Investigation into the role of volatile organic compounds, and abscisic acid in stomatal regulation, in the resurrection plant *Xerophyta humilis*

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DECLARATION

The experimental work described in this thesis was carried out in the Molecular and Cell Biology Department, University of Cape Town, South Africa and in the Consiglio Nazionale delle Ricerche, Italy under the supervision of Prof J.M Farrant and Dr F. Loreto, respectively, from January 2010 to August 2011.

I know the meaning of plagiarism and declare that all of the work in the dissertation, save for that which is properly acknowledged, is my own.

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ABSTRACT

Environmental stresses that inhibit electron transport to the carbon reduction cycle cause the formation of reactive oxygen species (ROS) by direct oxygen photoreduction. ROS are responsible for oxidative stress that impairs the photosynthetic apparatus. Drought exacerbates oxidative stress and plants growing in extremely dry conditions have evolved mechanisms to withstand the photo-oxidative stresses associated with dehydration. In the desiccation tolerant 'resurrection' plant *Xerophyta humilis,* chlorophyll is broken down and thylakoid membranes dismantled at relatively high water contents; a phenomenon known as poikilochlorophylly. This reduces the potential for oxidative damage, but there is also a simultaneous increase in antioxidant activity to quench ROS that do form. These antioxidant mechanisms include isoprenoids, such as carotenoids and tocopherols, which are conserved protective strategies amongst all plants. Therefore, carotenoid regulation was firstly investigated in *X. humilis*, simultaneously to re-examining photosynthetic regulation, chlorophyll content and fluorescent parameters during dehydration and rehydration. There was evidence for activation of the xanthophyll cycle during dehydration to dissipate excess energy and the antioxidant carotenoids lutein, zeaxanthin and β-carotene increased substantially during dehydration, and were subsequently present during early rehydration. Besides conserved mechanisms of protection, there are non-ubiquitous traits present in plants, such as volatile organic compound (VOC) production. Although the roles of VOCs are unclear, there is some evidence for an antioxidant function of these volatile molecules, particularly under various environmental stresses. In this study it was shown that *X. humilis* emitted isoprene, the most abundant VOC in the atmosphere, in relatively large amounts. This is the first time that isoprene emission is reported in a resurrection plant. Isoprene emission increased during initial dehydration, but decreased after drying below ca 60% relative water content. The decline in isoprene emission occurred after the onset of the decline in photosynthesis. Isoprene emission may indeed reduce ROS formation during the first phase of drought stress and stabilize membranes by enhancing hydrophobic interactions. A second signalling molecule, which plays a major role in plants' stress response to drought, is the phytohormone abscisic acid (ABA). It is well known that ABA causes stomatal closure in response to water deficit, which is advantageous to both drought-tolerant and drought-sensitive plants. However, the extent of stomatal regulation has not yet been characterized in resurrection plants, particularly once protective mechanisms have been laid down and water conservation is no longer necessary. The findings presented in this study give evidence for an ABA-mediated stomatal closure in response to initial dehydration of *X. humilis*, however, from 60% RWC onwards, stomata were observed to open again. This supplies further support for the hypothesis that resurrection plants

actively lose water once protection is accumulated in order to minimize ROS activity and put a stasis on unregulated metabolism. Interestingly, ABA and isoprene were observed to increase at the same stage. In relation to the putative mechanisms whereby resurrection plants attain desiccation tolerance, isoprene emission may indicate sustained formation and rapid turn-over of molecules which have isoprenoid moieties, namely ABA, chlorophylls and carotenoids, all of which are synthesized from the chloroplastic methyl-erythritol phosphate pathway.

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ABBREVIATIONS

A	Photosynthesis
ABA	Abscisic acid
ANOVA	Analysis of Variance
AP	Ascorbate peroxidase
Asc	Ascorbic acid
CAT	Catylase
C_i	Intercellular CO_2 concentration
DMAPP	Dimethylallyl diphosphate
DW	Dry weight
F_o	Minimum fluorescence
F_m	Maximum fluorescence
F_s	Steady state fluorescence
F_v/F_m	Maximum quantum yield of photosystem II photochemistry
Fosm	Fosmidomycin
FTSW	Fraction of transpirable soil water
GC-MS	Gas chromatography-mass spectrometry
GR	Glutathione reductase
GSH	Glutathione
HSP	Heat shock protein
IRGA	Infrared gas analyser
ISPS	Isoprene synthase
J_{max}	Maximum rate of electron transport during RuBP regeneration
LEA	Late embryogenesis abundant
LOX	Lipoxygenase
MDA	Malondialdehyde

MEP	Methylerythritol phosphate
NPQ	Non-photochemical quenching
OVOC	Oxygenated volatile organic compound
PAR	Photosynthetically active radiation
PEG	Polyethylene glycol
ppm	Parts per million
PSII	Photosystem two
ΦPSII	Quantum efficiency of PSII photochemistry
PTR-MS	Proton transfer reaction-mass spectrometry
qP	Photochemical quenching
ROS	Reactive oxygen species
Rubisco	Ribulose-1,5-bisphosphate carboxylase-oxygenase
RuBP	Ribulose 1,5-bisphosphate
RWC	Relative water content
SEM	Scanning electron microscopy
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TEM	Transmission electron microscopy
TPU	Triose phosphate utilization rate
$V_{c,max}$	Maximum rate of RuBP carboxylation
VOC	Volatile organic compound

CHAPTER 1

DESICCATION TOLERANCE IN RESURRECTION ANGIOSPERM PLANTS

1.1 INTRODUCTION

Water is fundamental to the growth and development of plants. Water deficit in plants is the result of transpiration rate exceeding water uptake and can occur due to a range of environmental stresses, such as drought, salinity and low temperature (Bray, 1997). Water deficit in vegetative tissues can have detrimental effects, resulting in cellular dehydration, damage and ultimately death. Many plants are able to withstand minor changes in water availability, and can reduce water loss by utilizing morphological and physiological adaptations. These adaptations include thickening of cell walls, sunken stomata, leaf hairs, reduced leaf surface area, growing in shady areas or in clumps, inhibition of photosynthesis and induction of stress-related genes (Bray, 1997, Lerner, 1999, Taiz and Zeiger, 2002). Although these adaptations may help reduce the severity of water stress, after prolonged periods of drought, the stresses associated with water loss will become too detrimental and the plants will still die. These stresses include mechanical stresses due to loss of turgor and cell volume (Iljin, 1957), metabolic stresses due to increased free radical activity and concentration of solutes (Farrant, 2007) and the loss of membrane integrity and denaturation of proteins (Oliver, 2007). The plant's capability to survive depends on the stress responses induced at the whole plant and cellular level and there are only a few plants that are able to survive extreme desiccation (Bray, 1997). Desiccation tolerance is defined as "the ability of an organism to dry to equilibrium with dry air and to resume full metabolic function on rehydration" (Bewley, 1979). Although complete water loss is a common occurrence in the development of seeds and pollen (Vicre et al., 2004) and in a large number of lichen and bryophyte species (Oliver et al., 2000, Oliver et al., 2005, Proctor and Pence, 2002), there are only a few higher order plants that are able to survive extreme water deficit in their vegetative tissues. Desiccation tolerance has only been observed in about 350 angiosperm plants and they are termed "resurrection plants", the majority of which are found in tropical and sub-tropical zones in southern Africa (Gaff, 1977). In this review emphasis will be placed on the resurrection plant, *Xerophyta humilis,* a species endemic to Southern Africa. It is a relatively well studied monocotyledonous species, in which physiological, biochemical and molecular studies have previously been performed (Dace et al., 1998, Farrant, 2000, Farrant et al., 1999).

1.2 DESICCATION TOLERANT MECHANISMS UTILIZED IN DEALING WITH THE ASSOCIATED STRESSES

In order to be desiccation tolerant, resurrection plants must limit the damage associated with severe water stress to a state that is repairable, preserve cellular integrity whilst in the dried state and induce mechanisms upon rehydration to repair the damage associated with drying and rehydration (Oliver, 2007).

Mechanical stress arises from a decrease in turgor and cell volume as water is lost. The loss of water from the vacuoles and cytoplasm results in increasing tension and the plasmalemma ruptures (Farrant, 2007). This is thought to be one of the main sources of irreparable damage induced by drought stress in plants, often leading to cell death (Iljin, 1957). The tissues of resurrection plants still shrink as water is lost, however these plants are able to actively mechanically stabilize the cells therefore preventing the plasmalemma from rupturing and collapsing (Farrant, 2008). There are two protective mechanisms which enable this in resurrection plants. Firstly, active and reversible cell wall folding, most common in the *Craterostigma* species (Vicre et al., 2004, Vicre et al., 1999), and secondly, replacement of water in the vacuoles with non-aqueous substances, such as occurs in the *Xerophyta* species (Farrant, 2000, Farrant, 2007, Mundree and Farrant, 2000). While the above mentioned species mainly utilize one of these two mechanisms, many such as *Myrothamnus flabellifolia* and *Eragrostis nindensis,* utilize both mechanisms (Moore et al., 2007b, Moore et al., 2006, Vander Willigen et al., 2004).

The second consequence of a reduction in cell volume due to water loss is that the cytoplasm becomes more viscous and cellular contents become more concentrated. This results in promotion of membrane adhesion among organelles and inappropriate molecular interactions leading to protein denaturation and membrane damage. Metabolism is increasingly disrupted as electron transport is uncoupled, resulting in increasing damaging free radical activity (Vertucci and Farrant, 1995, Farrant et al., 2011). To ensure the viability of cells upon rehydration, a controlled down-regulation of metabolism is required, coupled with the synthesis of antioxidants to reduce free radical activity (Bartels and Salamini, 2001, Mundree and Farrant, 2000, Oliver et al., 1998). An adaptation of desiccation-tolerant systems is that water is thought to be replaced with hydrophilic molecules to bring about stabilization of macromolecules (Crowe et al., 1998). These water replacement molecules include sugars, especially sucrose and oligosaccharides (Bartels and Salamini, 2001, Hoekstra et al., 2001, Illing et al., 2005, Ingram and Bartels, 1996, Peters et al., 2007), hydrophilic proteins, particularly late embryogenesis abundant (LEA) proteins (Collett et al., 2004, Ingram and Bartels, 1996), small heat shock proteins (HSPs) (Vierling, 1991, Wehmeyer et al., 1996), and other compatible solutes such as amino acids (Gaff and McGregor, 1979, Ramanjulu and Bartels, 2002).

Another source of severe damage during desiccation is free radical stress. Reactive oxygen species (ROS) occur as a natural consequence of metabolism in general but accumulate particularly in the mitochondria and chloroplasts as a result of electron transport during these metabolic processes, primarily photosynthesis. Although oxygen is necessary for metabolism in all aerobic systems, it can easily form reactive oxygen species such as singlet oxygen ($^1O_2$), superoxide (O_2^-), the hydroxyl radical (OH^-) and also reactive nitrogen species, such as nitric oxide (NO). The unpaired electrons of these molecules cause them to be highly reactive (Halliwell and Gutteridge, 1999) and they are therefore very damaging to cellular components and macromolecules, for example, enzymes, membranes and chromosomes (Dean et al., 1993, Dizdaroglu, 1994, Halliwell and Gutteridge, 1999, Møller et al., 2007). Under normal hydrated conditions, ROS activity is counteracted by a number of free radical scavenging systems. Firstly, there are enzymes that scavenge ROS, such as superoxide dismutase (SOD), ascorbate peroxidase (AP), glutathione reductase (GR) and catalase (CAT), and in addition there are non-enzymatic antioxidants, such as glutathione (GSH), ascorbic acid (Asc), tocopherols and β-carotene (Munné-Bosch and Alegre, 2002, Noctor and Foyer, 1998, Telfer, 2002).

In vegetative tissues, severe water stress results in a disruption of the electron transport and therefore disequilibrium between ROS production and scavenging ensues. Not only is there an excess of ROS produced from respiratory metabolism, but also from a disruption of photosynthesis and consequent inefficient use of light-generated photosynthetic electron transport. Excess energy from excited chlorophyll molecules is transferred to oxygen causing a rapid production of free radical species (Franca et al., 2007, Halliwell, 1987, Smirnoff, 1993). While desiccation-sensitive plants are unable to adequately deal with the surge in ROS production, which ultimately leads to cell death (Vicre et al., 2003, Smirnoff, 1993), resurrection plants have many mechanisms in place to firstly reduce ROS formation and, secondly, quench their activity.

Resurrection plants continue respiration to low levels of relative water content (RWC) (Farrant, 2000, Mundree et al., 2002, Tuba et al., 1998), and while this may provide the chemical energy needed to produce the molecules that help combat the effects of water stress, such as solutes, LEA proteins and HSPs, it also consequently exacerbates ROS production. However, the suggestion has been made regarding resurrection plants, that there are increased antioxidant processes, which are preserved even in the desiccated state, and also antioxidants which have only previously been seen in seeds, that help reduce ROS formation (Farrant, 2007, Illing et al., 2005).

Not only is there increased antioxidant activity, but ROS formation from photosynthesis is also minimized in resurrection plants by a down-regulation of photosynthesis at sub-critical water stress levels (Mundree et al., 2002, Tuba et al., 1998, Vicre et al., 2004). This is accomplished by one of two primary mechanisms, namely poikilochlorophylly or homoiochlorophylly (Farrant, 2000, Sherwin and Farrant, 1998, Tuba et al., 1996, Tuba et al., 1998). The first, poikilochlorophylly, involves the breakdown of chlorophyll and dismantling of thylakoid membranes during dehydration, resulting in a cessation of photosynthesis at water contents between 80% and 65% (Farrant, 2000, Sherwin and Farrant, 1998). This mechanism is common to the monocot *Xerophyta* species and *E. nindensis* and it is very effective in reducing photo-oxidative damage, allowing these plants to dehydrate and remain in the dried state for long periods of time. However, upon rehydration, these species have to subsequently resynthesize the photosynthetic apparatus causing relatively slower rates of rehydration (Sherwin and Farrant, 1996, Tuba et al., 1994, Tuba et al., 1993). Homoiochlorophyllous resurrection plants differ in that chlorophyll and the thylakoid membranes are retained in the desiccated state, however other mechanisms are employed to minimize ROS production during drying. These are typically dicots, such as the *Craterostigma* species and *Myrothamnus flabellifolia*, which undergo leaf folding and shading during drying therefore reducing the exposed surface area and thus chlorophyll-light interactions (Farrant, 2000, Moore et al., 2007a, Moore et al., 2007b, Sherwin and Farrant, 1998). Anthocyanin pigment accumulation occurs in the remaining exposed surfaces and these molecules are thought to shield chlorophyll from photosynthetically active light, thereby acting as antioxidants (Farrant, 2000, Farrant, 2007, Moore et al., 2007a, Sherwin and Farrant, 1998). This reduction in metabolism at lower RWC is also supported by evidence in resurrection plants for a down-regulation of specific genes involved in metabolism during desiccation stress (Farrant, 2007). These avoidance mechanisms, which commence during the early stages of drying, act together with the antioxidants to minimize the production of ROS and the associated damage which results from respiration continuing to low RWC levels (Bewley, 1979, Farrant, 2000, Hoekstra et al., 2001, Sherwin and Farrant, 1998, Tuba et al., 1998).

Xerophyta humilis, a poikilochlorophyllous species, also displays desiccation-induced morphological changes. Leaves are observed to fold in half along the midrib during drying, therefore enclosing the adaxial surface and enabling regulation of water loss from this surface during dehydration (Farrant, 2000, Oliver et al., 1998). It has been suggested that the morphological changes that occur during dehydration of resurrection plants are an adaptation to minimize exposed surface area and thus reduce water loss during the initial stages of drying (Vicre et al., 2004). This enables leaf water content to be maintained at near full turgor as has been observed in many resurrection plant species (Farrant, 2000, Farrant et al., 1999, Sherwin and Farrant, 1996). However, once the soil is dry, and the protective mechanisms against desiccation have been laid down, as outlined above, there is a rapid decline in water content of the plants in what is postulated to be an active loss of water in order to facilitate the formation of cytoplasmic glasses that minimize ROS activity and put a stasis on unregulated metabolism (Farrant, 2007, Farrant et al., 2011).

Clearly, there is a very controlled regulation of the dehydration process in resurrection plants in order to minimize the stress associated with desiccation, allowing the plants to recover full metabolism when water becomes available. The mechanisms discussed above are firstly protective against desiccation stress, and secondly they also help minimize and avoid the damages associated with desiccation stress. These adaptations separate resurrection plants from desiccation sensitive plants. However, there are various signalling molecules that function during drought stress in some desiccation sensitive plants which have not yet been fully investigated or investigated at all in resurrection plants. Two such molecules are the plant phytohormone, abscisic acid, and the volatile organic compound, isoprene.

1.3 POSSIBLE INVOLVEMENT OF SIGNALLING MOLECULES IN DROUGHT TOLERANCE

1.3.1 ABSCISIC ACID

Abscisic acid (ABA) is one of the major signals that mediates adaptive responses in plants to environmental water changes (Koorneef et al., 1998). It has been shown to increase by up to 30-fold during drought stress (Outlaw, 2003) and is also involved in responses to other environmental stresses such as salinity and freezing (Bray, 1997). ABA, furthermore, plays a critical role in many stages of the plant life cycle, such as seed maturation and dormancy, germination and seedling growth (Campalans et al., 1999, Farrant, 2007).

Soil drying subsequently results in dehydration of plant cells, this starting at the root level. A cellular perception of the dehydration stress, such as recognition of a decrease in turgor, cell volume or membrane area, stimulates the synthesis of ABA from carotenoid precursors (Acharya and Assmann, 2009, Bray, 2002, Wright, 1977). ABA accumulates in the roots and is transported by the xylem to the rest of the plant (Bray, 1997). ABA is thus a long-distance chemical signal that conveys the soil water status to the rest of the plant. Dehydration of leaf cells as a result of severe soil water shortages can also act as a stimulus for ABA synthesis (Wilkinson and Davies, 2002). Alternatively, there is evidence for a labile pool of ABA within the leaves, synthesized from the chloroplastic methyl-erythritol phosphate (MEP) pathway, that is able to cause stomatal closure in response to drought (Barta and Loreto, 2006).

The response mechanisms initiated by ABA to water deficit are complex and varied and only a few pertinent ones are highlighted here. ABA has been observed to regulate the expression of a variety of stress-related genes (Bray, 1997) and in response to water deficit conditions, there is a massive ABA-mediated change in gene expression (Ramanjula and Bartels, 2002). The survival and ability of the plant to function in water deficit conditions is enabled by changes in gene expression (Bray, 1997), as it allows the plant to respond accordingly to the stress. ABA upregulates antioxidative responses and the synthesis of detoxifying enzymes, namely ascorbate peroxidase and superoxide dismutase, in order to counter the increase in ROS production during water stress (Jiang and Zhang, 2001). ABA induces the expression of small HSPs and LEA proteins, both of which are involved in protection against dehydration during water deficit (Campalans et al., 1999, Oliver, 2007).

It has long been known that ABA strongly promotes stomatal closure (Jones and Mansfield, 1970). Stomata are microscopic pores found throughout the leaf epidermis, and each is surrounded by a pair of guard cells. Guard cells control CO_2 intake through the stomata and the rate of transpiration, which is the process whereby water is lost from the plant through stomata. Following water deficit perception and ABA synthesis in root cells (Zhang and Davies, 1989), ABA is transported through the leaf by the pull of transpiration until it reaches the stomatal guard cells (Wilkinson and Davies, 2002). These specialized cells modulate the control of stomatal aperture, thus regulating the rate of water loss by transpiration. Swelling or shrinking of guard cells, due to a change in turgor and volume, causes stomata to open or close, respectively (Acharya and Assmann, 2009). This is achieved primarily by transmembrane fluxes of potassium and anions (McAinsh et al., 1997). Guard cells contain external, and possibly internal, ABA receptors to which the hormone binds and induces an internal signalling transduction cascade consisting of a number of cellular and biochemical events leading to stomatal closure (Schroeder et al., 2001, Wilkinson and Davies, 2002). Thus, an increase in

ABA concentration in the leaves in response to water deficit prevents transpirational water loss by promoting stomatal closure (Jones and Mansfield, 1970).

Although a vast accumulation of evidence suggests that ABA acts as a major signalling molecule in the drought response of plants (Davies and Zhang, 1991), the role of ABA in drought tolerant plants is still being characterized. ABA is known to play an important role in the acquisition of desiccation tolerance in seeds, thus it is not unexpected that it also functions in inducing desiccation tolerance in vegetative tissues (Oliver, 2007). As in desiccation-sensitive plants, ABA upregulates a number of dehydration-regulated genes in resurrection plants (Vicre et al., 2004). It was noted that the upregulated genes are like those expressed in orthodox (desiccation tolerant) seeds (Campalans et al., 1999).

While desiccation-sensitive plants reduce water loss during drought, mainly through stomatal regulation, resurrection plants do not appear to conserve water to the same degree. During the initial stages of soil drying, leaf water content has been observed to be maintained at near full turgor in many resurrection plant species (Farrant et al., 1999, Sherwin and Farrant, 1996). However, once the soil is dry, and protective mechanisms against desiccation have been upregulated, there is a rapid decline in water content of the plants in what is postulated to be an active loss of water (Farrant, 2007). As has been discussed, under water stress conditions, stomatal closure and a down-regulation of photosynthesis causes a disruption of the electron transport chain, leading to a surplus production of ROS (Farrant, 2007). It is therefore deleterious for plants to be at intermediate water contents, and thus it is hypothesized that desiccation-tolerant plants actively lose water, once protection is established, to reduce the chance of excess ROS formation. Open stomata were observed to be present in both hydrated and desiccated plants of *Myrothamnus flabellifolius* (Moore et al., 2007a). Previous studies came to the same conclusion that severe dehydration of resurrection plants causes passive re-opening of stomata (Vicre et al., 2004). Preliminary studies using scanning electron microscopy (SEM) in the resurrection plant *Xerophyta humilis* found that stomata were closed during the early stages of drying but open again in the later stages (Chirese, 2006, unpublished data). However, it is not known to what extent ABA is involved in the stomatal regulation in resurrection plants during desiccation. Further investigation is required to fully characterize the role of ABA in stomatal regulation in resurrection plants and to what extent this regulation differs to desiccation sensitive systems.

1.3.2 VOLATILE ORGANIC COMPOUNDS

A second group of molecules reportedly involved in the response of some plants to environmental stresses are volatile organic compounds (VOC). Isoprene (C_5H_8, 2-methyl 1,3-butadiene) is a natural product of many organisms and is the most abundant VOC emitted by terrestrial plants (Guenther et al., 1995). Isoprene emission from plants requires de novo synthesis and it is synthesized from dimethylallyl diphosphate (DMAPP), catalyzed by the enzyme isoprene synthase (ISPS) (Schnitzler et al., 1996, Silver and Fall, 1995). This enzyme is located within the chloroplasts and requires a high pH optimum and Mg^{2+} (Mgaloblishvili et al., 1979, Wildermuth and Fall, 1998). The source of DMAPP within the chloroplast is the 2-deoxyxylulose 5-phosphate/2-methylerythritol 4-phosphate (MEP) pathway (Rohmer et al., 1993), and it has been shown that this pathway is responsible for isoprene synthesis (Schwender et al., 1997, Zeidler et al., 1998) (Figure 1-1).

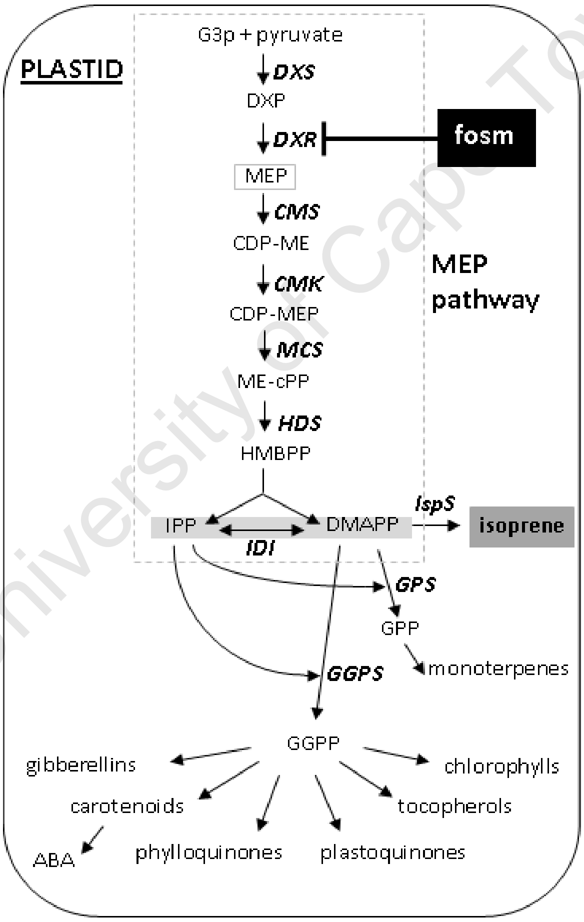

Figure 1-1: The MEP pathway is the source of DMAPP for isoprene synthesis as well as other monoterpenes, chlorophyll side chains, carotenoids and ABA. Also shown is fosmidomycin (Fosm), the chemical inhibitor of isoprene synthesis. G3P = glyceraldehyde 3-phosphate; DXS = deoxyxylulose 5-phosphate (DXP) synthase; DXR =DXP reductoisomerase; MEP methylerythritol 4-phosphate; CMS = diphosphocytidylyl methylerythritol (CDP-ME) synthase; CMK = CDP-ME kinase; CDP-MEP = CDP-ME 2-phosphate; MCS = methylerythritol 2,4-cyclodiphosphate (ME-cPP) synthase; HDS = hydroxymethylbutenyl diphosphate (HMBPP) synthase; IDP = isopentenyl diphosphate; DMADP = dimethylallyl diphosphate; IDI = IDP isomerase; IspS = isoprene synthase; GGPS = geranygeranyl pyrophosphate (GGPP) synthase; GPS = geranyl diphosphate (GPP) synthase.

Experiments with isotopically labelled carbon dioxide ($^{13}CO_2$) have shown that about 80% of the carbon in isoprene is derived directly from the Calvin Cycle of photosynthesis (Affek and Yakir, 2003, Mgaloblishvili et al., 1979, Sanadze et al., 1972), and during environmental stress conditions, such as drought stress or elevated temperature, the amount of carbon lost due to isoprene emission can increase by 50% when photosynthesis is depressed (Sharkey and Loreto, 1993). Many plants from a broad range of taxonomic groups emit isoprene, such as mosses, ferns, gymnosperms and angiosperms (Hanson et al., 1999, Sharkey et al., 2005, Tingey et al., 1987); however, there are also many members from these groups that do not emit isoprene. As the energy cost of isoprene emission is quite significant, especially under stress conditions (Sharkey et al., 2008, Sharkey et al., 2001), the benefit most probably outweighs the cost in the plants in which isoprene emission is favoured and has evolved. Researchers have therefore been intent on determining and investigating the benefits that accrue to plants that emit isoprene.

Thermotolerance is probably the most discussed advantage that plants gain from isoprene emission (Sharkey et al., 2008). A study showed that at 30°C, normally 2% of carbon fixed by photosynthesis was emitted as isoprene, but above 30°C, photosynthesis declined and isoprene emission increased, and at 40°C, about 15% of the carbon was emitted as isoprene (Sharkey et al., 1996). It was first proposed in 1995 that isoprene had some relationship to temperature, and more specifically, that isoprene helps protect photosynthesis against damage as a result of high leaf temperature (Sharkey and Singsaas, 1995a). Later on, the thermotolerance hypothesis was refined, namely that isoprene emission helped protect against heat flecks, which are large and rapid changes in leaf temperature, caused for example by changes in sunlight throughout the day (Hanson et al., 1999, Singsaas et al., 1999).

