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ADDENDUM

A number of images are repeated in this thesis, an explanation for this can be found on page 122-123. The following is a list of duplicated images in this thesis:

23f and 29
23i, 29f, 39 and 44h
24a and 46a
24c and 46f
24d, 45b and 45d
24e and 45e
24f and 46g

Figure 3 - Clockwise order of the line drawings pictured in 3d, beginning from the left, are 1, 5, 3, 7, 2, 4, and 6.

Figure 6 - Y-axis should read 'Relative Water Content (RWC) (%)'.

Figure 7 - Y-axis should read 'Relative Water Content (RWC) (%)'.

Figure 8 - delete 'pallisade' and read 'palisade'.

Page 48 - delete 'Normarski' and read 'Nomarski'.

Page 277 - Full title of journal is 'Endocytobiosis and Cell Research'.

Page 310 - Yatsu (1983), delete 'prepered' and read 'prepared'.

Maintenance of Ultrastructural Integrity during Dehydration in a Desiccation Tolerant Angiosperm as Revealed by Improved Preservation Techniques.

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ABSTRACT

Tolerance of extreme desiccation in certain higher plants has been attributed to various physiological mechanisms and a number of hypotheses have been put forward to explain alterations in cell structure during dehydration, as observed under the electron microscope. However, the quality of tissue preservation in electron microscope studies from such plants, particularly dehydrated or partially dehydrated tissue, has been the subject of considerable debate recently. This dissertation aimed to critically assess the impact of both traditional (aqueous and anhydrous chemical fixation) and untested (rapid cryofixation/freeze-substitution and phase-partition fixation) fixation techniques on leaf tissue from the desiccation tolerant angiosperm *Myrothamnus flabellifolia*.

Results revealed that traditional fixation methods altered leaf tissue ultrastructure, particularly in dehydrated leaf tissue. Following several types of aqueous chemical fixation, separation of the cell wall and the plasma membrane was frequently observed in dehydrated leaf tissue, subcellular organelles, particularly chloroplasts were swollen and cells were round in shape. In contrast, after rapid cryofixation and freeze-substitution, the cell walls and plasma membranes of dehydrated leaf tissue were in close apposition, cell walls were highly folded and cells appeared contracted. More importantly, membranes

remained intact after cryofixation and cell contents were not disrupted, implying that desiccation tolerant plants retain intact membranes and membrane compartments during dehydration. Phase-partition fixation also resulted in superior preservation compared to traditional fixatives but rapid cryofixation followed by freeze-substitution gave the most consistent results.

The hydrating effect of various fixatives and dehydrating agents was quantified by immersing dehydrated leaves for varying times. After 21 hours, leaves showed a 56% increase in weight after immersion in aqueous chemical fixatives. By contrast, dehydrated leaves immersed in acetone, which was found to be the best solvent for freeze-substitution of this tissue, showed only a 14% increase in weight.

Based on the disrupted appearance of dehydrated tissue following aqueous chemical fixation, it had been postulated that desiccation tolerant plant tissues must possess specific mechanisms for tissue repair during rehydration. However contrary to this hypothesis, the new techniques used in this thesis showed that ultrastructural integrity was maintained in dehydrated tissue. Hence, the focus of new research can shift to explore the physiological and cell biological mechanisms that maintain cellular integrity in these plants. Several new lines of research are discussed.

DECLARATION.

To the best of my knowledge, this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other institution, and does not contain any material previously published or written by another person, except where duly acknowledged or referenced.

Michaela Smith.

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Figure 57. - *Myrothamnus flabellifolia* leaf tissue at 27% RWC that was cryofixed and freeze-substituted in 4% paraformaldehyde in acetone.

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LIST OF COMMON ABBREVIATIONS.

RWC	Relative Water Content.
TEM	Transmission Electron Microscope.
DMP	2, 2-Dimethoxypropane.
DMSO	Dimethylsulphoxide.
OsO_4	Osmium.
FC-72	Perfluorocarbon (#72).
HFE 7100	Hydrofluoroether (#7100).
ABA	Abscisic Acid.
SM	Spongy Mesophyll Cells.
PM	Pallisade Mesophyll Cells.
VB	Vascular Bundle.
CW	Cell Wall.
V	Vacuole.
E	Electron Dense Material.
C	Chloroplast.
G	Grana.
S	Stroma.
SG	Starch Grain.
P	Plastoglobuli.
M	Mitochondria.
N	Nucleus.
Ne	Nucleolus.
R	Ribosomes.
Pd	Plasmodesmata.

Chapter 1 - Desiccation Tolerance

INTRODUCTION.

1.1 - Desiccation Tolerance.

Water is an integral part of life and is the main element in plant cells (Kramer and Boyer 1995, Close 1996, Monneveux and Belhassen 1996). In plants, water is a component of numerous physiological functions. At the cellular level, water orientates molecules in relation to hydrophobic and hydrophilic poles (Monneveux and Belhassen 1996) and it gives rise to membrane and protein structure (Sun and Leopold 1997). Water is a medium for many biochemical reactions and can also be a reactant in processes such as photosynthesis (Kramer and Boyer 1995). At the tissue level, water forms an apoplastic link between cells. This continuous liquid phase allows water to act as a solvent and results in the movement of various gases, minerals and other solutes (Bohnert *et al.* 1995, Kramer and Boyer 1995). At the whole plant level, water is essential for driving saps, containing nutrients and hormones, throughout the plant. It also cools organs through transpiration via stomata (Bohnert *et al.* 1995, Monneveux and Belhassen 1996). Finally, turgor maintenance is essential for cell enlargement, growth and the physical support of herbaceous plants (Kramer and Boyer 1995). Concomitant with this heavy reliance on water, if the water content of a plant drops much below 'normal' levels, water

stress is experienced. Water stress can modify or impair many biochemical and physiological processes (Hsiao 1973, Bohnert *et al.* 1995). Morphological and developmental changes can also result from water deficit (Close 1996, Jensen *et al.* 1996, Shinozaki and Yamaguchi-Shinozaki 1997).

In view of this dependence on water for the survival of plant life it is interesting to note that drought is a very common environmental stress (Jensen *et al.* 1996, Bockel *et al.* 1998). More than 35% of the world's land surface is categorized as either arid or semi-arid (Oliver and Bewley 1997) and it has been said that most plants experience at least some form of water stress at some stage during their life (Ingram and Bartels 1996). Also, it has been suggested that the species, type and quantity of vegetation occurring on various parts of the earth's surface depends more on the quantity of water available than on any other single environmental factor (Kramer and Boyer 1995).

There are three main strategies by which plants cope with real or potential water deficit and they are: 1) Drought evasion - such as seen in ephemeral or annual plants and is characterized by a short life cycle that is completed before water stress is experienced. 2) Drought avoidance or postponement - this strategy is seen in plants with various morphological or physiological adaptations that retard water loss or increase its absorption, thus

enabling the plant to endure the water stress. Such adaptations include thick cuticles, leaf rolling, stress-responsive stomata and deep root systems. 3) Protoplasmic drought tolerance - this is characterized by the ability to survive low water contents of the protoplasm without sustaining permanent injury (Kramer 1980, Jones *et al.* 1981). Desiccation tolerant vascular plants, which are often referred to as 'resurrection plants', are an extreme case where the protoplasm can become completely air-dry. It should be noted however that these three strategies represent an artificial classification system and they should not be considered mutually exclusive, as any one plant can possess more than one category of adaptation (Kramer 1980).

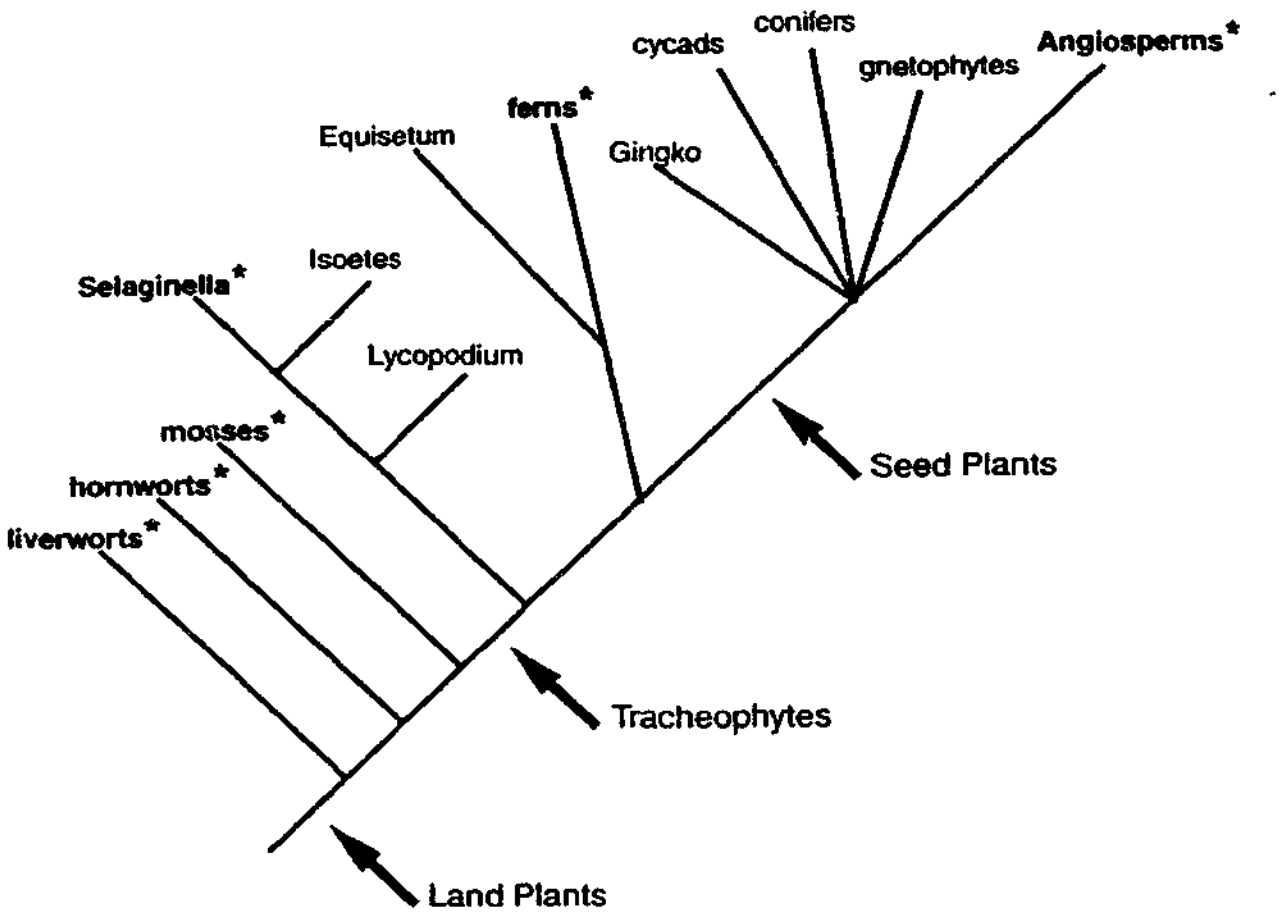
1.1.1 - Distribution of Desiccation Tolerance across the Plant Kingdom.

Desiccation tolerance is the ability of a cell or tissue to survive air-drying to levels at which the majority of cellular water is lost (Oliver 1996). These plants can survive the complete loss of all liquid phase water from their cells, which corresponds to a loss of 80-95% of cell water (Tuba *et al.* 1998). Desiccation tolerance is a near ubiquitous feature of the plant kingdom and is evident in a number of structures. The most common examples are the specialized structures produced by many

plants such as seeds, spores and pollen (Bewley and Krochko 1982, Platt *et al.* 1994, Alamillo *et al.* 1995). Less commonly, the whole vegetative plant body is desiccation tolerant. This form of desiccation tolerance has evolved independently on a minimum of twelve separate occasions (Oliver 1996) and whilst these plants are relatively few in number there are representatives in the majority of major plant divisions (Fig. 1). Most belong to the lower divisions such as algae, bryophytes and lichens (Bewley and Krochko 1982, Oliver 1996, Hartung *et al.* 1997). In the higher divisions, there are at least 60 species of ferns and fern allies and at least 60 species of angiosperms (Bewley and Krochko 1982). Gymnosperms are the only major plant group without a known representative (Gaff 1989, Oliver 1996).

Considering that water is a major component of plant cells (Monneveux and Belhassen 1996) and that it is involved in numerous cellular processes (Hsiao 1973, Bohnert *et al.* 1995, Close 1996), the ability of desiccation tolerant plants to survive reduced water content levels makes them truly remarkable plants. Bewley (1979) proposed that in order for a plant or tissue to survive severe dehydration it must exhibit several abilities. These are; 1) to limit damage during drying to a repairable level, 2) to maintain physiological integrity in the dry state and 3) to mobilize repair mechanisms upon rehydration which rectify damage suffered during

Figure 1. - A cladogram showing the evolution of land plants. Those clades that contain desiccation tolerant species are marked with a *. It has been suggested that at least one independent evolution of desiccation tolerance has occurred in *Selaginella*, the ferns and the angiosperms. Diagram taken from Oliver and Bewley (1997).


cycads
conifers
Angiosperms*
gnetophytes
ferns*
Equisetum
Gingko
Isoetes
Selaginella*
Lycopodium
mosses*
hornworts*
liverworts*
Seed Plants
Tracheophytes
Land Plants

desiccation. These parameters have since been condensed into the two concepts of cellular protection and cellular repair (Bewley and Oliver 1992).

In general, it is believed that lower order plants such as lichens, mosses and algae tend to rely more heavily on cellular repair mechanisms that are activated upon rehydration of the plant. This is because they are often poikilohydrous in nature; that is, their water content closely follows fluctuations of dryness in the environment (Walter 1955) and they generally possess no mechanisms to slow water loss. Consequently, the time for metabolic adjustment during drying can be quite limited, with desiccation for some species occurring in only a few minutes. Tolerance in these organisms may thus be a intrinsic property of the protoplasm with little or no adjustment required during drying (Gaff 1980, Reynolds and Bewley 1993a). In contrast, it is believed that higher order plants tend to rely more heavily on cellular protection, which is initiated during the drying process. In general, desiccation tolerant higher plants are able to survive desiccation only if water loss is slow, and not if water loss is rapid (Gaff *et al.* 1976, Gaff 1977, Hetherington and Smillie 1982, Bartels *et al.* 1990, Reynolds and Bewley 1993b, Farrant *et al.* 1999). Presumably this is because protective components take time to accumulate. Many desiccation tolerant higher plants also possess xeromorphic characteristics such as small or

needle-like leaves and pubescent or waxy leaf surfaces. These characteristics function in reducing the rate of water loss during water stress, thus extending the dehydration time of a plant which may possibly allow for the implementation of various protective mechanisms (Gaff 1980, Scott and Oliver 1994).

Within the variety of plants that exhibit vegetative desiccation tolerance it has been suggested that mechanisms of tolerance have evolved that span the range from a major dependence upon cellular protection to those that rely more heavily upon cellular repair. However, it appears unlikely that any plant relies upon one strategy to the exclusion of any other (Oliver 1996, Oliver, M. et *al*. 1998).

1.2 - Characteristic Features of Desiccation Tolerant Plants.

In the following section, characteristic features of desiccation tolerant plants are outlined, from the anatomical to molecular level, with particular focus on features that are thought to aid desiccation tolerance.

1.2.1 - Morphological Features.

There are no common or distinctive morphological features that are wholly characteristic of desiccation tolerance (Gaff 1977, Kappen and Valladares 1999). Due to

the ability of desiccation tolerant plants to survive water stress and their conspicuous habitation of arid environments it is often assumed that these plants possess xeromorphic features. This however is not the case for all such plants.

In general, plants of the lower orders such as lichens and bryophytes tend not to possess xeromorphic features. Gaff (1981) notes that many desiccation tolerant vascular plants are mesophytic in morphology and even hydrophytic in the case of the African plant *Chamaegigas intrepidus*. Ferns and angiosperms more frequently possess features that are considered xeromorphic that is they presumably function in the prevention of water loss (Gaff 1981). For example, *Myrothamnus flabellifolia* possesses small leathery leaves (Kappen and Valladares 1999) and *Borya nitida* has needle-like leaves (Gaff and Churchill 1976). *Xerophyta* species often possess massive sclerenchyma elements (Gaff 1977) and the stomata of *Borya nitida* are present in grooves (Gaff 1981). Leaf surfaces of desiccation tolerant plants may be pubescent, evident in many *Cheilanthes* species (Gaff 1977), they may be heavily enveloped with scales, illustrated by the leaves of *Paraceterach muelleri* (Gaff 1981), or they may have a waxy leaf surface as seen in *Craterocephalum conicum*, *Lunularia cruciata* (Proctor 1982) and *Scorpiurolus stapfianus* (Dalla Vecchia *et al.* 1998). Xeromorphic features however, are not ubiquitous in higher plants; for example, the dicotyledon *Craterostigma plantagineum* nas no

special anatomical features indicative of xeromorphic adaptations (Schneider *et al.* 1993).

There is some disparity in the literature as to the significance of xeromorphic features in desiccation tolerant plants. On one hand, Kappen and Valladares (1999) state that those plants that possess xeromorphic features are the exception rather than the rule and postulate that the possession of these features would be counterproductive during important rehydration periods. Gaff (1981) seems to concur with this point in his conclusion that desiccation tolerance is basically a property of the protoplasm and is largely unrelated to leaf morphology. On the other hand, Dalla Vecchia *et al.* (1998) state that the anatomy, morphology and ultrastructural characteristics of a leaf that serve to retard water loss are particularly significant as many desiccation tolerant plants are unable to survive dehydration if the rate of desiccation is too fast. A relationship between the rate of desiccation and survival has been found in a number of higher order desiccation tolerant plants (Gaff *et al.* 1976, Gaff 1977, Hetherington and Smillie 1982, Bartels *et al.* 1990, Reynolds and Bewley 1993b, Farrant *et al.* 1999) and also in the somatic embryos of carrot (Tetteroo *et al.* 1998, Wolkers *et al.* 1999). Some ecological observations are also consistent with a need for drying to be slow. For example many desiccation tolerant plants avoid dramatic changes in temperature and water availability by growing in

sheltered or shaded habitats. Muslin and Homann (1992) reported that *Polypodium polypodioides* has a preference for shaded habitats and Bewley and Krochko (1982) stated that many desiccation tolerant mosses grow on the north side of trees in the Northern Hemisphere and hence avoid exposure to direct sunlight.

Various types of leaf movements often accompany water loss (Gaff 1977) however, only a few plants such as *Boea hygroscopica* exhibit wilting responses that are more frequently seen in desiccation sensitive species (Gaff 1989). Common types of leaf movements include the inrolling of a plant's leaves to form a tight thread as found in *Coleochloa pallidior* (Gaff 1977) and *Oropetium thomaeum* (Rajeswari *et al.* 1993). Leaf curling is also frequently observed. For example, stems of *Selaginella lepidophylla* curl inward until the plant forms a tight ball with the old dead branches on the outside (Gaff 1989, Lebkuecher and Eickmeier 1991) and fronds of the fern *Ceterach officinarum* exhibit extensive folding so that there is curling along the frond axis (Schwab *et al.* 1989). *Tortula ruralis* (Tucker *et al.* 1975), *Ramonda nathaliae* (Müller *et al.* 1997), *Cheilanthes persica* (Gratani *et al.* 1998), *Craterostigma wilmsii* (Sherwin and Farrant 1996) and *Polypodium polypodioides* (Kappen and Valladares 1999) also exhibit leaf curling and/or rolling during dehydration. A variation on the leaf rolling and curling is seen in *Xerophyta scabrida* (Tuba *et al.* 1996b) and *Xerophyta*

viscosa (Sherwin and Farrant 1996). In these two species the leaves fold in half along the midrib. In other cases, leaves of desiccation tolerant plants turn upward during dehydration and form a decreasing angle with the stem until they enclose the apical bud. Species such as *Borya nitida* (Gaff 1981) and *Myrothamnus flabellifolia* (Sherwin and Farrant 1996) exhibit this feature.

There are a number of theories as to why leaf movements in drying desiccation tolerant plants are so frequently observed. A number of researchers have proposed that leaf and stem movements help reduce the rate of water loss (Gaff 1981, Gaff 1989, Rajeswari *et al.* 1993, Monneveux and Belhassen 1996, Tuba *et al.* 1996b, Gratani *et al.* 1998, Kappan and Valladares 1999). Stem curling has also been linked to protection against absorption of excessive light. Casper *et al.* (1993) found that stem curling in *Selaginella lepidophylla* reduced the impact of irradiance damage on the plant's photosynthetic tissues. They found that when stems were restrained from curling during dehydration, photoinhibitory damage resulted in a loss of photosystem II activity. Other authors have also proposed that leaf movements could reduce tissue damage from excessive irradiation (Gaff 1989 Lebkuecher and Eickmeier 1991, Kappen and Valladares 1999). Mechanisms to prevent photoinhibitory damage during desiccation will be discussed in further detail later in this chapter (refer to 1.2.2.3).

During desiccation and in the dehydrated state, changes in leaf colour are often observed. These colour changes can result from the loss of chlorophyll during desiccation or the presence of various water-soluble pigments (Gaff 1989). The loss of chlorophyll during dehydration occurs mainly in desiccation tolerant monocotyledons. Complete chlorophyll loss occurs in about 50% of monocots and the leaves of these species turn a yellowish, cream colour, such as seen in *Xerophyta viscosa* (Sherwin and Farrant 1996). Others, such as *Cropetium thomaeum* do not lose their chlorophyll during dehydration, the leaves of this species change to a dark violet in the dry state (Rajeswari *et al.* 1993). The water-soluble pigments that cause many of the observed colour changes are present in numerous species, including higher (Sherwin and Farrant 1996) and lower plants (Tucker *et al.* 1975), but they are not a universal feature of desiccation tolerant plants (Gaff 1989). Although Gaff (1977) stated that the formation of red and purple pigments is not needed for tolerance a number of other researchers concluded differently. They suggested that these pigments could play a role in the prevention of damage caused by excessive light whilst foliage is in the dehydrated state (Demmig-Adams and Adams 1992, Eickmeier *et al.* 1993, Sherwin and Farrant 1998). This hypothesis will be discussed in further detail later in the chapter (refer to 1.2.2.3).

1.2.2 - Physiological Changes between the Hydrated and Dehydrated States.

1.2.2.1 - Changes in Photosynthesis and Respiration During Dehydration.

When desiccation tolerant plants are dehydrated they enter a metabolic state known as anhydrobiosis. In this state they appear lifeless, they are as desiccated as dead plants and their metabolism is hardly detectable, however they are still viable (Keilin 1959, Crowe and Cooper 1971, Drazic *et al.* 1999). Anhydrobiosis is analogous to hibernation in animals and may be beneficial in the survival of desiccation. For example, it has been proposed that the slowed metabolism associated with anhydrobiosis might restrict the accumulation of toxins, the result of possible metabolic imbalances during dehydration, from reaching levels that are able to harm the plant (Gaff 1980). Leprince *et al.* (1995) concluded that a high and unabated metabolic activity during drying increases susceptibility to desiccation damage.

Before the state of anhydrobiosis is reached, cells undergo numerous physiological and molecular changes. Photosynthesis and respiration are two major physiological processes that are affected by dehydration, with respiration being less sensitive to desiccation than photosynthesis (Tuba *et al.* 1998, Kappen and Valladares

1999). In desiccation tolerant plants, changes in respiration do not follow a constant trend. Overall, respiration declines with dehydration however it can increase with moderate water stress (Gaff 1980). In contrast to this, a surge in respiration rate has been detected under severe water stress in the desiccation tolerant moss *Tortula ruralis* (Krochko *et al.* 1979). The persistence of respiration during dehydration is illustrated in *Craterostigma plantagineum* leaves, where respiration was unaffected by water stress until the leaf water content had dropped by about 70%. At a relative water content (RWC) of 15%, the leaf respiration rate was still approximately 30% of normal hydrated leaves (Schwab *et al.* 1989).

Photosynthesis also declines with dehydration however, in contrast to respiration, there is often an initial decrease in the rate of photosynthesis following stomatal closure (Schwab *et al.* 1989, Smirnoff 1993). Inhibition of photosynthesis is generally complete when water loss approaches 70-80% of the initial RWC of well-irrigated plants (Hartung *et al.* 1997).

The process of photosynthesis ceases in some species much more dramatically than in others and this is due, for the most part, to the organized breakdown of the chloroplast structure and chlorophyll in some species. In contrast, plants that retain their chlorophyll and chloroplast structure experience a more gradual decline and

cessation of photosynthesis (Tuba *et al.* 1996b). Such species include *Sphagnum capillifolium*, *Sphagnum magellanicum* and *Sphagnum fallax*, and whilst dehydration did not result in major changes either in the concentrations of photosynthetic pigments or in the pigment ratios, all three species were unable to photosynthesize in the desiccated state (Gerdol *et al.* 1996).

The reason for the persistence of respiration after photosynthesis has ceased is not fully understood. Tuba *et al.* (1996b) postulated that an active respiration system, that is functional until the end of the dehydration process, is necessary to supply the energy required for the metabolic degradation of chlorophyll and other thylakoid components. However, this theory does not explain why respiration activity persists longer than photosynthesis in both species that lose and retain their chlorophyll and chloroplast structure (Kappen and Valladares 1999).

1.2.2.2 - Changes in Photosynthesis and Respiration During Rehydration.

Massive uptake of water by desiccation tolerant plants is intrinsic to rehydration, but there is some disagreement about the details of this process. Sherwin and Farrant (1996) stated that in *Myrothamnus flabellifolia*, leaf rehydration only occurs via the xylem, as aerial parts of the plant do not take up water directly. However, Gaff

(1977) reported earlier that the rehydration of *Myrothamnus flabellifolia* was 3 times faster when the leaves were sprayed with water as well as the soil being watered, compared to when only the soil was watered. Bernacchia *et al.* (1996) found similar results in *Craterostigma plantagineum*. Tuba *et al.* (1994) also reported that rapid water uptake via the leaf surface in *Xerophyta scabrida* is of prime importance in the rehydration process and cannot be compensated for by dipping only the leaf base into water.

There is much variation in the rate at which desiccation tolerant plants recover from desiccation and attain fully hydrated water levels. These variations in recovery times can be explained, in part, by differences in plant morphology. For example, smaller, less complex plants generally require less time to reach full turgor. *Tortula ruralis* has one of the fastest recovery rates recorded and can resume the hydrated state within 90 seconds after contact with water (Scott and Oliver 1994). Other species such as *Cladonia convoluta* only take a few minutes to regain their normal water content levels (Tuba *et al.* 1998). Higher plants and those of a generally larger stature generally take longer to regain full turgor. Leaves of *Cratostigma wilmsii* were fully rehydrated after 48 hours while *Myrothamnus flabellifolia* leaves took 65 hours and those of *Xerophyta viscosa* took 92 hours to completely rehydrate (Sherwin and Farrant 1996). The

uptake of water is not the sole requirement for the successful hydration of desiccation tolerant plants, as dead plants do not lose their ability to rehydrate (Farrant *et al.* 1999). During and after the physical uptake of water a series of metabolic reactions must take place in order to ensure a successful recovery (Bernacchia *et al.* 1996).

Respiration recovers very rapidly with rehydration and the initial levels of respiration are often very intensive (Tuba *et al.* 1994). These intensive levels of respiration occur in numerous desiccation tolerant plant species and may last 6 to 10 times longer in plants that lose their chlorophyll during desiccation (Tuba *et al.* 1994). The re-activation of respiration and photosynthesis during rehydration does not necessarily require full turgidity. For example, in *Tortula ruralis* and *Cladonia convoluta* respiration resumed immediately on rehydration (Tuba *et al.* 1998) and respiration had completely recovered in *Craterostigma plantagineum* when its water content was only 20-25% of it's initial hydrated levels (Schwab *et al.* 1989, Hartung *et al.* 1997).

In comparison to respiration, the recovery times for photosynthesis are much more variable. One likely reason why photosynthesis frequently takes longer to resume than respiration is the need for some species to rebuild their chloroplasts. For example, in the first 10 hours of rehydration in *Xerophyta scabrida* there is a total absence

of chlorophyll and chlorophyll fluorescence signals and it is not until 72 hours after initial rehydration that chloroplasts are fully reconstructed and the normal rate of photosynthesis is obtained (Tuba *et al.* 1998). In those species that retain their chloroplast structure photosynthetic recovery times are relatively fast. For example, the normal rates of photosynthesis of *Tortula ruralis* and *Cladonia convoluta* are re-established within an hour (Tuba *et al.* 1998).

1.2.2.3 - Prevention of Damage from Photoinhibition.

The cessation of metabolic processes during dehydration is a potential source of problems for desiccation tolerant plants. One such problem is an increased susceptibility to photoinhibition (Kappen and Valladares 1999). This occurs when the photosynthetic apparatus absorbs more light than it can utilize in photochemical reactions (Muslin and Homann 1992). Under normal physiological conditions, various active oxygen species are produced; these include superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen (Navari-Izzo *et al.* 1997b). These oxygen species can be detrimental to numerous cellular structures and can produce chemical modifications and/or damage to proteins, lipids, carbohydrates and nucleotides (Tardieu 1996, Navari-Izzo *et al.* 1997b, Oliver and Bewley 1997). To prevent damage,

cells of aerobic organisms have evolved multiple means of maintaining the concentration of activated-oxygen species to non-harmful levels.

During desiccation the potential for production of free oxygen radicals is dramatically increased because photosynthesis is reduced and the light energy absorbed cannot be dissipated via the normal metabolic pathways (Eickmeier *et al.* 1993, Smirnoff 1993, Sherwin and Farrant 1996, Navari-Izzo *et al.* 1997b). Even under mild water stress conditions, stomatal closure can result in oxygen functioning as an alternative electron acceptor, which can cause the production of various activated-oxygen species (Smirnoff 1993, Di Blasi *et al.* 1998). Injuries that result from species of activated-oxygen can have important repercussions for survival of dehydration. For example, a link has been discovered between symptoms of activated-oxygen-induced injury and the loss of viability in dehydrating embryonic tissues and pollen (Leprince and Hoekstra 1998). Also, studies on the desiccation tolerant plants *Polypodium polypodioides* (Muslin and Homann 1992) and *Tortula ruralis* (Seel *et al.* 1992b) have shown that there is more damage, and recovery times are longer, when plants are dried under high light compared to low light conditions.

One mechanism by which plants are defended against activated-oxygen species is the production of compounds that react with active forms of oxygen, thus preventing

increases in their concentration. These compounds include enzymes such as peroxidase, catalase and superoxide dismutase along with antioxidant compounds such as ascorbic acid, α-tocopherol, carotenoids and glutathione. Other enzymes such as glutathione reductase, ascorbate peroxidase and mono- and dehydroreductases regenerate the above antioxidants (Smirnoff 1993, Navari-Izzo *et al.* 1997b, Oliver and Bewley 1997). The presence of antioxidants has been detected in many desiccation tolerant plants, and they also appear to respond to desiccation and rehydration. Desiccation tolerant species, which have been investigated in relation to their antioxidant defences, include *Sporobolus stapfianus* (Sgherri *et al.* 1994a), *Boea hygroscopica* (Sgherri *et al.* 1994b, Navari-Izzo *et al.* 1997a), *Xerophyta viscosa*, *Craterostigma wilmsii*, (Sherwin and Farrant 1998) and *Tortula ruraliformis* (Seel *et al.* 1992b).

It has also been suggested that leaf pigments, which determine the colour of dry desiccation tolerant plants (refer to 1.2.1), may play a role in protection against light induced damage. These pigments include the carotenoids and anthocyanins and are thought to aid in the removal of singlet oxygen (Demmig-Adams and Adams 1992, Seel *et al.* 1992a). They are also thought to be involved in the protection of chlorophyll through the absorption excess blue light, by reversibly combining with oxygen radicals to form xanthophylls and by acting as a filter

preventing excess light absorption (Hopkins 1999). It has been demonstrated that anthocyanin contents in *Xerophyta viscosa* and *Craterostigma wilmsii* increase four-fold and three-fold respectively during desiccation (Sherwin and Farrant 1998). However, upon rehydration, the pigment levels in *Xerophyta viscosa* remained high whereas in *Craterostigma wilmsii* they declined almost immediately. Sherwin and Farrant (1998) proposed that the retention of anthocyanins during rehydration of *Xerophyta viscosa* may be of benefit as they may afford additional protection against light damage during the re-assembly of thylakoid membranes and resynthesis of chlorophyll. Such protection is not needed in *Craterostigma wilmsii* as the photosynthetic system in this species does not appear to be broken down during desiccation.

Paraheliotropic leaf movements, which are triggered or intensified by water deficit, comprise another mechanism that has been proposed to be beneficial in defense against excess light absorption and activated-oxygen injury (Smirnoff 1993, Sherwin and Farrant 1998). Such leaf movements include leaf rolling in grasses and leaves being held in a vertical orientation in the dehydrated state (refer to 1.2.1). For example, it has been shown that leaf curling in *Polypodium polypodioides* is completed before the stage of drying that is most susceptible to photoinhibitory damage. Also, even at low light intensities in its natural habitat, *Polypodium polypodioides* experienced enough of a

photoinhibitory hazard to make leaf curling beneficial (Muslin and Homann 1992).

Finally, the dismantling of chloroplasts during dehydration is another mechanism that has been suggested to be a preventative measure against activated-oxygen damage (Sherwin and Farrant 1998). Because chloroplasts both consume and produce oxygen, and their thylakoids contain high concentrations of polar lipids and polyunsaturated fatty acid residues, both of which are susceptible to activated-oxygen attack, the chloroplast is particularly vulnerable to oxidative damage (Navari-Izzo *et al.* 1997b). Therefore, the active breakdown of chloroplasts in poikilohydrous plants has the potential not only to reduce the impact of activated-oxygen damage on the cell but also to reduce the levels of activated-oxygen able to be produced. The loss of chlorophyll during desiccation is not exclusive to those species that experience a complete breakdown of chlorophyll and dismantling of thylakoid membranes. Other species such as *Craterostigma wilmsii* only experience a partial breakdown of chlorophyll (Sherwin and Farrant 1998). Therefore, it appears that many desiccation tolerant plant species are potentially able to reduce the damaging impact of light-chlorophyll interactions during desiccation via this method.

1.2.2.4 - Production of Protective Sugars.

The hydrolysis of starch upon drying is one of the earliest perceived changes in both desiccation tolerant and desiccation sensitive plants (Gaff 1989) and concomitant with this is the frequent accumulation of high levels of soluble carbohydrates (Crowe *et al.* 1992, Sun and Leopold 1997, Crowe *et al.* 1998, Oliver, A. *et al.* 1998). The most common of these sugars in higher plants is sucrose (Ghasempour *et al.* 1998b, Oliver, A. *et al.* 1998). Desiccation tolerant plants that accumulate sucrose during drying include *Boea hygroscopica* (Bianchi *et al.* 1991b, Albini *et al.* 1999), *Craterostigma plantagineum* (Bianchi *et al.* 1991a), *Myrothamnus flabellifolia* (Suau *et al.* 1991, Bianchi *et al.* 1993, Drennan *et al.* 1993), *Ramonda nathaliae* and *Haberlea rhodopensis* (Müller *et al.* 1997). Not all plants accumulate sugars during desiccation. For example, whilst desiccation does not stimulate a difference in the total amount of sugars in *Selaginella lepidophylla*, it does stimulate a change in the relative levels of the component sugars (Adams *et al.* 1990). Changes in sugar levels and concentrations during drying are not limited to desiccation tolerant plants. During the maturation of various seeds, increases in the levels of certain sugars are frequently observed. For example, in desiccating wheat embryos, increases of sucrose and raffinose occur (Black *et al.* 1996).

Trehalose is another sugar that appears to have an analogous role to sucrose. High trehalose contents are essentially restricted to a variety of desiccation tolerant lower organisms such as spores, yeasts, microscopic animals and the more primitive phyla of vascular plants (Drennan *et al.* 1993, Müller *et al.* 1995, Sun and Leopold 1997). Whether or not trehalose is present in higher plants has been debated for a long time. Although Gussin (1972) was unable to detect trehalose in two species of beech, it has been positively identified in a few higher plants including the desiccation tolerant species *Myrothamnus flabellifolia* (Bianchi *et al.* 1993, Drennan *et al.* 1993, Müller *et al.* 1995), *Selaginella lepidophylla* (Adams *et al.* 1990) and various others (Ghasempour *et al.* 1998b, Iturriaga *et al.* 2000). It is interesting to note however, that the presence of trehalose in some species can be due to the biotransformation of other sugars by a fungal infection as reported when trehalose was detected in *Sporobolus stapfianus* samples (Murelli *et al.* 1996).

Recent research has supported the hypothesis that trehalose is involved in drought tolerance. For example, trehalose has been found to be an extremely effective carbohydrate in protecting enzyme activity during dry storage *in vivo* (Colaço *et al.* 1992). It is also an efficient sugar for membrane preservation (Sun and Davidson 1998). Furthermore, normally labile biological molecules can be dried *in vitro* in the presence of trehalose to

create stable enzymes that can be stored for extended periods of time (Colaço *et al.* 1992). Results from genetic studies also support the hypothesis that trehalose may be involved in desiccation tolerance. Trehalose can be produced by tobacco via the introduction of the gene encoding the yeast trehalose-6-phosphate synthase subunit by *Agrobacterium*-mediated gene transfer. The small amounts of trehalose produced in this way were correlated with an increase in the ability of the plant to avoid drought (Holmström *et al.* 1996, Goddijn *et al.* 1997).

There are two main hypotheses that attempt to explain the protective nature of sugars during desiccation. The first hypothesis is known as the 'water replacement theory' and it is based on the theory that if lipid membrane bilayers are dried in the absence of any added sugars, then as the water molecules are removed, the phospholipid headgroups are forced closer together (Wolkers and Hoekstra 1995, Oliver, A. *et al.* 1998). This causes the membranes to undergo various phase transitions during which they are unable to maintain their barrier properties, and leakage occurs. During tissue rehydration, this can result in cell death. However, when lipid bilayers are dried in the presence of sugar it is proposed that the sugar molecules may act as substitutes for water molecules around the polar headgroups in membranes. This allows for the maintenance of adequate spacing, which may prevent the membranes from undergoing phase transitions, thus ensuring that their

barrier properties are preserved and in turn prevent cell leakage (Albini *et al.* 1994, Crowe *et al.* 1998, Oliver, A. *et al.* 1998).

The alternative theory is based on the concept of 'vitrification'. This is the idea that sugars, including both sucrose and trehalose, are capable of forming glasses in the dry state. A glass is a liquid of high viscosity and is formed when a solution becomes so concentrated during drying that it precludes the diffusion of any more water and the solution assumes the properties of a plastic solid (Franks 1985). Glasses are thought to function in the immobilization of the cytoplasm, thus slowing down or even completely stopping the diffusion rate of molecules, therefore inhibiting deterioration processes that are time or diffusion dependent (Golovina *et al.* 1997, Hoekstra *et al.* 1997, Oliver, A. *et al.* 1998, Sun *et al.* 1998). Glasses may also prevent intracellular membrane fusion (Golovina *et al.* 1997, Hoekstra *et al.* 1997) by occupying the space in the cytoplasm that has been created by the loss of water. The formation of glasses has been postulated to help prevent cellular collapse and minimize stress associated damage (Koster 1991, Sun and Leopold 1997). It has thus been suggested that the glassy state may assure quiescence and stability in a living system for long periods (Crowe *et al.* 1998).

At the moment the only published data on glass formation in desiccation tolerant organisms are from seeds

(Bruni and Leopold 1991, Sun *et al.* 1994) and pollen (Buitink *et al.* 1996). Koster (1991) found that soluble sugars, similar to those found in desiccation tolerant embryos, were able to form glasses at ambient temperatures whereas those from desiccation sensitive embryos were only able to form glasses at subzero temperatures. From these findings it was concluded that dry desiccation tolerant embryos probably contained sugar glasses at ambient storage temperatures and water contents, but desiccation sensitive embryos probably did not. Results from soybean seed axes also support a correlation between cytoplasmic glass formation and desiccation tolerance. Bruni and Leopold (1991) and Sun *et al.* (1998) found that the loss of the glassy state in mung beans (*Vigna radiata*) was correlated with enhanced deterioration of proteins and the rapid loss of seed viability. However, Sun *et al.* (1994) found that in soybean seeds (*Glycine max*), glass formation alone was not sufficient for survival of desiccation. Although it does appear, at least in certain species, that the maintenance of the glassy state is associated with desiccation tolerance, additional data is required from other systems before conclusions can be drawn as to the overall influence that glasses have on desiccation tolerance (Crowe *et al.* 1998).

The two proposed protective mechanisms of 'vitrification' and 'water replacement' are not mutually exclusive and Crowe *et al.* (1998) believe that both are

required for preservation of labile components in cells in the dry state.

One aspect of sugar accumulation in desiccation tolerant plants that has received no real attention is its possible involvement in gene regulation. In higher plants, sugars not only sustain growth but can also regulate the expression of a variety of genes. The regulation of genes by sugars is a relatively new area of research and although at the moment no research has been conducted on the possible involvement of this type of regulation in desiccation tolerance it is possible that in the future some connections will be made. Evidence for the ability of sucrose to regulate various genes (Koch 1996) including genes involved in stress responses (Jang and Sheen 1994, Johnson and Ryan 1990, Ehness *et al.* 1997) does support the idea that gene regulation by sugars may be involved in desiccation tolerance.

1.2.3 - Molecular Responses and Hormone Changes during Desiccation.

Although plants vary in their sensitivity and responses to water stress, all plants appear to have encoded genetic information for stress perception, signaling, response and adaptation (Barrieu *et al.* 1999). These responses to water stress are often observed as changes in gene expression, with most plants producing an

array of proteins as part of a general stress response. Genes can be upregulated, downregulated or transiently expressed in response to water deficit (Bockel *et al.* 1998). It has been estimated that 800-3000 genes could be involved in the response of plants to desiccation (Hartung *et al.* 1997).

All desiccation tolerant plants undergo some type of genetic change in response to water deficit and most exhibit numerous changes during both drying and rehydration (Gaff *et al.* 1997, Kuang *et al.* 1995). *Craterostigma plantagineum* is a species that relies on the activation and synthesis of many transcripts and proteins during dehydration in order to survive desiccation (Ingram and Bartels 1996, Oliver and Bewley 1997, Bockel *et al.* 1998). Other species however, usually belonging to the lower plant orders, only exhibit genetic changes during rehydration. The desiccation tolerant moss *Tortula ruralis* is an example of such a species, as the onset of dehydration does not appear to trigger any changes in gene expression (Oliver 1991, Scott and Oliver 1994) however rehydration does (Oliver 1991, Oliver and Bewley 1984b, Wood *et al.* 1999). It has been suggested that the relatively fast rate of desiccation that these species can survive precludes any implementation of genetic change during drying and therefore the components necessary for desiccation tolerance must be constituitively present (Oliver 1991, Oliver *et al.* 1997).

Associated with the genetic changes observed during dehydration, an increase in abscisic acid (ABA) levels is also frequently observed. ABA is a plant hormone which has been implicated in the control of a wide range of physiological processes in higher plants such as stomatal closure and the inhibition of embryo germination (Hetherington and Quatrano 1991). Apart from these roles, it is also widely accepted that ABA mediates general adaptive responses to drought (Zeevaart and Creelman 1988). A role for ABA in the process of seed desiccation has been implied for a long time (Dure III 1975, Xu and Bewley 1995) and Tetteroo *et al.* (1995) found that application of exogenous ABA to carrot somatic embryos resulted in their acquisition of complete desiccation tolerance.

Dehydration and water stress has been correlated with a 2 to 7-fold increase in ABA levels in a number of desiccation tolerant plants including *Myrothamnus flabellifolia, Sporobolus stapfianus* and *Xerophyta humilis* (Hartung *et al.* 1997). Although researchers know that ABA increases in many plant species during water stress, there is limited evidence for a direct link between ABA and desiccation tolerance. Gaff and Loveys (1984) reported that there was an ABA-induced improvement in the desiccation tolerance of *Myrothamnus flabellifolia* and *Borya nitida* leaves when they were subjected to drying rates which would normally have resulted in injury. Also, Furini *et al.* (1997) found that in *Craterostigma*

plantagineum callus, constitutive over-expression of CDT-1, a gene that encodes a signaling molecule in the ABA transduction pathway, leads to desiccation tolerance in the absence of exogenous ABA.

In other cases, it appears that ABA may not be essential for desiccation tolerance. For example, ABA has not been detected in the desiccation tolerant moss *Tortula ruralis* nor does its exogenous application initiate the synthesis of desiccation-specific proteins (Bewley *et al.* 1993). Whilst in *Sporobolus stapfianus*, although there is a significant increase in endogenous ABA levels during drying and several genes have been shown to be induced by ABA, evidence suggests that ABA is not the primary mediator of desiccation tolerance. This is because detached leaves of this species are able to survive desiccation only if they are detached at a RWC of 61% or lower, which is before any significant increase in ABA levels (Gaff and Loveys 1992, Kuang *et al.* 1995). Furthermore, the application of exogenous ABA to detached leaves does not appreciably alter the extent of their tolerance (Ghasempour *et al.* 1998a). In the liverwort *Exormotheca holstii*, ABA application induces only fourteen out of the thirty-one desiccation-related polypeptides (Hellwege *et al.* 1994). From these findings, it appears that there are a number of ABA-independent gene expression mechanisms that are present in plants and that these may also play a role in water stress responses.

Most research on ABA-independent gene regulation has been performed on desiccation sensitive species (Creelman *et al.* 1990, Yamaguchi-Shinozaki *et al.* 1995, Gosti *et al.* 1995). The study by Frank *et al.* (2000) is one of the few to have identified a putative ABA-independent signalling pathway in a desiccation tolerant tissue. They identified an enzyme involved in phospholipid signalling that is stimulated by dehydration in *Craterostigma plantagineum*. The enzyme responded to drought within 10 minutes of the onset of drying although ABA could not induce its activation.

Other drought related parameters such as turgor loss (Guerrero *et al.* 1990) or mechanical stress (Ingber 1997) may trigger other alterations in metabolism and thereby contribute to the often complex genetic changes associated with desiccation (Hellwege *et al.* 1994).

1.2.4 - Changes in Cell Structure.

Desiccation tolerant plants often exhibit gross ultrastructural changes during dehydration. A reduction in cell volume is a frequent result of increasing cell water deficit. Cell shrinkage can result in mechanical stress, which occur as the central vacuole shrinks and the cell protoplast is drawn inwards. This creates tension between the plasma membrane and the cell wall, which is assumed to have limited elasticity (Vicré *et al.* 1999). It is

generally thought that coordination of dehydration between the protoplast and the cell wall is essential to prevent mechanical damage to the tissue, specifically in the prevention of disruption of the cell wall-plasma membrane association (Webb and Arnott 1982). The inward collapsing of the cell wall, otherwise known as 'cytorrhysis', occurs when the cell wall is relatively weak and is unable to resist the compressive stress during periods of negative turgor pressure (Scheidegger *et al.* 1995). Vicré *et al.* (1999) investigated the cell wall structure of hydrated and dry leaves of *Craterostigma wilmsii* and found that there was an increase in xyloglucans and unesterified pectins in the cell wall during drying. They hypothesized that the tensile strength associated with these compounds may be involved in allowing the cell wall to fold and collapse without allowing its total inward collapse. They also suggested that the increased tensile strength may be involved in the preventing the cell wall from unfolding and expanding too rapidly upon rehydration, thus allowing the plasma membrane-cell wall connections to be maintained.

In contrast to the theories of Vicré (1999), it is possible that some of the wall compounds synthesized during drying may function in firmly attaching the plasma membrane to the cell wall. For example, salt adapted tissues often cannot be plasmolyzed (Kargi and Dincer 2000), and as the cell loses water, the cell wall collapses inwards. It is

possible that a similar mechanism, to that observed in salt tolerant tissues, operates in desiccation tolerant tissues.

Cellular collapse in response to desiccation does not occur in all species. For example, Kappen and Valladares (1999) reported that the shape and appearance of some terrestrial unicellular algae and crustose lichens also did not visibly change with dehydration.

1.3 - Aims.

As illustrated above, research on desiccation tolerant tissues and plants has revealed a number of factors that appear to be related to desiccation tolerance. Despite these advances, our knowledge about the mechanisms of desiccation tolerance is still quite limited, especially in certain areas. In comparison to other areas of research, such as molecular and physiological studies, comparatively few ultrastructural studies have been conducted on desiccation tolerant plants. One possible reason for this is that dry tissue is very difficult to preserve for structural investigations. The accurate preservation of tissue in the dry state, and during desiccation, is central to understanding the process of desiccation tolerance. Many of the structural changes reported by earlier workers are contradictory. This may be due, in part, to the variety of fixation protocols used to preserve different species. Furthermore, 'traditional' fixation methods may

have created artefacts in dry tissue, caused by swelling and/or shrinkage at various steps during tissue processing. New, osmotically neutral fixation methods could eliminate many of these artefacts. As noted earlier, a major theory used to classify desiccation tolerant plants focuses on cellular protection versus cellular repair (refer to 1.1.1). If much of the damage that needs to be 'repaired' during dehydration is artefactual, the result of inappropriate fixation techniques, then this classification system must be questioned.

The primary aim of this dissertation is to, as accurately as possible, determine the ultrastructure of mesophyll leaf tissue of *Myrothamnus flabellifolia*. This will be achieved in two ways. First, traditional fixation techniques will be used as 'controls' to determine the 'current' ultrastructural view of *Myrothamnus flabellifolia* leaf tissue. Second, previously untested, osmotically neutral fixation techniques will be used to help determine whether the traditional fixation techniques affect the ultrastructure of *Myrothamnus flabellifolia* mesophyll cells. This comprehensive investigation into the impact of fixation techniques on the ultrastructural preservation of a single tissue type, in a single desiccation tolerant species, will enable the direct comparison of the various fixation techniques without species specific influences.

The use of osmotically neutral fixation techniques will also permit accurate preservation of tissue samples at

intermediate stages of desiccation. Prior attempts to investigate ultrastructural changes during desiccation have been fairly unsuccessful (refer to Chapter 5, 5.1). Finally if these techniques prove useful for this recalcitrant tissue, they may be successfully applied to other tissues that have, in the past, been very difficult to examine at the ultrastructural level.

Chapter 2 - An Evaluation of Fixation Techniques Previously Used on Dry Material

INTRODUCTION.

As noted in Chapter 1 (refer to 1.1.1), it has been proposed that the possession of three central abilities may account for the reason why desiccation tolerant plants are able to survive severe dehydration. Two of these abilities are: the ability to limit damage during drying to a repairable level and the ability to mobilize repair mechanisms upon rehydration which rectify damage suffered during desiccation. It is interesting that the prevention and repair of cellular damage are central concepts in desiccation tolerance since, in the literature, there is some debate as to the accuracy of previously obtained ultrastructural findings for dry biological material. This is because the fixation techniques used to date have the potential to induce ultrastructural alterations in the tissue samples. Thus, the question that must be asked is; are the observed ultrastructural changes authentic or are some of them artefacts of preservation resulting from inappropriate fixation techniques? This uncertainty in turn questions the basis of our understanding of desiccation tolerance. Before investigating new ultrastructural preservation techniques, I will review previously used fixation techniques and evaluate them in detail. The resulting findings will act as a 'control' with which the findings from the two new fixation techniques can be compared. Using the same plant material

throughout the study will help to ensure that any variation observed is due to differences in the fixation technique rather than the particular species used.

2.1 - Aqueous Chemical Fixation.

Generally, before biological tissue can be examined ultrastructurally with a transmission electron microscope (TEM), it must first be fixed, dehydrated and embedded in a resin. The purpose of fixation of biological tissue is primarily to stabilize proteins (Baker 1968) through chemical cross-linking that results in the formation of an insoluble, three-dimensional, macromolecular network (Hopwood 1985, Motte *et al.* 1988). Some fixatives, such as osmium tetroxide, react with the lipid components of membranes (Lindsay *et al.* 1995). The most common fixatives used in electron microscopy today are a group of chemicals known as aldehydes. Sabatini *et al.* (1963) were the first researchers to use aldehydes as fixatives for electron microscopy; prior to that, osmium tetroxide and to a lesser extent potassium permanganate were the fixatives primarily used. Once fixed, the tissue samples are dehydrated via an organic solvent series. The final stage of specimen processing is known as embedding, during which the tissue sample is infiltrated with a (generally) water-immiscible liquid resin, which is then solidified by polymerization.

Once in resin, ultrathin sections of tissue that can be penetrated by the electron beam can be cut.

The aim of tissue preparation for electron microscopy is to preserve the tissue in a state which is as near life-like as possible (Hopwood 1985, Elder 1989). This can be quite difficult as chemical fixatives by their very nature kill cells and are capable of producing numerous artefacts. Kellenberger *et al.* (1992) stated that the most important effect of fixatives in regards to the production of artefacts, is the perturbation of plasma membrane pumps and the resulting permeabilization of the membrane. The ensuing ion leakages profoundly alter the intracellular environment and this may result in numerous secondary effects on cellular ultrastructure. It has even been claimed that various structures including endoplasmic reticulum, Golgi bodies, cristae of mitochondria and nuclear pores are all artefacts and could not exist in living cells (Hillman 1980).

While it is generally accepted that fixatives can produce artefacts, other stages of tissue preparation can also cause problems. For example, whereas Motte *et al.* (1988) concluded that the choice of fixative markedly affected the nucleolus of *Zea mays* (maize), Dannenhoffer and Shen-Miller (1993) found that both fixative and buffer choice were important for the preservation of nucleolar fine structure in *Avena sativa* (oat) root tips. The dehydration stage of tissue preparation can result in a

rapid and marked shrinkage of tissue samples (Bahr *et al.* 1957) and it is during this stage of tissue processing that the morphology of the surface coat and glomerular basement membrane in rat kidneys is altered (Reale and Luciano 1993). Shrinkage or swelling of cells or cellular structures during polymerization of resins is also possible (Kellenberger *et al.* 1992). For example, methacrylate resins decrease in volume by 20% during polymerization (Bahr *et al.* 1957).

2.1.1 - Ultrastructure of Hydrated Tissue.

Most of the first ultrastructural studies of desiccation tolerant plants were conducted using the standard chemical fixatives as described above. When these techniques were used, hydrated tissue samples appeared similar to tissues of desiccation sensitive plants. Examination of the cellular ultrastructure of hydrated desiccation tolerant plants revealed no atypical structures that could account for the extreme desiccation tolerance observed in these species.

Hydrated leaf mesophyll cells are typically highly vacuolate with the cytoplasm occupying the periphery of the cell (Bartley and Hallam 1979, Hallam and Luff 1980a, Goldsworthy and Drennan 1991, Quartacci *et al.* 1997, Dalla Vecchia *et al.* 1998). There may also be a granular appearance to the vacuole (Hallam and Luff 1980b).

Chloroplasts are surrounded by a double membrane (Tucker *et al.* 1975, Bewley and Pacey 1978, Oliver and Bewley 1984a) and are generally elongated or ellipsoidal in shape (Hallam and Luff 1980a, Hetherington *et al.* 1982, Oliver and Bewley 1984a, Gerdol *et al.* 1996, Sherwin and Farrant 1996, Quartacci *et al.* 1997). Internal membranes of the chloroplast are well defined and they are separated into granal stacks of tightly packed thylakoid membranes (Tucker *et al.* 1975, Bewley and Pacey 1978, Bartley and Hallam 1979, Hallam and Luff 1980a, b, Hetherington *et al.* 1982, Oliver and Bewley 1984a, Rajeswari *et al.* 1993, Sherwin and Farrant 1996, Gerdol *et al.* 1996, Quartacci *et al.* 1997, Dalla Vecchia *et al.* 1998). Starch grains may be present (Tucker *et al.* 1975, Bewley and Pacey 1978, Bartley and Hallam 1979, Hallam and Luff 1980a, b, Hetherington *et al.* 1982, Oliver and Bewley 1984a, Gerdol *et al.* 1996, Sherwin and Farrant 1996) or absent (Rajeswari *et al.* 1993, Quartacci *et al.* 1997). Plastoglobuli may also be present (Bartley and Hallam 1979, Hallam and Luff 1980a, b, Oliver and Bewley 1984a, Rajeswari *et al.* 1993, Gerdol *et al.* 1996).

Mitochondria are also normal in appearance, that is, they contain distinct cristae and are bounded by a double membrane (Tucker *et al.* 1975, Bewley and Pacey 1978, Bartley and Hallam 1979, Hallam and Luff 1980a, b, Oliver and Bewley 1984a, Rajeswari *et al.* 1993).

The nucleus in these plants also appears normal (Oliver and Bewley 1984a, Rajeswari *et al.* 1993). Nuclei are bounded by a double membrane (Tucker *et al.* 1975) and the nuclear pores of the outer membrane may be visible (Bewley and Pacey 1978, Bartley and Hallam 1979, Oliver and Bewley 1984a). The chromatin of the nucleus can be visible and it is generally finely dispersed throughout the nucleoplasm although sometimes small aggregations may be observed (Tucker *et al.* 1975, Bartley and Hallam 1979, Hallam and Luff 1980a, b). Nucleoli may also be visible (Tucker *et al.* 1975, Bartley and Hallam 1979).

Other common structures observed in the hydrated mesophyll cells of these plants, include endoplasmic reticulum (Bewley and Pacey 1978, Bartley and Hallam 1979, Hallam and Luff 1980a, Oliver and Bewley 1984a), Golgi bodies (Bewley and Pacey 1978, Bartley and Hallam 1979) and ribosomes (Hallam and Luff 1980a).

As illustrated above, the general ultrastructure of hydrated mesophyll cells of desiccation tolerant plants do not differ significantly from the ultrastructure of hydrated mesophyll cells in desiccation sensitive species, such as the moss *Cratoneum filicinum* (Krochko *et al.* 1978) and *Fatsia japonica* (Lopez-Carbonell *et al.* 1994).

2.1.2 - Ultrastructure of Dehydrated Tissue.

As noted above, different species of desiccation tolerant and desiccation sensitive plants show ultrastructural similarities when hydrated. However, this is not the case for dehydrated tissue. With dehydration, many apparent differences in ultrastructure have been reported. Previous workers have claimed that these differences may represent relative levels of desiccation tolerance or are indicative of differing mechanisms for tolerance (maintenance versus repair). However, the different fixation and embedding techniques used in previous studies may explain the range of ultrastructural features observed.

The first ultrastructural studies of dry desiccation tolerant plants utilized aqueous chemical fixation, the standard technique of the day (O'Brien and McCully 1981). The following is a generalized description of the most frequent observations of dehydrated, mesophyll cells fixed using aqueous chemical fixation techniques.

There are regular references to cells that have a shriveled appearance with folds in the cell walls (Hallam and Gaff 1978, Hallam and Luff 1980b, Goldsworthy and Drennan 1991). The cytoplasm is usually condensed (Tucker *et al.* 1975, Bewley and Pacey 1978, Hetherington *et al.* 1982) and the plasma membrane can be either in close apposition to the cell wall (Bewley and Pacey 1978, Dace *et*

al. 1998, Farrant *et al.* 1999) or it can be pulled away from the cell wall to varying degrees (Gaff *et al.* 1976, Farrant *et al.* 1999). The plasma membrane and bounding membranes of organelles have been described as either intact (Altus and Hallam 1980, Rajeswari *et al.* 1993, Dace *et al.* 1998 Sherwin and Farrant 1998, Farrant *et al.* 1999) or disorganized, torn or absent (Gaff *et al.* 1976, Markovska *et al.* 1995, Gerdol *et al.* 1996).

Often there is mention of either a loss of vacuolation (Goldsworthy and Drennan 1991) or formation of numerous small vesicles postulated to arise from fragmentation of the single, large vacuole observed in hydrated cells (Gaff *et al.* 1976, Bartley and Hallam 1979, Altus and Hallam 1980, Quartacci *et al.* 1997, Dalla Vecchia *et al.* 1998, Farrant *et al.* 1999). The presence of osmiophilic material located within vacuoles in dehydrated material is occasionally reported (Wellburn and Wellburn 1976, Gaff *et al.* 1976, Bergstrom *et al.* 1982, Dace *et al.* 1998).

Chloroplasts are regularly observed to swell, become rounded in shape and frequently show extensive disorganization of their internal structure including expansion of thylakoid membranes and loss of grana (Tucker *et al.* 1975, Gaff *et al.* 1976, Bartley and Hallam 1979, Altus and Hallam 1980, Hallam and Luff 1980a, b, Hetherington *et al.* 1982, Bergstrom *et al.* 1982, Rajeswari *et al.* 1993, Tuba *et al.* 1993, Markovska *et al.* 1995, Gerdol *et al.* 1996, Sherwin and Farrant 1998, Dalla Vecchia

et al. 1998, Farrant *et al*. 1999). Plant species that lose their chlorophyll during desiccation usually show a greater loss of chloroplast structure than those species that retain their chlorophyll, however structural deterioration is not exclusive to these species. Starch grains are not observed after drought stress although often there is an increase in the number of plastoglobuli observed (Altus and Hallam 1980, Hallam and Gaff 1978, Tuba *et al*. 1993, Gerdol *et al*. 1996, Sherwin and Farrant 1998, Dace *et al*. 1998, Farrant *et al*. 1999).

Mitochondria are also observed to swell and exhibit a loss of their internal structure (Gaff *et al*. 1976, Bewley and Pacey 1978, Bartley and Hallam 1979, Hallam and Gaff 1978, Hallam and Luff 1980a, b, Altus and Hallam 1980, Rajeswari *et al*. 1993, Tuba *et al*. 1993, Dace *et al*. 1998). The cell nucleus is rarely mentioned and, when it is mentioned, it is described as being of normal appearance (Tucker *et al*. 1975, Altus and Hallam 1980). Lipid droplets may also accumulate in the cytoplasm, often at the periphery of the cell adjacent to the cell wall (Altus and Hallam 1980, Bergstrom *et al*. 1982).

In desiccation sensitive species that have been subjected to water stress (albeit less intense than the stress applied to desiccation tolerant plants) and have also been fixed using aqueous techniques, the ultrastructural observations are similar to those of desiccation tolerant plant species. The plasma membrane

separates from the wall and both the plasma membrane and the tonoplast develop breaks (Fellows and Boyer 1978). Chloroplast structure is dramatically affected by water stress, evident in their general swelling and roundness in shape (Ristic and Cass 1991, Lopez-Carbonell *et al.* 1994, Sherwin and Farrant 1996). Loss or breakage of the outer chloroplast envelope is seen (Ristic and Cass 1991, Lopez-Carbonell *et al.* 1994) as well as disruption of the internal membrane system (Ristic and Cass 1991, Lopez-Carbonell *et al.* 1994, Sherwin and Farrant 1996). Mitochondria also swell and there is a loss of cristae (Lopez-Carbonell *et al.* 1994).

Bermuda grass (*Cynodon dactylon*), although not considered a desiccation tolerant species, is relatively tolerant of water stress (Beyrouty *et al.* 1990). An ultrastructural investigation of water stressed plants, again revealed structural changes that were similar in nature to those observed in other desiccation tolerant and desiccation sensitive species (Utrillas and Alegre 1997). Observations included swelling of the chloroplast outer membrane, a reduction in granal stacking and the dilation of thylakoids. The mitochondrial matrix became progressively clear with the loss of cristae (Utrillas and Alegre 1997).

The above descriptions of dehydrated desiccation tolerant plant tissue fixed by aqueous chemical fixatives are indicative of initial attempts to preserve the

ultrastructure of these tissues. Using this type of fixation technique, there are a number of ultrastructural similarities between dry desiccation tolerant tissues and water stressed desiccation sensitive tissues. As will be discussed later in this chapter, a number of researchers began to question the validity of these results. The possible hydration of the dry tissue during fixation in an aqueous medium was a major concern for these workers. The following section outlines some of the initial attempts by researchers to prevent hydration during fixation of dry tissue samples.

2.2 - Anhydrous Chemical Fixation.

Apart from the theoretical possibility that dry biological tissue immersed in aqueous chemical fixatives would undergo rehydration, there was also some direct evidence that swelling did occur. For example, Öpik (1980, 1985) saw tissue samples visibly swell when placed in aqueous fixatives. Also, the use of Normarski optics, an interference-contrast microscopy technique that allows cells to be observed without the addition of fixatives, provided evidence of structural rearrangements that occurred during desiccation (Oliver and Bewley 1984a).

Swelling resulted in a number of structural changes including the straightening of cell walls, expansion of the cytoplasm and swelling of chloroplasts, mitochondria and

other organelles (Tucker *et al.* 1975, Hallam 1976, Öpik 1980, Webb and Arnott 1982, Yatsu 1983, Oliver and Bewley 1984a, Öpik 1985, Sack *et al.* 1988). Tucker *et al.* (1975) used a standard glutaraldehyde chemical fixation protocol for their investigation into the ultrastructure of the moss *Tortula ruralis* and concluded that their preparation of dehydrated material was unsuccessful, as the aqueous fixative did not preserve the cells in a dehydrated state. Other researchers have acknowledged the potential for swelling and state that their results must be viewed with caution (Morrison-Baird *et al.* 1979, Oliver and Bewley 1984a, Öpik 1985, Markovska *et al.* 1995). Despite these problems, aqueous fixatives are still frequently used to study dry or partially dry material (Rajeswari *et al.* 1993, Sherwin and Farrant 1996, Quartacci *et al.* 1997, Utrillas and Alegre 1997, Dalla Vecchia *et al.* 1998).

The disparity in the literature regarding the effect of aqueous fixatives on the cellular ultrastructure of dehydrated material may be related to whether fixation or hydration of the sample occurred first. Depending on one's point of view, the ultrastructural artefacts caused by aqueous fixation can be either severe or non-existent. Both Tucker *et al.* (1975) and Tiwari *et al.* (1990) found that ultrastructural changes occurred in less than 2 minutes in dry tissue of the desiccation tolerant moss *Tortula ruralis* and dry pollen, respectively. Öpik (1980) found that small pieces of dry rice coleoptile (Oryza

sativa L.) needed for TEM analysis required only a 10-second exposure to water to bring about structural changes. Platt *et al.* (1997) detailed three areas of concern when aqueous fixatives were used on dry and partially dry material. Firstly, hydration and swelling of the cellular structures may occur before stabilization by the fixative. Secondly, the physical surge of water or an aqueous fixative into dry and partially hydrated cells may disrupt cellular organization, particularly membranes; and finally, osmotic swelling of cells and membrane delimited organelles could occur during the fixation process (Platt *et al.* 1997).

On the other hand, Tuba *et al.* (1993) justified their use of aqueous fixation by stating that the short fixation time of 30 minutes did not apparently change the ultrastructure of *Xerophyta scabrida* cells.

Researchers concerned about the effects of aqueous fixation developed a number of anhydrous methods to prevent swelling of dry and partially dry tissues such as dry desiccation tolerant plants, seeds and pollen. Two main fixation strategies were developed; vapour fixation, using chemicals such as osmium or acrolein vapour (Perner 1965, Öpik 1980, Singh *et al.* 1984, Elleman and Dickinson 1986, Goldsworthy and Drennan 1991) and standard aldehyde fixatives dissolved in anhydrous liquids such as dimethylsulphoxide (DMSO) or glycerol (Hallam 1976, DeMason and Thomson 1981, Chabot and Leopold 1982, Grote 1992).

In dehydrated mesophyll cells fixed using both types of anhydrous fixation techniques, cell walls were described as highly folded (Hallam and Gaff 1978, Bartley and Hallam 1979, Hallam and Luff 1980b, Goldsworthy and Drennan 1991). Also, the plasma membrane was observed to be closely appressed to the cell wall (Hallam 1976, Hallam and Gaff 1978), although this was not always the case as Goldsworthy and Drennan (1991) observed the plasma membrane to be pulled away from the cell wall in places. Membranes were regularly observed to be intact in the dry state, including the plasma membrane (Goldsworthy and Drennan 1991) and the outer membranes of mitochondria and chloroplasts (Hallam and Gaff 1978, Bartley and Hallam 1979, Goldsworthy and Drennan 1991). A loss of mitochondrial internal membranes (Hallam and Gaff 1978, Bartley and Hallam 1979, Hallam and Luff 1980a, b) and grana, in those species that lose chlorophyll upon desiccation (Hallam and Gaff 1978, Bartley and Hallam 1979, Hallam and Luff 1980a, b), was often noted. Intact internal chloroplast membranes were observed in *Myrothamnus flabellifolia* (Goldsworthy and Drennan 1991), which retains substantial amounts of chlorophyll upon desiccation (Sherwin and Farrant 1996). Loss of vacuolization or the fragmentation of a large vacuole into numerous smaller vacuoles was also frequently mentioned (Hallam 1976, Bartley and Hallam 1979, Goldsworthy and Drennan 1991).

Similar ultrastructural differences, to those observed in aqueously and anhydrously fixed dehydrated desiccation tolerant tissue, were also observed in dry pollen and seeds tissues. When these tissues were fixed with various aqueous techniques, cell membranes were often disrupted, particularly the plasma membrane (Webster and Leopold 1977, Morrison-Baird *et al.* 1979, Heslop-Harrison 1979). Regular separation of the plasma membrane from the cell wall was observed (Webster and Leopold 1977, Morrison-Baird *et al.* 1979). Mitochondria and lipid droplets were spherical in shape whereas plastids were irregular in shape (Paulson and Srivastava 1968). However, in dry tissues fixed using anhydrous techniques cell membranes appeared intact (Buttrose 1973, Thomson 1979, Öpik 1980, Öpik 1985, Grote 1992) and the plasma membrane was closely appressed to the cell wall (Öpik 1980, Vigil *et al.* 1985). The smooth regular outlines of various organelles seen in aqueously fixed tissue were not seen in anhydrously fixed tissue. Nuclei, plastids, mitochondria and lipid bodies instead all showed irregular outlines (Öpik 1980, DeMason and Thomson 1981, Vigil *et al.* 1984, Öpik 1985).

Grote (1992) compared the ultrastructure of birch pollen after aqueous chemical fixation, anhydrous liquid fixation and vapour fixation. She concluded that the preservation of the pollen cytoplasm was very poor after aqueous fixation and the best results were obtained when vapour fixation was used.

2.3 - Partially Anhydrous Fixation Techniques.

Some methods that were described as 'anhydrous' however, did not eliminate all the water present during fixation. For example, aqueous solutions of glutaraldehyde were occasionally added to an anhydrous carrier (Hallam 1976, Bartley and Hallam 1979, Lott *et al.* 1984) or aqueous rinses followed an initial anhydrous fixation step (Thomson 1979, Smith 1991). Whilst Thomson (1979) acknowledged that a small amount of water had been added to the fixative in order to facilitate the solubilization of the paraformaldehyde. He justified this with the assumption that because the fixative contained at most 3-5% water, and this was less than the water content of dry seeds, it was doubtful that this would then cause hydration of the cells and their constituents. Lott *et al.* (1984) however, found that in solutions which had as little as 10% water, dry pea (*Pisum sativum*) cotyledon tissue swelled almost as much as tissue in 100% water. Thomson (1979) also used an anhydrous fixative but then rinsed dry *Vigna radiata* (L.) cotyledon tissue in an aqueous buffer. When Yatsu (1983) repeated this study he found that the buffer rinse induced as much swelling as an initial aqueous fixative.

2.4 - Post-Fixation Processing Difficulties.

Various processing difficulties have been reported when water is totally eliminated from fixation and processing techniques. These problems include difficulties with resin infiltration of the tissue and subsequent sectioning difficulties such as wetting of the block face and expansion of sections on the water surface (Öpik 1980, Lott *et al.* 1984). Lott *et al.* (1984) tested a number of anhydrous chemical fixatives, including a variety of solvents, and observed consistently poor resin infiltration. Yatsu (1983) postulated that poor infiltration into dry material may result because in dry material, minute pores in the cell wall are closed. However, when the dry material is wetted, the cell walls imbibe water, which open these pores in the wall matrix. Even when the tissues are subsequently dehydrated in anhydrous inorganic solvents, the pores remain open thus allowing infiltration of resin molecules. Yatsu (1983) surveyed the literature on dry seed ultrastructure, and found that in every case where anhydrous processing had been used, problems with resin infiltration and embedding were experienced. Conversely, in cases where aqueous processing techniques had been used there was no mention of difficulty in embedding the tissues.

2.5 - Aims.

The main differences observed in the structure of dehydrated mesophyll cells fixed using aqueous and anhydrous fixation techniques are as follows. In anhydrously fixed tissue, there is reduced separation of the plasma membrane from the cell wall and the bounding membranes of organelles are normally reported to be intact. There are also few, if any reports of organelle swelling in the dehydrated state when the tissue is fixed anhydrously, whereas organelle swelling is frequently reported in dry tissue that has been fixed aqueously. In tissues fixed using either technique there are reports of vacuole fragmentation and a loss of mitochondrial cristae.

There are a number of studies in which both aqueous and anhydrous techniques were used on the same species and ultrastructural differences between the two experimental techniques have been observed e.g. Nir *et al.* 1969, Hallam 1976, Hallam and Gaff 1978, Bartley and Hallam 1979, Hallam and Luff 1980a, b. DeMason and Thomson 1981, Webb and Arnott 1982, Chabot and Leopold 1982, Thomson and Platt-Aloia 1982, Yatsu 1983, Vigil *et al* 1984, 1985, Öpik 1985, Brown *et al.* 1987, Platt *et al.* 1997. Despite this abundance of evidence that indicates that fixation artefacts are a major problem when dealing with dry tissue, many researchers have paid little attention to them and have been happy to rely on structural information obtained

from potentially artefact-ridden work. This is probably because anhydrous fixation techniques, compared to aqueous fixation techniques, tend to be more technically difficult and more time consuming (Hallam 1976, Öpik 1980, Yatsu 1983). It appears that the ease with which experiments can be carried out has been a higher priority than the actual quality of the results obtained.

