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Exploring the Longevity of Dry *Craterostigma wilmsii* (homoiochlorophyllous) and *Xerophyta humilis* (poikilochlorophyllous) under Simulated Field Conditions

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Preface

Experimental work described in this thesis was supervised by Professor Jill M. Farrant and Dr Brigitte Hamman and conducted in the Department of Molecular and Cell Biology at the University of Cape Town, South Africa.

The results presented are original and have not been submitted in any form to another University. Where use was made of the work of others, it is duly acknowledged in the text.

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Jelena Bajic

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Abstract

In the field *C. wilmsii* (homoiochlorophyllous) and *X. humilis* (poikilochlorophyllous) plants do not experience drought longer than two and six months, respectively. In this study longevity of dry *C. wilmsii* and *X. humilis* plants was investigated for up to 10 months under four different sets of conditions. Some conditions resembled those under which the two species would experience drought in the field, but also included those that resemble the accelerated aging conditions used in seed studies. 'Artificial' drought conditions were included in order to test the ability of the two species to survive drought should climatic conditions change, as has been suggested by numerous studies. *C. wilmsii* plants survived no longer than three months in the dry state, and this was highly dependent on the climatic conditions, while *X. humilis* plants survived the 10 month dry period under all conditions.

C. wilmsii plants degraded both chlorophyll (a+b) and protective anthocyanins the longer they remained in the dry state, while *X. humilis* plants lost anthocyanins during 10 months of drought. The loss of the 'sun-block' anthocyanins could have attributed to the increasing damage observed in *C. wilmsii* plants while in the dry state. While MDA levels (measure of lipid peroxidation) did not increase during dry storage in *C. wilmsii* plants, a steady increase was observed in *X. humilis* plants over the 10 month dry period. MDA levels in dead tissues of both species were similar, suggesting that perhaps *X. humilis* plants are capable of tolerating greater ROS damage, as they rely, partially, on repair during rehydration.

Both species, under all conditions, accumulated sufficient sucrose to ensure survival in the dry state, and there was no indication that any loss of sucrose occurred during dry storage in either species. However, high sucrose levels did not ensure longevity of dry *C. wilmsii* plants for longer than three months. Although we cannot say that glasses were maintained (as no measure of glass formation was done), the glasses could have been breaking down due to instability in other factors which stabilise glasses such as proteins and oligosaccharides.

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The ability of both species to synthesise new RNA and proteins was not compromises during dry storage, indicating that the genome was well protected. *C. wilmsii* plants were capable of accumulating constituents necessary for the recovery of the photosynthetic machinery during rehydration, while drying and in the dry state, as indicated by their ability to rehydrate and resume photosynthesis in transcription and translation inhibitors. This species has shown the ability to repair if protection is inadequate and damage is incurred. *X. humilis* plants, however, accumulated mRNA necessary for the recovery of photosynthetic machinery, but the stability of these transcripts was compromised the longer the plants remained dry, as indicated by the inability of 10 month dry explants to regain necessary chlorophyll (a+b) levels for optimum PS II function. Although the stability of accumulated proteins and mRNA was tested in leaf explants only, due to the exclusion of inhibitors from the roots, intact 10 month dry *X. humilis* plants were capable of full recovery of the photosynthetic machinery. This identified the importance the roots play in the recovery of *X. humilis* plants following a prolonged drought.

Each species has evolved mechanisms that would ensure survival of drought in their natural environments. However, this study shows that, should drought persist for periods longer than the two species experience at present, *X. humilis* plants are better equipped to survive the prolonged dry storage.

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List of Abbreviations

AA	Accelerated aging
AP	Ascorbate peroxidase
AWC	Absolute water content
C. wilmsii	*Craterostigma wilmsii*
Chl (a+b)	chlorophyll (a+b)
d	Dead
DNA	Dioxyribonucleic acid
DT	Desiccation tolerant
DW	Dry weight
FM	Maximal fluorescence
FV	Variable fluorescence
FV/FM	Measure of quantum efficiency of photosystem II
GR	Glutathione reductase
H_2O	Water
HCl	Hydrochloric acid
HDT	Homoiochlorophyllous desiccation tolerant
HEPES	4-(2-Hydroxyethyl)-1-piperazine-ethane sulphonic acid
M	Month(s)
MDA	Malondialdehyde
MeOH	Methanol
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NaOH	Sodium hydroxide
PDT	Poikilochlorophyllous desiccation tolerant
PSII	Photosystem II
RH	Relative humidity
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	rotations per minute
RWC	Relative water content
SD	Summer natural drying

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SOD	Superoxide dismutase
TBA	2-thiobarbituic acid
TCA	Trichloroacetic acid
TEM	Transition Electron Microscope
WD	Winter natural drying
WGH	Winter green house
x	no sample
X. humilis	*Xerophyta humilis*

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wall. Chloroplasts (ch) were seen in the periphery of the cells. Thylakoid membranes (t) and starch (s) were present in the hydrated *C. wilmsii* and *X. humilis* leaf tissue. Following desiccation, in *C. wilmsii* the plasma membrane was seen to be withdrawn from the cell wall (cell wall had partially rehydrated due to the fixation process). Plastoglobuli (p) replaced starch, but the thylakoid membrane stacks were still present. Dry *X. humilis* exhibited numerous vacuoles. Starch had also been replaced by plastoglobuli, but the thylakoid membrane stacks were dismantled. The dead tissue of both species showed no cellular compartmentalisation, structure nor order. All micrographs are at magnification of x4000, except A1 at x5000.

Figure 3.1: Response of chlorophyll (a+b) [A], carotenoids (x+c) [B] and anthocyanins [C] to dehydration and prolonged desiccation (one, two and three months) in leaves of *C. wilmsii* plants. AA (accelerated aging); WGH (winter green house); SD (summer dehydration); WD (winter dehydration). 1, 2 and 3 refer to the drought period (months).

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plastoglobuli; v: vacuole; m - mitochondrion. All micrographs are at magnification of x4000.

Figure 5.8: Two month dry *C. wilmsii* leaf explants rehydrated in water (A), actinomycin-D (B) and cycloheximide (C). Images taken following 24 (1), 72 (2) and 144 (3) hours of rehydration. cw: cell wall; s: starch; t: thylakoid membrane stacks; p: plastoglobuli; v: vacuole. All micrographs are at magnification of x4000.

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CHAPTER ONE

General Introduction and Literature Review

1.1 Plant Stress

Stresses, or stressors, as defined by Lichtenthaler (1996), are any and all unfavourable substances and/or conditions that affect a plants' metabolism, growth or development. Lichtenthaler (1996) also suggested that stressors are dose-dependent. Stresses are not always destructive, and can be constructive and are selection factors in adaptive evolution (Larcher, 1987).

Once a biotic or abiotic change has been identified as stressful it can be subdivided into two categories, mild and severe. A mild stress can be favourable to a plant as it can activate certain aspects of cell metabolism or increase the physiological activity (Lichtenthaler, 1988). A severe stress can be lethal to a plant. The transition phase between mild and severe stress is reached once a threshold has been exceeded, and this is species specific. Lichtenthaler (1996) proposed that a short-term severe stress can be as destructive to a plant as a long-term mild stress. Tolerance to a stress depends on the plant species' pre-disposition (Lichtenthaler, 1996).

Plants are able to acclimatise to a variety of factors, such as high light, by physiological means such as adjusting leaf orientation and by biochemical means, such as adjusting the size of the antennae receptors of the photosynthetic pigments. Thus, factors such as this should not be considered as stresses, unless their changes are sudden and/or persist for a long period of time.

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1.2 Water Deficit Stress

Water constitutes more than 90% of the fresh weight in many plant organs (Meindner and Sheriff, 1976). Survival of most plants and/or plants organs is compromised if the relative water content (RWC) drops below 65% (reviewed by Farrant, 2000). The exceptions to this are spores, pollen, seeds and the vegetative tissue of desiccation tolerant (DT) plants, in which the RWC can drop below 5% without the loss of viability. In quiescent seeds, pollen, and dry DT plants, the water that remains is mostly adsorbed and thus not available for normal metabolic activity (Meindner and Sheriff, 1976).

Water is vital for plant survival and plays numerous vital roles within a plant cell (Meidner and Sheriff, 1976; Kramer, 1983; Vertucci and Farrant, 1995):

1. It keeps cells turgid and it provides structural support due to its incompressibility;
2. Due to the hydrophilic and hydrophobic properties of macromolecules, water stabilises their molecular structure;
3. It buffers temperature fluctuations; and
4. It is involved in numerous biochemical reactions in metabolism.

Shortage of water can, thus, greatly disturb the physical structure and the biochemical reactions within a cell (Leopold and Vertucci, 1986; Crowe *et al.*, 1992).

Drought is a meteorological term describing periods of no or minimal rainfall, where evaporation rate exceeds precipitation. It leads to reduced plant growth and/or yield due to an insufficient water supply (Passioura, 1996). Water deficit can be mild or

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severe. Plants that are tolerant to severe water deficit (desiccation) are termed desiccation tolerant (Gaff, 1971; Bewley and Oliver, 1992; Vertucci and Farrant, 1995; Ingram and Bartels, 1996; Mundree *et al.*, 2000).

According to Vander Willigen *et al.* (2001) 'drought tolerance' in plants entails avoiding the damage associated with water shortage by keeping the water potential above that of the environment. 'Desiccation tolerance', on the other hand, is the ability of a cell, tissue or the whole plant, to lose all free water without losing viability.

1.3 Desiccation Tolerant Plants

Plants that live in xeric environments can survive the shortage of water in one of three ways:

1. Evade drought by completing their life cycle during the rainy season;
2. Avoid drought by resisting water loss and/or increasing water uptake; and
3. Tolerate drought (Levitt, 1980).

In the plant kingdom, the ability of cells, organs and whole organisms to survive repetitive cycles of severe dehydration and rehydration has evolved in most systematic groups (Hartung *et al.*, 1998). DT occurs in heterotrophs, autotrophs, unicellular and multicellular organisms, as well as in plants with and without the vascular system and functional stomata (Proctor and Tuba, 2002). DT of vegetative tissues has only been observed in approximately 350 species. Almost all vascular plants have DT spores, pollen or seeds, therefore there is a great potential for DT in the plant kingdom. However, DT expression in vegetative tissues is less common

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(Proctor and Tuba, 2002). Oliver *et al.* (2000) have proposed that DT has re-evolved independently several times as mechanisms of DT among bryophytes, ferns and angiosperms differ greatly.

DT is only advantageous under extreme conditions. At the same time it is a selective disadvantage with respect to competition for resources and growth under non-extreme growth conditions (Hartung *et al.*, 1998). The means by which desiccation is tolerated in lower DT plants are different from those observed in angiosperms (Oliver and Bewley, 1997; Oliver *et al.*, 1998; Farrant, 2000). The former rely mainly on repair of rehydration-induced damage, while the latter utilise protective strategies during dehydration (Bewley and Oliver, 1992).

Lower order DT plants (such as mosses) have no control over water loss or gain and their water status changes rapidly (Proctor and Tuba, 2002). More than 95% of RWC can be lost or gained within minutes or hours. However, these plants are capable of storing water in the capillary space external to the photosynthesising cells. This water reservoir allows for free gas exchange, essential for photosynthesis (Dilks and Proctor, 1979; Proctor and Smith, 1995; Zotz *et al.*, 2000). Vascular DT plants, on the other hand have to refill xylem vessels and re-establish water conductivity within the plant, as well as return cellular metabolism to 'normal' (Proctor and Tuba, 2002). If plants are small, then embolisms can, with time, be repaired by root pressure (Proctor and Tuba, 2002). This is most probably the reason that there are no DT trees (Gaff, 1972).

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This physiological limitation in size, combined with morphological adaptations, enable DT plants to survive repetitive severe dehydration and rehydration cycles. Bryophytes, much like vascular DT plants, look vastly different wet and dry. The leaves fold (*Eragrostis nindensis*, *Myrothamnus flabellifolius*, *Xerophyta* spp.), curl (*Craterostigma* spp.) and shrink during desiccation, minimise the exposed surfaces to light, and thus decrease the levels of potential light-induced damage. In addition to these, leaves of vascular DT plants may also be hairy or scaly, they may lose chlorophyll or retain it, and, in addition, accumulate large amounts of 'sun-screen' pigments (reviewed by Farrant, 2000; Vicre *et al.*, 2004).

DT plants are usually pioneers in dry niches of areas that may or may not be generally arid. They are found in shallow soils, rocky outcrops and direct sunlight, experiencing water loss daily (Porembski, 2000). Vascular DT plants have no distinct morphologic feature in common (Gaff, 1977). There are approximately 100 known vascular DT species and more than two thirds of them are monocotyledonous; half of these are from the family Poaceae (Gaff and Loveys, 1984; Bewley and Krochko, 1982). Most dicotyledonous DT plants belong to the family Scrophulariaceae, where nine of 10 African *Craterostigma* species are desiccation tolerant (Fischer, 1992). Gaff (1977, 1987) reported that the majority of vascular DT plants occur in southern Africa, although several species are found in southern America and western Australia.

1.4 Stresses Associated with Water Deficit and Protection Mechanisms Believed to Protect Against them in Vascular Desiccation Tolerant Plants

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Vascular DT plants grow slowly and become quiescent in the desiccated state (Gaff, 1980). They usually reach air-dry state (where the water content of leaves equals that of the surrounding air) in two to three days once soil water is exhausted (Gaff, 1977). It has been suggested that when leaves reach the air-dry state, the plants can stay viable for a long time (Gaff, 1977; Scott, 2000). In order to survive severe dehydration and subsequent rehydration cycles, DT plants need to be able to limit the damage to a repairable level, maintain physiological integrity in the dry state and mobilise protective and reparative mechanisms during the loss and gain of water, respectively (Bewley, 1979; Oliver and Bewley, 1997; Oliver *et al.*, 2000).

The first line of defense is preventing the potentially harmful light-chlorophyll interaction through either chloroplast shading or chloroplast dis-assembly (Farrant, 2000). The ability of a DT plant to lose almost all chlorophyll upon desiccation and resynthesise it during rehydration was initially described by Vassiljev in 1931, but was later re-introduced and assigned terms poikilochlorophylly (loss of chlorophyll) and homoiochlorophylly (retention of chlorophyll) by Hambler in 1961. These are the main protective strategies employed by vascular DT plants in preventing photo-oxidative damage through reactive oxygen species (ROS) formation during dehydration and rehydration (reviewed by Farrant, 2000).

It is believed that poikilochlorophylly is evolutionarily the youngest strategy utilised by DT plants (Proctor and Tuba, 2002). The dismantling of the internal chloroplast structure during severe dehydration is an orderly process, as is the resynthesis and reassembling of thylakoid membranes upon subsequent rehydration (Sherwin and Farrant, 1996, 1998; Proctor and Tuba, 2002). This strategy minimises photo-

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oxidative damage without the added problem of maintaining intact chloroplasts during prolonged dry periods, as is done by homoiochlorophyllous plants.

Homoiochlorophyllous DT (HDT) plants, such as DT mosses, DT ferns, some DT monocotyledonous and all DT dicotyledonous plants keep most chlorophyll intact during dehydration and rehydration, but utilise other means to protect themselves against light-induced damage. As there is no need to resynthesise chlorophyll upon rehydration, HDT plants generally rehydrate faster than poikilochlorophyllous DT (PDT) plants (Hartung *et al.*, 1998). They accumulate anthocyanins ('sun-screen' pigment) and up-regulate antioxidant enzymes that prevent lipid peroxidation, thus minimising potential photo-oxidative damage, especially to membranes. In HDT plants, even though the photosynthetic apparatus is readily recoverable, some loss in chlorophyll does occur. This is species specific, and also highly dependent on environmental conditions (Farrant *et al.*, 1999, 2003; Proctor and Tuba, 2002).

A plethora of biochemical mechanisms are necessary for DT. Molecules that are synthesised and accumulated during unfavourable conditions are believed to be an important part of protection (Mundree *et al.* 2000). Some of these include the production of non-reducing sugars (Crowe *et al.*, 1984), synthesis of dehydrins and rehydrins (Close, 1996) and the components of the free radical scavenging system (Smirnoff, 1993; Kranner and Lutzoni, 1999). Accumulation of sucrose is an essential adaptation observed in all DT plants (Salisbury and Ross, 1992). During dehydration sucrose is believed to protect the intracellular components, such as enzymes and membranes, against damage (Ahmad *et al.*, 1979; Crowe *et al.*, 1984; Oliver and Bewley, 1997). The increased concentration of sucrose during dehydration leads to a

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drop in the osmotic potential (Salisbury and Ross, 1992) and the stability of the subcellular environment is improved by vitrification (Crowe *et al.*, 1992; Leopold *et al.*, 1992; Bianchi *et al.*, 1993; Dure, 1993; Schneider *et al.*, 1993; Ingram and Bartels, 1996; Oliver and Bewley, 1997; Ghasempour *et al.*, 1998; Oliver *et al.*, 1998).

Vascular DT plants have to avoid the following sub-cellular stresses in order to survive severe dehydration and subsequent rehydration:

1. Mechanical stress that occurs when the RWC is between 100 and 50% and the loss of turgour can cause the breaking away of the cell membrane from the cell wall (Vertucci and Farrant, 1995);
2. Oxidative stress that occurs due to the formation of free radicals as a result of chlorophyll-light interaction at low RWCs (Smirnoff, 1993);
3. Metabolic stress that causes degradation of macromolecules due to lack of water necessary for the hydrophilic and hydrophobic interactions (Leopold and Vertucci, 1989; Crowe *et al.*, 1992; Vertucci and Farrant, 1995).

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1.4.1 Mechanical Stress and Protection

Tension caused during dehydration between the cell wall and the cell membrane can force the plasma membrane to pull away from the cell wall as the cell dries (reviewed by Levitt, 1980; Gaff, 1989; Vertucci and Farrant, 1995; Farrant, 2000). Some species employ the cell wall folding mechanism in order to reduce the tension between the cell wall and the cell membrane during desiccation. This has been observed particularly in the *Craterostigma* species (Vicre *et al.*, 1999; 2003; 2004). This mechanism enables the cells to decrease in volume, with minimal pressure changes.

The elasticity of the cell wall of these species changes as the RWC decreases from rigid to elastic (Vicre *et al.*, 1999). However, this phenomenon is not only observed in vascular DT plants, but also in some desiccation-sensitive species that have specialised cells, such as the hydrenchyma in *Peperomia* species (Rabas and Martin, 2003).

The epidermis of many DT plants wrinkles during desiccation. At low water contents, cells contract due to tight connections between the cell wall and the plasma membrane (Hartung *et al.*, 1998). Transient membrane damage might occur, leading to solute leakage from cells (Levitt, 1980; reviewed by Farrant, 2000). This is particularly evident during rehydration when the structural arrangement of compounds within the cell membrane changes. Schwab (1986) compared the solute leakage of desiccation-sensitive and DT species and reported that the leakage from the former was significant and from the latter negligible. Similar work was done by Sherwin and Farrant (1996) and Farrant *et al.* (1999).

It has been observed that before acquiring DT, vacuoles of orthodox (DT) seeds are filled with storage proteins (reviewed by Vertucci and Farrant, 1995). Similar vacuole filling with compatible solutes has been observed in leaves of *Xerophyta viscosa* and bundle sheath cells of *E. nindensis*, and this is believed to provide the mechanical support by applying back pressure against the cell membrane and the cell wall preventing their collapse (Farrant and Sherwin, 1998; Vander Willigen *et al*, 2004). It has also been observed that some DT plants subdivide the vacuoles into many small vesicles, thus minimising the tension between the cell membrane and the cell wall (Farrant, 2000).

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1.4.2 Oxidative Stress and Protection

As DT plants enter anhydrobiosis, irreversible damage can be done to nucleic acids, lipids and proteins through Maillard reactions and reactive oxygen species (ROS), causing the subcellular structures to break down and denature (Kranner *et al.*, 2002). The resulting damage may be irreversible (Wolfe *et al.*, 1986).

Although ROS are produced when the plant is fully hydrated and metabolising optimally (Kranner *et al.*, 2002), their production increases as plants dry (Smirnoff, 1993; Elstner and Osswald, 1994; Kranner and Lutzoni, 1999) as well as upon the subsequent rehydration (Sgherri *et al.*, 1994). When water is sparse, the light energy that is harnessed by chlorophyll cannot be dissipated through photosynthesis, thus causing formation of oxygen free radicals (Larson, 1988; Smirnoff, 1993; Navari-Izzo *et al.*, 1997; Farrant, 2000). During photosynthesis and respiration, electron transport chain ROS are produced when electrons are transferred to ground state oxygen ($^3O_2$) (Halliwell, 1984). Photo-excited chlorophyll pigments transfer the excitation energy to $^3O_2$, forming a singlet oxygen ($^1O_2$). At photosystem II (PS II), superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are produced (McKersie and Leshem, 1994; Kranner and Lutzoni, 1999).

Vascular DT plants can minimise this free radical formation through poikilo- and homoiochlorophylly, as described earlier, and, in addition, by production of antioxidants (Tuba *et al.*, 1996; Sherwin and Farrant, 1998). Antioxidant enzymes

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such as ascorbate peroxidase (AP) and glutathione reductase (GR) minimise the potential ROS damage by interacting with free radicals and removing them from the cell environment. Production of antioxidants during dehydration is important as vascular DT plants need to be able to limit the damage done by free radicals to a repairable level, maintain physiological integrity in the air-dry state, as well as to be able to mobilise the repair mechanisms during rehydration to mend the damage that was done during desiccation, in the dry state, and possibly during rehydration (Bewley, 1979; Oliver and Bewley, 1997; Oliver *et al.*, 2000; Kranner *et al.*, 2002).

All vascular DT species studied to date show an upregulation of antioxidant systems which can account for some, but not all, free radical quenching (Smirnoff, 1993; Sgherri *et al.*, 1994; Kranner and Grill, 1997; Navari-Izzo *et al.*, 1997; Sherwin and Farrant, 1998; Farrant, 2000). In a report by Sherwin and Farrant (1998) AP activity was substantially higher in *C. wilmsii* than in *X. viscosa* once the plants reached air-dry state. A significant increase in AP activity was observed on partial dehydration (50 % RWC) of *C. wilmsii*, but AP activity decreased in the dry state and remained low during initial rehydration, recovering to control levels only in fully hydrated tissue. GR activity decreased on drying and increased to control levels on rehydration in *C. wilmsii*. Superoxide dismutase (SOD) activity declined during initial stages of drying in *C. wilmsii* but increased considerably during partial rehydration, and its activity declined to control levels in fully hydrated tissues. In *C. wilmsii* protection appears to be required during partial dehydration, when AP activity and anthocyanin content is elevated, and during partial rehydration when activities of GR and SOD are elevated (Sherwin and Farrant, 1998).

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C. wilmsii and *X. humilis* plants dried under light and dark conditions with restricted leaf movement showed an increase of AP activity by 80-100% in *C. wilmsii* during drying in all treatments, but only plants dried under light conditions with no restriction to leaf movement (natural drying regime) reduced activity to that of control levels upon subsequent rehydration (Farrant *et al.*, 2003). GR activity increased markedly (to 275% of control) during drying in leaves of plants dried naturally. On rehydration the activity of this enzyme dropped to control levels.

AP activity increased markedly in leaves of *X. humilis* dried in the light (300%). The activity of this enzyme returned to control levels during rehydration. GR activity was elevated (200%) during drying of plants under light conditions. Activity declined to control levels upon rehydration (Farrant *et al.*, 2003).

Vascular DT plants cannot stay in the dry state indefinitely due to loss of viability (Kranner *et al.*, 2002). One of the reasons for the loss of viability is an increase of photo-oxidative damage during severe dehydration (Farrant and Kruger, 2001).

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1.4.3 Metabolic Stress and Protection

At high relative water contents (>80%) the hydrophilic interaction of water molecules with proteins, lipid bilayer and nucleic acids ensures their functional conformation, but this stabilisation of macromolecules is compromised when relative water content drops below 65% (Farrant, 2000). Also, as RWC drops, the concentration of chaotropic ions increases. At lower concentrations the damage induced by these ions is reversible since damage done by them is based on competition for binding sites.

However, at higher concentrations they can promote membrane denaturation and dissociation of protein subunits.

In addition to homoiochlorophyllous and poikilochlorophyllous strategies, other protection mechanisms against damage to proteins and nucleic acids are possible through accumulation of low molecular weight compounds (Blackman *et al.*, 1992). Low molecular weight compounds help combat chaotropic ion damage by binding to vulnerable cellular components, stabilising their structure. The chemistry and the amount of compatible solute accumulation is species specific (Schwab and Gaff, 1986; Dietz and Keller, 1996).

Compatible solutes are a group of uncharged compounds that replace water molecules and bind to polar residues of proteins and phospholipids. This group of compounds comprises of polyols, betaines, polyamines and amino acids (Bohnert *et al.*, 1995). They contribute towards membrane stability and aide in vitrification. Studies have suggested that the compatible solutes may also aide in the retention of water through osmotic adjustment (Yancey *et al.*, 1982; Gaff, 1989), and may behave as antioxidants (Floyd and Nagy, 1984; Bohnert *et al.*, 1995).

At low RWC, accumulated sugars, mainly sucrose, form a highly viscous glassy state. This glass prevents crystalisation of protoplasmic constituents (Bruni and Leopold, 1991). Due to its high viscosity, the glass slows down chemical reactions and traps residual water molecules, minimising subcellular damage. Upon rehydration the glass melts, ensuring efficient recovery of metabolism.

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It has been suggested that sucrose is necessary in acquisition of desiccation tolerance as it stabilises membranes by preventing phase transition and fusion (Hoekstra *et al.*, 1997). However, more recent studies have shown that it is not so much the accumulation of sucrose that is necessary, but the removal of monosaccharides (which it turn form oligosaccharides) (McKersie and Leshem, 1994; Farrant and Sherwin, 1998). Monosaccharides are necessary for respiration (Leprince *et al.*, 1992) and their removal will slow down metabolism. By eliminating monosaccharides, Maillard reactions may also be minimised. Maillard reactions are particularly damaging as they result in cross-linking between glucose and proteins, causing their inactivation and irreversible damage (McKersie and Leshem, 1994; Farrant and Sherwin, 1998).

1.5 Longevity of Dry Desiccation Tolerant Systems

Longevity of air-dry spores, pollen, seeds and DT plants varies among species, but they are all highly dependent of water content and temperature (Hoekstra, 1986). In pollen, for example, at room temperatures and 40% RH, desiccation sensitive species last only hours, while tolerant species can last several months (Hoekstra, 1995). Lindsay *et al.* (1992) compared the storage longevity of spores of four fern species, in the air-dried state and in hydrated state, and found that hydrated, non-green spores remained viable for up to two years, while the viability of air-dried spores decreased with time. Also, green (chlorophyllous) spores were found to lose viability more quickly air-dried than hydrated. Conversely, the spores of mosses and fungi were found to lose viability faster in the hydrated state than in the air-dry state (Dalen and Soderstorm, 1999; Hoy *et al.*, 1993; Pfirter *et al.*, 1999; Beuchat, 1992; Nagtzaam and Bollen, 1994).

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Orthodox seeds are capable of drying to low moisture content and their longevity increases with a decrease in temperature and RWC (Roberts, 1973; Roberts and Ellis, 1982). Roberts and Ellis (1989) have proposed that there is an inverse relationship between moisture content of seeds and their longevity. This is attributed to the water status in the seed, namely if a seed is very dry (<5% RWC) the water present is bound and not available for chemical reactions (Murdoch and Ellis, 1992). Orthodox seeds induce desiccation tolerance early in their development, much earlier than when the drying event occurs, at their maturity (Wellington, 1956; Kermode and Bewley, 1985; Harlan and Pope, 1992).

The longevity of dry cryptogams has been researched extensively (reviewed by Bewley and Krochko, 1982; Lobban and Harrison, 1994; Davison and Pearson, 1996; Oliver and Bewley, 1997). Although some data are available on the longevity of excised dry leaves of several vascular DT species (Gaff, 1977; Tuba *et al.*, 1996, 1998), DT is a whole plant response, and so these data are not a true reflection of plant behaviour (Farrant and Kruger, 2001).

Much data have been produced in the last two decades, reporting on various aspects of DT in vascular plants. Studies thus far have mainly focused on mechanisms employed by several DT plant species with regard to surviving severe dehydration and/or subsequent rehydration (for reviews see Oliver and Bewley, 1997; Farrant, 2000). Almost no work has been done on the longevity of angiosperms in the dry state.

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No studies have been conducted on longevity of vascular DT plants in the field. Hartung *et al.* (1998) and Schiller *et al.* (1997) suggested that dry *Chamegegis intrepidis* survives for 11 months. Scott (2000) proposed that dry vascular DT plants can remain viable for 'several years', but no data were provided to support such a suggestion. Proctor and Tuba (2002) proposed that DT bryophytes and some vascular DT plants are capable of remaining in the dry state for six months, or even a year, without losing viability, but that this longevity is more limited than that of orthodox seeds. Farrant and Kruger (2001) explored the ability of intact dry *M. flabellifolius* plants to recover a prolonged drought. Their findings suggest that this woody DT plant is capable of full recovery following a six month simulated summer drought, and that thereafter viability is lost between nine and 12 months. Some studies have suggested that vascular DT plants cannot stay in the dry state indefinitely due to loss of viability that may be attributed to oxidative damage (Farrant and Kruger, 2001; Kranner *et al.*, 2002).

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1.6 Drying Speed of Desiccation Tolerant Systems

Plants and/or plant organs might have a genetic predisposition to DT, but DT is generally associated with a developmental stage of a plant or organ. DT is also highly dependent on the drying regime that the plant (or organ) is subjected to under laboratory conditions. Bewley and Black (1994) said that developing orthodox seeds acquire DT only once the reserves have been accumulated. Thus, if a developing orthodox seed is subjected to fast drying (over forced air) it will not survive, whereas if it is dried slowly (under laboratory temperature and relative humidity), it will (Kermode and Bewley, 1985; Bewley and Black, 1994). This has been attributed to the time required for DT mechanisms to be up-regulated.

Lower order DT plants seem to be tolerant to any drying regime. They employ protective and repairing mechanisms that ensure their survival upon rehydration (Oliver and Bewley, 1997), but those plants that are dried faster (at lower RH and higher temperatures) generally require a longer period for recovery and re-establishing of physiological and metabolic processes upon the return of non-stressful conditions (Schonbeck and Bewley, 1981).

Angiosperm DT plants are not as tolerant to all drying regimes. Cooper and Farrant (2002) showed that flash dried *C. wilmsii* leaves (homoiochlorophyllous) are capable of rehydrating, while *X. humilis* leaves (poikilochlorophyllous) subjected to the same drying regime died upon rehydration. The ability of vascular DT plant to survive fast drying (drying over silica gel) is species specific, however, all PDT plants are intolerant to this drying regime (Farrant *et al.*, 1999).

Even though lower order DT plants are capable of fast dehydration and rehydration, the survival of vascular DT plants, in general, is highly dependent on slow drying, due to their physiological complexity (Oliver *et al.*, 2000). The speed of drying is dependent on a number of environmental and physiological factors:

1. Relative humidity (RH) of the air surrounding drying plants. The lower the RH the faster the desiccation rate and the lower the RWC of leaves at the air-dry state;
2. Air temperature, as well as the temperature of the micro-climate immediately surrounding the aerial parts of plants; the higher the temperature the faster the rate of dehydration;

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3. Shape and size of the aerial parts and the composition of the protective outer layer (Pammenter *et al.*, 2002).

1.7 *Craterostigma wilmsii* and *Xerophyta humilis*

In order to survive desiccation *C. wilmsii* and *X. humilis* are believed to rely mainly on the protection of cellular components during dehydration, and are thus classified as modified DT plants. True DT plants, such as mosses, repair damage that has accumulated during dehydration and while in the dry state, once the water becomes available.

Craterostigma wilmsii Engl. (Scrophulariaceae) is a small, leafy, dicotyledonous DT plant that grows in direct sunlight and frequently experiences high water vapour deficit that could cause rapid drying (Farrant *et al.*, 1999). In the field, *C. wilmsii* plants undergo numerous cycles of drying and rehydration, thus rapid induction of protective mechanisms is essential to ensure survival (Farrant *et al.*, 1999).

C. wilmsii is homiochlorophyllous (Figure 1.1). This species keeps most of the chlorophyll and carotenoids during drying and in the dry state, but uses other means to protect itself from chlorophyll-light interaction, thus preventing photo-oxidation (Farrant, 2000). These include leaf curling and inner leaf shading, anthocyanin accumulation and antioxidant upregulation.

C. wilmsii are found in the NE South Africa where rain is not ample, but the rainy season lasts from September to June. Average minimum and maximum temperatures

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in summer are 13/26°C and in winter 7/20°C, respectively, and prolonged drought does not last longer than two months.

Figure 1.1: Hydrated (A) and desiccated (B) leaves of intact *C. wilmsii* plants. Purple colour of desiccated leaves indicates anthocyanin accumulation.

Xerophyta humilis (Bak.) Dur. and Schinz. (Velloziaceae) is a monocotyledenous DT species. It is poikilochlorophyllous and dismantles thylakoid membranes when water is sparse. It is found in northern South Africa on rocky outcrops known as inselbergs. During severe dehydration, leaf area decreases and leaf colour changes from green to yellow/brown (Figure 1.2).

