EFFECT OF LIGHT STRESS ON THE HOMOIOCHLOROPHYLLOUS RESURRECTION PLANT *CRATEROSTIGMA WILMSII*

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ABSTRACT

The effect of light stress during drying, experienced by the homoiochlorophyllous resurrection plant *Craterostigma wilmsii,* was examined in this study. Inner-whorl leaves, which become shaded from light when dry, and outer-whorl leaves, the abaxial surfaces of which receive light in the dry state, as well as leaves which had been restrained from curling during drying, were separately examined. Differences in chlorophyll (a +b) content, carotenoid (x + c) content, anthocyanin content, ascorbate peroxidase activity, and ultrastructure, were assessed. Shaded inner whorl leaves do not experience light-chlorophyll interactions, carotenoids and anthocyanin pigments do not accumulate and ascorbate peroxidase activity remains low in them during drying. Outer whorl leaves accumulate light-filtering pigments such as anthocyanins and carotenoids to mask their chlorophyll, and the activity of the enzyme ascorbate peroxidase is increased. Neither inner nor outer whorl leaves suffer subcellular damage during drying but leaves prevented from curling and shading their abaxial surfaces suffer damage to the subcellular environment on drying. Although such leaves accumulated reflective pigments and increase ascorbate peroxidase activity, these mechanisms are unable to provide adequate protection of the leaf tissue without concurrent shading provided by curling. Leaves that shaded their abaxial surfaces by curling and leaves that where shaded completely by other curled leaves decreased their content of chlorophyll in the dry state. Leaves that were restrained from curling had increased chlorophyll contents. Although chlorophyll decline in normal dry *C. wilmsii* leaves appears to be an artefact of shading, this decline in photopigment content concurrent with shading by leaf movements and light filtering pigments, is suggested to assist in protecting against free radical damage in the dry state.

INTRODUCTION

"Resurrection" plants are a unique groups of plants which are able to tolerate desiccation (< 5% Relative Water Content [RWC]) of their tissues (Child, 1960; Gaff 1977; Farrant and Sherwin, 1997). These plants are able to resume full metabolic competence on rehydration.

Resurrection plants, such as the angiosperm *Craterostigma* spp, occur in severe environments. *Craterostigma wilmsii* grows in direct sunlight on shallow soils and rocky outcrops (Child, 1960; Gaff, 1977; Farrant *et al*, 1999), where they frequently experience high water vapour deficits (Farrant *et al*, 1999). Thus the ability to survive frequent bouts of desiccation is vital to their survival. Desiccation tolerance occurs in the lower order plants, such as lichens, bryophytes and pteridophytes, and in the angiosperms (Hambler, 1961). Unless otherwise stated, the details discussed here are relevant to angiosperm resurrection plants.

Water has an array of biophysical and biochemical functions in vegetative tissue. An unmediated loss of cellular water can thus have catastrophic effects on the subcellular environment of a plant intolerant of such water loss (Vetucci and Farrant, 1995). The many stresses associated with desiccation are of a mechanical, chemical and metabolic nature. When severe drying occurs, cells lose turgidity and cell walls collapse. Denaturation of macromolecules results from the removal of water integrally associated with their surfaces. Interbilayer reactions occur between membranes that find themselves in close proximity; and phase transitions of lipids can occur (membranes, consisting of phospholipid bilayers, are consequently disrupted) (see reviews by Vetucci and Farrant, 1995; Pammenter and Berjak, 1999). As water is integral in many metabolic reactions, metabolism in the desiccated state is disrupted and chaotic (Farrant and Sherwin, 1997; Pammenter and Berjak, 1999). Disruption of metabolic pathways such as those of respiration and photosynthesis result in the production of free radicals, which are extremely damaging to the subcellular environment (Larson, 1988; Smirnoff, 1993; Vetucci and Farrant, 1995; Farrant and Sherwin, 1997, Sherwin and Farrant, 1998). It is of importance to note that it is at intermediate water contents that the metabolism is of an

unregulated nature (Pammenter and Berjak, 1999).

Light places an enormous stress on plants when water is limited. Chlorophylls (and other photoreceptors such as carotenes) continue to function under water stress, capturing energy from light. However, the photosynthetic pathways associated with these pigments are unable to receive this energy due to water limitations. This energy is instead dissipated as heat and fluorescence but also by the transformation of molecular oxygen into active oxygen (Larson, 1988). Active oxygen can cause the formation of organic free radicals (Smirnoff, 1993), which are extremely reactive and damage almost all biological molecules (Larson, 1988; Smirnoff, 1993). Free radicals can cause lipid peroxidation (hence damaging membranes), inactivation of enzymes (Smirnoff, 1993), damage to other proteins and DNA (Vetucci and Farrant, 1995; Pammenter and Berjak, 1999), and bleaching of carotenoids (Larson, 1988). (See reviews by Vetucci and Farrant, 1995; Pammenter and Berjak, 1999.)

The rehydration process also places other stresses on the plant tissues (Oliver *et al* 1998; Farrant and Sherwin, 1997). Damage inflicted during drying can be aggravated (physically) by a rapid influx of water (Farrant and Sherwin, 1997), and repair mechanisms are required to perform. It has been suggested that more destruction occurs during rehydration than dehydration of standard desiccation sensitive plants, such as *Pisum sativum*. This has been suggested to indicate that there is no mechanism for repair of damage in this desiccation sensitive plant (Sherwin and Farrant, 1996). It could also indicate that the plant hasn't the capacity to re-initiate protective mechanisms properly upon rehydration, to deal with the reactivated (yet still chaotic) metabolism.

