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**The effect of drying rate on the resurrection species
Craterostigma wilmsii (homoiochlorophyllous) and *Xerophyta
humilis* (poikilochlorophyllous)**

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**A dissertation presented for the degree of
Master of Science in Botany**

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PREFACE

Assoc. Prof. Jill Farrant supervised this thesis. It is hereby declared that this thesis, submitted for the degree of Master of Science at the University of Cape Town, is the result of my own investigation, except where the work of others is acknowledged.

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February 2001

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LIST OF ABBREVIATIONS

<i>18S</i>	<i>18S</i> rRNA
AP	Ascorbate peroxidase
asc.	Ascorbate
<i>B. hygroskopica</i>	<i>Boea hygroskopica</i>
<i>B. nitida</i>	<i>Borya nitida</i>
β -car.	β -carotene
Chl.	Chlorophyll
<i>C. nanum</i>	<i>Craterostigma nanum</i>
<i>C. plantagineum</i>	<i>Craterostigma plantagineum</i>
<i>C. wilmsii</i>	<i>Craterostigma wilmsii</i>
DIG	Digoxygenin
DNA	Deoxyribonucleic acid
dsp	Drought stress proteins
DW	Dry weight
F ₀	Ground state (initial) fluorescence
F _M	Maximal fluorescence
F _V	Variable fluorescence ($F_V = F_M - F_0$)
F _V /F _M	Measure of quantum efficiency of PSII
Glc-6-P	D-glucose-6-phosphate
H ₂ O ₂	Hydrogen peroxide
HEPES	4-(2-Hydroxyethyl)-1-piperazine-ethane sulphonic acid
LEA	Late embryogenesis abundant

<i>lhc</i>	Light harvesting complex (chlorophyll a/b binding protein)
<i>M. flabellifolius</i>	<i>Myrothamnus flabellifolius</i>
mRNA	Messenger ribonucleic acid
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NaOH	Sodium hydroxide
O ₂ ⁻	Superoxide free radical
OH	Hydroxyl group
PSII	Photosystem II
<i>rbcL</i>	Large subunit of Rubisco
<i>rbcS</i>	Small subunit of Rubisco
RNA	Ribonucleic acid
RNase	RNA-degrading enzyme
rRNA	Ribosomal ribonucleic acid
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RWC	Relative water content
³⁵ S	³⁵ Sulphur (radioactive isotope of sulphur)
³⁵ S-cys	³⁵ S-labelled cysteine
³⁵ S-met	³⁵ S-labelled methionine
SOD	Superoxide dismutase
<i>T. ruralis</i>	<i>Tortula ruralis</i>
UV	Ultra-violet (light)
Vis.	Visible (light)
<i>X. humilis</i>	<i>Xerophyta humilis</i>

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ABSTRACT

Craterostigma wilmsii and *Xerophyta humilis* are angiosperm resurrection species that employ different strategies in dealing with desiccation and the related light stress. *C. wilmsii* is homoiochlorophyllous in that it retains its chlorophyll throughout dehydration, while *X. humilis*, a poikilochlorophyllous resurrection plant, breaks down its photosynthetic apparatus during drying. Both species are classified as modified desiccation-tolerant plants as they are thought to rely primarily on protection of cellular components during drying to survive desiccation. The time taken for this protection is thought to preclude recovery after rapid drying. Thus the response of whole *C. wilmsii* and *X. humilis* plants to rapid dehydration was investigated in terms of survival and the effect on protective mechanisms.

In the first part of this study, the response of the photosynthetic apparatus to rapid drying was assessed by studying the quantum efficiency of photosystem II, changes in photosynthetic pigments, the presence of transcripts of major photosynthetic proteins and the cellular ultrastructure during dehydration and/or rehydration. Cellular ultrastructure, together with solute leakage from cells, was also used as an indication of the degree of damage incurred during slow and rapid drying. In addition, the effect of rapid drying on the accumulation of the protective molecule, sucrose, was determined.

Of the two species, only *C. wilmsii* was able to survive rapid drying. Although the protective mechanisms of both species were affected by an increased drying rate, with reduced sucrose accumulation and increased injury to cells, *C. wilmsii* was able to limit the damage and recover. Rapid dehydration prevented *X. humilis* from carrying

out complete dismantling of photosynthetic components. It is proposed that the light stress experienced as a result, and the impaired protection during drying, prevented survival of this species. *X. humilis* has morphological features which slow water loss and therefore protection can be more gradually induced. *C. wilmsii* is a plant with few features to retard water loss and thus rapidly instituted protection against desiccation and light stress may be necessary in order to survive. In addition, evidence suggests that there may be some repair, during rehydration, of the damage observed during dehydration.

In the second part of this study, the dependence of naturally and rapidly dried *C. wilmsii* on *de novo* transcription and translation during rehydration was compared with that of *X. humilis*. Recovery of leaf explants was determined by examining the quantum efficiency of photosystem II, changes in photosynthetic pigments and the subcellular organization after rehydration in water and metabolic inhibitors.

Unlike slowly dried *X. humilis*, which requires new protein synthesis on rehydration to re-establish the photosynthetic apparatus, slowly dried *C. wilmsii* requires no new transcription or translation during rehydration in order to recover. However, rapidly dried *C. wilmsii* does not survive without *de novo* mRNA and protein production on rewetting. Without inhibition (*ie.* water-rehydrated tissue), rapidly dried *C. wilmsii* survives, indicating that there is an induction of repair mechanisms during rehydration which enables the species to recover from rapid drying.

Thus, although *C. wilmsii* does rely almost entirely on protection during natural drying, it apparently has the ability to repair if protection is inadequate and damage is incurred. This may indicate a need to re-evaluate the general classification of all angiosperms as modified desiccation-tolerant plants.

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CHAPTER 1

INTRODUCTION

Imagine life without water. It makes up the greater part of plant and animal cell volume and indeed, life itself began in the medium of water. Although it covers three quarters of the Earth's surface, certain areas of land can experience severe water shortages for long periods of time. Most animals go to extreme lengths to avoid this very situation, travelling vast distances to find water sources to keep them alive. Plants are disadvantaged in that they cannot search for water beyond their immediate surroundings. Thus, when water becomes limited in their environment, plants may not be able to avert the loss of turgor in their cells. This loss of water is termed "water deficit stress", or simply "water stress" (review by Levitt, 1980), and it has an extreme effect on these organisms.

1. The effect of water stress on plants

1.1. Mechanical stress

When water is lost in significant quantities from plant cells, the immediate stress experienced as turgor is lost by the plant, is mechanical (Iljin, 1957; Levitt, 1980). As water leaves the cell, it begins to lose volume, resulting in plasmolysis, where the cell contents, in effect, "deflate" and the plasmamembrane withdraws from the cell wall, remaining attached only at the plasmodesmata. Since the cell wall is not as elastic as the cell membranes, this collapse places the plasmamembrane and tonoplast under tension, which can cause tearing (Leopold *et al.*, 1981; review by Stewart, 1990;

review by McKersie and Leshem, 1994). In addition, the extreme changes in volume resulting from the influx of water upon rehydration may exacerbate this damage (McKersie and Leshem, 1994; review by Oliver and Bewley, 1997).

Indeed, the very structure of the plasmamembrane is a consequence of the aqueous environment of the cell – the hydrophobic phospholipid tails are repelled by water, forming the bi-layer depicted in Figure 1.1A.

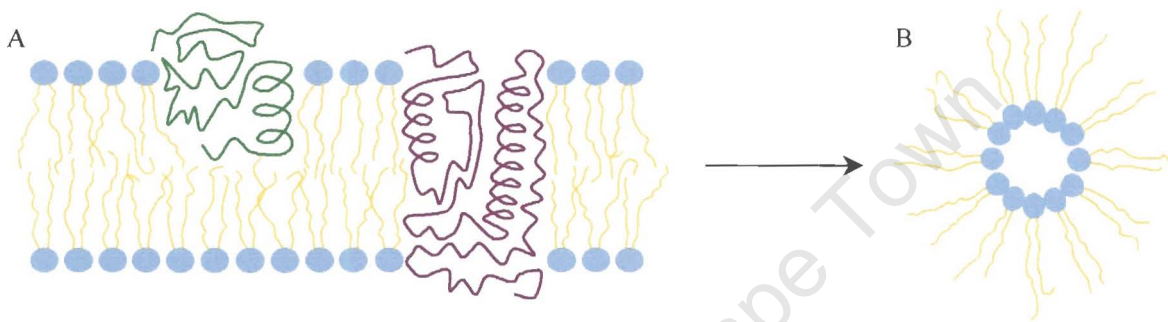


Figure 1.1. **(A)** Liquid crystalline structure of phospholipid membranes. Blue circles represent phospholipid polar head groups, while the fatty acid chains are shown as yellow lines. Membrane proteins are depicted by green and purple lines (from Singer and Nicolson, 1972); **(B)** Lipid micelle.

It has been found in animal cells that when the water medium of the cell is no longer present, there is a disruption of the bi-layer, the phospholipids instead forming micelles (Fig. 1.1B) (review by Simon, 1974; Levitt, 1980). However, microscopical observations suggest that this degree of membrane disruption does not occur upon desiccation in certain plant tissues, *viz.* some seeds (McKersie and Stinson, 1980; Seewaldt *et al.*, 1989). This is attributed to the presence of “bound” water – water that is not lost even during desiccation stress (Vertucci and Farrant, 1995; Seewaldt *et al.*, 1989; review by Leopold, 1990). Whether or not this disruption occurs in other plant tissues, it has been shown that severely water-stressed plants show a marked increase

in solute leakage, which is ascribed to membrane damage (Simon, 1974; Leopold *et al.*, 1981).

In addition, the tearing of either the plasmamembrane or the tonoplast causes a release of hydrolytic enzymes, which, upon loss of their compartmentalization, can autolyse cytoplasm and so exacerbate the damage already incurred in the cell (Salisbury and Ross, 1992). This damage inevitably disrupts, often permanently, the normal cellular metabolism.

1.2. Metabolic stress

Water, as has already been mentioned, constitutes the majority of the cell environment. It is the transport medium and solvent for all cellular reactions and processes (review by Bohnert *et al.*, 1995). Thus, when water is scarce, normal regulation cannot be maintained and metabolism ceases to operate effectively.

A loss of water from the cells also results in a concentration of solutes (review by Bray, 1997). Such a concentration of ions may become toxic to the cell, disrupting membranes and possibly causing protein denaturation (Garber and Steponkus, 1976; Heber *et al.*, 1979; review by Fitter and Hay, 1983). In addition, enzymes, which operate in specific optimal conditions, are susceptible to inhibition in such an environment (Fitter and Hay, 1983).

The dearth of water in the cell environment itself interferes with protein structure. Proteins comprise both hydrophobic and hydrophilic amino acids, and due to hydrophobic interaction with water, fold into characteristic conformations (Campbell, 1991). Without these interactions, the tertiary structure of proteins is lost and the proteins are denatured (Bray, 1997).

Another factor in metabolic damage during water stress is the degradation of nucleic acids, such as RNA (ribonucleic acid) and DNA (deoxyribonucleic acid). Kessler (1961) found increased RNase activity in water stressed leaves and hypothesized that it was due to the enzyme's release from the bound state. Similar findings regarding RNA degradation and RNase activity have been reported elsewhere (Levitt, 1980). However, it is likely that a major factor in the destruction of nucleic acids and other molecules is that of free radical activity. This is particularly marked at intermediate water contents, when metabolism appears to be unregulated and injurious free radical-mediated reactions occur (Vertucci and Farrant, 1995; review by Pammenter and Berjak, 1999). Such oxidative stress is inherent during a water stress in the natural environment.

1.3. Oxidative stress

Oxidative stress involves the formation of free radicals, particularly active oxygen species. It is the deleterious action of these molecules that can cause large-scale damage to cell structures (review by Smirnov, 1993). Such radicals may be formed in a number of sites (eg. mitochondria, endoplasmic reticulum, plasmamembranes) (McKersie and Leshem, 1994), however, in vegetative tissues of plants, it is thought that the most prevalent cause of oxidative stress during periods of water limitation, is the light-chlorophyll interactions which occur in the chloroplast (Kranner and Grill, 1997; Tuba *et al.*, 1998; Farrant, 2000).

As water becomes limited, the stomata close, preventing further water loss (review by Mansfield and Atkinson, 1990), but also limiting the carbon dioxide available for fixation by photosynthesis. This results in a decrease in quantum efficiency and an

excess of excitation energy at the reaction centres of the photosynthetic machinery (Hanson and Hitz, 1982; Stuhlfauth *et al.*, 1990). In this situation, NADP⁺ (electron acceptor in photosynthesis) becomes limited and ferredoxin selectively reduces oxygen instead, producing the highly reactive superoxide (O₂⁻) molecule (Smirnov, 1993; McKersie and Leshem, 1994). This is not the only free radical created. The decreased efficiency of photosynthesis (exacerbated by the aforementioned damage to membranes and proteins) means that activated chlorophyll may directly reduce oxygen, creating singlet oxygen (*O) (Seel *et al.*, 1992; Smirnov, 1993). The site of water splitting in photosystem II (PSII) may, too, give rise to the formation of superoxide and hydrogen peroxide (H₂O₂) (Smirnov, 1993; McKersie and Leshem, 1994).

Added to this, the above-mentioned free radicals, in reactions with ferric salts and each other, can generate hydroxyl radicals ([•]OH – an extremely powerful oxidising agent) (equations 1 & 2) and organic radicals ([•]ROH and [•]R *ie.* organic molecules with an unpaired electron) (equations 3 & 4).



Reactions amongst these molecules can, in turn, cause cross-linking of organic molecules (equation 5) and a chain reaction of destruction of organic molecules and

free radical formation (equations 6 & 7) (McKersie and Leshem, 1994). The latter is particularly destructive to the membranes. So-called lipid peroxidation is a chain reaction whereby a phospholipid reacts with a $\cdot\text{OH}$ radical, eventually creating a peroxy radical ($\text{ROO}\cdot$) (equation 6). The peroxy radical then reacts with an adjacent phospholipid, transforming it into another organic radical and in the process, becoming an unstable lipid hydroperoxide (equation 7) (McKersie and Leshem, 1994).



Radicals also attack proteins, causing fragmentation, cross-linking and altered electrical charge (McKersie and Leshem, 1994), while nucleic acids are subject to base degradation, strand breakage and cross-linkage to proteins due to free radical-mediated reactions (Imlay and Linn, 1986). It is because these radicals create other radical species and can be reformed indefinitely that they represent such a hazard to plants. Thus without adequate counter-measures, free radicals can create a disproportionate amount of damage to membranes and organelles in relatively low concentrations. Similarly to mechanical stress, it seems that when water is once again available following dehydration, free radical activity increases (Sgherri *et al.*, 1994).

2. Adaptations to cope with water deficit

In light of the above descriptions of the effects of water stress, it is essential that plants living in arid or xeric environments are equipped with means of dealing with water deficit. Escaping periods of water limitation is probably the simplest means of surviving a drought. Ephemeral plants evade water stress by completing their life cycle during the rainy season and thus escape water stress altogether (Levitt, 1980).

Alternatively, plants may adapt to drought in order to survive a paucity of water. This can be accomplished in two ways. Firstly, plants may avoid water stress, either by averting a loss of water during drought conditions or by taking up more water than is lost. Succulence is an example of the conservation of water, while increasing root biomass in order to “search” for water or decreasing the water potential of tissues to boost water absorption allows plants to replace lost water (Levitt, 1980).

The second strategy in dealing with drought is to tolerate water stress. These drought-tolerant plants are able to survive the loss of turgor and associated damage. There is a specialised and diverse group of plants, which exhibit extreme tolerance of drought. These are the desiccation-tolerant or resurrection plants, which have the ability to revive from an air-dry state (review by Gaff, 1989).

3. Resurrection plants

Resurrection plants encompass members of almost all plant classes, barring only the gymnosperms (review by Oliver, 1996). In light of the stresses experienced as a consequence of desiccation, tolerance requires either a protection against the stresses, repair of the resulting damage or a combination thereof. It has been suggested that lower orders (bryophytes, lichens and algae) chiefly repair damage associated with water loss, while higher orders (mainly pteridophytes and angiosperms) largely protect against desiccation damage (Oliver *et al.*, 1997, 1998; Farrant, 2000). The majority of studies on the repair occurring during rehydration have consequently focussed on lower plant taxa, particularly the mosses (Oliver and Bewley, 1997). Mosses, which lose water very rapidly in comparison to angiosperms, are thought to depend to a large extent on repair of desiccation damage, synthesizing several novel "rehydration" proteins or rehydrins in the first few hours of recovery (review by Bewley and Oliver, 1992). Synthesis of rehydrins in pteridophytes is slower and less prolific (Oliver and Bewley, 1997). Angiosperms, which have been termed modified desiccation-tolerant plants since their loss of water is slower, utilize a greater degree of protection during dehydration (Oliver, 1996). Since the species involved in this study are angiosperms, the following discussion will focus on the protective mechanisms induced in resurrection plants during drying to survive desiccation.

3.1. Avoidance of membrane rupture

The tension exerted upon the membrane during water loss (discussed earlier) is a result of the relative inelasticity of the cell walls (Levitt, 1980). Certain desiccation-tolerant plants circumvent this by collapsing or folding their cell walls (review by

Farrant and Sherwin, 1998; Vander Willigen *et al.*, 2001). Particularly noted for this folding are the members of genus *Craterostigma* (Sherwin, 1995; Vicré *et al.*, 1999).

The mechanical tension that causes tonoplast rupture can be minimised by subdivision of the vacuoles into numerous small vesicles, a phenomenon exhibited in several desiccation-tolerant species (Gaff, 1989; Farrant and Sherwin, 1998). In addition, some desiccation-tolerant plants appear to fill vacuoles with as yet unidentified substances (Farrant, 2000). *Xerophyta viscosa* and *X. humilis* maintain large vacuoles in the dry state, something which may be possible due to these vacuolar contents. Farrant and Sherwin (1998) have proposed that this not only stabilizes the vacuole, but provides a back-pressure against the cell wall, which prevents plasmamembrane collapse.

In addition to these mechanical modes of membrane protection, resurrection plants use chemical means to preserve membrane integrity and avoid metabolic disruption, which are discussed below.

3.2. Avoidance of metabolic disruption

3.2.1. Sugars and compatible solutes

It has been noted that all desiccation-tolerant plants exhibit a substantial increase in sugars during drying, particularly sucrose (Bianchi *et al.*, 1991; Bianchi *et al.*, 1993; Müller *et al.*, 1997; Ghasempour *et al.*, 1998). However, it has not yet been established what part they play in desiccation tolerance. There has been much study centering on the role of sugar accumulation in desiccation-tolerant (orthodox) seeds, which indicates that sugars play a role in membrane stabilization, as a replacement for water. Specifically, oligosaccharides such as sucrose, raffinose and stachyose are thought, in

effect, to substitute for water by interacting with membrane phospholipids, preventing fusion, phase transitions and phase separation (Hoekstra *et al.*, 1997; review by Kermode, 1997; review by Scott, 2000). The mechanism by which this interaction is accomplished is not yet known. Sugars were thought to bind to head groups of phospholipids, thus maintaining the bi-layer structure (review by Scott, 2000). However, Hoekstra *et al.* (1997) have shown that this binding is ineffective and have suggested instead, that amphipathic compounds may migrate into membranes, increasing stability and maintaining membrane integrity.

A further theory for the role of sugars suggests that the increase in sugar may cause vitrification of the cell environment, forming a stable glass that places the cells in a metabolic stasis (Koster and Leopold, 1988; review by Ingram and Bartels, 1996; Hoekstra *et al.*, 1997). Much more than mere membrane protection, this freezing of the metabolism is likely to benefit the plant by preventing the deleterious reactions involved in oxidative stress.

However, this theory of water replacement has yet to be proven and there is mounting evidence that sugar accumulation alone does not accord desiccation tolerance. Some sensitive tissues show comparable amounts of sugar, while other, tolerant species have low sugar concentrations when experiencing water stress (Farrant *et al.*, 1993). This suggests that sugars alone are insufficient to confer tolerance (Oliver and Bewley, 1997). A suggestion has been put forward that it is not the accumulation of oligosaccharides that is important, but rather the removal of monosaccharides (McKersie and Leshem, 1994; Farrant and Sherwin, 1998). Monosaccharides promote respiration (Leprince *et al.*, 1992), thus the elimination of these molecules (which combine to form oligosaccharides) removes respiratory substrates and thereby may place metabolism in stasis. The lack of monosaccharides

may also prevent deleterious Maillard reactions, a cross-linking between glucose and proteins that causes inactivation of the proteins (McKersie and Leshem, 1994; Farrant and Sherwin, 1998).

Sugars may also play a part in vacuolar stabilization as some of the unknown compounds that fill the vacuoles of some resurrection species, as mentioned previously. If, however, sugars are involved in protection of membranes and vacuoles, it is likely that they do so in combination with other compounds, such as proteins and “compatible solutes”, which have been found to accumulate in response to water and other stresses (Yancey *et al.*, 1982; Oliver and Bewley, 1997).

Compatible solutes, so-called because they do not interfere with normal metabolism, comprise uncharged compounds such as polyols (*eg.* sorbitol, mannitol, *myo*-inositol), betaines, polyamines and amino acids (Bohnert *et al.*, 1995). Much work has focussed on correspondence between the accumulation of these osmolytes (particularly proline, sorbitol and glycine betaine) and desiccation tolerance (Ahmad *et al.*, 1979; Tymms and Gaff, 1979; Schwab and Heber, 1984; review by Franco and Melo, 2000). In addition to the contribution to membrane stabilization and vitrification, these solutes have two further conceivable functions. Firstly, they may facilitate the retention of water by osmotic adjustment (Yancey *et al.*, 1982; Gaff, 1989; Bohnert *et al.*, 1995). Secondly, Floyd and Nagy (1984) have shown that proline forms long-lived adducts with hydroxyl radicals, which indicates that compatible solutes may possess free radical scavenging capabilities (Bohnert *et al.*, 1995). Although less work has focussed on sugars and compatible solutes in vegetative resurrection plants, it is thought that they play a similar role to that in orthodox seeds (Ingram and Bartels, 1996; Hoekstra *et al.*, 1997; Oliver and Bewley, 1997).

3.2.2. Proteins

There are several groups of proteins that, although present during normal metabolism, are upregulated in response to a variety of stresses, including water stress. Their capacity to confer desiccation tolerance has therefore been examined (review by Welch, 1993; Bewley and Oliver, 1992).

The accumulation of one of these groups was first observed in fruit fly metabolism, in response to a heat shock (Welch, 1993). These “heat shock proteins”, or stress proteins, were subsequently found in a number of organisms, plant tissue included, and were upregulated in response to a variety of stresses.

The principal function appears to be protein maintenance, the stress proteins acting as “molecular chaperones”, refolding denatured proteins and keeping incorrectly folded proteins out of cellular metabolism (Welch, 1993; review by Hartl, 1996; Rüdiger *et al.*, 1997). This enables the maintenance of metabolic processes, such as photosynthesis, in cells experiencing stress (Schroda *et al.*, 1999). In addition, some of these chaperonins may prevent aggregation of denatured proteins (Hartl, 1996), functioning similarly to another of the stress proteins, ubiquitin, which “collects” denatured proteins and transports them for resynthesis (Vierstra, 1993; Von Kampen *et al.*, 1995; Ingram and Bartels, 1996).

A further group of proteins, some of which may have similar functions to the stress proteins, were first described in seeds (Ingram and Bartels, 1996). These late embryogenesis abundant (LEA) proteins accumulate in seeds before and during maturation drying (Galau *et al.*, 1986; Vertucci and Farrant, 1995; Farrant and Sherwin, 1998). A family of LEA-like proteins called the dehydrins have been observed to accumulate in resurrection plants during dehydration (Schneider *et al.*, 1993; review by Close, 1996). Due to the hydrophilic nature of these proteins, Dure *et*

al. (1989) have suggested that they may play a role in water replacement or enhance water binding in the cell.

A number of other functions have been proposed for the LEA proteins and dehydrins. These include chaperonin- and ubiquitin-like roles in protein maintenance (Russouw *et al.*, 1997), as well as functioning as water channels (see below) (review by Bray, 1993; Dure, 1993a). The sequestration of ions (preventing toxicity), or the possible neutralization of ions by charged amino acids within the protein structure may also be carried out by the LEA's (Dure, 1993b; Schneider *et al.*, 1993). However, the exact mechanism by which these molecules aid desiccation tolerance has yet to be elucidated.

Another protein type that may play a role in preventing excessive damage to cell contents are the water channel proteins, or aquaporins. These are members of the major intrinsic superfamily of proteins that regulate the flux of water across membranes (review by Johansson *et al.*, 2000). It has been suggested that some of these, *eg.* γ -TIP (tonoplast intrinsic protein), control the water content of the vacuole and may be involved in osmotic adjustment in stressed cells (Bray, 1993).

Other proteins are upregulated in response to a specific stress, for example, several enzymes that function as antioxidants (see below).

3.3. Avoidance of oxidative stress

3.3.1. Avoidance of free radical formation

Because of the physiological problems associated with photosynthesis during water stress (see section 1.3), light represents a hazard for desiccation-tolerant plants (Muslin and Homann, 1992). Although resurrection plants do have means of dealing with free

radicals formed as a consequence of light stress, oxidative stress in general is avoided to some degree by desiccation-tolerant species (Farrant, 2000). Hallam and Capicchiano (1974) noted a general degradation of mitochondrial membranes, presumably removing a source of free radical formation. Bianchi *et al.* (1992) have found that an inhibitor of lipoxygenase, the activity of which results in lipid hydroperoxide formation, accumulates in the resurrection plant, *Craterostigma plantagineum*. However, the first defence against oxidative stress is simply to avoid the deleterious light-chlorophyll interactions that are the major cause of free radical formation. The strategy employed by resurrection plants to avoid photo-oxidative stress is used to classify them into two groups, *viz.* poikilochlorophyllous or homoiochlorophyllous.

Poikilochlorophyllous resurrection plants (or “chlorophyll-losers”) break down all chlorophyll and actively dismantle the thylakoid membranes of the chloroplasts (Gaff, 1977; Tuba, *et al.*, 1993a,b; Sherwin and Farrant, 1998; Farrant, 2000). Without the photosynthetic machinery, the reactions occurring in the chloroplast that create free radicals cannot occur. Upon rehydration, the photosynthetic apparatus is reconstituted and photosynthesis is resumed (Tuba *et al.*, 1993a,b; Sherwin and Farrant, 1996; Farrant, 2000). In order to accomplish this, it is likely that repair proteins are synthesized by the plants during rehydration (Oliver and Bewley, 1997).

