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Full Length Research Paper

Physiological response of selected eragrostis species to water-deficit stress

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Plant water-deficit stress is considered to be one of the greatest threats to world agriculture. In an effort to identify a better performing crop variety of *Eragrostis tef* under this stress, the responses of two varieties, white (*W*) and brown (*B*) seeded, were compared with those of the resurrection grass *Eragrostis nindensis* during a dehydration/rehydration cycle. After 6 days of dehydration, relative water content (RWC) dropped to 33%, 43% and 39% in *En*, *Et(B)* and *Et(W)* plants, respectively. This water loss was accompanied by a decline in transpiration and increased electrolyte leakage in the *Et* varieties. *Et(W)* did not recover from this level of drying when watered. *Et(B)* on the other hand recovered fully from 43% RWC, but lost viability after 9 days dehydration, RWC < 30%. *En* showed full metabolic recovery from drying to 10% RWC. Loss of viability in the *Et* varieties was accompanied by an increase in electrolyte leakage and irreversible decline in photosynthesis and transpiration. Ultrastructural study also indicated a drying-induced damage to membranes and organelles of *Et* tissues dehydrated for 6 days which was reversed in *Et(B),* 43% RWC, on rehydration. However, after 9 days of dehydration, (< 30% RWC), severe irreversible damage occurred to the entire subcellular organization of both *Et* varieties and was accompanied by loss of viability. This study shows that *En* is a true resurrection plant and both *Et* varieties are desiccation sensitive but drought tolerant to varying degrees. *Et(B)* is more drought tolerant and hence a better choice crop in drought prone areas. Comparative study of closely related plant species might be a better approach in finding adaptive characters in crop plants with respect to environmental stresses.

Key words: ***Eragrostis tef***, ***eragrostis nindensis***, dehydration, desiccation, gas exchange, leakage, ultrastructure.

INTRODUCTION

Plant water deficit stress is considered to be one of the greatest threats to world agriculture and, in the coming decades, is likely to be exacerbated by the effects of global climate change (FAO, 2008). It is predicted that by 2050, climate change in Africa will significantly affect agriculture and in some areas could lead to the complete abandonment of cropping (Thornton et al., 2009). A considerable increase in agricultural productivity can be brought about by the production of drought-tolerant crops and pasture grasses. Planting of such crops will increase both the length of the growing season and the area where such crops can be grown, and will accommodate fluctuations in climatic conditions associated with climate change. Currently, several different approaches are being taken to address the problem of decreased water availability for agricultural purposes, including conventional plant breeding, genetic modification, hormonal and chemical treatments. To date none of these have been successful in the long term (that is, for many successive generations) but most importantly, none thus far have been able to confer tolerance to severe drought. The ability to withstand severe water deficit (desiccation) is common in the seeds of most species but vegetative tissues of most plants are extremely sensitive to water deficit. There are, however, some 300 species of angiosperms, many endemic to Southern Africa, in which the vegetative tissues are tolerant of near complete water loss. These desiccation tolerant “resurrection plants”

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Abbreviation: RWC, Relative water content.

(Gaff, 1989) serve as ideal models for identifying the characteristics which enable tolerance of water deficit stress.

Research has been conducted on several species of resurrection plants in order to gain an understanding the mechanisms of desiccation tolerance (DT) in resurrection plants (for reviews see Gaff, 1989; Alpert, 2006; Farrant, 2007; Farrant et al., 2007; Moore et al., 2009; Oliver, 1996; Oliver et al., 2005; Vicar et al., 2004). To date such studies have been exclusively fundamental in nature and the research discipline specific, with most studies focusing exclusively on the molecular genetic changes (Collett et al., 2004; Rodriguez et al., 2010; Zhengbin et al., 2011), with others being either physiological and ultrastructural (Cooper and Farrant, 2004; Farrant, 2000; Farrant et al., 2003; Georgieva et al., 2007; Norwood et al., 1999; Sherwood and Farrant, 1996; Sherwood ***et al***., 1998; Tuba et al., 1996; Vander Willigen et al., 2001; 2004 ***inter alia***) or biochemical and metabolic (Dace et al., 1998; Kranner and Birtic, 2005; Moore et al., 2005, 2006, 2007; Peters et al., 2007; Whittaker et al., 2004; Shao et al., 2005) in nature. We use a systems biology approach in which we utilize several disciplines in attempt to achieve a greater understanding of the mechanisms of desiccation tolerance utilized by a variety of resurrection plants (reviewed in Farrant, 2007; Farrant et al., 2007; Moore et al., 2009; Moore and Farrant, 2011). Furthermore, we use such fundamental studies to identify key protectants that might be used for production of drought tolerant crops and pasture grasses using a bioengineering approach (Mundree et al, 2002; Gawe et al., 2006; Iyer et al., 2007; Moore and Farrant, 2011).

For production of drought tolerant crops using such an approach, it is important to use a resurrection plant model that is similar to the crop to be manipulated. Furthermore, in order to induce appropriate mechanisms for improved water deficit (drought) tolerance in a particular crop, it is important to understand the responses of that crop to water deficit stress. While some research has been conducted on the monocot resurrection plant ***X. viscose*** baker as a general model for monocot crops such as maize (Mundree and Farrant, 2000; Mundree et al., 2002; Garwe et al., 2006; Iyer et al,1007) none to date have been reported on closely related resurrection plants and crop species. This is because few crops have closely related resurrection plant relatives. One exception occurs in the genus ***Eragrostis***, in which ***E. nindensis*** has the properties of a resurrection plant (Gaff, 1977; Vander Willigen et al., 2001; 2004) and in which there are many desiccation sensitive (but with varying degrees of drought resistance/tolerance) species (Balsamo et al., 2006). Agriculturally, species such as ***E. curvula***, ***E. lehmanniana*** and ***E. tef*** are utilized as pasture grasses (van Oudtshoorn,1992) but the seed of the latter is also used as a cereal in many countries in Africa (Lester and Bekele, 1981) providing 2/3 of human nutrition in Ethiopia (Stallknecht et al., 1993; Zegeye, 1997). Although the seed of ***E. tef*** is exceptionally small and thus difficult to harvest for agricultural purposes, it has high nutritional quality with a reported protein content of 11%, 80% complex carbohydrate and 3% fat. It contains more lysine, calcium and potassium than barley, millet, and wheat and slightly less than rice or oats; and it is an excellent source of fibre and iron and other essential minerals (Yetneberk et al., 2004, Piccinin, 2002; Ketema, 1997; Mamo and Pearsons, 1987; Stallknecht et al., 1993). Futhermore, it is gluten free (Dekking et al., 2005), and thus is currently gaining popularity in the whole food and health food industries in the United States and Europe as an alternative grain for persons with gluten sensitivity (Piccinin, 2002; Hopman et al., 2008; Assefa et al., 2010). The objective of this study was to compare some physiological responses to water deficit of ***E. nindensis*** (Ficalho and Hiern) with that of two varieties of ***E. tef*** (Zucc.) Trotter (white and brown seeded) that are used as cereal crops in parts of Africa. The relative drought tolerance was assessed based on changes in transpiration, photosynthesis and respiration, and the ultrastructual consequences of such changes noted. The ultimate aim of this study was to identify characteristics that facilitate tolerance of water deficit stress in ***E. nindensis*** that can be utilized for improved drought tolerance in the tef varieties, either by conventional breeding or through biotechnological applications. In this regard, this study will form the basis for future molecular studies in which genes, proteins and metabolites facilitating desiccation tolerance in ***E. nindensis*** will be identified and characterized.