The following parts of this chapter investigate the impact of previously used aqueous chemical fixation techniques and anhydrous fixation techniques on hydrated and dehydrated leaf tissue of *Myrothamnus flabellifolia*. The potential impact of these fixation techniques will be assessed in two ways. Firstly, the weight gain of dehydrated leaves immersed in one of two routinely used chemical fixatives will be measured. Any weight gain observed will be taken as evidence of fixation-induced swelling. The second method is the visual analysis of TEM images of leaf tissue fixed using the same chemical fixatives. These images will be critically evaluated to help elucidate the effect of fixation techniques on the ultrastructure of *Myrothamnus flabellifolia* mesophyll leaf tissue.

The use of a single plant species for all experiments will eliminate any species-specific differences in response to fixation protocols. Moreover, the results from this chapter will act as a 'control' for subsequent fixation techniques that have been rarely used on tissue samples

from desiccation tolerant plants. These new fixation techniques will be examined in later chapters.

MATERIALS AND METHODS.

2.6 - Pre-Fixation Techniques.

2.6.1 - Plant Material.

The plant material used in this study was *Myrothamnus flabellifolia* Welw. (Myrothamnaceae) (Fig 2) (NB - this species is occasionally referred to as *Myrothamnus flabellifolius* (Sherwin *et al.* 1998)) a desiccation tolerant angiosperm (Fig. 3) native to Africa (Goldsworthy and Drennan 1991). This species exhibits plicate folding of the leaf (Fig. 4) and leaf colour changes (Fig. 5) in the dehydrated state. Hydrated and dehydrated plants were kept in a glasshouse under natural light conditions at a temperature of 23-29°C. To maintain full hydration plants were watered 3-4 times per week.

2.6.2 - Drying Rate of *Myrothamnus flabellifolia* Leaves.

Shoots of *Myrothamnus flabellifolia*, 5-10 cm long were detached from randomly chosen fully hydrated plants that had been selected from a total pool of about 20 individuals. The shoots were placed in empty petri dishes and were allowed to dry in natural light conditions at a temperature of 20-22°C. At regular intervals during the drying process individual leaves were removed from the

Figure 2. - *Myrothamnus flabellifolia* plants in the hydrated and dehydrated state.

Fig. 2a - A general view of a hydrated mature plant. The scale attached to the pot represents 1 cm lengths.

Fig. 2b - Magnified view of hydrated leaves. Bar represents 1 cm.

Fig. 2c - A general view of a dehydrated mature plant. The scale attached to the pot represents 1 cm lengths.

Fig. 2d - Magnified view of dehydrated leaves. Note the upward folding and positioning of the leaves against the stem in the dehydrated state. Bar represents 1 cm.

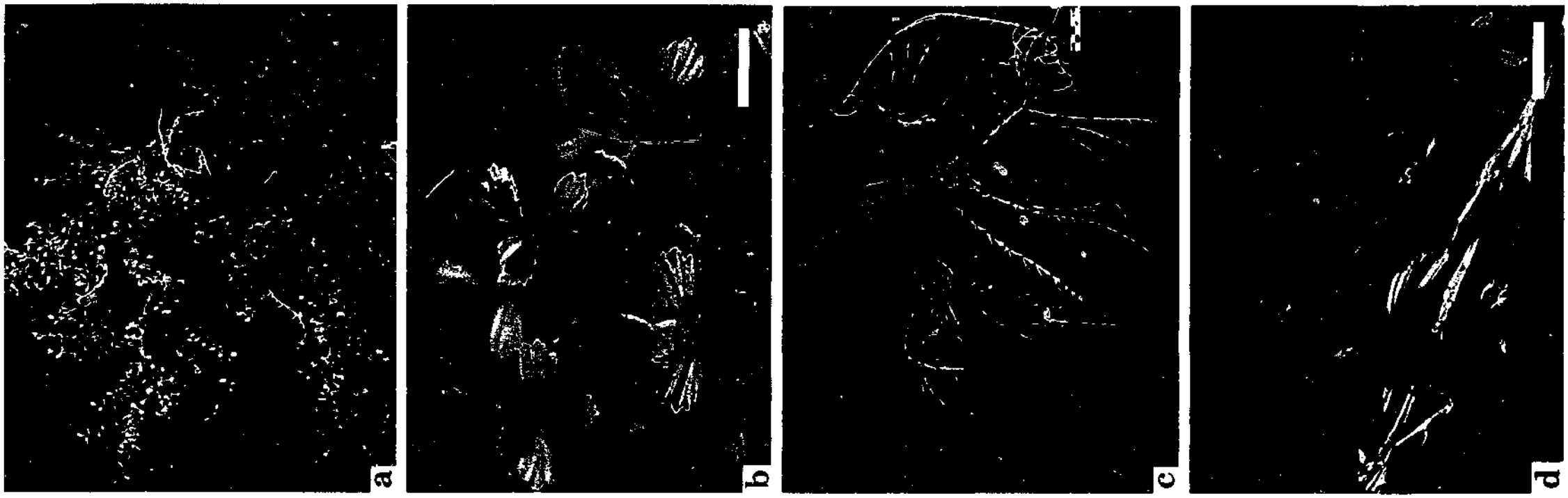

a
b
c
d

Figure 3. - *Myrothamnus flabellifolia* is an example of a dioecious plant, where male and female reproductive organs are separated on different individuals (Hutchinson 1973).

Fig. 3a - Magnified view of a female inflorescence. Each flower is enclosed by a single bract and the perianth is absent. They possess a superior, 3-lobed, 3-locular ovary. Locules contain numerous ovules in 2 rows attached to the inner angle of the loculi. Each flower possesses 3-6 free styles, stigmas have crinkled margins (Dyer 1975, Hutchinson 1973). Bar represents 2.5mm.

Fig. 3b - Magnified view of a male inflorescence. Male flowers posses 4-8 stamens, with connate or fused filaments that are free at the apex. The anthers are large and sub-quadrate and posses a connective that forms a short beak. (Dyer 1975, Hutchinson 1973). Bar represents 1.5mm.

Fig. 3c - The positioning of female inflorescences on the parent plant. Bar represents 1 cm.

Fig. 3d - Line drawings showing; an inflorescence (1), male flower (2), stamen (3), female flower (4), ovary (5), cross section through the ovary (6) and vertical section through the ovary (7) (Hutchinson 1973).

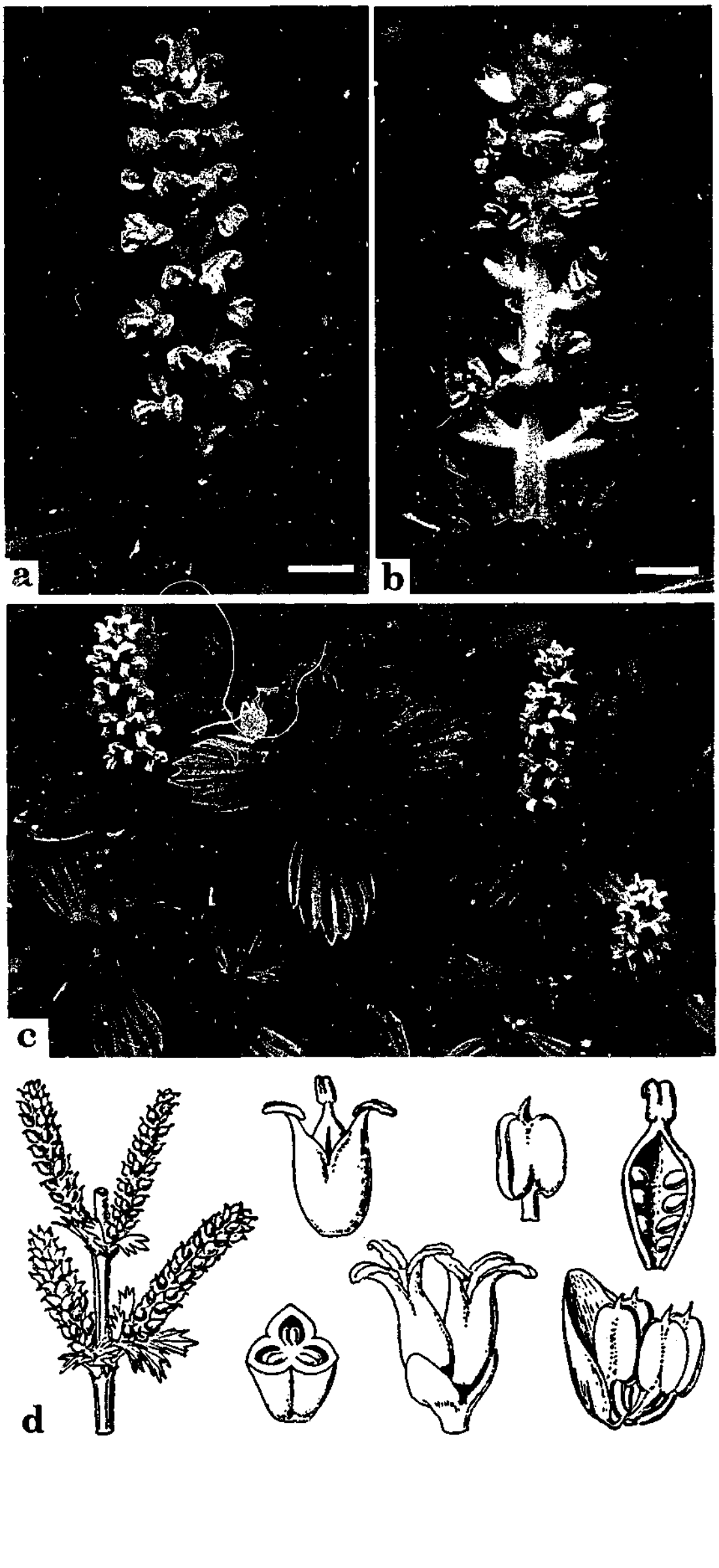

a
b
c
d

Figure 4. - Comparison of the shape of *Myrothamnus flabellifolia* leaves in the dehydrated and hydrated states. The hydrated leaf on the left shows a plicate structure, which is capable of folding during dehydration. The dehydrated leaf on the right is illustrative of a leaf in the folded state. Bar represents 1.5mm.

Figure 5. - Adaxial and abaxial surfaces of dehydrated *Myrothamnus flabellifolia* leaves. Note the retention of the green colouration on the adaxial surface of the left leaf in comparison to the brownish, red colouration of the abaxial surface of the right leaf. Bar represents 1.5mm.

shoots and their relative water content (RWC) was measured. The process by which a leaf's RWC is measured is explained below.

There are a number of different methods used to indicate the level of water stress experienced by a tissue (Barrs 1968), of which the RWC has been said to be the most generally applicable for comparing the responses of different plant cells to water stress (Kaiser 1987). The RWC of a leaf is an expression of the water content of a stressed leaf as a percentage of the leaf's water content when it is hydrated (Barrs 1968). A leaf's RWC is determined by measuring a) the initial fresh weight of a leaf just after removal from the plant, b) its fresh weight at full turgor, obtained by floating the leaf sample in distilled water overnight and c) the dry weight of the leaf after oven-drying at 70 °C for 2-3 days. The RWC is calculated with the following equation:

$$\frac{(a-c)}{(b-c)} \times 100\% = RWC$$

2.7 - Aqueous Chemical Fixation.

For all ultrastructural experiments during the course of this study, three to five randomly chosen leaves were obtained from two plants randomly chosen from a pool of about 20. Both hydrated and dehydrated leaves were sampled

from whole plants that were in either the fully hydrated or fully dehydrated state. A minimum of 10 and a maximum of 20 tissue samples were processed during each experiment and each experiment was repeated three times.

Leaf tissue samples of approximately 5mm x 5mm were fixed for 2 hours in either a) Karnovsky's fixative, a standard aqueous fixative which contains 4% paraformaldehyde, 4% glutaraldehyde and 1% caffeine in 0.08M phosphate buffer pH 6.8 (Karnovsky 1965) or b) a glutaraldehyde fixative, which contained 5% glutaraldehyde, 1% caffeine in 0.08M phosphate buffer pH 6.8. It is well known that tissue from different plant species can react differently to various fixatives, resulting in differences in structural preservation, it was for this reason that initial trials tested two different fixatives. Karnovsky's fixative has also been used previously on tissues from desiccation tolerant and water stressed plants (Kistic and Cass 1992, Platt *et al.* 1997).

Caffeine is added to fixatives to help stabilize phenolic compounds (Mueller and Greenwood 1978) and it had been previously added to fixatives used on desiccation tolerant plant material (Sherwin and Farrant 1996). In later trials however, the caffeine added to both of these fixatives was eliminated. This was because caffeine has been shown to disrupt microtubules (Paul and Goff 1973, Valster and Hepler 1997) and an initial aim of this investigation was to determine the effect of desiccation on

the cytoskeleton. The glutaraldehyde fixative, minus the caffeine, was also used in the study by Bartley and Hallam (1979). The elimination of caffeine from the two fixatives did not appear to affect the ultrastructural preservation obtained.

After fixation, samples were washed in 0.1M phosphate buffer for 30 minutes, during this time the buffer was replaced 3 times. The samples were then dehydrated through an ethanol series of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 95% ethanol. Each step in the series was for 15 minutes and this was followed by two 30-minute steps of 100% ethanol. Initially, samples were infiltrated in 25%, 50% and 75% Spurr's resin (Spurr 1969) for one day each, and the resin was changed twice per day. The samples were then placed in 100% Spurr's resin for 7 days and the resin was replaced twice each day. Due to inadequate resin infiltration, a longer resin infiltration schedule was used in later trials. The samples were first placed in 5% resin for 24 hours, then the resin concentration was raised by 5% every 24 hours. The samples were infiltrated in 100% resin for 7 days after which they were polymerized overnight at 60°C. This latter resin infiltration schedule became the standard schedule used during this study.

2.8 - Anhydrous Chemical Fixation.

Leaf tissue samples were fixed in one of two anhydrous fixatives consisting of either 2% paraformaldehyde powder (Sigma) or 2.5% anhydrous glutaraldehyde (Electron Microscopy Sciences - stock = 10% glutaraldehyde in acetone) dissolved in glycerol. The paraformaldehyde fixative had been previously used by Thomson (1979), Sack *et al.* (1988) and Skilnyk and Lott (1992). Glutaraldehyde dissolved in glycerol had not been tested before, only 70% glutaraldehyde dissolved in DMSO had been used previously (Hallam 1976, Hallam and Gaff Manston and Katchburian 1984, Lott *et al.* 1984, Horoyan *et al.* 1993). Although the degree of weight gain experienced by dehydrated *Myrothamnus flabellifolia* immersed in glycerol was not tested, other workers reported that glycerol did not induce swelling in dry tissues (Buttrose 1973, Yatsu 1983). The paraformaldehyde fixative was made by adding paraformaldehyde powder to glycerol and then gently heating the mixture on a hot plate until the paraformaldehyde dissolved. It was necessary to add 0.1 ml 1M NaOH to the paraformaldehyde to facilitate its solubilization and this did result in the addition of a small amount of water to the fixative (Thomson 1979, Sack *et al.* 1988).

Leaf tissue samples, approximately 5mm x 5mm, were fixed in either 2% paraformaldehyde or 2.5% anhydrous glutaraldehyde in glycerol for 2-3 hours. The samples were

then rinsed in 1:1 glycerol:ethanol for 15 minutes and then given a second 15 minute rinse in 100% ethanol. At this stage, the samples were split into two groups. Half of the samples from each fixative were rinsed for 15 minutes in 1,2-epoxy propane and were then infiltrated in Spurr's resin using the resin infiltration schedule referred to above (2.7). The remaining samples were post-fixed in 1% OsO_4 in ethanol for 1-2 hours at 0°C before the 1,2-epoxy propane rinse. This was then followed by infiltration and embedding in Spurr's resin as outlined above (2.7).

2.9 - **Partially Anhydrous Fixation Techniques.**

The first partially anhydrous fixation technique used was a modified version of Karnovsky's fixative used by Hallam and Luff (1980a). It consisted of 1% paraformaldehyde and 1% glutaraldehyde in DMSO and was made up as per the instructions contained in Hallam and Luff (1980a). This fixative was classed as partially anhydrous because the glutaraldehyde stock used was a 70% aqueous solution. Leaf tissue samples of approximately 5mm x 5mm were fixed under vacuum for approximately 10 minutes or until the samples had sunk to the bottom of the vial. The samples were left in the fixative for 2 hours at room temperature, then rinsed in two 20-minute changes of DMSO followed by one 10-minute wash in 100% chloroform. The samples were postfixed in 2% OsO_4 dissolved in chloroform

for 1 hour, rinsed briefly in 100% chloroform and then transferred to 100% ethanol. Finally the samples were infiltrated with Spurr's resin using the standard infiltration schedule as described in 2.7 and were polymerized overnight at 60°C.

A second partially anhydrous fixation technique was tested in which tissue samples were initially fixed anhydrously and were then processed through aqueous solutions. A number of researchers fixed dry tissue samples in anhydrous fixatives but then subsequently rinsed the tissue samples in an aqueous buffer and followed this with an aqueous secondary fixation step (Thomson 1979, Sack *et al.* 1988, Smith 1991). Instead of employing a fixative used by one of the previously mentioned authors, the anhydrous primary fixatives used in 2.8 were followed by aqueous fixation. After fixation in either 2% paraformaldehyde or 2.5% anhydrous glutaraldehyde in glycerol, the samples were rehydrated through an ethanol:water series (3:1, 1:1, 1:3) with each step lasting for 10 minutes. The samples were then placed in 100% water for 15 minutes and were then post-fixed in 1% OsO_4 in water for 1-2 hours. The samples were then dehydrated through a second ethanol:water series (1:3, 1:1, 3:1) with each step lasting for 10 minutes. After a final 15-minute rinse in 100% ethanol, they were infiltrated with Spurr's resin as per the standard infiltration method in 2.7, and polymerized overnight in a 60°C oven.

2.10 - Post-Embedding Techniques.

All tissues examined during the course of this study were sectioned with either a glass or diamond knife using a Reichert-Jung Ultracut E microtome. Due to the fragile nature of some of the sections it was necessary to use formvar-coated grids. Sections cut on a diamond knife were collected on water. Sections were stained with saturated uranyl acetate in 50% methanol for 10 minutes. This was then followed by staining in lead citrate for 10 minutes using the method of Reynolds (1963). A Jeol 200CX TEM at 80kV was used in the ultrastructural examination of all samples during the course of this study.

2.11 - Determination of the Percentage Weight Gain Experienced by Dehydrated Leaves Immersed in Various Solutions.

For all weight gain experiments, individual leaves were removed from a completely dehydrated plant and were weighed (initial weight). Each separate leaf was then placed in a screw-capped vial that contained 5 ml of a hydrating solution. For each hydrating solution examined, 10 replicates were tested. The leaves were left in the hydrating solutions overnight (approximately 18 hours) and were weighed for the second time in the morning (hydrated weight). After the hydrated weight of a leaf was known the

percentage increase in leaf weight was calculated using the following equation:

$$\frac{\text{hydrated weight - initial weight}}{\text{initial weight}} \times 100 = \text{percentage increase in weight}$$

An extension of the above experiment was used to determine whether the percentage water content of a solution was directly proportional to the amount of swelling produced. In this experiment, single dehydrated leaves were immersed overnight (approximately 18 hours) in screw-capped vials that contained 5 ml of solutions of varying water contents. A scale of increasing water content was used that went from 100% anhydrous solvent to 100% water. Ten leaves were examined at each point along the scale. The percentage increase in weight experienced by the leaves was calculated as above.

Four solvents were tested: acetone, ethanol, methanol and DMSO. DMSO was tested because it has been previously used as a solvent for anhydrous fixatives (Hallam 1976, Hallam and Gaff 1978, Manston and Katchburian 1984, Lott *et al.* 1984, Horoyan *et al.* 1993). The other three solvents were tested as they had either been used as anhydrous fixatives (Lott *et al.* 1984) or as carriers for other chemical fixatives (Peat and Potts 1987).

2.12 - Determination of the Percentage Weight Gain Per Hour Experienced by Dehydrated Leaves Immersed in Various Solutions.

The determination of the percentage weight gain per hour of dehydrated leaves was similar to the method outlined in 2.11. As with the experiments above, 10 separate leaves were used for each solution tested. The dehydrated leaves were removed from a whole plant and were weighed (initial weight). They were then each placed in a separate screw-capped vial that contained 5 ml of the hydrating solution. The weight of each leaf was then re-measured after 30 minutes, 2 and 21 hours (hydrated weights).

The percentage increase in weight per hour was calculated by first obtaining the weight gain and then the percentage increase in weight for each leaf tested (refer to 2.11). Once the percentage increase in leaf weight was known, the percentage increase per hour was calculated by the following equation:

$$\frac{\text{percentage increase in weight}}{\text{hours immersed in hydrating solution}} = \text{percentage increase in weight per hour}$$

2.13 - Statistical Analyses.

Statistical tests were performed on all data to determine the significance of any observed differences ($P<0.05$).

A standard one-way ANOVA followed by a Tukey's post-hoc test was used to determine the significance of differences in the percentage weight gain of dehydrated leaves immersed in various solutions. This test was also used to determine the significance of differences in weight gain for dehydrated leaves immersed in solutions of various water contents.

Paired t-tests were used to determine whether the rate of weight gain of dehydrated leaves immersed in different solutions differed between 30 minutes and 2 hours. Percentage data were arcsin transformed before analysis.

RESULTS.

2.14 - Leaf Water Content of Drying Leaves.

The dehydration rate of detached shoots of *Myrothamnus flabellifolia* was determined to identify the time at which the leaves became completely dehydrated and could be used in fixation experiments. Results of initial experiments revealed that the water content of hydrated detached leaves fell to a content level of approximately 5% by 48 hours (Fig. 6). A more detailed experiment revealed that within approximately 24 hours, leaf RWC dropped to approximately one third of initial levels and reached a constant level after approximately 40 hours (Fig. 7).

2.15 - Light Microscopy of *Myrothamnus flabellifolia* Leaf Tissue.

Fresh hand sections of hydrated *Myrothamnus flabellifolia* mesophyll leaf tissue were mounted in distilled water and viewed with a Leitz DM*IRB* inverted light microscope. Hydrated leaves were relatively flat in profile and they possessed prominent vascular bundles and bands of spongy and palisade mesophyll cells of approximately equal thickness (Fig. 8a). In contrast, fresh hand sections of dehydrated leaf tissue, which were mounted in glycerol, exhibited severe folding of the leaf.

Figure 6. - The rate of dehydration of hydrated detached leaves of *Myrothamnus flabellifolia* over a 156 hour period.

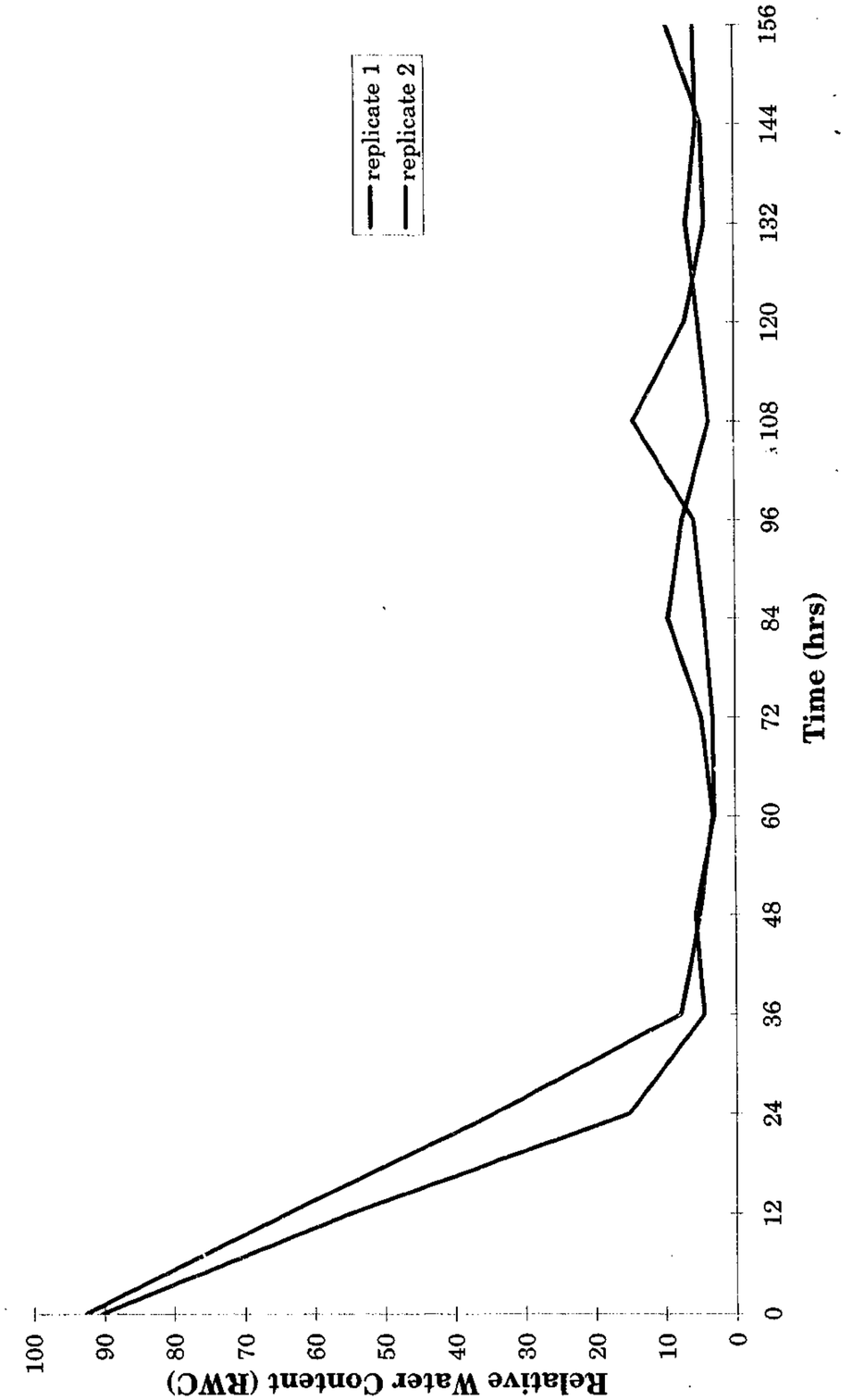


Figure 7. - The rate of dehydration of hydrated detached leaves of *Myrothamnus flabellifolia* over a 40 hour period.

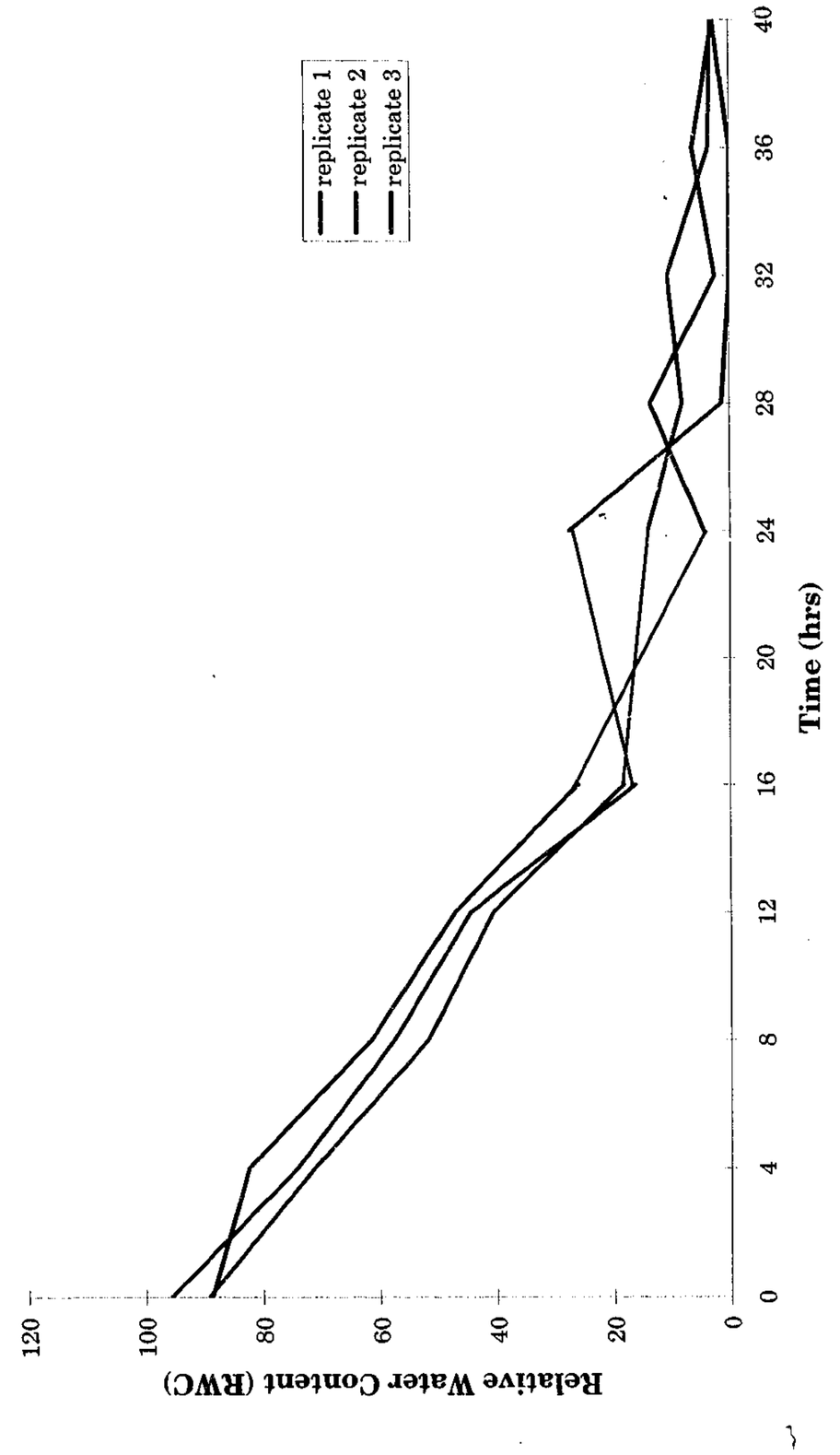
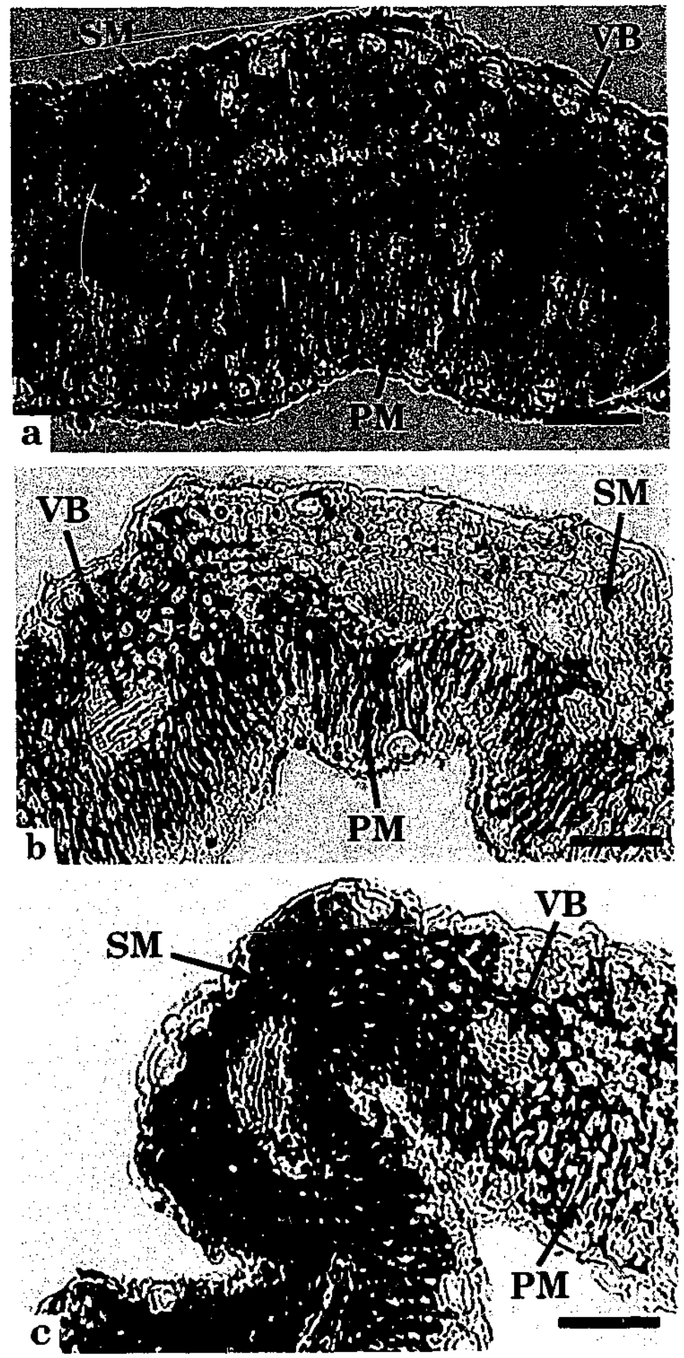


Figure 8. - Fresh hand sections of *Myrothamnus flabellifolia* leaf tissue.

Fig. 8a - Section of a hydrated leaf. Vascular bundles (VB) and spongy (SM) and pallisade mesophyll (PM) cell types are visible. The leaf is relatively flat in profile and there is no sign of any folding of the lamina. Bar represents 10 µm.

Fig. 8b - Section of a partially hydrated leaf showing spongy (SM) and pallisade mesophyll (PM) cell layers and vascular bundles (VB). The initial stages of leaf folding are evident with the leaf lamina curving upwards and enclosing the adaxial surface. Bar represents 10 µm.

Fig. 8c - Section through a dehydrated leaf illustrating the folding of the leaf lamina and the accumulation of xanthophyll pigments (red colouration) on the abaxial surface. Vascular bundles (VB), spongy (SM) and pallisade mesophyll (PM) cell types are visible. Bar represents 10 µm.



As indicated earlier, glycerol does not appear to induce swelling of dehydrated biological tissues (refer to 2.8). The outlines of the cells appeared compressed and they were not as clearly defined as in cells from hydrated plants. Red colouration of hypodermal cells at the abaxial surface was also seen (Fig. 8c). In partially dehydrated tissue, the degree of folding was intermediate to that of hydrated and dehydrated tissue, and cells showed intermediate levels of shrinkage (Fig. 8b).

2.16 - Ultrastructure after Aqueous Chemical Fixation.

2.16.1 - Hydrated Tissue.

Initial ultrastructural trials on hydrated leaf tissue using aqueous chemical fixatives produced the following results. Typically, mature leaf mesophyll cells had cell walls which had a smooth outline (Fig. 9a, 9b) and contained a large central vacuole that often had a fine granular appearance (Fig. 9a, 9b). The cytoplasm occupied the periphery of the cell (Fig. 9a, 9b) and the plasma membrane in these cells was closely appressed to the cell wall (Fig. 9a, 9b). Chloroplasts were elongated to elliptical in shape (Fig. 9c). The stroma contained a densely packed thylakoid membrane system composed mainly of grana (Fig. 9c). The grana exhibit the typical 'staircase' arrangement of thylakoids membranes evident in *Myrothamnus*

Figure 9. - Hydrated *Myrothamnus flabellifolia* leaf tissue fixed with aqueous chemical fixatives.

Fig. 9a - A general view of mesophyll cells fixed in 5% glutaraldehyde and 1% caffeine in 0.1 M phosphate buffer. Cells had a turgid appearance and the cell walls (CW) were smooth in contour. A large central vacuole (V) was present in which were large masses of electron dense material (E). Organelles such as chloroplasts (C) and mitochondria (M) were visible. Bar represents 4 µm.

Fig. 9b - A general view of mesophyll cells fixed in 5% glutaraldehyde and 1% caffeine in 0.1 M phosphate buffer. Like Fig. 9a, the cells appeared turgid and the cell wall (CW) was smooth in contour. A large central vacuole (V) was present. As with Fig. 9b, the central vacuole contained masses of electron dense material (E) however they were smaller in nature and lined the tonoplast as opposed to being centrally positioned. Chloroplasts (C) were also observed. Bar represents 3 µm.

Fig. 9c - Magnified view of chloroplasts in Fig. 9b. Thylakoid membranes (arrow heads) arranged in granal stacks (G) were visible as too were mitochondria (M), the tonoplast membrane (*) and electron dense material (E) present in the vacuole. Bar represents 1 µm.

Fig. 9d - Micrograph showing stacks of thylakoid membranes (arrow heads) forming grana (G) in leaf tissue fixed in Karnovsky's fixative. Bar represents 0.25 µm.

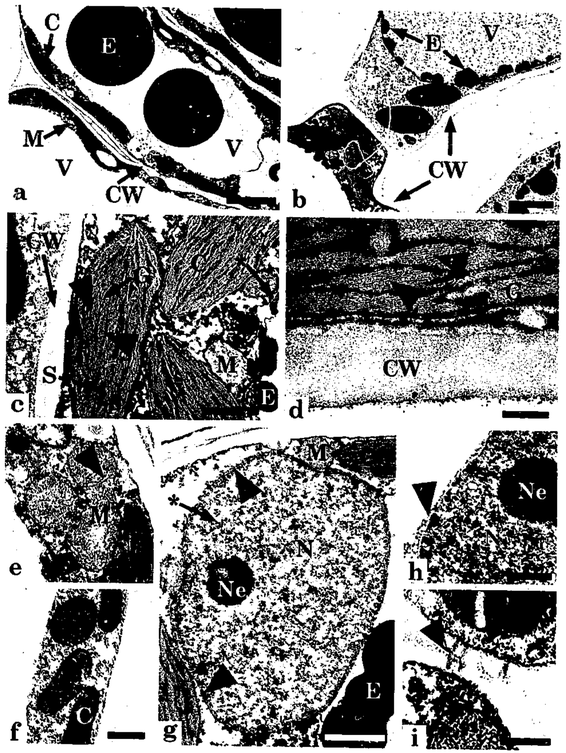
Fig. 9e - Lightly staining mitochondria (M) present in mesophyll cells fixed in 5% glutaraldehyde and 1% caffeine in 0.1 M phosphate buffer. Cristae can also be seen (arrow heads). Bar represents 0.5 µm.

Fig. 9f - Darker staining mitochondria (M) were also observed in mesophyll cells fixed in 5% glutaraldehyde and 1% caffeine in 0.1 M phosphate buffer. Bar represents 0.5 µm.

Fig. 9g - Nuclei (N) were round to oval in shape and were turgid in appearance. Nuclei frequently possessed a nucleolus (Ne) and the double bounding membrane can be discerned (arrow heads). The chromatin was visible and was lightly dispersed (*). Mitochondria (M), chloroplasts (C) and electron dense material (E) can also be seen. Tissue sample was fixed in 5% glutaraldehyde and 1% caffeine in 0.1 M phosphate buffer. Bar represents 2 µm.

Fig. 9h - A magnified view of a nucleus (N) showing the double bounding membrane (arrow head). The nucleolus can also be seen (Ne). Tissue was fixed in Karnovsky's fixative. Bar represents 1 µm.

Fig. 9i - Plasmodesmata (arrow head) were regularly observed in leaf tissue. Tissue was fixed in 5% glutaraldehyde and 1% caffeine in 0.1 M phosphate buffer. Bar represents 0.5 µm.



flabellifolia (Wellburn and Wellburn 1976) (Fig. 9d). Plastoglobuli were evident throughout the stromal matrix (Fig. 9d). Mitochondria were generally oval to round in shape and had a continuous bounding membrane (Fig. 9e, 9f). Cristae were observed in the mitochondrial matrix although their frequency was not great (Fig. 9e). The nucleus was surrounded by a continuous nuclear envelope (Fig. 9g, 9h). The chromatin was granular in appearance and nucleoli were observed (Fig. 9g, 9h). Osmiophilic material, presumed to be polyphenolic material, was observed in a number of cells either in the form of large central masses (Fig. 9a) or in smaller, more numerous masses aligned along the periphery of the tonoplast (Fig. 9b). Plasmodesmata were also observed (Fig. 9i). Refer to the end of this results section for a summary of these ultrastructural findings (Table 1).

2.16.2 - Dehydrated Tissue.

The mesophyll cells of dehydrated, mature leaves fixed in aqueous chemical fixatives had cell walls which occasionally showed slight folding (Fig. 10a), but in the majority of instances, the cell walls were smooth in profile (Fig. 10b, 10d), similar to those observed in hydrated tissue (cf. Fig. 9a, 9b). In some cases the plasma membrane was appressed to the cell wall, closely following the contours of the cell (Fig. 10a). However in

Figure 10. - Dehydrated *Myrothamnus flabellifolia* leaf tissue fixed with aqueous chemical fixatives.

Fig. 10a - A general view of leaf cells fixed with Karnovsky's fixative. Cell walls (CW) were generally convoluted in appearance and the plasma membrane (arrow heads) was closely appressed to the cell wall. A large central vacuole (V) was present and organelles such as chloroplasts (C) and mitochondria (M) can be seen. Bar represents 2 µm.

Fig. 10b - A general view of a leaf cell fixed in 5% glutaraldehyde in 0.1 M phosphate buffer. In this micrograph the plasma membrane and the cell wall have separated (*) resulting in the protoplasm being contracted in the centre of the cell. Chloroplasts (C), mitochondria (M), the nucleus (N) and a mass of electron dense material (E) were also present. Bar represents 2 µm.

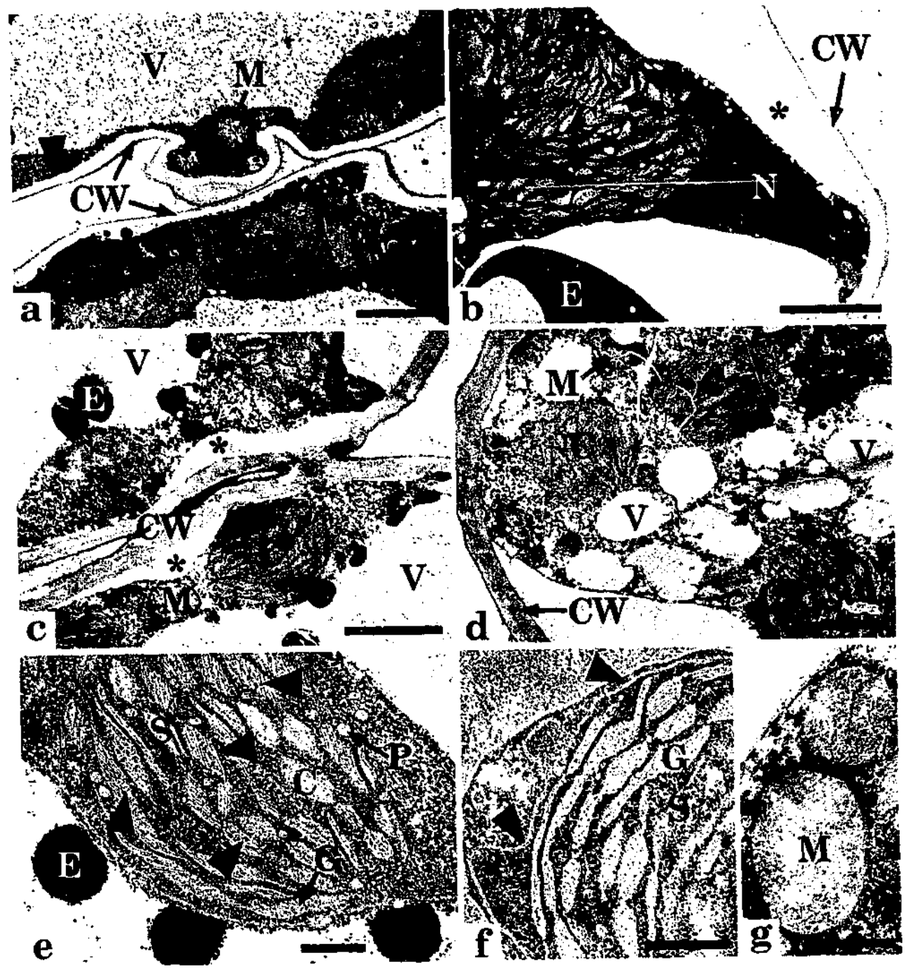
Fig. 10c - Areas of only slight separation of the cell wall and the plasma membrane (*) were regularly observed. Central vacuoles (V) which contained masses of electron material (E) were also present. Chloroplasts (C) and mitochondria (M) are also visible. Tissue sample was fixed in Karnovsky's fixative. Bar represents 2 µm.

Fig. 10d - Fragmentation of the single central vacuole into smaller, more numerous vacuoles (V) was occasionally seen. Chloroplasts (C) and mitochondria are also present. The fixative 5% glutaraldehyde in 0.1 M phosphate buffer was used to fix the tissue sample. Bar represents 2 µm.

Fig. 10e - Chloroplasts (C) were almost ubiqúitously round in shape in dehydrated leaf tissue fixed in aqueous chemical fixatives. The granal (G) and stromal (S) regions could be discerned and plastoglobuli (P) were present. Thylakoid membranes (arrow heads) were also visible. Tissue sample was fixed in Karnovsky's fixative. Bar represents 0.5 µm.

Fig. 10f - High magnification of a chloroplast. The granal (G) and stromal (S) regions can be distinguished as too can the double bounding membrane (arrow heads). Tissue was fixed with Karnovsky's fixative. Bar represents 0.5 µm.

Fig. 10g - Mitochondria (M) observed in dehydrated leaf tissue fixed with Karnovsky's fixative. Cristae could not be discerned. Bar represents 0.5 µm.



other instances, the plasma membrane had separated from the cell wall. In some cases, the separation created a small gap (Fig. 10c), whereas in other cases, the gap was quite large (Fig. 10b). When there was a large separation, the protoplast was frequently condensed in the centre of the cell and the large central vacuole seen in hydrated cells (Fig. 10a) was absent (Fig. 10b). Smaller, more numerous vacuoles were often dispersed throughout the centrally congregated cytoplasm (Fig. 10d). Abundant electron-dense material was observed in the vacuole, either as a large central mass (Fig. 10b) or as smaller, more numerous masses (Fig. 10c, 10e).

Chloroplasts in these cells were more rounded and spherical shape than the chloroplasts in hydrated tissue (Fig. 10b, 10e). Fine membrane structure was not very distinct in this tissue, however where it could be discerned, the granal stacks appeared shorter and more condensed compared to grana in hydrated tissue (Fig. 10e, 10f). The outer bounding membrane of the chloroplast when discernible, consisted of a double membrane structure (Fig. 10f).

Mitochondria were also evident (Fig. 10a, 10c) however the cristae were difficult to discern (Fig. 10g). The nucleus was compact and dense in nature and was irregular in shape (Fig. 10b).

During the actual processing of the samples, dehydrated tissue samples were observed to swell in size

and also change from a brown to a green colouration. A summary of these ultrastructural findings is contained in Table 1, which is located at the end of the results section.

2.17 - Weight Gain of Dehydrated Leaves Immersed in Various Solutions.

To quantify the observed visual impact of aqueous fixatives on dehydrated *Myrothamnus flabellifolia* tissue, detached, dehydrated leaves were floated in either Karnovsky's fixative or 5% glutaraldehyde in phosphate buffer overnight (approximately 21 hours). Changes in leaf weight were measured at various stages during this period and were compared to the leaves' initial weight. To act as a control, another group of leaves was floated in water for the same time period. Although it may be argued that measuring the actual volume change of a tissue sample is a more accurate measurement than measuring the change in leaf weight, Bahr *et al.* (1957) observed that as a rule, changes in weight closely parallel changes in volume. Leaves floated in all three solutions exhibited weight increases after 21 hours. The significance of the results obtained was statistically tested and the results of these tests are listed in Appendix E. There was no significant difference in the amount of swelling in leaves floated in either Karnovsky's fixative or 5% glutaraldehyde in phosphate

buffer (Appendix E - Table 6). Leaves immersed in either fixative increased in weight by 56% after 21 hours (Fig. 11). The level of swelling in aqueous chemical fixatives was less than the weight increase exhibited by leaves floated in water (84% weight increase). The difference between the weight increase of dehydrated leaves immersed in water and either Karnovsky's fixative or 5% glutaraldehyde in phosphate buffer was significantly different (Appendix E - Table 6).

The rate of weight increase per hour experienced by dehydrated leaves showed that there was initially a rapid rate of swelling which was followed by a slower rate of weight increase (Fig. 12). After 30 minutes of immersion, leaves in Karnovsky's fixative were increasing in weight by 24% per hour, those in 5% glutaraldehyde in phosphate buffer by 16% per hour, and leaves floating on water were increasing by 38% per hour. After 2 hours the rate of swelling had decreased, with leaves swelling at a rate of 5% per hour for Karnovsky's fixative and 5% glutaraldehyde in phosphate buffer and 13% per hour for leaves floated in water respectively. The differences in weight gain between 30 minutes and 2 hours were significant for each of the three solutions tested (Appendix E - Table 7). Finally after 21 hours, the swelling rate had decreased even further. Leaves in all three solutions were swelling at a rate of 2% per hour (Fig. 12). The statistical significance of the differences in the rate of weight gain

Figure 11. - A comparison of the average percentage increase in leaf weight of dehydrated *Myrothamnus flabellifolia* leaves immersed for 21 hours in either water, Karnovsky's fixative or 5% glutaraldehyde in phosphate buffer.

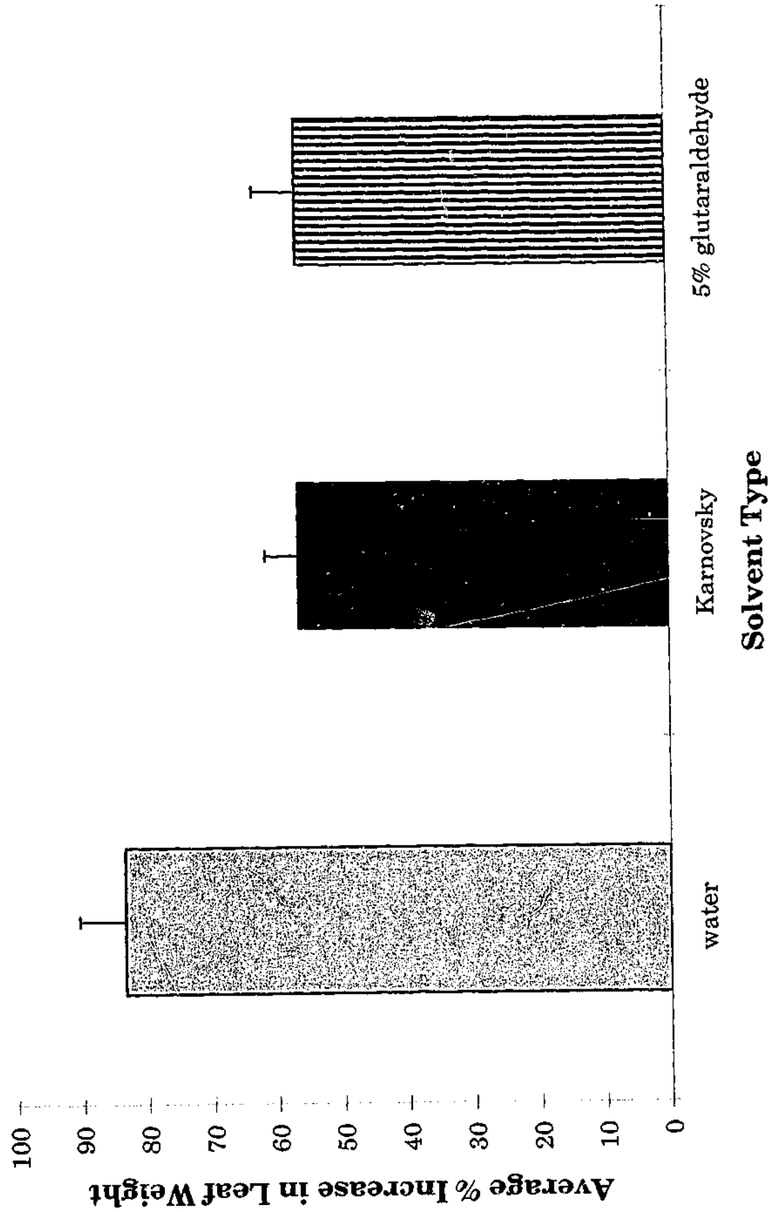
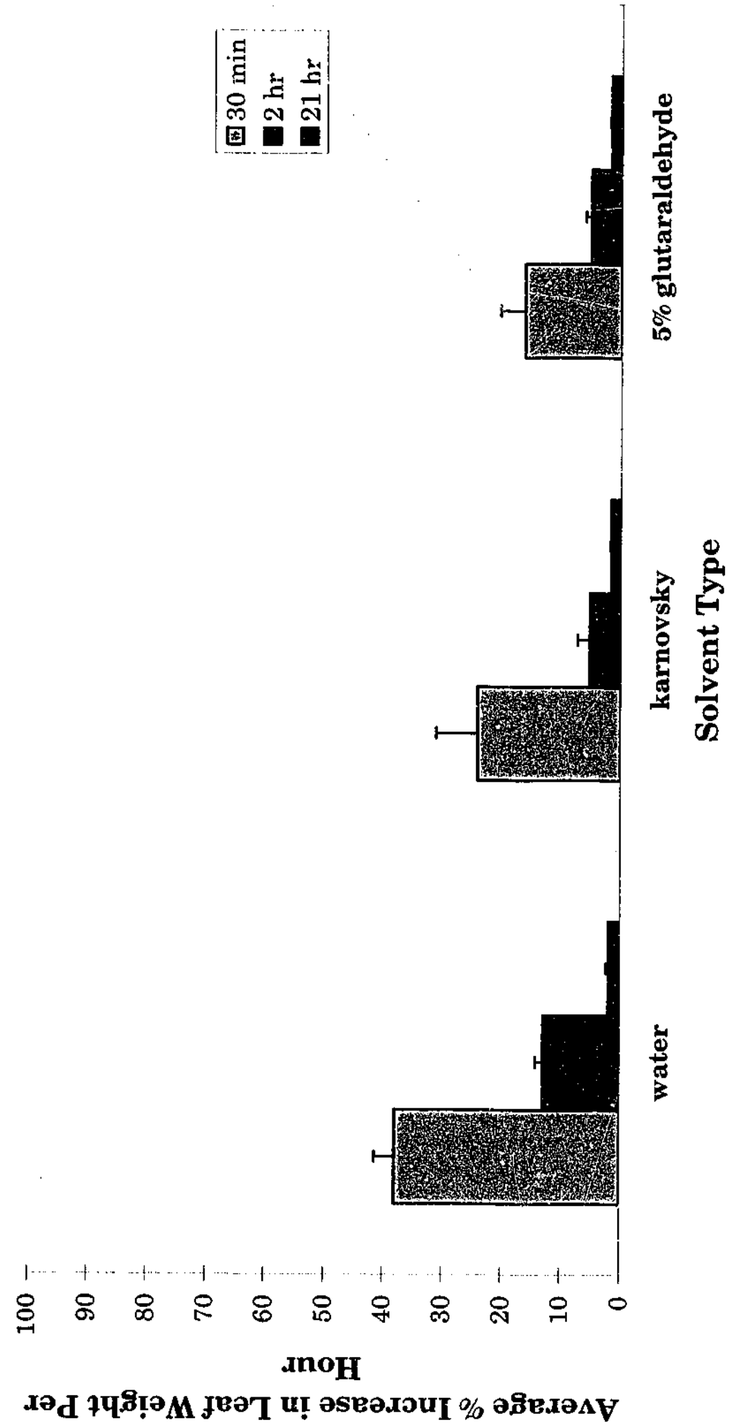


Figure 12. - A comparison of the average percentage increase in leaf weight per hour of dehydrated *Myrothamnus flabellifolia* leaves immersed for 21 hours in either water, Karnovsky's fixative or 5% glutaraldehyde in phosphate buffer.



between 2 hours and 21 hours was unable to be compared because of the presence of negative values in the 21-hour data set. Negative values cannot be arcsin transformed, which is required for analysis of data in percentage form.

2.18 - Ultrastructure after Anhydrous Chemical Fixation.

2.18.1 - Dehydrated Tissue.

Dehydrated tissue fixed in anhydrous fixatives showed distinctly different ultrastructure from dehydrated tissue fixed in aqueous fixatives. In anhydrously fixed tissue the cell walls were highly convoluted (Fig. 13a, 13b, 13c). The cytoplasm was intensely stained in comparison to hydrated leaf tissue (Fig. 13a, 13b, 13c) and the plasma membrane was mainly observed adjacent to the cell wall (Fig. 13a, 13b), although occasionally small areas of separation of the plasma membrane from the cell wall were seen (Fig. 13c). The plasma membrane was only occasionally observed (Fig. 13f) as it was frequently indiscernible from the darkly staining cytoplasm.

The central vacuole was observed in some micrographs and it frequently contained granular material (Fig. 13a, 13b). Electron-dense material was observed in the vacuole, either as spherical masses (Fig. 13a, 13c) or frequently lining the tonoplast (Fig. 13b, 13d).

Figure 13. - Dehydrated *Myrothamnus flabellifolia* leaf tissue fixed using the anhydrous chemical fixative 2% paraformaldehyde in glycerol.

Fig. 13a - A general view of dehydrated leaf tissue. A central vacuole (V) was present in the dehydrated state and it frequently contained electron-dense polyphenolic-like material (E). The cell walls (CW) were quite convoluted in nature. Chloroplasts (C) were also regularly observed. Bar represents 3 µm.

Fig. 13b - The plasma membrane (arrow head) was closely appressed to the cell wall (CW) which was convoluted. Central vacuoles (V) were regularly seen and chloroplasts (C) were regularly positioned along the periphery of the cell, adjacent to the cell wall (CW). Bar represents 2 µm.

Fig. 13c - Occasionally small areas of separation were observed between the plasma membrane and the cell wall (*). Bar represents 2 µm.

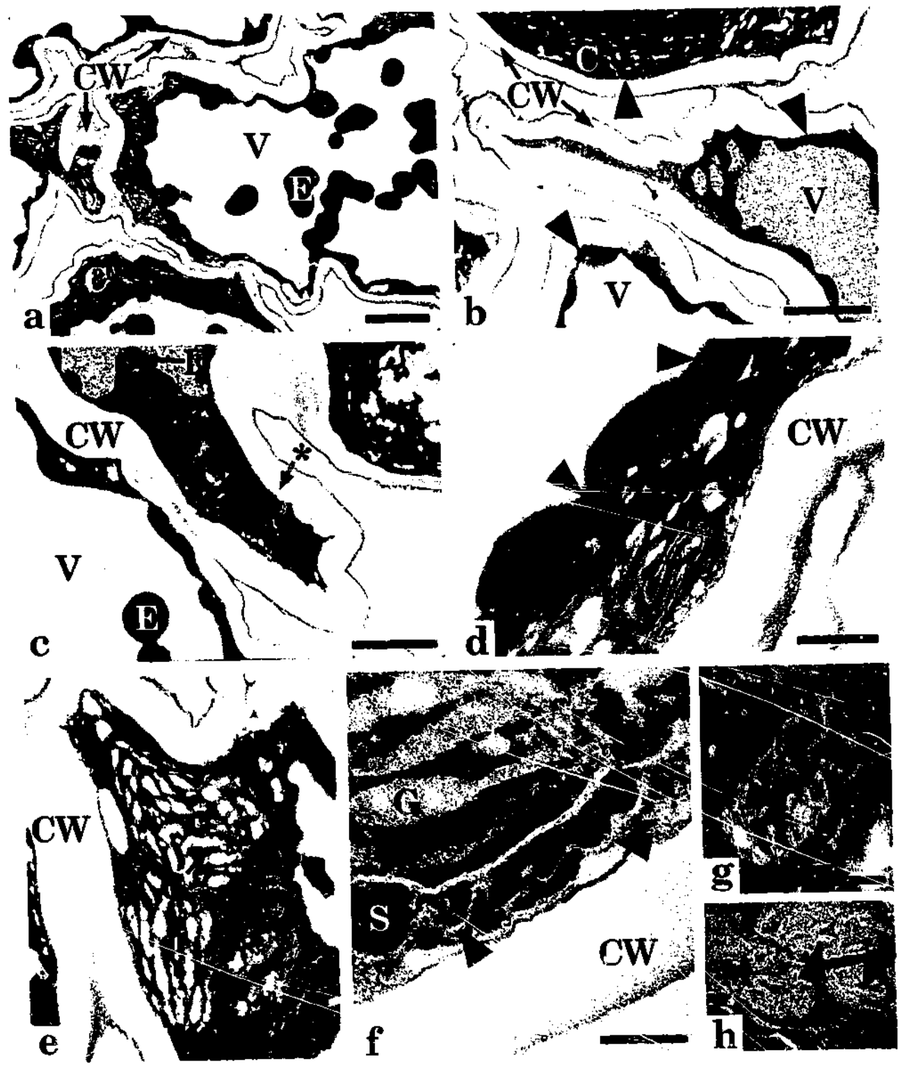
Fig. 13d - The tonoplast membrane (arrow heads) delineating the vacuole could be clearly seen whereas the plasma membrane was difficult to discern. Bar represents 0.5 µm.

Fig. 13e - Chloroplasts (C) were irregular in shape. Although the preservation of the internal membrane system was poor, granal stacks (G) were distinguishable as electron-lucent regions within the chloroplast and the stroma (S) was comparatively electron-dense. Bar represents 1 µm.

Fig. 13f - A high magnification view of a chloroplast showing the convoluted bounding membrane (arrow heads). Thylakoid membranes that comprise the granal stacks (G) could not be discerned. The stroma (S) was distinguishable as the electron-dense regions of the chloroplast. Bar represents 0.25 µm.

Fig. 13g - A mitochondrion with distinct cristae (arrow heads). Bar represents 0.25 µm.

Fig. 13h - A group of mitochondria (M) with distinct cristae (arrow heads). Bar represents 0.25 µm.



Chloroplasts in these cells were different from those observed in aqueously fixed hydrated tissue or dehydrated tissue. The chloroplasts were rounded in shape (Fig. 13e) and they possessed convoluted outer bounding membranes (Fig. 13f). They possessed numerous electron-translucent regions where granal stacks would be expected (Fig. 13e, 13f), other internal structures were difficult to discern.

Mitochondria of varying shapes from elongate to round were seen and cristae were present (Fig. 13g, 13h). Although nuclei would have been present in these cells, none were observed in these sections. It is possible that they were difficult to discern because of the dark cytoplasm or they may been situated in a part of the cell that was not sectioned. Refer to the end of this results section for a summary of these ultrastructural findings (Table 1).

2.18.2 - Hydrated Tissue.

Hydrated tissue fixed using anhydrous techniques showed signs of cell shrinkage. The walls of these cells varied from being quite smooth in shape (Fig. 14a, 14b) to being quite convoluted (Fig. 14c). The cytoplasm in general was poorly preserved. It was often greatly separated from the cell wall, with the protoplasm condensed in the centre of the cell (Fig. 14a, 14b). However, in

Figure 14. - Hydrated *Myrothamnus flabellifolia* leaf tissue fixed with anhydrous chemical fixatives.

Fig. 14a - A general view of a hydrated mesophyll cell fixed in the anhydrous chemical fixative 2% glutaraldehyde in glycerol. Cell walls (CW) were generally rounded in shape and areas of large separation between the protoplast and the cell wall were regularly observed (*). Although the general preservation of the cell contents was poor, a number of cellular structures could be discerned such as a central vacuole (V) and chloroplasts (C) that frequently possessed starch grains (SG). Bar represents 2 µm.

Fig. 14b - A second general view of hydrated mesophyll cells fixed in 2% paraformaldehyde in glycerol. As with Fig. 14a, the general cellular preservation using this fixative was poor. There was a large degree of separation between the plasma membrane and the cell wall (*). Various cellular structures however could be identified, these included chloroplasts (C), nuclei (N), vacuoles (V) and plasmodesmata (arrow head). Bar represents 2 µm.

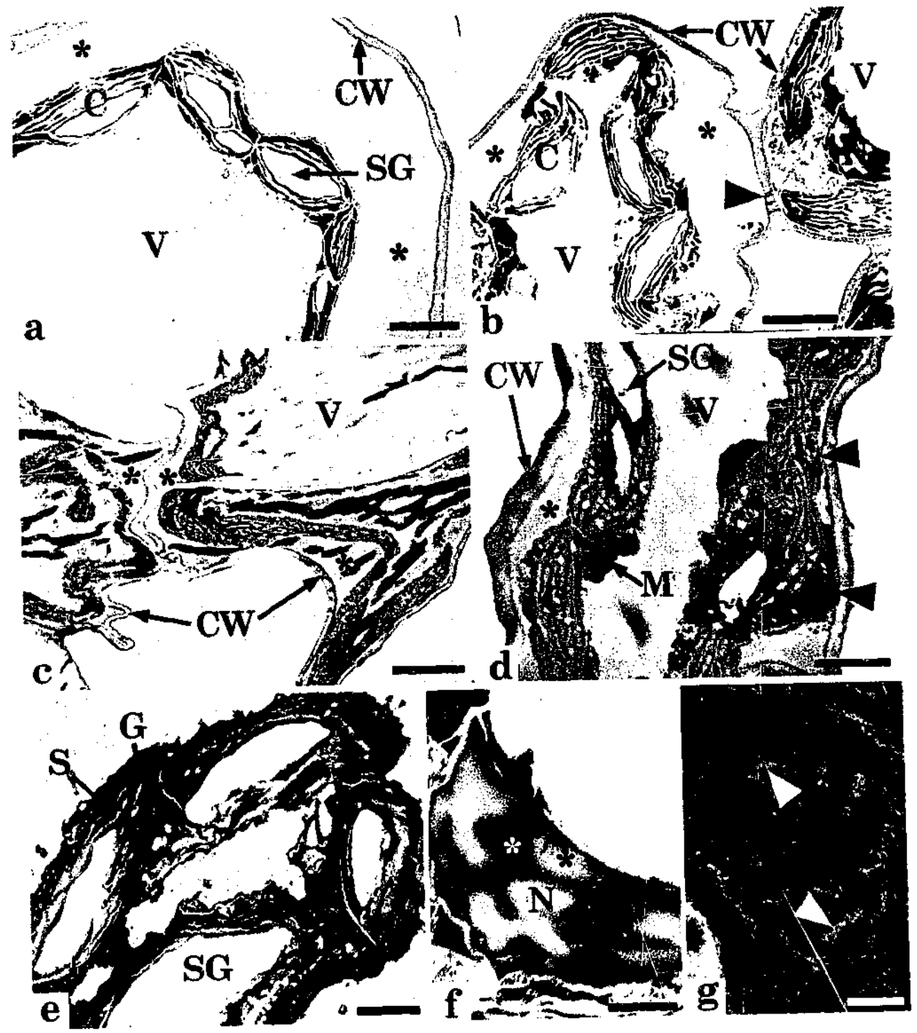
Fig. 14c - Occasionally hydrated cells were observed with convoluted cell walls (CW). In such tissue samples the plasma membrane was positioned relatively close to the cell wall although areas of separation (*) between the two structures were evident. Chloroplasts (C) and a vacuole (V) can also be seen. Tissue sample was fixed in 2% paraformaldehyde in glycerol. Bar represents 4 µm.

Fig. 14d - A magnified view of a hydrated cell fixed in 2% paraformaldehyde in glycerol. Only a small degree of separation (*) between the protoplast and the cell wall was evident. Various organelles could be identified including chloroplasts (C) that contain starch grains (SG) and mitochondria (M). Bar represents 1.5 µm.

Fig. 14e - Chloroplasts (C) were regularly observed to possess starch grains (SG). The granal (G) and stromal (S) regions of the chloroplast could be identified as light and dark staining regions respectively. Tissue sample was fixed using 2% glutaraldehyde in glycerol. Bar represents 1 µm.

Fig. 14f - A magnified view of the nucleus (N) in Fig. 14b. Nuclei were generally irregular in shape and possessed condensed chromatin that was separated into areas of electron-lucent (black *) and electron-dense (white *) material. Bar represents 0.5 µm.

Fig. 14g - A magnified view of the mitochondrion in Fig. 14d. Cristae (arrow heads) could occasionally be seen. Bar represents 0.1 µ.m



other instances, the degree of separation was not as severe and there was only a small gap evident (Fig. 14c, 14d).

The chloroplasts were oval to elongate in shape (Fig. 14a, 14b, 14c 14d) and frequently possessed large starch grains (Fig. 14a, 14b, 14f). The internal membranes of the chloroplast were poorly preserved (Fig. 14a, 14b) although the granal and stromal regions of some chloroplasts were able to be identified (Fig. 14d, 14e). Many chloroplasts contained electron-translucent plastoglobuli (Fig. 14d).

Nuclei were condensed in appearance and possessed light and dark staining regions of chromatin (Fig. 14f). Mitochondria containing cristae were observed (Fig. 14g), as were plasmodesmata (Fig. 14b). Table 1, which is located at the end of the results section, contains a summary of these findings.

2.19 - Partially Anhydrous Fixation Techniques.

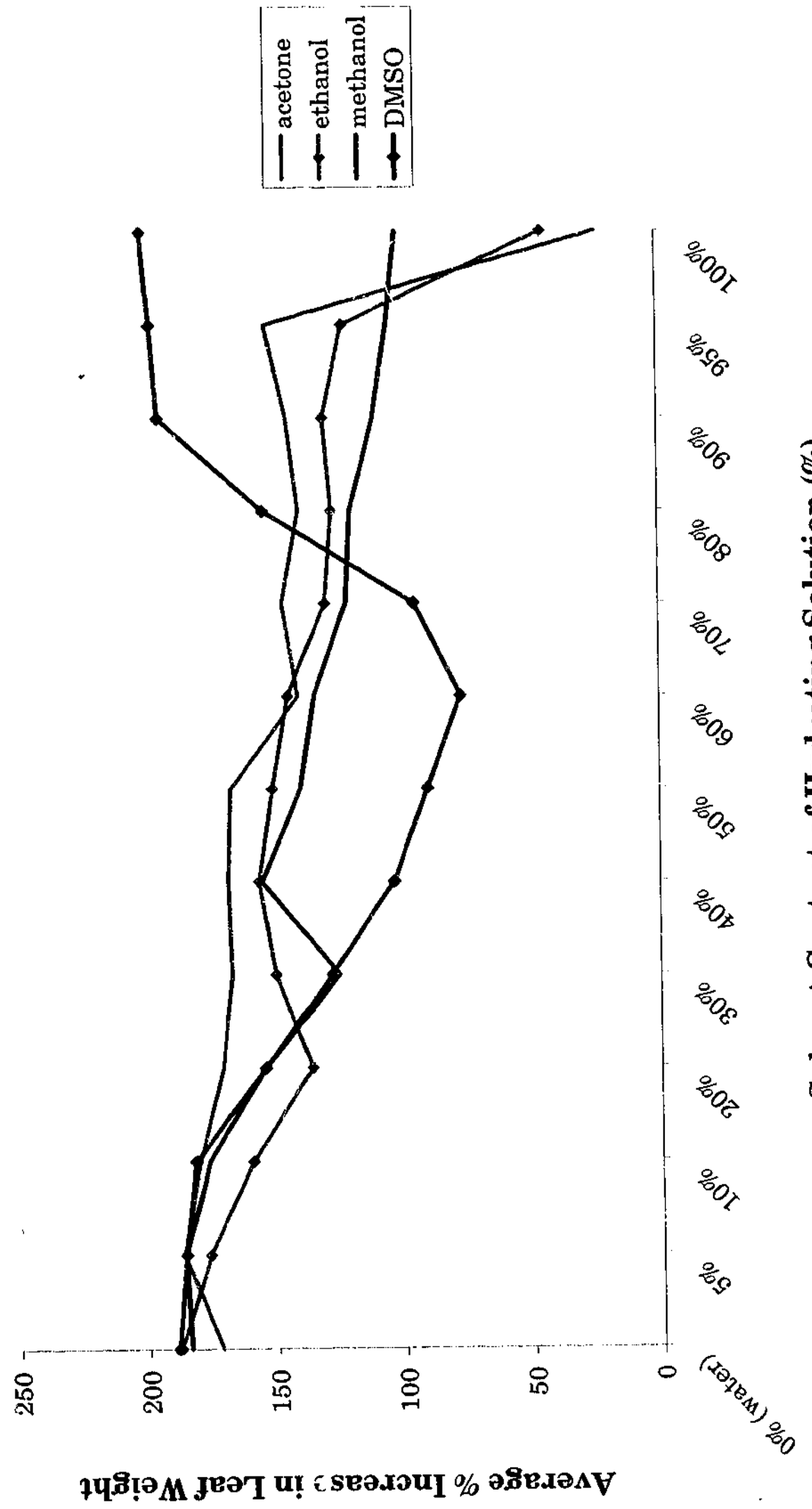
2.19.1 - The Relationship Between the Water Content of a Solution and the Degree of Swelling Experienced by a Dehydrated Leaf.

An analysis of reputedly anhydrous fixation protocols in the literature showed that some of these techniques still contained a small amount of water. As it had been shown that standard aqueous fixatives had drastic effects on cellular ultrastructure, an experiment was devised to

determine whether the percentage water content of a solution was directly proportional to the amount of swelling produced. Figure 15 shows that for solutions containing from 5% to 100% water, the different water contents of the solutions do not appear to markedly affect the total weight gain of dehydrated leaves. Leaves immersed in solutions of acetone, ethanol or methanol with varying water contents all had similar weight gains, no matter whether they were in a solution containing only 5% water or if they were in 100% water. Although there was an apparent slight reduction in weight gain between 100% water and 95% solvent, it was only when leaves were immersed in 100% acetone, ethanol or methanol that there was a substantially lower weight gain. Statistical tests revealed that the reduction in weight gain between 95% acetone and 100% acetone and between 95% ethanol and 100% ethanol were both significant (Appendix E - Tables 8 and 9). Samples immersed in 100% acetone and 100% ethanol had the two lowest levels of weight gain at 23% and 44% respectively. Samples immersed in 100% methanol had an average weight gain of 100%.

