# MECHANISMS OF DESICCATION TOLERANCE IN CRYPTOGAMS

by

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## DECLARATION

The experimental work described in this thesis was carried out in the Department of Botany, University of Natal, Pietermaritzburg, under the supervision of Prof RP Beckett.

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

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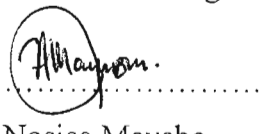

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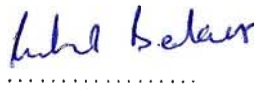

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*"The fear of the Lord is the beginning of all Wisdom - Praise the Lord, and Proclaim his name tell the nations what has done"*

## PUBLICATIONS
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## PRESENTATIONS AT CONFERENCES
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## ABSTRACT

In this study adaptations of bryophytes and lichens to desiccation stress were examined. The aim was to test whether desiccation tolerance in the selected species is constitutive or if desiccation tolerance could be induced by various hardening treatments. In addition, some putative tolerance mechanisms were investigated, including the accumulation of sugars, increase in ROS scavenging systems and other mechanisms e.g. energy dissipating processes. To determine if hardening treatments prior to desiccation stress increased desiccation tolerance, mosses and lichens were partially desiccated or treated with ABA. The effect of hardening treatments on the physiology of the moss *Atrichum androgynum* and lichens *Peltigera polydactyla, Ramalina celastri* and *Telochistes capensis* during a desiccation–rehydration cycle was investigated. Photosynthesis, respiration and chlorophyll fluorescence measurements were used as rapid tools to determine the metabolic activities in these lichens and moss species. In *A. androgynum* partial desiccation following slow drying at 52% RH increased the rate of recovery of net photosynthesis. Net photosynthesis recovered almost completely following slow drying in the material that was partially dehydrated and/or treated with ABA. This suggests that partial dehydration hardens the moss, and that ABA can fully substitute for partial dehydration. In *R celastri* and *P. polydactyla* both partial dehydration and ABA treatments displayed some improvement in desiccation tolerance depending on the duration and severity of stress. The reduction in the re-saturation respiration burst in *P. polydactyla*, although not quite significant, strongly suggests that hardening increases mycobiont tolerance. However, it is more difficult to establish whether the hardening treatments improve photobiont performance.

In the moss *A. androgynum* ABA treatment increased the rate of recovery of photosynthesis and PSII activity, and also doubled non-photochemical quenching (NPQ). Increased NPQ activity will reduce ROS formation, and may explain in part how ABA hardens the moss to desiccation. In ABA treated, but not untreated mosses, desiccation significantly increased the concentration of soluble sugars in *A. androgynum*. Sugar accumulation may promote vitrification of the cytoplasm and protect membranes during desiccation. Starch concentrations in freshly collected *A. androgynum* and *R. celastri* were only *c.* 40 and 80 mg $g^{-1}$ dry mass respectively,

and slightly rose during desiccation, but were only slightly affected by ABA pretreatment. ABA did not reduce chlorophyll breakdown during desiccation. In *P. polydactyla* ABA pretreatment had little effect on any of these parameters.

Changes in the activities of the free radical scavenging enzymes ascorbate peroxidase, catalase and superoxide dismutase were measured during wetting and drying cycles in the moss *A. androgynum* and in the lichens *P. polydactyla*, *R. celastri* and *T. capensis*. These species normally grow in the understorey of the Afromontane forest, moist, xeric, and extremely xeric microhabitats respectively. In *A. androgynum*, enzyme activity was measured shortly after collection, after 3 d storage following hardening by partial dehydration and/or 1 h treatment with ABA or distilled water and during desiccation and rehydration. In *A. androgynum* enzyme activities of CAT and SOD in untreated material were always higher than in the hardened treatments, while both partial dehydration and ABA treatments tended to reduce both CAT and the induction of SOD activity, although these effects were not significant between the treatments. This suggests that ABA may not be involved in the induction of free radical scavenging enzymes and probably these enzymes are not important in desiccation tolerance of *A. androgynum*. In lichens, the enzyme activity was measured shortly after collection, after hydration for 48 h at 100% RH, after desiccation for 14 d and 28 d, and during the first 30 min of hydration with liquid water. Enzyme activities tended to rise or stay the same following rehydration in all the species tested. After desiccation for 14 d, enzyme activities decreased, and then decreased further to very low values after 4 weeks desiccation. In all species, including *T. capensis* from an extremely xeric habitat, the activities of all enzymes remained at very low values during the 30 min following rehydration, and were therefore unavailable to remove any reactive oxygen species accumulating in lichen tissues as a result of desiccation. Results suggests that the enzymic antioxidants are more likely to be involved in removing reactive oxygen species produced during the normal metabolic processes of lichens than having a role in desiccation tolerance.

The Afromontane understorey moss *Atrichum androgynum* displayed an oxidative burst of $H_2O_2$ during rehydration following desiccation. Maximum rates of $H_2O_2$ production occur during the first 15 min of rehydration. While the production of $H_2O_2$ increases with increasing desiccation times, the moss produced significant

amounts of $H_2O_2$ during rehydration after desiccation for times that did not inhibit photosynthesis or cause $K^+$ leakage. *A. androgynum* may produce more $H_2O_2$ during desiccation than rehydration, because desiccation artificially induced using polyethylene glycol strongly stimulates production. Experiments involving inhibitors and exogenously supplied reductants indicate that peroxidases are responsible for the synthesis of $H_2O_2$. Factors that influence the rate of $H_2O_2$ production during rehydration include light and the hormone ABA. Patterns of $H_2O_2$ production are discussed in terms of their possible role as a defence against pathogenic fungi and bacteria.

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# CHAPTER 1

# 1. General introduction and literature review

## 1.1 Physiological and biochemical aspects of desiccation

Plants respond to water deficit at different levels: morphological, physiological, cellular and metabolic (Porembski and Barthlott 2000). These responses depend on the duration and severity of stress (Bray 1993). However, different species respond differently to water deficit based on their ability to resist stress. Studies on cellular responses to water stress mostly focus on what cells need to tolerate or resist water loss (Walters *et al.* 2002). Direct evidence concerning the damaging process is sparse, with the mechanisms of damage often made by inference from the presence of putative protectants. It is often unclear whether a change in morphology, ultrastructure or metabolism is a simple consequence of drying, a protective strategy or a sign of damage. For example cessation of metabolism is considered a component of all three, possibilities (Vertucci and Leopold 1986, Salmen Espindola *et al.* 1994, Leprince *et al.* 1999, 2000). Damage by desiccation is often measured by an irreversible change or a failure to revive metabolic activities of the cell upon rehydration. In order to survive, plants need to be resistant to oxidative stress (Smirnoff 1993). Resistant plants may avoid an internal water deficit through mechanisms, which promote water uptake, and retard water loss or they may tolerate protoplasmic dehydration (Bray 1993). There are several groups of plants, which are known to tolerate desiccation: the angiosperms called resurrection plants (Gaff 1980, Bewley and Krochko 1982, Ingram and Bartels 1996), a few ferns and algae, lichens and bryophytes (Oliver 1991). In order to be able to evaluate responses observed during periods of desiccation, plant resistance must be determined. It is also important to determine the response given by the resistant plant to water deficit. These responses may be either adaptive, contributing to the ability of the plant to withstand stress, or may result from injury (Bray 1993).

Most research on water deficit has been done on the responses of plants to relatively mild stress, which can only survive a limited water stress. However, under natural conditions, plants are often exposed to severe stress due to seasonal changes.

At whole plant level, the effects of stress are usually perceived as inhibition in the rate of photosynthetic activity, respiration and growth (Smirnoff 1993). However, in bryophytes and lichens, photosynthesis is strongly affected by desiccation because of the lack of cuticle to limit water loss (Kaiser 1987, Lange *et al.* 1996). When photosynthesis is inhibited during desiccation, the excess light energy absorbed by the photosynthetic pigments is either dissipated as chlorophyll fluorescence or heat (Lichtenthaler and Buschmann 1986). Modulated chlorophyll fluorescence measurements are now extensively used to investigate photosynthetic electron transport and associated physiological processes (Krause and Weis 1991, Schreiber and Bilger 1993, Edwards and Baker 1993). Empirical expressions representing photochemical quenching ($Q_p$), non-photochemical quenching (NPQ) and the quantum efficiency of photosystem II ($\Phi$PSII) were derived from measurements of $F_m$, $F_o$ and $F'_m$. These calculated parameters are now being used to provide information on rates of electron transport and the quantum yield of $CO_2$ assimilation, since they provide rapid and non-invasive means of gas exchange measurements (Schreiber *et al.* 1995c). To date, studies of fluorescence have been used predominantly as a means of providing insights into desiccation tolerance (Deltoro *et al.* 1998a, Dietz and Hartung 1998, 1999, Marschall and Proctor 1999, Bartošková *et al.* 1999, Csintalan *et al.* 1999).

Fluorescence techniques were originally developed in higher plants and there are differences in the organization of the photosynthetic apparatus of vascular plants and non-vascular plants that are reflected in their fluorescence characteristics (Schreiber *et al.* 1995a). Detailed studies of the fluorescence characteristics of eukaryotic algae, cyanobacteria and lichens (Büchel and Wilhelm 1993, Schreiber *et al.* 1995a, b, Campbell *et al.* 1998) have revealed that upon illumination with actinic light, the fast induction kinetics in cyanobacteria and micro-algae rise within approximately 200 ms to an initial peak (P), and then show a rapid decline to a quasi-stationary level (S). This P-S decline, the magnitude of which is determined by the $O_2$ content of the algal suspension, is much slower in higher plants. In higher plants, a number of alternative electron sinks may be responsible for the observations, including photorespiration and the reduction of molecular oxygen, $SO_4^{2-}$ and $NO_3^-$ (the Mehler reaction) (Polle 1996). The alternative means to dissipate energy might be a key factor in the desiccation tolerance of bryophytes. At present, the physiological

process that allows the photosynthetic apparatus to undergo water loss without experiencing photooxidative damage remains unclear in mosses and lichens. However, photodamage can occur as plants dry or while they are desiccated, due (at least in part) to light absorption without energy transfer to photosynthesis (Seel *et al.* 1992b, Gauslaa and Solhaug 1996). Desiccation tolerant plants show a variety of mechanisms likely to reduce photodamage, including leaf curling, accumulation of anthocyanin and carotenoids, and xanthophylls cycle metabolism (Muslin and Homann 1992, Eickmeier *et al.* 1993, Lebkeucher and Eickmeier 1993, Calatayud *et al.* 1997, Deltoro *et al.* 1998b, Beckett *et al.* 2000, Farrant 2000). Antarctic mosses, which could be subjected to photodamage during freezing, show reversible photoinhibition and zeaxanthin activity (Lovelock *et al.* 1995).

### 1.2 Effects of desiccation

Desiccation is characterized by the changes in the relative water content, photosynthesis, respiration, membrane structure, subcellular organelle ultrastructure and a decrease in cell volume (Smirnoff 1993, Farrant 2000). Photosynthetic activity of both lichens and bryophytes is strongly affected by desiccation (Kaiser 1987, Lange *et al.* 1996). The ability of these plants to survive desiccation and resume normal metabolic activity on rehydration is well known (Richardson 1993, Oliver and Bewley 1997). During desiccation photosynthetic activity and respiration of these plants are gradually inhibited (Lange *et al.* 1993, Sundberg *et al.* 1997b), and the light energy absorbed may exceed the level that this process is capable of dissipating (Beihler and Fock 1996). Under these conditions, plants need to employ mechanisms that protect photosynthetic apparatus during desiccation since it is very sensitive and liable to injury (Tuba *et al.* 1996).

#### 1.2.1 Factors affecting photosynthesis

Plants are often exposed to extreme environmental conditions, including high light intensity, temperature and water content. Under these conditions the formation of ROS increases and the rate of photosynthesis is inhibited (Smirnoff 1993, Elstner and Osswald 1994, Lawlor 1995, Bartosz 1997). It has been suggested that a decrease in photosynthesis may be due to a diffusive resistance of $CO_2$ (Lawlor 1976b, Ort *et al.* 1994), or due to impaired metabolism by direct inhibition of biochemical processes caused by ionic, osmotic or other conditions induced by loss of cellular water (Kaiser

*et al.* 1986, Gimenez *et al.* 1992). The latter is of particular significance when considering bryophytes and lichens due to the lack stomata's.

#### 1.2.1.1 Light intensity

Light is an important environmental factor in plants, but exposure to high light intensities may lead to a reduction in photosynthetic capacity resulting from the destruction of photosynthetic pigments (Asada and Takahashi 1987). Plants growing under natural or semi-natural conditions are exposed to a light environment that varies over time scale ranging from seconds to hours. This variability arises from the movement of the sun across the sky; changes in cloud cover and shade from neighbouring plants. The latter is of particular significance when considering woodland plants. The light environment of various types of woodland have been characterized (Pearcy 1983, Chazdon and Fetcher 1984) and bursts of direct sunlight, 'sunflecks' have been shown to represent a significant percentage of total daily photon flux for woodland floor species (Pearcy 1983, Chazdon and Fetcher 1984, Chazdon 1988). Many plant species are able to alter the composition of their photosynthetic apparatus to optimise photosynthesis for the light environment in which they are growing (Anderson *et al.* 1995).

Adaptation to excessive light is one of the requirements of survival particularly in poikilohydric organisms, which in contrast to the leaves of higher plants can tolerate complete desiccation (Heber *et al.* 2000). Water deficit usually occurs in the field associated with high light intensity. The interaction between light and water deficit has been examined in a few species (Somersalo and Krause 1990). In addition, light increases the amount of desiccation-induced damage as a result of oxidation (Smirnoff 1993). Some of the observed effects of water deficit may be the result of a stress-induced impairment of the biosynthetic machinery required for photosynthetic assimilation of carbon and its conversion to metabolically usable forms. Furthermore, survival of desiccation tolerant organisms under extreme light conditions may suggest a highly effective mechanism of photoprotection. Indeed, Deltoro *et al.* (1998a) have shown that a desiccation tolerant liverwort had a higher non-radiative dissipation of light energy than desiccation intolerant counterparts. In addition, Heber *et al.* (2000) also reported that chlorophyll fluorescence of lichens and mosses in their dehydrated state was extremely low, thus indicating effective

dissipation of absorbed light energy as heat. However, Bilger *et al.* (1989) suggested that in lichens the energy transfer between the pigment bed and PSII is impaired.

### 1.2.1.2 Temperature

Temperature is the most fundamental environmental factor controlling water loss and enzyme activity, thus affecting the rate of photosynthesis (Stirk *et al.* 1995). However, species from warm environments usually demonstrate noticeable heat-tolerance of their photosynthetic apparatus and superior photosynthetic performance at high temperatures (Larcher 1995a, b). Furthermore, plants from thermally contrasting habitats exhibit photosynthetic temperature responses that generally reflect an adaptation to their prevailing temperature regimes. Species that are native to cold environments generally show relatively high photosynthetic rates at low temperatures and lower photosynthetic temperature optima (Larcher 1995a).

Bryophytes become much more tolerant to extremes of temperature as they dry out (Meyer and Santarius 1998). Desiccation tolerant mosses and lichens, when dry, can survive extremely low temperatures. Bewley (1973b) have shown recovery of protein synthesis in *Tortula ruralis* shoots that have been cooled in the dry state to -196°C in liquid nitrogen. High temperatures may result to photosynthetic inhibition. However, different species have different optimum temperatures at which they show maximum photosynthetic activity. Lange *et al.* (1998) have shown that *Collema tenax* is adapted to higher temperatures than other lichens from the same habitat. The temperature optimum of this species lies above 30°C substantially higher than for *Diploschistes diacapsis* and *Psora cerebriformis*. In contrast to *C. tenax*, both these species have a much flatter temperature response curve (Lange *et al.* 1998).

### 1.2.1.3 Relative water content and humidity

Terrestrial bryophytes and lichens growing under mesic and xeric habitats face a limited and temporally variable water supply, and the water limitation has a strong impact on their performance (Proctor 1990). Bryophytes and lichens lack cuticle, and therefore are not able to prevent their water loss actively (Proctor 1990, Lange *et al.* 1996). Thus plants lose water under non-saturating conditions until the water potential of the tissue equilibrates with that of the ambient air (Proctor 1982, Proctor *et al.* 1998). Under field conditions, the ambient humidity decreases after a precipitation

event and the gradient in water potential between the moist thallus and the atmosphere results in loss of water through evaporation. Water retained in extracellular capillaries is lost first, giving reduced water content. On further desiccation, symplast water is lost and the cell water potential decreases. As the cell water potential is affected only at low water content in species with a relatively high external water holding capacity, bryophytes may maintain turgid cells at a range of thallus water contents (Dilks and Proctor 1979). Despite the lack of cuticle, differences in thallus, leaf and shoot morphology and packing produce several-fold differences in drying rates between different species of bryophytes and lichens (Gimingham and Smith 1971, Proctor 1982, Scott 1982, Vallandares 1994). Differences in morphological control of water loss may help explain differences in ability to colonize xeric microhabitats (Alpert and Oliver 2002).

Water is a prerequisite for photosynthesis and is strongly dependant on the thallus water content (Dilks and Proctor 1979) and as the species from mesic and xeric habitats spend much time desiccated (Proctor 1990), they have a low net photosynthesis per time unit. Most bryophytes and lichens are, however, physiologically and morphologically capable of taking maximum advantage of the beneficial periods, with rapidly resuming metabolism upon rehydration after rapid desiccation when cell water potential drops to suboptimal levels for net photosynthesis (Dilks and Proctor 1979). This adaptation optimises net photosynthesis. Many bryophytes tolerate extended periods at low water contents, but leakage of metabolites and a respiratory burst upon rehydration represent a direct cost of desiccation (Proctor 1990). Both desiccation and maximal hydration have a negative impact on the rate of photosynthesis in mosses and lichens (Lange *et al.* 1998). When water is taken up by the thallus, photosynthesis increases with increasing water content (WC) up to an optimum. With further increases in WC, many, but not all lichens and mosses display different degrees of depressed photosynthesis (Lange *et al.* 1993, Sundberg *et al.* 1997b). This depression may be reversed by increasing the external $CO_2$ concentration or by allowing the thallus to dry, losing some of the excess water (Rundel 1982). There is a huge variation among species in their ability for tolerance. Dilks and Proctor (1974) have shown that *Plagiothecium undulatum* is severely damaged at lower water potential, whilst *Racomitrium lanuginosum* easily survive at low water potentials. Scheidegger *et al.*

(1995) have shown that, many lichen species even at very low water contents display net photosynthesis. In mosses, rapid water loss causes more damage to the photosynthetic apparatus than slow drying (Krochko *et al.* 1979, Dhindsa and Matowe 1981, Dhindsa 1987).

The impact of the temporal variation in water supply on bryophyte and lichen performance can be summarized by three factors important for water balance of the thallus and colonies and the magnitude of the costs of desiccation. Species of humid habitats are often severely damaged by prolonged or intense desiccation, with high mortality as the result. Thalli of more desiccation tolerant species do, however, recover from desiccation, but recovery is generally more complete and rapid after short periods of desiccation (Dilks and Proctor 1974). The magnitude of the gradient in water potential between thallus and ambient air influences both the desiccation rate and the desiccation intensity (i.e. how rapidly the thallus loose water and how low the cell water potential drops during desiccation). Leakage of metabolites increases the more rapid or intense the desiccation is (Schonbeck and Bewley 1981a), and recovery is better with slow drying rate (Schonbeck and Bewley 1981a, Proctor 1982). Even though hardening in response to desiccation has been found (Schonbeck and Bewley 1981b, Proctor 1982), the costs associated with desiccation, the respiratory loss and leakage of metabolites upon rehydration should be expected to increase with an increased frequency of the dry periods.

### 1.2.2 Generation of active oxygen species

In plant and animal tissues, the generation of ROS is considered to be a primary event under a variety of stress conditions (Noctor and Foyer 1998). ROS are routinely generated at low levels in non-stressed plant cells in the chloroplasts and mitochondria (Halliwell 1987, Elstner 1991, Smirnoff 1993), and also by cytoplasmic, membrane-bound enzymes involved in redox reactions (Wojtaszek 1997). However, stresses e.g. desiccation greatly enhances the production of these toxic oxygen species (Smirnoff 1993, Sgherri *et al.* 1996, Kranner and Lutzoni 1999). ROS such as superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals ($^{\cdot}OH$) are produced by the sequential reduction of molecular oxygen during plant metabolism and are also induced by various stress factors such as light (Scandalios 1993), salt (Cella and Carbonella 1998) and desiccation (Smirnoff 1993). When molecular

oxygen is incorporated into organic molecules, ROS are often produced. These ROS can damage cell structure and metabolism either through their oxidizing activity or by inducing the formation of organic free radicals (Smirnoff 1993). An increase in active oxygen formation would either become manifest as oxidative damage to plants or would result in the activation of defence and repair mechanisms which could prevent damage from occurring. Severe dehydration could directly hinder antioxidant defence and leave the tissue exposed to oxidative deterioration (Smirnoff 1993).