Homoiochlorophyllous plants (“chlorophyll-retainers”) are those that retain chlorophyll during drying (Gaff, 1977; Bewley, 1979). Instead, the danger of chlorophyll-light interaction is evaded by “hiding” the chlorophyll. Curling or rolling of the leaves is recognised as an important mechanism to avoid light stress (Muslin and Homann, 1992; Lebkuecher and Eickmeier, 1993). *Craterostigma* sp. for example, curl all their leaves, so that only the abaxial surface of the outer whorl of leaves is

exposed to light and the inner leaves are completely shaded (Sherwin and Farrant, 1998; Farrant, 2000). In addition, leaves may accumulate anthocyanins, red-purple pigments that reflect those wavelengths absorbed maximally by chlorophyll. Sherwin and Farrant (1998) have suggested that these pigments act as a sunscreen, masking the chlorophyll during periods of possible light stress.

Another group of pigments, the xanthophylls, also aid photoprotection (Horton *et al.*, 1994; review by Gilmore, 1997). The xanthophylls (especially the violaxanthin-antheraxanthin-zeaxanthin cycle) serve to dissipate excess excitation energy and thus prevent photodamage (review by Demmig-Adams and Adams, 1996).

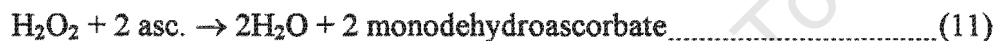
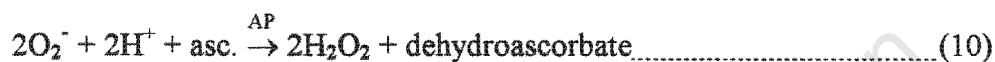
Despite these protective mechanisms, it is inevitable that free radical formation will occur as plants lose water. In addition to photo-oxidative injury, some angiosperm resurrection plants continue respiring until relatively low water contents (Tuba *et al.*, 1997, Farrant, 2000). Farrant (2000) has proposed that this provides the energy necessary to carry out the protective mechanisms needed for survival, however, this also creates a greater potential for free radical formation. Antioxidant enzymes and compounds are therefore essential as a means of coping directly with these radicals.

3.3.2. Antioxidants

The antioxidants comprise a large variety of compounds that interact with free radicals to remove them from the cell environment and lessen such damaging reactions as lipid peroxidation (Dhindsa and Matowe, 1981; review by Larson, 1988; McKersie and Leshem, 1994). The enzyme superoxide dismutase (SOD) reduces superoxide radicals (equation 8), while catalases and peroxidases rid the cell of hydrogen peroxide (equation 9) (Larson, 1988; Seel *et al.*, 1992; review by Scandalios, 1993).

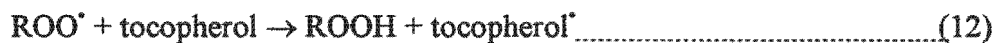


Superoxide is also the target of ascorbate (asc.), which removes hydrogen peroxide as well in a reaction catalyzed by the enzyme ascorbate peroxidase (AP) (equations 10 & 11) (Seel *et al.*, 1992; McKersie and Leshem, 1994).



Ascorbate is transformed into dehydro- and monodehydroascorbate due to these reactions and is therefore recycled either by spontaneous dismutation or by the enzymes dehydro- or monodehydroascorbate reductase acting in combination with glutathione (McKersie and Leshem, 1994; Sgherri *et al.*, 1994). Similarly, glutathione reduces several free radicals and is recycled by glutathione reductase (Sgherri *et al.*, 1994).

Tocopherol compounds, particularly α -tocopherol, donate an electron to peroxy radicals. The resultant tocopheroxy radical (tocopherol $^{\cdot}$) is reduced by ascorbate (equations 12 & 13) (McKersie and Leshem, 1994).



The carotenoids (mainly β -carotene (β -car.)) also scavenge peroxy radicals, as well as quenching triplet chlorophyll, a state of chlorophyll which leads to singlet oxygen formation (equations 14 & 15) (Larson, 1988; McKersie and Leshem, 1994).



Anthocyanin pigments are also thought to possess antioxidant capabilities, along with other flavonoids, some phenolic acids and alkaloids (Larson, 1988).

The level and activity of many of these compounds have been shown to increase in numerous resurrection plants in response to dehydration (Farrant and Sherwin, 1998) and likely play an important role in the tolerance of desiccation.

4. Study aims

As mentioned previously, distinctions have been made between “true” desiccation-tolerant plants and modified desiccation-tolerant plants. Typical desiccation-tolerant plants (comprised of the lower plant orders such as mosses and algae) lose water rapidly and rely largely on repair of desiccation-related damage on rehydration (Oliver, 1996). Those species classified as modified desiccation-tolerant plants (primarily comprised of the higher plant orders, viz. ferns, fern allies and angiosperms), however, place greater emphasis on protection during drying (Oliver and Bewley, 1997). It has been suggested that modified desiccation-tolerant species require a slower rate of water loss to allow the protective mechanisms to be instituted and that rapid drying would, thus, prevent survival (Gaff and Loveys, 1984; Bartels *et al.*, 1990; Oliver *et al.*, 1998; Tuba *et al.*, 1998). However, very little study has focussed on the effect of drying rate on the survival and protective mechanisms of angiosperm species, and no drying rate experiments have been performed on whole plants (as opposed to detached leaves or branches).

The aim of this study was to investigate the effect of rapid drying on the protective mechanisms and survival of two angiosperm resurrection plants which employ different strategies (homoiochlorophylly vs. poikilochlorophylly) in dealing with desiccation. These species were *Craterostigma wilmsii* Engl. (homoiochlorophyllous) and *Xerophyta humilis* (Bak.) Dub. and Schinz (poikilochlorophyllous), both of which grow in similar climates and experience like environmental conditions. Both species (pictured in Fig. 1.2 in the hydrated and dry states) are small, herbaceous plants and comparable in size. Due to their small size, it was possible to conduct drying rate experiments upon the whole plants.

The response of the two species to slow (*i.e.* natural) and rapid dehydration, as well as subsequent rehydration was assessed. Chlorophyll fluorometry was used to give an indication of photosynthetic changes during de- and rehydration. Additional data on the effect of drying rate on the photosynthetic apparatus was provided by studying the changes in photosynthetic pigment and the presence of transcripts of major photosynthetic proteins. The degree of damage to cell contents and membranes incurred during slow and rapid dehydration was investigated by examining the cell ultrastructure and solute leakage from the cells. In addition, the effect of rapid drying on the accumulation of the protective molecule, sucrose, was determined, in order to indicate whether protective mechanisms are impaired by an increased drying rate.

Finally, the dependence of slowly and rapidly dried *C. wilmsii* and *X. humilis* on *de novo* transcription and translation during rehydration was investigated using metabolic inhibitors. This provided insight into the extent of protein and/or mRNA accumulation during dehydration.



Figure 1.2. **A.** Hydrated *C. wilmsii* plants (100% RWC). 1cm=1.8cm. **B.** Dry *C. wilmsii* plants (<5% RWC). Note the curled leaves and accumulation of anthocyanin pigment on leaf undersides. 1cm=0.8cm. **C.** Hydrated *X. humilis* plants (100% RWC). 1cm=1.1cm. **D.** Dry *X. humilis* plants (<5% RWC). Note the lack of chlorophyll in the leaves. 1cm=1cm.

CHAPTER 2

THE EFFECT OF DRYING RATE UPON THE SURVIVAL AND PROTECTIVE MECHANISMS OF *C. WILMSII* AND *X. HUMILIS*.

1. Introduction

All desiccation-tolerant plants have a natural rate at which water is lost, varying between taxa and depending on the habitat of the plant. In the previous chapter, the division within resurrection plants based on the natural drying rate, *viz.* full desiccation-tolerance and modified desiccation-tolerance (Oliver, 1996), was mentioned. Bryophytes are classified as fully desiccation-tolerant plants, but, although they lose water extremely quickly relative to higher plant species, they are affected by an increased rate of water loss (Oliver and Bewley, 1997). Naturally desiccated gametophytic tissues of the moss *Tortula ruralis* showed a loss of polysomes corresponding to a decline in protein synthesis. However, during drying, mRNAs did accumulate, presumably to facilitate the rapid recovery of protein synthesis upon rewetting (Oliver and Bewley, 1997). An elevated rate of water loss in the desiccation-tolerant moss *T. ruralis* led to a retention of polysomes in the dry state and no accumulation of mRNAs (Oliver and Bewley, 1997). This resulted in a slower recovery of protein synthesis when rehydration occurred (Bewley and Oliver, 1992).

The modified desiccation tolerance of angiosperms is characterized by the ability to limit water loss and utilization of a greater degree of protection (Oliver and Bewley, 1997). These protective mechanisms (discussed previously) are generally induced during drying, and it has been suggested that the time taken for induction and

establishment precludes survival of rapid drying (Gaff and Loveys, 1984; Bartels *et al.*, 1990; Oliver *et al.*, 1996; Tuba *et al.*, 1998).

Very little research has focussed on the effect of drying rate on angiosperm resurrection species. Hetherington *et al.* (1982) found that chlorophyll degradation in the poikilochlorophyllous resurrection plant, *Borya nitida*, was impaired by a rapid drying rate. Upon rehydration, the leaves did not recover photosynthetic function and PSII reaction centres appeared to be detached from antennae. Conversely, slowly dried *B. nitida* lost all chlorophyll and recovered when rehydrated, regaining normal photosynthetic function.

Studies on the effect of drying rate upon homoiochlorophyllous resurrection plants are somewhat contradictory. Navari-Izzo *et al.* (1995) found that detached leaves of *Boea hygroskopica* did not survive rapid drying. The authors suggested that the increased rate of water loss caused irreversible changes to the thylakoid membranes. Bochicchio *et al.* (1998), however, found that detached leaves of *B. hygroskopica* could survive rapid drying and maintained that it is not the rate of drying that is important, but rather the water content to which the leaves are dried that is critical. They speculated that membranes undergo irreversible phase changes at very low water contents and that those leaves in the study of Navari-Izzo *et al.* (1995) were dried below a critical water content, resulting in the observed changes to membranes and loss of viability. However, recovery of excised leaves of another homoiochlorophyllous species, *Craterostigma nanum*, was not impaired by rapid dehydration to 1% RWC (Sherwin, 1995).

The aim of this study was to investigate the effect of drying rate on the recovery and protective mechanisms of the two resurrection plants, *C. wilmsii* and *X. humilis*

during dehydration and rehydration. These species employ different strategies in dealing with desiccation stress and in particular, light stress, *C. wilmsii* being homoiochlorophyllous, while *X. humilis* is poikilochlorophyllous (Farrant, 2000). Whole plants were rapidly and slowly dried and the following measurements and experiments were performed in order to establish the ability of either species to recover. The quantum efficiency of PSII was determined during dehydration, as well as rehydration, and the chlorophyll content of control, dry and rehydrated material was quantified in order to ascertain the effect of rapid drying on the photosynthetic apparatus of both species. Electrolyte leakage was measured during dehydration to provide a measure of membrane damage. Cellular ultrastructure, too, was examined during drying for both species as an indication of the amount of damage incurred during slow and rapid drying. The accumulation of sucrose, glucose and fructose was quantified at stages during dehydration to assess whether there is any curtailment of protective mechanisms due to rapid drying. Also the presence of some photosynthesis-related mRNA transcripts in dry and rehydrated tissue was determined in order to ascertain the extent to which the expression of photosynthetic components is affected by drying rate.

2. Materials and methods

2.1. Plant material

Whole plants of *X. humilis* (Velloziaceae) and *C. wilmsii* (Scrophulariaceae) were collected in the Pilanesberg Nature Reserve, Northwest Province and Buffelskloof Nature Reserve, Mpumalanga respectively. They were planted in a mixture of peat, river sand and potting soil and maintained in a greenhouse with no supplementary lighting at an average temperature of 28°C in summer and 15°C in winter. To ensure hardiness of the plants, they were subjected to regular cycles of desiccation and rewetting four to five times per year, in addition to drying for experimental purposes. Plants undergoing this cycle of desiccation were left in the dry state for no longer than two weeks.

2.2. Dehydration and rehydration

Whole plants were slowly dried by withholding water, allowing the plants to dry naturally. Rapid drying was accomplished by removing whole plants from the soil in which they were growing and placing them in a flash drying apparatus (Fig. 2.1). Dry, compressed air was passed via flexible polyvinyl chloride (PVC) tubing through a drying tube filled with silica gel. The air was diffused into a plastic receptacle containing silica gel, over which the plant was suspended, resting on nylon gauze. The air passed from the receptacle through its perforated lid.

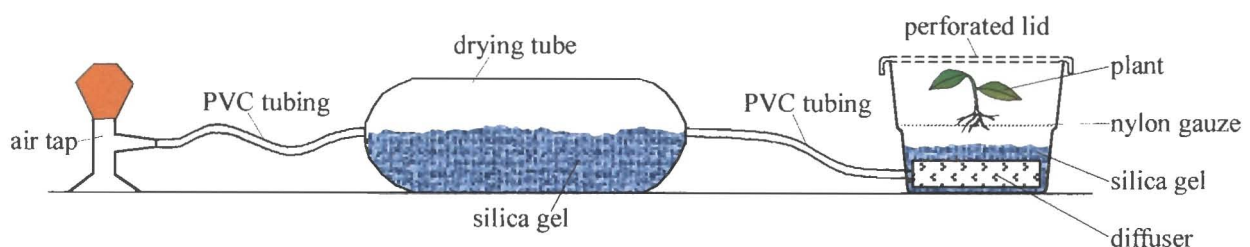


Figure 2.1. Flash-drying apparatus used to rapidly dry whole plants of *C. wilmsii* and *X. humilis*.

Whole plants were rehydrated by watering the soil in which the plants grew (in the case of slowly dried plants) or by placing the plants in water filled containers so that roots were immersed in water (in the case of rapidly dried plants). Testing of F_v/F_m , pigment content and cell ultrastructure were performed on leaves during rehydration. During removal of *C. wilmsii* plants from the soil for rapid drying, roots were damaged due to their soft and filamentous nature. The roots of *X. humilis*, however, are more robust and were not affected by removal from soil or rapid drying. Therefore, in the interest of consistency, leaf explants instead of whole plants were rehydrated and the aforementioned experiments were repeated. In addition, the slot blot analysis was performed using rehydrated leaf explants. Rehydration of these explants was accomplished by detaching individual dry leaves from the plants and placing them in Petri dishes containing water for 48 hours in a plant growth room at 25°C. The light intensity was $1200\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ on a 16/8 hour light-dark cycle.

Relative water content (RWC) was determined by the standard formula:

$$\text{RWC} = \frac{\text{water content} \times 100}{\text{water content at full turgor}}$$

Water content was determined gravimetrically by oven drying at 70°C for 48 hours.

2.3. Chlorophyll and carotenoid content

The content of photosynthetic pigments of four replicates of leaf tissue was determined for control, dry and rehydrated tissue. Leaves were cut into *ca.* 2mm^2 pieces and placed in 100% acetone overnight at 4°C. Thereafter, they were ground in the acetone to remove any excess pigment remaining in the leaf tissue and centrifuged at 4100g at 4°C for 10 minutes.

The absorbance of the extract at 661.6nm, 644.8nm and 470nm was measured using a Beckman DU 650 UV/Vis. spectrophotometer (Beckman, USA). Chlorophyll ($a+b$) and carotenoid ($x+c$) contents were calculated using extinction coefficients provided by Lichtenthaler (1987).

2.4. Quantum efficiency of photosystem (PS) II

Quantum efficiency of PSII was determined by chlorophyll fluorometry. Readings were taken at various stages during dehydration for both *C. wilmsii* and *X. humilis*. Five replicate leaves were dark adapted for 10 minutes using dark-adaptation cuvettes. F_v/F_M was measured using an OS-500 portable, modulated fluorometer (Opti-Sciences, USA) with a saturating light intensity of $7500\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ to obtain maximal fluorescence (F_M). F_v is the variable fluorescence and is defined as:

$$F_v = F_M - F_o \text{ (ground state fluorescence)}$$

This entire experiment was performed twice.

2.5. Electrolyte leakage

Electrolyte leakage was measured to give an indication of membrane integrity and damage in slowly and rapidly dried tissues. Five replicate leaves at various stages during dehydration were submerged in 10ml Milli-Q (Millipore) ultra-pure water and conductivity was measured at five-minute intervals for 30 minutes using a Jenway 4070 conductivity meter. The rate of leakage was calculated from the time course of conductivity and expressed per gram dry mass ($\mu\text{S}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ dry mass). The results are presented as a percentage of control (hydrated) electrolyte leakage.

2.6. Cellular ultrastructure

Cellular ultrastructure was investigated using transmission electron microscopy. Small pieces of leaf tissue (approximately 2mm²) were excised from at least four different leaves of at least two different plants. Samples were taken from hydrated, dry and rehydrated tissue, as well as three stages during dehydration. These drying stages were i) 85-70% RWC; ii) 65-50% RWC; iii) 40-25% RWC. The tissue was processed according to a method previously used for this tissue (Sherwin and Farrant, 1996). It was fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) containing 0.5% caffeine. The samples were then washed in 0.1M phosphate buffer and post-fixed in 1% osmium in phosphate buffer. After further washing with phosphate buffer, the samples underwent a graded, stepwise ethanol dehydration, finally being placed in 100% acetone. The acetone was gradually replaced with epoxy resin (Spurr, 1969) over four days to ensure complete resin infiltration into cells. The resin-impregnated samples were embedded in epoxy resin, hardened at 60°C for 16 hours and sectioned at a gold interference colour (95nm thick) using a Reichert Ultracut-S microtome. Sections were stained with 2% uranyl acetate and 1% lead citrate (Reynolds, 1963) and viewed with a Zeiss EM-109 transmission electron microscope (Zeiss, Germany). Observations were made of general cell structure in at least four different sections of all leaves prepared. As use of standard fixation techniques might have allowed slight rehydration of the desiccated leaves, results were compared with studies using cryofixation of dry leaves of *C. wilmsii* (Vicré *et al.*, 1999) and freeze substitution of dry tissue from both species used in this study (Wesley-Smith, pers. com.). These studies gave similar results to those observed in this study. However, those methods allowed preservation of only epidermis and one or two cell layers below that. In order

to view the subcellular changes in all leaf cells, standard fixation was used. Furthermore, since all treatments in the current study were fixed in the same manner, comparisons among them are valid.

2.7. Sugar extraction and quantitation

Sucrose, glucose and fructose was extracted from hydrated and dry tissue, as well at two stages during dehydration, *viz.* drying stage 1) 70-65% RWC; drying stage 2) 40-25% RWC. Tissue was ground in liquid nitrogen and mixed with cold extraction buffer consisting of 100mM NaOH in 50% ethanol (v/v). The volume of extraction buffer added was four (for hydrated tissue) to eight (for tissue at drying stages and dry tissue) times the weight of leaf material. This solution was placed in a 100°C water bath for 10 minutes and then rapidly cooled on ice. While mixing with a vortex mixer, 100mM HEPES in 100mM acetic acid was added to neutralise the extract (the volume needed for neutralisation to pH 7-8 was determined using litmus paper for each species). The extract was centrifuged at 28000g for 20 minutes and the supernatant retained. The remaining pellet was re-extracted using the same procedure as described above and the supernatants of the two extractions combined.

The quantitation of the sucrose, glucose and fructose was carried out using a D-glucose/D-fructose sugar assay kit (Boehringer Mannheim, Germany) based on the methodology of Bergmeyer and Bernt (1974). This involved enzymatic breakdown and/or conversion of sucrose, fructose or glucose to D-glucose-6-phosphate (Glc-6-P). Glc-6-P is converted to 6-phospho-gluconate, in the process reducing NADP to NADPH. The absorbance of the resultant NADPH was measured at 340nm with a Shimadzu UV-1601 vis./UV spectrophotometer (Schimadzu Scientific Instruments

Inc., USA). The production of NADPH is stoichiometrically equivalent to the amount of Glc-6-P in the sample, which in turn was used to determine the quantity of sugars in the sample (in $\mu\text{moles.g}^{-1}$ dry wt).

2.8. RNA isolation and slot blot analysis

Total RNA was isolated using plant mini RNeasy kits (Qiagen, Germany). Equal amounts of RNA from various treatments were blotted onto Hybond-N membrane (AEC Amersham, South Africa) using a slot blotting apparatus (Hoeffer Scientific Instruments, Germany). The blots were cross-linked with an UV cross-linker (AEC Amersham, South Africa). After prehybridisation of the blots in DIG (digoxigenin) Easy Hybridisation solution (Boehringer Mannheim, Germany) at 42°C for 30 minutes, 25ng of DIG-labelled probe (random priming) was added per ml of solution and hybridisation was performed for 20 hours at 42°C . Probes were detected using the chemiluminescent detection system carried out as per the DIG system user's guide (Boehringer Mannheim, Germany).

The oligo probes for *rbcS* (small subunit of Rubisco) and *lhc* (chlorophyll a/b binding protein) were supplied by Dr. S. Mackerness (Horticultural Research International, Warwick, UK), while the probes for *rbcL* (large subunit of Rubisco) and *18S* rRNA were supplied by Dr V. Abratt and Mr W. Mpoloka (Dept of Microbiology, University of Cape Town, SA).

3. Results

3.1. Drying rates

The two drying treatments resulted in markedly different rates of water loss for both *C. wilmsii* and *X. humilis* (Fig. 2.2 A & B). Slowly or naturally dried, *C. wilmsii* (Fig. 2.2 A) took approximately 8 days to reach an air-dry state, whereas loss of water in *X. humilis* (Fig. 2.2 B) was slower, drying to under 5% RWC in just over 12 days. Rapidly dried *C. wilmsii* and *X. humilis* however, achieved similar water contents in 17 and 25 hours respectively. In both cases the final water content achieved was similar for rapidly and slowly dried material (Table 2.1).

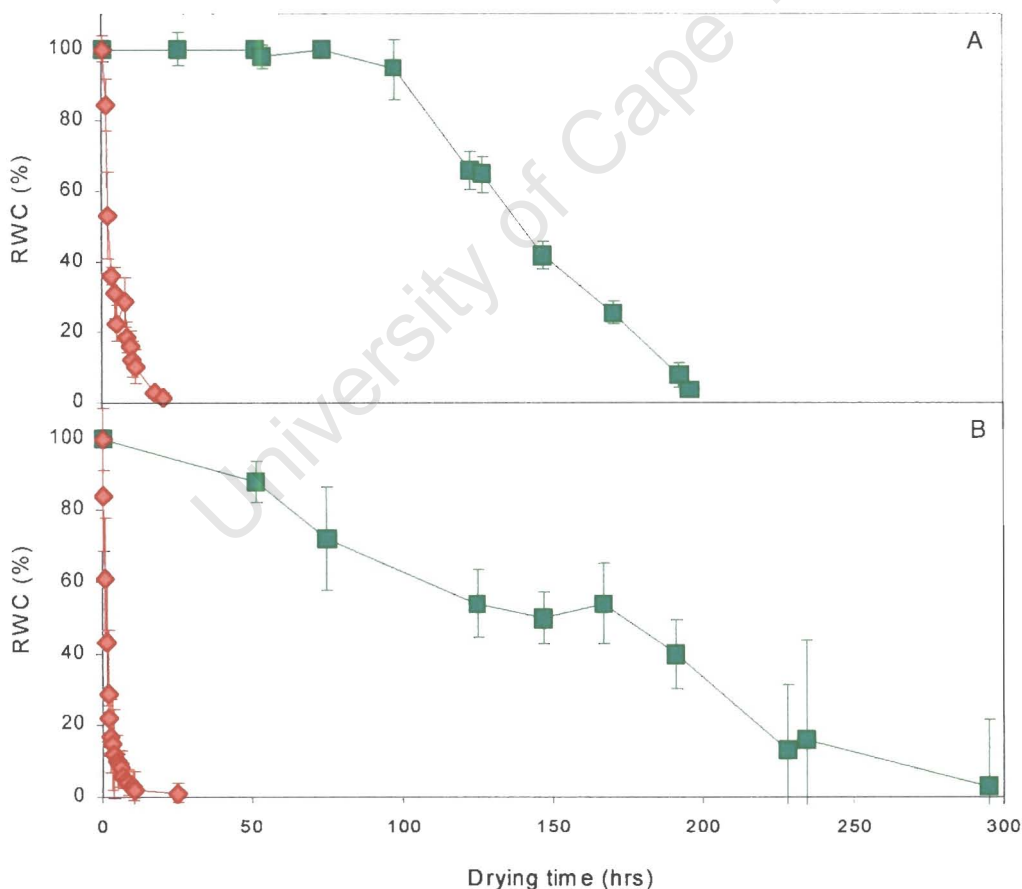


Figure 2.2. Drying rates of slowly (■) and rapidly (◆) dried whole plants of (A) *C. wilmsii* and (B) *X. humilis*.

Table 2.1. Final water contents ($\text{g H}_2\text{O} \cdot \text{g}^{-1}$ dry weight) and relative water content (%RWC) of leaves of *C. wilmsii* and *X. humilis* after slow and rapid drying.

Species	Slowly dried		Rapidly dried	
	RWC (%)	$\text{g H}_2\text{O} \cdot \text{g}^{-1}$ DW	RWC (%)	$\text{g H}_2\text{O} \cdot \text{g}^{-1}$ DW
<i>C. wilmsii</i>	3.60 (± 1.30)	0.19 (± 0.07)	3.10 (± 0.94)	0.16 (± 0.05)
<i>X. humilis</i>	3.00 (± 1.84)	0.10 (± 0.05)	3.00 (± 0.99)	0.09 (± 0.03)

Water uptake upon rehydration showed no variability among the species and drying treatments, with all leaves reaching approximately 100% RWC in 15 to 20 hours (not shown). However, the recovery of physiological functioning did not always correspond with the ability to rehydrate completely.

3.2. Chlorophyll and carotenoid content

Figure 2.3 depicts the change in photosynthetic pigment content of leaves of slowly and rapidly dried *C. wilmsii* and *X. humilis* after dehydration and rehydration. *C. wilmsii* (Fig. 2.3 A) lost approximately 60% and 25% of chlorophyll ($a+b$) and carotenoid ($x+c$) respectively during slow dehydration. When rapidly dried, leaves lost, on average, less chlorophyll and more carotenoid, but this was not a significant difference for either pigment. On rehydration, both drying treatments showed complete or almost complete (in the case of rapidly dried material) recovery of photosynthetic pigments.

X. humilis showed the loss of chlorophyll common to poikilochlorophylly, with only 2% of the control level chlorophyll remaining in the leaf (Fig. 2.3 B). Carotenoids, too, showed a substantial decrease of 75%. When rapidly dried, the breakdown of photosynthetic pigments appeared to be impaired, with a 50 to 55% decline of both chlorophyll and carotenoids. Upon rehydration of this material, there

was very little recovery. Only slowly dried material showed recovery of pigment to control level when rehydrated.

