

**TOWARDS AN UNDERSTANDING OF THE  
MECHANISMS OF DESICCATION TOLERANCE IN  
*Myrothamnus flabellifolius* (WELW.)**

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**A Dissertation Presented to the Faculty of Science  
University of Cape Town**

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**Submitted in the fulfillment of the requirements for the degree of  
Master of Science in Botany**

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**By  
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March 1998**

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*Happiness, for the bee  
as for the dolphin, is to exist.  
For man, it is to know existence  
and to marvel in it.*

*Jacques-Yves Cousteau*



## **Preface**

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

Signed by candidate

Lynette Anne Kruger

March 1998

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**List of abbreviations**

A	assimilation
ABA	abscisic acid
CK	cytokinin
DT	desiccation-tolerant
$F_v/F_m$	quantum efficiency
HDT	homoiochlorophyllous desiccation-tolerant
LEA	late-embryogenesis-abundant
PDT	poikilochlorophyllous desiccation-tolerant
PGR	plant growth regulator
PSII	photosystem II
$q_N$	non-photochemical quenching
$q_P$	photochemical quenching
$R_d$	dark respiration
RWC	relative water content
Y	yield

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## Abstract

To date, most of the studies on the homoiochlorophyllous desiccation-tolerant (HDT) plant *Myrothamnus flabellifolius* have been conducted on excised twigs or leaves. In this study drying (including prolonged exposure to the dry state), and recovery of whole plants was compared with that of detached twigs dried off the plant, and also with twigs excised after having dried on the plant. Study of the leaf response during drying and recovery in the presence and/or absence of roots, and following prolonged desiccation, can contribute towards understanding the mechanisms of desiccation tolerance in *M. flabellifolius*.

Ultrastructural studies and investigation into patterns of electrolyte leakage were undertaken in order to determine the subcellular response to mechanical stresses associated with water loss. Since photosynthesis and respiration are complex metabolic processes that depend on the functional integrity of biomembranes, metabolic functioning of these organelles was measured using CO<sub>2</sub> gas-exchange methods, chlorophyll fluorescence techniques and quantification of photosynthetic pigments. Two of the plant growth regulators known to drive metabolic processes (abscisic acid and cytokinins) were quantified.

The morphological feature of leaf reorientation, visible during drying and recovery of *M. flabellifolius* plants, may have protected the mesophyll tissue from damage associated with high irradiance. The photosynthetic and respiratory processes of intact *M. flabellifolius* plants were affected in a manner similar to that reported for other HDT plants during water loss and recovery (Schwab *et al.*, 1989). Plasmalemma integrity was maintained and the chloroplasts became rounded during changes in water content. The thylakoid membranes underwent alterations that inhibited photochemical activity at relatively high water contents during drying, and delayed recovery during rehydration. This may be another mechanism for reducing the potential hazard of disrupted photochemical activity at water contents below full turgor. The 40% loss of total chlorophyll during drying was recovered within 24-hours rehydration. The mitochondria were operational across a wider range of water contents, with respiratory activity measurable at lower water contents relative to that of the photosynthetic processes.

It was evident that *M. flabellifolius* has the ability to reversibly switch off metabolism. This metabolic quiescence may be mediated by abscisic acid (ABA), which accumulates in the leaves during the initial period of water loss. ABA may also play a role in gene induction,

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where it has been implicated in the production of proteins associated with the protection of cellular structures and/or ameliorate the effects of water stress. Ultimately the reversible shut-down of metabolism may be a strategy which prevents damage arising from unregulated metabolism at low water contents.

Desiccation tolerance of leaf material was affected by drying rate and the absence of roots during drying. Rapid drying of twigs in the absence of roots prevented successful leaf reorientation and resulted in high levels of damage including elevated solute leakage, loss of photosynthetic pigments, and subcellular disorganisation. These twigs did not recover on rehydration. The rehydration of twigs dried slowly in the absence of roots did not display more damage relative to twigs rehydrated after having dried on the plant (i.e. in the presence of roots). Both treatments underwent initial metabolic recovery on rehydration, suggesting that subcellular protection occurred during drying. That recovery took place in twigs dried off the plant suggests that roots were not required for this protection to be induced. Such protective mechanisms may again be attributable to ABA accumulation, which occurred in leaf tissue despite the absence of roots. This does not preclude the possibility that damage occurred during drying and rehydration, but this damage was, initially, repairable. Although roots were unnecessary for initial recovery of metabolism, they were essential for sustained recovery. It is possible that sustained metabolic recovery required novel transcription induced via chemical signals from the roots. These signals may have included cytokinins, which appear necessary for the induction of chlorophyll synthesis and new bud formation in *M. flabellifolius*.

Desiccation tolerance of leaf material was affected by long-term maintenance in the dry state, regardless of the presence and/or absence of roots during rehydration. Prolonged desiccation for periods exceeding 18 months resulted in irreparable levels of damage (such as elevated solute leakage and subcellular disruption). This damage prevented leaf rehydration in plants maintained in the dry state for 18 months. Intact plants rehydrated within 12 months of desiccation were able to recover through the survival of meristematic tissue, where new axial buds were produced. Twigs maintained in the dry state for 12 months were unable to stimulate new bud production, perhaps due to the lack of a signal (possibly cytokinins) produced in the roots during rehydration. Whole plants rehydrated within six months of drying survived, although metabolic recovery progressed more slowly, relative to the recovery of plants rewatered within one month of drying. This more gradual metabolic recovery associated with prolonged desiccation, as well as the limited recovery following 12 months desiccation, and lack of recovery after 18 months maintenance in the

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dry state, suggests that protective mechanisms require replenishment of constituents. As this cannot occur in the dry state, damage accumulates with time.

Since *M. flabellifolius* plants grow in annual rainfall areas, prolonged desiccation would be uncommon for plants growing in the field. Repair and/or protective mechanisms may thus have evolved in order to enable recovery from levels of damage associated with 6 to 8 months desiccation.

*Myrothamnus flabellifolius* displays both physical (leaf reorientation and subcellular reorganisation) and chemical strategies which may be constitutively expressed and/or induced with changes in water content. These mechanisms of desiccation tolerance are limited by drying rates, the length of time for which the plants are maintained in dry state, and the presence of roots during recovery.

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## Chapter One: General Introduction

Plant productivity and survival is limited by water deficit, with unstressed metabolism operating in a very restricted portion of the water potential range ( $> 1.5$  MPa (Gaff, 1989) or above 90% relative water content (Kaiser, 1987)). Plants have been divided into three categories depending on their survival during periods of water deficit: drought evaders (such as ephemeral annuals escaping periods of limited water availability by existing as seeds during the dry periods); drought-avoiders, (plants such as succulents, which exhibit features preventing the development of internal water deficits despite limited exogenous water-supply); and drought tolerators (a few unique plants able to tolerate extreme water loss) (Bewley and Krochko, 1981).

Plants able to tolerate extreme drying, popularly termed "resurrection" plants, have the ability to survive severe water loss (desiccation) in the vegetative state (Oliver 1996). Although few in number, desiccation tolerant plants are widespread, and represent most major classes of plants, including algae, bryophytes, lichens, ferns, fern allies, and angiosperms (Bewley and Krochko, 1981).

This thesis is aimed at gaining an understanding of how the resurrection plant *Myrothamnus flabellifolius* (Welw.) is able to adopt the strategy of desiccation tolerance. *M. flabellifolius* is a woody geophyte capable of drying to water contents below 7% (Gaff, 1977). It is distributed across southern Africa (Ross, 1972; Dyer, 1975; Mendes, 1978) and in South Africa it's distribution correlates with the granitic and quartzitic rocks of the Limpopo Metamorphic Province, the Bushveld Complex, and the Pongola, Barberton and Transvaal sequences (Goldsworthy, unpublished). The plants occur singly or in clusters, on shallow (often  $< 15$  cm), well-drained rocky outcrops (Child, 1960). Growing on the exposed rock faces, these plants withstand considerable local extremes in temperature and irradiance, as well as experiencing an erratic water supply (Child, 1960). Because of these growing conditions, the plants are reported to survive for a large part of the year in a dry state, rehydrating only after summer rains (November to March) (Child, 1960).

Understanding how plant cells revive from the air-dry state is likely to play an important role in the development of crops suited to grow in areas exposed to regular water deficits. This area of research has advanced, particularly in the molecular field, and many genes which may play a role in tolerance of water stress have been identified (see e.g. Ingram and Bartels, 1996, and Bray, 1997, for reviews). Understanding of the biochemical, cellular and physiological processes of desiccation tolerance lags behind the molecular technology

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being applied however, and there has been little success in the development of drought tolerant crop species to date (Oliver and Bewley, 1997).

### THEORIES OF DESICCATION TOLERANCE

Oliver (1996) has estimated the independent evolution of desiccation tolerance in land plants on a minimum of twelve separate occasions. Such diverse taxonomic origins argue against the assumption that the mechanisms of tolerance are identical, or even similar across members showing this ability. Nevertheless, several early theories were proposed in an attempt to explain the phenomenon of desiccation tolerance.

#### **Early theories of desiccation tolerance**

Inward contraction of the cytoplasm during water loss from the vacuole creates tension between the plasmalemma and the cell wall. This mechanical stress can induce cell wall collapse and rupturing of the plasmalemma, and was considered by Ijijin (1957) to be the prime cause of desiccation-sensitivity. This theory has been criticised for its simplicity (Levitt, 1972), not fully explaining the phenomenon of desiccation tolerance. Mechanical stresses developed during drying remain a valid consideration however.

Stocker (1960) suggested that rapid drying led to a disorganisation and loosening of the protein latticework which constituted the protoplasm. He concluded that the molecular organisation of the protoplasm was the key to desiccation tolerance, with damage during drying being a result of differential hydration of the components of the protoplasm. When drying was gradual enough, the latticework could then be re-organised, and bonds strengthened. Despite consideration of the effect of drying rate on desiccation tolerance, this theory does not specify the processes affected by drying rate.

Both Ijijin and Stocker had proposed theories which considered desiccation tolerance to be the ability for cells to avoid and/or prevent damage from occurring during drying and rehydration (Bewley and Krochko, 1981). Genkel and Pronina (1968, 1969) were the first to suggest that tissues could tolerate drying. Their conclusion was based on the fact that desiccation-tolerant plants do not resist dehydration, but rather lose water rapidly, as an apparently normal reaction (reviewed in Bewley and Krochko, 1981).

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### Current theories of desiccation tolerance

One of the problems associated with work in this field, is that workers simplify the aspect(s) which they study, to the exclusion of other factors, such that no all encompassing theory has been developed to consider all aspects of desiccation tolerance (Bewley *et al.*, 1993). Bewley (1979) suggested however, that plant structure must meet three criteria in order to survive severe protoplasmic water loss. These were the ability to (1) limit damage incurred during drying to a repairable level; (2) maintain physiological integrity in the dry state so that metabolism can be reactivated quickly; and (3) mobilise repair mechanisms for recovery from desiccation and rehydration damage.

These criteria have been simplified into the hypothesis that desiccation tolerance is a balance between two fundamental processes: firstly, cellular protection from desiccation- and rehydration- induced damage; and/or secondly cellular repair of the damage that does occur (Bewley and Oliver, 1992). Since desiccation tolerance has evolved on more than one occasion, it would be expected that different plants span the possible combinations of these two strategies. This would range from plants that rely heavily on cellular protection to those which rely more heavily on repair.

Recently Oliver *et al.*, (1998) and Oliver and Bewley (1997) published reviews concerning desiccation-tolerant plants. In these reviews they separate desiccation-tolerant (DT) plants into two groups according to the rate of water-loss from the plants once free surface water has evaporated. "Fully desiccation-tolerant plants" are defined as those which can withstand total loss of free protoplasmic water at any rate, and all these plants studied to date come from the lower order groups (algae, bryophytes or lichens) (Oliver *et al.*, 1998). These plants possess few morphological or physiological characteristics which would permit them to control or reduce tissue water loss, thus their internal water content rapidly equilibrates to the water potential of the environment (Bewley and Oliver 1992; Oliver *et al.*, 1998; Oliver and Bewley, 1997). As a result of this many of these plants experience extreme drying rates (minutes to hours). The speed of desiccation then precludes the induction of protective systems, thus it has been proposed that the mechanisms of desiccation tolerance within these less complex plant groups are constitutive (i.e. in a constant state of readiness), and/or coupled to rehydration-induced repair processes (Oliver *et al.*, 1998).

In contrast with "fully desiccation-tolerant plants", Oliver and Bewley (1997) and Oliver *et al.*, (1998) describe the second group as "modified desiccation-tolerant plants". The

majority of modified desiccation-tolerant plants are found within the more complex plant groups of pteridophytes and angiosperms (Bewley and Krochko, 1981; Oliver *et al.*, 1998; Oliver and Bewley, 1997), and experience relatively slower drying rates (12 to 48 hours or more). Angiosperms and ferns (and fern allies) then achieve slow drying rates by employing various morphological and physiological mechanisms to control their rate of water loss. Whether this classification is valid is debatable, since the slower drying rate of higher desiccation-tolerant plants may result purely from the anatomy and physiology of these plants, these features not specifically mechanisms "controlling" water loss.

Available evidence concerning desiccation tolerance among the higher plant groups strongly suggests that these plants utilise mechanisms that rely mostly on inducible protection systems (Oliver and Bewley, 1997). (For reviews see Gaff, 1989; Leopold, 1990; Bartels and Nelson, 1994; Bewley and Oliver, 1992; Crowe *et al.*, 1992; Bewley *et al.*, 1993; Close *et al.*, 1993; Bewley, 1995; Oliver 1996). The time involved in the induction and establishment of protective components is thought to be why these desiccation-tolerant plants do not survive rapid water loss (Oliver and Bewley, 1997).

#### PHYSIOLOGICAL RESPONSES TO WATER STRESS

Cellular water deficit can result in the concentration of solutes, changes in cell volume and membrane shape, disruption of water potential gradients, loss of turgor, disruption of membrane integrity, and denaturation of proteins (Bray, 1997). Responses to water deficit may occur within a few seconds (such as changes in phosphorylation status of a protein) to within minutes and hours (e.g. changes in gene expression) (Bray, 1997). In order to investigate the mechanisms of desiccation tolerance, possible sources of damage arising as a consequence of water loss from plant tissue need to be considered.

Damage can be induced from mechanical and metabolic stresses generated during water loss. At the cellular level mechanical stress (arising from tensions between the plasmalemma and cell wall during drying (Iljin, 1957)) can result in cell wall collapse and rupture of the plasmalemma. At the subcellular level, decreasing water status generates numerous metabolic stresses, often additive in their (deleterious) effects. For example, during cellular dehydration solute concentration increases, causing the fluidity of the aqueous medium to decline. This can disrupt metabolic pathways (such as protein synthesis and signal transduction) which will interfere with metabolic processes (including photosynthesis and respiration), ultimately inhibiting photoassimilation and generating activated oxygen species (free radicals) (Farrant and Sherwin, 1998). Further water loss

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(below 0.2 g H<sub>2</sub>O/g dry mass) can alter macromolecular conformation (Leopold, 1986; Crowe *et al.*, 1987) with irreversible denaturation being lethal (Vertucci and Farrant, 1995). Finally, damage may be induced as a consequence of cellular rehydration, with the influx of water into the cells able to exacerbate damage generated during water loss.

Resistance to water deficit occurs when a plant withstands the imposed stress, and may arise from either tolerance, or a mechanism that permits avoidance of the situation. The discussion which follows will consider mechanisms whereby desiccation tolerant plants in general, and *M. flabellifolius* in particular, survive mechanical and metabolic stresses.

### 1. Mechanical stresses

Leaves from desiccation-sensitive plants such as *Pisum sativum* display extensive mechanical damage during drying, with irreversible plasmalemma damage evident (Sherwin and Farrant, 1996). Desiccation tolerant plants in contrast, display mechanisms (which differ among them) to minimise mechanical stress associated with drying, and recover during rehydration (Sherwin and Farrant, 1996). Proposed mechanisms include the filling of vacuoles with non-aqueous substances in order to provide volume within the cytoplasm and a back pressure against the cell wall (e.g. *Xerophyta viscosa*) and cell wall folding (e.g. *Craterostigma wilmsii*) (Farrant and Sherwin, 1998). These mechanisms could then prevent plasmalemma withdrawal and cell wall collapse on drying, as well as disruption of the plasmalemma upon rehydration (Farrant and Sherwin, 1998).

Early ultrastructural studies on desiccation tolerant vegetative tissue reported that the cells, organelles and membranes were variously disrupted in the dry state or immediately upon rehydration and did not regain 'optimal' organisation until they have been fully hydrated for some time (Hallam and Gaff, 1978a,b; Gaff, 1980; Oliver and Bewley, 1984; Schneider *et al.*, 1993; Tuba *et al.*, 1993 a,b; Bewley, 1995).

The limitations of ultrastructural study of dried cells due to tissue-rehydration associated with fixative methodology, has confounded attempts to determine the degree of structural damage associated with desiccation. Platt *et al.*, (1994) showed, using freeze-substitution procedures, that for the bryophyte *Tortula ruralis* and spikemoss *Selaginella lepidophylla* cellular organisation and organelle integrity was maintained in dry leaves, even though the cells were collapsed and the cellular contents condensed and aggregated by the physical forces of dehydration (Thompson and Platt, 1997).

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The discrepancy between observed cellular integrity in dry freeze-substituted tissues and cellular disruption reported for rehydrating tissues prepared with aqueous fixatives indicates that desiccation-induced damage occurs either during rehydration of tissues or because of inadequate preservation by the fixation regime, or both (Platt *et al.*, 1997). Because of the maintenance of cellular integrity and lack of damage in *T. ruralis* and *S. lepidophylla* observed using cryopreservation techniques, Platt *et al.*, (1997) concluded that chemical fixation methodology is inadequate for structural preservation, and may have led to subcellular characterisation of artifacts developed during fixation.

Aqueous and vapour-fixing techniques were applied to hydrated and desiccated tissue of *M. flabellifolius* by Goldsworthy and Drennan (1991), and there was little difference in ultrastructural organisation between the two methods (Sherwin and Farrant, 1996). Both techniques resulted in the maintenance of membrane integrity and it was concluded that the membranes of *M. flabellifolius* were protected during desiccation, thus allowing rapid recovery during rehydration (Goldsworthy, 1992).

An alternative method for the investigation of plasmalemma integrity involves monitoring electrolyte leakage from tissues. Leakage from tissues has been explained in terms of phase changes which take place in the structure of membranes during water loss (Simon, 1974, 1978). Water loss is proposed to change the organisation of the membrane lipids, with evidence suggesting that they form a gel phase (Hoekstra *et al.*, 1991). This gel phase then alters the association between the integral and peripheral proteins with the membrane lipids, thereby altering solute transport across the plasmalemma. Measurement of electrolyte leakage has been used as an indicator of membrane organisation during stress (Leopold *et al.*, 1981; McKersie and Stinson, 1980; Seneratna and McKersie, 1983).

Sherwin and Farrant (1996) investigated the rate of electrolyte leakage from desiccated and hydrated leaves of three desiccation tolerant plants and one desiccation-sensitive species. The desiccation tolerant plants displayed very low levels of leakage, relative to the desiccation-sensitive species, indicating maintenance of membrane structure in the leaves of dry desiccation tolerant plants. The slightly elevated solute leakage from dry leaves of two desiccation tolerant plants was found to be reversible during rehydration. This suggests that some change in membrane conformation, which occurred on drying, was reversed and/or repaired on rehydration of these desiccation tolerant plants.

## 2. Metabolic stresses

All metabolism is affected by water deficit. Water deficit directly affects photosynthesis since water oxidation is the source of electrons which generate reductants and ATP, and which, in turn, sustain all other biological activities. The process of photosynthesis is also indirectly affected by water deficit since closure of stomata during dehydration limits availability of the terminal electron acceptor of photosynthesis ( $\text{CO}_2$ ). When carbon assimilation is limited, excitation energy can be transferred from chlorophyll to oxygen, enabling the subsequent formation of oxygen free radicals. These free radicals can then affect the functional integrity of the biomembranes on which photosynthetic and respiratory processes depend (Larson, 1988; Smimoff, 1993).

In desiccation-sensitive plants, photosynthesis was found more sensitive than respiration to water loss, declining under even mild water stress (Bradford and Hsiao, 1982; Harten and Eickmeier, 1986). Similarly the photosynthetic apparatus of desiccation tolerant plants has been found particularly susceptible to injury during water stress (Tuba *et al.*, 1996a,b). Two groups of desiccation tolerant (DT) plants can be established with respect to their differences in behaviour of the photosynthetic apparatus during desiccation. Desiccation tolerant plants which retain most of their chlorophyll and organisation of thylakoid membranes during drying are called homoiochlorophyllous plants (HDTs), whilst those which lose almost all their chlorophyll content and disassemble thylakoids are called poikilochlorophyllous plants (PDTs) (Hamblen, 1961; Gaff, 1977, 1989; Bewley, 1979). The HDTs preserve their photosynthetic apparatus in the desiccated state such that functional recovery is possible at rehydration, whereas the photosynthetic apparatus is broken down in PDT plants (Hetherington and Smillie, 1982; Tuba *et al.*, 1993a,b).

It has been suggested that the loss of chlorophyll and dismantling of thylakoid membranes in PDT plants are protective mechanisms preventing light-chlorophyll interactions during desiccation (Smimoff, 1993; Sherwin and Farrant, 1996; 1998). Many HDT plants undergo changes in leaf orientation (shielding adaxial leaf surfaces and apical meristems) and accumulate anthocyanins (which mask chlorophyll) to avoid light-chlorophyll interactions (Sherwin and Farrant, 1998). Goldsworthy (1992) found that the levels of anthocyanins did not accumulate in dry leaves of *M. flabellifolius*, although the location of these pigments in the sub-epidermal layers of the abaxial leaf surface may have contributed towards protection of the photosynthetic apparatus from excessive radiation.

In addition to these physical changes, the presence of antioxidant systems also afford protection against free radical damage. Antioxidant defense systems can be divided into two groups: systems which react with active forms of oxygen and keep them at a low level, and systems which generate oxidised antioxidants. Increased levels of antioxidants from within each system have been isolated for both PDT and HDT plants during periods of drying and rehydration (Muslin and Homann, 1992; Seel *et al.*, 1992; Sgheri *et al.*, 1994a,b). These are proposed to play a protective role, particularly during the period of drying and rehydration in which the photosynthetic apparatus is still exposed to light (Sherwin and Farrant, 1998).

Sugars and proteins have been recognised as major components involved in the induction of protection during drying of DT plants (for reviews see Bewley and Oliver, 1992; Ingram and Bartels, 1996; Oliver and Bewley, 1997). The protection of cellular integrity during drying would then minimise the need for cellular repair during recovery. Farrant and Sherwin (1998) propose that, in addition to the protective role of sugars and proteins on drying, mechanisms that prevent and/or reduce the accumulation of damage throughout drying and rehydration (such as chlorophyll masking by other pigments) also exist.

### *Sugars*

An accumulation of sugars during desiccation has long been positively correlated with the degree of stress tolerance in plants (Bewley, 1979; Bewley and Krochko, 1981; Stewart, 1989). Various sugars, most notably trehalose and sucrose, can act as a water replacement to maintain membrane phospholipids in the liquid-crystalline phase, and to prevent structural changes in soluble proteins (Crowe *et al.*, 1984; Leopold and Vertucci, 1986; Carpenter *et al.*, 1987). Although the disaccharide trehalose predominates in a variety of desiccation-tolerant lower organisms, it is rare in higher plants. Instead, sucrose has been suggested to have an analogous role (Hoekstra *et al.*, 1989).

Both sucrose and trehalose accumulate in hydrated and desiccated leaves of *M. flabellifolius* (Suau *et al.*, 1991; Bianchi *et al.*, 1993; and Drennan *et al.*, 1993). Drennan *et al.*, (1993) reported that trehalose levels, which were relatively high in the hydrated state, increased only slightly upon dehydration. Sucrose levels however, increased by almost 200% upon desiccation. Membranes are suggested to be stabilised by a sucrose:phospholipid ratio of 4:1 (Hoekstra *et al.*, 1989), and whilst this ratio is not reached

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in *M. flabellifolius*, it was proposed that the combined trehalose and sucrose concentrations of *M. flabellifolius* might be sufficient to stabilise its membranes during desiccation.

Sugars have also been implicated as solutes that promote vitrification (glass formation) of the cytoplasm (Burke, 1986), and although this occurs at ambient temperatures as water is removed from seeds (Koster, 1991), there is less certainty concerning glass formation in vegetative tissue. Glasses formed by sucrose do not appear to be stable, although it has been suggested that a combination of sugars, such as sucrose, stachyose or raffinose, may be sufficient to form protective glasses (Caffrey *et al.*, 1988).

Farrant and Sherwin (1998) have proposed that the accumulation of oligosaccharides may be a result of monosaccharide removal in desiccation tolerant tissues. Monosaccharides, as respiratory substrates, would promote mitochondrial electron transport (Leprince *et al.*, 1992), which would favour metabolism, energy production and the formation of free radicals. Loss of these reducing sugars would then impose metabolic quiescence (Farrant and Sherwin, 1998). Furthermore, the presence of monosaccharides such as glucose at low water contents may disrupt protein structure through Maillard-type reactions (Koster and Leopold, 1988; Vertucci and Farrant, 1995; Farrant and Sherwin, 1998).

An increased level of oligosaccharides does not necessarily confer tolerance however, and some desiccation tolerant plants, such as the DT moss *Tortula ruralis* for example, does not accumulate sugars during drying (Bewley and Krochko, 1981). Thus, although protection by sugars may contribute to desiccation tolerance of plant cells, it appears insufficient to explain tolerance fully, being only one of the many components required for desiccation tolerance (Farrant *et al.*, 1993).

### *Proteins*

Water stress has been shown to promote novel protein synthesis in desiccation tolerant plants (Bartels *et al.*, 1990, 1992, 1993; Reynolds and Bewley, 1993a; Bewley, 1995; Kuang *et al.*, 1995). While a large number of genes have been identified in association with water deficits, the functional role of many of the encoded proteins remains unknown (see Ingram and Bartels, 1996 for a review). Among the proteins which have been elucidated are aquaporins and chaperonins, both regular metabolic proteins, which also accumulate during water loss (Steudle and Henzler, 1995). Aquaporins are proposed to regulate water loss, and chaperonins recycle denatured proteins (Vierling, 1990).

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The genes encoding late-embryogenesis-abundant (LEA) proteins have received much attention regarding their role in desiccation tolerance, since LEA gene products are often upregulated during water stress (Ingram and Bartels, 1996). The hydrophilic and robust physical properties of LEA proteins has led to their proposed involvement in subcellular protection against desiccation-induced damage (Dure, 1993), although direct evidence for their role in desiccation tolerance does not exist (Ingram and Bartels, 1996). Several LEA-like proteins called dehydrins (Close *et al.*, 1993) have been reported to accumulate during dehydration of DT plants (Piatkowski *et al.*, 1990; Bewley *et al.*, 1993; Kuang *et al.*, 1995). Dehydrins also accumulate in desiccation-sensitive plants however (Close *et al.*, 1993), indicating that if, like sugars, these proteins play a role, it is only a part of the mechanism of tolerance (Farrant and Sherwin, 1998).

Farrant and Sherwin (1998) propose that part of the mechanism of desiccation tolerance may be the ability to prepare for recovery. Many of the mRNA's and proteins induced during the initial stages of drying may then be stored in the dry state, available for rapid utilisation upon rehydration (Bewley and Oliver, 1992; Kuang *et al.*, 1995; Dace *et al.*, 1998).

The accumulation of free proline in leaves under water stress has been widely reported in literature (Aspinall and Paleg, 1981; Paleg *et al.*, 1984; Stewart, 1989). Proline is the most stable amino acid and may provide a mechanism associated with decreasing toxicity arising from protein hydrolysis (Levitt, 1980; Aspinall and Paleg, 1981). Proline can act as a compatible solute that exists in high concentrations without disturbing the activity of the macromolecules; it may serve as reserve material for later use on emergence from the stress; and has also been suggested to increase bound water levels due to its highly hygroscopic nature (Savitskaya, 1976, cited in Levitt, 1980; Ahmad *et al.*, 1982; Laurie and Stewart 1990). However, proline accumulation may have no adaptive advantage, merely being a consequence of stress (Aspinall and Paleg, 1981).

Tymms and Gaff (1979), studying leaves from detached branches, and Goldsworthy (1992) studying whole plants, investigated the effect of desiccation on the proline levels in the dried, versus hydrated states of *M. flabellifolius*. Although proline levels did increase slightly in the dry state, *M. flabellifolius* was found a low accumulator of proline (Tymms and Gaff, 1979; Goldsworthy, 1992). Tymms and Gaff (1979), studying several desiccation tolerant species, concluded that the ability for resurrection plants to survive air-drying is not attributable to accumulation of free proline *per se*.

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*Plant growth regulators (PGRs)*

Plant growth regulators are thought to play an important role in governing the water economy of mesophytic plants. The role of PGRs in the regulation of stomatal conductance during water stress has received the most attention (see Mansfield, 1990 for a review). Abscisic acid (ABA) increases rapidly in leaf tissue exposed to water deficits, and is thought to induce stomatal closure. Auxins (IAA), which are also synthesised in the leaves, can stimulate stomatal opening, whilst cytokinins (CKs) (generally synthesised in root tips) may enhance stomatal opening in some species. Gibberellins have not been implicated as regulators of water balance. Unlike ABA, the relationship between stomatal movement and endogenous levels of IAA and CKs in water-stressed plants has not been clarified.

ABA has been reported to regulate gene expression in response to most stresses (Chandler and Robertson, 1994). In DT plants, ABA increases on drying (Gaff and Loveys, 1984, 1992; Bochicchio *et al.*, 1998; Farrant and Sherwin, 1998), and can induce LEA genes in vegetative tissues (Ingram and Bartels, 1996). Extensive research has been undertaken concerning the kinetics of gene expression during drying and the requirement for ABA in the induction of desiccation tolerance in the HDT plant *Craterostigma plantagineum* (Piatkowski *et al.*, 1990; Bartels *et al.*, 1990, 1992, 1993). These studies have led to the hypothesis that ABA coordinates the activation of genes leading to cellular tolerance of desiccation (Bartels *et al.*, 1990).

Gaff and Loveys (1984) investigated the role of ABA in desiccation tolerance of *Borya nitida* (PDT) and *M. flabellifolius* (HDT). Applied ABA increased desiccation tolerance in *B. nitida*, but not in *M. flabellifolius*, whilst endogenous ABA levels displayed a transitory increase with water stress. They concluded that although ABA could promote desiccation tolerance in some species, it did not appear to be the prime controlling factor. Instead, they proposed that the variable increase in endogenous ABA might serve as one mechanism for the perception of water stress. Perception of water loss from the cell triggers a cellular signal transduction pathway, with ABA being one of the major signals operating during drought stress (see Bray, 1997 for a review). Soil water status has been invoked by several authors as a trigger for the synthesis of root messages involved in the induction of a drought stress response in the leaves (Gollen *et al.*, 1986; Davies and Zhang, 1991). Roots are able to synthesise increased amounts of ABA in response to soil dehydration (Comish and Zeevaart, 1984) and increased quantities can be delivered to the shoot (see Jackson, 1997 for a review). ABA accumulation in the leaves could in turn initiate desiccation-specific gene transcription and stomatal closure.

Much less is known about the involvement of other plant growth regulators at low water potentials (Spollen *et al.*, 1993). It has been shown that drought stress reduced the production and transport of cytokinins from sunflower roots (Itai and Vaadia, 1965), and Davies and Zhang (1991) concluded that, in drying soil, a reduction in cytokinin supply, perhaps acting in concert with other signals, could play a role in cellular response to water stress. The role of PGRs in regulating plant adjustment to conditions of water stress other than stomatal control includes ABA stimulation of root growth (Watts *et al.*, 1981), root tissue permeability (Glinka and Reinhold, 1972) and root pressure (Mansfield, 1990).

#### LIMITS FOR CELLULAR SURVIVAL DESPITE THE PRESENCE OF DESICCATION TOLERANCE MECHANISMS

The mechanisms of desiccation tolerance described thus far (such as dedifferentiation of organelles, morphological and biochemical changes), prevent or limit the stresses arising from water loss. However, under certain circumstances these mechanisms may not be effective in preventing damage. For example if drying is too rapid, or if excised leaves are dried (rather than dried attached to plants), then the mechanisms of tolerance may not be effectively induced within the tissues.

Although it is well established that the rate of drying is crucial for the successful development of desiccation tolerance, few studies have investigated the response of intact plants to increased drying rates. Unpublished studies in which the effect of drying rates of the HDT *Craterostigma wilmsii* and PDT *Xerophyta humilis* plants were tested, found that only the former survived rapid drying (Cooper, 1997). It was suggested that the lack of recovery upon fast drying of the PDT species was a consequence of insufficient time for the breakdown of the photosynthetic apparatus during drying. That, in turn, was proposed to cause light-induced damage while the plant was in the dry state, resulting in irreparable damage and lack of recovery. A similar response was reported during the rapid drying of detached leaves from *Borya nitida* (PDT), where the retention of chlorophyll caused disassociation of photosystem II (PSII) reaction centres from the light harvesting system preventing recovery of photosynthesis on rehydration (Hetherington *et al.*, 1982). Damage of a similar nature occurs in drying (at any rate) of desiccation-sensitive plants (Sherwin, 1995).

Spraying isolated leaves with water at regular intervals during the initial period of exposure to desiccation can slow down the rapid drying rate arising from desiccation of single leaves detached from the parent plants. For some DT plants this enables cellular survival (see Schwab *et al.*, 1989), however slow dehydration of excised leaves does not necessarily confer desiccation tolerance. The leaves of *Sporobolus stapfianus* for example, are unable to survive desiccation, irrespective of drying rate, when dried off the parent plant (Gaff, 1989).

A recent study by Bochicchio *et al.*, (1998), compared the effect of drying rate and final moisture content during desiccation of detached leaves from the HDT grass *Boea hygroskopica*. They concluded that drying rate affected final leaf water content, and this final water content was the critical factor in determining desiccation tolerance (Bochicchio *et al.*, 1998).

The frequency of dehydration-rehydration cycles may also affect successful implementation of the mechanisms enabling cellular tolerance to extreme water loss. Bryophyte tissues for example, are subject to seasonal fluctuations and drought hardening (Dilks and Proctor, 1976a,b) with a strong correlation between desiccation tolerance and the frequency and duration of dry periods (Clausen, 1952; Busby *et al.*, 1978). The majority of vascular DT plants have not shown seasonal alterations in tolerance ability (Bewley and Krochko, 1981), although Alamillo and Bartels (1996) found that leaf developmental stage could affect desiccation tolerance. The effect of duration between rehydration events has not been examined within DT angiosperm plants. Some studies concerning desiccation tolerance of DT angiosperm species, (such as Gaff and Ellis, 1974; Schwab *et al.*, 1989; Navari-izzo *et al.*, 1994; Quartacci *et al.*, 1997; and Bochicchio *et al.*, 1998), provide no information concerning the duration for which the plants were maintained in the air-dry state prior to rehydration. Other studies have provided information concerning the duration for which plant material was maintained in the dry state prior to experimentation. For example, all the studies by Tuba *et al.*, (1993a,b, 1994, 1996b) have involved the use of dry leaf material collected in 1988, and stored in air-tight polythene bags until experimentation. Markovska *et al.*, (1994) collected dry whole-plants, which were stored at room temperature and humidity in the anabiotic state for 6-12 months before experimentation. Lastly, Sherwin and Farrant (1996) studied intact plants growing in a greenhouse and left in the dry state for between one and three months before rewatering.

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### AIMS OF THIS STUDY

The physiological response of *M. flabellifolius* to desiccation (and to a lesser extent rehydration) has been reported in literature. Grundell (1933), Vieweg and Ziegler (1968) and Genkel and Pronina (1968) investigated the metabolic processes of photosynthesis and respiration, whilst anatomical and ultrastructural studies have been undertaken by Vieweg and Ziegler (1968), Wellburn and Wellburn (1976); Goldsworthy and Drennan (1991) and Sherwin and Farrant (1996). The only chemical changes examined for *M. flabellifolius* are the effects of applied and endogenous levels of ABA and proline studied by Gaff and Loveys (1984) and Tymms and Gaff (1979) respectively. All these studies (excluding Sherwin and Farrant, 1996) examined either single leaves dried at different rates by equilibrating leaves in air of different relative humidities, or have used leaves dried and rehydrated on detached twigs under unknown laboratory conditions. Since successful implementation of the mechanisms enabling cellular tolerance to extreme water loss is limited by, amongst other factors, drying rate and signal transduction pathways from the roots, results derived from manipulation of detached leaves or branches of *M. flabellifolius* may be spurious.

