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A study on some of the physiological and ultrastructural changes occurring on desiccation and rehydration of *Myrothamnus flabellifolia.*

ABSTRACT

Some of the physiological and ultrastructural changes occurring during the dehydration and rehydration of the "resurrection" plant *Myrothamnus flabellifolia* were investigated. The changes in stem and leaf orientation through angles greater than 45 degrees were recorded, and these changes, along with the change in adaxial leaf surface pigmentation, may serve to limit bright-light and/or thermal damage to these plants. *M. flabellifolia* retained more than 70% total chlorophyll content, and there was little change in carotenoid content across changes in water status. The maintenance of membrane integrity throughout dehydration and rehydration was indicated by low levels of electrolyte leakage, as well as ultrastructural micrographs, although a slight increase in leakage at low water contents (<30%) during dehydration, might be taking place, and requires further investigation. A linear change in photochemical efficiency of PSII (Fv/Fm) between 40 and 80% relative water contents (RWC) suggests deliberate and controlled changes in the photosynthetic apparatus of the thylakoids during this time, whilst the stability of Fv/Fm at RWC <40% suggests photosystem stabilisation during desiccation. Ultrastructural studies indicate high levels of subcellular integrity, suggesting the possible role of cellular stabilisation in preventing damage during water stress.

INTRODUCTION

Water, although essential for growth, has been shown, for a limited number of organisms, not to be necessary for survival (Stewart, 1989). Extremely few angiosperms possess mature foliage that is desiccation-tolerant. The South African flora however, contains a unique abundance of higher plants which withstand almost complete desiccation (i.e. mature leaves which survive to an air-dry state between 15 and 0 percent relative humidity) (Gaff, 1971). These desiccation-tolerant, poikiloxerophytic (Genkel and Pronina, 1970), or poikilohydric (Bianchi *et al.,* 1993) plants, loosely defined by their unique ability to revive from an air-dry condition, have been described by various authors using a broad range of terminology, all attempting to define this still poorly understood process. The most general description applied to these plants involves their definition as 'resurrection plants': plants which, by various means, are able to recover from complete desiccation (Schroder, 1986). These plants are considered able to undergo dehydration into a state whereby metabolism is suspended, whilst the viability of the organism, and the entire cell organisation, is preserved (Genkel and Pronina (1970)).

A remarkable example of this habit is *Myrothmanus flabellifolia* Welw. (Maure, 1966). The only species of the Myrothamnaceae, it grows on exposed granite outcrops subject to extremes of available moisture, and is endemic to tropical, and adjoining southern Africa (Riley, 1963). In winter, during an absence of water, *M. flabellifolia* plants desiccate entirely, maintaining their leaves, which dry and fold close to the stems. Following the arrival of summer rains, these plants rehydrate. Half an hour after rewetting, respiration is induced, and, within seven hours, full photosynthetic recovery has taken place (Gaff, 1989). The mechanism by which *M. flabellifolia,* and other resurrection plants, are able to resume metabolism after long periods in the dry state is not yet known, however there is growing interest in the physiological processes associated with these plants (Suau *et al.,* 1991).

Injuries from desiccation are likely to arise from a combination of physical damage to cellular components following water removal, structural and metabolic consequences of increased solute concentration, and chemical damage resulting from metabolic dysfunction induced by dehydration (Stewart, 1989).

There has been much speculation on the mechanisms underlying desiccation tolerance in plant tissues (for reviews see Stewart, 1989; Bewley and Oliver, 1992, and Vertucci and Farrant, 1995). Theories are based on methods of stabilisation of the subcellular organisation, including the maintenance of membrane integrity, and the activity of repair systems. Biomolecules, such as sugars, proteins, and other low molecular weight compounds, have been involved in such protection by either water replacement (Crowe, 1971), chaperone behaviour (Bray, 1993), and/or vitrification (Vertucci and Farrant, 1995). While it is still not clear how, and to what extent, these molecules are involved in stabilisation of membranes, clearly the ability to maintain and repair membranes is an essential component in the mechanism of tolerance.

Another vital aspect to be considered, is the mechanical stabilisation required to prevent inward collapse of cytoplasm (and thus tearing of membranes as they pull away from the cell walls). In orthodox recalcitrant seeds, stabilisation is achieved by the filling of vacuoles with storage proteins, and packing the cytoplasm with numerous reserves, thereby providing outward pressure against the cell wall (Vertucci and Farrant, 1995). There has been little work on the stabilisation of foliar tissue during desiccation of resurrection plants. In the resurrection moss *Tortula ruralis,* cytoplasm shrinkage, accompanied by some membrane damage occurs (evident by considerable electrolyte leakage), but this is readily repaired on rehydration (Bewley, 1995). In the desiccation-tolerant angiosperm *Craterostigma nanum*, damage is prevented by cell wall folding (Sherwin, 1995).

A further, and important consideration, particularly in photosynthetic vegetative tissue, is the potential for free radical formation. In the dry state, photochemical energy cannot be dissipated in an orderly manner during normal CO_2 fixation, providing the opportunity for a number of biological oxidations, both enzymatic and spontaneous, to take place. These reactions are able to generate the cytotoxic free superoxide radical ($O_2^{\cdot}$), which, in turn, can react with H_2O_2 to produce singlet oxygen, and hydroxyl radicals, all highly potent oxidants (Fridovich, 1976). The oxidants can then induce considerable destruction, particularly to large polymers such as nucleic acids, proteins, polysaccharides, and membrane lipids. This is prevented in hydrated tissues, where free radical production is usually controlled by free radical absorbents or scavenging reactions (Fridovich, 1976). It is possible that water loss from cells of desiccation-intolerant plants can upset the balance between free radical producing and scavenging reactions in favour of the former, whereas in desiccation tolerant plants, the balance is somehow maintained (Bewley and Krochko, 1981). Resurrection plants appear to have two strategies for the prevention of damage to photosynthetic tissue from free radicals: either the deliberate breakdown of photosynthetic apparatus and associated loss of chlorophyll during desiccation (poikilochlorophyllis plants), or the retention of chlorophyll, and preservation of photosynthetic apparatus during desiccation, via mechanism(s) not yet understood (homiochlorophyllis plants) (Bewley, 1979).

The aim of this research was to investigate several of these issues operating in *M. flabellifolia* during both dehydration and rehydration. Membrane systems are considered to be particularly susceptible to damage from water stress (Steponkus and Webb, 1992). Water deficit stress is considered to amplify plant cell electrolyte leakage primarily as a result of alteration in membrane lipid and sterol composition (Zwiazek and Blake, 1990). According to this view, relative electrolyte leakage from cells or tissues during water stress is considered to be an indicator of dehydration tolerance (Martin *et al.,* 1987). The leakage of cytoplasmic solutes, in conjunction with studies of ultrastructural changes, were thus examined for *M. flabellifolia.*

The membrane-associated process of photosynthesis was also investigated since, although photosynthesis is inhibited during even mild water stress (Kaiser, 1989), the integrity of thylakoid membrane systems, (and thus their potential ability for electron transport,) might provide a key to understanding the amazing speed at which *M. flabellifolia* is able to recover. The accessory pigments, (chlorophyll *b* and carotenoids) capture light energy and direct it to the fluorescent chlorophyll *a* molecules of PSII (Hipkins and Baker, 1986). Changes in variable fluorescence (Fv), is indicative of inactivation in primary photochemistry of photosystem II, and the ratio Fv/Fm is proportional to the quantum yield of the photochemistry (Greer *et al.,* 1986). A decline in Fv/Fm is a good indicator of photo-inhibitory damage caused when plants are subjected to a wide range of environmental stresses (Sherwin, 1995). This study investigated changes in photochemical efficiency of photosystem II (Fv/Fm), as well as variation in chlorophyll *a,* chlorophyll *b,* and carotenoid contents during the dehydration and rehydration processes.

MATERIALS AND METHODS

Plant material

All experimentation was undertaken using well established *M. flabellifolia* plants growing in separate trays under greenhouse conditions. Plants were maintained in a fully hydrated state by watering three times weekly, and desiccation was induced by the exclusion of water to these plants. Plants maintained in a desiccated state for between 3 to 6 months were utilised for the rehydration experimentation.

Mature leaves (i.e. neither buds nor potentially senescent leaves) from the middle region of the plants, were consistently selected for experimentation. Samples were taken from fully turgid and completely desiccated plants, as well as during a number of stages intermediary between these two conditions. Threads of cotton were positioned on branches such that right-angled triangles were created. Angles, measured using a protractor, then allowed for the determination of change in stem position.

Triplicate samples were prepared for all experimentation, although the same three leaves were used for protocols which were non-destructive, (such as fluorescence and conductivity measures). Leaf dry weight and water content was measured in association with all protocols. This was determined gravimetrically by leaf drying in an oven at 103°C for 24 hours.

