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Protection mechanisms against excess light in the resurrection plants *Craterostigma wilmsii* and *Xerophyta viscosa*

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Abstract

Mechanisms of avoidance and protection against light damage were studied in the resurrection plants *Craterostigma wilmsii* and *Xerophyta viscosa*.

In *C. wilmsii*, a combination of both physical and chemical changes appeared to afford protection against free radical damage. During dehydration leaves curled inwards, and the abaxial surface became exposed to light. The tissue became purple/brown in colour, this coinciding with a three-fold increase in anthocyanin content and a 30% decline in chlorophyll content. Thus light-chlorophyll interactions are progressively reduced as chlorophyll became masked by anthocyanins in abaxial layers and shaded in the adaxial layers. Ascorbate peroxidase (AP) activity increased during this process but declined when the leaf was desiccated (5% RWC). During rehydration leaves uncurled and the potential for normal light-chlorophyll interaction was possible before full hydration had occurred. Superoxide dismutase (SOD) and glutathione reductase (GR) activities increased markedly during this stage, possibly affording free radical protection until full hydration and metabolic recovery had occurred.

In contrast, the leaves of *X. viscosa* did not curl, but light-chlorophyll interactions were minimised by the loss of chlorophyll and dismantling of thylakoid membranes. During dehydration, free radical protection was afforded by a four-fold increase in anthocyanin content and increased activities of AP, GR and SOD. These declined during rehydration. It is suggested that potential free radical damage may be avoided by the persistence of anthocyanins during the period of thylakoid membrane re-assembly and full chlorophyll restitution which only occurred once the leaves were fully rehydrated.

Abbreviations: RWC = (relative water content); AP = (ascorbate peroxidase); GR = (glutathione reductase); SOD = (superoxide dismutase); UV-Vis = (Ultraviolet – visible); HCL = (hydrochloric acid); [A] = (anthocyanin concentration); GSSG = (oxidised glutathione); SDS = (sodium dodecyl sulphate)

1. Introduction

Desiccation tolerant organisms are able to withstand a number of stresses [for reviews see references 1, 5, 19] brought about by, or in association with, extreme water loss. The presence of light during dehydration can be extremely damaging to photosynthetically active tissues. Under (even mild) water stress conditions, closure of stomata can result in excitation energy being transferred from chlorophyll to oxygen with the subsequent formation of oxygen free radicals. If unquenched, these molecules can cause considerable damage to the subcellular milieu [6, 16].

Plants cope with excess light energy in numerous ways. They can prevent the absorption of excess light by leaf movements, by the production of "sun screen" pigments (e.g. carotenoids and anthocyanins) and/or by the production of free radical quenching molecules and enzymes. However, under extreme conditions even these coping mechanisms can become depleted and

damage in the form of photo-bleaching of chlorophyll and lipid peroxidation of membranes occurs. (Refer to reviews by Larson [6] and Smirnoff [16] for details on mechanisms to cope with active oxygen in plants).

Most resurrection plants grow in shallow soils on rocky outcrops where there is little shade and water supply is more often than not limited. Thus these plants would be subject to light-related stress as they dehydrate, in the dry state, and during initial rehydration when, due to limited water availability, normal physiological activity is suspended. The periods of dehydration and rehydration are probably the most critical as, in the presence of limited water, the deleterious reactions can proceed, although the damage caused may not be readily repaired. As they are able to survive these conditions, such plants must have particularly good mechanisms for coping with this excess light energy.

Poikilochlorophyllous resurrection plants lose their chlorophyll and thylakoid membranes are dismantled during dehydration [3, 15, 18] and it has been suggested that this may be a protective mechanism to prevent photo-oxidation under conditions when photosynthesis is not possible [15, 16]. Homoiochlorophyllous plants presumably have alternative mechanisms to prevent photo-oxidation or are able to repair photo-oxidation related damage. Studies on the resurrection fern *Polypodium polypoides* [11] and on the resurrection moss *Tortula ruralis* [12] have shown that there was more damage, and recovery times were longer, when plants were dried under high light compared to low light conditions.