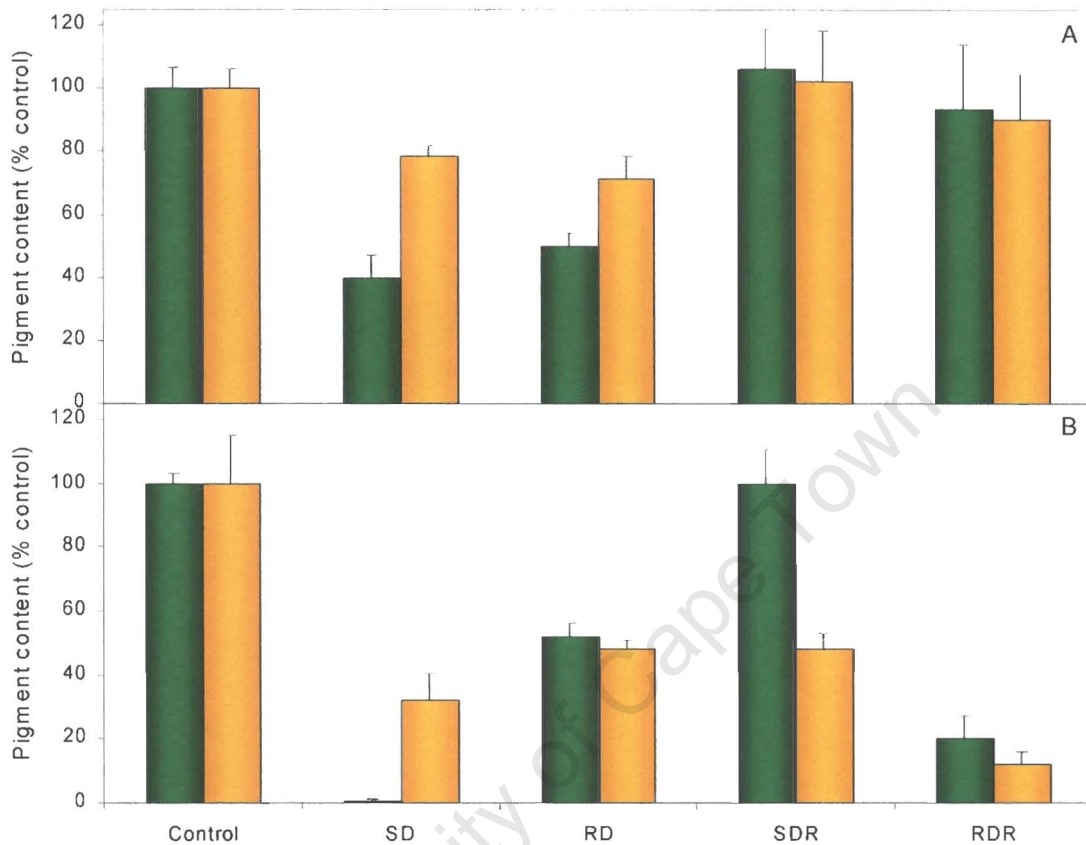


Figure 2.3. Chlorophyll (■) and carotenoid (□) content of slowly and rapidly dried leaves of (A) *C. wilmsii* and (B) *X. humilis*. Control – hydrated; SD – slowly dried; SDR – slowly dried, rehydrated; RD – rapidly dried; RDR – rapidly dried, rehydrated.

3.3. Quantum efficiency of photosystem II (PSII)

The changes in quantum efficiency of PSII (F_V/F_M) during dehydration and rehydration of *C. wilmsii* and *X. humilis* are shown in Figure 2.4. In *C. wilmsii* the trend in F_V/F_M as water content decreased is comparable for both slowly and rapidly dried material (Fig. 2.4 A(i)). F_V/F_M remained high until 20% RWC, after which it declined rapidly. Upon rehydration, recovery of PSII activity began between 20 and 40% RWC and showed full recovery after 80% RWC for both rapidly and slowly dried plants (Fig. 2.4 A(ii)).

Leaves of slowly and rapidly dried *X. humilis*, however, did not follow the same trend. Slowly dried *X. humilis* began to show a decline in F_v/F_M at approximately 60% RWC, corresponding with the breakdown of chlorophyll and photosynthetic membranes characteristic of poikilochlorophyllous plants (Fig. 2.4 B(i)). After rewetting, these leaves recovered, returning to normal PSII functioning after 80% RWC (Fig. 2.4 B(ii)). Rapidly dried material, however, showed a decline in quantum efficiency only after 25% RWC (Fig. 2.4 B(i)) and did not recover photosynthetic functioning upon rehydration (Fig. 2.4 B(ii)).

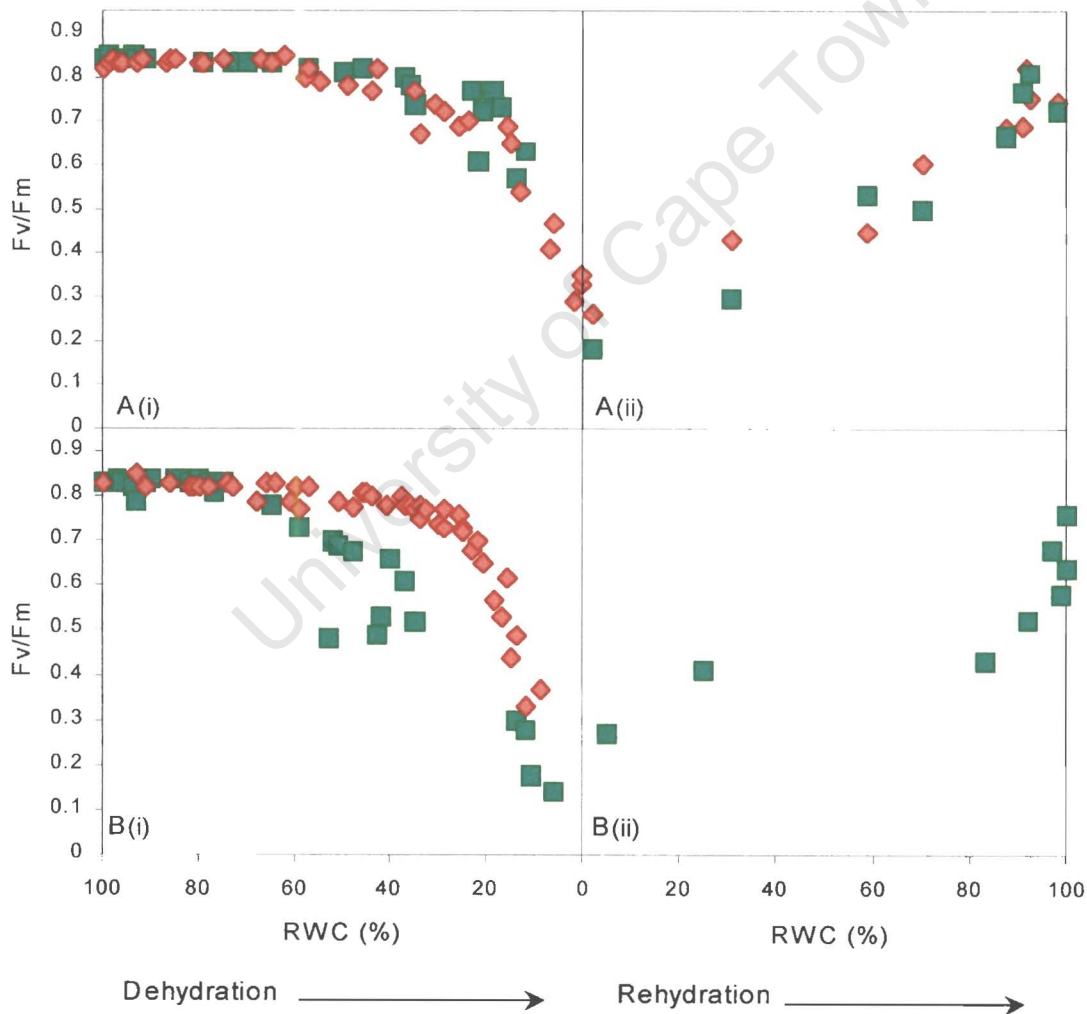


Figure 2.4. Changes in quantum efficiency of photosystem II for slowly (■) and rapidly dried (◆) leaves of (A) *C. wilmsii* and (B) *X. humilis* during dehydration (i) and rehydration (ii).

3.4. Electrolyte leakage

The electrolyte leakage of leaves from *C. wilmsii* and *X. humilis* is shown in Figure 2.5. The solute leakage of *C. wilmsii* leaves (Fig. 2.5 A) did not exhibit much variation, whether slowly or rapidly dried. Rapidly dried leaves of *X. humilis*, however, showed a marked increase in electrolyte leakage below 40% RWC, indicating that membrane integrity was not maintained during rapid drying (Fig. 2.5 B). Damage may have resulted due to the rehydration occurring during the measurement of conductivity.

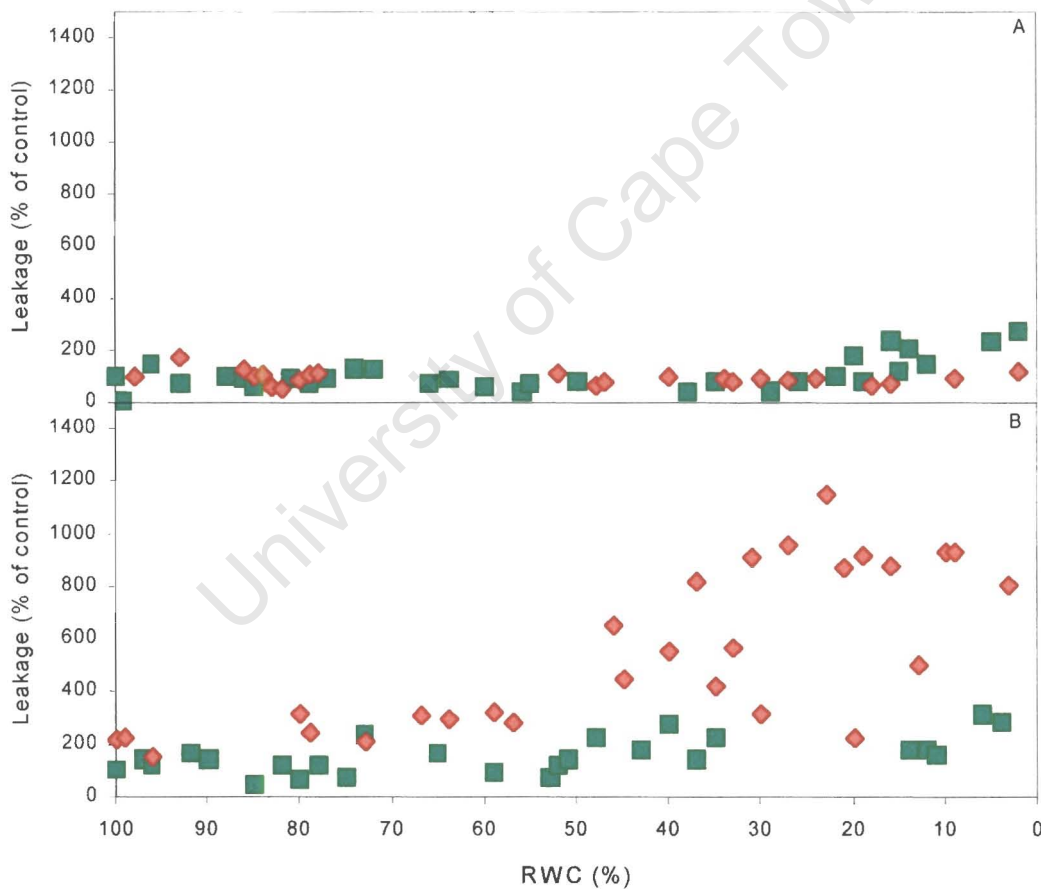


Figure 2.5. Electrolyte leakage from slowly (■) and rapidly (◆) dried leaves of (A) *C. wilmsii* and (B) *X. humilis* during dehydration.

3.5. Cellular ultrastructure

Figures 2.6 to 2.9 depict the subcellular organisation of *C. wilmsii* mesophyll cells in hydrated, dry and rehydrated states and at several stages during dehydration. Control (hydrated) *C. wilmsii* cells (Fig. 2.6 A & B) had a large central vacuole, with the cytoplasm and organelles situated at the periphery of the cell. Figures 2.6 C & D represent the typical cell appearance for slowly dried leaves at water contents between 85 and 70% RWC. The vacuoles had not undergone any visible loss of volume and, as a result, the plasmamembrane remained appressed to the cell wall. It is unlikely that the membranes were under substantial mechanical tension at this point and in fact, the appearance of the cell (Fig. 2.6 C) was practically unchanged from that of a fully hydrated cell. The chloroplasts (Fig 2.6 D), too, were visually consistent with those of cells at 100% RWC, except that there were no starch grains present, indicating that the plant had already hydrolysed these starch stores.

At high water contents (85 to 70% RWC) in rapidly dried *C. wilmsii* cells (Figs. 2.6 E & F), there was again no apparent membrane damage and no alteration in chloroplast structure.

Chloroplasts of cells of slowly dried *C. wilmsii* leaves at water contents between 65 and 50% RWC were not consistent in appearance. Some showed some displacement of internal membranes towards the characteristic U-shaped conformation of thylakoids (Sherwin and Farrant, 1998) seen in dry tissue (Fig. 2.7 A), while others still appeared unaffected (Fig. 2.7 B). There was a slight amount of wall folding (Fig. 2.7 C), the plasmamembrane had not yet withdrawn and there were no signs of damage in the cell. However, the fact that the chloroplasts were not pressed against the cell wall indicated that the vacuole had lost volume, presumably due to water loss.

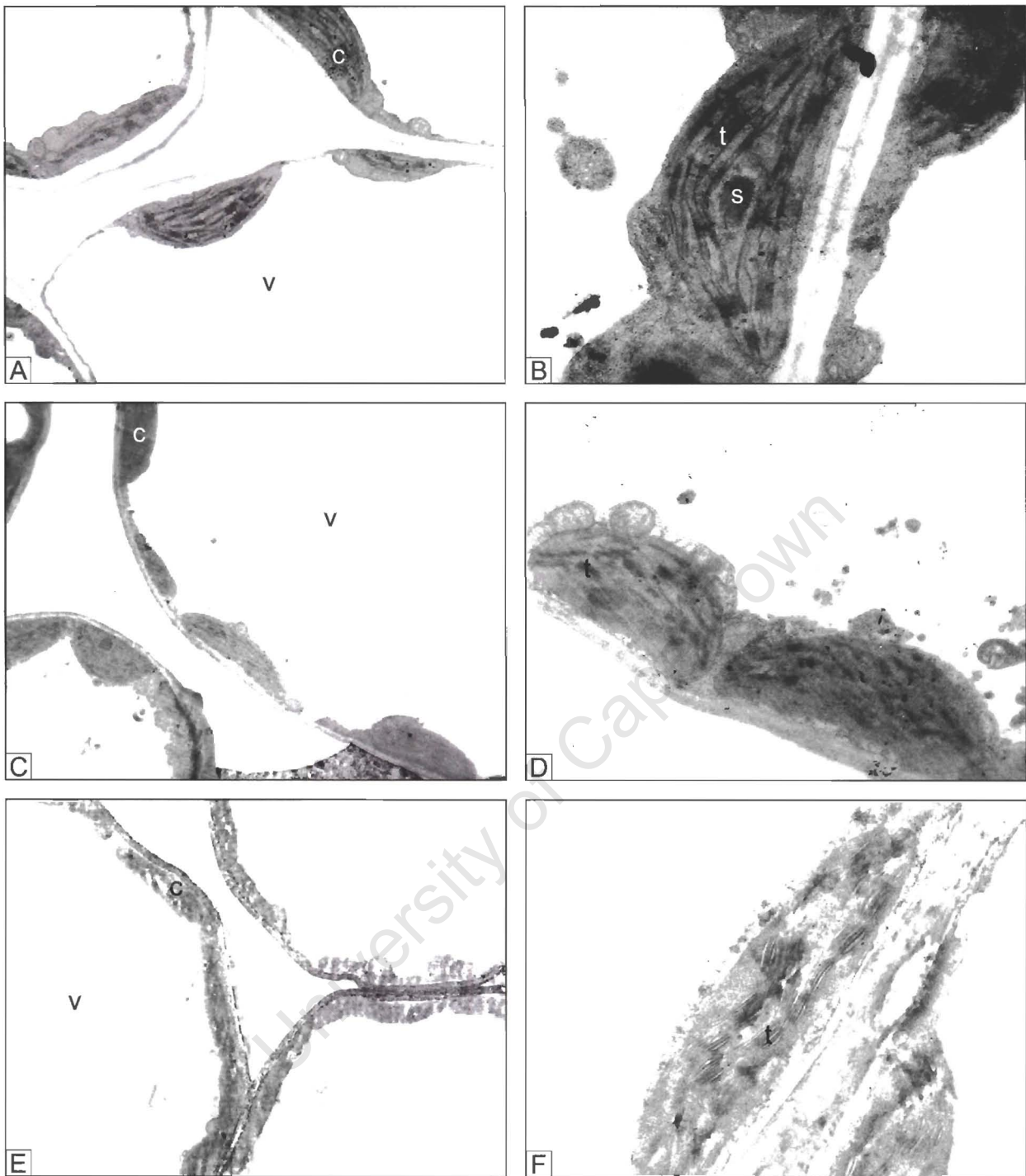


Figure 2.6 (A) Mesophyll cell of hydrated (control) *C. wilmsii* leaf. Cells are highly vacuolate (v) and the cytoplasm and chloroplasts (c) are situated at the cell periphery. $1\text{cm}=2.0\mu\text{m}$. (B) Chloroplast of a hydrated (control) *C. wilmsii* leaf mesophyll cell. Note the starch grain (s) and thylakoids (t) arranged in granal stacks. $1\text{cm}=0.6\mu\text{m}$. (C) Mesophyll cell from slowly dried *C. wilmsii* leaf at drying stage 1 (85 to 70% RWC). The appearance is virtually unchanged from that of the control cell with a large vacuole (v) present and no change to chloroplasts (c). $1\text{cm}=2.9\mu\text{m}$. (D) Chloroplasts of mesophyll cell from slowly dried *C. wilmsii* leaf at drying stage 1 (85 to 70% RWC). Thylakoids (t). $1\text{cm}=1.2\mu\text{m}$. (E) Mesophyll cell from rapidly dried *C. wilmsii* leaf at drying stage 1 (85 to 70% RWC). The vacuole (v) and chloroplasts (c) have the appearance of control and slowly dried cells. $1\text{cm}=2.5\mu\text{m}$. (F) Chloroplasts of mesophyll cell from rapidly dried *C. wilmsii* leaf at drying stage 1 (85 to 70% RWC). Thylakoids (t). $1\text{cm}=0.6\mu\text{m}$.

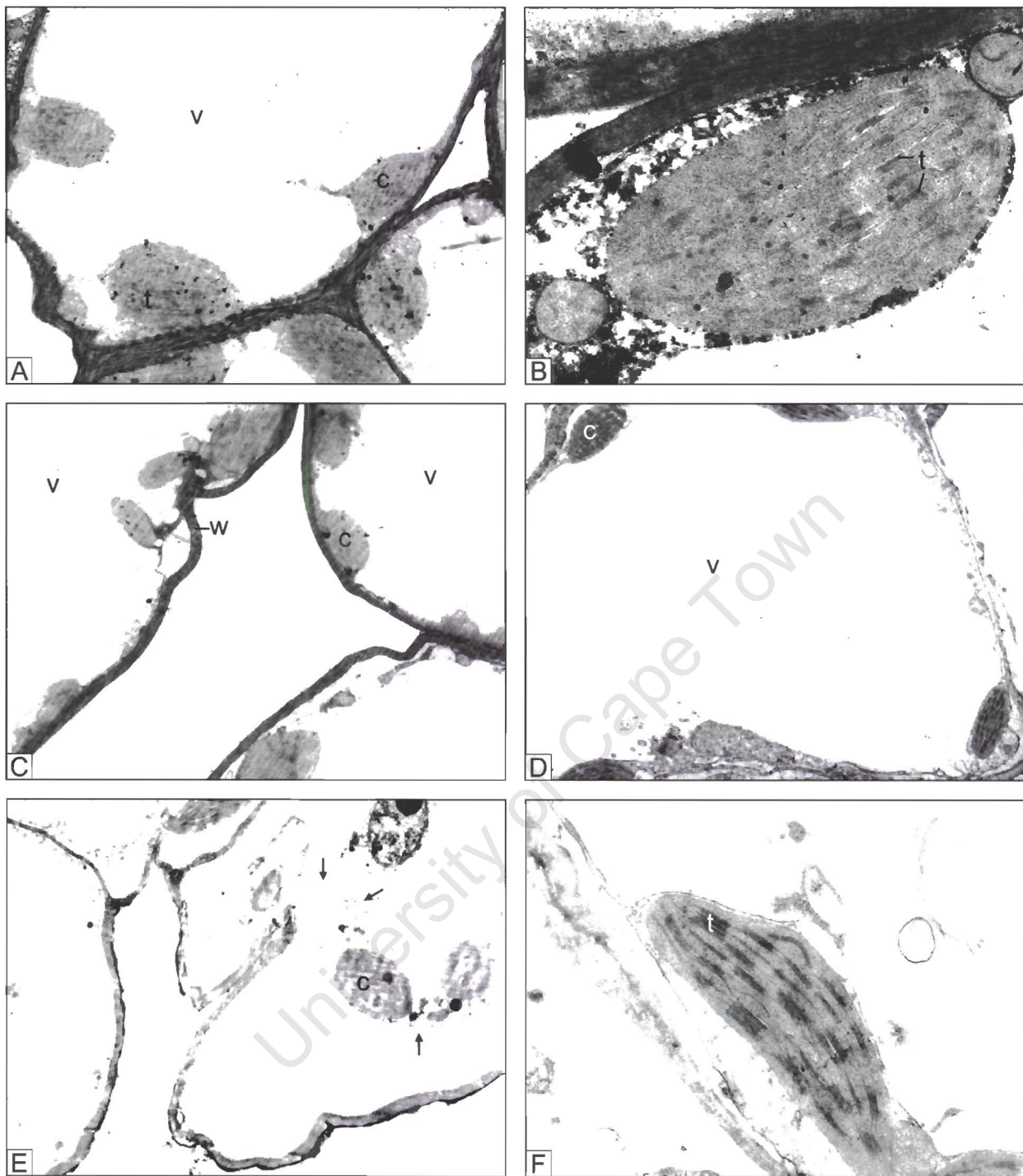


Figure 2.7 (A) Mesophyll cell from slowly dried *C. wilmsii* at drying stage 2 (65 to 50% RWC). Note the large vacuole (v) and slight displacement of thylakoids (t) in the some chloroplasts (c). 1cm=2.7 μ m. (B) Chloroplasts of mesophyll cell from slowly dried *C. wilmsii* at drying stage 2 (65 to 50% RWC). There is very little change the arrangement of thylakoid membranes (t). 1cm=0.6 μ m. (C) Cell wall (w) folding of a mesophyll cell from slowly dried *C. wilmsii* at drying stage 2 (65 to 50% RWC). Vacuole (v); chloroplasts (c). 1cm=2.6 μ m. (D) Undamaged mesophyll cell from rapidly dried *C. wilmsii* at drying stage 2 (65 to 50% RWC). The cells still possess a large central vacuole (v) and there is no damage visible. Chloroplasts (c). 1cm=2.9 μ m. (E) Damaged mesophyll cell from rapidly dried *C. wilmsii* at drying stage 2 (65 to 50% RWC). There are several membrane breaks (indicated by arrows). Chloroplasts (c). 1cm=3.0 μ m. (F) Chloroplasts of mesophyll cell from rapidly dried *C. wilmsii* at drying stage 2 (65 to 50% RWC). Thylakoids (t). 1cm=0.7 μ m.

At the same water content range in rapidly dried tissue, there was variability in the state of the cells (Figs. 2.7 D, E & F), with certain cells showing damage (Fig. 2.7 E), while others appeared intact (Fig. 2.7 D). The undamaged cell in Figure 2.7 D exhibited no obvious changes in organisation, but the cell walls did appear to have begun folding (indicated by the shape of the cell). Conversely, there was membrane breakage and marked plasmamembrane withdrawal in the damaged cell shown in Figure 2.7 E, although cell wall folding was also apparent. The chloroplasts in all cells (Fig. 2.7 F) remained unchanged, with no visible damage.

Extreme changes were observed in cellular ultrastructure between 40 and 25% RWC (Fig. 2.8 A & B) in slowly dried leaves. Cell wall folding was most noticeable at low water contents and there was substantial subdivision of the vacuoles (Fig. 2.8 A) and plasmamembrane withdrawal from the cell wall. The extent of this may have been an artefact of the aqueous processing of samples, but there was no breakage of the membranes. The majority of chloroplasts showed the U-shaped form of internal membranes (Fig. 2.8 A & B), with the thylakoid membranes still present.

At low water contents of rapidly dried leaves (Figs. 2.8 C, D & E), wall folding was more conspicuous, and as with the previous drying stage, certain cells showed membrane breakage (Fig. 2.8 C), while in the membranes of other cells appeared intact (Fig. 2.8 D). The majority of chloroplasts had adopted the U-shaped conformation (Fig. 2.8 D & E).

