Protection mechanisms against excess light in *Myrothamnus flabellifolius* (Welw.)

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Abstract

The effects of light stress during drying on excised twigs of the homoiochlorophyllous resurrection plant *Myrothamnus flabellifolius*, was examined in this study. Leaves were prevented from folding during drying and plants were dried in the light, and as a control, in the dark. The effects on the photoprotection mechanisms was assessed. Differences in water content, quantum efficiency of photosystem II, chlorophyll (a+b) content, carotenoid content, anthocyanin content were measured during dehydration and rehydration. Ultrastructural studies and examination of electrolyte leakage provided an indication of the subcellular changes in leaf tissues. There was no significant difference in the protection mechanisms of restrained and unrestrained leaves during dehydration and rehydration. Thus, it seems that leaf folding is not a critical factor in terms of achieving desiccation tolerance in *M. flabellifolius*, because both treatments were able to survive drying. Leaves that were dried in the dark showed extensive subcellular damage during dehydration and quantum efficiency of photosystem II did not recover fully on rehydration. It is suggested that some of the protection mechanisms against desiccation-induced damage can not be activated or sufficiently upregulated in the absence of light.

Table of Contents

Introduction

Water is crucial to the survival and productivity of a plant. Normally, metabolism operates in a very restricted water potential range (>1.5 MPa (Gaff, 1989), or above 90% relative water content (Kaiser,. 1987). Vegetative tissues of most angiosperms cannot survive desiccation but there are some which do. Such plants, termed resurrection plants (Gaff, 1971), are studied in an attempt to understand the mechanisms of desiccation tolerance, with the ultimate goal of being able to select for traits and/or genes for the production of drought-tolerant crops, either by traditional breeding or genetic engineering (Driouich *et al.*, 2000; Farrant, 2000).

Although few in number among higher plants, desiccation-tolerant plants are widespread, and represent most major classes of plants, including algae, bryophytes, lichens, ferns, fern allies, and angiosperms (Bewley and Krochko, 1982). Most angiosperm resurrection plants grow in shallow soils on rocky outcrops where there is little shade and water supply is frequently limited (Sherwin and Farrant, 1998). Thus these plants would be subjected to light-related stress as they dehydrate, and as they are able to survive these conditions, must have particularly good mechanisms for coping with this excess absorbed light (Sherwin and Farrant, 1998).

While the mechanisms enabling these plants to survive desiccation are still not fully understood, it is generally agreed that most utilize a strategy of physiological and biochemical protection, induced during drying, against damage (Gaff, 1989; Bewley and Oliver, 1992; Ingram and Bartels, 1996).

Bewley (1979) suggested 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) mobilize 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: first, cellular protection from desiccation- and rehydration- induced damage; and/or secondly cellular repair of the damage that does occur (Bewley and Oliver, 1992).

Mechanical stress

One of the earliest stresses brought about by dehydration is mechanical stress (Iljin, 1957). Inward contraction of the cytoplasm during water loss from the vacuole creates tension between the plasmalemma and the cell wall. This can induce cell wall collapse and rupturing of the plasmalemma (Iljin, 1957; Gaff, 1989; Vertucci and Farrant, 1995; Farrant, 2000).

Resurrection plants display various mechanism to minimize mechanical stress associated with drying, and recover during rehydration (Sherwin and Farrant, 1996; Farrant, 2000). 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*) and *Myrothamnus flabellifolius* (Farrant, 2000). These mechanisms could then prevent plasmalemma withdrawal and cell wall collapse on drying, as well as disruption of the plasmalemma upon rehydration (Farrant, 2000).

Oxidative stress

The presence of light during dehydration can be extremely damaging to photosynthetically active tissue (Demmig-Adams and Adams, 1996). Light stress results not from high light per sė, but rather from an excess of absorbed light beyond that utilized in photosynthesis (Demmig-Adams and Adams, 1992). Light energy captured by chlorophyll cannot be dissipated via photosynthesis under water limiting conditions (Larson, 1988), and instead is transferred to oxygen, resulting in the formation of oxygen free radicals (Larson, 1988; Smirnoff, 1993). These are highly reactive molecules and, if unquenched can cause considerable damage to proteins, enzymes, lipids and nucleic acids (Larson, 1988; Smirnoff, 1993).

Resurrection plants cope with excess light in numerous ways. Some, such as the *Xerophyta* species, limit chlorophyll-light interactions by losing their chlorophyll and dismantling the thylakoid membranes (Sherwin and Farrant, 1998). These are termed poikilochlorophyllous resurrection plants. Homoiochlorophyllous resurrection plants retain most of their chlorophyll and rather rely on other mechanisms to shade their chloroplasts (Sherwin and Farrant, 1998). The *Craterostigma* and *Myrothamnus* species, for example, use leaf movements to shade some of their leaves from light. In *C. wilmsii* the leaves curl inward during drying such that only the bottom (abaxial) surfaces of the outermost whorl of leaves are exposed to light (Sherwin and Farrant, 1998; Driouich *et al.*, 2000). In *M. flabellifolius*, each leaf folds laterally and against the stem, and the lateral branches curve upwards and inwards during drying, such that again only the abaxial surfaces of the leaves are exposed to light in the dry plant (Gaff, 1977; Kruger, 1998; Farrant, 2000).

Chemical protection against light involves several mechanisms in desiccation tolerant plants. Ultraviolet (UV)-radiation can react destructively with amino acids and membrane lipids due to its high energy status (Demmig-Adams and Adams, 1992; Hendry, 1993; Larson, 1988). Flavonoids, such as anthocyanins, are absorbent of UV-light, and act as light filters. In both, *M. flabellifolius* and *C. wilmsii* the cells near the leaf surface that remain exposed to light accumulate anthocyanin pigments, which act as "sunscreen pigments" and mask the chlorophyll (Larson, 1988; Hendry, 1993; Koonjul *et al.*, 1999; Farrant, 2000). They also act as antioxidants to stop further free radical production (Larson, 1988; Smirnoff, 1993). Other antioxidants, such as superoxide dismutase (SOD), ascorbate peroxidase (AP) and glutathione reductase (GR) also accumulate in leaves of these plants during drying (Sherwin and Farrant, 1998; Farrant, 2000).

Maintenance of physiological integrity

Membranes and macromolecules are held in physiologically active configuration due to the fact that they are in aqueous solution. When water levels are reduced below 20% (on a dry mass basis), the structure of nucleic acids, proteins and polar lipids are altered because the hydrophilic and hydrophobic interactions that stabilize their conformations are weakened (Vertucci and Farrant, 1995). Such denaturation is irreversible and lethal.

