

# **Desiccation tolerance and sensitivity of vegetative plant tissue**

by  
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## DECLARATION

These studies represent original work done by the author and have not been submitted in any form to another university. Where use was made of the work of others it has been duly acknowledged in the text.

A handwritten signature in cursive script, appearing to read 'H. Sherwin', written in black ink.

HW Sherwin

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## ABSTRACT

There is a great deal of work currently being done in the field of desiccation tolerance. Generally workers studying desiccation-tolerant plant tissues have concentrated on the mechanisms of desiccation tolerance without concomitant studies on why most plants cannot survive desiccation. The present study considers both a desiccation-tolerant plant as well as a range of desiccation-sensitive plants. The work incorporates physiological, biophysical, biochemical and ultrastructural studies in an attempt to get a holistic picture of vegetative material as it dries and then rehydrates.

The plant species used in this study are: *Craterostigma nanum*, a so-called resurrection plant; *Garcinia livingstonei*, a drought-tolerant small tree; *Isoglossa woodii*, an understory shrub which shows a remarkable ability to recover from wilting; *Pisum sativum* seedlings, which have a very high water content at full turgor; and finally, *Adiantum raddianum*, the maiden hair fern, which wilts very quickly and does not recover from wilting.

The desiccation-tolerant plant, *C. nanum*, had an unusual pressure-volume (PV) curve which indicated that while large volume changes were taking place there was little concomitant change in pressure or water potential. The unusual nature of this PV curve made it difficult to assess the relative water content (RWC) at which turgor was lost. The desiccation-sensitive plants exhibited standard curvi-linear PV curves. The amount of non-freezable water in the five species was studied and found to show no correlation with the ability to withstand dehydration or with the lethal water content. There were no differences in the melting enthalpy of tissue water between the tolerant and most of the sensitive plants. *Isoglossa woodii* had a lower melting enthalpy than the tolerant and the other sensitive species.

Survival studies showed that the desiccation-sensitive plants all had similar lethal RWCs. The tolerant plant survived dehydration to as low as 1% RWC, recovering on rehydration within 24 hours. Membrane leakage studies showed that the sensitive plants all exhibited membrane damage at different absolute water contents, but very similar RWCs and water

potentials. The increase in leakage corresponded to the lethal RWC for all the sensitive species. The desiccation-tolerant plant recovered from dehydration to very low water contents and did not show an increase in membrane leakage if prior rehydration had taken place. Without prior rehydration this tolerant plant exhibited an increase in leakage at similar RWCs and water potentials to that of the sensitive species. There did not appear to be much difference in the RWC at which damage to membranes occurred whether the material was dried rapidly or slowly.

Respiration and chlorophyll fluorescence were studied to determine what effect drying and rehydration have on the electron transport processes of the leaf. The chlorophyll fluorescence studies gave an indication of damage to the photosynthetic apparatus. Both qualitative changes as well as quantitative changes in fluorescence parameters were assessed. Characteristics like quantum efficiency ( $F_V/F_M$ ) remained fairly constant for a wide range of RWCs until a critical RWC was reached where there was a sharp decline in  $F_V/F_M$ . Upon rehydration, *C. nanum* recovered to pre-stress levels, *I. woodii* showed no recovery and no further damage on rehydration, whilst the other species exhibited even more damage on rehydration than they had on dehydration.

Respiration remained fairly constant or increased slightly during drying until a critical RWC was reached at which it suddenly declined. The RWC at which this decline occurred ranged from 15% and 20% in *P. sativum* and *C. nanum* respectively, to 50% for *G. livingstonei*. On rehydration respiration exceeded the levels measured in dehydrated material for the sensitive species.

Unsuccessful attempts were made to fix material anhydrously for ultrastructural studies so standard fixation was used. The ultrastructural studies revealed that changes had occurred in the ultrastructure of leaves of the sensitive species dried to 30% RWC particularly in *A. raddianum* and *P. sativum*. Drying to 5% RWC revealed extensive ultrastructural degradation which was worsened on rehydration in the sensitive species. The tolerant species showed ultrastructural changes on drying but these were not as severe as occurred in the sensitive species. The cell walls of the tolerant species folded in on drying. This folding was possibly responsible for the unusual PV curves found in this species. At 5%

RWC the cells were closely packed and very irregular in shape. The cell contents were clearly resolved and evenly spread throughout the cell. The large central vacuole appeared to have subdivided into a number of smaller vacuoles. On rehydration the cells regained their shape and the cell contents had moved towards the periphery as the large central vacuole was reformed. Beading of membranes, which was common in the sensitive species, was not found in the tolerant species suggesting that membrane damage was not as severe in the tolerant species.

Western Blot analysis of the proteins present during drying was performed to determine whether a class of desiccation-induced proteins, called dehydrins, were present. These proteins have been suggested to play a protective role in desiccation-tolerant tissue. It was found that *C. nanum* did, in fact, possess dehydrins, but so did *P. sativum*. The other three sensitive species did not show any appreciable levels of dehydrin proteins. The presence of dehydrins alone is, therefore, not sufficient to confer desiccation tolerance.

While physiologically the damage occurring in the sensitive plants was similar to that of the tolerant plant, at an ultrastructural level the damage appeared less in the tolerant plant. On rehydration from low RWCs damage appeared to become exacerbated in the sensitive plants. This was in contrast to the tolerant plant where damage was apparently repaired. There appears, therefore, to be a combination of protection and repair mechanisms responsible for the ability of *C. nanum* to tolerate desiccation. The lethal RWC of the sensitive species was higher than that at which protective mechanisms, such as water replacement, might come into play. So it is not just the possible ability to replace tightly bound water that set the tolerant plant aside. It must also have mechanisms to tolerate damage at the higher RWCs which were damaging and lethal to the sensitive plants. The lethal damage to sensitive species appeared to be related to a critical volume, thus it is concluded that the tolerant plant had the ability to tolerate or avoid this mechanical damage during drying as well as the ability to remain viable in the dry state. It is hypothesised that the ability of the walls to fold in and the unusual nature of the PV curve may provide some answers to the enigma of desiccation tolerance.

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## CHAPTER 1: GENERAL INTRODUCTION

Water is essential for plant life and productivity. Without water, living processes, at best, come to a halt. Water plays a number of important roles within a cell. Water fills cells, keeps them turgid and confers structure. Hydrophilic and hydrophobic interactions stabilize macromolecular conformations and allow for the sequestering of cellular constituents; therefore, water serves as a protectant of molecular structure (Vertucci & Farrant, 1995). The fluid environment provided by water allows for diffusion of substrates to the active sites of enzymes; thus water is an integral part of metabolic activity in plants (Clegg, 1978; Leopold & Vertucci, 1989) and acts as a general solvent. The loss of water, therefore, will profoundly affect the physical nature of, and the biochemical reactions taking place in, the cell (Crowe, Hoekstra & Crowe, 1992; Leopold & Vertucci, 1989). Usually, severe loss of water causes the irreversible cessation of plant growth. However, many plants can survive air-drying during part of their life cycle, as seeds, but in a few species the vegetative tissue can also survive air-drying, though these plants are an exception to the norm.

Plants growing in areas of frequent and severe water stress may be adapted to survive in three ways: they evade drought, avoid drought or tolerate it (Levitt, 1980). Drought evaders or escapers are the ephemeral annuals. These plants complete their life cycles in periods when water is available. Drought avoiders are adapted to resist water loss and/or increase water uptake. The third category is the drought tolerators, within which are a group of plants that can tolerate severe desiccation (Bewley, 1979). These are known as resurrection plants due to their ability to dehydrate to air-dry conditions and then regain turgor or "resurrect" when water becomes available. Resurrection plants are also referred to as poikilohydrous and, the preferred term for this thesis, desiccation-tolerant plants.

### **Desiccation-tolerant or resurrection plants**

Most plants wilt at water potentials around -1.5 MPa and die at water potentials below -15 MPa (Gaff, 1989). In the desiccation-tolerant plants, leaf tissue survives water potentials even lower than those experienced by air-dry seeds. Most survive equilibration to air of

0% relative humidity [RH] (Gaff, 1989). There are representatives of desiccation-tolerant species amongst all of the major plant divisions (Gaff, 1989). Some 60-70 monocotyledonous and dicotyledonous angiosperms, ferns and fern allies have been reported as being desiccation tolerant (Gaff, 1971; Gaff, 1977; Gaff & Churchill, 1976; Gaff & Ellis, 1974; Gaff & Latz, 1978) and there is a larger, but unknown, number of mosses, lichens and algae which can withstand air-drying (Bewley, 1979). Desiccation-tolerant species have been identified in only eight angiosperm families. The lack of close taxonomic relationships among many of the desiccation-tolerant species suggests that the ability to withstand air-drying has evolved several times independently (Bewley & Krochko, 1982). In many respects desiccation tolerance is a primitive feature, being more prevalent in prokaryotes than in eukaryotes. This could be due to the smaller size and simpler internal organization of the cells of the former, e.g. lack of cellular compartmentalization, and less complex membrane structures. The ability of mosses and lichens to act as primary colonizers in harsh habitats is intimately linked to their tolerance of severe environments (Bewley, 1979).

Ecologically, desiccation-tolerant angiosperms are predominantly pioneer species colonizing shallow rocky soils (Gaff, 1989). The majority of desiccation tolerant angiosperms have been reported only in the last two decades, mainly from seasonally arid tropical and sub-tropical regions, particularly in southern Africa, (Gaff, 1971; Gaff, 1977; Gaff & Ellis, 1974; Gaff & Latz, 1978). Many angiosperms possess the genetic information for protoplasmic desiccation tolerance in their seeds but it is not normally expressed in vegetative tissue (Gaff, 1989). There is evidence that desiccation tolerance enables plants to withstand not only water stress, but also the temperature extremes characteristic of dry habitats (Bewley & Krochko, 1982) although this may not be the case for all the tolerant species (Eickmeier, 1986). At least one third of the desiccation-tolerant monocotyledonous species are poikilochlorophyllous, that is, they suffer the loss of most of their chlorophyll, while retaining viability during drying (Bewley & Krochko, 1982). The rest of the desiccation-tolerant monocotyledonous angiosperms and all the dicotyledonous desiccation-tolerant angiosperms retain their chlorophyll on drying.

Desiccation-tolerant plants can occupy habitats that are unfavourable to sensitive plants

because: (1) they remain viable during extreme desiccation (and sometimes severe temperature stress); (2) some species can utilize dew for hydration; and (3) they can rapidly emerge from the quiescent state, and metabolism, including photosynthesis, recovers when water becomes available (Bewley & Krochko, 1982). These benefits must be considered against the potential costs associated with: (1) stored energy and/or time costs for physiological reactivation upon rehydration; (2) low photosynthetic rates; and (3) physiological deterioration associated with a possible decline in membrane integrity which permits carbohydrate and other solute loss during rehydration with liquid water. Different desiccation-tolerant species have evolved different ecological, morphological, and physiological adaptations that accomplish, ultimately, a net gain in their photosynthetic productivity (Bewley & Krochko, 1982).

There has been a great deal of work done on the effects of drought stress on plants (reviewed by Bradford & Hsiao, 1982; Levitt, 1980, and more recently by Jones & Corlett, 1992), particularly crop plants, and many advances have been made in breeding crops with increased drought resistance (Blum, 1988). Over the last two decades there has been increased interest in the extreme drought tolerance of resurrection plants. There is a belief that, by elucidating the underlying mechanisms of desiccation tolerance in plants, ultimately crop plants with the same degree of tolerance might be bred for dryer areas (Bartels & Nelson, 1994; Comis, 1992; Tomos, 1992). While caution must be applied in considering that a resurrection crop plant would be a productive plant (Hall, 1993) it is still an important line of research that could carry enormous benefits, particularly for more marginal, subsistence farmers. Breeding of desiccation-tolerant pasture grasses may also be an avenue worth exploring.

The possible mechanisms of desiccation tolerance in seeds has received much attention (reviewed by Leprince, Hendry & McKersie, 1993; Vertucci & Farrant, 1995). Orthodox seeds have the ability to tolerate desiccation whereas recalcitrant seeds cannot tolerate drying and therefore cannot survive long in storage. As many recalcitrant species are important cash crops (e.g. tea, cacao, mango and rubber) methods that might enable the germplasm to be stored have received a great deal of attention (Farrant, Pammenter & Berjak, 1988; 1993; Finch-Savage, 1992; Wesley-Smith *et al.*, 1992). The ability to

tolerate desiccation is not limited to orthodox seeds, it is also a characteristic of pollen, dormant buds and somatic embryos (reviewed by Bewley, 1979; Levitt, 1980; Bewley & Oliver, 1992; Crowe *et al.*, 1992).

In contrast to orthodox seeds which undergo desiccation in a programmed manner, vegetative tissues are subject to drying at unpredictable intervals and thus mechanisms of tolerance must be readily activated to cope with this stress. Despite this difference, the desiccation tolerance/protection mechanisms that evolved in the vegetative tissue may be an extension of those already in place which afford protection to tissues during embryonic maturation drying (Bewley, Reynolds & Oliver, 1993). It is for this reason that work done on seeds will be referred to in this thesis where appropriate; however, no attempt is being made to give a review of the work done on seeds.

Very little is known of the metabolic characteristics of desiccation-tolerant angiosperms as these plants have only recently been identified (Gaff, 1989), most of the earlier studies on desiccation tolerance having been undertaken on mosses and ferns. The most intensively studied desiccation-tolerant plant is probably the moss, *Tortula ruralis* (reviewed by Bewley, 1979 and Bewley & Krochko, 1982). Of the angiosperm species, the physiology and anatomy of *Borya nitida*, *Xerophyta villosa* and *Myrothamnus flabellifolia* have been studied (reviewed by Gaff, 1989). More recently, the molecular aspects of desiccation tolerance have been studied in *Craterostigma plantagineum* (Bartels *et al.*, 1990; Michel *et al.*, 1994; Piatkowski *et al.*, 1990).

### **Early theories of desiccation tolerance**

A number of early workers studying drought and desiccation tolerance proposed theories to account for their observations. These theories are reviewed in Levitt (1980) and Bewley and Krochko (1982). The three theories discussed here were proposed independently and while they have largely been ignored by later workers they are worth mentioning in terms of the development of thinking in this field.

### Iljin's mechanical stress theory

Iljin suggested that mechanical injury to the protoplast membranes during drying and re-moistening is the prime cause of desiccation sensitivity and that the protoplasm of all plants, theoretically, is capable of surviving complete dehydration if mechanical stresses are avoided (Iljin, 1957). That worker concluded that the lethal injuries in plants sensitive to drought are the consequence of tensions that develop during drying as the protoplasm is pulled inward by a shrinking vacuole and outward by attachments to a resisting cell wall. In tolerant plants, these stresses are minimized through various anatomical and morphological adaptations such as reduced cell size, increased cell surface/volume ratio, small or absent vacuoles, lack of plasmodesmata, reduced osmotic pressure, presence of insoluble constituents and easily deformed cell walls.

The most common criticism of Iljin's theory is that it is too simplistic (Levitt, 1980). However, mechanical stress developed during drying will certainly be deleterious. While Iljin's theory may not fully explain the phenomenon of desiccation tolerance it is worth considering when physical stress to plants and plant cells is being studied.

### Stocker's protoplasmic tension theory

Stocker (1960, reviewed by Levitt, 1980) suggested that the tensions occasioned by desiccation act not on the surface layer of the protoplasm but rather within the internal protoplasm. Stocker believed the protoplasm consists of a latticework of threadlike proteins interspersed with water, salts, sugars and other kinds of proteins. Damage during drying was suggested to be caused by differential hydration of these components and thus a disorganization of the latticework. If drying was gradual enough, Stocker was of the opinion that the latticework could be re-organized and bonds strengthened and the viscosity of the protoplasm increased (Bewley & Krochko, 1982).

While this theory went some way in explaining why fast drying was more deleterious than slow drying, it was still considered too simplistic to explain the complex phenomenon of desiccation tolerance.

### Genckel and Pronina's theory on desiccation as a normal event in tolerant plants

(Note that Genckel is often referred to as Henckel: however the papers referred to in this work have been published under the name Genckel, so this will be used in the present study. The problem may have come about in the transcription from the Cyrillic to our alphabet.) Genckel and Pronina (1968; 1969) have attributed to the desiccation-tolerant plants a specialized metabolism and structure which is neither susceptible to, nor altered by, extreme water stress. Their theory of desiccation-tolerance is based on the premise "that for this group of plants the dehydration process is not pathological, but a normal reaction" (reviewed by Bewley & Krochko, 1982). This was a complete change in thinking from the other two theories discussed above. This theory, however, has not really withstood testing as many later workers have shown that damage caused by drying is very similar in both desiccation-tolerant and sensitive species (Gaff, 1989).

In summary, these early theories are too general and lack specific details. They do not have a general applicability to such a diverse group as the desiccation-tolerant plants (Bewley & Krochko, 1982). As desiccation is a complex phenomenon which affects many aspects of a plant, it is unlikely that any one theory could adequately explain all the aspects of desiccation tolerance. There are likely to be a number of mechanisms by which desiccation is tolerated, and in some species one mechanism may be more important than another.

### **Responses to dehydration and current hypotheses**

Recent work on desiccation tolerance has tended to focus on specific aspects of desiccation stress and to develop theories attempting to explain how the plant would cope with that particular stress. While some important advances have been made in our understanding of the phenomenon of desiccation tolerance, there are still no holistic theories which can be applied to all aspects of desiccation tolerance (Bewley *et al.*, 1993). One of the problems with current work in this field is that the workers have tended to focus on specific aspects only, to the exclusion of others, and no real attempts have been made to link the different areas of study on desiccation tolerance. These different aspects will be discussed here in an attempt to give an overview of the work that is being done in this field. While some

aspects, such as membrane leakage, the electron transport processes of photosynthesis, respiration, presence of late embryogenesis abundant proteins, ultrastructure and mechanical stress, are dealt with in more detail in this thesis, other aspects, such as the accumulation of carbohydrates, lipid composition of membranes, antioxidant systems and plant growth regulators, are discussed only in this Introduction.

### Mechanical stress

One of the first visible indications of water stress in leaves is loss of turgor and wilting. As cells dry the walls are pulled inwards along with the cell contents. Addition of water should return the cell to its original shape (Levitt, 1980). Contraction of cells during dehydration can result in the loss of membrane material, (reviewed by Steponkus & Webb, 1992). Meryman (1974) proposed that cells could not contract beyond a "minimum critical volume" without loss of membrane function on rehydration. Iljin (1957) observed that cells of vegetative plant tissues which were tolerant to drying had only minor reductions in volume when desiccated; this was a consequence of the presence of small cells or cells in which dry matter occupied space previously filled with water and/or cells with small vacuoles.

When water is removed from cells, membrane systems become packed together and inter-bilayer interactions can cause membrane fusion and/or phase transitions (Steponkus & Webb, 1992). Organelles with high membrane contents such as chloroplasts and mitochondria would be particularly susceptible to this type of stress. Organisms that tolerate dehydration must be able to cope with the mechanical stress resulting from membrane apposition by avoiding the stress (spacers between membranes) or diminishing the strain (Vertucci & Farrant, 1995).

### Membrane integrity

Desiccation results in numerous physiological responses which suggest that membrane damage has occurred (Leopold, Musgrave & Williams, 1981). Membrane systems are considered to be particularly susceptible to dehydration damage (Crowe *et al.*, 1986; 1987; 1988; 1992; Leopold, 1986; Steponkus & Webb, 1992). When cells are dehydrated the structures of proteins and polar lipids are altered due to the weakening of hydrophilic and

hydrophobic interactions that stabilize conformations (Leopold, 1986; Crowe *et al.*, 1987; 1988). Leakage of cytoplasmic solutes after exposure to stress has become a standard procedure to measure membrane damage (Berjak, Vertucci & Pammenter, 1993; Hoekstra & van der Wal, 1988; Leopold *et al.*, 1981; McKersie & Stinson, 1980; Oliver, Mishler & Quisenberry, 1993; Senaratna & McKersie, 1986; Senaratna, McKersie & Stinson, 1986, among others).

A common feature of electrolyte leakage is that it is a transient phenomenon, lasting perhaps only a few minutes after addition of water, while prolonged loss of solutes is characteristic of irreversibly damaged tissues (Bewley & Krochko, 1982). Electrolyte leakage has been shown to occur in both desiccation-tolerant and sensitive bryophytes following desiccation (Gupta, 1977). However, prolonged loss of solutes occurs only when desiccation-sensitive material is severely stressed or desiccation tolerant-material is subjected to lethal drying rates (reviewed by Bewley & Krochko, 1982). Leakage from tissues has been explained in terms of phase changes which take place in the structure of membranes during water loss (Crowe, Hoekstra & Crowe, 1989; Hoekstra & van der Wal, 1988; Simon, 1974; 1978).

The loss of membrane function has been attributed to both "demixing" (segregation of the various components) of membrane constituents with different hydration characteristics and to phase transitions of the polar components (Crowe *et al.*, 1987; 1992; Steponkus & Webb, 1992; Webb, Hui & Steponkus, 1993). The composition of the membrane, in terms of degree of fatty acid saturation and the presence of proteins and sterols, appear to play a role in the type of phase transition that takes place during drying (Crowe, McKersie & Crowe, 1989; Hoekstra & McKersie, 1990; Hoekstra, Crowe & Crowe, 1991; Hoekstra *et al.*, 1992) The different types of phase transitions as well as the role of lipid composition in the susceptibility of the bilayers to phase transitions have been reviewed by Vertucci and Farrant (1995).

Leakage studies have been performed during the course of this study and further details of membrane phenomena are discussed in the Introduction and Discussion sections of Chapter 3.

### Accumulation of carbohydrates

Correlations between high concentrations of non-reducing sugars and desiccation tolerance have led to the suggestion that they may play a role in the mechanism of desiccation tolerance. This relationship has been noted in orthodox seeds (Blackman, Obendorf & Leopold, 1992; Koster & Leopold, 1988; Leprince, Bronchart & Deltour, 1990a), pollen (Hoekstra & van Roekl, 1988; Hoekstra, Crowe & Crowe, 1989), desiccation tolerant vegetative tissues (Adams, Kendall & Kartha, 1990; Bianchi *et al.*, 1991; 1993; Drennan *et al.*, 1993; Schwab & Gaff, 1986) and animal systems (Madin & Crowe, 1975). There are two hypotheses that have been proposed to account for possible roles for sugars in conferring desiccation tolerance. The first, known as the water replacement hypothesis, suggests that the hydroxyl groups of sugars substitute for water and provide the required hydrophilic interactions for membrane and protein stabilization (Clegg, 1986; Crowe *et al.*, 1992). In artificial membrane systems, the sugars bind to the polar headgroups resulting in the maintenance of the bilayer liquid-crystalline structure of the membrane even at very low hydration levels (Crowe *et al.*, 1987; 1988; 1992; Hoekstra *et al.*, 1989; 1991). The disaccharide, trehalose appears to be the most effective at maintaining conformation after desiccation (Crowe *et al.*, 1987; 1992). Trehalose is generally absent in plants (though it has been found in *Myrothamnus flabellifolia* (Bianchi *et al.*, 1993; Drennan *et al.*, 1993) and *Selaginella lepidophylla* (Adams *et al.*, 1990)), thus another sugar would need to perform this protective function. The high sucrose content in most angiosperms has led to the suggestion that under conditions precluding crystallisation, sucrose can also become hydrogen-bonded to the membrane surface, thus preventing lipid phase transitions (Crowe *et al.*, 1987; 1992; Hoekstra & van Roekl, 1988; Hoekstra *et al.*, 1989; 1991; Leopold & Vertucci, 1986).

The second hypothesis concerns glass formation. It has been proposed that sugars in the cytoplasm can result in the formation of a glass upon dehydration, which, due to its extreme viscosity, would prevent molecular movement and thus maintain cellular contents in a stasis. A glassy state would prevent degradative processes and may avoid alterations in pH and crystallization of cytoplasmic solutes (Leopold, 1990). Aqueous glasses have been detected in dried seed materials (Bruni & Leopold, 1991; 1992; Vertucci, 1990; Williams & Leopold, 1989) and in sugar mixtures of composition similar to that in maize

seeds (Koster, 1991). However there is limited evidence suggesting that sugars form glasses *in vivo*. Glasses formed with sucrose do not appear to be stable and tend to crystallize; this would defeat the object of the glass formation as it would need to be stable to protect the cellular structure (reviewed by Vertucci & Farrant, 1995). It is suggested that a combination of sugars such as sucrose and raffinose may be stable enough to produce protective glasses *in vivo* (Caffrey, Fonesca & Leopold, 1988; Koster, 1991; Koster & Leopold, 1988; Leopold & Vertucci, 1986).

While these hypotheses may go some way to explaining how tolerant plants survive extreme desiccation, they do not fully explain differences in desiccation tolerance amongst tissues with similar soluble sugar contents. High levels of soluble sugars, in the appropriate concentrations and ratios, are present in some developing and mature desiccation-sensitive (recalcitrant) seeds (Farrant, Pammenter & Berjak, 1992; 1993) and in desiccation-sensitive vegetative tissue during dehydration (Kaiser, Gaff & Outlaw, 1985). There is also little correlation between the moisture level at which desiccation-sensitive tissue becomes irreversibly damaged and the moisture level where protection in the form of water replacement and glass formation is expected (Berjak, Pammenter & Vertucci, 1992; Berjak *et al.*, 1993; Pammenter, Vertucci & Berjak, 1991). This implies that the possession of sugars in the correct concentrations and ratios is, on its own, inadequate to confer desiccation tolerance. Desiccation-tolerant tissues need some mechanism to cope with the damage at these higher water contents. Thus the water replacement and glass formation hypotheses cannot fully explain desiccation tolerance.

### Physiological stress

Photosynthesis and respiration are by far the most studied metabolic processes in desiccation-tolerant plants (reviewed by Bewley, 1979; Bewley & Krochko, 1982; Gaff, 1989). Photosynthesis is particularly susceptible to water stress and, even under mild water stress, photosynthesis declines (Bradford & Hsiao, 1982). Under mild water stress the primary cause of the inhibition of photosynthesis is likely to be stomatal closure, while more severe water stress probably directly affects the photosynthetic apparatus (Kaiser, 1987). Respiration appears to be less susceptible than photosynthesis to water stress (Harten & Eickmeier 1986; Seel, Hendry & Lee, 1992a). Respiratory activity continues

until tissues are dried below -11 MPa (Leopold & Vertucci, 1989); however, under severe water stress, respiration rates decline (reviewed by Bewley, 1979; Bewley & Krochko, 1982; Gaff, 1989). Desiccation tolerance will depend on the ability of the tissue to regain metabolic function rapidly on rehydration.

Aspects of both the light reactions of photosynthesis (measured by chlorophyll fluorescence) and respiration are examined in more detail in Chapter 4.

### Ultrastructure

A number of ultrastructural changes occur in desiccation-tolerant plant tissue on drying (reviewed by Bewley, 1979; Goldsworthy & Drennan, 1991; Hallam & Gaff, 1978; Hallam and Luff, 1980a; 1980b; Hetherington, Hallam & Smillie, 1982; Schneider *et al.*, 1993). During dehydration of these plants, chloroplasts and mitochondria swell and lose their internal organization and in some species vacuoles fragment, becoming smaller. Most of these changes are similar to those occurring in desiccation-sensitive tissues (Giles, Beardsell & Cohen, 1974; Giles, Cohen & Beardsell, 1976; Hsiao, 1970; Kurkova & Motorina, 1974, among others). In desiccation-tolerant plants that lose their chlorophyll on drying there are severe changes to the ultrastructure of the chloroplast on drying (Gaff, Zee & O'Brien, 1976; Hallam & Gaff, 1978; Hallam & Luff, 1980b; Hetherington *et al.*, 1982). In tolerant plants, the damage is repaired on rehydration while with the sensitive plants there is no return to the original state. Another important feature of desiccation-tolerant plants may be their ability to retain their nuclear integrity (Bewley, 1979). Iljin (1957) also suggested that small elongated cells with small vacuoles would be more tolerant to drying than larger cells with large vacuoles.

Ultrastructural studies are considered in Chapter 5.

### RNA and protein synthesis

There is evidence from a number of publications that very mild water stress reduces the level of protein synthesis in drought-sensitive vegetative tissues (reviewed by Hsiao, 1973; Bewley & Larsen, 1980; Dhindsa & Bewley, 1977). If dehydration has not been too severe there is a recovery of protein synthesis on rehydration in these desiccation-sensitive

plants. As with desiccation sensitive plants, a net loss of protein occurs in desiccation tolerant plants during air drying (Bewley, 1979) but the decline in total protein is usually less than in sensitive plants (Gaff, 1989). In sensitive plants the loss is predominantly from the water-soluble fraction, while in tolerant individuals it is mainly from the water-insoluble fraction (Gaff, 1989). Rehydration of tolerant material from the desiccated state results in the rapid recovery of protein synthesis (Eickmeier, 1982) and the synthesis of a number of rehydration proteins (Oliver, 1991). Our current understanding of the effects of desiccation on protein and associated RNA synthesis has come largely from studies on the gametophyte of the moss *Tortula ruralis* (reviewed by Bewley, 1979; Bewley & Krochko, 1982).

#### Late embryogenesis abundant (LEA) proteins and dehydrins

There are qualitative changes in protein synthesis during drying in desiccation-tolerant plants as well as those that are quantitative. Drought and desiccation stress have been shown to promote the synthesis of a number of proteins (reviewed by Bewley & Oliver, 1992; Bray, 1993; Skriver & Mundy, 1990), which were first identified in the maturing and desiccation-tolerant phase of seed embryos (Baker, Steele & Dure, 1988) and are often called LEA proteins. A subset of LEA proteins, known as dehydrins, has been reported to occur in a range of seed and vegetative tissues (Bartels *et al.*, 1990; Bewley *et al.*, 1993; Bradford & Chandler, 1992; Close, Fenton & Moonan, 1993; Finch-Savage, Pramanik & Bewley, 1994; Hong, Barg & Ho, 1992; Piatkowski *et al.*, 1990; Ried & Walker-Simmons, 1993). While the role of these proteins has not yet been elucidated, it has been hypothesised that they protect the membranes and other cellular constituents (Close, Kortt & Chandler, 1989; Dure *et al.*, 1989) and also that they sequester ions (Dure, 1993) and help renature unfolded proteins (Dure, 1993).

LEA-like proteins and dehydrins have, however, been found in desiccation-sensitive tissues (Blackman *et al.*, 1991; Finch-Savage *et al.*, 1994). It has been suggested that while these proteins may play a role in desiccation tolerance, they may not be sufficient to induce tolerance by themselves (Blackman *et al.*, 1991).

The presence of these proteins and their possible role in desiccation tolerance are discussed

further in Chapter 6 of this thesis.

### Plant growth regulators

The plant growth regulator, abscisic acid (ABA), appears to play an important role in the development of desiccation tolerance. ABA has been implicated in the tolerance of vegetative tissues to water stress and desiccation (Bewley & Oliver, 1992; Chandler, Munns & Robertson, 1993; Michel *et al.*, 1994; Piatkowski *et al.*, 1990; Ristic & Cass, 1993; Skriver & Mundy, 1990; Werner *et al.*, 1991, among others). ABA has been shown to induce tolerance in callus of *Craterostigma plantagineum* (Bartels *et al.*, 1990) and it also induces or turns on many genes which encode LEA-like and dehydrin proteins in a range of plant tissues (Bartels *et al.*, 1990; Galau, Hughes & Dure, 1986; Piatkowski *et al.*, 1990). The mechanism whereby ABA induces tolerance is not clear, but evidence suggests that it may be important in inducing or maintaining the post-abscission or pre-desiccation stages in orthodox seeds (Vertucci & Farrant, 1995). ABA may also act as a signal transducer for the transcription of protectants and LEA-like proteins (Bray, 1993; Chandler *et al.*, 1993; Dure *et al.*, 1989; Skriver & Mundy, 1990).

### Free radical attack and peroxidation

There have been suggestions over the years that deterioration resulting from oxidative and free radical reactions occurs in dehydrating and rehydrating tissue (Senaratna & McKersie, 1986; Senaratna, McKersie & Stinson, 1985; Smith & Berjak, 1995). The ability to avoid or repair such damage could contribute to desiccation resistance (reviewed by Bewley, 1979; Leprince, *et al.*, 1990b; 1992; 1994; Smirnov, 1993). In studies on desiccation-tolerant and sensitive mosses it was found that indications of lipid peroxidation were less in the tolerant than the sensitive moss (Dhindsa & Matowe, 1981; Seel, Hendry & Lee, 1992a; 1992b). The potential for active oxygen/free radical formation occurs in both the tolerant and sensitive mosses, but damage develops only in the sensitive species (Seel *et al.*, 1992a). Vegetative tissues with greater drought or desiccation tolerance appear to have more efficient anti-oxidant enzyme systems (Dhindsa, 1991; Pastori & Trippi, 1992). Studies on the presence of anti-oxidants have been performed on mosses and have been reviewed by Smirnov (1993). Very little is known about protection against the effects of oxygen free radicals in tolerant higher plants. *Craterostigma plantagineum*, a desiccation-

tolerant angiosperm, has been found to contain colneleic acid on drying (Bianchi *et al.*, 1992). Colneleic acid is a lipoxygenase inhibitor, thus its presence in a desiccation-tolerant plant may suggest that it is able to inhibit damaging peroxidation reactions.

Any oxidative damage which does occur during water deficit will be exacerbated in the light because excess excitation energy which cannot be used in photosynthesis, can lead to the formation of active oxygen species if the photo-protective systems are overwhelmed. During desiccation, leaf movements and folding are common amongst desiccation-tolerant moss and angiosperm species (Bartels *et al.*, 1990; Gaff, 1989; Muslin & Homann, 1992). Some angiosperms lose their chlorophyll on drying, which may be a mechanism to prevent photo-oxidative damage (Smirnoff, 1993). This may be particularly important for some poikilochlorophyllous species, such as *Xerophyta villosa*, which do not curl their leaves and are thus exposed to continuous high levels of incident radiation. Water-soluble pigments, which could possibly act as photo-protectants, are also present in the epidermal cells of some desiccation-tolerant plants (Gaff, 1989).

#### High ionic strength and the accumulation of compatible solutes

As cells dry, the intracellular solutions become more concentrated and ionic strength increases (Tomos, 1992). Accumulation of ions can destabilize membranes (Schwab & Gaff, 1986). Many workers studying the effects of drought on plants have shown that a decrease in osmotic potential results in the inhibition of photosynthesis (Berkowitz & Gibbs, 1982; 1983; Kaiser, 1982; Kaiser *et al.*, 1981). Tolerant plants must have a mechanism to cope with this increase in ionic strength. This is an area of study that has not received as much attention as the others mentioned above.

The accumulation of compatible solutes (solutes which are not toxic to metabolism and act to neutralise high ionic strength), such as proline, is a possible mechanism to cope with high ionic strength (Bartels & Nelson, 1994). Accumulation of free proline in leaves under water stress has been widely reported (reviewed by Bradford & Hsiao, 1982; Boggess & Stewart, 1976; Boggess *et al.*, 1976; Stewart, 1972, among others). It is thought that the accumulation of proline is advantageous to the plant during drought stress (Bradford & Hsiao, 1982). Tymms and Gaff (1979) studied proline concentration during drying of

desiccation-tolerant plants and found that levels increased in some plants, but remained constant in others. Proline accumulation could not, therefore, explain desiccation tolerance (Tymms & Gaff, 1979). Other compatible solutes may include dehydrins, as suggested by Close *et al.* (1993).

Another means of coping with high ionic strength is for the ions to be sequestered. Dure (1993) has suggested that this may be one of the roles of the LEA proteins. The formation of glasses in the cytoplasm may also help neutralise the problems associated with the concentration of ions (Leopold, 1990; Leprince *et al.*, 1994).

### Conclusion

Clearly there are a series of challenges associated with survival in an anhydrous environment. Truly desiccation-tolerant tissues will have mechanisms to tolerate all the physical, biochemical, physiological and biophysical changes that result when hydrated cells are dried. Consequently, desiccation tolerance must be viewed as a complex phenomenon with perhaps several interactive components (Vertucci & Farrant, 1995). Bewley (1979) outlined three major criteria that must be met for a plant to survive desiccation: (1) the ability to limit damage during drying to a repairable level; (2) physiological integrity in the dry state; and (3) the capacity to mobilise repair mechanisms upon rehydration to restore damage suffered during drying. Levitt (1980) and later Bewley and Oliver (1992) have also outlined two broad mechanisms of desiccation tolerance: protection and repair. There is evidence for repair mechanisms being important in the bryophytes (reviewed by Bewley & Oliver, 1992; Oliver *et al.*, 1993). Repair mechanisms would also need to be present in the higher plants which lose their chlorophyll on drying. Evidence for protection mechanisms in vegetative tissue, particularly higher plants, is largely indirect. There must be some form of cellular protection for the plants to be able to survive extended periods in the dry state and recover quickly on rehydration. It is most likely that there will be aspects of both protection and repair in most desiccation-tolerant plants (Bewley & Oliver, 1992).

An aspect that is little researched in the field of desiccation tolerance, is the potential for further damage caused by rehydration. In the initial period of rehydration, the passage to

a metabolically active state poses particular problems if metabolic "mayhem" is to be avoided (Stewart, 1990). Too-rapid rehydration has been shown to cause damage to dry seeds (Hoekstra & van der Wal, 1988; Simon, 1974). So while rehydration causes its own set of challenges to the desiccation-tolerant plant, it is also the period when repair of physical damage and the restoration of metabolic activity needs to take place. Thus in studying the phenomenon of desiccation tolerance one must not only study the drying process, but also rehydration. Another aspect often ignored is that to get to hydration levels where water replacement mechanisms come into play, tissues must pass through water contents damaging to sensitive plants. Thus water contents, higher than when water replacement becomes necessary, also need to be studied.

### **Aim of this study**

At the 1993 Riverside Symposium on cellular dehydration Bewley stated that "the precise mechanisms of desiccation tolerance remain to be elucidated, for few plants have been subjected to any exhaustive study on the structural, physiological, and molecular basis of tolerance. Consequently, few encompassing hypotheses on the nature of desiccation tolerance have been put forward. Desiccation tolerance as a research area is largely uncharted and provides numerous and interesting challenges" (Bewley *et al.*, 1993). Generally, the studies of desiccation tolerance have focused on one or two specific species and on only one or two aspects, such as physiology, biochemistry or molecular biology. It is for this reason that it was decided to undertake a broad study which combined physical, biochemical, physiological and ultrastructural aspects of desiccation tolerance. This study aimed to provide a holistic overview of the various factors involved in desiccation tolerance. It was not the aim of this work to deal exhaustively with any one of the constituent aspects. Some aspects, such as the anti-oxidant system, carbohydrate production and plant growth regulators, could not be studied due to time constraints. The investigations reported in this thesis should be seen as the start of a large comprehensive study on desiccation tolerance and sensitivity in vegetative plant tissue.

While Farrant, Pammenter & Berjak (1993) used a comparison of desiccation-tolerant and desiccation-sensitive seeds to evaluate current hypotheses concerning the mechanisms of

desiccation tolerance, a problem with studies of desiccation tolerance is that most workers have studied the material in its dry state and considered how it survives in that state. While this is a very important component to desiccation tolerance, desiccation-sensitive plants die at water contents higher than those where aspects like water replacement from membrane surfaces would be appropriate. Thus desiccation-tolerant tissue will have to survive the water contents that kill sensitive plants, as well as surviving in the very dry state. Furthermore, an understanding of desiccation tolerance requires an understanding of the damage caused by dehydration, i.e. an understanding of the deleterious processes against which protection must be provided. For these reasons it was decided to conduct studies with both desiccation-tolerant and desiccation-sensitive vegetative tissue. As particular desiccation-sensitive plants have been shown to vary in their abilities to tolerate water loss, a range of sensitive species were used.

Bewley and Oliver (1992) have outlined two broad mechanisms of desiccation tolerance: protection of cellular constituents in the dry state and repair of damage on rehydration. While most of the work reported in the literature has focused on protection, repair is also likely to play an important role in desiccation tolerance. After being in the dry state, tissues have also to survive rehydration, which has been shown to cause additional damage to sensitive species. As both repair and protection mechanisms are also likely to be important to desiccation tolerant tissues, both dehydration and rehydration were studied in an attempt to determine the relative importance of these two types of mechanisms.

#### Terminology and layout of thesis

The terms drought and desiccation have often in the past been used to mean the same thing. In this work drought refers to mild water stress where relative water content (RWC) is decreased to no more than 50%. Desiccation refers to severe water stress where RWC is decreased to less than 20%. Plants which can tolerate drying to air-dry states and survive on rehydration are referred to as desiccation tolerant and those which cannot survive this stress are referred to as desiccation sensitive. A drought-tolerant species is one which survives in arid areas, but is not desiccation tolerant.

Chapter 2 of this thesis describes the species used in the study. It then considers ways of

expressing water status (water content, RWC, water potential) and provides some information on physiological aspects of tissue water status (pressure volume curves and calorimetric properties). Chapter 3 deals with responses to desiccation at two different dehydration rates using electrolyte leakage as an expression of membrane damage. The water contents at which the sensitive plants die are also determined. Changes in physiological parameters (photosynthesis and respiration) of the leaf during both dehydration and rehydration are examined in Chapter 4. Chapter 5 then describes the ultrastructure of the leaves during drying and after rehydration. Chapter 6 examines the presence or absence of dehydrin-like proteins in the drying leaves. Finally, Chapter 7 consists of a general discussion, linking the work done in the different chapters and considers the results in relation to the body of knowledge of the subject matter.

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## CHAPTER 2: CHARACTERISATION OF EXPERIMENTAL MATERIAL

### 2.1 PLANTS CHOSEN FOR STUDY

The importance of studying desiccation-sensitive as well as tolerant plants while investigating the phenomenon of desiccation tolerance has been outlined in Chapter 1. When considering desiccation-sensitive plants it is difficult to isolate one particular species to study as these plants exhibit a range of responses to water stress. It is because of this range of responses that four desiccation-sensitive species were chosen for study in parallel with a desiccation-tolerant species. The sensitive species ranged in response to water stress from a drought tolerant tree, through an understorey shrub which can rapidly recover from wilting, seedlings of a crop plant to a highly water-stress sensitive fern. The desiccation-tolerant species used was a so-called "resurrection" plant, which can tolerate severe water stress. It was considered that comparing species, which have such a wide variety of responses to water stress, might shed light on how plants cope with severe water stress and desiccation and what allows some to cope with a level of water stress which kills most plants.

The species are discussed in order of increasing presumed desiccation tolerance.

#### *Adiantum raddianum*

The fern chosen for this work was *Adiantum raddianum* Presl; Schelpe, (Adiantaceae) commonly known as the maiden hair fern (Fig. 2.1). It is indigenous to South America but has become naturalised in South Africa (Jacobsen, 1983). It is a common house plant and requires regular watering for survival. The fronds are tufted and grow 200 - 400 mm long. It occurs naturally, growing always in shade along wet earthbanks, along streambanks and on wet forest floors (Jacobsen, 1983). The fronds of *A. raddianum* do not recover from wilting and the root stock usually dies if the plant is not supplied with water for about two weeks. The leaflets are broad and thin. Only the dark green fully matured leaflets were used as experimental material from potted specimens obtained from local nurseries. The soil in which they were growing was a garden loam with a high

humus content. The plants were kept indoors, well lit, but out of direct sunlight and were watered once a day.

### *Pisum sativum*

Seedlings of *Pisum sativum* L. (Fabaceae), the common garden pea, were also studied. This plant was chosen because it possesses leaves which have a high water content at full turgor. It is also very easy to grow. Seeds of the Greenfeast variety were sown in trays of vermiculite and seedlings used after fourteen days (Fig. 2.2). The material was kept in a greenhouse with no supplemented light. The two youngest fully expanded leaves were used for the experimental work performed in this study.

### *Isoglossa woodii*

*Isoglossa woodii* C.B. Cl. (Acanthaceae) (Fig. 2.3) is an understory shrub of coastal forests of south east Africa, which grows up to 2 metres in height (Ross, 1972). *Isoglossa woodii* has large broad leaves which wilt under mild water deficits or under conditions of high evaporative demand. Recovery from wilting is rapid. Stands of *I. woodii* growing naturally in a wooded area of the main campus of the University of Natal, Durban, were used for this study. Cuttings were grown in the same soils (Berea Red Sand) and kept outdoors shielded by shade cloth. They were watered once a day. The third leaves from the top were used as experimental material as these leaves were fully matured but did not yet show signs of ageing and senescence.

### *Garcinia livingstonei*

*Garcinia livingstonei* T Anders. (Clusiaceae) is a small- to medium-height tree occurring in sandy soils of riverine fringes and in open woodland in the eastern parts of southern Africa (Coates Palgrave, 1984). *Garcinia livingstonei* is considered to be drought tolerant as it grows in a habitat that is often dry. The leaves of *G. livingstonei* are thick and rigid. Two medium sized trees planted on the grounds of the University of Natal, Durban (Fig. 2.4) were studied in conjunction with ten potted specimens obtained from the Silverglen

Nature Reserve, Chatsworth, Durban. The trees were growing in direct sunlight in Berea Red Sand and were watered twice weekly. The potted specimens were grown in a 50:50 mixture of garden loam and Berea Red Sand in a greenhouse with no supplemental light and were watered every second day. The second and third leaves from the apex of branches were used as experimental material.

### *Craterostigma nanum*

The species *Craterostigma nanum* (Hochst.) (Scrophulariaceae) has been reported to be desiccation tolerant by Gaff (1977), i.e. it is a so-called "resurrection" plant. These plants have leaf material which can survive severe desiccation for a length of time and then recover upon watering. Leaves of *C. nanum* plants with water contents of less than 0.1 gram of water per gram dry weight, and that are capable of surviving upon rewatering, have been measured in the field (personal observations).

*Craterostigma nanum* (Figs. 2.5 and 2.6) grows in shallow (less than 10 cm deep) sandy soil in exposed rocky outcrops on the KwaZulu Natal South Coast, South Africa (Ross, 1972). The plants shown in Figure 2.5 were photographed during early summer when rainfall had been plentiful, while those shown in Figure 2.6 were photographed in winter after a number of very dry months. The soil, by its shallow and sandy nature is subject to rapid drying. It also becomes water-logged after heavy rains because there is no, or very little, drainage through the rocky substratum (personal observations). Samples were collected from the Umtavuna Nature Reserve (Natal Parks Board) and the farm belonging to Alec Wichman, on the eastern side of Yellowwood Gorge at Izingolweni (Fig. 2.7), about eight kilometres inland from Port Edward (30° 58'S 30° 95'E). The samples were collected from an altitude of approximately 800 m on Natal Group Sandstone of the Mzikaba formation. The area receives approximately 1 400 mm rainfall per annum, mainly in the spring months, and has an average summer maximum temperature of 26°C and an average winter maximum of 23°C (Michelin, 1990).

The collected plants were grown in trays containing soil collected from the same site. The trays were kept in a greenhouse and not given any supplementary light. They were

watered once a day. It was noted that under these conditions the leaves grew larger and longer than those observed in the field (Fig. 2.8). It was also observed that many of the plants, particularly those that had been produced by vegetative reproduction in the greenhouse, appeared to lose their ability to tolerate desiccation. It is for this reason that the plants used in this study were collected from the field and investigated within two to three months of having been collected.

This apparent loss of tolerance raises interesting questions which, unfortunately, were not part of the scope of this study, but should be investigated in the near future. Workers studying the resurrection plant, *Boea hygroskopica*, which is propagated using tissue culture and grown in greenhouses have also noted that less than 50% of their plants have the ability to tolerate desiccation (C.V. Vazzana, pers. comm.<sup>1</sup>). Slides shown in a presentation by Buitink and Hoekstra (1994) on the desiccation-tolerant plant *Craterostigma plantagineum*, indicate that the plants they were using (also products of tissue culture and greenhouse grown) have longer and broader leaves and do not curl up as tightly during drying as those observed in the field (personal observations). It would be of interest to know the mechanisms underlying any apparent loss of desiccation tolerance in these resurrection plants and also whether the ability to survive can be recovered with hardening the material.

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Figure 2.1: Fronds of a potted specimen of the desiccation-sensitive fern *Adiantum raddianum* Presl; Schelpe. (Adiantaceae).



Figure 2.2: Fourteen day old seedlings of the garden pea, *Pisum sativum* L. (Fabaceae), grown in vermiculite under greenhouse conditions.



Figure 2.3: Leaves of *Isoglossa woodii* C.B. Cl. (Acanthaceae) growing on the grounds of the main campus of the University of Natal, Durban.



Figure 2.4: Leaves of *Garcinia livingstonei* T Anders. (Clusiaceae) growing on the grounds of the main campus of the University of Natal, Durban.



Figure 2.5: Plants of *Craterostigma nanum* (Hochst.) (Scrophulariaceae) growing in their natural environment in Port Edward, KwaZulu Natal, South Africa after a period of good rains.

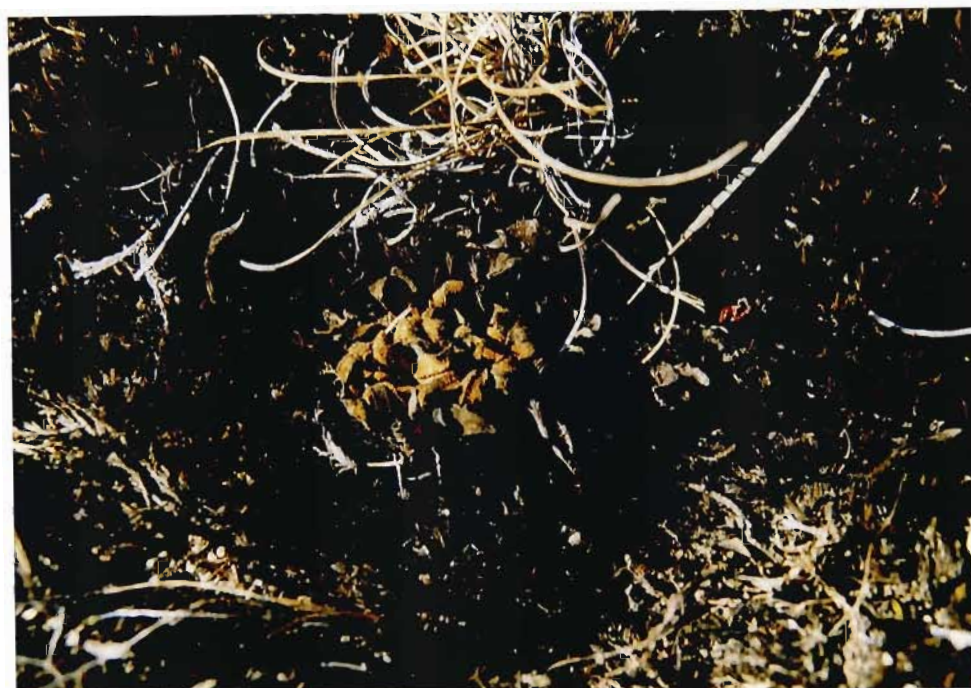


Figure 2.6: Plants of *Craterostigma nanum* (Hochst.) (Scrophulariaceae) growing in their natural environment in Port Edward, KwaZulu Natal, South Africa after a dry period. These plants recovered after they were rewatered.



Figure 2.7: The eastern side of Yellowwood Gorge at Izingolweni, Port Edward. This is the only location that plants of *C. nanum* have been recorded in the last 30 years. The rocky outcrops have shallow sandy soils.



Figure 2.8: Plants of *Craterostigma nanum* (Hochst.) (Scrophulariaceae) growing in a greenhouse with regular daily watering. The leaves are broader and longer than those found in the field (Fig. 2.5).

## 2.2 WATER STATUS

### **Introduction**

This section reports on the water status of the species used in this study in terms of their water content at full turgor, the relative water content (RWC) at which turgor is lost, their bulk modulus of elasticity and the amount of non-freezable water they possess.

### Measurements of water status

The appropriate measure for plant water status is of fundamental importance in the study of plant water relations (Schulte, 1992). There are a number of different measures used to describe the water status of plant tissue, the easiest and most direct of which is absolute water content, usually expressed on a gram water per gram dry mass basis. As an indicator of water stress, however, it is confounded by tissue size and dry matter content (Bradford & Hsiao, 1982). Relative water content (RWC) is a measure of the amount of water in a plant tissue as a percentage of the total possible amount it holds at full turgor. RWC is a useful measure in that it can be used to compare a number of plant tissues with different absolute water content at full turgor. It does, however, require accurate determination of the water content of the tissue at full turgor, which is not always easy to achieve (Bradford & Hsiao, 1982). RWC is also a measure of relative cell volume (Stadelman, 1984). Water potential is another commonly used measure of water status. It reflects the thermodynamic activity of the water in the tissue. However, it is not certain that water potential *per se*, is critical for physiological functions (Bradford & Hsiao, 1982). Percentage of water loss from the tissue has also been used by some workers (Levitt, 1980). Different measures of water status would possibly be useful for different purposes (Schulte, 1992). However, it is difficult to compare work done by different groups because of these different measures of water status. In order to express the water status in a number of ways in the present study, standard curves were drawn up which permit inter-conversion between absolute water content, RWC and water potential.

### Pressure volume curves

Two techniques are commonly used to determine water potential of leaf tissue. They are

the pressure chamber (Scholander *et al.*, 1964) and thermocouple psychrometry. Pressure volume (PV) curves, developed by Tyree and Hammel (1972), are a valuable tool in describing plant water relations. The measurement of a PV curve for a leaf or twig can be used to identify the relationship between water potential and RWC. From these curves the RWC at which turgor is lost, osmotic potential at full and zero turgor, the bulk modulus of elasticity ( $\epsilon$ ) and the amount of symplastic and apoplastic water can be determined. PV curves are curvi-linear with the curved portion describing water potential in terms of both osmotic and turgor potential. The curve becomes linear as turgor potential is lost and water potential has only the osmotic component.

Measurements of leaf tissue elasticity can be determined by analysing the turgor pressure-water content relation developed from PV curves (Roberts, Strain & Knoerr, 1981). A measurement, first calculated by Broyer (1952, cited in Stadelman, 1984), known as the bulk modulus of elasticity ( $\epsilon$ ) is defined as  $(dP/dV)V$  where P is the bulk turgor pressure and  $dV/V$  represents a differential change in tissue cell volume (Roberts *et al.*, 1981). A number of studies have shown  $\epsilon$  to vary with drought tolerance (Davies & Lakso, 1979; Kikuta & Richter, 1986; Roberts *et al.*, 1981; Schulte, 1992) with both high and low  $\epsilon$  described as being significant. A low  $\epsilon$  (elastic cell walls) is interpreted as meaning that a given loss of cell water will result in only a small change in tissue water potential and pressure potential, that is, turgor maintenance (Davies & Lakso, 1979; Kikuta & Richter, 1986). Maintenance of leaf turgor above species-dependent critical levels is particularly necessary for continued growth (Boyer, 1968). On the other hand, an increase in the elastic modulus (more rigid cell walls) is interpreted as leading to lower tissue water potential, increased ability to withdraw water from the soil, and the maintenance of a higher tissue water content (Cheung, Tyree & Dainty, 1975). In the former (elastic cell walls), the importance of maintaining cell water potential is emphasized over the water content, while the latter (more rigid cells), enhances water uptake and hence water content (mass of water) is emphasised at the expense of tissue water potential and its pressure component (Schulte, 1992).

### Calorimetry

At least five types of water can be distinguished from calorimetric and motional properties

in plant and animal tissue (Clegg, 1979; Vertucci, 1990). Type 5 is dilute solution water and is detected at water potentials greater than about -2 MPa. Type 4 is concentrated solution or capillary water and is detected at water potentials between -2 and -4 MPa. Type 3 is suggested to form bridges over hydrophobic moieties on macromolecules. It is detected at water potentials between -4 and -11 MPa. Type 2 water has glassy characteristics and is believed to have strong interactions with polar surfaces of macromolecules or hydroxyl groups of solutes. It is detected at water potentials of -12 to -150 MPa. Type 1 water corresponds to the theoretical level at which water binds to macromolecules as a structural component. Type 1 and type 2 water are constrained and do not undergo the freezing transition that is observed in "bulk" water (Vertucci, 1990). This water is often referred to as non-freezable water.

The role this non-freezable water plays in the ability of plant material to be tolerant of desiccation and freezing has received some attention (Pammenter, Vertucci & Berjak, 1991; 1992; Rascio, *et al.* 1992; Vertucci, Ellenson & Leopold, 1985; Vertucci & Leopold, 1987; Vertucci, 1989; 1990). Vertucci and Leopold (1987) suggested that tissues which were sensitive to desiccation had poor capacity to bind water tightly. Rascio *et al.* (1992) found that a drought tolerant wheat cultivar had a higher proportion of bound water than did the drought sensitive cultivar. This led to the thinking that the binding of water was a crucial component of desiccation tolerance. Comparisons of the properties of water in mature recalcitrant and orthodox seeds, however, showed no major difference between the embryos of these two seed types (Berjak, Pammenter & Vertucci, 1992; Berjak, Vertucci & Pammenter, 1993; Pammenter *et al.*, 1991; 1992). Pammenter *et al.* (1991) found that the amount of bound water was similar in the embryonic axis of both desiccation-tolerant and sensitive seeds (approximately  $0.28 \text{ g (gdw)}^{-1}$ ). This suggests that the proportion of non-freezable water is not critical in terms of desiccation tolerance; rather, it is the differing responses to removal of this type of water that distinguishes between desiccation-tolerant and sensitive seeds (Pammenter *et al.*, 1991).

The amount of bound water has been measured by the use of adsorption isotherms (Rascio *et al.*, 1992; Vertucci & Leopold, 1987; Vertucci *et al.*, 1985) and more recently by the use of differential scanning calorimetry (Farrant & Vertucci, 1995; Pammenter *et al.*,

1991; 1992; Vertucci, 1989; 1990). Differential scanning calorimetry (DSC) measures the enthalpy associated with phase transitions. If the enthalpy of freezing or melting of water is measured as tissue is dried, a water content is reached at which the water in the tissue is no longer observed to freeze. The water remaining in the tissue at this water content is referred to as non-freezable.

### Aim of study

The aim of this part of the work was to establish the inter-relationship of different measures of water status which could be used in later studies. A further aim was to characterise the plant water relations of the five species using PV curves to determine the bulk modulus of elasticity and the RWC at which turgor was lost. The amount of non-freezable water present in the five species was also determined. Differences between the tolerant plant and the sensitive plants were noted.

## **Materials and Method**

### Measurements of water status

#### Water content determination

Water content of the leaf tissue were determined by weighing the material and then placing it in an oven at 70°C for 24 hours to determine the oven dry weight.

#### Relative water content (RWC)

Relative water content was calculated by determining the water content of the leaf tissue as a percentage of the water content at full turgor. Full turgor was determined by placing the leaf petioles in a beaker of water and covering the beaker to prevent evaporative water loss. The covered beaker was placed in darkened cupboard for 24 hours. A standard curve of water content versus RWC was established which could allow inter-conversion from one parameter to the other.

#### Water potential

A standard curve of water potential versus RWC was set up using the following

procedure. Leaf material was brought to full turgor and placed above saturated salt solutions and allowed to equilibrate with the atmosphere above these solutions (Winston & Bates, 1960). The relative humidity (RH) of the atmosphere above the saturated salt solutions is known and the water potential of the leaf tissue can be calculated from these RHs. Table 2.1 shows the salt solutions used, the equilibrium RH and equivalent water potential at a temperature of 20°C. The sealed chambers with the saturated salt solutions in them were kept in an air-conditioned room at 20°C. Small electrical fans were placed in the chambers to keep the air circulating and thus prevent the development of a gradient in RH away from the solution and to increase the rate at which equilibrium was reached. The tissue was exposed to the salt solutions for a minimum of two days for the high RHs and up to two weeks for the low RHs.

**Table 2.1:** The saturated salt solutions used to calculate the water potential of leaf tissue equilibrated with this atmosphere. The RH of the atmosphere and the corresponding water potential of the leaf tissue is shown. Data was taken from Winston and Bates (1960).

Saturated salt solution	RH of atmosphere at 20°C (%)	Water potential (MPa)
$\text{CaSO}_4 \cdot 5\text{H}_2\text{O}$	98	-2.76
$\text{K}_2\text{SO}_4$	96.5	-4.86
$\text{Na}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$	95	-6.99
$\text{NH}_4\text{H}_2\text{PO}_4$	93.1	-9.75
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	90	-14.37
KCl	86	-20.57

### Pressure Volume curves

PV curves were constructed for the five species using thermocouple psychrometry. Leaf material of the five species was brought to full turgor as described above for the RWC measurements. Leaf tissue was then placed in WESCOR (Logan, Utah, USA) sample

holders (7 mm diameter x 2.5 mm deep) and then sealed in a WESCOR C-52 sample chamber and thermocouple psychrometer. Water potential was measured with a WESCOR HR 33T Dew Point Microvoltmeter. A standard curve using NaCl solutions of varying molalities, and thus varying water potentials, was established and water potential values of the leaf tissue were read from this curve. Once leaf tissue had been sealed in the chamber it was allowed to equilibrate with the atmosphere in the chamber. Equilibration times varied for the different species (7 hours for *A. raddianum* and *C. nanum* to 24 hours for *G. livingstonei*) and also increased with decreasing water content of the leaf tissue. Care was taken to ensure equilibrium had been reached, as insufficient time for equilibration is a potential source of error in the measurements of water potential using thermocouple psychrometers.

After the first measurement of water potential the material was removed from the chamber and reweighed. As the leaf tissue loses water while coming into equilibrium with the chamber atmosphere it is this weight which was used for the calculation of RWC. The leaf tissue was then allowed to dry slightly (with the higher RWCs just moving it between the chamber and the balance resulted in sufficient drying). The material was again sealed in the chamber, allowed to equilibrate and again a water potential and weight were determined. This process was repeated until the PV curve became linear at which point the leaf tissue was dried for 24 hours at 70°C to determine the dry weight. At least three replicates were done for each species and the data were pooled.

#### Measurements from the PV curves

Data from each species were plotted as the reciprocal of water potential versus the relative water content. The linear portion of the PV curve was fitted using a regression line while the curved portion was fitted by eye. The bulk modulus of elasticity was calculated for each species between 90% and 85% RWC. A further calculation was done for *C. nanum* between 65% and 55% RWC. Bulk modulus of elasticity ( $\epsilon$ ) was calculated using the formula:  $\epsilon = (dP/dV)V$  where V is equal to the cell volume. Where cell volume could not be accurately determined (*C. nanum* and *I. woodii*) or where the estimate of cell volume was greater than 100% RWC (*A. raddianum* and *P. sativum*), the value of V was taken as 1.

The RWC at which the curve became linear was taken as the point where turgor was lost. Extrapolation of the linear portion of the curve to 100% RWC gave the inverse of the osmotic potential at full turgor. Extrapolation of the linear portion to 1/water potential = 0 gave the amount of symplastic water in the tissue. It was not always possible to extrapolate this value due to the curve intersecting the abscissa at values less than zero RWC.

### Calorimetry

The amount of non-freezable water was measured using a Perkin-Elmer DSC-7 with temperatures calibrated between  $-95^{\circ}\text{C}$  and  $156^{\circ}\text{C}$  using methylene chloride and indium standards. Measurements were done on leaf material which had been air-dried to various water contents and sealed in aluminium sample pans. The pans were cooled at a rate of  $10^{\circ}\text{C min}^{-1}$  to  $-70^{\circ}\text{C}$  and then heated at the same rate to  $30^{\circ}\text{C}$ . After the thermal transitions were recorded, the pans were punctured and placed in an oven at  $70^{\circ}\text{C}$  for 24 hours and re-weighed. Dry weights and water contents of the material were determined.

Only melting thermal transitions were analysed due to their less complicated nature. The energy of the thermal transition was determined from the area of the peak above or below the baseline. Transition enthalpies expressed per unit dry weight of the sample were plotted against sample water content. A linear regression analysis was performed on the linear portion of the graph (all the points above zero). The intercept on the axis gives the amount of non-freezable water in the tissue under the conditions used here. The slope of the regression line gave the energy associated with the melting of water in  $\text{J (gH}_2\text{O)}^{-1}$ .

## **Results**

### Measures of water status

Figure 2.9 shows the standard curves set up which relate water content ( $\text{g (gdw)}^{-1}$ ) and RWC (%) for all five species. The solid line drawn in is the line obtained from a linear regression analysis of the data. The water content at full turgor (100% RWC) was very high for *P. sativum*, about  $12 \text{ g (gdw)}^{-1}$ , compared with *A. raddianum*,  $3.5 \text{ g (gdw)}^{-1}$  and *I. woodii*,  $4 \text{ g (gdw)}^{-1}$ . *Craterostigma nanum* had a water content of about  $7 \text{ g (gdw)}^{-1}$  at

full turgor and *G. livingstonei* had the lowest water content at full turgor (about 1.4 g (gdw)<sup>-1</sup>). The relationship between water potential (MPa) and RWC is shown in Figure 2.10. The RWC dropped steeply with only a slight change in water potential at water potentials above about -9 to -10 MPa (about 20% RWC) for all five species. Below -10 MPa there were only slight changes in water potential with changes in RWC.

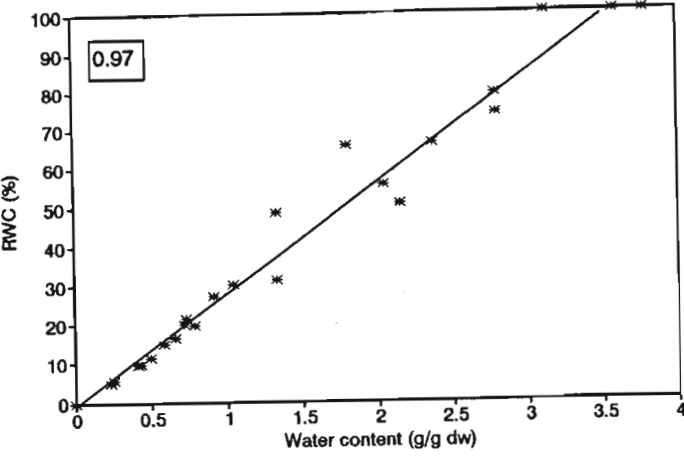
#### Pressure Volume curves

Figure 2.11 shows the PV curves for the five species with the regression lines for the linear portions of the curve drawn in. Table 2.2 summarises the data obtained from these curves.