The reduced level of weight gain observed in leaves immersed in solutions containing no water did not hold true for the solvent DMSO. Even though the solution 100% DMSO contained no water, the weight gain experienced by dehydrated leaves immersed in this solution was similar to the weight gains experienced by dehydrated leaves floated

Figure 15. - A comparison of the average percentage increase in leaf weight of dehydrated *Myrothamnus flabellifolia* leaves immersed in solutions containing a solvent and decreasing amounts of water from 100% water to 100% solvent.



in 100% water. The difference in weight gain between 100% water and 100% DMSO was not statistically significant (Appendix E - Table 11). For leaves floated in DMSO, solutions of 60% DMSO resulted in the smallest weight gains. Weight gains in 30% - 70% DMSO were significantly less than in 100% DMSO (Fig. 15).

These results highlight the importance of removing as much water as possible from a fixative to ensure that the least amount of swelling occurs in a tissue sample during fixation. These results should be borne in mind when comparing the ultrastructural descriptions in the following sections.

2.19.2 - Anhydrous Fixatives That Contain a Small Amount of Water.

Dehydrated *Myrothamnus flabellifolia* leaf tissue fixed in a partially anhydrous adaptation of Karnovsky's fixative (1% paraformaldehyde and 1% glutaraldehyde in DMSO) (refer to 2.9) had cell walls that were smooth in profile (Fig. 16a). The protoplast was separated from the cell wall and was often condensed in the middle of the cell (Fig. 16a, 16b). The plasma membrane was difficult to discern. Chloroplasts were rounded and swollen in appearance (Fig. 16a, 16b, 16c). The internal membranes of the chloroplast could not be discerned, and the grana appeared relatively short and swollen (Fig. 16c). Elongate to spherical shaped

Figure 16. - Dehydrated *Myrothamnus flabellifolia* leaf tissue fixed in an 'anhydrous chemical fixative' that contained a small amount of water. All tissue samples were fixed in an adaptation of Karnovsky's fixative which consisted of 1% paraformaldehyde and 1% glutaraldehyde in DMSO.

Fig. 16a - A general view of a dehydrated mesophyll cell. Cell walls (CW) were generally smooth in outline and large areas of separation (*) between the cell wall and the plasma membrane were regularly observed. The protoplast frequently appeared to be condensed in the centre of the cell. Organelles such as chloroplasts (C) and mitochondria (M) were able to be identified. Bar represents 1 µm.

Fig. 16b - A general view of a dehydrated *Myrothamnus flabellifolia* mesophyll cell similar in appearance to Fig. 16a. The plasma membrane and the cell wall were separated (*) from each other resulting in the protoplast being contracted in the centre of the cell. Chloroplasts (C) and mitochondria (M) could be seen. Bar represents 1 µm.

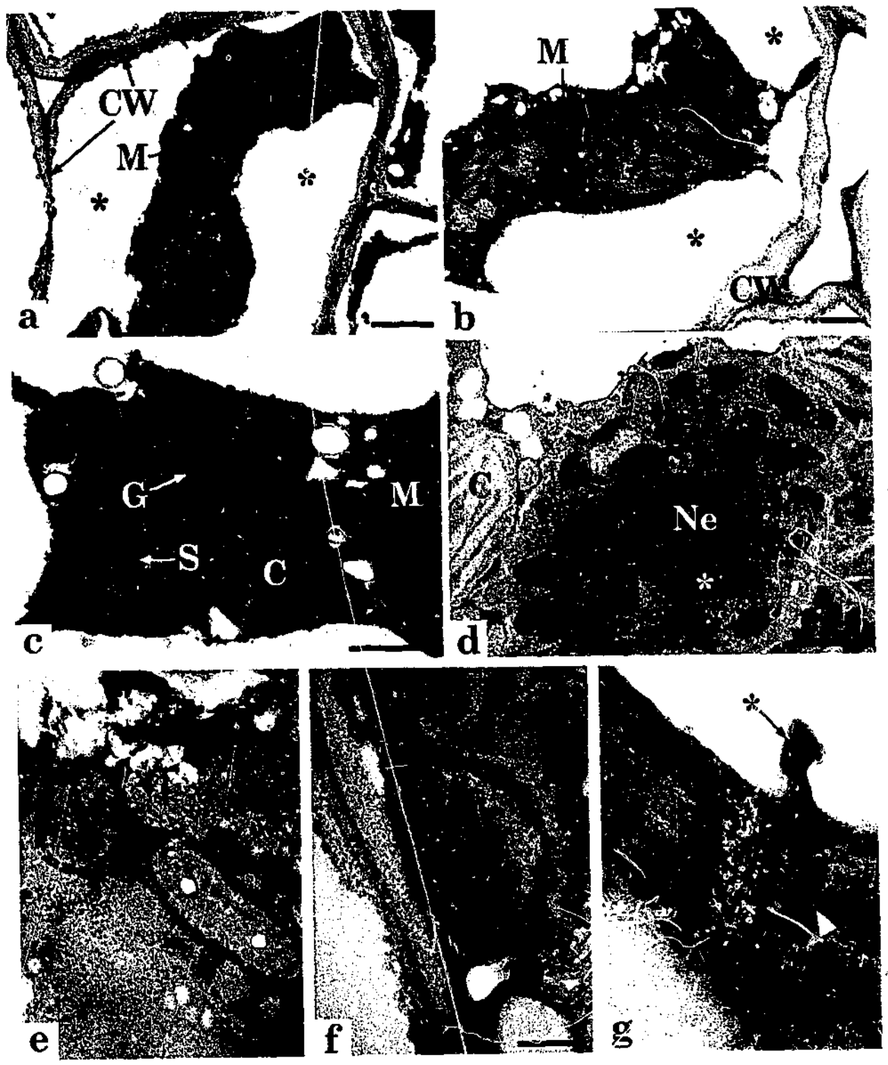
Fig. 16c - Chloroplasts (C) were quite round in shape and contained light and dark staining regions that corresponded with the grana (G) and stroma (S) respectively. The thylakoid membranes that comprise the granal stacks could not be distinguished. Mitochondria (M) could also be seen. Bar represents 1 µm.

Fig. 16d - Nuclei (N) in dehydrated tissue fixed in this manner contained condensed chromatin that was either electron-lucent (black *) or electron-dense (white *). Nuclei were frequently observed to contain a single nucleolus (Ne). Bar represents 1 µm.

Fig. 16e - Mitochondria (M) were generally round to oval in shape and cristae were not observed. Bar represents 0.25 µm.

Fig. 16f - Cytoplasmic ribosomes (R) could regularly be seen. Bar represents 0.25 µm.

Fig. 16g - Plasmodesmata (arrow head) were occasionally seen. In this micrograph, the remnant end of the endoplasmic reticulum (*) that runs through the plasmodesmata can be seen. It is possible that the endoplasmic reticulum was ruptured when the cell wall and the protoplast separated. Bar represents 0.1 µm.



mitochondria were observed although there was little evidence of cristae (Fig. 16e, 16f). The nucleus was relatively densely stained, had a convoluted outer membrane and possessed a nucleolus (Fig. 16d). Plasmodesmata (Fig. 16g) and cytoplasmic ribosomes (Fig. 16e, 16f) were also observed in these tissues.

Hydrated leaf material fixed in the above fixative had cell walls that were smooth in outline (Fig. 17a, 17b). The plasma membrane was mostly separated from the cell wall however it still occupied the periphery of the cell (Fig. 17a, 17b, 17c). In a few instances, what appeared to be connecting strands between the cytoplasm and the cell wall could be seen (Fig. 17c). The plasma membrane and tonoplast could not be distinguished. Chloroplasts were elongate in shape (Fig. 17b, 17c, 17d) and possessed electron-translucent plastoglobuli (Fig. 17d, 17e). The internal thylakoid membranes of the chloroplast could not be discerned. Mitochondria observed appeared to have few or no cristae (Fig. 17f, 17g). Some cells possessed masses of densely staining osmiophilic material in the central vacuole region of the cell (Fig. 17c). No nuclei were observed in these sections. It is possible that they were unable to be distinguished from the darkly stained cytoplasm. Refer to the end of this results section for a summary of these ultrastructural findings (Table 2).

Figure 17. - Hydrated *Myrothamnus flabellifolia* leaf tissue fixed in an 'anhydrous chemical fixative' that contained a small amount of water. All tissue samples were fixed in 1% paraformaldehyde and 1% glutaraldehyde in DMSO.

Fig. 17a - A general view of hydrated mesophyll cells. The cell walls (CW) were generally round and turgid in appearance. Cells possessed a large central vacuole (V) and the cell contents lined the periphery of the cell. There were regular separations (*) of the plasma membrane from the cell wall. Chloroplasts (C) comprised the majority of the cytoplasm. Bar represents 4 µm.

Fig. 17b - A hydrated mesophyll cell in which there was a relatively large degree of separation (*) between the plasma membrane and the cell wall. A large central vacuole (V) was regularly present and chloroplasts (C) could be seen. Bar represents 3 µm.

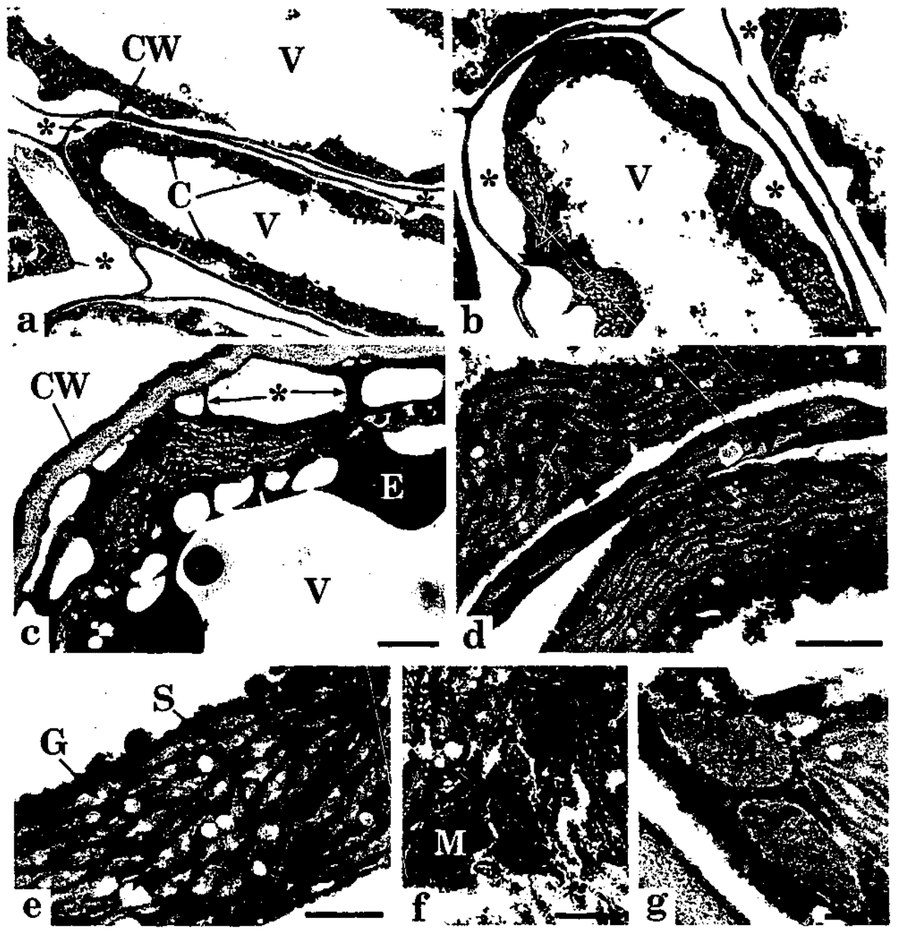
Fig. 17c - Electron-dense material (E) was frequently observed within the vacuole (V) and occasionally appeared to form a bridge (*) between the cell wall and the cytoplasm. Bar represents 1 µm.

Fig. 17d - Chloroplasts (C) were elongated in shape and often had folds or contortions (arrow head) along their length. Plastoglobuli (P) were also present. Bar represents 1 µm.

Fig. 17e - A magnified view of a chloroplast (C). Plastoglobuli (P) were present and granal regions (G) were identified as lightly staining regions in comparison to the darker staining stromal regions (S). Bar represents 0.5 µm.

Fig. 17f - Mitochondria (M) were regularly observed however cristae were not observed. Bar represents 0.5 µm.

Fig. 17g - As in Fig. 17f, mitochondria (M) containing cristae were not observed. Bar represents 0.25 µm.



2.19.3 - A Primary Anhydrous Fixative Followed by a Secondary Aqueous Fixative.

Hydrated mesophyll cells of leaf tissue that had been fixed in an anhydrous primary fixative and an aqueous secondary fixative had cell walls that were quite smooth in contour (Fig. 18a). In many instances, the plasma membrane had pulled away from the cell wall (Fig. 18b), however, in other instances it was closely appressed to the cell wall (Fig. 18a). The cytoplasm of these cells was poorly preserved and was observed to be either torn in appearance or had not been preserved during the fixation process (Fig. 18a, 18c). Chloroplasts were sometimes the only cellular structure readily discernible (Fig. 18b, 18c). Starch grains were present in many chloroplasts (Fig. 18b, 18c). Nuclei were generally oval to elongate in shape (Fig. 18d). They contained electron-dense material and nucleoli were observed (Fig. 18d). Plasmodesmata were also observed (Fig. 18e).

Dehydrated mesophyll cells of leaf tissue fixed using the same protocol also had cell walls that were smooth in contour (Fig. 19a, 19b). The plasma membrane of some cells had separated from the cell wall (Fig. 19a, 19c), however, in other cells, the cytoplasm lined the periphery of the cell and the plasma membrane was adjacent to the cell wall (Fig. 19b). The cytoplasm had a disrupted appearance (Fig. 19a, 19b) and few organelles could be identified.

Figure 18. - Hydrated *Myrothamnus flabellifolia* leaf tissue that was fixed in an anhydrous chemical fixative and was post-fixed in an aqueous chemical fixative. All tissue samples were initially fixed in 2% paraformaldehyde in glycerol and were post-fixed in 1% OsO_4 in water.

Fig. 18a - A general view of a hydrated mesophyll leaf cell. The cell wall (CW) was generally rounded in shape and a large central vacuole was frequently present. The plasma membrane lay closely appressed to the cell wall (arrow head), the preservation of the cell contents however was extremely poor (*). Very little cellular detail could be discerned. Bar represents 2 µm.

Fig. 18b - Chloroplasts (C) were one of the few organelles that could be identified. They often possessed starch grains (SG) but no other cellular detail could be identified. Bar represents 2 µm.

Fig. 18c - Another view of some chloroplasts (C) that had a fractured appearance as if they had been torn. Internal membranes of such chloroplasts could not be discerned. Bar represents 1 µm.

Fig. 18d - Nuclei (N) of hydrated cells fixed using this technique were relatively well preserved. They were relatively smooth in outline and possessed chromatin (*) that was relatively finely dispersed. Nucleoli (Ne) could also be identified. Bar represents 1 µm.

Fig. 18e - Plasmodesmata (arrow head) were also observed in tissue samples. Bar represents 0.25 µm.

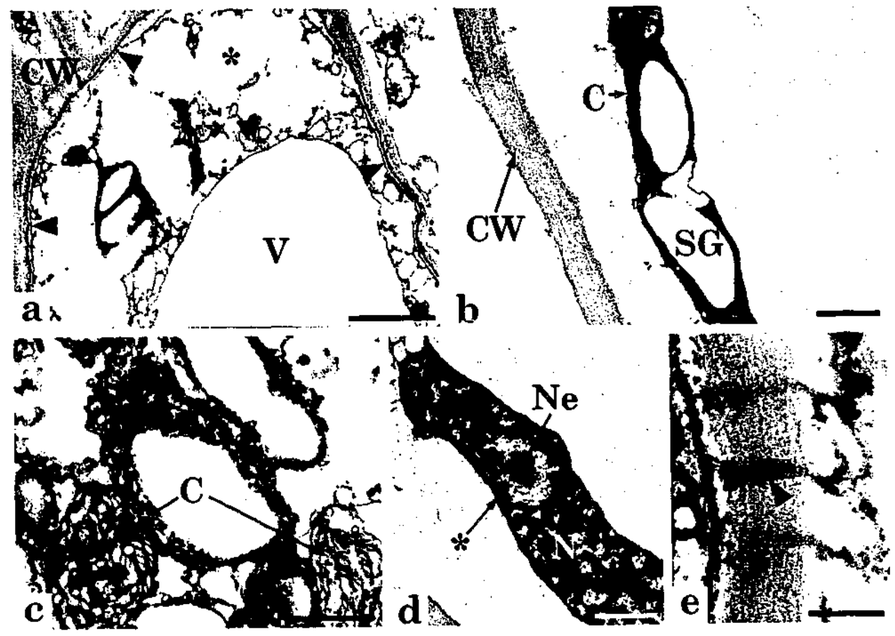


Figure 19. - Dehydrated *Myrothamnus flabellifolia* leaf tissue that was fixed in an anhydrous chemical fixative and was post-fixed in an aqueous chemical fixative. All tissue samples were initially fixed in 2% paraformaldehyde in glycerol and were post-fixed in 1% OsO_4 in water.

Fig. 19a - General view of dehydrated mesophyll cells. The cell walls (CW) of such cells were quite rounded in appearance. The general preservation of the cell was quite poor. In many instances the cell wall and the protoplast were in close alignment (arrow heads) and only occasionally were areas of cell wall and plasma membrane separation (*) observed. A membrane delimited vacuole (V) was regularly seen whereas on occasions the vacuole's bounding membrane was absent (*V). Bar represents 2 µm.

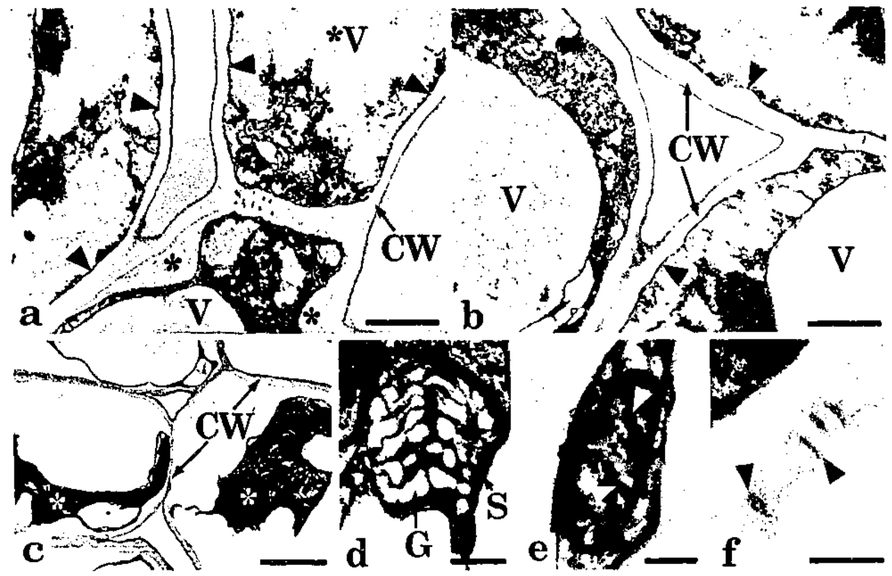
Fig. 19b - As in 19a, the cytoplasm of these cells was generally poorly preserved. The plasma membrane was generally adjacent to the cell wall (arrow heads) and large central vacuoles (V) were frequently observed. Bar represents 2 µm.

Fig. 19c - In some instances, the plasma membrane had dramatically separated from the cell wall and protoplast was often observed contracted in the centre of the cell (*). Again in these cases, the preservation of the cellular detail was poor. Bar represents 5 µm.

Fig. 19d - Chloroplasts were one of the few organelles that could be identified. The chloroplasts were generally round in shape and contained little internal detail. The granal (G) and stromal (S) regions could be identified as light and dark staining areas respectively. Bar represents 1 µm.

Fig. 19e - Mitochondria containing cristae (arrow heads) were observed. Bar represents 0.25 µm.

Fig. 19f - Plasmodesmata (arrow heads) were also observed. Bar represents 0.5 µm.



Chloroplasts were the most readily identifiable organelle, but they were frequently poorly preserved and most internal structure was lost (Fig. 19d). The thylakoid membranes of the chloroplast were not discernible although electron-translucent regions where one would expect the grana were apparent (Fig. 19d). Mitochondria were observed and they appeared to contain cristae (Fig. 19e), plasmodesmata were also regularly observed (Fig. 19a, 19f). A summary of these ultrastructural findings is contained in Table 2, which can be found at the end of the results section.

Table 1. - Summary of the ultrastructural findings for hydrated and dehydrated *Myrothamnus flabellifolia* leaf tissue fixed in aqueous or anhydrous chemical fixatives.

	Aqueous Fixative		Anhydrous Fixative	
	Hydrated	**Dehydrated**	**Hydrated**	**Dehydrated**
Cell Shape	Smooth outline.	Smooth outline, sometimes with slight folding of cell wall.	Smooth to convoluted cell walls.	Highly convoluted cell wall.
Protoplast	Adjacent to cell wall.	Plasma membrane sometimes separated from the cell wall. Cytoplasm was centrally positioned.	Separation of the cell wall and plasma membrane.	Generally adjacent to the cell wall.
Vacuole	Single, large, centrally positioned. Fine granular appearance, usually with masses of electron dense material.	Generally a large central vacuole present although occasionally smaller more numerous vacuoles present regularly contained electron dense material.		Central vacuole present which often contained electron dense material.
Chloroplast	Elongated to elliptical in shape, thylakoid membranes were visible and plastoglobuli present.	Round in shape with short granal stacks.	Oval to elongate in shape. Starch grains and plastoglobuli were present. Poor internal membrane preservation.	Round in shape with convoluted outer membranes. Poor preservation of internal membranes.
Mitochondria	Oval to round in shape. Cristae were occasionally observed.	Cristae were occasionally observed.	Oval to round in shape, cristae present.	Round to elongate in shape, with cristae.
Nucleus	Round in shape and contained granular chromatin. Nucleoli present.	Compacted and irregular in shape, contained condensed chromatin		Irregular in shape and contained condensed chromatin.

Table 2. - Summary of the ultrastructural findings for hydrated and dehydrated *Myrothamnus flabellifolia* leaf tissue fixed in anhydrous fixatives containing a small amount of water or a primary anhydrous fixative followed by an aqueous secondary fixative.

	Anhydrous Fixative with a Small Amount of Water		Primary Anhydrous Fixative Secondary Aqueous Fixative	
	Hydrated	**Dehydrated**	**Hydrated**	**Dehydrated**
Cell Shape	Smooth round cell walls.	Smooth round cell walls.	Smooth round cell walls.	Smooth round cell walls.
Protoplast	Separated from the cell wall although occupied the periphery of the cell.	Separated from cell wall and often occupied the centre of the cell.	Was sometimes adjacent to the cell wall and sometimes separated.	Was sometimes adjacent to the cell wall and sometimes separated.
Vacuole	Single, large vacuole that was centrally positioned. Sometimes contained electron dense material.	Generally absent (as protoplast often occupied centre of cell).	Single, large, centrally positioned vacuole.	Single, large, centrally positioned vacuole.
Chloroplast	Elongate in shape. Plastoglobuli were present. Poor internal membrane preservation.	Oval to round in shape. Poor internal membrane preservation. Grana were short in length.	Elongate to oval in shape. Starch grains were present. Poor internal preservation.	Round in shape. Poor internal membrane preservation.
Mitochondria	Oval to round in shape. Cristae were rarely seen.	Oval to round in shape. Cristae were rarely seen.	Oval to round in shape. Cristae were present.	Oval in shape. Cristae were observed.
Nucleus			Round in shape with an undulated outer membrane. Contained condensed chromatin. Nucleoli were observed.	Elongate in shape. Possessed a smooth outer membrane. Contained densely staining chromatin. Nucleoli were observed.

DISCUSSION.

In this study, both hydrated and dehydrated leaf tissue samples of *Myrothamnus flabellifolia* showed differences in ultrastructure depending on whether they were fixed using aqueous or anhydrous fixatives. Dehydrated tissue was particularly sensitive to any fixative that induced tissue swelling, including fixatives containing any residual water. Hence, this discussion will focus mainly on differences in the preservation of dehydrated leaf material.

2.20 - Leaf Dehydration Rate.

Before testing different fixation protocols, it was necessary to establish a consistent method for obtaining leaf material of a particular RWC. The RWC of detached shoots of *Myrothamnus flabellifolia* decreased at an almost linear rate until approximately 10-20% RWC. Below these RWCs the rate of water loss plateaued (Figs. 6 and 7). Sherwin *et al.* (1998) also found that the water content of *Myrothamnus flabellifolia* leaves dropped sharply once the water content of the soil had dried, although they used entire plants unlike this study which used detached shoots.

The desiccation tolerant plants *Xerophyta scabrida* (Tuba *et al.* 1996b), *Tortula ruralis*, and *Cladonia convoluta* (Tuba *et al.* 1996a) exhibited similar dehydration

rates to that described for *Myrothamnus flabellifolia*. Therefore, it was concluded that, *Myrothamnus flabellifolia* leaves, in regards to the rate of dehydration, was reasonably representative of desiccation tolerant plants.

2.21 - Effects of Aqueous Chemical Fixation.

Initial experiments compared the cellular ultrastructure after fixation by one of two aqueous chemical fixatives, either Karnovsky's fixative or 5% glutaraldehyde in phosphate buffer. There was no apparent difference in the quality of ultrastructural preservation of hydrated leaf tissue between these two fixatives. It was decided to use Karnovsky's fixative for future experiments because it is a fixative that has previously been used on other desiccation tolerant and water stressed plant material (Ristic and Cass 1992, Platt *et al*. 1997).

2.21.1 - Ultrastructure of Hydrated *Myrothamnus flabellifolia* Leaf Tissue.

Aqueously fixed hydrated leaf tissue of *Myrothamnus flabellifolia* showed typical ultrastructural characteristics seen in other plant species, both desiccation tolerant and desiccation sensitive. For example, the mesophyll cells possessed a large central vacuole and the cytoplasm occupied the periphery of the

cell (Table 1). Similar reports have been made for many other desiccation tolerant species (Bartley and Hallam 1979, Hallam and Luff 1980a, Goldsworthy and Drennan 1991, Quartacci *et al*. 1997, Dalla Vecchia *et al*. 1998).

Chloroplasts observed were elongate or ellipsoid in shape, as observed in a number of other desiccation tolerant species (Hallam and Luff 1980a, Hetherington *et al*. 1982, Oliver and Bewley 1984a, Gerdol *et al* 1996, Sherwin and Farrant 1996, Quartacci *et al*. 1997). Chloroplast internal membranes were well defined and were separated into granal stacks consisting of tightly packed thylakoid membranes (Table 1). Wellburn and Wellburn (1976) reported an unusual arrangement of *Myrothamnus flabellifolia* thylakoid membranes and referred to this arrangement as a 'staircase' arrangement. This study confirmed this observation. The general appearance of *Myrothamnus flabellifolia* chloroplasts did not differ significantly from descriptions of chloroplasts from desiccation sensitive species (Lopez-Carbonell *et al*. 1994).

Mitochondria of aqueously fixed hydrated leaf tissue contained distinct cristae and the nucleus contained visible chromatin that was finely dispersed (Table 1). Both the mitochondria and nuclei of *Myrothamnus flabellifolia* exhibited a normal appearance that did not differ from descriptions of these organelles in other desiccation tolerant plants (Tucker *et al*. 1975, Bewley and

Pacey 1978, Bartley and Hallam 1979, Hallam and Luff 1980a, b, Oliver and Bewley 1984a, Rajeswari *et al*. 1993).

As in previous studies on various desiccation tolerant plant species, the ultrastructure of *Myrothamnus flabellifolia* leaf tissue did not reveal any atypical structures that could possibly account for the extreme desiccation tolerance observed in this species.

2.21.2 - Absorption of Aqueous Chemical Fixatives by Dehydrated Leaf Tissue.

In the literature there is some debate about the impact that aqueous chemical fixatives have on the ultrastructure of desiccated material. During the initial fixation of dehydrated leaf material in aqueous chemical fixatives, a distinct re-greening and swelling (loss of the folded state evident during dehydration) was noticed. An attempt to quantify these observed changes was accomplished by measuring weight changes in dehydrated leaves floated in either Karnovsky's fixative or 5% glutaraldehyde in phosphate buffer over a period of 21 hours (refer to 2.17). Results from this experiment showed that *Myrothamnus flabellifolia* leaf tissue experienced weight gains when immersed in either of the two aqueous chemical fixatives. Similarly, Nir *et al*. (1969) found that segments of water stressed maize roots increased in weight by 23.5% after aqueous fixation. These findings support the idea that

quite substantial hydration may occur before the tissue is completely fixed.

Another important factor to consider is the speed at which dehydrated tissue gains weight when it is immersed in an aqueous chemical fixative. When the rate of weight increase per hour was measured, it was found that the rate of weight gain was particularly high in the first 30 minutes to 2 hours. The weight gain of dehydrated leaves at 2 hours is an interesting time to examine, as it is a frequently used time for standard chemical fixations. During the first 2 hours, 32-40% of the total increase in *Myrothamnus flabellifolia* leaf weight occurred (refer to 2.17). Smith (1991) also found that the rate of water uptake in imbibing seeds was characterized by an initial stage of rapid uptake followed by a slower rate of water uptake until full hydration was achieved. These findings are in direct contrast with the assumption used by Tuba *et al.* (1993) to justify their use of aqueous fixation. They stated that the short fixation time of 30 minutes did not apparently change the ultrastructure of *Xerophyta scabrida* cells. However, close examination of the single micrograph presented reveals possible evidence of tissue swelling (separation of the protoplast from the cell wall, refer to 2.21.3 for a detailed discussion of this observation) which questions the validity of their conclusion.

These results bring into question all previous results in which dry material was fixed in any aqueous solution.

The impact of aqueous fixatives on the ultrastructure of dehydrated *Myrothamnus flabellifolia* is discussed in the following section.

2.21.3 - Ultrastructure of Dehydrated *Myrothamnus flabellifolia* Leaf Tissue.

The ultrastructure of aqueously fixed dehydrated tissue was quite similar to hydrated tissue in some ways but it also exhibited a number of differences. The major similarity was the rounded appearance of the cells and the smooth profile of the cell walls (Table 1). Ultrastructural differences observed included the regular separation of the cell wall from the protoplast and the occasional appearance of smaller, more numerous vacuoles in dehydrated tissues compared to the close association of the cell wall and the protoplast and the large single vacuole seen in hydrated cells (Table 1). Organelles also exhibited differences between hydrated and dehydrated tissue that had been aqueously fixed. For example, the nucleus was irregularly shaped and had a dense appearance in dehydrated tissue whereas it was rounded in shape in hydrated tissue. Also, chloroplasts were round in shape in dehydrated tissue and elongated in shape in hydrated tissue (Table 1). Other researchers have reported similar findings. Öpik (1985) also noted that organelle outlines became rounded in dehydrated tissue that had been aqueously

fixed whereas in anhydrously fixed tissues organelles possessed irregular outlines. Vigil *et al.* (1984) reported that the nucleus of dehydrated seed tissue was more distended in aqueously fixed than in anhydrously fixed tissue samples.

As discussed earlier (refer to 2.2), a number of researchers suspected that aqueous fixatives could cause inadvertent rehydration during fixation and hence produce artefacts in dry tissue. In an attempt to avoid such artefacts aqueous fixatives were replaced by anhydrous chemical fixatives, such as those discussed below.

2.22 - Effects of Anhydrous Fixation Techniques.

2.22.1 - Ultrastructure of Dehydrated *Myrothamnus flabellifolia* Leaf Tissue.

Dehydrated leaf tissue of *Myrothamnus flabellifolia* fixed anhydrously showed a very different ultrastructure from that fixed aqueously. One of the most obvious differences was in cell shape. The cell walls of anhydrously fixed, dehydrated mesophyll cells were highly convoluted, in contrast to the smooth and rounded shape of dehydrated cells fixed aqueously (Table 1). Similar observations have been reported in seeds (Webb and Arnott 1982, Öpik 1985).

The high degree of cell wall folding in dehydrated tissues can also be observed in fresh sections mounted in an anhydrous solution such as glycerol or immersion oil and viewed under a light microscope. Webb and Arnott (1982) proposed that cell wall folding during desiccation is important for desiccation tolerant organisms as connections between the cell wall and the protoplasm are maintained and thus communication links between cells are preserved. If cells underwent plasmolysis during desiccation it is possible that these connections may not be saved, and could be difficult to re-establish upon rehydration.

Another major difference noted between aqueously and anhydrously fixed dehydrated leaf tissue was the degree of separation between the protoplast and cell wall. The protoplast of anhydrously fixed dehydrated cells was generally closely appressed to the cell wall, whereas in aqueously fixed dehydrated tissue, many separations between the cell wall and the protoplast were observed (Table 1). Other studies also noted these differences between aqueously and anhydrously fixed dehydrated material (Hallam 1976, Bartley and Hallam 1979, Öpik 1980, 1985). However, Goldsworthy and Drennan (1991) saw some separation of the cell wall from the protoplast in anhydrously fixed dehydrated tissue of *Myrothamnus flabellifolia*. This may have been an artefact of incomplete resin infiltration which is also evident in freeze-substituted material (refer to Chapter 4, 4.17). The fact that the cell walls were

still highly folded in Goldsworthy and Drennan's tissue samples indicates that the separation of the cell wall from the protoplast was not caused by hydration during fixation as then the cells walls would have been rounded and smooth in profile.

Vigil *et al* (1984) described cells as being plasmolyzed when the cell wall and the protoplast had separated. Öpik (1980) also described the protoplast of these cells as having pulled away from the cell wall. In contrast to these interpretations, Bartley and Hallam (1979) reported that this feature was the result of the cell wall having imbibed water and pulled away from the protoplast. Öpik (1985) supported the interpretation of Bartley and Hallam (1979) as opposed to her original interpretation (Öpik 1980). The interpretation by Bartley and Hallam (1979) seems more probable as a chemical fixative generally better stabilizes the cytoplasm of a cell than the cell wall. This is because chemical fixatives principally act on proteins and the cell wall is primarily composed of carbohydrates.

2.22.2 - Ultrastructure of Hydrated *Myrothamnus flabellifolia* Leaf Tissue.

Analogous to the observed hydration of dehydrated tissue during aqueous processing, it was postulated that hydrated tissue would undergo dehydration during anhydrous

fixation. Results revealed a number of ultrastructural differences between aqueously and anhydrously fixed hydrated tissue. For example, in anhydrously fixed leaf tissue, mesophyll cells possessed cell walls that were slightly convoluted. This contrasted to the rounded cells evident in aqueously fixed material and in fresh tissue viewed under the light microscope (Table 1).

The nucleus also exhibited differences in ultrastructure between aqueously and anhydrously fixed tissue. The nucleus of anhydrously fixed hydrated tissue appeared condensed and had regions of light and dark staining chromatin. This contrasted to nuclei in aqueously fixed tissue, which were round in shape and contained chromatin that was flocculent in appearance (Table 1).

These observations support the hypothesis that hydrated tissue undergoes dehydration during anhydrous fixation. For example, the slight folding of the cell walls in anhydrously fixed tissue samples gave the cells a flaccid appearance, which could have resulted from the loss of cellular water due to the osmotic potential of the fixative. Also, the nuclei in anhydrously fixed hydrated tissue appeared very similar to nuclei observed in dehydrated tissue. This too implies that a loss of cellular water occurred during the anhydrous fixation process and was probably the result of osmotic differences between the tissue sample and the fixative.

2.23 - Effects of Partially Anhydrous Fixation Techniques.

Some of the first attempts to develop liquid anhydrous fixatives saw a number of instances where, either accidentally or intentionally, the purported 'anhydrous' fixative contained a small amount of water. For example, Thomson (1979) used an 'anhydrous' fixative that contained approximately 3-5% water. Despite the fact that water was present in the fixative, Thomson justified its use on the grounds that hydration of the cells would not occur, as the water content of the fixative was less than the water content of dry seeds. Thus, he concluded that the osmotic flow of water would be from the tissue to the fixative. However, results in this thesis do not support the hypothesis of Thomson (1979) as *Myrothamnus flabellifolia* leaves exhibited similar weight gains whether they were immersed in solutions of 5% or 100% water (refer to 2.19.1). It was only when dehydrated leaves were immersed in completely anhydrous solutions that the percentage increase in leaf weight was greatly reduced. Moreover, only certain anhydrous solvents resulted in substantially reduced weight gains.

A possible reason why leaves with a 5-8% water content will swell in solutions with only 5% water is that when desiccation tolerant plants are completely dehydrated the small percentage of water that remains is not in the liquid

phase (Tuba *et al.* 1996a). Such water is termed 'bound water' and is closely associated with macromolecules in the cell. Water in such a state has different properties to liquid water (Vertucci and Leopold 1987). Thus, dehydrated leaves effectively contain no liquid water, and hence, will absorb liquid from a solution containing only 5% water.

Apart from highlighting the importance of the complete removal of water from fixatives when attempting to fix *Myrothamnus flabellifolia* leaf tissue, this experiment produced two other interesting findings. First, it was shown that dehydrated leaves experienced some degree of weight gain even when immersed in completely anhydrous solvents. This indicates that at least some degree of ultrastructural change will occur in any chemical fixation process. Second, it cannot be assumed that the use of anhydrous solvents will automatically result in a reduced degree of swelling, because dehydrated leaves may readily take up some solvents. In the most extreme case, leaves immersed in 100% DMSO showed weight gains comparable to leaves in 100% water.

The following two sections examine two 'partially anhydrous' fixation protocols that either contained water in the actual fixative or had an aqueous processing step after the initial anhydrous step. The ultrastructure of *Myrothamnus flabellifolia* leaf tissue fixed using 'partially anhydrous' fixatives was analyzed in order to determine whether or not small quantities of water affect

ultrastructural preservation and thus, in turn determine the need to remove all water from anhydrous procedures.

2.23.1 - Effect of Anhydrous Fixatives That Contain a Small Amount of Water.

The first 'partially anhydrous' fixative tested was an adaptation of Karnovsky's fixative which contained a small amount of water because 70% glutaraldehyde and not 100% glutaraldehyde was used to make up the fixative. Although this fixative contained only 0.5% water, the ultrastructure of dehydrated tissue fixed therein bore some similarity to hydrated leaf tissue. For example, the dehydrated *Myrothamnus flabellifolia* leaf tissue possessed cell walls that were quite round and smooth in shape (Table 2). There was a large degree of separation between the cell wall and the protoplast with the cytoplasm appearing isolated in the centre of the cell (Table 2). Similar observations were seen in dehydrated tissue that had been aqueously fixed (Table 1). This appears to indicate that the tissue experienced a certain degree of hydration during the fixation process. Bartley and Hallam (1979) who used a similar partially anhydrous fixative also observed condensation of the cytoplasm into a central mass that lay free within the walls of the cell.

Another ultrastructural similarity between tissue samples fixed using this partially anhydrous fixative and

dehydrated tissue fixed using aqueous chemical fixation is the round shape of chloroplasts (Table 2). It appears that this shape is the result of swelling, as chloroplasts in anhydrously fixed dehydrated tissue did not possess the distinct circular shape (Table 1). Similar findings were reported by Hallam and Luff (1980a, b).

These findings highlight that, at least for *Myrothamnus flabellifolia* leaf tissue, small amounts of water in a fixative can result in ultrastructural changes. Thus, these results support the findings of the previous weight gain experiments and highlight the importance that all water be removed from a fixative in order to prevent swelling artefacts.

It should be noted however that there is another factor in the 'partially anhydrous' Karnovsky's fixative that may have contributed to the ultrastructural changes observed. This is the use of DMSO as the solvent for the glutaraldehyde and paraformaldehyde. Dehydrated *Myrothamnus flabellifolia* leaves immersed in DMSO swelled significantly (refer to 2.19.1). Yatsu (1983) also found that DMSO caused swelling in dry peanut tissue and Öpik (1985) reported that the use of DMSO as a solvent for glutaraldehyde induced swelling and separation of protoplasts from cell walls, as well as some disruption of the cellular contents in dry seed tissue.

2.23.2 - Effect of a Primary Anhydrous Fixative Followed by a Secondary Aqueous Fixative.

The second 'partially anhydrous' fixation technique tested involved the use of an anhydrous fixative followed by an aqueous processing step, such as an aqueous buffer rinse or an aqueous secondary fixation. Such 'partially anhydrous' fixatives are frequently mentioned in the literature (Thomson 1979, Thomson and Platt-Aloia 1982, Chabot and Leopold 1982). When dehydrated *Myrothamnus flabellifolia* leaves were treated in this way, the cellular preservation was very poor, with the cytoplasm either torn in appearance or absent (Table 2). It is possible that the poor ultrastructural preservation observed in leaf tissue fixed in this way is the result of rehydration damage. Ultrastructural studies of the rehydration process of desiccation tolerant tissues frequently mention that membranes and organelles were disrupted in the early stages of rehydration and complete reorganization of the cellular structure was not regained for some time (Hallam and Gaff 1978, Bergstrom *et al*. 1982, Oliver and Bewley 1984a, Tuba *et al*. 1993).

Platt *et al* (1997) proposed that inadequacies of the fixation technique might be responsible for the observed cellular disruption in *Selaginella lepidophylla* tissue. There is some evidence that the primary fixation step does not necessarily fully stabilize the ultrastructure of a

cell. Orlovich and Ashford (1993) showed that semi-permeability to soluble phosphates and calcium ions was retained by vacuoles of the fungus *Pisolithus* for at least 1 hour in a glutaraldehyde fixative. Dong *et al.* (1994) also stated that membranes in red beet (*Beta vulgaris* L.) roots often remained semi-permeable for some time after stabilization of cellular matrix components. There is also evidence that a primary fixative does not always sufficiently stabilize tissue against osmotic effects of a secondary fixative. Thureson-Klein *et al.* (1975) found that 2-4% glutaraldehyde did not appreciably alter the sensitivity of noradrenergic vesicle membranes to subsequent volume changes, with the final size of the vesicle reflecting the composition of the secondary fixative. Eisenberg and Mobley (1975) also found that the primary fixative stage did not remove the osmotic response of *Rana pipiens* muscle fiber tissue and they concluded that the final volume of the muscle fibre was not fixed until the resin had been polymerized. *Myrothamnus flabellifolia* leaf tissue, in this study, was found to still increase in weight after 21 hours of fixation in two different aqueous fixatives. This indicates that although the tissue may have been completely fixed it retained a degree of osmotic sensitivity and was still able to take up water. Thus, immersing this tissue in a secondary aqueous fixative, after only two hours of anhydrous primary fixation, may

potentially result in various ultrastructural modifications.

Hydrated material fixed in this manner also showed very poor preservation. The cytoplasm of these cells, like that of dehydrated tissue, was torn in appearance (Table 2). The cellular damage observed here might also be a type of rehydration damage. It is possible that the primary anhydrous fixation resulted in the extraction of water from the hydrated tissue. As discussed previously, evidence suggests that fixatives may not fully stabilize tissue samples, thus when the secondary aqueous fixative was applied the tissue underwent rapid rehydration that resulted in the destruction of the tissue structure.

Other studies that have previously used anhydrous primary fixatives and secondary aqueous fixatives have not mentioned the severe ultrastructural damage that was observed in this study (Thomson 1979, Thomson and Platt-Aloia 1982, Chabot and Leopold 1982). However in these studies, various types of dehydrated seed tissue were examined. It is possible that seed tissues may be more resistant to rehydration damage as they may be physiologically programmed to take up water rapidly as they have to be ready to germinate fast. By contrast, leaf tissue may be programmed to take up water more slowly as the roots of a plant, as opposed to leaves, are probably the major source of water uptake. The artificial rehydration of dehydrated leaves in aqueous fixatives may

be faster than what the dehydrated leaves would normally encounter and therefore they may not be physically able to cope with the rapidity of the rehydration.

It appears from this study that primary anhydrous fixatives do not fully stabilize *Myrothamnus flabellifolia* leaf tissue and the use of a subsequent aqueous fixative therefore resulted in poor ultrastructural preservation. It was concluded that *Myrothamnus flabellifolia* leaf tissue was very sensitive to exposure to water at any stage during fixation, even when the tissue was already fixed. Thus it was necessary to ensure that any fixation technique used on dehydrated material was completely anhydrous.

2.24 - Processing Difficulties Associated with Anhydrous Techniques.

Although the results of this study showed that anhydrous fixation techniques were better able to preserve dehydrated leaf tissue than aqueous chemical fixation, some limitations were also encountered. A problem with incomplete resin infiltration was the main limitation detected. Increasing the length of time in which the tissue samples were infiltrated did improve the quality of the embedding obtained however imperfect infiltration was still occasionally encountered. Incomplete resin infiltration caused the tissue samples to fragment during sectioning, this made it extremely difficult to obtain

sufficient intact cells to assess the quality of preservation and observe cell ultrastructure.

This problem has plagued other investigators interested in dry seed ultrastructure (Hallam 1976, Khoo and Wolf 1970, Mollenhauer and Totten 1971, Paulson and Srivastava 1968, Swift and O'Brien 1972). On the other hand, workers who fixed seed tissues with aqueous fixatives did not mention encountering difficulties during embedding (Morrison Baird *et al.* 1979, Klein and Pollock 1968, Swift and O'Brien 1972, Yatsu 1983). Yatsu (1983) proposed that the compacting of the cell wall with the concomitant loss of microcapillary spaces is the major cause of difficulty of resin penetration into dry seeds. Yatsu (1983) found that in every case of anhydrous processing, plastic embedding of peanut tissue failed or at best was imperfect. Mollenhauer and Totten (1971) also proposed that the density of cell walls, the secretion of slimes and waxes, and the relatively dehydrated state of seed tissues could act as barriers to the penetration of fixatives and resins.

2.25 - A Comparison of Aqueously Fixed Hydrated Leaf Tissue and Anhydrously Fixed Dehydrated Leaf Tissue.

Analysis of the results in this chapter reveal that the best ultrastructural preservation of hydrated tissue was achieved using aqueous fixation and the best ultrastructural preservation of dehydrated tissue was

achieved using anhydrous fixation. Using this as a basis for comparison, the most obvious difference between hydrated and dehydrated tissue was the compacted and irregular shape of dehydrated cells in comparison to the smooth rounded shape of the cell walls in hydrated tissue (Table 1). A number of organelles also exhibited ultrastructural differences between hydrated and dehydrated tissue samples. For example, the nucleus was irregularly shaped and dense in appearance in dehydrated tissue whereas it was rounded in shape in hydrated tissue (Table 1). Chloroplasts in hydrated tissue were elongated and had a smooth outline whereas those in dehydrated tissue samples were oval with convoluted bounding membranes (Table 1). Goldsworthy and Drennan (1991) also noted that the plastid membrane of dehydrated *Myrothamnus flabellifolia* leaf tissue was convoluted after anhydrous vapour fixation. It is possible that the convoluted bounding membranes of dehydrated chloroplasts are indicative of a surplus of membrane material, the result of a contraction of the organelle. Tiwari *et al.* (1990) also hypothesized that the irregular outline of the plasma membrane and nuclear envelope in pear pollen may be a manifestation of the surplus membrane material. It is well known that during dehydration, cell and organelle matrices undergo a volumetric contraction, which produce a surplus of membrane material (Webb and Arnott 1982, Öpik 1985). Chloroplasts, plastids, mitochondria and nuclei are amongst a number of

cellular structures that are regularly described as being contracted when dehydrated (Yatsu 1965, Öpik 1980, 1985, Goldsworthy and Drennan 1991). The proliferation of vesicles in the cytoplasm is also thought to play a role in membrane preservation during dehydration (Singh *et al.* 1984, Tiwari *et al.* 1990). This was observed in *Tortula ruralis* in the dehydrated state (Singh *et al.* 1984), but no such observation was made during course of this thesis. It is possible that some of the protective mechanisms initiated in *Myrothamnus flabellifolia* during dehydration enable in situ preservation of membranes whereas the absence of such protective mechanisms in *Tortula ruralis* result in the dismantling of cellular membranes during dehydration.

Mitochondria did not appear to differ ultrastructurally between the hydrated and dehydrated states. In both tissue types, various-shaped mitochondria were observed and cristae were present in all cases (Table 1). Other studies also observed the presence of cristae in dehydrated tissue (Yatsu 1965, Vigil *et al.* 1984, Öpik 1985, Goldsworthy and Drennan 1991). Their findings are in contrast to the results of other workers who have noted an absence or reduction in number of mitochondrial cristae in dehydrated tissue (Thomson 1979, Bartley and Hallam 1979, Öpik 1980, Hallam and Luff 1980a, b, Demason and Thomson 1981, Vigil *et al.* 1984). A possible reason for this disparity is the use of aqueous fixatives to preserve

dehydrated tissue, as all studies that observed a lack of cristae used aqueous chemical fixatives and those studies that did detect cristae used anhydrous fixation techniques.

The above results illustrate a number of ultrastructural differences between hydrated and dehydrated tissue. Even though *Myrothamnus flabellifolia* tissue undergoes various ultrastructural changes during dehydration, the continued presence of various cellular structures in the dehydrated state and the ease with which they are able to be identified led to the conclusion that the structural integrity of *Myrothamnus flabellifolia* is maintained in the dehydrated state. Goldsworthy and Drennan (1991) drew the same conclusion for *Myrothamnus flabellifolia* leaf tissue.

The above conclusions contrast to the observations of other researchers who have described desiccation tolerant tissues in the dehydrated state as being structurally disorganized (Hallam 1976). It is possible that the use of inappropriate fixation techniques could have led to such conclusions, as this type of ultrastructural observation is generally associated with the fixation of dehydrated material with aqueous fixatives. Using such fixatives on dehydrated leaves of *Myrothamnus flabellifolia* also resulted in reduced levels of ultrastructural preservation.

2.26 - **Conclusions.**

Results from this chapter show the importance of matching the fixative to the tissue type, particularly for dehydrated tissues. Whilst the development and use of anhydrous fixatives has been instrumental in gaining a more accurate understanding of the ultrastructure of dehydrated biological tissue samples, it has served as only a partial solution to the problem of fixing dry and partially dry material. Similar to the swelling exhibited by dehydrated tissues in aqueous fixatives, correspondingly, hydrated tissues shrink when fixed in anhydrous fixatives. Thus, in studies that attempt to examine a series of tissue samples between complete hydration and dehydration, it is not clear when to change from aqueous to anhydrous fixatives. Platt *et al.* (1997) experimented with chemical fixatives of differing osmolarities, and found that tissues that were partially hydrated were the most sensitive to fixative osmolarity. Consequently, it appears that the safest use of both aqueous and anhydrous chemical fixation techniques is at the extremes of tissue hydration and dehydration respectively.

While much useful information has been gleaned by the application of conventional aqueous fixatives to dry seed tissues, significant artefacts have been reported. Anhydrous preparation methods avoid the artefacts associated with aqueous fixation of dehydrated tissue and

give a truer picture of organelle dimensions and membrane integrity. Despite the improved quality of ultrastructural preservation associated with the anhydrous fixation of dehydrated tissue, anhydrous techniques are still far from being routine procedures. A number of major problems are associated with anhydrous fixation techniques and these include incomplete resin infiltration and sectioning difficulties. Also, anhydrous techniques have the reputation of being technically difficult to apply (Öpik 1980, Grote *et al.* 1999). However for a more accurate representation of the structure and composition of tissues in the dry state, these procedures may be essential.

Since existing techniques give such variable results, it has been difficult to distinguish between ultrastructural changes that are a direct response to desiccation and those ultrastructural changes that are artefactual and result from the use of inappropriate fixation techniques. Thus, there is a need for the development of a fixation technique that is able to produce replicable results and eliminate uncertainty as to the cause of any observed ultrastructural changes. The use of a single fixation technique on both hydrated and dehydrated materials will help to distinguish those ultrastructural changes, if any, that are a direct response to desiccation. The next two chapters will examine two such fixation techniques, which have not previously been used on tissues from desiccation tolerant plants.

Chapter 3 - Phase-Partition Fixation

INTRODUCTION.

3.1 - Phase-Partition Fixation.

In the previous chapter, a range of anhydrous techniques were evaluated, each of which had limitations in the preservation of cell ultrastructure. This chapter evaluates a new technique, phase-partition fixation, not previously tested on desiccation tolerant plant tissue. This technique is completely anhydrous through all stages of processing from fixation to embedment.

Phase-partition fixation is based on the principle of solutes (i.e. aldehyde fixatives) being partitioned between two immiscible solvents (McFadden *et al.* 1988). A fixative in a water-immiscible solvent can partition into a tissue without any artefacts caused by hydration such as tissue swelling or to leakage of ions, solutes or other cell components into the fixative. Because aldehyde fixatives are unavailable in pure form (formaldehyde is a gas, glutaraldehyde a viscous liquid), before the tissue is fixed, the fixative must be partitioned from an aqueous solution into the water-immiscible solvent. The two immiscible solvents used in phase-partition fixation are an aqueous chemical fixative and a non-polar solvent. The immiscibility of the two solvents allows for the diffusion of the aldehyde fixatives between the two solvents but prevents the movement of water from the aqueous chemical

fixative into the non-polar solvent. Tissue samples are then fixed in the aldehyde-containing non-polar solvent. Osmotic artefacts are also prevented using this method as any water in the tissue samples is unable to diffuse out. Thus, phase-partition fixation allows the chemical fixation of tissue samples in an anhydrous environment.

The technique of phase-partition fixation has in the past been used mainly on animal tissues. It was developed by Zalokar (1971) in order to fix *Drosophila* eggs. Many insect eggs, including those of *Drosophila*, are surrounded by a membrane which prevents entry of water based fixatives. This precluded the use of standard aqueous fixatives unless the vitelline membrane was actively torn in order to expose the cytoplasm. This however, resulted in non-uniform penetration of the fixative and distortion of the cellular morphology (Hattel *et al.* 1983). Zalokar (1971) and Zalokar and Erk (1977) discovered that a fixative would permeate *Drosophila* eggs if previously dissolved in a non-polar solvent. Since its development, phase-partition fixation has been used mainly for the successful fixation of the mucus lining of animal airways (Sims *et al.* 1991, Lee *et al.* 1995, Geiser *et al.* 1997, Sims and Horne 1997). This is because conventional fixation methods are generally unable to preserve the mucus before it is either washed away or dissolved (Sims *et al.* 1991, Allan-Wojtas *et al.* 1997). Phase-partition fixation has also been used recently on intestinal mucus linings

(Allan-Wojtas *et al.* 1997) and the epithelial mucus coat of teleosts (Sanchez *et al.* 1997). Thurston *et al.* (1976) and Schürch *et al.* (1998) also utilized this technique for analysis of general, non-mucus lung ultrastructure. A few studies have utilized this method for the successful preservation of RNA for hybridization histochemistry in *Drosophila* (Hafen *et al.* 1983, Cabrera *et al.* 1987) and plant tissues (McFadden *et al.* 1988). The latter report was the only report found that described the use of phase-partition fixation on plant tissue.

A number of non-polar solvents have been used as the vehicle for the active compounds of the aqueous fixative. Heptane was the solvent used by Zalokar (1971) and Thurston *et al.* (1976) were the first group of researchers to use perfluorocarbons. Perfluorocarbons are derivatives of hydrocarbons and are produced by replacing all hydrogen molecules with fluorine molecules (Krafft and Riess 1998). This leads to an increase in the molecular mass and these solvents usually have a specific gravity approximately twice that of water. They are extremely stable, both chemically and thermally, owing to the strength of the carbon-fluorine bond (Lowe *et al.* 1998, Krafft and Reiss 1998). The high density, low viscosity and low surface tension of perfluorocarbons allow tissue surfaces to be evenly coated with the fixative and allow it to penetrate the entire surface of the tissue sample (Allan-Wojtas *et al.* 1997). Perfluorocarbons also have a high gas-

dissolving capability (Horne and Sims 1991, Krafft and Riess 1998) which has led to their use in the medical field. Geyer *et al.* (1968) first introduced perfluorocarbons as an oxygen carrying blood substitute, showing that rats can survive for hours after their red blood cells have been totally replaced by a perfluorocarbon in emulsion. Perfluorocarbons have since been used as a blood substitute in various physiological studies on animals (Schneeberger and Neary 1982) and have also been administered to humans as an immediate treatment for massive haemorrhaging or to patients who refused a blood transfusion on religious grounds (Mitsuno *et al.* 1984, Tremper and Cullen 1984).

Phase-partition fixation has a number of advantages over standard aqueous chemical fixation techniques. For example, it has been shown to significantly reduce volume changes in samples caused by the movement of water, both in gel blocks and in actual samples of liver tissue (Hattel *et al.* 1983). It should be noted however that phase-partition fixation does not completely eliminate all volume changes observed in fixed samples (Hattel *et al.* 1983). This is because shrinking or swelling of tissue samples during fixation is due not only to the osmotic movement of water but is also due to the effect of conformational changes in the fixed protein (Hattel *et al.* 1983). Nettleton and McAuliffe (1986) suggest that the post-fixation processes of dehydration and embedding may also affect tissue volume.

The majority of phase-partition fixation studies to date have followed the primary phase-partition fixation step with an aqueous secondary fixation step (Zalokar 1971, Thurston *et al.* 1976, Zalokar and Erk 1977, Hafen *et al.* 1983, McFadden *et al.* 1988, Sims *et al.* 1991, Lee *et al.* 1995, Sanchez *et al.* 1997, Geiser *et al.* 1997, Allan-Wojtas *et al.* 1997, Sims and Horne 1997). The impact of this aqueous step on volume change is not mentioned in any of these studies, probably because their main priority was not the maintenance of dehydrated tissue ultrastructure throughout the fixation process. Even so, there are number of instances in the literature where a primary anhydrous fixative step is insufficient to fully stabilize the tissue sample and secondary aqueous fixation steps result in various volume changes (refer to Chapter 2, 2.22).

Other reported advantages of phase-partition fixation include its ability to reduce leaching of components such as proteins (Mays *et al.* 1984) and lipids (Leist *et al.* 1986). It has been used to eliminate surface tension effects of aqueous fixatives that result in the incomplete fixation of some tissue samples (Thurston *et al.* 1976, Vaughn, K. pers. comm.). Phase-partition fixation of tissue also results in a stronger RNA hybridization signal compared to samples fixed in standard aqueous fixatives (McFadden *et al.* 1988).

These attributes make phase-partition fixation a potentially valuable technique for a wide range of

applications. Probably the main advantage that phase-partition fixation offers this study is its osmotically neutral nature and the resulting potential to reduce and/or eliminate volume changes caused by the osmotic movement of water. Hattel *et al.* (1983) pointed out that if the volume changes, which normally occur during fixation, are reflected in morphological alterations, phase-partition fixation may be of significant practical value for ultrastructural studies. Phase-partition fixation has been found to be suitable for general ultrastructural studies at both the light (Nettleton and Rice 1982) and electron microscope (McAuliffe and Nettleton 1984, McFadden *et al.* 1988) levels. Although it has been acknowledged previously that the attributes of phase-partition fixation would be advantageous for use on osmotically sensitive tissues (Horne and Sims 1991), to my knowledge, the following chapter describes the first time that this technique has been utilized to preserve the ultrastructure of dry tissues.

MATERIALS AND METHODS.

3.2 - Pre-Fixation Techniques.

The same plant material and pre-fixation techniques were used in this chapter as are outlined in the Materials and Methods section of Chapter 2 (refer to 2.6).

3.3 - Phase-Partition Fixation.

3.3.1 - Fixatives and Solvents Used.

The different fixatives used during the course of this study were: 1) 37% formaldehyde (neat concentration from bottle), 2) 25% glutaraldehyde (neat concentration from bottle), 3) Karnovsky's fixative, consisting of 4% paraformaldehyde, 4% glutaraldehyde in 0.08M phosphate buffer, 4) 1% osmium in distilled water and 5) 4% paraformaldehyde in 0.08M phosphate buffer.

A number of different non-polar solvents were also used, they were the perfluorocarbon - FC-72™ (3M), the hydrofluoroether - HFE 7100™ (3M) and n-heptane (Sigma). All of the solvents used are considered immiscible with water, with the solubility of water in FC-72 and HFE 7100 approximately 10 parts per million (3M Fluorinert information sheet) and 95 parts per million, respectively (3M HFE information sheet). N-heptane contains less than

300 parts per million of water at room temperature (Marsden and Mann 1963).

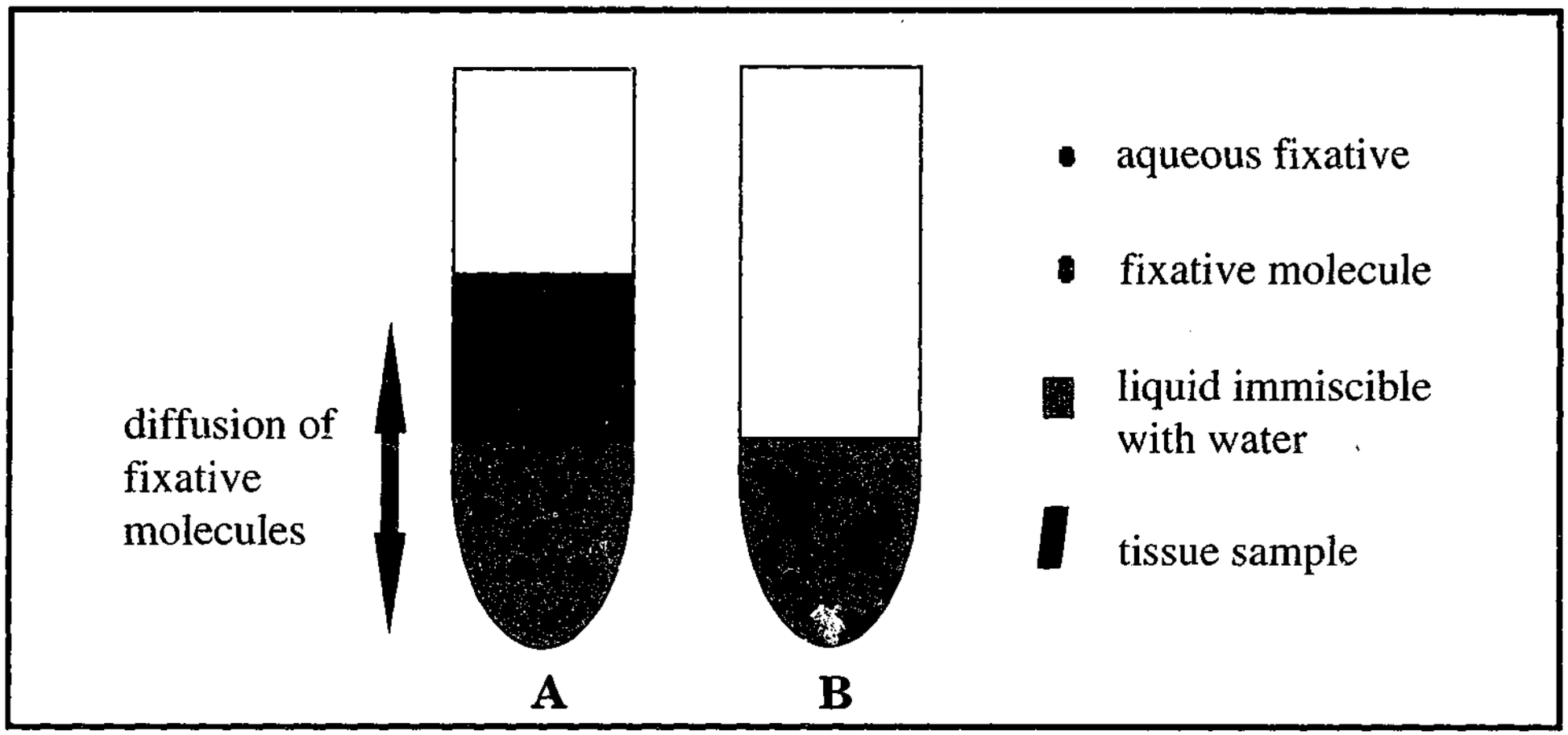
3.3.2 - Technique of Phase-Partition Fixation.

Equal volumes of fixative and the non-polar solvent were combined in a test tube and were shaken vigorously (Fig. 20). They were then left to stand overnight to allow the fixative to equilibrate between the two solutions. The aqueous fixative was then discarded (whether this was the top or the bottom solution depended on the relative density of the two solutions) and the tissue samples were immersed in the non-polar solvent/fixative solution. The samples were fixed for periods of 2, 6 or 24 hours and during this time they were gently shaken on an orbital shaker.

After fixation, the tissue samples were dehydrated either in acetone, or they were chemically dehydrated using 2,2-Dimethyoxypropane (DMP). The testing of two dehydration methods allowed me to compare the relative effectiveness of the two methods. There was no discernible ultrastructural difference between the two protocols so both methods were used during the course of this study. Hydrated and dehydrated samples that were transferred into acetone were processed slightly differently. Dehydrated samples were transferred into 100% acetone. The acetone was replaced every 30 minutes and after 2 hours the samples were placed in 5% Spurr's resin. Resin infiltration

Figure 20. - Diagram of the phase-partition fixation technique.

A standard aqueous chemical fixative is layered on top of a nonaqueous solvent that is immiscible with water (Test-tube A). The solutions are allowed to stand overnight in order for the complete diffusion of fixative molecules throughout both solutions. Water molecules are unable to diffuse out of the standard chemical fixative due to its immiscibility with the nonaqueous solvent. The aqueous fixative is then removed and the tissue samples to be fixed are immersed in the anhydrous solvent (Test-tube B). Depending on the specific gravity of the anhydrous solvent in comparison to water, the aqueous fixative may be either on top or below the chemical fixative.


diffusion of fixative molecules
A
B
aqueous fixative
fixative molecule
liquid immiscible with water
tissue sample

followed the standard method outlined earlier (refer to Chapter 2, 2.7). Hydrated samples on the other hand were placed in 70% acetone and went through a dehydration schedule of 70%, 80%, 90%, 95% acetone with 15 minutes in each step. The presence of water in the hydrated samples necessitated the samples undergoing the above described dehydration schedule. The hydrated samples were then placed in 100% acetone after which the processing of the samples was the same as for the dehydrated samples.

Chemical dehydration involves immersing tissue samples into acidified DMP (Muller and Jacks 1975, Kaeser 1989, Möller and Möller 1994). DMP is a solvent that chemically dehydrates tissue by the instant hydrolysis of water to form methanol and acetone. Acidified DMP is prepared by adding 0.05 ml concentrated HCl to 23 ml DMP (Muller and Jacks 1975, Kaeser 1989). After fixation, the non-polar solvent was removed and both hydrated and dehydrated samples were immersed in 2-5ml acidified DMP for approximately 15 minutes. Möller and Möller (1994) suggested that a suitable time for chemical dehydration in DMP would correspond to one step in a graded alcohol series whereas other researchers used longer dehydration times such as overnight (Grote 1992, Ristic and Ashworth 1993). After this, the samples were transferred into 100% acetone and processed as outlined above.

All samples were infiltrated in Spurr's resin using the standard resin infiltration technique (refer to Chapter 2, 2.7) and were then polymerized overnight in a 60°C oven.

3.4 - Determination of Weight Gain By Dehydrated Leaves Immersed in Various Solutions.

The methods used to determine the weight gain of dehydrated leaves immersed in different solutions were the same as outlined in the previous chapter (refer to Chapter 2, 2.11).

3.5 - Statistical Analyses.

All statistical methods used in this thesis are outlined in Chapter 2 (refer to 2.13).

RESULTS.

3.6 - Weight Gain of Dehydrated Leaves Immersed in Anhydrous Solvents.

The average increase in weight of a dehydrated leaf was 18% after immersion in FC-72 and was 12% after immersion in n-heptane for 21 hours (Fig. 21). The difference in weight gain between FC-72 and n-heptane was not significant. The weight increases exhibited by dehydrated leaves immersed in FC-72 and n-heptane were two of the three lowest values of all solutions tested during the course of this study. The weight gains in either FC-72 or n-heptane were significantly different to that of leaves immersed in either water or the aqueous chemical fixatives Karnovsky's fixative or 5% glutaraldehyde in phosphate buffer (Appendix E - Table 6).

The percentage increase in weight per hour shows that the greatest rate of weight gain occurred during the first stages of immersion. After 30 minutes of immersion leaves in FC-72 and n-heptane increased in weight at an average of 17% and 14% per hour, respectively (Fig. 22). After 2 hours of immersion these values had decreased to a rate of 2% per hour for leaves in FC-72 and 0.6% per hour for leaves in n-heptane. The difference in the rate of weight gain at 30 minutes and at 2 hours was significantly different for each of the solutions tested (Appendix E -

Figure 21. - A comparison of the average percentage increase in leaf weight of dehydrated *Myrothamnus flabellifolia* leaves immersed for 21 hours in either FC-72 or n-heptane.

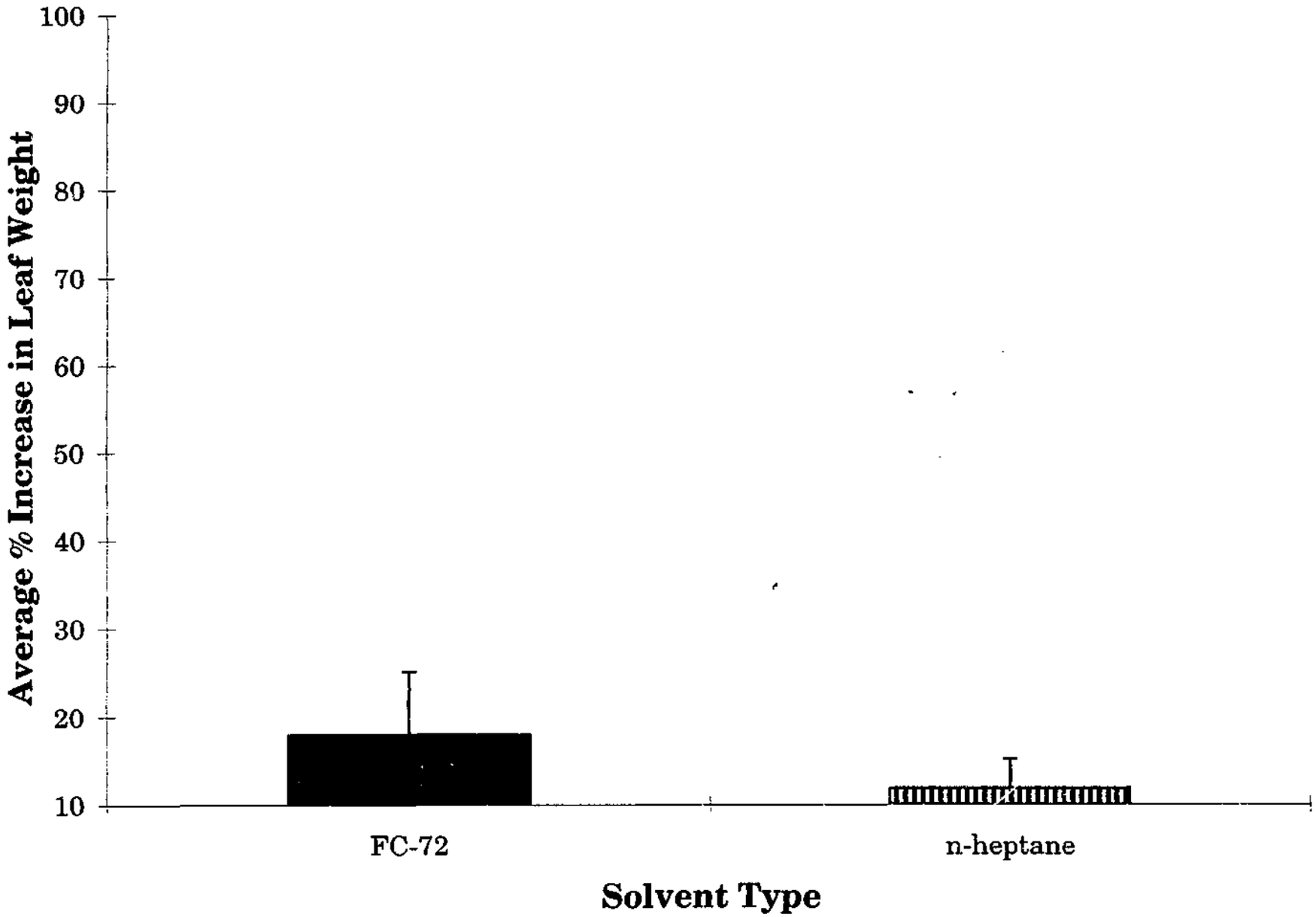

Average % Increase in Leaf Weight
100
90
80
70
60
50
40
30
20
10
FC-72
n-heptane
Solvent Type

Figure 22. - A comparison of the average percentage increase in leaf weight per hour of dehydrated *Myrothamnus flabellifolia* leaves immersed for 21 hours in either FC-72 or n-heptane.