Leaf water content, expressed as grams water per gram dry weight leaf material, was calculated using equation 1 below:

$$\frac{\text{fresh weight - dry weight}}{\text{dry weight}} \quad \text{.. Equation 1}$$

Although on average a water content between 1.5 to 2.5 grams water per gram dry weight was indicative of full turgor, natural variation in leaf size and organic matter content made comparison and interpretation difficult. For this reason leaf water status was converted to an estimated relative water content (RWC), calculated from equation 2:

$$\frac{\text{Water content}}{\text{Full turgor water content}} \times 100 \qquad \text{Equation 2}$$

Soil water status

Soil samples were extracted from the area directly below the main branches of the plant using a plastic tube with a 4 mm diameter. Soil water contents were calculated from the difference between fresh and dry mass, and expressed as a percentage using equation 3 below:

$$\frac{(\text{fresh mass} - \text{dry mass})}{\text{dry mass}} \times 100 \qquad \text{Equation 3}$$

Chlorophyll extraction

Standard methods of extraction and analysis of photosynthetic pigments were used (Lichtenthaler, 1987). Approximately 0.02-0.03g of mature leaf material was placed in 100% acetone, protected from air and light, and left at 4 °C overnight. This material was then ground in the original acetone, filtered, and centrifuged. The chlorophyll content was estimated using a UV-2201 Shimadzu UV-VIS recording spectrophotometer. Absorbance readings were recorded at 470, 661.6, and 644.8 nm wavelengths. Chlorophyll and carotenoid contents were calculated using the equations listed below.

Chlorophylls:

$$chl_a = 11{,}24A_{661.6} - 2.04A_{644.8} \qquad \text{Equation 4}$$

$$chl_b = 21.13A_{644.8} - 4.19A_{661.6} \qquad \text{Equation 5}$$

$$chl_{a+b} = 7.05A_{661.6} + 18.09A_{644.8} \qquad \text{Equation 6}$$

Total carotenoids: $C_{x+c} = (1000A_{470} - 1.90\,chl_a - 63.14chl_b) / 214$- Equation 7

The destructive nature of this process made it impossible to obtain the exact dry mass of the leaves used for the extraction process, thus an estimated dry weight was calculated from other sample leaves removed simultaneously with those detached for pigment analysis. It was then possible to calculate pigment concentration in milligrams chlorophyll per gram dry weight leaf material.

Chlorophyll fluorescence

Chlorophyll fluorescence was measured on an OS-500 Modulated Fluorometer (Opti-Sciences, Inc.), using the kinetic test mode. Leaves were 'dark adapted' for 5 minutes prior to the determining of minimal (Fo), and maximal (Fm) fluorescence yields. Modulation intensity and detector gain were optimised for each sample in order to obtain an instantaneous fluorescence signal (Ft) of between 150 and 250 counts. A saturation intensity of 200 (~7.84kuE), for a duration of one second, was found to be sufficient for ensuring maximal fluorescence without over-driving the photosystem. The intensity of the actinic source, with its peak wavelength of ~670nm, was set at 100 (~176.5uE), whilst the far-red (735nm) source was kept at an intensity of 120 (~7mW) for 3.5 seconds, and a second saturation pulse was set for 60 seconds into the run. "Auto-run" mode was selected such that, after a dark adapted measurement was taken, the Ft count was allowed to stabilise within ~1.2 counts of the Fo value, whereafter the kinetic pulse run could take place. The ratio of "variable" to maximal fluorescence (Fv/Fm) was calculated from the Fo and Fm parameters measures. (Fv being maximum variable fluorescence in the state when all non-photochemical processes are at a minimum (i.e. Fm-Fo) (van Kooten and Snel, 1990)).

Electrolyte leakage

Electrolyte leakage was measured using a CM100 Multiple Conductivity Meter (Reid and Associates, Durban). Wells were filled with water obtained from a Millipore purification system, and the meter was allowed to stabilised at 4 volts for 60 minutes. Leaf material was placed into the wells, and leakage was recorded (in uA) at 60 second intervals for one hour. Amount of leakage was calculated from a regression analysis of conductivity against time. These values were finally divided by the dry weight, in order to express leakage as microamperes per minute per gram dry weight.

In order to compare these leakage measures with total potential leakage, leaf tissue was subject one of the following treatments: (1) freeze-thaw cycling of leaf material, (cycled three times, being frozen at -80°C), (2) freezing of leaves in liquid nitrogen (-196K), or (3) desiccation of fresh leaf material in an oven for 24 hours at 103°C.

Ultrastructure

Due to time constraints and limited material availability, only four stages of the dehydration-rehydration series were examined. These were: (1) fully turgid leaf segments, (2) leaf material dehydrated to approximately 50% RWC, (3) completely desiccated leaf material, (~1% RWC,) and (4) rehydrating mesophyll tissue at approximately 50% RWC.

Leaf material was cut into small segments in a drop of 2.5% gluteraldehyde, (buffered in 0.1 M phosphate of pH 7.4 containing 0.5% caffeine), and fixed in this solution overnight. The segments underwent three successive washes (in caffeinated phosphate buffer), were post-fixed for one hour in 1% osmium tetroxide, and again washed three times in buffer. Samples were then dehydrated using a graded ethanol series (30, 50, 75, 80, 90, and 100 %) at room temperature. The final dehydration involved two changes of absolute alcohol for 10 minutes each. Material was placed in two changes of 100% acetone (10 minutes each), before being placed in a 50% resin, 50% acetone solution for 4 hours, followed by their placement in 100% resin overnight. Polymerization took place for 16 hours at 60°C. The embedded samples were sectioned on a Reichert Ultracut-S (Leica) ultramicrotome, stained in uranyl acetate and lead citrate (Reynolds 1963), and examined in a JEOL200CX transmission electron microscope.

RESULTS

OBSERVED CHANGES

Several distinct changes in plant physiology were observed in association with changes in water content, and are evident in figures 1(a) to (d). These changes include variation in stem and leaf position, as well as leaf pigmentation.

Figure 1: Observed changes in whole plant (i), and leaf (ii) physiology during rehydration and dehydration: (a) completely desiccated; (b) partially rehydrated; (c) full turgor; (d) partially dehydrated.

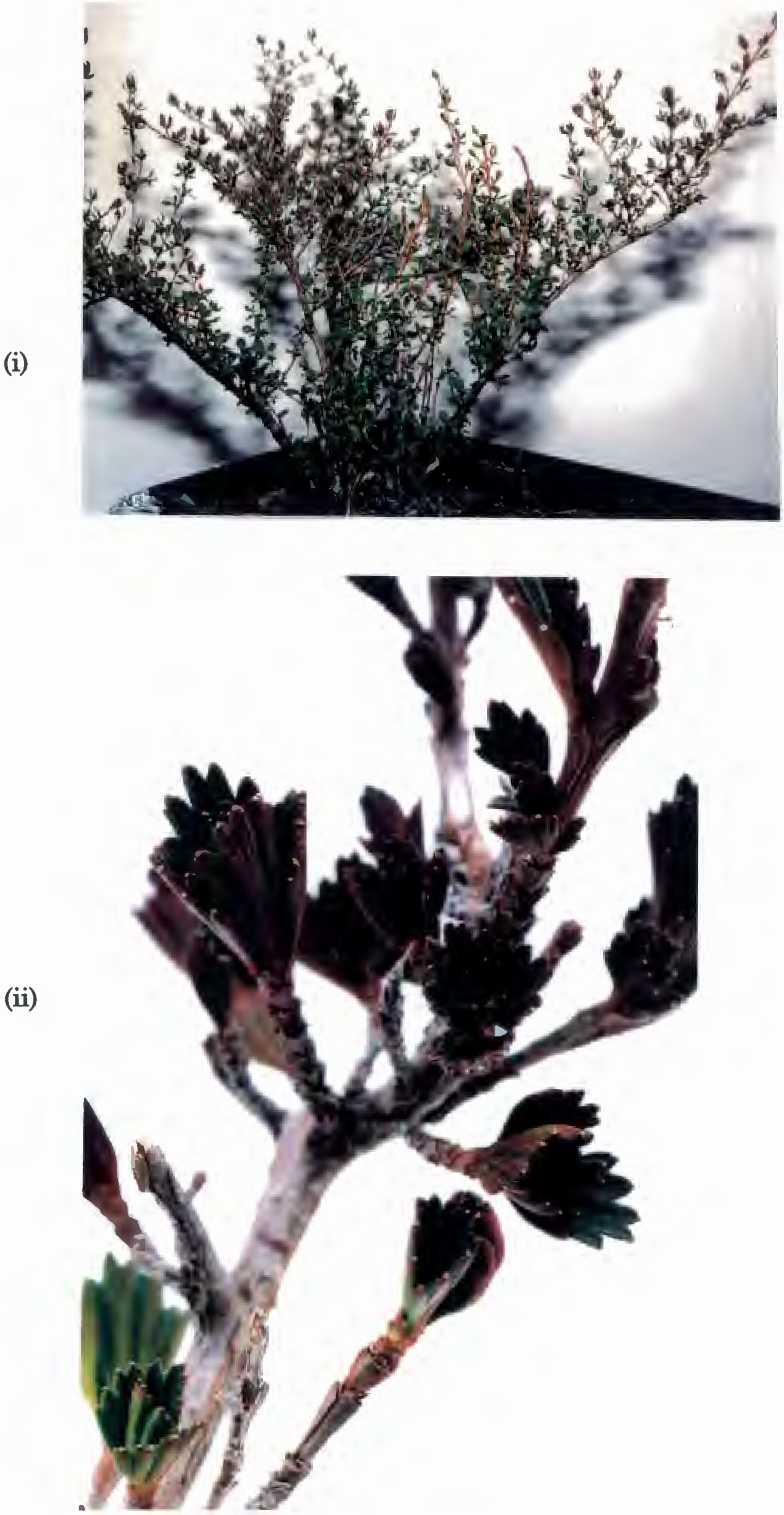


Figure 1 (b): Partial rehydration.