Rain is plentiful from October to March. Drought occurs during the remaining six months of the year. Average minimum and maximum temperatures in summer are 17/32°C and in winter 6/25°C, respectively.

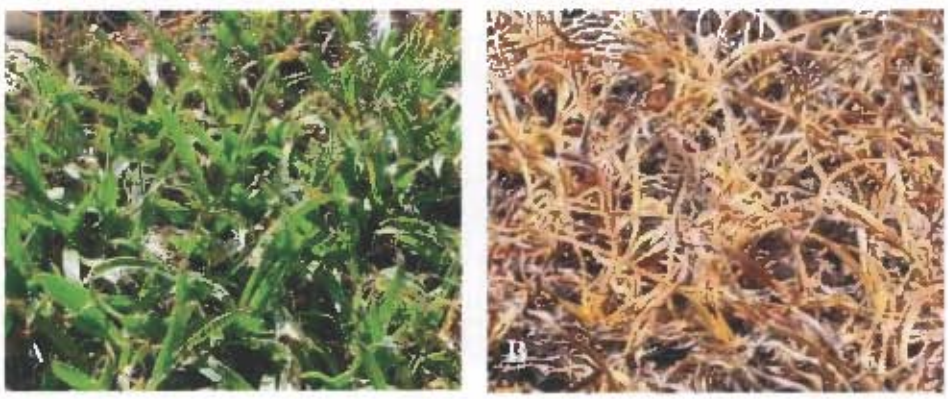

Figure 1.2: Hydrated (A) and desiccated (B) leaves of intact *X. humilis* plants. Yellow-brown colour of desiccated leaves is indicative of chlorophyll loss.

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1.8 Aims

DT systems cannot remain viable in the dry state forever (Walters *et al.*, 2005). Breakdown of systems and defense mechanisms is inevitable, but is highly dependent on the environmental conditions and the species pre-disposition (Walters *et al.*, 2005). Also, as suggested by Tuba *et al.* (1993, 1996), poikilochlorophyllous species might be able to remain viable in the dry state longer than homoiochlorophyllous species. In turn, this might be the influence of the amount of chlorophyll retained by the species while in the dry state (Sherwin and Farrant, 1998).

The aim of this study was to investigate the longevity of dry *C. wilmsii* (homoiochlorophyllous) and *X. humilis* (poikilochlorophyllous) species at varying light intensities, temperatures and RH under simulated field conditions. The two species are of similar size and naturally occur in environments where water deficit is frequent and where that water deficit varies between hours and many months. Yet each species has evolved an independent strategy in order to cope with such environmental conditions.

Numerous longevity studies have been conducted on excised leaves only, and only one study thus far (Farrant and Kruger, 2001) has reported on the longevity of an intact, dry vascular DT plant, *M. flabellifolius*. This study explored the suggestions put forward that viability of dry DT species is limited and that this limitation might be a result of the amount of chlorophyll retained by the species while in the dry state.

Thus, the present study examined the physiological and biochemical changes in dry *C. wilmsii* and *X. humilis* under a variety of simulated field conditions. These conditions

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put forward that viability of dry DT species is limited and that this limitation might be a result of the amount of chlorophyll retained by the species while in the dry state.

Thus, the present study examined the physiological and biochemical changes in dry *C. wilmsii* and *X. humilis* under a variety of simulated field conditions. These conditions mirrored those experienced by the two species in the field during the prolonged drought as well as conditions that might induce accelerated aging. The 'artificial' accelerated ageing conditions were introduced in order to explore the ability of these two species to survive drought at temperatures and relative humidity under which desiccation would not generally occur naturally. However, with the changing global climate due to the greenhouse effect, droughts, particularly in summer, are becoming more prevalent (www.ipcc.ch/pub/un/syreng/spm.pdf). Thus, while both species would naturally spend maximum time in the dry state under cool, low relative humidity conditions, they are increasingly facing dry periods under hot conditions with variable humidity.

How long can these two species remain dry under a variety of environmental conditions (natural and artificial) without losing viability? What causes loss of viability under these conditions? To answer these questions, the responses of *C. wilmsii* and *X. humilis* plants to dehydration, prolonged drought and subsequent rehydration explored included, among others:

1. The effect of temperature and RH on the drying speed of plants and the subsequent influence of the drying speed on longevity of the plants in the dry state;

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2. The species' ability to shut-down and restart photosynthesis by examining the photosynthetic pigment loss and gain during dehydration, in the dry state over a period of months and upon subsequent rehydration, respectively;

3. Protection of the subcellular components through carbohydrate analyses and membrane damage with regard to lipid peroxidation, as well as report on any changes in the carbohydrate status while the plants were dry;

4. The accumulation of proteins and mRNA during dehydration and their stability in dry tissues during prolonged desiccation.

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CHAPTER TWO

The Influence of Temperature, Light Intensity and Relative Humidity on Longevity of dry *Craterostigma wilmsii* and *Xerophyta humilis*

2.1 Introduction

Tolerance to desiccation, as well as the tolerance to prolonged desiccation, are some of the factors that limit the distribution of plant species (Bewley, 1979). Within the plant kingdom, the ability of DT systems to maintain viability while in the dry state for a prolonged period depends on the species, plant organ(s), plant/organ maturity and climatic conditions under which desiccation takes place.

The longevity of DT lichens in the dry state varies between weeks and many months (Rogers, 1971; Kappen, 1973). However, among algae, longevity in the dry state without loss of viability can reach decades (Becquerel, 1942; Bristol, 1919; Parker *et al.*, 1969), or even more than a century, as reported by Cameron (1962) for *Nostoc commune*. Although not all stages of their life cycle have the same tolerance for desiccation, those structures involved in the resting stages are more tolerant to prolonged desiccation (Bewley, 1979).

Longevity of dry pollen, seeds and DT plants varies among species, but it is highly dependent on their water content and ambient temperature (Hoekstra, 1986). Orthodox seeds are capable of drying to low moisture content and their longevity increases with the decrease in relative humidity and storage temperature, as defined

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by the viability equation (Roberts, 1973; Ellis and Roberts, 1980; Roberts and Ellis, 1982). Premature, or accelerated ageing (Delouhe and Baskin, 1973), may be induced in seeds by exposing them to high temperatures and high relative humidity. The ability of orthodox seeds to withstand these harsh conditions varies among species.

Longevity of dry DT plants has been poorly researched thus far. Gaff (1977) described the tolerance of nine vascular DT species to prolonged desiccation and argued that their longevity is species dependent, ranging from six months to more than five years. However, that study was conducted on excised leaves only. Scott (2000) proposed that dry vascular DT plants can remain viable for several years, but no data were provided to support such a suggestion. Proctor and Tuba (2002) offered that DT bryophytes and some vascular DT plants are capable of remaining in the dry state for six months, or even a year, without losing viability, but that in general longevity of DT plants is more limited than that of orthodox seeds. Only Farrant and Kruger (2001) have studied a response of a DT angiosperm to prolonged desiccation. This was done on *Myrothamnus flabellifolius* where this species was maintained dry for 12 months. That study showed that *M. flabellifolius* can recover fully after six months under simulated (summer) field conditions, but damage accrued thereafter with viability being lost between nine and 12 months.

In this chapter the longevity of dry *C. wilmsii* and *X. humilis* plants was explored. This was done under the natural conditions that the plants would experience (i.e. winter), but the effect of a range of other environmental conditions were also examined. It is important to study the effects of long-term drought under summer conditions in order to explore the survival ability of these two species should the

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climatic conditions in the field change. It was expected that the viability of these two species in the dry state would differ in time, similar to ranging long-term viability of orthodox seeds of different species. Thus 'accelerated aging' storage conditions were also used to get an estimate of how robust the protection mechanisms are under extreme environmental conditions. Although prolonged drought does not occur naturally in summer months in the field, *C. wilmsii* and *X. humilis* plants do however experience frequent severe short-term dehydration/rehydration cycles in summer, due to high temperatures and low relative humidity. The effects of such conditions on longevity in the dry state were thus also tested.

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2.2 Materials and Methods

2.2.1 Field Conditions and Environmental Chambers

Figure 2.1 shows the average minimum (min) and maximum (max) temperatures, as well as the average monthly rainfall for 2003. Rainfall patterns in 2003 differed from that of three previous years' rainfall in that the drought was most prominent, lasting months longer. As these conditions are determining factors in survival of these species, they were chosen in order to explore each species ability to maintain viability in the dry state for periods even longer than what the South African Weather Service data has suggested.

The average min/max temperatures that *C. wilmsii* plants experienced in the field (Figure 2.1A) were 13°C/26°C in summer and 7°C/20°C in winter, with no longer than two months of continuous drought in the winter months. *X. humilis* plants experienced average min/max summer temperatures of 17°C/32°C, and winter temperatures of 6°C/25°C, with up to six months of continuous drought in autumn and winter months (Figure 2.1B).

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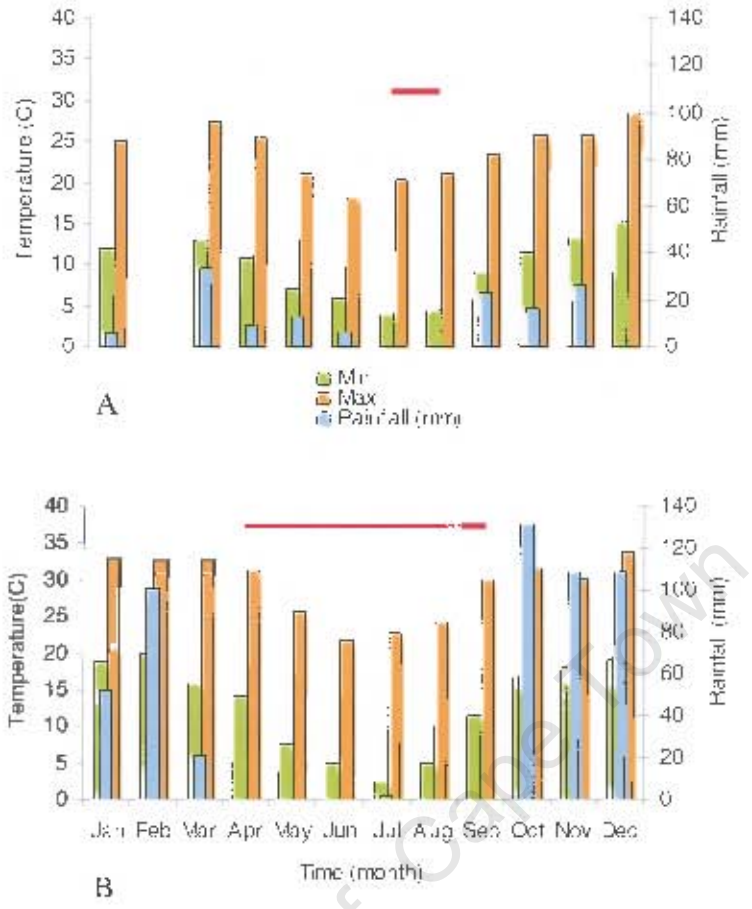

Figure 2.1: Summary of climatic field conditions *C. wilmsii* and *X. humilis* plants experience in the field near Lydenburg (A) and Rustenburg (B), respectively. The red horizontal line indicates the prolonged drought period. Lydenburg February data was missing from the South African Weather Service Database. Green bars – average minimum monthly temperature; orange bars – average maximum monthly temperature; blue bars – average monthly rainfall.

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Plants were subjected to prolonged desiccation under four different sets of simulated field conditions (Table 2.1). These included two sets of conditions that would resemble the natural field conditions (temperatures, light intensities and relative humidity) that the plants would experience at the start of a dry season, winter dehydration (WD), and the end of the dry season, summer dehydration (SD). Two further sets of conditions maintained the plants under artificial dehydrating conditions. These were introduced in order to explore each species ability to withstand prolonged dehydration under conditions during which prolonged

desiccation does not take place naturally in the field. One set of these conditions was similar to 'accelerated' aging (AA) conditions used in seed studies and is typified by high temperatures and high relative humidity. The other, winter green house conditions (WGH), maintained the plants under moderate temperatures, but varying relative humidity and light intensities. Cape Town winters are different from the winter conditions the plants would experience in the field. In the field winters are dry and sunny, while in Cape Town they can be wet and cloudy. Combinations of relative humidity, temperature and light intensity were implemented in order to explore the species' abilities to remain viable in the dry state. Table 2.1 summarises the conditions the plants were subjected to.

Table 2.1: Conditions under which *C. wilmsii* and *X. humilis* plants were dehydrated and then rehydrated after a period of time in the dry state

	Accelerated aging (AA)	**Summer drying (SD) conditions**	**Winter green house (WGH)**	**Winter natural drying (WD) conditions**
Temperature (°C)	high 20's to high 30's	25	mid teens to mid 20's	10 night/16 day
Light intensity ($\mu mol.m^{-2}.s^{-1}$)	high but variable >1200	350-400	variable moderate to low	>1000
RH (%)	high (>90)	50	variable	70
Day/Night length (h)	variable	16/8	variable	14/10

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2.2.2 Plant Material

Craterostigma wilmsii Engl. (Scrophulariaceae) and *Xerophyta humilis* (Bak.) Dub. and Schinz (Velloziaceae) plants were collected from the Buffelskloof Nature Reserve, Mpumalanga and Pilanesberg Nature Reserve, Northwest Province, respectively. Although the plants were collected in their native soil, most of the soil had to be removed for transport purposes. Once the plants were placed in the seedling trays, a mixture of peat, sand and potting soil was supplemented to the rooting support

mixture. The plants were kept in the green house at the University of Cape Town until required for the experimental studies. While in the green house, preceding dehydration, plants were watered every second day in order to maintain full turgour. Prior to the dehydration/rehydration cycles, the plants were moved into respective growth rooms and were acclimatised for two weeks. Once acclimatised, the plants were dried by withholding water. The plants were kept in the air-dried state for up to 10 months. Subsequently plants were watered at monthly intervals. Rehydration was done by soil watering until saturation and the high soil water content was maintained thereafter.

During dehydration leaf samples were taken from both species every day, at the same time of day, until the plants reached the air-dry state. Subsequent sampling was at monthly intervals, from dry and rehydrated plants. During rehydration sampling was done every six hours until plants reached 100% RWC. Leaves were sampled from three separate plants.

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2.2.3 Relative Water Content (RWC)

The leaf fresh weight (three replicates) was noted, and leaves were placed in the oven at 70°C for 48 hours in order to obtain dry weight. The following equation was used to calculate the absolute water content (AWC):

$$AWC = \frac{(\text{Fresh Weight} - \text{Dry Weight})}{\text{Dry Weight}}$$

AWC was converted to RWC by using the standard equation, as previously described by Sherwin and Farrant (1996):

$$RWC = (AWC_{sample} / AWC_{full\ turgour}) * 100,$$

where AWC at full turgour is the water content of fully hydrated leaves, brought to full turgour by bagging of the whole tray of plants watered to full soil saturation.

2.2.4 Ultrastructural Studies – Transmission Electron Microscopy

Leaves were excised from at least three different plants per species per treatment and were cut into small pieces (approximately 2 mm^2). Samples were taken from hydrated, dry, and dead leaves. Leaf tissue was prepared according to the method previously used for these species in a study by Sherwin and Farrant (1996). Samples were fixed for 18 hours in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) containing 0.5% caffeine. Thereafter, the samples were washed in 0.1M phosphate buffer. Post-fixation was done using 1% osmium tetroxide in 0.2M phosphate buffer (1:1, v/v). Samples were then washed with 0.1M phosphate buffer. The dehydration process was done using ethanol dilutions ranging from 30% to 100%. The last step of dehydration was conducted using 100% acetone. Acetone was gradually replaced with epoxy resin (Spurr, 1969) starting with 50% Spurr's resin solution (1 acetone : 1 Spurr's resin; v/v). After all acetone was replaced with resin, the samples were embedded in epoxy resin which was left to harden for 16 hours at 60°C.

Samples were sectioned using a Reichert Ultracut-S microtome to a thickness of 95nm. Sections were stained using 2% uranyl acetate and 1% lead citrate (Raynolds, 1963), and were viewed using LEO 912 TEM. Observations were made of the general cell structure, but particular notice was taken of membrane damage, chloroplast ultrastructure and the presence of mitochondria.

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Due to the nature of the fixation process used in this study, some rehydration of the dry samples did take place. However, since all samples were treated in the same manner, comparisons among treatments are believed to be valid.

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2.3 Results and Discussion

2.3.1 Longevity

Plants subjected to prolonged dehydration under four different sets of simulated field conditions survived this differently (Table 2.2).

Table 2.2: Survival of plants of *C. wilmsii* and *X. humilis* under various environmental conditions described in Table 2.1

	Accelerated aging (AA)	Summer drying (SD) conditions	Winter green house (WGH)	Winter natural drying (WD) conditions
C. wilmsii	1 month	2 months	2 months	3 months
X. humilis	≥10 months	≥10 months	≥10 months	≥10 months

C. wilmsii plants remained viable in the dry state for one month under accelerated aging (AA) conditions of high temperatures and high relative humidity, while plants that were subjected to moderate temperatures under summer drying (SD) and winter green house (WGH) conditions, lost viability after two months (Table 2.2). Although this species does not experience severe drought for longer than two months in the field (Figure 2.1 A), plants that were subjected to winter natural drying (WD) conditions remained viable in the dry state for three months (Table 2.2).

In the field, *X. humilis* plants may experience severe drought for up to six months (Figure 2.1 B). In this study the viability of this species in the dry state was explored for up to 10 months, and the plants under all abovementioned conditions survived this prolonged desiccation (Table 2.2).

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2.3.2 Dehydration

The time taken by *C. wilmsii* plants to reach the air-dry state depended greatly on temperature (Figure 2.2). Under accelerated aging (AA) conditions (high temperatures), as well as summer drying (SD) conditions (moderate temperatures), *C. wilmsii* plants reached the air-dry state within nine days (Figure 2.2) (similar results reported by Farrant *et al.*, 1999 and Cooper and Farrant, 2002). However, at lower temperatures (WGH and WD conditions), the air-dry state was reached only after 18 days (Figure 2.2). It appeared that the drying speed was not influenced by relative humidity, however, the RWC at the air-dry state was proportional to relative humidity (Figure 2.2).

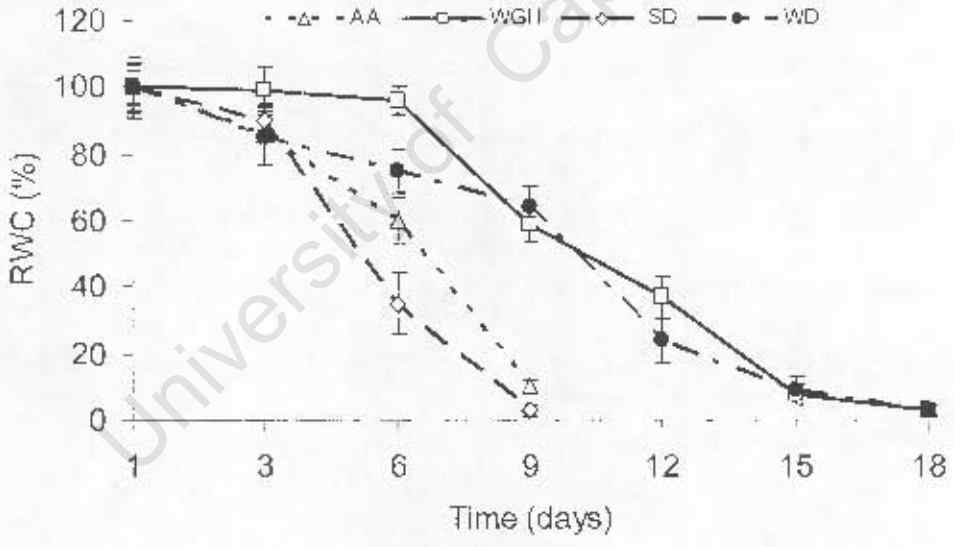

Figure 2.2: Dehydration speed of *C. wilmsii* plants under simulated field and artificial drying conditions. Plants maintained under AA and SD conditions dried within nine days, while plants that were kept under WGH and WD conditions dried within 18 days. Slower drying might be attributed to lower temperatures. AA (Δ) – accelerated aging conditions; WGH (□) – winter green house conditions; SD (◊) – summer dehydration conditions; WD (●) winter dehydration conditions.

The speed of dehydration may influence the extent of structural and compositional changes (protection mechanisms) necessary for survival of severe prolonged

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dehydration and subsequent rehydration (Navari-Izzo *et al.*, 1995). However, work done by Cooper and Farrant (2002) showed that independent of the drying speed, whether fast (over silica gel) or slow (natural), *C. wilmsii* plants were capable of inducing sufficient protective mechanisms thus ensuring minimal damage during dehydration and once desiccated. Data from the present study (see Chapters 3, 4 and 5) suggest that sufficient protection did accrue in all four treatments tested here and that loss of viability was due rather to deterioration during dry storage.

C. wilmsii plants that were subjected to conditions that would accelerate aging (AA) had a higher leaf relative water content (RWC) once the plants had reached the air-dry state, as expected due to higher relative humidity of the environmental conditions they were maintained under. The RWC was approximately 10%, while under all other conditions, SD, WGH and WD, the RWC of leaves at the air-dry state was approximately 3% (Figure 2.2). It is possible that this high leaf RWC in plants subjected to AA conditions contributed to this species inability to remain viable in the dry state longer than one month (Table 2.2).

The drying speed of *X. humilis* plants was independent of temperature, and the RWC at the air-dry state was independent of relative humidity. Irrespective of temperature, light intensity and relative humidity, once the plants reached the air-dry state the leaf RWC was approximately 4% and the plants remained viable for at least 10 months. However, maintenance under varying light conditions (WGH) resulted in this species reaching the air-dry state within 18 days (Figure 2.3 B), while under all other regimes within 11 to 12 days (Figure 2.3 A). It is possible that variable light during drying caused the WGH plants to delay the disassembly of the photosynthetic apparatus, an

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important protection strategy in this species (Farrant, 2000; Farrant *et al.*, 2003). It is possible that this species is capable of remaining viable in the dry state for even a longer period of time, but due to management constraints this study explored their longevity in the dry state for up to 10 months only.

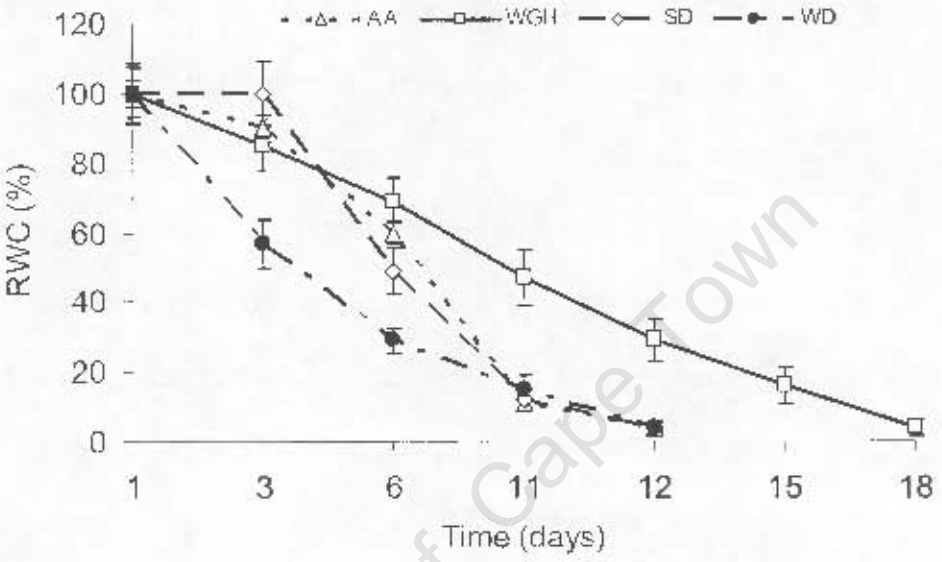

Figure 2.3: Dehydration speed of *X. humilis* plants under simulated field and artificial drying conditions. Plants maintained under AA and SD conditions dried within 11 days, while plants that were kept under WGH and WD conditions dried within 12 and 18 days respectively. Slower drying might be attributed to highly variable light conditions. AA (Δ) – accelerated aging conditions; WGH (□) – winter green house conditions; SD (◊) – summer dehydration conditions; WD (●) winter dehydration conditions.

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2.3.3 Rehydration

Upon rehydration, both *C. wilmsii* and *X. humilis* plants reached full turgour within two to three days at higher temperatures, but rehydration was a day slower under lower temperatures (Table 2.3). The somewhat slower rehydration at lower temperatures would possibly have ensured more time to repair and reconstitute photosynthetic apparatus and minimise potential ROS production during rehydration, especially in leaves of *C. wilmsii* plants (homoiochlorophyllous). In this species, the slower the rehydration, the longer the time taken by the outer leaves (with high levels

of 'sun screen' pigments) to uncurl and expose the inner green, and photosynthetically active leaves. It is possible that through this mechanism the plants ensure that the metabolic rates of inner leaves are at a level high enough to retain viability, increase antioxidant production (Sherwin and Farrant, 1998), and minimise rehydration-associated damage. Rehydration of *X. humilis* plants was marginally slower (Table 2.3) than that of *C. wilmsii* plants, possibly due to root embolisms. The rehydration speed of both species was not influenced by the prolonged desiccation.

Table 2.3: Time taken to reach full turgour (days) of *C. wilmsii* and *X. humilis* plants, after a range of time spent in the dry state

	AA						**SD**					
	Time in dry state (months)											
	1	**2**	**3**	4	**6**	**10**	**1**	**2**	**3**	**4**	**6**	**10**
C. wilmsii	2	d	X	X	X	X	2	3	d	X	X	X
X. humilis	3	3	X	7	3	2	3	2	X	2	3	3
	WGH						**WD**					
	Time in dry state (months)											
	1	**2**	**3**	**4**	**6**	**10**	**1**	**2**	**3**	**4**	**6**	**10**
C. wilmsii	2	4	d	X	X	X	3	4	2	d	X	X
X. humilis	3	4	X	3	**4**	4	6	4	X	3	4	3

d = dead; X = no samples were taken at that time

Bochicchio *et al.* (1998) showed that viability of dry *B. hygroscopica* was determined by the moisture content of leaves once they have reached the air-dry state. The water content of DT plants is invariably determined by the dryness of the surrounding air (Bewley, 1979). Although this does not hold true for *X. humilis* plants, as this study suggests, it is, however, in support of the findings for *C. wilmsii* plants. Under AA conditions, at such high RWC at the air-dry state (10%), *C. wilmsii* plants could have some degree of water activity (Type II water – Vertucci and Farrant, 1995). Although this water activity could be low, permitting limited metabolism, it is possible that toxic metabolic by-products could accumulate at this RWC but not be eliminated at a

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rate high enough to prevent damage and ensure viability (to be discussed in detail in Chapters 3, 4 and 5). It was interesting to note that this homoiochlorophyllous species was capable of surviving one month in the dry state under such damaging environmental conditions. Presumably, this could be attributed to effective levels of protective mechanisms up-regulated during dehydration (Cooper and Farrant, 2002).

Although the water content of air-dry plants is very low, it is possible that destructive processes do occur at a particularly slow rate, as has been previously suggested by Vertucci and Farrant (1995). These authors proposed that the speed of rehydration, and thus recovery, is dependent not only on plant morphology, but also on the amount of chlorophyll present in drying and dry tissues. They suggested that since removal of water results in a disruption of metabolic pathways, the energy absorbed by the photosynthetically active pigments cannot be dissipated in an orderly manner, and the consequence of this is the formation of the destructive ROS and free radicals. This is particularly evident in the time limit that *C. wilmsii* plants remained viable in the dry state. By retaining high levels of photosynthetically active pigments, the interaction between pigment and light is not easily preventable, other than through leaf curling and anthocyanin accumulation. It is possible that neither of these mechanisms is entirely effective for long-term desiccation. The rehydration speed of *C. wilmsii* plants might be highly dependent on the amount of damage accumulated during dehydration and in the dry state. Due to high chlorophyll levels in the dry leaves, *C. wilmsii* might be at a higher risk of photo-oxidative damage during drying and rehydration than poikilochlorophyllous *X. humilis*.

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Loss of viability, depicted in the ultrastructure of dead *C. wilmsii* and *X. humilis* leaves, is shown in Figure 2.4 below. An ultrastructural comparison was made among the fully hydrated, air-dry and dead leaves of each species. No noticeable differences were observed among treatments, and so only WD treated plants were used. Thylakoid membrane stacks and large starch granules were seen in both species at 100% RWC (Figure 2.4 A1 and B1). The cells were vacuolated with the cytoplasm appressed against the cell wall appearing as a narrow band at the cell periphery. Similar results have been reported previously by Sherwin and Farrant (1996; 1998), Farrant (2000) and Farrant *et al.* (1999; 2003).

Dehydrated, viable *C. wilmsii* plants retained thylakoid membranes (Figure 2.4 A2), while dry *X. humilis* plants dis-assembled them (Figure 2.4 B2). In *C. wilmsii* cell wall folding was evident and in *X. humilis* numerous vacuoles replaced the central vacuole. Similar results were previously reported by Farrant (2000) and Vicre *et al.* (2004). Smaller vacuoles reportedly store compatible solutes (Farrant, 2000). In both species the shape of chloroplast changed from elongated spherical to round.

Total destruction of the cellular components was observed in the dead tissues (Figure 2.4 A3 and B3). For *C. wilmsii* plants this might have been a result of accumulated oxidative damage due to retention of chlorophyll and breakdown of protective anthocyanins (see Chapters 3 and 4). *X. humilis* plants did not lose viability while dry, and so dead leaves off (10 month dry rehydrated) plants were used. This species does not retain any chlorophyll while dry, thus this extent of damage might be attributed to the overall cellular damage and slow macromolecular denaturation resulting from a prolonged drought period (> 10 months). However, it was also possible that some

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leaves of a plant were sacrificed in order to insure the survival of the plant. This has been observed particularly in *C. wilmsii* plants where the outer whorl leaves fail to rehydrate successfully due to damage accumulated while the plant was dry (reviewed by Farrant, 2000). As the outer whorl leaves fold over the inner, younger leaves, they are continuously exposed to environmental factors that may cause enough injury which might result in irreparable accrued damage.

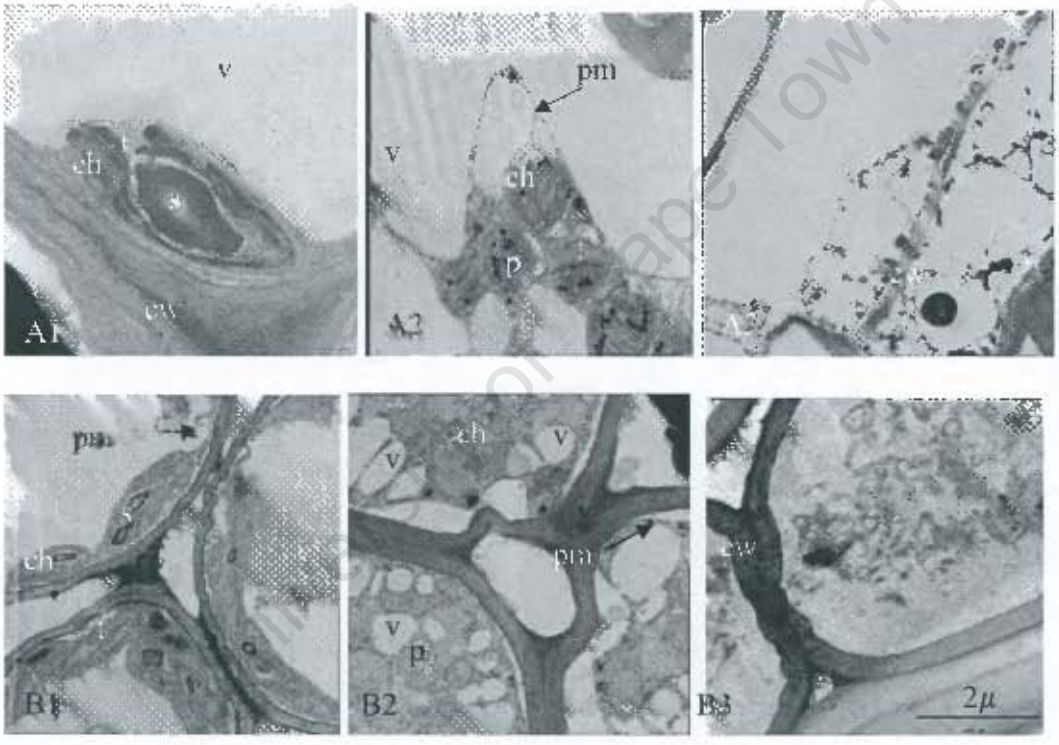

Figure 2.4: Ultrastructure of mesophyll cells of *C. wilmsii* (A) and *X. humilis* (B) hydrated (1), air-dry (2) and dead (3) leaves. In hydrated samples of both species only one vacuole (v) was present with the plasma membrane (pm) appressed to the cell wall. Chloroplasts (ch) were seen in the periphery of the cells. Thylakoid membranes (t) and starch (s) were present in the hydrated *C. wilmsii* and *X. humilis* leaf tissue. Following desiccation, in *C. wilmsii* the plasma membrane was seen to be withdrawn from the cell wall (cell wall had partially rehydrated due to the fixation process). Plastoglobuli (p) replaced starch, but the thylakoid membrane stacks were still present. Dry *X. humilis* exhibited numerous vacuoles. Starch had also been replaced by plastoglobuli, but the thylakoid membrane stacks were dismantled. The dead tissue of both species showed no cellular compartmentalisation, structure nor order. All micrographs are at magnification of x4000, except A1 at x5000.