There appears to be considerable variation in the antioxidant responses of resurrection plants to desiccation and rehydration. For example, desiccation of *Sporobolus stapfianus* resulted in increased glutathione reductase (GR) and decreased ascorbate peroxidase (AP) activity [13] while in *Boea hygroscopica*, GR activity decreased and AP activity remained constant on desiccation [14]. In *Tortula ruralis*, AP activity was higher in plants grown under high light and activity decreased on drying. Catalase activity also decreased while superoxide dismutase (SOD) activity did not change on drying [12]. All of these studies measured antioxidant activities in the control, dry and rehydrated state without any intermediate stages. There have been few, if any, studies on protective pigments, such as anthocyanins, in resurrection plants.

Besides a recent study on the comparative rehydration physiology of three different resurrection plants [15], there have been few attempts to compare tolerance mechanisms among different resurrection plants. The aim of the present study was to examine the responses of a homoiochlorophyllous species, *Craterostigma wilmsii* Engl., and a poikilochlorophyllous species, *Xerophyta viscosa* Baker, to desiccation and rehydration under high light conditions. Special emphasis was placed on characteristics which would minimise the effect of excessive light damage such as leaf movements, changes in protective pigments as well as the activities of some antioxidant enzymes.

2. Materials and methods

2.1 Materials

Plants were collected and maintained in a glasshouse as previously described [15]. The average midday light intensity during the drying and rehydration experiments was 1 200 μmol.m^{-2}.s^{-1}. Drying was initiated by withholding water from the soil. Once desiccated (<5% relative water content [RWC]) plants were left in the dry state for up to one month before rewatering to rehydrate the plants. The different treatments were as follows:

1) Fresh – leaves of well watered plants which had not been dried.
2) 50% RWC – leaves dried on the plant to 50% RWC (RWC = water content / water content at full turgor – expressed as a percentage).
3) Dry – leaves dried on the plant to an air-dried state (< 5% RWC).
4) Partial recovery – leaves taken from plants 24 and 48 h after watering for *C. wilmsii* and *X. viscosa* respectively. Leaves had usually regained 80% RWC by this time but had not recovered full physiological activity [15].
5) Full recovery – leaves taken from plants which had been rehydrated for 48 and 120 h for *C. wilmsii* and *X. viscosa* respectively.

Leaf movements and colouration in the wet and dry state were noted.

2.2 Methods

2.2.1 Ultrastructure of chloroplast

Leaf tissues were processed for transmission electron microscopy using the method previously reported for these tissues [15].

2.2.2 Pigment analysis
Assays were performed using a Cary UV – Vis Spectrophotometer (Varian, Australia). Five replicates were performed for each treatment (outlined in the materials section above). The plants were dehydrated and rehydrated twice, thus a total of ten measures per treatment were obtained.

2.2.2.1 Photosynthetic pigments
Photosynthetic pigments were extracted from leaf samples in 100% acetone. The absorbance of the extracts was measured at 470, 644.8 and 661.6 nm. Chlorophyll (a + b) and carotenoid (x + c) contents were calculated using adjusted extinction coefficients [7].

2.2.2.2 Anthocyanin content
Five mg lyophilised leaf material was extracted in 10 ml of acidified methanol (methanol:water:HCl [79:20:1]) for 48 h at 4 °C. The extract was then centrifuged and the supernatant was made up to 12 ml by the addition of acidified methanol. The absorbance was measured at 530 and 657 nm and the anthocyanin concentration [A] was determined by the formula [A] = A_{530} - ($\frac{1}{3}$ A_{657}). Results are presented as [A] per gram dry weight [9].

2.2.3 Enzyme Assays
Five different plants were dried down for each species and at least five leaf samples were taken per plant for each of the treatments. All extractions were performed on ice and solutions were cold.