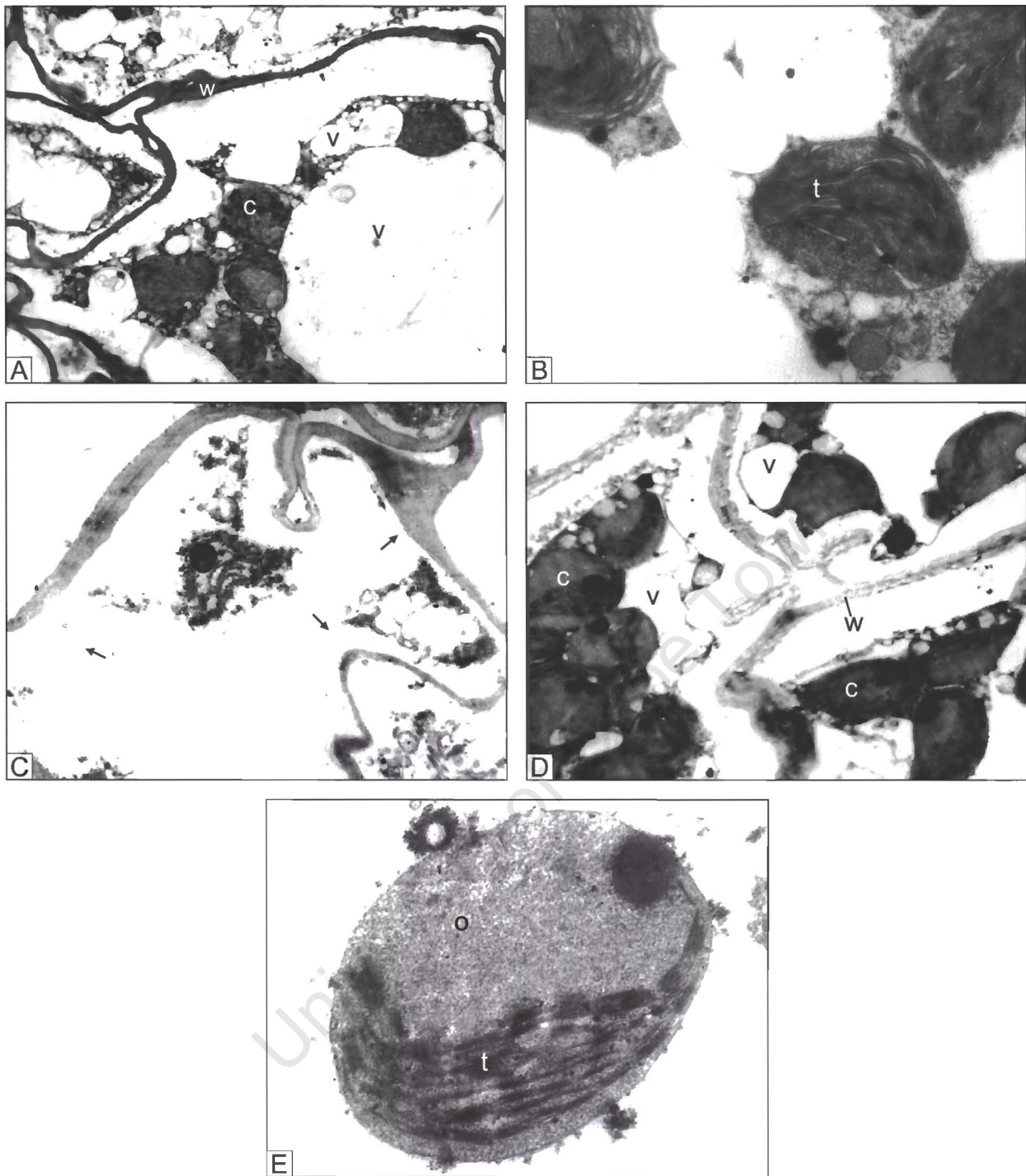


Figure 2.8 (A) Mesophyll cell from slowly dried *C. wilmsii* at drying stage 3 (40 to 25% RWC). Note the folding of the cell wall (w) and the numerous small vacuoles (v). Chloroplasts (c) show the U-shaped conformation of thylakoids. $1\text{cm}=2.8\mu\text{m}$. (B) Chloroplasts of mesophyll cell from slowly dried *C. wilmsii* at drying stage 3 (40 to 25% RWC). The thylakoids (t) show the U-shaped conformation. $1\text{cm}=1.0\mu\text{m}$. (C) Damaged mesophyll cell from rapidly dried *C. wilmsii* at drying stage 3 (40 to 25% RWC). The membrane has been almost completely destroyed (as indicated by arrows). $1\text{cm}=2.1\mu\text{m}$. (D) Undamaged mesophyll cell from rapidly dried *C. wilmsii* at drying stage 3 (40 to 25% RWC). As with slowly dried material, there is extensive cell wall (w) folding and subdivision of vacuoles (v). Chloroplasts (c). $1\text{cm}=2.7\mu\text{m}$. (E) Chloroplast of mesophyll cell from rapidly dried *C. wilmsii* at drying stage 3 (40 to 25% RWC). Note the U-shaped displacement of thylakoid (t) membranes. Stroma (o). $1\text{cm}=0.6\mu\text{m}$.

In the dry state, slowly dried (Fig. 2.9 A) and the majority of rapidly dried (Fig. 2.9 B) cells showed much the same appearance, with folded cell walls, U-shaped chloroplasts and numerous vesicles. There were, however, small pockets of cells in rapidly dried tissue that did show damage (Fig. 2.9 C). Upon rehydration, the tissue of both slow (Fig. 2.9 D) and rapid (Fig. 2.9 E) drying treatments reverted to the appearance of the control leaves, with no visible damage.

Figures 2.10 to 2.13 depict control, dry, rehydrated and the stages of dehydration of *X. humilis*. Figure 2.10 shows the appearance of a control *X. humilis* cell. There was a large central vacuole (Fig. 2.10 A) and chloroplasts (Fig. 2.10 B) exhibited internal membranes and starch grains.

At the first drying stage (85 to 70% RWC), the vacuoles of slowly dried leaf cells had already begun to subdivide, the plasmamembrane was still appressed to the cell wall and there was no sign of membrane injury (Figs. 2.10 C). Chloroplasts had already lost the starch grains seen in chloroplasts of fully hydrated cells, but the breakdown of the thylakoid membranes had not yet begun (Fig. 2.10 D).

In Figures 2.10 E & F, the typical appearance of rapidly dried tissues of *X. humilis* is depicted at water contents between 85 and 70% RWC. The vacuoles were still very large and there was no visible disruption of the cell components (Fig. 2.10 E). Like the chloroplasts from slowly dried tissues, the thylakoid membranes were intact, but unlike slowly dried tissues, the starch grains were still present (Fig. 2.10 F). Plastoglobuli, which may act as a storage medium for phospholipids (Hallam and Luff, 1980), were observed within the chloroplasts, which does suggest the onset of thylakoid dismantling.

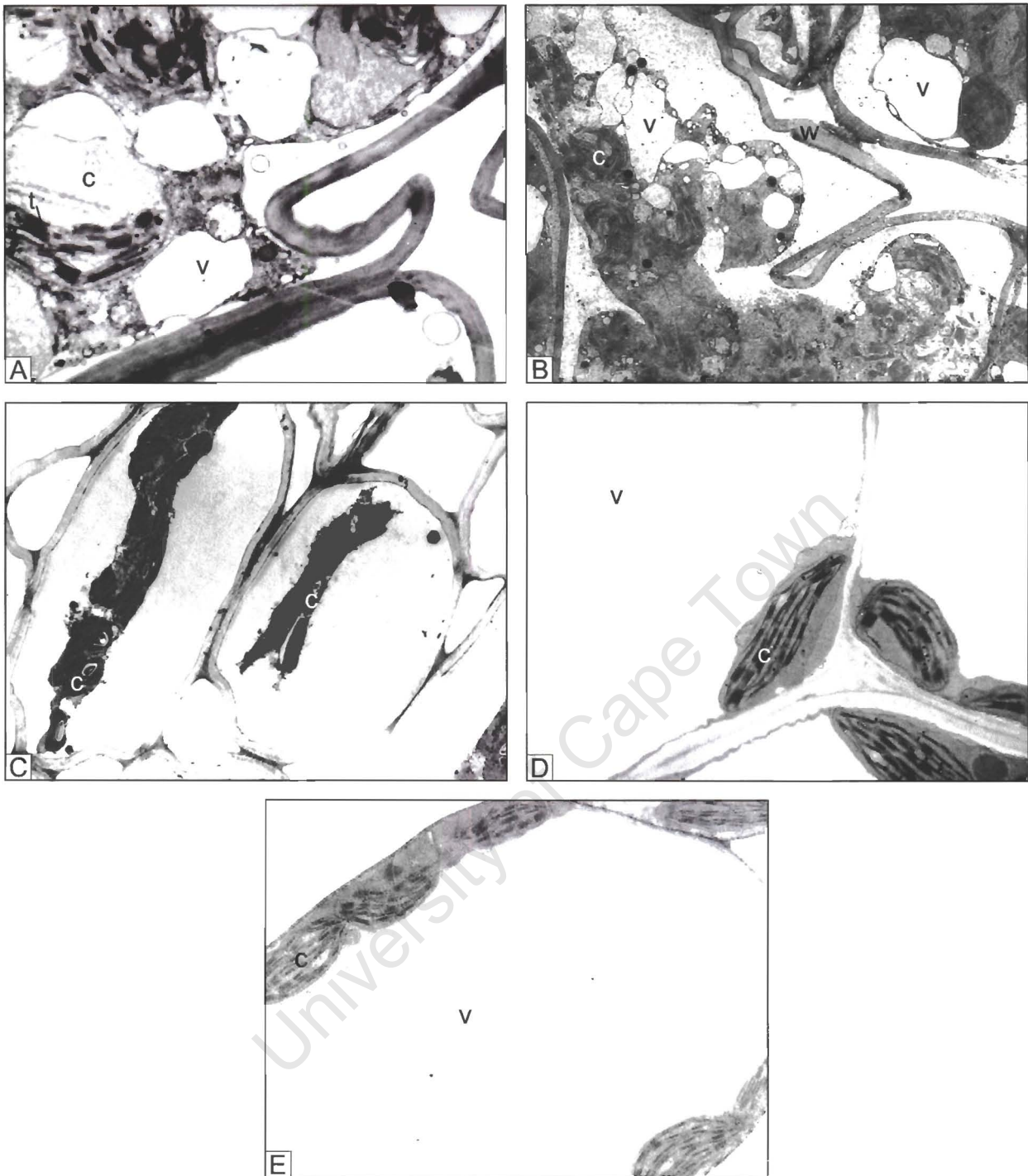


Figure 2.9 (A) Mesophyll cell of slowly dried *C. wilmsii*. Note the folding of the cell wall (w) and the numerous small vacuoles (v). Chloroplasts (c) show the U-shaped conformation of thylakoids (t). $1\text{cm}=1.3\mu\text{m}$. (B) Mesophyll cell from rapidly dried *C. wilmsii*. As with slowly dried material, there is extensive cell wall (w) folding and subdivision of vacuoles (v), as well as, “U-shaped” chloroplasts (c). $1\text{cm}=2.8\mu\text{m}$. (C) Damaged mesophyll cell from rapidly dried *C. wilmsii*. Note the extensive plasmamembrane withdrawal. Chloroplasts (c). $1\text{cm}=3\mu\text{m}$. (D) Mesophyll cell of rehydrated slowly dried *C. wilmsii*. The cell has the appearance of a control cell, with large vacuoles (v) and chloroplasts (c) are situated at the periphery of the cell. $1\text{cm}=1.5\mu\text{m}$. (E) Mesophyll cell of rehydrated rapidly dried *C. wilmsii*. There is a large central vacuole (v) and chloroplasts (c) are pressed against the cell wall. $1\text{cm}=3.1\mu\text{m}$.

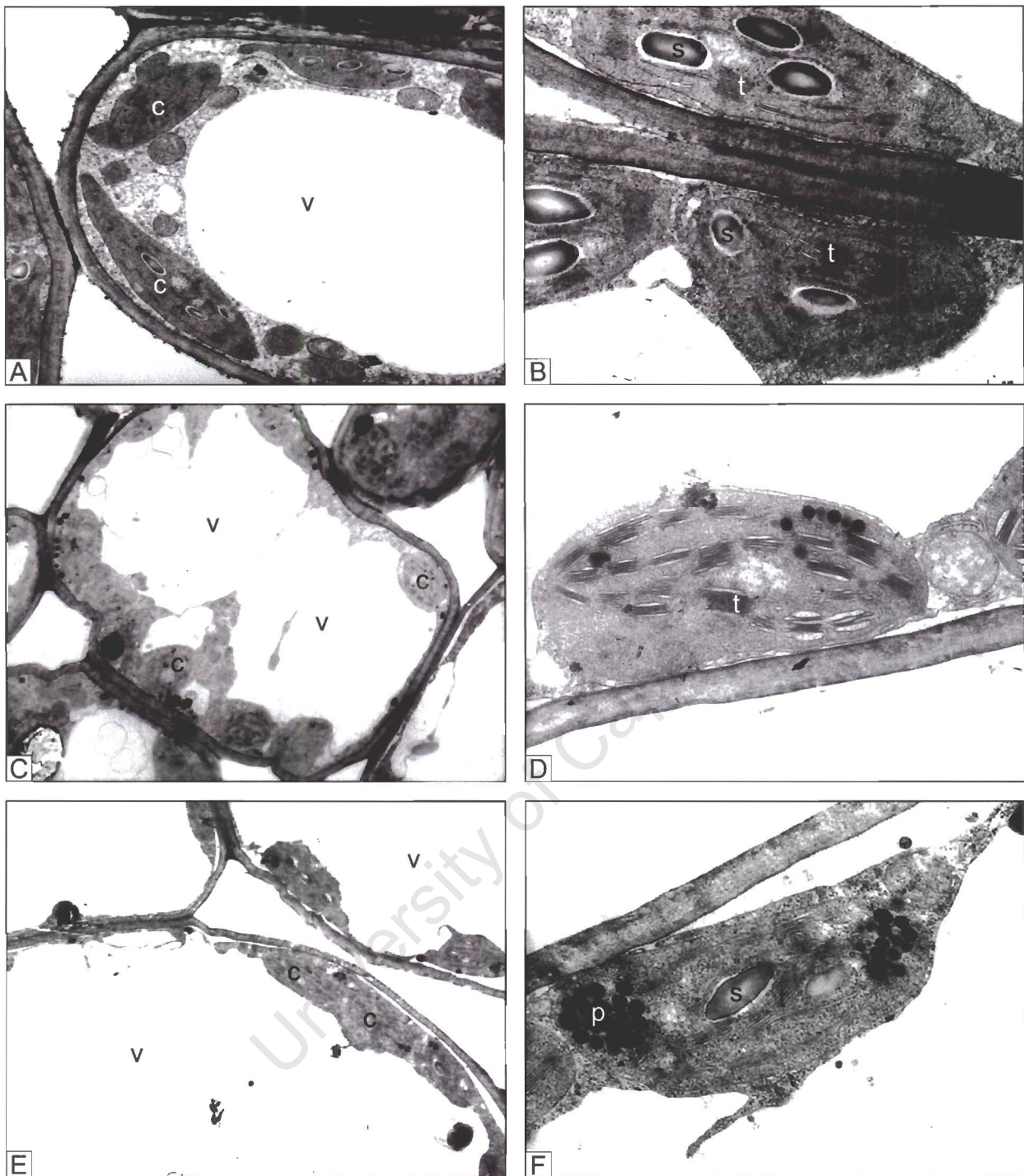


Figure 2.10 (A) Mesophyll cell of hydrated (control) *X. humilis*. Cells are highly vacuolate (v) and the cytoplasm and chloroplasts (c) are situated at the cell periphery. $1\text{cm}=1.6\mu\text{m}$. (B) Chloroplast of a hydrated (control) *X. humilis* mesophyll cell. Note the starch grains (s) and thylakoids (t) arranged into grana. $1\text{cm}=0.5\mu\text{m}$. (C) Mesophyll cell from slowly dried *X. humilis* at drying stage 1 (85 to 70% RWC). There is no sign of membrane rupture and the vacuole (v) has begun to subdivide. Chloroplasts (c). $1\text{cm}=2.9\mu\text{m}$. (D) Chloroplasts of mesophyll cell from slowly dried *X. humilis* at drying stage 1 (85 to 70% RWC). Note that the thylakoid membranes (t) are still visible, but starch grains are no longer present. Vacuole (v). $1\text{cm}=0.7\mu\text{m}$. (E) Mesophyll cell from rapidly dried *X. humilis* at drying stage 1 (85 to 70% RWC). Vacuoles (v) are still large and the chloroplast (c) are at the periphery of the cell. $1\text{cm}=3.9\mu\text{m}$. (F) Chloroplasts of mesophyll cell from rapidly dried *X. humilis* at drying stage 1 (85 to 70% RWC). Both thylakoids (g) and starch grains (s) are still present within the chloroplast, but there are numerous plastoglobuli (p). $1\text{cm}=0.6\mu\text{m}$.

During slow dehydration, following loss of approximately half the cellular water (drying stage 2), some chloroplasts still had not undergone full dismantling of thylakoid membranes (Fig. 2.11 A), while others had an appearance similar to those from dry tissue with very little thylakoid membrane structure within the chloroplast (Fig. 2.11 B). The latter had dismantled all photosynthetic membranes and exhibited the vesicles characteristic of dry *X. humilis* chloroplasts (Dace *et al.*, 1998; Farrant, 2000). Once again, the cells did not appear to have sustained any injury, there being no plasmamembrane withdrawal visible (Fig. 2.11 C).

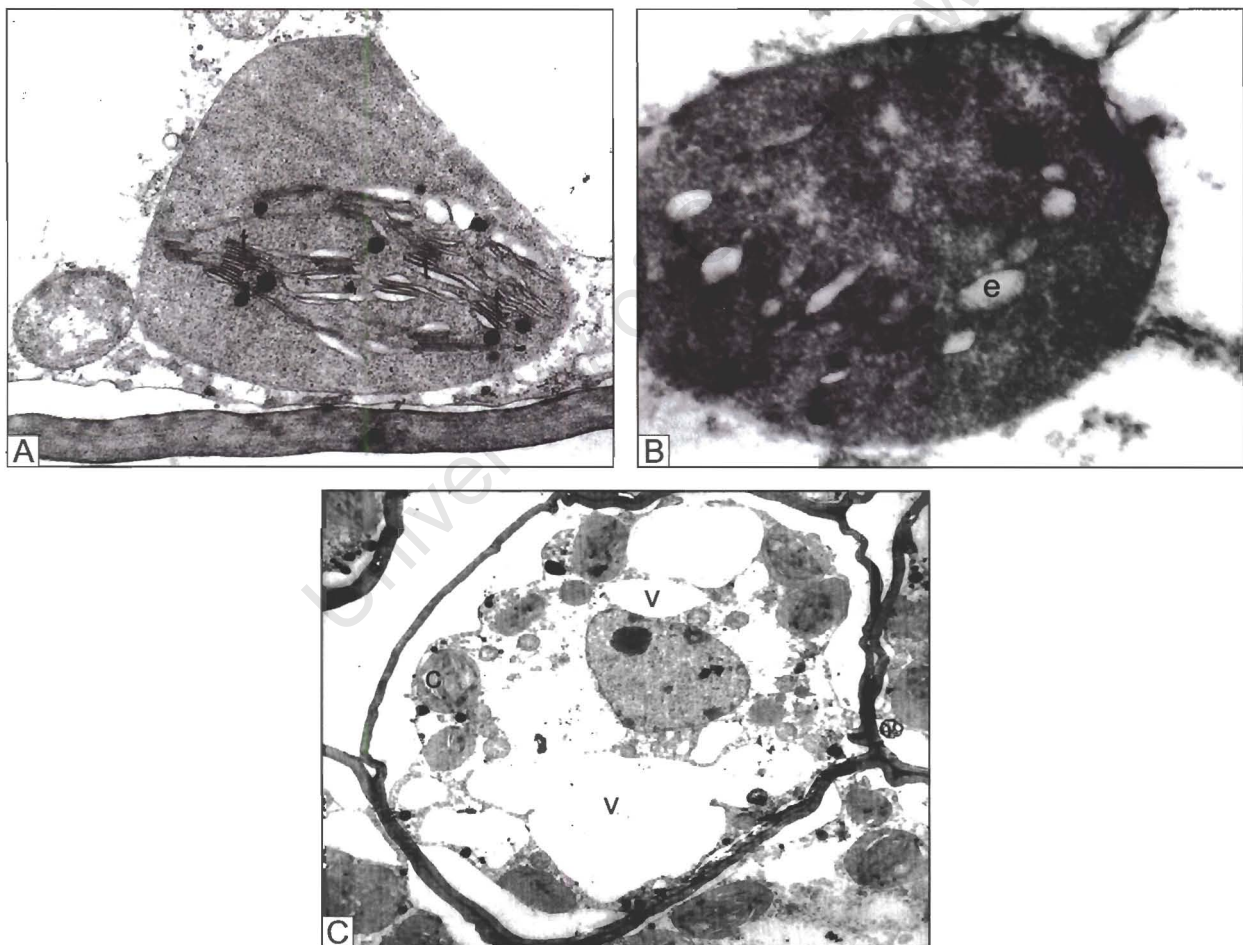


Figure 2.11 (A) Chloroplast of mesophyll cell from slowly dried *X. humilis* at 65 to 50% RWC. Note that thylakoid membranes (t) are still present. $1\text{cm}=0.6\mu\text{m}$. (B) Chloroplast of mesophyll cell from slowly dried *X. humilis* at 65 to 50% RWC. There is no arrangement into granal stacks and vesicles (e) are present within the chloroplast. $1\text{cm}=0.4\mu\text{m}$. (C) Mesophyll cell from slowly dried *X. humilis* at 65 to 50% RWC. Note the proliferation of small vacuoles (v). Chloroplasts (c). $1\text{cm}=2.9\mu\text{m}$.

As with the rapidly dried *C. wilmsii*, cells at intermediate drying stages (between 65 and 50% RWC) of rapidly dried *X. humilis* were not uniform (Figs. 2.11 D, E & F). Pockets of cells had the appearance of that in Figure 2.11 D, exhibiting no plasmamembrane withdrawal or vacuole shrinkage and there was no sub-division of vacuoles. The degree of damage was considerably greater in other cells, as shown in Figure 2.11 E, with membrane rupture and some plasmamembrane withdrawal. Chloroplasts, in general, appeared virtually unchanged by dehydration with starch grains and granal stacks clearly visible (Fig. 2.11 D & F). Some, however, seemed to have undergone some membrane degradation (Fig. 2.11 G).

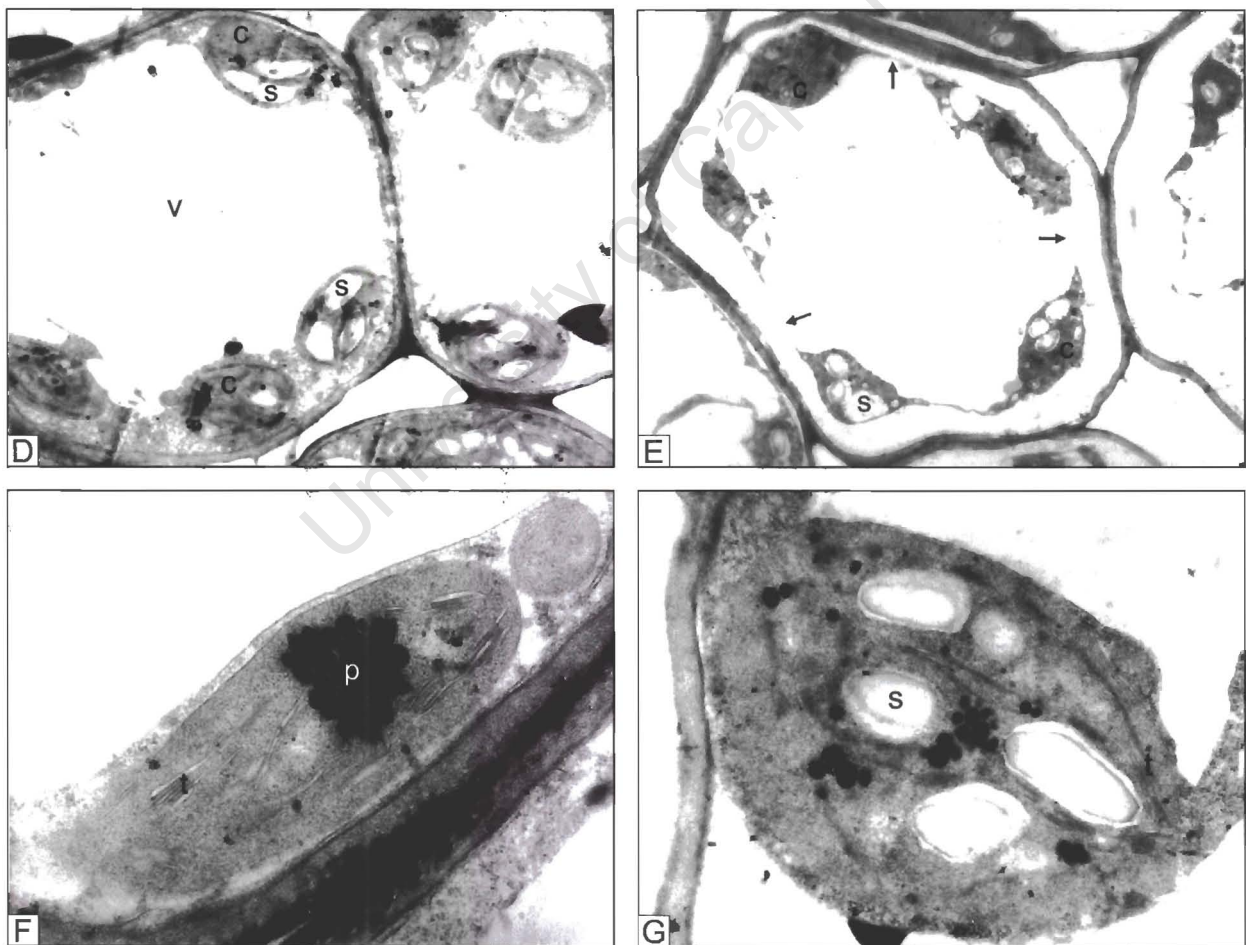


Figure 2.11 (D) Undamaged mesophyll cell from rapidly dried material. The large vacuole (v) is still present and there is no plasmamembrane withdrawal. Chloroplasts (c); starch grains (s). $1\text{cm}=2.3\mu\text{m}$. **(E)** Damaged mesophyll cell from rapidly dried *X. humilis* at 65 to 50% RWC. There are several areas of membrane rupture (indicated by arrows). Chloroplasts (c); starch grains (s). $1\text{cm}=2.8\mu\text{m}$. **(F)** Chloroplasts of mesophyll cell from rapidly dried *X. humilis* at 65 to 50% RWC. Thylakoids (t) are still present, although there are numerous plastoglobuli (p). $1\text{cm}=0.4\mu\text{m}$. **(G)** Chloroplast of mesophyll cell from rapidly dried *X. humilis* at 65 to 50% RWC. There is some thylakoid breakdown, but starch grains (s) are still present. Thylakoid membranes (t). $1\text{cm}=0.6\mu\text{m}$.

In slowly dried tissue between 40 and 25% RWC, there is again very little evidence of plasmamembrane withdrawal or rupture (Fig. 2.12 A). The vacuole had subdivided into numerous vesicles with tonoplasts intact. The chloroplasts of these cells (Fig. 2.12 B) were consistent with previous investigations (Farrant, 2000) - the thylakoid membranes had been almost entirely dismantled and there were numerous plastoglobuli present.

At water contents between 40 and 25% RWC, the majority of cells of rapidly dried tissue showed similar damage to those of the previous drying stage. Although vacuoles had subdivided, there were membrane breaks and in some cases, plasmolysis (Fig 2.12 C). Chloroplasts had retained their thylakoid membranes and starch grains (Fig. 2.12 D) and appeared in some cases to have lost their outer membrane (Fig. 2.12 C).