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2.4 Conclusion

Homoiochlorophyllous *C. wilmsii* may be described as an opportunistic DT species. It does not experience severe dehydration for longer than two months, but short-term dehydration/rehydration cycles are common, especially in summer months. It managed to up-regulate protective mechanisms during dehydration that shielded it from possible short-term stresses associated with severe dehydration (Farrant, 2000; Cooper and Farrant, 2002). However, this protection was insufficient for desiccation periods longer than three months. In contrast, *X. humilis*, a poikilochlorophyllous species, ensures that minimal damage occurs while in the dry state by dismantling almost all photosynthetic machinery during dehydration. It is possibly this strategy that ensures long-term (≥10 months) viability in the dry state under a range of temperatures, light intensities and relative humidity. Thus, as Tuba *et al.* (1996) proposed, poikilochlorophyllous DT species are capable of remaining in the dry state considerably longer than homoiochlorophyllous DT species, and this holds true for excised (Tuba *et al.*, 1993, 1996, 1998), as well as intact leaves.

Also, similar to the viability equation used for determining the long-term viability of orthodox seeds in storage, it might be possible to develop a comparable method to determine the viability of DT plants while in the dry state. The viability of both dry orthodox seeds and DT plants seems to be dependent on temperature and relative humidity. Variables that should be added into the potential viability equation for DT plants, and that are not included in the seed viability equation, are light intensity and chlorophyll content. As suggested by Sherwin and Farrant (1996), and as shall be discussed in the following chapters, chlorophyll content appears to be a factor that

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significantly influences the longevity of dry DT plants, particularly homoiochlorophyllous DT species.

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CHAPTER THREE

Photosynthetic Response of *Craterostigma wilmsii* and *Xerophyta humilis* to Dehydration, Prolonged Desiccation and Subsequent Rehydration

3.1 Introduction

Along with diurnal and seasonal changes in light quantity and quality, plants have evolved several physiological and biochemical mechanisms to optimise light harvesting (Müller *et al.*, 2001). Among them are physical movement of leaves and chloroplasts, phytochrome shade-avoidance responses, and adjustment of the size of light harvesting pigment antennae found within chloroplasts. However, under water limiting conditions, DT plants have also evolved strategies that minimise the potential damage of photo-oxidation in leaves (Tuba *et al.*, 1996; Sherwin and Farrant, 1998) by dismantling photosynthetic machinery (poikilochlorophylly), accumulating 'sun screen' pigments while retaining photosynthetically active pigments (homoiochlorophylly), and up-regulating antioxidants such as glutathione, catalase and ascorbate (reviewed by Farrant, 2000).

In order to minimise potential photo-oxidative damage, light harvesting needs to be balanced between absorption and utilisation. This balance is achieved not only through optimisation of light harvesting, but also through non-photochemical quenching (NPQ), such as chlorophyll fluorescence and thermal dissipation (Müller *et al.*, 2001). Maximum possible fluorescence used for photosynthesis is described by the F_V/F_M ratio (where F_V is the variable fluorescence defined as $F_M - F_O$, where F_M is

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the maximum fluorescence and F_O is the zero fluorescence). This is usually at approximately 80% efficiency of photosystem II (PS II) (Krause and Weis, 1991). As fluorescence is an indicator of the plants' health state, the F_V/F_M ratio will be inversely proportional to stress.

However, when water is sparse excess energy cannot be dissipated at a rate high enough to prevent damage. As a result, reactive oxygen species (ROS) are produced, and they, in turn, can cause irreversible damage to membranes, proteins and DNA. Vascular DT plants tend to minimise ROS formation through photosynthesis by avoiding light-chlorophyll interactions in the first instance and then by neutralising those that do form by antioxidants. There are two main means by which light-chlorophyll interactions are minimised (with variations in between). Homoiochlorophyllous DT plants (HDT) fold leaves to shade either whole rosettes of inner leaves e.g. in *C. wilmsii* (Sherwin and Farrant, 1998) or one leaf surface, usually abaxial, such as in *M. flabellifolius* (Farrant, 2000; Moore *et al.*, 2005). Chlorophyll is masked in those surfaces remaining exposed to light by anthocyanin accumulation. Poikilochlorophyllous DT plants (PDT) degrade chlorophyll and thylakoid membranes are dismantled during drying (Tuba *et al.*, 1996; Farrant, 2000). They also accumulate anthocyanins which mask chlorophyll during breakdown and resynthesis of the photosynthetic machinery. All vascular DT species studied to date show an upregulation of antioxidant systems during dehydration and early rehydration (Smirnoff, 1993; Sgherri *et al.*, 1994; Kranner and Grill, 1997; Navari-Izzo *et al.*, 1997; Sherwin and Farrant, 1998; Farrant, 2000). However, it has been shown that the extent of upregulation of the standard or "housekeeping" antioxidants (e.g. ascorbate, glutathione, SOD, catalase, GR, AP, tocopherols) is no more or different than that

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occurring in desiccation sensitive plants (Illing *et al.*, 2005) and thus this strategy will not be examined in this study. Rather, we will concentrate on the mechanisms peculiar to DT plants in the avoidance of ROS formation (leaf folding, chlorophyll and thylakoid dismantling, anthocyanin accumulation) in the first instance.

The physiological response of PDT and HDT vascular plants to dehydration and rehydration has been extensively reported for *Xerophyta humilis*, *X. viscosa*, *X. scabrida*, *Borya nitida* (all PDT), as well as *Craterostigma wilmsii* and *Myrothamnus flabellifolius* (both HDT) (Gaff and Churchill, 1976; Hetherington and Smillie, 1982; Hetherington *et al.*, 1982; Gaff and Loveys, 1984; Tuba *et al.*, 1993, 1994, 1996; Sherwin and Farrant, 1996; Farrant, 2000; Cooper, 2001; Cooper and Farrant, 2002). However, until 2001 no study had been conducted on the effect of long-term desiccation on the ability of these plants to recover physiologically. Only Farrant and Kruger (2001) have reported on an aspect in their study of the effect of longevity on physiological responses in *M. flabellifolius* plants in the dry state and upon subsequent rehydration. The mechanisms of tolerance in *M. flabellifolius* plants are intermediate between *X. humilis* and *C. wilmsii* plants, and although *M. flabellifolius* is typically homoiochlorophyllous, as it does not dismantle thylakoids, it may lose more than half of its chlorophyll while drying (Farrant *et al.*, 1999) and accumulate a powerful antioxidant in abaxial leaves that remain exposed to light while dry (Moore *et al.*, 2005). This species suffered damage to the photosynthetic apparatus only after three months in the dry state, but was able to repair damage and recover, with delay, after dry storage for up to nine months. Longer periods in the dry state lead to loss of photosynthetic capacity and viability (Farrant and Kruger, 2001).

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In this chapter, pigment level changes during dehydration, while dry and during subsequent rehydration, and photochemistry inhibition and re-initiation during dehydration and subsequent rehydration, respectively, were explored in *C. wilmsii* and *X. humilis* plants. As outlined above, *C. wilmsii* is a HDT species and maintains chloroplasts and most of the chlorophyll, but folds the outer leaves of the rosette to shade the inner ones (Farrant, 2000). *X. humilis* is a PDT species that breaks down chlorophyll and thylakoid membranes during dehydration. The ability to unfold leaves and resume full photosynthetic and metabolic activity after prolonged dehydration (*C. wilmsii*) and to resynthesise the photosynthetic apparatus (*X. humilis*) was followed biochemically, physiologically and microscopically.

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3.2 Materials and Methods

Plants were pre-equilibrated, dried and stored under the conditions outlined in Chapter 2. As before, for each dehydration experiment leaf samples were taken from both species every day, at the same time of day, until the plants reached the air-dry state. Subsequent rehydration sampling was done every six hours until plants reached 100% RWC. Sampling was done from three different plants.

3.2.1 Quantum Efficiency of Photosystem II

Chlorophyll fluorometry was used to determine the quantum efficiency of photosystem II (PS II) (Krause and Weis, 1991). Measurements were taken at various stages during dehydration and subsequent rehydration. For each stage leaves were dark-adapted for 10 minutes using dark-adaptation cuvettes. The F_V/F_M ratio was measured using a portable fluorimeter (OS-500: Opti-Science, USA), with a saturating light intensity of 7500 $\mu mol.m^{-2}.s^{-1}$, to obtain the maximal fluorescence (F_M). F_V is the variable fluorescence and is defined as:

$$F_V = F_M - F_O \text{ (ground state fluorescence)},$$

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3.2.2 Pigment Content

3.2.2.1 Chlorophyll (a+b) and Carotenoids (x+c)

Leaf samples were weighed and cut into 2 mm^2 pieces. The pieces of each sample were placed into individual 1.5 ml eppendorf tubes and 1 ml acetone was added to each of the tubes. The samples were kept in a fridge at 4°C in acetone until all pigment leached out of the tissue. Readings were taken using a spectrophotometer (Beckman DU 650 UV/Vis, Beckman, USA) at 661.6nm, 644.8nm and 470nm. The

following equations (Lichtenthaler, 1987) were used to calculate chlorophyll (a+b) and carotenoids (x+c) content:

$$\text{Chlorophyll a} = (11.24*\lambda_{661.6}) - (2.04*\lambda_{644.8})$$

$$\text{Chlorophyll b} = (20.13*\lambda_{644.8}) - (4.19*\lambda_{661.6})$$

$$\text{Chlorophyll a+b} = (7.05*\lambda_{661.6}) + (18.09*\lambda_{644.8})$$

$$\text{Carotenoids} = ((1000*\lambda_{470}) - (1.9*\text{chl a}) - (63.14*\text{chl b})) / 214$$

3.2.2.2 Anthocyanins

Leaf samples were weighed and cut into 2 mm^2 pieces. The pieces were placed into 1.5 ml eppendorf tubes and 1 ml acidified methanol (79 MeOH : 20 H_2O : 1 HCl) was added to each tube. The tubes were kept in the fridge at 4°C until all pigment leached out of the tissue. Readings were taken using a spectrophotometer (Beckman DU 650 UV/Vis, Beckman, USA) at 657nm and 530nm. The following equation (Lichtenthaler, 1987) was used to calculate the anthocyanin content in tissues:

$$\text{Anthocyanin} = \lambda_{530} - 1/3\lambda_{657}$$

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3.2.3 Ultrastructural Studies – Transmission Electron Microscopy

Method as described in Chapter 2.

3.3 Results and Discussion

3.3.1 Response of *C. wilmsii* plants to dehydration and prolonged drought

Figure 3.1, below, shows the effect of dehydration and prolonged drought on pigments of *C. wilmsii* plants. Pigment levels of hydrated plants are also included for comparison.

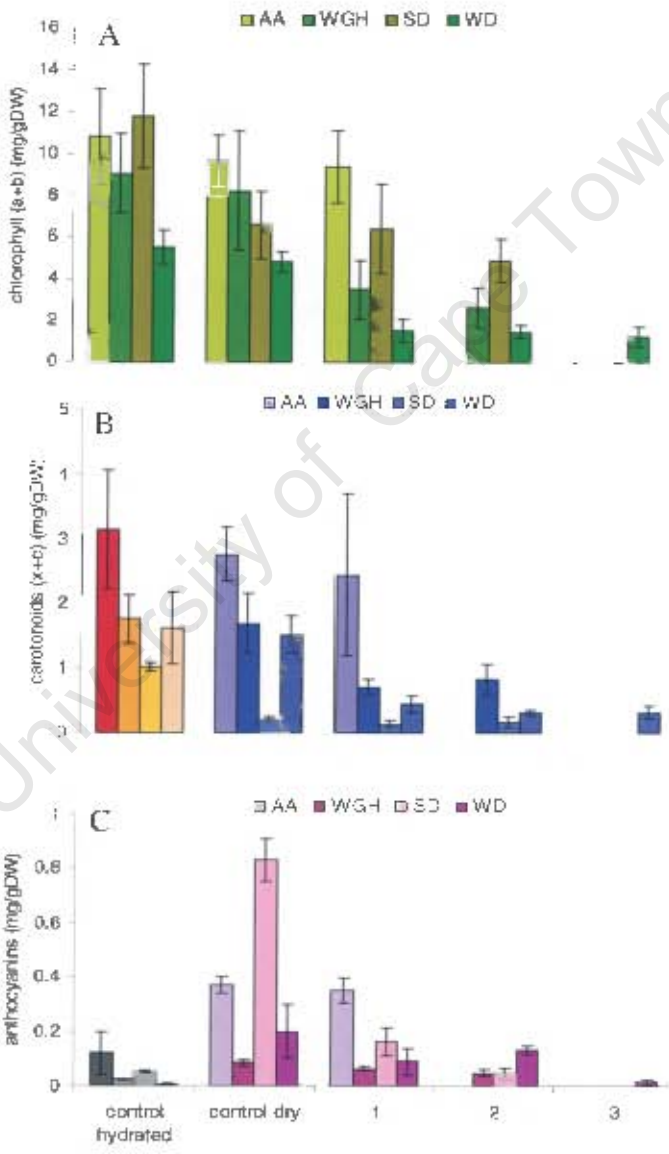

Figure 3.1: Response of chlorophyll (a+b) [A], carotenoids (x+c) [B] and anthocyanins [C] to dehydration and prolonged desiccation (one, two and three months) in leaves of *C. wilmsii* plants. AA (accelerated aging); WGH (winter green house); SD (summer dehydration); WD (winter dehydration). 1, 2 and 3 refer to the drought period (months).

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There were differences in the amount of chlorophyll (a+b) present in hydrated leaves from plants under different treatments (probably due to the different light intensities to which they had been acclimated prior to drying). Also, there were no significant differences between the chlorophyll (a+b) content in *C. wilmsii* plants prior to dehydration (control hydrated) and once the plants reached the air-dry state initially (control dry) in all plants maintained under AA, WGH nor WD (all high to moderate light conditions), but a 40% decrease was observed in *C. wilmsii* plants kept at SD (low light) conditions (Figure 3.1 A). However, following prolonged drought of one, two and three months, *C. wilmsii* plants that were subjected to WGH (moderate variable light intensity) and WD (high light intensity) conditions, suffered a further chlorophyll (a+b) loss of 10% and 40%, respectively (Figure 3.1 A). Similar trends were observed in carotenoid (x+c) levels (Figure 3.1 B). It is possible that this was a result of photo-oxidative damage to thylakoid membranes that in turn caused chlorophyll and carotenoid dissociation from the membranes, subsequently causing their breakdown by ROS.

C. wilmsii plants prevent the detrimental interaction between light and photosynthetic pigment by accumulating anthocyanins in the abaxial surfaces of outer, older leaves that remain exposed to light, and by curling these leaves over inner, green leaves during dehydration (reviewed by Farrant, 2000). Research done on HDT plants has shown that even though the photosynthetic apparatus is readily recoverable upon rehydration (Farrant, 2000; Farrant *et al.*, 1999; 2003), some loss in chlorophyll (a+b) content does occur and this is not only species specific, but also highly dependent on the simulated environmental conditions (Proctor and Tuba, 2002). This is particularly apparent in the work done on *C. wilmsii* plants. Cooper and Farrant (2002) found that

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this species lost approximately 60% chlorophyll (a+b) and 20% carotenoid (x+c) during dehydration. Sherwin and Farrant (1998) reported that *C. wilmsii* plants did not lose carotenoids (x+c), but lost 30% chlorophyll (a+b) during drying, while Farrant *et al.* (2003) also found that no carotenoids (x+c) were lost, but the chlorophyll (a+b) content decreased by 40% during dehydration. Another report stated that *C. wilmsii* lost 50% chlorophyll (a+b), but that there was no significant change in the carotenoid (x+c) levels during drying (Sherwin and Farrant, 1996), and in 1999 Farrant *et al.* reported that the chlorophyll (a+b) and carotenoid (x+c) concentration of *C. wilmsii* leaves declined by approximately 30 and 20%, respectively, during dehydration regardless of drying speed. In the present study the variation in light intensities among treatments will account for the variation in the amount of chlorophyll (a+b) and carotenoids (x+c) lost during dehydration. This identifies light, quantity and quality, as important factors that may determine the loss of photosynthetically active pigments during dehydration.

In hydrated, healthy, non-stressed leaves, high chlorophyll (a+b) and low anthocyanin levels in *C. wilmsii* plants would insure optimal chlorophyll fluorescence. However, chlorophyll masking by anthocyanin accumulation causes a decrease in quantum efficiency of photosystem II, indicating stressful conditions. Anthocyanins accumulate during dehydration in order to mask photosynthetically active pigments, thus interrupting the light-chlorophyll interaction and minimising the amount of energy available for photosynthesis. This in turn may limit water utilisation through photosynthetic pathways (Sherwin and Farrant, 1998; Farrant, 2000; Farrant *et al.*, 2003). At the air-dry state levels of anthocyanin of outer leaves of *C. wilmsii* plants from all treatments had increased by at least 2-fold compared to fully hydrated plants

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prior to dehydration (Figure 3.1 C). Most anthocyanin accumulated in leaves of plants maintained under SD conditions (low light) where levels increased by 16-fold during dehydration. However, anthocyanin levels decreased with time under all but AA conditions (Figure 3.1 C) while the plants were dry. In the field *C. wilmsii* is not exposed to continuous low light conditions and may find this situation to be stressful, thus accumulating large quantities of anthocyanins.

In *C. wilmsii* plants F_V/F_M decreased from 85% RWC and reached zero by 10% RWC in all treatments, although the decline was slower in SD plants (Figure 3.2 A). Anthocyanin levels initiated an increase from 85% RWC (Figure 3.2 B) in plants maintained under all conditions, except SD (low light) and thus it appears that anthocyanin accumulation is inversely proportional to chlorophyll fluorescence (F_V/F_M).

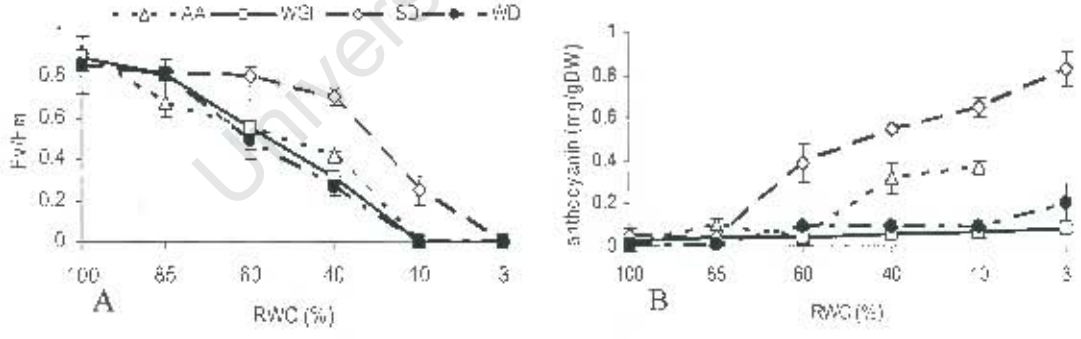

Figure 3.2: Changes in quantum efficiency of PS II (A) and anthocyanin levels (B) during dehydration of *C. wilmsii* plants. AA (Δ); WGH (□); SD (◊); WD (●).

In control, un-dried *C. wilmsii* leaves, chloroplasts were typical of hydrated plants in that the thylakoid membranes were regularly stacked and there was evidence of starch

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(Figure 3.3 A). Immediately after drying, and after one month of drought, the chloroplasts in the plants maintained under SD, WGH and WD conditions were more rounded although thylakoid stacking was still evident (Figure 3.3 B). This subcellular organisiation is typical of that previously reported for this species (Sherwin and Farrant, 1998; Farrant *et al.*, 2003). In AA plants after one month (and SD after two months), considerable damage to chloroplasts was evident (Figure 3.3 C).

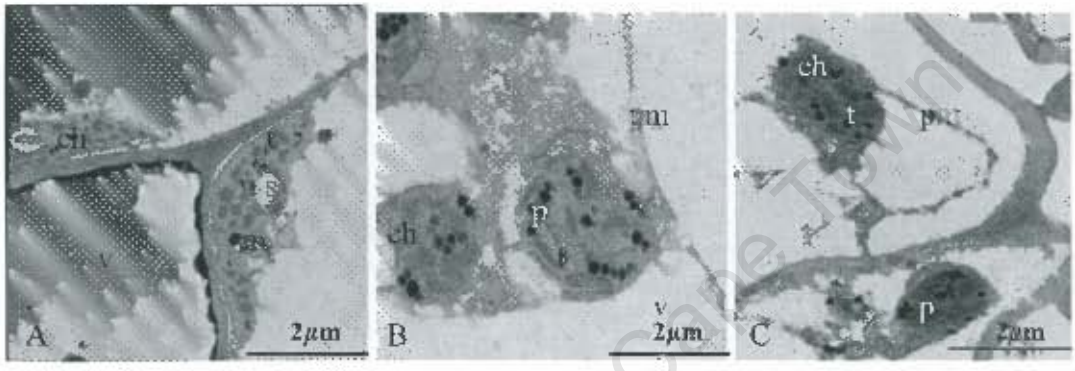

Figure 3.3: Ultrastructure detail of chloroplasts of control hydrated (A), control dry (B) and dry damaged (C) in *C. wilmsii* leaves. Ch – chloroplast; p – plastoglobuli; t – thylakoids; s – starch; pm – plasma membrane; v – vacuole; m – mitochondrion. All micrographs B and C are at magnification of x5000, and A is at x5000.

The longer the *C. wilmsii* plants remained dry, the lower the chlorophyll (a+b) levels measured in WGH and WD dry plants, but remained unchanged in AA and SD dry plants (Figure 3.1). TEM investigation has shown that the number of chloroplasts and thylakoid stacks decreased the longer the plants remained dry. This could attribute to the lower chlorophyll (a+b) content in the plants maintained under those conditions. The greater number of stacks per chloroplast in AA and SD plants might have contributed to the considerably faster loss of viability, than in WGH and WD plants (Chapter 2), as these plants would have been more vulnerable to ROS damage.

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3.3.2 Response of *X. humilis* plants to dehydration and prolonged drought

Among homoiochlorophyllous plants F_V/F_M is influenced largely by anthocyanin accumulation and leaf curling, but the main factor that disrupts the light-chlorophyll interaction among poikilochlorophyllous plants is the thylakoid dis-assembly and chlorophyll breakdown (reviewed by Farrant, 2000). In the current study, despite different initial chlorophyll (a+b) levels due to different light conditions plants were acclimated to, there was continual chlorophyll degradation in all treatments, approaching 0% chlorophyll (a+b) at 10% RWC (Figure 3.4). It was observed that the F_V/F_M remained close to the optimum in *X. humilis* plants until RWC decreased to 60% (Figure 3.4 A). At this stage the mean chlorophyll (a+b) content of leaves from all treatments was 4 $mg.g^{-1}$ DW (Figure 3.4 B). This may be a significant level of chlorophyll for optimal photosynthetic operation – as will be discussed in future chapters.

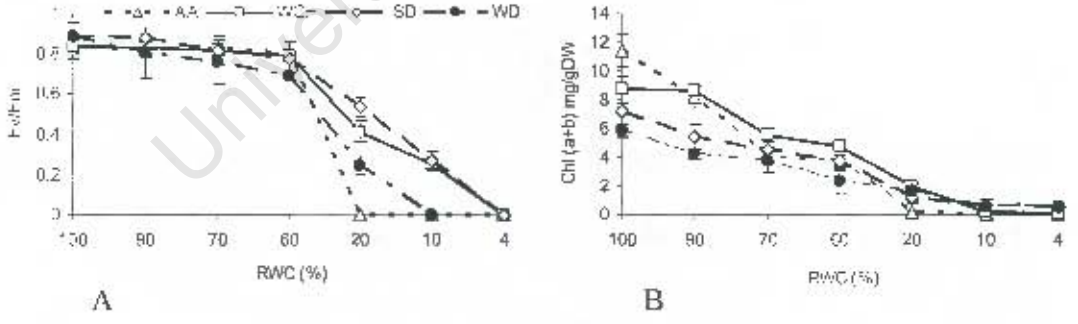

Figure 3.4: Changes in photochemistry (A) and chlorophyll (a+b) content (B) in leaves of *X. humilis* plants during dehydration. AA (Δ); WGH (□); SD (◊); WD (●).

Prior to dehydration carotenoids (x+c) levels in *X. humilis* plants were approximately 2 $mg.g^{-1}$ DW under all conditions except SD, where they were above 5 $mg.g^{-1}$ DW (to be discussed later on Figure 3.13). Once the plants reached the air-dry state,

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carotenoid (x+c) levels decrease by approximately 98% in all plants, except those maintained under WGH conditions, where carotenoid (x+c) levels dropped by only 85% (Figure 3.5). However, within the first month of dry storage this concentration dropped to that of others, and remained unchanged for the following nine months.

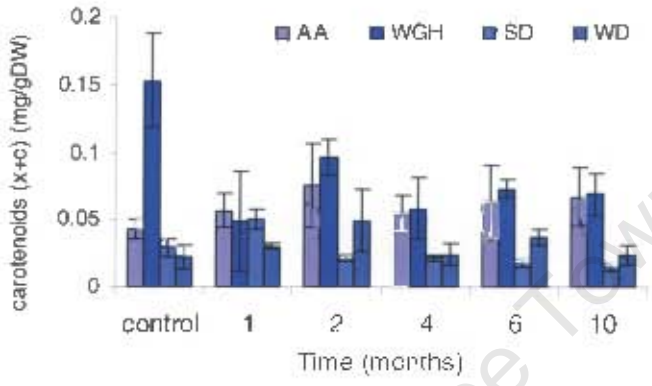

Figure 3.5: Carotenoid (x+c) levels in leaves of *X. humilis* plants in the dry state and each subsequent time point following a prolonged drought. Control refers to pigment levels initially at the air-dry state. AA (accelerated aging); WGH (winter green house); SD (summer dehydration); WD (winter dehydration).

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In a study by Farrant *et al.* (1999) it was reported that slowly (naturally) dried *X. humilis* plants lost nearly all chlorophyll (a+b), but that carotenoid (x+c) levels were reduced by only 70%. Work done by Tuba *et al.* (1993) on *X. scabrida*, a relative of *X. humilis*, showed that excised dry leaves contained no chlorophyll (a+b), but carotenoids (x+c) were present in large amounts. In that study, although carotenoids (x+c) levels were 72% lower than that of hydrated leaves, the amount of carotenoids (x+c) that remained was sufficient to protect the leaves from oxidative damage during subsequent rehydration (Tuba *et al.*, 1993). A study by Sherwin and Farrant (1996) reported that desiccated *X. viscosa*, another relative of *X. humilis*, lost almost all chlorophyll (a+b), but that carotenoid (x+c) levels were reduced by only 60%.

Relatively high carotenoids (x+c) levels in dry vascular DT plants might be important as carotenoids are important non-enzymatic antioxidants necessary for protection against oxidative damage during dehydration and subsequent rehydration. These antioxidants may be active in the chloroplasts where they react with singlet-oxygen preventing lipid peroxy radical formation (Burton and Ingold, 1984; Winston, 1990; Fryer, 1992). Thylakoid membranes have a high polyunsaturated fatty acid content, and are thus at high risk of singlet-oxygen peroxidation (Müller *et al.*, 2001). Carotenoid zeaxanthin, a potent antioxidant found in thylakoid membranes, blocks the singlet oxygen chain reaction, minimising lipid peroxidation. Work done by Sujak *et al.* (1998) showed that zeaxanthin and lutein decrease the rate of lipid peroxidation in artificial membranes. However, it is also possible that zeaxanthins become integrated into the membrane, thus reducing its fluidity, making the membrane less penetrable to ROS as suggested by Tardy and Havaux (1997). Carotenoids are also prone to oxidative damage, but only when they are associated with the thylakoid membranes (Liebler *et al.*, 1986; Eskling *et al.*, 1997). It is possible that the reason the initiation of thylakoid breakdown occurs at RWC below 60% during dehydration of *X. humilis* leaves, as this study shows, and so dis-associating carotenoids from the thylakoid membranes, suggesting that this species might be most susceptible to ROS damage at RWC higher than 60% during dehydration. Also, at this RWC partial chlorophyll degradation has occurred (Figure 3.4 B), thus ROS damage resulting from light-chlorophyll interaction is minimised. The carotenoids that dis-associate from the thylakoid membranes at this RWC, might remain inert, but undamaged in the chloroplast stroma, available for initial protection against photo-oxidative damage upon subsequent rehydration. Figure 3.5 suggests that the prolonged drought has had no influence on the level of carotenoids in dry leaves of *X. humilis*.

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At the air-dry state almost no chlorophyll (a+b) is available (Figure 3.6) for light-chlorophyll interactions, thus ROS production as a result of photo-oxidation, is minimised. In addition, some anthocyanin accumulation occurs during dehydration in *X. humilis* plants (Figure 3.7), generally at RWC prior to F_V/F_M deterioration (as shown in Figure 3.4 A), further preventing a possibility of photo-oxidative damage. As shown in Figure 3.6 some anthocyanin is degraded with time while the plants are dry. This was seen in plants maintained under all but AA conditions. This might be the reason why during subsequent rehydrations (one, four and 10 months) F_V/F_M increased at similar RWC at this set of conditions, while under other simulated environmental conditions, namely WGH, SD and WD, photochemistry was re-initiated at higher RWC the longer the plants remain in the dry state (as will be shown later in Figure 3.13). Although there was a decrease in chlorophyll (a+b) levels in leaves of plants maintained under SD conditions during the first month of desiccation, thereafter the pigment levels remained unchanged. Perhaps such low light is stressful to this species, and the stress is minimised through further loss of chlorophyll (a+b).

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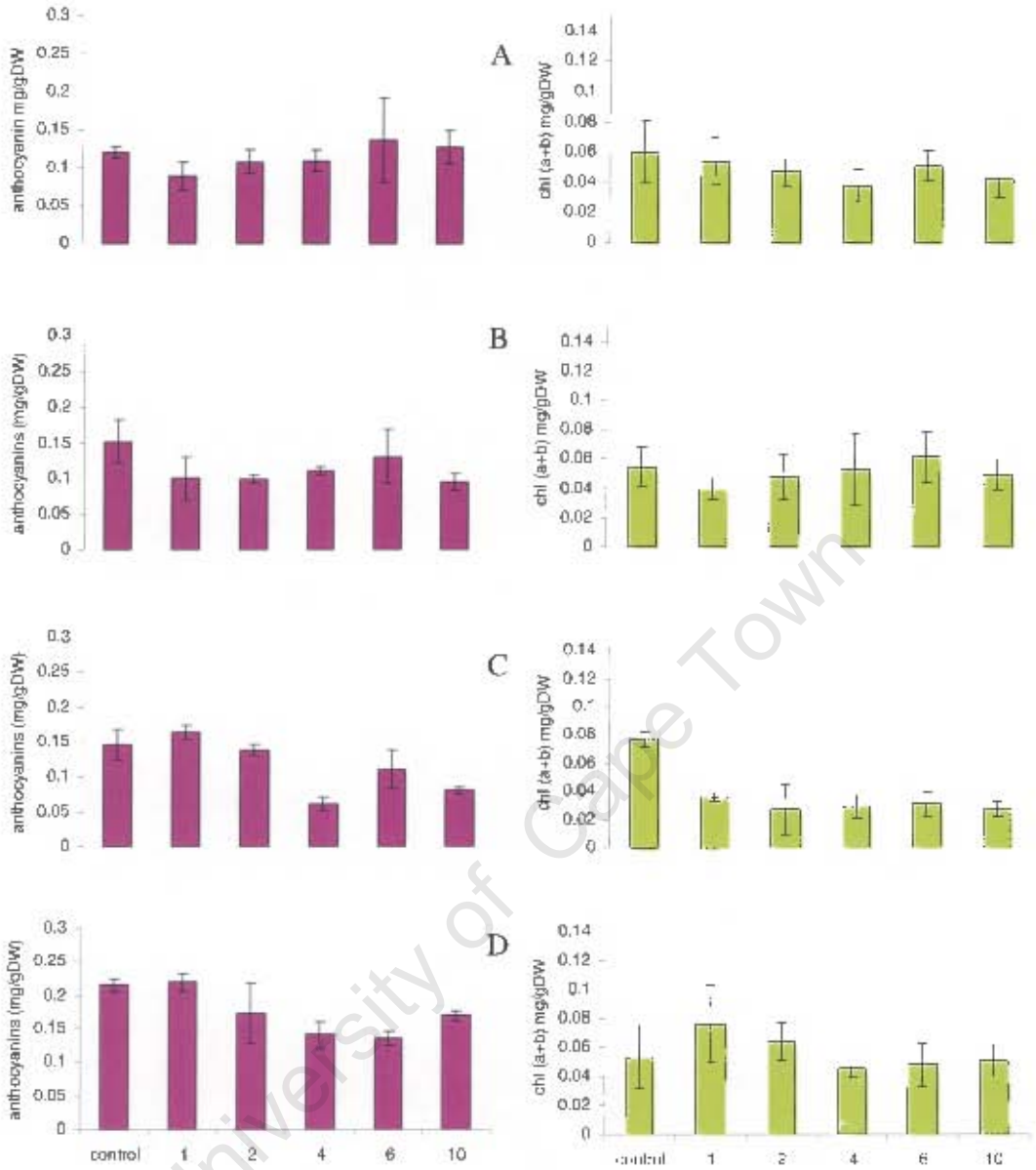

Figure 3.6: Anthocyanin and chlorophyll (a+b) levels in leaves of dry *X. humilis* plants. Control refers to initial pigment levels prior to rehydration. A: AA (accelerated aging); B: WGH (winter green house); C: SD (summer dehydration); D: WD (winter dehydration).

