Mohria caffrorum (L.) Desv.

A new, unique model-organism for the study of desiccation tolerance

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Abstract

Vegetative desiccation tolerance is the ability of a plant to dry to equilibrium with it's surrounding atmosphere and remain in this dry state for prolonged periods of time. Upon rehydration, these plants are able to regain full physiological functionality. Vegetative desiccation tolerance is common and partially understood in the less complex clades including the algae, bryophytes and lichens but is uncommon in the vascular land plants. Vegetative desiccation tolerance is partially understood in the angiosperms but there has been little work on desiccation tolerance in pteridophytes. *Mohria caffrorum* (L.) Desv. is one such desiccation tolerant pteridophyte. This fern has the unique characteristic of having both desiccation tolerant and desiccation sensitive forms, which alternate seasonally, which this study is the first to show. We show here that; 1) *M. caffrorum* is indeed a desiccation tolerant fern. 2) *M. caffrorum* has desiccation tolerant and desiccation sensitive frond types, which are morphologically distinct. 3) These morphological differences are directly related to the ability to survive desiccation as well as playing a role in the ecologies of the two growth forms. 4) The spores of *M. caffrorum* are also desiccation tolerant. A hypothesis is proposed detailing why *M. caffrorum* is able to change between being desiccation tolerant and desiccation sensitive.

Introduction

Most plants are sensitive to water stress and cannot persist in times of low water availability. However there are a few species of plants that can tolerate desiccation to an extent where almost all protoplasmic water is lost (Well documented examples are *Tortula caninervis, Craterostigma wilmsii, Myrothamnus flabellifolius* and *Xerophyta humilis*). Desiccation tolerance is common in bryophytes, but uncommon in both pteridophytes and angiosperms. There are no known gymnosperms that are desiccation tolerant (Alpert & Oliver, 2002). Desiccation tolerant "resurrection plants" (Gaff, 1971) are able to dry to less than 5% relative water content (RWC) for long periods of time (6 months to 5 years) and then, upon re-wetting, are able to rapidly (12 to 72 hours, depending on the species) regain full physiological functionality (Gaff, 1977). Although, some recent studies (e.g. Farrant & Kruger, 2001) show that the resurrection plants, under field conditions, seem to only be able to remain dry for up to about 12 months rather than 5 years, this is still a unique and

valuable mechanism of surviving extreme water stress. There is still much to learn before it is understood exactly how these plants are able to survive such adverse conditions. The ultimate goal behind many of the studies of desiccation tolerance in plants is to identify the genes responsible for this trait as well as the mechanisms of their activation. These genes can then possibly be used in transgenic food crops, which could increase drought tolerance (Farrant 2000), thus providing crops to the third world, which is plagued by frequent droughts. Most of these so-called 'resurrection plants' are endemic to Southern Africa (Gaff, 1971; Gaff, 1977; Alpert & Oliver, 2002) and therefore this provides a natural centre for research into these mechanisms.

The desiccation tolerance mechanisms of bryophytes are well documented (Oliver *et al*, 2005; Oliver *et al*, 2000; Proctor, 2001). Bryophytes rely mainly on rehydration-induced recovery and repair processes coupled with constitutive cellular protection mechanisms (Oliver *et al*, 2000). We are also beginning to understand and unravel the desiccation tolerant mechanisms of some of the angiosperms (e.g. Vertucci & Farrant, 1995; Alpert & Oliver, 2002; Sherwin & Farrant, 1996; Sherwin & Farrant, 1998; Farrant, 2000). In angiosperms, it is believed that extensive protection is laid down during drying, to minimize repair processes on rehydration and also to minimise the cost of having constitutively active protection mechanisms (Oliver *et al*., 1998; Farrant, 2000; Walters *et al*., 2002). Angiosperms thus rely on many different protection mechanisms to survive desiccation. These include: (1) the controlled loss (poikilochlorophyllous) or retention and protection (homoiochlorophyllous) of chlorophyll (e.g. Sherwin & Farrant, 1996), (2) the accumulation of free-radical quenching anti-oxidants (e.g. Smirnoff, 1993; Farrant, 2000), (3) the accumulation of sugars and proteins to maintain subcellular integrity (e.g. Vertucci & Farrant, 1995; Whittaker *et al*, 2001; Illing *et al*, 2005), and (4) many also undergo structural alterations to prevent the tearing of the plasmalemma from the cell wall during the resulting shrinkage from drying (Vicre *et al*, 2004; Farrant *et al*, 2003). Many plants use a combination of these mechanisms and they are not mutually exclusive. Most of these protection mechanisms are initiated during drying before the amount of water lost becomes damaging. The angiosperms therefore, rely predominantly on protection rather than repair mechanisms. It is believed that this is why most angiosperm desiccation tolerant species cannot withstand rapid dehydration (Oliver *et al*, 2005;

Balsamo *et al*, 2005). Pteridophyte desiccation tolerance has been observed and documented (Alpert & Oliver, 2002; Proctor & Pence, 2002; Muslin & Homann, 1992; Pessin, 1924; Lebkuecher & Eickmeier, 1993) but to date has not been studied in great depth. There has been some work on the characterization of protein synthetic changes and the effects of Abscisic Acid (ABA) on the desiccation tolerant fern *Polypodium virginianum* L. and it has shown that fern desiccation tolerance shares mechanisms with both desiccation tolerant Bryophytes as well as desiccation tolerant Angiosperms (Reynold & Bewley, 1993a, 1993b). This study aims to be one of the first to characterise the physiological aspects of desiccation tolerance in pteridophytes.

There are 3 main categories of stresses, which desiccation tolerant plants have to overcome:
(1) Oxidative stress related to the disruption of metabolism. As water is lost, electron transport is disrupted and this causes the formation of free radicals such as reactive oxygen species (ROS) commonly, O_2^- and OH^- (Smirnoff, 1993; Farrant, 2000).
(2) The loss of membrane integrity (Vertucci & Farrant, 1995) due to the absence of hydrophilic and hydrophobic interactions, which usually hold them together.
(3) Mechanical stress due to loss of turgor causing tension on the plasmalemma during shrinkage. There are also mechanical stresses associated with rehydration (Vertucci & Farrant, 1995).

This aim of this study is to characterise the nature of desiccation tolerance in the desiccation-tolerant fern, *Mohria caffrorum* (L.) Desv. (Gaff, 1971; 1977). *M. caffrorum* is common in Southern Africa and grows in semi-exposed to fully-exposed habitats on forest margins in low, moist fynbos (Roux, 1979). It occurs abundantly on the slopes of Table Mountain. Although reported anecdotally to be desiccation tolerant (Gaff 1971; 1977) there is no data if this is indeed so, and if so, what mechanisms of desiccation tolerance are employed by *M. caffrorum*.

From preliminary personal observations in January 2006, it appeared that *M. caffrorum*, like angiosperms, relies on some induced protection mechanisms to survive desiccation. During drying the fronds fold and curl to expose the scale covered abaxial surface of the frond to the incoming light, they also appear to retain

chlorophyll during dehydration (i.e. homoiochlorophyllous). Preliminary investigations throughout 2006 also showed that *M. caffrorum* appears to have different summer and winter frond types. This study showed that the summer growth form was desiccation tolerant while the winter form was not. This is a unique mechanism, which has not yet been described in any other resurrection plant. Therefore, the aim of this study was to characterise and describe of this unique mechanism of desiccation tolerance.

In particular, this project examined the physiological and morphological aspects of the two growth forms of *M. caffrorum's*. The 3 main objectives of this project were to:

1) Determine whether *Morhia caffrorum* is indeed a resurrection species and if the two frond types display different levels of desiccation tolerance.
2) Describe the morphological and physiological differences between the two frond types.
3) Test whether the spores of *M. caffrorum* are also desiccation tolerant.