In this study intact plants were subject to dehydration and rehydration under simulated field conditions, and the effects of changes in water content on leaf tissues were monitored throughout the range of water contents (Chapter 3). This provided insight into the leaf response during drying and recovery in the presence of roots. The leaf response to drying and rehydration on the whole-plant also served as a basis for comparison with drying and rehydration of excised stems and leaves as reported in literature.

Leaves of twigs detached from hydrated whole-plants, and allowed to desiccate under simulated field conditions, were also monitored during drying and recovery (Chapter 4). This provided not only a comparison with the results reported in literature, but also gave an indication of the role of roots in the ability for leaves to tolerate desiccation.

Since the frequency of dehydration-rehydration cycles has received little attention for DT angiosperms, the effect of prolonged desiccation on recovery of leaves from intact plants desiccated and stored under field conditions was examined (Chapter 5). Finally, leaves from twigs detached from desiccated whole-plants, and stored for one or twelve months under laboratory conditions were examined during rehydration (Chapter 6). This provided

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comparison with twigs dried in the absence of roots, and enabled comparison of recovery with whole plants after long term maintenance in the dry state.

In summary this study was aimed at contributing towards an understanding of the mechanisms of desiccation tolerance by investigating the leaf response during drying and recovery in the presence and/or absence of roots, and following prolonged desiccation.

Several techniques were applied in an attempt to investigate the leaf response to mechanical and metabolic stresses associated with desiccation. Ultrastructural studies and examination of electrolyte leakage patterns provided an indication of the subcellular response to the mechanical stresses of water loss. Since photosynthesis in the chloroplasts, and respiration in the mitochondria, are complex metabolic processes that depend on the functional integrity of biomembranes, these processes were recorded using CO<sub>2</sub> gas-exchange methods during drying and rehydration. Metabolic functioning was also examined using chlorophyll fluorescence techniques, (which provide an indication of photochemical efficiency within the chloroplast membranes), the quantification of photosynthetic pigments (indicating recovery of protein synthesis), and quantification of plant growth regulators (ABA and cytokinins).

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## **Chapter Two: MATERIALS AND METHODS**

### *Plant material*

Whole plants were collected from the Buffelskloof Nature Reserve near Lydenberg (25°40`S; 28°32`E, Mpumalanga Province, South Africa,). They were transplanted into pots using a mixture of peat, river sand, and potting soil. The plants were kept in a greenhouse, and subject to regular cycles of desiccation and rewatering for growth and maintenance of hardiness. All experimentation was done in a constant environment chamber in which conditions were maintained at 50% humidity, with a temperature range of 16-25°C, a daily light period of 12 hours, and light intensity of 1400  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . The plants were moved into this constant environment chamber at least one week before experimentation. Several separate dehydration and rehydration procedures were conducted on at least three plants. At regular intervals during the drying and rehydration processes, leaves (at least three per sample) were removed for analysis of the parameters (1-7) described below.

### ***Treatments***

#### *(a) Roots present*

Desiccated intact plants, dried by withholding water, were maintained in the dry state in the greenhouse for varying lengths of time (one, six, twelve and eighteen months). Dry plants were rehydrated through rewatering, where a spray was used to simulate rainfall. Plants were well watered on the first day, and the soil was kept damp for the duration of the experiment thereafter.

#### *(b) Roots absent*

The first detached-twig study (Chapter 4), involved dehydration of large (>10 g fresh mass), and small (<10 g fresh mass) twigs detached from hydrated whole plants. Within a week of drying, these twigs were rehydrated by the placement of the cut ends in water. For the second detached-twig study (Chapter 6), twigs of approximately 10 g were removed from dry whole plants, and subsequently stored for one or twelve months under ambient laboratory conditions before rehydration (by the placement of the cut end of the stem in water).

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A summary of the drying and rehydration treatments is in given in Table 2.1 below.

Table 2.1 A summary of whole-plant (roots present) versus detached-twig (roots absent) treatments which were compared during dehydration and rehydration.

Dehydration	Rehydration	1 months desiccation	12 months desiccation
roots present	roots present	Chapter 3	Chapter 5
roots absent	roots absent	Chapter 4	_____
roots present	roots absent	Chapter 6	Chapter 6

**Statistical Analyses**

Statistical analyses were performed using STATISTICA for Windows Release 5.1, Statsoft, Inc., USA. In all figures, (unless otherwise specified) averages were calculated and standard deviations are represented as vertical bars. Either linear or distance weighted least squares regressions are drawn and significance (at p<0.05) was determined using Anovas and the Lord's Multiple Range test.

**Parameters Measured**

1. Water content

Water content of leaf material was determined gravimetrically by oven drying at 70°C for 48 hours. Absolute leaf water content was calculated on a dry mass basis as g H<sub>2</sub>O.g<sup>-1</sup> dry mass (g/g DM), using the equation:

$$\frac{\text{Fresh mass} - \text{Dry mass}}{\text{Dry mass}} \dots\dots\dots(1)$$

Full turgor was calculated after the leaves had floated on water for 24 hours. Leaf relative water content (RWC) was then calculated (as a percentage) using the equation:

$$\frac{\text{Fresh mass} - \text{Dry mass}}{\text{Full turgor mass} - \text{Dry mass}} \times 100 \dots\dots\dots(2)$$

The relationship between water content and RWC, useful for comparison with other plant species, is given in Figure 2.1. All subsequent results are plotted against absolute water content.

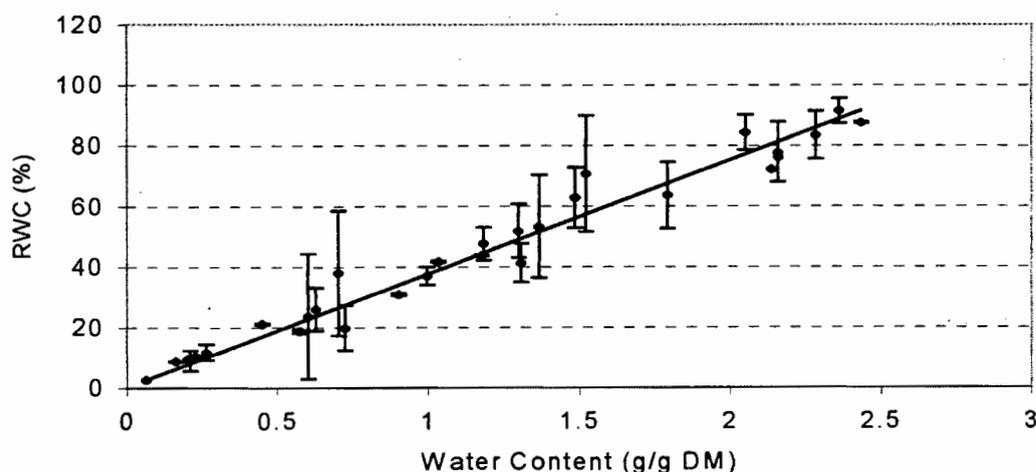


Figure 2.1 Relationship between leaf absolute water content (g/g DM) and relative water content (%). (Line calculated by linear regression,  $R^2 = 0.97$ ).

## 2. Leakage

Electrolyte leakage is expressed as a percentage of total electrolyte leakage (Yang *et al.*, 1996). Leaves of approximately 0.05 g fresh mass were placed in test tubes with 12 ml pure (distilled, deionised and filtered) water. Conductivity values of the bathing solution were recorded (using a Jenway 4070 conductivity meter) following initial leaf immersion ( $C_{\text{initial}}$ ). The samples were stored at room temperature for 3 h, and conductivity was again measured ( $C_{3\text{h}}$ ). This was compared with total leakage by measuring conductivity for the third time, once it had been autoclaved for 30 min, and allowed to cool to room temperature ( $C_{\text{autoclaved}}$ ). All subsequent electrolyte leakage data is expressed as a percentage of the total leakage, using the equation (3) below:

$$\text{Solute leakage (\%)} = [(C_{3\text{h}} - C_{\text{initial}}) / (C_{\text{autoclaved}} - C_{\text{initial}})] \times 100 \quad \dots\dots\dots(3)$$

### 3. Chlorophyll and carotenoid content

Photosynthetic pigments were extracted from 0.05 g leaf material using 100% acetone. The absorbencies at 470, 661.6, and 644.8 nm were measured using a Cary 1E, Varian Australia, double beam UV/VIS scanning spectrophotometer. Total chlorophyll (chlorophylls *a* and *b*), and total carotenoid (xanthophylls and carotene) content were calculated using the adjusted extinction coefficients according to Lichtenthaler (1987):

$$\text{chl}_a = 11.24A_{661.6} - 2.04A_{644.8} \dots\dots\dots(4)$$

$$\text{chl}_b = 21.13A_{644.8} - 4.19A_{661.6} \dots\dots\dots(5)$$

$$\text{chl}_{a+b} = 7.05A_{661.6} + 19.09A_{644.8} \dots\dots\dots(6)$$

$$C_{x+c} = (1000A_{470} - 1.90 \text{ chl}_a - 63.14 \text{ chl}_b) / 214 \dots\dots\dots(7)$$

### 4. Chlorophyll fluorescence

Fluorescence values were recorded from the adaxial leaf surface of leaves from whole plants and detached branches during both dehydration and rehydration processes. A modulated portable fluorometer (OS-500: Opti-Sciences, USA.) was used to measure chlorophyll fluorescence.

Leaves were dark-adapted for 5 minutes. Minimal fluorescence ( $F_o$ ), was set using a 660 nm modulation light source such that the modulation intensity was the highest possible before variable fluorescence was induced. Maximal fluorescence ( $F_m$ ) was induced using a saturating light intensity of 4 mmol photons  $\text{m}^{-2} \text{s}^{-1}$  for the duration of one second.

The level of fluorescence occurring during the operation of the Calvin Cycle ( $F_s$ ) was measured 3 minutes after maximal fluorescence. Finally, a saturating light pulse was again applied, this time to induce maximal fluorescence whilst the sample was undergoing photosynthesis ( $F_{ms}$ ). The actinic and saturation activity was then removed, and the sample exposed to a far-red source, thereby re-oxidising the photosystems, and calculating possible quenching of  $F_o$  ( $F_{od}$ ).

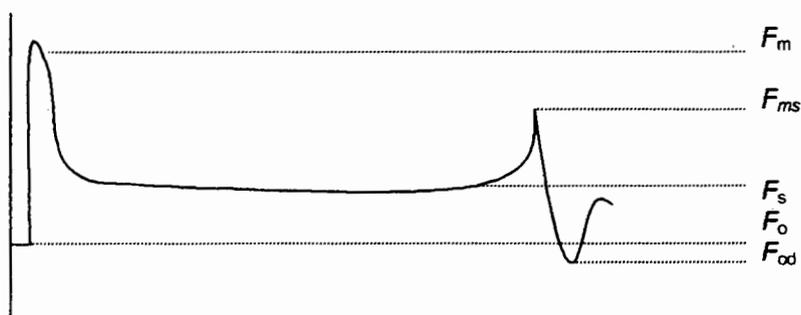


Figure 2.2 Schematic diagram of fluorescence parameters used in this study (Bolhar-Nordenkamp and Oquist, 1993).

Using these fluorescence values, (schematically represented in Figure 2.2), the following parameters were calculated (as described by Bolhar-Nordenkamp and Oquist, 1993):

$$\text{Quantum efficiency: } F_v / F_m = (F_m - F_o) / F_m \dots\dots\dots(8)$$

$$\text{Yield: } Y = (F_{ms} - F_s) / F_{ms} \dots\dots\dots(9)$$

$$\text{Photochemical quenching: } qP = (F_{ms} - F_s) / (F_{ms} - F_{od}) \dots\dots\dots(10)$$

$$\text{Non-photochemical quenching: } qN = (F_m - F_{ms}) / (F_m - F_{od}) \dots\dots\dots(11)$$

### 5. Measurement of CO<sub>2</sub> gas-exchange

The rates of dark respiration ( $R_d$ ), and light saturated net photosynthesis ( $A$ ), were measured using an LCA3-type (ADC Co. Ltd., Hoddesdon, UK) infrared gas analyser. This was operated in the differential mode at an ambient CO<sub>2</sub> concentration of 350 ppm, and the CO<sub>2</sub> gas-exchange parameters  $A$ , and  $R_d$ , were calculated according to the equations of von Caemmerer and Farquhar (1981).

### 6. Plant growth regulators

Leaf material, removed during the process of both dehydration and rehydration, was lyophilised, finely ground, and stored at  $-80$  °C until required. Analysis was on single samples (with internal replicates) selected at key water contents. Whole plants were sampled for both abscisic acid (ABA) and the cytokinins (CKs) zeatin and zeatin riboside, at water contents of 2.0, 1.6, 1.0, 0.5, and 0.06 g/g DM during drying and rehydration. A similar set of water contents were collected from large twigs during dehydration (1.3, 1.0, 0.3, 0.1 g/g DM); however only two water contents were sampled from the smaller twigs (1.3 and

0.1 g/g DM) during drying (this being due to the rapid rate of small twig dehydration (in <12 hours)). ABA and cytokinin content of rehydrating large twigs were examined at water contents of 1.7 g/g DM (36 hours following rewatering), and 1.0 g/g DM (this decrease due to dehydration 96 hours following rewatering). The ABA and cytokinin concentrations from twigs rehydrated detached from the whole-plant, (after having dried on the whole-plant for 1 or twelve months), were examined for at water contents of 1.2 g/g DM, and 0.5 g/g DM (for twigs dried for 12 months only).

Using the method described by Farrant *et al.*, (1993), samples (0.1 g dry mass) were extracted (24 hours at 4 °C, with stirring) in 2 ml of 70 % methanol, containing 20 mg/litre butylhydroxy toluene and 50 mg/litre sodium ascorbate. The samples were centrifuged at 20 000 g for 10 minutes, and the supernatant was passed through a C<sup>18</sup> Sep-Pak column. The filtrate was reduced to dryness in a Savant vacuum concentrator and stored at -20 °C before HPLC separation. Samples were redissolved in methanol, and triplicate aliquotes were separated by HPLC, following which radioimmunoassays were performed on them. The presence of interfering substances were tested for as described by Farrant *et al.*, (1993). Raw data were analysed using an on-line computer and the Securia data reduction radioimmunoassay package (Packard Instrument Company, 1986, publication No. 169-3016).

### 7. Ultrastructure

Leaves removed during the course of dehydration and rehydration were processed for transmission electron microscopy (TEM) using the method described by Sherwin and Farrant (1996) also working with this material. Platt *et al.*, (1997) investigated discrepancies in subcellular organisation of desiccated material that have been observed in association with freeze-substitution versus chemical fixation methodologies. They concluded that chemical fixation procedures were inadequate for structural preservation. However, Goldsworthy (1992) and Goldsworthy and Drennan (1991) investigated the ultrastructure of hydrated and desiccated tissue from *M. flabellifolius* using both aqueous and vapour techniques, and there were no significant differences between the two results (Sherwin and Farrant, pers. com.). If damage was caused by rehydration due to aqueous fixation, then any dehydration-associated damage which may have occurred would be exacerbated by this fixation protocol. Using this logic, aqueous fixatives will give an indication of potential damage, and further hydration will give an indication of the ability to repair this damage. As this method is most convenient, and because of the little difference in ultrastructural

organisation when using either aqueous or vapour fixed methods, chemical fixation methods were used in the current study.

Five mm<sup>2</sup> sections were fixed in 2.5% gluteraldehyde in 0.1M phosphate buffer (pH 7.2) and 0.5% caffeine, and post-fixated in 1% osmium tetroxide in 0.1M phosphate buffer. Following ethanol dehydration, the material was infiltrated and embedded in epoxy resin (Spurr, 1969). The tissue was sectioned at a gold interference colour (approximately 75 nm thick), using a Reichert Ultracut-S microtome. Sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) for 10 minutes each, and viewed with a Zeiss EM-109 transmission electron microscope.

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## **Chapter Three: Characterisation of the leaf response during drying and recovery of the whole plant**

### **INTRODUCTION**

Present understanding of desiccation-tolerance in *M. flabellifolius* has been based, to a large extent, upon studies of detached leaves or branches (Genkel and Pronina, 1968; Vieweg and Ziegler, 1968; Gaff, 1977). Because of the absence of roots, the response of isolated leaves, or leaves having dried on excised stems, might not accurately reflect the situation for leaves having dried on the whole plant. In the present study the changes occurring in leaf tissues dried on entire plants was followed.

Only two studies, have reported on material from whole plants of *M. flabellifolius*, have been reported. The first study (Goldsworthy, 1992) investigated some of the morphological, anatomical, ultrastructural and physiological responses from roots, stems and leaves of *M. flabellifolius* (as reported in Chapter 1). The second study (Sherwin and Farrant, 1996) examined leaf chlorophyll fluorescence characteristics across a range of water contents during whole plant rehydration.

This chapter reports on some physiological and ultrastructural changes occurring in the leaves of *M. flabellifolius* throughout a range of water contents during drying and rehydration.

### **RESULTS**

#### **3.1 DEHYDRATION OF THE WHOLE PLANT**

Visible signs of water stress, (observed as changes in leaf orientation), were evident several days (depending on plant size and soil volume) after water had been withheld from the plants. Following leaf reorientation, leaf water content took two to three days before attaining air-dryness. To normalize data among plants, time of drying was correlated with observations of leaf behaviour.

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### 3.1.1 Leaf reorientation with drying

Change in leaf water content with time during dehydration is given in Figure 3.1. Using changes in leaf orientation as visible indicators of water loss, drying could be divided into 3 stages:

- I. During the first  $15 \pm 4$  hours of drying, water content decreased slowly ( $0.03$  g/g DM/ h), from full turgor ( $2.0$  g/g DM) to  $1.6$  g/g DM. The leaves, initially lying perpendicular to the stem (Figure 3.2a), reorientated through  $90^\circ$ , becoming parallel to the stem axes and enclosing the apical buds (Figure 3.3a,b,c).
- II. The rate of water loss was at a maximum during the following  $12 \pm 4$  hours ( $0.05$  g/g DM/ h). The leaves folded along a series of leaf ridges and furrows (Figure 3.3d&e), and water content decreased to  $1.0$  g/g DM.
- III. Dehydration proceeded more slowly ( $0.03$  g/g DM/ h) during the following 24-36 hours, stabilising at air-dryness ( $0.2$  g/g DM). The lateral branches curved upward towards the main stem, and the desiccated leaves became reddish-brown in colour (Figure 3.2b).

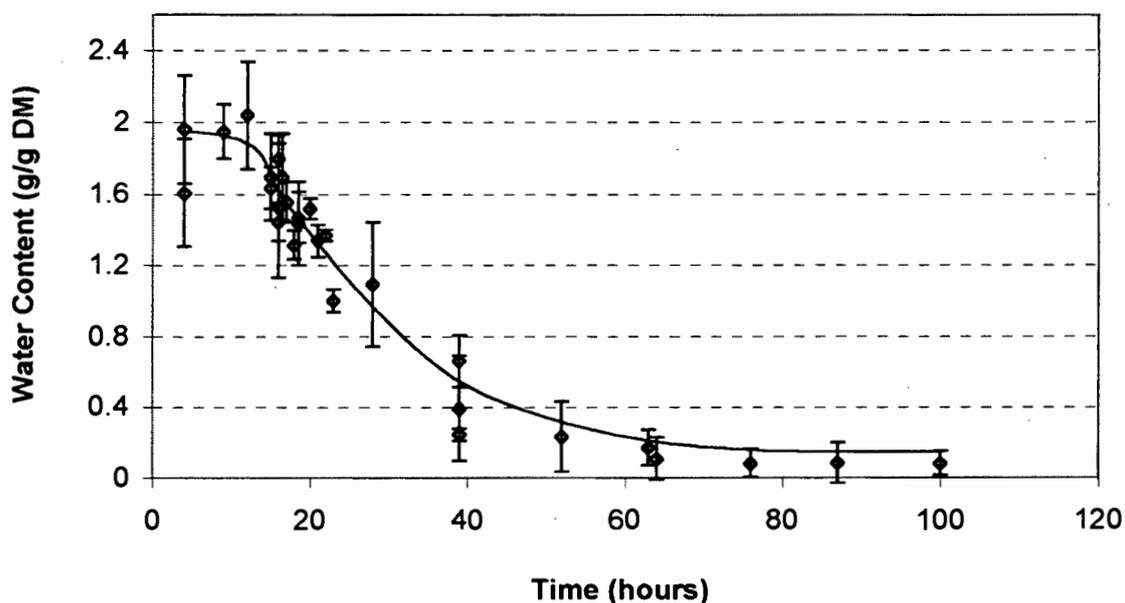


Figure 3.1 Leaf water loss with time during drying of the whole plant. (Data points represented as a scatterplot of means, bars indicating standard deviations, and trends investigated using distance weighted least squares regression analysis.)



Figure 3.2 Fully hydrated leaves (water content 2.0 g/g DM) several days after rewatering. Leaves orientated perpendicular to the stems, with some leaves yellowing before abscission (a). Desiccated leaves (water content 0.2 g/g DM) dried for one month, leaves orientated parallel to the stems (b). Scale bar = 5 mm

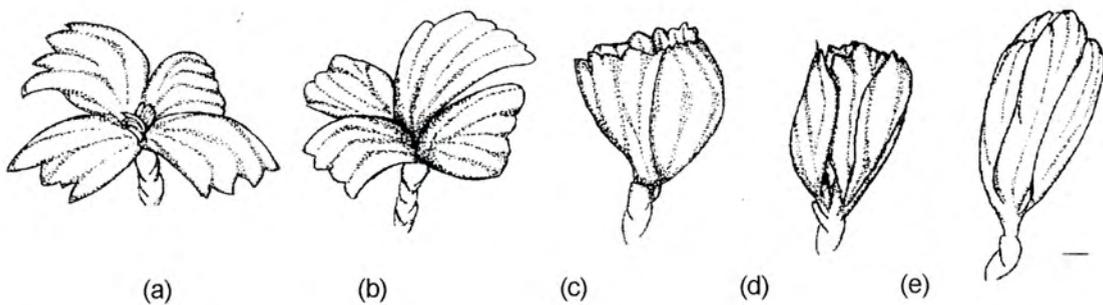


Figure 3.3 Sequence of leaf reorientation during drying of intact plants: leaves perpendicular to main stem (a) before reorienting through  $90^\circ$  and enclosing the apical bud (b&c), finally folding along a series of ridges to form a tight bud (d&e). Scale bar = 1 mm

### 3.1.2 Solute leakage

Leaf solute leakage, (as a percentage of total leakage), during dehydration of whole plants, is illustrated in Figure 3.4. Pre-stress leakage levels (from leaves of hydrated control plants), and leakage from leaves of plants maintained in the dry state for one month (dry control), are indicated in red.

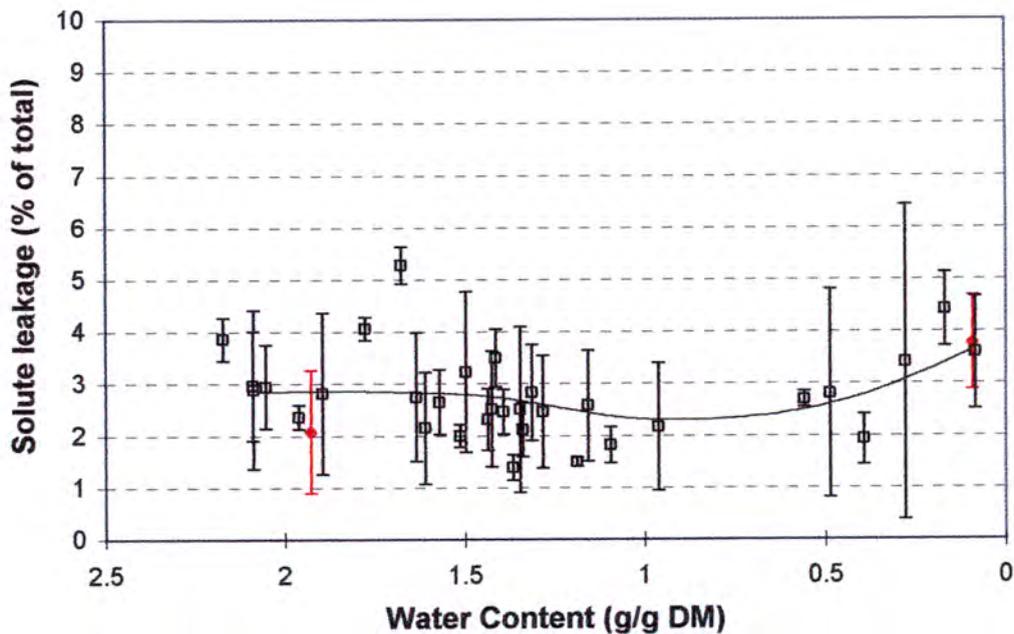


Figure 3.4 Solute leakage during dehydration of whole plants. Red symbols indicate average hydrated control (before experimentation) and dried (for one month) leakage values.

Solute leakage was low throughout dehydration, although leakage increased slightly during the final stages of water-loss, (water content  $<0.5$  g/g DM). Low values of leakage indicate that membrane damage did not occur during neither the dehydration process, nor after maintenance of the plants in the dry state for one month. This would imply that protective mechanisms exist which maintain membrane integrity throughout changes in water content.

### 3.1.3 Chlorophyll and carotenoid content

Although *M. flabellifolius* has been classified as a homoiochlorophyllous (chlorophyll-retaining) desiccation-tolerant (HDT) plant (Hoffman 1968; Gaff, 1977), both chlorophyll and carotenoid concentrations of the leaves decreased, by 42% and 28% respectively, following dehydration to less than 0.2 g/g DM (Figure 3.5). Upon attainment of air-dryness, there was no further loss of pigment content from *M. flabellifolius* leaves. Thus, after 60 hours dehydration, pigment concentrations had reached levels characteristic of plants maintained in the dry state for one month (pre-stress and dry (desiccated for one month) pigment levels indicated in red on Figure 3.5). This could indicate that enzymic breakdown of pigments cease once the plant has dried. Alternatively, pigments may be preferentially lost from some regions of the leaf, possibly from the abaxial surface, which discolours during drying.

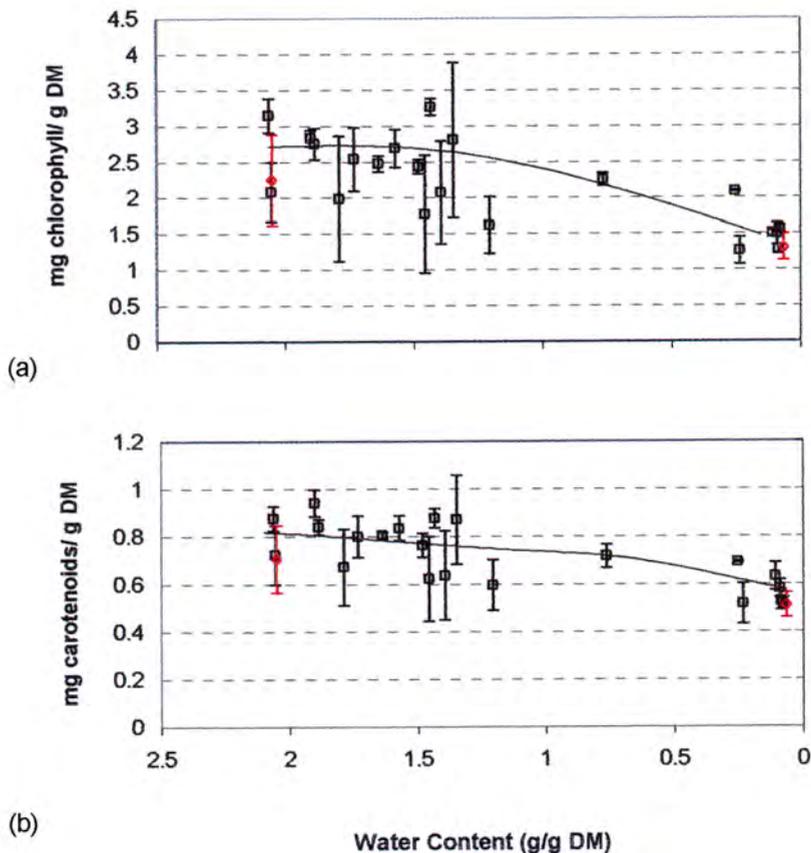


Figure 3.5 Total leaf chlorophyll (a), and carotenoid (b) concentrations, during dehydration of the whole plant. Red symbols indicate control (hydrated) and desiccated (for one month) leaf pigment concentrations.

### 3.1.4 Chlorophyll fluorescence

During the initial stages of drying, (i.e. during the reduction of water content from 2.0 to 1.6 g/g DM), quantum efficiency ( $F_v/F_m$ ), and photochemical quenching ( $qP$ ) remained near pre-stress levels (Figure 3.6a&c, pre-stress levels indicated in red). High  $F_v/F_m$  was indicative of functional chlorophyll, with the potential for electron transport through PSII. This contrasts with light-adapted PSII efficiency (Yield), which decreased immediately with water-loss (Figure 3.6b). The reduction in yield ( $Y$ ) may be the result of stomatal closure, restricting  $CO_2$  supply, or perhaps self-shading, through leaf reorientation.

Following a reduction in water content to values below 1.6 g/g DM, (around 15 hours following the onset of the first visible symptoms of water stress),  $F_v/F_m$  decreased markedly. This would appear to indicate that electron transport of PSII was down-regulated at relatively high water contents. Complete inhibition of all photochemical activity around water contents of 1.0 g/g DM further supports the suggestion that there is a controlled shut-down of this metabolism before water is limiting.

No clear trends were found for the non-photochemical quenching coefficient,  $qN$  during drying (Figure 3.6d). Non-photochemical quenching mechanisms would be expected to increase in order to provide alternate electron de-excitation pathways (necessary because of decreased electron transport within the chloroplasts). Self-shading (through leaf-folding) will decrease the amount of energy received however, thus non-photochemical quenching mechanisms may not be important during desiccation of this species.

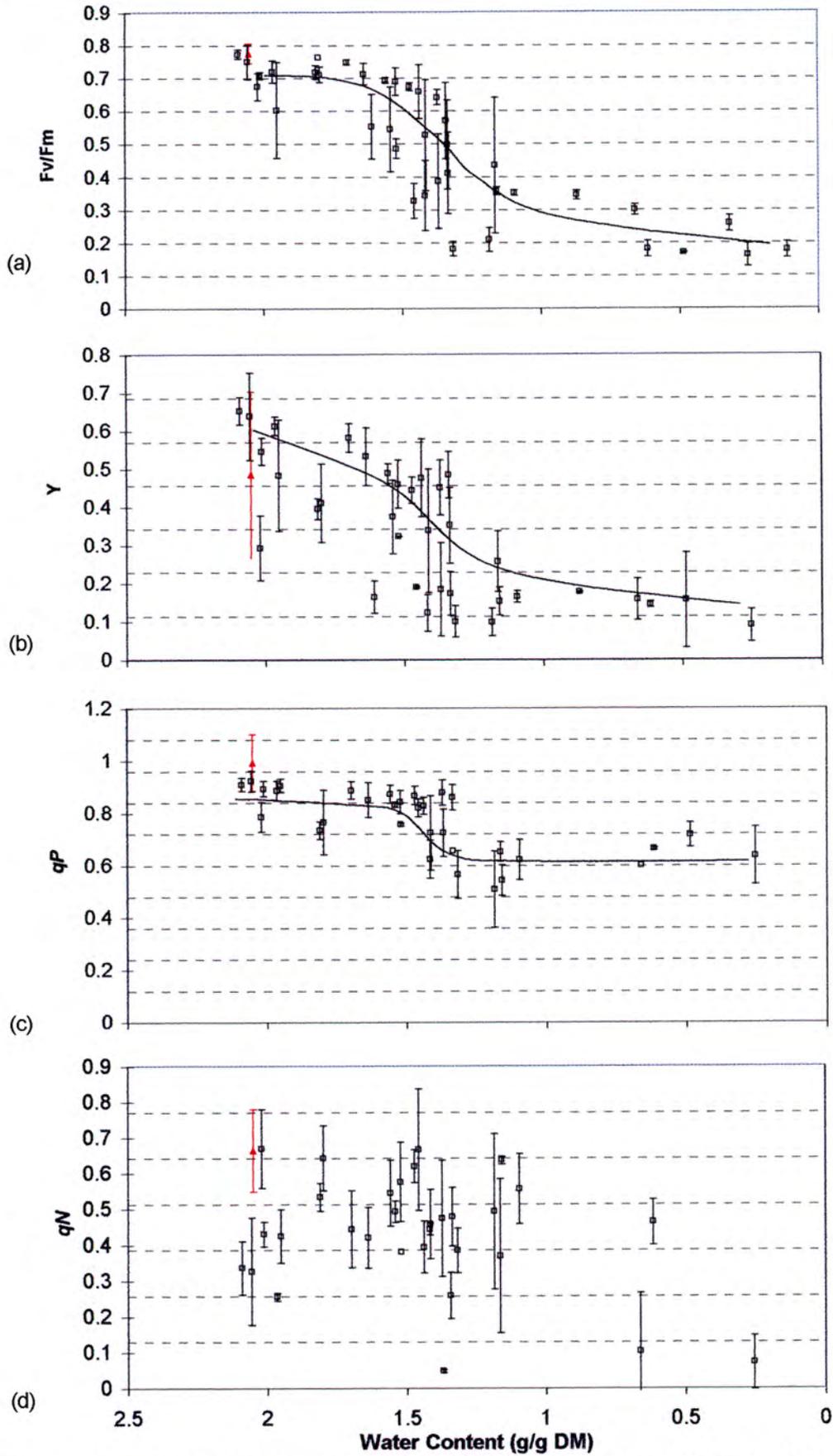


Figure 3.6 Leaf chlorophyll fluorescence during drying of the whole plant. ( $F_v/F_m$  (a), Yield (b), and the quenching coefficients  $q_P$  and  $q_N$  (c&d). Red symbols indicate control (hydrated pre-stress) values.

### 3.1.5 CO<sub>2</sub> gas-exchange

Net CO<sub>2</sub> assimilation (A) and dark respiration rates are given in Figure 3.7. Carbon assimilation decreased rapidly during the initial stage of drying, reaching zero net photosynthesis by the time water content had dropped to levels of 1.6 g/g DM (corresponding with the decrease in yield and leaf reorientation) (Figure 3.7a). The decrease in photosynthesis may again have arisen through reduced light-interception due to self-shading, although stomatal location in furrows of the abaxial surface (Goldsworthy, 1992), which became progressively more compact during leaf plication, could have reduced carbon assimilation by inhibiting gas exchange.

In contrast, dark respiration (Figure 3.7b) remained high during drying, decreasing only once water content had dropped below 1.0 g/g DM, and indicating incomplete stomatal closure during this period of water-loss. Respiration, despite water loss, might be necessary as an energy-source for processes such as the induction of protection proteins. No further respiration was measurable once water content had decreased below 0.5 g/g DM.