2.2.3.1 Ascorbate peroxidase (AP)
0.05 g of leaf material was ground using a pre-chilled mortar and pestle and mixed with 0.1 g polyvinyl pyrrolidine. Material was extracted in 4 mls of 50 mM Tris-HCl buffer pH 7.7 and 2 mls 2 mM sodium ascorbate. The reaction mixture was centrifuged and AP activity of the supernatant was assayed by measuring the oxidation of ascorbate at 290 nm [20]. The reaction mixture of 1 ml contained 25 mM potassium phosphate pH 6.1, 0.2 mM ascorbate, 0.1 mM H_2O_2 and 0.1 ml of crude extract. The reaction was started by adding H_2O_2. Reaction mixtures were warmed up to 22 °C before measurements were taken.

Bradford assays were performed on the crude extract to determine protein concentration and enzyme activity was expressed per mg protein.

2.2.3.2 Glutathione reductase (GR)
0.05 g of leaf material was ground with 0.1 g polyvinyl pyrrolidine in 4 mls of 1 mM potassium phosphate buffer pH 7.5 containing 0.4 mM EDTA and 9.94 mM sodium ascorbate. The extraction mixture was centrifuged and GR activity of the supernatant was assayed by measuring the oxidation of NADPH at 340 nm [13]. The reaction mixture of 1 ml contained 0.3 M potassium phosphate buffer pH 7.5, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.15 mM NADPH, 10 mM GSSG and 0.1 ml of crude enzyme extract. The reaction was started by adding GSSG. Corrections were made for any NADPH oxidation in the absence of GSSG. Reaction mixtures were warmed up to 22 °C before measurements were taken.

Bradford assays were performed on the crude extract to determine protein concentration and enzyme activity was expressed per mg protein.

2.2.3.3 CuZn Superoxide dismutase (SOD)
0.01 g of ground leaf tissue was extracted in 5 ml of 0.1 M potassium phosphate buffer pH 7.5, containing 0.1 mM EDTA, 0.01 g polyvinyl pyrrolidine, 1% w/v bovine serum albumin. The extract was filtered through Whatman GF/A glass fibre filters. Mn SOD was inactivated by sodium dodecyl sulphate (SDS). SDS was removed by precipitation with 3 M KCl at 6 °C and centrifugation at 20 000 × g [8]. SOD activity was assayed by measuring inhibition of nitrite formation from hydroxyl ammonium chloride oxidation at 530 nm [2]. 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 activity for SOD is defined as that amount which causes 50% inhibition of cytochrome c reduction [10]. The units of activity were divided by the dry weight of the sample and expressed as units per gram dry weight.

2.2.4 Statistics
Completely randomized ANOVAs and Duncan's Multiple Range tests were done on the pigment contents and enzyme activity data. Differences among the treatments as well as between the two species were tested for (95% confidence level).