MATERIALS AND METHODS

Mature plants of ***E. nindenses*** (***En)*** were collected from an Inselberg in the Northern Cape Province, South Africa and transferred to the glasshouse at the University of Cape Town where they were maintained as described in Vander Willigen et al. (2001). Two ***E. tef*** (Zucc.) Trotter varieties, identified as white and brown seeded, and referred to as ***Et(W)*** and ***Et(B)*** in this work, were also collected from the central high land province of Eritrea and sown directly into sand and potting soil in replicate pots and seedlings were maintained in a glasshouse as above for 4 weeks before start of further experimentation. A week prior to the start of the dehydration treatments, all plants species were transferred to a low light growth chamber for acclimation. The daytime temperature was set to 25°C during the 15 h photo-period, and 17°C, during a 9 h dark period with a relative humidity of 50%. Light was supplied by warm white fluorescent bulbs at a photon flux density of 300 µmol $m^{-2}s^{-}$

Dehydration/rehydration treatments

Dehydration of plants was achieved by cessation of soil watering for 3, 6, 9, 12 or 15 days prior to rehydration by soil watering. As a control, plants of the same batch were watered every other day until the end of the experiment. The parameters studied (RWC, gas exchange, leakage) were measured on each of three replicate trays (each comprising of 15 plants) every 3 days during dehydration,

and at 0, 1, 2, 3, 6 and 9 days during rehydration.

Determination of leaf relative water content (RWC)

Percent leaf RWC was determined on 3 replicates of leaf tissues from each tray using the standard formula: RWC = {(fresh weight-dry weight)/(weight at full turgor-dry weight)} × 100. Water contents were gravimetrically determined by oven drying of leaves at 70°C for 48 h, and full turgor was determined from plants that had been watered and kept overnight in plastic bags as described by Farrant (2007).

Gass exchange

Measurements of gas exchange were performed on 3 replicate plants from each treatment using LCA-3 infrared gas analyzer (IRGA) (Analytical Development Company Ltd., Hoddesdon,UK) operated in differential mode at an ambient CO_2 concentration of approximately 350 ppm. Measurements of net photosynthetic rate (A), and transpiration (E) were taken during the day (11:00 h), and respiration measurements were taken at night (20:00 h). As the small leaves of ***Eragrostis*** plants did not cover the area of the leaf chamber, leaf area used was determined as described by Gollan et al. (1985) and used for calculations.

Electrolyte leakage

Membrane integrity of leaf tissues was determined by measuring electrolyte leakage using a CM100 multiple cell conductivity meters (Reid and Associates, Durban, South Africa). Leaves were placed in 3 ml ultra pure water (milli-Q) and conductivity was read every minute for an hour. Rate of leakage was calculated as the slope of line generated from the time course of leakage and was corrected by leaf dry weight ($\mu S.gdwt^{-1}\ min^{-1}$). The experiment was performed in triplicate for each treatment.

Ultrastructural studies

Leaf bases (n = 5) which contained the meristem, from ***Et(W)*** and ***Et(B)*** dehydrated for 6 and 9 days, respectively, and rehydrated for a subsequent 3 days, were fixed in 2.5% gluteraldehyde with 0.5% caffeine according to the method described by Sherwin and Farrant (1996) for resurrection plants. After fixation, tissues were dehydrated using an ethanol gradient, infiltrated with epoxy resin (Spurr, 1969) over two days and polymerized for 16 h at 60°C. Sections (95 nm thick) were cut using a Reichart Ultracut-S (Leica, Vienna, Austria); mounted on copper grids and stained with uranyl acetate and lead citrate (Reynolds, 1963) for 10 min each. Sections were viewed with a transmission electron microscope (Zeiss109 TEM).

Statistical data analysis

T-test at 95% confidence level was used to analyze data for all the measurements taken unless indicated otherwise.

RESULTS

RWC and electrolyte leakage

Figure 1 shows the changes in leaf RWC during dehydration and rehydration treatments. For the first three days of dehydration there was no significant water loss from ***En*** or the ***Et*** varieties (RWC ~ 80%, Figure 1A).There was full recovery of all metabolism processes measured on re-watering (data not shown). After 6 days of drying, however, RWC had dropped to 33% in ***En*** and 39% and to 43% in ***Et(W)*** and ***Et(B)***, respectively ($p<0.05$, as compared to control levels), and by 9 days of dehydration, the RWC of all plants had dropped to 10% with no further water loss thereafter (Figure 1A). When the plants were watered after 6 days of dehydration, the RWC of ***En*** and ***Et(B)*** recovered to the control levels within 24 h (Figure1B) but ***Et(W)*** did not rehydrate. When the plants were watered after 9 days of dehydration only ***En*** was able to recover (Figure 1C).Upon dehydration there was a progressive increase in rates of leakage in both ***Et(W)*** and ***Et(B)***, with a maximum leakage of 21.9 and 18.8 $\mu S\ g\ DW^{-1}\ min^{-1}$ respectively occurring after 12 days of dehydration (Figure 2A). In comparison, leakage from ***En*** was low throughout the dehydration treatment and control plants showed no change in electrolyte leakage. On rehydration following 6 days of dehydration, electrolyte leakage rates continued to increase in ***Et(W)*** leaves (Figure 2B) suggesting that membrane damage had occurred on dehydration and this was exacerbated on rehydration. In comparison, leakage from ***Et(B)*** dropped to the control levels after 24 h, indicating little membrane damage and/or repair of potential damage on rehydration. Rehydration after 9 days of drying resulted in elevated leakage rates in both ***Et*** varieties (Figure 2C) while ***En*** maintained low leakage on rehydration (Figure 2 B and C).

Gas exchange characteristics

Transpiration (E)

During the first 3 days of dehydration treatment, there was no significant change in ***E*** in ***En*** nor in the ***Et*** varieties (Figure 3A). However, after 6 days ***E*** (all ***E*** in italics) in ***En*** leaves dropped to 0.78 mmol $H_2O\ m^{-2}s^{-1}$ (a 90% decline relative to the control) and values stayed low during further dehydration. The decline in ***E*** in ***Et*** plants was more gradual, with a 50% and 30% decrease in ***E*** relative to hydrated control plants occurring in ***E***t(B) and ***E***t(W) respectively after 6 days, but by 9 days ***E*** rates in both varieties was similar to that of ***En***. When plants were rehydrated after 6 days of dehydration, ***Et(***W) showed decreased rates of ***E*** but there was a progressive increase in ***E*** in leaves of ***En*** and ***Et***(B), with both reaching levels equivalent to pre-dried hydrated state by 3 days of rehydration. ***En*** had significantly higher rates of ***E*** than ***Et***(B) (Figure 3B). After 9 days of dehydration treatment only ***En*** recovered ***E,*** although, this was achieved more gradually than for plants rehydrated after 6 days of drying (Figure 3C).