It is generally accepted that the integrity of membranes and stabilization of the subcellular milieu in desiccation-tolerant organisms is achieved, in part, by the accumulation of "compatible solutes" (Vertucci and Farrant, 1995; Ingram & Bartels, 1996). In all the resurrection plants examined to date there are large increases in sucrose, and variable increases in other compatible solutes such as sugar alcohols, low-complexity carbohydrates (e.g. fructans) and amino acids (Crowe *et al.*, 1992; Vertucci and Farrant, 1995; Ingram & Bartels, 1996). These are thought to allow osmoregulation at intermediate water contents and, importantly, in dry plants to 1) replace water on membranes and macromolecules and so maintain their integrity and/or 2) vitrify the cytoplasm and so put a stasis on injurious metabolism (such as free radical formation). Sherwin and Farrant (1996; 1998) proposed that they also serve to fill vacuoles and prevent mechanical stress.

This study focuses on the homoiochlorophyllous resurrection plant *Myrothamnus flabellifolius* (Welw.). This species is a woody shrub which grows on shallow, well-drained rocky outcrops (Child, 1960), across a broad region of southern Africa (Goldsworthy, 1992). These plants withstand considerable local extremes in temperature and irradiance, as well as experiencing an erratic water supply (Child, 1960). During drying the leaves fold up against the stem so that only the abaxial surfaces remain exposed to light and it has been proposed that this is a mechanism to minimize light-chlorophyll interactions in the adaxial surfaces, and thus reactive oxygen species (ROS) formation and damage, during dehydration (Farrant *et al.*, 1999; Farrant, 2000). Furthermore biochemical protectants such as antioxidant systems and light-filtering pigments (Farrant *et al.*, 1999; Farrant, 2000) accumulate to minimize the extent of light-chlorophyll interaction and protect against free radical damage while in the dry state.

The aim of this study was to assess whether light is indeed a stress during drying, and whether the leaf folding and biochemical mechanisms proposed by Farrant (2000) do protect against this stress. Thus leaves were restrained from folding during drying and the effects monitored in plants dried in the light and dark. Unrestrained leaves were also dried in the light and dark. Changes in water content and concentration of chlorophyll, carotenoids and anthocyanins were measured. Metabolic functioning of photosystem II was examined using chlorophyll fluorescence techniques. Ultrastructural studies and measurement of electrolyte leakage patterns provided an indication of the subcellular changes in leaf cells and the extent of membrane damage, respectively, during desiccation.

Materials and Methods

Plant material

Plants were collected in the field and maintained in a glasshouse, as described by Sherwin and Farrant (1996). All experimentation was done in a controlled environment chamber (phytotron). Conditions were maintained at 60% relative humidity, a temperature range of 16°C minimum and 26°C maximum, a photoperiod of 14 hours light, 10 hours dark, and a maximum light intensity of 1200 µmol.m^{-2}.s^{-1}. The plants were acclimatized in the phytotron at least two weeks before experimentation started. Plants were well watered.

Methods

In order to prevent leaves from folding naturally during drying, twigs were used rather than whole plants. Farrant *et al.* (1999) have shown that excised twigs of *M. flabellifolius* do survive desiccation. Twigs of 4-5 cm were excised from the hydrated plants and divided into two treatments: restrained and unrestrained. In the restrained twigs the leaves were pinned with staples onto a foamolite board (covered with nylon mesh to decrease reflection) so that the abaxial surface was facing upwards (see Fig. 1) to prevent them from folding during drying. The leaves of the unrestrained twigs were allowed to fold naturally during drying. Half of the unrestrained and restrained leaves were subjected to high light conditions (1200 µmol.m^{-2}.s^{-1}) and the other half were kept in the dark. All twigs were air dried for 150 h and then rehydrated in the light under the same environmental conditions used for dehydration by releasing the restrains and placing the cut end of the stem in water for 72 h. Leaves were removed at regular intervals during the drying as well as upon rehydration and assessed as described below.

1. Water content

Water content of leaf material was determined gravimetrically by oven drying at 70°C for 48 h. Due to the small leaf size, three to five leaves had to be pooled to obtain a manageable sample of dry leaf material. Absolute water content was calculated on a dry mass basis as g $H_20.g^{-1}$ dry mass (g/g DW), using the equation:

$$WC = \frac{\text{Fresh mass} - \text{Dry mass}}{\text{Dry mass}}$$

Full turgor was calculated on fresh leaves after they had been enclosed in a plastic bag for 24 h. Relative water content (RWC) was then calculated as percentage using the equation:

$$RWC = \frac{\text{Fresh mass} - \text{Dry mass}}{\text{Full turgor mass} - \text{Dry mass}} \quad x100$$

2. Chlorophyll fluorescence

Chlorophyll fluorescence was measured on 6 different leaves for each treatment, accounting for 5 replicates within. Leaves were dark-adapted for 15 min. A modulated portable fluorometer (OS-500; Opti-Science, USA) was used to measure the quantum efficiency (Fv/Fm) of leaves at various stages of dehydration and rehydration, using a saturation light intensity of 4 mmol photons m^{-2} s^{-1} for the duration of 1 s.

3. Pigment analysis

Three leaves were analyzed for each treatment. Each leaf was cut longitudinally in half: one side was used to determine chlorophyll and carotenoid content, and the other to determine anthocyanin content. The extractions were done separately for each half.

3.1. Chlorophyll and Carotenoids

Chlorophyll (a+b) and carotenoids were extracted in 100% acetone for 48 h at 4°C in the dark. The samples were centrifuged for 10 min to remove debris and absorbances were then read at 470, 648.8 and 661.6 nm on a Beckman DU-64 spectrophotometer (Fullerton, California, USA). Chlorophyll (a + b), and carotenoid (x + c) content were calculated using the adjusted extinction coefficients according to Lichtenthaler (1987).

3.2. Anthocyanin content

Leaf material was extracted in acidified methanol (methanol:water:HCl [79:20:1]) in the dark for 48 h at 4°C. The extract was then centrifuged for 10 minutes to remove any debris. The absorbance of the extract was measured at 530 and 657 nm. Anthocyanin concentration was determined according to Mancinelli *et al.* (1975).

5. Electrolyte leakage

Membrane integrity was assessed as a percentage of maximum electrolyte leakage. Leakage measurements were performed on dehydrated leaves (< 5% RWC) and after 12 h rehydration. Five replicates of individual leaves were used for each treatment. These were placed in 30 ml Milli-Q (Millipore) ultra-pure water and conductivity was monitored at 5 min intervals for 30 min using a Jenway 4070 conductivity meter. Leakage rate was calculated according to the methods in Vander Willigen *et al.* (2000).