Methods

Approximately 30 plants from Eastern slopes of Table Mountain were originally collected for this study in April and May 2006. The plants were potted with as much soil from where they were transplanted from, and the rest was topped up with potting soil. They were watered regularly and allowed to acclimatise for at least two weeks, in the greenhouse located on the grounds of the University of Cape Town, before experimentation. It was initially not known that *M. caffrorum* had different frond types. Thus, by the time experimentation began on the potted plants in July 2006, all initial work was done on a single frond type, which appeared not to be desiccation tolerant. In September 2006, it was noted that the wild populations of *M. caffrorum* began to resemble those first observed in January 2006 and that there were two morphologically distinct frond types present. The two frond types were later identified by the presence (apparently tolerant summer fronds) or absence (apparently sensitive winter fronds) of abaxial scales. With the most apparently tolerant fronds appearing to have a dense, brown layer of abaxial scales. These two frond types will be referred to as SP (Scales Present) and SA (Scales Absent) for the remainder of the paper. To

compare between the SP and SA frond types all experiments on the SP form were performed on fronds collected from wild populations of *M. caffrorum* as time did not allow for the re-collection and acclimatisation of new plants. As all greenhouse plants only had SA fronds, experiments on SA fronds were performed on a mixture of fronds collected from both wild and the greenhouse individuals as it was not known whether the apparent sensitivity to desiccation was due to plants being grown in greenhouse conditions. In all experiments, there was no apparent difference in the response of the SA fronds from wild or greenhouse populations (not shown) so all data for SA fronds was combined. Where possible, only fully expanded, mature fronds of roughly equal size were used for the following experiments. Wild fronds and plants were collected from two main sites on the Eastern Slopes of Table Mountain. The first site was located just above the contour path, above Kirstenbosch Gardens and the second was located in Waterfall ravine near Rhodes Memorial.

Drying Course

Five fronds of each frond type (SP and SA) were sampled from different individuals to ascertain a drying course. The fronds were removed from the plants, weighed and then allowed to dry in lab conditions (20°C – 25°C, 45% - 55% RH). The fronds were then weighed at regular intervals during dehydration. After 22 hours, once the mass of the fronds had appeared to have stabilised, the base of each frond was placed into distilled water. The water level was adjusted so that the bottom two pinnae of each frond were submerged, and the fronds were allowed to rehydrate for a further 22 hours. The fronds were weighed at regular intervals during rehydration. Before weighing, excess surface water was removed by dabbing the fronds with paper towel. After the final measurement, the fronds were oven dried at 70°C for 48 h and then weighed to obtain a dry mass.

The common method of measuring relative water content RWC (Explained in Farrant, 2000) did not give accurate results in the excised frond of *M. caffrorum* as the full turgor water content was found to be lower than the maximum water content of either of the frond types in the above experiment. This may be an artefact of performing the experiments with excised fronds instead of whole plants where the root system may moderate water uptake. Therefore the modified method described below was used.

The absolute water content (AWC) of the frond at each time interval was calculated gravimetrically using the following formula, AWC = (M - DM)/DM where M = mass and DM = dry mass. This measurement was then expressed as a percentage of the maximum AWC of that frond type (SP or SA) to give relative water content (RWC) for that frond type, which was then plotted against time.

Ultrastructural Studies

Scanning electron microscopy (SEM) was used to investigate the fine surface details of wet and dry fronds. Three fronds from three different individuals were investigated for each of the frond types. The abaxial and adaxial surfaces of fronds were examined to view the status, morphology and orientation of the cells and scales of dehydrated and hydrated fronds as well as gross morphological differences between SP and SA fronds. The SEM was also used to investigate the surface structures of the fertile fronds to attempt to confirm if they originated from SP or SA fronds. Samples were fixed in 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.2). Following alcohol dehydration, the samples are critical point dried and then mounted on aluminium stubs. Finally, the samples are sputter coated with gold palladium before viewing with the SEM (LEO fully analytical S440 Scanning Electron Microscope, Cambridge, UK). Wet and dry samples were also cryo-fixed by plunging into liquid nitrogen and then viewed using the SEM. Freeze fracturing was used to view cross-sections of frond samples.

Light microscopy was also used to investigate the morphological differences between the SP and SA fronds, in both the dehydrated and hydrated state. The morphology of the fertile fronds was also examined. A minimum of 3 fronds from separate individuals were investigated. No fixation was used and whole fronds or hand sectioned tissue slices were examined. A Nikon SMZ1500 Stereoscopic Zoom Microscope (Nikon Corporation, Tokyo, JAPAN) was used for all of the light microscopy.

Time Lapse Photography

Time lapse photography was used to help visualise the drying and rehydration of both SP and SA fronds. It was also used to illustrate the finding that the SP fronds rehydrate while the SA fronds do not. A single frond of each frond type (SA and SP)

was used for the time lapse photography. Each frond was photographed before dehydration and then allowed to dry in lab conditions (20°C – 25°C, 45% - 55% RH) for 24 hours. The fronds were then rehydrated by placing the base of the frond into water. The water level was adjusted so that the bottom two pinnae of each frond were submerged. A Co\anon Powershot S2 IS (Canon Inc., Tokyo, Japan) digital camera was used to photograph the fronds at 10second intervals during the rehydration process. Once the fronds had rehydrated, the images were used to make a time-lapse movie with one image used per frame using Adobe Premiere Pro 2.0 (Adobe Systems Inc. San Jose, CA.). The frame rate of the movie was set to 27 frames per second and compressed to standard windows .AVI format.

Specific Leaf Area

The specific leaf area (SLA) has been shown to be a good estimator of frond thickness. SLA is measured by dividing the frond area by the frond dry mass (Vile *et al*, 2005). Fronds were placed on a white background with a 1cm^2 black reference square. The fronds were then photographed with a Canon Powershot S2 IS (Canon Inc., Tokyo, Japan) digital camera. Images were analysed in Adope Photoshop CS2 Version 9.0 (Adobe Systems Inc. San Jose, CA.) and the relative size of the frond to the reference square was measured to give a frond area in cm^2. The frond was then oven dried at 70°C for 48 h and then weighed to obtain dry mass. A total of five SP and five SA fronds were used for the measurements of SLA. An analysis of variance (ANOVA) was performed on the SLA data to investigate whether there was a significant difference in SLA's between the SP and SA fronds.

Electrolyte leakage

The membrane integrity of fronds was assessed by measuring electrolyte leakage from frond samples over time. Electrolyte leakage of pinnae sampled from dehydrated as well as fully hydrated SP and SA fronds samples was measured using a portable, Autoranging Microprocessor Conductivity/TDS meter (Model HI 9835, Hanna Instruments, Rhode Island, USA). The conductivity of distilled, de-ionised and filtered water was measured every 5minutes for 40minutes to identify any background electrolyte signal before samples were added. Samples were then added and the conductivity was again measured every 5minutes for 60minutes. The samples were

then oven dried to obtain a dry mass and the conductivity measurements were then corrected for leaf dry mass (Sherwin & Farrant 1996) and expressed as µS/g dry mass. All measurements were performed in triplicate. An ANOVA was performed to investigate whether there was a significant difference in electrolyte leakage between the samples and, if a significant difference was found, a post-hoc Tukey HSD test was also performed to tell which samples were responsible for the significant difference.

Spore Germination

The following methods for spore germination were modified from Dyer, 1979. Spores were collected by removing fertile fronds and placing them on clean, white paper with the sporangia facing down. Each frond was then covered with a petri-dish and left for 24hours to release spores. Spores were then collected into Eppendorf tubes (1.5ml) and stored in the refrigerator. Before collection of the spores, the fronds were also agitated to help release any remaining spores from the sporangia. This process was carried out using a total of three fronds from separate plants. The spores were then combined and mixed so that both the treatments would be carried out on a homogenous mixture of spores to nullify the effects of variation between different individuals. Half of the spores were left to dry over silica-gel for a further 24hours to remove all moisture while the other half was left in a sealed Eppendorf tube in the refrigerator, these two sets of spores were used to investigate whether the spores of *Mohria caffrorum* are desiccation tolerant. Spores were then plated on sterilised, full strength, MS (Murashige & Skoog, 1962) agar plates and covered. A total of eight plates were cultured for each of the two treatments as well as four control plates with no spores. The culture plates were placed in a culture room at 25°C under a 24hour light cycle. After 30 days, the culture plates were viewed under a dissecting microscope and the presence or absence of germinated spores was noted.

