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**COMPARISONS OF THE RESURRECTION
GRASS, *ERAGROSTIS NINDENSIS*, WITH
THE RELATED DESICCATION-SENSITIVE
SPECIES, *E. CURVULA***

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Thesis submitted for the Degree of
Doctor of Philosophy
in the Department of Botany
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पदं पदं प्रतिपदं अर्हति इति
प्रातिपदिकम् ॥

Sanskrit Grammatical Sūtra

paḍaṃ paḍaṃ pratipaḍaṃ arhati iti prātipadikaṃ

Sanskrit transliterated into Roman script

Step by step, action by action, word by word,
at every word, action or step, there stands
waiting, quite still, that which is appropriate
to each word, action or step.

English translation

Claim nothing. Enjoy!
(Eesha Upanishad)

Preface

The experimental work described in this thesis was carried out at the University of Cape Town from January 1999 to August 2001 under the supervision of Associate Professor Jill M. Farrant, Doctor Sagadevan G. Mundree (Department of Molecular and Cellular Biology, University of Cape Town) and Associate Professor Norman W. Pammenter (School of Life and Environmental Sciences, University of Natal, Durban).

It is hereby declared that this thesis, submitted for the degree of Doctor of Philosophy in Botany at the University of Cape Town, is the result of my own investigations, except where the work of others is acknowledged.

Signed by candidate

Clare Vander Willigen

September 2001

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University of Cape Town

Abstract

Desiccation tolerance of the inner leaves of *Eragrostis nindensis* is compared with the desiccation sensitivity of the outer leaves, as well as those of the closely related species, *E. curvula*. Both *E. nindensis* and *E. curvula* dehydrate to a relative water content (RWC) of less than 5% in two weeks. Photosynthetic activity in *E. curvula* is maintained down to 40% RWC, after which further drying results in a sudden irreversible breakdown of the photosynthetic system and its pigments. At this point, electrolyte leakage increases and F_v/F_M decreases such that below *ca.* 40% RWC, metabolism is irreparably damaged. Ultrastructural studies using freeze-substitution of these leaves revealed the maintenance of cellular organization in the dry state but confirmed that irreparable damage occurs but this is evident only on rehydration. During dehydration of *E. nindensis* (inner leaves) there is a controlled loss of photosynthetic pigments, paralleled by a gradual shutdown in gas exchange. On rewatering, most *E. nindensis* leaves (except the older, outer ones) resume normal metabolic activity within a few days. Respiration resumes almost immediately but photosynthesis only restarts at 70% RWC, by which time chlorophyll has been resynthesised and anthocyanin content reduced. Although *E. nindensis* is unique as the only known poikilochlorophyllous resurrection grass, some chloroplast structure is maintained in the dry state. In addition leaf rolling and anthocyanin accumulation, features associated with homoiochlorophylly (the strategy used predominantly in resurrection grasses) were observed. Interestingly, the older outer leaves of most tillers of *E. nindensis* do not rehydrate. These leaves show signs of membrane damage and curl in an irregular manner, similar to those of *E. curvula*, during dehydration. Ultrastructural observations reveal that this damage (particularly in the cell walls) is incurred during dehydration, which probably prevents the initiation of many of the inherent protective mechanisms necessary to ensure desiccation tolerance in these tissues.

Pressure-volume (PV) curves of *E. nindensis* and *E. curvula* were compared with two other resurrection angiosperms: *Craterostigma wilmsii* and *Xerophyta humilis*. The shape of curves for *E. nindensis* and *C. wilmsii* differed from the usual curvi-linear form. Over the RWC range of *ca.* 70% to 25%, there was a negligible change in water potential,

corresponding with considerable cell volume reduction (a consequence of cell wall folding). Water potentials were always higher than directly-measured water activity on frozen-thawed tissue confirming that negative turgor did not develop. The wall folding of these two species possibly prevents this stress and thus assists in their tolerance of desiccation. *X. humilis*, *E. curvula* and the outer desiccation-sensitive leaves of *E. nindensis* showed normal PV curves. The cell wall fractures in the desiccation-sensitive outer leaves of *E. nindensis* support these suggestions. The absence of wall folding and volume reduction in *X. humilis* is probably due to maintenance of vacuolar volume within these cells during desiccation – an alternative strategy to wall folding as a means to reduce the physical stresses associated with desiccation.

The appearance of abundant small vacuoles in the bundle sheath cells of dehydrated desiccation-tolerant leaves of *E. nindensis* coincided with the presence of an α -tonoplast intrinsic protein (TIP), which was observed to be associated with these vacuolar membranes using immunofluorescence. This is the first report of such proteins in vegetative tissues and suggests a storage role in these vacuoles, similar to those found in seeds. Differences in expression of δ -TIP with dehydration in both *E. nindensis* and *E. curvula* were observed but the purpose of these proposed aquaporins in water stress is unclear.

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Chapter 1:

Introduction

All organisms, including plants, are entirely dependant on water for life. Water also constitutes the major component of any growing plant (over 80% in herbaceous tissues) thus a water deficit would be critical (Kramer, 1983). However, because plants are sessile and cannot translocate from such pressures imposed on them by their environment, some ingenious and fascinating mechanisms to survive water shortages have been developed.

The effects of a water shortage in a plant are numerous and varied since water has many biological properties. Not only does water buffer temperature fluctuations, act as a reactant in hydrolysis, provide a medium for most biochemical reactions and transport of solutes, but its incompressibility means that it is also important in structural support (Meidner & Sheriff, 1976; Kramer, 1983; Vertucci & Farrant, 1995). The impact of a water deficit on these features has and continues to give rise to a considerable number of studies from an ecological to a molecular level (reviewed by Kozlowski, 1972; Levitt, 1980; Turner & Kramer, 1980; Tomos, 1988; Bray, 1997; Mager *et al.*, 1998).

1.1 Classification of water stresses

Since this is such a vast field involving a number of different disciplines, a variety of different meanings of the terminology have developed. To an agronomist, a drought is any water shortfall that reduces plant productivity (Jones, 1992) but the definitions in most other fields do not usually make any reference to plant yield. Levitt (1980) defined “drought stress” as any water deficit stress and “desiccation stress” as an artificially imposed water stress. The term “desiccation stress” has also been used to describe the stress associated with dehydration of a plant to the air-dry state (Gaff, 1980). The range of definitions and the substitution of the terms with each other can be misleading and confusing with respect to the ability of a plant to survive water deficits.

For the purposes of this investigation, “desiccation” involves the loss of all free water and thus “desiccation tolerance” is defined as the ability to survive such a stress. Drought tolerance involves a different strategy, whereby a plant tends to avoid the strain associated with a water deficit, usually maintaining a water potential above that of the environment (Figure 1.1). Because the vegetative tissues of most drought tolerant plants cannot survive the extreme loss of water associated with desiccation, “drought” is often considered as a moderate water deficit (Hoekstra *et al.*, 2001). The different drought tolerance strategies are summarised in Figure 1.1 (terminology adapted from Levitt (1980) and Jones (1992)).

Whereas many vegetative tissues are able to survive mild or moderate water stresses, few plants are able to survive extended periods of water loss or desiccation (Gaff, 1980; Proctor, 2000). Although there are a few apparent similarities between the mechanisms to achieve drought tolerance and the initial stages of desiccation tolerance in a few species, these two phenomena generally involve very different strategies and most drought tolerant plant parts are in fact desiccation sensitive (Gaff & Ellis, 1974; Bradford & Hsiao, 1982). Desiccation tolerance has been described as a drought avoidance mechanism (Mittler *et al.*, 2001) since neither turgor nor cellular metabolism is maintained under water stress. However it is debatable whether desiccation-tolerant plants can survive for extended periods at intermediate water contents. Even though desiccation-tolerant plants are able to survive extreme drought they are slow growing and dormant in the desiccated state (Gaff, 1980). Thus they are of no immediate economic value to the agricultural industry. Nevertheless, a greater understanding of this extreme in plant water stress may provide further insight into possible mechanisms for improving crop performance. The demand for higher productivity to feed the ever-expanding human population has increased the pressure on existing farmlands and the potential for increased irrigation is limited. Since water is the primary limiting factor of plant productivity, the importance of understanding plant water stress is a necessity in order to provide a sustainable food source for the future (Richards *et al.*, 1997; Holmberg & Bulow, 1998).

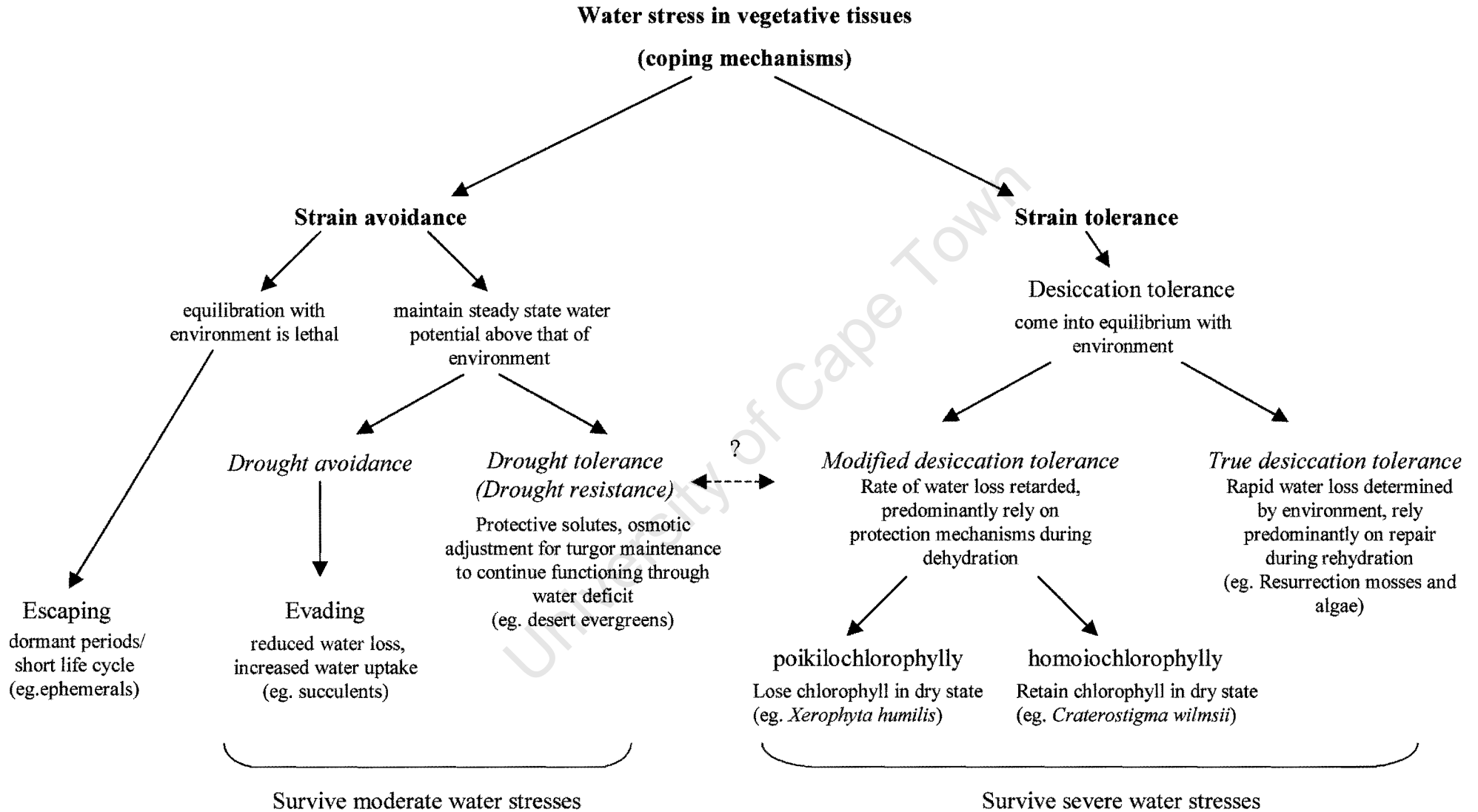


Figure 1.1 Classification of mechanisms utilized by plants in coping with water stress

1.2 Desiccation tolerance

Desiccation tolerance is common in prokaryotes, lower eukaryotes, orthodox seeds and pollen but is rare in vegetative tissues of higher plants (Bewley & Krochko, 1982). However, a small group of vascular plants are also able to survive extended periods of desiccation. Since all these so called “resurrection plants” are taxonomically diverse, desiccation tolerance is either inherent but suppressed in most plants or it must have evolved independently numerous times (Oliver & Bewley, 1997; Hartung *et al.*, 1998; Oliver *et al.*, 2000). Since desiccation tolerance is present in the seeds of most angiosperms, this phenomenon is intrinsic to those plants. However the diverse mechanisms employed by the few species that are able to survive desiccation in the vegetative state argues for the latter.

Severe water deficits impose structural, physical and metabolic stresses on a plant and survival from these stresses necessitates changes at all levels of organization: physiological, biochemical, morphological, ultrastructural and molecular (Gaff, 1980; Oliver *et al.*, 2000). Since there are numerous stresses associated with desiccation, tolerance necessitates the involvement of a number of strategies. In addition there are a variety of mechanisms utilized by different resurrection plants to confer desiccation tolerance to each of the stresses (Oliver & Bewley, 1997; Farrant, 2000). It is generally accepted that desiccation tolerance requires both some protection of cellular constituents during desiccation and in the dry state, as well as repair of damage on rehydration (Oliver *et al.*, 1998).

It has been proposed that resurrection plants that are able to survive rapid dehydration, (at a rate determined by their environment) are called “true” desiccation-tolerant plants, whereas plants that utilize some mechanism to retard water loss during desiccation in order to avoid irreparable desiccation-induced damage are referred to as “modified desiccation-tolerant plants” (Figure 1.1; Oliver & Bewley, 1997). True desiccation-tolerant plants (including most of the lower order resurrection plants such as algae and bryophytes) are generally less complex and small and are thus unable to

significantly retard water loss and rely predominantly (but not solely) on repair of desiccation-induced damage during rehydration (Oliver, 1996). These plants generally experience desiccation more frequently than the modified desiccation-tolerant plants and constitutively express some genes thought to be necessary to prevent irreparable dehydration damage (Oliver *et al.*, 1998). Although some angiosperm resurrection plants are able to survive rapid drying rates of 4-8 hours (Farrant *et al.*, 1999; Cooper, 2001) it is assumed that most cannot (Oliver & Bewley, 1997). These plants initiate protection strategies during drying and generally rely less on repair-based mechanisms during rehydration.

1.3 Water deficit-associated stresses

Since water assumes numerous vital roles in plants, the process of conferring desiccation tolerance requires a number of different mechanisms to cope with the associated stresses during both dehydration and rehydration. Although these stresses generally occur in synchrony with one another, it is simpler to consider them separately. While the stresses experienced by all resurrection plants are similar, the mechanisms to survive them differ (Oliver & Bewley, 1997; Farrant, 2000). In this review most attention is paid to strategies employed by resurrection angiosperms. Where appropriate, comparisons to drought tolerance are given.

a) Structural stresses

Water provides support for the components of a cell. Often the first sign of a water deficit is a loss in turgidity (Iljin, 1957; Levitt, 1980). The loss in volume as water leaves the cell usually results in plasmolysis; the plasma membrane is thought to withdraw from the cell wall. The tension that results from the loss of volume can cause tearing of cell membranes (Leopold *et al.*, 1981). In addition, the influx of water into the cell on rehydration can exacerbate this damage, often measured as an increase in solute leakage (McKersie & Stinsin, 1980; Leopold *et al.*, 1981; Oliver & Bewley, 1984). Membrane disruption would also cause a lack of compartmentalization within

the cell; autolytic enzymes would no longer be confined to lytic vacuoles and intracellular compartments and there would be a lack of organization for normal cellular metabolism (Wolkers *et al.*, 1998; Hoekstra *et al.*, 2001).

Since much of this damage is irreversible, resurrection plants have developed various methods to reduce or avoid this stress. Because it is the rigidity of the cell wall (compared with that of the membranes) that often causes the initial tension some resurrection plants, particularly those from the genus *Craterostigma*, change the biochemical properties of their cell walls on dehydration (Vicré *et al.*, 1999; Vicré, 2001). This enables reversible cell wall folding to occur on dehydration (Sherwin, 1995; Vicré, 2001). Another strategy used extensively by *Xerophyta* species involves substituting the water in the vacuoles with nonaqueous material (Farrant & Sherwin, 1998; Farrant, 2000) in order to maintain cellular structure and volume and prevent collapse during desiccation in a similar manner to the theories proposed by Iljin (reviewed by Levitt, 1980).

Sugars and other compatible solutes are known to accumulate in many resurrection plants during desiccation (Schwarb & Gaff, 1986; Bianchi *et al.*, 1991; 1992; 1993; Albin *et al.*, 1994; Ghasempour *et al.*, 1998a; Scott, 2000; Whitaker *et al.*, 2001). Considerable research into the roles of similar compounds in dry seeds has suggested that sugars, particularly oligosaccharides, act in stabilizing membranes in the absence of water, preventing phase transition and separation (Crowe *et al.*, 1987; Caffrey *et al.*, 1988; Hoekstra *et al.*, 1997; 2001). This is known as the “water replacement hypothesis” whereby sugars substitute for water during desiccation (Womersley, 1981). It has also been proposed that similar compounds may fill the vacuoles of desiccated cells of resurrection plants such as *Xerophyta viscosa* and *X. humilis* in order to prevent cellular collapse (Farrant & Sherwin, 1998; Farrant, 2000).

b) Metabolic stresses

Resurrection plants cope with the metabolic stresses associated with desiccation using a number of strategies. Modified desiccation-tolerant plants tend to switch off metabolism during dehydration to prevent damage associated with unregulated metabolism and thus minimize repair during rehydration (Farrant & Sherwin, 1998; Pammenter & Berjak, 1999).

Since even mild water stresses affect metabolism and thus plant growth, there have been extensive investigations into the effect of moderate water stresses in many commercially important plants (reviewed by Losch, 1996). As with many other water stressed plants, water loss is reduced by stomatal closure in modified desiccation-tolerant plants. Consequently, photosynthesis decreases initially as a result of the restricted gas exchange (Dietz & Herber, 1983; Kaiser, 1987; Tuba *et al.*, 1996a; 1996b; 1998). The artificial removal of this limitation to gas exchange in *Craterostigma plantagineum* has shown that in this species, photosynthetic capacity is not reduced until moderate to severe water stress (Dietz & Herber, 1983). In another resurrection angiosperm, *Romonda serbica*, the CO₂ fixation pathway was found to change from C3 to CAM during desiccation (Markovaska *et al.*, 1997).

In addition to acting as a solvent for many metabolic reactions, water also stabilizes the tertiary structure of proteins (Bray, 1997). The stabilization of proteins in the dry state is proposed as another role of the accumulation of sugars during desiccation (Crowe *et al.*, 1987). Furthermore vitrification of these sugars has been suggested (Ingram & Bartels, 1996). The formation of glasses in seeds is thought to suspend cellular activity and prolong the life of a cell in the dry state (Williams & Leopold, 1989; Koster, 1991) and it may well perform a similar function in desiccated vegetative tissues.

As water is lost from a cell, the osmotic concentration will also tend to increase. Increases in ionic strength can be toxic, denaturing proteins and destabilizing membranes (Schwarb & Herber, 1984; Schwarb & Gaff, 1986). However, compatible

solutes (eg. proline, sorbitol and glycine betaine) which are not toxic to metabolism, are reported to accumulate in a number of desiccation-tolerant plants during desiccation and may play a role in neutralizing high ionic strength (Tymms & Gaff, 1979; Bradford & Hsiao, 1982; Bartels & Nelson, 1994).

Oxidative stress

In addition to the general disruption to metabolism, damage due to the formation of free radicals has the potential to result in considerable cellular damage. Free radicals, especially reactive oxygen species are known to cross-link and damage both proteins and nucleic acids as well as disrupt membrane integrity via lipid peroxidation (Smirnov, 1993; McKersie & Lesham, 1994) in positive feedback cycles which are particularly prevalent at intermediate water contents during both dehydration and the subsequent rehydration period (Smirnov, 1993; Vertucci & Farrant, 1995).

Unlike dehydrating seeds, in which the formation of free radicals is largely restricted to mitochondria, endoplasmic reticulum and plasma membranes, the most abundant and dangerous site in water-stressed vegetative tissues is the chloroplast (Smirnov, 1993; Kranner & Grill, 1997; Sherwin & Farrant 1998). Three sites of free radical formation exist as result of the membrane disruption and reduced quantum efficiency of the photosystems caused by water stress *vis.* 1) activated chlorophyll can reduce oxygen directly, forming singlet oxygen, 2) as NAPH⁺ becomes limited, ferredoxin reduces oxygen forming superoxide and 3) the splitting of water itself can also give rise to superoxide and hydrogen peroxide (Smirnov, 1993). In addition to these, free radicals can react with each other and ferric salts to create further unstable radicals (Smirnov, 1993; McKersie & Lesham, 1994).

The most effective means of minimizing free radical damage is prevention of free radical production. Resurrection plants generally use one of two strategies to avoid light-associated photooxidative stress: shade their chlorophyll from the light

(homoiochlorophyllous plants) or lose their chlorophyll (poikilochlorophyllous plants) during desiccation (Figure 1.1; Bewley, 1979).

Poikilochlorophyllous resurrection plants such as *Xerophyta humilis* actively disassemble their chlorophyll and thylakoid membranes during dehydration (Hambler, 1961; Gaff, 1977; Hetherington & Smillie, 1982). This is likely to minimize free radical formation in the chloroplasts. On rehydration, the chlorophyll and thylakoids are reassembled and photosynthesis then resumes. Homoiochlorophyllous resurrection plants (for example *Craterostigma wilmsii*) use a number of different methods to mask their chlorophyll from the light when water is limited and free radical formation is likely (Gaff, 1977; Bewley, 1979; Sherwin & Farrant, 1998). Many of these plants curl or roll their leaves on drying, such that only the outermost adaxial surface is exposed to light (Farrant, 2000). In addition, the pigment anthocyanin, which reflects light of similar wavelengths to those usually trapped by chlorophyll (Gould *et al.*, 1995; Cooper-Driver, 2001), accumulates particularly in the leaves which are exposed to the sun (Sherwin & Farrant, 1998).

Nevertheless, some free radical damage is inevitable and both types of resurrection plants utilize additional mechanisms to limit oxidative damage. The accumulation of xanthophylls (especially zeoxanthin and violaxanthin) aids in the dissipation of excess excitation energy at the photosystem reaction centers (Demming-Adams *et al.*, 1996), and a variety of antioxidants are known to increase during desiccation. Antioxidants such as superoxide dismutase react with superoxide radicals directly whereas glutathione reductase, peroxidases, catalases, tocopherols and carotenoids are known to quench other free radicals such as hydrogen peroxide and peroxy radicals (Larson, 1988; Young, 1991; Seel *et al.*, 1992; Sgherri *et al.*, 1994a; 1994b). Anthocyanin and some phenolic compounds, which have also been shown to accumulate during desiccation of many resurrection plants, are also thought to have antioxidant properties (Cooper-Driver & Bhattacharya, 1998; Wang *et al.*, 1999; Cooper-Driver, 2001).

c) *Physical stresses*

To date, little attention has focused on the physical stress of severe water deficits and plant water relations in desiccation tolerance. The physical processes such as translocation and water transport are also obviously interrupted as a consequence of desiccation. Recently there has been some interest in the mechanisms employed by the only woody resurrection plant, *Myrothamnus flabellifolius* to reestablish a water column in its dry xylem conduits (Canny, 2000; Schneider *et al.*, 2000; Wagner *et al.*, 2000) however the means by which this is achieved is debated (Tyree, 2001). Nevertheless, this aspect of desiccation is probably the determining factor with respect to the maximum height a resurrection plant can attain (Sherwin *et al.*, 1998). In fact it has been suggested that this height limitation is the reason there are no resurrection gymnosperms (Oliver, 1996).

Other aspects of plant water relations are also affected by severe water stresses. It has already been mentioned that turgor is lost and osmotic potentials increase as water becomes limiting. Water potentials of less than -160 MPa have been reported in dry leaves of resurrection plants (Gaff, 1980). Furthermore the loss of membrane organization and the dehydration of the apoplast result in the cessation of normal water movement. Although very little is known of the specific role of aquaporins in plant membranes during desiccation, the expression of some of these water channel proteins has been reported to change with desiccation (Mariaux *et al.*, 1998; Neale *et al.*, 2000). With their ability to dynamically alter and regulate transmembrane water movements, it is likely that they are important, particularly during the rehydration of desiccated cells (Hartung *et al.*, 1998).

1.4 Desiccation-related proteins and genes

Many of the response mechanisms associated with conferring desiccation tolerance have been described in context of the stresses outlined above. However, a number of genes and

proteins have also been shown to be up- and down-regulated as a consequence of water stress.

Two examples are heat shock and LEA (late embryogenesis abundant) proteins (Schneider *et al.*, 1993; Ingram & Bartels, 1996; Black *et al.*, 1999). Heat shock proteins act as molecular chaperones preventing the aggregation of denatured proteins and assisting in protein refolding (reviewed by Hartl, 1996). A subclass of LEA proteins particularly associated with desiccation tolerance (dehydrins) are thought to function primarily in water replacement, whereas other classes of LEA proteins are thought to be involved in a variety of other mechanisms suggested to minimise water-stress induced damage (reviewed by Dure, 1997). Heat shock and LEA proteins were first discovered in heat shocked fruit flies and developing seeds, respectively (Galau *et al.*, 1986; Close, 1996; Hartl, 1996). A full description of the role of these and the many other genes and proteins thought to be involved in desiccation tolerance will not be discussed since this aspect of desiccation tolerance is not the main focus of this thesis.

Cross-talk between genes and proteins from different species but related stresses (eg. temperature, osmotic, salinity, drought) is common (Sabehat *et al.*, 1998; Shinozaki & Yamaguchi-Shinozaki, 2000; Grover *et al.*, 2001). This discovery has prompted studies into the possibility of conferring multiple stress tolerance in crops (Khanna-Chopra & Sinha, 1998; Nuccio *et al.*, 1999). Mass screening molecular biology techniques have also enabled the identification of a vast array of many as yet uncharacterised desiccation-related genes and proteins (Frank *et al.*, 1998; Blomstedt *et al.*, 1998; 2000; Mundree & Farrant, 2000, Neale *et al.*, 2000). However, as it has been shown that not all desiccation tolerance mechanisms (particularly those occurring on rehydration) require *de novo* transcription or translation (Dace *et al.*, 1998; Cooper, 2001), studies on gene expression may not be adequate to fully explain desiccation tolerance and a broader, more holistic approach to the phenomenon is required.

1.5 Desiccation sensing and signalling

Unlike the constitutive expression of various genes important in conferring desiccation tolerance in true desiccation-tolerant plants, the initiation of many of the mechanisms possessed by resurrection angiosperms are triggered only as a consequence of drying (Oliver & Bewley, 1997). Efforts focussed on identifying regulatory proteins such as protein kinases, phosphatases and transcription factors which are thought to mediate gene expression in response to water stress (Jonak *et al.*, 1996; Shinozaki *et al.*, 1998) in resurrection plants have recently been undertaken. Several abscisic acid responsive elements (Ingram & Bartels, 1996) have been identified in *Craterostigma plantagineum*. Further discoveries in the signalling of desiccation tolerance are likely.

A change in osmotic potential is thought to be one of the main points of perception of water stress (Urao *et al.*, 1999) and various plant growth regulators have been shown to be important in the initiation of many of the response mechanisms (Gaff & Loveys, 1984a; Chandler & Robertson, 1994; Bochicchio *et al.*, 1998). Abscisic acid is known to increase significantly during water loss and is thought to be involved in stomatal closure (Mansfield & Atkinson, 1990; Rock, 2000) as well as initiate many genes involved in conferring desiccation tolerance in some resurrection plants (Bartels *et al.*, 1990; 1992; Ingram & Bartels, 1996; Velasco *et al.*, 1998) but not others (Gaff & Loveys, 1984). The concentration of other plant growth regulators such as cytokinins and auxins also change during water stress (Hare *et al.*, 1997; Kruger, 1998; Pospisilova *et al.*, 2000).

The discovery of dehydration responsive elements (eg. DRE) and their corresponding DNA-binding proteins (eg DREB) which also trigger the expression of a host of other ABA-independent stress-related genes in other plants (Yamaguchi-Shinozaki & Shinozaki, 1994) suggests that similar processes are very likely in conferring desiccation tolerance as well. Furthermore cross-talk between these signalling pathways from comparable stresses (Shinozaki & Yamaguchi-Shinozaki, 2000) has highlighted these as important elements in the genetic transformation of crops for multiple stress resistance (Grover *et al.*, 2001).

1.6 This study

Of the approximately 100 resurrection angiosperms identified to date, more than two thirds are monocotyledonous (Porembski & Barthlott, 2000) and nearly half of these are from the family Poaceae – all of which are from the subfamily Eragrostoideae. *Eragrostis nindensis* Ficalho & Hiern, formally *E. denudata*, is one such grass (Figure 1.2). As with many of the resurrection angiosperms, early reports of this species note its “drought hardiness” (Walter & Volk, 1954), but it was not until Gaff pioneered expeditions to identify such species that its ability to resurrect from the air-dry state was recorded (Gaff & Ellis, 1974). His efforts continue to identify desiccation tolerance in vascular plants, with *Sporobolus atrovirens* from Mexico, another resurrection grass from the Eragrostoideae subfamily being amongst the latest additions (Iturriaga *et al.*, 2000). Interestingly all the resurrection grasses identified to date are restricted to three closely related tribes, Eragrostoideae, Sporoboleae and Chlorideae (Gaff & Ellis, 1974). These authors speculated that since desiccation tolerance is confined to only to these grass tribes, this trait has evolved relatively recently in these species.