6. Ultrastructure

Leaf segments (ca 5 mm^2) were fixed in 2.5% gluteraldehyde in 0.1 M phosphate buffer containing 0.5% caffeine and post fixated in 1% osmium tetroxide in 0.1 M phosphate buffer, following the protocol previously used for this tissue (Sherwin and Farrant, 1996). After ethanol dehydration, the material was infiltrated and imbedded in epoxy resin (Spurr, 1969). Sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) for 10 minutes each, and viewed with a Jeol 200CX transmission electron microscope (Akishma, Japan).

Statistics

Because plant material was limited, there was insufficient replication for detailed statistical analyses. However, means and standard deviations were calculated for all figures, and regression lines fitted where appropriate. Tukey's Multiple Range Test was performed on electrolyte leakage data using Statgraphics version 7.0.

Results

Leaf orientation and colour change

During dehydration unrestrained leaves folded up against the stem so that only the abaxial surface of the outer leaves was exposed to the light (Fig. 1a, b) as occurs during natural drying of whole plants (Farrant, 2000) and excised twigs (Farrant et al., 1999). The light-exposed surface of the unrestrained leaves became purple/brown in colour during dehydration (Fig. 1b). During rehydration these leaves became green again (Fig. 1c). The restrained light-exposed leaves were green on the exposed adaxial surface (Fig. 1d) and somewhat purple on the concealed abaxial surface (Fig. 1e) at dehydration. Upon rehydration they had a blotched yellowish appearance (Fig. 1f). The unrestrained and restrained leaves that were dried in the dark turned pale-green during dehydration (Fig. 2a and 2b) and returned to a deep green when rehydrated (Fig. 2c).

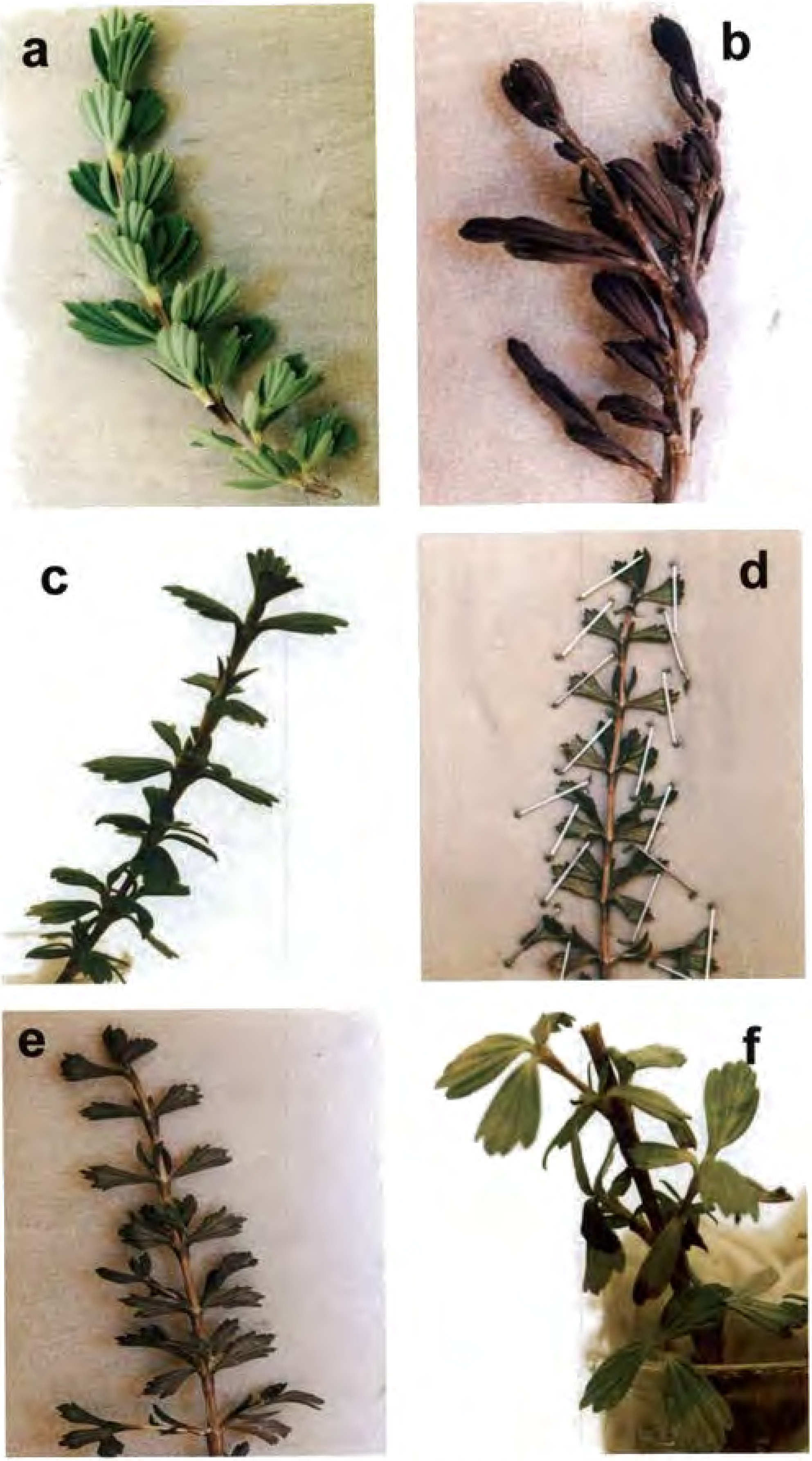

Fig. 1: Photographs of unrestrained light-exposed twigs after 2 h dehydration (a), 6 days dehydration (b) and 48 h rehydration (c), and restrained light-exposed twigs after 6 days dehydration (d), adaxial side and (e), abaxial side and 48 h rehydration (f).

