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An Overview of Mechanisms of Desiccation Tolerance in Selected Angiosperm Resurrection Plants

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ABSTRACT

The vegetative tissues of resurrection plants, like seeds, can tolerate desiccation to 5% relative water content (RWC) for extended periods and yet resume full metabolic activity on re-watering. In this review we will illustrate how this is achieved in a variety of angiosperm resurrection plants, our studies ranging from the ecophysiological to the biochemical level. At the whole plant level, leaf folding and other anatomical changes serve to minimise light and mechanical stress associated with drying and rehydration. The mechanisms of cell wall folding are described for *Craterostigma wilmsii* and *Myrothanmus flabellifolia*. Free radicals, radical oxygen species (ROS) usually generated under water-deficit stress by photosynthesis, are minimised by either homoiochlorophylly (e.g. *C. wilmsii* and *M. flabellifolia*) or poikilochlorophylly (e.g. *Xerophyta* sp.). The antioxidant systems of these plants effectively deal with ROS generated by other metabolic processes. In addition to antioxidants common to most plants, resurrection plants also accumulate polyphenols such as 3, 4, 5 tri-*O*-galloylquinic acid in *M. flabellifolia*, and seed-associated antioxidants (e.g. 1-cys-peroxiredoxin and metallothionines) as effective ROS scavengers. Sucrose accumulates at low RWC, presumably protecting the sub-cellular milieu against desiccation-induced macromolecular denaturation.

Keywords: *Craterostigma*, *Eragrostis*, *Myrothamnus*, Xerophyta

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INTRODUCTION

Desiccation tolerance is the ability of an organism to survive the loss of most (>95%) of its cellular water for extended periods and to recover full metabolic competence upon rehydration. Such anhydrobiosis is a relatively rare trait except in the reproductive structures of most plants (pollen, spores, seeds). Desiccation tolerance only occurs in a few species of nematodes and bdelloid rotifers and the vegetative tissue of a few plants. Vegetative desiccation tolerance is more common in less complex plants such as bryophytes (Proctor 1990) and lichens (Kappen and Valadares 1999) but is relatively rare in pteridophytes and angiosperms (Gaff 1977, 1989; Porembski and Barthlott 2000; Alpert and Oliver 2002) and absent from gymnosperms (Gaff 1989). The mechanisms of desiccation tolerance differ between the extant lower orders and the angiosperms. In the former, desiccation occurs very rapidly and protection prior to drying is minimal and constitutive. Survival is thought to be based largely on rehydration-induced repair processes (Oliver *et al*. 1998; Alpert and Oliver 2002). In angiosperm vegetative tissues, while some repair is probably inevitable, considerable and complex protection mechanisms are laid down during drying to minimize the need for extensive repair (Gaff 1989; Farrant 2000; Scott 2000; Alpert and Oliver 2002; Vicre *et al*. 2003, 2004a; Bartels 2005; Illing *et al*. 2005; Farrant 2007). In common with those produced in orthodox seeds, these include *inter alia* the accumulation of sucrose and other oligosaccharides (reviewed in Pammenter and Berjak 1999; Scott 2000; Farrant 2007), the production of late embryogenesis abundant (LEA) proteins (e.g. Russouw *et al*. 1995; Wolkers *et al*. 1998; Illing *et al*. 2005), the upregulation of "housekeeping" antioxidants and the appearance of novel antioxidants that are apparently unique to desiccation-tolerant organisms (Aalen 1999; Illing *et al*. 2005; Farrant 2007). All of these contribute to protecting the subcellular *milieu* (reviewed by Berjak 2006). We are interested in understanding the protection mechanisms associated with acquisition of vegetative desiccation tolerance in angiosperm resurrection plants because we believe that this will allow identification of characteristics that might be important for the ultimate development of drought tolerant crops. We have thus conducted research on a range of resurrection plants as models for various crop species. Since most staple food crops are monocots, we use the monocotyledonous resurrection plants *Xerophyta* sp. as primary models, but also the resurrection grass *Eragrostis nindensis* as a model for development of drought-tolerant pasture grasses

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Invited Review

Fig. 1 Hydrated (A, C, E) and dry (≤5% RWC (B, D, F)) monocotyledonous resurrection plants *X. viscosa* (A, B) and *X. humilis* (C, D) and the grass *E. nindensis* (E, F). Scale bars: A, B, D, E, F = 10 cm; C = 1 cm.

(**Fig. 1**). Models for dicot crops are the herbacious *Craterostigma wilmsii* and the woody shrub *Myrothamnus flabellifolia* (**Fig. 2**). In the following review, we will identify and compare some of the mechanisms of protection accumulated in response to drying in leaves of these various resurrection plants. For comparison, where applicable, the responses of selected desiccation-sensitive species will be reviewed. For example, in the genus *Eragrostis* there are species with differing degrees of tolerance to water deficit which serve as a good comparative model system. *E. nindensis* (**Fig. 1E, 1F**) is the only resurrection species, tolerating drying to 5% RWC, but *E. curvula*, *E. teff* and *E. capensis* have critical water contents below which they cannot be dried of 45, 50 and 65% respectively (Balsamo *et al*. 2005, 2006).

Oliver *et al*. (1998) have proposed that vegetative desiccation tolerance is the ancestral state for early land plants (e.g. bryophytes) but was lost early in the evolution of tracheophytes. The subsequent successful radiation of vascular plants on land was probably a consequence of the evolution of desiccation tolerance in seeds, in parallel to the evolution of structural and morphological modifications in vegetative tissue which allowed greater control of water status. Oliver *et al*. (1998) speculate that the emergence of desiccation tolerance in seeds was a modification of vegetative desiccation tolerance in early ancestors. They suggest furthermore that vegetative desiccation tolerance in angiosperms subsequently re-evolved independently at least eight times as an adaptation of seed desiccation tolerance. Our work supports these hypotheses, as there are considerable differences among the various angiosperm resurrection plants in their mechanisms of protection against desiccation. We have also shown that there are a number of similarities in putative protection mechanisms among orthodox seeds

Fig. 2 Hydrated (A, C) and dry (≤5% RWC (B, D)) dicotylendous resurrection plants *C. wilmsii* (A, B) and *M. flabellifolia* (C, D). Inset to D: cross section of dry leaves of *M. flabellifolia* showing leaf curling and retention of chlorophyll in the shaded adaxial surfaces and waxy anthocyanin in the outer abaxial surfaces. Scale bars = 1 cm.

and vegetative tissues of species such as *Xerophyta humilis* (Illing *et al*. 2005). While we will allude briefly to the latter, this review will concentrate mainly on the differences among resurrection plants.

Stresses associated with desiccation and mechanisms of amelioration

Water plays many and varied roles in plant tissues. It is involved in metabolism as both a reactant and a product of many processes and it is the medium in which the intracellular *milieu* is suspended. By providing hydrophobic and hydrophilic interactions, it determines conformation of macromolecules and membranes and controls and maintains intracellular distances between them (Vertucci and Farrant 1995; Hoekstra *et al*. 2001; Buitink *et al*. 2002; Walters *et al*. 2002).

Mechanical stress

Mechanical stress resulting from the decreased turgor and cell volume as water is lost has been proposed by Iljin in 1957 to be one of the major causes of irreversible desiccation-induced damage in plants. At the cellular level, loss of water from vacuoles and cytoplasm causes tension on the plasmalemma as it shrinks from plasmadesmatal attachments to the cell wall. Increasing compaction of organelles and macromolecules and ultimate rupture of the plasmalemma, allowing entry of extracellular hydrolases, results in lethal damage and cell death (Walters *et al*. 2002).