(i)

(ii)

Figure 1 (c): Full turgor.

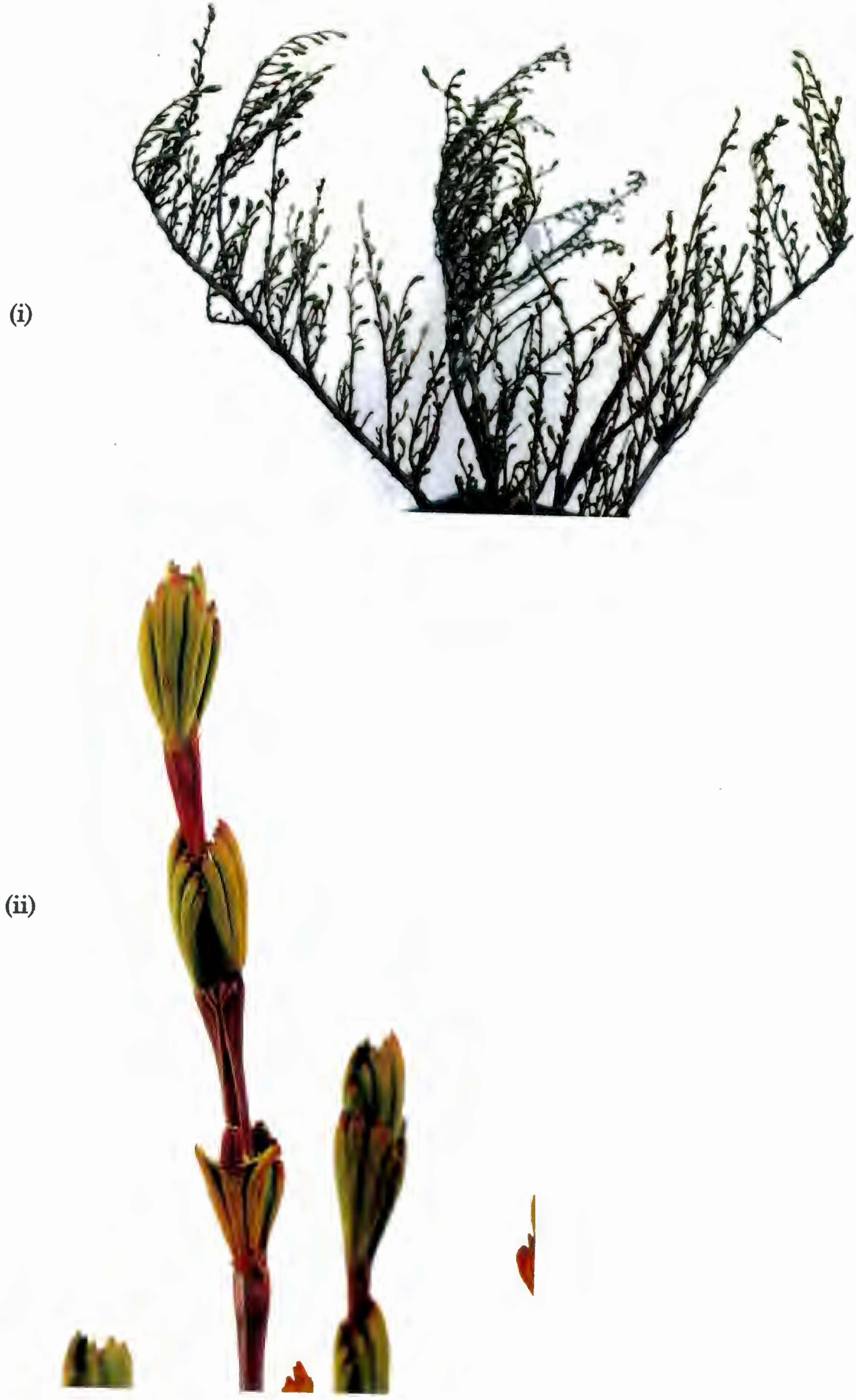


Figure 1 (d): Partial dehydration.

A comparison between the completely desiccated plant, Figure 1(ai), with the fully turgid plant, Figure 1(ci), shows that the main stems moved through an angle of between 5 and 45 degrees in association with changes in water content. During dehydration, the stems drew away from the ground, towards a central vertical axis, whilst upon rehydration, these stems opened out, thereby spanning a larger surface area.

Figures 1a (ii), through to Figure 1 d (ii) show the change in leaf angle with desiccation and rehydration. In the dry state (Figure 1a (ii)), the leaves are folded tightly against the stem, enclosing the apical bud. Rehydration causes the leaves nearest to the base of the plant to open first, followed by those higher up the stem (Figure 1b (ii)). Within 24 hours of watering, all the leaves are fully opened, and RWC has returned to between 80 and 100% (Figure 2). After 48 hours, the plant has recovered to a fully turgid state (RWC at 100%), all leaves having opened through 90 degrees (Figure 1c (ii)).

The time sequence for both dehydration and rehydration (Figure 2), showed that water content changed very rapidly during both these processes. There was a paucity of data for relative water contents between 10 and 40% because, during this time, no clear external differences in leaf orientation or colour took place, making it difficult to judge when to sample material for physiological studies.

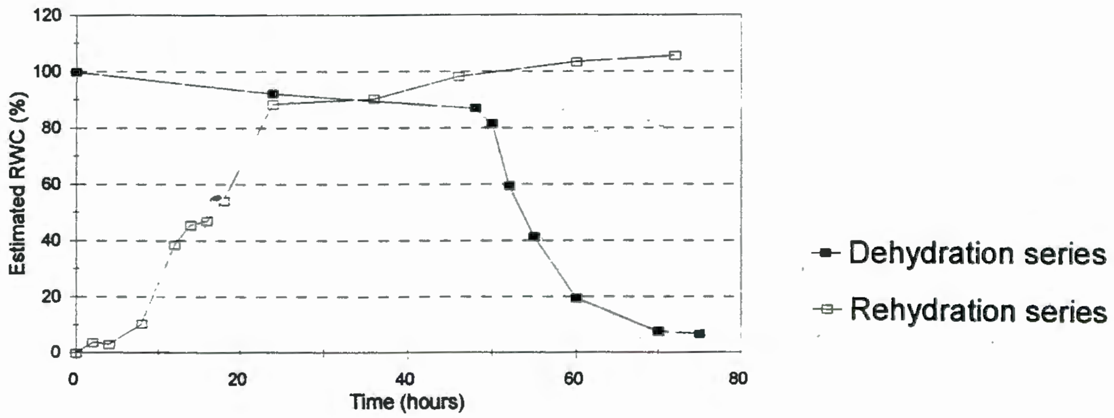
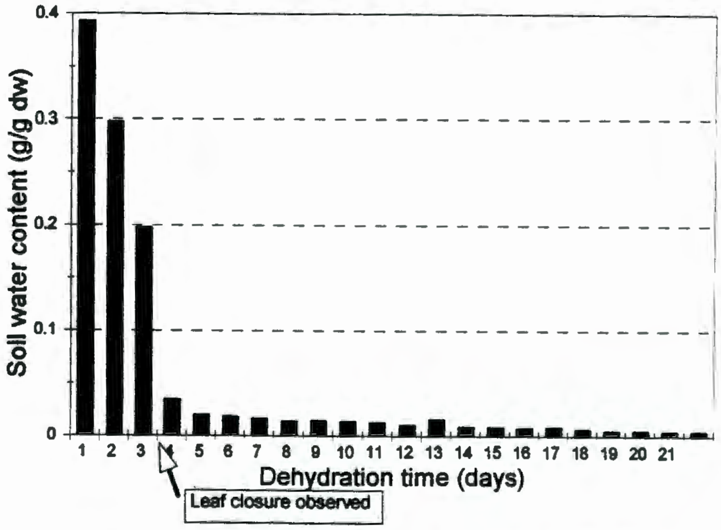


Figure 2: Time sequence of rehydration and dehydration series.

From Figure 2, it is evident that the dehydration process did not occur as rapidly as did that of rehydration. This was because dehydration is dependent upon the temperature and humidity of the surrounding air during the period of limited water availability, since this will affect soil water status and transpiration rate. What could be determined however, was that, following a decrease of relative water content to below 80%, and soil water status to less than 0.05 g.g^{-1}dw^{-1} (Figure 3), leaf closure occurred. Within 12 hours thereafter, RWC had decreased to less than 10% (Figure 2), and the leaves moved through an angle of 90° (Figure 1d (ii)). The leaf closing process involved plicate folding of the lamina, such that the furrows were protected from the external environment, whilst the ribs were exposed.