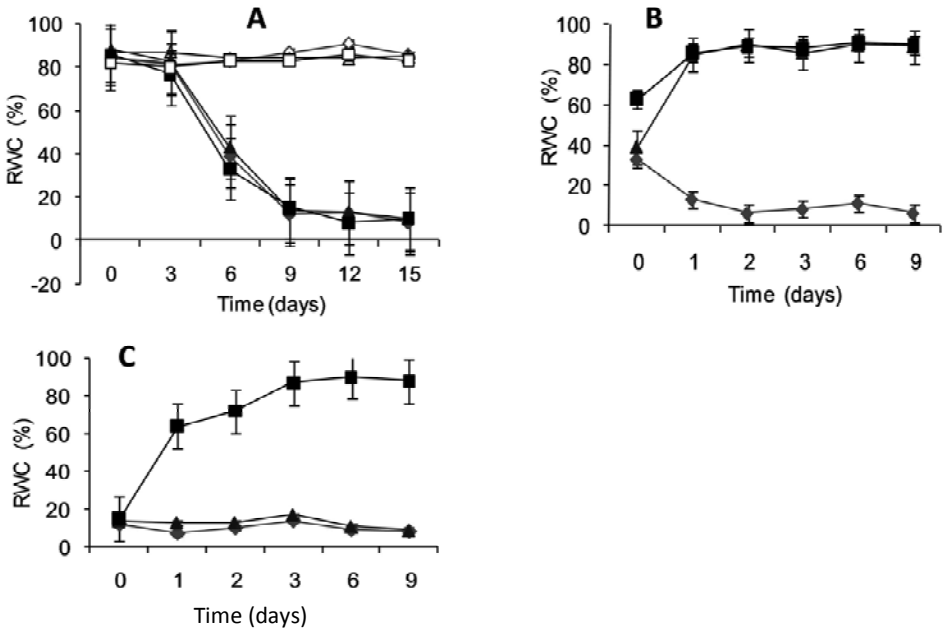

Figure 1 Leaf relative water content (% RWC) of plants measured during dehydration (A), and during two step rehydration: (B) after 6 days dehydration and (C) after 9 days of dehydration (♦ = ***Et(W)*** treatment, ◊ = ***Et(W)*** control, ▲ = ***Et(B)*** treatment, ∆ = ***Et(B)*** control, ■ = ***En*** treatment, □ = ***En*** control).

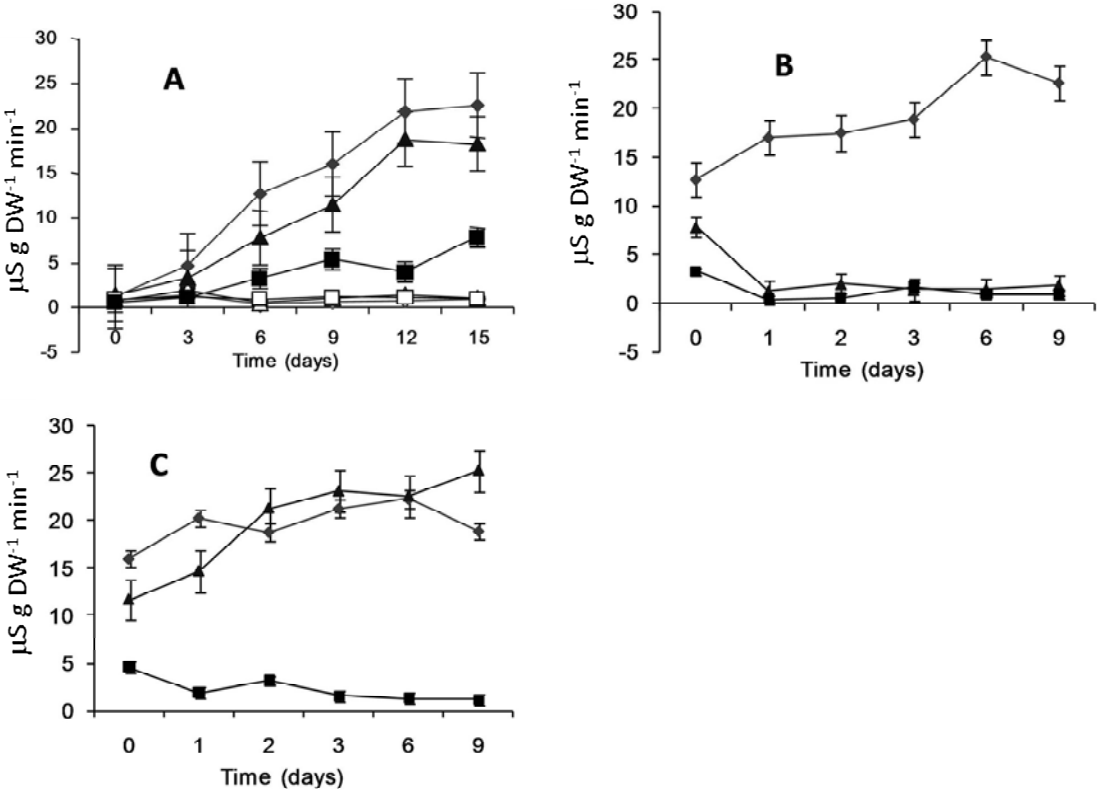

Figure 2. Rate of electrolyte leakage (µS g DW^{-1} min^{-1}) from plants measured during dehydration (***A***), and during two step of rehydration: (B) after 6 days dehydration and (C) after 9 days of dehydration (♦ = ***Et(W)*** treatment, ◊ = ***Et(W)*** control, ▲ = ***Et(B)*** treatment, ∆ = ***Et(B)*** control, ■ = ***En*** treatment, □ = ***En*** control).

