsis thaliana*	5	0.38	23824 Da
	P06378	Chloroplast 50S Ribosomal protein L2 *Marchantia polymorpha*	5	0.38	17791 Da
E (10kDa)	P30828	RuBisCO large chain fragment *Magnolia latahensis*	7	0.64	12053 Da

Table 2.3. Content of various saccharides and metal ions present in the leaves of hydrated and desiccated *M. flabellifolia* plants. Data represents the means and standard deviations of the mean from two independent experiments with at least two replicates per assay.

Compound	Unit of Measure	Hydrated	Desiccated
Saccharides			
Glucose	$mg.g.dw^{-1}$	14.9 ± 2.3	13.1 ± 6.0
Fructose	$mg.g.dw^{-1}$	22.7 ± 4.7	7.5 ± 3.8
Sucrose	$mg.g.dw^{-1}$	18.1 ± 0.6	42.0 ± 10.8
Trehalose	$mg.g.dw^{-1}$	26.9 ± 19.8	41.1 ± 7.3
Raffinose	$mg.g.dw^{-1}$	0.2 ± 0.2	2.4 ± 1.6
Stachyose	$mg.g.dw^{-1}$	4.9 ± 2.7	1.8 ± 1.5
Cations			
Potassium	$\mu g.g.dw^{-1}$	12261.1 ± 4677.6	6332.4 ± 670.6
Calcium	$\mu g.g.dw^{-1}$	4920.4 ± 1049.5	4231.2 ± 566.2
Magnesium	$\mu g.g.dw^{-1}$	986.1 ± 208	1497.3 ± 139.5
Sodium	$\mu g.g.dw^{-1}$	345.2 ± 193.6	29.6 ± 2.6
Iron	$\mu g.g.dw^{-1}$	155.4 ± 89.7	180.7 ± 116.4

2.4 DISCUSSION

Desiccation causes three main types of stress in *M. flabellifolia* leaves; these are mechanical stress, photo-oxidative stress and metabolic stress. Mechanical stress is caused by the effects of severe water loss on hydrated tissue structure which results in tension developing on the cell wall and plasma membrane (Walter et al., 2002). Photo-oxidative stress results from the combination of high light intensities and desiccation-induced cessation of photosynthesis leading to the production of dangerous free radical species (Dalton, 1995; Farrant et al., 2003). Metabolic stress occurs when desiccation-induced osmotic imbalances affect normal cellular processes (Walters et al., 2002). *M. flabellifolia* leaves appear to have evolved a number of adaptations to counteract these stresses.

The general morphology of *M. flabellifolia* leaves seems to be governed by a rigid network of vascular and sclerenchymous tissue which supports the surrounding mesophyll and epidermal tissue during desiccation. The sclerenchymous ribs are largely responsible for the fan-like leaf shape and also appear to allow morphology to be rapidly regained upon 'resurrection'. Numerous gland cells, scattered on both adaxial and abaxial leaf surfaces, are likely responsible for the production of the previously characterised essential oils present in this species (Fahn, 1982; Viljoen et al., 2002). Furthermore, these gland cells maintain their shape upon desiccation whilst the adjacent epidermal cells fold around them. Thus, it is possible that these cells have an additional function in supporting leaf shape during desiccation. The cell walls of the bulk of the mesophyll and epidermal cells are capable of extensive desiccation-induced folding and appear to possess an inordinate degree of flexibility (see Chapter 4). The network pattern of wax-like nano-fibres or filaments observed to occur between leaf mesophyll cells in both hydrated and

desiccated tissue is extremely unusual. No other reports of such structures have been found in the literature. It is known that the vascular tissue of the stem of *M. flabellifolia* accumulates lipid like material which has been proposed to control the spatial rate of water movement during rehydration (Wilson and Drennan, 1992; Wagner et al., 2000; Schneider et al., 2003). It has also been suggested that these lipids may act to separate cells and tissues during desiccation (Schneider et al., 1999). It is possible that this wax-like filamentous network serves a similar role in desiccated leaves. The combination of these unique structural and ultra-structural features appears to prevent the mechanical damage associated with desiccation.

Due to its habitat on bare rock outcrops, *M. flabellifolia* is regularly exposed to high light intensities (Glen et al., 1999) which can be particularly detrimental to the plant as photosynthetic processes may become uncoupled resulting in the generation of dangerous free radicals (Dalton, 1995; Farrant et al., 2003). *M. flabellifolia* appears to utilise multiple mechanisms to ameliorate this photo-oxidative stress. Upon desiccation, the leaf folds against the stem exposing its abaxial surface and concomitantly minimising its face area by closing its leaf ribs together. The abaxial surface is well protected being composed of broad ribs covered with a thick layer of wax. This wax is arranged in longitudinal striations and may function to reflect excess light from the leaf surface (Juniper and Jeffree, 1983). Light reflection is usually mediated by leaf hairs (Fahn, 1982) which are conspicuously absent in *M. flabellifolia.* An additional function for the wax could be in facilitating water run-off from the leaf surface. *M. flabellifolia* rehydrates from the roots (Child, 1960; Glen et al., 1999) as do other resurrection plant species (Gaff, 1977). It has been reported that water applied to the leaf surface is not absorbed in this species (Child, 1960) It is possible that the absorption of water via the leaf surface may be detrimental to proper 'resurrection' in *M. flabellifolia.* In addition to constitutive protective measures against excess light, such as wax layering, we have also shown that the anthocyanin pigment, cyanidin 3-glucoside, accumulates predominately in the spongy mesophyll layer adjacent to the abaxial surface and surface wax

layers. It is proposed that anthocyanins act as protective agents against excess light in plant leaves (Chalker-Scott, 1999). Anthocyanins have also been shown to co-pigment with other phenolic compounds particularly phenolic acids such as chlorogenic acid (Brouillard and Mazza, 1990; Bobbio et al., 1994). We have previously shown that the purified cyanidin 3-glucoside co-pigments with the phenolic compounds (data not shown; Moore et al., 2004) present in *M. flabellifolia*, identified as galloylquinic acids (Moore et al., 2005) (see chapter 3). We propose that this pigment likely functions to protect the underlying chlorophyll and carotenoid pigments of the palisade mesophyll tissue of *M. flabellifolia* leaves which do not change concentration during desiccation.

We have also shown that photosynthetic complex proteins, such as Rubisco, and chloroplast associated ribosomal proteins are stored in the leaves during desiccation. This suggests that *M. flabellifolia* is able to regain the ability to photosynthsise and initiate protein synthesis without the need to rebuild its photosynthetic complexes such as is required in poikilochlorophyllous resurrection plant species (Farrant et al., 2003). Furthermore, *M. flabellifolia* is amphistomatic which allows the plant to take advantage of the high light intensities when sufficient water is available to in order to maximise CO_2 fixation for rapid growth during the rainy season (Parkhurst, 1978; Mott et al., 1982; Goldsworthy, 1992). Moreover, it has been suggested that the position of the stomata in the furrows may allow the plant to regulate transpiration during leaf drying and thereby reduce the rate of leaf dehydration (Goldsworthy, 1992). This could be important as *M. flabellifolia* and other resurrection plants have been shown to be sensitive to rapid drying rates (Farrant et al., 1999).

The extreme water loss associated with desiccation can lead to damage of plant membranes and cell walls (Walters et al., 2002). One of the proposed mechanisms believed to be utilised by desiccation-tolerant organisms to protect membranes is to increase the soluble sugar to cation

ratio in tissues and cells (Schwab and Gaff, 1986; Goldsworthy, 1992). Maintaining a high amount of saccharide whilst diminishing the effective free cation concentration is believed to result in membrane protection (Schwab and Gaff, 1986; Goldsworthy, 1992). *M. flabellifolia* responds to desiccation in a similar manner by increasing the amounts of soluble saccharides such as sucrose and trehalose while concomitantly decreasing the cation concentration in the leaves. Hydathodes have previously been reported to secrete amino acids such as aspartic acid, glutamine and histidine as well as cations such as sodium and potassium from *Arabidopsis* leaves via guttation (Pilot et al., 2004). The hydathode-like stomata present at the leaf apices in this species may regulate the salt content of the leaves similarly through salt secretion upon desiccation. In addition to regulating cation concentration, the vitrification of cell cytoplasm is also believed to be important in stabilising the dehydrated cytoplasm of desiccation tolerant organisms (Hoekstra et al., 2001). Although saccharides, such as sucrose and trehalose, are involved in this process (Hoekstra et al., 2001) we have no evidence that such a mechanism occurs in this species. Finally, it is interesting to note that the application of exogenous sucrose to radish hypocotyls resulted in increased anthocyanin production as well as the upregulation of mRNA transcripts involved in anthocyanin biosynthesis (Hara et al., 2004). It is possible that sucrose and anthocyanin accumulation in *M. flabellifolia* leaves in response to desiccation is similarly co-regulated.

In summary, we have identified a number of possible anatomical, ultrastructural and physiological adaptations that *M. flabellifolia* utilizes in order to survive periods of desiccation and hydration. It is likely that *M. flabellifolia*, due to its basal taxonomic position, having diverged from its sister group *Gunnera* 120 million years ago, has evolved unique solutions to desiccation tolerance not found in other resurrection plants. *M. flabellifolia* appears to utilise a strategy of controlled rehydration and rapid recovery of photosynthetic capacity in order to efficiently capitalise on its erratic water supply.

CHAPTER THREE

THE ROLE OF POLYPHENOLS IN THE DESICCATION TOLERANCE OF *Myrothamnus flabellifolia*

3.1 INTRODUCTION

The broad term 'Polyphenol' refers to a structurally diverse group of compounds produced as secondary metabolites in plant and microbial cells (Haslam and Lilley, 1985; Haslam, 1989). Compounds identified as polyphenols possess an aromatic ring bearing one or more hydroxyl substituents as their defining structural unit (Thomson, 1968; Haslam and Lilley, 1985; Haslam, 1989; Harborne, 1998). Many plant biosynthetic compounds can be classified as polyphenols and so the polyphenol group encompasses a great diversity of structures (Thomson, 1968). Plant polyphenols can be subdivided into a number of sub-groups including the widely distributed 'flavonoids' (Harborne, 1964; 1984), the 'quinones' which are found in primary metabolism (Harborne, 1964; 1998) and the coloured 'anthocyanins' present in flower petals and leaves (Harborne and Simmonds, 1968, Chalker-Scott, 1999). Polyphenols are generally believed to be located in plant cell vacuoles although there is some evidence for organelle specific compounds (Conn, 1984; Hrazdina and Wagner, 1985). The polymeric polyphenols, such as lignin, are extremely hydrophobic and insoluble and are found associated with the cell walls of plants (Higuchi, 1990; Brett & Waldron, 1996). Various forms of biosynthetic modification of polyphenols occur in plant cells such as glycosylation and methylation (Harborne, 1998; Pichersky and Gang, 2000). The functional significance of these modifications is uncertain although it has been proposed that they may act to enhance or diminish solubility in certain cell compartments such as the vacuole or apoplast (Harborne, 1964; Barz et al., 1985). The biosynthetic pathways leading to the various classes of phenolic compounds inevitably share an

evolutionary history (Harborne, 1984) hence similarities between compound classes reflect this fact, with some compounds being widely distributed and ancestral and others being more restrictive appearing in only a few plant groups (Harborne & Simmonds, 1968; Harborne 1998). Many pathways in phenolic metabolism, as in primary metabolism, share precursors and so the final structure of each class is dependent as much on the primary phenolic precursor utilised as it is on the extent of enzymatic modification employed during biosynthesis (Barz et al., 1985; Pichersky and Gang, 2000). The polyphenol biosynthetic pathway is linked to primary carbohydrate and protein metabolism via the gateway enzyme phenylalanine ammonia lyase (PAL) (Boudet et al., 1985). The primary shikimic acid – cinnamate pathway via the chrosimate pool from carbohydrate metabolism is the main supplier of carbon skeletons to the cinnamate reservoir (Boudet et al., 1985; Garrett and Grisham, 1995). From these metabolic junctions, various enzymatic and chemical modifications result in each different plant taxa specialising in the exclusive production of a certain class of phenolic compound (Harborne, 1984; Pichersky and Gang, 2000)

Originally the general belief was that a large majority of phenolic compounds were waste products which the plant siphoned by a process called 'local excretion' into the nearest inert compartments for storage (Boudet et al., 1984; Barz et al., 1985). The two major 'apparently inert' compartments in a plant cell are the cell wall and the cell vacuole, both critical to plant functioning (Boudet et al. 1984; Hrazdina and Wagner, 1985; Brett and Waldron, 1996). It has been shown that exogenously fed xenobiotic compounds, toxic to plant growth, are metabolized and modified afterwhich they are found to accumulate in the cell vacuole and/or cell wall (Barz et al., 1985). Certainly local excretion may have been necessary in plant evolution but this compartmentation allows for selection of new functions and now plants deliberately synthesize and store specific compounds in certain compartments during plant growth and development (Haslam and Lilley, 1985; Grassmann et al., 2002). Evidence indicates that the majority of

phenolic compounds in the plant cell are initially synthesized in the endoplasmic recticulum (ER) where most phenolic compound pathways share their initial precursors and early stages of metabolism (Boudet et al., 1985; Pichersky and Gang, 2000). Phenylalanine ammonia lyase (PAL), the gateway enzyme to the phenylpropanoid pathway, seems to be primarily located on the cytosolic side of the ER where it has access to the amino acid biosynthetic products produced by primary metabolism in the cell cytoplasm (Boudet at al., 1985; Garrett and Grisham, 1995). Moreover, most of the phenylpropanoid pathway enzymes seem to be membrane bound, located at the ER (Boudet at al., 1985). Hence plant secondary metabolites are deposited far from the sites of original synthesis such as in plant vacuoles and so the need for feedback inhibition mechanisms in plant secondary metabolism is much reduced as compared to other organisms (Boudet et al., 1985).

Vacuoles are now known to be much more dynamic entities of the plant cell than what was originally thought (Ferry and Ward, 1959; Boudet at al., 1984; Hrzadina and Wagner, 1985; Buchanan et al., 2002). The vacuole was the first plant organelle to be studied and the initial major functions ascribed to it included the maintenance of turgor pressure as well as osmotic balance (Boudet et al., 1984; Hrzadina and Wagner, 1985). The vacuole itself possesses no anabolic functions but has the ability to actively and passively transport molecules across its membrane (Hrzadina and Wagner, 1985). A range of anthocyanins, flavonoids, tannins and other phenolic compounds have all been shown to accumulate in the plant cell vacuole (Boudet at al., 1984; Conn, 1984; Hrzadina and Wagner, 1985). Plant vacuoles are capable of reversible and irreversible storage of a variety of compounds (Boudet et al., 1984; Hrzadina and Wagner, 1985). In addition to vacuoles there is some evidence of accumulation in the chloroplast of flavonoids and anthocyanins (Harborne and Simmonds, 1968; Hrzadina and Wagner, 1985).

Polyphenols have been extensively researched with regards to biotic stress; particular examples include herbivory and pathogen invasion where more obvious correlations exist between enhanced levels of a particular phenolic and a biotic stimulus (Weier, 1982; Friend, 1985; Winkel-Shirley, 2002). In contrast, research into the role of phenolic compounds in plant abiotic stress tolerance has not been undertaken to any great extent (Grassmann et al., 2002). Plants are subject to a range of abiotic stresses and because of their sedentary nature they must develop mechanisms to survive these periods (Galston, 1994). The main types of abiotic stresses plants encounter include drought, freezing, UV light, osmotic, anoxia, oxidative and heavy metal toxicity (Levitt, 1980; Galston, 1994). A role for polyphenols in plant abiotic stress tolerance, particularly light and oxidative stress, has more recently begun to be recognized and appreciated (Yamasaki et al., 1997; Cockell and Knowland, 1999; Grassmann et al., 2002).

The flavonoid metabolic and genetic pathway has been shown to be dependent on UV light stimulation (Jenkins, 1999; Pichersky and Gang, 2000). Certain genes involved in biosynthesis of phenolic compounds are regulated by light such as the enzyme Chalcone Isomerase which requires intermediates to undergo light induced isomerisation before conversion to the next metabolic intermediate, which seems to indicate an evolutionary dependence on light stimulus (Towers et al., 1985; Jenkins, 1999). But although the light connection exists with phenolic metabolism it is still poorly understood and there is a lack of sufficient evidence demonstrating how polyphenol compounds function in relation to light stress (Cockell and Knowland, 1999). It has been shown that *Arabidopsis* mutants with defective phenolic genes display different degrees of UV tolerance which may indicate different hierarchies of polyphenols with respect to their efficacy in ameliorating UV damage (Landry et al., 1995; Winkel-Shirley, 2002). Flavonoids in many plant leaves are found in the epidermal layers where they are believed to function as 'sunscreens' against photo-oxidative damage produced by excess light falling on the photosynthetic apparatus (Landry et al., 1995; Yamasaki et al., 1997).

The evolution of aerobic metabolism has been a double edged sword to aerobic organisms since the generation of oxygen radicals, as a side reaction of metabolism, is detrimental and damaging to the very organisms that require oxygen to maintain primary metabolism (Garrett and Grisham, 1995; Dalton, 1995). Hence organisms in parallel to the acquisition of aerobic metabolism have had to evolve the capability to detoxify or remove these dangerous free radicals (Dalton, 1995; Larson, 1995). The main antioxidant pathway in many organisms is the Haliwell-Asada 'ascorbic acid and glutathione' redox cycle which involves the linking of reducing equivalents in primary metabolic pathways to the antioxidants ascorbic acid and glutathione (Asada, 1994; 1996). These antioxidants function to reduce ROS preventing damage to the organism and they are then regenerated by enzymes with reducing co-factors hence linking the pathway to the reducing power generated from primary metabolism (Asada, 1994; 1996). Polyphenols are powerful antioxidants and have been shown to prevent ROS damage in a number of *in vitro* and *in vivo* biological systems (Larson, 1995; Karpinski et al., 1999; Grassmann et al., 2002). The question whether this antioxidant capability is utilized by the plants in protecting themselves against oxidative stress has been largely unexplored whereas much research has focused on the beneficial effects of plant polyphenols in health and medicine (Grassmann et al., 2002). There has been some research which indicates a potential link between the primary anti-oxidant Haliwell-Asada cycle and the vacuolar antioxidant plant polyphenols (Yamasaki et al., 1997; Pérez et al., 2002) but further work is necessary to substantiate and explore this potentially promising area of plant research.

No detailed chemical analysis has been performed to elucidate the chemical structure(s) of the main polyphenol(s) present in the leaves of *Myrothamnus flabellifolia*. In the present study, we report on the structure and cellular location of the main polyphenols present in hydrated and dehydrated leaves of *M. flabellifolia*. In addition, we present data suggesting that this class of

polyphenols is able to protect membranes against desiccation and also against free radical-induced damage. Furthermore, we demonstrate that the leaf galloylquinic acid composition varies between populations of *M. flabellifolia* in Southern Africa and reflect on the significance of these observations in respect of desiccation tolerance in this species (Moore et al., 2005b). We also investigated the genetic variation between populations based on nucleotide sequence comparisons of regions on the chloroplast genome and relate these data to the phenolic analysis performed.

3.2 MATERIALS AND METHODS

3.2.1 Plant Material

Plant material for dehydration/rehydration experiments: *M. flabellifolia* plants were collected near Outjo (Namibia) and from Vaalwater (South Africa). Whole plants were transported to Cape Town and maintained in a glasshouse as described previously (Sherwin and Farrant, 1996). These were dehydrated by withholding water for approx. 2 weeks until the plants had reached an air-dry state, with approx. 12.5 % RWC (relative water content). Plants were maintained in the dry state for at least 6 months before sampling. Water content was determined gravimetrically by oven drying at 70°C for 48 h and RWC was calculated as described previously (Cooper and Farrant, 2002). Desiccated plant material for molecular and chemotaxonomic analyses was obtained from populations (see Figure 3.1 for a map) occurring in Namibia (N1 to N5), namely, the Komas Hochland, near the Cunene river and around Outjo as well as populations in South Africa (S1 to S4), namely, the Limpopo province, near Lydenberg, and the Blouberg mountains, Mpumalanga province. The dry leaf material was stored at 20 °C in the laboratory until use.

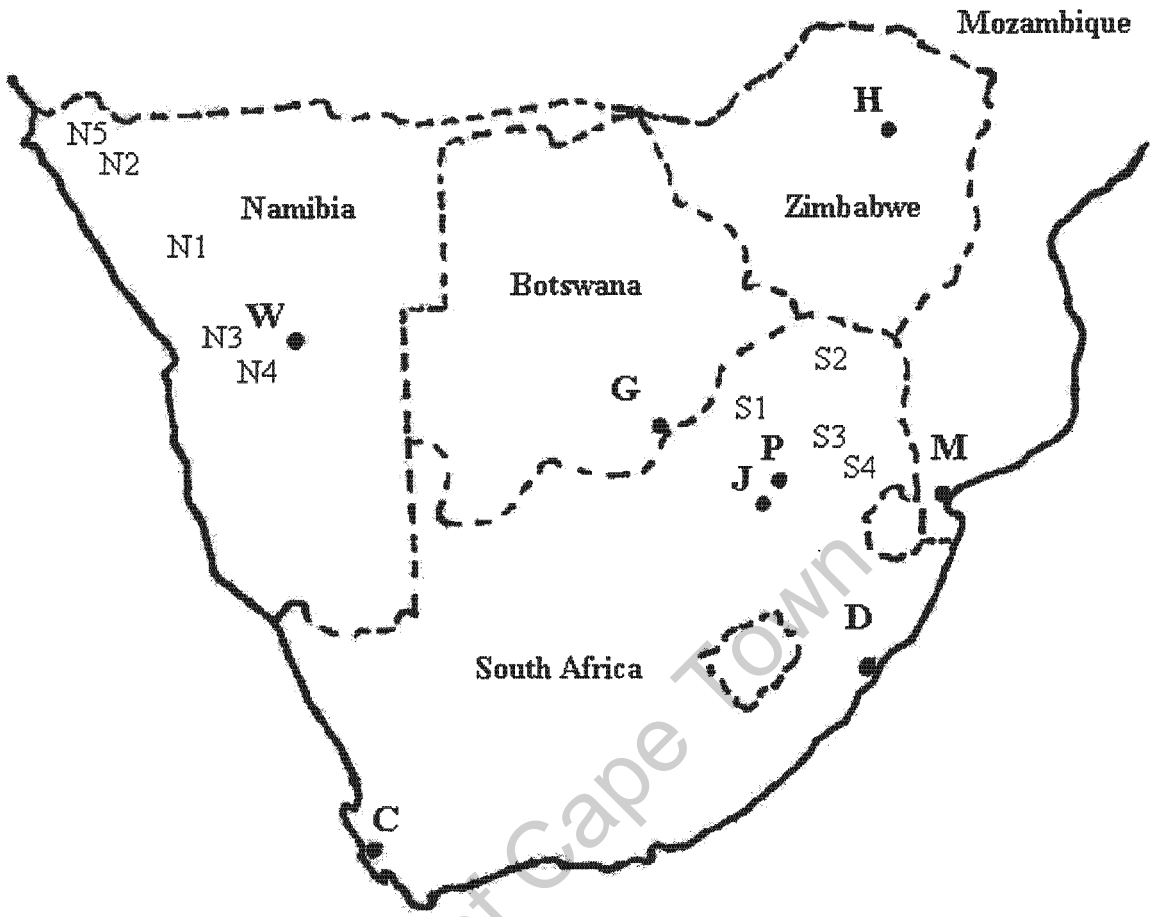


Figure 3.1. A map of southern Africa displaying the locations of the populations of *Myrothamnus flabellioflius* in South Africa (S1-S4) and Namibia (N1-N5) used in the study of genetic and galloylquinic acid variation. Key: C, Cape Town; D, Durban; G, Gaborone; H, Harare; J, Johannesburg; M, Maputo; P, Pretoria; W, Windhoek.

3.2.2 Polyphenol purification

Leaves (5 g) from dehydrated plants were ground to a fine powder and extracted using the serial reflux extraction at 60°C with 100 ml of heptane (twice) followed by 70% methanol (twice). The latter extracts were pooled to yield approx. 90% of the total extractable UV absorbing material in this fraction. Preliminary preparative separation of the main polyphenols from *M. flabellifolia* was performed using isocratic cellulose column chromatography using 5% acetic acid as the eluant. Selected column fractions were further purified using low-pressure liquid chromatography in 0.1% TFA (trifluoroacetic acid) using Waters RP-C18 resin. Fractions were eluted using a linear gradient between 0.1% TFA and 100% acetonitrile and 0.1% TFA for 40 min at a flow rate of 0.7 ml/min. Final purification was by preparative HPLC using a Higgins Analytical RP-C18 column and using the same buffer system but at a flow rate of 2.5 ml/min. Elution (Figure 3.2) yielded approx. 2 mg of an amorphous white powder (peak 1) after freeze-drying.

3.2.3 Quantitative and compositional analysis of polyphenols

Dehydrated and hydrated leaves were dissected longitudinally with a razor blade. One half was used to determine leaf RWC, whereas the other half was freeze-dried to a stable dry weight and then analysed for polyphenol content. Phenolic extraction was performed by homogenizing leaf material for 5 min in 70% aqueous methanol using a Polytron Homogeniser (Kinematica PT 2000). The homogenate was then subjected to sonication (Bandelin Sonorex bath sonicator) for 5 min after which it was centrifuged at ~ 12000 g for 5 min. The pellet was twice re-extracted using 3 volumes solvent per mass of sample. The supernatants were pooled, the spectrum between 200 nm and 400 nm determined, and 20 µl was subjected to analytical HPLC. Selected fractions were collected, dried and analysed by MALDI-TOF-MS. Mild acid hydrolysis was performed using 2M HCl at 80°C for 30 min.

3.2.4 High Performance Liquid Chromatography

High Performance Liquid Chromatography was performed on a Shimadzu LC-10A binary gradient system equipped with Photo-Diode Array detection (Shimadzu Corporation, Kyoto, Japan). Fractions were eluted from a Jones chromatography RP-C18 (250 x 4 mm) column using a linear gradient between 0.1% TFA and 100% acetonitrile with 0.1% TFA over 40 min at a flow rate of 0.7 ml/min.