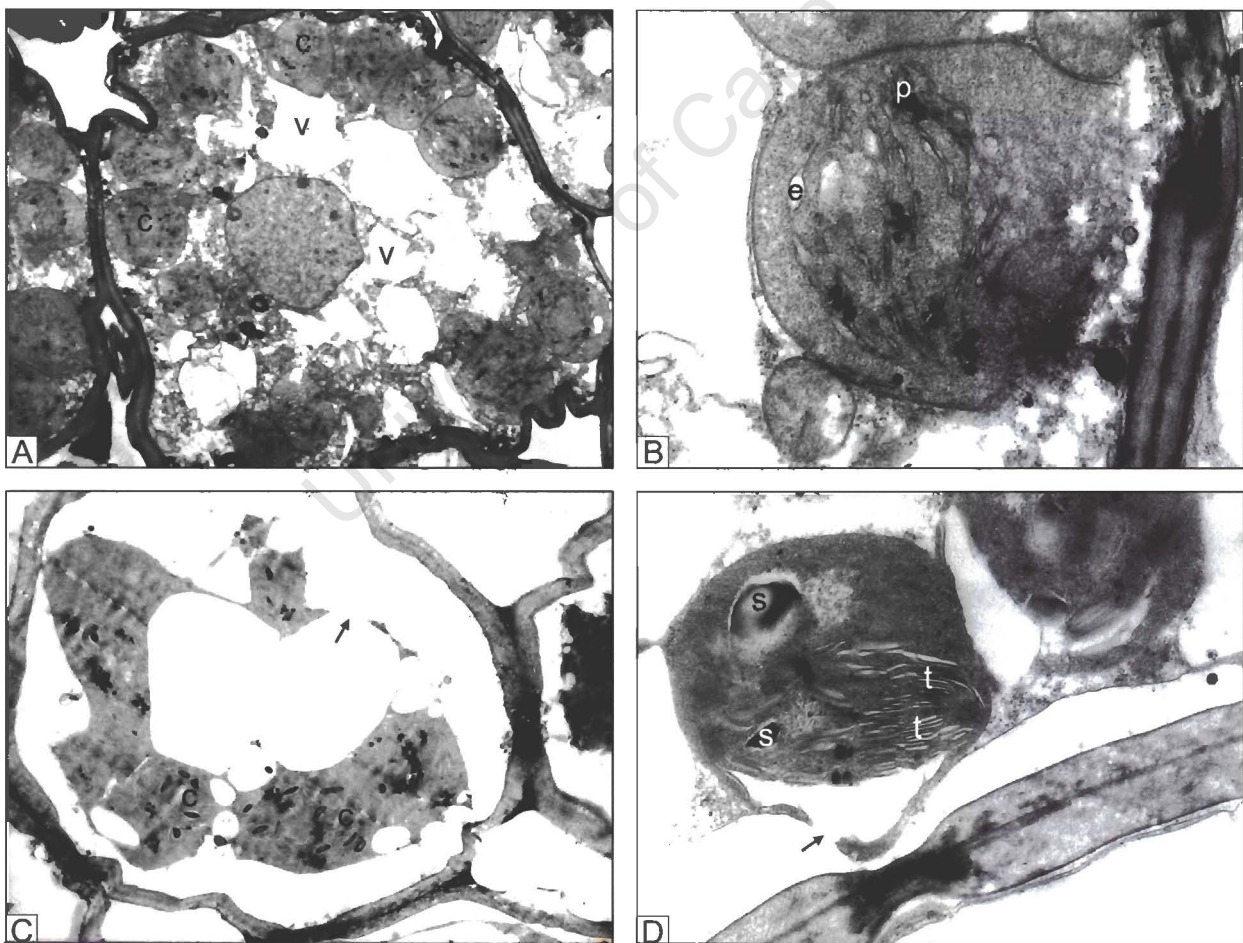


Figure 2.12 (A) Mesophyll cell from slowly dried *X. humilis* at drying stage 3 (40 to 25% RWC). There is no plasmamembrane withdrawal and the vacuole (v) has sub-divided. Chloroplasts (c). 1cm=3.0 μ m. (B) Chloroplasts of mesophyll cell from slowly dried *X. humilis* at drying stage 3 (40 to 25% RWC). Very little membrane structure exists in the chloroplast and there are several vesicles (e) and plastoglobuli (p) present. 1cm=0.6 μ m. (C) Mesophyll cell from rapidly dried *X. humilis* at drying stage 3 (40 to 25% RWC). There are membrane breaks (indicated by arrows) and chloroplasts (c) have lost their double membrane. 1cm=2.3 μ m. (D) Chloroplasts of mesophyll cell from rapidly dried *X. humilis* at drying stage 3 (40 to 25% RWC). Note the thylakoid membranes (t) and starch grains (s). Membrane breakage is indicated with an arrow. 1cm=0.8 μ m.

Cells from slowly dried *X. humilis* had intact membranes and organelles in the dry state (Fig. 2.13 A). The plasmamembrane remained appressed to the cell wall and large vacuoles were visible, the content being granular (electron-dense) in appearance. Chloroplasts exhibited the vesicles resulting from thylakoid breakdown and there were no starch grains present (Fig. 2.13 B). Upon rehydration, the cell reverted back to the appearance of the control cells, with one large central vacuole (Fig. 2.13 C). The photosynthetic membranes were reconstituted and starch grains were once again visible (Fig. 2.13 C).

When rapidly dried, however, the majority of *X. humilis* cells showed plasmamembrane withdrawal and tearing (Fig. 2.13 D). Chloroplasts had undergone very little breakdown of the photosynthetic membranes and the thylakoids appeared to be disarranged within the chloroplasts. In some cases, the outer membrane of the chloroplast appeared to be damaged as well (Fig. 2.13 D). Upon rehydration, there was almost complete dissolution of cell contents (Fig. 2.13 E).

A common feature of all the cells represented here was that vacuoles did not show accumulation of any electron-dense contents during the drying stages, even those at lower water contents. Only in dry tissue, could granular material be seen. This is noteworthy, since the presence of electron dense material within vacuoles (observed in *C. wilmsii* and *X. humilis*) may indicate stabilisation of the tonoplast, and the cell as a whole, by the filling of vacuoles with sugars, proteins and/or compatible solutes (Farrant, 2000).

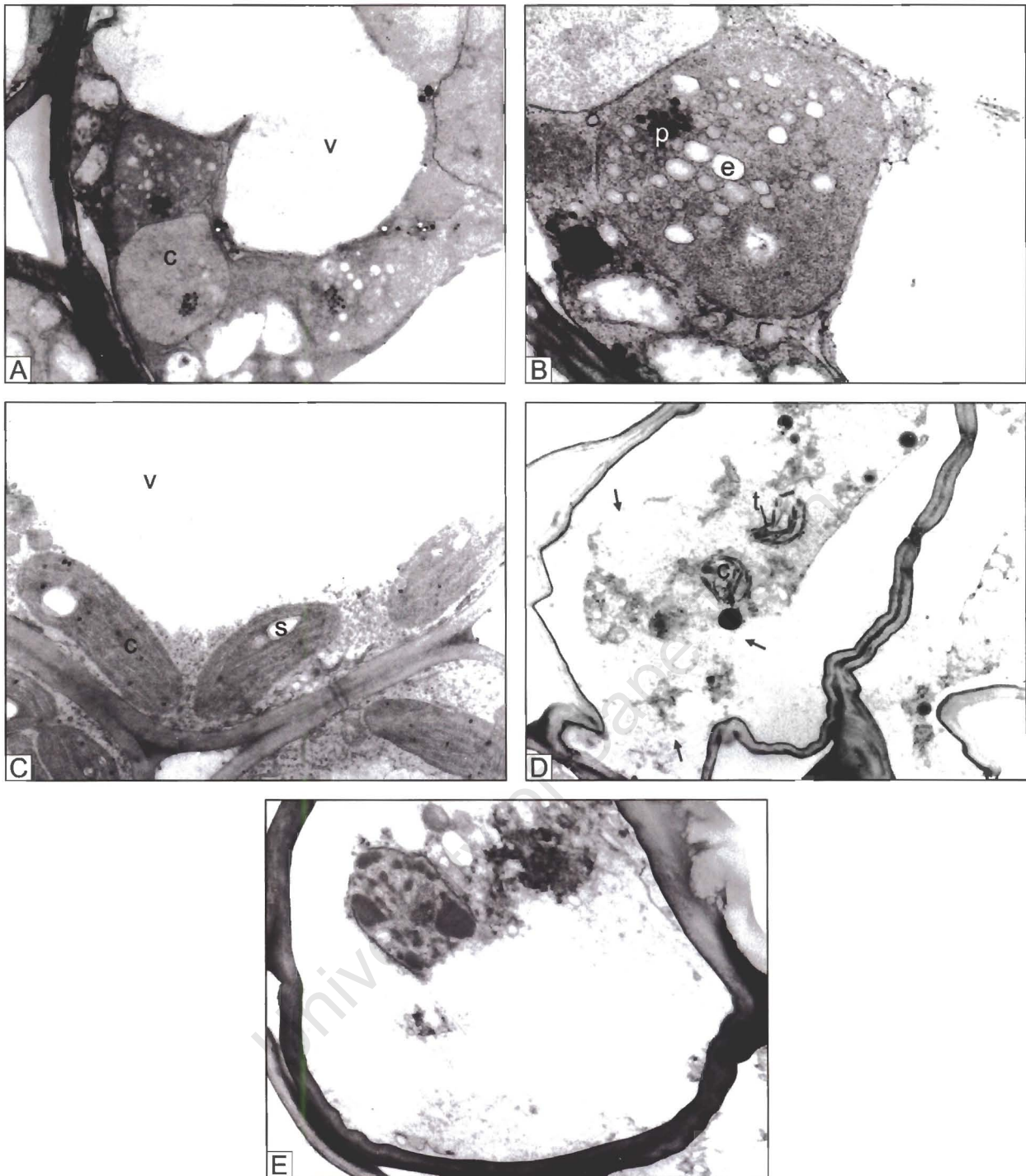


Figure 2.13 (A) Mesophyll cell of slowly dried *X. humilis*. The plasmamembrane is appressed to the cell wall and there is electron dense material in the vacuole (v). Chloroplasts (c). $1\text{cm}=1.3\mu\text{m}$. (B) Chloroplasts of mesophyll cell of slowly dried *X. humilis*. The thylakoids have been broken down and only small vesicles (e) and plastoglobuli (p) are present. $1\text{cm}=0.6\mu\text{m}$. (C) Mesophyll cell of rehydrated slowly dried *X. humilis*. The cell has the appearance of a control cell with a large vacuole (v) and chloroplasts (c) situated at the cell periphery. Starch grains (s). $1\text{cm}=1.3\mu\text{m}$. (D) Damaged mesophyll cell of rapidly dried *X. humilis*. There are membrane breaks (indicated by arrows) and plasmamembrane withdrawal. In chloroplasts (c) of rapidly dried *X. humilis* mesophyll cells, thylakoids (t) are still present and the outer membrane appears to be damaged. $1\text{cm}=2.8\mu\text{m}$. (E) Mesophyll cell of rehydrated rapidly dried *X. humilis*. There has been almost complete cellular dissolution. $1\text{cm}=1.4\mu\text{m}$.

3.6. Sugars

Figures 2.14 A, B & C show the contents of glucose, fructose and sucrose respectively for rapidly and slowly dried leaves of *C. wilmsii* and *X. humilis*. The glucose and fructose contents remained low in both species during rapid and slow drying. The major trend was a decrease in both glucose and fructose from hydrated to dry tissue (Fig. 2.14 A & B), although largely, the differences are not significantly different. The glucose content of slowly dried *C. wilmsii* and fructose content of rapidly dried *C. wilmsii* did show an increase in dry tissue, but again the difference was not significant. However, the unusual decrease in both hexose sugars during the intermediate drying stages (stage one and two) can be seen over all drying treatments.

In general, the quantities of all sugars (glucose, fructose and sucrose) were greater than previously reported for other resurrection plants by Ghasempour *et al.* (1998) and Norwood *et al.* (1999), although the ratios of Glu:Suc and Fru:Suc fell in to the same range (Glu:Suc = 1:1.1 – 1:5.7 and Fru:Suc = 1:0.4 – 1:10.8 for hydrated material; Glu:Suc = 1:3.3 – 1:49.1 and Fru:Suc = 1:2 – 1:136 for dry material). This difference is attributable to the environment in which the experimental plants used in the studies of Ghasempour *et al.* (1998) and Norwood *et al.* (1999) were maintained prior to and during desiccation. The light intensities used were low and possibly affected the sugar metabolism.

Sucrose, however, showed a substantial increase in both drying treatments of *C. wilmsii* and *X. humilis* (note the difference in scale of y-axes between Fig 2.14 C and Figs. 2.14 A & B). This increase was most marked in slowly dried *C. wilmsii*, which showed an accumulation of sucrose that was more than two-fold greater than that of slowly dried *X. humilis*. In fact, even rapidly dried *C. wilmsii* accumulated more

sucrose than slowly dried *X. humilis*. Both species accumulated less sucrose when rapidly dried. However, relative to *X. humilis*, *C. wilmsii* showed a proportionally larger reduction in sucrose accumulation due to rapid dehydration. In both species, the induction of sucrose accumulation occurred at a late stage in dehydration, particularly in the case of *C. wilmsii*, where this accumulation was considerable.

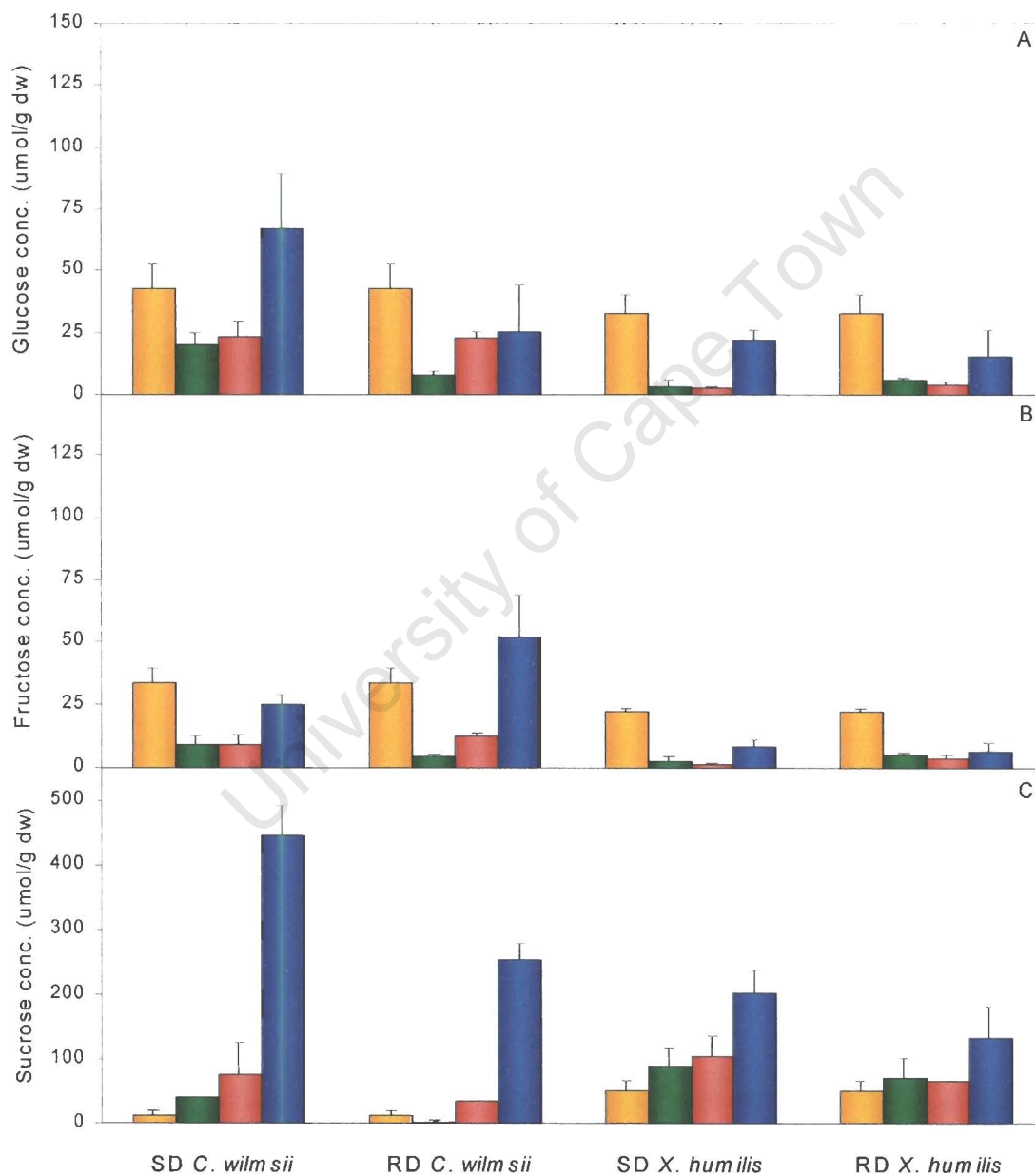


Figure 2.14. (A) Glucose, (B) fructose and (C) sucrose content of slowly (SD) and rapidly dried (RD) leaves of *C. wilmsii* and *X. humilis* at various stages during dehydration. (■) – Control/hydrated tissue ; (■) – Tissue at drying stage 1; (■) – Tissue at drying stage 2; (■) – Dry tissue. Note the difference in y-axis scale.

3.7. Slot blot analysis

Figure 2.15 A & B depicts the results of the slot blot analysis performed on *C. wilmsii* and *X. humilis*. The *rbcL*, *rbcS* and *lhca* genes, which were tested for, encode chloroplast-localised proteins. *rbcS* and *lhca* are two nuclear-encoded multi-gene families, the former coding for the small subunit of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase), while the latter encodes for a chlorophyll a/b binding protein associated with PSII. The *rbcL* gene, which codes for the large subunit of Rubisco, is chloroplast-encoded. The 18S rRNA was included as a loading control.

Interestingly, there were no *rbcS* transcripts detected at all, except in extremely low quantities in slowly dried *X. humilis* that had been rehydrated for 24 hours. It is uncertain whether this was due to the inadequate binding of probes to the transcripts (there was also no transcript present in control material), or whether there was, in actuality, no new expression of the gene. If the latter is true, accumulation of the protein must occur during drying, being stored in the dry state for use upon rehydration.

C. wilmsii showed the presence of both *lhca* and *rbcL* transcripts, but substantially more of the latter were present. The expression of the *lhca* genes increased substantially during drying (both slowly and rapidly), however and there were little or no transcripts after rehydration for 24 hours, suggesting that the majority or all of those *lhca* transcripts present in the dry tissue had been translated and the transcript degraded. After 48 hours the transcript was once again present. Rapid drying did not impair expression of the gene, and again there was very little new transcription during rehydration.

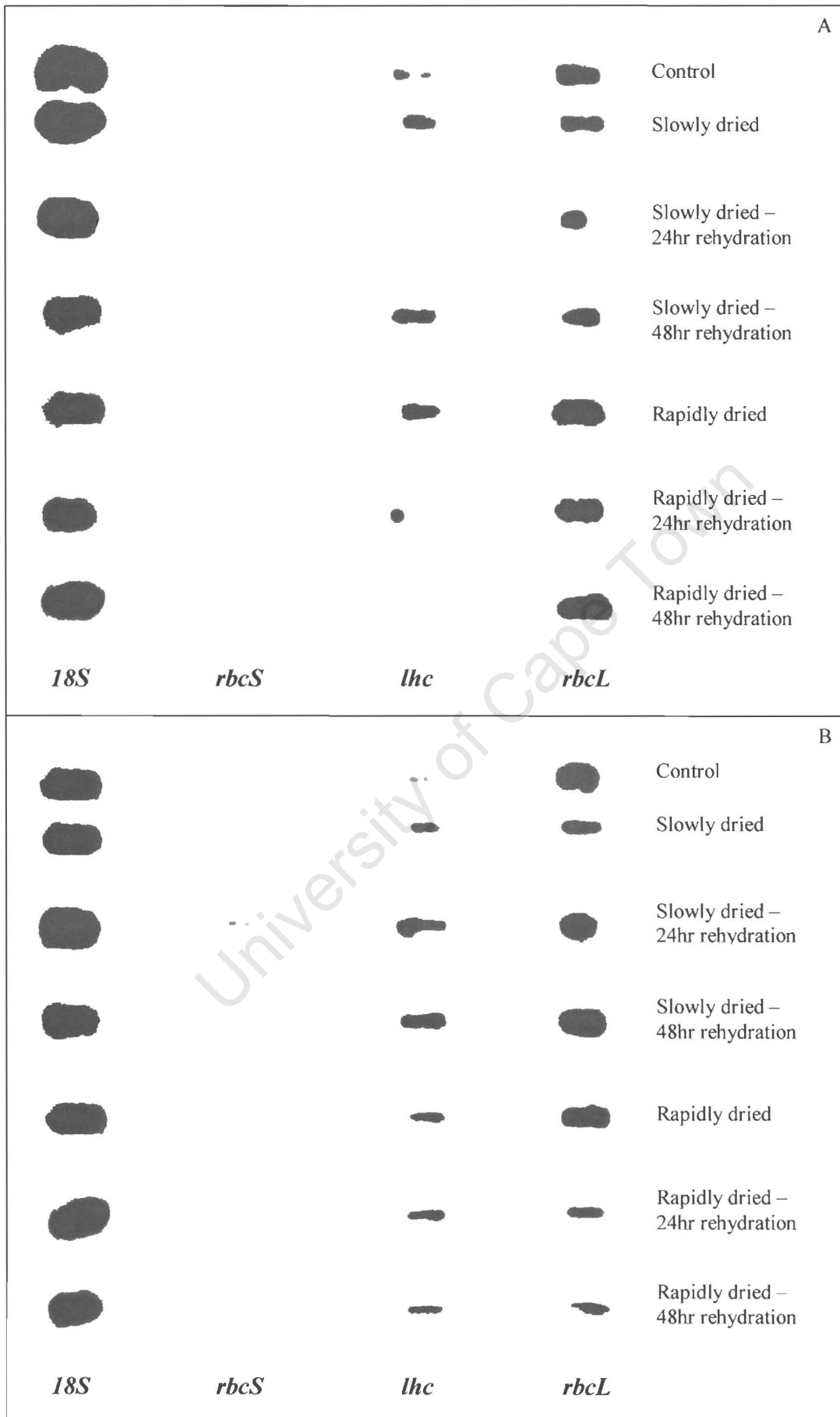


Figure 2.15. Expression of *rbcS*, *lhcb* and *rbcL* genes in slowly and rapidly dried leaf tissue of **(A)** *C. wilmsii* and **(B)** *X. humilis* plants (dry, rehydrated for 24 hours and rehydrated for 48 hours). Slot blot analysis was performed using 1 µg total RNA for each blot.

The *rbcL* transcript was present in dry tissue and both the rehydration stages. However, rapidly dried tissues exhibited a noticeably larger degree of transcript accumulation, both in dry and rehydrated leaves.

X. humilis followed a similar trend to *C. wilmsii* in that, in general, there was more expression of the *rbcL* gene than the *lhc*. The *lhc* transcript, in fact, was present in extremely low quantities in fully hydrated tissue. The expression of this gene was upregulated during rehydration of slowly dried tissue, however, and was present at both 24 and 48 hours. During rehydration of rapidly dried material, the quantity of *lhc* transcripts appeared to be reduced, although they were present in both rehydration stages.

The *rbcL* gene was expressed in fully hydrated *X. humilis* in comparable amounts to *C. wilmsii* at the same water content. The transcript was still observed in slowly dried tissue of *X. humilis* and appeared to be upregulated during rehydration, to a greater extent than *C. wilmsii*. Although rapidly dried *X. humilis* leaves had accumulated the transcripts, the concentration of the transcript was considerably reduced at both rehydration stages.

4. Discussion

Both *C. wilmsii* and *X. humilis* are classified as modified desiccation-tolerant plants, in that they are thought to rely primarily on protection during dehydration to survive the desiccated state. Previous research has indeed found that protective mechanisms play a significant role in the survival of these two resurrection species (Tuba *et al.*, 1993a,b; Farrant and Sherwin, 1998; Sherwin and Farrant, 1998; Vicré *et al.*, 1999; Farrant, 2000). Oliver *et al.* (1998) have suggested that this reliance on protection necessitates slow drying in order to allow sufficient time to institute the required mechanisms.

However, these data show that of the two species investigated in this study, *C. wilmsii* does survive rapid drying in the order of 6-8 hours. Both rapidly and slowly dried leaves showed similar changes in photosynthetic functioning (both quantum efficiency and pigment content, as well as expression patterns of *lhc* and *rbcL* genes) during drying and recovery of these parameters on rehydration were comparable as well. Folding of the cell walls, too, occurred similarly in both slowly and rapidly dried *C. wilmsii*. This protective mechanism occurred in the second drying stage (65 to 50% RWC) and appears to have been rapidly induced, with almost complete wall folding visible in the 40 to 25% RWC range.

The rapidly dried tissue, however, showed localised areas where cells had incurred some damage, indicated by membrane tearing and some derangement of cell contents. Thus, not all cells were able to institute suitable protective mechanisms to maintain membrane integrity. However, damaged cells were of a sufficiently small number so as not to significantly increase electrolyte leakage.

The possibility of impairment of protection would seem to be supported by the pattern of sucrose accumulation in rapidly dried and slowly dried leaf tissue. Sucrose accumulation occurred, as would be expected, in both rapidly and slowly dried *C. wilmsii*, but rapid dehydration resulted in a pronounced reduction in the amount of sucrose accumulated. In both drying treatments, this accumulation occurred relatively late in dehydration, which may correlate with the lack of electron density seen in the vacuoles during dehydration up to and including the 40 to 25% drying stage for both *C. wilmsii* and *X. humilis*.

Glucose and fructose, conversely, were present in small quantities throughout the drying treatments. This follows the trend observed in *C. plantagineum*, where the quantity of these two hexose sugars remained low in fully hydrated and dry tissue (Norwood *et al.*, 1999). However, the unusual decrease in both sugars seen in drying stages one and two has not yet been observed in any other species. It has been reported by Whittaker *et al.* (2001) that in the two resurrection plants *Xerophyta viscosa* and *Sporobolus stapfianus*, the level of invertase activity increases in the initial stages of dehydration and remains constant until at least 29% RWC. Invertase is responsible for the hydrolysis of sucrose and its presence during dehydration is evidence of some sucrose cycling even while sucrose is being accumulated (Whittaker *et al.*, 2001). Thus, the accumulation of hexose sugars that occurs after drying stage two may be due to residual invertase activity.