An advancement in the studies of isoprene emission and its hypothesized benefits was the discovery that feeding plants with low levels of fosmidomycin, the inhibitor of 2-deoxyxylulose 5-phosphate reductoisomerase (Figure 1-1), caused isoprene emission to be inhibited (Zeidler et al., 1998). It was also shown that photosynthesis was unaffected by fosmidomycin (Sharkey et al., 2001). Studies showed that leaves in which isoprene emission was inhibited by fosmidomycin suffered much more heat damage and the recovery was greatly retarded, compared to leaves which were not fed fosmidomycin (Velikova and Loreto, 2005). Sharkey et al (2001) also showed that feeding isoprene to leaves in which isoprene emission had previously been inhibited by fosmidomycin, restored thermoprotection, thus confirming the thermotolerance role of isoprene in plants.

The second hypothesized role for isoprene in plants, more pertinent to this study, is its function in tolerance of ozone stress and other reactive oxygen species. Isoprene has been shown to protect the photosynthetic apparatus against ozone damage, quench ozone products, such as hydrogen peroxide, and help reduce lipid peroxidation of membranes from ozone (Loreto et al., 2001, Loreto and Velikova, 2001). Isoprene has been shown to reduce a loss in photosynthesis due to ROS stress, such as treatment with singlet oxygen (Velikova et al., 2008, Penuelas et al., 2005, Velikova et al., 2004).

In terms of thermal and oxidative stress tolerance, there are two main hypotheses for the way isoprene exerts a protective action. Firstly, membrane stabilization was suggested as a mode of action for isoprene (Sharkey and Singsaas, 1995b). Isoprene is lipophilic and could therefore integrate in the bilipid layer of membranes thereby stabilizing hydrophobic interactions (Vickers et al., 2009). A study observed that when isoprene was dissolved in a model membrane, there was a subsequent increase in membrane order equivalent to a 10°C decrease in temperature and it was therefore concluded that isoprene stabilizes bilipid membranes (Siwko et al., 2007). The second mechanistic hypothesis is that isoprene directly acts as an antioxidant and scavenges ROS through the conjugated double bond system (Loreto et al., 2001, Loreto and Velikova, 2001, Velikova et al., 2004). Nonvolatile isoprenoids, such as tocopherols, zeaxanthin and β-carotene, form an integral part of the non-enzymatic oxidative defense system in all plants (Demmig-Adams and Adams III, 1996, Munné-Bosch and Alegre, 2002, Telfer, 2002), however, volatile isoprenoids, such as isoprene, may form part of an additional protective system against oxidative stress, which is not conserved amongst all plants.

The sources of stress discussed up until now are that of heat and ozone stress. More recently, isoprene emission has been implicated in drought stress. Water stress has been shown to uncouple the emission of isoprene from photosynthesis. Early studies, which were subsequently later confirmed, showed that a short period of drought caused a significant decrease in photosynthesis; however, isoprene emission remained constant or only decreased slightly (Pegoraro et al., 2004, Sharkey and Loreto, 1993). Studies have been conducted on a range of plant species and all found that isoprene emission was less responsive to drought than that of photosynthesis and stomatal conductance (Brilli et al., 2007, Delfine et al., 2005, Fang et al., 1996, Loreto and Sharkey, 1993, Pegoraro et al., 2004). Brilli et al. (2007) demonstrated in *Populus alba* saplings that as a function of the fraction of transpirable water (FTSW), photosynthesis decreased at FTSW of 30%, whereas isoprene emission only decreased towards the end point of FTSW. As a consequence, the amount of assimilated carbon lost due to isoprene emission increased during drought stress. This is confirmed by earlier findings by Pegora et al. (2004), who showed that during severe drought stress when photosynthesis has decreased to zero, up to 50% of assimilated carbon is lost. Brilli et al. (2007)

suggest that the maintenance of high emission rates of isoprene, even once photosynthesis has declined, could be due to the use of alternative carbon sources.

During recovery from water stress, photosynthesis has been observed to return to pre-stress levels, however, isoprene emission reached rates that are generally higher than in pre-stress or control plants (Brilli et al., 2007, Fang et al., 1996, Sharkey and Loreto, 1993). Brilli et al. (2007) showed, by way of a $^{13}CO_2$ labeling experiment, that the source for isoprene emission upon recovery was once again mainly photosynthesis. It is still unclear as to why there is an increase in carbon invested in isoprene synthesis after stress, and that this increase is only transient.

The studies discussed above investigated the response of isoprene, in conjunction with photosynthesis, to water stress over a relatively long time period, ranging between 10 to 35 days. Resurrection plants reach a fully desiccated state over a much shorter time period, ranging between 2 to 5 days. Many plants emit isoprene, however not all and the phylogenetic distribution of isoprene emission is mostly amongst woody plants (Vickers et al., 2009). It is not known whether resurrection plants emit volatile isoprenoids, namely isoprene, and if so, nor is it known how the emission compares to other desiccation-sensitive plants. As isoprene emission seems to convey an adaptive advantage to plants to survive in adverse environmental conditions, investigating VOCs in resurrection plants could uncover an additional mechanism in desiccation tolerance.

1.4 AIMS OF THE CURRENT WORK

Examining non-ubiquitous traits, which enable certain species to adapt to conditions where others would not survive, is important to understand the adaptations which confer desiccation tolerance in resurrection plants. Therefore, the aim of this thesis was to firstly fully investigate photosynthetic regulation in *X. humilis* and some of the known conserved antioxidant processes. Under stress conditions, the production of ROS from photosynthetic processes becomes perturbed, however, resurrection plants are able to minimize and avoid the damaging effects of oxidative stress (discussed above). Thus a further aim was to investigate whether *X. humilis* emits any VOCs and to characterize their role in this resurrection plant, thereby also confirming the function VOCs, namely isoprene, in drought stress in general. Lastly, as there is no need for resurrection plants to attempt to conserve water for as long as possible during drought, stomatal regulation could be significantly different in desiccation tolerant compared to desiccation sensitive species. This thesis also characterized the regulation of stomata in *X. humilis* and the role of abscisic acid in the process. In completion, this study will aim to clarify the role of the various signalling molecules in *X. humilis* and possibly highlight how the processes are unique in resurrection plants.

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CHAPTER 2

REGULATION OF PHOTOSYNTHESIS AND PIGMENTS IN *XEROPHYTA HUMILIS*

2.1 INTRODUCTION

Excess excitation energy resulting from uncoupled photosynthesis is one of the major causes of damage to plants under abiotic stress conditions (Smirnoff, 1993). *Xerophyta humilis* reduces the potential for oxidative damage as it is poikilochlorophyllous and therefore breaks down chlorophyll and dismantles thylakoid membranes during dehydration and resynthesizes the apparatus during rehydration. This mechanism has been well studied in resurrection plants (Farrant, 2000, Hoekstra et al., 2001, Mundree and Farrant, 2000, Farrant, 2007, Farrant et al., 2011, Farrant et al., 2003). It is necessary to firstly investigate and confirm how photosynthesis and the known associated pigments are regulated in *X. humilis*, before further exploring the roles of other molecules which have not yet been studied in *X. humilis*, but which have been shown to enable other plants to adapt to adverse environmental conditions. Although the nature of poikilochlorophylly has been previously studied in *X. humilis* (Farrant, 2000, Farrant et al., 2003), these experiments will be repeated in light of this study. Secondly, the aim is to use alternative techniques, such as gas exchange and chlorophyll fluorescence, to assess the many parameters associated with photosynthesis and the photosynthetic electron transport rate. The response of photosynthesis (A) to increasing internal CO_2 concentrations (Ci) will be assessed during different stages of dehydration and rehydration. An example of a typical A/Ci response curve is given in Figure 2-1.

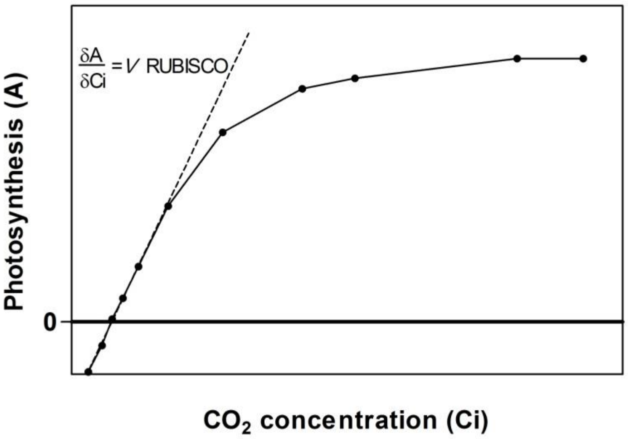


Figure 2-1: Hypothetical A/Ci response curve showing change in photosynthesis as internal CO_2 concentration increases. The slope of the initial linear section of the curve (δA/δCi) is strongly influenced by Rubisco activity.

These response curves have not yet been performed in a resurrection plant and will provide information on the optimal conditions for photosynthesis, and on photosynthetic limitations, particularly on the regulation of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco). Rubisco catalyzes the first step of carbon fixation in photosynthesis, supplies acceptor molecules for the products of the light reactions and is able to regulate the amount of key photosynthetic intermediaries by changes in its activity (Sharkey, 1989). Therefore, investigating Rubisco activity with A/Ci response curves in *X. humilis* will add to the existing knowledge of photosynthetic regulation in resurrection plants. Carotenoids are known for their antioxidant activity within the chloroplasts, as well as the radiationless dissipation of excess heat through activation of the xanthophyll cycle (Demmig-Adams and Adams Iii, 1996, Larson, 1988, Munné-Bosch and Alegre, 2000). Previous studies have only looked at total carotenoid content in *X. humilis* (Farrant, 2000). Therefore this study will aim to investigate how individual carotenoids in *X. humilis* change in response to dehydration and rehydration, thereby providing a more complete understanding of poikilochlorophylly before additional or alternative protective mechanisms are investigated.

2.2 METHODOLOGY

2.2.1 PLANT MATERIAL

Xerophyta humilis plants were collected in the Pilanesberg Nature Reserve, South Africa, and maintained in a glasshouse as previously described (Sherwin and Farrant, 1996). Trays of plants (15 cm x 20 cm with a soil depth of about 5 cm) were used for the procedures described below. For experimental purposes, fully hydrated plants were transferred to a constant environment room in which the conditions were maintained at 55% relative humidity with a 14 h photoperiod and 17 ˚C (dark): 25 ˚C (light) temperature cycle. The light intensity was 400 µmol photons $m^{-2}s^{-1}$. Plants were allowed to acclimatize for 4 to 5 days prior to commencing experiments.

2.2.2 RELATIVE WATER CONTENT (RWC) DETERMINATION

Trays were well watered to ensure plants were fully hydrated at commencement of an experiment. Thereafter, whole plants were dehydrated by withholding water and allowing plants to dry naturally. Soil was watered to field capacity to allow for rehydration.

Water content was determined gravimetrically on a dry weight (DW) basis by oven drying at 70˚C for 48 h. RWC was measured using the standard formula: RWC = water content/water content at full turgor and was expressed as a percentage. Full turgor was achieved as previously described (Martinelli, 2008).

2.2.3 GAS EXCHANGE MEASUREMENTS

Rate of photosynthesis was measured using an LI-6400 (LI-COR Biosciences Inc., Nebraska, USA) infrared gas analyser (IRGA). The equations used to calculate photosynthesis and transpiration were those previously derived (von Caemmerer and Farquhar, 1981). Readings were taken for three to four leaves, selected randomly from three trays, each day of dehydration and rehydration, between 3 and 4 hours after dawn. Readings were taken over a time period of 5 minutes and the average of the technical repeats (at least 3) for each leaf was calculated. A small section of each leaf was cut for relative water content determination.

The response of photosynthesis (A) to intercellular CO_2 concentration (C_i) was determined. The light source was set at 700 µmol $m^{-2}s^{-1}$ and a leaf enclosed in a chamber was exposed to a range of external CO_2 concentrations. The measurements were started at ambient CO_2, then CO_2 was decreased so as to have 5 points below 400 µmol mol^{-1} (ambient) CO_2 , then increased, stopping at ambient CO_2 again, and ending at 1500 µmol mol^{-1} so as to have 5 points above ambient CO_2. Photosynthesis was allowed to stabilize at each concentration before recording the value. The total time taken to perform all measurements was 45-60 minutes. The A/Ci curves were used to estimate the maximum rate of ribulose-1,5-bisphosphate (RuBP) carboxylation ($V_{c,max}$), the maximum rate of electron transport driving RuBP regeneration (J_{max}) and the triose phosphate utilization rate (TPU), using an Excel utility (Sharkey et al., 2007). Three biological repeats were performed, and the same leaves were measured during dehydration and rehydration.

Similarly, photosynthetic light response curve data were obtained. External CO_2 concentration was set at 400 µmol mol^{-1}, and the enclosed leaf was firstly adapted to a light intensity of 1000 µmol photons m^{-2}s^{-1} and then exposed to a range of light intensities, namely 0, 50, 100, 150, 200, 400, 600, 800, 1000 and 1200 µmol photons m^{-2}s^{-1}. Photosynthesis was recorded once it had stabilized at each stage of light intensity (usually 3-5 minutes). Three biological repeats were performed.

2.2.4 MEASUREMENT OF CHLOROPHYLL FLUORESCENCE

A Maxi-Imaging-PAM-fluorometer (Heinz Walz GmbH, Effeltrich, Germany) was used for chlorophyll *a* fluorescence measurements. The MAXI version of the IMAGING-PAM M-Series employs a very compact and powerful 300W LED array for homogeneous illumination of up to 10 x 13 cm areas with pulse-modulated excitation, actinic light and saturation pulses. The charge-coupled device (CCD) camera has a resolution of 640 x 480 pixels. Pixel value images of the fluorescence parameters were displayed using a false colour code ranging from black (0.000) through to red, yellow, green, blue and pink (1.000) (Berger et al., 2004).

Plants were dark adapted for at least 20 minutes prior to the determination of F_o and F_m (minimum and maximum fluorescence, respectively). The maximum quantum yield of photosystem two (PSII) photochemistry (F_v/F_m) was determined as $(F_m - F_o)/F_m$. Leaves were adapted to the specific light level and a saturating pulse of 0.8 s was applied in order to determine the maximum fluorescence (F'_m) and the steady-state fluorescence (F_s) during the actinic illumination. The quantum efficiency of PSII photochemistry, ΦPSII, was calculated using the formula: $(F'_m - F_s)/F'_m$ (Genty et al., 1989). The coefficient of photochemical quenching, qP, is a measurement of the fraction of open centers calculated as $(F'_m - F_s)/ (F'_m - F'_o)$(Schreiber et al., 1986). The value of F'_o was estimated using the approximation, $F'_o = F_o/(F_v/F_m + F_o/F'_m)$ (Oxborough and Baker, 1997). Calculation of quenching due to non-photochemical dissipation of absorbed light energy (NPQ) was determined at each saturating pulse, using the equation $NPQ = (F_m - F'_m)/ F'_m$ (Bilger and Björkman, 1991). The measured values of NPQ were divided by four to display values less than 1.000 so that the values fell within the range of the colour scale used for the other parameters. Chlorophyll fluorescence determinations were obtained from n = 7 leaves, selected at random on the images of whole plants. Images were taken at the following light intensities: 0, 55, 185, 335, 460, 610 and 700 µmol $m^{-2}s^{-1}$. Images at 610 µmol $m^{-2}s^{-1}$ were used to generate dehydration and rehydration curves as this was the light intensity closest to ambient light levels. Data from increasing light intensities was used to plot light response curves at ambient CO_2 concentration for various chlorophyll fluorescence parameters.

2.2.5 Confocal Microscopy

Confocal microscopy was used to observe chlorophyll fluorescence at different stages of drying, namely 100%, 75% and 10% RWC. Fresh leaves were excised from the plant and placed in a tin foil mold. 2% agarose in double distilled water was added to the molds and left to set with the leaf upright. It was then unmolded and affixed to a Perspex holder on a Vibratome Series 1000 (Technical Products International Inc., Missouri, USA). Sections were cut at 80 µm and a bath of double distilled water was used to catch sections. Sections were placed on a slide and immersed in 100% glycerol before covering with a cover slip.

The confocal microscope system used was a Zeiss LSM 510 Meta NLO (Carl Zeizz Inc., USA) equipped with a colour AxioCam HR and Axiovision 4.7 supporting software. To acquire images, the 488 nm laser line was used to scan at 5% intensity and fluorescence was detected with the filter adjusted to the red range (677-700 nm). Transmitted light images were recorded using the 488 nm laser in combination with the transmitted light confocal detector.

2.2.6 Pigment Analyses

Individual carotenoids were identified and quantified as reported in García-Plazaola and Becerril (1999). Fresh leaf material (120-150 mg) was extracted with 2 × 4 mL acetone (added with 0.5 g L^{-1} $CaCO_3$) and 15 µL aliquots were injected in a Perkin-Elmer Flexar chromatograph equipped with a 21 quaternary 200Q/410 pump and LC 200 diode array (DAD) detector (all from Perkin-22 Elmer, Bradford, CT, USA).

Photosynthetic pigments were separated by a 250 × 4.6 mm Waters Spherisorb ODS1 (5 µm) column operating at 30°C, eluted with a linear gradient solvent system, at a flow rate of 1.2 mL min^{-1}, consisting of $CH_3CN/MeOH/H_2O$ (8.4/0.8/0.7, A) and MeOH/Ethyl acetate (6.8/3.2, B) during an 18 min run: 0-12 min from 100% to 0% A; 12-18 min at 0% B.

Violaxanthin cycle pigments, lutein and α-, β-carotene were identified using visible spectral characteristics and retention times. The compounds were calibrated as such: Neoxanthin, violaxanthin and antheraxanthin with the calibration curve of lutein at lower concentration points (from Extrasynthese, Lyon-Nord, Genay, France); lutein with the calibration curve of lutein (from Extrasynthese, Lyon-Nord, Genay, France); zeaxanthin with the calibration curve of zeaxanthin (from Extrasynthese, Lyon-Nord, Genay, France); and α-, β- carotene with the calibration curve of β- carotene (from Extrasynthese, Lyon-Nord, Genay, France). Chlorophyll a and b were quantified by spectrophotometric analysis (Lichtenthaler and Buschmann, 2001). The dehydration-rehydration experiment was repeated twice and at least three biological replicates were obtained for each RWC point plotted.

2.2.7 STATISTICAL ANALYSES

Means and standard errors were calculated with Graph Pad Prism (Version 5). Biological replication varied by experiment and is indicated in the appropriate figure legend. The significance of differences between means at different relative water contents was analyzed using Student's *t*-test.

2.3 Results and Discussion

2.3.1 Nature of Dehydration and Rehydration

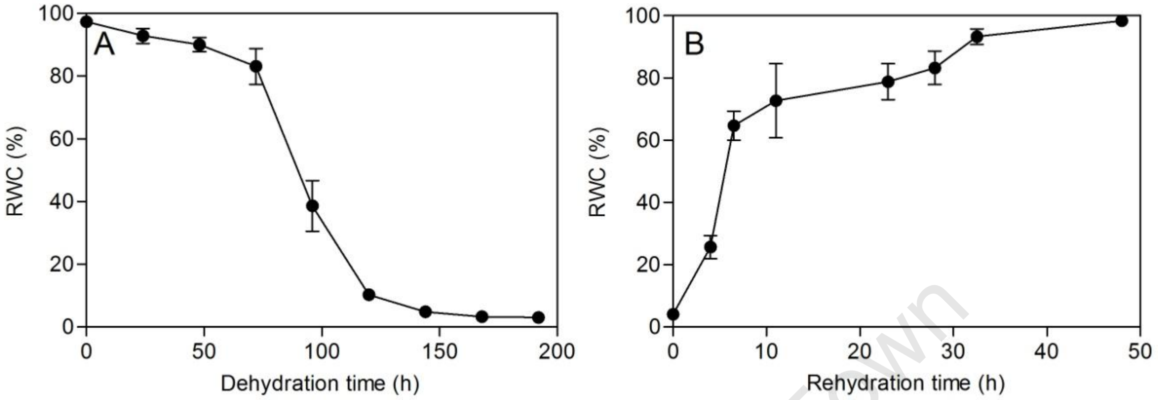


Figure 2-2: Time course for, A) dehydration, once plants had been left to dry naturally, and B) rehydration, once plants had been watered to pot capacity and maintained moist throughout rehydration (mean ± SEM, n=10).

Previous studies have investigated the time course for dehydration and rehydration for *X. Humilis* and other resurrection plants (Farrant et al., 1999, Sherwin and Farrant, 1996). However, the dehydration rate of plants will differ slightly depending on the size of the trays, soil depth and the amount of water plants receive. It was therefore necessary to establish a drying curve, with the specified measurements of tray size, soil depth and water to be received by the plants, so that this could be used as a reference for future experiments. As can be seen in Figure 2-2A, the plants maintained a high RWC for the first 72 hours after water had been withheld. Subsequently, there was a sudden decrease from 80 % to 30 % RWC within 24 to 48 hours, and thereafter the RWC decreased slightly as the plants lost the remaining water over three to four days. There were differences in drying rate between trays due to slight variations in soil depth.

The rate of drying in resurrection plants is very important in determining their survival (Farrant et al., 1999). The authors showed that *X. humilis* plants that were rapidly dried did not rehydrate and they lacked characteristics that normally provided mechanical stabilization and reduced oxidative damage. It is hypothesized that the rate of drying is important to allow sufficient time for plants to lay down protective mechanisms. It was therefore vitally important to allow plants to dry naturally in subsequent experiments. The rapid loss of water is unique to resurrection plants, and is hypothesized to happen only once protection has been accumulated in the first few days of drying (0 to 80 hours in Figure 2-2A) (Farrant, 2000, Farrant, 2007). The stomatal regulation during this period of dehydration will be investigated and discussed in Chapter 4.

Once plants received water again, rehydration took place steadily, as can be seen in Figure 2-2B, and the plants had reached full water content again within 48 hours. The rate of rehydration in resurrection plants differs amongst the various species (Sherwin and Farrant, 1996). *X. humilis* rehydrates relatively fast compared to the bigger *X. viscosa*, although rehydration of these poikilochlorophyllous plants is not as fast as in homoiochlorophyllous plants which do not need to reconstitute the photosynthetic apparatus (Sherwin and Farrant, 1996). Although dehydration of resurrection plants is the period when protective mechanisms against damage are laid down, rehydration is also challenging and is when repair and reconstitution must take place (Oliver et al., 1998). Rehydration is therefore a vital part of acquiring desiccation tolerance, and consequently, rehydration studies will also be performed in this thesis.

2.3.2 PHOTOSYNTHESIS

OPTIMIZATION OF CONDITIONS

Prior to conducting drought treatments and monitoring photosynthesis, the optimum conditions were determined. Figure 2-3A shows the response of CO_2 assimilation (A) to intercellular CO_2 (Ci), which is typical of an A/Ci curve (Long and Bernacchi, 2003). Initially, when Ci is low, the rate of photosynthesis predicts Rubisco activity, if there is a saturating supply of the substrate, RuBP. This is indicated by the initial linear portion of the graph (Figure 2-3A). As Ci increased further, the graph became curvilinear from 400 µmol mol^{-1} onwards, indicating the passage to photosynthesis limitation set by the rate of regeneration of RuBP, which is in turn controlled by light intensity. Theoretically, a third state is then reached at high CO_2 concentrations, called TPU limitation, where photosynthesis does not respond to increasing CO_2, and a plateau is reached (Long and Bernacchi, 2003, Sharkey et al., 2007). However, TPU limitation is often not seen in nature, and does not seem to occur even at very high CO_2 concentrations in *X. humilis*, as photosynthesis was still increasing slightly as Ci increased.

An external CO_2 concentration of 400 µmol mol^{-1} was used for future experiments as this is the ambient CO_2 concentration. Moreover, at this concentration, A is approaching the maximum and Rubisco activity is not the main limitation of photosynthesis anymore. A/Ci response curves will be discussed in relation to dehydration and rehydration later on.

To determine the light intensity at which photosynthesis is saturated, a light response curve was plotted (Figure 2-3B). Initially as light intensity increased from its minimum, photosynthesis increased rapidly and reached a plateau between 350 and 800 µmol photons m^{-2}s^{-1}. A light source with an intensity of 700 µmol photons m^{-2}s^{-1} was therefore used for all future experiments. This was also near to the average light intensity in the greenhouse at which plants were maintained. Photosynthesis was plotted as a percentage of the maximum obtained for the A/Ci and light response curves. This was due to considerable natural variation between leaves, with the maximum photosynthetic rate ranging between 12 and 20 µmol CO_2 m^{-2} s^{-1}.

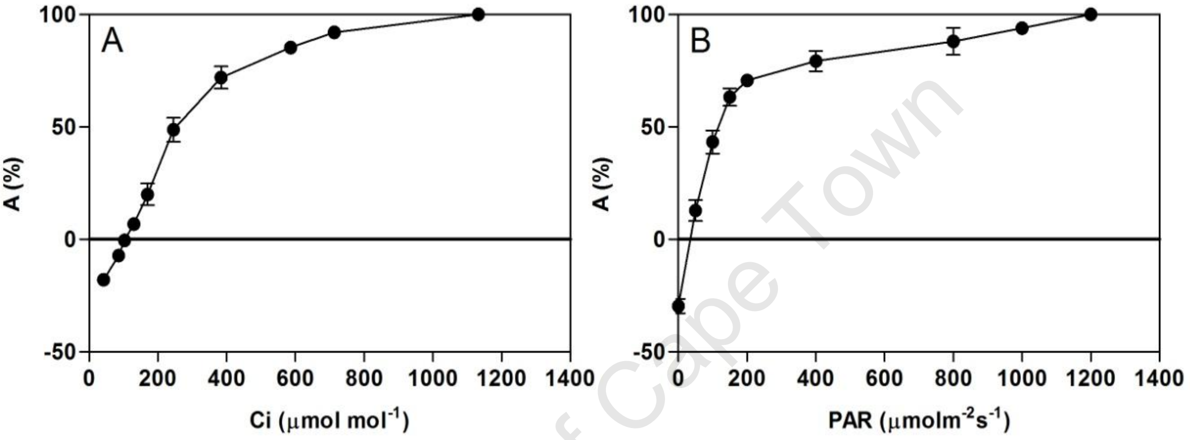


Figure 2-3: Response of photosynthesis as C_i increases through a range of concentrations (A), and the light source increases through a range of intensities (B), (mean ± SEM, n=3).

Regulation of Photosynthesis during Dehydration and Rehydration

Figure 2-4A shows how photosynthesis decreased during dehydration. Net photosynthesis started to decrease at relatively high water contents, between 80% and 75% RWC, and had ceased by 57% RWC. Previous studies in *X. humilis* have shown that cessation of photosynthesis is not limited by CO_2 availability, as respiration continued to much lower water contents compared to photosynthesis (Farrant, 2000). Rather, the reduction in photochemical activity is due to chlorophyll degradation or, as in some other resurrection plants, chlorophyll masking by anthocyanins (Sherwin and Farrant, 1996, Tuba et al., 1996). Therefore, chlorophyll content was also measured during dehydration. As can be seen in Figure 2-5A, chlorophyll content started to decrease at the onset of dehydration, and by 60% RWC, the total chlorophyll content was less than half the content at 100% RWC. The sudden decrease in chlorophyll content between 90% and 60% RWC coincides with the decrease in photosynthesis. Chlorophyll had been entirely degraded by the time the plants were completely dry.

These results are supported by numerous other studies in the *Xerophyta* species (Sherwin and Farrant, 1998, Tuba et al., 1996, Tuba et al., 1998). It is thought that poikilochlorophylly, as seen in these results, is a protective strategy to reduce photo-oxidation under water-limiting conditions (Farrant, 2000), and it is one of the major mechanisms that makes resurrection plants unique in their ability to survive drought.