E. nindensis, a perennial growing in exposed areas in shallow sandy soils across a broad region of southern Africa (Gibbs Russell *et al.*, 1990) is unlike most other resurrection grasses in that it loses its chlorophyll on dehydration (Figure 1.2b, Gaff & Ellis, 1974). Apart from a few isolated studies incorporating a number of the resurrection grasses (Sutaryono & Gaff, 1992; Ghasempour *et al.*, 1998a), little is known of these resurrection angiosperms with the single exception of *Sporobolus stapfianus* (Kuang *et al.*, 1995; Quartacci *et al.*, 1997; Ghasempour *et al.*, 1998b; Neale *et al.*, 2000 *inter alia*).

The purpose of this study was to investigate the desiccation tolerance of *E. nindensis*. Since it is well known that desiccation tolerance involves a variety of mechanisms which differ among species, this work will broaden the base of resurrection angiosperms considered to date. Furthermore, since the resurrection grasses are the most similar of the desiccation-tolerant plants to many commercially important crop plants, a greater understanding of desiccation tolerance in these species may advance the development of



Figure 1.2 *Eragrostis nindensis* photographed on a hill at Witsand Nature Reserve (a) and on the Gamsberg inselberg (b, c) in the Northern Cape Province, South Africa in the hydrated (a), dehydrated (b) and partially rehydrated (c) state.

improving water stress tolerance in crops and is thus of interest to the agricultural industry.

E. curvula (Schrad.) Nees, a relatively drought tolerant species and well-known pasture grass from the same genus, has been used during the course of this investigation for comparative purposes. *E. curvula*, a widespread southern African grass which generally occurs in high rainfall areas (Gibbs Russell *et al.*, 1990) was recorded as desiccation sensitive by Gaff and Ellis (1974).

Since so little is known about *E. nindensis* and desiccation tolerance is a complex phenomenon involving a number of different aspects, the scope of a study such as this is enormous. I have chosen to direct this investigation towards some of the least studied aspects of this field: plant water relations and the role of aquaporins in desiccation tolerance.

In order to form a framework from which these two aspects could be approached, it was first necessary to characterise some of the key physiological responses of *E. nindensis* to desiccation. A general overview of photosynthesis and membrane integrity during both dehydration and rehydration of both *E. nindensis* and *E. curvula* are present in the next chapter. Thereafter a study of various components of water potential of these two species during dehydration was undertaken. This aspect of plant water relations is presented in Chapter 3. A number of other resurrection angiosperms were included in this part of the study in order to substantiate the findings which are described in context with the morphological changes associated with dehydration. In the fourth chapter, two water channel proteins, α - and δ -TIP (tonoplast intrinsic proteins) are characterised during dehydration and the subsequent rehydration of both *E. nindensis* and *E. curvula*. The following chapter explores freeze-substitution as a method of preparing dry plant material for transmission electron microscopy and presents an ultrastructural overview of the two species in light of the data present in the previous chapters. A general overview of these findings and their relevance to desiccation tolerance is discussed in the final chapter.

Chapter 2:**Physiology of *E. nindensis* and *E. curvula* during desiccation and rehydration****2.1 Introduction**

It is well reported that desiccation-tolerant angiosperms have developed a variety of mechanisms to tolerate extreme desiccation (Bewley, 1979; Gaff, 1989; Oliver *et al.*, 1998; Farrant 2000). Although *Eragrostis nindensis* is reported to be desiccation tolerant (Gaff & Ellis, 1974), the mechanisms by which this resurrection grass achieves this have not been characterised. From the review of desiccation tolerance presented in the previous chapter it is clear that a broad approach to desiccation tolerance is required in order to critically assess desiccation-related responses in context.

In this chapter some of the physiological features of the desiccation response of *E. nindensis* and *E. curvula* are characterised so as to provide a framework within which other data from these species can be interpreted. In order to assess the effects of desiccation on cellular degradation, membrane integrity (measured as electrolyte leakage) and the quantum efficiency of photosystem II were recorded during both dehydration and rehydration of *E. nindensis*. Dark respiration was measured to assess metabolic activity. During the course of this study it was observed that the outer leaves of this species are desiccation sensitive (Figure 2.1). Thus these leaves, as well as inner leaves from the fairly drought tolerant yet desiccation-sensitive relative, *E. curvula*, were also assessed for comparative purposes.

Since *E. nindensis* is poikilochlorophyllous (Gaff & Ellis, 1974), CO₂ assimilation and changes in pigment contents during desiccation were measured to characterise this phenomenon. Some general physiology of many other resurrection plants is known (Gaff, 1989; Hartung *et al.*, 1998; Farrant, 2000). From this accumulation of information it may be possible that further trends in the physiology of desiccation tolerance may be found.

2.2 Materials and methods

2.2.1 Plant material

Seeds of *E. nindensis* and *E. curvula* were sown in seedling flats and individual seedlings transplanted into 1 l bags (1:1 potting soil: river sand mix) and maintained in a glasshouse for six months. All seed was obtained from the Grasslands Science Department, Agricultural Research Council, Pretoria, South Africa. Two weeks prior to experimentation plants were transferred to a controlled environment chamber (16 h light ($2\,000\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$), 26°C; 8 h dark, 15°C, 50-65% RH) where they remained for the duration of the experimental period.

The physiological parameters detailed below were recorded at regular intervals on more than five separate plants during both dehydration (watering withheld) and rehydration (daily watering to field capacity resumed). Plants remained in a dry state for at least two weeks prior to rewatering. All measurements detailed below were taken on the same piece of leaf tissue from a region *ca.* 2 cm distal from the leaf sheath. Both the oldest leaf on a tiller (referred to as “outer leaf”) and the next leaf on a sheath (referred to as “inner leaf”) were analysed in *E. nindensis* (Figure 2.2). Only inner leaves of *E. curvula* were assessed. After rehydration, it was confirmed that the remaining proximal part of desiccation-tolerant inner leaves and the outer desiccation-sensitive leaves of *E. nindensis* used in the study did and did not recover, respectively.

2.2.2 Relative water content (RWC)

RWC was calculated as the water content, determined gravimetrically, divided by that estimated at full turgor according to the equation below. Mean moisture content at full turgor was calculated separately for outer and inner leaves of *E. nindensis* as well as for inner leaves of *E. curvula*.

$$\text{RWC} = (\text{fresh weight} - \text{dry weight}) / (\text{fresh weight at full turgor} - \text{dry weight}) \times 100$$

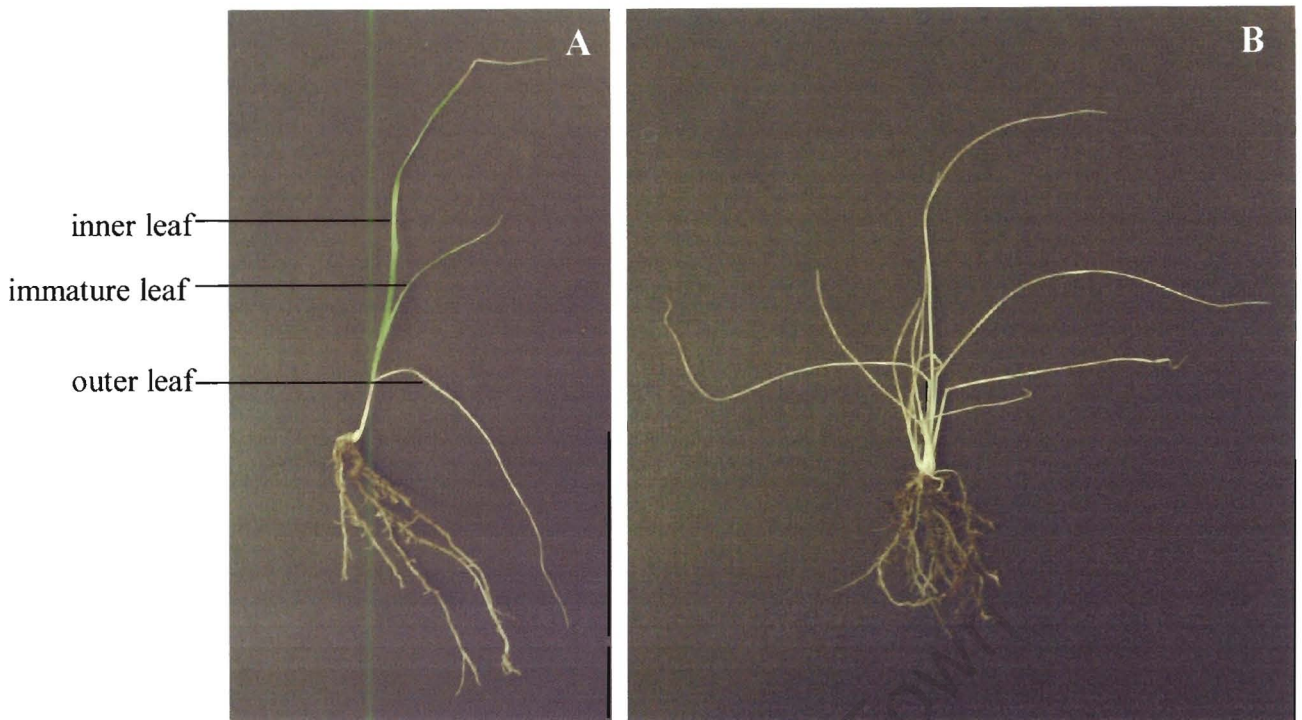


Figure 2.1. Tillers of *E. nindensis* (a) and *E. curvula* (b) after 72 h rehydration. Note that only the inner and immature leaves of *E. nindensis* (a) regreen.

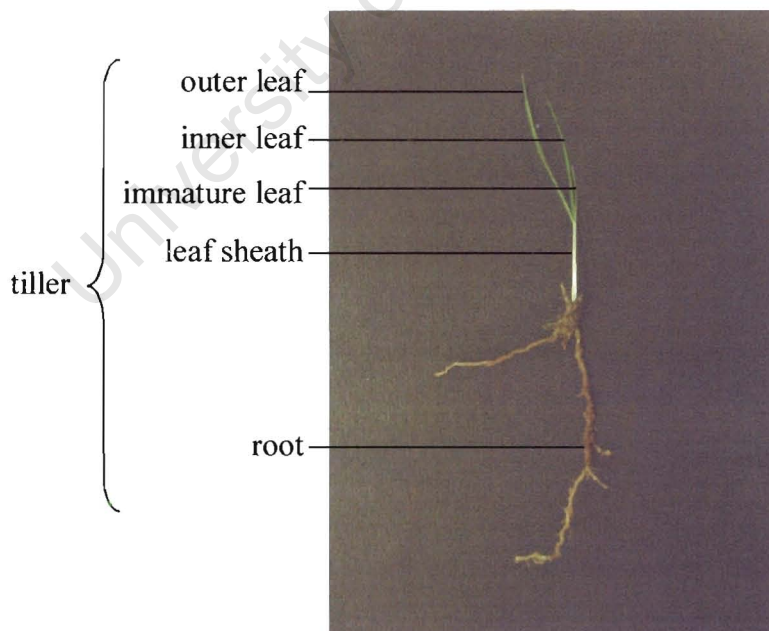


Figure 2.2. *Eragrostis nindensis* tiller showing inner, outer and immature leaves

This was determined using more than 30 representative leaf samples from hydrated plants which had been covered overnight with plastic bags. Water contents were determined gravimetrically by oven drying at 70°C for 48 h.

2.2.3 Soil water status

Whole potted plants were weighed regularly. Soil dry weight was estimated from subsamples from each bag at the completion of the experiments. At this time total plant biomass was also estimated for each time point from the final plant biomass and the change in plant water content during the course of the experiment. It was assumed that root water content would have been similar to that of the leaves. Soil water potential was measured on subsamples during both dehydration and after rewatering in C52 sample chambers using a HR-33T thermocouple psychrometer in the dew point mode (Wescor, Logan, Utah, USA).

2.2.4 Quantum efficiency of photosystem II

Quantum efficiency (F_v/F_M) was measured on dark adapted leaves using an OS-500 Modulated Fluorometer (Opsci-Sciences, Haverhill, USA) using a saturating light intensity of *ca.* 4 mmol photons $m^{-2} s^{-1}$ and a duration of 1 s.

2.2.5 Gas exchange parameters

Net CO₂ assimilation was measured across a range of light intensities from dark to an intensity of 2 000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ using a Ciras-1 infrared gas analyser with a Parkinson's Leaf Cuvette and in-built illumination unit (PP Systems, Hertfordshire, UK) operated in the differential mode at an ambient CO₂ concentration of 350 ppm and 22°C (50% RH). Stomatal conductance and transpiration rates were also recorded and gas exchange parameters calculated according to the manufacturer's instructions.

2.2.6 Electrolyte leakage

Membrane integrity was assessed as a percentage of maximum electrolyte leakage. Initial leakage was measured as the change in conductivity during a 40 min period using a CM100 conductivity meter (Reid and Associates, Durban, South Africa). Maximum electrolyte leakage was measured after repeated snap freezing of the leaf samples in liquid N₂.

2.2.7 Pigment content

The absorbance of leaf extracts in 100% acetone was measured at 470.0, 644.8 and 661.6 nm on a Beckman DU-64 spectrophotometer (Fullerton, California, USA). Total chlorophyll ($a+b$) and carotenoid ($x+c$) contents were then calculated (Lichtenthaler, 1987) and expressed on a dry weight basis. Anthocyanin concentration was determined from the absorbance of leaf extracts in acidified methanol (methanol:water:HCl (79:20:10)) at 530 and 657 nm (Mancinelli *et al.*, 1975). At least two internal replicates on each extract were recorded.

2.3 Results

At full turgor absolute water contents were 3.54 ± 0.42 and 3.15 ± 0.56 g g⁻¹ dry mass for inner and outer leaves of *E. nindensis* respectively. The turgid inner leaves of *E. curvula* had an absolute water content of 3.28 ± 0.35 g g⁻¹ dry mass.

2.3.1 Dehydration

16 d after water was withheld, RWC for all leaves of both *E. nindensis* and *E. curvula* was less than 5% (Figure 2.3a). There was a slightly more gradual decline in RWC of leaves of *E. nindensis* compared with those of *E. curvula*. Once water was withheld, soil water content dropped to less than 0.2 g g⁻¹ (-1 MPa) in approximately 5 d.

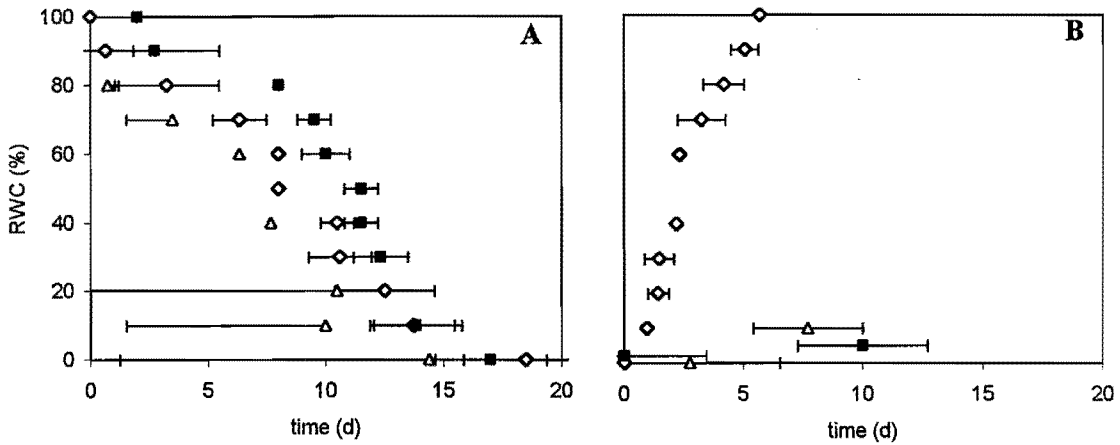


Figure 2.3 Time course of the changes in relative water content during dehydration (a) and rehydration (b) of inner (◊) and outer (Δ) leaves of *E. nindensis* and inner leaves of *E. curvula* (■). The data has been grouped into 10% RWC intervals. Errors bars represent standard errors.

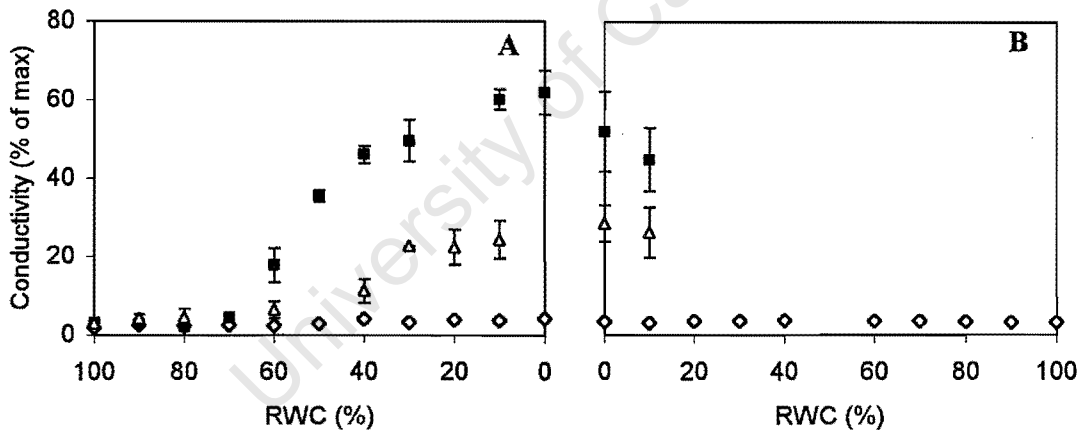


Figure 2.4 Electrolyte leakage during dehydration (a) and rehydration (b) of inner (◊) and outer (Δ) leaves of *E. nindensis* and inner leaves of *E. curvula* (■). The data has been grouped into 10% RWC intervals. Errors bars represent standard errors.

Concomitant with a precipitous decrease in soil water potential at this point, RWC of all leaves from both species started to decline.

During dehydration most electrolyte leakage occurred from the leaves of *E. curvula* (Figure 2.4a). It was found that these leaves were able to recover from a loss of water down to a RWC of *ca.* 40% (data not shown). This critical point, below which further dehydration was lethal, corresponded to a 50% increase in electrolyte leakage. At this time, both edges of the leaves started rolling inwards (Figure 2.5). There was also an increase in electrolyte leakage in the outer leaves of *E. nindensis* upon drying below 40% RWC, indicating some loss of membrane integrity in those leaves. Similar irregular leaf inrolling was also observed (Figure 2.5). There was no apparent membrane damage to inner leaves of this species (Figure 2.4a). These leaves rolled inwards from one edge to form a tight cylinder on drying (Figure 2.5).

There were no differences in quantum efficiency of photosystem II between the species or with leaf insertion level (Figure 2.6a). As RWC declined below 70%, F_v/F_M dropped such that at RWCs less than 30% there was minimal electron transport through photosystem II in either species. However, differences in pigment composition were observed (Figure 2.7a,c,e). Chlorophyll and carotenoid contents decreased gradually in all leaves of *E. nindensis* (Figure 2.7a,c). In *E. curvula* carotenoid content declined gradually but chlorophyll was retained until the critical 40% RWC, at which point there was a sudden loss in pigmentation. Anthocyanins were synthesised only in the inner leaves of *E. nindensis* from a RWC of 70% (Figure 2.7e). HPLC analysis of this sample established that cyanidin 3-galactoside was the predominant form.

Only at high RWCs was there an immediate stomatal response to changes in light intensity in all species. Once water became limiting, stomatal conductance decreased and no longer showed any significant response to changes in light intensity. However, as leaf water content dropped, the sequence of gas exchange shutdown differed both between species and with leaf age (Figure 2.8a,c,e). When soil water became limited, the greatest restriction in transpiration was observed in *E. curvula*, as seen by the

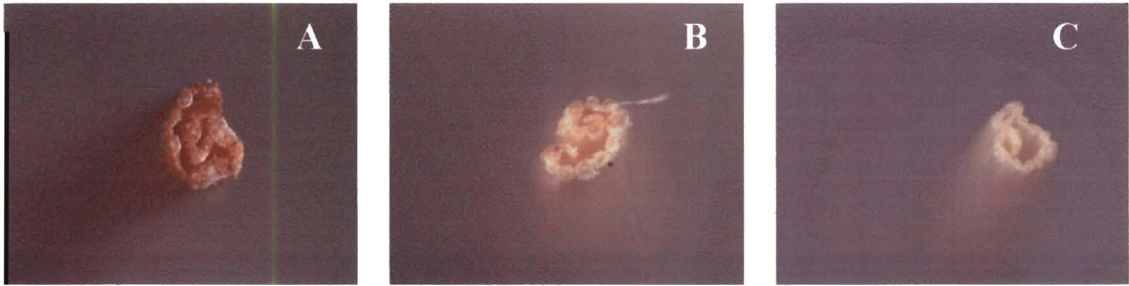


Figure 2.5. Transverse cuts through a desiccation-tolerant inner leaf (a) and a desiccation-sensitive outer leaf (b) of *E. nindensis* and a desiccation-sensitive inner leaf of *E. curvula* (c). Note the spiral folding of (a) compared with the irregular folding from both edges in (b) and (c).

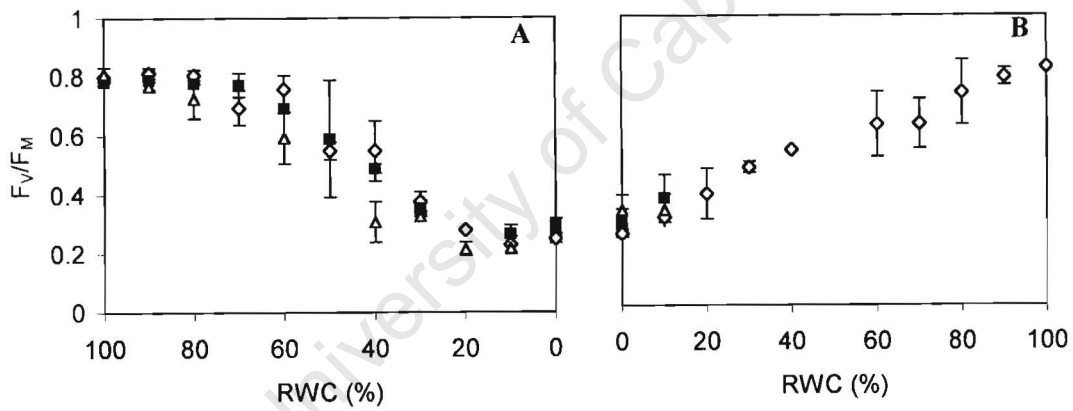


Figure 2.6 Quantum efficiency of photosystem II during dehydration (a) and rehydration (b) of inner (◇) and outer (△) leaves of *E. nindensis* and inner leaves of *E. curvula* (■). The data has been grouped into 10% RWC intervals. Errors bars represent standard errors.

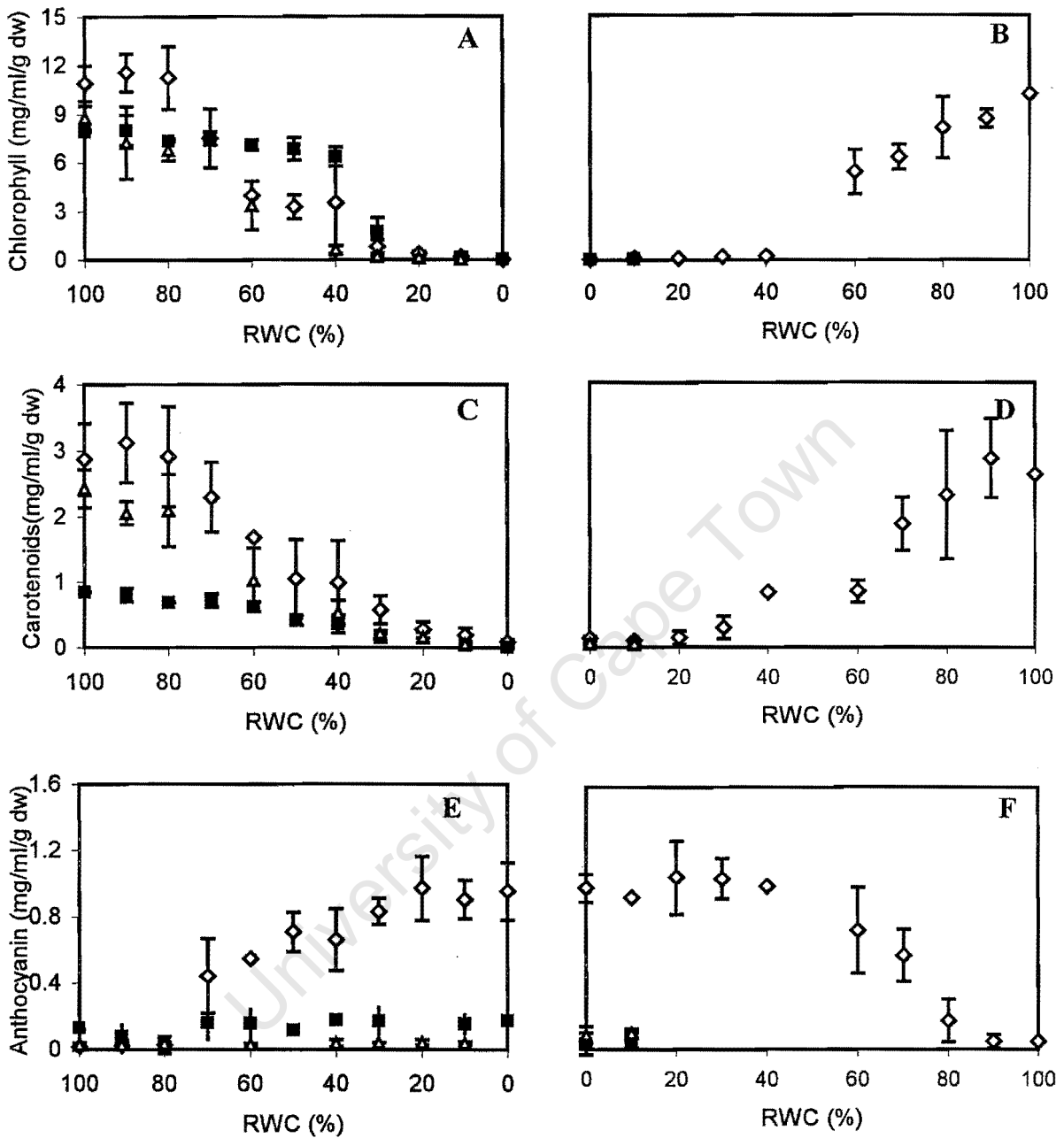


Figure 2.7 Pigment composition of inner (◇) and outer (Δ) leaves of *E. nindensis* and inner leaves of *E. curvula* (■). Total chlorophyll (a and b), carotenoids (c and d) and anthocyanin (e and f) are plotted against RWC during dehydration and rehydration respectively. The data has been grouped into 10% RWC intervals. Error bars represent standard errors.