Results

The drying and rehydration course of the SP fronds follows the pattern typical of desiccation tolerant plants, with a steady decrease in water content down to a minimum level and then, following rehydration, the water content increases to (and above) initial levels. In the SA fronds however, one can see a similar dehydration, but following rehydration, the plant only takes up a minimal amount of water and does not recover water contents to initial levels. The small amount of water uptake in the SA fronds is most probably an artefact of the first two pinnae of the frond being submerged during rehydration and thus being able to absorb a small amount of water.

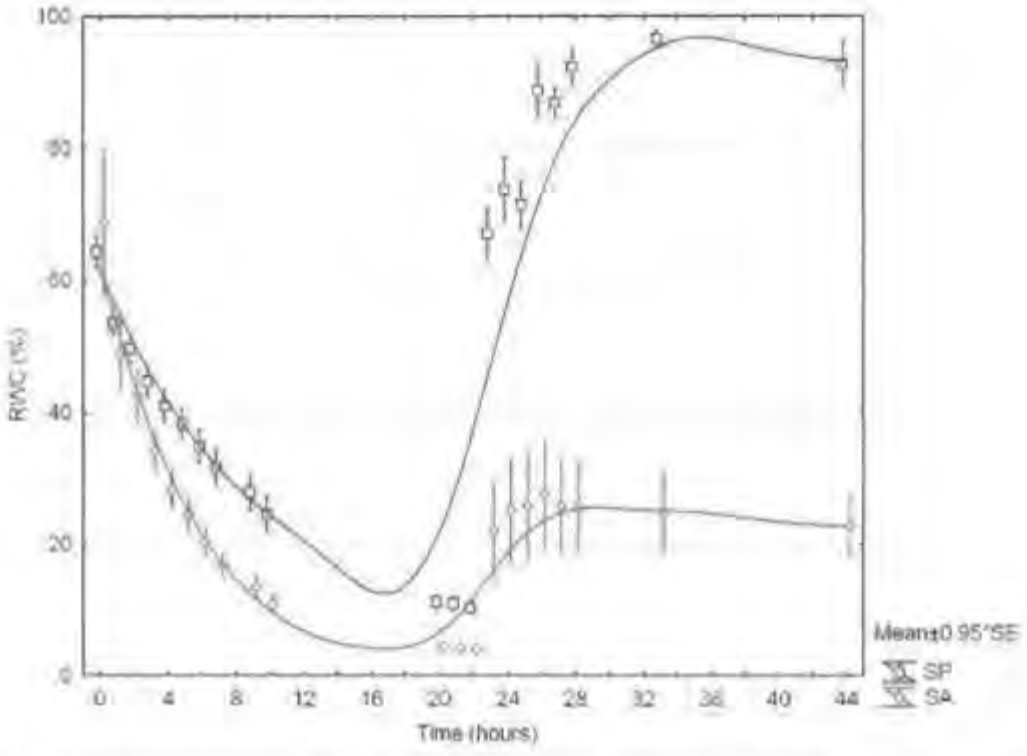

Figure 1: Relative water content of SP (open squares) and SA (open diamonds) fronds during a course of dehydration and rehydration. The relative water content is expressed as a percentage of the highest water content measured for that specific frond type.

Plate 1 shows that the entire abaxial surface of the SP fronds (A) is characterised by being covered by a layer of scales, which cover the pinnae as well as the stipe of the fronds. The adaxial surfaces (B) have no visible scales or hairs but are characterised by having round tipped pinnules. The abaxial surface of the SA fronds (C) have only a few, fine, hair like scales but are otherwise very similar to the adaxial surfaces of the

SA fronds. The adaxial surface of the SA frond (D) is similar to that of the SP frond in that there are no obvious surface structures, but, the tips of the pinnules are pointed rather than rounded. Thus, the main characteristic difference between the SP and SA fronds was the complete absence of the scales on the abaxial surface of the SA fronds. This is also why it was used as a simple character to differentiate the fronds and also used as a diagnostic for collection in the field.

Plate 2 shows that both the abaxial (A) and adaxial (B) surfaces of the immature fertile fronds are very similar to that of the SA fronds. The abaxial surfaces (both immature and mature) only have a small number of fine, hair-like scales. The developing sporangia occur on the margin of the pinnules. As the fertile fronds mature and becomes dry, the fronds fold to expose the sporangia (C and D). The fronds fold in such a way that sporangia are exposed on both the abaxial and adaxial surfaces. As the sporangia dry, they split open and the spores are released (C and D). The mature fertile fronds are more similar in morphology to the SA fronds, with few fine scales, and are unlike the SP fronds.

Plate 3 shows scanning electron microscope images of hydrated SP (A and B) and SA (C and D) fronds. In A, one can see the fine detail of the dense covering of plate-like scales on the abaxial surface of an SP fronds. The plate-like scales can be seen to cover almost the entire surface and very little of the actual frond surface cells can be seen. The inset shows how the plate-like scales form a protective layer over the underlying cells. It also shows the complex, multicellular nature of these scales as well as showing a close-up of one of the many gland-like structures found on both the abaxial and adaxial surfaces of all fronds. The density of these gland-like structures appears to be higher on the SP fronds than the SA fronds. The adaxial surface of the SP fronds (B) displayed the highest density of the gland-like structures as well as the round tipped pinnules. There were also few, fine hair-like scales on the adaxial surface of the SP frond, which resembled those found on the SA frond (C). The inset shows the base of one of the fine scales as well as the complex 'puzzle-piece' structure of the surface cells of the frond. The abaxial surface of the SA frond (C) had none of the same plate-like scales that could be seen on the SP frond. Instead, there was only a sparse covering of fine hair-like scales. A sparse distribution of the gland-like structures was also apparent. The inset shows the structure of the fine hair-like

scales, which appear to comprise of a single row of cells. They may also have branched tips (not shown). There were very few structures on the adaxial surfaces of the SA fronds (D). There were some gland-like structures and no scales were present. The inset shows a high magnification image of one of the gland-like structures as well as showing that the shape of the surface cells of the SA fronds was similar to those of the SP fronds.

Figure A of plate 4 shows the nature of the folding in a dry SP frond. The frond folded in such a way that the scales of the adaxial surface were exposed while the adaxial surface of the frond was concealed. The inset shows the extent of the covering of the scales and their effectiveness at concealing the adaxial surface cells. The scales could be seen to curl around the edge of the pinnules to completely cover the surface cells. Figure B shows the adaxial surface of the dry SP frond. This was exposed by hand sectioning a dry, folded pinnule prior to cryo-fixation. The immense folding of the surface cells is evident as well as the flattening of the gland-like structures. The stomata appear to remain open in the dry state. The inset for figure B shows the folding of the surface cells, which appears to allow for compression during the folding of the frond as well as decrease mechanical stress within the cells. The cells appear to extend vertically from their centre and are compressed laterally. The inset also shows a higher magnification image of open stomata and a portion of a flattened gland-like structure. Figure C shows the abaxial surface of a dry SA frond. The fine scales on the surface of the frond appear to have become flattened and compressed upon drying. Even though the frond is desiccated, there was no evidence of structural folding. From the inset, it is evident that the cells folded in much the same was as in the desiccation tolerant frond, but the overall frond did not fold in any apparently regulated way. The abaxial surface of the fertile frond (D) was very similar to that of the SA frond except that it did undergo some structural folding to expose the sporangia on the abaxial surface of the pinnules. The scales on the fertile frond were similar to those of the SA frond. The inset shows a higher magnification image of the open sporangium with spores inside.