Leaf and root tissues of angiosperm resurrection plants undoubtedly undergo considerable shrinkage (**Figs. 3, 4**) and morphological change during drying (**Figs 1-4**), the degree of shrinkage being greater in dicots, where wall folding plays an important role in mechanical stabilisation. They are able to survive these changes by active induction of protection mechanisms that allow avoidance of plasmalemma rupture and wall collapse.

There appear to be two general mechanisms employed by angiosperm resurrection plants to avoid mechanical stress: 1) active and reversible wall folding as seen in the *Craterostigma* sp. (**Fig. 5A**; Vicre *et al*. 1999, 2003, 2004b)

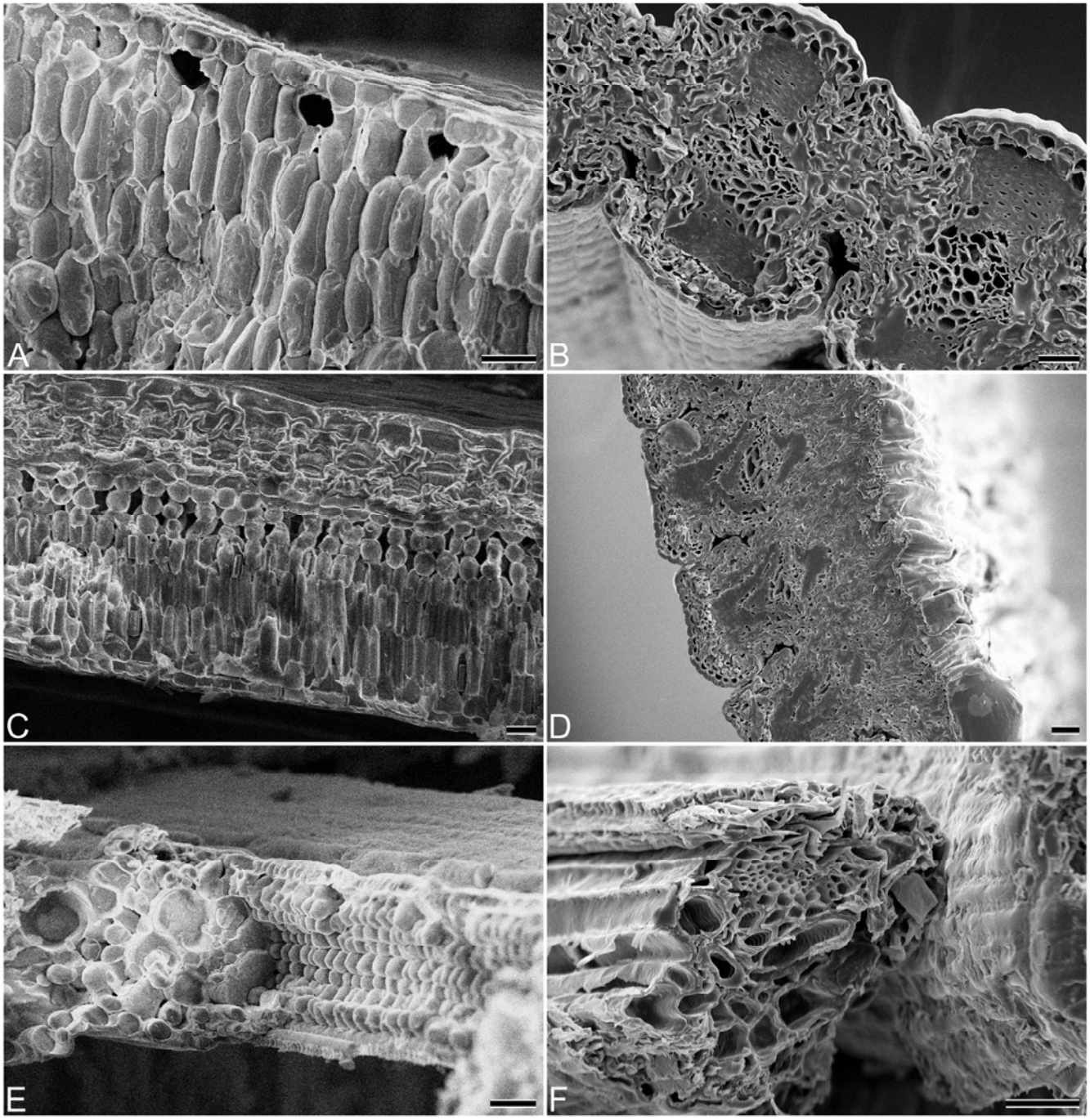

Fig. 3 Scanning electron microscopical images of hydrated (A, C, E) and dry (≤5% RWC (B, D, F)) leaves of the monocots *X. humilis* (A, B), *X. viscosa* and *E. nindensis* (E, F). Scanning electron microscopy was performed using a Leica Stereoscan 440 digital scanning electron microscope equipped with a Fisons LT7400 Cryo Transfer System. Leaves from hydrated and desiccated plants were frozen using liquid nitrogen and viewed directly or after freeze-fracturing. Scale bar for all images = 20 μm.

and 2) increased vacuolation with water replacement in vacuoles by non-aqueous substances such as in the *Xerophyta* sp. (**Fig. 5B**; Farrant 2000; Mundree and Farrant 2000). Some species, such as *M. flabellifolia* (**Fig. 5C, 5D**) and *E. nindensis* (**Fig. 5E, 5F**) use both mechanisms, usually in different tissues. In the grasses, wall folding occurs in the mesophyll and vacuole filling in the bundle sheath cells (van der Willigen *et al*. 2003, 2004). Desiccation-sensitive species show neither mechanism and sub-cellular damage is lethal, as is illustrated in **Fig. 6** for *E. capensis*. While resurrection plants adopt one (or both) of these general strategies, the manner in which they achieve it varies among the species, which probably reflects multiple evolutions of the same strategy.

Thus in those species employing wall folding, there appears to be no uniformity among them in the manner in which reversible wall folding is achieved during drying. Indeed their overall wall composition is similar to other related desiccation-sensitive species, but the resurrection species have utilized inherent wall characteristics, with only slight modifications during drying, to achieve stable and reversible conformational changes (Vicre *et al*. 1999, 2003, 2004a, 2004b; Moore *et al*. 2006). Comprehensive biochemical and immunocytological investigation of leaf wall changes during drying and rehydration of *C. wilmsii* (**Fig. 5A**) has shown that the major difference between dry and hydrated walls lay only in the hemicellulose wall fractions (Vicre *et al*. 1999, 2004b). There was a reduction in glucose and an increase in galactose substitutions in the xyloglucans (XG) from dry walls compared to hydrated walls. We have proposed that cleavage, or partial cleavage of the long-chained XG units during drying into shorter, more flexible ones, allows for wall folding. Secondary ion mass spectrometry (SIMS) revealed a marked increase in