The low levels of glucose and fructose suggest that they play a minor role, if at all, in sucrose mobilisation. It has been found by Bianchi *et al.* (1991) that in both *C. wilmsii* and *C. plantagineum*, the main carbohydrate source at 100% RWC is 2-octulose, and the authors propose that this is the main source of sucrose within the

leaves. Contradicting this are observations made by Norwood *et al.* (1999), which indicate that 2-octulose is not the main source of carbon for sucrose. They suggest that a more likely explanation is that sucrose is mobilised in older leaves and translocated to younger leaves, resulting in the older leaves being more desiccation sensitive. However, given the belated accumulation of sucrose during dehydration, the translocation of reserves at low water contents does not seem feasible for *C. wilmsii*.

In light of the impairment of sucrose accumulation in *C. wilmsii*, it is likely that other protective mechanisms were also affected by rapid drying as well. However, the impairment of protection by rapid drying was not sufficient to cause widespread damage to *C. wilmsii* tissue (as indicated by the solute leakage of the leaves) and this low level of injury did not compromise the survival of this species. In addition, it would appear that, on rehydration, there is little evidence of the subcellular damage seen during rapid drying, which may indicate some form of repair process.

X. humilis, despite reaching similar final water contents whether rapidly or slowly dried, did not recover from rapid dehydration. The breakdown of thylakoids, so important to the survival of *X. humilis* (Tuba *et al.*, 1993a,b; Sherwin and Farrant, 1998), was fairly gradual in slowly dried tissue of this species, occurring over a large range of water contents. Given the time taken for this degradation, it is not surprising that rapidly dried cells could not carry out the procedure adequately. By the last drying stage it was clear that most cells of rapidly dried material had not instituted the dismantling of the photosynthetic apparatus. Added to this, the degradation of chlorophyll was impaired by the rapid dehydration. This inability to carry out the dismantling of the photosynthetic components is reflected in the delayed decline in F_V/F_M of rapidly dried *X. humilis* leaves.

The accumulation of *lhc* transcripts, too, was affected by rapid drying in *X. humilis*. The concentration of this transcript was reduced in rapidly dried tissue, while the *rbcL* expression appeared to be upregulated during rapid drying. There was a noticeable upregulation of both genes in rehydrated leaves of slowly dried plants, corresponding with the reconstitution of the photosynthetic machinery. This did not occur in rapidly dried material on rehydration, however. There may be two reasons for this. First, the incomplete dismantling of thylakoids, and resultant light stress, may prompt a down-regulation or inhibition of gene products associated with photosynthesis. It has been found that high irradiance does dampen the circadian rhythms of transcript accumulation observed in gene expression (Mansfield and Snaith, 1984; Hennessey *et al.*, 1993), however, the expression patterns of the *lhc* gene would likely follow the same trend. The second and more likely cause is that the lack of transcripts, particularly the *rbcL* transcript, may have resulted from damage to the cell and especially the chloroplast, which would be a primary site of free radical injury resulting from light stress (the *rbcL* protein is chloroplast-encoded, while *lhc* is encoded by nuclear genes). The high expression of *rbcL* in rapidly dried material may also result from increased damage in the chloroplast, incurred during drying. In addition, the reduced expression of the *lhc* transcript in rapidly dried tissue suggests that there was damage to the nuclear genome as well.

Neither slowly nor rapidly dried *C. wilmsii* showed expression of the *lhc* gene after 24 hours of rehydration, suggesting that it is rapidly turned over and not re-transcribed during rehydration. In general, the expression of the *lhc* gene was lower than that of the *rbcL*. A possible explanation for this and the reduced accumulation in slowly dried material is that of feedback repression of the expression of the *lhc* protein. Sheen

(1990) found that the transcriptional activity of several photosynthetic gene promoters was inhibited by the presence of glucose, fructose and sucrose. Thus, if sucrose levels remain high immediately following rehydration, the transcription of certain genes may be affected. In addition, Van-Oosten and Besford (1994) found that feeding detached leaves with sucrose or glucose resulted in down-regulation of the *rbcS* gene. The lack of *rbcS* transcripts in both species may be attributable to this feedback repression. Bernacchia *et al.* (1996) found that this gene is down-regulated during dehydration in *C. plantagineum* and appears at later stages of rehydration. This down-regulation may, alternatively, indicate that the *rbcS* subunit is stored in a stable form during rehydration, or is not required by the plants immediately after rehydration. The latter, however, seems unlikely, particularly in the case of the poikilochlorophyllous *X. humilis*, which must reconstitute its dismantled photosynthetic apparatus upon rehydration.

In general, the rapidly dried cells of *X. humilis* again showed the disparity of cell status with respect to damage, as seen in rapidly dehydrated *C. wilmsii*. At lower water contents, however, the damage to cells was more widespread in *X. humilis* than in *C. wilmsii*. This increased damage to membranes was manifested as the rise in solute leakage from the rapidly dried leaves. Unlike *C. wilmsii*, *X. humilis* could not limit injury to membranes at low water contents.

Rapidly dried *X. humilis*, too, did not show the filling of vacuoles seen in dried mesophyll cells of slowly dried material, proposed to be important in mechanical stabilisation of cells (Farrant, 2000). This was the case for both resurrection species, but the cell wall folding of *C. wilmsii* may give it an advantage in terms of mechanical protection (Vicré *et al.*, 1999). In addition, *C. wilmsii* showed much larger sucrose

accumulation, with rapidly dried *C. wilmsii* leaves exhibiting a higher concentration of sucrose than slowly dried *X. humilis*. Whether this higher concentration is associated with a greater degree of protection, or whether the degree of protection afforded is relative to the species concerned, is unknown. In both drying treatments, this accumulation occurred relatively late in dehydration, which may correlate with the lack of electron density seen in the vacuoles of the drying treatments for both *C. wilmsii* and *X. humilis*.

Thus, it seems that the strategy of protection employed by *X. humilis* is adversely affected by rapid drying. The avoidance of light stress by dismantling the photosynthetic apparatus is a gradual process, which requires much time, while the proposed primary mechanism of mechanical stabilisation (vacuole filling) occurs at a late stage in dehydration. Thus, rapid drying gives this species insufficient time to carry out these mechanisms, resulting in membrane breakage and extensive free-radical mediated damage (due to light stress).

The morphology of *X. humilis* has xerophytic features which function to retard water loss. It has sclerenchymatous leaves and grows in dense leaf mats (including the old, dead leaf bases), all of which slows the rate of water loss. This, under natural circumstances, provides this species with enough time to carry out the processes it requires to survive.

C. wilmsii, however, has a swiftly instituted protection strategy, involving leaf curling and accumulation of anthocyanins to avoid light stress (Sherwin and Farrant, 1998; Farrant, 2000), as well as cell wall folding in order to avoid mechanical strain (Vicré *et al.*, 1999; Farrant, 2000). *C. wilmsii* has few features to prevent excessive water loss – its leaves are broad, consist largely of spongy parenchyma and have few

leaf hairs. In the harsh environment in which these plants occur, water loss may be relatively rapid. Although rapid drying does affect certain protective mechanisms (resulting in damage), *C. wilmsii* apparently possesses enough rapidly instituted protection to limit the damage and survive. Indeed, the injury to cell ultrastructure during rapid dehydration as compared with that perceived when this material was rehydrated, indicates that there is some degree of repair carried out by *C. wilmsii* on rehydration. In an environment where plants experience constant cycles of drying and rewetting, this strategy of rapidly induced protection in combination with repair would be essential to survival. In this respect, *C. wilmsii* does not conform to the character of a typical modified desiccation-tolerant plant.

CHAPTER 3

THE EFFECT OF METABOLIC INHIBITORS ON THE RECOVERY OF SLOWLY AND RAPIDLY DRIED *C. WILMSII* AND RAPIDLY DRIED *X. HUMILIS*.

1. Introduction

It has already been established that during desiccation, resurrection plants undergo metabolic changes in order to survive. In recent years, a most productive means of studying these changes has been to study patterns of gene expression during water stress (review by Ingram and Bartels, 1996). Those genes upregulated presumably code for proteins that play a role in the plant's ability to survive desiccation. But, concerning angiosperms, very little is known about what is required upon rehydration. Since angiosperms are modified desiccation-tolerant plants, most work regarding desiccation tolerant angiosperms has focussed on protection occurring during dehydration, as this is proposed to be critical in order to survive desiccation. Very little work has therefore centred on the extent to which angiosperms utilize or require protection and repair in order to recover.

Since poikilochlorophyllous resurrection plants require the reconstitution of photosynthetic components, it follows that they may require upregulation of genes during rehydration to facilitate this. Dace *et al.* (1998) investigated the temporal organization of gene expression in *X. humilis* by determining the extent to which initial recovery was dependent on *de novo* transcription or translation during rehydration. They found that while inhibition of transcription on rehydration of leaf explants has no apparent effect on initial recovery of the explants, inhibition of

translation prevented the reconstitution of the photosynthetic apparatus and thus, the leaves were not able to regain viability. They propose that mRNAs for chlorophyll synthesis and recovery of electron transport in chloroplasts are stored in a stable form in the dried leaves and are translated upon rehydration. However, evidence suggested that new genomic transcription was necessary after approximately 18 hours for complete recovery of PSII functioning. *C. wilmsii* is homoiochlorophyllous, however, and requires no reconstitution of the photosynthetic apparatus. The first aim of this study, therefore, was to determine the reliance of *C. wilmsii* on *de novo* transcription and translation upon rehydration after slow drying. In order to compare the results with those of *X. humilis* adequately, data from Dace *et al.* (1998) will be included.

It has been shown in the previous chapter that *X. humilis* could not survive rapid drying, ostensibly due to the time required to institute protective mechanisms. *C. wilmsii*, conversely, did survive rapid drying, and it was proposed that this was due to the rapidly induced protection and possible repair ability of the species. However, both species showed impairment of at least some protective mechanisms during rapid drying and this impairment may extend to protein synthesis. The second aim of this experiment was to assess the importance of *de novo* synthesis of mRNA and proteins on rehydration of rapidly dried *C. wilmsii* and *X. humilis*.

2. Materials and Methods

2.1. Plant material

Plants were collected and maintained as described in section 2.1. in Chapter 2.

2.2. Dehydration and rehydration

Plants were slowly dried by withholding water from the soil and allowing them to reach an air-dry state. Rapid drying was accomplished using the flash drying apparatus described in Chapter 2 (section 2.2).

Individual dry leaves of *C. wilmsii* (leaf explants) were rehydrated for 48 hours in water or in one of the inhibitor solutions (the concentrations of which were determined as described below). *X. humilis* leaf explants were rehydrated in a $20\mu\text{g.ml}^{-1}$ actinomycin D solution and a $20\mu\text{g.ml}^{-1}$ cycloheximide solution (taken from Dace *et al.* (1998)). In addition, hydrated explants, which had not been dried, were incubated for 48 hours in water and inhibitor solutions to assess the affect of prolonged inhibition on normal (hydrated) leaf metabolism.

During rehydration, the quantum efficiency of photosystem II, photosynthetic pigment content and cellular ultrastructure were investigated.

2.3. Determination of concentration needed for inhibitor solutions

Naturally (slowly) dried leaf explants were incubated for 48 hours in a series of radioactive solutions of differing concentrations ($0.1\text{--}1.5\mu\text{l}$ label/ml water), in order to determine the concentration which provided optimal radioactive labeling of mRNA transcripts and proteins during rehydration. For labeling of RNA, tritiated uridine

(Sigma) was used, while protein labeling was achieved with a ^{35}S -met, ^{35}S -cys mixture (Promix, Sigma). Total RNA and protein were extracted using Trireagent (Sigma) following the protocol detailed in the Trireagent manual. The degree of labeling was determined by counting the disintegrations per minute of radioactive isotopes using a Beckman LS 5000 TD scintillation counter (Beckman, USA).

Once the optimal labelling concentration of the isotopes had been determined, the concentration of inhibitor required for transcriptional or translational inhibition was ascertained. Dry leaves were then rehydrated in solutions of either distamycin A or cycloheximide at different concentrations and containing the pre-determined quantity of labeling isotopes. Dace *et al.* (1998) made use of actinomycin D as a transcriptional inhibitor for *X. humilis*. However, it is an extremely large molecule and was not able to prevent radioactive labeling of RNA in *C. wilmsii*, suggesting that it was not able to effectively penetrate the cells. For this reason, distamycin A, a smaller molecule, was substituted as an inhibitor of transcription. No such difficulty was experienced concerning cycloheximide, an inhibitor of protein translation. Total RNA and protein were again extracted using Trireagent and the degree of radioactive labeling determined as described above. From this, the necessary concentrations of both inhibitors to prevent the majority of translation and transcription were ascertained, viz. $310\mu\text{g}\cdot\text{ml}^{-1}$ for distamycin A and $220\mu\text{g}\cdot\text{ml}^{-1}$ for cycloheximide.

2.4. Quantum efficiency of photosystem (PS) II

Quantum efficiency of PSII was determined by chlorophyll fluorometry. Readings were taken from leaves rehydrated for 2, 4, 6, 8, 12, 15, 20, 24, 48 and 72 hours for slowly dried *C. wilmsii*. For leaves of slowly dried *X. humilis*, readings were taken

from leaves rehydrated for 2, 4, 8, 12, 18, 24 and 48 hours (Dace *et al.*, 1998). In addition, fluorescence was determined for hydrated *X. humilis* leaves incubated for 18, 24 and 48 hours in water and inhibitor solutions (Dace *et al.*, 1998). For rapidly dried material of both species, readings were taken three to five times during the first five hours of rehydration and subsequently at 8, 12, 24 and 48 hours of rehydration. No further readings were taken, as the photosynthetic pigment of leaves rehydrated in inhibitors had degraded.

Readings were performed as described in Chapter 2 (section 2.4).

2.5. Chlorophyll and carotenoid content

The content of photosynthetic pigments of four replicates of leaf tissue was determined after full rehydration (48 hours) for slowly and rapidly dried *C. wilmsii* and rapidly dried *X. humilis* and after a further day in inhibitor solutions (72 hours). Chlorophyll content of slowly dried *X. humilis* was determined for leaves rehydrated for 48 hours as well as hydrated explants incubated for 48 hours in water and inhibitor solutions (Dace *et al.*, 1998).

Extraction and determination of concentration were carried out as described in Chapter 2 (section 2.3).

2.6. Cellular ultrastructure

The ultrastructure of cells from slowly and rapidly dried leaves of *C. wilmsii* and *X. humilis* rehydrated in each treatment was investigated by transmission electron microscopy. Sections were prepared and viewed as described in Chapter 2 (section 2.6).

3. Results

3.1. Rehydration

Figure 3.1 A shows the rehydration time courses for slowly dried *C. wilmsii* leaves that were rehydrated in water and the two metabolic inhibitors, distamycin A and cycloheximide. All three treatments exhibited comparable rehydration rates, reaching 100% RWC between approximately 15 and 20 hours. There was considerable variability in RWC of leaves during the early stages of rehydration in all rehydration treatments, including water.

Figure 3.1 B shows the rehydration courses of *X. humilis* leaves rehydrated in water, actinomycin D (inhibitor of transcription) and cycloheximide (inhibitor of translation). Again, all treatments reached 100% RWC at the same rate, in approximately 12 hours (Dace *et al.*, 1998).

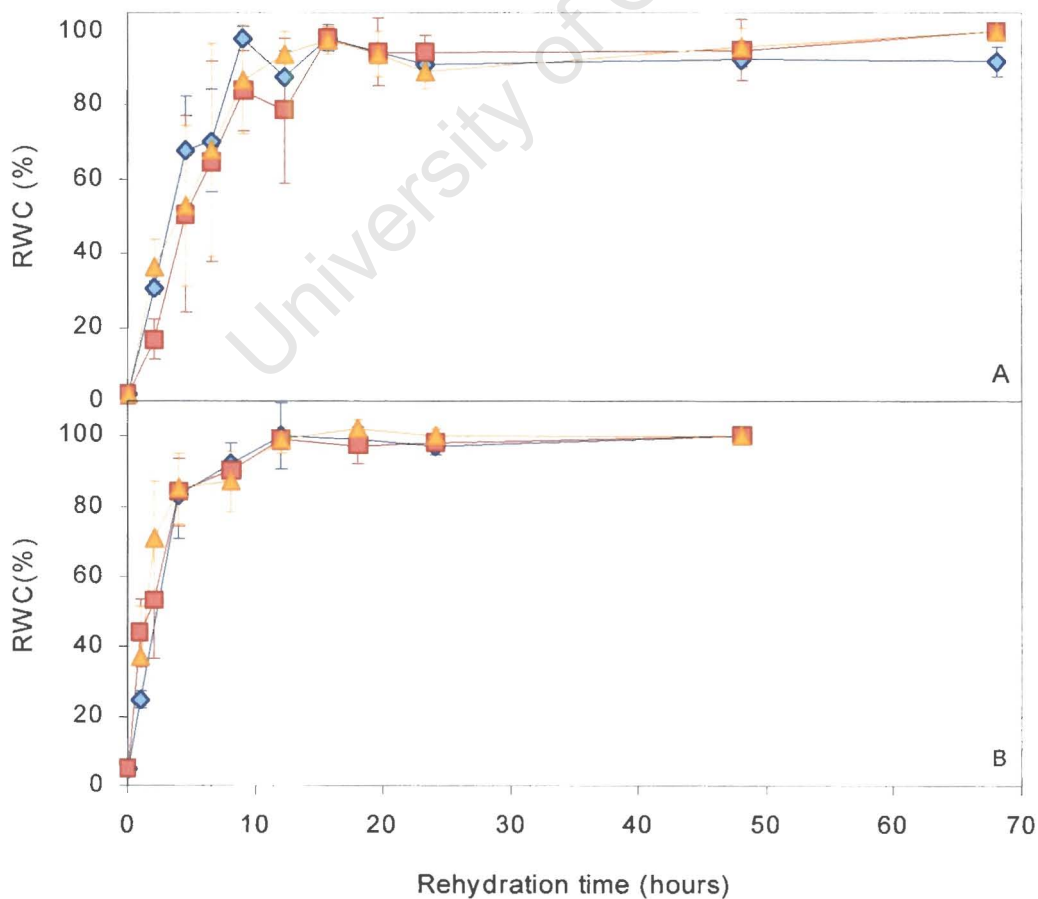


Figure 3.1. Time course for (A) slowly dried *C. wilmsii* and (B) slowly dried *X. humilis* leaves rehydrated in water (◆), distamycin A (actinomycin D for *X. humilis*) (■) and cycloheximide (▲). Data for *X. humilis* after Dace *et al.* (1998).

3.2. Quantum efficiency of PSII

Similarly to the rehydration courses, there was very little difference in the recovery of PSII functioning between the three treatments of slowly dried *C. wilmsii*, shown in Figure 3.2 A. The similarity of the curves indicates that the inhibitor solutions had no deleterious side-effects on the leaves. As leaves rehydrated, F_v/F_M increased to control levels (>0.7). The variability seen in the initial stages of rehydration corresponds with the aforementioned variability of RWC before 20 hours of rehydration. In addition, there is no drop in quantum efficiency for up to 72 hours of rehydration. This suggests that sufficient protein was present to maintain normal photosynthetic metabolism for a considerable period subsequent to rehydration.

There was little increase in F_v/F_M in the first ten hours of rehydration of slowly dried *X. humilis* explants (Fig. 3.2 B(i)) (Dace *et al.*, 1998). Those leaves rehydrated in water, however, showed increasing quantum efficiency thereafter, with full recovery after approximately 48 hours. The quantum efficiency of leaves incubated in water was maintained for at least 48 hours (Fig. 3.2 B(ii)). Those leaves rehydrated in cycloheximide showed essentially no recovery at all ($F_v/F_M < 0.4$) (Fig. 3.2. B(i)) and after 24 hours of incubation of hydrated leaves in this inhibitor solution, there was a considerable decline in F_v/F_M (Fig. 3.2 B(ii)). Thus, *de novo* protein synthesis was required by *X. humilis* for recovery on rehydration and during normal metabolism, new protein synthesis is required after approximately 24 hours. As with the water treatment, there was an initial increase in actinomycin D-rehydrated leaves, but after 18 hours, there was also a decline in quantum efficiency to approximately 0.4 (Fig. 3.2 B(i)). Hydrated leaves, incubated in actinomycin D, however, showed no significant decline in F_v/F_M after 48 hours (Fig. 3.2 B(ii)).

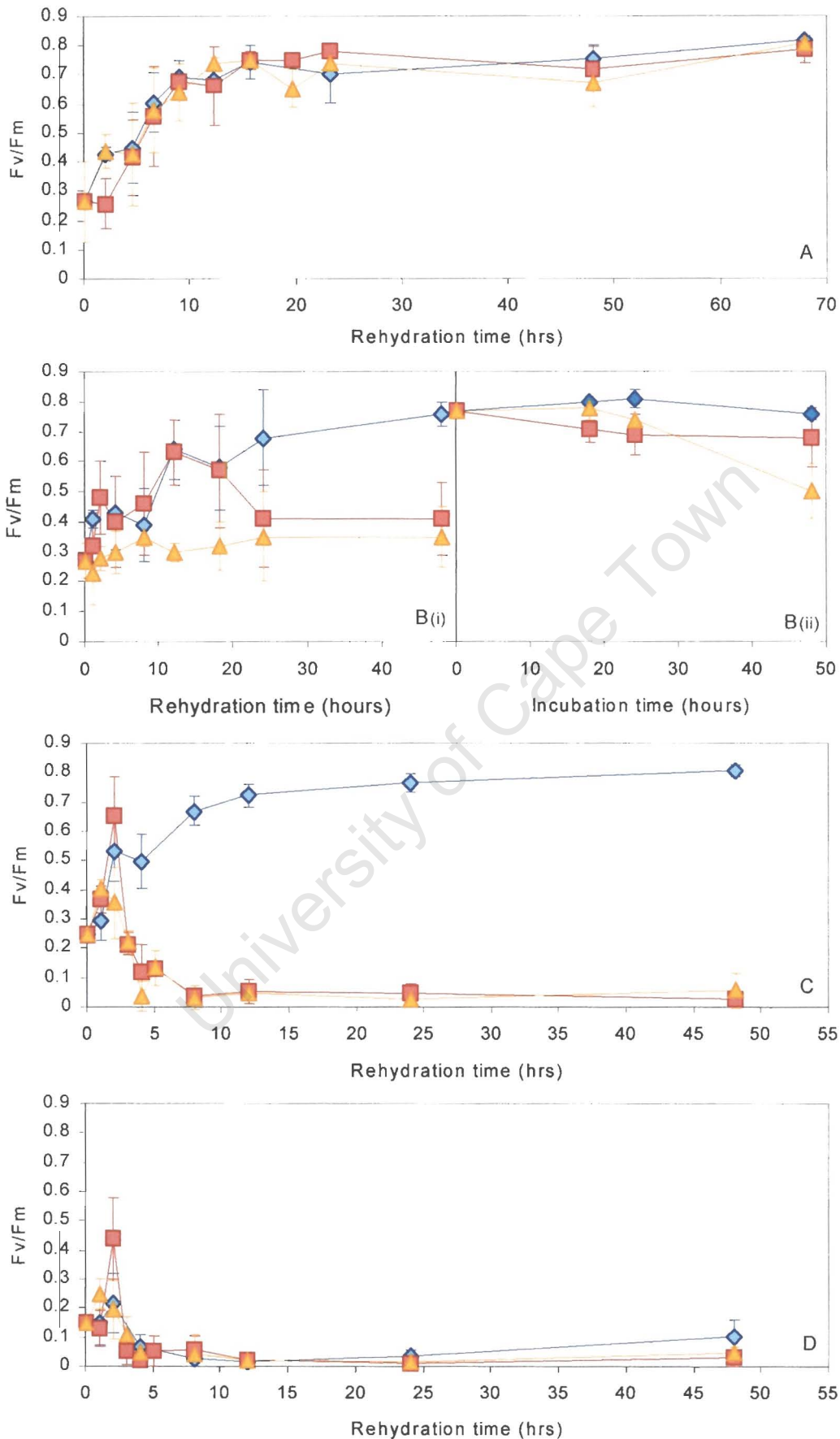


Figure 3.2. Changes in quantum efficiency of photosystem II for (A) slowly dried *C. wilmsii*, (B (i)) slowly dried *X. humilis*, (B (ii)) hydrated *X. humilis*, (C) rapidly dried *C. wilmsii* and (D) rapidly dried *X. humilis* rehydrated/incubated in water (◆), distamycin A (actinomycin D for *X. humilis*) (■) and cycloheximide (▲). Data for slowly dried *X. humilis* after Dace *et al.* (1998).

Figure 3.2 C & D shows the pattern of chlorophyll fluorescence during rehydration of rapidly dried *C. wilmsii* and *X. humilis*. Of the two species, only *C. wilmsii* showed any survival and only when rehydrated in water. During the recovery of the water-rehydrated *C. wilmsii*, quantum efficiency increased more gradually than observed with slowly dried material (Fig. 3.2 A). Although both inhibitors (distamycin A and cycloheximide) prevented eventual recovery of F_v/F_M , sufficient transcripts were present to allow an initial increase of photosynthetic functioning in those leaves rehydrated in distamycin A (which was significantly different from that shown by cycloheximide-rehydrated leaves). These leaf explants exhibited essentially no recovery upon rehydration (Fig. 3.2 C), suggesting that when dried rapidly, protein translation on rehydration is vital for recovery of *C. wilmsii*.

As expected, no rehydration treatments of rapidly dried *X. humilis* survived. Although there appeared to be marginally more recovery of leaves rehydrated in the transcriptional inhibitor (actinomycin D), this was not significantly different (Fig. 3.2 D).

3.3. Chlorophyll and carotenoid contents

Again, there was very little variation in chlorophyll content at the various rehydration stages for each of the three treatments (Fig. 3.3 A) of slowly dried *C. wilmsii*. The dry leaves did lose chlorophyll and carotenoids as has been previously reported, and upon rehydration, there was some resynthesis of both photosynthetic pigments observed in leaves subjected to all treatments. It therefore seems that the proteins required for synthesis and/or maintenance of photosynthetic pigments are laid down during drying.

As previously reported, slowly dried *X. humilis* contained little chlorophyll. Leaf explants rehydrated in both water and actinomycin D recovered chlorophyll to control levels, but the cycloheximide treatment prevented this recovery (Fig. 3.3 B). Loss of chlorophyll from hydrated leaves incubated in inhibitor solutions was not significantly greater than that of explants maintained in water (Figure 3.3 B) (Dace *et al.*, 1998).

The pattern of chlorophyll and carotenoid content of rapidly dried *C. wilmsii* and *X. humilis* leaves upon rehydration is shown in Figures 3.3 C & D. As previously reported, rapidly dried *C. wilmsii* does break down some chlorophyll, but on rehydration in water, regains these photosynthetic pigments to control level (Fig. 3.3 C). When rehydrated in either inhibitor, there was no pigment recovery and, in fact, there was almost total loss of chlorophyll and carotenoids. This was most likely due to bleaching caused by free radical action.