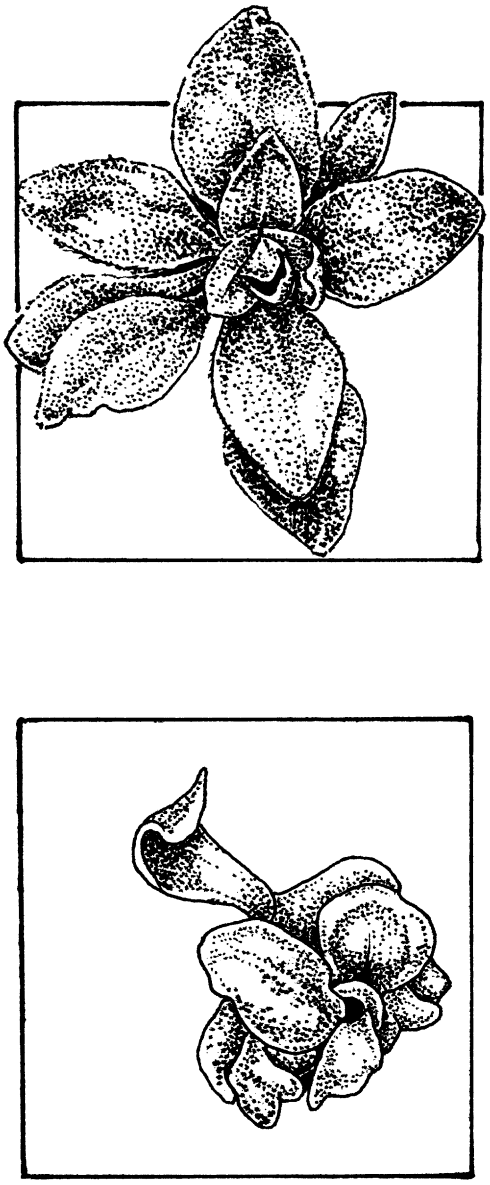

Figure 1. A diagrammatic representation of a hydrated (a) and dry (b) plant of *Craterostigma wilmsii*. Plants are drawn to scale to show leaf movements and decrease in leaf area exposed to light.

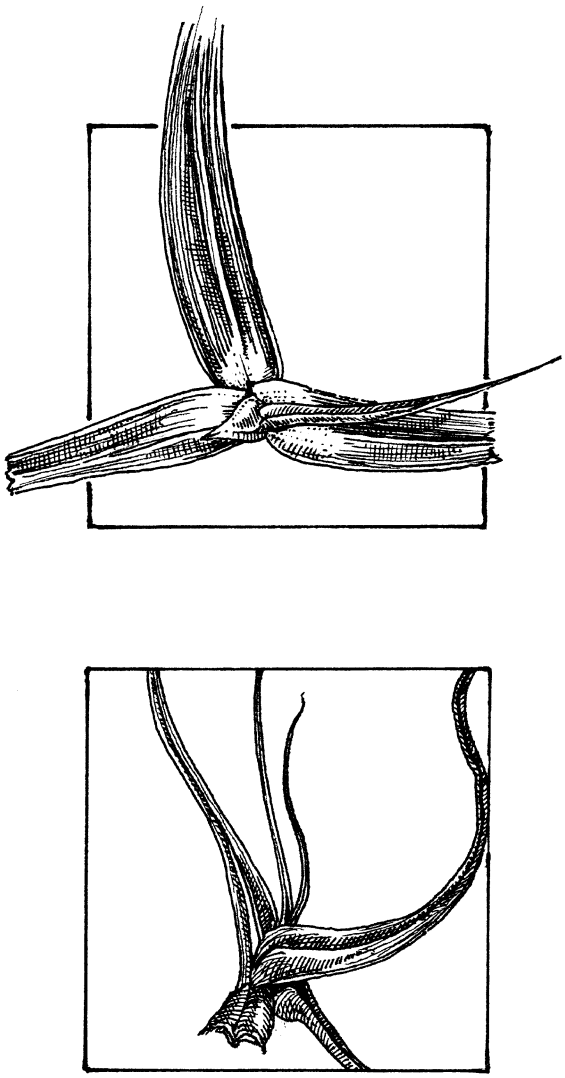

Figure 2. A diagrammatic representation of a hydrated (a) and dry (b) plant of *Xerophyta viscosa*. Plants are drawn to scale to show leaf movements and decrease in leaf area exposed to light.

3. Results

Figure 1 shows the appearance of *C. wilmsii* and Figure 2 that of *X. viscosa* in the wet (a) and dry (b) state. They are drawn to scale to show the reduction in leaf surface area which is exposed to light when the plant is in the dry state. On drying, the leaves of *C. wilmsii* curl tightly and only the abaxial surface of the outer whorl of older leaves are exposed to light (Figure 1). These become red in colour and have a hairy reflective surface. On drying the leaf blades of *X. viscosa* fold in half along the midrib, with only the abaxial surface being exposed to the light (Figure 2). The leaves initially become yellow as they loose their chlorophyll and then they turn dark purple in the dry state. The abaxial surfaces have a reflective sticky coating which may serve to reduce light absorbed by the leaf.