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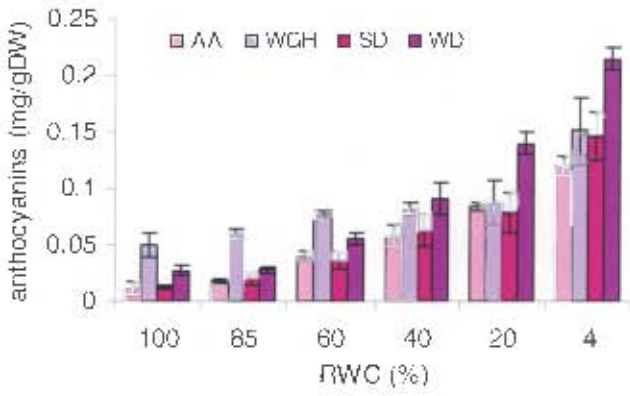

Figure 3.7: Anthocyanin accumulation in leaves of *X. humilis* plants during dehydration.

The ultrastructure of *X. humilis* plants was independent of the environmental conditions and differences were only observed in the length of time the plants had remained dry. Comparisons for *X. humilis* plants were made between the one and 10 month rehydrated plants from the AA treatment only.

In control, un-dried leaves, the chloroplasts were typical of hydrated plants in that the thylakoids were regularly stacked and there was evidence of starch (Figure 3.8 A). Once desiccated, the leaves of *X. humilis* plants lost most chlorophyll (a+b) (as shown in Figure 3.4 B). This was visually supported through the evidence of the vesiculation of thylakoid membranes (Figure 3.8 C). Starch was hydrolysed and fewer scattered plastoglobuli appeared in chloroplasts. The shape of chloroplasts changed from elongated to round during dehydration (Figure 3.8 A and C).

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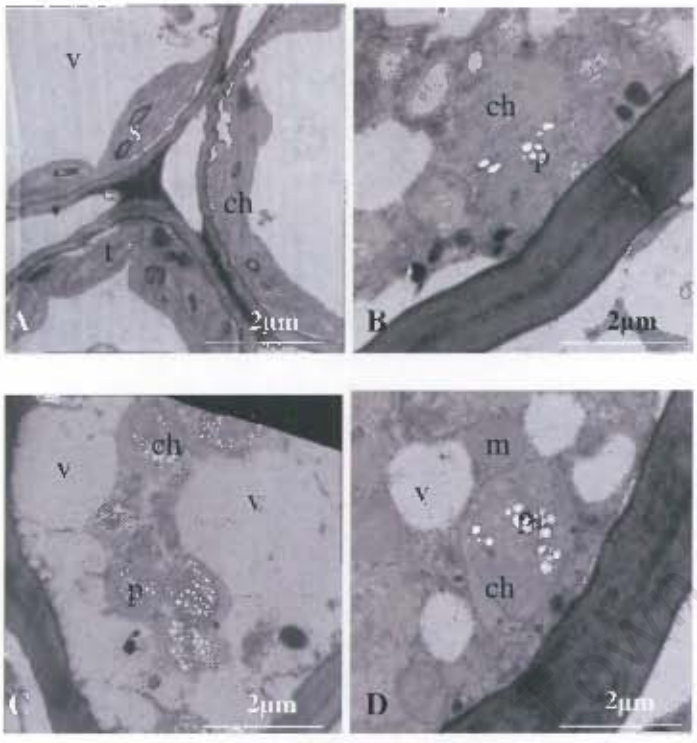

Figure 3.8: Ultrastructure detail of chloroplasts of control hydrated (A), damaged dry (B) one month dry undamaged (C) and 10 month dry undamaged in *X. humilis* plants. Ch – chloroplast; v – vacuole; s – starch; t – thylakoids; p – plastoglobuli; m – mitochondrion. Micrographs A and C are at magnification of x4000, and B and D at x5000.

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The accumulation of anthocyanins (Figure 3.7) as well as the loss of chlorophyll (a+b) (Figure 3.4 B) seemed to successfully minimise the potential for damage by ROS during the 10 month drought as the cellular ultrastructure of one month and 10 month dry samples was similar (Figure 3.8 C and D). In those plants where damage had accumulated over the prolonged drought chloroplasts lacked boundary membranes, similar to that of *C. wilmsii* plants, as seen in Figure 3.8 B.

3.3.3 Response of *C. wilmsii* plants to rehydration following a prolonged drought

The RWC at which photochemistry was re-initiated after subsequent rehydration was dependent on the simulated environmental conditions, chlorophyll (a+b) content, as well as time spent in the dry state. Photochemistry was fully functional by 80% RWC in *C. wilmsii* plants that remained dry for one month, under all, but WD conditions (high light, low temperature) (Figure 3.9 A). Here, F_V/F_M reached optimum only at full turgour. However, the longer the plants remained dry, the higher the RWC at which photochemistry reached full recovery (Figure 3.9). This may be a result of cumulative damage to the photosynthetic machinery that needed to be repaired.

As mentioned earlier, the longer the *C. wilmsii* plants remained dry the lower the number of chloroplasts and thylakoid stack per chloroplast were observed in WGH and WD plants. Also, as shown in Figure 3.9, recovery of Fv/Fm of WD *C. wilmsii* plants was much slower than that of AA plants.

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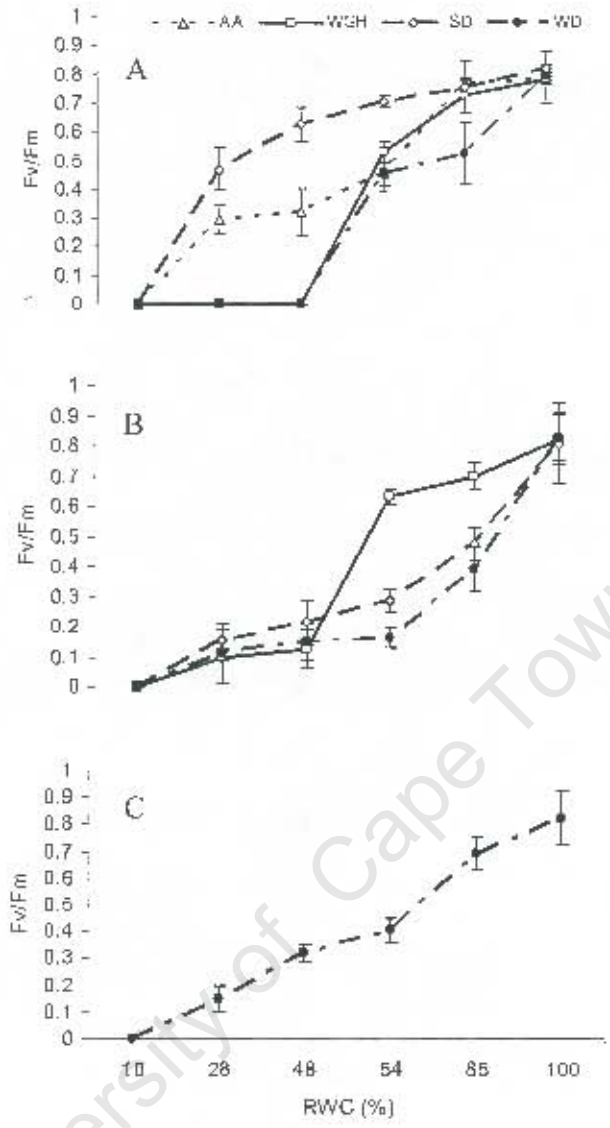

Figure 3.9: Re-initiation of photochemistry in *C. wilmsii* plants after a prolonged drought. (A) One month, (B) two months and (C) three months of dry storage. AA (Δ); WGH (□); SD (◊); WD (●).

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Cooper and Farrant (2002) observed that in *C. wilmsii* plants F_V/F_M dropped at RWC below 20%, and was re-initiated at 40% RWC upon rehydration and was fully functional at 80% RWC. Sherwin and Farrant (1996) reported that *C. wilmsii* recovered photochemical functioning before the chlorophyll (a+b) content had fully recovered, similar to the results of this study. These authors, as well as Farrant *et al.* (1999), also reported that the F_V/F_M recovered before the plants reached 100% RWC.

Although the latter are similar to the findings of this study, chlorophyll (a+b) recovery and anthocyanin loss during rehydration was highly variable and dependent on the climatic conditions *C. wilmsii* plants were subjected to and the time spent in the dry state (Figure 3.10), as discussed earlier.

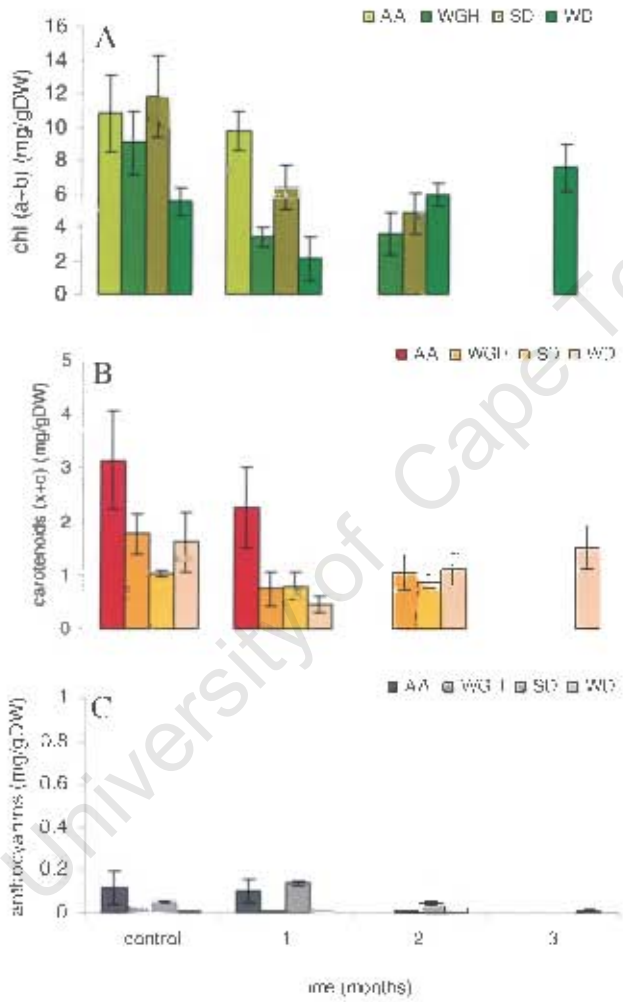

Figure 3.10: Response of chlorophyll (a+b) [A], carotenoids (x+c) [B] and anthocyanins [C] to rehydration following a prolonged drought (one, two and three months) in leaves of *C. wilmsii* plants. Control refers to the pigment levels prior to dehydration. AA (accelerated aging); WGH (winter green house); SD (summer dehydration); WD (winter dehydration).

Once the plants reached full turgour, following the one month drought, only the plants that were maintained dry under AA conditions measured chlorophyll (a+b) levels to

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that of the control. After two months of dry storage only WD plants recovered chlorophyll (a+b) to the levels of pre-dried plants (Figure 3.10). Anthocyanin levels returned to that of the control after rehydration in plants maintained dry under all conditions, except for the SD plants following a one month drought (Figure 3.10). Earlier it was mentioned that these plants, in the dry state, had a much higher anthocyanin level than the plants kept under other conditions. The higher anthocyanin levels at full turgour suggest that the low light conditions (SD) are stressful to *C. wilmsii* upon rehydration.

The chloroplast ultrastructure of *C. wilmsii* plants differed among treatments and was dependent on the time spent in the dry state. For the ultrastructural comparisons for *C. wilmsii* plants, comparisons were made between the one month rehydrated and dead plants from the AA treatment and the two month rehydrated and dead plants from the WD treatment.

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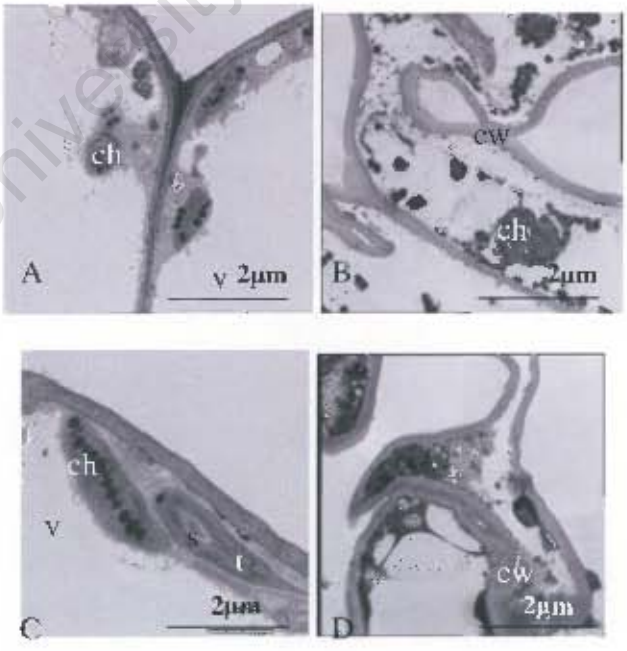

Figure 3.11: Ultrastructure detail of chloroplasts of one month rehydrated (A) and dead (B) AA, and two month rehydrated (C) and dead (D) WD *C. wilmsii* plants. Ch – chloroplast; t – thylakoids; v – vacuole; cw – cell wall; s – starch. All micrographs are at magnification of x4000.

Upon rehydration at full turgour, subcellular organisation of AA treated plants appeared irregular. Cytoplasm and organelles were situated at the periphery of the cell, and chloroplasts regained the elongated shape, however, numerous mitochondria and granular vacuoles, indicate some damage (compare to Figure 3.3 C for dry AA tissue). Two month WD rehydrated plants show a typical subcellular organisation of hydrated plants. Although few plastoglobuli were still present in some chloroplasts, others showed starch and regularly stacked thylakoid membranes. Starch was observed only in two month rehydrated *C. wilmsii* plants, suggesting that some metabolic enzymes were damaged during dry storage under AA conditions. The ultrastructure of dead leaf tissue showed severe plasmolysis, no subcellular organisation, great membrane damage and loss of the chloroplast boundary membranes.

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3.3.4 Response of *X. humilis* plants to rehydration following a prolonged drought

Upon rehydration, anthocyanin levels remain high initially (Figure 3.11), inhibiting light-chlorophyll interaction, until chlorophyll (a+b) levels were high enough to re-initiate photosynthesis at a rate at which light absorption and utilisation could be balanced. Once the plants reached full turgour, anthocyanin levels generally returned to that on pre-dried plants under all but WGH conditions, where anthocyanin levels decreased to below the control levels (Figure 3.12).

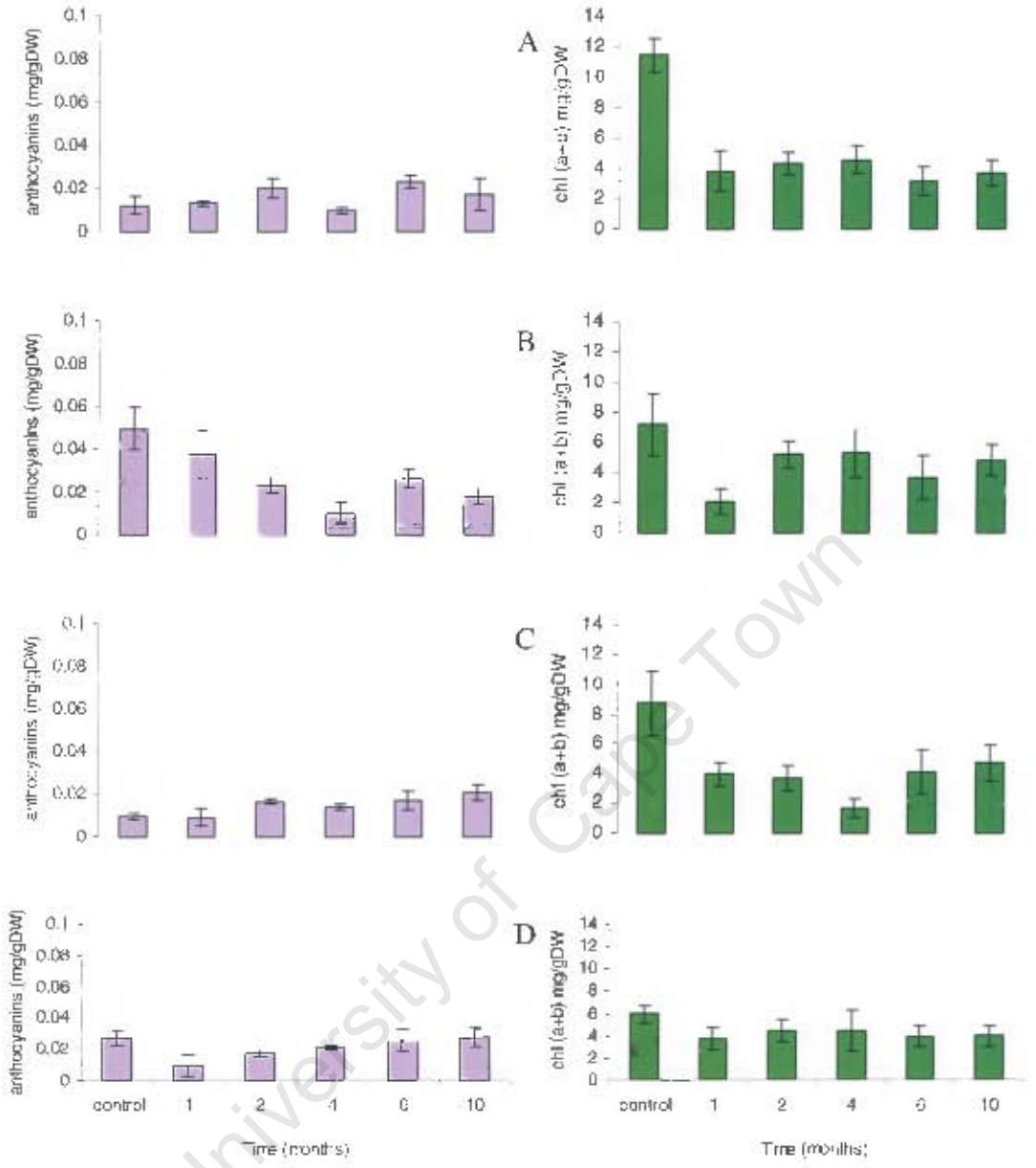

Figure 3.12: Chlorophyll (a+b) and anthocyanins levels in leaves of *X. humilis* plants. Control refers to initial pigment levels prior to dehydration. A: AA (accelerated aging); B: WGH (winter green house); C: SD (summer dehydration); D: WD (winter dehydration).

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At full turgour, following prolonged drought of one, two, four, six and 10 months, the mean chlorophyll content of leaves from all treatments was 4 $mg.g^{-1}$ DW (Figure 3.12), similar to that described earlier where during dehydration F_V/F_M remained optimal until chlorophyll content decreased below 4 $mg.g^{-1}$ DW. Perhaps *X. humilis* plants require this amount of chlorophyll for optimal photochemistry. This is not true

for *C. wilmsii* plants, where optimal photochemistry is reached at chlorophyll levels between 2 and 10 $mg.g^{-1}$ DW (Figure 3.9 and Figure 3.10).

Upon subsequent rehydrations, carotenoid (x+c) levels at full turgour varied among treatments (Figure 3.13), but the plants maintained under AA conditions had a higher carotenoid (x+c) level at full turgour the longer they remained in the dry state. Plants maintained under SD conditions had the highest carotenoid (x+c) level, and together with the plants kept under WGH and WD conditions generally were able to recover carotenoids to control levels.

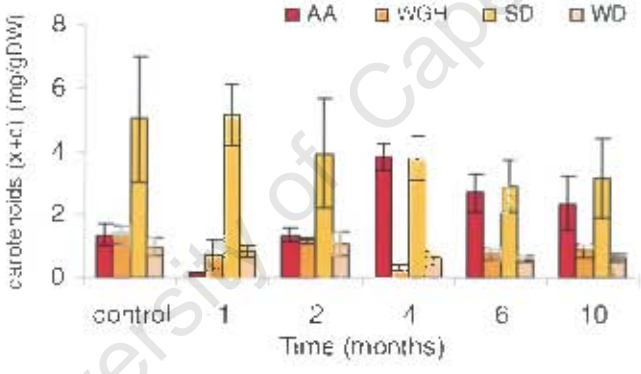

Figure 3.13: Carotenoid (x+c) levels in leaves of *X. humilis* plants at full turgour following months of drought. Control refers to initial pigment levels prior to dehydration. AA (accelerated aging); WGH (winter green house); SD (summer dehydration); WD (winter dehydration).

The photochemistry of *X. humilis* plants was able to recover to optimal levels independent of the treatment and time spent in the dry state (Figure 3.14). However, plants maintained under AA conditions initiated photochemical recovery at RWC above 50%, while under all other conditions this initiation occurred at RWC above 35%. Perhaps the combination high light, high temperature and high relative humidity slowed down the recovery of the photosynthetic machinery at the lower RWC.

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Irrespectively, at 100% RWC the photochemistry of all *X. humilis* plants under all conditions had fully recovered (Figure 3.14).

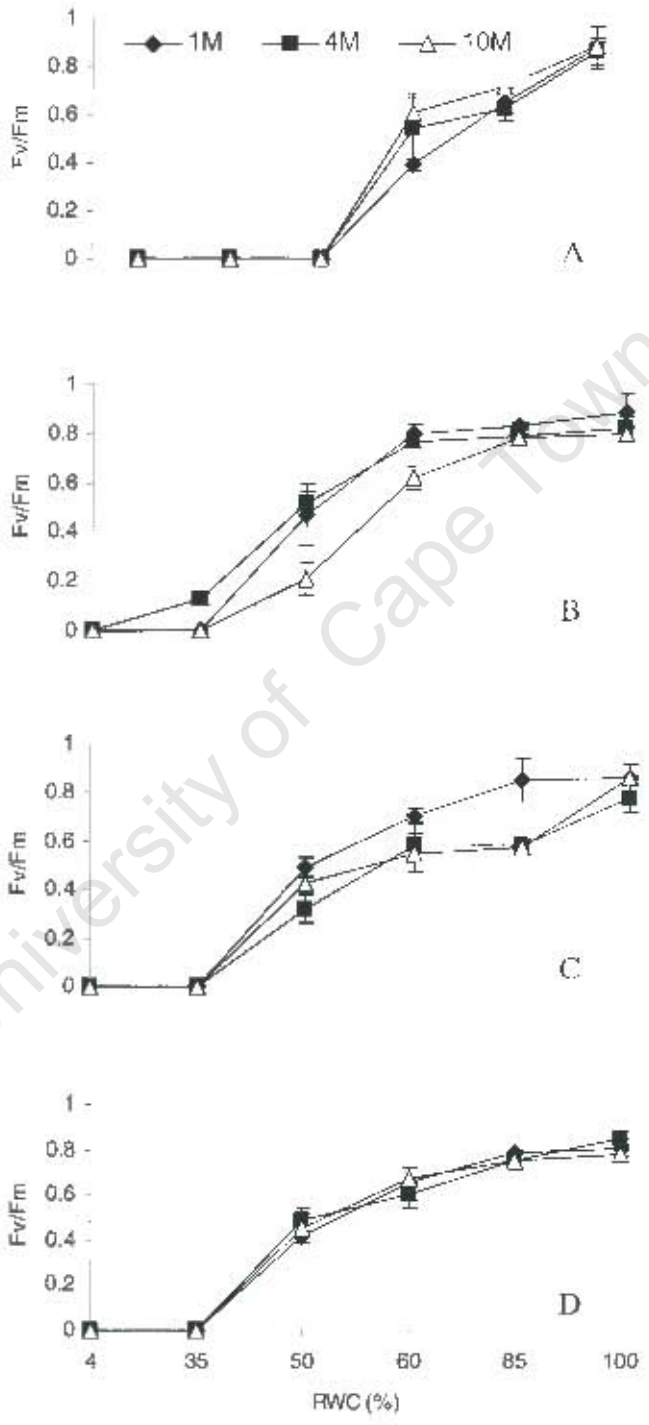

Figure 3.14: Photochemistry re-initiation in leaves of *X. humilis* plants following prolonged drought of one (●), four (■) and ten months (Δ). (A) AA; (B) WGH; (C) SD; (D) WD.

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The ultrastructure of *X. humilis* plants was independent of the environmental conditions and differences were only observed in the length of time the plants had remained dry. Comparisons for *X. humilis* plants were made between the one and 10 month rehydrated plants from the AA treatment only. Following the one month drought, upon rehydration the chloroplasts seemed to regain the characteristic shape of hydrated and non-stressed chloroplasts (Figure 3.15 B). Thylakoid membranes re-organised into granal and inter-granal areas and starch was evident. However, after a 10 month drought, although thylakoid membranes re-stacked, starch was present in small amounts (Figure 3.15 D), and some plastoglobuli were still present. The process to full recovery might have been delayed in these plants as they had been exposed to stressful conditions for longer, thus needing a longer time to regain the appearance typical of hydrated plants.

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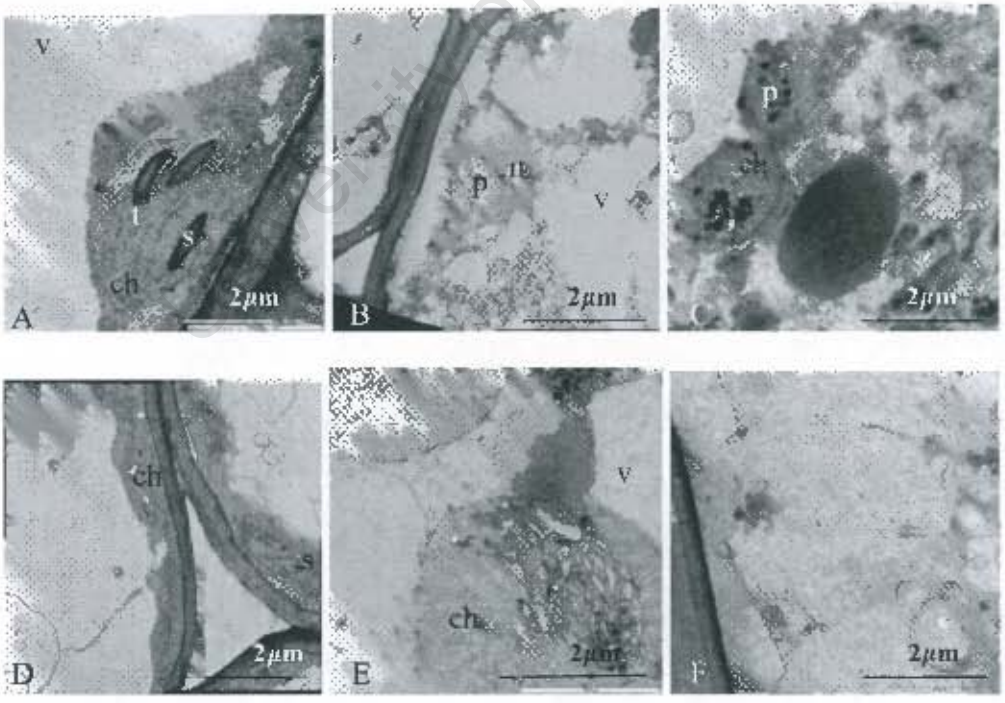

Figure 3.15: Ultrastructure detail of chloroplasts of one month rehydrated undamaged (A), rehydrated damaged (B) and dead (C), and 10 month rehydrated undamaged (D), rehydrated damaged (E) and dead (F) in *X. humilis* plants. Ch – chlorophyll; t – thylakoids; s – starch; v – vacuole; p – plastoglobuli. All micrographs, other than B and D are at magnification of x5000. B and D are at x4000.

Damaged chloroplasts of one month rehydrated *X. humilis* plants showed clustered plastoglobuli and an intact chloroplast boundary layer (Figure 3.15 B). On the contrary, damaged chloroplasts of 10 month rehydrated *X. humilis* plants showed a broken boundary membrane and irregular thylakoid membranes (Figure 3.15 E). There was a complete loss of plasmalemma and organellar integrity in dead leaves (Figure 3.15 C and F).

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3.4 Conclusions

C. wilmsii, a homoiochlorophyllous DT species, accumulates anthocyanins during dehydration to block light-chlorophyll interaction, and in turn minimise potential photo-oxidative damage. Initially anthocyanin levels remained high while the plants were dry, but decreased with time. This trend was also seen in chlorophyll (a+b) and carotenoid (x+c) levels. Over time, this species become increasingly damaged, possibly due to oxidative damage, through the loss of 'sun-block' pigments, and it is possible that this was one of the factors contributing to the relatively short maximum time *C. wilmsii* plants are capable of remaining viable in the dry state (no longer than three months).

During dehydration, poikilochlorophyllous *X. humilis* plants break down chlorophyll and disassemble thylakoid membranes (reviewed by Farrant, 2000). This mechanical method of preventing photosynthetically active light interacting with photosynthetic pigments plays an important role in the long-term viability of dry *X. humilis* plants. While the plants were in the dry state chlorophyll (a+b) and carotenoid (x+c) levels did not decrease with time (possibly due to their breakdown during dehydration, with minimal pigment level retention), but protective anthocyanin levels did. Although light-chlorophyll interaction in dry leaves was minimised through thylakoid dis-assembly, loss of anthocyanins over prolonged drought could prove lethal. However, for a period of 10 months, *X. humilis* plants seem to be unaffected by the slow decrease in anthocyanin levels.

Although this study demonstrates that poikilochlorophylly is a superior strategy for long-term desiccation, as previously suggested by Tuba *et al.* (1996), each species has

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evolved mechanisms that would protect it against prolonged drought within their natural environments.

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CHAPTER FOUR

Biochemical Response of *Craterostigma wilmsii* and *Xerophyta humilis* to Dehydration, Prolonged Desiccation and Subsequent Rehydration

4.1 Introduction

In seeds, and possibly in all DT systems, cell deterioration and loss of viability are influenced by storage conditions, namely temperature and relative humidity (RH) (Hoekstra, 1986). At low temperatures, deterioration might be mainly due to free radical damage, and at high temperatures melting of the protective intracellular glasses may occur which may result in crystalisation causing irreversible damage to macromolecules (reviewed by Vertucci and Farrant, 1995). At higher water contents, metabolically active cells play a role in aging through enzymatic reactions, while at lower water contents, non-enzymatic reactions may contribute towards cell deterioration (Priestly, 1986; Wettlaufer and Leopold, 1991). It is likely that, similar to seeds, the elements that contribute towards the deterioration of DT vegetative tissues seem to involve changes in the biophysics and biochemistry of cells during water loss and subsequent water gain (Murthy and Sun, 2000). Although research has identified several stresses associated with water loss and gain, it is difficult to distinguish among these while attributing a particular damage to a specific stress. Similar is true for protective mechanisms induced within an organism by these stresses (Walters *et al.*, 2001).

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It has been suggested that the accumulation of non-reducing sugars, such as sucrose, may be correlated with DT, as the accumulation of non-reducing sugars has been observed in all DT systems namely orthodox seeds, DT pollen, DT plants and DT animals during dehydration (reviewed by Vertucci and Farrant, 1995). In order to support this suggestion several theories have been proposed. One is the 'Water Replacement Hypothesis' which suggests that membranes and proteins are stabilised by non-reducing sugars through sugar hydroxyl group stabilisation of water, thus providing necessary hydrophilic interactions (Clegg, 1986; Crowe *et al.*, 1992). Another hypothesis is the 'Glass Formation Hypothesis' which suggests that water and sugar hydroxyl groups may interact in a non-specific manner when a solution is dried or cooled sufficiently thus changing from super-saturated liquid to super-viscous fluid (Franks, 1982). Due to this super-viscous state the glass may prevent molecular movement, thus suspending metabolism (Burke, 1986; reviewed by Vertucci and Farrant, 1995).

Considerable research has been done on sugars in DT plants and other desiccation tolerant organisms (for reviews see Vertucci and Farrant, 1995; Pammenter and Berjak, 1999; Farrant, 2000). Crowe *et al.* (1992) suggested that during dehydration, oligosaccharides bind to the phospholipid phosphate polar head groups, preventing the acyl chains from packing tightly together and thus increasing the gel-to-crystalline phase transition temperature (Hoekstra *et al.*, 1997). Phase transition can be debilitating to cells, as the co-existence of the solid and liquid phases (Crowe *et al.*, 1989) causes an increase in permeability. This is minimised by the presence of sugars. The presence of any small carbohydrate will protect membranes to a certain RWC (Crowe *et al.*, 1992; Hoekstra *et al.*, 1997). However, it is the presence of a stable

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glass that is as important as the ability of a sugar to bind to the phosphate polar head groups. A non-reducing sugar such as sucrose is capable of both (Hoekstra *et al.*, 1997).