3.2.5 Mass Spectrometry

ESI (electrospray ionization) mass spectra were obtained using a Fisons VG Quattro mass spectrometer equipped with a triple quadropole analyser. Sample aliquots in 50% acetonitrile were injected directly into the spectrometer, which was operated in negative-ion mode using a capillary voltage of -2.7 kV and a cone voltage of 40 V. In-flight fragmentation was achieved using argon gas collision-induced fragmentation in the quadropole analyser. A collision energy of 13 eV and an argon collision gas pressure of 2×10^{-3} bar were used.

MALDI-TOF mass spectra were obtained on a Perseptive Biosystems DE-PRO MALDI mass spectrometer equipped with a TOF analyzer (Perseptive Biosystems, Framingham, Massachusetts, USA). A 1 µl sample aliquot was dissolved in a 50 % aqueous acetonitrile solution, mixed with 1 µl 2, 5 dihydroxybenzoic acid matrix and applied to a gold sample plate. The mixture was air-dried prior to use. The spectrometer was operated in positive and negative ion modes.

3.2.6 Lipid oxidation studies

Linoleic acid oxidation was performed using AAPH [2,2- azobis(2-amidino-propane) dihydrochloride] as a free radical source. Oxidation was determined by an increase in absorption at 234 nm, which monitors the production of conjugated diene hydroperoxides (ε =2.8×10^4 M^{-1} cm^{-1}) (Liegois et al., 2000).

3.2.7 Scanning and transmission electron microscopy

Leaves from five fully hydrated (~ 95 % RWC) and five dry (~ 5 % RWC) plants were cut into 4 mm^2 segments and were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) supplemented with 0.5% caffeine. Dehydration was performed using a graded ethanol series and specimens were infiltrated and then embedded in epoxy resin (Spurr, 1969). Leaf segments (1 - 2 mm^2) were excised from the mid-blade of hydrated or desiccated leaves and fixed overnight at 4 °C in 0.1M phosphate buffer pH 7.4 containing 2.5 % glutaraldehyde supplemented with 0.5 % caffeine. Certain aldehyde-fixed specimens were post-fixed in a 1 % solution of osmium tetroxide. Fixed samples were dehydrated in ethanol and embedded in epoxy resin (Spurr, 1969). Thin sections (90 – 100 nm) were cut using a Reichert Ultracut-S ultramicrotome and collected on 200 µm mesh copper grids. For transmission electron microscopy sections were prepared using a Reichert Ultracut-S microtome after which they were stained with uranyl acetate and lead citrate (Reynolds, 1963). Transmission electron microscopy was performed using a LEO 912 transmission electron microscope equipped with CCD camera. Certain resin blocks were polished with alumina slurry gamma (0.5 µm) and viewed using a fully analytical Leica Stereoscan 440 digital scanning electron microscope equipped with a 4 quadrant backscatter detector. For elemental analysis certain specimens were coated with gold-palladium or carbon prior to viewing.

3.2.8 DNA extraction, PCR amplification, purification and sequencing

Leaf material was frozen, lyophilized and ground in a mortar using liquid nitrogen prior to DNA extraction. Total DNA was extracted using a modified CTAB DNA extraction protocol (Gawel and Jarret, 1991). The standard CTAB buffer (5 ml) (Gawel and Jarret, 1991) was supplemented with 20 mg PVP (poly-(1-vinyl-pyrrolidine-2)) (Merck) and 1 ml of 0.1 % solution of aqueous 2-mercaptoethanol (Gawel and Jarret, 1991). DNA purity was assessed using the A_{260}:A_{280} ratio and by agarose gel electrophoresis. The PCR reaction mixture consisted of SuperTherm buffer, 0.025 Units SuperTherm *Taq* polymerase, 5 mM $MgCl_2$, 0.1 mM dNTP, 0.33 µM each forward and reverse primers and 3 µl template DNA in a total volume of 30 µl. The amplification program consisted of: 95 °C for 2 min; 30 cycles of 95 °C for 1 min, 52 °C for 1 min and 72 °C for 2 min with a final extension of 72 °C for 7 min. The forward and reverse primers, designed to amplify the *psbA-trnH* region on the chloroplast genome (Aldrich et al., 1988), were 5'GTTATGCATGAACGTAATGCTC3' and 5'CGCGCATGGTGGATTCACAAATC3' respectively. PCR reactions were supplemented with various amounts of soluble PVP (Merck) to reverse polyphenol inhibition (Koonjul et al., 1999). Amplified products were purified using a QIAquick PCR purification kit (Qiagen Corp. 2002). Product quantity and purity was approximated using agarose gel electrophoresis. Purified products were sequenced on a MegaBACE 1000 sequencer. DNA sequences were edited using Chromas 2.01. Alignment of sequences was performed using DNAMAN and MegAlign 3.08.

3.3 RESULTS

3.3.1 Characterisation of the polyphenols present in the leaves of *M. flabellifolia*

The purified polyphenol compound was characterised to be 3,4,5 tri-*O*-galloylquinic acid (Figures 3.2 and 3.3) from the ^{1}H and ^{13}C one- and two-dimensional NMR spectra from previous analysis (Moore, 2001). To confirm the identity of the purified polyphenol as 3,4,5 tri-*O*-galloylquinic acid, the purified polyphenol was subjected to mass spectral analysis using MALDI-TOF-MS to determine the mass of the parent compound. ESI-MS was also performed to show that the fragmentation pattern was consistent with the proposed structure (Figure 3.3), which would have a molecular mass of 648 Da (all values ± 1 Da). The MALDI-TOF positive ion mass spectrum displayed peaks at *m/z* values of 671, 688 and 694 corresponding to the sodium $[M+Na]^+$, potassium $[M+K]^+$ and di-sodium $[M-H+2Na]^+$ adducts respectively. The MALDI-TOF negative ion spectrum displayed a peak at *m/z* 647, which was considered to represent the deprotonated parent ion $[M-H]^-$. The ESI-MS spectra of 3,4,5 tri-*O*-galloylquinic acid gave two peaks $[M-2H]^{2-}$ and $[M-H]^-$ at *m/z* values of 323 and 647 respectively. The most abundant species was the doubly charged *m/z* 323 ion indicating the tendency of the molecule to readily acquire a second charge during ionization. Collision-induced dissociation fragmentation of this ion resulted in two fragment ions at *m/z* 247 and 169. Further fragmentation of the *m/z* 169 ion produced a prominent *m/z* 125 ion, which was interpreted to cleavage of one of the C-1– C-7 galloyl carbon–carbon bonds releasing a charged pyrogallol ring. The *m/z* 169 ion was considered to have resulted from cleavage of the C-3, C-4 or C-5 carbon– oxygen bonds releasing charged gallic acid ions (Figure 3.3). The *m/z* 247 ion was deduced to represent the doubly charged parent molecule with one of its galloyl groups cleaved at the labile ester between C-7 of a galloyl group and the C-3, C-4 or C-5 oxygen atom of the quinic acid moiety (Figure 3.3). Since the MS data

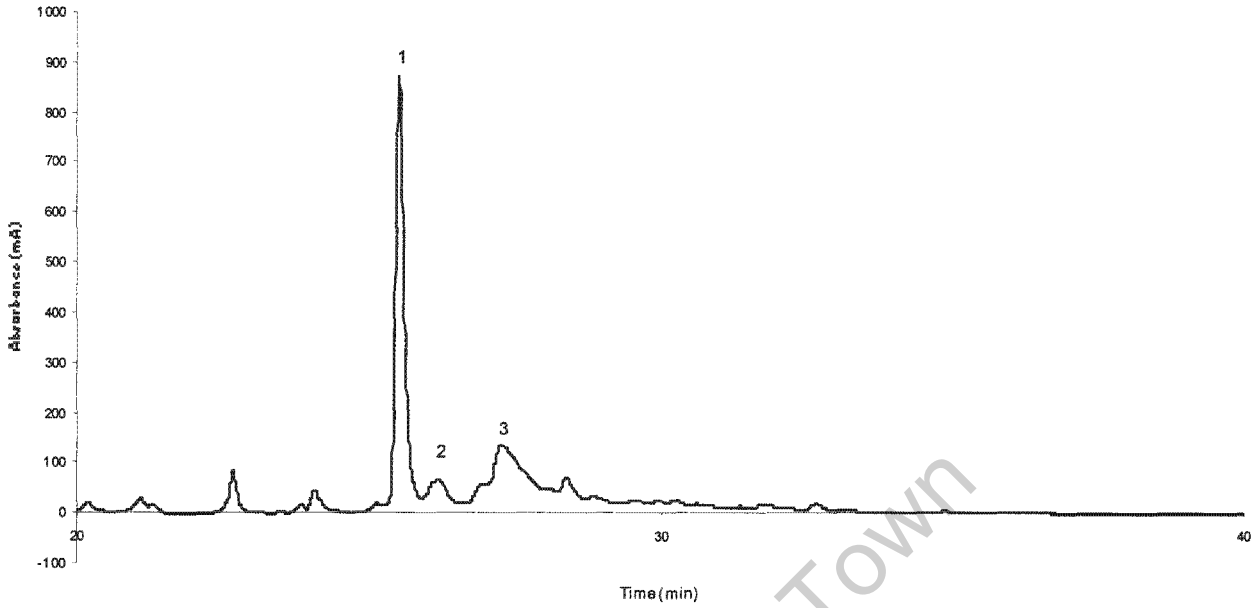


Figure 3.2. Elution profile of total polyphenols eluted from the C_{18} HPLC column using a 0-100 % gradient of acetonitrile in 0.1 % TFA. Peaks 1-3 were pooled and freeze-dried before analysis. The absorbance is shown in milli-absorbance units (mAbs).

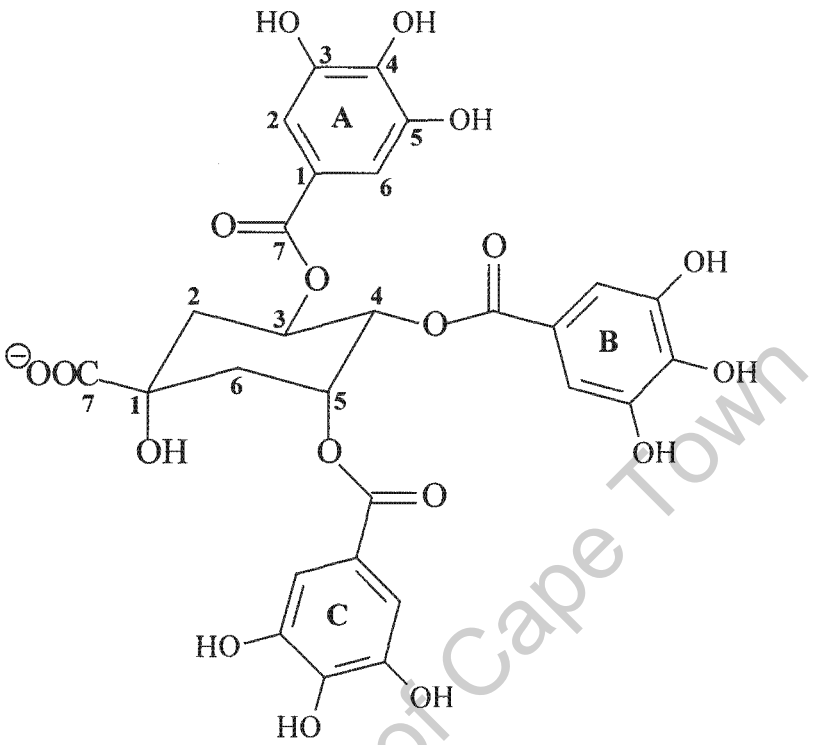


Figure 3.3. Structure of 3, 4, 5 tri-*O*-galloylquinic acid isolated from *M. flabellifolia.* The galloyl rings were annotated A-C to aid in the interpretation of the mass spectral data (from Moore et al. 2005a).

were consistent with the structure assigned from the NMR spectra, we concluded that the proposed structure was indeed 3,4,5 tri-*O*-galloylquinic acid.

Galloylquinic acid derivatives have been reported to be present in other plants. The ethyl ester of 3,4,5 tri-*O*-galloylquinic acid together with other quinic acid gallates have been reported to be present in *Guiera senegalensis* (Bouchet et al., 1998), tetragalloylquinic acid has been reported to be present in *Galphimia glauca* (Neszmelyi et al., 1993) and gallotannins have been reported to be present in *Lepidoptrys staudtii* (Bokesch et al., 1996). These compounds were determined to be galloylquinic acids from their ^{1}H and ^{13}C one dimensional NMR spectra and the fast atom bombardment mass spectra. However, no complete structure was assigned as two dimensional NMR spectral analyses were not performed.

The presence of additional polyphenols but in significantly lower abundance were observed during the isolation of 3,4,5 tri-O-galloylquinic acid from the leaves of *M. flabellifolia* (Figure 3.2). To investigate these polyphenols, total polyphenol extracts were subjected to HPLC analysis and selected fractions were analysed by MALDI–TOF-MS. This showed that the polyphenols eluted from the HPLC column in the order of increasing molecular mass with masses at *m/z* 647, 786, 799, 936, 951, 1103, 1255, 1407, 1570 and 1722 (all ± 1 Da) being observed (Table 3.1). Since the cleavage mass of the galloyl ion is *m/z* 152, this series at approx. 152 Da intervals suggested multiple galloylation of 3,4,5 tri-O-galloylquinic acid with the ions at *m/z* 647, 799, 951, 1103, 1255 and 1407 deduced to represent the tri- to octa-galloylquinic acids respectively, although the peaks with masses of 1103, 1255 and 1407 Da were present in low abundance. The latter two masses of 1570 and 1722 Da were interpreted to be ellagic acid tannins derived from hexa- and hepta-galloylquinic acids respectively brought about by the oxidative addition of gallic acid moieties (Figure 3.4) (Nonaka, 1989). This was confirmed by mild acid hydrolysis of the total polyphenol extract. This resulted in gallic acid together with 3,4,5 tri-*O*-galloylquinic acid as

Table 3.1

Molecular ion species present in various HPLC fractions of polyphenols extracted from Namibian and South African plants (see text for details).

Fraction number	R_t	Galloyl groups	Quinic acid groups	M_r	Molecular ion
1#	25.8	3	1	647*	$[M-H]^-$
		4	1	675*	$[M-C_6H_5O_3-H]^-$
2#	26	3	1	647*	$[M-H]^-$
		4	1	675*	$[M-C_6H_5O_3-H]^-$
		4	1	783*	$[M-OH]^-$
		5	1	936*	$[M-OH]^-$
		9	1	1570*	$[M-H]^-$
		10	1	1722*	$[M-H]^-$
3#	27	4	1	783*	$[M-OH]^-$
		4	1	799*	$[M-H]^-$
		5	1	936*	$[M-OH]^-$
		5	1	953*	$[M-H]^-$
		9	1	1570*	$[M-H]^-$
		10	1	1722*	$[M-H]^-$
4	29	4	1	767	$[M-2OH]^-$
		4	1	783*	$[M-OH]^-$
		5	1	936*	$[M-OH]^-$
		6	1	1171	$[M+CO-H]^-$
		7	1	1322	$[M+CO-H]^-$
		9	1	1570*	$[M-H]^-$
		10	1	1722*	$[M-H]^-$
		11	1	1874	$[M-H]^-$
5	29.7	4	0	636	$[M+CO-OH]^-$
		4	1	675*	$[M-C_6H_5O_3-H]^-$
				751	unassigned
		6	0	912	$[M-OH]^-$
		5	1	936*	$[M-OH]^-$
		6	1	1088	$[M-OH]^-$
		7	1	1322	$[M+CO-H]^-$
		9	1	1570*	$[M-H]^-$
		10	1	1722*	$[M-H]^-$
		12	1	2054	$[M+K-2H]^-$
		13	1	2203	$[M+K-2H]^-$
6	30.1	4	0	636	$[M+CO-OH]^-$
		4	1	675*	$[M-C_6H_5O_3-H]^-$
				751	unassigned
		5	0	882	$[M-CO-H]^-$
		5	1	936*	$[M-OH]^-$
		6	1	1088	$[M-OH]^-$
		7	1	1322	$[M+CO-H]^-$
		9	1	1570*	$[M-H]^-$
		10	1	1722*	$[M-H]^-$
		13	1	2203	$[M+K-2H]^-$

and * represent the respective peak fractions and molecular ions which are common to both South African and Namibian plants

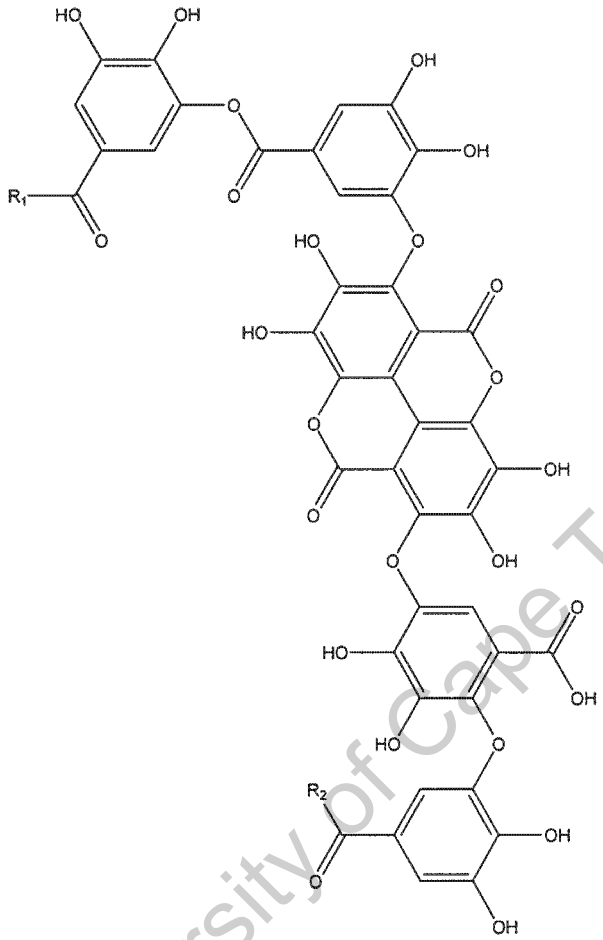


Figure 3.4. Putative substructure of one of galloylquinic acids identified using HPLC and MS data. The structure reveals the polygalloyl ester chains and ellagic acid moiety inferred to be present based on the mass spectral data. R_1 and R_2 represent the 3, 4, 5 tri-*O*-galloylquinic acid core.

the major hydrolysis products (results not shown). The depside ester bonds of the gallic acid polyester chains have been reported (Mueller-Harvey, 2001) to be more labile to hydrolysis than the quinic–gallic ester bonds and thus the core molecule would remain largely intact after mild acid hydrolysis. We therefore consider that the major polyphenol found in *M. flabellifolia* is 3,4,5 tri-*O*-galloylquinic acid and that, in addition, a number of higher molecular mass gallic acid polyesters are present, but at markedly lower concentrations. These are due to multiple galloylation of the central 3,4,5 tri-*O*-galloylquinic acid core.

Quantitative analysis of total leaf polyphenols was undertaken to determine whether a difference in polyphenol content existed between fully hydrated (~ 90 % RWC) and desiccated (~ 12 % RWC) leaves (Table 3.2). Hydrated leaves were found to contain ~ 40 % (g.g dry wt^{-1}) of 3,4,5 tri-*O*-galloylquinic acid whilst the content in desiccated leaves increased slightly to ~ 45-55 %. This would indicate that galloylquinic acids are the main compounds in *M. flabellifolia* at 25-50 % w/w they far in excess of the previously analysed saccharides such as trehalose and sucrose which amount to around 4 % w/w of the leaf dry weight.

3.3.2 Functional roles for galloylquinic acids in membrane protection against desiccation and free-radical induced oxidation

Polyphenols have been shown to protect membranes against osmotic stress and desiccation (Won-Huh et al., 1996; Hincha et al., 1999). Furthermore a recent study has revealed that 3,4,5 tri-*O*-galloylquinic acid isolated from *M. flabellifolia* is able to protect a model membrane system against desiccation (Westall, 2002). In this study various quantities of this compound was added to liposomes made from phosphatidylcholine and cholesterol encapsulating the fluorescent dye

Table 3.2.

Percentage polyphenol content of hydrated and desiccated leaves of *M. flabellifolia* from South African and Namibian locations.

Location	Hydrated		Desiccated	
	RWC	% polyphenol content	RWC	% polyphenol content
South Africa				
Vaalwater	86.0 ± 11.7	39.3 ± 16.7	9.8 ± 2.3	56.9 ± 18.2
Magaliesberg	90 ± 8.9	37 ± 2.7	12.3 ± 3.3	42.5 ± 4.9
Namibia				
Outjo	86.4 ± 9.7	44.3 ± 11.9	12.8 ± 4.5	56.5 ± 8.1

Polyphenol content is expressed in TGA (tri-O-galloylquinic acid) equivalents in % g polyphenol per g dry wt. RWC is expressed as percentage g H_2O per g H_2O at full turgor. Data represent the mean and standard deviation from two independent experiments with at least three assays per experiment.

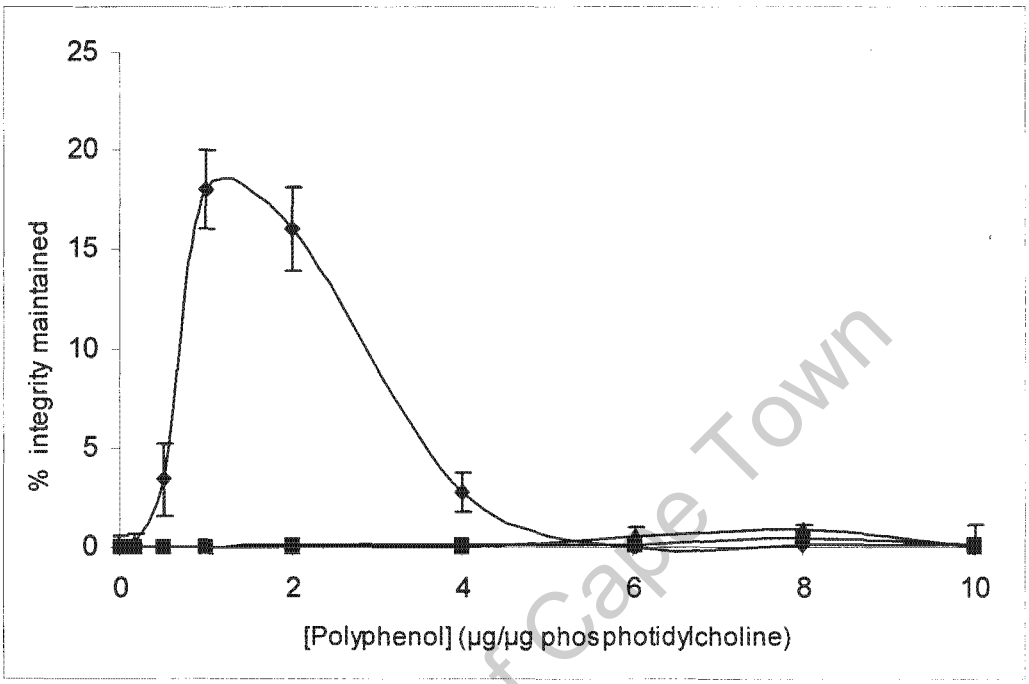


Figure 3.5. Liposome structural integrity after desiccation and subsequent rehydration determined by calcein leakage as a function of polyphenol addition (from Westall, 2002; Moore et al., 2005a). ♦, 3, 4, 5 tri-*O*-galloylquinic acid; ■, arbutin; ▲, gallic acid

calcein, before the liposomes were subjected to desiccation and subsequent rehydration (Westall, 2002). It was found (Figure 3.5) that the addition of 3,4,5 tri-*O*-galloylquinic acid indeed protected liposomes against desiccation with a maximum protection observed at a polyphenol/phospholipid ratio of 1 (w/w) (Westall, 2002). Lowering or increasing the polyphenol/phospholipid ratio resulted in decreased protection being observed (Westall, 2002). In contrast, the addition of equivalent concentrations of gallic acid to the liposome preparation resulted in no protection being observed (Figure 3.5). Similar results demonstrating maximum protection at low concentrations of the polyphenol with no protection being observed at higher concentrations were reported for arbutin (4-hydroxyphenyl-β-glucopyranoside) binding to liposomes containing monogalactosyldiacylglycerol (Hincha et al., 1999). It has been reported (Suau et al., 1991) that arbutin is present in significant quantities in *M. flabellifolia*, although we were not able to detect any trace of this compound. Furthermore, the study also reported that the addition of arbutin to the liposome preparation resulted in no protection being observed (Figure 3.5). This result agrees with previous work that demonstrated that the addition of arbutin to liposome preparations only resulted in protection against desiccation when the chloroplast lipid monogalactosyldiacylglycerol was present (Hincha et al., 1999).

A possible explanation of these data is that 3,4,5 tri-*O*-galloylquinic acid binds exclusively to liposomes at low polyphenol/phospholipid ratios, but as the ratio increases, the liposome 'sites' become saturated and 3,4,5 tri-*O*-galloylquinic acid appears in solution (Figure 3.6) (Westall, 2002). Since 3,4,5 tri-*O*-galloylquinic acid is amphipathic, micelle formation occurs and the binding of 3,4,5 tri-*O*-galloylquinic acid molecules to one another to form a micelle is stronger than the interaction between 3,4,5 tri-*O*-galloylquinic acid and liposomal lipids resulting in the removal of the 3,4,5 tri-*O*galloylquinic acid from the liposome preparation (Figure 3.6) (Westall, 2002). Circumstantial evidence in favour of this model is that an aqueous solution of 3,4,5 tri-*O*-galloylquinic acid frothed when shaken, a behaviour typical of detergents in solution.