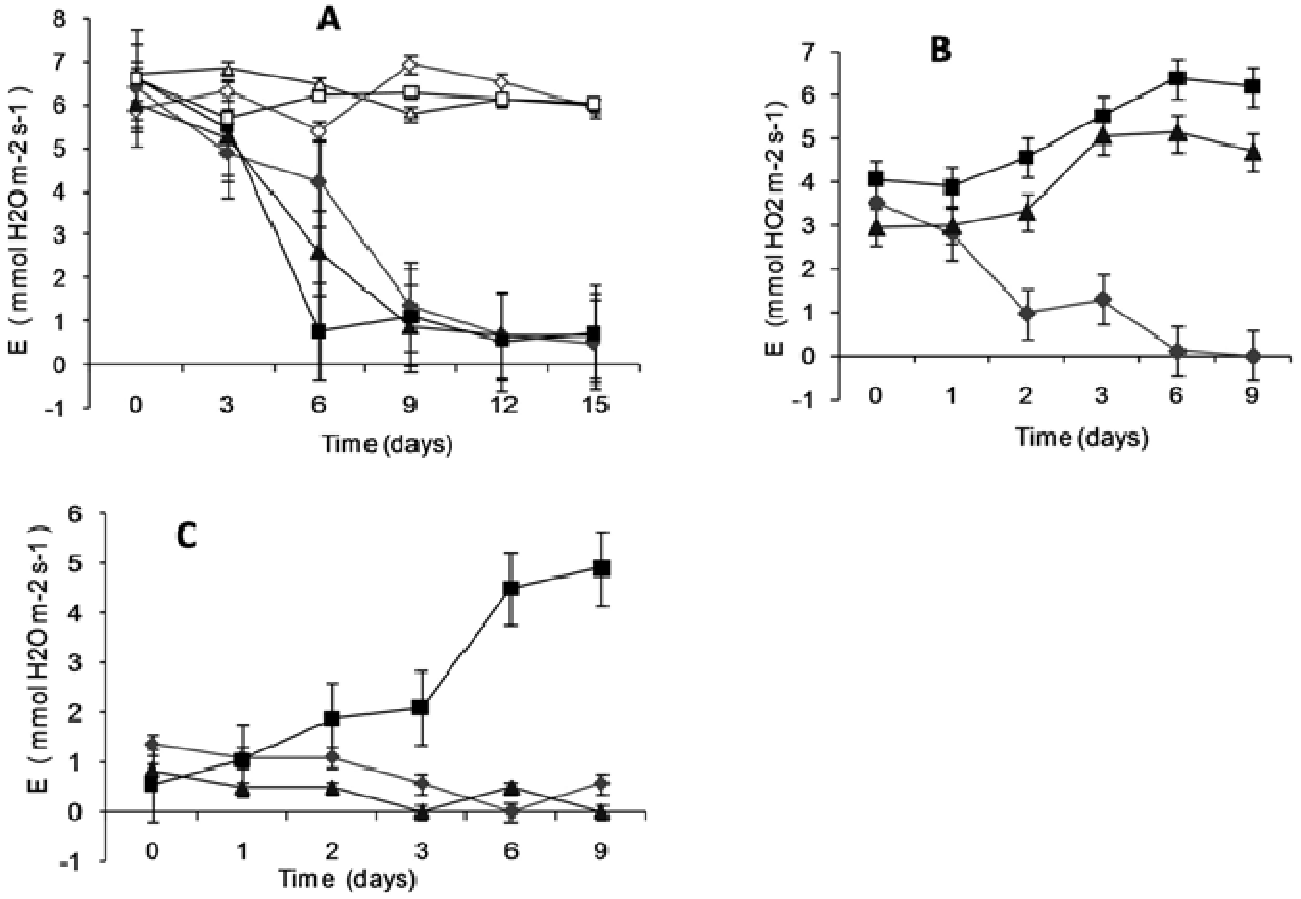

Figure 3. Transpiration rate (mmol H_2Om^{-2} s^{-1}) of plants measured during dehydration (A), and during two step rehydration: (B) after 6 days dehydration and (C) after 9 days of dehydration (♦ = ***Et(W)*** treatment, ◊ = ***Et(W)*** control, ▲ = ***Et(B)*** treatment, ∆ = ***Et(B)*** control, ■ = ***En*** treatment, □ = ***E. nidensis*** control.

Assimilation (A)

Initial net photosynthetic rates (***A***) of 12.1 µmol m^{-2} s^{-1}, 11.9 µmol $m^{-2}.s^{-1}$ and 11.0 µmol $m^{-2}.s^{-1}$ were recorded from ***Et(W)***, ***Et(B)*** and ***En*** respectively and there was no significant change in ***A*** during the first 3 days of drying (Figure 4A).

After 6 days of drying, there was a 40% decrease in ***A*** in ***En*** and ***Et(B)*** and a 70% decrease in ***Et(W)*** compared to the hydrated control plants***.*** After 9 days of dehydration, ***A*** had dropped to 0.6 µmol $m^{-2}s^{-1}$ in ***En*** and ***Et(W)***. Furthermore, the decline of ***A*** between days 6 and 12 during the dehydration treatment was more gradual in ***Et(B)*** compared to ***En and Et(W)***. There was a gradual increase in ***A*** in plants of ***En*** that had been dried for 6 days to levels equivalent to those of control undried plants when watered after 6 days of soil watering (Figure 4B). While there was an initial decline in ***A*** in leaves of ***Et(B)*** during the first 2 days following watering, ***A*** increased to similar levels recorded for ***En*** thereafter. After 6 days of drying, assimilation further declined in ***Et(W)*** when rehydrated. On rehydration after 9 days of dehydration, ***A*** showed recovery in ***En*** only (Figure 4C).

Respiration

There was a gradual but progressive decline in respiration upon drying in all plants with very low levels being recorded by 12 days (Figure 5A). On rehydration after 6 days of drying, respiration increased within 24h in ***Et(B)*** and ***En t***o that of pre-drying controls (Figure 5B). In ***Et(W)*** respiration declined on rehydration of plants watered after 6 days of drying. After 9 days of dehydration, only ***En*** showed full recovery of respiration when rehydrated (Figure 5 C).

Cellular ultrastructure

Representative images of mesophyll cells from leaf bases of ***Et(W)***, ***Et(B)*** and ***En*** upon dehydration and rehydration