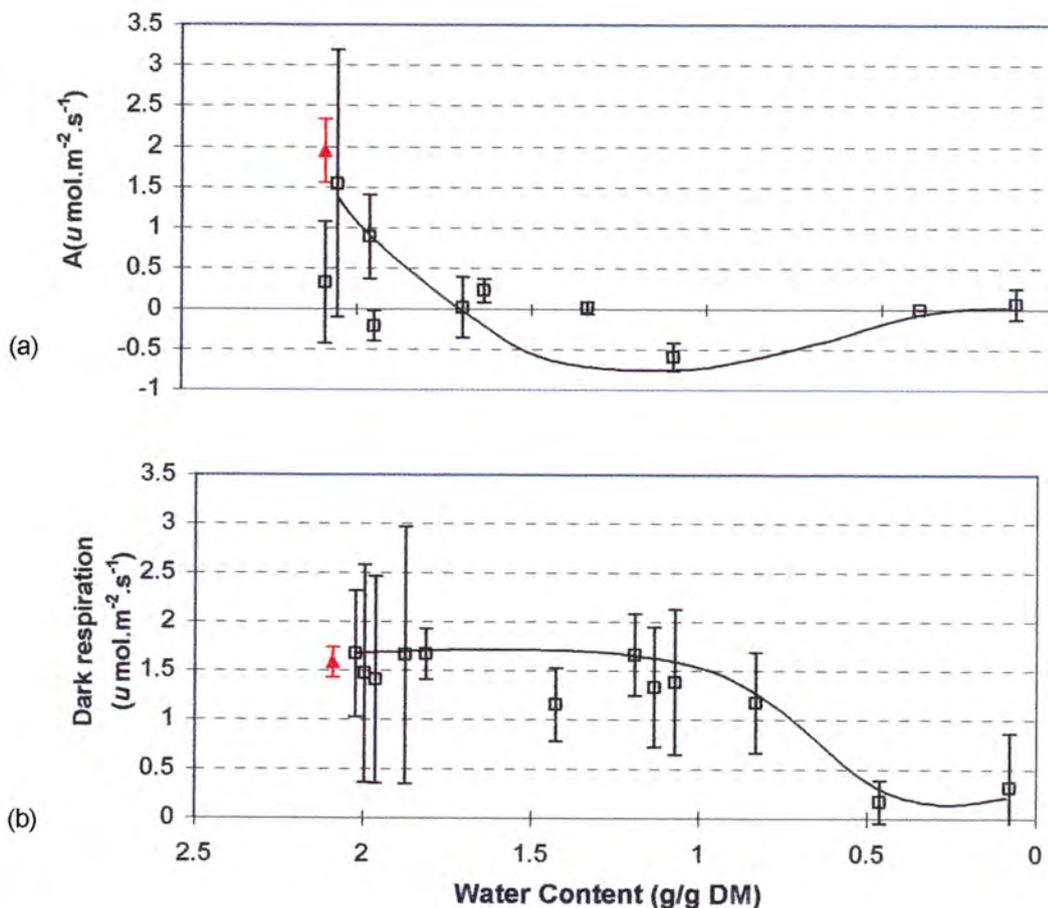


Figure 3.7 CO<sub>2</sub> assimilation, A (a), and Dark Respiration,  $R_d$  (b) from leaves during whole plant dehydration. Red symbols indicate hydrated control (pre-stress) values.

### 3.1.6 Plant growth regulators

Abscisic acid (ABA) and cytokinin (zeatin and zeatin riboside) concentrations were sampled at four stages of dehydration, as illustrated in Figure 3.8. The levels of ABA were within the range recorded for *M. flabellifolius* by Gaff and Loveys (1984), and Goldsworthy (1992). ABA concentrations increased as water content dropped from full turgor to 1.6 g/g DM (coincident with leaf reorientation and plication). ABA has been associated with the induction of genes encoding polypeptides which may be involved in the protection of cellular structures and/or ameliorate the effects of drought stress (reviewed by Ingram and Bartels, 1996). Increased levels of ABA during water-loss from *M. flabellifolius* might then imply that ABA plays a role in the induction of desiccation tolerance for this species.

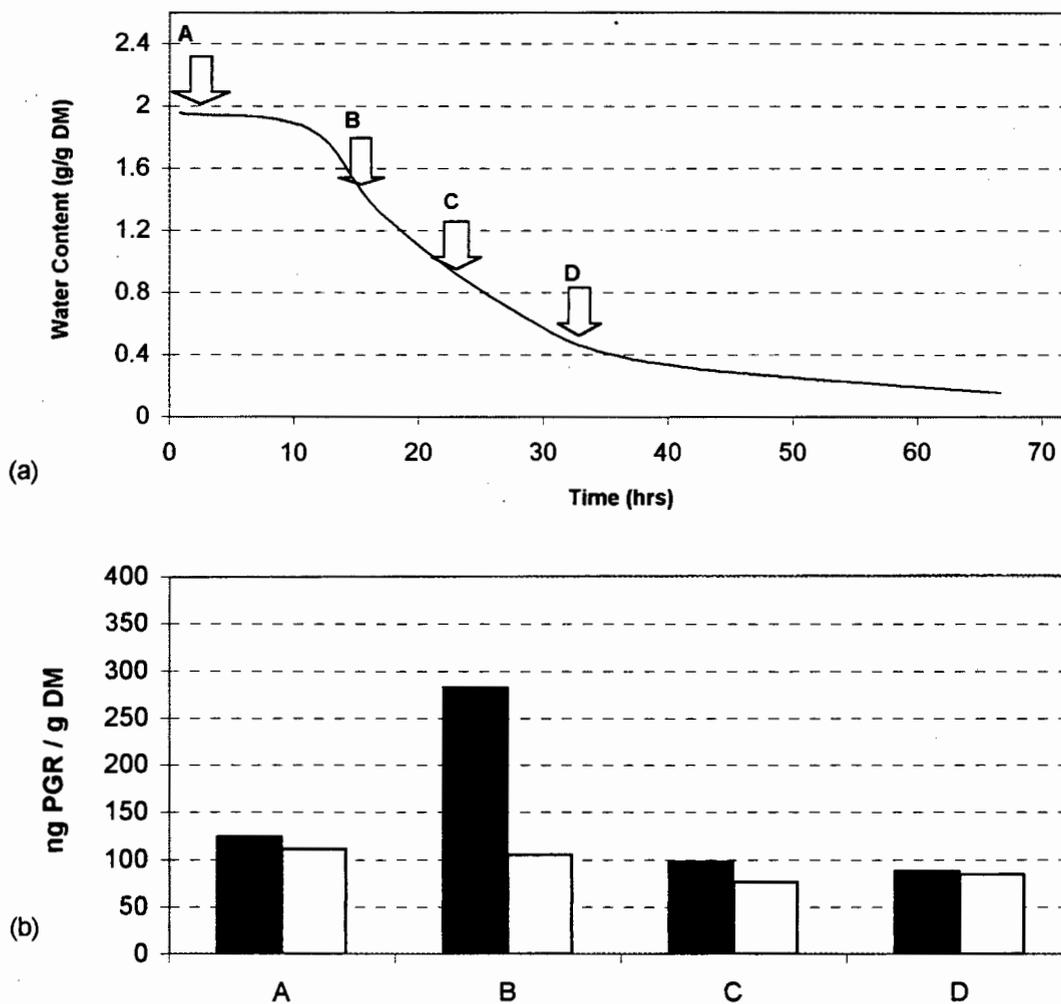


Figure 3.8 Water content and time during which leaves were sampled for the presence of the plant growth regulators (a); and total abscisic acid (ABA, solid bars) and cytokinins (CKs, blank bars) extracted at these water contents during whole plant dehydration (b).

ABA levels decreased with subsequent water-loss, stabilising at levels slightly below hydrated control values as the leaves became air-dry. This indicates, as suggested by Gaff and Loveys (1984), that ABA does not have to be present at continuously high levels in order for the leaves to tolerate desiccation.

In contrast with the rise in ABA concentration during the first 15 hours of water stress, cytokinins remained relatively constant throughout the drying process. Cytokinins (CK), ABA, and the interaction of these two growth regulators, have been implicated as root signals that modulate responses of shoot growth and stomatal aperture to soil drying (Zhang and Davies, 1991; Tardieu and Davies, 1992).

Increased ABA alone, or elevated ABA interacting with low CKs, have been found to promote stomatal closure of plants subjected to a shortage of soil water (Mansfield and Atkinson, 1990). Stomatal studies of this species (Goldsworthy, 1992) show that stomatal closure did not occur coincident with changing ratios of ABA and CK during the drying of *M. flabellifolius*. This would indicate that these plant growth regulators were not be involved in this behaviour. Very little work has been reported concerning the role of cytokinins during drying and subsequent rehydration of DT angiosperm plants. It would not be surprising however, that desiccation-tolerant plants utilise different mechanisms of coping with water loss, stomatal control being of lesser importance in these species.

### 3.1.7 Ultrastructure

The micrographs in Figure 3.9 depict subcellular organisation of fully hydrated mesophyll cells. These were highly vacuolated, with chloroplasts aligned along the edges of the cells (Figure 3.9i). Chloroplasts were typically elongated, with well-defined granal stacks, appressed thylakoid membranes and starch was present (Figure 3.9ii). Thylakoid membranes had an unusual 'staircase' stacking arrangement (Figure 3.9iii), as described by Wellburn and Wellburn (1976), and always appeared negatively stained. Mitochondria had well-defined cristae, which was indicative of active respiratory activity (Figure 3.9iv).

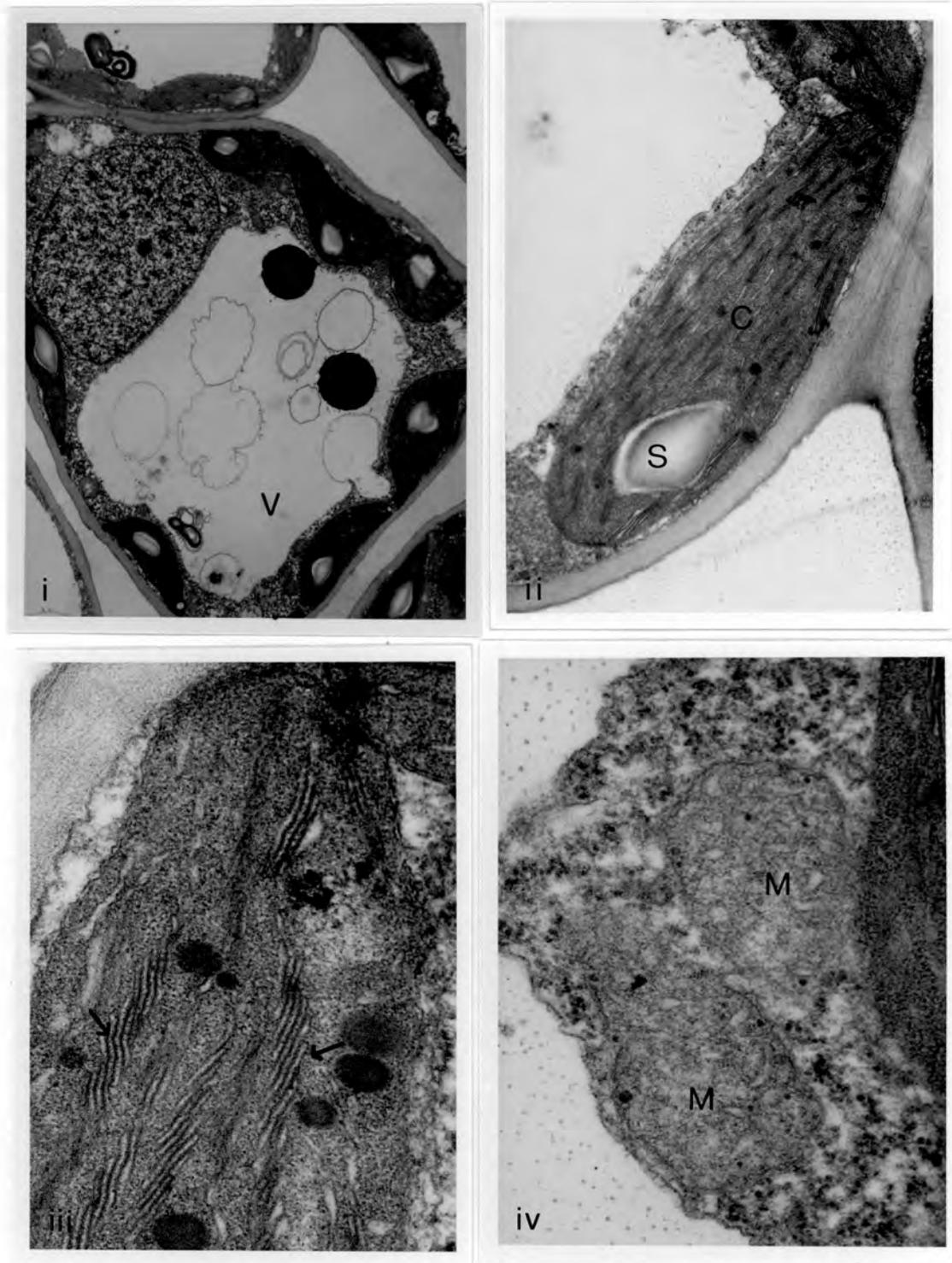


Figure 3.9 Micrographs of highly vacuolated (V) mesophyll tissue at full turgor ( $\times 3000$ ) (i). Chloroplasts (C) typically elongated with starch (S) present ( $\times 12\ 000$ ) (ii). Thylakoid "staircase" (arrowed) structure ( $\times 36\ 000$ ) (iii). Mitochondria (M) with well-defined cristae ( $\times 45\ 000$ ) (iv).

There were no ultrastructural changes evident during the initial stages of dehydration, (i.e. in response to leaf reorientation parallel to the main stem axes and water content decreasing from full turgor to 1.6 g/g DM). Ultrastructural changes became evident during the period of maximum water loss and leaf folding (Figure 3.10). During this period the central vacuoles were still present. Chloroplasts became rounded and, although remaining appressed, the thylakoids separated into loosely arrayed, smaller granal stacks, spread evenly within the stroma. This physical separation and reduction in granal size may have contributed to the inhibition of photochemical activity and carbon assimilation. Starch was no longer evident within chloroplasts, perhaps having been metabolised as an energy-source necessary for the induction of protection mechanisms, alternatively perhaps being reduced to sugars which may act as a protection agent during desiccation. Mitochondrial matrices had started to become electron-transparent, the cristae no longer easily visible, and this coincided with the measured reduction in respiratory activity around 1.0 g/g DM.

Upon air-dryness (0.2 g/g DM), the mesophyll cells were reduced in size, probably due to some cell wall folding, and the central vacuole became fragmented into smaller compartments (Figure 3.11i). The plasmalemma was withdrawn from the cell wall, except at some plasmodesmata, but there was no evidence of membrane disruption. This reduction in vacuolar volume, together with plasmalemma withdrawal, may reduce mechanical stress, preventing cell wall collapse and rupture of the plasmalemma. The chloroplasts remained rounded, and the thylakoid membranes were no longer appressed (Figure 3.11ii).



Figure 3.10 Ultrastructural organisation of mesophyll cells during the period of maximum water loss (water content decreasing from 1.6 to 1.0 g/g DM) ( $\times 7\ 500$ ). Chloroplasts became rounded, with displaced thylakoids, the mitochondria were less electron dense, and the central vacuole was still present.

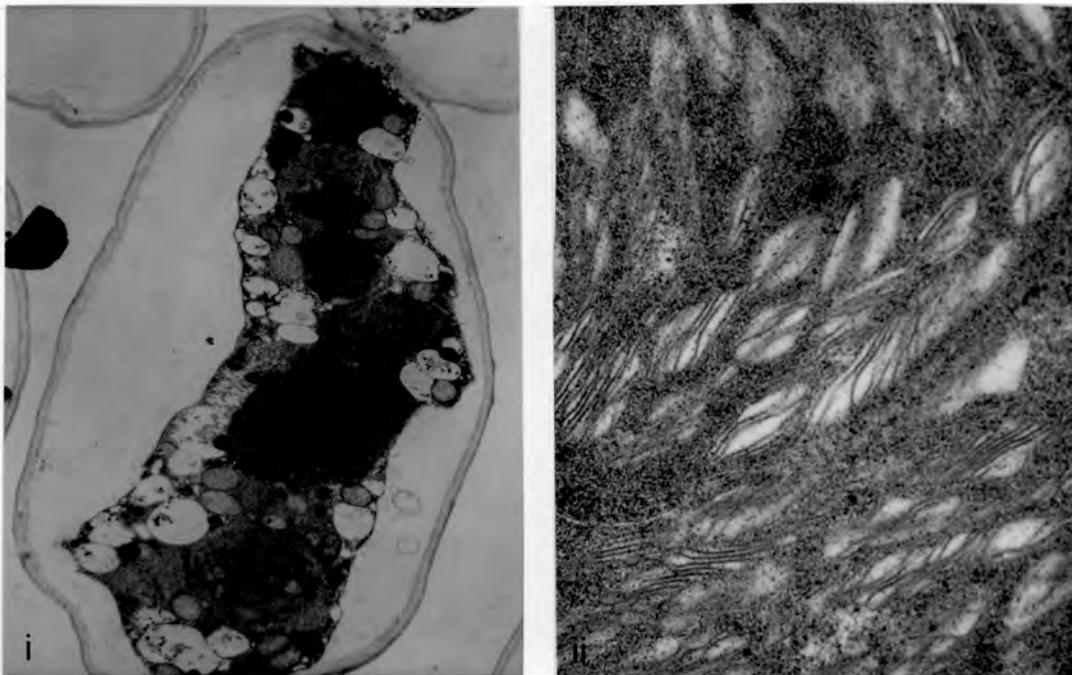


Figure 3.11 Ultrastructural organisation of air-dry (0.2 g/g DM) mesophyll cells. The cytoplasm withdrawn from the cell wall and densely stained ( $\times 4\ 500$ ) (i). Thylakoid membranes within the chloroplasts no longer appressed ( $\times 54\ 000$ ) (ii).

## 3.2 REHYDRATION OF THE WHOLE PLANT

Leaf water content recovered to pre-stress levels within 24 hours of rewatering. Because physiological activity continued to recover after the plants had attained full turgor, the data is presented as a function of time as well as water content.

Leaves of *M. flabellifolius* are abscised throughout the growing season (Child, 1960). In the current study, it was found that some of the leaves which had re-greened, yellowed and abscised four to five days following rehydration. It was not possible to predict which leaves would abscise, and the inclusion of these leaves in physiological measurements may be responsible for the variation evident within the data.

### 3.2.1 Leaf reorientation with rehydration

There was no change in leaf water content during the first 6 hours following rewatering, possibly a result of gradual root rehydration and repair, as well as xylem refilling (Figure 3.12). During the following nine hours leaf water content increased rapidly (0.15 g/g DM/ h), reaching 1.6 g/g DM after 15 hours rehydration. Within this nine-hour period the lateral branches and leaves had reoriented perpendicular to the stem axes, and the leaves revealed a green adaxial surface. (Figure 3.13). Recovery of leaf water content then proceeded more slowly (0.05 g/g DM/ h). Full turgor was reached within 24 hours of rewatering, and new axial buds were visible four to five days following rewatering.

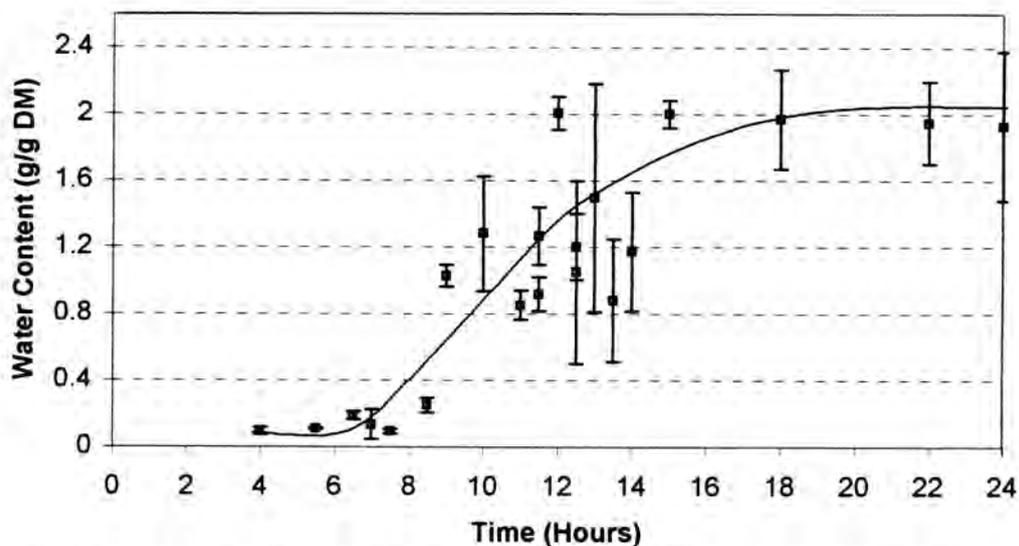


Figure 3.12 Recovery of leaf water content with time during whole plant rehydration.



Figure 3.13 Leaf reorientation (revealing a green adaxial surface) during leaf recovery to full turgor with whole plant rehydration. Scale bar = 5 mm

### 3.2.2 Solute leakage

Solute leakage during leaf recovery to full turgor was variable, (this variability probably arising from the leaves which would abscise shortly after rehydration), however leakage remained within a consistently low range, irrespective of water content (Figure 3.14). This suggests that membrane configuration remained unchanged during rehydration.

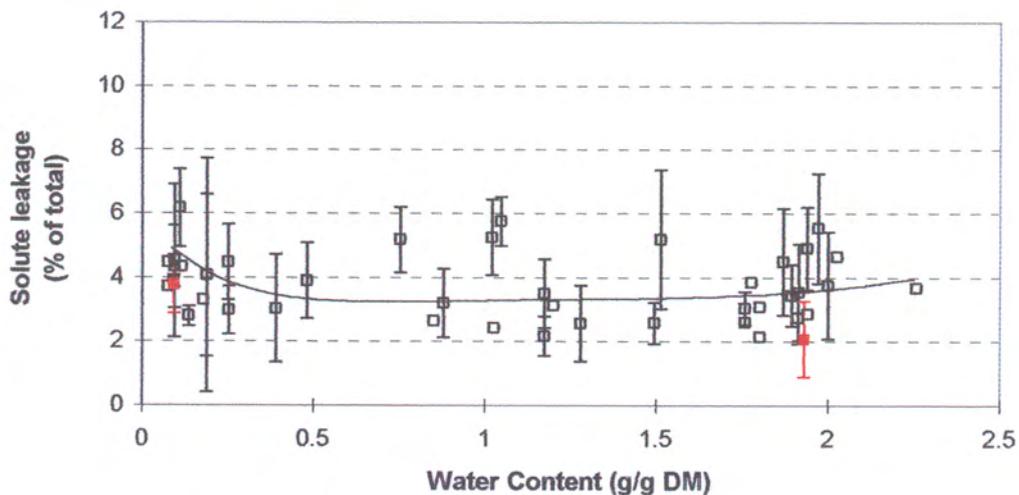


Figure 3.14 Solute leakage from leaves removed from whole plants during rehydration. Red symbols indicate average pre-stress (hydrated control) and dried (for one month) leakage values.

### 3.2.3 Chlorophyll and carotenoid content

Within 24 hours following rewatering, both chlorophyll and carotenoid concentrations had recovered to levels characteristic of pre-stressed (hydrated control) leaves (Figure 3.14, pre-stress levels indicated with red symbols). Thus pigment recovery was coincident with attainment of full turgor. Both pigments continued to increase linearly with time, exceeding control levels during the following 48 hours.

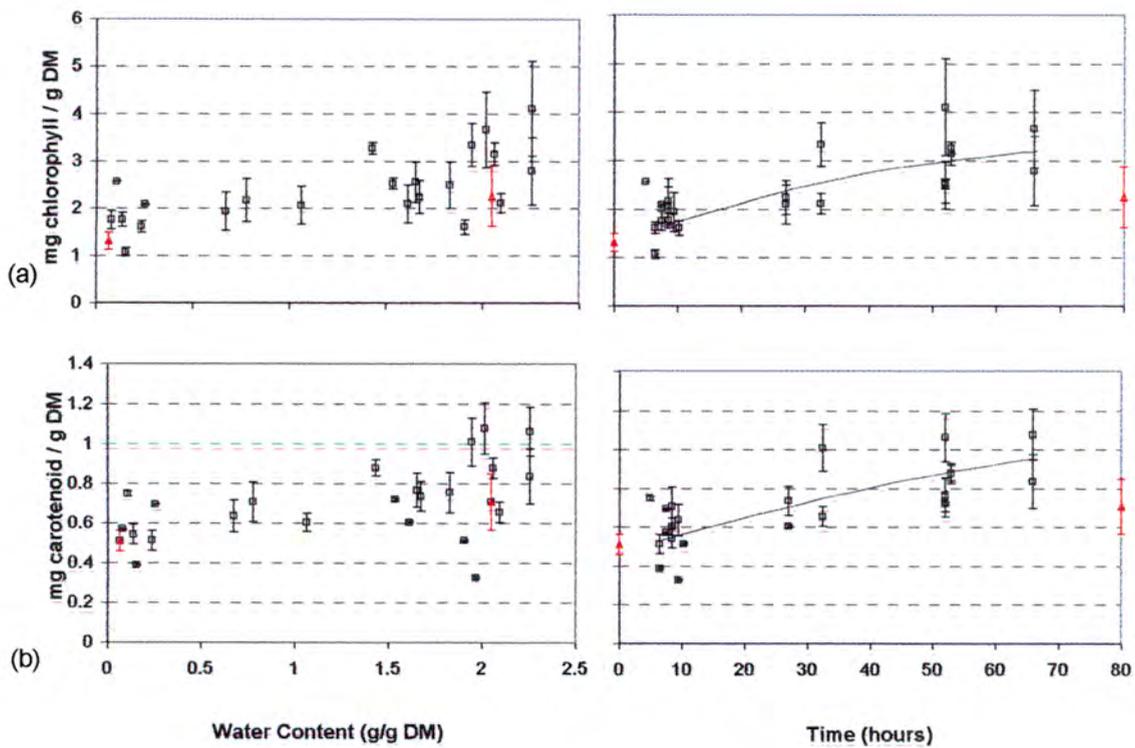


Figure 3.14 Total leaf chlorophyll (a) and carotenoid (b) concentrations with respect to changes in water content (left column) and time (right column) during rehydration. Red symbols indicate pre-stress (hydrated control before experimentation) and dried (after maintenance in the dry state for one month) pigment concentrations.

### 3.2.4 Chlorophyll fluorescence

Both  $F_v/F_m$  and light-adapted PSII efficiency (Yield) increased linearly with time (Figure 3.15a&b). Photochemical recovery was slow, with pre-stress values attained 60 hours following rewatering, and 48 hours after the recovery of chlorophyll to pre-stress levels. Recovery of PSII activity thus required more than just the availability of sufficient chlorophyll, again indicating strict control of the photosynthetic process in these plants. Similarly, Calvin Cycle activity, as derived from yield measurements, recovered only 60 hours after rewatering. Slow recovery of photochemistry might indicate that despite possible protection mechanisms induced on drying, repair processes are likely to be necessary upon subsequent rehydration. It is also possible however, that the inhibition of photochemical activity until complete cellular recovery, was a deliberate strategy to prevent damage arising from unregulated metabolic activity.

The large amount of variation observed for both  $qP$  and  $qN$  during the early stages of rehydration was reduced 24 hours following rehydration (Figure 3.15c&d). Both  $qP$  and  $qN$  recovered towards pre-stress levels, and remained near these levels 36 hours after rewatering.

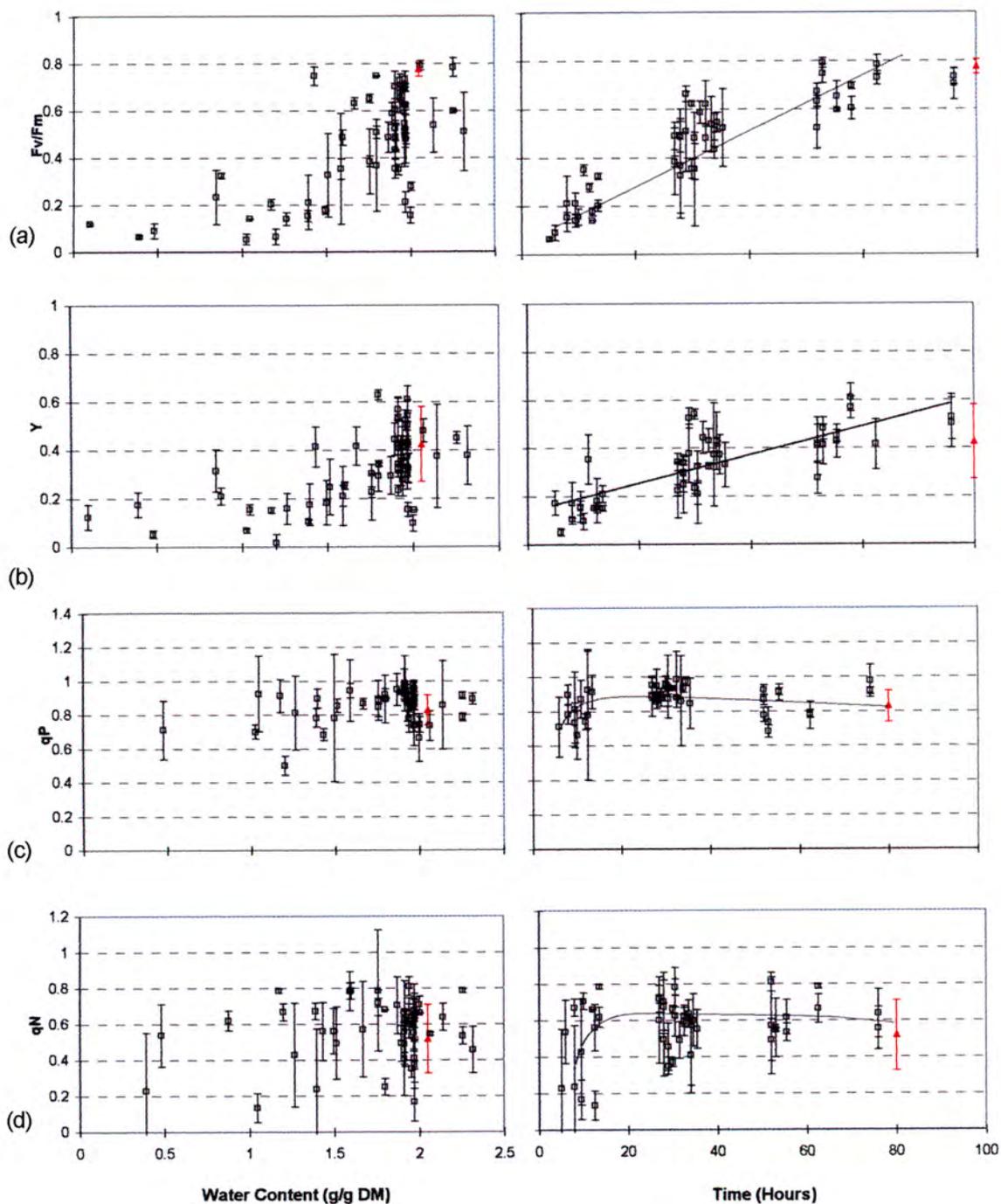


Figure 3.15 Changes in whole plant chlorophyll fluorescence with change in water content (left column) and time (right column) during rehydration. ( $F_v/F_m$  (a), yield (b), and the quenching coefficients  $qP$  and  $qN$  (c and d). Red symbols indicate hydrated control (pre-stress) values).

### 3.2.5 CO<sub>2</sub> gas-exchange

Variation amongst the data, particularly at low water contents, was large, making interpretation of trends difficult. Dark respiration was initiated within 7 hours of rewatering (Figure 3.16a), coincident with the gradual increase in leaf water content. Respiration continued to increase, stabilising near pre-stress levels within 24 hours (coincident with the attainment of full turgor and leaf reorientation). This would indicate that the mitochondria and respiratory enzymes did not suffer irreversible damage during drying, and the rapid rate of recovery would support the conclusion suggested by Bewley (1979), that most of the mitochondrial and respiratory enzymes of HDT plants, are retained on desiccation. These results do not show an initially high rehydration dark respiration rate, as recorded during the rehydration of the PDT angiosperm *Xerophyta scabrida* (Tuba *et al.*, 1994), and observed to occur as “resaturation respiration” in lichens (Smith and Molesworth, 1973).

Like photochemical activity, there was a delay in the resumption of carbon assimilation (A) during the first 36 hours following rewatering (Figure 3.16b). Assimilation proceeded to increase rapidly during the 24 hours thereafter, reaching a maximum and stabilising after 60 hours (i.e. concomitant with the recovery of photochemical efficiency).

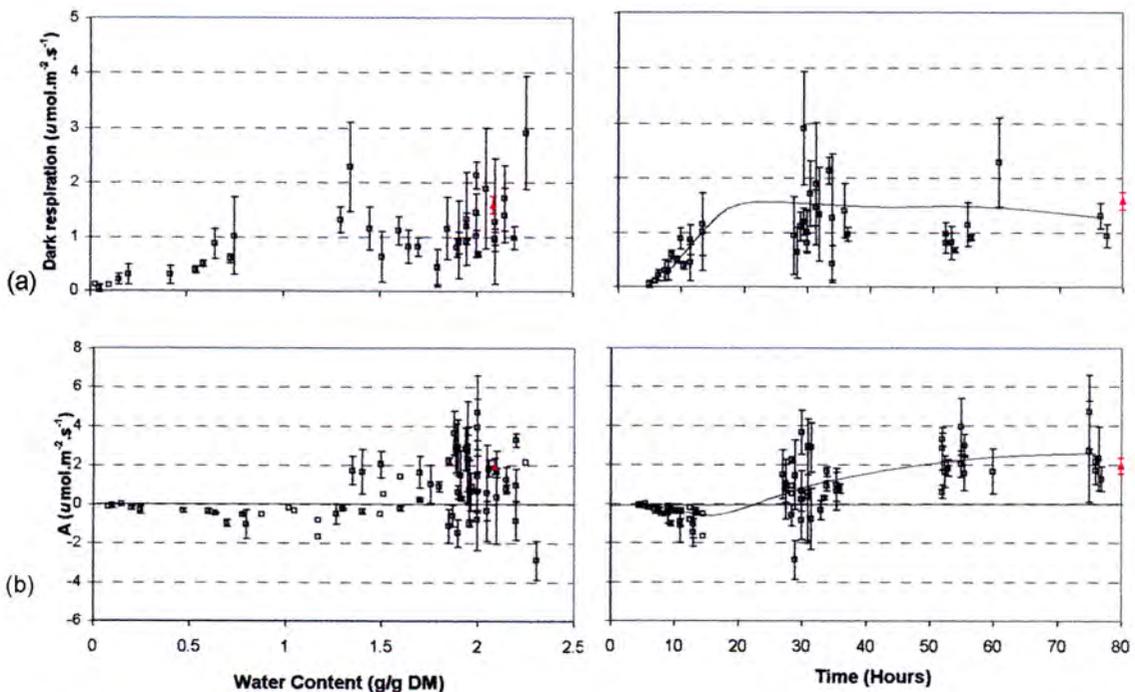


Figure 3.16 Dark respiration (a), and net assimilation, A, (b), with increasing water content (left column) and time (right column) during whole plant rehydration. (Red symbols indicate hydrated control (pre-stress) values.)

### 3.2.6 Plant growth regulators (PGRs)

ABA and CK concentrations were sampled at four stages of rehydration, as illustrated in Figure 3.17. ABA concentrations in leaves of plants which had been kept in the dry state for one month, were lower than those recorded for similar water contents (0.4 g/g DM) after 36 hours drying. Thus some ABA degradation had occurred while the plant was in the dry state. ABA levels increased markedly after 10 hours of rehydration but declined when the plant regained full turgor. Cytokinins followed a similar trend.

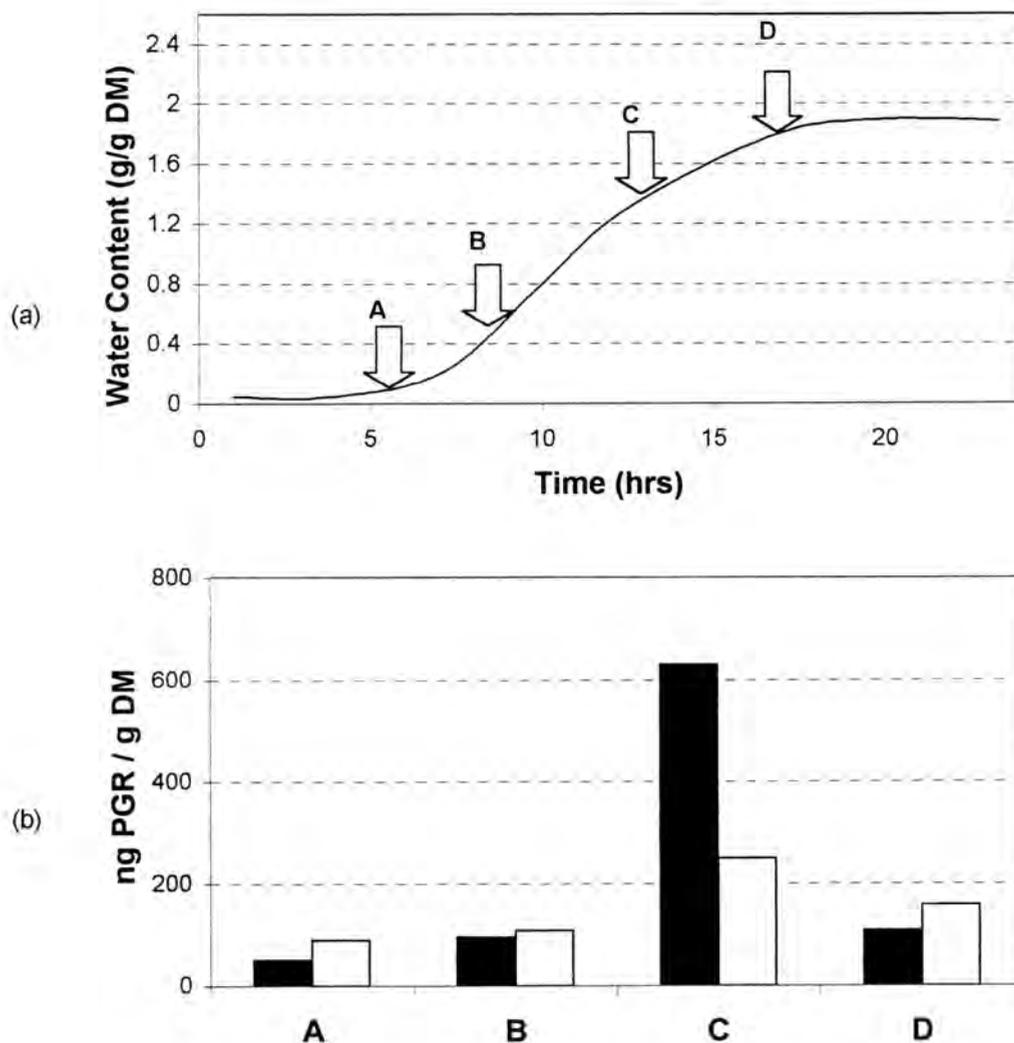


Figure 3.17 Water content and time during which leaves were sampled for the presence of the plant growth regulators (a); ABA (solid bars) and CK (empty bars) concentrations extracted at these water contents during whole plant rehydration (b).