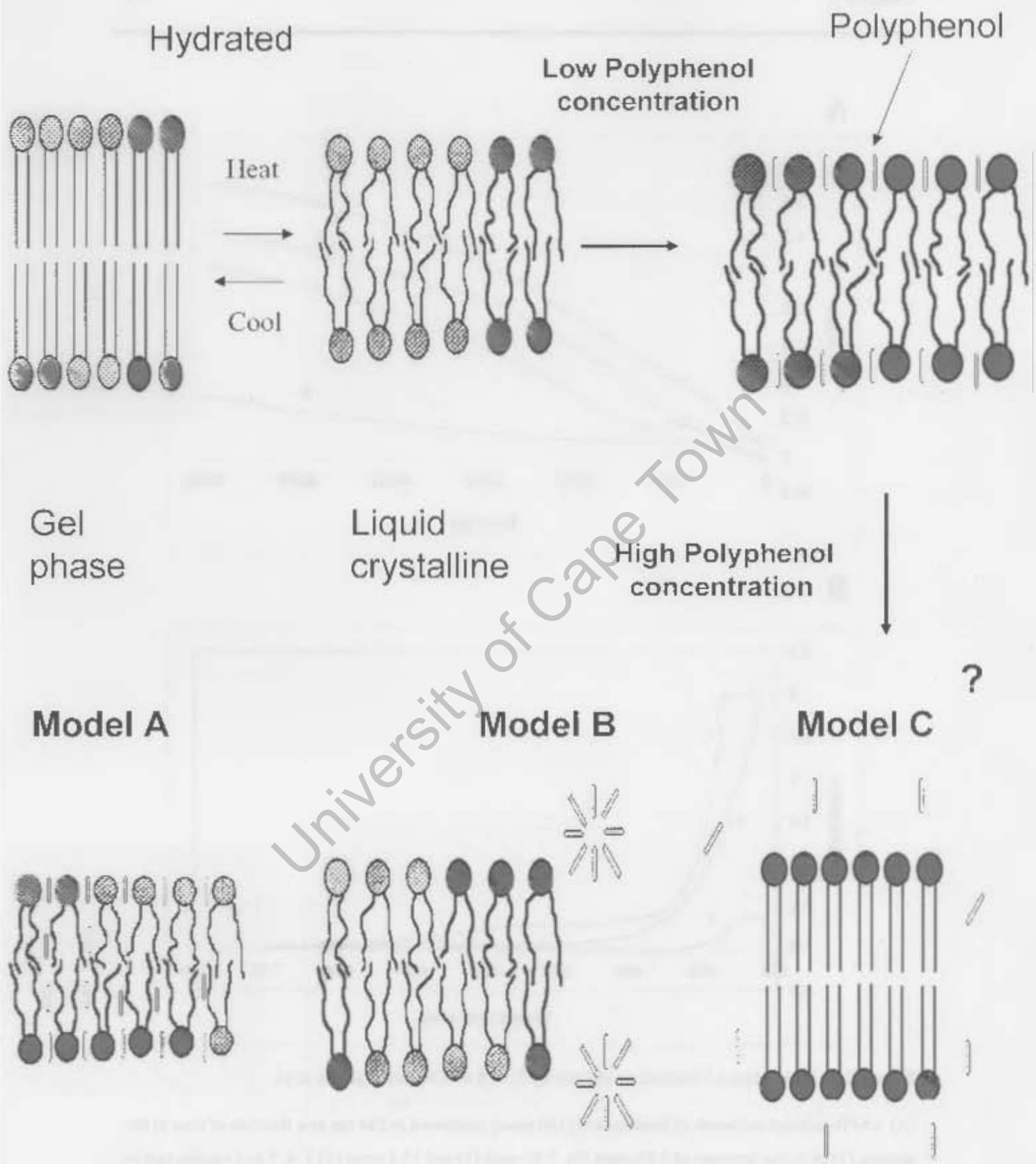


Figure 3.6. Models for polyphenol interactions with biological membranes

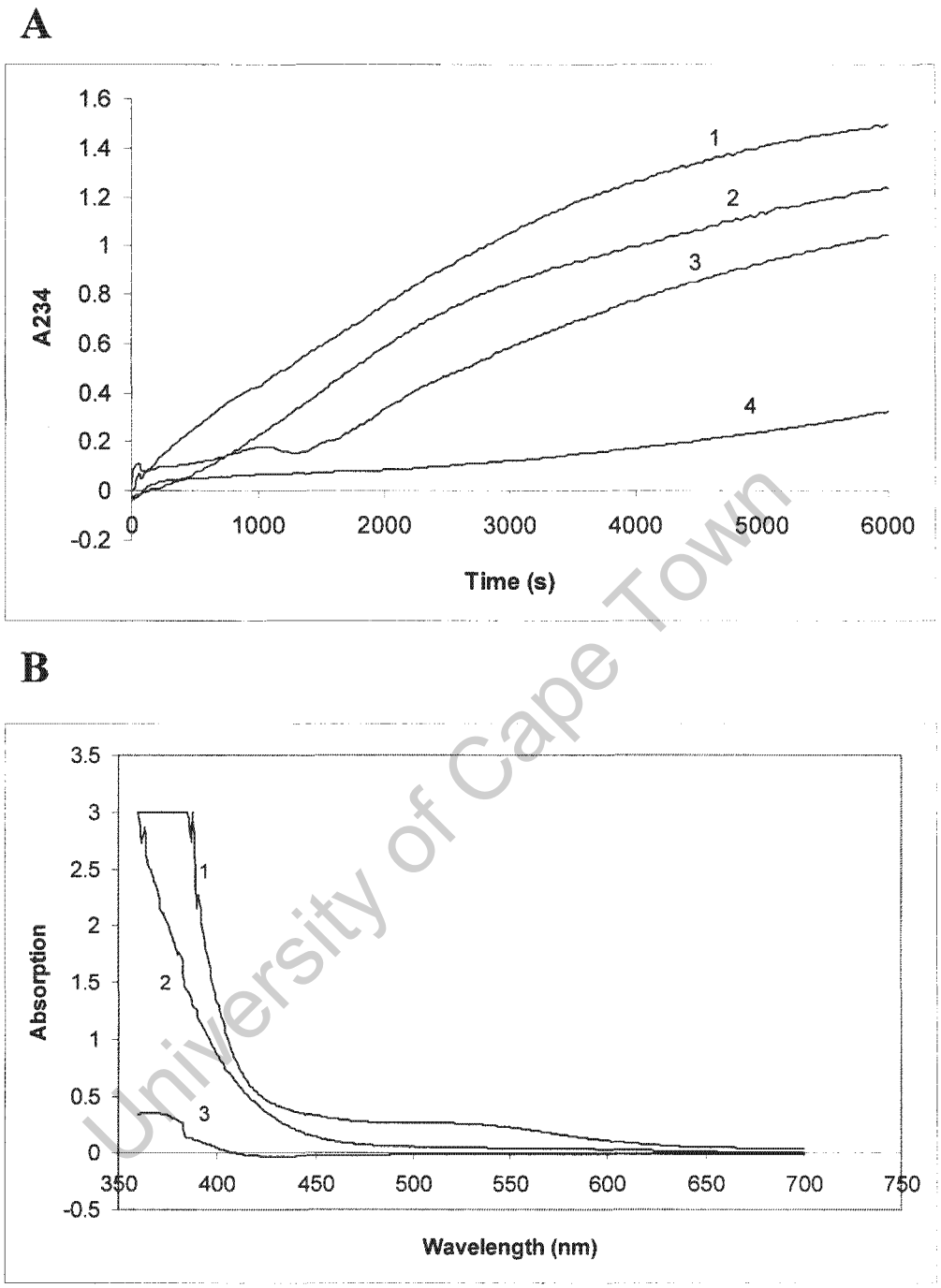


Figure 3.7. Prevention of linoleic oxidation by 3, 4, 5 tri-*O*-galloylquinic acid.

(A) AAPH-induced oxidation of linoleic acid (160 nmol) monitored at 234 nm as a function of time in the absence (1) or in the presence of 3.85 nmol (2), 7.70 nmol (3) and 15.4 nmol (4) 3, 4, 5 tri-*O*-galloylquinic acid . (B) Spectra of 3, 4, 5 tri-*O*-galloylquinic acid after (1) and before (2) AAPH-induced oxidation of linoleic acid. The spectrum of AAPH is also shown (3).

Polyphenols have been proposed to act as antioxidants in plant cells. The results of the polyphenol-liposome study (Westall, 2002) demonstrated an interaction between 3,4,5 tri-*O*-galloylquinic acid and liposomes and suggested a possible additional role for this compound in protecting membrane lipids against oxidation. Therefore we investigated whether 3,4,5 tri-*O*-galloylquinic acid would protect linoleic acid (C18:3) against oxidation. The free radical initiator AAPH was therefore added to an aqueous solution of linoleic acid with or without 3,4,5 tri-*O*-galloylquinic acid, and the oxidation was monitored at 234 nm as a function of time (Figure 3.7).

We found that the addition of increasing concentrations of 3,4,5 tri-*O*-galloylquinic acid resulted in a decreased rate of linoleic acid oxidation. The addition of 15.4 μM 3,4,5 tri-*O*-galloylquinic acid, approximately one tenth the concentration of the linoleic acid, resulted in a reduction in the rate of linoleic acid oxidation by approx. 95%. Ascorbic acid is a well-known reducing agent in many tissues, and n-propyl gallate is commercially used as an antioxidant. Therefore we compared the inhibition of AAPH-mediated oxidation of linoleic acid brought about by ascorbic acid and n-propyl gallate with that observed for 3,4,5 tri-*O*-galloylquinic acid. We found that equivalent molar concentrations (13.8 μM) of ascorbic acid and n-propyl gallate were less effective antioxidants when compared with the same molar concentration of 3,4,5 tri-*O*-galloylquinic acid with the AAPH-mediated oxidation of linoleic inhibited by 58 ± 4 % and 40 ± 0.5 % respectively compared with 98 ± 0.6 %. The inhibition mediated by an equivalent molar concentration of gallic acid was also investigated and found to be 29 ± 2%. This clearly demonstrated that 3,4,5 tri-*O*-galloylquinic acid is a far better antioxidant than the other known antioxidants tested and suggested that this compound is capable of efficiently preventing linoleic acid oxidation. To confirm that the redox state of the 3,4,5 tri-*O*-galloylquinic acid changed as a result of AAPH-induced oxidation, the spectrum of 3,4,5 tri-*O*-galloylquinic acid was determined in the presence and absence of AAPH (Figure 3.7). This showed that the absorbance in the region

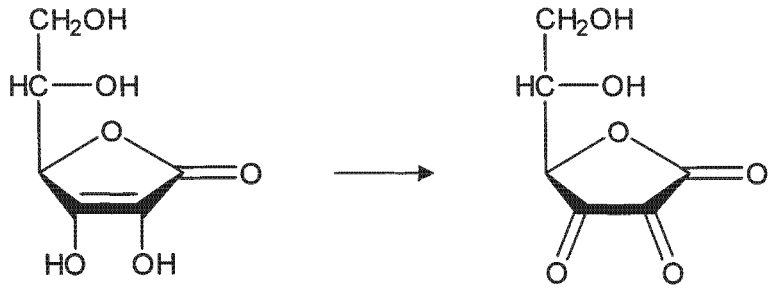
450–500 nm was markedly reduced by AAPH, which we interpreted to demonstrate that 3,4,5 tri-*O*-galloylquinic acid protected linoleic acid against oxidation by itself becoming oxidized.

An additional study was undertaken to confirm the relative antioxidant potential of the phenolic antioxidants tested previously in relation to each other as well as ascorbic acid. The auto-oxidation of ascorbic acid in the presence of Cu^{2+} ions by atmospheric oxygen was used as a standard system to test the antioxidant efficacy of various compounds. Oxidation of 130μM of ascorbic acid to its oxidised dehydroascorbate form was monitored in solution at 263nm in the presence and absence of 0.5 nM of $Cu(NO_3)_2$ (Figure 3.8). The reaction mixture was incubated with the specific phenolic antioxidant and the reaction rate obtained was compared with the control reaction rate. It was found that the addition of 0.1μM of gallic acid and n-propyl gallate resulted in a 25 % reduction in the oxidation rate of ascorbic acid relative to the control. In contrast, addition of only 0.033μM of 3,4,5 tri-*O*-galloylquinic acid resulted in an 85 % reduction in the ascorbic acid oxidation rate (Figure 3.8). Thus, this study revealed that the phenolic antioxidants tested possessed markedly better reducing ability than ascorbic acid. In addition our results demonstrated that 3,4,5 tri-*O*-galloylquinic acid was a significantly better antioxidant than both gallic acid and n-propyl gallate.

Since 3,4,5 tri-*O*-galloylquinic acid is able to protect membranes against oxidation and is a markedly better antioxidant than the common antioxidants tested, we wished to understand the structural and mechanistic features of this molecule which might to contribute towards its antioxidant properties. Inspection of a molecular computer model of 3,4,5 tri-*O*-galloylquinic acid revealed unique structural features which may promote intramolecular oxidation in this molecule (Figure 3.9). It was noted that the galloyl B and C rings are in close spatial proximity due to the stereochemistry of the substituted hydroxyl moieties of the quinic acid core of 3,4,5 tri-*O*-galloylquinic acid (Figure 3.9). We therefore propose a reaction scheme whereby oxidation of

3,4,5 tri-*O*-galloylquinic acid proceeds by a carbon-carbon bond forming between the galloyl rings B and C resulting in the loss of 2 protons and electrons (Figure 3.9). This carbon-carbon bond formation results in a phenolic hydroxyl of the C galloyl ring attacking the electrophilic carbonyl carbon of carboxylic ester of the B galloyl ring resulting in cleavage of the B galloyl ring from the quinic acid (Figure 3.9). Subsequent rotation around the carbon-carbon bond formed during the initial oxidation results in the formation of a lactone forming between the free carboxylic acid of the B galloyl ring and the free phenolic hydroxyl of the C ring (Figure 3.9). Simultaneously the converse reaction occurs whereby the electrophilic carbonyl carbon of the carboxylic acid ester of the C ring is attacked by the nucleophilic phenolic hydroxyl of the B galloyl ring resulting in cleavage of the covalently B and C linked lactone from the quinic acid (Figure 3.9). The covalently linked B and C ring undergo complete lactonisation resulting in the formation of ellagic acid (Figure 3.9). In order to provide some experimental evidence for our hypothetical oxidation reaction mechanism for 3,4,5 tri-*O*-galloylquinic acid, we subjected an aqueous solution of 3,4,5 tri-*O*-galloylquinic acid buffered with phosphate ions to pH 8 to oxidation via atmospheric oxygen. We analysed the reaction products formed by HPLC after time intervals of 0, 24 and 48 hrs (Figure 3.10). After 24 hrs it was noted that the peak corresponding to 3,4,5 tri-*O*-galloylquinic acid was significantly reduced and in addition, a major later eluting peak was present in the chromatograph (Figure 3.10). After a further 48 hrs of oxidation the 3,4,5 tri-*O*-galloylquinic acid peak was no longer present in the chromatograph and instead the later eluting peak had significantly increased in concentration becoming the dominant peak in the chromatograph (Figure 3.10). This later eluting peak was identified as ellagic acid by comparison of retention time and spectral properties with a commercial standard of ellagic acid (not shown). It was also shown that ellagic acid when incubated in an aqueous solution buffered to pH 8 with phosphate ions for 48 hrs became significantly reduced in concentration. Together these data suggest that 3,4,5 tri-*O*-galloylquinic acid is oxidised to ellagic acid as the major reaction product and that the proposed oxidative reaction scheme proposed for this molecule is quite plausible.

A



λmax 263 nm
molar extinction coefficient
E = 16000 E= 0

B

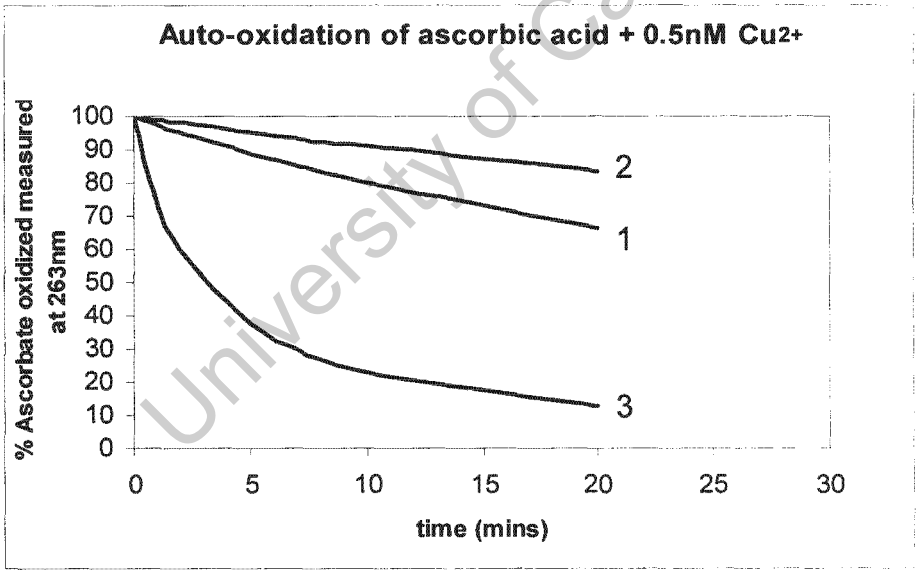


Figure 3.8. Auto-oxidation of ascorbic acid in the presence of Cu^{2+} ions by atmospheric oxygen.

(**A**) Oxidation of ascorbic acid (130 μM) and absorption properties of the oxidised and reduced forms. (**B**) Oxidation of ascorbic acid in the absence of Cu^{2+} ions (1), presence of 0.033μM 3, 4, 5 tri-*O*-galloylquinic acid (2) and the presence of Cu^{2+} ions (3).

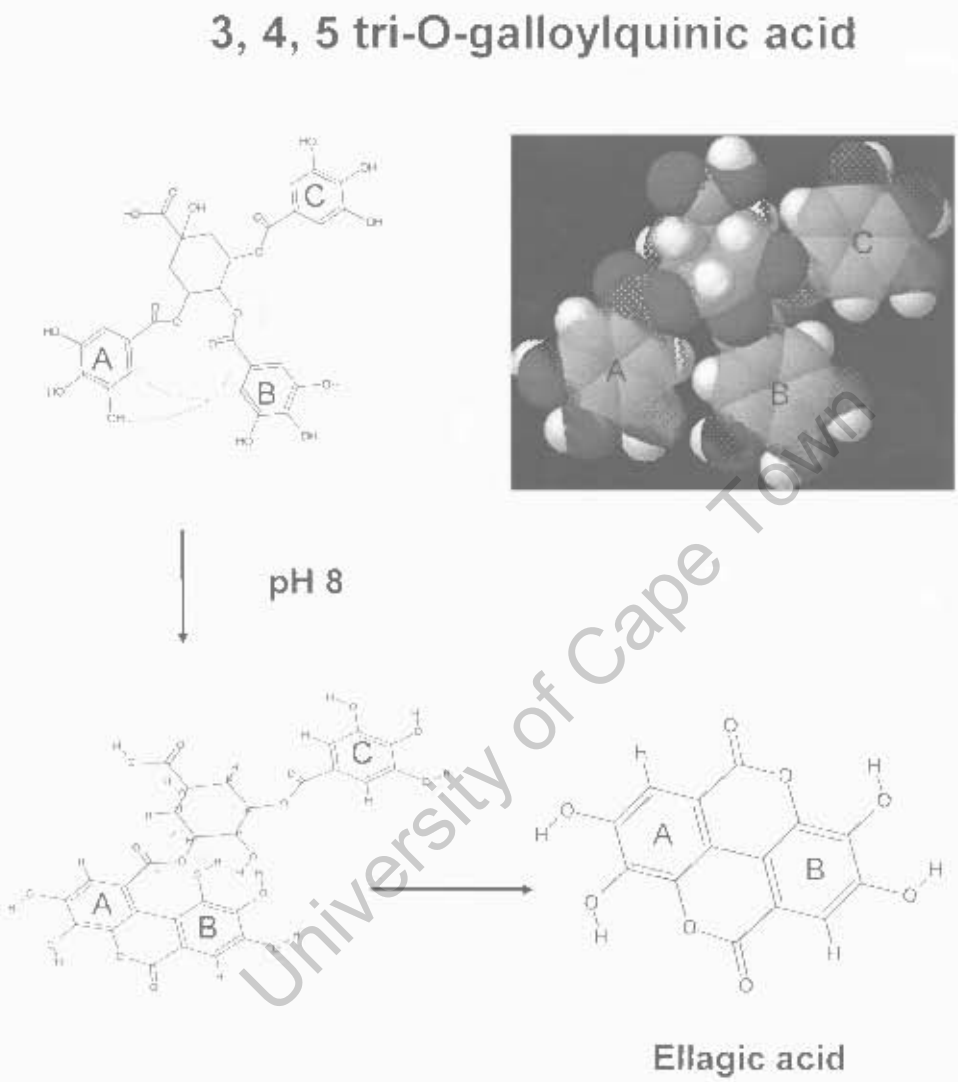


Figure 3.9. Molecular model of the 3, 4, 5 tri-*O*-galloylquinic acid molecule (upper right). Proposed reaction for the oxidative rearrangement and fragmentation of 3, 4, 5 tri-*O*-galloylquinic acid at pH 8 to produce ellagic acid (upper left and bottom).

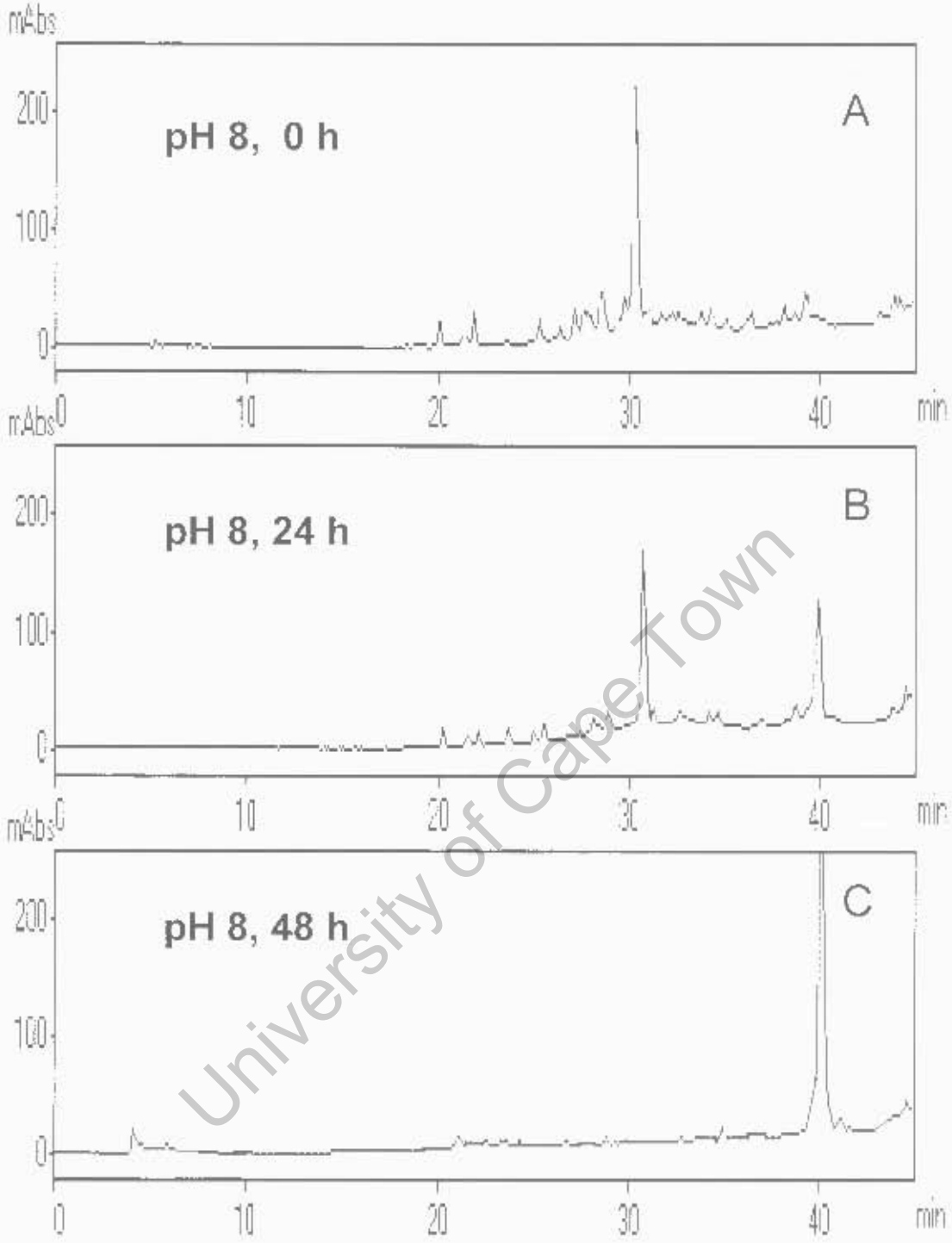


Figure 3.10. HPLC analysis of reaction products produced after the oxidation of 3, 4, 5 tri-*O*-galloylquinic acid by atmospheric oxygen at pH 8 after 0 hrs (A), 24 hrs (B) and 48 hrs (C). Peaks at approximately 30 and 40 mins correspond to 3, 4, 5 tri-*O*-galloylquinic acid and ellagic acid respectively.

In addition, these data also show that ellagic acid itself is oxidised to further product(s) which are not resolvable by HPLC. It is likely that ellagic acid is oxidised to higher molecular weight polymers which are insoluble in the solvents used for analysis. Ellagic acid itself is not very soluble in aqueous solvent systems and is likely to precipitate during the oxidation of 3,4,5 tri-*O*-galloylquinic acid. This may provide an additional rationale for the high antioxidant efficacy of 3,4,5 tri-*O*-galloylquinic acid, since if its oxidation product(s) is removed from the reaction equilibrium due to precipitation then according to Le Chatelier's principle (Ebbing, 1993) the forward oxidation reaction should be considerably favoured relative to the reverse reduction reaction. Perhaps a combination of rapid intramolecular oxidation and oxidation product removal contributes to the antioxidant efficiency of 3,4,5 tri-*O*-galloylquinic acid.