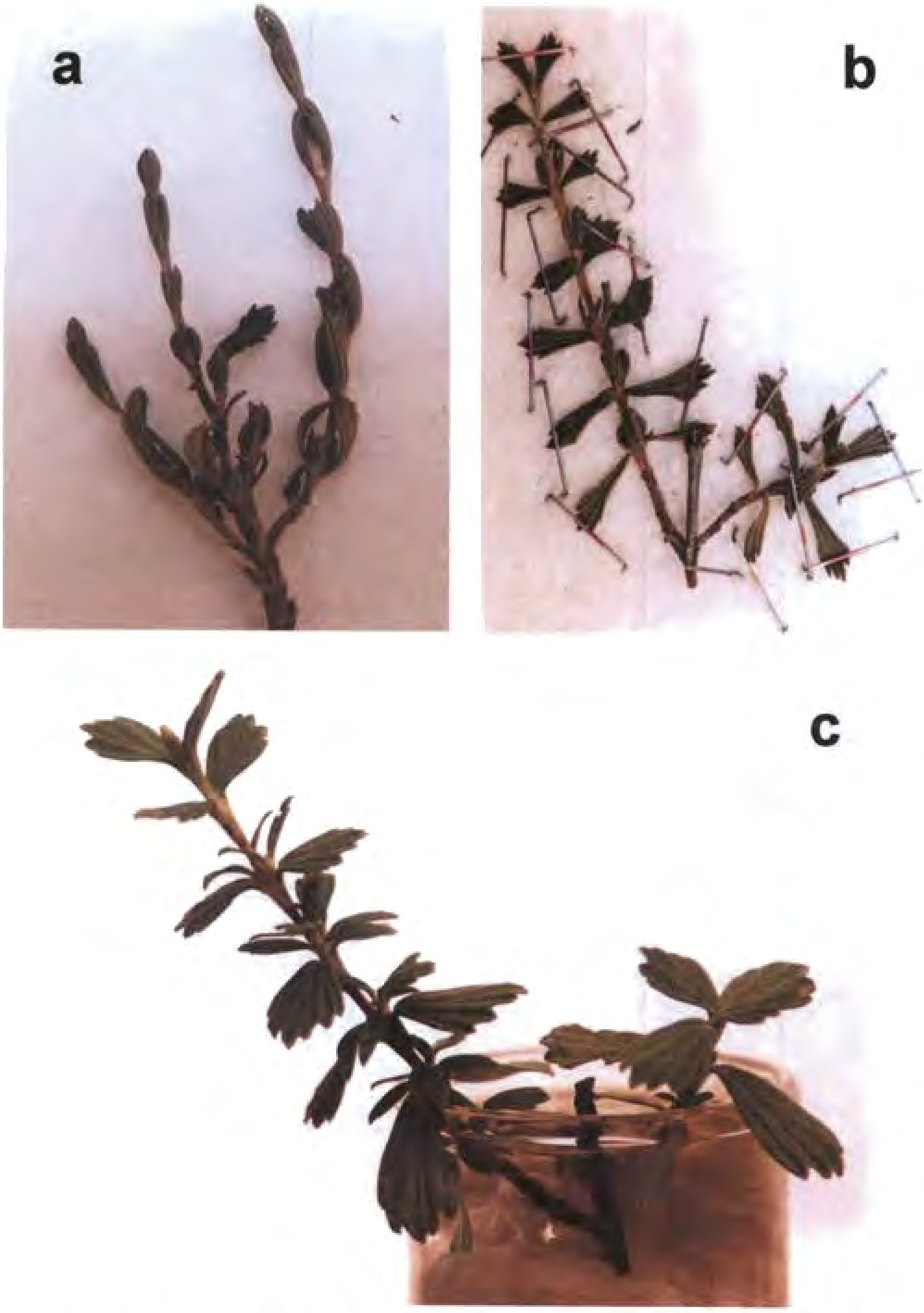

Fig. 2: Photographs of unrestrained dark-treated twigs after 6 days dehydration (a) and restrained dark-treated twigs after 6 days dehydration (b) and 48 h rehydration (c).

RWC

Once the branches were excised relative water content dropped rapidly (Fig. 3). In the leaves exposed to the light RWC dropped below 20% in 24 h and slightly less rapidly in the dark. Upon rehydration all four treatments were able to absorb and transport water to their leaves, although water uptake in the unrestrained dark-treated leaves was initially delayed. After 72 h of rehydration all leaves had recovered to 80% RWC and above.

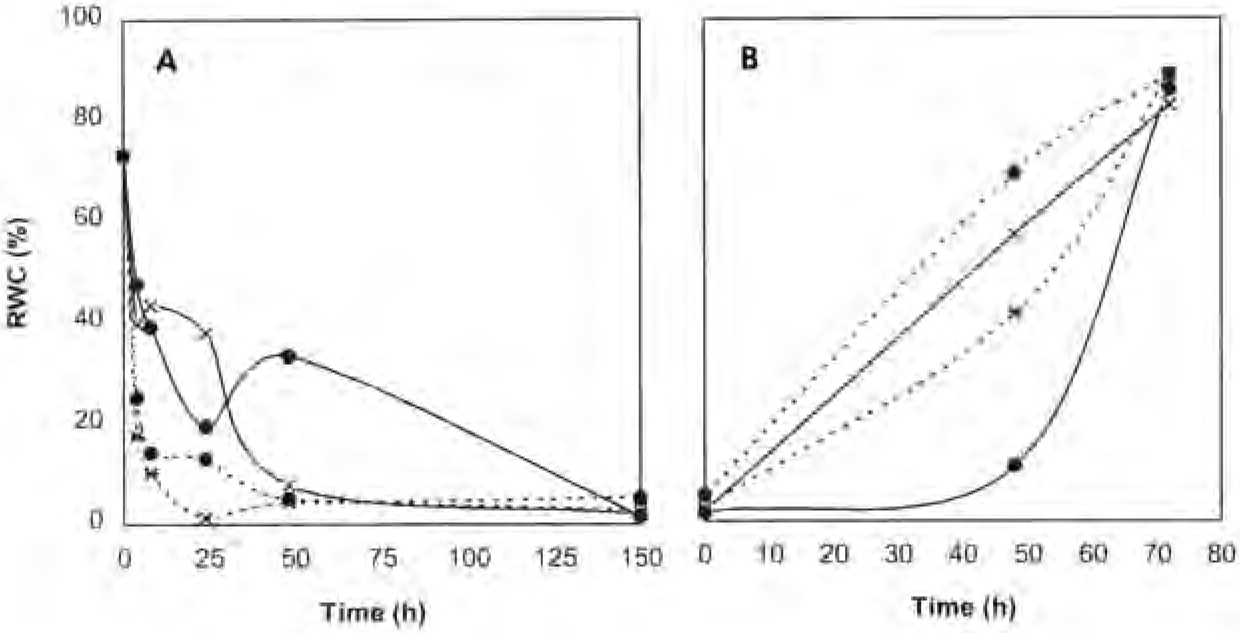

Fig. 3: Changes in relative water content with time during dehydration (A) and rehydration (B) of restrained (x) and unrestrained (•) leaves in the light (broken lines) and in the dark (solid lines).

Chlorophyll fluorescence

Quantum efficiency (Fv/Fm) of photosystem II decreased in all treatments during drying (Fig. 4) but began to decline at a higher water content in light-treated leaves than in dark-treated leaves. This is probably because leaves that were dehydrated in the light dried quicker than those in the dark. Re-initiation of PS II activity upon rehydration was slowest in the unrestrained leaves in the light. Full recovery had occurred at 90% RWC in the leaves that dried in the light, but those exposed to dark during dehydration did not recover fully.