The chloroplasts from hydrated leaves of both species were typical of those from photosynthetically active tissues (Figure 3a and b). In the dry leaves of *C.*

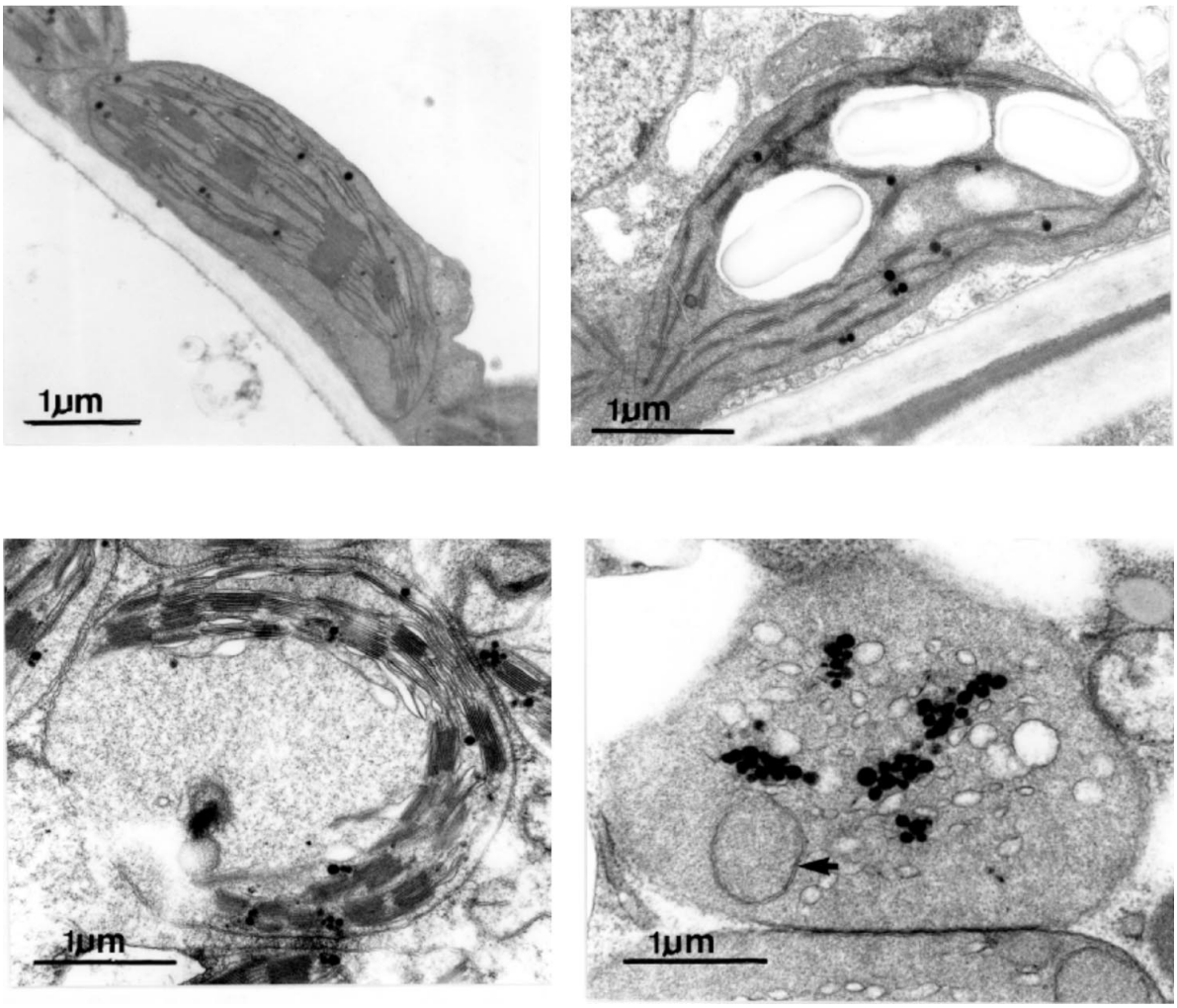

Figure 3. The chloroplasts of a hydrated leaf of *Craterostigma wilmsii* (a) and *Xerophyta viscosa* (b), and those from dry leaves of *C. wilmsii* (c) and *X. viscosa* (d). The chloroplasts from the dry leaves show the changes occurring on dehydration as well as the differences between the chloroplasts of the two species in the dry state. Arrows (d) point to circular membraneous structures thought to be a consequence of invaginations of the chloroplast boundary membranes.