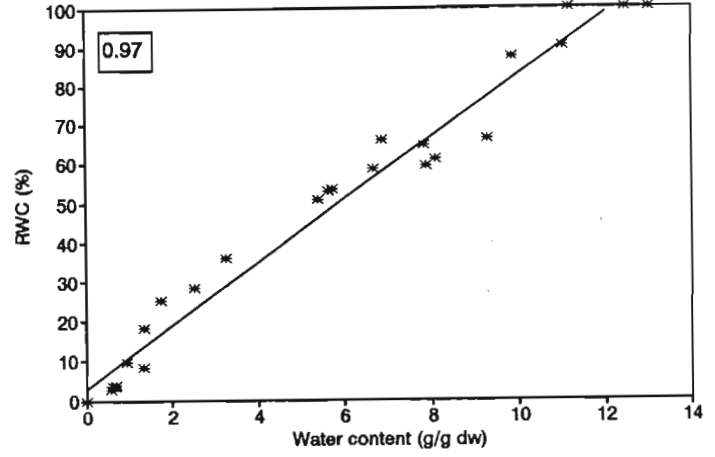
The PV curves shown in Figure 2.11 are the typical curvi-linear shape for the four desiccation-sensitive species. The tolerant species, *C. nanum*, however had an unusual shape. The initial decrease was similar to that of the sensitive species but then there appeared to be very little change in pressure with change in volume between 70% and 50% RWC. Below about 37% RWC there was a linear decrease in pressure with decreasing volume.

The only species for which the percentage of symplastic and apoplastic water was readily determined was *G. livingstonei*, which had a symplastic water content of 80% of its total water content. For both *I. woodii* and *C. nanum* the symplastic water content was very close to 100% of the total water content. It was not possible to use the PV curves to determine the percentage of symplastic water for *A. raddianum* and *P. sativum* as the curve never intercepted the abscissa. As *G. livingstonei* is a woody species one would expect the symplastic volume to be less than that for herbaceous species.

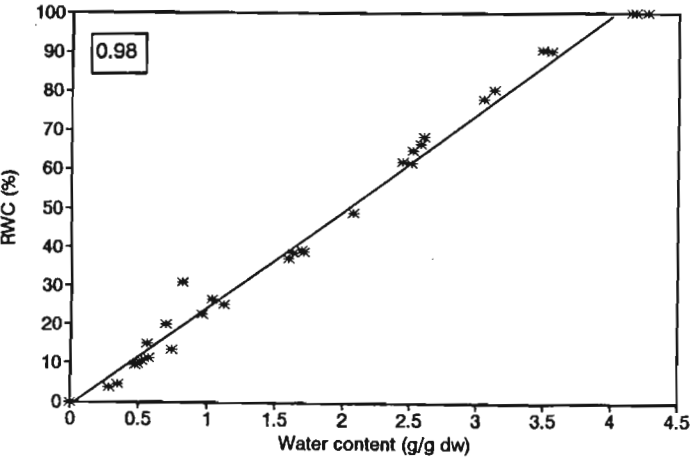
*Adiantum raddianum*



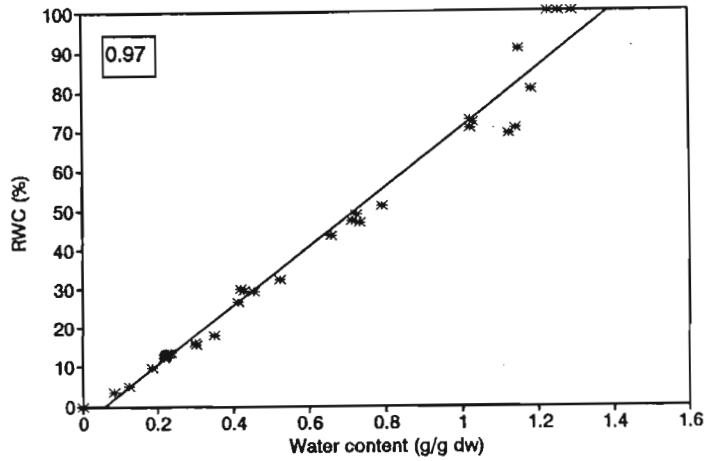
*Pisum sativum* 48



*Isoglossa woodii*



*Garcinia livingstonei*



*Craterostigma nanum*

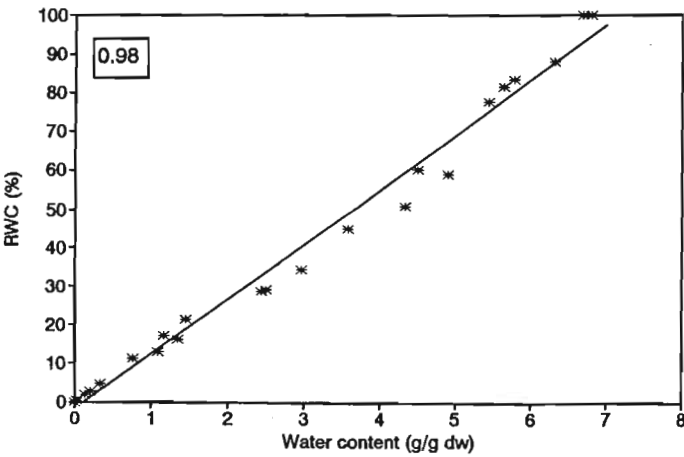
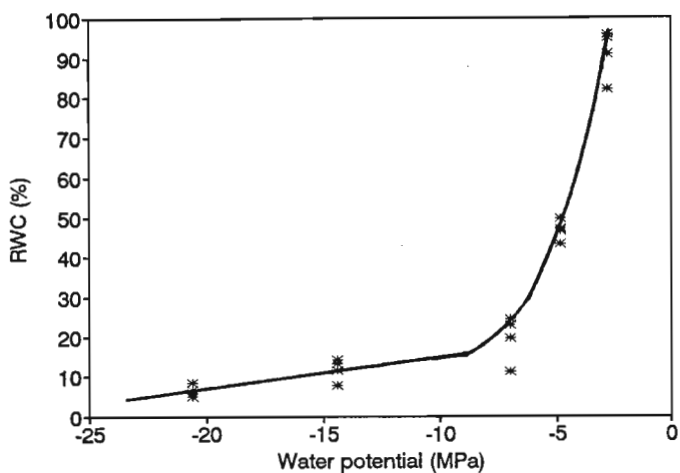
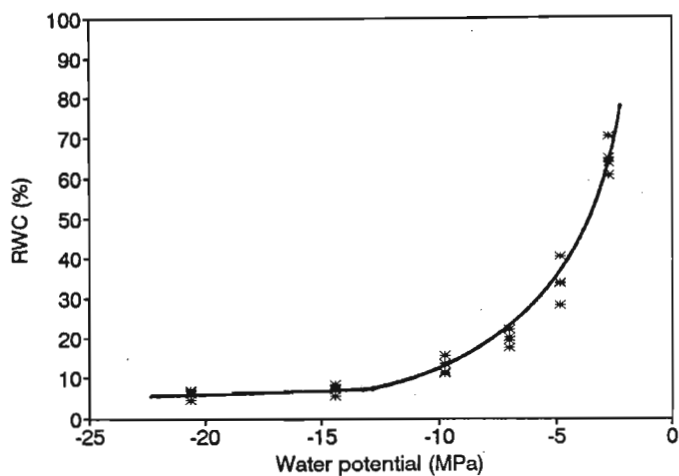


Figure 2.9: Relative water content (RWC) versus water content ( $\text{g}(\text{gdw})^{-1}$ ) for the five species. Regression analysis was done on the data and the resultant regression line is shown in the graph. The R squared value (goodness of fit) is shown in the top left hand corner of the graph. These graphs were used to convert from water content to RWC in later studies.

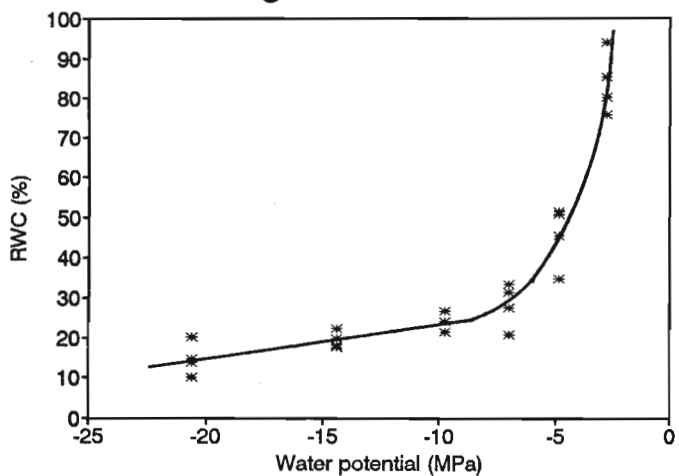
*Adiantum raddianum*



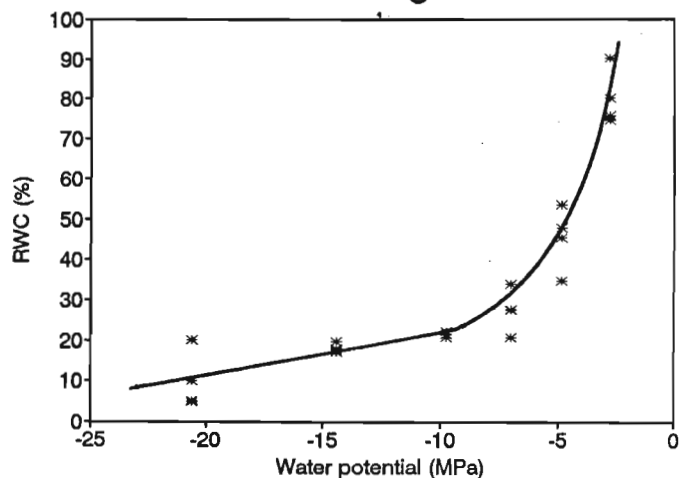
*Pisum sativum* 49



*Isoglossa woodii*



*Garcinia livingstonei*



*Craterostigma nanum*

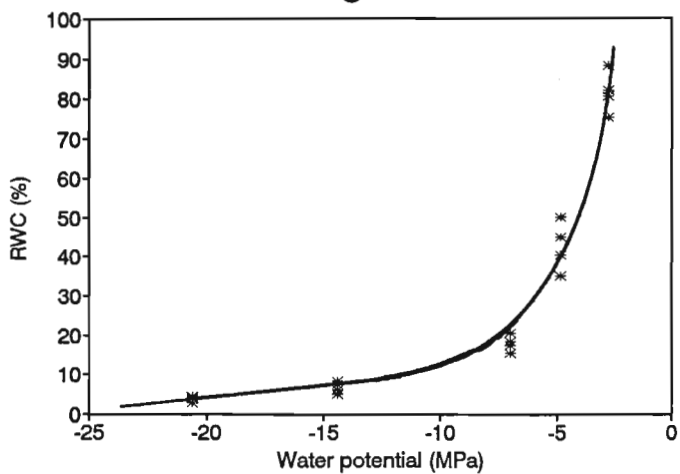
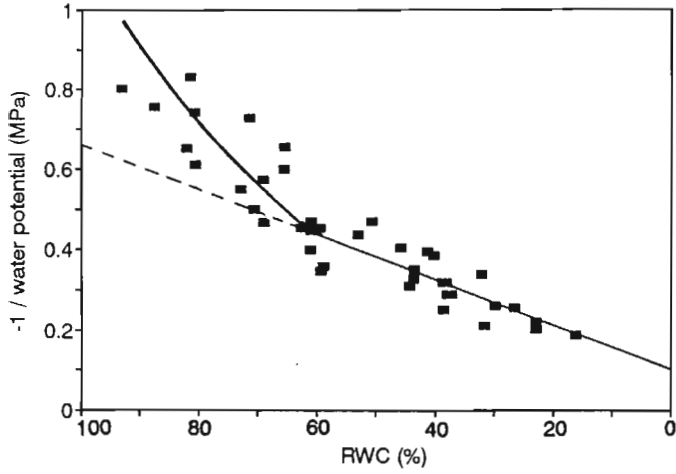
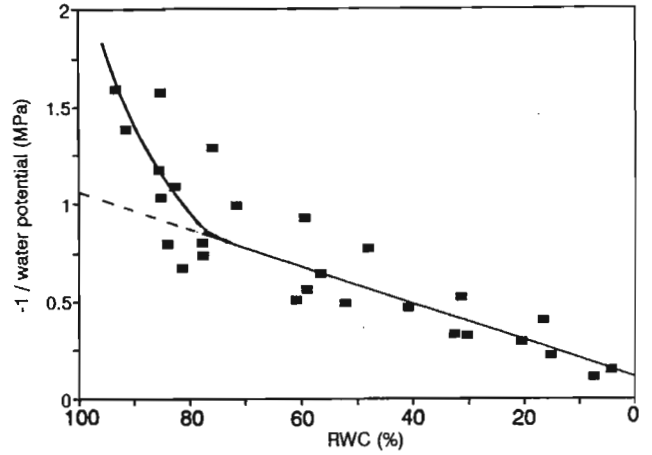
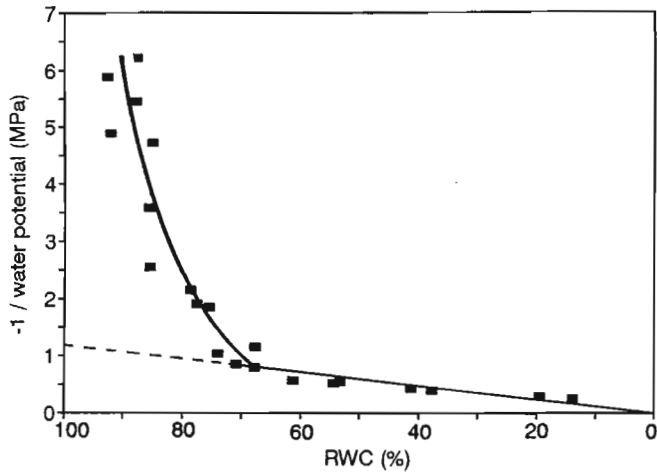
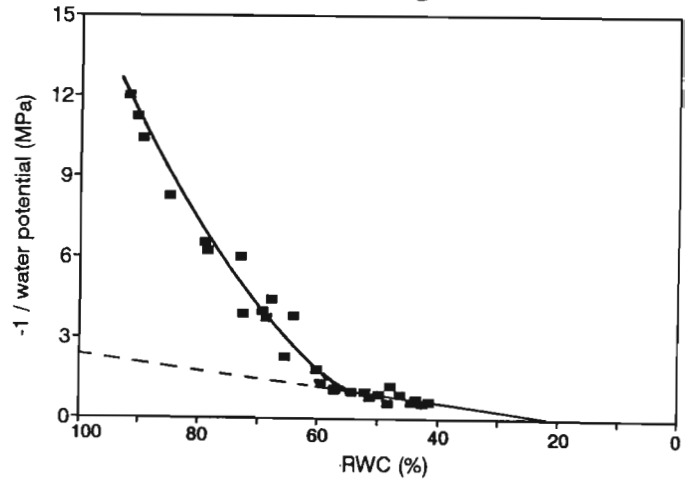
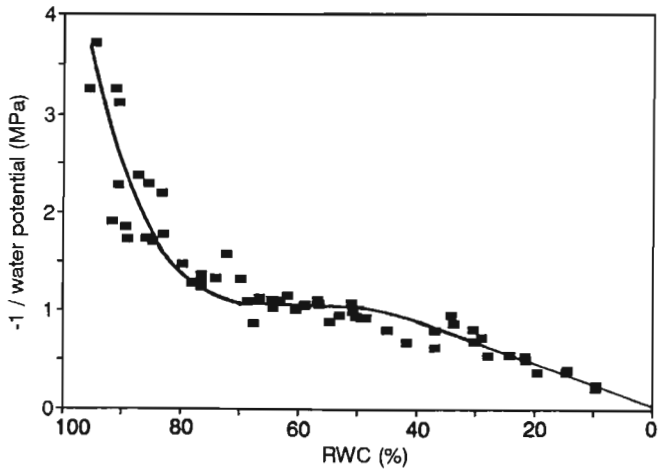


Figure 2.10: Relative water content (RWC) versus water potential (MPa) for the five species. Water potential was determined by placing leaf tissue over various saturated salt solutions. The curves have been fitted by eye.

*Adiantum raddianum**Pisum sativum**Isoglossa woodii**Garcinia livingstonei**Craterostigma nanum*

**Figure 2.11:** The pressure-volume curves for the five species. Regression analyses were performed on the linear portions of the curves and the resultant regression line has been drawn in and extrapolated to 100% RWC and  $1/\text{water potential} = 0$  (if possible).

**Table 2.2:** The RWC at zero turgor, osmotic potential ( $\Psi_{\pi}$ ) at full and zero turgor and the bulk modulus of elasticity ( $\epsilon$ ) of the four desiccation-sensitive species. These data were derived from the pressure-volume curves shown in Figure 2.11. Due to the unusual nature of the PV curve for *C. nanum*, it was not possible to accurately determine the RWC at which turgor was lost and the osmotic potential at full and zero turgor. A turgor loss point for *C. nanum* has, however, been estimated. Two values have been given for the bulk modulus of elasticity for *C. nanum*. The first (13 MPa) was calculated between RWC of 90% and 85% and the second (0.5 MPa) was calculated between RWC 65% and 55%. The percentage of apoplastic water is also presented for the species for which it could be determined from the PV curves.

Species	RWC at zero turgor	$\Psi_{\pi}$ at full turgor (MPa)	$\Psi_{\pi}$ at zero turgor (MPa)	$\epsilon$ (MPa)	Apoplastic water (%)
<i>A. raddianum</i>	63%	-1.5	-2.2	2.6	-
<i>P. sativum</i>	78%	-0.94	-1.1	5	-
<i>I. woodii</i>	70%	-0.84	-1.25	15	3
<i>G. livingstonei</i>	58%	-0.42	-1.3	20	21
<i>C. nanum</i>	37%	-	-1.25	13 & 0.5	-

*Isoglossa woodii* and *P. sativum* lost turgor at similar RWCs and water potentials (75%, -1.1 MPa and 78%, -1.25 MPa respectively). The RWC and water potential at which *A. raddianum* lost turgor was lower than this (63%, -2.2 MPa) and *G. livingstonei* lost turgor at an even lower RWC (58%), however the water potential at which turgor was lost in this species (-1.3 MPa) was similar to that of *I. woodii* and *P. sativum*. The estimated RWC at which turgor was lost in *C. nanum* (37%) was very low, but corresponded to a water potential of about -1.25 MPa, which was similar to that of the sensitive species. The osmotic potential of *A. raddianum* was lower than that of the other species, even at full

turgor, while that of *G. livingstonei* was higher. The bulk modulus of elasticity ( $\epsilon$ ) was lowest for *A. raddianum* and highest for *G. livingstonei*. This indicates that *G. livingstonei* had the most rigid cell walls followed by *I. woodii* which also had relatively rigid cell walls, while those of *A. raddianum* and *P. sativum* were elastic.

Due to the unusual nature of the PV curve for *C. nanum* it was difficult to determine where turgor was lost. There are two possibilities. The first is at a RWC of 70%, where the curve flattens out and the second possibility is at about 37%, where the curve becomes linear. It appears as if the elasticity of the cell walls undergoes a drastic change between RWCs of 70% and 45%. Two values were calculated for  $\epsilon$ . The first was taken from the sharp decrease in pressure with decreasing volume. This is where  $\epsilon$  was measured for the sensitive species. This value of  $\epsilon$  was relatively high (13 MPa) and thus indicated rigid cell walls. The second value for  $\epsilon$  taken from the flat portion of the curve was very low (0.5 MPa), indicating that at these RWC the cell walls were very elastic. This second value is relevant only if turgor is lost at 37% RWC and not 70% RWC. The standard convention of extrapolating the linear portion of the PV curve to RWC = 100% to give osmotic potential at full turgor could not be followed done as this would have meant that the osmotic potential was greater than total water potential for a range of RWCs, which is not possible.

### Calorimetry

Figure 2.12 shows typical melting curves for dehydrating plant tissue. Data for *C. nanum* were used to construct these curves. The area below the peak was reduced as water was lost from the plant tissue. All five species showed similar patterns in their melting curves. Figure 2.13 shows the enthalpy per unit dry matter associated with the melting of tissue water versus the water content. The lines do not pass through the origin but have a positive water content intercept. This indicates that there is a certain amount of water in each of the five species which is not freezable. The slopes of the lines shown in Figure 2.13 are equivalent to the enthalpy of melt of tissue water. Table 2.3 gives the water contents, RWCs and water potentials of the non-freezable water as well as the melting enthalpy of the tissue water.

**Table 2.3:** Water content, RWC and water potential of non-freezable water present in the leaf tissue of the five species studied. The estimations of water potential were not precise as the values were taken from the lower ends of the graphs shown in Figure 2.10. The melting enthalpies, determined by the slope of the regression lines in Figure 2.13 is also presented.

Species	Water content (g (gdw) <sup>-1</sup> )	RWC (%)	Water potential (MPa)	Melting enthalpy J(gH <sub>2</sub> O) <sup>-1</sup>
<i>A. raddianum</i>	0.43	10	-15	245
<i>P. sativum</i>	0.49	7	-18	282
<i>I. woodii</i>	0.27	6.5	< -25	172
<i>G. livingstonei</i>	0.3	18	-17	254
<i>C. nanum</i>	0.26	4	-19	266

From Table 2.3 it can be seen that the absolute amount of non-freezable water is relatively similar for all five species even though they have very different water contents at full turgor. The highest amount of non-freezable water occurs in *P. sativum* and the lowest in *I. woodii* and *C. nanum*. The amount of non-freezable water on a RWC basis is similar for *P. sativum* and *I. woodii*. *Garcinia livingstonei* has a greater proportion of its water as non-freezable water, as shown by the high RWC of non-freezable water (18%), than the rest of the sensitive species. The desiccation-tolerant species, *C. nanum*, had the smallest proportion of non-freezable water. The water potential at which non-freezable water is found was similar for all the species except *I. woodii*, which was substantially lower than the rest. It was difficult to determine the exact water potential at which non-freezable water occurred in *I. woodii*, but it would have been below -25 MPa. These estimates of water potential were not precise as the values were taken from the lower ends of the graphs shown in Figure 2.10. The water potentials at which non-freezable water

occurs fall in the range of water potentials suggested for type 2 water by Clegg (1979).

The melting enthalpies of all the species (Table 2.3) were lower than the melting enthalpy for pure water ( $333 \text{ J (gH}_2\text{O)}^{-1}$ ) reported in Farrant and Vertucci (1995). The melting enthalpy of the desiccation-tolerant species *C. nanum* ( $266 \text{ J (gH}_2\text{O)}^{-1}$ ) was similar to that of three of the sensitive species, *A. raddianum* ( $245 \text{ J (gH}_2\text{O)}^{-1}$ ), *P. sativum* ( $282 \text{ J (gH}_2\text{O)}^{-1}$ ) and *G. livingstonei* ( $254 \text{ J (gH}_2\text{O)}^{-1}$ ). The melting enthalpy of *I. woodii* ( $172 \text{ J (gH}_2\text{O)}^{-1}$ ) was lower than those of the other sensitive species and that of the tolerant species. The low melting enthalpy may be the result of a highly viscous and concentrated cell solution.

## Discussion

The water content at full turgor for *P. sativum* was much higher than that of any of the other species, while that of *G. livingstonei* was much lower. This is possibly due to pea plants relying on water for physical support while the drought-tolerant tree, *G. livingstonei*, has thick, rigid leaves. The water content at full turgor of the desiccation-tolerant plant *C. nanum* fell within the range found in the sensitive species.

One area where the tolerant species was very different from the sensitive species was in its water relations as measured using pressure-volume curves. The PV curve for the desiccation-tolerant plant, *C. nanum*, was different from the curves for the sensitive species and the standard PV curves reported in the literature (Tyree & Hammel, 1972). The PV curve for *C. nanum* indicated that there is a change in cell wall elasticity at RWCs below 70%. Similar results have been obtained for PV curves of *Myrothamnus flabellifolia*, a desiccation-tolerant species (R. Beckett pers. comm.<sup>2</sup>). Another desiccation-tolerant plant, *Xerophyta villosa*, had curvi-linear PV curves similar to those of the desiccation-sensitive species (R. Beckett, pers. comm.). *Xerophyta villosa* is a monocotyledonous species which loses its chlorophyll on drying and may thus have different adaptations to tolerate desiccation compared with the two dicotyledonous,

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<sup>2</sup> R. Beckett, Botany Department, University of Natal, Pietermaritzburg, South Africa.

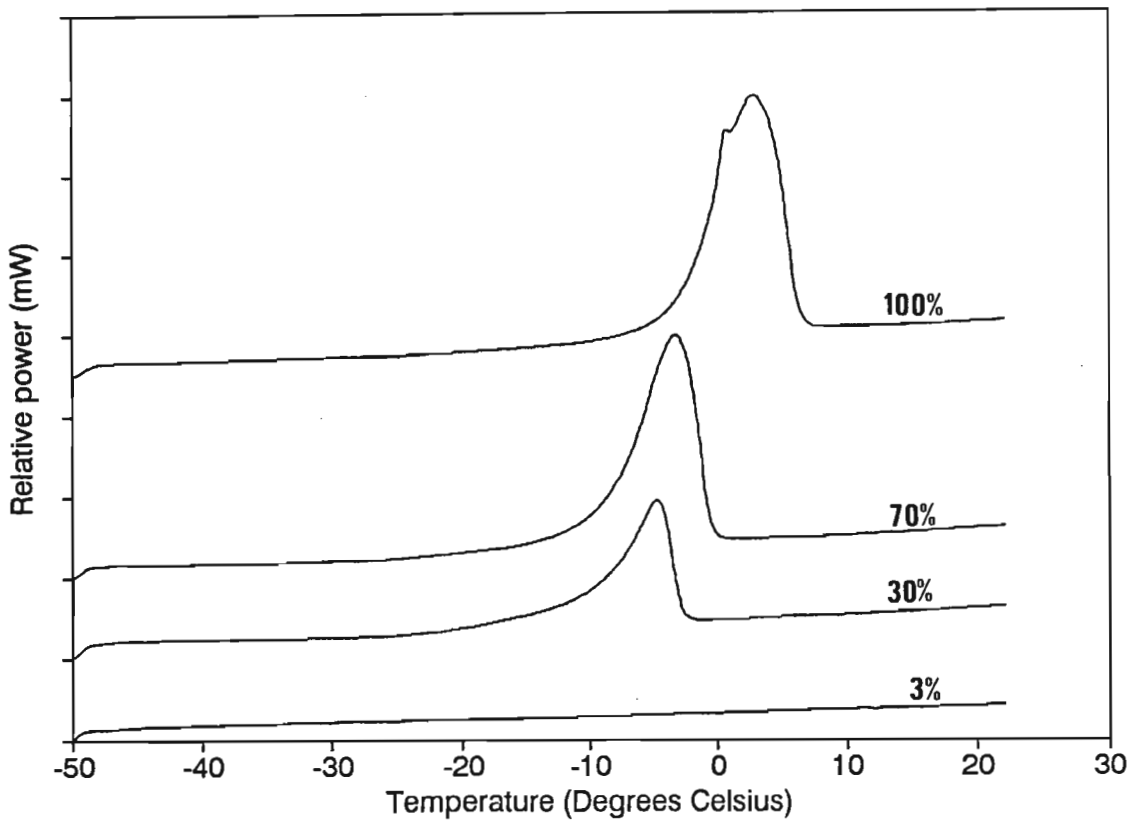


Figure 2.12: Typical DSC melting thermograms of leaf tissue at different relative water contents. *Craterostigma nanum* leaves were used to generate these melting curves. Samples were scanned at  $10^{\circ}\text{C min}^{-1}$  from  $-70^{\circ}\text{C}$  to  $30^{\circ}\text{C}$ . The RWC at which these measurements were taken are shown on the different curves.

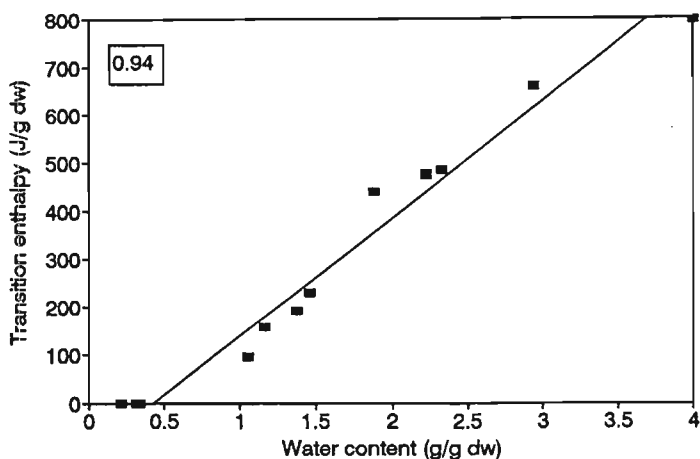
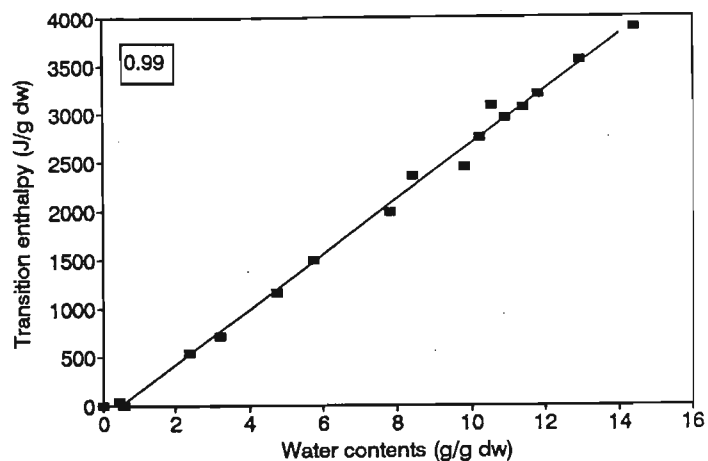
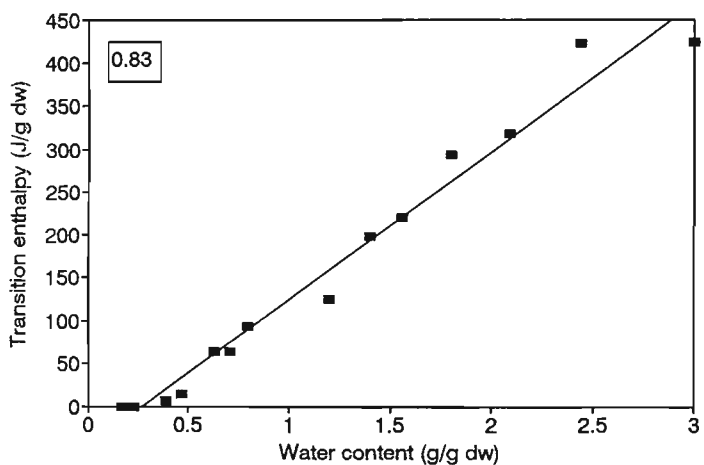
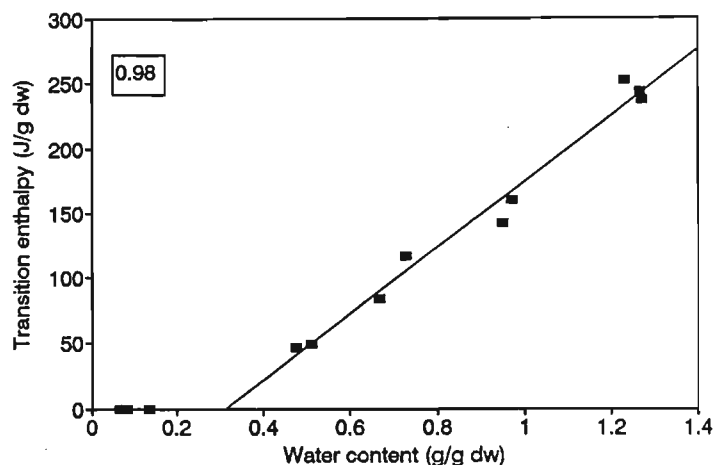
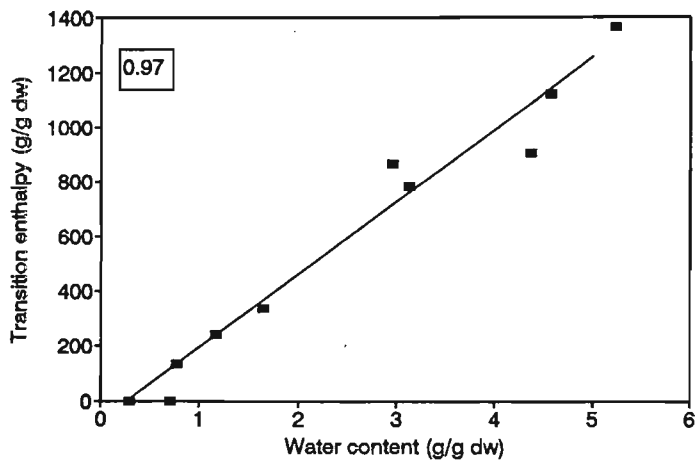
*Adiantum raddianum**Pisum sativum**Isoglossa woodii**Garcinia livingstonei**Craterostigma nanum*

Figure 2.13: The effect of moisture content on the enthalpy of the melting transitions in the five species studied. The lines represent the linear regression analysis of the points and the  $R^2$  value is shown in the top left hand corner of the individual graphs.

chlorophyll-retaining species, *C. nanum* and *M. flabellifolia*. Davies and Lakso (1979) state that the development of more elastic tissues under water stress as RWC decreases is highly desirable as this allows for turgor maintenance under stress conditions. Such changes have been noted with more drought tolerant species (Davies & Lakso, 1979; Roberts *et al.*, 1981; Saliendra & Meinzer, 1991) but the changes were not as marked as those shown for *C. nanum* and *M. flabellifolia*. The response of these two desiccation-tolerant species may be an extreme of the drought response. The drought tolerant species used in this study, *G. livingstonei*, had rigid cell walls, which is typical of drought-tolerant tissue (Cheung, Tyree & Dainty, 1975). In this species, however, there did not appear to be marked changes in cell wall elasticity at lower RWCs as has been reported for other drought tolerant species (Davies & Lakso, 1979; Roberts *et al.*, 1981; Saliendra & Meinzer, 1991).

The values for bulk modulus of elasticity ( $\epsilon$ ) obtained for the species used in this study were similar to those obtained for other plant species (Dainty, 1976; Davies & Lakso, 1979; Roberts, *et al.*, 1981). The higher values for  $\epsilon$  of *G. livingstonei* and *I. woodii* are probably related to their habitat. *Garcinia livingstonei* is a drought-tolerant tree and the rigid cell walls may be an adaptation that prevents severe water loss by leading to marked decreases in turgor with only minor changes in RWC and thus increased water uptake (large gradient) or reduced water loss by causing stomatal closure. The rigid walls of *I. woodii* may enhance its ability to extract water from the soil as it is often subjected to daily wilting. The elastic cell walls of the other two species are probably a result of their growing in moist habitats. The pea seedlings, in particular, grow very fast and probably have high transpiration rates. Elastic cell walls would allow for a certain amount of water loss before severe turgor loss.

Loss of turgor potential is deleterious to a cell in that turgor is needed to maintain growth. Turgor is lost at lower RWCs for *G. livingstonei* and *A. raddianum* than the other two sensitive species. If the estimate of turgor loss for *C. nanum* is correct, then this desiccation-tolerant plant loses turgor at very low RWCs. The RWC at which turgor is lost has been reported in the literature for a number of crop and some tree species; it ranges between about 65% and 90% (Cheung *et al.*, 1975; Kikuta & Richter, 1986; Saliendra &

Meinzer, 1991; Stadelmann, 1984). Thus it appears that the desiccation-tolerant species can maintain turgor till very low RWCs compared to sensitive species. Even though the RWC at which turgor is lost in *C. nanum* was very low, the water potential at which turgor was lost was similar to that of the sensitive species and those reported in the literature for sensitive species (Boyer & Potter, 1973; Cheung *et al.*, 1975; Kikuta & Richter, 1986; Saliendra & Meinzer, 1991; Stadelmann, 1984). The water potential at which turgor was lost was lowest in the fern *A. raddianum*. Why turgor is lost at lower RWCs and water potentials in *A. raddianum* than *P. sativum* and *I. woodii* is not known. It could be because the fern cannot recover from wilting whereas the other two species can. It would thus be advantageous to maintain turgor to lower RWCs.

There was very little to distinguish the tolerant and sensitive species in terms of non-freezable water. These results indicate that the absolute amount of non-freezable water present in a leaf does not play a role in desiccation tolerance. These observations are similar to those made on seeds by Pammenter *et al.* (1992). It has been suggested that tolerant seeds can withstand the loss of a substantial portion of this non-freezable water whereas sensitive seeds are damaged when non-freezable water is removed (Pammenter *et al.*, 1991). However, membrane damage in sensitive seed tissues has been recorded at higher water contents than the water content of non-freezable water (Pammenter *et al.*, 1992).

The absolute amounts of non-freezable water present in these leaf tissues were similar to that observed for a range of orthodox and recalcitrant seed species (Farrant & Vertucci, 1995; Pammenter *et al.*, 1991; 1992; Vertucci, 1989; 1990), although the amount of non-freezable water in the pea and fern was higher than that recorded for the seed material. The results for the DSC studies showed that there was only little difference in the amount of non-freezable water present in the desiccation-tolerant and desiccation-sensitive species studied, despite large differences in the amount of water that the different species possessed at full turgor.

The water potentials at which non-freezable water occurs in this study, whilst not being very accurate estimates, fall within the range of type 2 water (Clegg, 1979). This is where

they would be expected to fall. Type 2 water has solvent properties and it has the ability to form glasses, but it is not observed to show freezing or melting transitions (Vertucci, 1990).

The melting enthalpies for all the species except *I. woodii*, shown in Table 2.3, were similar to the melting enthalpies of Type 4 water in pea and soybean cotyledons (Vertucci, 1990). The melting enthalpies are lower than those for pure water which is not surprising given that the water in leaf tissue contains various solutes. The melting enthalpy of *I. woodii* was lower than that for the other species and similar to water at hydration level 3 in pea cotyledons (Vertucci, 1990). Water that melts with low enthalpies have also been found in mature orthodox seeds (Vertucci, 1990), in the more desiccation-tolerant stages of *Aesculus hippocastanum* seed development (Farrant & Vertucci, 1995) and in embryos of the intermediate seed type *Zizania* (Vertucci *et al.*, 1994). This low melting enthalpy is an indication of a concentrated solution and the formation of glasses (Vertucci, 1990). Why the tissue water of *I. woodii* should have these properties which have been associated more with desiccation tolerance is unknown. The osmotic potentials at full and zero turgor were not lower than the other species (Table 2.2). The habitat of this species results in the plants experiencing water stress almost daily. For the species to be competitive it needs to recover rapidly from wilting which *I. woodii* does. The unusual thermal nature of the water in the leaf may play a role in the ability of this species to survive wilting and to recover rapidly from wilting. It is interesting to note that the desiccation-tolerant plant, *C. nanum*, does not have water with unusual thermal properties as do some other desiccation-tolerant tissues (Farrant & Vertucci, 1995; Vertucci, 1990; Vertucci *et al.*, 1994).

The work discussed in this Chapter provides some background information on the water relations of the species used in this study. The amount of water at full turgor and the amount of non-freezable water does not distinguish the tolerant from the sensitive species. The shape of the PV curve, however, is clearly different between the tolerant and sensitive species used in this study.

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## CHAPTER 3: DESICCATION SENSITIVITY

### Introduction

In order to assess sensitivity and/or tolerance to desiccation one needs to establish the water content at which the material becomes damaged and when that damage becomes lethal. The most direct means of doing this is to dry the plant to various water contents and then assess whether the leaves regain turgor and the plant continues to grow on re-watering. These survival studies were correlated with electrolyte leakage experiments, a commonly used approach to identify desiccation-tolerance at a more biochemical level.

### Membrane damage

Exposure of plants to various environmental stresses often results in damage to cell membranes. Contraction of cell contents during drying places considerable physical stress on the plasmalemma, particularly if cell walls are rigid (Levitt, 1980). Leakage of solutes from dry seeds, pollen and other plant material upon rehydration has been well documented and is thought to indicate membrane damage, of the plasmalemma in particular (Dhindsa & Bewley, 1977; Leopold, Musgrave & Williams, 1981; McKersie & Stinson, 1980; Senaratna & McKersie, 1983; Simon, 1974). Leakage is not limited to either tolerant or sensitive tissues; it has been recorded in both desiccation-tolerant and sensitive bryophytes upon rehydration following desiccation (Gupta, 1976; 1977), in drought-stressed leaves of *Populus deltoides* (Gebre & Kuhns, 1991) and bur oak, *Quercus macrocarpa*, (Kuhns, Stroup & Gebre, 1993) as well as in desiccation-tolerant and sensitive seeds (Berjak, Vertucci & Pammenter, 1993; Senaratna & McKersie, 1983; Senaratna, McKersie & Stinson, 1984). If the membrane damage is reversible, leakage is a transient phenomenon, lasting only a few minutes after the addition of water. This is well documented in seed tissue and is often referred to as imbibitional leakage. Prolonged loss of solutes, however, is characteristic of irreversibly damaged tissues (Bewley & Krochko, 1982).

Simon (1974; 1978) proposed that conformational changes in membrane structure on dehydration lead to the dry membranes becoming more porous because, in the dry state, membranes cannot retain their bilayer arrangement and form a hexagonal arrangement. The formation of hexagonal phases is associated with irreversible damage because it leads to membrane fusion, irreversible membrane disorganization and loss of cell compartmentation (Crowe *et al.*, 1986). The reversible increase in leakage exhibited by plant tissue then, is unlikely to be caused by the formation of hexagonal phases.

McKersie and Stinson (1980) using x-ray diffraction, showed that phospholipids from both desiccation-tolerant and sensitive tissue remained in a lamellar structure even at water contents as low as 5%. They concluded that membrane damage and the leakage of solutes from seeds could not be explained by the formation of a hexagonal phase by membrane phospholipids. The formation of a hexagonal phase has also been shown only for bulk phospholipid extracts and has not been reported in phospholipid bilayers as thin as biological membranes (Bewley & Krochko, 1982). Another less destructive type of phase transition, a liquid crystalline to gel phase transition, has been reported in desiccation-tolerant organisms (Hoekstra, Crowe & Crowe, 1991; Crowe, Hoekstra & Crowe, 1992).

The possibility of membrane phase transitions and the nature of these transitions are determined by the molecular geometry of the polar lipid components, the presence of other membrane constituents (proteins and/or sterols), the degree of curvature of the bilayer, the temperature and the degree of dehydration (Steponkus & Webb, 1992). Since phase transitions are reversible they may not be lethal in themselves. However, "demixing" of membrane constituents can lead to the irreversible efflux of membrane bound proteins (Senaratna, McKersie & Borochoy, 1987). Demixing may also lead to the formation of non-bilayer structures (Crowe *et al.*, 1986; McKersie, Crowe & Crowe, 1989, among others).

In dehydrated tissue the initial stages of rehydration pose particular problems for the cell. Imbibitional damage occurs when dry material is placed directly into water. This is physical damage to the membrane which results in an increase in membrane leakage. Hoekstra & van der Wal (1988) discuss the mechanisms of imbibitional damage and report

that prehumidification in a moist atmosphere is an accepted technique to lessen this damage.

### Rate of drying

Levitt (1980) reported some early work done on rates of dehydration and the effect they had on the survival of the plant. The results shown were contradictory in that some species were adversely affected by fast drying but not slow drying while other species showed no difference in survival on fast or slow drying. More recent work by Hetherington, Smillie and Hallam (1982) has also shown differences in survival of plant tissue with different rates of drying. These workers showed that fast drying was deleterious to the desiccation-tolerant plant, *Borya nitida*. This plant loses chlorophyll during slow, viable, drying whilst it retains chlorophyll during fast non-viable drying. The fast drying does not allow sufficient time for the dismantling of the chlorophyll and the chloroplast membranes which are necessary for survival of desiccation in this species. The work done on the desiccation-tolerant moss *Tortula ruralis* (reviewed by Bewley, 1979; Bewley & Krochko, 1982) has shown that, although both slow and rapidly dried moss survived desiccation, there appeared to be more damage occurring in the rapidly dried moss. Isolated axes from desiccation-sensitive seeds have been shown to survive to lower moisture contents if dried rapidly. However, such tissues do not survive unless immediately rehydrated or cryopreserved. It has been interpreted that survival is due only to the fact that water is removed too rapidly to allow damage to accumulate (Pammenter, Vertucci & Berjak, 1991).

Due to the reported significance of drying rates, it was decided to establish two different drying rates, one fast, using excised material, and a slower one achieved by drying the whole plant. When studying excised material one is removing any interaction or signals from the rest of the plant. It is a common feature of desiccation-tolerant higher plants that their leaves can tolerate desiccation whether attached to the plant or detached from the plant, provided they are dried slowly enough (Bartels *et al.*, 1990; Hetherington *et al.*, 1982). Thus the ability to tolerate desiccation in these leaves is not reliant on signals from the rest of the plant.

### Aim of study

The aim of this study was to assess the desiccation tolerance and sensitivity of the five species by determining to what water contents the material could be dried to and survive. Damage to membranes at various water contents, RWCs and water potentials was assessed using electrolyte conductivity and this was related to the water contents to which they could be dried to and survive. The nature of electrolyte leakage measurements is such that they are performed on rehydrated material, thus the extent of damage to leaf tissue in the dry state cannot be fully determined from these measurements.

There are a number of different damage parameters that can be obtained from studies on electrolyte leakage from cells. One of the oldest measurements is specific conductance, introduced by Dexter, Tottingham and Graber (1930), which just measured the electrical conductance of the bathing solution. Flint, Boyce and Beattie (1966, cited in Whitlow *et al.*, 1992) using Stuart's work (1939 cited in Whitlow *et al.*, 1992) introduced an index of injury which related the conductivity of tissue to its total possible conductivity as a percentage. This is still a commonly used measurement. More recently Whitlow *et al.* (1992) introduced a tissue ionic conductance which took into account variations in tissue thickness and electrolyte concentration.

Because comparisons of the absolute amount of leakage among the five species was not the aim of this work, it was decided only to measure the conductivity of the bathing solution over a period of time and obtain a rate of electrolyte leakage. This rate was divided by the dry weight of the material on which the measurements were taken. This rate of leakage per unit dry weight was measured over a range of water contents to assess changes in membrane permeability as the material was dried. This method has been used by Berjak, Pammenter and Vertucci (1992), Berjak *et al.* (1993), Pammenter *et al.*, (1991), Vertucci (1989) and others.

## **Materials and Method**

### Dehydration rates

Two dehydration methods were used. The first was fast dehydration or flash drying. This

was achieved by placing whole leaves or leaflets (in the case of *A. raddianum*, *P. sativum* and *C. nanum*) or leaf discs of 10 cm<sup>2</sup> (*G. livingstonei* and *I. woodii*) in chambers which had dry air circulating through them. The slow drying was achieved by withholding water from the potted specimens thus allowing the soil to dry out slowly. The rates of slow drying were plotted from the time when water contents of the leaves or leaflets started decreasing. At least ten leaves or potted plants were used per treatment.

### Survival studies

Potted material was well watered on day zero. Water was then withheld from the plants. After various periods leaf material was excised to measure water contents and some of the pots were re-watered. This was continued until the dried pot plants no longer recovered when re-watered. Survival was indicated by the regaining of turgor by the leaves and continued growth of the plant.

### Electrolyte leakage

Fast and slow dried leaves and leaf discs of the five species were slowly rehydrated, in order to prevent imbibitional damage, by placing them in a moist atmosphere overnight. The material was then placed in 30 ml of de-ionised water and electrical conductance was measured using a conductivity meter. Conductance was measured every five minutes for forty minutes to obtain a rate of leakage. Figure 3.1 shows a typical data set obtained from electrolyte leakage measurements over a period of forty minutes. The rate of leakage was obtained by plotting conductivity against time and a linear regression analysis was performed on the data from the linear portion of the slope (indicated in Fig. 3.1). This rate of leakage that was plotted was then divided by the dry weight of the material to give a rate of electrolyte leakage in units of  $\mu\text{S min}^{-1} \text{g}^{-1}$  dry weight. This rate of leakage was plotted as a function of water content.

To assess the extent of the possible imbibitional damage a further set of leaf material was flash dried and placed straight into the de-ionised water without prior rehydration in a moist atmosphere. Electrolyte leakage was measured as above.

At least ten replicates were examined for each treatment.

### Data analysis

The data sets were analyzed in the manner outlined by Berjak *et al.* (1992). Leakage rates were plotted against water content. Regression analyses were performed on the straight line portions of the graphs and a point of intersection was recorded. This point of intersection was recorded as the water content at which membrane leakage increased.

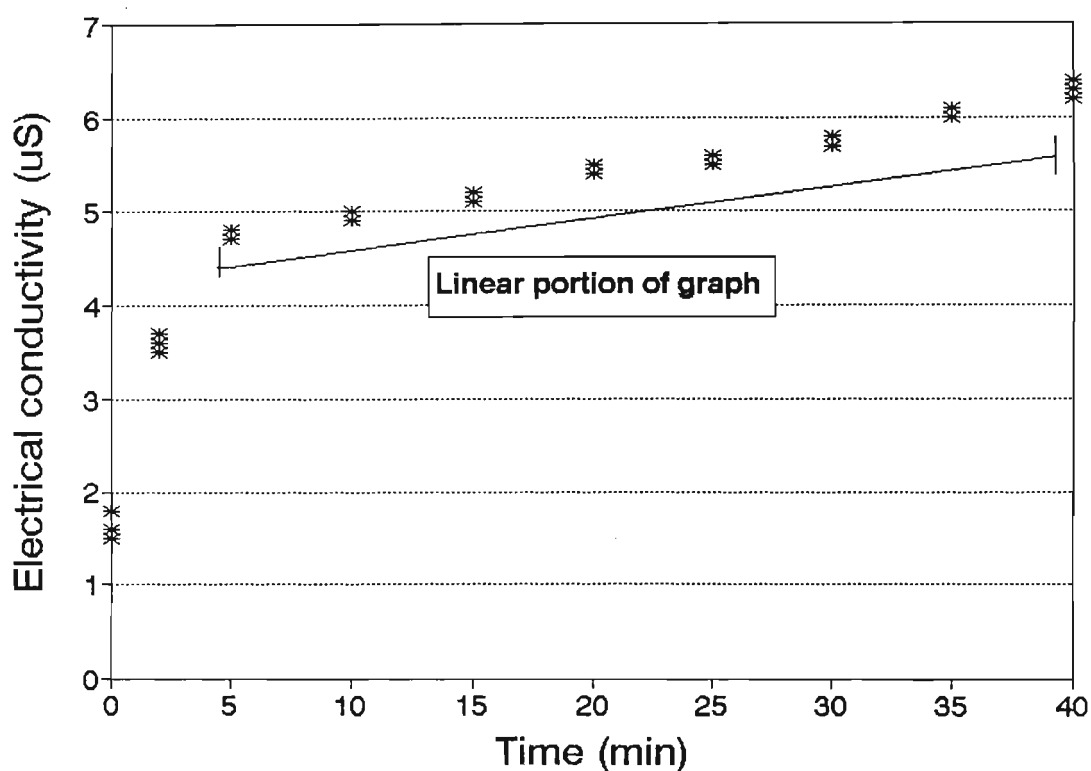


Figure 3.1: A representation of a typical data set obtained from measuring electrical conductivity of the bathing solution for forty minutes. The linear portion of the graph occurs after an initial rapid efflux of ions into the solution. It is this linear portion of the graph which is used to calculate a rate of electrolyte leakage. The data set shown in this figure was taken from *G. livingstonei* leaves at water contents of  $1 \text{ g (gdw)}^{-1}$ .

## Results

### Dehydration rates

The rapid and slow dehydration rates for the five species are shown in Figures 3.2 and 3.3, respectively. In the fast drying treatment (Fig. 3.2) the leaves initially lost water quite rapidly and then as water contents declined they lost water at a slower rate. *Adiantum raddianum*, *I. woodii* and *C. nanum* all reached water contents of slightly below 0.5 g (gdw)<sup>-1</sup> (10% RWC for *A. raddianum* and *I. woodii* and 5% RWC for *C. nanum*) within 35 hours. *Garcinia livingstonei* reached water contents of as low as 0.16 g (gdw)<sup>-1</sup> (5% RWC) within 30 hours. *Pisum sativum*, on the other hand, reached a water content of 2 g (gdw)<sup>-1</sup> (20% RWC) after 45 hours of flash drying. Drying for a further 45 hours was needed before it reached water contents of about 0.5 g (gdw)<sup>-1</sup> (5% RWC). The fact that leaves of *P. sativum* contain so much water at full turgor probably accounts for this slower drying.

The slow drying rates (Fig. 3.3) also showed differences in the time taken for leaf tissue to dehydrate. The fern, *A. raddianum*, took only four days to reach a water content of 0.15 g (gdw)<sup>-1</sup> (about 2% RWC). The desiccation-tolerant plant *C. nanum* took 12 days to reach water contents lower than 0.1 g (gdw)<sup>-1</sup> (less than 2% RWC). The other three desiccation-sensitive species took 25 to 30 days to reach very low water contents. The rate of slow drying would have depended on the ratio of leaf surface area to soil volume.

### Survival studies

The results of the survival studies of the five species are given in Tables 3.1 - 3.5. The terms slightly wilted, wilted and very wilted are used to describe the leaves or leaflets. The term wilted is used when the leaves or leaflets were drooping at their petioles and were flaccid. The term slightly wilted refers to the start of this process when the leaves or leaflets were no longer turgid and firm in appearance, but had not yet become flaccid and drooping. The term very wilted refers to when the leaves or leaflets had drooped and had started curling inwards and in some cases started becoming brittle and dry.

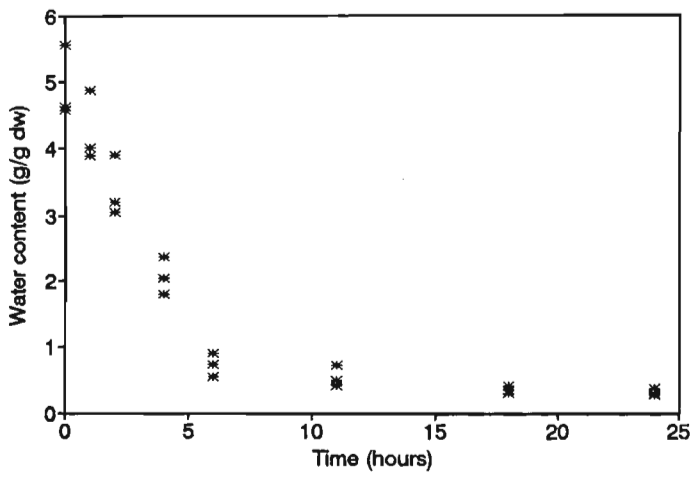
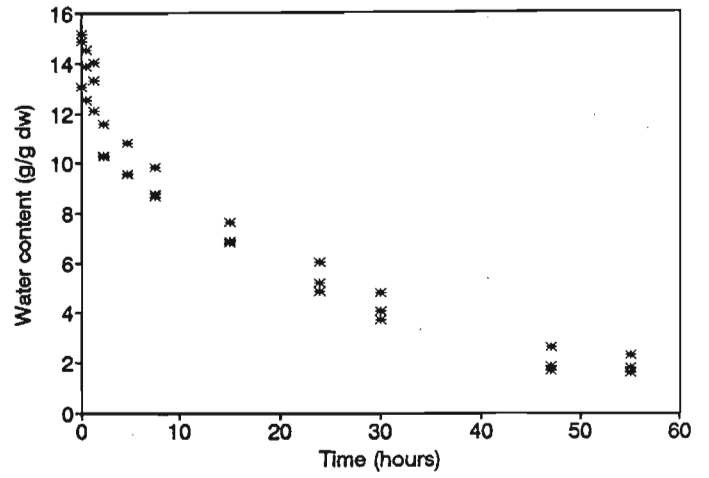
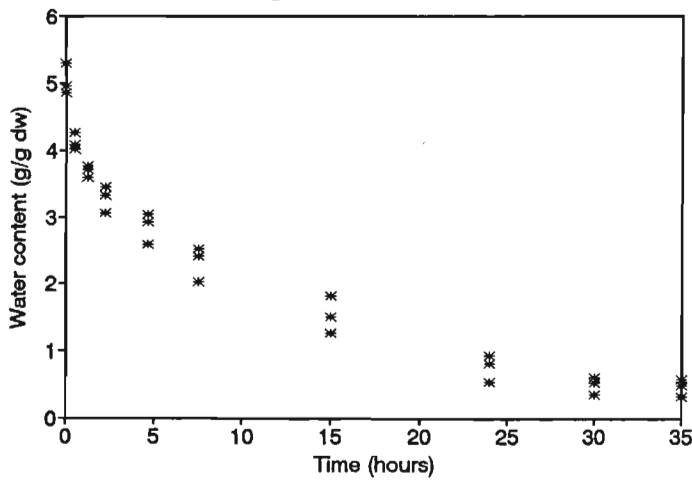
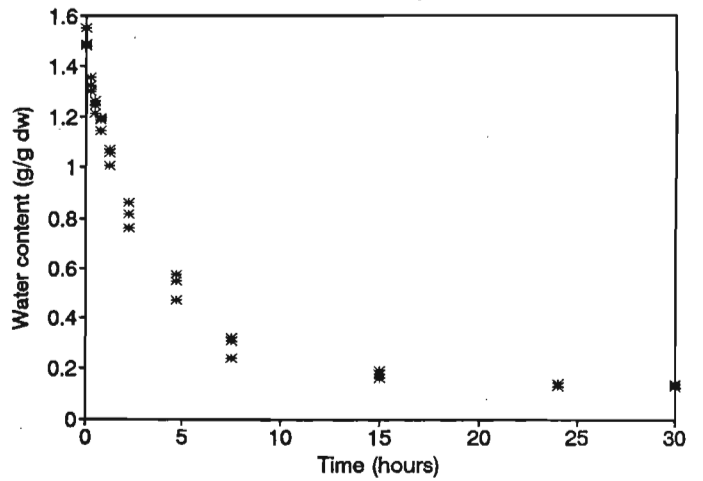
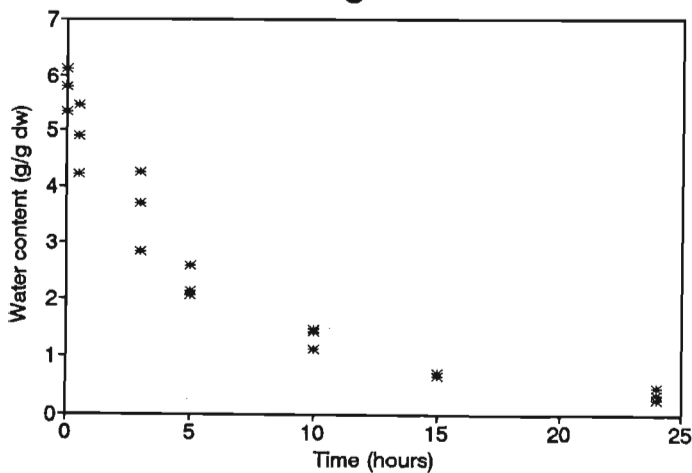
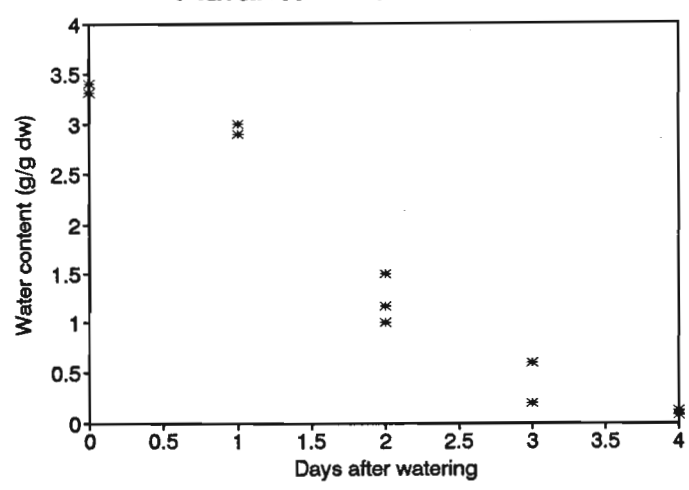
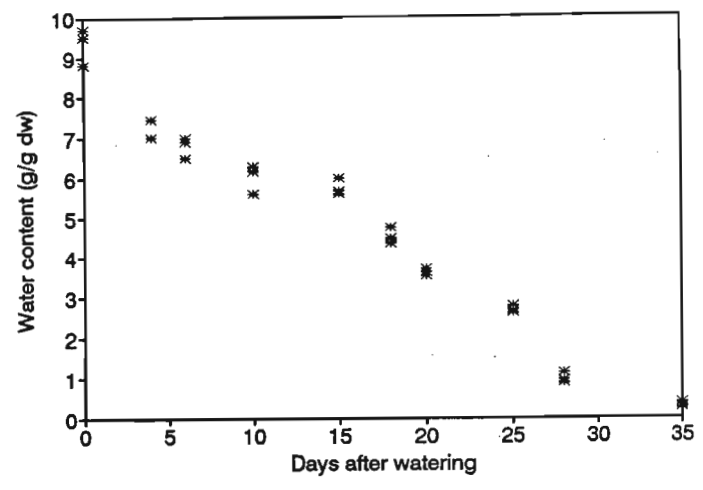
*Adiantum raddianum**Pisum sativum**Isoglossa woodi**Garcinia livingstonei**Craterostigma nanum*

Figure 3.2: Rapid drying rates for excised leaf tissue of the five species studied. Rapid drying was achieved by placing the leaf tissue over a dry air-stream.

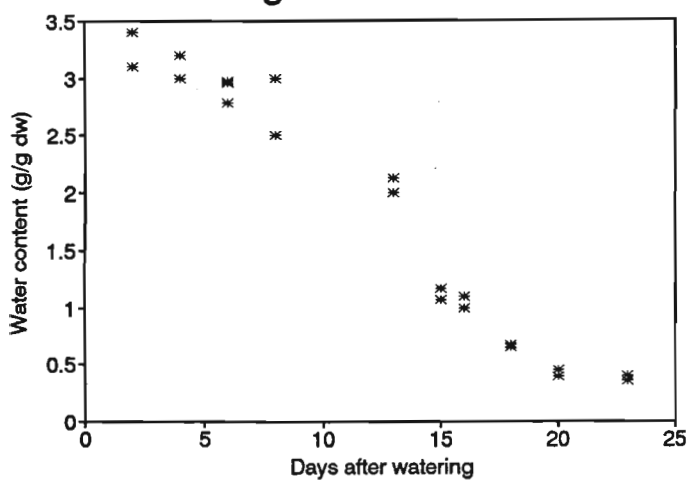
*Adiantum raddianum*



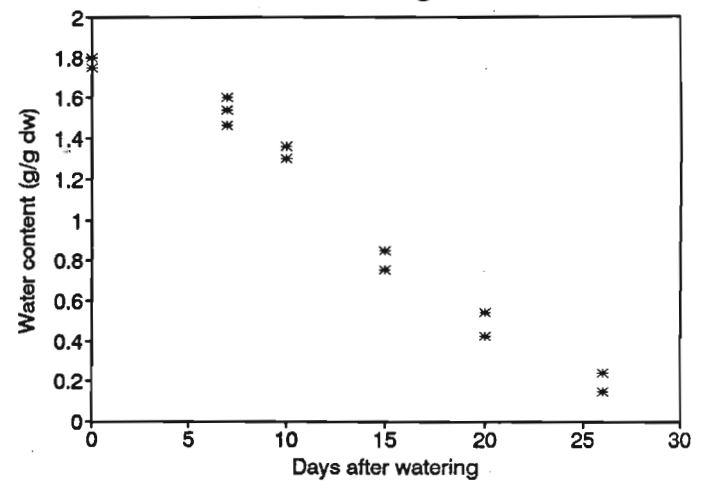
*Pisum sativum*



*Isoglossa woodii*



*Garcinia livingstonei*



*Craterostigma nanum*

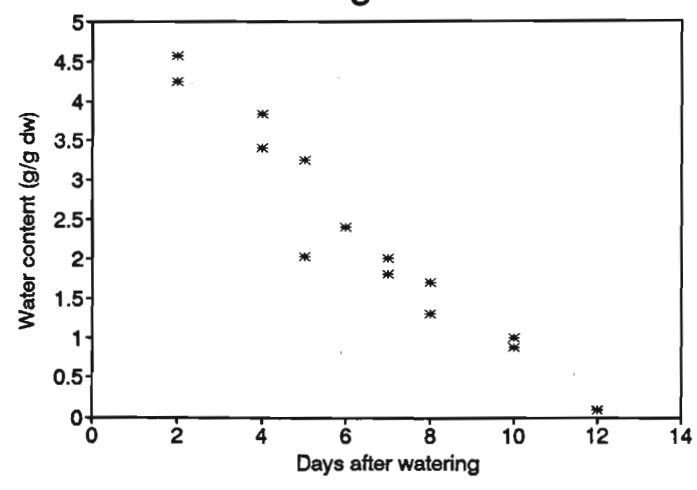


Figure 3.3: Slow drying rates for leaf tissue of the five species studied. Slow drying was achieved by withholding water from the potted specimens of the five species. Data was recorded only once the leaves or leaflets had started showing decreases in water contents.

Table 3.1 gives the results of the slow drying survival studies for the fern *A. raddianum*.

Table 3.1: Results of the survival studies of the maiden hair fern *Adiantum raddianum*.

A number of potted plants were well watered on day 0 and then left to dry slowly by withholding water. Recovery was established by the fronds regaining turgor and continuing growth.

Days after watering	Water content g (gdw) <sup>-1</sup>	RWC (%)	Water potential (MPa)	Condition of plant	Recovery of fronds on re-watering
0	4.2	100	-0.25	Fronds green and fully turgid	yes
3	2.8	80	-3.5	Older leaflets starting to wilt	yes
5	1.2	35	-5.5	All fronds slightly wilted	yes
6	0.8	22	-7	Whole plant wilted	no
7	0.1	5	-21	Whole plant very wilted	no

From Table 3.1 it can be seen that the fronds did not survive dehydration to as low a water content of 0.8 g (gdw)<sup>-1</sup> (RWC 22% and water potential of -7 MPa). Once general wilting of the fronds had occurred they did not have the ability to recover. If the plant was not watered within a week or two of the fronds wilting, the rootstock also died.