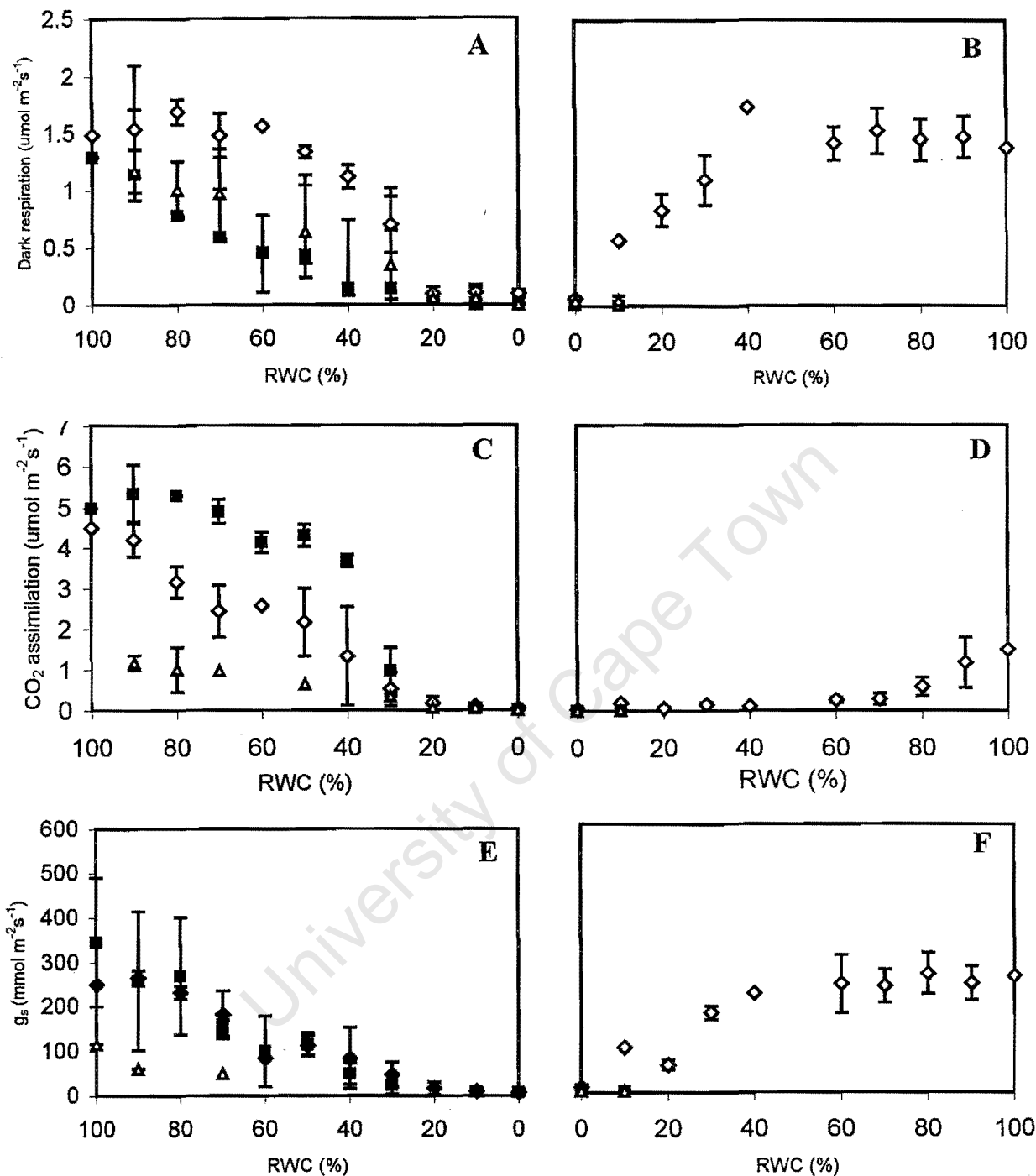


Figure 2.8 Gas exchange at a light intensity of $2\ 000\ \text{mmol m}^{-2}\ \text{s}^{-1}$ of inner (\diamond) and outer (Δ) leaves of *E. nindensis* and inner leaves of *E. curvula* (\blacksquare): dark respiration (a and b), net photosynthesis (c and d) and stomatal conductance (e and f) during dehydration and rehydration respectively. The data has been grouped into 10% RWC intervals. Errors bars represent standard errors.

large decline in stomatal conductance between 90 and 80% RWC (Figure 2.8e). These leaves continued to assimilate CO₂ until leaf death occurred at *ca.* 40% RWC, although respiration declined more gradually from higher RWCs (Figure 2.8a,c). By 40% RWC, stomatal conductance had declined to low levels in all leaves from both species (Figure 2.8e). At full turgor both net CO₂ assimilation and stomatal conductance of the outer leaves of *E. nindensis* were considerably lower than the other leaf types (Figure 2.8c,e). Net CO₂ assimilation declined gradually in both the inner and outer leaves of *E. nindensis* (Figure 2.8c) but respiration continued until 40% RWC in the inner leaves only, after which there was a gradual shutdown in this activity in these leaves as well (Figure 2.8a). Light response curves for both species were typical of C₄ plants, showing no light saturation.

The hydrated vascular bundles of *E. nindensis* contain a ring of bundle sheath cells with single large vacuoles and centrifugally arranged chloroplasts (Figure 2.9), this Kranz anatomy being indicative of NAD-ME (nicotinamide adenine dinucleotide- malic enzyme) type C₄ photosynthesis. Vascular bundles of *E. curvula* have a similar arrangement (Ellis, 1977).

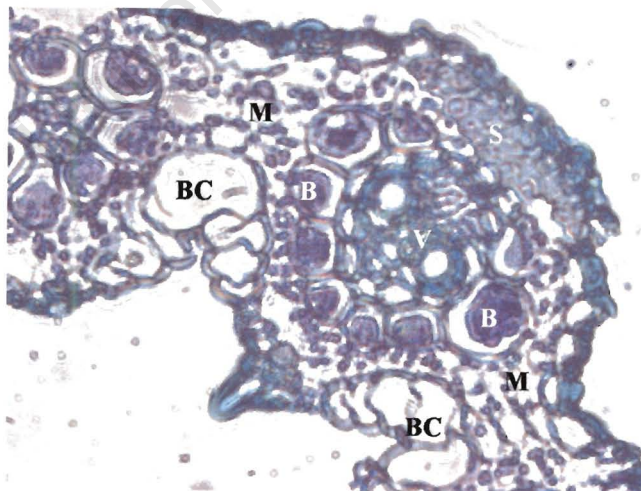


Figure 2.9 Light micrograph of a transverse section through a hydrated *Eragrostis nindensis* leaf. A vascular bundle with bundle sheath cells (B) enclosing vascular tissue (V) is visible. Mesophyll (M), bulliform cell (BC), sclerophyllous tissue (S). Preparation of this toluidine blue stained wax-embedded section is described in Chapter 4.2.6 (X 450)

2.3.2 Rehydration

Soil water content and water potential rose to previous maximum levels within hours of rewatering (data not shown). Approximately 75% of all the leaves of *E. nindensis* recovered to at least 95% RWC after 6 d (Figure 2.3b). The inner and immature leaves rehydrated in a front; the tips often remained dry. The outer leaves of *E. nindensis* and all leaves of *E. curvula* did not rehydrate (Figure 2.1).

There was no change in electrolyte leakage during rehydration in either species from that measured during dehydration (Figure 2.4b). Cells from inner leaves of *E. nindensis* appear to have maintained membrane integrity but outer leaves which had incurred membrane damage during dehydration did not recover. Inner leaves of *E. nindensis* showed a gradual restoration of the quantum efficiency of photosystem II after 40% RWC had been attained (Figure 2.6b). This corresponded with the resynthesis of chlorophyll and carotenoids (Figure 2.7b,d) and the breakdown of anthocyanin (Figure 2.7f) in these leaves. There was no change in pigment composition or F_V/F_M in outer leaves of *E. nindensis* or those of *E. curvula* (Figure 2.6b, 2.7b,d,f).

Gas exchange resumed as soon as rehydration began in the inner leaves of *E. nindensis* (Figure 2.8b,d). At 40% RWC, respiration peaked. Thereafter stomatal conductance and respiration stabilised at rates recorded for hydrated (control) tissue (Figure 2.8b,f compared with Figure 2.8a,e). Net CO₂ assimilation resumed only at 70% RWC (Figure 2.7d) and reached maximal rates more than 5 d after full turgor had been reached (data not shown). No gas exchange was recorded in the outer leaves of this species, or in *E. curvula* (Figure 2.8b,d,f).

2.4 Discussion

Both *E. nindensis* and *E. curvula* have Kranz anatomy typical of all C₄ species (Figure 2.9; Gaff & Ellis, 1974; Puliga *et al.*, 1996). Anatomical observations suggest that *E. nindensis* belongs to the NAD-ME biochemical subtype (Prendergast & Hattersley, 1987) unlike the

desiccation-tolerant *S. stapfianus*, which uses the PEP-CK (phosphoenolpyruvate carboxylkinase) pathway (Dalla Vecchia *et al.*, 1998). The desiccation-sensitive *E. curvula* has the same biochemical pathway as *E. nindensis* (NAD-ME (Gutierrez *et al.*, 1974)). Interestingly, of these two C₄ subtypes found in Eragrostoideae, the NAD-ME pathway is more prevalent in low rainfall regions (Ellis, 1980).

This is in keeping with the observation that *E. curvula* is relatively drought tolerant and can continue growing even at relatively low leaf water potentials of *ca.* -1.4 MPa (Puliga *et al.*, 1996). The leaves of this grass restrict water loss by reducing transpiration as soon as the RWC drops below about 90%, but they do not have the capacity to survive severe desiccation and remain metabolically active until irreparable damage is incurred at RWCs below about 40% (Figures 2.1, 2.4, 2.6, 2.8). It was noticed that a colouration suggestive of anthocyanins did accumulate around the base of tillers, but no new growth was observed from the meristem when plants were rewatered following severe water stresses.

E. nindensis is reported to be poikilochlorophyllous (Gaff & Ellis, 1974) and this study confirms that chlorophyll is lost (Figure 2.7a). The ability to reversibly switch off metabolism has been suggested to be part of the mechanism of desiccation tolerance (Farrant *et al.*, 1997; Sherwin & Farrant, 1998; Pammenter & Berjak, 1999; Farrant, 2000). Thus the dismantling of the photosynthetic apparatus, associated with the cessation of photosynthesis, constitutes a part of a programmed suspension of physiological activity. In *E. curvula* CO₂ assimilation remains at relatively high values until a RWC of 40%, after which further drying probably causes damage to the photosynthetic apparatus and assimilation ceases (Figure 2.8). The damage is evidenced by the inability of this process to recover. Poikilochlorophylly is an effective mechanism to prevent the consequences of photo-oxidation at intermediate and low water contents (Sherwin & Farrant, 1998; Tuba *et al.*, 1998). However, because of the necessity to resynthesise and reassemble the photosynthetic apparatus, it results in a lag before photosynthesis can resume (Figures 2.6b, 2.7d; Tuba *et al.*, 1996a; 1996b). Most other resurrection grasses are homiochlorophyllous (Gaff & Ellis, 1974) or intermediate (*Sporobolus stapfianus* (Quartacci *et al.*, 1997)) and hence probably resume full metabolic activity sooner.

E. nindensis (inner leaves) is thus truly desiccation tolerant, whereas *E. curvula* is not. In addition to the light protection mechanisms, the former has many features in common with other resurrection plants that account for its ability to survive drying. These include the maintenance of membrane integrity (Figures 2.4a (Dace *et al.*, 1998)) and the ability to switch off metabolism (Figure 2.8a,c respiration and photosynthesis) and recover it on rehydration (Figure 2.8b,d (Vertucci & Farrant, 1995; Farrant *et al.*, 1997; Pammenter & Berjak, 1999)). However, like *S. stapfianus* (Gaff & Loveys, 1984a; Kuang *et al.*, 1995), the inner desiccation-tolerant leaves are desiccation sensitive if they are dehydrated detached from the parent plant (personal observation). These leaves remained green on drying. Detached leaves of other resurrection plants can survive desiccation detached (Navarri-Izzo *et al.*, 1994; Sgherri *et al.*, 1994b).

It has been suggested that because of the small proportion of desiccation-tolerant species in the *Eragrostis* genus, tolerance is a relatively recent evolutionary adaptation (Gaff, 1989; Oliver *et al.*, 2000). Furthermore, species in which desiccation tolerance is restricted to the basal meristematic or immature tissue (for example, *E. hispida*) are said to be less advanced than those in which mature tissues can rehydrate (Gaff & Ellis, 1974). It is thus interesting that although the seed and mature plants of *E. nindensis* are desiccation tolerant, the seedlings are not (personal observation). Although leaf senescence is common to all plants, like some other resurrection plants, dehydration also precipitates premature death in the oldest outer leaves of *E. nindensis* (Figure 2.1, 2.4, 2.8; Tymms *et al.*, 1982; Gaff, 1989; Norwood *et al.*, 2000). Unlike the young tissue of the seedlings, the immature leaves which are wrapped with the sheath of the inner leaves during desiccation (Figure 2.1), are also tolerant. It is possible that the desiccation tolerance of these immature leaves is due primarily to the shading and reduced rate of water loss that the older “inner” leaf would provide. However, it was not established whether seedlings (which would be of a similar developmental age as these immature leaves) would survive desiccation under similar protective conditions. It may be that the long delay (few months) required for seedling establishment is however necessary in order to achieve desiccation tolerance in the adult plant.

The older desiccation-sensitive leaves of *E. nindensis* are usually less photosynthetically active than the younger leaves (Figure 2.8c). During dehydration chlorophyll degradation and gas exchange patterns in the outer leaves follow the controlled shutdown observed in younger leaves (Figures 2.7a, 2.8a,c,e). Although this seems to suggest that the ability to suspend metabolic activity observed in younger leaves has not been entirely lost, anthocyanin is not synthesised (Figure 2.8e) and membrane damage is incurred in the outer leaves (Figure 2.4a) during dehydration. Furthermore, like the desiccation-sensitive species, *E. curvula*, the outer leaves curl irregularly (Figure 2.5). Leaf rolling in monocotyledonous plants is thought to be regulated by the bulliform cells of the abaxial epidermal cell layer (Cutler, 1978). Even though it is a feature more commonly associated with homiochlorophyllous resurrection plants, the ability of the inner leaves of *E. nindensis* to form a tight spiral, with anthocyanin abundant in the exposed adaxial tissue, possibly assists in the protection from irreparable light damage during dehydration (Figure 2.5, 2.7e). The leaves of the resurrection grass *Sporobolus stapfianus* do not form tight spirals in the dry state (Dalla Vecchia *et al.*, 1998). Since *E. nindensis* is poikilochlorophyllous and *S. stapfianus* is intermediate (Quartacci *et al.*, 1997), it is surprising that, if leaf curling is an important light protective mechanism, it is not present in *S. stapfianus*. The uncontrolled leaf curling in the older leaves of *E. nindensis* may be a consequence rather than cause of irreparable membrane damage on drying.

The desiccation sensitivity of rapidly-dried or detached leaves of some resurrection plants is a phenomenon that has been used in studying the mechanisms which confer tolerance in the natural situation (Kuang *et al.*, 1995; Gaff *et al.*, 1997; Quartacci *et al.*, 1997; Farrant *et al.*, 1999). The comparison of desiccation-tolerant (inner) and desiccation-sensitive (outer) leaves on the same plant (*E. nindensis*) may constitute an even better system for investigating the mechanisms underlying this unusual phenomenon. Throughout this study the desiccation-tolerant inner leaves of *E. nindensis* are compared with the desiccation-sensitive outer leaves of the plant. Desiccation tolerance in this species is also compared with the drought tolerant, but desiccation-sensitive related species, *E. curvula*.

Chapter 3:

Plant water relations in desiccation tolerance

3.1 Introduction

The previous chapter provided a framework from which further investigations into the mechanisms through which desiccation tolerance in *Eragrostis nindensis* and other resurrection plants might be achieved. Mechanical damage associated with loss of turgor is proposed to be one of the major causes of irreversible desiccation-induced damage in plants (Iljin, 1957; Vertucci & Farrant, 1995). Tearing of membranes is thought to occur as result of tension imposed on membranes as cytoplasm contracts beyond the point of zero turgor. Since resurrection plants, including the inner leaves of *E. nindensis*, are able to survive dehydration to an air-dry state (Chapter 2, Gaff, 1971; Bewley & Krochko, 1982 *inter alia*), these plants must thus either tolerate symplastic tension or avoid it. Desiccation tolerance involves repair processes during rehydration (observed mainly in lower order desiccation-tolerant plants) and/or protection mechanisms during dehydration (reviewed by Gaff, 1989; 1997; Oliver & Bewley, 1997; Farrant 2000), including protection against the physical stresses associated with water loss.

Pressure-volume (PV) curves are a valuable tool in describing plant water relations; the isotherms characterise the relationship between the inverse of water potential (measured as the negative of the applied pressure) and relative water content (Tyree & Hammel, 1972). Although plant water relations have received little attention in the study of desiccation tolerance of vegetative tissues to date, two previous studies have revealed that some desiccation-tolerant plants have atypical PV curves as illustrated in Figure 3.1 (Sherwin, 1995; Beckett, 1997).

Beckett (1997) proposed that the unusual isotherms indicate that negative turgor develops during dehydration of the resurrection plants with these unusual curves. However, two different linear extrapolations to describe the osmotic component of water potential are possible (Figure 3.1). Beckett (1997) fitted a spline function to the PV curves and from

this analysis proposed extrapolation (i) in Figure 3.1. Since this line illustrates a range of water contents over which the osmotic component is greater than that of the water potential, this author suggested the development of negative turgor in those resurrection plants with atypical curves. However, no direct evidence or functional significance of the phenomenon was given. The alternative extrapolation for these anomalous PV curves, illustrated by line (ii) in Figure 3.1 suggests a period of water loss with little attendant change in water potential. This alternative has not been explored.

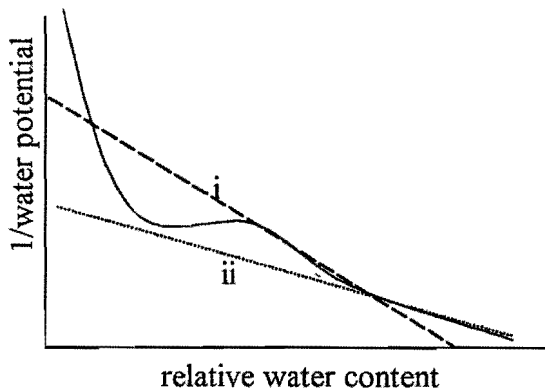


Figure 3.1 Diagram illustrating the atypical PV curve. Lines i and ii indicate possible extrapolations from the "linear" part of the curve; (i) suggests development and release of negative turgor and (ii) suggests cell volume reduction and hence water loss with little attendant change in water potential.

In this chapter the plant water relations of inner desiccation-tolerant leaves of *E. nindensis* are compared with the outer desiccation-sensitive leaves of this species as well as those of the desiccation-sensitive grass, *E. curvula*. I will argue that negative turgor does not develop in desiccation-tolerant angiosperms and thus membrane damage during desiccation is prevented. In order to substantiate my explanation for the anomalous PV curves, anatomical changes and direct measurements of water activity were recorded during dehydration. Furthermore, the data from this part of the study are substantiated with that from two other resurrection angiosperms, *Craterostigma wilmsii* and *Xerophyta humilis*. The desiccation tolerance of these two species is described elsewhere (Sherwin & Farrant 1996; 1998; Farrant *et al.*, 1999; Vicré *et al.*, 1999; Cooper, 2001).

3.2 Material and Methods

3.2.1 Plant material

Eragrostis nindensis and *E. curvula* plants were grown from seed as described in Chapter 2.2. Whole plants of *Xerophyta humilis* (Bak.) Dur. and Schinz and *Cratogeostigma wilmsii* Engl. were collected from nature reserves in the north eastern regions of South Africa and transplanted into seedling flats (1:1 potting soil: river sand mix) as described by Sherwin and Farrant (1996). All plants were maintained in a glasshouse with no additional lighting.

3.2.2 Pressure-Volume curves

Well-watered potted plants were fully hydrated overnight in plastic bags to bring leaves to full turgor. Leaf discs (0.5 cm²) from mature leaves (second outer-most whorl (*C. wilmsii*, *X. humilis*) or segments from a region *ca.* 2 cm distal to the leaf sheath on the inner and outer leaves (*E. nindensis*) and inner leaves (*E. curvula*)) were used. Water potentials were measured using C52 sample chambers and an HR-33T microvoltmeter (Wescor, Logan, Utah) in the dew point mode. Leaf material and instruments were kept at 25°C for the duration of the experiment. The following procedure was used to construct PV curves. Leaf tissue was weighed at full turgor, sealed in the sample chambers, and water potential was measured after an appropriate equilibration period. The sample holder was then removed from the chamber and the tissue allowed to dry slightly. Tissue was weighed immediately after measuring the water potential (in case slight water loss occurred during the equilibration period). This process was repeated until the tissue lost no more weight. Generally only short times of bench drying between measurements were necessary, although this increased as the tissue dried. Equilibration time required varied among the species and increased (up to 6 h) as tissue water content decreased. When there was no further decrease in weight, leaf explants were dried for 48 h at 70°C to determine dry weight. Between seven and 10 measurements were recorded for each tissue piece, and tissue was taken from four or more different plants for each species. All the data were combined to draw a single PV curve. To verify the unusual

shape of the curves in *E. nindensis* and *C. wilmsii*, water potentials and RWC of leaves that were excised from both dehydrating and rehydrating plants, were also measured. These data were combined with the previous measurements from the bench-dried samples for the PV curves presented for these two species.

3.2.3 Direct measurement of 'osmotic' potential (water activity)

Fully hydrated plants were allowed to dry naturally by withholding water. At regular intervals during dehydration, leaf samples were taken. Leaf water potential and sample weight were recorded as described above. Thereafter, the leaf sample and sample holder, wrapped in at least four layers of Parafilm™ (American National Can) and covered with aluminium foil, were plunged five times into liquid N₂ for approximately 30 s over a period of 10 min. Between immersions, the samples were warmed to room temperature. After the sample and cup had warmed to *ca.* 25°C, they were uncovered and replaced in C52 sample chambers. After an equilibration period, water potential and weight were recorded again. Dry weight of the leaf sample was measured after oven drying at 70°C for 48 h. Measurements were taken from at least 15 different leaf segments from each of at least four different plants for each species. The water potential measured after freezing and thawing of tissue is generally considered to be the osmotic potential if apoplastic water is negligible (Jones & Rawson, 1979). However, at the low RWCs achieved in this experiment it is highly likely that the system deviated from ideal behaviour. Thus the term 'water activity' (measured by vapour phase equilibration) is used.

3.2.4 Anatomical studies

Leaves were sampled at regular intervals from plants which were allowed to dry naturally. For each leaf sectioned RWC was determined on a portion of the leaf immediately distal to that sampled for microscopy. Water content and dry weight (oven-dried for 48 h at 70°C) were determined gravimetrically and RWC calculated from this and the mean water content at full turgor as described in Chapter 2.2.2.

Transverse hand sections of leaf tissue were viewed with a light microscope (Ascoscope; Zeiss, Hallbergmoos, Germany) and photographed. Cell wall perimeter and area of the region enclosed by the walls were measured from electronic images using an image analysis program (AnalySiS, Soft Imaging Software). At least 10 cells on each of the approximately 20 images from four replicate leaves of two different plants for each species, were measured. Vascular tissue was not measured.

Care was taken to maintain the leaf tissue at the RWC to which it had dried. Preliminary experiments were undertaken to ensure that the RWC of the tissue did not change during sectioning and viewing. Fully hydrated tissues were cut and viewed in deionised water. Tissues of RWCs from 80% to 50% were hand-cut and viewed in a solution of sucrose of equal osmotic potential (measured psychrometrically) to that of the tissue. Hand sections of tissues of 40% RWC and lower were cut dry and viewed in a solution of PEG 6 000 (polyethylene glycol) of comparable osmotic potential (measured psychrometrically). The osmotic potential of the sucrose and PEG 6 000 solutions were remeasured after viewing to confirm that there had been no change.

3.3 Results

Pressure-volume curves of the desiccation-tolerant (inner) and -sensitive (outer) leaves of *E. nindensis* and those of *E. curvula* are illustrated in Figure 3.2. Apoplastic water, assessed as the intercept on the RWC axes, showed negative values. This has been observed before on woody tissue, using both the pressure chamber and thermocouple psychrometry. The desiccation-sensitive leaves of *E. nindensis* and *E. curvula* had typical curvilinear isotherms, with turgor loss points of -1.95 and -1.25 MPa respectively. However, there was a clear deviation in the shape of the PV isotherm of the desiccation-tolerant leaves of *E. nindensis* such that it was not possible to determine a point at which turgor was lost. After approaching loss of turgor there was a period during which tissue water content decreased with little change in water potential (70% to 45% RWC). Thereafter a linear relationship between tissue volume and the inverse of water potential was observed. This phenomenon was observed in desiccation-tolerant leaf tissues of *E.*

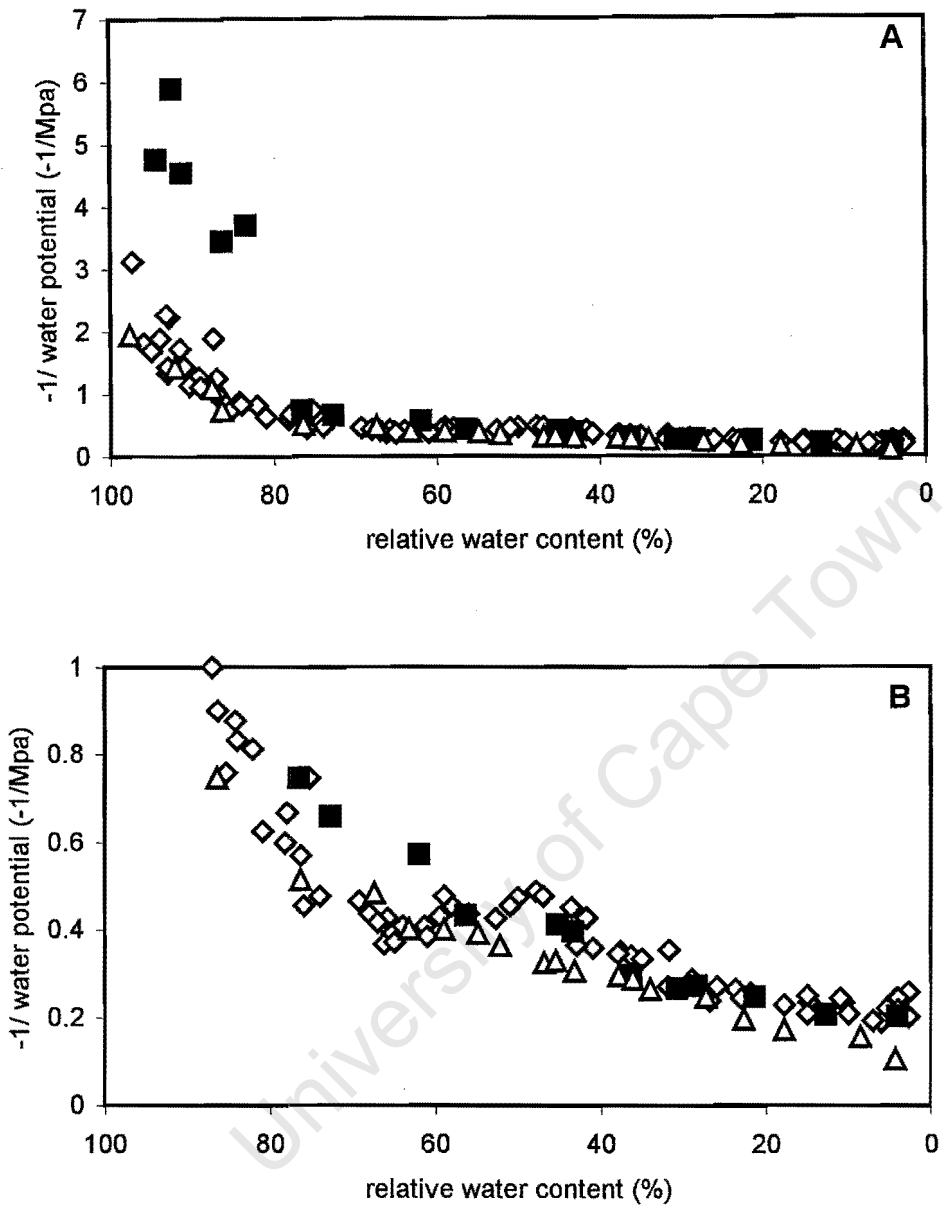


Figure 3.2 a) Pressure-volume curves of *E. nindensis* (inner and outer leaves) and *E. curvula* (inner leaves). (b) Enlargement of (a) between 0 and 1 MPa⁻¹(a).

nindensis, whether they were dried detached (regular measurements with bench drying) or measurements were taken at various time intervals from samples dehydrated and also rehydrated on the plant.

Due to the unusual shape of the PV curve of the desiccation-tolerant inner leaves of *E. nindensis*, the water relations of two other resurrection angiosperms were explored. The PV curve of *C. wilmsii* showed a similar deviation to that *E. nindensis* (Figure 3.3) but *X. humilis* did not (Figure 3.4).

In order to examine this phenomenon further, the osmotic component of the water potential (measured as water activity) of *E. nindensis* (desiccation-tolerant leaves only, Figure 3.5) and *E. curvula* (desiccation-sensitive leaves, Figure 3.6) as well as the other two resurrection angiosperms (Figures 3.3, 3.4), were measured. In all species, a linear relationship between the negative inverse of the direct measurements of the water was found (Table 3.1, Figures 3.3 to 3.6). These data correlated well with extrapolations of the linear portion of the PV curves at RWCs below the range where deviation from the norm was observed. Direct measurements of water activity were consistently slightly more negative than values predicted from the respective PV curves (Figures 3.3 to 3.6, Table 3.1; this result is not because of dilution effects consequent on membrane rupture as that effect would be in the opposite direction). Ultrastructural examination of tissue samples on which water activity had been measured confirmed that in these samples, cellular membranes were ruptured (data not shown).