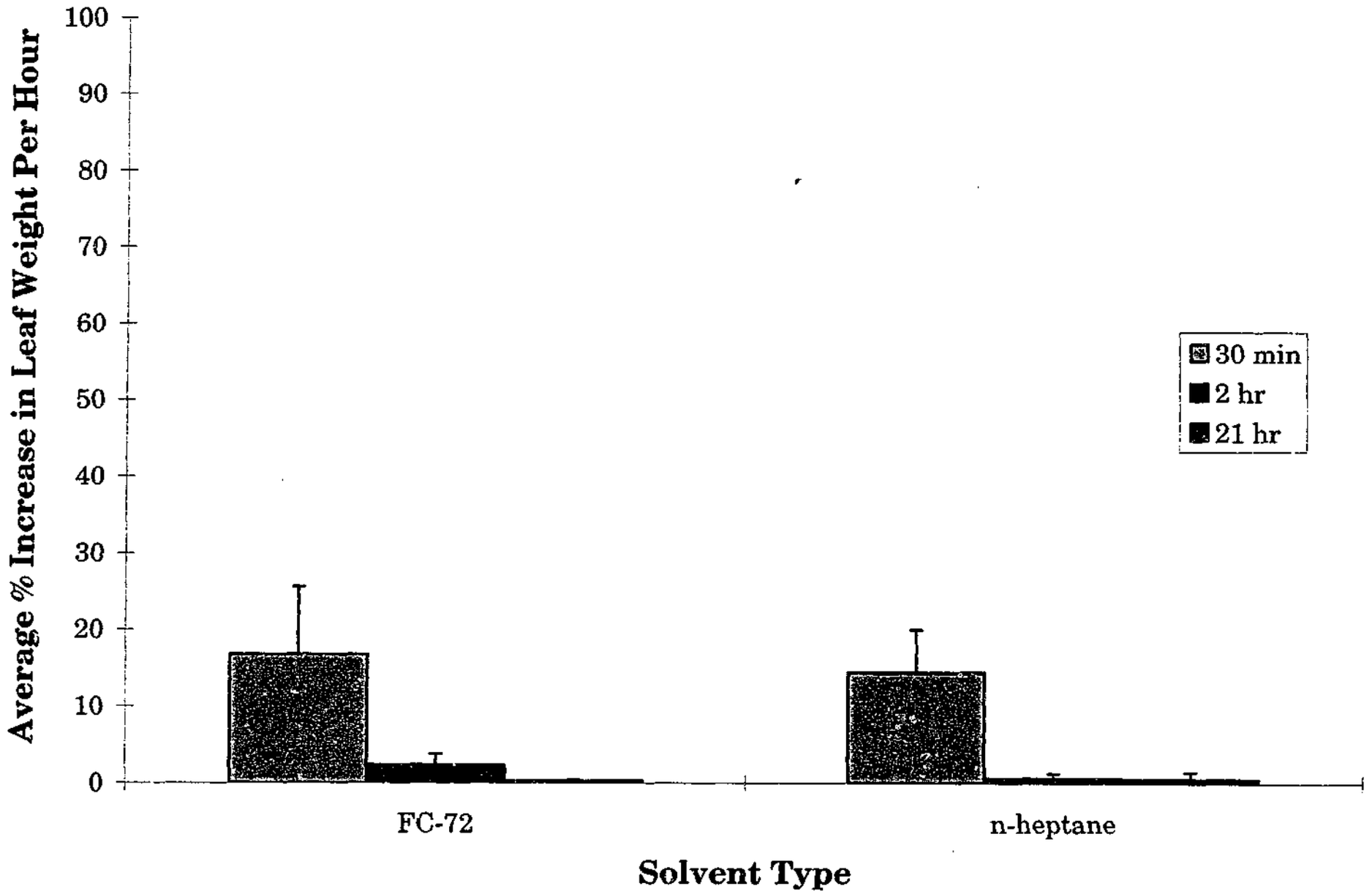

Average % Increase in Leaf Weight Per Hour
100
90
80
70
60
50
40
30
20
10
0
30 min
2 hr
21 hr
FC-72
n-heptane
Solvent Type

Table 12). After 21 hours of immersion, these values had further decreased to an average weight gain of a 0.3% per hour for FC-72 and 0.5% for n-heptane (Fig. 22). The significance of the difference in the rate of weight gain between 2 hours and 21 hours was unable to be tested because of the negative values in the 21-hour data set. Negative values are unable to undergo the required arcsin transformation which is necessary for data in percentage form.

NB - there is no data on the weight gain of dehydrated *Myrothamnus flabellifolia* leaves immersed in HFE 7100 because this solvent was not used for ultrastructural experiments until after the above data had been collected. The solvents FC-72 and HFE 7100 are not routinely used in ultrastructural studies and are sold in commercial quantities that far exceed the needs of any histology laboratory. Their prohibitive expense meant that I relied on small samples of these solvents donated by 3M. Unfortunately the quantities of HFE 7100 supplied did not allow me to conduct any weight gain experiments for this solvent.

3.7 - The Ultrastructure of Phase-Partition Fixed *Myrothamnus flabellifolia* Leaf Tissue.

3.7.1 - Hydrated Tissue.

Hydrated mesophyll leaf tissue generally possessed cell walls that were smooth in outline (Fig. 23a) however, cell walls that were convoluted in shape were occasionally seen (Fig. 23b). The cytoplasm generally occupied the periphery of the cell, with the central region of the cell occupied by vacuoles (Fig. 23a, 23b). On occasions, areas of the plasma membrane had separated from the cell wall (Fig. 23a, 23b).

Cells usually possessed a single, round to oval shaped nucleus (Fig. 23a, 23b, 23c) which often contained a single nucleolus (Fig. 23b, 23c). The chromatin was visible and was generally finely dispersed (Fig. 23a, 23b, 23c). A double membrane bounding the nucleus was observed (Fig. 23d).

Chloroplasts from hydrated tissue were generally elongate or oval in shape (Fig. 23a, 23b, 23c, 23e) and regularly possessed starch grains (Fig. 23a, 23b, 23e) and plastoglobuli (Fig. 23e). The internal membranes of the chloroplast were generally difficult to discern however on occasions thylakoid membranes were observed (Fig. 23f). Normally, the granal stacks could only be differentiated by a staining difference between the granal and non-granal

Figura 23. - Hydrated *Myrothamnus flabellifolia* mesophyll tissue fixed using phase-partition fixation.

Fig. 23a - General view of a cell fixed in 25% glutaraldehyde in FC-72. Cells were relatively turgid in appearance however in many instances, the plasma membrane was observed to have separated from the cell wall (*). Sometimes a number of vacuoles (V) were present in the cell. A number of organelles were routinely seen, these included chloroplasts (C), mitochondria (M) and nuclei (N). Bar represents 1 µm.

Fig. 23b - General view of a cell fixed in Karnovsky's fixative in n-heptane. Cells appeared slightly flaccid as the walls were slightly convoluted (CW). Like Xa, the plasma membrane had separated from the cell wall in a number of instances (*). Large vacuoles (V) were present in the central region of the cell and organelles such as chloroplasts (C) and the nucleus (N) were regularly observed. Bar represents 2 µm.

Fig. 23c - Nuclei (N) were usually oval to round in shape and had a turgid appearance. The chromatin (*) was visible and was generally finely dispersed. Nucleoli (Ne) were frequently seen. Sample was taken from tissue fixed in 4% paraformaldehyde in HFE 7100. Bar represents 1 µm.

Fig. 23d - High magnification view of a nucleus showing the double bounding membrane (arrow heads). Tissue was fixed in Karnovsky's fixative in n-heptane. Bar represents 0.25 µm.

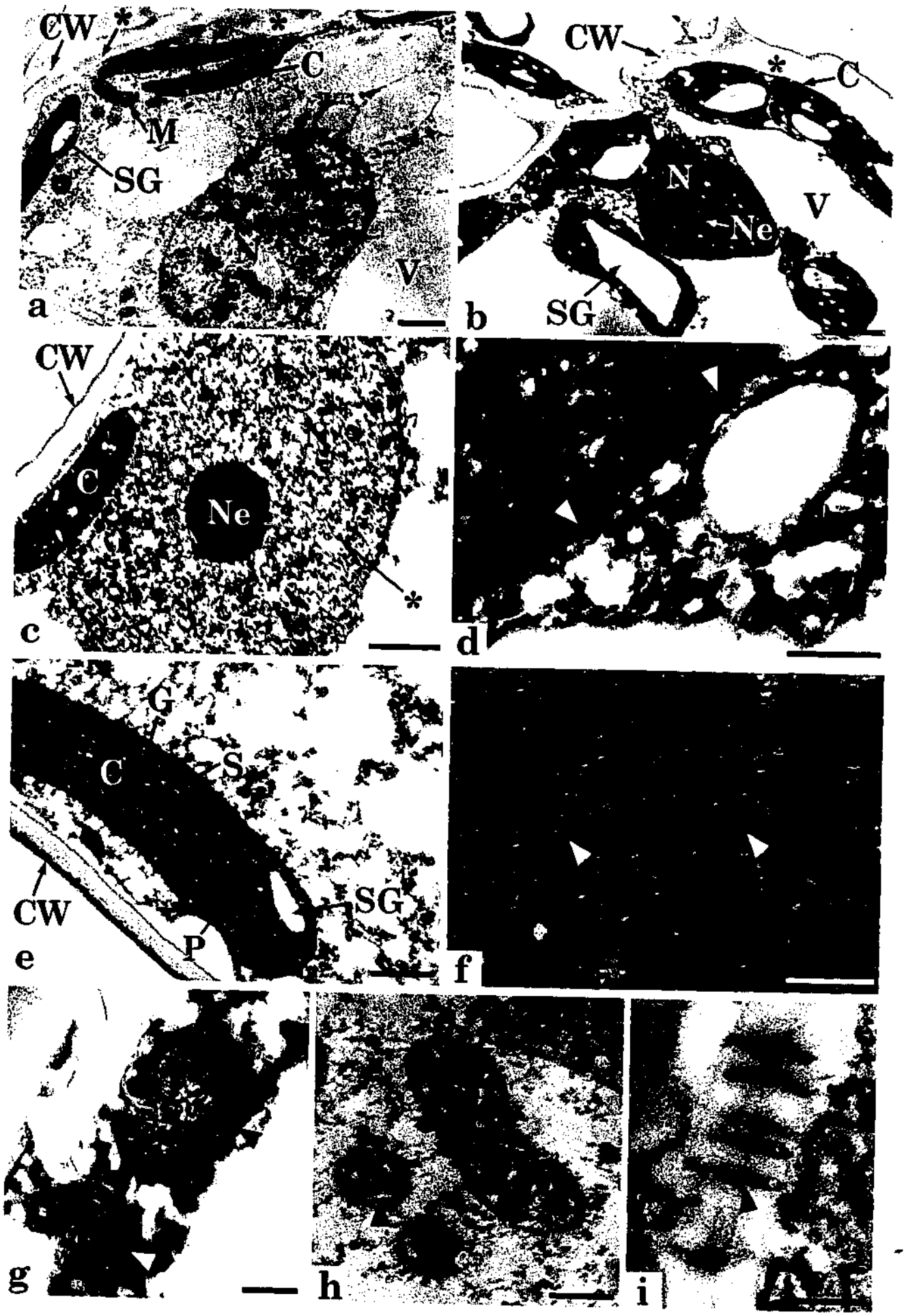
Fig. 23e - Chloroplasts (C) were usually oval to elongate in shape. The granal (G) and stromal (S) regions could be identified by differences in staining intensity with the granal regions being lighter in colouration compared to the stromal region. Starch grains (SG) and plastoglobuli (P) were frequently seen. Tissue was fixed in 37% formaldehyde in FC-72. Bar represents 0.5 µm.

Fig. 23f - High magnification view of the thylakoid membranes (arrow heads) of a chloroplast from tissue fixed in 1% OsO_4 in HFE 7100. Bar represents 0.05 µm.

Fig. 23g - Mitochondria (M) observed were usually round to oval in shape. The outer membranes (arrow heads) in this micrograph are well preserved although the internal cristae are difficult to discern. Tissue sample was fixed in 37% formaldehyde in FC-72. Bar represents 0.25 µm.

Fig. 23h - Occasionally more elongate mitochondria (M) were observed. Cristae (arrow head) were regularly observed. Sample was from tissue fixed in 25% glutaraldehyde in FC-72. Bar represents 0.25 µm.

Fig. 23i - Plasmodesmata (arrow head) were frequently observed. Tissue was fixed in 1% OsO_4 in n-heptane. Bar represents 0.25 µm.


CW
C
M
SG
V
a
CW
C
N
Ne
V
SG
b
CW
C
Ne
c
d
G
C
S
CW
P
SG
e
f
g
h
i

regions, with the granal stacks being less dense (Fig. 23e).

The mitochondria of hydrated mesophyll cells were generally round to oval in shape (Fig. 23g, 23h), however occasionally more elongate shapes were observed (Fig.23h). Only occasionally could the internal cristae membranes of the mitochondria be observed (Fig. 23g), in most cases cristae membranes were only distinguishable as light and dark staining regions (Fig. 23h). Plasmodesmata were also observed (Fig. 23i). Refer to the end of the results section for a summary of the ultrastructural findings for hydrated leaf tissue (Table 3).

3.7.2 - Dehydrated Tissue.

Dehydrated leaf mesophyll cells possessed cell walls that were generally convoluted in nature (Fig.24a, 24b). In the best-preserved cells, the cytoplasm appeared to occupy the entire cell and the plasma membrane was closely appressed to the cell wall (Fig. 24a, 24b). In some cells, the central vacuole was visible, with the cytoplasm occupying the periphery of the cell (Fig. 24a). In other cells, there was no evidence of a central vacuole but the cytoplasm often contained numerous small vacuoles (Fig. 24b). Electron dense material was observed in the vacuoles of some cells (Fig. 24a).

Figure 24. - Dehydrated *Myrothamnus flabellifolia* mesophyll tissue fixed using phase-partition fixation.

Fig. 24a - A general view of a mesophyll cell fixed in Karnovsky's fixative in n-heptane. Cells generally had convoluted cell walls (CW) and the plasma membrane was normally closely aligned to the cell wall (*). A large central vacuole (V) was occasionally present and within it were large masses of electron dense material (E) were frequently seen. Bar represents 1 µm.

Fig. 24b - General view of a cell fixed in 25% glutaraldehyde in n-heptane. The plasma membrane was regularly in close apposition with the cell wall (*). There is no evidence of a large central vacuole however there are many small vacuoles (V) dispersed throughout the cytoplasm. Chloroplasts (C) and nuclei (N) were routinely observed. Bar represents 1 µm.

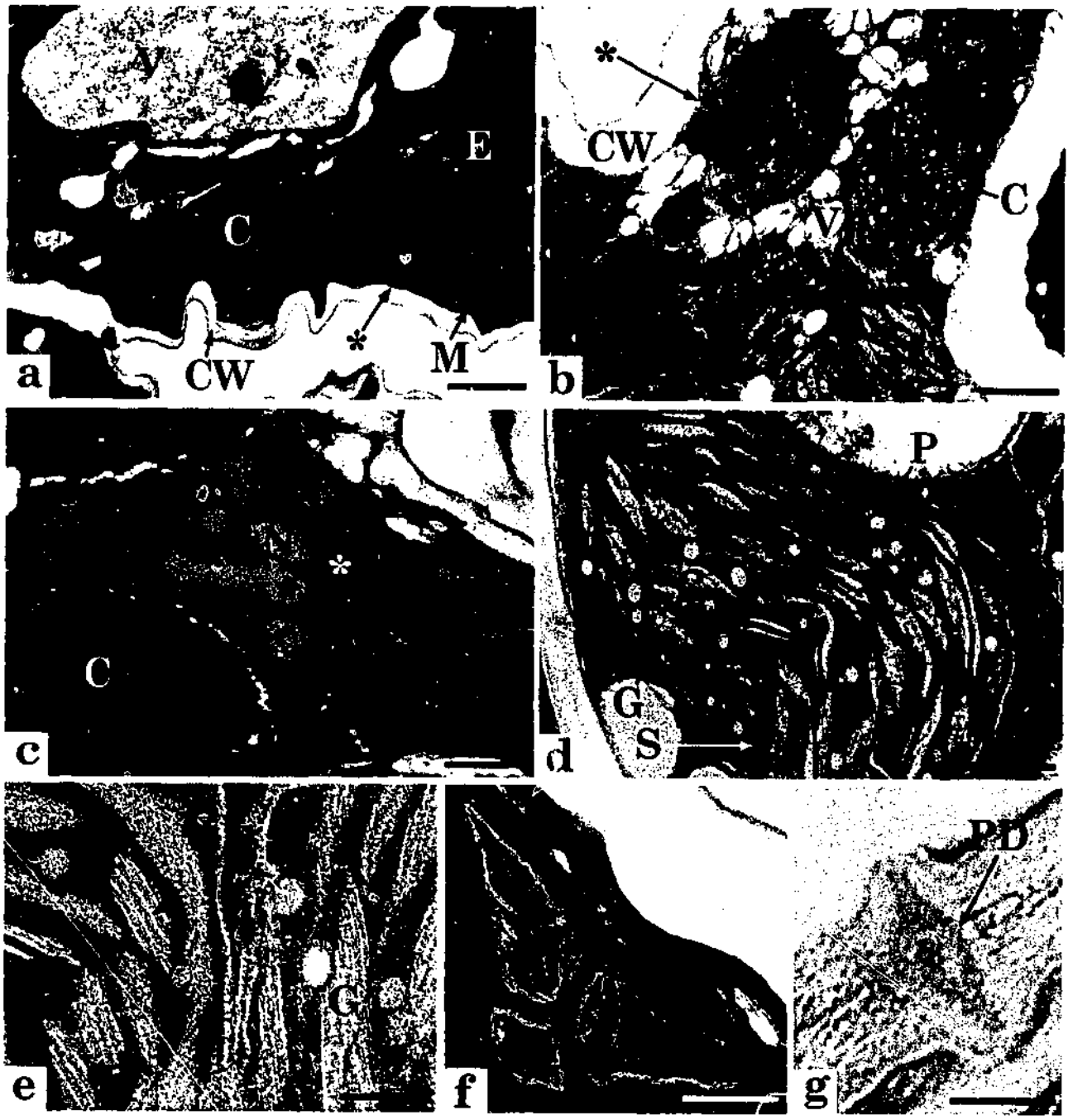
Fig. 24c - Nuclei (N) observed in phase-partition fixed tissue were irregular in shape. The chromatin of the nucleus was extremely condensed and could be separated into electron-lucent (black *) and electron-dense (white *) staining regions. Tissue was fixed in Karnovsky's fixative in n-heptane. Bar represents 0.5 µm.

Fig. 24d - Chloroplasts (C) were usually round in shape and possessed a wavy outer membrane (arrow head). The granal stacks (G) were quite short in length and were lighter in colour compared to the stromal (S) region. Numerous plastoglobuli (P) were seen. Tissue was fixed in 37% formaldehyde in FC-72. Bar represents 0.5 µm.

Fig. 24e - High magnification view of a chloroplast fixed in 25% glutaraldehyde in FC-72 showing the thylakoid membranes (arrow heads) that comprise the granal stacks (G). The stromal (S) region is also visible as too are plastoglobuli (P). Bar represents 0.25 µm.

Fig. 24f - Mitochondria (M) were generally irregular in shape and contained distinct cristae (arrow head). Tissue was fixed in Karnovsky's fixative in n-heptane. Bar represents 0.25 µm.

Fig. 24g - Plasmodesmata (Pd) were frequently observed. Tissue was fixed in 25% glutaraldehyde in FC-72. Bar represents 0.1 µm.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Nuclei were irregular in shape and contained densely staining chromatin that was distinguishable into light and dark regions (Fig.24c, 24c).

Chloroplasts were generally rounded in shape (Fig.24a, 24b, 24d) and the outer bounding membrane appeared ruffled (Fig. 24b, 24d). The internal thylakoid membranes of the chloroplast could occasionally be discerned (Fig.24e) and the grana were short and compact in shape. In the majority of cases, the internal chloroplast membranes could not be discerned. However, light-staining regions did serve to identify the granal stacks (Fig. 24b, 24c, 24d).

Mitochondria were irregular in shape and contained well-defined cristae (Fig. 24f). Plasmodesmata were also observed (Fig.24g). A summary of these ultrastructural findings is contained in Table 3, which can be found at the end of the results section.

3.8 - Effect of Fixative Type on Cellular Ultrastructure.

Three different types of fixative were used during the course of this study, these being glutaraldehyde, formaldehyde and osmium. To act as controls, tissue samples also underwent phase-partition fixation in the absence of any chemical fixatives.

NB - Some of the images contained in the following figures are contained in other figures. These figures

examine particular aspects of the phase-partition process in detail and I decided it would be simpler to combine the relevant images in one figure instead of referring to various images contained in different figures.

3.8.1 - **Hydrated Tissue.**

Organelles such as nuclei from hydrated leaf tissue fixed using paraformaldehyde (Fig. 25a, 25c (4%) 26a, 26e, 26f (37%)), glutaraldehyde (Fig. 27a), Karnovsky's fixative (Fig. 28b, 28e, 28f) or osmium (Fig. 29c) were not markedly different in appearance. They were circular in cross section and frequently possessed a single nucleolus. Nuclei of hydrated cells fixed using paraformaldehyde (Fig. 25c (4%) 26f (37%)) and Karnovsky's fixative (Fig. 28f) were observed to possess double bounding membranes.

Chloroplasts from hydrated tissue samples fixed using paraformaldehyde (Fig. 25a, 25b (4%) 26a, 26b, 26c, 26d (37%)), glutaraldehyde (Fig. 27a, 27b, 27c), Karnovsky's fixative (Fig. 28a, 28b, 28c, 28d) or osmium (Fig. 29a, 29c) were also very similar in appearance. They were generally oval to elongate in shape and regularly possessed plastoglobuli. Occasionally more rounded chloroplasts were observed (Fig. 29b). The internal membranes of the chloroplast were difficult to distinguish although occasionally granal stacks of thylakoid membranes were observed (Fig. 27d, 29d). Generally, good quality

Figure 25. - Hydrated *Myrothamnus flabellifolia* mesophyll tissue fixed in 4% paraformaldehyde in HFE 7100.

Fig. 25a - The general quality of preservation using the fixative 4% paraformaldehyde was relatively poor. However, various organelles could still be distinguished including the nucleus (N), chloroplasts (C) and mitochondria (M). Bar represents 2 μm.

Fig. 25b - Chloroplasts (C) were usually oval in shape. The composite structures of the chloroplast however could not be discerned except for the presence of plastoglobuli (P). Bar represents 0.5 μm.

Fig. 25c - Nuclei (N) frequently contained a single nucleolus (Ne) and the chromatin was relatively dense in appearance (*). The double bounding membrane (arrow heads) could also be regularly observed. Bar represents 1 μm.

Fig. 25d - Plasmodesmata (arrow heads) were also seen. Bar represents 0.1 μm.

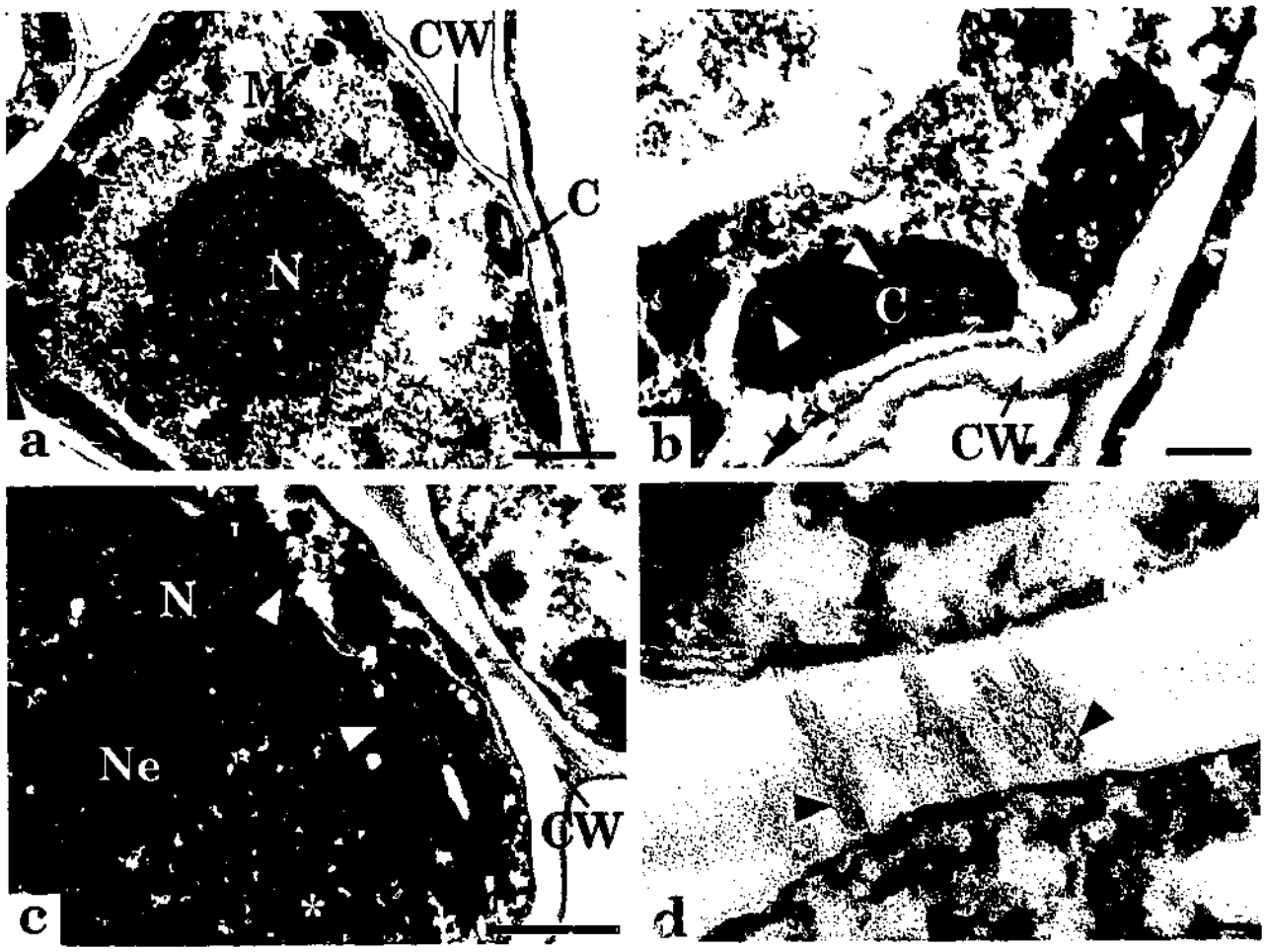
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Figure 26. - Hydrated *Myrothamnus flabellifolia* mesophyll tissue fixed in 37% formaldehyde in FC-72.

Fig. 26a - A general view of hydrated mesophyll cells. Most cells possessed a large central vacuole (V) and the plasma membrane was generally in close alignment with the cell wall (*). Cells were turgid in appearance and had rounded cell walls (CW). Chloroplasts (C) and mitochondria were regularly observed. Bar represents 3 µm.

Fig. 26b - A number of organelles could easily be identified in the cytoplasm of hydrated cells fixed in this manner. Such organelles included chloroplasts (C), mitochondria (M) and nuclei (N). Bar represents 1 µm.

Fig. 26c - Chloroplasts (C) were usually elongate in shape. They often possessed starch grains (SG) and regularly contained plastoglobuli (P). Bar represents 0.5 µm.

Fig. 26d - Thylakoid membranes usually could not be discerned although the granal regions (G) could still be identified as they were lighter colour than the stromal (S) region. Plastoglobuli (P) were also visible. Bar represents 0.25 µm.

Fig. 26e - Nuclei (N) were usually oval in shape and had a turgid appearance. They contained chromatin (*) which was finely dispersed. Nucleoli (Ne) were regularly seen. Bar represents 1 µm.

Fig. 26f - High magnification view of a nucleus (N). The double bounding membrane (arrow heads) was routinely seen as too were nucleoli (Ne). Chromatin (*) was visible and was finely dispersed. Bar represents 0.25 µm.

Fig. 26g - Plasmodesmata (arrow heads) were regularly visible. Bar represents 0.25 µm.

Fig. 26h - Mitochondria (M) were generally round to oval in shape. Cristae were sometimes difficult to see. Bar represents 0.25 µm.

Fig. 26i - Occasionally more elongate forms of mitochondria (M) were observed. Cristae (arrow heads) were generally observed. Bar represents 0.25 µm.

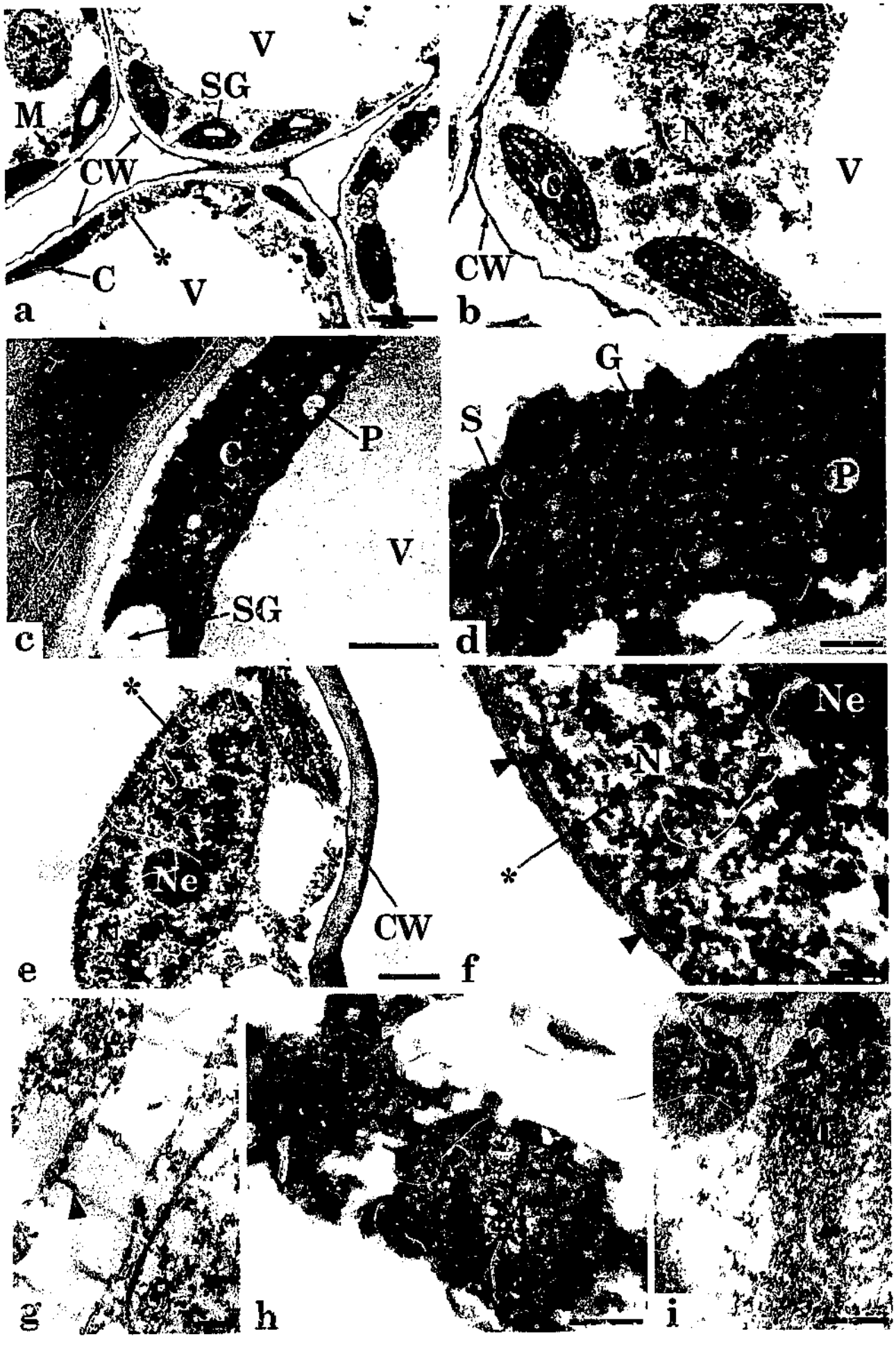
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Figure 27. - Hydrated *Myrothamnus flabellifolia* mesophyll tissue fixed in 25% glutaraldehyde in FC-72.

Fig. 27a - General view of a hydrated mesophyll cell. The plasma membrane was observed to have separated from the cell wall on a number of occasions (*). Occasionally, a number of vacuoles (V) were present throughout the cytoplasm. Chloroplasts (C), mitochondria (M) and nuclei (N) could all be regularly seen. Bar represents 2 µm.

Fig. 27b - A large central vacuole (V) was present in most cells. Chloroplasts (C) were generally oval to elongate in shape, generally possessed plastoglobuli (P) and often contained starch grains (SG). Mitochondria (M) were generally round in shape. Bar represents 1 µm.

Fig. 27c - Mitochondria (M) observed were mainly round in shape although more elongated mitochondria were also seen. Cristae (arrow heads) were generally present and could be distinguished as light staining areas within the mitochondria. Bar represents 0.5 µm.

Fig. 27d - Occasionally the actual cristae membranes (arrow heads) of the mitochondria could be distinguished. Cytoplasmic ribosomes (R) were also seen. Granal stacks (G) in chloroplasts (C) were generally long and thin in shape. Bar represents 0.2 µm.

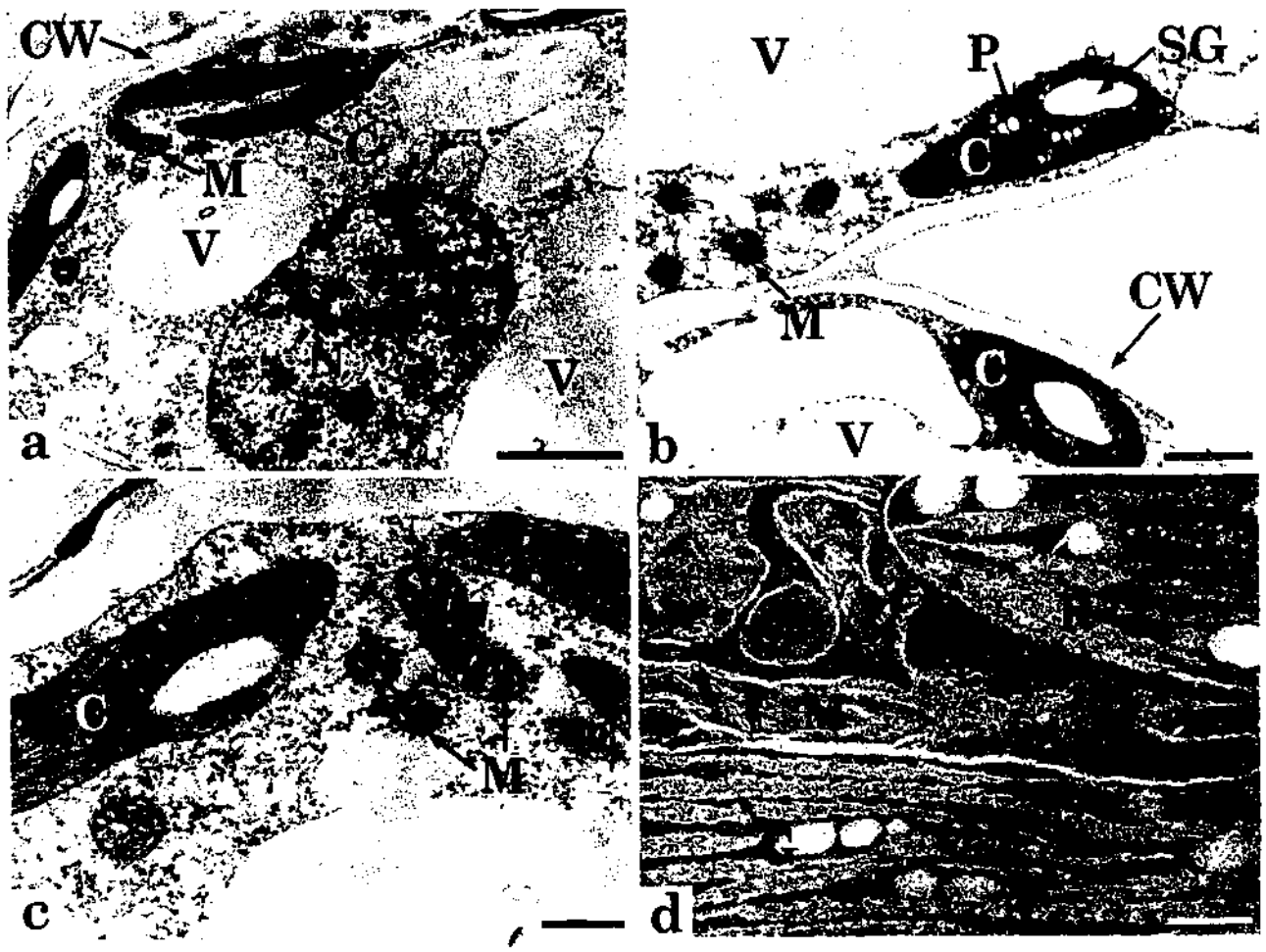
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Figure 28. - Hydrated *Myrothamnus flabellifolia* mesophyll tissue fixed in Karnovsky's fixative in n-heptane.

Fig. 28a - Hydrated mesophyll cells were quite shrunken in appearance and had convoluted cell walls (CW). Areas of separation (*) between the plasma membrane and the cell wall were frequently apparent. In the centre of the cell was often a large central vacuole (V). Chloroplasts (C) were easily identified. Bar represents 3 μm.

Fig. 28b - Organelles such as chloroplasts (C) and nuclei (N) were able to be distinguished. Bar represents 2 μm.

Fig. 28c - In a number of cells there was no evidence of a large central vacuole, instead the cell contained smaller more numerous vacuoles (V) distributed throughout the cytoplasm. Such cells had a shrunken appearance to them and had quite convoluted cell walls (CW). Bar represents 4 μm.

Fig. 28d - Chloroplasts (C) often possessed starch grains (SG). The internal membranes of the chloroplast could not be distinguished although the granal (G) and stromal (S) areas could be identified through staining differences with the granal regions staining slightly lighter than the stromal regions. Mitochondria (M) were generally round in shape and cristae were difficult to discern. Plas ta could also be seen (arrow head). Bar represents .

Fig. 28e - Nuclei (N) were turgid in appearance and had smooth rounded outlines (black *). A nucleolus (Ne) was regularly observed and the chromatin (white *) was relatively dense. Bar represents 1 μm.

Fig. 28f - A high magnification view of a nucleus reveals the double bounding membrane (arrow heads). Bar represents 0.2 μm.

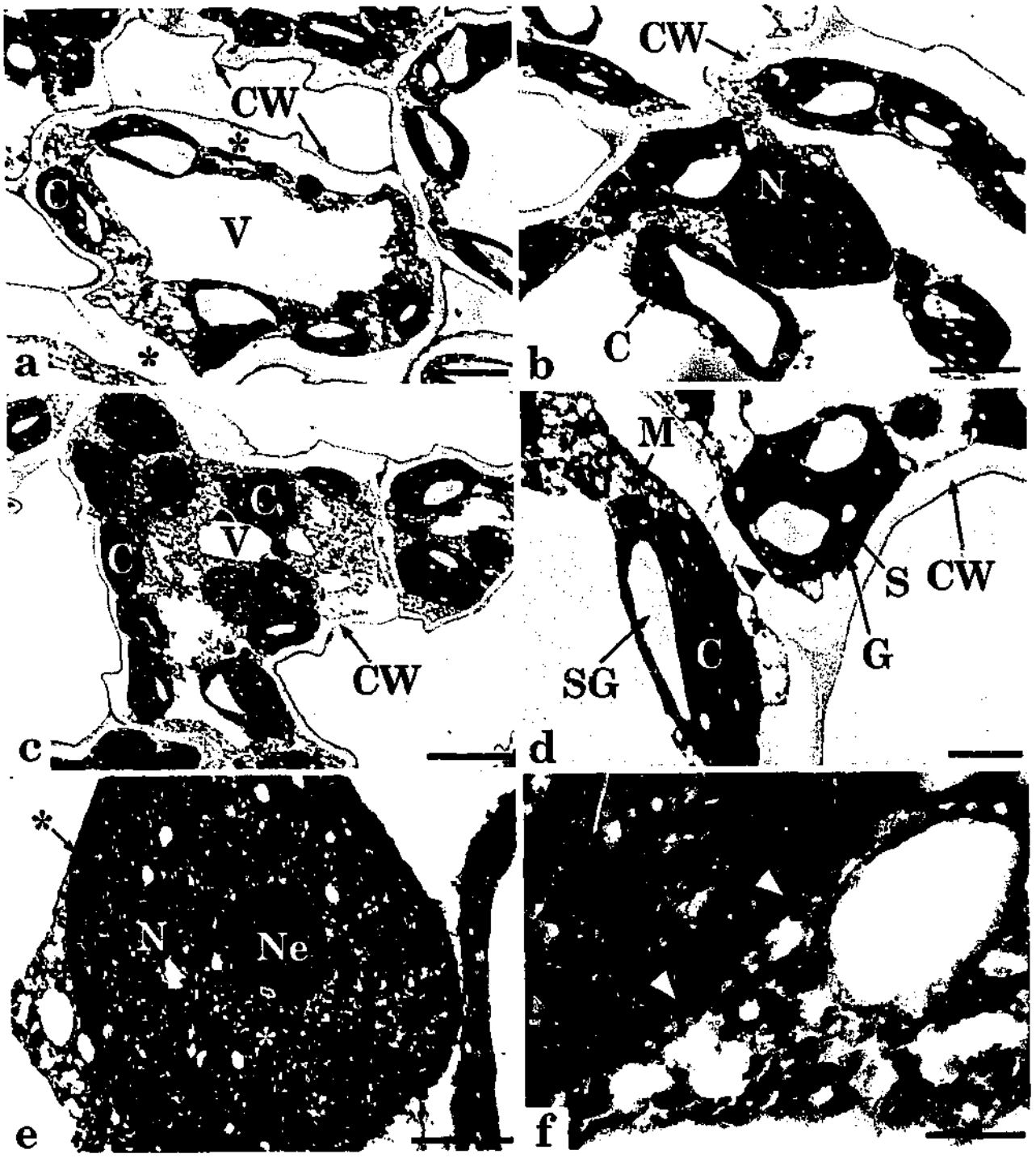
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Figure 29. - Hydrated *Myrothamnus flabellifolia* mesophyll tissue fixed in 1% OsO_4 in either HFE 7100 (a, c, d) or n-heptane (b, e, f).

Fig. 29a - General view of a hydrated mesophyll cell. Cells had a flaccid appearance, as the cell wall (CW) appeared slightly shrunken. The plasma membrane was positioned adjacent to the cell wall (*) and large masses of electron dense material (E) were regularly observed in the central vacuole. Chloroplasts (C) that were elongated in shape were regularly seen. Bar represents 2 µm.

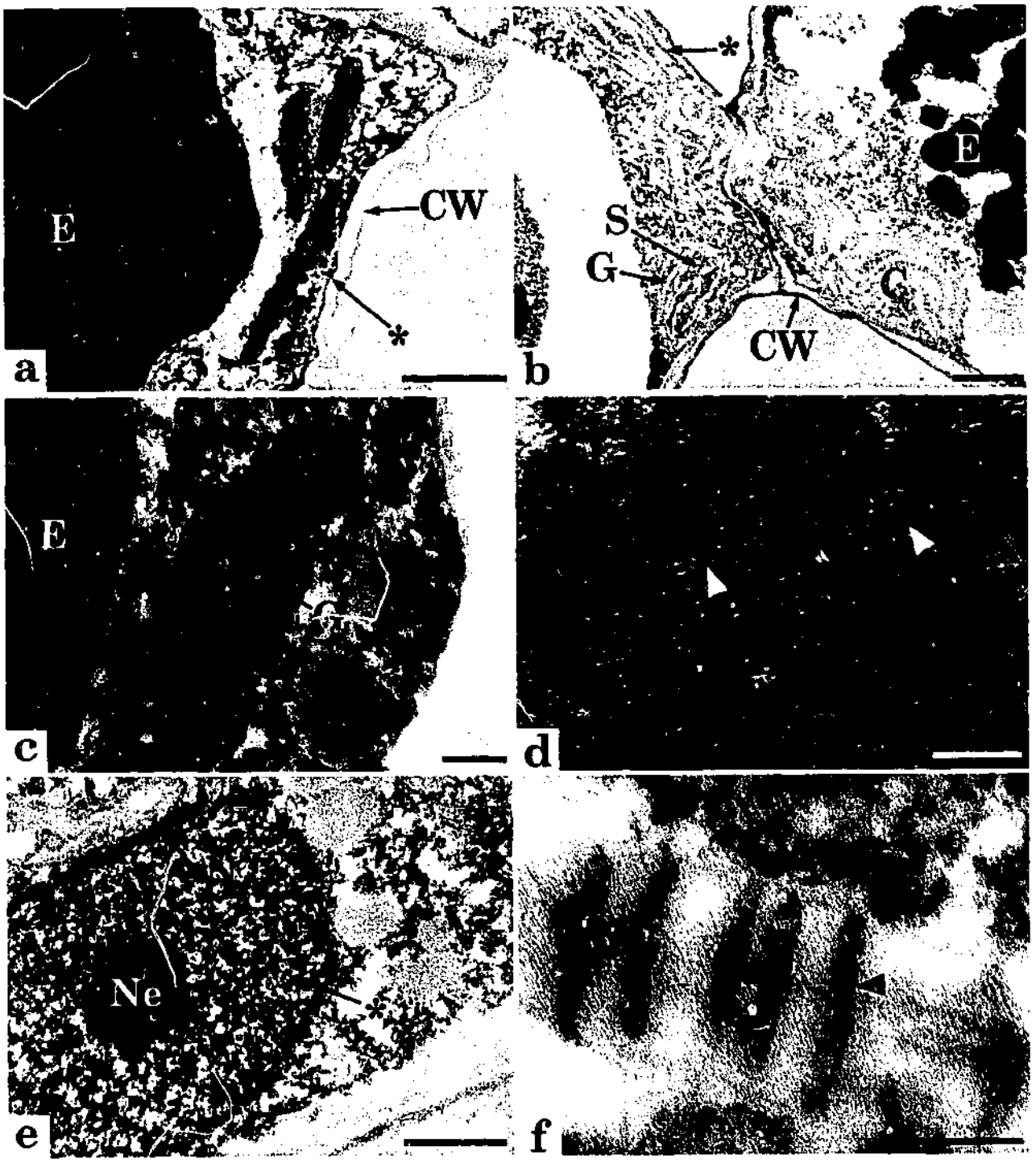
Fig. 29b - Chloroplasts (C) that were more rounded in shape were also seen. Although the granal (G) and stromal (S) regions could be identified, the internal chloroplast membranes could not be identified. Again, these cells were flaccid looking in appearance and had slightly convoluted cell walls (CW). The plasma membrane was in close apposition to the cell wall (*) and masses of electron-dense material (E) was present in the central vacuole region. Bar represents 2 µm.

Fig. 29c - Chloroplasts (C) that were more elongated in shape had better-preserved internal membranes. The fine lines (arrow heads) present in the granal stacks (G) are its composite thylakoid membranes. The granal stacks (G) were generally long and thin in shape. Bar represents 0.25 µm.

Fig. 29d - A high magnification view of 29c, showing a granal stack and the constituting thylakoid membranes (arrow heads). Bar represents 0.05 µm.

Fig. 29e - Nuclei (N) were generally oval in shape and had a turgid appearance. They contained chromatin (*) which was finely dispersed throughout the organelle and nucleoli (Ne) were frequently observed. Bar represents 1 µm.

Fig. 29f - Plasmodesmata (arrow head) were also routinely observed. Bar represents 0.25 µm.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preservation only allowed for observations of light and dark staining regions which separated the chloroplast into granal and stromal regions respectively (Fig. 26d), frequently not even this level of preservation was achieved (Fig. 25b, 28d).

Mitochondria from hydrated tissue samples fixed using paraformaldehyde (Fig. 26a, 26b, 26h, 26i (37%)), glutaraldehyde (Fig.27b, 27c) or Karnovsky's fixative (Fig. 28d) were comparable in appearance. They were generally round to oval in shape and possessed light staining regions where one would expect cristae to be located. Occasionally, mitochondria resembled those seen in dehydrated tissue (Fig. 27d cf. 33e, 34e, 33f, 31d).

Plasmodesmata were also observed in hydrated samples fixed using paraformaldehyde (Fig. 25c, 25d (4%) 26g (37%)), Karnovsky's fixative (Fig. 28d) and osmium (fig. 29f).

3.8.2 - **Dehydrated Tissue.**

Dehydrated cells such as those fixed in paraformaldehyde (Fig. 30b - 4%, 31b - 37%), 25% glutaraldehyde (Fig. 32a, 32b) or Karnovsky's fixative (Fig. 33a) generally had cell walls that were wavy and convoluted. Occasionally however, cells were observed that had extremely convoluted cell walls and were shrunken in appearance (Fig. 34a).

Figure 30. - Dehydrated *Myrothamnus flabellifolia* mesophyll tissue fixed in 4% paraformaldehyde in HFE 7100.

Fig. 30a - The use of the fixative 4% paraformaldehyde resulted in poor quality preservation of dehydrated mesophyll tissue. Some cellular detail could be discerned however including, the occasional presence of a central vacuole (V). Chloroplasts (C) were one of the few organelles that could be easily identified, they were round in shape and generally possessed plastoglobuli (P). Bar represents 1 µm.

Fig. 30b - The internal membranes of the chloroplast (C) could not be distinguished however the wavy outer membrane (*) was frequently observed. Granal (G) and stromal (S) regions could be distinguished by differences in staining intensity with the granal stacks being lighter in colouration compared to the stromal regions. Bar represents 1 µm.

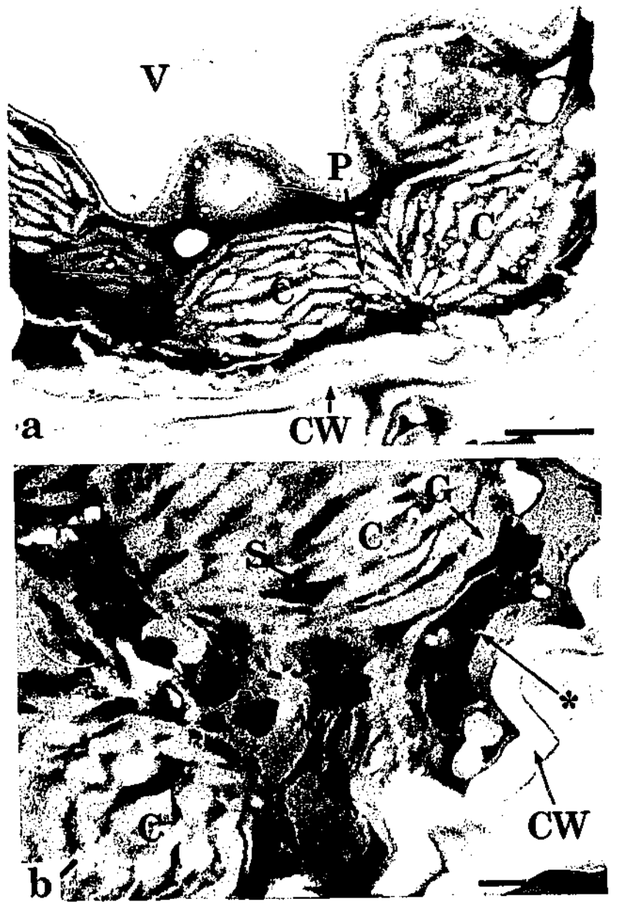


Figure 31. - Dehydrated *Myrothamnus flabellifolia* mesophyll tissue fixed in 37% formaldehyde in FC-72.

Fig. 31a - General view of a dehydrated mesophyll cell. Cells were relatively turgid in shape with only a few convolutions of the cell wall (CW) present. Separations (*) of the cell wall and the plasma membrane could be seen in many samples. A large central vacuole (V) was regularly observed. Bar represents 2 µm.

Fig. 31b - Apart from regions where the cell wall and the plasma membrane had separated (*) regions where they were adjacent to each other (arrow head) were also visible. A larger central vacuole (V) and a number of smaller vacuoles (*V) were regularly present in the cell. Chloroplasts (C) were routinely observed. Bar represents 1 µm.

Fig. 31c - Micrograph showing the condensed nature of the cytoplasm. Chloroplasts (C) comprised a major portion of the cytoplasm and were mainly round in shape. They possessed a wavy outer membrane (*) however the inner membranes could not be discerned. Granal (G) and stromal (S) regions of the chloroplast were identified by differences in staining intensity with the granal regions staining a lighter colour than the stromal region. Bar represents 1 µm.

Fig. 31d - Irregular shaped mitochondria (M) were regularly seen. Cristae (arrow heads) were nearly always observed. Bar represents 0.25 µm.

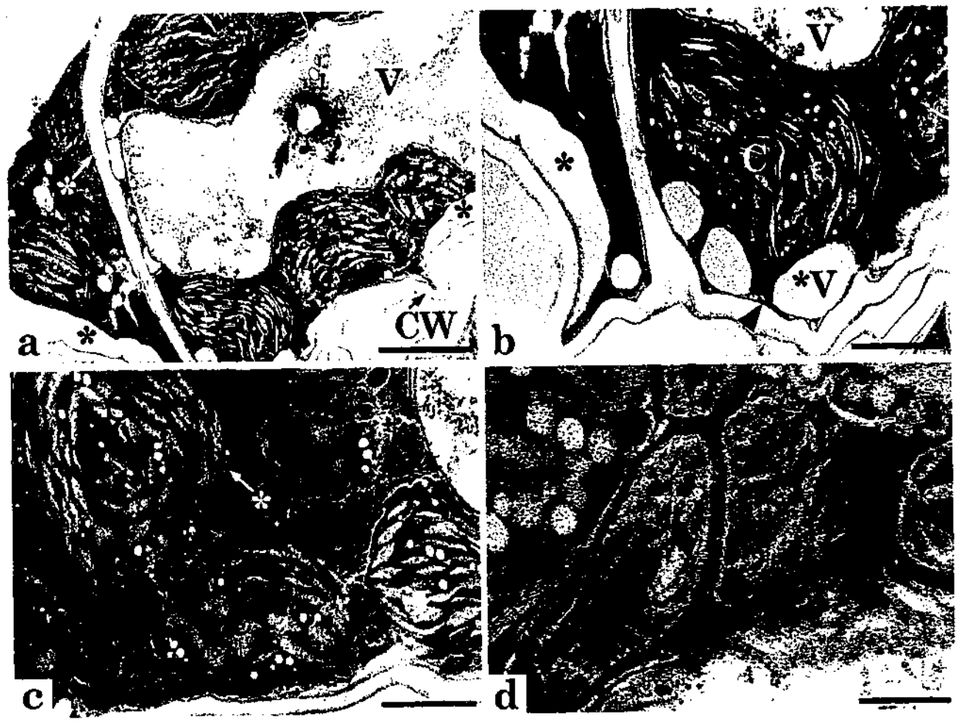


Figure 32. - Dehydrated *Myrothamnus flabellifolia* mesophyll tissue fixed in 25% glutaraldehyde in either FC-72 (a, c, d, e, h) or n-heptane (b, f, g).

Fig. 32a - In many dehydrated mesophyll cells the cell wall and the protoplasm were seen to have separated (*) leaving the cytoplasm situated in the middle of the cell. There was regularly no evidence of a central vacuole but rather many smaller vacuoles (V) were present throughout the cytoplasm. Chloroplasts (C) were routinely observed. Bar represents 2 µm.

Fig. 32b - In other dehydrated mesophyll cells the plasma membrane remained in close contact with the cell wall (*). As with Fig. 32a there was no evidence of a single central vacuole but there are many smaller vacuoles distributed throughout the cytoplasm (V). Chloroplasts (C) comprised a large portion of the cytoplasm and were round in shape. Bar represents 1 µm.

Fig. 32c - A high magnification view of a chloroplast. The thylakoid membranes (arrow heads) that comprise the granal stacks were visible and so was the chloroplasts' wavy outer membrane (*) and a number of plastoglobuli (P). Bar represents 0.25 µm.

Fig. 32d - The chromatin of nuclei (N) was very dense in nature and was separated into regions of electron-lucent (black *) and electron-dense (white *) staining material. Bar represents 0.5 µm.

Fig. 32e - Various shaped mitochondria (M) were seen. Distinct cristae (arrow head) were present in the majority of mitochondria observed. Bar represents 0.2 µm.

Fig. 32f - An irregular shaped mitochondria (M) with cristae (arrow heads). Bar represents 0.1 µm.

Fig. 32g - Plasmodesmata (arrow heads) were regularly seen. Bar represents 0.25 µm.

Fig. 32h - A plasmodesma (arrow head) seen in dehydrated mesophyll tissue. Bar represents 0.1 µm.

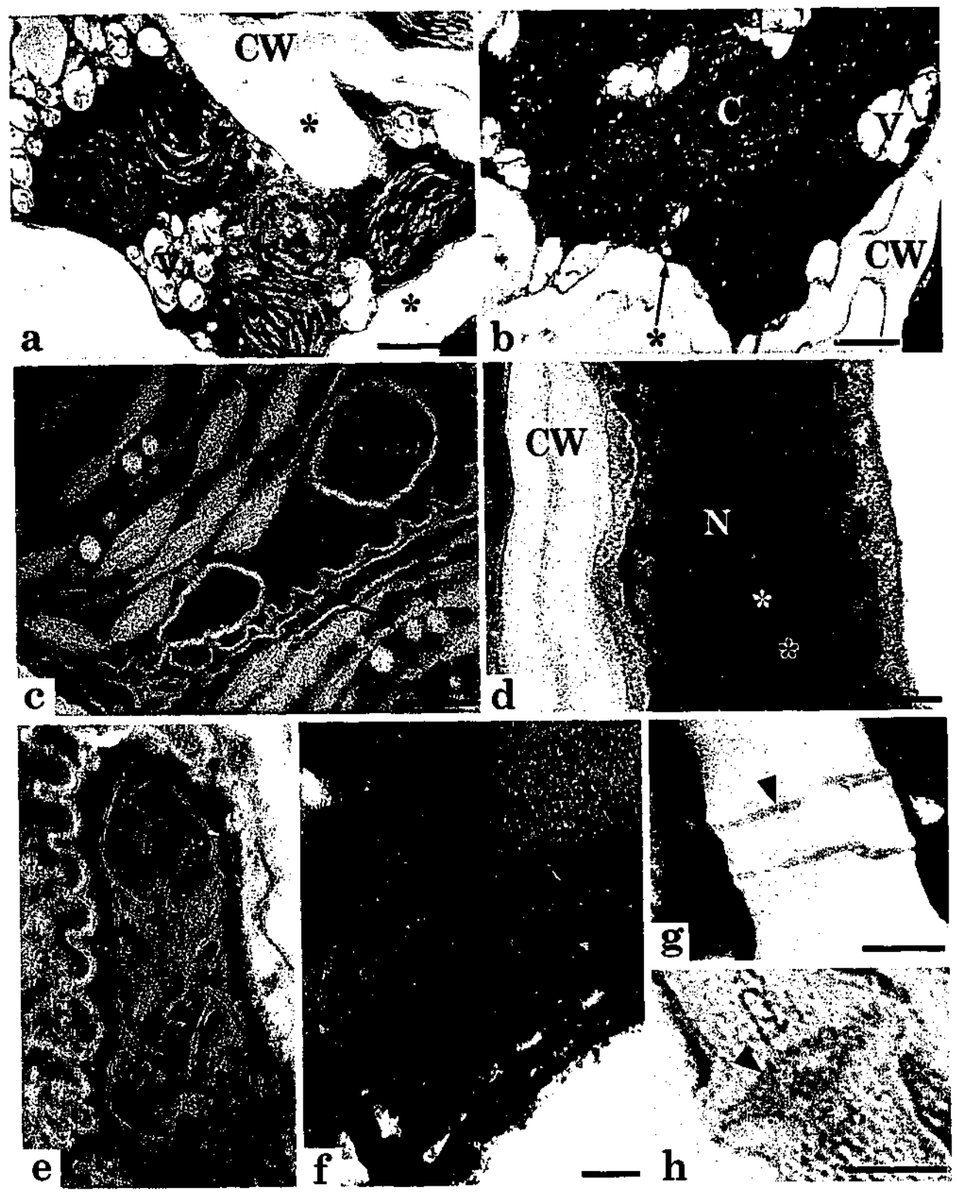


Figure 33. - Dehydrated *Myrothamnus flabellifolia* mesophyll tissue fixed in Karnovsky's fixative in n-heptane.

Fig. 33a - A general view of a dehydrated mesophyll cell. Cell walls (CW) were convoluted in nature and the plasma membrane was closely aligned to the cell wall (*). A large central vacuole (V) occasionally observed within which were large masses of electron-dense material (E). Chloroplasts (C) and mitochondria (M) were routinely observed. Bar represents 1 μm.

Fig. 33b - As well as the presence, in many cells, of a central vacuole (*V), a number of smaller vacuoles (V) were regularly seen dispersed throughout the cytoplasm. Bar represents 1 μm.

Fig. 33c - The nuclei (N) in such cells were generally irregular in shape and contained dense masses of chromatin which is separated into electron-lucent (black *) and electron-dense (white*) staining areas. Bar represents 1 μm.

Fig. 33d - The cytoplasm of dehydrated cells was very dense. However, many organelles could be discerned including chloroplasts (C), mitochondria (M) and plasmodesmata (arrow heads). Chloroplasts (C) were typically oval in shape. Bar represents 1 μm.

Fig. 33e - The granal (G) and stromal (S) regions of the chloroplast were distinguishable by staining differences with the granal region staining a lighter colour than the stromal region. The thylakoid membranes (arrow heads) that comprise the granal stacks could regularly be distinguished. Plastoglobuli (P) were generally present. Bar represents 0.2 μm.

Fig. 33f - Various shaped mitochondria (M), which contained cristae, were regularly observed. Bar represents 0.2 μm.

Fig. 33g - Plasmodesmata (arrow head) were also frequently seen. Bar represents 0.2 μm.

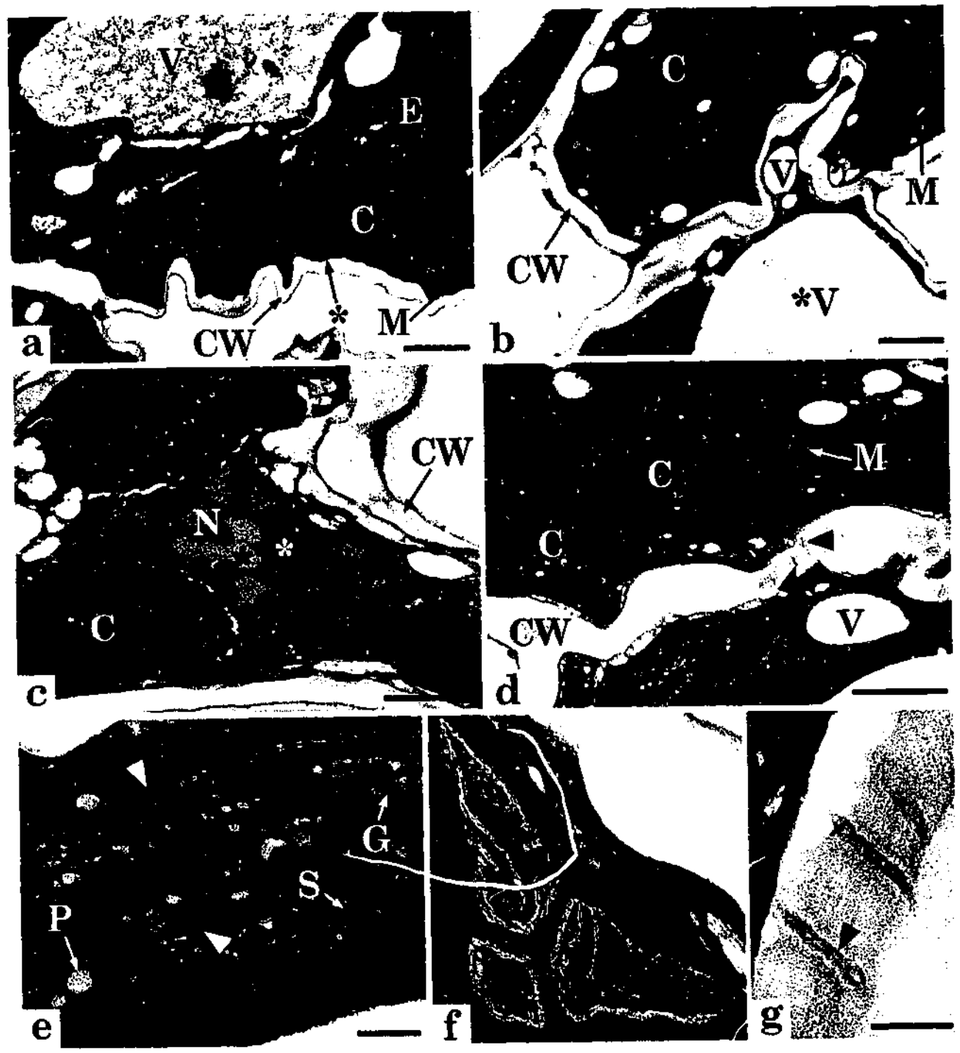


Figure 34. - Dehydrated *Myrothamnus flabellifolia* mesophyll tissue fixed in 1% OsO_4 in either HFE 7100 (a) or n-heptane (b, c, d, e).

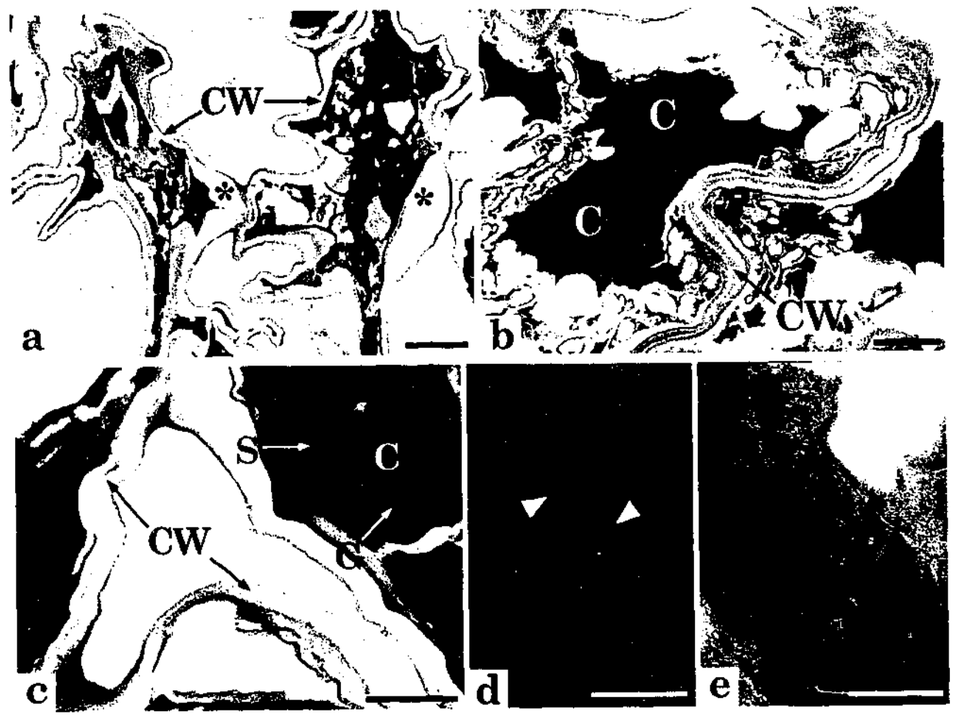
Fig. 34a - The use of this technique on dehydrated mesophyll cells resulted in poor quality fixation. Cells were highly shrunken in nature and had extremely convoluted cell walls (CW). Areas where the plasma membrane had separated (*) from the cell wall were observed. Bar represents 2 µm.

Fig. 34b - When areas of cytoplasm were successfully preserved they were extremely electron-dense. Chloroplasts (C) were regularly identifiable. Bar represents 1 µm.

Fig. 34c - Chloroplasts (C) were one of the few organelles that could be identified. Although the outer bounding membranes could not be easily distinguished, granal (G) and stromal (S) regions were apparent. Bar represents 1 µm.

Fig. 34d - Despite the poor quality of preservation, occasionally the thylakoid membranes (arrow heads) that comprise granal stacks could be distinguished. Bar represents 0.1 µm.

Fig. 34e - Mitochondria (M) containing cristae (arrow heads) were also seen. They were mainly irregular in shape. Bar represents 0.25 µm.



Nuclei from dehydrated tissue did not appear to be affected by the type of fixative used as evidenced by samples fixed in either glutaraldehyde (Fig. 32d) or Karnovsky's fixative (Fig. 33b, 33c). The nuclei of dehydrated tissue were irregular in shape and were densely stained. The chromatin was condensed into light and dark staining regions.

Fixative type did not appear to influence the ultrastructure of chloroplasts from dehydrated tissue. Chloroplasts fixed with paraformaldehyde (Fig. 30a, 30b (4%) 31a, 31b, 31c (37%)), glutaraldehyde, (Fig. 32a, 32b) or Karnovsky's fixative (Fig. 33a, 33c, 33d) were similar in appearance. They were round in shape and frequently possessed a ruffled bounding membrane. The granal membranes could only occasionally be identified (Fig. 34d). Generally, only the appearance of lighter staining regions in the chloroplast separated the granal region from the stromal region (Fig. 30a, 30b, 31a, 31b, 31c, 33c, 32a, 32b, 32c, 33d, 33e, 34c).

Mitochondria from dehydrated tissue samples that had been fixed in paraformaldehyde (Fig. 31d - 37%), glutaraldehyde (Fig. 32e, 32f), Karnovsky's fixative (Fig. 33f) or osmium (Fig. 34e) all appeared very alike. Mitochondria from dehydrated tissue were irregular in shape and possessed distinct cristae.

Ribosomes were observed in dehydrated tissue samples that had been fixed using glutaraldehyde (Fig. 32e).

Plasmodesmata were also observed in dehydrated samples fixed using glutaraldehyde (Fig. 32g, 32h) and Karnovsky's fixative (Fig. 33g).

3.8.3 - Effect of Phase-Partition Treatment in the Absence of Chemical Fixatives.

Tissue fixed in the absence of a chemical fixative, i.e. fixed in 100% n-heptane or 100% HFE 7100, acted as controls for the above experiments. Tissue samples fixed in this way were of a poorer quality than tissue that had been fixed in the presence of aldehyde fixatives. Using this method, dehydrated tissue was better preserved than hydrated tissue (cf. Figs. 35 and 36).

In spite of the absence of a fixative, a number of organelle types such as nuclei (Fig. 35c), chloroplasts (Fig. 35a, 35b, 36a, 36b, 36c, 36e, 36f, 36g) and mitochondria (Fig. 36a, 36d, 36h) were still discernible. Cytoplasmic ribosomes (36d) and plasmodesmata (Fig. 35d) were also seen.

3.9 - Effect of Fixation Duration on Cellular Ultrastructure.

During the course of this study, tissue samples were fixed for varying lengths of time. Samples of leaf tissue were fixed in either 25% glutaraldehyde or 37% formaldehyde

Figure 35. - Hydrated *Myrothamnus flabellifolia* mesophyll tissue fixed using the technique of phase partition fixation without the addition of any fixatives. Samples fixed in HFE 7100.

Fig. 35a - General view of a hydrated mesophyll cell. The overall quality of cellular preservation was extremely poor with very little cellular detail being able to be discerned. The cell wall (CW) was flaccid in appearance and aggregations of cytoplasm (*) could be seen throughout the cell. Bar represents 2 µm.

Fig. 35b - Detail of what is possibly a chloroplast (C). No bounding or internal membranes can be discerned. Bar represents 0.5 µm.

Fig. 35c - The nucleus (N) was one of the few organelles that could be readily identified. It was turgid in appearance and contained finely dispersed chromatin (*). Bar represents 2 µm.

Fig. 35d - Plasmodesmata (arrow heads) were also identified. Bar represents 0.25 µm.

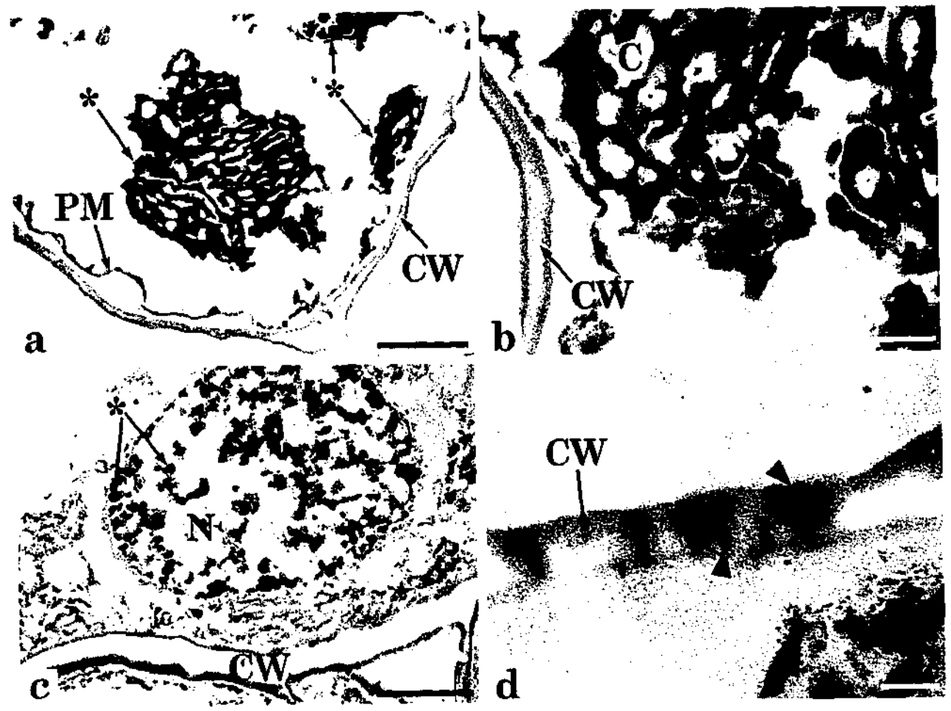


Figure 36. - Dehydrated *Myrothamnus flabellifolia* mesophyll tissue fixed using the technique of phase-partition fixation without the addition of any fixatives. Samples fixed in either HFE 7100 (a, c, e) or n-heptane (b, d, f).

Fig. 36a - General view of the cellular ultrastructure of dehydrated mesophyll cells 'fixed' in 100% HFE 7100. The overall quality of cellular preservation was not good however a number of organelles could be discerned such as chloroplasts (C) and mitochondria (M). Bar represents 0.5µm.

Fig. 36b - General view of the cellular ultrastructure of dehydrated mesophyll cells 'fixed' in 100% n-heptane. As with Fig. 36a, the quality of preservation was not good. Although the cytoplasm was very densely stained organelles such as chloroplasts (C) could be discerned. The cytoplasm in many cases appeared to have tears in it (*). Bar represents 1 µm.

Fig. 36c - High magnification view of a chloroplast (C). The bounding membrane of the chloroplast was wavy in appearance (arrow heads). No thylakoid membranes could be observed although the granal regions (G) could be identified as light staining regions compared to the darker staining stromal (S) regions. Tissue sample was 'fixed' in 100% HFE 7100. Bar represents 0.5µm.