Plants that are able to survive the extreme stresses of desiccation have a number of mechanisms for preventing and/or repairing the damage caused through a desiccation cycle (drying down, existing in the dry state, and rehydrating). These responses differ (to greater or lesser degrees) between different resurrection species (Farrant and Sherwin, 1997). Desiccation tolerance is a complex, multifaceted phenomenon, with many processes at the preventative and repair levels (protection and repair are relied upon to different extents in different plants (Bewley and Oliver, 1992)). Angiosperms rely predominantly on protective mechanisms, which are required for maintenance of

physiological integrity while in the desiccated state, as well as during drying and rehydration (Oliver *et al*, 1998). Because drying of vegetative tissues can occur at unpredictable intervals, these mechanisms of stress tolerance must be activated readily (Vetucci and Farrant, 1995).

Mechanisms include accumulation of soluble reserves within vacuoles and changes in degree of vacuolation, which aids in preventing damage through loss of turgidity (Farrant and Sherwin, 1997). Organelles can be de-differentiated (Sherwin and Farrant, 1996), which causes metabolic quiescence (Farrant and Sherwin, 1997; Pammenter and Berjak, 1999). Cell walls undergo folding in some plants (eg *Craterostigma* spp), to prevent mechanical stress (Farrant *et al*, 1999; Vicre` *et al*, 1999). LEA-like proteins (Late Embryogenesis Accumulated proteins), also known as 'dehydrins', as well as sugars (sucrose and oligosaccharides) accumulate within the cells. These molecules are suggested to be protective in nature. (See Oliver *et al*, (1998) and Pammenter and Berjak, (1999) for reviews.)

Desiccation tolerant plants also, very vitally, seek to protect themselves from the damages associated with production of free radicals. Light stress associated with drying is one of the major causes of the production of free radicals (as has already been discussed). Protection against free radical damage operates at two levels; initial protection against the production of free radicals, and secondly the presence of antioxidant systems to deal with these molecules when they are produced. The protection against initial free-radical production is primarily achieved by limiting light-chlorophyll interactions (Farrant and Sherwin, 1997; Sherwin and Farrant 1998). The antioxidant systems act to mitigate the effects of free radical production by preventing them from reacting with organic molecules within the subcellular environment (Larson, 1988).

Resurrection plants are divided into two groups according to the predominant physical method for limiting light-chlorophyll interactions. Poikilochlorophyllous (Hambler, 1961) desiccation tolerant (PDT) plants break down chlorophyll and de-differentiate their chloroplasts (dismantle thylakoid membranes), and homoiochlorophyllous (Hambler, 1961) desiccation tolerant (HDT) plants have leaf movements which act in shading the chlorophyll retained by these plants. HDT plants also retain the structure of their

chloroplasts (Sherwin and Farrant, 1998). *Xerophyta viscosa* is an example of a PDT plant, which breaks down its chlorophyll and chloroplasts during dehydration (Sherwin and Farrant, 1998). *C. wilmsii* (HDT) curls its leaves up on dehydration. The leaves of *C. wilmsii* grow in a rosette, with younger leaves in the inner whorls. Leaf curling is thought to shade these inner-whorl leaves (Sherwin and Farrant, 1998) as only the abaxial surfaces of outer-whorl (older) leaves remain exposed to light. The HDT plant *Myrothamnus flabellifolius* folds its leaves against the stem, and the lateral branches curve upwards and inwards during drying (Gaff, 1977; Kruger, 1998; Sherwin and Farrant, 1996; Farrant *et al*, 1999). These resurrection plants decrease light-exposed leaf area by changing leaf-shape and position (Gaff, 1977). Although HDT plants maintain most of their chlorophyll, they do show some reduction in leaf photo-pigment content during drying. Drying of *C. wilmsii* caused leaf chlorophyll content to decline by about 30 %, in a study by Sherwin and Farrant (1998). Kruger (1998) and Koonjul *et al* (1999) found total chlorophyll of *M. flabellifolius* to decline by 40% during drying, and Koonjul *et al* (1999) have reported that chlorophyll loss ranges from 30 - 50 %, depending on light levels during drying. *C. wilmsii* has been found to retain its carotenoids during drying (Sherwin and Farrant, 1998) but also to loose carotenoids (ca. 20%) during drying (Farrant *et al* 1999). *M. flabellifolius* has also been found to loose carotenoids during drying (Koonjul *et al* 1999). Losses of pigment in desiccation tolerant plants are not permanent. When the plants rehydrate, their levels of chlorophylls and carotenoids increase again. The reduction in photo-pigment concentration may be another mechanism for reducing the hazard of chaotic photochemical activity (Kruger, 1998).

Chemical protection against light involves several systems in dessication tolerant plants. Some light-chlorophyll interactions are chemically limited by 'sun screen' pigments (e.g. anthocyanins and carotenoids) (Larson, 1988; Hendry, 1993; Koonjul *et al* 1999). UV radiation can react destructively with amino acids and membrane lipids due to its high-energy status. Flavanoids, such as anthocyanins, are absorbent of UV (ultraviolet)-radiation, and act as light filter. They mask the chlorophyll within the leaf tissue, limiting the absorption of light energy by the photosystems, and thereby limit free radical production. A colour-change of the leaf tissue is associated with these pigments

(Koonjul *et al* 1999) which accumulate in many resurrection plants (Gaff, 1977). Sherwin and Farrant (1998) have shown them to accumulate in significant quantities in *C. wilmsii,* and *X. viscosa* during a dehydration cycle.

Larson (1988) and Smirnoff (1993) have reviewed the antioxidant systems which are involved in limiting damage from free radicals produced in spite of limited light-chlorophyll interactions: Plant peroxidases, such as ascorbate peroxidase (AP), protect against oxidation by free radicals. AP is an enzyme which catalyses the scavenging of the free radical, hydrogen peroxide, by ascorbate. AP is thus essential to the functioning of ascorbate in antioxidant activities. Superoxide dismutase (SOD), catalase, ascorbate and tocopherols, such as α-tocopherol, are also systems that react with active forms of oxygen to keep them at low levels. Other protective systems regenerate oxidised antioxidants, allowing them to continue functioning. Glutathione reductase (GR), ascorbate, and mono- and dehydroascorbate belong to this second group of antioxidant regenerators. Not only are flavanoids (such as anthocyanins) absorbent of UV, they also act as antioxidants, as do the light-capturing pigments β-carotene and chlorophyll 'a' (chlorophyll 'a' inhibits autooxidation when in the dark). The biosynthesis of flavanoids can be increased by light. In a study on the resurrection plants *C. wilmsii* and *X. viscosa*, Sherwin and Farrant (1998) have shown that both plants experience a significant increase in AP, GR and SOD during the desiccation cycle, indicating that protection is received by the plants from these systems.