**Table 3.2:** Results of the survival studies of the green pea, *Pisum sativum*. Fourteen day old seedlings were well watered on day 0 and then left to dry slowly by withholding water. Recovery was established by the younger leaves regaining turgor and continued growth of the plants. Water content measurements were taken on the two youngest sets of leaves.

Days after watering	Water content g (gdw) <sup>-1</sup>	RWC (%)	Water potential (MPa)	Condition of plant	Recovery on re-watering
0	12.5	100	-0.2	Leaves green and fully turgid	yes
7	6.2	55	-3	Older leaves starting to wilt, young leaves turgid	yes
18	4.5	40	-4.5	Young leaves starting to wilt	yes
22	3.8	32	-5	Young leaves very wilted	yes
28	1.8	18	-8	Young leaves turning yellow, very wilted	no
35	0.7	5	-21	Top leaves pale yellow and very brittle	no

Table 3.2 shows that the pea seedlings did not survive dehydration to water contents of 1.8 g (gdw)<sup>-1</sup> (18% RWC and water potential of -8 MPa). The leaves of *P. sativum*, unlike those of *A. raddianum*, can recover from wilting. They were very wilted at a water

content of  $3.8 \text{ g (gdw)}^{-1}$  (32%) but on re-watering they regained turgor. Once chlorophyll loss was noted the leaves could not recover from the water stress. Seedlings of *P. sativum* died at higher water contents, but similar RWCs and water potentials, to *A. raddianum*.

**Table 3.3:** Results of the survival studies of *Isoglossa woodii*. Potted plants were well watered on day 0 and then left to dry slowly by withholding water. Recovery was established by the leaves regaining turgor and continued growth of the plant. The third set of leaves from the apex were used to measure water contents and recovery.

Days after watering	Water content $\text{g (gdw)}^{-1}$	RWC (%)	Water potential (MPa)	Condition of plant	Recovery on re-watering
0	3.8	100	-0.3	Leaves green and turgid	yes
5	2.8	70	-3.5	Leaves starting to wilt	yes
7	2.2	55	-4	Leaves wilted	yes
10	1.6	40	-5.5	Leaves very wilted	yes
12	1.1	25	-10	Leaves very wilted, curling inwards	no
18	0.35	5	< -25	Leaves curled inwards and brittle	no

*Isoglossa woodii* leaves could also recover from a very wilted state (Table 3.3). The leaves started to wilt at relatively high RWCs (70%). The leaves, however, survived at water

contents as low as 1.6 g (gdw)<sup>-1</sup>. The lethal RWC and water potential were similar to the other two species discussed this far.

**Table 3.4:** Results of the survival studies of *Garcinia livingstonei*. Potted plants were well watered on day 0 and then left to dry slowly by withholding water. Recovery was established by the leaves regaining turgor and the continued growth of the plant. The second and third youngest set of leaves were used to measure water content and recovery.

Days after watering	Water content g (gdw) <sup>-1</sup>	RWC (%)	Water potential (MPa)	Condition of plant	Recovery on re-watering
0	1.5	100	-0.15	Leaves green and fully turgid	yes
15	1.3	90	-2	No change in leaves	yes
25	0.8	55	-4	Leaves drooping at petiole, no wilting, some abscised	yes
30	0.4	30	-6.5	No wilting, leaves left on plant drooping at petiole	no
45	0.18	5	-25	Leaves left on plant turned brown	no

The leaves of *G. livingstonei* showed no signs of wilting even though they were dried to water contents lower than that which was lethal (Table 3.4). This was probably due to the

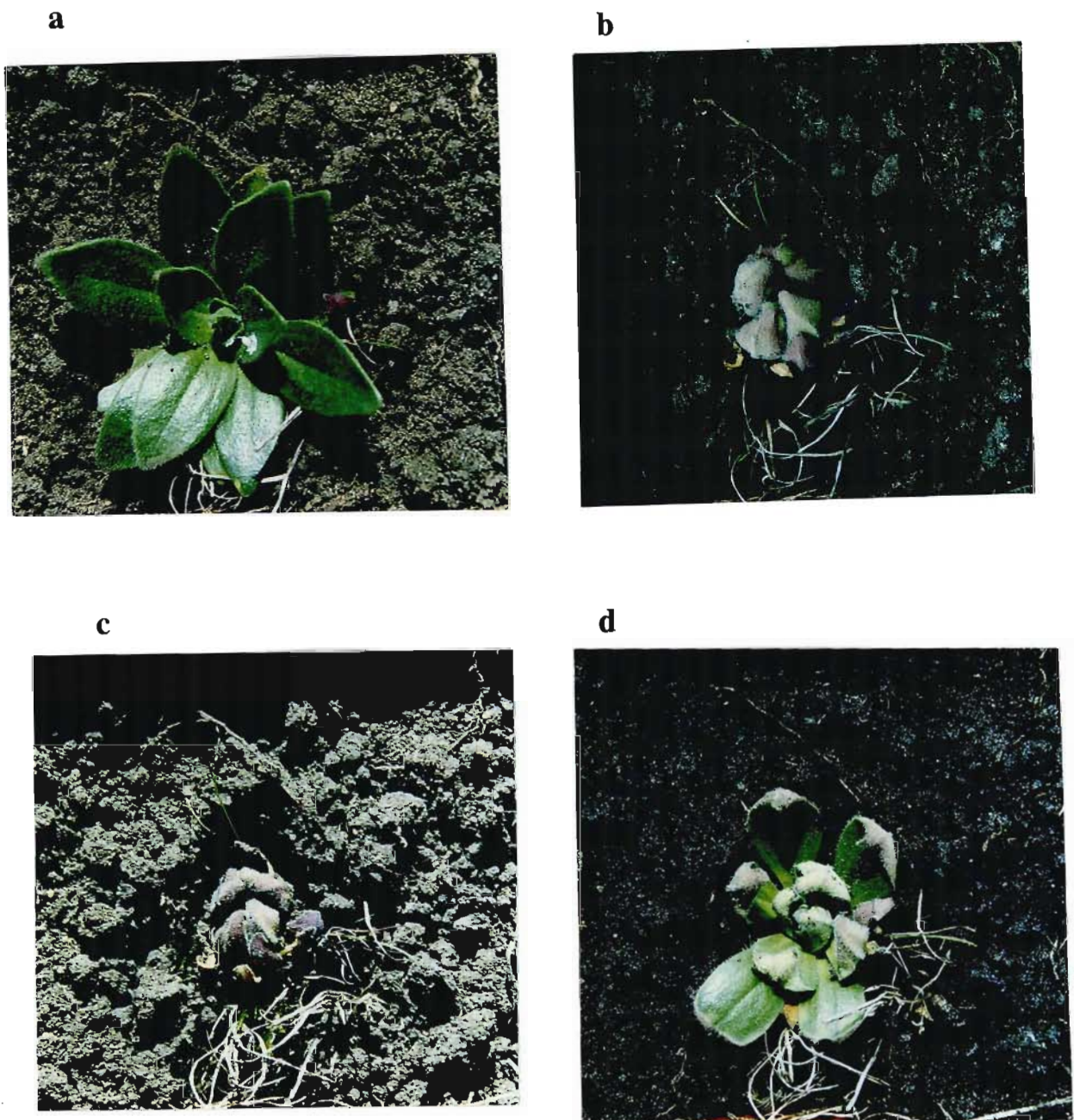
thick, sclerophyllous nature of the leaves. The leaves survived to water contents of  $0.8 \text{ g (gdw)}^{-1}$ , which is lower than that of the other species. However, the lethal RWC and water potential were similar to those of the other three desiccation-sensitive species. Some leaves abscised from the plants. In plants where abscission occurred in most or all of the leaves, the rootstock survived and the plant produced new buds and leaves during the next growing season. The plants on which the dead leaves were retained did not produce new growth. The leaves eventually lost their chlorophyll and became dry and brittle as did the stems.

Table 3.5 shows that even though *C. nanum* was dried to water contents as low as  $0.2 \text{ g (gdw)}^{-1}$  (1% RWC) the plants still recovered on re-watering. The leaves showed signs of water stress at water contents of  $4.2 \text{ g (gdw)}^{-1}$  (RWC of about 57%, water potential of about -4 MPa) by wilting and curling inwards. This inward curling of the leaves probably helped reduce the rate of water loss from the leaf tissue as well as protected the inner leaves from exposure to excessive light. The outer leaves turned a reddish/purple colour. This was probably due to the accumulation of anthocyanins which would also protect the plant from photo-oxidative damage. As the plant grows in a very exposed habitat, these are probably important features enabling it to survive dry periods.

The photographs in Figure 3.4 show *C. nanum* during a drying and re-watering cycle. Recovery on re-watering was rapid. Within 24 hours most of the uncurling had taken place and after 48 hours the appearance of the plants was similar to that before they were dried. The tips of the leaves often did not survive drying, giving the fully turgid and green leaves ragged tips.

**Table 3.5:** Results of the survival studies of *Craterostigma nanum*. Potted plants were well watered on day 0 and then left to dry slowly by withholding water. Recovery was established by the leaves regaining turgor and continued growth of the plant. The second and third ring of leaves from the outside were used to measure water content and determine recovery.

Days after watering	Water content g (gdw) <sup>-1</sup>	RWC (%)	Water potential (MPa)	Condition of plant	Recovery on re-watering
0	6.5	100	-0.2	Leaves green, turgid and expanded	yes
5	4.2	60	-3	Leaves curling inwards, starting to wilt	yes
7	2.2	30	-6	Leaves curled tightly inwards	yes
9	1.7	18	-7	Outer leaves turning red/purple on adaxial side, abaxial side still green	yes
15	0.5	5	-21	Plant in tightly curled ball, outer leaves red	yes
30	0.2	1	< -25	No change from above	yes



**Figure 3.4:** The dehydration and subsequent "resurrection" of *Craterostigma nanum*.

- a) Plant at full turgor after being well watered
- b) Plant after 7 days of drying - leaves curling inwards (30% RWC)
- c) Plant after 14 days of drying - leaves tightly curled, soil very dry (5% RWC)
- d) Plant 24 hours after re-watering - leaves have uncurled and regained turgor

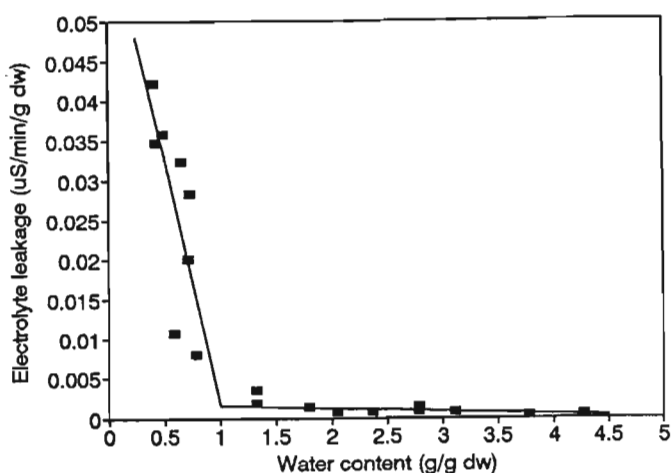
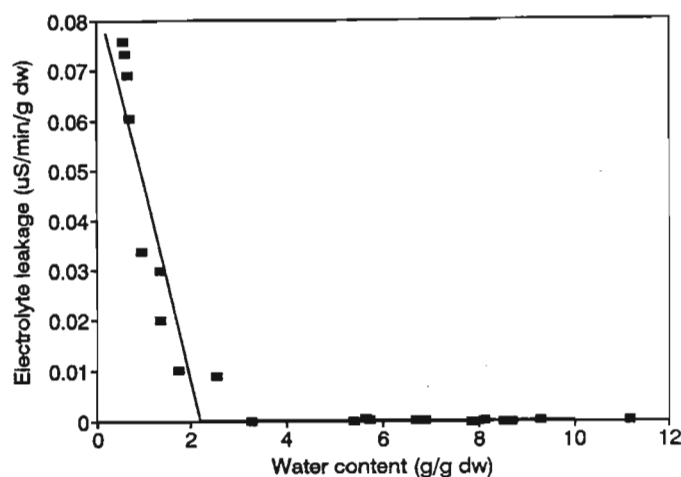
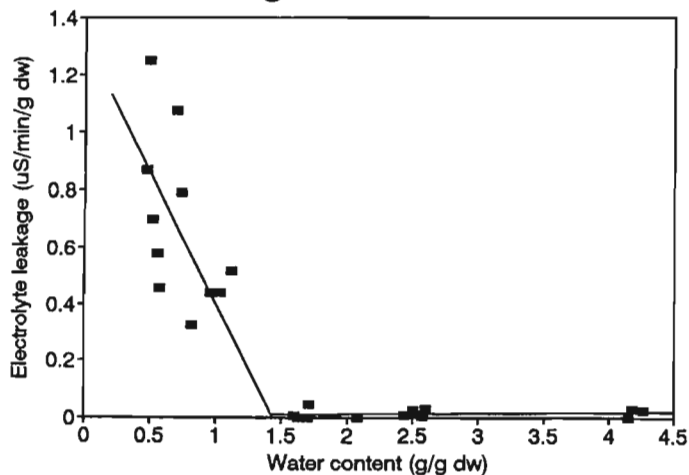
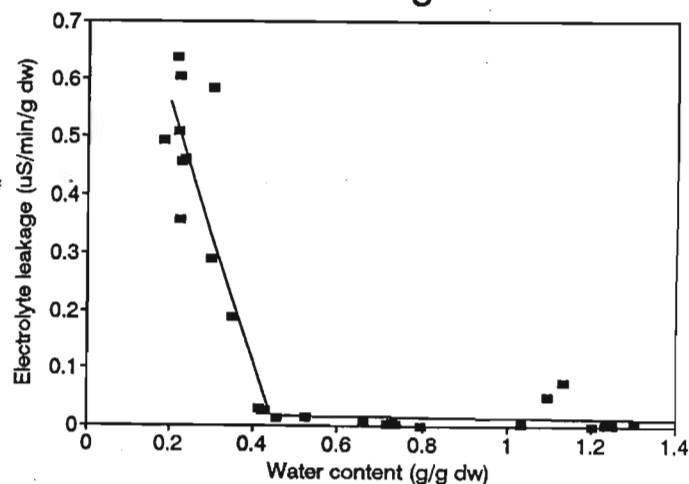
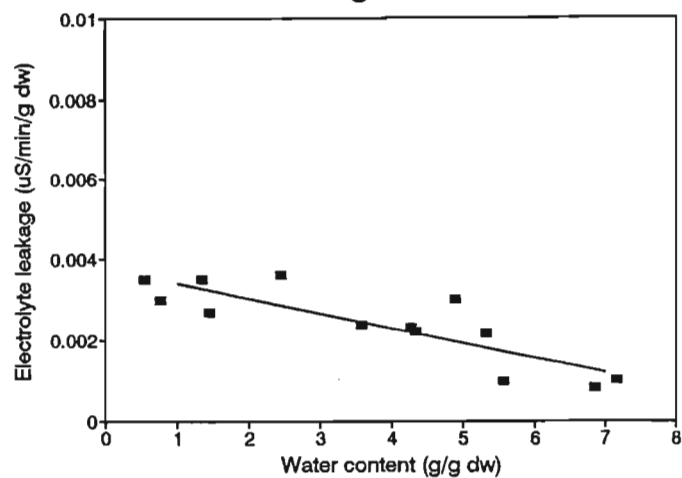
The rate of electrolyte leakage versus water content for rapidly dried tissue is shown in Figure 3.5. These graphs show that the rate of leakage remained at a constant low level for a wide range of water contents until a water content was reached where this rate sharply increased. The water content, RWC (from Fig. 2.9) and water potential (from Fig. 2.10) at which leakage increased sharply are shown in Table 3.6.

**Table 3.6:** The water content, RWC and water potential at which the rate of electrolyte leakage increased after the material had been flash dried and slowly rehydrated in a moist atmosphere for 24 hours. This increase is thought to be related to the point at which membrane damage becomes severe.

Species	Water content (g (gdw) <sup>-1</sup> )	RWC (%)	Water potential (MPa)
<i>A. raddianum</i>	1	28	-6.7
<i>P. sativum</i>	2.2	22	-7.5
<i>I. woodii</i>	1.4	30	-7.5
<i>G. livingstonei</i>	0.45	30	-7
<i>C. nanum</i>	none	none	none

The desiccation-tolerant plant, *C. nanum*, does not show the sudden increase in leakage found in the four desiccation-sensitive species; instead leakage increased slightly at a fairly constant rate as the leaves were dried. This indicates that membrane damage associated with drying either did not occur in this species or that it was repaired during the slow rehydration process. From Table 3.6, it can be seen that the absolute water content at which electrolyte leakage increased differed amongst the four desiccation-sensitive species but that the RWC and water potential at which this increase occurred was similar amongst the sensitive species.

Figure 3.6 shows electrolyte leakage as a function of water content for leaves which were dried slowly on the plant. There does not appear to be much difference in the pattern of leakage changes with dehydration in *A. raddianum*, *G. livingstonei* and *C. nanum* between

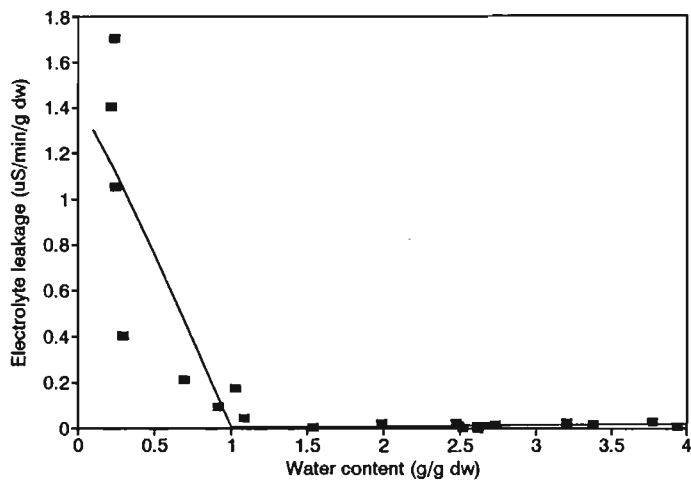
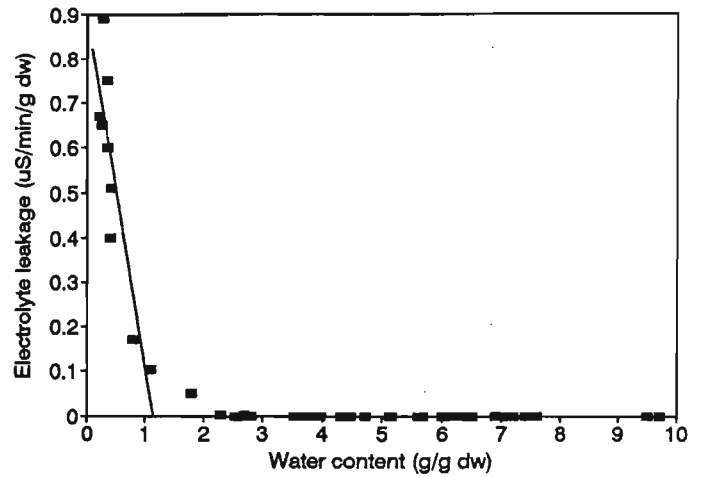
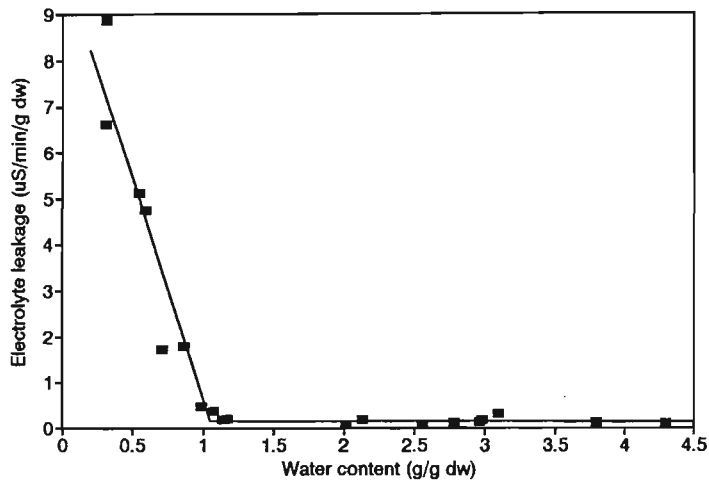
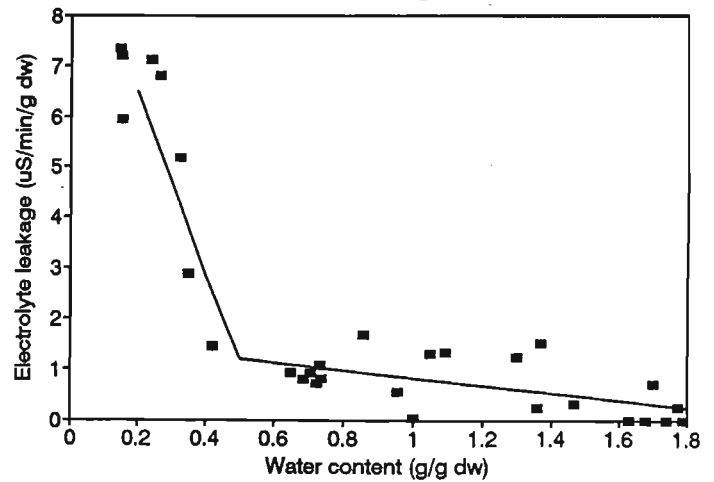
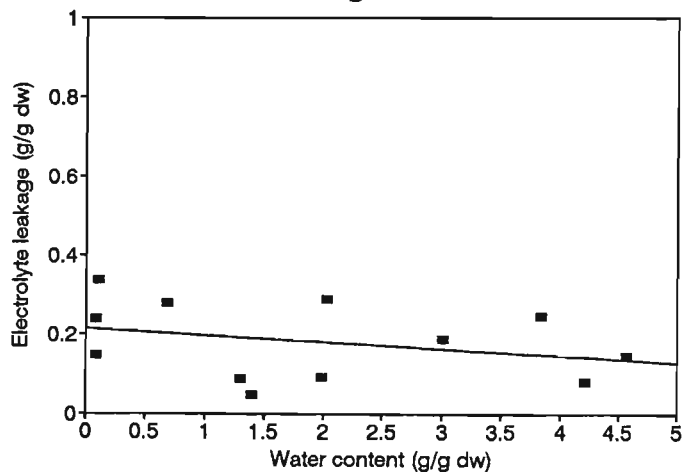
*Adiantum raddianum**Pisum sativum**Isoglossa woodii**Garcinia livingstonei**Craterostigma nanum*

**Figure 3.5:** Changes in the rate of electrolyte leakage during dehydration of rapidly dried leaf tissue. Prior rehydration took place overnight in a moist atmosphere. The solid lines were fitted by linear regression analysis and the intercept was taken as the water content at which electrolyte leakage increased sharply.

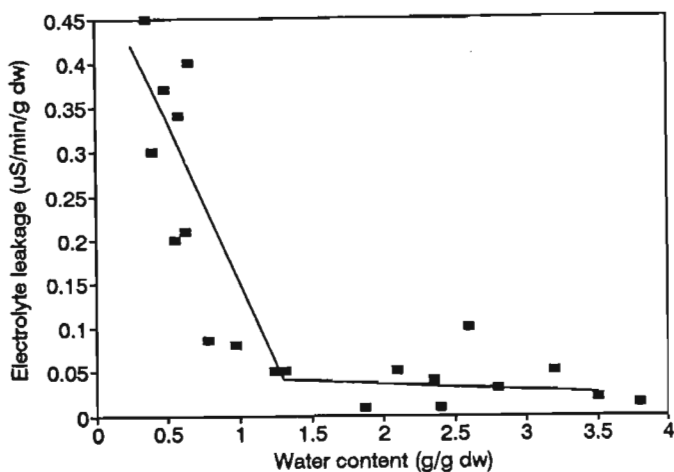
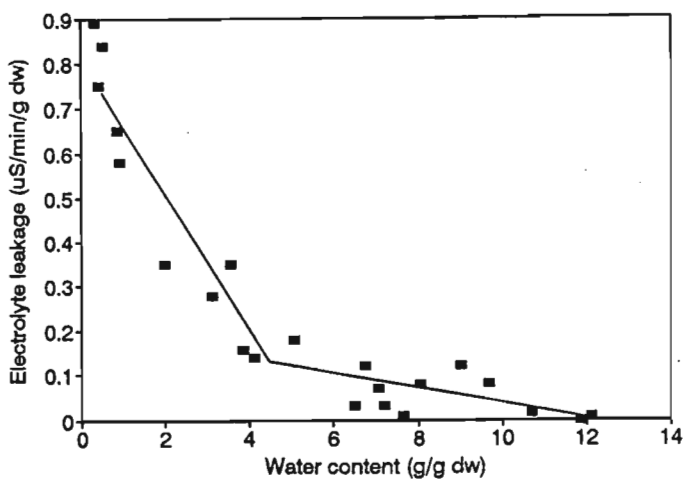
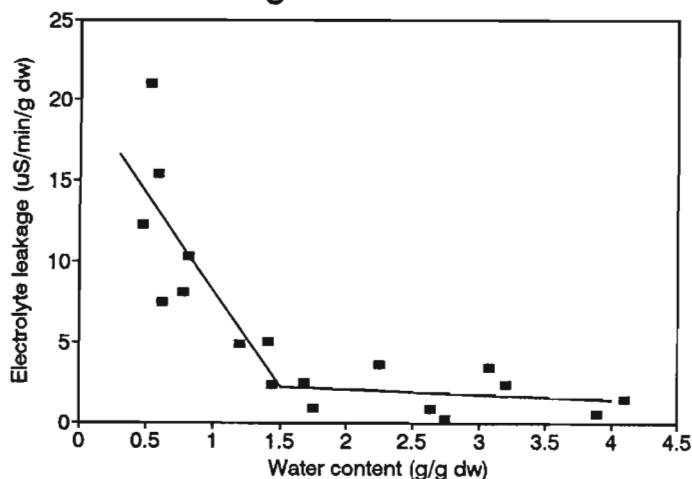
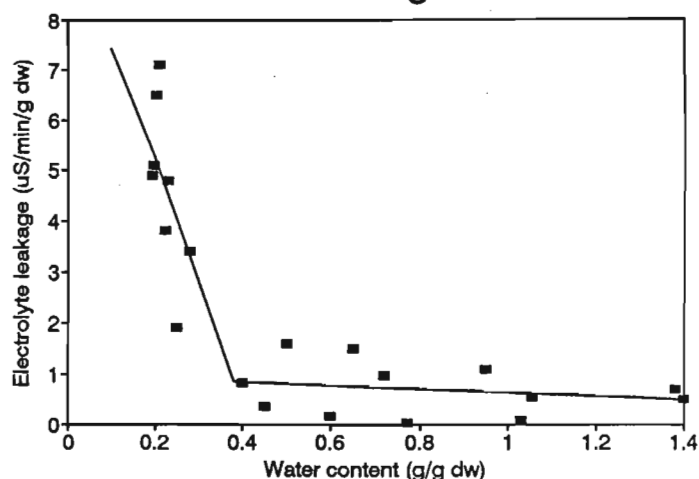
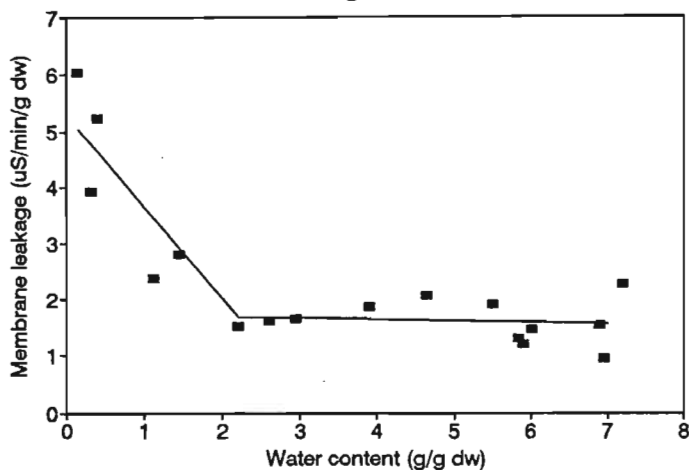
the fast and slow drying treatments. For *P. sativum* and *I. woodii* the increase in leakage occurred at a lower water content if leaves were dried slowly attached to the plant, compared with rapid drying of excised material ( $1.0 \text{ g (gdw)}^{-1}$  and  $2.2 \text{ g (gdw)}^{-1}$  for slow and rapid drying of *P. sativum* and  $1.1 \text{ g (gdw)}^{-1}$  and  $1.4 \text{ g (gdw)}^{-1}$  for slow and rapid drying of *I. woodii*). This indicates that damage was caused at higher water contents with rapid drying in these two species. The absolute amount of leakage was at least a factor of ten for the sensitive species, and a factor of one hundred, for *C. nanum*, higher for the slowly dried material. This indicates that leaf material leaked more solutes after slow drying than after rapid drying. This could indicate greater damage during slow drying than rapid drying. It is well known that response to a stress is not only dependent on the magnitude of that stress but also on the amount of time for which the stress was applied. In terms of the slow dried material, water stress was applied over a number of days and not hours as was the case in the rapidly dried material. The slow drying also took place on a whole plant basis. It is possible that the leaves dried on the parent plant would contain more solutes due to absorption by roots and production and/or concentration of certain solutes. Differences in absolute amounts of solute leakage need to be interpreted with caution, however, as it would be the percentage of the total amount of solutes leaked that would be a better measure for comparing different treatments within a species. The percentage was not determined in this case as it was the water content at which leakage increased that was thought to be the most meaningful measure of where damage was occurring.

The water contents below which the sensitive species did not survive (Tables 3.1, 3.2, 3.3 & 3.4) were similar to the water content where the sharp increase in electrolyte leakage was noted (Figs. 3.5 & 3.6 and Table 3.6). Thus severe membrane damage, as shown by a sharp increase in electrolyte leakage, was a good indication of lethal damage to the sensitive plants.

Figure 3.7 shows the rate of leakage as a function of water content of rapidly dried tissue when the tissue was not placed in a moist atmosphere overnight prior to taking measurements. For both *I. woodii* and *G. livingstonei*, the water content at which leakage increased was the same as for material that had been rehydrated slowly in a moist

*Adiantum raddianum**Pisum sativum**Isoglossa woodii**Garcinia livingstonei**Craterostigma nanum*

**Figure 3.6:** Changes in the rate of electrolyte leakage during dehydration of leaf tissue which had been slowly dried on the parent plant. Prior rehydration took place overnight in a moist atmosphere. The solid lines were fitted by linear regression analysis and the intercept was taken as the water content at which electrolyte leakage increased sharply.

*Adiantum raddianum**Pisum sativum**Isoglossa woodii**Garcinia livingstonei**Craterostigma nanum*

**Figure 3.7:** Changes in the rate of electrolyte leakage during dehydration of rapidly dried leaf tissue. The material was flash dried to the various water contents. No prior rehydration took place, the material was placed straight into the bathing solution. The solid lines were fitted by linear regression analysis and the intercept was taken as the water content at which electrolyte leakage increased sharply.

atmosphere before leakage measurements were taken. For the fern, *A. raddianum*, the increase occurred at a slightly higher water content ( $1.3 \text{ g (gdw)}^{-1}$ ) for the material with no rehydration as opposed to the  $1.0 \text{ g (gdw)}^{-1}$  for material that had been rehydrated prior to measuring conductivity. The increase in leakage for *P. sativum* also occurred at higher water contents with no prior rehydration ( $4.0 \text{ g (gdw)}^{-1}$ ) as opposed to  $2.2 \text{ g (gdw)}^{-1}$ ). This could indicate a degree of repair during slow rehydration or that rapid rehydration caused further damage to these two species. The absolute rates of leakage in material that had not been previously hydrated were much higher than if prior slow rehydration had taken place before immersion into the bathing solution. There was also a lot more noise in the data set which came from material that did not have prior rehydration.

For the desiccation-tolerant plant, *C. nanum*, there was a distinct difference between the material subjected to prior rehydration or not. With prior rehydration no increase in electrolyte leakage was noted. When there was no prior rehydration an increase in leakage, similar to that shown in the sensitive species, was shown in the tolerant species. The increase in leakage occurred at a water content of about  $2.2 \text{ g (gdw)}^{-1}$  (RWC of 30% and a water potential of  $-5.7 \text{ MPa}$ ) which was similar to the RWC and water potential at which the increase was noted for the sensitive species.

## Discussion

The results of the survival studies indicate that the desiccation sensitive species all die at similar RWC's and water potentials. A lethal RWC of about 30% has been reported for a number of species (Conroy *et al.*, 1988; Flower & Ludlow, 1986; Hall, 1993; Ludlow & Muchow, 1990). The lethal water potential of approximately  $-7 \text{ MPa}$  is higher than the  $-15 \text{ MPa}$  reported by Gaff (1989) to be lethal to desiccation-sensitive plants. However, Hall (1993) reported that the lethal water potential for millet was about  $-3 \text{ MPa}$  and for peanut about  $-9 \text{ MPa}$ . The lethal water potential for the sensitive species coincided with the estimated water potential of Type 3 water (Clegg, 1986; Vertucci, 1990). Thus the sensitive plants were dying before the removal of any tightly bound (Type 2 or Type 1) water. Death in the sensitive species, occurs at higher water contents than that which consists of non-freezable water (refer Chapter 2). This indicates that sensitive plants die

before this non-freezable water is removed. These results are similar to those obtained by Pammenter *et al.* (1992). This finding has implications in the applicability of the water-replacement hypothesis. While water replacement may play a role in the survival of tolerant plants in the dry phase it does not explain why sensitive plants die. There appears to be a range in water content between that at which the sensitive plants die (about 30% RWC) and water replacement becomes necessary (about 8% RWC), which must also be critical for a tolerant plant to survive.

There were also markedly different morphological responses of the different plants to severe water stress. Species like *I. woodii* and *P. sativum* had the ability to wilt and recover from the wilt, whilst *A. raddianum* could not recover from wilting. *G. livingstonei* showed no signs of wilting, possibly due to its sclerophyllous leaves. Abscission of leaves on water stress is a common response of woody species to drought and is referred to as drought deciduousness (Abrams, 1990). This loss of leaves is important to the survival of the *G. livingstonei* plants; the leaves which did not abscise did not show recovery nor did regrowth of the plant occur, whereas the plants in which the leaves had abscised resprouted and continued growing. All these morphological features, however, are a consequence of the physical nature of the leaf and are not related to desiccation sensitivity which appeared similar in all the desiccation-sensitive species despite the presumed differences outlined in Chapter 2.

The RWC at which turgor was lost (Table 2.2, Chapter 2) did not appear to correlate with the water content at which the plants died. Flower and Ludlow (1986) have also shown that the turgor loss point for pigeonpea (*Cajanus cajan* L.), 80% RWC, is far higher than the lethal RWC (32%) and concluded that the death of the leaves had nothing to do with turgor loss. The RWC at which *I. woodii* wilted was 70% and turgor was lost at 75% RWC (Table 2.2). While these RWCs were similar, *P. sativum* showed loss of turgor at 78% RWC but showed visible signs of wilting below only 50% RWC. The rigid cell walls of *G. livingstonei* were possibly responsible for the leaves showing no visible signs of wilting even at very low water contents. The RWCs over which there was little change in water potential in the PV curve of *C. nanum* (Fig. 2.11) appear to correspond with the RWCs at which the leaves of *C. nanum* begin to wilt and curl inwards.

Leopold *et al.* (1981) demonstrated that rehydrating leaf discs of a desiccation-sensitive plant (*Vigna sinensis* [L.] Endl.) showed increasing leakiness in proportion to the extent of prior dehydration, whereas the desiccation-tolerant plant, *Selaginella lepidophylla* Spring., showed no such increase in leakiness. These observations are similar to the results presented in this study which show an increase in leakage with severe dehydration in the desiccation-sensitive plants, while the desiccation-tolerant plant showed no such increase in leakage. This was the case as long as rehydration in a moist atmosphere occurred prior to imbibition. The water content at which the increase in electrolyte leakage was noted correlated well with the water content at which the sensitive plants did not recover from slow dehydration in all species. The method used to represent electrolyte leakage was, therefore, a very useful indicator of severe and often lethal damage to the sensitive plant species.

The species used in this study showed membrane damage and death at very similar RWCs and water potentials even though this damage occurred at different absolute water contents. The fact that the increase in membrane leakage occurred at similar RWCs in all five species suggests that a critical volume is reached where membrane damage takes place. The appearance of lethal damage at similar RWCs has also been shown by other workers. Conroy *et al.* (1988) found that a RWC of 36% proved deleterious to sunflower plants (*Helianthus annuus*). The plants survived until this RWC below which permanent damage was noted. Flower and Ludlow (1986) and Ludlow and Muchow (1990) reported that for pigeonpea (*Cajanus cajan* (L.) millsp.) death occurred at a RWC of 32% when plants were subjected to different drying regimes. The same authors reported similar results for several forage grass species.

Hall (1993) discusses the similarity in RWC at which death occurs for many species, suggesting that there may be a "critical" cell volume which reflects an irreversible loss of cellular function due to a catastrophic inward collapse of cell walls resulting from a critical level of negative turgor (also proposed by Tomos, 1988). Meryman (1974) also proposed that cells could not contract beyond a "minimum critical volume" without loss of membrane function on rehydration.

In contrast to the above findings work on 21 lines of *Sorghum bicolor* (L.) by Basnayake *et al.* (1993) have shown that the RWC at which leaves of the different lines died varied considerably. It must be borne in mind that water stress is a function not only of the intensity of the stress but also of the time for which the stress was applied. It is very difficult to maintain excised leaves, let alone whole plants at specific water contents. (An attempt was made to maintain leaves at specific RHs but because of the nature of the local climate, fungal growth tended to kill the leaves before meaningful measurements could be taken.) It is possible that, on instantaneous drying, cells can decrease in volume until a RWC of 30% before irreversible mechanical damage is noted. However, plants maintained at higher RWCs could show irreversible damage caused by biochemical processes, such as lipid peroxidation, over a period of time.

The results presented in this study show no significant differences between fast and slow drying in the pattern of electrolyte leakage in relation to water content. However, the absolute amount of leakage is shown to be higher in the slow dried material. This may be due to the fact that in the slow drying treatments the plants were exposed to stress for longer periods. The slow drying treatment was also conducted on the whole plant, though how this would lead to an increase in the amount of solutes leaked cannot be determined.

Work done on fast and slow drying in mosses showed that leakage of both tolerant and sensitive material was more severe on rapid drying compared with slow drying (Dhindsa & Bewley, 1977). Damage measured by leakage (Bewley & Krochko, 1982) and by decreases in ability to synthesise proteins (Dhindsa, 1987; Gwóźdz & Bewley, 1975; Gwóźdz, Bewley & Tucker, 1974), appeared less for slow, than for fast dried material. This was thought to be due to fast drying causing more cellular damage than slow drying (Bewley & Oliver, 1992). Similar results have also been obtained for work done on fast and slow drying of *Selaginella lepidophylla* (Eickmeier, 1987) and on ferns (Penny & Bayfield, 1982). Rapid drying for the mosses used in these studies took place within an hour while slow drying took place over a period of four to six hours (Gwóźdz *et al.*, 1974). Even the slow drying rate for these mosses and ferns was much faster than the rapid drying of the leaves used in this study. It is possible that the rapid drying rate used in this study was not fast enough to result in more severe damage to the plants than slow

drying.

There are contradictory reports of damage on fast drying in desiccation-tolerant angiosperms. *Borya nitida*, a poikilochlorophyllous resurrection plant, appears to be more deleteriously affected by rapid drying than slow drying (Hetherington *et al.*, 1982). The rapid drying rate used in the experiments with *B. nitida* was similar to that used in this study (15 hours, compared to 18 hours for *C. nanum*). In rapid drying experiments, *B. nitida* did not lose its chlorophyll and did not survive subsequent rehydration. It is possible that the rapid drying did not provide sufficient time during drying for the changes that needed to take place in this poikilochlorophyllous plant which enable it to survive dehydration. This would implicate the importance of protection mechanisms for this species. As mentioned in Chapter 1, the loss of chlorophyll in itself may be a protective mechanism for the poikilochlorophyllous plants.

However, another poikilochlorophyllous plant, *Sporobolus stapfianus*, did not appear to be affected by similar rapid drying rates (C.V. Vazzana, pers. comm.<sup>3</sup>). However Vazzana did not indicate whether *Sporobolus stapfianus* lost or retained its chlorophyll during rapid drying. Another desiccation-tolerant plant, *Boea hygroskopica*, does not lose its chlorophyll during drying, but does not recover from rapid drying (Vazzana).

Thus there is no clear relationship between drying rate and survival in higher plants. It is possible that plants which rely heavily on protection mechanisms would be deleteriously affected by too rapid a drying rate as this may not afford sufficient time to entrain these measures. The rapid drying rate used in this study may have afforded *C. nanum* sufficient time to institute protection mechanisms and this species may also rely more on repair mechanisms than those desiccation-tolerant plants which are damaged by the same drying rates.

The experiment in which conductivity of dehydrated material was measured without prior rehydration in a moist atmosphere, showed that for *A. raddianum* and *P. sativum*, the rate

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<sup>3</sup> C. V. Vazzana, Dipartimento di Agronomia e Produzioni Erbacee, Universita di Firenze, Firenze, Italy.

of leakage increased at higher water contents than if prior rehydration had taken place. This could indicate that both these species were damaged by rapid rehydration or that they have the ability to repair some of the damage, caused by dehydration, during slow rehydration. The amount of leakage was also much higher in the leaf tissue which had no prior rehydration than the tissue which was rehydrated slowly in a moist atmosphere. This again indicated that more damage had occurred during this process. Levitt (1980) also reported work that showed that the rapid addition of water to dried seedlings is more harmful than slow re-wetting, while still other plants showed no difference in damage between fast and slow re-wetting.

Rate of electrolyte leakage with rapid drying and no prior rehydration resulted in the desiccation-tolerant plant, *C. nanum*, showing an increase in leakage at a similar RWC and water potential to the desiccation-sensitive species. The fact that this damage also occurred at a RWC of around 30% for *C. nanum*, further emphasises that this RWC may be critical. At this stage it is not known whether it was the fast rehydration that caused the membrane damage or whether the slow rehydration allowed damage occurring during dehydration to be repaired. Subsequent experiments should clarify when this damage occurred (see later). No literature was found which reported rapid rehydration causing damage in desiccation-tolerant plants.

The work discussed in this chapter shows that measuring the increase in electrolyte leakage is a suitable measure of desiccation damage as it correlates well with death of the plant on slow drying. This damage occurs at very similar RWCs and water potentials for the sensitive species, and for the tolerant species in the absence of slow rehydration. This suggests that a critical volume may be reached, beyond which the sensitive species die but the tolerant species can repair damage and recover. Iljin (1957) suggested that mechanical injury was the primary cause of desiccation damage. While it may not be the only type of injury or even the main injury caused by desiccation, there does appear to be a mechanical component to the sensitivity of plants to desiccation. Iljin (1957) also stated that survival of dehydration is possible if mechanical stresses are avoided. The PV curve of *C. nanum* shows that over a range of RWCs there are changes in the properties of the cell walls such that the pressure inside the cell is maintained even though volume is decreasing. This

occurs at higher RWCs than that which appears to be the critical volume of the sensitive cells. At lower water contents where pressure again starts to decrease, other biochemical and biophysical factors permit the leaf to withstand desiccation.

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## CHAPTER 4: ELECTRON TRANSPORT PROCESSES: PHOTOSYNTHESIS AND RESPIRATION

### Introduction

Photosynthesis and respiration are fundamental physiological processes. Both are membrane associated processes and so effects of dehydration on membranes should be manifested as effects on photosynthesis and respiration. Conversely, effects on photosynthesis and respiration could yield information on the effects of dehydration on membranes. Photosynthesis and respiration have been extensively studied in desiccation-tolerant plants (for reviews see Bewley, 1979; Bewley & Krochko, 1982; Gaff, 1989). Harten and Eickmeier (1986), studying the enzymes of respiration and photosynthesis of a desiccation-tolerant fern, found that respiratory enzymes were more conserved during drying than were those of the photosynthetic system. Seel, Hendry and Lee (1992) in their studies of desiccation-tolerant and sensitive mosses also showed that respiration was less sensitive to desiccation than was photosynthesis .

### Photosynthesis

Photosynthesis is particularly susceptible to water stress. During even mild water stress photosynthesis is inhibited. There is a debate whether this inhibition of photosynthesis during mild water stress is due to a direct effect on the photosynthetic apparatus or due to stomatal closure and thus a shortage of CO<sub>2</sub>, or both (for example: Björkman & Powles, 1984; Boyer, 1970; Bradford & Hsiao, 1982; Kaiser, 1987; Ögren, 1988; 1990; Sharkey, 1990; Sharkey & Seeman, 1989, among others). However, during severe desiccation the photosynthetic apparatus will almost certainly be affected. Kaiser (1987) suggested that at RWCs above 70% the primary cause of a decrease in photosynthesis is stomatal closure, between 30% and 70% RWC both the dark and light reactions of photosynthesis are affected, and below 30% RWC there would be irreversible reduction in photosynthesis caused by membrane damage in desiccation-sensitive tissue.

### Chlorophyll fluorescence

When green leaves are illuminated, chlorophyll molecules are excited. This excitation energy drives photosynthesis. Some energy is also dissipated as heat and a smaller fraction as red light (chlorophyll *a* fluorescence) as electrons drop back to the ground-state orbitals (Seaton & Walker, 1990). Chlorophyll fluorescence is, therefore, in competition with other processes such as the photochemical reaction, thermal deactivation, and transfer of excitation energy to non-fluorescing pigments. Thus chlorophyll fluorescence changes can indicate variations in photosynthetic activity (Bohlàr-Nordenkampf & Öquist, 1993; Briantais *et al.*, 1986). It has long been accepted that there must be an inverse relationship between fluorescence yield and photosynthesis (Seaton & Walker, 1990).

Since Kautsky and Hirsch's discovery (1934, cited in Bohlàr-Nordenkampf & Öquist, 1993) of the nature of the fluorescence induction signal when dark-adapted leaves are exposed to light, there has been remarkable progress in the understanding and practical use of chlorophyll fluorescence in plant science. Measurements of chlorophyll fluorescence have become a very attractive means of obtaining rapid, semi-quantitative information on photosynthesis (van Kooten & Snel, 1990). Because the measurement of chlorophyll fluorescence is both non-destructive and non-invasive, it has considerable potential for both field and laboratory use (Bohlàr-Nordenkampf *et al.*, 1989). Chlorophyll fluorescence has been widely used in laboratory studies in understanding the mechanism of *in vivo* photosynthesis (Briantais *et al.*, 1986; Krause & Weis, 1991; Renger & Schreiber, 1986) particularly the relationship between the light and dark reactions (Sivak & Walker, 1985; Walker *et al.*, 1983). Fluorescence has also been used to study the mechanisms by which a range of environmental factors alter photosynthetic capacity.

It is well documented that the effective functioning of thylakoid membranes is sensitive to several environmental stresses. Photosystem II (PSII), including the water oxidising step, appears to be particularly sensitive to a number of stress factors (Bohlàr-Nordenkampf & Öquist, 1993). Fluorescence induction kinetics and the parameters derived from the induction curves are a useful and sensitive indicator of damage to photosynthesis and to the physiology of the plant in general, and are thus widely used for characterising different stress effects. Chilling and freezing stress have been studied using chlorophyll

fluorescence as a tool by, amongst others, Hetherington and Öquist (1988), MacRae, Hardacre and Ferguson (1986) and Smillie and Hetherington (1983). Chlorophyll fluorescence has also been used extensively to study photoinhibition (Demmig & Björkman, 1987; Greer, Berry & Björkman, 1986; Muslin & Homann, 1992; Ögren & Öquist, 1984). The effect of drought and dehydration stress on the photosynthetic system has been studied by Ögren (1990), Stuhlfauth, Scheuermann and Fock (1990) and Toivonen and Vidaver (1988). Chlorophyll fluorescence has also been used to study desiccation tolerance in mosses (Seel, Baker & Lee, 1992a), ferns (Muslin & Homann, 1992) and the resurrection plant *Borya nitida* (Hetherington, Smillie & Hallam 1982). Björkman and Powles (1984), Havaux (1992) and Seel *et al.* (1992a) have studied the effects of both high light and water stress on the photosynthetic apparatus using various chlorophyll fluorescence parameters. Due to the fact that all these stresses affect the functioning of PSII, chlorophyll fluorescence can be used as a tool to both quantify the stress response and reveal stress response mechanisms (Bohlär-Nordenkampf & Öquist, 1993).

#### The nature of the fluorescence signal

Upon a dark-light transition the chlorophyll fluorescence intensity of green plants undergoes characteristic variations with time (Kautsky effect). One can distinguish rapid transients (completed within a few seconds) and slow transients (extending to several minutes). The rapid transients reflect reactions close to the primary photo-reactions, whereas the slower transients are an expression of secondary dark processes involving activation of Calvin cycle enzymes and ion fluxes through the thylakoid membrane (Schreiber, 1983). Measurements of fluorescence are, therefore, essentially divided into two: fast induction kinetics and slow induction kinetics. Superimposed on the slow induction kinetics, the contributions of photochemical and non-photochemical quenching of chlorophyll fluorescence, can be studied.

Details of the nature and interpretation of the chlorophyll fluorescence signal are outlined in reviews by Papageorgiou (1975) and more recently by Bohlär-Nordenkampf and Öquist (1993), Briantais *et al.* (1986) and Krause and Weis (1991). The nomenclature used in this study is that outlined by van Kooten and Snel (1990) and is based on the qualified

endorsement of the OIDPSMT nomenclature by Lavorel and Etienne (1977) and others (Figure 4.1). Alongside developments in the understanding of the nature of chlorophyll fluorescence have been developments in the instruments to measure fluorescence. The recent development of pulse amplitude modulated (PAM) fluorometers has led to an even greater understanding of the nature of fluorescence quenching mechanisms (Schreiber, Schliwa & Bilger, 1986). For a review on the instrumentation refer to Schreiber (1983).

The chlorophyll fluorescence signal emitted by green plants is a complex one and, despite its widespread use, full interpretation of this signal is still uncertain (Krause & Weis, 1991). The measurements used in this study will reflect those that are currently used by the majority of workers in this field. The interpretation of these results will reflect the current consensus by workers using chlorophyll fluorescence to study stress responses in plants.

#### Chlorophyll fluorescence parameters

Besides the qualitative changes that can be seen on Kautsky curves when a stress is applied, a number of useful parameters can also be quantified using fluorescence induction kinetics. They include the minimal or "constant" fluorescence level,  $F_0$ , which is theoretically the fluorescence emission when all reaction centres are open. The  $F_0$  level provides a useful signal for the standardisation of other fluorescence signals (Bohlàr-Nordenkampf & Öquist, 1993; Bohlàr-Nordenkampf, *et al.*, 1989; Briantais, *et al.*, 1986; van Kooten & Snel, 1990). The  $F_0$  level is obtained when the exciting light is low enough to prevent detectable photochemistry from occurring (Bohlàr-Nordenkampf & Öquist, 1993). However,  $F_0$  is not always constant. Increases may indicate damage to PSII reaction centres, or that the transfer of excitation energy from the antenna to the reaction centres is impeded (Bohlàr-Nordenkampf, *et al.*, 1989).

The maximum fluorescence value, or  $F_M$ , is the fluorescence intensity with all the PSII reaction centres closed (van Kooten & Snel, 1990).  $F_M$  is achieved when the plant is given a strong pulse of actinic light (Bohlàr-Nordenkampf *et al.*, 1989).  $F_M$  will decrease if there is damage to processes related to PSII function. The variable fluorescence, or  $F_v$ , is obtained by subtracting  $F_0$  from  $F_M$  (Krause & Weis, 1991). Changes in  $F_v$  reflect an

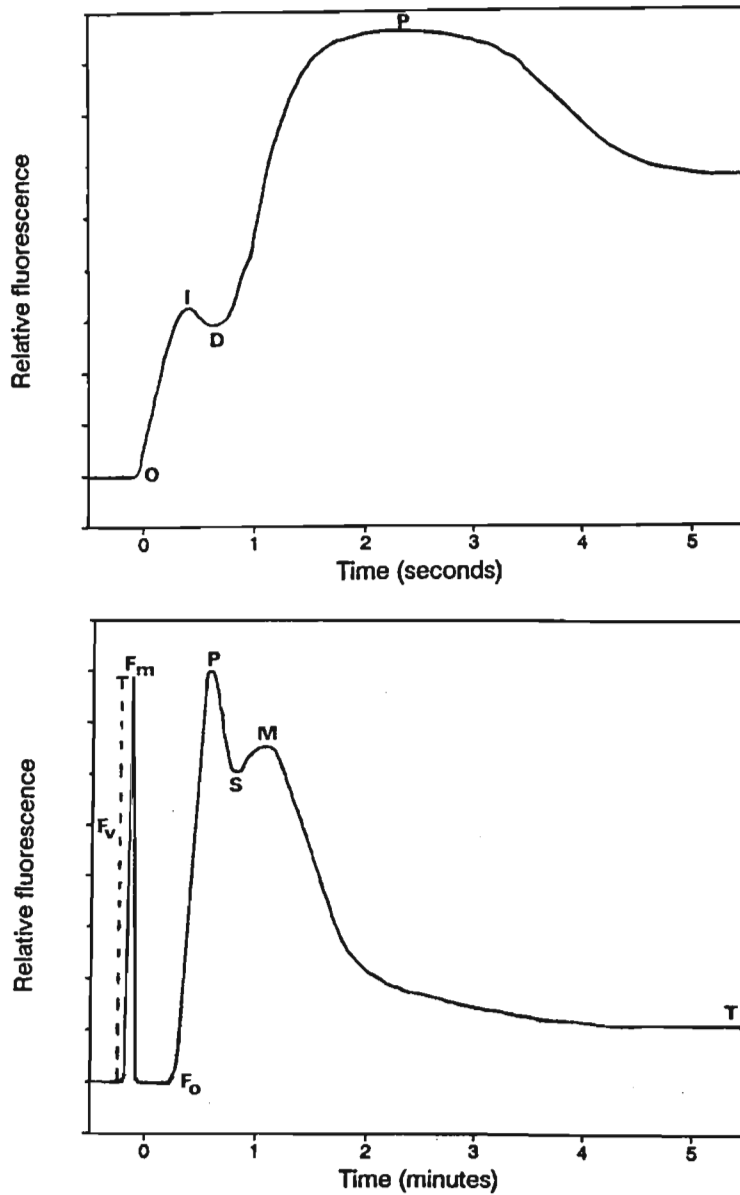


Figure 4.1: Fast (a) and slow (b) induction kinetics curves (Kautsky curves) showing the use of the OIDPSMT terminology as outlined in Bohlär-Nordenkamp and Öquist (1993). O, also known as  $F_O$ , is the baseline fluorescence when all reaction centres are open; I, inflection or intermediate level and D, dip or plateau, are associated with the transfer of light via  $Q_B$  to the plastoquinone pool; P, peak level, occurs when most of the reaction centres are closed, it is the same as  $F_M$  if the light is strong enough; S, slope and M, secondary maximum, are both associated with the onset of  $CO_2$  fixation; T, is the terminal or steady-state level. The fluorescence maximum ( $F_M$ ) produced by a saturating light pulse, is superimposed on the slow induction kinetics curve (b). Variable fluorescence ( $F_V$ ) is the difference between  $F_M$  and  $F_O$ .

inactivation of the primary photochemistry of PSII (Greer *et al.*, 1986). The ratio  $F_v/F_M$ , typically in the range of 0.75 to 0.85, is proportional to the quantum yield of the photochemistry (Butler & Kitajima, 1975). A decline in  $F_v/F_M$  is a good indicator of photo-inhibitory damage caused by light when plants are subjected to a wide range of environmental stresses (Adams *et al.*, 1990; Björkman & Demmig, 1987; Bohlár-Nordenkampf *et al.*, 1989). However, in examining changes in  $F_v/F_M$  it is important to distinguish increases in  $F_o$  from decreases in  $F_v$ . An increase in  $F_o$  is characteristic of destruction of PSII reaction centres, whereas a decline in  $F_v$  may indicate an increase in non-photochemical quenching; photoinhibition produces both of these changes (Björkman, 1987).

#### Quenching of chlorophyll fluorescence

The steady state fluorescence (T) in a healthy leaf will be very different from the height of the peak (P). This difference occurs due to quenching of chlorophyll fluorescence. It is well established that the yield of chlorophyll fluorescence is determined by two distinct processes, photochemical ( $q_p$ ) and non-photochemical ( $q_N$ ) quenching (Bradbury & Baker, 1981). Photochemical quenching occurs due to the conversion of energy at PSII into the photochemical reactions of photosynthesis. Other processes, such as photorespiration and the antioxidant system, that use electrons which pass through the electron transport chain, will also contribute to  $q_p$ . Non-photochemical quenching is due to the conversion of energy into energy-dissipating processes such as heat and the energization of the thylakoid membranes (Bilger & Schreiber, 1986).

It is possible to measure fluorescence quenching using a technique developed by Bradbury and Baker (1981). It was, however, not feasible to measure the quenching parameters during this study because the nature of the dehydration treatment was such that photochemical quenching would have stopped at fairly high RWC due to stomatal closure, and therefore the only component that would have been measured for most of the study would have been  $q_N$ . During previous quenching studies on salt treated and desiccated material (Pammenter, unpublished data; Sherwin & Pammenter, unpublished data) it was found that  $F_o$  changed during the measurement of quenching thus resulting in false readings for  $q_p$  and  $q_N$ .

### Respiration

The influence of drought stress on the respiration of plants has been widely studied for many years (see review by Hsiao, 1973). These studies dealt mainly with mild water stress. Recently studies on the effect of desiccation (severe water stress) on respiration have been carried out (for reviews see Bewley, 1979 and Bewley & Krochko, 1982; Bewley & Thorpe, 1974; Harten & Eickmeier, 1986; Seel *et al.*, 1992b). It has been implied that desiccation-induced changes in the membranes of the mitochondria are responsible for the decrease in respiration with increasing desiccation (Bewley, 1979). Leprince *et al.* (1994) showed that free radical production is closely linked to respiration on dehydration of germinating, desiccation-sensitive, maize seeds. Unquenched free radical production will result in extensive peroxidation damage of cellular membranes, resulting in loss of semi-permeability and changes in phase properties (Senaratna, McKersie & Borochoy, 1985) and impairment of several mitochondrial enzyme complexes (Leprince *et al.*, 1992).

Genkel & Pronina (1969) suggested that desiccation-tolerant and sensitive species are distinguished from each other because in the tolerant species the respiration (oxygen uptake) rate falls continually with water loss, whereas in the sensitive species, it rises during the period when most water is lost and then declines sharply. Bewley (1979), however, pointed out that this idea needs more testing. Gaff (1989) reported that, for both tolerant and sensitive plants, respiration remained fairly constant or increased with increasing dehydration until the material was very dry after which respiration decreased sharply.

### Aim of this study

The aim of this part of the study was to examine the effects of desiccation on the oxidative and photosynthetic electron transport processes of the five plant species. It was decided to measure photosynthetic activity in terms of chlorophyll fluorescence as the nature of the desiccation treatment is such that the photosynthetic apparatus itself and not just the gas exchange characteristics would be affected. Respiration was measured by CO<sub>2</sub> production using an infra-rad gas analyzer (IRGA). Due to the non-invasive nature of chlorophyll fluorescence and respiration measurements, it was possible to study leaves in both a

dehydrated and rehydrated state. This allows comparisons, between the sensitive and tolerant species, of damage occurring during drying, as well as the response on subsequent rehydration of the desiccated material. The effect of rapid rehydration on the tolerant plant *C. nanum* was also investigated to determine whether the increase in electrolyte leakage noted in Chapter 3 (Fig. 3.7) was a direct effect of rehydration or if it was a consequence of insufficient time being allowed for the repair of damage to the membranes.

Fast and slow chlorophyll fluorescence induction kinetics were studied and  $F_0$ ,  $F_v$  and  $F_v/F_M$  were measured to determine the effects of drying and subsequent rehydration on the photochemical apparatus, particularly PSII. It is well known that high light in combination with desiccation results in more severe damage to the photosynthetic apparatus (Björkman & Powles, 1984; Havaux, 1992; Seel *et al.*, 1992a). The aim of this study was to investigate desiccation injury, and so to eliminate the complicating factor of light induced damage, material was treated and handled in the dark or very low light conditions.

CO<sub>2</sub> production was used as a measure of respiration. Oxygen consumption may have been a better measure of respiration, but a working oxygen electrode was not available. It is acknowledged that other metabolic reactions, such as the Maillard reactions, produce CO<sub>2</sub> and by measuring changes in CO<sub>2</sub> production one cannot distinguish between sources of CO<sub>2</sub> production. It is, however, thought that most of the CO<sub>2</sub> production would come from respiration.

## **Materials and Method**

### Leaf material

Whole leaves or leaflets of the five plant species were brought to full turgor by placing their leaf bases in a beaker of water. The beakers with the leaves in them were placed in a moist atmosphere in a dark cupboard overnight. The leaves were weighed and this weight was taken to be the full turgor weight. Chlorophyll fluorescence and respiration were measured on these fully turgid leaves as outlined below. Due to there being little or no difference in the water content at which membrane leakage increased between the fast and slow drying treatment, it was decided to perform the rest of the experiments on fast

dried material due to the shorter time needed for drying the leaves.

The leaves were flash dried in a dark container for various lengths of time (to give a variety of water contents) and fluorescence and respiration were measured at specific water contents. After measuring the various parameters of the leaves, they were then rehydrated to full turgor, or as close to full turgor possible, by first placing them in a moist atmosphere overnight and then immersed in a beaker of water for one to three hours. Chlorophyll fluorescence and respiration were then measured on the rehydrated leaves. Thus for each leaf there would be measurements of it at full turgor, a specific water content and a further measurement after rehydration.

#### The chlorophyll fluorescence measuring system

The details of a standard chlorophyll fluorescence measuring system have been outlined in a technical review by Schreiber (1983). Modulated chlorophyll fluorescence was measured using the PAM Fluorometer 101 (Walz, Effeltrich, Germany). This allowed the measurement of continuous fluorescence signals from a leaf exposed to light. This is achieved by using a weak modulated light source in conjunction with a fluorescence system, which monitors only the fluorescence emitted at the frequency and phase of the modulated light. Actinic light was provided by Schott 1500 KL lamps (Schott, Stafford, UK) using a Schott KG1 heat filter and a Phillips 15V 150W halogen reflector bulb. The PAM Fluorometer was controlled by the program DA100 run on a personal computer. Measurements were recorded also using this program.

The leaves were placed in a cuvette which was sealed such that no light, other than that provided by the fibre-optic tubes, could penetrate. Ambient air taken from outdoors was pumped over the leaves to prevent any build up of CO<sub>2</sub> or O<sub>2</sub>. Water at a temperature of 25°C was circulated through the water jacket of the cuvette to maintain a constant temperature.

#### Induction kinetics

A leaf was placed in the cuvette and dark adapted for 10 minutes. This was deemed sufficient time as the leaf had been kept in the dark for at least 18 hours prior to

measurement and had been transferred to the cuvette under low light conditions. To set the  $F_o$  value, the pulsed light was turned on at 1.6 kHz and the intensity and gain were increased to give an output of about 0.3 (about  $0.05 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). This was done for the fully turgid leaf. The gain and intensity were then left at that setting and the subsequent readings of  $F_o$  were compared with the value of 0.3.

For induction kinetics the intensity and duration of the light pulse was set using the PAM 102 control unit. The PAM 102L LED lamp was used as a light source. For fast induction kinetics a 5 s pulse was applied and the resultant induction curve was recorded. The leaf was then dark-adapted again, for 20 min. Slow induction kinetics were then measured using the same procedure as for fast induction kinetics except the light was turned on manually and left on for 5 min. A light intensity setting of 11 (about  $180 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) was used for both fast and slow induction kinetics.

#### Determination of $F_M$

A dark adapted leaf was placed in the modulated beam and  $F_o$  was recorded. The leaf was then exposed to a brief saturating light pulse from the FL500 lamp which was controlled by the DA100 program. Settings of time, 0.6 s; pulse rate, 20 s; and decay time, 1.2 s were used. These settings gave the type of output required for the correct measurement of  $F_M$  as outlined in the DA100 manual. The saturating flash from the FL500 lamp was at an intensity of about  $3500 \mu\text{mol m}^{-2} \text{s}^{-1}$ . The value of  $F_M$  was then recorded.

#### Parameters measured and data analysis

Qualitative changes in the fast and slow induction kinetics were recorded. As most of the more noticeable changes had occurred by RWCs of 50%, 30% and 5%, these curves were considered to be adequate to represent the changes taking place during dehydration and subsequent rehydration. In all cases measurements were done on at least five different leaves or leaflets. Changes in the values of  $F_o$ ,  $F_v$  and  $F_v/F_M$  with dehydration and rehydration were measured. Graphs were plotted of the fluorescence parameters versus RWC. Regression analysis was done on the straight line portions of these graphs as described in Chapter 3. In some cases up to three regression lines were fitted. The intersection of two regression lines was taken to be the RWC where the fluorescence

parameters changed.

#### Rehydration time course of *Craterostigma nanum*

In this experiment leaves of *C. nanum* were dried to 5% RWC and placed straight into water to rehydrate, with no prior rehydration in a moist atmosphere. Fast and slow induction kinetics were then measured on these leaves at various time intervals after rehydration. This experiment was performed to test whether the fast rehydration, i.e. putting the leaves directly into water, caused any damage. By following the time course after rapid rehydration it could be ascertained whether the damage was permanent, or whether time was required for repair processes to become operational. The standard method of slow rehydration minimises imbibitional damage, and material rehydrated in this manner was also studied.

