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# A comparison of mechanisms of desiccation tolerance among three angiosperm resurrection plant species

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*Key words:* Antioxidants, Mechanical stress, Oxidative stress, Photosynthesis, Vacuolation

**Abstract**

The mechanisms of protection against mechanical and oxidative stress were identified and compared in the angiosperm resurrection plants *Craterostigma wilmsii, Myrothamnus flabellifolius* and *Xerophyta humilis*. Drying-induced ultrastructural changes within mesophyll cells were followed to gain an understanding of the mechanisms of mechanical stabilisation. In all three species, water filled vacuoles present in hydrated cells were replaced by several smaller vacuoles filled with non-aqueous substances. In *X. humilis*, these occupied a large proportion of the cytoplasm, preventing plasmalemma withdrawal and cell wall collapse. In *C. wilmsii*, vacuoles were small but extensive cell wall folding occurred to prevent plasmalemma withdrawal. In *M. flabellifolius*, some degree of vacuolation and wall folding occurred, but neither were sufficient to prevent plasmalemma withdrawal. This membrane was not ruptured, possibly due to membrane repair at plasmodesmata junctions where tearing might have occurred. In addition, the extra-cytoplasmic compartment appeared to contain material (possibly similar to that in vacuoles) which could facilitate stabilisation of dry cells.

Photosynthesis and respiration are particularly susceptible to oxidative stress during drying. Photosynthesis ceased at high water contents and it is proposed that a controlled shut down of this metabolism occurred in order to minimise the potential for photo-oxidation. The mechanisms whereby this was achieved varied among the species. In *X. humilis,* chlorophyll was degraded and thylakoid membranes dismantled during drying. In both *C. wilmsii* and *M. flabellifolius*, chlorophyll was retained, but photosynthesis was stopped due to chlorophyll shading from leaf folding and anthocyanin accumulation. Furthermore, in *M. flabellifolius* thylakoid membranes became unstacked during drying. All species continued respiration during drying to 10% relative water content, which is proposed to be necessary for energy to establish protection mechanisms. Activity of antioxidant enzymes increased during drying and remained high at low water contents in all species, ameliorating free radical damage from both photosynthesis and respiration. The nature and extent of antioxidant upregulation varied among the species. In *C. wilmsii*, only ascorbate peroxidise activity increased, but in *M. flabellifolius* and *X. humilis* ascorbate peroxidise, glutathione reductase and superoxide dismutase activity increased, to various extents, during drying. Anthocyanins accumulated in all species but this was more extensive in the homoiochlorophyllous types, possibly for protection against photo-oxidation.

## Introduction

Most plants are unable to survive desiccation to the air-dry state. There are, however, a small number of species from every major class of plant except gymnosperms that do tolerate desiccation (Bewley & Krochko 1982). Such plants, termed resurrection plants (Gaff 1971), are studied in attempts to understand the mechanisms of desiccation tolerance, often with the ultimate aim of identifying genes which can be used to bioengineer crops for improved tolerance to water stress.

The mechanisms of desiccation tolerance in lower order resurrection plants (lichens, algae and bryophytes) differ from those present in angiosperms (Oliver & Bewley 1997 1997; Oliver et al. 1998)

and since crops are angiosperms, we are interested in mechanisms present in the latter. In those plants, tolerance is achieved largely by induction of protection mechanisms during dehydration (reviewed by Gaff 1989; Farrant & Sherwin 1997; Oliver & Bewley 1997; Oliver et al. 1998) although some repair of desiccation-and/or rehydration-induced damage is necessary.

Drying causes a number of subcellular stresses which must be ameliorated if plants are to survive. These include:

- mechanical stress associated with turgor loss (Iljin 1957) which occurs in the water potential range $-1.5$ to $-3$ Mpa or at relative water contents (RWC) of ca. 100–50% (Vertucci & Farrant 1995);
- free radical mediated damage (oxidative stress) brought about by unregulated metabolism at intermediate water contents ($-11$ to $-3$ Mpa or 45–25% RWC) (Hendry 1993; Smirnoff 1993; Vertucci & Farrant 1995; Navari-Izzo et al. 1997b); and
- the destabilisation or loss of macromolecular integrity at low water contents ($-150$ Mpa; $< 10$% RWC) (Leopold & Vertucci 1986; Crowe et al. 1992; Vertucci & Farrant 1995).

The majority of studies reported to date have concentrated on mechanisms whereby loss of integrity of membranes and macromolecules might be prevented (see reviews by Vertucci & Farrant 1995; Oliver & Bewley 1997; Oliver et al. 1998; Berjak & Pammenter 1999). In this regard, it is widely believed that protection is afforded by the accumulation of various proteins, sugars and 'compatible solutes' which serve to replace water and/or stabilise the subcellular milieu by vitrification (Crowe et al. 1992; Bianchi et al. 1991, 1993; Leopold et al. 1992; Dure 1993; Schneider et al. 1993; Ingram & Bartels 1996; Oliver & Bewley 1997; Ghasempour et al. 1998; Oliver et al. 1998).