The increase in PGRs coincided with the most rapid period of leaf rehydration (i.e. corresponding with leaf reorientation perpendicular to stem axes). It was possible that this increase was due to the PGR transport, moving in the xylem from the roots to the shoots.

The role of ABA during rehydration of desiccation-tolerant plants is unclear. It may be predicted that increased ABA concentrations inhibited stomatal opening, however the recovery of respiratory activity shortly thereafter does not support this suggestion. Since ABA affects gene expression during drying (Bartels and Ingram, 1996) it may also be involved in the promotion of gene transcription for proteins involved in cellular repair and/or recovery.

The role of cytokinins during rehydration of DT plants has received little attention. Cytokinins have been implicated in the stimulation of cell division and bud break (see Davies, 1990, for a review). As new bud formation was evident following recovery of *M. flabellifolius*, it is possible that elevated CK levels could account for this phenomenon. Cytokinins also promote chlorophyll formation (Davies, 1990). Increased CK levels were coincident with increasing chlorophyll concentrations (12 hours after rewatering), thereby implicating cytokinins in this process.

### 3.2.7 Ultrastructural changes

As the plants hydrated to 1.6 g/g DM (during the 7-15 hour period following rewatering), the plasmalemma became appressed to the cell wall, the central vacuole reformed, and the organelles were aligned along the edges of the cell. The mitochondria had started to become more electron dense, coinciding with the initiation of respiratory activity, however the chloroplasts remained similar to those observed in the dry state, with considerable distortion of the thylakoids (Figure 3.18). The discontinuity between adjacent thylakoid membranes, and their reconstitution would contribute towards the delay in recovery of photosynthesis.

Thirty-six hours following rewatering (i.e. 12 hours after recovery of full turgor), the subcellular organisation had recovered to that typical of hydrated mesophyll tissue (Figure 3.19i). Most of the chloroplasts had recovered their elongated shape with starch was present in the stroma. The granal stacks had re-formed, and this recovery coincided with increased photosynthesis (Figure 3.19ii).

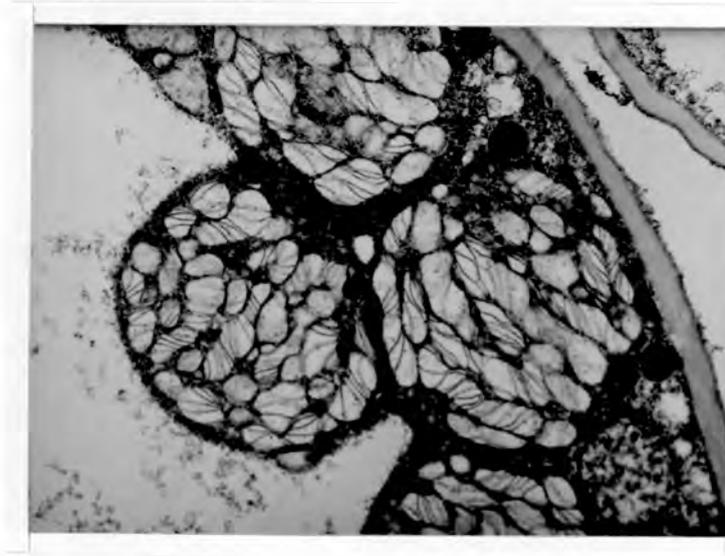


Figure 3.18 Ultrastructural organisation of mesophyll cells during the 7 to 15 hour period following rewatering (water content increasing from air-dryness to 1.6 g/g DM). The plasmalemma was appressed to the cell wall, the central vacuole reformed, and the organelles were aligned along the edges of the cell. Chloroplasts remained rounded with distorted thylakoid membranes and cristae became visible within the mitochondria (x8 000).

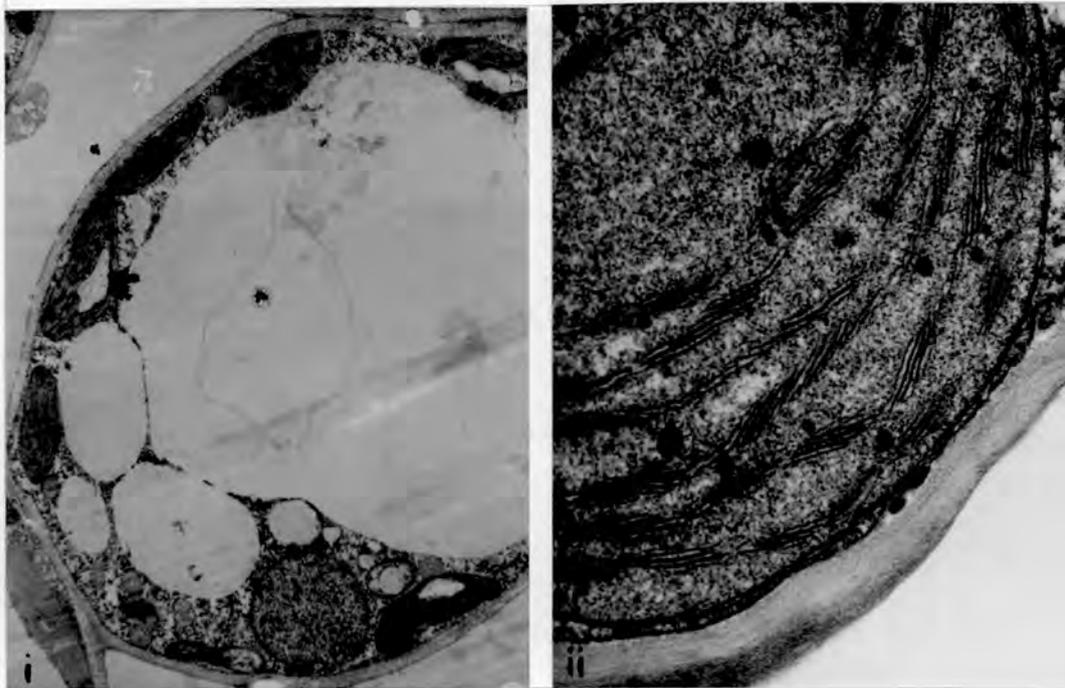


Figure 3.19 A typical hydrated mesophyll cell 36 hours after rewatering. Recovery of elongated chloroplast shape with starch present (x 3 500) (i) as well as re-establishment of the "staircase-like" thylakoid arrangement (x12 000) (ii).

## DISCUSSION

There was no indication of plasmalemma damage throughout the continuum of leaf water contents during drying and recovery (as investigated by electrolyte leakage and ultrastructural studies). This suggests that considerable protection of membranes is afforded, and that at least this part of the mechanism of desiccation tolerance in *M. flabellifolius* is constitutive.

Leaf orientation provided the first visible symptom of water stress. Changes in leaf orientation also provided the first indication that part of the protection mechanism associated with desiccation tolerance may be behavioural, in that leaf folding would decrease light-chlorophyll interactions and so facilitate the inhibition of photosynthesis. Photosynthesis was inhibited at relatively high water contents (>1.6 g/g DM), although the potential for electron transport was initially maintained, and complete stomatal closure was not observed (since respiratory activity remained high). It might be suggested that the immediate shutdown of photosynthetic activity was a deliberate strategy protecting cells from the potential hazards associated with disrupted photosynthesis (such as free radical formation) during drying. Following the inhibition of photosynthesis, the chloroplasts became rounded, with the thylakoid membranes, whilst still appressed, being separated into smaller granal stacks. This reduction and separation of the thylakoid stacks may have been responsible for the subsequent shut-down of electron transport, which also occurred at high water contents (>1.0 g/g DM) (relative to photochemical inhibition of desiccation-sensitive species (Sherwin, 1995)). Inhibition of photochemical activity may be a further mechanism preventing the potential hazards of disrupted photochemical activity with water-loss. Furthermore, the granal stack separation during drying may also provide protection during rehydration (when leaves unfold and the adaxial surfaces become exposed to light).

As the leaves became air-dry, the abaxial leaf surface (exposed to light) became purple/brown in colour. This may be due to anthocyanin accumulation in the sub-epidermal layers of the abaxial surface, as reported by Goldsworthy (1992), and/or a result of the 40% decline in chlorophyll content. The reduction in total chlorophyll content may reflect a preferential loss of chlorophyll from the abaxial surface of the leaves, as photographs taken during rehydration revealed a green adaxial surface, whilst the abaxial leaf surfaces were brown (Figure 3.13). It is possible that light-chlorophyll interactions in the leaves of *M. flabellifolius* were progressively reduced as chlorophyll was either degraded, masked by anthocyanins in the abaxial surface, or shaded in the adaxial surface. It might be suggested

then, that, as found for the HDT *Craterostigma wilmsii* (Sherwin and Farrant, 1998), the leaves of *M. flabellifolius* combine both physical and chemical changes in order to afford protection against light-induced free radical damage.

The increase in ABA coincided with leaf reorientation and inhibition of photosynthesis, but not with the loss of chlorophyll, nor complete stomatal closure (although both of these processes have been associated with increased ABA levels in desiccation-sensitive species (Davies, 1990)). Chlorophyll degradation of *M. flabellifolius* occurred slowly with time, and complete stomatal closure did not occur throughout dehydration (Goldsworthy, 1992). ABA has been implicated as a chemical signal produced by the roots, in response to soil dehydration, before moving into the transpiration stream and inducing a water stress response via its accumulation in the shoots (Zhang and Davies, 1987; Zhang *et al.*, 1987; Neales *et al.*, 1989; Davies and Zhang, 1991). The site of ABA synthesis could not be determined from the current study. Gaff and Loveys (1984) found an accumulation of ABA during the desiccation of isolated leaves, thus ABA may not play a role in root perception of water stress.

A rise in ABA content in the leaves of the HDT plant *Craterostigma plantagineum* is thought to induce genes encoding for protectants of subcellular organisation (Piatkowski *et al.*, 1990; Bartels *et al.*, 1993; Dure *et al.*, 1993). A similar function could be invoked for ABA in *M. flabellifolius* during the initial stages of water-loss. Gaff and Loveys (1984) also found a correlation between ABA levels and the survival of isolated leaves dried at different relative humidities, providing further evidence for tolerance induction during desiccation of this species.

Starch was no longer evident within the chloroplasts during the period of maximum water loss. The loss of starch might indicate that carbohydrate metabolism provided an energy source for processes (such as the synthesis of protection proteins described above), initiated in response to desiccation (Womersely, 1981). It is also possible that the starch was broken down into sugars, which could then play a more direct role in cellular protection, such as glass formation (Burke, 1986) and water replacement (Close *et al.*, 1993) during desiccation.

The leaves became air-dry 36-48 hours after the first visible symptoms of water stress. The vacuoles in the dry leaves had sub-divided into a number of smaller vacuoles and there was substantial plasmalemma withdrawal from the cell walls without loss of membrane integrity. Reduction in vacuolar volume is considered to be essential for the attainment of

desiccation tolerance (Bewley, 1979) but generally this is related to prevention against tearing of the plasmalemma from its association with the wall. The ability for the plasmalemma of *M. flabellifolius* to withdraw without tearing is unusual, and presumably, the plasmodesmata linkages have the ability to re-seal after withdrawal. Finally, the lack of cell wall collapse during desiccation indicates considerable cell wall strength.

Following an initial lag in leaf rehydration, probably a result of the time necessary for root and stem rehydration and repair, leaf rehydration proceeded rapidly. Within 24 hours the leaves had recovered full turgor, reorientated perpendicular to the stem, and recovered subcellular organisation (although the chloroplasts remained rounded and the thylakoid membranes non-appressed in the stroma). Neither photochemical activity nor photosynthesis had been restored, despite water availability and exposure of the green adaxial leaf surface (containing the 60% chlorophyll retained in the dry state). It is possible that the limited recovery of the granal structure in the chloroplasts was not merely a consequence of the inflow of water during rehydration, but rather a strategy to prevent damage from light-chlorophyll interactions arising during rehydration. Chloroplasts would then only recover following sufficient opportunity for repair and reconstitution of metabolism.

The increase in both ABA and cytokinins during the most rapid period of leaf water-uptake (i.e. corresponding with leaf reorientation perpendicular to stem axes) might implicate these PGRs in root signaling to the shoots. Increased ABA may then prevent stomatal opening (thus inhibiting photosynthetic and respiratory recovery) and/or promote the synthesis of proteins associated with cellular repair and recovery.

There is little understanding concerning the role of cytokinins in desiccation-sensitive species during water stress, and even less information related to fluctuations of this PGR in desiccation-tolerant plants. Cytokinins studied in desiccation-sensitive species have been implicated in bud formation and chlorophyll synthesis. A similar role may be associated with CKs in *M. flabellifolius*, since chlorophyll content recovered and apical buds became visible following an increase in CK concentration during rehydration of this species.

In contrast with the gradual recovery of photosynthesis, low levels of carbon evolution were measurable shortly after rewatering (indicating that if increased ABA was a deliberate mechanism promoting stomatal closure, it was not completely effective). Measurable levels of respiratory activity at lower water contents than were observed for photosynthesis might indicate that the respiratory machinery was more tolerant to water stress. This may be necessary for the provision of energy for repair and recovery of metabolism. There was no

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distinct peak of elevated respiratory activity ("rewetting respiration") during initial rehydration as reported by Tuba *et al.*, (1994), Smith and Molesworth, (1973), and Bewley *et al.*, (1978) studying a variety of DT plants. Sherwin (1995) working with the HDT *Craterostigma nanum* also found that "rewetting respiration" was not evident during rehydration, and was not necessarily characteristic of DT plants.

Vieweg and Ziegler (1968) and Genkel and Pronina (1968) both measured CO<sub>2</sub> gas-exchange of rehydrating twigs from *M. flabellifolius*. Their results correspond with those from this study, whereby a delay between the induction of photosynthesis, relative to respiration, was reported during rehydration. The results from this study also support those reported by Goldsworthy (1992) studying intact plants of *M. flabellifolius*, where it was shown that the rate of CO<sub>2</sub> assimilation declined rapidly during drying, and recovered gradually following rehydration.

From this study it can be concluded that some constitutive level of cellular protection exists in leaves of *M. flabellifolius*, enabling the plasmalemma to maintain integrity across the entire span of water contents. Furthermore, the coincidence of physical (leaf reorientation and subcellular reorganisation) and chemical (changes in plant growth regulator concentrations) changes with alterations in photosynthetic and respiratory metabolism, implicate both physical and chemical strategies as mechanisms induced to promote desiccation tolerance of this species.

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## **Chapter 4: Characterisation of the leaf response during drying and recovery of detached twigs**

### **INTRODUCTION**

As mentioned in previous chapters, most reported studies on the response of *M. flabellifolius* to desiccation have been based upon studies of excised leaves or twigs (Genkel and Pronina, 1968; Vieweg and Ziegler, 1968; Gaff, 1977; Gaff and Loveys, 1984). In this aspect of the current study, the response of leaves from excised twigs were compared with those from the whole plant during drying and recovery. Thus the response of leaves, during their dehydration and recovery on twigs dried after excision from the (hydrated) whole plant, was investigated. The current study will also provide an indication of the role of roots in the ability for *M. flabellifolius* to tolerate desiccation.

During the course of the investigation it was found that large twigs (>10 g fresh mass) dried more slowly than small (<10 g fresh mass) twigs. Gaff and Loveys (1984) found that rapid drying of isolated leaves of *M. flabellifolius* induced damage, whereas slow drying rates were not deleterious. The effect of drying rate on the leaf response during drying and recovery of excised twigs was thus also taken into consideration in the current study.

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## RESULTS

### 4.1 DEHYDRATION OF DETACHED TWIGS

Dehydration was initiated immediately following the detachment of twigs from the hydrated whole plants. Large twigs (>10 g fresh mass) took 24 hours before water content had dropped below 0.6 g/g DM, and a further 24 hours before air-dryness was reached (Figure 4.1a). In contrast only six hours dehydration of the small (<10 g fresh mass) twigs reduced water content to 0.6 g/g DM, and air-dryness was attained after 36 hours (Figure 4.1b):

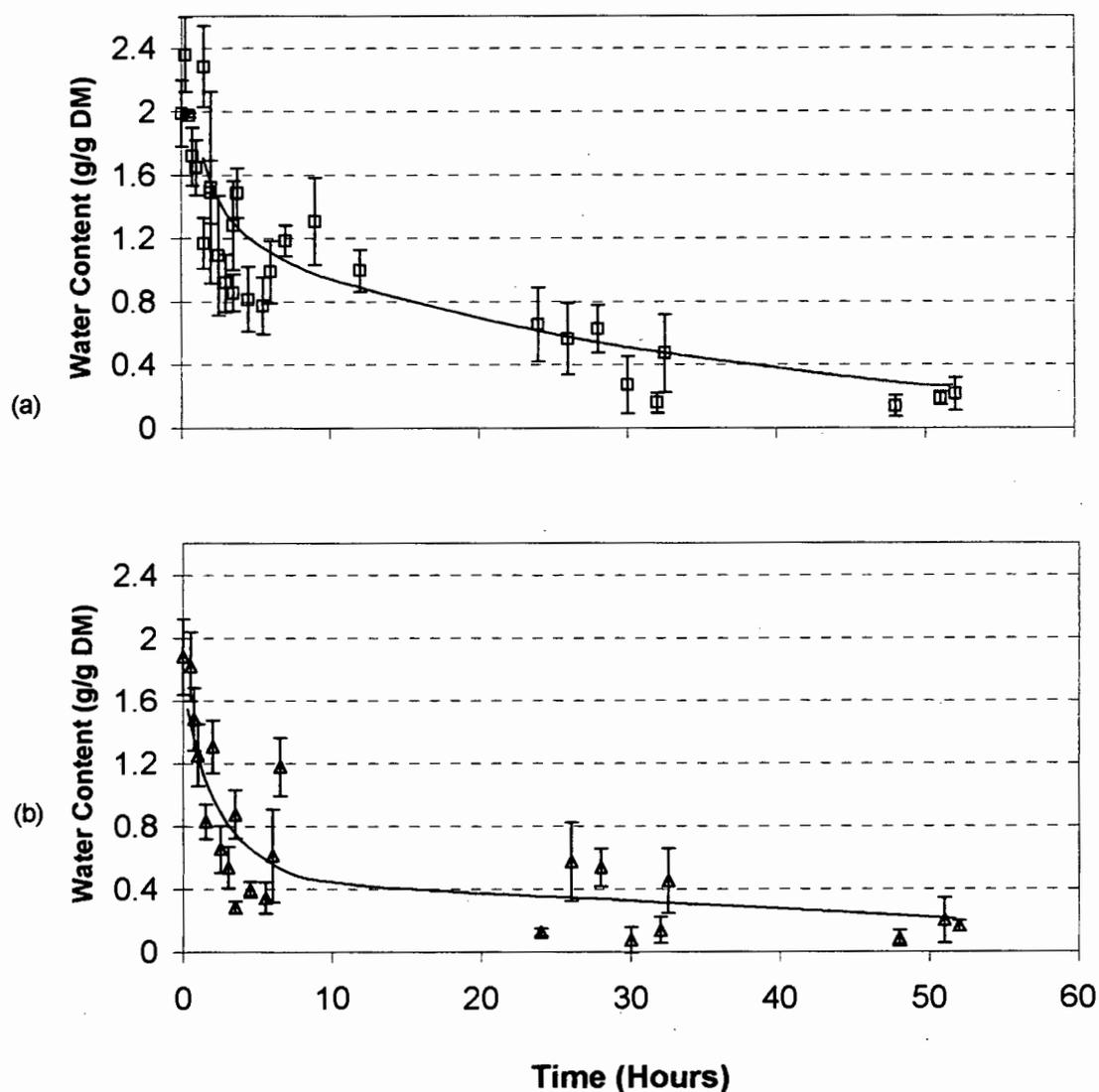


Figure 4.1 Leaf water loss with time during drying of large twigs (>10 g) (a), and small twigs (<10 g) (b). (Data points represented as a scatterplot of means, bars indicating standard deviations and trends investigated using distance weighted least squares regression analysis.)

#### 4.1.1 Leaf reorientation with drying

Leaves of large twigs dried five times faster (0.1 g/g DM/ h) than those from whole plants during the early stage of water loss (water content decreasing from 2.0 to 1.0 g/g DM). Despite an increased rate of water loss, the leaves from the large twig treatments were able to reorientate parallel to the stem and form a tight bud as was observed during whole plant dehydration (Figure 3.2b). Small twigs lost water more rapidly than was observed during both whole plant and large twig dehydration. The rapid rate of water loss throughout small twig dehydration prevented successful leaf folding and plication (Figure 4.2), and resulted in leaves being unable to form the tight bud characteristic of desiccated whole plant material. The abaxial leaf surface of both twig treatments became reddish-brown in colour after 60 hours dehydration.



Figure 4.2. Incomplete leaf reorientation and folding after 60 hours small twig dehydration.

Scale bar = 10 mm

### 4.1.2 Solute leakage

Solute leakage from leaves of large twigs (Figure 4.3a) occurred within the range reported for leaves dried on intact plants. This suggests that protective mechanisms of membrane structure were operational in the large twigs.

Higher levels of solute leakage, particularly at water contents below 1.0 g/g DM, were evident during the dehydration of small twigs (Figure 4.3b). These levels of solute leakage derived from small twig treatments were still low (<12% of total leakage) relative to desiccation-sensitive species (Sherwin and Farrant, 1996), as were leakage values obtained from dead leaf material (which generated leakage values below 20% of the total solute leakage (data not shown)). Consistently low levels of leakage indicate that some level of plasmalemma protection exists despite a lack of cellular survival (see later). Nevertheless, the slight increase in solute leakage arising during desiccation of the small twigs may indicate some plasmalemma damage manifest upon rapid drying.

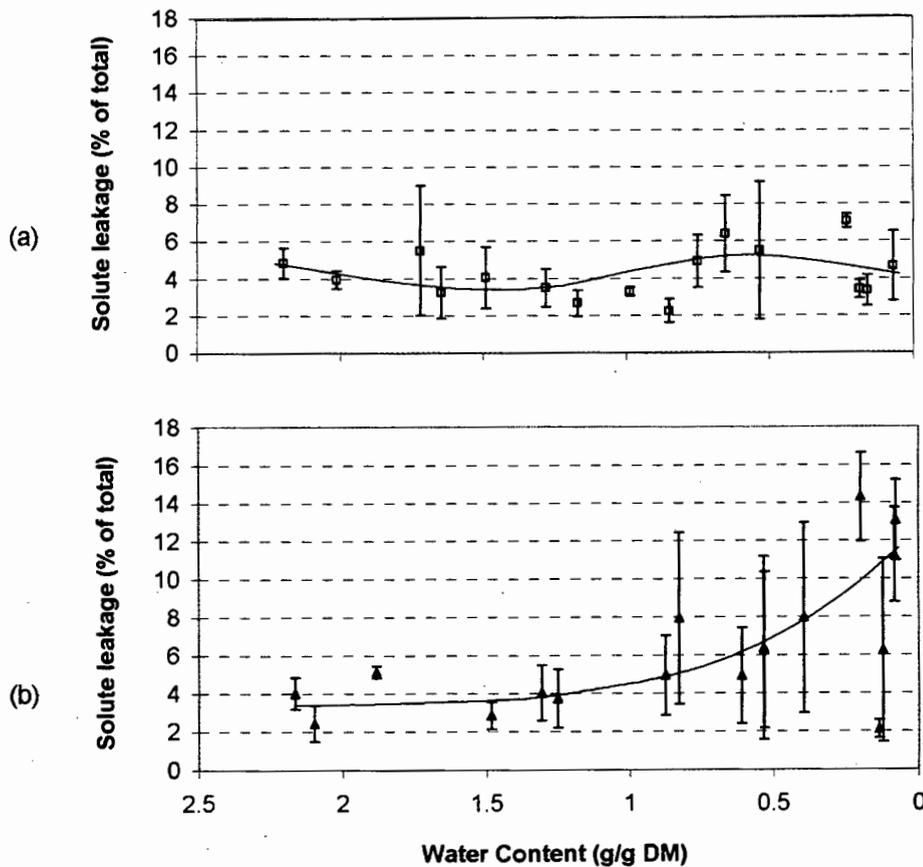


Figure 4.3. Solute leakage (as a percentage of total leakage) during dehydration of large (>10 g) (a) and small (<10 g) (b) twigs.

### 4.1.3 Chlorophyll and carotenoid content

Changes in chlorophyll and carotenoid concentrations extracted from the leaves of both twig treatments during drying are presented in Figure 4.4. Large twigs (Figure 4.4a) displayed a gradual decrease in pigment concentrations with decreasing water content, this trend similar to that reported during whole plant dehydration. Small twigs maintained constant pigment levels throughout water loss until water contents had dropped below 0.5 g/g DM (Figure 4.4b). Below this water content a sharp drop-off occurred coincident with the final stages of drying.

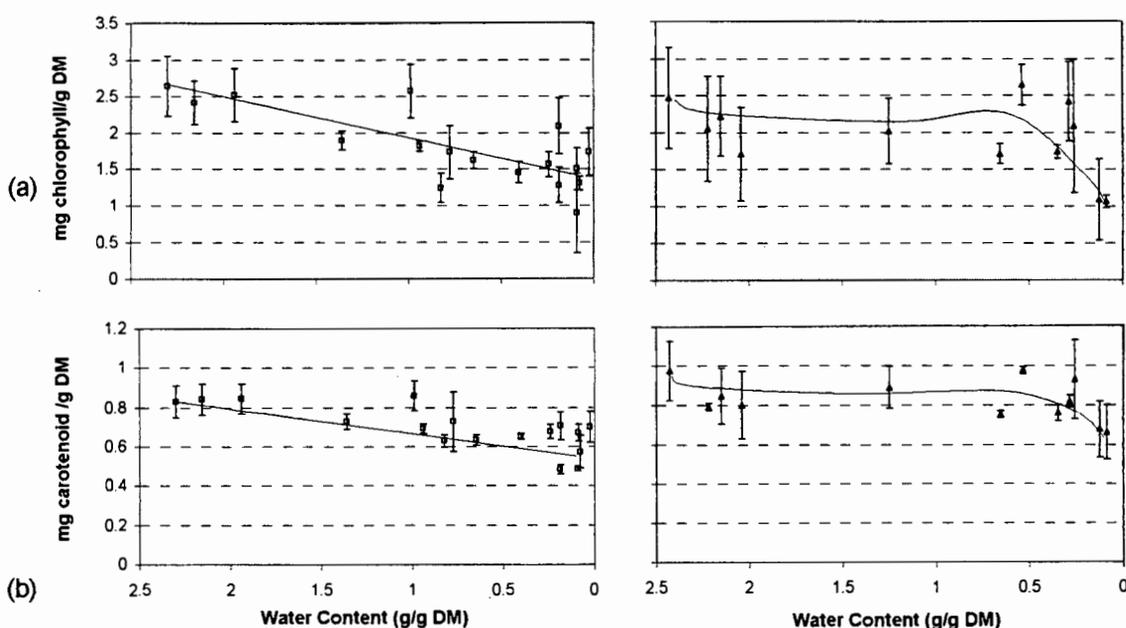


Figure 4.4. Total leaf chlorophyll (a) and carotenoid (b) concentrations during dehydration of large (left column) and small (right column) twigs.

Figure 4.5 shows total pigment concentrations (expressed as a percentage of the hydrated control) from air-dried leaves detached after 60 hours desiccation. The 40% decrease in total chlorophyll content during dehydration of large twigs was similar to the decrease reported during whole plant drying. This contrasts with the carotenoid content, which decreased by 14% from pre-stress levels during the dehydration of large twigs. Small twigs lost slightly, but insignificantly, greater quantities of both chlorophyll (52%) and carotenoid (32%) pigments relative to the whole plant and large twig treatments (Anova and Lord's multiple range test).

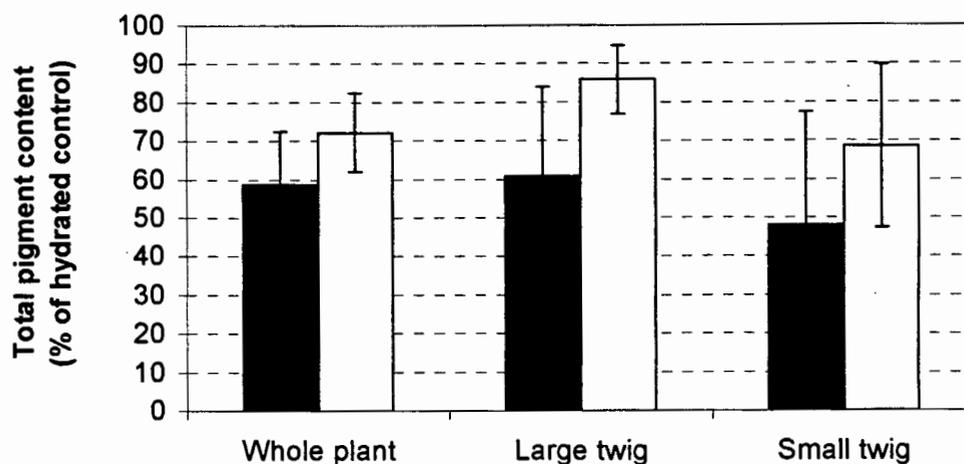


Figure 4.5. Comparison of total chlorophyll (solid bars) and carotenoid (empty bars) content (expressed as a percentage of the hydrated control) between treatments of air-dry leaves after 60 hours dehydration. (Anova and Lord's Multiple range test indicated no significant differences at  $p < 0.05$ .)

#### 4.1.4 Chlorophyll fluorescence

The photochemical response of leaves examined from twigs during dehydration (Figure 4.6) was similar between twig treatments, and followed the same trend as evidenced during whole plant dehydration (i.e. decreasing photochemical activity with decreasing water content). Unlike the whole plant however, the twig treatments displayed a more gradual and delayed decrease in photochemical activity during dehydration. Quantum efficiency of PSII ( $F_v/F_m$ ), Yield ( $Y$ ), and photochemical quenching ( $qP$ ) from both twigs started to decline at water contents of 1.5 g/g DM, however the most rapid drop-off in photochemical activity occurred when water content dropped below 1.0 g/g DM. Only non-photochemical quenching of both twigs remained high until water content dropped below 1.0 g/g DM.

The immediate drop-off in yield at water contents around 2.0 g/g DM evident during whole plant dehydration was not induced during the dehydration of both large and small twigs. Similarly the complete down-regulation of quantum efficiency of PSII ( $F_v/F_m < 0.3$ ), between water contents of 1.6 and 1.0 g/g DM, evident during whole plant dehydration was not observed during the drying of both twig treatments. It is possible that the rapid rate of water loss caused the twigs to reach lower water contents before photochemical activity was inhibited. Alternatively, the absence of roots may have prevented chemically signaling necessary for the down-regulation of photochemical activity in the leaves of the twigs.

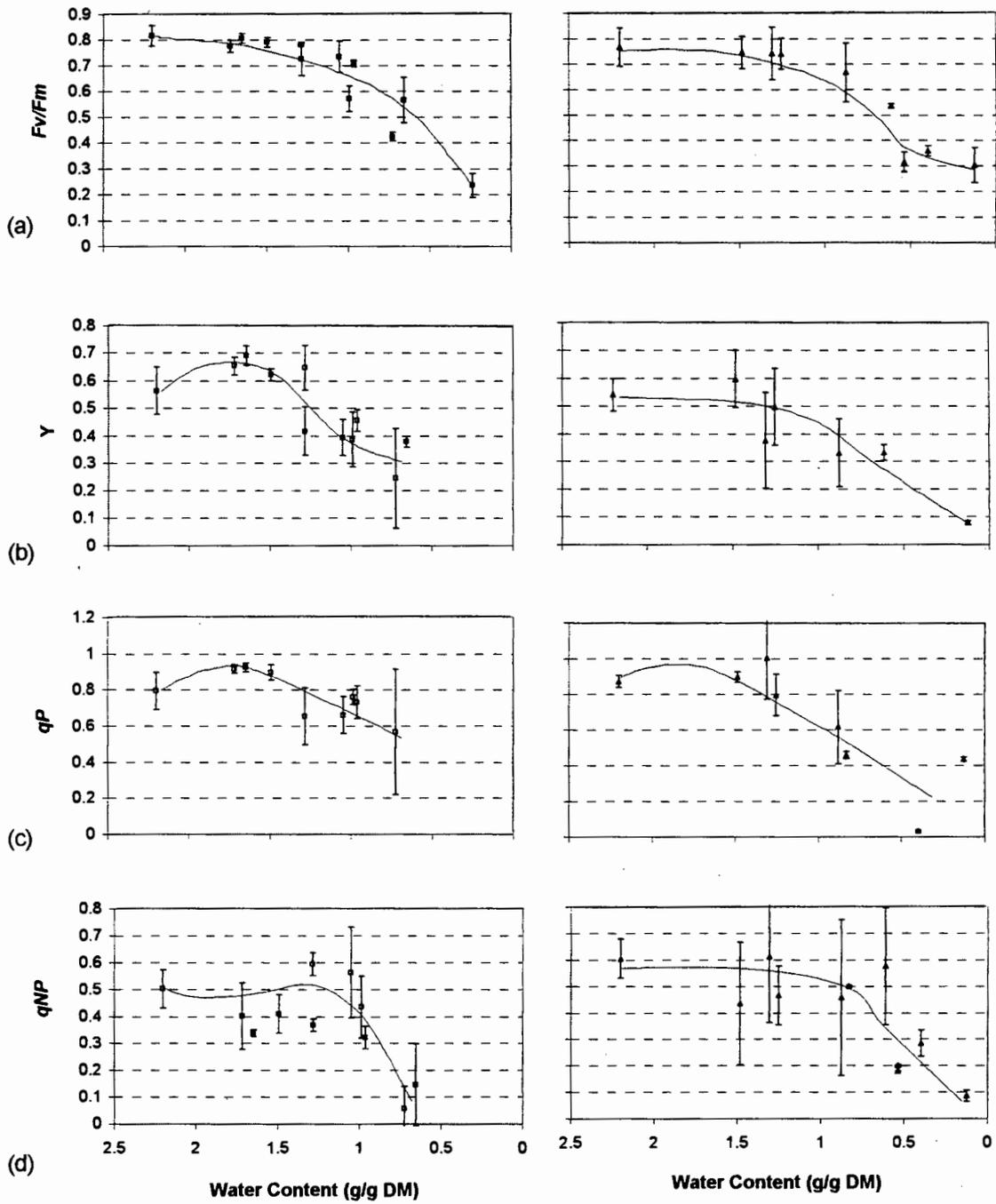


Figure 4.6. Changes in leaf chlorophyll fluorescence characteristics during dehydration of large (left column) and small (right column) twigs.  $F_v/F_m$  (a), Yield (b), and quenching coefficients  $q_N$  and  $q_P$  (c and d).

### 4.1.5 CO<sub>2</sub> gas-exchange

As observed during the study of photochemical activity, the inhibition of CO<sub>2</sub> gas-exchange (Figure 4.7), relative to the whole plant response, was delayed during dehydration of both large and small twig treatments. Photosynthetic activity of both twig treatments was maintained during the initial stages of water loss (in contrast with the immediate decrease observed following the first visible symptoms of water stress during whole plant dehydration). Again, this may be attributed to the time factor associated with more rapid water loss, all activity being reduced at lower water contents. There was a sharp decrease in photosynthesis of both large and small twig treatments as leaf water content dropped below 1.5 g/g DM, and carbon assimilation was completely inhibited around water contents of 1.3 g/g DM. Essential components of the photosynthetic pathway may be limited by water contents below 1.3 g/g DM, since whole plants also displayed inhibition of photosynthetic activity around these water contents.