Rapidly dried *X. humilis* retained just over 50% of control chlorophyll (Fig. 3.3 D). This is in stark contrast to the almost total chlorophyll loss seen in naturally dried plants. After rehydration for 48 hours, some pigment was still present, but by 72 hours of rehydration, virtually all pigment had been lost. There was large variability in the leaves rehydrated in metabolic inhibitors, as can be seen in Figure 3.3 D. Some leaves were completely bleached, while others were severely damaged only in certain areas (while the rest of the leaf remained green). By 72 hours, as with water-rehydrated leaves, the majority of pigment had been lost. Again, this was presumably due to free radical action within the cells, resulting in bleaching of the leaf pigment.

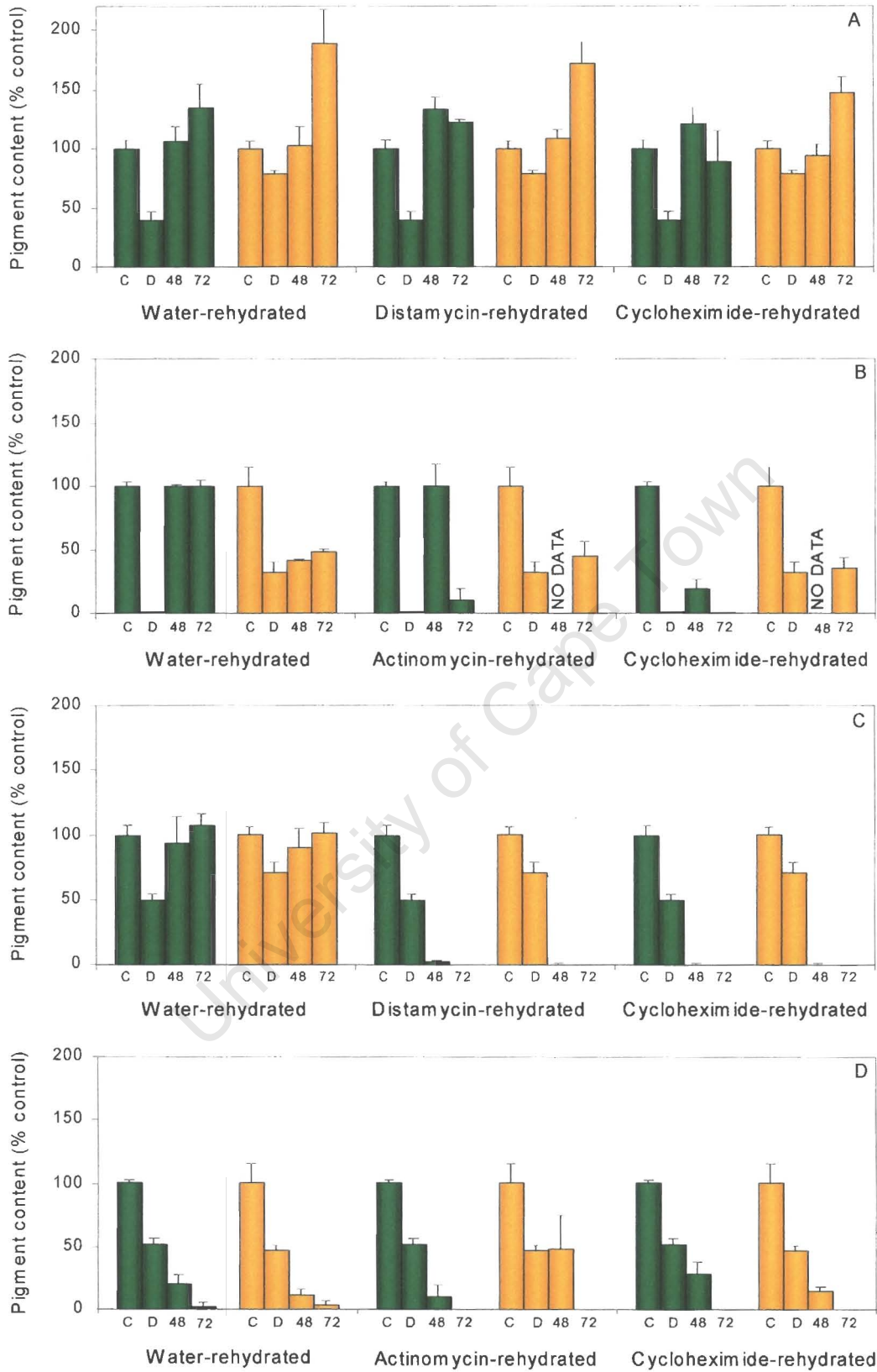


Figure 3.3. Chlorophyll (■) and carotenoid (■) content of (A) slowly dried *C. wilmsii*, (B) slowly dried *X. humilis*, (C) rapidly dried *C. wilmsii* and (D) rapidly dried *X. humilis* rehydrated for 48 and 72 hours. Data for slowly dried *X. humilis* after Dace *et al.* (1998). C – control (undried) tissue; D – dry tissue; 48 – rehydrated for 48 hours; 72 – rehydrated for 72 hours.

3.4. Cellular ultrastructure

The cellular organisation of mesophyll cells of leaves rehydrated in water, distamycin A and cycloheximide are shown in Figures 3.4 to 3.6 respectively. In general, the rehydrated leaves of resurrection plants exhibited cells identical in appearance to control (hydrated) leaves, as can be seen in Figure 3.4 A, depicting a water-rehydrated mesophyll cell. These cells were dominated by a large, central vacuole that pushed all other cell contents (cytoplasm and organelles) to the periphery of the cell. There was no visible damage to membranes or the thylakoids of the chloroplasts (Fig. 3.4 B). Also clearly visible in the chloroplasts were starch grains.



Figure 3.4 (A) Mesophyll cell of slowly dried *C. wilmsii* rehydrated in water. The cell has the appearance of a control cell, with large vacuoles (v) and chloroplasts (c) are situated at the periphery of the cell. 1cm=1.5 μ m. (B) Chloroplast of slowly dried *C. wilmsii* rehydrated in water. Thylakoids (t) and starch grains (s) are present 1cm=0.5 μ m.

Ultrastructural observations of the whole cells and chloroplasts of leaves rehydrated in both distamycin A (Figs. 3.5 A - D) and cycloheximide (Figs. 3.6 A - D) showed almost identical results to those rehydrated in water. Again, cells exhibited large vacuoles and the plasmamembrane remained appressed to the cell wall, having

incurred no visible damage (Figs. 3.5 A and 3.6 A). Thylakoids were intact within the chloroplasts and starch grains were visible (Figs. 3.5 B and 3.6 B). Membrane structure, too, was visible within the mitochondria (Figs. 3.5 C and 3.6 C). Possibly the only visible difference was in the cell walls of leaves rehydrated in the metabolic inhibitors, which exhibited slight folding (Figs. 3.5 D and 3.6 D), something not evident in leaves rehydrated in water. In addition, there appeared to be osmophillic material within the vacuole in both distamycin A- and cycloheximide-rehydrated tissue (Figs 3.5 A and 3.6 A).

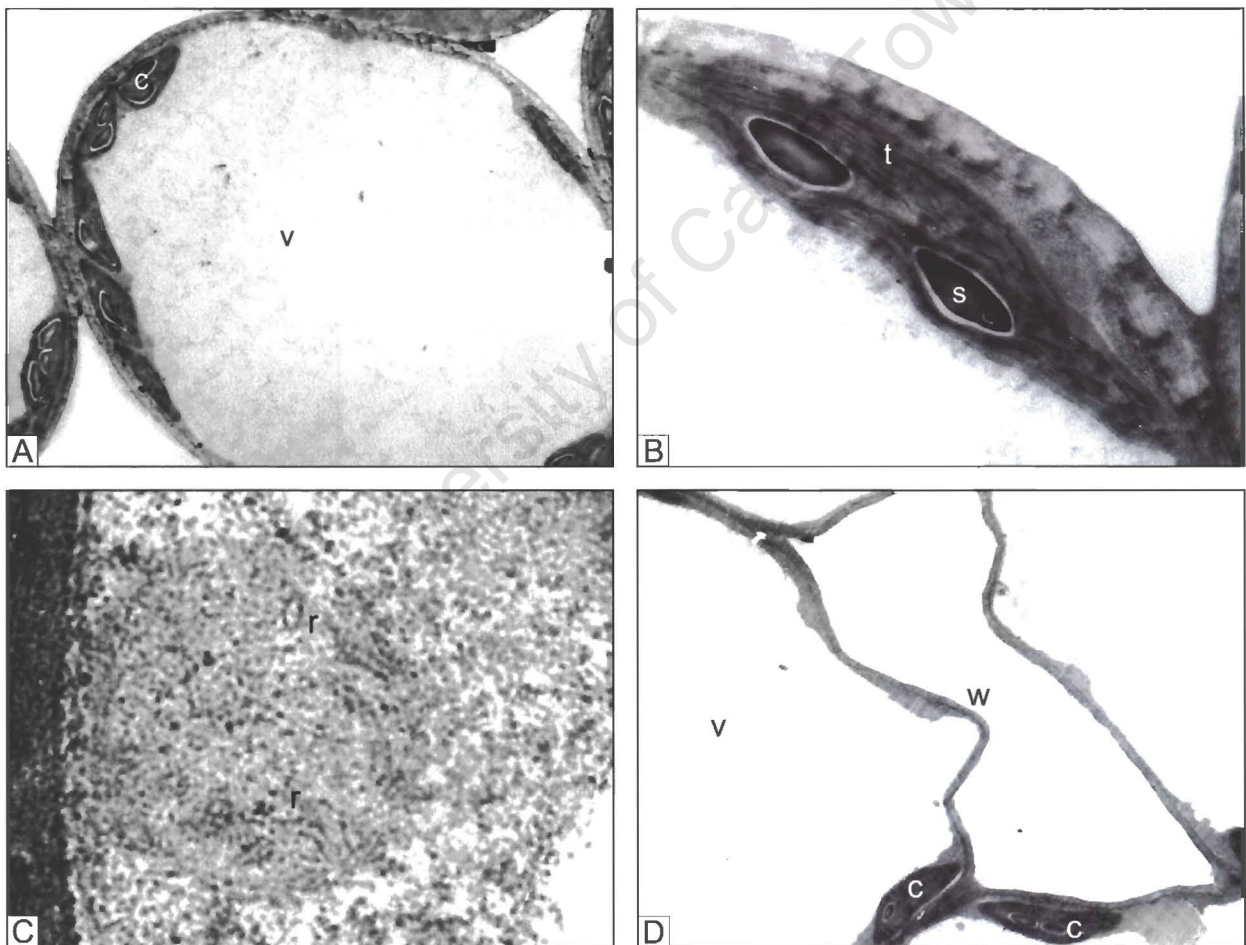


Figure 3.5 Slowly dried *C. wilmsii* tissue rehydrated to 100% RWC in distamycin A: **(A)** Mesophyll cell. There is a single, central vacuole (v) and chloroplasts (c) are situated at the periphery of the cell. Note the electron dense material in the vacuole. 1cm=2.9 μ m. **(B)** Chloroplast of mesophyll cell with thylakoid membranes (t) and starch grains (s) visible within. 1cm=0.7 μ m. **(C)** Mitochondrion of mesophyll cell. Mitochondria had the appearance of those in water-rehydrated cells. Cristae (r). 1cm=0.1 μ m. **(D)** Folding of the mesophyll cell wall (w). Vacuole (v); chloroplasts (c). 1cm=2.6 μ m.

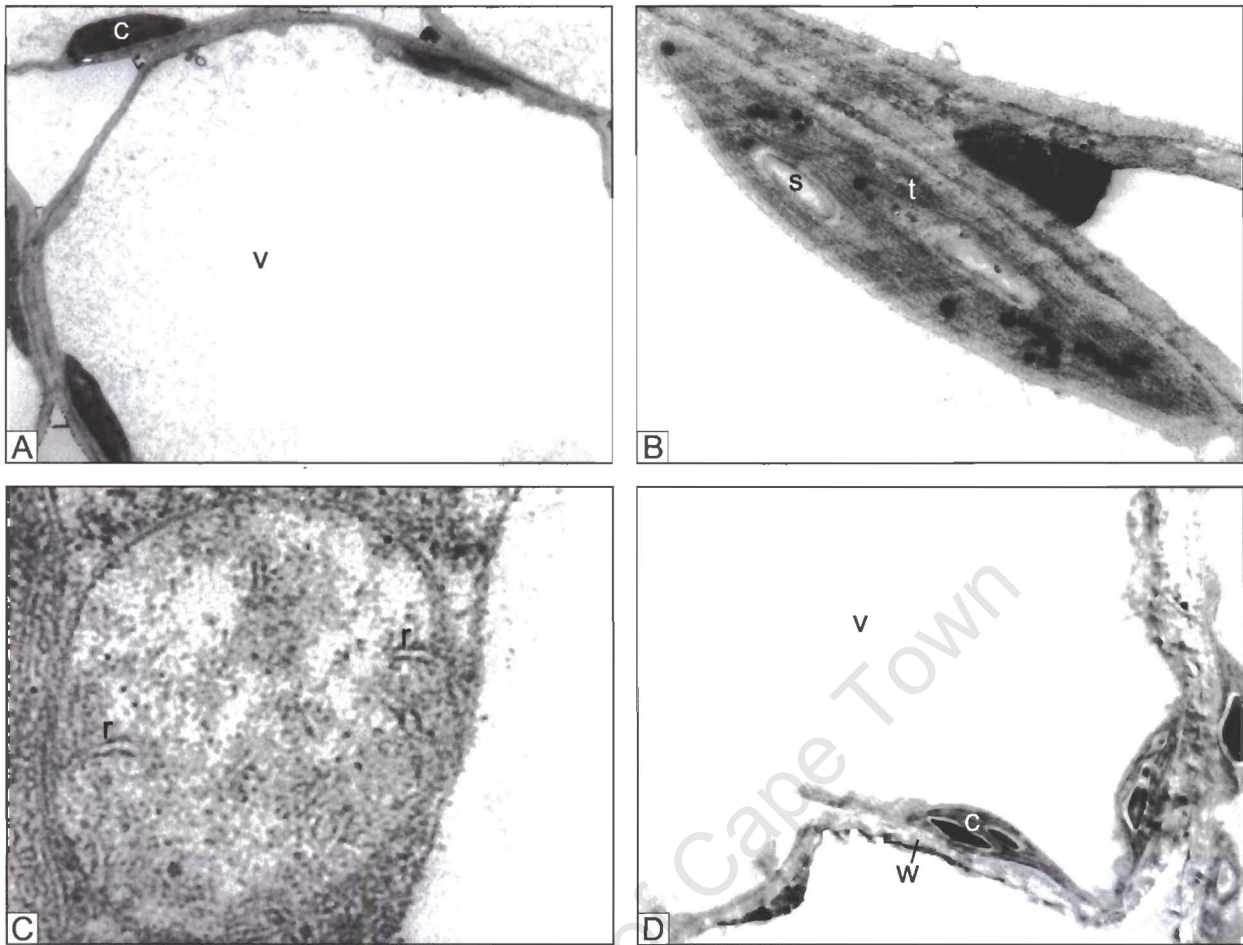


Figure 3.6 Slowly dried *C. wilmsii* tissue rehydrated to 100% RWC in cycloheximide: **(A)** Mesophyll cell. The vacuole (v) is large and chloroplasts (c) are situated at the periphery of the cell. Note the electron dense material in the vacuole. 1cm=2.3 μ m. **(B)** Chloroplast of mesophyll cell. Both starch grains (s) and thylakoid membranes (t) arranged into grana are present. 1cm=0.7 μ m. **(C)** Mitochondrion of mesophyll cell. Cristae (r) are visible within the mitochondrion. 1cm=0.2 μ m. **(D)** Folding of mesophyll cell wall (w). Vacuoles(v); chloroplasts (c). 1cm=2.4 μ m.

After 48 hours of rehydration in water, most cells of slowly dried *X. humilis* leaves showed a large, central vacuole, reassembled thylakoid membranes and starch grains within the chloroplasts, as reported previously (Dace *et al.*, 1998). Mitochondria had well-developed cristae, suggesting that the tissue was metabolically active (Fig. 3.7 A). Mitochondria with cristae, were present, too, in leaves rehydrated in inhibitor solutions, and there was no apparent damage to membranes (Fig. 3.7 B & C). Chloroplast structure, however, was abnormal and differed depending on the inhibitor treatment. Cycloheximide-treated cells exhibited membrane-like structures, not

organised into granal stacks, apparently formed by a coalescing of the internal vesicles seen in dry tissue (Fig. 3.7 B) (Dace *et al.*, 1998). Following rehydration in actinomycin D (Fig. 3.7 C), some chloroplasts resembled those described for cycloheximide (Fig. 3.7 B). Others, however, were lobed and appeared to have pockets of cytoplasm within them, often containing mitochondria (Fig. 3.7 D). It is not clear whether these pockets were contained within the mitochondria, or whether this was an artefact created by the two-dimensional image that is produced by thin sections, the mitochondria being situated in depressions of the chloroplast lobes (Dace *et al.*, 1998).

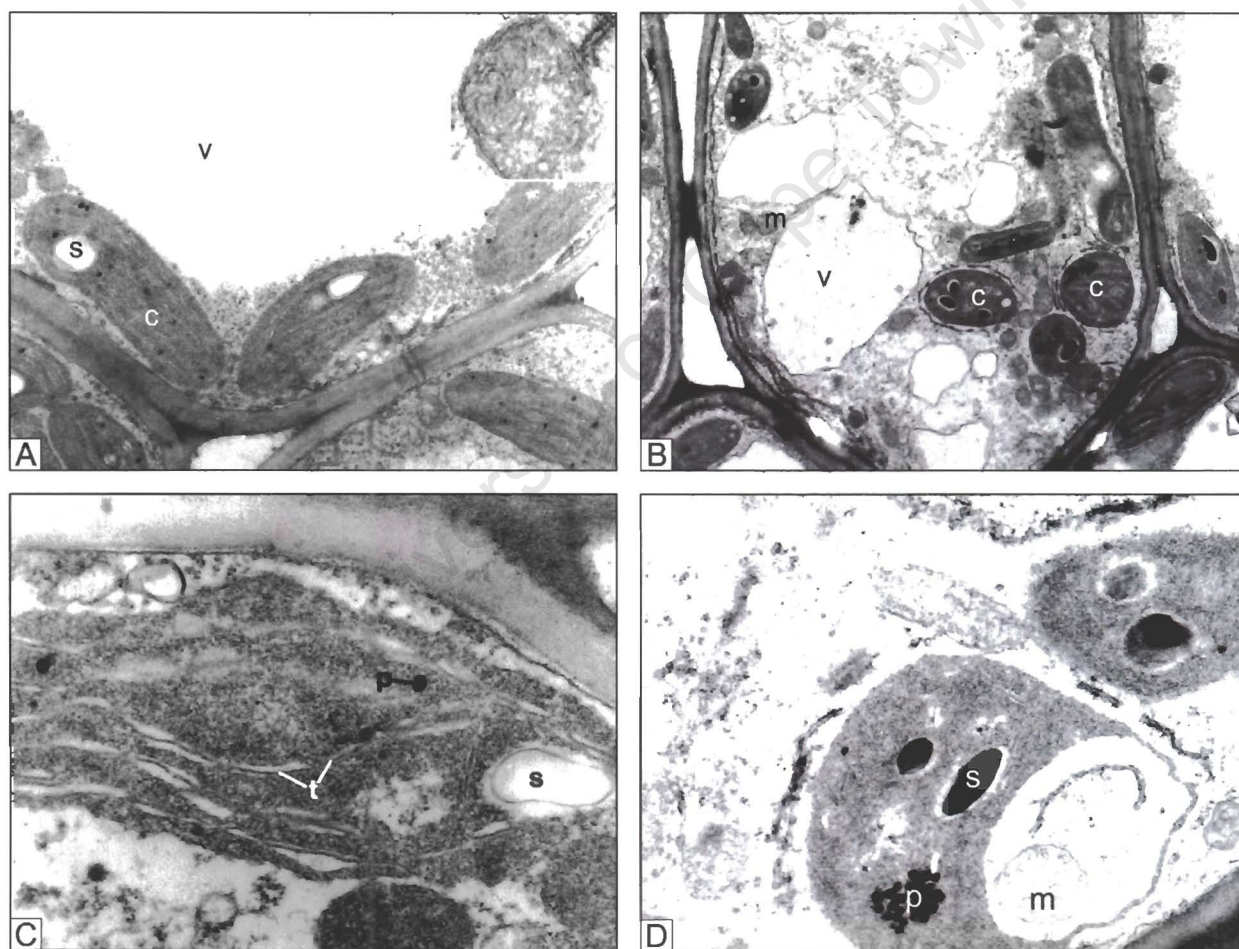


Figure 3.7 (A) Mesophyll cell from *X. humilis* leaf explant rehydrated in water. Chloroplasts contain starch grains (s) and well developed thylakoid membranes. A single central vacuole (v) is present. 1cm=1.3 μ m. Inset – a mitochondrion from this tissue. Note the internal membranes, indicating that it is active. 1cm=0.4 μ m. (B) Mesophyll cell from *X. humilis* leaf explant rehydrated in cycloheximide. The plasmamembrane is appressed to the cell wall and the cytoplasm is vacuolate (v). Starch and plastoglobuli are present in chloroplasts (c), but thylakoids had not reassembled into granal stacks. Mitochondria (m) were similar in appearance to those in water controls. 1cm=2.1 μ m. (C) Chloroplast of mesophyll cell from *X. humilis* leaf explant rehydrated in actinomycin D. Thylakoids were partially reassembled but not typically into granal stacks. Thylakoids (t); starch grains (s); plastoglobuli (p). 1cm=0.2 μ m. (D) Chloroplast from *X. humilis* leaf explant rehydrated in actinomycin D. Note the apparent inclusion of a mitochondrion (m) and endoplasmic reticulum strand. Mitochondria possessed cristae. Starch grain (s); plastoglobuli (p). After Dace *et al.* (1998). 1cm=0.6 μ m.

The cellular ultrastructure of rapidly dried *C. wilmsii* and *X. humilis* rehydrated in water, transcriptional inhibitor or translational inhibitor is shown in Figures 3.8 and 3.9. The cells of rapidly dried *C. wilmsii* rehydrated in water had the appearance of control (hydrated) cells (Fig. 3.8 A). The vacuole comprised most of the cell volume, the plasma membrane was appressed to the cell wall and the cell contents were situated at the periphery of the cell. There was no visible membrane damage in the chloroplasts (Fig 3.8 B) or in the cell as a whole.

However, the cells of those *C. wilmsii* leaves rehydrated in metabolic inhibitors did not show this recovery (Figs. 3.8 C - F). Both treatments exhibited considerable folding of cell walls and neither appeared to have reconstituted the several small vesicles into a single central vacuole. In each case, cells had suffered severe plasmolysis with numerous membrane breaks visible and blistering of the thylakoid membranes in the chloroplasts. Indeed, in some cases, there were few intact organelles present at all.

The cells of rapidly dried *X. humilis* showed similar injury to membranes regardless of the treatment to which they were subjected (Figs. 3.9 A - E). There was general derangement (and in some cases total dissolution) of cell contents. Those chloroplasts that were visible no longer possessed the thylakoids seen in rapidly dried tissue.

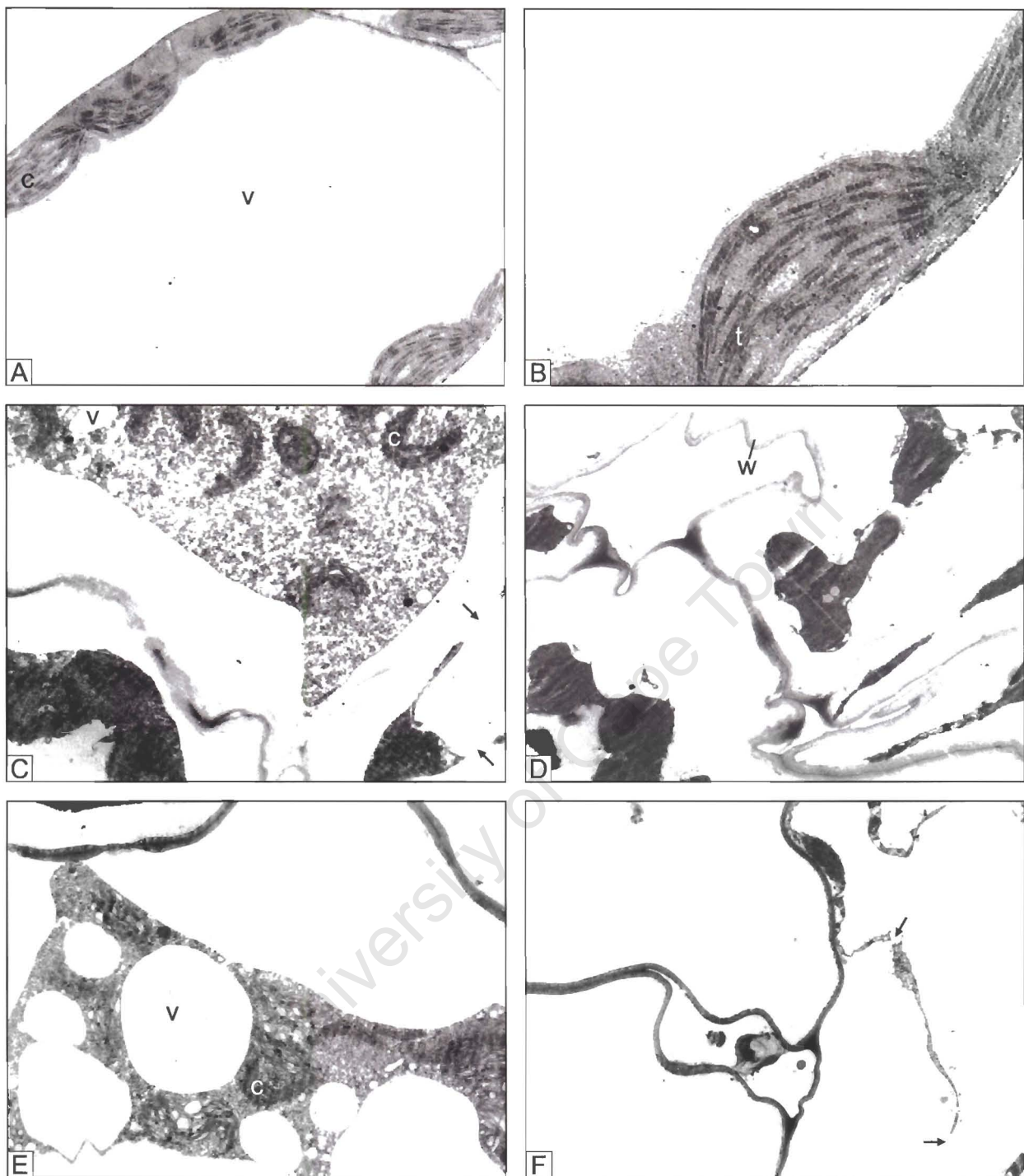


Figure 3.8 (A) Mesophyll cell of rapidly dried *C. wilmsii* rehydrated to 100% RWC in water. The vacuole (v) is large and the chloroplasts (c) are situated at the periphery of the cell. $1\text{cm}=3.1\mu\text{m}$. (B) Chloroplast of rapidly dried *C. wilmsii* cell rehydrated to 100% RWC in water. Thylakoid membranes (t) arranged into granal stacks are visible. $1\text{cm}=1.1\mu\text{m}$. (C) Mesophyll cell and chloroplasts of rapidly dried *C. wilmsii* rehydrated to 100% RWC in distamycin A. There is severe plasmamembrane withdrawal, vacuoles (v), where still intact, have not coalesced and chloroplasts (c) lack boundary membranes. Membrane breaks are indicated with arrows. $1\text{cm}=3.3\mu\text{m}$. (D) Mesophyll cell of rapidly dried *C. wilmsii* rehydrated to 100% RWC in distamycin A. There is very little subcellular organization and cell walls (w) are still folded. $1\text{cm}=3.1\mu\text{m}$. (E) Mesophyll cell and chloroplasts of rapidly dried *C. wilmsii* rehydrated to 100% RWC in cycloheximide. There is severe plasmamembrane withdrawal, vacuoles (v), where still intact, have not coalesced and chloroplasts (c) lack boundary membranes. $1\text{cm}=3.1\mu\text{m}$. (F) Mesophyll cell of rapidly dried *C. wilmsii* rehydrated to 100% RWC in cycloheximide. There is very little subcellular structure left. Membrane breaks are indicated with arrows. $1\text{cm}=2.9\mu\text{m}$.