3.3.3 Ultrastructural location of polyphenols in the leaves of *M. flabellifolia*

To determine the location of the polyphenols within *M. flabellifolia* leaves, an ultrastructural investigation was performed using scanning and transmission electron microscopy after fixing the tissue with glutaraldehyde and caffeine (Figures 3.11 and 3.12). In addition, certain glutaraldehyde fixed hydrated and dehydrated specimens were post-fixed with osmium tetroxide (Figures 3.11A, C, E and 3.12A, B). Osmicated sections revealed electron dense precipitates within the vacuoles of the leaf mesophyll cells (Figures 3.11A, C, E and 3.12A, B). Phenolic compounds are known to oxidise and cross-link in the presence of osmium tetroxide into hard complexes (Schneider, 1976; Hayat, 1981). These electron dense complexes were shown to contain > 90% Osmium using EDAX analysis with the scanning electron microscope. In comparison, non-osmicated leaf sections (Figures 3.11 B, D, F and 3.12 C, D) displayed diffuse precipitate complexes occupying the vacuolar space of the leaf mesophyll cells in both hydrated and dehydrated states. Caffeine has been reported to precipitate polyphenols at their cellular location (Hayat, 1978; Mueller and Greenwood, 1978; Haslam and Lilley, 1985). These

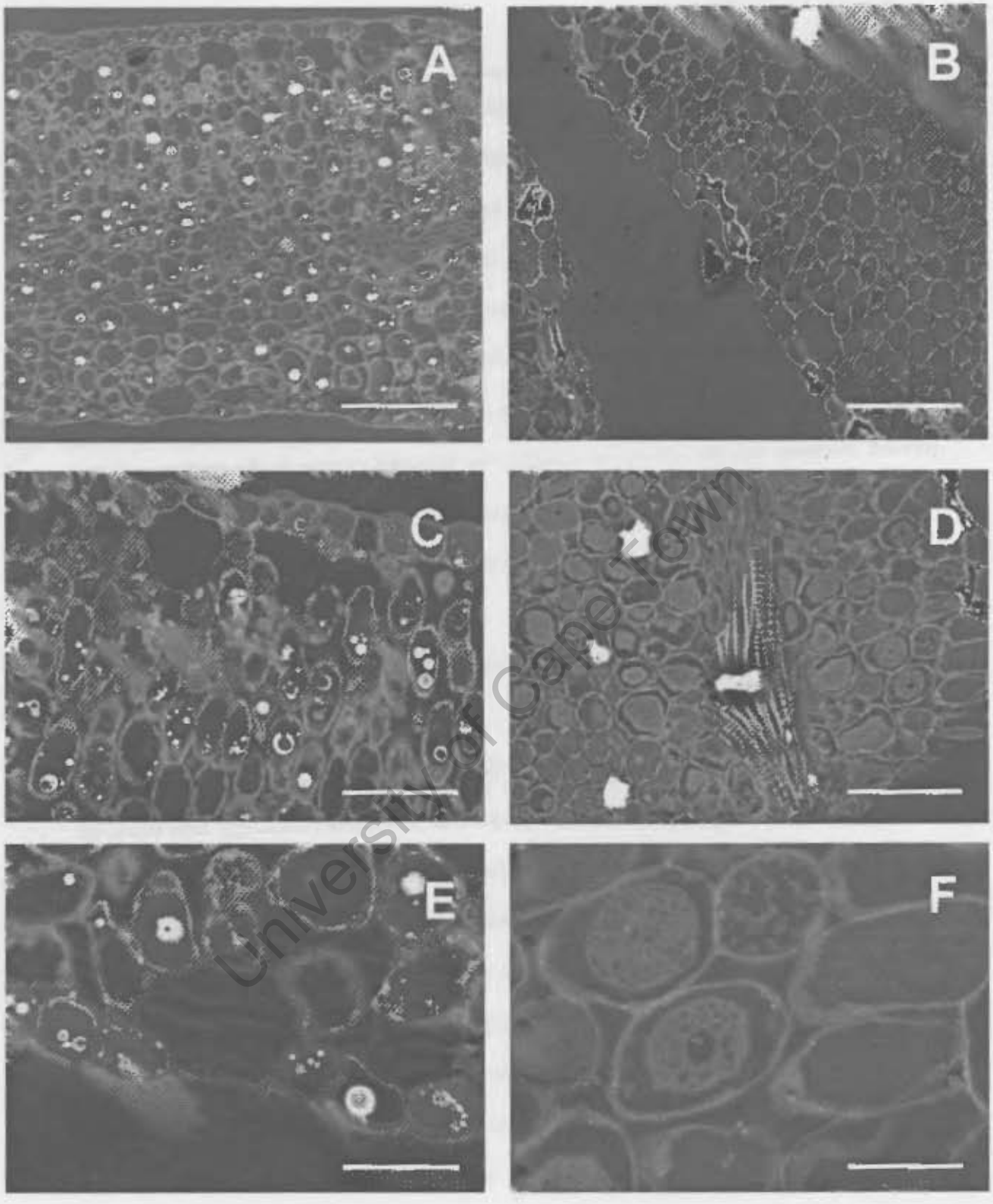


Figure 3.11. Scanning electron micrographs of leaf tissue of *M. flabellifolia* fixed with (A, C, E) and without (B, D, F) osmium tetroxide. White bodies present in micrographs A, C and E represent osmium tetroxide polypehnol complexes. [Scale bars, a,b = 21µm; c, d = 10µm; e, f = 6 µm]

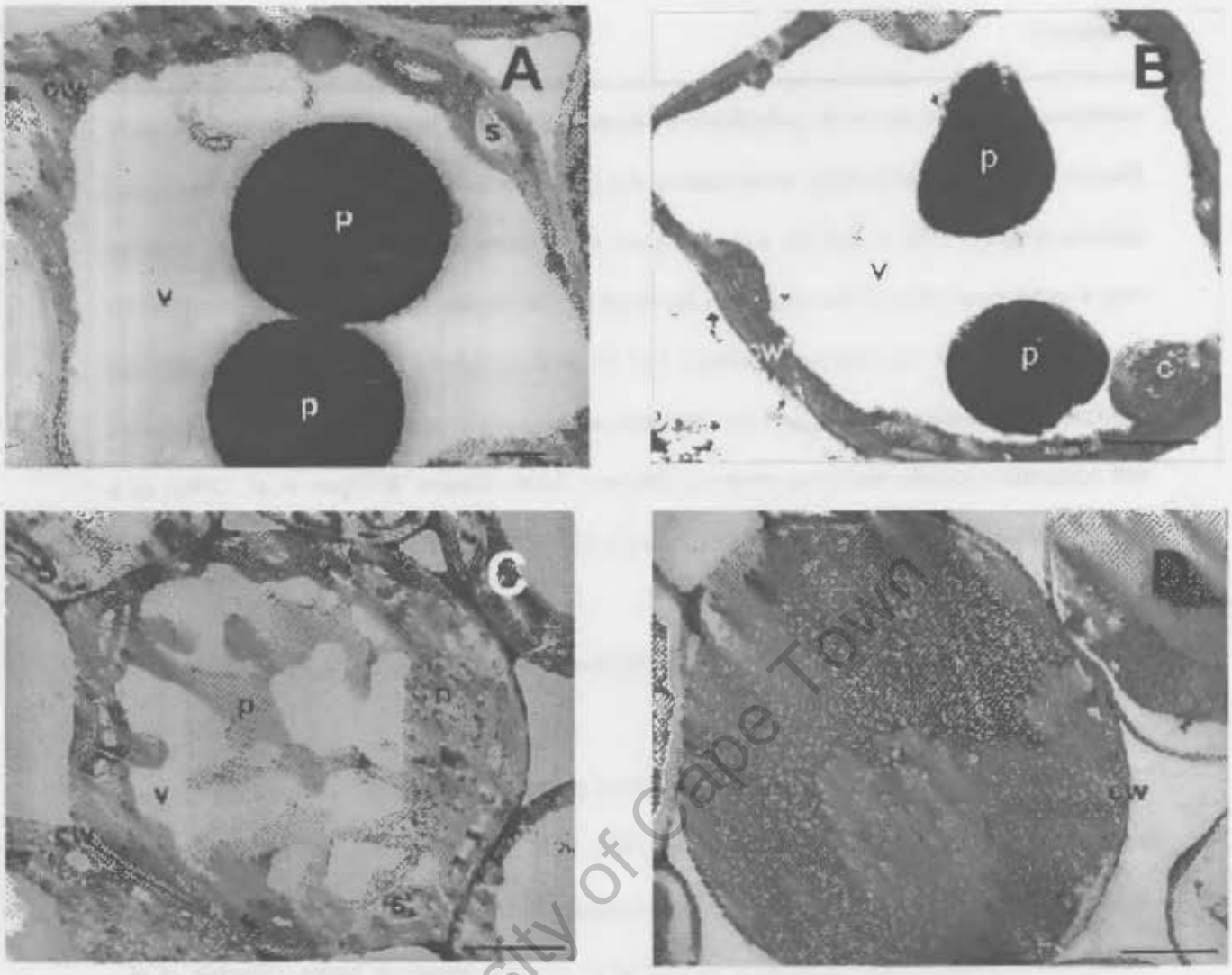


Figure 3.12. Transmission electron micrographs of hydrated (A and C) and desiccated (B and D) leaf mesophyll cells of *M. flabellifolia* treated with (A and B) or without (C and D) osmium tetroxide. [key: p, polyphenols; cw, cell wall, c, chloroplast; s, starch granule; n, nucleus; v, vacuole. Scale bars - 3µm]

complexes are believed to be polyphenol-caffeine precipitates, which in the absence of post-fixation are less electron dense. In contrast to the dense compact complexes found in osmicated sections (Figures 3.12 A and B), non-osmicated tissue revealed that the polyphenols occupied only a small proportion of the vacuole in hydrated leaves (Figure 3.12 C), whereas in dry leaves they filled the entire vacuolar space (Figure 3.12 D). In this capacity they could interact with and stabilize the tonoplast in the desiccated state. The replacement of water in vacuoles with proteins and compatible solutes has been proposed (Farrant, 2000; Vander Willigen et al., 2003) as a mechanism for the prevention of mechanical injury (Iljin, 1957) during desiccation.

3.3.4 Galloylquinic acid and genetic variation in populations of *M. flabellifolia*

The Namibian and the Zimbabwean/South African populations of *M. flabellifolia* are not only separated by the Kalahari Desert (Glen et al., 1999; Puff, 1978) but these two populations also occur in different biomes, with the Namibian population occurring in a dry Karoo biome and the Zimbabwean/South African population occurring in a moist savannah biome (Sinclair et al., 2002). We initially quantified the total leaf polyphenol content from plants collected in Namibia and in South Africa. Leaf material from each population was exhaustively extracted in 70 % aqueous methanol and the polyphenol content determined from the UV absorption at 280 nm (Altmann and Falk, 1995) (Table 3.3). A significant difference in the total average polyphenol content was observed between plants from Namibia and from South Africa. Plants collected in Nambia were found to contain 33 ± 5 % in contrast to plants collected in South Africa which were found to contain 24 ± 2 % (Table 3.3).

HPLC analysis of the extracted polyphenols was performed in order to determine whether South African *M. flabellifolia* plants contained the same polyphenol species as plants collected in Namibia. The chromatographs obtained (Figure 3.13A and 3.13B) revealed distinct compositional

differences, the major difference being an abundance of a single polyphenol in Namibian plants (Moore et al., 2005), whereas South African plants contained a wide variety of different polyphenols. The main polyphenol present (peak 1) in Namibian plants was ascribed to 3, 4, 5 tri-O-galloylquinic acid and confirmed by mass spectrometry of the leaf extract; this yielded a mass of 647 Da. The remaining phenolic peaks (peaks 2 and 3) in the chromatograph represented gallic and ellagic acid esters of 3,4,5 tri-O-galloylquinic acid. These assignments were made after mass spectrometry of the material eluting in these peaks yielded a mass of 799 Da for peak 2 and a range of masses, namely 799, 936, 953, 1570 and 1722 Da for peak 3 respectively (Table 3.1, Figure 3.14). These latter masses represent tetra-, penta-, hexa- and hepta-galloylquinate esters (Table 3.1, Figure 3.14). In contrast, HPLC analysis of polyphenols extracted from South African *M. flabellifolia* plants showed that no single polyphenol was present in abundance. Instead, a variety of polyphenols were present, some of which were identical to those present in Namibian plants but the majority of which eluted from the HPLC column with higher retention times (Figure 3.13B). Fractions 1, 2 and 3 from plants collected in South Africa had identical polyphenols present to these fractions from Namibian plants with polyphenols eluted in fractions 4 to 6 unique to South African plants. These later eluting polyphenol factions, as expected, had higher masses, brought about by the addition of either gallic acid or ellagic acid moieties to the core molecule, 3,4,5 tri-O-galloylquinic acid (Table 3.1, Figure 3.14). A range of masses was evident at m/z 647, 799, 936, 953, 1088, 1570, 1722, 1874, 2050 and 2203. These masses varied from one another by an integral mass of either 152 Da, the mass addition of one extra galloyl group to the core molecule, or 298 Da, the mass addition of one extra ellagic acid moiety to the parent structure. Peaks displaying masses of 1874, 2050 and 2203 Da represent novel octa-, nona- and deca-galloylquinate ester molecules (assuming 3,4,5 tri-O-galloylquinic acid as the core molecule) respectively, each with the addition of a single ellagic acid moiety (Table 3.1, Figure 3.14). These species were only found to be present in South African plants (Figure 3.13A, Table 3.1). The final major peak eluting after approximately 30 min displayed masses of 1088 and 1322

Da. The mass of 1088 Da represents a hexa-galloylquinic acid, not found in plants collected in Namibia. The mass of 1322 Da cannot be assigned to the addition of gallic acid or ellagic acid moieties to the core molecule, although peaks of 1171 Da and 1474 Da present at low abundance in the mass spectrum (Figure 3.14) revealed that this ion represents a galloylated molecule (Table 3.1, Figure 3.14). The masses of 1088 and 1322 Da possibly represent partial products of fragmentation of an unknown parent molecule containing hexa-galloylquinic acid with a mass predicted to be greater than 2203 Da (Table 3.1, Figure 3.14). It was noted that there was no change in galloylquinate ester composition between desiccated and hydrated plants (data not shown).

Table 3.3.

Percentage polyphenol content in desiccated leaves of plants collected from various locations in Namibia (N1-N5) and South Africa (S1-S4)

Location	% polyphenol content	Location	% polyphenol content
N1 Outjo	41.7 ± 6.7	S1 Limpopo	26.7 ± 0.7
N2 Cunene	30.1 ± 5.2	S2 Mpumalanga	21.7 ± 5.3
N3 Komhochsland	28.3 ± 1.3	S3 Lydenberg	25.3 ± 4.6
N4 Komhochsland	32.7 ± 6.7	S4 Mpumalanga	22.9 ± 1.0
N5 Cunene	30.9 ± 0.1		
Average	32.7 ± 5.2	Average	24.4 ± 2.2

Polyphenol content is expressed in TGA equivalents in % g polyphenol per g DW. A minimum of 3 plants from each population were analysed in duplicate.

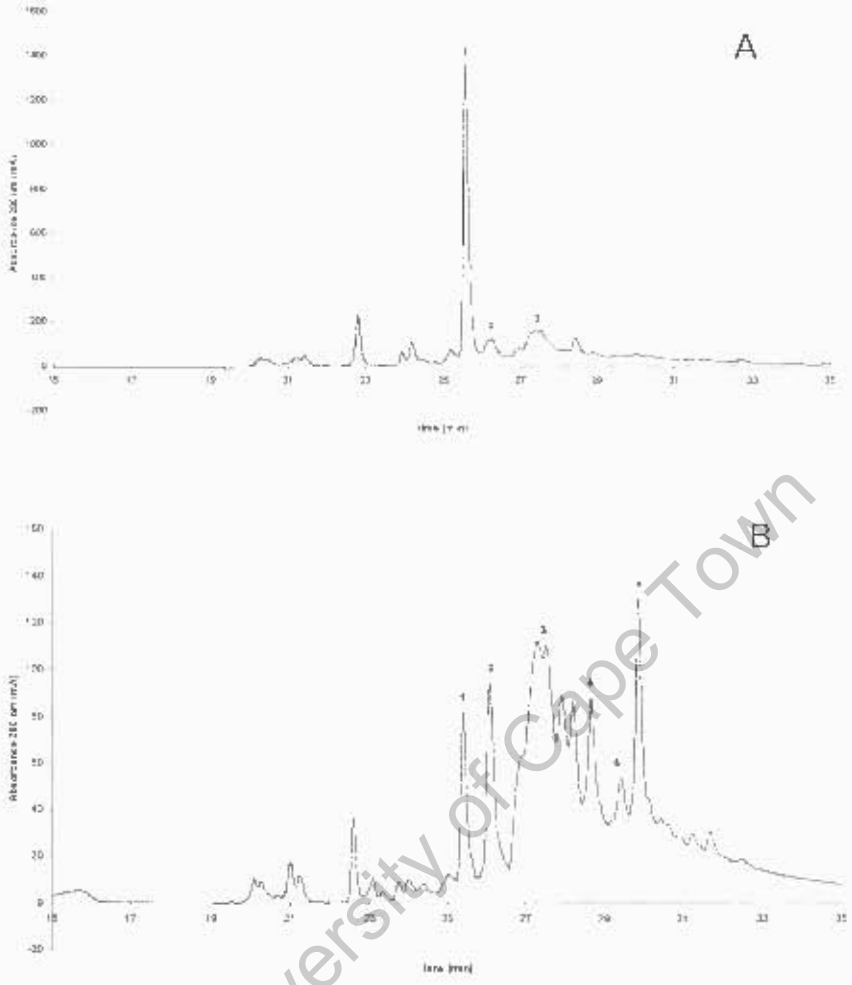


Figure 3.13. HPLC elution profile of polyphenols extracted from plants collected in Namibia (A) and South Africa (B). Fractions were pooled and lyophilized prior to analysis. The absorbance is shown in milli-absorbance units (mA).Note that the ordinate scale of (B) is 10^{-1} times of that shown in (A).

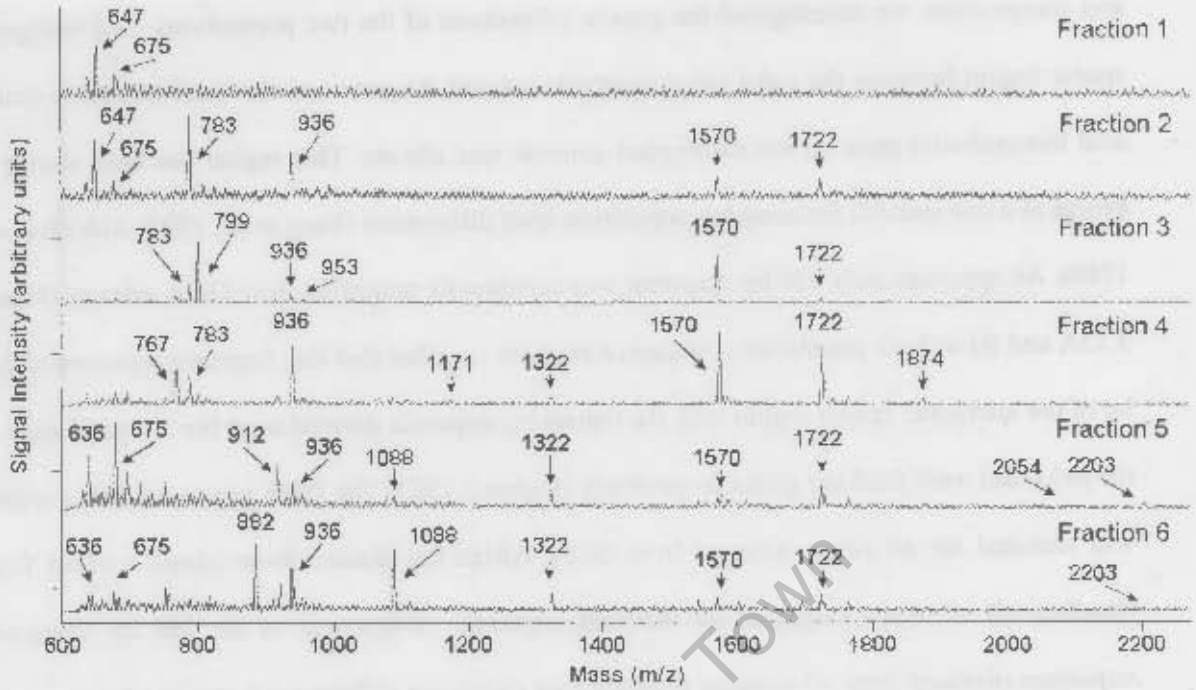
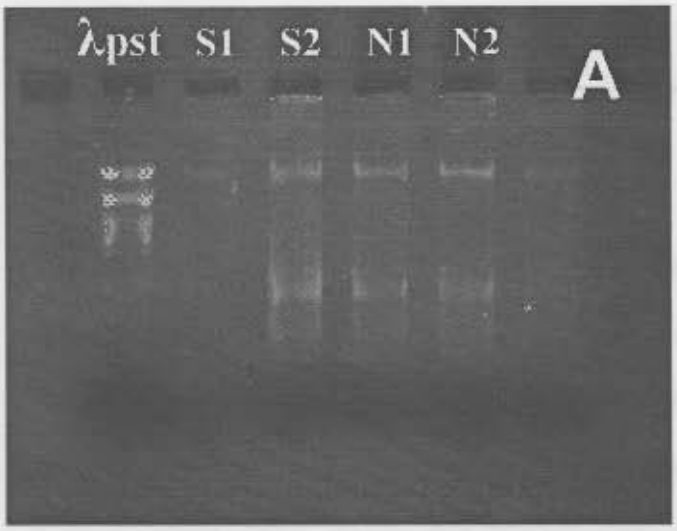
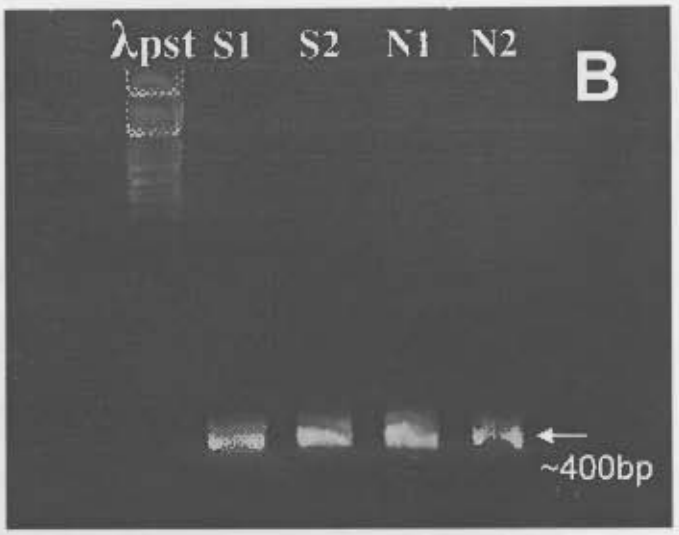


Figure 3.14. MALDI TOF mass spectral analysis of polyphenol fractions separated by HPLC from South African *M. flabellifolia* populations. (see **Table 3.1** for fraction retention times and mass assignments)

Since the two *M. flabellifolia* populations have distinct differences in their polyphenol content and composition, we investigated the genetic relatedness of the two populations. The intergenic spacer region between the *psbA* (photosynthetic subunit A) gene and the *trnH* (histidine amino acid biosynthesis) gene on the chloroplast genome was chosen. This region has been shown to evolve at a rate suitable for assaying population level differences (Sang et al., 1997; Aldrich et al., 1988). An approximately 400 bp fragment was consistently amplified from DNA extracts (Figure 3.15A and B) of both populations. Sequence analysis revealed that this fragment represented 288 bp of the intergenic spacer region with the remaining sequence derived from the 3' and 5' ends of the *psbA* and *trnH* flanking genes respectively (Figure 3.15C). The DNA sequence of this region was identical for all plants sourced from South Africa but distinct from plants sourced from Namibia, all of which displayed an identical sequence. Alignment of the 288 bp intergenic sequences obtained from all samples revealed four consistent differences between plants sourced from Namibia and from South Africa. These differences occurred at positions 88, 154, 176 and 221 and represented a sequence divergence of 1.4 % between the two populations. It was also noted that an insertion of 12 bp occurred in the *psbA* coding region of all plants sourced from Namibia (Figure 3.15C).

The genus *Myrothamnus* is related to the genus *Melianthus*, which has been proposed to have diverged from its sister group *Greyia* at a mutation rate of 0.0035 substitutions per site per 10^6 years (Hennings, 2003). Assuming an equal mutation rate, a divergence rate of 0.013 substitutions per site yields a time of divergence for the Namibian and South African *M. flabellifolia* populations of approximately 4 million years.





C

```
S   CTAGCTGCTGTCGAAG.............CTCCATCGTACAA   28
N   ----------------CCCCATCTACAA------------   40

S   ATGGATAAGACTTTGGTCTTAGTGTATACGAGTTTTTGAA   68
N   ----------------------------------------   80
                    3'↓
S   CTTAAAGGAGCAATAACCAATTTCTTGTTCTATCAAGAGG   108
N   ----------------------------------------   120

S   GTTGGTATTGCTCCTTTATTTAGTATCTTTTATTCTAT   148
N   ----------------------------------------   160

S   TTTCTTTTCATTTCTTTGCTTCAGCATAAGAAAAAAGAAA   188
N   ----------------------------------------   200

S   AAGTATTCTTATGGGTATTGAGTATCATACTTTTTTTCTG   228
N   ----------------------------------------   240

S   TACTAATCTCTAATTTTTCAAATAGAAAAATATTTTTCTG   268
N   ---------G----------------C---------   280

S   ACTAATATTTGTATCTAAGAAGGAAGATAAGAAAGACTAA   308
N   ----------------------------------A---   320

S   ATGAAATAATTATAAATGGAATCCTTTCTAATTTGTAACT   348
N   ----------------------------------------   360
                              ↓5
S   TTCTAATTTGTAAATAGTATAGGGCGGATGTAGCCAAGT   388
N   ----------------------------------------   400

S   GGATCAAGGCAGT   401
N   -------------   413
```


Figure 3.15. Gel photographs of DNA extracts (A) and PCR products (arrow) (B) from plants collected in South Africa (S1,S2) and Namibia (N1,N2). DNA sequence (C) of the 3' end of the psbA gene, the intergenic spacer and the 5' end of the trnH gene from plants collected in South Africa (S) and Namibia (N). The arrows denote the 3' end of the psbA gene and the 5' end of the trnH gene respectively.