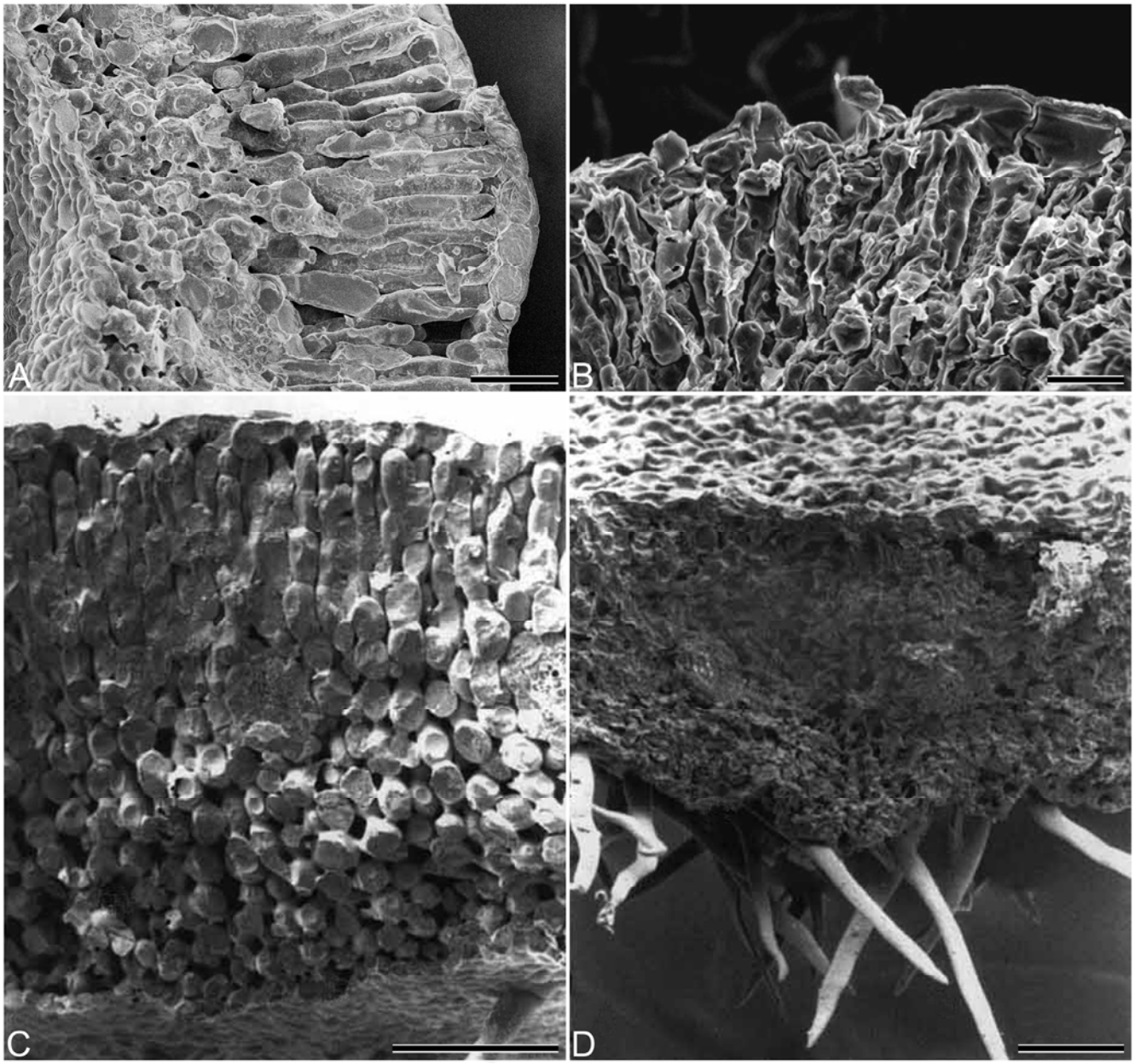

Fig. 4 Scanning electron microscopical images of hydrated (A, C) and dry (≤5% RWC (B, D)) leaves of the dicots *C. wilmsii* (A, B) and *M. flabellifolia* (C, D). Scale bar in A, B = 50 μm; C, D = 200 μm.

wall-associated Ca^{2+}, but only at the final stages of drying. Since this ion plays an important role in cross-linking wall polymers, such as acid pectins, we propose that this serves to stabilize walls in the dry state and, more importantly, prevent mechanical stress of rehydration. *C. wilmsii* is a small plant, and rehydration is rapid and is initially mainly apoplastic (Sherwin and Farrant 1996). If walls hydrate and unfold before cell volume is regained, plasmalemma tearing and further sub-cellular damage could occur (reviewed in Vicre *et al*. 2003, 2004a). Jones and McQueen-Mason (2004) have shown an increase in abundance of an α-expansin transcript during drying and rehydration in leaves of *Craterostigma plantigineum* that correlated with changes in wall extensibility in that species. Expansins are proposed to be involved in wall loosening via disruption of non-covalent bonds between polysaccharides (McQueen-Mason and Cosgrove 1995) and this could be an additional or alternative mechanism whereby wall folding might be facilitated in the *Craterostigma* species.

A similar biochemical, immunocytological study was conducted on leaf wall changes in *M. flabellifolia* (Moore *et al*. 2006). In this species, wall folding occurs in the epidermis (around seemingly less flexible stomata and gland cells) and in the immediately adjacent mesophyll cells (Moore *et al*. 2007b; **Figs 2C, 2D, 5C**). The more centrally located mesophyll cells show less wall folding and mechanical stabilisation is almost entirely due to vacuole filling (**Fig. 5D**). In this species, there were no significant changes in wall components during drying, but the walls contained an unusually high amount of arabinose, probably as arabinan polymers, and in arabinogalactin-rich wall proteins. Arabinose polymers are highly mobile and allow wall flexibility (Foster *et al*. 1996; Renard and Jarvis 1999) and have a high water absorbing capacity (Goldberg *et al*. 1989; Belton 1997) which would be important for rehydration. We propose that arabinans are constitutively synthesised in leaf cell walls of *M. flabellifolia* and that their presence allows constant preparedness for dehydration-rehydration cycles in this species (Moore *et al*. 2006).

Wall folding also occurs in mesophyll cells of the grass *E. nindensis* (**Fig. 5E**) but the biochemical nature of wall changes have not yet been analysed. In the bundle sheath cells of these species (**Fig. 5F**), as in mesophyll cells of the *Xerophyta* sp. (**Fig. 5B**) and *M. flabellifolia* (**Fig. 5D**), the large central vacuole present in hydrated tissues (not shown) is replaced by a number of smaller vacuoles, which serve to fill the cytoplasm, minimising organelle compaction and membrane appression and preventing plasmalem-