Fewer studies have been done on mechanisms of alleviating oxidative stress, with most reporting only on the production of antioxidants (our use of the term includes enzymic and non-enzymic forms) involved in scavenging of free radicals produced by unregulated metabolism (Smirnoff 1993; Sgherri et al. 1994a,b; Navari-Izzo et al. 1997b; Kranner & Grill 1997; Sherwin & Farrant 1998). There appears to be variation in the nature of antioxidant response to drying among the angiosperm resurrection plants. Desiccation of *Sporobolus stapfianus* results in increased glutathione reductase (GR) and decreased ascorbate peroxidase (AP) activity (Sgherri et al. 1994a), whereas in *Boea hygroscopica*, glutathione accumulates, GR activity declines and AP activity remains constant (Sgherri et al. 1994b; Navari-Izzo et al. 1997a). In *Xerophyta viscosa*, AP and superoxide dismutase (SOD) activity increases but GR activity remains constant during drying (Sherwin & Farrant 1998). In *Craterostigma wilmsii* AP activity was maximal midway (50% RWC) during dehydration, but SOD and GR activity was greatest midway during rehydration (Sherwin & Farrant 1998).

Chloroplasts are particularly sensitive to (photo) oxidative damage (Halliwell 1987; Kaiser 1987). Light energy harnessed by chlorophyll cannot be dissipated via photosynthesis under water limiting conditions and can lead to formation of oxygen free radicals (Larson 1988; Smirnoff 1993; Navari-Izzo et al. 1997b). There is some evidence that, in addition to production of antioxidants, angiosperm resurrection plants employ physical means to prevent free radical formation. In poikilochlorophyllous resurrection plants, chlorophyll is degraded and thylakoid membranes are dismantled during drying (Tuba et al. 1996, Sherwin & Farrant 1998). Homoiochlorophyllous species retain chlorophyll, but use various mechanisms to prevent light-chlorophyll interaction while plants are dry (Sherwin & Farrant 1998; Koonjul et al. 2000).

Mechanical injury is the rupture of the plasma membrane due to the tension created by its contraction from the cell wall upon drying and its attachment to it via the plasmodesmata (reviewed by Levitt 1980; Gaff 1989; Vertucci & Farrant 1995). Sherwin (1995) and Vicre et al. (1999) have shown that mesophyll cell walls of *Craterostigma nanum* and *C. wilmsii* fold during drying and this may prevent development of tension between the plasmalemma and the wall. It is not yet clear how other angiosperm resurrection plants prevent plasmalemma rupture.

There is increasing evidence that there are differences among the angiosperm resurrection plants in their mechanisms of coping with a given stress. The variations in response to oxidative stress has been discussed above. Farrant et al. (1999) have shown that *Craterostigma wilmsii*, *Myrothamnus flabellifolius* and *Xerophyta humilis* differ in their ability to tolerate rapid drying and have suggested that this might be related to differences in the nature of protection which must be induced during drying. There have been few other comparative studies on protection mechanisms in angiosperm resurrection plants.

The occurrence of different mechanisms of protection to a given stress has implications for bioengineering work, in that the genetic pathways needed for successful transformation to drought tolerance are likely to differ among crops. An understanding of the different mechanisms of tolerance which do exist might allow for more informed decisions as to which ones might be more effective in a particular crop.

The aim of this work was to review and compare the mechanisms of protection against mechanical and oxidative stresses which occur in the resurrection plants, *Craterostigma wilmsii* Engl., *Myrothanmus flabellifolius* (Bak.) and *Xerophyta humilis* (Bak.) Dur. and Schinz. Transmission electron microscopy was used to characterise the subcellular changes in leaf cells during desiccation as a means of understanding mechanisms associated with alleviation of mechanical stress. Photosynthesis and respiration, the reactions of which are particularly prone to the production of oxygen free radicals under conditions of water stress (Larson 1988; Hendry 1993; Smirnoff 1993; Navari-Izzo et al. 1997b), were measured to determine the water contents at which such metabolism was switched off and for correlation with changes in free radical scavenging activities. The latter included measurement of changes in (i) antioxidant enzyme activity; (ii) concentration of photopigments and anthocyanins, (iii) subcellular organisation of chloroplasts and mitochondria and (iv) leaf movements which would minimise light-chlorophyll interaction. Since in angiosperms resurrection plants protection is induced during dehydration, we characterised only the changes occurring during water loss.

## Methods

### *Plant material*

Plants were collected and maintained in a glasshouse as previously described (Sherwin & Farrant 1996). Drying was initiated by withholding water from the soil. The average midday light intensity during the drying time course was 1200 $\mu$mol $m^{-2}$ $s^{-1}$. Leaves from at least 5 different plants from each of the species were sampled at regular intervals during drying for the procedures described below.

### *Relative water contents (RWC)*

RWC was measured using the standard formula: RWC = water content/ water content at full turgor and was expressed as a percentage. Water content was determined gravimetrically by oven drying at 70 °C for 48 h.

### *Ultrastructural studies*

Mesophyll leaf segments (approximately 5 $mm^2$) were processed for transmission electron microscopy (TEM) using the method previously reported for these tissues (Sherwin & Farrant 1996). Tissues were fixed in 2.5% gluteraldehyde in 0.1 M phosphate buffer (pH 7.2) containing 0.5% caffeine. Post-fixation was in 1% osmium tetroxide in phosphate buffer. Following ethanol dehydration, the material was infiltrated and embedded in epoxy resin (Spurr 1969). Tissues were sectioned using a Reichert Ultracut-S microtome, stained with uranyl acetate and lead citrate (Reynolds 1963) and viewed with a Jeol CX TEM. The proportional area contributions of the cell, cytoplasm and vacuoles were measured using a grid overlay (Berjak et al. 1992) on a total of 20 mesophyll cells from wet and dry plants, respectively.