wilmsii the chloroplasts became rounded, membranes remained intact but the thylakoids were displaced to one side (Figure 3c). On dehydration of *X. viscosa* the outer membranes of the chloroplast, or desiccoplast [18], remained intact, but the thylakoid membranes were broken down to small vesicles (Figure 3d). Circular membranous structures (arrowed) were also present in most chloroplasts. These are thought to be a consequence of invagination of the boundary membrane. Plastoglobuli accumulated in chloroplasts of dry leaves.

Figure 4 shows the changes in chlorophyll (a + b) [a], carotenoid (x + c) [b] and anthocyanins [c] which occurred on drying and rehydration of the two species. Virtually all chlorophyll (Figure 4a) was lost during dehydration of *X. viscosa* and chlorophyll content decreased by 30% in *C. wilmsii*. Chlorophyll recovery upon rehydration took 48 h in *C. wilmsii* and 120 h in *X. viscosa*. There was no change in carotenoid content (Figure 4b) on drying of *C. wilmsii* whereas there was a 60% reduction in *X. viscosa*, this taking in excess of 48 hours to recover. Anthocyanin contents (Figure 4c) increased on drying in both *C. wilmsii* and *X. viscosa*. Anthocyanin contents remained high in *X. viscosa* on rehydration but declined almost immediately in *C. wilmsii*.

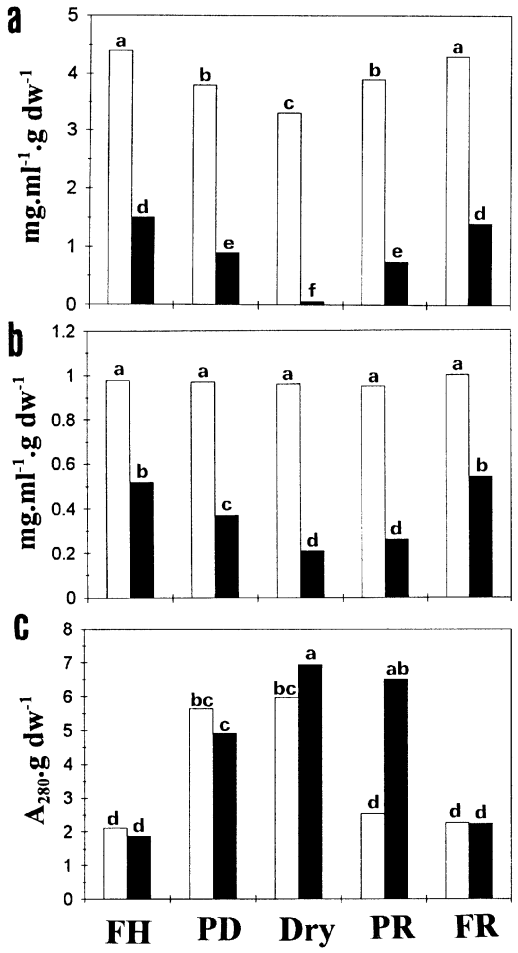

Figure 4. Changes in chlorophyll[a + b] (a), carotenoid[x + c] (b) and anthocyanin (c) contents during dehydration and rehydration of *Craterostigma wilmsii* and *Xerophyta viscosa*. The empty (□) bars represent *C. wilmsii* and the solid (■) bars represent *X. viscosa*. FH refers to fully hydrated tissue; PD refers to leaves dried to 50% RWC; PR refers to partially recovered leaves (24 hours of rehydration for *C. wilmsii* and 48 hours rehydration for *X. viscosa*); FR refers to fully recovered leaves (48 hours of rehydration for leaves of *C. wilmsii* and 120 hours for leaves of *X. viscosa*). The letters above the bars indicate significant differences (95% confidence level) among the treatments and between the species.