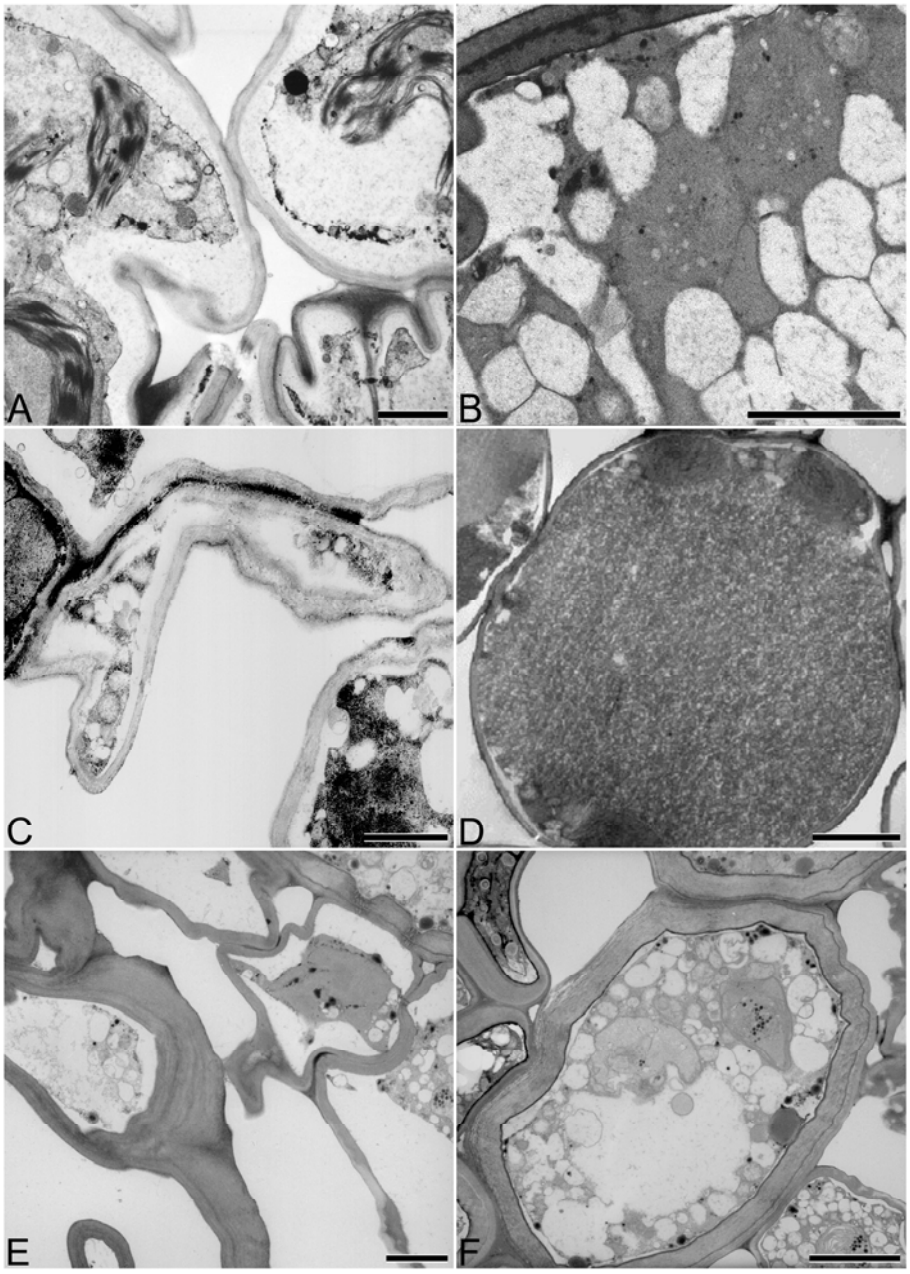

Fig. 5 Transmission electron micrographs of mesophyll tissue from dry leaves (≤5% RWC) of *C. wilmsii* (A), *X. humilis* (B), *M. flabellifolia* (C, D) and *E. nindensis* (E, F). Wall folding is evident in plates A, C and E and vacuole filling is evident in plates B, D and F. Segments (1-2 mm^2) were excised from the mid-blade of dehydrated leaves and processed by the method of Sherwin and Farrant (1996). Microscopy was performed using a LEO 912 transmission electron microscope equipped with CCD camera. Scale bar for all images = 2 μm.

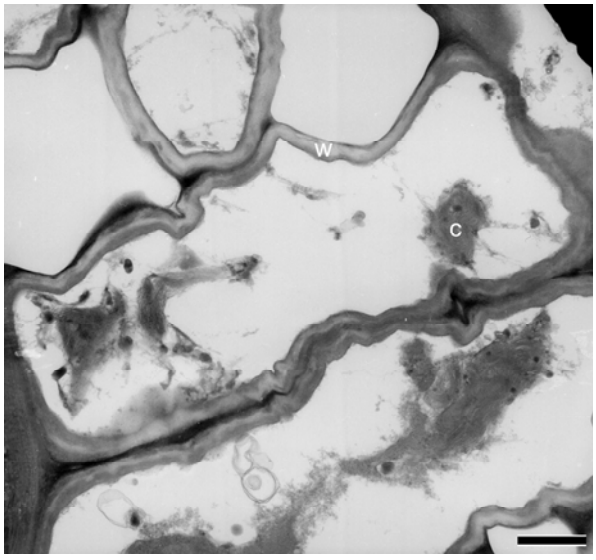

Fig. 6 Sub-cellular damage associated with desiccation in leaves of *Eragrostis capensis*. Note that the plasmalemma and tonoplast are disrupted and the organelles are totally degraded. Fixation and viewing as described in **Fig. 5**. C and W refer to the chloroplast and cell wall, respectively. Scale bar = 2 μm.

ma withdrawal.

The content of desiccated *E. nindensis* vacuoles has been analysed after non-aqueous extraction (van der Willigen *et al*. 2004). These were found to contain proline, sucrose and protein in equal proportions (van der Willigen *et al*. 2004). Similarly vacuoles from both hydrated and dry leaves of *M. flabellifolia* (Moore *et al*. 2005a, 2005b, 2007b) were found to contain 3,4,5 tri-*O*-galloylquinic acid. The concentration of this polyphenolic increased on desiccation to fill the vacuole (**Fig. 5D**) thereby stabilising the sub-cellular *milieu* against mechanical stress.

Metabolic stress

As water is lost from the sub-cellular milieu, metabolism is increasingly perturbed resulting in, *inter alia*, increasing free radical activity. Cellular contents become concentrated, increasing the chances of molecular interactions that can cause denaturation and membrane fusion. Ultimately, the lack of sufficient water to surround macromolecules causes sub-cellular denaturation. The ability to withstand such water loss therefore requires adaptations to protect against these stresses.

Free radical stress (ROS)

Free radicals are atoms or molecules with an unpaired electron, which is readily donated and thus highly reactive. Oxygen, albeit absolutely necessary for metabolism in all aerobic life forms, is a highly oxidizing molecule and readily forms radicals such as singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet -}$), the hydroxyl radical (•OH) and nitric oxide (NO•). These are collectively termed reactive oxygen species (ROS) (Halliwell and Gutteridge 1999). ROS cause damage to all macromolecules and subcellular components (reviewed by Hendry 1993; Pammenter and Berjak 1999; Mundree *et al*. 2002; Walters *et al*. 2002; Vicre *et al*. 2004a; Berjak 2006) and it is thus not surprising that ROS are frequently cited in both seeds (Hendry 1993; Kranner *et al*. 2006) and resurrection plants (Smirnoff 1993; Kranner and Grill 1996; Kranner and Birtić 2005; Kranner *et al*. 2006) as being the most damaging consequence of desiccation stress. Because of their highly reactive nature, the accumulation of the products of ROS-associated damage together with the up-regulation of antioxidants to quench ROS activity is normally assayed. However, there is also recent convincing evidence for a role for ROS in intracellular signalling (Finkel and Holbrook 2000; Apel and Hirt 2004; Bailly 2004; Laloi *et al*. 2004). While we have little information on how ROS might play a role in signalling associated with desiccation tolerance, angiosperm resurrection plants appear to go to great lengths to minimize ROS formation and to quench their activity. It is also evident that the ability to maintain antioxidant potential in the dry state is essential for recovery upon rehydration. For example, Illing *et al*. (2005) and Farrant (2007) have shown that antioxidant enzymes remain undenatured during desiccation, so that the same enzymes can function to prevent ROS damage during rehydration.