However, it has been reported that non-reducing sugars, on their own, do not provide all the necessary protection to membranes. Amphiphilic compounds, such as flavonols, may interact more efficiently with membranes after partitioning into the membrane under low water contents when they become dissociated (Hoekstra *et al.*, 1996). Flavonols have strong antioxidant properties (Wang and Zheng, 1992; Terao *et al.*, 1994) and they prevent sugars from being excluded from the bilayer (Hoekstra *et al.*, 1997). The migration of flavonols into the membranes during dehydration and out upon rehydration (Hoekstra *et al.*, 1997) might account for the transient membrane leakage (Pammenter and Berjak, 1999) which is observed in DT seeds, pollen and vascular plants. The incorporation of flavonols into membranes is of great importance as it is believed that they maintain membrane integrity due to lowering the water content at which membrane lipids change from the liquid crystalline to the gel phase (Hoekstra *et al.*, 1997). Flavonol association with the membranes during dehydration is reversible upon rehydration.

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It is also believed that in addition to the accumulation of critical amounts of non-reducing sugars, their very formation leads to a reduction in the amounts of reducing sugars, such as glucose. This, in turn, minimises the harmful Maillard and Amadori reactions that cause denaturation of macromolecules (Berjak *et al.*, 1989; Farrant *et al.*, 1992; Finch-Savage, 1992; Ooms *et al.*, 1992).

During dehydration lipid components of cell membranes change. The nature and the extent of these changes depend on the intensity and duration of this stress, the combination of the stresses associated with water loss and subsequent gain, as well as the genetic predisposition to dealing with the changing environment (Chetal *et al.*, 1983; Navari-Izzo *et al.*, 1989, 1993; Pham Thi *et al.*, 1990). Membrane protection of desiccation tolerant systems is believed to be facilitated by a monosaccharide content decrease and oligosaccharide content increase during dehydration (reviewed by Vertucci and Farrant, 1995).

Literature suggests that glucose and fructose levels decrease, while sucrose levels increase during dehydration of DT vascular plants (reviewed by Farrant, 2000). Reverse is true for rehydration studies. However, this chapter aims at attempting to answer what, if any, changes occur in mono- and di-saccharide levels and lipid peroxidation levels while the plants remain dry during a prolonged desiccation period. To do this, analyses were made of changes in membrane integrity, monosaccharide and oligosaccharide concentration changes, and lipid peroxidation in leaves of *C. wilmsii* and *X. humilis* plants. Measurements were done during dehydration, while the plants remained in the dry state for a period of up to 10 months, and during the subsequent rehydration.

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4.2 Materials and Methods

C. wilmsii and *X. humilis* plants were maintained as described in Chapter 2. During dehydration leaf samples were taken every day, at the same time of day, while during rehydration leaf samples were taken every six hours until plants reached full turgour. Sampling was done from three different plants.

4.2.1 Electrolyte Leakage

CM100 1.5 ml trays were filled with Milli-Q (Millipore) ultra pure water. Five mm^2 leaf segments were placed into the wells and readings were taken every 60 seconds for 90 minutes, using the Jenway 4070 conductivity meter. In order to obtain maximum leakage, the tissue segments were then plunged into liquid nitrogen and placed back into the CM100 tray wells for further measurement. Readings were taken every 60 seconds for 30 minutes. Measured leakage was expressed as a percentage of total according to the equation below:

Electrolyte leakage (%) = sample leakage / total leakage * 100

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4.2.2 Carbohydrates

Leaf tissues from each sampling point were weighted, snap frozen in liquid nitrogen and ground using a mortar and pestle. Extraction was in cold 100mM NaOH in 50% ethanol (v/v), the volume of the extract being 40 times the weight of hydrated leaves, and 80 times the weight of dry leaves. The mixture was vortexed and the extracts were neutralised by adding 100mM HEPES in 100mM acetic acid (pH 7-8 was determined using litmus paper). This mixture was transferred into centrifuge tubes, and was centrifuged for 20 minutes at 12 000 rpm. The supernatant was retained, and

the remaining pellet was re-extracted following the protocol described above. The two supernatants were combined. Sucrose, glucose and fructose content were quantified using a D-glucose / D-fructose sugar assay kit (Boehringer Mannheim, Germany). The absorbance of the extracts was measured at 340nm (production of NADPH) using (Beckman DU 650 UV/Vis, Beckman, USA), and the results were used to calculate the quantity of sucrose in samples. The following equations were used to calculate the sugar content:

Glucose:

$$\Delta \text{ abs} = \text{abs (glucose final)} - \text{abs (glucose initial)}$$

To convert the measurements to $\mu mol.ml^{-1}$, the following equation was used:

$$\frac{\Delta \text{ abs x total volume in cuvette}}{\text{sample volume in cuvette x extinction coefficient for NADPH}}$$

$$= \frac{\Delta \text{ abs x } 1000}{25\mu l \text{ x } 6.22}$$

Fructose:

$$\Delta \text{ abs} = \text{abs (fructose final)} - \text{abs (glucose final)}$$

Same formula was used in order to convert fructose measurements into $\mu mol.ml^{-1}$ as used for glucose.

Sucrose:

$$\Delta \text{ abs} = \text{abs (total glucose final)} - \text{abs (glucose initial)}$$

Final concentrations were calculated as for glucose and fructose using the same formula. Final sucrose concentrations were calculated as follows:

Sucrose = (total glucose content) – (glucose content)

4.2.3 Lipid Peroxidation Quantification through Malondialdehyde (MDA) Assays

A malondialdehyde (MDA) assay was done in order to determine the extent of lipid peroxidation during desiccation, while the plants were dry, and during subsequent

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rehydration. The method was adapted from Heath and Packer (1968). Triplicate samples of 1 g leaf tissue were ground in 5 ml distilled water. The tissue was then homogenised in an equal volume if 0.5% 2-thiobarbituic acid (TBA) in 20% trichloroacetic acid (TCA). The extract was incubated for 30 minutes at 95°C. Once cooled to room temperature the samples were centrifuged for 30 minutes at 16 000 g. The supernatant was filtered to remove pigment and the absorbance of the extract was read at 532nm and 600nm using the Beckman DU 650 UV/Vis, Beckman, USA. The following equation was used to calculate the amount of MDA:

$$MDA = (((\lambda_{532} - \lambda_{600})/155)*\text{volume of extract})/\text{g DW},$$

where $155 mM^{-1}cm^{-1}$ is the extinction coefficient.

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4.3 Results and Discussion

4.3.1 Membrane damage in drying, dry and rehydrating *C. wilmsii* and *X. humilis* plants

During dehydration changes in electrolyte leakage (EL) were observed in leaves of both species (Figure 4.1). There was a slight increase in EL in *C. wilmsii* at 35% RWC (Figure 4.1 A). In *X. humilis* initial EL (at full turgour) was higher than that at the air-dry state (Figure 4.1 B). The lower EL at the air-dry state might be attributed to the stabilisation of the membranes by glass formation. Similar results were observed among treatments and so only WD *C. wilmsii* and AA *X. humilis* plants were used to demonstrate these changes.

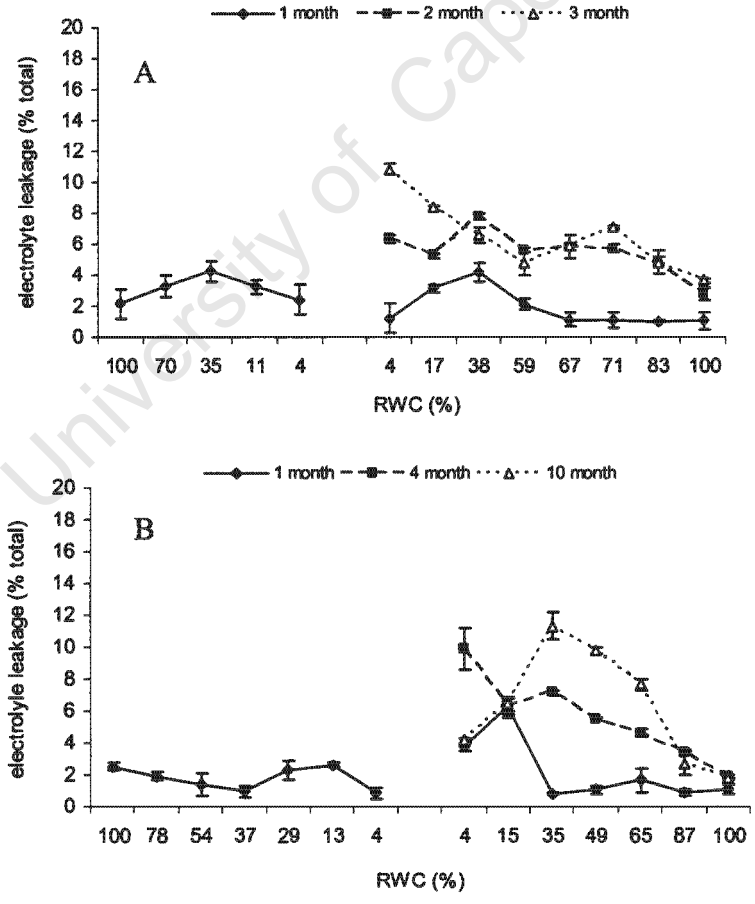

Figure 4.1: Electrolyte leakage (% total) in *C. wilmsii* (A) and *X. humilis* (B) leaves during dehydration and subsequent rehydration. One month (◊); two/four month (■); three/10 month (Δ) drought.

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The longer the plants remained dry, the higher the EL in both species at the onset of rehydration, except for the one month *C. wilmsii* plants. Following a one month drought, during the initial stages of rehydration, EL was found to increase in both species (Figure 4.1). In *C. wilmsii* (Figure 4.1 A) a steady increase in EL was observed up to 38% RWC and in *X. humilis* leaves this was observed up to 15% RWC (Figure 4.1 B). Thereafter EL returned to control levels in both species. In *C. wilmsii* the increase in EL occurred at similar RWC during both de- and rehydration. This increase in EL could be attributed to re-organisation of membranes during rehydration, as previously reported by Hoekstra *et al.* (1997) and Farrant (2000).

Following the two month drought, *C. wilmsii* plants had a much higher EL during the initial rehydration stages, as did the three month dry plants. In both instances, EL at full turgour was higher than that of one month dry plants (Figure 4.1 A). Similar findings were observed for *X. humilis* plants, however, once fully rehydrated, this species was able to restore its membranes, as EL returned to that observed in one month dry plants.

The longer the drought period, the higher the EL in both species (Figure 4.1). Although there is much higher solute leakage in the three month dry *C. wilmsii* than in one month dry *C. wilmsii*, similarly for 10 month dry *X. humilis* and one month dry *X. humilis*, past reports have shown that these higher EL measurements are within the range of EL associated with membrane re-organisation during rehydration in these species (Dace *et al.*, 1998; Farrant *et al.*, 2003).

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Lipid-protein, protein-protein and membrane bound enzyme activities are influenced by the composition and physical state of the lipid bilayer, which in turn is influenced by the hydration state (Navari-Izzo and Rascio, 1999; Navari-Izzo *et al.*, 2000; Kerkeb *et al.*, 2001). Work done by Sherwin and Farrant (1996) showed that *C. wilmsii* showed no significant difference in electrolyte leakage during dehydration or rehydration indicating maintenance of membrane integrity. However, the electrolyte leakage in *X. humilis* and *M. flabellifolius* did increase, but in both species leakage returned to control levels on rehydration. Drying appeared to cause some change in membrane configuration, but this was reversed on rehydration (Sherwin and Farrant, 1996). Farrant *et al.* (1999) reported that the extent of electrolyte leakage from leaves of *C. wilmsii* remained unchanged during both rapid and slow drying indicating that drying, regardless of the drying speed, did not affect membrane integrity in leaves of this species. Also, in a study by Farrant *et al.* (2003) there was no difference in extent of leakage, compared to hydrated material, in leaves of *C. wilmsii* that have been dried unrestrained in the light, nor upon their rehydration, suggesting that little membrane damage occurred during these treatments.

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No significant increase in MDA concentration was observed in either of the species while the plants were drying (Figures 4.2 and 4.3). In *C. wilmsii* plants the MDA concentration remained unchanged as long as plants remained viable while dry, but the MDA concentrations of dead leaves were significantly higher that that of viable dry leaves (Figure 4.2).

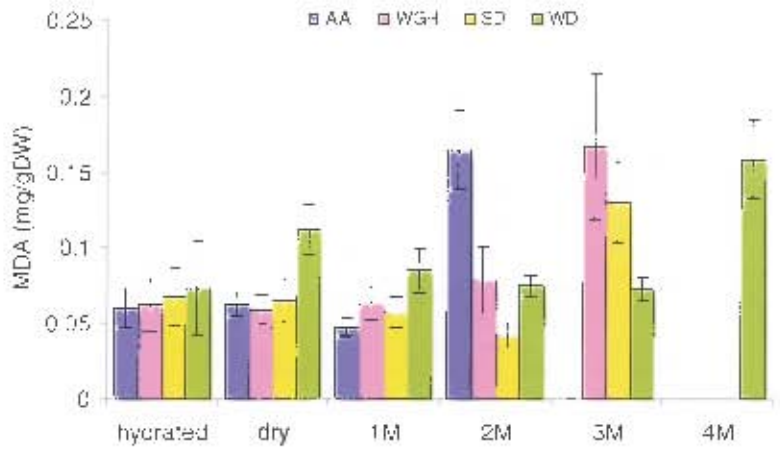

Figure 4.2: MDA (mg.g^{-1} DW) levels in hydrated, dry (M - months) and dead (AA – 2M; WGH and SD – 3M; WD – 4M) *C. wilmsii* leaves.

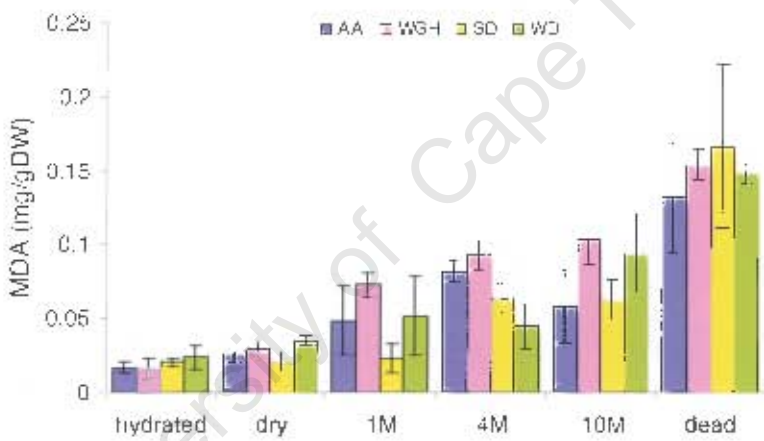

Figure 4.3: MDA (mg.g^{-1} DW) levels in hydrated, dry (1M, 4M and 10M – M (months)) and dead *X. humilis* leaves.

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In *X. humilis* plants there was a steady increase in MDA concentration the longer the plants remained in the dry state, indicating slow lipid peroxidation (Figure 4.3). MDA concentration in *X. humilis* plants was considerably lower than in *C. wilmsii* plants during the first few months in the dry state, and increased to approach levels in *C. wilmsii* plants between four and 10 months of dry storage. This suggests that damage was accruing in dry stored *X. humilis* plants although not reflected in other measures.

Once *X. humilis* plants reached the same MDA concentrations as those observed in *C. wilmsii* plants (between four and 10 months of drought, Figure 4.3) this species was still capable of maintaining viability for some time. Although in this study the time limit during which *X. humilis* might lose viability was not reached, the steady increase in MDA concentration might indicate that a further few months of desiccation would have ultimately proven lethal to this species. The MDA concentrations in dead leaves of both species were similar, thus indicating that the level of lipid peroxidation that both species could tolerate was below 0.15 $mg.g^{-1}$ DW.

Figure 4.2 is in support of the EL data. The EL remained within range of the norm for *C. wilmsii*, and the MDA concentrations did not increase while the plants were dry. In *X. humilis* plants, however, although MDA concentrations increased the longer the plants remained dry, EL did not exceed what is perceived to be acceptable for this species, indicating that membranes were protected sufficiently to withstand this level of damage.

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4.3.2 Changes in sugar levels in drying, dry and rehydrating *C. wilmsii* and *X. humilis* plants

Sucrose levels in *C. wilmsii* plants remained unchanged while the plants were maintained dry under all conditions (Figure 4.4). During subsequent rehydrations, sucrose levels returned to that of the control, but only in plants maintained under AA and SD conditions for one and two months, respectively. In plants maintained under WGH and WD conditions, the longer the plants remained dry, the higher the sucrose levels at full turgour, following rehydration. This also might indicate a steady

accumulation of damage while the plants were in the dry state, thus higher sucrose levels might be a result of damage to enzymes.

During dehydration sucrose levels in *X. humilis* plants increased from <50mg.g^{-1} DW to approximately 150mg.g^{-1} DW. While in the dry state, sucrose concentrations in *X. humilis* plants remained unchanged independent of the time spent in the dry state and independent of the simulated field conditions (Figure 4.5). Following the one month drought period, upon rehydration to full turgour, sucrose levels decreased to below that of the control.

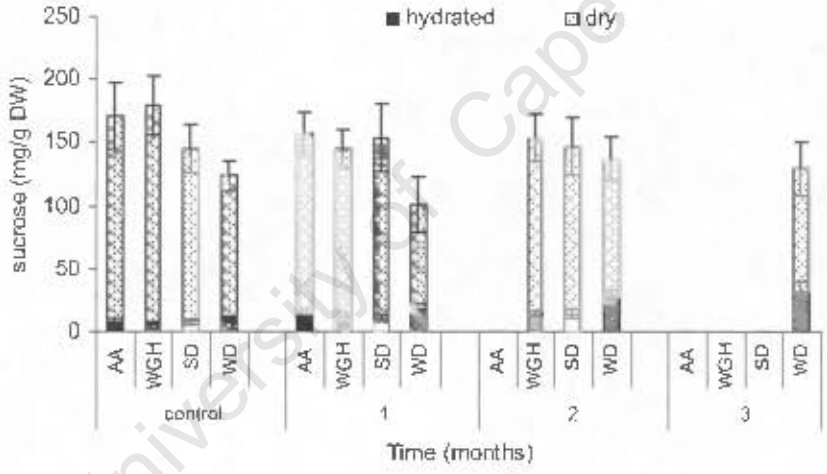

Figure 4.4: Sucrose concentration in leaves of *C. wilmsii* plants in the dry and hydrated state. Control refers to either the immediately air-dry state or 100% RWC, and 1, 2 and 3 refer to the number of months the plants remained dry prior to sampling and rehydration.

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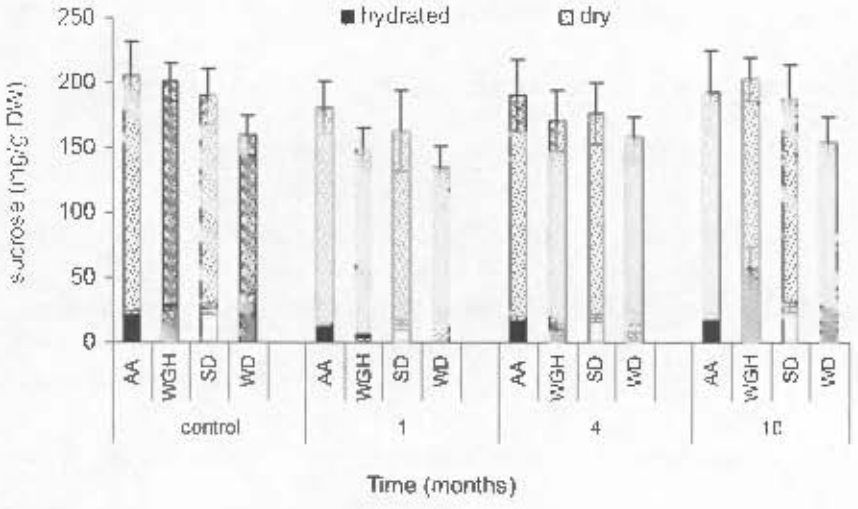

Figure 4.5: Sucrose concentration in leaves of *X. humilis* plants in the dry and hydrated state. Control refers to either the immediately air-dry state or 100% RWC, and 1, 4 and 10 refer to the number of months the plants remained dry prior to sampling and rehydration.

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However, the longer the plants remained dry, upon subsequent rehydrations sucrose levels at full turgour did return to control levels, except for the 10 month plants maintained under WGH conditions (Figure 4.5). In those plants sucrose levels were twice the control, possibly indicating some kind of damage which resulted in the inability to metabolise sucrose on rehydration.

It has been suggested that accumulation of sucrose alone cannot ensure long term viability of seeds. It is the combination of sucrose and proteins that are important for keeping glasses stabilised (Horbowicz and Obendorf, 1994; Leopold *et al.*, 1994; Walters et *al.*, 2005). This proposition might apply for the long term viability of some DT vascular plants, as suggested by the *C. wilmsii* sucrose data (Figure 4.4). Here, although sucrose levels remained unchanged throughout the prolonged drought, *C. wilmsii* plants lost viability. This might apply also to *X. humilis* dry storage, however the viability time limit for this species was not attained in this study.

Prior to drying, glucose levels in *C. wilmsii* plants were higher than that in *X. humilis* plants (Figures 4.6 and 4.7), but levels reduced to those typical of *X. humilis* during dry storage. In *C. wilmsii* glucose levels remained low during dry storage but returned to the control levels on rehydration. Following the two and three month rehydrations, *C. wilmsii* plants that were kept under WD conditions had a much higher glucose concentration than the control (Figure 4.6).

Glucose levels in dry *X. humilis* plants did change while the plants were dry (Figure 4.7). This was particularly evident in plants maintained under AA and WGH conditions. This increase in glucose levels while the plants were dry might account for the increase in MDA levels during the prolonged drought, as glucose fuels the damaging Maillard and Amadori reaction. Also, the longer the plants remained in the dry state, the higher the glucose concentrations once the plants rehydrated to full turgour. Most significant increase was observed after the rehydration following the four months of dry storage in plants maintained under all but AA conditions. Here, glucose levels were approximately twice the control in both SD and WD plants, and only partially higher in plants maintained under WGH conditions. However, the rehydration of the four month *X. humilis* plants resulted in sucrose levels being somewhat lower than that of the control, possibly accounting for some of the extra glucose in the rehydrated tissues.

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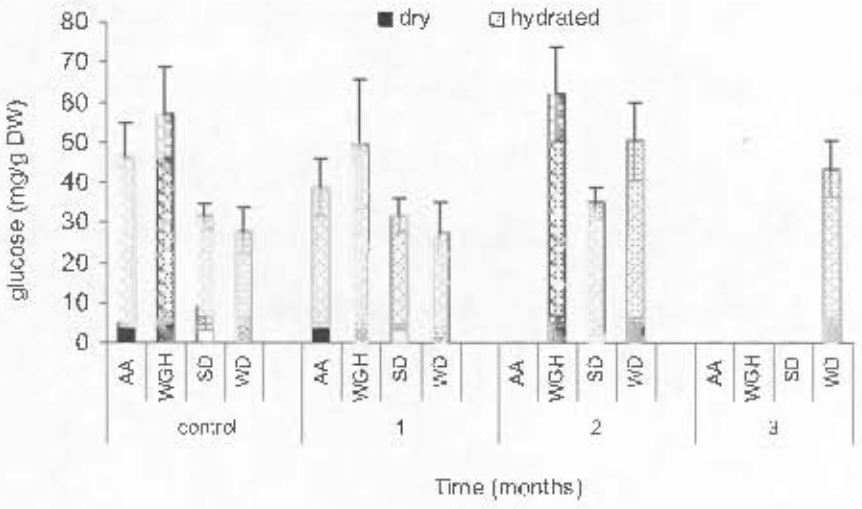

Figure 4.6: Glucose concentration in leaves of *C. wilmsii* plants in the dry and hydrated state. Control refers to either the immediately air-dry state or 100% RWC, and 1, 2 and 3 refer to the number of months the plants remained dry prior to sampling and rehydration.

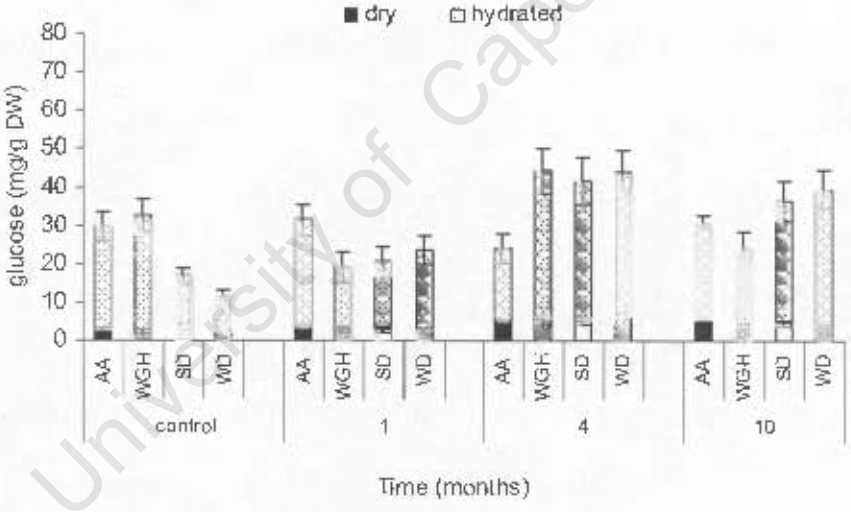

Figure 4.7: Glucose concentration in leaves of *X. humilis* plants in the dry and hydrated state. Control refers to either the immediately air-dry state or 100% RWC, and 1, 4 and 10 refer to the number of months the plants remained dry prior to sampling and rehydration.

Figure 4.8 shows that even at full turgour, following each rehydration, fructose concentrations in *C. wilmsii* plants return to control levels in all plants maintained under all but WGH conditions. Under these conditions, it seemed that the longer the plants remain in the dry state the lower the fructose level at full turgour. In *X. humilis*

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after the 10 month rehydration plants maintained under WGH conditions had a lower fructose concentration than the control. But under all other conditions and after each rehydration, fructose did return to control levels at full turgour (Figure 4.9).

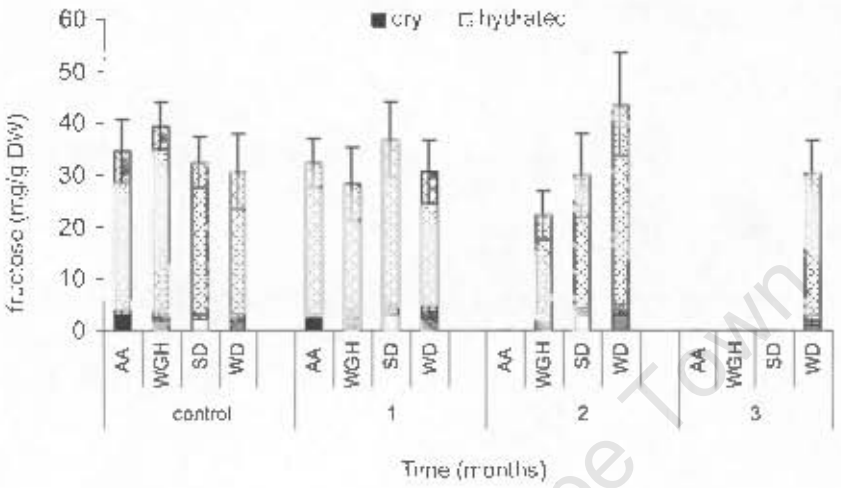

Figure 4.8: Fructose concentration in leaves of *C. wilmsii* plants in the dry and hydrated state. Control refers to either the immediately air-dry state or 100% RWC, and 1, 2 and 3 refer to the number of months the plants remained dry prior to sampling and rehydration.

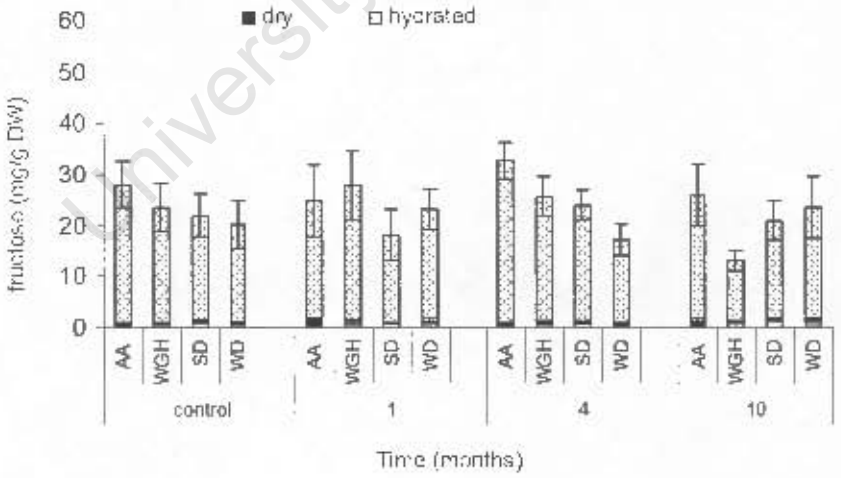

Figure 4.9: Fructose concentration in leaves of *X. humilis* plants in the dry and hydrated state. Control refers to either the immediately air-dry state or 100% RWC, and 1, 4 and 10 refer to the number of months the plants remained dry prior to sampling and rehydration.

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Accumulation of non-reducing sugars has been associated with DT (reviewed by Vertucci and Farrant, 1995). During dehydration non-reducing sugars are thought to replace water associated with membranes (Clegg, 1986). By associating with the membrane polar head groups, these sugars prevent liquid crystalline to gel phase transition (Crowe *et al.*, 1992). Sucrose has been identified as the most abundant non-reducing sugar in DT seeds, pollen and vascular DT plants (reviewed by Pammenter and Berjak, 1999; Farrant, 2000). However, other non reducing sugars are also accumulated, namely stachyose, raffinose, trehalose, and fructans (Ingram and Bartels, 1996; Norwood *et al.*, 2000; Loewus and Murthy, 2000; Corbineau *et al.*, 2004; Kamer *et al.*, 2004). These sugars vitrify the aqueous phase and form a highly viscous, super-saturated solution (glass) (Caffrey *et al.*, 1988; Koster and Leopold, 1988; Leopold *et al.*, 1994). Due to their high viscosity, glasses minimise molecular mobility and protect macromolecules from damage, but this protection is not indefinite. Seed research has shown that there appears to be a strong correlation between the oligosaccharide:sucrose ratio and storage longevity of dry seeds (Horbowicz and Obendorf, 1994; Steadman *et al.*, 1996; Sun and Leopold, 1997). The presence of a glassy state has been shown to improve storage stability and longevity of dried food products (Slade *et al.*, 1993; Slade and Levine, 1994) and is believed to play a similar role in dry DT systems (Sun and Leopold, 1994).

As shown above the concentration of sugars changes during dehydration and upon rehydration. This study investigated only three such sugars, however many others are accumulated during water loss and gain (as indicated above). Figures 4.4 to 4.9 have shown some inconsistencies in sugar levels. For instance, in dry *X. humilis* plants maintained under AA, WGH and WD conditions, sucrose and fructose levels

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remained constant throughout the drought period, but glucose levels increased the longer the plants remain dry. Higher glucose levels could be a result of damage to glucose polymers which, as a result, break down increasing the free glucose content. MDA data suggests that the presence of glasses does not prevent molecular mobility, but merely slows it down, and so macromolecular damage results, as will be further discussed in Chapter 5.

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4.4 Conclusion

The longevity in the dry state did not prove to increase membrane damage to either of the species. Although higher EL was observed the longer the plants remained dry, this can be attributed to the temporary membrane dis-organisation known to occur during both dehydration and rehydration. Also, the EL reported in this study is considered to be within the range of membrane leakage which has been previously reported to be the norm for these two species (Dace *et al.*, 1998; Farrant *et al.*, 2003).

Although *X. humilis* is capable of disassembling most of its chlorophyll and accumulating non-reducing sugars, such as sucrose, at the expense of monosaccharides, *X. humilis* plants are still susceptible to lipid peroxidation. The longer the drought period, the higher the lipid peroxidation (MDA) levels that were observed. *C. wilmsii* on the other hand maintained constant levels of MDA but its survival in the dry state was more limited than that of *X. humilis*. This suggests that *C. wilmsii* is capable of tolerating higher initial levels of damage to its membranes, but for a shorter period of time, while *X. humilis* might fall victim to slowly progressing and accumulating lipid peroxidation, which could be a result of the slow but steady increase of glucose known to fuel Amadori and Maillard reactions.

This study explored the changing concentration of three sugars, however, many more are known to be accumulated during dehydration and metabolised during rehydration in DT organisms. These sugars include, among others, stachyose, trehalose, raffinose, mannitol and fructans. Oligosaccharides such as these have been reported to be important for DT as their ratio to sucrose seems to correlate with storage longevity. However, these oligosaccharides could have been somehow damaged while the plants

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were dry, resulting in the observed increase in glucose at full turgour following a prolonged drought. Perhaps future studies could explore the oligosaccharide:sucrose ratio and its contribution to longevity of dry *C. wilmsii* and *X. humilis* plants.