The homoiochlorophyllous resurrection plant *Craterostigma wilmsii* Engl. (Scrophulariaceae) is the subject of this study. This plant uses morphological changes (such as leaf curling), and biochemical protectants (such as antioxidant systems and light-filtering pigments), to minimise the extent of light-chlorophyll interaction and protect against free radical damage while in the dry state. It has been suggested that leaf curling causes shading of the inner leaves which prevents light-chlorophyll interactions in these leaves and so minimises free radical formation in them (Sherwin and Farrant, 1998). This study will attempt to assess whether outer-whorl leaves, which remain exposed to light, have different levels or mechanisms of protection to those present in the shaded

inner-whorl leaves, when in the dry state. Ultrastructure and certain aspects of the biochemistry of *C. wilmsii* are examined in this study to assess if differences occur between inner- and outer-whorl leaves. The following questions are asked: Do shaded inner leaves retain more chlorophyll than outer leaves because they have less light-stress on drying? Do the levels of chemical protectants such as anthocyanins, carotenoids and ascorbate peroxidase, differ between inner- and outer-whorl leaves? Is there a difference in chloroplast structure (in the dry state) between inner- and outer-whorl leaves? If leaves are prevented from curling (and thus do not shade their own adaxial surfaces), what effects does this have on the protection mechanisms invoked during drying?

In this study, light-exposed and shaded (concealed beneath outer leaves, in the dry state) *C. wilmsii* leaf tissue, as well as leaf tissue that has been prevented from curling, were examined. Water, chlorophyll, carotenoid and anthocyanin contents were determined and differences among the three tissue types (outer leaves, inner leaves, and restrained leaves) were noted, as were differences in ascorbate peroxidase activity. Tissue ultrastructure was examined to assess the subcellular response to the various treatments.

MATERIALS AND METHODS

Materials

Plants were collected and maintained in a glasshouse, as described by Sherwin and Farrant (1996). Experiments were done during winter, and plants were thus maintained in a controlled environment chamber (phytotron) to obtain conditions of high light intensity. The midday light intensity was that of full sunlight (2 000 $\mu mol.m^{-2}.s^{-1}$) as would be experienced in their natural environment, and temperature was 26°C. All plants used were placed in the chamber in a hydrated state, and drying down was thus initiated under conditions of high light intensity. Drying was initiated by withholding water from the soil. Control plants, maintained in a hydrated state, were kept under the same light conditions as those that were dried down. Three of the plants that were dried were dried

with some of their leaves restrained from curling (see Fig. 1). Three leaves per plant were restrained with laboratory film (Parafilm, American National Can), covering a minimum of leaf surface-area. The remaining leaves of the 'restrained' plants' were unrestricted and allowed to curl in a normal fashion.

Figure 1: *Craterostigma wilmsii*, in the dry state, showing position of leaves restrained from curling by parafilm ties (arrowed). Leaves decreased in size during drying and eventually moved out from under the restraints, but did not curl. Arrows indicate (p) parafilm restraints, and (a) anthocyanins in the margins of restrained leaves.

Methods

The leaves of *C. wilmsii* grow in a rosette of inner and outer leaves (Fig. 3). All experiments were performed separately on inner and outer leaves of three dry (< 5 % RWC) and three hydrated plants, and on the dry, restrained leaves of three such-treated plants.

1. *Water content*

Dry mass water content (g $H_2O.g^{-1}$ tissue) was determined gravimetrically for hydrated, dry, and dry-restrained leaves by oven drying fresh leaves for 48h at 70°C.

2. *Pigment determinations*

2.1. *Photosynthetic pigments*

In determining pigment content, each leaf was cut longitudinally in half: One side was used to determine photo-pigment content, and the other to determine anthocyanin content. The photosynthetic pigments, chlorophylls (a + b) and carotenoids (x + c), were extracted in 100% acetone, for 24 h at 4° C. The extractions were done separately for each leaf, with 3 internal replicates per leaf. Samples were centrifuged to remove debris and absorbances were then read at 470, 648.8 and 661.6 nm on a Shimadzu UV-1601 light-UV spectrophotometer (Shimadzu Scientific Instruments Inc.,USA). Chlorophyll (a + b) and carotenoid (x + c) contents were calculated using adjusted extinction coefficients (Lichtenthaler, 1987)

2.2. *Anthocyanin content*

Anthocyanins were extracted in acidified methanol (methanol:water:HCL [79:20:1]) in the dark, at 4°C for 48 h. The absorbance of the extract was read at 530 and 657nm. The formula [A] = A530 - (1/3 A657) was used to determine anthocyanin concentration, and these results are presented as [A] per gram dry weight (Mancinelli *et al*, 1975)

3. *Ascorbate Peroxidase (AP)*

AP assays were performed as described by Sherwin and Farrant (1998). At least four internal replicates were obtained for each of the dry and hydrated plants. Due to limited plant material, the AP assay was performed on the leaves from only one restrained plant, with four internal replicates. 0.05g (dry mass) of leaf tissue was ground using a chilled mortar and pestle. Tissue was ground in 0.2 g polyvinylpyrrolidone. Material was extracted in 1 ml of 2mM sodium ascorbate and 4 ml of Tris-HCL buffer pH 7.7. The

solution was centrifuged to remove cellular debris. 100µl of the supernatant containing the enzyme was then added to 700ml of 35mM K_2HPO_4 pH6.1 (final molarity 25mM) and 100ml of 20mM sodium ascorbate (final molarity 2mM). Mixtures were kept chilled during extraction, and were warmed up to room temperature before reading on a Shimadzu spectrophotometer. 100ml of 0.1mM H_2O_2 was used to initiate the oxidation of ascorbate, and hence the activity of AP. The activity was read over one minute. The enzyme activity per gram dry mass was determined using the formula: $((\Delta[A]/\Delta t) / 0.0028) * (1/ g DM)$.