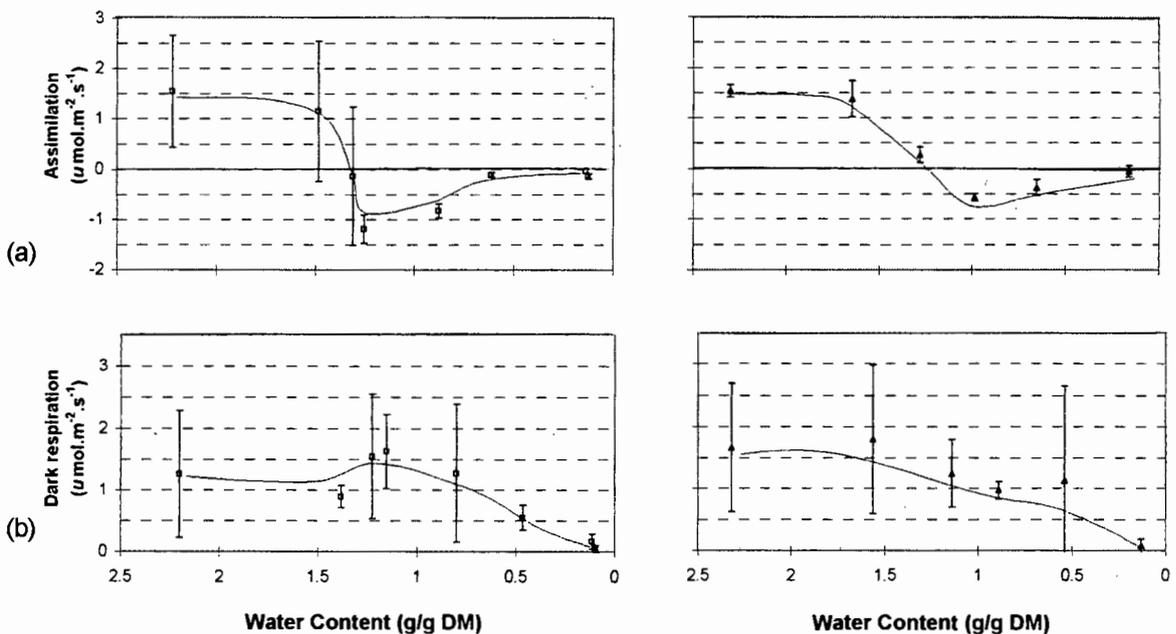


Figure 4.7 Changes in carbon assimilation (a) and dark respiration (b) during dehydration of large (left column) and small (right column) twigs.

During the early stages of water loss, dark respiration from both twig treatments displayed a pattern similar to that of whole plant dehydration, whereby respiration remained constant, only decreasing once CO<sub>2</sub>-fixation had been inhibited. Once photosynthesis was inhibited however, whole plant respiration decreased rapidly, whereas the large, and particularly the small twigs, displayed a gradual decrease in respiration, with measurable CO<sub>2</sub>-evolution being recorded around water contents as low as 0.3 g/g.

#### 4.1.6 Plant growth regulators (PGRs)

Total ABA and cytokinins (zeatin and zeatin riboside) extracted from large twigs were sampled at four stages of dehydration, as illustrated in Figure 4.8. Because small twigs did not survive drying, studies were not conducted on these tissues.

ABA and cytokinin (CK) concentrations remained near pre-stress levels recorded from whole plants during the first 5 hours of dehydration. As water content dropped to 1.0 g/g DM, ABA concentrations decreased. With further water loss however, (water contents at 0.6 g/g DM), ABA concentrations in the leaves of the large twigs increased towards levels similar to those recorded at water contents of 1.6 g/g DM during whole plant drying. This might indicate that the rapid drying rate caused a rise in ABA to occur at a lower water content during twig dehydration, relative to whole plant dehydration. After 48 hours dehydration, twig ABA levels had again decreased, however these values exceeded those recorded from air-dry whole-plant material.

The increased levels of ABA evident (although delayed) during large-twig dehydration, indicates that roots were not essential for ABA accumulation in the leaves. ABA accumulation, independent of transport from the roots to the leaves in the xylem stream, might indicate that 'root messages' via ABA do not play an essential role during dehydration. As mentioned previously (see Chapter 3), ABA does not appear to play a role in stomatal closure of *M. flabellifolius*. This conclusion is supported in the current study, since the rapid rate of water loss, as well as sustained photosynthetic and respiratory activity throughout dehydration of these twigs, suggests little stomatal control during drying.

Aside from the decrease in cytokinins (coincident with the decrease in ABA concentrations) around 1.0 g/g DM, cytokinin concentrations extracted from the leaves of twigs during drying remained relatively constant, as was observed during whole plant dehydration. This supports the conclusion derived from the whole-plant study (Chapter 3) where, unlike desiccation-sensitive plants, *M. flabellifolius* may not require cytokinins for the suppression of stomatal opening in leaves of these twigs in response to water stress.

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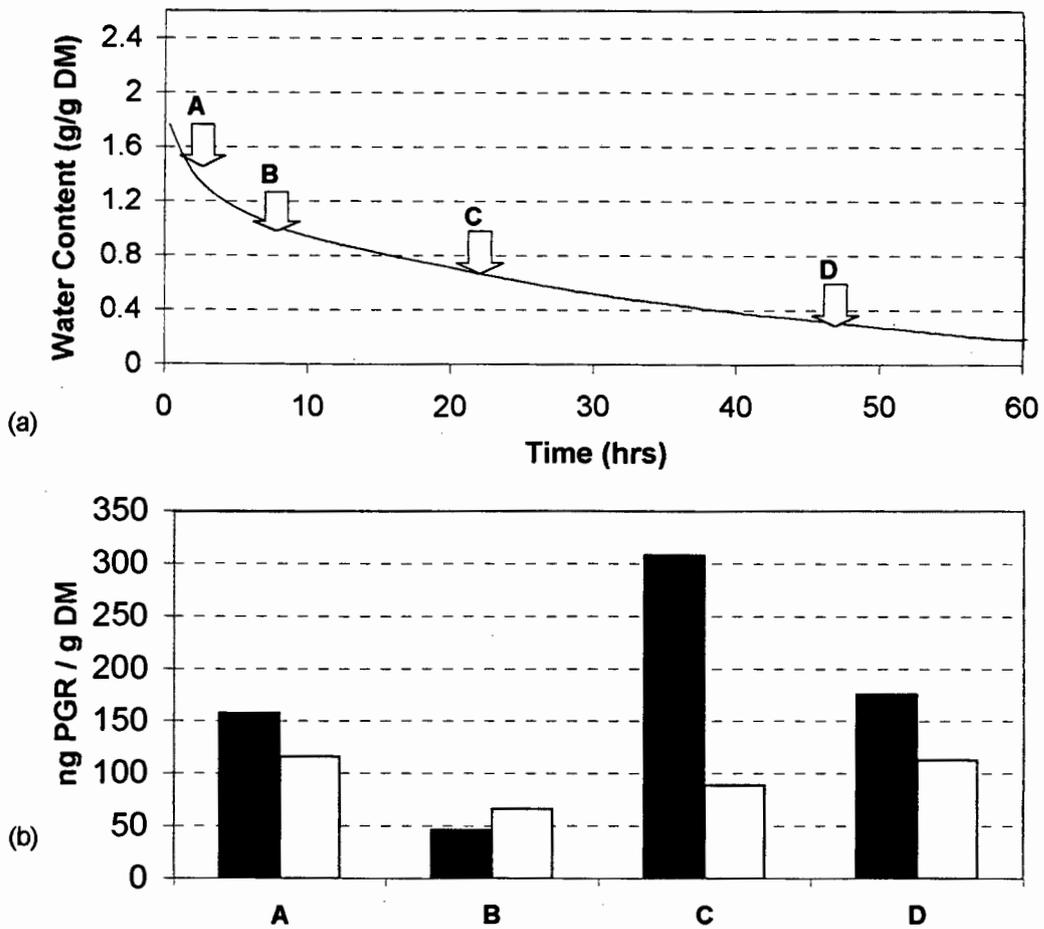


Figure 4.8 Water content and time during which leaves were sampled for the presence of the plant growth regulators (a); ABA (solid bars) and CKs (empty bars) extracted at these water contents during large twig (>10 g) dehydration (b).

#### 4.1.7 Ultrastructure

As water content decreased to around 1.0 g/g DM (during the first 6- or 2-hours of large and small twig drying respectively), some of the chloroplasts became rounded, although unlike the whole plant, there was no separation of the granal stacks into smaller stacks, and there was less evidence for dedifferentiation of the cristae (Figure 4.9i). In the chloroplasts which became rounded (arrowed in Figure 4.9i), the granal stacks were oriented in a half-moon shape along the edge of the chloroplasts. Chloroplasts which did not become rounded lost their granal stacking arrangement, and the thylakoid membranes became loosely arrayed in the stroma (Figure 4.9ii). These differences between the twig, versus whole plant, subcellular reorganisation during drying may be ascribed to insufficient time available for subcellular re-arrangement during twig dehydration. This would account for the prolonged maintenance of metabolism associated with twig desiccation, rather than the shut-down of respiratory and photosynthetic processes at relatively high water contents during whole plant dehydration.

Upon further dehydration towards air-dryness, the large twigs displayed ultrastructural characteristics similar to that observed during whole plant drying. The central vacuole was divided into numerous smaller compartments, the plasmalemma withdrew from the cell wall, and the thylakoids were no longer appressed within the stroma of the rounded chloroplasts (Figure 4.10). In contrast, the small twig treatments displayed subcellular disruption with continued water loss (water content < 0.4 g/g DM after 24 hours drying). There was a loss of plasmalemma integrity and release of cell contents inside the cell (Figure 4.11).

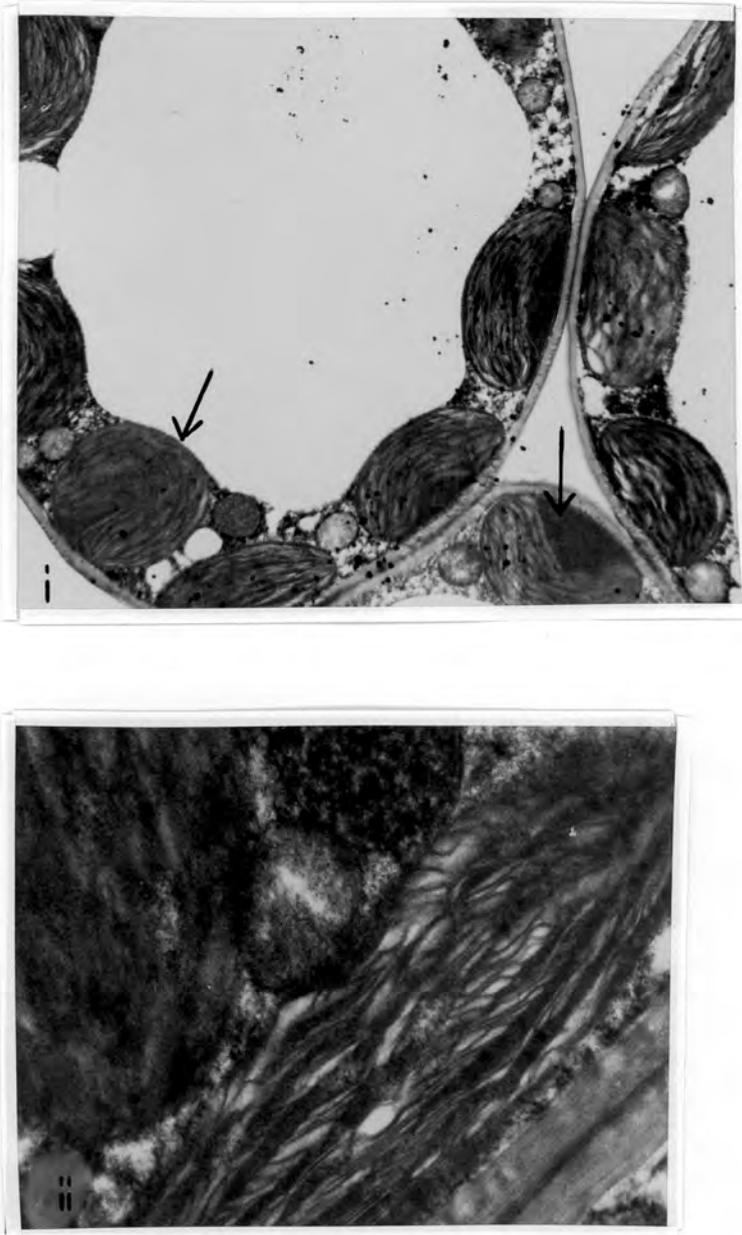


Figure 4.9 Ultrastructural organisation of mesophyll cells during initial twig dehydration (water content decreasing from 2.0 to 1.0 g/g DM). A few chloroplasts became rounded (arrowed), whereupon the granal stacks orientated in a half-moon shape along the edge of the chloroplasts and the thylakoid membranes remained appressed ( $\times 5\ 000$ ) (i). In the chloroplasts which remained elongated, the granal stack arrangement was lost and the thylakoid membranes were loosely arrayed within the stroma ( $\times 20\ 000$ ) (ii).

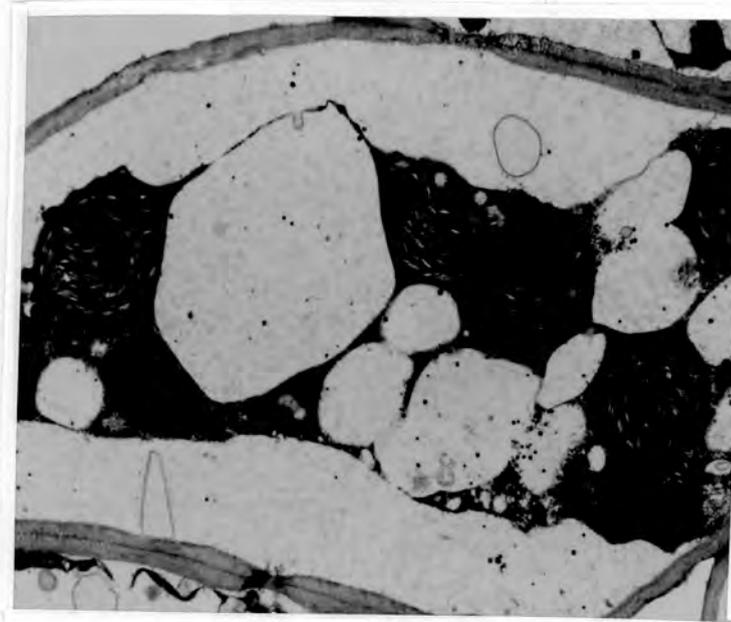


Figure 4.10 Ultrastructural organisation of air-dry mesophyll cells from large twigs at water contents below 0.4 g/g DM after 48 hours dehydration. Subcellular characteristics typical of desiccated whole-plant cells, the plasmalemma withdrawn from the cell wall, cytoplasm densely stained, and vacuole divided into numerous smaller vacuoles (x 8 000).

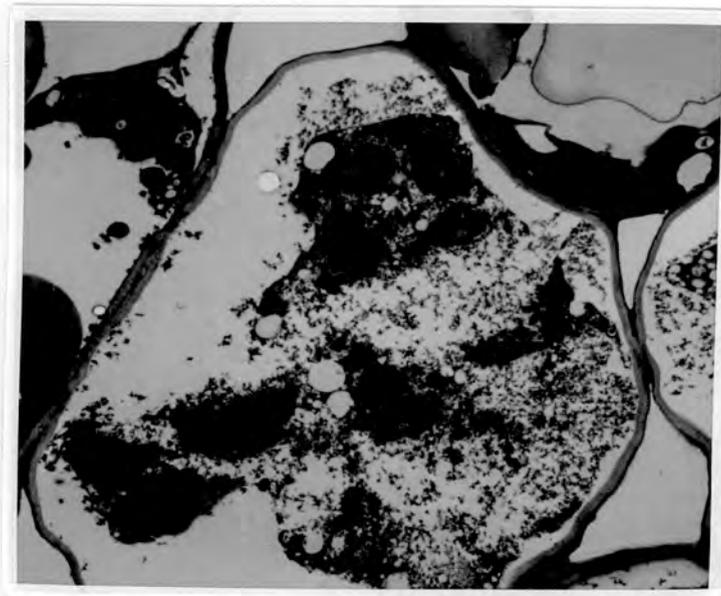


Figure 4.11 Ultrastructural organisation of air-dry mesophyll cells from small twigs at water contents below 0.4 g/g DM after 24 hours dehydration. There was subcellular disruption and disintegration of the plasmalemma (x 4 400).

## 4.2 REHYDRATION OF DETACHED TWIGS

### 4.2.1 Water content

The leaves of small twigs did not rehydrate, whilst the leaves of the large twigs took twice as long (36–48 hours) as was necessary for whole plant recovery to full turgor (Figure 4.12). The lack of small twig rehydration may be a result of xylem breakdown, or perhaps the formation of an abscission zone in the leaves, preventing water movement into the leaves.

That the large twigs were able to undergo initial recovery might indicate that the rate of drying was more important than the absence of roots for the development of desiccation tolerance in *M. flabellifolius*. Water content of the large twigs remained high 24 to 36 hours following recovery to full turgor, whereafter the leaves dried out once more (i.e. the leaves became air-dry five days after rehydration, despite the continued availability of water).

The slow rate and short duration of recovery to full turgor associated with leaf rehydration of the large twig treatments may indicate that protection mechanisms were not successfully induced during drying. Alternatively, protection mechanisms may have been induced during desiccation, but repair processes were unable to be maintained, thereby preventing complete recovery and survival from the desiccation event.

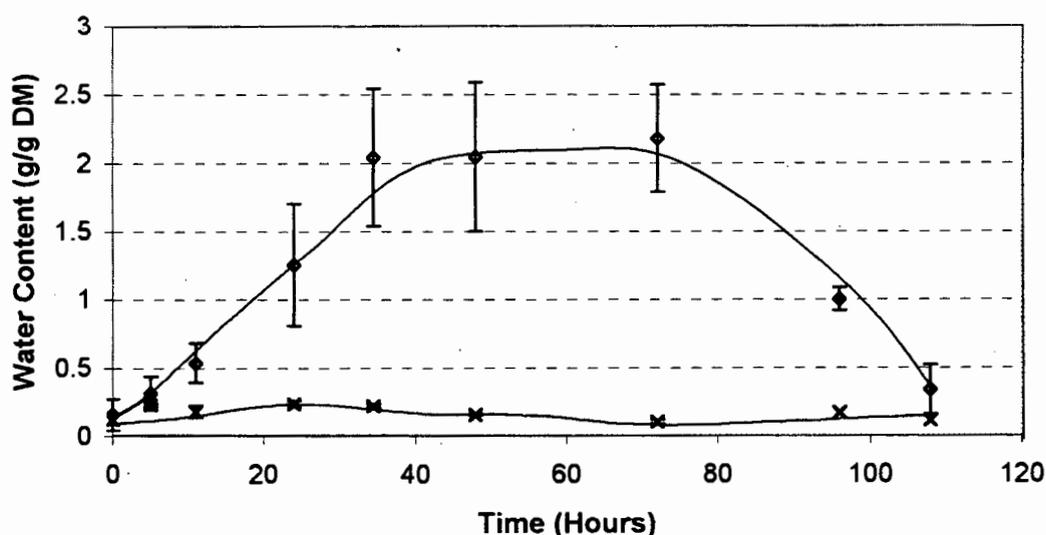


Figure 4.12 Rehydration of leaves from large (squares) and small (crosses) twigs with time.

The small twig treatments were unable to rehydrate, thus (excluding initial measurement of small twig solute leakage), the remaining results pertain only to large twig recovery.

#### 4.2.2 Solute leakage

The high levels of leakage recorded during attempted rehydration of the small twigs (Figure 4.13) provides a quantitative indication of the level of membrane damage observed in the ultrastructural studies during the final stages of desiccation (Figure 4.11). In contrast, solute leakage from the large twig treatments (Figure 4.13) remained within the range observed during whole plant rehydration (i.e. <5% total leakage). These results indicate that although too rapid a drying rate disrupts membrane structure, the presence of roots during drying is not essential for maintenance of membrane integrity upon rehydration. Furthermore, the continued low levels of solute leakage despite subsequent leaf water loss (five days after rehydration), suggests some constitutive level of protection enabling maintenance of plasmalemma integrity.

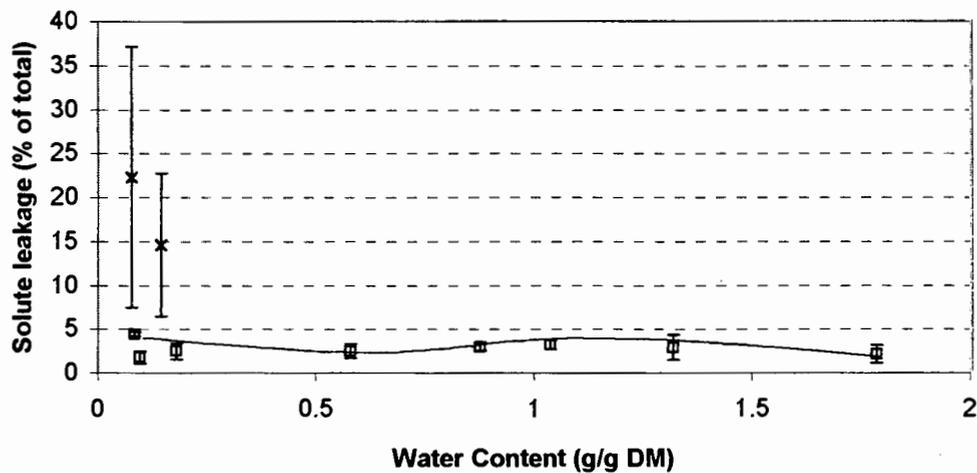


Figure 4.13 Solute leakage (as a percentage of maximum leakage) during rehydration of large (squares) and small (crosses) twigs.

#### 4.2.3 Chlorophyll and carotenoid content

Both chlorophyll and carotenoid concentrations remained constant during leaf rehydration to full turgor (Figure 4.14), with no evidence of chlorophyll synthesis as observed during whole plant recovery. Photosynthetic pigment concentrations decreased 60 hours following rewatering, and this drop-off was concomitant with subsequent leaf water loss.

This might indicate that roots were essential for pigment synthesis during leaf recovery. Since cytokinin (CK) biosynthesis occurs in the root tips of most plants, where it is transported via the xylem from the roots to the shoots (Davies, 1990), it is possible that the absence of cytokinins inhibited chlorophyll production. Cytokinins have been implicated in translation by the activation of polysomes in such a manner that increased recruitment of untranslated mRNA occurs (McGaw, 1990). Cytokinins may then be necessary as a signal for the translation of mRNA for chlorophyll synthesis during rehydration. It is also possible however, that cytokinins were present, but mRNA was not laid down during drying, thereby preventing the CK stimulation of chlorophyll synthesis. The chlorophyll degradation with subsequent leaf water loss may have been caused by exposure of the chloroplasts to light (upon leaf reorientation), increasing damaging light-chlorophyll interactions such as free radical formation.

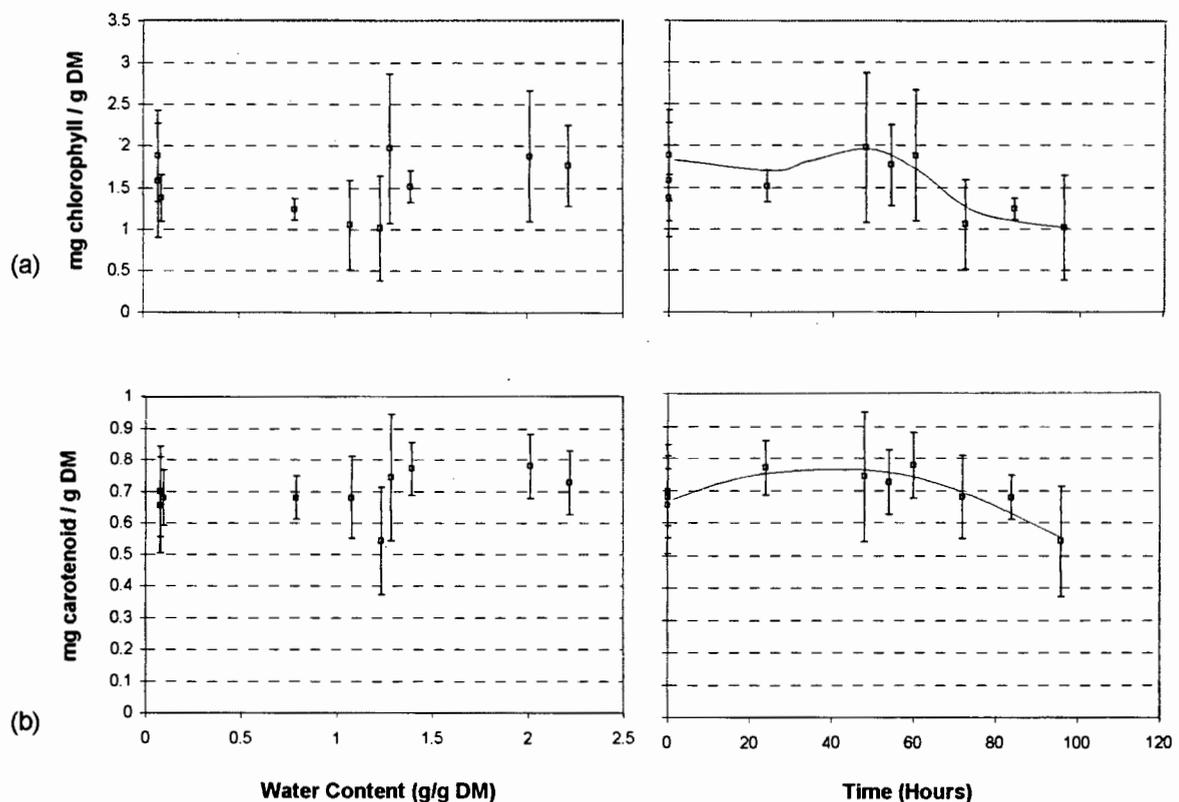


Figure 4.14 Total leaf chlorophyll (a) and carotenoid (b) concentrations with respect to changes in water content (left column) and time (right column) during large twig rehydration.

#### 4.2.4 Chlorophyll fluorescence

As found during whole-plant rehydration,  $F_v/F_m$  and Yield increased linearly with water content and reached pre-stress levels 12 hours after recovery to full turgor (Figure 4.15a&b). This suggests that neither the presence of roots, nor associated chlorophyll synthesis, were necessary for initial photochemical recovery during rehydration. Rather, photochemical activity may recover immediately following subcellular re-organisation of the chloroplasts, (and possibly the thylakoid arrangement in particular). This apparent recovery of photochemical activity towards pre-stress levels, despite a 40% loss of total chlorophyll, may not reflect the entire leaf response however, since fluorescence characteristics were measured from the upper leaf surface only. Should chlorophyll be preferentially lost from the lower leaf surface, abaxial chlorophyll fluorescence analyses might display limited recovery of the photochemical activity. Nevertheless, these results show that the enzymes necessary for photochemical activity (in the upper leaf surface at least), were able to function on rehydration, and thus must have been protected in the dry state and/or repaired upon rehydration.

Disrupted photochemical activity at the low water contents interfered with the quenching coefficients in a manner similar to that observed during the initial stages of whole plant rehydration (Figure 4.15c&d). Upon rehydration to full turgor,  $qP$  remained high, as expected, since fluorescence yield had recovered and photosynthesis had been initiated. Non-photochemical quenching mechanisms ( $qN$ ), which had increased during initial rehydration, decreased steadily following leaf recovery of full turgor. All photochemical activity was then inhibited concomitant with subsequent water loss (60 hours after rewatering).

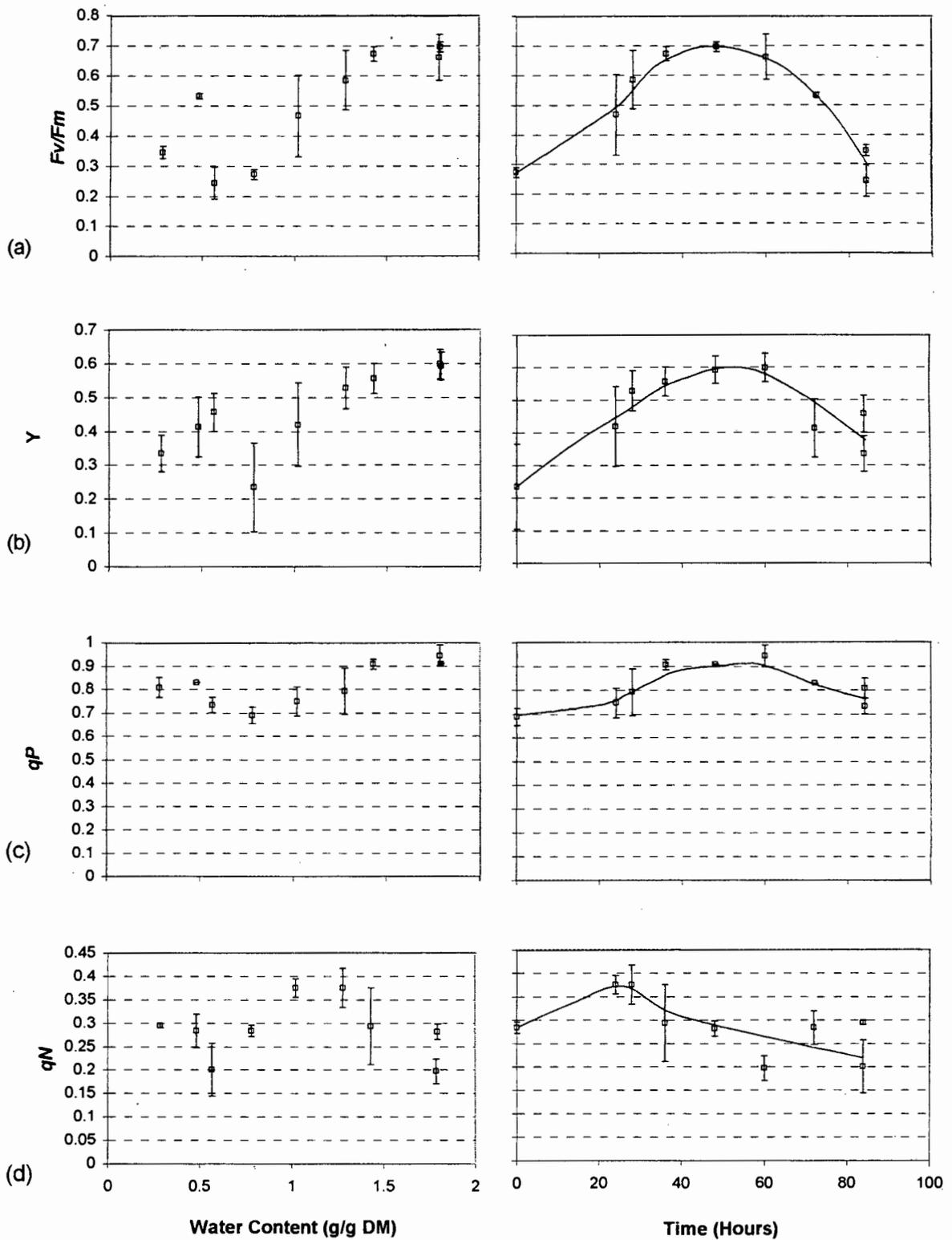


Figure 4.15 Changes in chlorophyll fluorescence characteristics with respect to changes in water content (left column), and time (right column), during large twig rehydration.  $F_v/F_m$  (a), Yield (b), and the quenching coefficients  $qN$  and  $qP$  (c&d).

### 4.2.5 CO<sub>2</sub> gas-exchange

Dark respiration recovered in a manner similar to that recorded during whole plant rehydration, being initiated within six hours of rewatering, and continuing to increase until full turgor had been attained (i.e. after 36 hours) (Figure 4.16a). Respiration then remained constant, although there appeared to be a slight increase in association with subsequent water loss, this CO<sub>2</sub>- evolution perhaps due to cellular damage.

There was no recovery of photosynthesis during the first 24 hours of rehydration (Figure 4.16b), although low levels of photosynthetic activity were induced during the following 24-hour period. It is possible that water was limiting (due to the brief period of full turgor) on recovery, thus insufficient time was available for the induction of positive carbon assimilation. Lower (60% of total) levels of chlorophyll may also have contributed towards the reduced photosynthesis, relative to the whole plant response during rehydration.

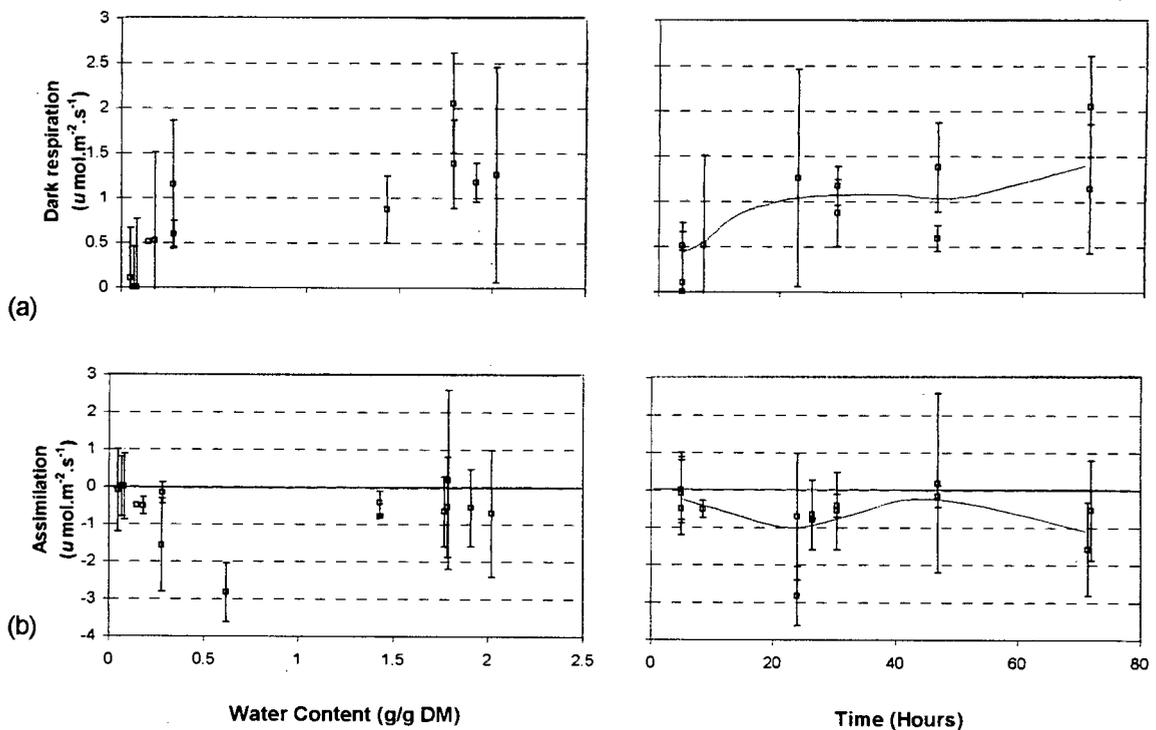


Figure 4.16 Changes in dark respiration (a) and assimilation (b) with respect to changes in water content (left column), and time (right column), during rehydration of leaves from large twigs.

#### 4.2.6 Plant growth regulators

Total ABA and cytokinin concentrations extracted from large twigs were examined at two stages of rehydration, as illustrated in Figure 4.16. After 36 hours rehydration of the large twigs ABA levels increased towards levels similar to that recorded 12 hours after whole plant rehydration (600 ng ABA/g DM). Although this increase in ABA concentration was delayed relative to the whole-plant response, it occurred at similar water contents (1.6 g/g DM). Increased ABA levels in the leaves of detached twigs indicates that, as observed during twig drying, ABA may be synthesised in the leaves on rehydration.