#### Respiration

CO<sub>2</sub> production of the leaves dried to various RWCs was measured with an Analytical Development Company, or ADC, (Hoddson, United Kingdom) Mark III infra-red gas analyzer (IRGA) using an ADC cuvette. The window of the cuvette was covered with black cloth to prevent light reaching the leaf material and resulting in photosynthetic CO<sub>2</sub> production. Leaf material dehydrated to a range of water contents was left in the cuvette until a constant rate of CO<sub>2</sub> production was measured. The material was then removed from the cuvette and weighed so as to ascertain the exact water content. This leaf material was then rehydrated as described above. This rehydrated material was placed back into the cuvette and CO<sub>2</sub> production was again measured to give a rate of CO<sub>2</sub> production on rehydration.

Data were analysed as for leachate conductivity (refer to Chapter 3). Linear regressions were performed for the straight line portions of the graphs. The point of decline in respiration was taken to be the point where the two regression lines intersected.

## Results

### Rehydration

The rehydration protocol did not result in the desiccation-sensitive leaves regaining full turgor, particularly those dried to low RWCs. The leaves of *P. sativum* and the leaflets of *A. raddianum* did not rehydrate well, even compared to *I. woodii* and *G. livingstonei*. The leaves of the tolerant plant, *C. nanum*, however, regained full turgor on rehydration.

### Chlorophyll fluorescence

#### Fast and slow induction kinetics

Representations of the typical Kautsky curves produced during fast and slow induction kinetics are presented in Figures 4.2 to 4.6. The results of both dehydration and rehydration are shown on these curves. Figure 4.7 shows how rehydration time affected the fluorescence parameters of *C. nanum*. Tables 4.1 to 4.6 summarise the main induction parameters. In all cases the rehydration results refer to leaves which had been dried to specific RWCs and then subsequently rehydrated as close to full turgor as possible.

Figure 4.2 shows the fast (a) and slow (b) induction curves of the fern *A. raddianum*. The parameters derived from the curves are summarised in Table 4.1. During dehydration there was a drop in the fluorescence peak (P), indicating damage to the processes relating to PSII function. The point at which the inflection (I) occurred decreased only at RWCs below 5%. The rise in fluorescence from O to I is associated with the water splitting reactions (Bohlár-Nordenkampf & Öquist, 1993), thus a decrease in I probably reflects a breakdown of this process. During slow induction, the secondary maxima or plateau (M) decreased when leaves were dried to 50% RWC and M was not evident on the curves of leaf material dried to 30% RWC and below. This plateau is associated with carbon fixation (Bohlár-Nordenkampf & Öquist, 1993) and thus it appears that no carbon fixation was taking place at 30% RWC and below. There was a rise in the final steady state fluorescence (T) with drying, indicating an increase in the dissipation of energy as fluorescence rather than other processes such as photosynthesis. At 5% RWC T dropped again but not below the 50% RWC level. The decrease in the value of T with drying could

be due to less energy being absorbed by the leaflets rather than increased dissipation by other pathways. An important parameter is P/T. A high value, as shown at full turgor, indicates that the quenching mechanisms were working efficiently. Photosynthetic quenching ( $q_p$ ) was probably responsible for a considerable portion of the decline from P to T at full turgor. The rise in the value of T relative to P was possibly due to a decrease in  $q_p$ , even though  $q_N$  would have increased. P/T having a value of one, as occurred with the 5% RWC and the rehydrated 30% and 5% RWC treatments, shows that no quenching mechanisms were operational and all light energy absorbed was being dissipated as fluorescence. The value of P/T decreased drastically on dehydration due to both a decrease in P and an increase in T.

**Table 4.1:** A summary of the fast and slow induction parameters derived from the Kautsky curves for *A. raddianum*. The height of the peak (P) was taken as a value of 1 and other features were calculated relative to this. The fast induction peak ( $P_{fast}$ ) was used for calculating the inflection (I). The slow induction peak ( $P_{slow}$ ) was used to calculate the secondary peak or plateau (M), terminal fluorescence (T) and P/T.

RWC (%)	$P_{fast}$	I	$P_{slow}$	M	T	P/T
100	1	0.4	1	0.9	0.06	16.6
50	0.85	0.4	0.9	0.76	0.1	9
50 rehyd.	0.7	0.4	0.9	0.76	0.1	9
30	0.5	0.3	0.67	-	0.3	2
30 rehyd.	0.27	0.14	0.2	-	0.2	1
5	0.2	0.14	0.2	-	0.2	1
5 rehyd.	0.04	0.03	0.03	-	0.03	1

There was little difference in the leaf material that had been dried to 50% RWC and dried to this level and rehydrated, indicating no further damage on rehydration but no recovery

to full turgor levels as a result of the rehydration protocols used here. There were, however, marked differences between leaf material dried to 30% RWC and this same material subsequently rehydrated. During both fast and slow induction kinetics the leaflets dried to 30% RWC and subsequently rehydrated showed similar patterns to leaflets dehydrated to 5% RWC. This indicates that further damage occurred on rehydration of leaflets dried to 30% RWC. The fluorescence signal for the rehydrated 5% RWC leaflets barely rose above the base line level, indicating the absence of primary photosynthetic reactions. These leaflets were also more damaged than in the dehydrated state.

**Table 4.2:** A summary of the fast and slow induction parameters derived from the Kautsky curves for *P. sativum*. The height of the peak (P) was taken as a value of 1 and other features were calculated from this. The fast induction peak ( $P_{fast}$ ) was used for calculating the inflection (I) and P/I. The slow induction peak ( $P_{slow}$ ) was used to calculate the secondary peak or plateau (M), terminal fluorescence (T) and P/T.

RWC (%)	$P_{fast}$	I	$P_{slow}$	M	T	P/T
100	1	0.4	1	0.9	0.13	7.7
50	0.88	0.3	0.9	0.9	0.2	4.5
50 rehyd.	0.75	0.3	0.8	0.7	0.25	3.2
30	0.5	0.2	0.56	0.5	0.1	5.6
30 rehyd.	0.2	0.14	0.35	-	0.08	4.3
5	0.1	0.1	0.08	-	0.06	1.3
5 rehyd.	0.04	0.03	0.01	-	0.01	1

The fast induction kinetics for *P. sativum* (Fig. 4.3a) were very similar to those for *A. raddianum*. The slow induction kinetics (Fig. 4.3b) were, however, different in a number of aspects. The parameter M ( $CO_2$  fixation), was still visible at 30% RWC and there was a slight decrease in T as the leaves were dried to 30% RWC. A further drop occurred at

5% RWC. The P/T value, however, was higher at 30% RWC than 50% RWC but then decreased substantially once the leaves were dried to 5% RWC.

There were differences which indicated further damage had occurred (decrease in P and rise in T) between the leaves at 50% RWC and those that had been dried to 50% and then rehydrated. This shows that *P. sativum* is more susceptible to rehydration damage than *A. raddianum* which showed this damage only at 30% RWC.

**Table 4.3:** A summary of the fast and slow induction parameters derived from the Kautsky curves for *I. woodii*. The height of the peak (P) was taken as a value of 1 and other features were calculated from this. The fast induction peak ( $P_{fast}$ ) was used for calculating the inflection (I). The slow induction peak ( $P_{slow}$ ) was used to calculate the secondary peak or plateau (M) and terminal fluorescence (T) and P/T.

RWC (%)	$P_{fast}$	I	$P_{slow}$	M	T	P/T
100	1	0.4	1	0.8	0.1	10
50	0.68	0.35	0.8	0.7	0.08	10
50 rehyd.	0.6	0.3	0.8	0.7	0.08	10
30	0.47	0.27	0.6	0.6	0.3	2
30 rehyd.	0.4	0.2	0.6	0.6	0.3	2
5	0.06	0.06	0.05	-	0.03	1.6
5 rehyd.	0.02	0.02	0.01	-	0.01	1

Fast induction curves of *I. woodii* (Fig 4.4a) showed similar trends to both *A. raddianum* and *P. sativum* during dehydration. On rehydration, however, the induction parameters (Table 4.3) were similar to those of the dehydrated material. Rehydration damage became evident only at RWCs of 5%. There was little difference in leaves at full turgor and those dried to 50% RWC in the slow induction curves (Fig. 4.4b). The value of T started increasing in leaves dried to 30% RWC. Leaves dried to 5% RWC showed a decrease in

T, similar to the other species and the P/T value was close to one. The P/T value decreased only at 30% RWC for *I. woodii* indicating that this species is less affected by drying to 50% RWC than the others discussed so far.

**Table 4.4:** A summary of the fast and slow induction parameters derived from the Kautsky curves for *G. livingstonei*. The height of the peak (P) was taken as a value of 1 and other features were calculated from this. The fast induction peak ( $P_{fast}$ ) was used for calculating the inflection (I). The slow induction peak ( $P_{slow}$ ) was used to calculate the secondary peak or plateau (M), terminal fluorescence (T) and P/T.

RWC (%)	$P_{fast}$	I	$P_{slow}$	M	T	P/T
100	1	0.23	1	0.95	0.068	14.5
50	-	0.15	0.75	0.7	0.23	3.2
50 rehyd.	-	0.14	0.4	-	0.07	5.5
30	-	0.13	0.5	0.47	0.37	1.3
30 rehyd.	0.02	0.02	0.06	-	0.04	1.5
5	0.06	0.05	0.1	-	0.08	1.25
5 rehyd.	0.01	0.01	0.007	-	0.007	1

The fast and slow induction curves and parameters for *G. livingstonei* are shown in Figures 4.5a and 4.5b and Table 4.4. The 50% and 30% RWC and the rehydrated 50% RWC treatments did not reach a peak (P) in fluorescence within the 5 seconds that the fast induction kinetics were conducted. The parameter M (associated with the dark reactions of photosynthesis) was still present at 50% RWC during drying but was not evident on rehydration from 50% RWC. Leaves of *G. livingstonei* showed damage during rehydration from RWCs as high as 50%. This was particularly noticeable with the slow induction kinetics where the patterns of leaves rehydrated from 50% RWC were very different from those of leaves dehydrated to 50% RWC. The leaves rehydrated from 30% RWC showed similar trends to the material dried to 5% RWC, with only a slight rise in fluorescence

above the base line level.

During dehydration, the fast and slow induction curves of *C. nanum* (Figs. 4.6a & b) showed trends similar to the other four species. The decrease in parameters such as P and the increase in T (Table 4.5) were not as marked as with some of the other species. The major difference between *C. nanum* and the desiccation-sensitive species occurred on rehydration. With *C. nanum* the rehydrated leaves showed similar curves to the full turgor and the dehydrated 50% RWC leaves, this held true even for the leaves dried to 5% RWC. This indicated recovery from the injury occurring during dehydration. There are still some features, such as M (carbon fixation) and P/T, which had not recovered to the full turgor level during the time these leaves were given to rehydrate. Both these parameters would be reliant on the dark reactions of photosynthesis, which possibly take longer to recover because of CO<sub>2</sub> limitations consequent upon stomatal closure.

**Table 4.5:** A summary of the fast and slow induction parameters derived from the Kautsky curves for *C. nanum*. The height of the peak (P) was taken as a value of 1 and other features were calculated from this. The fast induction peak ( $P_{fast}$ ) was used for calculating the inflection (I). The slow induction peak ( $P_{slow}$ ) was used to calculate the secondary peak or plateau (M), terminal fluorescence (T) and P/T.

RWC (%)	$P_{fast}$	I	$P_{slow}$	M	T	P/T
100	1	0.15	1	0.6	0.18	5.5
50	0.9	0.15	0.9	0.9	0.16	5.6
50 rehyd.	0.9	0.15	0.9	0.9	0.2	4.5
30	0.73	0.2	0.8	0.8	0.4	2
30 rehyd.	0.87	0.2	0.9	0.9	0.2	4.5
5	0.1	0.06	0.1	0.07	0.07	1.4
5 rehyd.	1	0.17	0.85	0.8	0.2	4

The recovery of induction kinetics after various periods of rehydration of *C. nanum* dehydrated to 5% RWC are shown in Figures 4.7a and 7.4b and Table 4.6. The leaves that had been placed in a moist atmosphere overnight showed a slightly lower P than that of the fully turgid leaves. The material that had been placed straight into water and rehydrated for one hour showed slight recovery over the 5% dehydrated level. Six hours of rehydration in water led to further recovery while being left in water overnight led to almost complete recovery. This experiment showed that being placed straight into water did not cause further damage to the leaves. However, a period of 18 hours is required for almost complete recovery, whether rehydration occurs directly in water, or with slow prior rehydration in a moist atmosphere.

**Table 4.6:** A summary of the fast and slow induction parameters derived from the Kautsky curves for *C. nanum* taken during rehydration in water and in after 18 hours in a moist atmosphere. The height of the peak (P) was taken as a value of 1 and other features were calculated from this. The fast induction peak ( $P_{fast}$ ) was used for calculating the inflection (I). The slow induction peak ( $P_{slow}$ ) was used to calculate the secondary peak or plateau (M), terminal fluorescence (T) and P/T.

Rehydration	$P_{fast}$	I	$P_{slow}$	M	T	P/T
100% RWC	1	0.2	1	0.6	0.1	10
5% RWC	0.06	0.04	0.12	0.07	0.07	1.7
1 hour	0.2	0.1	0.22	0.18	0.18	1.2
6 hours	0.6	0.17	0.7	0.45	0.36	2
18 hours	1	0.18	0.98	0.56	0.1	9.8
18 hours moist atm.	1	0.16	0.9	0.63	0.11	9

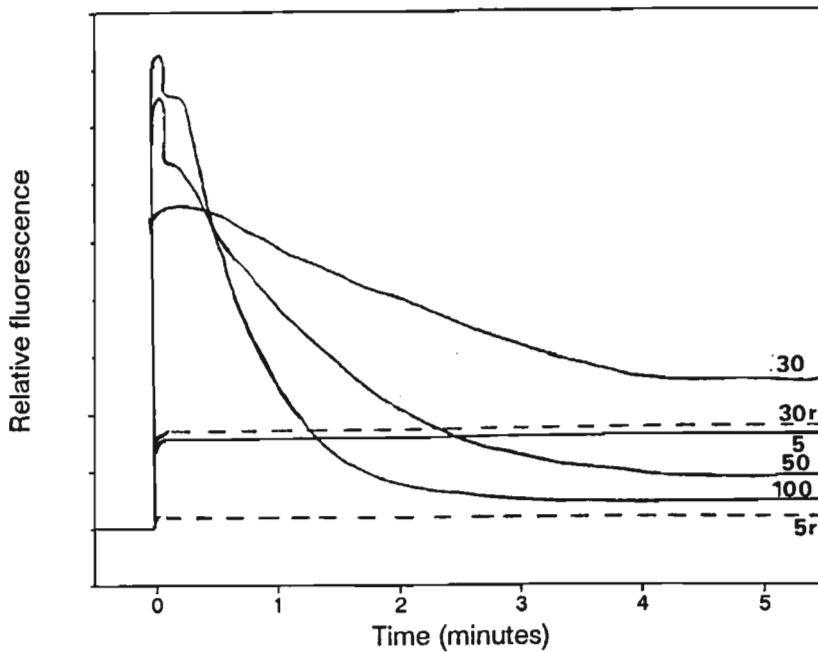
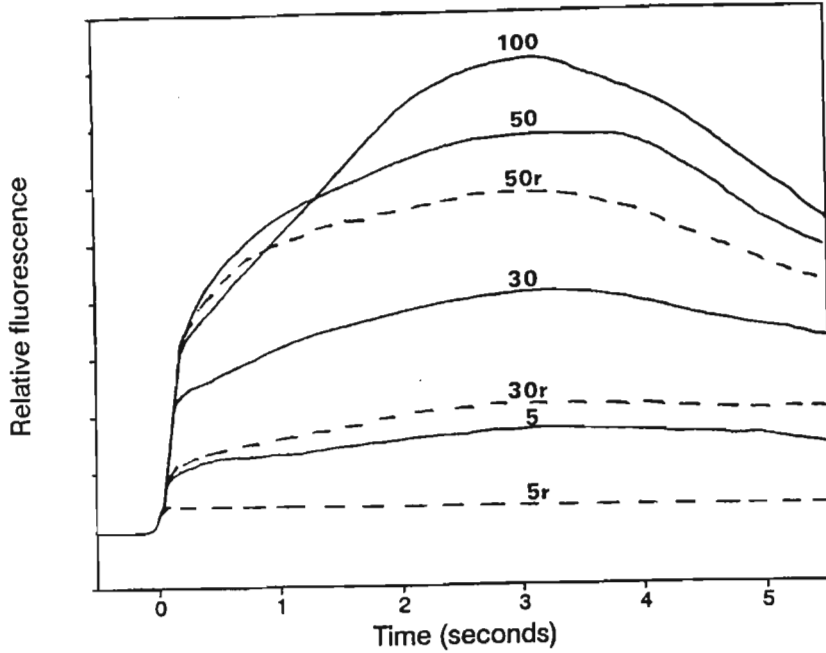


Figure 4.2: Fast (a) and slow (b) induction kinetics of *A. raddianum* at full turgor, 50%, 30% and 5% RWC and after dehydration to those RWCs followed by complete rehydration. The curves of the dehydrated material are solid and those of the rehydrated material are dashed. The slow induction curves for leaves dehydrated to 50% RWC, and leaves dehydrated to 50% RWC and then rehydrated, are superimposed because there was no difference between these two treatments.

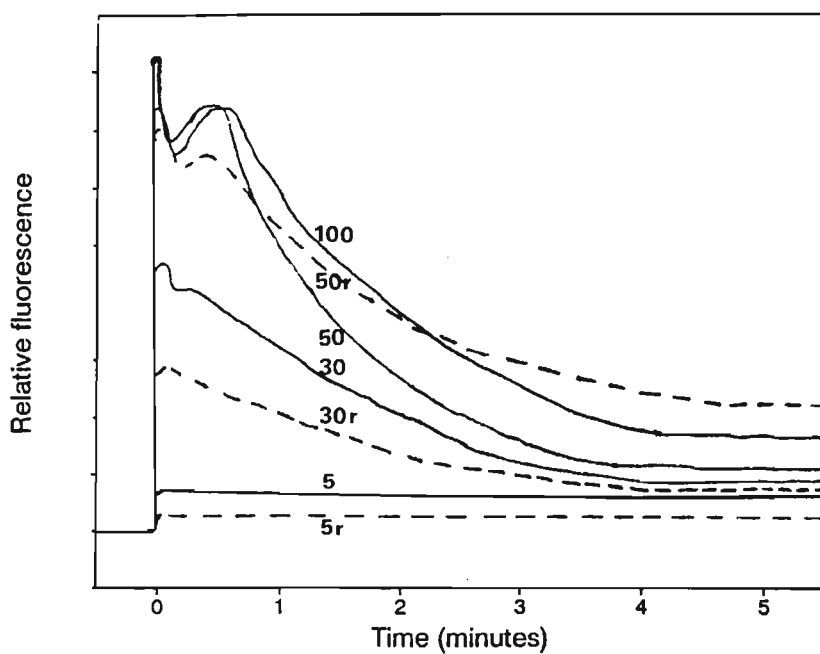
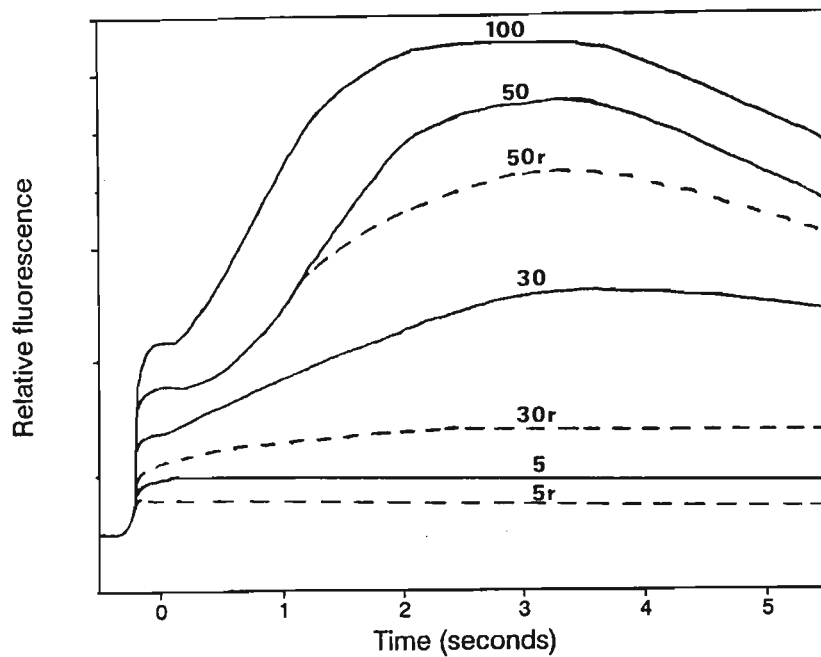
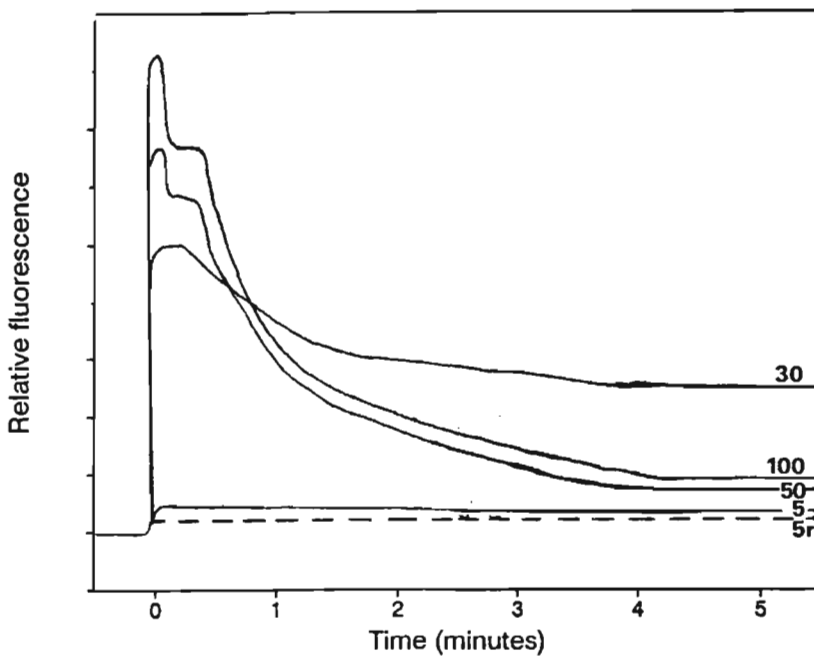
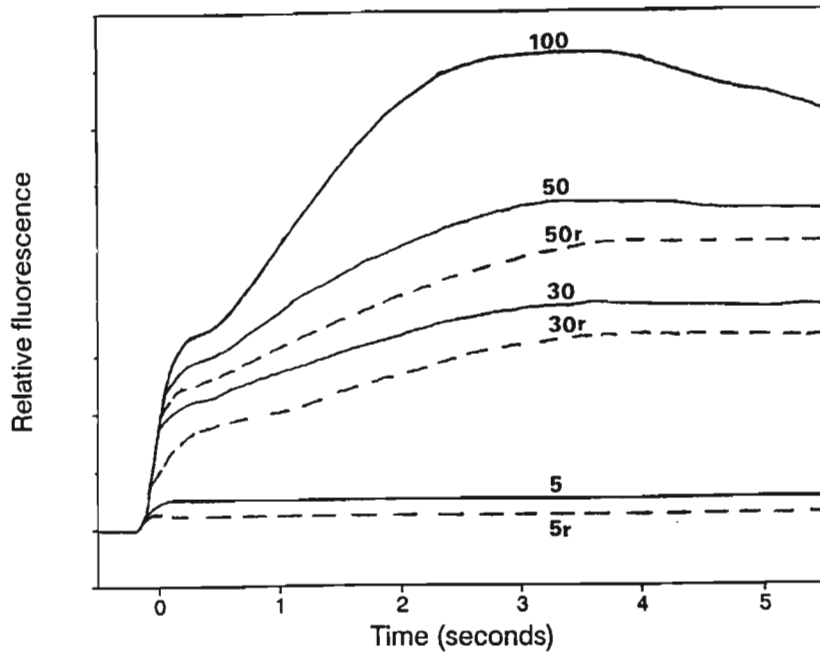


Figure 4.3: Fast (a) and slow (b) induction kinetics of *P. sativum* at full turgor, 50%, 30% and 5% RWC and after dehydration to those RWCs followed by complete rehydration. The curves of the dehydrated material are solid and those of the rehydrated material are dashed.



**Figure 4.4:** Fast (a) and slow (b) induction kinetics of *I. woodii* at full turgor, 50%, 30% and 5% RWC and after dehydration to those RWCs followed by complete rehydration. The curves of the dehydrated material are solid and those of the rehydrated material are dashed. The slow induction curves for leaves dehydrated to 50% RWC, and leaves dehydrated to 50% RWC and then rehydrated, are superimposed because there was no difference between the two treatments. This was also the case for leaves dehydrated to 30% RWC and leaves rehydrated from 30% RWC.

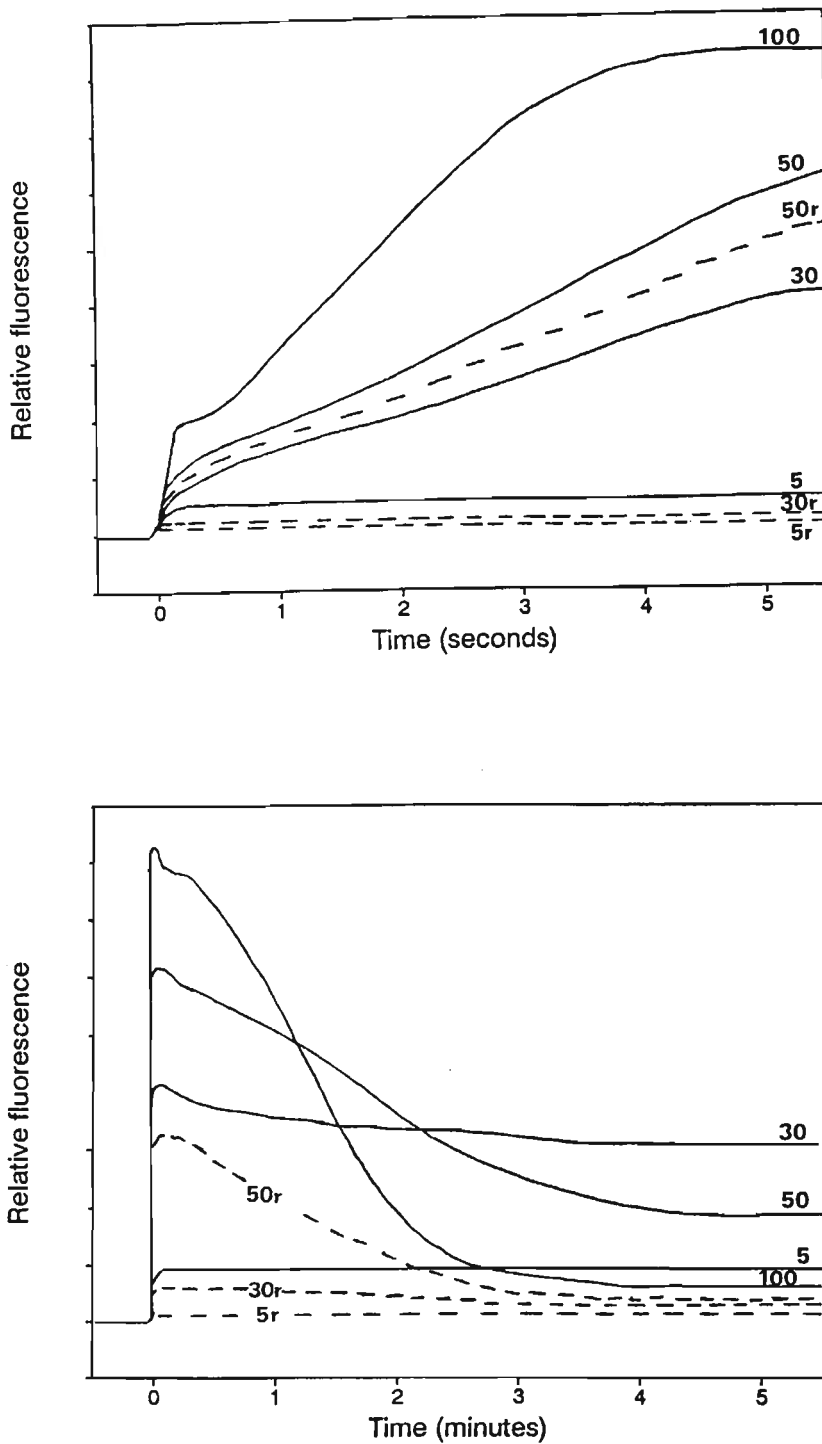
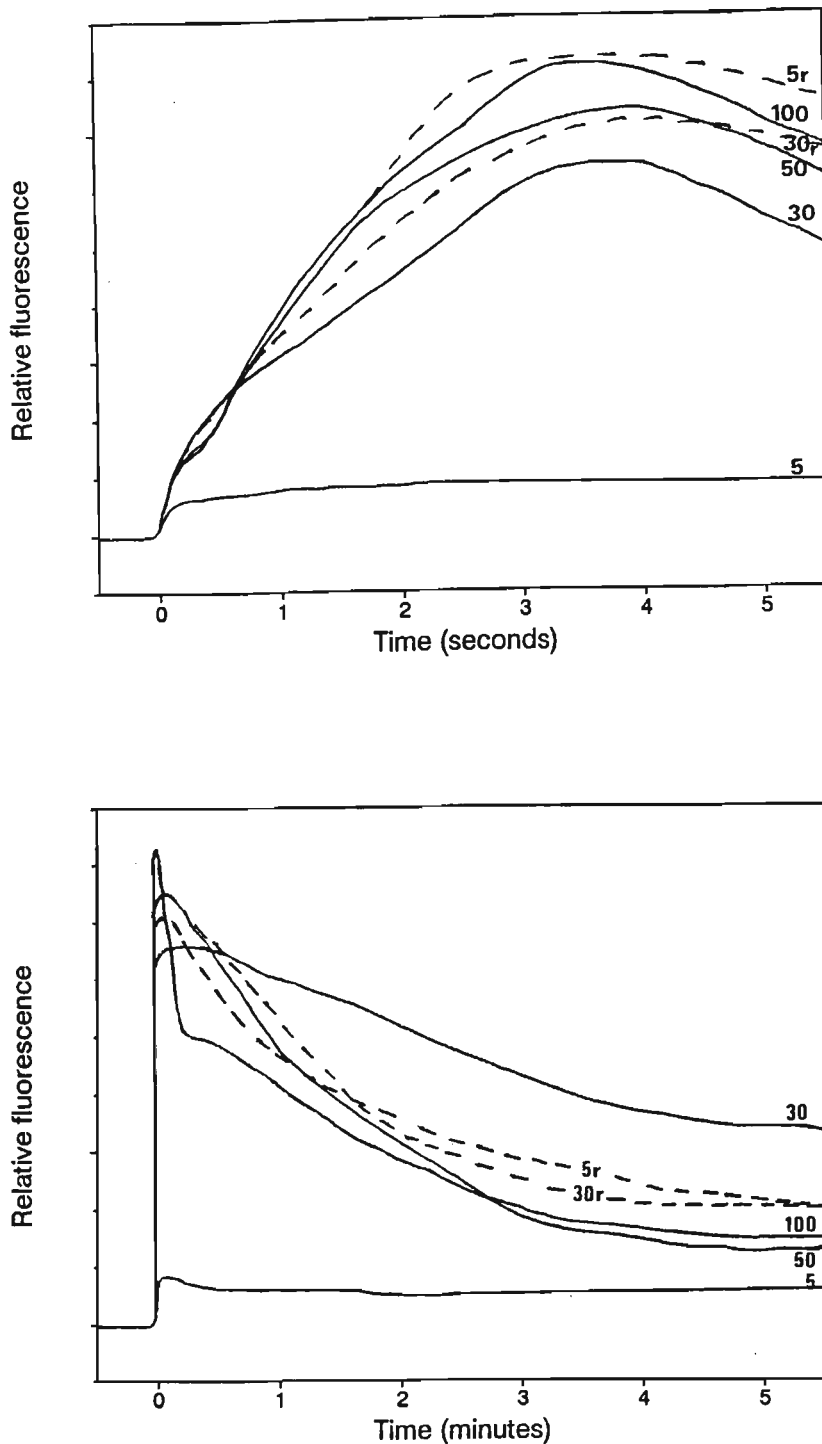
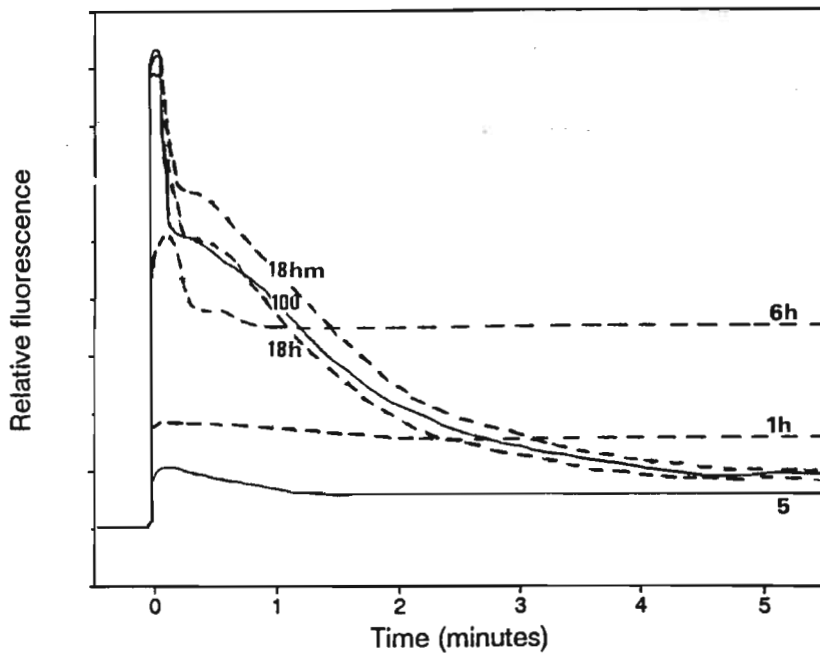
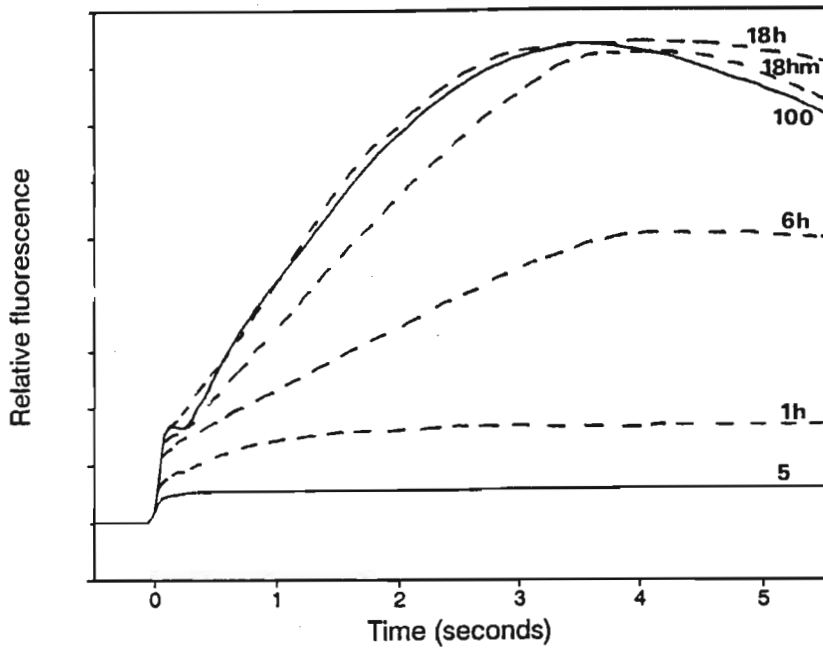


Figure 4.5: Fast (a) and slow (b) induction kinetics of *G. livingstonei* at full turgor, 50%, 30% and 5% RWC and after dehydration to those RWCs followed by complete rehydration. The curves of the dehydrated material are solid and those of the rehydrated material are dashed.



**Figure 4.6:** Fast (a) and slow (b) induction kinetics of *C. nanum* at full turgor, 50%, 30% and 5% RWC and after dehydration to those RWCs followed by complete rehydration. The curves of the dehydrated material are solid and those of the rehydrated material are dashed. The slow induction curves for leaves dehydrated to 50% RWC, and leaves dehydrated to 50% RWC and then rehydrated, are superimposed because there was no difference between these two treatments.



**Figure 4.7:** Fast (a) and slow (b) induction kinetics of *C. nanum* at full turgor (control) and leaves dried to 5% RWC, in their dry state and then their recovery with differing rehydration treatments and times. The curves of the dehydrated material are solid and those of the rehydrated material are dashed. The values and letters 1h, 6h and 18h refer to the leaves that were placed directly in water for 1 hour, 6 hours and 18 hours, while 18hm refers to the material that was slowly rehydrated for 18 hours in a moist atmosphere. 100 and 5 refer to material at 100% RWC and 5% RWC respectively.

$F_V/F_M$ ,  $F_O$  and  $F_V$

The RWC at which  $F_V/F_M$  and  $F_V$  decreased during drying and subsequent rehydration are summarised in Table 4.7. The data for  $F_O$  was not summarised this way as it was difficult to determine clear trends from the data shown in Figure 4.9.

**Table 4.7:** The RWC of material at which the  $F_V/F_M$  ratio and variable fluorescence ( $F_V$ ) decreased sharply during dehydration and after being dehydrated and rehydrated to as close to full turgor as possible. If a steady decrease and not a sharp one was observed then a dash was used to denote this. An R is used to denote recovery in *C. nanum*.

Species	$F_V/F_M$	$F_V/F_M$ rehyd.	$F_V$	$F_V$ rehyd.
<i>A. raddianum</i>	35%	42%	70%	-
<i>P. sativum</i>	48%	50%	-	42%
<i>I. woodii</i>	21%	21%	45%	40%
<i>G. livingstonei</i>	25%	58%	30%	75%
<i>C. nanum</i>	18%	R	60%	R

The change in the  $F_V/F_M$  ratio during dehydration is shown in Figure 4.8. For the four desiccation-sensitive species the  $F_V/F_M$  remained fairly constant over a wide range of RWCs and then decreased sharply. The RWC at which this decrease occurred was at about 35% for *A. raddianum*, 48% for *P. sativum*, 20% for *I. woodii* and 25% for *G. livingstonei*. The  $F_V/F_M$  for the desiccation-tolerant plant, *C. nanum*, however, showed a shallow decline from about 55% RWC and then a sharp decline from around 18% RWC. This shallow decline may indicate an ordered shut-down process occurring in the desiccation-tolerant plant that does not occur in the sensitive plants. As the  $F_V/F_M$  is a good indication of quantum efficiency it appears that photosynthesis in *P. sativum* is the most susceptible to dehydration damage followed by that of *A. raddianum*.

The results indicate that, after rehydration, further damage occurred, to a limited extent in *P. sativum* and *A. raddianum*, and to quite a large extent in *G. livingstonei*. Leaves of *G. livingstonei* dried to 30% RWC did not show decreases in  $F_v/F_M$  in the dehydrated state. However, after they were rehydrated, the  $F_v/F_M$  was much lower than that of the dehydrated leaves. The leaves of *I. woodii* do not appear to be further damaged during rehydration. However, there was no recovery of the leaves almost to full turgor levels as was the case with *C. nanum*. Leaves of *C. nanum*, although they exhibited a similar decline in  $F_v/F_M$  (or PSII efficiency) during dehydration to the sensitive species, were able to repair that damage during rehydration. These results support the conclusions drawn from the fast and slow induction kinetics curves.

Changes in the  $F_v/F_M$  ratio can be brought about by changes in  $F_0$  (base line fluorescence) or variable fluorescence  $F_v$ . Figure 4.9 shows the change in the  $F_0$  during dehydration. There did not appear to be clear trends for changes in  $F_0$  during drying as there were for other fluorescence parameters measured. One of the problems is that  $F_0$  is dependent on leaf geometry and as different leaves were used for each RWC it is not surprising that absolute values of  $F_0$  varied among samples. There are, however, trends in some of the species which are worth further discussion.

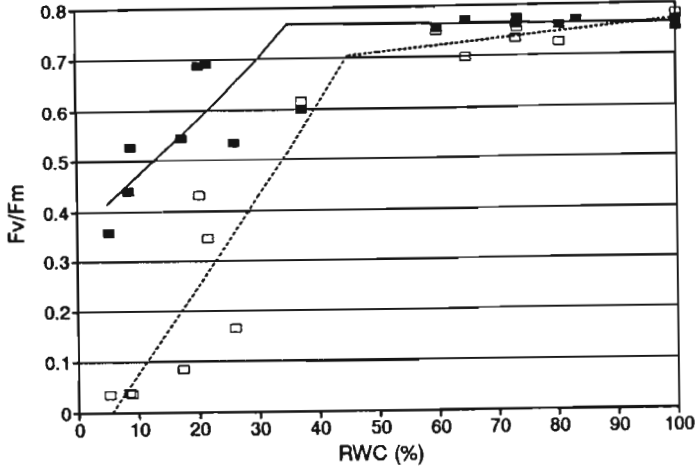
During dehydration there was an increase in  $F_0$  below RWCs of 25% for *A. raddianum* and *G. livingstonei*. For rehydrated *A. raddianum* leaflets, which had been dried below 50% RWC, the  $F_0$  level dropped below the  $F_0$  value of the dehydrated leaflets. It is not known whether this indicates recovery or further damage for *A. raddianum*. With *G. livingstonei*, however, the rehydrated leaves had  $F_0$  values higher than those of the dehydrated leaves, indicating further damage had occurred. For *P. sativum*, the  $F_0$  values during dehydration appear to decrease at a constant rate.  $F_0$  appeared to remain fairly constant during dehydration in *I. woodii*. Leaves that were dried below 30% RWC and then rehydrated, however, had  $F_0$  values quite a lot lower than the dehydrated value. The drier the leaves the greater was this discrepancy between the dehydrated and rehydrated leaves. This was the only fluorescence parameter of *I. woodii* which showed a difference between dehydrated and rehydrated leaves. Leaves of *C. nanum* showed a decrease in  $F_0$  with dehydration below about 70% RWC. The  $F_0$  values, however, recovered to close to

the full turgor level during rehydration. Increases in  $F_o$  values are thought to indicate physical damage to the reaction centres, though why the  $F_o$  values should decrease in some species and increase in others is not known.

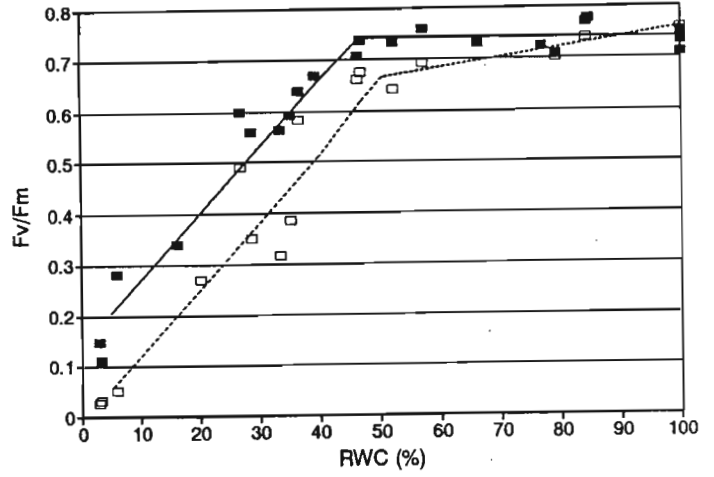
Figure 4.10 shows the changes in the variable fluorescence  $F_v$  with dehydration.  $F_v$  is the height of the fluorescence peak above the baseline level  $F_o$ , or alternatively it is the difference between the maximum fluorescence ( $F_M$ ) and  $F_o$ . During dehydration of *A. raddianum* leaflets,  $F_v$  decreased at a fairly constant rate below 70% RWC. This is in contrast to  $F_v$  remaining at a fairly constant level and then declining sharply as for *I. woodii* and *G. livingstonei*. There was no clear trend for the changes in  $F_v$  for *P. sativum* although it appeared to decrease fairly slowly until 20% RWC from where on it decreased at a slightly faster rate but did not show the sharp decline of the other two species. The  $F_v$  of *C. nanum* showed a similar decrease to *A. raddianum* below RWCs of about 60%. Again *C. nanum* leaves appeared to recover (though not to previous full turgor levels) once rehydrated, while those of *G. livingstonei* showed marked differences between the dehydrated and rehydrated state indicating further damage caused during rehydration.

Changes in  $F_v/F_M$ , therefore, appear to have been brought about by both damage to reaction centres (changes in  $F_o$ ) and the inactivation of the primary photochemistry of PSII (changes in  $F_v$ ).

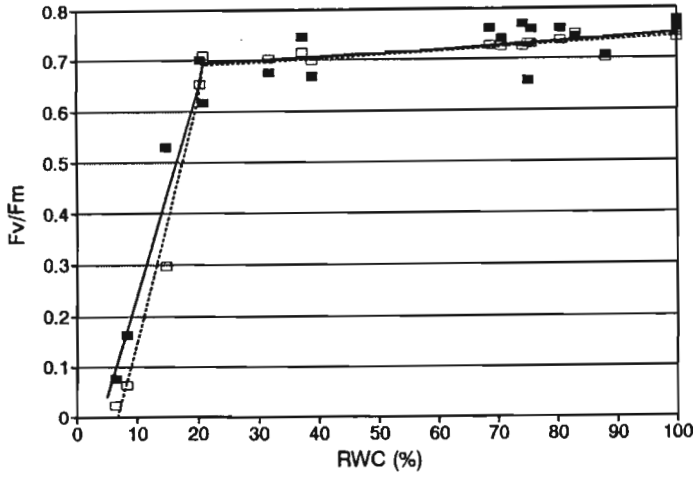
*Adiantum raddianum*



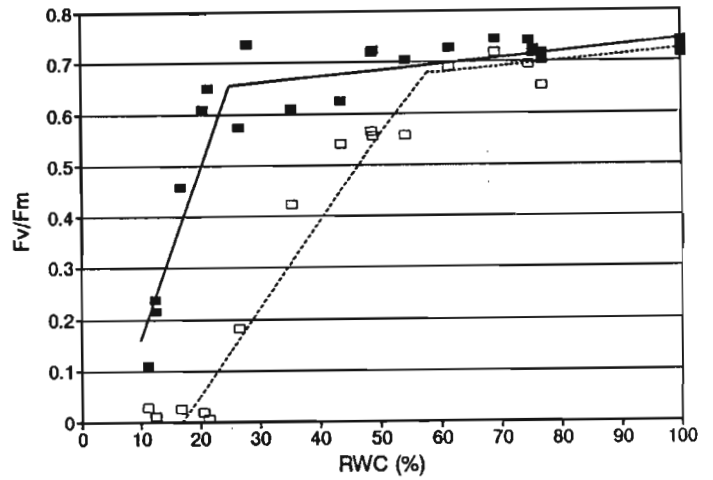
*Pisum sativum* 123



*Isoglossa woodii*



*Garcinia livingstonei*



*Craterostigma nanum*

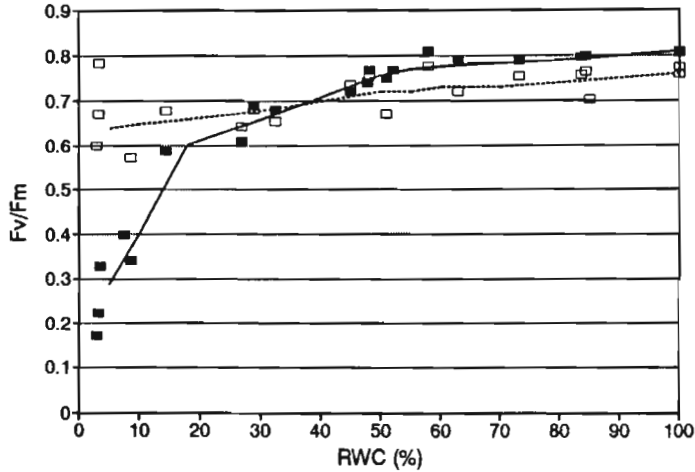
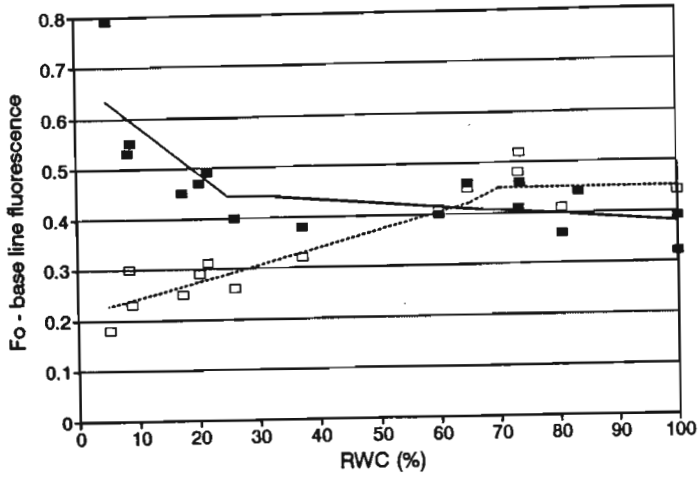
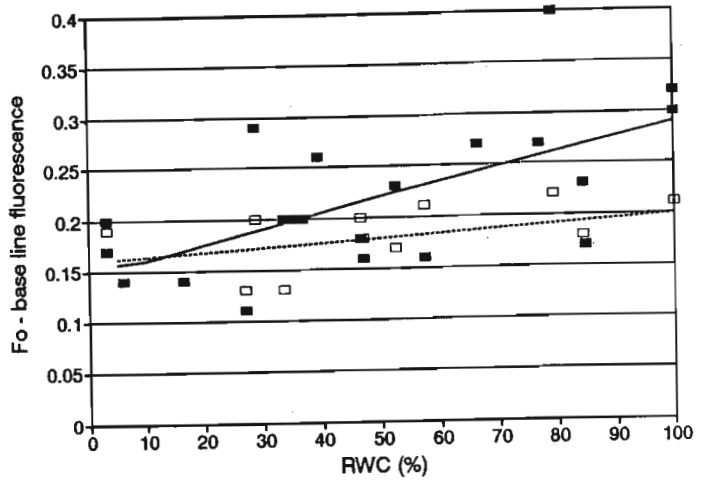


Figure 4.8: Changes in  $F_V/F_M$  (quantum efficiency) during dehydration and subsequent rehydration for all five species. ■ and solid lines refer to the dehydrated leaf material, while □ and dashed lines refer to the material dried to that particular RWC and rehydrated to as close to full turgor as could be achieved.

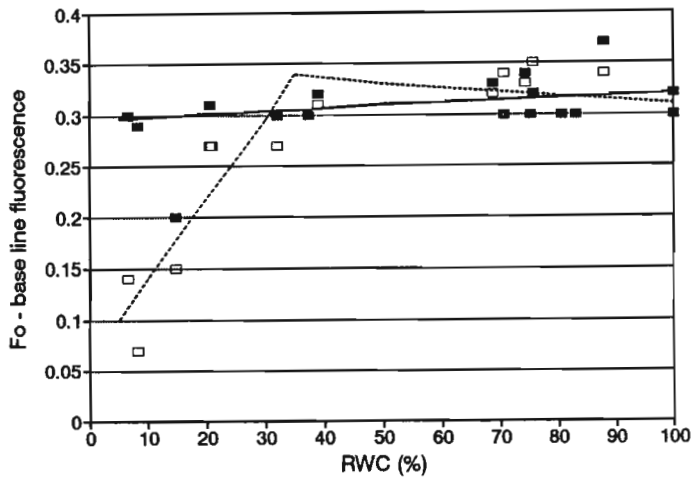
*Adiantum raddianum*



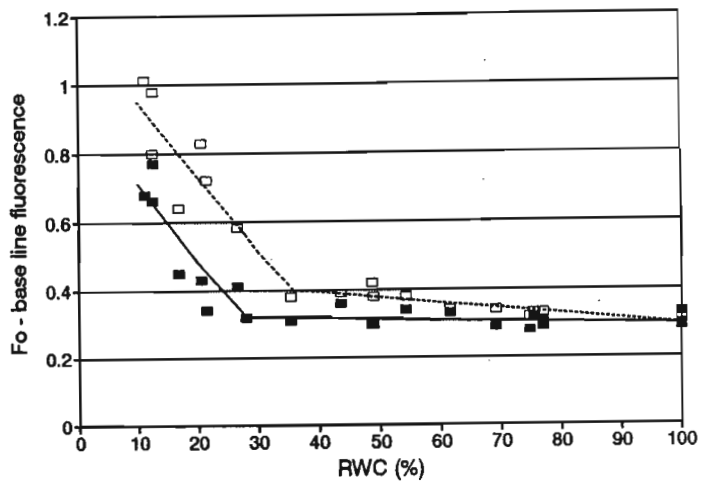
*Pisum sativum* 124



*Isoglossa woodii*



*Garcinia livingstonei*



*Craterostigma nanum*

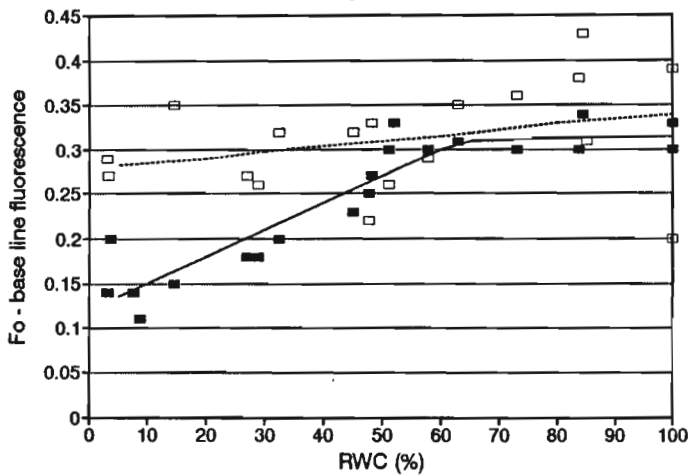
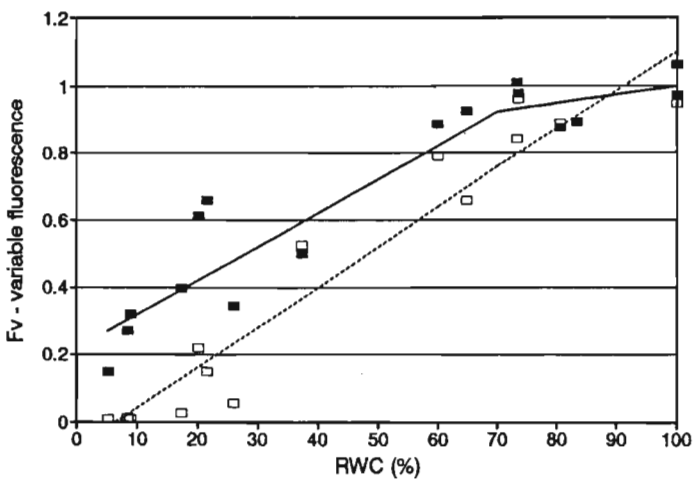
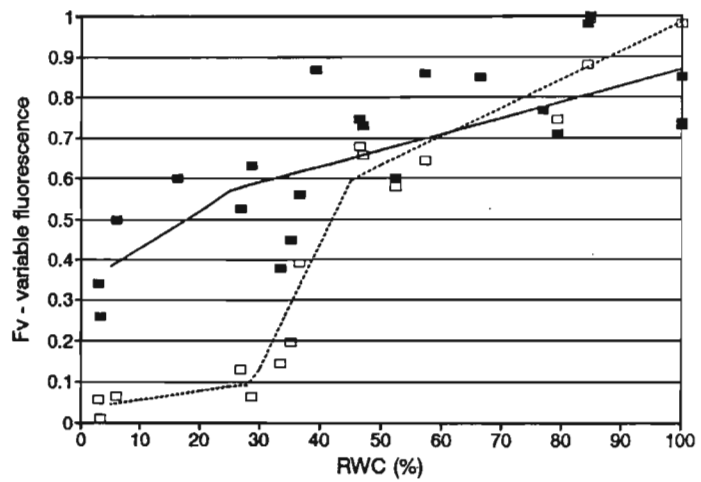


Figure 4.9: Changes in  $F_0$  (baseline fluorescence) during dehydration and subsequent rehydration for all five species. ■ and solid lines refer to the dehydrated leaf material, while □ and dashed lines refer to the material dried to that particular RWC and rehydrated to as close to full turgor as could be achieved. Changes in  $F_0$  are an indication of damage to the PSII reaction centre.

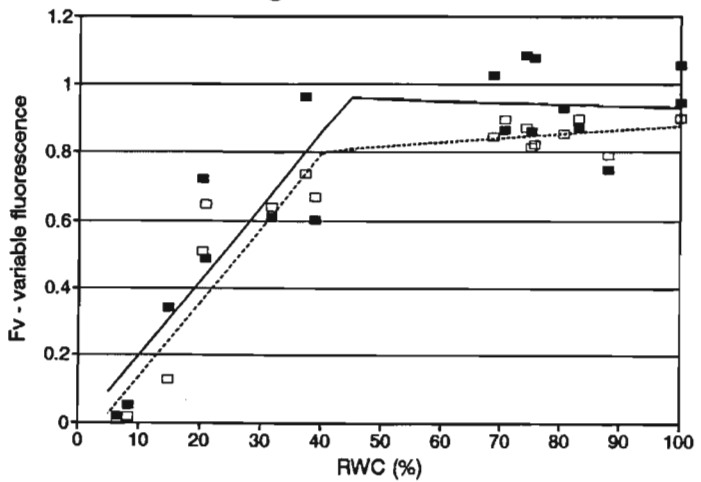
*Adiantum raddianum*



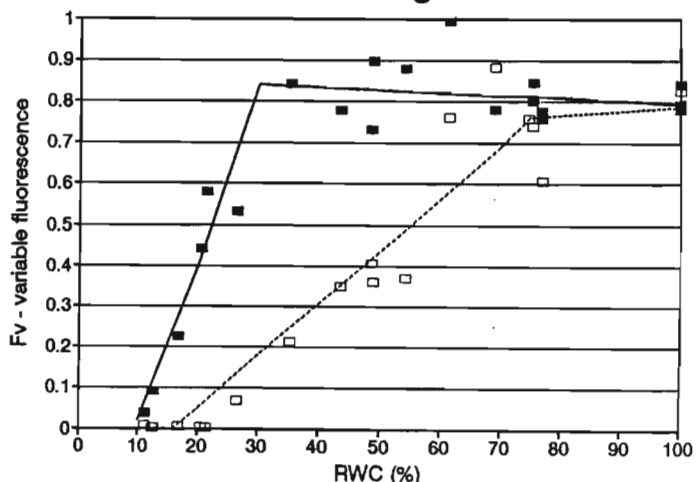
*Pisum sativum*



*Isoglossa woodii*



*Garcinia livingstonei*



*Craterostigma nanum*

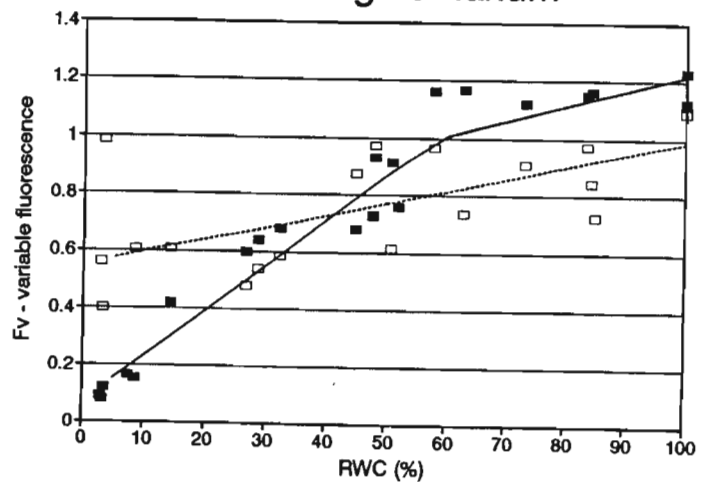


Figure 4.10: Changes in  $F_v$  (variable fluorescence or primary photochemistry) during dehydration and subsequent rehydration for all five species. ■ and solid lines refer to the dehydrated leaf material, while □ and dashed lines refer to the material dried to that particular RWC and rehydrated to as close to full turgor as could be achieved.

### Respiration

Figure 4.11 shows the respiratory rates (measured in  $\mu\text{mols CO}_2 \text{ s}^{-1} (\text{gdw})^{-1}$ ) with decreasing RWC and upon subsequent rehydration. Table 4.8 summarises the RWCs at which changes in respiratory rate were measured.

**Table 4.8:** The RWCs at which respiration decreased sharply during dehydration and after subsequent rehydration to as close to full turgor as could be achieved.

Species	RWC at which respiration decreased during dehydration.	RWC at which respiration decreased after rehydration.
<i>A. raddianum</i>	30%	20%
<i>P. sativum</i>	15%	10%
<i>I. woodii</i>	40%	20%
<i>G. livingstonei</i>	50%	40%
<i>C. nanum</i>	20%	10%

Respiratory rates decreased slightly with drying until critical RWCs after which a sharp decline was noted for *A. raddianum*, *P. sativum* and *I. woodii*. Respiration rates of *G. livingstonei* and *C. nanum* tended to increase on drying and then also reach a point where they declined sharply. The decline in respiratory rates was sharpest in *P. sativum* and *C. nanum* and the RWCs at which this decline occurred was lowest in these two species (15% for *P. sativum* and 20% for *C. nanum*) indicating that the respiratory apparatus of these two species is the most resistant to drying. The highest RWC at which a marked decline in respiration occurred was found in *G. livingstonei* (50%).

Upon rehydration of the desiccation-sensitive species, respiratory rates generally rose to levels higher than those occurring in the dehydrated material. For the tolerant species, *C. nanum*, respiratory rates of dried and rehydrated material were similar. In all species, respiration could be measured after rehydration from lower RWCs than the RWC at which

respiration was absent from dry material. This was true even for *G. livingstonei* which has been shown to be further damaged by rehydration (Fig. 4.5 and Table 4.4).

The data in general were noisy and in some cases, particularly with rehydration, it was difficult to determine trends. The respiratory rates of *P. sativum* were far higher than for the other species and those for *G. livingstonei* much lower. This could reflect different growth rates of the species, or could be because most of the dry weight of *G. livingstonei* is taken up by structural material and not metabolically active material, whereas with pea there is very little structural material, and most of the material will be metabolically active resulting in high respiratory rates per unit dry weight.

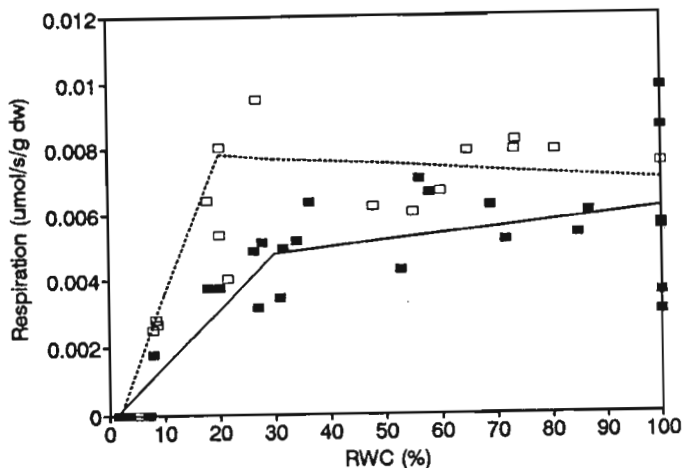
## Discussion

### Chlorophyll fluorescence

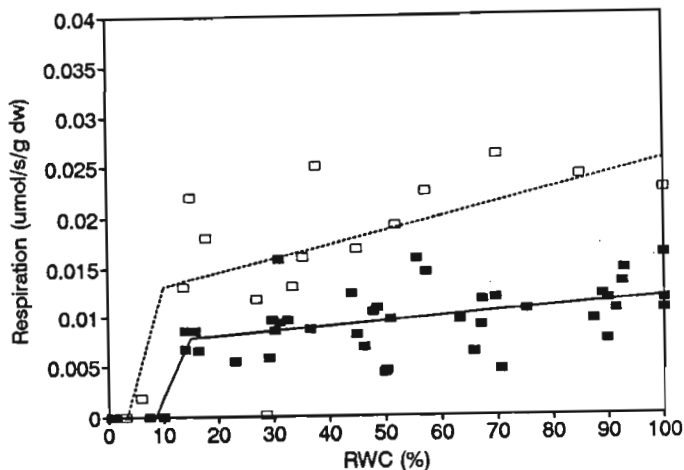
From the work done in this study it appears that dehydration adversely affects the photosynthetic apparatus in a number of ways. It is also apparent that subsequent rehydration has a deleterious affect on *A. raddianum*, *P. sativum* and particularly *G. livingstonei*. Rehydration does not seem to affect *I. woodii* adversely. In *C. nanum*, rehydration from very low RWCs resulted in recovery of the leaf to pre-dehydration levels provided sufficient time was given for repair to occur.