### *Measurement of photosynthesis and respiration*

Rates of dark respiration ($R_d$) and light saturated net photosynthesis (A) were measured using an LCA3 (ADC Co. Ltd. Hoddesdon, UK) infrared gas analyser (IRGA), operated in differential mode at an ambient $CO_2$ concentration of 350 ppm. The parameters A and $R_d$ were calculated according to the equations of von Caemmerer & Farquhar (1981). Measurements were taken on 5 individual plants and repeated during at least two cycles of drying and rehydration. For comparison among the species, data are expressed as a percentage of the metabolism measured in control (hydrated, undried) tissues.

### *Pigment analysis*

Chlorophyll was extracted from 5 unpooled leaf samples in 100% acetone. The absorbance of extracts was measured at 470, 644.8 and 661.6 nm and chlorophyll ($a + b$) content was calculated using adjusted extinction coefficients (Lichtenthaler 1987). Total anthocyanin content was determined as previously described (Sherwin & Farrant 1998). Extraction was from 5 mg of tissue (from each of 5 leaves) in 10 ml of acidified methanol (methanol:water:HCl [79:20:1]) for 48 h at 4 °C. The extract was centrifuged and the supernatant made up to 12 ml by the addition of acidified methanol. The absorbance was measured at

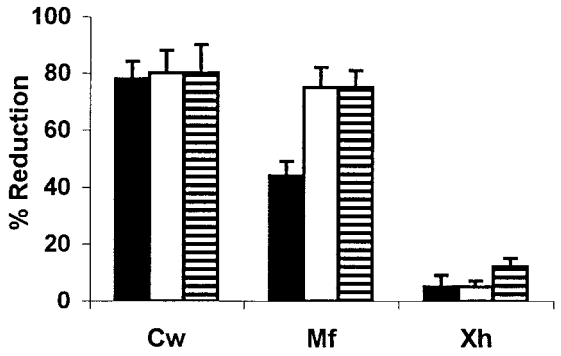


*Figure 1.* Percent reduction in mesophyll cell area (solid bars), cytoplasmic area (open bars) and vacuolar area (hatched bars) after drying to 5% RWC. Cw= *C .wilmsii* , Mf = *Myrothamnus flabellifolius*, *Xh* = *Xerophyta humilis*. Area proportions were calculated using a grid overlay technique. Cell area is that delimited by the cell wall; cytoplasmic area is that within the plasmalemma and vacuolar area is the sum of the areas within tonoplast membranes.

530 and 657 nm and the anthocyanin concentration [A] was determined by the formula $[A] = A_{530} - (\frac{1}{3}A_{657})$. Results were calculated as [A] per g dry mass (Mancinelli et al. 1975) and are presented as a percentage of the control. In the homoiochlorophyllous species, anthocyanins appeared to visibly accumulate more in leaves which remain exposed to light during drying than in those which became shaded from light. We thus determined the anthocyanin content of shaded and exposed leaves separately.

*Antioxidant enzyme assays*

Activities of ascorbate peroxidase (AP), glutathione reductase (GR) and CuZn superoxide dismutase (SOD) were determined as described previously (Sherwin & Farrant 1998). For AP determination, the rate of oxidation of ascorbate was measured at 290 nm (Wang et al. 1991) and activity was calculated as umol ascorbate mg protein$^{-1}$. GR activity was determined from the rate of oxidation of NADPH measured at 340 nm (Sgherri et al. 1994a) and calculated as nm NADH mg protein$^{-1}$. SOD activity was assayed by measuring inhibition of nitrite formation from hydroxyl ammonium chloride oxidation at 530 nm (Elstner & Heipel 1976). Units of activity were calculated from a standard curve obtained by treating a known concentration range of SOD from horseradish (Sigma) as described above. A unit of enzyme activity is defined as that amount which causes 50% inhibition of cytochrome c reduction (McCord & Fridovich 1969) per gram dry mass of leaf tissue extracted. For each enzyme assayed, triplicate extractions were performed from leaves of 5 plants

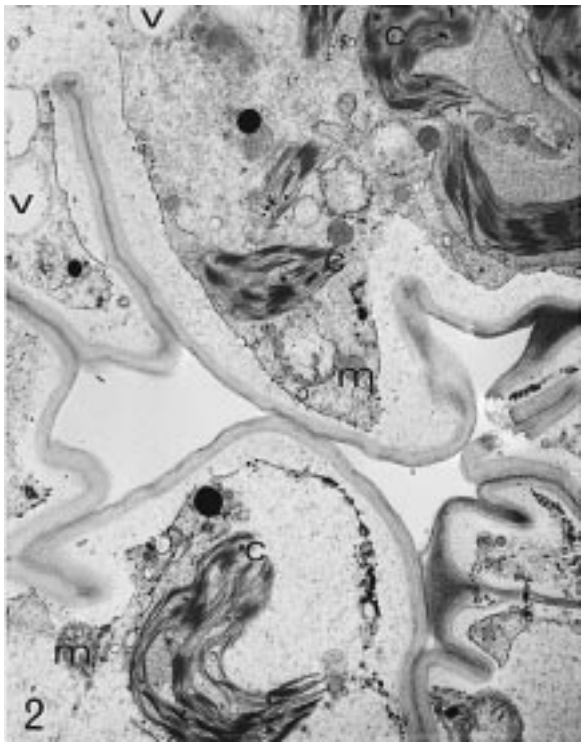


*Figure 2.* Subcellular organisation of dry (5% RWC) mesophyll cells of *C. wilmsii*. Note the folded cell walls, small vacuoles (v) and chloroplasts (c) with well defined thylakoid membranes. m, mitochondrion. × 5 681.

and enzyme activities are presented as a percentage of activity present in control tissue.