Leaf closure observed

Figure 3: Soil water profile during a dehydration series.

The changes in leaf pigmentation with change in water status are illustrated in the Figures 1a (ii), b(ii), c(ii) and d(ii). In the fully turgid state, the leaves were a bright-green colour (Figure 1c (ii)). Leaf closure, in association with dehydration, resulted in the underside of the leaves becoming a yellow-green colour (Figure 1d (ii)), this adaxial surface gradually turning a reddish-maroon shade (Figure 1d (iii)). Finally, following a week of exposure to normal light intensities and zero water availability, the adaxial surface became a dark red shade (Figure 1a (ii)).

CHLOROPHYLL AND CAROTENOID CONTENT

Changes in photosynthetic pigment concentration with relative water content are presented in Figure 4. The mean total chlorophyll concentrations obtained during the rehydration series were consistently lower than those calculated during the dehydration period (Figure 4a). Both showed, however, an average change of 2 $mg.g^{-1}dw^{-1}$ between the desiccated and fully turgid states. This suggests that between a RWC of 10 and 60%, *M. flabellifolia* lost approximately 32% of its total chlorophyll content. This chlorophyll was rapidly recovered, since approximately 80% of total chlorophyll was present by the time a 70% relative water content was reached.

From Figure 4b it can be noted that chlorophyll *a* concentrations, (during both the rehydration and dehydration series,) were consistently between one and a half, to twice as great as those measured for chlorophyll *b*.

Total carotenoid concentrations (Figure 4c), showed a trend towards increased carotenoid levels with increasing relative water content. Both the dehydration and rehydration sequences showed a decrease of approximately 40% total carotenoid content by a 5% RWC, with this loss occurring between 10 and 70% relative water contents.

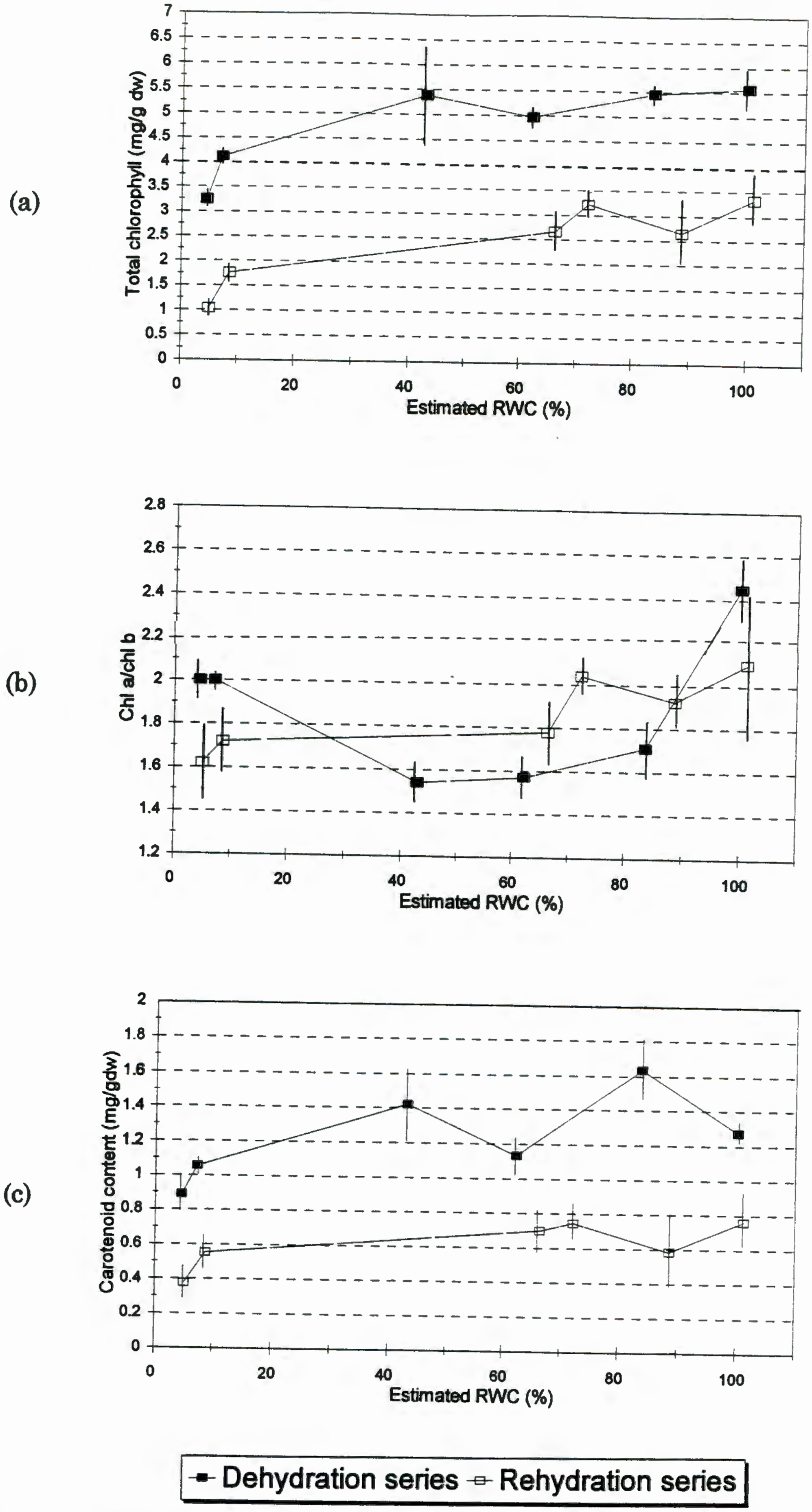


Figure 4: Mean pigment content: (a) total chlorophyll; (b) chlorophyll *a* to chlorophyll *b* ratio; and (c) total carotenoid content.

LEAKAGE DATA

Maximum leakage of 93 uA.min^{-1}g^{-1}dw^{-1} was achieved only when fully turgid leaf material was placed in liquid nitrogen (Figure 5). All other treatments were unable to induce leakage above 10 uA.min^{-1}g^{-1}dw^{-1}, suggesting that these treatments did not perturb the plasmalemma of the leaf tissue.

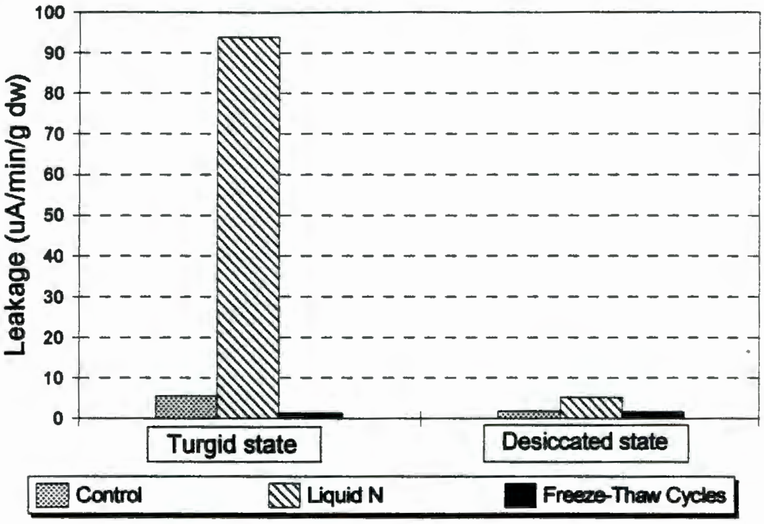


Figure 5: Average maximum leakage.

Electrolyte leakage, expressed relative to maximum leakage, is shown in Figure 6. There was very little leakage (averaging 12.6 uA.min^{-1}g^{-1}dw^{-1}), regardless of water status during the rehydration series. In contrast, the dehydration series appeared to show a slight increase in leakage when relative water content dropped below 20%.

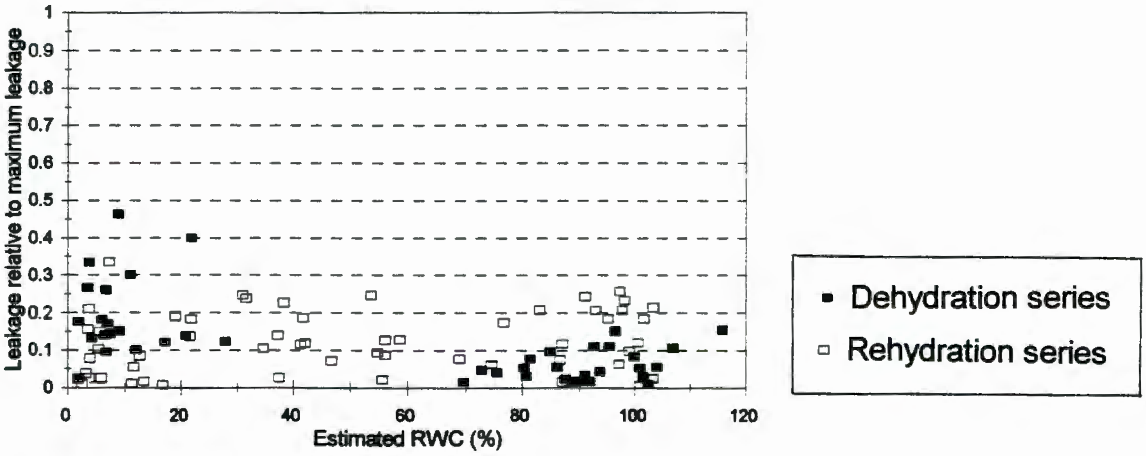


Figure 6: Leakage as a proportion of maximum leakage.