In all plants, ROS form as a natural consequence of metabolic processes involving electron transport and thus mitochondria and chloroplasts are major sites of ROS production. Under hydrated conditions, their activity is neutralized and homeostatic control realised by what has been referred to as the "classical" (Kranner and Birtić 2005) antioxidants such as the water-soluble glutathione (γ-glutamyl-cysteinylglycine; GSH) and ascorbic acid (Asc) (Noctor and Foyer 1998), the lipid soluble tocopherols and β-carotene (Munne-Bosch and Alegre 2002) together with enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (AP), other peroxidases, mono- and dehydroascorbate reductases, glutathione reductase (GR) and catalase (for an overview see Elstner and Osswald (1994)). However, under severe water stress conditions, disruption of electron transport results in excess ROS production. While ROS accrue mainly from respiratory metabolism in seeds (Hendry 1993; Bailly 2004), there is an additional critical contribution from disruption of photosynthesis in vegetative tissues. Excess energy from excited chlorophyll molecules rapidly results in formation of ROS (Halliwell 1987; Seel *et al*. 1992a, 1992b; Smirnoff 1993) which are inadequately dealt with by desiccation-sensitive plants, ultimately causing loss of viability (reviewed by Smirnoff 1993; Hendry 1993; Vicre *et al*. 2003; Bailly 2004; Vicre *et al*. 2004b). In contrast, resurrection plants maintain respiration to low levels of RWC (Schwab *et al*. 1989; Hartung *et al*. 1998; Tuba *et al*. 1998; Farrant 2000; van der Willigen *et al*. 2001; Mundree *et al*. 2002), giving a relatively large window of opportunity for unregulated ROS production. It is well documented that ROS activity can and does occur at low water contents, even at hydration levels I and II in which tissues are considered to be in a glassy state (Vertucci and Farrant 1995; Walters *et al*. 2002, 2005). We presume that antioxidant capacity, via both "classical" and additional antioxidant processes (see below) are able to quench this ROS production. ROS production from photosynthesis is minimized at high RWC (Tuba *et al*. 1998; Farrant 2000; Mundree *et al*. 2002; Farrant *et al*. 2003) and, in all species examined, photosynthesis is switched off at water contents between 80% and 65% RWC (Sherwin and Farrant 1998; Farrant 2000; van der Willigen *et al*. 2001; Mundree *et al*. 2002; Farrant *et al*. 2003). This, together with up-regulation of antioxidants, minimizes ROS-associated damage. This down-regulation of photosynthesis is achieved by two primary mechanisms, termed poikilochlorophylly and homoiochlorophylly (Gaff 1989; Smirnoff 1993; Tuba *et al*. 1993a, 1993b, 1994; Sherwin and Farrant 1998; Farrant 2000).

Poikilochlorophyllous species, many of which are monocots such as *Xerophyta* sp. and *E. nindensis* (**Fig. 2**) break down chlorophyll and dismantle thylakoid membranes during dehydration (Tuba *et al*. 1993a, 1993b; Sherwin and Farrant 1998; Farrant 2000; Mundree and Farrant 2000). This strategy is highly effective in minimizing photosynthetically associated ROS production and has been proposed to be a major reason why poikilochlorophyllous species are able to remain viable in the dry state for far longer than homoiochlorophyllous ones (Tuba *et al*. 1998). The potential disadvantage of this strategy is the need to resynthesize the photosynthetic machinery *de novo* upon rehydration, thus retarding recovery. However, in *X. humilis*, RNA coding for chlorophyll synthesis and thylakoid re-

Table 1 Total phenolic content of leaves of resurrection plants and their antioxidant potential as determined by the Ferric Reducing Antioxidant Power (FRAP) and DPPH[2] assays. 500 mg of dry leaf tissue from each of 5 plants were used for phenols extraction with heptane under nitrogen and using ultrasound at 120W for 30 min at room temperature. The mixture was centrifuged at 11,000 × *g* for 10 min at 4°C and the pellet dried. A second extraction from the pellet was done using 70% acetone as solvent and the total soluble polyphenols were spectrophotometrically (Slinkard and Singleton, 1977) using gallic acid (GA) as a standard and the results expressed as mg GA equivalents per g dry weight (mg GAE/g DW). The free radical (electron) scavenging activities were evaluated by the DPPH[1] assay according to the method of Brand-Williams et al. (1995) and the FRAP assay by the method of Benzie and Strain (1996). Standard deviation given in parenthesis (n= 5).

Resurrection plants	**Total phenolics (mg GAE/g DW)**	**FRAP[a] PAC[b]**	**(mmol Fe^{2+}/L)**	**% inhibition of PARC[d]**	**DPPH[c]**
M. flabellifolius	247.1 (15.9)	25.1 (0.8)	0.7	94.8 (0.4)	0.4
C. wilmsii	47.9 (1.3)	11.5 (0.4)	1.6	47.7 (0.1)	1.0
C. plantigineum	43.4 (5.1)	10 .9 (0.4)	1.7	54.3 (1.3)	1.2
C. pumilum	41.5 (2.3)	7.8 (0.2)	1.3	40.0 (1.4)	1.0
X. humilis	38.9 (0.6)	7.7 (0)	1.4	31.7 (2.4)	0.8
X. viscosa	39.6 (1.5)	8.0 (0.3)	1.4	36.1 (0.6)	0.9
X. schlecterii	45.8 (5.1)	8.7 (0)	1.3	24.0 (2.6)	2.3
E. nindensis	10.5 (1.1)	3.4 (0.1)	2.3		
DS plants					
E. curvula	6.8 (1)				
Aspalathus xxx honeybush					

[1]DPPH 1.1 diphenyl-2-picrylhydrazyl .
[a]FRAP – ferric reducing/antioxidant power
[b]PAC – phenol antioxidant coefficient, calculated as FRAP/total phenolcontent
[c]PARC – phenol antioxidant coefficient, calculated as percent inhibition of DPPH radical/total phenol content