Molecular oxygen is not particularly reactive although it is a free radical with two unpaired electrons; they are of parallel spin (Smirnoff 1995). However, oxygen can become more reactive when transformed into more reduced or electronically excited state. The sources of superoxide are electron transport activities in which electrons are diverted from normal metabolic course. Chloroplasts are able to produce $O_2^{\cdot-}$ by the reduction of $O_2$ at photosystem I and II (Asada and Takahashi 1987). However, superoxide itself is short-lived not highly reactive and damage arises from subsequent formation of hydrogen peroxide and even more from hydroxyl radicals (Halliwell and Gutteridge 1989). $H_2O_2$ is a relatively stable ROS, being not very reactive and electronically neutral, $H_2O_2$ is able to pass through cell membranes and reach cell locations remote to the site of its formation (Wojtaszek 1997). Furthermore, $H_2O_2$ can inactivate a number of Calvin cycle enzymes (Charles and Halliwell 1980).

The formation of these species from superoxide starts with the dismutation of superoxide to hydrogen peroxide and water. Superoxide radical and hydrogen peroxide react to form hydroxyl radicals (Haber-Weiss reaction) the most reactive species among ROS. On the other hand, significant levels of hydroxyl radicals could be formed through the cycle reactions involving oxidation of transition metals, such as iron and copper (Fenton reaction), and subsequent regeneration of the oxidized ions to their reduced state via reactions with superoxide (Wojtaszek 1997). The hydroxyl radical with its ability to initiate radical chain reactions is believed to be a major ROS responsible for the irreversible damage of organelles. Furthermore, singlet oxygen is produced under water stress when photosynthesis is inhibited and unable to dissipate the excess light absorbed by the photosynthetic pigments. The absorption of excess light by chlorophyll may result in the formation of triplet state chlorophyll, which can then pass excitation energy to $O_2$ resulting in the formation of singlet oxygen

(Smirnoff 1993). The consequences of ROS formation depend on the severity of stress and on the physiochemical conditions in the cell (i.e. antioxidant status, redox state, and pH).

### 1.2.3 Lipid peroxidation

Plants respond to water stress by modifying aspects of their lipid metabolism, and these depend on the plant species and the conditions of desiccation (Liljenberg 1992). Lipid peroxidation (LP) products have a pronounced effect on membrane orientational order, packing and interaction of phospholipids (Dhindsa *et al.* 1981). A well-known effect of water stress is lipid oxidation that can lead to disruption or destabilization of membrane structure (Dhindsa *et al.* 1981, Liljenberg 1992, Seel *et al.* 1992b). This oxidation is caused by the generation of ROS during desiccation (Smirnoff 1993, Jiang 1999). LP is easily ascribed to oxidative damage but its symptoms are not easily measured. These include protein denaturation and damage to nucleic acids (Kunert and Enderer 1985). Iron can influence peroxidation by enhancing hydroxyl radical production in the Harber-Weiss reaction or it may react with lipid hydroperoxidases to form alkoxy and peroxy radicals. These can draw an extra hydrogen atom from other fatty acids (Smirnoff 1993). Interestingly, LP products do not affect the fluidity of the membranes, but membrane fluidity has been shown to affect the rate of LP propagation stage (Shewfelt and Purvis 1995). The water stress-induced changes in lipid metabolism may reflect direct effects of ROS or may be a result of the need to repair damaged membrane lipids (Guschina *et al.* 2002).

Not only can lipid biosynthesis be changed by water stress, but degradation of polar lipids may also be altered (Sahsah et al. 1998). Recently, scientists have realized that lipid metabolism is a key factor in adaptation mechanisms of many organisms to environmental stress. In lichens, several workers have studied the importance of lipids in response to environmental factors such as light, temperature and humidity (Shapiro *et al.* 1998, Bychek-Guschina *et al.* 1999). Dhindsa and Matowe (1981) and Seel *et al.* (1992a) have suggested that in mosses oxidative damage due to LP occurs during desiccation. By measurement of malondialdehyde, an inductor of LP, Seel *et al.* (1992b) demonstrated that the relatively desiccation intolerant moss exhibited increased LP following desiccation, while the tolerant species *Tortula ruraliformis* did not. Interestingly, the extent of LP, whether in hydrated or desiccated gametophytes,

was five to six-folds higher in the intolerant species. This indicates an inherent protection against LP in tolerant species (Oliver *et al.* 2000).

### 1.2.4 Membrane damage and ion leakage

Mechanisms of desiccation tolerance are sometimes based on membrane behaviour. Cell membranes have been regarded for a long time as an important site of desiccation injury, mainly because the earliest symptom of injury is enhanced leakage of cytoplasmic solutes during rehydration (Simon 1974). Ultrastructural studies of dry tissue confirm that membrane disorganization is a common phenomenon, but only if desiccation is lethal, i.e. in cells that are dried at a tolerant stage remain organized (Crèvecoeur *et al.* 1976, Dasgupta *et al.* 1982). The disorganization of membranes and their apparently enhanced permeability may be simply a consequence of physical rupture of the membrane due to tearing during cellular collapse, or alternatively, it may involve more subtle changes in the physical organization of lipid or protein components in an otherwise intact membrane. All desiccation tolerant tissues leak solutes during rehydration (Simon 1978, Bewley 1979, Bewley and Krochko 1982), indicating that the cell membrane has been compromised. Intolerant cells suffer extensive leakage of solutes when rehydrated (Smirnoff 1993). LP is considered a very important process underlying membrane degradation during dehydration-rehydration period of the plant tissue. This is evident from ultrastructural studies showing progressive deteriorative changes in organelles and membranes of plant tissue and also from membrane permeability studies that indicate increased solute leakage. Large changes in the physical properties of membrane leakage accompanying rehydration appear to contribute to the loss of permeability and are also correlated with a decline in membrane lipids due to LP (Dhindsa *et al.* 1981, Guschina *et al.* 2002).

Susceptibility of plants to ion leakage through membranes after rehydration is an important component of the overall desiccation tolerance in mosses. Furthermore, Beckett and Hoddinott (1997) showed large seasonal changes in $K^+$ leakage following desiccation in a moss *Atrichum androgynum*. *A. androgynum* lost much less $K^+$ during the dry winter months than the moist summer months suggesting that unlike *T. ruralis*, *A. androgynum* has an inducible protection based tolerance mechanism. Membrane deterioration of plant tissue is also associated with the breakdown of fatty

acids, particularly the unsaturated fatty acids, and the products of peroxidation such as aldehyde and malondaldehyde (MDA) (Paulin *et al.* 1986). These products accumulate in membranes during dehydration-rehydration period and appear to cause destabilization of membrane bilayer structure and loss of membrane function (Barber and Thompson 1983). Jiang and Zhang (2002) reported that water stress led to a significant increase in the percentage of electrolyte leakage, but did not affect the MDA content. However, pretreatment with diphenylene iodonium (DPI) or tungstate increased the content of MDA and the percentage of electrolyte leakage in the water stressed maize leaves. While the application of ABA fully prevented the increase caused by tungstate in the stressed leaves.

### 1.3 Protection against stress

Desiccation tolerant plants exhibit cellular changes, some of which can be described as extensive damage, during and following desiccation (Bewley 1979). The plant or tissue must limit damage to a repairable level, maintain its physiological integrity in the dried state and mobilize mechanisms upon rehydration that repair damage suffered during desiccation and rehydration (Alpert and Oliver 2002). Desiccation tolerant plants employ mechanisms that protect them from the rigours of the extensive water loss and also mechanisms, at least in the case of vegetative cells, that repair damage suffered during desiccation and rehydration (Bewley and Oliver 1992). Cellular repair, as a component of desiccation tolerance mechanisms, is more easily defined in desiccation tolerant bryophytes. Desiccation tolerance in bryophytes is thought to employ a mechanism for desiccation tolerance that represents the most primitive form expressed in land plants (Oliver *et al.* 2000). Because bryophytes have little in the way of adaptations to retain water within the plant and, as a result, the internal water content of these plants rapidly equilibrates to the water potential of the environment (Proctor *et al.* 1998). A consequence of this is that many bryophytes experience drying rates that are extreme and therefore have insufficient time to induce and set in place protective measures, especially species occurring in xeric habitats. It appears that bryophytes have evolved constitutive protective mechanisms for desiccation tolerance, one that has protective measures that are always in place (Alpert and Oliver 2002). Evidence concerning desiccation tolerance of modified desiccation tolerant plants strongly suggests that tissues and plants utilize mechanisms that rely heavily on inducible cellular protection systems (Bartels and Nelson 1994, Bartels *et al.* 1993,

Bewley *et al.* 1993, Bewley and Oliver 1992, Close *et al.* 1993, Crowe *et al.* 1992, Dure 1993a, Oliver and Bewley 1997). However, desiccation tolerant moss *T. ruralis* displays a constitutive protection mechanism (Close 1996). The constitutive protection mechanism appears to be particularly effective in preventing damage to the photosynthetic apparatus, as evidenced by the rapid recovery of photosystem II activity (Tuba *et al.* 1996, Csintalan *et al.* 1999, Proctor and Smirnoff 2001). Among the metabolic changes that take place prior to or during drying is the synthesis of proteins and sugars. These two have long been postulated to form the basis of a series of overlapping protective mechanisms that limit damage to cellular constituents (Bewley 1979, Leprince *et al.* 1993, Oliver and Bewley 1997). Also they have since been widely implicated as being critical for desiccation tolerance in all plant cells including vegetative cells (Ingram and Bartels 1996, Oliver and Bewley 1997, Scott 2000).

### 1.3.1 Sugars

Accumulation of soluble sugars is strongly correlated to the acquisition of desiccation tolerance in plants and other organisms (for reviews see Crowe *et al.* 1992, Leprince *et al.* 1993, Vertucci and Farrant 1995). Plants and animals that can survive dehydration accumulate high concentrations of disaccharides in their cells and tissues during desiccation (Oliver *et al.* 2000). Many lower organisms are desiccation tolerant, but there are only a small number of higher plants, the so-called resurrection plants, that can withstand complete desiccation during vegetative growth (Bewley and Krochko 1982, Ingram and Bartels 1996). Is this resistance towards desiccation linked to a rapid accumulation of compatible solutes, especially carbohydrates? Solute accumulation decreases cell osmotic potential, which in turn allows turgor or cell volume to be maintained or increased relative to non-stressed plants (Morgan 1984). In case of lower organisms, besides other compatible solutes, the disaccharide trehalose is thought to play a central role as a protectant against desiccation (Crowe *et al.* 1984, Wiemken 1990). Interestingly, while trehalose is generally absent in higher plants (Müller *et al.* 1995), three resurrection plants have been reported to accumulate trehalose upon desiccation, the pteridophyte *Selaginella lepidophylla* (Adams *et al.* 1990), the angiosperm *Myrothamnus flabellifolius* (Bianchi *et al.* 1993, Drennan *et al.* 1993) and *Sporobolus staptranus* (Albini *et al.* 1994). In resurrection angiosperms desiccation tolerance appears to involve an increase in sucrose during desiccation,

presumably to promote vitrification of the cytoplasm and protect membranes (Scott 2000). Sucrose is the only free sugar available for cellular protection in desiccation tolerant plants e.g. mosses including *T. ruraliformis* and *T. ruralis* (Bewley *et al.* 1978). However, the preliminary studies of Smirnoff (1992) showed no increase in sugar concentration during desiccation in a range of mosses. Neither drying nor rehydration in the dark or light resulted in a change in sucrose concentration suggesting it is important for cells to maintain sufficient amounts of this sugar (Bewley *et al.* 1978). In other cases, stress-induced changes may reflect adaptation for stress tolerance. For example, cyclic carbohydrates have been reported to accumulate during drought (Keller and Ludlow 1993, Wannek and Richter 1997), and sugars are only present at low concentrations in bryophytes.

### 1.3.2 Proteins

In addition to sugars, proteins are considered to be another component of desiccation tolerance. Various types of drought-inducible proteins have been identified (Skriver and Mundy 1990, Shinozaki and Yamaguchi-Shinozaki 1997). Among a number of plant genes induced by water stress are ABA-inducible *cis*- and *trans*-acting factors, a number of genes for protein kinases and enzymes involved in phosphatidylinositiol turnover (Shinozaki *et al.* 1998) In addition, among these, dehydrins are known to accumulate immediately before desiccation during seed development (Close *et al.* 1989, Dure 1993c). Dehydrins also occur in mature pollen grains (Michel *et al.* 1994, Wang and Cutler 1995). It has been suggested that these proteins play a role in protecting plant structures during water loss. During abiotic stresses, plants synthesize new proteins, which are to be important for the survival of these plants. A synthesis of large number of new proteins during rehydration and the existence of rehydrins in desiccation tolerant tissues of bryophytes has only recently been reported (Oliver *et al.* 1994). Whilst rehydrins are initiated during rehydration, the synthesis of another form of proteins called hydrins is inhibited (Oliver *et al.* 1994). Furthermore, Oliver *et al.* (1994) showed that during dehydration bryophytes synthesize a new class of proteins, the dehydrins, and later Oliver (1996) suggested that they are synthesized from mRNA stored on ribonuclear particles. Dehydrins can be induced in vegetative tissues by a variety of environmental stresses (e.g. drought, cold, salt) and by the external application of ABA (Dure 1993b). Various stresses cause dehydration of the plant tissue, which respond by producing dehydrins. Dehydrin expression, have been

shown to be related to situations that induce cellular dehydration (Reviewed by Close 1996). Similarly, the accumulation of dehydrin-like proteins in birch buds was correlated with the cellular water status (Welling *et al.* 1997), but only if desiccation was due to photoperiod. Dehydrins may protect cells against dehydration damage, although the mechanism had not yet been established (Close 1996). Some species possess dehydrin proteins that may act as surfactants, thus inhibiting the coagulation of a range of macromolecules (Hellwege *et al.* 1994, 1996). *T. ruralis* possesses two major dehydrins (80-90KD and 35KD). Rather surprisingly, unlike most other systems investigated, these two dehydrins are present in hydrated vegetative tissue, and do not appear to increase or decrease during rapid or slow drying (Bewley *et al.* 1993). Similar results have been obtained with the desiccation tolerant moss *Thuidium delacatulum* (Oliver *et al.* 1997). Thus, in desiccation tolerant species, proteins appear to possess a constitutive protection system and a rehydration-induced recovery mechanism.

### 1.4 Antioxidants as defence against stress

In the oxidative stress-associated disorder the main function of low molecular antioxidants is assumed to be ROS scavenging. However, antioxidants such as ascorbate, glutathione and tocopherols have a number of other important physiological functions. Elucidation of the relationship between scavenging of ROS and other functions of antioxidants in association with stress-induced metabolic changes is of particular interest, since it has been shown recently that initiation of a signal transduction is due to the combination of different physicochemical factors (Clement *et al.* 1998). Because susceptibility to peroxidation may increase with drying (See Vertucci and Farrant 1995, Guschina *et al.* 2002), one may reason that free radical scavenging systems are an important component among the mechanisms of desiccation tolerance. ROS are natural by-products of metabolism, which are particularly present in chloroplasts and mitochondria (Halliwell 1987, Elstner 1991, Smirnoff 1993). Thus, plants are well endowed with antioxidant molecules and scavenging systems (Larson 1988, Hendry 1993). Several studies have demonstrated the link between tolerance of oxidative stress induced by water deficit and rise in antioxidant systems (Winston 1990, Price and Hendry 1991). In vegetative tissues, removal of the cytotoxic products resulting from oxidative events is considered to be of prime importance for survival of desiccation stress because genes encoding

enzymic antioxidants become up-regulated during drying (Ingram and Bartels 1996). Over the years it has also been clear that the synthesis of antioxidants and enzymes involved in oxidative metabolism also play a critical role in cellular protection and desiccation tolerance. Oxygen is potentially toxic since it can be transformed by metabolic activity into reactive forms (Halliwell and Gutteridge 1989). Plant cells contain a number of protective and repair systems, which minimize the occurrence of oxidative damage. The defence mechanisms are divided into two groups: the enzymic and non-enzymic antioxidant systems. Free radical scavenging enzymes include superoxide dismutase (SOD), peroxidases (PO) and catalases (CAT) and the non-enzymic antioxidants include ascorbate, α-tocopherol and glutathione. Antioxidant systems, both enzymic and non-enzymic interact to reduce the risk of damage by ROS (Bowler *et al.* 1992). Without these defences plants could not remove the excessive free radicals that are generated as a product of normal metabolism or increased under stressful conditions. Under normal metabolic conditions the formation of ROS and peroxidation of membrane lipids are in dynamic equilibrium with the activity of the antioxidant systems. Any changes in environmental conditions may alter this equilibrium and lead to oxidative stress such alteration may be achieved either by enhanced ROS formation, or by slowing down its removal, i.e. inhibition of antioxidant systems.

### 1.4.1 Non-enzymic antioxidants

#### 1.4.1.1 Ascorbic acid

Ascorbic acid (AA) has been shown to have an essential role in several physiological processes in plants, including growth, differentiation and metabolism (Foyer 1993). Ascorbate functions as a reductant for many free radicals, thereby minimizing the damage caused by oxidative stress but ascorbate may have other functions, which remain undefined. Ascorbate can directly scavenge oxygen free radicals with and without enzyme catalysts and can indirectly scavenge them by recycling tocopherol to the reduced form (Thomas *et al.* 1992). By reacting with activated oxygen more readily than any other aqueous component, ascorbate protects critical macromolecules from oxidative damage. The reaction with the hydroxyl radical is limited only by diffusion. The indirect role of ascorbate as an antioxidant is to regenerate membrane-bound antioxidants, such as α-tocopherol, that scavenge peroxy radicals and singlet oxygen, respectively. However, in the presence of metal ions it can act as an

antioxidant (Foyer *et al.* 1991). In addition, ascorbate takes part in the regulation of the cell cycle affecting the progression from G1 to S phase, and it has been implicated in the regulation of cell elongation (Smirnoff 1996). Furthermore, ascorbate is also implicated in the regulation of photosynthetic light harvesting, because it is a cofactor for violaxanthin de-epoxidase (Bratt *et al.* 1995, Smirnoff 1996). Under high light intensity, low temperatures and desiccation stress, when $CO_2$ fixation is limited, the excess excitation energy is dissipated as heat by the zeaxanthin and ascorbate, which acts as a photoprotectant (Smirnoff 1996). Not only does the chloroplast ascorbate pool detoxify hydrogen peroxide, thereby preventing enzyme inactivation and the generation of more dangerous radicals, it also allows flexibility in the production of photosynthetic assimilatory power (Foyer *et al.* 1990, Foyer 1997).

### 1.4.1.2 Carotenoids

Carotenoids not only function as accessory pigments in photosynthesis but also protect plants from photooxidative damage by scavenging active oxygen species. For protection against singlet oxygen in the chloroplasts, carotenoids were found to be the most competent scavengers (DiMascio *et al.* 1989). Carotenoids protects plants in two ways: either by converting the excitation energy of triplet state chlorophyll before it can interact with oxygen back to the ground state or by de-excitation of any singlet oxygen that may be formed (Young and Britton 1990). When normally protective carotenoids are absent or ineffective, de-excitation of triplet chlorophyll may not occur and the protective system may become overloaded (Bramley 1993). In this case, accumulation of singlet oxygen may lead to subsequent destruction of photosynthetic membranes (Young and Britton 1990, Sharma and Hall 1997). On the other hand, it is now widely accepted that zeaxanthin is involved in the de-excitation of excess energy via non-radiative dissipation in the pigment bed (Demming-Adams and Adams 1992). There is evidence that zeaxanthin and antheraxanthin may be responsible for the development of non-radiative energy dissipation (Horton *et al.* 1996, Gilmore 1997, Niyogi *et al.* 1998). The xanthophyll cycle is a well known mechanism that protects photosystems against excess light by facilitating the thermal dissipation of excess light energy, thus avoiding formation of singlet oxygen (Siefermann-Harms 1990, Demming-Adams and Adams 1996, Kranner and Lutzoni 1999).