Changes occurred in both the fast and the slow induction kinetics during dehydration of all five species. As fast induction kinetics are largely related to the primary processes of PSII and the slow induction kinetics are related more to the interaction between processes in the thylakoid membranes and metabolic processes in the stroma (Bohlàr-Nordenkampf & Öquist, 1993) it appears that both the primary membrane and the metabolic processes are adversely affected by drying. The trends shown in the induction curves are fairly similar to those given by Hetherington *et al.* (1982) working on *Borya nitida*, a desiccation-tolerant plant that loses its chlorophyll on drying. They also showed a decrease in P, a loss of the SM portion and an increase in T with drying. Hetherington & Smillie (1982) also showed that T increased with increasing dehydration but then decreased sharply as dehydration became severe. The changes in fast induction were also similar to

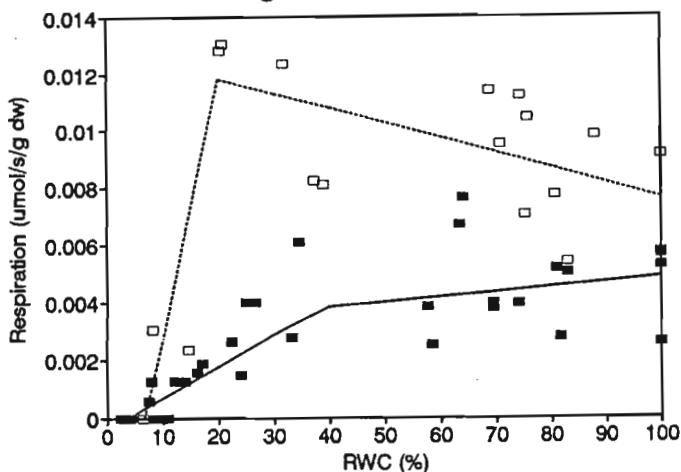
*Adiantum raddianum*



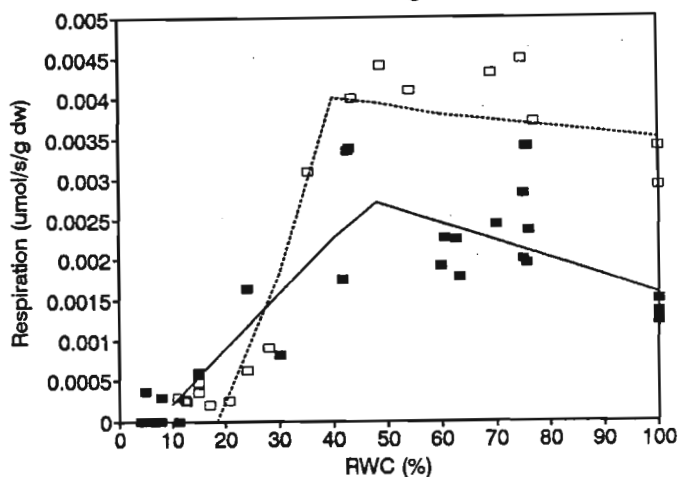
*Pisum sativum*



*Isoglossa woodii*



*Garcinia livingstonei*



*Craterostigma nanum*

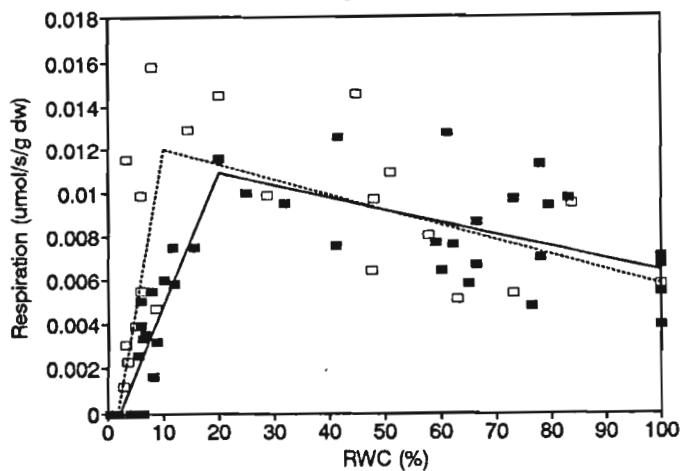


Figure 4.11: Changes in respiration, measured as  $\mu\text{mols CO}_2 \text{ s}^{-1} (\text{gdw})^{-1}$ , with decreasing RWC. ■ and solid lines refer to the dehydrated leaf material, while □ and dashed lines refer to the material dried to that particular RWC and rehydrated to as close to full turgor as could be achieved.

those shown by Smillie & Hetherington (1983) in their work on chilling. During mild water stress of *Nerium oleander* plants, Govindjee *et al.* (1981) showed a similar decrease in the height of P. They attributed this decline in P to the inhibition of the electron donation (or water oxidation) side of PSII.

The  $F_v/F_M$  ratio at full turgor for all the species was similar to those reported by Bohlár-Nordenkamp *et al.* (1989) and Demmig and Björkman (1987). The decrease in  $F_v/F_M$  was thought to indicate a decrease in the quantum yield of photosynthesis. Seel *et al.* (1992a), studying desiccation-tolerant and sensitive mosses, showed sharp decreases in  $F_v/F_M$  with desiccation of both tolerant and sensitive species. Muslin and Homann (1992) studied fluorescence during drying in a desiccation-sensitive and tolerant fern. They showed a fairly shallow decrease in  $F_v/F_M$  after drying below 50% water content (comparable to the 50% RWC reported here) for the sensitive species, *Asplenium resiliens*. This was at a higher RWC than the decrease in  $F_v/F_M$  of the sensitive species used in this study. Muslin and Homann (1992) also showed a sharp decrease below 30% water content (comparable to the present 30% RWC) in the tolerant species *Polypodium polypodioides* L. The decrease in  $F_v/F_M$  of the desiccation-tolerant *C. nanum* occurred at lower RWC (18%) than shown for this fern. As with *C. nanum*, both the desiccation-tolerant moss and fern showed a recovery of  $F_v/F_M$  with subsequent rehydration. The shallow decline followed by the sharp decline has not been reported for other desiccation-tolerant species on which fluorescence studies have taken place. Such shallow declines in physiological processes with drying in desiccation-tolerant plants are thought to indicate an orderly shut-down process (Genkel & Pronina, 1969).

Csapó *et al.* (1991) showed increases in  $F_o$  with chilling and Larcher, Wagner and Thammathaworn (1990) showed a similar increase in  $F_o$  with an increase in temperature. They report that this reflects alterations in pigment contents and arrangements. Demmig and Björkman (1987) report that the response of  $F_o$  to high light was complex. In some species  $F_o$  increased, in others it decreased, and in yet others it showed no marked consistent changes. This was similar to the results obtained in this study. Demmig and Björkman (1987) explained their results for changes in  $F_o$  as follows: an increase in  $F_o$  is thought to arise from physical damage to the reaction centres and the decrease in  $F_o$

results from an increased non-radiative energy dissipation.

In the fluorescence induction curve, the rise from  $F_0$  to  $F_M$  (the variable fluorescence  $F_V$ ) reflects the reduction of  $Q_A$ . Changes in  $F_V$  reflect an inactivation of the primary photochemistry of PSII (Greer *et al.*, 1986). Csapó *et al.* (1991) reported a decrease in  $F_V$  with chilling, while Demmig & Björkman (1987) have shown that exposure of leaves to excessive light results in a decrease in  $F_V$ . A reduction in  $F_V$  does not exclusively or necessarily reflect a detrimental effect on PSII but may result from protective regulatory processes that serve to dissipate excess excitation energy (Demmig & Björkman, 1987). In the present study dehydration (in some cases mild dehydration) resulted in similar decreases in  $F_V$ . At higher RWC the decrease was possibly due to protective processes (non-photosynthetic quenching); however at low RWCs, damage to PSII would have occurred.

Conroy *et al.* (1988) also measured fluorescence as a function of RWC in *Helianthus annuus* and found that electron transport activity, inferred by chlorophyll *a* fluorescence remained functional until tissue was desiccated to the lethal RWC, which was a RWC of 36%. This correlates well with some of the present results as it appears from both the fast and slow induction curves and from measurements of  $F_V/F_M$  that RWCs of about 30% and below are the most detrimental to the leaves. Studies of recovery during rehydration were done using desiccation-tolerant species (Hetherington & Smillie, 1982; Hetherington *et al.*, 1982; Muslin & Homann, 1992; Seel *et al.*, 1992a). From these studies it could be seen that *C. nanum* showed similar recovery of fluorescence characteristics to these other desiccation-tolerant species. No reports were found showing the further damage during rehydration, as was shown for three of the species studied here, though Seel *et al.* (1992a) and Muslin and Homann (1992) showed that sensitive species did not recover on rehydration after being desiccated.

The major damage to the photosynthetic apparatus seemed to occur at a similar RWC to that at which increases in membrane leakage were shown and where the sensitive species showed no or very little recovery (Chapter 3). This supports the hypothesis of Kaiser's (1987) that irreversible damage occurs to the photosynthetic apparatus below RWCs of

30%. The RWC at which irreversible damage occurs are higher than the levels of non-freezable water (Chapter 2). The RWCs at which turgor was lost (Chapter 2) would probably have had an impact on the gas exchange characteristics of photosynthesis, but did not effect the membrane associated processes examined using chlorophyll fluorescence.

### Respiration

In some instances (*A. raddianum* and *G. livingstonei*) the variation in the data made it difficult to identify the RWC at which respiration rate declined. However, there appeared to be no distinct difference in responses of respiration to desiccation between the sensitive and tolerant plants used in this study. They showed a response similar to that suggested by Genkel and Pronina (1969) for sensitive plants, in that respiration remained constant or increased with drying, until a critical RWC below which a sharp decline in respiration occurred.

A sharp decline in respiration below RWCs of about 20% has also been reported by Gaff (1989) for the desiccation-tolerant plants *Talbotia elegans* and *Xerophyta villosa*. This is similar to the decline in *C. nanum*. That author also reported that the response of respiration to desiccation was similar for both sensitive and tolerant plants. As with the study of Bewley and Thorpe (1974), no respiration was measurable in very desiccated material.

The sharp decline in respiration occurred at RWCs similar to that at which electrolyte leakage increased in *A. raddianum*, *I. woodii* and *C. nanum* (Chapter 3). The increase in leakage occurred at higher RWCs (25%) than the decrease in respiration (15% RWC) of *P. sativum*. With *G. livingstonei*, on the other hand, respiration decreased at higher RWC (50%) than the increase in leakage (30% RWC). This discrepancy may be due to the difficulty in analysing the respiration data (particularly that for *G. livingstonei*).

The data for the rehydrated material showed, in all cases, that respiratory activity was occurring in rehydrated material when none was measured in the dry state at those specific RWCs. Whether this indicates a recovery upon rehydration at marginal RWCs, or CO<sub>2</sub> production due to cellular damage, is not known. Krochko, Winner & Bewley (1979)

found that respiration upon rehydration reached and surpassed control levels within 30 minutes in the desiccation-tolerant moss *Tortula ruralis*. For the sensitive material used in this study it was shown that respiration of rehydrated material generally exceeded that of the dehydrated material. The respiration rate of the desiccation-tolerant *C. nanum*, however, did not seem to be substantially higher than that of the dehydrated material.

### Conclusion

Respiration has been shown by many workers to be less sensitive to the effects of desiccation than photosynthesis (Bewley, 1979; Dilks & Proctor, 1979; Harten & Eickmeier, 1986; Seel *et al.*, 1992b). In comparing respiration with photosynthesis (in terms of quantum efficiency or  $F_v/F_M$ ) it appeared that respiration was more tolerant to drying than  $F_v/F_M$  in *A. raddianum* and *P. sativum*. The decrease in  $F_v/F_M$  and respiration occurred at similar RWCs in *C. nanum*, while in *I. woodii* and *G. livingstonei* respiration decreased at higher RWC than those at which  $F_v/F_M$  decreased during drying. However, most measurements of photosynthesis have been done on gas exchange phenomena and it would be expected that stomatal closure would lead to significant decreases in photosynthesis at RWCs higher than those recorded for decreases in parameters such as  $F_v/F_M$ . It was also difficult to resolve trends in some of the respiration results which complicated interpretation of the data.

The physiological processes of photosynthesis (in terms of chlorophyll fluorescence) and respiration generally appear to be able to continue until lethal RWCs are reached in the desiccation-sensitive species. In terms of ability to withstand damage to the photosynthetic and respiratory apparatus with drying, the drought tolerant tree, *G. livingstonei*, appears to be most sensitive in terms of susceptibility to irreversible damage. Of the sensitive species, *I. woodii* appeared to be the most resistant to damage, largely due to its ability to limit rehydration damage. This species is an understory shrub which is subject to daily wilting from which it recovers very quickly. The ability to withstand rehydration damage would facilitate this quick recovery from wilting. The melting enthalpy of tissue water in *I. woodii* was typical of water which had glassy characteristics (Chapter 2). This feature may protect *I. woodii* during dehydration and subsequent rehydration.

In the desiccation-tolerant species, *C. nanum*, there do not appear to be major differences in chlorophyll fluorescence parameters or respiration during drying compared with the sensitive species. The main difference is that *C. nanum* can recover upon rehydration, whereas rehydration appears to result in further damage to most of the sensitive species. The unusual nature of the PV curve (Chapter 2), which may indicate the avoidance of mechanical damage between RWCs of about 70% and 37%, does not appear to confer any special abilities of *C. nanum* to withstand damage to the photosynthetic apparatus or the respiratory pathway.

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## CHAPTER 5: ULTRASTRUCTURE

### Introduction

Ultrastructural studies allow one to visualise the state of plant cells and the various organelles and relate their appearance to a variety of physiological processes. A study which assesses physiological damage to a leaf would be incomplete without attempting to visualise that damage at an ultrastructural level.

#### Ultrastructure and water stress

The effects of mild water stress on ultrastructure have been studied using a number of desiccation-sensitive crop plants (Giles, Beardsell & Cohen, 1974; Giles, Cohen & Beardsell, 1976; Hsiao, 1970; Kurkova & Motorina, 1974, among many others). There have also been a number of ultrastructural studies of desiccation-tolerant (resurrection) plants. These studies have been conducted both on plants that lose chlorophyll during drying (*Borya nitida*; Gaff, Zee & O'Brien, 1976; Hetherington, Hallam & Smillie, 1982 and *Xerophyta villosa*; Hallam & Gaff, 1978; Hallam and Luff, 1980b), and on plants that retain their chlorophyll during drying (*Talbotia elegans*; Hallam & Luff, 1980a and *Myrothamnus flabellifolia*; Goldsworthy & Drennan, 1991; Wellburn & Wellburn 1976). More recently Schneider *et al.* (1993) have used immunocytochemical methods to localise proteins produced on desiccation in *Craterostigma plantagineum*.

Several of the workers mentioned above have shown that both mild water stress and more severe desiccation results in changes in cell ultrastructure, e.g. disruption of internal lamellar structures of chloroplasts, rounding or swelling of mitochondria, loss of definition of cristae and condensation of chromatin (reviewed by Bewley, 1979). The plasmalemma of desiccated tolerant and sensitive leaves has also been shown to have many discontinuities. After relief from non-lethal water deficits, structures return to normal within a few hours. Sensitive plants stressed to lethal water deficits are unable to reverse these changes, and increasing fragmentation of organelles and membrane structures occurs (Bewley, 1979).

Ilijin (1957) hypothesised that mechanical stress was the primary cause of injury to the dehydrating cell. That author proposed that mechanical stress could be minimized through various anatomical and morphological adaptations such as reduced cell size, increased cell surface:volume ratio, small or absent vacuoles, lack of plasmodesmata, reduced osmotic pressure, presence of insoluble constituents and easily deformed cell walls. Other workers have observed a correlation between desiccation tolerance in lower plants and reduced cell size and small or absent vacuoles (reviewed by Bewley, 1979; Bewley & Krochko, 1982; Ilijin 1957). In desiccation-tolerant seeds, the packaging of reserves in the cytoplasm and the consequent reduction in total vacuolar volume may alleviate some of the mechanical stresses imposed by dehydration (Berjak, Dini & Pammenter, 1984; Bewley, 1979; Levitt, 1980). The highly recalcitrant (desiccation-sensitive) seeds of *Avicennia marina* remain highly vacuolated throughout development, while the less sensitive seeds of *Auracaria angustifolia* are minimally vacuolated (Farrant, Pammenter & Berjak, 1992; 1993). There thus may be a correlation between extent of vacuolation (and the amount of complex reserve accumulated) and the degree of sensitivity to desiccation in seed tissue.

From the work described in the previous chapters of this thesis it can be seen that dehydration causes damage to the plasmalemma (solute leakage), chloroplasts (decrease in  $F_V/F_M$  and changes in  $F_O$ ) and the mitochondria (decrease in respiration). It has also been shown that the desiccation-tolerant material behaves in a manner similar to the desiccation-sensitive material during drying, but that on rehydration the tolerant leaves can recover while most of the sensitive leaves show further damage. Ultrastructural studies can be used to investigate the physical damage occurring, during dehydration and after rehydration, to the organelles and the cell in general. The pressure volume (PV) curves described in Chapter 2, indicate that the tolerant plant, *C. nanum*, is unusual in that it can undergo marked changes in volume without concurrent changes in pressure over a certain range of RWCs. The possible explanation of this phenomenon could be that the cell walls are collapsing or folding inwards, resulting in substantial changes in volume but little associated change in turgor pressure. Desiccation-induced cell wall collapse has been shown for the most studied desiccation-tolerant angiosperm species (*Myrothamnus flabellifolia*, Goldsworthy & Drennan, 1991; *Talbotia elegans*, Hallam & Luff, 1980a; *Xerophyta villosa*, Hallam & Luff, 1980b; and *Craterostigma plantagineum*, Schneider *et*

*al.*, 1993).

#### Problems with tissue preparation

One of the main problems in studying the ultrastructure of plants in the desiccated state is that some hydration of the tissue occurs during aqueous fixation (Hallam & Luff, 1980a; 1980b). Öpik (1980) reported that cell walls straighten out and shrunken organelles become rounded. This problem has been partly overcome with the use of anhydrous fixatives which appear to provide better preservation of dry material (Hallam, 1976). The most commonly used anhydrous fixatives are vapours of acrolein and osmium tetroxide. These techniques, however, often result in low contrast (Öpik, 1980).

Ultra-rapid freezing (cryofixation) of biological material yields preparations in which all components are immobilized in a life-like state (Edelmann, 1991). A technique, therefore, based on freezing plant tissue and fixing it anhydrously should result in the best preservation of cellular ultrastructure in the dry state. Such a technique, called freeze-substitution, has been developed. This is a chemical dehydration process in which ice in frozen-hydrated specimens is removed and replaced by an organic solvent (Echlin, 1992). The organic solvent is then replaced by resin as in standard fixation. Fernandez-Moran in 1957 was the first to apply freeze-substitution techniques to ultrastructural studies (Echlin, 1992). Other workers further developed the freeze-substitution protocols (Kaeser, 1989; Müller, Marti & Kriz, 1980, and others cited in Echlin, 1992). The use of freeze-substitution has led to the visualization of structures, such as aggregation-sensitive DNA (Kellenberger, 1991) and the pleomorphic canalicular system, which is present in living tissue but drastically altered in conventionally fixed tissue (McCully & Canny, 1985). The technique was not specifically developed for dry plant tissue but there are many advantages in employing it for such tissue. The material will be frozen in the dry state and therefore preserved in this state with no introduction of additional water. The substitution medium will then replace the water in the tissues without the introduction of more water.

Freeze-substitution does, however, have a number of problems associated with it. The freezing step can result in the formation of ice crystals and therefore ice crystal damage

if the material is not frozen quickly enough. Due to the size of even small pieces of leaf tissues, freezing will be good only at the surface zone of 20-30  $\mu\text{m}$  (Kaeser, 1989). While there has been some success in obtaining good ultrastructural preservation of animal tissues using freeze-substitution, only poor ultrastructural preservation of plant material has been achieved (Kaeser, 1989). Edelman (1991) also found that subcellular redistribution cannot be ruled out even if most of the diffusible ions are retained within the freeze-substituted specimen.

Even though it is well known that aqueous fixation does introduce a certain amount of rehydration and therefore distortion in dry tissue, many workers studying desiccation-tolerant plants have continued to employ aqueous fixatives because of the ease of use. Workers studying desiccation-tolerant plants have used osmium vapour (Goldsworthy & Drennan, 1991; Hallam & Luff, 1980a), while others have employed dimethylsulphoxide, or DMSO, (Hallam & Luff, 1980a; 1980b) or aqueous fixation (Gaff *et al.*, 1976; Hetherington *et al.*, 1982; Schneider *et al.*, 1993; Wellburn & Wellburn, 1976). In the latest study on desiccation-tolerant plant ultrastructure Schneider *et al.* (1993) use standard aqueous fixation followed by progressive lowering of temperature (PLT). The PLT would have been used to facilitate immunocytochemical studies (Echlin, 1992); it is not a technique that preserves material in its dry state.

#### Aims of this study

The aim of this part of study was to visualise the damage occurring in the leaf tissue during drying and after subsequent rehydration and to relate the ultrastructural observations to the results achieved in the previous studies. An attempt was made to use freeze-substitution as an anhydrous fixation technique, as it was thought that it would best preserve the dry leaf tissue. However, due to many technical difficulties, and difficulties with the freeze-substitution technique itself, it was decided to fix the material using standard aqueous techniques as well. Time did not permit the utilisation of another anhydrous fixation technique.

Leaf material at full turgor, 30% and 5% RWC was used as these appear to be the RWCs at which changes in physiological processes occur (Chapter 4). Material was fixed in its

dry state as well as after complete rehydration. The rehydration took place in a moist atmosphere for 18 hours and subsequent immersion in water until it was as close to full turgor as could be achieved.

## **Materials and Methods**

Leaves of the five plant species were flash dried to 30% and 5% RWC. Leaves at these two RWCs and at full turgor were used for this part of the study. The leaves were divided into three portions, one of which was freeze-substituted, another was put straight into standard aqueous fixative and the last portion was rehydrated (using the rehydration protocol outlined in Chapter 4) and then placed in standard aqueous fixative.

### Freeze-substitution

#### Substitution medium - 1 (DMP medium)

Glutaraldehyde (25% aqueous) was shaken with acidified dimethoxypropane (DMP) to eliminate the water from the former. The medium was made up as follows: 0.6 ml of glutaraldehyde was shaken with 6 ml of acidified DMP, the final solution was clear; 3 ml acetone, 2.25 ml methanol and 0.75 ml of 10% uranyl acetate in methanol were added to this mixture. Osmium tetroxide crystals were added to the solution (0.76%) and the mixture was cooled in liquid nitrogen to  $-196^{\circ}\text{C}$ . DMP reacts with water in an equimolar reaction to yield methanol and acetone and thus the amount of DMP in the final substitution medium needs to be sufficient to eliminate the amount of water in the material. The details of this substitution medium are outlined in Kaeser (1989).

#### Substitution medium - 2 (methanol medium)

The details of this medium are described by Müller *et al.* (1980). The medium consists of methanol, containing 3% glutaraldehyde, 1% osmium tetroxide, 0.4% uranyl acetate and 3% water. Water was introduced using a 50% aqueous glutaraldehyde solution.

#### Freezing protocol - 1

The material that was to be freeze-substituted was first cut into very small squares,

approximately 0.5 mm<sup>2</sup>. The tissue samples need to be as small as possible for optimal rapid cooling and to present a short diffusion path for the substitution and embedding chemicals (Echlin, 1992). For *A. raddianum*, leaflet pieces were mounted on aluminium pins using double sided adhesive tape and were rapidly plunged into subcooled liquid nitrogen (-210°C). The rate of freezing is critical. If the freezing rate is not fast enough, the formation of ice-crystals will damage the sample (Echlin, 1992). The material was then transferred into the first substitution medium (Kaesler, 1989) at -196°C. It was then placed in a -80°C deep freeze and allowed to equilibrate to this temperature for one week.

#### Freezing protocol - 2

Small pieces of *A. raddianum* leaflets and *C. nanum* leaves were frozen using isopentane at its melting point. To do this, a container with isopentane was held in contact with liquid nitrogen which resulted in the isopentane freezing. A metal rod was forced into the frozen isopentane to melt a well of about 10 mm in diameter. Before the isopentane could re-freeze, an orange stick (sharpened to a fine point) carrying approximately 10 pieces of leaf tissue adhering to a thin coating of glycerol was quickly introduced and kept there for 15 seconds. The material was then transferred into the substitution medium 2 (Müller *et al.*, 1980) at -196°C. It was then placed in a -80°C deep freeze and allowed to equilibrate at this temperature for one week.

#### Progressive raising of temperature

After one week at -80°C the temperature of the substitution media and leaflets was raised to -60°C over a 12 hour period. It was held at -60°C for 6 hours. The temperature was then raised to -40°C over a 12 hour period and held there for 6 hours, after which it was raised to -20°C over a 12 hour period and held there for 6 hours. The temperature was then raised to 0°C over a 12 hour period and held there for 12 to 24 hours.

#### Infiltration

The leaf material was then slowly brought to room temperature and infiltrated with epoxy resin (Spurr, 1969). Infiltration was carried out over a period of two days, first using 25% resin in acetone for 12 hours, followed by 50% resin for 12 hours and finally 100% resin for 24 hours. The resin-embedded material was polymerised at 70°C for 12 hours.

### Standard fixation

Material for standard fixation was cut into small pieces and left overnight in a 2.5% solution of glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) containing 0.5% caffeine. The material was washed three times for five minutes each, using 0.1 M phosphate buffer (pH 7.2) and then post-fixed for 1 hour in 0.5% osmium tetroxide. After washing (as described above) the material was dehydrated in an acetone series, starting from 30% acetone through 50%, 75%, 90% and finally 100% acetone. The dehydrated material was then infiltrated (50% resin in acetone for 4 hours followed by 100% resin for 18 hours) and embedded in epoxy resin (Spurr, 1969). The resin was polymerised for 12 hours at 70°C.

### Light microscopy

Leaf cross-sections (1  $\mu\text{m}$  thick) were stained with toluidine blue and viewed with a Nikon Biophot photomicroscope. Transverse-sections of the leaves were photographed using Pan-F film. Images of the leaf transverse-sections were also recorded digitally in TIFF format using a computer programme (Movie Machine). At least two different images were captured for each of 3 different leaf cross-sections.

### Transmission electron microscopy

Leaves were sectioned at a gold interference colour and picked up on copper grids. Sections were stained with lead citrate (Reynolds, 1963) for 15 minutes and viewed with a JEOL 1010 transmission electron microscope (TEM). Micrographs and thermal images were taken and used for analysis. Observations were made on at least 3 sections of three different leaf samples. Both the freeze-substituted and the standard fixed material were sectioned this way.

### Measurements

Measurements of cell perimeter to area ratios were done on the captured images of the leaf transverse-sections, using an image analysis programme (MicroImages Inc.: The Map and Image Processing System 3.33). At least five cells were measured for each captured image. The data were analysed for significant differences using a completely randomized ANOVA and a Duncan's Multiple Range test (using the program Costat). Chloroplast

length to breadth ratios were measured on the full turgor and dehydrated leaf material in order to determine changes in shape with drying.

## Results

### Freeze-substitution

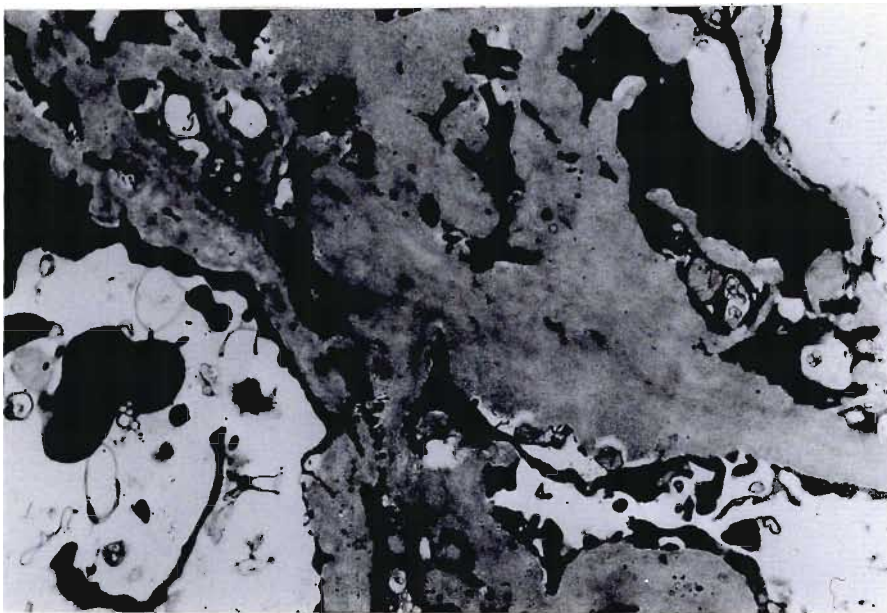
Leaflets of *A. raddianum* fixed using freeze-substitution medium 1 (Kaeser, 1989) and freezing protocol 1 showed poor tissue preservation. From Figure 5.1 it can be seen that organelles were not clearly distinguishable, there was also very little contrast in the section. Because of the poor results obtained using medium 1 and freezing protocol 1 more *A. raddianum* leaflets and leaves of *C. nanum* were fixed using freezing protocol 2 and substitution medium 2 (Müller *et al.*, 1980). This protocol yielded better results with organelles such as chloroplast being recognisable (Figs. 5.2 and 5.3).

Thylakoid membranes and large starch grains were visible in the chloroplasts of *A. raddianum* leaflets dried to 30% RWC (Fig. 5.2a). The plasmalemma appeared to have pulled away from the cell wall, though whether this was due to drying or the fixation protocol is not known. Further drying to 5% RWC yielded cells with recognisable chloroplasts (Fig. 5.2b) even though these organelles contained no starch and were not elliptical. There was clear contraction of the plasmalemma from the cell wall. There are structures (labelled in Fig. 5.2c) which were probably mitochondria, although no cristae were evident. Again this could have been due to damage which occurred during drying or because of the fixation method employed. Membranes were, however, preserved using this freeze-substitution protocol. Contrast was also quite high although the sections were very delicate and easily damaged in the TEM.

Figure 5.3 shows *C. nanum* leaves dried to 30% RWC (Figs. 5.3a & b) and 5% RWC (Fig. 5.3c) fixed anhydrously using substitution medium 2 and freezing protocol 2. The preservation of cell structure appeared not to be as good in *C. nanum* as it was for *A. raddianum*. The chloroplasts were not as clearly defined and the thylakoid membrane structure was less evident. The plasmalemma appeared to have pulled away from the cell walls and was discontinuous. Membranes within the cytoplasm did, however, seem to be

preserved with numerous membrane bound structures visible. Contrast was very low and it was difficult to obtain much detail of organelles at higher magnifications (Fig. 5.3b). Drying to 5% RWC resulted in densely stained cell contents with little or no detail visible (Fig. 5.3c). The cell walls were very irregular and folded inwards. This could have been a result of drying, as cell walls of resurrection plants have been shown to collapse inwards on desiccation (Hallam & Luff, 1980a; 1980b; Schneider *et al.*, 1993). The PV curves (Chapter 2) for *C. nanum* indicated a reduction in volume with little change in pressure starting at about 70% RWC. The inward folding of the cell walls in this species, is possibly the reason for this phenomenon.

Leaflets of *A. raddianum* showed better preservation than leaves of *C. nanum* using freeze-substitution. This is most probably due to the fact that the fern leaflets are very thin, often only two cell layers thick, thus allowing effective freezing to take place. Due to the disappointing preservation achieved from the freeze-substitution protocols, and the time constraints, it was decided to study the ultrastructure of the five species using standard fixation methods.



**Figure 5.1:** Transmission electron micrograph of a transverse-section of *A. raddianum* leaflets dried to 30% RWC. The leaflets were fixed anhydrously using freeze-substitution. Freezing protocol 1 and substitution medium 1 (DMP - Kaeser, 1989) were used. Ultrastructural preservation was poor and discreet organelles could not be distinguished.

Magnification x 5 200

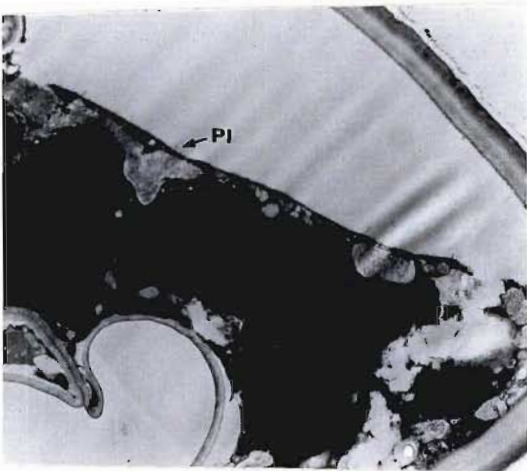
Figure 5.2: Transmission electron micrographs of *A. raddianum* leaflets fixed anhydrously using freeze-substitution. Freezing protocol 2 and substitution medium 2 (methanol medium - Müller *et al.*, 1980) were used. Leaflets were dried to 30% RWC (a, mag. x 26 000) and 5% RWC (b, mag. x 6 350 and c, mag. x 20 000). Preservation using this substitution medium and freezing protocol was better than that obtained for medium 1 and freezing protocol 1. Organelles were distinguishable. The plasmalemma appears to have contracted away from the cell wall at both RWCs.

T - thylakoid stack; S - starch; Pl - plasmalemma; M - possible mitochondrion

**a**



**b**



**c**

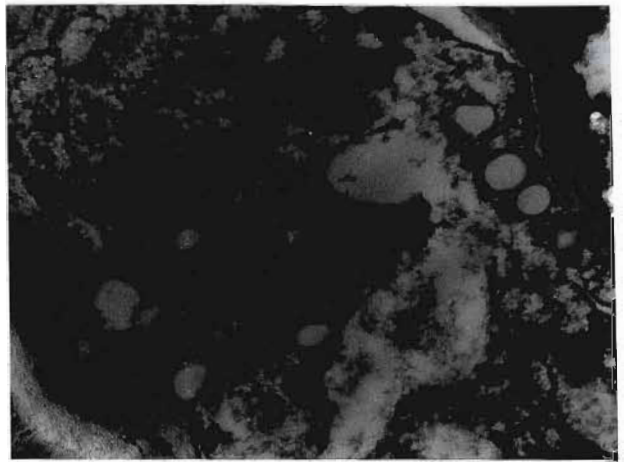
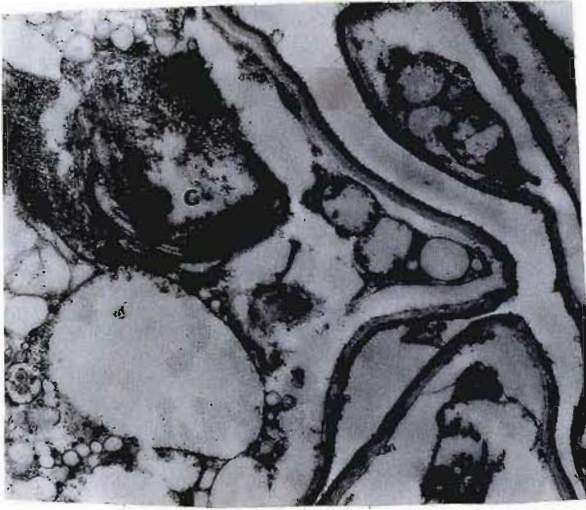


Figure 5.3: Transmission electron micrographs of *C. nanum* leaves fixed anhydrously using freeze-substitution. Freezing protocol 2 and substitution medium 2 were used (methanol medium - Müller *et al.*, 1980). Leaves were dried to 30% RWC (a, mag. x 6 500 and b, mag. x 13 000) and 5% RWC (c, mag. x 52 000). General ultrastructural preservation was not very good. Chloroplasts were distinguishable in leaves dried to 30% RWC, but were not well preserved. The cell walls appeared to fold inwards, particularly so at 5% RWC.  
C - chloroplast; W - cell wall

**a**



**b**



**c**



## Standard fixation

### Light microscopy

Figures 5.4 to 5.9 are light micrographs of leaf transverse sections of the five species. Each figure consists of five micrographs showing the leaf tissue at full turgor, 30% RWC, rehydrated from 30% RWC, 5% RWC and rehydrated from 5% RWC. Table 5.1 gives the results of the perimeter:area ratio studies performed on captured images of leaf transverse sections.

The fern, *A. raddianum*, showed no significant differences in the perimeter:area ratios between cells in leaves dried to 30% RWC and those which were fully hydrated (Table 5.1). Cells from leaves dried to 5% RWC, however, had larger perimeter:area ratios than the cells at full turgor. This indicates that there had been some cell shrinkage with little or no change in perimeter. Once rehydrated, these cells regained their shape as indicated by the decrease in the perimeter:area ratio. The leaflets were thin and often consisted of only two layers of cells and there were large air spaces between the two cell layers. At full turgor the chloroplasts were appressed to the cell walls facing the inside of the leaflet (Fig. 5.4a). Drying to 30% RWC (Fig. 5.4b) disrupted the internal organisation of the cells. The chloroplasts were now in the middle of the cells or closer to the cell walls facing the outside of the leaf. Rehydration from 30% RWC (Fig. 5.4c) appeared to lead to a degree of recovery as some cells regained the full turgor appearance, but other cells showed chloroplasts that were irregularly scattered. Drying to 5% RWC (Fig. 5.4d) showed disruption of cellular organisation, particularly chloroplast location, and no recovery was evident on rehydration (Fig. 5.4e).

Cells of *P. sativum* showed significant increases in perimeter:area ratios with drying (Table 5.1). Rehydration from 30% RWC resulted in the area of these cells increasing and a rounding of the cells so that the perimeter:area ratios decreased. There was no significant difference between the material dehydrated to 5% RWC and that dehydrated to 5% RWC and subsequently rehydrated. At full turgor (Fig. 5.5a) the cells had a large central vacuole and the chloroplasts were peripherally located. There were comparatively small intercellular air spaces in these leaves. A similar arrangement was seen after drying

to 30% RWC (Fig. 5.5b) although the cells appeared smaller. Rehydration of this material (Fig. 5.5c) resulted in disruption of cellular constituents. Drying to 5% RWC (Fig. 5.5d) resulted in a fairly compact arrangement of shrunken cells with chloroplasts displaced towards the centre and few air spaces. After rehydration (Fig. 5.5e) the cells regained some shape but there was disruption of cellular organisation characterised by scattered organelles.

**Table 5.1:** The perimeter:area ratios of mesophyll cells of the five species studied. The measurements were taken from captured images of leaf transverse sections using an image analysis programme. Letters next to the figures refer to significant differences amongst the different treatments within a species (Duncan's Multiple Range Test  $P \leq 0.05$ ,  $n = 25$  for all treatments). For *I. woodii* and *G. livingstonei* data for both spongy and palisade mesophyll cells are given separately as these two types of cells were clearly distinguishable irrespective of the degree of dehydration.

Species	Full turgor	30% RWC	Rehydrated from 30% RWC	5% RWC	Rehydrated from 5% RWC
<i>A. raddianum</i>	0.0614 <sup>a</sup>	0.0676 <sup>ab</sup>	0.0689 <sup>ab</sup>	0.0707 <sup>b</sup>	0.0696 <sup>ab</sup>
<i>P. sativum</i>	0.0695 <sup>a</sup>	0.084 <sup>bc</sup>	0.0787 <sup>b</sup>	0.0893 <sup>c</sup>	0.0895 <sup>c</sup>
<i>I. woodii</i> (spongy)	0.1376 <sup>a</sup>	0.1727 <sup>b</sup>	0.11 <sup>c</sup>	0.189 <sup>d</sup>	0.1604 <sup>b</sup>
<i>I. woodii</i> (palisade)	0.1379 <sup>a</sup>	0.1644 <sup>b</sup>	0.0944 <sup>c</sup>	0.1902 <sup>d</sup>	0.1283 <sup>a</sup>
<i>G. livingstonei</i> (spongy)	0.1262 <sup>a</sup>	0.1367 <sup>a</sup>	0.141 <sup>a</sup>	0.1274 <sup>a</sup>	0.1327 <sup>a</sup>
<i>G. livingstonei</i> (palisade)	0.2056 <sup>b</sup>	0.193 <sup>bc</sup>	0.1951 <sup>bc</sup>	0.1798 <sup>cd</sup>	0.1648 <sup>d</sup>
<i>C. nanum</i>	0.0446 <sup>a</sup>	0.0543 <sup>b</sup>	0.0454 <sup>a</sup>	0.08 <sup>c</sup>	0.0456 <sup>a</sup>

Cells of *I. woodii*, similar to those of *P. sativum*, showed an increase in perimeter:area ratio with drying to 30% RWC (Table 5.1) indicating cellular shrinkage. Rehydration of the dried leaves resulted in some recovery of cellular area. Although there were clear spongy and palisade layers of mesophyll cells present, there were no significant differences in the perimeter:area ratios at full turgor or after drying or rehydration between these two cell types. As with *P. sativum*, chloroplasts of *I. woodii* at full turgor were peripherally located and the cells had large central vacuoles (Fig. 5.6a). The contrast of Figure 5.6a does not allow easy comparison with Figures 5.6b - 5.6e. However, there was little difference in leaves at full turgor and those dried to 30% RWC (Fig. 5.6b) and those rehydrated from 30% RWC (Fig. 5.6c). Figure 5.6d, material dried to 5% RWC, indicated that the cell vacuole was effectively eliminated and the plasmalemma had withdrawn from the cell wall. Rehydration (Fig. 5.6e) did not appear to improve the cellular organisation, though the perimeter:area ratio increased indicating that the cells had regained some shape. There were large intercellular air spaces in the leaves which were still present after drying to 5% RWC.

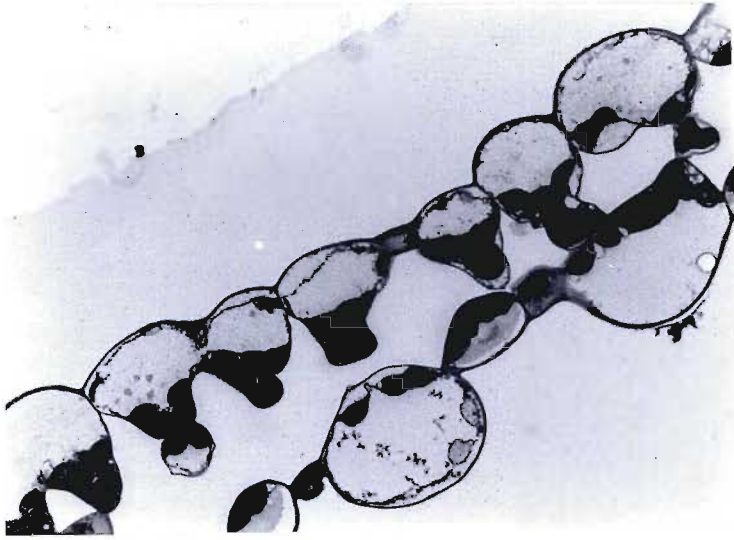
There was also a distinct spongy and palisade layer of mesophyll cells in *G. livingstonei*. During drying, even to as low as 5% RWC, there was no change in perimeter:area ratios of the spongy mesophyll cells (Table 5.1). The cells did not appear to shrink, this is also apparent from the micrographs (Fig. 5.7). The perimeter:area ratios of the palisade mesophyll cells were significantly different from that of the spongy cells at all levels of hydration. The palisade cells showed slight decreases in perimeter:area ratios with drying. This is in contrast to the general increase in this ratio found in the species discussed thus far. This decrease in ratio was the result of the palisade cells rounding with drying. Rehydration of the leaves did not significantly affect these perimeter:area ratios. The micrographs of the leaf transverse-sections clearly show the two types of mesophyll cells. There were substantial deposits of what appeared to be polyphenolics in the cells generally and in some vacuoles. Leaves of *G. livingstonei* had large intercellular air spaces at all levels of hydration. At full turgor (Fig. 5.7a) and at 30% RWC (Fig. 5.7b) both types of cell had a large central vacuole with peripherally orientated chloroplasts. Rehydration of leaves dried to 30% RWC (Fig. 5.7c) resulted in cellular disruption, particularly of the spongy cells, as evidenced by the location of the chloroplasts. Drying to 5% RWC (Fig.

5.7d) resulted in shrinkage of the vacuoles, particularly in the spongy mesophyll, and the resultant movement of chloroplasts towards the centre of the cell. Rehydration (Fig. 5.7e) resulted in total disorganisation of the cellular contents.

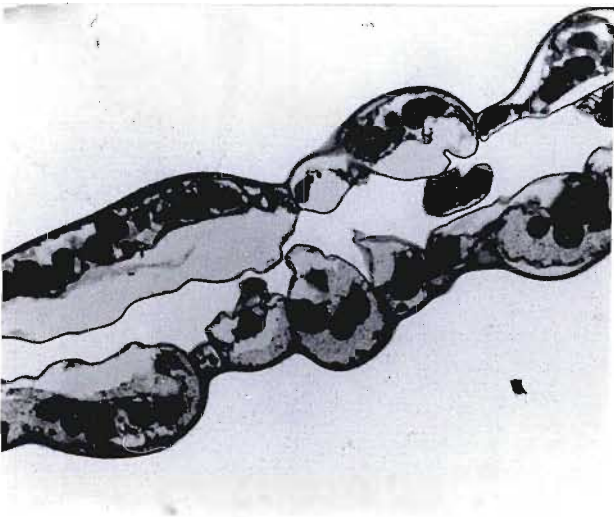
The perimeter:area ratios of *C. nanum* (Table 5.1) show the ability of these cells to shrink on drying (the ratio almost doubles from that of the hydrated tissue to that dried to 5% RWC) and their ability to recover after rehydration. This is clearly shown in the micrographs of the leaf transverse-sections (Fig. 5.8). The cells of *C. nanum* were also larger than the cells of the other four species studied and the intercellular air spaces were lost during drying. At full turgor (Fig 5.8a) the cells were large, circular and had a prominent central vacuole. The chloroplasts were closely associated with the cell periphery. Drying to 30% RWC (Fig. 5.8b) resulted in marked cell shrinkage, but the large central vacuole was maintained. The chloroplasts, while still peripheral, were not as close to the cell wall as they were at full turgor. The cells recovered their size after rehydration (Fig. 5.8c) although the chloroplasts appeared rounded. After drying to 5% RWC cells were irregular and closely packed, resembling an assembled jigsaw puzzle (Fig. 5.8d). The chloroplasts and cell contents were displaced to the centre of the cells and there were no clear vacuoles. Rehydration (Fig. 5.8e) resulted in the cells regaining their shape and the large central vacuole reconstituting. The chloroplasts also appeared rounded.

Figure 5.4: Light micrographs of transverse-sections of *A. raddianum* leaflets. The micrographs are of leaflets at full turgor (a), dried to 30% RWC (b), rehydrated from 30% RWC (c), dried to 5% RWC (d) and rehydrated from 5% RWC (e). Standard aqueous fixation was used. As vacuoles shrank during drying so the chloroplasts moved from the periphery to the centre of the cell. Apparent recovery of some cells was noted on rehydration of tissue dried to 30% RWC. Leaf tissue dried to 5% RWC showed cellular disorganisation which was not repaired on rehydration. Magnification x 730.

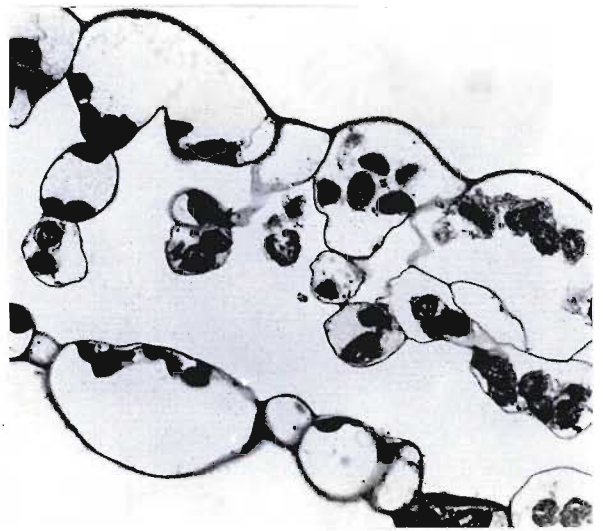
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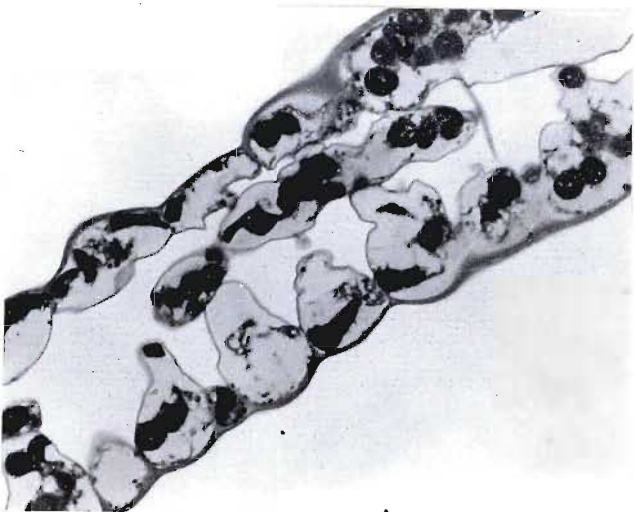
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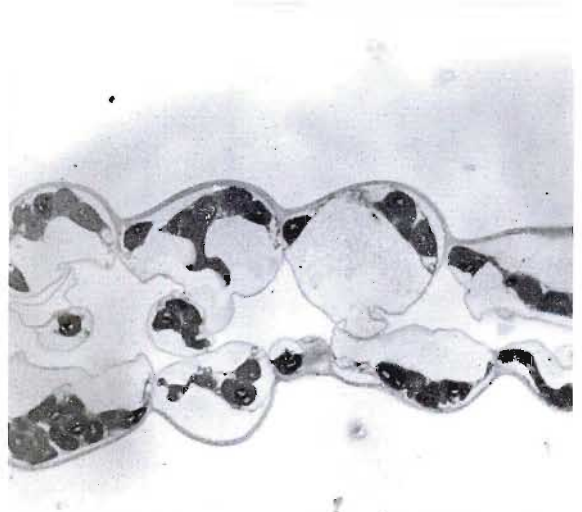
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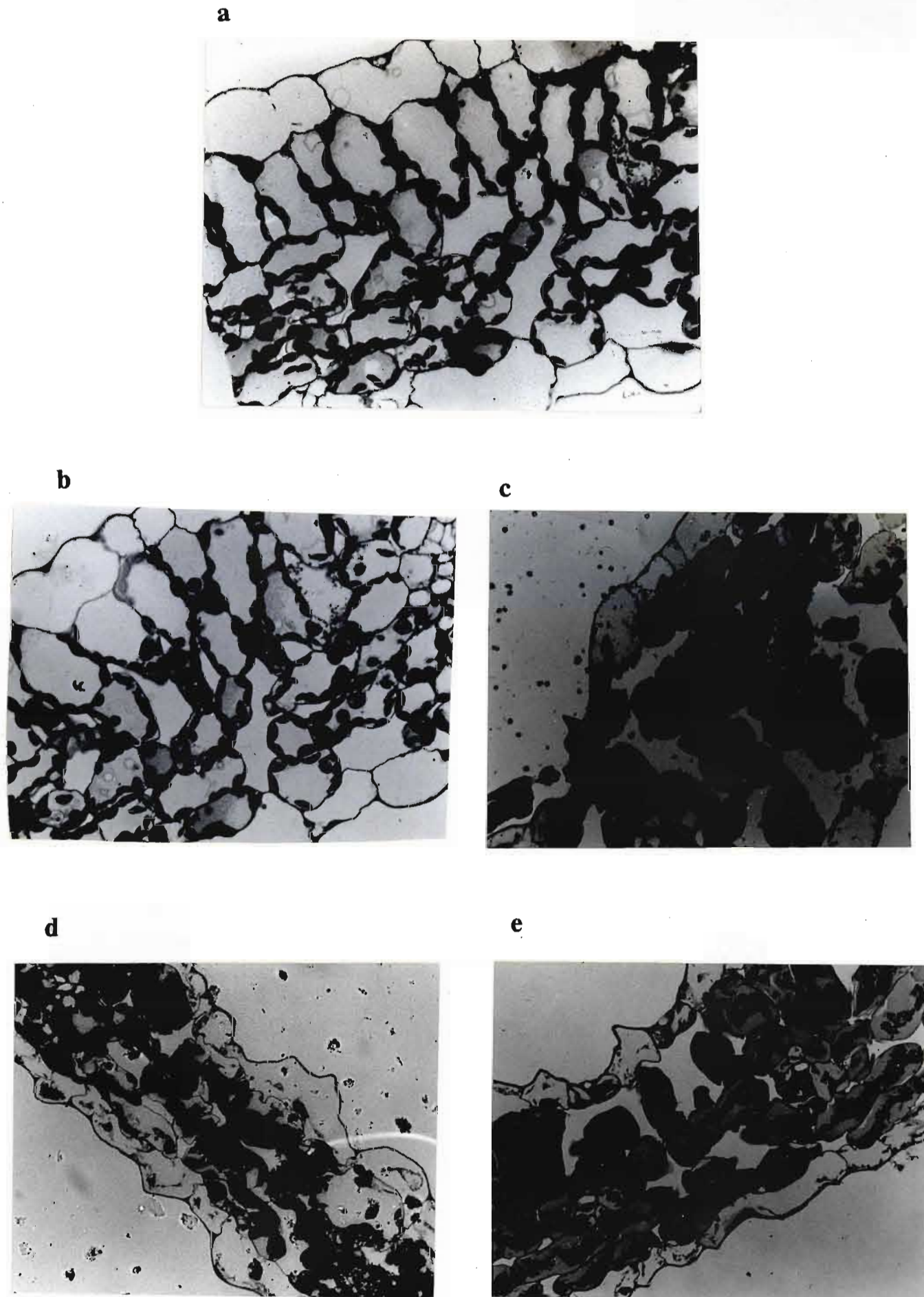


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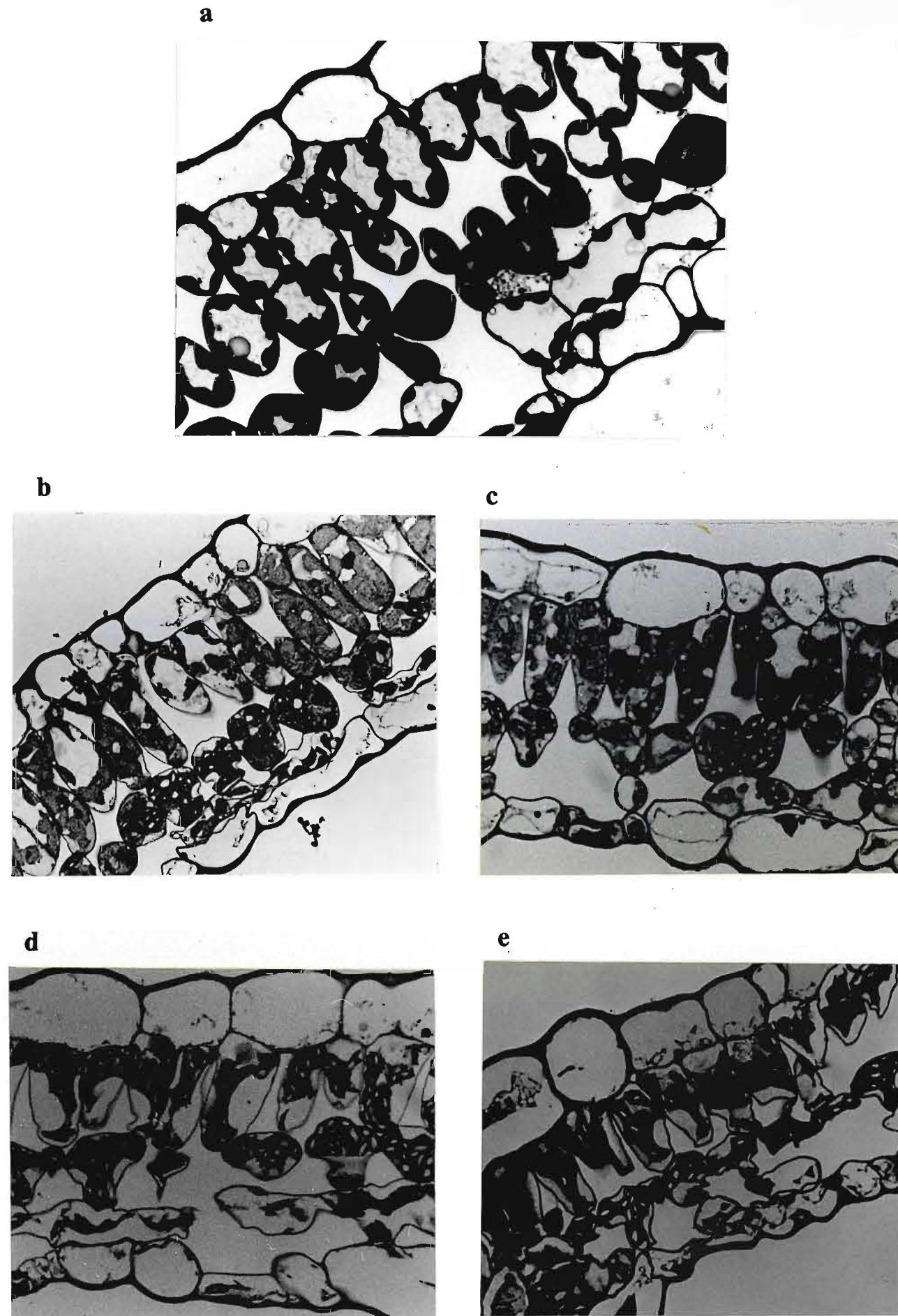


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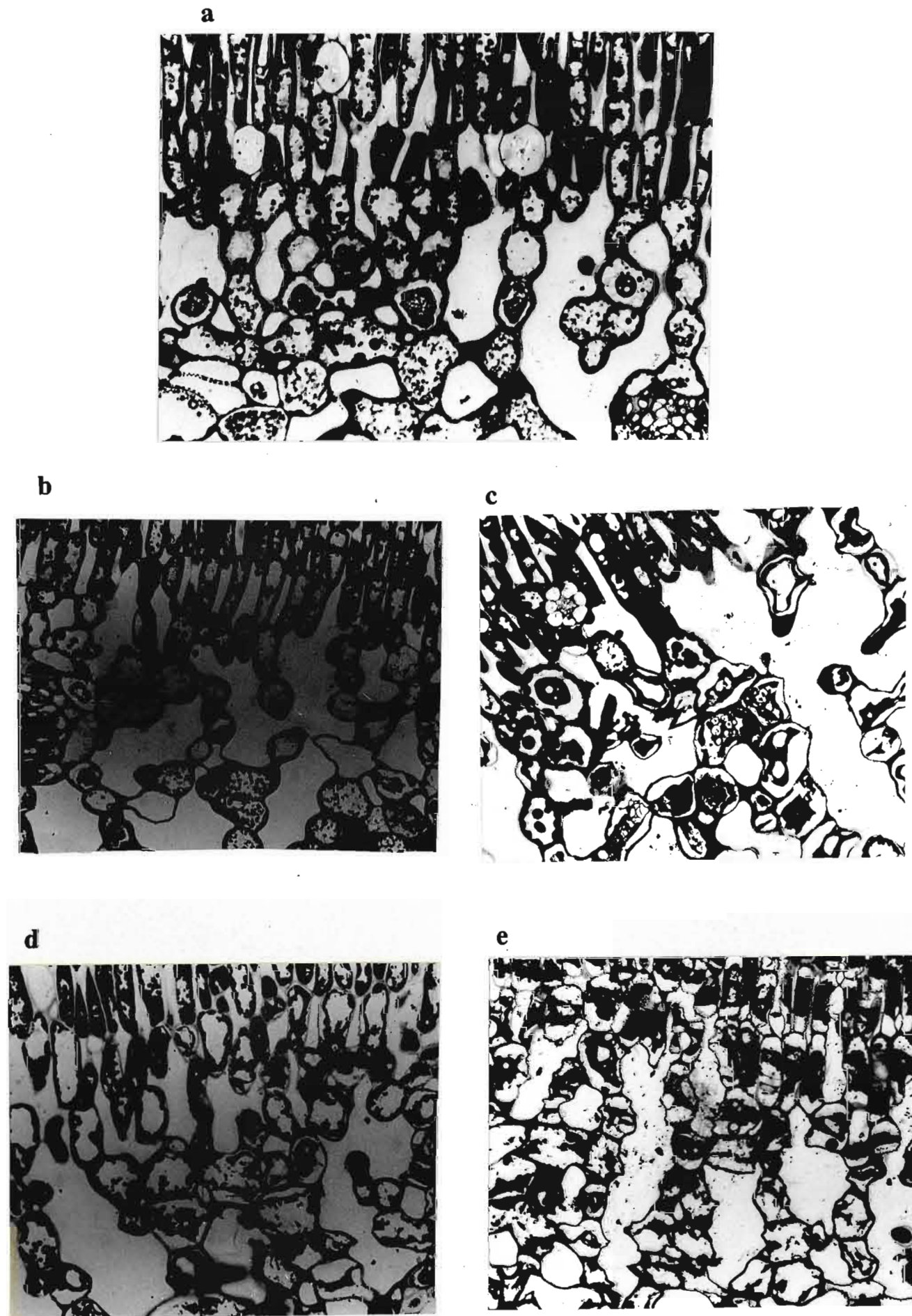




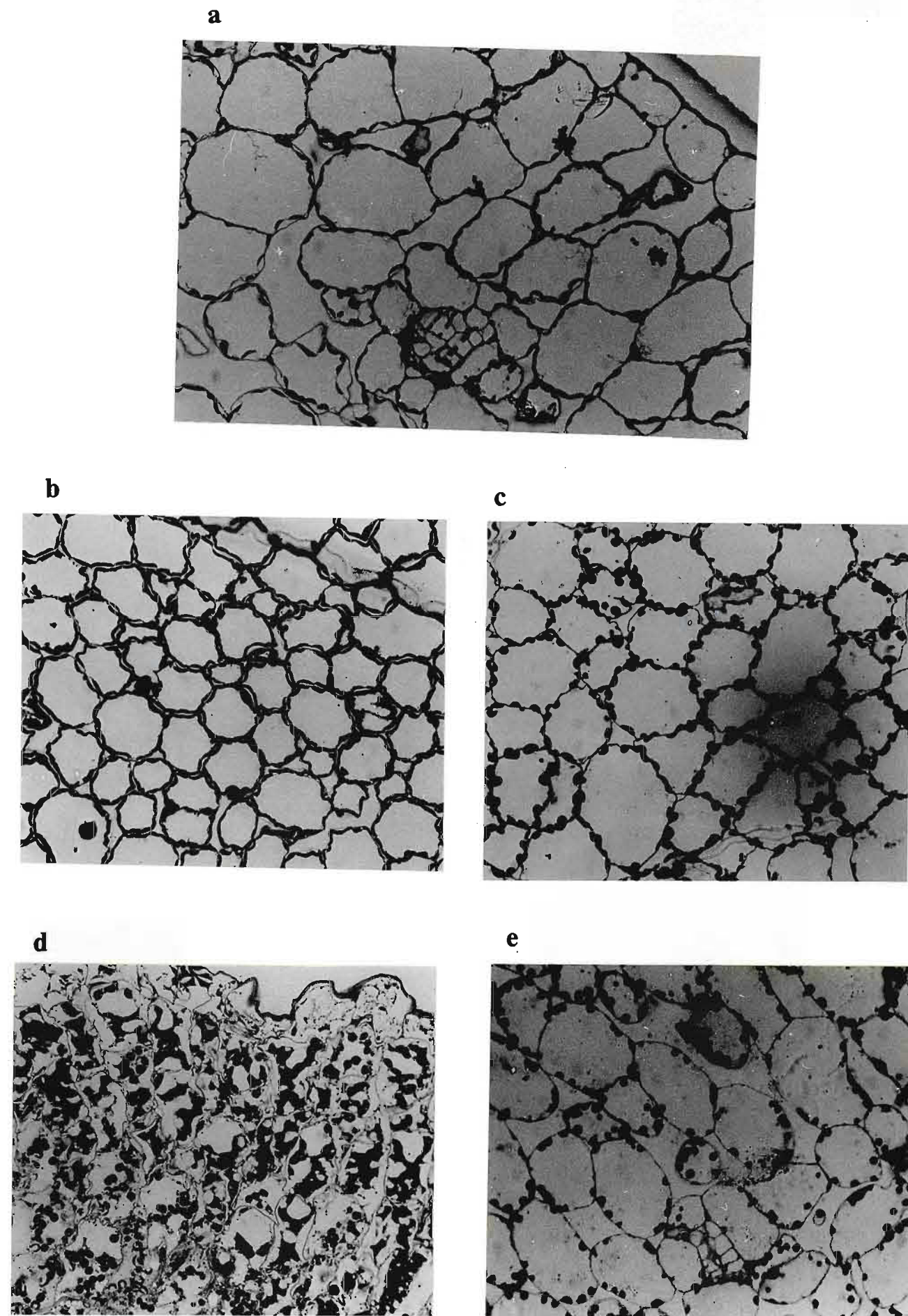
**Figure 5.5:** Light micrographs of transverse sections of pea, *P. sativum*, leaves. The micrographs are of leaves at full turgor (a), dried to 30% RWC (b), rehydrated from 30% RWC (c), dried to 5% RWC (d) and rehydrated from 5% RWC (e). Standard fixation was used. Turgid cells had large central vacuoles and peripherally located chloroplasts. Rehydration of leaves from 30% RWC resulted in disruption of cellular constituents, even though in the dehydrated state the organelles were still peripherally located. Marked cell shrinkage was noted at 5% RWC and while the cells regained shape after rehydration, cellular disruption was evident. Magnification x 730.



**Figure 5.6:** Light micrographs of transverse-sections of *I. woodii* leaves. The micrographs are of leaves at full turgor (a), dried to 30% RWC (b), rehydrated from 30% RWC (c), dried to 5% RWC (d) and rehydrated from 5% RWC (e). Standard fixation was used. Chloroplasts contracted towards the centre of the cells during dehydration. Dehydration to 5% RWC resulted in cellular disruption which was not repaired on rehydration. Magnification x 730.



**Figure 5.7:** Light micrographs of transverse sections of *G. livingstonei* leaves. The micrographs are of leaves at full turgor (a), dried to 30% RWC (b), rehydrated from 30% RWC (c), dried to 5% RWC (d) and rehydrated from 5% RWC (e). Standard fixation was used. Clear palisade and spongy mesophyll cells were evident. The dark-staining granular inclusions have the typical appearance of polyphenolics. Rehydration of leaves dried to 30% RWC resulted in disruption in a number of cells. Dehydration to 5% RWC resulted in cellular disruption which appeared to have been exacerbated on rehydration. Magnification x 400.



**Figure 5.8:** Light micrographs of transverse-sections of *C. nanum* leaves. The micrographs are of leaves at full turgor (a), dried to 30% RWC (b), rehydrated from 30% RWC (c), dried to 5% RWC (d) and rehydrated from 5% RWC (e). Standard fixation was used. Leaves dried to 30% RWC retained large central vacuoles and a peripheral arrangement of organelles although the cells were shrunken compared with those in fully hydrated tissue. Dehydration to 5% RWC resulted in closely packed cells with irregular walls and centrally located organelles. Rehydration from both these RWCs resulted in recovery of cell shape, the large central vacuole and peripherally located chloroplasts. Magnification x 400.