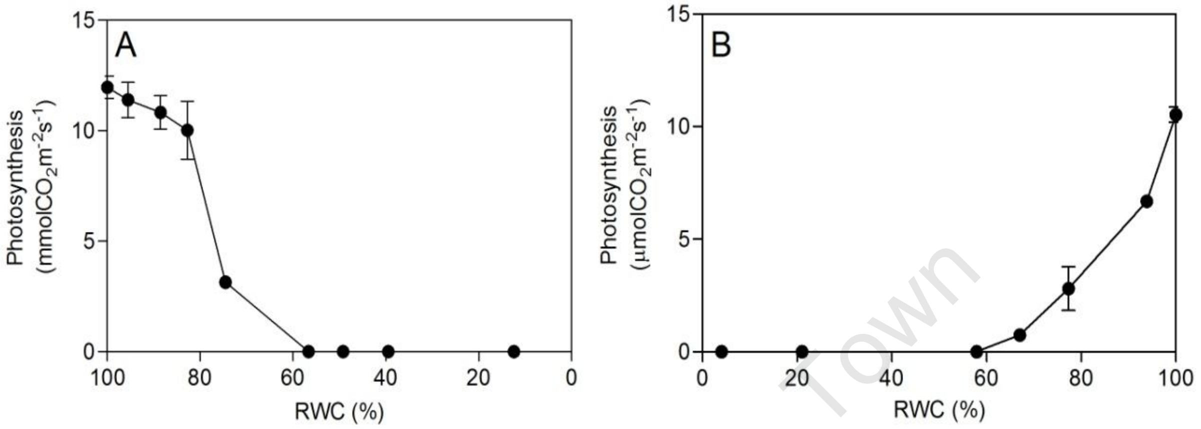


Figure 2-4: Change in photosynthetic rate during dehydration (A) and rehydration (B) of *X. humilis* (mean ± SEM, n=5), with a light intensity of 700 µmol m^{-2}s^{-1} and CO$_2$ set at 400 µmol mol^{-1}.

During rehydration, photosynthesis recovered once plants reached 60% RWC, as seen in Figure 2-4B. This corresponds to between 24 and 48 hours after re-watering, if Figure 2-2B is referred to. The RWC increased first whilst photosynthesis remained below zero. This is because chlorophyll molecules must first be regenerated and thylakoid membranes reassembled (Farrant, 2000). It is advantageous for water content to first increase before the photosynthetic rate has fully recovered to reduce the possibility for generation of ROS during rehydration. As can be seen in Figure 2-5B, there was initially a lag in chlorophyll regeneration, but once water content had recovered, the chlorophyll content rapidly increased from 80% RWC onwards (Figure 2-5B) and photosynthesis concurrently increased (Figure 2-4B).

Differences in recovery rate of metabolic activities in resurrection plants is thought to depend on the strategy employed to minimize stress associated with excess light, namely that poikilochlorophyllous plants, such as *X. humilis* in this study, will take longer to recover than homoiochlorophyllous plants which do not break down the photosynthetic apparatus (Sherwin and Farrant, 1998). Not only is the dismantling and reassembling of the photosynthetic apparatus observed in physiological studies, such as in this thesis and others as discussed, but it is also supported by molecular evidence (Collett et al., 2003). The authors showed that photosynthetic genes are actually differentially transcribed during dehydration and rehydration, which did not happen in the *Arabidopsis thaliana* control.

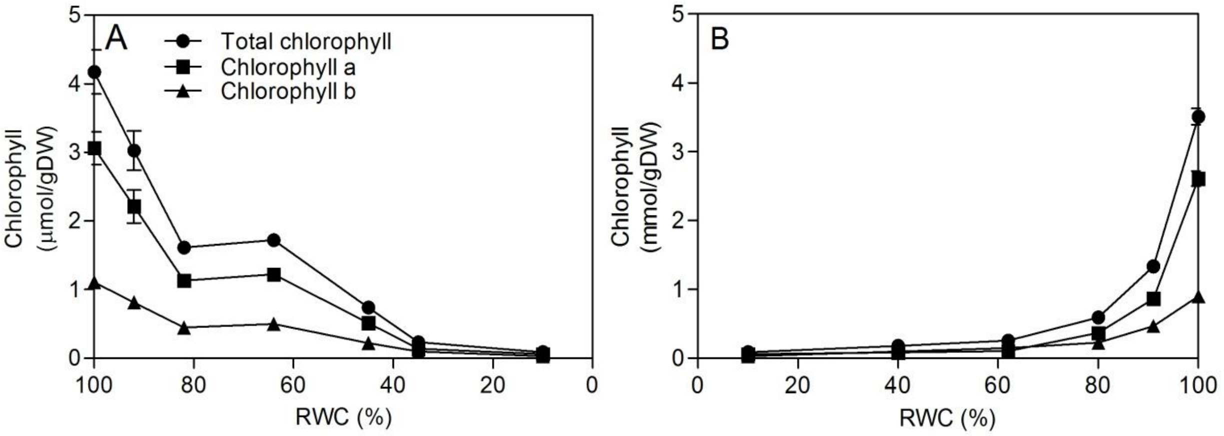


Figure 2-5: Change in total chlorophyll content, chlorophyll *a* and chlorophyll *b* during dehydration (A) and rehydration (B) of *X. humilis*, (mean ± SEM, n = 3).

A/Ci Response Curves during Dehydration and Rehydration

The response of photosynthesis to changing CO_2 concentration can provide information on a number of parameters related to leaf physiology (Sharkey et al., 2007). Once A/Ci curves had been performed under control conditions (Figure 2-3A), the curves were then performed during the initial stages of dehydration (Figure 2-6A) and the later stages of rehydration (Figure 2-6B). During the later stages of dehydration and the early stages of rehydration, photosynthesis and stomatal conductance were too low to accurately carry out the curve responses.

When Ci is low and the biochemical reactions of photosynthesis are considered to be limited by Rubisco, the initial slope of the curve gives a measure of the activity of Rubisco. As can be seen in Figure 2-6A, the slope of the control plants is slightly steeper than that of the plants at 95% RWC, indicating that at 95% RWC, Rubisco activity has been slightly inhibited. The second difference between control plants and those at 95% RWC is that the maximum photosynthesis reached is lower at 95% RWC. When CO_2 concentration is high, it does not limit photosynthesis anymore, and the difference in maximum photosynthesis reached is due to the different efficiency of light use, as light becomes the limiting factor. As light use depends on pigment concentration and functionality, it is likely that the difference in amount of chlorophyll affects this parameter in poikilochlorophyllous resurrection plants exposed to dehydration. Indeed, at 95% RWC the chlorophyll has already begun to be degraded. The compensation point gives the CO_2 concentration at which photosynthesis is equal to photorespiration and is determined by the point at which the graph intersects the x-axis. Normally, it is reported to be between 40 and 60 µmol mol^{-1} (Wullschleger, 1993). However, in this experiment, the compensation point for plants under control conditions and at 95% RWC was slightly higher at 80 µmol mol^{-1}. This could be due to the plants being at a higher temperature than is assumed by the LICOR gas exchange system. In particular, it may indicate that the mesophyll temperature was higher than indicated by the thermocouple gently pressed to the epidermal layer of the abaxial leaf side.

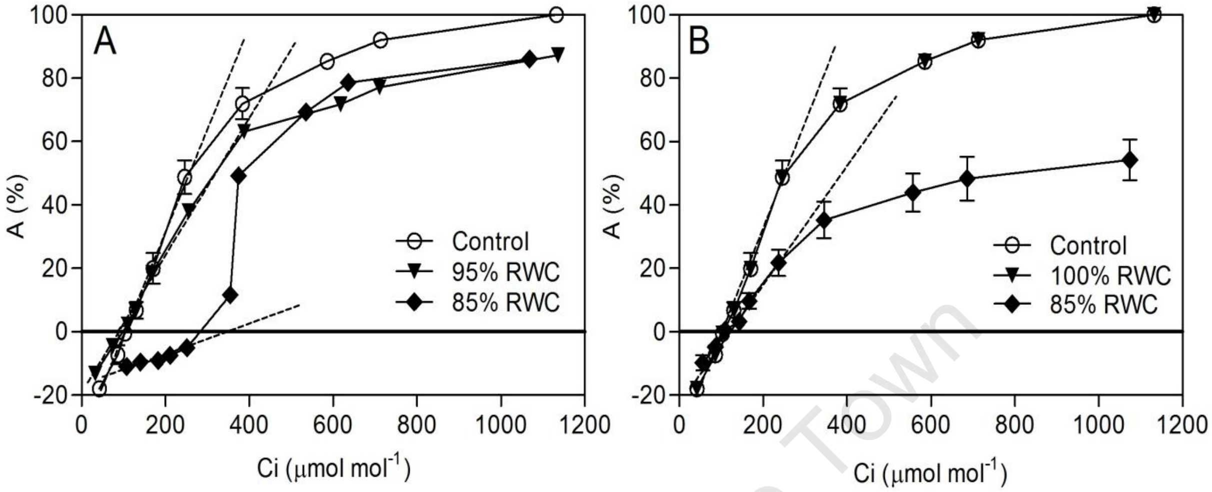
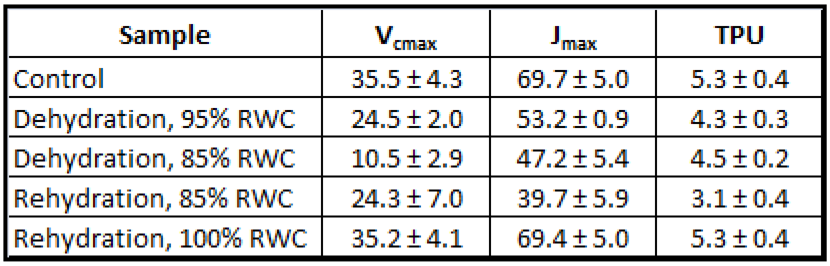


Figure 2-6: The response of net photosynthesis, A, to variation in intercellular CO_2, Ci, at various relative water contents during dehydration (A) and rehydration (B) of *X. humilis*, slopes of initial linear section of curves indicated by dotted lines (mean ± SEM, n=3).

The A/Ci response curve of plants at 85% RWC, seen in Figure 2-6A, is very interesting. The initial slope of the graph is greatly reduced compared to those at control conditions and 95% RWC. This shows that already at 85% RWC, Rubisco activity has decreased greatly. However, when CO_2 concentration is high, the plants at 85% RWC are still able to reach a relatively high maximum photosynthesis, comparable to plants at 95% RWC. This could also indicate that at 85% RWC, there is an internal resistance to CO_2 diffusion, which reduces CO_2 within the chloroplasts, compared to that measured in the intercellular spaces (Ci). When the external supply of CO_2 is increased, then the internal resistances could be overcome and photosynthesis is restored as in control plants, as seen for the curve of 85% RWC in Figure 2-6A. Another striking difference at 85% RWC is that the compensation point has been shifted to the right to 280 µmol mol^{-1}. This highlights that due to the decrease in Rubisco activity and degradation of chlorophyll at 85% RWC, a higher concentration of CO_2 is required for photosynthesis to firstly negate photorespiration and mitochondrial respiration (at the compensation point), and then overcome the respiratory processes and increase above 0. The decrease in Rubisco activity from early on, namely 85% RWC, corresponds to the rapid decrease in photosynthesis seen in Figure 2-3A. The impairment of Rubisco activity during the early stages of dehydration could limit photosynthesis in *X. humilis*; however as to whether this is true in all plants is still under debate. For example, in contrast to these findings, a study in 2004 found that the impairment of Rubisco activity and RuBP content in five plant species did not limit photosynthesis until drought was very severe, and photosynthesis was rather down-regulated by stomatal closure (Bota et al., 2004), however, these plants were desiccation sensitive. Stomatal regulation in *X. humilis* will be discussed in Chapter 4.

Plants were allowed to dehydrate completely and then after re-watering, the A/Ci response curve was performed again at 85% RWC (Figure 2-6B). The response at 85% RWC during dehydration is very different to that at 85% RWC during rehydration. As can be seen in Figure 2-6B, the slope of the graph for 85% RWC is only slightly lower than that for control plants, indicating that Rubisco activity is reduced, but not as severely as in plants at 85% RWC during dehydration. Secondly, the maximum photosynthesis reached is lower at 85% RWC during rehydration compared to the plants at 85% RWC during dehydration. This is because at 85% RWC during rehydration, the chlorophyll is still being regenerated. As seen in Figure 2-5B, the chlorophyll content is still very low at 85% RWC. The A/Ci curve was also performed once plants had reached 100% RWC again, 3 days after re-watering. As seen in Figure 2-6B, the curve for plants at 100% RWC after a complete cycle of dehydration and rehydration overlaps that for the plants at control conditions. This highlights that resurrection plants make a complete recovery after desiccation, whereas in desiccation sensitive plants, as has been shown in clover, long term water stress inactivates Rubisco (Medrano et al., 1997).

The Curve Fit Analysis Tool developed by Sharkey et al. (2007), gave estimates of the values for $V_{c,max}$ J_{max} and TPU, the outputs of which are summarized in Table2-1. The changes in these values during dehydration and rehydration emphasize what has been discussed above. Firstly, $V_{c,max}$ decreased rapidly during the initial stages of dehydration from 35.5 μmolm^{-2}s^{-1} at control conditions to 24.5 μmolm^{-2}s^{-1} at 95% RWC to 10.5 μmolm^{-2}s^{-1} at 85% RWC. This shows how the rate of Rubisco activity decreased rapidly from the start of dehydration. After rehydration $V_{c,max}$ returned to 35.5 μmolm^{-2}s^{-1}, indicating that Rubisco activity had fully recovered. TPU values did not change hugely, however did decrease slightly during dehydration and then increase again on rehydration.



Sample	V_{cmax}	J_{max}	TPU
Control	35.5 ± 4.3	69.7 ± 5.0	5.3 ± 0.4
Dehydration, 95% RWC	24.5 ± 2.0	53.2 ± 0.9	4.3 ± 0.3
Dehydration, 85% RWC	10.5 ± 2.9	47.2 ± 5.4	4.5 ± 0.2
Rehydration, 85% RWC	24.3 ± 7.0	39.7 ± 5.9	3.1 ± 0.4
Rehydration, 100% RWC	35.2 ± 4.1	69.4 ± 5.0	5.3 ± 0.4

Table 2-1: Summary of outputs obtained by the curve fit from the A/Ci curves. Values are in μmol m^{-2} s^{-1}, (mean ± SEM, n=3). Each column was analyzed using a one-way ANOVA and the means were found to be statistically significantly different (V_{cmax}: $p<0.05$, J_{max}: $p<0.01$, TPU: $p<0.01$).

J_{max} also decreased during dehydration and increased again during rehydration. The ratio J_{max}: $V_{c,max}$ gives an indication of the balance between RuBP regeneration and carboxylation (Ethier and Livingston, 2004, Wullschleger, 1993). The ratio at control conditions is 1.96 whereas at 85% RWC during dehydration it is much higher at 4.4. This once again indicates the great decrease in Rubisco activity at 85% RWC during dehydration.

2.3.2 CHLOROPHYLL FLUORESCENCE

CHANGING PARAMETERS DURING DEHYDRATION AND REHYDRATION

Light energy absorbed by chlorophyll molecules in a leaf will excite electrons and subsequently enters one of three processes, which are in competition. Either the energy in converted to chemical energy to drive photosynthesis, known as photochemistry, or excess energy, which is damaging to the leaf, can be emitted as heat, known as NPQ, or re-emitted as light, also called chlorophyll fluorescence. As the processes are in competition, by measuring changes in chlorophyll fluorescence, the efficiency of photochemistry and NPQ can indirectly be assessed (Maxwell and Johnson, 2000). Fluorescence analyses can give insights into a plant’s capacity to withstand environmental stresses and also give a measure of the amount of damage a stress has caused to the photosynthetic apparatus. In particular, fluorescence imaging allows an assessment of the heterogeneity of photosynthetic efficiency throughout a leaf during stress development and recovery. Therefore, a study into photosynthesis is more complete with fluorescence data and thus the technique was used in *X. humilis*, which had not been done before.

The change in chlorophyll fluorescence during dehydration can be seen in the graphs in Figure 2-7, with representative images of chlorophyll fluorescence of a plant taken at 100%, 60% and 25% RWC. F_o, measured when the plant had been dark-adapted and all reaction centers are assumed to be open, decreased slightly at the onset of dehydration, and then evened out for the remainder of dehydration (Figure 2-7A). F_m, measured during the high intensity rapid flash of light which causes all reaction centers to close, is reached in the absence of photochemical and non-photochemical quenching. As can be seen in Figure 2-7B, F_m follows a similar trend to F_o during dehydration, although the initial decrease is extended over a longer period of time. As can be seen in the images for F_m, there is heterogeneity in the leaves’ ability to reach maximal fluorescence. This is most evident at 100% RWC, as some areas of the leaves are green whilst most leaves are yellow-orange. This is natural variation within the leaves of a single plant, and hence readings were taken from several leaves.

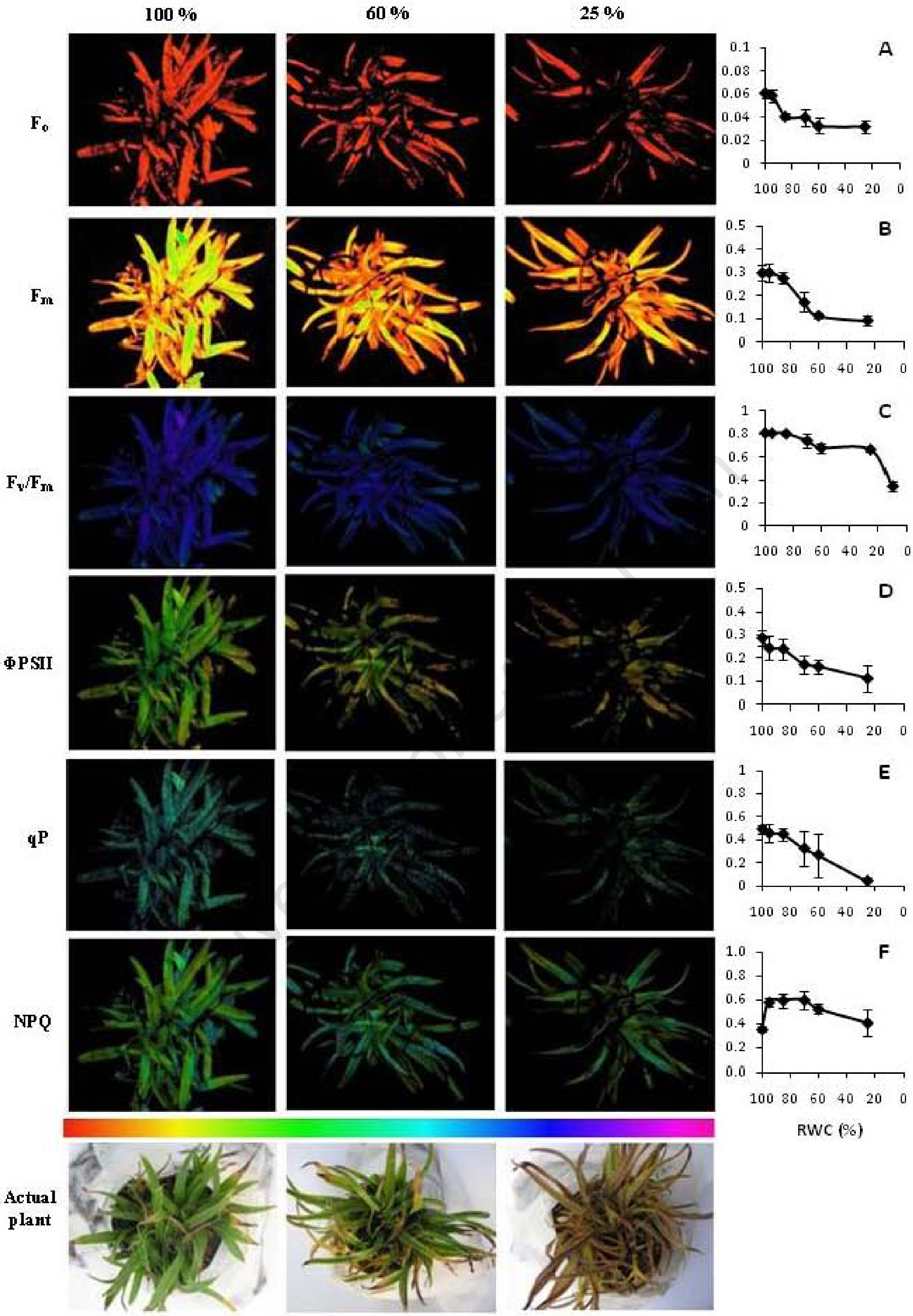


Figure 2-7: Selected chlorophyll fluorescence images of F_o, F_m and F_v/F_m in dark-adapted plants and ΦPSII, qP and NPQ at steady-state actinic illumination of 610 µmol photons.m^{-2}.s^{-1} measured at 100%, 60% and 25% RWC during dehydration of *X. humilis*, with photographs inserted of the actual plants. The false colour code depicted ranges from 0 (black) to 1 (pink). Graphs show changes in above mentioned parameters during dehydration, measured at 100%, 95%, 85%, 70%, 60% and 25% RWC (mean ± SE, n=7).

F_v/F_m, the maximum efficiency of PSII in dark-adapted leaves, remained relatively high during the initial stages of dehydration and started to decline from about 50% RWC with a rapid decline from 20% RWC onwards (Figure 2-7C). As can be seen in the images for F_v/F_m, the false colour remained blue-purple even until 25% RWC, indicating at this stage that the efficiency of PSII, if all reaction centers were open, was still relatively high. The response curve of F_v/F_m to dehydration in this study corresponds to that seen in Farrant et al. (1999), where F_v/F_m also started to decline from 50% RWC onwards and in another study conducted in the resurrection plant *Selaginalla lipidophyll* where F_v/F_m remained high through a range of RWC and only decreased from 40% RWC (Eickmeier et al., 1993).

Figure 2-7D shows the true efficiency of photosystem II (ΦPSII) during dehydration. ΦPSII measures the proportion of light absorbed by PSII when leaves are illuminated and photosynthesis is activated, and therefore measures the rate of linear electron transport rate driving photosynthesis and photorespiration. As such, it estimates overall photosynthesis and therefore had a similar trend to that of the photosynthetic rate (Figure 2-4A).The images for ΦPSII clearly show how the efficiency of PSII rapidly declined during dehydration, with the false colour changing from green to orange, as well as an evident decrease in surface area from which fluorescence could be measured.

Photochemical quenching (qP) decreased steadily during dehydration (Figure 2-7E). qP estimates the proportion of reaction centers that are open, and therefore shows that although the possible maximum efficiency (F_v/F_m) remained relatively high until the late stages of dehydration (Figure 2-7C), indicating the efficiency if all reaction centers were open, it is the closure of the reaction centers, indicated by the decrease in qP, that alters the actual efficiency of PSII (Figure 2-7D). This closing of reaction centers, together with the breakdown of chlorophyll, as discussed previously in Figure 2-5A, contributes to the decline in PSII.

Molecular studies in *X. humilis* and *X. viscosa* have shown that during dehydration, there is a down-regulation of genes (Collett et al., 2004, Collett et al., 2003) and proteins (Ingle et al., 2007) involved in PSII assembly and functionality. This supports the physiological observations seen in this study and others (Farrant et al., 1999, Sherwin and Farrant, 1996), that poikilochlorophyllous plants actually actively deactivate the photochemistry of photosynthesis during dehydration.

Non-photochemical quenching (NPQ) is the mechanism whereby plants convert excess excitation energy to heat, thereby reducing the chance for damage, especially in high light conditions. NPQ initially increased as the plants dehydrated from 100% to 80% RWC (Figure 2-7F). From 60% RWC onwards, NPQ then decreased, which coincides with the decrease in photosynthesis as chlorophyll is broken down.

As can be seen in the fluorescent images in Figure 2-7, there seems to be a relatively large heterogeneity in some fluorescent parameters, both between leaves and even within some leaves. This is most obvious for the parameters F_m and NPQ, where there are at least 2 or 3 false colours displayed in the images. This provided a difficulty when calculating the mean value of a fluorescent parameter for the whole plant. To overcome this, the colour most prevalent in the image was chosen and then 7 leaves, each of which showed minimal heterogeneity, were chosen to measure and used to calculate the mean. The possible reasons for the heterogeneity is that leaves of different ages could dehydrate at different rates and also the tips and edges of the leaves are older and therefore could dehydrate quicker, resulting in the variation within some leaves. This should be taken into account in future gas exchange studies in the *Xerophyta* species, in that leaves of a similar age should be used and the number of leaves measured should be increased to account for the heterogeneity between leaves and also within leaves in this species. This finding highlights the value of imaging fluorescence.

The photographs of the actual plants in Figure 2-7 show how the colour of the plants changed from green to yellow to brown during dehydration, highlighting the breakdown in chlorophyll. As can be seen in these images, the leaves also began to fold in half along the midrib as the RWC decreases. This is thought to be a protective mechanism of *X. humilis* to cope with drought stress (Farrant, 2000), and will be discussed in more detail in Chapter 4.

During rehydration, there was a recovery of the chlorophyll fluorescence parameters as seen in Figure 2-8. By 60% RWC, plants had begun to recover the maximum efficiency of PSII, and F_v/F_m had fully recovered before water content had reached 100% (Figure 2-8C). The complete recovery of F_v/F_m indicates that chloroplasts became fully functional once again after rehydration. This concurs with previous work done in *X. viscosa* (Sherwin and Farrant, 1996) and *X. humilis* (Ingle et al., 2008). Another study in *X. humilis* found that there was little increase in F_v/F_m in the first 10 hours of rehydration (Dace et al., 1998). Although these results are expressed as a function of increasing RWC (Figure 2-8C), the results are comparable to Dace et al. (1998) if the time course for rehydration is noted (Figure 2-2B).