### 1.4.1.3 Alpha tocopherol

Tocopherol is actually a family of antioxidants (Hess 1993) that includes four tocols. α-Tocopherol is generally considered to be the most active form of the tocols; the other forms may be biosynthetic precursors. α-Tocopherol is well established as a membrane stabilizing agent and is a lipophilic membrane located compound, which can scavenge singlet oxygen, although it may have a more important role in scavenging lipid peroxy radicals, thus preventing LP (Kunert and Böger 1984, Winston 1990, Fryer 1992). α-Tocopherol is an effective protector against peroxidation *in vitro* (Tappel 1980) and is located in the biomembranes. Further, a high concentration of α-tocopherol has been found in the chloroplast envelope (Lichtenthaler *et al.* 1981). In the thylakoid membranes, α-tocopherol is the major mobile lipophilic antioxidant, which blocks the chain propagation reactions of LP (Larson 1988, Fryer 1992). α-Tocopherols also inhibit oxidation induced singlet oxygen by physical quenching or by chemical reactions (Kamal-Eldin and Appelquist 1996). In addition, α-tocopherol has non-antioxidant functions in cell membranes, which are less known. They seem to modulate membrane structure, thus altering membrane permeability and phase transition (Wassal *et al.* 1986). They also protect integral membrane proteins (Takenaka *et al.* 1991) and have been identified as the endogenous free radical scavengers slowing down the aging in plants (Dhindsa *et al.* 1981). It has been demonstrated that α-tocopherols can protect biological membranes against phospholipases and their hydrolysis products, free fatty acids and lysophospholipids, which are characteristically produced in large amounts in several stress situations (Blokhina *et al.* 2000). It has been suggested that a fundamental part of the biological action of tocopherol is due to its ability to physically stabilize membrane structure (Fryer 1992).

### 1.4.1.4 Glutathione

Glutathione is a potent cellular reductant, is easily oxidised, and can function as an antioxidant in many ways. It acts as a scavenger of peroxides and serves as a storage and transport form of reduced sulphur (May *et al.* 1998). It has been also shown that glutathione acts as a regulator of gene expression (Baier and Dietz 1997), and is a precursor of phytochelatins (Grill *et al.* 1989). Due to the redox potential of the active thiol group, reduced glutathione (GSH) may be involved in the regulation of the cell

cycle and act as a defence compound against oxidative stress (Cittero *et al.* 1994). GSH is probably the most important antioxidant, which can directly react chemically with singlet oxygen, superoxide and hydroxyl radicals and therefore function directly as a free radical scavenger and during drying may protect, via thiol-disulfide exchange, the thiol status of proteins. This maintains their metabolically active form and the activity of enzymes that possess exposed thiol groups (Gilbert *et al.* 1990). GSH may stabilize membrane structure by removing acyl peroxides formed by LP reactions (Price *et al.* 1990). GSH is the reducing agent that recycles ascorbic acid from its oxidized to its reduced form by the enzyme dehydroascorbate reductase (Loewus 1988). GSH can also reduce dehydroascorbate by a non-enzymic mechanism at pH > 7 and GSH concentrations greater than 1 mM. This may be a significant pathway in chloroplasts whose stromal pH in the light are about 8 and GSH concentrations may be as high as 5 mM (Foyer and Halliwell 1976, Noctor *et al.* 1998).

$H_2O_2$ levels have been found to increase during water stress (Sgherri *et al.* 1994b). GSH may also metabolise $H_2O_2$ by participating in ascorbate-glutathione cycle that eliminates the risk of oxidation of enzymes by $H_2O_2$ in the chloroplast. This cycle involves reactions of glutathione, ascorbic acid, glutathione reductase (GR), ascorbate peroxidase (APX) and mono- and dehydroascorbate reductases (Kunert and Foyer 1993), or in the reaction catalysed by glutathione peroxidase (GP) (Droter *et al.* 1985, Holland *et al.* 1993). The scavenging of free radicals by the antioxidant glutathione can lead to oxidation of GSH to glutathione disulphide (GSSG). In unstressed tissues, glutathione is maintained in the reduced state by the action of NADPH-dependant glutathione reductase (GR), and in higher plants, accumulation of GSSG is often correlated with increased stress. In lichens, the shifts in the redox status of the antioxidant glutathione are part of the response process of desiccation and rehydration (Kranner 2002). Furthermore, GP and glutathione transferase (GT) reduce organic peroxides, thus protecting cell proteins and membranes from oxidation (Bartling *et al.* 1993, Navari-Izzo *et al.* 1994). Although very few studies have been carried out on lichens, Kranner and Lutzoni (1999) showed that GSH probably play a pivotal role in conferring desiccation tolerance in these organisms. Recently, Kranner (2002) studied glutathione-related mechanisms of coping with desiccation in three lichens (*Pseudevernia furfuraca*, *Peltigera polydactyla* and *Lobaria pulmonaria*) with

different degrees of tolerance. Results showed that following long-term desiccation, *P. furfuraca* rapidly increased GSH when rehydrated in liquid water or water vapour, whilst *L. pulmonaria* regenerated initial concentrations of GSH only when rehydrated in liquid water and in *P. polydactyla* neither method of rehydration re-established the initial GSH pool (Kranner 2002).

### 1.4.2 Enzymic antioxidants

#### 1.4.2.1 Superoxide dismutase

Superoxide dismutases are a group of metallo enzymes that catalyse the disproportionation of superoxide radicals generated in different cellular compartments to $H_2O_2$ and $O_2$ (for review see Smirnoff 1993). Since SOD is present in all aerobic organisms and most (if not all) subcellular compartments that generate activated oxygen, it has been assumed that SOD has a central role in the defense against oxidative stress (Beyer *et al.* 1991, Bowler *et al.* 1992, Scandalios 1993). High SOD activity maintains a low steady state concentration of superoxide radical and therefore minimizes hydroxyl radical formation. There are three distinct types of SOD classified on the basis of the metal cofactors: the copper/zinc (Cu/Zn - SOD), the manganese (Mn-SOD) and the iron (Fe-SOD) isozymes (Bannister *et al.* 1987). These isozymes can be separated by native polyacrylamide gel electrophoresis, their activity detected by negative staining and identified on the basis of their sensitivity to KCN and $H_2O_2$. The Mn-SOD is resistant to both inhibitors, whereas the Cu/Zn-SOD is sensitive to both inhibitors; Fe-SOD is resistant to KCN, and sensitive to $H_2O_2$. The subcellular distribution of these isozymes is also distinctive. The Mn-SOD is found in the mitochondria of eukaryotic cells; some Cu/Zn-SOD isozymes are found in the cytosol, others in the chloroplasts of higher plants (Navari-Izzo *et al.* 1998). The Fe-SOD isozymes are often not detected in plants, but when detected, Fe-SOD is usually associated with the chloroplast compartment (Bowler *et al.* 1992).

To date it has been shown that SOD activity is increased in cells in response to diverse environmental and xenobiotic stresses including paraquat, high light, water logging and drought. Apparently, each of the SOD isozymes are independently regulated according to the degree of oxidative stress experienced in the respective subcellular compartments, but how this is communicated at the molecular level is unknown. Bowler *et al.* (1992) have suggested that this role may be served by unique

LP products from each organelle that diffuse from the site of oxidative damage to the nucleus where they would enhance transcription of specific SOD genes. Several reviews on SOD have recently been published which describe the characteristics of the enzymes, the cloned cDNA sequences and genes, and the effects of over expression in transgenic plants (Bowler *et al.* 1994, Doke *et al.* 1994, Foyer *et al.* 1994, Gressel and Galun 1994, Scandalios 1993, Van Camp *et al.* 1994). In mosses the dependence of desiccation tolerance on the ability to process active oxygen species has been reported (Seel *et al.* 1992a, Dhindsa and Matowe 1981). The distinction between desiccation tolerance and intolerance in mosses may be a function of various molecular defences against active oxygen species formed by the reduction of oxygen (Seel *et al.* 1991, 1992a). SOD activity responds differently to water deficit in different species. Desiccation led to a significant increase in SOD activities in *T. ruraliformis*, a tolerant species and little or no effect in SOD in *Dicranella palustris*, a sensitive moss (Seel *et al.* 1992a). Similar results were reported in mosses desiccated under high irradiance (Dhindsa and Matowe 1981). SOD activity in *Armeria maritima* and *Deschampsia flexuous* increased in response to water stress but not in *Cochlearia atlantica* and a number of other grass species (Price and Hendry 1989, Buckland *et al.* 1991). An increase in SOD activity has been observed in barley (Smirnoff 1993). However, in tomatoes only the cytoplasmic Cu/Zn-SOD was induced by water stress (Bowler *et al.* 1992). SOD protects plant cells from the action of ROS in response to a number of oxidative stresses (Rabinowitch and Fridovich 1983). However, the activation of SOD under water deficit and the possible relationship between activation and the overall tolerance is still controversial (Dhindsa *et al.* 1981, Luna *et al.* 1985). The increased SOD activity in some species (Badiani *et al.* 1990) can imply a water stress-induced superoxide formation whereas in others, the control of an increased superoxide radical production, if any, could rely primarily on some scavenging systems other than SOD. It is therefore, difficult to interpret changes in SOD activity.

### 1.4.2.2 Peroxidases

Plant peroxidases are divided into two groups: the peroxidases whose primary function is not hydrogen peroxide scavenging, these serve a physiological function (e.g. lignification). The other group consists of enzymes whose primary function is hydrogen peroxide scavenging, these includes ascorbate peroxidase (Asada 1992). No relationship has been reported for guaiacol peroxidase as far as hydrogen peroxide

scavenging. Thylakoid-bound APX catalyses the reduction of $H_2O_2$ by ascorbate, producing the monodehydroascorbate (MDA) radical, which in turn is reduced to ascorbate using electrons from photo-reduced ferrodoxin or from NAD(P)H through monodehydrascorbate reductase (MDAR) (Miyake and Asada 1994). Relatively few measurements of APX have been reported during water deficit (Smirnoff 1993). APX increases in *Eragrostis tef* but not significantly in barley during water deficit (Smirnoff and Colombe 1988). An increase in APX has been reported in *Lycopersicon esculentum* and *Lycopersicon chiliense* during water stress but in *Cochearia atlantica* and *Armeria maritima* APX activity was not affected (Buckland *et al.* 1991). The increased or maintained activity of APX could indicate an enhanced production of $H_2O_2$ by the plants under stress (Badiani *et al.* 1990). Animals have a selenocysteine-containing glutathione peroxidase which catalyses the oxidation of GSH by $H_2O_2$ and this has a major antioxidant function (Smirnoff 1993). GSH peroxidase activity is absent or very low in higher plants although it has been reported occasionally (Droter *et al.* 1985, Holland *et al.* 1993). Furthermore, peroxidase activity has been positively correlated with plants resistance to pathogen attack. However the precise role of peroxidase in the plants defence reaction in response to pathogens is not clearly understood.

### 1.4.2.3 Catalases

Catalase is a heme-containing enzyme that catalyses the dismutation of hydrogen peroxide into water and oxygen. The enzyme is found in all aerobic eukaryotes and is important in the removal of hydrogen peroxide generated in peroxisomes (microbodies) by oxidases involved in ß-oxidation of fatty acids, the glyoxylate cycle (photorespiration) and purine catabolism. There is no marked response to water deficit reported in relation to catalase activity (Gamble and Burke 1984, Badiani *et al.* 1990, Buckland *et al.* 1991). A decrease in CAT activity in response to a short-term water deficit has been reported in mung bean seedlings (Mukherjee and Choudhuri 1983) and in sunflower (Quartacci and Navari-Izzo 1992). Seel *et al.* (1992a) reported a decrease in CAT activity in a desiccation tolerant moss *T. ruraliformis* and a decrease in the more sensitive moss *D. palustris* following desiccation.

## 1.5 Oxidative burst in response to stress

Plants are endangered by a large number of bacterial and fungal pathogens and have

to be protected. Several observations point to a possible molecular mechanism shared by biotically and abiotically induced oxidative stress (Baker and Orlandi 1995, Van Camp *et al.* 1998). The production of ROS not only acts as toxic by-products, but also as an important component of the plant defence response to pathogenic infections (Wojtaszek 1997, Allan and Fluhr 1997, Bolwell 1999, Bolwell *et al.* 1999). However, the source of ROS in the oxidative burst is only partially elucidated. Controversy exists as to which of the ROS constitute the oxidative burst in plant cells. The oxidative burst is generally defined as a rapid production of high levels of ROS in response to the external stimuli (Mendy 1994). It is believed to be an important part of the defence response of plants against pathogens including programmed cell death, also called hypersensitive response (Mittler and Zilinskas 1994, Wojtaszek 1997). High rates of superoxide and hydrogen peroxide production create an oxidizing film on the surface of a plant, inhibiting the germination of fungal spores and bacterial cell division (Murphy *et al.* 1998). Most of the data seems to indicate that the major ROS forming the oxidative burst is hydrogen peroxide, with possible participation of superoxide. However, the inherent relationship between hydrogen peroxide and superoxide generation makes it difficult to identify clearly the ROS behind the oxidative burst. Most studies have emphasized that, pathogens or elicitors derived from them, can induce extracellular ROS production (Wojtaszek 1997). However, evidence is accumulating that extracellular production of ROS can also be induced by abiotic stresses that leave plants vulnerable to bacteria or fungi e.g. mechanical disruption or UV light (Murphy *et al.* 1998). Interestingly, Chandra *et al.* (2000), studying the oxidative burst in cultured higher plant cells, recently showed that communication occurs between elicitor molecule and mechanical stimuli, indicating that significant cross talk exists between the different signalling pathways. Experimental data suggests that the main sources of ROS produced during oxidative burst in plant cells are enzymes localized at the external surface of the plant cells, specifically NADPH-, NADH-, or other oxidases, and/or cell wall peroxidases (Lüthje *et al.* 1997, 2000, Bolwell *et al.* 1998, Bolwell 1999).

### 1.6 Strategies of desiccation tolerance and abscisic acid

The plant hormone abscisic acid (ABA) modulates a wide spectrum of responses, including gene activation and repression, and photosynthesis inhibition, under multiple environmental stress conditions such as drought, cold, salinity and

desiccation (Davies 1991, Bray 1997, Leung and Giraudat 1998). Desiccation tolerance in the highly desiccation tolerant bryophytes appears to be largely constitutive (Oliver and Bewley 1997, Oliver *et al.* 1998). However, in many species of more mesic habitats, tolerance is induced to varying degrees. ABA-induced tolerance (involving synthesis of specific proteins during drying) has been demonstrated in higher plants, ferns and bryophytes (Werner *et al.* 1991, Bopp and Werner 1993, Reynolds and Bewley 1993, Hellwege *et al.* 1993, Leung and Giraudat 1998, Muchaka *et al.* 1999, Lichi *et al.* 2000). Beckett (1999) showed that partial dehydration of the moss *A. androgynum* increased resistance to desiccation-induced ion leakage tolerance, and that exogenously supplied ABA produced the same effect. ABA also is associated with dehydration-regulated gene expression (Bellaire *et al.* 2000, Guan *et al.* 2000, Philips *et al.* 2002). It plays a pivotal role in desiccation tolerance (Oliver and Bewley 1997), and induces the formation of a set of proteins in the xerophilic liverwort *Exormetheca holstii* (Hellwege *et al.* 1994). Hellwege *et al.* (1996) have shown that ABA can bring about the transition from the aquatic to land form of *Riccia fluitans*. The fluctuations of endogenous ABA during desiccation and rehydration seem to be sufficiently high indicating the role of ABA as a stress hormone and thereby as an indicator of stress-related protein synthesis. Exposure to exogenous ABA causes the induction of genes that otherwise are activated by dehydration. In most bryophytes, limited information is available on the endogenous levels of ABA and how they change under different environmental conditions (see Bopp and Werner 1993, Christianson 2000). Slow drying of *Funaria hygrometrica* increased its endogenous ABA content six fold, and was correlated with increased tolerance to rapid drying. Moreover, exogenously applied ABA could also increase tolerance to rapid drying (Werner *et al.* 1991). The ability to respond to ABA with enhanced desiccation tolerance thus seems likely to be widespread among bryophytes, but how widely endogenous ABA occurs within the bryophytes is unknown. ABA is undetectable in *T. ruralis*, in which a high level of desiccation tolerance is constitutive (Oliver and Bewley 1997).

Most genes that respond to drought are also abscisic acid (ABA)-responsive (Ingram and Bartels 1996, Bray 1997). In well-watered thalli, desiccation tolerance disappears due to the inability to synthesize stress-induced ABA. However, under such conditions exogenous application of ABA may trigger the synthesis of

desiccation-related proteins (Oliver *et al.* 1997, Reynolds and Bewley 1993). Knowledge of the role of ABA in lichens is very limited (Dietz and Hartung 1998, 1999). For the resurrection angiosperms, ABA was reported to induce the formation of new proteins responsible for desiccation tolerance (Bartels *et al.* 1990). The question arises whether ABA affects mosses and lichens in a similar manner. However, Smirnoff (1995) suggested that ABA might be involved in the alteration of cell wall extensibility, which occurs under water deficit. Recently, Guan and Scandalios (1998) reported that two structurally similar cytosolic SOD genes could be regulated in part by ABA during late embryogenesis. Moreover, in two jute species, *Corchorus capsularis* and *Corchorus olitorius*, subjected to water stress, foliar sprays of ABA increased the activity of the ROS scavenging enzymes, SOD and CAT, and reduced MDA formation. However, low concentrations of ABA (10 - 100 μM) induce an oxidative defence response against oxidative damage, but higher concentrations of ABA cause an excessive generation of ROS that may lead to oxidative damage in plant cells (Bueno *et al.* 1998, Guan and Scandalios 1998a, b, Bellaire *et al.* 2000, Guan *et al.* 2000, Jiang and Zhang 2001). In addition, ABA treated cells maintain the ability to scavenge ROS and remain viable (Fath *et al.* 2001). This suggests that part of the role of ABA is to activate signal transduction pathways that reduce ROS formation which will, in turn, reduce lipid peroxidation and membrane damage (Chowdhury and Choudhuri 1989). Furthermore, ABA may be involved in hardening responses. Mild stress induces ABA, which then induces other responses such that the plant gives into a "stress adapted state". It has been suggested that ROS play an important intermediary role in the ABA signal transduction pathway leading to the induction of the antioxidant defence system (Bellaire *et al.* 2000, Guan *et al.* 2000, Jiang and Zhang 2001).

### 1.7 Introduction to the present study

Most bryophytes and lichens are desiccation tolerant organisms, and can survive in the air-dried state for long or short periods even at relative water contents (RWC) below 10%, a state that would be lethal to all non-desiccation tolerant plants (Gaff 1997, Kranner and Lutzoni 1999). Beckett and co-workers have used the moss *A. androgynum* as a model system for studying desiccation tolerance in mosses. This species, which is common in the understorey of Afromontane forests, combines the upright habit of *Polytrichum* with the fragile textured leaves of *Mnium*. In initially,

Beckett and Hoddinott (1997) used a simple ion leakage assay to measure desiccation tolerance, which involves measuring potassium ($K^+$) loss 30 min after rehydration following desiccation of fully hydrated material over silica gel. Large seasonal changes in $K^+$ leakage occur in *A. androgynum* following desiccation. Mosses lose much less $K^+$ during the dry winter months than the moist summer months, indicating the presence of inducible tolerance mechanisms that reduce desiccation-induced damage. Later studies showed that this species could be hardened to desiccation stress under controlled conditions. Reducing the RWC to *c.* 0.6 for 3 d increases tolerance, even if plants are then stored fully hydrated for 24 h before assaying for desiccation tolerance (Beckett 1999). Furthermore, exogenous applications of ABA followed by storage for 3 d can fully substitute for partial dehydration. Presumably, in the field, partial dehydration will often precede a more severe desiccation stress, inducing ABA synthesis that in turn activates signal transduction pathways that increase desiccation tolerance. These results, and other limited data available in the literature, strongly suggest that ABA plays an important role in the induction of genes that harden *A. androgynum*, and at least some other bryophytes, to desiccation stress (Hartung *et al.* 1998).

Beckett *et al.* (2000) used chlorophyll fluorescence to show that in the closely related species *A. undulatum* ABA pretreatment speeds up the recovery of photosystem II (PSII) during rehydration following desiccation, and also increases NPQ. If desiccation reduces Calvin cycle activity more than membrane-bound electron transport, excess excitation energy can pass to oxygen from photo-excited chlorophyll pigments, forming singlet oxygen, while superoxide and hydrogen peroxide are produced at PSII (McKersie and Lesham 1994). Under these conditions enhanced NPQ may reduce potentially harmful ROS formation, and may explain how ABA increases the desiccation tolerance of PSII and reduces ion leakage. When stress damages the defence systems, or exceeds their capacity to remove ROS, damage can occur. However, ROS are not only the toxic by-products of stress. Within the last 15 years it has become apparent that the rapid production of extracellular ROS, often called the "oxidative burst", also plays an important role in plant defence against pathogenic infections. Most studies have emphasized that pathogens, or elicitors derived from them, can induce extracellular ROS production (Wojtaszek 1997). However, extracellular production of ROS can also be induced by abiotic stresses that

leave plants vulnerable to bacteria or fungi e.g. mechanical disruption or UV light (Murphy *et al.* 1998). In addition, the severe mechanical and physiological damage that accompanies desiccation and subsequent rehydration may make them vulnerable to infection by pathogens (Wojtaszek 1997).