FLUORESCENCE ANALYSIS

The ratio of variable to maximum fluorescence described an S-shaped curve when plotted against RWC (Figure 7). The rehydration and dehydration data were congruent, with changes in quantum efficiency occurring at the same relative water contents, regardless of whether material underwent a dehydration or rehydration process. Above 80% RWC, the ratio of variable to maximum fluorescence averaged at 0.76. Between 40 and 80% RWC, the Fv/Fm values decreased in a linear fashion, whilst below 40% RWC, this value stabilised around 0.3.

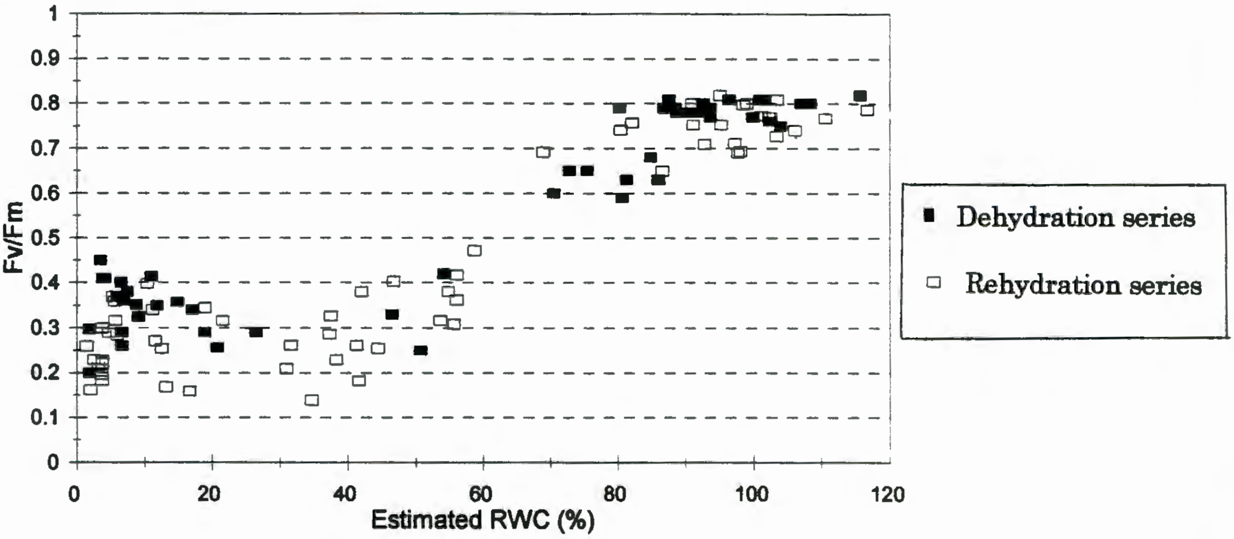


Figure 7: Changes in Fv/Fm with RWC.

ULTRASTRUCTURE

One of the problems associated with visualisation of ultrastructural status for *M. flabellifolia* involves the potential for rehydration during the aqueous fixation procedure. Since standard methods were applied, the possibility for changes in, or damage to cell ultrastructure, should be considered when interpreting these micrographs.

The micrographs in Figure 8 show the ultrastructural status of fully turgid mesophyll cells (100% RWC). These were highly vacuolated, with some vacuoles containing a densely staining material which has been termed “osmiophilic vacuolar material” (OVM), (Wellburn and Wellburn (1976); Goldsworthy and Drennan (1991)) (Figure 8a). Chloroplasts, with a novel thylakoid stack arrangement (Figure 8 b & c), were distributed around the perimeter of the cell (Figure 8a). Each stack was offset relative to the adjacent thylakoids, causing the granal stacks to “lean” from one side of the chloroplast to the other, often traversing a major part of the length of the plastid. The direction of leaning varied within a plastid, and this arrangement has led to the description of these stacks as “staircase” grana (Wellburn and Wellburn (1976)). The cytoplasm had a granular appearance, and the nuclear material was evenly dispersed (Figure 8a). Mitochondria did not have well defined cristae, but they were densely stained, indicating an active state (Figure 8b).

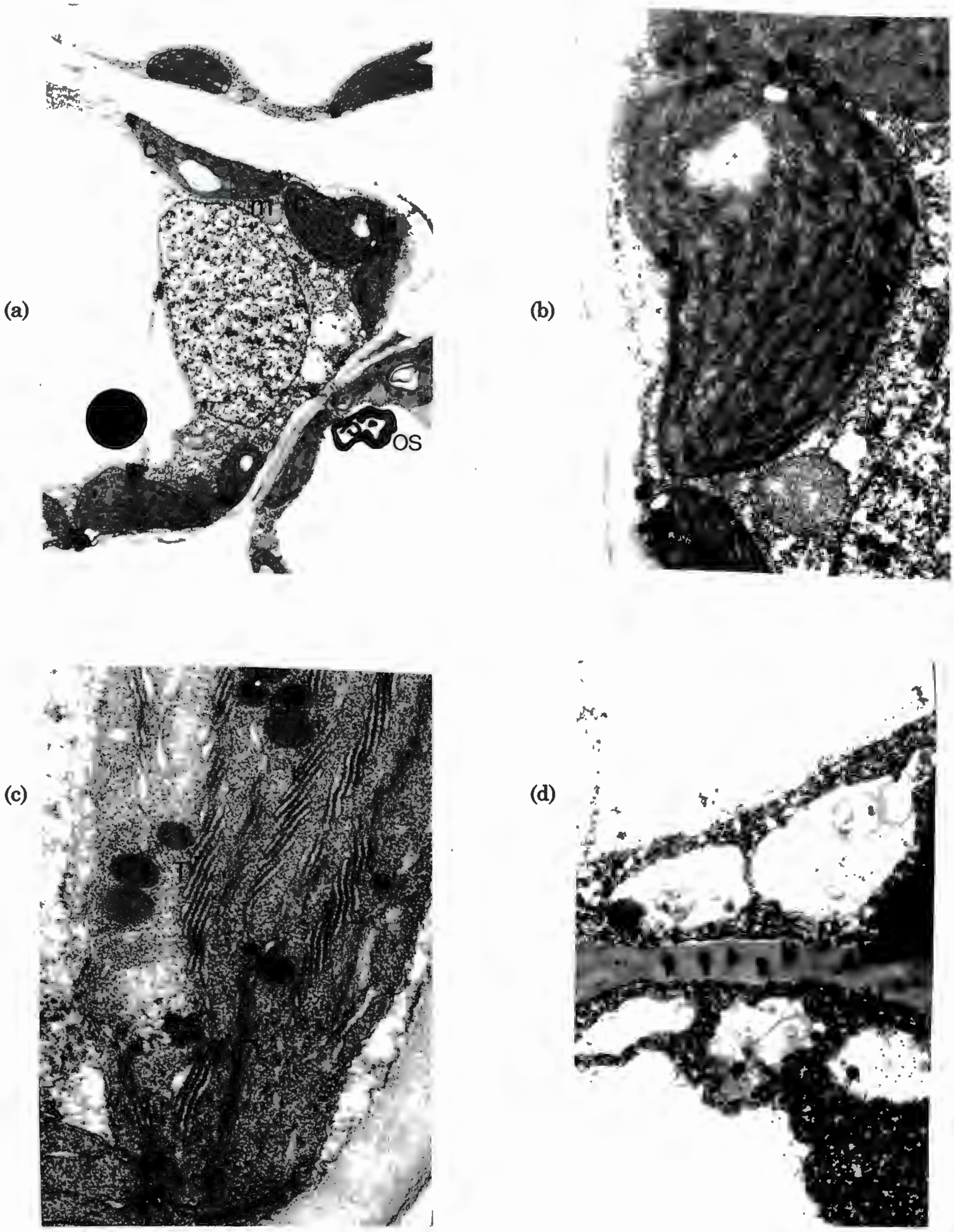


Figure 8: Fully turgid *M. flabellifolia* mesophyll tissue: (a) low power electron micrograph of adjacent cells with osmiophilic vacuolar material (**os**), chloroplasts (**c**), and mitochondria (**m**) at perimeter of cells. X3 000 ; (b) distinct membranes surround chloroplasts, mitochondria, and the nuclear membrane. X15 000; (c) "staircase grana" constituting the thylakoid stacks (**t**) X45 000; and (d), granular cytoplasm and plasmodesmata. X20000.

As dehydration occurred, the chloroplasts became compacted together, and assumed a rounded shape (Figure 9a). The staircase granal structure of the chloroplasts remained intact (Figure 9b), whilst the mitochondria became electron transparent, and cristae were not readily visible (Figure 9c). A sheath-like structure also appears to define the chloroplasts and mitochondria during this stage of dehydration.