Fig. 36d - A magnified view of a chloroplast (C) 'fixed' in 100% n-heptane. Granal (G) and stromal (S) regions could be distinguished and the outer bounding membrane was generally wavy (arrow head). Plastoglobuli (P) could also be discerned. Bar represents 0.25µm.

Fig. 36e - Mitochondria (M) of various shapes were seen, cristae (arrow heads) were also present. Cytoplasmic ribosomes (R) were regularly observed. Tissue sample was 'fixed' in 100% HFE 7100. Bar represents 0.25µm.

Fig. 36f - Mitochondria (M) 'fixed' in 100% n-heptane were also irregular in shape and possessed cristae (arrow heads). Bar represents 0.25µm.

partitioned into FC-72 for times of either 1, 6 or 24 hours.

3.9.1 - Hydrated Tissue.

The ultrastructure of the nucleus from hydrated leaf tissue fixed for 1 hour (Fig. 37e) did not appear different from tissue that had been fixed for 6 hours (Fig. 38a, 38c) or 24 hours (Fig. 39b, 39g, 39h, 39i). In all instances, the nuclei were round and smooth in shape and they contained finely staining chromatin.

Nor was there any apparent difference in the ultrastructure of chloroplasts from hydrated tissue fixed for 1 hour (Fig. 37a, 37b, 37c), 6 hours (38a, 38b) or 24 hours (39a, 39b, 39c, 39d, 39e, 39f). Such chloroplasts were oval or elongate in shape. The internal membranes of chloroplasts from hydrated leaf tissue were difficult to distinguish and only occasionally could the thylakoid membranes of the granal stacks be distinguished (Fig. 37d). However the granal and stromal regions of the chloroplasts could be distinguished as they exhibited a difference in staining intensity (Fig. 37c, 38b, 39e, 39f).

Mitochondria from hydrated tissue also did not appear to be influenced by the length of fixation as evidenced by micrographs of mitochondria fixed for 1 hour (Fig. 37b, 37f), 6 hours (Fig. 38a, 38d) and 24 hours (Fig. 39c). Despite the fact that the mitochondria were fixed for

Figure 37. - Hydrated *Myrothamnus flabellifolia* mesophyll tissue fixed using the phase-partition fixation technique for 1 hour.

Fig. 37a - A hydrated mesophyll cell fixed in 37% formaldehyde in FC-72. Cells were relatively turgid with the cell wall (CW) outline being quite smooth in contour. A large central vacuole (V) was generally present however the tonoplast membrane was sometime absent. The plasma membrane was generally adjacent to the cell wall (*) and chloroplasts (C) and mitochondria (M) were regularly observed. Bar represents 2 µm.

Fig. 37b - Mesophyll cells fixed in 25% glutaraldehyde in FC-72. As with Fig. 37a, the plasma membrane is in close apposition to the cell wall (*) and a large central vacuole is present (V). Chloroplasts (C) that possess starch grains (SG) can be seen and so can mitochondria (M). Bar represents 1 µm.

Fig. 37c - A magnified view of a chloroplast (C) fixed in 25% glutaraldehyde in FC-72. The chloroplast is elongated in shape and long, thin granal stacks (G) can be seen although the composite thylakoid membranes cannot be distinguished. The stroma (S) is also visible. Bar represents 0.25 µm.

Fig. 37d - A magnified view of the cytoplasm of a hydrated mesophyll cell fixed in 25% glutaraldehyde in FC-72. Mitochondria (M) containing cristae (arrow heads) could be seen as too could cytoplasmic ribosomes (R). The granal (G) and stromal (S) regions of chloroplasts (C) can also be seen. Bar represents 0.25 µm.

Fig. 37e - A nucleus (N) in a mesophyll cell fixed in 37% formaldehyde in FC-72. The bounding membrane was evident (arrow heads) and it forms a smooth outline. Finely dispersed chromatin (*) was generally visible. Bar represents 1 µm.

Fig. 37f - Mitochondria (M) seen in a hydrated cell fixed in 25% glutaraldehyde in FC-72. Mitochondria were usually turgid in appearance and were round to oval in shape. This is in contrast to the mitochondria seen in Fig. 37d. Cristae were difficult to distinguish. Bar represents 0.25 µm.

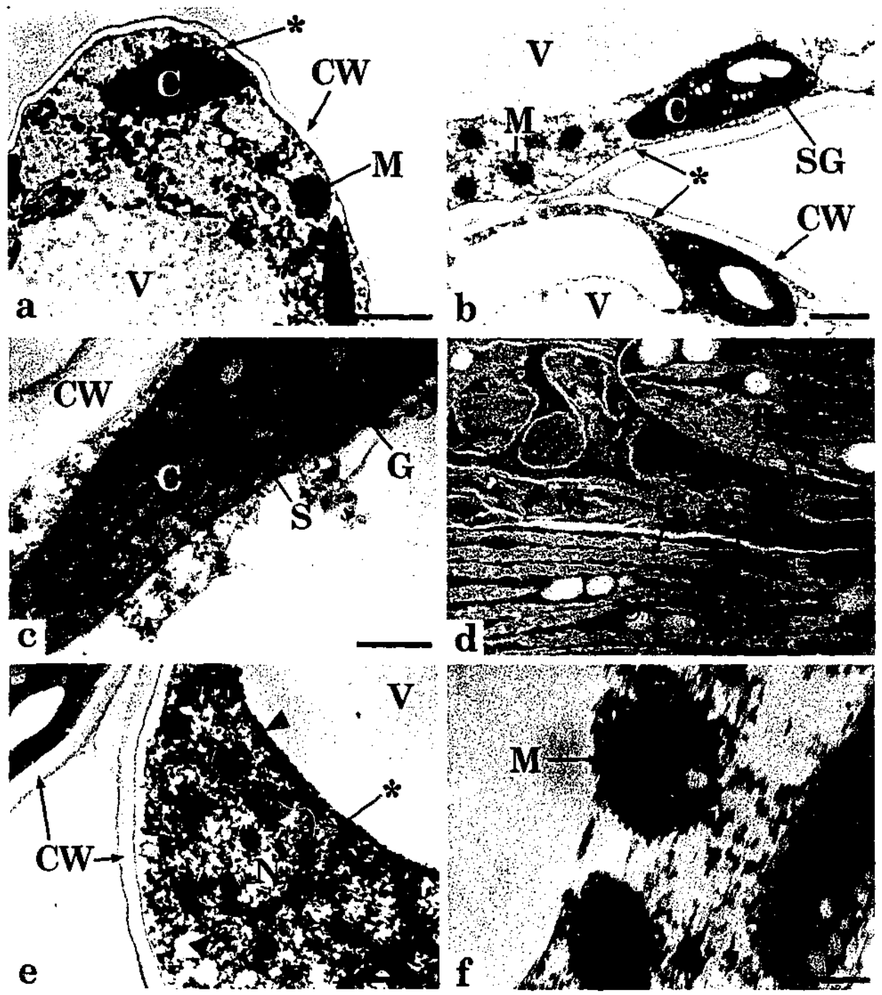


Figure 38. - Hydrated *Myrothamnus flabellifolia* mesophyll tissue fixed using the phase-partition fixation technique for 6 hours.

Fig. 38a - View of hydrated mesophyll cells fixed in 37% formaldehyde in FC-72. The plasma membrane and the cell wall were generally in close contact with each other (arrow heads). A single large vacuole (V) was observed to occupy the centre of the cell. Chloroplasts (C), nuclei (N) and mitochondria (M) are some of the organelles that were regularly observed. These organelles were turgid in appearance. Bar represents 1 µm.

Fig. 38b - Chloroplasts (C) regularly contained starch grains (SG). The granal (G) and stromal (S) regions of the chloroplasts could easily be identified. The granal stacks (G) were generally long and thin in shape. The thylakoid membranes that comprise the granal stacks were difficult to distinguish. Plastoglobuli (P) were regularly present. Bar represents 0.5 µm.

Fig. 38c - The nucleus (N) contained chromatin (*) that was generally finely dispersed. A nucleolus (Ne) was regularly observed as too were the double bounding membranes (arrow heads) of the nucleus. Bar represents 0.25 µm.

Fig. 38d - Mitochondria (M) were regularly round in shape. The bounding membrane was well preserved (arrow heads) although cristae were difficult to discern. Bar represents 0.5 µm.

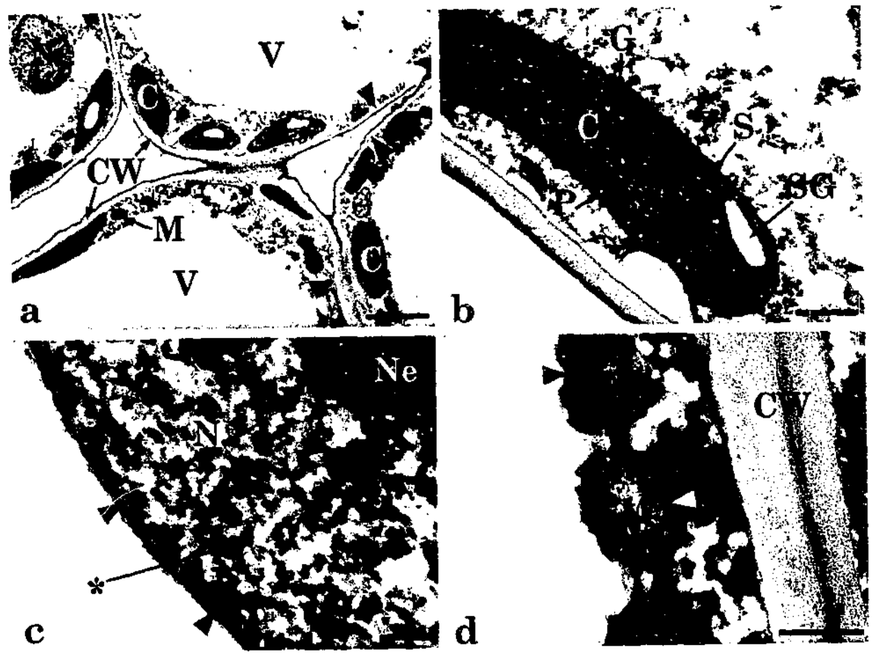


Figure 39. - Hydrated *Myrothamnus flabellifolia* mesophyll tissue fixed using the phase-partition fixation technique for 24 hours.

Fig. 39a - General view of a hydrated mesophyll cell fixed in 1% OsO_4 in HFE 7100. Cells were often flaccid in appearance with the cell wall (CW) being slightly convoluted. The plasma membrane was generally in close alignment with the cell wall (arrow heads). A large amount of electron-dense material (E) was frequently seen in the central vacuole region of the cell. Bar represents 2 µm.

Fig. 39b - A general view of a hydrated mesophyll cell fixed in 25% glutaraldehyde in FC-72. In contrast to Fig. 39a, the cell was turgid in appearance with the cell wall (CW) being smooth in outline. In a number of areas the plasma membrane and the cell wall have separated (*). Cell structures such as chloroplasts (C), the nucleus (N), mitochondria (M) and vacuoles (V) were regularly identifiable. Bar represents 2 µm.

Fig. 39c - A high magnification view of Fig. 39b. The plasma membrane and the cell wall have separated (*). Chloroplasts (C) were generally elongated in shape and often possessed starch grains (SG). Round and elongated shaped mitochondria (M) that possessed cristae (arrow heads) were regularly observed. Bar represents 0.5 µm.

Fig. 39d - A detail of a chloroplast (C) fixed in 25% glutaraldehyde in FC-72. Chloroplast were elongate in shape and contained plastoglobuli (P), no other details could be determined. The plasma membrane could also be seen in close apposition to the cell wall (arrow heads). Bar represents 0.5 µm.

Fig. 39e - A detail of a chloroplast fixed in 1% OsO_4 in HFE 7100. Chloroplasts (C) were elongated and the thylakoid membranes (arrow heads) were visible. Electron dense material (E) was often present in the central vacuole. Bar represents 0.5 µm.

Fig. 39f - Detail of a chloroplast (C) fixed in 37% formaldehyde in FC-72. Outer membranes of chloroplasts could be seen (arrow heads) as too could the granal (G) and stromal (S) regions. Plastoglobuli (P) were also present. Bar represents 0.5 µm.

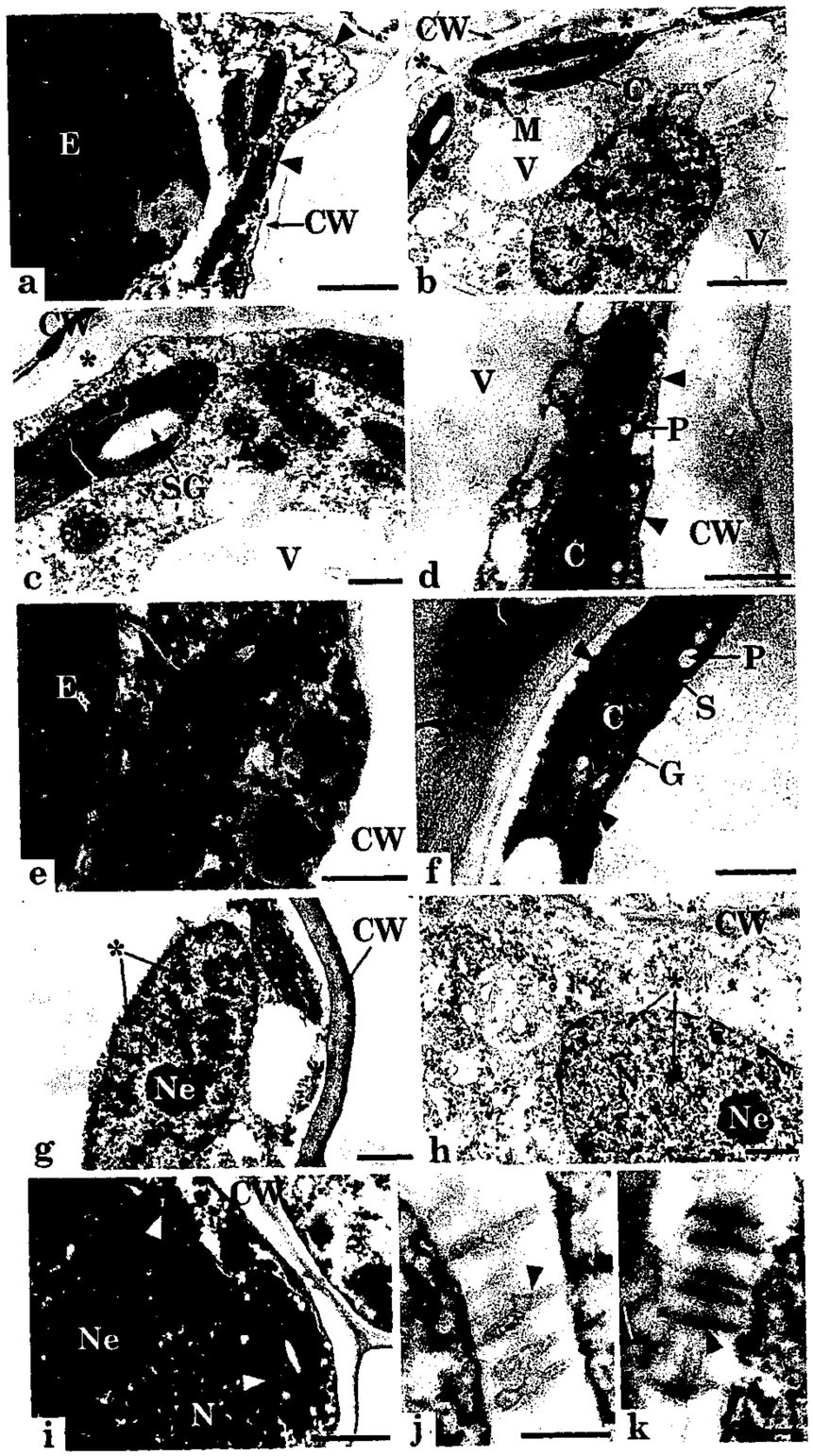
Fig. 39g - A nucleus (N) and nucleolus (Ne) in a hydrated cell fixed in 37% formaldehyde in FC-72. Nuclei were generally round to oval in shape and contained finely dispersed chromatin (*). Bar represents 1 µm.

Fig. 39h - A nucleus (N) and nucleolus (Ne) fixed in 1% OsO_4 in n-heptane. As in Fig. 39g, nuclei were generally round to oval in shape and contained finely dispersed chromatin (*). Bar represents 1 µm.

Fig. 39i - Nucleus (N) and nucleolus (Ne) fixed in 4% paraformaldehyde in HFE 7100. The outer membrane of the nucleus can be seen (arrow heads). Bar represents 1 µm.

Fig. 39j - Plasmodesmata (arrow heads) were frequently observed. Tissue sample was fixed in 4% paraformaldehyde in HFE 7100. Bar represents 0.25 µm.

Fig. 39k - Plasmodesmata (arrow heads) observed in tissue fixed in 1% OsO_4 in n-heptane. Bar represents 0.25 µm.



different lengths of time, they all possessed the same smooth, round shape. Sometimes more elongate forms were seen (Fig. 39c). Cristae were also obvious as light staining regions of the mitochondria (Fig. 39c).

Plasmodesmata were observed in tissue samples that had been fixed for 24 hours (Fig. 39j, 39k).

3.9.2 - **Dehydrated Tissue.**

The nucleus of dehydrated cells did not appear any different whether it was fixed for 1 hour (Fig. 40f) or 24 hours (Fig. 42b, 42c). In both instances it was irregular in shape and contained densely staining chromatin.

There also did not appear to be any obvious change in the ultrastructure of chloroplasts of dehydrated tissue fixed for either 1 hour (Fig. 40a, 40b, 40c), 6 hours (Fig. 41a) or 24 hours (Fig. 42a, 42b, 42c, 42d, 42e). Chloroplasts were usually round in shape and regularly possessed ruffled bounding membranes (40c, 41b, 41c, 42e, 42f). Thylakoid membranes were occasionally observed (Fig. 42f), however, in general, the granal region could only be distinguished as a less densely staining area within the stroma (Fig. 40c, 40e, 41b, 41c, 42b, 42c, 42d, 42e).

Mitochondria from dehydrated tissue also did not appear to be influenced by the duration of fixation. Mitochondria fixed for 1 hour (Fig. 40c, 40d, 40e), 6 hours

Figure 40. - Dehydrated *Myrothamnus flabellifolia* mesophyll tissue fixed using the phase-partition fixation technique for 1 hour.

Fig. 40a - A general view of dehydrated mesophyll cells fixed in 37% formaldehyde in FC-72. The cell wall (CW) was generally convoluted and was shrunken in appearance. The plasma membrane was observed adjacent to the cell wall in some areas (arrow heads) whereas in other areas the cell wall and the plasma membrane had separated (*). Vacuoles (V) were seen throughout the cytoplasm and chloroplasts (C) were also regularly seen. Bar represents 1 µm.

Fig. 40b - A general view of a mesophyll cell showing regions where the cell wall and the plasma membrane had separated (*) whereas in other regions they remained in close contact (arrow heads). A large central vacuole (V) was regularly seen as too were chloroplasts (C) and mitochondria (M). Tissue sample was fixed in 37% formaldehyde in FC-72. Bar represents 1 µm.

Fig. 40c - Chloroplasts (C) in dehydrated mesophyll cells fixed in 37% formaldehyde in FC-72 were mostly round in shape and possessed wavy outer membranes (arrow heads). Granal (G) and stromal (S) regions could be distinguished as light and dark regions of the chloroplast respectively however the internal membranes cannot be distinguished. Plastoglobuli (P) were generally present. Mitochondria (M) could also be seen. Bar represents 1 µm.

Fig. 40d - High magnification view of the cytoplasm of a dehydrated mesophyll cell fixed in 25% glutaraldehyde in FC-72. Thylakoid membranes (arrow heads) of chloroplasts could be discerned as too could plastoglobuli (P). Numerous cytoplasmic ribosomes (R) were regularly seen and mitochondria (M) were also present although cristae were occasionally difficult to distinguish. Bar represents 0.25 µm.

Fig. 40e - Various shaped mitochondria (M) were observed and they usually contained distinct cristae (arrow heads). Bar represents 0.5 µm.

Fig. 40f - Nuclei (N) contained condensed chromatin and were very dense in appearance. The chromatin could be distinguished into regions that were electron-dense (white *) and electron-lucent (black *). Bar represents 0.5 µm.

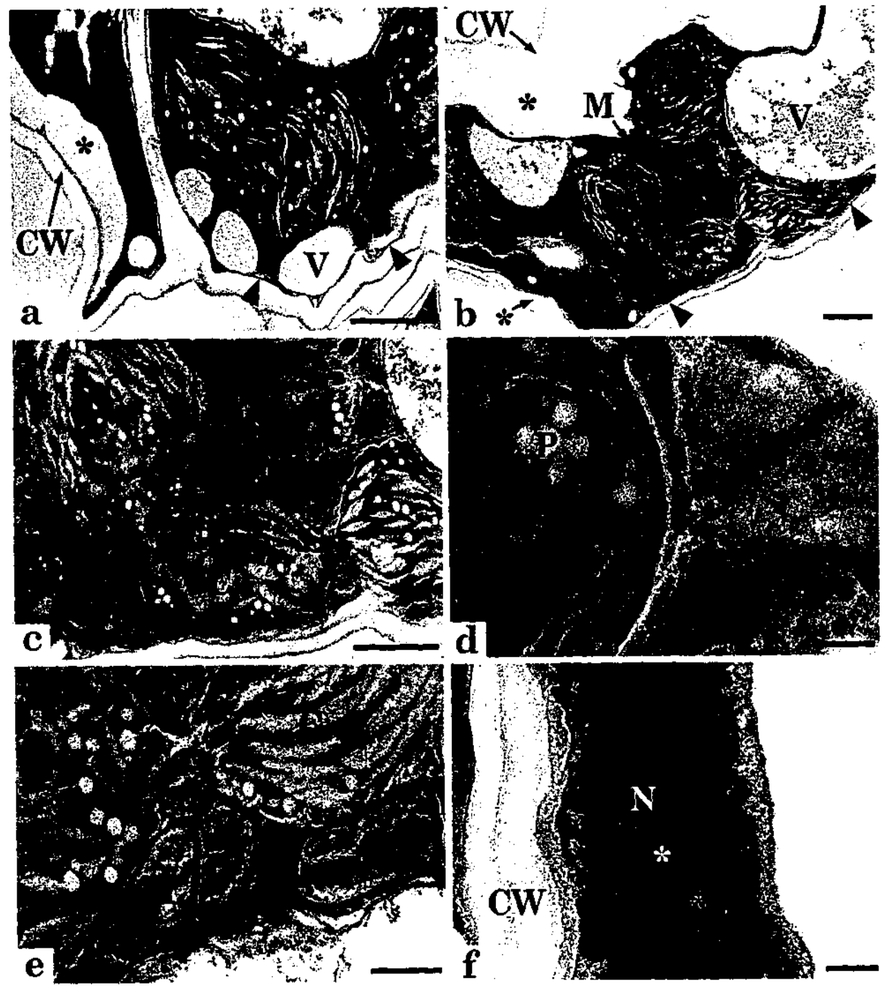


Figure 41. - Dehydrated *Myrothamnus flabellifolia* mesophyll tissue fixed using the phase-partition fixation technique for 6 hours.

Fig. 41a - General view of dehydrated mesophyll cells fixed in 25% glutaraldehyde in FC-72. The cell walls (CW) were relatively smooth in contour and the plasma membrane was regularly closely appressed to the cell wall (*). A central vacuole (V) was present and chloroplasts (C) were round in shape. Bar represents 2 µm.

Fig. 41b - In a number of cases, the plasma membrane was seen to have separated (*) from the cell wall. Chloroplasts (C) possessed granal (G) and stromal (S) regions with the grana being quite sort in length. Plasmodesmata (arrow head) were frequently observed. Bar represents 0.5 µm.

Fig. 41c - Chloroplasts also possessed a wavy outer membrane (*) and plastoglobuli (P) were frequently present. The thylakoid membrane (arrow heads) that comprised the granal stacks (G) could be seen in some samples. The stromal (S) region was also identifiable. Bar represents 0.25 µm.

Fig. 41d - Various shaped mitochondria (M) that possessed cristae (arrow heads) were regularly observed. Cytoplasmic ribosomes (R) were also viewed as too was the tonoplast membrane (*) that bounds the vacuole (V). Bar represents 0.25 µm.

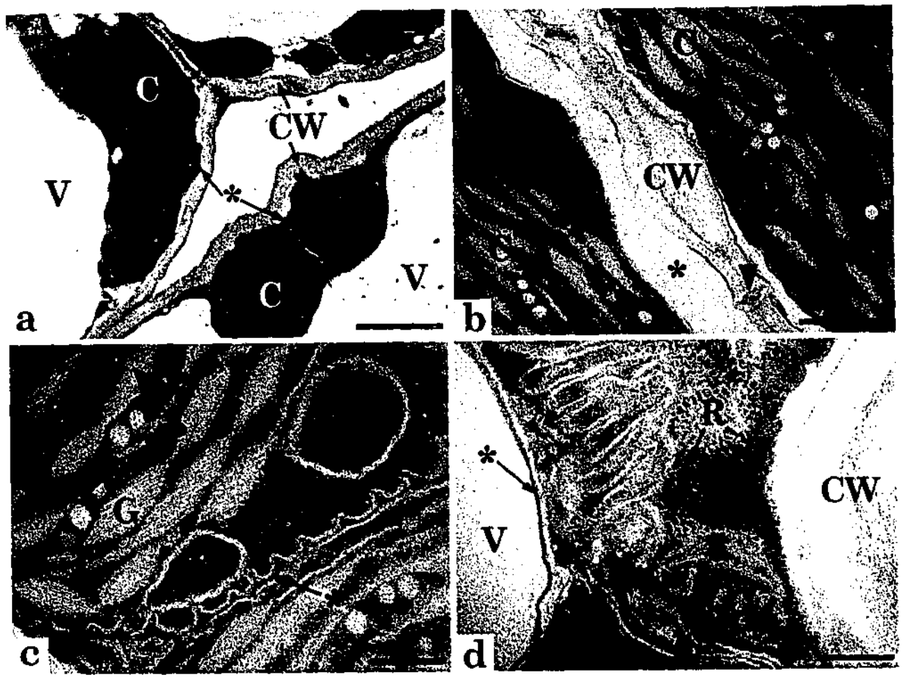


Figure 42. - Dehydrated *Myrothamnus flabellifolia* mesophyll tissue fixed using the phase-partition fixation technique for 24 hours.

Fig. 42a - General view of a dehydrated mesophyll cell fixed in 1% OsO_4 in n-heptane. The cell walls (CW) and the plasma membrane were generally closely appressed together (arrow heads). However some areas of cell wall and plasma membrane separation (*) could be seen. The cytoplasm was densely stained which obscured a lot of cellular detail although chloroplasts (C) could be discerned. Bar represents 1 µm.

Fig. 42b - General view of a dehydrated mesophyll cell fixed in 25% glutaraldehyde in n-heptane. Cell walls (CW) were flaccid and wavy in appearance and the plasma membrane was closely adhered to the cell wall (arrow heads). There was no evidence of a central vacuole but smaller more numerous vacuoles (V) were observed positioned throughout the cell. Bar represents 1 µm.

Fig. 42c - In a number of cases the plasma membrane of some cells had separated from the cell wall (*), leaving the cytoplasm positioned in the centre of the cell. Tissue sample was fixed in 25% glutaraldehyde in FC-72. Bar represents 2 µm.

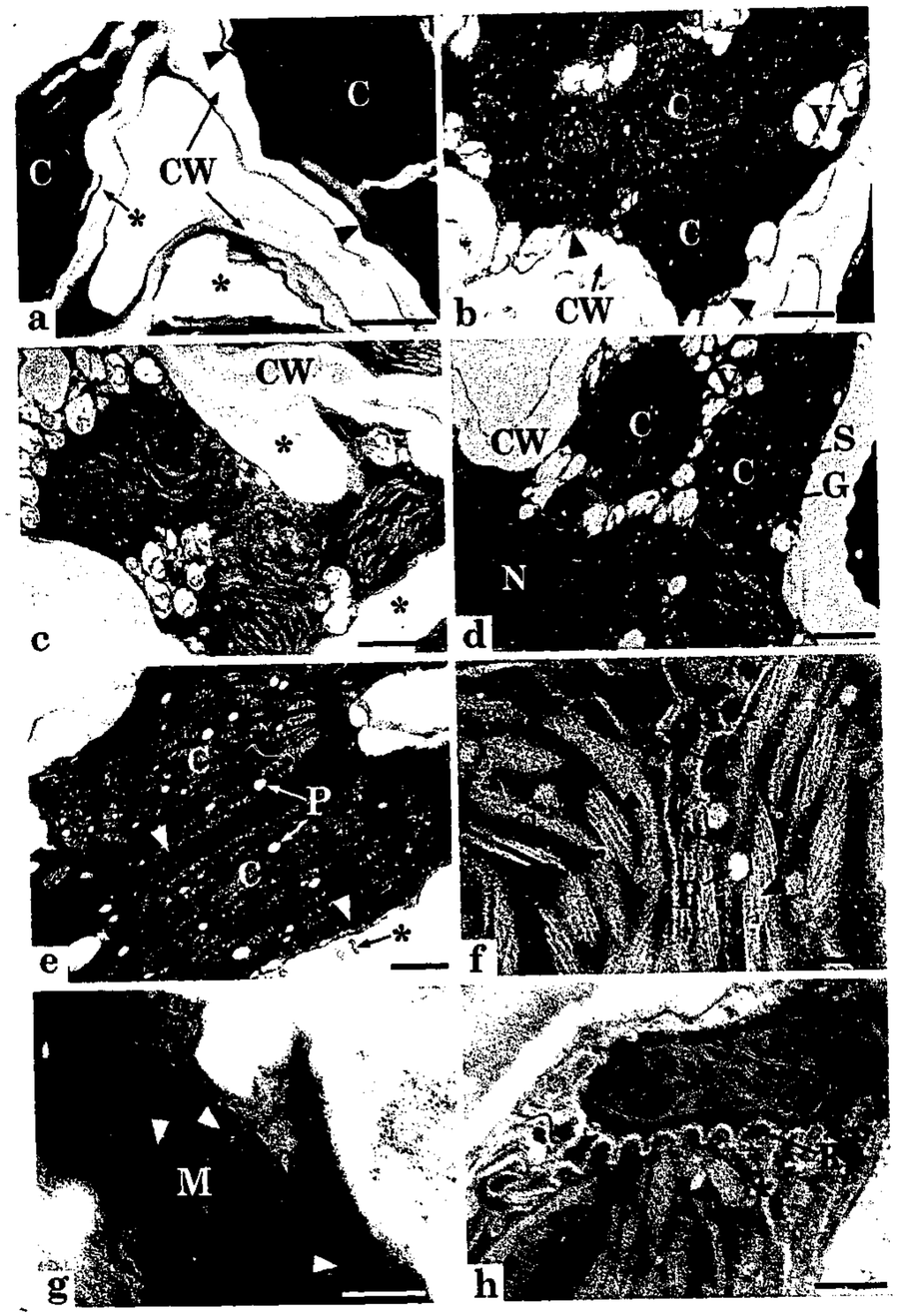
Fig. 42d - Chloroplasts (C) were usually round in shape and had light and dark staining regions which corresponded with the granal (G) and stromal (S) regions respectively. The nucleus (N) was irregular in shape and had a wavy outline. The chromatin was condensed and is electron-dense. Tissue sample was fixed in 25% glutaraldehyde in n-heptane. Bar represents 1 µm.

Fig. 42e - Chloroplasts (C) possessed a wavy outer membrane (arrow heads) and also contained plastoglobuli (P). Thylakoid membranes could not be discerned. Plasmodesmata were regularly observed (*). Sample taken from tissue fixed in 25% glutaraldehyde in n-heptane. Bar represents 0.5 µm.

Fig. 42f - Occasionally the thylakoid membranes (arrow heads) that comprise the granal stacks (G) of the chloroplast could be discerned. The stromal areas (S) could also be discerned. Tissue sample was fixed in 25% glutaraldehyde in FC-72. Bar represents 0.25 µm.

Fig. 42g - Various shaped mitochondria (M) were seen in tissue fixed in 1% OsO_4 in n-heptane. Cristae (arrow heads) were generally present. Bar represents 0.25 µm.

Fig. 42h - A mitochondrion (M) in a tissue sample fixed in 25% glutaraldehyde in FC-72. Cristae (arrow heads) could be seen as too could cytoplasmic ribosomes (R). Wavy cell walls (*) of chloroplasts (C) were also evident. Bar represents 0.25 µm.



(Fig. 41d) or 24 hours (Fig. 42g, 42h) were all irregular in shape and generally possessed distinct cristae.

3.10 - Effect of Fixative Concentration on Cellular Ultrastructure.

Two of the fixatives used during this study were at concentrations much higher than would be routinely used in aqueous chemical fixation, these being 25% glutaraldehyde and 37% formaldehyde. The other three fixatives were used at concentrations that would be considered 'normal' for use in laboratory experiments, these were Karnovsky's fixative, 4% paraformaldehyde and 1% osmium. A comparison was made between the various fixatives to determine whether high or low concentration fixatives influenced ultrastructural preservation.

3.10.1 - Hydrated Tissue.

Nuclei from hydrated tissue that had been fixed in high concentration fixatives 25% glutaraldehyde and 37% formaldehyde (Fig. 43a, 43d, 43i) did not appear any different from nuclei fixed in the lower concentration fixatives, i.e. 4% paraformaldehyde, Karnovsky's fixative or 1% osmium (Fig. 44a, 44f, 44g). No matter what concentration fixative the hydrated leaf tissue samples had

Figure 43. - Hydrated *Myrothamnus flabellifolia* mesophyll tissue fixed in high concentration fixatives.

Fig. 43a - A general view of a hydrated mesophyll cell fixed in 25% glutaraldehyde in FC-72. Chloroplasts (C), mitochondria (M), nuclei (N) and vacuoles (V) were some of the structures that were routinely seen. Bar represents 2 µm.

Fig. 43b - A general view of a hydrated mesophyll cell fixed in 37% formaldehyde in FC-72. Cells generally had cell walls (CW) that were smooth and rounded. A large central vacuole (V) was regularly present and the plasma membrane was in close apposition to the cell wall (*). Chloroplasts (C) and nuclei were also observed. Bar represents 3 µm.

Fig. 43c - A micrograph of a tissue sample fixed in 25% glutaraldehyde in FC-72 in which the cell wall and the plasma membrane had separated (*). A central vacuole (V) was regularly visible. Chloroplasts (C) were generally elongated in shape and frequently contained starch grains (SG). Both round and elongated mitochondria (M) were observed, cristae (arrow heads) were visible as electron-lucent areas. Bar represents 0.5 µm.

Fig. 43d - Nuclei (N) were generally round to oval in shape and had a turgid appearance. They contained finely dispersed chromatin (*) and nucleoli (Ne) were regularly observed. Tissue sample was fixed in 37% formaldehyde in FC-72. Bar represents 1 µm.

Fig. 43e - Chloroplasts (C) fixed in 25% glutaraldehyde in FC-72 were generally elongated in shape. Plastoglobuli (P) could be seen as too could the granal (G) and stromal (S) regions. Mitochondria (M) were regularly visible although cristae were sometime difficult to distinguish. Bar represents 1 µm.

Fig. 43f - Chloroplasts fixed in 37% formaldehyde in FC-72 were also elongated in shape. The outer bounding membrane (arrow head) was regularly visible although the thylakoid membranes were not. The granal stacks (G) could be discerned as the lighter staining region of the chloroplast in comparison to the darker staining stromal (S) region. Plastoglobuli (P) were routinely present. Bar represents 0.25 µm.

Fig. 43g - Plasmodesmata (arrow head) were regularly observed. Tissue sample fixed in 37% formaldehyde in FC-72. Bar represents 0.5 µm.

Fig. 43h - A high magnification view of a chloroplast (C) from tissue fixed in 25% glutaraldehyde in FC-72. Granal stacks (G) were generally long and thin and plastoglobuli (P) were also present. Mitochondria (M) with cristae (arrow head) were rarely observed. Bar represents 0.25 µm.

Fig. 43i - The double outer bounding membrane (arrow heads) of some nuclei (N) could be seen. Nucleoli (Ne) were also visible as too was finely dispersed chromatin (*). Sample was taken from tissue fixed in 37% formaldehyde in FC-72. Bar represents 0.25 µm.

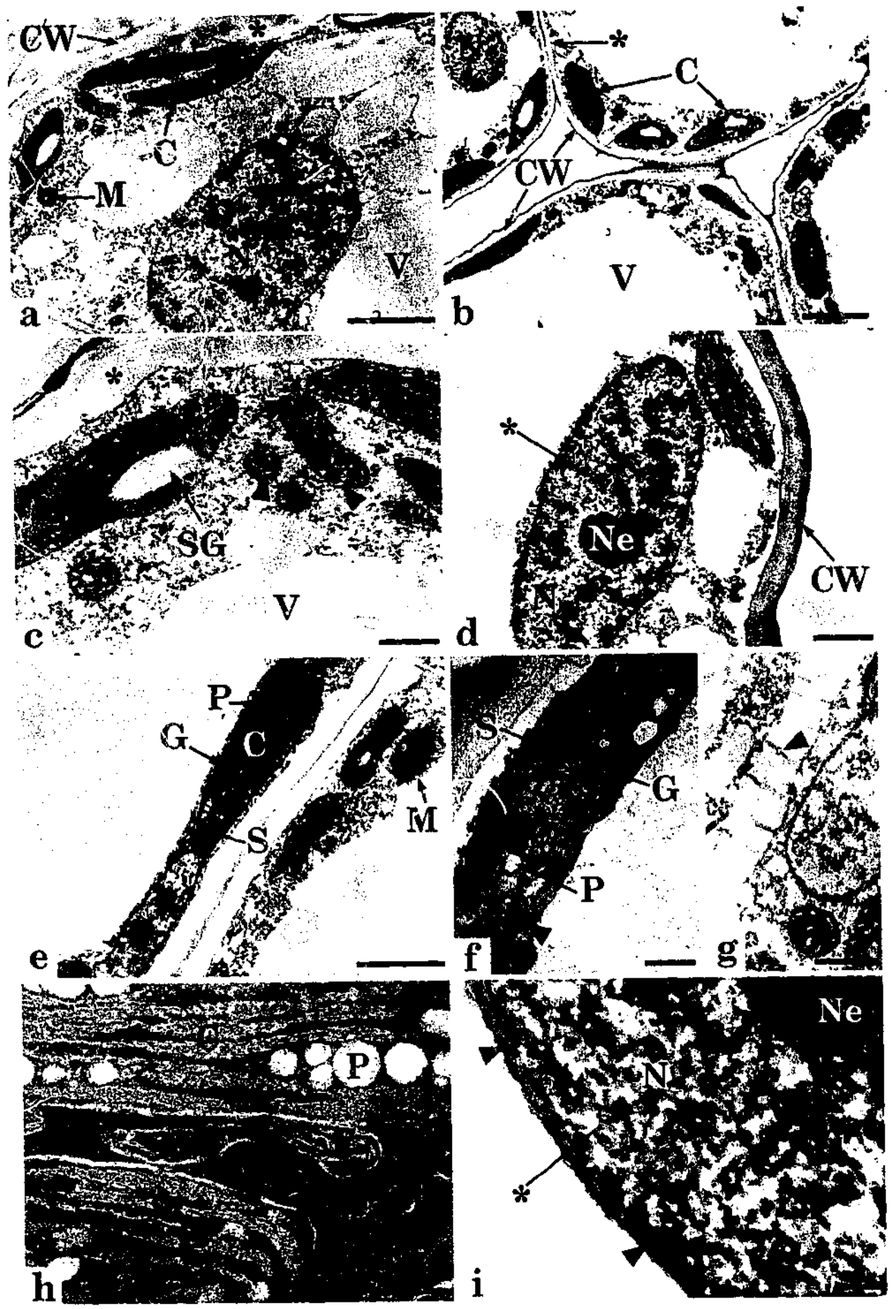


Figure 44. - Hydrated *Myrothamnus flabellifolia* mesophyll tissue fixed in low concentration fixatives.

Fig. 44a - A general view of a hydrated mesophyll cell fixed in Karnovsky's fixative in n-heptane. Cells appeared flaccid, as they possessed cell walls (CW) that were slightly convoluted. Chloroplasts (C) tended to be oval in shape and regularly possessed starch grains (SG). Nuclei (N) regularly contained a nucleolus (Ne). Bar represents 2 µm.

Fig. 44b - Tissue samples fixed in 1% OsO4 in n-heptane were also flaccid looking and had cell walls (CW) that were slightly convoluted. The plasma membrane was in close apposition to the cell wall (*) and large masses of electron-dense material (E) were regularly observed in the central vacuole of these cells. Chloroplasts (C) were generally elongated in shape. Bar represents 2 µm.

Fig. 44c - Granal (G) and stromal (S) areas of the chloroplast (C) were identified by differences in the staining intensity with the granal stacks staining lighter than the stromal area. Plasmodesmata (Pd) were regularly identified. Chloroplasts (C) frequently possessed starch grains (SG). Tissue sample was fixed in Karnovsky's fixative in n-heptane. Bar represents 1 µm.

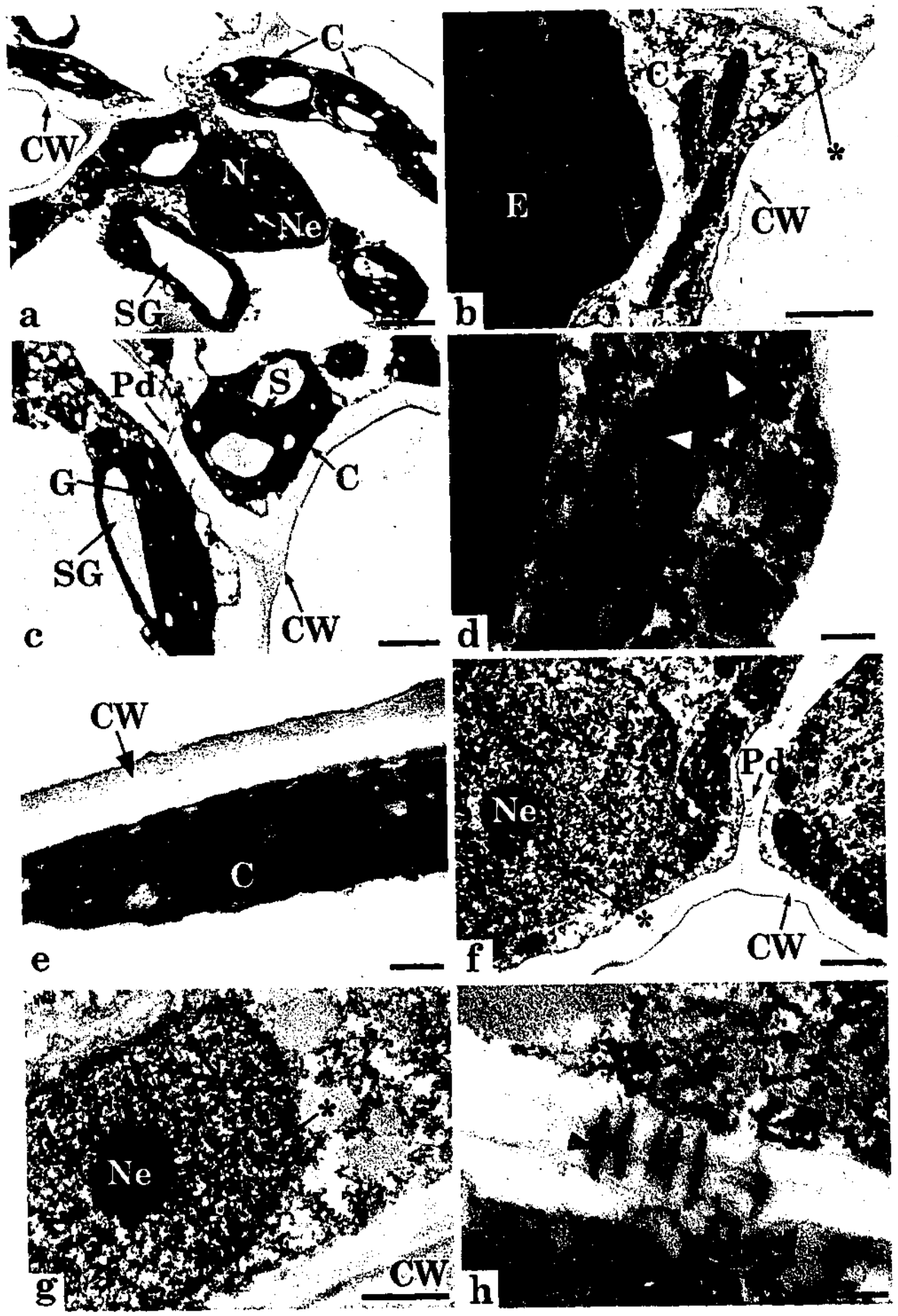
Fig. 44d - High magnification view of a chloroplast (C) fixed in 1% OsO_4 in HFE 7100. Chloroplasts were normally elongate in shape and had long slender granal stacks (G). Occasionally it was possible to observe fine lines within the granal stacks, which were the thylakoid membranes (arrow heads) that comprise these stacks. Bar represents 0.25 µm.

Fig. 44e - Chloroplasts (C) fixed in 4% paraformaldehyde in HFE 7100 were also elongated in shape. The overall quality of preservation was poor and the internal structures were difficult to identify. Bar represents 0.25 µm.

Fig. 44f - Nuclei (N) were generally oval to round in shape and were smooth in contour. They contained finely dispersed chromatin (*) and nucleoli were also regularly observed (Ne). Tissue was fixed in 4% paraformaldehyde in HFE 7100. Bar represents 1 µm.

Fig. 44g - Nuclei (N) of similar appearance to that described in Xf were also observed in tissue fixed in 1% OsO_4 in n-heptane. Again a nucleolus (Ne) is present and the chromatin (*) is visible and is finely dispersed. Bar represents 1 µm.

Fig. 44h - Plasmodesmata (arrow head) were frequently observed. Tissue was fixed in 1% OsO_4 in n-heptane. Bar represents 0.25 µm.



been fixed in, the nuclei were round in shape and had a smooth outline.

Chloroplasts from hydrated tissue that had been fixed in high concentration fixatives (Fig. 43a, 43b, 43c, 43d, 43e, 43f) also did not appear to differ from chloroplasts that had been fixed in the low concentration fixatives (Fig. 44a, 44b, 44c, 44d, 44e). Chloroplasts from hydrated leaf tissue were oval to elongate in shape and regularly possessed starch grains and plastoglobuli. Grana were generally long and thin in shape.

Mitochondria from hydrated tissue fixed in high concentration fixatives (Fig. 43c, 43b, 43e, 43g) were similar in appearance to those fixed using the low concentration fixative, Karnovsky's fixative (Fig. 44c). Mitochondria were generally round in shape and cristae were evident as electron-translucent regions. Occasionally mitochondria, which were more similar in appearance to those generally seen in dehydrated tissue, were observed (Fig. 43h). Plasmodesmata were seen in tissues fixed in both low (Fig. 44c, 44f, 44h) and high (Fig. 43g) concentration fixatives.

3.10.2 - **Dehydrated Tissue.**

Dehydrated cells generally had mildly convoluted cell walls no matter whether they were fixed in a high concentration fixative (Fig. 45d) or a low concentration

fixative (Fig. 46a, 46d). Although occasionally cells were observed that had extremely convoluted cell walls and were shrunken in appearance (Fig. 46b).

Nuclei from dehydrated tissue did not differ ultrastructurally in relation to the concentration of the fixative. Nuclei fixed in the high concentration fixative, 25% glutaraldehyde (Fig.45g) were very similar in appearance to those fixed in the low concentration Karnovsky's fixative (Fig. 46f). In both cases, they were generally irregular in shape and contained densely staining highly compacted chromatin.

Chloroplasts from dehydrated leaf tissue also did not appear to be influenced by fixative concentration. Chloroplasts were generally circular in shape and had similar degrees of internal membrane preservation no matter whether they were fixed at high (Fig. 45a, 45b, 45c, 45d, 45e, 45f) or low (Fig. 46a, 46c, 46d, 46e, 46f) concentrations. Only occasionally could the thylakoid membranes of the granal stacks be discerned (Fig. 45e). Plastoglobuli were frequently observed in tissue samples fixed in both high concentration (Fig. 45d, 45e, 45f) and low concentration (Fig. 46e) fixatives.

The appearance of mitochondria in dehydrated tissue also seemed unaffected by the concentration of the fixative used. Examples from high concentration fixatives (Fig. 45h, 45i) and low concentration fixatives (Fig. 46g, 46h) illustrate the general appearance of mitochondria from

Figure 45. - Dehydrated *Myrothamnus flabellifolia* mesophyll tissue fixed in high concentration fixatives.

Fig. 45a - General view of a dehydrated mesophyll cell fixed in 25% glutaraldehyde in FC-72. The cell wall (CW) was only slightly convoluted in appearance and the protoplast was contracted in the centre of the cell leaving large areas of separation (*) between the cell wall and the plasma membrane. Numerous small vacuoles (V) were observed distributed throughout the cytoplasm. Bar represents 1 µm.

Fig. 45b - General view of a mesophyll cell fixed in 37% formaldehyde in FC-72. The cell wall was slightly convoluted in some areas (CW) whereas in other areas it appeared relatively turgid (*CW). Areas where the plasma membrane had separated from the cell wall (*) could regularly be seen. A large central vacuole (V) was usually present. Bar represents 1 µm.

Fig. 45c - In contrast to Fig. 45a, the cell wall and plasma membrane of some cells fixed in 25% glutaraldehyde in n-heptane remained closely appressed to each other (*). Numerous small vacuoles (V) were seen throughout the cytoplasm. Chloroplasts (C) were generally round in shape and nuclei (N) were irregular and flaccid in appearance. Bar represents 1 µm.

Fig. 45d - Small vacuoles (V) distributed within the cytoplasm of cell were also seen in tissue fixed in 37% formaldehyde in FC-72. Chloroplasts (C) generally possessed a wavy outer membrane (*) and were round in shape. Bar represents 1 µm.

Fig. 45e - High magnification view of a chloroplast taken from tissue fixed in 25% glutaraldehyde in FC-72. The thylakoid membranes (arrow heads) that comprise the granal stacks of the chloroplast could occasionally be seen. A wavy outer membrane of the chloroplast (*) was regularly observed. Bar represents 0.25 µm.

Fig. 45f - A chloroplast fixed in 37% formaldehyde in FC-72. The thylakoid membranes cannot be discerned however the granal stacks (G) can still be identified as they stained a lighter colour compared to the stromal (S) region. Plastoglobuli (P) were routinely observed. Bar represents 0.5 µm.

Fig. 45g - Nuclei (N) observed were very condensed in nature and contained regions of condensed chromatin that was either electron-lucent (black *) or electron-dense (white *). Bar represents 0.5 µm.

Fig. 45h - Mitochondria observed were usually irregular in shape and possessed cristae (arrow head). Tissue sample was fixed in 37% formaldehyde in FC-72. Bar represents 0.25 µm.

Fig. 45i - Mitochondria of a similar appearance to Fig. 45h were observed in tissue fixed in 25% glutaraldehyde in FC-72. Cristae were regularly seen (arrow head). Bar represents 0.25 µm.

Fig. 45j - Plasmodesmata (arrow head) were frequently observed. Tissue was fixed in 25% glutaraldehyde in n-heptane. Bar represents 0.1 µm.

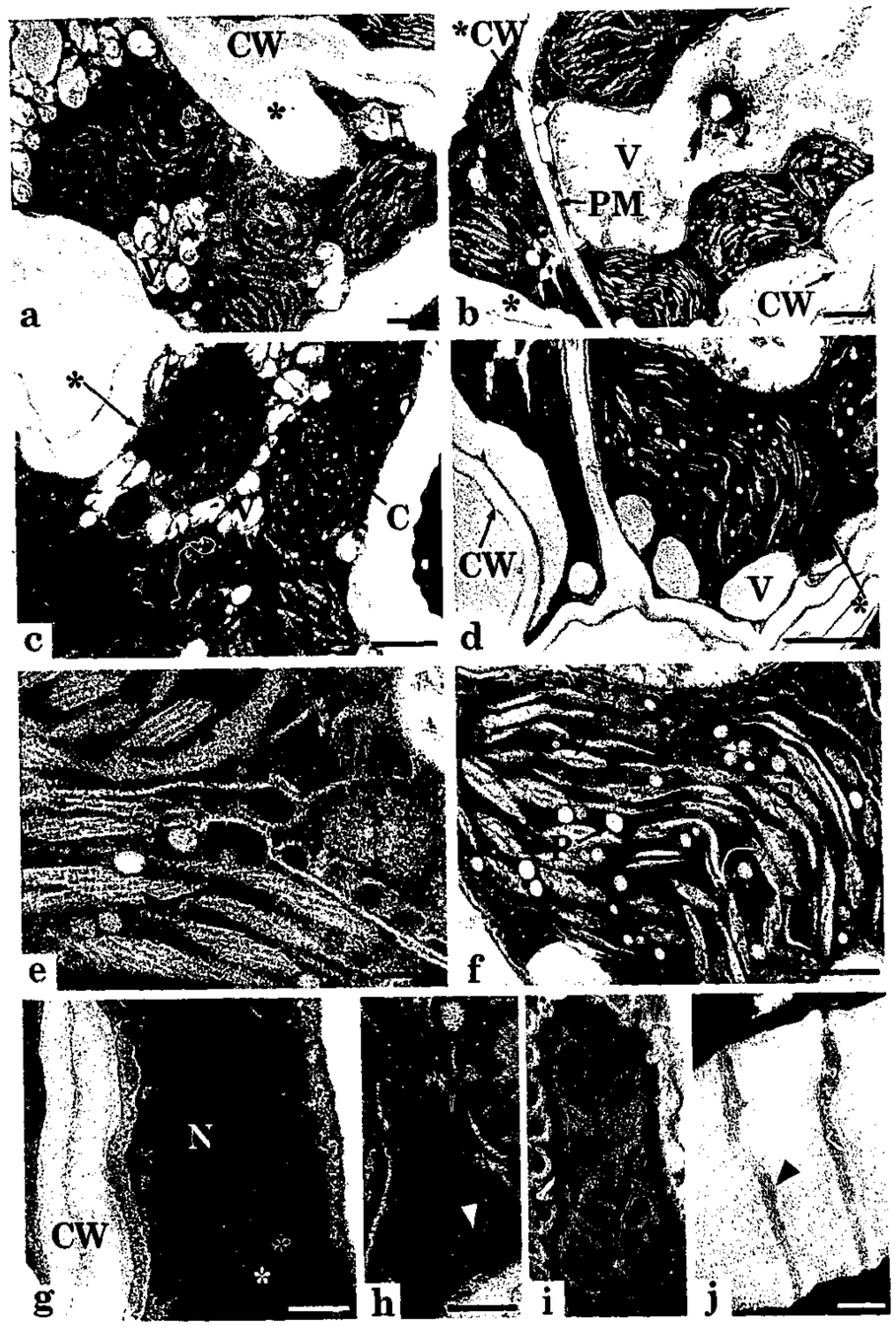


Figure 46. - Dehydrated *Myrothamnus flabellifolia* mesophyll tissue fixed in low concentration fixatives.

Fig. 46a - General view of a dehydrated mesophyll cell fixed in Karnovsky's fixative in n-heptane. The cell walls (CW) were quite convoluted and the plasma membrane was generally in close contact with the cell wall (*). A large central vacuole (V) was regularly observed in which there was a large mass of electron-dense material (E). Chloroplasts (C) and mitochondria (M) were regularly seen. Bar represents 1 µm.

Fig. 46b - Dehydrated mesophyll cells fixed in 1% OsO_4 in HFE 7100. Cells were extremely shrunken and had highly convoluted cell walls (CW). Little structural detail could be determined, as the quality of preservation was very poor. Areas where the cell wall and the plasma membrane had separated were observed (*). Bar represents 2 µm.

Fig. 46c - The cytoplasm of tissue samples fixed in 1% OsO4 in n-heptane stained very intensely and little structural detail could be determined. Chloroplasts (C) were one of the few structures that could be easily identified even though they were poorly preserved. They appeared to be relatively round in shape and although it was difficult to distinguish the outer bounding and internal membranes the granal (G) and stromal (S) regions could be distinguished. Bar represents 1 µm.

Fig. 46d - Chloroplasts (C) seen in tissue samples fixed in Karnovsky's fixative in n-heptane were oval to round in shape and possessed a wavy outer membrane (*). Bar represents 1 µm.

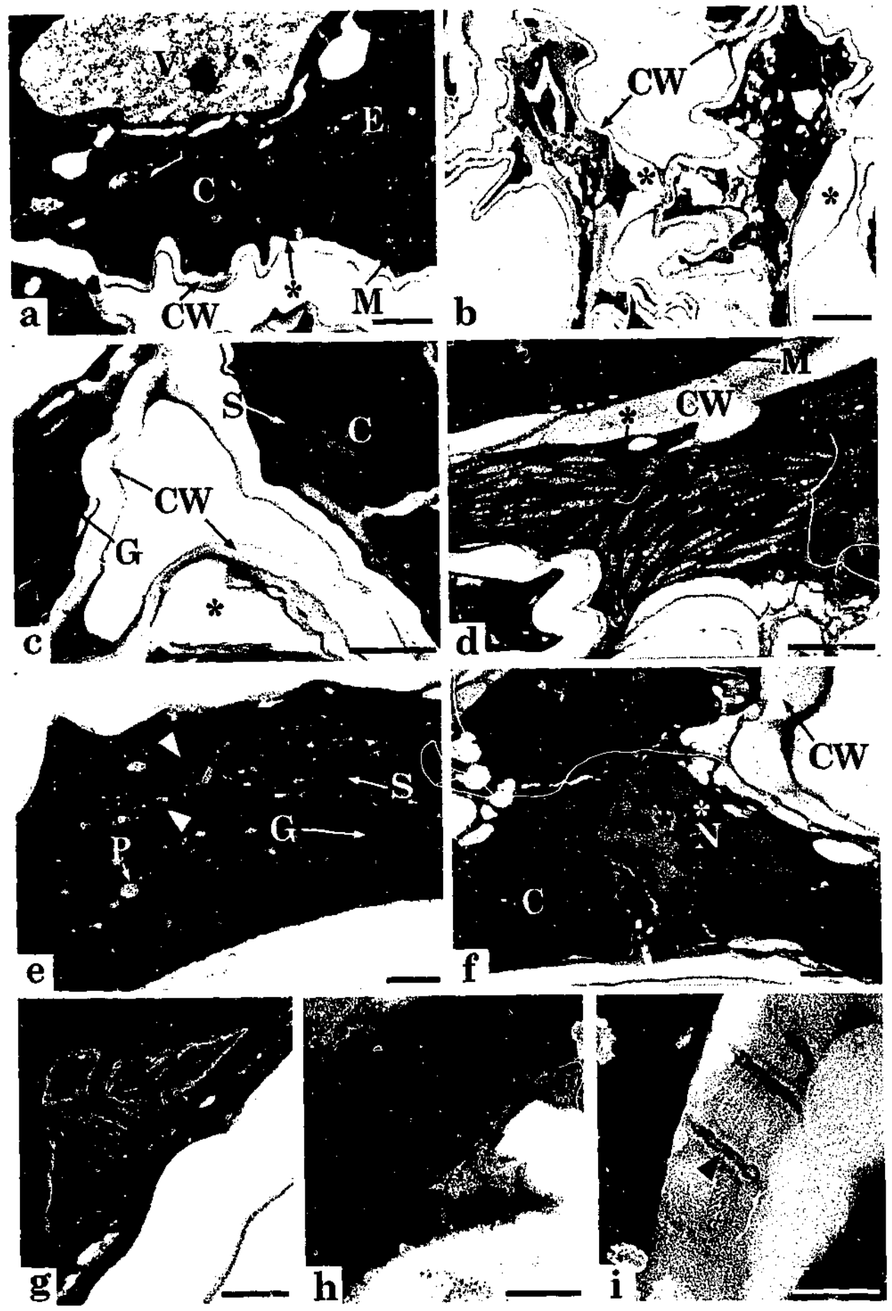
Fig. 46e - A high magnification view of a chloroplast fixed in Karnovsky's fixative in n-heptane. Occasionally it was possible to observe faint lines in the granal regions (G) of chloroplasts, these lines were the thylakoid membranes (arrow heads) that comprise the granal stacks (G). The stromal (S) regions could also be identified. Plastoglobuli (P) were also frequently present. Bar represents 0.25 µm.

Fig. 46f - Nuclei (N) were irregular in shape, with a wavy outline (arrow heads) and had a condensed appearance. The nucleus contained condensed chromatin which was either electron-lucent (black *) or electron-dense (white *). Tissue was fixed in Karnovsky's fixative in n-heptane Bar represents 1 µm.

Fig. 46g - Various shaped mitochondria (M) were observed which almost always contained distinct cristae (arrow heads). Tissue samples was taken from leaves fixed in Karnovsky's fixative in n-heptane. Bar represents 0.25 µm.

Fig. 46h - Irregular shaped mitochondria (M) containing cristae (arrow head) were also observed in tissue samples fixed in 1% osmium in n-heptane. Bar represents 0.25 µm.

Fig. 46i - Plasmodesmata (arrow head) were regularly observed. Tissue was fixed in Karnovsky's fixative in n-heptane. Bar represents 0.25 µm.



dehydrated tissue. In all instances, mitochondria from dehydrated tissue were irregular in shape and possessed distinct cristae.

Plasmodesmata were seen in tissue samples of both high (Fig. 45j) and low (Fig. 46i) concentration fixatives.

Table 3. - Summary of the ultrastructural findings for phase-partition fixed hydrated and dehydrated *Myrothamnus flabellifolia* leaf tissue.

	Hydrated	Dehydrated
Cell Shape	Smooth in outline.	Convoluted cell walls.
Protoplast	Adjacent to cell wall although occasional separation was observed.	Adjacent to cell wall.
Vacuole	Central region contained numerous vacuoles.	Sometimes possessed a central vacuole other times more numerous vacuoles were observed.
Chloroplast	Elongate to oval in shape. Starch grains and plastoglobuli were present. Thylakoid membranes were visible and grana were long and thin in shape.	Round in shape. Possessed a ruffled outer membrane. Grana were short and compact in shape.
Mitochondria	Round to oval in shape, although sometimes more elongated forms were seen. Cristae were occasionally observed.	Irregular in shape and contained distinct cristae.
Nucleus	Round to oval in shape. Nucleoli were observed and chromatin was finely dispersed.	Irregular in shape and contained densely staining chromatin.

DISCUSSION.

In this chapter the technique of phase-partition fixation was successfully used, for the first time, to preserve cellular ultrastructure in dehydrated tissue samples for TEM examination.

3.11 - Phase-Partition Fixation as a Technique for the Preservation of Hydrated and Dehydrated *Myrothamnus flabellifolia* Leaf Tissue.

As mentioned previously (refer to 3.1), the technique of phase-partition fixation has been successfully utilized for the preservation of cellular ultrastructure at both the light (Nettleton and Rice 1982, Nettleton and McAuliffe 1986, Horne and Sims 1991, Sims and Horne 1997) and TEM levels (Thurston *et al.* 1976, Zalokar and Erk 1977, McAuliffe and Nettleton 1984, McFadden *et al.* 1988, Horne and Sims 1991, Sims *et al.* 1991, Rostgaard *et al.* 1993, Lee *et al.* 1995, Geiser *et al.* 1997, Sanchez *et al.* 1997, Sims and Horne 1997, Schürch *et al.* 1998). Results from this study indicate that phase-partition fixation can also be used to preserve the ultrastructure of hydrated and dehydrated leaves of *Myrothamnus flabellifolia*.

A comparison of the cellular ultrastructure of hydrated and dehydrated *Myrothamnus flabellifolia* mesophyll tissue revealed numerous differences between the two tissue

types. The overall shape of hydrated and dehydrated cells was dramatically different with hydrated cells having a rounded appearance in comparison to the shrunken, convoluted shape of cells from dehydrated tissue (Table 3). Hydrated cells generally possessed a large central vacuole whereas in dehydrated cells smaller more numerous vacuoles were often observed (Table 3). Differences between hydrated and dehydrated tissue were also seen in the ultrastructure of various organelles. For example, the nucleus in hydrated tissue was round in shape and contained loosely dispersed chromatin whereas the nucleus in dehydrated tissue was irregular in shape and contained condensed chromatin (Table 3). Differences were also observed between chloroplasts from hydrated and dehydrated tissue. Chloroplasts observed in hydrated tissue were elongated in shape whereas chloroplasts from dehydrated tissue were round in shape (Table 3).

Overall the quality of ultrastructural preservation of *Myrothamnus flabellifolia* leaf tissue produced when using phase-partition fixation was satisfactory. As described above there were a number of differences observed between hydrated and dehydrated tissue samples fixed in this manner. These differences are indicative of the successful preservation of the tissue in its native state as it has been found that fixation induced artefacts tend to make hydrated and dehydrated tissue samples more similar in appearance (refer to Chapter 2, 2.25). It is difficult to

compare my findings with the results of other researchers as almost all previous work using this technique has concentrated on the preservation of mucous layers in mammalian tissues (Sims *et al.* 1991, Lee *et al.* 1995, Geiser *et al.* 1997, Sims and Horne 1997, Allan-Wojtas *et al.* 1997, Sanchez *et al.* 1997). Only a few studies reported any ultrastructural details apart from the preservation of mucous linings. McFadden *et al.* (1988) mentioned that phase-partition fixation resulted in adequate ultrastructural preservation, with major organelles such as nuclei, plastids and mitochondria readily identifiable. Thurston *et al.* (1976) noted that the membranes and cristae of mitochondria from lung tissue were well preserved using phase-partition fixation. It is possible to surmise from the lack of discussion about ultrastructural detail that it is very similar to that observed in conventionally fixed material.

3.12 - A Comparison of the Ultrastructural Preservation of Phase-Partition Fixed Tissue and Tissue Fixed Using Aqueous and Anhydrous Chemical Fixatives.

A comparison of the results of phase-partition fixed tissue and tissue fixed using the previously used techniques of aqueous and anhydrous chemical fixation, outlined in Chapter 2, revealed a number of differences.

3.12.1 - **Hydrated Tissue.**

A loss of the smooth, rounded shape of hydrated *Myrothamnus flabellifolia* cells was occasionally observed in phase-partition fixed material. There was also evidence of separation of the plasma membrane from the cell wall, which was not noted in tissue fixed by aqueous chemical fixation (refer to Table 3 and Chapter 2, Table 1). These observations are indicative of cellular shrinkage and are similar to the changes observed in hydrated tissue fixed using anhydrous chemical fixation (refer to Chapter 2, Table 1).

Nettleton and McAuliffe (1986) also found that in some cases, phase-partition fixation failed to prevent shrinkage and swelling artefacts even though fixation of the tissue blocks was apparently complete. They postulated that the shrinkage artefacts might have resulted from the post-fixation dehydration of the tissue samples. This may also explain the shrinkage artefacts observed in the hydrated leaf tissue of *Myrothamnus flabellifolia*. Therefore, one may need to test variations in the post-fixation procedure, such as the use of different solvents or dehydration times, to optimize the phase-partition protocol for particular tissues.

Previous studies have shown that longer fixation times (Hattel *et al.* 1983) and higher fixative concentrations (Hattel *et al.* 1983, Nettleton and McAuliffe 1986) may be

required for successful phase-partition fixation as there is speculation that phase-partitoned fixatives may penetrate more slowly than when in an aqueous medium (Hattel *et al.* 1983). This study compared the effect of fixation duration times (refer to 3.12.2) and fixative concentrations (refer to 3.12.3) and did not find any obvious ultrastructural differences. Virtually all researchers who have used the technique of phase-partition fixation have subsequently followed it with a secondary aqueous fixation technique. It is possible that phase-partition fixed tissue may retain its ability to respond osmotically even after fixation thus, as found by Nettleton and McAuliffe (1986), post-fixation processing can induce structural changes. This potential for structural change may have led some researchers to believe that the tissue had not been fixed properly and so they extended the fixation times and/or fixative concentrations. In contrast to previous uses of phase-partition fixation, in this thesis, no secondary aqueous fixation step was used. The elimination of this major source of ultrastructural artefacts (refer to chapter 2, 2.23) may have removed the need to use increased fixation times and/or fixative concentrations.

Although phase-partition fixation was not detrimental to the ultrastructure of most organelles in hydrated tissue, there were some obvious fixation-induced artefacts. Before there can be widespread use of this technique, it is

feasible that modifications to certain aspects of the fixation process may be required to reduce or eliminate these artefacts.

3.12.2 - **Dehydrated Tissue.**

A number of differences were also observed when comparing dehydrated leaf tissue fixed using either phase-partition fixation or anhydrous chemical fixation. Numerous small vacuoles were regularly observed in dehydrated leaf tissue after phase-partition fixation, whereas after anhydrous chemical fixation, a large central vacuole was generally seen (refer to Table 3 and Chapter 2, Table 1). Numerous small vacuoles have been observed in dehydrated tissue of a number of desiccation tolerant plants (Gaff *et al.* 1976, Bartley and Hallam 1979, Altus and Hallam 1980, Quartacci *et al.* 1997, Dalla Vecchia *et al.* 1998) including *Myrothamnus flabellifolia* (Farrant *et al.* 1999). These vacuoles are generally thought to arise from the breakdown of the single large vacuole present in hydrated tissue. Another possible explanation for the presence of the small vacuoles in dehydrated tissue is the fact that small vacuoles always precede the formation of the large central vacuole in cell development. Young cells are highly cytoplasmic and become vacuolate as they expand. It would seem logical that this process would be reversed, as water is lost from the cell during dehydration. As the

cells shrink, the cytoplasm would naturally make up a higher proportion of the cell volume, and the cytoplasmic strands that normally traverse the large vacuole may simply become larger, relative to the vacuole, until they eventually subdivide it into smaller vacuoles.

Altus and Hallam (1980) noted that vacuole fragmentation was more pronounced in some cells of *Sporobolus stapfianus* leaves than in others, which was also seen in dehydrated *Myrothamnus flabellifolia* leaf tissue after phase-partition fixation. Therefore it is possible that the small vacuoles may have been present in dehydrated tissue fixed anhydrously, but none of these cells were observed under the TEM (refer to Chapter 2, Table 1).

It is possible that the presence of the small vacuoles may be the result of a processing artefact, perhaps caused during transfer to the intermediate solvents after fixation. Finally, it is possible that the small vacuoles observed in the phase-partition fixed tissue may not be vacuoles but rather small holes. Thurston *et al.* (1976) and Sims (1991) found that the solvent FC-80 could interfere with the curing of resin, and holes could be produced in the cured block if the solvent was not fully eliminated from the tissue after fixation. However FC-72, the solvent used in this thesis, was found to be easier to remove from tissue after fixation than FC-80 (Sims *et al.* 1991), which would possibly reduce the likelihood of such holes forming. As the small vacuoles seen do not look like

holes in the resin, I do not think that it is very likely that the vacuoles seen are in fact holes in the resin.

Another difference observed, was that in dehydrated tissue fixed anhydrously, the protoplast remained closely appressed to the cell wall, whereas areas of separation were frequently seen in phase-partition fixed material (refer to Table 3 and Chapter 2, Table 1). This type of separation was also seen after aqueous chemical fixation (refer to Chapter 2, Table 1) and it appears to be correlated with tissue swelling during processing. It is possible that the dehydrated tissue absorbed minute residues of water which were left after the removal of the aqueous fixative during partitioning into the non-aqueous solvent, or the cells may have swollen in the intermediate solvent (acetone) during post-fixation processing.

Chloroplast internal membrane structure was better preserved in phase-partitioned fixed tissue than in tissue fixed by anhydrous chemical fixation (refer to Table 3 and Chapter 2, Table 1). There appeared to be no difference in the ultrastructure of the nucleus or the mitochondria in dehydrated tissue fixed by either of these two techniques (refer to Table 3 and Chapter 2, Table 1).

Overall, the above results indicate the potential of phase-partition fixation for use with both hydrated and dehydrated *Myrothamnus flabellifolia* leaf tissue. The ability to fix both hydrated and dehydrated specimens with the same fixation technique is certainly an advantage over

using the separate techniques of aqueous and anhydrous chemical fixation.

3.13 - Differences in Phase-Partition Fixative Technique and Their Effect on Ultrastructural Preservation.

Three phase-partition protocols were used to preserve *Myrothamnus flabellifolia* mesophyll leaf tissue. The impact of the different phase-partition fixation techniques on the preservation of cellular ultrastructure was analyzed.

3.13.1 - The Effect of Fixative Type.

There is some difference of opinion in the literature as to the suitability of various fixatives for phase-partition fixation. On one hand, Hattel *et al.* (1983) found that phase-partition fixation could be accomplished using both formalin and glutaraldehyde in all of the non-polar solvents tested, which included heptane, xylene, Mediflor (perfluorobutyl tetrahydrofuran) and Omnisolve (1,1,2-trichlorotrifluoroethane). Zalokar and Erk (1977) also found that glutaraldehyde partitioned well into heptane. On the other hand, Sims and Horne (1997) stated that glutaraldehyde and formaldehyde do not dissolve into perfluorocarbons and osmium is the only fixative that can be successfully partitioned into the perfluorocarbon FC-72.

None of these studies elaborated on how they came to the above conclusions.

One factor that could possibly explain the above findings is differences in the type of solvent used. To date the two major solvents that have been used are heptane and the perfluorocarbon FC-72. In previous studies, aldehyde fixatives were used solely with the solvent heptane and osmium was used solely with FC-72. The reason why this is the case is not mentioned in any of the studies except that Sims and Horne (1997) stated that glutaraldehyde and formaldehyde were unable to be partitioned successfully into perfluorocarbons.