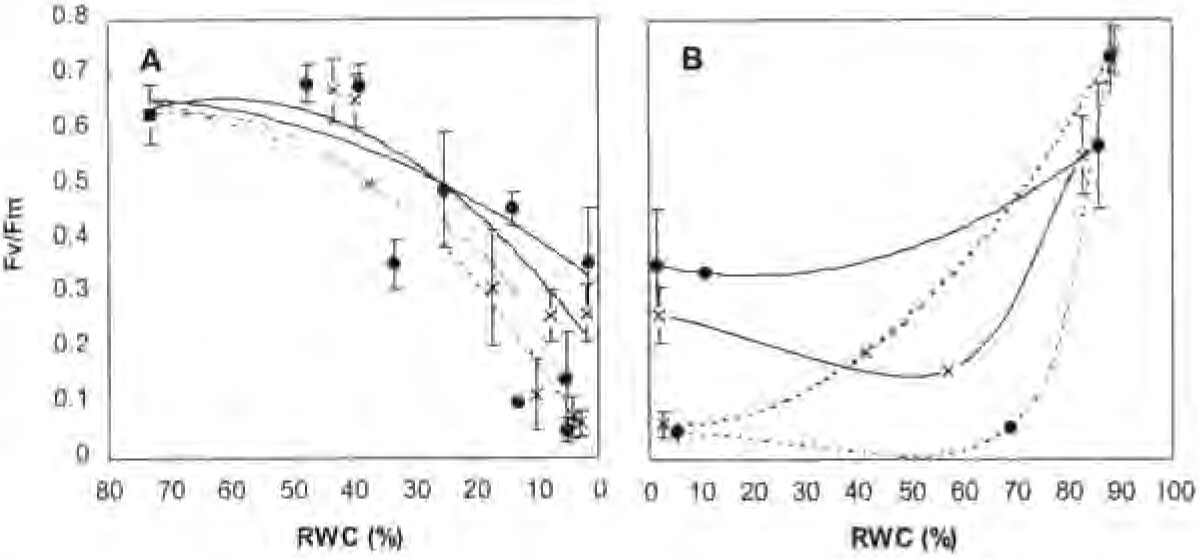

Fig. 4: Changes in quantum efficiency during dehydration (A) and rehydration (B) of restrained (x) and unrestrained (•) leaves in the light (broken lines) and in the dark (solid lines).

Pigment concentrations

Changes in photosynthetic pigments are shown in Fig. 5. There was a general decline in chlorophylls and carotenoids during dehydration. However, there was a consistent (experiment was repeated twice) increase in chlorophylls and carotenoids at approximately 12% RWC in the restrained leaves dehydrated in the light. These leaves then continued to lose

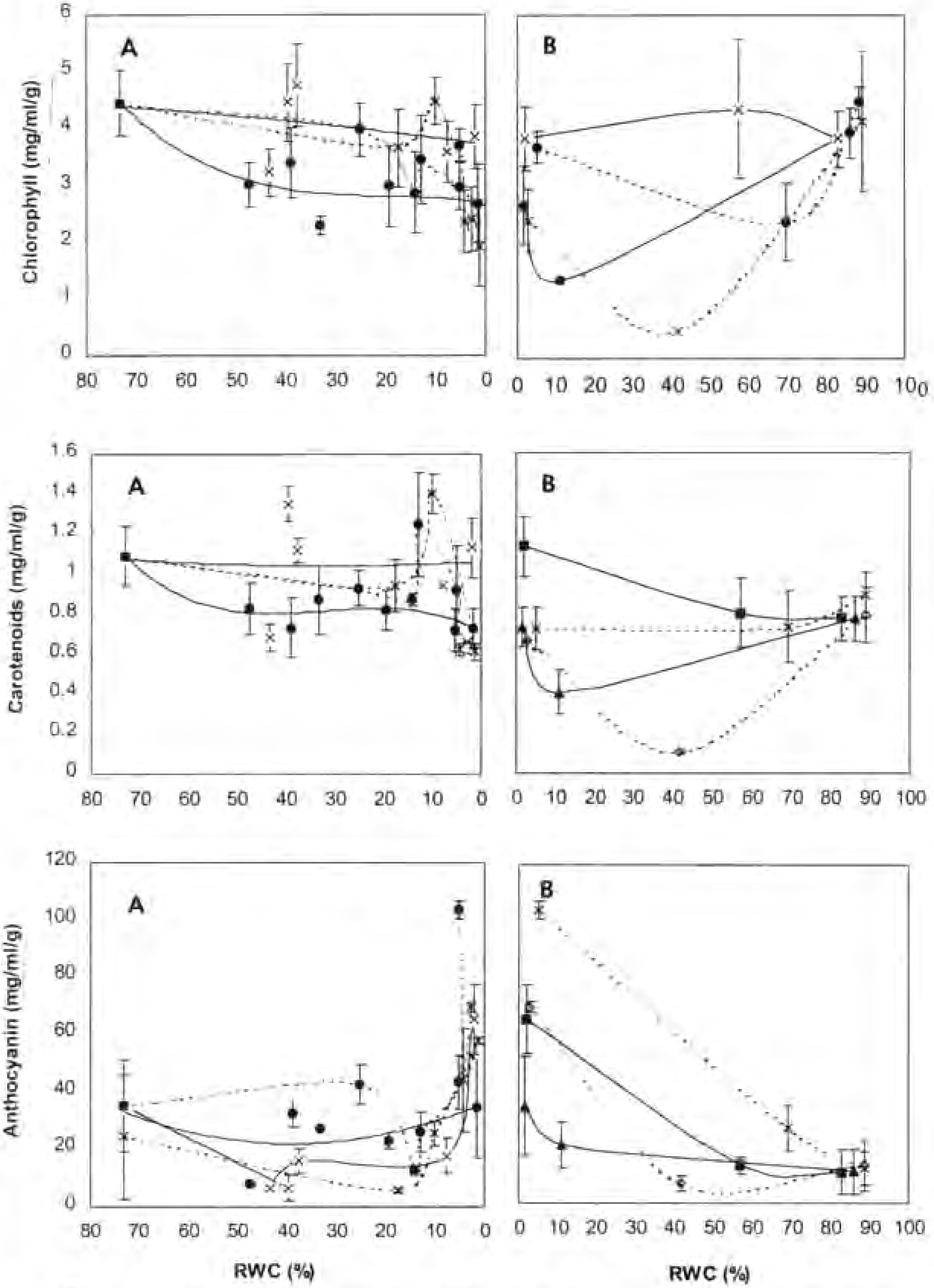

Fig. 5: Changes in chlorophyll, carotenoid and anthocyanin levels during dehydration (A) and rehydration (B) of restrained (x) and unrestrained (•) leaves in the light (broken lines) and in the dark (solid lines).

chlorophyll and carotenoids during rehydration, increasing only after recovery to 40% RWC. In the unrestrained light-exposed leaves chlorophyll and carotenoid levels changed by 22% and 38% respectively during dehydration, and recovered to control values upon rehydration. In the dark-treated leaves chlorophyll and carotenoids declined slightly (unrestrained) or stayed approximately the same (restrained) during dehydration but did not recover to control values upon rehydration.