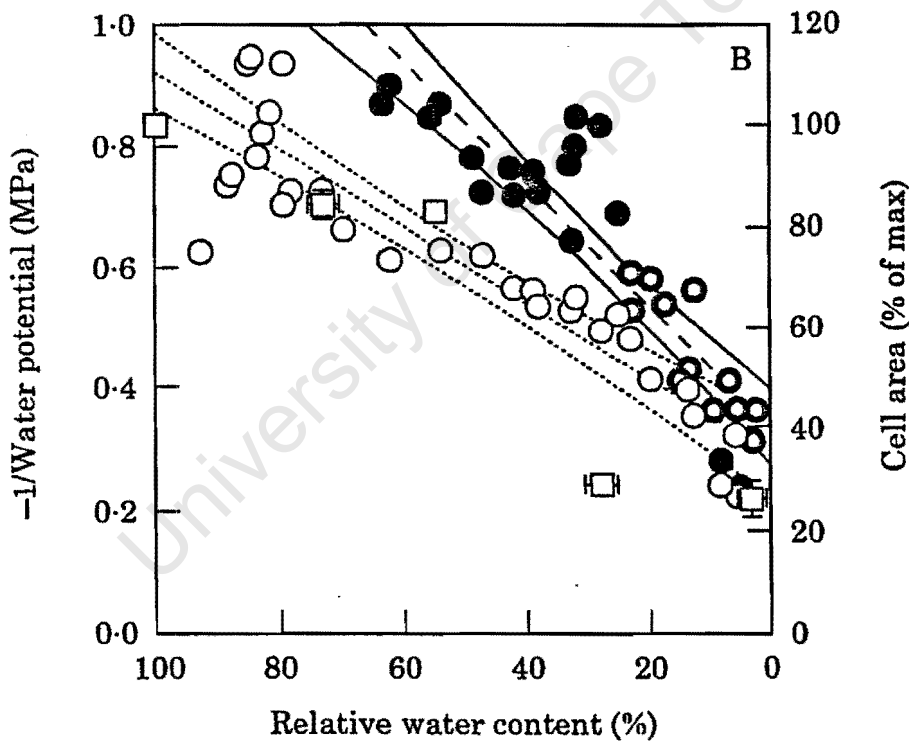
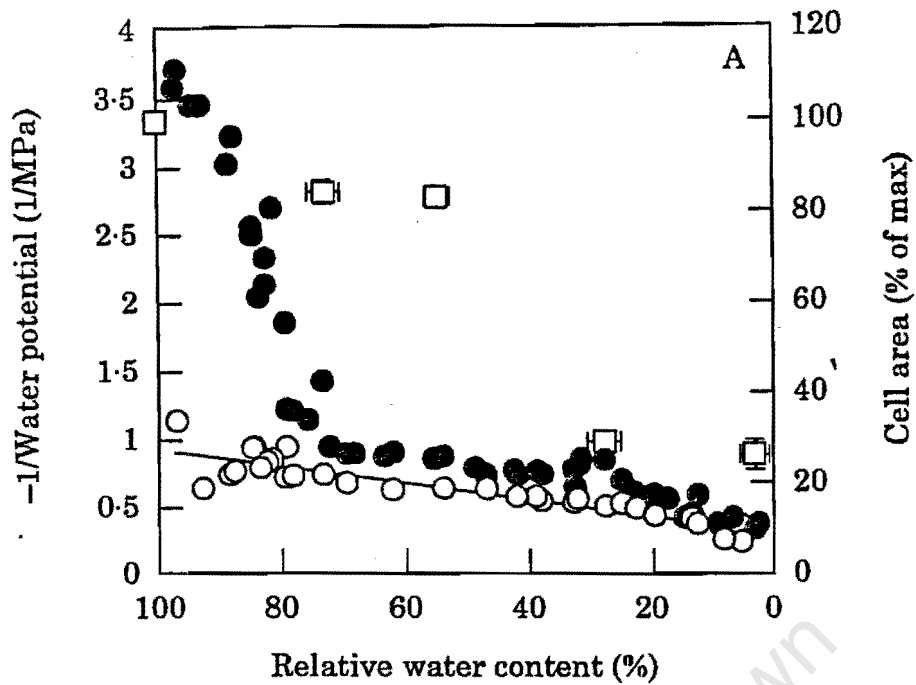


Figure 3.3. Pressure-volume curve of *Craterostigma wilmsii* (●). Open circles indicate water activity measured on freeze-thawed tissues. Areas enclosed by the cell walls indicated by open squares. Error bars indicate standard deviations. (b) is an enlargement of (a) between 0 and 1 MPa⁻¹. (a) Dotted lines indicate the linear regression and 95% confidence limits for the water activity data. Solid lines indicate the linear regressions and 95% confidence limits for the water potential data (closed circles marked with a white dot).

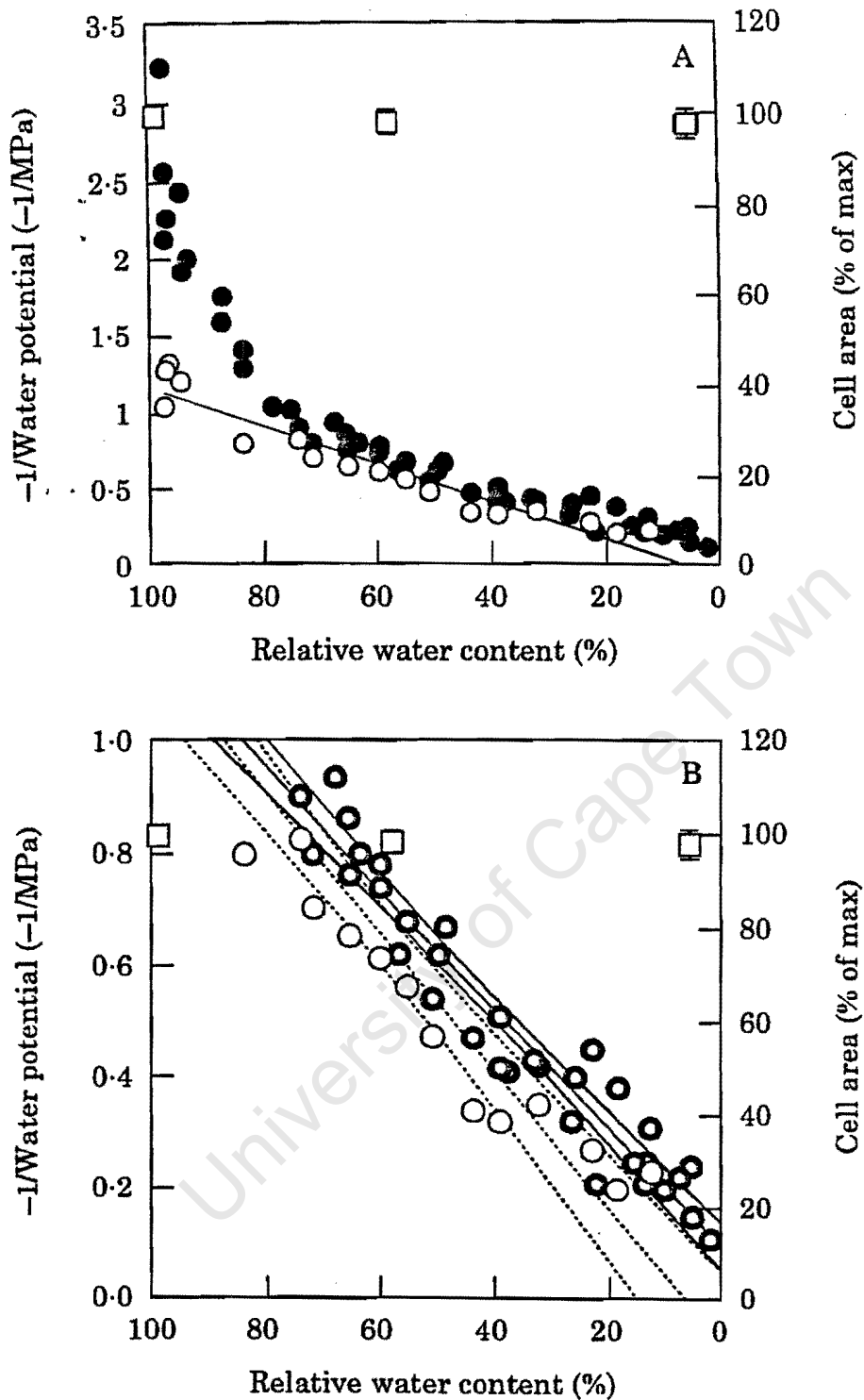


Figure 3.4. Pressure-volume curve of *Xerophyta humilis* (●). Open circles indicate water activity measured on freeze-thawed tissues. Areas enclosed by the cell walls indicated by open squares. Error bars indicate standard deviations. (b) is an enlargement of (a) between 0 and 1 MPa⁻¹. (a) Dotted lines indicate the linear regression and 95% confidence limits for the water activity data. Solid lines indicate the linear regressions and 95% confidence limits for the water potential data (closed circles marked with a white dot).

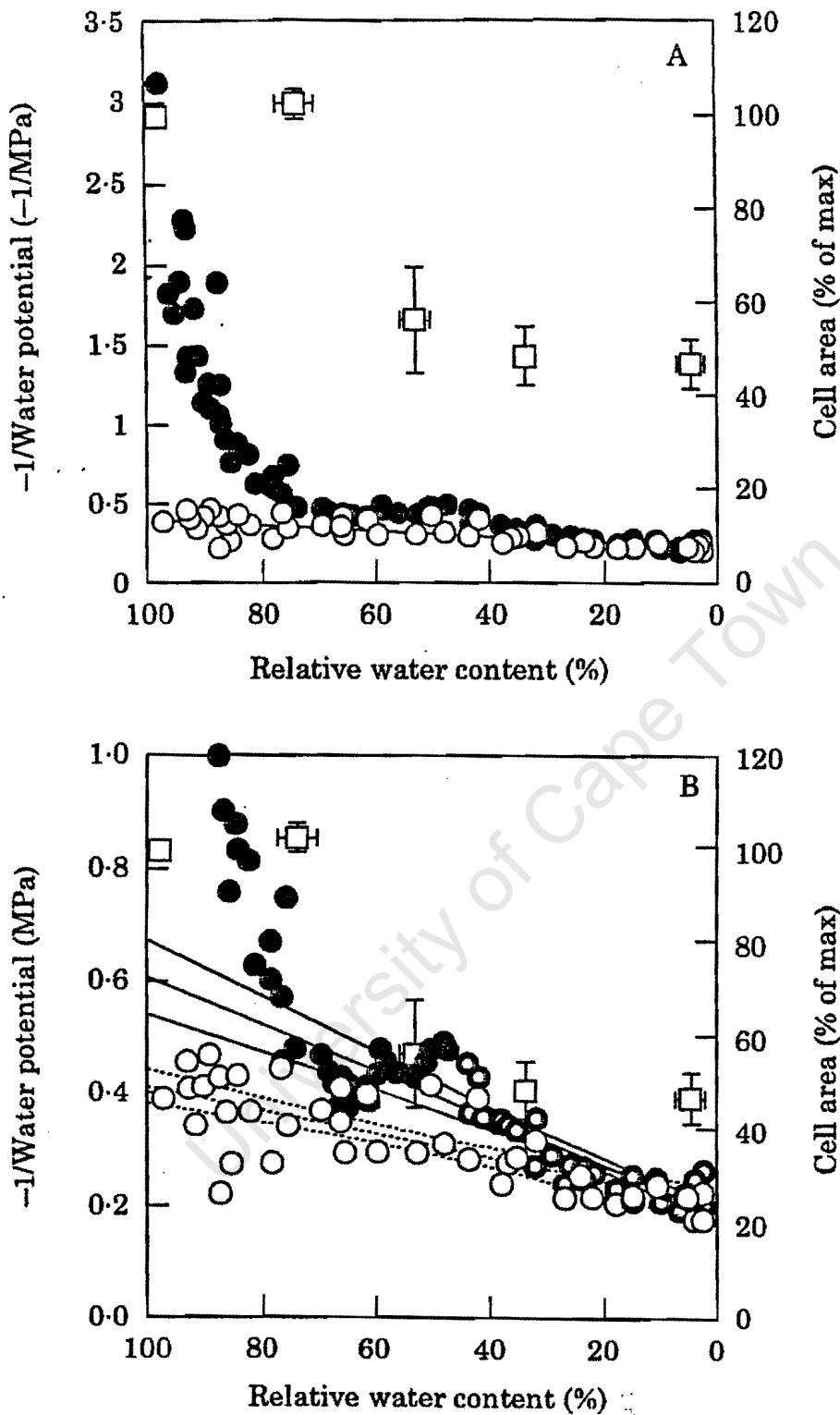


Figure 3.5. Pressure-volume curve of the inner desiccation-tolerant leaves of *E. nindensis* (●). Open circles indicate water activity measured on freeze-thawed tissues. Areas enclosed by the cell walls indicated by open squares. Error bars indicate standard deviations. (b) is an enlargement of (a) between 0 and 1 MPa⁻¹. (a) Dotted lines indicate the linear regression and 95% confidence limits for the water activity data. Solid lines indicate the linear regressions and 95% confidence limits for the water potential data (closed circles marked with a white dot).

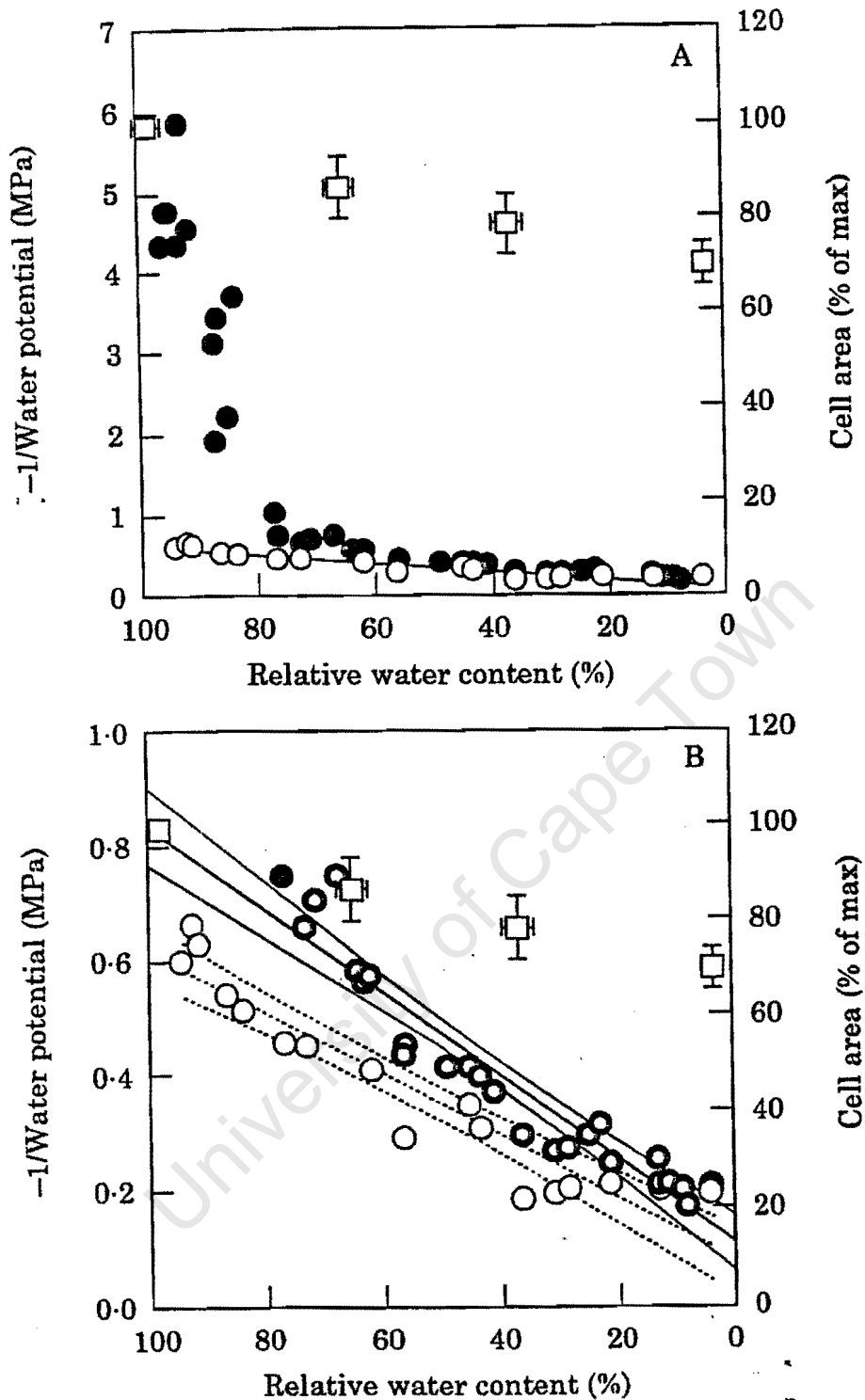


Figure 3.6. Pressure-volume curve of *E. curvula* (●). Open circles indicate water activity measured on freeze-thawed tissues. Areas enclosed by the cell walls indicated by open squares. Error bars indicate standard deviations. (b) is an enlargement of (a) between 0 and 1 MPa⁻¹. (a) Dotted lines indicate the linear regression and 95% confidence limits for the water activity data. Solid lines indicate the linear regressions and 95% confidence limits for the water potential data (closed circles marked with a white dot).

Table 3.1. Linear regressions ($-1/WP = m \cdot RWC + c$) derived from the linear portion of the PV curve (limited RWC range) and direct measurement of water activity from the desiccation-sensitive leaves of *E. curvula*, and the desiccation-tolerant leaves of *E. nindensis*, *C. wilmsii* and *X. humilis*. There were no significant differences between the slopes of the regressions within each species (analysis of co-variance (ANCOVA), $p < 0.05$, $n > 30$).

Species	Data source	m	c	R ²
<i>E. curvula</i>	PV curve (70% – 0%)	7.27	0.11	0.90
	Direct measurement	5.40	0.08	0.90
<i>E. nindensis</i> (inner leaves)	PV curve (45% - 0%)	4.20	0.19	0.78
	direct measurement	2.11	0.20	0.62
<i>C. wilmsii</i>	PV curve (25% - 0%)	0.33	0.01	0.81
	direct measurement	0.29	6.38	0.82
<i>X. humilis</i>	PV curve (70% - 0%)	0.01	0.10	0.94
	direct measurement	0.01	-0.08	0.92

Changes in cell volume during dehydration, measured as a proportion of cell area (area enclosed within the cell wall) at full turgor, are also illustrated for *E. curvula* and *E. nindensis* (inner leaves) as well as the other two resurrection angiosperms in Figures 3.3 to 3.6. The outer desiccation-sensitive leaves of *E. nindensis* were not examined further in this part of the study due to technical difficulties in measuring cell volume in this tissue. Examples of sections illustrating the shrinkage from a hydrated to dehydrated state in the four species are illustrated in Figure 3.7. There were no differences in mean cell wall perimeter at any point during dehydration in all species ($p > 0.05$, $n > 170$, data not shown). There was a slight decrease in cell area in the desiccation-sensitive species, *E. curvula* (Figures 3.6, 3.7a). In the desiccation-tolerant tissues, there was either no change (*X. humilis*, Figures 3.4, 3.7d) or a dramatic reduction in the cell area (by 53% in *E. nindensis*, Figures 3.5, 3.7b and by 74% in *C. wilmsii*, Figure 3.3, 3.7d) on drying. The change in cell volume in the latter two species occurred over the range of RWCs during

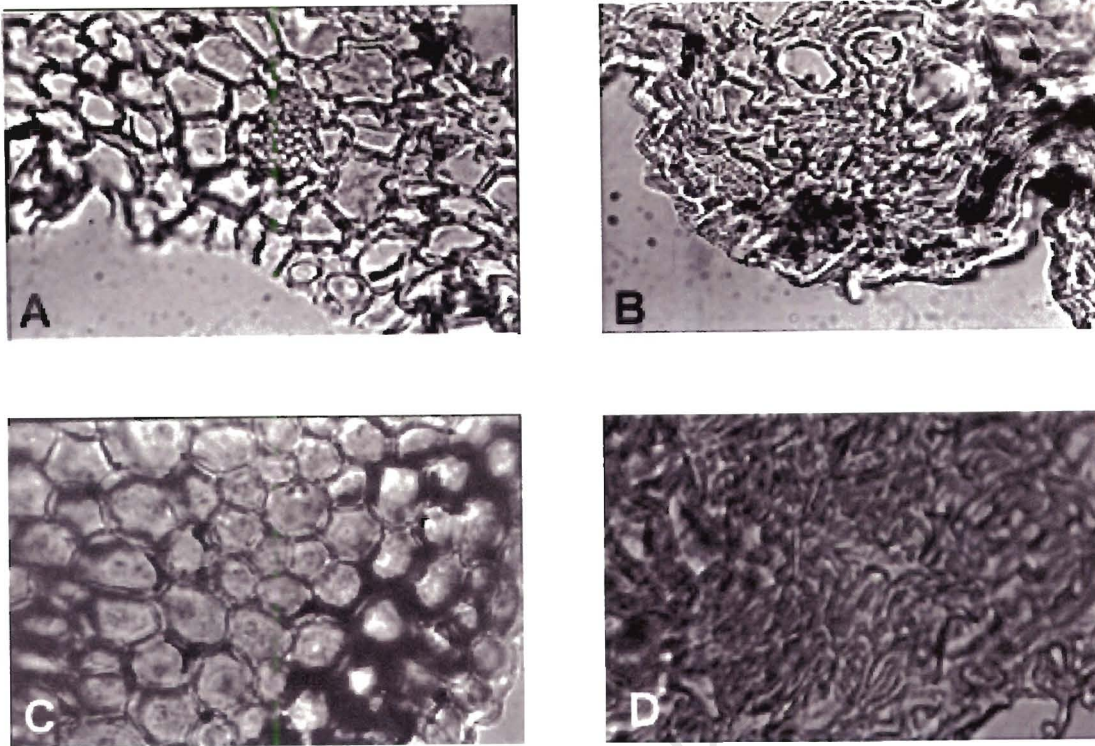


Figure 3.7 Light micrographs of mesophyll of (a) *E. curvula*, (b) *E. nindensis*, (c) *X. humilis* and (d) *C. wilmsii* photographed in isosmotic PEG 6000 solutions at ca. 5% RWC ($\times 1\,380$ for all images).

which no or minimal change in water potential was recorded, the region of the PV curves which deviated from the typical shape. Although there are numerous errors associated with measuring cell volumes (Zimmermann *et al.*, 1981; Malone & Tomos, 1990), these data were used for comparative purposes only.

3.4 Discussion

Unusual PV curves similar to those found in this study (Figures 3.3 and 3.5) have been reported before, using both pressure chambers (Oertli, 1993) and thermocouple psychrometry (Sherwin, 1995; Beckett, 1997). Two possible interpretations of curves of

this type are illustrated in Figure 3.1. The first interpretation is that removal of water beyond the turgor loss point leads to the development of negative turgor. Cavitation or cytorrhysis (cell wall collapse) then releases water which increases the turgor of neighbouring cells (Oertli, 1989; 1993; Beckett, 1997). Although Tyree (1976) disputed early studies predicting negative turgor from osmotic and water potential measurements, this author did suggest that if negative turgor was possible in living plant cells, the PV curve would indeed have to deviate from the usual shape described by Tyree and Hammel (1972). However, even with advances in the pressure probe technique (reviewed by Tomos and Leigh, 1999), negative turgor has yet to be measured in living tissues. The second interpretation is that negative turgor does not develop, and there are three possible mechanisms by which this could occur. Firstly, there could be more than one population of cell types, each having a different turgor loss point, such that the resultant PV curve is in fact a compilation of numerous PV curves. Secondly, close to the turgor loss point there is a reduction in the volume enclosed by the cell wall (wall folding), such that the tissue is maintained at zero to slightly positive turgor and negative turgor does not develop as considerable water is lost. Thirdly, the membrane permeability of some or all the cells could change with dehydration, possibly due to changes in aquaporin expression or regulation (Chrispeels & Maurel, 1994; Kjellbom *et al.*, 1999; Chrispeels *et al.*, 2001, reviewed in Chapter 4) such that changes in water potential are experienced as water is lost. Although there is some evidence that aquaporins can influence water potential under moderate water stress (Johansson *et al.*, 1996; Sarda *et al.*, 1997; Liu *et al.*, 1994), the effect of these water channel proteins on PV curves has not been explored. However, it is unlikely that membrane permeability will influence water potential during desiccation (as compared with moderate water stresses) because of the very limited amount of free water.

Beckett (1997) proposed that resurrection plants with unusual PV curves had more rigid cells walls, that negative turgor developed in the tissues, and that the cells initially resisted collapse until after a period of negative turgor. However, unlike the irreparable damage that can be caused by extensive cytorrhysis, tissues in resurrection plants do recover from cell wall folding which appears to be a regulated process associated with

changes in the biochemical properties of the cell walls at specific stages of desiccation (Hallam & Luff, 1980a; Goldsworthy & Drennan, 1991; Vicré *et al.*, 1999; Farrant, 2000). Beckett (1997) did not present actual data points, only the results of a spline fit to the data, and so direct comparison of the data presented here and that of Beckett (1997) is not possible.

It is thus proposed that the unusual PV curves found in many resurrection plants are not a consequence of cavitation and water release i.e. negative turgor does not develop. There are a number of lines of evidence to support this hypothesis. (i) Even though the solute concentration would be high and possibly non-ideal at low RWCs, direct measurements of water activity from frozen-thawed tissue were always below the measured water potential values. This is indicative that negative turgor does not develop. (ii) Extrapolating a straight line from the linear portion of the PV curve data (indirect estimate of 'osmotic potential') at low relative water contents (below the RWCs corresponding to the deviation) yields a line statistically indistinguishable from that determined by direct measurement of water activity (Table 3.1). This indirect estimate of water activity also suggests that negative turgor does not occur. (iii) If in fact the deviation from the normal PV curve is a consequence of cavitation or cytorrhysis, extensive membrane damage and hence electrolyte leakage would be expected. There is, in fact, very little leakage of electrolytes from dehydrated leaves of both species which have unusual curves (*C. wilmsii*, Farrant *et al.*, 1999; *E. nindensis*, Figure 2.4), whereas considerable leakage occurs from *E. curvula* and the desiccation-sensitive leaves of *E. nindensis* (Figure 2.4).

Consequently there has to be an alternative explanation for the unusual curves which does not suggest negative turgor. Unfortunately it was not possible to identify clearly the point of plasmolysis during the anatomical analysis of the tissue at the various water contents and thus the possibility that numerous PV curves have been superimposed to give the anomalous shape cannot be excluded. It would however be unlikely, as the cells of *C. wilmsii*, one of the species with an unusual curve, are fairly uniform in their appearance and physical properties. The experimental techniques to accurately determine

membrane permeability have been developed for hydrated tissues (Steudle, 1989) and consequently it is not yet possible to explore possible (but unlikely) changes in the regulation/ expression of aquaporins which might have an effect on water potential during desiccation. However, I propose that the unusual curves are predominantly the result of a reduction in the volume enclosed by the walls. Firstly, the extensive cell wall folding and hence reduction in cell volume measured in this study occurred over the range of RWCs in which the PV isotherms deviate from the norm (Figures 3.3 and 3.5). Secondly, the shape of the PV curves could imply considerable changes in the elastic properties of the tissue. No attempt was made to measure elastic moduli; however, changes in cell wall chemistry during dehydration of *C. wilmsii* have been observed (Vicré *et al.*, 1999; Vicré, 2001). Such changes, which are likely to affect physical properties of cell walls, are coincident with wall folding (Vicré *et al.*, 1999).

Tissues that are tolerant of desiccation must prevent mechanical damage associated with the shrinkage consequent upon the removal of water. One way by which this may be achieved is the accumulation of insoluble material that effectively replaces the water that is lost. This phenomenon has been shown to occur in *X. humilis*; during drying the vacuoles become packed with insoluble material (Farrant, 2000). This species yields normal PV curves (Figure 5). An alternative possibility is a reduction in cell volume by cell wall folding. It is suggested that the wall folding observed in a number of resurrection plants (Farrant, 2000), which is associated with atypical PV curves in *C. wilmsii* (Figure 3.3) and *Myrothamnus flabellifolius* (Beckett, 1997), is a phenomenon that reduces physical damage on drying and contributes to the desiccation tolerance of these plants. The fact that there are changes in cell wall chemistry in *C. wilmsii* (Vicré *et al.*, 1999) suggests that this is a regulated phenomenon.

The development of single cell sampling and direct measurements of turgor using the pressure probe (reviewed by Tomos and Leigh, 1999) provide the opportunity to analyse the unusual water relations of resurrection plants in more detail. However, as yet these techniques are limited to peripheral cell layers and since individual cell volume changes cannot be accurately quantified during dehydration, PV curves and measurements of

elastic properties of individual cells within complex tissues is not possible. However, measurements of turgor from both pressure probes and psychrometric techniques do agree (Nonami *et al.*, 1987) and so whole tissue investigations will continue to provide holistic interpretations of a system.

University of Cape Town

Chapter 4:**Tonoplast intrinsic proteins in desiccation tolerance****4.1 Introduction****4.1.1 Membrane water permeability**

Water transport across membranes is of importance in plants during desiccation and rehydration from this stress. Although the physiological aspects of plant water relations have been researched and described using PV curves for many decades (discussed in the previous chapter), it was not until the discovery of a group of water transport proteins that membrane water permeability was extensively explored (reviewed by Chrispeels & Agre, 1994; Chrispeels & Maurel, 1994; Kjellbom *et al.*, 1999; Tyerman *et al.*, 1999; Maurel & Chrispeels, 2001 *inter alia*).

Water movement across membranes is a consequence of the turgor and osmotic gradients. The osmotic component ($\Delta\Psi_o$) of this phenomenon is determined by the solute concentration gradient (ΔC) and the solute reflection coefficient (σ), which is a measure of membrane selectivity for a solute, according to the equation:

$$\Delta\Psi_o = \sigma RT\Delta C \quad \dots\dots\dots 1$$

where R is the universal gas constant and T is the absolute temperature (Finkelstein, 1987).