## Results

*Cellular changes during dehydration: minimising mechanical stress*

The subcellular organisation of mesophyll cells from hydrated leaves of all three resurrection species were typical of most other angiosperms, having a single central vacuole with cytoplasm occurring peripherally and adjacent to the cell wall (not shown). Dehydration to 5% RWC resulted in a 78% and 44% reduction in cell area in *C. wilmsii* and *M. flabellifolius* respectively, but only a 3% reduction in *X. humilis* (Figure 1). The reduction in cell size in *C. wilmsii* was entirely due wall folding which occurred in all mesophyll cells (Figure 2). In *M. flabellifolius*, wall folding was less extensive and did not occur in all cells (Figures 4A and 4B). No wall folding occurred in *X. viscosa* (Figure 3). In *C. wilmsii* and *X. humilis* the area occupied by cyto-

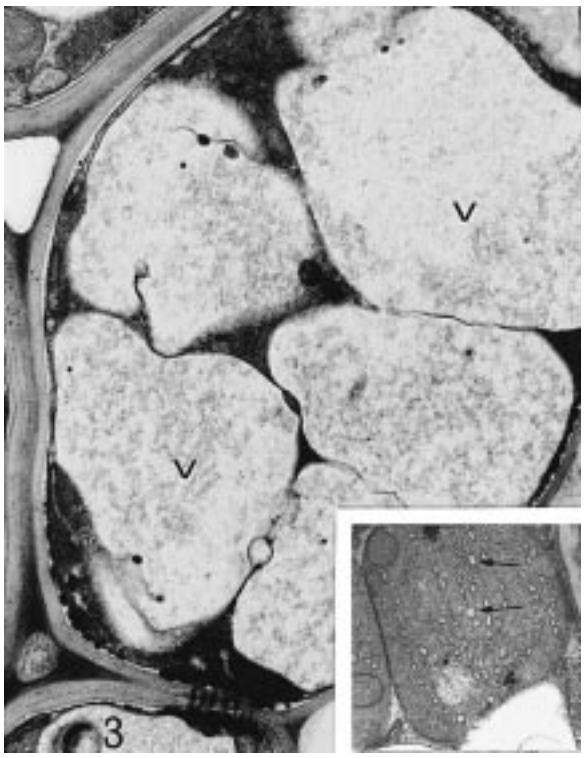


*Figure 3.* Subcellular organisation of dry (5% RWC) mesophyll cells of *X. humilis*. No plasmalemma withdrawal is evident. Vacuoles (v) occupy a large proportion of the cytoplasm. × 8 194. Inset: Chloroplast from a dry leaf of *X. humilis*. Thylakoid membranes are vesiculated (arrowed) × 7 500.

plasm declined by a similar amount as that of the cell wall boundary (Figure 1) and thus plasmalemma withdrawal from the wall was negligible (Figures 2 and 3). The plasmalemma remained intact and there was no electrolyte leakage from dry tissues (not shown), suggesting that little mechanical stress had occurred in these species. In *M. flabellifolius* there was considerable reduction in cytoplasmic area relative to that of the cell wall (Figure 1) and plasmalemma withdrawal had occurred in most cells (Figure 4). However, the plasmalemma appeared intact, even at sites where plasmodesmata had previously attached to the wall (arrowed, Figure 4). Low levels of electrolyte leakage occurred from dry leaf tissues ($< 5$ $\mu$Siemen g dry mass$^{-1}$ in both wet and dry leaves), confirming plasmalemma integrity. The extra-cytoplasmic space had an electron dense appearance similar to that of the vacuoles from dry tissue.

During drying of all three species, the central vacuole divided into a number of smaller ones, the content of which became increasingly electron opaque as water content declined (Figures 2–4). The size of vacuoles present in dry leaves varied among the species, with those in *X. humilis* being larger and occupying more of the cytoplasmic volume than those in *C. wilmsii* and *M. flabellifolius* (Figures 1–4). The nature of the vacuolar content is not known as yet, but it is unlikely to be water, since these vacuoles were present in leaves in which the only water present (ca. 0.1 g $H_2O$ g dry mass$^{-1}$) must have been strongly bound to surfaces (Vertucci & Farrant 1995). The replacement of water in vacuoles with non-aqueous substances would serve to maintain volume within cytoplasm and prevent undue plasmalemma withdrawal and tearing upon drying. As such vacuoles made up a considerable proportion of the cytoplasm in dry mesophyll cells in *X. humilis* they are likely to have contributed substantially to the mechanical stabilisation this tissue.

*Photosynthesis and respiration during dehydration: Minimising oxidative damage*

Figure 5 shows that photosynthesis (A) ceased at far higher RWCs than respiration (B) in all three species. Net assimilation started to decline between RWC of 80 and 75% RWC and had ceased by 55% (1.5 g $H_2O$ g$^{-1}$ dry mass) in *M. flabellifolius*, 47% in *X. humilis* (1.2 g $H_2O$ g$^{-1}$ dry mass ) and 40% (2.0 g $H_2O$ g$^{-1}$ dry mass) in *C. wilmsii*. Respiration declined only after drying to 40% RWC in *X. humilis* and *M. flabellifolius* and to 20% in *C. wilmsii*, and ceased in all three species at RWC of $\leq$10% ($\pm$ 0.2 g $H_2O$ g$^{-1}$ dry mass). Mitochondria retained integrity and had defined cristae during drying (see, e.g., Figure 2). Since these plants survived disruption of these metabolic activities, we assume that free radicals were either prevented from forming and/or their damage was alleviated by antioxidants. The nature of such protection mechanisms were investigated.