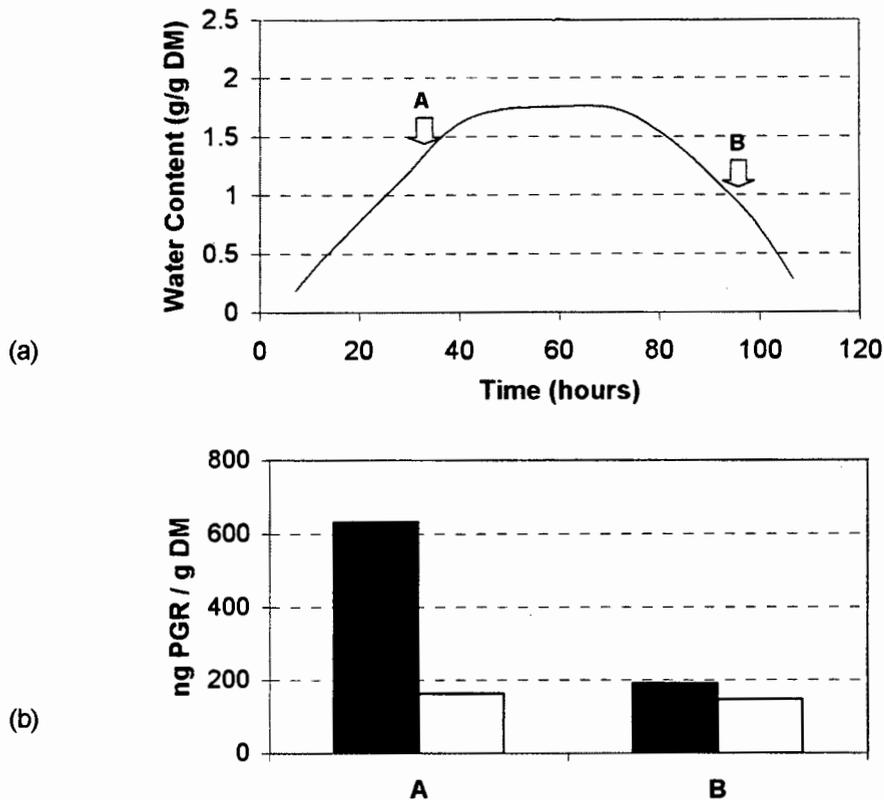


Figure 4.16 Water content and time during which leaves were sampled for the presence of the plant growth regulators (a); ABA (solid bars) and cytokinin (empty bars) concentrations extracted at these water contents during large twig rehydration (b).

As observed during whole plant rehydration, ABA accumulation during rehydration of detached twigs did not induce the effects typical of elevated ABA in desiccation-sensitive plants (e.g. stomatal closure and chlorophyll degradation). It is possible however, that increased ABA during twig recovery might contribute to the inhibition of photosynthesis before recovery of metabolic competence, or perhaps induce the transcription of proteins necessary for cellular repair and/or recovery.

ABA levels extracted from the leaves of large twigs after 96 hours rehydration were slightly higher than those obtained from whole-plant material after 36 hours rehydration. It is possible that this higher level of ABA was a result of water limiting ABA metabolism, since during this period the leaves of the large twig treatments were again dehydrating. The high levels of ABA might also have contributed to leaf senescence observed to occur following subsequent large twig dehydration.

Cytokinin (CK) levels remained constant during recovery and subsequent dehydration. Although absolute CK levels were slightly higher than those recorded during whole plant rehydration, there was no increase (concomitant with the rise in ABA levels), as was observed during whole plant rehydration. Since cytokinins are generally synthesised in the roots, the lack of elevated CK levels is a likely consequence of root absence. Exogenous application of CK leads to an accumulation of chlorophyll in desiccation-tolerant species (Parthier, 1979), thus it is possible that the lack of cytokinins prevented chlorophyll re-synthesis in the leaves of detached twigs.

#### 4.2.7 *Ultrastructure*

As found during whole plant rehydration, there was recovery of subcellular organisation in the leaves of the large twig treatments following rehydration to full turgor. The plasmalemma became appressed to the cell wall, the central vacuole re-formed, and organelles were aligned along the edges of the cells (Figure 4.17). The mitochondria had become electron dense indicating the formation of cristae, and this was coincident with recovery of respiratory activity. The thylakoid membranes recovered their staircase-like arrangement (concomitant with the induction of photosynthesis), and starch was present.

Upon subsequent leaf water loss (80 hours following rewatering), most of the cells from the leaves of the large twigs appeared similar to that typical of the whole plant during drying. The plasmalemma generally remained intact but had withdrawn from the cell wall. Unlike whole plant dehydration however, some cell wall rupture was evident, and there was a greater abundance of starch present within the chloroplasts (Figure 4.18). The maintenance of plasmalemma integrity throughout large twig rehydration and subsequent water loss correlates with the low levels of electrolyte leakage, and continues to imply a degree of constitutive membrane protection.

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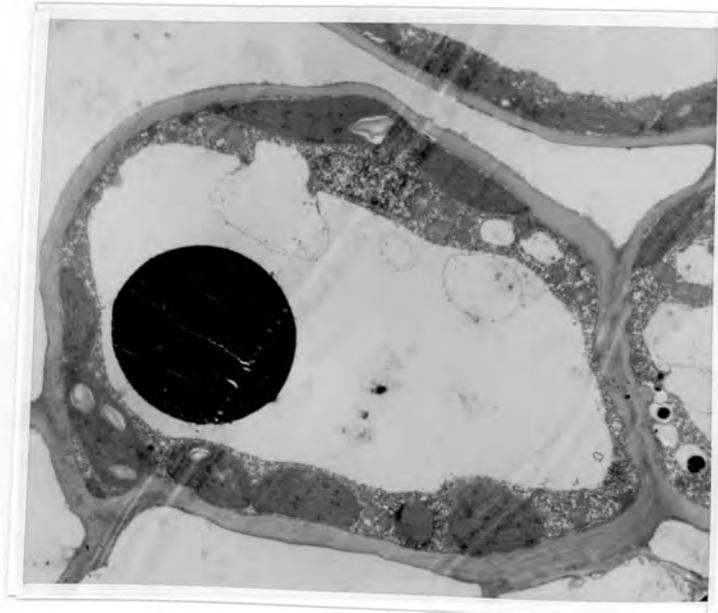


Figure 4.17 Recovery of subcellular organisation following rehydration of large twigs to full turgor (i.e. 36 hours following rewatering, water content 1.8 g/g DM) ( $\times 3\ 000$ ). The central vacuole reformed and organelles were aligned along the edges of the cells.

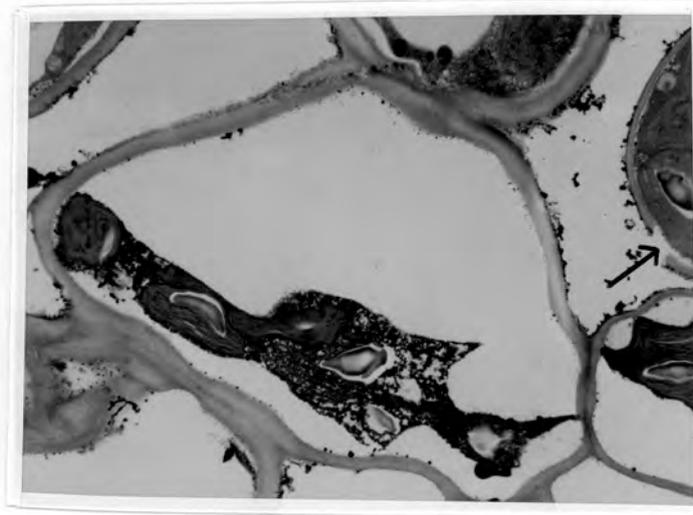


Figure 4.18 Subcellular dehydration 80 hours after large twig recovery. Plasmalemma integrity was maintained despite withdrawal from the cell wall. Some cell wall rupture was evident (arrowed) and starch was present in the chloroplasts ( $\times 3\ 000$ ).

## DISCUSSION

Despite the elevated drying rate during the first eight hours of large twig dehydration these twigs, like the whole plant, then gradually desiccated towards air-dryness, reaching water contents of 0.4 g/g DM within 36-48 hours drying. The small twigs in contrast, reached water contents of 0.4 g/g DM within 10 hours dehydration. The rapid rate of small twig dehydration to air-dryness could have prevented the induction of tolerance mechanisms within the cells of these leaves. In addition, rapid drying could have caused irreparable damage; both effects resulting in a lack of survival in the leaves of small twigs.

Evidence supporting the suggestion that drying rate affected small-twig desiccation tolerance was first observed as incomplete leaf folding. The inhibition of physical protection mechanisms such as leaf folding may have resulted in increased light-chlorophyll interactions and associated damage. The physiological data provided evidence of damage, with increased levels of solute leakage, pigment degradation and subcellular disruption. Ultimately however, the inability for the leaves of small twigs to rehydrate confirmed irreparable cell damage and absence of desiccation tolerance.

In contrast, dehydration of large twigs resulted in the maintenance of membrane integrity and a loss of chlorophyll similar to that reported during whole plant drying. Although these twigs did not ultimately survive, they did recover some metabolism (such as photosynthesis and respiration), suggesting that either there was more protection afforded during drying, and/or less damage accrued during slow drying.

The different drying rates between the two twig treatments did not induce different photochemical responses during the early stages of desiccation. Both treatments displayed a delayed reduction in net photosynthesis and photochemical activity relative to the whole plant. This similar pattern eliminates the suggestion that self-shading (through leaf folding) was responsible for the initial inhibition of photosynthesis, as was proposed during whole plant dehydration in Chapter 3. (If leaf reorientation was responsible for the immediate reduction in photosynthesis evident during whole plant dehydration, then large twig treatments would be expected to show, like the whole plants, reduced photosynthesis at high water contents.) Ultrastructural studies indicate that the thylakoids in the chloroplasts of both twig treatments did not separate into smaller stacks as evidenced during whole plant dehydration. This may be a result of the rapid drying rate providing insufficient time for

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subcellular re-organisation, or due to the absence of a chemical signal from the roots. Sustained levels of photosynthesis and photochemistry during leaf dehydration may be a consequence of the maintained granal arrangement, and this, in turn, may have induced free radical damage and limited recovery during rehydration.

ABA accumulation during the drying of leaves from large detached twigs indicates that ABA synthesis can occur in leaf tissue. Furthermore, the accumulation of ABA in leaf tissue, as found during whole plant drying, was not purely a signal from the roots to the shoots in response to water stress (although this does not preclude the possibility that chemical signals and/or compounds from the roots might be necessary for subsequent recovery). The increased level of ABA during drying supports the possibility (suggested in Chapter 3) that ABA plays a role in the induction of protection mechanisms during water stress.

The presence of roots during drying and rehydration would not appear to be essential for initial recovery of *M. flabellifolius*, since the leaves of the large twigs were able to rehydrate and initiate a low level of metabolism. The mechanisms of xylem recovery from water stress in *M. flabellifolius* have recently been reported (Sherwin *et al.*, 1998). These authors found that water uptake appeared to be rapid, and due mainly to capillary action, hence the inability for small twig rehydration is inexplicable. Unfortunately, stem water content was not investigated in the current study. It might be speculated that water was transported up the stem of the small twig treatments, but was unable to enter the leaves, perhaps as a result of an abscission signal initiated during the rapid drying of these small twigs.

In contrast with whole plant recovery, (which described an S-shaped pattern of leaf rehydration over time, see Figure 3.12), the large twig treatments displayed a linear, but slower, rate of leaf water uptake over time. These differences in patterns of leaf water-uptake can be ascribed to the absence of roots during twig rehydration. The initial lag in water uptake evident during whole plant recovery but absent during large twig rehydration may be a likely consequence of root rehydration and repair necessary before water transport up the stem. Similarly, the faster rate of whole plant leaf rehydration (relative to large twig leaf recovery) following the initial lag in water uptake could then be ascribed to root pressure.

The leaves of large twigs were able to rehydrate and initiate photochemical activity, albeit at a slower rate, and for a limited period of time relative to the whole plant response. It is possible that chlorophyll retained in the leaves on drying, along with enzymes necessary for

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photosynthesis, were protected, enabling the brief recovery of metabolic activity associated with initial large-twig rehydration. The absence of chlorophyll synthesis however, combined with minimal carbon assimilation during twig rehydration, suggests that either the rate of drying, and/or the absence of roots prevented complete (i.e. sustained) metabolic recovery. Since cytokinins have been implicated in the promotion of mRNA transcription necessary for chloroplast development, and are generally produced in the roots of plants, the absence of roots coincident with the inability for chlorophyll synthesis suggests that roots were essential for sustained recovery. It is possible however, that mRNA was not laid down due to the rapid rate of drying, or that excessive levels of damage limited recovery.

An explanation for subsequent water loss five days following large twig rehydration remains elusive, although it may be speculated that a lack of root signaling prevented ongoing transcription/translation, thereby preventing sustained recovery. Alternatively, a senescence signal may have been induced in the leaves, once damage was found to exceed repairable levels.

In summary these results indicate that protective mechanisms exist, despite the absence of roots and the increased drying rate, thereby enabling a degree of large twig recovery. Leaf reorientation and ABA accumulation during drying might indicate that part of the protection mechanism was induced. Ultimately however, the slow rate and short duration of twig recovery indicates that roots, certainly during rehydration, were essential for sustained recovery.

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## **Chapter Five: Characterisation of the leaf response during recovery following prolonged desiccation of the whole plant**

### **INTRODUCTION**

No studies have been reported concerning the length of time for which field-dried desiccation-tolerant plants are able to remain viable in their natural environment. Although bryophytes have shown a strong correlation between desiccation-tolerance and the frequency and duration of dry periods (Busby *et al.*, 1978; Clausen, 1952), these effects have not been examined amongst desiccation-tolerant angiosperm plants.

It has been estimated by both Child (1960), and Gaff (1977), that *M. flabellifolius* exists in the dry state for at least half of the year, during the dry winter season which lasts from at least May to September. Sherwin (pers. com.) has found however, that plants were still dry in October, and had often become desiccated by March, with perhaps a single rehydration-dehydration event before drying out again for winter. The possible importance of a single rehydration event in the maintenance of desiccation tolerance of this species remains unknown.

Studies using field-dried leaves of *M. flabellifolius*, which had been excised and sealed in polythene bags, suggested that leaves could remain viable for up to 3.5 to 5 years (Gaff, 1977). In these studies relative humidity was maintained below 50%, and survival was inferred (after 24 hours immersion of the detached leaves in water) by the exclusion of Evan's blue dye from the cells (Gaff and Okong 'O-Gola, 1971). Determination of survival, based on the retention or loss of the semi-permeable properties of the plasmalemma might produce unreliable results however, since it was shown (Chapter 4 of this thesis) that mere membrane integrity of *M. flabellifolius* does not provide an indication of metabolic recovery.

In the present study whole plants were allowed to desiccate and were maintained in the dry state (under simulated natural conditions, midday PPFD  $1400 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , relative humidity 50%) for 6, 12 and 18 months. Solute leakage levels and leaf photosynthetic pigment concentrations were determined for comparison with desiccated material not exposed to prolonged desiccation, whilst the nature of recovery after rewatering was investigated using the parameters described in chapter 2.

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## RESULTS

### 5.1 DESICCATED PLANT MATERIAL

Electrolyte leakage from leaf material increased with the length of time plants were kept desiccated (Figure 5.1). Plants dried for 6 and 12 months displayed slightly elevated levels of leakage than was recorded for material kept dry for one month, whilst plants dried for 18 months had significantly higher leakage levels (up to 28% of total leakage). These increased levels of leakage suggest plasmalemma damage, and a loss of constitutive membrane protection, with time. This might indicate that continual replacement and/or repair, which was unable to occur in the dry state, was necessary for the maintenance of membrane protection mechanisms.

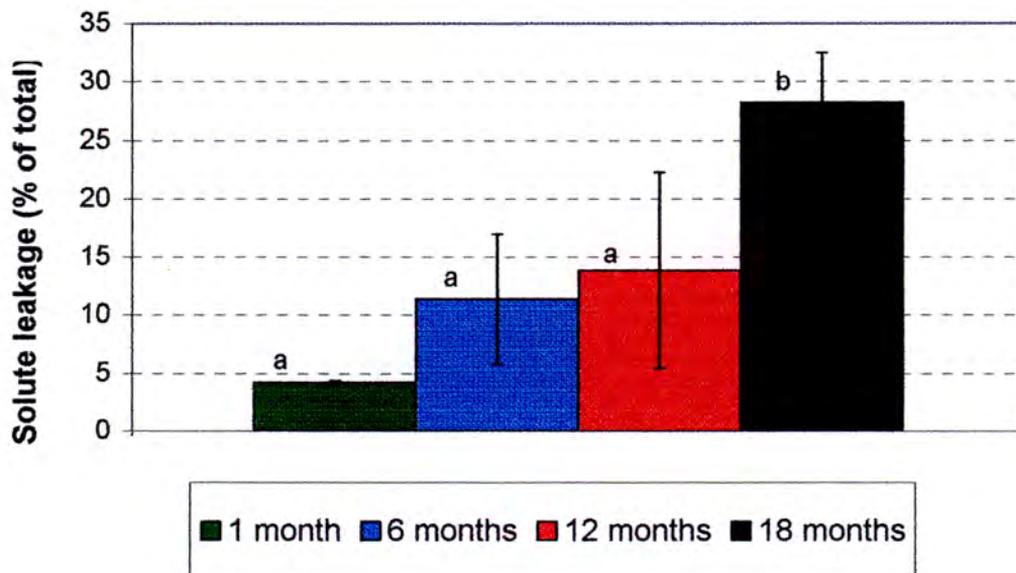


Figure 5.1 Solute leakage from leaves after whole plants kept dry for 6, 12 and 18 months. Values obtained from air-dry leaves after whole plant desiccation for one month (Chapter 3) provided for comparison. Different letters indicate significant differences between treatments (Lords Multiple Range Test,  $p < 0.01$ ).

Photosynthetic pigment concentrations following prolonged desiccation did not differ significantly between desiccation treatments (Figure 5.2). This indicates that whilst a degree of pigment loss occurred during dehydration, (as noted in Chapter 3), increased degradation was not evident with prolonged exposure to the dry state.

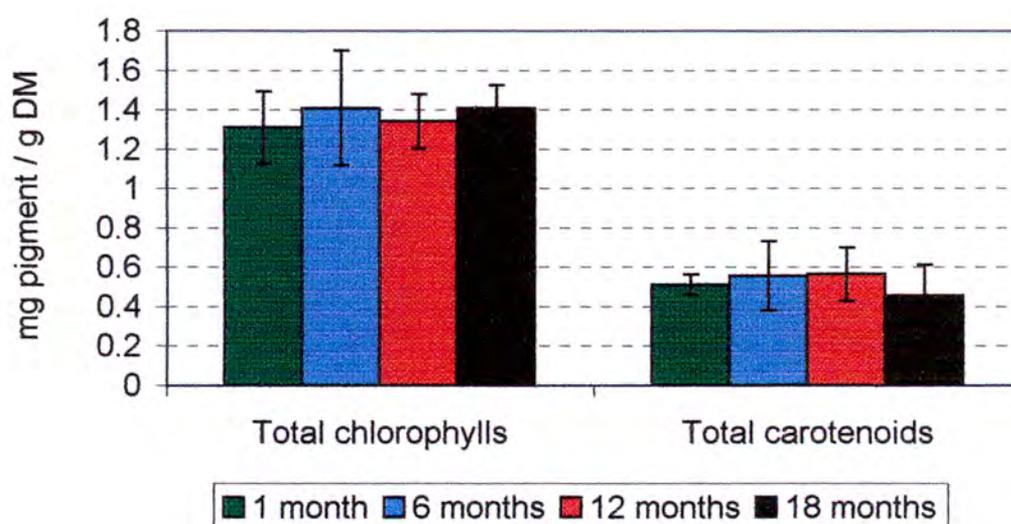


Figure 5.2 Photosynthetic pigment concentrations extracted from air-dry leaves of plants desiccated for 6, 12 and 18 months. Values from air-dry leaves after whole plant desiccation for one month (Chapter 3) provided for comparison. No significant differences at  $p < 0.05$  (Anova and Lords multiple range test)

## 5.2 REHYDRATION

### 5.2.1 Water content

Whole plants exposed to desiccation for 6 and 12 months rehydrated more slowly than plants desiccated for one month (Figure 5.3). This slower rehydration rate was associated with an extended lag-phase in water-uptake. Both the 6 and 12 month treatments required 36 hours before leaf rehydration to full turgor (in contrast with the 18 hours necessary for leaf rehydration of plants after one month of desiccation). From this it might be suggested that root cells and/or xylem might have suffered some damage during prolonged maintenance in the dry state. The delay in water uptake and transport then being due to the need for some repair before the onset of renewed metabolism.

Upon rehydration, leaves from plants air-dried for six months maintained full turgor, and new shoots were produced several days following rewatering (in a manner similar to that observed during rehydration of plants within one months desiccation). In contrast the leaves of plants rehydrated after 12 months desiccation lost leaf water content after attaining full turgor, despite the continued availability of water at the root level (i.e. in a manner similar to that reported during large twig rehydration (Chapter 4)). Both the large twig treatments and the whole plants dried for 12 months lost their leaves upon subsequent dehydration, but the whole plants, (unlike the twigs,) then produced new axial shoots (Figure 5.4). This new shoot production suggests that the continuity of the transpiration stream was maintained, and that root and stem recovery was possible after 12 months maintenance in the dry state.

As found during attempted rehydration of small twigs (Chapter 4), plants desiccated for 18 months were unable to recover leaf water content. Although not examined in this study, it is possible that root damage exceeded repairable levels, therefore preventing water transport up the stem. Alternatively, it is also possible that roots did take up water, which was transported up the stem, but unable to enter the leaf tissue due damage and senescence of the leaves.

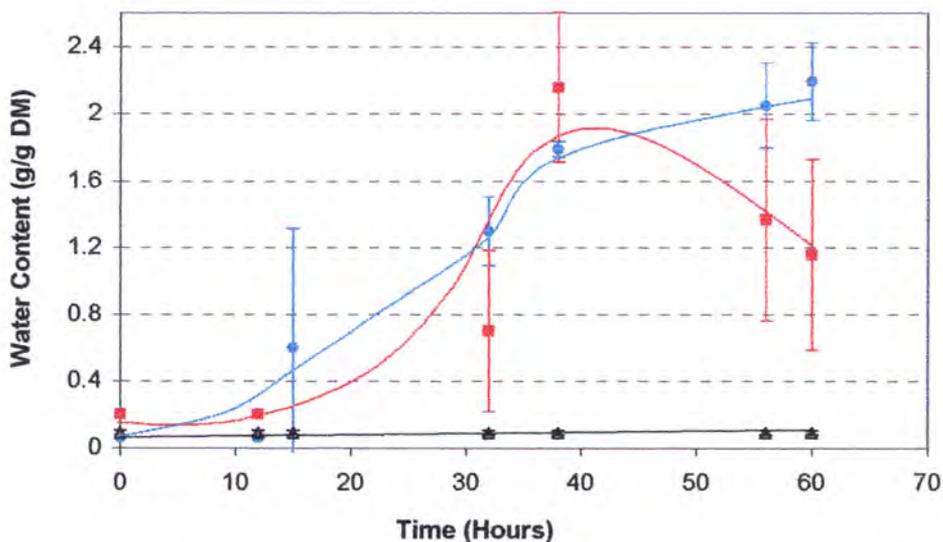


Figure 5.3 Leaf water uptake following 6 (blue symbols), 12 (red symbols) and 18 (black symbols) months desiccation of plants. (Data points represented as a scatterplot of means, with bars indicating standard deviations, and trends investigated using distance weighted least squares regression analysis.)



Figure 5.4 New shoot formation several days following the rewatering of plants which had been maintained in the air-dry state for 12 months. Scale bar = 10 mm

### 5.2.2 Solute leakage

Solute leakage from whole plants during rehydration following prolonged desiccation is shown in Figure 5.5. Solute leakage from plants dried for 6 months was consistently low, this being indicative of limited membrane damage.

During the first 12 hours of rehydration, leakage from plants dried for 12 months was slightly higher than pre-stress levels, however leakage decreased 24 hours following rewatering. This might be associated with membrane repair during restoration of full turgor. However, after 52 hours, (when the leaves had again started to dehydrate), solute leakage increased once more. It is possible that initial repair of membranes occurred due to the presence of stored components (or components translated from stored mRNA's), but continued repair may have required *de novo* transcription. If transcription was impaired (as a result of damage to the genome), further repair would be inhibited, and this damage could be evidenced as increased leakage.

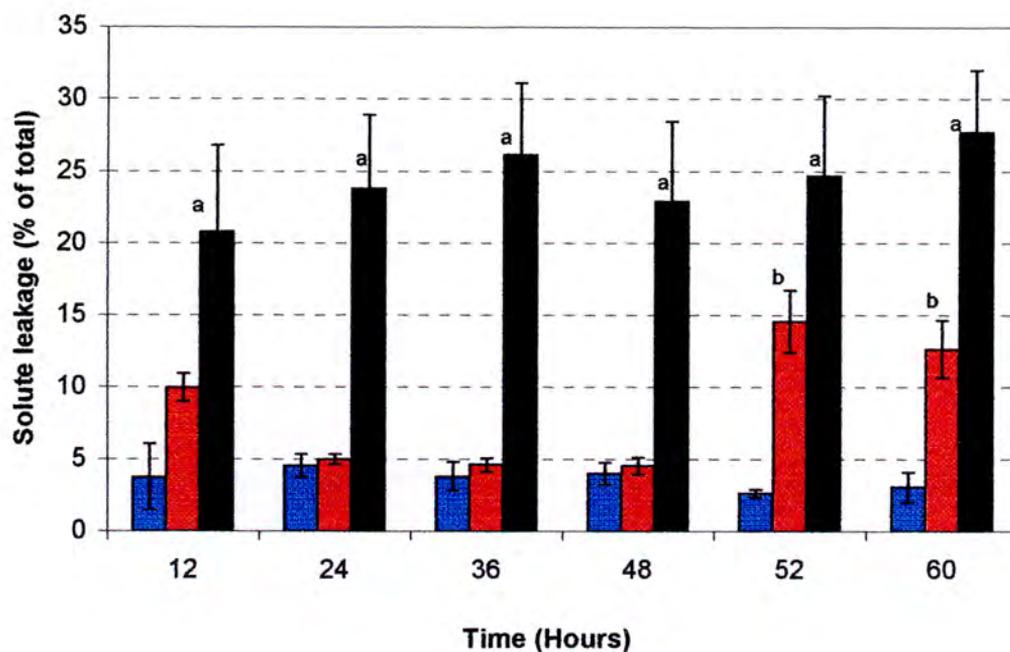


Figure 5.5 Solute leakage over time, during rehydration of whole plants following 6 (blue shading), 12 (red shading) and 18 (black shading) months exposure to desiccation. (Differences between letters indicate significant differences (Anova and Lords Multiple Range test,  $p < 0.05$ )).

Continuously high levels of solute leakage recorded during whole plant rehydration following 18 months desiccation was similar to those values recorded during small twig desiccation (Chapter 4). These results suggest that extensive damage, which was not repairable, had occurred, and thus prevented recovery from the air-dry state.

### 5.2.3 Chlorophyll and carotenoid contents

Photosynthetic pigment concentrations after 60 and 120 hours rehydration are given in Figure 5.6. Pigment concentrations extracted from plants dried for 6 months increased to control levels five days after rehydration. Plants desiccated for 12 and 18 months were unable to recover pre-stress pigment concentrations.

It was proposed in Chapter 4 that roots were necessary for chlorophyll production in the leaves of *M. flabellifolius*. The lack of chlorophyll production in whole plants maintained in the dry state for 12 and 18 months might refute this suggestion. However, it is likely that roots of the plants dried for 18 months were themselves damaged, and could not send appropriate signals for chlorophyll production. The situation for plants maintained in the dry state for 12 months is less clear. Roots did survive, as was evidenced by the production of new axial buds and continued survival of the whole plant. But, as the existing leaves of the 12 month treated plants senesce, root signals for chlorophyll production within these leaves may either not be sent, or not be utilised in these leaves.

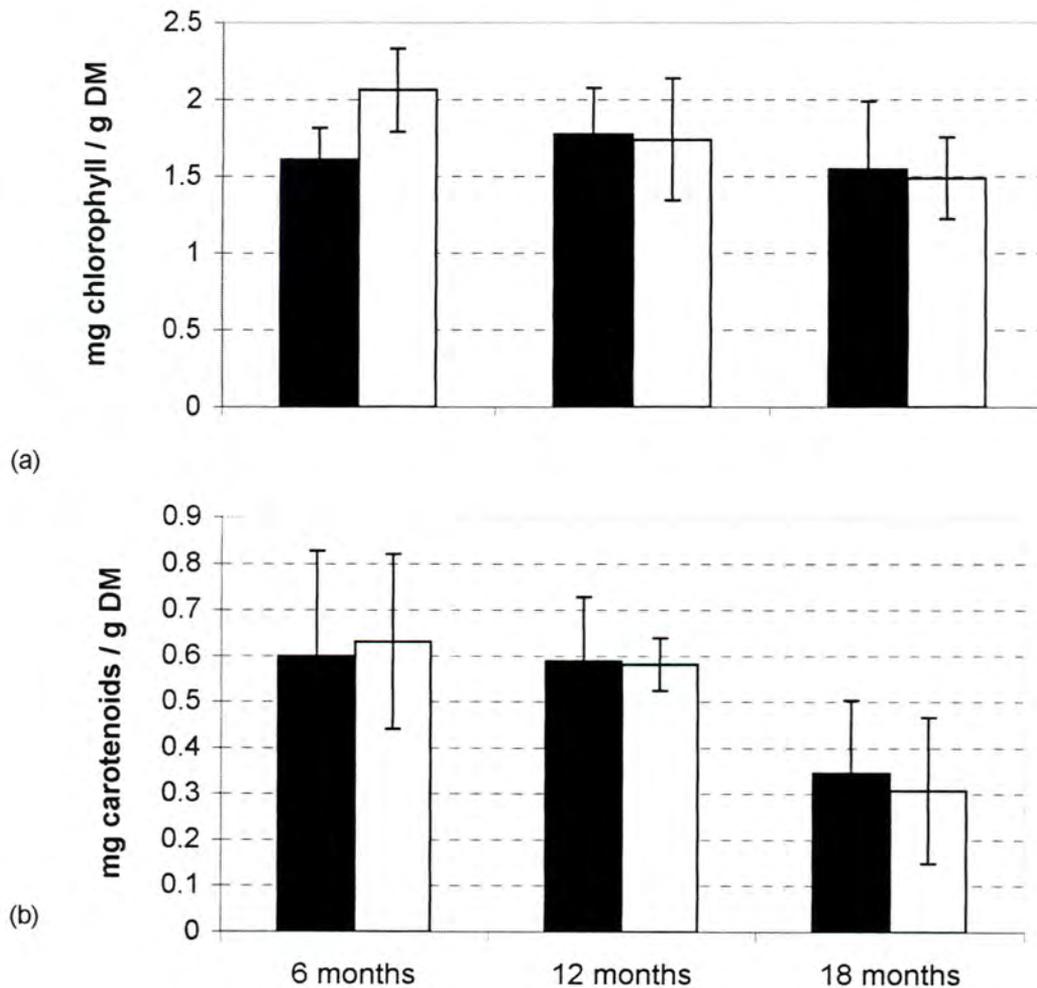


Figure 5.6 Chlorophyll (a) and carotenoid (b) concentrations extracted from leaves after 60 (solid bars) and 120 (empty bars) hours rehydration of plants following 6, 12 and 18 months exposure to desiccation.

### 5.2.4 Chlorophyll fluorescence

Plants rehydrated after 6 months in the dry state gradually recovered photochemical activity to levels characteristic of leaves from hydrated control plants (Figure 5.7). Whole plants rehydrated after one months' desiccation (Chapter 3) recovered photochemical activity 36 hours following leaf rehydration to full turgor (i.e. 60 hours following rewatering). In contrast, photochemical recovery of leaves from plants dried for 6 months recovered towards hydrated control levels 84 hours after leaf rehydration to full turgor (i.e. 120 hours after rewatering). This might indicate that enzymes necessary for electron transport were damaged during 6 months desiccation, such that repair processes were necessary before photochemistry could be induced. Even more damage could have been associated with plants desiccated for 12 months since they did not recover photochemical activity, before declining in response to subsequent water loss (Figure 5.7). This suggests that repair processes (including stored mRNA's) present in dried tissues and used in short-term (one month) dried material for recovery of photosynthetic processes themselves must be damaged following long-term maintenance of leaf material in the dry state.

No measurable levels of photochemical activity were recorded during rehydration of plants maintained in the dry state for 18 months. This is likely to be a consequence of the inability for leaf rehydration, although considerable damage to leaf tissues must also have occurred during this prolonged maintenance in the dry state.

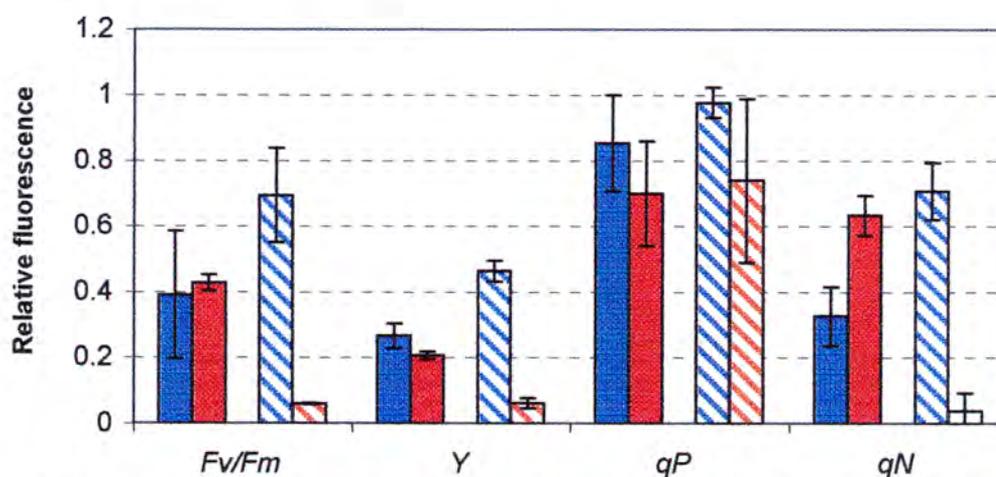


Figure 5.7 Chlorophyll fluorescence after 60 (solid bars) and 120 hours (patterned bars) rehydration of whole plants maintained in the dry state for 6 (blue shading) and 12 months (red shading). (The fluorescence parameters quantum efficiency of PSII ( $F_v/F_m$ ), Yield ( $Y$ ) and photochemical and non-photochemical quenching coefficients,  $qP$  and  $qN$  respectively, were recorded.)

### 5.2.5 CO<sub>2</sub> gas -exchange

CO<sub>2</sub>-evolution from plants desiccated for 6 months increased with water content reaching a maximum (near hydrated control levels) upon attainment of full turgor (i.e. after 36 hours) (Figure 5.8a). Carbon assimilation also recovered to control levels, although this recovery was delayed relative to recovery from plants rehydrated within one month of drying, possibly a result of the more gradual uptake of water following six months desiccation (Figure 5.8b).

Whole plants dried for 12 months showed an initial increase in dark respiration, but pre-stress levels were never attained, and positive levels of CO<sub>2</sub>-assimilation were not induced (Figure 5.8a&b). No gas-exchange was associated with plants maintained in the dry state for 18 months, again due to the lack of leaf rehydration.