4. Ultrastructure

Leaf segments (5 mm^2) from the outer and inner leaves of a dry plant, the outer leaf of a hydrated plant, and a restrained, dry leaf were fixed in 2.5% gluteraldehyde in 0.1M phosphate buffer (pH 7.2) with 0.5% caffeine. Material was washed in 0.1M phosphate buffer and post fixing was then done in 1% osmium tetroxide in phosphate buffer. Material was subsequently dehydrated through an ethanol series, and then infiltrated and imbedded in Spurr's epoxy resin (Spurr, 1969). Sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and viewed with a Jeol 200CX transmission electron microscope.

RESULTS

(Due to the small sample sizes used in this study, statistics other than the determination of standard deviations from the mean could not be performed on these results.)

Pigment concentrations

Figure 2 shows the differences in total chlorophyll (a + b), carotenoid (x + c) and anthocyanin content for inner- and outer-whorl leaves and restrained leaves in the dry state, compared with control (hydrated) tissue. Total chlorophyll (a + b) (figure 2a) did

not differ between inner- and outer leaves of hydrated tissue but decreased overall by 46% in dry tissue. Chlorophyll content of dry inner-whorl leaves decreased by more than half that of control leaves whereas outer-whorl leaves decreased by just over a third. Restrained leaves didn't loose any chlorophyll at all on drying, and chlorophyll appears instead to have increased from control levels. The chlorophyll content for these leaves was 2,5 times higher than the chlorophyll content of outer leaves allowed to curl in the normal fashion. Carotenoid (x + c) content (figure 2b) was also higher in restrained leaves, increasing to 186% of the levels found in hydrated outer-whorl leaves. An increase was similarly seen in dry outer leaves, which contained about 130 % more carotenoids than in hydrated tissue. Interestingly hydrated, outer-whorl leaves had a third less carotenoids than the younger inner-whorl leaves and drying caused no change in carotenoids of the inner leaves. Anthocyanin levels (figure 2c) showed similar trends to carotenoids in the various tissue-types. A distinct purple colour was observed to accumulate in the abaxial surfaces of dry outer leaves (figure 3), concurrent with a doubling of the amount of anthocyanin found in hydrated outer leaves. Dry inner leaves showed a slight decrease in anthocyanin content and no notable accumulation of purple colouring. The dry restrained tissue (unexpectedly) contained about 400% more of this sunscreen pigment than control tissue; 127% higher than normally curling dry outer leaves. This tissue showed very little coinciding colour changes: The light-exposed adaxial leaf-surfaces had no purple colour in them except for occasionally around the periphery of the leaf (figure 1, arrow indicates purple 'border'). Analysis of anthocyanin content for dry, restrained leaves was repeated due to the unusual initial results The values for the second analysis were much closer to the results for dry outer leaves, differing by less than 10%.

Ascorbate peroxidase

Activity of the enzyme ascorbate peroxidase was very variable within some tissue types (Figure 4). Dry outer leaves varied greatly in their AP content, but had a mean content 65% greater than the content of AP in hydrated outer leaves. The levels of AP in dry inner leaves was 66 % *lower* than hydrated inner leaves, and the net effect was that total

AP content of dry tissue was the same as total AP content of control (hydrated) tissue. Enzyme activity from inner and outer hydrated leaves differed only by about 10 %. AP in dry restrained leaves was similar to that in dry outer leaves, being about 20 % higher than hydrated tissue.

Ultrastructure

Figures 5, 6 and 7 show the ultrastructural characteristics of cells from the different tissue types. Figure 5 shows mesophyll cells from control tissue and is typical of that of hydrated, photosynthetically active *C. wilmsii* leaves (Sherwin and Farrant, 1996, 1998). Vacuoles are large, and chloroplasts are elongated and situated along the cell walls. Cells from dry outer leaves (figures 6a, and 6b) show much vacuolation (figure 6b) and some cell wall folding is apparent. Chloroplasts are generally rounded and granal stacks are peripherally displaced in some. Boundary and thylakoid membranes are intact, and a small amount of thylakoid-membrane 'blistering' has occurred. Chloroplasts from dry inner mesophyll leaf cells (figures 6c and 6d) also assume a rounded conformation, but thylakoids appear not to be laterally displaced within the organelles, and no blistering is evident. Considerable cell wall folding is apparent (figure 6c). As was the situation in outer leaves, inner leaves were vacuolated but the cells appeared more 'compact' than those of dry outer leaves (figure 6b). Leaves that were restrained on drying (figure 7b) had some cells with evidence of subcellular damage, and some cells were typical of those from normal dry *C. wilmsii* leaf tissues. The cells exhibited wall folding, and some vacuolation had occurred in some cells, but not in others (figure 7b) Some membrane rupture (arrowed, figure 7b) had occurred. Restrained leaves that have been rehydrated show much subcellular damage. Starch bodies are present in some chloroplasts, and some chloroplasts have resumed a 'normal' appearance and positioning (figure 7a), but in the main cell plasmalemma and tonoplast membranes were ruptured; chloroplast outer membranes were broken and thylakoid damage was evident (figure 7c). Accumulation of lipophillic debris along cell walls was apparent in most cells (figure 7a and 7c). Many mitochondria were associated with the chloroplast in the damaged tissue.