Cessation of photosynthesis was unlikely to be due to limited $CO_2$ availability, since respiration (and thus gas exchange) continued to low water contents. In some resurrection plants, reduction in photochemical activity has been associated with chlorophyll degradation and/or masking (Sherwin & Farrant 1998; Tuba et al. 1996; 1998). The changes in chlorophyll content during drying are given in Figure 6A. In *X. humilis* chlorophyll was completely degraded during drying, its loss being initiated coincident with the onset of decline in net assimilation. In addition, thylakoid membranes were dismantled, and were completely vesiculated in dry leaves (inset, Figure 3) and this

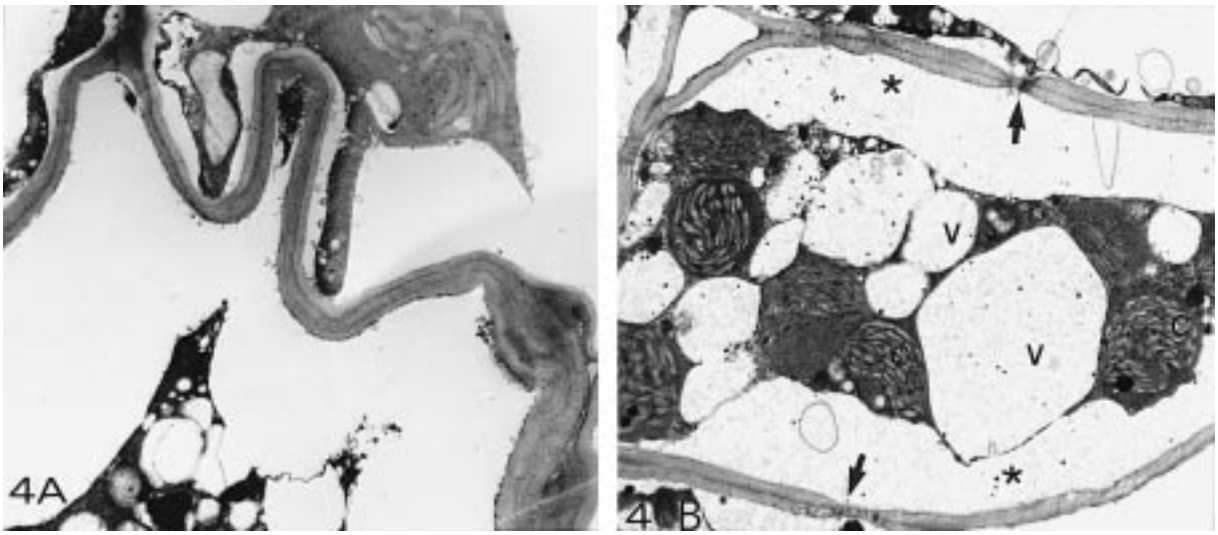


*Figure 4.* Subcellular organisation of dry (5% RWC) mesophyll cells of *M. flabellifolius*. Wall folding (A, × 6300) occurs in some cells but not others (B, × 5 256). Plasmalemma withdrawal occurs in all cells but no tearing of this membrane is evident. Arrows indicate plasmodesmata connections which were present in hydrated tissue. Note the electron dense appearance of the extra-cytoplasmic space (*) which is similar in appearance to the vacuolar (v) content. Thylakoid membranes have a 'blistered' appearance. c, chloroplast.

species is thus typically poikilochlorophyllous. The dismantling of the photosynthetic apparatus is likely to have put a stasis on photosynthesis which, since it occurred at relatively high water contents, would have minimised the potential for photo-oxidation (Sherwin & Farrant 1998; Tuba et al. 1998).

*C. wilmsii* and *M. flabellifolius* retained 82 and 60% chlorophyll respectively during drying (Figure 6A) being therefore typically homoiochlorophyllous. In these species leaf folding occurred during drying (Figure 7) which served to shade all of the inner rosette leaves of *C. wilmsii* and the adaxial surfaces of leaves of *M. flabellifolius* from light during the later stages of dehydration. This shading would stop photosynthesis from occurring in those tissues and indeed, assimilation ceased when folding was nearing completion (compare Figures 5A and 7). Thylakoid membranes from chloroplasts of both shaded and exposed leaf surfaces of *C. wilmsii* remained intact during drying although they became displaced to one side of the chloroplast (Figure 2). In *M. flabellifolius* thylakoid membranes became increasingly 'blistered' during drying (Figure 4), which could have interfered with the membrane-associated photosynthetic reactions (Koonjul et al. 2000) and so also have played a role in stopping photosynthesis.

Anthocyanins reflect photosynthetically active light and can prevent light-chlorophyll interaction and thus photosynthesis. Furthermore, being antioxidants, they can minimise damage from free radicals which are produced (Larson 1988; Smirnoff 1993). Anthocyanin content increased markedly during drying of all species (Figure 6C), but was greatest in the homoiochlorophyllous species, where accumulation occurred preferentially in leaves which remained exposed to light throughout dehydration. In *C. wilmsii*, anthocyanin content of the outer leaves of the rosette increased markedly during drying from 80 to 50% RWC, reaching a maximum with the onset of leaf folding (Figure 7). Thus when abaxial surfaces of the outer leaves became exposed to direct light for the first time they had a high anthocyanin content which could serve to reflect light and mask chlorophyll and so minimise photo-oxidation. In *M. flabellifolius* anthocyanin accumulation occurred in abaxial surfaces during leaf folding, with maximum levels being present at 40% RWC when these surfaces were fully exposed to light (Figure 7). In the poikilochlorophyllous *X. humilis* anthocyanin accumulation was also complete by 40% RWC, the accumulation having an inverse trend to that of chlorophyll breakdown (compare Figures 6A and 6).