Until modern methods of measuring membrane permeability had been developed (Steudle, 1989), the more crude estimates suggested that diffusion through the lipid bilayer accounted for symplastic water movement (Dainty, 1963; Stadelmann, 1969) and early suggestions of the existence of water pores in membranes were marginalized (Maurel, 1997). However, membrane water pores do exist. The first clear physiological evidence for this in animals was reported more than 40 years ago in erythrocytes

(Paganelli & Solomon, 1957), but has only been recently been conclusively shown in plants (Henzler & Steudle, 1995).

Probably because aquaporins (water-transport proteins) are in fact incredibly abundant in plants, the characterisation of many of their protein and DNA sequences were reported before their function was known (Maurel, 1997). To date, approximately 1220 aquaporins have been sequenced, of which more than two thirds are from plants (National Library of Medicine, 2001). The integration of the cellular and molecular biology of aquaporins with plant water relations has led to a new combined approach to understanding the regulation of water movement in plants.

4.1.2 Major Intrinsic Protein (MIP) superfamily

Aquaporins are part of the major intrinsic protein (MIP) superfamily (Agre *et al.*, 2001). MIPs have been reported in bacteria, yeast, plants and animals (Kaldenhoff & Eckert, 1999) and are thought to be evolutionarily very old and highly conserved (Reizer *et al.*, 1993). Consequently, they have recently formed the basis of numerous phylogenetic studies (Chaumont *et al.*, 2001; Zardoya & Villalba, 2001).

This family of proteins has been sub-divided into three major groups, namely, animal aquaporins, aquaglyceroporins and plant aquaporins based on their localisation and permeability to various solutes (Figure 4.1). Three groups within the plant aquaporins are recognised: nodular-like membrane proteins (NLPs) and the plasma membrane intrinsic proteins (PIPs) and tonoplast intrinsic membrane proteins (TIPs) which are found in plasmalemmas and vacuolar membranes, respectively (Paris *et al.*, 1996 Kaldenhoff *et al.*, 1998).

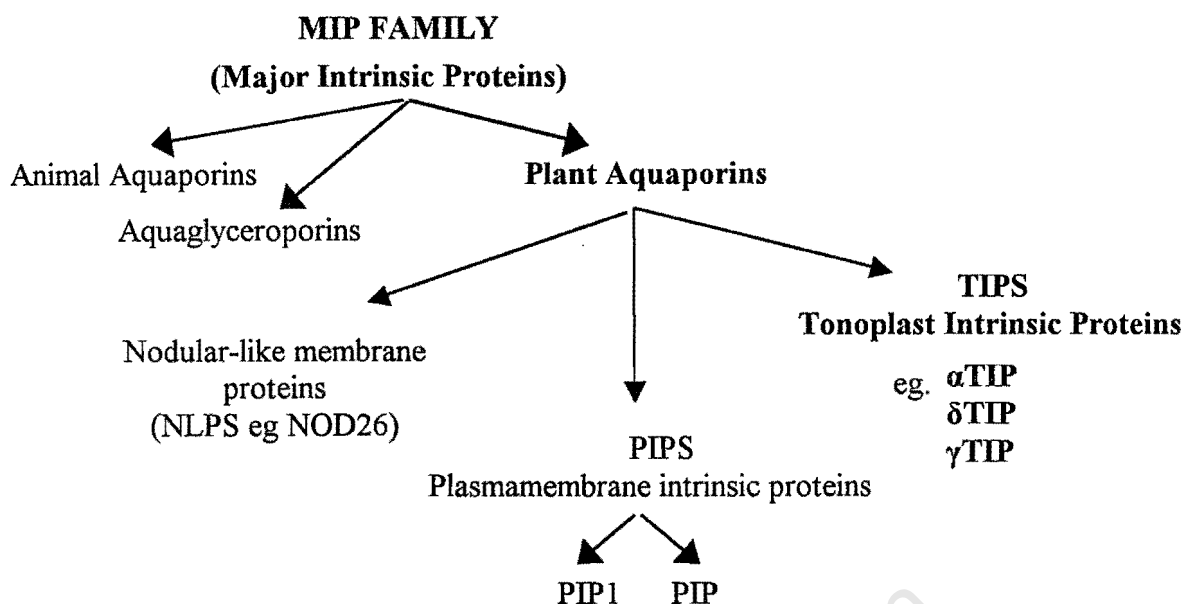


Figure 4.1. Classification of the membrane intrinsic protein superfamily. The two groups to the left are found in animals while the other three are predominantly found in plants.

4.1.3 Aquaporin structure and function

Although the nucleotide sequence identity of the known aquaporins is less than 25% (Maurel, 1997), all aquaporin mRNAs code for proteins (25- 30 kDa) with numerous highly conserved domains (Figure 4.2). There are six membrane-spanning α helices with the amino- and carboxyl- termini both positioned on the cytoplasmic side of the membrane (Reizer *et al.*, 1993) and numerous conserved residues including two NPA (asparagine-proline-alanine) motifs. These two motifs are located on the loops which fold inwards to form a narrow hydrophobic pore (*ca.* 3 Å diameter and *ca.* 30 Å long) in an hourglass model (Figure 4.3) which has recently been confirmed by 2-dimensional crystallography, high resolution x-radiography and electron microscopy (Fu *et al.*, 2000; Murata *et al.*, 2000; Ren *et al.*, 2000). Daniels *et al.* (1999) discovered that aquaporins form 6 nm square tetramers. From the pore size, it has been predicted that *ca.* 0.5×10^9 water molecules can diffuse through a single aquaporin every second (Weiss, 1996), far exceeding the maximum transport rate of an ion pump (Sansom & Law, 2001).

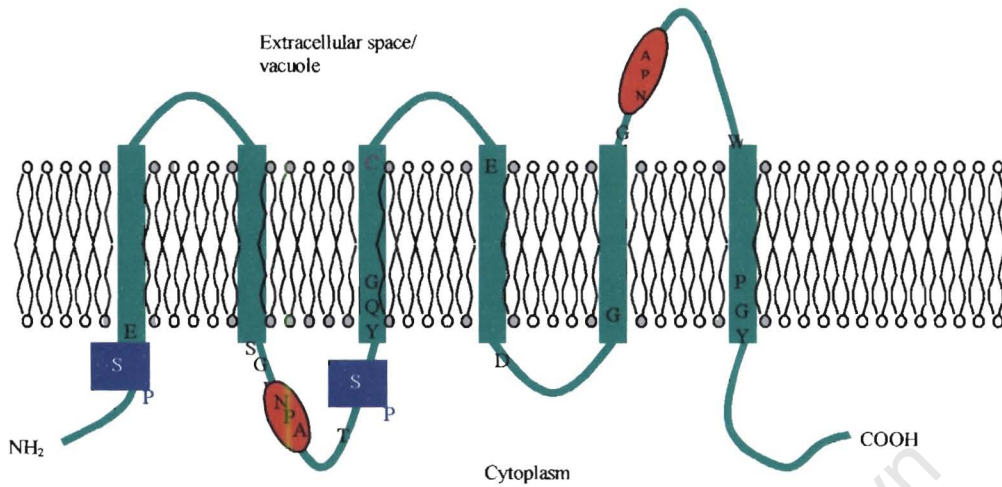


Figure 4.2. The two-dimensional structure of a plant aquaporin showing the six membrane spanning sequences and the highly conserved residues. Note the extrinsic cysteine residues, thought to be associated with mercury sensitivity. (Modified from Chrispeels & Maurel, 1994).

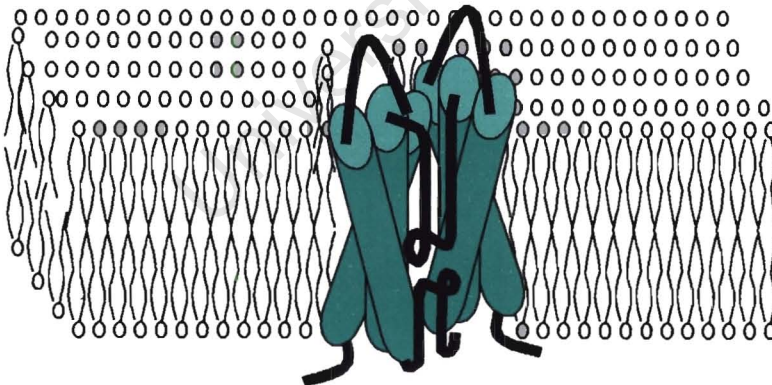


Figure 4.3. The hourglass model of a plant aquaporin (modified from Maurel & Chrispeels, 2001).

The expression of aquaporins in *Xenopus* oocytes has been the predominant technique used to measure water permeability of aquaporins across membranes (Preston *et al.*, 1992; Maurel *et al.*, 1994). In addition to water, some aquaporins have been reported to be conductive to other solutes including urea (Echevarria *et al.*, 1994; Ishibashi *et al.*, 1997), ions (Weaver *et al.*, 1994), glycerol (Biela *et al.*, 1999), formamide (Rivers *et al.*, 1997), H₂O₂ (Henzler & Steudle, 2000) and CO₂ (Nakhoul *et al.*, 1998). Mercury has been shown to block the pores of many aquaporins, binding to the cysteine residues illustrated in Figure 4.2 (Daniels *et al.*, 1996; Maurel, 1997; Martre *et al.*, 2001) whereas phosphorylation has been demonstrated to stimulate activity in response to increased turgor pressure (Maurel *et al.*, 1995). The volume of research on aquaporins in the last decade has escalated, and apart from their proposed function in water transport and osmoregulation (Kaldenhoff & Eckert, 1999, Tyerman *et al.*, 1999 *inter alia*), aquaporins have also been suggested to be involved in stomatal movement (Tyerman *et al.*, 1999), seed maturation and germination (Guerrero *et al.*, 1990; Yamamoto *et al.*, 1991; Maeshima *et al.*, 1994), spore dormancy (Mitra *et al.*, 2001), cell elongation (Ludevid *et al.*, 1992; Kaldenhoff *et al.*, 1995; Chaumont *et al.*, 1998) and water uptake of roots (Maggio & Joly, 1995; Sarda *et al.*, 1999, *inter alia*).

4.1.4 Tonoplast intrinsic proteins (TIPs)

Tonoplast intrinsic proteins (TIPs), which are unique to plants, are not only the most abundant proteins found in tonoplasts (Maeshima, 2001), but also constitute 48 and 35% of all the aquaporins identified in *Arabidopsis thaliana* and *Zea mays*, respectively (Weig *et al.*, 1997; Chaumont *et al.*, 2001). Of the two groups of plant aquaporins, TIPs are smaller (23-26 kDa) than PIPs (30kDa) and although they share many identical residues, the amino acid sequence homology can be as low as 40% between the two groups (Santoni *et al.*, 2000).

The different isoforms of the TIPs tend to be spatially and temporally expressed and there have been suggestions that the abundance and wide variety of isoforms may play an important role in defining vacuolar function (Jauh *et al.*, 1998; 1999; Jiang & Rogers,

1998). There are three main TIP isoforms: α -TIPs, δ -TIPs and γ -TIPs. α -TIP, the first aquaporin to be identified in plant tissue (Johnson *et al.*, 1990), is associated with protein storage vacuoles (PSVs) of seeds (Johnson *et al.*, 1989; Hofte *et al.*, 1992); β -TIP is a subtype of α -TIP (Maeshima, 2001). Vegetative storage proteins are stored in vacuoles which are characterised by the presence of δ -TIPs whereas γ -TIPs are abundant on the tonoplasts of lytic vacuoles (Hoh *et al.*, 1995; Paris *et al.*, 1996; Jauh *et al.*, 1998) and are thought to gradually replace the α -TIPs during seed germination (Maurel *et al.*, 1997).

4.1.5 Desiccation tolerance and aquaporins

Since changes in hydraulic conductivity of many plant tissues have been extensively reported to be associated with water stress (Cruz *et al.*, 1992; Lo Gullo *et al.*, 1998; Vander Willigen & Pammenter, 1997 *inter alia*) and aquaporins play a crucial role in water movement, their behaviour under water stress conditions is starting to receive attention (Yamaguchi-Shinozaki *et al.*, 1992; Yamada *et al.*, 1995; 1997; Kirch *et al.*, 2000; Steudle, 2000; Mitra *et al.*, 2001). There are now some reports of changes in the expression of aquaporins under stress conditions which influence not only hydraulic conductivity but also water potential via aquaporin phosphorylation (Liu *et al.*, 1994; Johansson *et al.*, 1996; Sarda *et al.*, 1997; Johansson *et al.*, 1998; Barrieu *et al.*, 1999).

Not long after the discovery of water channel proteins in plants (Johnson & Chrispeels, 1992), their role in desiccation tolerance was suggested (Gaff, 1997; Hartung *et al.*, 1998). Five aquaporins have been isolated from resurrection angiosperms: three PIPs and a TIP from *Craterostigma plantagineum* (Mariaux *et al.*, 1998) and one γ -TIP from *Sporobolus stapfianus* (Neale *et al.*, 2000). Changes in the expression of these five genes during desiccation were reported, however no protein studies on these TIPs or their role in desiccation tolerance have been undertaken. Most of the studies on the role of aquaporins in water stress have focussed on the roles of PIPs during a water stress; very few studies have considered the function of TIPs in water limited environments.

It was shown in the previous chapter that the leaf water relations in some resurrection plants differ from that observed in desiccation-sensitive tissues. Although PIPs are more likely to have an effect, if any, on water potential during desiccation (discussed in the previous chapter), TIPs may also play a role in conferring desiccation tolerance in resurrection plants. Vacuoles have been proposed to assist in maintaining cellular integrity during desiccation (Farrant, 2000) and thus the intrinsic proteins of the tonoplasts of these vacuoles may be important in this process and possibly other aspects of tolerance. In this chapter the protein expression and immunolocalization of α -TIP and δ -TIP during the desiccation and rehydration of *E. nindensis* and *E. curvula* is presented. The possible roles of these two proposed tonoplastic intrinsic proteins are discussed.

4.2 Materials and Methods

4.2.1 Plant material

E. curvula plants germinated from seed were grown to maturity in a greenhouse as described previously (Chapter 2.2.1). *E. nindensis* plants from an inselberg located in a north western region of South Africa were potted in the sandy soil from the site and translocated to a greenhouse at the University of Cape Town. All plants remained hydrated for at least one month prior to any experimentation. So as to avoid additional variation as a result of changes in the experimental design which are known to affect the physiology of the plants (Pammenter *et al.*, 2001), the same drying and rehydrating procedures as those used in the previous chapters (sections 2.2.1, 3.2.1) were used.

Inner and outer leaves from *E. nindensis* and inner leaves of *E. curvula* from at least three different plants were harvested at intervals during desiccation (withholding of watering) from plants which were initially hydrated to full turgor. After two weeks in the dry state, plants were rewatered and held at field capacity during regular sampling over a rehydration period. Relative water contents were determined gravimetrically at each point, as described previously (Chapter 2.2.2). The mean RWC for all plants at each sampling point was calculated. Because diurnal changes in the expression of TIPs have

been reported (Henzler *et al.*, 1999; Clarkson *et al.*, 2000), tissue for RNA and protein extractions was always harvested at 9h00, immediately plunged into liquid nitrogen and stored at -80°C . Since the sampling intervals during rehydration were less than 24 h, sampling times are reported in Table 4.2. Two reference plant tissues were used for the Western blots: Bean (*Phaseolus vulgaris*) seed were soaked in water for 48 h prior to RNA and protein extractions and roots were harvested from radishes (*Raphanus sativus* L.) purchased from a local vegetable market.

4.2.2 Material (TIP antibodies)

The TIP antibodies used in this study were kindly provided by Dr JC Rogers, (Institute of Biological Chemistry, Washington State University, USA). The α -TIP and δ -TIP were isolated from bean seed and radish roots respectively. Since the amino acid sequence of the carboxyl-terminal, cytoplasmic tails of these two TIP isoforms are conserved across species (Jauh, *et al.*, 1998), antibodies were raised in rabbits against polypeptides synthesised from the following two sequences: HQPLAPEDY (α -TIP) and CHVPLASADF (δ -TIP; Jauh *et al.*, 1998). Antibodies raised against the α -TIP isolated from *P. vulgaris* (Johnson *et al.*, 1989) were also used.

4.2.3 Protein and RNA extraction

Leaf segments (from 0.5 to 4 cm distal to the leaf sheath) of *E. nindensis* and *E. curvula*, *P. vulgaris* and *R. sativus* roots were ground in liquid nitrogen and homogenised in Trizol (Life Technologies, GIBCO BRL) for 5 min at room temperature with vortexing (*ca.* 0.1 g tissue in 1 ml Trizol per microcentrifuge tube) according to the methods of Chomezynski and Sacchi (1987). Thereafter 200 μl chloroform was added per tube, gently agitated and left to stand for 5 min before centrifugation at 12000 g for 15 min at 4°C . The aqueous phase containing the RNA was then transferred to clean microcentrifuge tubes and incubated with 500 μl of isopropanol for 15 min, to precipitate the RNA. The RNA pellets, following centrifugation at 12000 g for 10 min at 4°C , were washed with 75% ethanol (RNAase-free water) and air-dried. RNA pellets were

resuspended in formamide or DEPC- (diethyl pyrocarbonate) treated water and stored at -80°C . During this procedure 300 μl ethanol was also mixed with the remainder of the homogenate in the original tubes that contained the DNA and protein. After 2 min incubation and 5 min centrifugation at 2 000 g (4°C), the precipitate containing DNA was discarded while the supernatant was incubated for a further 10 min with 1.5 ml isopropanol in clean microcentrifuge tubes to precipitate the protein extract. The Chomezynski (1993) protein extraction method was modified slightly by washing the pellets three times with cold 0.1 M ammonium acetate and once with cold acetone (centrifugation at 12000 g for 10 min at 4°C and 5 min incubation periods between washes). Air-dried protein samples were stored at -20°C .

4.2.4 Protein quantification

The fresh mass of ground plant tissues used in RNA and protein extractions were measured gravimetrically. Resuspended proteins were quantified according to the methods of Bradford (1976) using a protein assay kit (Pierce, Rockford USA). The absorbance of 40 μl protein samples that had been incubated with 2 ml of the diluted Bradford reagent for 2 min, was read at 595 nm (Beckman DU 530 spectrophotometer, Fullerton, CA, USA). Protein concentrations were calculated from a standard curve, constructed using bovine serum albumin (BSA). This data is present in Tables 4.1 and 4.2.

Table 4.1. Changes in the total protein concentration of leaves from *E. nindensis* and *E. curvula* with relative water content (RWC) during a two week dehydration period. The rate of dehydration is given in Figure 2.3. Standard deviations given in parentheses. n=4 (RWC); n=6-10 (protein concentration).

<i>E. nindensis</i> inner leaves		<i>E. nindensis</i> outer leaves		<i>E. curvula</i> inner leaves	
RWC (%)	Protein conc. (mg. g d mass ⁻¹)	RWC (%)	Protein conc. (mg. g d mass ⁻¹)	RWC (%)	Protein conc. (mg. g d mass ⁻¹)
96.7 (3.4)	31.64 (6.95)	87.2 (6.7)	30.7 (4.82)	95.1 (3.2)	49.1 (4.93)
78.3 (4.3)	29.7 (5.38)	45.7 (7.1)	39.6 (5.91)	56.3 (7.5)	45.3 (5.29)
46.6 (4.3)	32.6 (4.01)	5.2 (2.6)	11.4 (6.72)	20.8 (4.2)	32.1 (3.05)
26.3 (5.1)	53.9 (4.52)	-	-	-	-
11.4 (3.2)	53.9 (3.85)	-	-	-	-
4.2 (2.1)	56.7 (5.44)	-	-	-	-

Table 4.2. Changes in the total protein concentration (mg. g dry mass⁻¹) against relative water content (RWC) during the rehydration of desiccated leaf tissue from *E. nindensis* and *E. curvula* measured at regular time intervals. Standard deviations given in parentheses. n=4 (RWC); n=6-8 (protein concentration).

<i>E.nindensis</i> inner leaves			<i>E.nindensis</i> outer leaves			<i>E.curvula</i> inner leaves		
Time (h)	RWC (%)	Prot. Conc.	Time (h)	RWC (%)	Prot. Conc.	Time (h)	RWC (%)	Prot. Conc.
4	14.1 (5.6)	57.8 (6.33)	24	8.4 (5.9)	7.4 (5.61)	24	6.9 (4.5)	12.7 (5.92)
8	23.5 (7.9)	53.7 (4.59)	-	-	-	-	-	-
12	41.1 (8.2)	58.1 (5.60)	-	-	-	-	-	-
24	57.3 (7.8)	59.3 (5.28)	-	-	-	-	-	-
72	86.9 (8.3)	51.2 (6.82)	-	-	-	-	-	-

dash - no data since no rehydration.

4.2.5 Western blot analysis

Proteins (15 µg per lane) were incubated at 37°C for 10 min and electrophoresed on 20% SDS-PAGE gels containing 0.1% bis acrylamide. Higher incubation temperatures cause TIP dimers to form (Johnson *et al.*, 1989). The gel, nitrocellulose membrane, Whatmann 3MM paper and foam pads (all cut to the same size as the gel) were soaked in transfer buffer (192 mM glycine, 25 mM tris, 3.5 mM SDS, 20% (v/v) methanol). The gel and membrane were sandwiched between the paper and foam pads. Care was taken to avoid air bubbles. The proteins were transferred to the PVDF Western blotting membrane (Boehringer Mannheim) (nearer the cathode) for 1 h at 30 V in a precooled Western

blotting tank (Hoefer, San Francisco, USA). The membrane was rinsed with TBS (50 mM Tris, 150 mM NaCl, pH 7.5) and non-specific binding sites were blocked with 0.5% w/v skim milk powder in TBS for 1 h at room temperature with shaking. The membrane was then incubated overnight with the primary antibody (α -TIP: 1:2 000 dilution (polypeptide); 1:3 000 dilution (entire protein) or δ -TIP: 1:2 500 dilution) diluted with 0.5% w/v skim milk powder at 4°C with shaking. After two washes with TBS containing 0.1% Tween 20 for 10 min, the membrane was incubated with the secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase) at 1:1 000 dilution for 1 h at room temperature with shaking. The membrane was thoroughly washed with TBS containing 0.1% Tween 20 four times for 15 min each before detection using chemiluminescence (Duvant & Furler, 1994) according to the manufacturer's instructions (Amersham Pharmacia, Buckinghamshire, UK). Blots were developed on high performance chemiluminescence film (Amersham Pharmacia, Buckinghamshire, UK), scanned and analysed using Biorad Quantity One 4.4 software (Biorad, California, USA). Duplicate gels were routinely stained with Coomassie Brilliant blue R-250 (0.25% (w/v) in 50% (v/v) methanol, 7% acetic acid (v/v)) to confirm equal protein loading. All blots were repeated at least twice on separately extracted protein samples from different plants.

4.2.6 Immunofluorescence studies

Leaf segments (*ca.* 5 mm² pieces) from 2 cm distal to the leaf sheath were fixed in 4% (w/v) paraformaldehyde in 0.1 M Sorensens phosphate buffer (pH 7.2) overnight at 4°C. Specimens were then dehydrated through an alcohol series (50%, 70%, 95% and 100% ethanol, 100% isopropanol, 100% butanol) and embedded in paraffin wax at 60°C overnight. Thick wax embedded transverse sections (10 μ m) were cut on a Leitz rotary microtome (Vienna, Austria) and attached to APTES- (3-aminopropyltriethoxysilane) coated glass microscope slides by heating to 60°C for 1 h. After dewaxing with xylol and rehydrating through an ethanol gradient, the sections were washed with PBS (50 mM NaH₂PO₄, 150 mM NaCl, pH 7.4) and blocked with 1% BSA in PBS for 1 h in a moist chamber at room temperature for 1 h. Sections were incubated with either primary

antibody (α -TIP: 1:100 dilution, δ -TIP: 1:75 dilution – used only polypeptide antibodies) or 1% BSA in PBS overnight at 4°C in a moist chamber. Thereafter, the sections were jet-washed with 0.1% Triton X-100 in PBS and then rinsed for a further 15 min in the buffer before incubating in the fluorochrome tagged secondary goat anti-rabbit IgG antibody (Alexa fluor 568, Molecular Probes, Orgeon USA: dilution 1:1000) for 2 h in a moist chamber at room temperature in the dark. The slides were then thoroughly washed with PBS containing Triton X-100. Sections were mounted with ProLong Antifade (Molecular Probes, Oregon, USA) and viewed with an inverted flourescent microscope (Nikon, Tokyo, Japan) under oil emersion with a B2 DM510 epi-fluorescence filter (Zeiss, Tokyo, Japan). The approximate absorption and fluorescence emission maxima for Alexa fluor 568 are 578 nm and 603 nm, respectively (Molecular Probes, Inc. 2000). A confocal laser scanning microscopy (550 Confocal, Microscope, Leica, Cambridge, UK) was used to confirm the results, using a narrower range of wavelengths (600 – 610 nm). Images were captured with an Axiocam digital camera (Zeiss, Hallbergmoos, Germany) using AxioVision 2.05 software (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). Care was taken to use the identical exposure times and images were not adjusted thereafter for comparative purposes.

4.3 Results

The antibodies raised against polypeptides from the α -TIP and δ -TIP cytoplasmic tails of *P. vulgaris* seed and *R. sativus* roots (Jauh *et al.*, 1998) cross-reacted with only proteins of an equivalent size (*ca.* 26 kDa) in both *E. nindensis* and *E. curvula*, respectively (Figure 4.4). These antibodies were thus used to characterise the proposed α - and δ -TIPs found in the study plants.

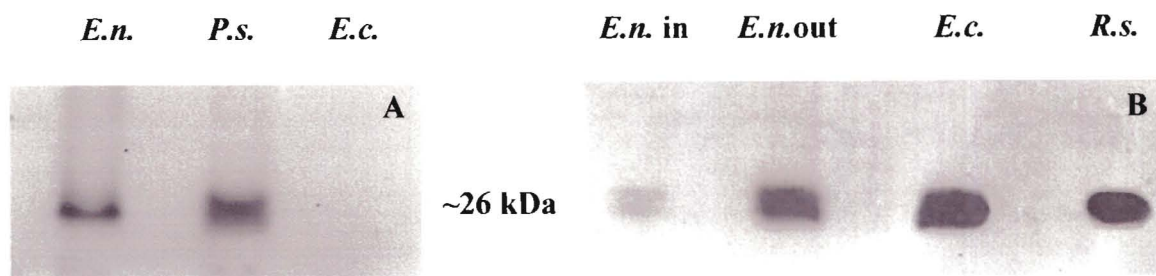


Figure 4.4 Western blots of α -TIP (a) and δ -TIP (b) with protein extracts from source (*Phaeolus vulgaris* seeds – *P.s.* and *Raphanus sativus* roots – *R.s.* respectively) and experimental material: (a) desiccated leaves of *E. nindensis* (*E.n.*) and *E. curvula* (*E.c.*) and (b) hydrated inner (*E.n. in*) and outer (*E.n. out*) *E. nindensis* leaves and *E. curvula* (*E.c.*) leaves. 15 μ g of protein loaded in each lane.

4.3.1 Characterization of α -TIP

Western blots of α -TIP with the desiccation-tolerant inner leaves of *E. nindensis* suggest that the protein is only present in the desiccated state (Figure 4.5a). It appears that α -TIP is translated very late during dehydration, once the leaves are less than 0.43 ± 0.22 g $\text{H}_2\text{O} \cdot \text{g dmass}^{-1}$ (11.4% RWC) and that it is degraded immediately on rehydration. This result was confirmed using the α -TIP antibodies raised against the entire protein (Johnson *et al.*, 1989 – data not shown). Like all other vegetative tissues studied to date (Jauh *et al.*, 1998), α -TIP is not found in the desiccation-sensitive leaves of either *E. curvula* or *E. nindensis* (Figure 4.5b). Positive controls were done simultaneously to confirm this negative result.

Immunofluorescence studies confirmed that α -TIP was only present in dry inner leaves of *E. nindensis* (Figure 4.6c-f). The localization of the fluorescent probe to the abundant

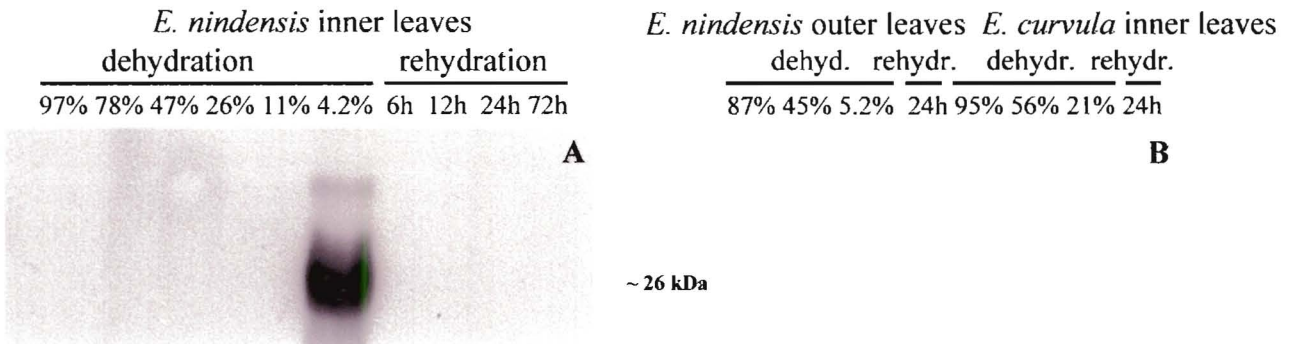


Figure 4.5. Western blots of *E. nindensis* (inner (a) and outer (b) leaves) and *E. curvula* (inner leaves (b)) with α -TIP antibodies. Proteins (15 μ g/lane) were extracted from tissue at a range of water contents during dehydration and rehydration (RWC and time denoted on top of the gel).