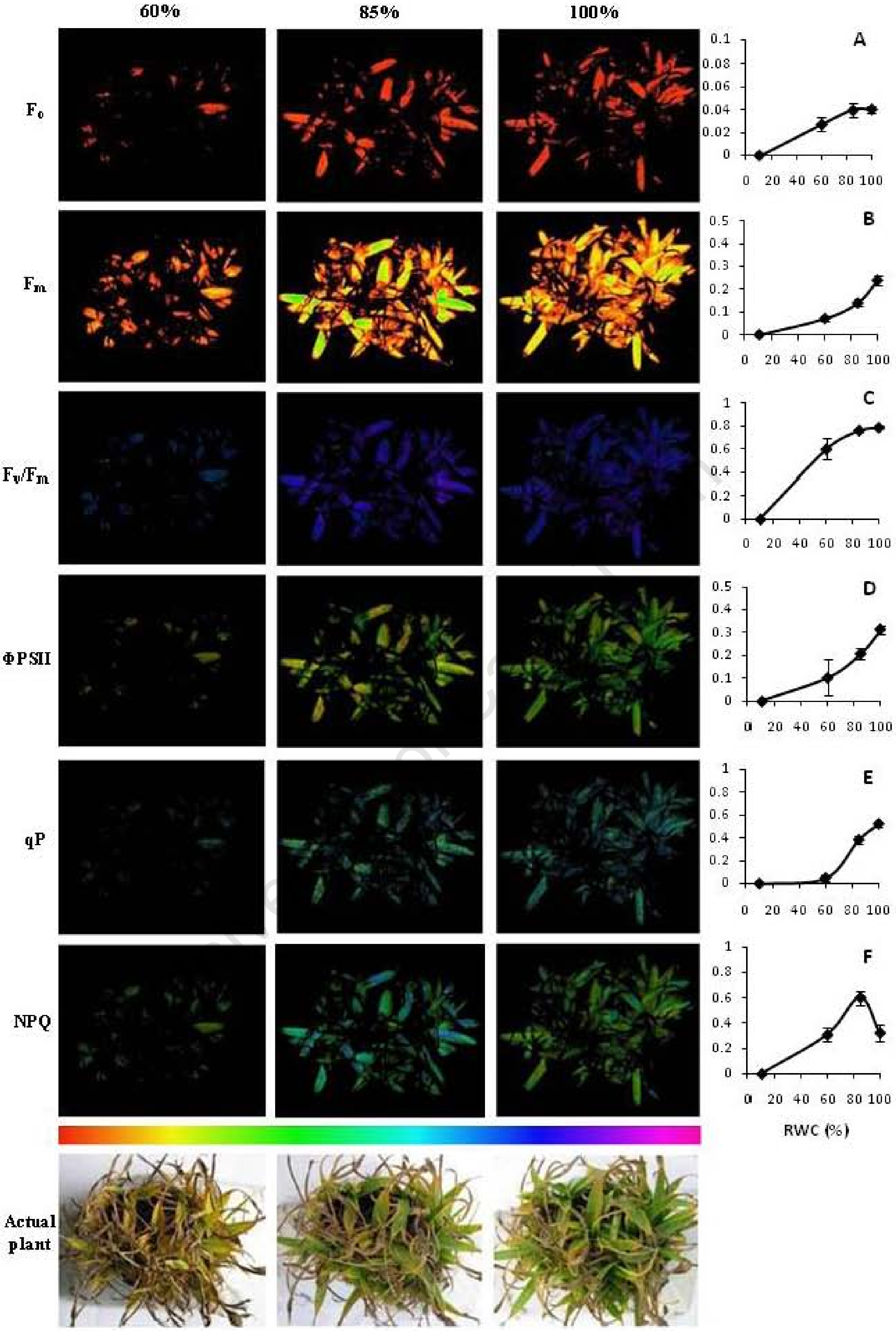


Figure 2-8: Selected chlorophyll fluorescence images of F_o, F_m and F_v/F_m in dark-adapted plants and ΦPSII, qP and NPQ at steady-state actinic illumination of 610 µmol photons.m^{-2}.s^{-1} measured at 60%, 85% and 100% RWC during rehydration of *X. humilis*, with photographs inserted of the actual plants. The false colour code depicted ranges from 0 (black) to 1 (pink). Graphs show changes in above mentioned parameters during rehydration, measured at 10%, 60%, 85% and 100% RWC (mean ± SE, n=7).

Figure 2-8D shows how ΦPSII increased during rehydration. Unlike F_v/F_m which increased rapidly, there was a lag in the initial increase of ΦPSII. Although the theoretical maximum efficiency of PSII had started to recover by 60% RWC (Figure 2-8C), the actual PSII efficiency in illuminated leaves (ΦPSII) was still very low at 60% RWC (Figure 2-8D). This is possibly due to the limit of the number of reaction centers that were open, indicated by qP (Figure 2-8E), which had not begun to increase significantly by 60% RWC. Ingle et al. (2008) studied the biogenesis of chloroplasts in *X. humilis* and observed a recovery of ΦPSII within 12 hours of re-watering and had almost fully recovered by 15 hours. If Figure 2-2B is used to compare time after re-watering and recovery of RWC, it can be seen that the results in this study for ΦPSII correspond to those of Ingle et al. (2008).

NPQ increased steadily during rehydration from 0% to 85% RWC (Figure 2-8F). However, from 80% RWC until plants had completely rehydrated, NPQ actually decreased again to 0.4, which corresponds to the reading prior to dehydration (Figure 2-8F). This is clearly illustrated in the images for NPQ, where the leaves in the image at 85% RWC are mostly blue, whereas at 100% RWC, the leaves are mostly green, indicating a lower value on the false colour code. The values for NPQ have been shown after the initial values have been divided by 4 to fit within the false colour code. The drop in NPQ is interesting as it represents the moment in which PSII becomes fully operational again and the leaves therefore do not need to dissipate excess energy as heat anymore.

The photographs of the plants in Figure 2-8 illustrate how *X. humilis* leaves rehydrate from the base to the tip. The leaves in the photograph of the plant at 60% RWC are still mostly yellow as chlorophyll had not been regenerated yet, although RWC had begun to recover. By 85% RWC the leaves are green, although ΦPSII had not fully recovered yet, as discussed previously. The ends of some of the leaves still had not rehydrated by 100% RWC in this plant, as seen in the photograph at 100% RWC. This could be due to old age as after repeated cycles of drying and rehydrating, the leaves lose their capacity for rehydration and senesce.

LIGHT RESPONSE OF CHLOROPHYLL FLUORESCENCE PARAMETERS

The onset of dehydration had an effect on the light response curve for ΦPSII (Figure 2-9A). Under low light conditions, between 0 and 110 µmol $m^{-2}s^{-1}$, ΦPSII remained similar during the initial stages of drying, as the graphs of 100%, 85% and 60% RWC are overlapping. However, as light intensity increased above 110 µmol $m^{-2}s^{-1}$, dehydration had a bigger effect on ΦPSII. Under severe dehydration, as shown by the graph for 25% RWC in Figure 2-9A, the efficiency of PSII is affected even under low light conditions.

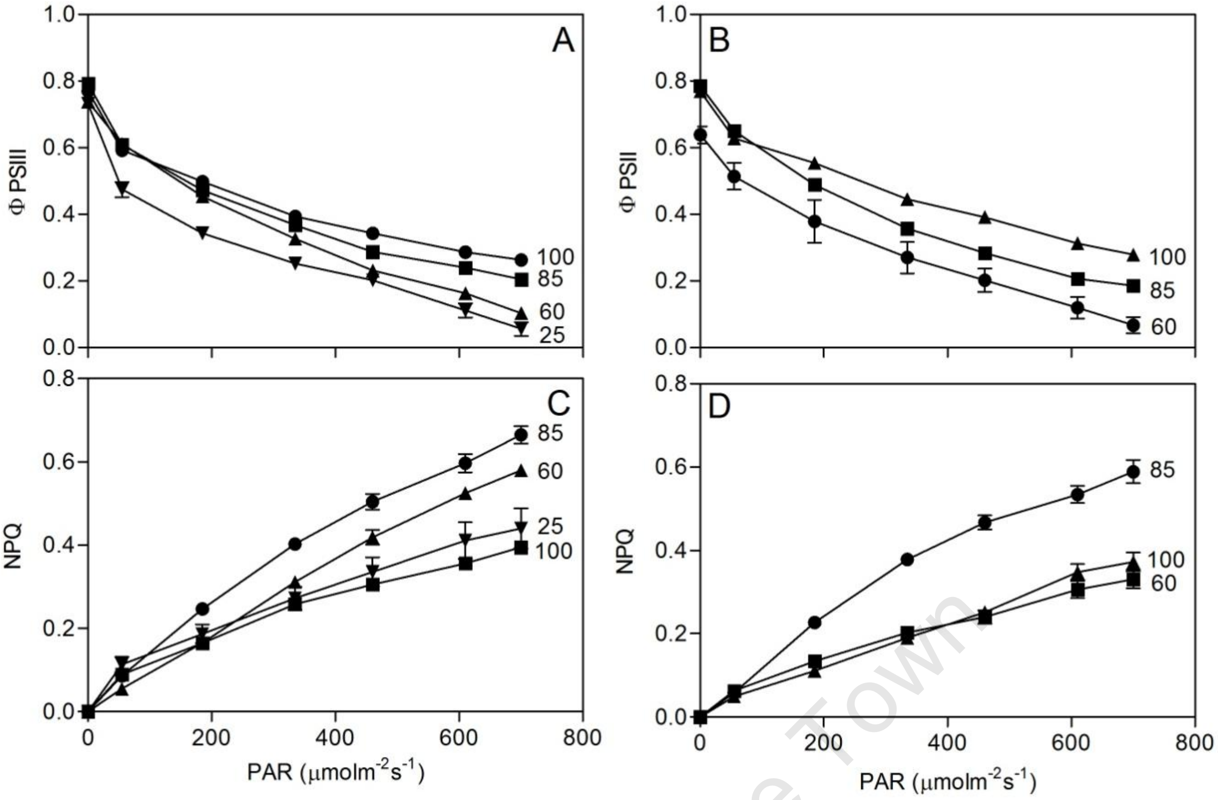


Figure 2-9: Light response of the chlorophyll fluorescence parameters ΦPSII (A, B) and NPQ (C. D), in *X. humilis* at various relative water contents during dehydration (A, C) and rehydration (B, D), (mean ± SEM, n=7).

In Figure 2-9C, it is possible to see how the plants developed non-photochemical quenching as light intensity increased at different stages of dehydration. These results are very interesting as the plants at 100% RWC (fully hydrated) and those at 25% RWC (dehydrated) have a similar response to increasing light intensity. Moreover, these two response curves are below those for the plants at intermediate water contents, namely 60% and 85% RWC. Possible explanations for these observations are that at 100% RWC, the plants were unstressed and therefore NPQ was low, however, as RWC decreased to 85%, NPQ increased to help dissipate excess energy as the chlorophyll content was still relatively high at this RWC (Figure 2-5A). At 60% RWC, NPQ was lower than at 85% RWC, but still higher than fully hydrated plants (Figure 2-9C). This might be because chlorophyll was significantly reduced at 60% RWC (Figure 2-5A) and various other protective mechanisms had been upregulated at this stage. For example, the carotenoids lutein and zeaxanthin started to accumulate at this stage, as discussed below, and as Farrant (2000) found, anthocyanin content accumulation was also almost complete by this stage of dehydration of *X. humilis*. There was therefore less reliance on NPQ to dissipate excess energy at 60% RWC compared to 85% RWC. At 25% RWC, chlorophyll was completely degraded (Figure 2-5A), and all other protective mechanisms had been laid in place. For example, lutein and zeaxanthin were highest in severely dehydrated tissues, as discussed in the following section. Therefore at such a severely dehydrated state

(25% RWC), plants lose the capacity to dissipate excess energy as heat due to the already large loss of chlorophyll molecules and rely on alternative protective mechanisms, resulting in an NPQ response similar to that of fully hydrated plants, as seen in Figure 2-9C.

During rehydration, under low light conditions, ΦPSII was already lower at 60% RWC compared to 85% and 100% RWC, which overlapped initially, however at high light intensities, ΦPSII was lower at 85% RWC (Figure 2-9B). The response of NPQ to increasing light intensities during rehydration is also interesting (Figure 2-9D). The response curves for plants at 60% and 100% RWC overlap, whilst that for 85% RWC is considerably higher. This could be because at 60% RWC, chlorophyll was still very low (Figure 2-5B) and therefore there was no need to dissipate excess energy as heat. However, as plants rehydrated further to 85% RWC, and chlorophyll content (Figure 2-5B) and photosynthesis (Figure 2-4B) increased, NPQ subsequently increased to dissipate excess energy which formed when plants were not yet fully rehydrated and still in a stressful condition.

VISUALIZATION OF CHLOROPLASTS

Chlorophyll autofluorescence can be detected on a confocal microscope, as can be seen in the images of leaf sections at different stages of dehydration in Figure 2-10. When the sections were excited at 488nm, a small portion of the light, which is not absorbed by the photosynthetic pigments, is de-excited and emitted as red chlorophyll fluorescence. The images in Figure 2-10 show how this fluorescence decreased during dehydration as chlorophyll was broken down. The fluorescence also indicates the position of chloroplasts. This is clearly seen in the leaf section at 100% RWC (Figure 2-10A), where the chloroplasts are lining the periphery of the cells. This is to maximize the exposure of the chloroplasts to sunlight for photosynthesis (Taiz and Zeiger, 2002).

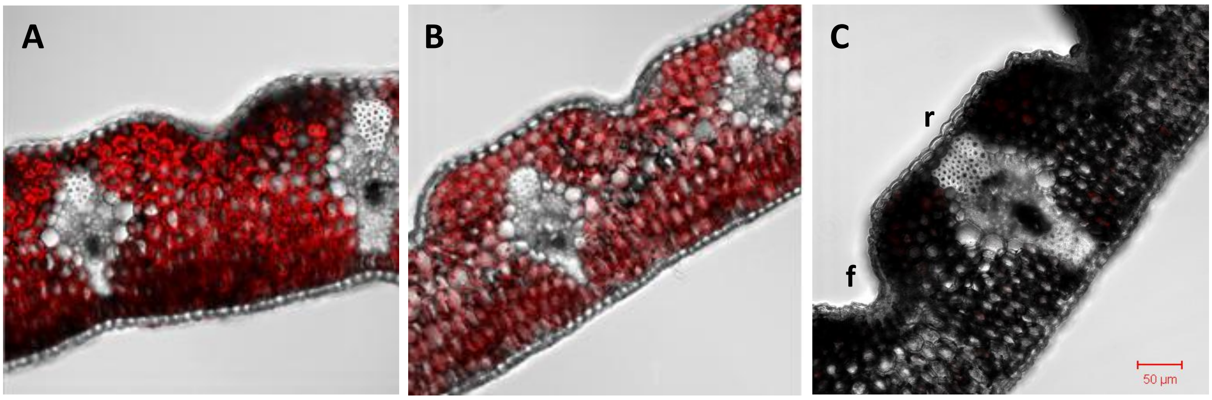


Figure 2-10: Confocal microscope images showing chlorophyll fluorescence detected in the red range (677-700nm), at 100% RWC (A), 75% RWC (B) and 10% RWC (C). r, ridge; f, furrow.

The leaves of *X. humilis* are characterized by ridges and furrows. The ridges become more pronounced on drying and the furrows deepen as the ridges draw closer together. This is evident in a comparison between a fully hydrated leaf (Figure 2-10A) and a dry leaf (Figure 2-10C) in which the furrows are much deeper. This will be discussed in more detail in Chapter 4.

2.3.4 Carotenoid Pigment Analyses

Carotenoids play a significant role in photoprotection as they can quench reactive oxygen species, thereby reducing permanent damage associated with excess excitation energy under stress situations, namely drought (Demmig-Adams and Adams Iii, 1996, Larson, 1988, Munné-Bosch and Alegre, 2000). Therefore, in order to fully understand the nature of photosynthetic regulation and poikilochlorophylly in X. *humilis*, various carotenoids were quantitatively analysed during dehydration and rehydration. The data are expressed firstly relative to dry weight, as this is the protocol followed for resurrection plants (Farrant et al., 2003, Georgieva et al., 2008), and secondly the data are expressed relative to total chlorophyll content as is the accepted protocol universally. Both sets of results are given as different points can be made from each, as will be discussed.

Xanthophylls

During the xanthophyll cycle, violoaxanthin undergoes stepwise removal (de-epoxidation) of the epoxy groups resulting in antheraxanthin and then zeaxanthin, and solar radiation is dissipated as heat (Figure 2-11) (Demmig et al., 1988, Demmig-Adams and Adams Iii, 1996). During dehydration of *X. humilis*, it appears as though violaxanthin is converted to zeaxanthin. This is seen in Figure 2-12A, as violaxanthin decreased and had almost disappeared at the end of dehydration, whilst zeaxanthin increased during dehydration. The cycling of violaxanthin to zeaxanthin exceeded the reverse reaction, which regenerates violaxanthin, and zeaxanthin therefore accumulated during dehydration.

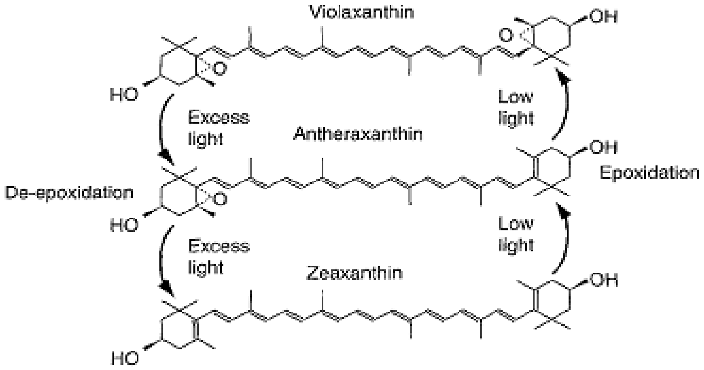


Figure 2-11: Scheme of the xanthophyll cycle (Figure taken from Demmig-Adams and Adams (1996).

Previous studies in resurrection plants have expressed carotenoid content relative to dry weight, as in Figure 2-12, rather than total chlorophyll content as is universally accepted, as during dehydration and rehydration, chlorophyll content changes drastically in resurrection plants. However, expressing carotenoids in resurrection plants relative to total chlorophyll content also provides valuable, interesting information as it highlights how although chlorophyll content is decreasing during dehydration, some carotenoids actually increase in concentration, which is unusual when compared to a desiccation-sensitive system where carotenoid content is tightly linked to chlorophyll content. When the carotenoids are expressed relative to total chlorophyll content, it can be seen in Figure 2-13A, that the increase in zeaxanthin during dehydration is very significant, especially from 40% to 20% RWC (two-tailed t-test, t=5.344, df=4, p<0.001). This great increase corresponds with the initial increase in NPQ during dehydration (Figure 2-7F).

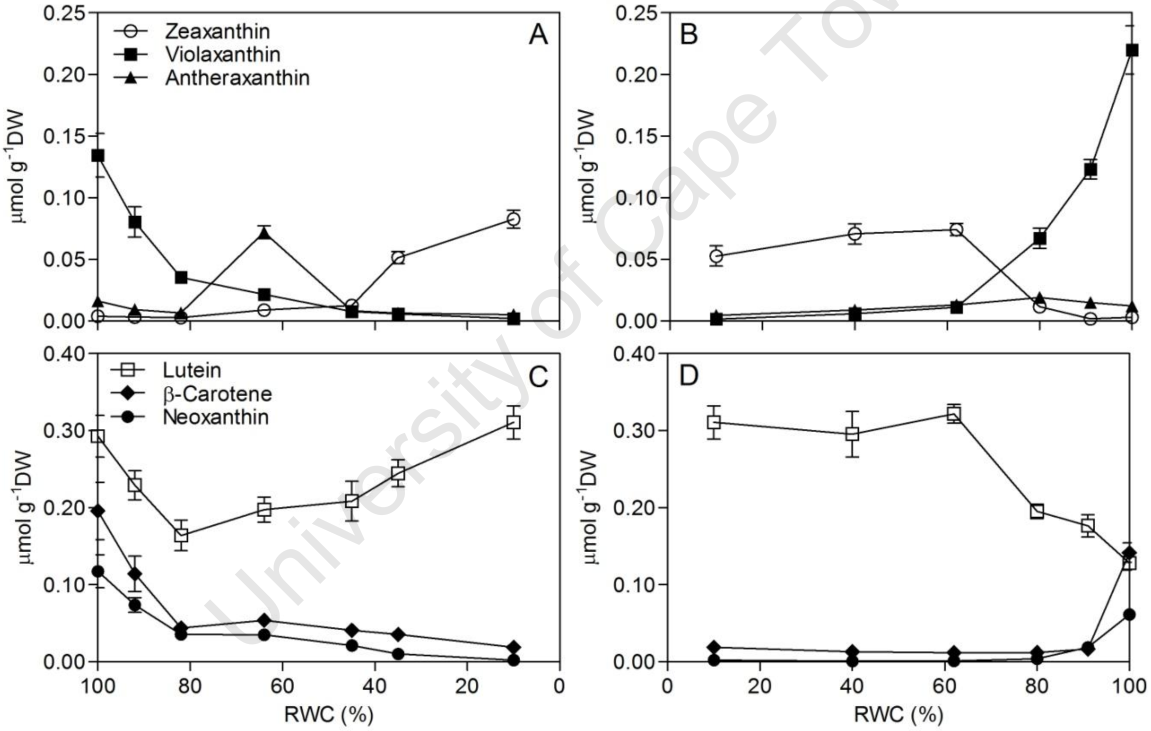


Figure 2-12: Change in carotenoid pigments during dehydration (A, C) and rehydration (B, D) of *X. humilis* relative to dry weight, (mean ± SEM, n=3).

Although the xanthophyll cycle is triggered by excess light (Figure 2-11), a study done in 1988 by Demmig et al. showed that in response to high light, the zeaxanthin content increased significantly more in water stressed plants of *Nerium oleander* compared to well-watered plants. This concurs with the profound increase in *X. humilis* in response to dehydration. The level of antheraxanthin showed a less pronounced change during dehydration and remains low (Figure 2-12A). However, there was a peak in antheraxanthin at 65% RWC. This corresponds to the period in which zeaxanthin and violaxanthin are both relatively low and could reflect the de-epoxidation of violaxanthin to zeaxanthin via antheraxanthin.

Resurrection plants that are homoiochlorophyllous, unlike *X. humilis,* generate a vast amount of ROS during dehydration due to retaining chlorophyll, and therefore utilize the xanthophyll cycle to help reduce ROS formation (Kranner et al., 2002). Although *X. humilis* is poikilochlorophyllous, it still generates ROS, especially during the early stages of dehydration when chlorophyll has not been completely degraded (Farrant, 2000). Therefore, the xanthophyll cycle provides one mechanism to reduce ROS formation during dehydration, as zeaxanthin and antheraxanthin appear to quench the singlet excited state of chlorophyll (Demmig-Adams and Adams, 1992). Previous studies (Farrant, 2000, Farrant et al., 2003) have reported a decrease in total carotenoid content for *X. humilis*, which may be the overall scenario. As can be seen in Figure 2-12A and C, most carotenoids do decrease. However, certain carotenoids increase, namely zeaxanthin which is most obvious in Figure 2-12A. Possible reasons for the differences in these results are that in the previous studies, total carotenoid content was determined spectrophotometrically, whereas in this study individual carotenoids were quantified by high performance liquid chromatography, therefore enabling individual trends in carotenoids to be distinguished.

During rehydration, the reverse reaction took place. As can be seen in Figure 2-12B, when expressed relative to dry weight, zeaxanthin levels remained constant during the initial stages of rehydration and then decreased rapidly from 60% RWC onwards with violaxanthin increasing from 60% RWC onwards. At the end of rehydration violaxanthin content was higher than it was prior to dehydration. Zeaxanthin possibly remained high until 60% RWC to reduce the formation of active oxygen species which may form when water content is still low and chlorophyll is being resynthesized again. Activation of the epoxidation of zeaxanthin back to violaxanthin seems to occur at 60% RWC during rehydration. Antheraxanthin levels did not change significantly. Figure 2-13B clearly shows how zeaxanthin, which was very high in desiccated tissues, decreased back to control levels during rehydration, with a significant decrease early in rehydration from 10% to 40% RWC (two-tailed *t*-test, $t=4.156$, $df=4$, $p<0.05$). A study done in *Rosmarinus officinalis* found that the de-epoxidation of violaxanthin to zeaxanthin increased during drought, however, when the rainy season returned, the authors noted a reversal of the cycle and regeneration of violaxanthin (Munné-Bosch and Alegre, 2000). This supports the findings in this study, although rehydration occurred from a completely desiccated state.

Neoxanthin decreased during dehydration, and upon rehydration it increased again and reached a level comparable to what it was before dehydration (Figure 2-12C and D). As seen in Figure 2-11C, when lutein is expressed relative to dry weight, the concentration initially decreased as the plants dehydrated from 100% to 90% RWC, and from then on it increased slightly again. Although at the end of dehydration, the level was still relatively high. However, if lutein is expressed relative to total chlorophyll content as seen in Figure 2-13C, it can be seen that as plants dehydrated from 45% to 20% RWC, there was a huge increase in lutein (two-tailed *t*-test, t=7.25, df=4, p<0.01). During rehydration, the level of lutein decreased as is seen in both Figure 2-12D and Figure 2-13D.

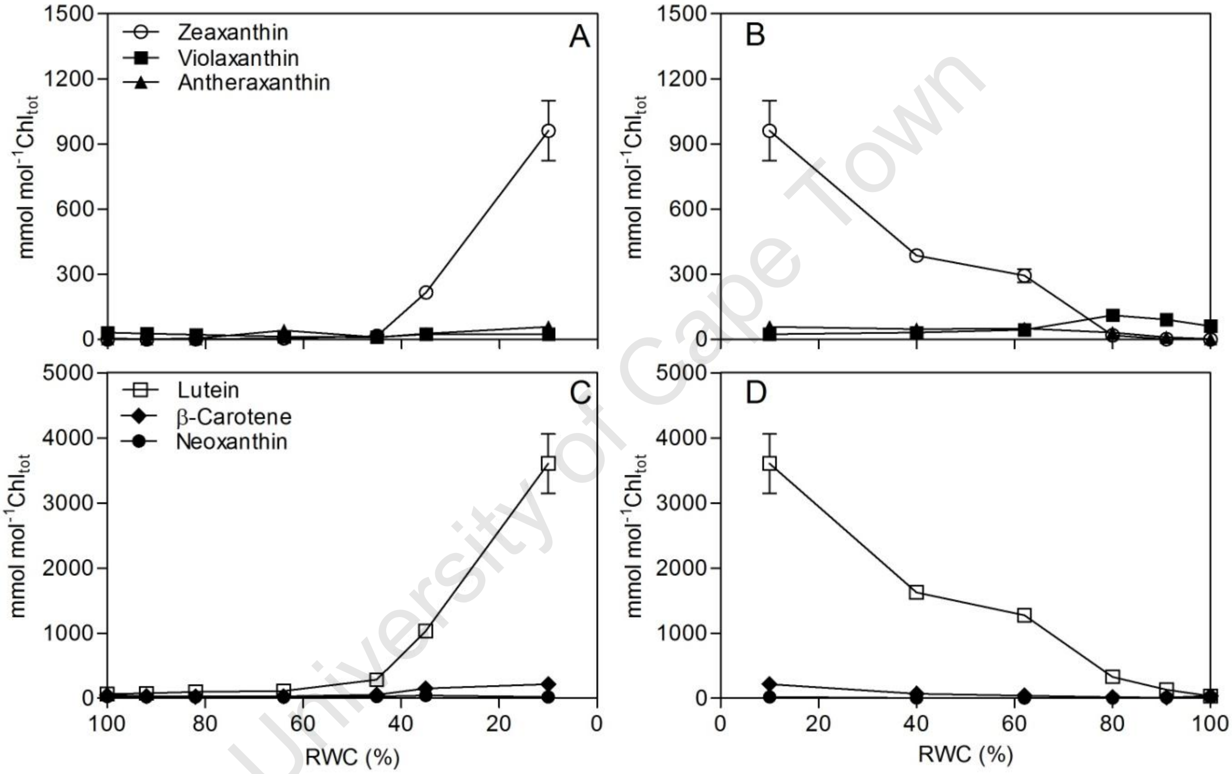


Figure 2-13: Change in carotenoid pigments during dehydration (A, C) and rehydration (B, D) of *X. humilis* relative to total chlorophyll content, (mean ± SEM, n=3).

When results are expressed on a chlorophyll basis, as opposed to dry weight, the 60% RWC threshold is lost and both lutein and zeaxanthin seem to change more gradually, particularly during rehydration. When expressed as dry weight, zeaxanthin and lutein remained high until 60% RWC during rehydration and then decreased (Figure 2-12B and D), but when expressed as total chlorophyll, zeaxanthin and lutein content decreased steadily from the beginning of rehydration (Figure 2-13B and D). These differences can possibly be explained due to the synthesis of chlorophyll during rehydration of *X. humilis* and therefore the increase in the total amount of chlorophyll which was used to normalize carotenoid contents.