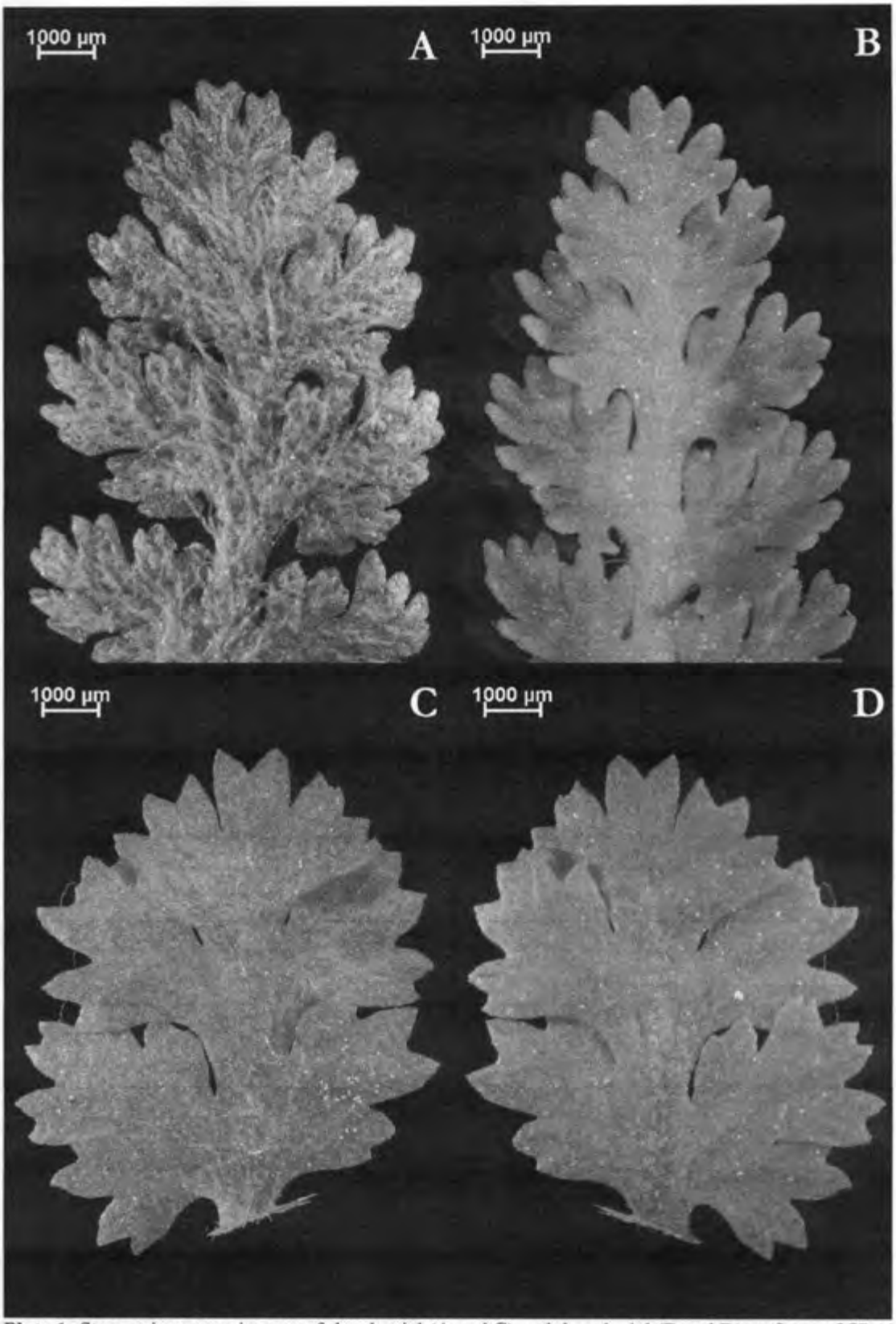

Plate 1: Stereomicroscope images of the abaxial (A and C) and the adaxial (B and D) surfaces of SP (A and B) and SA (C and D) fronds of M. caffrorum. The images were taken at a magnification of 0.75X

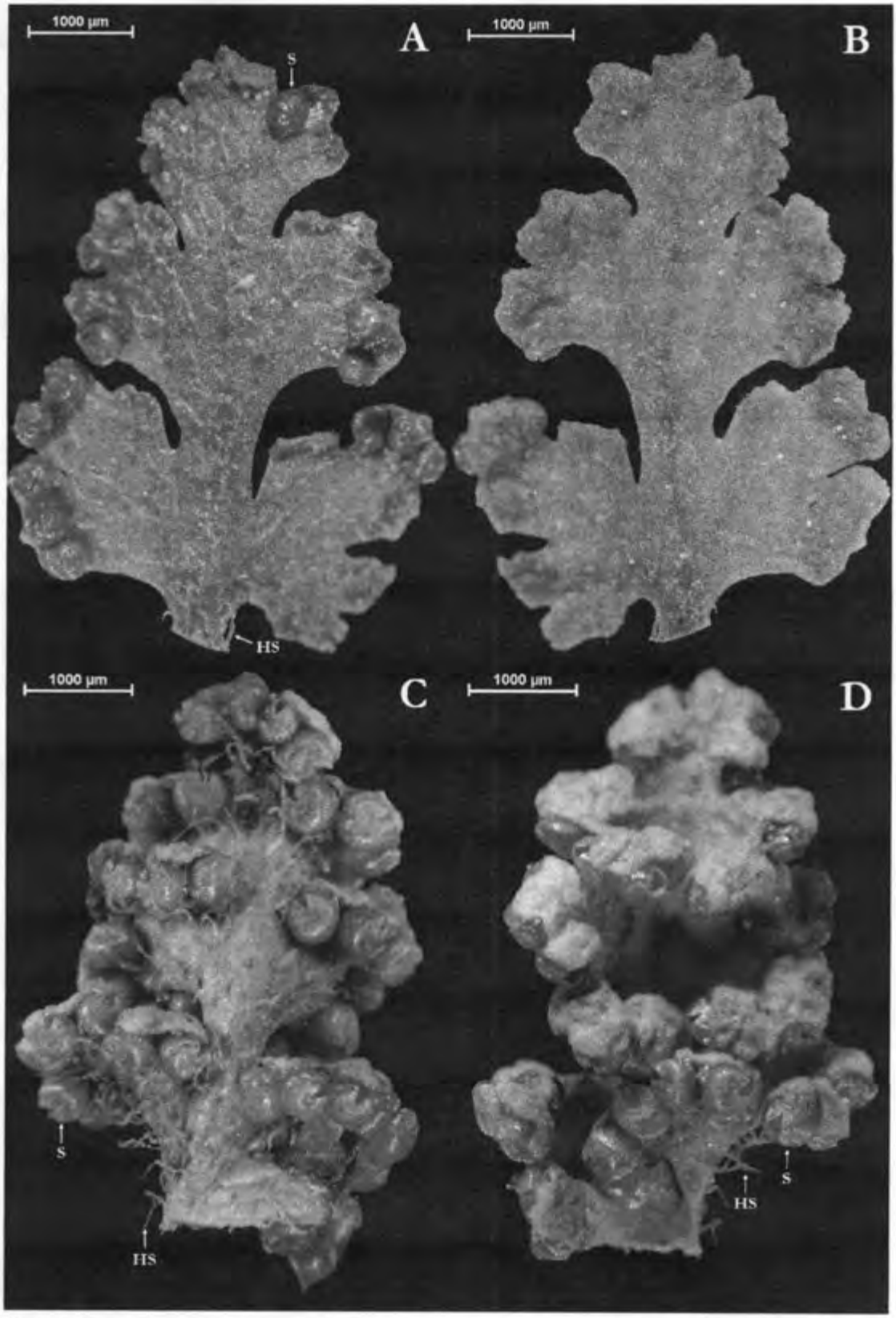

Plate 2: Stereomicroscope images of the abaxial (A and C) and the adaxial (B and D) surfaces of immature (A and B) and mature (C and D) fertile fronds of M. caffrorum. The images were taken at a magnification of 0.75X. S = Sporangium; HS = Hair-like Scale