TEM - *A. raddianum*

Table 5.2 summarises the observations made of the ultrastructure on drying and subsequent rehydration of *A. raddianum* leaflets. The general ultrastructure of the cells of *A. raddianum* (Fig. 5.9) shows how the cell appearance changed from normal, at full turgor (Fig. 5.9a), to the entire contents, including the plasmalemma withdrawing from the cell walls at 30% RWC (Fig. 5.9b). This was also shown in the freeze-substituted leaflets at 30% RWC (Fig. 5.2a). The plasmalemma appeared "beaded". This indicates damage to the phospho-lipid bilayer and could be a consequence of lipids having coalesced into droplets. The plasmalemma was discontinuous in about 30% of the cells (Table 5.2). The partial recovery after rehydration, as shown in Figure 5.4c, is also evident in Figure 5.9c in terms of the cell regaining its original shape and peripheral location of chloroplasts. However, the tonoplast was discontinuous in about 50% of the cells (Table 5.2) and this would have led to the further breakdown of cell structure. Cells dried to 5% RWC (Fig. 5.9d) showed physical discontinuities of the plasmalemma and damage to the organelles. The plasmalemma and tonoplast were discontinuous in all the cells viewed (Table 5.2). Rehydration of these leaves resulted in further damage as evidenced in Figure 5.9e. The leaflets dried to 5% RWC and freeze-substituted (Fig. 5.2b) were similar in appearance to those that were fixed using standard means. The plasmalemma and cytoplasm of the freeze-substituted leaflets appeared to have been better preserved than those fixed using the standard procedure.

The chloroplast ultrastructure (Fig. 5.10) showed similar trends, however, the damage to chloroplast ultrastructure at 30% RWC (beaded outer membranes, disruption of thylakoid membranes) shown in Figure 5.10b was partially removed on rehydration (Fig. 5.10c). The severe damage at 5% RWC (Fig. 5.10d) was worsened by rehydration (Fig. 5.10e). The outer membrane of the chloroplast became "beaded" on drying. It was discontinuous in about 30% of the chloroplasts per cell when the leaflets were dried to 30% RWC and in about 75% of the chloroplasts per cell at 5% RWC (Table 5.2). Thylakoid membranes were slightly separated in the leaflets dried to 5% RWC (Fig. 5.10d) but became essentially amorphous on rehydration (Fig. 5.10e). The change in chloroplast shape with drying is shown in Figure 5.11. The range in chloroplast length:width ratios was small. There was not much change in chloroplast shape with drying, though at 30% RWC (Fig.

5.11b) there appeared to be some elongation of the chloroplasts, indicated by an increase in the percentage of chloroplasts with higher length: breadth ratios.

The mitochondria of *A. raddianum* leaflets at full turgor had clear cristae and both outer and inner membranes were well resolved (Fig. 5.12a). The dense matrix is consistent with activity. There was no difference in the mitochondria in leaves dried to 30% RWC and those dried to 30% RWC and rehydrated. Figure 5.12b shows mitochondria from a cell that had been dried to 30% RWC and then rehydrated. The cristae remained well resolved, but there was some breakdown of the outer membrane in about 20% of the mitochondria per cell (Table 5.2). Mitochondria were not distinguishable in leaflets dried to 5% RWC. After rehydration many clear circular profiles, like those shown in Figure 5.12c, were visible. These were possibly mitochondria which had been extensively degraded by drying to 5% RWC and subsequent rehydration.

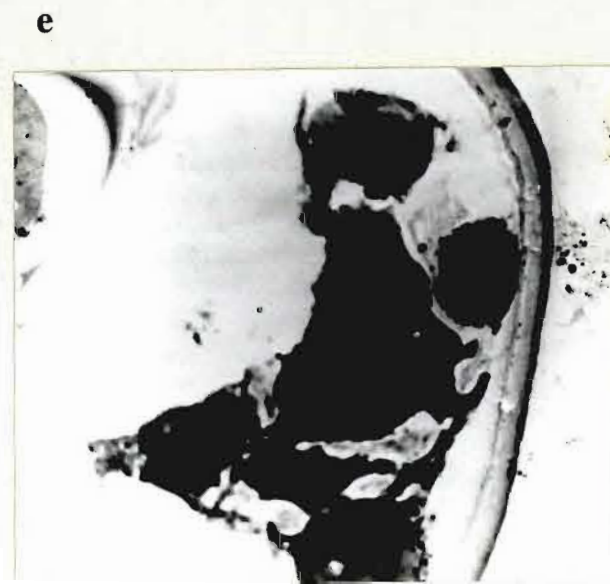
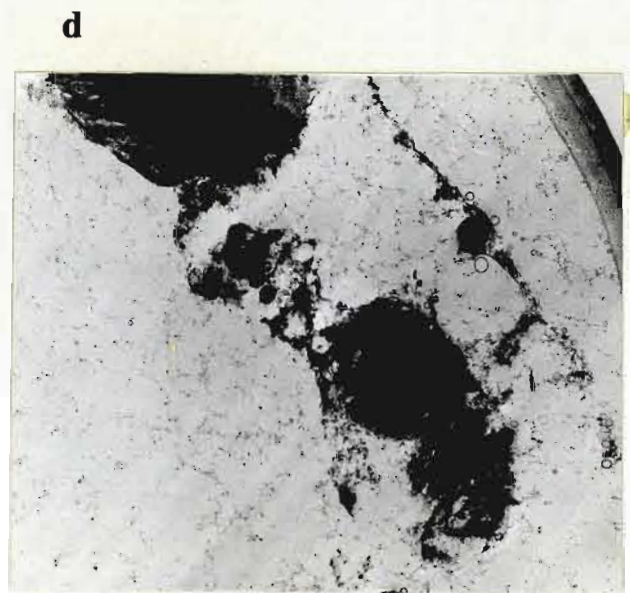
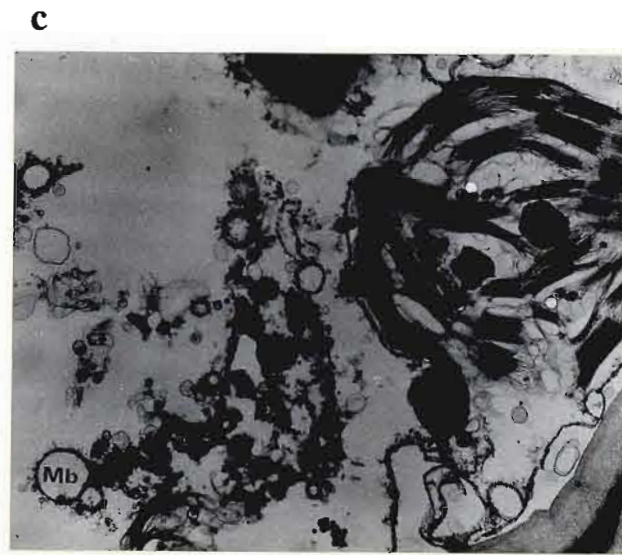
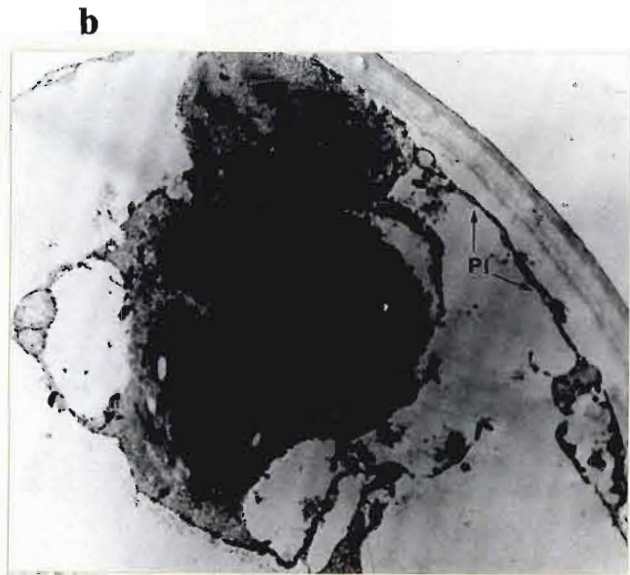
The nuclei of leaflets of *A. raddianum* at full turgor (Fig. 5.13a) appeared normal with some unusual, densely stained, hexagon shaped structures that might have been the nucleoli. After drying to 30% RWC there was a great deal of chromatin clumping (Fig. 5.13b). Nuclei in leaves dried to 30% RWC and rehydrated were similar in appearance to those in leaves at 30% RWC. No bodies which could be recognised as nuclei were visible at 5% RWC or after rehydration from this RWC.

### Summary

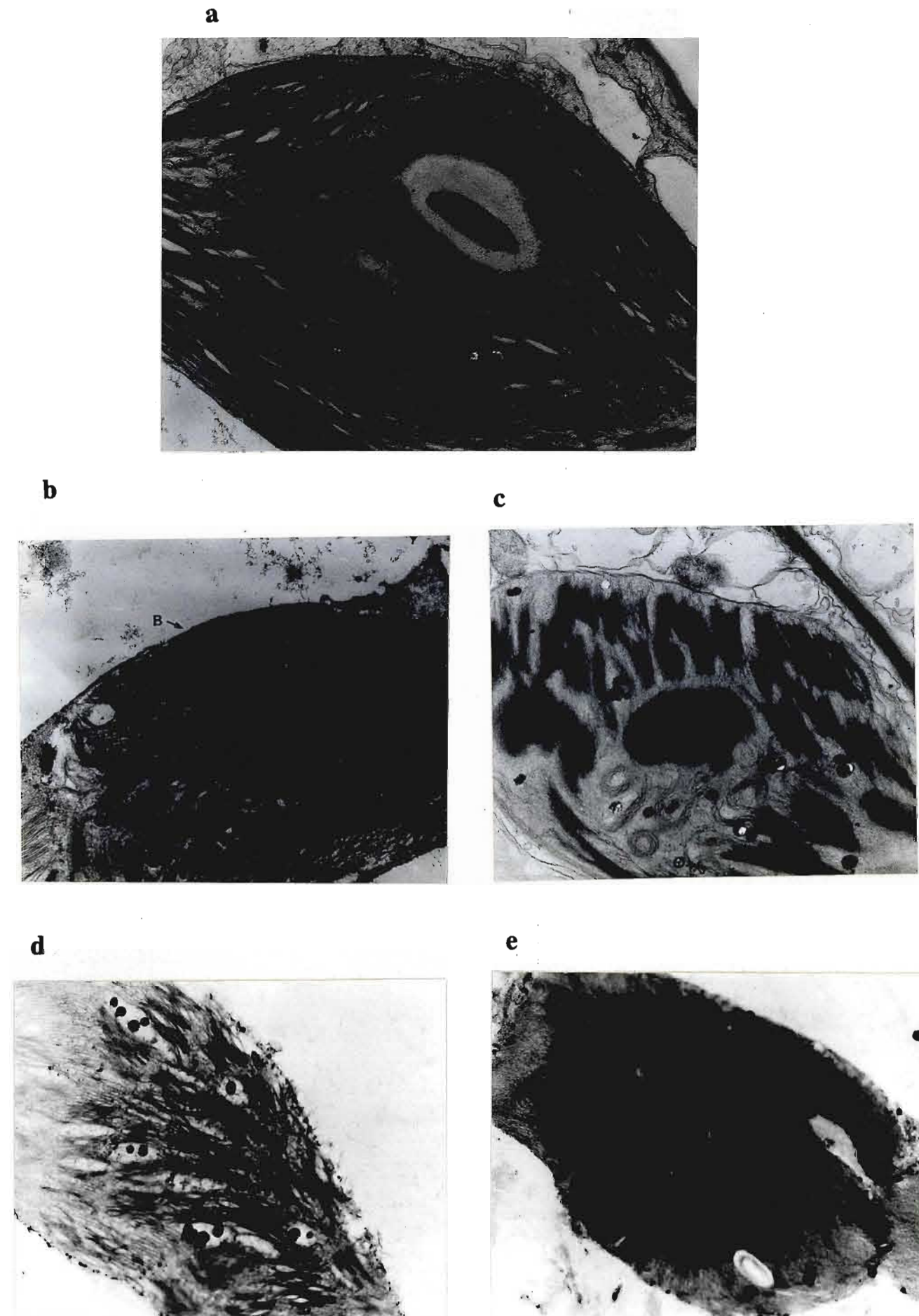
As leaflets of *A. raddianum* were dried so their vacuoles contracted and the plasmalemma withdrew from the cell wall. The plasmalemma was discontinuous in about 20% of the cells at 30% RWC and at 5% RWC it was discontinuous in all of the cells. The tonoplast broke down in the majority of the cells after drying to 5% RWC, being ultrastructurally intact in 50% of the cells dried to 30% RWC (Table 5.2). Rehydration from 30% RWC lead to further tonoplast breakdown. Rehydration of leaflets dried to 5% RWC also resulted in further damage to the cells and organelles.

**Table 5.2:** A summary of the observations made of the ultrastructure of leaves of *A. raddianum* at full turgor, 30% RWC, rehydrated from 30% RWC, 5% RWC and rehydrated from 5% RWC. These observations were made on leaf material that was fixed by standard procedures. Where possible observations were quantified.

Organelle/structure	Full turgor	30% RWC	Rehydrated from 30% RWC	5% RWC	Rehydrated from 5% RWC
Plasmalemma	100% intact Flat against the cell wall	30% discontinuous Inwardly withdrawn Beaded	30% discontinuous Inwardly withdrawn Slight improvement	All discontinuous Beaded	Similar to situation at 5% RWC
Chloroplast	Outer membranes intact Clear thylakoid stacks	30% outer membranes discontinuous Outer membranes beaded Clear thylakoid stacks	20% outer membranes discontinuous Clear thylakoid stacks Slight improvement	75% outer membranes discontinuous Outer membranes beaded Thylakoids separating	All outer membranes discontinuous Appearance fuzzy Damage worse than at 5% RWC
Mitochondria	Dense matrix Cristae present and clear Outer membranes intact	Matrix not very dense Fewer cristae visible 30% outer membranes discontinuous	Dense matrix Cristae present and clear Outer membranes intact Better than at 30% RWC	No mitochondrion-like structures found	Matrix not dense No cristae 80% outer membranes discontinuous
Nucleus	Membrane intact Dense nucleoplasm	Membrane intact Chromatin clumping	Membrane intact Chromatin clumped Dense nucleoplasm	No nucleus-like structures resolved	No nucleus-like structures resolved
Vacuole	Large central vacuole Tonoplast intact Contained some debris	Smaller than at full turgor Tonoplast intact 70% of cells Contained some debris	Smaller than at full turgor Tonoplast intact 50% cells Contained a lot of membranous debris	No vacuole evident Tonoplast discontinuous in all cells	Similar to situation at 5% RWC

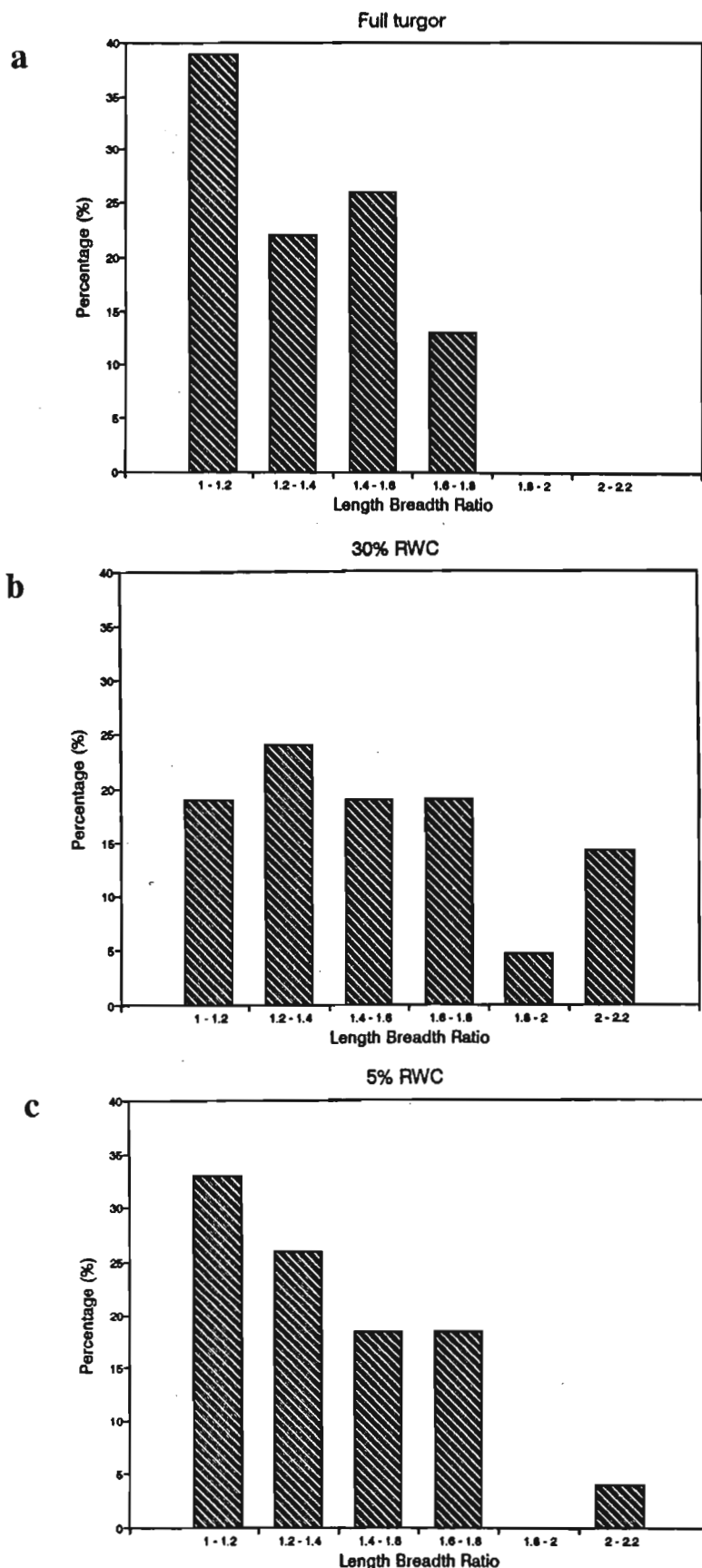


**Figure 5.9:** Transmission electron micrographs of the general cell appearance of leaflets of *A. raddianum* at full turgor (a, mag. x 10 400), 30% RWC (b, mag. x 6 500), material rehydrated from 30% RWC (c, mag. x 7 800), 5% RWC (d, mag. x 6 500) and material rehydrated from 5% RWC (e mag x 4 800). Standard fixation was used. Membrane-bound structures were visible in the tissue dried to 30% RWC and rehydrated. The cell membranes became beaded on drying and by 5% RWC little ultrastructural detail was preserved. Rehydration of material dried to 5% RWC resulted in further degradation of cellular contents. Pl - plasmalemma; Mb - clear membrane-bound structures

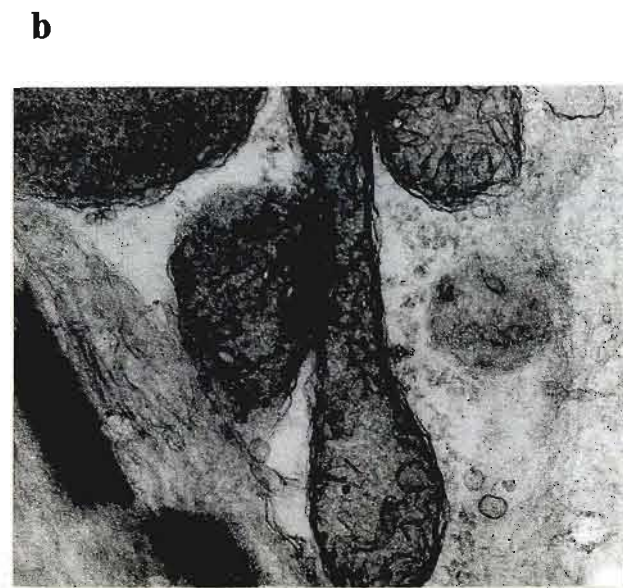
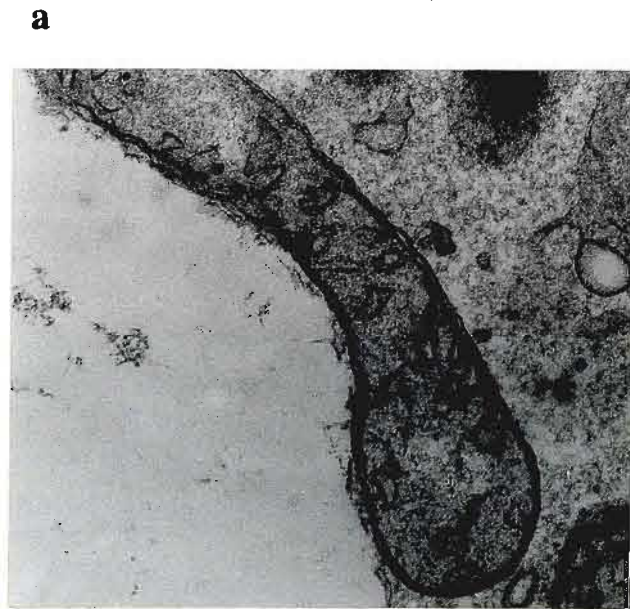


**Figure 5.10:** Transmission electron micrographs of the chloroplasts of *A. raddianum* leaflets at full turgor (a, mag. x 15 600), 30% RWC (b, mag. x 15 600), material rehydrated from 30% RWC (c, mag. x 13 000), 5% RWC (d, mag. x 15 600) and material rehydrated from 5% RWC (e, mag. x 10 400). Some of the damage shown in chloroplasts of leaves dried to 30% RWC (b) was removed upon rehydration (c). Outer membranes became beaded on drying. Thylakoid membranes were not clearly distinguishable in material that had been dried to 5% RWC and then rehydrated.

B - beaded membrane

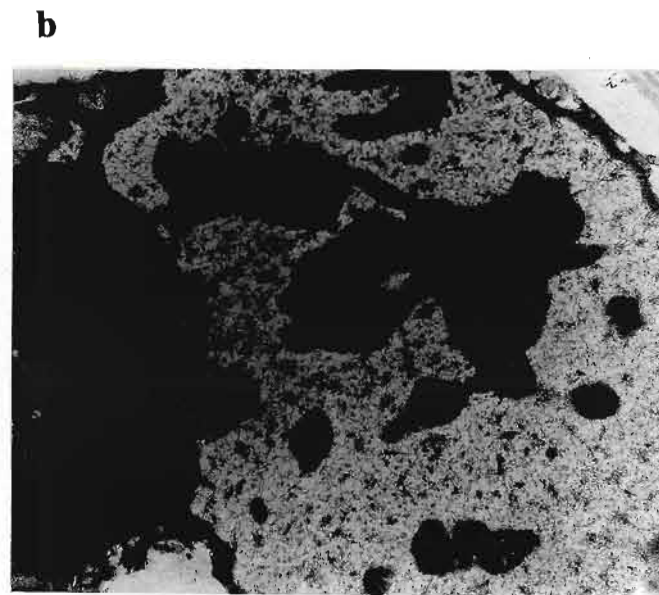
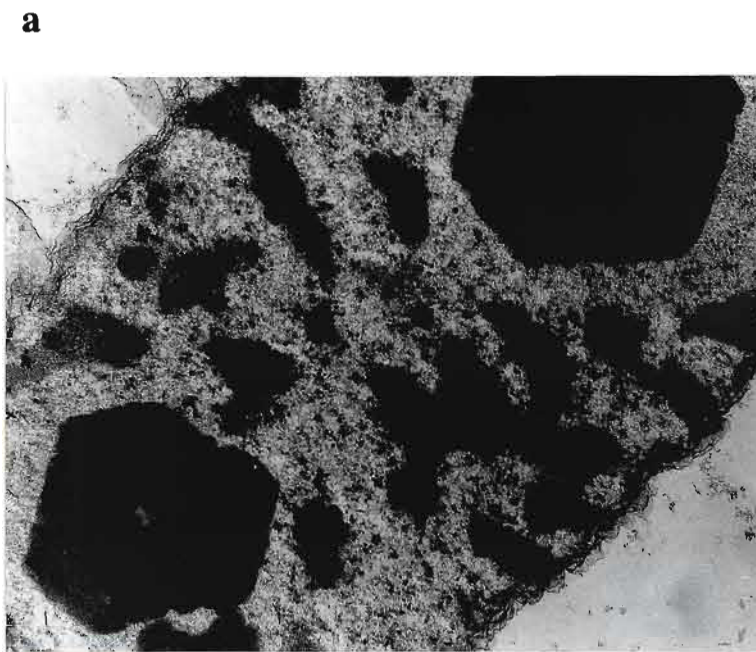


**Figure 5.11:** The chloroplast length:breath ratios of *A. raddianum* leaflets at full turgor (a) and dried to 30% RWC (b) and 5% RWC (c). Measurements were taken from thermal prints of material that had been fixed by standard means and sectioned for TEM.



**Figure 5.12:** Transmission electron micrographs of the mitochondria of *A. raddianum* leaflets at full turgor (a, mag. x 40 000), material rehydrated from 30% RWC (b, mag. x 40 000) and material rehydrated from 5% RWC (c, mag. x 30 000). There was no difference in mitochondrial structure between leaves dried to 30% RWC and dried to this RWC and rehydrated, thus Figure 5.12b represents both these treatments. The outer membranes had become less distinct in these treatments. No structures resembling mitochondria were resolved in leaflets dried to 5% RWC. Only degraded structures, as shown in 5.12c, were found in material rehydrated from 5% RWC. These possibly represent extensively degraded mitochondria.

C - cristae



**Figure 5.13:** Transmission electron micrographs of the nucleus of *A. raddianum* leaflets at full turgor (a, mag. x 7 800) and at 30% RWC (b, mag. x 10 400). No difference was noted in the nuclei of leaves dried to 30% RWC and those rehydrated from 30% RWC, thus Figure 5.13b represents both these treatments. Chromatin clumping was observed in these treatments. No nucleus-like structures were identified in leaflets dried to 5% RWC and those dried to 5% RWC and rehydrated.

N - possible nucleolus; C - chromatin clumping

TEM - *P. sativum*

Table 5.3 summarises the observations made on the ultrastructure of *P. sativum* leaves with drying and with subsequent rehydration. The general cell ultrastructure of fully hydrated leaves of *P. sativum* is shown in Figure 5.14. Cells at full turgor (Fig. 5.14a) showed a large central vacuole with clearly visible and distinguishable organelles. Drying to 30% RWC (Fig. 5.14b) led to some cell shrinkage and rounding of many of the chloroplasts. Rehydration from 30% RWC resulted in the expansion of cells and the walls appeared to pull away from the cell contents (Fig. 5.14c). The plasmalemma was intact in all the cells viewed at 30% RWC and in 80% of those rehydrated from this RWC (Table 5.3). Leaves dried to 5% RWC showed clear inwards contraction of the plasmalemma and a compaction of cellular contents as the vacuole disappeared (Fig. 5.14d). The plasmalemma was discontinuous in 80% of the cells viewed (Table 5.3) and also became beaded. Rehydration of these leaves led to total breakdown of the organelles and cell structure (Fig. 5.14e). The plasmalemma was discontinuous in all of the cells viewed (Table 5.3).

There were numerous membranous structures visible in cells at 30% RWC (Fig. 5.15a), which possibly originated from invaginated plasmalemma and/or degenerated organelles and endoplasmic reticulum. The cell walls at 5% RWC appear to be folded inwards (Fig. 5.15b) and even though the plasmalemma had pulled away from the walls there were large stretches where this membrane remained close to the cell wall. Rehydration of leaves from 5% RWC resulted in further damage (Fig. 5.15c). Degraded chloroplasts were visible and there were many darkly staining lipid bodies.

The ultrastructure of the chloroplasts (Fig. 5.16) confirmed these general trends. Thylakoid structure appeared disorganised after drying to 30% RWC (Fig. 5.16b) and after rehydration from this RWC (Fig. 5.16c). The outer chloroplast membranes were intact in leaves dried to 30% RWC (Table 5.3), while outer membranes of the chloroplasts of leaves dried to 5% RWC had broken down in 60% of the chloroplasts per cell (Table 5.3) and had a beaded appearance (Fig. 5.16d). This damage was worsened by rehydration (Fig. 5.16e) as the internal membranes of the chloroplast broke down and the outer membranes were ruptured. The change in shape of the chloroplasts with drying is shown

in Figure 5.17 which shows the chloroplast length:breadth ratios. There was a clear rounding of chloroplasts, shown by the marked increase in the percentage of chloroplasts with length:breadth ratios close to 1. At 5% RWC the chloroplasts tended towards a more elongated shape.

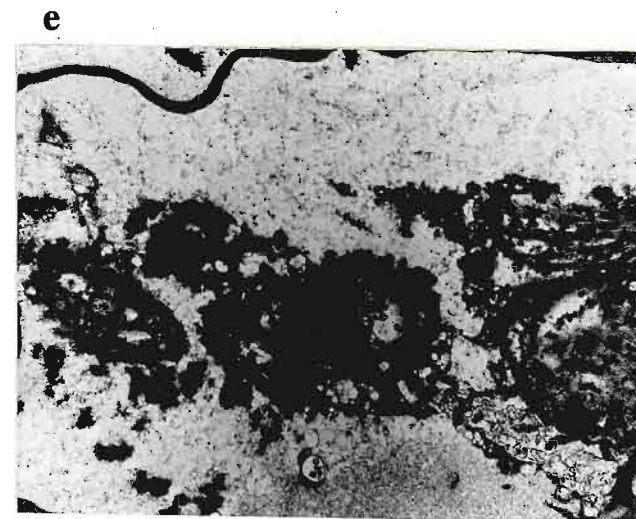
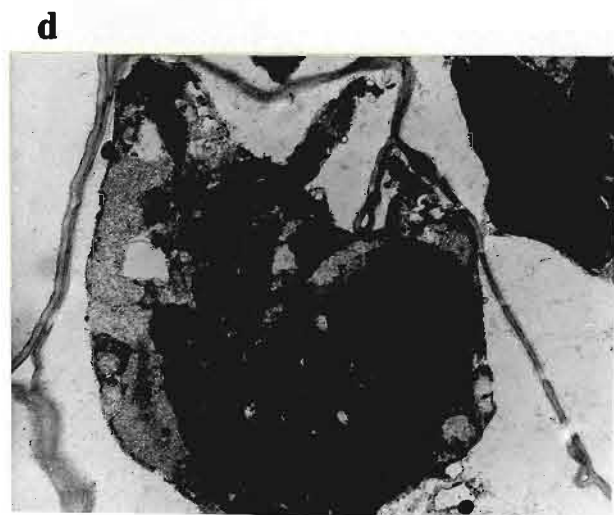
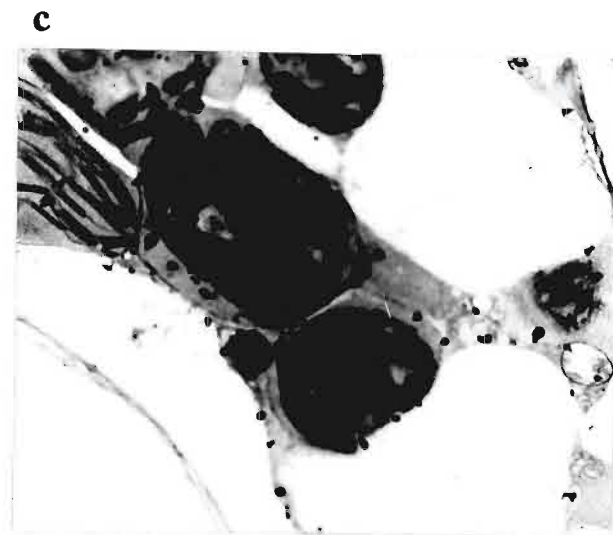
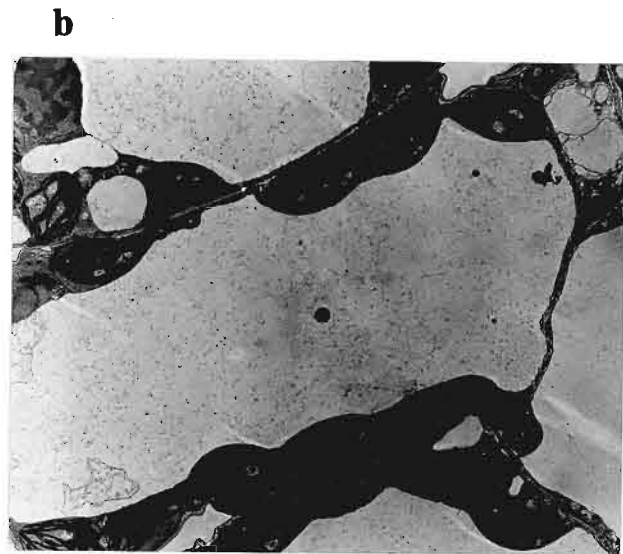
Mitochondria were also damaged by dehydration (Fig. 5.18). The cristae were not as numerous at 30% RWC (Fig. 5.18b) as they were at full turgor and appeared more slender (Fig. 5.18a), while the matrix was less dense. The outer membranes were ruptured in about 20% of the mitochondria per cell (Table 5.3). There was no obvious difference in the mitochondria in leaves dried to 30% RWC and those after rehydration. At 5% RWC (Fig. 5.18c) cristae were still visible but had assumed unusual configurations. The internal matrix also appeared to have degenerated. The outer membranes were ruptured in about 80% of the mitochondria visible per cell (Table 5.3). No structures which could be recognised as mitochondria were visible after leaves dried to 5% RWC were rehydrated. There appeared to be little ultrastructurally resolvable difference in nuclei of *P. sativum* mesophyll cells dried to 30% RWC and those in fully hydrated tissue (Figs. 5.19b and 5.19a respectively). No nuclei were visible in leaves dried to 5% RWC and those which had been rehydrated from 5% RWC.

### Summary

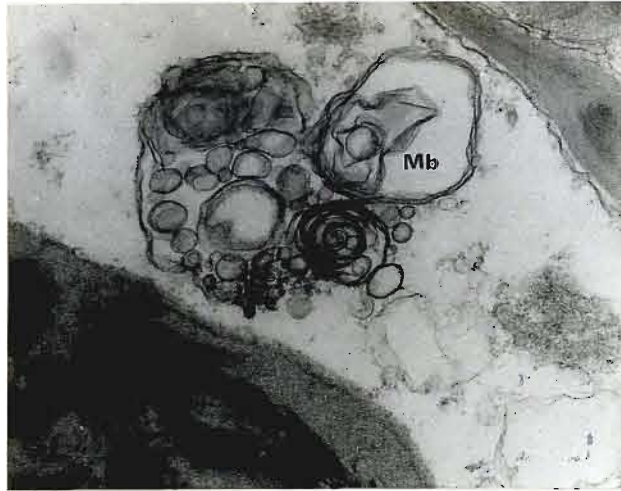
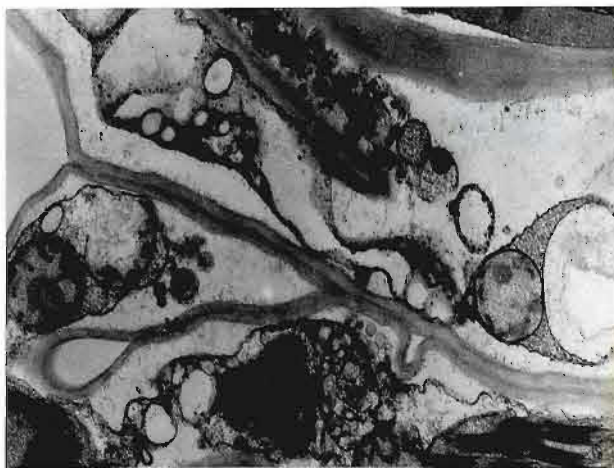
As with *A. raddianum*, the damage caused by drying leaves to 30% RWC appeared to be slightly exacerbated on rehydration. Rehydration of leaves dried to 5% RWC resulted in further damage to the cells. Leaves of *P. sativum* appeared to be more resistant to drying than those of *A. raddianum*, the plasmalemma remaining apparently continuous to below 30% RWC in *P. sativum*. The tonoplast also showed discontinuity only below 30% RWC and the chloroplast and mitochondrial outer membranes appeared less damaged at 30% RWC in *P. sativum*. The damage that occurred at 5% RWC in *P. sativum* appeared less severe than that which occurred in *A. raddianum* at 5% RWC. The collapsing inwards of the cell walls was not seen in *A. raddianum*.

**Table 5.3:** A summary of the observations made of the ultrastructure of leaves of *P. sativum* at full turgor, 30% RWC, rehydrated from 30% RWC, 5% RWC and rehydrated from 5% RWC. These observations were made on leaf material that was fixed by standard procedures. Where possible observations were quantified.

Organelle/structure	Full turgor	30% RWC	Rehydrated from 30% RWC	5% RWC	Rehydrated from 5% RWC
Plasmalemma	100% intact Flat against the cell wall	100% intact Slight withdrawal from cell wall	80% intact Not closely associated with cell walls	80% discontinuous Withdrawn to centre of cell Beaded	100% discontinuous Withdrawn to centre Beaded
Chloroplast	Outer membranes intact Clear thylakoid stacks Elongated	Outer membranes intact Thylakoid membranes separating Rounded	Outer membranes intact Granal stacks displaced Thylakoid membranes separating	60% outer membranes discontinuous Outer membranes beaded Thylakoids separating	All outer membranes discontinuous Appearance fuzzy Damage worse than in dry state
Mitochondria	Dense matrix Cristae present and clear Outer membranes intact	Matrix less dense Fewer cristae visible 20% outer membranes discontinuous	Similar to situation at 30% RWC	Matrix not dense Few cristae present 80% outer membranes discontinuous	No mitochondrion-like structures resolved
Nucleus	Membrane intact Dense nucleoplasm	Similar to situation at full turgor	Similar to situation at full turgor	No nucleus-like structures resolved	No nucleus-like structures resolved
Vacuole	Large central vacuole Tonoplast intact	Smaller than at full turgor Tonoplast intact Contains some membranous debris	Similar to situation at 30% RWC	No vacuole evident Tonoplast discontinuous in all cells	Similar to situation at 5% RWC

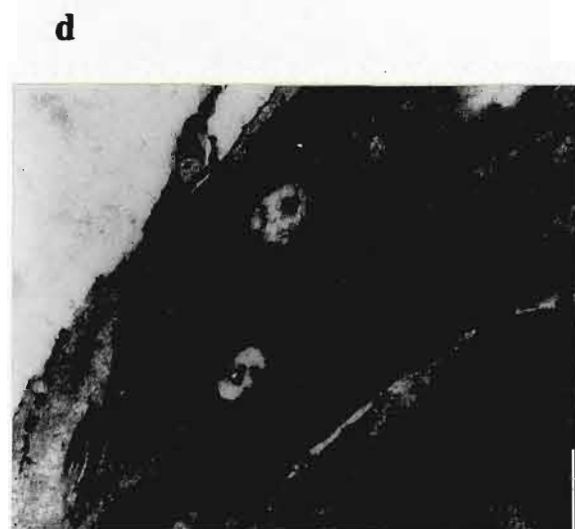
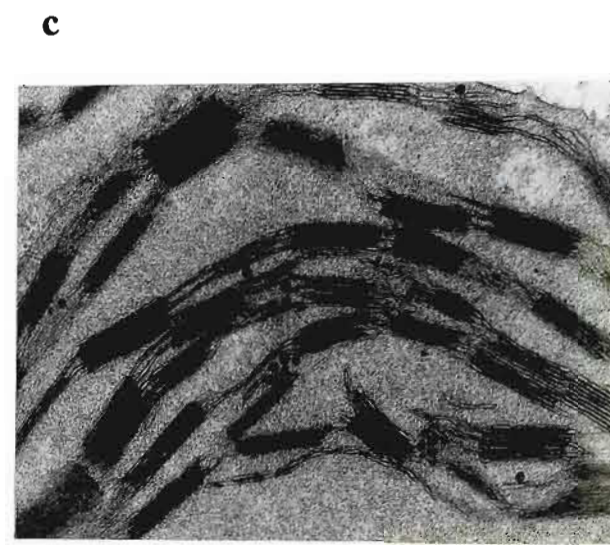
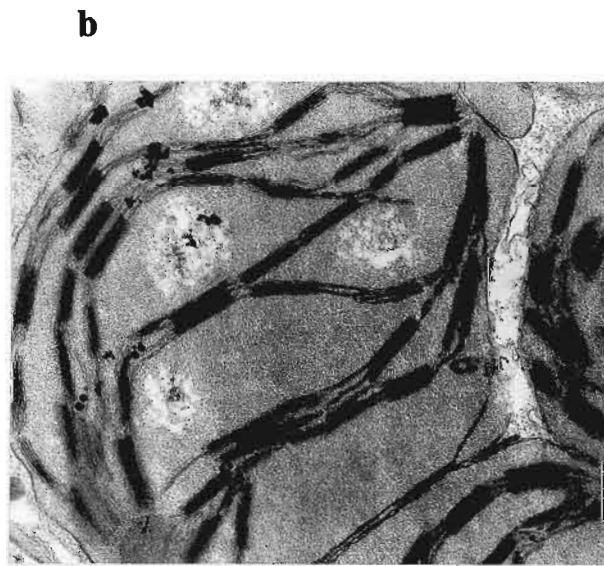
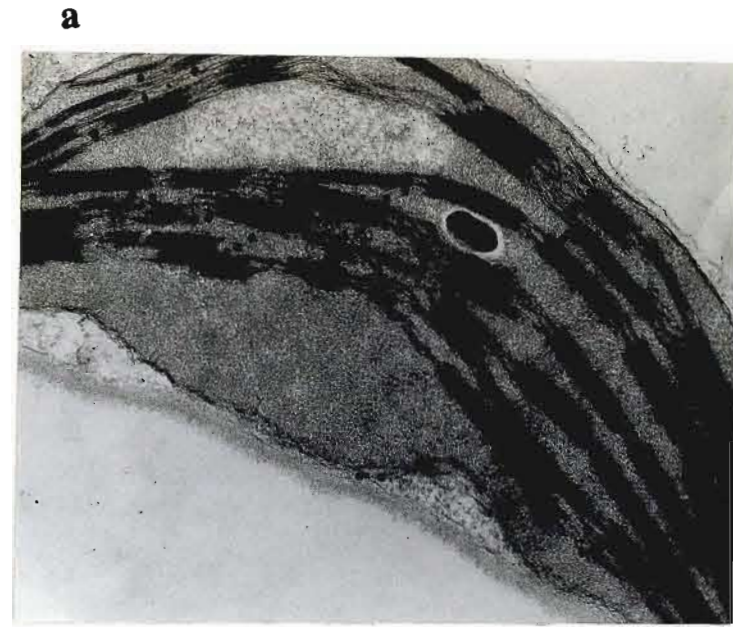


**Figure 5.14:** Transmission electron micrographs showing the general cell appearance of leaves of *P. sativum* at full turgor (a, mag. x 3 250), 30% RWC (b, mag. x 7800), rehydrated from 30% RWC (c, mag. x 2 600), 5% RWC (d, mag. x 7 800) and rehydrated from 5% RWC (e, mag x 4 000). The contraction of the cell contents towards the centre of the cell is evident in material dried to both 30% RWC and 5% RWC. Material rehydrated from 30% RWC and from 5% RWC showed more extensive damage than that in the dry state.

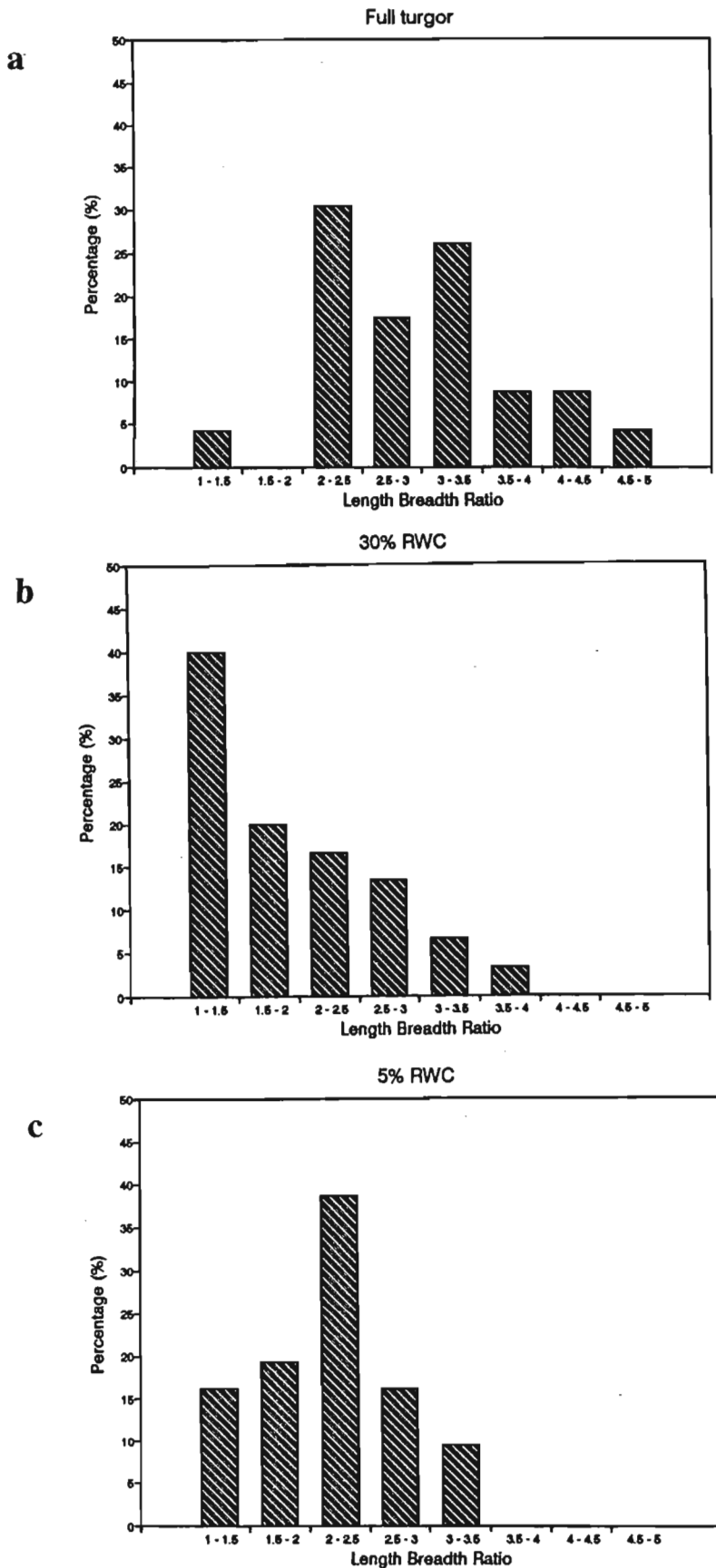
**a****b****c**

**Figure 5.15:** Transmission electron micrographs of *P. sativum* leaves dried to 30% RWC (a, mag. x 19 500) showing membranous structures which possibly originated from invaginated plasmalemma and/or degenerated organelles and endoplasmic reticulum. Leaves dried to 5% RWC (b, mag. x 15 600) showed cell walls folding in and continuous stretches of plasmalemma. Leaves dried to 5% RWC and then rehydrated (c, mag. x 13 000) show few recognisable organelles with breakdown of internal membranes and the accumulation of lipid.

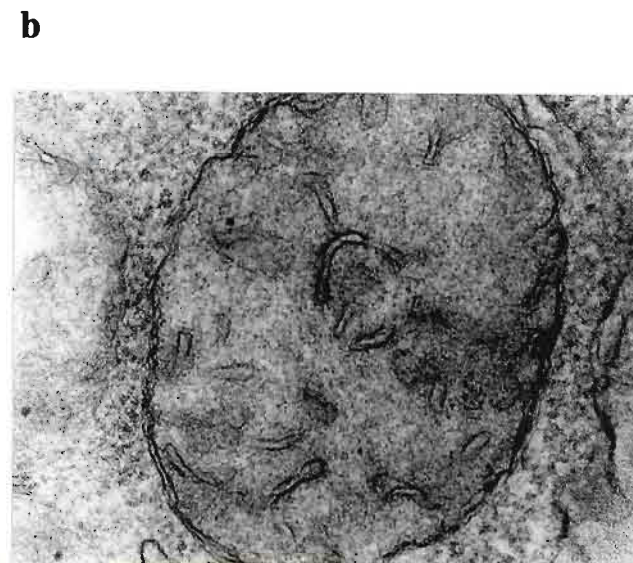
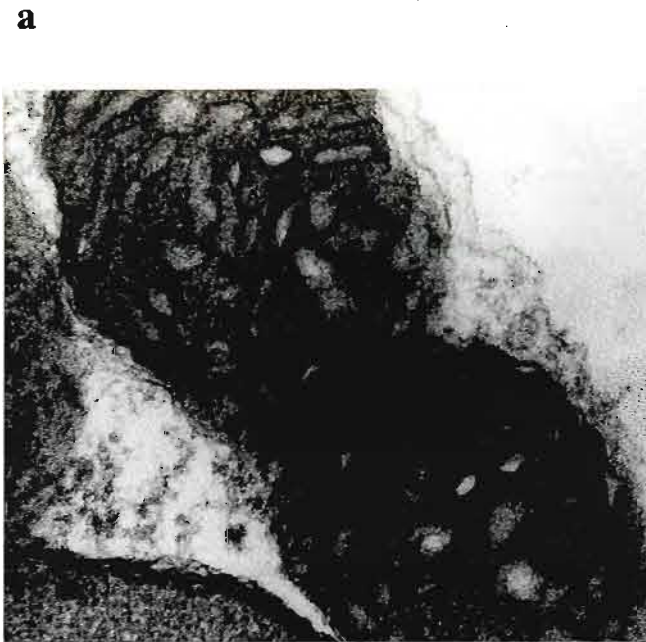
Mb - membranous structures; W - folded cell walls; L - lipid accumulations



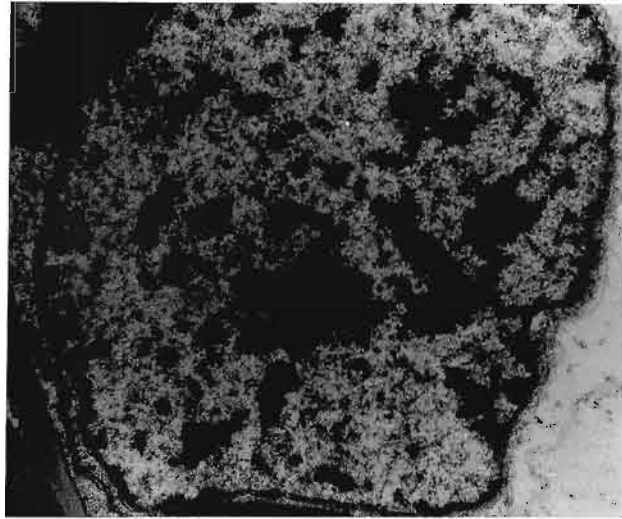
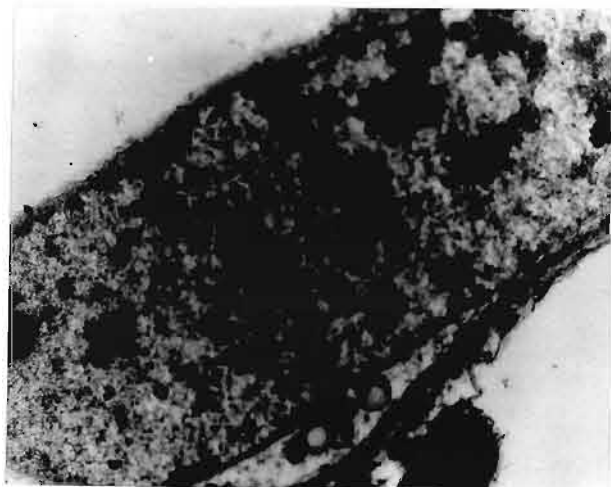
**Figure 5.16:** Transmission electron micrographs of the chloroplasts of *P. sativum* leaves at full turgor (a, mag. x 15 600), 30% RWC (b, mag. x 19 500), rehydrated from 30% RWC (c, mag. x 26 000), 5% RWC (d, mag. x 26 000) and rehydrated from 5% RWC (e, mag. x 26 000). The chloroplasts rounded on drying to 30% RWC. Thylakoid membranes were still clearly distinguishable at 5% RWC but were not resolved after the material had been rehydrated from 5% RWC.



**Figure 5.17:** The chloroplast length:breath ratios of *P. sativum* leaves at full turgor (a) and dried to 30% RWC (b) and 5% RWC (c). Measurements were taken from thermal prints of material that had been fixed by standard means and sectioned for TEM.



**Figure 5.18:** Transmission electron micrographs of the mitochondria of *P. sativum* leaves at full turgor (a, mag. x 65 000), rehydrated from 30% RWC (b, mag. x 65 000) and dried to 5% RWC (c, mag. x 52 000). There were no obvious differences in the mitochondria of leaves dried to 30% RWC and those dried to this RWC and rehydrated, thus Figure 5.18b represents both these treatments. There were fewer cristae and the cristae were more slender at 30% RWC and after rehydration from this RWC than they were at full turgor. Mitochondria in material dried to 5% RWC showed few cristae and the matrix had started breaking down. No structures resembling mitochondria were found in leaves rehydrated after being dried to 5% RWC.

**a****b**

**Figure 5.19:** Transmission electron micrographs of the nucleus of *P. sativum* leaves at full turgor (a, mag. x 10 400) and 30% RWC (b, mag. x 13 000). There were no obvious differences in the nuclei of leaves dried to 30% RWC and those dried to this RWC and rehydrated, thus Figure 5.19b represents both these treatments. There was also not much visible difference between these nuclei and those in fully hydrated leaves. No structures resembling nuclei were found in leaflets dried to 5% RWC and those rehydrated from 5% RWC.

TEM - *I. woodii*

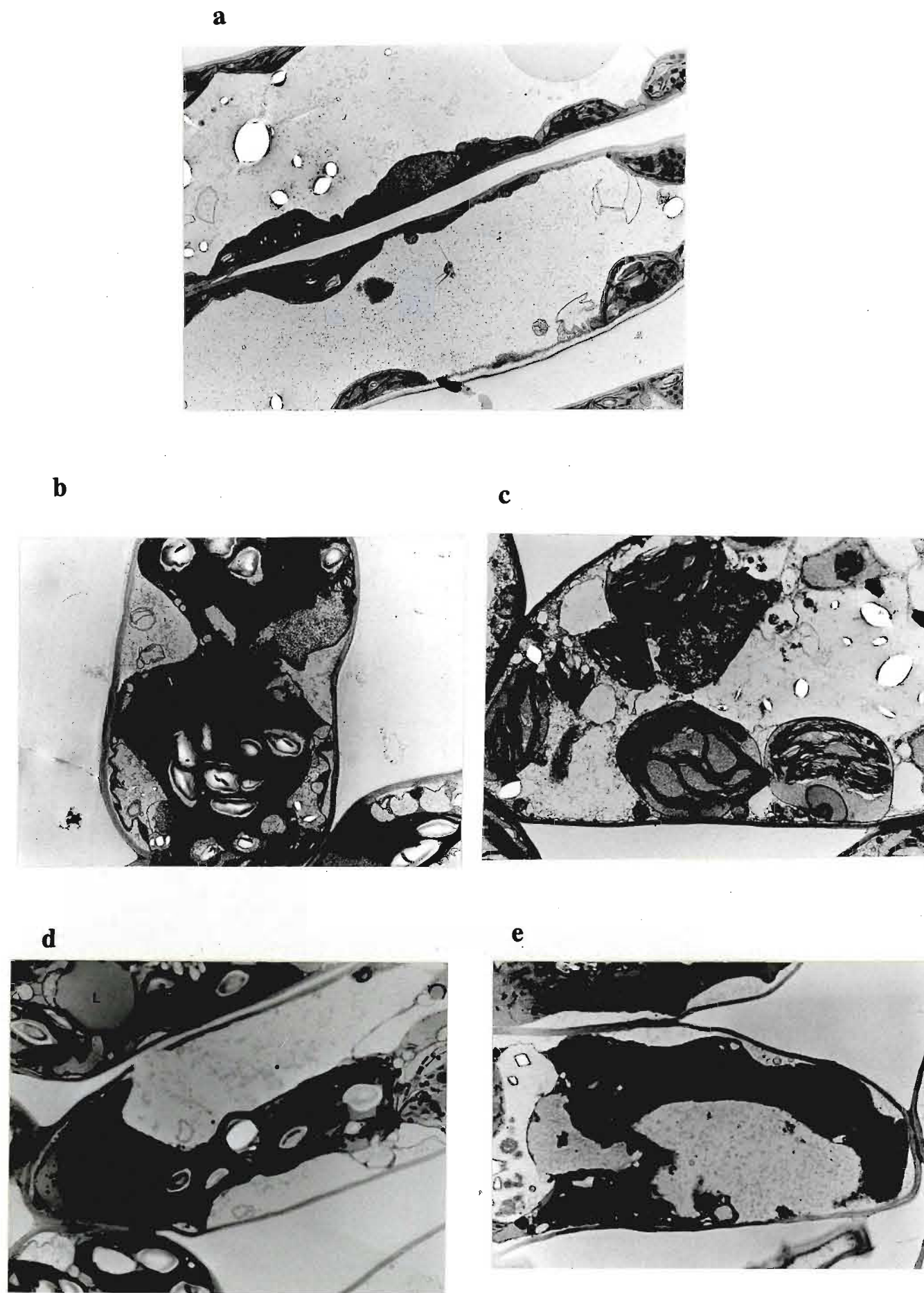
Table 5.4 summarises the observations made of the ultrastructure of *I. woodii* leaves with drying and subsequent rehydration. The general cell ultrastructure of leaves of *I. woodii* is shown in Figure 5.20. As the cells dried the vacuoles contracted and the plasmalemma contracted away from the cell wall. The plasmalemma was discontinuous in about 30% of cells dried to 30% RWC and about 20% of cells rehydrated from 30% RWC (Table 5.4), while the plasmalemma was discontinuous in 80% of cells that had been dried to 5% RWC and those rehydrated from 5% RWC. There appeared to be a redistribution of organelles in leaves rehydrated from 30% RWC (Fig. 5.20c), though whether this indicates recovery is not known. There appeared to be further contraction of cell contents in leaves dried to 5% RWC. Large intracellular lipid aggregations were found in these leaves. Cells of leaves rehydrated from this RWC appeared to be autolysed and thus little by way of organelle structure could be resolved (Fig. 5.20e). The folding in of the cell walls shown in *P. sativum* did not occur in *I. woodii*.

Chloroplasts tended to round with drying to 30% RWC (Fig. 5.21b and Fig. 5.22b). The double membrane was still present and apparently intact in all the cells viewed (Table 5.4), although slight beading had occurred. There was no difference in chloroplasts when material had been dried to 30% RWC and in that dried to this RWC and rehydrated. The thylakoid membrane systems remained intact and appeared to be centred in the chloroplast. Drying to 5% RWC (Fig. 5.21c and Fig. 5.22c) showed chloroplasts to be less rounded. The thylakoid organisation had degenerated to a certain extent and the stacks appeared much smaller. The outer membrane was discontinuous in 50% of the chloroplasts per cell (Table 5.4). As with *A. raddianum* and *P. sativum*, there was evidence of further damage to the chloroplast ultrastructure after rehydrating leaves from 5% RWC.

Mitochondria of leaves of *I. woodii* dried to 30% RWC (Fig. 5.23b) showed fewer cristae than were present at full turgor (Fig. 5.23a) and about 20% of outer membranes were ruptured (Table 5.4). Rehydration of these leaves resulted in curious circular cristal profiles. At 5% RWC no obvious mitochondrion-like structures were evident. After rehydration, however, organelles that were possibly degenerated mitochondria were visible (Fig. 5.23d), although there were no cristae evident in these structures. These structures

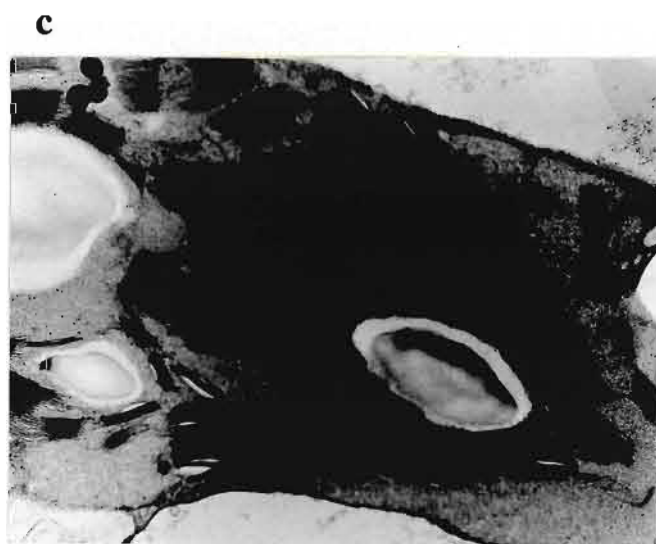
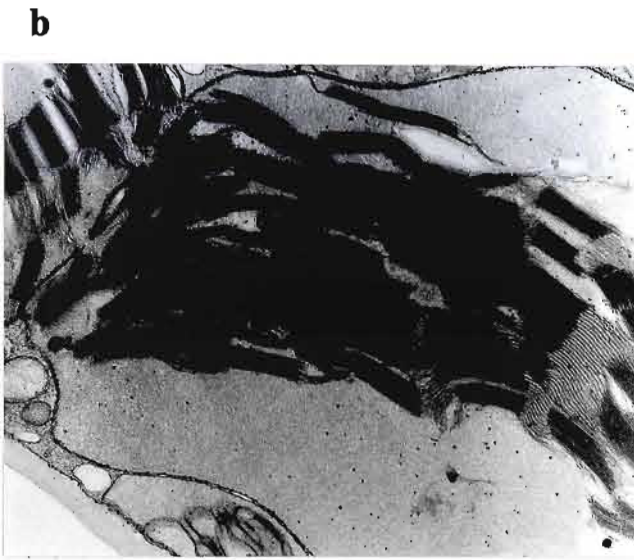
**Table 5.4:** A summary of the observations made of the ultrastructure of leaves of *I. woodii* at full turgor, 30% RWC, rehydrated from 30% RWC, 5% RWC and rehydrated from 5% RWC. These observations were made on leaf material that was fixed by standard procedures. Where possible observations were quantified.

Organelle/structure	Full turgor	30% RWC	Rehydrated from 30% RWC	5% RWC	Rehydrated from 5% RWC
Plasmalemma	100% intact Flat against the cell wall	30% discontinuous Contraction from cell wall	20% discontinuous Closer to cell wall than at 30% RWC	80% discontinuous Withdrawn to centre of cell	80% discontinuous Withdrawn to centre Beaded
Chloroplast	Outer membranes intact Clear thylakoid stacks Elongated	Outer membranes intact, but beaded Thylakoid membranes separating Rounded	Similar to situation at 30% RWC	50% outer membranes discontinuous Outer membranes beaded Thylakoids separating	Difficult to resolve any detail Damage worse than at 5% RWC
Mitochondria	Dense matrix Cristae present and clear Outer membranes intact	Matrix less dense Fewer cristae visible 20% outer membranes discontinuous	Similar to situation at 30% RWC	No mitochondrion-like structures resolved	Only degenerated featureless organelles resolved
Nucleus	Membrane intact Dense nucleoplasm	Membrane intact Chromatin clumping	Similar to situation at 30% RWC	No nucleus-like structures resolved	Membrane discontinuous Large chromatin clump Nucleoplasm fibrous
Vacuole	Large central vacuole Tonoplast intact Contents granular	Smaller than at full turgor Tonoplast intact	Larger than at 30% RWC Tonoplast intact Slight improvement	No vacuole evident Tonoplast discontinuous in all cells	Similar to situation at 5% RWC

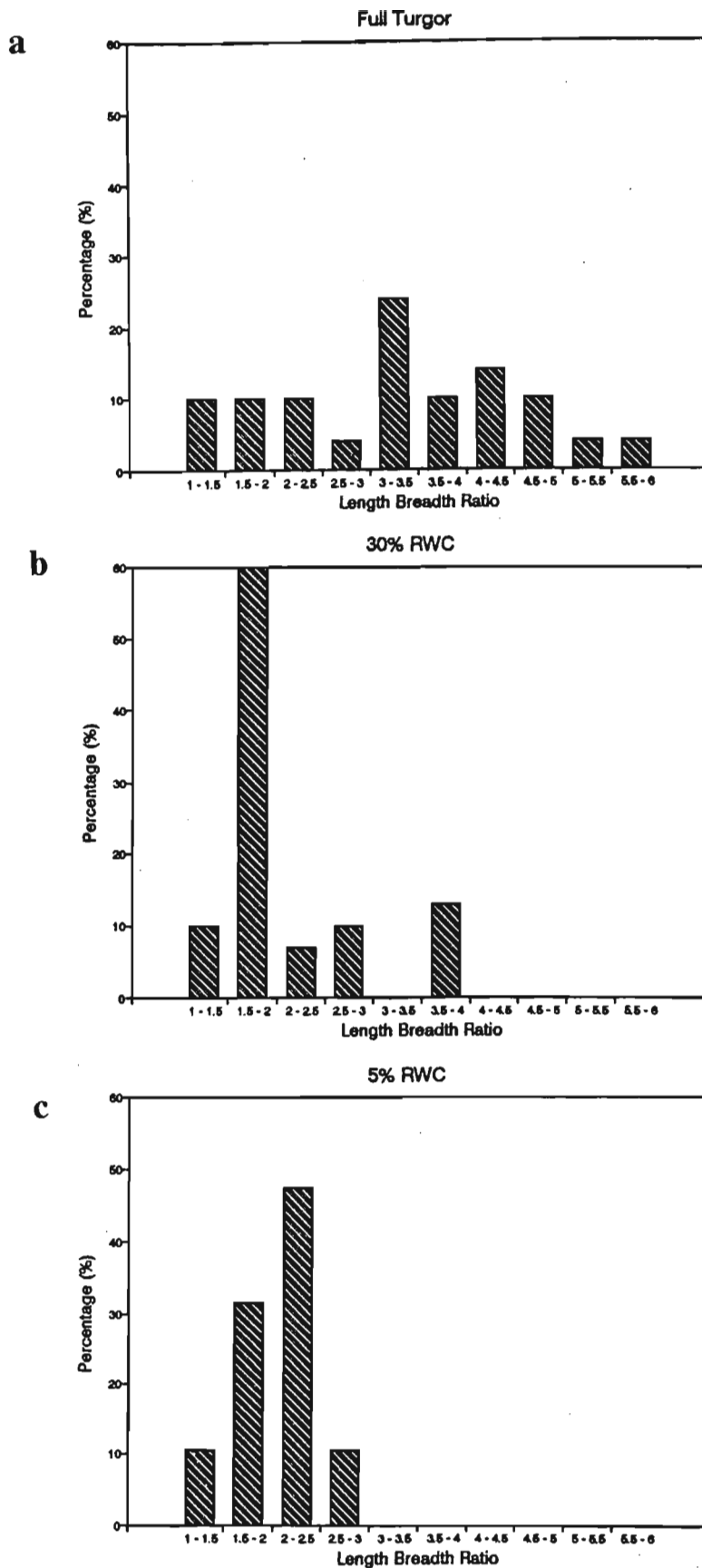


**Figure 5.20:** Transmission electron micrographs of the general cell appearance of leaves of *I. woodii* at full turgor (a, mag. x 3 600), 30% RWC (b, mag. x 4 000), rehydrated from 30% RWC (c, mag. x 4 000), 5% RWC (d, mag. x 3 250) and rehydrated from 5% RWC (e, mag x 4 000). Cell contents contracted towards the centre of the cell on drying. Large lipid aggregations were seen in material dried to 5% RWC. Rehydration from 5% RWC appeared to result in autolysis of the cell contents.