In this study, initial experiments investigated the *Atrichum* model in more detail. The first aim of the work presented here was to compare the time courses of the recovery of respiration and carbon fixation with PSII activity during rehydration following desiccation. Results obtained confirmed that PSII activity recovers much faster than C fixation, suggesting that NPQ acts as a "safety valve", reducing ROS production when Calvin cycle activity is impaired. Secondly, to test if desiccation tolerance can be induced, the effect of ABA pretreatment on photosynthesis, respiration, chlorophyll fluorescence, and on the concentrations of carbohydrates and chlorophylls in a moss *A. androgynum* and lichens *P. polydactyla* and *R. celastri* during desiccation and rehydration was also investigated. It was then hypothesized that, in addition to its effects on NPQ, ABA treatment could increase the pool of soluble sugars available during desiccation. As discussed above, sugar accumulation will probably help vitrify the cytoplasm or preserve membranes. In addition, ABA treatment could preserve chlorophylls during desiccation.

As a defence strategy plants use a complex interplay of mechanisms rather than one simple adaptive response. It was therefore, hypothesized that ABA and other hardening treatments up-regulates the free radical scavenging enzymes, thus reducing ROS damage. The aim of the experiments was to measure the changes in the activity of the antioxidant enzymes APX, CAT and SOD during desiccation and rehydration in *A. androgynum* and lichens *P. polydactyla*, *R. celastri* and *T. capensis*. Furthermore, It is shown that in the moss *A. androgynum* an oxidative burst of $H_2O_2$ rather than $O_2^{\cdot-}$ occurs during rehydration following desiccation. This experiment, reports the basic kinetics of the oxidative burst, and describes the preliminary findings on the nature of the enzymes responsible for $H_2O_2$ formation. The overall aim of these experiments was to investigate further the mechanisms of desiccation tolerance in a moss *A. androgynum* and lichens *P. polydactyla*, *R. celastri* and *T. capensis* from contrasting water status.

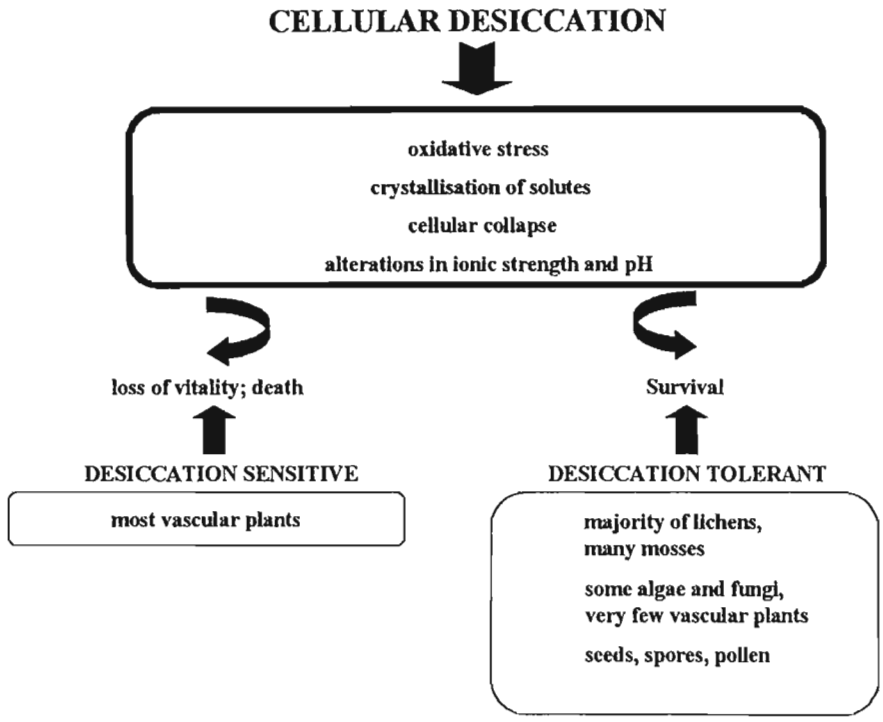



**Figure 1.1:** Effects of cellular desiccation in plants.

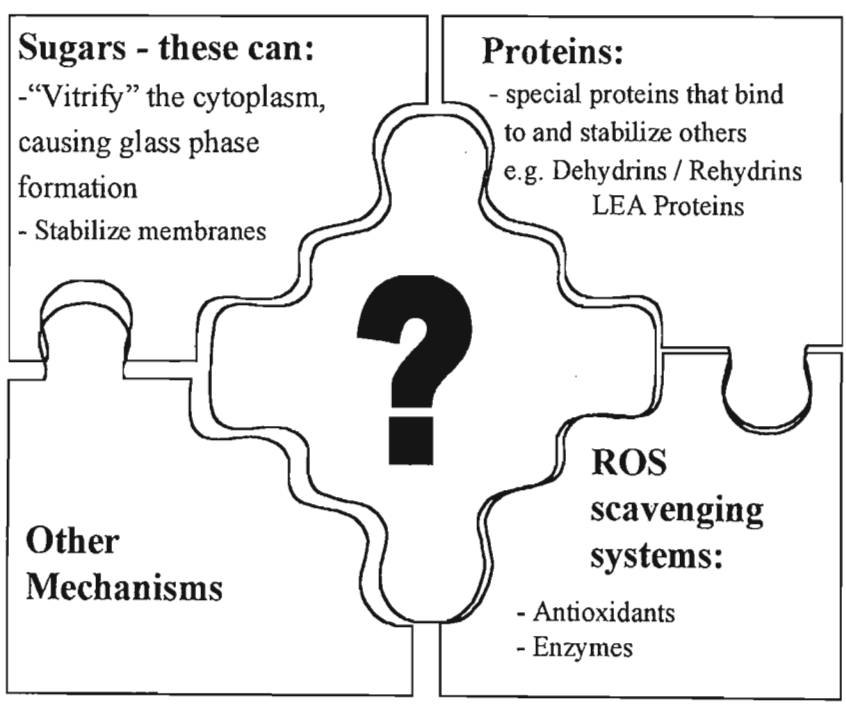



**Figure 1.2:** Proposed mechanisms of desiccation tolerance.

# CHAPTER 2

# 2. Materials and Methods

## 2.1 Plant material

*Atrichum androgynum* (C. Mull.) A. Jaeger was collected from the understorey of the Doreen Clarke Nature Reserve, Hilton (29°39' South and 30°17' East), or Fern Cliffe Nature Reserve, Pietermaritzburg (29°31' South, 30°20' East), KwaZulu-Natal Province, Republic of South Africa. These nature reserves form small pockets of Afromontane forest in the mist belt region of KwaZulu-Natal. Once collected, material was stored on wet filter paper for a minimum of two and a maximum of seven days in a controlled environment chamber at 15 - 20°C and a photosynthetic photon fluence rate (PPFR) of 75 μmol photons $m^{-2}s^{-1}$ under continuous fluorescent light. For photosynthetic and respiration measurements, ten apical 2 cm segments were used. Each replicate typically had a fresh mass of 160 mg and a dry mass of *c.* 50 mg. For soluble sugar and starch analyses, 50 mg fresh mass of material was used, and for chlorophyll analyses 200 mg fresh mass was used. For experiments on $H_2O_2$ determination *c.* 100 mg fresh mass plant material of 7 apical 2 cm segments were used. In all these experiments each treatment comprised five replicates. All light intensities used were measured across photosynthetically active wavebands using the light meter in the Parkinson leaf chamber of an ADC (Analytical Development Corporation) Mark III portable infrared gas analyser (IRGA).

Lichens were collected from contrasting habitats: *Peltigera polydactyla* (Necker) Hoffm. from moist covered boulders under a tree canopy, Drakensberg, Cathedral peak, KwaZulu-Natal Province, Republic of South Africa (28°45' South, 29°10' East); *Ramalina celastri* (Sprengel) Krog and Swinscow, from exposed tree branches of the Fern Cliffe Nature Reserve, Pietermaritzburg, KwaZulu-Natal Province, Republic of South Africa (29°31' South, 30°20' East) and *Teloschistes capensis* (L.f.) Vain. from an extremely xeric habitat in the Wlotzkasbaken, Namib Desert, Namibia (22°30' South, 14°25' East). Once collected, lichens were gradually rehydrated using air at a relative humidity of 100% for 24 h (over distilled water) at 20°C, and a PPFR of 135 μmol $m^{-2}s^{-1}$, followed by contact with wet filter paper for a further 24 h. For photosynthetic measurements, in *P. polydactyla* and *R. celastri*, 20 X

2 cm discs of *c.* 200 mg fresh mass and *c.* 200 mg fresh mass of thallus segments were used respectively. Each treatment comprised of three replicates. The RWC after drying was estimated as (fresh mass after drying – dry mass) / (turgid mass – dry mass).

### 2.2 Measurement of Photosynthesis and Respiration

Net photosynthesis (A) and respiration were measured at 25°C and relative humidity (RH) of 50% using an ADC Mark III portable IRGA with a barrel-shaped Parkinson leaf chamber, modified with a water-cooled jacket. The flow rate through the leaf chamber was 120 ml $min^{-1}$. Normally plants were rehydrated under dim laboratory lighting (PPFR approx. 5 - 10 µmol photon $m^{-2}s^{-1}$). However, in experiments that measured respiration plants were rehydrated in complete darkness. In addition for photosynthetic measurements, during the time while actual readings were taking place, *A. androgynum, P. polydactyla* and *R. celastri* were exposed to various PPFR's of 150, 350 and 500 µmol photons $m^{-2}s^{-1}$ respectively. In lichens, *R. celastri* and *P. polydactyla* the maximal rates of photosynthesis were achieved at RWC of 0.5 and 0.8 respectively. These RWC's and PPFR's were used throughout the experiments. Equilibrating samples in the IRGA for 10 min in *A. androgynum* and *P. polydactyla* and 5 min in *R. celastri was* found to give steady state rates of gas exchange without causing enough water loss to reduce photosynthesis.

### 2.3 Chlorophyll Fluorescence Measurements

When working with the moss *A. androgynum*, chlorophyll fluorescence was measured using a Hansatech (King's Lynn, UK) FMS1 modulated fluorometer. When working with the lichens the FMS 2 was used. Preliminary investigations showed that actinic light at 50 µmol photons $m^{-2}s^{-1}$ gave a good balance between photochemical and non-photochemical quenching in the moss while the corresponding value was 33 µmol photons $m^{-2}s^{-1}$ in lichens. To take a measurement, each replicate was placed in the fluorometer, and $F_o$ and $F_m$ were recorded. The actinic light was then switched on, the initial peak ($F_p$) noted, and $F_s$, $F'_m$, and (with the actinic light switched off) $F'_o$ recorded after 5 min in *A. androgynum* and *P. polydactyla* and 15 min in *R. celastri*, by which time a steady state had been reached. Calculation of fluorescence parameters and conventions for symbols follow Schreiber and Bilger (1993) and Schreiber *et al.* (1995a); the Stern-Volmer quotient NPQ was used as a measure of

non-photochemical quenching. Briefly, $F_v / F_m = (F_m - F_o) / F_m$; $\Phi PSII = (F'_m - F_s) / F'_m$; non-photochemical quenching (NPQ) = $F_m / F'_m - 1$; $F_o$ quenching = $(F_o - F'_o) / F_o$.

### 2.4 Desiccation-induced $K^+$ Loss

Mosses were desiccated for 2, 4, 8, 16, 24 and 32 h by placing the stem segments in 2 x 5 cm specimen bottles over silica gel at 20°C and a PPFR of 75 μmol $m^{-2}s^{-1}$ under continuous fluorescent light, and then rehydrated at a PPFR of 5 - 10 μmol photons $m^{-2}s^{-1}$ by shaking for 0.5 h in 10 ml of deionized distilled water. The cellular location of $K^+$ was determined using a simplification of the method of Brown and Buck (1979). The mosses were removed from the rehydration solution, and extracellular $K^+$ was then displaced by incubating the moss in 10 ml of 20 mM $NiCl_2$ for 0.5 h. After a further wash with deionized distilled water for 0.5 h, mosses were dried to constant mass at 80°C, weighed, and intracellular $K^+$ displaced by incubation in 10 ml of 1 M $HNO_3$ for 1 h. All samples were spiked with 1000 μg $g^{-1}$ $Cs^+$, and $K^+$ was determined by atomic absorption spectrophotometer in an air/acetylene flame. The $K^+$ leakage was calculated as 100 x ($K^+$ lost into the incubation solution + extracellular $K^+$) / (total thallus $K^+$ content).

### 2.5 Determination of soluble sugars and starch

For soluble sugar analysis, 0.5 g fresh mass plant material was ground in liquid nitrogen then extracted three times with 5 ml 80% ethanol at 75°C for 5 min, centrifuging after each extraction. The supernatants were pooled and made up to 25 ml with distilled water. For starch analysis, 5 ml distilled water was added to the remaining pellet, the tubes were incubated in a water bath at 100°C for 5 min, then 5 ml of 60% perchloric acid added. Tubes were then centrifuged at 1500 G for 30 min, the supernatant decanted, and the pellet washed in 10 ml of 15% perchloric acid. Tubes were re-centrifuged; the supernatants pooled and then made up to 25 ml with distilled water. For both sugar and starch analyses, 0.4 ml 2% phenol and 2 ml conc. $H_2SO_4$ were added to 0.4 ml of extract and the absorbance was measured at 490 nm ($A_{490}$).

### 2.6 Determination of chlorophylls

In the moss *A. androgynum* chlorophylls were extracted by grinding 200 mg fresh mass plant material in liquid nitrogen using a pre-chilled pestle and mortar, then

extracting in 5 ml of 80% acetone. Extracts were centrifuged at 1500 G and 4°C for 6 min, and total chlorophylls (a + b) measured spectrophotometrically using the equations of Lichtenthaler (1987). In lichens chlorophyll content was determined by extracting 200 mg fresh mass of lichen powder in $CaCO_3$-saturated dimethylsulfoxid (DMSO) solution after grinding in liquid nitrogen. Samples were incubated in a water bath at 60°C for 40 min. Extracts were centrifuged as above and total chlorophyll (a + b) were measured in the green algal lichen, *R. celastri* and calculated as above. However, in cyano-lichen, *P. polydactyla* chlorophyll a was measured and calculated according to Palmquist and Sundberg (2002).

### 2.7 Soluble enzyme extraction and protein determination

Initially, lichens were homogenised in pre-chilled pestle and mortar. Later, to improve the efficiency of extraction mosses were placed in small paper bags, immersed in liquid nitrogen for few minutes, and then freeze-dried for 24 h. Mosses were then removed from the drier and placed in sealed double plastic bags with silica gel to absorb excess moisture during storage. Samples were stored at -20°C for a maximum of 1 week before extraction. All samples (mosses and lichens) were extracted in chilled 0.2 M Tris/HCl buffer (pH 7.8) containing 1 mM DTT, 2 mM NaEDTA and 2% PVP at a ratio plant material: buffer = 1:3. Homogenates were filtered through 4 layers of muslin cloth and centrifuged at 12000 G for 5 min. The supernatant containing soluble proteins was then collected in 2 ml eppendorf tubes and stored for a maximum of 1 month at -20°C for further analysis. Protein estimation was carried out spectrophotometrically following Bradford (1976) using 1% bovine serum albumin. A series of standardised solutions containing 10 – 100 µg protein were prepared and the absorbance was measured at 595 nm ($A_{595}$), then the calibration curve was constructed.

### 2.8 Enzyme assays

The activities of APX (EC 1.11.1.11), CAT (EC 1.11.1.6) and SOD (EC 1.15.1.1) were measured spectrophotometrically. APX activity was determined by monitoring the decrease in absorbance of ascorbic acid at 270 nm as described by Nakano and Asada (1981). One unit of APX was defined as the amount of enzyme that hydrolyses 1 µmol of ascorbic acid $min^{-1}$ at room temperature, and activity expressed as units $mg^{-1}$ protein $min^{-1}$. CAT activity was determined following the consumption of $H_2O_2$ at

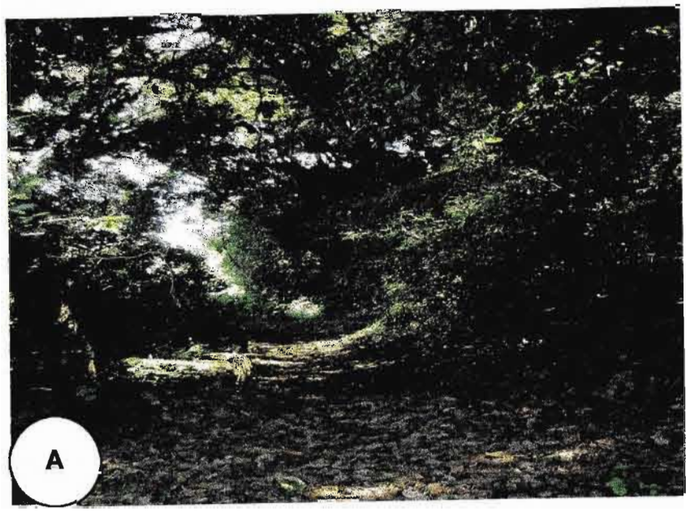
240 nm according to Chance and Meahly (1955). One unit of CAT was defined as the amount of enzyme that hydrolyses 1 μmol of $H_2O_2$ $min^{-1}$ at room temperature and activity expressed as units $mg^{-1}$ protein $min^{-1}$. SOD activity was detected by monitoring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) at 560 nm (Beauchamp and Fridovich 1971). One unit SOD was defined as the amount of enzyme causing 50% inhibition of formazin formation, and activity expressed as units $mg^{-1}$ protein. This assay determines all the isozymes of SOD.

### 2.9 Hydrogen peroxide Assay

Production of $H_2O_2$ was assayed using xylenol orange assay (Gay and Gebicki 2000). Briefly, two reagents were prepared, reagent A comprising 25 mM $FeSO_4$, 25 mM $(NH_4)_2SO_4$ and 2.5 M $H_2SO_4$, and reagent B comprising 125 μM xylenol orange (Sigma, St Louis, MO, USA) and 100 mM sorbitol. Working reagent comprised of 0.1 ml of reagent A and 10 ml of reagent B. In a typical assay, 100 mg moss was desiccated, then rehydrated in 5 ml of distilled water at a PPFR of 5 - 10 μmol photons $m^{-2}s^{-1}$ for 15 min. Working reagent (3 ml) was added to 0.6 ml of incubation solution, the resulting mixture was then kept for 1 h, and then $H_2O_2$ was determined spectrophotometrically by measuring the absorbance at 560 nm ($A_{560}$). A standardized 10 μM solution of $H_2O_2$ was used to construct a calibration curve. Specificity of the assay was confirmed by the inhibition of $H_2O_2$ production where 500 units $ml^{-1}$ of catalase was added to the rehydration medium of mosses desiccated for 8 h and 32 h (Able *et al.* 2000; Table 7.1).

### 2.10 Statistical analysis

In *A. androgynum* the significance between the treatments was tested using the student's *t*-test with the use of MINI TAB. In *R. celastri* and *P. polydactyla* the data was statistically analysed using a two-way analysis of variance (ANOVA) in a randomised complete design (RCD) with the use of a Genstat® computer package for windows ™ (Lane and Payne 1996). The separation of means was done by Fisher's Least Significant Difference (*LSD*) test at 5% (Gomez and Gomez, 1983).





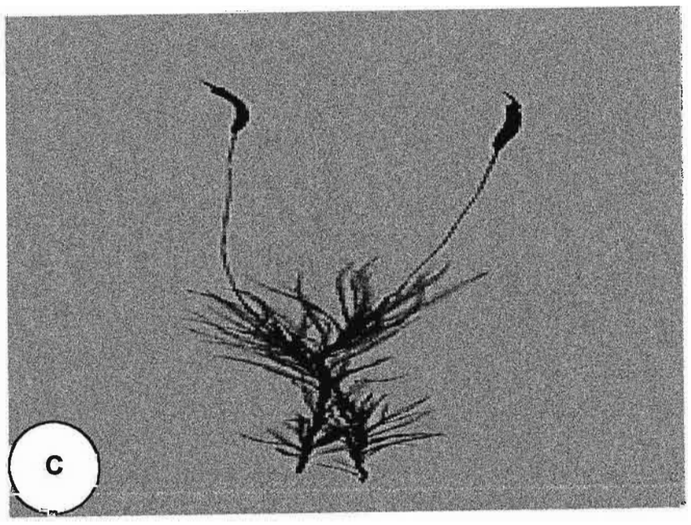



**Plate 2.1:** (A) Picture showing the Afromontane forest (*A. androgynum* habitat).
(B) *A. androgynum* on the forest floor.
(C) Close up picture of *A. androgynum*.