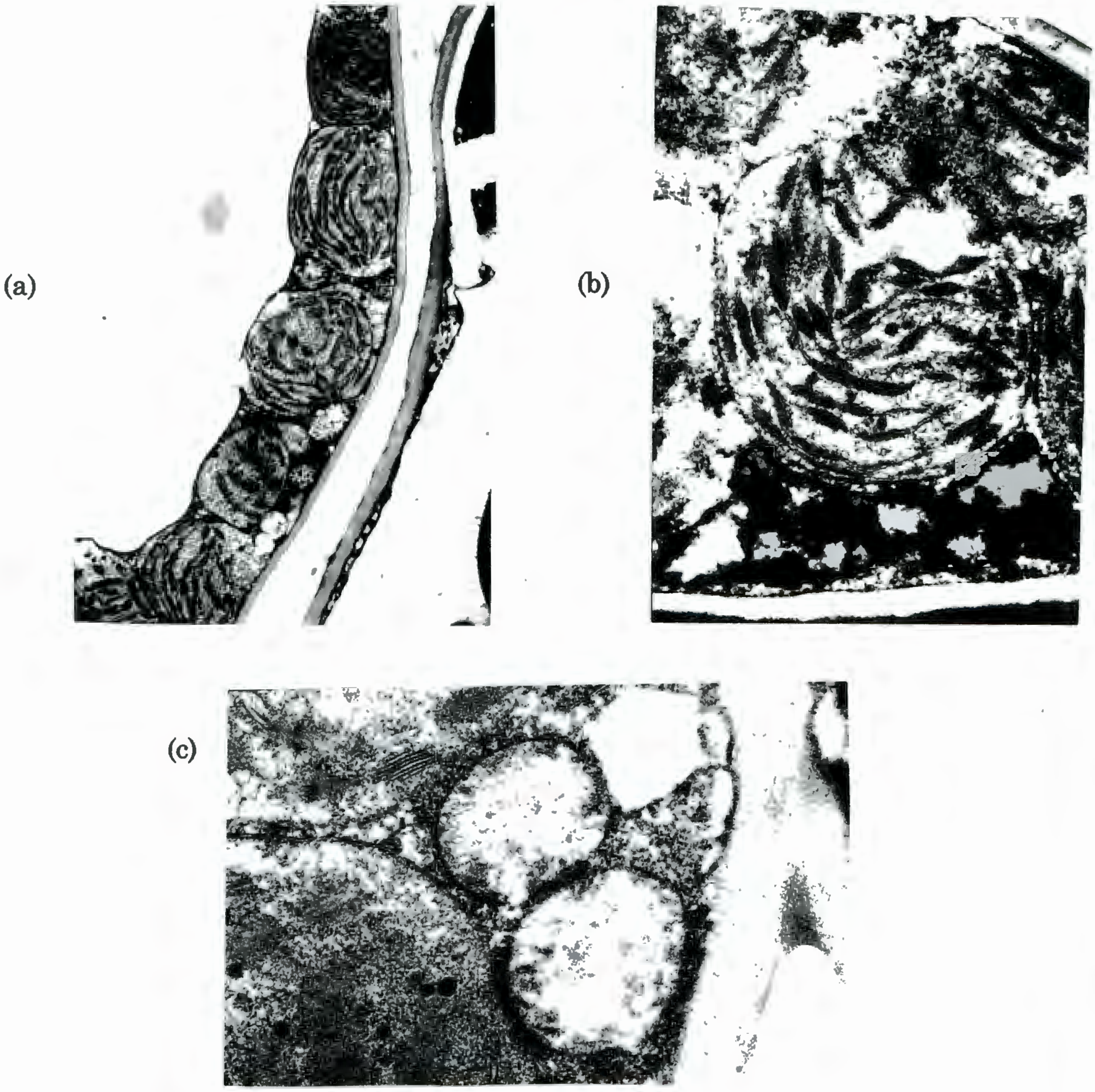


Figure 9: Mesophyll tissue dehydrated to *ca* 50% RWC: (a) chloroplasts rounded and compacted together X6 000; (b) thylakoid stacks maintained “staircase” structure X15 000; and (c), mitochondria became electron transparent X45 000.

In desiccated material (*ca* 1% RWC), cells were reduced in size, due to cell wall folding (Figure 10a&b). The central vacuole became fragmented into smaller compartments (Figure 10a), and the plasmalemma became separated from the cell wall in places, but remained continuous at cell-to-cell junctions (Figure 10a&b). Distinct surfaces outlining the chloroplasts and mitochondria were still evident. The chloroplasts remained circular, and the thylakoids became swollen in appearance (Figure 10c&d). Although the chloroplasts did not retain their "staircase" arrangement, the thylakoid membranes remained intact. In contrast to these thylakoid membranes, cristae were no longer evident inside the mitochondria (Figure 10d).

Following rehydration to 50% RWC, the large central vacuole had reappeared, and the plasmalemma had once more become appressed to the cell wall (Figure 11a&b). There was little evidence of membrane damage in any organelles (Figure 11). Nuclear material was somewhat coagulated relative to the fully hydrated state (Figure 11a), and mitochondria still had electron transparent regions within them (Figure 11b). The thylakoid membranes remained swollen, and had not returned to their staircase arrangement (Figure 11 b&c).

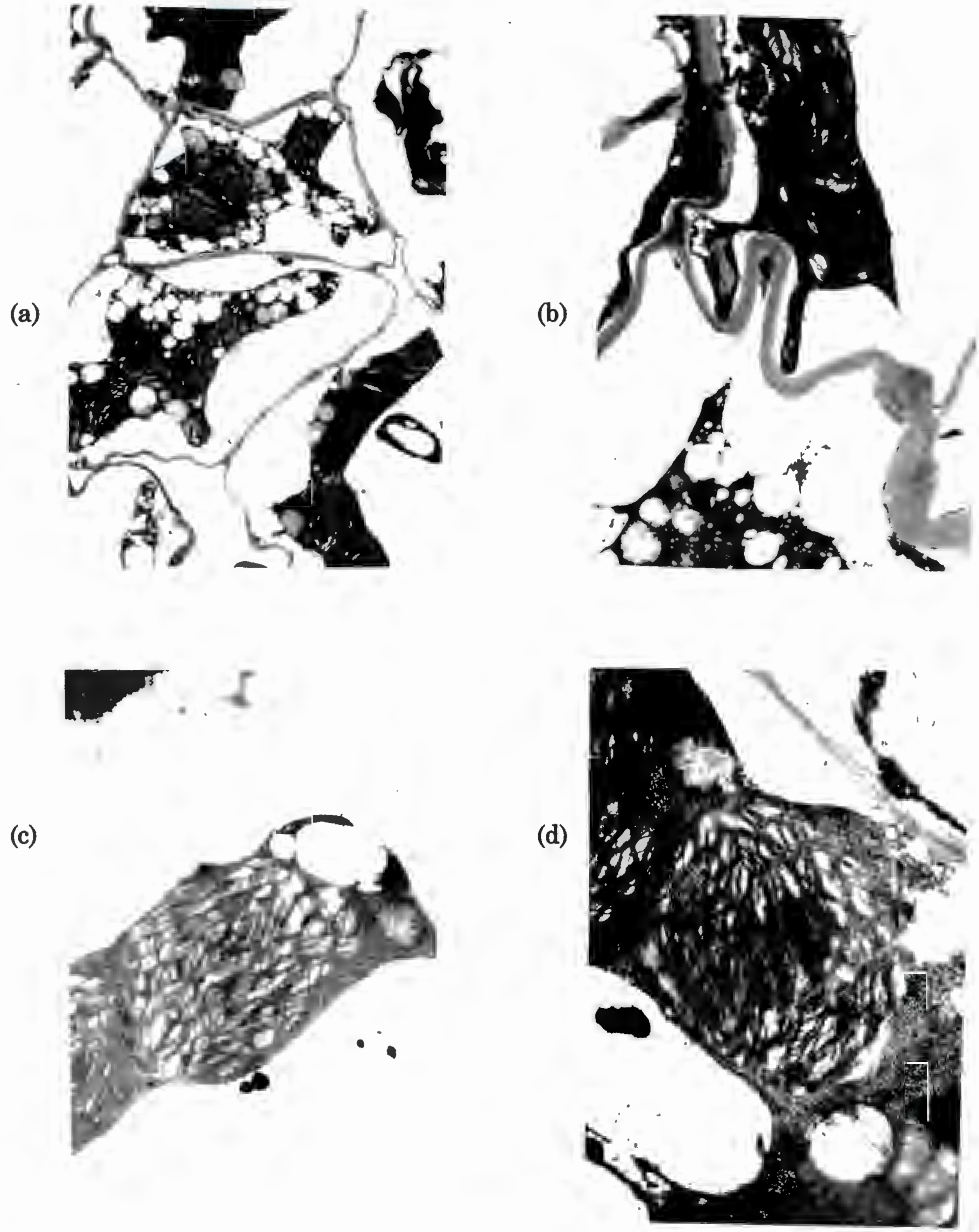


Figure 10: Mesophyll tissue dehydrated to *ca* 1% RWC: (a) several cells with numerous small vacuoles X3 700; (b) cell wall folding, with plasmalemma intact X7 300; (c) cellular contents maintained in centre of cell, away from cell wall X5000; and (d), thylakoid stacks arranged to fill entire chloroplast X15 000.