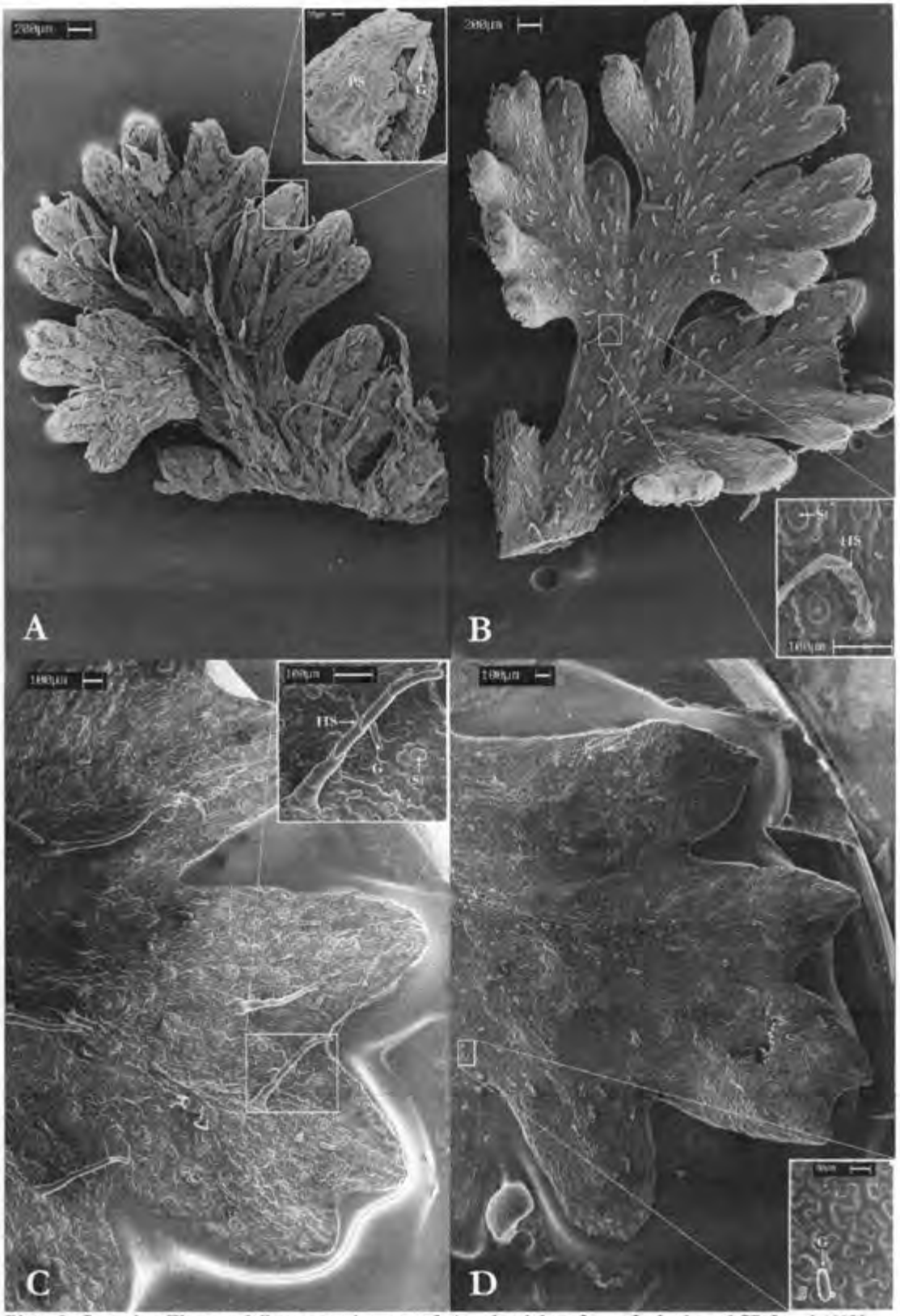

Plate 3: Scanning Electron Microscope images of A - abaxial surface of a hydrated SP frond (69X Mag.), the inset shows a close-up of the abaxial scale (384X Mag.) B - adaxial surface of a hydrated SP frond (67X Mag.) with an inset illustrating the shape of the cells (352X Mag.) C - abaxial surface of a hydrated SA frond (117X Mag.) with an inset showing the structure of the abaxial scale (489X Mag.) D - adaxial surface of a hydrated SA frond (86X Mag.) with an inset showing the shape of the cells (600X Mag.). St = Stomata; HS = Hair-like Scale; PS = Plate-like Scale; G = Gland

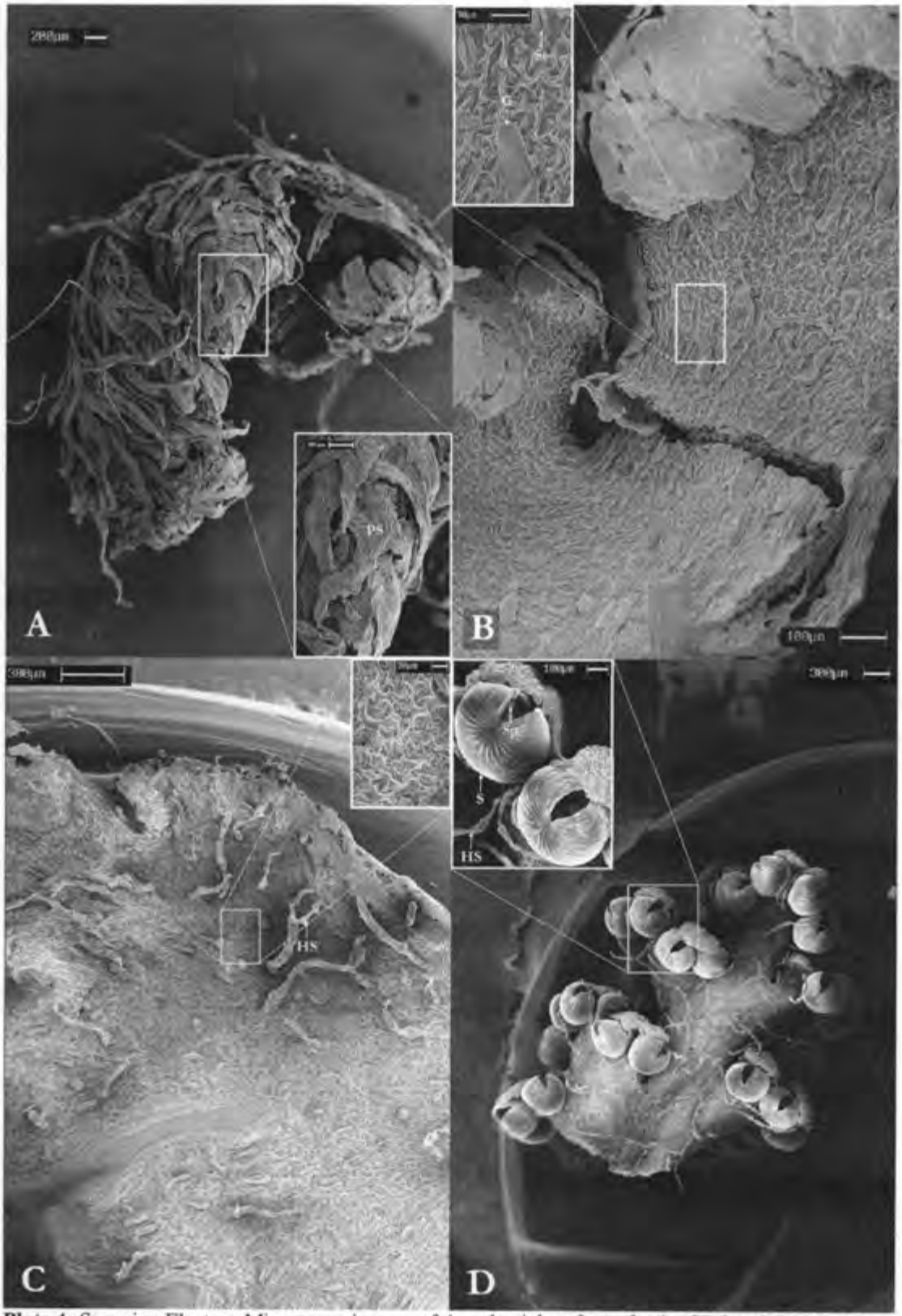

Plate 4: Scanning Electron Microscope images of A – abaxial surface of a dry SP frond showing the curling of the pinnae (65X Mag.) with an inset illustrating the extent of the coverage of the abaxial scales (350X Mag.) B - adaxial surface of a dry, SP frond (272X Mag.) with an inset to show the extent of the folding and the open stomata (1150X Mag.) C - abaxial surface of a dry SA frond showing limited folding (124X Mag.) with an inset showing the folding of the cells (495X Mag.) D - abaxial surface of a fertile frond showing the folding of the pinnae as well as the abaxial scales (51X mag) inset showing open sori and exposed spores (154X Mag.). St = Stomata; HS = Hair-like Scale; PS = Plate-like Scale; G = Gland; S = Sporangium; Sp = Spores