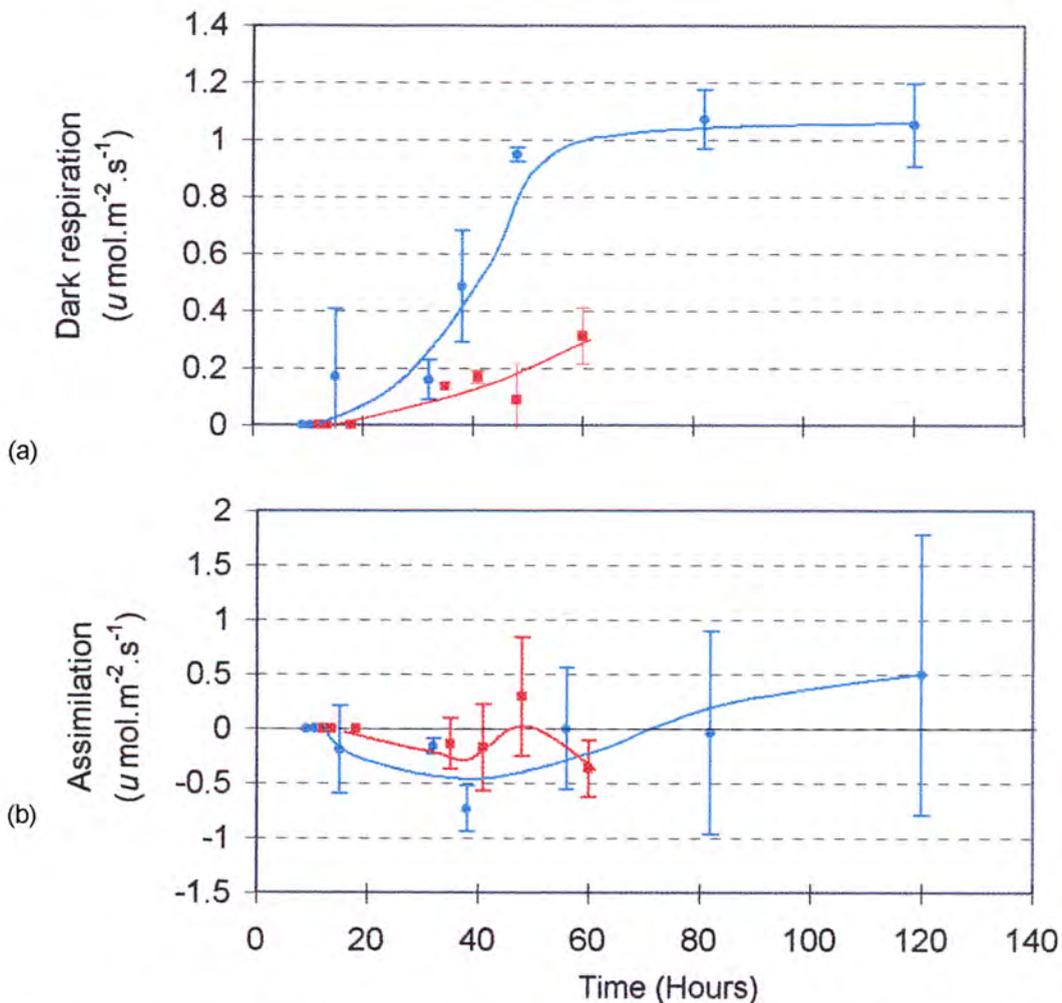


Figure 5.8 Changes in dark respiration (a) and net assimilation (b) during rehydration of whole plants dried for 6 (blue shading) and 12 (red shading) months.

### 5.2.5 Ultrastructure

#### Whole plants desiccated for 6 months:

During the first twelve hours following rewatering, leaf water content increased towards 0.6 g/g DM, but the subcellular organisation of leaves remained typical of air-dry material (Figure 5.9). The central vacuole was divided into numerous small vacuoles and the plasmalemma was withdrawn from the cell wall. After 60 hours rehydration (water content 2.0 g/g DM) subcellular recovery had progressed such that the central vacuole had reformed and the plasmalemma was continuous with the cell walls (Figure 5.10i). The chloroplasts were aligned along the edges of the cell, however they remained rounded with a discontinuous thylakoid membrane arrangement. One hundred and twenty hours following rewatering the chloroplasts had recovered a more elongated shape (this correlating with photochemical recovery) and starch was present within the stroma (Figure 5.10ii)

#### Whole plants desiccated for 12 months:

Thirty-six hours following rewatering (water contents near 1.5 g/g DM) the central vacuole had started to reform, however the plasmalemma was not yet completely aligned with the cell wall. The chloroplasts were rounded and the thylakoids were not appressed inside the stroma (Figure 5.11). Upon subsequent water loss, (60 hours after rewatering, water contents decreasing towards 1.2 g/g DM), the plasmalemma had still not become aligned with the cell wall (Figure 5.12i), and the thylakoid membranes were no longer visible within the more densely stained chloroplasts (Figure 5.12ii). Small vesicles, perhaps arising from the damaged thylakoid membrane system, were visible in the stroma (arrowed in Figure 5.12ii). The lack of subcellular reorganisation correlates with the inability for recovery of the metabolic processes such as photosynthesis and respiration.

#### Whole plants desiccated for 18 months:

Mesophyll cells from the leaves of plants dried for 18 months had ruptured (Figure 5.13i). The chloroplasts were densely stained with distorted thylakoid membranes and vesicles present in the stroma (Figure 5.13ii). This evidence of extensive subcellular damage correlates with the lack of metabolic recovery and high levels of solute leakage.

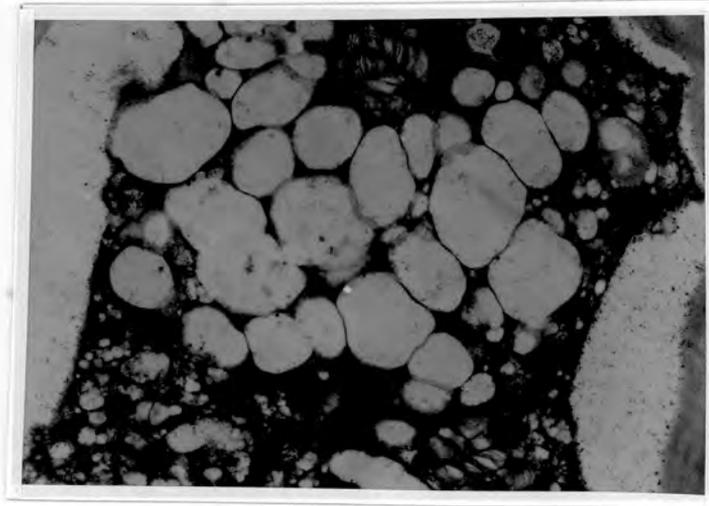


Figure 5.9 Initial (12 hour) recovery of subcellular organisation of leaves from whole plants following 6 months desiccation (water content 0.6 g/g DM). The central vacuole was subdivided into a number of smaller vacuoles and the plasmalemma was withdrawn from the cell wall ( $\times 6500$ ).

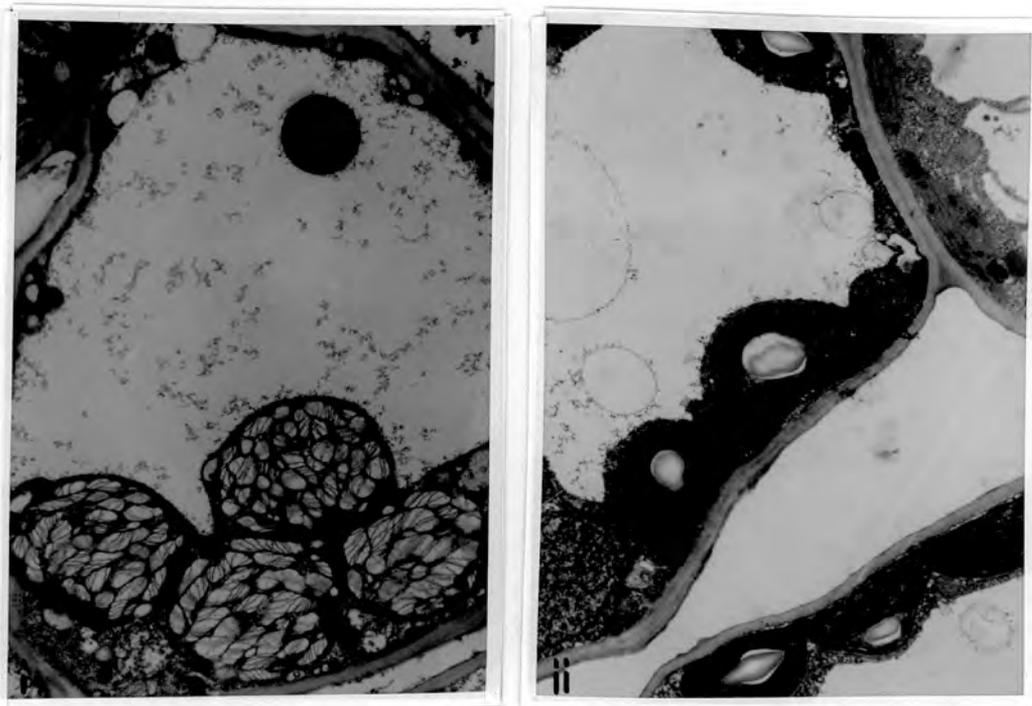


Figure 5.10 After 60 hours rehydration (water content 2.0 g/g DM), the central vacuole had re-formed, although the chloroplasts remained rounded with a non-appressed thylakoid arrangement ( $\times 6000$ ) (i). The chloroplasts recovered their elongated shape 120 hours following rewatering ( $\times 5500$ ) (ii).

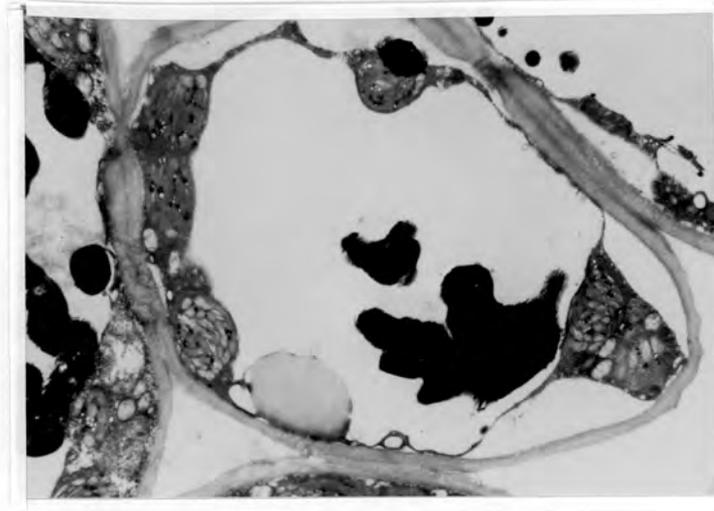


Figure 5.11 Subcellular organisation following 36 hours rehydration (water content 1.5 g/g DM) of leaves from whole plants desiccated for 12 months. The central vacuole had started to reform, however the plasmalemma was not aligned with the cell wall (x3 500).

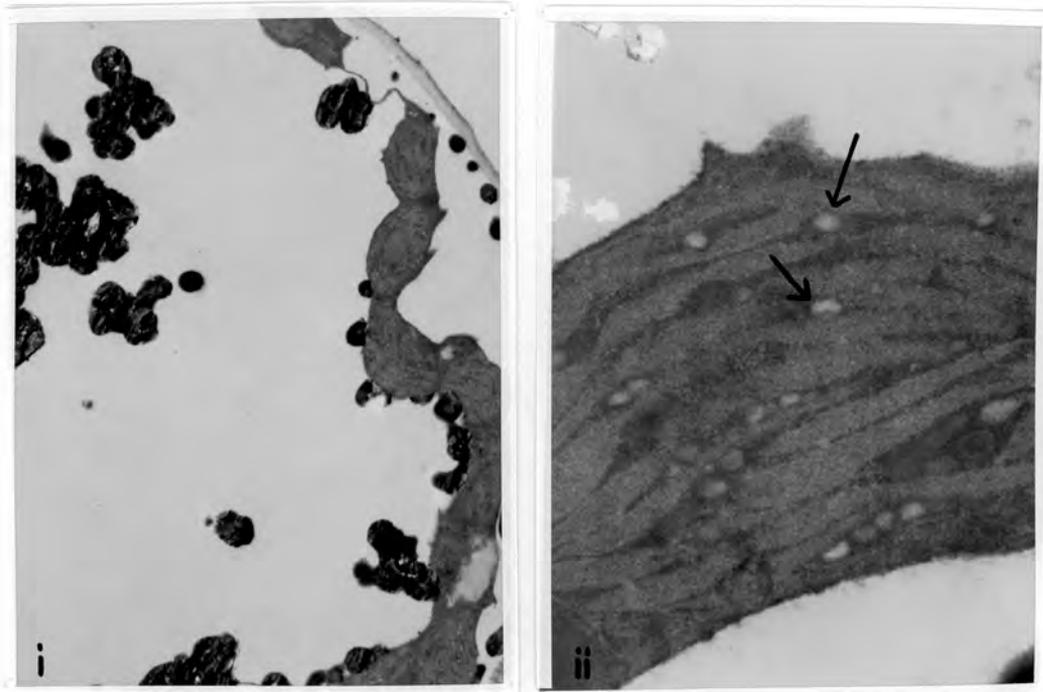


Figure 5.12 Subcellular organisation following 60 hours rehydration (water content decreasing towards 1.2 g/g DM), indicating little recovery (x3 750) (i). The remaining chloroplasts were densely stained, with the thylakoid membrane system less distinct, and small vesicles (arrowed) evident within the stroma (x45 000) (ii).

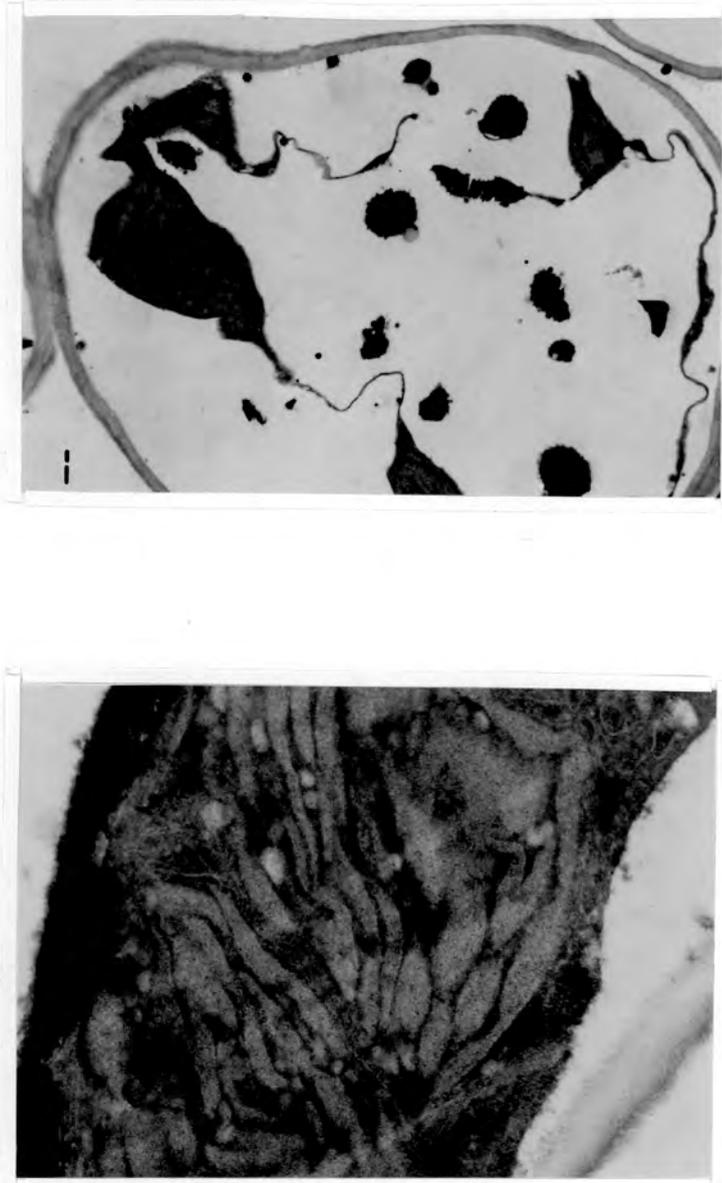


Figure 5.13 Rehydration (after 36 hours, water content  $<0.2$  g/g DM) of leaves from whole plants following 18 months desiccation. Complete plasmalemma degradation and subcellular disruption had occurred (i) ( $\times 4\ 400$ ). The chloroplasts were densely stained and small vesicles were evident in the stroma whilst the remaining thylakoid membrane structure was distorted ( $\times 20\ 000$ ) (ii).

## DISCUSSION

Experimentation in which desiccated leaves of *M. flabellifolius* were repeatedly freeze-thawed or immersed in liquid nitrogen resulted in consistently low levels of leakage (data not shown). From this it was deduced that the plasmalemma of this species was extremely resistant to damage. From the current study it became evident however, that maintenance in the dry state for more than six months resulted in increased solute leakage, cellular disruption and reduced photochemical recovery.

Plants dried for 6 months were able to recover full metabolic activity although this recovery process occurred more slowly relative to plants rehydrated within one month of desiccation. The more gradual recovery following 6 months desiccation may have been a consequence of delayed water transport to the leaves, implying some root and/or xylem damage that had to be repaired before successful water transport up the stem. The delay in photochemical recovery also suggests that gradual recovery was a consequence of some subcellular damage in the mesophyll tissue, with macromolecular and membrane replacement and/or repair processes within the mesophyll tissue also necessary before the recovery of metabolism.

Exposure to the dry state for 12 months resulted in even more gradual leaf rehydration, again implying root damage. Root repair was possible however, since the leaves from these treatments were able to rehydrate and axial buds developed into new shoots. Ultrastructural studies, and the inability for the maintenance of full turgor in the leaves of the plants dried for 12 months, suggests that more damage occurred in plants desiccated for this period of time (relative to plants dried for 6 months). This would imply that subcellular damage of the mesophyll tissue had exceeded repairable levels, and possibly led to the abscission of the leaves.

Plants exposed to 18 months desiccation did not survive. The leaves were unable to rehydrate, and extensive subcellular damage of the mesophyll tissue, including disruption of the plasmalemma, was evident. An inability for leaf rehydration might imply that plants desiccated for 18 months experience damage beyond repairable levels in not only leaf, but also root tissue, thereby preventing recovery.

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These results imply that membranes can resist damage in the hydrated and recently desiccated state, but in order to maintain structure, constant replacement of constituents (or repair) is necessary. As this cannot occur in the dry state, damage accumulates with time. This damage associated with long-term drying could include damage to stored mRNA components needed for initial recovery, and ultimately may preclude *de novo* transcription and translation, preventing repair and resulting in cell death.

Root tissue appears more tolerant of prolonged desiccation, being able to recover after 12 months desiccation. However even these organs were irreparably damaged after 18 months maintenance in the dry state. Increased damage in leaf, relative to root tissue after 12 months desiccation, might imply that the exposure of desiccated material to light increases the amount of damage induced in the dry state, perhaps through the induction of lipid peroxidation through free-radical formation. It is also possible however, that, whilst both leaf and root material was exposed to a range of environmental factors, including temperature, moisture, and microflora, leaf material was exposed to wider fluctuations within these factors, which may have promoted cellular deterioration.

From this study it can be concluded that desiccated *M. flabellifolius* plants, growing in their natural environment, experience unavoidable and continuous damage accumulation if the period in the dry state is prolonged. Although the cause of membrane and macromolecular destabilisation remains uncertain, part of the desiccation-tolerance mechanism is the ability to recover from such deterioration. Since this plant occurs in an environment which usually experiences at least a single rehydration-dehydration event within 6-8 months of drying, it has probably evolved repair and/or protective strategies enabling recovery from associated levels of damage. Less frequent periods of rehydration are uncommon in the field, thus, upon prolonged desiccation, repair capabilities may themselves be damaged, thereby limiting the desiccation-tolerant ability of *M. flabellifolius*.

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## **Chapter Six: Effect of desiccation on recovery of detached twigs dried on the whole plant and stored for one or twelve months**

### **INTRODUCTION**

The recovery of intact plants following their maintenance in the dry state for one (Chapter 3), or twelve (Chapter 5) months has already been discussed. In order to investigate the role of roots during rehydration this chapter will characterise the response of twigs, detached from dry plants, and stored for one or twelve months before their rehydration by placement of the cut stems in water. These desiccated twigs were stored under ambient laboratory conditions in a manner similar to that applied by Genkel and Pronina (1968). The effect of these conditions on recovery will also be taken into consideration.

To simplify discussion within this chapter, twigs Dried Intact (i.e. on the whole plant) and allowed to Rehydrate Detached (i.e. off the whole plant) after one month's maintenance in the dry state, will be described as the DIRD-1 treatments. Similarly, twigs Dried Intact and allowed to Rehydrate Detached after twelve months desiccation will be described as the DIRD-12 treatments.

### **RESULTS**

#### **6.1 REHYDRATION**

##### *6.1.1 Water content*

Leaf rehydration following storage in the dry state for one or 12 months is presented in Figure 6.1. Both treatments displayed initial recovery. However neither were able to sustain full turgor. The inability for all excised twig treatments to maintain full turgor indicates that roots were essential for sustained leaf recovery.

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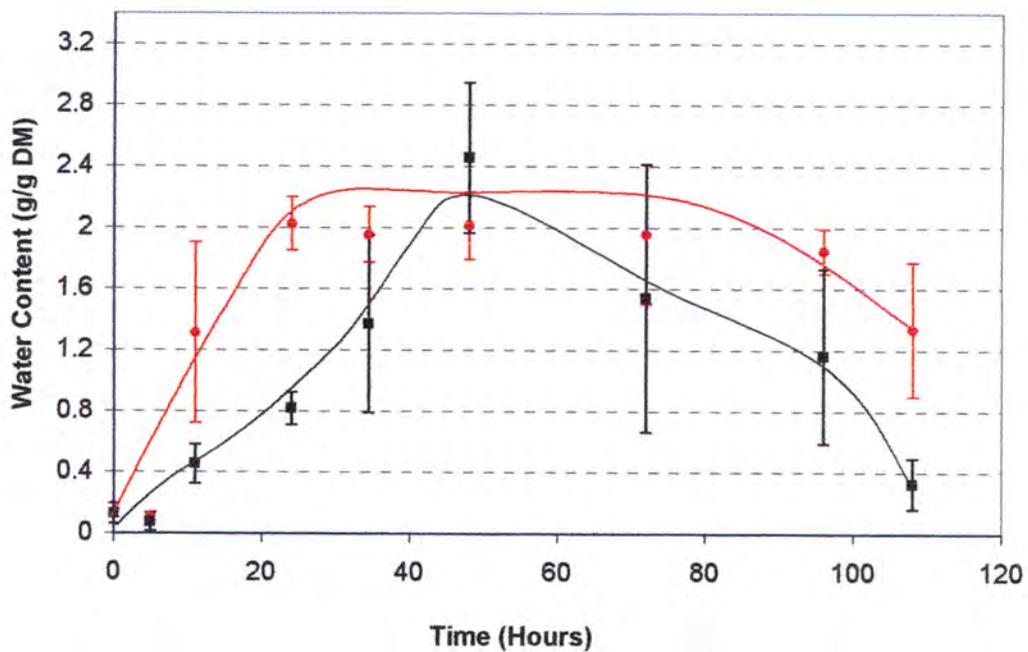


Figure 6.1 Rehydration of leaves from detached twigs following desiccation on the whole plant, and maintenance in the dry state for one (red symbols), or twelve (black symbols) months. (Data points represented as a scatterplot of means, bars indicating standard deviations, and trends investigated using distance weighted least squares regression analysis.)

Leaf water contents of DIRD-1 treatments, like those observed during the rehydration of twigs dried in the absence of roots (Chapter 4), decreased after 80 hours rehydration. DIRD-12 treatments in contrast, lost leaf water content almost immediately following recovery to full turgor, suggesting increased levels of damage relative to the DIRD-1 treatments. Increased levels of damage following 12 months desiccation supports the conclusions reached in Chapter 5, where it was found that damage accumulated with time.

### 6.1.2 Solute leakage

As found for all treatments in which plants were rehydrated within 6 months (dried and rehydrated with or without roots), solute leakage from DIRD-1 treatments were consistently low, (Figure 6.2, red symbols), indicating the likely maintenance of plasmalemma integrity.

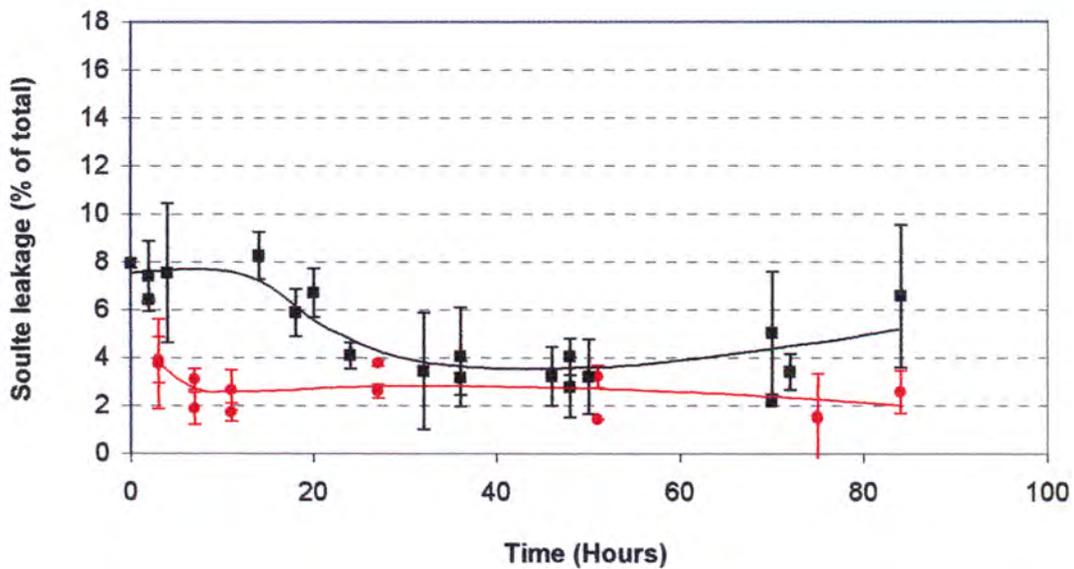


Figure 6.2 Solute leakage during rehydration of leaves from detached twigs following desiccation on the whole plant, and maintenance in the dry state for one (red symbols), or twelve (black symbols) months.

Leaves from DIRD-12 treatments displayed a pattern of solute leakage (Figure 6.3, black symbols) similar to that observed during recovery of whole plants dried for 12 months (Chapter 5, Figure 5.5). During the first 24 hours of rehydration solute leakage was slightly elevated. This decreased with recovery to full leaf turgor, before increasing with time when the leaves desiccated once more. These results might indicate that some damage has accrued within the 12 month period, however this could initially be repaired (by stored material in the cell or perhaps through short-term translation of stored mRNA). Damage to the genome after 12 months may be such that new transcription, necessary for the continued repair of membranes (and all other aspects of metabolism) cannot occur, ultimately causing increased solute leakage and resulting in cell death.

### 6.1.3 Chlorophyll and carotenoid content

Chlorophyll and carotenoid concentrations during rehydration of both the DIRD-1 and DIRD-12 treatments (Figure 6.3) were similar to those observed during the rehydration of twigs dried detached from the whole plant (Chapter 4). Pigment levels remained constant and decreased following leaf recovery to full turgor.

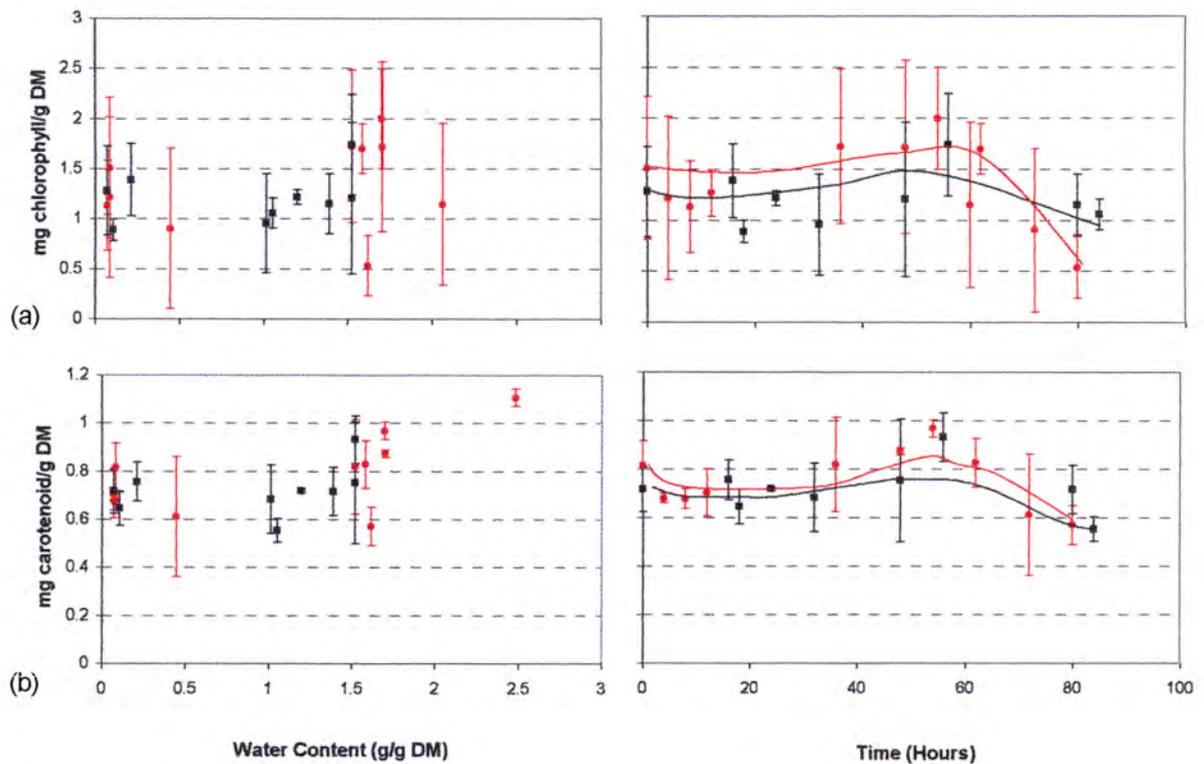


Figure 6.3 Chlorophyll (a) and carotenoid (b) concentrations during rehydration of leaves from detached twigs which had dried on the whole plant and maintained in the dry state for one (red symbols), or twelve (black symbols) months. Pigment concentrations plotted against water content (left column) and time (right column).

All treatments rehydrated in the absence of roots (irrespective of root presence/absence during drying, and the duration of twig exposure to the dry state before rehydration), were unable to recover hydrated control pigment levels. These results suggest that the presence of roots were essential for regaining and maintaining the full pigment complement.

#### 6.1.4 Chlorophyll fluorescence

Photochemical activity during leaf rehydration is given in Figure 6.4. As found for all treatments rehydrated within six months of drying (Chapter 3 and Chapter 4), photochemical activity of the DIRD-1 treatment increased linearly with time, reaching pre-stress levels 12 hours after recovery to full turgor. This indicates that the initial recovery of PSII does not require the presence of roots.

Recovery of DIRD-1 photochemical activity was temporary, being almost immediately followed by a decrease in  $F_v/F_m$ ,  $Y$ , and  $qN$ , in a manner similar to that described during the rehydration of twigs dried off the whole plant (Chapter 4). This inhibition of photochemistry of DIRD-1 treatments, preceded a drop-off in leaf water content, (again, as found during rehydration of twigs dried off the whole plant (Chapter 4)), indicating that water was not the limiting factor causing photochemical inhibition during twig rehydration. Since intact plants (rehydrated within six months of desiccation) were able to sustain photochemical activity, it might be suggested that the presence of roots were essential for continued recovery, perhaps through the induction of chlorophyll synthesis via a signal from the roots.

Photochemical activity of DIRD-12 treatments never recovered, indicating that damage to enzymes involved in photochemical processes had occurred, and that initial repair of membranes and enzymes necessary for electron transport could not be induced. This indicates that neither stored material within the cell, nor short-term translation of mRNA, could be utilised following 12 months desiccation. This inability for photochemical recovery correlates with a similar lack of recovery following rehydration of intact plants which had been desiccated for 12 months, confirming the conclusion that damage in leaf tissue accumulates with the length of time it is maintained in the dry state.

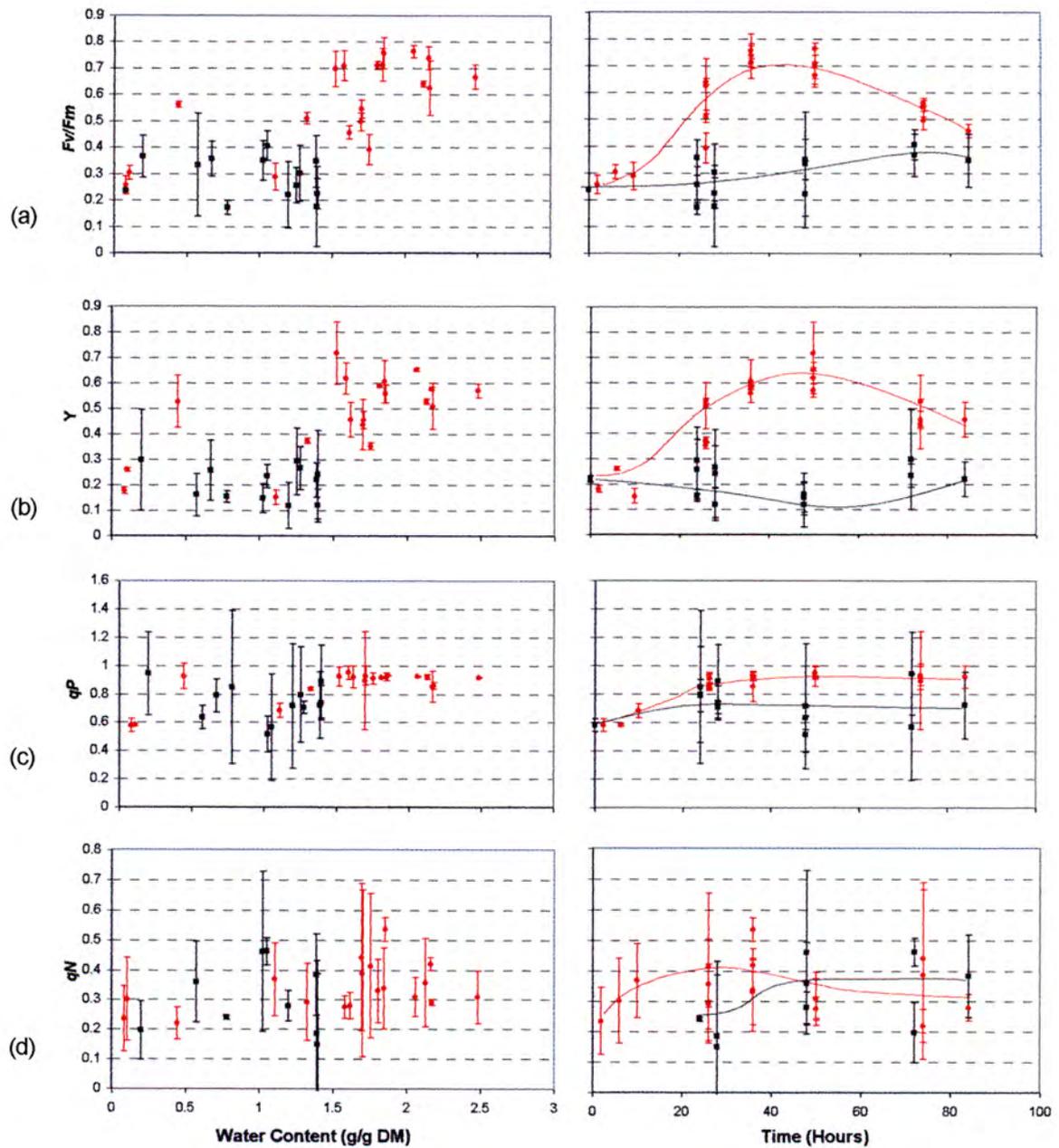


Figure 6.4 Photochemical activity during rehydration of leaves from detached twigs desiccated on the whole plant and maintained in the dry state for one (red symbols), or twelve (black symbols) months. ( $F_v/F_m$ , (a), Yield (b), photochemical ( $qP$ ) and non-photochemical ( $qN$ ) quenching coefficients c and d respectively)). Photochemical activity plotted against water content (left column) and time (right column).

### 6.1.5 CO<sub>2</sub> gas-exchange

As found for all treatments examined, regardless of the presence/absence of roots during drying, and irrespective of the time spent in the desiccated state (excluding material desiccated for 18 months), respiration from both DIRD-1 and DIRD-12 treatments increased with time. Respiration then reached a maximum following attainment of full turgor (although the DIRD-12 treatment retained full turgor for only a brief period, and variation within the data was large) (Figure 6.5). This suggests firstly, that the respiratory enzymes and mitochondria were less sensitive to damage associated with both water stress and prolonged desiccation, and secondly, that roots were unnecessary for the induction of respiration during recovery. Since repair processes are likely to be an essential part of recovery from desiccation, and respiration is essential for the conversion of photoassimilate into substances usable for plant maintenance and growth, it would not be surprising if respiratory mechanisms were constitutively protected.