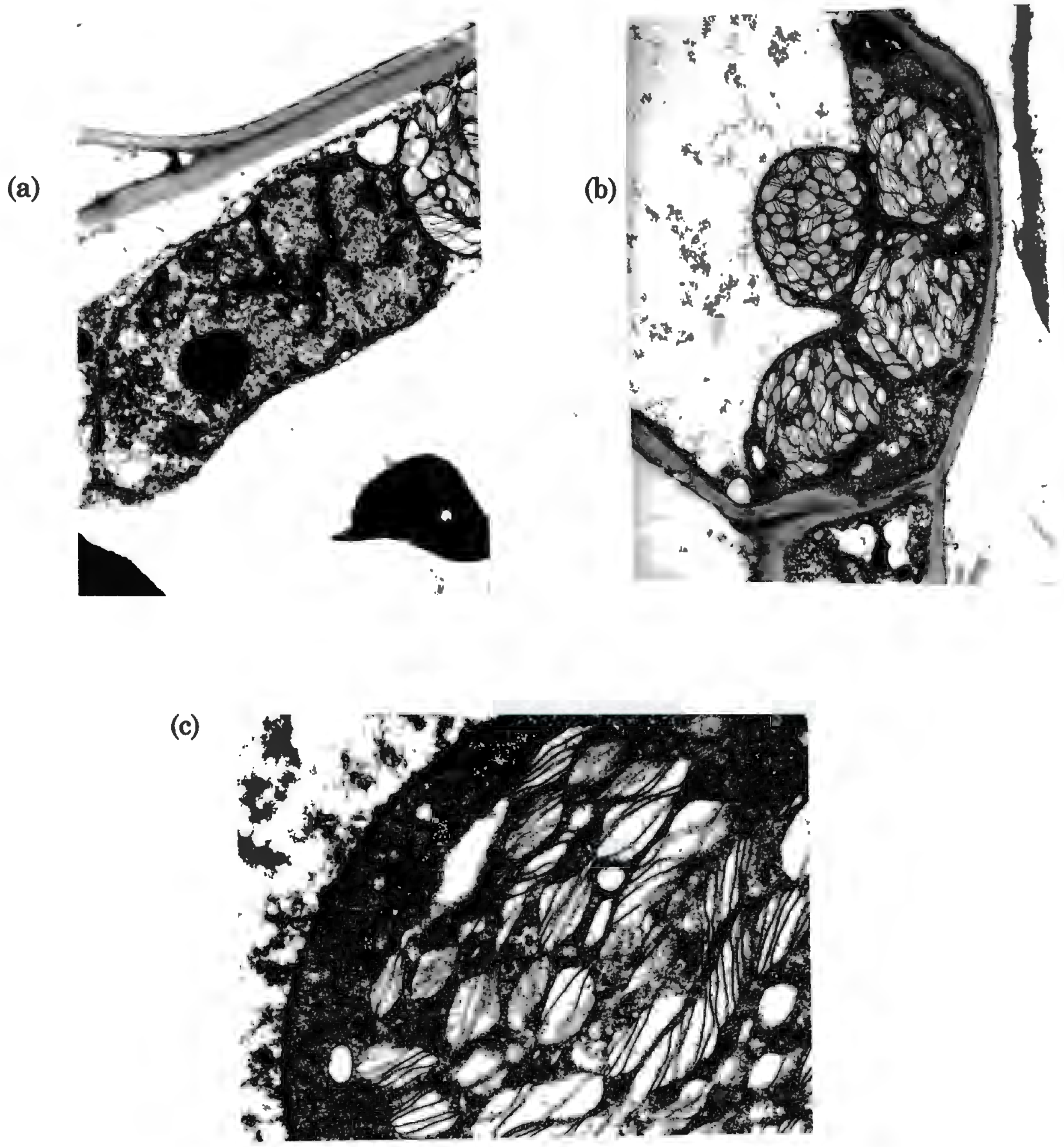


Figure 11: Mesophyll tissue rehydrated to *ca* 50% RWC: (a) intact nucleus and nucleolus X20 000; (b) thylakoid stacks swollen inside the chloroplasts X6 000; and (c) swollen thylakoid stacks, X30 000.

DISCUSSION

The ability for *M. flabellifolia* to tolerate desiccation below 5% RWC must impact upon a number of physiological processes. From this study, several patterns concerning the physiological responses of *M. flabellifolia* to water stress were investigated.

The most dramatic changes were those involving alteration in stem position, leaf orientation, and leaf pigmentation. The changes in both leaf and stem position would appear to reduce irradiance levels during the period of desiccation, and during early stages of rehydration, suggesting its possible role in the protection of microphyll surfaces from photoinhibition. Both these processes appear to be physical phenomena, since 'dead' leaves (those which had been oven-dried for 48 hours), were also able to return to their original shape when placed in water (not shown). Never-the-less, this physical process suggests that change in leaf and stem orientation might be important in *M. flabellifolia's* management of light stress during dehydration, and maximisation of light capture in the fully hydrated state.

Water stress predisposes plants to photoinhibition (Bjorkman and Powles, 1984). It would be predicted therefore, that mechanisms associated with decreasing surface exposure to high levels of irradiance would be initiated under waters stress conditions. Leaf rolling and stem curling, along with other changes causing reduction in surface area, have been documented as a first line of defense in reducing photoinhibition (Ehleringer and Cooper, 1992; Pereira and Chaves, 1993), and is supported from the results of this study.

The necessity for instigation of processes associated with the dissipation of light energy might also provide an explanation for the changes in pigment concentrations observed across the various water contents. Although there is a distinct change in pigment colour on the adaxial surface of the leaves of *M. flabellifolia* during dehydration, the abaxial surfaces (observed from the deliberate unrolling of the leaves,) remained a dark green colour.

VARIATION IN PIGMENT CONTENT

M. flabellifolia's ability to retain 70% of its total chlorophyll content during desiccation confirms its status as a homoiochlorophyllous desiccation-tolerant plant. Total chlorophyll content changed only slightly at water contents above 60%, with the 30% loss occurring at low water contents (<40% RWC). Total chlorophyll content recovery by 50% RWC (which was shown to occur within 12 hours of rehydration), might suggest the possibility that the chlorophyll undergoes a chemical conversion into a structure which was not discernible in the spectrophotometry analysis. An alteration of chlorophyll structure, into a form which is protective against photoinhibition, but able to convert back to its original state, might partially explain the rapid recovery of total chlorophyll concentrations upon rehydration.

The consistently lower levels of chlorophyll and carotenoid concentrations recorded for the rehydration, relative to the dehydration series, is possibly attributable to chlorophyll degradation (such as chlorophyll bleaching), associated with the length of time the plants had been maintained in the dried state, as well as the light levels to which they were exposed during this period. The difference between these pigment levels during the dehydration and rehydration series were not shown to be significant however (student t-test, $p>0.1$).

As has been commonly reported for angiosperms, chlorophyll *a* concentrations were consistently found to be twice as high as those values recorded for chlorophyll *b*. Neither chlorophyll *a* nor chlorophyll *b* levels show any wide fluctuations relative to each other in association with changes in leaf water status, although chlorophyll *a* appeared to decrease slightly when water content dropped below 80% during the dehydration series. Whether this indicates an increase in pigments associated with light-protection, rather than light-harvesting, is difficult to conclude, since it did not occur during the rehydration process, and a wide standard deviation was observed for these results. Never-the-less, since chlorophyll *a*/*b*-protein complexes are the major light-harvesting components in the photosynthetic membranes (Markwell, 1986), and since photosynthesis is rapidly able to recover, it is not surprising that these pigment levels to not significantly change with water content.

Carotenoids, the second most prominent pigment after chlorophylls, contribute to both light absorption and energy dissipation (Farquhar *et al.*, 1989). Carotenoid levels were not greater than those found for non-resurrection plants, occurring around a 4:1 chlorophyll:carotenoid ratio, and remaining constant, with only a decrease at relative water contents below 5%. This was surprising, since carotenoids are able to quench the excited states of chlorophyll which result in singlet oxygen formation, as well as being able to react with singlet oxygen (Stewart, 1989), thus providing a dual protective role expected to be necessary during the changes in RWC experienced by *M. flabellifolia.* This study indicates that such a system of protection is not operational in *M. flabellifolia,* rather, the chlorophyll is protected from damage by avoidance of light as the leaves fold tightly against the stem. The xanthophyll cycle has also been proposed as a mechanism for the orderly dissipation of energy in excess of that required by photosynthesis (Demmig *et al.,* 1987). Further research concerning the interconversion of xanthophylls might be helpful in unraveling possible processes associated with protection of *M. flabellifolia* from photoinhibition.

LEAKAGE

The inability to induce maximum leakage from even turgid leaves, along with the very low level of leakage noted across all water contents, indicates that little damage to the plasmalemma of *M. flabellifolia* was able to take place. This might suggest that some mechanism of membrane stabilisation is present, or perhaps that there are no configurational changes in membrane structure.