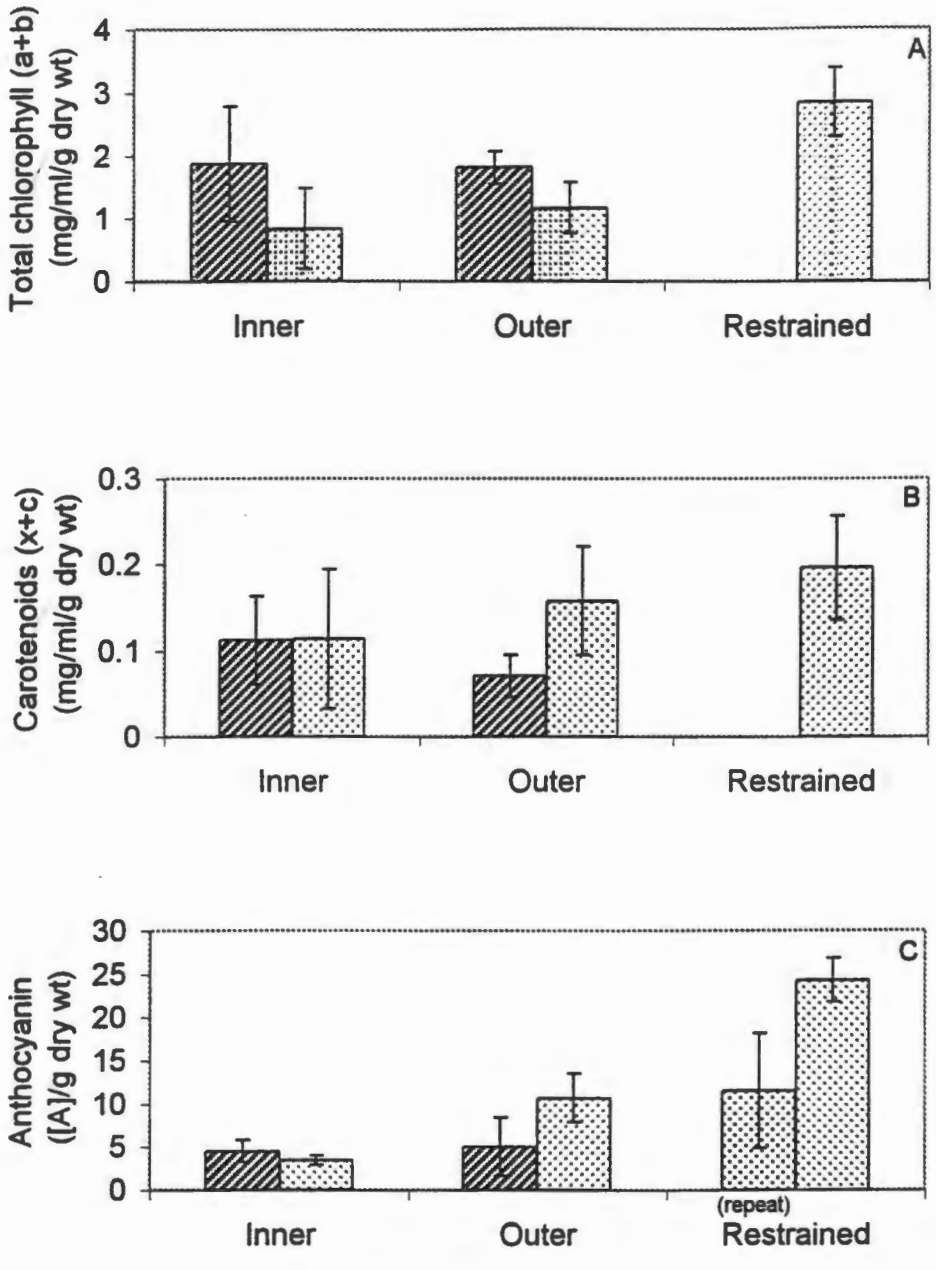

Figure 2: Pigment contents of inner-whorl, outer-whorl and restrained leaves of *Craterostigma wilmsii* in the hydrated (▨) (control) and dry (<5% Relative water content) (⁘) state. (A) Chlorophyll (a + b) content, (B) carotenoids (x + c), (C) Anthocyanin content.

Figure 3: *Craterostigma wilmsii* in the (A) dry state and (B) partially rehydrated state. Purple colouration of anthocyanin accumulation in the abaxial surfaces of outer-whorl leaves, is evident. The lack of purple (anthocyanins) in the adaxial surfaces is evident in (B) where leaves have begun to uncurl but have not yet lost anthocyanin.

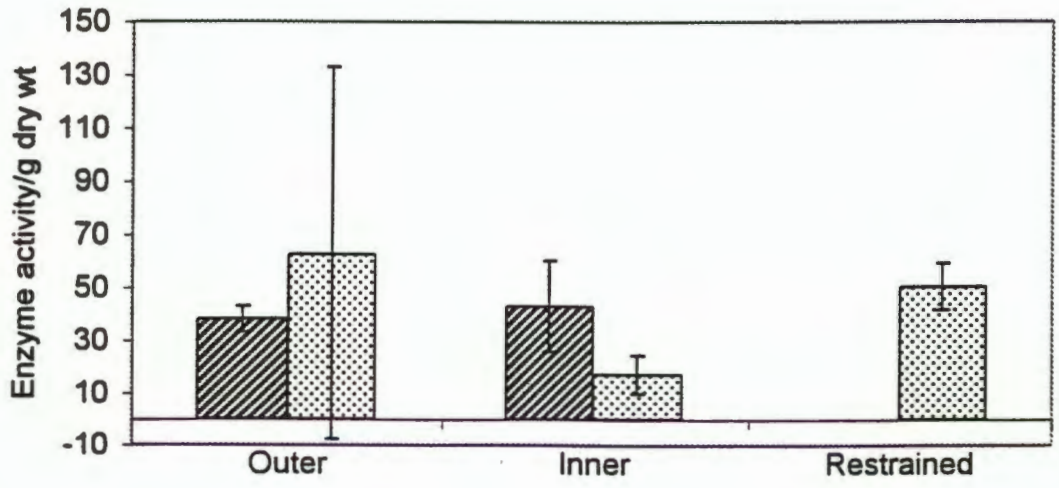

Figure 4: Ascorbate peroxidase activity of inner-whorl, outer-whorl and restrained leaves of *Craterostigma wilmsii*, in the hydrated (▨) and dry (<5% RWC) (▩) state.