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CHAPTER FIVE:

Protein and RNA Stability in Dry *Craterostigma wilmsii* and *Xerophyta humilis* Plants Following a Prolonged Drought

5.1 Introduction

Craterostigma wilmsii and *Xerophyta humilis* have been classified as modified DT species (Oliver, 1996) as their survival of desiccation relies largely on protection of the subcellular milieu during drying. True DT plants, such as the moss *Tortula ruralis*, survive the water deficit stress through the ability to repair the dehydration and/or rehydration induced damage upon the return of favourable conditions.

Previous research has confirmed that some, if not all, angiosperm DT species' survival relies on the induction of protective mechanisms induced during dehydration (Farrant and Sherwin, 1998; Sherwin and Farrant, 1998; Farrant *et al.*, 1999; Vicre *et al.*, 1999; Cooper and Farrant, 2002). As discussed in previous chapters these mechanisms include the accumulation of anthocyanins, break-down of photosynthetic machinery (poikilochlorophylly), leaf folding and curling to shade chlorophyll (homoiochlorophylly), accumulation of sucrose, as well as the reduction of monosaccharide concentrations, and up-regulation of antioxidant systems. In addition to these, other putative mechanisms of protection include the accumulation of compatible solutes such as proline and glycine betaine, as well as the accumulation of heat shock and late embryogenesis abundant (LEA) proteins (reviewed by Farrant, 2000).

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However, in addition to the abovementioned protection mechanisms that are induced during dehydration, *C. wilmsii* and *X. humilis* plants also accumulate gene products necessary for recovery of metabolism upon early rehydration, especially with regard to the photosynthetic machinery in the poikilochlorophyllous types (Dace *et al.*, 1998; Cooper and Farrant, 2002; Collett *et al.*, 2003). Dace *et al.* (1998) found that in *X. humilis* plants initial photosynthetic recovery was independent of *de novo* transcription, but that new protein synthesis was necessary in order for the plants to regain fully functional photosystem (PS) II and optimal photosynthesis. These researchers observed that messenger RNA (mRNA) for chlorophyll biosynthesis was accumulated during dehydration and stored in a stable state and that this stored mRNA was transcribed upon rehydration. Cooper and Farrant (2002) showed that unlike *X. humilis*, *C. wilmsii* plants did not rely on *de novo* transcription nor translation upon rehydration, and that complete photosynthetic functioning was regained at full turgour and remained at optimum for at least 72 hours. However, these studies were conducted on plants that were maintained dry no longer than two weeks, as was the case for the *X. humilis* study, and no longer than a few days for the *C. wilmsii* study.

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Following the work done by Dace *et al.* (1998), Collett *et al.* (2003) identified several transcripts associated with the photochemistry of PS II that are stored in dry leaf tissues of *X. humilis* plants. These transcripts included *psb*A, *psb*P and *psb*R. *psb*A codes for the D1 protein that binds chlorophyll and is a core component of PS II reaction centre (He and Vermaas, 1998). *psb*P codes for a protein that forms a part of the oxygen evolving complex of PS II, while *psb*R is presumed to play a role in the water excitation within PS II and forms an important link in this reaction centre for

stable assembly of psbP (Suorsa *et al.*, 2006). In *X. humilis* plants *psb*R and *psb*P transcript levels were found to decrease during dehydration but then increased within the first 12 hours upon rehydration (Collett *et al.*, 2003). *psb*A transcript levels also decreased during dehydration, but increased within the first 24 hours of rehydration. *psb*A and *psb*P transcripts were found to be stably stored while the plants were dry (Collett *et al.*, 2003).

In this study leaves of desiccated *C. wilmsii* and *X. humilis* plants were rehydrated in water (as control), as well as transcription (distamycin-A and actinomycin-D, for *C. wilmsii* and *X. humilis*, respectively) and translation (cycloheximide) inhibitors, in order to determine to what extent initial photosynthetic recovery of the one and two, and one and 10 month dry *C. wilmsii* and *X. humilis* plants, respectively, depended on *de novo* transcription and translation. This study aims at exploring the stability of the stored mRNA and proteins during a prolonged drought.

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5.2 Materials and Methods

In this study, dry leaf explants of *C. wilmsii* plants from WGH treatment only were used to test the stability of stored protectants. WGH treatment was chosen as under these conditions *C. wilmsii* plants were capable of surviving in the dry state for two months, the same time span the plants would experience drought naturally in the field. Although this species probably does not naturally experience prolonged drought under the conditions we used for WGH treatment (see Table 2.1 in Chapter 2; which might be considered to be more damaging due to variability in light intensity and temperature), this treatment was chosen for the current work to test the robustness of their protective mechanisms and to see which ones might fail under adverse environmental conditions. For *X. humilis*, leaf explants from only AA treated plants were chosen to test the stability of stored mRNA, as although *X. humilis* plants behaved similarly among all other treatments, and survived drought for 10 months, under AA conditions there was a suggestion of initiation of damage (see Chapter 4). Thus again, the metabolism most susceptible to damage can be identified in this chapter. In the figures that follow all *C. wilmsii* results refer to the WGH treatment and all *X. humilis* results refer to the AA treatment.

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5.2.1 Initiation of *de novo* transcription and translation using radiolabels and without the use of inhibitors

The rate of protein synthesis was calculated as the rate of incorporation of ^{3}H-leucine into proteins using the method of Mans and Novelli (1961). Excised dry leaves of one and two month dry *C. wilmsii*, and one and 10 month dry *X. humilis* plants were rehydrated in water containing 1μCi/ml ^{3}H leucine. Samples were taken every six hours for the first 24 hours, and further sampling was done twice, at 36 and 48 hours.

For each time point proteins were extracted from three leaves per treatment. Protein extraction was carried out using 0.2M Tris-HCl (pH 7). 100μl of extract was placed on 15mm diameter discs of 3MM Whatmann filter paper, and proteins were precipitated in cold 10% trichloroacetic acid (TCA) for one hour. The amount of radiolabel incorporated into proteins was measured using a scintillation counter.

The rate of RNA synthesis was calculated as the rate of ^{3}H-uridine incorporation into RNA using an adaptation of the method of Mans and Novelli (1961). Excised dry leaves of one and two month dry *C. wilmsii*, and one and 10 month dry *X. humilis* plants were rehydrated in water containing 1μCi/ml ^{3}H-uridine. Samples were taken every six hours for the first 24 hours, and further sampling was done twice, at 36 and 48 hours. For each time point RNA was extracted from three leaves per treatment. RNA extraction was carried out using 0.2 M Tris-HCl (pH 7). 100μl of extract was placed on 15mm diameter discs of 3MM Whatmann filter paper, and RNA was precipitated in cold 0.5 M ammonium acetate (in ethanol) for one hour. The amount of radiolabel incorporated into RNA was measured using a scintillation counter.

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5.2.2 Transcription and Translation Inhibition

Leaf explants of *C. wilmsii* were taken from one and two month dry plants, and *X. humilis* explants were taken from one and 10 month dry plants. The leaves were placed into Petri-dishes filled with either water (control), or distamycin-A (as a transcription inhibitor for *C. wilmsii*) or actinomycin-D (as a transcription inhibitor for *X. humilis*) or cycloheximide (as a translation inhibitor for both species). For *X. humilis* explants concentrations of 20 $\mu g.ml^{-1}$ of actinomysin-D and cyclohximide was previously determined to be sufficient to inhibit incorporation of radioactive

precursors into RNA and proteins respectively (Dace *et al.*, 1998). Cooper (2001) showed that these concentrations were too low to inhibit transcription and translation in *C. wilmsii* plants, and thus determined that 310 $\mu g.ml^{-1}$ and 220 $\mu g.ml^{-1}$ of distamycin-A and cycloheximide, respectively, were requited for inhibition. Actinomycin-D was found to be too large a molecule to be incorporated into *C. wilmsii* and thus distamycin-A was used instead (Cooper and Farrant, 2002).

The explants were left to rehydrate in these solutions and in water (control) for six days. Chlorophyll fluorescence, chlorophyll (a+b), carotenoid (x+c), anthocyanin levels were measured (as previously described in Chapter 3) and the subcellular organization was observed at 24 hours, 72 hours and six days.

5.2.3 Ultrastructural Studies – Transmission Electron Microscopy

The effect of inhibitors on subcellular organization during rehydration was analysed through the use of Transmission Electron Microscopy. Method as described in Chapter 2.

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5.3 Results and Discussion

Due to the necessity to use explants rather than whole plants (see Materials and Methods), it must be noted that rehydration of leaf explants was much faster than that of intact plants. However, since explants reached full turgour within 12 hours, similar to the findings of Dace *et al.* (1998) for *X. humilis* leaf explants and to the findings of Cooper and Farrant (2002) for *C. wilmsii* leaf explants, rehydration speed is not illustrated here.

5.3.1 *De novo* transcription and translation in *C. wilmsii* and *X. humilis* leaf explants

The timing of the initiation of *de novo* transcription and translation measured through radiolabel incorporation of ^{3}H-uridine and ^{3}H-leucine, respectively, is shown in Figure 5.1. In order to understand how the plants recovered during rehydration, *de novo* transcription and translation were followed for 48 hours.

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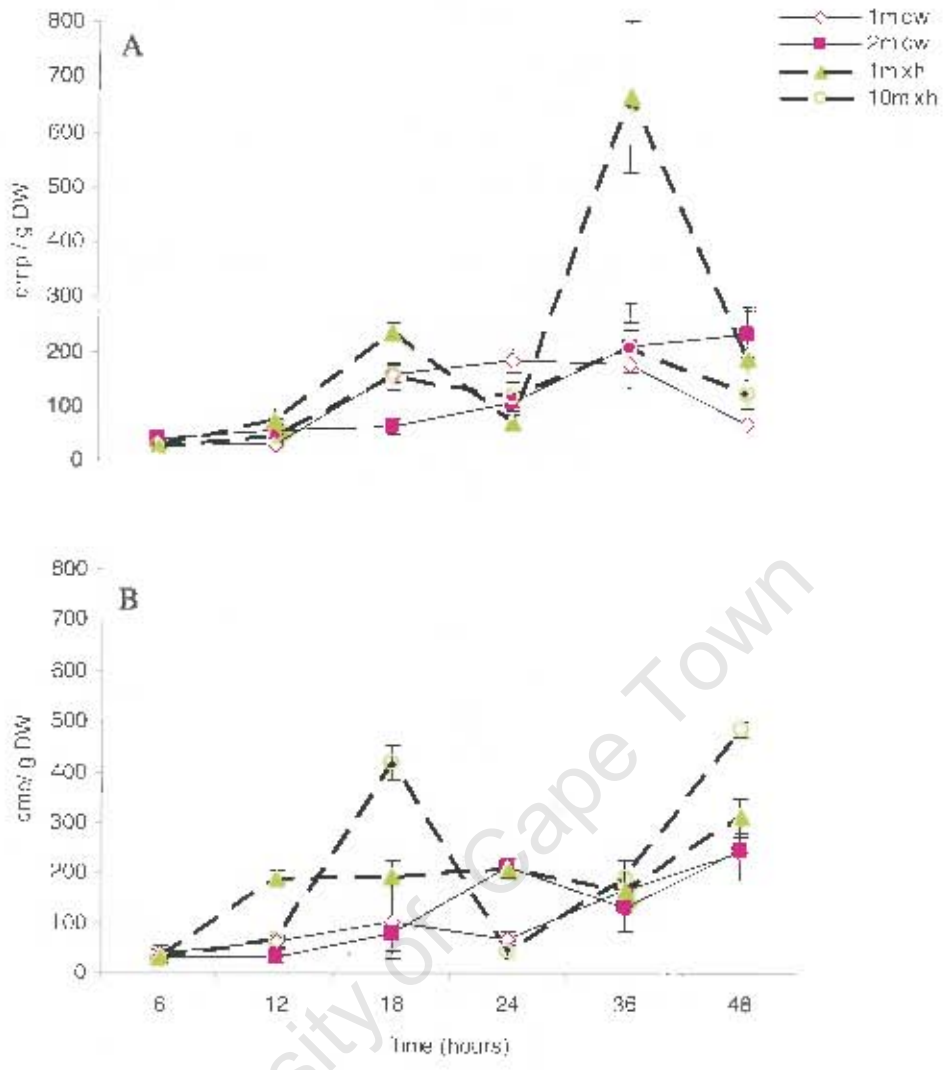

Figure 5.1: Rate of *de novo* mRNA (A) and protein (B) synthesis in one and two month (m) dry *C. wilmsii* (cw) leaf explants and one and 10 month dry *X. humilis* (xh) leaf explants during 48 hours of rehydration. ▲ – one month dried *X. humilis*; ○ – 10 month dried *X. humilis*; ◇- one month dried *C. wilmsii*; ■ – two month dried *C. wilmsii*.

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During the first 12 hours of rehydration no significant *de novo* transcription occurred in either species (Figure 5.1A). After 18 hours, *de novo* transcription was observed in both one and 10 month dried *X. humilis* and one month dried *C. wilmsii* explants. Perhaps the delayed response of the two month dried *C. wilmsii* explants was a result of accrued damage to nuclear material or enzymes needed for transcription, or both. Transcription was higher in one month dried *X. humilis* explants than in the 10 month

dried samples, possibly indicating some damage to enzymes needed for transcription in the 10 month dried samples. At 24 hours of rehydration, one month dried *X. humilis* explants decreased transcription, presumably a result of accumulation of sufficient transcript levels necessary to sustain protein synthesis (Figure 5.1B). One month dried *C. wilmsii* and 10 month dried *X. humilis* explants maintained a constant transcription rate, while two month dried *C. wilmsii* explants increased transcription (Figure 5.1A). At 36 hours one month dried *X. humilis* explants increased *de novo* transcription again to three times the rate observed at 18 hours. This increase in transcription might indicate that all stored mRNA was used and/or that new transcripts (not stored) that were necessary for protein synthesis were transcribed at this stage. By 48 hours transcription in all explants, except two month dried *C. wilmsii*, had decreased. The increased transcription in the two month dried *C. wilmsii* explants at 48 hours is probably the result of the delayed transcription due to accrued damage in dried tissues, and thus the need for repair. Both one and 10 month dried *X. humilis* explants demonstrated cyclical transcription trends, possibly reacting in response to the diurnal cycle.

C. wilmsii explants showed no significant *de novo* translation within the first 18 hours of rehydration (Figure 5.1B). In one month dried *C. wilmsii* explants *de novo* translation was observed only after 36 hours of rehydration. By that time, *de novo* transcription was significant for at least 18 hours (Figure 5.1A). This might suggest that the delayed protein synthesis was a result of either post-transcription modification preventing immediate translation until 36 hours of rehydration, and/or that stored proteins were sufficiently protected while the plants were dry and used for recovery during early rehydration when new synthesis was required. But new protein synthesis

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was required by 36 hours, when *de novo* translation was initiated, and sufficient transcript levels were accumulated to sustain increased protein synthesis observed at 48 hours. The initiation of *de novo* translation was observed earlier in two month dried *C. wilmsii* explants than in one month explants, possibly suggesting that damage to dry tissues was greater after two months of dry storage, thus the need for new proteins for repair. Also, *de novo* transcription increased in two month dried samples by 24 hours, providing necessary transcripts for protein synthesis.

One month dry *X. humilis* explants showed *de novo* translation between six and 12 hours during rehydration, but the rate of translation remained elevated and constant thereafter (Figure 5.1B). *De novo* transcription data (Figure 5.1A) shows that no new transcripts were made during that time, indicating that *de novo* translation was probably from stored mRNA. Ten month dry explants, however, showed a delayed, but much higher rate of protein synthesis than one month dried explants, as indicated by the peak at 18 hours. Higher protein synthesis was probably the result of much needed repair after a long dry period. This data coincides with the induction of *de novo* transcription (Figure 5.1A). Much like mRNA synthesis of 10 month dried leaf explants, protein synthesis also showed a cyclical pattern that corresponded with that of the mRNA synthesis.

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5.3.2 Use of inhibitors during rehydration

5.3.2.1 Recovery of photosystem (PS) II in *C. wilmsii* and *X. humilis* leaf explants

The stability of stored proteins and mRNA and their need for the recovery of PS II of dry *C. wilmsii* and *X. humilis* leaf explants, respectively, was examined through the

use of transcription and translation inhibitors. Figures 5.2 and 5.3, below, illustrate the findings.

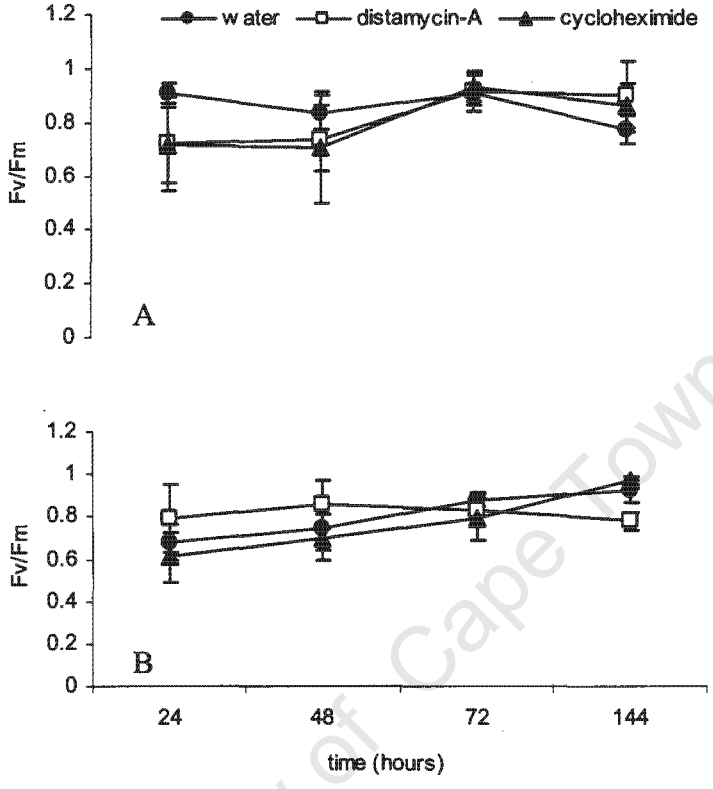

Figure 5.2: Recovery of photosystem II as indicated by the F_V/F_M ratio in excised leaves of WGH treated *C. wilmsii* plants following a one (A) and two (B) month drought. Water (●); distamycin-A (□); cyloheximide (▲).

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Leaf explants from the one and two month dry *C. wilmsii* plants behaved similarly among the three treatments, in that in all cases F_V/F_M recovered completely (Figure 5.2). This suggests that no or little repair was needed for full function of the PS II for up to six days, as previously reported for this species (reviewed by Farrant, 2000). It is possible for *C. wilmsii* to initiate photosynthesis early during the rehydration process as it maintains a sufficient amount of chlorophyll in the dry state, being homoiochlorophyllous. As shown in Chapter 3, some loss of chlorophyll (a+b) was observed under these conditions (WGH) after two months of drought, but chlorophyll

(a+b) levels were still probably high enough to maintain F_V/F_M at optimum. In that chapter it was discussed that *C. wilmsii* plants were capable of optimal PS II function at chlorophyll (a+b) levels between 2 and 10 $mg.g^{-1}DW$. In two month dried explants F_V/F_M in water and distamycin-A rehydrated samples reached optimum only after 72 hours and maintained it for the following three days. Again, although the initial low F_V/F_M possibly indicates that reorganisation of the photosynthetic apparatus was required. After two months of dry storage the full activity of PS II was restored without the need for de novo transcription and translation.

These data suggest that little damage to PS II had occurred during the prolonged drought in *C. wilmsii* plants. Previous research has shown that the transcription and translation inhibitors do not influence the recovery of PS II in naturally dried *C. wilmsii* plants (maintained dry for a couple of days only, Cooper and Farrant, 2002), and similarly, this study suggests that the inhibitors do not affect the recovery process of the photochemistry in any way in plants that were maintained dry for up to two months under WGH conditions. This suggests that the accumulated proteins were sufficiently protected while the plants were dry.

When Cooper and Farrant (2002) compared the effect of transcription and translation inhibitors on the photosynthetic pigments association with the PS II of slowly (naturally) and fast dried *C. wilmsii* plants, their data showed that the recovery of slowly dried plants was not affected by either of the inhibitors, while fast dried plants were affected by both. This indicated that the speed of drying affected the full laying down of protective mechanisms. However, fast dried plants were capable of regaining a fully functional PS II when rehydrated in water. This suggests that although some components for the synthesis of proteins associated with PS II might have survived

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fast drying, it was the effect of the transcription and translation inhibitors that disabled the leaf explants from regaining a fully functional PS II during rehydration. The water treatment allowed for *de novo* mRNA and protein synthesis necessary for the complete recovery of PS II.

X. humilis leaf explants that were kept dry for one month recovered F_V/F_M completely within 24 hours in both water and actinomycin-D (transcription inhibitor) (Figure 5.3 A). This high efficiency of PS II was maintained for at least six days. Explants treated with cycloheximide (translation inhibitor) never completely recovered the function of PS II, and F_V/F_M remained at 0.5 (Figure 5.3 A). This suggests that *de novo* protein synthesis was required to recover photosynthesis, as has been previously reported (Dace *et al.*, 1998) The transcription inhibitor did not hinder the recovery of PS II in one month dried explants indicating that stored mRNA was sufficient for the recovery of PS II after one month of dry storage.

X. humilis plants that were kept dry for 10 months behaved differently to those kept dry for one month. F_V/F_M of 10 month leaf explants never reached optimal levels (0.8) in either water or actinomycin-D treatments (Figure 5.3 B). In these treatments partial recovery of PS II was observed within the first 48 hours of rehydration, but thereafter F_V/F_M declined to 0.5 or below. This suggests that some necessary mRNA was present in dry tissues, but that it was not sufficient for the complete recovery of PS II. Explants treated with cycloheximide maintained F_V/F_M at 0.5 for the first 48 hours, but thereafter declined to below 0.3 (Figure 5.3 B).

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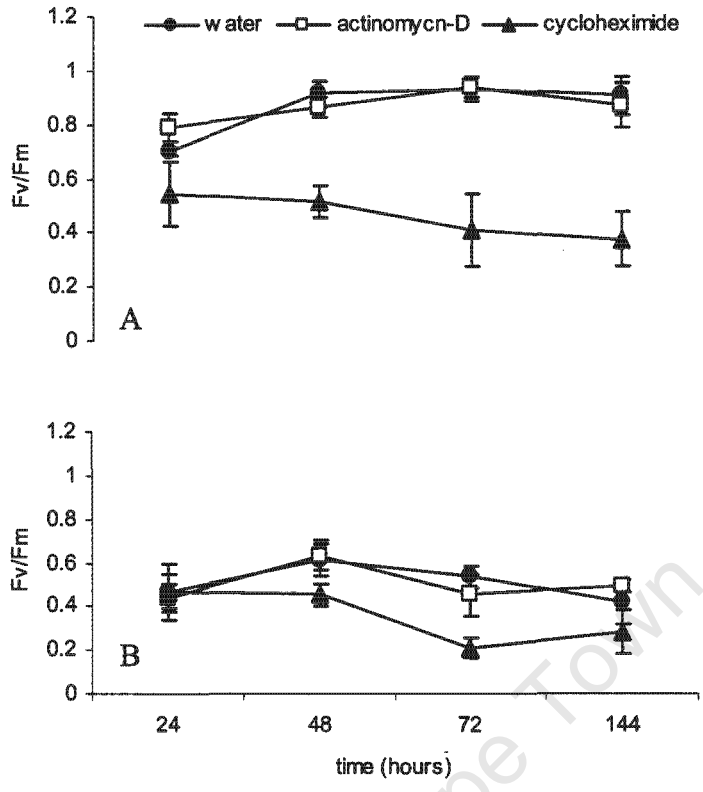

Figure 5.3: Recovery of photosystem II as indicated by the F_V/F_M ratio in excised leaves of AA treated *X. humilis* plants following a one (A) and 10 (B) month drought. Water (●); actinomycin-D (Í); cycloheximide (▲).

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These data indicated that mRNA necessary for the recovery of PS II was more abundant, or less degraded, in one month dry explants than 10 month dry explants. This suggests that mRNA degraded or lost stability over time and that following 10 months of dry storage mRNA that remained was insufficient for PS II recovery. Although Figure 5.1 suggests that 10 month dried *X. humilis* explants are capable of *de novo* transcription and translation, there is no evidence that suggests that newly synthesised transcripts and proteins are utilised for the recovery of chlorophyll and PS II. On the contrary, F_V/F_M data above suggest that *de novo* mRNA and proteins were probably not used in the recovery of photochemical machinery as water rehydrated explants (control) were incapable of recovering a fully functional PS II. However, intact *X. humilis* plants did regain a fully functional photochemistry, as described in

Chapter 3. This suggests that the roots of this species could play a significant role in rehydration following a prolonged drought, either through stimulating the recovery of photosynthetic machinery with plant growth regulators such as cytokinins, or through storing mRNA necessary for PS II recovery upon rehydration, or both.

5.3.2.2 Recovery of pigments in *C. wilmsii* and *X. humilis* leaf explants

It must be noted that the control refers to the pigment levels in intact plants, while this series of results was conducted on explants. Perhaps anomalies could be explained by the absence of roots. During dry storage *C. wilmsii* plants lost chlorophyll (a+b) during the first month, but levels remained unchanged thereafter (Chapter 3). After one and two months of dry storage intact *C. wilmsii* plants had approximately 3.5 $mg.g^{-1}$DW chlorophyll (a+b) in the air-dry state (Figure 5.4A). Explants rehydrated in water were able to recover chlorophyll (a+b) to above that of the control within 24 hours, indicating that the recovery of chlorophyll was not compromised by the prolonged dry storage. Explants treated with distamycin-A and cycloheximide showed that the inhibitors affected the recovery of chlorophyll (a+b) in some way. By 72 hours chlorophyll levels in water and the transcription inhibitor showed similar chlorophyll levels, and by 144 hours chlorophyll (a+b) levels among the three treatments were comparable, supporting previous suggestions (Copper and Farrant, 2002) that *C. wilmsii* accumulate enough protection during drying necessary for the ability to regain full chlorophyll function on rehydration, as neither of the inhibitors prevented chlorophyll recovery over 144 hours of rehydration. Figure 5.2A suggests that although chlorophyll (a+b) levels in the inhibitors were initially low, PS II was fully functional supporting suggestions from Chapter 3 that *C. wilmsii* plants do not require high chlorophyll levels for optimal PS II functioning.

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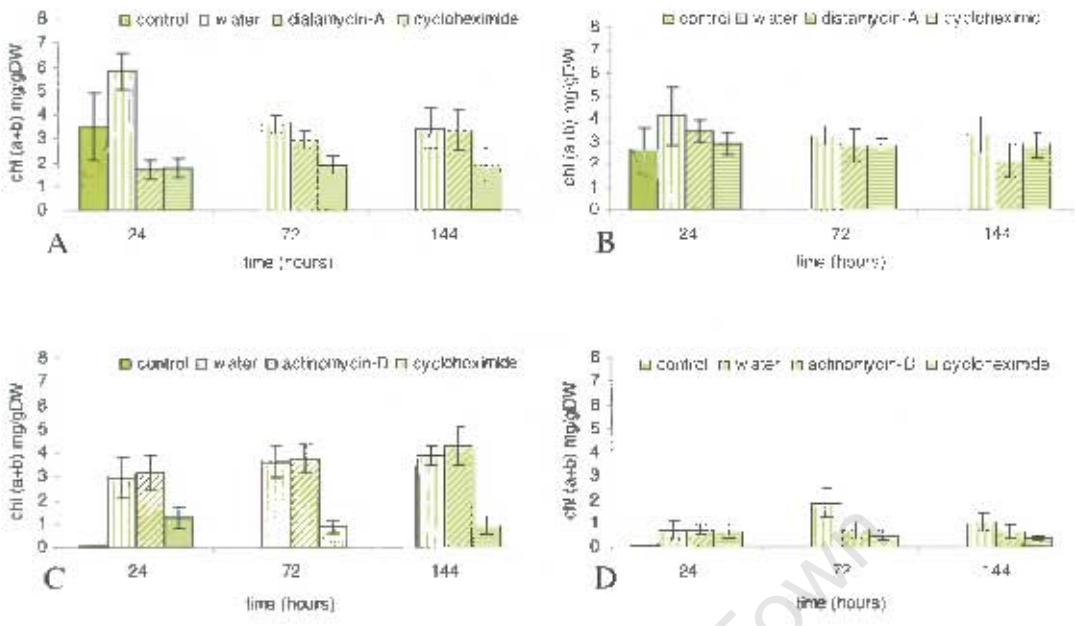

Figure 5.4: Chlorophyll (chl) (a+b) content in leaf explants rehydrated in water, transcription inhibitor (distamycin-A for *C. wilmsii* and actinomycin-D for *X. humilis*) and translation inhibitor (cycloheximide) of one month dry *C. wilmsii* plants (A), two month dry *C. wilmsii* plants (B); one month dry *X. humilis* plants (C) and 10 month dry *X. humilis* plants (D), after 24, 72 and 144 hours of rehydration. Control refers to chlorophyll (a+b) levels measured in intact plants (in the air-dry state) following that drought period under the same conditions. Full squares (control); vertical lines (water); diagonal lines (distamycin-A for *C. wilmsii* and actinomycin-D for *X. humilis*); horizontal lines (cycloheximide).

After two months in dry storage, *C. wilmsii* explants regained approximately 3 $mg.g^{-1}$ DW chlorophyll (a+b) within the first 24 hours of rehydration under all treatments, and maintained these levels for six days (Figure 5.4B). These levels were similar to those of the control indicating that within 144 hours of rehydration no extra chlorophyll was made by two month dried explants.

As previously showed by Dace *et al.* (1998) *X. humilis* excised leaves rehydrated in water and actinomycin-D recovered similar chlorophyll (a+b) levels, but cycloheximide rehydration prevented chlorophyll recovery. Similar results were observed in this study for *X. humilis* leaf explants rehydrated after one month of dry storage (Figure 5.4 C). However, plants that were maintained dry for 10 months

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showed a slightly higher chlorophyll (a+b) recovery in plants rehydrated in water than in the inhibitors (Figure 5.4 D). Still, these chlorophyll (a+b) levels were significantly lower than those measured in plants that were kept dry for one month (also reflected in Figure 5.3, where PS II did not show signs of recovery in 10 month dried explants). This suggests that the stability of stored mRNA was compromised between one and 10 months of dry storage. Thus, mRNA that was present in one month dry plants was used for the recovery of PS II, but that those mRNA levels decreased during the prolonged dry storage and were insufficient for chlorophyll (a+b) and PS II recovery. Figure 5.1 also suggests that while *de novo* translation in 10 month dried explants was high, the newly synthesised proteins were not utilised for the recovery of PS II, as water rehydrated explants did not recover optimal F_V/F_M.

Dace *et al.* (1998) showed that *X. humilis* leaf explants rehydrated in both water and the transcription inhibitor reached chlorophyll (a+b) levels of approximately 4 $mg.g^{-1}$ DW at full turgour. Similar results were observed in this study and follow the hypothesis proposed in Chapter 3. *X. humilis* plants seem to require a minimum of approximately 4 $mg.g^{-1}$ DW chlorophyll (a+b) in order to attain a fully functional PS II. Figures 5.4 C and D suggest that although chlorophyll levels reached the 4 $mg.g^{-1}$ DW in one month dried leaf explants rehydrated in water and the transcription inhibitor, these chlorophyll (a+b) levels were not attained in one month dried explants rehydrated in the translation inhibitor, nor in any of the explants rehydrated following the 10 month drought.

Carotenoid (x+c) levels in one and two month dried *C. wilmsii* leaf explants rehydrated in water and the inhibitors were alike (Figure 5.5A). Also, these carotenoids (x+c) levels were comparable to those of the control. During 144 hours of

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rehydration, carotenoid (x+c) levels did not change drastically in explants rehydrated in either inhibitor or in water. Clearly, this pigment was not affected by drying, nor was *de novo* synthesis required for recovery up to 144 hours.

Carotenoid data from *X. humilis* explants from one month dry plants followed similar trends as the chlorophyll data. Explants rehydrated in water increased carotenoid levels until 72 hours of rehydration, but these levels declined thereafter (Figure 5.5 C). Carotenoid (x+c) levels in explants rehydrated in the transcription inhibitor remained unchanged, but those rehydrated in the translation inhibitor decreased over the six days of rehydration. Even though water rehydrated explants lost some carotenoids in the latter three days of rehydration, these levels still matched those of the transcription inhibitor at 144 hours (Figure 5.5 C). Carotenoid (x+c) levels were significantly higher than the control, indicating that despite the inhibition of transcription and translation, explants were able to recover at least some carotenoids. This suggests that no *de novo* transcription and translation were necessary for the recovery of these pigments. Although proteins necessary for the synthesis of carotenoids were present in dry tissues, as indicated by the ability of explants to recover carotenoids levels higher than the control in the translation inhibitor, these protein levels declined over the six day rehydration, suggesting the need for carotenoid turnover.