3.4 DISCUSSION

In the present study, we have confirmed the identity of the main polyphenol constituent of *M. flabellifolia* leaves as 3,4,5 tri-*O*-galloylquinic acid in relation to previous analysis (Moore et al., 2001). In addition, smaller quantities of higher molecular mass gallic acid polyesters were also present, the result of multiple galloylation of the central 3,4,5 tri-*O*-galloylquinic acid core. These compounds are collectively present in very high concentrations with between one quarter to one half of the leaf dry mass being due to the presence of these compounds. Electron microscopy demonstrated that the polyphenols are present in the leaf pallisade and spongy mesophyll cell vacuoles in both hydrated and dehydrated leaves. Mechanical stabilization, by replacement of water in vacuoles with compatible solutes during dehydration, has been reported to occur in other resurrection plants (Farrant, 2000; Vander Willigen et al., 2003) and it is possible that 3,4,5 tri-*O*-galloylquinic acid plays a similar role in *M. flabellifolia*. These compounds absorb UV light with the maximum molar absorption coefficient of ~ 2.1×10^4 observed at 280 nm (Altmann and Falk, 1995). Therefore a role for the absorbing light thereby preventing UV-induced free radical damage may also be inferred. The results presented here suggest that 3,4,5 tri-*O*-galloylquinic acid interacts with membranes in two ways. Its one role is to protect membranes against desiccation by presumably intercalating into the lipid bilayer and reducing the Tm of the transition between the liquid crystalline and the gel phases. Such a behaviour, which has been reported to occur for the polyphenol arbutin (Oliver et al., 1998), allows successful desiccation and subsequent rehydration to occur. Whereas arbutin has been shown to have a specific lipid requirement in the target membranes, the liposomes used in this study comprised only egg yolk phosphatidylcholine and cholesterol suggesting a non-specific interaction of 3,4,5 tri-*O*-galloylquinic acid with membranes. The other suggested role is to protect membranes against free radical-induced damage. This would be an important property since the antioxidant status of *M. flabellifolia* has been shown to correlate with the duration of viability of the plant in the

desiccated state (Kranner et al., 2002). The results reported in this study agree with that of other authors who have shown gallotannins to be potent antioxidants (Haslam and Lilley, 1985; Nonaka, 1989; Pérez et al., 2002). Our results indicated that using the oxidation of linoleic acid as a model system, 3,4,5 tri-*O*-galloylquinic acid displays significantly greater antioxidant properties when compared with ascorbic acid and the commercially used n-propyl gallate as well as gallic acid itself. Since only very low relative concentrations of 3,4,5 tri-O-galloylquinic acid are required for it to act as an antioxidant protecting unsaturated lipids and since the activity observed in protecting liposomes against desiccation only occurred over a limited concentration range, we would postulate that the *M. flabellifolia* cells must display some mechanism for concentration dependent release and sequestration of the polyphenol. This might depend on the solubility equilibrium between the soluble and insoluble states, which might be influenced by the metabolic status of the plant affecting parameters such as the intra-vacuolar pH as well as the presence of metal ions. We are currently investigating whether such mechanisms control the effective biological concentration of 3,4,5 tri-*O*-galloylquinic acid in the cell. The site of biosynthesis of this molecule has not been determined but is likely to be the endoplasmic reticulum or cytoplasm and so must be transported to the vacuole for deposition probably via vesicle bodies. Although 3,4,5 tri-*O*-galloylquinic acid accumulates predominantly in the vacuole it is possible that it could be present at lower concentrations in other cell compartments where it could function in a protective role.

An extract of *M. flabellifolia* leaves has been reported to be used by indigenous people in Namibia as an aid in wound healing and to treat asthma and general chest ailments (Van Wyk et al., 1997). In keeping with this, gallotannins are known for their anti-burn properties (Onayade et al., 1996) and galloylquinic acids have been identified as possessing high activity against bronchial hyper-reactivity and allergic reactions (Neszmelyi et al., 1993, Onayade et al., 1996). A group of galloylquinic acids has also been shown to display anti-HIV RT (reverse transcriptase)

and anti-HIV activity (Bokesch et al., 1996, Nishizawa et al., 1989). The reverse transcriptase inhibition by galloylquinic acids correlates with the difficulties encountered using Molecular Biology techniques, e.g. RT–PCR, to study this plant (Koonjul et al., 1999).

This work reports that the Namibian and South African populations of *M. flabellifolia* have distinct differences in their polyphenol content and composition. Whereas plants collected in Namibia essentially contained a single polyphenol, 3, 4, 5 tri-O-galloylquinic acid, South African plants contained a mixture of polyphenols which were derivatives of this molecule. Although both the Namibian and South African populations occur in similar geological environments, the rainfall patterns of the regions in which these populations occur are very different. South African plants grow in a region which experiences dry winters and regular annual summer rainfall. In contrast, Namibian plants grow in a far more arid region of dry winters and irregular summer rainfall patterns. It has been reported that South African *M. flabellifolia* plants are only capable of surviving 9 months in a desiccated state (Farrant and Kruger, 2001). Whereas South African plants must only survive the dry winter months, Namibian plants must be capable of surviving not only a dry winter but also the ensuing dry summer and the following dry winter. In this context it must be understood that dry means that no rainfall whatsoever is experienced. Anecdotal evidence suggests that Namibian *M. flabellifolia* plants are capable of surviving for extended periods (three years and more) in a quiescent state. We propose that the presence of the single polyphenol 3, 4, 5 tri-O-galloylquinic acid contributes towards this degree of desiccation tolerance.

M. flabellifolia in Africa south of the Sahara was originally divided on morphological grounds into 3 subspecies, namely *M. flabellifolia sensu stricta, elongata* and *robusta,* the latter only occurring north of the Zambesi river in Zambia and Mozambique (Weimarck, 1936). A later revision resulted in the abandonment of *M. flabellifolia elongata* as a subspecies due to it being

indistinguishable from *M. flabellifolia sensu stricta* (Puff, 1978). Since polyphenols do not display rapid turnover during plant growth and metabolism (Boudet et al., 1985; Haslam and Lilley, 1985) these molecules serve as useful chemotaxonomic markers for plant systematic and evolution studies. Our data therefore suggest a new division of *M. flabellifolia* based on the phenolic compounds. This division would correlate with the biogeography of the species. We propose that there are two distinct populations on either side of the Kalahari Desert respectively. This hypothesis is supported by the genetic data presented here which showed that plants collected in Namibia were genetically identical and distinct from plants collected in South Africa, which were also genetically identical. The 1.4 % genetic variation within the *psbA/trnH* intergenic region corresponds to a time of divergence of approximately 4 million years. This figure correlates with the climate change that occurred approximately 5 million years ago when the Karoo region along the western part of Southern Africa changed from a wet to an arid region (Klak, 2004). Our hypothesis is that this climate change resulted in a requirement for Nambian population of *M. flabellifolia* to remain in a quiescent state for extended periods. The separation of the two populations by over 1000 km of desert presumably prevented cross-pollination and wind dispersal of the seeds (Child, 1960) resulting in two separate populations. Current research is focused on the relationship between the polyphenol content and the ability of Namibian plants to survive in a quiescent state.

CHAPTER FOUR

THE RESPONSE OF THE LEAF CELL WALL TO DESICCATION IN THE RESURRECTION PLANT *Myrothamnus flabellifolia*

4.1 INTRODUCTION

Plant cell walls are dynamic structures that play an essential role in the biology of green plants (Fry, 1988; Carpita and Gibeaut, 1993; Brett and Waldron, 1996). They fulfil many functions in the life processes of plants including the provision of structural support to growing cells, acting as a barrier against invading pathogenic organisms, facilitating the transport of metabolites and hormones (via the apoplast) and so are vital for normal plant functioning (Brett and Waldron, 1996; Cosgrove, 1997). As opposed to the traditional view of the cell wall as static skeleton, it has become increasingly apparent that the wall needs to be able to constantly change and adapt in response to the current environmental and developmental conditions experienced by the plant (Brett and Waldron, 1996). The cell wall itself is a composite structure constituted of a skeletal framework of cellulose microfibrils which is cross-linked and intermeshed within a network of pectic and hemicellulosic polymers (Brett and Waldron, 1996). This complex multi-network structure of the cell wall allows it to resist mechanical stresses imposed on it from multiple directions (Brett and Waldron, 1996). This is necessary considering the compressive and expansive forces experienced by the cell wall during normal growth and development (Brett and Waldron, 1996; Cosgrove, 1997).

Although the cell wall has been extensively researched with regards to its biochemical composition (McNeill et al., 1984; Carpita and Gibeaut, 1993), controversy still surrounds the exact higher order structure of the plant cell wall (Cosgrove, 2001). A number of macromolecular models, representing the *in vivo* architecture of the cell wall, have been proposed (Cosgrove, 2001). Which if any of the models and subsequent refinements provide an accurate molecular approximation of the *in planta* state remains unclear (Cosgrove, 2001). Recently a new proposal has come to light suggesting that homogalacturonans may actually be side chains of rhamnogalacturonan I (Vincken et al., 2003). This new cell wall model is an interesting departure from the standard models previously proposed in the literature (Cosgrove, 2001). The polymer networks that constitute the cell wall are broadly divided into the cellulose microfibrils, hemicellulosic polysaccharides, such as xyloglucan and xylan, and the pectic polysaccharides, composed of homogalacturonan and rhamnogalacturonan I and II (Brett and Waldron, 1996). The individual polymer networks are reported to possess diverse functions ranging from structural support by xyloglucan polymers to the water and metal ion binding properties of the pectin matrix (Brett and Waldron, 1996). How these various polymers interact with each other in the wall and in so doing facilitate wall functions is still imperfectly understood.

To ascertain specific wall polymer functions and thereby unravel cell wall dynamics, a number of techniques have been developed to investigate cell wall structure (Fry, 1988; Reiter, 1997; Knox, 1997; Jarvis and McCann, 2000; Willats et al., 2000; McCann et al., 2001). This is important, as a more complete model of cell wall architecture is required to understand the role played by the cell wall in the adaptation of plants to environmental stresses (Pennel, 1998; Vicré et al., 2004b). The number of environmental stresses experienced by plants and the subsequent adaptations believed to involve the cell wall constitute a diverse range including wounding (Cardemil and Riquelme, 1991), osmotic stress (Iraki et al., 1989a,b,c; Wakabayashi et al., 1997), cold acclimation (Weiser

at al., 1990), drought tolerance (Zwiazeck, 1991) and pathogen invasion (Boudart et al., 1998). Changes in plant cell walls in response to environmental stress are highly varied and stress specific (Vicré, 2001). For example it was found that tobacco cells responded to osmotic stress increasing the amounts of weakly held pectin (polygalacturonic acid and rhamnogalacturonan polymers) in their cell walls (Iraki et al., 1989b) whilst drought-stressed spruce needles accumulated high amounts of cell wall xyloglucan polymers (Zwiazeck, 1991). In order to investigate cell wall structure and its response to stresses, a range of techniques have been developed which span the scientific disciplines of chemistry, biochemistry, physics, genetics and microscopy (Fry, 1988; McCann et al., 2001; Vicré, 2001).

The traditional method used to analyse the plant cell wall involves a combination of cell wall fractionation and carbohydrate chemistry (Selvendran, 1985; Selvendran and O'Neill, 1987). Cell wall fractionation involves separating the various polymer networks from the cell wall using a serial extraction regime (Jarvis et al., 1981; Selvendran, 1985). The standard method involves using a strong chelator to solubilise pectin followed by increasing concentrations of a strong alkali, such as potassium hydroxide, to liberate polysaccharides from the cellulose microfibrils (Selvendran, 1985; Selvendran and O'Neill, 1987). Collected fractions are hydrolysed with acid to their constituent monosaccharides which are then separated and quantified chromatographically (Selvendran, 1985; Zablackis et al., 1995). This is a very powerful technique as it provides both qualitative and quantitative data on the wall fractions analysed. Further polysaccharide enrichment and purification, from the individual fractions, allows for the use of advanced techniques in determining the molecular structures of the carbohydrates present (Jarvis et al., 1981; Fry, 1988). The main disadvantages of these techniques are that they require substantial amounts of cell wall sample, are invasive and disrupt polymer structure during isolation (alkali fractionation results in bond breakage) and because it involves analysing a

mixture of walls from different cell types provides only an average representation of wall structure (Jarvis et al., 1981; Fry, 1988; Jarvis and McCann, 2000). The lack of sensitivity of these methods can be overcome by using radiolabelling techniques to study cell walls (Fry, 1988). Significant progress in radiolabelling studies of plant cell walls have been made using plant cell suspension systems whilst research using whole plant systems are possible although many problems still need to be resolved (Fry, 1988).

Immunocytochemistry is an extremely useful technique to provide spatial data on specific polymer epitopes present in the cell wall and the changes the associated polymers undergo during plant growth and development (Knox, 1997; Willats et al., 2000). Designer polyclonal and monoclonal antibodies have been raised against a large number of cell wall polymer and protein epitopes (Knox, 1997). Studies, performed using a variety of cell wall antibodies, have revealed the previously unknown cell specific and *in muro* location specific occurence of certain wall polymers in plant tissues (Willats et al., 1999; McCartney and Knox, 2002; Orfila et al., 2002). Disadvantages of immunocytochemical techniques are that they are semi-quantitative and only a limited range of antibodies is available (Knox, 1997). Another problem is that novel polymers must be characterised and purified using standard biochemical techniques before antibodies can be raised against them (Willats et al., 2000).

Additional non-invasive techniques which have been employed to study plant cell walls include FTIR (Fourier transform infra-red) microspectroscopy and solid state NMR (nuclear magnetic resonance) spectroscopy (Jarvis and McCann, 2000). Advances in instrumentation have allowed the devopment of a FTIR microspectroscope which combines the spatial resoultion of a microscope with the sensitivity of FTIR (Jarvis and McCann, 2000). This combination of features allows for the spatial determination of different chemical groups associated with cell walls within

a plant tissue sample. This complements invasive chemical techniques and has found use as a rapid means to screen for plant cell wall mutants in genetic studies (Jarvis and McCann, 2000). Solid state NMR spectroscopy provides a means to non-invasively probe a cell wall preparation and determine the presence of various polymer systems (Jarvis and McCann, 2000). It also allows for the determination of inter-spatial distance of the polymers within the wall as well as being able to determine the mobility of the different cell wall polymer systems (Fenwick et al., 1997; Fenwick et al., 1999). The disadvantages of solid state NMR spectroscopy are that it requires large amounts of wall material and is destructive (Jarvis and McCann, 2000). It is therefore evident that the use of a range of different scientific techniques is necessary so as to provide a holistic description of the dynamic nature of the plant cell wall and its important role in plant stress and adaptation.

In this study we have investigated the composition and molecular architecture of the cell wall of *M. flabellifolia* leaves before and after desiccation using biochemical and immunocytochemical approaches. The results of our ultrastructural study revealed folded cell walls in the majority of desiccated leaf cells of leaf tissue implicating wall folding as the major mechanism by which *M. flabellifolia* minimises mechanical damage. The biochemical and immunocytochemical analysis showed that the cell wall *M. flabellifolia* underwent some desiccation-induced modifications but for the most part remained unchanged on desiccation. The *M. flabellifolia* leaf cell wall conformed to the standard dicotyledonous pattern except it was noted that the wall contained an unusual abundance of pectin-associated arabinan polymers. We propose that this arabinan rich wall is well adapted to be able to withstand cycles of desiccation and hydration.

4.2 MATERIALS AND METHODS

4.2.1 Plant Material

M. flabellifolia plants, collected from the Buffelskloof Nature Reserve, Mpumalanga Province, South Africa, were maintained in a glasshouse at the Botany department, University of Cape Town. Desiccation of whole plants was performed by withholding water and allowing the plants to dry naturally under ambient environmental conditions. Relative water content measurements were performed as outlined previously (Sherwin and Farrant, 1996).

4.2.2 Scanning and transmission electron microscopy

Scanning electron microscopy was performed using a Leica Stereoscan 440 digital scanning electron microscope equipped with a Fisons LT7400 Cryo Transfer System. Leaves from hydrated and desiccated plants were frozen using liquid nitrogen and viewed directly or after freeze-fracturing. Transmission electron microscopy was performed using a LEO 912 transmission electron microscope equipped with a CCD camera. Leaf segments (1 - 2 mm^2) were excised from the mid-blade of hydrated or desiccated leaves and fixed overnight at 4 °C in 0.1M phosphate buffer pH 7.4 containing 2.5 % glutaraldehyde supplemented with 0.5 % caffeine. Fixed samples were dehydrated in ethanol and embedded in epoxy resin (Spurr, 1969). Thin sections (90 – 100 nm) were cut using a Reichert Ultracut-S ultramicrotome and collected on 200 µm mesh copper grids. Sections were stained with uranyl acetate and lead citrate as described previously (Reynolds, 1963).

4.2.3 Isolation and fractionation of cell wall material

Cell walls were prepared from flash-frozen and lyophilised hydrated (RWC ~ 86 %) and desiccated (RWC ~ 9 %) leaves from hydrated and desiccated plants. The lyophilate was ground to a fine powder using a pestle and mortar and suspended in boiling ethanol (1 % w/v) for 15 min to deactivate any enzymes present. The powder was recovered by filtration and subjected to a series of extractions to remove lipids, polyphenols and other low molecular weight metabolites. Briefly, the residues were extracted for 12 hours at room temperature twice with methanol/chloroform (1:1 v/v), twice with methanol/acetone (1:1 v/v) and finally with acetone/water (4:1 v/v). The residue was air-dried at 80 °C, suspended in 50 mM acetate pH 5.4 and de-starched at 80 °C using a thermostable α-amylase and amyloglucosidase preparation (Megazyme International, Ireland, EC 3.2.1.1). Approximately 35 % of the lyophilised leaf weight was recovered.

A scheme outlining the fractionation procedure, based on that developed by Selvendran (Selvendran, 1985; Selvendran and O'Neill, 1987) has been provided (Figure 4.1). Briefly, cell wall material was subjected to a sequential extraction regime using 0.1 M phosphate pH 6 at 80 °C (this includes the de-starched extract), 50 mM CDTA pH 6.5, 50 mM KOH, 1 M KOH and 4 M KOH. All extractions were for 2 hours at room temperature except where stated. After extraction, suspensions were centrifuged at 5000 rpm for 10 min and residues collected on sintered glass fibre filters for re-extraction. All extract suspensions were at a concentration of 1 % w/v. CDTA extracts were dialysed against 1M NaCl to exchange the CDTA anion for the chloride anion. Alkaline extractions supplemented with 1 % $NaBH_4$ were performed in a nitrogen atmosphere after which the pH was adjusted to pH 4.5 with glacial acetic acid. All extracts were dialysed against distilled water, concentrated by rotary evaporation before being lyophilised.

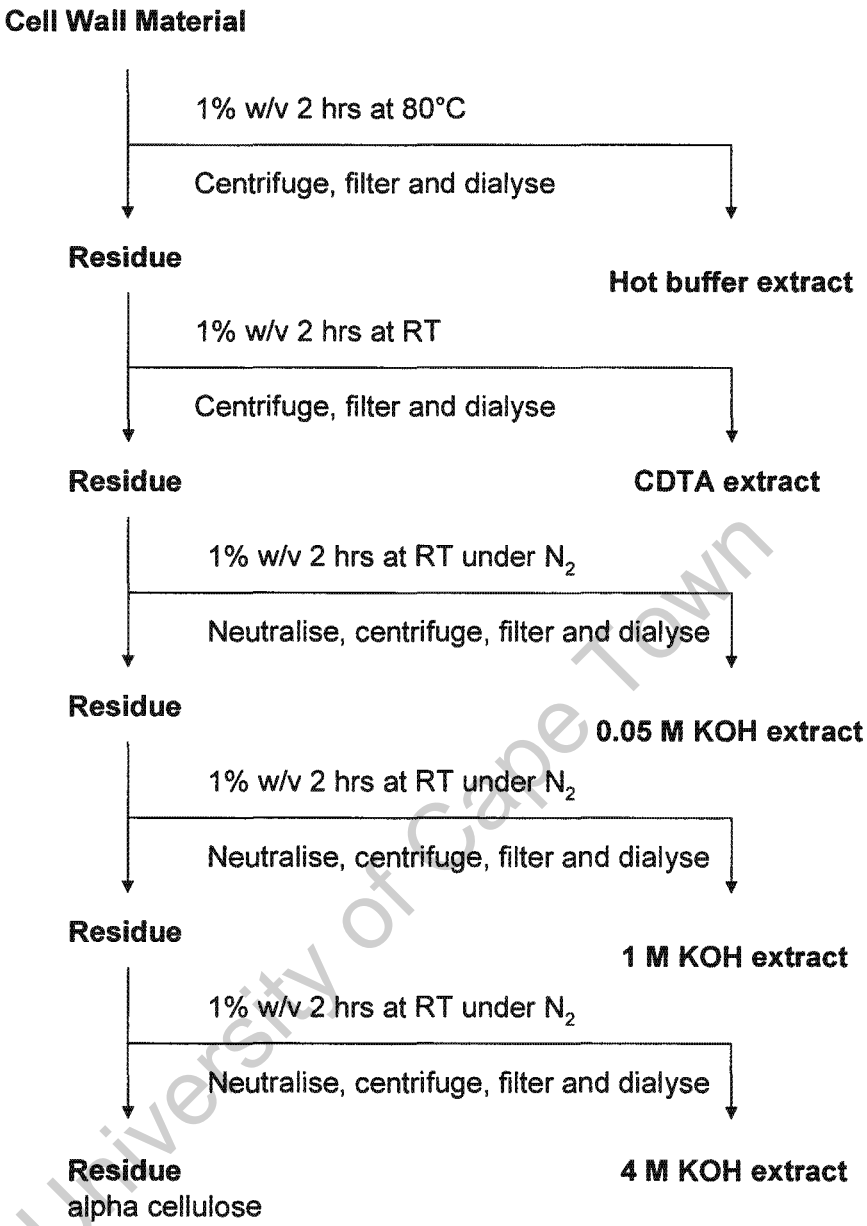


Figure 4.1. Flow diagram of the cell wall fractionation procedure. All extractions were for 2 h at room temperature (RT), except the hot buffer extraction which was at 80 °C. Alkaline extractions were supplemented with 1 % $NaBH_4$ and performed under a nitrogen atmosphere.

4.2.4 Monosaccharide composition analysis

The monosaccharide composition of each cell wall fraction apart from the sulphuric acid hydrolysate was analysed by GLC (York et al., 1985) using mannitol as the internal standard. The sulphuric acid hydrolysate was analysed by GLC (York et al., 1985) using inositol as the internal standard. Briefly, each fraction (5 - 10mg) was hydrolysed (2 M TFA, 110 °C, 2 h) and the liberated monosaccharides converted to the methoxy-sugars by incubation at 80 °C for 24 h in 1 M methanolic HCl. After silylation at 80 °C for 30 min, samples were dried, dissolved in cyclohexane/pyridine (50:1 v/v) and analysed using a GC 3800 Varian gas chromatography system equipped with a flame ionisation detector. A Varian WCOT fused silica column (dimensions 25m x 0.25mm i.d.) coated CP-SIL 5CB (DB1) was used for analysis. The following temperature gradient was employed: 120°C for 2 min then to 160°C over 4 min, to 220°C over 40 min and finally to 280°C over 8 min. Helium was used as carrier gas. This temperature program was optimised for the separation of the most common cell wall monosaccharides, specifically: arabinose (Ara), fucose (Fuc), galactose (Gal), galacturonic acid (GalA), glucose (Glc), glucuronic acid (GlcA), mannose (Man), rhamnose (Rha), xylose (Xyl) as well as the internal standards inositol and mannitol. Chromatographic data was analysed and integrated using GC Star Workstation (Varian) software with each monosaccharide being corrected according to its response factor.

4.2.5 Xyloglucan analysis by enzymatic hydrolysis

Xyloglucan polymer fragments were generated by digestion at 37 °C for 24 h with 5 - 10 units of endo-β-(1,4) glucanase (Megazyme International, Ireland, EC 3.2.1.4) in 1 ml of 50 mM sodium acetate pH 5.4. These fragments was separated using a DIONEX HPLC (see 4.2.5) and the masses determined by MALDI-TOF mass spectrometry (see 4.2.6) using a Micromass (Manchester, UK) mass spectrometer using dihydroxybenzoic acid as the matrix. Standards were prepared by digestion of tamarind seed (York et al., 1993) and *Argania spinosa* (Ray et al., 2004) xyloglucan.

4.2.6 DIONEX HPLC analysis

HPLC analysis of partial enzyme hydrolysis products was preformed on a DIONEX HPAC workstation equipped with pulsed amperometric detection. Eluent A consisted of 100 mM NaOH and eluent B consisted of a solution of 1 M NaOAc and 100 mM NaOH. Both buffers were degassed under nitrogen prior to use. A linear gradient from 0 – 8 % buffer B over 30 min was used to separate xyloglucan derived oligosaccharides. Fractions were collected, frozen and lyophilised for storage at -20°C.

4.2.7 Mass spectrometry

Mass spectrometry was performed by mixing 1µl of sample and 1µl of a matrix (dihydroxybenzoic acid (DHB)), 10 mg/ml). One µl of this solution was loaded onto a stainless steel plate and induced to crystallise under vacuum. Analysis was performed on a Micromass (Manchester, UK) Tof spec E MALDI-TOF mass spectrometer and spectra were acquired in reflectron mode.

4.2.8 Amino acid analysis

Amino acid analysis of cell walls from hydrated and desiccated leaf material was performed by hydrolysing the samples with 6 M HCl to their constituent amino acids (Cohen et al., 1984). Amino acids present in the lysates were separated, identified and quantitated using a Waters Amino Acid Analyser using the OPA/ion-exchange method (Klapper, 1982).

4.2.9 Analysis of Arabinogalactan proteins

Arabinogalactan proteins were extracted from lyophilised hydrated and desiccated leaf material as described previously (Schultz et al., 2000) before Yariv precipitable arabinogalactan proteins were selectively precipitated (Ding & Zhu, 1997; Schultz et al., 2000) using the β-D-glucosyl Yariv reagent (Yariv et al., 1962) (Biosupplies, Australia). Purified Yariv precipitable arabinogalactan proteins were hydrolysed with TFA and analysed for monosaccharide composition as described previously for cell wall material.

4.2.10 Immunocytochemistry

Chemical fixation and preparation of leaf tissue for immunogold labelling experiments were performed as outlined in previous cell wall immunocytochemical studies (Vicré et al., 1999; Vicré, 2001). Briefly, 1 – 2 mm^2 segments were excised from the mid-blade of hydrated and desiccated leaves and fixed overnight at 4 °C in 0.1M phosphate buffer pH 7.4 containing 4 % paraformaldehyde and 0.5 % glutaraldehyde supplemented with 0.5 % caffeine. Fixed samples were dehydrated in ethanol, placed in beem capsules to exclude air and progressively infiltrated with LR White resin before being hardened by heating overnight at 60 °C. Ultra-thin sections (90 - 100 nm) were prepared from resin embedded tissue blocks using a Reichert Ultracut-S ultramicrotome and collected on 200 μm mesh formvar-backed nickel grids.