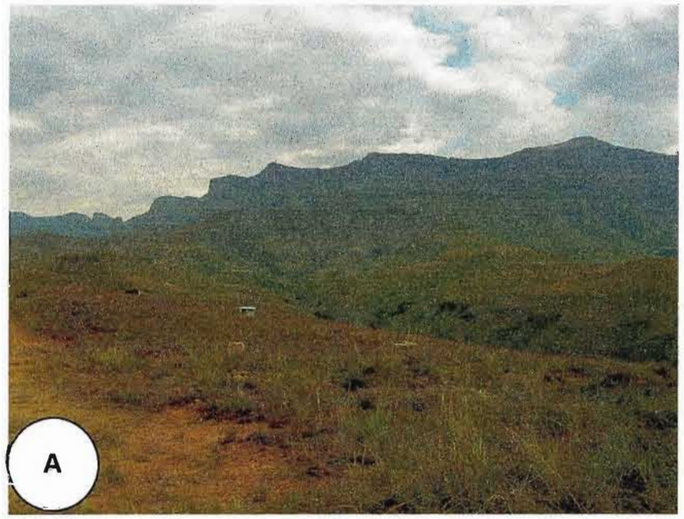
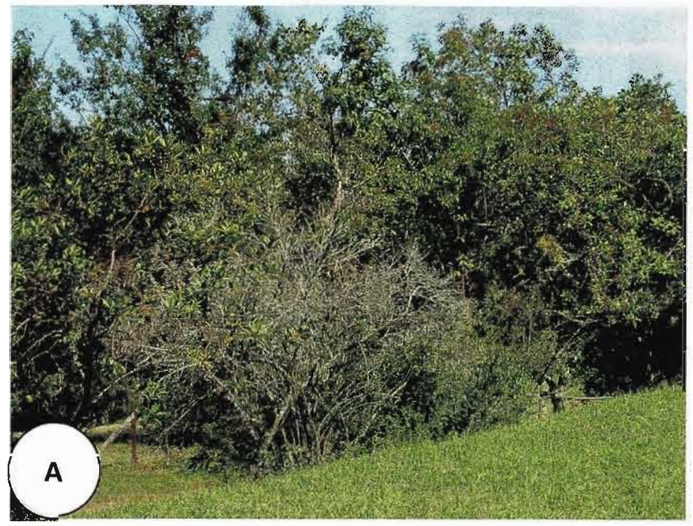





**Plate 2.2:** (A) Picture showing the Drakensburg (Cathedral Peak, *P. polydactyla* habitat).

(B) Desiccated *P. polydactyla* in the nature.

(C) Hydrated *P. polydactyla* in the nature.





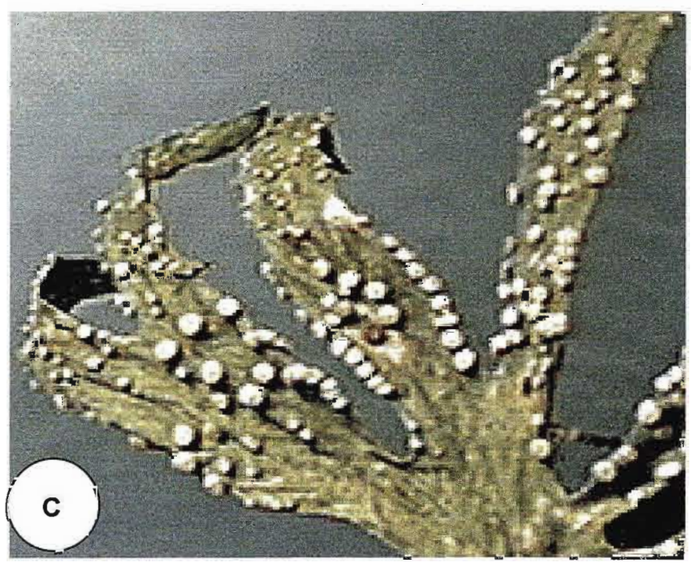



**Plate 2.3:** (A) Picture showing trees in the Fern Cliffe (habitat for *R. celastri*).

(B) *R. celastri on* the tree branches.

(C) Close up picture of *R. celastri* in the nature.



**Plate 2.4:** (A) Picture showing *T. capensis* on the floor of Namib Desert, Wlotzkasbaken, Namibia.

(B) Close up picture of *T. capensis*.

# CHAPTER 3

## 3. The Effect of ABA pretreatment on desiccation tolerance of photosynthesis and chlorophyll fluorescence in the moss *Atrichum androgynum*

### 3.1 Introduction

As part of the survival strategy, bryophytes appear to employ a complex interplay of many mechanisms rather than one simple adaptive feature (Oliver 1996, Oliver and Bewley 1997, Oliver and Wood 1997, Oliver *et al.* 1997, 1998, Alpert and Oliver 2002) to survive stress. These include protein synthesis, antioxidant systems, sugars and/or xanthophyll cycle. In some cases exogenous application of ABA is known to be involved in many processes that harden plants to desiccation stress, and may induce desiccation tolerance of photosynthesis. Beckett *et al.* (2000) used chlorophyll fluorescence to show that in the closely related species *A. undulatum* ABA pretreatment speeds up the recovery of photosystem II (PSII) during rehydration following desiccation, and also increases NPQ. If desiccation reduces Calvin cycle activity more than membrane-bound electron transport, excess excitation energy can pass to oxygen from photo-excited chlorophyll pigments, forming singlet oxygen, while superoxide and hydrogen peroxide are produced at PSII (McKersie and Lesham, 1994). Under these conditions enhanced NPQ may reduce potentially harmful ROS formation, and may explain how ABA increases the desiccation tolerance of PSII and reduces ion leakage. The initial objective of the work presented here was to compare the time courses of the recovery of respiration and C fixation with photosystem II activity during rehydration following desiccation. Secondly, the effects of ABA pretreatment on the concentrations of soluble sugars and chlorophylls during desiccation and rehydration were studied. It was hypothesized that in addition to its effects on NPQ, ABA treatment could increase the pool of soluble sugars available during desiccation. As discussed in chapter 1, sugar accumulation will help vitrify the cytoplasm or preserve membranes. In addition, ABA treatment could preserve chlorophylls during desiccation, which will enable the plants to recover photosynthesis and regain positive energy balance upon rehydration. The overall aim of this section was to investigate further how ABA increases desiccation tolerance in *A. androgynum*.

### 3.2 Materials and Methods

Mosses were desiccated for 16 h by placing the stem segments in 2 x 5 cm specimen bottles in a desiccator over silica gel at 20°C and a PPFR of 75 μmol photons $m^{-2}s^{-1}$ under continuous fluorescent light (see Figure 5.2 for a graph of RWC as a Function of time). The mosses were suddenly rehydrated by addition of 10 ml distilled water. Rehydration was probably complete within 2 minutes. In preliminary experiments, mosses were desiccated for 8, 16, 24 and 30 h to determine the most appropriate time for desiccation stress. Results indicated that desiccation for 16 h significantly inhibited photosynthesis during the first few hours of rehydration, but after 8 h rehydration net photosynthesis partially recovered (Figure 3.1b). Desiccation for 16 h was used in all subsequent experiments. During desiccation, the RWC of plants dropped to 0.15 after 8 h and to 0.04 after 16 h. Endogenous ABA levels were not measured. Partial dehydration prior to desiccation induced desiccation tolerance in a related species *A. undulatum* (Beckett 1999). Therefore, to confirm the relevance of hardening process to field situations, the ability of both partial dehydration and ABA (100 μM) treatments to increase the desiccation tolerance of photosynthesis was tested. Partial desiccation was achieved by placing stem segments on dry filter paper for 3 d at 100% RH, then the material was transferred to a wet filter paper for 1 d before desiccation instead of ABA treatment at the same intervals as above. Because net photosynthesis only partially recovered during rehydration following rapid drying over silica gel, mosses were desiccated by storage for 32 h at 52 % RH, then suddenly rehydrated with deionised distilled water.

### 3.3 Results

In preliminary experiments, photosynthesis was found to saturate at 150 μmoles photons $m^{-2}s^{-1}$ (Figure 3.1a). During storage for 3 d following treatment with distilled water or ABA, respiration increased slightly, declined to zero following desiccation, and then increased rapidly during the first 20 min of rehydration (Figure 3.1c). In untreated material, the rate of respiration increased from *c.* 1.0 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$ before desiccation to *c.* 3.0 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$ after 20 min rehydration, while in ABA treated material respiration was significantly less at only *c.* 1.7 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$ (Student's *t*-test, $P < 0.01$). After rehydration for 2 h, respiration in material from both treatments recovered to similar values as those occurring before desiccation.

The rate of net photosynthesis at the start of the experiment was *c.* 4.0 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$ (Figure 3.1d). The rate declined slightly during storage following treatment with distilled water or ABA, and was slightly lower in ABA treated material. Immediately following rehydration, a burst of $CO_2$ was released. The rate of $CO_2$ release was *c.* 6.0 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$ in untreated material, but was significantly less in ABA treated material at *c.* 3 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$ (Student's *t*-test, $P < 0.001$). Rates of $CO_2$ release were much faster than the respiratory rates of material rehydrated in the dark. Net photosynthesis recovered more slowly than respiration. ABA treated material required 2 h to reach positive carbon balance, while untreated material needed 8 h. Recovery was incomplete after 8 h; even ABA treated material only achieved a net photosynthetic rate of *c.* 1 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$.

During storage after distilled water or ABA pretreatment, minimum fluorescence ($F_o$), maximum fluorescence ($F_m$) and the optimal quantum yield ($F_v$ / $F_m$) all declined slightly, while other fluorescence parameters remained unchanged (Figure 3.2a-f). Desiccation sharply reduced all parameters except $F_v$ / $F_m$. During rehydration, $F_o$ and $F_m$ recovered quickly, $F_m$ recovering slightly faster in ABA treated plants. $F_v$ / $F_m$ did not recover in untreated plants, but increased slightly in ABA treated plants. The actual quantum yield of PS II (ΦPSII) initially declined further during rehydration, then partially recovered, but was consistently higher in ABA treated plants. NPQ and $F_o$ quenching recovered quickly, and were always considerably higher in ABA treated plants.

The concentration of soluble sugars was initially *c.* 59 mg $g^{-1}$ dry mass, and increased slightly during storage (Figure 3.3a). Following desiccation, the concentration significantly increased to *c.* 106 mg $g^{-1}$ dry mass in ABA treated material (Student's *t*-test, $P < 0.01$), but to only 86 mg $g^{-1}$ dry mass in untreated material (not significant, $P > 0.1$). After rehydration for 1 h, soluble sugar concentrations declined in both treatments, but remained higher in ABA treated material. Starch concentrations in freshly collected *A. androgynum* were only *c.* 40 mg $g^{-1}$ dry mass, and were only slightly affected by ABA treatment or desiccation (Figure 3.3b). Desiccation reduced the total concentration of chlorophylls a and b from *c.* 6.0 to *c.* 3.5 mg $g^{-1}$ dry mass (Figure 3.3c). No significant differences existed

in the responses of chlorophylls to desiccation in ABA and untreated material.

During dehydration, the rate of photosynthesis declined rapidly in both treatments during the first 4 h to *c.* 0.2 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$, then to zero in 32 h (Figure 3.4a). In Figure 3.4b showing the rate of photosynthesis during rehydration following dehydration at 52% RH, the rate of photosynthesis slowly recovered to *c.* 1.2 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$ and 2.5 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$ in distilled water and ABA treated material respectively in the first 20 minutes. Significant differences existed between the treatments, starting after 4 h rehydration onwards. ABA treated material needed 4 h to reach its original rate of photosynthesis while in untreated material the recovery was incomplete even after 6 h then slowly declined in both treatments in the next 18 h (Figure 3.4b).

In material hardened by partial desiccation, net photosynthesis recovered immediately in both hardened and non-hardened material, but more rapidly in hardened material (Figure 3.5). Net photosynthesis recovered almost completely in hardened material, whilst in non-hardened material recovered only partially even after 24 h rehydration. Significant differences existed between the treatments immediately upon rehydration. The rate of photosynthesis recovered almost completely following slow drying at 52% RH in both ABA treated material (Figure 3.4b) and in material hardened by partial dehydration (Figure 3.5) compared to rapid drying over silica gel (Figure 3.1d).

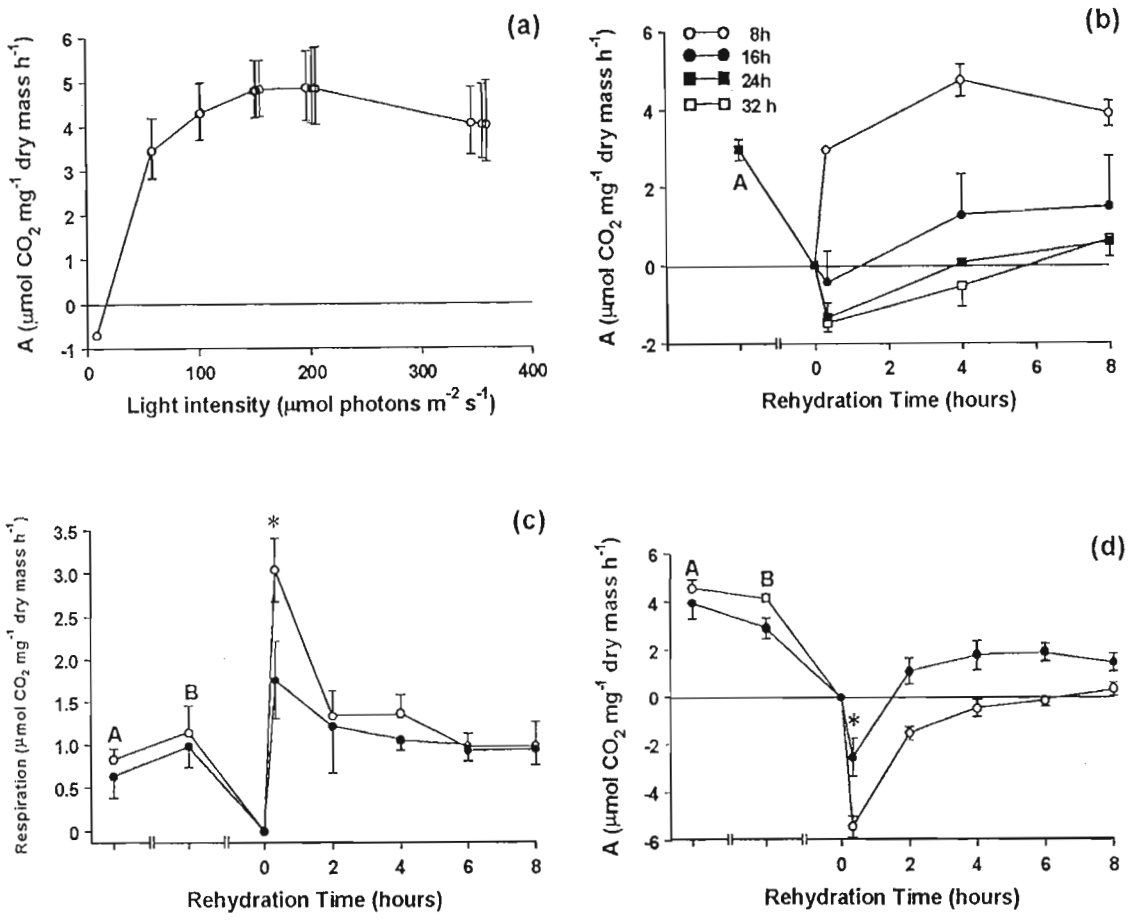



**Figure 3.1:** (a) The effect of light intensity on photosynthesis in the moss *A. androgynum*. Points represent fitted values with 95% confidence limits calculated using "Spline" program of Hunt and Parsons (1974); (b) The effect of desiccation for a range of times on photosynthesis during rehydration in the moss *A. androgynum*. Overlapping error bars have been removed, (c) The effect of treatment with distilled or 100 μM ABA for 1 h followed by storage for 3 d and then desiccated for 16 h on respiration and (d) net photosynthesis during rehydration in the moss *A. androgynum*. In these figures (c, d) and Figure 3,5a an asterisk above selected points indicates that a significant difference exists between distilled water and ABA treated mosses (Student's *t* - test $P < 0.05$). In c and d symbols: solid circles, ABA treated material and open circles, distilled water. Letters: A, start of the experiment; B, after 3 d storage following treatment.

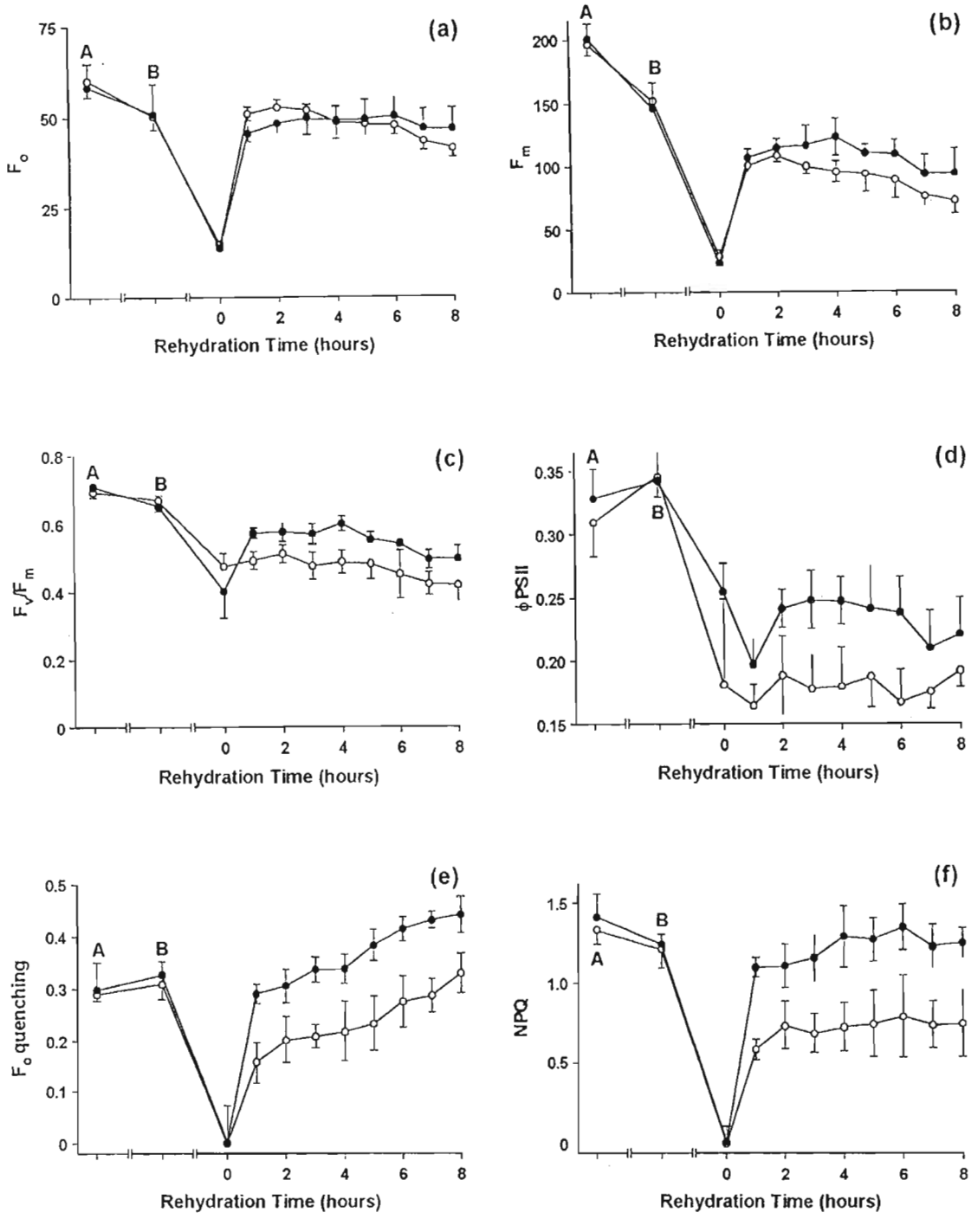



**Figure 3.2:** The effect treatment with distilled water or 100 μM ABA for 1 h followed by storage for 3 d and then desiccated for 16 h on $F_0$ (a), $F_m$ (b), $F_v / F_m$ (c), $F_0$ quenching (d), ΦPSII (e) and NPQ (f) during rehydration in the moss *A. androgynum*. Symbols: solid circles, ABA treated and open circles, distilled water. Letters: A, start of the experiment; B, after 3 d storage following treatment.