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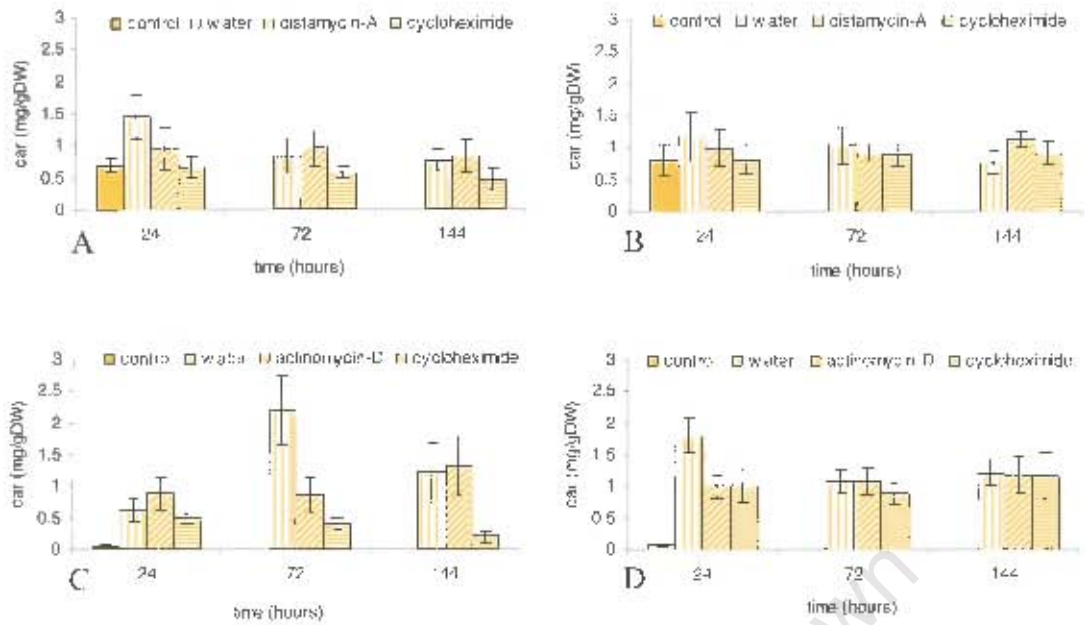

Figure 5.5: Carotenoid (car) (x+c) content in leaf explants of one month dry *C. wilmsii* plants (A), two month dry *C. wilmsii* plants (B); one month dry *X. humilis* plants (C) and ten month dry *X. humilis* plants (D), after 24, 72 and 144 hours of rehydration. Control refers to carotenoid (x+c) levels measured in intact plants (in the air-dry state) following that drought period under the same conditions. Full squares (control); vertical lines (water); diagonal lines (distamycin-A for *C. wilmsii* and actinomycin-D for *X. humilis*); horizontal lines (cycloheximide).

Ten month dry *X. humilis* leaf explants had similar carotenoid (x+c) levels among treatments, except that following the 24 hour water rehydration explants had higher carotenoid (x+c) levels than those measured in other treatments (Figure 5.5 D). However, water rehydrated explants lost some carotenoids during the remainder of the rehydration, showing similar levels to those measured in other treatments after 72 and 144 hours (Figure 5.5 D). This suggests that components required for the recovery of carotenoids were sufficiently protected and stable during 10 months of dry storage. Also, the inability of translation inhibitor rehydrated explants to reduce carotenoid levels over six days (similar to the one month cycloheximide rehydrated explants) suggest damage to enzymes needed for carotenoid turnover.

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Anthocyanin levels of one month dry *C. wilmsii* explants were greater in leaves rehydrated in the transcription and translation inhibitors, than in those rehydrated in water (Figure 5.6 A). Similar trends were observed in leaves of the two month dry explants, however the anthocyanin levels in explants rehydrated in the inhibitors were initially significantly lower than those in one month dried plants (Figure 5.6 B).

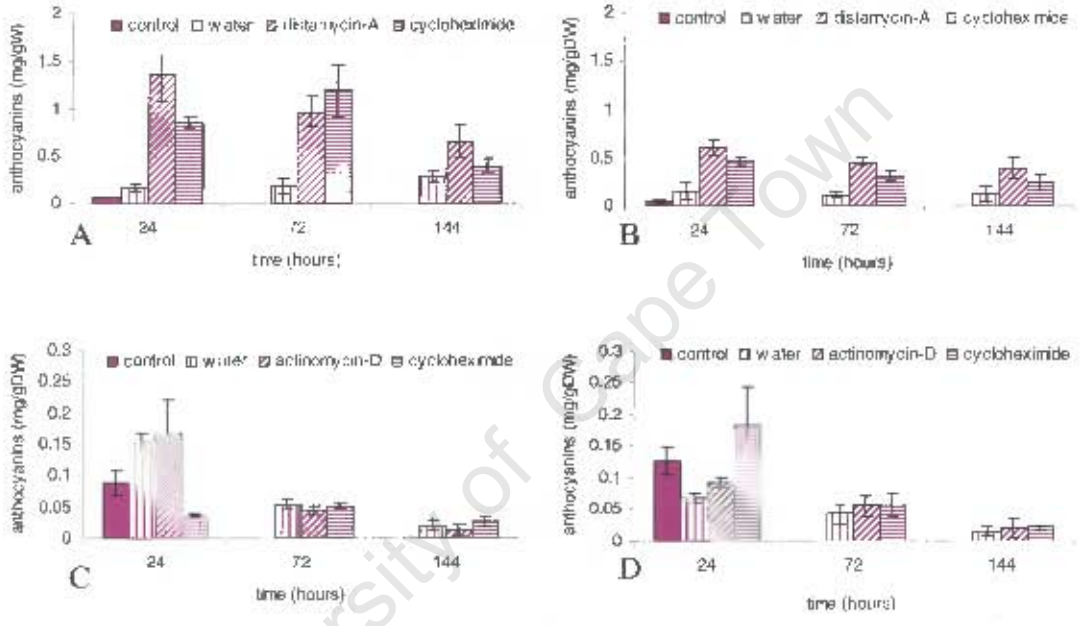

Figure 5.6: Anthocyanin content in leaf explants of one month dry *C. wilmsii* plants (A), two month dry *C. wilmsii* plants (B); one month dry *X. humilis* plants (C) and ten month dry *X. humilis* plants (D), after 24, 72 and 144 hours of rehydration. Note the differences in scale for *C. wilmsii* and *X. humilis* explants. Control refers to anthocyanin levels measured in intact plants (in the air-dry state) following that drought period under the same conditions. Full squares (control); vertical lines (water); diagonal lines (distamycin-A for *C. wilmsii* and actinomycin-D for *X. humilis*); horizontal lines (cycloheximide).

The high anthocyanin levels indicate some photo-oxidative stress associated with the rehydration of explants in the inhibitors. Anthocyanins are known to play a significant role as antioxidants (Neill *et al.*, 2002) and were present in high amounts possibly due to ROS attack. *C. wilmsii* plants require high anthocyanin levels during partial rehydration when chlorophyll is still exposed to light, and when relative water content

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is high enough for ROS formation, but not for antioxidant upregulation. It is possible that transcription and translation inhibitors might have prevented the breakdown of anthocyanins, hence the lower amounts were observed in the water rehydrated explants. This also indicates that enzymes necessary for anthocyanin biosynthesis were accumulated prior to dehydration and were better protected or less damaged in tissues of one month dried explants than two month dried explants. However, when intact *C. wilmsii* plants were rehydrated after one and two months of dry storage (WGH conditions), they were capable of reducing anthocyanin levels to below that of the control (Chapter 3), but this was not observed in water rehydrated explants. Perhaps the higher anthocyanin levels in explants were a consequence of the absence of roots.

Anthocyanin levels in *X. humilis* leaf explants were significantly lower that those observed in *C. wilmsii* (note the differences in scale, Figure 5.6). In one month dried explants, within the first 24 hours of rehydration, water and transcription inhibitor explants behaved similarly, both showing significantly higher anthocyanin levels than the explants rehydrated in the translation inhibitor, and the control (Figure 5.6 C). But, by 72 hours, explants from all three treatments had similar pigment levels, which had decreased further by 144 hours under all treatments (Figure 5.6 C), and were lower than the control. Neither transcription nor translation were needed to break down anthocyanins during rehydration in the long term. The rehydration of 10 month dry explants showed somewhat similar trends to that of the one month dry plants in that by 72 hours anthocyanin levels decreased below that of the control (Figure 5.6 D). Although water and transcription inhibitor rehydrated explants still behaved similarly, their anthocyanin levels were significantly lower than that of the translation

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inhibitor rehydrated explants (Figure 5.5 D) and the control. This suggests that anthocyanins were utilised or broken down during early rehydration (first 12 hours). Enzymes necessary for the breakdown of anthocyanins were not compromised while the plants were dry and were available even when transcription and translation were inhibited, suggesting that they were stored in dry tissues.

5.3.2.3 Subcellular recovery in *C. wilmsii* and *X. humilis* leaf explants

Figures 5.7 to 5.10 depict the subcellular organisation of *C. wilmsii* and *X. humilis* leaf explants following a prolonged maintenance in the dry state. The effect of rehydration in transcription and translation inhibitors on the cellular organisation was followed from 24 through to 144 hours for both species. Samples rehydrated in water were compared to those rehydrated in the inhibitors. From the F_V/F_M data above (Figure 5.2), it was expected that *C. wilmsii* leaf explants would be able to fully recover chloroplast function in both one and two month dried plants. *X. humilis* F_V/F_M data suggests that one month dry leaf explants would be able to recover chloroplast function when rehydrated in water and transcription inhibitor, but not in the translation inhibitor, while leaf explants from 10 month dry plants would not regain chloroplast function under any treatment.

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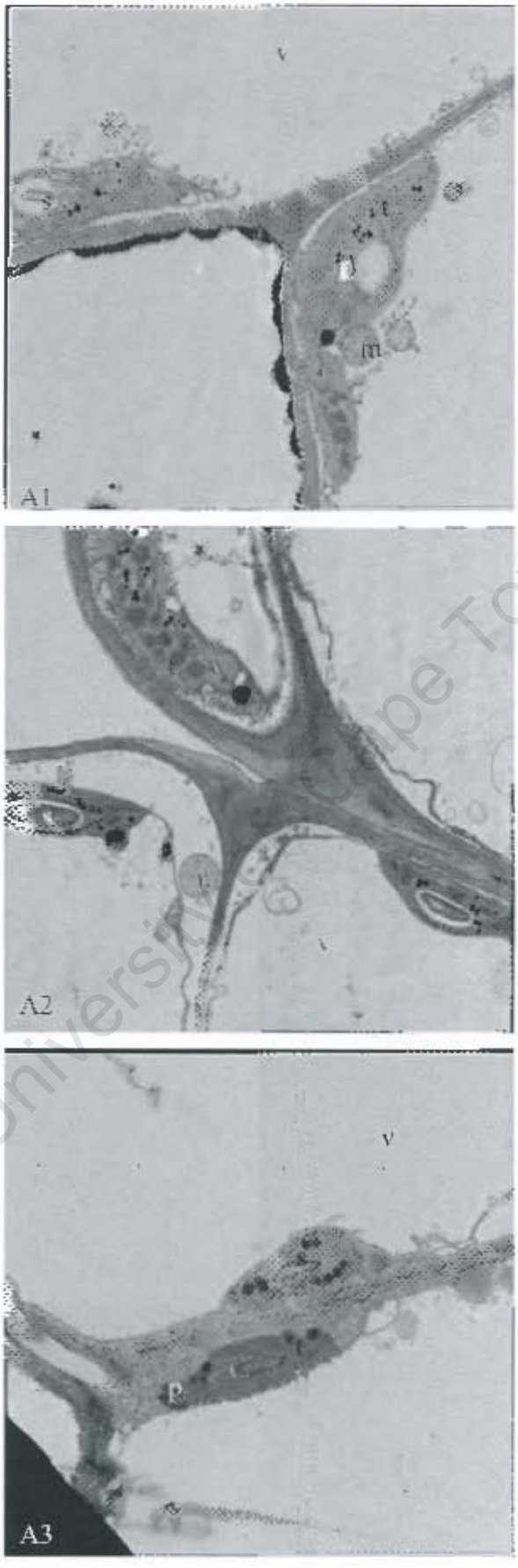
One month dry *C. wilmsii* leaf explants rehydrated in water and in the inhibitors showed well developed chloroplasts after the 24 hour rehydration and the structure appeared maintained after 72 and 144 hours (Figure 5.7). Both starch and few plastoglobuli were present in chloroplasts. Thylakoid membrane stacks were evident and in conjunction with F_V/F_M data (Figure 5.2A) were presumably functional.



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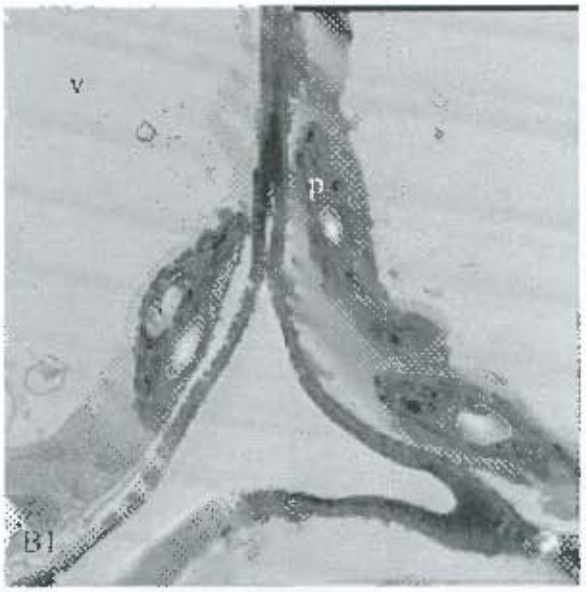

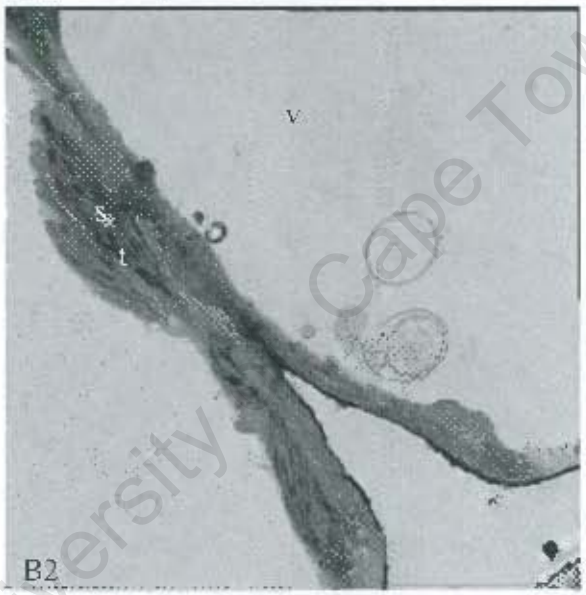

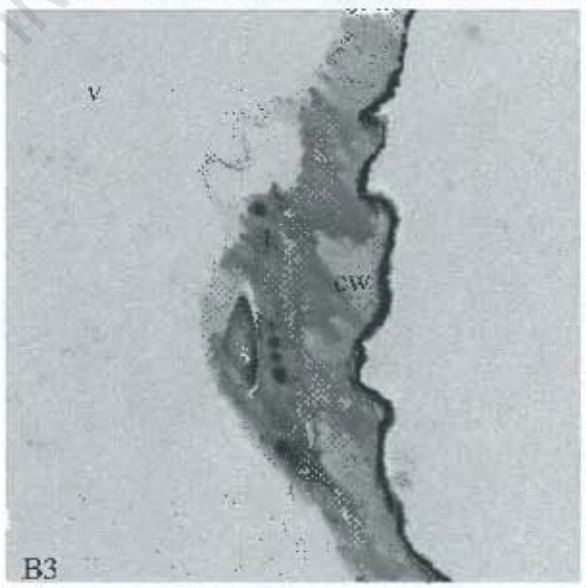

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Figure 5.7: One month dry *C. wilmsii* leaf explants rehydrated in water (A), actinomycin-D (B) and cycloheximide (C). Images taken following 24 (1), 72 (2) and 144 (3) hours of rehydration. cw: cell

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wall; s: starch; t: thylakoid membrane stacks; p: plastoglobuli; v: vacuole; m - mitochondrion. All micrographs are at magnification of x4000.

After the 24 hour rehydration of two month dry *C. wilmsii* explants in all three treatments, some overall subcellular damage was observed (Figure 5.8A). There was evidence of cytoplasmic debris in the vacuoles, suggesting some cytolysis has occurred. Under all treatments chloroplasts were typical of hydrated plants in that thylakoid membranes were regularly stacked, and appeared functional, and there was evidence of starch. Although the explants were able to rehydrate completely by this stage, repair of damage was complete by 72 hours of rehydration (Figure 5.8B), as suggested by the absence of debris observed in vacuoles at 24 hours. This is also supported by the delay in PS II recovery as shown in Figure 5.2B. The subcellular integrity remained similar at 144 hours. Transcription and translation inhibitors did not hinder the recovery of chloroplasts, suggesting that *C. wilmsii* plants do not require *de novo* transcription and translation for chloroplast recovery during the first 144 hours of rehydration. Figure 5.1 suggested that *de novo* transcription and translation was delayed in two month dried explants, as evident in Figure 5.8 where full subcellular reconstitution was observed by 72 hours of rehydration in water rehydrated explants, unlike that of one month dried explants that showed recovery by 24 hours.

It has been reported that plastoglobuli are composed of α-tocopherol, triacylglycerols and plastoquinone (Tevini and Steinmüller, 1985). α-Tocopherol is a lipid-soluble antioxidants and is perhaps required in large amounts in rehydrating explants for protection. Triacylglycerols may indicate that thylakoids have not fully recovered, perhaps an indication of damage during dry storage, as they were more abundant in two month dried explants.

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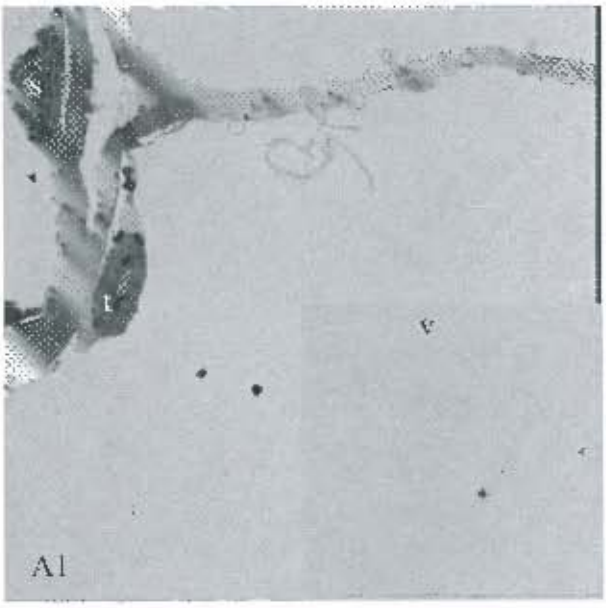

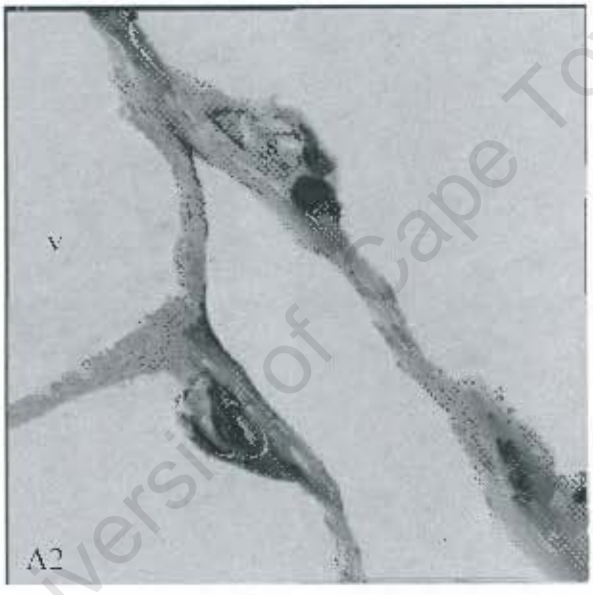

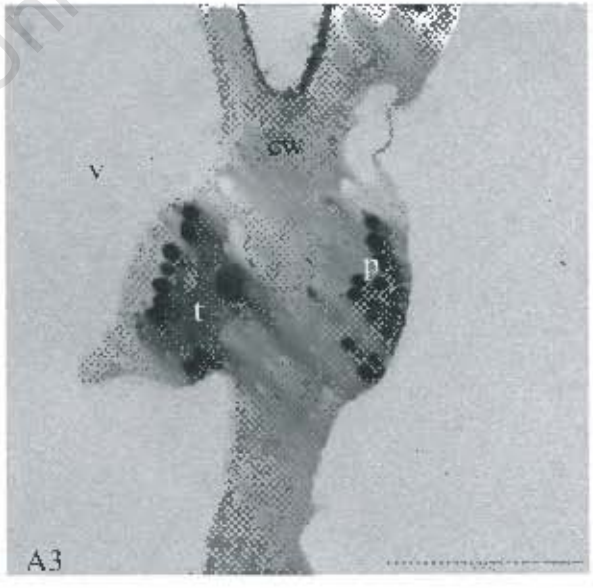

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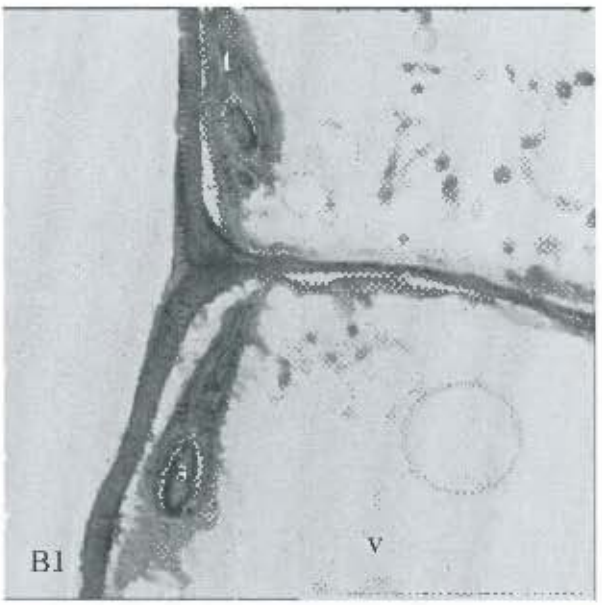

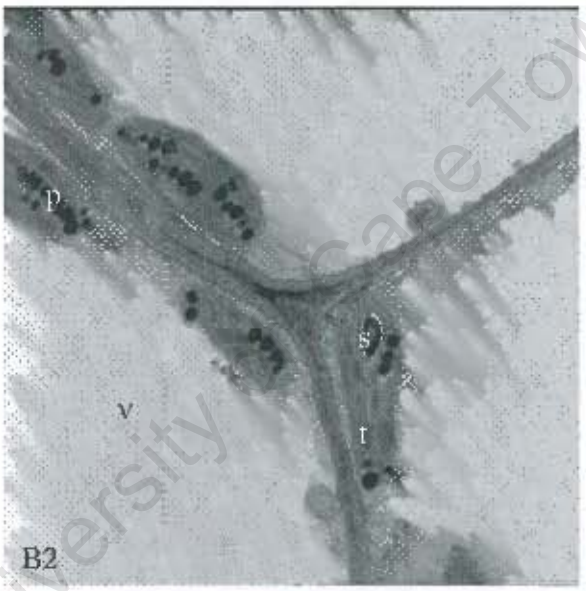

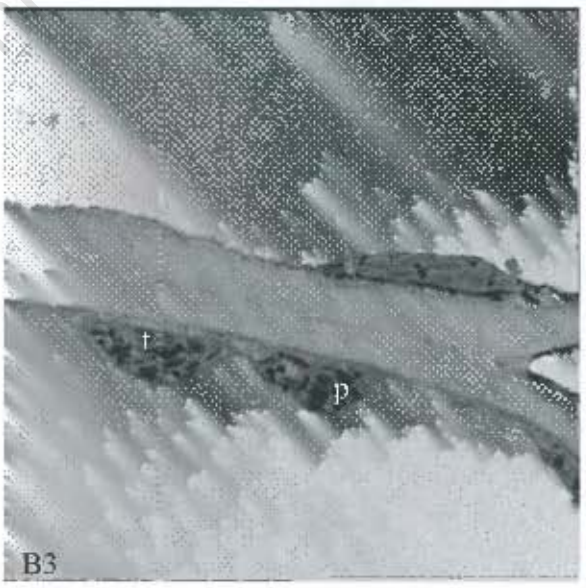

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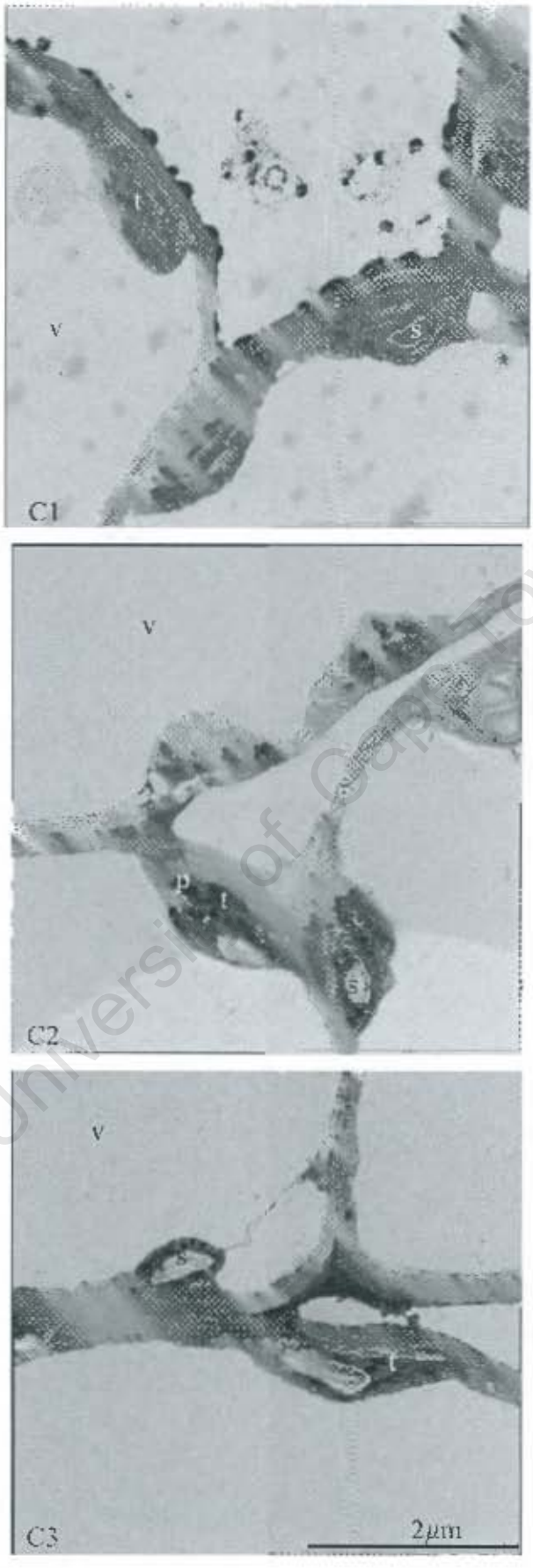

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Figure 5.8: Two month dry *C. wilmsii* leaf explants rehydrated in water (A), actinomycin-D (B) and cycloheximide (C). Images taken following 24 (1), 72 (2) and 144 (3) hours of rehydration. cw: cell wall; s: starch; t: thylakoid membrane stacks; p: plastoglobuli; v: vacuole. All micrographs are at magnification of x4000.

One month dry *X. humilis* leaf explants rehydrated in water showed fully reconstituted chloroplasts with reassembled thylakoid membrane stacks and starch after 24 hours (Figure 5.9 A1). This recovery was maintained at 72 and 144 hours. Leaf explants rehydrated in the transcription inhibitor showed delayed, but none-the-less recovered subcellular organisation (Figure 5.9B). Although mRNA necessary for the reconstitution of chloroplasts was stored and was sufficient for initial recovery, *X. humilis* explants seem to benefit from extra transcription observed in water rehydrated explants. By 24 hours, starch was observed, indicating some recovered starch metabolism. By 72 and 144 hours, some deterioration in chloroplast structure was evident, although other subcellular organisation was observed, indicating that explants were somewhat capable of maintaining recovery. It appears that recovery of chloroplasts is possible from stored mRNA, but that *de novo* transcription is needed for a fully maintained function (as indicated by the slow deterioration of chloroplast structure in the transcription inhibitor). As expected, chloroplasts in leaf explants rehydrated in the translation inhibitor showed no signs of recovery (Figure 5.9 C), other than starch accumulation, indicating that enzymes for starch synthesis were accumulated prior to dry storage. These data support the F_V/F_M results (Figure 5.3A) that showed that PS II did not recover in the translation inhibitor. Translation is clearly necessary for the reconstitution of functional chloroplasts as indicated by the water rehydrated explants (Figure 5.9 A).