Lutein helps to protect photosynthesis under environmental stresses (Demmig-Adams and Adams, 2002) and plays an important role in NPQ (Niyogi et al., 1997). The increase in lutein during dehydration is beneficial to the plants as it will help to reduce oxidative damage during dehydration. Lutein will also subsequently be present upon rehydration, as indicated in Figure 2-12D and Figure 2-13D, to protect photosynthesis as it is re-established under stressful conditions. These results differ to those of other findings, for example, in the resurrection plant *Myrothamnus flabellifolia*, lutein was found to decrease during dehydration and then increase again upon rehydration (Kranner et al., 2002). So too during severe drought stress of *Rosmarinus officinalis*, the authors noted a depletion in lutein (Munné-Bosch and Alegre, 2000).

β-Carotene

During dehydration, β-carotene was progressively decreased (Figure 2-12C) and during the very late stages of rehydration, the level recovered (Figure 2-12D). β-carotene probably decreased during dehydration as it was expended due to its action as an antioxidant and quenching ROS or absorbing excess light and therefore protecting the plant from oxidative damage (Telfer, 2002, Larson, 1988). During rehydration β-carotene is re-synthesized so that it can also scavenge ROS that may be formed under high light. β-carotene is also an accessory pigment to photosynthesis (Demmig-Adams and Adams, 2002) which may explain why there was a sudden increase in β-carotene only at the end of rehydration once photosynthesis had begun to recover during rehydration. These trends in β-carotene are comparable with those observed in other resurrection plants (Kranner et al., 2002).

2.4 CONCLUSION

Xerophyta humilis has very effective mechanisms to reduce and alleviate oxidative damage, arising from an uncoupling of photosynthesis during dehydration. In response to dehydration these include, a controlled deactivation of photosynthesis resulting in cessation at about 50% RWC; a breakdown of chlorophyll; a change in the regulation of carotenoids to improve antioxidant responses including activation of the xanthophyll cycle. During rehydration, chlorophyll is resynthesized resulting in metabolic activities resuming and various carotenoids are available to assist with any ROS formation, especially during the early stages of rehydration. During the stressful period when plants dehydrated from 100% to 85% RWC, and subsequently when they rehydrated from 60% to 85% RWC, chlorophyll content was still relatively high and other antioxidant mechanisms were likely to not be fully active, the plants relied on non-photochemical quenching to dissipate excess energy resulting from an uncoupling of photosynthesis under water deficit conditions. As discussed, numerous studies have been conducted to investigate these mechanisms and the results presented here concur with what has been deduced before. However, there are possible additional mechanisms employed by *X. humilis* to reduce oxidative stress and regulate photosynthesis which have not yet been studied. These will be investigated in the following chapters.

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CHAPTER 3

VOC EMISSION FROM *XEROPHYTA HUMILIS* DURING DEHYDRATION AND REHYDRATION

3.1 INTRODUCTION

Plants have evolved a number of different strategies for relieving the excess energy in photosynthetic membranes under stressful conditions, thereby reducing photo-oxidative damage. Some of these mechanisms include isoprenoids. Biosynthesis of non-volatile isoprenoids, such as the carotenoids β-carotene and zeaxanthin, and the tocopherols which are conserved mechanisms of photoprotection amongst all plants (Peñuelas and Munné-Bosch, 2005). However, some plants produce additional isoprenoids which are emitted as volatile organic compounds (VOCs), such as isoprene and monoterpenes. Isoprene has been found to be emitted by a number of different species (Kesselmeier and Staudt, 1999), however the emission of volatile isoprenoids is not conserved amongst all plants. Considerable investigation has been aimed at discovering whether the cost of losing carbon through VOC emission is outweighed by an advantageous function within plants. It has been suggested that the non-ubiquitous emission of VOCs may increase the plasticity in photoprotection in plants which have adapted to extreme conditions (Peñuelas and Munné-Bosch, 2005). *Xerophyta humilis* makes use of the photoprotective role of the conserved isoprenoids, such as β-carotene, lutein and the xanthophyll cycle, as has been discussed in Chapter 2, however, what is not known is whether it emits any VOCs. As *X. humilis* is desiccation tolerant, the plant tissues and photosynthetic machinery are subject to extreme conditions. In light of the hypothesized roles of VOCs in plants, especially isoprene, they could provide additional protection against lipid peroxidation and oxidative damage during desiccation of *X. humilis*. This chapter therefore aims to determine if *X. humilis* emits any VOCs and subsequently attempt to elucidate the potential roles of VOCs in desiccation tolerance.

3.2 METHODOLOGY

3.2.1 EXPERIMENTAL DESIGN

The conditions under which plants were maintained in a greenhouse during analyses were as previously described (Sherwin and Farrant, 1996). When gas exchange and VOC measurements were performed, plants were transferred to the laboratory only for the duration of the measurement and kept under a light source with an intensity of 700 µmol photons $m^{-2}s^{-1}$, equivalent to the light intensity in the greenhouse. Dehydration protocols and relative water content determination were performed as in Chapter 2. Gas exchange and VOC emission measurements were performed simultaneously on leaves of *X. humilis* grown in soil. Leaves were marked with small adhesive labels and the same leaves were followed during dehydration and rehydration. The photosynthetic rate and isoprene emission for each sample was then calculated as a percentage of the maximum achieved during the complete dehydration-rehydration cycle for that particular sample. The experiment was repeated 3 times and at least 3 or 4 biological repeats (different leaves from different plants) were obtained for each RWC plotted. For assessment of the role of isoprene during desiccation, plants were grown under aeroponics conditions as described below. Assessment of leaf RWC during dehydration and rehydration was done using the oven drying method described in Chapter 2. The fraction of transpirable soil water (FTSW) was determined according to Brilli et al. (2007).

3.2.2 GAS EXCHANGE MEASUREMENTS

Gas exchange was measured using the LI-6400 Portable Photosynthesis System (Li-Cor, Lincoln, NE, USA). A leaf was clamped in a gas-exchange cuvette and exposed to a 0.44 L min^{-1} flow of contaminant-free air with 400 ppm CO_2. The relative humidity within the chamber was controlled at 45-60%. Measurements were done at a photosynthetically active radiation (PAR) of 700 µmol photons $m^{-2}s^{-1}$, equivalent to the light intensity at which plants were maintained in the greenhouse. Photosynthesis, transpiration, and stomatal conductance were calculated using the LI-6400 software from the difference between CO_2 and H_2O concentration at the cuvette inlet and outlet. To measure VOC emissions the outlet of the cuvette was disconnected from the LI-6400 system and the flow was diverted into a silcosteel cartridge packed with 200 mg of Tenax (Agilent, Cernusco sul Naviglio, Italy). A volume of 8 L of air was pumped through the trap at a rate of

150 mL min^{-1}. The cartridge was analyzed by gas chromatography-mass spectrometry (GC-MS). Data is expressed as a percentage of maximum photosynthesis achieved under fully hydrated conditions.

3.2.3 VOC EMISSION MEASUREMENTS

GC-MS

GC-MS analyses were performed with an Agilent 6850 gas chromatograph coupled to an Agilent 5975C Mass Selective Detector (Agilent Technologies, Wilmington, DE, USA). The GC was supplied with a thermal desorber UNITY (Markes International Limited). The GC was equipped with a splitless injector and an HP-5MS capillary column (30 m in length, 250 µm in diameter and 0.25 µm film thickness). The column oven temperature was kept at 40°C for the first 5 min, then increased by 5°C min^{-1} to 250°C, and maintained at 250°C for 2 min. Helium was used as carrier gas. The concentration of each volatile was calculated by comparison with the peak area of a gaseous standard. The GC-MS was calibrated weekly using cylinders with standard mixtures of the main isoprenoids emitted by plants at an average concentration of 60 ppb (Rivoira, Milan, Italy). Compounds were identified using the NIST library provided with the GC/MS ChemStation software (Agilent). GC peak retention time was substantiated by analysis of parent ions and main fragments of the spectra.

PTR-MS

Isoprene emission was also measured on-line, by diverting the air at the exit of the gas-exchange cuvette into a Proton Transfer Reaction-Mass Spectrometer (PTR-MS, Ionicon, Innsbruck, Austria), which allowed fast detection of isoprene (Tholl et al., 2006). This was ideal to perform an isoprene light response experiment. Once the exit of the gas exchange system had been connected to the PTR-MS and a steady state in isoprene emission had been reached, the cuvette containing the leaf was covered with a black cloth until the emission reached the lowest possible level (approximately 400 seconds), and the cloth was then removed again. The PTR-MS was operated in a single-ion mode to detect isoprene (protonated $m/z = 69$). Calibrations using an isoprene gaseous standard (60 nL L^{-1}) were performed daily before measurements. Details on isoprene analysis by PTR-MS can be found in Tholl et al. (2006).

3.2.4 AEROPONICS GROWTH SYSTEM

Xerophyta humilis plants were grown in an aeroponics chamber according to a previous study performed on *Xerophyta viscosa* (Kamies et al., 2010). The aeroponic plant growth chamber(s) (Figure 3-1) consisted of a polyurethane black plastic box (0.4m x 0.3m and 0.4m high) with tight fitting lids containing 15 plant holders. The roots were misted by 20 cm high rigid riders with attached 360° rotating micro-jet sprays. Each rigid rider was inserted into agricultural piping (diameter 10 mm) which was connected to a 45 watt submersible pump (Aqua H_2O submersible pump- APH 2500) with attached filter which was used to pump Hoagland's nutrient solution (Hoagland and Arnon, 1950), from a nearby 25 L reservoir at a flow rate of 2,700 L h^{-1}. The nutrient solution was recirculated into the reservoir and renewed once a week. A 24 h electrical timer (Major tech) was connected to the submersible pump and controlled the pumping of nutrient solution through the system at set time intervals.

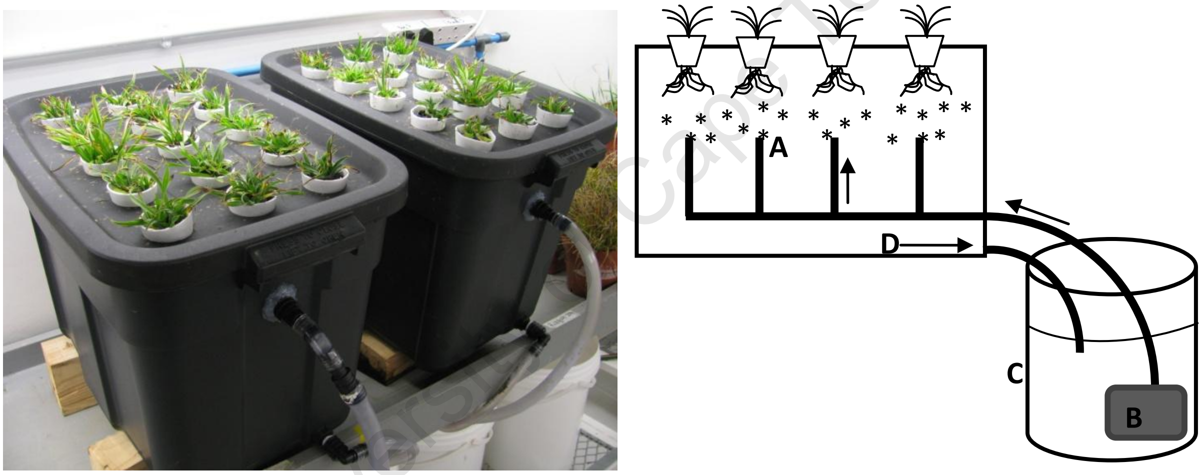

Figure 3-1: Photograph and diagram showing the aeroponic system used, the plants within the photograph being *X. humilis* in the fully hydrated condition. Plant roots were sprayed with Hoagland's nutrient solution through irrigation spouts (A), regulated by a timed electronic submersible pump (B), situated in an adjacent reservoir (C). Nutrient solution was re-circulated back into the tank through an outlet pipe (D).

Transfer of *X. humilis* plants to the aeroponics system was achieved with minimal damage to the roots. Plants were removed from the soil growth medium, separated into individual plants and excess soil was gently shaken from the roots prior to washing with water to dislodge adhering particles. Plants were inserted into polystyrene holding collars in the lid of the aeroponics system. Plant roots were sprayed with Hoagland nutrient solution at 0700, 1300 and 1900 hours for 15 minutes to maintain root hydration. After 3 weeks under these conditions treatments were commenced.

Dehydration of aeroponically grown plants was initiated by progressively reducing the number of times per day that the root tissues were sprayed with plant nutrient solution. This was achieved over 3 days, in which roots were sprayed twice a day (0700 hours and 1900 hours) for one day, then once a day (0700 hours) before withholding spraying on the third day. No further spraying occurred until plants were fully dehydrated and then were rehydrated by resumption of spraying (3 times daily as described above). Control plants grown in soil, control plants grown in aeroponics and treated plants grown in aeroponics were sampled for MDA and H_2O_2 as described below.

Plants grown in soil and plants grown in the aeroponics systems were maintained at the same conditions in a constant environment room in which the conditions were 55% relative humidity with a 14 h photoperiod and 17 ˚C (dark): 25 ˚C (light) temperature cycle. The light intensity was 400 µmol photons $m^{-2}s^{-1}$. Plants were allowed to acclimatize to the aeroponics system for 4 weeks prior to commencing dehydration.

3.2.5 FOSMIDOMYCIN TREATMENT

According to previous studies which tested the effect of fosmidomycin treatment on the inhibition of isoprene, the minimum concentration found to inhibit isoprene by more than 90%, was 20 µM (Loreto and Velikova, 2001). As an aeroponics system was utilized in these experiments, the concentration of fosmidomycin was increased to 100 µM. Prior to instigation of dehydration as outlined above, the nutrient solution in the reservoir for the designated treated plants was exchanged for nutrient solution including 100 µM fosmidomycin. The treated plants were subsequently sprayed with nutrient solution containing fosmidomycin for the 3 day dehydration procedure, whilst control plants were sprayed with nutrient solution only.

3.2.6 DETERMINATION OF H_2O_2 CONTENT

Hydrogen peroxide levels were determined as described by Loreto and Velikova (2001). Leaf tissue (0.1g) was ground in liquid nitrogen and added to 1 ml 0.1% (w/v) trichloroacetic acid (TCA). The solution was centrifuged at 12 000 x g for 15 minutes and 0.5ml of the supernatant was added to 0.5ml 10mM potassium phosphate buffer (pH 7) and 1ml 1M KI. The absorbance of the supernatant was read at 390 nm. The content of H_2O_2 was given on a standard curve. Control plants grown in soil, control plants grown in aeroponics and treated plants grown in aeroponics were sampled during dehydration and samples were grouped together into a range of RWC, with at least 15 biological replicates within each range.

3.2.7 MALONYLDIALDEHYDE ESTIMATION

To measure lipid peroxidation in leaves, the thiobarbituric acid (TBA) test, which determines malonyldialdehyde (MDA) as an end product of lipid peroxidation (Heath and Packer, 1968), was used. Leaf material (0.1 g) was ground in liquid nitrogen and added to 1 ml 0.1% (w/v) TCA solution. The solution was centrifuged at 12 000 x g for 15 minutes and 0.5ml of the supernatant was added to 1 ml 0.5% (w/v) TBA in 20% TCA. The mixture was incubated in boiling water for 30 minutes and the reaction stopped by transferring the reaction tubes to an ice bath. The samples were then centrifuged at 10 000 x g for 5 minutes and the absorbance of the supernatant was read at 532 nm. The value of non-specific absorption at 600nm was subtracted. The results were recorded as thiobarbituric acid reactive substances (TBARS), which represent MDA equivalents. The amount of MDA was calculated from the extinction coefficient 155 $mM^{-1}cm^{-1}$. Control plants grown in soil, control plants grown in aeroponics and treated plants grown in aeroponics were sampled during dehydration and samples were grouped together into a range of RWC, with at least 15 biological replicates within each range.

3.2.8 STATISTICAL ANALYSES

Means and standard errors were calculated with Graph Pad Prism (Version 5). Biological replication varied by experiment and is indicated in the appropriate figure legend. Statistical analysis was performed using analysis of variance (ANOVA). The significance of differences between means at different relative water contents was analyzed using Student's *t*-test.

3.3 RESULTS AND DISCUSSION

3.3.1 ISOPRENE EMISSION FROM *X. HUMILIS* IS LIGHT DEPENDENT

From preliminary experiments, *Xerophyta humilis* was found to emit the volatile organic compound isoprene. The nature of isoprene emission was consequently investigated. Firstly, the light-dependency of isoprene emission was established by darkening a plant with a black cloth and measuring the isoprene emission with a PTR-MS. This allowed a real-time observation of the rate of change of isoprene emission in response to changing light conditions. As can be seen in Figure 3-2, at 100 seconds the plant was covered and the rate of isoprene emission changed rapidly and decreased within 200 seconds. Once the lowest emission had been reached the black cloth was removed and the plant responded by increasing isoprene emission again. The response in *X. humilis* was very rapid when compared to other species. For example, a study done in red oak found that 15 minutes of darkness were required in order for isoprene emission to cease and once illuminated again, isoprene emission only reached control levels in 45 minutes (Loreto and Sharkey, 1990).

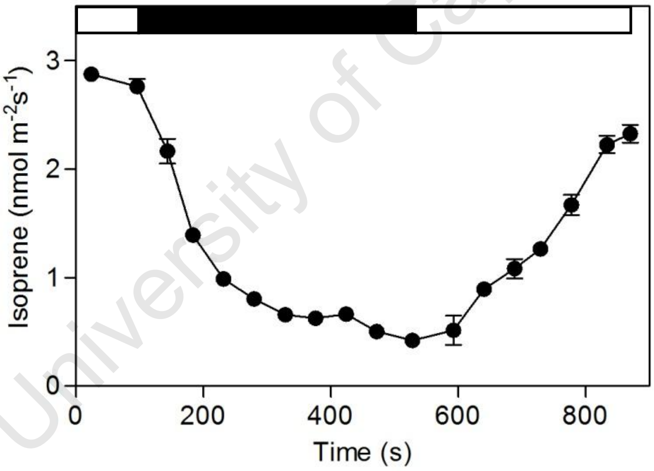

Figure 3-2: Response of isoprene emission from *X. humilis* to a light-dark-light cycle, measured online with a PTR-MS, (mean ± SEM, n=10). White blocks indicate light periods, black box indicates dark period.

Isoprene emission requires de-novo synthesis and it has been shown that the methylerythritol phosphate (MEP) pathway (Figure 1-1) in plastids is responsible for the synthesis of most of the isoprenoids (Lichtenthaler et al., 1997). Isoprene emission is therefore dependent on photosynthesis, and more specifically the Calvin Cycle. Therefore, the results outlined above support the light-dependency of isoprene emission. Previous studies have described the light dependency of isoprene emission (Sanadze, 1969, Sanadze and Kursanov, 1966) and have observed that the wavelength dependence is similar to that of photosynthesis (Rasmussen and Jones, 1973).

Often, isoprene emission is saturated at the same light level as photosynthesis (Rasulov et al., 2009, Sharkey and Loreto, 1993, Sharkey et al., 1991), but sometimes isoprene emission increases as light intensity increases, although photosynthesis has already been saturated. It is thought that the increase in isoprene emission is a result of light activation of isoprene synthase, or activation of the MEP pathway, or even a combination of both (Fall and Wildermuth, 1998).

3.3.2 Isoprene Emission versus Photosynthesis during Dehydration and Rehydration

Once it had been established that *X. humilis* emitted isoprene, the aim was to see how the emission changed during dehydration and rehydration in relation to photosynthetic changes. As can be seen in Figure 3-3A, the isoprene emission rapidly increased during the initial stages of dehydration. The increase in isoprene emission as the RWC decreased from 100% to 90% was highly significant (two tailed *t*-test, t=4.326, df=6, p<0.001). From 90% RWC onwards, the photosynthetic rate started to decrease as has been seen before and cessation occurred at 60% RWC. The isoprene emission, however, remained high until 75% RWC and only then decreased at a slower rate than photosynthesis and cessation occurred at 50% RWC. The ratio of isoprene emission to photosynthesis increased in response to initial dehydration and as a result, the amount of carbon lost as isoprene increased drastically as RWC decreased from 100% to 62%, as seen in Figure 3-4. At 62% RWC the amount of carbon lost as isoprene was very high at 26%. This is because at this RWC, photosynthesis was almost zero, whilst isoprene was still being emitted. When calculating the percent of carbon lost as isoprene, the number of carbons in an isoprene molecule (5) was taken into account.

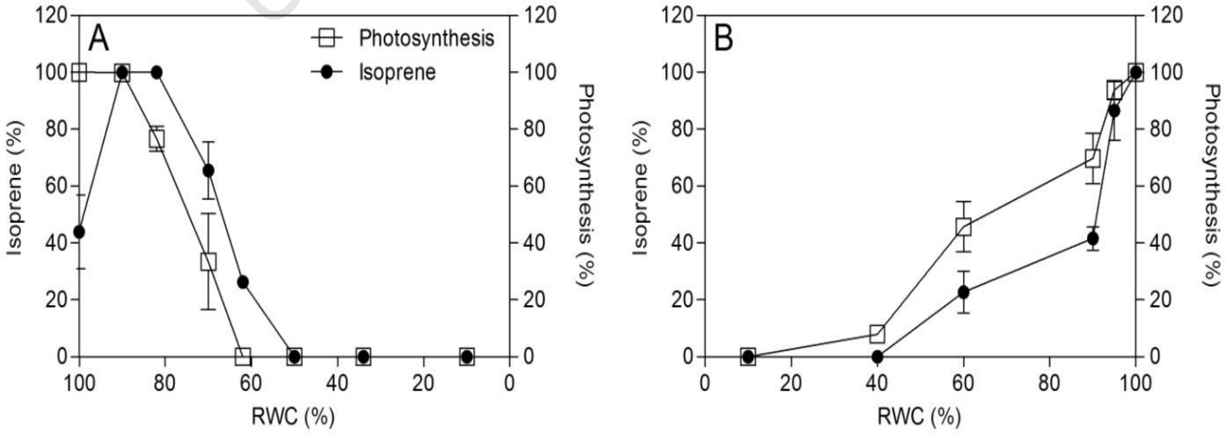

Figure 3-3: The response of isoprene emission and photosynthesis, measured with a GC-MS and IRGA gas exchange machine respectively and expressed as a percentage of the maximum achieved, during dehydration (A) and rehydration (B) of *X. humilis* plants, (mean ± SEM, n=3-4).

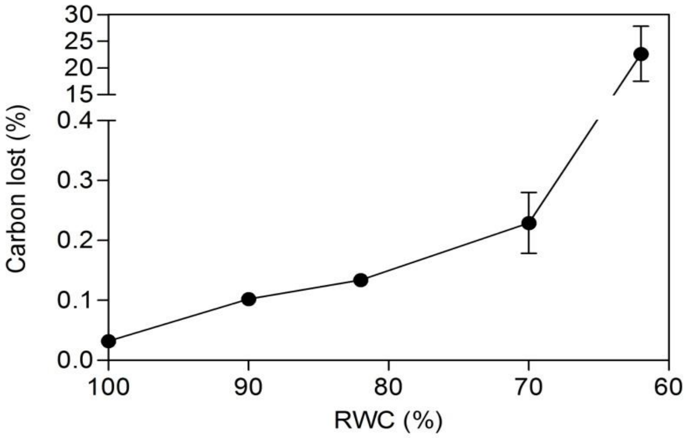

Figure 3-4: Carbon lost as isoprene during the initial stages of dehydration, (mean ± SEM, n=3-4).

The findings in *X. humilis* in this study agree with previous studies in desiccation sensitive plants which have been subjected to a drought stress, namely that isoprene emission responds slower in time to drought than photosynthesis as the emission is not limited by stomatal conductance as photosynthesis is (Fang et al., 1996, Loreto and Sharkey, 1993, Pegoraro et al., 2004, Brilli et al., 2007). Subsequently the amount of carbon lost increased by up to more than 250% at 62% RWC when photosynthesis was almost zero, which has also been reported by Brilli et al. (2007) for white poplar, whilst Pegora et al (2004) reported a 50% increase in carbon lost as isoprene when *Quercus virginiana* plants were water stressed.

Brilli et al. (2007) suggest that isoprene emission could be maintained once photosynthesis has stopped due to the contribution of alternative carbon sources. The authors showed by labeling with ^{13}C that carbon recently assimilated accounted for 78% to 90% of the isoprene produced in fully hydrated plants under control conditions, whilst under severe drought stress, it only accounted for 16% to 42% of the molecule. Similarly, Funk et al. (2004) showed that recently fixed carbon accounted for 84% to 88% of the isoprene produced in control plants of *Quercus ruber*, which decreased to 62% in plants under drought stress. The hypothesis is that during severe drought stress, a large contribution of the carbon incorporated into isoprene is not directly from photosynthesis but rather comes from extra-chloroplastic sources (Brilli et al., 2007, Funk et al., 2004, Karl et al., 2002). It is not known whether *X. humilis* uses carbon with extra-chloroplastic origins to maintain isoprene emission, especially in the period between 65% and 50% RWC when photosynthesis has stopped and isoprene is still being emitted. This is scope for future experimentation.

In this study, dehydration was monitored by measuring leaf RWC, and the decrease in RWC in resurrection plants is only observed after three to four days of withholding water (Figure 2-2A). However, in previous studies on the effects of drought on isoprene emission, the fraction of transpirable soil water (FTSW) is often used as a measure of drought severity (Brilli et al., 2007, Delfine et al., 2005) or else the number of days after withholding water is used (Fortunati et al., 2008, Pegoraro et al., 2004). This results in difficulties in comparing the rate of change of isoprene emission and photosynthesis in response to drought in this study with previous studies. For example, Brilli et al (2007) found that once soil water started to decrease below a FTSW of 50%, which was considered to be a severe level of drought, isoprene emission then started to decline. As can be seen in Figure 3-5, where an experiment was conducted on *X. humilis* plants to compare FTSW to RWC, *X. humilis* plants were still fully hydrated at a FTSW of 50% and therefore isoprene emission and photosynthesis were still high and had not yet started to decrease.

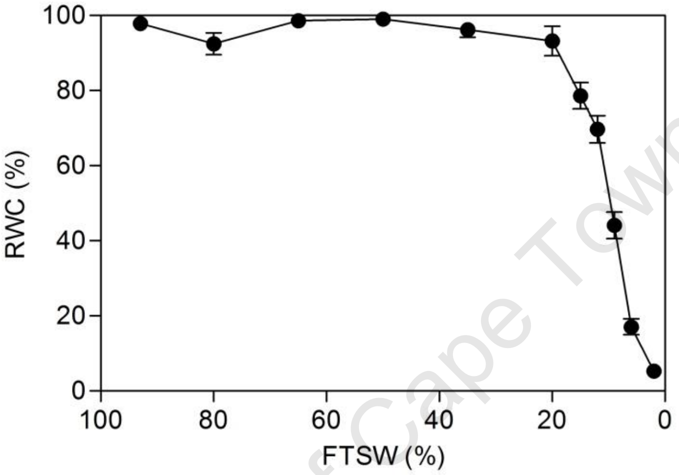

Figure 3-5: The change in RWC of *X. humilis* plants as FTSW decreased once water had been withheld, using the method outlined in Brilli et al. (2007) for FTSW calculation, (mean ± SEM, n=6).

Another study on *Q. ruber* showed that once drought was severe enough to limit photosynthesis, isoprene emission also decreased, but only 2 weeks after photosynthesis started to decline (Funk et al., 2005). This is contrasting to the results for *X. humilis* as the subsequent decrease in isoprene emission was slower than the reduction in photosynthesis, but only by several hours as the decrease in RWC occurred at a much faster rate once soil had dried. However, various other studies also show an initial stimulation of isoprene emission followed by a sudden and dramatic decrease when water stress was severe (Loreto and Sharkey, 1993, Pegoraro et al., 2004, Funk et al., 2004), which concurs with the trend seen in *X. humilis*. An alternative hypothesis for the initial stimulation seen in *X. humilis* in response to a decrease in RWC (Figure 3-3A), is that it could be due to an increase in leaf temperature during water deficit stress. As was seen in Chapter 2, there was also an increase in NPQ as plants dehydrated from 100% to 85% RWC (Figure 2-9C), indicating there was an increase in dissipation of excess energy as heat and this coincides with the increase in isoprene emission. Isoprene emission has been shown to be very sensitive to temperature changes within the leaf (Loreto et al., 2006, Loreto and Sharkey, 1993).