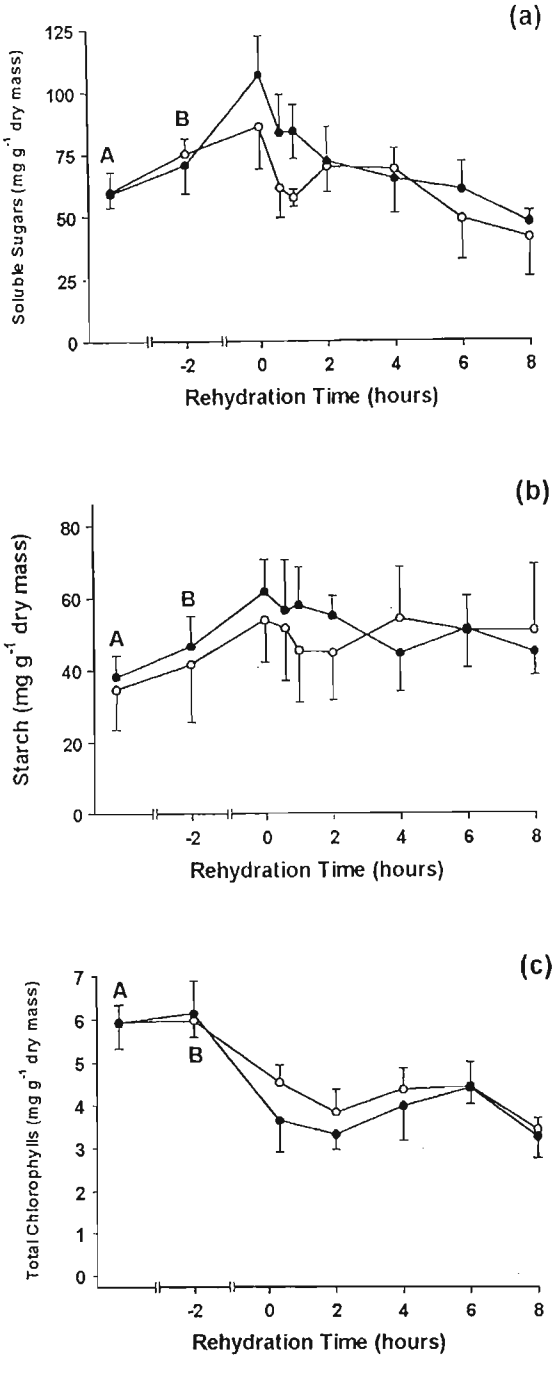



**Figure 3.3:** The effect of treatment with distilled water or 100 μM ABA for 1 h followed by storage for 3 d and then desiccated for 16 h on (a) Soluble sugars, (b) Starch, and (c) Total chlorophylls (a+b) during rehydration in the moss *A. androgynum*. Symbols: solid circles, ABA treated material and open circles, distilled water. Letters: A, start of the experiment; B, after 3 d storage following treatment.

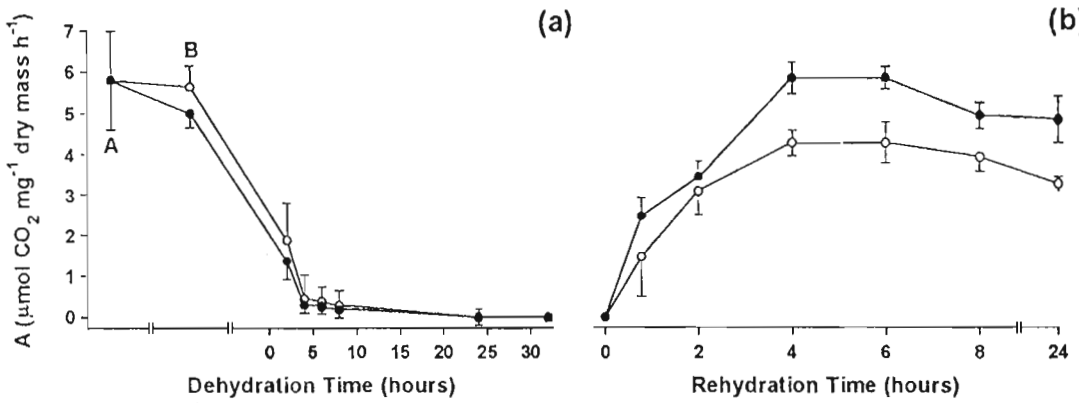



**Figure 3.4:** The effect of treatment with distilled water or 100 µM ABA for 1 h followed by storage for 3 d on photosynthesis during dehydration at 52% RH (a) and rehydration (b) in the moss *A. androgynum*. Symbols: solid circles, ABA treated and open circles, distilled water. Letters: A, start of the experiment; B, after 3 d storage following treatment.

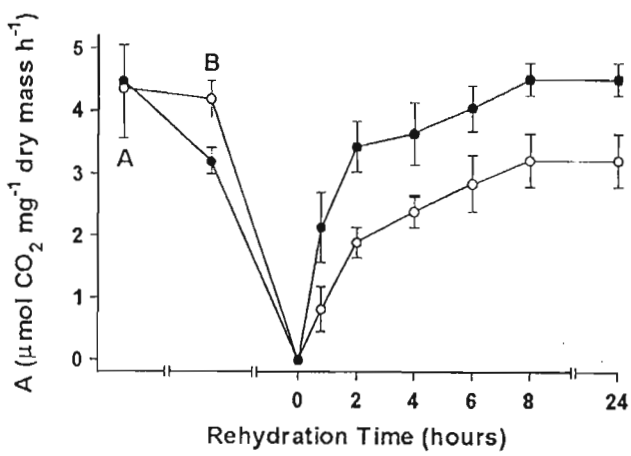



**Figure 3.5:** The effect of partial desiccation for 3 d on dry filter paper over 100% RH followed by 1 d on wet filter paper before desiccation for 32 h at 52% RH on net photosynthesis in the moss *A. androgynum*. Symbols: solid circles, hardened material and open circles, non-hardened material. Letters: A, start of the experiment; B, after 3 d storage following treatment.

### 3.4 Discussion

Results presented here clearly indicate that in the moss *A. androgynum* PSII activity recovers much more quickly than photosynthetic carbon fixation during rehydration following desiccation. Although limited data is available suggesting that PSII activity is more resistant to desiccation than carbon fixation in bryophytes (e.g. Proctor 2000a, Proctor and Smirnoff 2000), the present study is one of the first to make a clear comparison using the same species stressed in the same way. As discussed in the Introduction, rapid recovery of PSII activity but slow recovery of carbon fixation can cause ROS production (McKersie and Lesham 1994). Under these conditions, NPQ may considerably reduce the amount of oxidative damage. Pretreatment with ABA both increases NPQ (Figure 3.2f) and improves the tolerance of *Atrichum* to desiccation-induced damage e.g. ion leakage (Beckett 1999, 2001), and reductions in PSII activity (Beckett *et al.* 2000). It seems likely that in *A. androgynum* some of these beneficial effects are a consequence of increased NPQ activity. This is supported by the observation of Proctor and Smirnoff (2000) that $F_v$ / $F_m$ declines if the moss *Racomitrium* is rehydrated with the xanthophyll cycle inhibitor dithiothreitol in the light, but not in the dark. The likely "cost" of increased NPQ is reduced rates of photosynthesis at non-saturating light intensities (Niyogi *et al.* 1998, Niyogi 2000). It is worth noting that in addition to these effects, ABA pretreatment also considerably reduces the respiratory burst of *A. androgynum* rehydrated in the dark (Figure 3.1c). The observation that ABA increases desiccation tolerance even in the absence of light implies that ABA can improve tolerance in ways in addition to beneficial effects of increased NPQ. However, ABA-induced increases in NPQ are likely to harden bryophytes to stresses that inhibit carbon fixation more than PSII activity.

Few measurements of endogenous levels of ABA in bryophytes exist in the literature. In the present study, ABA was applied at 0.1 mM because Beckett (1999) found that this concentration significantly increased tolerance to desiccation-induced ion leakage during rehydration in *A. androgynum*. This concentration is about one hundred times greater than that found in desiccated thalli of the liverwort *Exomotheca holsii* by Hellwege *et al.* (1994). Possibly, low rates of ABA uptake or alternatively rapid metabolism of supplied ABA (not measured) explain why high exogenous concentrations were needed to elicit a response. To confirm the role of ABA in hardening the moss *A. androgynum* in field situations, our future work will focus on

endogenous ABA metabolism.

ABA probably increases NPQ activity by increasing the concentrations of xanthophyll cycle pigments (for review see Gilmore 1997). Li *et al.* (2000) showed that the chlorophyll-binding proteins associated with the photosystem II contributes to the photoprotective energy dissipation rather than photosynthetic light. However, the exact role of these proteins, remain unclear. Similarly, Bukhov *et al.* (2001a, b) showed that the main mechanism for dissipating light energy in the moss *Rhytidiadelphus* was zeaxanthin-dependent quenching in the antenna of photosystem II. Few studies exist on the effects of ABA on NPQ, although Ivanov *et al.* (1995) showed that ABA treatment increased both NPQ and the levels of xanthophyll cycle pigments in barley seedlings. Interestingly, Zorn *et al.* (2001) showed that slow desiccation and rehydration of the highly desiccation tolerant lichen *Ramalina massiformis* causes conversion of zeaxanthin to violaxanthin and also *de novo* synthesis of violaxanthin. This presumably hardens the lichen to a subsequent more severe stress (e.g. longer desiccation or more rapid rehydration). However, it is unknown whether ABA is involved in desiccation tolerance in lichens (Dietz and Hartung 1998, 1999). In future we will investigate the mechanism of increased NPQ by studying the effect of ABA on xanthophyll cycle pigments in *Atrichum*.

Respiration rates measured during the drying and rehydration cycle also suggest that light-induced free radical production is responsible for some of the harmful effects of desiccation. The respiratory burst was much larger when *A. androgynum* was rehydrated in the light than in the dark (Figures 3.1c, d). Compared with undesiccated material, the increases in the rates of respiration during the early stages of rehydration in mosses not treated with ABA were 5.0 and 2.0 μmoles $CO_2$ $g^{-1}$ dry mass $h^{-1}$ in the light and the dark respectively. In ABA treated material the increases were only 2.0 and 0.7 μmoles $CO_2$ $g^{-1}$ dry mass $h^{-1}$ respectively. Other workers have also found that rehydration of mosses in the light is more harmful than in the dark. For example, Seel *et al.* (1992b) found rehydration in the light increased chlorophyll breakdown in the moss *Dicranum*, strongly suggesting that light-generated free radicals are responsible for at least some of the harmful effects of desiccation on bryophytes. Hardening by partial desiccation before drying seemed to induce the desiccation tolerance in a moss *A. androgynum*. The ability of ABA to

substitute for partial dehydration provides evidence for the involvement of ABA in signal transduction pathways that can increase tolerance to desiccation (Beckett 1997).

ABA treatment stimulates the accumulation of soluble sugars during desiccation from 59 to 106 mg $g^{-1}$ dry mass (Figure 3.3a), while in untreated material the concentration only increased to 86 mg $g^{-1}$ dry mass. This experiment was repeated three times, and in every case the accumulation of soluble sugars during desiccation was always much higher in ABA treated moss. According to Santarius (1994), freshly collected material of the closely related species *A. undulatum* contains *c.* 80 mg $g^{-1}$ dry mass sugars, within the ranges of concentrations reported here for *A. androgynum*. Most of the sugar was sucrose, but plants also contained small quantities of fructose and glucose. As discussed in the Introduction, sugars can promote vitrification of the cytoplasm and protect membranes (Scott 2000). Increases in soluble sugars following desiccation in ABA treated material were proportionally similar to those reported in the resurrection angiosperms *Myrothamnus fabellifolia* and *Xerophyta villosa*, but were much less than those found in *Craterostigma plantagineum* (for review see Scott 2000). Smirnoff (1992) found no increase in sugars during desiccation in a range of mosses, similar to the response of non-ABA treated material in the present study. However, Schwall *et al.* (1995) reported that ABA could induce sucrose accumulation in *Craterostigma plantagineum*. Possibly, the ability to accumulate sugars during desiccation is only expressed in hardened mosses. The accumulation of soluble sugars may play a small, but possibly significant role in the ABA-induced increase in desiccation tolerance in *A. androgynum*.

Oliver *et al.* (1998) divide desiccation tolerant plants into firstly those that survive if drying is slow enough to induce mechanisms that either protect the plants during desiccation or facilitate recovery during dehydration, and secondly those that tolerate rapid drying. These two strategies may be termed "constitutive" or "inducible" respectively. The advantage of inducible systems is that, unlike constitutive mechanisms, they do not divert energy away from growth and reproduction. The disadvantage is that a sudden, severe drought may not allow time for the induction of tolerance and thus plants may not survive. Oliver *et al.* (2000) argue that constitutive tolerance is likely to be ancestral. It was hypothesized that

inducible tolerance mechanisms will be selected for in environments that are usually moist, and in which mosses only occasionally (and probably slowly) desiccate on a predictable seasonal basis. The latter corresponds to the habitat in which *A. androgynum* grows (Beckett and Hoddinott 1997). It was envisaged that bryophytes with largely constitutive mechanisms would include species like *Tortula* that grow on exposed rocky surfaces. Although even in this genus apparently some induction of tolerance can occur (Schonbeck and Bewley 1981a, b), rather than involving ABA, hardening seems to occur by the accumulation of "recovery" mRNA transcripts into ribonuclear protein particles (Wood *et al.* 2000). It is worth noting that the overall desiccation tolerance of species with inducible tolerance mechanisms appears to be less than that of those with constitutive mechanisms. Comparing the tolerance to desiccation of photosynthesis in *A. androgynum* with that of other moss species (Proctor 2000a, b), suggests that it is rather sensitive. Further work is needed to assess the distribution of constitutive or inducible tolerance mechanisms in other bryophytes.

ABA is clearly involved in many responses that harden the moss *Atrichum* to desiccation stress. However, it should be noted that in both higher plants (Bray 1997) and bryophytes (Hellwege *et al.* 1994, 1996) ABA does not regulate all genes induced by drought or desiccation. In *A. androgynum*, ABA pretreatment increases NPQ activity during the subsequent rehydration, and also slightly increases the concentrations of soluble sugars during desiccation. However, ABA does not affect starch metabolism or protect chlorophyll from breakdown during desiccation. Further studies on the effect of ABA on antioxidant enzymes, SOD and CAT were conducted and results showed that ABA tended to reduce both CAT activity and the induction of SOD activity (Chapter 5). Future progress in understanding the mechanism of desiccation tolerance in bryophytes will come by studying the gene products induced by ABA and other hardening treatments. In addition to the already investigated ABA- and desiccation-induced dehydrin-like proteins, the present study suggests that genes involved in the xanthophyll cycle are good targets for molecular investigation.

# CHAPTER 4

# 4. The effect of ABA treatment and hardening by partial dehydration on the desiccation tolerance of photosynthesis and chlorophyll fluorescence in lichens from contrasting habitats

## 4.1 Introduction

The extent to which lichens can tolerate desiccation stress is partly related to the moisture conditions to which they are adapted in their natural habitat (Buck and Brown 1979). The desiccation tolerance of lichens probably involves desiccation tolerance of both the mycobiont and the photobiont, and is moreover a trait that the lichen bionts share with many free-living relatives (Kranner and Lutzoni 1999). However, the physiological and biochemical mechanisms behind desiccation tolerance in lichens and their photobionts are far from understood and require much further study (Bewley and Krochko 1982, Kranner and Lutzoni 1999, Kranner 2002). The aim of this study was to test whether lichens have 'constitutive' desiccation tolerance mechanism, or if desiccation tolerance could be induced by various hardening treatment. In order to determine if hardening treatments prior to desiccation stress increased the desiccation tolerance, lichens were partially dehydrated or treated with exogenously supplied ABA. The green-algal containing lichen, *R. celastri* from more exposed habitat was compared with the cyanobacterial lichen *P. polydactyla* from a more shaded habitat. Photosynthesis, respiration and chlorophyll fluorescence measurements were used as rapid tools to determine the metabolic activity of the photobiont in the lichen symbiosis.

## 4.2 Materials and Methods

Lichens were hardened by partial desiccation or pretreated with 100 μM ABA or distilled water for 1 h and stored hydrated for 3 d at 20°C and a PPFR of 75 μmoles photon $m^{-2}s^{-1}$. Partial desiccation was achieved by placing the material on dry filter paper for 3 d, and then placed on a wet filter paper for 1 d at 100% RH (over water in a desiccator) before desiccation. Lichens were then desiccated for 15 and 30 d by placing thalli in 2 x 5 cm specimen bottles in a desiccator over silica gel at 20°C and a PPFR of 75 μmoles photon $m^{-2}s^{-1}$ under continuous fluorescent light. Lichens were rapidly rehydrated in of 10 ml of distilled water. Photosynthesis, chlorophyll

fluorescence parameters, and the concentrations of soluble sugars, starch and chlorophylls were measured in freshly collected material, after pretreatment with ABA or distilled water for 3 d, then at intervals during the 8 h following rehydration.

### 4.3 Results

Figure 4.1a shows that in *R. celastri* the rate of photosynthesis increases with the increase in RWC up to 0.5. A further increase in RWC decreased the rate of photosynthesis. Net photosynthesis saturated at a PPFR of *c.* 500 μmol photon $m^{-2}s^{-1}$ (Figure 4.1b). The rate of photosynthesis in *R. celastri* at the start of the experiment was *c.* 5.0 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$ (Figure 4.2a). Treatment with distilled water or ABA followed by storage for 3 d had little effect on photosynthesis (Figure 4.2a). Net photosynthesis recovered immediately during rehydration following both 15 and 30 d desiccation. ABA treated material recovered more rapidly than the material in distilled water following 15 and 30 d desiccation, although the recovery was incomplete even after 8 h. (Figure 4.2a, b). Following both 15 d desiccation ABA treatment increased the rate of photosynthetic recovery at $P < 0.001$ (Appendix 1A). After 30 d desiccation, ABA treatment increased the rate of recovery significantly at $P < 0.05$ (Appendix 1B)

Photosynthesis in material hardened by partial desiccation before drying recovered at similar rates to non-hardened material during rehydration following desiccation for 15 d, and both hardening by partial dehydration and the interaction between the treatments (time.ABA) displayed no significant difference (Appendix 2A). Photosynthesis had only recovered to *c.* 3.0 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$ even after 8 h rehydration (Figure 4.2c). However, after 30 d desiccation, hardened material recovered faster than the non-hardened material although recovery was again only partial (to *c.* 3.0 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$ and *c.* 1.8 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$ in non-hardened and hardened material respectively) (Figure 4.2d).

During storage after distilled water or ABA pretreatment, the optimal quantum yield ($F_v$ / $F_m$), the actual quantum yield of PS II (ΦPSII) and non-photochemical quenching (NPQ) increased slightly (Figure 4.3 a, c, e). Desiccation sharply reduced all parameters. During rehydration following desiccation for 15 and 30 d, $F_v$ / $F_m$ recovered almost completely, and, no significant differences existed between the treatments (Figure 4.3a, b). ΦPSII immediately recovered to *c.* 0.25 following 15 d

desiccation and *c.* 0.2 following 30 d in both treatments, with ABA treated material slightly higher than distilled water, although, differences were not significant (Figure 4.3c, d). NPQ recovered quickly, and was significantly higher ($P < 0.001$) in ABA treated material for both 15 and 30 d desiccation (Figure 4.3e, f; Appendix 3A, B). However, the CV% (the percentage that the standard deviation is of the mean) was high showing that there was a big variation within the measurements, probably caused by the differences in the ages of the plant material used. Following 15 d desiccation NPQ recovered almost completely in ABA treated material (Figure 4.3e), but only partially following 30 d desiccation (Figure 4.3f).

Initially, the concentration of soluble sugars in *R. celastri* were approx. 15 mg $g^{-1}$ dry mass (Figure 4.4a). Concentrations increased slightly in untreated and ABA treated material after desiccation for 15 d then declined during rehydration to below the original levels (Figure 4.4a). Following desiccation for 30 d, the level of soluble sugars increased to *c.* 50 mg $g^{-1}$ dry mass in both treatments then declined during rehydration (Figure 4.4b). However, no significant differences existed between the treatments. Starch concentrations in freshly collected material were approx. 80 mg $g^{-1}$ dry mass. Concentrations increased slightly during storage and slowly declined during desiccation to below the original levels in both treatments. However, in ABA treated material concentrations were slightly higher than untreated material. During rehydration following desiccation for 15 d, concentrations remained more or less the same throughout rehydration period in untreated material, whilst in ABA treated material declined even further (Figure 4.4c). A slight increase in starch concentrations occurred after 30 d desiccation in untreated material, while rehydration in ABA treated material showed a slight decline in starch concentrations during the first 20 min. Starch concentration remained almost constant thereafter in both treatments (Figure 4.4d). Desiccation for 15 and 30 d reduced total chlorophyll a and b from *c.* 6.0 to 4.5 mg $g^{-1}$ dry mass in ABA treated and 3.6 mg $g^{-1}$ dry mass in untreated material (Figure 4.4e), whilst desiccation for 30 d reduced the chlorophylls to *c.* 4.0 mg $g^{-1}$ dry mass in both treatments (Figure 4.4f). No statistically significant differences existed between the treatments. Chlorophyll content did not recover during rehydration following desiccation for 15 or 30 d.

In *P. polydactyla*, net photosynthesis increased with increasing RWC reaching a maximum at 0.8 (Figure 4.5a). Photosynthesis saturated at a PPFR of 150 μmol photons $m^{-2}s^{-1}$ (Figure 4.5b). The rate of net photosynthesis at the start of the experiment was *c.* 4.0 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$ (Figure 4.6a). Photosynthesis slightly declined during storage following treatment with distilled water or ABA. During the first 20 min of rehydration, ABA treated material recovered a positive carbon balance following 15 d desiccation. Material pretreated in distilled water initially displayed net $CO_2$ release. After rehydration for 2 h ABA treated material photosynthesized significantly faster than untreated material. However, following 4 h rehydration photosynthesis in both distilled water and ABA treated material completely recovered. Following 30 d desiccation, both distilled water and ABA treated material initially displayed net $CO_2$ release. The release was smaller, but not significantly so in ABA treated material. However, following 8 h of rehydration photosynthesis in lichens receiving both treatments recovered almost completely (Figure 4.6b).