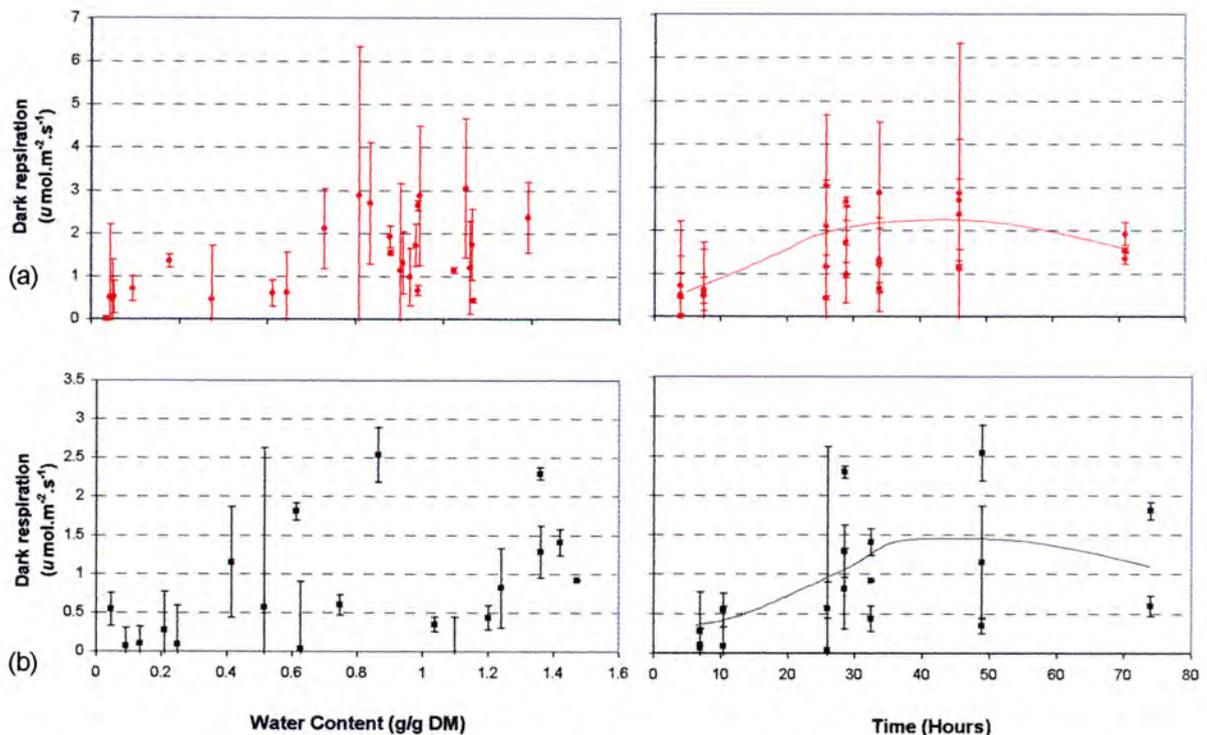


Figure 6.5 Dark respiration during rehydration of leaves from detached twigs, following their desiccation and maintenance on the whole plant for one (a), or twelve (b) months, plotted against water content (left column), and time (right column).

Positive levels of carbon assimilation were induced 36 hours following rewatering of the DIRD-1 treatments (Figure 6.6a). This coincided with the recovery of photochemical activity to pre-stress levels (12 hours following rehydration to full turgor). Photosynthesis did not recover to pre-stress levels however, decreasing almost immediately after the induction of positive net assimilation (despite sustained leaf water contents). This pattern was similar to that observed during the rehydration of twigs dried off the whole plant (Chapter 4). These results support the photochemical data, which suggested that some degree of protection exists, enabling the initial induction of photosynthetic activity, however roots were necessary (possibly for the induction of chlorophyll synthesis) in order for recovery to be sustained.

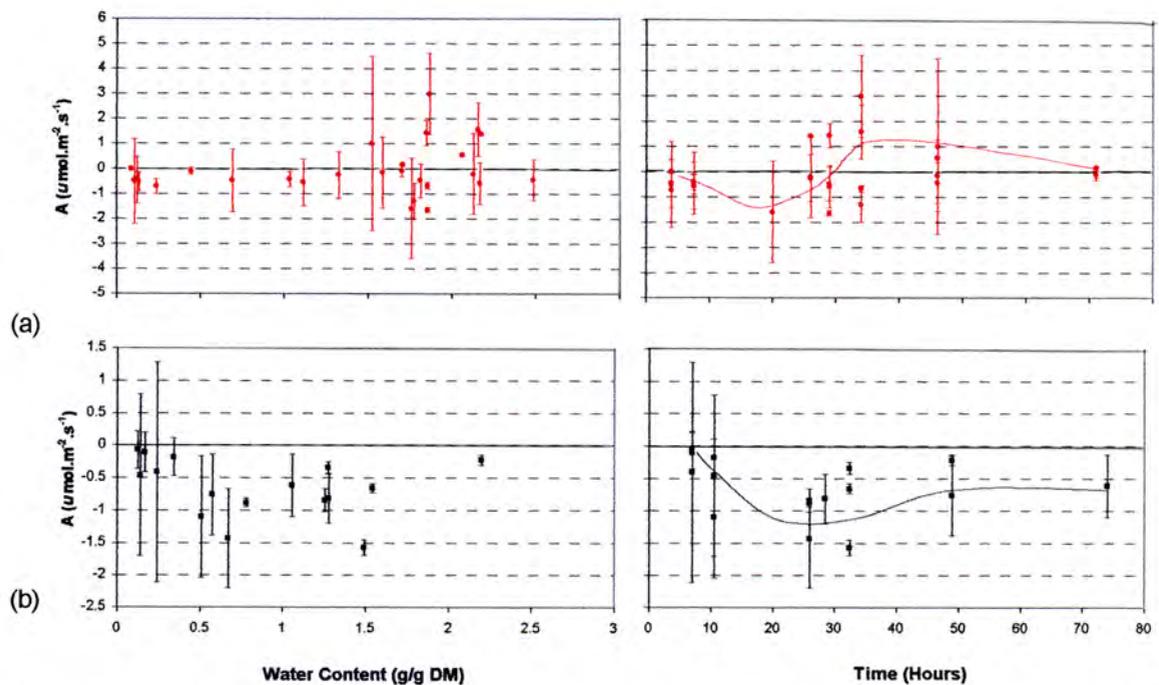


Figure 6.6 Net carbon assimilation (A) in leaves during rehydration of detached twigs which had dried on the whole plant and maintained in the dry state for one (a), or twelve (b) months, plotted against water content (left column), and time (right column).

As found during the rehydration of whole plants dried for 12 months, DIRD-12 treatments were unable to recover positive levels of carbon assimilation (Figure 6.6b). This inability for carbon assimilation correlates with the photochemical results, and again indicates that damage following 12 months desiccation exceeds repairable levels necessary for the induction of photosynthetic activity.

### 6.1.6 Plant Growth Regulators

Total ABA and cytokinin concentrations extracted from DIRD-1 and DIRD-12 treatments were examined at various stages of rehydration as illustrated in Figure 6.7. The increased level of ABA (at 1.5 g/g DM), visible during recovery of all treatments associated with rehydration within one month of desiccation, (regardless of the presence/absence of roots), was not evident during the rehydration of neither DIRD-1 nor DIRD-12 treatments. Although ABA levels from these treatments were slightly higher than hydrated control levels, they did not increase towards the 600 ng/g DM levels recorded during all previous treatments rehydrated after one month's desiccation (Chapters 3 and 4). It is possible that ABA levels did increase during rehydration of these treatments, however this increase, which has already been shown to be transient (Chapter 3), may have been overlooked due to limited sampling.

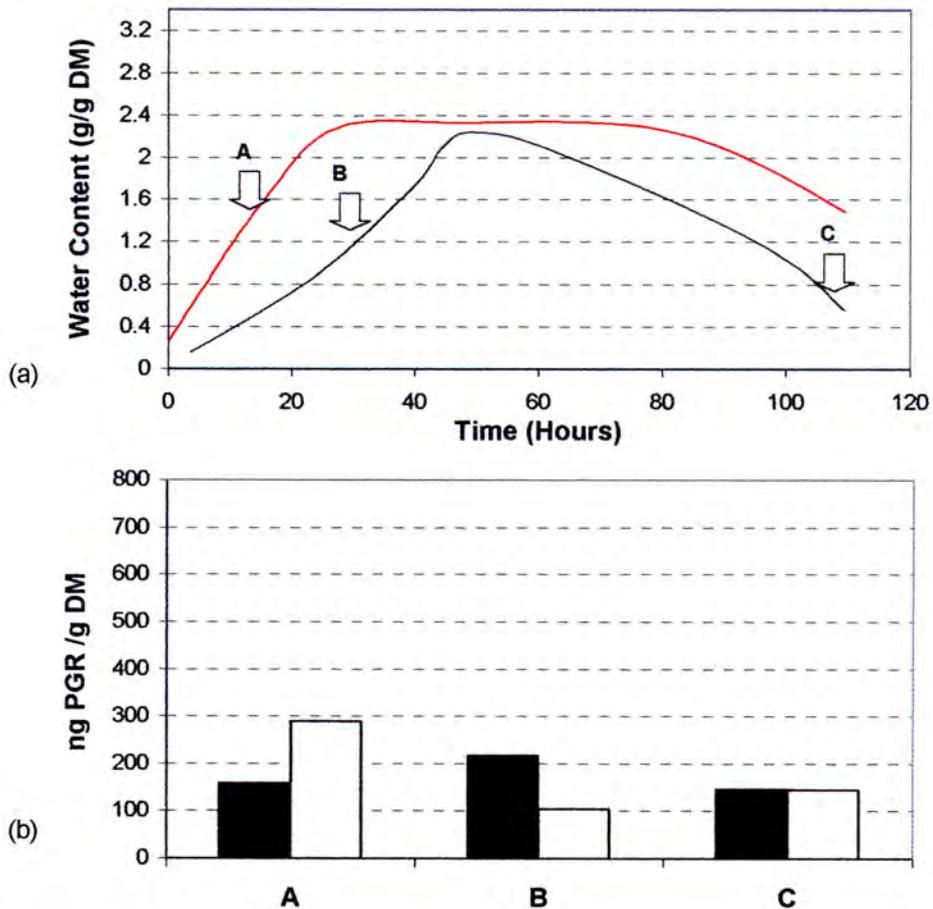


Figure 6.7 Water content and time during rehydration of twigs dried on the whole plant, and rehydrated after one (red line) and twelve (black line) months desiccation, arrows indicating points during which leaves were sampled for the presence of the plant growth regulators abscisic acid (ABA), and cytokinins (CK) (a); and total ABA (solid bars) and CK (empty bars) concentrations extracted at these water contents (b).

Cytokinins extracted from leaves of DIRD-1 treatments at water contents around 1.5 g/g DM were higher than hydrated control levels, reaching values similar to those obtained during whole-plant rehydration after one month's desiccation (Chapter 3). This increase in the leaves of detached twigs was inexplicable, since cytokinins are thought to be synthesised in the root tips (although some controversy exists concerning alternate sites of CK synthesis in plants (Mansfield, 1990)). In contrast with the DIRD-1 treatment, the DIRD-12 treatment displayed continuously low levels of CKs, as was obtained during the rehydration of twigs dried in the absence of roots (Chapter 4). These low levels of cytokinins could then account for the lack of photosynthetic recovery (since cytokinins promote chlorophyll synthesis) associated with rehydration in the absence of roots.

### 6.1.7 Ultrastructure

#### Desiccation for 1 month (DIRD-1):

Upon rehydration to full turgor, subcellular organisation was similar to that observed for all treatments (regardless of the presence/absence of roots) rehydrated within one month of desiccation (Chapters 3 and 4). Plasmalemma integrity was maintained (correlating with the solute leakage results reported in section 6.1.2), and the central vacuole was restored (Figure 6.8i). Mitochondria had become more electron dense, and the chloroplasts were re-aligned along the edge of the cells. The ultrastructural organisation of the chloroplasts was typical of the hydrated control condition, although the granal stacks were smaller, and more thinly spread across the stroma (Figure 6.8ii). The initial recovery of photochemical activity and carbon assimilation confirms the lack of immediate structural damage precluding recovery during rehydration (Figure 6.8ii).

The cells from the DIRD-1 treatment then lost water in a manner similar to the leaves of twigs dried off the whole plant (Chapter 4). The cytoplasm became more dense, and the plasmalemma, which drew away from the cell wall, appeared broken in places (Figure 6.9). The organisation of the chloroplasts was indicative of damage, thereby correlating with the inhibition of photosynthetic activity observed in the physiological data.

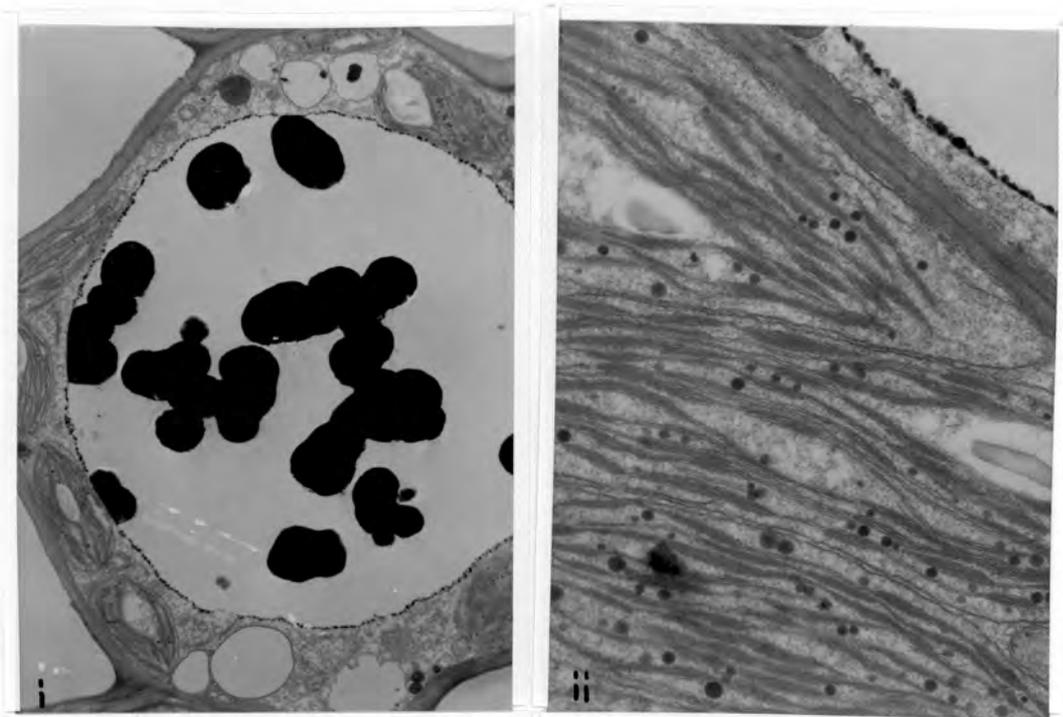


Figure 6.8 Recovery of subcellular organisation in leaves 36 hours after rehydration of twigs dried on the whole plant and maintained in the dry state for 1 month (water content 1.8 g/g DM,  $\times 4\ 200$ ) (i). Mitochondria more electron dense and chloroplasts aligned along the edge of the cells. The thylakoids recovered their 'staircase' arrangement, and starch was present within the stroma ( $\times 15\ 500$ ) (ii).

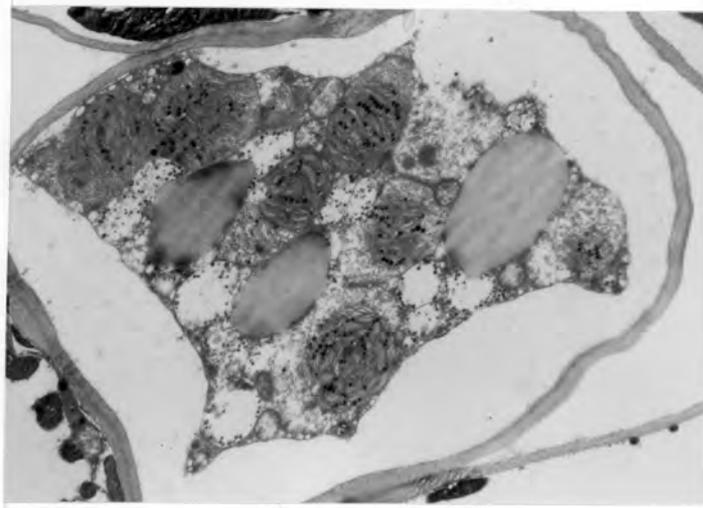


Figure 6.9 Subcellular organisation upon subsequent leaf dehydration (84 hours after the rehydration of detached twigs dried on the whole plant and maintained in the dry state for one month,  $\times 3\ 000$ ).

12 months desiccation (DIRD-12):

As found during whole plant recovery following desiccation for 12 months (Chapter 5), the mesophyll cells from DIRD-12 treatments displayed extensive subcellular damage (Figure 6.10). There was considerable disruption of the membranes (Figure 6.10i), and the chloroplasts remained rounded with the thylakoid membranes separated throughout the stroma (Figure 6.10ii). These results provided visual evidence for the high levels of solute leakage, and limited recovery of photosynthetic activity, associated with initial rehydration of DIRD-12 treatments.

A unique feature evident during rehydration of this (DIRD-12) treatment was the apparent concentration of plastoglobuli within the stroma of the chloroplasts, (rather than their less obvious, random distribution across the stroma of all other treatments) (arrowed in Figure 6.10ii). The role of the plastoglobuli is unclear, although they may be the result of lipid breakdown from accumulated damage associated with the length of time the material had been maintained in the dry state.

There was little further change in subcellular organisation following subsequent leaf water loss (after 96 hours rehydration) (Figure 6.11i). The plasmalemma and vacuolar membranes were disrupted, although the chloroplastic and thylakoid membranes appeared to have remained intact (Figure 6.11ii). This subcellular disruption correlates with the ultrastructural studies of intact plants rehydrated within 12 months of desiccation, and suggests that damage occurs with time maintained in the dry state and is exacerbated on rehydration and the subsequent dehydration that occurs.

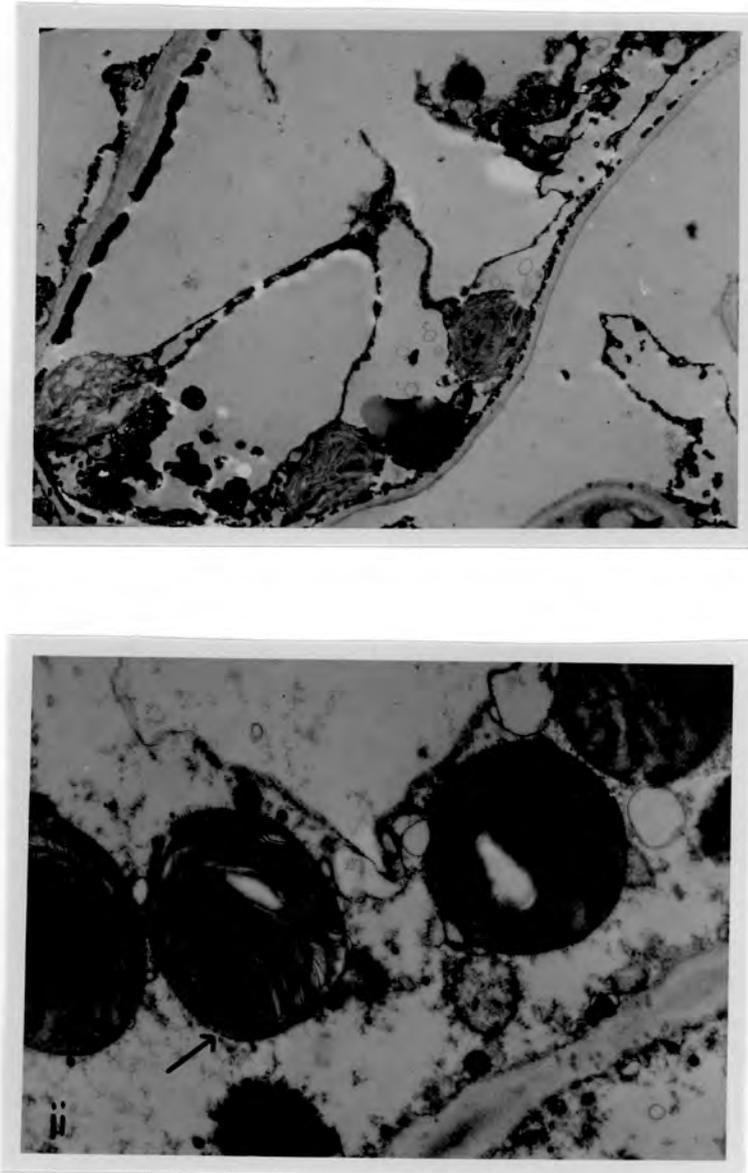


Figure 6.10 Subcellular organisation of leaf tissue during initial rehydration (water content 1.5 g/g DM) 36 hours following rewatering of detached twigs dried on the whole plant and maintained in the dry state for 12 months. A large number of cells had burst ( $\times 4\,500$ ) (i), while others were unable to recover their central vacuole, with the chloroplasts remaining rounded, the thylakoid membranes separated across the stroma, and starch bodies present. A unique feature during the rehydration of this treatment was the apparent clumping of plastoglobuli (arrowed) ( $\times 10\,000$ ) (ii).

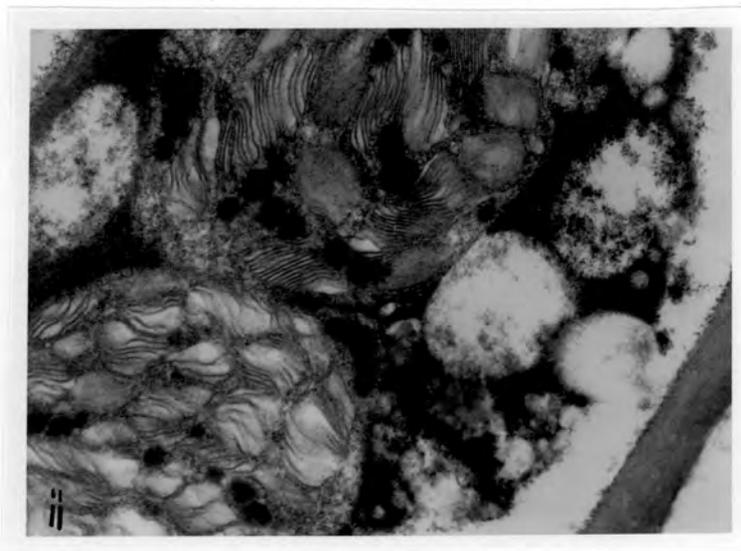
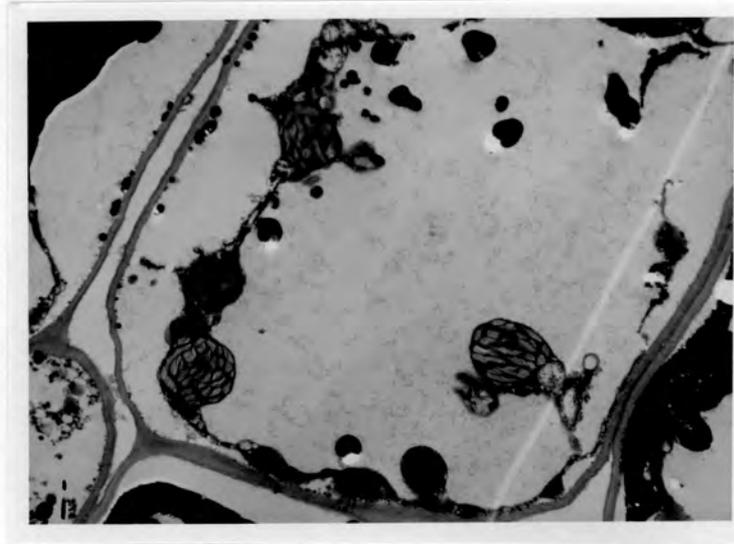


Figure 6.11 Complete cellular disruption 96 hours following the rehydration of detached twigs dried on the whole plant and maintained in the dry state for 12 months (water content 0.5 g/g DM). Plasmalemma disintegration occurred, such that only the larger organelles remained inside the cells (x3 800) (i). Despite cellular disruption, the chloroplastic and thylakoid membranes appeared to have remained intact (x30 000) (ii).

## DISCUSSION

Prolonged desiccation reduced the ability for leaf recovery, as was evidence by increased levels of damage (elevated solute leakage, low levels of photochemical activity and widespread subcellular disruption) from the DIRD-12, relative to the DIRD-1, treatments. These levels of damage evident during DIRD-12 recovery were similar to those reported upon rehydration of intact plants following 12 months desiccation. This implies that neither the presence of roots, nor more ambient conditions (of the laboratory) under which the twigs were stored, reduced the effects of prolonged desiccation. Rather, it would appear that general deterioration occurred over time in both intact plants and detached twigs. Roots were necessary for survival however, since intact plants were able to recover from desiccation through axial bud formation, whilst the DIRD-12 treatments underwent subsequent water loss and cell death. This might imply that signal transduction from the roots promotes bud break, and this signal may involve cytokinins, since low levels in excised twigs correlates with their inability for new shoot development.

Fluctuating laboratory conditions did not appear to effect the recovery of DIRD-1 treatments, since these treatments recovered in a manner similar to that observed during recovery of the large twigs dried off the plants (Chapter 4) under greenhouse conditions (i.e. low solute leakage and recovery of photochemical activity). DIRD-1 leaves were able to attain slightly higher levels of carbon assimilation however, and it cannot be determined whether this was attributable to slightly cooler laboratory conditions or the slower rate of drying associated with leaf desiccation on intact plants.

Initial recovery of excised twig treatments rehydrated with or without roots within one month of drying, implies that roots were not essential for early recovery. This suggests that protection of organelles and repair mechanisms induced during drying is constitutively expressed and maintained in the dry state, so enabling initial metabolic recovery. The inability for the induction of initial recovery following 12 months desiccation of both the detached twigs and intact plants implies that damage accumulates with time, with 12 months desiccation causing damage accumulation to the point that mechanisms involved with initial recovery are no longer operational and/or damaged themselves. It is possible that after 12 months desiccation the stored constituents and/or mRNAs usually induced with initial rehydration, as well as the genome itself, might have been damaged, preventing transcription necessary for sustained recovery.

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Finally, no detached twig treatments were able to maintain metabolic activity, indicating that roots were essential for sustained recovery. It is possible that roots are necessary for the induction of signals associated with the transcription of new compounds necessary for continued growth and repair of the leaves. The lack of elevated cytokinin concentrations might suggest that this plant growth regulator, amongst others, may be necessary for the induction of chlorophyll synthesis and bud break (both processes being inhibited during excised twig rehydration).

These results indicate that previous studies based on the rehydration of excised twigs (such as that of Genkel and Pronina, 1968), yield valid insights into only the initial stages of leaf recovery, provided the plant material had not been stored in the dry state for prolonged periods of time (i.e. less than 12 months).

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## Chapter Seven: General Discussion and Conclusion

*M. flabellifolius* has an unusual habit amongst desiccation-tolerant plants, being woody and growing up to 1.5 meters in height (Child, 1960). The leaf response of this species during drying and recovery can now be added to our understanding of the leaf response derived from similar studies of smaller, low-growing homoiochlorophyllous desiccation-tolerant (HDT) plants (Schwab *et al.*, 1989).

The respiratory and photosynthetic response of *M. flabellifolius* during drying and recovery was consistent with that reported by Schwab *et al.*, (1989) for other HDT plants, although *M. flabellifolius* did not display "rewetting" respiration (elevated respiratory activity exceeding control levels during the early stages of rehydration) as observed by Schwab *et al.*, 1989. Tuba *et al.*, (1994) studying detached leaves of the poikilochlorophyllous desiccation tolerant (PDT) *Xerophyta scabrada* also reported sharp increases in CO<sub>2</sub> during the first 2 hours following rehydration. Sherwin (1995) however, studying leaves of excised plants of the HDT species *Craterostigma nanum* during drying and rehydration also found no evidence for elevated respiratory rates. It might be suggested then, that "rewetting" respiratory activity is not necessarily characteristic of desiccation-tolerant plants.

Whereas mitochondria appeared to be well protected during drying and rehydration, the thylakoid membranes undergo alterations which become evident as a delay of recovery during rewatering (although this delay is short-lived relative to the recovery rate of PDT species (see Tuba *et al.*, 1993, 1994a,b)). The results from this study correspond with those from other HDT species where it was found that Calvin-cycle activity was reduced before membrane-bound electron transport decreased (Schwab *et al.*, 1989). This might indicate that Calvin-cycle enzymes in the aqueous phase of the chloroplast stroma were more affected by dehydration than membrane-bound electron-transport constituents. However, it has been shown that the loss of activity of stroma enzymes *in vitro* at high osmotic potentials is fully and immediately reversible when the osmotic potential returns to normal (Kaiser *et al.*, 1981b). Therefore, it appears that the slow recovery of light-dependant CO<sub>2</sub> uptake is a consequence of a delay in the recovery of thylakoid properties. The complex process of carbon assimilation cannot proceed until functional thylakoid activity has been restored.

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Discontinuity between adjacent thylakoid membranes apparent at the ultrastructural level during drying and rehydration may be part of a strategy preventing light-chlorophyll interactions before the induction of complete protective or recovery mechanisms. However, it is also possible that these ultrastructural observations were artifacts of chemical fixation, since the half-strength gluteraldehyde (2.5%) used throughout this study was found by Platt *et al.*, (1997) to induce differences in subcellular organisation relative to freeze-substitution methodologies. Platt *et al.*, (1997) concluded that the rounded chloroplasts and loosely arranged photosynthetic membrane system, coincident with the "swelling and disruption of compartments" often reported with rehydration of desiccated material was caused by the hypotonic nature of the fixative. Artifacts from chemical fixation may well result in spurious conclusions regarding the functional significance of subcellular organisation, and this requires greater consideration in future ultrastructural studies of not only *M. flabellifolius*, but desiccation-tolerant material in general.

Although damage arising as a consequence of water deficit has received the most attention in this study, water deficits in nature are usually accompanied by high irradiance. Water stress, coupled with high-light stress, can present a serious environmental stress. There may be several periods of heightened photosystem II sensitivity to high-light exposure during desiccation and rehydration. Light absorption at critical stages during desiccation and hydration may be reduced by the physical action of leaf folding and stem curling characteristic of many desiccation-tolerant plants (Eickmeier *et al.*, 1992) and also occurring in *M. flabellifolius*. Lebkuecher and Eickmeier (1993) showed that when the fronds of the PDT pteridophyte *Selaginella lepidophylla* were allowed to curl normally under high light intensities, significantly less photosystem II damage and chlorophyll photooxidation was induced relative to plants artificially restrained. Similarly, restraint had no effect on levels of damage during desiccation under low light intensities. Although no leaf-restraining studies have been undertaken for *M. flabellifolius*, the correlation between the shut-down of carbon assimilation and leaf reorientation during drying of this species may be part of a protection mechanism associated with reducing light-stress and promoting desiccation tolerance during dehydration.

Studies of the leaf response during whole plant drying and recovery have shown that *M. flabellifolius* has the ability to reversibly switch off metabolism. The shut-down of metabolic activity may be induced through elevated levels of abscisic acid (ABA) during drying, although ABA may also play a role in the induction of genes encoding proteins associated with tolerance to water stress (Ingram and Bartels, 1996). Farrant and Sherwin (1998)

propose that controlled metabolic quiescence is not merely a consequence of drying, but rather a strategy to prevent damage arising from unregulated metabolism due to water stress. For *M. flabellifolius* it is clear that whilst the mechanisms of cellular tolerance to extreme water loss are not yet understood, they are highly effective.

The mechanisms of desiccation tolerance were affected by not only drying and rehydration in the absence of roots, but also by recovery following prolonged desiccation. These limitations to desiccation tolerance will be summarised in turn below.

### **1. The effect of drying and rehydration (within one month of desiccation) in the presence and/or absence of roots**

The absence of roots during drying affected detached twig recovery. Rapid drying, (achieved only when small twigs were dried (Chapter 4)), prevented the induction of physical protection mechanisms including leaf folding and subcellular re-organisation. If chemical protection mechanisms were induced or constitutively present, these mechanisms were insufficient for the prevention of extensive subcellular damage evident as elevated solute leakage, increased loss of photosynthetic pigments, and complete subcellular breakdown. Ultimately damage during rapid dehydration prevented small twig leaf rehydration.

Large twigs detached from hydrated plants and allowed to desiccate (Chapter 4) dried faster than twigs dried on the plant (Chapter 5, DIRD-1 treatments). This increased drying rate did not appear to result in more damage during rehydration, although a lower level of carbon assimilation during recovery of the leaves from twigs dried off the plant was apparent, and perhaps a result of damage following an increased rate of water loss. Furthermore, these twigs (dried on/off intact plants) displayed similar patterns of initial (but unsustainable) photochemical recovery and a lack of chlorophyll synthesis. These results indicate that roots were not essential for initial recovery of metabolism.

Initial re-activation of photochemical activity and carbon assimilation during recovery of excised twigs indicates that subcellular organisation and the components of metabolism were protected during drying. These protection mechanisms may have been induced through ABA accumulation in the leaves desiccated on and off intact plants. Any damage, which may have occurred during drying and subsequent rehydration, must have been repairable, perhaps involving the use of stored material in the cell, or short-term translation

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of stored mRNA, such that initial recovery could take place. The presence and/or absence of roots during drying and recovery were unnecessary for the maintenance of plasmalemma integrity and respiratory activity. Plasmalemma integrity was maintained throughout initial recovery and later water loss, whilst respiratory activity was measurable at low water contents associated with subsequent water loss. Continual maintenance of membrane integrity and energy supply may be essential for the induction of both protection and repair mechanisms. It is likely therefore, that these processes are constitutively protected in the leaves.

Roots were essential for sustained and complete leaf recovery, since no excised twig treatments were able to survive. It is possible that new transcription is required (for the maintenance of membranes and all other aspects of metabolism), and this transcription is induced via chemical signals from the roots. These signals include, possibly amongst others, cytokinins necessary for the induction of chlorophyll synthesis and new bud formation.

## **2. The effect of prolonged desiccation on recovery in the presence or absence of roots**

Long-term maintenance of leaf material in the dry state results in damage (evidenced as increased solute leakage, cellular disruption, and reduced photochemical recovery), regardless of the presence or absence of roots on rehydration. Ingram and Bartels (1996) suggested that this damage might have arisen from the modification of protein residues by chemical processes such as deamination, isomerization, or oxidation, which can occur under desiccated conditions.

Complete, but slow, metabolic recovery (evidenced as delayed water uptake, and gradual photochemical recovery) of intact plants following 6 months desiccation, suggests that membranes and enzymes require replenishment or replacement of components after approximately six months. Prolonged desiccation beyond six months resulted in the accumulation of damage over time.

Following 12 months desiccation, whole plant recovery was possible through the survival of the meristematic tissue, as new axial buds were produced. The absence of roots during recovery following 12 months desiccation (Chapter 5, DIRD-12 treatments) resulted in levels of damage similar to that reported from intact plants dried for this period, although the

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twigs were unable to induce bud growth and thus died. This supports the earlier suggestion that signals from roots (including cytokinins necessary for the induction of bud break) are essential for desiccation tolerance.

Whole plants maintained in the dry state for 18 months were unable to recover, with even the meristematic tissue unable to survive.

## Conclusion

A complete understanding concerning the mechanisms of desiccation tolerance for *M. flabellifolius* remains elusive. This thesis has shown that mechanisms of desiccation tolerance in this species include both physical and chemical strategies that may be constitutively expressed and/or induced with changes in water content.

The initial recovery of metabolism following leaf rehydration was indicative of subcellular protection during drying. This protection enables the immediate initiation of metabolism following the release of water stress, and may be an essential strategy for survival (since reproduction and growth can only occur during this unpredictable and transitory period of water availability).

Survival of leaf material in the dry state is limited by firstly prolonged desiccation, and secondly rapid rates of water loss during desiccation. Since these conditions are unlikely to exist in the field, desiccation tolerance of leaf material of *M. flabellifolius* would not be limited in its natural environment. Identifying these limitations indicates nevertheless, that desiccation tolerance involves the induction of protection and repair mechanisms on drying and rehydration, and that these mechanisms have a limited period in which they remain effective.

This study has shown that research concerning the mechanisms of desiccation tolerance in *M. flabellifolius* should be undertaken on intact plants, as roots are essential for complete metabolic recovery. Furthermore, future studies of desiccation tolerance for this species in particular, as well as for desiccation-tolerant plants in general, should consider the frequency and duration of dehydration-rehydration cycles, since prolonged dryness reduces desiccation tolerance.

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