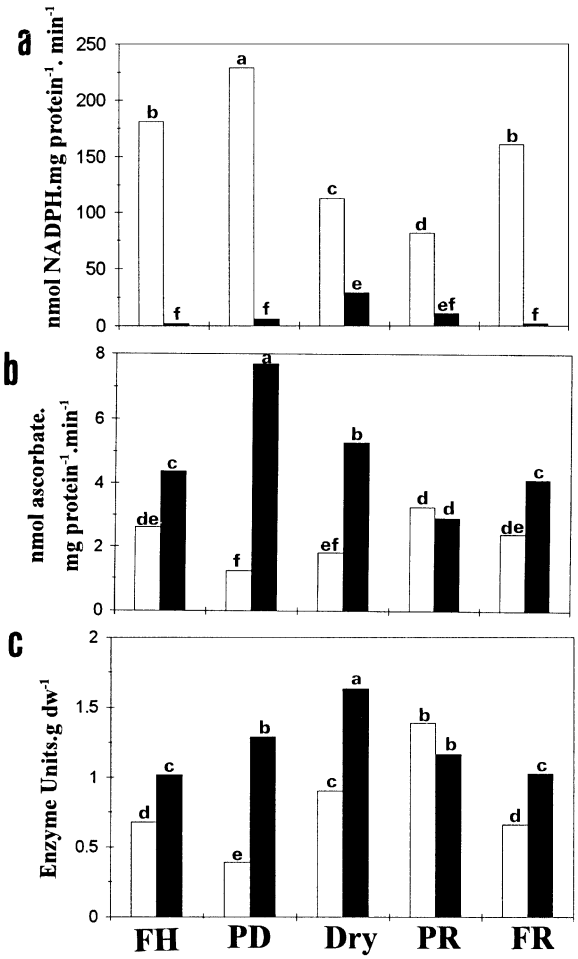

Figure 5. Changes in activity of the anti-oxidant enzymes ascorbate peroxidase (a), glutathione reductase (b) and superoxide dismutase (c) during dehydration and rehydration of *Craterostigma wilmsii* and *Xerophyta viscosa*. The empty (□) bars represent *C. wilmsii* and the solid (■) bars represent *X. viscosa*. FH refers to fully hydrated tissue; PD refers to leaves dried to 50% RWC; PR refers to partially recovered leaves (24 hours of rehydration for *C. wilmsii* and 48 hours rehydration for *X. viscosa*); FR refers to fully recovered leaves (48 hours of rehydration for leaves of *C. wilmsii* and 120 hours for leaves of *X. viscosa*). The letters above the bars indicate significant differences (95% confidence level) among the treatments and between the species.

The changes in activities of three anti-oxidant enzymes during drying and rehydration of the two species is given in Figure 5. AP activity (Figure 5a) was substantially higher in *C. wilmsii* than in *X. viscosa*. There was a significant increase in AP activity on partial dehydration (50% RWC) of *C. wilmsii* but AP activity decreased in the dry state and remained low during initial rehydration, recovering to control levels only in fully hydrated tissue. *X. viscosa*, on the other hand, showed a significant increase in AP activity during dehydration and this gradually returned to normal during rehydration. GR activity (Figure 5b) decreased on drying and increased to control levels on rehydration in *C. wilmsii*. In *X. viscosa* GR activity increased on drying, showing a significant increase at 50% RWC, and decreased to control levels during rehydration. SOD activity (Figure 5c) declined during initial stages of drying in *C. wilmsii* and increased considerably during partial rehydration. Activity declined

to control levels in fully hydrated tissues. In *X. viscosa* trends in SOD activity were similar to those shown for AP and GR, with activity increasing with progressive dehydration and declining to control levels during rehydration.