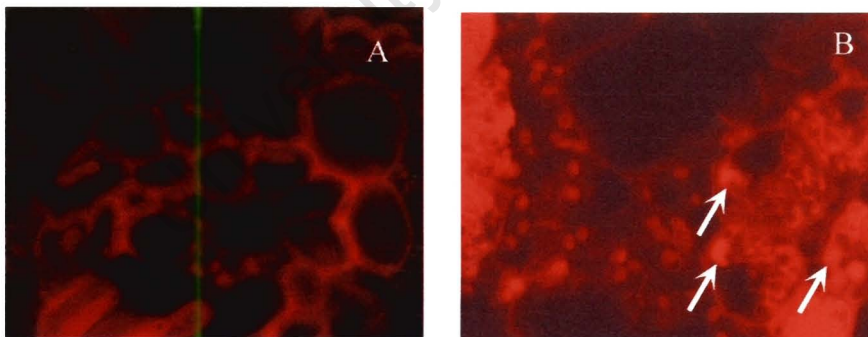


Figure 4.6 Immunolocalization of α -TIP in desiccation-tolerant inner leaves of *E. nindensis* (5% RWC). (a) immunological control in which BSA was substituted for α -TIP. Alexafluor 568 labelled vacuoles (red) are arrowed (b). (x 1690 for both images).

small vacuoles present predominately in the bundle sheath cells of this tissue confirms the specificity of the antibody to an α -TIP in *E. nindensis* (Figures 6c-f). Unfortunately, the wavelength and intensity of the background fluorescence changed with hydration state and could thus not be eliminated completely (Figures 4.5a and b). This is likely to be a consequence of changes in pigment composition and concentration with desiccation (Figure 2.7). However, the intensity and location of the fluorescent marker was clearly distinguishable in tissue sections in which the protein was present at 603 nm and was also confirmed using confocal microscopy (data not shown).

4.3.2 Characterization of δ -TIP

Like α -TIP, the expression of δ -TIP was also found to change during both dehydration and rehydration of all three leaf types studied (Figures 4.6a and b). This TIP, suggested to be associated with vacuoles storing vegetative storage proteins (Jauh *et al.*, 1998), was found to be present in the fully hydrated leaves of both *E. nindensis* and *E. curvula*. During desiccation, the protein expression drops initially and then peaks at *ca.* 50% and 20% RWC in *E. nindensis* (inner leaves) and *E. curvula*, respectively. Even though *E. nindensis* was more than 80% hydrated after 72 h rehydration, δ -TIP, which is not present at RWC lower than 20%, had not yet been retranscribed in these tissues (Figure 4.7a). Unlike the α -TIP in *E. nindensis*, it appears that the *E. curvula* δ -TIP is not degraded during desiccation and low concentrations of the protein were still present in the dead leaves which had been rehydrated for 24 h (Figure 4.7b). There was no δ -TIP expression in the outer leaves of *E. nindensis* once desiccation commenced (Figure 4.7b). This might be contributory to the inability of these leaves to resurrect.

The acrylamide gels were loaded with equal amounts of total protein extract, however, protein concentration was found to increase (*E. nindensis* inner leaves) and decrease (*E. curvula* and outer leaves of *E. nindensis*) during desiccation (Table 4.1). This phenomenon has been reported before in resurrection plants (Gaff & McGreggor, 1979; Gaff, 1980; Tymms *et al.*, 1982; Kuang *et al.*, 1995). The relative intensities of the bands, calculated as an average from three different blots for each lane, are plotted against the

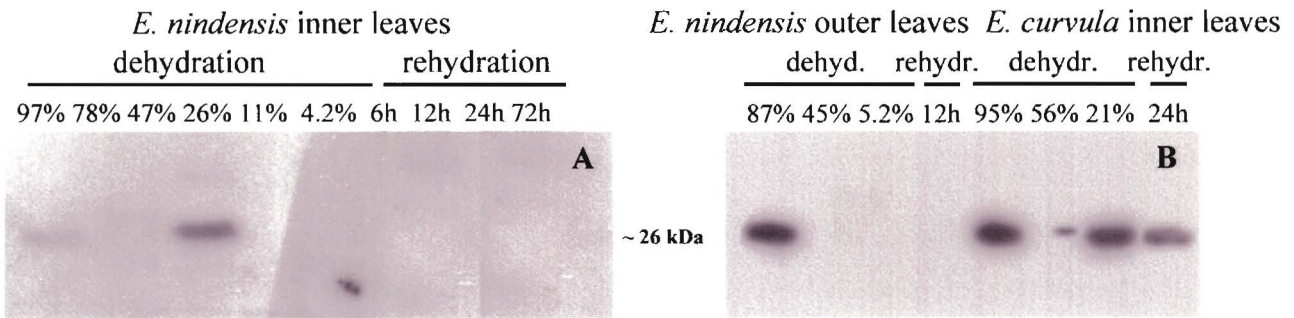


Figure 4.7. Western blots of *E. nindensis* (inner (a) and outer (b) leaves and *E. curvula* (inner leaves (b)) with δ -TIP antibodies. Proteins (15 μ g/ lane) were extracted from tissue at a range of water contents during dehydration and hydration (RWC and time denoted on top of the gel).

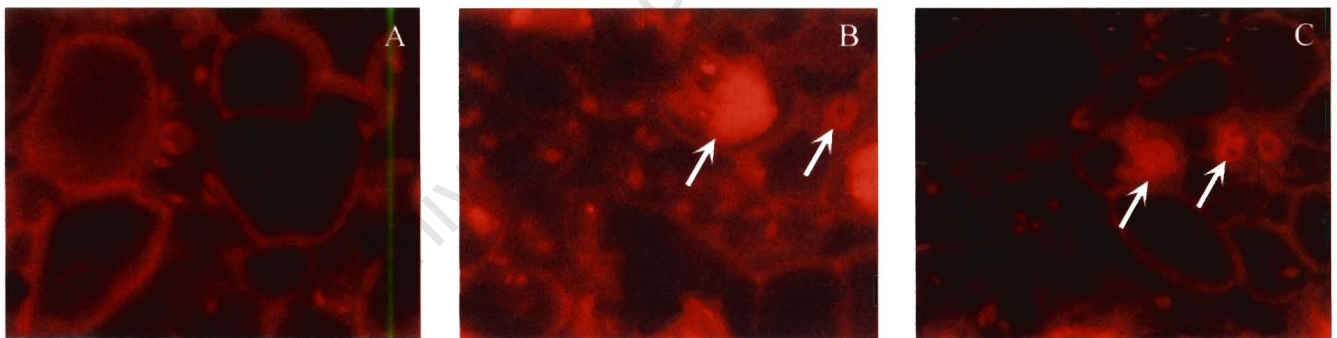


Figure 4.8 Immunolocalization of δ -TIP in desiccation-tolerant inner leaves of *E. nindensis*. (a) immunological control in which BSA was substituted for δ -TIP. Alexafluor 568 labelled vacuoles (red) at 97% RWC (b) and 47% RWC (c), are arrowed. (x 1690 for all images).

relative water content of the leaves on both a protein and dry mass basis in Figure 4.9. This gives some measure of the proportion of δ -TIP as a function of total dry mass, a variable which is more constant during desiccation. The pattern of expression calculated in this manner follows the same trend as that of the δ -TIPs identified in this study, however the relative changes are different. Since no purified δ -TIP of known concentration was available, this data cannot be quantified. A dramatic increase in δ -TIP at ca. 46% RWC in *E. nindensis* is highlighted if the δ -TIP concentration is set at 100% for the hydrated leaves in both species and leaf types. In addition, it is clear that this increase in expression is proportionally greater on a dry mass basis compared with the smaller increase measured on protein content because of the general increase in protein accumulation during desiccation (Figure 4.8). However, from the blots (Figures 4.4b, 4.7a and b), it appeared that the concentration of δ -TIP was considerably lower in the hydrated inner leaves of *E. nindensis* compared with the desiccation-sensitive leaves in both species. The reason for this is unknown.

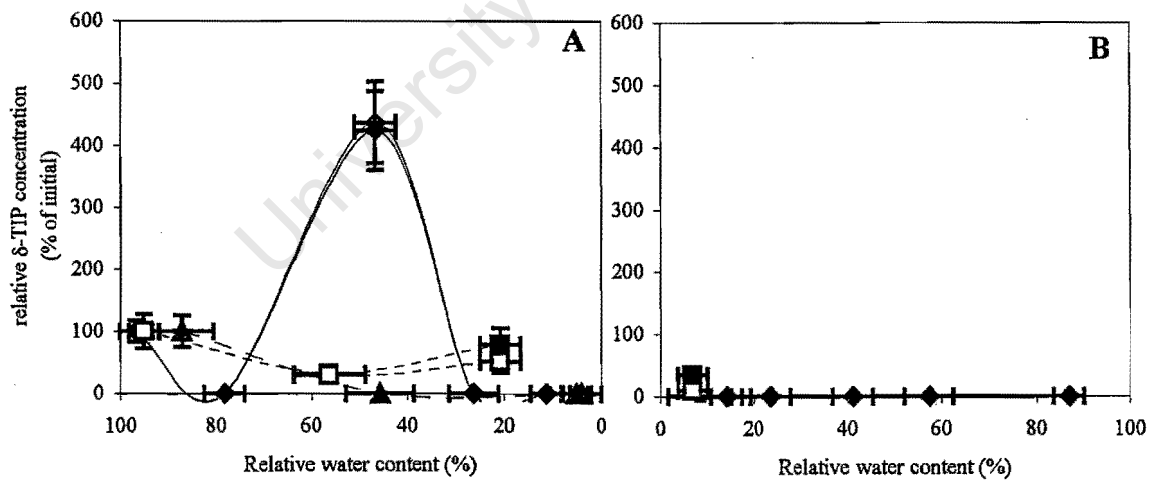


Figure 4.9 Relative proportions of δ -TIP expressed on a dry mass (open symbols) and protein concentration (closed symbols) for *E. nindensis* inner leaves (\diamond), outer leaves (Δ) and *E. curvula* inner leaves (\blacksquare) during dehydration (a) and rehydration (b).

The δ -TIP antibodies targeted tonoplasts at comparable hydration states with those seen in the protein blots for the various leaves. δ -TIP was localized to both large and smaller vacuoles in hydrated tissues of both *E. nindensis* and *E. curvula* (Figures 4.6c and d) at the peak in expression during desiccation (50 and 20% RWC respectively, Figures 4.7a and b).

4.4 Discussion

Although there are a few reports of changes in the expression of individual TIP isoforms during water stress (Maurel *et al.*, 1997; Barrieu *et al.*, 1999), this is the first report of different TIP isoforms being translated only at particular stages of desiccation. Notably, this is only true of the desiccation-tolerant tissue used in this study and thus it reiterates previous findings, both in the preceding two chapters and in many other studies: the mechanisms employed by resurrection plants which enable them to revive from extreme water stress differ from the unsuccessful strategies attempted by desiccation-sensitive tissues to avoid drought-related death (reviewed by Gaff, 1980).

Similar differences in the presence and degradation of different TIP isoforms observed during dehydration and subsequent rehydration of the desiccation-tolerant leaves of *E. nindensis* (Figures 4.5a and 4.6a), have been reported during orthodox seed development and germination (Figures 4.5a and 4.6a; Hofte *et al.*, 1992; Maurel *et al.* 1997; Jauh *et al.*, 1999). During the hydration of desiccation-tolerant tissues (orthodox seeds and resurrection plants), osmotic adjustment and the maintenance of membrane integrity are essential (Vertucci & Farrant, 1995; Pammenter & Berjak, 1999). In seeds, various TIP isoforms, including both α - and δ -TIPs, are proposed to act in synchrony to assist in these processes (Maurel, 1997; Gao *et al.*, 1999). Since the δ -TIP identified in *E. nindensis* in this study is absent in the dry state and during the initial stages of rehydration (Figure 4.7a) and the *E. nindensis* α -TIP is degraded early during rehydration (Figure 4.5a), it is likely that TIP isoforms other than those identified in this study may be involved in similar processes during the rehydration of vegetative desiccation-tolerant tissues.

In addition to the conclusive evidence that the α -TIP isolated from *P. vulgaris* is an aquaporin since it is permeable to water, the activity of which is regulated at the protein level by turgor-dependant phosphorylation and dephosphorylation (Maurel *et al.*, 1995), there have been suggestions that it may be permeable to glycerol and small proteins (Maurel & Chrispeels, 2001). Since the α -TIP identified in *E. nindensis* is only observed in dry leaf tissues (>11% RWC) and its permeability to water or other solutes has not yet been established, it is unclear what the role of this TIP might be. Other α -TIPs expressed during the dehydration stages of seed maturation have been proposed to be involved in maintaining tonoplast integrity during this phase (Maurel *et al.*, 1995). During germination, α -TIPs from seeds are thought to be associated with the mobilization of the contents of the protein storage vacuoles with which they are associated (Johnson *et al.*, 1990; Maurel *et al.*, 1997). The α -TIP found in *E. nindensis* is associated with the abundant small vacuoles seen in the desiccated state (Figures 4.6 e-f) which are thus likely to be similar to the protein storage vacuoles characterised in dry seed tissues (Johnson *et al.*, 1989) and hence a similar function is proposed. However, since the expression of the *E. nindensis* α -TIP is more transient than those characterised in seeds (Johnson *et al.*, 1990; Hofte *et al.*, 1992), its role in desiccation tolerance cannot yet be conclusively deduced. Farrant (2000) proposed that the vacuoles seen in the dry states of many resurrection plants store non-aqueous substances to reduce mechanical stress. It is thus possible that α -TIP is involved in the stabilization and/or mobilization of these vacuoles in the dry state and on rehydration, respectively. Jauh *et al.* (1999) proposed that some TIPs play a role in determining the function of a vacuole; the α -TIPs seen on protein storage vacuoles are gradually replaced by γ -TIPs during seed germination in *P. vulgaris*, redefining the function and structure of those vacuoles. α -TIP in *E. nindensis* might have a similar function.

The appearance of an α -TIP in desiccated inner leaves of *E. nindensis* is the only account of this TIP isoform in vegetative tissues. To date, no expression of all the other α -TIPs reported in the seeds of numerous plants have been expressed subsequent to seed maturation (Maurel *et al.*, 1997). The absence of any cross reactivity between the *P.*

vulgaris α -TIP antibodies with proteins from *E. curvula* leaves at any time during desiccation might suggest that this antibody is not specific to this species, and does not exclude the possibility that an α -TIP does exist in those tissues (Figure 4.5b). Even though there are no other reports of α -TIP in vegetative tissues, desiccated plant tissues were not examined in those studies. However, the specificity and cross-reactivity of this antibody to α -TIPs to the seed but not any vegetative tissues from a number of species has been tested (Jauh *et al.*, 1998) and hence this is unlikely.

The correlation between the increase in expression of δ -TIP with increased vesiculation during desiccation (Figures 4.7b, 4.8a-c) has been reported in an unrelated TIP isoform expressed in cauliflower (Barrieu *et al.*, 1999). In that study, there was a gradual increase in the translation of the TIP rather than the depression in expression observed in both the *E. nindensis* and *E. curvula* δ -TIPs (Figures 4.7b, 4.9). The reason for this pattern of expression is unknown, but even though the magnitude of the increased presence in *E. nindensis* is significantly greater than that in *E. curvula*, the δ -TIPs in both species seem to respond to water stress in a similar manner and hence this phenomenon cannot be unique to resurrection plants. δ -TIPs characterised in other plants have been proposed to be associated with specialized storage vacuoles (Jauh *et al.*, 1999) are known to be water permeable aquaporins (Maurel *et al.*, 1995). The function of the δ -TIPs characterised in this study are still unknown, however it might be worth noting that the accumulation of anthocyanin during desiccation (Figure 2.7e) occurs in vacuoles other than those bounded by tonoplasts with the δ -TIPs seen in this study (data not shown).

In conclusion, it is clear from the pattern of expression of both the α - and δ -TIPs identified in the desiccation-tolerant leaves of *E. nindensis* that these TIPs both respond to desiccation and are thus likely to be involved in conferring desiccation-tolerance in this tissue. This is the first report of an α -TIP in vegetative tissue; it is unique to the desiccation-tolerant leaves at very low water contents and is likely to play a critical role in maintaining membrane integrity in the dry state, being associated with protein storage-like vacuoles. The peak in δ -TIP expression was at a similar water content to the unusual shape in the PV curve of *E. nindensis* (Figures 3.5 and 4.7) and thus it is possible that this

TIP may also contribute in some way to this phenomenon. However, the atypical shape of the PV curve was observed during both dehydration and rehydration (Chapter 3.3), whereas δ -TIP was not expressed at similar RWCs during rehydration. Consequently, that correlation does not hold.

Chapter 5:**Ultrastructural studies in desiccation tolerance:
fixation techniques and cellular observations****5.1 Introduction**

Ultrastructural evidence is an important tool in the understanding of desiccation tolerance. The ability to observe structural changes in tissues under water stress has contributed considerably towards confirming and explaining the physiological, biochemical and molecular processes that have been analysed during both dehydration and rehydration (Hallam 1976; Gaff, 1980; Platt *et al.*, 1997; Sherwin & Farrant, 1998 *inter alia*). Electron microscopy provides the means to examine tissues at an ultrastructural level.

The initial step in the traditional method of preparation for transmission electron microscopy (TEM) involves chemical fixation. There are however a number of artefacts which are proposed to be caused by this technique. Leaching of solutes and ions (Morgan 1980; Coetzee & van der Merwe, 1984; Gilkey & Staehelin, 1986) as well as changes in leaf morphology (Klein *et al.*, 1992) have been reported. Furthermore it is thought that oxygen dissolved in glutaraldehyde can cause cell and organelle swelling and membrane disruption (Gavin *et al.*, 1991). The primary cause of many of these artefacts is the long time (minutes/ hours) it takes chemical fixatives to penetrate and immobilise a specimen (Roos, 1991; Kiss & McDonald 1993) during which stage cellular activity and destruction can occur (Mersey & McCully, 1978). The most recent alternative to chemical fixation is physical fixation by cryoimmobilisation. Although this procedure is extremely rapid (milliseconds) and thus usually eliminates the development of the artefacts mentioned above, the prevention of ice damage on freezing presently limits the use of this technique. It is often necessary to impregnate hydrated biological tissues with sugars prior to ultra-rapid freezing, which has to be executed using either specialized high pressure freezing or jet propulsion equipment to obtain good results (Ding *et al.*, 1991; Parthasarathy, 1995; Ryan & Mangel, 1997).

Additional artefacts in the form of artificial rehydration of desiccated tissues during aqueous chemical fixation have hampered ultrastructural investigations of desiccation in both seeds (Swift & Buttrose, 1972; Buttrose, 1973) and vegetative tissues (Hallam & Luff, 1980a; 1980b; Hetherington *et al.*, 1982; Schneider *et al.*, 1993; Vicié *et al.*, 1999; Koonjul *et al.*, 2000). Attempts to ensure isotonic conditions between the chemical fixative with the tissue has reduced these artefacts in partially dehydrated tissues (Fellows & Boyer, 1978; Platt *et al.*, 1997), but it is not successful in totally desiccated tissues (Platt *et al.*, 1997). Anhydrous chemical fixation using osmium vapour of dry tissues has also been attempted with limited success (Hallam, 1976; Opik, 1980; Webb & Arnott, 1982; Goldsworthy & Drennan, 1991; Smith, 1991; Vicié, 2001). However, the absence of free water in desiccated tissues makes these specimens ideal candidates for cryofixation. Ice damage cannot occur without any free water in the tissue and consequently many of the precautions required for hydrated tissues are unnecessary (Tiwari *et al.*, 1990; Thompson & Platt, 1997; Wesley-Smith, 2001). The high concentration of sugars in the dry state of many resurrection plants (Scott, 2000) probably aids this process as well.

Following conventional chemical fixation, specimens are usually dehydrated with organic solvents, embedded in resin for sectioning and then stained with heavy metals (Hayat, 1989). After cryofixation, samples can be prepared using a number of low-temperature techniques: cryosectioning, freeze-fracturing or cryodehydrating (Quintana, 1994). Freeze-fracturing is considered to produce the most reliable ultrastructural preservation (Buttrose, 1973, Platt *et al.*, 1994) but desiccated samples often fragment extensively in irregular, unpredictable planes making the location of cells in these samples difficult (Fincher-Chabot & Leopold, 1982; Vigil *et al.*, 1985). Alternatively, freeze-substitution and resin embedding of cryofixed samples combines the convenience of conventional sectioning and viewing techniques with the advantages of the improved fixation (Parthasarathy, 1995). Freeze-substitution entails the replacement of solidified water in the sample by an anhydrous solvent at low temperatures at which ice crystal formation can be avoided. This technique has been used successfully in many hydrated plant tissues

(Mersey & McCully, 1978; Kiss *et al.*, 1990; Ding *et al.*, 1991; Davidson & Newcomb, 2000) and is recommended for immunocytological studies as one of best methods for sample preparation (Kiss & McDonald, 1993; Bendayan, 2001).

Cryofixation and freeze-substitution have been used successfully on desiccated plant tissues including pollen (Tiwari *et al.*, 1990) and a desiccation-tolerant spikemoss, *Selaginella lepidophylla* (Thomson & Platt, 1997). Not only is this technique highly suited to desiccated tissues but it circumvents the rehydration artefacts associated with aqueous chemical fixation (Thomson & Platt, 1997; Wesley-Smith, 2001). In this chapter, the development of cryofixation and freeze-substitution for partially and fully desiccated leaf tissues is discussed and compared with conventional methods. The morphological and ultrastructural changes during both dehydration and rehydration of the desiccation-tolerant inner leaves of *E. nindensis* and the sensitive leaves of *E. nindensis* and *E. curvula* are also presented and discussed in relation to the physiological and molecular aspects examined in the preceding three chapters.

5.2 Material and Methods

5.2.1 Plant material

E. nindensis and *E. curvula* plants were grown to maturity from seed in seedling flats as described previously (Chapter 2.2.1). Additional *E. nindensis* plants were also transplanted from two field sites, one in north-western South Africa and the other in southern Namibia (Chapter 4.2.1). All microscopical analyses were performed on leaf segments from *ca.* 2 cm distal to the leaf sheath from inner and outer leaves of *E. nindensis* and inner leaves of *E. curvula*. RWC of these leaf segments was calculated as the mean of the two leaf segments either side of that to be viewed. Measurement of RWC is described in Chapter 2.2.2. Samples were prepared for transmission electron microscopy using two different techniques: conventional fixation (CF) and cryofixation followed by freeze-substitution (FS).

Leaf samples were excised and prepared conventionally from hydrated plant tissues, as well as at regular intervals during both dehydration and rehydration in all leaf types. Only leaves which contained less than *ca.* 1.5 g H₂O. g dmass⁻¹ were prepared using freeze-substitution. These included fully desiccated tissues, as well as leaves in the later stages of dehydration and early stages of rehydration. Although hydrated plant tissue can be prepared using this technique (Parthasarathy, 1995), this requires specialized ultra-rapid freezing equipment which was not available. The environmental conditions as well as dehydration and rehydration regimes were kept consistent with those used in the previous experiments (Chapter 2.2.1, 3.2.1, 4.2.1) to avoid introducing additional variability.

5.2.2 Conventional fixation

Leaf segments (approx 2 mm²) were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and 0.5% caffeine according to the methods of Sherwin and Farrant (1996). Caffeine has been found to ensure better preservation of tissues containing phenolic compounds (Mueller & Greenwood, 1978) which includes resurrection plants. After post-fixation in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h, the leaf tissue was dehydrated using an ethanol gradient, infiltrated with epoxy resin (Spurr, 1969) over two days and polymerised for 16 h at 60°C. Sections (100 nm thick) were cut using a Reichart Ultracut S (Leica, Vienna, Austria), mounted on copper grids and stained sequentially with uranyl acetate and lead citrate (Reynolds, 1963) for 10 min each. A JEM 200CX transmission electron microscope (JEOL, Tokyo, Japan) was used to view the samples.

5.2.3 Cryofixation

Very small (*ca.* 0.5 mm²) leaf segments were mounted on nickel grids with a minimal amount of Tissue Tek adhesive (Miles Scientific, USA) and immediately plunge frozen into liquid propane (-185°C) using a Reichert KF8 cryofixation instrument (Leica, Vienna, Austria).

5.2.4 Freeze-substitution

Three different modifications to a freeze-substitution protocol for plants tissues (Ding *et al.*, 1991) were tried. The only difference between the first two protocols described below was the solvent that was used: methanol or acetone.

Cryofixed specimens were immersed in the precooled (-80°C) solvent (acetone/methanol) and transferred to a Leica EM AFS automatic freeze-substitution system (Leica, Vienna, Austria). Both solvents had been previously dried using a 3\AA pore size molecular sieve. The samples were then transferred to a precooled solution of 0.1% tannic acid in the anhydrous solvent where they remained at -80°C for 24 h. After the samples had been rinsed with the anhydrous solvent they were left in a precooled freeze-substitution solution containing 2% (w/v) anhydrous glutaraldehyde (Electron Microscopy Sciences, Fort Washington, USA), 2% (w/v) uranyl acetate and 2% (w/v) osmium tetroxide made up in the dry solvent, for 48 h at -80°C . Because the anhydrous glutaraldehyde was made up in methanol, the final solution contained at least 20% methanol. The temperature was then set to increase up to 20°C by 1°C every hour. The samples were then washed with the solvent and detached from the nickel grids before infiltrating with increasing concentrations of low viscosity epoxy resin (Spurr, 1969) over at least 5 days. The infiltrated specimens were embedded in the resin and polymerised at 60°C for 48 h. The long infiltration time of the low viscosity resin were necessary to ensure sufficient penetration of the specimens with resin. Since cryofixation beyond a depth of $40\ \mu\text{m}$ is poor (Parthasarathy, 1995), ultrathin sections (120- 150 nm) from the outermost parts of the embedded tissue were cut on a Reichart Ultracut S (Leica, Vienna, Austria) and mounted on Formvar-coated copper grids (300 mesh). Sections were briefly (less than 1 min) stained with 2% uranyl acetate and then lead citrate (Reynolds, 1963) and viewed with a JEM-200CX transmission electron microscope (JEOL, Tokyo, Japan).

Although methanol is thought of as a superior and more efficient freeze-substitution solvent (Steinbrecht & Muller, 1987; Thomson & Platt, 1997), the increase of the infiltration period and low viscosity of the resin eliminated many of the problems

associated with acetone as a solvent. In the dry leaf tissues freeze-substituted in this study, the quality of sections substituted in acetone was better than those in methanol (Figure 5.1). However, both these protocols produced very darkly stained sections (Figure 5.1) and thus a further modification to the original protocol to reduce the time the samples were exposed to osmium tetroxide was undertaken.

Anhydrous acetone was the solvent of choice in the third freeze-substitution protocol. This method commenced as before: cryofixed samples were transferred in acetone at -80°C to the automatic freeze-substitution system and left in the initial medium containing 0.1% tannic acid for 24h at -80°C . The freeze-substitution solution was prepared as previously described, but osmium tetroxide was not added to this medium. The freeze-substitution of samples using this osmium-free medium (48 h at -80°C followed by a 1°C rise per hour) was briefly interrupted for a 2 h period at -20°C . At that temperature, the samples were transferred to the original freeze-substitution solution containing 2% osmium tetroxide. Thereafter the samples were allowed to warm to room temperature in fresh freeze-substitution solution (without osmium) which had been precooled to -20°C . Specimens were infiltrated with low viscosity resin, sectioned, stained and viewed as described previously.

5.2.5 Image analysis

The number of cell wall fractures in fully desiccated tissues were counted using a grid overlay technique (Berjak *et al.*, 1992) in all three leaf types. At least 15 grid blocks from each of four different sections from at least two different specimens per sample were analysed.

The number of vacuoles/ cell, their size, volume, and the perimeter of the tonoplasts in each bundle sheath cell of desiccation-tolerant inner leaves of *E. nindensis* were measured at a few stages of dehydration and rehydration from electronic scans of transmission electron microscope images using AxioVision 2.05 image analysis software




Method	Representative image	Comment
1-Methanol + osmium		- Grainy image - Cytoplasm stained too darkly
2-Acetone + osmium		- Clear image - Cytoplasm stained too darkly
3-Acetone + brief osmium		- Clear image - Good contrast

Figure 5.1. Summary of three freeze-substitution protocols undertaken on desiccated leaf tissues of *E. nindensis* (inner leaves).