Antibodies used were selected to recognize specific epitopes. The polyclonal anti-polygalacturonic acid/ rhamnogalacturonan I (PGA/RG1) antibody has been reported to recognise the non-esterified form of homogalacturonans in pectin (Moore et al., 1986; Lynch and Staehelin, 1992), the monoclonal antibody JIM 5 has been reported to recognise homogalacturonans displaying a relatively low degree of methylesterification (VandenBosch et al., 1989; Knox et al., 1990; Willats et al., 2000; Clausen et al., 2003), the polyclonal anti-XG antibody has been reported to recognise β(1→4)-glucans in the xyloglucan backbone (Moore et al., 1986), the monoclonal antibody LM6 has been reported to specifically recognise the linear chains of (1→5)-α-L-arabinan which are known to be associated with pectin and AGPs (Knox et al., 1997; Willats et al., 1998). The monoclonal antibody JIM 13 has been shown to recognise polysaccharide epitopes of arabinogalactan-proteins (Knox et al., 1991; Knox, 1997; Yates et al., 1996).

Immunocytochemistry was performed essentially as described previously (Vicré et al., 1999). Briefly, sections mounted on nickel grids were blocked in 1 % w/v BSA in Tris buffered saline TBS (50 mM Tris HCl pH 7.2 and 150 mM NaCl) for 30 min. Sections were then incubated overnight at 4°C in the antibody solution. Certain antibody solutions were diluted with TBS prior to incubation (specifically PGA/RG1 1:20 and anti-XG 1:10). The sections were then washed with TBS supplemented with 0.1 % v/v tween 20 and then incubated in 1:20 dilution (using TBS) of secondary antibody (goat anti-rat or goat anti-rabbit) coupled to 10 nm colloidal gold (Sigma) for 1 hr. Sections were stained in uranyl acetate and lead citrate (Reynolds, 1963). All washing, incubation and staining steps were performed at room temperature unless otherwise indicated. Labelled grids were viewed using a LEO 912 transmission electron microscope equipped with CCD camera. The density of labeling, expressed as the number of gold particles/μm^2, was obtained by counting the number of particles present in 10 micrographs each from three independent experiments (Vicré et al., 1999).

4.3 RESULTS

4.3.1 Ultrastructural characterisation of desiccation-induced cell wall folding in *M. flabellifolia* leaves

The pronounced morphological changes observed on desiccation of *M. flabellifolia* are similar to those observed in other resurrection plants undergoing the same process (Alpert and Oliver, 2002; Vicré et al., 2004b). Dehydrating *M. flabellifolia* plants undergo curling of both their stem segments as well as their leaves (see chapter 2). In particular, the leaves fold upwards to a position where the adaxial surfaces are appressed against the stem (see chapter 2) and the sclerenchymous leaf ribs (Grundell, 1933) move closer to one another thereby closing the leaf face, which has been proposed to minimise the exposure of the desiccated leaf to light (Farrant et al., 2003). Electron microscopy of hydrated and desiccated leaves revealed further morphological changes that occurred upon desiccation (Figures 4.2 and 4.3). Cryo-scanning electron microscopy of the epidermal surface of hydrated leaves revealed a distinctive network pattern of stomata and gland cells inter-dispersed between regular epidermal cells (Figure 4.2A). Upon desiccation, these epidermal cells underwent extensive cell wall folding and shrinkage around the seemingly less flexible stomata and gland cells (Figure 4.2B). The stomata in particular were clearly visible in the desiccated state displaying little of the extreme morphological changes present in desiccated epidermal cells (Figure 4.2B). Freeze fracture cross-sections through cryo-fixed leaves showed that considerable changes in tissue and cellular structure occurred associated with the desiccation process (Figures 4.2C – 4.2F). Electron microscopy of a cross-section through a frozen hydrated leaf (Figures 4.2C and 4.2E) revealed clear anatomical features such as the epidermis, the spongy mesophyll layer, the palisade mesophyll layer as well as vascular cells. In contrast, electron

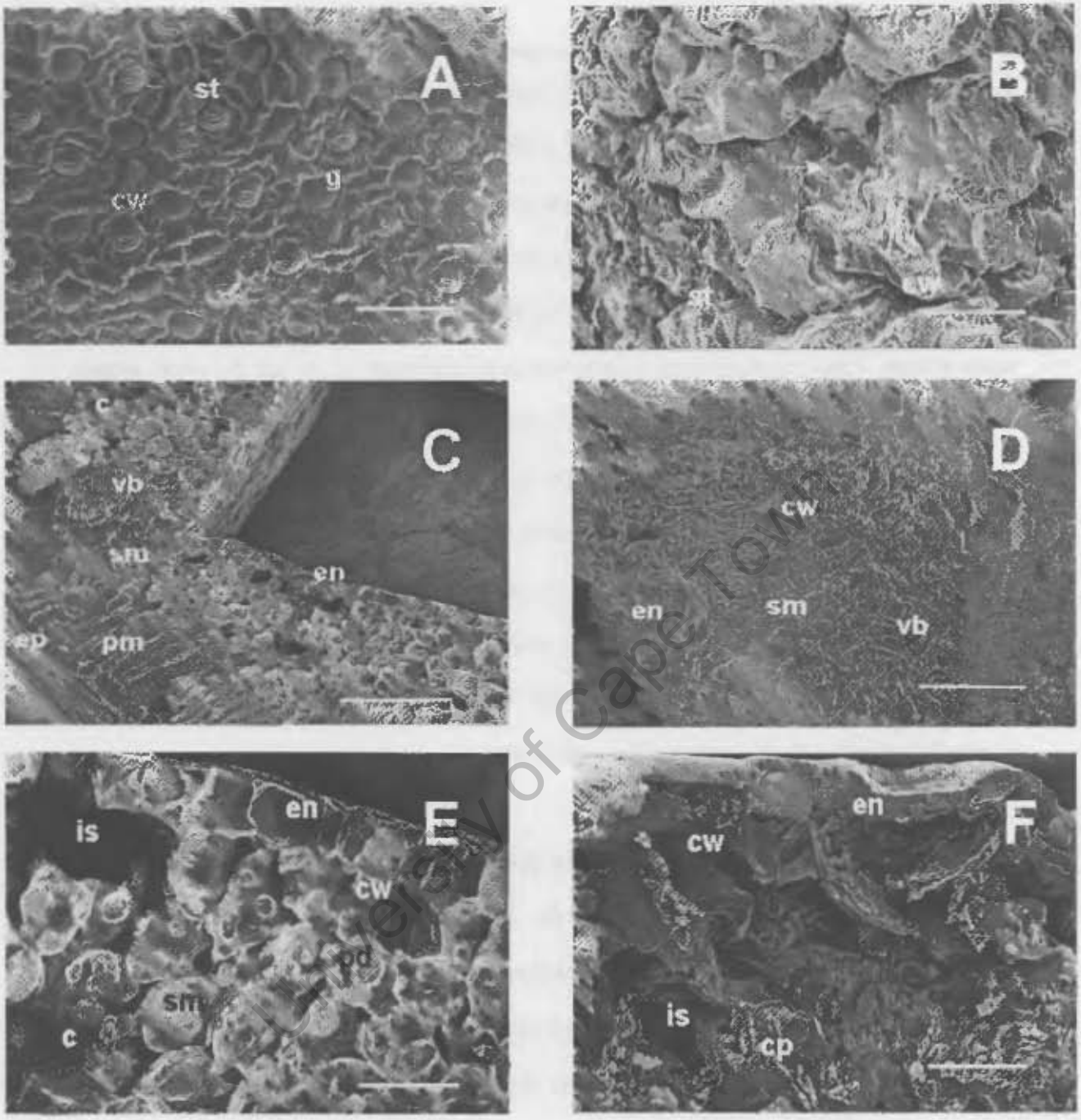


Figure 4.2. Scanning electron micrographs of hydrated (A, C, E) and desiccated (B, D, F) *M. flabellifolia* leaves. Surface view of the epidermal surface of hydrated and desiccated leaves (A & B); transverse sections of hydrated and desiccated leaves (C - H). [key: c: calcium oxalate crystal; chl: chloroplast; cp: cytoplasm; cw: cell wall; en: endodermis; g: gland; is: intercellular space; pd: plasmodesmata; pm: palisade mesophyll; st: stomata; sm: spongy mesophyll; vb: vascular bundle. Scale bars: a, b = 70 μm; c = 100 μm, d – 80 μm ; e, f = 40 μm.]

microscopy of a cross-section through an identically treated desiccated leaf showed no discernable anatomical organisation (Figures 4.2D and 4.2F). It would appear that cell wall folding had occurred during desiccation resulting in a compacted, wrinkled appearance of the leaf cell layers. Only sclerenchymous and vascular cells resistant to folding as well as epidermal cells were clearly identifiable (Figures 4.2D and 4.2F). Higher magnification examination of hydrated tissue (Figure 4.2E) revealed turgid endodermal cells and small (~ 30 μm diameter) spongy mesophyll cells with multiple plasmodesmata connections to adjacent cells. Several intercellular airspaces and calcium oxalate druse crystals (Grundell, 1933) were also clearly visible. Desiccated leaf tissue at the same magnification (Figure 4.2F) showed none of the features evident in hydrated tissue. Only folded cell wall fragments, most likely the result of mechanical fracturing during sample preparation, a greater number of intercellular airspaces and amorphous deposits between cell wall layers were observed. These amorphous deposits were possibly the result of desiccation of salts and cytosolic constituents.

Examination of chemically fixed hydrated and desiccated leaf tissue by transmission electron microscopy provided further evidence for the changes observed using scanning electron microscopy (Figure 4.3 A - D). Transverse sections of hydrated leaves (Figures 4.3 A and C) showed turgid mesophyll cells typical of turgid tissue, with distinct cell walls, plasmodesmata, cytoplasm and associated constituents such as chloroplasts, starch granules and large central vacuoles. The cytoplasm of these cells occurred on the periphery of the cells adjacent to the cell wall. In contrast, transverse sections of desiccated leaves (Figures 4.3 B and D) showed cells with folded cell walls (Figure 4.3 B) and a compact dense cytoplasm separated from the cell wall in manner which resembled that observed in plasmolysed plant cells, although the plasmalemma remained intact. The plasmolysed appearance of desiccated cells might have been brought about

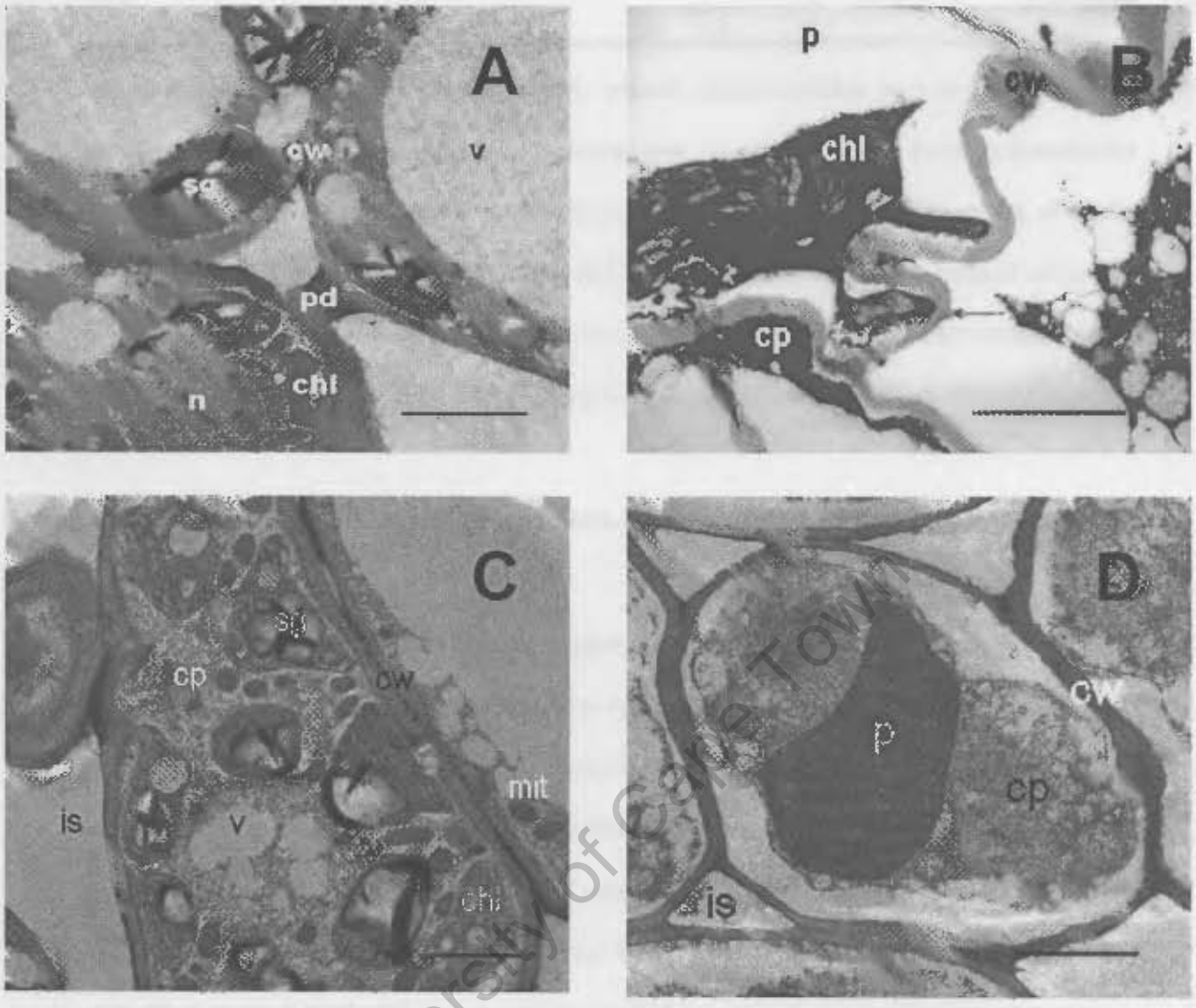


Figure 4.3. Transmission electron micrographs of transverse sections through hydrated (A, C) and desiccated (B, D) *M. flabellifolia* leaves. [key: chl: chloroplast; cp: cytoplasm; cw: cell wall; is: intercellular space; mit: mitochondria; n: nucleus; p: polyphenols; sg: starch granule; v: vacuole; Scale bars: a-d = 3 µm.]

by use of an aqueous aldehyde-based fixative (Hayat, 1981). This would preferentially fix cytoplasmic proteins rather than the cell wall carbohydrates as the former have a higher number of amino groups (Hayat, 1981). Since desiccated cell walls would presumably have swollen during the fixation process, our observations of cell wall folding in fixed desiccated cells provide strong evidence that cell wall folding occurs *in vivo*, and this is probably the main mechanism of mechanical stabilization of the mesophyll cells of this species.

4.3.2 Biochemical analysis of hydrated and desiccated leaf cell walls

Since the cell wall of *M. flabellifolia* might be unique on account of its taxonomic position (Qiu et al., 1998) being basally situated in angiosperm evolution, we next investigated the composition of the cell wall of both hydrated and desiccated leaves. Hydrated or desiccated leaf material was serially extracted with a variety of organic solvents (see Methods) before being air-dried; this material represented total cell walls of these leaves. No difference was detected in the recovery of cell wall material from hydrated leaves (42 ± 5 %) and desiccated leaves (40 ± 5 %). The total sugar composition of these cell walls was determined (Figure 4.4) after TFA hydrolysis (York et al., 1985). This analysis, which excluded the TFA-resistant α-crystalline cellulose, revealed arabinose, xylose and galacturonic acid as the predominant monosaccharides, representing approximately 75 % of the total hydrolysable sugar present in both hydrated and desiccated leaves (Figure 4.4). The other monosaccharides present, in decreasing order of concentration, in both hydrated and desiccated leaves were found to be galactose, glucose, rhamnose, mannose, glucuronic acid and fucose. Of all these monosaccharides, only xylose and galacturonic acid displayed any concentration differences between hydrated and desiccated leaves with an elevated level of xylose and a decreased level of galacturonic acid present in desiccated leaves.

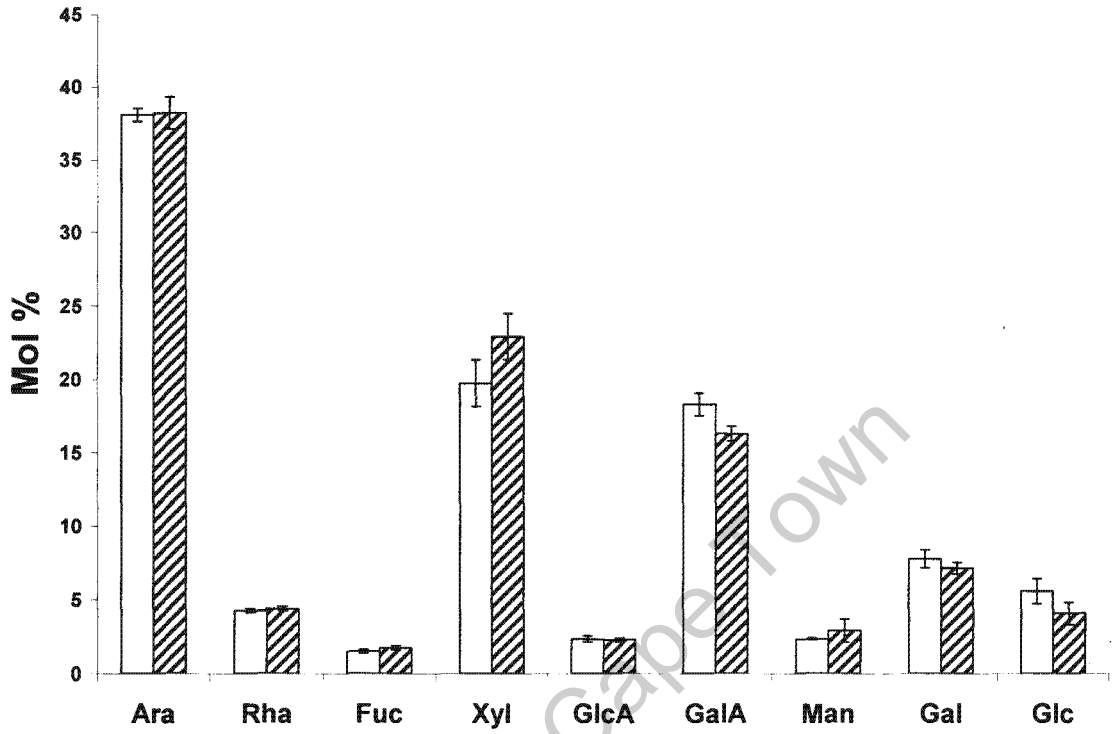


Figure 4.4. Monosaccharide composition of total cell walls of hydrated (empty bars) and desiccated (hatched bars) leaves. Monosaccharides analysed were arabinose (Ara), rhamnose (Rha), fucose (Fuc), xylose (Xyl), glucuronic acid (GlcA), galacturonic acid (GalA), mannose (Man), galactose (Gal) and glucose (Glc). Error bars represent standard deviations of the mean from two independent experiments with at least three replicates per experiment.

Table 4.1.

Quantitation of the amino acid content of cell walls isolated from hydrated and desiccated leaves of *M. flabellifolia*. Amino acid quantity present in hydrated and desiccated cell walls is presented as mol % and is calculated with respect to the equivalent dry weight of isolated wall material. The means and error values representing standard deviations of the mean from two independent experiemnts with at least two replicates per experiment are indicated.

Amino Acid	Code	Total	Hydrated	Desiccated
Polar		~ 26 - 28 %		
Glycine	G		10.9 ± 0.4	12.0 ± 0.6
Serine	S		6.2 ± 0.2	5.9 ± 0.3
Threonine	T		5.2 ± 0.1	5.9 ± 0.3
Tyrosine	Y		3.5 ± 0.3	3.5 ± 0.1
Cysteine	C		1.0 ± 0.3	0.6 ± 0.3
Non-polar		~ 36 - 40 %		
Leucine	L		10.1 ± 0.4	11.0 ± 0.5
Alanine	A		7.9 ± 0.8	9.3 ± 0.7
Valine	V		7.3 ± 0.4	7.8 ± 0.2
Isoleucine	I		5.3 ± 0.4	5.5 ± 0.2
Phenylalanine	F		4.4 ± 0.2	4.7 ± 0.5
Methionine	M		1.2 ± 0.1	1.4 ± 0.3
Basic		~ 10 - 12 %		
Arginine	R		5.0 ± 0.3	6.1 ± 0.6
Lysine	K		4.9 ± 0.3	6.1 ± 0.7
Histidine	H		----------	----------
Acidic		~ 20 - 23 %		
Glutamic acid	E		11.0 ± 0.3	12.8 ± 0.6
Aspartic acid	D		9.3 ± 0.6	10.1 ± 0.5

As cell wall associated proteins have been implicated in the response of the plant cell wall to osmotic stress (Iraki et al. 1989c) we performed a comparative analysis of amino acid composition and quantity in hydrated and desiccated *M. flabellifolia* cell walls. No significant differences in amino acid content and quantity were observed between hydrated and desiccated leaf cell walls of *M. flabellifolia* (Table 4.1). The *M. flabellifolia* cell wall was composed of high amounts of glycine, leucine, alanine, glutamic acid and aspartic acid (Table 4.1).

We next fractionated the total cell walls prepared from hydrated and desiccated *M. flabellifolia* leaves (Table 4.2, Figure 4.1) by serial extraction initially with phosphate buffer at 80 ^{0}C, then with CDTA and finally with increasing concentrations of KOH. These latter extractions were all performed at 20 ^{0}C. Each fraction extracted (Figures 4.5 A – 4.9 B, Table 4.2) was gravimetrically analysed before being hydrolysed with TFA; insoluble material remaining after TFA hydrolysis of the fraction represented as Figure 4.9 A was hydrolysed with H_2SO_4 (Fraction 9 B) (Figure 4.9 B). These hydrolysates were then analysed for the presence of individual monosaccharides (Figure 4.5). Gravimetric analysis of lyophilised fractions recovered from serial fractionation (Table 4.2) yielded three significant differences in certain fractions (Figures 4.5 B, 4.6 B and 4.7 A) and between hydrated and desiccated leaves. CDTA extraction (Table 4.2, Figure 4.5 B) extracted more material from hydrated (10.9 ± 0.4 %) than from desiccated (8.0 ± 0.5 %) samples; more 0.05 M KOH insoluble material (Table 4.2, Figure 4.6 B) was present in desiccated (12.3 ± 1.5 %) compared with hydrated samples (9.7 ± 0.4 %) and 1 M KOH (Table 4.2, Figure 4.7 A) extracted almost double the material from hydrated (8.2 ± 1.6 %) than from desiccated samples (4.1 ± 0.6 %).

The monosaccharide composition of each fraction obtained after cell wall fractionation was determined (Figures 4.5 A – 4.9 B) allowing the ability to infer the main polymers present.

Table 4.2.

Gravimetric analysis of material isolated as a result of the fractionation procedure (Figure 4.1) from hydrated and desiccated leaf cell walls. Figures 4.5 – 4.9 represent cell wall fractions which were hydrolysed with TFA; alkali insoluble material remaining after TFA hydrolysis (Figure 4.9 A) was hydrolysed with H_2SO_4 (Figure 4.9 B). KOH A and KOH B represent soluble and insoluble components respectively. The data represent g per 100 g dry cell wall material of duplicate samples and error bars represent standard deviation of the mean. Component extracted describes the major polymers proposed to be present in each fraction.

Fraction	Extraction method	Component extracted	Hydrated	Desiccated
Figure 4.5 A	Hot buffer	Proteins and pectin	6.9 ± 2.1	7.6 ± 2.8
Figure 4.5 B	CDTA	Pectin and neutral polymers	10.9 ± 0.4	8.0 ± 0.5
Figure 4.6 A	0.05 M KOH A	Pectin and neutral polymers	3.4 ± 1.7	6.0 ± 2.0
Figure 4.6 B	0.05 M KOH B	Pectin and neutral polymers	9.7 ± 0.4	12.3 ± 1.5
Figure 4.7 A	1 M KOH A	Xylans and neutral polymers	8.2 ± 1.6	4.1 ± 0.6
Figure 4.7 B	1 M KOH B	Xylans	5.1 ± 0.3	4.9 ± 0.2
Figure 4.8 A	4 M KOH A	Xyloglucan	6.3 ± 0.3	7.2 ± 1.1
Figure 4.8 B	4 M KOH B	Xylans and neutral polymers	5.4 ± 0.7	4.4 ± 1.1
Figure 4.9 A and B	Insoluble residue (incl. TFA extract)	Cellulose and neutral polymers	44.2 ± 5.6	45.6 ± 7.4

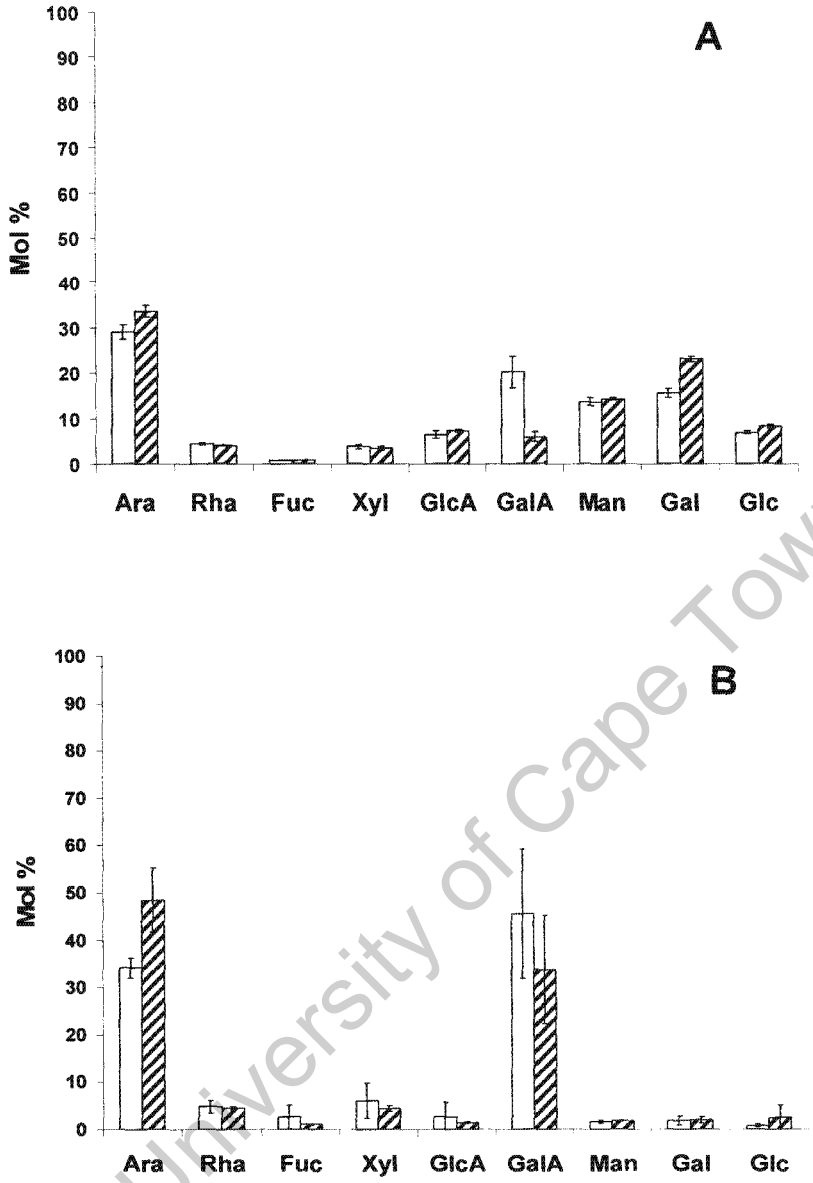


Figure 4.5. Monosaccharide composition (see Figure 4.4 for abbreviations used and statistics) of cell wall fractions extracted using hot buffer (A) and CDTA (B) (Figure 4.1) from hydrated (empty bars) and desiccated (hatched bars) leaves.