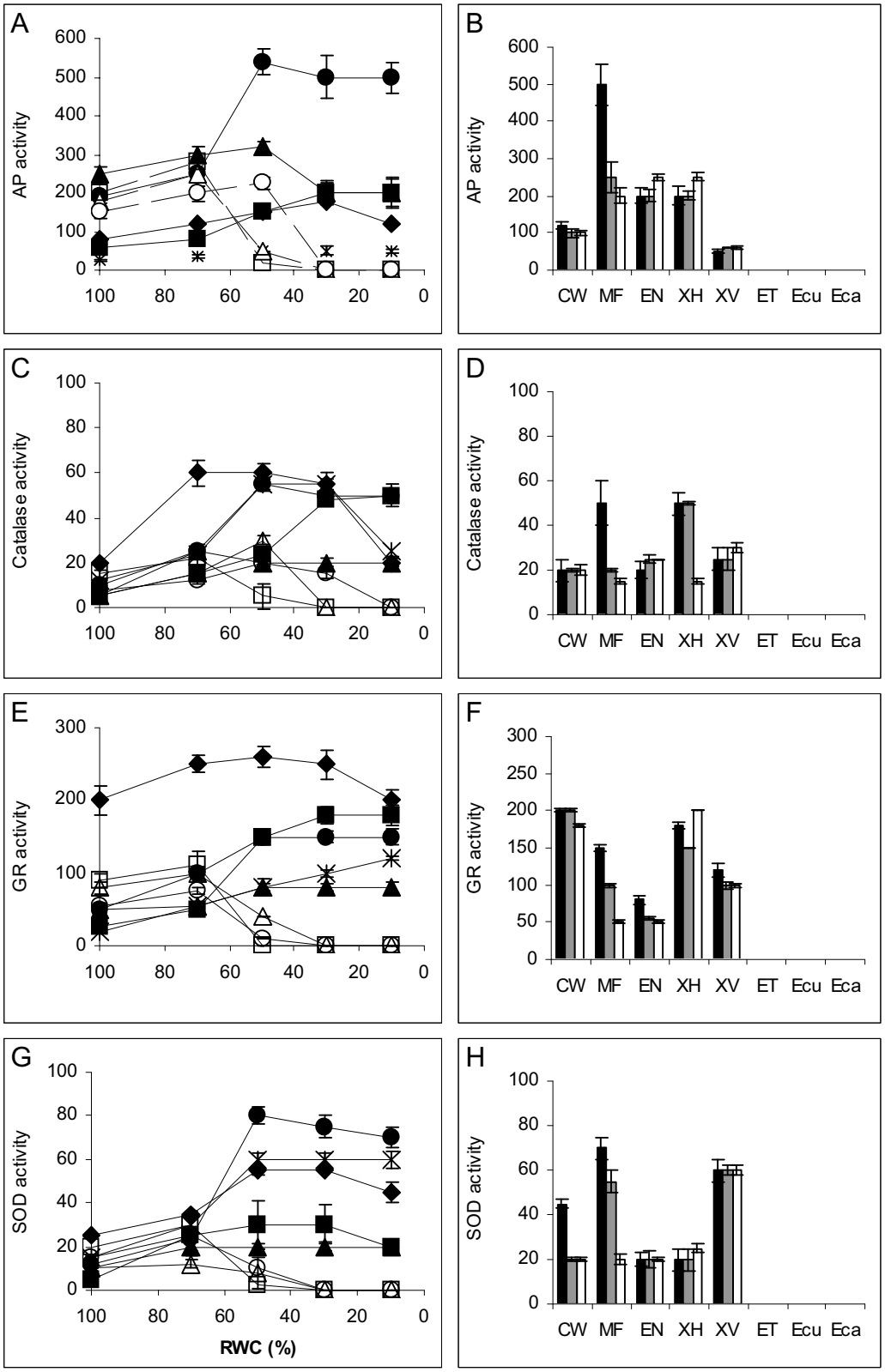

Fig. 7 Activities (nmol.min^{-1}.mg protein^{-1}) of the antioxidant enzymes ascorbate peroxidase (A, B), catalase (C, D), glutathione reductase (E, F) and superoxide dismutase (G, H) during dehydration (A, C, E, G) and during rehydration (B, D, F, H). Dehydration series: *C. wilmsii* (◆; CW), *M. flabellifolia* (●), *X. humilis* (■), *Eragrostis nindenis* (▲), *X. viscosa* (X), *E. teff* (△), *E. curvula* (○), *E. capmensis* (□). For the rehydration series, the enzyme activities of dry (black bars) partially rehydrated (grey bars) and leaves that had recovered full turgor (open bars) are shown. None of the desiccation-sensitive species recovered enzymatic activity upon rehydration when previously desiccated to 5% RWC. Antioxidant enzymes were extracted from leaf tissues at various stages of dehydration and rehydration and analysed using the protocols described in Farrant *et al*. (2004).

constitution is transcribed during drying, stably stored in the dry state, and translated immediately on rehydration, even before reactivation of the nuclear genome (Dace *et al*. 1998; Collett *et al*. 2003).

Homoiochlorophyllous species, typically dicots such as *Craterostigma* sp. and *M. flabellifolia* (**Fig. 2**) retain most of their chlorophyll (the amount retained depending on the light levels under which the plants are dried) and thylakoid membranes in the dry state. Various mechanisms are used to prevent ROS production during drying and rehydration (Sherwin and Farrant 1998; Farrant 2000; Farrant *et al*. 2003) such as leaf folding and shading of the inner leaves

(*Craterostigma* sp.) or the adaxial surfaces (*M. flabellifolia*) from light (**Fig. 2**). In addition, anthocyanin pigments (**Table 1**) accumulate in those surfaces that remain exposed to light in the dry state. It has been suggested that these molecules act as 'suncreens' reflecting back photosynthetically active light, masking chlorophyll and acting as antioxidants (Smirnoff 1993; Sherwin and Farrant 1998; Farrant 2000; Farrant *et al*. 2003; Moore *et al*. 2007a, 2007b). Homoiochlorophyllous species accumulate far more anthocyanins than poikilochlorophyllous ones (**Table 1**), affirming that these pigments may indeed play an important role in the prevention of ROS damage.

Resurrection plants, like desiccation-sensitive types, also upregulate antioxidants to quench ROS that are produced on drying. However, the difference between desiccation-tolerant and desiccation-sensitive species appears to be in their ability to maintain oxidative potential of ubiquitous antioxidants during dehydration as well as the ability to produce, *de novo,* antioxidants that previously have been reported to occur only in seeds (Mowla *et al*. 2002; Illing *et al*. 2005). Considerable variation exists between desiccation-tolerant species with respect to the extent of up-regulation of the various antioxidants, and the RWC at which this occurs (reviewed e.g. in Farrant 2000; Farrant *et al*. 2003). Although some of this variation might be due to differences in the collection and reporting of data, work in our laboratory where conditions were standardised and full dehydration/rehydration time courses were followed (**Fig. 7**) suggests that some variation indeed occurs. All four antioxidant enzymes investigated were active in hydrated tissues from both the desiccation-tolerant and desiccation-sensitive species tested and all these species were able to upregulate antioxidant enzymes on initial drying, although with individual differences (**Fig. 7**, left hand panel). Importantly, however, only the resurrection plants were able to retain enzyme activity at lower RWC and through rehydration to full turgor (**Fig. 7**, right hand panel). Presumably the enzymes are not susceptible to damage during desiccation in desiccation-tolerant plants but not in desiccation-sensitive plants (reviewed further below).

Kranner and Birtic (2005) and Kranner *et al*. (2006) have also postulated that maintenance of the antioxidant potential, particularly that of glutathione, is key to survival for a variety of desiccation-tolerant systems. These authors have demonstrated that the half-cell redox potential ($E_{GSSG/2GSH}$) can be used as a marker for plant stress, and more specifically, when $E_{GSSG/2GSH}$ exceeds -160 mV, stress becomes lethal and programmed cell death ensues. Interestingly, they have demonstrated that longevity of *M. flabellifolia* in the dry state was lost after 8 months, in agreement with our own longevity studies on *M. flabellifolia* (Farrant and Kruger 2001), when $E_{GSSG/2GSH}$ values exceeded - 160 mV (Kranner and Birtic 2005). Furthermore, loss of viability in dry, stored *C. wilmsii* (3 months) and *X. humilis* (10 months, under the most adverse conditions) plants coincided with loss of activity of the antioxidant enzymes GR, catalase and SOD, even though $E_{GSSG/2GSH}$ did not exceed - 160 mV (unpublished observations). Since regeneration of GSH (and presumably other antioxidants such as ascorbate and tocopherol) is dependant on enzymatic activity, protection of these enzymes against ROS activity must be of prime importance during drying and early rehydration.