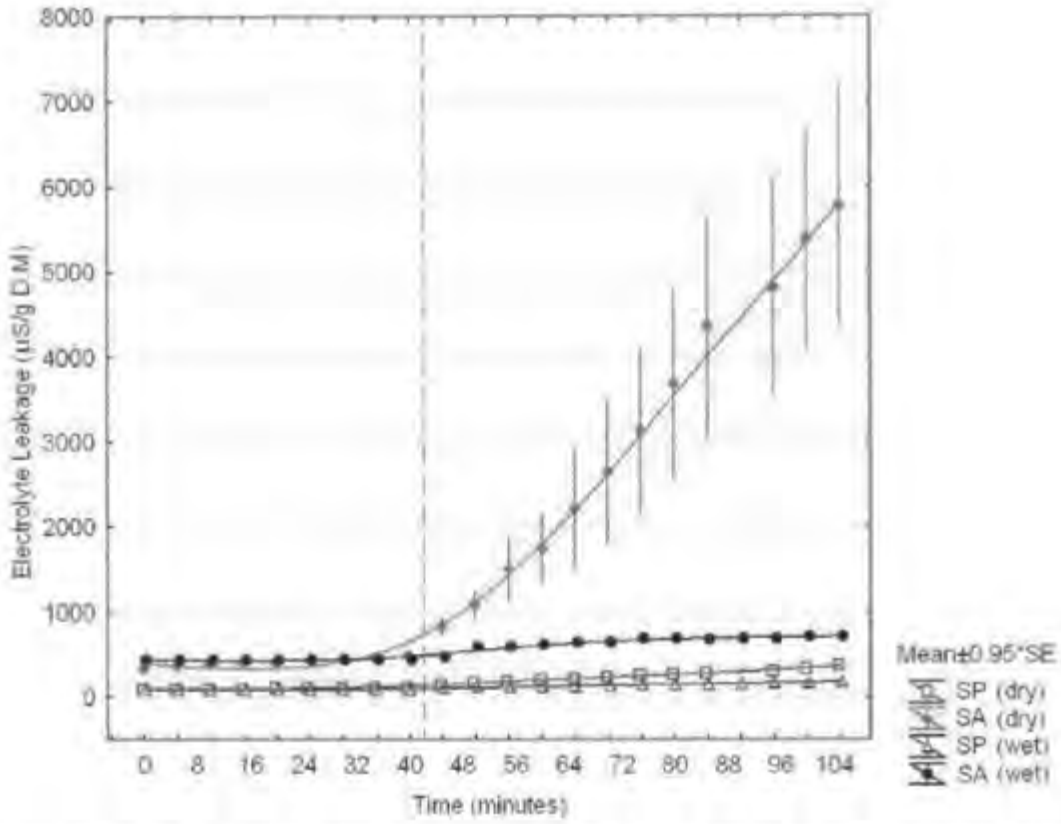

Figure 2: Change in electrolyte leakage, measured as µS per gram dry mass, over time of SP wet (open triangles) and dry (open squares) and SA wet (Closed circles) and dry (closed diamonds) fronds. All values to the left of the vertical dotted line are the initial, control measurements without samples and all the measurements to the right of the dotted line are measurements after the frond samples were added. ANOVA shows a significant difference and ($D.F = 3$; $P < 0.0000$) a post-hoc Tukey HSD test shows that the dry, SA frond has significantly more electrolyte leakage than all other fronds at the 95% confidence level ($D.F = 92$, $P < 0.00015$).

The conductivity of the water before the samples were added remained unchanged for the duration of the testing period. Thus, any change in the conductivity of the solution was due to the samples being added and not from contamination due to solutes, which may have already have been present in the solution and/or sample tubes. Once the leaf samples were added, the electrolyte leakage of the SP (wet and dry) and the SA (wet) samples increased slightly but remained relatively constant over time indicating very little damage or change in permeability of the membranes in these samples. However after addition of the dry SA samples the electrolyte leakage increases rapidly and is significantly greater than that of the other sample types ($D.F = 92$, $P < 0.00015$). This indicates that the dry SA fronds suffered membrane damage (or re-organisation) allowing leakage of the electrolytes from within the cells of the fronds.

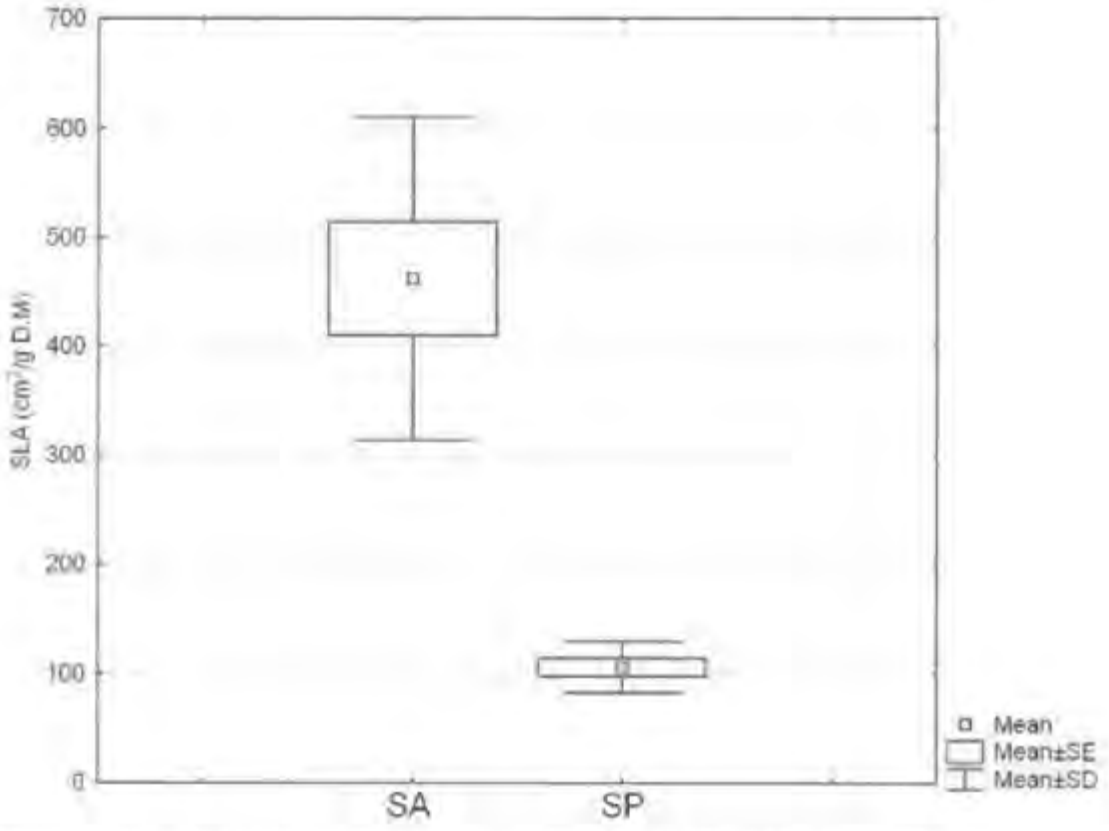

Figure 3: Box and whisker plot of the SLA (cm^2/g Dry Mass) of SP and SA fronds. There was a significant difference between the SP and SA fronds ($P < 0.000$).

The box and whisker plot in Figure 3 shows the difference in SLA between the SP and SA fronds with the SA fronds having a far greater SLA than the SP fronds. This difference was statistically significant at the 95% confidence limit ($F = 45.31$; $P < 0.0000$).

Table 1: Spore germination expressed as % of plates which contained germinated spores. The absolute water content expressed as grams of water per gram dry mass of spores is also shown for each of the two treatments.

	Dried Spores	Control Spores
Abs. Water Content (g H_2O/g D.M)	0.04	0.06
No. of Plates with Germinated Spores	75%	87.5%

Plates with no spores did not contain anything resembling germinated spores and the results found were therefore not a result of contamination. There was very little difference in the absolute water content of the artificially dried spores and the freshly collected spores and there was also little difference in the number of plates (75% vs. 87.5%), which germinated for each of the two treatments.