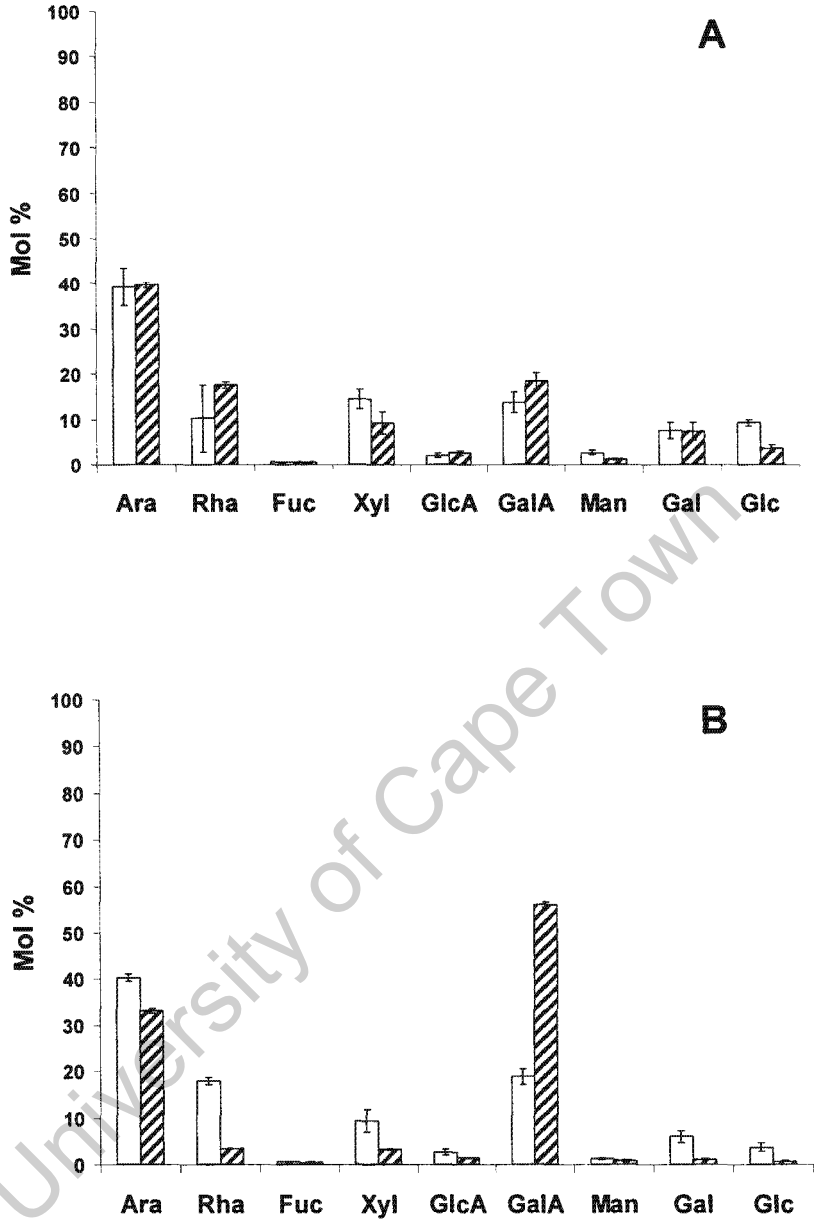


Figure 4.6. Monosaccharide composition (see Figure 4.4 for abbreviations used and statistics) of cell wall fractions extracted using 50 mM KOH (Figure 4.1) from hydrated (empty bars) and desiccated (hatched bars) leaves. A and B represent soluble and insoluble material respectively.

Fraction 5 A (Figure 4.5 A) contained predominantly arabinose, galacturonic acid, mannose and galactose, consistent with manno-proteins, arabinogalacto-proteins, soluble pectin and other glycoproteins being extracted with phosphate buffer at 80 ^{0}C (Brett and Waldron, 1996). There were significant differences in the levels of galacturonic acid, galactose and arabinose between hydrated and desiccated leaves with hydrated leaves found to contain increased levels of galacturonic acid and decreased levels of galactose and arabinose. Fraction 5 B (Figure 4.5 B) contained predominantly arabinose and galacturonic acid, consistent with CDTA-mediated solublisation of pectin. The presence of arabinose suggested the association of neutral arabinan chains together with the pectin, in agreement with previous data presented (Figure 4.4) which revealed a high arabinose and galacturonic acid content in the cell wall. Furthermore, less arabinose was present in hydrated compared with desiccated leaves. Fraction 6 A (Figure 4.6 A) contained arabinose, galacturonic acid, glucose, rhamnose, and xylose as the major monosaccharides present. Of these monosaccharides, relatively increased amounts of xylose and glucose and a decreased amount of galacturonic acid were found to be present in hydrated leaves. Fraction 6 B (Figure 4.6 B) contained arabinose, galacturonic acid and rhamnose as the most abundant monosaccharides present with increased levels of arabinose and rhamnose and a decreased level of galacturonic acid found in hydrated leaves. The high amounts of arabinose and galacturonic acid present in these fractions (6 A and B) (Figures 4.6 A and B) indicated that additional pectic material is present in desiccated leaves associated with arabinans tightly bound to the cell wall and only released by strong alkali extraction. Rhamnogalacturonan polymers were also inferred to be present due to the presence of rhamnose in both extracts. Fraction 7 A (Figure 4.7 A) contained primarily xylose with lesser amounts of arabinose and glucose together with small amounts of the other monosaccharides, consistent with the presence of arabinoxylans in this fraction. Hydrated leaves were found to contain increased xylose and glucose and decreased arabinose concentrations. Fraction 7 B (Figure 4.7 B) contained almost exclusively xylose with

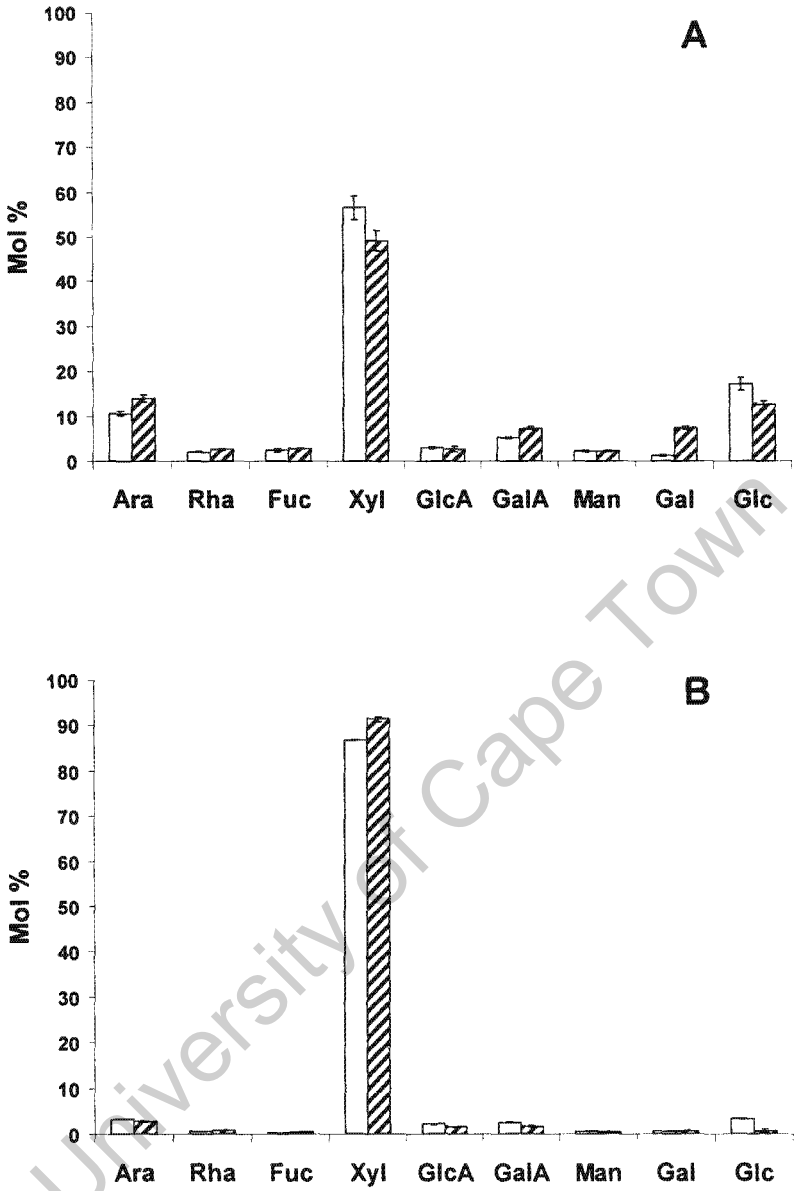


Figure 4.7. Monosaccharide composition (see Figure 4.4 for abbreviations used and statistics) of cell wall fractions extracted using 1 M KOH (Figure 4.1) from hydrated (empty bars) and desiccated (hatched bars) leaves. A and B represent soluble and insoluble material respectively.

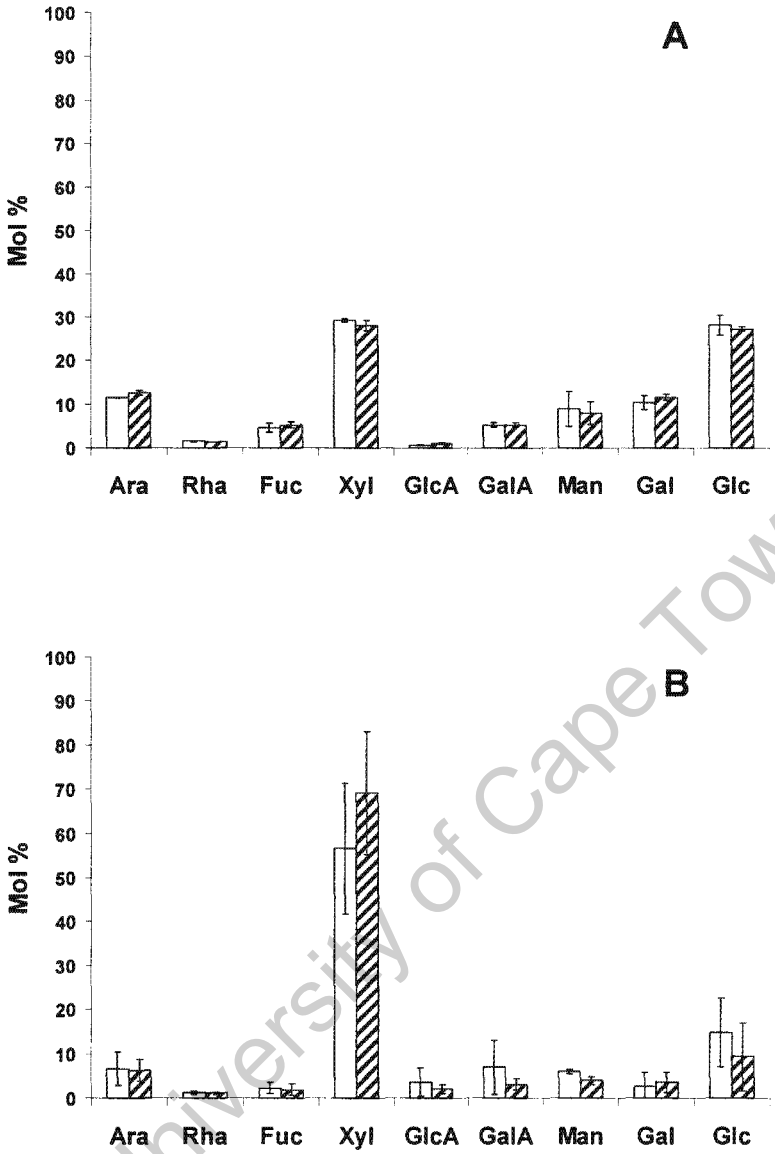


Figure 4.8. Monosaccharide composition (see Figure 4.4 for abbreviations used and statistics) of cell wall fractions extracted using 4 M KOH (Figure 4.1) from hydrated (empty bars) and desiccated (hatched bars) leaves. A and B represent soluble and insoluble material respectively.

no significant difference in concentration between the hydrated and desiccated states. The high concentration of xylose in both the soluble and insoluble 1M KOH extracts (fractions 7 A and B) (Figures 4.7 A and B) suggested the presence of the more soluble arabinoxylans in fraction 7 A (Figure 4.7 A) and the insoluble crystalline xylans polymers in fraction 7 B (Figure 4.7 B). Continued serial extraction with 4 M KOH and subsequent TFA hydrolysis failed to reveal any significant differences between hydrated and desiccated leaves. Material extracted with 4M KOH (fraction 8 A) (Figure 4.8 A) contained mainly xylose and glucose together with lesser amounts of arabinose, mannose and galactose, consistent with the presence of xyloglucan polymers. Insoluble material after extraction with 4M KOH (fraction 8 B) (Figure 4.8 B) contained chiefly xylose suggesting the presence of residual insoluble crystalline xylans. The alkali-insoluble residue remaining after serial extraction was finally subjected to successive acid hydrolysis using first TFA (fraction 9 A) (Figure 4.9 A) and then H_2SO_4 (fraction 9 B) (Figure 4.9 B). Fraction 9 A (Figure 4.9 A) was found to contain high amounts of arabinose together with lesser amounts of galacturonic acid and xylose suggesting the presence of residual tightly bound pectic arabinans and xylans. Alternatively this could also indicate the presence of extension proteins in Fraction 9 A. Fraction 9 B (Figure 4.9 B) contained mostly glucose, most likely derived from crystalline cellulose. Mannans have been reported to be associated with cellulose (Fry, 1988); the presence of mannose in this fraction suggested such an association in *M. flabellifolia* leaves.

4.3.3 Xyloglucan structure in *M. flabellifolia* cell walls

In order to characterise the xyloglucan component of *M. flabellifolia* leaf hemicellulose, the major load bearing polymers of the plant cell wall, isolated cell walls were subjected to enzymatic degradation and analysis. Released oligosaccharides were identified by HPLC (data not shown)

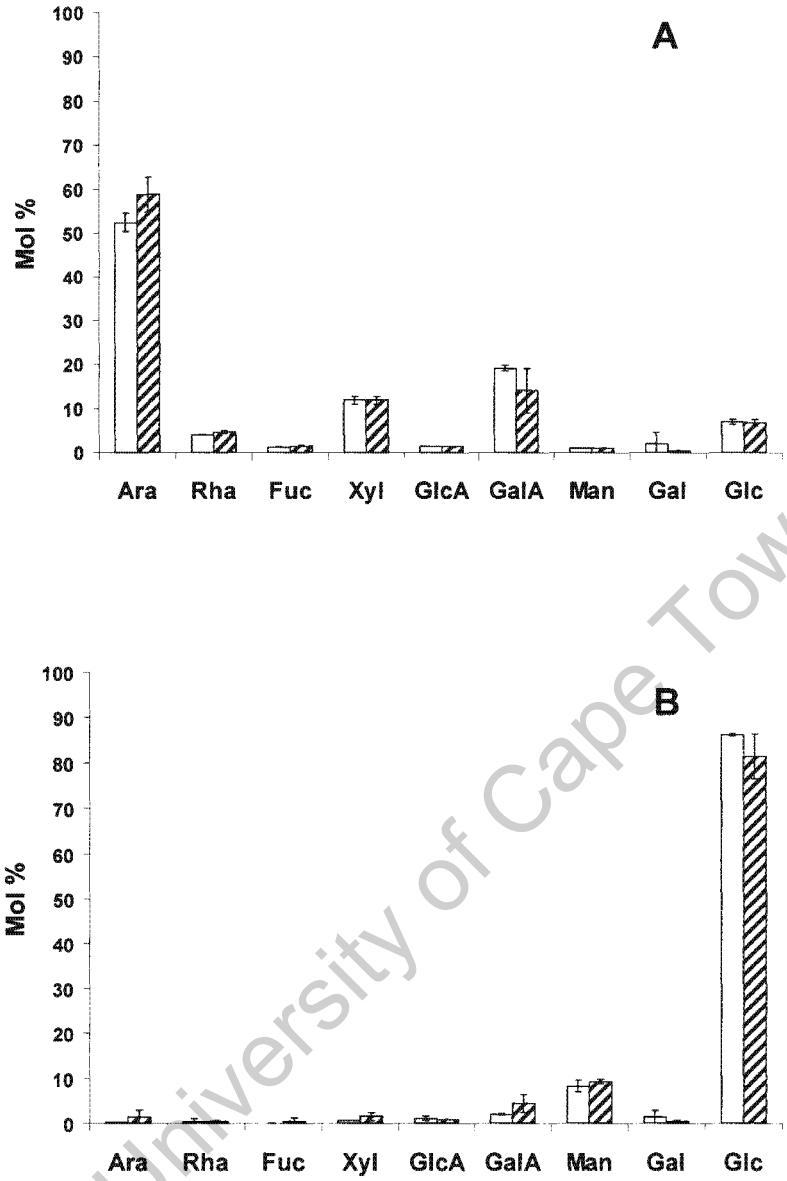


Figure 4.9. Monosaccharide composition (see Figure 4.4 for abbreviations used and statistics) of the alkali insoluble residue prepared (see Figure 4.1) from hydrated (empty bars) and desiccated (hatched bars) leaves. A and B represent the TFA and H_2SO_4 hydrosylates respectively.

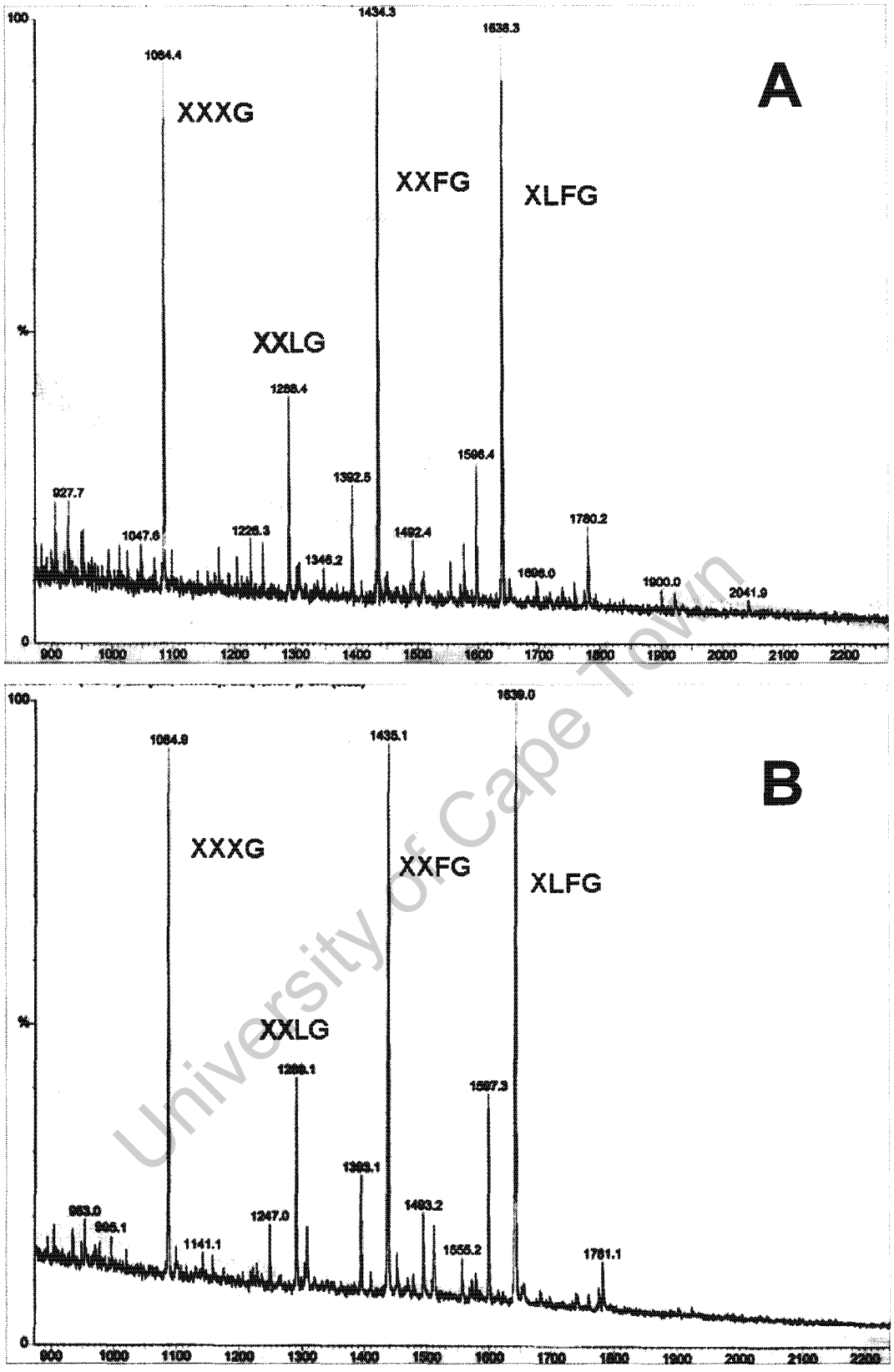


Figure 4.10. Mass spectral analysis of xyloglucan fragments of hydrated (A) and desiccated (B) leaves. XXXG, XXLG, XXFG and XLFG denote hepta-saccharide, octa-saccharide, nona-saccharide and deca-saccharide fragments respectively according to the standard xyloglucan nomenclature (Fry et al., 1993). The ordinate denotes the relative percentage ion abundance

with further confirmation by MALDI-TOF mass spectrometry. Enzymatic degradation resulted in the release of four predominant xyloglucan derived oligosaccharides with m/z 1084, 1288, 1435 and 1639 (Figure 4.10). The m/z 1084 ion was assigned to XXXG (nomenclature according to Fry et al. 1993) with the m/z 1288, 1435 and the m/z 1639 ions assigned to mono-acetylated XXLG, mono-acetylated XXFG and di-acetylated XLFG respectively. These were confirmed by MALDI-TOF mass spectrometry after de-acetylation using sodium hydroxide that resulted in ions of m/z 1247, 1393 and m/z 1555 respectively (data not shown). Traces of these unacetylated parent oligosaccharides ions are present in the original mass spectrum (Figure 4.10). Although the position of acetylation was not determined, it is likely to occur on galactose residues by analogy with previous studies on xyloglucan acetylation (Fry, 1988). No major differences in ion composition or intensity were observed between enzymatic extracts sourced from hydrated or desiccated cell walls (Figure 4.10).

4.3.4 Analysis of arabinogalactan proteins isolated from hydrated and desiccated leaf material

Arabinogalactan proteins have been reported to be associated with the cell wall and plasma membrane of plant cells (Knox, 1997) where it has been proposed that they likely function in a structural capacity to facilitate important processes in plant growth and development (Showalter, 2001). A study of the monosaccharide content of the arabinogalactan proteins isolated using the Yariv reagent (Yariv et al., 1962) from hydrated and desiccated *M. flabellifolia* leaf tissue was performed. This showed an abundance of arabinose, galactose, galacturonic acid and mannose and in from both hydrated and desiccated leaves with elevated levels of galacturonic acid and galactose observed in hydrated samples (Figure 4.11). In addition, quantification of Yariv

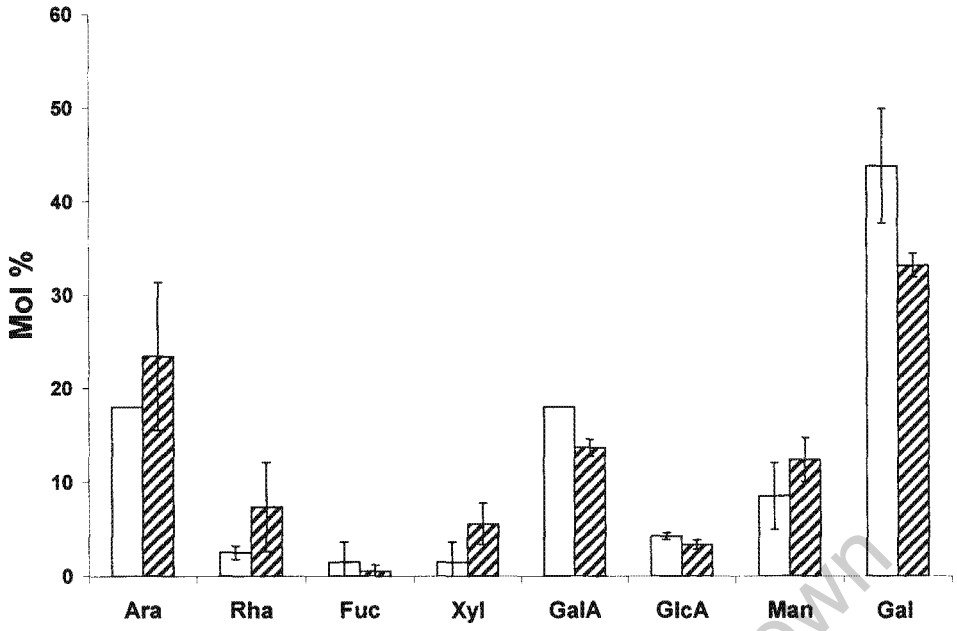


Figure 4.11. Monosaccharide composition (see Figure 4.4 for abbreviations used and statistics) of purified arabinogalactan proteins isolated from hydrated (empty bars) and desiccated (hatched bars) leaves.

precipitable arabinogalactan proteins using rocket electrophoresis did not reveal any qualitative or quantitative differences between hydrated and desiccated samples (data not shown).