The leakage data provides no insight into the status of other subcellular membranes, since leakage would only be recorded upon damage to the plasmalemma. An almost sheath-like structure surrounding the chloroplasts and mitochondria can be observed from the ultrastructural micrographs during desiccation, perhaps having a role in preventing fusion of organelle membranes in the dehydrated state.

The nature of plasmalemma stability for *M. flabellifolia* remains unknown. Whether it is an integral part of this plants' membrane structure, (thus being independent of water status,) or whether it involves expression of new genetic information during water-stress, requires further investigation. Since the only protocol able to induce maximum leakage involved exposing hydrated tissue to liquid nitrogen, it would appear that the rate at which the stress is induced might be important. The possibility that gradual water loss allows for controlled changes in the configuration of macromolecules, which favour their stability, or which facilitate limited chemical reactions (thereby increasing resistance to disruption by dehydration), has been suggested (Bewley and Krochko, 1981). Experimentation involving rapid artificial dehydration might provide insight into these processes.

FLUORESCENCE ANALYSIS

The Fv/Fm ratio for *M. flabellifolia* at relative water contents above 80%, were similar to those reported for a variety of plants at full turgor (Sherwin, 1995). Averaging at 0.76, this is indicative of intact and fully functional photosynthetic apparatus.

In contrast to the changes in quantum efficiency with decreasing RWC reported for non-resurrection plants, (i.e. where photosynthetic efficiency is maintained until 30% RWC, whereafter it declines suddenly (Sherwin, 1995)), Fv/Fm for *M. flabellifolia* shows a linear decrease with decreasing RWC between 80 and 40%. Fv/Fm for *M. flabellifolia* is also unusual in its stability around 0.3 at water contents below 40%, compared with those of non-resurrection plants, which continue their linear decrease to values around 0.1 (Sherwin, 1995).

The linear relationship of Fv/Fm between 40 and 80% RWC, during both the dehydration and rehydration processes, may be indicative of an ordered sequence of events enabling *M. flabellifolia* to prepare its photosynthetic apparatus for either desiccation or photosynthesis. The deliberate deactivation of photosystems, and their arrangement into a stable state before water content has dropped to levels too low for any metabolic activity (appearing from this data to be at water contents below 40%,) might be part of the mechanism by which *M. flabellifolia* is able to tolerate repetitive desiccation and rehydration. A shallow decrease between 55 and 18% RWC reported for the resurrection plant *Craterstigma nanum* was also suggested to be indicative of a deliberate shut-down process (Sherwin, 1995), however no stabilisation below 18% RWC was observed for this plant.

The stability of Fv/Fm for water contents below 40%, would appear to indicate that light stress is not responsible for the decrease in this ratio during the intermediary water conditions. If photoinhibitory effects were responsible for decreasing Fv/Fm, these effects would be expected to be manifest below 40% relative water contents. This data suggests that although disruption of photosynthesis, and deactivation of PSII complexes does occur, these changes are induced, (or removed) around 80% RWC. If damage is taking place, then the staircase granal structure may be important in allowing for rapid repair, since, it has been speculated (Baker, 1993)that the complexes must migrate from an appressed thylakoid domain to a non-appressed region, where the protein can be degraded by a membrane-bound protease, and the staircase granal arrangement effectively decreases appressed thylakoid surface area.

ULTRASTRUCTURE

From the leakage, as well as the fluorescence results discussed above, it can be concluded with a relatively high level of certainty that the plasmalemma, and possibly the thylakoid membranes, remain intact regardless of plant water status. The ultrastructural study supports these conclusions, showing that membranes of organelles appear to remain intact, there being particularly noticeable sheath-like structures around both the chloroplasts and mitochondria during both the dehydration and dehydration processes. Only in the completely dehydrated state do these outlining surfaces become less discernible, largely due to the more compact nature of the cytoplasm, rather than disappearance of these structures. This maintenance of ultrastructural integrity confirms the purely ultrastructural study undertaken by Goldsworthy and Drennan (1991) on the rehydration process of *M. flabellifolia*, and infers the possibly crucial contribution which the maintenance of structural integrity plays in allowing rapid recovery of this plant following rewetting.

The novel chloroplast ultrastructure of *M. flabellifolia* suggests that it may contribute towards this plants ability to tolerate desiccation. In the turgid state, the offset position of the grana increases contact with the stroma, whilst decreasing the appressed membrane surface area. Whether this is involved in a reduction of energy transfer between PSI and PSII is an interesting possibility, considering that this mechanism might decrease the ability for the occurrence of hazardous reactions associated with excess irradiance.

During dehydration the chloroplasts become swollen, but maintain their outer membrane structure, whilst the thylakoid membranes remain intact, losing their staircase arrangement. These distinct changes in chloroplast structure might be part of a protective process occurring in *M. flabellifolia.* The dedifferentiation of the granal stacks, reducing appressed and non-appressed surfaces, and rather widening to fill the entire chloroplast volume, might be indicative of mechanical support and chloroplast packaging, conferring protection during desiccation and subsequent rehydration.

During water stress, the cell walls undergo some folding which decreases cell volume, and probably contributes to the prevention of plasmolysis during the low water contents experienced by the cells. As noted earlier in the discussion, there is no disruption of the plasmalemma, although it undergoes localised separation from the cell wall, maintaining contact at cell-to-cell junctions. The apparent reduction in number of plasmodesmata present in these cells might also be part of the mechanisms minimising plasmalemma disruption during dehydration. The mitochondria of *M. flabellifolia* do not show any novel changes during the dehydration-rehydration processes, however the development of electron transparent spaces within them suggests that respiratory processes might be halted in the desiccated state.

The unusually granular nature of the cytoplasm, regardless of water content, as well as the presence of densely staining material (OVM), makes one curious as to their role in stress tolerance. Wellburn and Wellburn (1976), suggested that OVM is lipophyllic in nature, thus it may have a role in membrane stabilisation, alteration, or repair. This requires further investigation.

FUTURE RESEARCH

M. flabellifolia has shown a number of unusual features, raising interesting evolutionary, and physiological questions for the future. Future studies should consider the possible relationship between membrane permeability and abscisic acid (ABA) levels since, depending on the species and conditions, ABA has been reported to increase or decrease membrane permeability (Tan and Blake, 1993). The possible role of the unusual sugars found in *M. flabellifolia* (Bianchi *et al.,* 1993; Suau *et al.,* 1991; Drennan *et al.,* 1993; and Tymms and Gaff, 1979), also shows potential for improving our understanding concerning the role of these substances during conditions of osmotic stress. The glucoside arbutin is already under scrutiny, comprising 20% of *M. flabellifolia* leaves in the desiccated state (Suau *et al.,* 1993), and being found in an increasing number of plants adapted to stress conditions such as arctic low temperatures, (*Vaccinium* spp., *Arctostaphylos* spp., (Holopainen *et al.,* 1988)), and drought stress (*Leucodendron,* (Glennie, 1980), and *M. flabellifolia* (Suau *et al.,* (1993)). Research into the mechanisms preventing free radical damage is also likely to arise from future investigations into *M. flabellifolia,* Suau *et al.,* (1993) having noted that the two-ring hydroxylated structure of the arbutin molecule reduces the rate of peroxidation of unsaturated plant membrane lipids.

CONCLUSION

This study has highlighted some of the remarkable aspects associated with desiccation tolerance of *M. flabellifolia.* The structural stability, shown across the complete range of water conditions, can only be described as incredible. Although it is not yet possible to suggest an all-encompassing strategy operating in this plant, modifications preventing deterioration of the membranes would appear to be superbly effective, as is the maintenance of chlorophyll content, allowing rapid recovery to maximum levels of plant productivity upon availability of water.

Knowledge of how plants avoid irreversible damage from dehydration is important for the preservation of seeds and germplasm. Its possible application in cereal crops, where drought is one of the most important factors limiting crop yields worldwide, further highlights the potential contribution arising from research in this field. Understanding, and exploiting, the resistance of some plants to environmental factors such as not only drought, but also water-logging, temperature extremes, and high salinities, are regarded not simply as physiological and ecological problems, but increasingly as important goals of internationally economic, political, and humanitarian significance (Wyn Jones and Pritchard, 1989).

Improved knowledge obtained from investigation into the physiological processes observed from resurrection plants such as *M. flabellifolia,* can only further our understanding of the role played by water in maintaining structural and functional integrity of membranes and macromolecules. The controlled metabolic shutdown which occurs as plants pass into a state of anhydrobiosis may provide valuable insights into the functional significance of the metabolic responses of water deficits in mesophytes.

Finally, this study has served to expose our vast ignorance concerning drought physiology, indicating the enormous amount of research necessary to begin to unravel the complex processes associated with "resurrection" ability. Information such as root signaling in recognition of water deficit; hormone control in regulation of plant developmental response; as well as xylem function and cavitation, are all areas which require explanation. Since none of this work has been undertaken on *M. flabellifolia,* these mysteries provide a fertile field for study, promising to yield possibly complicated, but no less amazing discoveries.

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