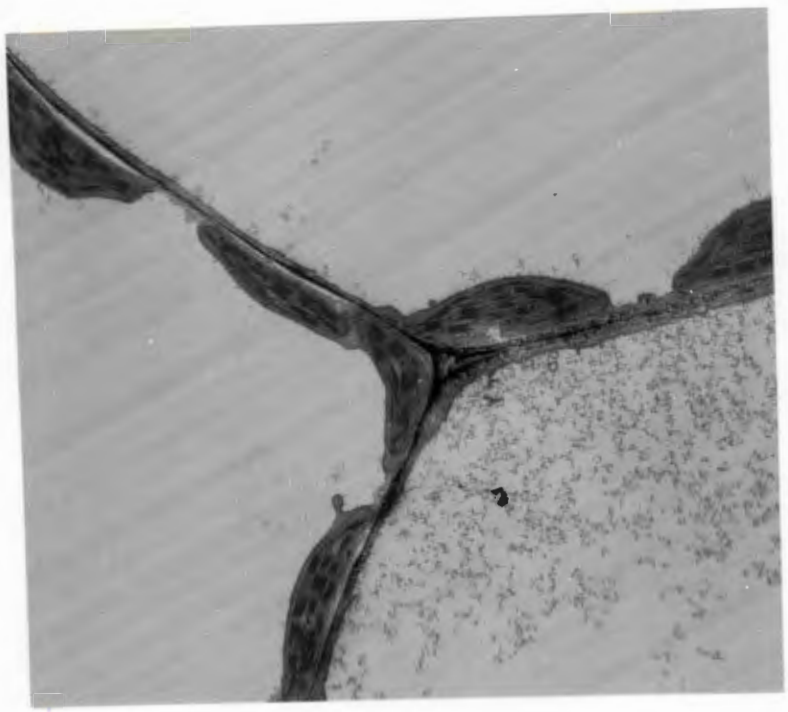

Figure 5: Subcellular organization of hydrated tissue of *Craterostigma wilmsii* mesophyll tissue cells (x 5640), typical for hydrated, photosynthetically active tissue for this species.

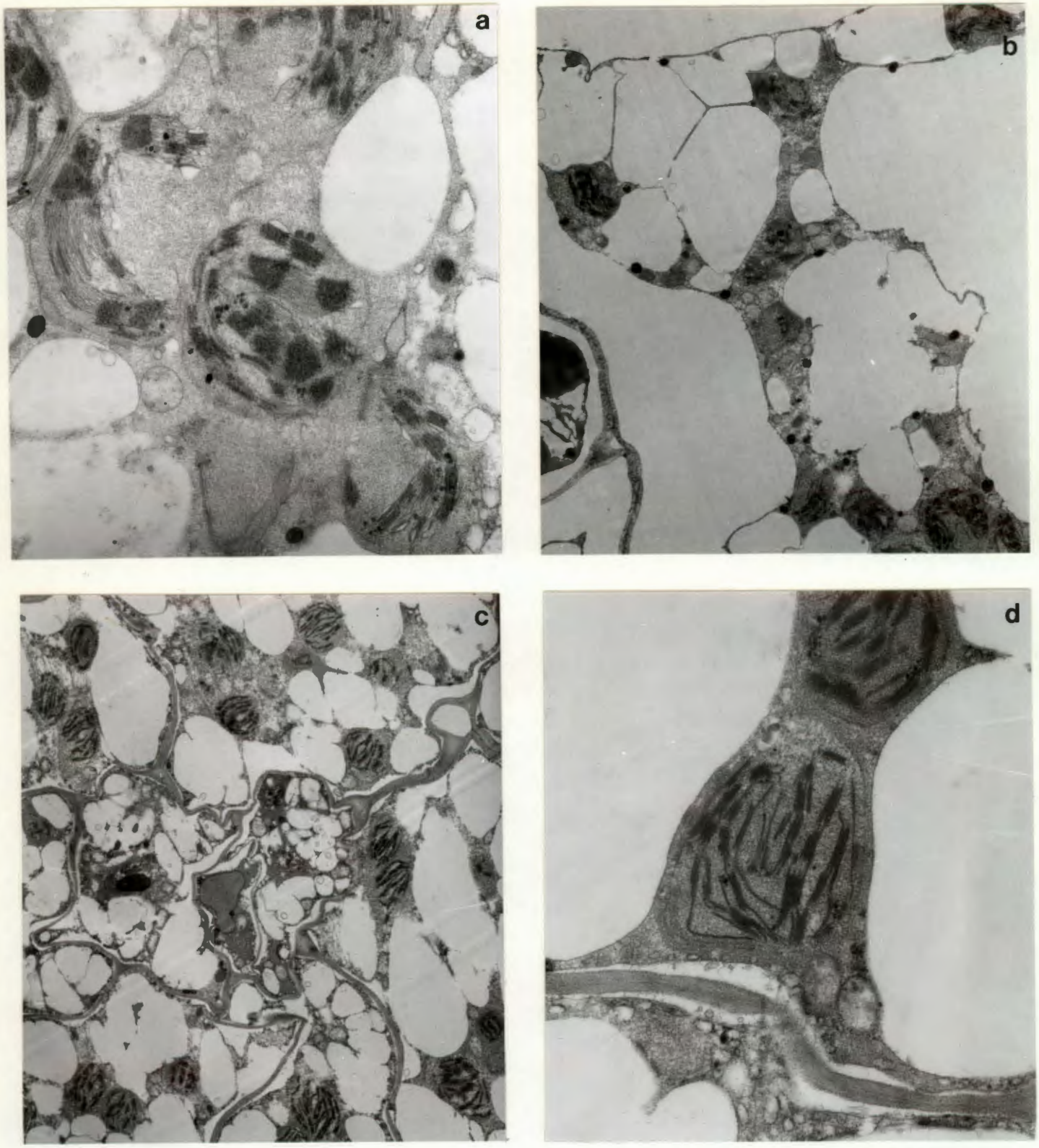

Figure 6: Subcellular organization of dry inner and outer-whorl mesophyll cells of *Craterostigma wilmsii*. Figure 6a (x 14000), and 6b (x 4500), show dry outer leaf cells. Much vacuolation, and some cell wall folding is evident. Figure 6c (x 4590), and 6d (x 21960) show dry inner leaf cells Considerable vacuolation is apparent, and cells appear more 'compact' than outer-leaf cells.