One aim of this thesis was to clarify these inconsistencies and to determine the suitability of various fixatives for use with phase-partition fixation. Results from this study showed that both formaldehyde and glutaraldehyde used in conjunction with FC-72 produced good quality ultrastructural preservation (refer to Figs. 26, 27, 31, 32) which does not support the findings of Sims and Horne (1997). Osmium and n-heptane, the other previously unused fixative and solvent combination, also gave reasonable ultrastructural preservation (refer to Figs. 41, 34).

To confirm that these fixatives did in fact partition into the various non-polar solvents, a second set of experiments was devised. In these experiments, *Myrothamnus flabellifolia* leaf tissue was 'fixed' in the phase-

partition solvents in the absence of standard chemical fixatives. The two solvents tested in this way were n-heptane and HFE 7100. After this treatment, ultrastructural preservation was reduced, implying that glutaraldehyde and formaldehyde are in fact able to partition successfully into perfluorocarbons (refer to Fig. 35, 36).

The results of this thesis showed that the type of fixative used did not appreciably affect the quality of the ultrastructure in *Myrothamnus flabellifolia* leaves. Moreover, numerous combinations of solvent and fixative can be used successfully for the phase-partition fixation of *Myrothamnus flabellifolia* leaf tissue, including combinations, previously thought to be unsuitable. Such combinations include glutaraldehyde or formaldehyde partitioned in the perfluorocarbon FC-72.

3.13.2 - The Effect of Fixation Duration.

Various fixation times from 1 to 24 hours were used during the course of this study, however they did not appear to affect the quality of the ultrastructural preservation. In contrast to my findings, Hattel *et al.* (1983) found that the rate of penetration of fixatives from the solvent was slower than from the corresponding aqueous fixative, hence a 48-hour fixation time was necessary for liver tissue. Hattel *et al.* (1983) tested the penetration

rate of fixatives by measuring the depth of fixative-induced coagulation of protein gel blocks at various times between 1 and 25 hours. The findings of Hattel *et al.* (1983) are in direct contrast to those of McFadden *et al.* (1988) who found that phase-partition fixation improved that rate of fixative infiltration compared to aqueous fixatives. McFadden *et al.* (1988) did not elaborate on how they came to this conclusion.

One difference between the two studies that may account for the two different results is the type of tissue used in the experiment. Hattel *et al.* (1983) experimented on animal tissue whereas McFadden *et al.* (1988) used plant material. It is well known that cell walls and hydrophobic waxy cuticles in plant cells can greatly retard the penetration of aqueous chemical fixatives (O'Brien and McCully 1981, Hayat 1989). Thus, it is possible that the highly volatile nature of heptane allows it to penetrate the cell wall of the plant tissue more easily than the water contained in aqueous fixatives. That *Myrothamnus flabellifolia* leaf tissue did not require extended fixation times supports the findings of McFadden *et al.* (1988). These findings strengthen the argument that phase-partition fixatives are able to penetrate samples of plant tissue with relative ease.

3.13.3 - The Effect of Fixative Concentration.

The effects of high concentrations of formaldehyde and glutaraldehyde were investigated because unusually high concentrations of aldehyde fixatives were found by Hattel *et al.* (1983) and Nettleton and McAuliffe (1986) to penetrate samples at a rate comparable to that of lower concentrations, of the same fixative, in aqueous solutions. Although the above findings led these authors to pursue the use of high concentration fixatives, phase-partition fixation was still able to be accomplished using lower concentrations of both formaldehyde and glutaraldehyde (Hattel *et al.* 1983). Results from my study showed that high fixative concentrations were not necessary for successful phase-partition fixation of *Myrothamnus flabellifolia* leaf tissue. This finding complements the findings outlined above (refer to 3.12.3), that is, high fixative concentrations were not necessary because the actual fixative itself was able to penetrate the samples of *Myrothamnus flabellifolia* leaf tissue with relative ease.

Overall, this study found that fixative concentration did not appreciably affect the quality of ultrastructural preservation of *Myrothamnus flabellifolia* leaf tissue.

3.14 - Conclusions.

The results of this study indicate that phase-partition fixation is a viable alternative to aqueous and anhydrous chemical fixation. Dehydrated leaf tissue of *Myrothamnus flabellifolia* was generally better preserved using phase-partition fixation than was hydrated leaf tissue. It should be noted however, that reproducible results were relatively difficult to obtain compared to aqueous or anhydrous chemical fixation.

The main disadvantage encountered when using phase-partition fixation was the difficulty in successfully preserving hydrated leaf tissue as apparent shrinkage artefacts were regularly observed. It is possible that these artefacts resulted from the dehydration of the samples before resin embedding. If the fixed tissue samples had retained some osmotic capabilities (refer to 3.12.1), then they could have been able to respond to the pre-resin infiltration dehydration step. Shrinkage artefacts regularly observed included the convoluted shape of the cell walls and the regular separation of the plasma membrane from the cell wall. Another potential problem was that if the non-polar solvent was not completely removed after fixation it could result in resin infiltration and sectioning problems such as the production of holes in the resin.

Phase-partition fixation however does have a number of advantages. The main advantage of this technique was the ability to fix both hydrated and dehydrated leaf tissues in exactly the same manner. This is in contrast to aqueous and anhydrous chemical fixation where different fixative carriers need to be used for hydrated and dehydrated tissue, this in turn introduces variation into the study. Phase-partition fixation also potentially allows one to fix tissue samples of intermediate water contents. This is difficult to achieve using the techniques of aqueous and anhydrous chemical fixation (refer to Chapter 5, 5.1). Finally, phase-partition fixation is advantageous in that it requires no special equipment and is relatively inexpensive, unlike the final technique examined in the next chapter.

Chapter 4 - Rapid Cryofixation/ Freeze-Substitution

INTRODUCTION.

4.1 - Rapid Cryofixation.

The aim of cryofixation is to immobilize specimens in their native or near native state through the physical means of heat extraction, i.e. rapid freezing (Elder and Robards 1988). This contrasts to standard fixation techniques which rely on the much slower diffusion of chemical fixatives through the specimen and may induce artefacts as discussed in Chapter 2 (refer to 2.1) (Studer *et al.* 1989). There are a number of methods that have been used to rapidly freeze biological specimens, these include: 1) plunging - where a specimen is immersed into a liquid cryogen, 2) slamming - where a specimen is impacted against a cooled metal block, 3) jet freezing - where a cryogen is streamed in a jet onto the specimen and 4) spray freezing - where small specimens are sprayed into a cryogen (Ryan and Knoll 1994). The relative simplicity of plunge freezing in comparison to other freezing methods makes it the most frequently used method of cryofixation (Quintana 1994). A number of liquids have been used as cryogens, these include liquid nitrogen, ethane and propane. The large temperature range over which propane is a liquid and its good conduction of heat out of the specimen makes liquid propane one of the most popular (Quintana 1994) and one of the most efficient (Ryan *et al.* 1987) cryogens used to date.

Propane is a gas at room temperature and must be cooled in order to condense it into liquid (propane is a liquid between -42°C and -187°C). Liquid nitrogen (-196°C) is commonly used to cool gaseous propane (Galway *et al.* 1995).

The aim of cryofixation is to rapidly freeze cellular water so that ice crystal growth does not occur or is minimized. This is essential for good ultrastructural preservation as primary ice crystal formation results in the disruption of cellular ultrastructure.

4.2 - **Freeze-Substitution.**

Freeze-substitution is the link between cryofixation and resin embedding. It is the process by which the frozen cellular water is gently dissolved from the frozen specimen and replaced by an organic solvent (Hayat 1989, Robards 1991). Freeze-substitution is conducted at low temperatures (-80°C to -90°C) in order to prevent secondary ice crystal growth (Robards and Sleytr 1985, Echlin 1992, Hippe-Sanwald 1993, Nicolas and Bassot 1993, Parthasarathy 1995). The most common solvents used as substituting fluids are acetone and methanol, although ethanol is also occasionally used (Nicolas 1991, Robards 1991). Immersion in a solvent during the substitution process not only functions to remove cellular water but also stabilizes the sample through protein denaturation (Harvey 1982) and prevents enzyme activity (Kellenberger *et al.* 1992).

The time required for the cellular ice to be fully dissolved varies as it depends primarily on the water absorbing capacity of the solvent. According to Steinbrecht and Müller (1987) methanol has a better ability to dissolve ice than does acetone, thus shorter substitution times can be used with methanol. At -90°C the substitution rate in acetone is considerably reduced by the presence of as little as 1% water whereas methanol will substitute specimens fairly rapidly even in the presence of 10% water (Humbel *et al.* 1983, Humbel and Müller 1984, Steinbrecht and Müller 1987). Thus it is necessary to add a form of desiccant to acetone to ensure the complete removal of absorbed cellular water from the solvent (Monaghan 1995, Parthasarathy 1995). In general, substitution times of up to 7 days for acetone and 2-3 days for methanol are frequently used. Because of these differences in the rate of substitution between methanol and acetone, Moor (1987) suggested that acetone was more gentle than methanol in the removal of cellular water. Honegger *et al.* (1996) also found that the size of the sample influenced the time required for complete substitution. They found that single cell layers required a substitution time of 7-14 days whereas thin fragments of lichen thalli required 40-116 days and even then not all cells were properly substituted.

When the samples are completely substituted they can be infiltrated with resin, which can be done at room

temperature or lower. Grote *et al.* (1999) stated that low temperature embedding should be abandoned for difficult to embed samples, such as dry plant material, in favour of carrying out all the dehydration and infiltration steps at room temperature. This is because resin infiltration of recalcitrant samples may be enhanced by the reduced viscosity of resins at room temperature. After polymerization of the resin, the samples can be sectioned, stained and examined as per conventional processing techniques.

4.2.1 - Advantages of Rapid Cryofixation/Freeze-Substitution.

Rapid cryofixation and freeze-substitution are said to possess a number of advantages over conventional chemical fixation. A major advantage is the speed at which cryofixation fixes specimens. Chemical fixatives penetrate cells relatively slowly, taking several minutes to immobilize cellular structures (Gilkey and Staehelin 1986, Studer *et al.* 1989; Ding *et al.* 1991, Ryan and Knoll 1994). Robards (1984) calculated that fixatives penetrate cells at a rate of about 140 μm min^{-1} or less, so that in a cell 1.5 mm from the nearest cut surface, it would take at least ten minutes before fixation commenced. Mersey and McCully (1978) also reported fixative penetration rates in plant hair cells to be approximately 140 μm min^{-1} and found that

it took an average of 15 minutes to immobilize the cytoplasm by glutaraldehyde fixation and 30 minutes using formaldehyde fixation. Coetzee and van der Merwe (1985) point out that penetration times for most samples will be much slower than those quoted by Mersey and McCully (1978) as normally blocks of tissue are fixed rather than individual cells. In contrast, cryofixation can physically stabilize cellular components in a few milliseconds (Plattner and Bachmann 1982, Robards 1984, Escaig 1984, Menco 1986, Gilkey and Staehelin 1986, Baatsen 1993). There are a number of estimates of the actual degree, to which cryofixation is faster than conventional chemical fixation, these include 10^4 to 10^5 times faster (Elder and Bovell 1988), by at least 10^3 times faster (Gilkey and Staehelin 1986) and approximately 10^6 times faster than chemical fixation (Robards 1984).

The rapidity of cryofixation is beneficial in a number of ways. Firstly, it substantially reduces the possibility of cellular changes occurring before complete fixation (Gilkey and Staehelin 1986, Hyde *et al.* 1991b, Hoch 1991). Ryan and Knoll (1994) list many examples of both structural and chemical changes observed in cells during the lag time between contact with the fixative and the actual immobilization of the sample. They concluded that cells, which are observed under the electron microscope following chemical processing, are often highly modified from their *in vivo* state, in comparison to cells that have been

cryofixed. Also, the rapid nature of cryofixation enables the study of dynamic events (Gilkey and Staehelin 1986, Erk *et al.* 1998, de Felipe *et al.* 1997). For example Plattner (1989) states that cryofixation permits the study of dynamic processes such as exocytosis which occurs in the time frame of 0.1 millisecond.

A further benefit of cryofixation is that it is able to arrest all cellular processes (Nicolas 1991). Chemical fixation, on the other hand, is unable to achieve this, as chemical fixatives do not react with all types of molecules (Gilkey and Staehelin 1986, Nicolas 199i). For example, aldehyde fixatives can crosslink only those molecules with free amino groups and react poorly with lipids and nucleic acids (Baker 1968, Hasty and Hay 1978, Bullock 1984). Another common fixative, osmium tetroxide, can crosslink lipids and molecules with free amino groups, but reacts poorly with proteins, nucleic acids and saturated lipids (Bullock 1984). Kellenberger (1991) stated that the first action of chemical fixatives in aqueous solutions is on the pumps and gates of the plasma membrane, which can result in the complete disorganization of ion and water pumping. These changes occur before the fixative has a chance to penetrate the cell and immobilize molecules and can lead to fundamental changes of structure and displacement of cellular constituents. Kellenberger *et al.* (1992) showed that even in good conditions enzymes can stay active and

produce structural changes before they are inactivated by fixation.

Cryofixation followed by freeze-substitution is a fixation technique that over recent years has been lauded by many researchers for numerous reasons. Areas in which freeze-substitution is believed to be superior to standard chemical fixation include, general preservation of cell structure (McCully and Canny 1985, Lancelle *et al.* 1986, Cresti *et al.* 1987, Howard and O'Donnell 1987, Kiss *et al.* 1990, Hyde *et al.* 1991b, Kaminskyj 1992, Hippe-Sanwald 1993, Ng *et al.* 1994, Baskin *et al.* 1996, Platt *et al.* 1997, Erk *et al.* 1998, Neuhaus *et al.* 1998, Porta and López-Iglesias 1998), capture of rapid cellular dynamics and labile structures such as cytoskeletal components (Howard and Aist 1979, Tiwari and Gunning 1986, Lancelle *et al.* 1987, Ding *et al.* 1992a, b, Ryan and Knoll 1994, Kaneko and Walther 1995, Royer and Kinnamon 1996, Babuka and Pueschel 1998) and increased ion and antigen retention (Kellenberger 1987, Monaghan and Robertson 1990, Hippe-Sanwald 1993, Nicolas and Bassot 1993, Orlovich and Ashford 1995, Moreira *et al.* 1996, Baskin *et al.* 1996, Lonsdale *et al.* 1999).

4.2.2 - Limitations of Rapid Cryofixation/Freeze-Substitution.

Nevertheless rapid cryofixation is not without its limitations. The major problem associated with cryofixation is the formation of ice crystals (Chan and Inoue 1994, Royer and Kinnamon 1996). Up to nine different types of ice can form depending on the cooling technique used (Monaghan 1995). Most forms of ice have a crystalline structure and when ice crystals grow they expand in size which results in cellular disruption of the sample (Robards and Sleytr 1985, Quintana 1994, Monaghan 1995). Also, as ice crystals grow they exclude solutes contained in the cellular water. This is known as phase separation and results in an increase in the concentration of solutes contained within a sample (Robards and Sleytr 1985). The increasing concentration of solutes in the liquid phase can result in functional and chemical changes in the sample (Quintana 1994). Vitreous or amorphous ice however, does not possess a crystalline structure and thus its production does not disrupt the cellular structure of a sample (Monaghan 1995). The production of vitreous ice depends on the speed of freezing, as it is produced only at very high cooling rates (Nicolas and Bassot 1993, Monaghan 1995).

Whilst in theory, the production of vitreous ice appears to be the solution to the problem of ice crystals disrupting the cellular ultrastructure of tissue samples,

the fact that water is a poor heat conductor prevents researchers from obtaining high freezing rates in the centre of specimens above a certain size (Robards and Sleytr 1985, Dahl and Staehelin 1989). Good cellular preservation can be achieved only within the outermost 10-20 μm of samples frozen at atmospheric pressure (Tiwari and Gunning 1986, Lancelle *et al.* 1986, Gilkey and Staehelin 1986, Moor 1987, Dahl and Staehelin 1989, Monaghan and Robertson 1990, Ding *et al.* 1991, Hernandez-Verdun *et al.* 1991, Robards 1991, Nicolas 1991, Kaneko and Walther 1995). At depths greater than 20 μm, ice crystal damage is observed.

Indeed, there is much debate as to whether vitreous ice can be produced at all during cryofixation. Dubochet *et al.* (1987) state that vitrification can occur to the depth of several μm from the surface of slam frozen tissue, whereas Elder (1989) considers that so called 'vitreous' ice is probably in a micro-crystalline state. Robards (1991) stated that producing vitrified ice for most microscopists working with higher plants is not a realistic option because higher plant tissue samples are generally relatively large which precludes the vitrification of water and the presence of thick cell walls can hinder rapid freezing.

There are a number ways in which the quality of cryofixation can be assessed. Such criteria include

specific measurements of acceptable ice crystal size, with values of <2 nm (Quintana 1994), <10 nm (Robards and Sleytr 1985, Robards 1991), 10 to 15 nm (Dahl and Straehelin 1989) and 20 nm (Moor 1987) considered acceptable by these authors. McDonald and Morphew (1993) used a different criterion, they stated that there should be no evidence of ice crystals at a magnification of 100,000 x. Other researchers mention specific aspects of cellular ultrastructure that can be used to assess the quality of the cryofixation achieved, such as smoothness of membranes (Dahl and Staehelin 1989, Porta and López-Iglesias 1998). Brand and Arnold (1986) state that tightly adjacent thylakoid membranes are extremely sensitive to even the smallest amount of freezing damage and thus these organelles can be used to evaluate the quality of freezing.

Other limitations of cryofixation include the need to use minute pieces of cellular material to increase the surface area to volume ratio, which improves the cooling rate of the specimen (Plattner and Bachmann 1982) and thus the quality of structural preservation. However, the need to dissect small pieces of tissue can induce artefacts such as desiccation and ionic changes (Ryan and Knoll 1994) as cells and tissues can respond to trauma within milliseconds (Radford *et al.* 1998). A compromise must be made between the necessity to excise a piece of tissue small enough to freeze and the inevitable mechanical and other damage that will be done to cells by the sampling process (Robards

1991). A reduction in specimen contrast (Fisher 1975, Ding *et al.* 1991, Hoch 1991, Galway *et al.* 1993, Royer and Kinnamon 1996, Erk *et al.* 1998, Babuka and Pueschel 1998) and resin infiltration problems (Howard and O'Donnell 1987, Robards 1991, Babuka and Pueschel 1998) are two more problems frequently associated with cryofixation. Finally, successful cryofixation of higher plant cells is frequently difficult to achieve when compared to other cell types as plant cells are more difficult to freeze quickly. Lancelle *et al.* (1986) postulated that this is because of their relatively large size and high water content, especially in vacuolate cells.

4.3 - The Suitability of Freeze-Substitution for Use on Dehydrated Plant Material.

An important benefit of cryofixation and freeze-substitution is that they are osmotically neutral techniques. This aspect makes them particularly suitable for investigating desiccated materials. When specimens are processed using these techniques they do not experience osmotic movement of water (Nicolas and Bassot 1993, Platt *et al.* 1997). Cryofixation has also been shown to prevent shrinkage and distortion of cellular structures (Van Harreveld *et al.* 1974, Boyde *et al.* 1977, Sjöstrand 1990).

Two other factors make cryofixation and freeze-substitution especially suitable for the preservation of

dry desiccation tolerant plant material. First, the sugar content of a specimen frequently increases during desiccation. These sugars act as a naturally occurring cryoprotectant and depress the freezing temperature required to produce high quality ultrastructural preservation. Gilkey and Staehelin (1986) state that the presence of these naturally occurring cryoprotectants can increase the depth of well-frozen tissue sevenfold. Whilst the increase in solute concentration at the whole organism level is relatively rare, the cryoprotective nature of sugars has been noticed in particular cell types such as phloem sieve elements (Browning and Gunning 1977, Fisher 1975, Ding *et al.* 1992a, Galway *et al.* 1995). The second feature of desiccation tolerant plants that makes cryofixation particularly suitable is the simple fact that in their dehydrated state they have a low water content. Schultz *et al.* (1973) suggest that the low water content of dormant spores enabled them to be one of the few higher plant tissues that could be successfully frozen.

4.3.1 - Previous Uses of Cryofixation on Dehydrated Biological Material.

Despite the theoretical benefits of cryofixation and freeze-substitution for use with dry and partially dry material, these techniques have, for the most part, remained unexplored. Before commencing this study, only 2

papers were found that had utilized the techniques of freeze-substitution on dry plant material, these papers examined desiccated pollen (Tiwari *et al.* 1990) and the lichens *Xanthoria parietina* and *Parmelia sulcata* (Honegger 1995). A third paper utilized freeze-substitution for examining the ultrastructural changes in rye leaves due to water stress following freezing (Harvey and Pihakaski 1989). At the commencement of this study the techniques of cryofixation and freeze-substitution had not been used on desiccation tolerant plant material. It was only during the course of this study that two papers were published that used cryofixation and freeze-substitution on the desiccation tolerant plant *Selaginella lepidophylla* (Platt *et al.* 1997, Thomson and Platt 1997). Another report using cryofixation and freeze-substitution on pollen was also published (Hoekstra *et al.* 1999). The desiccation tolerant plants *Tortula ruralis* and *Selaginella lepidophylla* had also been studied using the technique of freeze-etching (Platt *et al.* 1994).

The above mentioned studies utilized the techniques of cryofixation and freeze-substitution in order to prevent rehydration of the desiccated materials during aqueous chemical fixation. This has been shown to be a major problem for the successful preservation of the ultrastructure of dry biological material, as discussed in detail earlier (refer to Chapter 2, 2.1.2).

4.3.2 - **Hydrated Tissue.**

The one study to date that examined hydrated, mesophyll cells of a desiccation tolerant plant (*Selaginella lepidophylla*) fixed by cryofixation and followed by freeze-substitution found that cells had smooth membranes including both the plasma membrane and the tonoplast (Platt *et al.* 1997). The plasma membrane was also observed to be in close contact with the cell wall (Platt *et al.* 1997). This description is consistent with the findings of Honegger *et al.* (1996) who also found that the plasma membrane of the fungal and algal cells of a number of lichens was smooth and in close contact with the cell wall. They also found the thylakoids in cells fixed in this manner cells, were without any signs of deformity.

4.3.3 - **Dehydrated Tissue.**

Previous reports of dehydrated mesophyll cells fixed by rapid cryofixation followed by freeze-substitution describe cells as being shrunken in appearance with highly convoluted walls (Platt *et al.* 1994, Platt *et al.* 1997, Thomson and Platt 1997). The cytoplasm was condensed and had a relatively high degree of electron density (Platt *et al.* 1997). The plasma membrane was intact and in continuous apposition with the cell wall (Platt *et al.* 1997, Thomson and Platt 1997). Chloroplasts were

compacted, had an intact bounding, double membrane and possessed well-organized grana and there was no sign of swelling of the thylakoids (Platt *et al.* 1994, Platt *et al.* 1997). Mitochondria were irregular in outline (Platt *et al.* 1994, Thomson and Platt 1997) but possessed cristae and had an intact bounding membrane (Platt et al. 1997, Thomson and Platt 1997). Nuclei also had irregular outlines (Thomson and Platt 1997). Osmiophilic material was present in the vacuoles (Platt *et al.* 1997) and lipid droplets were seen to be associated with the plasma membrane (Platt *et al.* 1994, Thomson and Platt 1997). In these studies it was concluded that the structural integrity of the cell's organelles was maintained. Similar observations were seen in the photobionts of a number of desiccation tolerant lichens and Honegger (1995) and Honegger *et al.* (1996) concluded that all cellular organelles were in a well preserved state.

These findings are not exclusive to desiccation tolerant vegetative tissue, other desiccation tolerant tissues that have been examined using cryofixation techniques were also seen to possess, amongst other things, highly folded walls (Kerhoas *et al.* 1987) and the plasma membranes that were observed were both continuous (Thomson and Platt-Aloia 1982, Bliss *et al.* 1984, Vigil *et al.* 1985, Platt-Aloia *et al.* 1986) and in close apposition to the cell wall (Platt-Aloia *et al.* 1986, Kerhoas *et al.* 1987, Tiwari *et al.* 1990). Membranes, including the plasma

membrane, were observed to possess the normal lipid bilayer organization in the dry state (Vigil *et al.* 1985, Platt-Aloia *et al.* 1986). Lipid bodies were closely appressed to the plasma membrane (Thomson and Platt-Aloia 1982, Vigil *et al.* 1985) and were described as angular in outline (Vigil *et al.* 1984). Nuclei were also irregular in shape (Vigil *et al.* 1984). No microtubules or microfilaments were observed (Tiwari *et al.* 1990).

The above descriptions reveal distinct differences from descriptions of dehydrated tissue fixed by aqueous chemical fixation (refer to Chapter 2, 2.1.2). The differences in ultrastructure of dehydrated tissue fixed by two different techniques highlights not only the degree and ease with which fixation artefacts may be produced but also brings into question theories surrounding desiccation tolerance, especially those that have been based on ultrastructural observations using conventional fixation techniques.

4.4 - Aims.

As has been highlighted in this introduction, the techniques of cryofixation and freeze-substitution possess a number of benefits that make them superior to standard aqueous chemical fixation techniques, e.g. the rapidity of the fixation process and simultaneous arresting of all cellular processes. Moreover, desiccation tolerant plant

material is particularly suitable for cryofixation and freeze-substitution in that it usually has a relatively high sugar content and a low water content in the dehydrated state.

The osmotically neutral nature of cryofixation and freeze-substitution is another feature that makes these techniques suitable for use on both hydrated and dehydrated leaf tissue of *Myrothamnus flabellifolia*. The technique of phase-partition fixation investigated in Chapter 3 is another technique that is osmotically neutral, however, the quality of the results obtained using this technique was not completely satisfactory in that a number of artefacts were seen (refer to Chapter 3, 3.11). The apparent swelling of chloroplasts in phase-partition fixed dehydrated tissue and the relative difficulty in preserving hydrated leaf tissue resulted in the need to continue the search for an alternative fixation technique that would be suitable for use on both hydrated and dehydrated leaf tissue.

The following chapter examines the ultrastructure of hydrated and dehydrated leaf tissue of *Myrothamnus flabellifolia* fixed using the techniques of cryofixation and freeze-substitution.

MATERIALS AND METHODS.

4.5 - Pre-Fixation Techniques.

The same plant material and pre-fixation techniques used in this chapter are outlined in the Materials and Methods section of Chapter 2 (refer to 2.6).

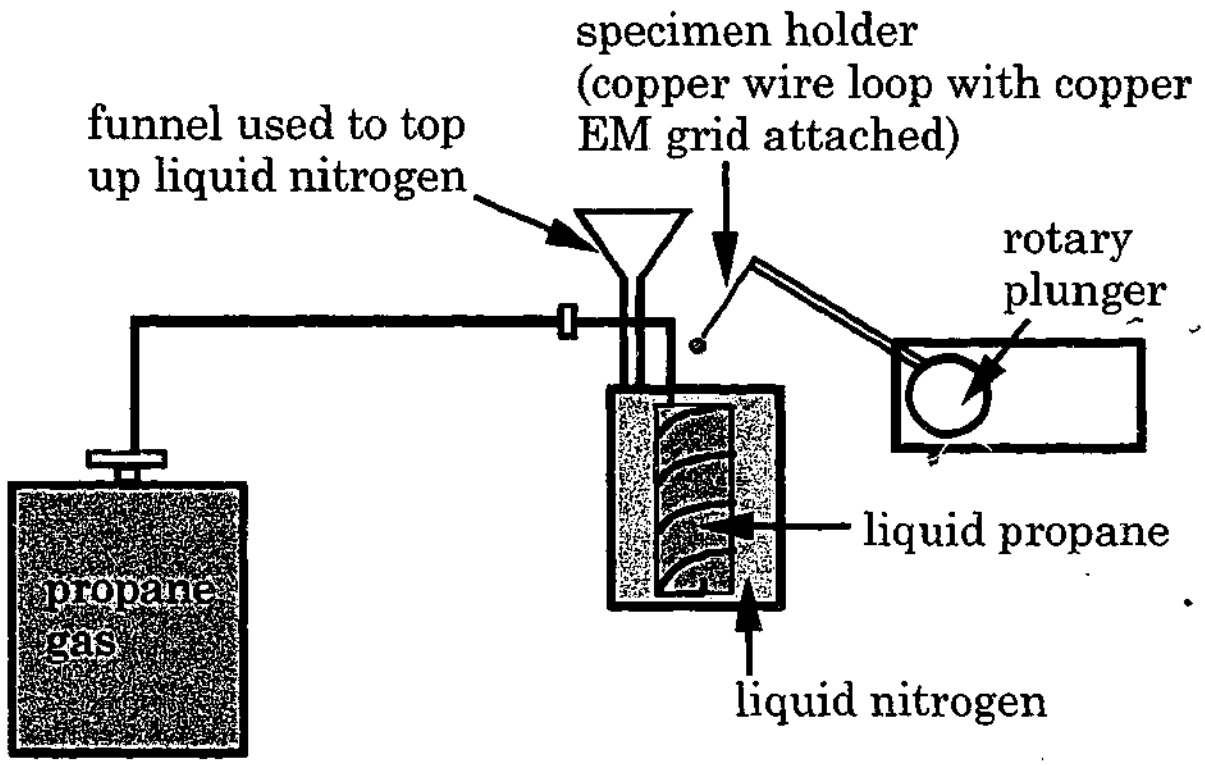
4.6 - Rapid Cryofixation.

A plunge method of cryofixation was used in this study (Fig. 47a). Samples of leaf tissue were cut into pieces approximately $1mm^2$. This small size was necessary to obtain good quality ultrastructural preservation. The tissue samples were attached to a specimen holder using a drop of glycerol. Any extra glycerol was wicked off using a piece of filter paper. Glycerol was used as it did not elicit swelling of peanut tissue even after soaking (Yatsu 1983). The specimen holder consisted of a copper EM grid glued to a small copper loop approximately 4-5 mm in diameter. The loop had a 'tail' approximately 10 cm long. The copper loops were attached by their 'tails' to a plunge device which consisted of a rotary solenoid similar to that described by Lancelle *et al.* (1986). The loops were then plunged into -180°C liquid propane for approximately 5-10 seconds. The whole process from sample dissection to the completion of plunging into the cryogen was done as quickly

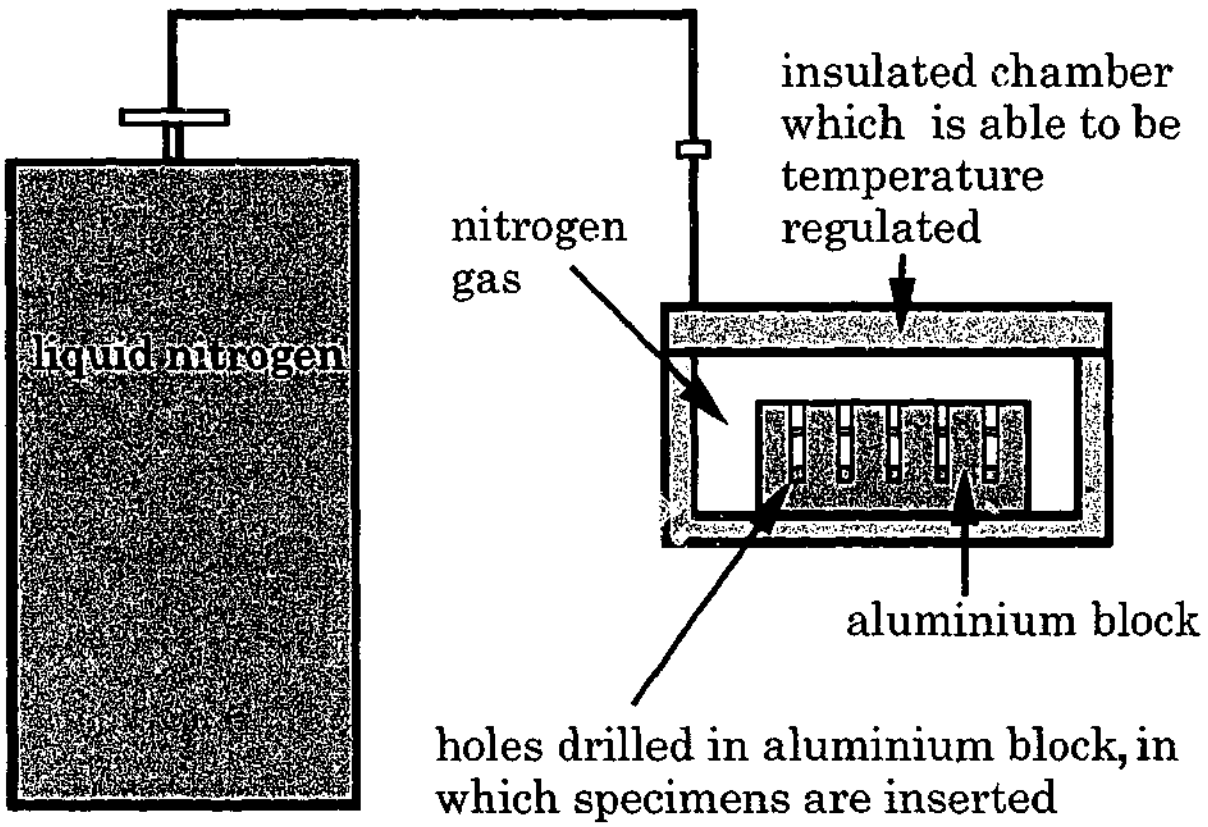
Figure 47. - Diagrams of the equipment used in rapid cryofixation and freeze-substitution.

Fig. 47a - Simplified diagram of the equipment used in rapid cryofixation. Tissue samples were attached to a specimen holder. A plunging device (rotary plunger) was used to immerse the samples into the cryogen (liquid propane). After the samples had been cryofixed they were then freeze-substituted.

Fig. 47b - Simplified diagram of the equipment used to raise the temperature of the tissue samples from -82°C to room temperature. Vials containing the freeze-substituted samples were inserted into specially drilled holes in an aluminum block. The aluminum block was positioned in a pre-cooled insulated chamber that was able to be temperature regulated. Over a set period of time the temperature in the chamber was slowly raised to room temperature after which the tissues samples were infiltrated with resin.


specimen holder
(copper wire loop with copper
EM grid attached)
funnel used to top
up liquid nitrogen
rotary
plunger
propane
gas
liquid propane
liquid nitrogen

A


insulated chamber
which is able to be
temperature
regulated
nitrogen
gas
liquid nitrogen
aluminium block
holes drilled in aluminium block, in
which specimens are inserted

B

as possible so as to avoid any possible artefacts. Such artefacts include the dehydration of samples as they wait to be plunged and ice crystal growth that can result from the specimen being held for too long too close to the cryogen before plunging (Robards and Sleytr 1985). After plunging, the samples were quickly transferred to a container of liquid nitrogen where they were stored until all the samples had been plunged.

4.7 - Freeze-Substitution.

The specimens on the copper loops were then transferred to vials that contained a pre-cooled substitution fluid (-82°C) and a desiccant. Pre-cooling of the substitution fluid was achieved by placing it in a low temperature freezer 24 hours before the experiment was performed. Either molecular sieve or acidified 2,2-Dimethoxypropane (DMP) was used as the desiccant. DMP chemically dehydrates tissue by the instant hydrolysis of DMP by water to form methanol and acetone (Muller and Jacks 1975; Kaeser 1989). Acidified DMP is prepared by adding 0.05 ml concentrated HCl to 50ml DMP (Muller and Jacks 1975; Kaeser 1989). The samples remained in the freezer (-82°C) for 3-7 days during substitution.

A number of solvents were used as substitution fluids, these included 100% acetone, 100% ethanol, and 100% methanol. Because *Myrothamnus flabellifolia* leaf tissue

showed an intermediate amount of swelling in 100% ethanol, compared to 100% acetone and 100% methanol, ethanol was not used in further experiments. These solvents were occasionally combined with fixatives such as 1% OsO4, 2.5% anhydrous glutaraldehyde (Electron Microscopy Sciences) or 4% paraformaldehyde. Paraformaldehyde powder is not soluble in acetone so a 20% solution of paraformaldehyde in anhydrous methanol was made by gently warming the solution on a heated magnetic stirrer. The solution was heated until condensation was observed at the upper edges of the beaker, then 1/4 to 1/2 pellet of NaOH was added to clear the solution (Morphew 1997). The solution was then diluted to the desired concentration with acetone. It is possible that the use of methanol may have induced a certain degree of tissue swelling however, if this was the case, it was not to such an extent that it was able to be observed by the naked eye, as was the case when dehydrated leaf tissue was fixed with an aqueous chemical fixative (refer to Chapter 2, 2.16.2).

4.8 - Resin Infiltration and Embedding.

After the substitution period, the samples were gradually brought up to room temperature using one of two methods. In the first method, the sample vials were placed in a polystyrene box containing metal plates that had been pre-cooled to -82°C. The metal plates acted to cool the

air temperature in the box and slow the rise in temperature experienced by the samples. The box was then transferred to a -20°C freezer where the samples were left to equilibrate for 24 hours. The box was then transferred to a 4°C cool room where the vials were again left for 24 hours to equilibrate: Finally, the box was brought up to room temperature (20°C) by leaving it on the laboratory bench for approximately 6 hours. In the second method, the vials were placed in a controlled-temperature chamber pre-cooled to -82°C (Fig. 47b). The vials were placed in an aluminium block in which holes, the same size as the vials, had been drilled. The vials containing the samples were inserted into these holes. The aluminium block acted to minimize any temperature fluctuations. The temperature in the chamber was then set to warm up from -82°C to 20°C over a period of 72 hours.

Once at room temperature all samples were twice rinsed in a fresh change of the respective substitution fluid. The substitution fluid was then gradually replaced with 1,2-epoxy propane over a period of 1-2 hours. Once the samples were in 100% 1,2-epoxy propane they were given a further two changes of epoxy propane. The samples were then placed in a solution of 5% Spurr's resin in epoxy propane. The resin infiltration schedule was the same as that described in Chapter 2 (refer to 2.7). After

completion of the resin infiltration stage, the samples were polymerized overnight in a 60°C oven.

4.9 - Post-Embedding Techniques.

The procedures for sectioning and staining the freeze-substituted resin blocks were the same as those outlined in Chapter 2 (refer to 2.10).

4.10 - Determination of the Weight Gain Experienced by Dehydrated Leaves Immersed in Various Solutions.

The methods used to determine the weight gain of dehydrated leaves immersed in different solutions were the same as outlined in Chapter 2 (refer to 2.11).

4.11 - Statistical Analyses.

All statistical tests used in this chapter are outlined in the Materials and Methods section of Chapter 2 (refer to 2.13).

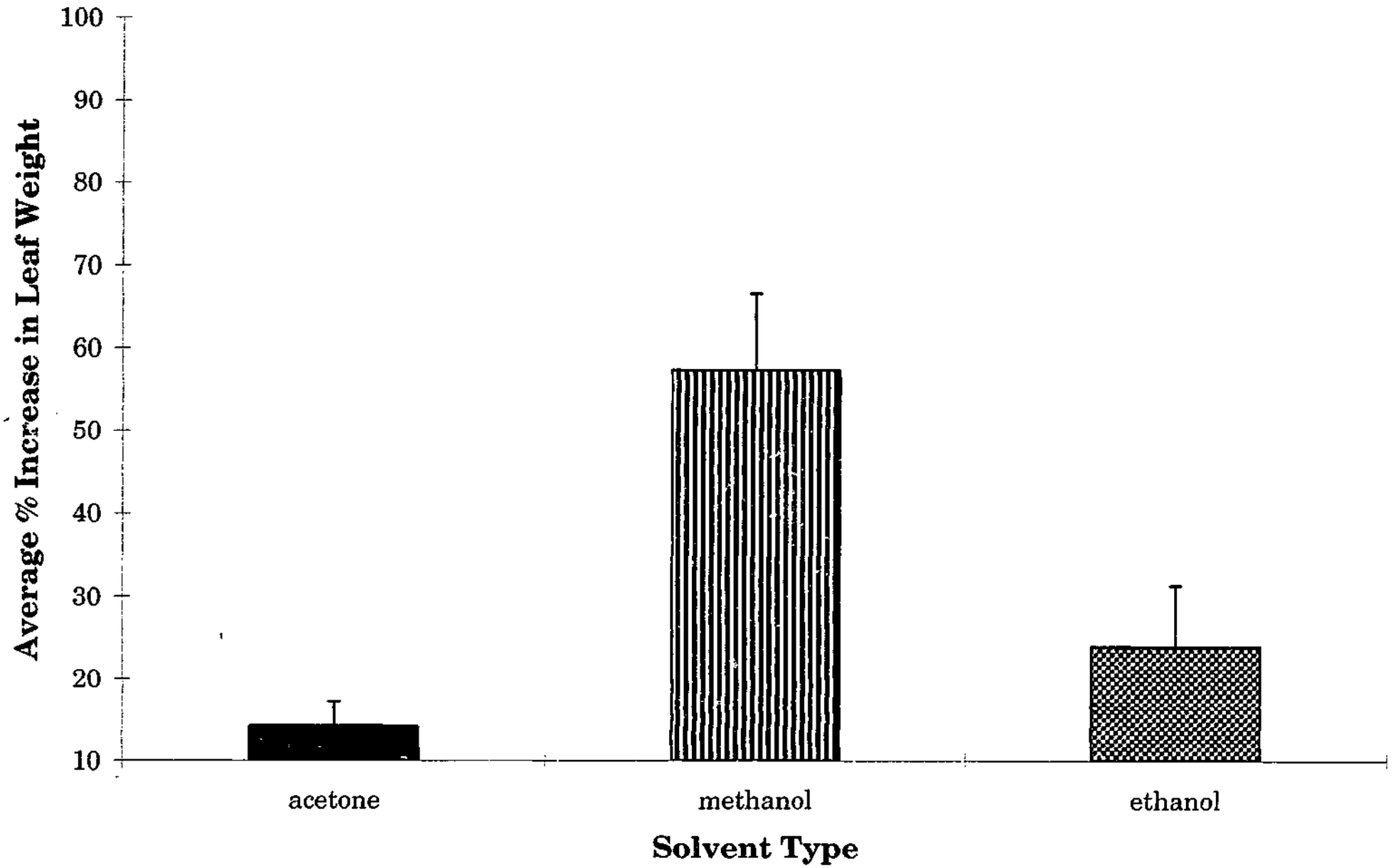
RESULTS.

4.12 - Weight Gain of Dehydrated Leaves Immersed in Various Solutions.

Detached dehydrated leaves were floated for 21 hours in various solvents that have frequently been used as freeze-substitution fluids. The leaves, depending on the solvent in which they were floated, exhibited variable degrees of swelling. Acetone caused the least weight gain, followed by ethanol, with methanol causing the greatest weight gain of any solvent. The average percentage weight increase exhibited by these leaves was 14%, 24% and 57% respectively. The difference in weight gain by dehydrated leaves immersed in acetone and ethanol was not significant whereas the differences between leaves immersed in acetone or methanol versus leaves immersed in ethanol or methanol were significant (Appendix E - Table 6). In comparison to the solutions used in the previous chapters, acetone produced one of the smallest weight gains in dehydrated leaves, whereas methanol produced one of the greatest weight gains (Fig. 48).

To ensure that the organic solvents were completely anhydrous, the chemical dehydrant DMP was added to a replicate set of vials. Similar weight increases were recorded for leaves in each solvent with or without DMP. Methanol was the only solvent in which the addition of DMP

Figure 48. - A comparison of the average percentage increase in leaf weight of dehydrated *Myrothamnus flabellifolia* leaves immersed for 21 hours in either acetone, methanol or ethanol.


Average % Increase in Leaf Weight
100
90
80
70
60
50
40
30
20
10
acetone
methanol
ethanol
Solvent Type

reduced the average weight gained. In contrast, leaves immersed in acetone or ethanol plus DMP had higher weight gains than in each solvent alone (Fig. 50). In no case was there a significant difference in weight gain plus or minus DMP (Appendix E - Table 6).

The percentage weight increase per hour followed the trend shown in previous chapters where the greatest rates of weight gain occurred during the initial period of immersion, and decreased over the next 21 hours. Leaves immersed in methanol experienced the greatest rates of weight gain, peaking at 83% after 30 mins. Leaves floated in acetone and ethanol gained weight at rates of 15% and 20% respectively in the same time period. After 2 hours of hydration, leaves floated in methanol had a rate of weight gain of 10%, leaves floated in acetone were increasing in weight at 2% per hour and leaves immersed in ethanol were increasing in weight at 5% per hour (Fig. 49). The differences in the rate of weight gain between 30 minutes and 2 hours were significant for all the solutions tested (Appendix E - Table 13). After 21 hours, the rates of weight gain per hour had decreased to -0.58%, 0.14% and 0.23% for methanol, acetone and ethanol respectively. The significance of the difference in the rate of weight gain between 2 hours and 21 hours was unable to be tested as there were negative numbers in the data set for 21 hours. Negative values are unable to undergo the required arcsin transformation required for data in percentage form.

Figure 49. - A comparison of the average percentage increase in leaf weight per hour of dehydrated *Myrothamnus flabellifolia* leaves immersed for 21 hours in either acetone, methanol or ethanol.

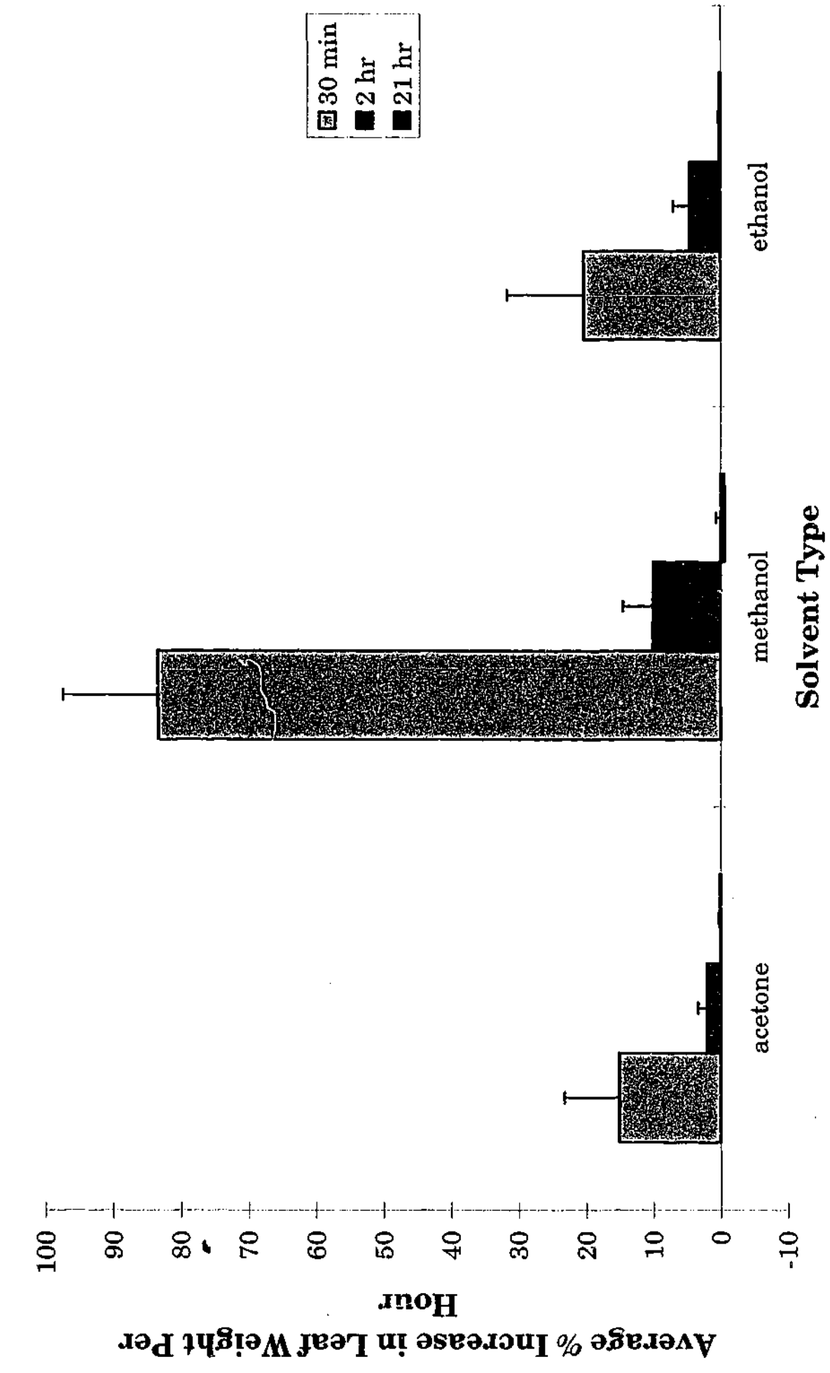
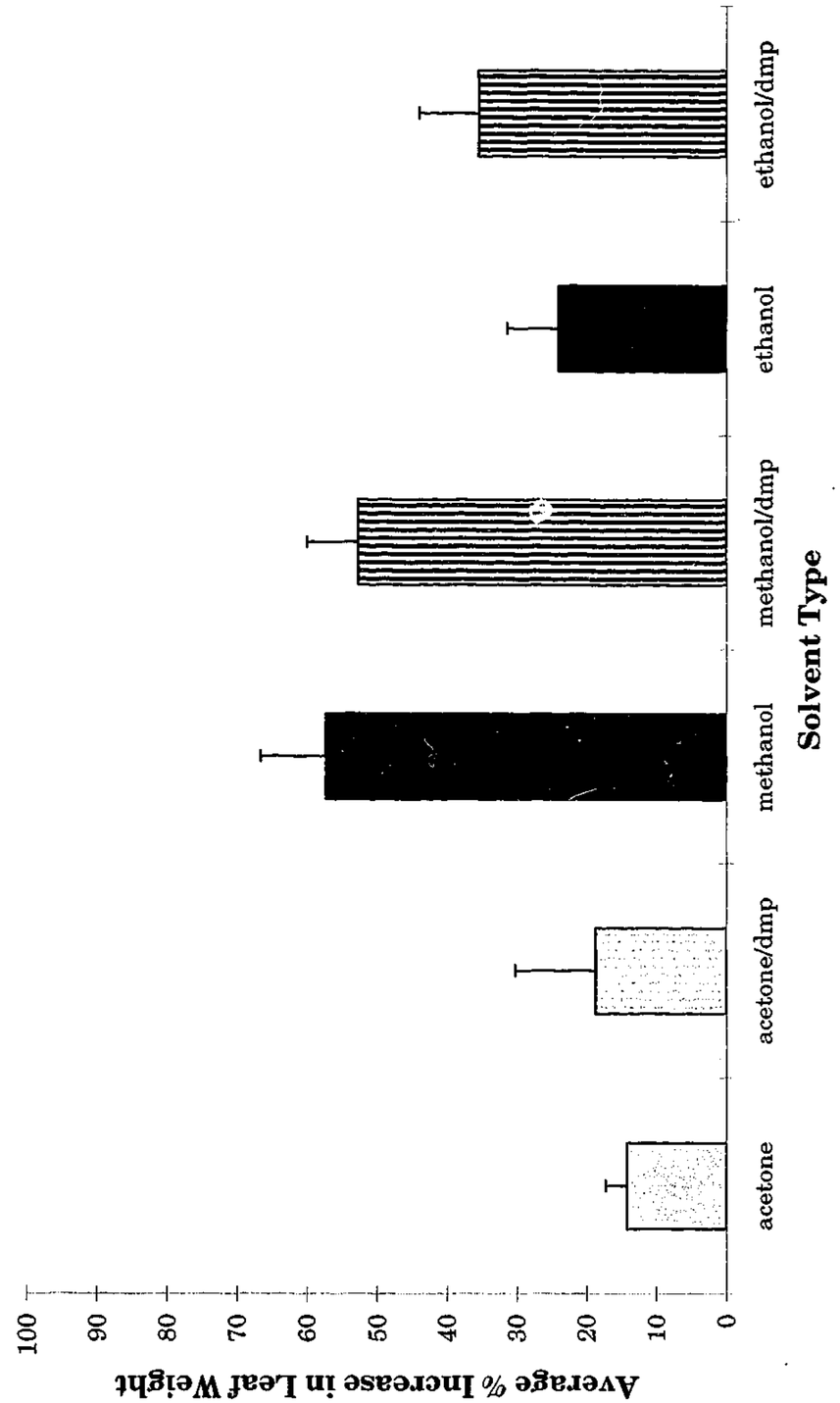


Figure 50. - A comparison of the average percentage increase in leaf weight of dehydrated *Myrothamnus flabellifolia* leaves immersed for 21 hours in either an anhydrous solvent or the solvent and the dehydrating agent DMP.



4.13 - The Ultrastructure of *Myrothamnus flabellifolia* Leaf Tissue Fixed by Rapid Cryofixation/Freeze-Substitution.

4.13.1 - Hydrated Tissue.

The following descriptions apply to tissue samples substituted in various substitution fluids, both with and without the addition of chemical fixatives. Specific details about a particular substitution fluid used are noted only when the structural detail appears to be peculiar to the solvent in question.

Hydrated mesophyll cells that had been plunge frozen and freeze-substituted had rounded cell walls (Fig. 51a, 51b, 52a), the cytoplasm in methanol substituted cells occupied the periphery of the cell, adjacent to the cell wall (Fig. 51a, 51b) as a large vacuole was positioned centrally within the cell. Frequently, the vacuole contained a small amount of granular material (Fig. 51a, 51b, 52a, 52c). In many acetone-substituted cells however, the cytoplasm was observed to have separated from the cell wall (Fig. 52a, 52b). Chloroplasts were elongated or ellipsoid in shape (Fig. 51a, 51b, 51c, 52a, 52b, 52c) and possessed electron-translucent plastoglobuli (Fig. 51d, 51e, 52c, 52d). Starch grains were also present (Fig. 51a, 52a, 52c). Thylakoid membranes were visible in granal

Figure 51. - Hydrated *Myrothamnus flabellifolia* leaf tissue that was plunge frozen and freeze substituted in 100% methanol.

Fig. 51a - General view of hydrated cells. Cells appeared turgid and had rounded cell walls (CW). Most cells possessed a large central vacuole (V). The plasma membrane was frequently adjacent to the cell wall (arrow heads) however there were instances where the cell wall and the plasma membrane had separated (*). Chloroplasts (C), mitochondria (M) and a nucleus (N) can also be seen. Bar represents 4 µm.

Fig. 51b - A number of organelles could be discerned in the cellular matrix including chloroplasts (C) and mitochondria (M). Cells possessed a central vacuole (V) and the cytoplasm lined the periphery of the cell with the plasma membrane generally being in close apposition to the cell wall (arrow heads). Bar represents 3 µm.

Fig. 51c - Chloroplasts (C) were elongated in shape and possessed distinct granal (G) and stromal (S) regions. Grana (G) were generally long and thin in shape. Bar represents 2 µm.

Fig. 51d - High magnification view of a granal stack and its composite thylakoid membranes (arrow heads). Bar represents 0.05 µm.

Fig. 51e - Mitochondria (M) were generally oval in shape. Cristae (arrow heads) could be discerned. Bar represents 0.25 µm.

Fig. 51f - The majority of nuclei (N) observed were round in shape. They contained visible chromatin (*) that was finely dispersed. Bar represents 1 µm.

Fig. 51g - Occasionally nuclei (N) were seen that had an irregular outline and contained condensed areas of chromatin that were either electron-lucent (black *) or electron-dense (white *). Bar represents 1 µm.

Fig. 51h - Plasmodesmata (arrow heads) were also observed. Bar represents 0.1 µm.

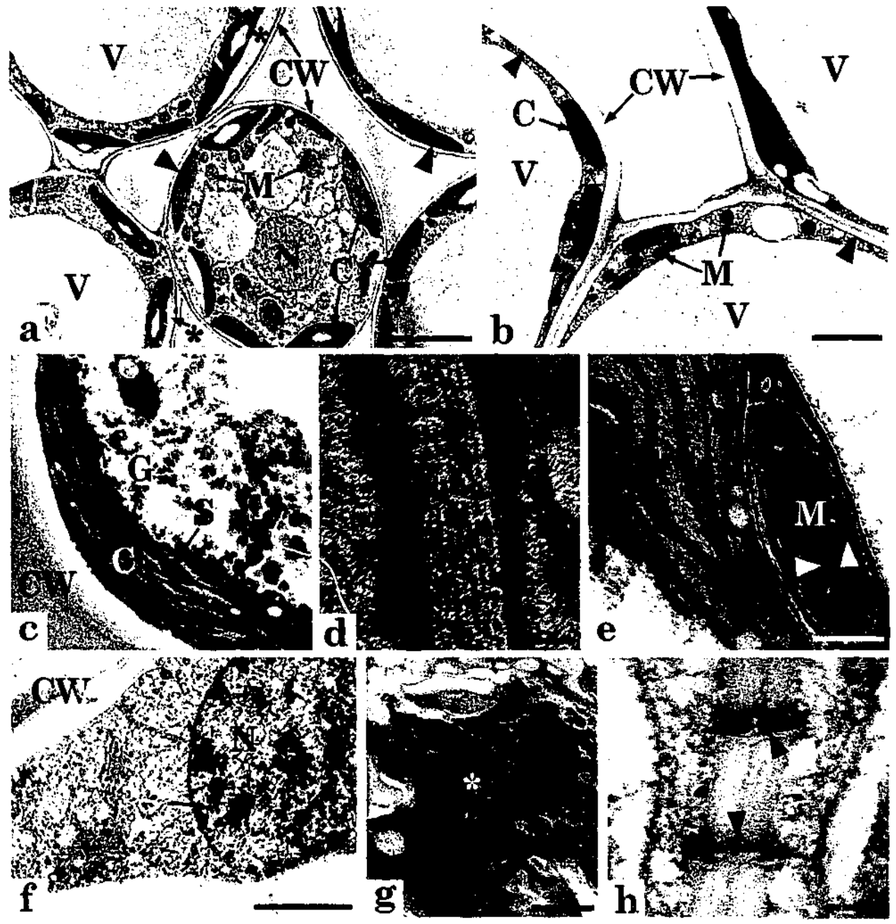


Figure 52. - Hydrated *Myrothamnus flabellifolia* leaf tissue that was plunge frozen and freeze substituted in acetone containing 4% paraformaldehyde.

Fig. 52a - A general view of a hydrated mesophyll. Cells had smooth cell walls (CW). There was a large degree of separation (*) of the cell wall from the plasma membrane. Separation of this kind was frequently observed in acetone substituted hydrated tissue. Cells possessed a large central vacuole (V) containing finely dispersed electron-dense material. Chloroplasts (C) were regularly observed to contain starch grains (SG). Mitochondria (M) were also observed. Bar represents 1 μm.

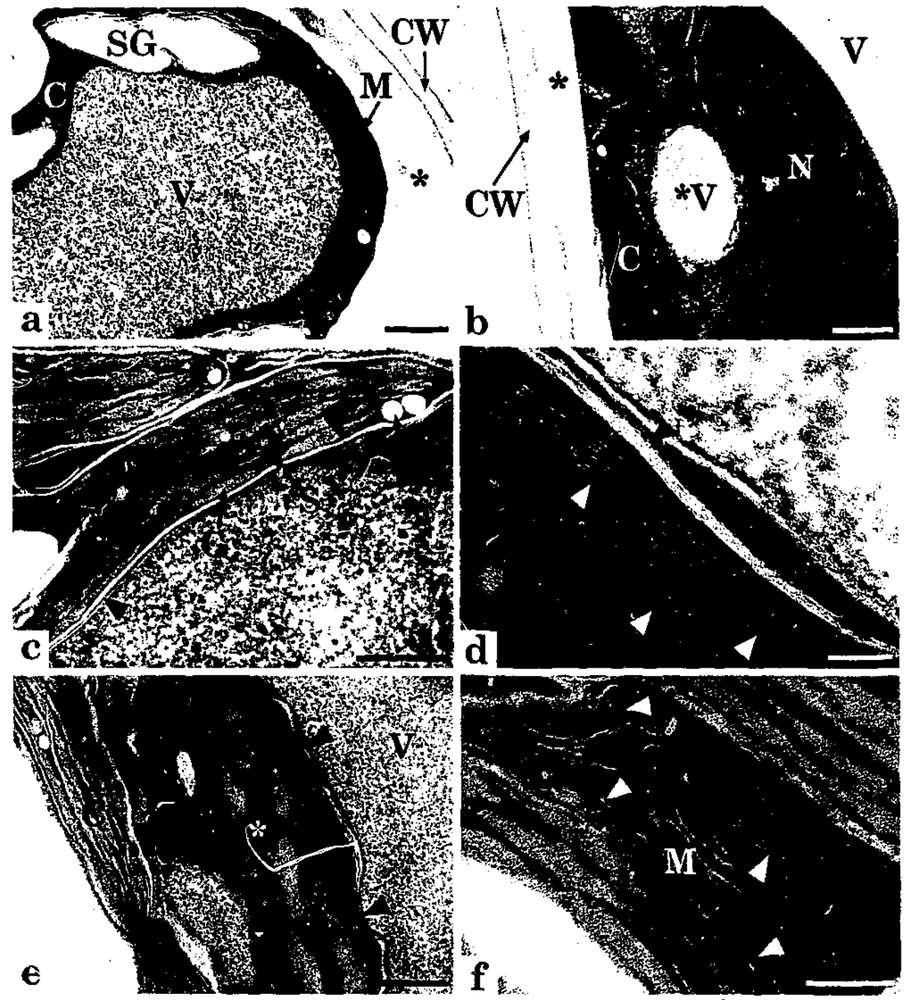
Fig. 52b - A magnified view of a hydrated cell in which there was a separation (*) of the cell wall and plasma membrane. A large central vacuole (V) was generally present and occasionally smaller vacuoles (*V) within the cytoplasm were seen. Chloroplasts (C), mitochondria (M) and nuclei (N) could also be identified. Bar represents 0.5 μm.

Fig. 52c - Chloroplasts (C) were generally elongated in shape. The high magnification shows the chloroplast's bounding membrane (arrow heads), the grana (G) and stromal (S) regions and a number of plastoglobuli (P). Bar represents 0.5 μm.

Fig. 52d - Detail of a chloroplast. Thylakoid membranes (white arrow heads) that comprise the granal stacks and their 'staircase' arrangement could clearly be seen. The bounding membrane of the chloroplast could also be seen (black arrow head). Bar represents 0.1 μm.

Fig. 52e - Nuclei (N) were occasionally observed that appeared contracted and were irregular in shape. This appearance contrasted to the more rounded shape of the nucleus in Fig. 52b. Areas of electron dense (white *) and electron-lucent (black *) chromatin were observed. The bounding membrane of the nucleus can also be seen (arrow heads). A chloroplast (C) and a portion of the central vacuole (V) can also be seen. Bar represents 0.5 μm.

Fig. 52f - Mitochondria (M) varied in shape but were usually elongated. Cristae (arrow heads) were almost universally seen with this fixation technique. Bar represents 0.25 μm.



stacks with a 'staircase' arrangement (Fig. 51d, 52d). The outer bounding membrane was also present (Fig. 51e, 52c, 52d). Chromatin in methanol substituted tissue was evenly dispersed throughout the nucleus and was quite flocculent in appearance, no nucleoli were observed (Fig. 51a, 51f). Nuclei in acetone substituted material were more dense in appearance and distinct light and dark regions of chromatin could be seen (Fig. 52b, 52e). Occasionally nuclei more similar in appearance to those seen in acetone substituted tissue were observed in methanol substituted tissue samples (Fig. 51g). Mitochondria were numerous, varied in shape (Fig. 51b, 52a) and they had well-developed cristae (Fig. 51e, 52f). Plasmodesmata were also observed (Fig. 51h). These results are summarized in Table 4, which can be located at the end of the results section.

4.13.2 - Dehydrated Tissue.

Freeze-substituted, dehydrated mesophyll cells had extremely convoluted cell walls, but this convolution was not as pronounced in methanol substituted cells (Fig. 53a) as in acetone substituted cells (Fig. 54a, 54b). The protoplast and the cell wall of acetone substituted cells was adjacent to the cell wall (Fig. 54a, 54b, 54c) whereas the protoplast of methanol substituted cells was frequently observed to have separated from the cell wall (Fig. 53a, 53b). There appeared to be no vacuole in acetone

Figure 53. - Dehydrated *Myrothamnus flabellifolia* leaf tissue that was plunge frozen and freeze substituted in 100% methanol.

Fig. 53a - A general view of dehydrated mesophyll cells. Cells were shrunken in appearance the cell walls (CW) were convoluted. The Plasma membrane had separated from the cell wall in many places (*). Organelles such as chloroplasts (C) and mitochondria (M) can be seen. Bar represents 2 µm.

Fig. 53b - An area of separation (*) between the cell wall and the plasma membrane can be seen. Chloroplasts (C) and the nucleus (N) can also be seen. Bar represents 2 µm.

Fig. 53c - Chloroplasts (C) were generally round in shape and possessed a wavy bounding membrane (arrow heads). Plastoglobuli (P) were frequently present and the granal (G) and stromal (S) regions of the chloroplast could be distinguished as light and dark staining regions respectively. Bar represents 0.5 µm.

Fig. 53d - A high magnification view of a chloroplast. A wavy outer bounding membrane (*) was often observed as too were the granal (G) and stromal (S) regions of the chloroplast and the thylakoid membranes (arrow heads) that comprise the granal stacks. Plastoglobuli (P) could also be seen. Bar represents 0.25 µm.

Fig. 53e - Nuclei (N) were generally irregular in shape and contained condensed masses of chromatin that was either electron-dense (white *) or electron-lucent (black *). Bar represents 0.5 µm.

Fig. 53f - Mitochondria (M) were irregular in shape and contained cristae (arrow heads). Cytoplasmic ribosomes (R) were also regularly observed. Bar represents 0.25 µm.

Fig. 53g - Plasmodesmata (arrow heads) were frequently seen. Bar represents 0.1 µm.

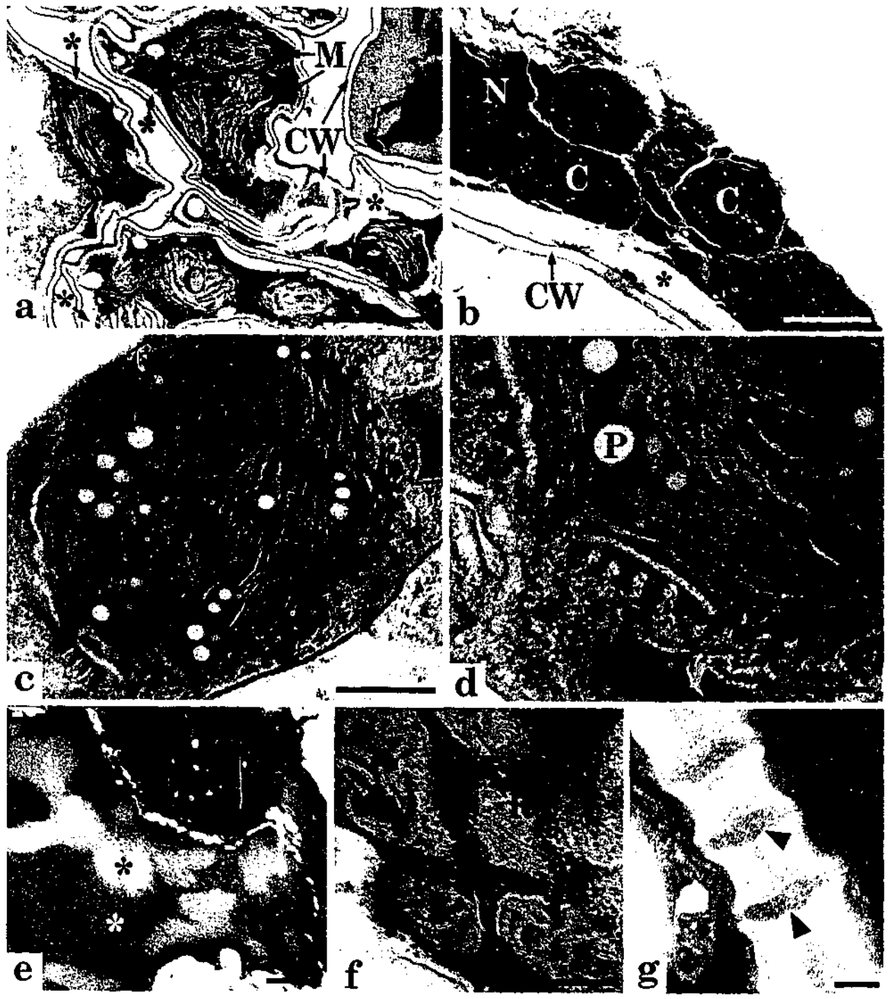


Figure 54. - Dehydrated *Myrothamnus flabellifolia* leaf tissue that was plunge frozen and freeze substituted in acetone containing 1% osmium except where indicated.

Fig. 54a - General view of dehydrated mesophyll cells of *Myrothamnus flabellifolia*. Cells were shrunken in appearance and cell walls (CW) were contorted. The cytoplasm was densely staining and was closely appressed to the cell wall (arrow heads). Chloroplasts (C) could be discerned within the condensed masses of cytoplasm. Bar represents 2 µm.

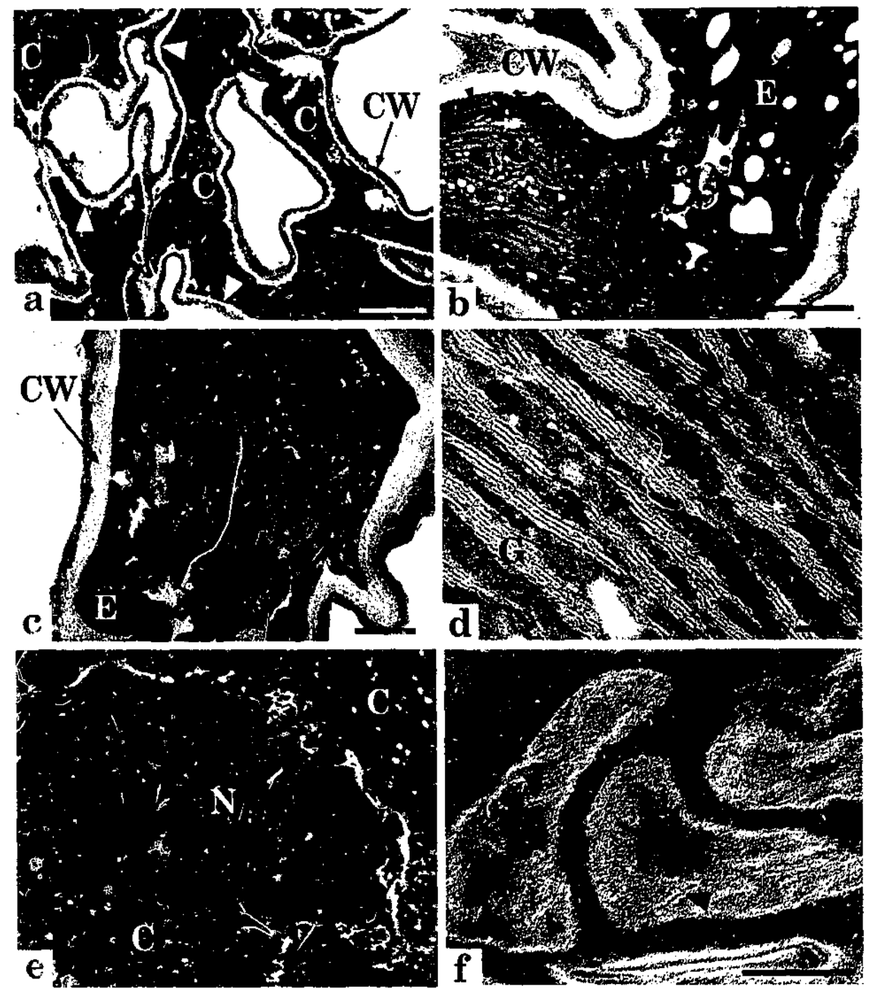
Fig. 54b - In low magnification views (Fig. 54a) no vacuoles could be discerned. At higher magnifications, vacuoles were seen which were filled with electron-dense material (E). Chloroplasts (C) were observed and they generally had irregular outlines (arrowheads). Bar represents 1 µm.

Fig. 54c - Electron-dense material (E) also lined the periphery of the cell adjacent to the cell wall (CW). Bar represents 0.5 µm.

Fig. 54d - High magnification view of a chloroplast revealed granal stacks (G) and thylakoid membranes (arrow heads). The stroma (S) could also be seen. Bar represents 0.1 µm.

Fig. 54e - Nuclei (N) contained condensed chromatin and had an irregular outline. Bar represents 0.5 µm.

Fig. 54f - Mitochondria (M) were irregular in shape and contained distinct cristae (arrow heads). This sample was substituted in 100% acetone without osmium. Bar represents 0.25 µm.



substituted cells (Fig. 54a, 54b) or it contained electron-dense material (Fig. 54c). The electron-dense material observed in the vacuoles of some acetone substituted cells was also seen adjacent to the cell wall (Fig. 54d). Chloroplasts in dehydrated acetone substituted cells were irregular in shape and contracted (Fig. 54c, 54d, 54e) however, chloroplasts in methanol substituted cells were distinctly round in shape (Fig. 53b, 53c) and they also frequently possessed a wavy outer bounding membrane (Fig. 53c, 53d). Chloroplasts substituted in both types of fluids possessed distinct thylakoid membranes (Fig. 53d, 54f). Nuclei in acetone substituted cells were difficult to characterize. They were extremely dense in nature and appeared slightly contracted (Fig. 54g). Nuclei in methanol substituted cells were similarly dense in appearance although they possessed distinct light and dark staining regions. They were also irregular in shape (Fig. 53e). Mitochondria were similar in appearance in both acetone and methanol substituted cells, varying in shape and possessing distinct cristae (Fig.53f, 54h). Ribosomes were observed in both types of substituted tissue (Fig. 53f, 54h). Plasmodesmata were also seen (Fig. 53g). Refer to the end of this results section for a summary of these ultrastructural findings (Table 4).

Table 4. - Summary of the ultrastructural findings for hydrated and dehydrated *Myrothamnus flabellifolia* leaf tissue that has been cryofixed and freeze-substituted.