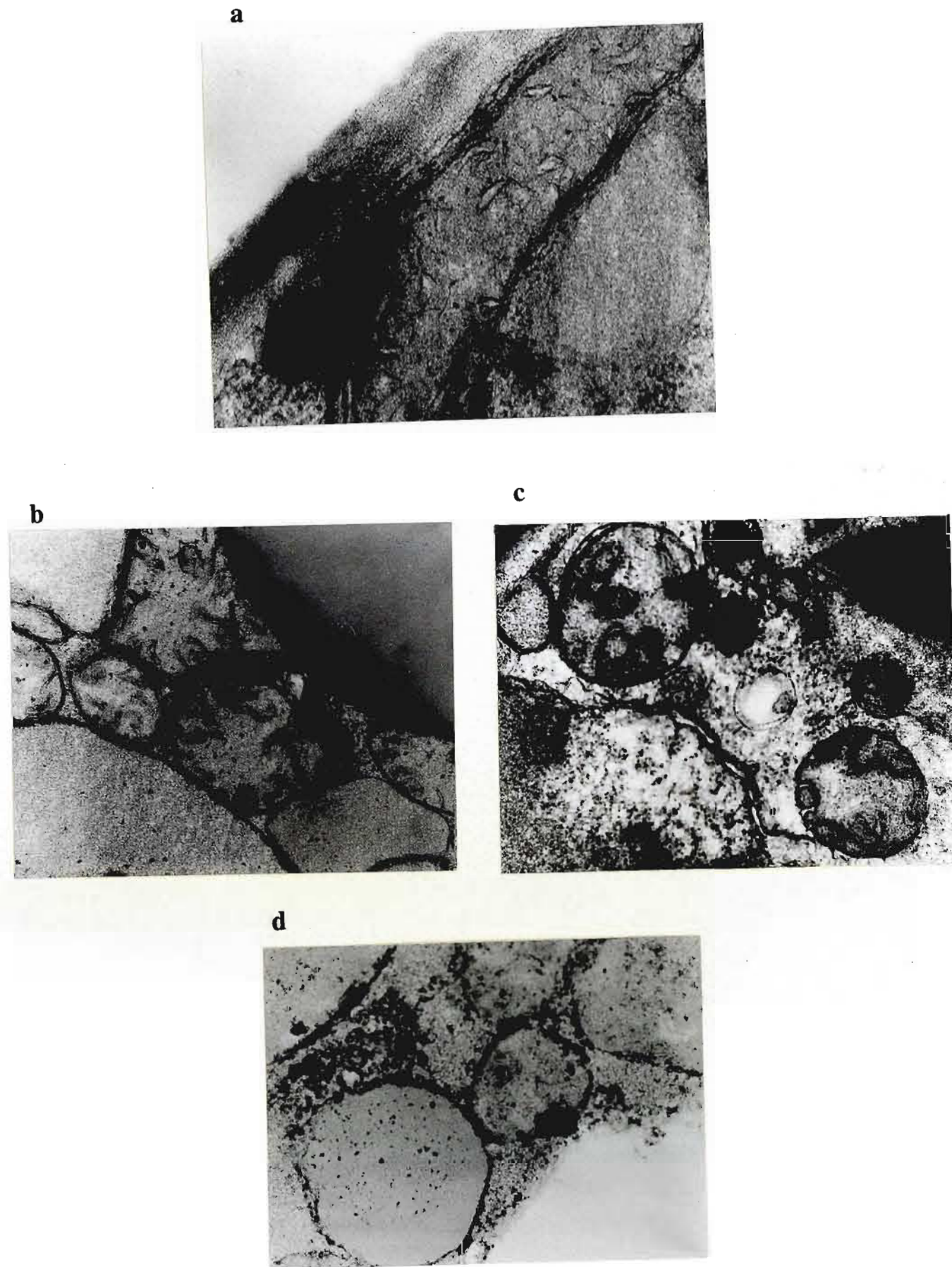
L - lipid aggregation



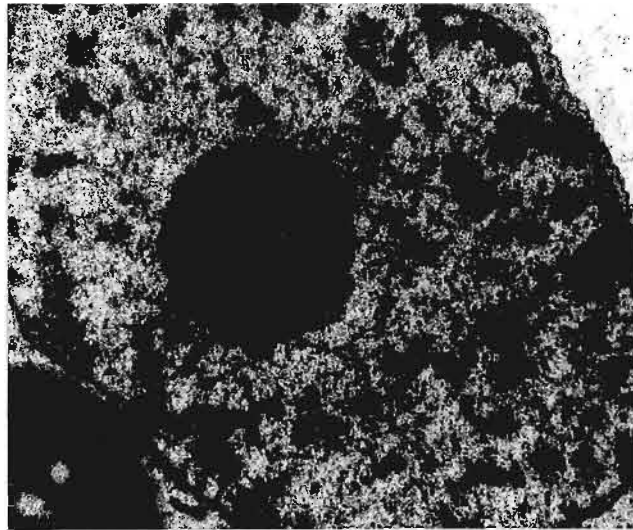
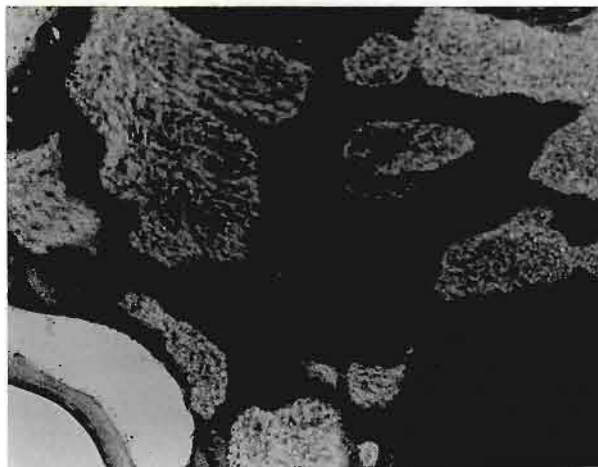
**Figure 5.21:** Transmission electron micrographs of the chloroplasts of *I. woodii* leaves at full turgor (a, mag. x 15 600), 30% RWC (b, mag. x 15 600) and 5% RWC (c, mag. x 13 000). There was no difference in the chloroplasts of leaves dried to 30% RWC and those dried to this RWC and rehydrated, thus Figure 5.21b represents both these treatments. Chloroplasts of leaves rehydrated from 5% RWC had little internal structure and were very darkly stained. Because this can be seen in Figure 5.20e, and there was difficulty in getting good resolution of these chloroplasts at high magnifications, no additional micrograph has been included here.



**Figure 5.22:** The chloroplast length:breath ratios of *I. woodii* leaves at full turgor (a) and dried to 30% RWC (b) and 5% RWC (c). Measurements were taken from thermal prints of material that had been fixed by standard means and sectioned for TEM.



**Figure 5.23:** Transmission electron micrographs of the mitochondria of *I. woodii* leaves at full turgor (a, mag. x 52 000), 30% RWC (b, mag. x 48 000), rehydrated from 30% RWC (c, mag. x 40 000) and rehydrated from 5% RWC (d, mag. x 52 000). Some outer membranes became discontinuous and cristae decreased in number on drying to 30% RWC. Rehydration resulted in cristae with unusual circular profiles. No obvious mitochondrion-like structures were visible at 5% RWC. Remnants of degraded mitochondria were all that were visible in material that had been rehydrated from 5% RWC.

**a****b****c**

**Figure 5.24:** Transmission electron micrographs of the nucleus of *I. woodii* leaves at full turgor (a, mag. x 13 000), 30% RWC (b, mag. x 13 000) and rehydrated from 5% RWC (c, mag. x 15 600). There was no difference in the nuclei of leaves dried to 30% RWC and those dried to this RWC and rehydrated, thus Figure 5.24b represents both these treatments. Chromatin clumping was observed in these treatments. No clear nucleus-like structures were visible in leaves dried to 5% RWC. Those in leaves rehydrated from this RWC showed extensive chromatin clumping with unusual fibrous nucleoplasm.  
C - chromatin clumping; Np - nucleoplasm

### TEM - *G. livingstonei*

Table 5.5 summarises the observations made of the ultrastructure of *G. livingstonei* leaves with drying and subsequent rehydration. The general cell ultrastructure of *G. livingstonei* during drying and rehydration is shown in Figure 5.25. In terms of the cell periphery and central vacuole, there did not appear to be much difference in the cells at full turgor (Fig. 5.25a), those dried to 30% RWC (Fig. 5.25b) and those rehydrated from this RWC (Fig. 5.25c). The large central vacuole persisted and the plasmalemma, which was still intact in all the cells viewed (Table 5.5), had not contracted from the cell wall. Drying to 5% RWC, however, showed marked damage of cell ultrastructure (Fig. 5.25d). The vacuole was no longer visible, the chloroplasts appeared to have broken down and the plasmalemma was discontinuous in all the cells viewed (Table 5.5). The plasmalemma also became beaded suggesting damage to the lipid bilayer. Rehydration from this RWC (Fig. 5.25e) resulted in further damage with membranous structure breaking down.

The chloroplast ultrastructure (Fig. 5.26) showed these same trends. The chloroplasts of leaves at full turgor (Fig. 5.26a), 30% RWC and rehydrated from 30% RWC (Fig. 5.26b) had clearly resolved, apparently intact, outer membranes and thylakoid stacks (Table 5.5). Drying to 5% RWC (Fig. 5.26c) resulted in a breakdown of the outer membranes and separation of the thylakoid membranes in 80% of organelles viewed. Rehydration from this RWC (Fig. 5.26d) yielded compact, dense chloroplasts which lacked clearly visible internal and bounding membranes (Table 5.5). The length:breadth ratios (Fig. 5.27) show that there was a slight rounding of chloroplasts with drying but this is not nearly as marked as with the other species discussed thus far.

The mitochondria (Fig. 5.28) showed few changes from full turgor (Fig. 5.28a), dried to 30% RWC and rehydrated from 30% RWC (Fig. 5.28b). The bounding double membrane and the cristae were clearly visible and the matrix was relatively dense. Drying to 5% RWC (Fig. 5.28c) resulted in regression of cristae, though the outer membranes were still visible. Rehydration from this RWC yielded cells in which no mitochondrion-like structures were visible.

As leaves of *G. livingstonei* were dried so the chromatin of their nuclei appeared to

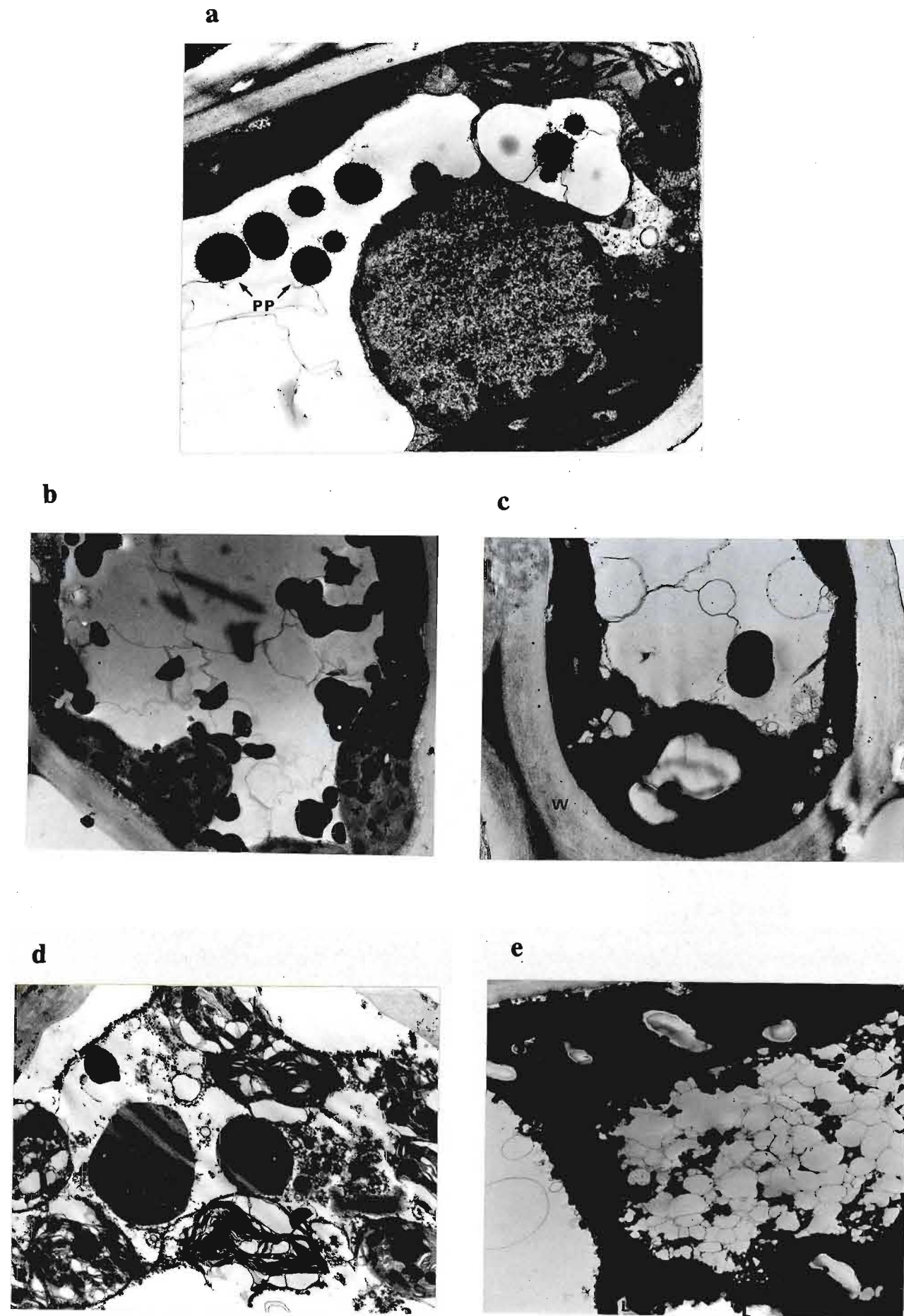
become more clumped (Fig. 5.29). Clearly distinguishable nuclei were still evident at 5% RWC (Fig. 5.29c). Rehydration from this RWC resulted in severe abnormality of the nuclei (Fig. 5.29d). The chromatin was clumped in the centre of the nucleus, the outer membrane was discontinuous and the nucleoplasm was fibrous. These were similar to the nuclei observed in *I. woodii* after rehydration from 5% RWC (Fig. 5.24c).

### Summary

Leaves of *G. livingstonei* appear to be more resistant to damage occurring during drying to 30% RWC than the other sensitive species. Damage at 5% RWC, however, appeared to be as extensive, if not more so, than that occurring in the other species. Rehydration from this RWC also resulted in further damage. The cell walls of *G. livingstonei* were thick and there was no folding inwards as there was with *P. sativum*. The spongy mesophyll cells appeared to be more susceptible to dehydration damage than palisade mesophyll cells.

**Table 5.5:** A summary of the observations made of the ultrastructure of leaves of *G. livingstonei* at full turgor, 30% RWC, rehydrated from 30% RWC, 5% RWC and rehydrated from 5% RWC. These observations were made on leaf material that was fixed by standard procedures. Where possible observations were quantified.

Organelle/structure	Full turgor	30% RWC	Rehydrated from 30% RWC	5% RWC	Rehydrated from 5% RWC
Plasmalemma	100% intact Flat against the cell wall	100% intact Contraction from cell wall	Similar to 30% RWC	80% discontinuous Contracted to centre of cell Beaded	100% discontinuous Contracted to centre cell Beaded
Chloroplast	Outer membranes intact Clear thylakoid stacks Elongated	Similar situation to full turgor	Similar to states at full turgor and 30% RWC	70% outer membranes discontinuous Outer membranes beaded	Difficult to resolve detail Damage worse than at 5% RWC
Mitochondria	Dense matrix Cristae present and clear Outer membranes intact	Similar to situation at full turgor	Similar to states at full turgor and 30% RWC	50% outer membranes discontinuous Few cristae	No mitochondrion-like structures resolved
Nucleus	Membrane intact Dense nucleoplasm	Membrane intact Nucleoplasm not very dense	Similar to situation at 30% RWC	No nucleus-like structures resolved	Membranes discontinuous Large chromatin clump Nucleoplasm fibrous
Vacuole	Large central vacuole Tonoplast intact	Smaller than at full turgor Tonoplast intact Membranous debris	Similar to situation at 30% RWC	No vacuole evident Tonoplast discontinuous in all cells	Similar to 5% RWC



**Figure 5.25:** Transmission electron micrographs of the general cell appearance of leaves of *G. livingstonei* at full turgor (a, mag. x 6 500), 30% RWC (b, mag. x 6 500), rehydrated from 30% RWC (c, mag. x 7 800), 5% RWC (d, mag. x 6 500) and rehydrated from 5% RWC (e, mag x 7 800). There were numerous polyphenolic inclusions in the cells of all treatments. There was little contraction away from the cell wall in leaves dried to 30% RWC. Dehydration to 5% RWC resulted in degeneration of the cell contents as the plasmalemma and organelle membranes ruptured. Rehydration from this RWC resulted in autolysis of the cells with little cellular detail remaining visible. Plastoglobuli accumulated in the remains of the chloroplasts in this treatment. The cell walls of this species were very thick.

PP - polyphenolics; L - lipid accumulations; W - cell wall



b



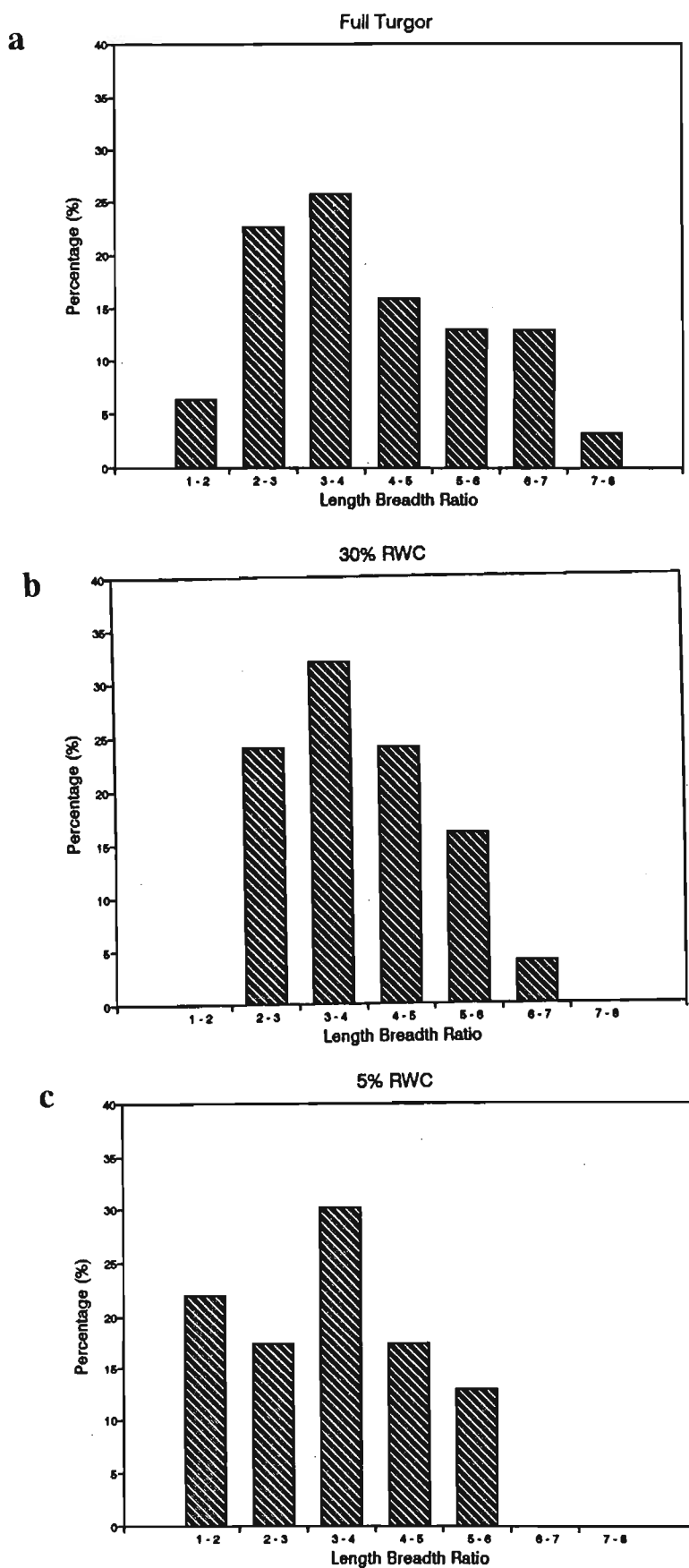
c



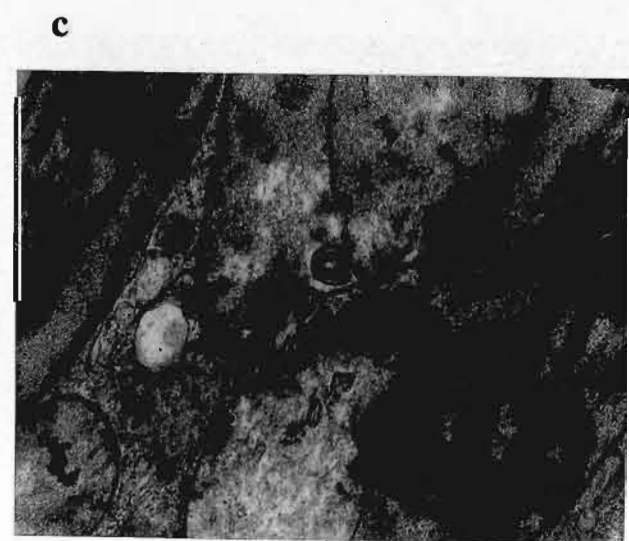
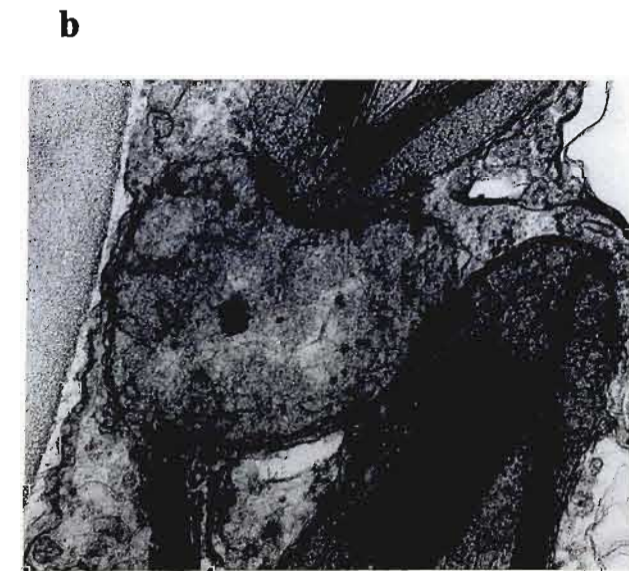
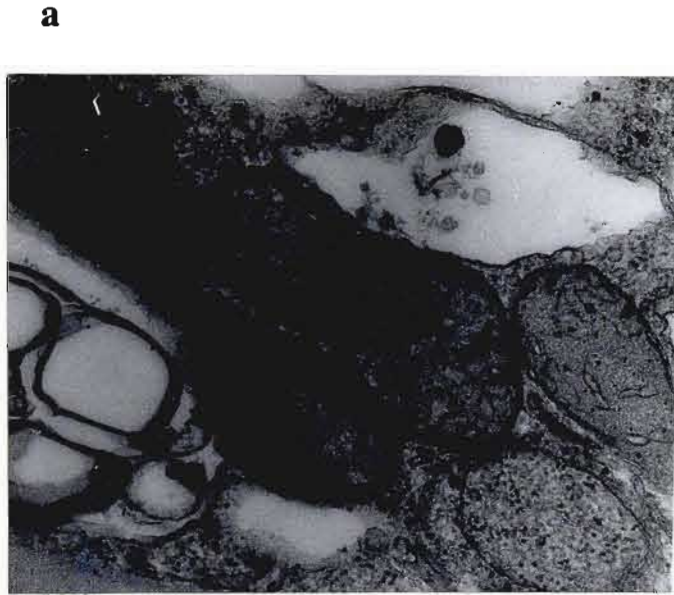
d



**Figure 5.26:** Transmission electron micrographs of the chloroplasts of *G. livingstonei* leaves at full turgor (a, mag. x 19 500), rehydrated from 30% RWC (b, mag. x 15 600), dried to 5% RWC (c, mag. x 26 000) and rehydrated from 5% RWC (d, mag. x 19 500). There was no difference in the chloroplasts of leaves dried to 30% RWC and those dried to this RWC and rehydrated, thus Figure 5.26b represents both these treatments. The chloroplasts in leaves subjected to these treatments were also similar to those in fully hydrated leaves. Chloroplasts of leaves dried to 5% RWC showed disruption of thylakoid and outer membranes. Rehydration from 5% RWC resulted in loss of internal structure of the chloroplast.

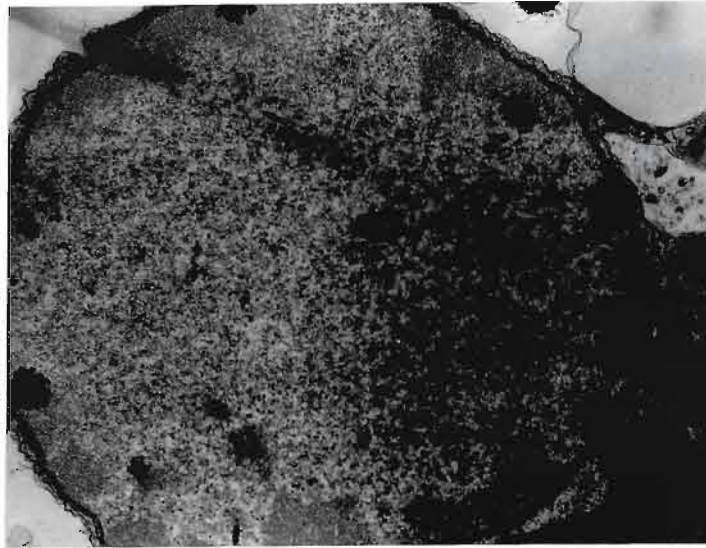


**Figure 5.27:** The chloroplast length:breadth ratios of *G. livingstonei* leaves at full turgor (a) and dried to 30% RWC (b) and 5% RWC (c). Measurements were taken from thermal prints of material that had been fixed by standard means and sectioned for TEM.

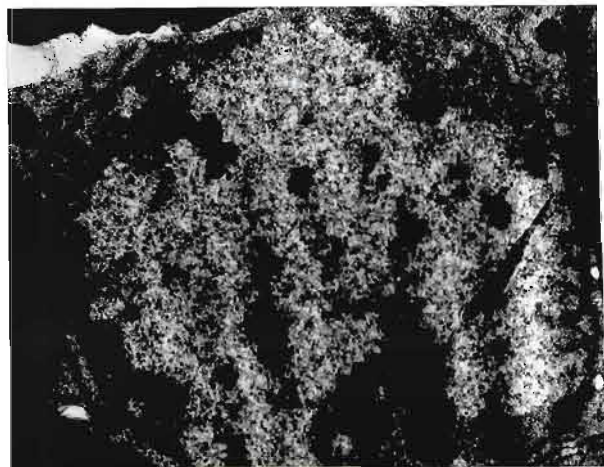


**Figure 5.28:** Transmission electron micrographs of the mitochondria of *G. livingstonei* leaves at full turgor (a, mag. x 52 000), rehydrated from 30% RWC (b, mag. x 52 000) and 5% RWC (c, mag. x 40 000). There was no difference in the mitochondria of leaves dried to 30% RWC and those dried to this RWC and rehydrated, thus Figure 5.28b represents both these treatments. There did not appear to be much difference between the mitochondria of leaves of these two treatments and those in fully hydrated leaves. There were fewer cristae at 5%RWC and some of the outer membranes had ruptured. No clear mitochondrion-like structure was visible in leaves dried to 5% RWC and rehydrated.

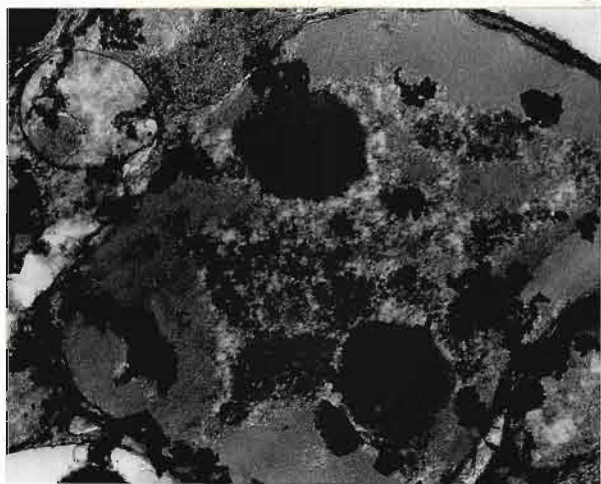
a



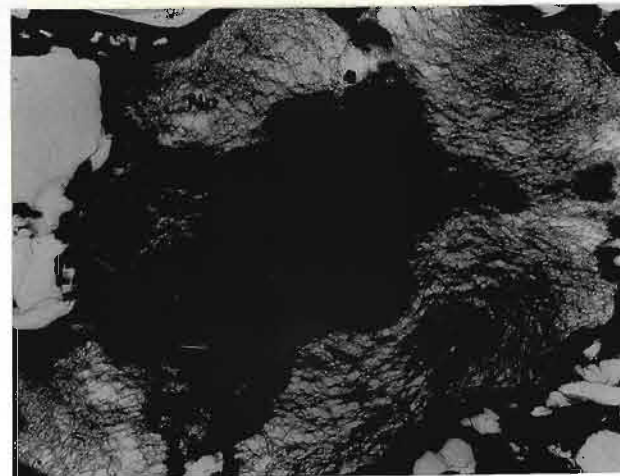
b



c



d



**Figure 5.29:** Transmission electron micrographs of the nucleus of *G. livingstonei* leaves at full turgor (a, mag. x 13 000), rehydrated from 30% RWC (b, mag. x 15 600), 5% RWC (c, mag. x 13 000) and rehydrated from 5% RWC (d, mag. x 15 600). There was no difference in the nuclei of leaves dried to 30% RWC and those dried to this RWC and rehydrated, thus Figure 5.29b represents both these treatments. The nucleoplasm appeared to have become more granular in these two treatments than at full turgor. There appeared to be more extensive chromatin clumping in nuclei from leaves dried to 5% RWC. The nucleoplasm of leaves rehydrated from 5% RWC showed extensive degradation, with one central clump of chromatin and a fibrous nucleoplasm.

C - chromatin clumping; Np - nucleoplasm

TEM - *C. nanum*

Table 5.6 summarises the observations made of the ultrastructure of *C. nanum* leaves with drying and subsequent rehydration. Drying *C. nanum* leaves to 30% RWC (Fig. 5.30b) resulted in cell shrinkage but the maintenance of the large central vacuole and peripheral location of cell contents. The plasmalemma had pulled away from the cell wall in places but was still continuous. At slightly higher magnifications (Fig. 5.31a) it can be seen that organelles and cell contents were still peripherally located and are clearly discernable. This is in contrast to the apparent situation in the freeze-substituted specimens (Fig. 5.3a & b) where the plasmalemma was not continuous and organelle structure was not visible. The plasmalemma was intact in all the cells viewed (Table 5.6). Drying to 5% RWC (Fig. 5.30c & Fig. 5.31b) resulted in the cell walls folding inwards as was shown in the light micrographs (Fig. 5.8d) and in the freeze-substituted specimen (Fig. 5.3c). The organelles were located in the middle of the cells as the large central vacuole had disappeared. The large vacuole appeared to have subdivided into a number of small vacuoles which were not always clearly distinguishable. At higher magnifications (Fig. 5.31b) the space between the plasmalemma and the cell wall was seen to contain granular material. The cytoplasm was relatively dense and the organelles were closely packed together. After rehydration the cell regained its original shape and size and the plasmalemma was again generally associated with the cell wall (Fig. 5.31c).

The ultrastructure of the chloroplasts (Fig. 5.32) showed that there was some separation of the thylakoid membranes after drying to 30% RWC (Fig. 5.32b). These irregularities in the thylakoids were still present after these leaves had been rehydrated (Fig. 5.32c). The outer membranes of the chloroplasts were intact in all the cells viewed (Table 5.6). Drying to 5% RWC resulted in the chloroplasts becoming rounder (Fig. 5.33c). The thylakoids were not in the middle of the stromal space as with other species that showed rounding of chloroplasts, but rather the membranes were displaced to one side. About 70% of the outer membranes of the chloroplasts viewed per cell appeared ruptured (Table 5.6). Pronounced rounding occurred at 5% RWC compared with the situation in *P. sativum* and *I. woodii* where this rounding occurred at 30% RWC. Leaves dried to 5% RWC and then rehydrated showed recovery of the chloroplasts (Fig. 5.32e). The organelles elongated, but not to full turgor dimensions in the time allowed for rehydration.

The stroma contained some clear membrane bound structures which may represent either degenerated or reconstituting or new thylakoids. At no stage in the drying of the cells did the chloroplast outer membranes appear beaded, although about 20% of the outer membranes of chloroplasts per cell appeared discontinuous in the leaves rehydrated from 5% RWC (Table 5.6).

The mitochondria showed similar trends to the chloroplasts during drying. Mitochondria at 30% RWC (Fig. 5.33b) did not have as dense a matrix as did the mitochondria at full turgor (Fig. 5.33a). Cristae persisted and both bounding membranes were clearly resolved. After rehydration from 30% RWC (Fig. 5.33c) the matrix had not regained the density it had at full turgor. Drying to 5% RWC (Fig. 5.33d) resulted in marked changes to the mitochondria. There did not appear to be any cristae and the matrix was clear in places. The bounding membranes remained visible though discontinuity was seen in both the inner and outer membrane in about 50% of the mitochondria per cell (Table 5.6). After rehydration (Fig. 5.33e) the mitochondria had reconstituted cristae and appeared similar to the mitochondria of the leaves dried to 30% RWC.

The nuclei of *C. nanum* leaves at full turgor (Fig. 5.34a) had little visible aggregated chromatin, its apparently being uniformly dispersed in the nucleoplasm. Drying to 30% RWC (Fig. 5.34b) did not result in chromatin clumping as it did with most of the desiccation-sensitive species, the nucleoplasm appearing to become more finely grained. Rehydration from this RWC yielded nuclei which were similar in appearance to those in the dry state. Nucleus-like structures were not resolvable in the densely packed cytoplasm of leaves dried to 5% RWC in the dry state. After rehydration, however, nuclei that were apparently similar to those found at full turgor were present (Fig. 5.34c). However, the nucleoplasm was less dense than that at full turgor.

### Summary

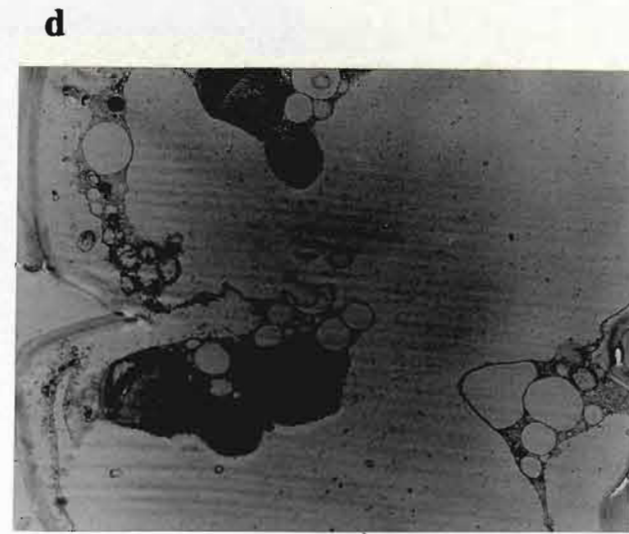
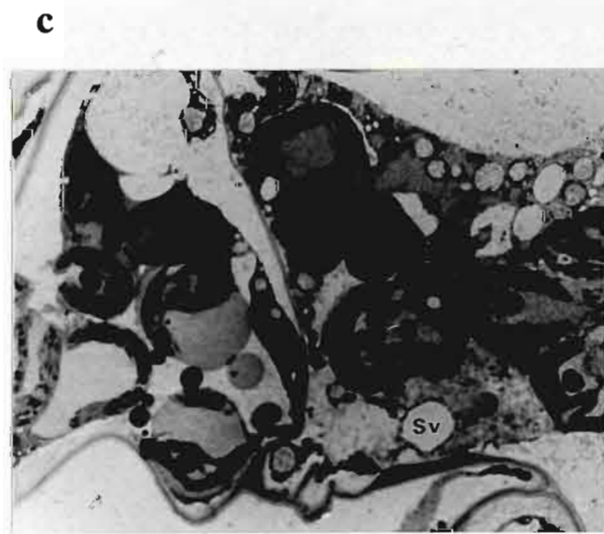
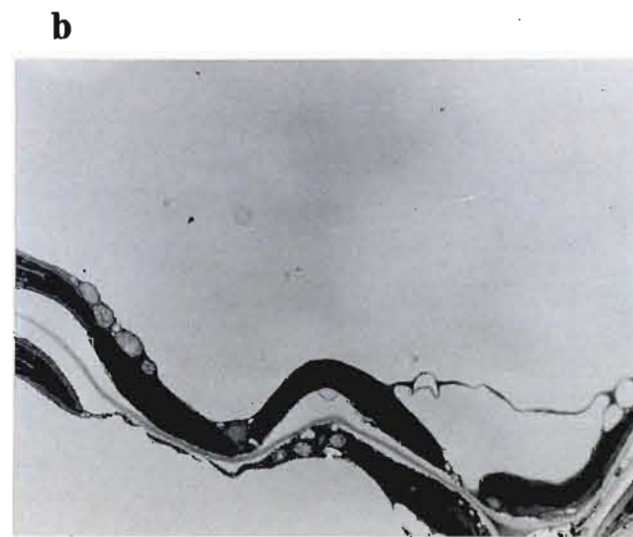
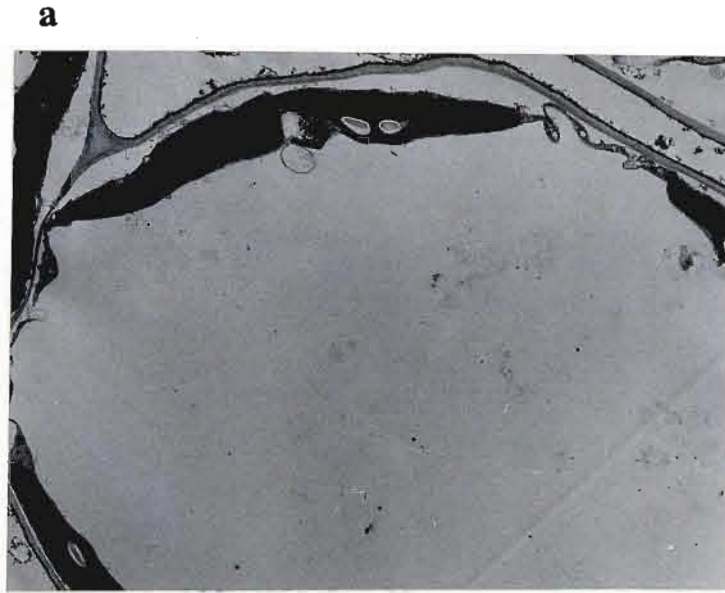
The ability of the leaves of this species to recover from severe desiccation was clearly visible at an ultrastructural level, although some damage during drying was similar to, but not as severe as, that of the sensitive species. No beading of membranes was observed at any stage in *C. nanum* which is interpreted as an ability to prevent membrane

deterioration. At 30% RWC the appearance of the cells was very similar to that at full turgor. In most sensitive species this was not the case as cell contents had started contracting inwards by this RWC. In *C. nanum*, however, the cells were smaller than at full turgor due to the folding of the cell wall but the large central vacuole and the peripheral location of the organelles was maintained.

The appearance of the cells at 5% RWC was very different from that of the sensitive species. The internal structure of the cells was intact but the contents were densely packed. The cell walls also collapsed inwards, far more so than in *P. sativum*, the only other species to show this phenomenon. Another difference noted between the tolerant and sensitive species was the formation of numerous small vacuoles when dried to 5% RWC in the tolerant species. The vacuoles of the sensitive species appeared simply to have broken down. The nuclei of *C. nanum* were also different in appearance from those of the sensitive plants.

**Table 5.6:** A summary of the observations made of the ultrastructure of leaves of *C. nanum* at full turgor, 30% RWC, rehydrated from 30% RWC, 5% RWC and rehydrated from 5% RWC. These observations were made on leaf material that was fixed by standard procedures. Where possible observations were quantified.

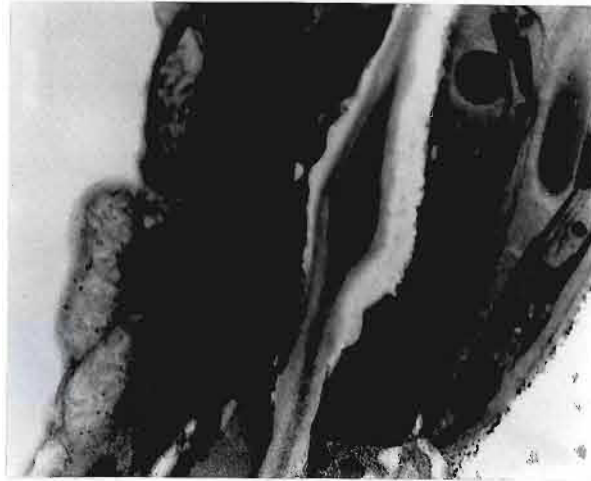
Organelle/structure	Full turgor	30% RWC	Rehydrated from 30% RWC	5% RWC	Rehydrated from 5% RWC
Plasmalemma	100% intact Flat against the cell wall	100% intact Slight contraction from cell wall	10% discontinuous Slight contraction from cell wall	50% discontinuous Contracted to centre of cell	10% discontinuous Slight contraction from cell wall
Chloroplast	Outer membranes intact Clear thylakoid stacks Elongated	10% outer membranes discontinuous Thylakoid membranes separated	Outer membranes intact Thylakoid membranes separated	70% outer membranes discontinuous Thylakoid membranes separated Rounded	20% outer membranes discontinuous Degenerated or reconstituting or newly forming thylakoids present
Mitochondria	Dense matrix Cristae present and clear Outer membranes intact	Similar to situation at full turgor but matrix less dense	Similar to state at full turgor	50% outer membranes discontinuous Few cristae Degenerating matrix	10% outer membranes discontinuous Cristae present
Nucleus	Membrane intact Nucleoplasm not very dense	Membrane intact Nucleoplasm more finely grained than at full turgor	Similar to state at 30% RWC	No nucleus-like structures resolved	Membrane intact Nucleoplasm less dense than at full turgor
Vacuole	Large central vacuole Tonoplast intact	Smaller than at full turgor but still central Tonoplast intact	Large central vacuole similar to situation at full turgor	A number of small vacuoles Tonoplast intact	Large central vacuole similar to state at full turgor



**Figure 5.30:** Transmission electron micrographs of the general cell appearance of leaves of *C. nanum* at full turgor (a, mag. x 4 000), 30% RWC (b, mag. x 4 000), 5% RWC (c, mag. x 3 250) and rehydrated from 5% RWC (d, mag. x 3 000). The leaves rehydrated from 30% RWC were similar in appearance to the leaves at full turgor. Dehydration to 5% RWC was accompanied by the formation of numerous small vacuoles, presumably by subdivision of the original large vacuoles. Leaves rehydrated from 5% RWC had regained their shape, although the organelles had not regained their original structure and peripheral location in the 22 hours they were given for rehydration.

Sv - small vacuole

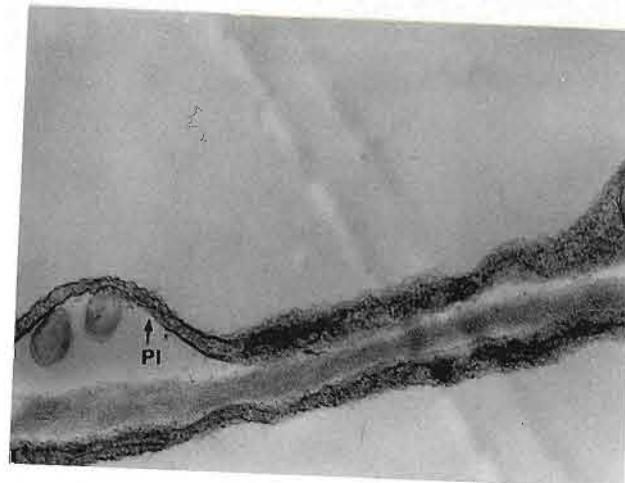
a



b

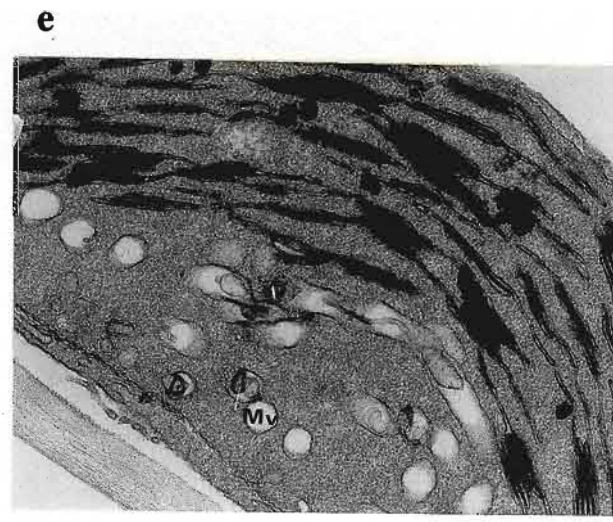
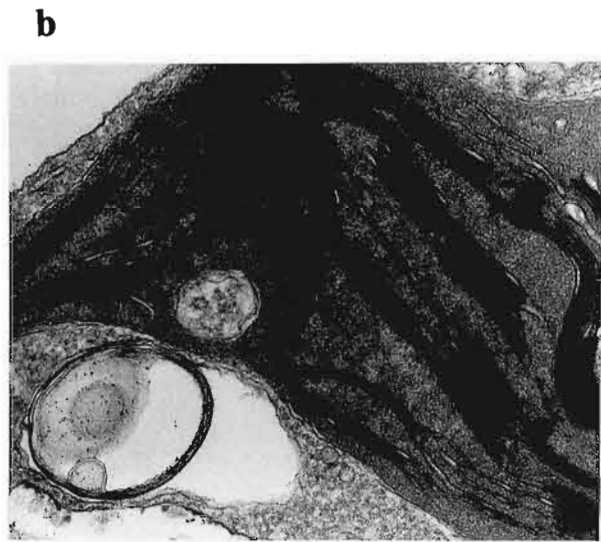
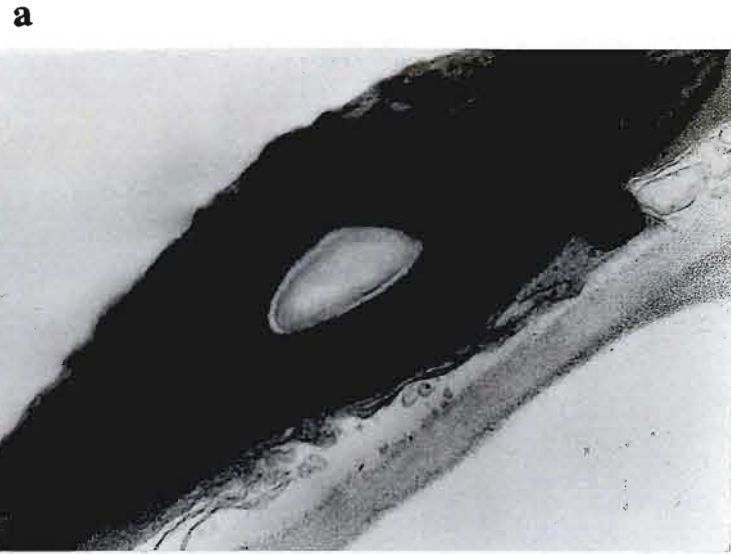


c

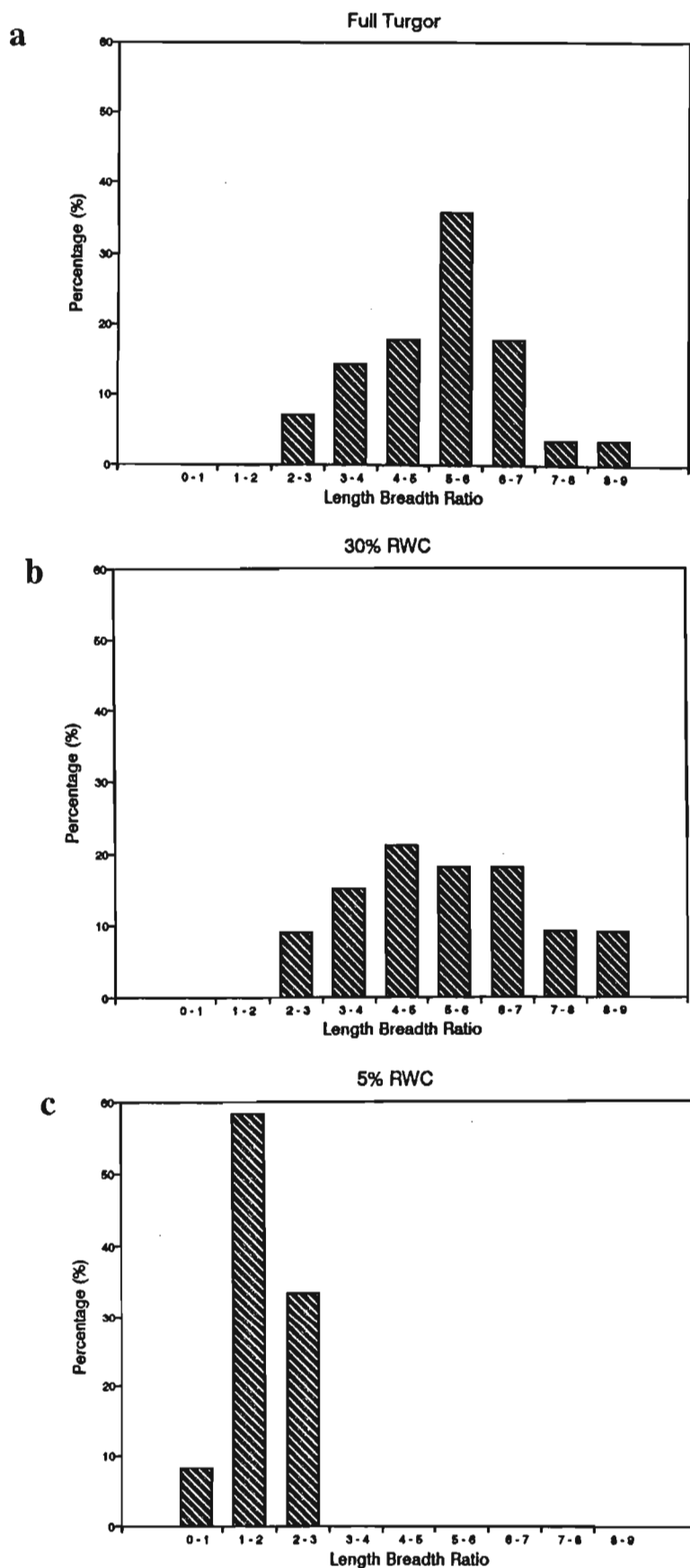


**Figure 5.31:** Transmission electron micrographs of the leaves of *C. nanum* dried to 30% RWC (a, mag. x 19 500), 5% RWC (b, mag. x 6 500) and rehydrated from 5% RWC (c, mag. x 26 000). Some contraction away from the cell wall was noted in the plasmalemma of leaves dried to 30% RWC, although this was very slight compared with the sensitive species. At 5% RWC cell contents were granular, large stretches of plasmalemma were continuous and the cell walls were folded inwards (similar to that shown in Figs. 5.3c and 5.8d). After rehydration from this RWC the plasmalemma was reconstituted and was again closely associated with the cell wall.

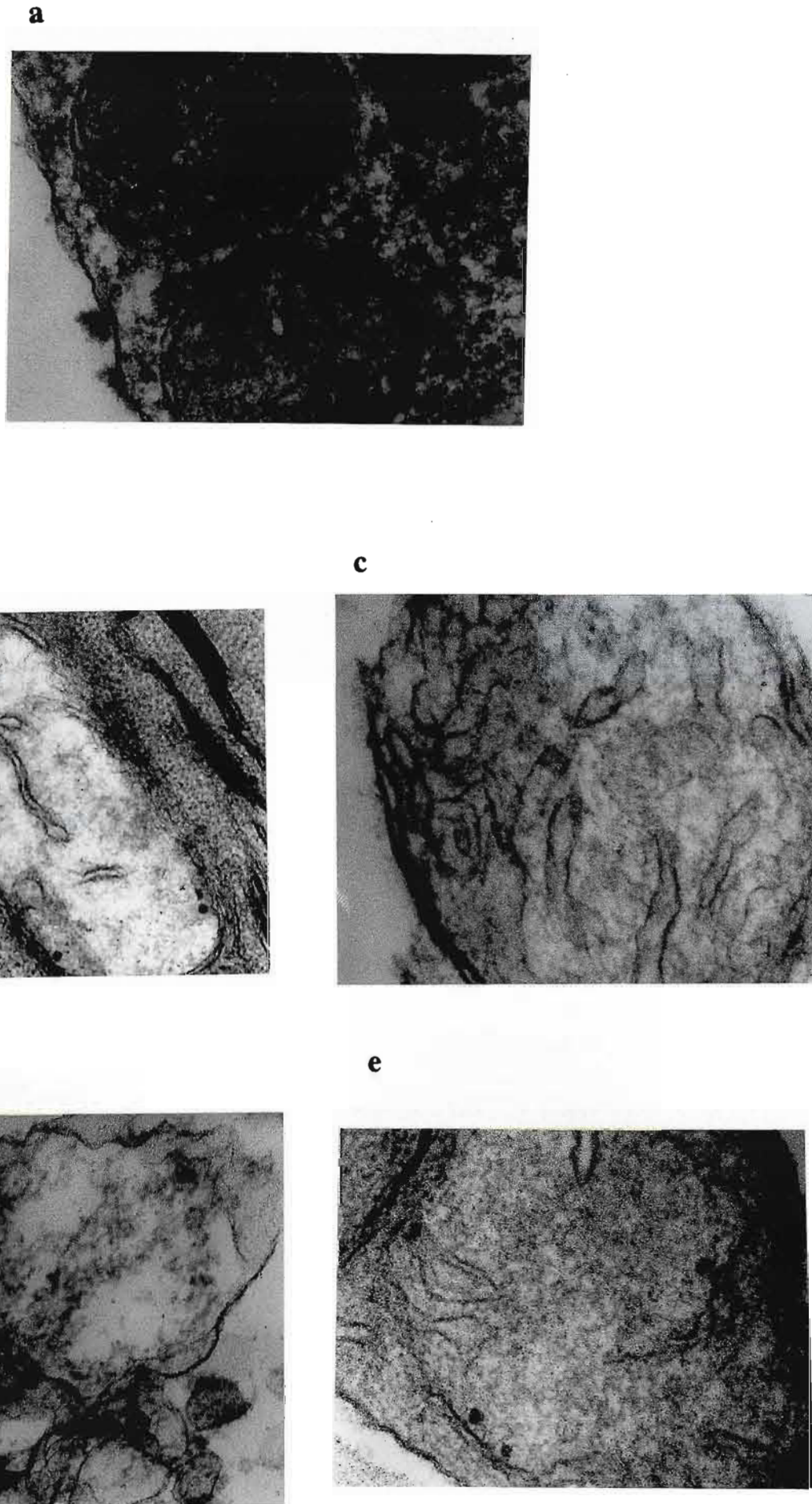
W - cell walls; PI - plasmalemma



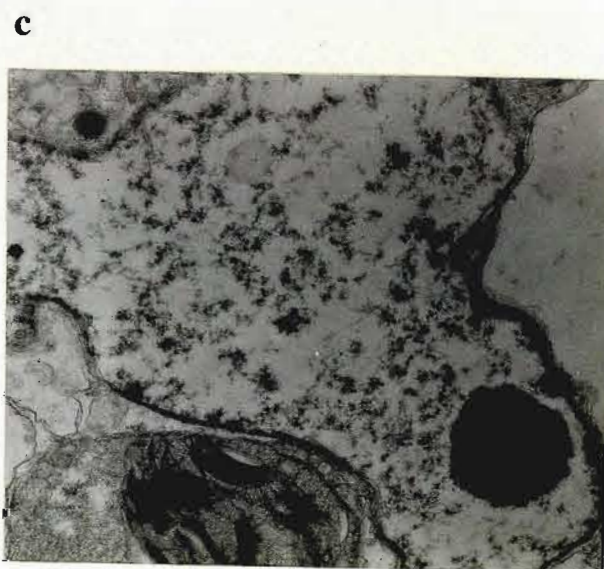
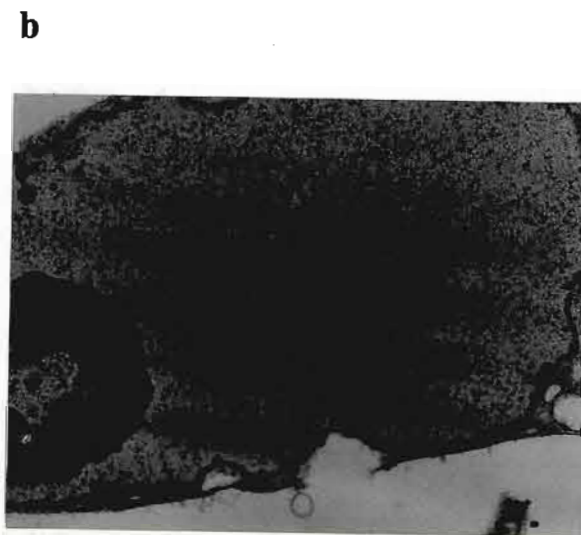
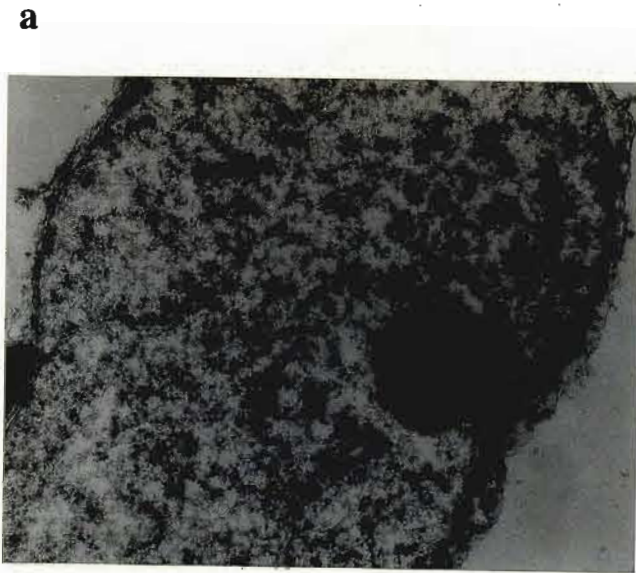
**Figure 5.32:** Transmission electron micrographs of the chloroplasts of *C. nanum* leaves at full turgor (a, mag. x 36 000), 30% RWC (b, mag. x 40 000), rehydrated from 30% RWC (c, mag. x 32 500), 5% RWC (d, mag. x 16 500) and rehydrated from 5% RWC (e, mag. x 32 500). There was some separation of apposing thylakoid membranes in material dried to 30% RWC which was still apparent in material rehydrated from 30% RWC. Chloroplast from leaves dried to 5% RWC appeared rounded with the thylakoids displaced to one side. The separation of thylakoid membranes was more severe at this RWC. After rehydration from 5% RWC the chloroplast had become more elongated and membrane bound vesicles were present, which could be degenerated or new or reconstituting thylakoids. Mv - membrane bound vesicle, possibly degenerated or new or reconstituting thylakoids



**Figure 5.33:** The chloroplast length:breath ratios of *C. nanum* leaves at full turgor (a) and dried to 30% RWC (b) and 5% RWC (c). Measurements were taken from thermal prints of material that had been fixed by standard means and sectioned for TEM.



**Figure 5.34:** Transmission electron micrographs of the mitochondria of *C. nanum* leaves at full turgor (a, mag. x 100 000), 30% RWC (b, mag. x 100 000), rehydrated from 30% RWC (c, mag. x 100 000), 5% RWC (d, mag. x 100 000) and rehydrated from 5% RWC (e, mag. x 100 000). The mitochondrial matrix became less dense on drying. The matrix of the mitochondria from the rehydrated leaves was denser than in the dehydrated state (both 30% and 5% RWC) but was not as dense as in the fully hydrated tissue. At 5% RWC the outer bounding membranes appear to be discontinuous in about 50% of mitochondria viewed per cell.



**Figure 5.35:** Transmission electron micrographs of the nucleus of *C. nanum* leaves at full turgor (a, mag. x 15 600), 30% RWC (b, mag. x 15 600) and rehydrated from 5% RWC (c, mag. x 13 000). As there was no difference in the nuclei of leaves dried to 30% RWC and those dried to 30% RWC and rehydrated Figure 5.35b represents both these treatments. The nucleoplasm was more finely grained in these treatments than it was at full turgor. There was no evidence of chromatin clumping. No nucleus-like structures were visible at 5% RWC, though in leaves rehydrated from this RWC, nuclei with rather dispersed nucleoplasm were present.

## Discussion

While the freeze-substitution protocols used in this study did not result in good preservation, the results achieved using freezing protocol 2 and substitution medium 2 with the thin fern leaves were promising. For this tissue, preservation was as good as, if not better than, that achieved using standard aqueous fixatives. Membrane integrity appeared to be retained better in the freeze-substituted samples than those fixed by standard means. This is possibly a result of slight imbibitional damage due to rehydration taking place in the tissues fixed by standard means. Öpik (1980) found that membrane integrity was maintained with anhydrous fixation but not so with aqueous fixatives and suggested that this was possibly due to stresses set up between the plasmalemma and the cell wall as the cells are rehydrated during fixation.

The results achieved for freeze-substitution in *C. nanum* were poor. This could have been due to the thickness of the leaf which may have resulted in ice-crystal damage and/or poor infiltration of both fixative and embedding resin. Internal detail was not clear and contrast was also very low. What freeze-substitution did show clearly was the folding of the cell walls which was also evident in standard aqueous-fixed material.

Although it is generally held that preparation by freeze-substitution is preferable, perhaps the imposition of stresses by aqueous fixatives more adequately reveals damage that has been accumulated, i.e. had there been no "weaknesses" then events such as membrane rupture would have been unlikely to have occurred. Therefore, although the results achieved using aqueously fixed material are treated with circumspection, because of rehydration, some interesting trends were observed, which may well reliably represent the consequences of the dehydration treatments. Damage occurring when leaves were dried to 30% RWC was exacerbated in *A. raddianum* and *P. sativum* whereas in *I. woodii* and *G. livingstonei* there was little difference in material dried to 30% RWC and that rehydrated from 30% RWC. Damage occurring on drying to 5% RWC, however, was further exacerbated on rehydration in all the sensitive species.

Studies of other desiccation-sensitive species subjected to water stress reveal trends similar

to those shown here. In sorghum, bean and maize leaves, subjected to severe dehydration, chloroplasts were shown to swell and membranes become discontinuous (Giles, *et al.*, 1974; 1976; Kurkova & Motorina, 1974). Increases in stromal space and thylakoid separation were also shown in maize (Kurkova & Motorina, 1974). In studies on desiccation-sensitive material it was found that as long as tonoplast integrity was maintained the cells survived on rehydration (Giles *et al.*, 1974; 1976). A similar observation was noted in this study. When damage was severe at 5% RWC and no tonoplast was visible rehydration resulted in further damage. This is not surprising, considering that vacuoles are the major lytic compartments of plant cells (Matile, 1975). Thus autolysis would invariably be associated with tonoplast discontinuity. In the desiccation-tolerant plant the vacuole became subdivided into a number of smaller vacuoles, thus retaining the tonoplast intact. This is probably a survival mechanism as tonoplast breakdown is associated with irreversible destruction of cell contents.

The extensive chromatin clumping seen in *A. raddianum*, *I. woodii* and *G. livingstonei* has also been shown to occur in the desiccation-tolerant plants *Talbotia elegans* (Hallam & Luff, 1980a) and *Xerophyta villosa* (Hallam & Gaff, 1978). This clumping, although not visible in *C. nanum*, does not, therefore, represent a phenomenon peculiar to sensitive species. It may also not be damage-related as chromatin naturally condenses, e.g. prior to mitosis. The critical feature regarding nuclear integrity, is that normal functioning of the nucleus is either retained during drying or regained on rehydration in the tolerant species, while in the sensitive species it is probably lost.