Anthocyanin levels increased in all leaves as RWC decreased (Fig. 5), but were highest in the unrestrained light-exposed leaves at 5% RWC. Anthocyanin content dropped during rehydration.

Electrolyte leakage

Solute leakage in dry leaves (>5% RWC) was 50-60% higher in leaves that were kept in the dark compared to the leaves dried in the light (Fig. 6), regardless of being restrained or not. This suggests that the integrity of the plasmamembrane had not been maintained in the dark-treated leaves during dehydration. This was exacerbated upon rehydration.

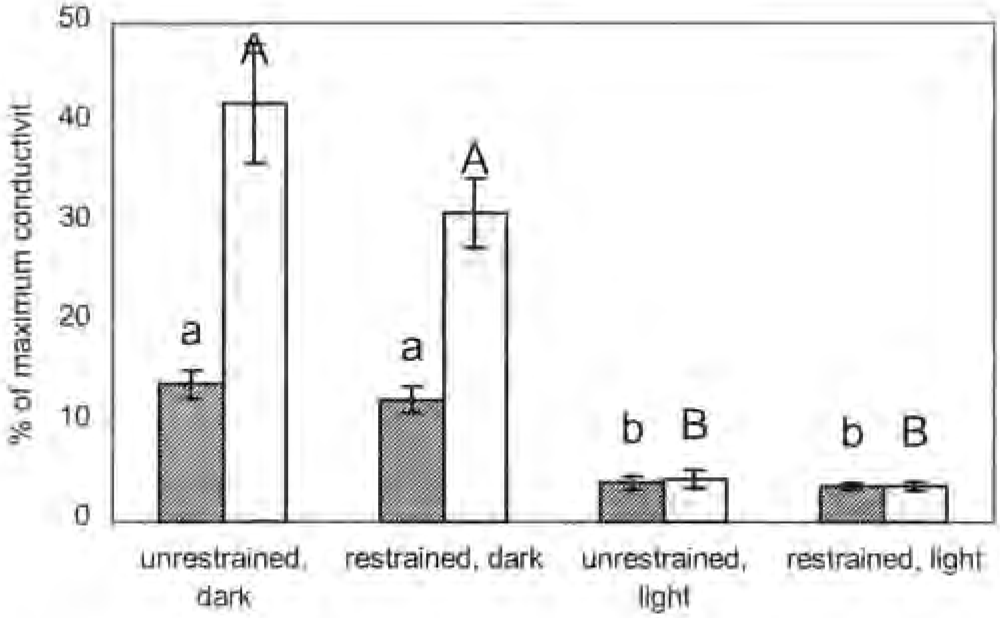

Fig. 6: Electrolyte leakage expressed as percentage maximum conductivity after 150 h dehydration (hashed) and 72 h of rehydration (dotted). Tukey Multiple Range Test, showing comparisons within dehydration (lower case lettering) and rehydration (upper case lettering) treatments separately ($p<0.05$, $n\leq4$).

Ultrastructure

Subcellular organization of hydrated (starting) material was typical of that of hydrated, physiologically active whole plants, and therefore this data is not given.

Dried tissue of restrained and unrestrained light-exposed leaves was mostly intact (Fig. 7a, b). There was considerable withdrawal of the plasma membrane from the walls, but no tearing was evident. Thylakoid membranes were generally still intact, although there was a small degree of blistering. This has been reported to occur during natural drying of whole plants (Koonjul *et al*., 1999; Farrant, 2000) and thus is not considered pathological here, especially since Fv/Fm (Fig. 4) recovered in these leaves. Only a few cells showed plasmalemma tearing and some organelle disintegration (Fig. 7b). On rehydration the cells from all light treatments (restrained and unrestrained) appear to have recovered the subcellular organisation. Vacuoles expansion had occurred (Fig. 7c) and chloroplasts had recovered thylakoid orientation and many contained starch grains (Fig. 7d). Active mitochondria were observed (Fig. 7d).

Dried restrained dark-treated tissue showed considerable subcellular damage in a large proportion of cells (Fig. 8a, b) but not all (Fig. 8c). In the damaged cells plasmamembrane and tonoplasts were ruptured (Fig. 8a) and many chloroplasts had damaged outer membranes and blistered thylakoids (Fig. 8b). Many cells did appear to "recover" upon rehydration (Fig. 8d), but many showed signs of damage obvious in dry cells.