	Hydrated Tissue	Dehydrated Tissue
Cell Shape	Round in shape.	Possessed convoluted cell walls, which were more pronounced in acetone substituted cells.
Protoplast	Adjacent to cell wall (some separation was observed in acetone substituted cells).	Adjacent to cell wall (some separation was observed in methanol substituted cells).
Vacuole	Large single vacuole positioned centrally. Contained some granular material.	Frequently observed in methanol substituted cells whereas only occasionally observed in acetone substituted cells. Contained electron dense material.
Chloroplast	Elongate to ellipsoid in shape. Possessed plastoglobuli and starch grains. Thylakoid membranes were visible.	Contracted and irregular in shape (acetone substituted cells). Round in shape and possessed a wavy outer membrane (methanol substituted cells). Thylakoid membranes were visible.
Mitochondria	Various shapes observed. Contained distinct cristae.	Various shapes observed. Contained distinct cristae.
Nucleus	Chromatin was condensed (acetone substituted cells). Evenly dispersed flocculent chromatin (methanol substituted cells).	Contained condensed chromatin and was contracted in appearance.

DISCUSSION.

Rapid cryofixation and freeze-substitution have rarely been used for the preservation of dry biological material. The studies by Platt *et al.* (1997) and Thomson and Platt (1997) are the only instances where rapid cryofixation and freeze-substitution have been used previously for the preservation of a desiccation tolerant plant. This is despite the numerous features of this technique that make it particularly suitable for use with dehydrated biological material.

***4.14* - Ultrastructural Preservation of Hydrated and Dehydrated *Myrothamnus flabellifolia* Leaf Tissue After Rapid Cryofixation and Freeze-Substitution.**

There were dramatic differences between hydrated and dehydrated mesophyll leaf tissue preserved by rapid cryofixation and freeze-substitution. For example, the overall shape of hydrated and dehydrated cells was distinctly different with hydrated cells having rounded cell walls whereas the cell walls of dehydrated cells were extremely convoluted (Table 4). The cytoplasm of dehydrated cells appeared very condensed and was electron dense in comparison to cells from hydrated tissue. Frequently there was no evidence of a central vacuole in

the cells of dehydrated tissue whereas in hydrated cells the central vacuole was regularly observed (Table 4).

Differences were also observed in organelle structure between hydrated and dehydrated tissue. Chloroplasts showed the most marked changes from the hydrated to the dehydrated state. In hydrated tissue, chloroplasts were slender and elongated in shape whereas in dehydrated tissue they had irregular outlines and were less elongated (Table 4). The regular folding of the outer membrane in chloroplasts in dry tissue is an interesting finding. It is possible that it is a way of conserving membranes during dehydration so that they do not need to be resynthesized during rehydration. Membrane ruffling in animal cells has also been linked to the process of signal transduction (Ridley 1994) so it is possible that it may also perform a similar role in plant cells. Thylakoid membranes were retained in chloroplasts from both hydrated and dehydrated tissue (Table 4). Other researchers have also observed thylakoids in freeze-substituted dehydrated tissue (Honegger and Peter 1994, Thomson and Platt 1997). There was a difference however, in the shape of the grana with the grana from hydrated tissue being long and thin whereas the grana from dehydrated tissue were shorter (Table 4).

Koonjul *et al.* (2000) in an investigation of *Myrothamnus flabellifolia* chloroplasts did not note the above-mentioned change in thylakoid membrane length, observed during the course of experiments for this thesis.

Instead, they reported that during drying the previously appressed thylakoid membranes became 'blistered' as they pulled apart. They argued that this was not due to the use of an aqueous fixative as vapour fixation used by Goldsworthy and Drennan (1991) resulted in similar ultrastructural preservation. However, the thylakoids appeared closely appressed in images of Goldsworthy and Drennan (1991), who noted that the grana were intact in desiccated leaf material. Goldsworthy and Drennan's results, together with results in this thesis, strongly suggest that the blistering of thylakoid membranes, observed by Koonjul *et al.* (2000) was in fact an artefact of their aqueous fixation protocol. Similar observations to those of Koonjul *et al.* (2000) were made during the course of this present study and they were generally associated with a very poor level of preservation, as such, these results were not included in this thesis.

Nuclei in hydrated and dehydrated cells also appeared different. In hydrated cells, the chromatin of the nucleus was generally quite flocculent in appearance whereas, in dehydrated tissue, the nuclei were electron-dense in appearance. Nuclei in dehydrated cells were also contracted and irregular in shape, whereas nuclei in hydrated leaf tissue were rounded in shape (Table 4). Thomson and Platt (1997) and Hallam and Luff (1980a) observed similar differences in nucleus shape and chromatin condensation between hydrated and dehydrated tissue samples

of the desiccation tolerant plants *Selaginella lepidophylla* and *Xerophyta villosa* respectively. Deltour (1985), in a review of nuclear activity in higher plant embryos, reported that chromatin condensation was a common feature of dry quiescent embryos. Chromatin condensation has been found to be reversible upon rehydration in desiccation tolerant plants and irreversible in desiccation sensitive tissues (Crèvecoeur *et al*. 1988).

During the course of this thesis there were occasions when chromatin condensation was observed in hydrated tissue. This observation indicates that the hydrated tissue may have experienced some degree of dehydration during the fixation process and it appeared to be associated with the use of acetone as a substitution fluid. Previous results from this thesis also indicate that the use of acetone may have resulted in the condensed appearance of the nuclei as it was found that dehydrated leaves immersed in acetone resulted in small weight gains (refer to 4.12). An analysis of the effects of various freeze-substitution solvents is discussed below (refer to 4.15).

Dehydration did not affect the appearance of all organelles, as mitochondria did not appear any different between hydrated and dehydrated leaf tissue of *Myrothamnus flabellifolia*. In both instances various shaped mitochondria were observed and they possessed distinct cristae (Table 4). Mitochondria containing cristae were

also recognizable in both hydrated and dehydrated tissue samples of *Selaginella lepidophylla* that had been cryofixed and freeze-substituted (Thomson and Platt 1997).

The differences in appearance between hydrated and dehydrated leaf samples, outlined above, suggest that cryofixation followed by freeze-substitution was able to successfully preserve hydrated and dehydrated leaf tissue samples of *Myrothamnus flabellifolia* close to their native states. Previous experiments in this thesis indicated that dehydrated *Myrothamnus flabellifolia* leaf tissue was very sensitive to the presence of water and was capable of significant and rapid rehydration (refer to Chapter 2, 2.17 and 2.19). In dehydrated leaf tissue, there was no indication of the swelling artefacts apparent after all other fixation protocols used.

4.15 - Differences in Ultrastructural Preservation that are the Result of Different Substitution Solvents.

In initial trials, samples of dehydrated tissue became swollen and regained their green colouration when transferred to cold methanol. Such changes did not occur after specimen transfer into acetone. An experiment was then set up to quantify the amount of swelling experienced by dehydrated leaves when immersed overnight in either acetone, ethanol or methanol, which are three relatively common substitution fluids. Results from this experiment

confirmed that dehydrated tissue floated overnight in methanol gained more weight than tissue floated in either ethanol or acetone. Although this experiment was not conducted at the subzero temperatures used in freeze-substitution it is possible that similar results could have been produced as the rehydration of the samples could have occurred during the time samples were warmed to room temperature and before they were embedded in resin. In subsequent experiments, only acetone and methanol were used as substitution fluids, because tissue gained the least or most weight, respectively, in these solvents. There appears to be no consensus as to which substitution fluid would theoretically be more appropriate. On one hand, Baskin *et al.* (1996) reported that hydrated *Arabidopsis* roots substituted in acetone showed superior preservation to roots substituted in either methanol or ethanol. Parthasarathy (1995) and Porta and López-Iglesias (1998) also reported that acetone was better than methanol as a substitution fluid for plant cells and the green algae *Jaagiella* respectively. On the other hand, Humbel *et al.* (1983) and Humbel and Müller (1984), who studied animal tissues, suggested that methanol is generally better for structural studies.

Thomson and Platt (1997) in their work on the desiccation tolerant plant *Selaginella lepidophylla*, also tested a number of substitution fluids. They concluded that acetone and ethanol were unsuitable as substitution

fluids as infiltration of both fixatives and resin into the samples was incomplete and thin sectioning was virtually impossible. It is possible that if Thomson and Platt (1997) had used a longer substitution and/or infiltration time they would not have encountered these problems. Instead, they deemed methanol to be most suitable as its use resulted in fairly good infiltration of both fixatives and resin into the sample and thin sections were readily obtainable. This finding by Thomson and Platt (1997) may have been the result of the samples absorbing the methanol, thus facilitating fixative and resin penetration. In an examination of dry seed literature, Yatsu (1983) noted that every time anhydrous processing was used problems with resin infiltration and embedding were encountered whereas when aqueous processing was used, no such problems were mentioned. Thus, if methanol also produced swelling in *Selaginella lepidophylla* tissue, as it did in *Myrothamnus flabellifolia* (refer to 4.12), it may explain the relative ease of resin infiltration encountered by Thomson and Platt (1997) in comparison to acetone and ethanol substituted tissue.

The observation that dehydrated leaf tissue of *Myrothamnus flabellifolia* swells when immersed in anhydrous solutions (refer to Chapter 2, 2.19.1) is quite important because, while it is generally recognized that dehydrated leaves will swell when fixed in aqueous fixatives (Yatsu 1983, Orlovich and Ashford 1995), it has generally been

assumed that anhydrous fixation would eliminate this problem. This is despite evidence that other anhydrous solvents can cause cell wall rehydration. For example, Öpik (1985) found that DMSO caused wall hydration in dry seed tissue.

Great care was taken to ensure that the solvents used during freeze-substitution were completely anhydrous because previous experiments had shown that tissue samples of *Myrothamnus flabellifolia* were capable of swelling significantly even when they were exposed to solutions containing only 5% water (95% solvent) (refer to Chapter 2, 2.19.1). The anhydrous nature of the solvents was maintained by adding either molecular sieve or the chemical dehydrant DMP (refer to 4.7) to the substitution fluids. It was discovered however, that when DMP was added to acetone or ethanol, dehydrated leaves gained more weight (refer to 4.12). This finding was surprising since one would have expected a decrease in weight gained in all the solvents as any water present would have been removed by the DMP. One possible explanation for this observation is that the dry tissue may have absorbed the methanol produced when DMP reacts with water. As noted earlier, dehydrated leaves of *Myrothamnus flabellifolia* swelled much more in methanol than in ethanol or acetone (refer to 4.12). Hence, one should be cautious when using DMP as it has the potential to cause artefacts in dehydrated leaf tissue.

4.16 - The Effect of Chemical Fixatives Added to the Substitution Solvent.

In this study, freeze-substitution of tissue samples was conducted in solvents both with and without the addition of chemical fixatives. Fixatives are generally added to the substitution medium in order to optimize the preservation of cellular details (Monaghan and Robertson 1990) by preventing extraction of cell components by the solvent (Harvey 1982). In this study, the addition of a fixative to the substitution fluid did not improve the already good ultrastructural preservation. Although in the past the inclusion of a fixative with the substitution solvent has been standard practice (Harvey 1982, Hippe-Sanwald 1993), a growing number of studies have found that it is not necessary (Monaghan and Robertson 1990, Lancelle and Hepler 1991, Nicolas and Bassot 1993, Baskin *et al.* 1996).

One reason why fixatives are said to be required for some samples but not for others is related to the temperature at which resin infiltration is conducted. Porta and López-Iglesias (1998) found that the addition of a fixative was only necessary for structural studies when resin infiltration was conducted at room temperature. In the absence of any chemical fixative, marked extraction of cytoplasmic material occurred in the soil green algae *Jaagiella*. Monaghan (1995) also noted that fixatives

should be used when embedding at room temperature although he did not elaborate on the reason why.

Furthermore, considerable evidence suggests that chemical fixatives only become functional as the tissue samples are gradually warmed before resin infiltration (Robards and Sleytr 1985, Nicolas and Bassot 1993). For example, Harvey and Pihakaski (1989) found that specimens were not significantly fixed by osmium after 5 days at temperatures below -20°C and Hereward and Northcote (1972) state that reduction of osmium does not take place below 0°C. Moreover, Steinbrecht and Müller (1987) conclude that our understanding of the effects of chemical fixatives at subzero temperatures is incomplete. A comparison between substitution fluids, both with and without chemical fixatives, used to test the necessity of fixatives at low temperatures, would be rendered worthless if the fixative was not functional at low temperatures. This could then result in the erroneous conclusion that fixatives are unnecessary when fixation and embedding occurs at low temperatures.

The association between embedding temperature and the need for a chemical fixative is not universal. Baskin *et al.* (1996) found that the structural improvements seen in cryofixed and freeze-substituted fresh *Lilium longiflorum* pollen and *Tradescantia virginiana* plants, compared to aqueous chemical fixation, occurred regardless of whether a fixative was included in the substitution fluid or whether

resin infiltration was performed at room or lower temperatures.

In this study, neither the inclusion nor absence of aldehyde fixatives in the substitution fluids, nor the type of fixative added to the substitution fluid appeared to influence the quality of preservation. Honegger et al. (1996) also used the technique of freeze-substitution and obtained similar findings in their investigation of drought-induced structural alterations in foliose macrolichens. They concluded that no optimal or ideal preparative protocol, which could be generally recommended for macrolichens, had emerged from their experiments in spite of the fact that over 60 different preparative protocols had been used. It is possible that these findings may, in part, be due to the dehydrated state of the samples investigated. As discussed earlier, rapid cryofixation and freeze-substitution are particularly suitable for use with dehydrated biological materials that contain only relatively small amounts of water (refer to 4.2.1 and 4.3). This in turn leads to intrinsically good quality preservation. Because of the relative ease with which dehydrated samples are cryofixed, it is possible that this acts to reduce any beneficial action of a chemical fixative as the physical means of fixation already leaves the tissue samples well preserved.

4.17 - Post-Freeze-Substitution Problems.

Poor resin infiltration appears to be a common problem after freeze-substitution (Howard and O'Donnell 1987, Ding *et al.* 1991, Hoch 1991). It also appears to be predominantly associated with plant tissue samples (Hoch 1991, Ristic and Ashworth 1993, Parthasarathy 1995). Parthasarathy (1995) found that freeze-substituted plant cells generally require more gradual and prolonged infiltration times than those that are chemically fixed. Baskin *et al.* (1996) and Thomson and Platt (1997) found that artefacts caused by poor resin infiltration were commonly associated with acetone substituted tissue in contrast to methanol substituted tissue.

The reason for these infiltration problems is not known. Ristic and Ashworth (1993) suggested that it may be related to the effects of phosphate buffers on cell wall permeability as it has been reported that during chemical fixation cell wall components are often removed and this facilitates resin infiltration (Howard and O'Donnell 1987). Virk and Cleland (1988) observed that phosphate buffers extracted cell wall calcium, and induced loosening of the cell wall matrix.

Ristic and Ashworth (1993) incorporated two additional steps after their standard freeze-substitution protocol, a rehydration step and a sodium phosphate buffer rinse. They found that these two steps increased the degree of resin

infiltration into their samples and there was no mention of any change in the quality of ultrastructural preservation. The freeze-substitution protocol of Ristic and Ashworth (1993) was tested as part of this study, on hydrated and dehydrated leaf tissue of *Myrothamnus flabellifolia*. The phosphate buffer rinse did not appear to increase resin infiltration in dehydrated tissue and it resulted in severe tearing of the cytoplasm. These results showed that *Myrothamnus flabellifolia* leaf tissue was still highly responsive to the presence of water even after substitution and therefore one needs to be vigilant for the inadvertent creation of swelling-induced artefacts at all processing steps. The results obtained using the method of Ristic and Ashworth (1993) were so poor that the protocol was immediately abandoned and the results were not incorporated into this thesis. Similar effects were seen in dehydrated tissue samples after anhydrous chemical fixation with a post-fixation aqueous buffer rinse (refer to Chapter 2, 2.19.3 and 2.23.2).

Freeze-substitution does not always result in resin infiltration problems. Such problems were not observed during the course of this dissertation, despite the fact that acetone was the main substitution fluid used. One explanation for this is that because resin infiltration problems were encountered, even after aqueous chemical fixation, an extended resin infiltration protocol (refer to Chapter 2, 2.7) was used throughout this study. Indeed,

prolonged infiltration may solve some of the problems experienced by other workers. England *et al.* (1997) concluded that rapid cryofixation and freeze-substitution of sugarcane stem tissue actually improved resin infiltration. Once again, because sugarcane is an intrinsically difficult tissue to embed, their resin infiltration schedule may have already been extended enough to overcome any additional limitations posed by rapid cryofixation and freeze-substitution.

4.18 - A Comparison of the Ultrastructural Preservation of Rapid Cryofixed/Freeze-Substituted *Myrothamnus flabellifolia* Leaf Tissue with Leaf Tissue Fixed Using Either Aqueous or Anhydrous Chemical Fixatives or Phase-Partition Fixation.

The following sections compare the results obtained using rapid cryofixation/freeze-substitution and the fixative techniques of aqueous chemical fixation, anhydrous chemical fixation and phase-partition fixation. For the reasons outlined above (refer to 4.15) the following discussion refers to hydrated leaf tissue freeze-substituted in methanol and dehydrated leaf tissue freeze-substituted in acetone.

4.18.1 - Hydrated Tissue.

Hydrated *Myrothamnus flabellifolia* leaf tissue that had been cryofixed and freeze-substituted bore the greatest similarity to hydrated tissue that had undergone aqueous chemical fixation. Such tissues contained cells that were turgid looking and had smooth walls that were round to oval in shape. Also, there was a close alignment between the cell wall and the protoplast (refer to Table 4 and Chapter 2, Table 1). These observations are in contrast to hydrated tissue that had been fixed by phase-partition fixation. In many instances, the cell walls appeared wavy which gave the cells a flaccid appearance and occasionally separation of the protoplast and the cell wall was observed (refer to Table 4 and Chapter 2, Table 1 c.f. Chapter 3, Table 3). These features were taken to be artefacts for two reasons. First, they were not ubiquitously seen; in a number of cases, phase-partition fixed tissue was similar in appearance to cryofixed and aqueously fixed tissue samples. Secondly, such observations were similar to the changes observed in hydrated tissue fixed using anhydrous chemical fixation (refer to Chapter 2, Table 1).

Although there were some ultrastructural differences between the various fixation techniques there were also a number of similarities. For example, nuclei, mitochondria and chloroplasts appeared unaffected by the fixation technique used. In each treatment nuclei were rounded in

shape, contained finely dispersed chromatin, possessed a double bounding membrane and often contained a nucleolus. Chloroplasts were elongated in shape and possessed distinct granal and stromal regions and mitochondria were oval to round in shape and regularly contained cristae (refer to Table 4, Chapter 2, Table 1 and Chapter 3, Table 3).

It is interesting that the type of fixation technique did not influence the ultrastructure of these organelles whereas the cell wall was quite sensitive to the fixation protocol. In theory, phase-partition fixation is an osmotically neutral technique (refer to Chapter 3, 3.1), therefore, what appeared to be dehydration-associated artefacts in phase-partition fixed tissue was a surprise. This suggests that post-fixation processing may have caused these artefacts, for example, dehydration artefacts may have resulted when the samples were transferred to 70% solvent prior to resin infiltration. Altering these steps may improve ultrastructural preservation and hence, make this a more viable technique for use with dehydrated and hydrated tissue.

From the above results, it was concluded that rapid cryofixation followed by freeze-substitution produced results that were comparable in quality to hydrated tissue fixed by aqueous chemical fixation. A comparison of various fixation techniques, during the course of this thesis, had previously considered the aqueous chemical fixation of hydrated *Myrothamnus flabellifolia* leaf tissue

to result in the best quality of ultrastructural preservation. Therefore, rapid cryofixation/freeze-substitution can be considered as a viable alternative to aqueous chemical fixation, for the preservation of hydrated leaf tissue of *Myrothamnus flabellifolia*.

4.18.2 - Dehydrated Tissue.

Dehydrated leaf tissue of *Myrothamnus flabellifolia* that had been cryofixed and freeze-substituted was similar in structure to tissue that had been fixed anhydrously. Samples fixed using these techniques possessed cell walls that were extremely convoluted and possessed an irregularly shaped central vacuole that was reduced in size and contained electron-dense material (refer to Table 4 and Chapter 2, Table 1). There were also similarities in chloroplast structure between these two fixation techniques. Chloroplasts fixed in such a way were irregular in shape and were shorter compared to chloroplasts in hydrated tissue (refer to Table 4 and Chapter 2, Table 1). There was a difference however between the two techniques, in the preservation of the internal structure of the chloroplast, with the use of rapid cryofixation resulting in a better overall preservation quality.

In comparison, after phase-partition fixation, cell walls were not as convoluted. Chloroplasts were short but

they were distinctly round in shape (refer to Table 4 and Chapter 2, Table 1 c.f. Chapter 3, Table 3). Nevertheless, both rapid cryofixation and phase-partition fixation successfully preserved the internal structure of the chloroplast (Refer to Table 4 and Chapter 3, Table 3). Finally, the degree separation of the protoplast from the cell wall was more severe and more frequently observed when the tissue had undergone phase-partition fixation rather than the other two protocols (refer to Table 4 and Chapter 2, Table 1 c.f. Chapter 3, Table 3).

There is a large amount of evidence that implicates rehydration or solvent absorption as a possible cause for the disparity between the results of the various techniques. The majority of ultrastructural differences observed in dehydrated tissue that has been fixed with phase-partition fixation have also been observed in dehydrated tissue fixed using techniques that induce swelling. For example, rounded chloroplasts were also observed in dehydrated tissue that had been fixed with an aqueous fixative, in a partially anhydrous chemical fixative and in cryofixed tissue that had been substituted in methanol. Both the presence of methanol and the actual presence of water in a fixative have been associated with the swelling of dehydrated tissue (refer to Chapter 2, 2.16.2, 2.19 and Chapter 4 4.12 respectively). The separation of the protoplast from the cell wall has also been associated with other fixation techniques that induce

the rehydration of dehydrated tissue (refer to Chapter 2, 2.16.2, 2.19.2 and Chapter 4, 4.13.2).

The above results indicate that rapid cryofixation followed by freeze-substitution and anhydrous chemical fixation, are similar in their capability of successfully preserving dehydrated leaf tissue of *Myrothamnus flabellifolia*. Before the fixation technique of rapid cryofixation/freeze-substitution was examined, anhydrous chemical fixatives were considered superior to other fixation techniques tested during the course of this thesis. The ability of rapid cryofixation/freeze-substitution to successfully preserve the internal membranes of the chloroplast means that it should be considered as best fixation method for the preservation of dehydrated *Myrothamnus flabellifolia* leaf tissue.

4.19 - Conclusions.

Results from this study revealed that the fixation technique of rapid cryofixation followed by freeze-substitution is particularly successful in preserving both hydrated and dehydrated *Myrothamnus flabellifolia* leaf tissue. This is an important finding as it is one of the first times where a single fixation technique has been successfully used on both hydrated and dehydrated tissue samples. In general, other fixation techniques have only been successful on either hydrated or dehydrated tissue

samples. The ability to use a single fixation technique means that any differences observed between hydrated and dehydrated tissue samples can be attributed to the effects of desiccation with more certainty.

The osmotic neutrality of rapid cryofixation and freeze-substitution is the reason that both hydrated and dehydrated tissue samples can be preserved with this single technique. This technique can be extended further to allow researchers to analyze tissue samples at intermediate stages of dehydration. Whilst no one has previously taken advantage of this capability of rapid cryofixation/freeze-substitution, Honegger and Peter (1994) did acknowledge the technique's potential for such research. Thus, the following chapter is the first time that the techniques of rapid cryofixation and freeze-substitution have been utilized to examine the ultrastructure of biological tissue at intermediate water contents between hydrated and dehydrated.

Chapter 5 - Ultrastructure of *Myrothamnus flabellifolia* Leaf Tissue at Different Stages during Desiccation

INTRODUCTION.

Previous chapters established that many of the reported characteristic ultrastructural features of dry desiccation tolerant tissues were actually fixation artefacts. Artefacts such as membrane rupture and separation of the plasma membrane from the cell wall were incorporated into the theories about desiccation tolerance. Significant improvements in ultrastructural preservation were achieved with the use of the technique of rapid cryofixation followed by freeze-substitution. In light of these improvements, we therefore now need to revise our theories about both desiccation and rehydration, and in this chapter, rapid cryofixation and freeze-substitution will be used to examine the ultrastructural changes in *Myrothamnus flabellifolia* leaf tissue during these processes.

5.1 - Previous Research Examining Desiccation Tolerant Plant Material at Intermediate Water Contents between Hydrated and Dehydrated.

Of the various ultrastructural studies on desiccation tolerant tissues, the majority focussed exclusively on either completely hydrated or completely dehydrated tissues. Few studies investigated tissue ultrastructure at intermediate stages during desiccation or rehydration. Of

those, the majority examined only seeds and pollen (Klein and Ben-Shaul 1966, Klein and Pollock 1968, Kerhoas *et al.* 1987, Perdomo and Burris 1998), there has been little work on vegetative tissues such as leaves.

Whilst some researchers have examined changes in the ultrastructure of desiccation tolerant plants during rehydration (Hallam and Gaff 1978, Gaff *et al.* 1976, Bergtrom *et al.* 1982, Tuba *et al.* 1993, Markovska *et al.* 1995, Sherwin and Farrant 1996, Quartacci *et al.* 1997, Dalla Vecchia *et al.* 1998), investigations of ultrastructural changes during dehydration are fewer in number (Bartley and Hallam 1979, Hallam and Luff 1980a, Hetherington *et al.* 1982). Other tissues that have been examined during dehydration include desiccation tolerant seeds (Ciamporová 1987) and various desiccation sensitive tissues (Nir *et al.* 1969, Giles *et al.* 1974, Freeman and Duysen 1975, Fellows and Boyer 1976, 1978, Lopez-Carbonell *et al.* 1994).

One reason for the paucity of research in this area is that it is very difficult to evaluate effects of the fixative regime on the ultrastructure of dry or partially dry tissues. As was discussed in Chapter 2, the type of fixative used can have a dramatic impact on cell ultrastructure (refer to Chapter 2, 2.2; 2.21.3; and 2.23.2). Using aqueous chemical fixatives with hydrated tissue and anhydrous chemical fixatives with dehydrated tissue can reduce the effect of osmotic differences between

the fixative and the tissue sample. However, how tissues with intermediate water contents respond to different fixatives is, for the most part, unknown.

In the few published studies, two methods have been used in an attempt to counteract this problem. In the first method, aqueous fixatives were used on tissue samples of high water contents and anhydrous fixatives were used on tissue samples of low water contents. However, there appears to be no consistent water content at which the switch from aqueous to anhydrous fixatives is made. For example, Hallam and Luff (1980 a, b) fixed samples with water contents below 26% RWC anhydrously, but Bartley and Hallam (1979) used anhydrous fixation for samples below 35% RWC. Nir *et al.* (1969) used anhydrous vapour fixation for samples that had lost 42% or more of their fresh weight. Finally, Hallam and Luff (1980a) stated that if aqueous fixatives were used on tissues with RWCs below 40%, cell walls would rehydrate more rapidly than the cytoplasm, thus resulting in the separation of the two structures.

The different levels of dehydration at which these researchers switched to using anhydrous fixatives highlights some of the uncertainty surrounding this approach. Although it can be assumed that aqueous chemical fixatives will induce swelling in some samples and anhydrous chemical fixatives will induce shrinkage in other samples during dehydration, what is not understood is at what levels of dehydration the aqueous fixatives cease

acting as hydrating agents and at what levels of dehydration the anhydrous fixatives will not dehydrate the tissue. It is also debatable whether there is a distinct point at which an aqueous fixative rather than an anhydrous fixative can be used, since the use of either aqueous or anhydrous fixatives may impact on the ultrastructure of the tissue.

The second approach used to counteract the effect of osmotic differences between the tissue sample and the fixative has been to use iso-osmotic fixatives. Fellows and Boyer (1976, 1978) added sucrose to aqueous fixatives to bring the fixative to within 1 bar of the water potential of the sample being fixed. They reported that this resulted in improved structural integrity compared to previous studies that used fixatives without added osmotica. Hallam and Luff (1980b) used water/DMSO combinations to match the water content of the fixative with the osmotic potential of the drying tissue. They observed better preservation of fine structure than in dry tissue fixed in totally aqueous fixatives. However, results from previous chapters show that this method can still generate artefacts. Firstly, dehydrated leaves of *Myrothamnus flabellifolia* immersed in 100% DMSO still showed considerable swelling, resulting in the production of ultrastructural artefacts (refer to Chapter 2, 2.19.1). Secondly, dehydrated leaves, which had a water content of approximately 5%, when immersed in solutions containing

only 5% water showed similar swelling to leaves immersed in solutions containing 50% water (refer to Chapter 2, 2.19.1).

The poor results obtained, and difficulty obtaining reproducible images of partially dehydrated tissues fixed by traditional aqueous and anhydrous methods, were probably the main reasons why this research was virtually abandoned. Most papers on ultrastructural changes during dehydration are over 20 years old. Osmotically neutral fixatives, such as rapid cryofixation, have the potential to eliminate such uncertainties.

5.2 - Ultrastructural Changes in Drying Tissue.

The following is a summary of various changes observed in plant tissues during dehydration however it should be noted that many of the ultrastructural changes that are described below may be artefacts because the majority of studies used aqueous chemical fixation techniques.

Chloroplasts are one of the first cellular structures to show any ultrastructural changes during dehydration and these changes have been widely examined in comparison to other organelles. During the early stages of dehydration (78-76% RWC), the thylakoid system is regularly reported to separate and become more widely spaced than in hydrated tissue (Bartley and Hallam 1979, Hallam and Luff 1980a), although this is not always the case. For example, the

thylakoid membranes of *Talbotia elegans* only became disorganized at 50% RWC (Hallam and Luff 1980b) and it was only after 27 hours of dehydration that most thylakoids were distended in *Borya nitida* (Hetherington *et al.* 1982). The disruption of thylakoids is not a response exclusive to species that are desiccation tolerant (Freeman and Duysen 1975, Lopez-Carbonell *et al.* 1994).

Changes in the overall shape of chloroplasts are also regularly observed during the process of dehydration. Many researchers have reported that chloroplasts of various species become more rounded as desiccation proceeds (Bartley and Hallam 1979, Hallam and Luff 1980b, Hetherington *et al.* 1982). These responses have been reported to occur as early as 76% RWC in *Coleochloa setifera* (Bartley and Hallam 1979) too as late as 38% RWC in *Talbotia elegans* (Hallam and Luff 1980b). Similar responses have also been made in desiccation sensitive plants (Kurkova and Motorina 1974, Fellows and Boyer 1976).

Mitochondria also undergo ultrastructural changes during dehydration with the loss of cristae being the most frequent change reported. This response does not occur until the middle to late stages of dehydration and is first observed at 50% RWC in *Talbotia elegans* (Hallam and Luff 1980b), at 42% RWC in *Xerophyta villosa* (Hallam and Luff 1980a) and at 35% RWC in *Coleochloa setifera* (Bartley and Hallam 1979). Mitochondria may also exhibit various shape changes during dehydration. For example, in *Xerophyta*

villosa, mitochondria become circular in cross section at RWCs of 42% and below (Hallam and Luff 1980a). Mitochondrial shape changes have also been observed in maize seeds. During the early stages of dehydration mitochondria may take upon the shape of a dumb-bell or become irregularly elongated (Ciamporová 1987). Similar ultrastructural changes have been observed in desiccation sensitive species (Nir *et al.* 1969, Lopez-Carbonell *et al.* (1994).

Condensation of the chromatin in the nucleus is another frequently observed change during dehydration. The beginnings of such changes were seen at 78% RWC in *Xerophyta villosa* (Hallam and Luff 1980a) and were reported to be complete at 26-35% RWC (Bartley and Hallam 1979, Hallam and Luff 1980a). As with other reported desiccation-related changes, the condensation of chromatin has also been observed in desiccation sensitive tissues (Nir *et al.* 1969, Ciamporová 1987).

Vacuoles may also exhibit various changes during dehydration and fragmentation of the vacuole is one of the most commonly reported changes. Vacuole fragmentation does not appear to occur at a particular time during dehydration. For example, vacuole fragmentation occurred at 26% RWC in *Xerophyta villosa* (Hallam and Luff 1980a), at 50% RWC in *Talbotia elegans* cells (Hallam and Luff 1980b) and at 76% RWC in *Coleochloa setifera* (Bartley and Hallam 1979). Vacuole fragmentation has also been reported in the

desiccation sensitive species *Fatsia japonica* (Lopez-Carbonell *et al.* 1994).

Finally, the appearance of lipid droplets and electron dense material in the cytoplasm and vacuoles of cells is another regular observation during dehydration. Such deposits have been observed at 76% RWC in *Coleochloa setifera* (Bartley and Hallam 1979), at 59% RWC in *Talbotia elegans* (Hallam and Luff 1980b) and at 42% RWC in *Xerophyta villosa* (Hallam and Luff 1980a). In desiccation sensitive species the accumulation of lipids and electron dense material has also been noted (Nir *et al.* [illegible], Mayaux and Boyer 1978).

5.3 - Aims.

Whilst the above summaries of ultrastructural changes observed in both desiccation tolerant and desiccation sensitive plant species during dehydration give an important account of the process of desiccation, they are also problematic in that the majority of studies used aqueous chemical fixation techniques. Although a few of the studies attempted to compensate for possible osmotic differences between the fixative and the tissue (refer to 5.1), work in previous chapters of this thesis suggests that ultrastructural artefacts are likely to have occurred (refer to Chapter 2, 2.19.1). The use of osmotically neutral techniques such as rapid cryofixation and freeze-

substitution have the potential to eliminate ultrastructural changes to a degree that has never before been achieved (refer to Chapter 4, 4.1 and 4.2). To date, the advantages of osmotically neutral fixation techniques have not been used to examine the ultrastructure of tissue at various stages during dehydration. Honegger and Peter (1994) applied the techniques of rapid cryofixation and freeze-substitution to several species of lichen. Whilst they only examined specimens that were either hydrated or dehydrated they did acknowledge that all intermediate levels of desiccation could be explored in this way. This portion of the research aims to examine for the first time the ultrastructural changes in mesophyll cells of *Myrothamnus flabellifolia* leaves during various stages of desiccation.

MATERIALS AND METHODS.

5.4 - Plant Material and Tissue Sampling.

Leaf tissue of *Myrothamnus flabellifolia* (refer to Chapter 2, 2.6.1) at various stages of dehydration was used during the course of this chapter. The determination of leaf's relative water content is outlined in Chapter 2 (refer to 2.6.2).

All experimental tissue was obtained from hydrated plants. Small shoots approximately 5-10 cm in length were excised and placed in an empty petri dish. The petri dishes containing the shoots were placed on a windowsill and were allowed to dry under natural conditions at a constant temperature of 21°C.

Excised shoots of *Myrothamnus flabellifolia* were used as the experimental material rather than whole plants as they are able to survive dehydration after excision (Gaff and Loveys 1984). This enabled a more accurate determination of leaf RWC during drying as the RWC of a whole plant only begins to drop when the soil has dried out. Thus different water retention properties of the soil of different plants means that although two plants may have been drying for the same period of time their RWC would not necessarily also be the same (Sherwin *et al.* 1998). By excising shoots at different times before fixation, a range of tissue samples at various RWCs could easily be obtained.

5.5 - Tissue Fixation.

All tissue samples were cryofixed and freeze-substituted in acetone as outlined in the Material and Methods section of Chapter 4 (refer to 4.5, 4.6 and 4.7).

RESULTS.

5.6 - The Ultrastructure of *Myrothamnus flabellifolia* Leaf Tissue at Various Stages during Dehydration.

5.6.1 - 70% RWC.

At 70% RWC, mesophyll cell walls had become slightly convoluted (Fig. 55a, 55b). A large central vacuole was generally present (Fig. 55a, 55b, 55c) and the cytoplasm occupied the periphery of the cell (Fig. 55a, 55b).

Chloroplasts were usually elongated in shape (Fig. 55a, 55b, 55c, 55e) and possessed distinct granal and stromal regions (Fig. 55e). Thylakoid membranes were regularly observed (Fig. 55f) and plastoglobuli were also frequently present (Fig. 55e).

Nuclei were generally irregular in shape and contained regions of electron-dense and electron-translucent chromatin (Fig. 55d).

Mitochondria were elongated in shape and possessed cristae (Fig. 55g). Plasmodesmata were also observed (Fig. 55h).

Refer to the end of this results section for a summary of the ultrastructural findings contained in this chapter (Table 5).

Figure 55. - *Myrothamnus flabellifolia* leaf tissue at 70% RWC that was cryofixed and freeze-substituted in 4% paraformaldehyde in acetone.

Fig. 55a - A general view of a mesophyll cell at 70% RWC. Cell walls (CW) were slightly convoluted thus giving the cell an overall flaccid appearance. Large central vacuoles (V) were present and the protoplast occupied the periphery of the cell. In many instances, the cell wall and the protoplast had separated (*). Chloroplasts (C) were regularly observed to contain grains of starch (SG). Bar represents 1 µm.

Fig. 55b - A second generalized view of a mesophyll cell at 70% RWC. As in Fig. 55a, the cells had a flaccid appearance, as the cell walls (CW) were wavy in contour. Each cell possessed a large central vacuole (V) and chloroplasts (C) were generally the most predominant organelles observed. Bar represents 1 µm.

Fig. 55c - Various organelles were regularly observed including chloroplasts (C), nuclei (N), mitochondria (M) and golgi apparatus (G). Areas of plasma membrane separation from the cell wall (CW) were frequently observed (*). Bar represents 0.5 µm.

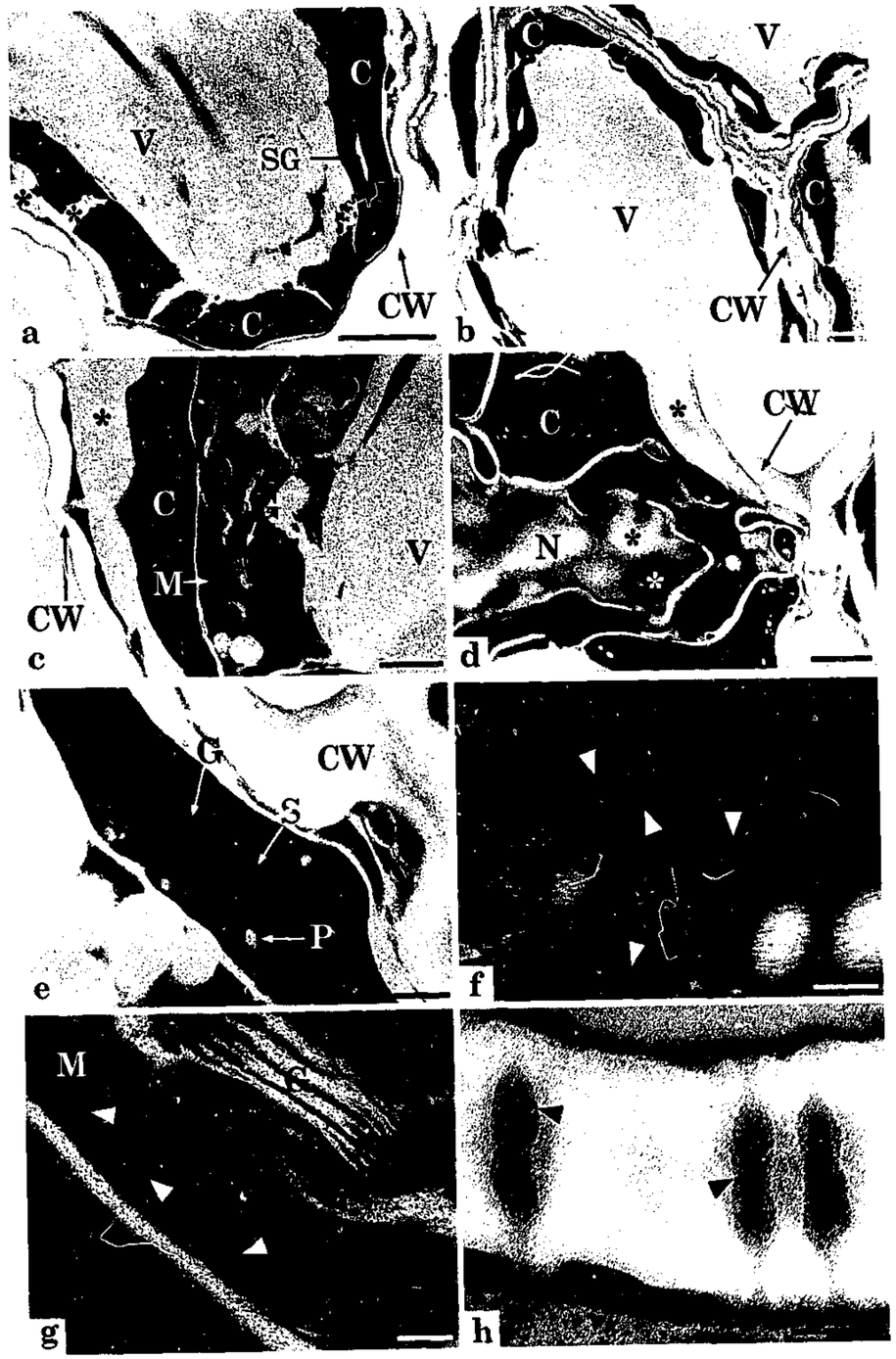
Fig. 55d - Nuclei (N) were generally irregular in shape and contained chromatin that was either electron-dense (white *) or electron-lucent (black *). Bar represents 0.5 µm.

Fig. 55e - Chloroplasts (C) were usually elongate in shape. Granal (G) stromal (S) areas could be discerned as could plastoglobuli (P). Bar represents 0.25 µm.

Fig. 55f - The thylakoid membranes (arrow heads) that comprise the granal stacks were readily observed. Bar represents 0.05 µm.

Fig. 55g - Mitochondria (M) were generally elongate in shape and contained distinct cristae (arrow heads). Golgi apparatus (G) were also observed. Bar represents 0.1 µm.

Fig. 55h - Plasmodesmata (arrow heads) were regularly seen at this stage of dehydration. Bar represents 0.1 µm.



5.6.2 - 49% RWC.

At 49% RWC, mesophyll cell walls were quite convoluted (Fig. 56a, 56b). A central vacuole was present within which had accumulated large masses of electron-dense material (Fig. 56a, 56b). In the majority of cases, the cell wall and the protoplast were in close alignment (Fig. 56a, 56b) however in some cases areas of separation were observed (Fig. 56b).

Chloroplasts were rounded in shape and contained distinct granal and stromal regions (Fig. 56c). The composite thylakoid membranes of the granal stacks were frequently able to be seen (Fig. 56d).

Nuclei were irregular in shape and contained chromatin that was condensed in nature (Fig. 56b, 56e). The chromatin was distinguishable into light and dark staining regions (Fig. 56e).

Mitochondria were elongated in shape (Fig. 56c, 56f) and possessed cristae (Fig. 56f).

5.6.3 - 27% RWC.

At 27% RWC the protoplast of mesophyll cells of *Myrothamnus flabellifolia* was predominantly positioned adjacent to the cell wall (Fig. 57a) however on some occasions separation of the protoplast and the cell wall was observed (Fig. 57b). A central vacuole was

Figure 56. - *Myrothamnus flabellifolia* leaf tissue at 49% RWC that was cryofixed and freeze-substituted in acetone either with or without fixatives added.

Fig. 56a - A general view of a mesophyll cell at 49% RWC. Cell walls (CW) had a wavy contour giving them a flaccid appearance. A central vacuole (V) that contained electron-dense material were regularly present. Chloroplasts (C) and mitochondria (M) could also be discerned. Tissue sample substituted in 100% acetone. Bar represents 0.5 µm.

Fig. 56b - Another general view of a mesophyll cell. As in Fig. 56a, the cell wall (CW) is quite convoluted giving the cell a shrunken appearance. A central vacuole (V) is present in which masses of electron-dense material have accumulated. Small areas of cell wall and protoplast separation (*) could occasionally be seen. A nucleus (N) and mitochondria (M) can also be seen. Tissue sample substituted in 100% acetone. Bar represents 1 µm.

Fig. 56c - Chloroplasts (C) and mitochondria (M) were two frequently observed organelles. Tissue sample substituted in 2.5% glutaraldehyde in acetone. Bar represents 0.5 µm.

Fig. 56d - A high magnification micrograph showing the thylakoid membranes (arrow heads) that comprise chloroplast granal stacks. Tissue sample substituted in 2.5% glutaraldehyde in acetone. Bar represents 0.02 µm.

Fig. 56e - Nuclei (N) were irregular in shape (arrow heads) and contained chromatin that could be separated into electron-dense (white *) and electron-lucent (black *) areas. Tissue sample substituted in 2.5% glutaraldehyde in acetone. Bar represents 0.25 µm.

Fig. 56f - Mitochondria (M) were generally elongate in shape and contained distinct cristae (arrow heads). Tissue sample substituted in 2.5% glutaraldehyde in acetone. Bar represents 0.1 µm.

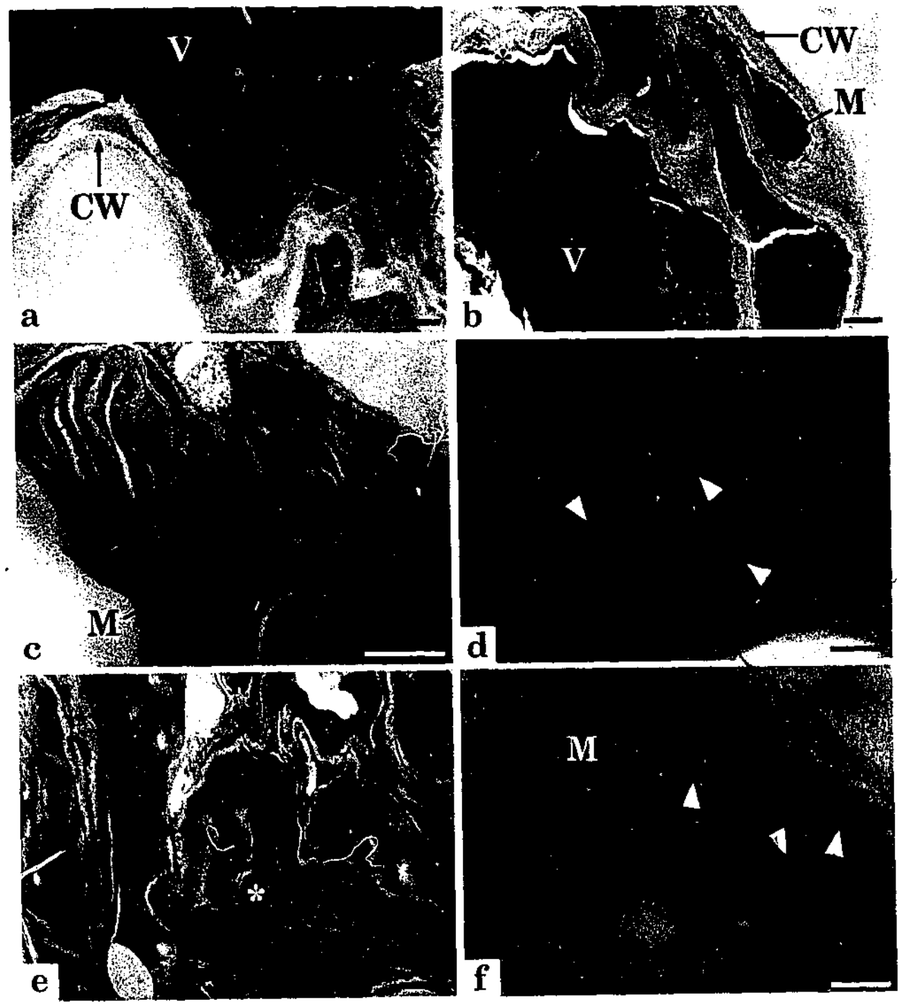


Figure 57. - *Myrothamnus flabellifolia* leaf tissue at 27% RWC that was cryofixed and freeze-substituted in 4% paraformaldehyde in acetone.

Fig. 57a - A general view of a mesophyll cell at 27% RWC. The protoplast and the cell wall (CW) were for the most part in close alignment with each other (arrow heads). Chloroplasts (C) generally comprised a dominant proportion of the cytoplasm. Irregularly shaped central vacuole (V) could be seen in which there was often an accumulation of electron-dense material. Bar represents 1 µm.

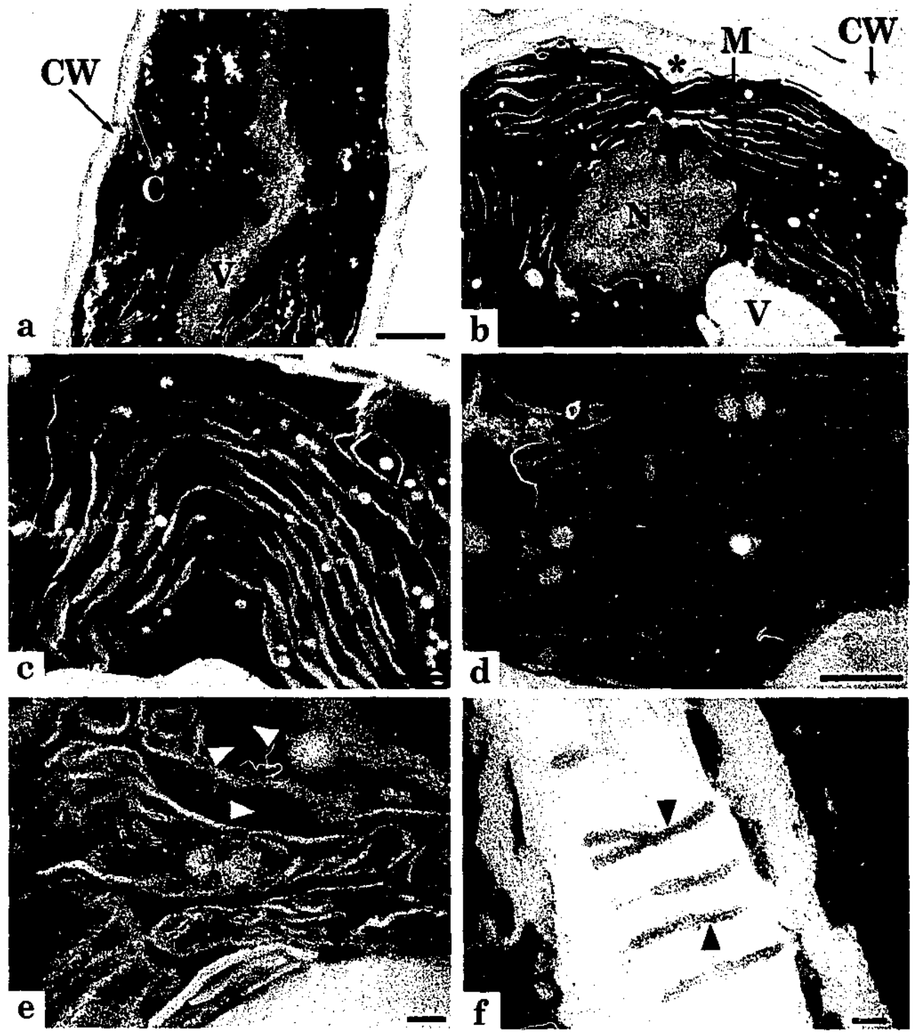
Fig. 57b - In some instances, separation of the cell wall and the protoplast was observed (*). Various organelles such as chloroplasts (C), mitochondria (M) and nuclei (N) were regularly observed. A small central vacuole can be seen, however in contrast to Fig. 57a, no electron-dense material can be seen. Bar represents 1 µm.

Fig. 57c - Chloroplasts (C) had an irregular outline and were frequently rounded in shape (arrow heads). Granal (G) and stromal (S) regions could be discerned, as could plastoglobuli (P). Bar represents 0.5 µm.

Fig. 57d - A high magnification view of a chloroplast showing the granal (G) and stromal (S) regions. The thylakoid membranes that comprise the granal stacks can be seen in some grana (arrow heads). Bar represents 0.25 µm.

Fig. 57e - Mitochondria (M) were generally elongate in shape and had an irregular contour. Cristae (black arrow heads) were almost universally observed. Cytoplasmic ribosomes (white arrow heads) were also frequently seen. Bar represents 0.1 µm.

Fig. 57f - Plasmodesmata (arrow heads) were regularly observed. Bar represents 0.1 µm.



distinguishable (Fig. 57a, 57b) and in some cases it contained an accumulation of electron-dense material (Fig. 57a) whereas in other cases it appeared translucent (Fig. 57b).

Chloroplasts were irregular in shape and were less elongated than chloroplasts observed at higher RWCs (Fig. 57a, 57b, 57c), they also possessed distinct granal and stromal areas (Fig. 57c, 57d). The thylakoid membranes of the chloroplast were occasionally observed (Fig. 57d). Plastoglobuli were routinely observed (Fig. 57c, 57d).

Nuclei were also irregular in shape and contained chromatin that was condensed in appearance (Fig. 57b).

Mitochondria were elongated in shape and possessed cristae (Fig. 57e). Plasmodesmata were regularly seen (Fig. 57f).

Table 5. - Summary of the ultrastructural changes observed in *Myrothamnus flabellifolia* leaf tissue during dehydration.

	70% RWC	49% RWC	27% RWC
Cell Shape	Cell walls were slightly convoluted and had a flaccid appearance.	Cells walls were quite convoluted in shape.	Cell walls were quite convoluted in shape.
Protoplast	The cytoplasm occupied the periphery of the cell although separation of the protoplast and the cell wall was frequently observed.	The cell wall and the protoplast were generally in close alignment however sometimes areas of separation were seen.	The cell wall and the protoplast were generally in close alignment however sometimes areas of separation were seen.
Vacuole	A single, large, centrally positioned vacuole was generally present.	A central vacuole was present within which had accumulated masses of electron dense material.	A central vacuole was generally present, within which, in some cases, electron dense material had accumulated.
Chloroplast	Elongate in shape and possessed distinct granal and stromal regions. Thylakoid membranes were observed as too were plastoglobuli.	Round in shape and contained distinct granal and stromal regions. Thylakoid membranes were frequently discernable.	Were irregular to circular in shape and possessed distinct granal and stromal regions. Thylakoid membranes were occasionally observed. Plastoglobuli were routinely seen.
Mitochondria	Elongate in shape and possessed cristae.	Were elongate in shape and possessed cristae.	Were elongate in shape and possessed cristae.
Nucleus	Generally irregular in shape and contained regions of electron-dense and electron-lucent chromatin.	Irregular in shape. Chromatin was condensed and was distinguishable into electron-dense and electron-lucent regions.	Were irregular in shape and contained condensed masses of chromatin.

5.7 - Changes in the Ultrastructure of *Myrothamnus flabellifolia* Leaf Tissue during Dehydration.

At 70% RWC, very few ultrastructural differences were evident when compared to hydrated tissue. The main change evident was in the cell wall, which was wavy in contrast to the smooth round cell walls evident in hydrated tissue (refer to Table 5 and Chapter 4, Table 4). Such observations are likely to be a direct result of the initial loss of water.

The nucleus also showed some ultrastructural changes. At 70% RWC it was irregular in shape and contained condensed chromatin, similar to nuclei in dehydrated tissue (refer to table 5 and Chapter 4, Table 4). In contrast, nuclei in hydrated tissue had smooth outlines and were round to oval in shape. It is possible that this observation is the result of using acetone as the substitution fluid as hydrated tissue that was freeze-substituted in methanol contained chromatin that was finely dispersed whereas chromatin in acetone-substituted tissue was more condensed in appearance (refer to Chapter 4, 4.13.1 and 4.13.2). However, chromatin condensation has frequently been reported as a response to dehydration, for example, Hallam and Luff (1980a) observed condensed chromatin in *Xerophyta villosa* at 78% RWC. While it is not

known whether chromatin condensation is purely a response to water loss or whether it serves a particular function it is a well-known symptom of genetic inactivity (Deltour 1985).

Other aspects of cellular ultrastructure, such as the presence of a large central vacuole, the elongated shape of the chloroplasts, the presence of thylakoid membranes, the elongated shape of the mitochondria and the presence of criscae remained unchanged from those observed in hydrated tissue (refer to Table 5 and Chapter 4, Table 4).

At 49% RWC, further ultrastructural changes were evident, in particular, an increase in the convolutions of the cell wall. Mesophyll cell walls were no longer slightly wavy, but were shrunken in appearance (Table 5).

Although a large central vacuole was present in the vast majority of cells at 49% RWC, unlike the vacuoles observed at 70% RWC, they contained large masses of electron-dense material (Table 5). This has been observed in other plant species, including both desiccation tolerant (Bartley and Hallam 1979, Hallam and Luff 1980a, b) and desiccation sensitive species (Nir *et al.* 1969, Fellows and Boyer 1978) and it has been postulated that it may help protect the plant from ultraviolet light (Hallam and Luff 1980a, b).

Chloroplasts also exhibited structural changes between 70% RWC and 49% RWC. Whereas chloroplasts at 70% RWC were elongated in shape, at 49% RWC they were more round in

shape (Table 5). Numerous other researchers have also reported this ultrastructural change in desiccation tolerant species including *Talbotia elegans* (Hallam and Luff 1980b), *Borya nitida* (Hetherington *et al.* 1982) and *Coleochloa setifera* (Bartley and Hallam 1979). Hetherington *et al.* (1982) proposed that the increase in salt concentration observed during dehydration in *Borya nitida* may osmotically draw water into the chloroplast which in turn results in swelling of the organelle.

The thylakoid membranes of *Myrothamnus flabellifolia* at 49% RWC were also shorter than those observed at 70% RWC (Table 5). Changes in thylakoid membrane composition are frequently mentioned in relation to ultrastructural changes during dehydration however the majority of researchers have reported that there is a loss of integrity of the granal stacks (Bartley and Hallam 1979, Hallam and Luff 1980b, Hetherington *et al.* 1982). Semenova *et al.* (1994) reported that dehydration could act as a thylakoid membrane destabilizer and postulated that the destabilization could be due to protein denaturation and/or to the disturbance of hydrogen bonds. Only Navari-Izzo *et al.* (2000) reported similar findings to those found during the course of this study, namely an increase in the extent of thylakoid membrane overlap during dehydration. They suggested that membrane and/or environmental changes could have weakened the repulsive forces between the membrane surfaces. This response may also be beneficial in preventing

photoinhibition (refer to Chapter 1, 1.2.2.3). Because thylakoid membranes are still present in mesophyll cells of *Myrothamnus flabellifolia* during dehydration this may mean that they are still potentially susceptible to photoinhibitory damage. It is possible that the shorter length of the granal stacks acts to reduce the amount of light absorbed by the cell and thus limits the potential for creating active oxygen species. I was unable to find any research that supported this theory.

Other organelles did not appear to be affected by dehydration. For example, mitochondria and nuclei were similar in appearance to those observed at 70% RWC (Table 5).

The major ultrastructural change, observed at 27% RWC compared to 49% RWC, was a reduction in vacuole size, and the vacuole also tended to be more irregular in shape (Table 5). In other plant species, the vacuole was reported to fragment, forming numerous smaller vacuoles (Gaff *et al.* 1976, Hallam and Luff 1980b, Markovska *et al.* 1994, Quartacci *et al.* 1997). Vacuole fragmentation has been suggested to play a role in preventing the rupture of the plasma membrane, caused by tension generated during its withdrawal from the cell wall except in areas where it is attached to the plasmodesmata. According to this hypothesis, the small vacuoles arising from fragmentation of the large central vacuole provide backpressure to prevent cell wall collapse and withdrawal of the plasma

membrane (Hallam 1976, Farrant 2000). However, it is difficult to imagine any situation in which this theory has physiological relevance. Many salt-tolerant plants accumulate substantial ion concentrations in their vacuoles without it fragmenting, and vacuole fragmentation is not seen during plasmolysis of cells with any type of salt or sugar. Instead, results in this chapter strongly suggest that any withdrawal of the membrane for the cell wall is artefactual, and it is possible that vacuole fragmentation is the result of another fixation or tissue processing artefact. Indeed, because the walls of *Myrothamnus flabellifolia* display such a high degree of folding during dehydration, such a mechanism would appear unnecessary, at least for this tissue. Cell wall folding has also been proposed to aid the cell in the prevention of plasma membrane rupture in other tissues (Farrant 2000).

Apart from the changes to the vacuole described above, other organelles remained unchanged at 27% RWC from what was observed at 49% RWC. In the majority of cases, mesophyll cells at 27% RWC had an overall shrunken appearance which was also evident at 49% RWC. Chloroplasts were rounded in shape and mitochondria were elongated in shape and contained distinct cristae (Table 5).

5.8 - Conclusions.

The results contained in this chapter have, for the first time, utilized rapid cryofixation and freeze-substitution to preserve the ultrastructure of desiccation tolerant leaf material at intermediate levels of desiccation. Although this study has been relatively brief it has illustrated the suitability of this technique for the examination of partially dry tissue samples. As highlighted by Hoekstra *et al.* (2001), research into this aspect of desiccation tolerance is particularly important as desiccation sensitive organisms usually die when the water content is still relatively high [(0.5-2 (gH_20)(g dry weight)$^{-1}$]. Thus, mechanisms to protect against tissue damage are likely to be triggered while tissue water content is still fairly high. Ultrastructural results reported here, and by Hallam and Luff (1980b) also show that most changes are seen between the hydrated state and 50% RWC. To date most biophysical investigations have focused primarily on the dehydrated state. The use of fixation techniques such as rapid cryofixation and freeze-substitution should facilitate investigations into a wider range of dry and semi-dry tissues

Chapter 6 - General Discussion

6.1 - Purpose of this Study.

The primary aim of this thesis was to re-examine the ultrastructure of desiccation tolerant plant tissues, particularly in the dehydrated state. Changes in ultrastructure during dehydration and rehydration, and differences between the dehydrated and hydrated state have been used to develop theories about how different plants withstand extreme drying. It was thought that if these new techniques could overcome some or most of the known artefacts, improved preservation could shed light on the diversity of physiological mechanisms responsible for plant desiccation tolerance. Previous work also used a variety of plant tissues, fixed and processed in a variety of ways, making it hard to compare the different results. In this study, just one species was used, this allowed the direct comparison of results from each different fixation technique, thus enabling a more accurate assessment of preservation in each case.

6.2 - A Comparison of the Results from this Study with Previous Ultrastructural Observations of *Myrothamnus flabellifolia*.

6.2.1 - Hydrated Tissue.

The characteristic ultrastructure of hydrated *Myrothamnus flabellifolia* leaf tissue following aqueous chemical fixation (refer to Chapter 2, 2.16.1), phase-partition fixation (refer to Chapter 3, 3.6.1) or rapid cryofixation followed by freeze-substitution (refer to Chapter 4, 4.13.1) appeared no different to that reported previously. For example, mesophyll cells were vacuolate (Goldsworthy and Drennan 1991), chloroplasts were elongate in shape (Sherwin and Farrant 1996, Farrant *et al.* 1999) and exhibited the characteristic 'staircase' arrangement of the thylakoids (Wellburn and Wellburn 1976, Goldsworthy and Drennan 1991, Sherwin and Farrant 1996, Farrant *et al.* 1999). As noted earlier, the ultrastructure was also similar to that of other desiccation tolerant plants in the hydrated state (refer to Chapter 2, 2.1.1). Hence, good preservation of such material can be achieved using a wide variety of fixation and embedding protocols, unless they all suffer from a consistent artefact or set of artefacts.

6.2.2 - **Dehydrated Tissue.**

The similarity in findings between this dissertation and previous studies does not continue for descriptions of dehydrated *Myrothamnus flabellifolia* leaf tissue. The findings of this thesis revealed that *Myrothamnus flabellifolia* leaves retained a high degree of structural integrity in the dry state, especially those leaves fixed with anhydrous chemical fixatives (refer to Chapter 2, 2.18.1) or by cryofixation followed by freeze-substitution (refer to Chapter 4, 4.13.2). Goldsworthy and Drennan (1991) used vapour fixation to preserve *Myrothamnus flabellifolia* leaves and they reported similar findings. They postulated that the preservation of structural integrity in the dry state might enable a more rapid recovery during rehydration.

The main features considered to represent good quality preservation of *Myrothamnus flabellifolia* dry leaf tissue are as follows. Firstly, the maintenance of a tight cell wall-membrane association appears critical to desiccation tolerance, and this was seen consistently after freeze-substitution and also after phase-partition and some anhydrous methods. Vapour fixation also gives good results (Goldsworthy and Drennan 1991). However, aqueous fixation gave very poor results in this thesis, and poor preservation was also seen in Farrant *et al.* (1999). From these results, I conclude that it is highly likely that

most if not all, desiccation tolerant plants maintain a tight wall-membrane association when in the dry state. As a consequence, cell walls become highly folded in order to accommodate the irregular shape of the shrunken protoplast. This is contrary to the conclusions of previous workers. Hence, preservation techniques that produce wall swelling and separation from the plasma membrane are producing an artefact, and should be abandoned for dry tissue.

Secondly, during drying the chloroplasts become contracted with a regularly ruffled outer membrane. This was seen in this thesis after freeze-substitution, phase-partition and some anhydrous fixation protocols, and after vapour fixation by Goldsworthy and Drennan (1991). Almost all other work on *Myrothamnus flabellifolia* has reported rounded chloroplasts in dry tissue. I conclude that chloroplast swelling and rounding is an artefact of aqueous fixation, or of processing solvents that are taken up by dry tissues. The regular ruffles formed in the outer membrane are probably an efficient mechanism for membrane 'storage', this would accommodate chloroplast swelling during rehydration without the need for immediate synthesis or remobilization of membrane lipids and proteins. Membrane tubules have been seen coating organelles in dry seed tissues after vapour fixation (Öpik 1985), and he suggests that this may be a strategy for membrane storage that enables rapid seed swelling without membrane rupture upon rehydration. Any membrane rupture during rehydration

of seed or other tissues would potentially be very damaging, and it would be surprising if the tissues relied on repair of such damage rather then prevention of membrane rupture in the first place.

Thirdly, thylakoid membranes and grana structure remained intact and compressed with good preservation seen in this thesis and by Goldsworthy and Drennan (1991). In other work, thylakoids appeared swollen in tissue from *Myrothamnus flabellifolia* and numerous other species (Sherwin and Farrant 1996, Farrant *et al*.1999, Koonjul *et al*. 2000). I therefore conclude that it appears that at least some desiccation tolerant plants are able to maintain thylakoid integrity during dehydration and in the dry state, and propose that at least some of the observations of disrupted chloroplast structure could be artefactual. This is in contrast to many other previous generalizations about chloroplast behaviour and the maintenance of chloroplast integrity in dry tissues. However, to completely clarify the issue of chloroplast structure in the dry state more work needs to be done. The information gained from a comprehensive study of desiccation tolerant plants that both retain and lose their chlorophyll during dehydration, using the techniques of rapid cryofixation and freeze-substitution, would prove valuable for our future understanding of how desiccation affects the chloroplast.

In conclusion, from the evidence presented here and from other work, rapid cryofixation followed by freeze-

substitution is the best method to use with plant tissues at any stage of desiccation. Key to the successful use of this technique is patience, as considerably longer substitution and resin infiltration times are required than have been used by many in the past. Specific substitution fluids need to be tested on each species examined, to ensure swelling artefacts are kept to a minimum, and other fluids not tested in this thesis may also be useful, for example, diethyl ether and chloroform. It may also be useful to test the degree of swelling over longer times and at -82°C to faithfully represent tissue treatment during freeze-substitution. Other fixatives could also be tested in the substitution fluid, for example, acrolein. Finally, it may be worth testing whether completely anhydrous processing, into anhydrous Cl-free resin, followed by dry sectioning in a low humidity environment and viewing completely dry, improves tissue preservation. This has certainly proved useful for other critical applications, such as limiting redistribution of mobile ions during processing (Orlovich and Ashford 1995).

6.3 - How Do the Results From This Study Impact on the Current View of Desiccation Tolerance?

Early ultrastructural investigations of dry tissues regularly contained observations of cellular disruption and disorganization in desiccation tolerant plants (Hallam

1976, Gaff *et al*. 1976, Hallam and Gaff 1978, Bewley 1979, Oliver and Bewley 1984a, Schneider *et al*. 1993) and seeds (Webster and Leopold 1977, Morrison-Baird *et al*. 1979). These observations appeared to contradict several early theories on the mechanisms of desiccation tolerance, which emphasized the importance of maintaining cellular integrity in the dry state. For example, Iljin (1957) proposed that mechanical injury to protoplast membranes during drying and remoistening was the prime cause of desiccation sensitivity and if mechanical stress could be overcome then any cell could tolerate desiccation. Keilin (1959) also considered the retention of structural integrity as fundamental for survival of desiccation.

These theories were, for the most part, replaced by one put forward by Bewley (1979) (refer to Chapter 1, 1.1.1) in which he suggested that the maintenance of structural integrity during dehydration was only one component of desiccation tolerance. The maintenance physiological integrity in the dry state and the ability to repair damage upon rehydration were the other two factors Bewley proposed to be involved in the process of desiccation tolerance. Support for Bewley's theory came, in part, from ultrastructural observations of desiccation tolerant plants. When Bewley (1979) first proposed his hypothesis on desiccation tolerance, most ultrastructural studies utilized aqueous chemical fixation techniques. Evidence of ultrastructural damage in dry desiccation

tolerant tissues was prevalent in tissues fixed using these techniques. This made it difficult to distinguish between actual damage that was the result of the dehydration process and artefactual damage caused by the use of aqueous chemical fixation techniques (Sack *et al.* 1988, Oliver and Bewley 1997, Platt *et al.* 1997, Hoekstra *et al.* 1999).

The ability to determine what degree of damage occurs, if any, and when it occurs during dehydration and/or subsequent rehydration, is crucial to ascertain the importance of structural integrity in the dry state. The use of new fixation techniques, which minimize the production of rehydration related artefacts, allowed researchers to gain an understanding of how structural integrity is involved in desiccation tolerance. These techniques revealed that the ultrastructure of some desiccation tolerant biological materials is more intact in the dry state than previously thought. Some of the first results showing that structural integrity was maintained during dehydration were in seed tissues fixed in osmium vapour fixation (Buttrose 1973, Thomson 1979, Öpik 1980, 1985) or freeze-fractured (Chabot and Leopold 1982, Bliss *et al.* 1984, Vigil *et al.* 1985). Subsequent research, using osmotically neutral fixation techniques, also demonstrated a high level of structural integrity in the vegetative tissues of some desiccation tolerant plants during dehydration (Honegger 1995, Honegger *et al.* 1996, Thomson and Platt 1997, Platt *et al.* 1997). The results of

this thesis (refer to Chapter 4, 4.3.1, 4.13.2 and 4.18 - acetone substituted tissue), add further support to the theory that ultrastructural integrity is generally maintained in desiccation tolerant plants during dehydration.

There is also some evidence that the maintenance of cellular integrity is essential for desiccation tolerance. For example, Giles *et al.* (1974) reported that mesophyll cells of *Zea mays*, in which the tonoplast remained intact during dehydration, had no lasting structural damage whereas those cells in which the tonoplast was disrupted showed no signs of recovery. From this finding they proposed that the maintenance of tonoplast integrity could be crucial for mesophyll cells to withstand water stress. Ristic and Cass (1991) found that although water stress affected both drought resistant and drought sensitive maize lines, there were obvious differences in chloroplast structure between these lines, with chloroplasts in the drought resistant line being less affected. Similarly, Vieira da Silva (1976) found that modifications in cellular ultrastructure including the cytoplasm, chloroplasts and mitochondria were more obvious in the drought-sensitive *Gossypium hirsutum* than in the drought-resistant *Gossypium arientinum* (Vieira da Silva 1976). Finally, Tetteroo *et al.* (1996) concluded that the integrity of the plasma membrane has to be preserved for carrot somatic embryos to survive dehydration to low moisture contents. These

findings add further weight to the idea that the maintenance of structural integrity in the dehydrated state is essential for desiccation tolerance.

Another current hypothesis is that higher plants rely mainly on cellular protection for desiccation tolerance whereas lower order plants rely on cellular repair (refer to Chapter 1, 1.1.1). The concept of cellular repair during rehydration implies that cellular disruption occurs during dehydration. However, the seedless vascular plant *Selaginella lepidophylla* showed good ultrastructural integrity in the dry state after rapid cryofixation and freeze-substitution (Thomson and Platt 1997, Platt *et al.* 1997) as also seen in *Myrothamnus flabellifolia*. Therefore, even though there are other differences in desiccation tolerance strategies between higher and lower plants (refer to Chapter 1, 1.2) it would appear advantageous for all such plants to retain as much structural integrity as possible during dehydration. Possible benefits of this strategy include an increased recovery rate and/or reduced energy expenditure to fuel the rehydration process.

The evidence outlined above together with the ultrastructural findings reported in this thesis indicate that, contrary to previous opinion, ultrastructural integrity is routinely maintained in desiccation tolerant plants during dehydration and in the desiccated state. Support for this proposal requires re-examination of

ultrastructure in other higher and lower plants using improved techniques, especially rapid cryofixation and freeze-substitution. Rather than trying to elucidate rehydration repair mechanisms, a fruitful area of research would focus on understanding how these plants maintain cell structure during severe drying. These mechanisms are likely to be similar to those in other desiccation tolerant structures, such as seeds and pollen: for example vitrification of the cytoplasm (refer to Chapter 1, 1.2.2.4).

6.4 - Other Possible Fixation Methods Suitable For Use With Dehydrated Biological Material.

Over and above the various fixation methods described in this dissertation, there are other fixation methods described in the literature, which may prove suitable for use on desiccation tolerant plant material.