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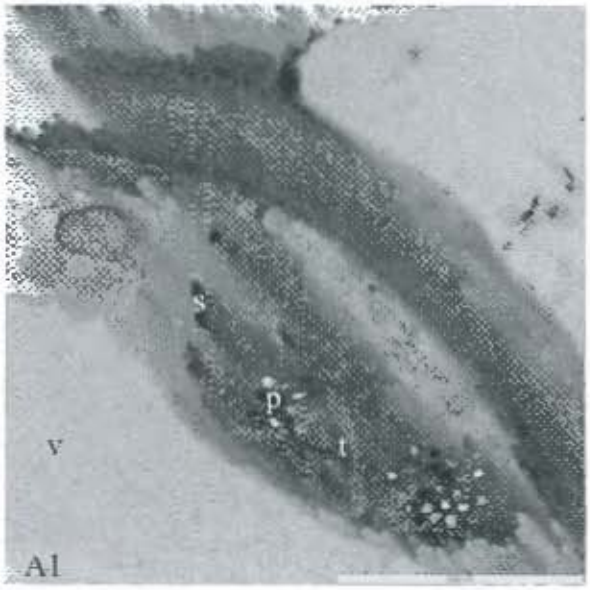

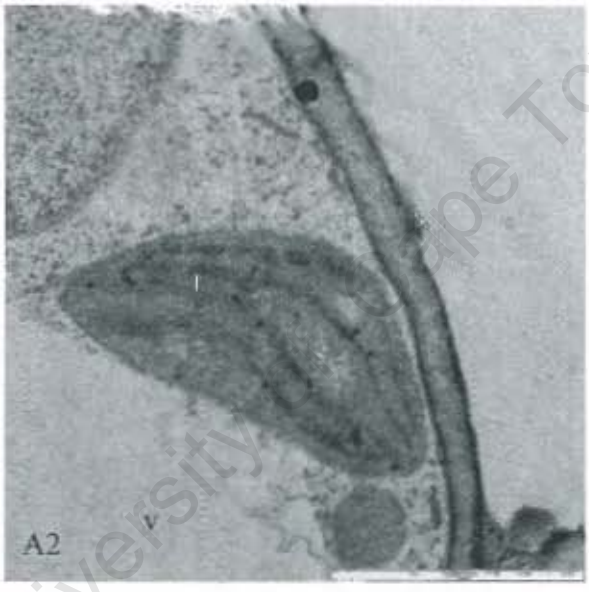

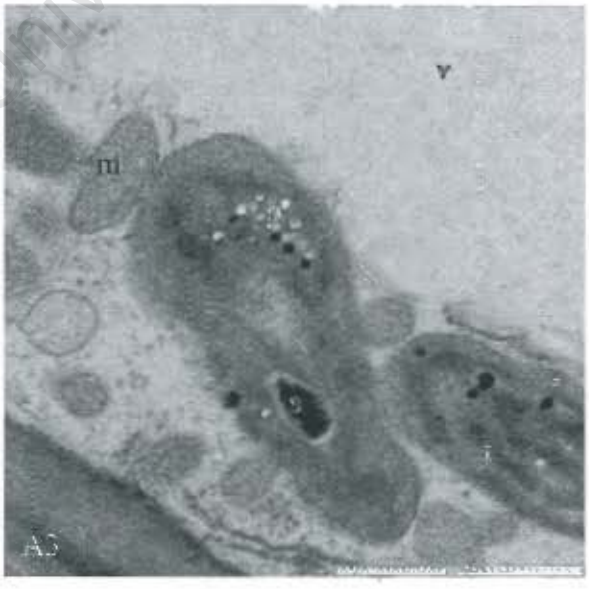

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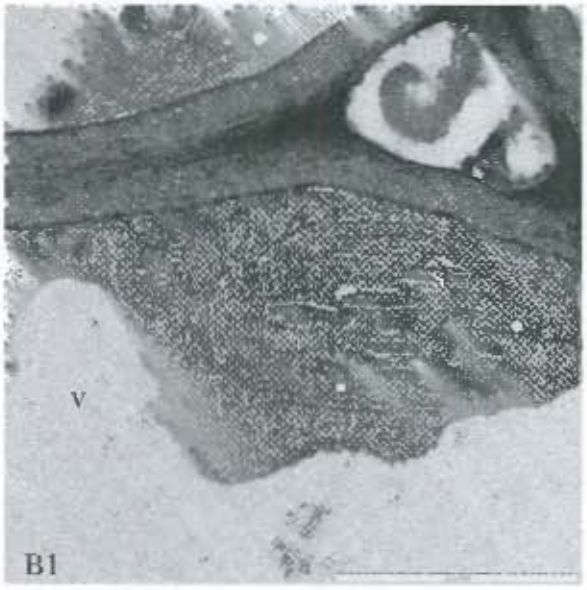

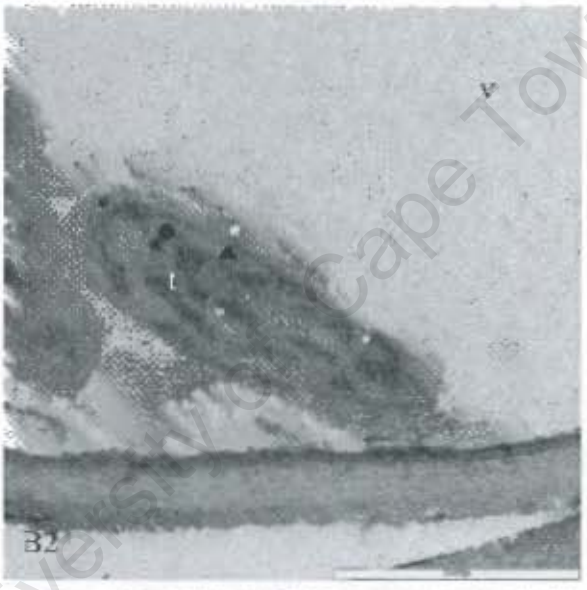

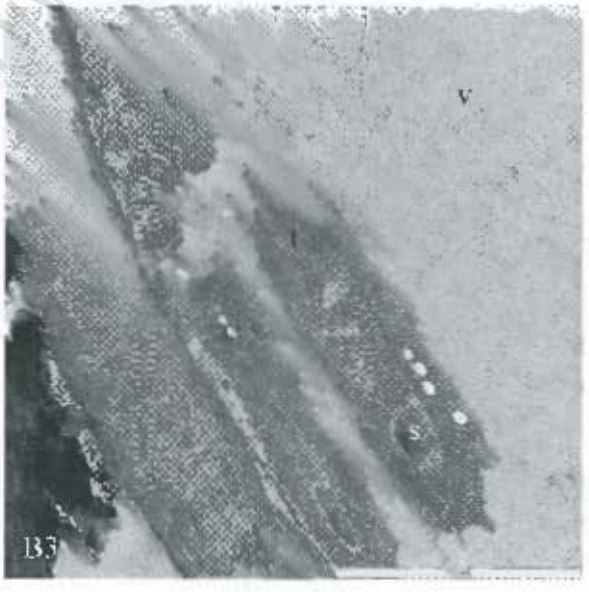

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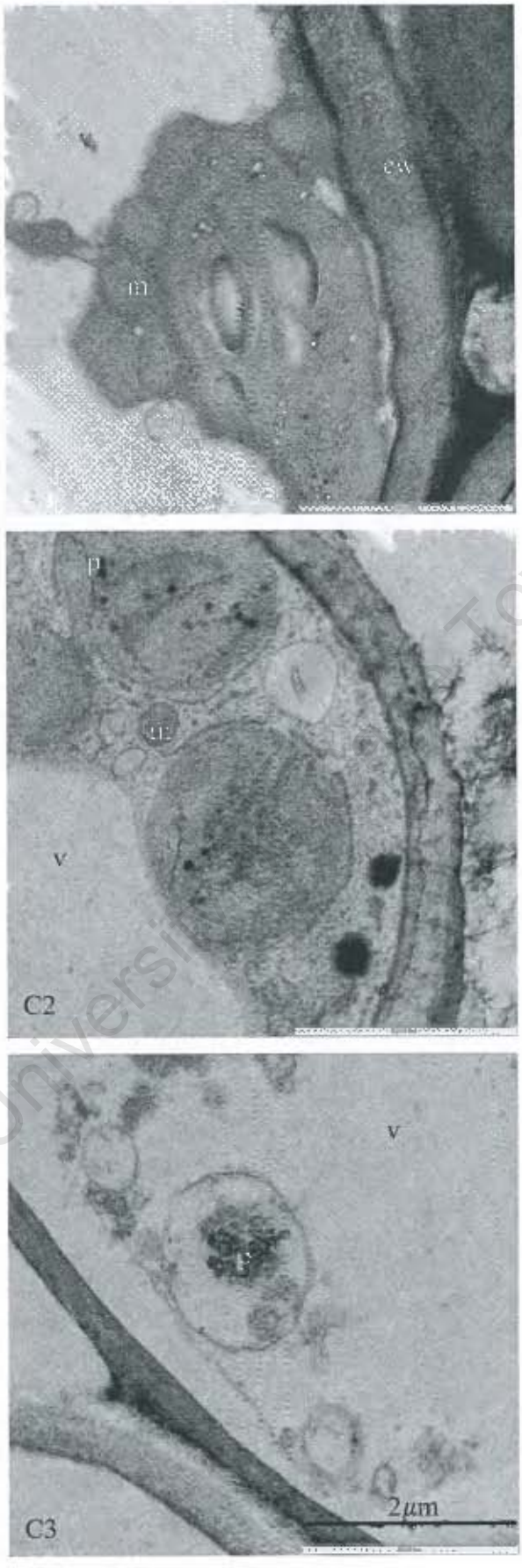

Figure 5.9: One month dry *X. humilis* leaf explants rehydrated in water (A), actinomycin-D (B) and cycloheximide (C). Images taken following 24 (1), 72 (2) and 144 (3) hours of rehydration. cw: cell

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wall; s: starch; t: thylakoid membrane stacks; p: plastoglobuli; v: vacuole; m: mitochondrion. All micrographs at magnification of x5000.

After the 24 hour rehydration of leaf explants from 10 month dry *X. humilis* plants, the subcellular disorganisation under all three treatments is depicted in Figure 5.10. In water rehydrated explants chloroplasts partially regained their hydrated structure in that starch was visible, but no thylakoid membrane stacks were observed. Also, the vacuole was not reconstituted as numerous smaller vacuoles were observed. After 72 hours of rehydration in water, although the vacuole was reconstituted, chloroplasts appeared more damaged. Starch was metabolised and chloroplasts appeared rounded with poorly developed thylakoids (no stacking observed). By 144 hours severe plasmolysis was observed. Figure 5.1 suggested that 10 month dried *X. humilis* explants were capable of *de novo* transcription and translation, but water rehydrated explants showed little evidence of recovery. Although *de novo* transcription and translation were measured for only the first 48 hours of rehydration, when significant protein synthesis was observed, water rehydrated explants in Figure 5.10 below, suggest that initial protein synthesis might have contributed to the reconstitution of chloroplasts. However, after 72 hours of rehydration in water it appears that either translation was compromised, or that damage was so severe that the rate of protein synthesis could not ensure recovery. This was indicated by the deterioration of the chloroplast structure, and the general subcellular organisation.

Explants rehydrated in the transcription inhibitor for 24 hours showed incomplete subcellular reconstitution evident by two large vacuoles that appeared to contain debris (Figure 5.10B). Chloroplasts did not recover as suggested by the absence of starch and thylakoid membrane stacks. Similar results were observed after 72 hours of rehydration, but chloroplast boundary membrane appeared damaged by this time.

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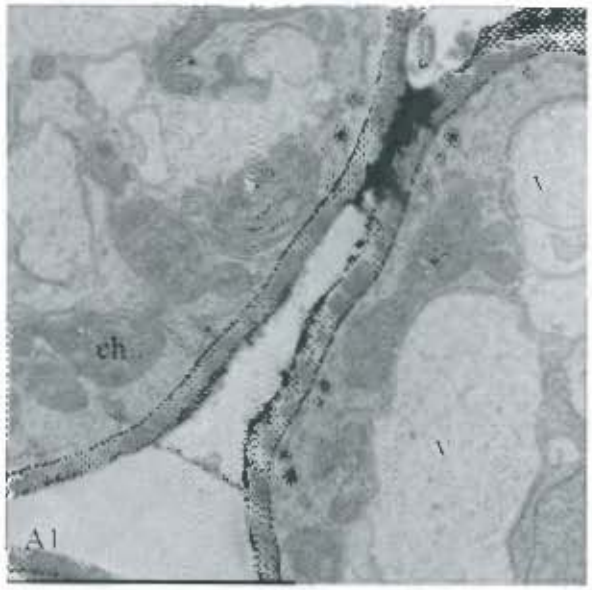

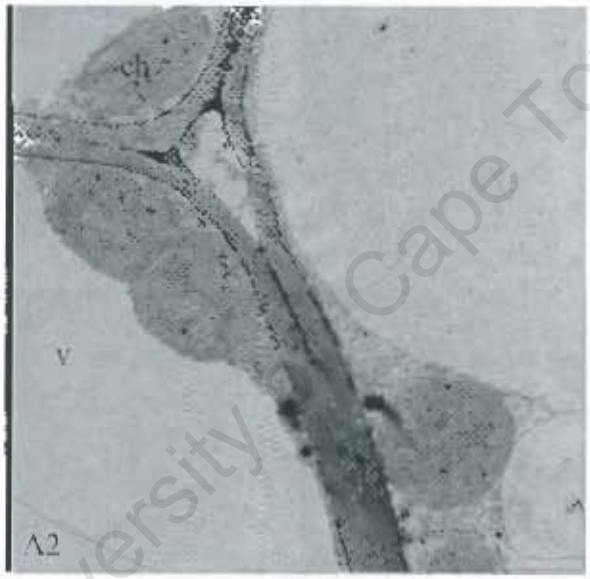

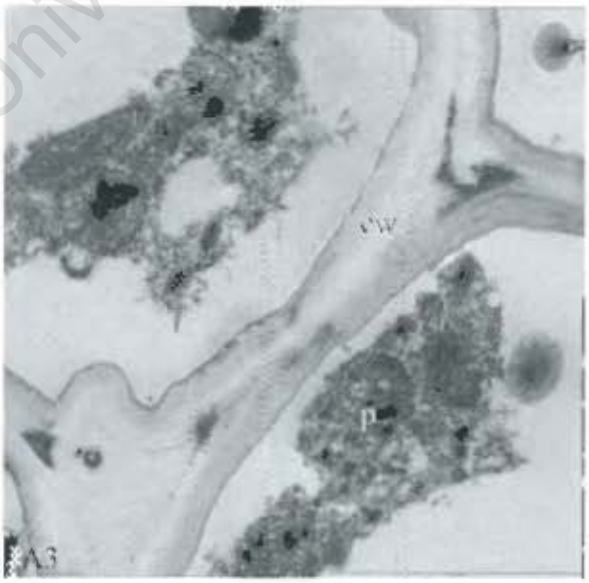

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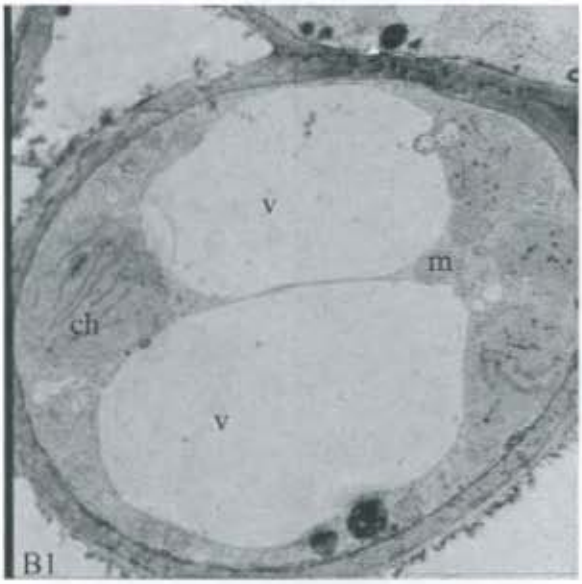

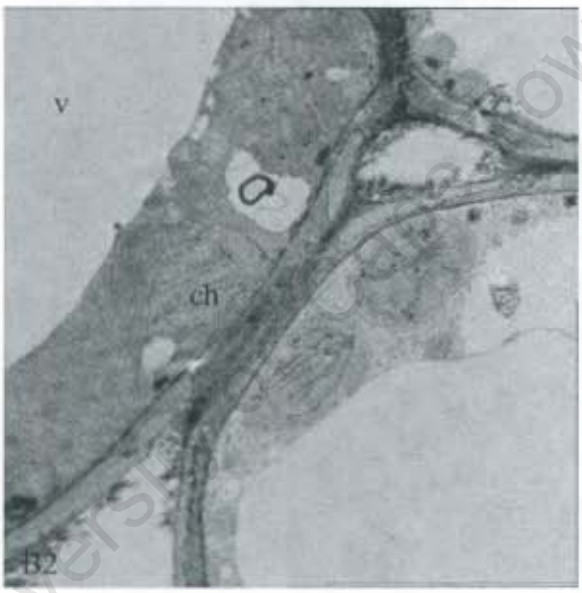

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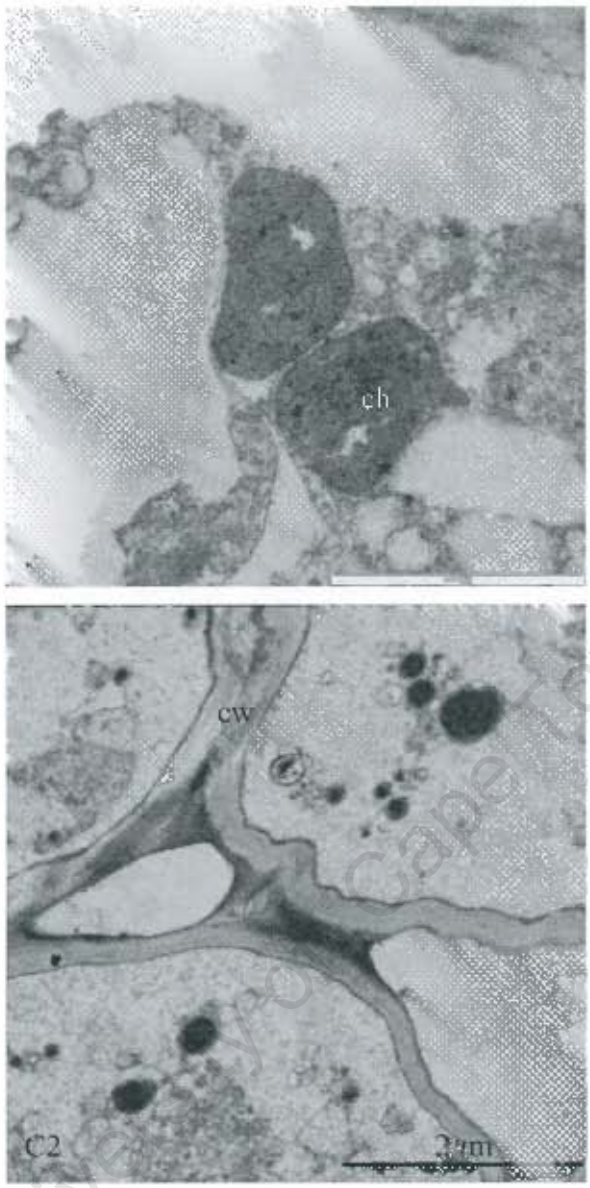

Figure 5.10: 10 month dry *X. humilis* leaf explants rehydrated in water (A), actinomycin-D (B) and cycloheximide (C). Images taken following 24 (1), 72 (2) and 144 (3) hours of rehydration. Samples rehydrated in cycloheximide had partially disintegrated by 72 and 144 hours, thus no ultrastructural images are shown. cw: cell wall; ch: chloroplast; p: plastoglobuli; v: vacuole; m - mitochondrion. All micrographs are at magnification of x4000.

Explants rehydrated in the translation inhibitor showed no signs of recovery. The vacuole was not reconstituted and chloroplasts lacked both starch and thylakoid membrane stacks. Severe plasmolysis was observed by 72 hours of rehydration, where no subcellular compartmentalisation was evident. These data provide visual

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evidence supporting F_V/F_M results (Figure 5.3B), which indicated that PS II function could not recover in leaf explants of 10 month dried *X. humilis* under any treatment.

As mentioned earlier, 10 month dry *X. humilis* intact plants were capable of full photosynthetic recovery (also see Chapter 3). It appears that without roots this species is incapable of reconstituting chloroplasts, and thus PS II, after prolonged dry storage. *X. humilis* plants are capable of accumulating and storing mRNA necessary for the recovery of photosynthetic machinery in the leaf tissues (Figure 5.3A and Figure 5.9), but this mRNA is stable in dry tissues for a period less than 10 months. Perhaps this species has two possible recovery strategies. Firstly, if mRNA stored in leaves is not damaged by prolonged dry storage, *X. humilis* plants are able to repair the photosynthetic machinery through the translation of this mRNA. Secondly, if stored mRNA is damaged, the roots provide some sort of signal (whether in the form of plant growth regulators, or otherwise) that then enables the plants to repair and recover a fully functional photochemistry.

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5.4 Conclusion

As previously reported by Cooper and Farrant (2002) on fast and slow dried *C. wilmsii* plants, this species is capable of synthesising and accumulating all components necessary for complete protection and a successful restoration of the full metabolism during the initial rehydration, while drying. And, in addition, *C. wilmsii* plants are capable of protecting these accumulated components during a prolonged period in the dry state, for at least two months, as this chapter, and the study in general, has shown. This ensured fast recovery upon rehydration without need for *de novo* transcription nor translation during the initial stages of rehydration.

X. humilis plants accumulated mRNA needed for the reconstitution of at least the photosynthetic machinery during dehydration (Dace *et al.*, 1998). Cooper (2001) has shown that this accumulation was dependent on the speed of drying, which indicated that *X. humilis* relies somewhat on repair upon rehydration. This study has shown that although some mRNA is accumulated during dehydration, its protection and stability in the dry state becomes compromised the longer the plants remain dry. The photochemistry of one month dry leaf explants was able to recover fully in water and the transcription inhibitor, but in 10 month dried leaf explants the photosynthetic machinery never recovered under any rehydration treatment, indicating that the stored mRNA was damaged or present in insufficient amounts. After such long storage in the dry state these plants were still capable of *de novo* transcription and translation for at least 48 hours during rehydration, suggesting that the genome had not suffered irreparable damage. However, F_V/F_M and chlorophyll (a+b) data of water rehydrated explants suggest that PS II could not recover in 10 month dried explants, thus new mRNA and proteins were utilised elsewhere. But, as shown in Chapter 3, 10 month

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dried intact plants were capable of complete recovery of photochemistry signifying the importance the roots play in rehydration of this species.

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CHAPTER SIX

General Conclusions

Maintaining *C. wilmsii* and *X. humilis* plants under different conditions confirmed that the time taken to reach the air dry state followed that of the soil water loss, as previously suggested (Farrant *et al.*, 1999; Farrant, 2000). Climatic field data suggested that *C. wilmsii* plants experience dry spells in winter and do not generally experience prolonged drought for longer than one month, but a two month drought period has occurred at least once in the past decade (Chapter 2, Table 2.1A). Survival of *C. wilmsii* plants under AA and SD conditions (i.e. those in which natural drying for long periods would not normally occur) was compromised after one and two months, respectively. Although the two month dry storage under SD conditions matched the maximum time this species experiences drought in the field, those plants that were kept dry under WD conditions (high light, low temperature) managed to remain viable dry for three months. Longer storage in the dry state led to a total loss of stability under even the most benign of environmental conditions. This suggests that the protection this species up-regulated during drying was sufficiently robust to protect the plants for only up to three months, but less during unfavourable conditions.

In the field *X. humilis* plants usually experience drought no longer than six months (Chapter 2, Table 2.1B). Under simulated field conditions survival in dry storage in this species was tested for up to 10 months, and the plants survived this dry period under all conditions. This suggests that *X. humilis* plants were either sufficiently protected to remain viable while dry for a longer period of time regardless of

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environmental conditions, or are capable of tolerating greater damage as they rely, somewhat, on repair during rehydration (Dace *et al.*, 1998), or both.

Sucrose is considered to be the universal protectant in dry resurrection plants (Pammenter and Berjak, 1999; Farrant, 2000) as it assists *inter alia* in glass formation (Franks, 1982; Horbowicz and Obendorf, 1994) which facilitates longevity in the dry state (Leopold and Vertucci, 1986; Leopold *et al.*, 1994; Walters *et al.*, 2005). Both species, under all conditions, accumulated sufficient sucrose to ensure survival in the dry state, and there was no indication that any loss of sucrose occurred during dry storage in either species. Although *C. wilmsii* plants accumulated higher sucrose levels under harsh AA conditions, probably due to high light under which it dried, this high sucrose level did not protect them sufficiently to survive dry storage for longer than one month. However, under AA conditions higher RWC was observed in the air-dry state. This could have accounted for the faster loss of viability while the plants were dry. Localised increases in water content can cause increased intermediate metabolism (Vertucci and Farrant, 1995; Walters *et al.*, 2001; 2005) which is sufficient to allow for elevated ROS activity, but not elevated repair (such as antioxidants) to enable accrual of damage and ultimate loss of viability. Although we cannot say that the glasses were maintained (since no measure of glass formation was done here) and while sucrose was still present in *C. wilmsii* plants, the glasses could still have been breaking down due to instability in other factors which stabilise glasses such as the presence of proteins (Wolkers *et al.*, 2001) and oligosaccharides (Caffrey *et al.*, 1988; Koster and Leopold, 1988; Koster, 1991; Leopold *et al.*, 1994).

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Both species were capable of shutting down photosynthesis (F_V/F_M data) in a controlled manner as previously reported (Sherwin and Farrant, 1998; Farrant, 2000; Farrant *et al.*, 2003). Also, the respective strategies, homoiochlorophylly and poikilochlorophylly, were able to operate successfully during drying in all treatments. However, it appears that homoiochlorophylly was less successful for long-term dry conditions. In the longer stored plants breakdown of chlorophyll (a+b) in *C. wilmsii* was observed, and this was coincident with the loss of viability, possibly suggesting ROS damage. We cannot state with certainty that the decline was due to inherent instability of chlorophyll over the long dry storage period, or due to its breakdown due to ROS. Certainly the fact that there was a progressive decline in chlorophyll (a+b) levels suggests inherent instability. This instability appears to be exacerbated under the harsher conditions of WGH and SD. *C. wilmsii* plants were capable of resynthesising lost chlorophyll if they remained viable (two months SD and WGH and three months WD conditions) suggesting ability to retain machinery for repair or resynthesis of chlorophyll (a+b) from *de novo* transcription and translation (Cooper and Farrant, 2002). Breakdown in chlorophyll and anthocyanins (thus protection against light-chlorophyll interaction) correlated with the increased damage to chloroplasts (Chapter 3, Figure 3.3). Work by Farrant *et al.* (2003) suggested that *C. wilmsii* plants are highly sensitive to light stress if leaf movement was restrained during drying. This resulted in the inability of this species to resynthesise the chlorophyll that had broken down indicating severe damage due to light stress. This study has shown that although loss of chlorophyll was observed during dry storage, these plants were capable of recovering optimal PS II functioning (Chapter 5, Figure 5.2) once rehydrated, even when transcription and translation were inhibited. However, inhibitors prevented the recovery of chlorophyll to levels above the control

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(chlorophyll levels in dry tissues; Chapter 5, Figure 5.4 A and B). *C. wilmsii* plants maintain chlorophyll intact while they are dry, thus ensuring that photosynthesis can recover early during rehydration. Although chloroplasts appeared more damaged the longer the plants remained dry, they were able to recover, presumably as a result of stored proteins, even when transcription and translation were inhibited (Chapter 5, Figure 5.8).

Poikilochlorophylly appeared to be more successful for long-term dry storage. *X. humilis* plants lost most chlorophyll (a+b) during dehydration, but accumulated mRNA necessary for chlorophyll and photochemistry recovery upon rehydration, while drying, or prior to dehydration. There was no evidence that suggested that while the plants were dry further loss of chlorophyll occurred (except under SD conditions within the first month of dry storage). Loss of chlorophyll during dehydration ensured minimal photo-oxidative damage while the plants were dry, and hence the amount of anthocyanin accumulated during dying was significantly lower than that measured in *C. wilmsii* plants. However, ROS attack was not completely eliminated, as MDA levels increased during prolonged dry storage (Chapter 4, Figure 4.3). This suggested that metabolism was not entirely suspended in dry plants, and molecular mobility was possible, as suggested by Vertucci and Farrant (1995) and Walter *et al.* (2005). Inhibitor studies have shown that although *X. humilis* plants accumulate transcripts necessary for the reconstitution of photochemistry during dehydration, the stability of these transcripts became compromised the longer the plants remained dry. One month dry *X. humilis* leaf explants were capable of recovering chlorophyll (a+b) levels in water and the transcription inhibitor, while explants from 10 month dried plants were not. This suggests that mRNA levels that were present in one month dried leaves were

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less abundant, or more damaged, in 10 month dried leaves. The machinery necessary for *de novo* transcription and the translation of stored transcripts was not damaged during dry storage, as 10 month dried explants were capable of both *de novo* transcription and translation (Chapter 5, Figure 5.1). However, newly synthesised proteins were not utilised for the recovery of photochemical machinery, as indicated by the inability of water rehydrated 10 month dried explants to regain optimal PS II functioning, recover chlorophyll (a+b), or reassemble thylakoid membrane stacks.

It has been shown that antioxidant enzymes ascorbate peroxidase and glutathione reductase increased in activity during dehydration in *C. wilmsii* and *X. humilis* plants (Farrant *et al.*, 2003). The antioxidant enzymes were readily available in air-dry tissues, and were stable for the one week the plants remained dry. However, the stability of these antioxidants during the prolonged drought in *C. wilmsii* and *X. humilis* plants has not been evaluated thus far, but the increasing levels of lipid peroxidation in the latter suggest that perhaps their stability was compromised the longer the plants remained in the dry state. Kranner and Birtić (2005) suggested that as long as antioxidants and the photoprotective machinery remain undamaged, DT plants will be able to survive drought. These researchers also mentioned that fatal damage to leaves can be correlated with the breakdown of antioxidants. This study has shown that in HDT *C. wilmsii* plants MDA accrual was minimal and that whatever damage occurred to PS II could be repaired upon rehydration in the short-term dried plants. However, storage under harsher environmental conditions (AA, WGH and SD) for longer periods has shown that repair upon rehydration was not possible, indicating damage to photosynthetic machinery, and thus failure of protective mechanisms. In PDT *X. humilis* plants lipid peroxidation continued while

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the plants were dry despite no apparent lack of stability, as indicated by the ultrastructure (Chapter3, Figure 3.8). This would suggest that antioxidants in *X. humilis* plants could cope with damage, or repair increasing damage during the 10 month dry period, while in *C. wilmsii* plants the three month limit the plants could remain viable while dry is determined by other factors, such as the breakdown of photoprotective machinery. Future studies should examine the role of antioxidants in these species during prolonged dry storage.

In *C. wilmsii* plants, the outer leaves accumulated high anthocyanin levels in order to protect the inner rosettes of leaves from light-chlorophyll interaction and photo-oxidative damage. However, as this work has shown, anthocyanins degraded the longer the plants remain in the dry state. Further, antioxidant capacity was probably low at these water levels, however it has been reported that free radicals can continue to operate in dry tissues (Vertucci and Farrant, 1995; Walters *et al.*, 2001; 2005). The loss of this sunscreen pigment could lead to great ROS damage to the outer leaves, and this could be irreversible, resulting in loss of viability. Also, as the outer leaves are 'sacrificed', the inner green leaves become more susceptible to damage.

During drying and rehydration, while chlorophyll remains exposed, antioxidant activity is high, so minimising ROS damage (Sherwin and Farrant, 1998; Farrant, 2000; Farrant *et al.*, 2003). In the field, *C. wilmsii* plants experience frequent, short dehydration/rehydration cycles, but prolonged drought (greater than one month) is very rare. Perhaps this is supported by the evidence that within the first month of dry storage some loss of pigment was observed in this species. Minimal repair (Cooper and Farrant, 2002) further lessens chances of recovery. We cannot rule out other

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damaging reactions that might contribute towards the loss of viability in the dry state, but certainly having chlorophyll is a liability.

It is believed that *X. humilis* plants accumulate and store some mRNA in ribonuclear particles (Dace *et al.*, 1998; Collett *et al.*, 2003). Any damage to these particles would expose the stored mRNA making it more susceptible to damage and prevent full recovery. Both *C. wilmsii* and *X. humilis* plants showed active metabolism in dry plants, albeit at a slow rate, as indicated by the breakdown of chlorophyll (a+b) and anthocyanins, and accumulation of MDA, respectively. Accumulation ROS, although believed to be directly correlated with degenerative processes (Marx, 1985; Skulachev, 2000, 2002), may also play beneficial roles as signals (Kranner and Birtić, 2005). The inability of 10 month dried *X. humilis* explants to resynthesise chlorophyll (a+b) when rehydrated in water and the transcription inhibitor (that was possible in one month dried explants) suggests that mRNA suffered increased damage while the plants remained dry for that period of time. Also, since mRNA is believed to be stored and protected by ribonuclear particles and yet it was unavailable in sufficient amounts for recovery, suggests that protection mechanisms in leaves deteriorated while the plants were dry. This was further supported by the ultrastructural evidence that showed inability of 10 month dried explants to reconstitute and recover thylakoid membranes in water rehydrated explants, as was possible in one month dried explants. The failing of protection mechanisms indicated that even though the loss of viability of this species was not attained in this study (for 10 months the plants were maintained dry) perhaps it could have been achieved had the plants been kept dry for a few months longer. Although leaf explants from 10 month dried plants were unable to recover chlorophyll (a+b) levels and optimal PS II function in the water treatment,

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intact *X. humilis* plants were capable of recovery. Thus, roots seem to play a significant role in the recovery of *X. humilis* plants following prolonged dry storage.

In the absence of literature on storage of dry DT angiosperms, the only comparisons that can be made, and are believed to be relevant, are those with seed storage literature. During storage of both dry orthodox seeds and dry DT angiosperms deterioration increases with storage time, and loss of viability is inevitable. For both, it is not the initial exposure to drying, but rather the aging stresses associated with dry storage that incur death (Walters *et al.*, 2005). It is also possible that the mechanisms of protection that are induced during drying are not the same mechanisms that are required for maintenance of viability in dry tissues for an extended period of time (Walters *et al.*, 2005). The rate of aging in both dry orthodox seeds and dry DT angiosperms depends on storage conditions. In seeds it is relative humidity and temperature that play significant roles, but in DT angiosperms light could also affect the viability of dry tissues, as some maintain chlorophyll intact. Walters *et al.* (2005) believe that it is an innate factor that determines the ability of a species to remain viable in the dry state for an extended period of time, and to date this has not been identified for any species.

As *C. wilmsii* plants do not normally experience drought for longer than two months in the field, the mechanisms that they have evolved that would ensure their recovery upon the return of favourable conditions are those concerned mainly with protection. They maintain chlorophyll intact and protect from light-chlorophyll interaction (believed to be damaging during dehydration) by accumulating high levels of anthocyanins. However, some constituents for the recovery of photosynthetic

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machinery are accumulated during dehydration (Cooper and Farrant, 2002). Maintaining chlorophyll intact has been shown to be a liability, and its degradation was observed the longer the plants remained dry. The stability of stored constituents became compromised during dry storage, as plants maintained under all simulated field conditions lost viability, and this loss of viability was dependent on relative humidity, temperature and light intensity, as well as time period the plants were maintained in dry storage. Loss of protection mechanisms and minimal repair capabilities decreased chances of recovery.

X. humilis plants, however, experience longer drought periods in the field than *C. wilmsii* plants, and have adapted to these long droughts by protection during dehydration, but also significant repair upon subsequent rehydration. This species dismantles the photosynthetic machinery during dehydration, decreasing potential for photo-oxidative damage. Minimal levels of anthocyanins are accumulated during drying, presumably for their antioxidant capacity, but possibly also for the protection of whatever chlorophyll remains in dry tissues, or for upon rehydration when chlorophyll is resynthesised. Recovery of photochemistry is ensured through the accumulation of relevant mRNA which is transcribed upon rehydration. However, the stability of stored mRNA becomes compromised after 10 months of dry storage, but the machinery for *de novo* transcription and translation remains intact, indicating little or no damage to the genome.

DT is a multi-faceted response to water deficit stress. Research has suggested that unless all necessary conditions and mechanisms are fulfilled, DT would not be possible (Leprince *et al.*, 1993). Poikilochlorophylly might be considered to be a safer

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strategy due to breakdown of the photosynthetic machinery, and thus removal of the potential for ROS formation. PDT species have the ability to resynthesise and repair, allowing recovery of carbon metabolism. Although this study shows that poikilochlorophylly is a superior strategy for long-term desiccation (as previously proposed by Tuba *et al.*, 1996), each species has evolved mechanisms that would protect it against drought within their natural environments. However, should field climatic conditions change, as has been proposed (www.ipcc.ch/pub/un/syreng/spm.pdf), this study suggests that *X. humilis* plants are better equipped to survive drought should it occur for longer periods of time or during seasons when drought is not prevalent at present.

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