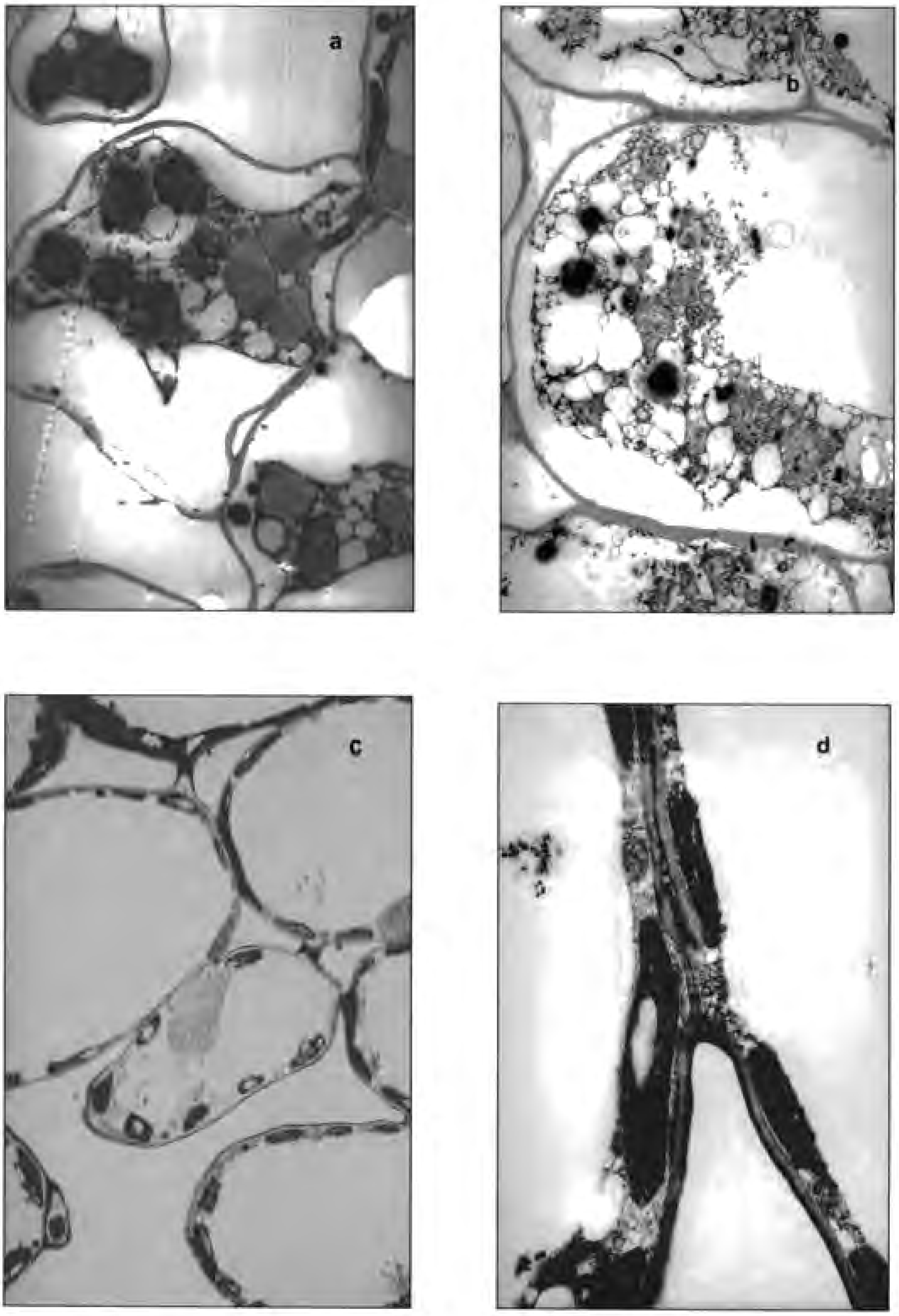

Fig. 7: Subcellular organization of restrained, light-exposed leaves in the dry state (fig. 7a, x3000 and 7b, x2000) and in the rehydrated state (fig. 7c, x2263 and fig.7d, x10 000).

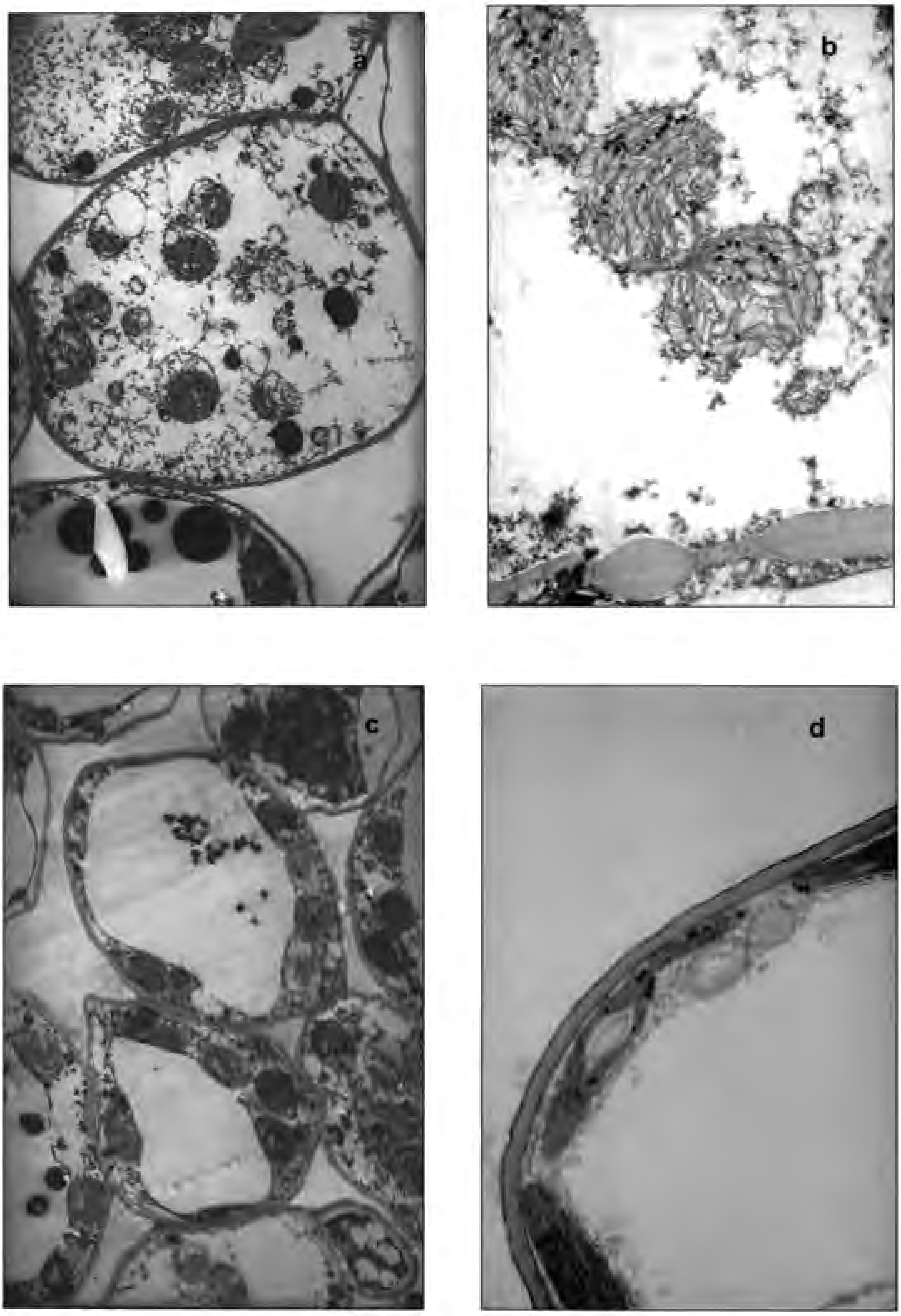

Fig. 8: Subcellular organization of dark-treated restrained leaves at dehydration (fig. 8a, x3078, fig.8b, x10 000 and fig. 8c, x3000) and rehydration (fig.8d, x10 000).