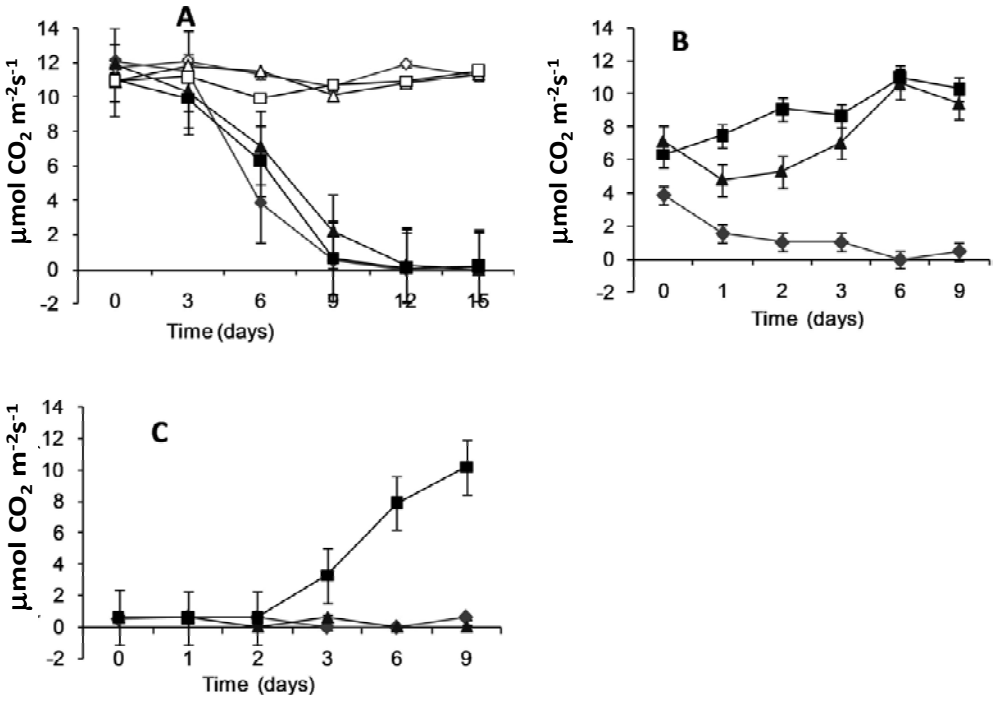

Figure 4. Net photosynthetic rate (***A***) (µmol $m^{-2}.s^{-1}$) of plants measured during dehydration (A), and during two step rehydration: (B) after 6 days of dehydration and (C) after 9 days of dehydration (♦ = ***Et(W)*** treatment, ◊ = ***Et(W)*** control, ▲ = ***Et(B)*** treatment, ∆ = ***Et(B)*** control, ■ = ***En*** treatment, □ = ***En*** control).

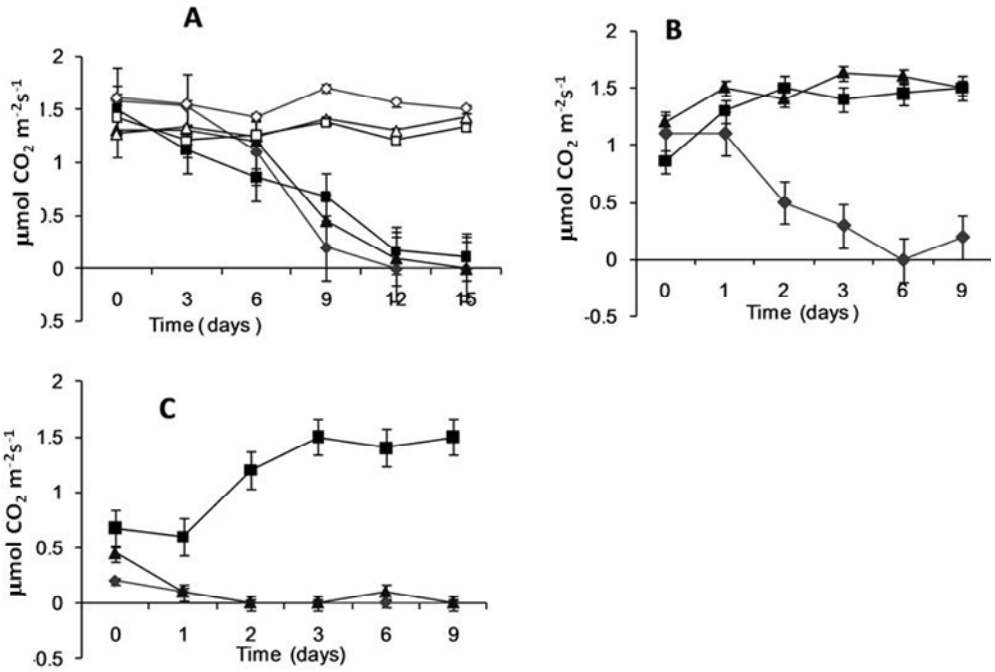

Figure 5. Respiration rate (µmol CO_2 $m^{-2}s^{-1}$) of plants measured during dehydration (A), and during two step rehydration: after 6 days of dehydration (B) and after 9 days of dehydration (C). (♦ = ***Et(W)*** treatment, ◊ = ***Et(W)*** control, ▲ = ***Et(B)*** treatment, ∆ = ***Et(B)*** control, ■ = ***En*** treatment, □ = ***En*** control).