4. Discussion

Photosynthetic tissues of resurrection plants are capable of withstanding the stresses associated with desiccation in the presence of light. The species studied here appear to employ a number of strategies to cope with such stress. Both utilise leaf movements to reduce the surface area exposed to light during dehydration and in the dry state. Surfaces which are exposed to light are reflective to further reduce light absorbed and possibly facilitate temperature control. We have observed similar changes in several other resurrection plants studied in our laboratory (unpublished observations).

Leaf curling may have an additional function in *C. wilmsii*. As only the older, outermost leaves are exposed to light, the younger inner leaves are effectively shielded from deleterious light-chlorophyll interactions and light-induced damage is avoided. Chlorophyll may thus be retained and chloroplast ultrastructure maintained in these leaves in the dry state. Chloroplast ultrastructure appears to be equally well preserved in outer and inner leaves. The ability to retain chloroplast integrity and a considerable amount of chlorophyll enables a rapid recovery of photosynthesis on rehydration. The elongated and sclerophyllous nature of *X. viscosa* leaves probably prevents them from curling. Thus each leaf of *X. viscosa*, despite folding in half along the midrib, still has a large leaf area exposed to light in the dry state. The dismantling of chloroplasts and the loss of chlorophyll in this species may be necessary to prevent excess light absorption. The cost to a poikilochlorophyllous species like *X. viscosa*, is in the longer recovery associated with repair and resynthesis once water becomes available.

Pigments such as carotenoids and anthocyanins have been implicated in protection against light damage [6, 4]. Levels of carotenoids remain unchanged in *C. wilmsii* and the decline during dehydration of *X. viscosa* is more in keeping with the dismantling of the photosynthetic apparatus to prevent light-interactions. It is not known if they afford protection during desiccation in either of these species. Anthocyanins have been shown to accumulate in vacuoles of plants in response to light and temperatures stress [17] and are thought to mask chlorophyll and/or act as filters preventing excess light absorption by the leaf [4]. They have also been suggested to play an anti-oxidant role [6]. The accumulation of anthocyanins during dehydration of both *C. wilmsii* and *X. viscosa* leaves may serve as protection both at the level of light absorption and free-radical quenching. The retention of anthocyanins during rehydration of *X. viscosa* may be of benefit in affording additional protection against light damage during the reassembly of thylakoid membranes and synthesis of chlorophyll. In rehydrating *C. wilmsii*, where such reconstitution is not required, anthocyanin contents declined rapidly. Thus, protection during rehydration in this species appears to rather be afforded by antioxidant systems.

The results obtained for the anti-oxidant enzyme assays did not show any clear trends and interpretation of these results was complex. This is similar to other work on the antioxidant enzymes of resurrection plants [12, 13, 14]. There were, however, some interesting trends. Activities of all three enzymes studied increased during dehydration of *X. viscosa*. It is likely that these antioxidant systems are required to protect against light- (and other, eg mitochondrial-) induced free radical damage which could occur when water becomes limiting and components of the photosynthetic and respiratory apparatus are still sufficiently intact so as to allow electron flow. With the exception of GR, enzyme activities and anthocyanin content remain elevated during rehydration and return to control levels only once the plant is fully rehydrated and the photosynthetic apparatus is reassembled. In *C. wilmsii* protection appears to be required during partial dehydration, when AP activity and anthocyanin content is elevated, and during partial rehydration when activities of GR and SOD are elevated.

In summary, both species reported on here appear to withstand damage caused by light by a combination of avoidance and protective mechanisms. In *C. wilmsii* leaf curling prevents light stress to inner leaves and enables maintenance of the photosynthetic apparatus in those leaves. In *X. viscosa* light stress is avoided by the dismantling of the photosynthetic apparatus. Intermediate stages of dehydration and rehydration are seen as critical, as limited water prevents full physiological activity but enables deleterious reactions to proceed. During these stages protection is afforded by elevated levels of anthocyanins and increased activities of some antioxidant enzymes.

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