A study done on Black Poplar found that during drought stress, mRNA transcript level, and protein concentration of isoprene synthase (ISPS) decreased in unison with isoprene emission (Fortunati et al., 2008), although, the authors noted that ISPS activity decreased before isoprene emission during drought. They suggested that this indicates control of the emission at a transcriptional or post-transcriptional level during drought stress. However, the drought stress in the experiment conducted by Fortunati et al. (2008) developed over a period of 30 days, whereas in this study, the rate of dehydration of *X. humilis* is much faster and plants were completely desiccated within two days once RWC started to decrease. It will therefore be interesting to see whether there is also evidence for a transcriptional control of isoprene emission in *X. humilis* which displays a very rapid dehydration-rehydration cycle, compared to other desiccation sensitive plants which have been monitored for isoprene emission.

During rehydration, photosynthesis recovered to pre-stress levels (Figure 3-3B), as has already been demonstrated in Chapter 2. However, isoprene emission recovered at a slower rate than photosynthesis, but also reached levels higher than it was prior to dehydration. As can be seen in Figure 3-3B, isoprene emission reached 100% once fully rehydrated. A 100% emission rate is equivalent to a range between 3.5 and 4.2 nmol $m^{-2}s^{-1}$, whereas in fully hydrated plants prior to dehydration, as seen in Figure 3-3A, the emission rate was at 42%, equivalent to a range between 0.8 and 1.6 nmol $m^{-2}s^{-1}$.

A striking difference between this study and others is that in the latter, isoprene emission was observed to recover before photosynthesis after a drought stress (Brilli et al., 2007, Fang et al., 1996, Fortunati et al., 2008, Loreto and Sharkey, 1993, Pegoraro et al., 2004), whereas in this study photosynthesis recovered first. However, in the desiccation sensitive plants used in the previous studies, desiccation was not as severe as drying to 5% RWC, as is the case here for *X. humilis*, and thus rehydration, as shown in Figure 3-3B, was from the desiccated state. Consequently, the photosynthetic machinery first had to be resynthesized, as was discussed in Chapter 2, before carbon could be assimilated for the production of isoprene from the MEP pathway. As Brilli et al. (2007) showed by radioactive labeling, the main source of carbon for isoprene synthesis once again becomes photosynthesis following a drought stress. This could account for the contrasting result in this study with those reported in desiccation sensitive plants.

After rehydration, isoprene emission reached levels much higher than it was prior to dehydration in *X. humilis*, and this is supported by previous findings in other plant species (Brilli et al., 2007, Fang et al., 1996, Loreto and Sharkey, 1993). However, the reason for this transient increase in isoprene emission following drought stress is still not known. Brilli et al. (2007) suggested that it could be due to the observed maintenance of a relatively high ISPS concentration during drought stress, which resulted in the high ISPS activity observed after re-watering as the ISPS protein was reactivated. The difference in *X. humilis* is that although the ISPS protein may still be present over the entire stress period and after recovery, the photosynthetic machinery firstly needs to be reassembled before isoprene can be synthesized. This is purely speculative in *X. humilis* and needs to be confirmed with genomic and proteomic studies, such as functional protein studies and quantitative real time polymerase chain reaction, to evaluate the change in protein expression of ISPS during dehydration.

3.3.3 EMISSION OF LIPOXYGENASE PRODUCTS

Analysis of the samples of air surrounding leaf surfaces, which were collected during dehydration and rehydration, using a GC-MS also revealed the emission of oxygenated volatile organic compounds (OVOCs), namely hexanal, a C_6-aldehyde, as seen in Figure 3-6. The biochemical pathway leading to the formation of these OVOCs has been well documented (Croft et al., 1993, Hatanaka, 1993) and is summarized in Figure 3-7. Lipoxygenases (LOX) catalyze the addition of oxygen to polyunsaturated fatty acids to produce an unsaturated fatty acid hydroperoxide. In plants, the substrates for LOX are linoleic and linolenic acid, which are common constituents of the plant membranes (Croft et al., 1993). LOX enzymes are reported to preferentially act on free fatty acids, which are generated from cell membranes in response to ROS accumulation under stressful conditions (Porta and Rocha-Sosa, 2002, Beauchamp et al., 2005). LOX activity produces 9- or 13hydroperoxylinoleic or -linolenic acid, or a mixture, and the degradation of the hydroperoxides leads to the formation of volatile C_6 compounds (Heiden et al., 2003). These volatile LOX products, such as hexanal, can therefore be quantified by GC-MS analysis to give a measure of LOX activity.

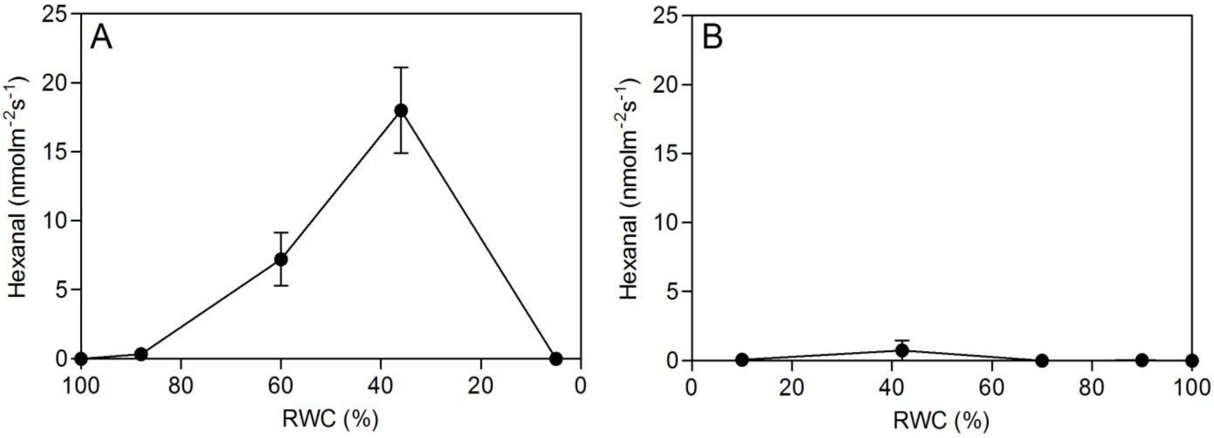

Figure 3-6: Emission of hexanal during dehydration (A) and rehydration (B) from *X. humilis* plants, quantified using a GC-MS, (mean ± SEM, n=3).

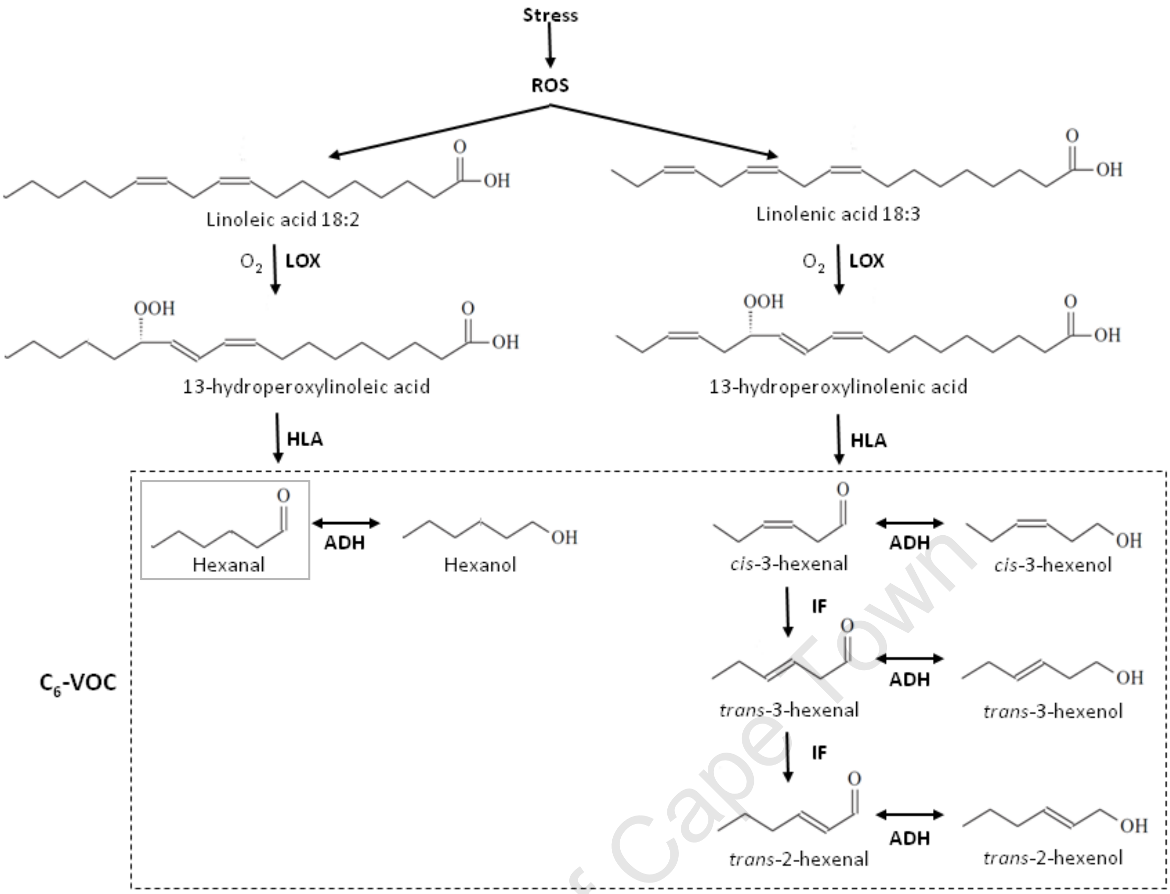

Figure 3-7: Diagram of the processes linking plant stress and LOX product emission. Under stressful conditions, such as water deficit, ROS accumulate, which act as a signal for the formation of free fatty acids. The free fatty acids linoleic and linolenic acid, constituents of plant membranes, are then oxidized by lipoxygenases. The destruction of the hydroperoxides formed by lipoxygenases leads to the formation of volatile LOX products. Grey box indicates compound identified by GC-MS analysis to be emitted by *X. humilis* during dehydration. LOX, lipoxygenase; HLA, hydroperoxide lyase; ADH, alcohol dehydrogenase; IF, isomerisation factor. The diagram is drawn according to Croft et al. (1993), Hatanaka (1993) and Beauchamp et al. (2005).

As can be seen in Figure 3-6A, the emission of hexanal increased in response to dehydration and peaked at 35% RWC, before decreasing rapidly during the very late stages of dehydration. This suggests that LOX activity is stimulated by dehydration in *X. humilis* plants. The increase in hexanal could be related to a breakdown of thylakoids during desiccation as it is at these RWCs that both breakdown of thylakoids and a concomitant increase in hexanal occurred. A study conducted in clover also found that the aldehyde (Z)-3-hexenal and the alcohol (Z)-3-hexenol were produced during the drying process (de Gouw et al., 1999) and LOX activity has been monitored in olive trees and shown to increase during the progression of water deficit (Sofo et al., 2004). During rehydration, there was no emission of hexanal detected from *X. humilis* (Figure 3-6B), suggesting that LOX activity is not stimulated during the recovery process and hexanal is presumably not required during rehydration.

LOX gene expression has been shown to be regulated by different types of stress, such as wounding, and more relevantly, water stress (Porta et al., 1999, Gigon et al., 2004). Gigon et al. (2004) found that LOX gene expression was stimulated under slight and moderate water deficit stress in *Arabidopsis thaliana*, but it decreased sharply under severe water stress. The authors considered a severe water stress to be after 14 days of withholding water and RWC had declined to 20%. This was a much slower dehydration compared to that which occurs in *X. humilis* once water has been withheld, however, the emission of the LOX product, hexanal, in *X. humilis* corresponds to the trend in LOX gene expression in *A. thaliana*, although it may occur over different time scales.

The timing of emission of hexanal can be related to the change in isoprene emission. As can be seen in Figure 3-3A, isoprene emission remained high during the initial stages of dehydration and then started to decrease from 70% RWC. Interestingly, hexanal emission was low during the initial stages of dehydration and only increased from 60% RWC onwards, once isoprene emission started to decrease, and peaked at 35% RWC, once isoprene emission had been completely inhibited.

One of the proposed roles of isoprene during stress is that as a small lipophilic molecule, it might enhance hydrophobic interactions within membranes or protein complexes (Sharkey and Yeh, 2001, Singsaas et al., 1997). Indeed, there is accumulating evidence for the hypothesized role of stabilizing chloroplast membranes, especially during high temperature and ozone stress (Velikova et al., 2008, Velikova and Loreto, 2005). It could be hypothesized that in *X. humilis*, isoprene may also stabilize membranes during the initial stages of drying to possibly maintain chloroplast membrane structure and therefore preserve photosynthesis until water loss becomes more severe and subsequent breakdown of thylakoids occurs to minimize photosynthetically associated ROS production. Furthermore, isoprene would then initially prevent the formation of free fatty acids by stabilizing membranes, until later stages when thylakoids are actively broken down and the free fatty acids are subsequently oxygenated by LOX enzymes, once isoprene emission has decreased.

Gigon et al. (2004) demonstrated in *A. thaliana* that LOX gene expression peaked between 82% and 73% RWC and there was a subsequent decrease in total fatty acid content. Although the pattern of LOX gene expression was not measured in *X. humilis*, it can be seen that hexanal, a LOX product, only peaked at a much lower RWC than when the peak in LOX gene expression was observed in *A. thaliana*, which does not emit isoprene. Therefore, LOX gene expression could be stimulated in *X. humilis* during dehydration, as in *A. thaliana*, but the presence of isoprene could help to reduce lipid peroxidation of membrane fatty acids during the initial stages of dehydration. This is speculative and needs to be confirmed with future studies in *X. humilis* where the LOX gene expression is followed during dehydration and total lipid and fatty acid content is analysed.

Various types of stresses have been shown to induce LOX activity and subsequent emission of volatile LOX products, such as exposure to high ozone concentrations (Beauchamp et al., 2005, Heiden et al., 2003), pathogen attack (Croft et al., 1993, Porta et al., 1999), wounding (de Gouw et al., 1999, Heiden et al., 2003) and drought (Gigon et al., 2004, Pham Thi et al., 1985, Porta et al., 1999). However, it has also been shown that the products of lipid degradation in response to biotic and abiotic stresses can also act as secondary messengers of stress-response signal transduction pathways (Munnik et al., 1998). For example, the oxylipin pathway is stimulated in response to various environmental stresses ranging from wounding, to pathogen attack, to drought and UV light exposure (Blée, 2002, Howe and Schilmiller, 2002). The biologically active compounds that subsequently form from oxidation of fatty acids, termed oxylipins, are responsible for a range of functions within plants, such as regulating stress-induced gene expression (Howe and Schilmiller, 2002, Schaller, 2001).

Gigon et al. (2004) hypothesize that the early stimulation of LOX gene expression stimulated by drought could be an adaptive response in *A. thaliana*, as in light of the literature discussed, the LOX products formed could then act as secondary messengers. The difference between *A. thaliana* which is desiccation sensitive and does not emit isoprene, and *X. humilis* which is desiccation tolerant and does emit isoprene, is that LOX enzymes could be activated in *X. humilis* in response to early dehydration, however, the peak in isoprene emission could help to stabilize membranes and prevent fatty acid peroxidation during the initial stages of dehydration. However, as isoprene decreased and LOX enzymes then oxygenate free fatty acids resulting in LOX product formation, these molecules could then act as secondary signalling molecules during the later stages of dehydration. Further work is required to clarify this hypothesis, such as monitoring gene expression, especially LOX gene expression and other genes which have previously been found to be induced by LOX products.

3.3.4 FURTHER INVESTIGATION INTO THE ROLE OF ISOPRENE IN *X. HUMILIS*

Under stressful conditions, carbon is redirected in order to form VOCs, as is seen in Figure 3-4, where the amount of carbon lost as isoprene in *X. humilis* increased drastically during dehydration. In order to justify the metabolic expense of producing VOCs, such as isoprene during stress, experiments must be conducted in isoprene-emitting species where the emission is modulated or inhibited. The most effective way of modulating isoprene emission has been to use fosmidomycin, a specific inhibitor of the deoxy-xylulose-phosphate pathway of isoprenoid biosynthesis (Lichtenthaler et al., 1997), shown in Figure 1-1. When fosmidomycin is fed through the leaf petiole, isoprene emission is inhibited by more than 90% in about 1 hour (Zeidler et al., 1998, Possell et al., 2010), whereas photosynthesis remains stable for several hours thereafter (Sharkey and Yeh, 2001). Many studies have made use of this and demonstrated the hypothesized roles of isoprene, namely, stabilizing membranes and acting directly as an antioxidant by scavenging ROS, under high temperature stress (Velikova and Loreto, 2005, Velikova et al., 2006, Velikova et al., 2005, Sharkey et al., 2001) and oxidative stress resulting from increased ozone exposure (Loreto et al., 2001, Loreto and Velikova, 2001, Velikova et al., 2004, Velikova et al., 2008).

In order to further clarify the role of isoprene in desiccation tolerance, the protocol and logic of the above studies was followed. However, as drought stress was to be investigated, fosmidomycin could not be fed to *X. humilis* plants in solution as in previous studies. Therefore, plants were grown in an aeroponics system and the spraying protocol was followed as outlined in the Methodology. MDA, used as a measure of lipid peroxidation, and H_2O_2 were measured to assess whether inhibiting isoprene in *X. humilis* had an effect on membrane stabilization and the oxidative state during dehydration and the results are shown in Figure 3-8.

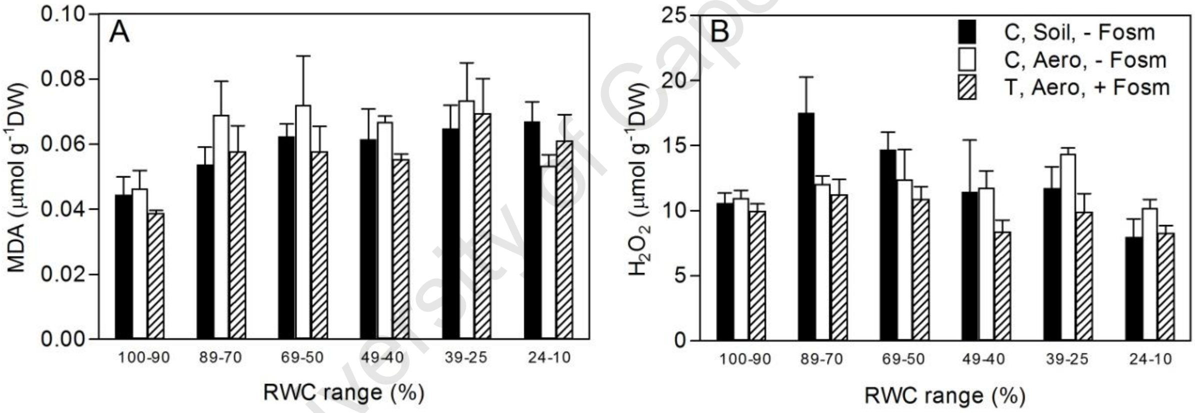

Figure 3-8: The effect of dehydration on the content of MDA (A) and H_2O_2 (B) in the leaves of *X. humilis* control plants grown in soil and watered with Hoagland's solution without fosmidomycin (solid bars); in control plants grown in aeroponics and sprayed with Hoagland's solution without fosmidomycin (open bars); and in treated plants grown in aeroponics sprayed with Hoagland's solution with 100 μM fosmidomycin according to the protocol outlined in Methods, (mean±SEM, n=15-25).

The measurements were performed in untreated plants grown in soil and untreated plants grown in aeroponics to firstly establish that the aeroponics systems did not cause any added stress to the plants during dehydration. There was no significant difference (two way ANOVA, $p>0.05$) in the MDA content (Figure 3-8A) and H_2O_2 content (Figure 3-8B) between control plants grown in the soil or aeroponics system. This indicated that plants in the aeroponics system were unstressed and any differences seen in treated plants would be due to the fosmidomycin treatment. However, when plants were sprayed with fosmidomycin during the initial dehydration process before being left to dry, there was no significant difference in MDA and H_2O_2 contents between treated and untreated plants grown in aeroponics (two way ANOVA, $p>0.05$).

This was not expected as previous studies in isoprene-emitting species have clearly shown that when isoprene is inhibited, indicators of the destructive effects of stress are significantly higher in fosmidomycin treated plants compared to untreated plants. For example, Loreto and Velikova (2001) demonstrated in *Phragmites australis* that leaves in which isoprene had been inhibited by fosmidomycin were much more sensitive to elevated ozone than leaves in which isoprene was emitted. Similarly, Velikova et al. (2005) found that MDA and H_2O_2 contents were higher in isoprene-inhibited leaves than isoprene-emitting leaves when exposed to high temperature.

The initial conclusions which could be drawn from the results presented in this study are that, if isoprene had been inhibited in the treated plants, then it does not play any role in reducing lipid peroxidation by stabilizing membranes during dehydration, and nor does it act directly as an antioxidant and scavenge ROS, such as H_2O_2. However, as isoprene emission was not monitored in this experiment, as the relevant equipment was not available in South Africa at this time, it was not known whether isoprene was indeed inhibited by fosmidomycin during dehydration.

In previous studies, isoprene emission was monitored and shown to be reduced within 60 minutes of feeding fosmidomycin through the petiole in solution (Loreto et al., 2001, Zeidler et al., 1998). Stress treatments were then subsequently performed within a short time period. The difficulty with drought stress is that fosmidomycin cannot be supplied in solution and possibly after the last spraying with 100 µM fosmidomycin in Hoagland's solution, until the time that plants started to dehydrate, the competitive inhibition of isoprene had worn off. Therefore, isoprene could still have been emitted in the treated plants which could account for there being no difference between untreated and treated plants as indicated in Figure 3-8. Consequently, conclusions about the possible roles of isoprene in desiccation tolerance cannot be drawn from this experiment.

A possible solution could be to grow plants in hydroponics and simultaneously treat with fosmidomycin to inhibit isoprene and polyethylene glycol (PEG), to induce osmotic stress causing dehydration from plant tissues, therefore simulating drought stress. This would enable fosmidomycin to be continually taken up by the roots that are in the solution, but simultaneously, plant tissues would dehydrate. However, it has been noted that the stress caused from drought and that from PEG-mediated osmotic stress are essentially different. This system would therefore first need to be evaluated in resurrection plants to see if PEG-induced dehydration has the same effect as natural dehydration resulting from withholding water, before further experiments could be conducted. Another concern in using fosmidomycin to evaluate the role of isoprene in drought stress, and therefore also desiccation tolerance, is that drought stress accumulates over a much longer time period than, for example a high ozone or temperature stress. In previous studies, fosmidomycin was only applied for short periods while the stress was being administered so that it did not have any lasting effects on photosynthesis and the synthesis of carotenoids. As seen in Figure 1-1, treating with fosmidomycin inhibits the MEP pathway which is also responsible for producing other molecules, such as carotenoids, tocopherols and chlorophyll. Recently, Possel et al. (2010) demonstrated in the white poplar and tobacco that photosynthesis was drastically reduced after treating with fosmidomycin for several hours, most likely as a result of the inhibition of many MEP products, subsequently causing photoinhibition and photo-damage. In order to avoid misinterpretation of results in drought experiments where the stress develops over days rather than hours and fosmidomycin would therefore have to be used for extended periods of time, these secondary effects of fosmidomycin would have to be accounted for in the experimental design and analysis. Alternatively, the direct antioxidant hypothesis of isoprene could be further tested in a desiccation tolerant system by rather searching for specific reaction products of isoprene oxidation from the reaction with ROS.

3.4 CONCLUSION

This is the first report in which isoprene emission has been reported in a resurrection plant and therefore has implications for investigating the role of isoprene in drought stress and subsequently desiccation tolerance. Isoprene emission was found to increase significantly during initial dehydration whereas photosynthesis decreased rapidly. This resulted in an exponential increase in the carbon lost as isoprene during early dehydration. Once photosynthesis had stopped, isoprene emission also decreased drastically. Further VOC analysis found that hexanal, a product of lipoxygenase activity, increased during dehydration, however, only once isoprene emission had started to decrease. As reported in many previous studies, it has been hypothesized that isoprene could therefore also stabilize membranes in *X. humilis* during the initial stages of dehydration, preventing lipoxygenases from oxidizing free fatty acids. Thereafter, hexanal could act as a secondary messenger during the later stages of dehydration. However, this still needs to be confirmed. Further investigation into the role of isoprene in *X. humilis* during dehydration by following the change in hydrogen peroxide and malonyldialdehyde, a measure of lipid peroxidation, in control and fosmidomycin treated plants was unsuccessful. The most likely explanation was that isoprene was not inhibited by the time plants had started to dehydrate, resulting in insignificant differences between control, untreated and treated plants. Various alterations to the experimental design have been suggested and further investigation is required to fully understand whether the hypothesized roles of isoprene stabilizing membranes and acting directly as an antioxidant under stress, plays a significant role in affording desiccation tolerance to resurrection plants.

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CHAPTER 4

ABA-MEDIATED STOMATAL REGULATION IN *XEROPHYTA HUMILIS*

4.1 INTRODUCTION

Desiccation tolerant plants' striking difference to those which are desiccation sensitive is that under drought conditions, once protective mechanisms have been laid down, conservation of water is no longer required. As stomatal regulation plays a major role in regulating water loss in desiccation sensitive plants, especially during adverse environmental conditions, it is of interest to investigate the nature of stomatal regulation in resurrection plants. This is especially interesting in the stage in which resurrection plants might actively remove water. The aim was therefore to investigate how stomata are regulated in *Xerophyta humilis* during dehydration. Given the morphological changes that occur in *X. humilis* during dehydration and rehydration, various techniques to view stomata were investigated to elucidate the most appropriate for this species. Secondly, the plant hormone ABA was measured and related to stomatal aperture regulation. ABA is one of the major signalling molecules in plants that mediates adaptive responses to environmental water changes, from upregulating gene expression to inducing stomatal closure to prevent water loss (Koorneef et al., 1998). As with the previous signalling molecules discussed in this thesis, such as isoprene and hexanal, the role of ABA has been extensively studied in desiccation sensitive systems, however, not yet in the unique resurrection plant system. Therefore, the aim of this aspect of the current study was to extend the knowledge of ABA functioning in *X. humilis*, with specific reference to stomatal regulation.

4.2 METHODOLOGY

The conditions under which plants were maintained during analyses, dehydration protocols and relative water content determination were performed as in Chapter 2.

4.2.1 MICROSCOPY

Various microscopy techniques were investigated to determine the most appropriate and efficient method to view and assess stomatal apertures during stages of dehydration and rehydration of *X. humilis*. Stereomicroscopy allows for an examination of the surface of specimens and can therefore provide an overview of the morphological changes that occur during drying. Microscopy techniques, such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM), have been used extensively for viewing leaf surfaces and cell structure, respectively, in plants, and were therefore investigated for viewing stomata and the desiccation-induced changes that occur during dehydration.

LIGHT MICROSCOPY

Micrographs of whole leaf specimens were taken at 10x and 40x magnifications using a Nikon Stereoscopic Zoom Microscope SMZ1500 equipped with a Nikon DS Camera Control Unit DS-U2 and DS-5M Camera head.