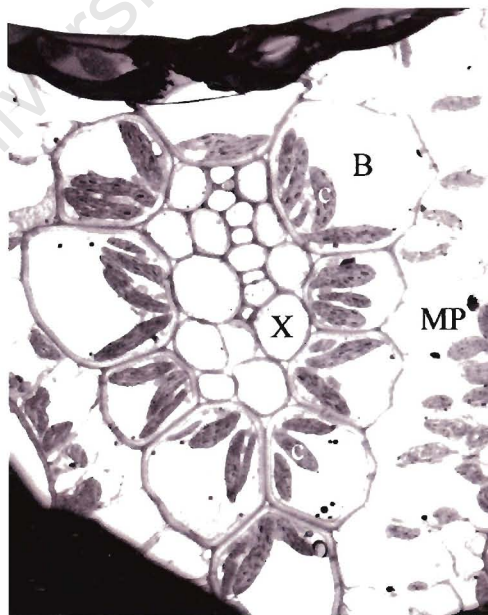


Figure 5.2. Transmission electron micrograph of a transverse section of a hydrated vascular bundle of *E. nindensis* (x 1 340). Chloroplasts (c) are centripetally arranged in the bundle sheath cells (BS) which surround the vascular tissues (x). Mesophyll cells (MP) border the vascular bundles.

(Carl Zeiss Vision GmbH, Hallbergmoos, Germany). These measurements were repeated on at least 10 bundle sheath cells from at least two different specimens at each water content. Statistical analyses were conducted with Statgraphics Plus software (Statistical Graphic Corporation, 1993). One-way analysis of variance (ANOVA) followed by Scheffé's multiple range tests at $p < 0.05$ were conducted to compare each parameter at different stages of dehydration and rehydration.

5.3 Results

As was shown in the light micrograph in Figure 2.9, grass leaves consist of numerous vascular bundles separated by mesophyll tissue. The vascular bundles of *E. nindensis* showed a typical Kranz arrangement with each bundle consisting of a ring of bundle sheath cells with centripetally arranged chloroplasts, enclosing the vascular tissue (Figures 2.9, 5.2). The arrangement of these vascular bundles was the same in both *E. nindensis* and *E. curvula* (Figure 2.9; Ellis, 1977). For the purpose of the ultrastructural studies, observations were predominantly confined to bundle sheath cells, the chloroplasts within them, and mesophyll tissue.

5.3.1 The hydrated state

Hydrated tissues were prepared using conventional fixation only. Hydrated tissues of inner and outer leaves of *E. nindensis* were indistinguishable (data not shown). The bundle sheath cells of *E. nindensis* and *E. curvula* were of a similar size, but the density of chloroplasts within them was higher in *E. curvula* than in *E. nindensis* (Figure 5.3a,b). The central vacuole was particularly obvious in *E. nindensis* bundle sheath cells (Figure 5.3a), with abundant active mitochondria present in these cells of both species (Figure 5.3a,b, c, d). Numerous large starch grains were characteristic of the chloroplasts of *E. curvula* (Figure 5.3b) with the chloroplasts of *E. nindensis* being considerably longer and narrower (compare Figure 5.3a,c with 5.3b,d). In both species, there were numerous thylakoids and conspicuous granal stacks (Figure 5.3c,d). The mesophyll cells in both species had large vacuoles with the organelles confined to the periphery (Figure 5.3e,f).

E. nindensis

E. curvula

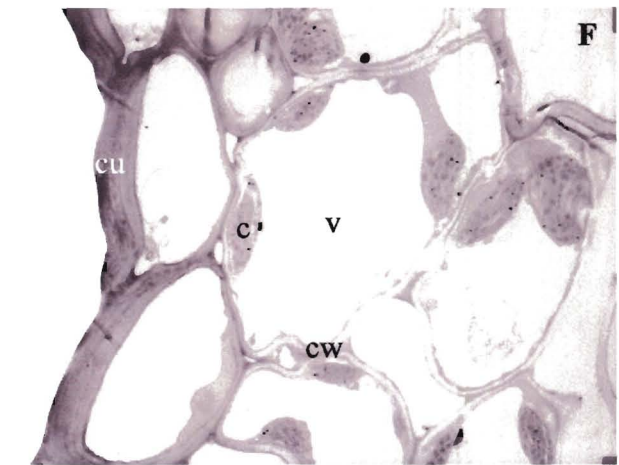
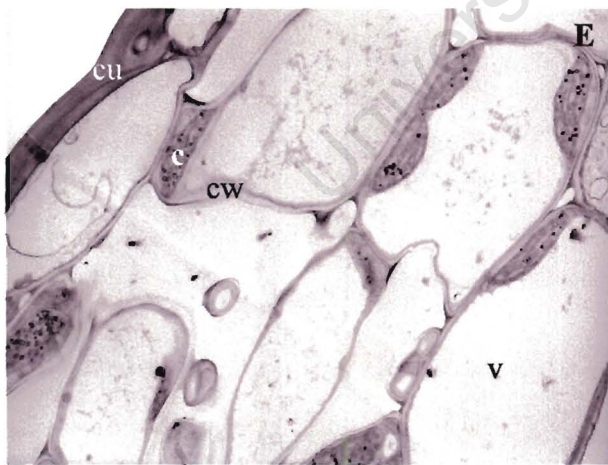
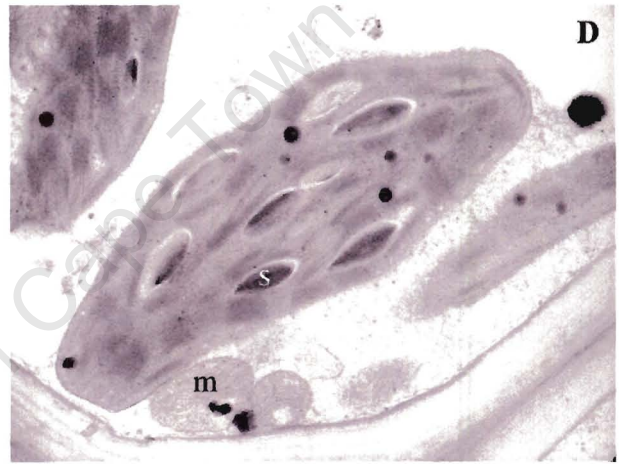
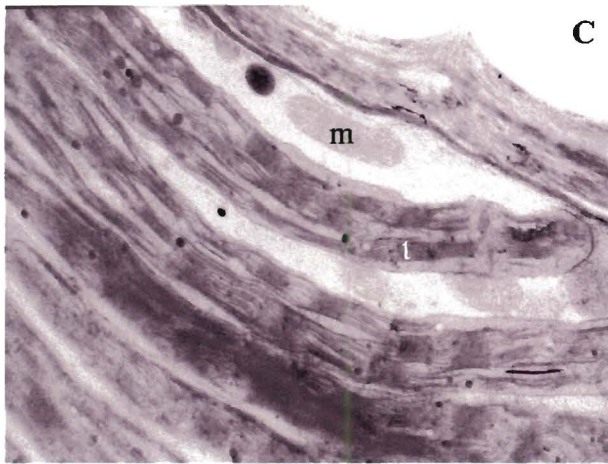
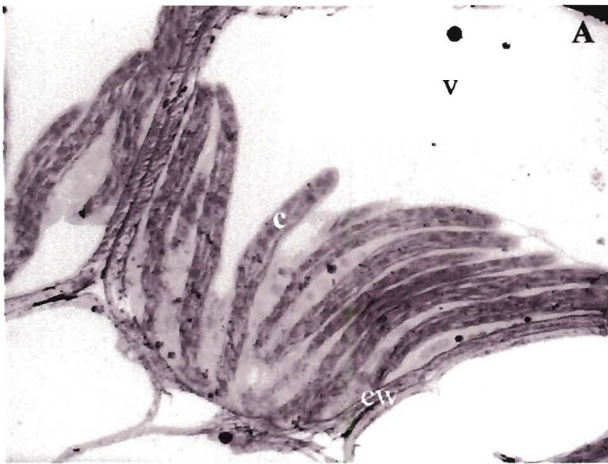


Figure 5.3. Transmission electron micrographs of hydrated leaf tissues in *E. nindensis* (a,c,e) and *E. curvula* (b,d,f). Micrographs (a) and (b) depict bundle sheath cells, (c) and (d) depict bundle sheath chloroplasts and (e) and (f) depict mesophyll tissue in *E. nindensis* and *E. curvula* respectively. Chloroplast (c); vacuole (v); cell wall (cw); mitochondrion (m); starch grain (s); cuticle (cu). Magnification: (a) x 3 200, (b) x 3 100, (c) x 24 600, (d) x 28 700, (e) x 5 300, (f) x 6 100.

The cell walls of these cells were considerably thinner than those of the bundle sheath cells (compare Figure 5.3e,f with a,b). Chloroplasts in mesophyll cells were smaller and less abundant than those in the bundle sheath cells with no difference in the appearance of the mesophyll chloroplasts in either species (Figure 5.35e,f). Mitochondria were present, but few starch grains were observed (Figure 5.3e,f). The cuticles in both species were *ca.* 1.4 nm thick. The cuticles from xeric plants tend to range from 1.5 to 2.5 nm thick (Jordaan & Kruger, 1998).

5.3.2 The dehydrated state

Dehydrated leaf tissues (RWC less than 30%, *ca.* 1.5 g. g dry mass⁻¹) of both *E. nindensis* and *E. curvula* were prepared using both conventional fixation and cryofixation and freeze-substitution. Of the three variations to the freeze-substitution technique that were attempted (section 5.2.4), the final method, in which acetone was used as the solvent and minimal osmium fixation was used, was chosen for ultrastructural assessment of dehydrated leaves.

a) Desiccation-sensitive leaves of E. curvula

There was a remarkable difference in the appearance of the desiccated inner leaves of *E. curvula* between the two fixation methods (compare Figure 5.4a,e with 5.4b,d,f). In most conventionally fixed bundle sheath cells, the plasma membrane seemed to be intact, having withdrawn from the cell wall (Figure 5.4a). However, no components of any of the cells were distinguishable in these sections (Figure 5.4a,e). Consequently no chloroplast-like structures could be photographed (Figure 5.4c).

In contrast, freeze-substitution revealed that only minimal cellular degradation occurred on dehydration of these tissues (Figure 5.4b,d,f). The bundle sheath cells were particularly well preserved with little apparent subcellular disorganization (Figure 5.4b). Although no mitochondria were found there were numerous chloroplasts with clearly defined thylakoid membranes showing no signs of disintegration (Figure 5.4b,d). Most damage was evident in the mesophyll cells, which had collapsed (Figure 5.4f). Only

E. curvula dehydrated

Conventional fixation

Freeze substitution

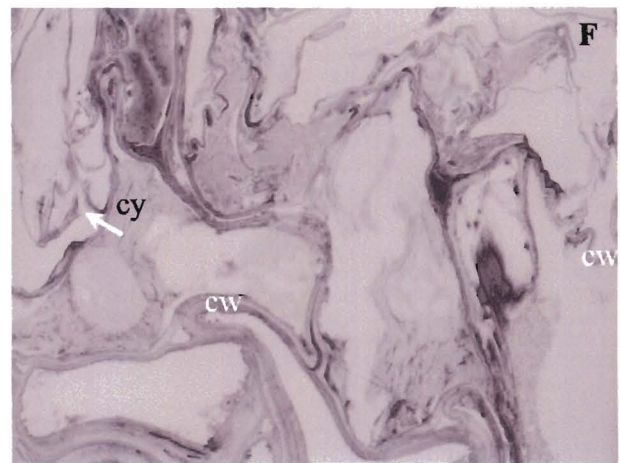
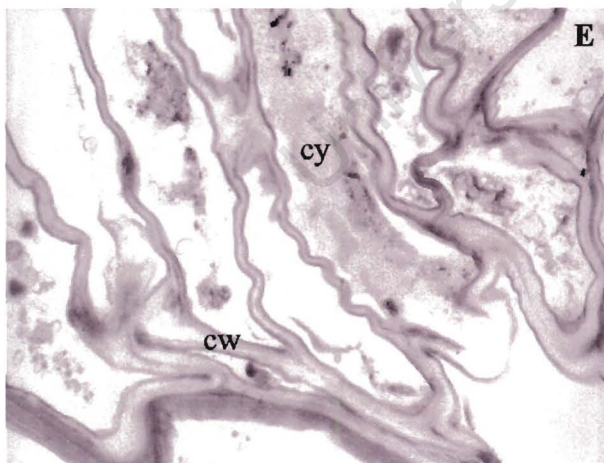
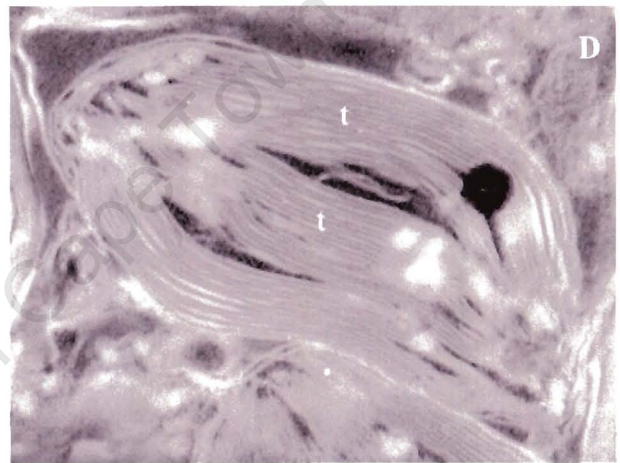
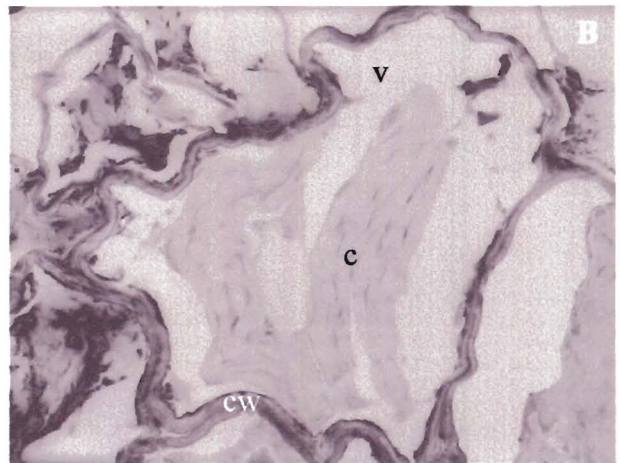
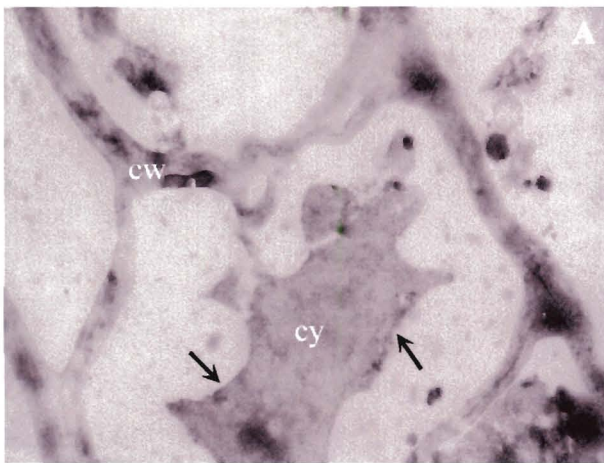


Figure 5.4. Transmission electron micrographs of dehydrated leaf tissues in *E. curvula* prepared by conventional fixation (a,e) and freeze-substitution (b,d,f). Micrographs (a) and (b) depict bundle sheath cells, (d) depicts a bundle sheath chloroplast (none distinguishable in conventionally fixed tissues i.e no image (c)) and (e) and (f) depict mesophyll tissues. Plasma membrane withdrawal (black arrows); plasma membrane break (white arrow) chloroplast (c); cytoplasm (cy); vacuole (v); cell wall (cw); thylakoids (t). Magnification: (a) x 3 900, (b) x 3 100, (d) x 31 000, (e) x 4 200, (f) x 5 200.

isolated organelles were distinguishable in these cells and plasma membranes were broken (Figure 5.4f). No fractures in the cell walls were observed in any of these tissues.

b) Desiccation-sensitive leaves of E. nindensis

There was little difference in the preservation between the conventionally fixed and freeze-substituted tissues of the dehydrated desiccation-sensitive outer leaves of *E. nindensis* because of the minimal cellular integrity of these tissues (compare Figure 5.5a,c,e with 5.5b,d,f). Since freeze-substituted samples with a RWC of *ca.* 27% showed similar damage to that of the desiccated tissues (data not shown), these tissues have probably been irreparably damaged during desiccation. Slightly more cellular detail was apparent in the freeze-substituted tissue, with segments of membranes and remnants of some organelles being distinguishable (Figure 5.5d). The most notable feature of these tissues using either preparation method was vast number of breaks in the cell walls (*ca.* 1 wall fracture/ 50 μm^2 (Figure 5.5e,f)). Since the various tissues were virtually unrecognisable, it was impossible to confirm whether the breaks were specific to a particular cell type. However, the only cells which were identifiable by their shape and size, were the bundle sheath cells and the walls of all these cells seemed to be intact (Figure 5.5a,b).

c) Desiccation-tolerant leaves of E. nindensis

The ultrastructure of air-dry desiccation-tolerant inner leaves of *E. nindensis* is depicted in Figure 5.6. There are a number of obvious rehydration artefacts in those micrographs taken from conventionally fixed material (Figure 5.6a,c,d). The plasma membranes had withdrawn from the cell walls in all cell types (Figure 5.6a,e). The chloroplasts were swollen and the number of thylakoids was diminished and had become blistered (Figure 5.6c). and the cell walls had become smooth with less folding being clearly evident (Figure 5.6a,e).

In contrast, the freeze-substituted sections of the same tissue showed no rehydration artefacts (Figure 5.6b,d,f). The walls of the bundle sheath cells were crenulated with the closely associated plasma membranes assuming the same shape as that of the walls.

E. nindensis (outer leaves) dehydrated

Conventional fixation

Freeze substitution

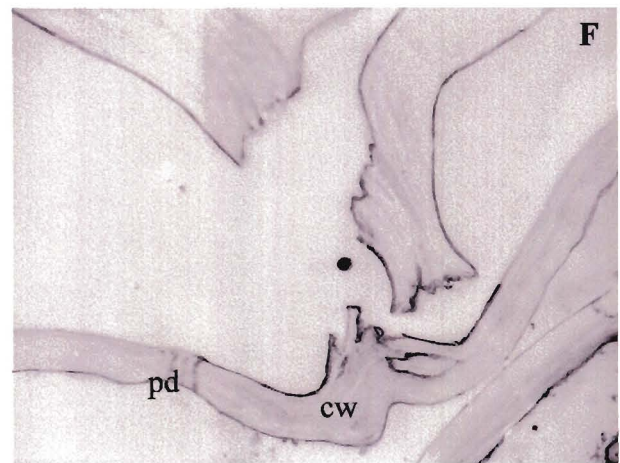
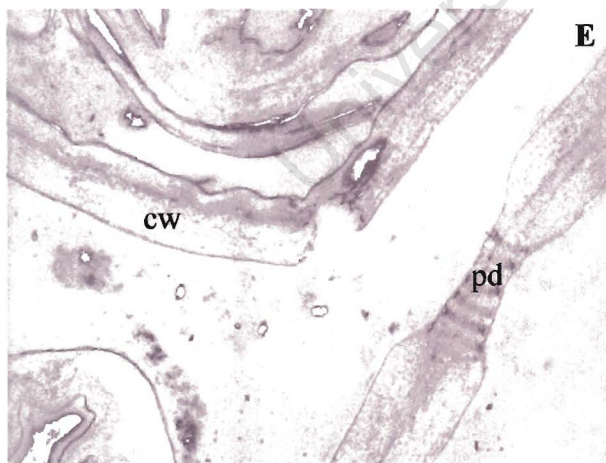
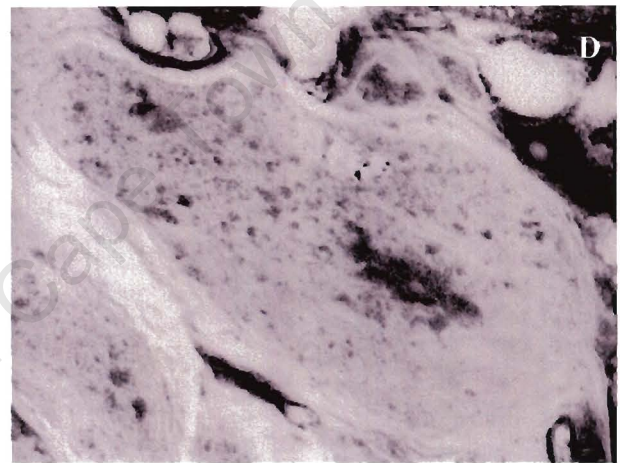
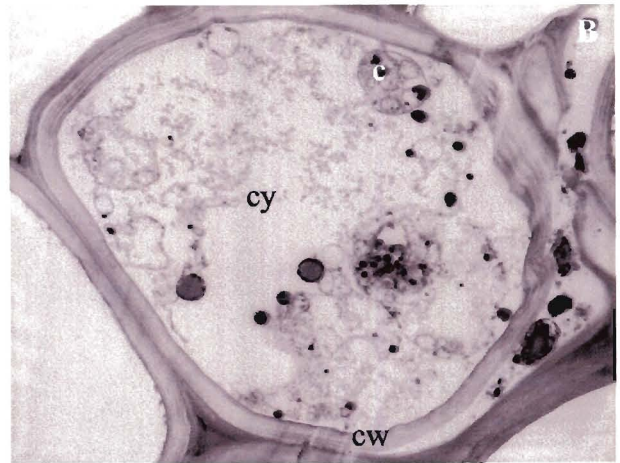


Figure 5.5. Transmission electron micrographs of dehydrated tissues from the outer desiccation-sensitive leaves of *E. nindensis* prepared by conventional fixation (a,e) and freeze-substitution (b,d,f). Micrographs (a) and (b) depict bundle sheath cells, (d) depicts a possible bundle sheath chloroplast (none distinguishable in conventionally fixed tissues i.e. no image (c)) and (e) and (f) depict mesophyll tissues. Chloroplast (c); cytoplasm (cy); cell wall (cw); plasmodesmum (pd). Magnification: (a) x 3 780, (b) x 3 400, (d) x 32 400, (e) x 14 500, (f) x 15 600.

E. nindensis (inner leaves) dehydrated

Conventional fixation

Freeze substitution

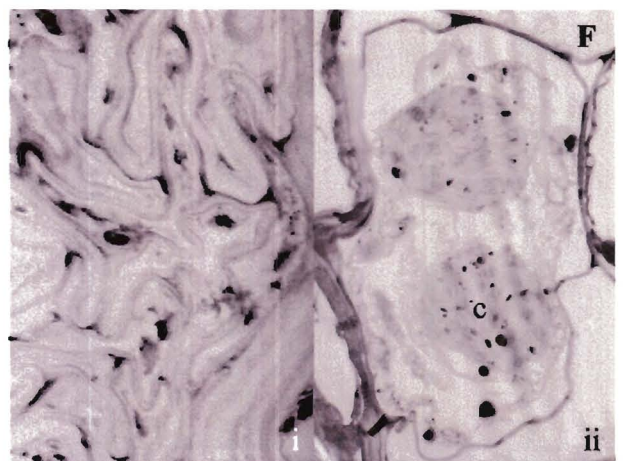
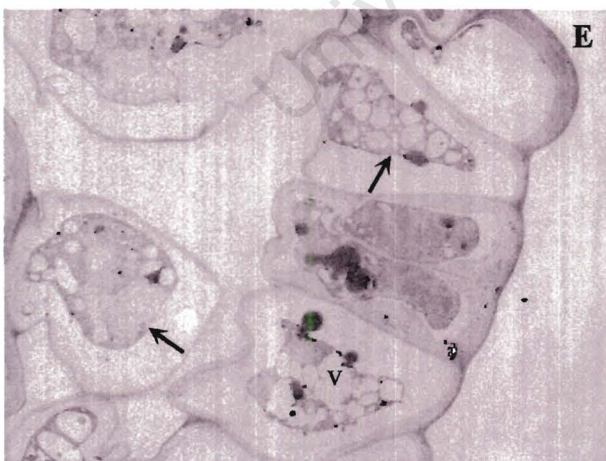
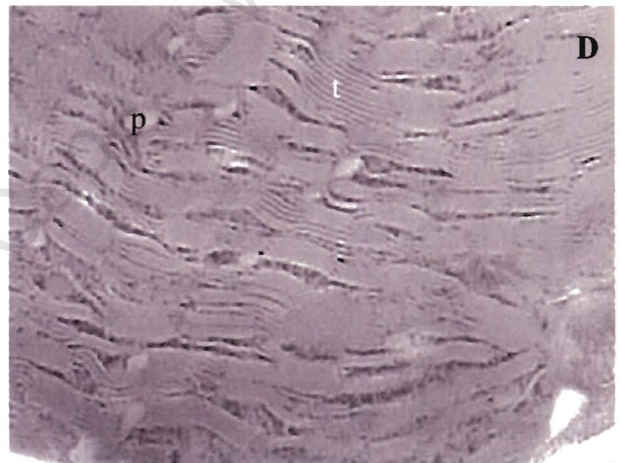
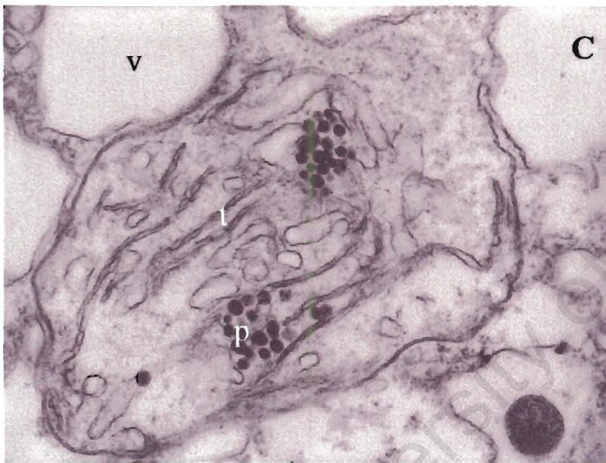
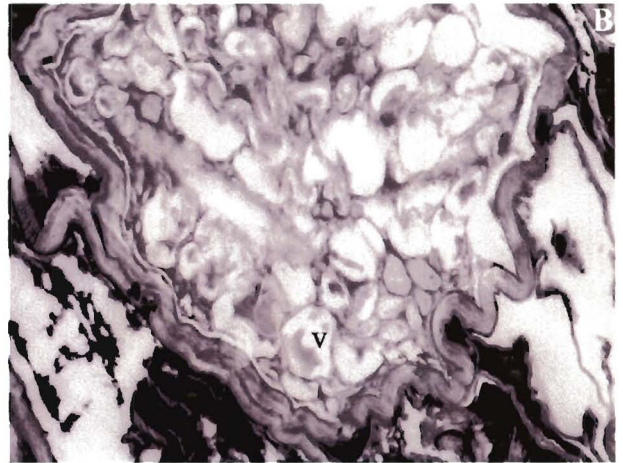
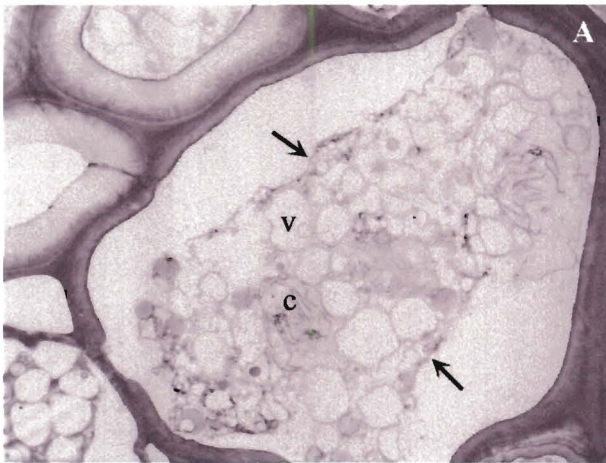


Figure 5.6. Transmission electron micrographs of dehydrated tissues from the inner desiccation-tolerant leaves of *E. nindensis* prepared by conventional fixation (a,c,e) and freeze-substitution (b,d,f). Micrographs (a) and (b) depict bundle sheath cells, (c) and (d) depict bundle sheath chloroplasts and (e) and (f) depict mesophyll tissues. plasmamembrane withdrawal (arrowed); chloroplast (c); thylakoid (t), plastoglobuli (p); vacuole (v). Magnification: (a) x 3 500, (b) 3 600, (c) x 33 400, (d) x 32 000, (f) x 4 100, (f - i) x 6 200, (f -ii) x 7 800.

These cells were densely packed with small vacuoles whose contents had different degrees of electron density (Figure 5.6b). The number and size of the chloroplasts per bundle sheath cell was visibly reduced compared with the hydrated state (Figure 5.3b), but the thylakoids and granal stacks were still intact (Figure 5.6d). Numerous translucent plastoglobuli were evident. The cell volume of the mesophyll cells was much reduced compared with the hydrated state and extensive cell wall folding was observed (Figure 5.6f). The cytoplasm had become compacted. Although the central vacuole (Figure 5.3 a,b) had reduced in size during desiccation, numerous small vacuoles, as occurred in the bundle sheath cells (Figure 5.6a,b), were not apparent (Figure 5.6f). A small number of chloroplasts were observed in the mesophyll cells; these had become rounded with some thylakoid dismantling being evident (Figure 5.6f ii).

5.3.3 Intermediate water contents

Extensive ice damage was evident in partially hydrated leaf samples containing more than *ca.* 1.5 g.g dry mass⁻¹ (*ca.* 30% RWC in all leaves) using the plunge freezing method of cryofixation (data not shown). Consequently only the later stages of desiccation and the early stages of rehydration were intensively studied using this method.