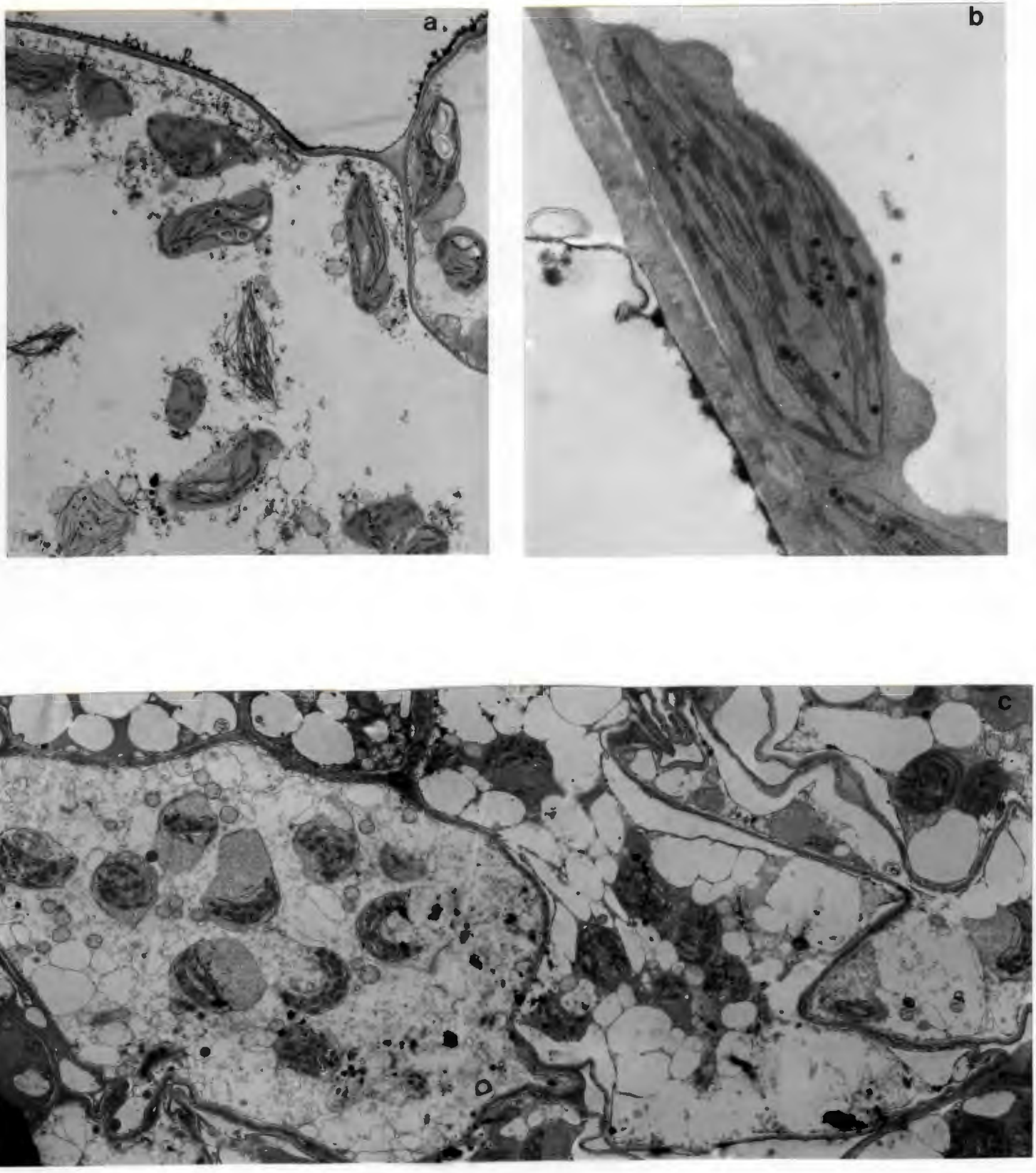

Figure 7: Subcellular organization of leaves that were restrained from drying; (figure 7b, x 4714) in the dry state, and (figures 7a [x 2180] and 7c [x 4330]) after rehydration. Cells from dry restrained leaves exhibit normal cell wall folding and some vacuolation. Some cells are damaged. Cells from rehydrated restrained leaves exhibit much damage, and accumulation of lipophillic debris along most cell walls.

DISCUSSION

When allowed to dry without interference, tissues of *C. wilmsii* survive light-induced stress. The mechanisms employed by this species to protect against light damage (reported by Sherwin and Farrant, 1998) are found in this study to differ between the young inner-whorl leaves that are shaded from light in the dry state, and the older outer-whorl leaves that curl over and shade the leaves below. Sherwin and Farrant (1998) have noted the importance of leaf movements in reducing leaf area exposed to light and the current study reinforces the importance of shading.