One such fixation method is high-pressure rapid cryofixation. High-pressure rapid cryofixation is similar to the rapid cryofixation technique described in Chapter 4 (refer to 4.1) except that the tissue is frozen at pressures up to 2500 bar (Studer *et al.* 1992), rather than being plunged into the cryogen at ambient pressure. High-pressure freezing enables good ultrastructural preservation to depths greater than 20-40 μm as it inhibits the expansion of water during freezing which hinders the

production of ice crystals and it also reduces the cooling rate required for satisfactory freezing (Dahl and Staehelin 1989). It has been claimed that high-pressure freezing results in improved ultrastructural preservation of both plant and animal tissues (Studer *et al.* 1992, Thijssen *et al.* 1997). Therefore, high-pressure cryofixation would be a valuable technique to test on desiccation tolerant plant material. It should be noted however, that there are reports of high-pressure freezing causing the disruption of cellular structures. For example, Hyde *et al.* (1991a) noted that high-pressure freezing led to the degradation of large peripheral vesicles in the sporangia of *Phytophthora*. Many of the artefacts associated with high pressure freezing have been associated with fluid membranes in 'wet' tissues so the membranes in dry tissue, which are no longer fluid, may not be affected by the use of this technique.

Another fixation technique that may prove suitable for use on desiccation tolerant plant tissues is one recently published by Grote *et al.* (1999) who fixed dry bean seeds (*Phaseolus vulgaris*) in acrolein vapour. After fixation, the samples were dehydrated in DMP and embedded in Lowicryl K4M resin. All stages of processing were conducted at room temperature until resin polymerization when the temperature was lowered to -35°C. Grote *et al.* (1999) concluded that using this technique the ultrastructural preservation was excellent for both desiccated cells and organelles. They

also reported that large sections were routinely obtained from easily sectionable tissue blocks.

6.5 - Future Research Prospects.

This dissertation is one of the most comprehensive studies of *Myrothamnus flabellifolia* leaf ultrastructure to date and the results obtained have greatly contributed to our understanding of the ultrastructure of desiccation tolerant plants during dehydration. Further work is needed to validate the findings and claims of this study, and to further advance our understanding of the mechanisms of desiccation tolerance, some possible avenues of research are outlined below.

6.5.1 - Is Structural Integrity Maintained During Dehydration in Other Desiccation Tolerant Plants?

As noted in other chapters, perhaps the most beneficial experiment would be to test the fixation techniques used here on a selection of desiccation tolerant plants encompassing the range of orders across the plant kingdom. This would indicate whether maintenance of ultrastructural integrity is a necessary component of desiccation tolerance in all species. It would be particularly useful to compare closely related species,

such as the desiccation tolerant *Sporobolus stapfianus* and the desiccation sensitive *Sporobolus pyramidalis*.

These experiments may also help to identify genuine ultrastructural changes. Previously it was difficult to distinguish desiccation-induced changes from rehydration-induced cellular damage. The identification of genuine changes will help to elucidate the processes of desiccation tolerance. Organelle de-differentiation is one phenomenon that could be clarified. This has been observed in desiccation tolerant vegetative tissues and seeds, but interpreted in two different ways. In vegetative tissues, it has been said to indicate damage during dehydration (Gaff *et al.* 1976, Bewley and Pacey 1978, Bewley *et al.* 1978, Bewley 1979, Hallam and Luff 1980a, b), but in seed tissues it was thought to be intrinsic to desiccation tolerance (Vertucci and Farrant 1995, Pammenter and Berjak 1999). In the absence of rehydration-induced cellular changes, organelle de-differentiation may, instead, be considered part of a controlled and programmed ultrastructural change, central to desiccation tolerance in vegetative tissues.

6.5.2 - Localization of Molecular Changes.

Improved fixation techniques will also benefit researchers in other fields such as molecular biology. To date, the primary goal of molecular research with respect

to desiccation tolerance, has been the identification of the numerous genes and gene products that respond to water stress. A number of studies have produced lists of genes and/or gene products that respond to desiccation (Scherer and Potts 1989, Bartels *et al.* 1990, Oliver 1991, Reynolds and Bewley 1993a, Hellewege *et al.* 1994, Kuang *et al.* 1995, Bockel *et al.* 1998, Wood *et al.* 1999, Machuka *et al.* 1999) however, even though a gene has been isolated and characterized, its function is often not fully determined. Of the many genes that have been identified as responding to desiccation it is possible that only some will be directly involved with the induction of desiccation tolerance, other genes may only be involved in secondary reactions or they may not be involved at all.

It is only quite recently that researchers have begun to try to elucidate some of the functions of these genes, with the majority of work done on *Craterostigma plantagineum* (Furini *et al.* 1994, Alamillo *et al.* 1995, Bernacchia *et al.* 1995, Ingram *et al.* 1997, Furini *et al.* 1997, Heino *et al.* 1998) and a small amount of work on *Sporobolus stapfianus* (Blomstedt *et al.* 1998). Clues to the function of a gene may be obtained by identifying the timing of expression, and its location at the organ, cellular and sub-cellular levels (Bray 1994). Successful localization of gene expression will depend on improved fixation protocols like those described in this thesis. Techniques such as *in situ* hybridization,

immunocytochemistry and tissue printing are used to determine the specific location of transcripts or proteins in particular tissues, cells or organelles. Some research in this area has been conducted on the desiccation tolerant plant *Craterostigma plantagineum*. Research has included investigations into a desiccation and ABA-responsive promoter (Michel *et al.* 1993), the increases in mRNA and enzyme activity levels of cytosolic GAPDH (Velasco *et al.* 1998), and the isolation and expression analysis of two stress-responsive sucrose-synthase genes (Kleines *et al.* 1999). Accurate ultrastructural preservation is crucial here, as ultrastructural artefacts can affect the localization of various protein antigens or transcripts.

6.5.3 - Cellular Mechanisms Involved in Maintaining Structural Integrity.

Further investigation is needed into mechanisms and cell processes that both maintain structural integrity during dehydration and help regain cell structure during rehydration. One cell component that may be involved in this process is the cytoskeleton, which in plants comprises actin filaments and microtubules. These dynamic structures are composed of monomers of actin and tubulin, respectively, and are involved in maintaining organelle positioning, moving organelles within the cell, cell shape and cell division. The cytoskeleton also responds to a

wide range of external and internal stimuli, including light (Nick *et al.* 1991, Han *et al.* 1991, Eun and Lee 1997), gravity (Nick *et al.* 1991, Baluška and Hasenstein 1997), wounding (Hush *et al.* 1990, Foissner and Wasteneys 1994), temperature (Bartolo and Carter 1991, Aström *et al.* 1991, Chu *et al.* 1993), electrical fields and mechanical force (Hush and Overall 1991), fungal attack (Kobayashi *et al.* 1992, 1994, Gross *et al.* 1993, Škalamera and Heath 1998) and various plant hormones (Shibaoka 1991).

There is some evidence that the cytoskeleton also changes during desiccation. In desiccation tolerant tissues, tubulin levels generally drop with the onset of desiccation (de Castro *et al.* 1995, Bino *et al.* 1996, Górnik *et al.* 1997, Portis *et al.* 1999) whereas actin levels were shown to remain unchanged (Kuang 1995). In desiccation sensitive tissues however, actin levels were found to decrease with dehydration (Creelman and Mullet 1991, Kuang 1995) and tubulin levels have been observed to either decrease (Creelman and Mullet 1991) or remain relatively constant (Kuang 1995).

Visualization of the cytoskeleton during dehydration has been attempted in some plant species. Bartolo and Carter (1991) found that there was a depolymerization of cortical microtubules in spinach mesophyll cells during dehydration and Pammenter and Berjak (1999) discovered that the extensive rays of actin and microtubule filaments are

dismantled during dehydration of the desiccation sensitive *Quercus robur* seeds.

It is not known why the cytoskeleton appears to respond to desiccation nor whether these responses are direct or indirect responses to desiccation. Tiwari and Polito (1988) investigated the spatial organization of actin during hydration, activation and germination of pollen in *Pyrus communis* and observed a rapid reorganization of actin during the first few minutes of hydration and activation. They claimed that technical difficulties prevented them from investigating actin in dry pollen, but they did not elaborate as to what these difficulties were. It is likely though, that their use of an aqueous fixative prevented them from successfully observing actin in dry pollen. Their admission that hydrated pollen grains had probably undergone the first stages of activation whilst in the fixative supports this hypothesis.

The evidence presented above supports the hypothesis that the cytoskeleton in desiccation tolerant tissues may respond to dehydration and is illustrative of how the research contained in this thesis will enable the pursuit of numerous other areas of research.

6.6 - Conclusions.

Results contained in this dissertation demonstrate that fixation techniques can have a dramatic impact on the ultrastructure of *Myrothamnus flabellifolia* leaf tissue, particularly when dehydrated. Aqueous chemical techniques were found to give the worst preservation of dehydrated leaf tissues. Anhydrous chemical fixatives and phase-partition fixation gave somewhat improved preservation, but rapid cryofixation followed by freeze-substitution was the most successful fixation technique. These improved methods, combined with new molecular and physiological information, will begin to shed further light on the processes underlying desiccation tolerance in plants.

APPENDIX - A.

Dehydration Rate of Detached Shoots of *Myrothamnus flabellifolia* (Fig. 6).

			initial weight (g)	hydrated weight (g)	dehydrated weight (g)	RWC
Day 1	**9:00 AM**	replicate 1	0.022	0.0239	0.0064	90.30%
		replicate 2	0.0113	0.0119	0.0038	92.60%
	9:00 PM	replicate 1	0.0152	0.0228	0.0059	55%
		replicate 2	0.0091	0.0122	0.0035	64%
Day 2	**9:00 AM**	replicate 1	0.0068	0.0197	0.0045	15.10%
		replicate 2	0.0024	0.0045	0.0013	34.40%
	9:00 PM	replicate 1	0.0063	0.0212	0.0056	4.50%
		replicate 2	0.003	0.0089	0.0025	7.80%
Day 3	**9:00 AM**	replicate 1	0.0031	0.0114	0.0026	5.70%
		replicate 2	0.0025	0.0081	0.0022	5.10%
	9:00 PM	replicate 1	0.0037	0.0138	0.0034	2.90%
		replicate 2	0.003	0.0092	0.0028	3.10%
Day 4	**9:00 AM**	replicate 1	0.0023	0.0085	0.0021	3.10%
		replicate 2	0.0029	0.0091	0.0026	4.80%
	9:00 PM	replicate 1	0.0017	0.0061	0.0015	4.30%
		replicate 2	0.0045	0.0132	0.0036	9.40%
Day 5	**9:00 AM**	replicate 1	0.0024	0.0089	0.002	5.80%
		replicate 2	0.0013	0.0038	0.0011	7.40%
	9:00 PM	replicate 1	0.0013	0.0037	0.0009	14.30%
		replicate 2	0.0024	0.0077	0.0022	3.60%
Day 6	**9:00 AM**	replicate 1	0.003	0.0096	0.0025	7%
		replicate 2	0.0015	0.0052	0.0013	5.10%
	9:00 PM	replicate 1	0.0046	0.0161	0.0041	4.20%
		replicate 2	0.0023	0.0078	0.0019	6.80%
Day 7	**9:00 AM**	replicate 1	0.0015	0.0056	0.0013	4.70%
		replicate 2	0.0016	0.0051	0.0014	5.40%
	9:00 PM	replicate 1	0.0012	0.0041	0.0009	9.40%
		replicate 2	0.0025	0.0075	0.0022	5.70%

APPENDIX - B.

Dehydration Rate of Detached Shoots of *Myrothamnus flabellifolia* (Fig. 7).

			initial weight (g)	hydrated weight (g)	dehydrated weight (g)	RWC
Day 1	**6:00 AM**	replicate 1	0.0139	0.015	0.0048	89.20%
		replicate 2	0.0066	0.0068	0.0023	95.60%
		replicate 3	0.0058	0.0063	0.0019	88.60%
	10:00 AM	replicate 1	0.0076	0.0095	0.0029	71.20%
		replicate 2	0.0091	0.0111	0.0034	74.10%
		replicate 3	0.0022	0.0025	0.0008	82.40%
	2:00 PM	replicate 1	0.0026	0.004	0.0011	51.70%
		replicate 2	0.0046	0.0066	0.0019	57.50%
		replicate 3	0.0138	0.0188	0.0058	61.50%
	6:00 PM	replicate 1	0.0027	0.0046	0.0014	40.60%
		replicate 2	0.0014	0.0024	0.0006	44.40%
		replicate 3	0.0126	0.0199	0.0061	47.10%
	10:00 PM	replicate 1	0.0027	0.0063	0.0019	18.20%
		replicate 2	0.0017	0.0043	0.0012	16.10%
		replicate 3	0.0075	0.0149	0.0049	26%
Day 2	**6:00 AM**	replicate 1	0.0028	0.0071	0.0021	14%
		replicate 2	0.0043	0.0088	0.0026	27.40%
		replicate 3	0.0021	0.0064	0.0019	4.40%
	10:00 AM	replicate 1	0.0026	0.0072	0.0022	8%
		replicate 2	0.0035	0.0111	0.0034	1.30%
		replicate 3	0.002	0.0052	0.0015	13.50%
	2:00 PM	replicate 1	0.0043	0.012	0.0034	10.50%
		replicate 2	0.0019	0.0062	0.0019	0%
		replicate 3	0.0019	0.006	0.0018	2.40%
	6:00 PM	replicate 1	0.0025	0.0073	0.0023	3.60%
		replicate 2	0.0017	0.0054	0.0017	0%
		replicate 3	0.0007	0.0022	0.0006	6.30%
	10:00 PM	replicate 1	0.0014	0.0045	0.0013	3.10%
		replicate 2	0.0052	0.0159	0.0049	2.70%
		replicate 3	0.0015	0.0053	0.0016	2.70%

APPENDIX - C.

Weight Gains of Dehydrated *Myrothamnus flabellifolia* Leaves Immersed in Various Solutions over Time.

water (Figs. 11 & 12).

	weight (g) at: initial	30 min	2 hr	21 hr
replicate 1	0.0054	0.0067	0.0091	0.015
replicate 2	0.0042	0.0052	0.007	0.0114
replicate 3	0.004	0.0049	0.0065	0.0108
replicate 4	0.0044	0.0057	0.0081	0.0136
replicate 5	0.0048	0.0058	0.0076	0.0132
replicate 6	0.0056	0.0068	0.0094	0.0155
replicate 7	0.0049	0.006	0.0084	0.0103
replicate 8	0.0062	0.0075	0.0095	0.0174
replicate 9	0.0058	0.0072	0.0097	0.0168
replicate 10	0.0051	0.0064	0.0087	0.0143
	percentage increase in weight at: initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0	19.4030	26.37363	39.33333
replicate 2	0	19.2308	25.714285	38.59649
replicate 3	0	18.3673	24.615385	39.814815
replicate 4	0	22.8070	29.629629	40.411765
replicate 5	0	17.2414	23.684211	42.42424
replicate 6	0	17.6471	27.659574	39.354838
replicate 7	0	18.3333	28.571428	18.446602
replicate 8	0	17.3333	21.052632	45.402298
replicate 9	0	19.4444	25.773196	42.261905
replicate 10	0	20.3125	26.43678	39.160839
	percentage increase in leaf weight per hour. initial	**30 min**	**2 hr**	**21 hour**
replicate 1	0	38.806	13.186815	1.8730157
replicate 2	0	38.4616	12.857145	1.837928
replicate 3	0	36.7346	12.307695	1.895944
replicate 4	0	45.614	14.814815	1.92437
replicate 5	0	34.4828	11.842105	2.020202
replicate 6	0	35.2942	13.829785	1.87404
replicate 7	0	36.66667	14.285715	0.8793333
replicate 8	0	34.666667	10.526315	2.1620143
replicate 9	0	38.8888	12.8866	2.0124719
replicate 10	0	40.625	13.21839	1.8648019

Karnovsky's fixative (Figs. 11 & 12).

	weight (g) at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0.0037	0.004	0.0043	0.0065
replicate 2	0.0034	0.0039	0.0045	0.0066
replicate 3	0.0042	0.0048	0.0058	0.008
replicate 4	0.0034	0.0039	0.0044	0.0066
replicate 5	0.005	0.0058	0.0067	0.0103
replicate 6	0.0033	0.0036	0.004	0.0064
replicate 7	0.0043	0.005	0.0056	0.0086
replicate 8	0.0036	0.004	0.0045	0.0073
replicate 9	0.0039	0.0043	0.0044	0.0067
replicate 10	0.0029	0.0036	0.0039	0.0058
	percentage increase in weight at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0	7.5	6.976744	33.84615
replicate 2	0	12.82051	13.3333	31.81818
replicate 3	0	12.5	17.241379	27.5
replicate 4	0	12.820513	11.363636	33.3333
replicate 5	0	13.793103	13.43284	34.951456
replicate 6	0	8.3333	10	37.5
replicate 7	0	14	10.714286	34.88372
replicate 8	0	10	11.11111	38.3562
replicate 9	0	9.302326	2.272727	34.32836
replicate 10	0	19.444444	7.692308	32.75862
	percentage increase in leaf weight per hour.			
	initial	**30 min**	**2 hr**	**21 hour**
replicate 1	0	15	3.488372	1.611721429
replicate 2	0	25.641	6.6665	1.5151429
replicate 3	0	25	8.62069	1.30952381
replicate 4	0	25.641	5.68182	1.5873
replicate 5	0	27.5862	6.71642	1.6643552
replicate 6	0	16.6666	5	1.7857142
replicate 7	0	28	5.357145	1.6611295
replicate 8	0	20	5.55555	1.8264857
replicate 9	0	18.6046	1.1363635	1.6346838
replicate 10	0	38.888	3.846154	1.55993428

5% glutaraldehyde (Figs. 11 & 12).

	weight (g) at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0.0041	0.0046	0.0052	0.0091
replicate 2	0.0043	0.0048	0.0055	0.0096
replicate 3	0.0046	0.005	0.0056	0.0084
replicate 4	0.0055	0.0058	0.0064	0.0099
replicate 5	0.0054	0.0058	0.0062	0.0099
replicate 6	0.0047	0.0051	0.0056	0.0083
replicate 7	0.0047	0.0052	0.0059	0.0093
replicate 8	0.0069	0.0073	0.0081	0.0125
replicate 9	0.0052	0.0056	0.0062	0.0111
replicate 10	0.0053	0.0059	0.0067	0.011
	percentage increase in weight at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0	10.86	11.53846	42.857143
replicate 2	0	10.41667	12.72727	42.70833
replicate 3	0	8	10.714286	33.3333
replicate 4	0	5.172414	9.375	35.3535
replicate 5	0	6.896552	6.451613	37.37374
replicate 6	0	7.843137	8.92857	32.53012
replicate 7	0	9.61538	11.864405	36.55912
replicate 8	0	5.479452	9.876534	35.2
replicate 9	0	7.142857	9.677419	44.144144
replicate 10	0	10.16	11.940298	39.09091
	percentage increase in leaf weight per hour.			
	initial	**30 min**	**2 hr**	**21 hour**
replicate 1	0	21.72	5.76923	2.04081619
replicate 2	0	20.8334	6.363635	2.03373
replicate 3	0	16	5.357145	1.5873
replicate 4	0	10.3448	4.6875	1.6835
replicate 5	0	13.7932	3.2258065	1.7797019
replicate 6	0	15.6862	4.464285	1.54905333
replicate 7	0	19.2308	5.932205	1.740910476
replicate 8	0	10.959	4.938267	1.676190476
replicate 9	0	14.2858	4.8387095	2.102101905
replicate 10	0	20.32	5.97015	1.8614719

FC-72 (Figs. 21 & 22).

	weight (g) at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0.0027	0.0028	0.0028	0.0029
replicate 2	0.0064	0.0074	0.0077	0.0081
replicate 3	0.0041	0.0045	0.0049	0.0052
replicate 4	0.0039	0.0041	0.0044	0.0046
replicate 5	0.0053	0.006	0.0062	0.0066
replicate 6	0.0051	0.0059	0.0062	0.0066
replicate 7	0.0047	0.0054	0.0059	0.0063
replicate 8	0.0051	0.0055	0.0058	0.0061
replicate 9	0.003	0.0032	0.0034	0.0035
replicate 10	0.0033	0.0034	0.0034	0.0035
	percentage increase in weight at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0	3.5714	0	3.4482759
replicate 2	0	13.5135	3.896104	4.938272
replicate 3	0	8.8889	8.163265	5.769231
replicate 4	0	4.8780	6.8181818	4.347826
replicate 5	0	11.6667	3.225806	6.060606
replicate 6	0	13.5593	4.838709	6.060606
replicate 7	0	12.9630	8.474576	6.349206
replicate 8	0	7.2727	5.1724138	4.918032
replicate 9	0	6.2500	5.882353	2.857143
replicate 10	0	2.9412	0	2.8571428
	percentage increase in leaf weight per hour.			
	initial	**30 min**	**2 hr**	**21 hour**
replicate 1	0	3.5714	0	0.164204
replicate 2	0	27.027	1.948052	0.7113463
replicate 3	0	17.7778	4.0816325	0.5000003
replicate 4	0	9.756	3.409091	0.2070393
replicate 5	0	23.3334	1.612903	0.2886003
replicate 6	0	27.1186	2.4193545	0.2886003
replicate 7	0	25.926	4.237288	0.302343
replicate 8	0	14.5454	2.586207	0.234192
replicate 9	0	12.5	2.9411765	0.1360544
replicate 10	0	5.8824	0	0.1360544

n-heptane (Figs. 21 & 22).

	weight (g) at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0.005	0.0056	0.0058	0.006
replicate 2	0.0043	0.0048	0.0048	0.005
replicate 3	0.0051	0.0055	0.0055	0.0059
replicate 4	0.0026	0.0027	0.0028	0.0029
replicate 5	0.0045	0.005	0.005	0.0052
replicate 6	0.003	0.0032	0.0032	0.0034
replicate 7	0.0056	0.0058	0.0059	0.006
replicate 8	0.005	0.0052	0.0053	0.0054
replicate 9	0.0045	0.0049	0.0049	0.0051
replicate 10	0.0056	0.0061	0.0062	0.0062
	percentage increase in weight at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0	10.714286	3.448276	3.33333
replicate 2	0	10.416667	0	4
replicate 3	0	7.272727	0	6.77966
replicate 4	0	3.703703	3.571429	3.448276
replicate 5	0	10	0	3.846154
replicate 6	0	6.25	0	5.88235
replicate 7	0	3.448276	1.694915	1.66667
replicate 8	0	3.846154	1.886792	1.851852
replicate 9	0	8.163265	0	3.921569
replicate 10	0	8.1967213	1.612903	0
	percentage increase in leaf weight per hour.			
	initial	**30 min**	**2 hr**	**21 hour**
replicate 1	0	21.4286	1.724138	0.15872857
replicate 2	0	20.8334	0	0.19047619
replicate 3	0	14.5454	0	3.38983
replicate 4	0	7.4074	1.7857145	0.164203619
replicate 5	0	20	0	0.18315019
replicate 6	0	12.5	0	0.280111904
replicate 7	0	6.8966	0.847475	0.0793666
replicate 8	0	7.6924	0.943396	0.0881834
replicate 9	0	16.3266	0	0.18674138
replicate 10	0	16.3934	0.8064515	0

acetone (Figs. 48 & 49).

	weight (g) at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0.0027	0.0031	0.0033	0.0032
replicate 2	0.0041	0.0045	0.0045	0.0046
replicate 3	0.0026	0.0031	0.0031	0.003
replicate 4	0.003	0.0032	0.0034	0.0034
replicate 5	0.0049	0.0053	0.0055	0.0058
replicate 6	0.0049	0.0051	0.0054	0.0058
replicate 7	0.0046	0.0048	0.0049	0.005
replicate 8	0.0035	0.0037	0.0039	0.0042
replicate 9	0.0049	0.0052	0.0053	0.0057
replicate 10	0.0082	0.0086	0.0092	0.0098
	percentage increase in weight at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0	12.903226	6.060606	-3.125
replicate 2	0	8.888889	0	2.173913
replicate 3	0	16.129032	0	-3.33333
replicate 4	0	6.25	5.88235	0
replicate 5	0	7.547169	3.63636	5.172414
replicate 6	0	3.921568	5.55556	6.896552
replicate 7	0	4.16667	2.040816	2
replicate 8	0	5.405405	5.1282051	7.14285714
replicate 9	0	5.769231	1.8867925	7.017544
replicate 10	0	4.651163	6.521739	6.122449
	percentage increase in leaf weight per hour.			
	initial	**30 min**	**2 hr**	**21 hour**
replicate 1	0	25.8064	3.030303	-0.1488095
replicate 2	0	17.7778	0	0.10351966
replicate 3	0	32.258	0	-0.158728
replicate 4	0	12.5	2.941175	0
replicate 5	0	15.0944	1.81818	0.2463054
replicate 6	0	7.8432	2.77778	0.3284072
replicate 7	0	7.8432	1.020408	0.095238
replicate 8	0	10.8108	2.5641025	0.340136047
replicate 9	0	11.5384	3.773586	0.3341687
replicate 10	0	9.3024	3.2608695	0.29154519

methanol (Figs. 48 & 49).

	weight (g) at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0.0019	0.0035	0.004	0.0037
replicate 2	0.0029	0.0047	0.0062	0.0056
replicate 3	0.0047	0.0099	0.0109	0.01
replicate 4	0.0028	0.0051	0.0057	0.0052
replicate 5	0.0025	0.0052	0.0058	0.0053
replicate 6	0.006	0.01	0.0131	0.0126
replicate 7	0.0055	0.0084	0.0124	0.012
replicate 8	0.0036	0.0053	0.0078	0.0074
replicate 9	0.0067	0.0113	0.0146	0.0139
replicate 10	0.0054	0.0085	0.0112	0.0138
	percentage increase in weight at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0	45.714286	12.5	-8.10811
replicate 2	0	38.29787	24.19355	-10.71429
replicate 3	0	52.52525	9.1743119	-9
replicate 4	0	45.09804	10.52632	-9.61538
replicate 5	0	51.923077	10.34483	-9.43396
replicate 6	0	40	23.66412	-3.96825
replicate 7	0	34.52381	32.25806	-3.33333
replicate 8	0	32.07547	32.05128	-5.40541
replicate 9	0	40.70796	22.60274	-5.03597
replicate 10	0	36.47059	24.107143	18.84058
	percentage increase in leaf weight per hour.			
	initial	**30 min**	**2 hr**	**21 hour**
replicate 1	0	91.4286	6.25	-4.054055
replicate 2	0	76.5958	12.096775	-0.51020476
replicate 3	0	105.0506	4.587156	-0.42857143
replicate 4	0	90.196	5.26316	-0.45787524
replicate 5	0	103.8462	5.172415	-0.449236
replicate 6	0	80	11.83206	-0.18896429
replicate 7	0	69.0476	16.12903	-0.15873014
replicate 8	0	64.151	16.02564	-0.25740048
replicate 9	0	81.416	11.30137	-0.2398081
replicate 10	0	72.9412	12.05357	0.897170476

ethanol (Figs. 48 & 49).

	weight (g) at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0.0026	0.0029	0.003	0.0031
replicate 2	0.0023	0.0028	0.003	0.0031
replicate 3	0.0029	0.0029	0.003	0.0033
replicate 4	0.003	0.0034	0.0037	0.0038
replicate 5	0.0044	0.0051	0.0053	0.0055
replicate 6	0.0049	0.0054	0.0061	0.0064
replicate 7	0.0062	0.0075	0.0086	0.009
replicate 8	0.0061	0.0069	0.0084	0.009
replicate 9	0.0061	0.0065	0.0074	0.0078
replicate 10	0.0051	0.0053	0.006	0.0063
	percentage increase in weight at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0	10.344828	3.33333	3.225806
replicate 2	0	17.857143	6.66667	3.225806
replicate 3	0	0	3.333333	9.090909
replicate 4	0	11.764705	8.108108	2.631579
replicate 5	0	13.72549	3.773585	3.63636
replicate 6	0	9.2592593	11.475409	4.6875
replicate 7	0	17.33333	12.79069	4.444445
replicate 8	0	11.594203	17.857143	6.66667
replicate 9	0	6.153846	12.162162	5.128205
replicate 10	0	3.773585	11.66667	4.761095
	percentage increase in leaf weight per hour.			
	initial	**30 min**	**2 hr**	**21 hour**
replicate 1	0	20.6896	1.66665	0.1536098
replicate 2	0	35.7142	3.33335	0.1536098
replicate 3	0	0	1.6665	0.432900428
replicate 4	0	23.5294	4.054054	0.125313285
replicate 5	0	27.451	1.8867925	0.17316
replicate 6	0	18.5186	5.737705	0.223214285
replicate 7	0	34.6666	6.395345	0.211640238
replicate 8	0	23.1884	8.92857	0.317460333
replicate 9	0	12.3076	6.08108	0.244200238
replicate 10	0	7.5472	5.833335	0.226718809

acetone and DMP (Fig. 50).

	weight (g) at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0.0036	0.0039	0.0041	0.0042
replicate 2	0.0053	0.0055	0.0057	0.0079
replicate 3	0.0042	0.0046	0.0048	0.0047
replicate 4	0.0049	0.006	0.006	0.0061
replicate 5	0.003	0.0032	0.0031	0.0034
replicate 6	0.0036	0.004	0.0042	0.0046
replicate 7	0.0053	0.006	0.0065	0.0073
replicate 8	0.0046	0.0048	0.0056	0.0063
replicate 9	0.0039	0.0042	0.0044	0.0038
replicate 10	0.004	0.004	0.0043	0.0047
	percentage increase in weight at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0	7.6923077	4.878048	2.380952
replicate 2	0	3.636363	3.508772	27.848101
replicate 3	0	8.6956522	4.16667	-2.12766
replicate 4	0	18.33333	0	1.639344
replicate 5	0	6.25	-3.22581	8.823529
replicate 6	0	10	4.761905	8.695652
replicate 7	0	11.66667	7.692307	10.958904
replicate 8	0	4.16667	14.285714	11.11111
replicate 9	0	7.142857	4.54545	-15.78947
replicate 10	0	0	6.97674	8.510638

methanol and DMP (Fig. 50).

	weight (g) at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0.0047	0.0097	0.0106	0.01
replicate 2	0.0039	0.008	0.0084	0.0076
replicate 3	0.003	0.0064	0.0068	0.0062
replicate 4	0.0022	0.0042	0.0045	0.0045
replicate 5	0.0021	0.0044	0.0046	0.004
replicate 6	0.0085	0.0147	0.0173	0.0168
replicate 7	0.0079	0.0117	0.0159	0.0172
replicate 8	0.0081	0.0141	0.0173	0.0169
replicate 9	0.0052	0.0084	0.0101	0.0103
replicate 10	0.0051	0.0066	0.0094	0.0088
	percentage increase in weight at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0	51.5463917	8.490566	-6
replicate 2	0	51.25	4.761905	-10.52632
replicate 3	0	53.125	5.882353	-9.677419
replicate 4	0	47.61905	6.6667	0
replicate 5	0	52.272727	4.347826	-15
replicate 6	0	42.176871	15.028902	-2.97619
replicate 7	0	32.478632	26.41509	7.558139
replicate 8	0	42.55319	18.497109	-2.36686
replicate 9	0	38.095238	16.83168	1.9417476
replicate 10	0	22.72727	29.78723	-6.8182

ethanol and DMP (Fig. 50).

	weight (g) at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0.0027	0.0031	0.0034	0.0042
replicate 2	0.0032	0.0041	0.0043	0.0055
replicate 3	0.0028	0.0033	0.0036	0.0038
replicate 4	0.0038	0.0045	0.0051	0.0053
replicate 5	0.0036	0.0041	0.0042	0.0047
replicate 6	0.0041	0.0045	0.0049	0.0055
replicate 7	0.0052	0.0059	0.0067	0.0081
replicate 8	0.0051	0.0063	0.0071	0.0087
replicate 9	0.0038	0.0042	0.0045	0.0053
replicate 10	0.0049	0.0055	0.0061	0.0068
	percentage increase in weight at:			
	initial	**30 min**	**2 hr**	**21 hr**
replicate 1	0	12.903226	8.823529	19.04762
replicate 2	0	21.951219	4.651163	21.81818
replicate 3	0	15.151515	8.3333	5.263157
replicate 4	0	15.55556	11.764706	3.773585
replicate 5	0	12.1951219	2.380952	10.63829
replicate 6	0	8.888889	8.163265	10.90909
replicate 7	0	11.864406	11.94029	17.28395
replicate 8	0	19.047619	11.267606	18.390804
replicate 9	0	9.523809	6.66667	15.09434
replicate 10	0	10.90909	9.836065	10.294117

APPENDIX - D.

Percentage Increase In Weight of Dehydrated *Myrothamnus flabellifolia* Leaves Immersed in Solutions Containing a Solvent and Decreasing Amounts of Water from 100% Water to 100% Solvent (Fig. 15).

	initial weight (g)	hydrated weight (g)	% weight increase		initial weight (g)	hydrated weight (g)	% weight increase
water				**5% acetone**			
replicate 1	0.0052	0.0137	163.461539	replicate 1	0.0022	0.0068	209.090909
replicate 2	0.0072	0.0209	190.277778	replicate 2	0.006	0.0173	188.333333
replicate 3	0.0048	0.0133	177.083333	replicate 3	0.0045	0.0122	171.111111
replicate 4	0.004	0.013	225	replicate 4	0.0054	0.0152	181.481482
replicate 5	0.0058	0.018	210.344828	replicate 5	0.0048	0.015	212.5
replicate 6	0.0054	0.0085	57.4074074	replicate 6	0.0066	0.0183	177.272727
replicate 7	0.0042	0.0119	183.333333	replicate 7	0.0046	0.0132	186.956522
replicate 8	0.0102	0.0279	173.529412	replicate 8	0.0093	0.0258	177.419355
replicate 9	0.0049	0.0128	161.22449	replicate 9	0.0052	0.0146	180.769231
replicate 10	0.0042	0.0116	176.190476	replicate 10	0.004	0.0111	177.5

	initial weight (g)	hydrated weight (g)	% weight increase		initial weight (g)	hydrated weight (g)	% weight increase
10% acetone				**20% acetone**			
replicate 1	0.011	0.0236	114.545455	replicate 1	0.0075	0.0175	133.333333
replicate 2	0.0058	0.0163	181.034483	replicate 2	0.0038	0.0116	205.263158
replicate 3	0.004	0.0116	190	replicate 3	0.003	0.0077	156.666667
replicate 4	0.0067	0.0191	185.074627	replicate 4	0.0064	0.0179	179.6875
replicate 5	0.0074	0.0209	182.432432	replicate 5	0.0055	0.0163	196.363636
replicate 6	0.0068	0.0186	173.529412	replicate 6	0.0064	0.0165	157.8125
replicate 7	0.0041	0.012	192.682927	replicate 7	0.0047	0.0135	187.234043
replicate 8	0.0023	0.0074	221.73913	replicate 8	0.0081	0.0231	185.185185
replicate 9	0.0062	0.0168	170.967742	replicate 9	0.0036	0.0089	147.222222
replicate 10	0.0072	0.0208	188.888889	replicate 10	0.0051	0.0131	156.862745
30% acetone				**40% acetone**			
replicate 1	0.0061	0.0156	155.737705	replicate 1	0.0028	0.0076	171.428571
replicate 2	0.009	0.0257	185.555556	replicate 2	0.0039	0.0106	171.794872
replicate 3	0.0046	0.0132	186.956522	replicate 3	0.0061	0.0164	168.852459
replicate 4	0.0045	0.0132	193.333333	replicate 4	0.004	0.0116	190
replicate 5	0.0069	0.0167	142.028986	replicate 5	0.0055	0.014	154.545455
replicate 6	0.007	0.0193	175.714286	replicate 6	0.0056	0.0139	148.214286
replicate 7	0.0047	0.0128	172.340426	replicate 7	0.0068	0.0191	180.882353
replicate 8	0.0049	0.0128	161.22449	replicate 8	0.005	0.0146	192
replicate 9	0.0075	0.0203	170.666667	replicate 9	0.0047	0.0128	172.340426
replicate 10	0.0035	0.0078	122.857143	replicate 10	0.0047	0.0107	127.659575

	initial weight (g)	hydrated weight (g)	% weight increase		initial weight (g)	hydrated weight (g)	% weight increase
50% acetone				**60% acetone**			
replicate 1	0.0073	0.0194	165.753425	replicate 1	0.0031	0.0064	106.451613
replicate 2	0.0079	0.0215	172.151899	replicate 2	0.0041	0.0097	136.585366
replicate 3	0.0039	0.0107	174.358974	replicate 3	0.0061	0.0151	147.540984
replicate 4	0.0055	0.0164	198.181818	replicate 4	0.0022	0.0054	145.454546
replicate 5	0.0052	0.0124	138.461539	replicate 5	0.006	0.015	150
replicate 6	0.0044	0.0128	190.909091	replicate 6	0.007	0.0152	117.142857
replicate 7	0.0048	0.0116	141.666667	replicate 7	0.0044	0.0106	140.909091
replicate 8	0.0061	0.0152	149.180328	replicate 8	0.0061	0.0148	142.622951
replicate 9	0.0062	0.018	190.322581	replicate 9	0.0049	0.012	144.897959
replicate 10	0.0035	0.0086	145.714286	replicate 10	0.0069	0.0187	171.014493
70% acetone				**80% acetone**			
replicate 1	0.0056	0.0136	142.857143	replicate 1	0.0046	0.0116	152.173913
replicate 2	0.0083	0.0207	149.39759	replicate 2	0.0096	0.0232	141.666667
replicate 3	0.0036	0.0088	144.444444	replicate 3	0.0037	0.0096	159.45946
replicate 4	0.00	0.0164	134.285714	replicate 4	0.0101	0.0243	140.594059
replicate 5	0.006	0.0158	163.333333	replicate 5	0.0064	0.014	118.75
replicate 6	0.0051	0.0132	158.823529	replicate 6	0.0063	0.0156	147.619048
replicate 7	0.0065	0.0159	144.615385	replicate 7	0.0058	0.0124	113.793103
replicate 8	0.0034	0.0082	141.176471	replicate 8	0.0053	0.0131	147.169811
replicate 9	0.004	0.0099	147.5	replicate 9	0.0044	0.0109	147.727273
replicate 10	0.0047	0.0111	136.170213	replicate 10	0.0053	0.012	126.415094

	initial weight (g)	hydrated weight (g)	% weight increase		initial weight (g)	hydrated weight (g)	% weight increase
90% acetone				**95% acetone**			
replicate 1	0.0064	0.017	165.625	replicate 1	0.0043	0.0111	158.139535
replicate 2	0.009	0.0211	134.444444	replicate 2	0.0067	0.018	168.656716
replicate 3	0.0044	0.0109	147.727273	replicate 3	0.0048	0.012	150
replicate 4	0.0076	0.0186	144.736842	replicate 4	0.0069	0.0179	159.42029
replicate 5	0.0036	0.0085	136.111111	replicate 5	0.0048	0.0125	160.416667
replicate 6	0.0062	0.016	158.064516	replicate 6	0.0064	0.0163	154.6875
replicate 7	0.0046	0.0105	128.26087	replicate 7	0.0084	0.0211	151.190476
replicate 8	0.0061	0.0154	152.459016	replicate 8	0.0039	0.0096	146.153846
replicate 9	0.0051	0.0122	139.215686	replicate 9	0.0045	0.0102	126.666667
replicate 10	0.0041	0.0096	134.146342	replicate 10	0.005	0.0124	148
100% acetone				**100% acetone + DMP**			
replicate 1	0.0044	0.0057	29.5454546	replicate 1	0.0038	0.0084	121.052632
replicate 2	0.0056	0.0065	16.0714286	replicate 2	0.0077	0.0092	19.4805195
replicate 3	0.0038	0.0044	15.7894737	replicate 3	0.0037	0.0046	24.3243243
replicate 4	0.011	0.0142	29.0909091	replicate 4	0.0076	0.0108	42.1052632
replicate 5	0.0045	0.0053	17.7777778	replicate 5	0.0038	0.0048	26.3157895
replicate 6	0.0034	0.0039	14.7058824	replicate 6	0.0049	0.0052	6.12244898
replicate 7	0.0071	0.0093	30.9859155	replicate 7	0.0055	0.0066	20
replicate 8	0.007	0.009	28.5714286	replicate 8	0.0044	0.0054	22.7272727
replicate 9	0.0041	0.0047	14.6341463	replicate 9	0.0052	0.0069	32.6923077
replicate 10	0.0046	0.006	30.4347826	replicate 10	0.0038	0.0054	42.1052632

	initial weight (g)	hydrated weight (g)	% weight increase		initial weight (g)	hydrated weight (g)	% weight increase
water				**5% methanol**			
replicate 1	0.007	0.0139	98.5714286	replicate 1	0.0034	0.0103	202.941177
replicate 2	0.0054	0.0157	190.740741	replicate 2	0.0113	0.0233	106.19469
replicate 3	0.0047	0.0126	168.085106	replicate 3	0.006	0.0198	230
replicate 4	0.0098	0.0296	202.040816	replicate 4	0.0096	0.0261	171.875
replicate 5	0.0046	0.0124	169.565217	replicate 5	0.0033	0.01	203.030303
replicate 6	0.0033	0.0103	212.121212	replicate 6	0.0065	0.0175	169.230769
replicate 7	0.0054	0.017	214.814815	replicate 7	0.0052	0.0149	186.538462
replicate 8	0.0049	0.0143	191.836735	replicate 8	0.004	0.0114	185
replicate 9	0.0054	0.0154	185.185185	replicate 9	0.0064	0.0189	195.3125
replicate 10	0.0041	0.0125	204.878049	replicate 10	0.0087	0.0267	206.896552
10% methanol				**20% methanol**			
replicate 1	0.0054	0.0148	174.074074	replicate 1	0.0033	0.0088	166.666667
replicate 2	0.0041	0.0133	224.390244	replicate 2	0.0048	0.0114	137.5
replicate 3	0.0086	0.0238	176.744186	replicate 3	0.0061	0.0147	140.983607
replicate 4	0.0049	0.0122	148.979592	replicate 4	0.0042	0.0116	176.190476
replicate 5	0.0055	0.0161	192.727273	replicate 5	0.0037	0.01	170.27027
replicate 6	0.0066	0.0183	177.272727	replicate 6	0.0058	0.0153	163.793103
replicate 7	0.0044	0.0123	179.545455	replicate 7	0.0078	0.0166	112.820513
replicate 8	0.009	0.0241	167.777778	replicate 8	0.0068	0.0155	127.941177
replicate 9	0.0053	0.0152	186.792453	replicate 9	0.0028	0.0082	192.857143
replicate 10	0.0078	0.0183	134.615385	replicate 10	0.0114	0.0287	151.754386

	initial weight (g)	hydrated weight (g)	% weight increase		initial weight (g)	hydrated weight (g)	% weight increase
30% methanol				**40% methanol**			
replicate 1	0.0045	0.0091	102.222222	replicate 1	0.0035	0.0092	162.857143
replicate 2	0.0061	0.013	113.114754	replicate 2	0.005	0.011	120
replicate 3	0.0042	0.0101	140.476191	replicate 3	0.003	0.0091	203.333333
replicate 4	0.0034	0.0082	141.176471	replicate 4	0.0056	0.0149	166.071429
replicate 5	0.0053	0.0098	84.9056604	replicate 5	0.0065	0.0138	112.307692
replicate 6	0.0062	0.017	174.193548	replicate 6	0.0047	0.0129	174.468085
replicate 7	0.0034	0.0073	114.705882	replicate 7	0.0032	0.0073	128.125
replicate 8	0.0056	0.013	132.142857	replicate 8	0.0077	0.0192	149.350649
replicate 9	0.0041	0.0098	139.02439	replicate 9	0.0086	0.0226	162.790698
replicate 10	0.0085	0.0175	105.882353	replicate 10	0.0043	0.01 6	169.767442
50% methanol				**60% methanol**			
replicate 1	0.0046	0.0114	147.826087	replicate 1	0.005	0.0119	138
replicate 2	0.0069	0.0182	163.768116	replicate 2	0.0096	0.0207	115.625
replicate 3	0.0038	0.0097	155.263158	replicate 3	0.0064	0.0152	137.5
replicate 4	0.0058	0.0154	165.517241	replicate 4	0.0085	0.0203	138.823529
replicate 5	0.0082	0.0196	139.02439	replicate 5	0.0059	0.014	137.288136
replicate 6	0.0025	0.006	140	replicate 6	0.0054	0.0139	157.407407
replicate 7	0.0038	0.0078	105.263158	replicate 7	0.0069	0.0161	133.333333
replicate 8	0.0054	0.0115	112.962963	replicate 8	0.0041	0.0093	126.829268
replicate 9	0.0071	0.0153	115.492958	replicate 9	0.0037	0.0085	129.72973
replicate 10	0.0068	0.017	150	replicate 10	0.0051	0.0116	127.45098

	initial weight (g)	hydrated weight (g)	% weight increase		initial weight (g)	hydrated weight (g)	% weight increase
70% methanol				**80% methanol**			
replicate 1	0.0048	0.011	129.166667	replicate 1	0.0035	0.0073	108.571429
replicate 2	0.0065	0.014	115.384615	replicate 2	0.0057	0.0132	131.578947
replicate 3	0.0051	0.0115	125.490196	replicate 3	0.0056	0.0125	123.214286
replicate 4	0.0079	0.0173	118.987342	replicate 4	0.0077	0.0162	110.38961
replicate 5	0.0089	0.0187	110.11236	replicate 5	0.0039	0.0087	123.076923
replicate 6	0.0063	0.0138	119.047619	replicate 6	0.0074	0.0176	137.837838
replicate 7	0.0081	0.0186	129.62963	replicate 7	0.0078	0.017	117.948718
replicate 8	0.0052	0.0116	123.076923	replicate 8	0.0036	0.0076	111.111111
replicate 9	0.0042	0.0094	123.809524	replicate 9	0.006	0.0135	125
replicate 10	0.0069	0.015	117.391304	replicate 10	0.0058	0.0119	105.172414
90% methanol				**95% methanol**			
replicate 1	0.004	0.0085	112.5	replicate 1	0.0064	0.0132	106.25
replicate 2	0.008	0.0153	91.25	replicate 2	0.0078	0.0132	69.2307692
replicate 3	0.0037	0.0079	113.513514	replicate 3	0.0063	0.0128	103.174603
replicate 4	0.0078	0.016	105.128205	replicate 4	0.0043	0.0086	100
replicate 5	0.0056	0.0126	125	replicate 5	0.0065	0.0136	109.230769
replicate 6	0.0092	0.0199	116.304348	replicate 6	0.0098	0.0209	113.265306
replicate 7	0.0106	0.0217	104.716981	replicate 7	0.0071	0.0139	95.7746479
replicate 8	0.0057	0.012	110.526316	replicate 8	0.0049	0.0106	116.326531
replicate 9	0.0047	0.0096	104.255319	replicate 9	0.0068	0.0145	113.235294
replicate 10	0.0063	0.0136	115.873016	replicate 10	0.0045	0.0098	117.777778

	initial weight (g)	hydrated weight (g)	% weight increase		initial weight (g)	hydrated weight (g)	% weight increase
100% methanol				**100% methanol + DMP**			
replicate 1	0.0064	0.0131	104.6875	replicate 1	0.0048	0.0085	77.0833333
replicate 2	0.0092	0.0167	81.5217391	replicate 2	0.0037	0.008	116.216216
replicate 3	0.0041	0.008	95.1219512	replicate 3	0.0053	0.0104	96.2264151
replicate 4	0.0073	0.0147	101.369863	replicate 4	0.0045	0.0089	97.7777778
replicate 5	0.0069	0.0143	107.246377	replicate 5	0.0053	0.0104	96.2264151
replicate 6	0.0067	0.0145	116.41791	replicate 6	0.0077	0.0137	77.9220779
replicate 7	0.0054	0.0109	101.851852	replicate 7	0.0064	0.0125	95.3125
replicate 8	0.0042	0.0075	78.5714286	replicate 8	0.0041	0.0086	109.756098
replicate 9	0.0046	0.0094	104.347826	replicate 9	0.0054	0.0111	105.555556
replicate 10	0.0055	0.0118	114.545455	replicate 10	0.0047	0.0108	129.787234

	initial weight (g)	hydrated weight (g)	% weight increase		initial weight (g)	hydrated weight (g)	% weight increase
water				**5% ethanol**			
replicate 1	0.0057	0.0167	192.982456	replicate 1	0.0052	0.014	169.230769
replicate 2	0.0087	0.025	187.356322	replicate 2	0.0028	0.0069	146.428571
replicate 3	0.0071	0.0203	185.915493	replicate 3	0.0041	0.0115	180.487805
replicate 4	0.004	0.0116	190	replicate 4	0.0072	0.0215	198.611111
replicate 5	0.005	0.0141	182	replicate 5	0.0054	0.0146	170.37037
replicate 6	0.0076	0.0212	178.947368	replicate 6	0.0085	0.021	147.058824
replicate 7	0.0045	0.0123	173.333333	replicate 7	0.0052	0.0151	190.384615
replicate 8	0.0056	0.0157	180.357143	replicate 8	0.0035	0.0104	197.142857
replicate 9	0.0066	0.0191	189.393939	replicate 9	0.0043	0.0126	193.023256
replicate 10	0.0057	0.018	215.789474	replicate 10	0.0072	0.0195	170.833333
10% ethanol				**20% ethanol**			
replicate 1	0.0038	0.0112	194.736842	replicate 1	0.0073	0.0185	153.424658
replicate 2	0.006	0.0164	173.333333	replicate 2	0.0076	0.0208	173.684211
replicate 3	0.0044	0.0112	154.545455	replicate 3	0.0035	0.0081	131.428571
replicate 4	0.0058	0.0129	122.413793	replicate 4	0.0091	0.021	130.769231
replicate 5	0.0043	0.0129	200	replicate 5	0.0087	0.0172	97.7011494
replicate 6	0.0063	0.0154	144.444444	replicate 6	0.0103	0.0273	165.048544
replicate 7	0.0074	0.0192	159.45946	replicate 7	0.0057	0.0145	154.385965
replicate 8	0.0066	0.015	127.272727	replicate 8	0.0032	0.0076	137.5
replicate 9	0.0065	0.0158	143.076923	replicate 9	0.0047	0.011	134.042553
replicate 10	0.0033	0.009	172.727273	replicate 10	0.005	0.009	80

	initial weight (g)	hydrated weight (g)	% weight increase		initial weight (g)	hydrated weight (g)	% weight increase
30% ethanol				**40% ethanol**			
replicate 1	0.0037	0.0097	162.162162	replicate 1	0.0058	0.0154	165.517241
replicate 2	0.0051	0.011	115.686275	replicate 2	0.009	0.0208	131.111111
replicate 3	0.0035	0.0085	142.857143	replicate 3	0.0045	0.0117	160
replicate 4	0.0091	0.0247	171.428571	replicate 4	0.0066	0.0177	168.181818
replicate 5	0.0056	0.0146	160.714286	replicate 5	0.0054	0.0132	144.444444
replicate 6	0.0074	0.0196	164.864865	replicate 6	0.0054	0.0147	172.222222
replicate 7	0.0059	0.0163	176.271186	replicate 7	0.0078	0.0208	166.666667
replicate 8	0.0038	0.0099	160.526316	replicate 8	0.0053	0.0141	166.037736
replicate 9	0.0032	0.0082	156.25	replicate 9	0.0029	0.0073	151.724138
replicate 10	0.0045	0.0084	86.6666667	replicate 10	0.0046	0.0108	134.782609
50% ethanol				**60% ethanol**			
replicate 1	0.0037	0.0097	162.162162	replicate 1	0.0044	0.0115	161.363636
replicate 2	0.0071	0.0163	129.577465	replicate 2	0.0099	0.0205	107.070707
replicate 3	0.0061	0.0158	159.016393	replicate 3	0.0054	0.0137	153.703704
replicate 4	0.0065	0.0173	166.153846	replicate 4	0.0116	0.029	150
replicate 5	0.0052	0.0136	161.538462	replicate 5	0.0038	0.009	136.842105
replicate 6	0.0048	0.0127	164.583333	replicate 6	0.0061	0.0151	147.540984
replicate 7	0.005	0.0125	150	replicate 7	0.005	0.0123	146
replicate 8	0.0033	0.0087	163.636364	replicate 8	0.0041	0.0109	165.853659
replicate 9	0.0051	0.0119	133.333333	replicate 9	0.0044	0.0111	152.272727
replicate 10	0.0054	0.0117	116.666667	replicate 10	0.0053	0.0118	122.641509

	initial weight (g)	hydrated weight (g)	% weight increase		initial weight (g)	hydrated weight (g)	% weight increase
70% ethanol				**80% ethanol**			
replicate 1	0.0058	0.0128	120.689655	replicate 1	0.004	0.0092	130
replicate 2	0.0058	0.0117	101.724138	replicate 2	0.0065	0.0144	121.538462
replicate 3	0.0052	0.0131	151.923077	replicate 3	0.0048	0.011	129.166667
replicate 4	0.0053	0.0123	132.075472	replicate 4	0.0056	0.0126	125
replicate 5	0.0052	0.0118	126.923077	replicate 5	0.0056	0.0124	121.428571
replicate 6	0.0034	0.009	164.705882	replicate 6	0.0058	0.0138	137.931035
replicate 7	0.0038	0.0088	131.578947	replicate 7	0.0062	0.0142	129.032258
replicate 8	0.0035	0.0081	131.428571	replicate 8	0.0039	0.0088	125.641026
replicate 9	0.0041	0.0091	121.95122	replicate 9	0.0045	0.0106	135.555556
replicate 10	0.0056	0.0119	112.5	replicate 10	0.0035	0.0074	111.428571
90% ethanol				**95% ethanol**			
replicate 1	0.007	0.0165	135.714286	replicate 1	0.0038	0.0078	105.263158
replicate 2	0.0084	0.0153	82.1428571	replicate 2	0.0052	0.0123	136.538462
replicate 3	0.0041	0.0098	139.02439	replicate 3	0.0025	0.0054	116
replicate 4	0.0048	0.0107	122.916667	replicate 4	0.0108	0.0232	114.814815
replicate 5	0.0056	0.0126	125	replicate 5	0.0042	0.0089	111.904762
replicate 6	0.005	0.012	140	replicate 6	0.0109	0.0242	122.018349
replicate 7	0.0052	0.0121	132.692308	replicate 7	0.0051	0.0109	113.72549
replicate 8	0.0035	0.0081	131.428571	replicate 8	0.0066	0.0151	128.787879
replicate 9	0.0037	0.0091	145.945946	replicate 9	0.0069	0.0162	134.782609
replicate 10	0.0056	0.0136	142.857143	replicate 10	0.0038	0.0089	134.210526

	initial weight (g)	hydrated weight (g)	% weight increase		initial weight (g)	hydrated weight (g)	% weight increase
100% ethanol				**100% ethanol + DMP**			
replicate 1	0.0088	0.0137	55.6818182	replicate 1	0.0067	0.0101	50.7462687
replicate 2	0.0115	0.0162	40.8695652	replicate 2	0.008	0.014	75
replicate 3	0.0065	0.0086	32.3076923	replicate 3	0.006	0.0064	6.66666667
replicate 4	0.0068	0.0105	54.4117647	replicate 4	0.0057	0.0094	64.9122807
replicate 5	0.005	0.0071	42	replicate 5	0.0042	0.0073	73.8095238
replicate 6	0.0042	0.0064	52.3809524	replicate 6	0.0056	0.009	60.7142857
replicate 7	0.0059	0.0081	37.2881356	replicate 7	0.0047	0.0078	65.9574468
replicate 8	0.005	0.0063	26	replicate 8	0.0052	0.0076	46.1538462
replicate 9	0.0059	0.0086	45.7627119	replicate 9	0.0056	0.0081	44.6428571
replicate 10	0.004	0.0062	55	replicate 10	0.0035	0.0049	40

	initial weight (g)	hydrated weight (g)	% weight increase		initial weight (g)	hydrated weight (g)	% weight increase
water				**5% DMSO**			
replicate 1	0.0056	0.0153	173.214286	replicate 1	0.0044	0.013	195.454546
replicate 2	0.0034	0.0106	211.764706	replicate 2	0.0046	0.0123	167.391304
replicate 3	0.0068	0.0193	183.823529	replicate 3	0.0059	0.0168	184.745763
replicate 4	0.0042	0.012	185.714286	replicate 4	0.0052	0.0151	190.384615
replicate 5	0.0065	0.0196	201.538462	replicate 5	0.0072	0.0191	165.277778
replicate 6	0.0057	0.0165	189.473684	replicate 6	0.0097	0.0289	197.938144
replicate 7	0.0044	0.0133	202.272727	replicate 7	0.006	0.0169	181.666667
replicate 8	0.0058	0.0169	191.37931	replicate 8	0.0056	0.0162	189.285714
replicate 9	0.0051	0.0132	158.823529	replicate 9	0.0046	0.0138	200
replicate 10	0.0044	0.0127	188.636364	replicate 10	0.0055	0.0155	181.818182
10% DMSO				**20% DMSO**			
replicate 1	0.0054	0.0144	166.666667	replicate 1	0.0038	0.0108	184.210526
replicate 2	0.0072	0.0199	176.388889	replicate 2	0.0037	0.0079	113.513514
replicate 3	0.0054	0.0155	187.037037	replicate 3	0.0045	0.0127	182.222222
replicate 4	0.005	0.0136	172	replicate 4	0.0059	0.0156	164.40678
replicate 5	0.0067	0.0192	186.567164	replicate 5	0.0026	0.0077	196.153846
replicate 6	0.0061	0.0171	180.327869	replicate 6	0.0092	0.0215	133.695652
replicate 7	0.0037	0.0108	191.891892	replicate 7	0.0094	0.0192	104.255319
replicate 8	0.0076	0.0211	177.631579	replicate 8	0.0081	0.0172	112.345679
replicate 9	0.0116	0.0311	168.103448	replicate 9	0.0056	0.0142	153.571429
replicate 10	0.0061	0.0188	208.196721	replicate 10	0.0027	0.008	196.296296

	initial weight (g)	hydrated weight (g)	% weight increase		initial weight (g)	hydrated weight (g)	% weight increase
30% DMSO				**40% DMSO**			
replicate 1	0.0041	0.0091	121.95122	replicate 1	0.0039	0.0075	92.3076923
replicate 2	0.0058	0.0164	182.758621	replicate 2	0.0042	0.0085	102.380952
replicate 3	0.0067	0.019	183.58209	replicate 3	0.0076	0.0161	111.842105
replicate 4	0.0084	0.0194	130.952381	replicate 4	0.0064	0.0125	95.3125
replicate 5	0.0058	0.0099	70.6896552	replicate 5	0.0065	0.0125	92.3076923
replicate 6	0.007	0.017	142.857143	replicate 6	0.0059	0.0101	71.1864407
replicate 7	0.0064	0.0145	126.5625	replicate 7	0.0051	0.0114	123.529412
replicate 8	0.0048	0.0092	91.6666667	replicate 8	0.0049	0.0104	112.244898
replicate 9	0.0047	0.0099	110.638298	replicate 9	0.0049	0.0125	155.102041
replicate 10	0.0041	0.0088	114.634146	replicate 10	0.0074	0.013	75.6756757
50% DMSO				**60% DMSO**			
replicate 1	0.0092	0.0143	55.4347826	replicate 1	0.0032	0.006	87.5
replicate 2	0.0069	0.0114	65.2173913	replicate 2	0.0076	0.0136	78.9473684
replicate 3	0.004	0.0101	152.5	replicate 3	0.0094	0.0165	75.5319149
replicate 4	0.0082	0.0133	62.195122	replicate 4	0.0054	0.0098	81.4814815
replicate 5	0.0053	0.0071	33.9622642	replicate 5	0.0065	0.0098	50.7692308
replicate 6	0.0056	0.0109	94.6428571	replicate 6	0.0067	0.0116	73.1343284
replicate 7	0.0053	0.0136	156.603774	replicate 7	0.0097	0.021	116.494845
replicate 8	0.0043	0.01	132.55814	replicate 8	0.0034	0.0064	88.2352941
replicate 9	0.0036	0.0067	86.1111111	replicate 9	0.0034	0.0055	61.7647059
replicate 10	0.0062	0.0099	59.6774194	replicate 10	0.0048	0.0074	54.1666667

	initial weight (g)	hydrated weight (g)	% weight increase		initial weight (g)	hydrated weight (g)	% weight increase
70% DMSO				**80% DMSO**			
replicate 1	0.0037	0.0061	64.8648649	replicate 1	0.0062	0.0117	88.7096774
replicate 2	0.0052	0.0139	167.307692	replicate 2	0.0085	0.0194	128.235294
replicate 3	0.0057	0.0126	121.052632	replicate 3	0.005	0.0152	204
replicate 4	0.0066	0.0107	62.1212121	replicate 4	0.0039	0.0107	174.358974
replicate 5	0.0079	0.0133	68.3544304	replicate 5	0.0066	0.0181	174.242424
replicate 6	0.0047	0.0097	106.382979	replicate 6	0.0047	0.0125	165.957447
replicate 7	0.0064	0.0096	50	replicate 7	0.0078	0.0212	171.794872
replicate 8	0.0034	0.0084	147.058824	replicate 8	0.0034	0.0078	129.411765
replicate 9	0.0074	0.0122	64.8648649	replicate 9	0.0043	0.0121	181.395349
replicate 10	0.0053	0.0103	94.3396226	replicate 10	0.0051	0.0111	117.647059
90% DMSO				**95% DMSO**			
replicate 1	0.0028	0.0084	200	replicate 1	0.0028	0.0089	217.857143
replicate 2	0.0092	0.0278	202.173913	replicate 2	0.0065	0.0202	210.769231
replicate 3	0.0077	0.0235	205.194805	replicate 3	0.0069	0.0207	200
replicate 4	0.0035	0.0107	205.714286	replicate 4	0.0072	0.0218	202.777778
replicate 5	0.0065	0.0204	213.846154	replicate 5	0.0055	0.0166	201.818182
replicate 6	0.0056	0.0185	230.357143	replicate 6	0.0054	0.0168	211.111111
replicate 7	0.0081	0.0224	176.54321	replicate 7	0.0059	0.0171	189.830509
replicate 8	0.0046	0.0121	163.043478	replicate 8	0.0039	0.0113	189.74359
replicate 9	0.0045	0.0126	180	replicate 9	0.0044	0.0123	179.545455
replicate 10	0.0044	0.0115	161.363636	replicate 10	0.0051	0.0134	162.745098

	initial weight (g)	hydrated weight (g)	% weight increase		initial weight (g)	hydrated weight (g)	% weight increase
100% DMSO				**100% DMSO + DMP**			
replicate 1	0.0034	0.0094	176.470588	replicate 1	0.0065	0.0206	216.923077
replicate 2	0.0053	0.0164	209.433962	replicate 2	0.0044	0.0139	215.909091
replicate 3	0.0127	0.0367	188.976378	replicate 3	0.0108	0.0309	186.111111
replicate 4	0.0051	0.0149	192.156863	replicate 4	0.0048	0.0147	206.25
replicate 5	0.0061	0.0193	216.393443	replicate 5	0.0043	0.0132	206.976744
replicate 6	0.0062	0.0236	280.645161	replicate 6	0.0066	0.0195	195.454546
replicate 7	0.0058	0.0177	205.172414	replicate 7	0.0031	0.0076	145.16129
replicate 8	0.006	0.0182	203.333333	replicate 8	0.007	0.0193	175.714286
replicate 9	0.0044	0.0118	168.181818	replicate 9	0.0059	0.0146	147.457627
replicate 10	0.0076	0.0196	157.894737	replicate 10	0.0042	0.0111	164.285714

APPENDIX - E.

Table 6. - One-way AVOVA comparing the percentage increase in leaf weight of dehydrated *Myrothamnus flabellifolia* leaves immersed for 21 hours in various solutions.

Source of variation	df	MS	F-ratio	P
Grouped data	15	3051.84	88.62	<0.00
Error	143	34.44		

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
(1)															
(2)	0.00003														
(3)	0.72213	0.00003													
(4)	0.84164	0.00003	1.00000												
(5)	0.00003	0.99977	0.00003	0.00003											
(6)	0.99988	0.00003	0.99826	0.99978	0.00003										
(7)	0.99994	0.00003	0.16075	0.25166	0.00003	0.89938									
(8)	0.00003	0.90897	0.00003	0.00003	0.99999	0.00003	0.00003								
(9)	0.93775	0.00003	0.00943	0.01937	0.00003	0.33321	0.99999	0.00003							
(10)	0.00072	0.00003	0.00003	0.00003	0.00003	0.00003	0.04214	0.00003	0.28269						
(11)	0.00003	0.90577	0.00003	0.00003	0.99999	0.00003	0.00003	1.00000	0.00003	0.00003					
(12)	0.00003	0.00003	0.00003	0.00003	0.00003	0.00003	0.00003	0.00035	0.00003	0.00012	0.00036				
(13)	0.93651	0.00003	1.00000	1.00000	0.00003	0.99999	0.39490	0.00003	0.04278	0.00003	0.00003	0.00003			
(14)	0.00003	0.00003	0.00003	0.00003	0.00003	0.00003	0.00003	0.00013	0.00003	0.00035	0.00013	1.00000	0.00003		
(15)	0.00003	0.00003	0.00003	0.00003	0.00003	0.00003	0.00003	0.00013	0.00003	0.00034	0.00013	1.00000	0.00003	1.00000	
(16)	0.00003	0.00003	0.00003	0.00003	0.00003	0.00003	0.00003	0.00003	0.00003	0.01092	0.00003	0.99963	0.00003	0.99999	0.99999

significant difference non-significant difference

(1) FC-72, (2) water, (3) epoxy propane, (4) epoxy propane/DMP, (5) 95% acetone, (6) acetone, (7) acetone/DMP, (8) 95% ethanol
(9) ethanol, (10) ethanol/DMP, (11) 95% methanol, (12) methanol, (13) n-heptane, (14) karnovsky, (15) 5% glutaraldehyde
(16) methanol/DMP

Table 7. - Paired t-tests comparing the weights gained by individual *Myrothamnus flabellifolia* leaves after immersion in either water, Karnovsky's fixative or 5% glutaraldehyde in phosphate buffer after 30 minutes and 2 hours of rehydration.

	df	t	P
Water	9	35.07	<0.00000
Karnovsky	9	10.84	<0.00000
5% glutaraldehyde	9	13.14	<0.00000

Table 8. - One-way AVOVA comparing the percentage increase in leaf weight of dehydrated *Myrothamnus flabellifolia* leaves immersed for 21 hours in solutions containing acetone and decreasing amounts of water from 100% water to 100% acetone.

Source of variation	df	MS	F-ratio	P
Grouped data	13	25114.15	51.79	<0.00
Error	126	484.93		

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
(1)														
(2)	0.97320													
(3)	0.99990	0.99999												
(4)	1.00000	0.94856	0.99954											
(5)	1.00000	0.77413	0.98567	1.00000										
(6)	1.00000	0.84000	0.99362	1.00000	1.00000									
(7)	1.00000	0.77593	0.98594	1.00000	1.00000	1.00000								
(8)	0.07935	0.00027	0.00415	0.11251	0.29115	0.22755	0.28942							
(9)	0.34540	0.00389	0.03854	0.43069	0.72254	0.64117	0.72057	0.99999						
(10)	0.06378	0.00020	0.00307	0.09172	0.24923	0.19196	0.24766	1.00000	0.99999					
(11)	0.21751	0.00153	0.01811	0.28487	0.56163	0.47580	0.55945	1.00000	1.00000	1.00000				
(12)	0.78351	0.03752	0.21499	0.85252	0.97549	0.95440	0.97503	0.99474	0.99999	0.99088	0.99991			
(13)	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002		
(14)	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.98995	

significant difference non-significant difference

(1) water, (2) 5% acetone, (3) 10% acetone, (4) 20% acetone, (5) 30% acetone, (6) 40% acetone, (7) 50% acetone, (8) 60% acetone, (9) 70% acetone, (10) 80% acetone, (11) 90% acetone, (12) 95% acetone, (13) 100% acetone, (14) 100% acetone/DMP

Table 9. - One-way AVOVA comparing the percentage increase in leaf weight of dehydrated *Myrothamnus flabellifolia* leaves immersed for 21 hours in solutions containing ethanol and decreasing amounts of water from 100% water to 100% ethanol.

Source of variation	df	MS	F-ratio	P
Grouped data	13	16391.85	45.72	<0.00
Error	126	358.5		

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
(1)														
(2)	0.98881													
(3)	0.04983	0.75143												
(4)	0.00002	0.00016	0.24304											
(5)	0.00066	0.09366	0.99790	0.33368										
(6)	0.04147	0.48306	1.00000	0.48464	0.99998									
(7)	0.00108	0.12623	0.99928	0.89548	1.00000	0.99996								
(8)	0.00005	0.01145	0.89530	0.99928	0.99996	0.98361	0.99997							
(9)	0.00002	0.00003	0.03093	0.99998	0.49143	0.09668	0.41214	0.89969						
(10)	0.00002	0.00002	0.00917	0.99855	0.26454	0.03418	0.20757	0.71184	1.00000					
(11)	0.00002	0.00003	0.03376	0.99999	0.51099	0.10400	0.43082	0.90966	1.00000	1.00000				
(12)	0.00002	0.00002	0.00085	0.93192	0.05914	0.00411	0.04200	0.30234	0.99975	0.99999	0.99966			
(13)	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002		
(14)	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.00002	0.99913	

significant difference non-significant difference

(1) water, (2) 5% ethanol, (3) 10% ethanol, (4) 20% ethanol, (5) 30% ethanol, (6) 40% ethanol, (7) 50% ethanol, (8) 60% ethanol, (9) 70% ethanol, (10) 80% ethanol, (11) 90% ethanol, (12) 95% ethanol, (13) 100% ethanol, (14) 100% ethanol/DMP

Table 10. - One-way AVOVA comparing the percentage increase in leaf weight of dehydrated *Myrothamnus flabellifolia* leaves immersed for 21 hours in solutions containing methanol and decreasing amounts of water from 100% water to 100% methanol.

Source of variation	df	MS	F-ratio	P
Grouped data	13	9162.57	20.35	<0.00
Error	126	449.68		

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
(1)														
(2)	1.00000													
(3)	0.99995	0.99939												
(4)	0.09652	0.05306	0.52304											
(5)	0.00002	0.00002	0.00002	0.10889										
(6)	0.12260	0.06925	0.58874	1.00000	0.08521									
(7)	0.00028	0.00011	0.00797	0.96122	0.95767	0.94039								
(8)	0.00004	0.00003	0.00002	0.70427	0.99939	0.64169	0.99999							
(9)	0.00002	0.00002	0.00002	0.03485	1.00000	0.02598	0.81033	0.98528						
(10)	0.00002	0.00002	0.00002	0.01804	0.99999	0.01315	0.68362	0.95579	1.00000					
(11)	0.00002	0.00002	0.00002	0.00029	0.95415	0.00020	0.09944	0.36552	0.99600	0.99934				
(12)	0.00002	0.00002	0.00002	0.00004	0.66854	0.00003	0.01552	0.09465	0.88982	0.95204	0.99999			
(13)	0.00002	0.00002	0.00002	0.00002	0.37090	0.00002	0.00322	0.02662	0.64686	0.77769	0.99944	1.00000		
(14)	0.00002	0.00002	0.00002	0.00002	0.34416	0.00002	0.00273	0.02318	0.61719	0.75217	0.99914	1.00000	1.00000	

significant difference non-significant difference

(1) water, (2) 5% methanol, (3) 10% methanol, (4) 20% methanol, (5) 30% methanol, (6) 40% methanol, (7) 50% methanol, (8) 60% methanol, (9) 70% methanol, (10) 80% methanol, (11) 90% methanol, (12) 95% methanol, (13) 100% methanol, (14) 100% methanol/DMP

Table 11. - One-way AVOVA comparing the percentage increase in leaf weight of dehydrated *Myrothamnus flabellifolia* leaves immersed for 21 hours in solutions containing DMSO and decreasing amounts of water from 100% water to 100% DMSO.

Source of variation	df	MS	F-ratio	P
Grouped data	13	20233.03	24.73	<0.00
Error	126	817.87		

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
(1)														
(2)	1.00000													
(3)	0.99999	1.00000												
(4)	0.27564	0.44380	0.67084											
(5)	0.00017	0.00054	0.00207	0.72418										
(6)	0.00002	0.00002	0.00002	0.00540	0.82100									
(7)	0.00002	0.00002	0.00002	0.00007	0.15646	0.99899								
(8)	0.00002	0.00002	0.00002	0.00002	0.00549	0.72690	0.99915							
(9)	0.00002	0.00002	0.00002	0.00030	0.35333	0.99999	1.00000	0.98278						
(10)	0.25399	0.41624	0.64293	1.00000	0.74974	0.00628	0.00008	0.00002	0.00036					
(11)	1.00000	0.99999	0.99955	0.10316	0.00004	0.00002	0.00002	0.00002	0.00002	0.09264				
(12)	0.99999	0.99984	0.99626	0.05431	0.00002	0.00002	0.00002	0.00002	0.00002	0.04815	1.00000			
(13)	0.99985	0.99761	0.97758	0.02362	0.00002	0.00002	0.00002	0.00002	0.00002	0.02067	1.00000	1.00000		
(14)	1.00000	1.00000	1.00000	0.40872	0.00044	0.00002	0.00002	0.00002	0.00002	0.38202	0.99998	0.99992	0.99848	

significant difference non-significant difference

(1) water, (2) 5% DMSO, (3) 10% DMSO, (4) 20% DMSO, (5) 30% DMSO, (6) 40% DMSO, (7) 50% DMSO, (8) 60% DMSO, (9) 70% DMSO, (10) 80% DMSO, (11) 90% DMSO, (12) 95% DMSO, (13) 100% DMSO, (14) 100% DMSO/DMP

Table 12. - Paired t-tests comparing the weights gained by individual *Myrothamnus flabellifolia* leaves after immersion in either FC-72 or n-heptane after 30 minutes and 2 hours of rehydration.

	df	t	P
FC-72	9	8.91	<0.00001
n-heptane	9	8.55	<0.00001

Table 13. - Paired t-tests comparing the weights gained by individual *Myrothamnus flabellifolia* leaves after immersion in acetone, methanol or ethanol after 30 minutes and 2 hours of rehydration.

	df	t	P
acetone	9	5.18	<0.00580
methanol	7	11.46	<0.00001
ethanol	9	3.95	<0.00330

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