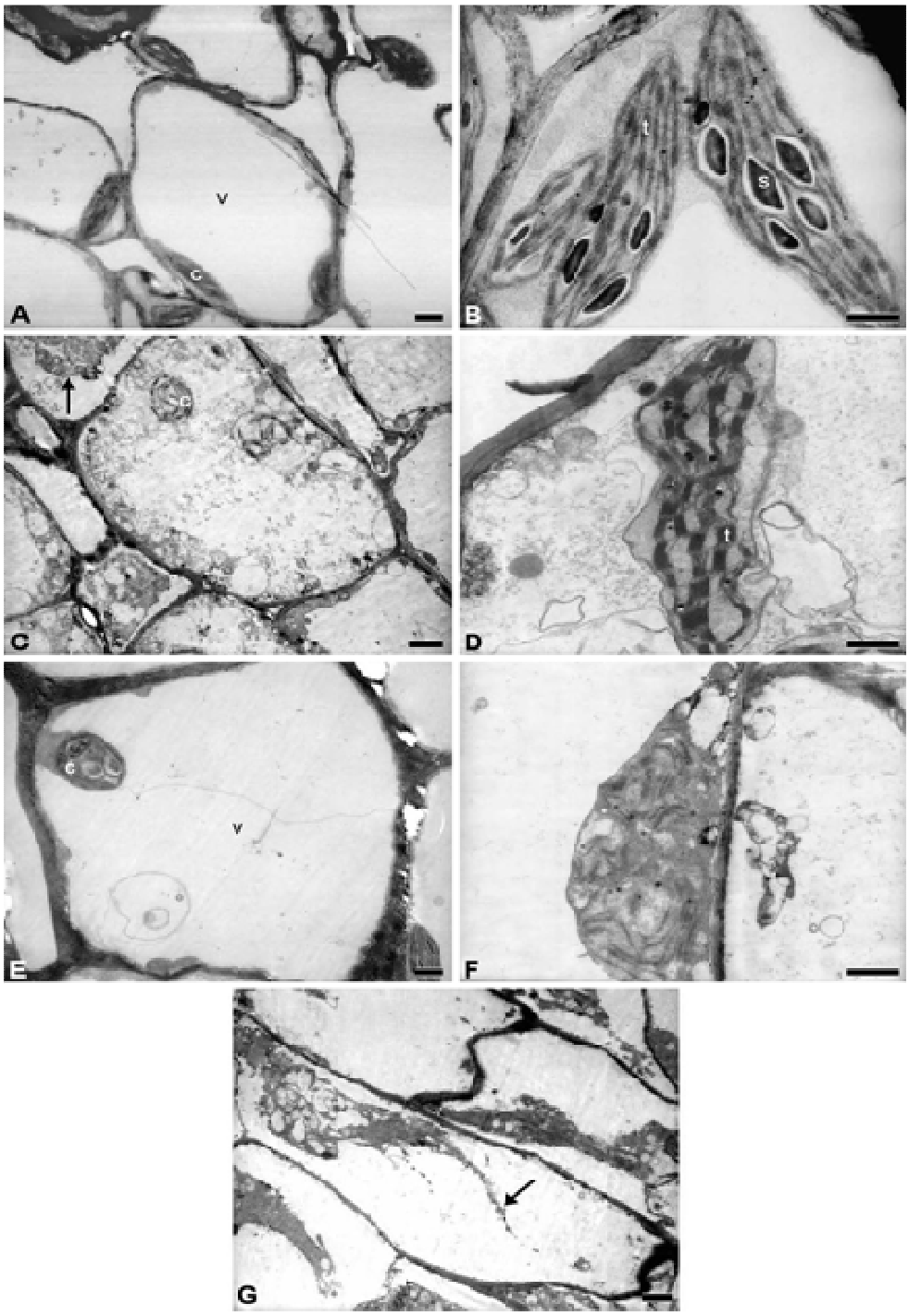

Figure 6. Ultrastructural changes in mesophyll cells of ***Et(W)*** during drying and rehydration. A and B show mesophyll cells and typical chloroplast organization, respectively from hydrated leaves; C and D represent those cells from leaves dried for 6 days and E and F show ultrastructual details typical of cells rehydrated after 6 days of drying. G, mesophyll cells after 9 days of dehydration. Chloroplast (c); starch (s); thylakoid membranes (t); vacuole (v). Scale bar = 1 µm.

are shown in Figures 6, 7 and 8 respectively. Prior to initiation of drying the ultrastructural organization was typical of hydrated fully turgid tissues in ***En*** and both ***Et*** varieties. Cells contained a large central electron transparent vacuole and cytoplasm and organelles were confined to the cell periphery (Figures 6A, 7A and 8A).

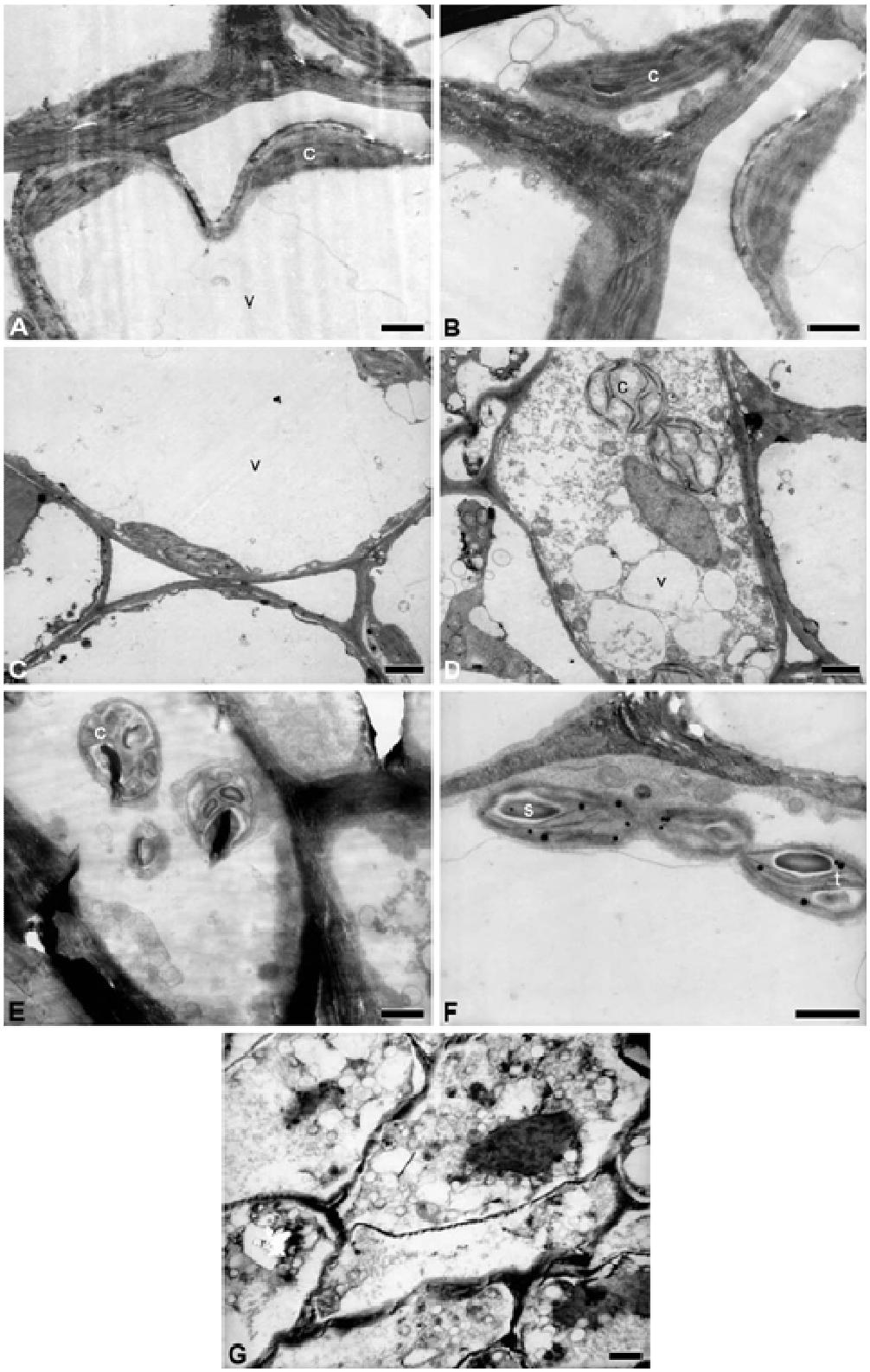

Figure 7. Ultrastructural changes in mesophyll cells of ***Et(B)*** during drying and rehydration. A and B show mesophyll cells and typical chloroplast organization respectively from hydrated leaves. C and D show different types of subcellular organization found in leaves from plants dried for 6 days. E and F show ultrastructural detail typical of cells rehydrated after 6 days of drying. G, mesophyll cells after 9 days of dehydration. Chloroplast (c); starch (s); thylakoid membranes (t); vacuole (v). Scale bar = 1 µm.