The activities of the anti-oxidant enzymes AP, GR and SOD during drying of the three species are given in Figure 8. The data indicate that there were differences among the species with respect to which of the antioxidants were upregulated, and the extent of upregulation which occurred during drying. In *C. wilmsii*

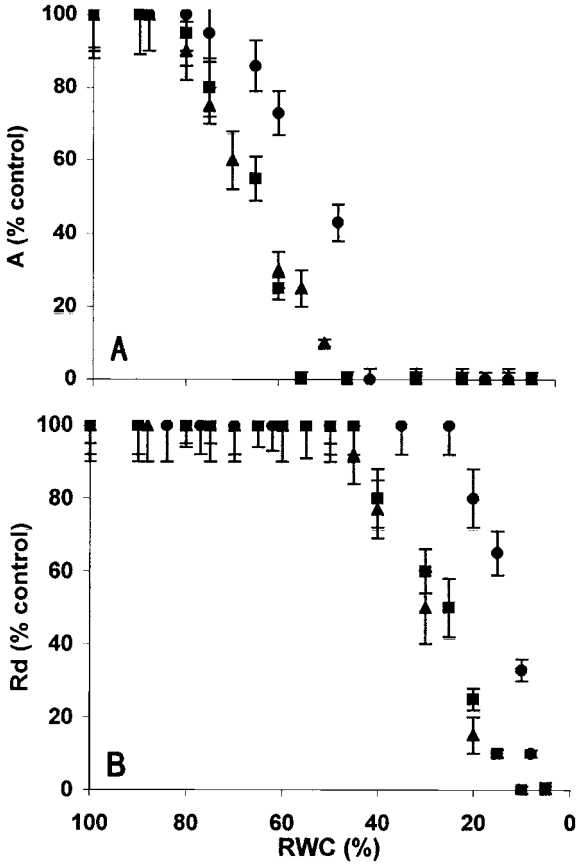


*Figure 5.* Changes in A: net assimilation [A]; and B, dark respiration [$R_d$], expressed as a percentage of metabolism typical of hydrated control tissue, during drying of *C. wilmsii* (●), *M flabellifolius* (■) and *X. humilis* (▲).

only AP activity increased (Figure 8A). As for the trend in anthocyanin accumulation, the increase in AP activity occurred at RWC before the onset of leaf folding. Activity was maximal at 40% RWC, coincident with the initiation of leaf folding, and declined with further water loss. This trend suggests involvement of AP in scavenging free radicals generated by photo-oxidation. However, because of the almost universal subcellular presence of this enzyme, and because activity remained elevated relative to control (undried) tissue, it is likely to also have played a role in protection against free radicals generated by respiration and other metabolism which continued to low water contents.

The activity of all of the anti-oxidant enzymes measured increased during drying of *M. flabellifolius* (Figure 8B) and *X. viscosa* (Figure 8C) and, unlike the situation in *C. wilmsii*, their activities did not

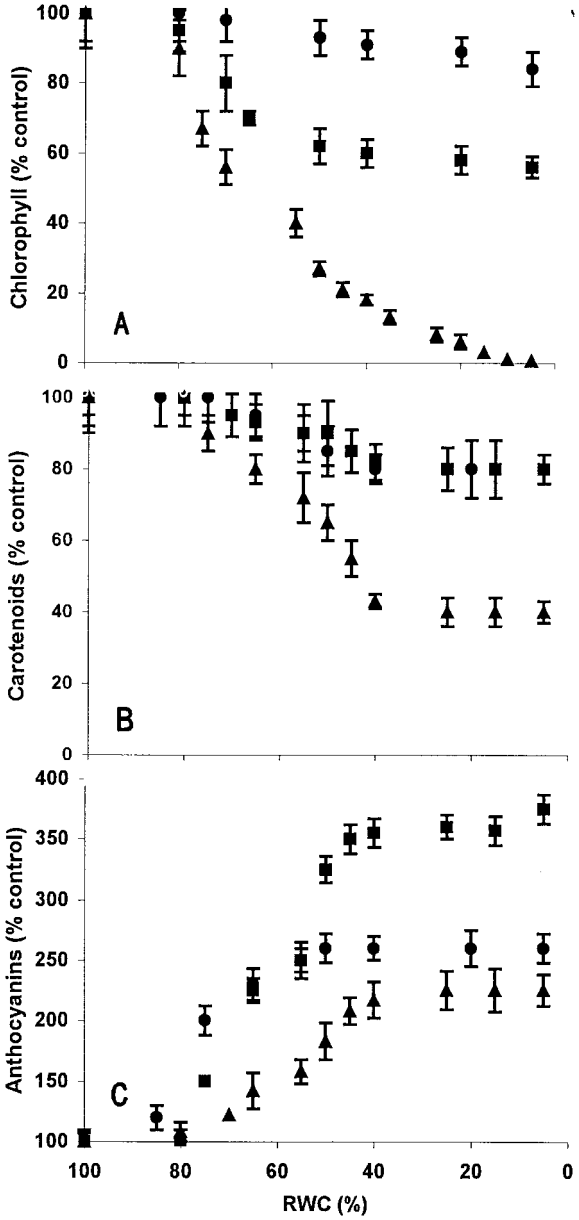


*Figure 6.* Changes in A, chlorophyll; B, carotenoid; and C, anthocyanin content, expressed as a percentage of pigment present in hydrated control tissue, during drying of *C. wilmsii* (●), *M flabellifolius* (■) and *X. humilis* (▲). Note: anthocyanin contents given for *C. wilmsii* and *M. flabellifolius* are those measured in the outermost layer of rosette leaves of *C. wilmsii* and abaxial surfaces of *M. flabellifolius* only. Since there were no changes in anthocyanin content of inner rosette leaves of *C. wilmsii* and adaxial surfaces of leaves of *M.flabellifolius*, these are not given.