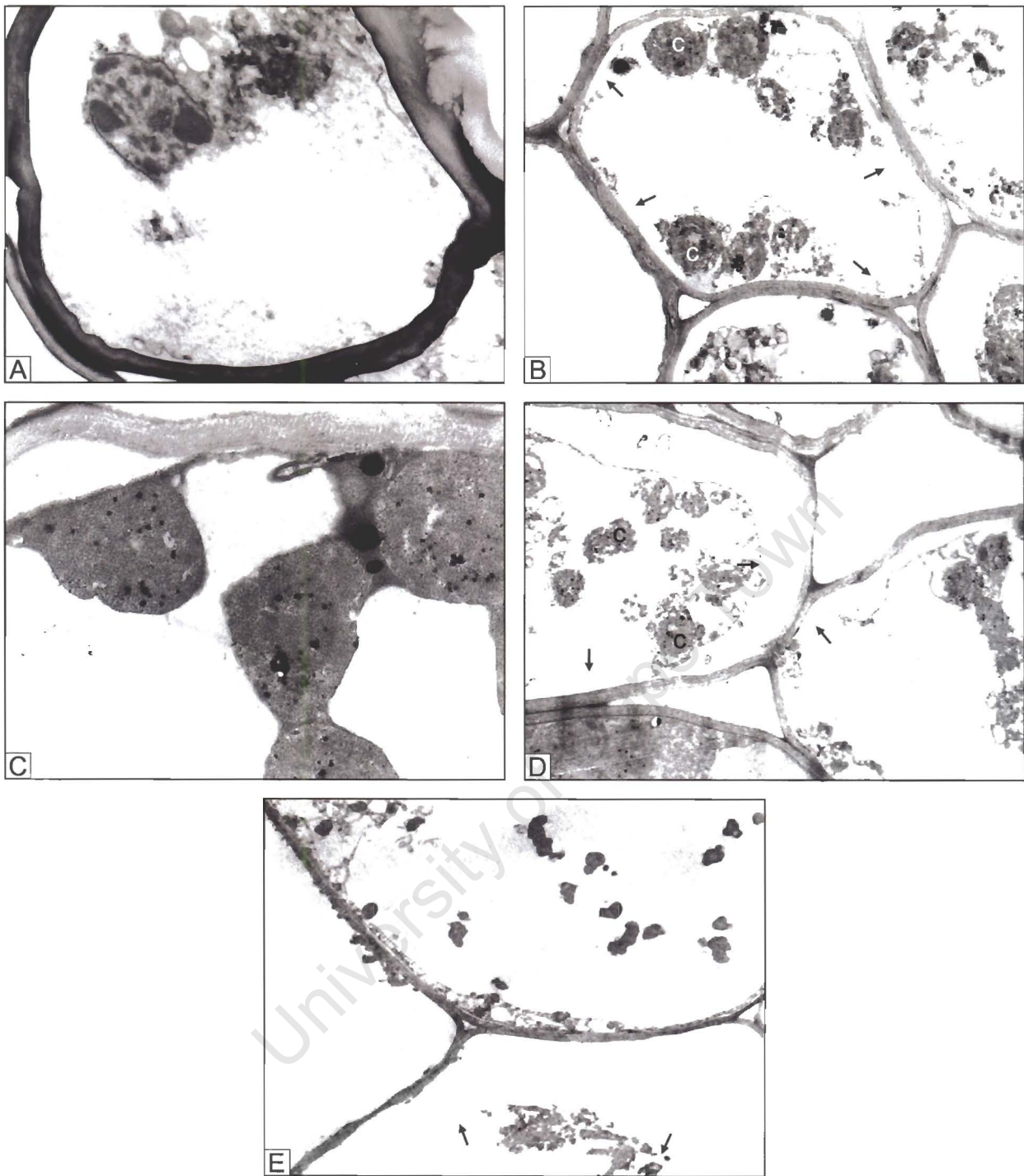


Figure 3.9 (A) Mesophyll cell of rapidly dried *X. humilis* rehydrated to 100% RWC in water. There has been almost complete cellular dissolution. $1\text{cm}=1.4\mu\text{m}$. (B) Mesophyll cell and chloroplasts of rapidly dried *X. humilis* rehydrated to 100% RWC in actinomycin D. There is severe membrane rupture (indicated by arrows) and chloroplasts (c) lack boundary membranes. $1\text{cm}=2.8\mu\text{m}$. (C) Chloroplasts of rapidly dried *X. humilis* rehydrated to 100% RWC in actinomycin D. No membrane organization is visible within the chloroplasts. $1\text{cm}=1.0\mu\text{m}$. (D) Mesophyll cell and chloroplasts of rapidly dried *X. humilis* rehydrated to 100% RWC in cycloheximide. The plasmamembranes has been ruptured (indicated by arrows) and chloroplasts (c) lack boundary membranes. $1\text{cm}=3.0\mu\text{m}$. (E) Mesophyll cell of rapidly dried *X. humilis* rehydrated to 100% RWC in cycloheximide. There is very little subcellular structure left. Membrane breaks are indicated with arrows. $1\text{cm}=2.7\mu\text{m}$.

4. Discussion

C. wilmsii is classified as a modified desiccation-tolerant plant as previously mentioned (Oliver, 1996). As such, its strategy in surviving desiccation is primarily to protect its cells to limit the damage incurred (Oliver, 1996). In addition, it is homoiochlorophyllous and although some chlorophyll is lost, its photosynthetic apparatus remains intact throughout dehydration. Consequently, it requires no reconstitution of chloroplastic membranes upon rehydration, unlike the poikilochlorophyllous *X. humilis* (Tuba *et al.*, 1993a; b; Sherwin and Farrant, 1998; Farrant, 2000). The data presented here show that leaves rehydrated in distamycin A (inhibitor of transcription) and cycloheximide (inhibitor of translation) recovered completely, indicating that slowly dried *C. wilmsii* has very little need for either transcription or translation to resume photosynthetic functioning and normal metabolism on rehydration.

As seen with leaves rehydrated in water (discussed in previous chapter), slowly dried *C. wilmsii* leaves rehydrated in inhibitors regained the photosynthetic pigments lost during desiccation. This supports the notion that the accumulation of gene products required for chlorophyll and carotenoid biosynthesis occurs prior to complete desiccation in this species. Slowly dried *X. humilis*, too, shows recovery of photosynthetic pigments, but only with rehydration in actinomycin D (transcriptional inhibitor). This species could not recover photosynthetic pigment or reconstitute chloroplastic membranes with inhibition of translation and thus showed no recovery of PS II quantum efficiency when rehydrated in cycloheximide. Therefore, it seems that *X. humilis* accumulates the mRNA's required for chlorophyll and carotenoid synthesis during slow drying, but requires new translation of these mRNA transcripts on

rehydration (Dace *et al.*, 1998). The temporal difference of expression between *C. wilmsii* and *X. humilis* may be due to the scale of resynthesis of chlorophyll, which in *C. wilmsii* is far smaller than that of *X. humilis*. This allows the former to adequately synthesise the necessary proteins during drying without expending excessive energy. *X. humilis*, conversely, must invest much energy in the dismantling of thylakoids and degradation of chlorophyll upon desiccation (Tuba *et al.*, 1997), and may therefore postpone the synthesis of elements required for reconstitution of the photosynthetic apparatus until rehydration. It therefore required *de novo* translation of stored mRNA transcripts in order to re-establish its photosynthetic machinery and regain chlorophyll (Dace *et al.*, 1998).

There are, however, similarities between the species in gene expression during drying. As with slowly dried *X. humilis*, the mitochondria of slowly dried *C. wilmsii* resynthesised cristae, and starch grains were present in the chloroplasts upon rehydration in both transcriptional and translational inhibitors. This indicates that the protection of respiratory components is completed during drying and *de novo* synthesis of elements in this metabolism is not required for recovery. For *X. humilis*, this prompt supply of energy may be required for partial assembly of the photosynthetic apparatus on rehydration and may explain the close association of mitochondria and chloroplasts seen in actinomycin D-rehydrated tissue (Dace *et al.*, 1998). The enzymes involved in starch synthesis within the chloroplast, too, are presumably independent of transcription and translation.

Although slowly dried *C. wilmsii* seems almost unaffected by inhibitor treatments, an exception was the mechanism of cell wall unfolding. The folding of cell walls is a controlled process, which is thought to involve the accumulation during drying of

components that increase tensile strength (Vicré *et al.*, 1999). Upon rehydration, the quantity of these molecules (*eg.* xyloglucan and pectins) decline and the walls “unfold” (Vicré *et al.*, 1999). It is conceivable that although the proteins needed for the reversal of this process are for the large part synthesised before or during desiccation, further protein synthesis is required upon rehydration for completion. Similarly, the components needed to rid the vacuole of the various proteins, sugars or compatible solutes accumulated during dehydration may be synthesised only after rehydration as they are not essential for survival, since vacuoles retained their electron dense appearance in cells rehydrated in distamycin A and cycloheximide.

In general, however, it would appear that the recovery of slowly dried *C. wilmsii* is, to a large extent, independent of both transcription and translation. It has been shown in *Tortula ruralis* (a bryophyte) that both proteins and mRNA transcripts are maintained in a stable form during the dry state (mRNA being stabilised in polysomes) (Dhindsa and Bewley, 1977; review by Oliver and Bewley, 1997). It therefore seems likely that *C. wilmsii* upregulates the expression and translation of molecules needed for desiccation-tolerance during dehydration and stores the gene products in the dry state. As a consequence of this, in combination with the protection of cellular components, *C. wilmsii* requires very little *de novo* gene induction upon rehydration. This is consistent with the findings of Bernacchia *et al.* (1996) that in *C. plantagineum*, there were only a small number of rehydration-specific proteins synthesised on rewetting, and some production of hydration-related gene products at a later stage of rehydration. The authors propose that most of the events essential for the acquisition of desiccation-tolerance occur during dehydration and the rehydration events only contribute to full metabolic recovery. It is clear in this study that

protection during dehydration was complete and no repair was needed by slowly dried *C. wilmsii* on rehydration, since it continued to metabolise without new transcription or translation during early rehydration. Interestingly, it has recently been demonstrated in some bryophytes that rapid recovery of photosystems on rehydration was independent of new protein synthesis in the short term (the first 40 minutes of rehydration), despite the fact that they are thought to rely on repair for survival (Proctor and Smirnov, 2000). However, translation was required at later stages of rehydration, but only in the presence of light (Proctor and Smirnov, 2000).

Also apparent, was the ability of *C. wilmsii* to maintain photosynthetic functioning for a considerable period of time with incubation in both metabolic inhibitors. This may indicate persistence of dehydration-accumulated proteins during and after rehydration. Indeed, Schneider *et al.* (1993) found that translation products accumulated during drying remained for many hours after rehydration in the tissues of *C. plantagineum*. Leaves of slowly dried *X. humilis*, although they recovered with rehydration in the transcriptional inhibitor, required new transcription after 12 hours of rehydration as indicated by the decline in F_v/F_m . However, when hydrated leaves were incubated in the inhibitor solutions, results indicated that, similar to *C. wilmsii*, there was little need for transcription and translation for maintenance of metabolism for up to 24 hours (Dace *et al.*, 1998).

Both *C. wilmsii* and *X. humilis* show some induction of gene expression during natural drying, apparently accumulating the necessary mRNAs for recovery, and, in the case of *C. wilmsii*, the requisite proteins as well. Conversely, when rapidly dried, it would appear that both transcription and translation are needed by *C. wilmsii* and *X. humilis* in order to recover.

The failure of rapidly dried *X. humilis* to regain normal metabolic functioning was anticipated since even those leaves rehydrated in water were unable to survive, as rapid drying did not provide enough time to protect the cells, particularly against light stress (as reported in the previous chapter). The quantum efficiency of leaves subjected to water and inhibitor treatments showed essentially no recovery, despite the fact that chlorophyll was present in the dry leaves. This, and the complete bleaching of photosynthetic pigments after three days, indicates substantial rehydration damage. The damage incurred could be seen in the cell ultrastructure, where there was extensive membrane disruption. In addition, those chloroplasts still visible no longer possessed the thylakoids seen in dry tissue. This is indicative of some free radical activity, which resulted in the destruction of the chloroplastic membranes and may have contributed to the damage seen in the cell in general.

Rapidly dried *C. wilmsii*, as reported in the previous chapter, recovered on rehydration in water. When rehydrated in metabolic inhibitors, however, there was little recovery of photosynthesis. Rapidly dried *C. wilmsii* does not have sufficient time to institute mechanisms needed to protect the cells completely, resulting in injury during desiccation (discussed in previous chapter). With rehydration in water, proteins that play a role in repair (and probably limitation of further damage) are apparently synthesised, but when rehydrated in inhibitors, no such synthesis can take place, and the plant cannot survive. Thus it would appear that both transcription and translation of repair-related proteins are needed by this species to allow recovery from rapid drying. Those leaves rehydrated in distamycin A did exhibit an initial recovery, which paralleled that of leaves rehydrated in water. This suggests that there was some accumulation of mRNA transcripts during drying which were translated in the first

few hours of rehydration, but thereafter, new genomic transcription was required as evidenced by the drop in F_V/F_M . It is also possible that this drop in fluorescence was due to damage incurred within the chloroplast. Indeed, the pigment in both treatments was lost after several days of rehydration in inhibitors. Both inhibitor treatments resulted in severe cellular damage for leaves of rapidly dried *C. wilmsii*. There was widespread membrane breakage and in some cases, almost complete cellular dissolution, something not seen in those cells rehydrated in water. This, too, is indicative of some degree of gene expression in rapidly dried *C. wilmsii* tissue, which occurs upon rehydration in water and is involved in repair of the injury caused by inadequate protection due to rapid drying.

Free radical action may be involved in the failure to recover, although the fine structure of some of the inhibitor-rehydrated cells of *C. wilmsii* was still intact to some extent. This may be due to dehydration-accumulated antioxidant defences. Alamillo and Bartels (1996) have shown, however, that young *C. plantagineum* plants showed reduced accumulation of desiccation stress proteins (dsp's). The authors proposed that these plants dried more rapidly than the adult plants and therefore could not accumulate the typical quantity of dsp's. The inability to recover when rehydrated in inhibitors indicates a similar impairment of protein synthesis during rapid dehydration, which is likely to extend to antioxidant defence systems (since enzymes form a major part in plant antioxidant defence). It therefore seems unlikely that adequate free radical defence could be made ready to completely protect the plant on rehydration. Thus, the damage seen must be due, primarily, to a lack of protection on drying. Some induction of protective mechanisms may occur during rehydration in water of rapidly dried

material, which prevents additional damage, however, there is, as yet, no evidence for this hypothesis.

Also suggestive of impaired protein accumulation during rapid dehydration was the observation that in inhibitor-rehydrated rapidly dried *C. wilmsii*, cell walls remained folded and the small vacuoles, though still present in some cells, had not yet coalesced. Thus, it seems that cell wall unfolding (as was shown with slowly dried leaves) and reconstitution of the central vacuole require some *de novo* gene expression during dehydration in order to allow complete recovery, something that is impaired by rapid dehydration.

In conclusion, the survival strategy of *C. wilmsii* places almost complete emphasis on protection during desiccation, something that enables an extremely rapid recovery when water is once again available. It is not known how genomic DNA is protected during desiccation, but it may incur some injury or undergo some degree of reorganisation. The independence of *C. wilmsii* from transcription and translation would enable the plant to repair any possible damage or simply reorganise the DNA into an active state upon rehydration, with no immediate requisite for *de novo* expression of transcripts or proteins. This accumulation of proteins on dehydration, as well as the protective mechanisms, appears to be hindered by rapid drying. This study indicates that *C. wilmsii* may have a latent ability to induce repair processes, something that occurs during rehydration if the plant has incurred damage. Since rapidly dried plants rehydrated in inhibitors showed no recovery, clearly, components of the repair metabolism are laid down during dehydration. When rehydrated in inhibitors of transcription and translation, these repair processes cannot be effected and the plant cannot survive.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

As indicated in previous chapters, distinctions have been made as to what constitutes a desiccation-tolerant plant versus a modified desiccation-tolerant plant. Resurrection plants are thought to either protect cellular integrity during dehydration and in the desiccated state in order to limit the damage incurred (modified desiccation-tolerance), or repair the de- and rehydration-induced damage (desiccation-tolerance) (Bewley and Oliver, 1992). The strategy employed is largely dictated by the complexity of the plant concerned and the environment in which it evolved (Oliver *et al.*, 1997). It is probable that resurrection plants use aspects of both strategies (Oliver *et al.*, 1997), although the ratio of protection to repair is likely to increase as plants become more complex. Angiosperms, with the highest level of complexity, rely mainly on protection (Tuba *et al.*, 1993a,b; Farrant and Sherwin, 1998; Sherwin and Farrant, 1998; Vicré, *et al.*, 1999; Farrant, 2000) and are thus classified as modified desiccation-tolerant plants (Oliver, 1996). It is possible that this inducible protection evolved only in plants capable of limiting water loss by morphological or physiological means (Oliver *et al.*, 1997), thereby slowing dehydration and providing sufficient time to institute the required mechanisms. When the rate of water loss is increased, protection and in turn, survival, is likely to be compromised (Gaff and Loveys, 1984; Bartels *et al.*, 1990; Oliver *et al.*, 1998; Tuba *et al.*, 1998).

It is clear that rapid drying does, indeed, impair the protection instituted by *C. wilmsii* and *X. humilis*. There was reduced accumulation of the protective molecule, sucrose, and injury to the subcellular structure during rapid dehydration of both

species. However, on rehydration, only *C. wilmsii* recovers, apparently due to this species' ability to limit damage. In comparison, the inability of *X. humilis* to recover may be attributable to the different strategies employed by *C. wilmsii* and *X. humilis*. Since *X. humilis* is poikilochlorophyllous, part of its approach in dealing with desiccation is to dismantle its photosynthetic apparatus, and this, like other protective measures, is hindered by rapid drying. Thus, although, like *C. wilmsii*, it may be capable of limiting damage even when dried rapidly, it is the additional perturbation of light stress and free radical formation during desiccation and rehydration that prevents survival. Similarly, in certain bryophytes, it is apparently light stress which necessitates the need for repair-based protein synthesis during rehydration (Proctor and Smirnov, 2000).

For *C. wilmsii*, the primary light-avoiding mechanisms, as well as the prevention of mechanical strain on membranes by cell wall folding, are not impaired by rapid drying. However, the rehydrated tissue of rapidly dried *C. wilmsii* seemingly showed a lesser degree of injury than would be expected from observation of *C. wilmsii* cells during rapid dehydration. From this, as well as the low solute leakage from rapidly dried material, the tentative conclusion is made that *C. wilmsii* induces some form of repair of the damage incurred during dehydration.

The use of metabolic inhibitors provides further evidence for this conclusion. It is clear from the rehydration of slowly dried *C. wilmsii* in solutions of distamycin A and cycloheximide that this species requires almost no new protein synthesis on rehydration, *ie.* components needed for protection during dehydration and full recovery (*ie.* reversal of protection mechanisms instituted during dehydration) are laid down almost completely during drying.

Conversely, when rapidly dried, there is extensive need for *de novo* transcription and protein synthesis during rehydration of *C. wilmsii*, as indicated by the failure of rapidly dried *C. wilmsii* to recover with rehydration in metabolic inhibitors. Since water-rehydrated tissue, when rapidly dried, recovers completely, there must be some induction of gene expression in *C. wilmsii* during rehydration, which counters the injury caused by rapid drying. The nature of this expression is largely the subject of speculation. There may be an upregulation of protective mechanisms (which, if not for the increased rate of drying, would have been established prior to desiccation), in order to reduce rehydration-incurred damage due to the sudden influx of water. However, rehydration is a fairly rapid occurrence, with most rehydration-related damage occurring in the initial stages of rewetting (Pammenter and Berjak, 1999) and it is unlikely that protective mechanisms alone could adequately counter this injury in such a short space of time. Thus, the nature of the rehydration-induced expression is likely to include repair-based mechanisms as well.

One can therefore conclude that although *C. wilmsii* relies almost entirely on protection, its lack of xerophytic morphological features may require that it: (i) can limit damage incurred when rapidly dried by utilising rapidly inducible protective mechanisms and; (ii) can repair damage if it is incurred.

This is a departure from the conventional assumptions regarding classifications of desiccation-tolerance and may extend to other desiccation-tolerant species. Certainly, the findings of Proctor and Smirnoff (2000) indicate that the photosystems of “fully” desiccation-tolerant bryophytes are kept intact during desiccation and that repair systems are not required for initial recovery of PS II quantum efficiency. Only with long term rehydration in light conditions does the need for repair arise, most likely due

to photo-oxidative stress. Although increased complexity of angiosperm resurrection plants does indeed engender a greater degree of protection, rapid drying does not necessarily constrain these mechanisms to such a degree as to prevent survival. In addition, the role of repair in these species may have been underestimated. In light of these findings, it seems appropriate to re-evaluate the general classifications of modified versus full desiccation-tolerance.

Future study

It is known that resurrection plants show acclimation to desiccation with repeated drying cycles. It would therefore be beneficial to study the effect of repeated cycles of dehydration on the amount of damage incurred during rapid drying of both *C. wilmsii* and *X. humilis* and on the latter's ability to survive. During the course of drying rate experiments, it was found that *X. humilis* plants that apparently possess a lower content of chlorophyll (as determined from casual observation of leaf colour) in the control/hydrated state survived rapid drying. These plants were kept in high light conditions and ostensibly lost some photosynthetic pigment as a result. The chlorophyll content of dry material, although higher than that of slowly dried tissue, appeared to be lower than that of "normal" rapidly dried material. When rehydrated, F_v/F_M recovered eventually, although more gradually than slowly dried leaves (Fig. 4.1).

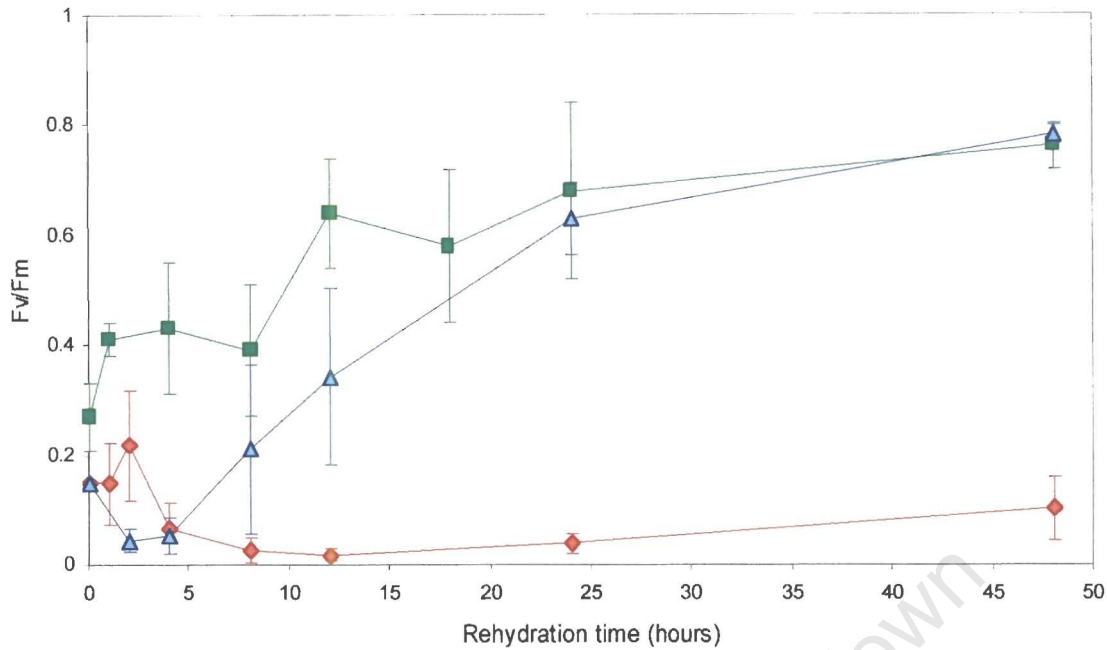


Figure 4.1. Changes in quantum efficiency of photosystem II for slowly (■) and rapidly (◆) dried *X. humilis* leaves and rapidly dried *X. humilis* leaves that had a lower control content of chlorophyll (▲).

Although the experiment is incomplete, this suggests that, with partial acclimation to high light such that there is reduced light stress, *X. humilis* might survive rapid drying and may, too, possess an ability to repair. Partial acclimation to dehydration, particularly directly after natural drying when chlorophyll content is still low, may also facilitate the recovery of rapidly dried tissue. It would be extremely worthwhile to continue this line of experimentation.

Also, with the finding that *C. wilmsii* possesses the ability to repair on rehydration, certain questions arise regarding the extent of repair possible for this species. And what potential do other resurrection plants have for repair when it is required? In order to elucidate this issue, it is essential that future work focuses on characterisation of genes and gene products induced on rehydration in modified desiccation-tolerant plants with rapid dehydration.

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