Discussion

There was no significant difference in the survival of restrained and unrestrained leaves during dehydration and rehydration. This could imply that leaf folding is not a critical factor in terms of achieving desiccation tolerance in *M. flabellifolius*. Drying in the dark, however, resulted in some damage and loss of leaf viability.

Considering the emphasis that has in the past been placed on the importance of leaf movements in reducing leaf area exposed to light (Farrant & Sherwin, 1998) it was unexpected to find that the restrained leaves exposed to the light during drying were not more damaged than the unrestrained ones. Both showed recovered electron transport (Fig. 4), photosynthetic pigments (Fig. 5) and ultrastructural integrity (Fig. 8, 9). Thus, whatever damage had occurred was obviously repaired. A similar study on *Craterostigma wilmsii* reports that leaves prevented from curling suffered damage to the subcellular environment on drying (Loffell, 1999). Although such leaves accumulated reflective pigments (anthocyanins and carotenoids) and increased the activity of the antioxidant ascorbate peroxidase (AP), these mechanisms were unable to provide adequate protection of the leaf tissue without concurrent shading provided by leaf-curling (Loffell, 1999).

The decline in RWC during dehydration was rapid in all four treatments, but slightly more so in the light than in the dark. A previous study by Farrant *et al* (1999) showed that the drying rate during dehydration plays an important role in the survival of desiccation tolerant angiosperms. It was reported that rapidly dried leaves of *M. flabellifolius* could not induce changes such as leaf folding and accumulation of anthocyanins, and did not survive desiccation (Farrant *et al.*, 1999). The changes in RWC in this study corresponds to those of slowly-dried twigs in other studies (Kruger, 1998; Farrant *et al.*, 1999). Damage to the subcellular tissues should thus not have resulted from too rapid drying even in plants dried in the light.

Chlorophyll concentrations declined by 55% in the restrained light-exposed leaves and 35% in the unrestrained light-exposed tissues. Reduction of chlorophyll in *M. flabellifolius* is a normal response to dehydration under high light intensities and is thought to prevent hazardous chlorophyll-light interactions (Kruger, 1998; Sherwin and Farrant, 1998; Farrant *et al.*, 1999; Driouich *et al.*, 2000). In *M. flabellifolius* the amount of chlorophyll reduction during drying varies between 30% - 50%, depending on the light intensity (Koonjul *et al.*, 1999). Possibly chlorophyll decline was more intensive in the restrained leaves because they could not protect photosynthetic tissue by leaf folding or because chlorophyll in adaxial surfaces, which normally would be shaded from light in the dry state, was photo-oxidised when continually exposed to light. A study on *Craterostigma wilmsii* (Loffell, 1999) showed that restrained leaves had higher chlorophyll concentrations. The author suggested that the tissue may not have been able to break down chlorophyll when remaining exposed to light, but instead continues to synthesize it.

Carotenoids are photosynthetic pigments and have also been implicated in photoprotection; as a sunscreen pigment, and as an antioxidant (Larson, 1988; Hendry, 1993, Demmig-Adams and Adams, 1996). Chlorophylls absorb most of the light in the red-orange region (690-650 nm) and the blue-violet region (480-400 nm) of the light spectrum, whereas carotenoids reflect the yellow-orange to red wavelengths of light (Hendry, 1993). Although it has been shown that carotenoids decline in certain resurrection plants during drying under high light intensities (Kruger, 1998; Sherwin and Farrant, 1998; Farrant *et al.*, 1999; Farrant, 2000). Loffell (1999) reported increases in carotenoids in dry outer leaves (which remain exposed to light) and restrained leaves of *C. wilmsii*, which did not occur in the inner (shaded) leaves. In *Myrothamnus flabellifolius* leaves that were dried in the light increased carotenoid contents at 12% RWC. Thus, as suggested by Loffell (1999), carotenoid accumulation could be induced by the presence of light and may well be a protection mechanism.

The upregulation of anthocyanins during drying under high light intensities is a well-documented response of *M. flabellifolius* (Sherwin and Farrant, 1998; Farrant *et al.*, 1999; Farrant, 2000). Anthocyanins reduce light-chlorophyll interactions by reflecting photosynthetically active light, by absorbing the UV-light and can also act as free radical scavengers (Larson, 1988; Smirnoff, 1993). With regard to this theory it would be expected that there would be higher anthocyanin concentrations in the restrained leaves. However, restrained light-exposed leaves had lower anthocyanin concentrations than unrestrained leaves. Furthermore it was observed that the adaxial surface of the restrained leaves was still green in the air-dry state, but the abaxial surface - although not exposed to the light – was purple. A possible explanation for this observation might be that the upregulation of anthocyanins during drying happens primarily in the abaxial leaf surface, since this is also the surface which remains exposed to the light under normal conditions.

Interestingly anthocyanin levels increased in the dark-treated leaves during drying. Until now it has been believed that this is a light-induced response (Sherwin and Farrant, 1998; Farrant *et al.*, 1999; Driouich *et al.*, 2000; Farrant, 2000). It is suggested that in this study it was in response to the drying of the tissue. These results are in contrast with a study on *Craterostigma wilmsii* which shows that shaded inner whorl leaves do not accumulate photosynthetic pigments and anthocyanins and do not upregulate their antioxidants (Loffell, 1999; Farrant, 2000).

Carotenoids and chlorophylls did not decline to any great extent in the dark-dried leaves. This is despite the observation that a lack of light often induces photosynthetic pigment loss (Larson, 1988; Smirnoff, 1993; Demmig-Adams and Adams, 1996).

Leaves dried in the dark showed considerable subcellular damage (increased electrolyte leakage, Fig. 6 and ultrastructural studies, Fig 8b, 9b and did not fully recover photosynthetic activity (Fv/Fm) Fig. 4b). Why would leaves that have been dehydrated in the dark be so much more damaged than light-dried leaves? Possibly light triggers biochemical responses such as hormones and enzymes that in turn upregulate protection mechanisms. In the absence of light these mechanisms may not be activated, so causing subcellular damage. In particular the leaves may not be able to accumulate "compatible solutes" such as sugars, protectants of subcellular membrane integrity (Crowe *et al.*, 1988) to prevent mechanical damage as there is no light to photosynthesize. Furthermore the excised twigs cannot rely on roots for sources of sugars or for energy to upregulate protective mechanisms and to protect membranes. Examination of whole plants would give further insight into the relative importance of roots. It is also important to gain an understanding of the activity of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (AP) and glutathione reductase (GR) since they also might play a role in protection against free radical damage (Driouich *et al.*, 2000; Farrant, 2000).

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