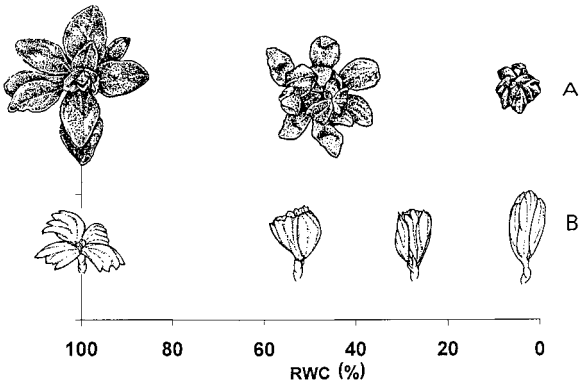


*Figure 7.* Diagrammatic representation showing the nature of leaf folding in *C. wilmsii* (A) and *M. flabellifolius* (B) during drying.

decline during the final stages of dehydration. In *M. flabellifolius*, AP activity increased most markedly (> 650%), whereas GR and SOD increased by 50 and 100%, respectively. The activity of all the enzymes reached a maximum by 40% RWC. The trend in GR and SOD activity in *X. humilis* was similar to that of *M. flabellifolius* (Figure 8C). AP activity also increased markedly in this species, but its activity continued to increase throughout dehydration. As for *C. wilmsii*, the trend of increasing antioxidant activity, coincident with the occurrence of other mechanisms for minimising light-chlorophyll interaction, suggests their participation in protection against photo-oxidation. However, the continued elevation of anti-oxidant enzyme activity at low water contents also suggests their participation in protection against mitochondrial (and other) free radical production.

## Discussion

Although there are shared features among the three species examined in this study, there are also significant differences in the mechanisms of protection induced against mechanical and oxidative stress. With respect to mechanical stabilisation, all the species retain a number of non-aqueous vacuoles in the dry leaves. These serve to occupy space in the cytoplasm, reducing the extent of plasmalemma withdrawal (and so tearing) from the cell wall during drying. The three species differ in the extent of such vacuolation and in other aspects of mechanical stabilisation.

In dry cells of *X. humilis* non-aqueous vacuoles fill most of the cytoplasmic space. These are proposed to create a sufficient back-pressure against the wall to prevent its collapse and plasmalemma withdrawal.

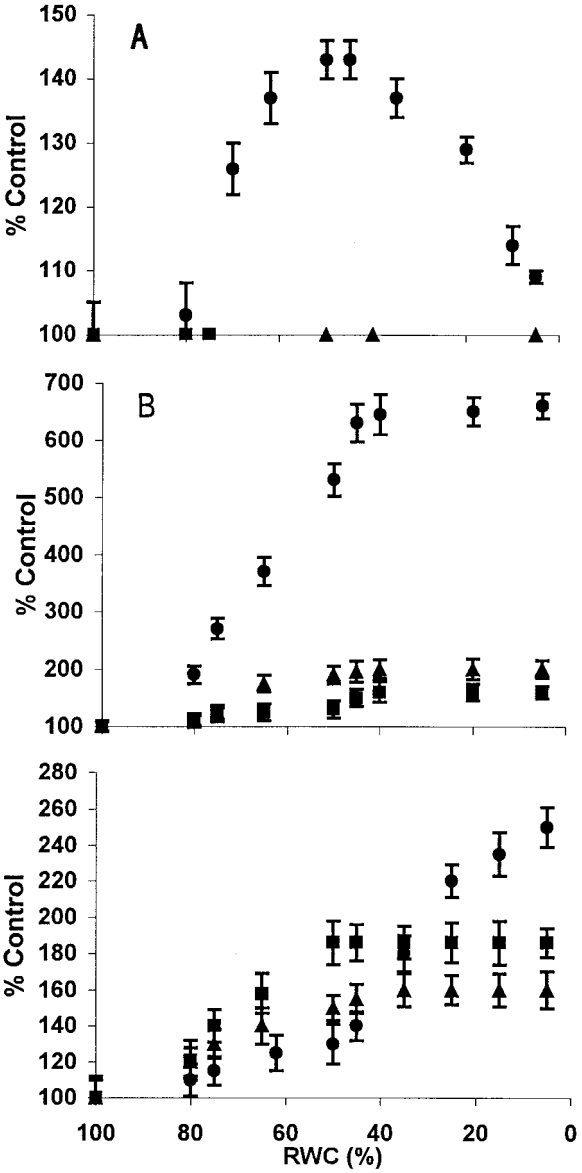


*Figure 8.* Changes in activity of the antioxidant enzymes ascorbate peroxidase (●), glutathione reductase (■) and superoxide dismutase (▲), expressed as a percentage of activity in hydrated control tissue, during drying of *C. wilmsii* (A), *M. flabellifolius* (B) and *X. humilis* (C).