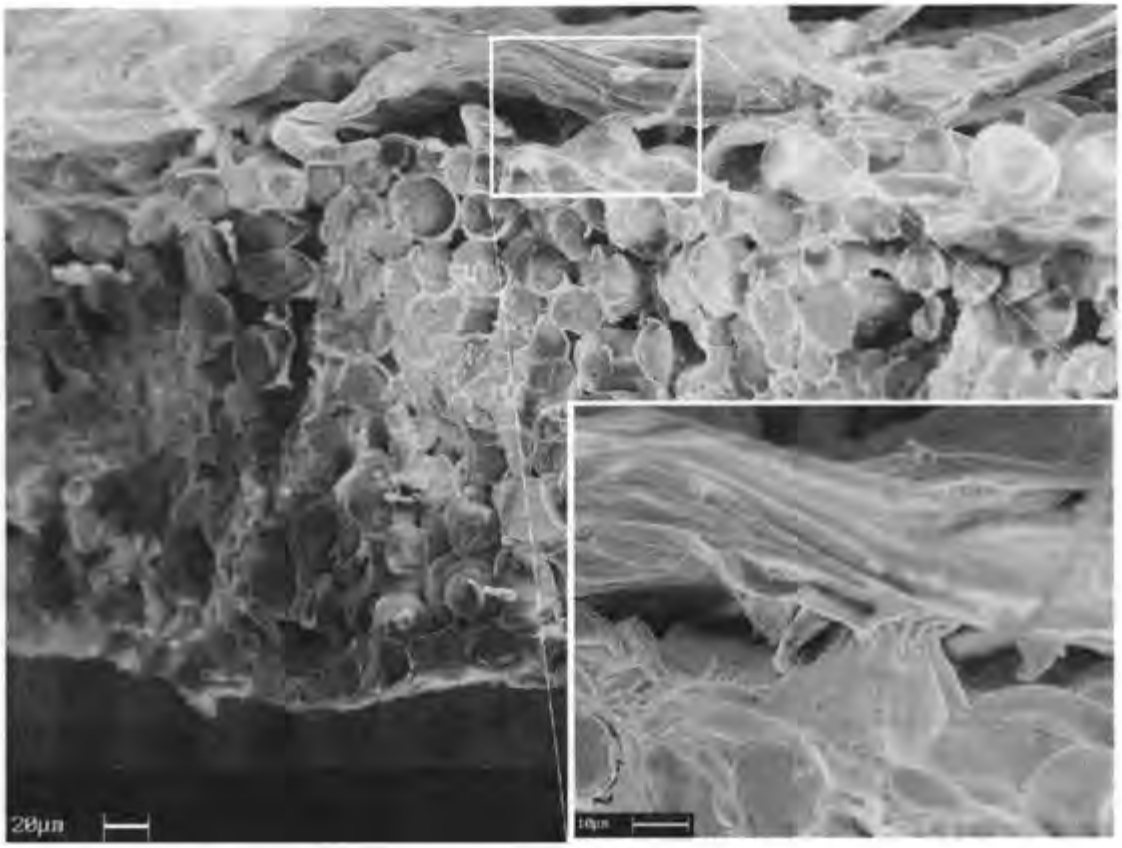

Figure 4: Scanning Electron Microscope image (771X Mag.) of a freeze fractured, wet, SP frond showing a transverse section of the frond with the abaxial surface at the top of the image and the adaxial surface at the bottom of the image. Inset shows the airspace between the abaxial scale and the abaxial surface of the frond (3190X Mag.)

The result of the time laps photography can be found on the CD attached on the back, inside cover of this manuscript. For optimal playback, it is recommended that the file (Mohria caffrorum time lapse video.avi) is copied off of the CD onto a computer before viewing with Windows Media Player or other compatible software. The video demonstrates the difference in rehydration in the two frond types. The SA frond (left) does not rehydrate while the SP frond (right) rehydrates rapidly. The total time taken for rehydration was 8 hours. The abaxial surface of the SP frond (right), does not appear to be brown, as expected but this is partly due to the lighting and partly due to the frond being harvested early in September 2006 when the SP fronds were first emerging.

Discussion

From the drying course in Figure 1 and the electrolyte leakage data in Figure 2, one can see that the SP fronds show evidence towards being desiccation tolerant while the SA fronds do not. After drying, the SP fronds are able to recover while the SA fronds are not (see attached CD) indicating that the former may be desiccation tolerant while the later may not be desiccation tolerant. The slight increase in water content in the SA fronds can be explained by simple diffusion of water into the submerged portions of the frond but no further recovery was observed (see attached CD). The SP fronds however, were able to recover to initial levels and higher.

The water contents of dry SP fronds is not as low (±15% RWC) as those typically reached by most desiccation tolerant plants (< 5% RWC, e.g. Sherwin & Farrant, 1996) but this may be an artefact of the different methods used to obtain these figures. It would be interesting to determine whether the SP fronds of *M. caffrorum* could tolerate further drying and still be able to recover. It would also be important to investigate this phenomenon on whole plants instead of removed fronds as there may be fundamental differences in the extent of desiccation tolerance between the two. The extent of increase in water content of the SP fronds post rehydration (to level higher than those of the freshly removed fronds) can possibly be explained by the action of water being taken up by capillary action in the space between the abaxial scales and the abaxial surface of the frond. This space can be clearly seen in Figure 4 with a space of ±10-20µm between the bottom of the abaxial scale and the abaxial surface of the frond and it is hypothesised here that this space would allow water to be passively taken up by capillary action and then absorbed through the abaxial surface of the frond, into the cells of the plant. During rehydration, the water can be seen rapidly rising up the dry SP fronds as the abaxial surface of the dry frond become progressively wetter. This can be seen on the SP frond (right) in the first few seconds of the time lapse video (see attached CD) and may be one of the fundamental functions of the scales on the abaxial surface of the SP fronds.

The electrolyte leakage data also provides support for above suggestion that the SP fronds are desiccation tolerant while the SA fronds are not. The extremely high leakage of the dry SA fronds compared to that of the wet SA and SP fronds as well as

the dry SP frond shows that there is possibly substantial membrane damage (or re-organisation) in the dry SA fronds. The electrolyte leakage of the dry SP frond is comparable to that of the wet SA and SP fronds and this shows that the membrane damage incurred by the SP frond during drying is minimal. Similar data has been shown in the comparison between the desiccation tolerant *Eregrostis nindensis* and the desiccation sensitive *Eregrostis curvula* (Vander Willigen *et al*, 2004).

Plate 1 shows some of the gross morphological differences between the SP and SA fronds and also displays the characteristics that were used to distinguish the different frond types in the field. The main defining characteristic between the SP and SA frond are the different scale types. SA fronds have a sparse covering of fine, hair-like scales and these scales are morphologically very distinct from those found on the abaxial surface of the SP fronds, which are broader, larger and plate-like. We proposed (above) that the scales may play a role in the absorption of water through capillary action. An additional function may be that the broad scales on the SP fronds appear to serve a protective function against light during dehydration as well as in the dry state. It is hypothesised that these scales, combined with the folding of the fronds, would act in much the same way as anthocyanin accumulation and leaf folding in the resurrection angiosperms *Craterostigma wilmsii* and *Myrothamnus flabellifolius* (Farrant, 2000; Farrant et al, 2003; Sherwin & Farrant, 1998). In the dry state, the scale covered abaxial surface would be the only surface exposed to the light and thus the plant would be able to prevent adverse interactions of chlorophyll, in adaxial surfaces, with light at intermediate water contents and therefore decrease the chance of the formation of reactive oxygen species (ROS). The extent to which the abaxial scales cover the abaxial surface can also be seen on plates 2 and 3. Figure A on plate 2 shows how even in the hydrated state, the abaxial surface of the frond is completely covered by scales and that almost none of the surface cells of the frond can be seen. Figure A on plate 3 shows how the combination of abaxial scales and frond folding acts to completely screen the 'naked' adaxial surface from any possible exposure to incoming light. Future studies would need to investigate the efficiency of the abaxial scales of the SP fronds in blocking light. The function of the fine, hair-like scales (Plate 1 Figure C) on the SA fronds is unknown but may play an anti-herbivory role.