Resurrection plants also utilize additional antioxidants, such as 1- and 2-cys-peroxiredoxins, glyoxalase I family proteins, zinc metallothioine and metallothionine-like antioxidants (Blomstedt *et al*. 1998; Mowla *et al*. 2002; Collett *et al*. 2004) that have been reported to be important for desiccation tolerance of orthodox seeds but are never found to be up-regulated in desiccation-sensitive vegetative tissues (Aarlen 1999; Stacey *et al*. 1999). Various polyphenols have also been proposed to protect against ROS (Smirnoff 1993; Wang *et al*. 1996; Kahkonen *et al*. 1999). Resurrection plants contain different amounts of polyphenols, the potential antioxidant capacities of which are given in **Table 1**. In general, these are higher than those recorded for closely related desiccation-sensitive species, and equivalent to the antioxidant capacity of the commercial teas *Aspalathus linearis* ('rooibos') and *Cyclopia intermedia* ('honeybush tea') and the medicinal plant *Mellisa officinalis* (Katalinic *et al*. 2005), all of which are valued for their antioxidant properties. Leaves of *M. flabellifolia* contain a high proportion (up to 50% of the leaf dry weight) of 3, 4, 5 tri-*O*-galloylquinic acid which acts as a potent antioxidant *in vitro* (Moore *et al*. 2005a). Despite this polyphenol being predominantly located in the vacuole and cell wall, we think that these reservoirs act to absorb electrons from the cytoplasmically located antioxidants. A potential link between the primary antioxidants in the Haliwell-Asada cycle and the vacuolar antioxidant plant polyphenols has been proposed in desiccation-sensitive plants (Takahana and Oniki 1997; Yamasaki and Grace 1998). The extreme quantities of polyphenols in *M. flabellifolia* and other resurrection plants would greatly increase the antioxidant potential of these plants compared to their desiccation-sensitive relatives (**Table 1**).

The total antioxidant potential, the extent of up-regulation of antioxidant enzymes (**Fig. 7**) together with the potential polyphenol antioxidant capacity and anthocyanin protection (**Table 1**), of the homoiochlorophyllous species (*M. flabellifolia* and the *Craterostigma* sp.) is greater than that of the poikilochlorophyllous species (*Xerophyta* sp. and *E. nindesis*). This supports the contention that homoiochlorophyllous resurrection plants might require greater protection against ROS than the poikilochlorophyllous plants, since the latter better avoid ROS formation due to their dismantling the photosynthetic apparatus (Tuba *et al*. 1998; Farrant 2000; Farrant *et al*. 2003).

Denaturation and sub-cellular perturbations

As water is progressively lost, the cytoplasm becomes increasingly viscous. Moreover loss of water promotes protein denaturation and membrane fusion, processes that start to occur at water contents of below 50% RWC or 0.3 $g.g^{-1}$ (loss of type III and some of type II water) (Vertucci and Farrant 1995; Walters 1998). Upon further water loss to 10% RWC, $\leq$0.1 $g.g^{-1}$ (loss of type II and some type I water) the hydrophobic effect of water that is essential in the maintenance of macromolecular and membrane structure is lost and irreversible sub-cellular denaturation occurs. It is generally thought that desiccation-tolerant systems substitute water with hydrophilic molecules that form hydrogen bonds to stabilize macromolecular interactions in their native configuration (Crowe *et al*. 1998, *inter alia*). In addition to this water replacement, further stabilization of the sub-cellular *milieu* is thought to be brought about by vitrification of the cytoplasm by the same water replacement molecules (Leopold 1986; Vertucci and Farrant 1995; Walters 1998; Hoekstra *et al*. 2001, *inter alia*). Typical water replacement molecules include sugars, particularly sucrose together with oligosaccharides (reviewed e.g. in Scott 2000; Berjak 2006), hydrophilic proteins, particularly late embryogenesis abundant (LEA) proteins (reviewed e.g. by Mwtisha *et al*. 2006) and small heat shock proteins (Almogeura and Jordano 1992; Mtwisha *et al*. 2006) and compatible solutes, including amino acids such as proline (e.g. Gaff and McGregor 1979; Tymms and Gaff 1978) and amphiphiles (Golovina and Hoekstra 2000; Hoekstra *et al*. 2001). While we have not yet done exhaustive metabolomic studies on the various resurrection plants, we have considered the role of sugars, sucrose in particular, in sub-cellular protection against desiccation (**Figs 8, 9; Table 2**).

Sucrose is apparently accumulated in the leaves and roots of all angiosperm resurrection plants examined to date (**Fig. 8**; Bianchi *et al*. 1991; Ghasempour *et al*. 1998; Norwood *et al*. 2000; Bartels and Salamini 2001; Whittaker *et al*. 2001; Norwood *et al*. 2003; Whittaker *et al*. 2004; Peters *et al*. 2007). Oligosaccharides also accumulate in resurrection plants during drying, but always to a lesser extent than that of sucrose (**Table 2**). Sucrose accumulation

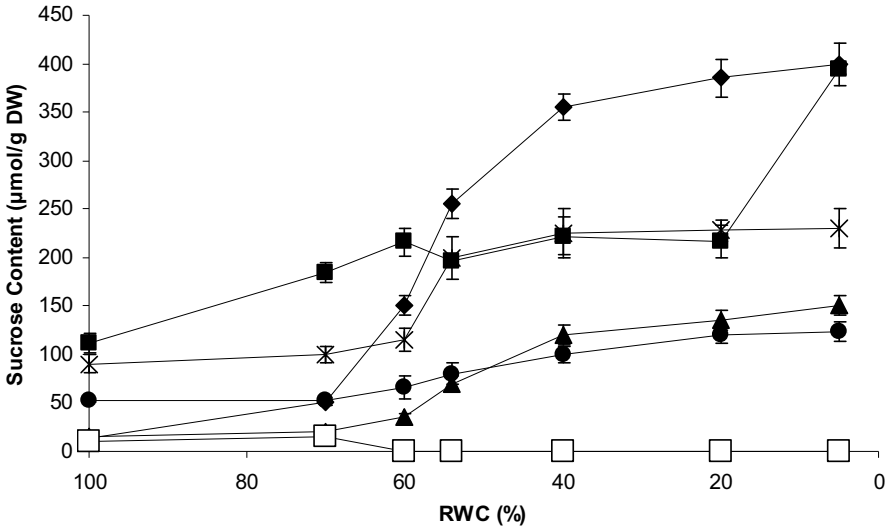

Fig. 8 Changes in leaf sucrose content during drying of resurrection plants *C. wilmsii* (●), *M. flabellifolia* (■), *X. humilis* (▲), *X. viscosa* (◆), *E. nindenis* (○); *S. stapfianus* (X) and the desiccation sensitive species *E. curvula* (□). Sucrose was extracted from leaves and quantified as previously reported (Illing *et al*. 2005).