A similar mechanism occurs in many desiccation-tolerant seeds, in which protein-filled vacuoles afford such mechanical stabilisation (Bewley & Black 1994). Dry tissues of *C. wilmsii* have only a small degree of vacuolation but the major contribution towards mechanical stabilisation appears to be the folding of cell walls during drying. A similar mechanism has been shown to occur in *C. nanum* (Sherwin 1995). Vicre

et al. (1999) have shown that wall folding is accompanied by changes in wall architecture and chemistry, and it is thus a controlled process rather than a consequence of wall collapse upon drying. Dry tissues of *M. flabellifolius* have a small degree of vacuolation and some wall folding but neither mechanism appears to be sufficiently developed to prevent plasmalemma withdrawal. Nevertheless, membrane rupture does not occur, even at points where it was previously attached to the wall via plasmadesmata. Thus part of the survival mechanism may be the ability to repair the plasmalemma at these sites. The electron dense appearance of the extra-cytomic space might suggest the presence of materials, possibly similar to those in the vacuoles, in this compartment. This would contribute considerably to the stabilisation of the subcellular milieu in this species.

The metabolism associated with photosynthesis and respiration is particularly sensitive to free radical production under conditions of water stress (Larson 1988; Hendry 1993; Smirnoff 1993; Navari-Izzo et al. 1997b), but oxidative damage is effectively prevented in resurrection plants. In the three species studied here, photosynthesis ceases at far higher water contents than respiration. This has also been reported for other angiosperm resurrection plants (Tuba et al. 1996b; 1997; 1998). Tuba et al. (1998) have suggested that photosynthesis is more susceptible than respiration to oxidative damage, and that continuation of the latter to low water contents is necessary to provide energy for the acquisition of subcellular protection. We concur with those authors and propose that a controlled shut-down of photosynthesis occurs (see discussion below) at relatively high water contents in order to minimise photo-oxidation. We further suggest that the need for respiration to continue at water contents, where metabolism is likely to become unregulated, requires the presence of efficient antioxidant systems to ameliorate free radical production. Again, we have observed differences among the resurrection plants in the mechanisms used to shut down photosynthesis and in the nature of antioxidant response to drying.

Cessation of photosynthesis is achieved in the different species by physical and/or chemical means. In *X. humilis*, chlorophyll is degraded and thylakoids dismantled, which effectively puts a stasis on photosynthesis at leaf RWC's of $< 50\%$. A similar mechanism (i.e., poikilochlorophylly) has been reported for several of the other *Xerophyta* species and in *Borya nitida* (Hetherington et al. 1982; Gaff 1989; Tuba et al. 1996; Sherwin & Farrant 1998). *C. wilmsii* and *M. flabellifolius* retain most of their chlorophyll during drying, but photosynthesis is switched off due to chlorophyll shading through leaf folding and anthocyanin accumulation. In addition to this, in *M. flabellifolius* separation of thylakoid membranes occurs which may contribute to the cessation of photochemical activity. Thus even among these homoiochlorophyllous species, there are differences in response to light stress during drying. It is not known to what extent other homoiochlorophyllous species use chlorophyll shading and thylakoid re-arrangement as mechanisms to stop photosynthesis and so minimise photo-oxidation, as no studies have reported specifically on this. Some degree of leaf folding and re-organisation of thylakoid ultrastructure occurs in *Sporobolus stapfianus* (Altus & Hallam 1980; Dalla Vecchia et al. 1998) which might play a role in this regard.

Antioxidant protection, in the form of enzyme activity and anthocyanin production, increases markedly during drying of the three species examined in this study, confirming the need for antioxidant processes in these plants. Data from other studies suggest that this is indeed true for most other resurrection plants (Smirnoff 1993; Sgherri et al. 1994a,b; Kranner & Grill 1997; Navari-Izzo et al. 1997a,b; Sherwin and Farrant 1998). In all three species, anthocyanin accumulation occurs in parallel with the onset of mechanisms which switch off photosynthesis, and thus these chemicals are likely to specifically ameliorate photo-oxidative damage. Although there were also correlations in patterns of antioxidant enzyme activity with changes in photosynthetic or respiratory activity, it is not possible to interpret with confidence, the exact nature of such relationships. What is apparent from this study however, is that there are differences among species in the type of antioxidant enzymes upregulated and the timing and extent of the antioxidant response. For example (Figure 8), in *C. wilmsii* only AP activity is upregulated, whereas in *M. flabellifolius* and *X. humilis* AP, GR and SOD activities increase during drying. The trends in AP activity in relation to water content differ among the species. In *C. wilmsii*, activity increases upon drying to 50% RWC after which it declines. In *M. flabellifolius* activity increases markedly during drying to 40% RWC but does not change upon further water loss and in *X. humilis* activity increases sigmoidally with declining water content. The increase in AP activity is greatest in *M. flabellifolius* (ca. 650%) and least in *C. wilmsii* (ca. 150%). Although few other studies have compared

antioxidant responses among different resurrection plants, comparison of reports on individual species suggest that the nature of antioxidant responses do vary widely among species (see, for example, Sgherri et al. 1994a,b; Navaro-Izzo et al. 1997a,b; Sherwin & Farrant 19989). We measured the activity of only a few enzymes involved in antioxidant processes; a large number of other potential antioxidants (including many of the phenolics and compatible solutes [cited in Smirnoff 1993; Kranner & Grill 1997]) and enzymes are reported to occur in plants. Thus the variation in antioxidant responses of resurrection plants is likely to be considerable. However, we propose that for each resurrection species there is a well balanced interplay of antioxidants and enzymes required to scavenge free radicals generated during desiccation, although the exact nature of these and timing of their upregulation will vary among different resurrection plant types.

Our understanding of desiccation tolerance in angiosperm resurrection plants is far from complete, but it is clearly a complex process, requiring the induction and interaction of numerous processes. In the present study we have shown that these can vary among species, which will indeed impact on bioengineering work.

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