Although chlorophyll generally is maintained in homoiochlorophyllous resurrection plants, this study and others (Sherwin and Farrant, 1998; Koonjul *et al* 1999) have shown that there is some reduction during drying. When dried down at an average midday light intensity of 1 200 $\mu mol.m^{-2}$, the decrease in chlorophyll content of *C. wilmsii* was found to be about 30 % (Sherwin and Farrant, 1998). This is less than the reduction found in the current study (ca. 45 %) where midday light intensities during drying where higher (2 000 $\mu mol.m^{-2}$). The amount of chlorophyll reduction during drying of the HDT plant *M. flabellifolius* has also been reported to differ when light levels during drying differed - a greater decline in chlorophyll content occurring when light levels were greater (Koonjul, *et al* 1999). Higher light intensities during drying appear to cause greater losses of chlorophyll. This may be a mechanism to prevent hazardous light-chlorophyll interactions in the higher light. However, the shaded inner leaves of *C. wilmsii* had a greater decline in chlorophyll than that occurring in outer leaves, suggesting that this might not be the case. Very high levels of chlorophyll were found in the restrained leaves that were unable to curl over and shade their adaxial surfaces. It has been reported by Krol *et al* (1999) that light is required for chlorophyll synthesis in angiosperms, and the restrained tissue may not have been able to break down chlorophyll while remaining exposed to light, but instead continued to synthesise it. If this is the case, it may suggest that breakdown of chlorophyll in adaxial surfaces is not a normal part of the mechanism

to cope with light during dehydration.

Carotenoids are photosynthetic pigments and have also been implicated in photoprotection; as a 'sunscreen' pigment, and as an antioxidant (Larson, 1988; Hendry, 1993). Chlorophyll absorbs most of the light in the red-orange region and also the blue-violet region of the light spectrum (690-650 nm, and 480-400 nm respectively), whereas carotenoids *reflect* the yellow-orange to red wavelengths of light (Hendry, 1993). Carotenoid content did not change during drying in leaves of *C. wilmsii* that where dried down under light levels of 1 200 $\mu mol.m^{-2}$ (Sherwin and Farrant, 1998). Under the higher light levels of this study increases in carotenoids in dry outer leaves and dry-restrained leaves occurred, which did not occur in the inner leaves that were shaded. This indicates that in drying, carotenoid accumulation or biosynthesis is induced by the light-exposure of the leaves and may well be a protection mechanism in this species.

The upregulation of the other light filtering pigment analysed in this study, anthocyanin, is also induced by light-exposure during drying. Anthocyanins reflect light that is pale blue to mauve, appearing purple (Hendry, 1993). The shaded inner leaves showed no increase in anthocyanin content, whereas large increases occurred in the exposed outer and restrained leaves. Inner leaves that *are* exposed to light during drying acquire a very definite purple colouration (Fig. 3b). The lack of anthocyanin accumulation in inner leaves in this study is thus not due to an inability of young leaves to upregulate the pigment, but due to shading. Interactions of light with the chlorophyll that remains in shaded leaves is screened out by the curled-over outer leaves, and chemical light filters (in the form of anthocyanins or carotenoids) are apparently not required. The exceptionally high anthocyanin content initially found in dry restrained leaves was anomalous, considering the lack of purple colour in either the exposed adaxial layer, or in the abaxial layer lying against the soil. Results from the repeated experiments indicate that anthocyanins *are* upregulated in this tissue, but perhaps not to the same degree as initially indicated. Purple colouration present in dry leaves (that have been exposed to light and allowed to curl normally) is very distinctly in the abaxial layer, indicating that adaxial mesophyll cells may be unable to accumulate anthocyanins. If the adaxial mesophyll cells *cannot* accumulate anthocyanins, the pigment that is present may

be in the central cells of the leaves, concealed by the large amounts of chlorophyll remaining in this tissue (accounting for the lack of purple but presence of pigment).

Pigment analyses for inner, outer and restrained leaves of *C. wilmsii* indicate that self-shading through leaf movements (and the associated partial breakdown of chlorophyll) is an important part of these plants' protection mechanisms. Self-shading is very important for inner-whorl leaves, which do not even need to accumulate light filtering pigments as a consequence. Outer-whorl leaves accumulate light-filtering pigments, as they are still exposed to light. Shading of the adaxial leaf surfaces of the outer leaves may be an important protection mechanism anyway. The restrained tissue, which could not shade either the adaxial surface through curling or with anthocyanins, and did not break down any chlorophyll, was damaged by the light-exposure (figure 7).

Ascorbate peroxidase activity was found by Sherwin and Farrant (1998) to be the similar and low in hydrated and dry tissue, but upregulated during the drying process. It is during drying when disrupted metabolic processes can be expected to be most severe, and the need for antioxidant enzymes highest (Pammenter and Berjak, 1999). The mean level of AP activity for dry tissue did not differ from hydrated tissue in this study (figure 4), in keeping with the results of Sherwin and Farrant (1998). This mean level masks the interesting difference between inner and outer leaves. The high levels of AP activity in dry, light-exposed leaves (outer curled, and outer restrained) indicate that free radical production is probably still occurring in exposed leaves inspite of light avoidance strategies (curling and the related chlorophyll breakdown, anthocyanin production and carotenoid production). Low levels of AP activity in shaded leaves indicates that shading is indeed effective in preventing free radical production, negating the need for high levels of this antioxidant enzyme.

The leaves that were restrained from curling incurred damages during drying that are unexpected in resurrection plants and the damage was exacerbated during rehydration. These leaves had high chlorophyll levels, which increase the risk of free radical production, and hence damage to cells, which they were unable to shade through leaf movements. Large amounts of membrane disruption incurred during drying of restrained

leaves became further evident on rehydration. Such disruption is an artefact of unmediated free radical levels (Larson, 1988; Smirnoff, 1993), indicating that the increased carotenoids, anthocyanins and AP activity were not sufficient for preventing damage by free radicals.

In HDT plants, self-shading is an important protection mechanism during desiccation that is aided by other biochemical changes that occur in leaf tissue. Outer-whorl leaves that remain exposed to light, and shaded inner-whorl leaves have very different levels of the other biochemical protectants. The virtual lack of increase in biochemical protectants in shaded tissue, indicates that a) shading is sufficient as a protective mechanism, and b) biochemical protectants are induced by light-stress during drying, and not by drying alone. Examination of plants dried under very low light levels would give further insight into the relative importance of shading and biochemical changes for protection of drying *C. wilmsii*.

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