Material hardened by partial desiccation before drying immediately displayed net $CO_2$ fixation during rehydration following desiccation for 15 d whilst non-hardened material displayed net $CO_2$ release during the first 20 min of rehydration (Figure 4.6c). No significant differences existed between the treatments after 2 h of rehydration. Both hardened and non-hardened material had completely recovered after 8 h. Following 30 d desiccation, hardened material initially showed almost no $CO_2$ release whilst non-hardened material showed net $CO_2$ release of *c.* 1.8 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$. During subsequent rehydration, there were no significant differences between the treatments, and photosynthesis in both sets of material almost recovered to initial values (Figure 4.6d).

During storage for 3 d following hardening by partial dehydration and/or treatment with distilled water or ABA, respiration increased slightly, declined to zero following desiccation, and then increased rapidly during the first 20 min of rehydration (Figure 4.6e,f). Following desiccation for 15 d, the rate of respiration in untreated material was always higher than in hardened treatments. Hardening as a factor alone had no significant effect on the rate of respiration. However, the interaction between time and hardening was significant at $P = 0.01$ (Appendix 6).

Following 30 d desiccation the rate of respiration increased to *c.* 2.3 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$ in material hardened by partial dehydration and untreated material during the first 20 min of rehydration. However, no significant differences existed between the treatments. After rehydration for 8 h, respiration only recovered partially to *c.* 1.5 μmol $CO_2$ $mg^{-1}$ dry mass $h^{-1}$ following 30 d desiccation.

Desiccation reduced $F_v$ / $F_m$, ΦPSII and NPQ following 15 d desiccation. During rehydration following 15 d desiccation $F_v$ / $F_m$ and ΦPSII slowly recovered almost completely in both treatments with no significant differences. NPQ was very low in *P. polydactyla.* NPQ recovered more rapidly in ABA treated material up to 4 h rehydration, and then declined rapidly in the last 2 h of rehydration to same levels as the untreated material. During the first 6 h of rehydration NPQ in ABA treated material was significantly higher than in untreated material. Following desiccation for 30 d, all the parameters only recovered partially during rehydration even after 8 h (Figure 4.7b, d, f). No significant differences existed between treatments.

Desiccation had little effect on the concentration of soluble sugars and starch in *P. polydactyla* although some starch accumulation occurred during the pretreatment (Figure 4.8). Desiccation for 15 d slightly reduced chlorophyll concentrations, but concentrations recovered after rehydration for 8 h (Figure 4.8e). Desiccation for 30 d reduced chlorophyll concentrations to slightly more than half, and these did not recover during rehydration (Figure 4.8f). ABA pretreatment had little effect on any these parameters.

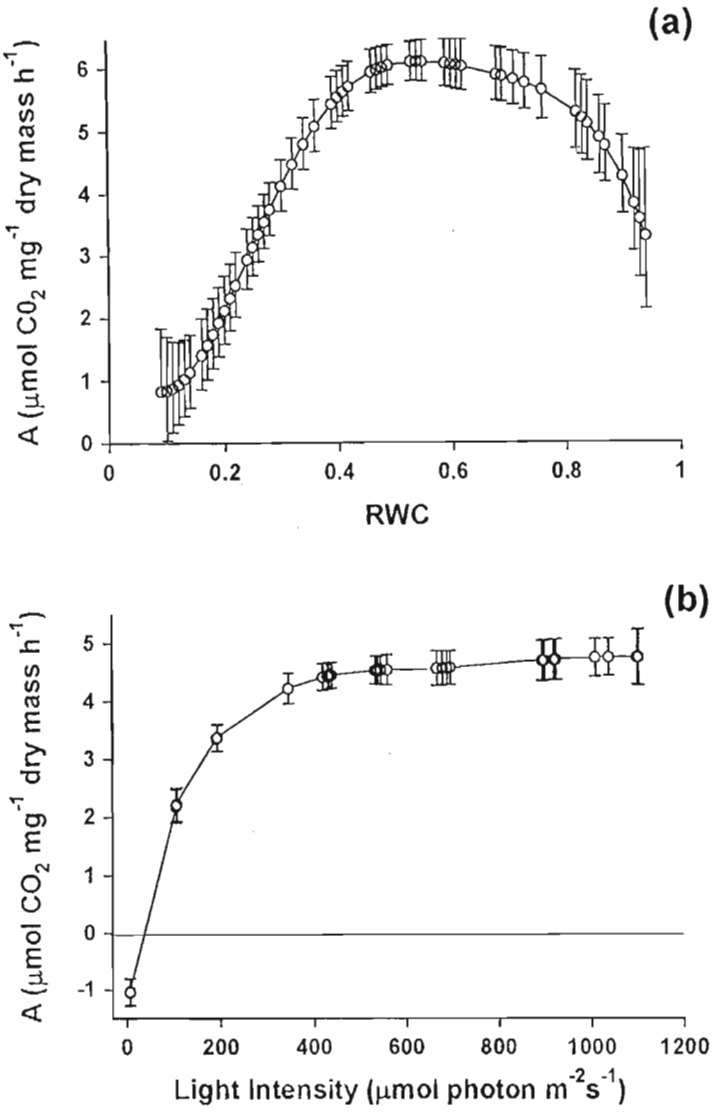



**Figure 4.1:** The effect of RWC (a) and light intensity (b) on photosynthesis in the non-hardened material in the lichen *R. celastri* measured at saturating light intensity and RWC respectively. Points represent fitted values with 95% confidence limits calculated using "Spline" program of Hunt and Parsons (1974).

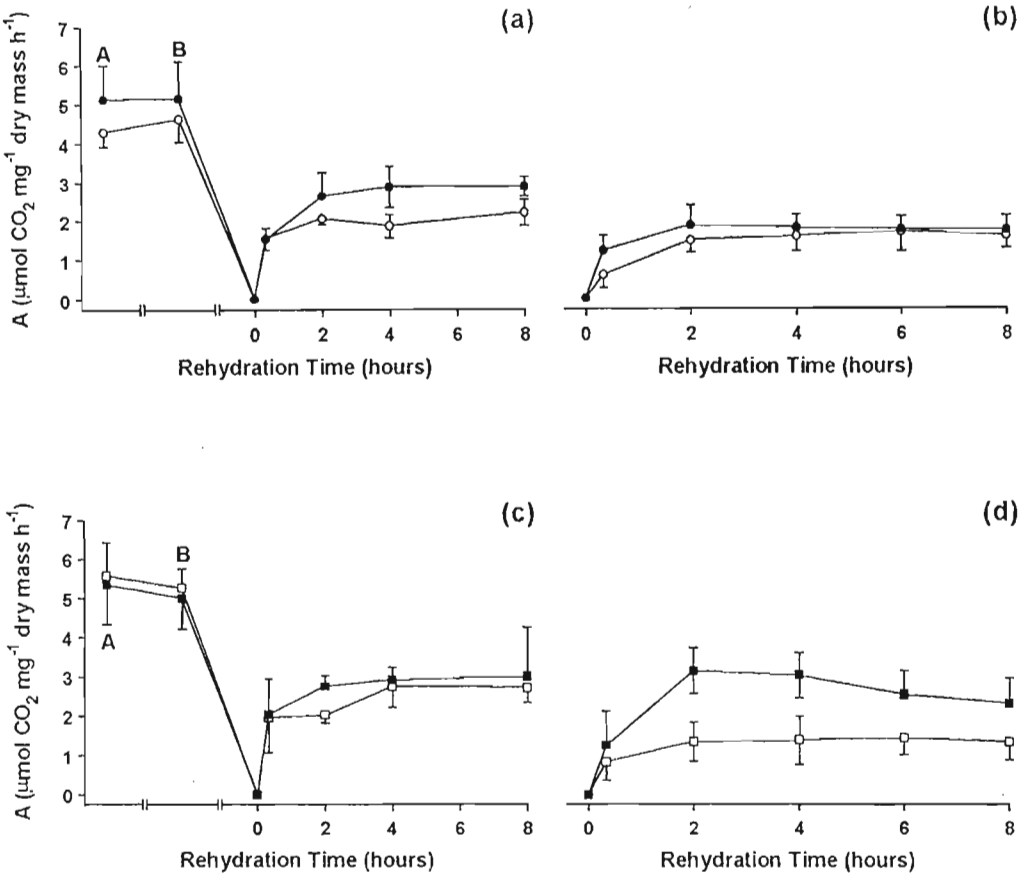



**Figure 4.2:** The effect of treatment with distilled water or 100 μM ABA for 1 h followed by storage for 3 d (a) and (b), and the effect of hardening by partial dehydration (c) and (d) on photosynthesis during rehydration following desiccation for 15 d and 30 d in the lichen *R. celastri*. Symbols: solid circles, ABA-treated and open circles, distilled water; solid squares, hardened by partial dehydration; opens squares, non-hardened. Letters: A, start of the experiment; B, after 3 d storage following treatment.

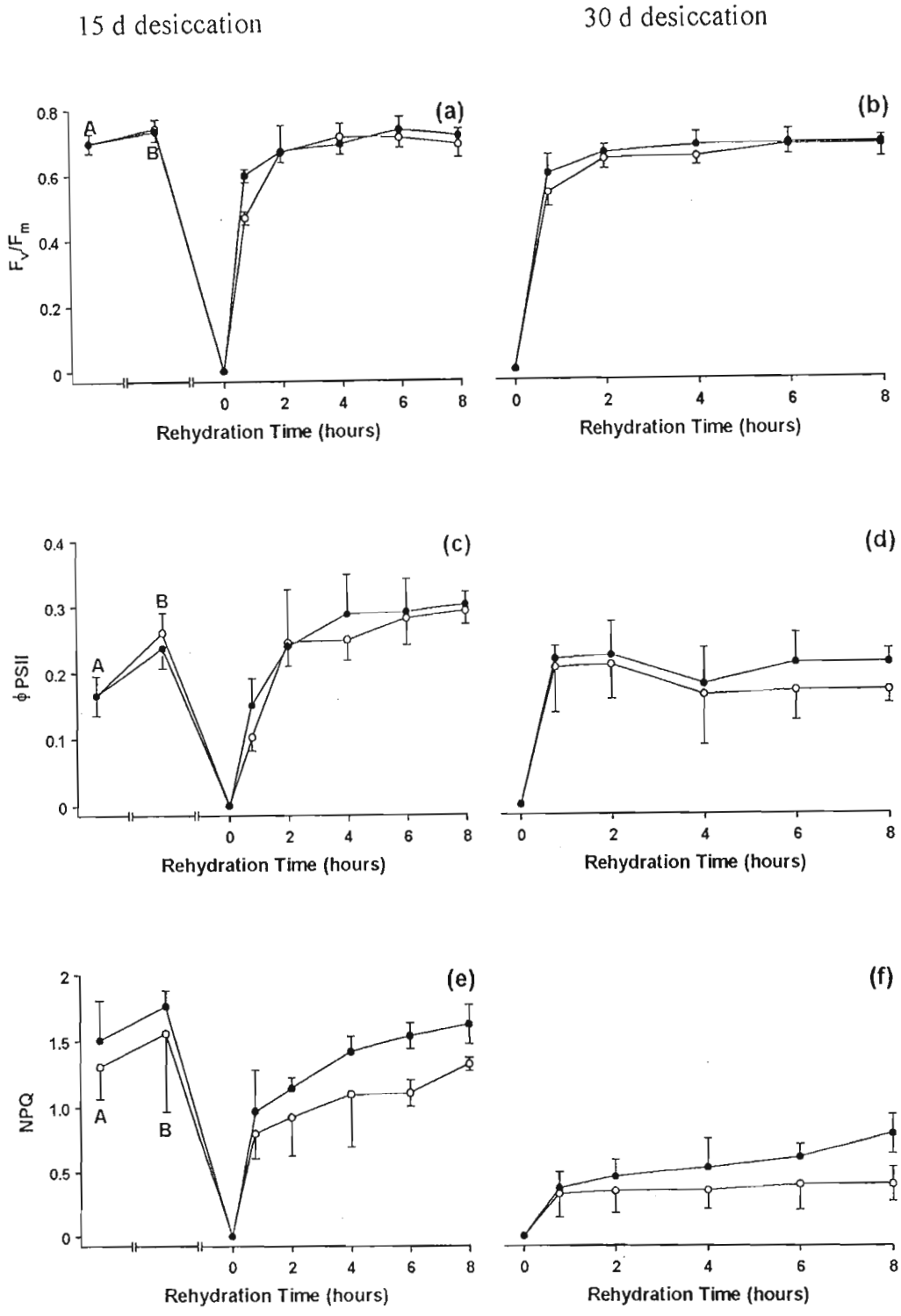



**Figure 4.3:** The effect of treatment with distilled water or 100 µM ABA for 1 h followed by storage for 3 d then desiccation for 15 d (a, c, e) and 30 d (b, d, f). (a) and (b), $F_v/F_m$; (c) and (d), Φ PSII; (e) and (f), NPQ during rehydration in the lichen *R. celastri*. Symbols: solid circles, ABA-treated and open circles, distilled water. Letters: A, start of the experiment; B, after 3 d storage following treatment.

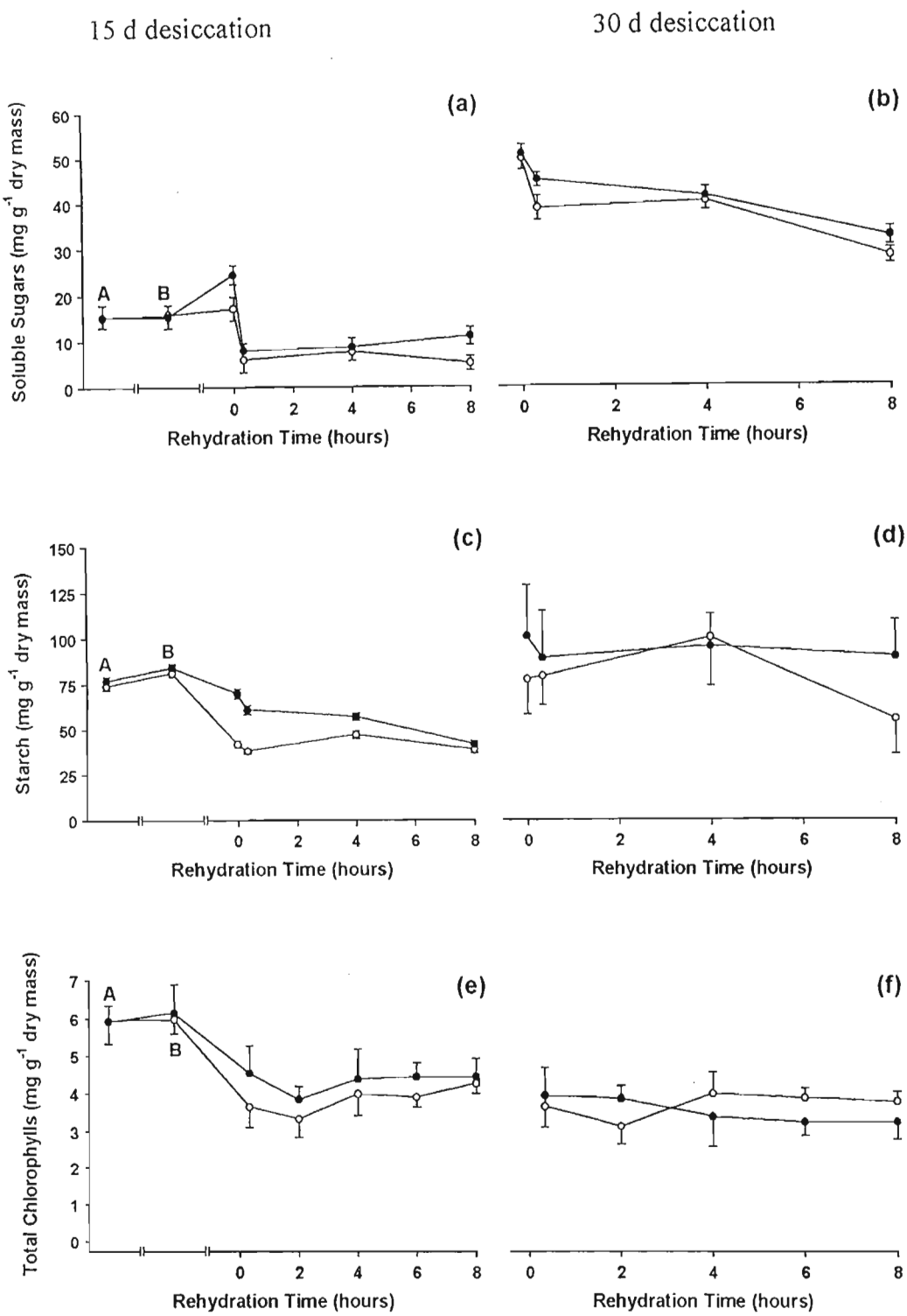



**Figure 4.4:** The effect of treatment with distilled water or 100 μM ABA for 1 h followed by Storage for 3 d and then desiccated for 15 d (a, c, e) and 30 d (b, d, f). (a) and (b), Soluble Sugars; (c) and (d), Starch; and (e) and (f), Total chlorophylls (a+b); during rehydration in the lichen *R. celastri*. Symbols: solid circles, ABA-treated and open circles, distilled water. Letters: A, start of the experiment; B, after 3 d storage following treatment.

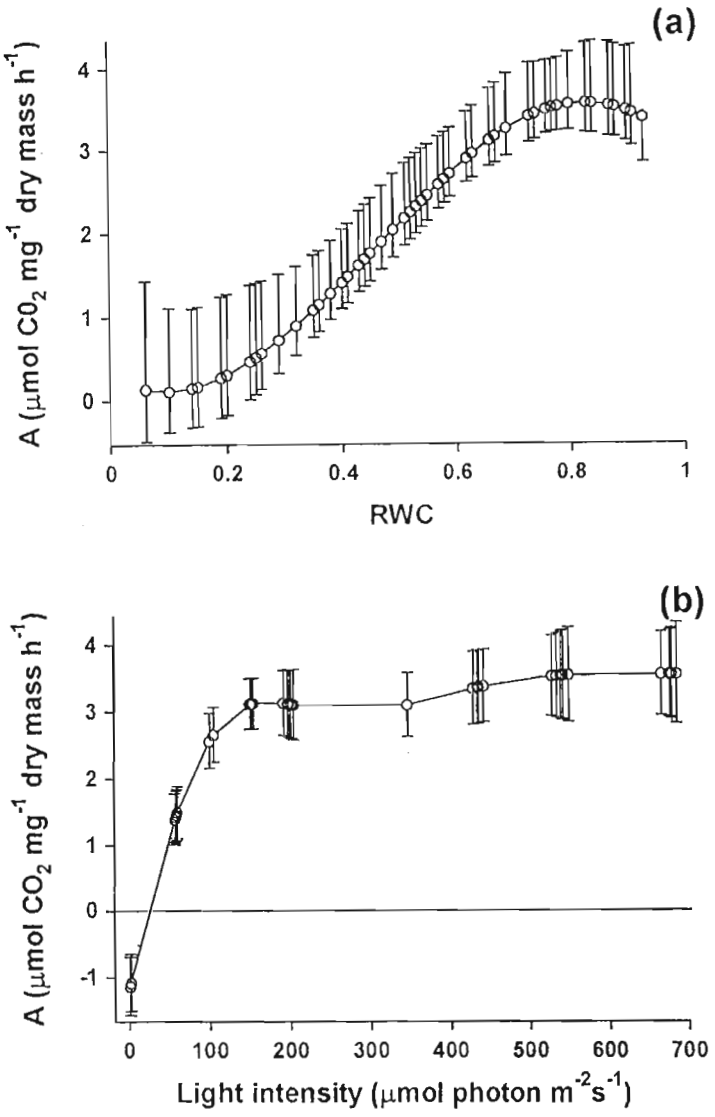



**Figure 4.5:** The effect of RWC (a) and light intensity (b) on photosynthesis in the non-hardened material in the lichen *P. polydactyla* measured at saturating light intensity and RWC respectively. Points represent fitted values with 95% confidence limits calculated using "Spline" program of Hunt and Parsons (1974).