ELECTRON MICROSCOPY

Leaf segments (approximately 25 mm^2 for SEM and 5 mm^2 for TEM) were excised from approximately midway up the leaf of fully hydrated and desiccated leaves at 3 hours after dawn. Samples for scanning electron microscopy were fixed to an aluminum stub, flash frozen in liquid nitrogen and viewed directly using a LEO fully analytical S440 scanning electron microscope. Samples for transmission electron microscopy were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) containing 0.5% caffeine. Post-fixation was in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4). Following graded ethanol dehydration, the material was infiltrated by gradually replacing the solvent with epoxy resin (Spurr, 1969). The samples were then embedded in Spurr's resin by oven baking at 60 ˚C for 16 hours. Tissues were sectioned using a Reichert Ultracut-S microtome, stained with uranyl acetate and lead citrate (Reynolds, 1963) and viewed using an LEO912 transmission electron microscope.

4.2.2 NAIL POLISH PEELS

Nail polish peels were used to monitor stomatal aperture during dehydration and rehydration of *X. humilis* plants. Peels of the adaxial and abaxial surfaces of *X. humilis* leaves were made over a period of eight days during the course of dehydration. Ten to twelve leaves were selected at random from different plants each day between 3 and 4 hours after dawn. Half of the leaves were used to make peels of the adaxial surface and half were used for the abaxial surface. It was found that clear nail polish diluted to 85% with acetone provided the best quality peels. A film of polish was applied directly to the leaf using the brush provided with the polish bottle and allowed to dry for 2 to 4 hours. A small section of the same leaf was cut for relative water content determination. A piece of adhesive tape was used to peel the film off and it was adhered directly to a glass slide with the attached peel. Peels were examined with a Nikon Eclipse 50i Compound Microscope equipped with a Nikon DS Camera Control Unit DS-U2 and DS-5M Camera head. Three micrographs of different fields of view for each peel were taken at 400x magnification with 640 x 480 pixel resolution. The percentage of open stomata was calculated for each by manually counting open and closed stomata on large printed images and an average percentage of the three technical repeats for each peel was calculated. Micrographs of whole leaf specimens were taken at 10x and 40x magnifications using a Nikon Stereoscopic Zoom Microscope SMZ1500 equipped with a Nikon DS Camera Control Unit DS-U2 and DS-5M Camera head.

4.2.3 ABA EXTRACTION AND QUANTIFICATION

At various intervals during the dehydration time course, leaf tissue was collected at 3 hours after dawn, frozen in liquid N_2 and stored at -70˚C. Three trays were used and the sample collected from each consisted of 10 to 12 leaves selected at random. Three of the leaves were used to determine the average relative water content for the sample, and the remaining leaves were used for ABA extraction and quantification. ABA was extracted as previously described (Walker-Simmons, 1987). Samples were ground in liquid N_2 and powdered tissue was suspended in methanol containing 0.5 g/L citric acid monohydrate and 100 mg/L butylated hydroxytoluene at a ratio of 10 mg of powdered tissue per 0.1 ml of methanol solution. Suspensions were stirred in sealed tubes for 36 h at 4˚C in the dark and centrifuged at 1500g. The supernatants were recovered, adjusted to 70% methanol using a 62.5% solution of the extracting methanol, and passed through a Sep-Pak C18 cartridge (Waters). The eluates were dried in a Speed Vac and resuspended in 400 uL TBS-buffer (tris buffered saline; 150 mmol/L NaCl, 1mmol/L $MgCl_2$, 50 mmol/L tris) with 5% methanol. The concentration of ABA in the samples was analysed using a Phytodetek ABA ELISA test kit (Agdia Inc., Elkhart, IN, USA) using a monoclonal antibody prepared against *cis/trans* (+)-ABA (Sigma, CA 4906), as per kit instructions. Two technical repeats for each sample were performed. Readings not within the specified optimum sensitivity range were excluded. Readings were normalized against DW of the sample.

4.2.4 GAS EXCHANGE MEASUREMENTS

Rates of photosynthesis and transpiration were measured using an LI-6400 (LI-COR Biosciences Inc., Nebraska, USA) IRGA. The equations used to calculate photosynthesis and transpiration are those derived by von Caemmerer and Farquhar (1981). Readings were taken for five to seven leaves each day of dehydration and rehydration between 3 and 4 hours after dawn. Readings were taken over a time period of 5 minutes and the average of the technical repeats for each leaf was calculated. After readings had been taken, the leaf was removed, traced on paper to calculate the leaf surface area and a nail polish peel was made to examine stomata. A small section of the each leaf was cut for relative water content determination.

4.2.5 STATISTICAL ANALYSES

Means and standard errors were calculated with Graph Pad Prism (Version 5). Biological replication varied by experiment and is indicated in the appropriate figure legend. Statistical analyses were performed using analysis of variance (ANOVA). The significance of differences between means at different relative water contents was analyzed using Student's *t*-test.

4.3 RESULTS AND DISCUSSION

4.3.1 STRUCTURAL AND ULTRASTRUCTURAL STUDIES

DESICCATION-INDUCED MORPHOLOGICAL CHANGES

In the hydrated state, *X. humilis* leaves were flat and green (Figure 4-1A, B) and desiccated leaves (Figure 4-1G, H) were reddish-brown and corrugated. The surface areas of the leaves decreased on drying, as can be seen in a comparison of hydrated leaves (Figure 4-1A, B) and desiccated leaves (Figure 4-1G, H).

In hydrated leaves, the stomata were distributed evenly over the adaxial surface (pale dots arrowed in Figure 4-1C), arranged in bands running parallel to the midrib. The abaxial surface (Figure 4-1D) was characterised by ridges and furrows, with the stomata clustered in the furrows. During desiccation, the ridges became more pronounced on the abaxial surface (Figure 4-1J), resulting in a corrugated appearance, compared to the adaxial surface (Figure 4-1). The ridges were closer together in the desiccated leaves, resulting in the decreased surface area, previously mentioned. This corresponds to the confocal images in Chapter 2, where the ridges and furrows are clearly seen in a cross-section of the leaf (Figure 2-7). During dehydration, leaf folding occurred along the midrib. As can be seen in Figure 4-1E, the leaf has already begun to fold at 75 % RWC. The photographs of whole plants in Figure 2-6 clearly indicate the leaf folding during dehydration.

Scanning electron microscopy studies confirmed the differences between adaxial and abaxial surfaces noted above and the desiccation-induced structural changes can clearly be seen in Figure 4-2. The stomata on the adaxial surface (Figure 4-2A) were more evenly distributed than those on the abaxial surface (Figure 4-2B), which were observed to be clustered in and around the furrow. A closer view of the adaxial surface of a desiccated leaf (Figure 4-2C), reveals extensive folding of the epidermal cells, with stomata barely visible. A comparison of the abaxial surface of a hydrated (Figure 4-2B) and desiccated (Figure 4-2D) leaf shows how the furrow became narrower on drying, bringing the ridges closer together, reducing surface area and enclosing the stomata.

In most angiosperm plants, stomata are characteristically found on the abaxial leaf surface. This protects stomata from direct sunlight thus reducing water loss by transpiration (Taiz and Zeiger, 2002). However, as observed in this study of *X. humilis*, the majority of stomata are found on the adaxial surface and those on the abaxial surface are found clustered in furrows. The desiccation-induced morphological changes outlined above, and previously (Oliver et al., 1998, Sherwin and Farrant, 1998), help to explain the differential distribution of stomata on the adaxial and abaxial

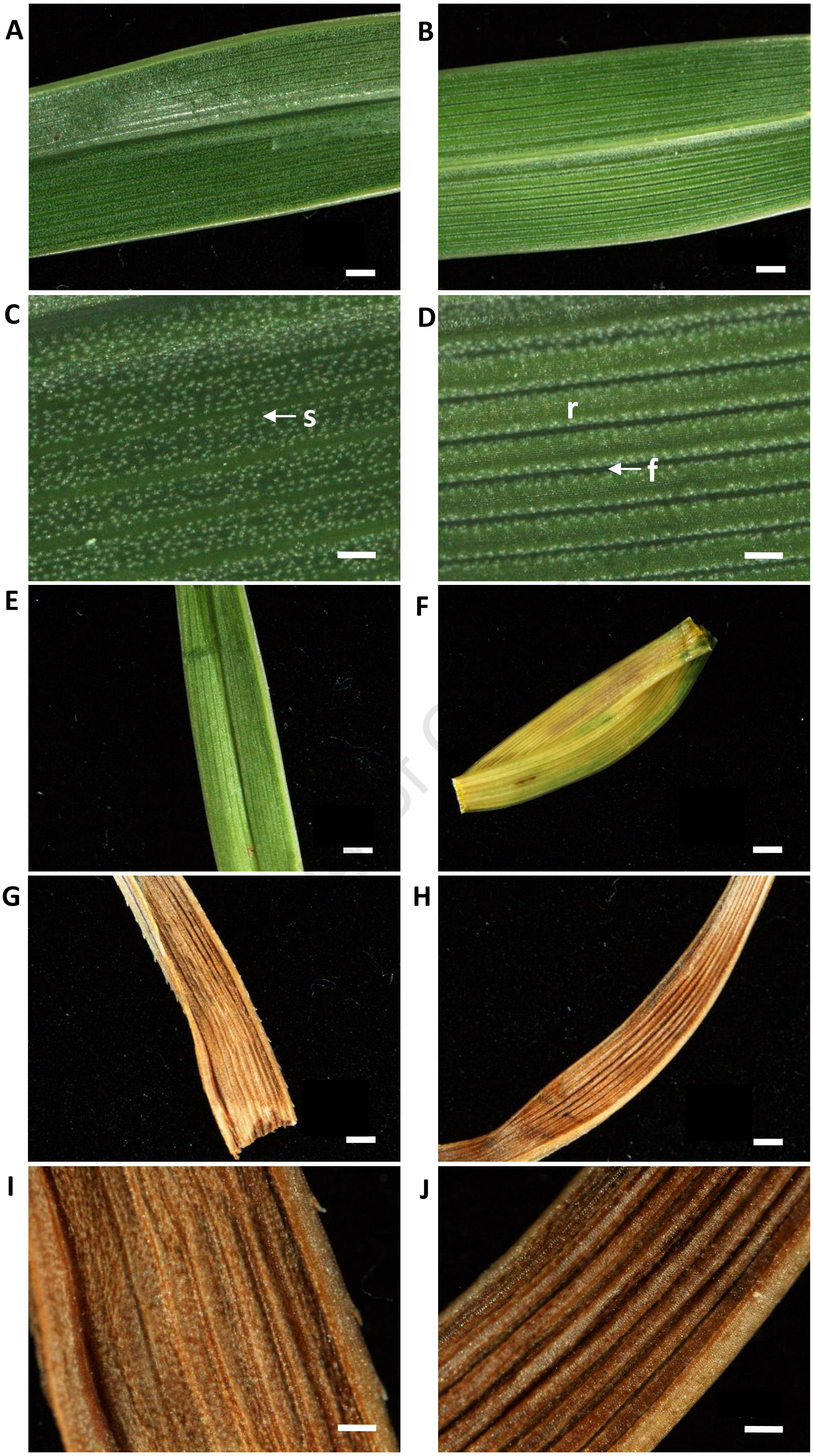

Figure 4-1: Light micrographs of the adaxial (A, C, E, G, I) and abaxial (B, D, F, H, J) surfaces of *X. humilis* leaves at 100% RWC (A, B, C, D), 75% RWC (E), 30% RWC (F) and 6% RWC (G, H, I, J). s, stomata; r, ridge; f, furrow. Scale bars: 1000 µm (A, B, E, F, G, H); 300µm (C, D, I, J).

surfaces. In hydrated plants, the adaxial surface is exposed to sunlight, accounting for more stomata on this surface to allow for maximum photosynthesis. During dehydration, the adaxial surface is enclosed and the abaxial surface remains exposed to the environment as the leaves fold in half. Therefore, it is beneficial to the plant to have the majority of stomata on the adaxial surface as they are therefore protected from the environment, thus reducing water loss from the adaxial stomata. The abaxial surface is left exposed during desiccation-induced leaf folding and thus clustering of stomata in furrows serves to regulate water loss even from the abaxial surface by reducing transpiration (Farrant, 2000). It has been suggested that the morphological changes that occur during dehydration of resurrection plants are an adaptation to minimize the exposed surface area and thus reduce water loss during the initial stages of drying (Vicre et al., 2004).

Although these results provide valuable observations into the morphological changes induced during drying of *X. humilis*, it is not clear whether the stomata are open or closed, and this technique could not be used to assess stomatal aperture. Despite previous studies reporting open stomata in scanning electron micrographs of other species of resurrection plants (Moore et al., 2007a), it was not observed in this study. A possible explanation is that samples were cryofixed and there was a slight delay between a sample being cut from a leaf and cryofixation in liquid nitrogen. The stomata on the cut sample could have responded rapidly to the stress and therefore closed.

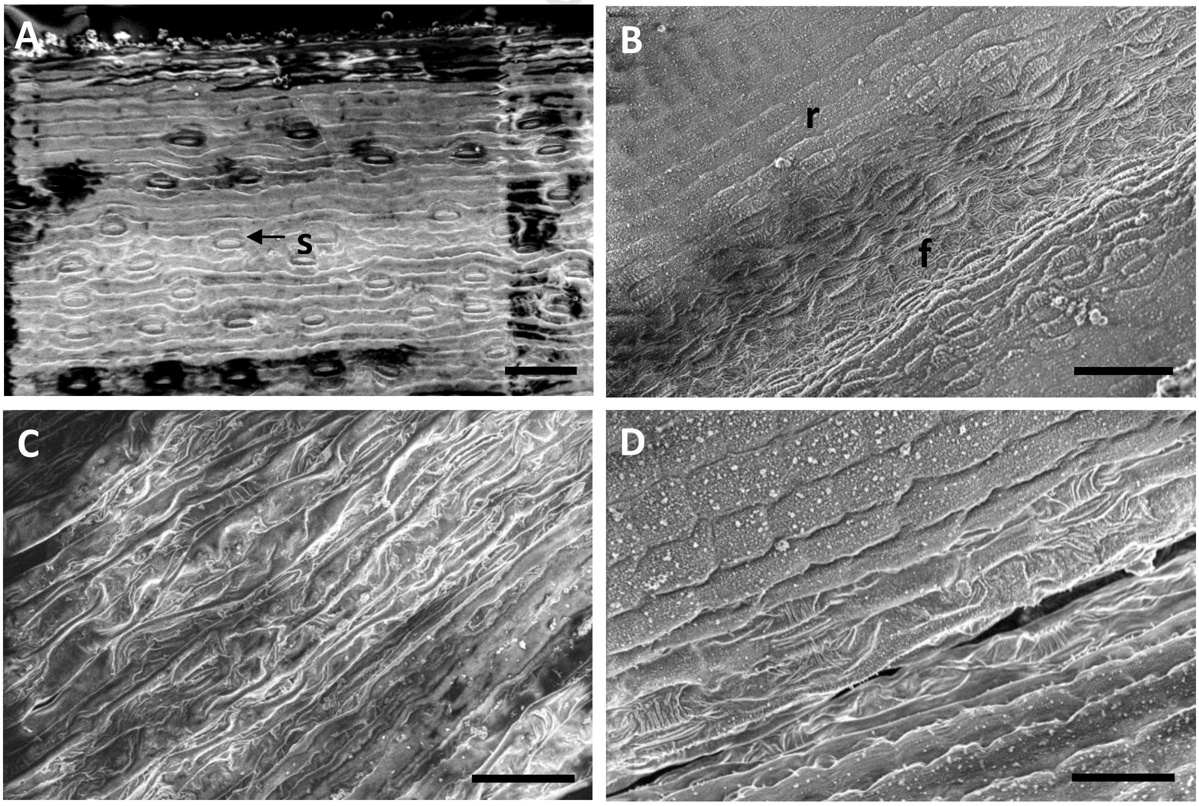

Figure 4-2: Scanning electron micrographs showing stomata of hydrated (A, B) and desiccated (C, D) leaves of *X. humilis* on the adaxial (A, C) and abaxial (B, D) surfaces. s, stomata; r, ridge; f, furrow. Scale bars: 60 µm (A, B); 30 µm (C, D).

X. humilis has adaptations in place to limit water loss, some of which have been discussed, for example, the folding of leaves during dehydration, the different arrangement of stomata on the two surfaces of the leaves and the clustering of abaxial stomata in furrows. However, the postulate is that it is advantageous to actively and rapidly lose water during drought conditions (Farrant, 2007). This seems contradictory; however, perhaps this could be explained in an evolutionary context. The water-loss limiting adaptations could have evolved first, before plants became desiccation tolerant and it was subsequently unnecessary to maintain a high water content during drought conditions. However, this is speculative and needs to be confirmed.

ULTRASTRUCTURAL CHANGES

Ultrastructural studies enabled an investigation into the cellular morphology of stomata. The differences in adaxial and abaxial guard cells and the effect of desiccation on guard cell morphology were investigated using TEM. There were no major differences observed between the adaxial (Figure 4-3A, C) and abaxial (Figure 4-3B, D) guard cells. However, the appearance of the guard cells was affected by desiccation. The observations made in this study are consistent with what has been previously reported for mesophyll cells of this species and other desiccation-tolerant species (Farrant, 2000; Moore *et al*., 2007).

The guard cells in the hydrated tissue were flush with the leaf surface whereas they appeared slightly sunken in the desiccated tissue due to the convoluted and folded epidermis of dried leaves, as can be seen in Figure 4-3D. This could be an adaptation to reduce water loss during the initial stages of drying as the stomata are protected from the drying wind, therefore reducing transpiration rate, which is a common trait in desert plants (Ashby, 1932).

The large starch grains seen in the hydrated tissue are not present in the desiccated guard cells. This is due to the desiccation-induced catabolism of starch to provide energy to lay down protective mechanisms during dehydration (Dace et al., 1998, Farrant, 2000). There were many vacuoles present in the guard cells of hydrated tissue (Figure 4-3A), as has been previously reported for the cells of this and other *Xerophyta* species (Dace et al., 1998, Sherwin and Farrant, 1998). There were also vacuoles present in the desiccated tissue, however their content is postulated to not be water (as RWC < 5%), but rather to have been replaced with non-aqueous substances (Dace et al., 1998, Farrant, 2000, Mundree and Farrant, 2000). This serves to maintain cytoplasmic volume, thus providing mechanical stabilization for the tissue during drying (Farrant, 2000, Sherwin and Farrant, 1998). As can be seen in Figure 4-3C and D, the presence of numerous, large vacuoles has prevented the plasmalemma from withdrawing from the cell wall and possibly tearing during dehydration. The purpose of a TEM study was to examine the ultrastructure of stomatal guard cells. However, it was not possible to view many stomata on a leaf surface to calculate the percentage that were open, and it was therefore not appropriate for investigating stomatal regulation during dehydration.

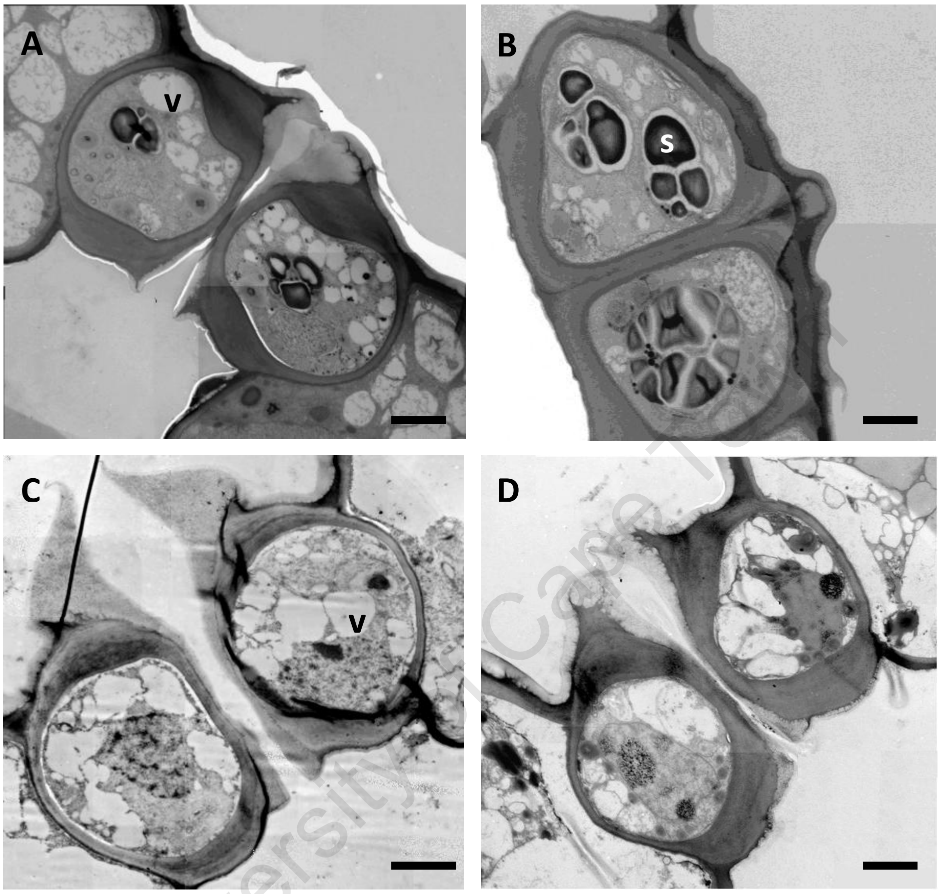

Figure 4-3: Transmission electron micrographs showing stomata of hydrated (A, B) and desiccated (C, D) leaves of *X. humilis* on the adaxial (A, C) and abaxial (B, D) surfaces. v, vacuole; s, starch grain. Scale bar: 2000nm.

4.3.2 STOMATAL REGULATION DURING DEHYDRATION

NAIL POLISH PEELS OF LEAF SURFACES

Nail polish was originally used to examine stomata of corn leaves (Miller and Ashby, 1968) and has since been used as a fast, easy, inexpensive method to study all epidermal features. It is a crude technique compared to electron microscopy but has advantages. The polish can be applied directly to the leaves whilst they are still attached to the plant, thus eliminating the chance for stomata to respond to the stress of a sample being cut from the plant, as is the case for TEM and SEM.

Figure 4-4 shows a representative of peels of adaxial and abaxial surfaces of leaves at various relative water contents. The low magnification micrographs of the abaxial (Figure 4-4A) and adaxial

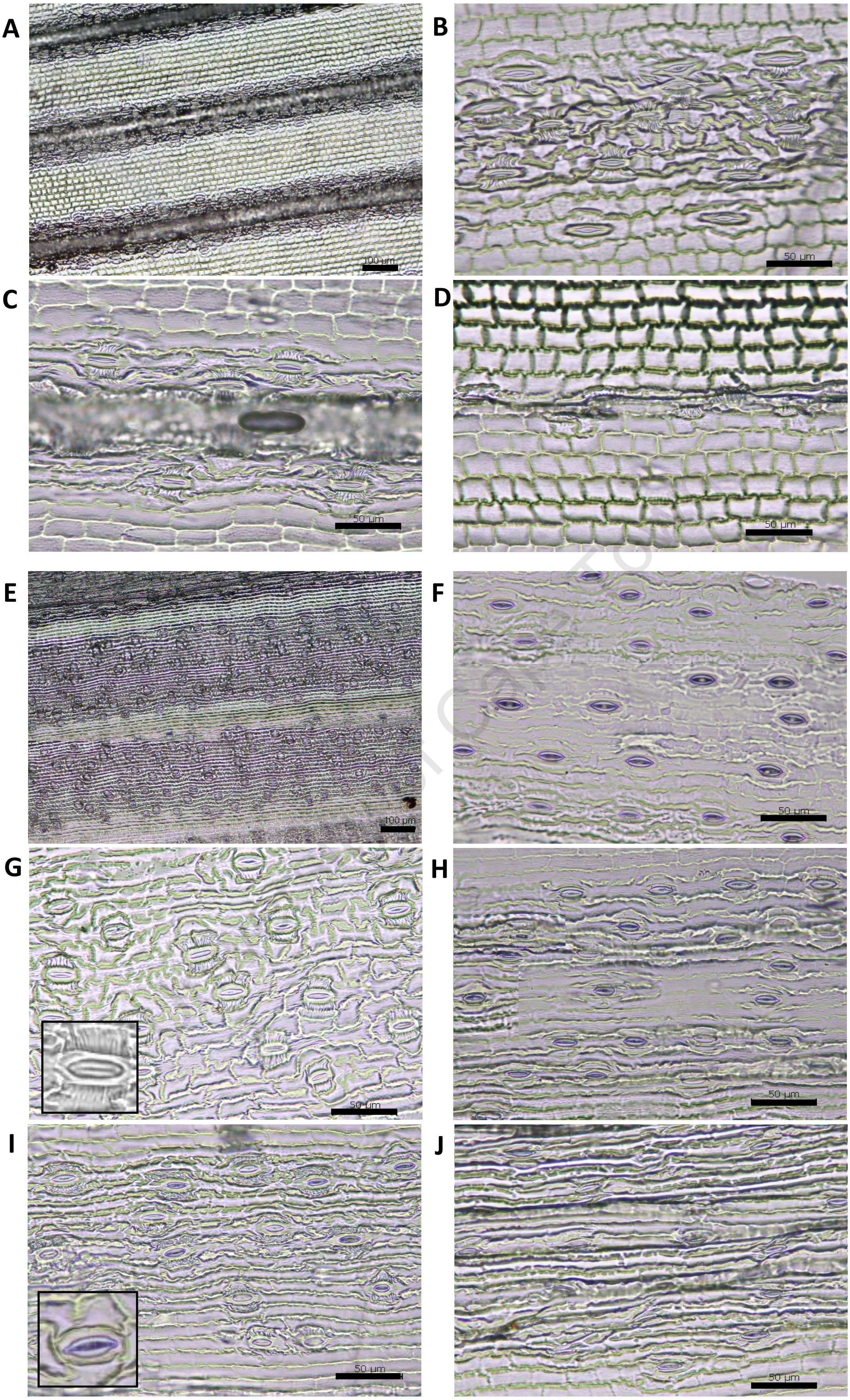

Figure 4-4: Light micrographs of nail varnish peels showing abaxial (A, B, C, D) and adaxial (E, F, G, H, I, J) surfaces of *X. humilis* leaves. RWC: (A, B) 100%; (C) 80%; (D) 55%; (E, F) 100%; (G) 85%; (H) 60%; (I) 55%; (J) 30%. Scale bars: 100 µm (A, E); 50 µm (B, C, D, F, G, H, I, J). To assess stomatal aperture, photos with a higher fold magnification were used (for example, see the not scaled inserts in G and I).

(Figure 4-4E) surfaces highlight the differences, as shown before, between the arrangements of stomata on the two surfaces. The stomata on the adaxial surface were distributed more evenly over the leaf surface in broader bands compared to the abaxial surface. As can clearly be seen in Figure 4-4B and F, stomata were closed on the abaxial surface and open on the adaxial surface. However, the images in Figure 4-4 are small resulting in it being difficult to classify stomata as open or closed, and larger images were used to assess stomatal aperture. Due to the two dimensional nature of the micrographs, areas such as the furrows were out of focus (Figure 4-4C) making it difficult to view stomata on the abaxial surface, especially during the later stages of drying (Figure 4-4D). However, the technique still provided good quality images and it was a fast, efficient method of obtaining an image of a leaf surface.