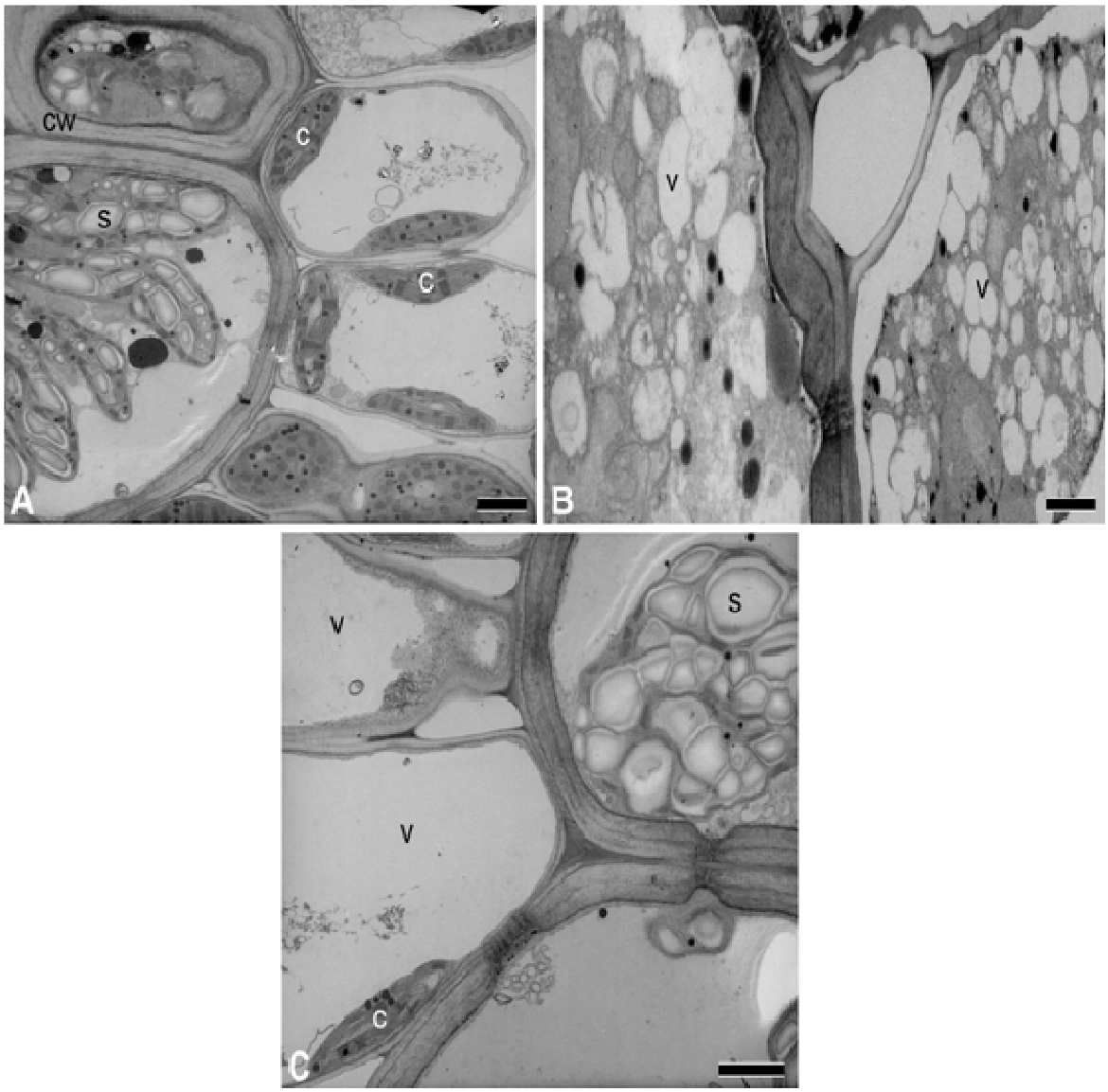

Figure 8. Mesophyll cells of fully hydrated (A) and dehydrated (B) leaves of ***En***. Details of subcellular organization of cells of plants rehydrated after drying to 10% RWC for 15 days are shown in (C) with several small vacuoles occupying most of the cytoplasmic space. Chloroplast (c); starch (s); thylakoid membranes (t); vacuole (v); cell wall (CW).

Chloroplasts had well defined thylakoid membranes and contained considerable amounts of starch and mitochondria had the appearance indicative of active tissue. Drying for 3 days resulted in little change in ultrastructure of ***En*** or ***Et*** plants (data not show).

After dehydration for 6 days (RWC, 39%) vacuoles of most mesophyll cells of ***Et (W)*** were reduced in size and both cytoplasm and vacuole content had an electron diffuse appearance (Figure 6C). Starch was absent from chloroplasts and thylakoids appeared distended, although, remained organized around areas where presumably starch grains had been present (Figure 6D). Leaf mesophyll cells of ***Et(B)*** after 6 days drying (RWC 43%) had varied subcellular organization. In some sections, the vacuole still occupied a large proportion of the cell, the content of which was electron transparent, and chloroplasts within the peripheral cytoplasm had reduced amounts of starch compared to the fully hydrated state but thylakoid membrane organization was regular (Figure 7C). In other cells, there were several smaller vacuoles with well-defined tonoplast membranes and relatively electron transparent content (Figure 7D). Chloroplasts in these cells contained no starch and thylakoid membranes were spatially separated. Upon rehydration after 6 days, drying mesophyll cells from both tef varieties showed restoration of the central electron transparent vacuole and peripheral location of the cyptoplasm (Figure 6***E*** and 7***E***). In ***Et(W)***, however, the

organelles and particularly the chloroplasts within many cells had poorly defined outer membranes and thylakoids were separated, with little evidence of granal stacking (Figure 6***E***). In ***Et(B)*** organelles were well defined. Chloroplasts had stacked thylakoid membranes and starch and some plastoglobuli were evident (Figure 7***E***). After 9 days of dehydration, the subcellular organization of tissues in both ***Et*** species looked severely compromised (Figure 6F and 7F). Plasma lemma withdrawal and rupture had occurred and there was general loss of organelle integrity. Rehydration resulted in loss of cytoplasmic content, probably the result of leaching upon entry of water into the tissues, with no subcellular detail being evident.

Mesophyll cells from dry leaves (RWC 10%) of ***En*** showed altered organization from the hydrated state but full subcellular integrity was evident (Figure 8B). The plasmalemma was intact, although withdrawn from the cell wall in places. Several small vacuoles occupied most of the cytoplasmic space. Chloroplasts were intact but thylakoid stacking was not evident. Instead numerous small vesicles and plastoglobuli were present. On rehydration, mesophyll cells appeared similar to that of hydrated tissue, containing a central electron transparent vacuole and chloroplasts with well defined thylakoids, many of which contained starch (Figure 8C).

DISCUSSION

The physiological data presented here for ***En*** support earlier reports that this plant is indeed a resurrection species (Gaff, 1977; Vander Willigen et al., 2001, 2004). In this study, ***En*** survived drying to 10% RWC, showed little increase in electrolyte leakage, suggesting that membranes remained intact and this was confirmed by ultrastructural studies which showed little damage to the plasmalemma or organelle boundary membranes in mesophyll cells, neither in the dry state nor on rehydration. Such organization has been reported for poikilochlorophyllous resurrection plants (Dace et al., 1998; Farrant, 2000, 2007). All plants maintained full hydration for only 3 days after cessation of watering after which there was a 70% loss of water over the next 3 days, reaching an air-dry state within 12 days. Although, transpiration rates declined rapidly as the bulk of water was being lost from leaves, it is unlikely that this was an attempt to retard water loss. Since assimilation rates remained relatively high, it would suggest that stomata are open. Furthermore, in other resurrection species, such as ***Myrothamnus flabellifolia*** (Moore et al., 2007) stomata remain open during this period of rapid water decline. The latter has been proposed to be a strategy to allow rapid loss of water, since the bulk of protection mechanisms are in place and it is dangerous, even with these in place, to be held at intermediate water contents where damaging reactions such as free radical proliferation can occur but perhaps antioxidant capacity (or mobility) is hampered (Farrant, 2007). It is possible that this is also a strategy used by ***En*** on drying***. Et(W)*** and ***Et(B)*** lost viability when dried below 39 and 43% RWC, respectively indicating that ***Et*** is not desiccation tolerant, and that ***ET(W)*** is more sensitive to water loss than ***Et(B).*** The rate of decline in water content in the ***Et*** varieties was similar to that of ***En.*** This together with the observation of low level transpiration and assimilation that continued over time, indicating open stomata, suggests that ***Et*** does not resist water loss but rather tolerates it to some degree; ***Et(B)*** to a greater extent than ***Et(W).*** Given the amount of water loss tolerated by ***Et(B)*** (ca 57%) before loss of viability, our work confirms suggestions by Shiferaw and Baker (1996) and conclusions drawn by Balsamo et al., (2006) based on the tensile properties of leaves of this species, that some varieties of ***E. tef*** are indeed relatively drought tolerant. However, from the data presented in our study, the ***Et(W)*** variety would be classified as less drought tolerant.