The specific leaf area (SLA) is also significantly different between the two frond types. The higher SLA of the SA frond equates to a thinner frond with less structural carbon investment per unit of frond area. While the SP fronds, with a lower SLA, are thicker, equating to a higher investment in structural carbon per unit of frond area. This measurement helps clarify the ecological role of each of the two frond types. The SP frond is made to survive longer and has a high investment in structural carbon as well as other mechanisms to help protect it against desiccation (eg. Plate-like abaxial scales). This type of frond spends a large portion of the dry season in the desiccated state where it is not photosynthesising and these fronds are therefore possibly structurally more suited to survive desiccation rather than photosynthesise and create energy. The SA fronds on the other hand are thin and not apparently desiccation tolerant. It has been shown that thinner leaves are more efficient at absorbing and using light (Agusti et al, 1994). Their role is, presumably, to photosynthesise as much and as efficiently as possible during the wet season so that the plant can gain enough energy to complete its life-cycle. SA fronds do not persist for long periods and appear to be replaced by SP fronds at the onset of the dry season (Pers. Obs).

The fertile fronds appear to be modified SA fronds. Figure A on plate 3 shows the developing fertile frond. It can be seen to have the same hair-like scales on the abaxial surface as can be seen on the regular SA fronds. These hair-like scales can also be seen on the abaxial surface of the mature fertile frond. There is no sign of the plate-like hairs, which are present on the SP fronds. Field observations, combined with these morphological observations, confirm that the fertile fronds of *Mohria caffrorum* are modified SA fronds. Unlike the regular SA fronds however, the fertile fronds do fold when dehydrated. This is done to expose the sporangia on the margins of the abaxial surface of the fertile fronds. Upon further dehydration, the sori split open and release the spores (Figure D, Plate 2 and Figure D, Plate 4). Following the release of spores, these fronds die back.

From the above information, coupled with information from field observations, *Mohria caffrorum* can be seen to go through the following seasonal changes, which are described in Figure 5:

In the dry season, the plant has desiccation tolerant, SP fronds (1). This allows the plant to persist through the dry season by drying to equilibrium with the atmosphere and remaining in a folded, dormant state (2). At the onset of the rainy season, the desiccation tolerant, SP fronds are able to rapidly recover (3) and begin photosynthesising. This gives the plant an advantage over other, annual, herbaceous species which have to produce new plants *de novo*. During the rainy season, SA fronds are produced photosynthesis as much and as efficiently as possible during the wet season (4). Nearing the end of the wet season, a portion of the SA fronds begin to produce sporangia (5). When the fertile fronds desiccate they fold and the sporangia rupture to release desiccation tolerant spores (6). The plant then goes on to produce new desiccation tolerant, SP fronds before the onset of the dry season (1).

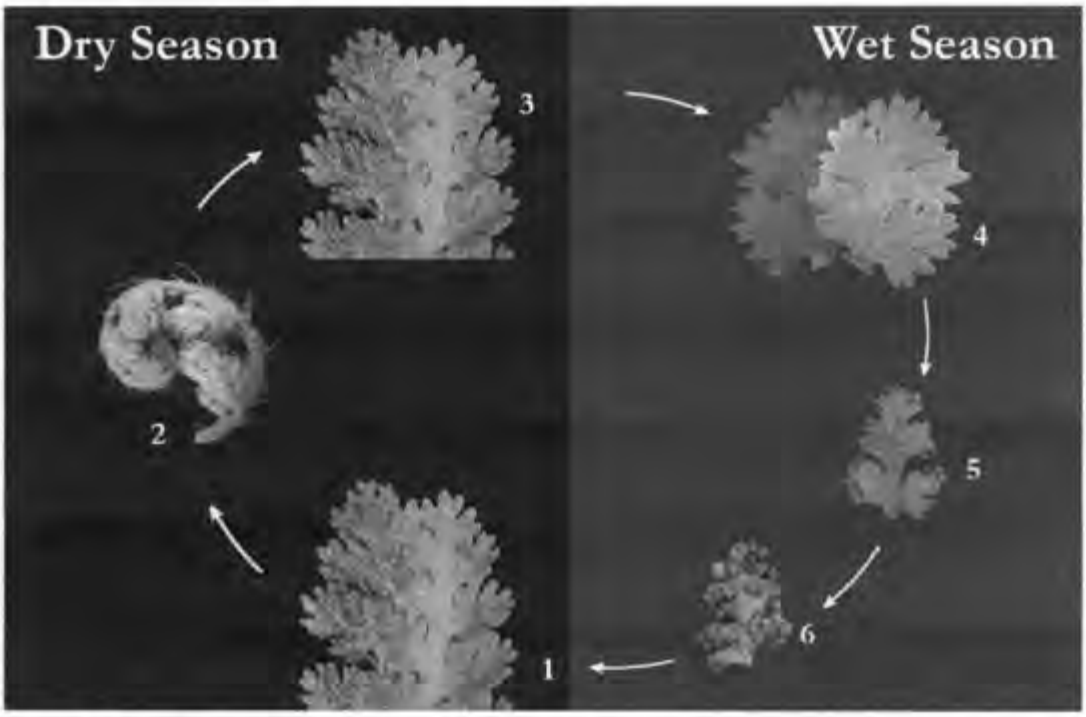

Figure 5: Seasonal cycle of the M. caffrorum showing the transitions between desiccation tolerant and desiccation sensitive forms

Having a single species which changes between being desiccation tolerant and desiccation sensitive, depending on the season, could provide valuable information in terms of investigating the cost of desiccation tolerance. Many papers (e.g. Oliver *et al*, 2000) report that desiccation tolerance comes at a cost but this has not yet been shown conclusively. The fact that a single species has both tolerant and sensitive fronds, should mean that change seen between the two frond types should be as a result of the

frond acquiring or losing desiccation tolerance and therefore could give insights into the cost of desiccation tolerance. This species also provides a unique chance to study the molecular mechanisms behind desiccation tolerance as it would allow investigators to more easily differentiate between molecules, genes and pathways activated solely for the acquisition of desiccation tolerance and those used for general house-keeping purposes. Evolutionarily, it would be important for the plant to upregulate only those mechanisms, which are essential for the acquisition of desiccation tolerance, during the desiccation tolerant phase of its life cycle as to not waste energy unnecessarily. As the spores of *M. caffrorum* are also desiccation tolerant, it would also be important to investigate the mechanisms of their desiccation tolerance to investigate whether they are shared with those of the vegetatively desiccation tolerant plants. This could help investigate the hypothesis proposed by Oliver *et al*, 2000 that genes responsible for vegetative desiccation tolerance in the primitive clades of land plants were recruited for other processes (such as propagule desiccation tolerance e.g. spores and seeds) as vegetative desiccation tolerance was lost in the evolution of the vascular plants. Once these genes were present in the desiccation tolerant propagules, they were available for re-induction into vegetative desiccation tolerance in higher plants and that this would be evident in the similarities between vegetative and propagules desiccation tolerance. Evidence supporting this hypothesis has been demonstrated in Illing *et al*, 2005. *M. caffrorum* provides another model system in which this can be investigated but it is unique in that it can be investigated whether the genes, which are activated, in the production of desiccation tolerant spores are the same as those activated in producing the desiccation tolerant vegetative tissue.

Future investigations will need to characterise the growth parameters required to keep *M. caffrorum* plants in artificially regulated environments as they did not survive growth room conditions. This would provide a means to investigate the environmental cues used by the plant to change between being desiccation tolerant and desiccation sensitive without having to wait for a seasonal change. Desiccation tolerance in other plant systems (vegetative and propagules desiccation tolerance) has been shown to be developmentally regulated (Illing *et al*, 2005) while the desiccation tolerance in *M. caffrorum* appears to be environmentally regulated. This could provide a valuable research tool in terms of being able to investigate the environmental and genetic

triggers, which are used to regulate the desiccation tolerance in *M. caffrorum* as this change could hypothetically be induced as required in controlled growth room conditions. *M. caffrorum* could therefore prove to be a valuable model system in the investigation desiccation tolerance in terms of being able to induce desiccation tolerance in non-tolerant organisms, which is the ultimate goal of research in this unique field. For example, if the mechanisms of spore desiccation tolerance and vegetative desiccation tolerance are similar and induced by similar genes, it would provide valuable information into being able to induce desiccation tolerance present in orthodox seeds of desiccation sensitive plants into the desiccation sensitive vegetative tissue.

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