The freeze-substituted sections of partially dehydrated (*ca.* 30% RWC) outer leaves of *E. nindensis* showed similar damage and cell wall breakages to that depicted in the dry state (Figure 5.5). Further investigation of this tissue was not pursued.

A RWC of 32% is beyond the lethal water stress limit for *E. curvula* (discussed in Chapter 2, section 2.3.1). At that point during dehydration, that leaf tissue appeared as it did in the dry state (Figure 5.4): numerous chloroplasts were present and intact in the bundle sheath cells, although no starch was visible (Figure 5.7a) but some membrane disruption was evident particularly in the mesophyll (Figure 5.7a). Freeze-substituted samples of *E. curvula* which had been rehydrated for 6 h revealed considerable subcellular damage. No organelles or membrane structures could be distinguished (Figure 5.7b).

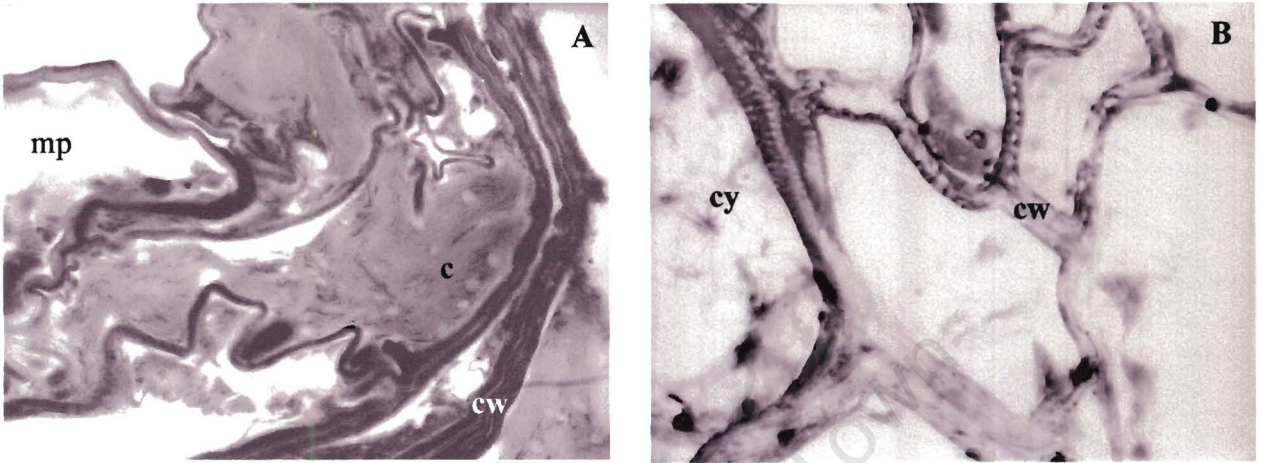


Figure 5.7. Transmission electron micrographs of tissues from inner desiccation-sensitive leaves of *E. curvula* prepared by freeze-substitution; (a) dehydrated bundle sheath cell at 32% RWC (x 3 400) and (b) after 6 h rehydration (3 900). Chloroplast (c), Cytoplasm (cy); cell wall (cw); mesophyll cell (mp).

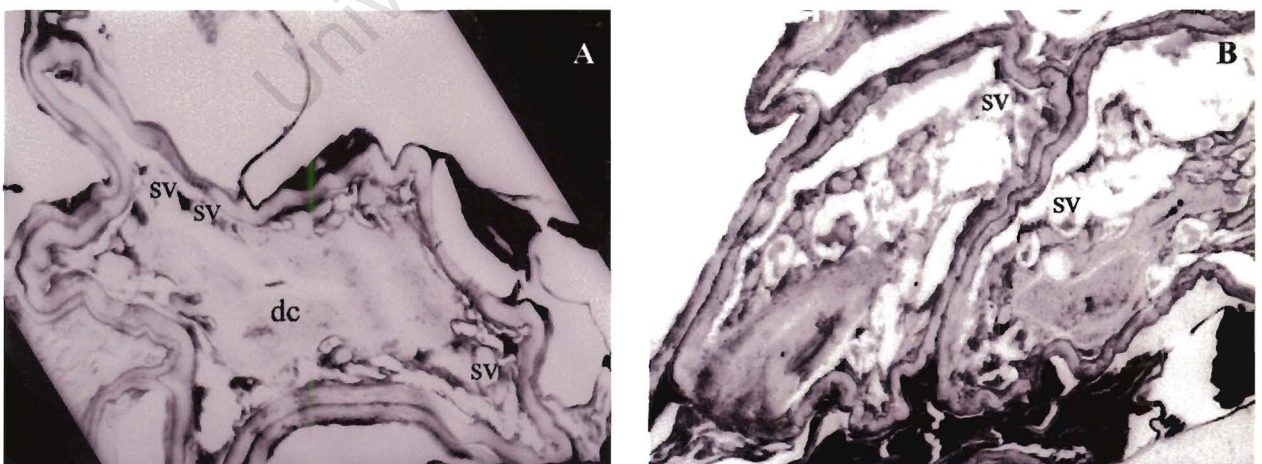


Figure 5.8. Transmission electron micrographs of a bundle sheath cell from (a) dehydrated (26% RWC, x 2 800) and (b) rehydrated tissue (15% RWC, x 3 200) of inner desiccation-tolerant *E. nindensis* leaves prepared by freeze-substitution. Small vacuole (sv); dismantled chloroplast (dc).

Cell wall folding in the mesophyll of partially (26% RWC) dehydrated desiccation-tolerant leaves of *E. nindensis* was evident (Figure 5.8). Although the bundle sheath cells of this tissue were of a similar shape to those at 5% RWC, the size of the large central vacuole had started to diminish and a number of smaller vacuoles were evident (compare Figure 5.6a with Figure 5.8a). It is unclear whether this change in the size and number of the vacuoles

was due to fragmentation of the central vacuole or *de novo* synthesis. However, although vacuolar volume of the bundle sheath cells did not change during desiccation, the amount of tonoplast increased (Table 5.1). After a 6 h rehydration period, the number of vacuoles present had reduced by 66% compared with the number in the dry state (Figure 5.8b, Table 5.1). Cell wall folding was also less pronounced (Figure 5.8b).

Table 5.1 Characteristics of vacuoles of the bundle sheath cells of the desiccation-tolerant inner leaves of *E. nindensis* during dehydration and rehydration. Standard deviations given in parentheses. Letters represent the mean separation by Scheffe's multiple range tests, showing comparisons between each parameter independently ($p < 0.05$, $n = 20-25$)

RWC	98%	26%	4%	15%
Hydration state, fixation method	Hydrated, CF	Partially dehydrated, FS	Dehydrated, FS	Partially rehydrated, FS
Number of vacuoles/ cell	1 (0) a	17.4 (2.3) b	67.3 (3.1) c	23 (6.2) b
Ratio of large: small vacuoles	1:0	1: 15.4	0: 67.3	1: 21.7
Total vacuolar volume ($\mu\text{m}^2/\text{cell}$)	38.4 (2.5) a	36.9 (3.1) a	33.8 (2.7) a	34.8 (3.5) a
Total length of tonoplast ($\mu\text{m}/\text{cell}$)	74.43 (5.34) a	82.57 (6.31) a	125.32 (13.5) b	94.51 (8.64) ab

CF – conventional fixation, FS – cryofixation and freeze-substitution.

5.4 Discussion

5.4.1 Conventional fixation versus cryofixation and freeze-substitution

Freeze-substitution is regarded by many as a considerable improvement in TEM sample preparation (Roos, 1991; Parthasarathy, 1995; Kiss and McDonald, 1993 *inter alia*). This study confirms that this is undoubtedly the case in the analysis of partially or fully desiccated tissues. Blatant rehydration artefacts of aqueous conventional fixation can distort cellular structure significantly. Cell walls tend to imbibe the aqueous fixatives more rapidly than the cytoplasm, becoming less convoluted while the plasma membrane and cytoplasm are cross-linked by the fixative more quickly and thus maintain a volume similar to that at the point of fixation (Figures 5.4a,e, 5.6a,e; Gaff, 1980; Hetherington *et al.*, 1982; Wesley-Smith, 2001). In desiccation-sensitive tissues, it is impossible to distinguish between injury caused as a result of dehydration and that incurred during the influx of water on rehydration (Figures 5.4, 5.5). It could be tempting to consider conventionally fixed water-stressed tissues as if they had been briefly rehydrated at the time of fixation. However, the property of aqueous chemical fixatives to immobilise some cellular structures (protoplasm) more so than others (cell walls) means that this analogy poorly represents this process.

5.4.2 Ultrastructural evidence of damage in desiccation-sensitive tissues

The physiological evidence presented in Chapter 2 illustrated that neither the outer leaves of *E. nindensis* nor any of the leaves of *E. curvula* survive desiccation. Membrane integrity, assessed as a measure of electrolyte leakage on immersion in water (Figure 2.4), identified the RWC at which extensive membrane damage was measured. However it is only with the ultrastructural evidence using freeze-substitution, that it is possible to determine that the desiccation-induced damage in the outer leaves of *E. nindensis* is incurred as water is lost from the tissues, whereas the irreparable damage in *E. curvula* occurs on rehydration (Figures 5.4, 5.5). Since cellular organisation is maintained during dehydration in *E. curvula*, it is probably the inability of these tissues to prevent and/ or

repair membrane damage on rehydration which is one of the features which excludes this species from surviving desiccation.

Although cell wall fractures are often considered to be an artefact of the fixation procedure (Hayat, 1989), the consistency of this phenomenon in the outer leaves of *E. nindensis* using two independent fixation procedures (Figure 5.5d,e) but not in any of the other desiccated tissues prepared and examined in this study, suggests that even if this damage is artefactual, there must be some inherent weakness in the walls of these cells. The regulated cell wall folding observed during the dehydration of numerous resurrection plants is thought to be important in attaining tolerance in those species (Vicré *et al.*, 1999; Farrant, 2000). The cell wall defect in the outer leaves of *E. nindensis* may be contributory to the desiccation sensitivity of these leaves. Furthermore, if turgor is lost on desiccation of these cells as a result of cell wall damage, the atypically-shaped PV curves associated with the desiccation-tolerant inner leaves (Figure 3.2) would not be possible in these leaves (Figure 3.2). The PV curve of the outer desiccation-sensitive leaves of this species are of a usual shape (Figure 3.2) and hence this structural evidence (Figure 5.5e,f) thus strengthens the theory presented in Chapter 3. It is possible that the tension (negative turgor) caused by the apparent inability of these cell walls to fold on drying was contributory to the demise of these leaves.

5.4.3 Ultrastructural analysis of desiccation tolerance in *E. nindensis*

In the hydrated state, the leaf tissues of *E. nindensis* appear remarkably similar to those of *E. curvula* (Figure 5.2). However, on dehydration a number of ultrastructural changes were observed in the inner leaves of *E. nindensis* which are proposed to be involved in conferring desiccation tolerance in these leaves (Figures 5.6, 5.7, Table 5.1).

Cell wall folding was observed during dehydration from a RWC of *ca.* 60%. This corresponded to the significant reduction in cell volume illustrated in Figure 3.5, which is proposed to be associated with the unusual shaped PV curves of these leaves (discussed in Chapter 3), and is likely to reduce the mechanical strain associated with water loss. As

in another resurrection plant, *Myrothamnus flabellifolius*, cell wall folding was not uniform across cell types (Figure 5.6a,b,e,f; Farrant, 2000). In the current study, wall folding was predominantly confined to the thinner-walled mesophyll cells with no wall folding being observed in the xylem and sclerophyllous tissues. Minimal cell wall folding was observed in the thicker-walled bundle sheath cells.

The maintenance of the shape and volume of the bundle sheath cells is possibly important in the desiccation tolerance of this tissue. These cells are likely to be the most photosynthetically and metabolically active (Figure 5.3ab) and probably play an important role in the protection of these leaves against desiccation-induced damage as well as the resumption of metabolic activity on rehydration. Although *E. nindensis* loses chlorophyll on dehydration (Figure 2.7) and is proposed to be poikilochlorophyllous, some of the thylakoids in chloroplasts of the bundle sheath cells are not dismantled on dehydration (Figure 5.6a-d). Most resurrection grasses are homoiochlorophyllous and *Sporobolus stapfianus* is intermediary retaining some chlorophyll and chloroplast structure (Gaff & Ellis, 1974; Dalla Vecchia *et al.*, 1998). It is thus probable that the closely related *E. nindensis* would adopt a similar approach. A number of features associated with protection against light-induced damage in homoiochlorophyllous resurrection plants have been observed in *E. nindensis*. Anthocyanin accumulates (Figure 2.7) and leaf rolling (Figure 2.4) occurs on drying. Detached inner leaves of *E. nindensis* are desiccation sensitive and do not lose chlorophyll on drying (personal observation) which suggests that, as in *S. stapfianus* (Gaff & Loveys, 1984a), this phenomenon may require some feature only present if the leaves are attached to the rest of the plant.

Compatible solutes and sugars are thought to replace water lost on dehydration in order to maintain cell structure and membrane integrity (Crowe *et al.*, 1987; Farrant & Sherwin, 1998; Whittaker *et al.*, 2001). Sugars (Ghasempour *et al.*, 1998a) and proline (Tymms & Gaff, 1979) are known to accumulate in *E. nindensis* on drying. Many of these compounds are thought to accumulate in vacuoles (Farrant, 2000) and as such retain cell volume and shape (Farrant & Sherwin, 1998; Pammenter & Berjak, 1999). Although the central vacuole in the mesophyll cells was retained on drying, it was reduced in volume

concomitant with the extensive cell wall folding observed in these cells (Figure 5.6c,d). The bundle sheath cells tend to retain their shape on drying (Figure 5.6b). In these cells, the central vacuole maintained its original volume until less than *ca.* 26% RWC, by which time cell wall folding (Figure 5.6) and cell volume reductions (Figure 3.5) would have already occurred. However, only after this point is the formation of abundant small vacuoles observed (Figures 5.6a,b, 5.8a, Table 5.1). Numerous small vacuoles have been observed during the desiccation of several other resurrection plants (Gaff *et al.*, 1976; Quartacci *et al.*, 1997; Dalla Vecchia *et al.*, 1998) as well as in water-stressed desiccation-sensitive tissues (Barrieu *et al.*, 1999). Since changes in cell volume and shape would have occurred prior to the formation of these small vacuoles in *E. nindensis*, it can be deduced that their formation does not affect the retention of cell structure during dehydration. However, the maintenance of total vacuolar volume during this process may be critical to the preservation of cell shape (Table 5.1).

It is presently unclear whether these vacuoles are formed *de novo* or from the subdivision of the central vacuole. However, since there is an increase in the amount of vacuolar membrane in the desiccated bundle sheath cells of *E. nindensis* compared with that in the hydrated state (Table 5.1), some synthesis must occur. The expression of a Rab protein (associated with vesicle trafficking) during desiccation of *Sporobolus stapfianus* (O'Mahony & Oliver, 1999) was suggested to show a stimulation of endomembrane synthesis during dehydration of this resurrection grass. A similar process may be involved in the formation of small vacuoles of the bundle sheath cells of the desiccation-tolerant leaves of *E. nindensis*.

The presence of small vacuoles in the bundle sheath cells of the *E. nindensis* desiccation-tolerant leaves corresponds with the expression and immunolocalization of α -TIP in these tissues (Figures 4.4, 5.6a,b, 5.8). It is thus very likely that these proteins are expressed in the tonoplasts of these vacuoles. Just as the purpose of the formation of the vacuoles in the desiccated state is unclear, so is the association of the α -TIP with them. Even though it is not known what substances fill these vacuoles, it is possible that as in the protein bodies found in dry seeds (Mettler & Beevers, 1979; van der Wilden *et al.*, 1980;

Pernollet, 1985), these vacuoles may store compounds necessary for the resumption of metabolism on rehydration. Their presence in the bundle sheath cells, which are usually the most metabolically active, supports this hypothesis. α -TIP might then be involved in the release of these compounds on rehydration. Alternatively they may play a role in defining the fate and function of these vacuoles and possibly also the reformation of central and other vacuoles during rehydration, similar to that which has been proposed by Jiang and Rogers (1998) and Jauh *et al.* (1999). This was discussed previously (Chapter 4.4).

Chapter 6:

General overview and conclusion

6.1 Discussion

The purpose of this investigation was to characterise some physiological and biochemical/molecular aspects of the desiccation tolerance of one of the less well studied resurrection angiosperms, *E. nindensis*, in order to broaden the knowledge of this phenomenon.

In Chapter 1 the various stresses associated with desiccation as well as the wide variety of mechanisms various resurrection plants employ to prevent or recover from them, were reviewed. Although this study has focussed predominately on the physical stresses associated with plant water relations during desiccation, and considered only a few aspects within these, the data confirm that desiccation tolerance is a complex phenomenon which can be explained neither by a few traits nor a single strategy.

An overview of the physiology of *E. nindensis* and *E. curvula* during desiccation and rehydration, presented in chapter 2, confirmed that *E. nindensis* is desiccation tolerant whereas although *E. curvula* is relatively drought tolerant, this species cannot revive from the air dry state (Figures 2.2, 2.7). The differences between these two closely related species enabled a useful comparison between drought and desiccation tolerance. Furthermore, the observation that the outer leaves of *E. nindensis* were desiccation sensitive offered a further evaluation of the mechanisms necessary to confer desiccation tolerance on the same plant (*E. nindensis*) under natural conditions.

In poikilochlorophyllous resurrection plants, chlorophyll is lost during dehydration and the resumption of photosynthesis during rehydration is consequently delayed (Gaff, 1977; Bewley, 1979). Although both these characteristics were recorded in the inner desiccation-tolerant leaves of *E. nindensis*, (Figures 2.6a, 2.7f), a number of bundle sheath chloroplasts with well developed thylakoids were observed in desiccated samples

of these leaves (Figure 5.6). Chloroplast dismantling is a common feature of most other poikilochlorophyllous plants, a consequence of which is thought to be a reduction in the formation of free radicals by photosynthetic processes during water stress (Bewley, 1979; Hetherington & Smillie, 1982). *E. nindensis* displayed other characteristics which are often associated with homiochlorophylly: the accumulation of anthocyanin on drying (Figure 2.6e) and leaf rolling (Figure 2.4) both of which are thought to shade chlorophyll from light and so reduce light-associated free radical formation (Bewley, 1979; Farrant, 2000). Although most monocotyledonous resurrection plants are poikilochlorophyllous (Hartung *et al.*, 1998), amongst the resurrection grasses poikilochlorophylly is unique to *E. nindensis*, the others being intermediate (retaining some of their chlorophyll eg. *Sporobolus stapfianus*; Quartacci *et al.*, 1997) or homiochlorophyllous (eg. *Colechloa setifera* (Gaff & Ellis, 1974). Since *E. nindensis* is probably most closely related to the other resurrection grasses than to any of the other desiccation-tolerant angiosperms, the data suggest a slight evolutionary deviation in the strategy of this resurrection grass to prevent light-associated free radical formation on drying compared with the other resurrection grasses. It also highlights that at least in this group of desiccation-tolerant plants there is no clear distinction between homiochlorophylly and poikilochlorophylly but rather a continuum, from the one approach to the other.

The physiology of the desiccation-sensitive leaves (*E. nindensis* outer and *E. curvula*) in this study contrasted with each other: *E. curvula* leaves retained chlorophyll and continued to photosynthesise until the lethal water content limit for this species of 40% RWC (Figures 2.6a, 2.7e) whereas the outer leaves of *E. nindensis* started to degrade their chlorophyll and reduce CO₂ assimilation during the early stages of dehydration similar to that observed in the desiccation-tolerant leaves of this species (Figures 2.6a, 2.7e). The expression in sensitive outer leaves of *E. nindensis* of one of the proteins characterised later in this study (δ -TIP) also differed from that of *E. curvula* (Figures 4.7, 4.8), with no further expression of this protein in the outer leaves of *E. nindensis* once dehydration began but continued expression in *E. curvula*. Ultrastructural studies highlighted further differences between these two desiccation-sensitive leaf types. Freeze-substituted samples of these leaves in the desiccated state revealed that

desiccation-induced damage was incurred during dehydration of the outer of leaves of *E. nindensis* (Figure 5.4) whereas the damage observed in *E. curvula* was apparent only on rehydration (Figure 5.5). As with the phenomenon of desiccation tolerance, these data suggest differences in the cause of sensitivity among species. Outer leaves of *E. nindensis* appear to have initiated some of the characteristics observed in the desiccation-tolerant leaves, but are, none-the-less sensitive to desiccation. Although *E. curvula* continues metabolism until water is no longer able to sustain it and none of the mechanisms thought to add to the protection against desiccation-induced damage were observed during the dehydration experiments undertaken in this study, this species does show the ability to recover (possibly involving some repair) from moderate water stresses (Chapter 2.3.1).

There are possibly some common features between the drought tolerance of *E. curvula* and the desiccation tolerance of *E. nindensis* (eg. expression of δ -TIP during dehydration, Figure 4.7). Similarities such as this suggest that there may be a few common traits between drought and desiccation tolerance (Figure 1.1). I would suspect that if this is true, these two strategies are more likely to share common repair processes once water is replenished. However, although the two species used in this study would serve as a useful model for such investigations, this was not the focus of this study.

It is possible that some of the strategies associated with desiccation tolerance in the inner leaves of *E. nindensis* are initiated during the early stages of dehydration of the outer leaves of this species. However, the irreparable damage incurred by these leaves during dehydration (particularly in the cell walls) possibly prevents them from continuing and/or executing the processes necessary to protect themselves against the stresses associated with desiccation and rehydration. An increase in electrolyte leakage was observed from ca. 50 RWC (Figure 2.4), the water content after which no further regulated cellular activity is possible. The absence of the two TIPs in the outer leaves of *E. nindensis*, expressed during dehydration of the desiccation-tolerant inner leaves of this species (Figures 4.5, 4.7) is one such example.

To date, the water relations of resurrection plants have not been well characterised. The relationship between the negative inverse of water potential and RWC, referred to as a PV curve, was found to be particularly interesting in this unique group of plants. Atypical curves were observed in the desiccation-tolerant inner leaves of *E. nindensis* (Figure 3.2). This phenomenon was also observed in some but not other resurrection plants (Figures 3.3 to 3.5; Sherwin, 1995; Beckett, 1997). Beckett (1997) proposed that negative turgor developed during dehydration of the plants which had these anomalous curves. However, direct measurements of water activity in this study suggest this is not the case (Figures 3.3, 3.5). There is a dramatic reduction in cell volume as consequence of extensive cell wall folding in the resurrection angiosperms known to produce these unusual PV curves (*E. nindensis*, *C. wilmsii*, *C. nanum* and *M. flabellifolius*) (Figures 3.3, 3.5, 3.6, 5.6, Sherwin, 1995; Farrant, 2000). In the *Craterostigma* species this wall folding is pronounced in all cells types however, in *E. nindensis* (inner leaves) the cell walls of the mesophyll fold considerably (Figure 5.6f) whereas those of the bundle sheath cells and vascular tissues do not (Figure 5.6b, 5.7, 5.8). This distinction between the degree of cell wall folding has been observed in other resurrection plants (*M. flabellifolius*, Farrant, 2000; *X. villosa*, Hallam & Luff, 1980b). It is suggested that the reduction in the cell volume in some or all of their cells of these plants prevents negative turgor from developing, minimising the physical stress on the protoplasm during the absence of water. The considerable cell wall fracturing observed in the outer desiccation-sensitive leaves of *E. nindensis*, which produce normal PV curves (Figure 3.2), supports the suggestion that the anomalous PV curves are a consequence of wall folding, a feature that contributes to desiccation tolerance by avoiding mechanical stresses associated with dehydration.

The change in the number and size of vacuoles observed during the dehydration of the desiccation tolerant inner leaves of *E. nindensis* (Figures 5.6, 5.7, 5.8) has also been reported in other resurrection plants (*X. villosa*, Gaff *et al.*, 1976; *Sporobolus stapfianus*, Quartacci *et al.* 1997; *X. viscosa*, Mundree & Farrant, 2000). Protein storage vacuoles are abundant in orthodox seeds where they are proposed to provide a source of protein and energy to the embryo on germination (Mettler & Beaver, 1979; Pernollet, 1985) but the

purpose of these small vacuoles in desiccated vegetative tissues is unknown. They may well serve a number of possible functions: (i) providing energy required to initiate metabolism on rehydration. This may be particularly important in poikilochlorophyllous resurrection plants such as *E. nindensis* which cannot resume photosynthesis until some time after rehydration occurs (Figure 2.6). However, although they are not as abundant similar small vacuoles are also observed in some homoiochlorophyllous species as well (eg. *Craterostigma wilmsii*, Cooper, 2001). These vacuoles could also be involved in (ii) maintaining cell volume and integrity to prevent mechanical stress in the dry state and (iii) acting as osmolyte stores to control osmotic and water potentials during dehydration and rehydration. The association of α -TIP, a tonoplast intrinsic protein exclusively found in the tonoplasts of protein storage vacuoles of seeds (Jauh *et al.*, 1999), with the vacuoles in *E. nindensis* (Figure 4.6) is not only the first account of this protein in vegetative tissues but also strengthens the suggestion that the small vacuoles in the desiccated bundle sheath cells of *E. nindensis* are storage vacuoles.

Preliminary investigations into the accumulation of proline during desiccation have revealed a 33 fold increase in the desiccation-tolerant leaves of *E. nindensis* but only a 5 fold increase in the desiccation-sensitive outer leaves of this species (measured according to the methods of Magne & Larher, 1992) Proline accumulation during water stress is widely reported (Henckel, 1964; Tymms & Gaff, 1979; Gaff, 1980, Bandurska, 2000) but it not been established where in the tissue it accumulates. Since it has been suggested that aquaporins may be permeable to small compatible solutes such as proline (Tyerman *et al.*, 1999), it is possible that the α -TIP characterised in *E. nindensis* is permeable to proline and the accumulation of this compound occurs in the small vacuoles found in the desiccated bundle sheath cells of *E. nindensis* inner leaves.

The α -TIP characterised in this study is observed only in the very late stages of dehydration and early stages of rehydration of *E. nindensis* inner leaves and hence is unlikely to play a major role in transmembrane water movement. However, δ -TIP, is found to be differentially expressed during dehydration of both *E. nindensis* and *E.*

curvula (Figures 4.7, 4.8), although the role of this proposed aquaporin in water stress is unknown.

6.1.1 Future research

As with many scientific studies, the results obtained to answer the original question generate several more questions in turn.

A number of these further studies suggested by the original investigation described in this thesis are already under way:

- Since the cell walls of the three leaf types used in this study varied from being brittle (*E. nindensis* outer leaves) to remarkably flexible (*E. nindensis* inner leaves), and the manner in which the leaves curled on desiccation differed, the elastic modulus, tensile strength and strain failure of the leaves at different water contents is currently being measured. This data will also compliment the unusual PV curves from which bulk moduli of elasticity could not be calculated.
- It is necessary to analyse the TIPs characterised in this study (Chapter 4) further in order to determine whether they are aquaporins (i.e. permeable to water) and what their role in desiccation tolerance might be. To this end, the genes of the α - and δ -TIPs identified in Chapter 4 of this study are being isolated from *E. nindensis* and Northern membranes have been prepared to characterise the expression of these genes. Thereafter further studies as to the purpose of these TIPs are possible. It will be particularly interesting to discover whether the same or different α -TIP isoforms are present in the dry desiccation-tolerant seeds as where found in the desiccated tolerant leaf tissues of *E. nindensis*. In addition, the small vacuoles observed in the desiccated inner leaves of *E. nindensis* are being isolated and their contents analysed biochemically.
- A cDNA expression library of the genes expressed during the dehydration of *E. nindensis* was constructed during the course of this research. This will be screened for

genes which are differentially expressed during desiccation using microarray technology. The isolation of any genes which may confer increased drought tolerance to *E. curvula* as well as other grass-like crops could then be transformed for further evaluation.

In addition to these, a number of other specific projects which I have not begun, are suggested:

- The development of freeze-substitution as the preferred preparation method of desiccated tissues for ultrastructural examination has provided an invaluable tool in the study of desiccation tolerance. Since this technique is particularly well suited for immunocytochemical work (Bendayan, 2001), it will now be possible to observe the TIPs characterised in this study, as well as other proteins present in desiccated vegetative tissues with greater resolution of transmission electron microscopy.
- The use of a pressure probe may assist in understanding the unusual PV curves observed in some resurrection plant including *E. nindensis* at the cellular level.
- The early seedling stage of *E. nindensis* is desiccation sensitive; study of the stage is likely to provide clues as to the conferring of desiccation tolerance on most of the vegetative tissues of the mature plant. A detailed study of the seedling stage, compared with the desiccation tolerance of both the seed and the mature plant may also provide useful information with respect to differences in desiccation tolerance as observed in seeds relative to that which is observed in vegetative tissues of resurrection plants.
- Further analysis, particularly into desiccation sensing and signalling using both the detached inner leaves and attached outer leaves of *E. nindensis* as sensitive controls to the desiccation-tolerant inner leaves, may provide invaluable information with respect to triggering stress response mechanisms which have been highlighted as specific areas of relevance to economically important crops.

Chapter 7:**References**

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