After 6 days of drying, there were elevated leakage rates in both ***Et*** varieties compared to ***En***, suggesting some membrane re-arrangement to increase porosity in ***Et***. Ultrastructural studies showed considerable changes in subcellular organization on drying in both varieties, but this had occurred in the majority of mesophyll cells in ***Et(W)*** but only in some in ***Et(B)*** possibly accounting for the elevated leakage in the former relative to the latter. On rehydration, leakage was exacerbated in ***Et(W)*** suggesting membrane damage had indeed occurred and this was confirmed by ultrastructural studies showing considerable subcellular damage (Figure 6 E and F). In ***Et(B)*** leakage, declined on rehydration indicating that either membranes had re-aligned or any damage that had occurred on drying was repaired. Ultrastructural studies showed full recovery of subcellular organization in ***Et(B)*** variety (Figure 7 E and F). Such data suggests that the increased degree of drought tolerance in ***Et(B)*** is related to ability to restrict degree of subcellular damage on drying and to repair damage to those cells that were compromised. It is interesting that vacuole organization in mesophyll cells that had altered subcellular organization after 6 days drying in ***Et(B)*** cells was similar to that of ***En***, in that several small vacuoles were evident (Figure 7D) rather than just a reduced volume in the large central vacuole evident in ***Et(W)*** cells (Figure 6E). In resurrection plants, such vacuole formation is reported to be accompanied by replacement of water in these with compatible solutes, allowing prevention of plasmolysis and cytorrhysis in the dry state (Farrant, 2000, 2007). Whether this is indeed happening in ***Et(B),*** or is related to the ability to initially minimize cytorhesis (and thus plasmalemma rupture) on drying and rehydration should be tested. But ultimately, loss of viability in ***Et(B)*** was also characterized by subcellular damage evident in most of the mesophyll cells together with increased leakage with both leakage and damage being exacerbated on rehydra-

tion. After 6 days of drying, ***Et(W)*** had the highest rate of transpiration, which might suggest that this variety is not able to regulate stomatal aperture as ***Et(B).*** Interestingly, the assimilation rates at this stage were lowest in ***Et(W).*** We propose that this is due to damage to the photosynthetic apparatus rather than limited CO_2 availability. It is well known that reactive oxygen species (ROS) are formed during photosynthesis and if not sufficiently quenched by antioxidants cause subcellular (and particularly chloroplastic) damage (Schwab and Haber, 1984; Smirnoff, 1993; Farrant et al. 2003; Kranner and Birtic, 2005; Scheibe et al. 2005). In resurrection plants, excess ROS formation due to photosynthesis is minimized by the use of either homoiochlorophylly (keeping chlorophyll but hiding it from light by leaf shading and anthocyanin production) or poikilo-chlorophylly (breakdown of chlorophyll and dismantling of thylakoids) (Tuba et al., 1996; Farrant, 2000, 2007; Farrant et al., 2003). In some homoiochlorophyllous species, separation of thylakoids on drying has been noted and suggested to be a mechanism to minimize photosynthetically associated ROS production (Farrant, 2000; 2007; Benko et al., 2002). ***En*** appears to separate thylakoids, even dismantle most (Figure 8B) and the slow recovery of A upon rehydration after drying to 10% RWC in ***En*** (Figure 4C) is probably due to the time required to reconstitute the photosynthetic apparatus, as has been reported for other poikilochlorophyllous resurrection plants (Sherwin and Farrant, 1996; Farrant, 2000; Farrant et al., 2003). While chloroplasts from both ***Et*** varieties showed irregular thylakoid arrangements on drying, recovery of arrangement upon rehydration (Figure 7E) together with resumption of assimilation (Figure 4B) occurred only in ***Et(B)***, and this was possible only if dried to ca 43% RWC. Drying below this RWC level resulted in damage to chloroplasts and irreversible loss of photosynthetic capacity (Figure 4C).Nevertheless, this data suggests that this variety has the better ability to initially minimize ROS damage and/or repair such damage. If ROS formation had indeed compromised chloroplast integrity, it is likely also to have caused damage to other subcellular organelles including the plasmalemma and contributed to the elevated electrolyte leakage and loss of viability in both varieties. Respiration rates remained high in ***En*** and both ***Et*** varieties for at least 6 days of drying before declining (Figure 5A), but recovery upon rehydration followed the same trends as those of photosynthesis and transpiration (Figure 5B C). Several studies on plant gas exchange parameters have reported that respiration is the last parameter to be affected by water deficit (Farrant and Kruger, 2001; Vander Willigen et al., 2001; Lawlor and Cornic, 2002; Ribas-Carbo et al., 2005) and it has been suggested that in resurrection plants, it is important to retain the ability to provide ATP for laying down of protection and/or repair of stress induced damage (Farrant et al., 2007). This may well be the situation during initial stages of water deficit in the ***Et*** varieties, however, it is only in ***En*** that respiration is recovered on drying to below 30% RWC. If elevated respiration in the ***Et*** varieties is involved in facilitating accumulation of subcellular protection, it is either insufficient, or the protection mechanisms are simply inadequate, to protect at low water contents in this species. Furthermore, respiration is another source of ROS formation and maintenance of elevated respiration rates in ***Et*** varieties for up to 6 to 9 days of drying that may have ultimately exacerbated rather than facilitated survival.

In summary, ***En*** is a typical resurrection plant surviving drying to 10% RWC. ***Et*** is desiccation sensitive but does not appear to resist water loss, rather tolerating some short term water deficit. The differences among the varieties in extent of water loss tolerated appears to be related to ability to initially restrict the amount of damage occurring and to repair such damage on rehydration. However, if these were indeed effective in the short term, they were insufficient to restrict damage incurred on drying below ca 43% RWC to repairable levels. For improved drought tolerance, mechanisms to further restrict subcellular damage and to repair incipient damage must be introduced into ***Et*** varieties.

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