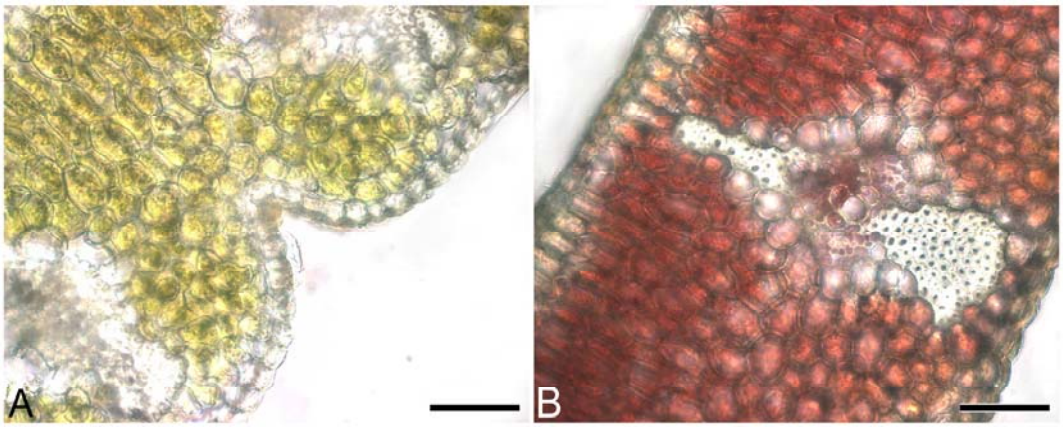

Fig. 9 Sucrose localization in hand cut, unfixed, cross sections of partially dehydrated (RWC = 20%) leaves of *X. humilis*. Sucrose was visualized using the colorimetric method of Martinelli (2007) in which the presence of sucrose was identified by red formazan precipitation after reduction of iodonitrotetrazolium chloride (**B**). The enzyme cocktail was omitted in the case of the control section shown (**A**). Scale bar = 100 µm.

occurs relatively late in the dehydration process, usually initiated below a leaf RWC of 60% although in some species such as *X. humilis*, the majority of accumulation occurs at ≤20% RWC (**Fig. 8**). Since accumulation generally occurs after cessation of photosynthesis (Mundree *et al*. 2002), the source of carbon has been debated. In *C. plantigineum*, octulose and stachyose decline in leaves and roots respectively as sucrose accumulates suggesting that these oligosachharides are converted into sucrose during drying (Norwood *et al*. 2000, 2003). Sucrose is also universally accumulated in orthodox seeds (Amuti and Pollard 1977; Koster and Leopold 1988; Vertucci and Farrant 1995; Pammenter and Berjak 1999; Berjak 2006) suggesting that sucrose plays an important role in desiccation tolerance in general. Sucrose in vegetative tissue is mainly cytoplasmic, predominantly in mesophyll and cortical parenchyma of leaf and root tissues respectively (**Fig. 9**), although it is also present as a minor constituent of vacuoles in those species in which water replacement in vacuoles occurs during drying (van der Willigen *et al*. 2004). We propose that this ubiquitous presence of sucrose plays an important role in "glass" formation and stabilisation of the sub-cellular *milieu* during maintenance in the dry state.

Trehalose is used as a water replacement molecule in animal systems (Crowe *et al*. 1998) and has been shown to be exceptional at membrane stabilisation (Kaushik and Bhat 2003). In resurrection plants, trehalose has only been shown to accumulate in *M. flabellifolia,* but the extent of accumulation is insufficient to serve either function. It is widely held in the seed literature that the raffinose series of oligosachharides (RFOs), particularly raffinose and stachyose, may play an important role in stabilization of the subcellular *milieu* by either water replacement or vitrification (for reviews, see e.g. Buitink *et al*. 2002; Kermode and Finch-Savage 2002). These two sugars are most commonly accumulated in resurrection plants examined to date (**Table 2**). However, the variability in amounts accumulated is such that we consider that oligosaccharides and various compatible solutes may interchangeably serve to afford protection, and that the particular metabolite accumulated is species specific and reflects the predominant metabolism associated with the hydrated condition. The protection functions they could serve are the facilitation of glass formation as well as preventing sucrose crystallisation, the filling of vacuoles in species that use this means of mechanical stabilisation, the removal of monosaccharides in the process of their formation, and as an additional carbon source for metabolic synthesis during rehydration. The monosaccharide content almost universally declines during drying, and in many species the oligosaccharide content also declines (**Table 2**; Vertucci and Farrant 1995; Walters *et al*. 2002). The loss of oligosaccharides can be due to the use of their C skeletons for the formation of sucrose. The reduction in monosaccharides during drying is thought to limit respiration and associated ROS production and to induce the metabolic quiescence required in the desiccated state (Vertucci and Farrant 1995; Farrant *et al*. 1997). Furthermore, since monosaccharides participate in Maillard-type reactions, and by binding to proteins can cause their glycation, their removal during drying can limit these damaging reactions (Vertucci and Farrant 1995; Mtwisha *et al*. 2006).

CONCLUDING STATEMENTS

The work outlined above indicates that there are some key differences among resurrection plants in their responses to desiccation, but also some unequivocal similarities, particu-

Table 2 Contents of various saccharides in hydrated and dry leaves of various resurrection plants.

Species		Trehalose	Octulose	Raffinose	Starch	Sucrose	Fructose	Glucose	References
C. wilmsii	F	ND		0.5 (0.01)	5.6 (0.5)	13 (0.3)	92 (5)	112 (2)	Sherwin and Farrant
	D	ND		2.5 (0.02)	16.6 (0.8)	400 (13)	4 (0.1)	2.2 (0.2)	1998; Farrant *et al*. 2003; Farrant unpublished
C. plantigineum	F	ND	620	NR	NR	2000	104.2	105	Bianchi *et al*. 1991
	D	ND	51			73	8	135	
C. plantigineum roots	F	ND	61.9 (10)	82.5 (2.9)	614 (20)	36.9 (7.7)	0 (0)	4.2 (1.2)	Norwood *et al*. 2003
	D	ND	4.9 (0.7)	36.9 (0.5)	259 (16)	111 (8)	12.2 (0.6)	10.6 (0.9)	
M. flabellifolius	F	45.8 ±2	ND	0.4 (0.2)	7.4 (2.7)	52 (1)	113 (5)	73 (2.3)	Moore *et al*. 2007b
	D	70 ± 5	ND	4.8 (1.6)	2.7 (1.5)	123 (10)	39 (4)	67 (6)	
E. nindensis	F	1.0 ± 0.14	ND	0.0 (0)	0 (0)	15 (0.1)	1.6 (0.1)	4.6 (0.2)	Ghasempour *et al*. 1998; van der Willigen *et al*. 2001; Illing *et al*. 2005
	D	1.2 ± 0.16	ND	3.0 (0.04)	1.63 (0.09)	150 (12)	9.4 (0.1)	6.8 (0.2)	
X. viscosa	F	ND	ND	9.9 (0.2)	3.6 (0.2)	90 (8)	10 (0.2)	18 (0.3)	Peters *et al*. 2007
	D	ND		39.4 (2)	26.5 (0.5)	230 (11)	4 (0.02)	5 (0.1)	Whittaker *et al*. 2001

F = fully hydrated leaves; D = air dry leaves. Sugar contents expressed as μmol.g.dw^{-1}. Mechanisms of extraction and quantification are as in the references given. ND, not detected; NR, not reported. Standard deviation given in parentheses (n=5)

larly at the biochemical level. With the advent of more transcriptome, proteome and metabolome studies, these similarities will probably become increasingly apparent.

Desiccation tolerance is a complex phenomenon and involves a great deal more than what is outlined above. We know little about the control mechanisms involved, from the environmental sensing of water deficit to the pre- and post-transcriptional and -translational control. We need a greater understanding of the full spectrum of protectant metabolites involved and of the role of repair mechanisms, both during drying and rehydration. To date, more focus has been placed on mechanisms of desiccation tolerance in leaves than in roots and we need to start gaining an understanding of the whole plant integrative responses to desiccation.

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