4.3.5 Immunocytochemical analysis of hydrated and desiccated leaf cell walls

The distribution of the various carbohydrate epitopes present in or near the cell walls of hydrated and desiccated *M. flabellifolia* leaves was next investigated using immunocytochemistry using antibodies specific for epitopes associated with pectin, arabinogalactan proteins and xyloglucan. Two antibodies, PGA/RG1 and JIM 5, were used to locate pectin epitopes. The polyclonal PGA/RG1 antibody was found to label throughout the cell walls of both hydrated (Figure 4.12 A and C) and desiccated (Figure 4.12 B and D) tissue whereas the monoclonal JIM 5 antibody was found to specifically label the middle lamella region in both hydrated and desiccated cell walls (Figure 4.12 E and F). The location of xyloglucan epitopes was investigated using an anti-XG polyclonal antibody. This antibody revealed material throughout hydrated and desiccated cell walls (Figure 4.13 A and B) but failed to detect xyloglucan epitopes in the middle lamella zone and cell junctions (Figure 4.13 A). The monoclonal anti-arabinan LM 6 antibody directed against the (1→5)-α-L-arabinan epitope (Willats et al., 1998) was shown to label material in the cell walls of both hydrated and desiccated leaf tissue in close proximity to the cytoplasm (Figure 4.13 C and D). Finally, the monoclonal anti-arabinogalactan protein JIM 13 antibody was shown to predominately label the cell cytoplasm near the plasma membrane (Figure 4.13 E and F) as opposed to the cell wall in both desiccated and hydrated leaf tissue. Our data showed that whereas LM 6 labels in the cell wall adjacent to the plasma membrane (Figure 4.13 C and D), in contrast JIM 13 labels the cytoplasm and plasma membrane near the cell wall (Figure 4.13 E and F).

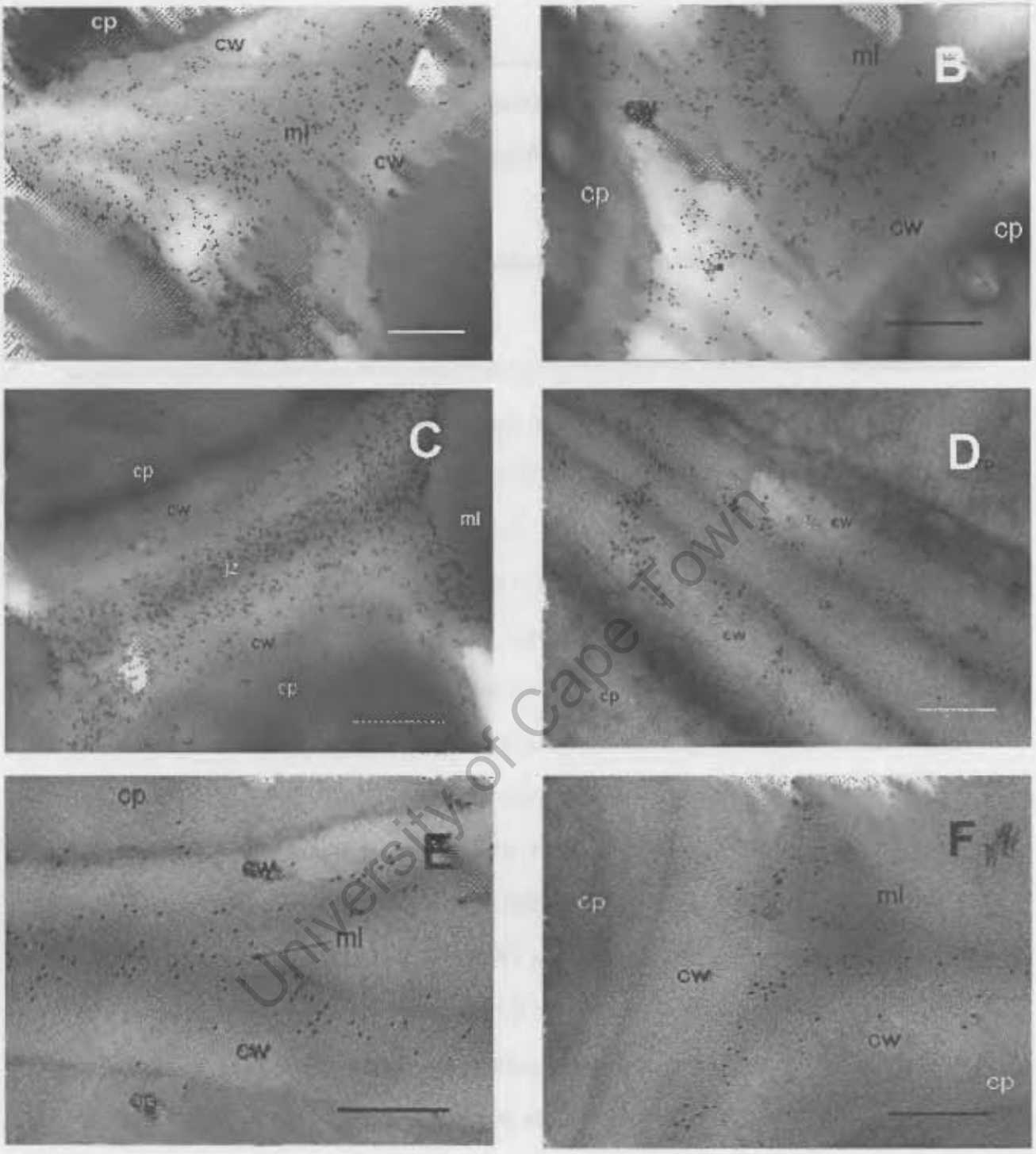


Figure 4.12. Immunogold labelling of pectin (homogalacturonan) epitopes, using the PGA/RG1 (A-D) and JIM 5 (E-F) antibodies, present in hydrated (A, C, E) and desiccated (B, D, F) leaf cell walls. [key: cp: cytoplasm; cw: cell wall; is: intercellular space; ml: middle lamella; jz: junction zone Scale bars a, b = 400 nm; c, d, e, f = 300 nm.]

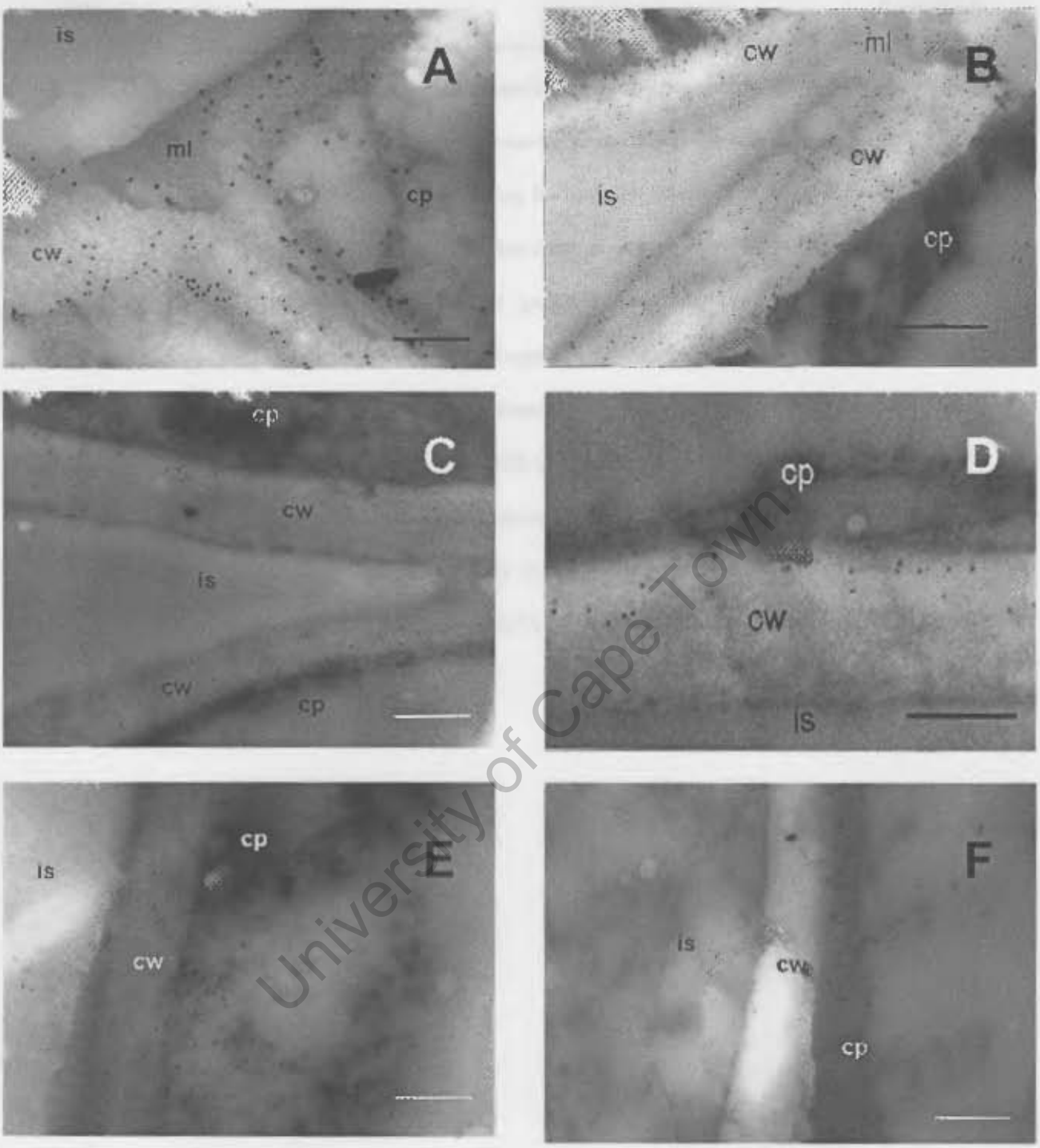


Figure 4.13. Immunogold labelling of xyloglucan epitopes, using the anti-XG antibody (A, B); of pectin (arabinan) epitopes, using the LM 6 antibody (C, D) and of arabinogalactan protein epitopes, using the JIM 13 antibody (E, F), present in hydrated (A, C, E) and desiccated (B, D, F) leaf cell walls. [key: cp: cytoplasm; cw: cell wall; is: intercellular space; ml: middle lamella. Scale bars: a = 500 nm; b = 200 nm; c = 300 nm; d = 150 nm; e, f = 300 nm.]

Additional analysis of pectin epitopes was performed (data not shown) using the monoclonal JIM 7 antibody which detects homogalacturonans possessing a relatively high level of methylesterification with flanking unesterified galacturonic acid residues (Willats et al., 2000; Clausen et al., 2003) as well as the monoclonal LM7 antibody, which recognises relatively unesterified homogalacturonans with flanking methyl-esterified galacturonic acid residues (Clausen et al., 2003). These antibodies displayed a very low density of labelling and did not provide any location data additional to that found with the other antibodies already used (not shown). Finally we attempted to quantitate any differences in the concentrations of these various cell wall polymers between hydrated and desiccated tissue by determining the density of gold particles observed in a number of micrographs (Table 4.3). No significant difference in the number of gold particles per μm^2 was observed (Table 4.3).

Table 4.3.

Quantitation of immunogold labelling with cell wall antibodies in hydrated and desiccated leaves of *M. flabellifolia*. The column 'Antibody' represents the specific antibody used in that study (see methods for corresponding references). The column 'Antigen/Epitope' denotes the target polymer for the specific antibody. The column 'Characteristics' indicates the method of antibody production. The density of labelling is expressed as the number of gold particles per μm^2 obtained from at least 10 micrographs and three independent labelling studies. The means and standard deviation are indicated.

Antibody	Antigen/Epitope	Characteristics	Hydrated	Desiccated
Anti-PGA/RG1	Homogalacturonans (unesterified)	Polyclonal (rabbits)	204.3 ± 40.7	168.6 ± 71.1
JIM 5	Homogalacturonans (unesterified)	Monoclonal (rats)	33.8 ± 10.6	43.1 ± 14.5
Anti-XG	β(1→4) glucose of the xyloglucan backbone	Polyclonal (rabbits)	166.9 ± 64.2	203.2 ± 65.1
LM 6	(1→5)-α-L-arabinans in pectin/AGPs	Monoclonal (rats)	20.3 ± 9.3	24.6 ± 8.1

4.4 DISCUSSION

Electron microscopic analysis of desiccation-induced *M. flabellifolia* leaf cell wall folding revealed distinct folding in mesophyll and epidermal cells accompanied by cytoplasm shrinkage and possible crystallization, presumably due to water loss. We believe that the absence of folding seen with gland, vascular and sclerenchyma cells, is due to these cells having thick possibly reinforced walls. In particular, the majority of the cells where wall folding occurs upon desiccation sit between the sclerenchymous ribs (Grundell, 1933), which form a fan-like structure. It is likely that these thick-walled cells and tissues provide structural support to the leaf during desiccation thereby allowing leaf morphology to be rapidly regained upon rehydration.

Dicotyledonous plants generally contain primary cell walls with about one-third each of cellulose, hemicellulose and pectin components (Brett and Waldron, 1996). In contrast, *M. flabellifolia* cell walls were found to contain approximately 45 %, cellulose 25 % hemicellulose and 30 % pectin. We propose that the vascular tissue and the ribs contributed to the large cellulose fraction observed. Analysis of the alkali insoluble pectin showed a marked increase in galacturonic acid content upon desiccation. This is interesting as it correlates with an immunocytochemical study of desiccation-induced changes in the cell wall of the resurrection plant *Craterostigma wilmsii* which showed that pectin epitopes increased upon desiccation (Vicré et al., 1999). A further biochemical study of the cell wall of *C. wilmsii* revealed no overall change in galacturonic acid content between states but did reveal a change in solubility of pectin polymers as determined by differential fractionation from hydrated and desiccated cell walls (Vicré et al., 2004b). In relation to *M. flabellifolia* the additional pectin material recovered might be due to an increased solublilisation of pectins from cell walls of desiccated plants. As proposed for *C. wilmsii* (Vicré et

al., 2004b), our data suggests that there is a change in the physical properties of the cell wall of *M. flabellifolia* following desiccation. In addition, an unusual abundance of arabinose, possibly in the form of arabinans, was observed associated with the pectic polymers. Precipitation of arabinogalactan-proteins using the Yariv reagent failed to reveal any major differences in quantity and composition between hydrated and desiccated leaf tissue. The presence of significant amounts of galacturonic acid present in the arabinogalactan-protein fraction may indicate a co-precipitation (and possible association) of arabinogalactan polymers with pectin These data do not rule out changes in quantity and composition of arabinogalactan-proteins that were not precipitated using the Yariv reagent. It is known for example that the Yariv reagent does not precipitate arabinogalctan polymers from Larch and small Hyp-arabinogalactan polysaccharides (Lamport et al., 2006). It has also recently come to light that rare arabinogalactan-proteins with small glycomodules that lack rhamnose residues may not precipitate with Yariv phenylglucosides (J. Xu et al., unpublished, cited in Lamport et al., 2006).

Our immunological data using the LM 6 antibody showed that arabinan epitopes were present in the primary cell walls of the leaf tissue, whereas the data using the JIM 13 antibody showed that the AGP epitopes were present in the cytoplasm adjacent to the plasma membrane. Vascular and sclerenchyma tissue normally contains thick secondary walls with high amounts of cellulose and lignin relative to pectin, and therefore should not contain much pectin-associated arabinose polymers. Since no arabinan epitopes were detected in vascular and sclerenchyma tissue using the LM 6 antibody, it is unlikely that these tissues contain high quantities of arabinose-containing polymers. The hemicellulose component of the cell wall of *M. flabellifolia*, the composition of which did not change upon desiccation, was found to be composed of a large amount of xylan and lesser amounts of xyloglucan polymers, which showed a high degree of acetylation as has been

shown in other dicotyledonous plants. Neither the composition nor the structure of the xyloglucan present changed upon desiccation.

The locations of the various polymers were investigated by immunocytochemistry. No major deviations from the normal patterns of distribution found in other plants were observed in *M. flabellifolia* leaves and no major change in the location of polymers between hydrated and desiccated material was noted. The presence of xyloglucan epitopes reinforces the biochemical analysis previously discussed. The arabinan epitopes were detected adjacent to the cytoplasm and not near the homogalacturonan rich middle lamella as would be predicted from the biochemical analysis performed. This pattern of distribution has been observed previously with the LM 6 antibody in other plant species (Willats et al., 1998; Orfila et al., 2001).

M. flabellifolia leaves were found to have an unusually large amount of arabinose present, presumably in the form of arabinan polymers. Arabinose polymers have been shown to be highly mobile (Foster et al., 1996; Renard and Jarvis, 1999) and to have a high water-absorbing capacity (Goldberg et al., 1989; Belton, 1997), properties ideally suited to cell wall rehydration. Our data showed no overall difference in the arabinose levels between hydrated and desiccated leaves suggesting that arabinose is constitutively synthesized and that the *M. flabellifolia* cell wall is continuously prepared for loss of water. This is interesting as a study of the cell wall response to hypersalinity, 0.4 M NaCl for two weeks, in the drought and salt resistant *Mesembryanthemum crystallinum* showed that some wall remodeling occurred but the major observation was a high concentration of arabinose, around 60 % of the non-cellulosic sugars, consistently present in the cell wall of both treated and non-treated plants (Galkina et al., 2001). It has recently been shown that arabinan polymers act as plasticizers increasing cell wall flexibility and diminishing strong interactions between homogalacturonan chains in pectin (Jones et al., 2003). Recently, potato

tuber tissue genetically engineered to be deficient in arabinan and galactan side chains in cell wall pectin was shown to be significantly more brittle to imposed stress, highlighting the critical role that these side chains play in controlling the biophysical properties of the cell wall (Ulvskov et al., 2005). In addition, arabinogalactan-polymers in the form of arabinogalactan-proteins are believed to function in cell lubrication (Yates et al., 1996) and recently have been proposed to act as cell wall plasticisers (Lamport, 2001; Lee et al., 2005). A similar role has been proposed for the stress response protein Hsp 12 in the yeast *Saccharomyces cerevisiae* (Motshwene et al., 2004; Karreman et al., 2005). Another possible role for a protein in wall flexibility was recently found in the resurrection plant *Craterostigma plantagineum,* where α-expansin mRNA was demonstrated to be upregulated in response to desiccation (Jones and McQueen-Mason, 2004). These findings might therefore indicate a role for arabinogalactan-proteins in enhancing cell wall flexibility. We believe that the high concentration of arabinose polymers associated with the pectin matrix in the *M. flabellifolia* cell wall is likely to be one of the major components that allow the wall to desiccate and fold without any apparent damage.

CHAPTER FIVE

CONCLUSIONS AND FUTURE PROSPECTS

This thesis has investigated the mechanisms implicated in the desiccation tolerance of the resurrection plant *Myrothamnus flabellifolia*. The main focus of the research considered the role that polyphenols and the cell wall present in the leaves of *M. flabellifolia* played in the response of this unique plant to desiccation and its associated stresses. It was revealed that *M. flabellifolia* appears to employ both constitutive and desiccation-induced mechanisms to achieve desiccation tolerance. As observed in other angiosperm resurrection plants, *M. flabellifolia* utilises a prevention of damage strategy in response to desiccation (Farrant, 2000; Farrant et al., 2003). The general stress countermeasures mounted by this plant include those against mechanical (e.g. cell wall rupture), photo-oxidative (e.g. chlorophyll bleaching) and metabolic stress (e.g. cell osmotic imbalance). It appears that achieving tolerance to desiccation necessitates the evolution of a multi-faceted strategy in order to be successful.

The first part of this thesis involved the study of the anatomical and ultrastructural adaptations of the leaves of *M. flabellifolia* as well as their response to desiccation. In addition, an analysis of biochemical components such as saccharides, proteins, pigments and metal ions present in the leaves of *M. flabellifolia* before and after desiccation was carried out. It was observed that the general leaf anatomy and morphology of *M. flabellifolia* changes upon desiccation. The leaves which resemble a fan close their ribs together upon desiccation minimising the exposure of the leaf to the environment. Constitutive protective measures such as a thick waxy coating likely protects the leaves from excess light and uncontrolled rehydration as well as a network of vascular and sclerenchyma tissue which may help to maintain leaf shape during desiccation. Desiccation-induced measures including anthocyanin and saccharide accumulation with the

concomitant removal of excess metal ions help to protect the leaves from photo-oxidative and metabolic stress respectively. Ultrastructural observations revealed desiccation-induced tissue compaction and internal wax networks suggesting that *M. flabellifolia* leaf tissue is well adapted to desiccation. *M. flabellifolia* is homoiochlorophyllous and retains photosynthetic proteins and pigments during desiccation. *M. flabellifolia* is also amphistomatic with leaf stomata arranged in unique patterns on both the adaxial and abaxial surfaces. It would seem that *M. flabellifolia* employs a prevention of damage strategy upon desiccation coupled with a rapid recovery of photosynthesis and associated metabolism when rehydration occurs.

The second component of this thesis was an investigation into the polyphenols present in the leaves of *M. flabellifolia* and their functional role in this plant, especially in relation to desiccation tolerance. A further study of polyphenol composition and genetic differences at the population level in *M. flabellifolia* was also conducted. This study has shown that the main polyphenols present in the leaves of *M. flabellifolia* are galloylquinic acids comprised of 3, 4, 5 tri-O-galloylquinic acid as their central core molecule. These molecules are present at high concentrations ranging from 25 to 50 % of the leaf dry weight in both hydrated and desiccated leaves. An ultrastructural study revealed that polyphenols were located in the central cell vacuoles of predominately the mesophyll cells in both the hydrated and desiccated states. These molecules had been shown to protect liposomes against desiccation and were shown here to protect lipid mixtures against oxidation. 3, 4, 5 tri-O-galloylqinic acid possesses marked antioxidant ability in comparison to known commercial antioxidants. We suggest that *M. flabellifolia* may utilise galloylquinic acids to protect its cell structures against the damaging effects of desiccation and associated oxidative damage. Furthermore, galloylquinic acids possess known medicinal properties (Neszmelyi et al., 1993; Bokesch et al., 1996; Onayade et al., 1996) which may explain the use of *M. flabellifolia* in traditional medicine formulations (Van Wyk et al., 1997). An additional systematic study of *M. flabellifolia* revealed consistent differences in galloylquinic acid

and genetic composition between populations occurring in South Africa versus those in Namibia. These differences suggest that *M. flabellifolia* may not be a uniform species across its range and that Namibian populations may be able to remain in a quiescent (desiccated) state for longer periods than those found in South Africa.

The final section of the thesis involved a thorough microscopic, biochemical and immunocytochemical study of the leaf cell wall of *M. flabellifolia* and its response to desiccation. The study revealed that *M. flabellifolia* responds to desiccation by folding most of its leaf cell walls. Only the thick-walled vascular and sclerenchymous cells do not fold and may act to support the surrounding folded tissue during desiccation. The biochemical and immunocytochemical analyses revealed that desiccation caused minimal changes in the cell wall composition and the spatial distribution of wall polymer epitopes. The *M. flabellifolia* resembled a dicotyledonous wall by being comprised of a third each of pectin, hemicellulose and cellulose (Brett and Waldron, 1996). The hemicellulose component, which did not change upon desiccation, consisted of high amounts of xylan with lesser amounts of xyloglucan. The pectin matrix underwent some desiccation-induced changes but the most conspicuous observation was a high concentration of arabinose, likely in the form of arabinose polymers, associated with the pectin in both hydrated and desiccated states. We propose that the highly mobile arabinose polymers, associated with the pectin matrix, impart the necessary flexibility to the *M. flabellifolia* leaf cell wall to allow it to desiccate and fold without any apparent damage.

This work provides a foundation for future studies on the desiccation tolerance of *M. flabellifolia.* In order to gain a better understanding of the mechanisms employed by this species in countering desiccation and associated stresses, a number of avenues of research are now possible. These avenues include:

1. Performing a more thorough molecular and proteomic study of changes in the respective mRNA transcript and protein levels in *M. flabellifolia* leaves between desiccated and hydrated states. This study is now possible as this thesis has provided a method for protein extraction and analysis from *M. flabellifolia* leaves. In addition, this thesis has confirmed previous methods (Koonjul et al., 1999) to successfully reverse the phenolic inhibition of molecular manipulations of nucleic acid extracts from the leaves of this plant.

2. Undertaking a more thorough investigation of the polyphenols (galloylquinic acids) isolated from the leaves of *M. flabellifolia*. These investigations could include determining their efficacy in stabilising cellular structures *in vivo* in addition to the previously discussed *in vitro* demonstrations of protecting artificial membranes (liposomes) against desiccation and oxidation. Non-desiccation related research might involve ascertaining the utility of using galloylquinic acids in medicinal formulations for treatment against HIV, cancer and respiratory diseases. The systematic study of phenolic and genetic variation amongst populations of *M. flabellifolia* has highlighted the feasibility and necessity of performing a more thorough systematic analysis of the genus *Myrothamnus* across its African and Madagascan range. This is important as longevity in the desiccated state appears to be correlated with the geographical distribution of *M. flabellifolia*.

3. Investigating the role of arabinose polymers and their possible role in providing cell wall flexibility to this species in response to desiccation. More sophisticated techniques, requiring specialised equipment and expertise, such as atomic force microscopy (AFM) and solid state nuclear magnetic resonance (NMR) spectroscopy could be extremely useful in testing our previously discussed 'arabinose polymer' hypothesis.

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