CHANGES IN STOMATAL APERTURE DURING DEHYDRATION

Changes in stomatal aperture during dehydration were assessed using the nail polish technique. Figure 4-5A shows the change in percentage of open stomata during dehydration for the adaxial and abaxial leaf surfaces. A one-way ANOVA was performed and the significant p-value ($p<0.0001$) indicates that RWC has a significant effect on stomatal aperture. The stomata were initially open when the plant was fully hydrated (see example in Figure 4-4F). This is as expected because when water is available in the surrounding environment, stomata are open to allow for gaseous exchange and plants remain hydrated due to the continual uptake of water by the roots (Taiz and Zeiger, 2002). As the relative water content of the plants decreased, the adaxial stomata appeared mainly closed, as is seen in Figure 4-5A, this occurring when soil moisture had been depleted. The difference in the percentage of open stomata in the RWC ranges of 100-97% and 96-90% was highly significant (two-tailed *t*-test, $t=9.4$, $df=37$, $p<0.0001$), highlighting the sudden closure of stomata in response to initial dehydration. Leaves that were in the range of 80-73% RWC had a very low percentage of open stomata. This can be seen in Figure 4-4G; the leaf is at 80% RWC and all the stomata are closed. This effectively reduces water loss from the leaves by transpiration, and maintains RWC near full turgor in the presence of an environmental water shortage. The sudden closure of stomata in response to drought is thought to be one mechanism by which resurrection plants delay drying in order to lay down protective mechanisms.

However, as the relative water content decreased further, beyond 60%, the stomata were observed to open again (Figure 4-4H and I; Figure 4-5A). The sudden opening of stomata is seen in the highly significant difference between the percentage open at 79-73% and those leaves at 60-45% RWC (two-tailed *t*-test, $t=11.8$, $df=25$, $p<0.0001$). The range of relative water contents at which the stomata were observed to open again corresponds to the rapid decrease in water content between 80 and 120 hours after the dehydration treatment began (Figure 2-2A). This is unique in resurrection plants compared to desiccation-sensitive plants as the latter conserve water for as long as possible during drought with stomata remaining closed (Schulze, 1986). However, resurrection plants, such as *X. humilis* have established desiccation tolerance by upregulating protective mechanisms during the early stages of drying, such that they do not need to conserve water thereafter (Farrant, 2007). The subsequent opening of stomata in the later stages of drying is consistent with previous studies which have also reported open stomatal pores in both hydrated and desiccated tissue (Moore et al., 2007a, Vicre et al., 2004). When the relative water content decreased below 30%, it became difficult to view stomata, which were sunken in folds and it was difficult to make a clear impression of the ridged leaf surface.

Unlike on the adaxial surface, the trend is not as apparent on the abaxial surface. Although the trend is statistically significant (one-way ANOVA, F=5.911, p<0.0001), one has to bear in mind the technical difficulties in observing stomata on this surface in desiccated tissues. However, it did appear that some stomata did open slightly when water content decreased below 60% (Figure 4-5A). Stomata on the abaxial surface were mostly closed, even in fully hydrated leaves (Figure 4-4B). As can be seen in Figure 4-5A, the percentage of open stomata on the abaxial surface of fully hydrated leaves (100-97% RWC) was 20% whereas that of the adaxial surface was 80%. This is possibly because abaxial stomata are not exposed to as much direct sunlight as the adaxial surface, limiting photosynthesis and therefore gaseous exchange, and thus stomata are mostly closed. This indicates a noticeable difference in regulation of stomata on the adaxial and abaxial surfaces of the leaves.

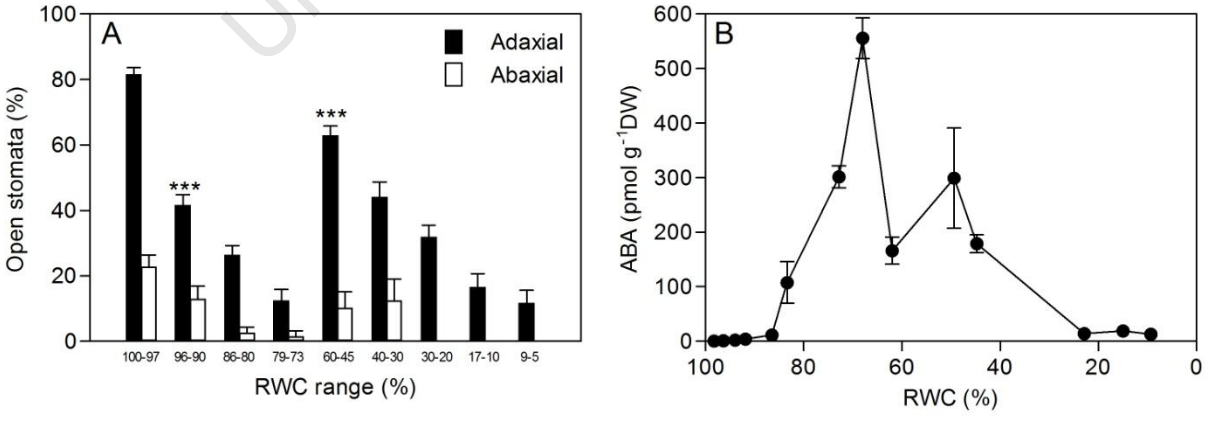

Figure 4-5: (A) The change in percentage of open stomata on the adaxial (solid bars) and abaxial (open bars) surfaces of *X. humilis* leaves during dehydration. Values are means of the percentage of open stomata visible on light micrographs of leaf peels within the indicated RWC range (mean ± SEM, n=10-25), highly significant differences in means between RWC ranges are indicated by *, with p < 0.0001. (B) The change in ABA concentration within leaves during dehydration (mean ± SEM, n=3).**

ABA AND STOMATAL APERTURE DURING DEHYDRATION

The ABA concentration was measured for samples of varying RWC during dehydration, in order to relate it to the timing of opening and closing of stomata. Figure 4-5B shows that ABA concentration remained stable as plants decreased in RWC from 100% to 90%. However, as plants decreased in water content from 86% to 69% RWC, there was a dramatic increase in ABA concentration, from 15 to 549 picomoles ABA g^{-1}DW, (two tailed *t*-test, t=14.4, df=4, p<0.0001), indicating a 35-fold increase.

Root to shoot signalling is vital for plants to respond and adapt to drought conditions, and ABA is known to be one of the major chemical signals, conveying the soil water status to the rest of the plant (Schachtman and Goodger, 2008, Zhang et al., 2006). The rapid increase in ABA concentration in the leaves of *X. humilis* corresponds to drying of the soil, and the plants subsequently started to decrease in RWC. This provides evidence that in response to drought and a decrease in RWC, there is an increase in ABA concentration in the leaves of *X. humilis*, supporting ABA as a key regulator of the drought response in resurrection plants.

ABA is mostly synthesized in the roots, but also in the leaves. Therefore, the ABA concentration in the roots correlates significantly with the soil water status and the RWC of the roots of many plants species (Zhang and Davies, 1989). The ABA content was only measured in the leaves in this study and thus it is not clear whether the increase in ABA in the leaves is a result of ABA synthesis in the roots and transport to the leaves via the xylem only, or whether ABA is also synthesized in the leaves of resurrection plants. Previous studies have found that xylem ABA concentration is low during the early stages of soil drying, followed by an increase in concentration during periods of water deficit in both woody (Auge et al., 2000, Jackson et al., 1995) and herbaceous species (Correia and Pereira, 1995, Liu et al., 2005). Lui et al. (2005) found that xylem sap ABA was 70 pmol ml^{-1} in well-watered plants and had increased 33-fold by the end of a drought stress treatment. Although the authors measured xylem ABA concentration and the present study measured leaf ABA concentration, the fold increase in ABA concentration is comparable in both studies.

The change in ABA concentration during dehydration can be related to the timing of closing and opening of stomata. The sudden increase in ABA concentration, beginning at about 90 % RWC, correlated with the closing of stomata on the adaxial surface (Figure 4-5A). Stomata began to close rapidly at 96-90% RWC and were maximally closed in the range of 80-73% RWC. During the very early stages of dehydration, when stomata were observed to start to close, there was no significant change in ABA concentration, and there might be another mechanism involved during this stage. The subsequent decrease in ABA concentration during the later stages of drying, from 60% RWC onwards, corresponded to when stomata opened again.

It is well known that ABA causes stomatal closure in desiccation sensitive plants (Schroeder et al., 2001). In a study of quinoa (*Chenopodium quinoa*), it was observed during drying that root-sourced ABA resulted in stomatal closure, enabling a maintenance of leaf water potential (Jacobsen et al., 2009). The authors concluded that ABA regulation seemed to be a major mechanism employed by quinoa during drought to induce a decrease of turgor in stomatal guard cells, thereby closing stomata. This is supported by another study in the mango (*Mangifera indica*) (Zaharah and Razi, 2009). In the present study, the increase in ABA concentration in the initial stages of drying (Figure 4-5B) correlates with the closing of adaxial stomata (Figure 4-5A).

In some plant species, the MEP pathway produces a labile pool of ABA in the leaves and it has been shown that this source of ABA responds rapidly to environmental changes and also causes stomatal closure in response to drought (Barta and Loreto, 2006). As the authors noted, this pool is able to respond quickly to frequent changes in the availability of water. As *X. humilis* dehydrates very rapidly and the increase in ABA is seen to respond accordingly, there could also be a labile pool of ABA within *X. humilis* leaves, generated from the MEP pathway, in order to accommodate such rapid changes in RWC. However, this needs to be confirmed with further studies.

There seems to be a second closure of stomata in the later stages of drying (Figure 4-5A). It is not clear if this closure is due to regulation by ABA, as it was for the induced closure in the early stages of drying. There is a second peak in ABA concentration in the leaves at 50% RWC, however the biological variation at this RWC was large and therefore it is inconclusive if the apparent second peak is physiologically significant. The apparent closure of stomata during the late stages of dehydration could alternatively be due to physical changes brought about by drying, rather than chemical signals. Severe drying could result in collapse of the guard cells, causing stomatal closure. Although, this is not evident in the TEM images of guard cells, which, as discussed previously, show little change in size between hydrated and desiccated tissue. In the later stages of dehydration, there is folding of the epidermal cells and furrows become more pronounced, mostly on the abaxial but also on the adaxial surface. These desiccation-induced morphological changes could result in stomatal closure as the surface becomes more convoluted and there is severe water loss.

Transport of ABA throughout the plant relies on the transpiration stream and movement of water through the xylem (Brodribb, 2009, Schachtman and Goodger, 2008). In the early stages of drying of *X. humilis* there is sufficient water to transport the ABA, allowing for ABA regulation of stomatal closure during this stage of dehydration. However, during the later stages of drying, there may not be sufficient water to transport ABA through the plant (changes in transpiration rate will be discussed in the next section). Resurrection plants may then possibly rely on the inherent desiccation-induced morphological changes to close stomata during the late stages of drying, thus still enabling regulation of water loss. Lui *et al*. (2005) investigated the stomatal control and water use efficiency of the soybean (*Glycine max*) and indicated a similar finding. Their results suggested that at mild soil water deficits, stomatal conductance to water vapour was controlled by root-originated ABA, but at severe soil water deficits, it was most probably controlled by the leaf water potential. However, a major difference is that while desiccation-sensitive plants (such as the soybean) may not be able to provide stomatal regulation at severe soil and plant water deficits, in resurrection plants it is essentially not necessary, as desiccation tolerance has already been achieved.

This study provides evidence that there is indeed regulation of stomatal aperture during the initial stages of drying when it is advantageous to maintain a high RWC to accumulate protection, and stomata therefore close rapidly. However, once protection is acquired, stomatal regulation is seemingly no longer necessary, and water is rapidly lost. It is also deleterious to plants to have intermediate water contents due to the production of ROS and free radicals and thus it is beneficial to resurrection plants to rapidly lose water once desiccation tolerance has been acquired, to minimize damage (Farrant, 2007). It is still not clear whether the subsequent re-opening of stomata is an active or rather a passive process. The severe dehydration of guard cells (and therefore loss of turgor and shrinkage of cells) could result in a passive re-opening of stomata in the later stages of desiccation (Moore et al., 2007b, Vicre et al., 2004). However, the TEM images do not seem to agree with this hypothesis as there is no significant change in guard cell size between the hydrated and desiccated tissue.

4.3.4 STOMATAL REGULATION INFLUENCES GAS EXCHANGE

Gas exchange measurements were made and related to the percentage of open stomata during dehydration to examine the effect of stomatal closure on photosynthesis and to investigate whether it is possible to observe the hypothesized rapid loss of water when stomata open again from 60% RWC onwards.

PHOTOSYNTHESIS

Figure 4-6A shows the change in photosynthetic rate during dehydration. As can be seen, there was a rapid decrease in photosynthetic rate as the plants dehydrated from 100% to 80% RWC. Beyond 50% RWC, the photosynthetic rate measured below zero. This is in agreement with Figure 2-4A and previous studies (Farrant, 2000). The rapid decrease in photosynthesis corresponded with the closure of stomata. This is expected as stomata allow for gaseous exchange necessary for photosynthesis and thus closure of stomata will contribute to a decrease in photosynthetic rate. However, as the observed closure of stomata seems to be regulated by ABA and happens during the early stages of dehydration of *X. humilis*, it could be another mechanism employed to in turn regulate photosynthesis.

A study conducted on a wide range of C3 plants showed that under mild drought conditions, stomatal closure was the first response and main limiting factor of photosynthesis (Flexas and Medrano, 2002). However, the authors noted that at under severe drought, the increasing inhibition and down-regulation of metabolic processes was the main limiting factor of photosynthesis. Other studies have also found that there is a very strong correlation with stomatal closure and decreasing photosynthesis in the early stages of mild drought (Medrano et al., 2002, Cornic, 2000). In this study, it is clear that the decrease in photosynthesis is also tightly linked to the closure of stomata in the early stages of drying (Figure 4-6A). However, as discussed in Chapter 2, there also seems to be a decrease in Rubisco activity which is evident from the early stages of dehydration, namely 85% RWC (Figure 2-6A). Previous studies have also shown that RuBP regeneration and a decrease in Rubisco activity occurs during drought which subsequently limit photosynthesis (Castrillo and Calcagno, 1989, Gunasekera and Berkowitz, 1993, Medrano et al., 1997). This highlights the debate as to whether photosynthesis is limited during drought mainly by stomatal closure or by metabolic impairment. In the case of resurrection plants, the initial decrease in photosynthesis seems to be a result of both factors. The difference being that photosynthesis in *X. humilis* is subsequently also actively deactivated as chlorophyll is broken down, and recovers to full capacity during rehydration, as has been shown in Chapter 2.

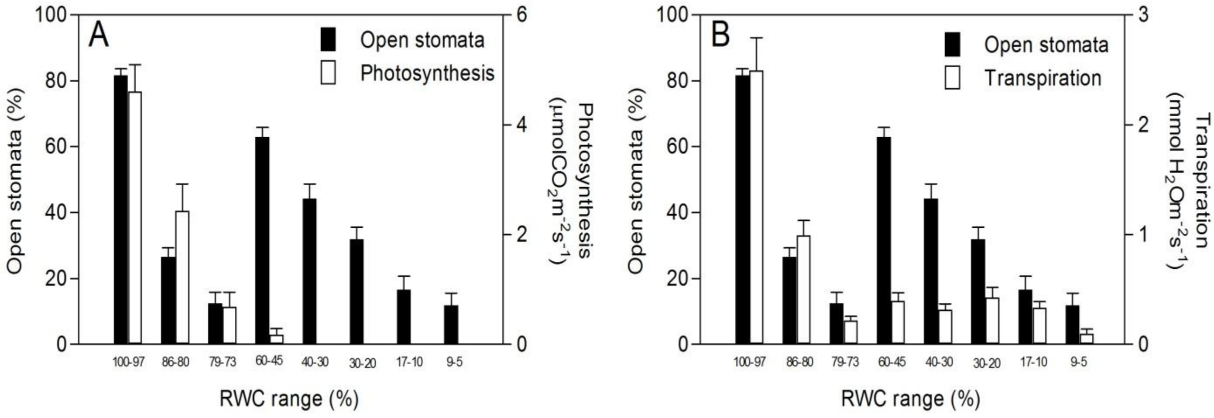

Figure 4-6: The change in photosynthetic rate (A) and transpiration rate (B) in relation to stomatal regulation on the adaxial leaf surface during dehydration of X. humilis plants (mean ± SEM, n=8).

If photosynthesis is compared to the change in total chlorophyll content (Figure 2-5A), it can be seen that the photosynthetic rate decreased more rapidly than the rate at which chlorophyll was degraded during dehydration. For example, at 80% RWC, the photosynthetic rate had decreased 6-fold, whereas the chlorophyll content at 80% RWC had only decreased about 2-fold. It seems that during the early stages of dehydration, the decrease in photosynthesis is more tightly correlated with the closure of stomata than with the degradation of chlorophyll. However, during the later stages of dehydration, when stomata open again, photosynthesis is rather inhibited by the dismantling of the photosynthetic apparatus characteristic of poikilochlorophyllous plants. In the above mentioned studies (Cornic, 2000, Flexas and Medrano, 2002, Medrano et al., 2002), the authors discussed how during the later stages of severe drought, photosynthesis was limited by the breakdown of metabolic processes, which were irreparable. Thus, resurrection plants, such as *X. humilis*, may be similar to desiccation-sensitive plants during mild drought, where photosynthesis is down-regulated by the closure of stomata. However, during the later stages, photosynthesis in resurrection plants is rather actively limited by the degradation of chlorophyll and dismantling of thylakoid membranes.

TRANSPIRATION

The rapid decrease in transpiration rate from 100% to 80% RWC, seen in Figure 4-6B, was expected, as *X. humilis* efficiently reduces water loss during early stages of dehydration. This confirms the observation that stomata initially respond to a decrease in plant and environmental water by closing. However, as can be seen in Figure 4-6B, the transpiration rate remained low throughout the later stages of drying, fluctuating around 0.5 mmol H_2O $m^{-2}s^{-1}$, even though stomata were observed to open again below 60% RWC (Figure 4-5A). This is not what was expected. As postulated, an opening of stomata in the later stages of drying is thought to be an active and sudden loss of water (Farrant, 2007), whereas the readings in Figure 4-6B do not reflect this.

The opening of stomata below 60% RWC during dehydration correlates with the sudden and rapid decrease in RWC between 80 and 120 hours after dehydration had begun (Figure 2-2A). However, this is not supported by the results obtained for gas exchange studies of transpirational water loss. This result could be in opposition to the hypothesis, suggesting that there is no active loss of water. But, this is unlikely and it is more probable that this is a result of technical short comings. The IRGA, and the equations used to calculate transpiration (von Caemmerer and Farquhar, 1981), rely on the assumptions that the plant is fully hydrated and the internal water vapour of the plants is 100%, whereas in this study the water content and internal water vapour decreased during dehydration. Another possible explanation is that when fully hydrated plants are transpiring, a considerable amount of water is lost from the leaves. The postulated active loss of water from the plants below 60% RWC may be crucial in the dehydration process; however, it is not significant when compared to the loss of water by transpiration from fully hydrated plants. The IRGA may not be adequately sensitive to measure the slight changes in water vapour in the chamber in the later stages of dehydration. Therefore, in order to test the hypothesis that water is actively lost from resurrection plants, once desiccation tolerance is established, it is suggested that one has to determine if there are pumps in the cells that actively cause a loss of water and to locate these in the genome.

4.4 CONCLUSION

X. humilis had many adaptations in place to limit water loss during the initial stages of drying, whether it is desiccation-induced changes in the morphology of the plant, such as folding of the leaves and clustering stomata in furrows, or a change in regulation of hormones such as ABA, which in turn causes stomata to close. As stomata close, photosynthesis is also limited. These responses to mild drought are similar to desiccation-sensitive plants. However, resurrection plants such as *X. humilis*, need only maintain water content initially whilst protective mechanisms against drought-induced damage are established. Once this is successful, stomata open again from about 60% RWC onwards and plants rapidly lose water. This is the first study in which the changes in ABA concentration have been investigated in resurrection plants. Although a correlation between ABA concentration and stomatal closure has been shown, further investigation is needed in order to determine whether ABA is definitely required for stomatal closure. An ABA inhibitor, such as fluridone, could be employed to treat *X. humilis* plants and subsequently assess stomatal aperture during dehydration to compare to untreated plants. It is also hypothesized here that during dehydration of *X. humilis*, the photosynthetic rate is initially limited by stomatal closure during mild drought, however, later on under severe drought conditions it is limited by the controlled dismantling of the photosynthetic machinery.

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CHAPTER 5

CONCLUDING REMARKS

Water deficit stress poses a huge threat to plants as excess energy resulting from disrupted photosynthesis can have detrimental effects if the situation becomes too severe. Plants have evolved several mechanisms to cope with such stress (reviewed in Chapter 1), which from work conducted here, appear to include increased ABA to promote stomatal closure and water conservation and the involvement of isoprenoid compounds with a photoprotective role, although further work is required to confirm these mechanisms.

All photosynthesizing organisms make use of the conserved mechanisms of photoprotection, for example carotenoids, such as β-carotene and zeaxanthin, and tocopherols. Specifically, β-carotene and zeaxanthin have strong antioxidant capacities as they quench singlet oxygen and other ROS, and the activation of the xanthophyll cycle, namely de-epoxidation of violaxanthin to zeaxanthin, to dissipate excess light energy as heat. As has been shown, these mechanisms of photoprotection are also present in *Xerophyta humilis.*

However, there are additional or alternative photoprotective mechanisms which have not been conserved amongst all plants, and these include the volatile isoprenoids. It is thought that these mechanisms allow an increase in the plasticity in photoprotection in certain plants, therefore allowing these species to adapt to adverse environmental conditions, such as high temperatures and elevated ozone (Peñuelas and Munné-Bosch, 2005). From the work presented in Chapter 3, it has now been shown that *Xerophyta humilis* emits the volatile organic compound isoprene, and there is evidence for an antioxidant role of isoprene as well as lipid stabilization. The unique capacity of resurrection plants to reduce photooxidative damage during severe dehydration, by one of two mechanisms, namely homoiochlorophylly or poikilochlorophylly, as in *X. humilis*, has been well studied, and also discussed in the thesis. This highlights that resurrection plants make use of alternative, non-ubiquitous strategies to minimize damage during dehydration. The finding in this study that *X. humilis* also emits isoprene, which has been shown to have a photoprotective role, suggests that this is an additional mechanism employed when the well-conserved mechanisms are not adequate to sufficiently protect against oxidative damage in order to attain desiccation tolerance.

The phytohormone ABA is well known to increase in response to drought in plants thereby inducing gene expression and causing stomatal closure. In *X. humilis,* as stomata were observed to close in response to early dehydration, this coincided with a peak in foliar ABA concentration. However, unlike desiccation sensitive plants which attempt to conserve water for as long as possible, stomata in *X. humilis* open again from 60% RWC onwards, thus providing further evidence for the hypothesis that there is an active loss of water in resurrection plants, once protective mechanisms have been accumulated early on.

Interestingly, the peak in ABA coincided with the peak in isoprene emission during dehydration of *X. humilis*. Both isoprene and ABA are formed from the methyl-erythritol phosphate (MEP) pathway in the chloroplast. There are multiple pools of ABA within plants, and therefore ABA is not only produced in the leaves, but can also accumulate in the roots and subsequently be transported up the xylem to the rest of the plant. It has been shown that the MEP pathway may produce a part of the leaf ABA in isoprene emitting and non-emitting species; that the emission of volatile isoprenoids is directly linked to foliar ABA concentration, and that the ABA pool formed by the MEP pathway causes stomatal closure in response to drought (Barta and Loreto, 2006).

Cleavage of the xanthophylls, neoxanthin and violaxanthin, has been shown as another pathway for the synthesis of ABA in leaves (Tan et al., 1997). As was seen in this study, neoxanthin and violaxanthin decreased during the initial stages of dehydration, which coincided with the sudden and rapid increase in ABA concentration. Xanthophylls are also formed in the chloroplasts via the MEP pathway (Figure 1-1).

Barta et al. (2006) suggest that alterations in the MEP pathway activity may result in a change in the labile pool of ABA within the leaves, which in response to water deficit, is also involved in stomatal regulation. As the MEP pathway is also responsible for volatile isoprenoid production, the change in isoprene emission could act as a proxy of the activity of the MEP pathway, and therefore ABA (Barta and Loreto, 2006). The authors also suggest that this labile pool of ABA within the leaves, which is able to rapidly respond to environmental stresses, has evolved in plants which are repeatedly exposed to these stresses.

In light of the results presented in this study, a hypothesis is that the flow of carbon through the MEP pathway in *X. humilis* is modulated in response to dehydration. Consequently, there is the increase in isoprene emission observed in *X. humilis* in response to early dehydration, and the subsequent rapid increase in ABA content, causing stomatal closure. The fact that the ABA content increased so rapidly within the leaves in response to early drought suggests that the metabolism could be linked to the chloroplast rather than cytosolic metabolism alone. So too are the individual carotenoids in *X. humilis* modulated during dehydration and rehydration, which are also formed from the MEP pathway. However, as *X. humilis* is poikilochlorophyllous, chloroplasts are broken down during dehydration, which may result in cessation of the MEP pathway. This could also explain the sudden decrease in isoprene emission and ABA concentration observed from about 60% RWC onwards, which coincided to a 50% decrease in chlorophyll content. Therefore, isoprene and ABA could act as signalling molecules during the early stages of dehydration of *X. humilis*, however, once chloroplasts are dismantled, these signalling molecules have already indirectly set secondary processes in place. For example, the subsequent re-opening of stomata due to a decrease in ABA allows for the rapid and active loss of water and the decrease in isoprene emission which then allows for an increase in lipoxygenase activity to produce LOX products, such as hexanal, to act as secondary messengers.

Furthermore, isoprene could initially stabilize thylakoid membranes during the initial stages of dehydration, until water deficit stress becomes severe enough to subsequently actively breakdown thylakoid membranes. Thereafter, free fatty acids become available for oxygenation by lipoxygenase enzymes, resulting in the peak in hexanal seen in the later stages of dehydration. Isoprene may also quench ROS that form, particularly during the initial stages of dehydration when photosynthesis is still taking place and protective mechanisms possibly have not been fully laid down.

During rehydration, isoprene is clearly needed as it increased to much higher levels during the later stages, possibly when antioxidant protection and thylakoid membrane stabilization was once again required due to reconstitution of the photosynthetic pathways. Chlorophyll content increased rapidly from 85% RWC onwards during rehydration as thylakoid membranes were reassembled. However, plants were not yet fully rehydrated and there is therefore potential for oxidative damage until full hydration is achieved. These hypotheses are speculative and need to be confirmed with future studies.

Further experiments to clarify these hypotheses have already been suggested in this thesis. Firstly, these include studies to clarify the role of isoprene in *X. humilis*, and therefore desiccation tolerance, whether it is by inhibiting isoprene with fosmidomycin or looking for products of isoprene oxidation. Secondly, the role of LOX activity should be further investigated by monitoring LOX gene expression and subsequently considering other genes which have previously been found to be induced by LOX products to confirm whether LOX products act as secondary signals in *X. humilis* during dehydration. The hypothesis of an active loss of water from resurrection plants, evidence for which is seen in the subsequent re-opening of stomata in *X. humilis*, should be further clarified by searching for possible pumps within the cell walls. Further studies should also be conducted to compare foliar ABA with root ABA during dehydration and rehydration to determine whether the rapid increase in ABA observed in *X. humilis* leaves in response to early dehydration is in fact derived from a labile pool of ABA within the leaves and whether it is this pool of ABA that regulates stomatal aperture during dehydration, rather than ABA synthesized in the roots. Finally, isotopically labeled carbon can be used to determine whether there are alternative carbon sources for isoprene formation within *X. humilis*, especially during dehydration and rehydration. The incorporation of ^{13}C into ABA and the various carotenoids during dehydration (and rehydration), could also be detected to assess how the flow of carbon is partitioned through the MEP pathway and how this flux changes during dehydration and rehydration. Ultimately, these studies will hopefully provide insightful information about some of the mechanisms which are unique to resurrection plants and also additional to the well-conserved strategies seen in all plants.

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