It was interesting to note that ultrastructurally visible damage in *G. livingstonei* was not as severe at 30% RWC as it was in other sensitive species, *A. raddianum* and *I. woodii* in particular, and that no further damage was noted to have occurred during rehydration of material dried to 30% RWC. According to the physiological studies (Chapter 4), *G. livingstonei* is the species most sensitive to rehydration damage and chlorophyll fluorescence parameters were affected at RWCs as high as 50% after rehydration. This damage could not, however, be ascribed to such ultrastructural changes as were visible with the standard fixation techniques but are suggested to reflect changes in membrane molecular architecture, chlorophyll and associated molecules. The thick cell walls of this

drought tolerant species are the basis for its high bulk modulus of elasticity (refer Chapter 2).

*Isoglossa woodii* showed no visible rehydration damage after dehydration to 30% RWC. This is similar to what was observed in the chlorophyll fluorescence studies (Chapter 4). There were no ultrastructural features (like glassy states) which related to the low melting enthalpy reported in Chapter 2.

Damage occurring during drying in the tolerant species *C. nanum* in terms of the deterioration of outer membranes and of inner detail of organelles was similar to that of the sensitive species. The membranes, however, never had the beaded appearance which was common amongst the sensitive species. It is possible that this beaded appearance and the formation of lipid droplets was caused by the breakdown of the phospho-lipid bilayer. If this is the case, the tolerant species must have some mechanism that prevents this. The tolerant species, as shown by the physiological studies, could repair damage accumulated during drying. During the time given the material to rehydrate, the chloroplasts and mitochondria had not assumed all the visible attributes of these organelles at full turgor. This could explain the chlorophyll fluorescence results (Chapter 4) which show marked recovery of fluorescence parameters but not to prior dehydration levels. The results obtained for *C. nanum* were similar to those obtained for *Craterostigma plantagineum* by Schneider, *et al.* (1993) who found similar disorganisation of the cell contents followed by recovery on rehydration, but not to full turgor levels in the eight hours of rehydration that these workers employed. Further studies need to be conducted to relate changes in ultrastructure to changes in physiological characteristics during rehydration, particularly to the early stages of rehydration.

The extensive infolding of the cell wall has also been found to occur in several desiccation-tolerant plants: *Xerophyta villosa* (Hallam & Luff, 1980b); *Craterostigma plantagineum* (Schneider, *et al.*, 1993) and *Myrothamnus flabellifolia* (Goldsworthy & Drennan, 1991). This ability would allow the cell to undergo relatively large volume changes without changing its water potential as the plasmalemma would still be associated with the wall. This cell wall folding is probably the cause of the unusual PV curves

reported in Chapter 2. It is interesting that walls in *P. sativum* also have the ability to fold, although folding to the extent that was observed in *C. nanum* was not evident in pea. *Pisum sativum* tissue does not, however, have the ability to recover from severe desiccation. While this infolding may prevent excessive stress building up between the cell wall and the plasmalemma during drying as the vacuoles shrink, it alone is probably not sufficient to enable recovery of the cell. Dehydration to 30% RWC did not result in the plasmalemma of pea becoming discontinuous as it did with some of the other sensitive species at this RWC. However, below 30% RWC, the plasmalemma became discontinuous in most cells despite cell wall folding.

In all sensitive species, damage at 5% RWC was extensive and was exacerbated on rehydration. The damage in *C. nanum*, in contrast, did not appear as severe at 5% RWC as was the case in the sensitive species. A greater percentage of mitochondrial and plastid outer membranes were intact and the tonoplast was intact even though the large central vacuole had subdivided into a number of small ones. Hallam and Luff (1980a; 1980b) showed that for the desiccation-tolerant plants, *Talbotia elegans* and *Xerophyta villosa*, vacuoles subdivided into a number of smaller ones and that the cytoplasm was densely granular. Schneider *et al.* (1993) state that the presence of LEA type proteins in the resurrection plant *Craterostigma plantagineum* could protect the cellular structure and facilitate repair once rehydrated. Sugars have also been implicated in the provision of protection to cell contents in the dry state. However, no single parameter has been unequivocally identified as the major factor conferring desiccation tolerance. Nevertheless, *C. nanum* must have some sort of protection mechanism as the appearance of cells at 5% RWC is far better than those of the sensitive material at the same RWC.

The retention of the outer membranes of the chloroplasts even though internal structure had broken down was observed in *Xerophyta villosa* (Hallam & Gaff, 1978). Similar spherical vesicles in the chloroplasts were shown to occur in *X. villosa*, which loses chlorophyll on dehydration. It was shown that these spherical vesicles swell, flatten out and attach themselves to the thylakoid system (Hallam & Gaff, 1978). This may be occurring in *C. nanum* and even though this species does not lose chlorophyll on drying, the mechanisms of chloroplast restitution may be similar. Hallam and Gaff (1978) also

found that, in *X. villosa*, mitochondria reformed cristae after rehydration, which is similar to the situation found for *C. nanum* in this study. Goldsworthy and Drennan (1991) found that, in *Myrothamnus flabellifolia* the space between the plasmalemma and the cell wall contained granular material as was found with *C. nanum*. The significance of this is not presently known.

Dehydration in *Myrothamnus flabellifolia* (Wellburn & Wellburn, 1976) does not appear to result in as severe damage as that noted in *C. nanum* and other desiccation-tolerant plants (Hallam & Gaff, 1878; Hallam & Luff, 1980a; 1980b; Hetherington *et al.*, 1982; Schneider *et al.*, 1993). This may explain the faster recovery rates of *M. flabellifolia* compared with other desiccation-tolerant plants (Wellburn & Wellburn, 1976).

### Conclusion

While it may have been preferable to fix the dry material with non-aqueous fixatives, the use of standard aqueous fixatives resulted in good preservation of material. The use of freeze-substitution as a non-aqueous fixation protocol has great potential if the problems of quick freezing and slow infiltration of thick specimens can be solved. The information obtained from aqueous fixation is, however useful in that even though partial rehydration of tissue may have occurred during aqueous fixation, any rehydration damage that may have occurred would have been the result of weakness caused by dehydration.

The ultrastructural studies have shown that similar damage occurs during drying and rehydration in all the sensitive species. The tolerant species does not show the same degree of derangement at an ultrastructural level during drying and also has the ability to reverse this damage upon rehydration. Rehydration times longer than 18 hours would be necessary for complete recovery. This is known from the data reported in Chapter 4 regarding the rehydration time course of *C. nanum*. The elasticity of the cell walls of *C. nanum* may be a component of the mechanism preventing excessive damage during dehydration. The inward folding of the walls could permit considerable volume changes while avoiding excessive stresses between plasmalemma and cell wall. The "beading" of membrane structures, observed in the desiccation-sensitive species is probably an indication of damage to the phospholipid component of the membranes. The fact that this was not

apparent in *C. nanum* suggests some membrane protection mechanisms.

Iljin (1957) suggested that plants with small cells and vacuoles, no plasmodesmata and easily deformed cell walls would enable cells to reduce mechanical stress associated with drying. Of these features *C. nanum* has only easily deformed cell walls. The cells and vacuoles were larger than those present in sensitive plant tissue. However, the dehydration response of subdivision of the vacuolar compartment could be taken as equivalent to the possession of small vacuoles, *per se*. Plasmodesmata were also clearly evident in *C. nanum* leaf tissue. While these features listed by Iljin (1957) may be important for plants to survive water stress, the ability to survive desiccation requires different and additional features.

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## CHAPTER 6: DETECTION OF DEHYDRIN PROTEINS IN DESICCATION-TOLERANT AND SENSITIVE VEGETATIVE PLANT TISSUE

### Introduction

Upon exposure to stresses, such as heat, cold, toxic metals and desiccation, organisms from bacteria to higher plants and mammals respond by producing a range of stress proteins (Welch, 1993). The most widely studied of these proteins in plants are the so-called heat-shock proteins (reviewed by Harlt, Hlodan and Langer, 1994; Lindquist, 1986; Vierling, 1991). Drought and desiccation stress have also been shown to promote the production of a number of proteins in response to the stress (reviewed by Bewley & Oliver, 1992; Skriver & Mundy, 1990; Vertucci & Farrant, 1995). These proteins and their genes were first identified in the maturing and desiccation-tolerant phase of seed embryos (Baker, Steele & Dure, 1988) and are often called LEAs (Late Embryogenesis Abundant).

Messenger RNAs for LEAs and LEA-homologues (dehydrins [Close, Kortt & Chandler, 1989]) have since been found to be induced by water stress and ABA (which is known to play an important role in water stress) in other (non-seed) tissues of many species, for example, in cyanobacteria (Close & Lammers, 1993), in some angiosperm seedlings (Bradford & Chandler, 1992; Hong, Barg & Ho, 1992; Ried & Walker-Simmons, 1993) and in the leaves and callus tissue of the desiccation-tolerant plant *Craterostigma plantagineum* (Bartels *et al.*, 1990; Piatkowski *et al.*, 1990). The presence of LEAs and LEA homologues in desiccation-tolerant tissues and their induction upon drying has led to the belief that these proteins play an important role in desiccation tolerance. Their specific function, however, has not been elucidated. Functions for these proteins have been predicted from their deduced amino acid sequences (Bray, 1993). These functions include the protection of membranes and other cellular constituents (Close *et al.*, 1989; Dure *et al.*, 1989), the sequestration of ions (Dure, 1993) and the renaturation of unfolded proteins (Dure, 1993). Although LEAs might fulfil these functions under severe stress conditions it is not known to what extent, or what function they have, under mild stress conditions

(Bray, 1993).

Farrant, Berjak and Pammenter (1992) have shown no changes in patterns of protein synthesis during late stages of development, and deduced therefore that LEAs were presumably not present, for the highly desiccation-sensitive seeds of *Avicennia marina*. These authors suggested that the lack of desiccation tolerance may be due to an absence of these proteins. However dehydrin homologues have been demonstrated in the desiccation-sensitive seeds of some temperate recalcitrant species (Finch-Savage, Pramanik & Bewley, 1994). Blackman and co-workers (1991) have shown that the presence of LEAs alone are not sufficient to confer tolerance in desiccation-tolerant soybean seeds. This conclusion has been borne out by the work of Iturriaga *et al.* (1992) who found that transgenic tobacco plants, having genes for LEA-type proteins, did not become any more tolerant to drying than the wild-type. It has been suggested that such proteins form only part of a more complex mechanism of desiccation tolerance (Vertucci & Farrant, 1995).

Dehydrins (also referred to as LEA D-11 [Close *et al.*, 1989]) are a family of proteins that accumulate in plants in response to desiccation, regardless of whether the stimulation is evaporation, chilling or a decrease in external osmotic potential (Skriver & Mundy, 1990). They are characterised by the consensus amino acid sequence domain EKKGIMDKIKEKLPG found at, or near, the carboxy terminus; the core of this domain (KIKEKLPG) may be repeated from one to many times within the complete polypeptide (Close, Fenton & Moonan, 1993). Close and co-workers (1993) have made an antibody specific to the KIKEKLPG core sequence. This antibody has been used to test for the presence of dehydrin-like proteins in a wide range of plant species (Bewley, Reynolds & Oliver, 1993; Close *et al.*, 1993; Finch-Savage *et al.*, 1994; Gee, Probert & Coomber, 1994).

As dehydrins appear to play some role in desiccation tolerance it was decided to determine if similar proteins were present, or could be induced on drying, in the five species used in this study. Leaf material at full turgor, 30% and 5% RWC was used as these appear, from the physiological studies, to be important RWCs. The characteristic of heat stability was used to separate dehydrin-like proteins from total soluble proteins (Blackman, *et al.*,

1991; Ried & Walker-Simmons, 1990). Dehydrin-homologues were identified by use of the KIKEKLPG consensus sequence antibody developed by Close *et al.* (1993).

## Materials and Methods

Leaves of the five plant species were flash dried to 30% and 5% RWC. Leaves at these two RWCs and at full turgor were used for this part of the study. The leaf material was frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further processing.

### Protein extraction

Extractions were done in triplicate on unpooled samples. Frozen leaf material (0.1 g) was ground to a fine powder and the proteins were extracted in 500  $\mu\text{l}$  of 10 mM Tris buffer (pH 7.0) containing 2.5% Triton-X and 0.7 M sucrose. An equal volume of water-saturated phenol was added and the mixture was incubated on ice for 10 minutes. Phenol extraction has been shown to facilitate the extraction of membrane associated proteins (Hurkman & Tanaka, 1986). The homogenate was centrifuged at 14 000 g for 20 min at  $4^{\circ}\text{C}$ . The proteins were precipitated from the phenol phase by addition of five times the volume of 0.1 M ammonium acetate in methanol at  $-20^{\circ}\text{C}$  overnight. The pellet obtained after centrifugation, was washed five times with cold acetone and dried under vacuum. The protein pellets were redissolved in water and separated into two equal portions, one of which was heated at  $80^{\circ}\text{C}$  for 10 min to obtain heat-stable proteins. Denatured proteins were removed by centrifugation. The other served as a non-heated control. Equal volumes of SDS buffer (50 mM Tris-HCl [pH 6.8], 20% glycerol, 10% 2-mercaptoethanol and 4% SDS) were added to the supernatant of both samples. These were heated for 2 min at  $100^{\circ}\text{C}$ . Equal volumes of supernatant from each treatment were separated by 1-D SDS PAGE (Laemmli, 1970). Electrophoresis was performed in duplicate. One of the gels was stained with Coomassie brilliant blue (0.25% in 50% methanol/10% acetic acid, destained with 10% acetic acid 5% methanol) and the other used for Western-blotting. Each of these treatments was repeated in triplicate.

### Western-blot analysis

Following electrophoresis, proteins were electroblotted onto a 0.45  $\mu\text{m}$  pore-size

nitrocellulose membrane by standard methods (Harlow & Lane, 1988). Air-dried membranes were incubated for 30 min at room temperature in phosphate-buffered-saline (PBS) pH 7.4: containing 50 mM phosphate, 150 mM NaCl and 5% fat-free milk. Primary antibody (donated in the concentrated form to J.M. Farrant<sup>4</sup> by T.J. Close<sup>5</sup>) was added to this solution to give a dilution of 1:1000. Incubation was for 2 hours at room temperature. Membranes were then washed 3 times with PBS buffer containing 0.05% Tween 20. The membrane was further incubated for 1 hour at room temperature in PBS containing the secondary antibody diluted to 1:500 (goat anti-rabbit coupled to alkaline phosphatase). Membranes were washed as described above with the addition of a fourth wash in 10 mM Tris-HCl (pH 7.4) with 150 mM NaCl. The blot was finally incubated in a 0.1 M Tris buffer (pH 9.5) containing 100 mM NaCl and 100 mM MgCl<sub>2</sub>. For signal development 0.05% nitroblue tetrazolium (NBT) in 70% di-methyl-fluoride (DMF) and 0.05% 5-bromo-4-chloro-3-indoyl phosphate (BCIP) in 100% DMF were added. After correct colour development membranes were air-dried and photographed.

As a control for the Western blotting methodology, protein extracts of pea, maize and barley seed material were treated in the protocols outlined above. These seeds are known to have dehydrin-like proteins (Close *et al.*, 1993; Russouw, 1993).

## Results

Figure 6.1 shows the Western-blot obtained from a separation of pea, maize and barley seed proteins. The antibody clearly detected dehydrins in these tissues indicating that the antibody dilutions and detection protocols used in the present study were appropriate. Figures 6.2 - 6.6 show the Western-blots and stained gels for *A. raddianum*, *P. sativum*, *I. woodii*, *G. livingstonei* and *C. nanum*.

In *A. raddianum*, *I. woodii* and *G. livingstonei* there were no proteins present that were immunologically detected by the antibody used in the present study (Figs. 6.2a, 6.4a &

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6.5a). This was regardless of the degree of desiccation of the leaves. Coomassie stained gels of *A. raddianum* (Fig. 6.2b) and *G. livingstonei* (Fig. 6.5b) showed that there was no change in banding patterns or protein intensities (in either the heat-stable or control separations) as the leaf tissues were dried. Thus, although these species were killed by desiccation, there was no apparent breakdown of proteins by protease action. In *I. woodii*, drying to 30% RWC showed an increase in protein staining intensity (Fig. 6.4b). This could be a concentration effect or be due to intensified protein metabolism. Further drying to 5% RWC, however, resulted in virtually complete dissolution of leaf proteins. Only a few low molecular weight bands were visible as discrete entities, these probably being a result of breakdown of larger proteins. The "smear-like" appearance of the protein separation from severely desiccated leaves (lane 6 in Fig. 6.4b) is typical of that obtained when proteins have been progressively degraded by proteases. Plant tissues contain high levels of proteases and it is possible that dehydration of leaves of *I. woodii* causes release of such proteases which ultimately results in autolysis of subcellular proteins. The other two sensitive species do not show this pattern of gel separation and presumably their proteases must in some way be contained during drying.

Both *P. sativum* and *C. nanum* had proteins with antigenic sites which were detected by the antibody (Figs. 6.3a & 6.6a respectively). In *P. sativum*, five dehydrin homologues (labelled ps1 - 5, Fig. 6.3a) were present. Protein bands ps1 and ps2 had molecular weights of between 55 and 66 kDa. These were present in hydrated leaves and leaves dried to 30% RWC. They were also present at 5% RWC, but were very faint in the gels. Protein bands ps3 - 5 (with molecular weights slightly lower than 30 kDa) were not present at full turgor but were present in dried leaves. There was no apparent difference in band intensity between the 30% and 5% RWC.

The significance in the decline of bands ps1 and ps2 at lower RWCs was not clear. There are three possible reasons for this decline 1) proteins were denatured and the antibody no longer recognised them; 2) they had broken down into smaller subunits and 3) they had declined to such low levels that they were no longer detectable by the antibody. These proteins did decline in intensity in the stained gels (Fig. 6.3b) but there was no concomitant increase in intensity of smaller subunits: thus they are unlikely to have broken

down. Furthermore their degree of staining indicated that there was still sufficient quantity present to have been detectable by the antibody should they have retained their antigenicity.

The appearance of bands ps3 - 5 on drying is of interest in the light of current hypotheses. Following the argument outlined above, it is unlikely that these bands appear as a consequence of the breakdown of the immunogenic bands ps1 and ps2. Stained gels showed that these bands also declined on drying. Furthermore protein bands ps3 - 5 are not newly synthesised on drying: stained gels show them to be present even at full turgor. This can be interpreted as an increase in antigenicity of these proteins with drying. This could occur if drying induces some conformational change in the protein so it exposes more antigenic sites. Dehydrins have been hypothesised to sequester ions during drying which might minimise their toxicity in the subcellular milieu (Dure, 1993). This process may bring about a conformational change in the protein as ions are accumulated and in so doing expose more antigenic sites.

Stained gels showed a general decline in protein banding intensity with drying of the leaf material. Unlike the gels from preparations of *I. woodii*, there was no gel graininess indicative of protein lysis.

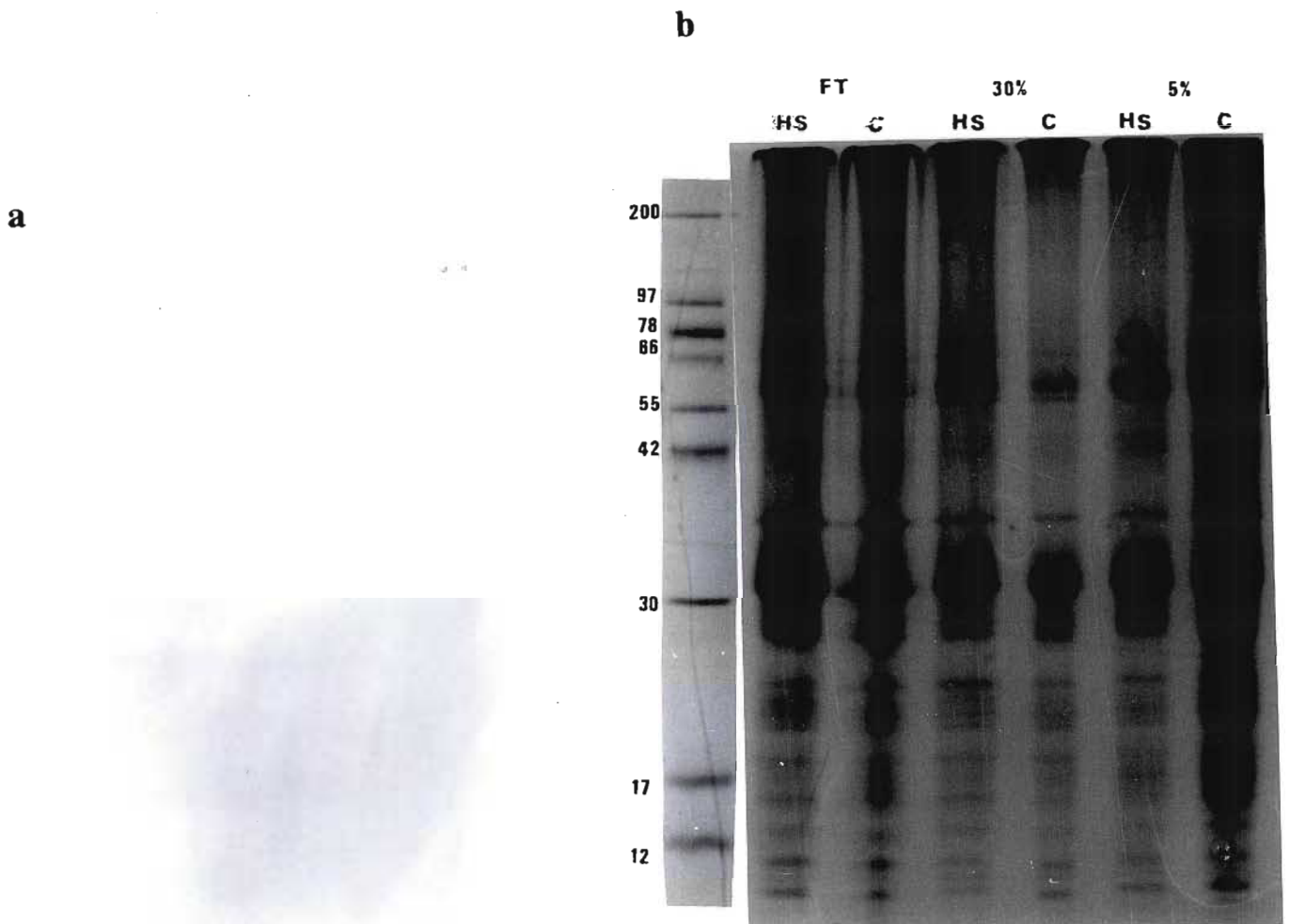
In the desiccation-tolerant plant, *C. nanum*, two dehydrin-homologues became apparent when the tissues were dried to 5% RWC (Fig. 6.6a). The protein band labelled cn1 had a molecular weight of between 55 and 66 kDa, similar to proteins bands ps1 and ps2 in *P. sativum*. Protein band labelled cn2 had a molecular weight of about 30 kDa, slightly higher than that of protein bands ps3 - 5 of *P. sativum*. Coomassie blue stained gels (Fig. 6.6b) showed that, as with *P. sativum*, these proteins were present at full turgor and were thus not newly synthesised on drying. Unlike the situation of *P. sativum*, stained gels showed that these proteins increased in intensity with drying. Thus the detection at 5% RWC could be due to increased levels of these proteins and/or an increase in antigenicity. Furthermore, other proteins (not detected by immunological techniques) also increased in intensity when leaf tissues were dried. This could be due to ongoing protein metabolism, occurring even at 5% RWC, and/or a concentration effect, as more protein is present in

0.1 g of dry tissue than tissue at full turgor. This is in contrast to the gels of *A. raddianum* and *G. livingstonei* which showed no change in band intensity and *I. woodii* which, although staining intensity was enhanced at 30% RWC, showed protein breakdown at 5% RWC.

Figures 6.3c & 6.6c show the pre-immune blots for *P. sativum* and *C. nanum*. No proteins were detected with pre-immune serum, indicating that the bands detected by the immune serum were real. The only difference between the heat stable protein and the control protein lanes was one of staining intensity, with the control lane being more intense than the heat stable lane. There did not appear to be any qualitative differences in the proteins detected.



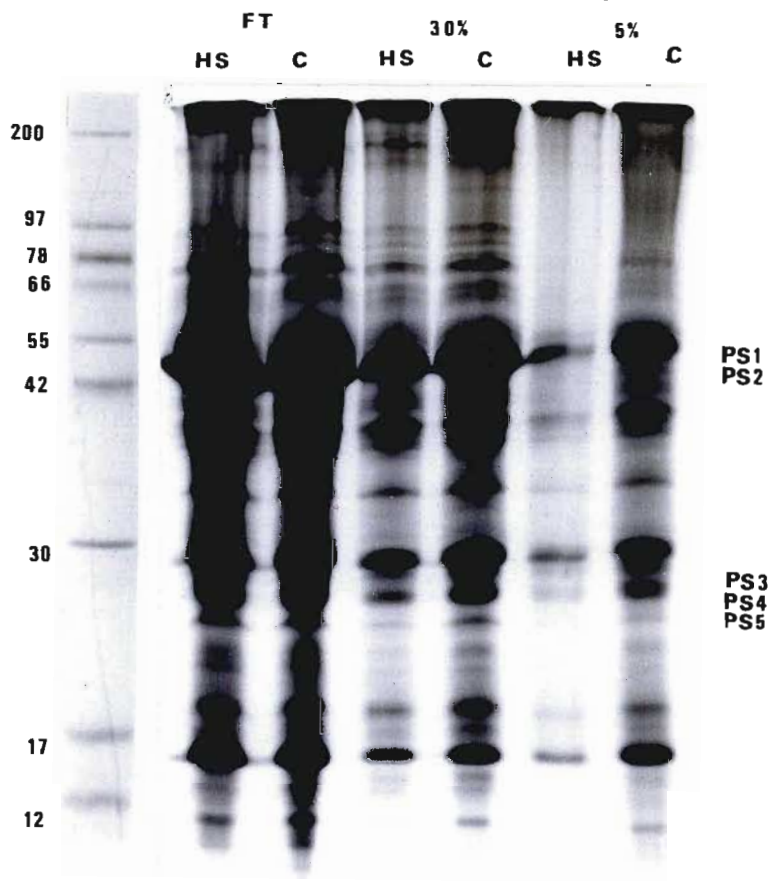
**Figure 6.1:** Western-blot of seed material of barley (B), maize (M) and pea axes (Pa) and pea cotyledon (Pc).



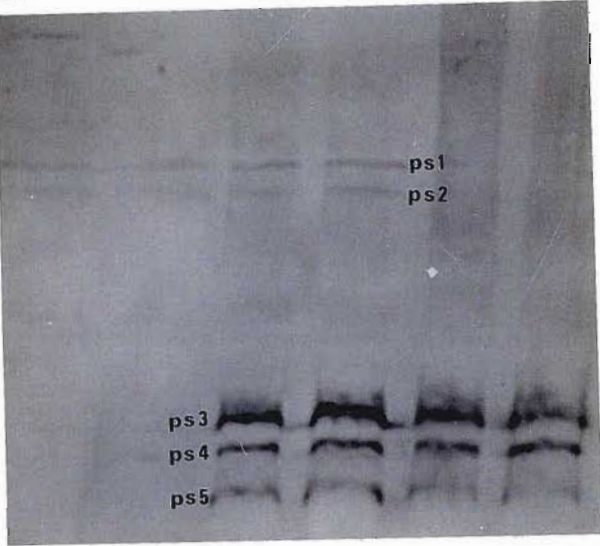
**Figure 6.2:** The Western-blot (a) and Coomassie blue stained gel (b) of proteins of *A. raddianum*. The Western-blot shows no antigenic protein bands. The labelled lanes in b refer to protein extractions from leaves at full turgor (FT) and leaves dried to 30% RWC (30%) and 5% RWC (5%). Both a heat stable sample (labelled HS) as well as a control sample (C) were run. Molecular weight markers were run on the same gel as the protein extractions and are shown in the first lane. Due to the low staining intensity they were printed separately.

**Figure 6.3:** The Western-blot (a) and the 1-D Coomassie blue stained gel (b) of *P. sativum*. The Western-blot shows a number of antigenic protein bands. The labelled lanes in a and b refer to protein extractions of leaves at full turgor (FT) and leaves dried to 30% RWC (30%) and 5% RWC (5%). Both a heat stable sample (labelled HS) as well as a control sample (C) were run. Molecular weight markers were run with the gel and are shown in the first lane. Due to the low staining intensity they were printed separately. Protein bands labelled ps1 - 5 are the bands corresponding to the same labelled protein bands shown in the Western-blot (a). The pre-immune blot (c) shows no protein bands were picked up by the pre-immune serum.

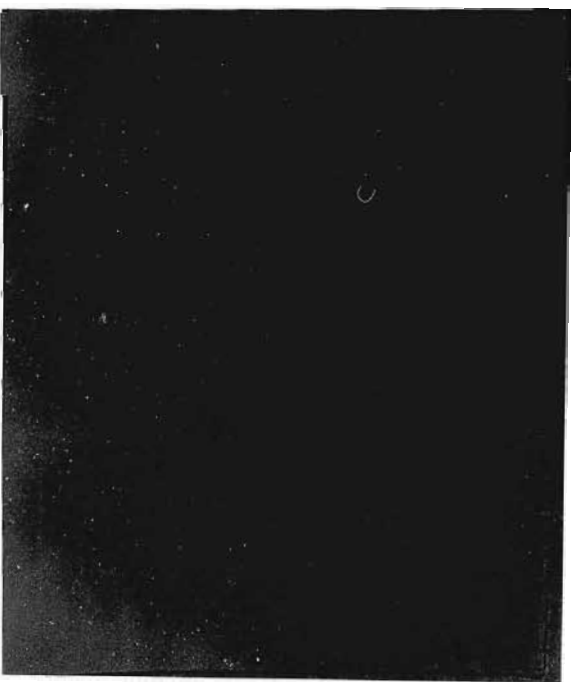
b

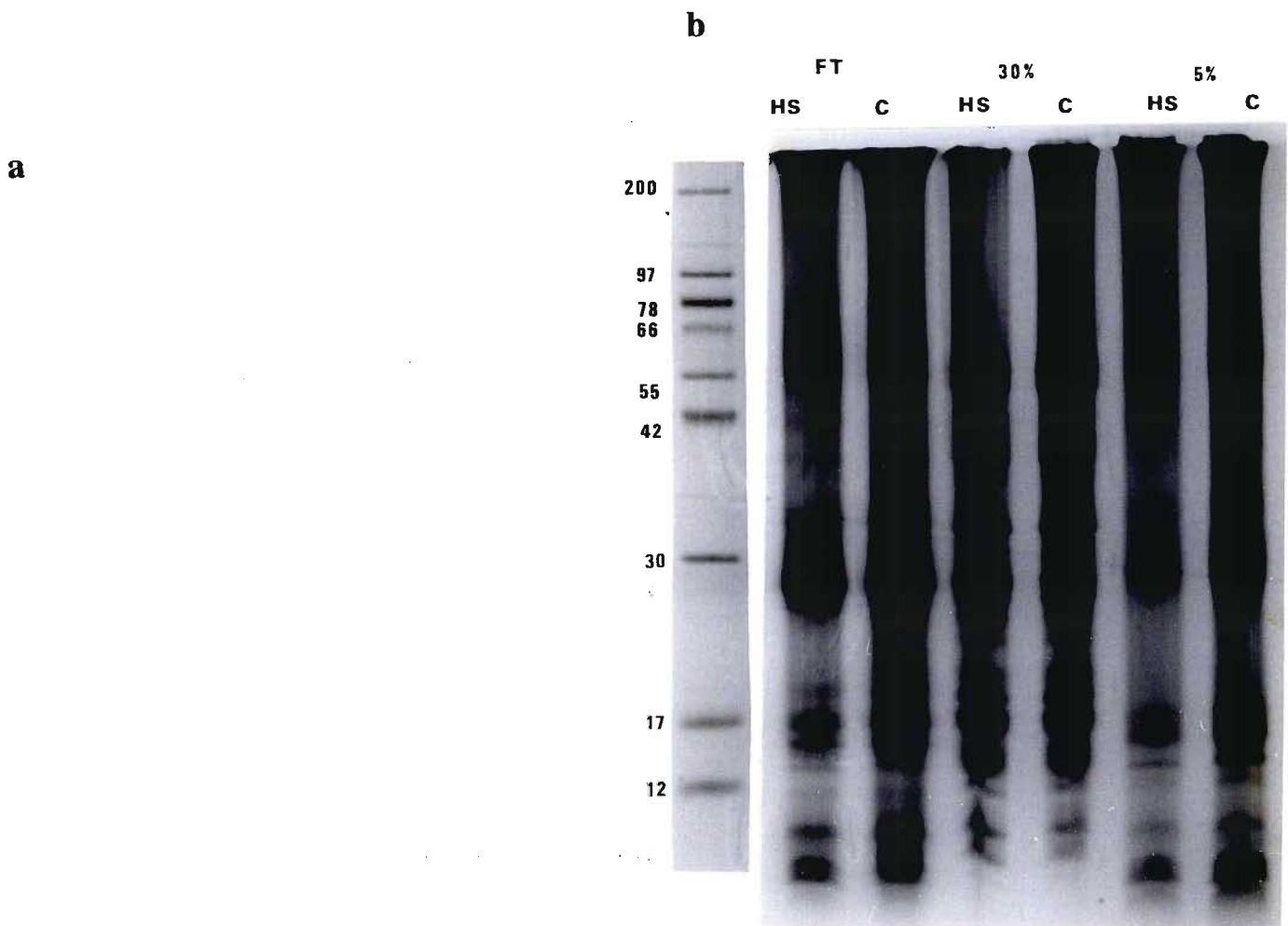


a



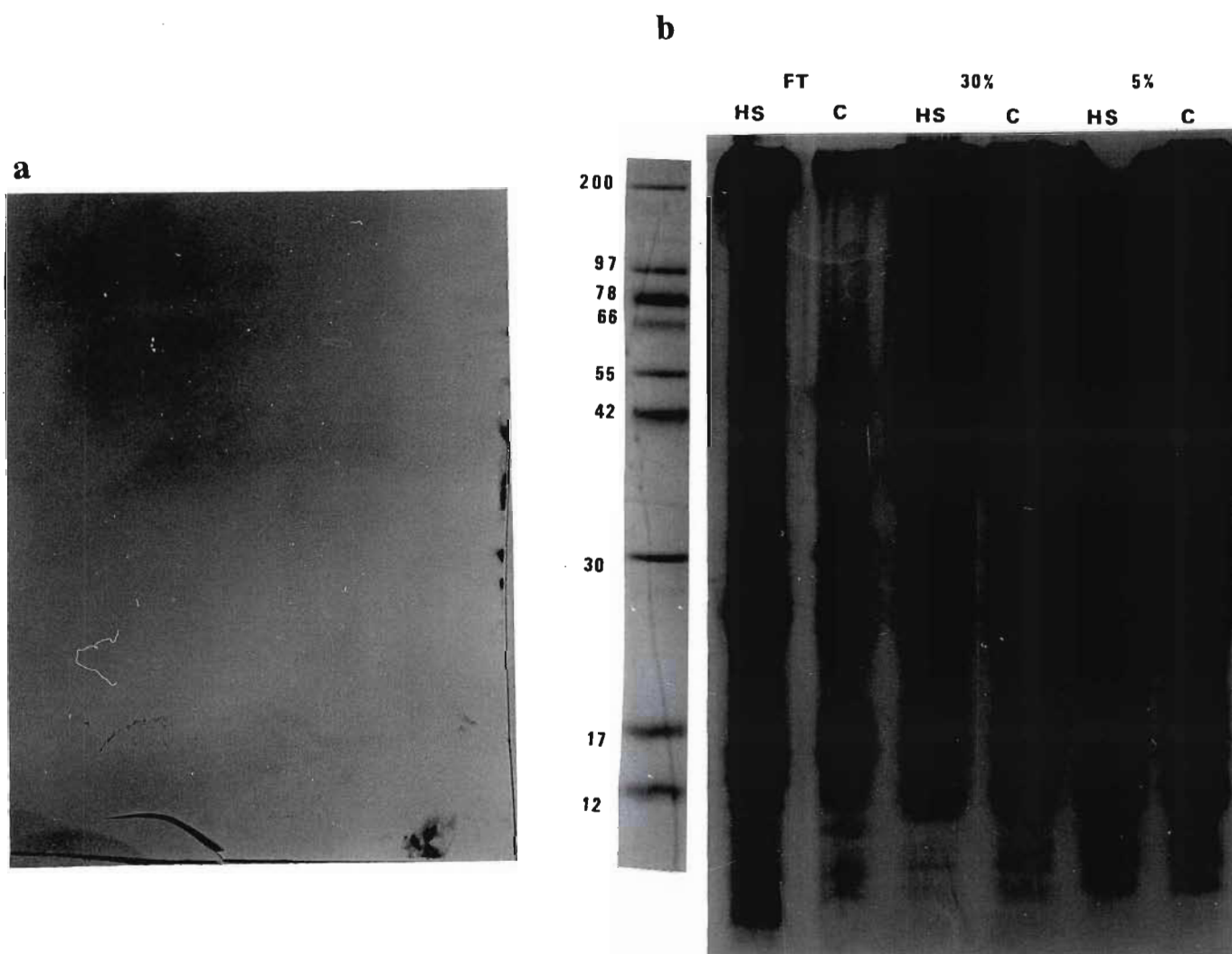
c





**Figure 6.4:** The Western-blot (a) and the 1-D Coomassie blue stained gel (b) of *I. woodii*.

The Western-blot shows no antigenic protein bands. The labelled lanes in b refer to protein extractions of leaves at full turgor (FT) and leaves dried to 30% RWC (30%) and 5% RWC (5%). Both a heat stable sample (labelled HS) as well as a control sample (C) were run. Molecular weight markers were run with the gel and are shown in the first lane. Due to the low staining intensity they were printed separately.

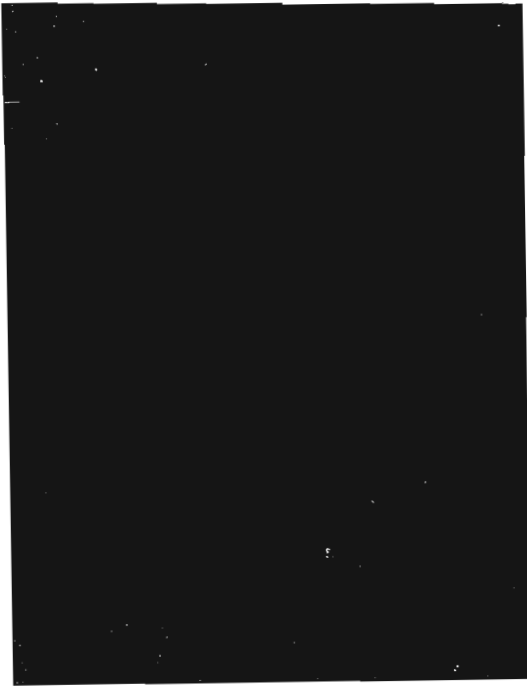


**Figure 6.5:** The Western-blot (a) and the 1-D Coomassie blue stained gel (b) of *G. livingstonei*. The Western-blot showed no antigenic protein bands. The labelled lanes in 6.5b refer to protein extractions of leaves at full turgor (FT) and leaves dried to 30% RWC (30%) and 5% RWC (5%). Both a heat stable sample (labelled HS) as well as a control sample (C) were run. Molecular weight markers were run with the gel and are shown in the first lane. Due to the low staining intensity they were printed separately.

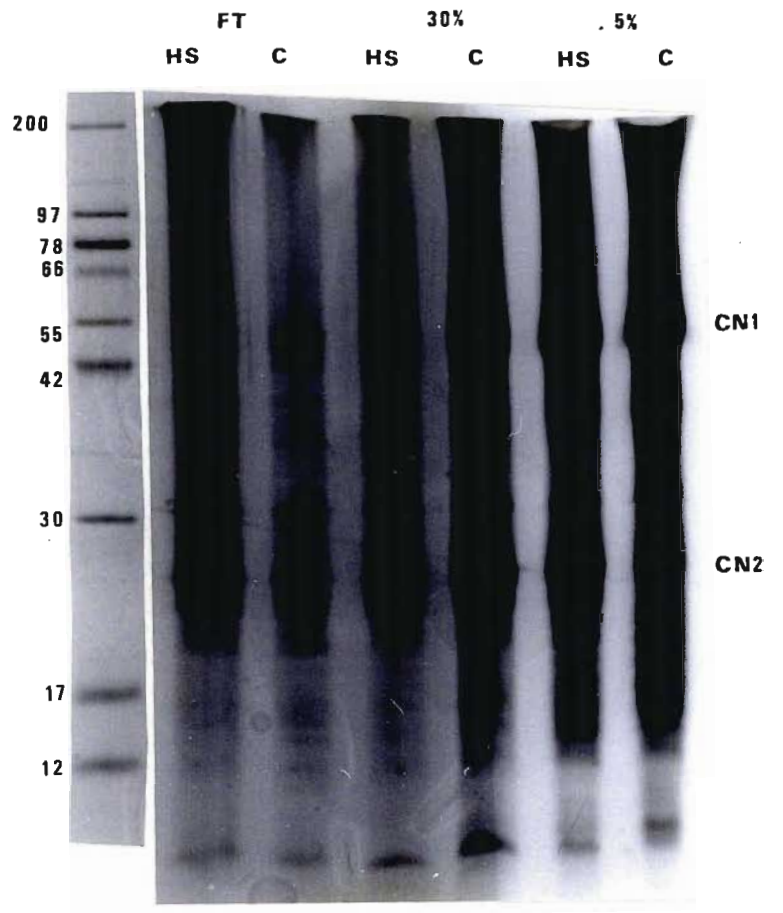
**Figure 6.6:** The Western-blot (a) and the 1-D Coomassie blue stained gel (b) of *C. nanum*.

The Western-blot shows two antigenic protein bands. The labelled lanes in a and b refer to protein extractions of leaves at full turgor (FT) and leaves dried to 30% RWC (30%) and 5% RWC (5%). Both a heat stable sample (labelled HS) as well as a control sample (C) were run. Molecular weight markers were run with the gel and are shown in the first lane. Due to the low staining intensity they were printed separately. Protein bands labelled cn1 and cn2 are the bands corresponding to the same labelled protein bands shown in the Western-blot (a). The pre-immune blot (c) shows no protein bands were picked up by the pre-immune serum.

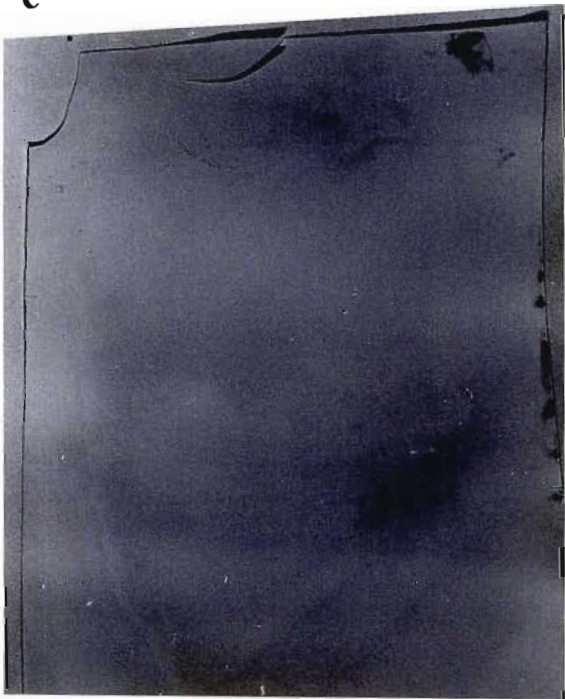
**a**



**b**



**c**



## Discussion

The results show that only *P. sativum* and *C. nanum* possessed proteins which were homologs of dehydrins. Of these two species only *C. nanum* is desiccation tolerant. Dehydrin-type proteins have been shown to be present in another species of *Craterostigma*, viz. *C. plantagineum* (Piatkowski, *et al.* 1990). The presence of dehydrins in *C. nanum* is not surprising in terms of current hypotheses on the role of these proteins in desiccation tolerance. It is uncertain what the precise role of these proteins would be, particularly as *C. nanum* behaves in a manner similar to the sensitive species in terms of its physiology during drying. At an ultrastructural level, however, there does appear to be fairly good preservation of organelle structure during drying in *C. nanum*, and the dehydrins may play a role in protecting the cell contents as suggested by Dure (1993). There are also quantitative changes in other proteins during drying which are not detected by the antigen. These proteins may also play a role in desiccation tolerance.

It is interesting to note that these dehydrin-type proteins are not newly synthesised on drying. They are present at full turgor but increase in quantity during drying. Alternatively drying could induce conformational changes of the protein which facilitate antibody recognition of it. Bewley *et al.* (1993) in their work on the desiccation-tolerant moss, *Tortula ruralis*, found that dehydrins were also present when the moss was in the hydrated state. This is in contrast to work on wild rice (Bradford & Chandler, 1992) and other species (*Craterostigma plantagineum*, Bartels *et al.*, 1990; barley, Close *et al.*, 1989; and wheat, Ried & Walker-Simmons, 1993) which suggest that the dehydrin-type proteins are newly synthesised on drying.

From the chlorophyll fluorescence and respiration studies (reported in Chapter 4) it appears that the ability to recover from desiccation damage appears to be important for *C. nanum*. From the ultrastructural studies (Chapter 5) it appears that *C. nanum* can limit ultrastructurally visible damage during drying. The tonoplast was retained intact and the plasmalemma and other internal membranes were never beaded in appearance which could indicate that the membranes of this plant were protected. It is possible that the dehydrins, and some of the other proteins that are produced, could play some role in the ability of

*C. nanum* both to limit damage during dehydration and to repair any damage on rehydration. There is obviously more than dehydrins involved in desiccation tolerance as their presence does not confer similar abilities on desiccation-sensitive *P. sativum*. This is in accord with the view of Blackman *et al.* (1991) who also suggest that the presence of dehydrins is not in itself sufficient to confer desiccation tolerance.

The presence of these proteins in *P. sativum* is interesting, especially since *P. sativum* shows very similar physiological and ultrastructural characteristics to the other sensitive species and has a lethal RWC similar to the others (around 30% RWC). The fact that seedlings of *P. sativum* were used could account for the presence of these dehydrin-type proteins. Other workers have shown that seedlings of some crop plants have these proteins: *Zizania palustris* or wild rice (Bradford & Chandler, 1992), barley (Hong *et al.*, 1992) and wheat (Ried & Walker-Simmons, 1993). The seedlings used in all those studies were a lot younger than the *P. sativum* seedlings used here (approximately 4 days as opposed to 14 days). Wheat seedlings have been shown to be desiccation tolerant for up to 4 days (Ried & Walker-Simmons, 1993), while pea seedlings are not (personal observations). The other work done on seedlings (Bradford & Chandler, 1992; Hong *et al.*, 1992) did not indicate if the seedlings survived desiccation. *Pisum sativum* is a commercial crop plant and is highly bred for protein content for the diet of both humans and other animals. It is possible that dehydrin type proteins have been unselectively bred in.

Most dehydrins reported in the literature to be associated with desiccation seem to be in the low molecular weight range of ca. 20 kDa and 60 kDa. For example, the dehydrin-type band identified by Bradford and Chandler (1992) in wild rice corresponded to a molecular weights of 20 kDa; Finch-Savage *et al.* (1994) found bands of 20, 32 and 56 kDa, among others, in seeds of recalcitrant species, using the same antibody; dehydrins of similar molecular weight were also found in the desiccation-tolerant moss *Tortula ruralis* (Bewley *et al.*, 1993) using the Close antibody. These bands are of similar molecular weights to those shown in both *P. sativum* and *C. nanum*.

While only a limited amount of qualitative and quantitative information on protein content

can be obtained from 1-D gels, it is interesting to note some differences among the species studied. The increase in band intensity with drying in leaves of *C. nanum* (Fig. 6.6b) and *I. woodii* (Fig. 6.4a) indicates that either the protein in the leaves is maintained and the intensity increase is probably a concentration increase with drying, or possibly an increase in protein metabolism with drying. The other species show a decline in band intensity, indicating a reduction in protein content with drying. The protein bands of *C. nanum* continue increasing in intensity down to RWCs of 5% whereas *I. woodii* shows protein destruction at 5% RWC. The ability to respond, in terms of retention of proteins or increasing protein metabolism, to drying to 30% RWC, may enable *I. woodii* to prevent the rehydration damage shown by the other three sensitive species (refer to chlorophyll fluorescence studies in Chapter 4 and ultrastructure in Chapter 5).

Despite the presence, and even the increase, of dehydrin-type proteins with drying, *P. sativum* shows a decrease in protein band intensity on drying. Leaves of *P. sativum* show similar responses to those of other sensitive species during dehydration in terms of electrolyte leakage (Chapter 3) and changes in chlorophyll fluorescence and respiration (Chapter 4) and degeneration of cell ultrastructure (Chapter 5). Rehydration also appeared to result in further damage in this species, thus the presence of the dehydrin-type proteins does not appear to have a role in preventing any of this type of damage. Whether the dehydrins play some role during milder water stress is not known.

This study was not meant to be an exhaustive characterization of protein metabolism on drying, it was meant to determine presence or absence of dehydrin-type proteins on drying. It was useful in terms of adding to the number of species reported which produce dehydrins on drying and also showed that dehydrins are not present only in desiccation-tolerant tissue as did Finch-Savage *et al.* (1994). It is clear that protein metabolism holds some useful clues in trying to understand desiccation tolerance and sensitivity, thus the pattern of protein metabolism during drying as well as rehydration needs to be more intensively studied for all these species. Two-dimensional gel electrophoresis as well as autoradiography can be utilised in these studies. Of particular interest is what, if any, proteins are produced on rehydration in *C. nanum*.

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## CHAPTER 7: GENERAL DISCUSSION

This thesis reports on some of the physiological, ultrastructural, biophysical and biochemical changes which accompany dehydration and rehydration of the desiccation-tolerant (resurrection) plant *C. nanum* in comparison with those occurring in a range of desiccation-sensitive species. This was done in order to gain an understanding of the mechanisms whereby plant vegetative tissue, as opposed to specialised structures such as seeds, react to severe desiccation.

### Overview of results obtained

The results reported in Chapter 2 showed that the desiccation-tolerant plant, *C. nanum*, had an unusual pressure volume (PV) curve. The desiccation-sensitive species had typical curvilinear PV curves while that of *C. nanum* seemed to have a plateau at relative water contents (RWC) between 70% and 50% where there was little or no change in pressure (or water potential) with a fairly large volume change. PV curves similar to that reported here for *C. nanum* have also been shown in another desiccation-tolerant plant, *Myrothamnus flabellifolia* (R. Beckett, pers. comm.<sup>6</sup>). These unusual PV curves are difficult to interpret and it is not known at what RWC turgor is lost. Loss of turgor *per se* may not play a role in the RWC to which plants can survive, but it may be important in maintaining the ability of the tolerant plant to grow and remain photosynthetically active at low RWCs.

There were no distinct differences in the amount of non-freezable water between the tolerant and sensitive species, thus it is unlikely that the amount of non-freezable water plays a role in the ability to tolerate desiccation. This was in accordance with results obtained by Pammenter, Vertucci & Berjak (1991; 1992) for seeds.

Damage, presumably to membranes, as measured by electrolyte leakage, was found in

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both the tolerant and sensitive species during drying (Chapter 3). Membrane damage, as indicated by a sharp increase in electrolyte leakage, was recorded in the tolerant species only if slow rehydration in a moist atmosphere prior to leakage measurements was not carried out. This indicated that either slow rehydration or a longer period of time was needed, to repair the damage caused by dehydration in this tissue. While the absolute water content at which leakage increased differed among the species, the RWC and water potential at which leakage increased was similar (average RWC was 27% and average water potential was -7 MPa). This suggests that a critical volume was reached below which membrane damage was severe. The tolerant species, while also showing this damage, appeared to be able to repair it if slow rehydration prior to water immersion took place.

The RWC at which the sensitive species did not recover (lethal RWC) was similar amongst *A. raddianum*, *P. sativum*, *I. woodii* and *G. livingstonei* despite perceived differences in tolerance to water stress. The supposedly drought tolerant tree, *G. livingstonei*, was the only species that showed drought induced leaf abscission when the plants were dried. This abscission occurred at higher RWCs than those at which an increase in leakage was recorded in excised leaves and in leaves which did not abscise when the whole plant was dried. Leaf abscission appeared to be important for the survival of *G. livingstonei* when water stress was severe. The lethal RWC was also similar to the RWC at which electrolyte leakage increased, indicating that measurements of increased leakage were good indicators of severe and often lethal water stress.

The lethal RWC of leaf tissue found in this study was similar to the lethal RWCs for a range of other desiccation-sensitive species, such as sunflower (Conroy *et al.* 1988) and pigeonpea (Flower & Ludlow, 1986; Ludlow & Muchow, 1990) and several forage grasses (Flower & Ludlow, 1986; Ludlow & Muchow, 1990). The lethal water potential (about -7 MPa) for the sensitive species used in this study, was higher than the -15 MPa suggested by Gaff (1989). Lethal water potentials have, however, been shown to vary considerably among species; -3 MPa for millet and about -9 MPa for peanut (Hall, 1993), this could be due to differences in the component potentials. It must be stressed however, that the duration as well as the intensity of the stress will play a role in survival. In this

experiment leaf tissue was allowed to dry slowly attached to the plant and also fairly quickly when excised. There did not appear to be much difference in the RWC at which damage occurred between these two treatments. If material had been maintained stressed for longer periods at RWCs higher than those shown here to be lethal, damage, such as that caused by the build up of free radicals, could become lethal over time and the leaves (or plants) could die at higher RWCs than those recorded here.

Studies of respiration and chlorophyll fluorescence (Chapter 4) showed that the desiccation-tolerant plant, *C. nanum*, suffered a certain degree of damage on dehydration. This damage was similar to that shown by the desiccation-sensitive species: the maximum fluorescence decreased; the final steady state fluorescence increased and became similar to the maximum value; the quantum yield of PS II ( $F_v/F_M$ ) decreased; and respiration decreased. The RWC at which these changes were noted differed slightly among the species, with the supposedly drought-tolerant tree, *G. livingstonei*, showing damage at higher RWCs than the other species. There was no marked difference between the tolerant and sensitive species, although the decreases in the various parameters generally occurred at slightly lower RWCs in *C. nanum* than for most of the sensitive species. Changes in photosynthetic parameters generally occurred at slightly higher or similar RWCs to that at which electrolyte leakage increased.

Rehydration from low RWCs had a deleterious affect on the photosynthetic apparatus of *A. raddianum*, *P. sativum* and particularly *G. livingstonei*, such that tissue appeared to be damaged at higher RWCs than those at which damage was noted during drying. Rehydration did not seem to affect the photosynthetic apparatus of *I. woodii* adversely which may be related to its habitat in which it frequently experiences severe wilting. In *C. nanum*, rehydration from very low RWCs resulted in recovery of the leaf to pre-dehydration levels provided sufficient time was given for repair to occur. The rehydration time course experiment (Chapter 4) showed that after one hour there was slight improvement in the chlorophyll fluorescence parameters of *C. nanum*. After six hours there was further improvement and after 18 hours the fluorescence parameters of the leaves had recovered to almost pre-dehydration levels.

Damage was also shown at an ultrastructural level during drying in all five species (Chapter 5). The damage, however, did not appear to be as severe in the tolerant species as that shown by all the sensitive species, particularly at 5% RWC. In *C. nanum*, organelles still appeared intact at a RWC of 5% whereas little was resolved at this RWC in the sensitive species. Extensive wall folding was noted in *C. nanum* and the vacuole appeared to subdivide into a number of smaller vacuoles. The tolerant species also never showed the membrane "beading", indicative of severe membrane damage, that was shown even at 30% RWC in the sensitive species. Rehydration from very low RWCs resulted in recovery in the tolerant species, whilst in the sensitive species further damage was noted.

Desiccation induced proteins, dehydrins, were found in *C. nanum*, and in the desiccation-sensitive pea seedlings (Chapter 6). It is possible that their presence in the latter is due to the highly bred nature of pea and/or the fact that pea seedlings were used and thus due to developmental plasticity the dehydrins were still present. Although the expression of dehydrins is unlikely to be the sole factor conferring desiccation tolerance, the presence of these proteins in *C. nanum* is possibly one of the factors facilitating survival to, and recovery from, very low RWCs.

#### **Assessment of hypotheses relating to desiccation tolerance**

Current hypotheses relating to desiccation tolerance have dealt largely with the ability of the tissue to survive in the very dry state. The water replacement hypothesis proposes that, in order to retain their structural integrity in the dry state, water molecules bound to the surface of membranes and macromolecules will be replaced by another molecule, such as sucrose or trehalose (Clegg, 1986; Crowe, Hoekstra & Crowe, 1992), or possibly by the dehydration proteins (Close, Kortt & Chandler, 1989; Dure *et al.*, 1989). Another hypothesis proposes that if the proportions and concentrations of oligo- and monosaccharides are correct, on dehydration, vitrification of the cytoplasm (glass formation) may occur. The viscosity of the glass is such that the rate of deleterious processes would be considerably reduced. The occurrence of sucrose and/or trehalose (Bianchi *et al.*, 1991; 1993; Drennan *et al.*, 1993; Hoekstra, Crowe & Crowe, 1989; Koster & Leopold, 1988; Schwab & Gaff, 1986, and others) and the LEA proteins

(reviewed by Bewley & Oliver, 1992; Bray, 1993; Skriver & Mundy, 1990) in a number of desiccation-tolerant plant species and tissue types has strengthened the hypothesis that these sugars and proteins are involved in the protection of cellular structure.

This study did not investigate the carbohydrate composition of the leaf tissue but dehydrin-like proteins were found both the desiccation-sensitive species, *P. sativum* and in the desiccation-tolerant species, *C. nanum*. From the ultrastructural studies it appears that there must be a certain amount of protection of cellular contents in *C. nanum* that did not occur in the sensitive tissue. However, the presence of dehydrins alone cannot account for this protection as they were also present in *P. sativum*. The dehydrins could also prevent the toxic build up of ions by sequestering them in these plants, as suggested by Dure (1993).

However, while protection may be an important factor in the survival of tolerant plants in the dry state, it does not explain why sensitive species die at water contents far higher than that at which tightly bound water would need to be replaced. From the investigations reported in this work it appears that a critical volume is reached below which sensitive plants cannot recover. Thus mechanical stress at RWCs of about 30% and below contribute to lethal damage in sensitive plants. This idea is not new: it was originally proposed by Iljin (1957) who worked on drought-tolerant species and proposed that any cell could survive dehydration if the mechanical stress could be eliminated by features such as small cells and vacuoles and thick cell walls. While this view is perhaps too simplistic, as damage caused by processes such as lipid peroxidation and lipid phase changes would almost certainly be a problem even if mechanical stress is avoided, the importance of dealing with mechanical stress is an important feature of desiccation tolerance. Meryman (1974) working on freezing injury also found that there appeared to be a critical volume below which cells succumbed to damage.

Thus to understand this aspect of desiccation tolerance one must also be able to explain how the tolerant plant prevents this mechanical damage which appears to be lethal to sensitive plants. Desiccation-tolerant tissues seem to withstand mechanical stress by a number of mechanisms. In orthodox seeds the packing of the vacuoles with food reserves

possibly prevents excessive mechanical stress (Vertucci & Farrant, 1995). Some of the tolerant mosses appear to have cells and particularly vacuoles of small size, and vacuolar contents may even solidify on drying (reviewed by Bewley, 1979; Iljin, 1957; Levitt, 1980). The desiccation-tolerant plant *C. nanum*, however, has very large cells with a very large central vacuole. The vacuole does, however, subdivide into a number of smaller vacuoles at very low water contents. This feature and the ability of the cell walls of this plant to fold inwards during drying may be the mechanisms by which excessive mechanical stress is prevented. The unusual PV curve showing changes in volume with little decrease in pressure is probably a consequence of this cell wall folding. If this is the case then the folding possibly starts at the point where turgor would be lost in the sensitive species.

The ability to minimise the mechanical stress associated with drying would be critical in survival of the tolerant tissue to lower water contents where protection of membranes and macromolecular structure would be necessary. To understand the whole process of tolerance, therefore, it is important to not just study tissue in the dry state, but to also understand how it survives the higher water contents which normally kill sensitive tissue.

Bewley and Oliver (1992) proposed that protection and repair were two important processes that must occur in tolerant tissues and that some tissues would have the tendency to employ one process more than the other. Studies reported on here show that damage does occur to *C. nanum* on drying (decrease in respiration and quantum efficiency, Chapter 4, and degradation of cell contents, Chapter 5). This damage is rapidly repaired on rehydration, whereas in the sensitive species the damage is worsened by the addition of water. Thus, although *C. nanum* apparently entrains repair mechanisms in the desiccation-tolerance strategy, there must also be protection mechanisms in play as the damage at the ultrastructural level in this species was less than in the sensitive ones. Even if the strategy was to employ mostly repair mechanisms, these repair mechanisms would need to be protected in the dry state.

### **Future work**

As stated by Bewley, Reynolds & Oliver (1993), the field of desiccation tolerance is largely uncharted and offers many interesting challenges. The present work is only the start of a broader and holistic study on aspects of desiccation tolerance. This study has established that: 1) desiccation-sensitive and desiccation-tolerant species suffer damage at the membrane level on drying; 2) this damage occurs at a similar RWC (critical volume) and thermodynamic status of water (water potential) for all sensitive species, irrespective of their habitat, growth form and perceived drought tolerance; and 3) the tolerant species show cell wall properties that could reduce mechanical stress associated with considerable water loss. The biophysical and biochemical nature of the damage suffered by the sensitive species has not been established, nor have the protective measures in the tolerant species, except for the cell wall properties and the ability to repair.

Further investigations into the pressure volume relationship are needed. If it is the cell wall folding which allows for this unusual PV curve then cell wall compositional changes on drying need to be studied as there appeared to be changes in the elasticity with drying. The extent to which the unusual PV curves are common among desiccation-tolerant plants also needs to be established.

To truly understand desiccation tolerance the processes occurring in sensitive tissues, particularly at water contents higher than that at which the proposed tolerance mechanisms would come into play, need to be studied. The deleterious effects of free radicals generated by the oxidative processes and the mechanisms used by tolerant plants to cope with them need to be examined. Some of this work has been done on desiccation-tolerant and sensitive mosses (Seel, Hendy & Lee, 1992) but there is little information on these processes in tolerant higher plants. The difference in strategy between poikilochlorophyllous and homoiochlorophyllous plants is also an interesting avenue to be explored in relation to the dissipation of excess excitation energy.

The ability to retain viability in the dry state is an area which has received attention but is still open to further study. Of particular interest is the role of protectant molecules such

as carbohydrates and proteins and the occurrences and ramifications of glass formation.

Another often understudied aspect of desiccation tolerance is the repair process. Processes occurring on rehydration have received attention by only a few workers (Bewley & Oliver, 1992; Oliver, 1991; Oliver, Mishler & Quisenberry, 1993). Some of the questions needing to be answered are: How does plant tissue repair the damage that occurred during dehydration and how does it prevent further damage as water becomes available? What new proteins, if any, are produced during rehydration and what are their roles? Again the difference in strategy between the plants that lose chlorophyll and those that retain it will be an interesting study.

These are just a few of the studies which need to be carried out in order to understand the phenomenon of desiccation tolerance in plant tissue. It is unlikely that there is one single factor that makes one plant tolerant and another sensitive. Desiccation tolerance will more than likely involve a whole series of mechanisms which come into play at different water contents as the plants dry and as they rehydrate. There is also an important difference in the tolerance mechanisms of seeds and of vegetative tissue. While some of the tolerance mechanisms may be the same or similar, seeds undergo a programmed dehydration, while vegetative tissue is subject to drying at unpredictable intervals and durations, and often to a number of desiccation events. The mechanisms in tolerant vegetative tissue would need to be present all the time or quickly switched on when drying occurred. There are also a number of different strategies involved in different desiccation-tolerant higher plants. Most notable are the differences in plants which retain chlorophyll and those which lose their chlorophyll. There are also likely to be differences between plants like *Myrothamnus flabellifolia* which are woody and have an extensive xylem system and other plants such as the *Lindernia* species which grow in pools of water on granite outcrops.

Desiccation-tolerant plants have been put in the category of drought tolerators (Bewley, 1979). While there is no doubt that they can tolerate drought, it may be more correct to put them in their own category. Drought tolerance is often characterised by features such as thick walled cells, drought induced leaf abscission, small cells with small vacuoles, etc. (Hall, 1993; Iljin, 1957; Levitt, 1980). While some desiccation-tolerant plants have some

of these features many of the desiccation-tolerant angiosperms possess a different suite of characters to withstand the stresses of desiccation. Mechanisms conferring desiccation tolerance should perhaps not be seen as the extreme of drought tolerance mechanisms but rather as separate and unique strategies.

### **In conclusion**

While physiologically the damage occurring in the desiccation-tolerant plant, *C. nanum*, is similar to that in the sensitive plants, at an ultrastructural level the damage appears less in the tolerant plant. On rehydration from low RWCs, damage appeared to worsen in the sensitive plants. This was in contrast to the tolerant plant where damage was repaired. There appeared, therefore, to be a combination of protection and repair mechanisms responsible for the ability of *C. nanum* to tolerate desiccation. The lethal RWC of the sensitive species was higher than that at which protective mechanisms, such as water replacement, would come into play. So it is not just the ability to replace tightly bound water that sets the tolerant plant aside; it must also have mechanisms to cope at the higher RWCs which are lethal to sensitive plants. The lethal damage to sensitive species appeared to be related to a critical volume and thus the tolerant plant must have the ability to cope with this mechanical stress during drying as well as being able to remain viable while in the dry state. It is hypothesised that the ability of the walls to fold in and the unusual nature of the PV curve may provide some answers to the enigma of desiccation tolerance, at least in *C. nanum*.

This work has raised more questions than it has answered. What is needed is, as Bewley *et al.* (1993) stated, "an exhaustive study on the structural, physiological and molecular basis of tolerance". This study would need to combine studies with a range of desiccation-sensitive plants and also a range of desiccation-tolerant plants as there is unlikely to be a single answer to the question of what makes one plant tolerant and another plant sensitive.

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