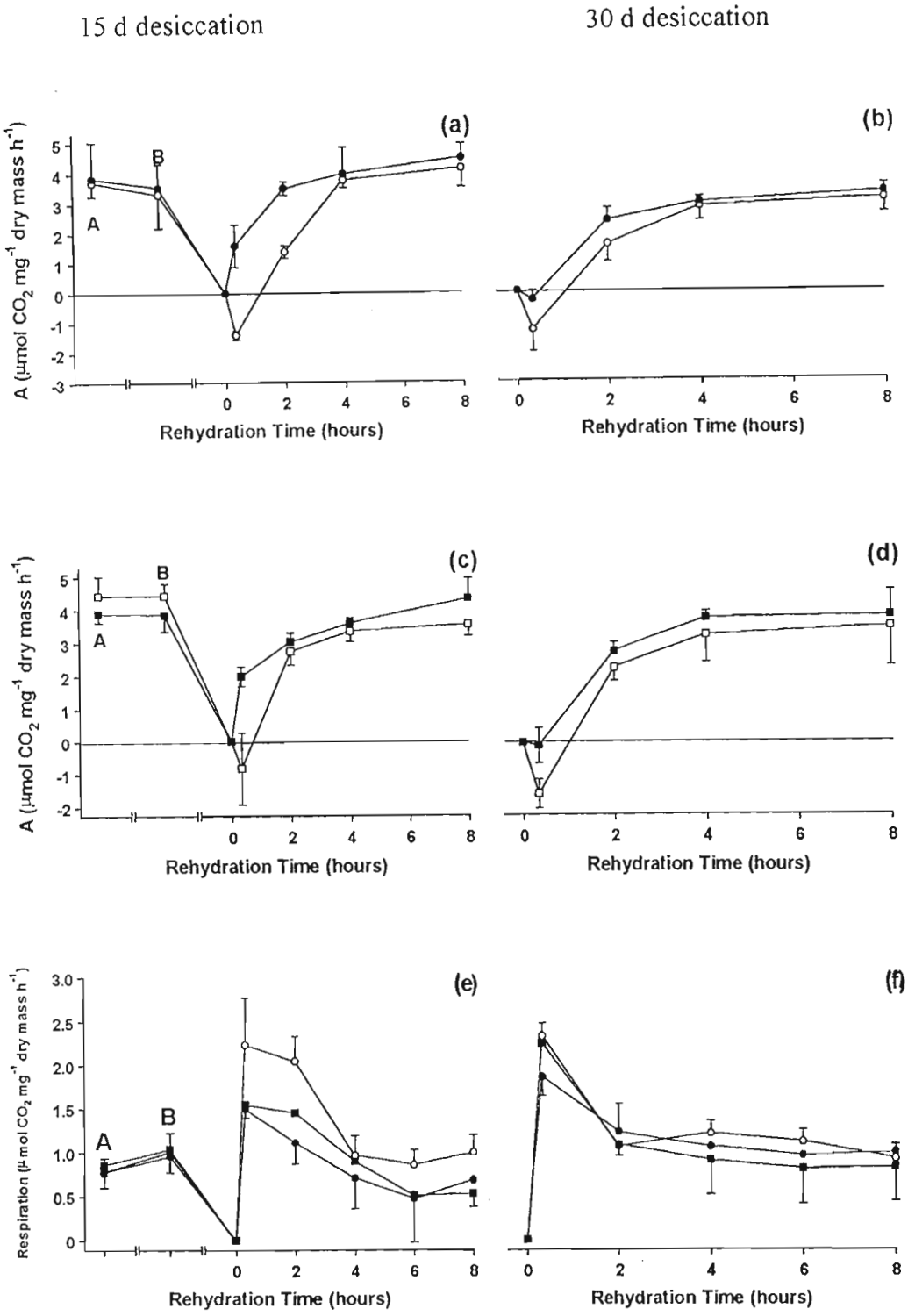



**Figure 4.6:** The effect of treatment with distilled water or 100 μM ABA for 1 h followed by storage for 3 d during rehydration following desiccation for 15 d (a) and 30 d (b), and the effect of hardening by partial dehydration 15 d (c) and 30 d (d) on photosynthesis, and respiration 15 d (e) and 30 d (f) in the lichen *P. polydactyla.* Symbols: solid circles, ABA - treated and open circles, distilled water, solid squares, hardened by partial dehydration; opens squares, non-hardened. Letters: A, start of the experiment; B, after 3 d storage following treatment.

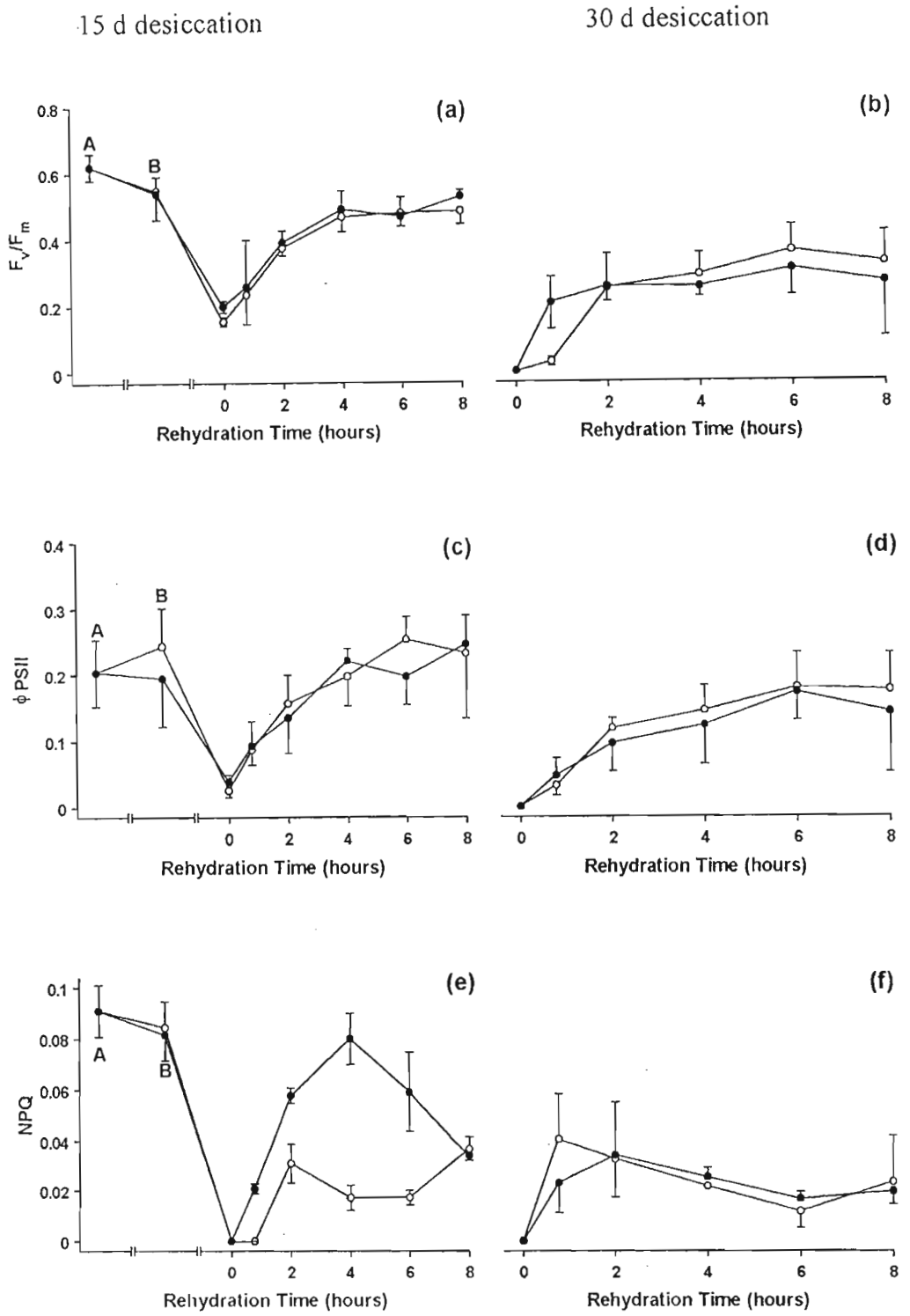



**Figure 4.7:** The effect of treatment with distilled water or 100 µM ABA for 1 h followed by storage for 3 d then desiccation for 15 d (a, c, e) and 30 d (b, d, f). (a) and (b), $F_v / F_m$; (c) and (d), ΦPSII; (e) and (f), NPQ during rehydration in the lichen *P. polydactyla*. Symbols: solid circles, ABA- treated and open circles, distilled water. Letters: A, start of the experiment; B, after 3 d storage following treatment.

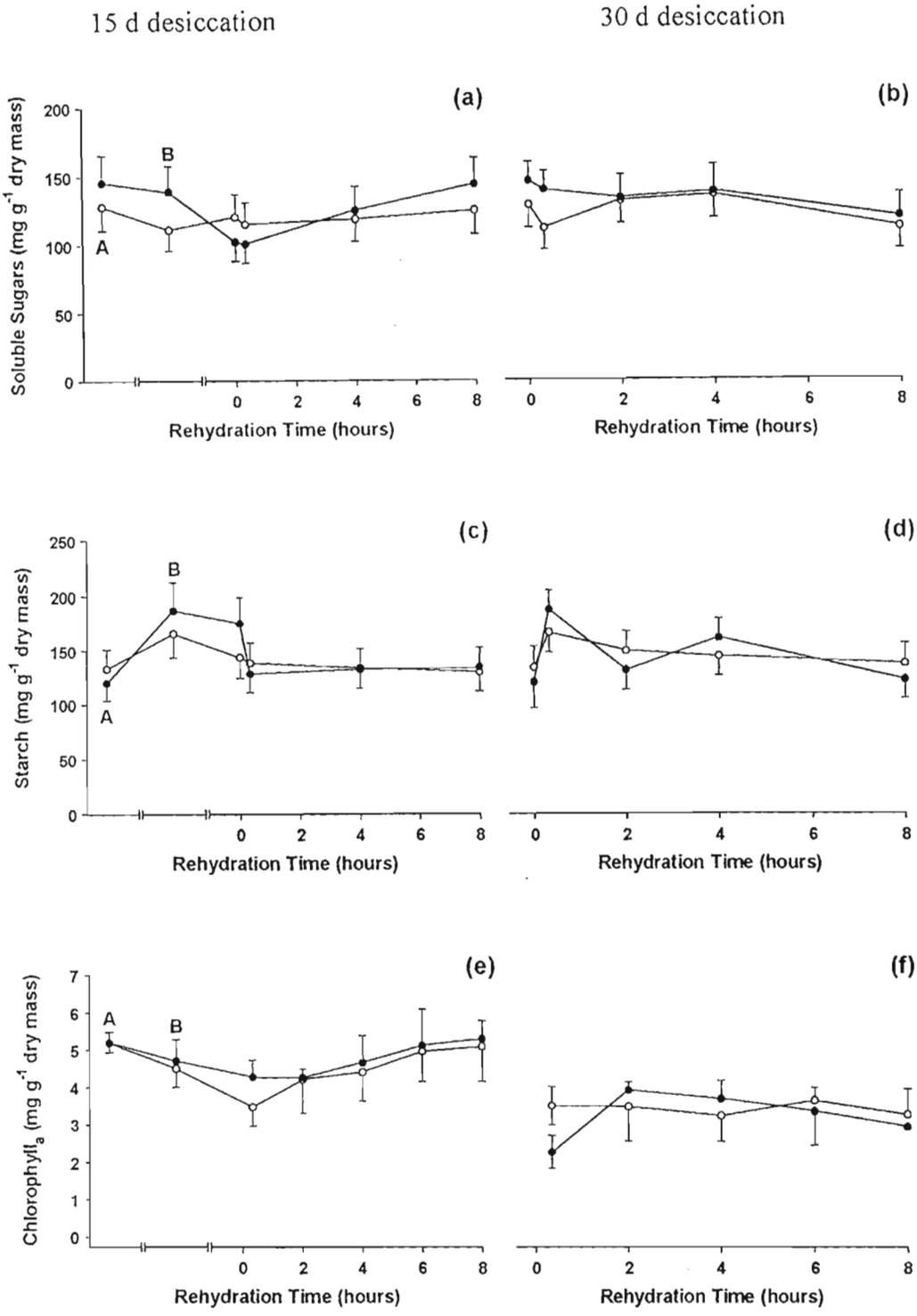



**Figure 4.8:** The effect of treatment with distilled water or 100 μM ABA for 1 h followed by storage for 3 d and then desiccation for 15 d (a, c, e) and 30 d (b, d, f). (a) and (b), Soluble sugars; (c) and (d), Starch; and (e) and (f), Chlorophyll (a); during rehydration in the lichen *P. polydactyla*. Symbols: solid circles, ABA-treated and open circles, distilled water. Letters: A, start of the experiment; B, after 3 d storage following treatment.

## 4.4 Discussion

Results presented here clearly illustrate that hardening treatments can increase desiccation tolerance of net photosynthesis in both *R. celastri* and *P. polydactyla*. In *R. celastri*, the benefits of ABA pretreatment decreased during recovery following 30 d desiccation, but lichens still displayed some improvement in tolerance (Figure 4.2b). Based on chlorophyll fluorescence measurements, Dietz and Hartung (1999) suggested that ABA does not play a role in desiccation tolerance in lichens. However, the present investigation shows that simply measuring chlorophyll fluorescence parameters is inadequate, and more thorough gas exchange data are needed in order to conclude about the effects of ABA. The reduction in the re-saturation respiration burst in *P. polydactyla*, although not quite significant, strongly suggests that hardening increases mycobiont tolerance (Figure 4.6e, f). It is most difficult to establish whether the hardening treatments improve photobiont performance. Hardening increased photosynthesis during rehydration much more than it decreased re-saturation respiration (compare Figures 4.6a-d with 4.6e-f). It is therefore tempting to speculate that hardening treatments also improved the desiccation tolerance of the photobiont. However, it is highly likely that lichens will experience greater stress if they are rehydrated in the light rather than the dark (Seel *et al.* 1992a, Proctor 2000a, Chapter 3). Measuring photosynthesis obviously involves illuminating lichens, while respiration is measured in the dark. Therefore the observation that the difference in photosynthesis between hardened and non-hardened material exceeds the difference in respiration is not unequivocal evidence that photobionts can be hardened. For example, additional ROS produced by algae during rehydration in the light may cause more damage to the fungi than would have occurred if rehydration had taken place in darkness. While ABA has certainly been detected, and probably plays an essential metabolic role, in most algae (including cyanobacteria, see Minorsky [2002] for review), further experiments are needed to determine whether it is involved in signal transduction pathways that increase the desiccation tolerance in lichen photobionts.

The recovery of photosynthesis depended on the duration of the preceding dry periods and there were clear differences between the two species. *R. celastri* from an exposed habitat was surprisingly more sensitive to prolonged desiccation than *P. polydactyla* from shaded habitats. Generally, desiccation tolerance in lichens is correlated to the water availability in their natural habitats (Buck and Brown 1979). In

the present study, while *P. polydactyla* may be desiccated for the most of the winter season, *R. celastri* from the mist belt is probably periodically wetted even in winter. In field situations, *P. polydactyla* may slowly dry inducing ABA synthesis, which in turn activates signal transduction pathways that increase desiccation tolerance. The effect of ABA suggests that *P. polydactyla* possesses an inducible protection mechanism. It was envisaged that lichens with largely constitutive mechanisms would include species like *R. celastri* from exposed habitats. Surprisingly, results presented here show that desiccation tolerance in *R. celastri* is at least to some extent inducible (Figure 4.2d).

The rapid recovery of PSII but slow recovery of carbon fixation can cause rapid production of ROS (Mckersie and Lesham 1994). Under these conditions NPQ may considerably reduce the amount of oxidative damage. Pretreatment with ABA both increase NPQ (see chapter 3) and improves the tolerance of the moss *Atrichum* to desiccation-induced damage e.g. ion leakage (Beckett 1999, 2001), and reductions in PSII activity (Beckett *et al.* 2000). In the green-algal lichen, *R. celastri* NPQ was higher than in cyanobacterial lichen, *P. polydactyla*. Therefore, *R. celastri* with high NPQ may stand a better chance of scavenging the potential toxic oxygen species than *P. polydactyla*. In addition, soluble sugars are elevated after 30 d desiccation in hardened plants. These could help the recovery of these plants following prolonged desiccation period. Demming-Adams and co-workers revealed the importance of xanthophyll cycle for scavenging activated oxygen in lichens. Researchers showed that zeaxanthin frequently occurs in lichens with cyanobacterial photobionts that also contain remarkable amounts of ketocarotenoids (Adams *et al.* 1993). In *P. polydactyla* NPQ was very low may be the xanthophylls cycle is not the only way to remove the ROS produced around the photosystems. After desiccation for 15 d, ABA treatment increased NPQ in the first 6 h of rehydration.

Photosynthesis recovered remarkably well during rehydration in *P. polydactyla*, which suggests that the photosynthetic apparatus of these plants were not irreversibly damaged by desiccation *per se*. However, in *R. celastri* photosynthesis recovered only partially suggesting that desiccation caused a severe damage, and that the photosynthetic apparatus was obviously compromised. Desiccation of material from both species reduced chlorophyll contents, but the photosynthetic activity was

able to recover, although only partially in *R. celastri*. This may be as a result of reduced chlorophyll levels during desiccation, but only in part. The reductions in chlorophylls were much less than in photosynthesis in both lichen species.

Desiccation greatly increased the levels of soluble sugars during prolonged desiccation from *c.* 15 to 50 mg $g^{-1}$ dry mass (Figure 4.4b) in *R. celastri* whilst soluble sugars in *P. polydactyla* remained almost constant. Possibly in *R. celastri* photosynthesis occurs to some extent during storage in the 'conviron' (even over silica gel), and the ordinary metabolic activity may be unable to use the resulting sugars, which thus accumulate. Also it is necessary to note that the absolute sugar levels were much higher in *P. polydactyla*. In *P. polydactyla*, sugar maintenance may be important in desiccation tolerance or help during recovery. Sugar accumulation may confer a selective advantage to the lichens. Non-reducing sugars may substitute for water by forming hydrogen bonds, thereby maintaining hydrophilic structures in their hydrated orientation (Crowe *et al.* 1992), or sugars can promote vitrification of the cytoplasm and protect membranes (Scott 2000). Increases in soluble sugars in *R. celastri* following desiccation were proportionally similar to those reported in resurrection angiosperms *Myrothamnus fabellifolia* and *Xerophyta villosa*, but were much less than those found in *Craterostigma plantagineum* (for review see Scott 2000). Owing to high viscosity of the cytoplasm, chemical reactions are strongly slowed down. Consequently, degenerative processes, such as alteration in ionic strength and pH, and crystallization of solutes, are prevented (Kranner and Lutzoni 1999

Data presented here clearly shows that *P. polydactyla* display an inducible protection mechanism in addition to any constitutive mechanisms. However, it is interesting to find out that NPQ was very low in *P. polydactyla*, assuming that the increase in NPQ allows the rapid recovery of photosynthesis. The mechanism behind the rapid recovery of photosynthesis in this species may be due to their degree of tolerance. It is important to note that NPQ around PSI would not be detected by the Hansatech device, but could protect the photosnthetic apparatus from damage. Unlike *R. celastri*, they are desiccated for most of the winter season and could have some constitutive mechanisms that take place. *P. polydactyla* belongs to the cyano-lichens, of which the desiccation tolerance is generally lower than that of lichens with green

algal photobionts (Buck and Brown 1979, Beckett 1995). Some of these results are difficult to explain and several of the explanations provided above remain speculative. It would have been interesting to check a lichen occupying a niche like the highly desiccation tolerant *T. ruralis* e.g. *Parmelia* species. Probably in this kind of habitat one would not expect to find any inducible tolerance (or less so than *Peltigera* at least). Understanding the photochemical processes that contribute to the PSII reactions of photosynthesis and the non-photochemical reactions, with which they interact in the thylakoid membranes of the chloroplasts, is a major goal in studies of photosynthetic physiology. This information could have important implications for increasing efficiency and understanding the complex relationship between plant photosynthesis and the environment. However, both present knowledge of the ecophysiology of bryophytes and the above discussion indicate that ABA is involved in desiccation tolerance of lichens.

# CHAPTER 5

# 5. Effects of desiccation on free radical scavenging enzymes in a moss *Atrichum androgynum*

## 5.1 Introduction

Recently, much knowledge has been gained about the basic mechanisms behind desiccation tolerance in bryophytes (Smirnoff 1993, 1995, Proctor and Smirnoff 2000, Proctor and Pence 2002). The electron transfer mechanism of green plant photosynthesis is capable of generating ROS (Smirnoff 1993, 1995, Proctor and Smirnoff 2000, Proctor and Pence 2002). Selection has favoured plants with sophisticated protection systems consisting of low molecular weight antioxidants and antioxidant enzymes (Foyer *et al.* 1994, Allen 1995). Their function is to remove ROS, which are also produced at high levels during abiotic and biotic stress. ROS can oxidize a large number of molecules and are thus potentially hazardous to the cells if not removed immediately. As discussed previously, desiccation tolerance of the moss *A. androgynum* can be improved by various hardening treatments. Presumably, in the field, partial dehydration will often precede a more severe desiccation stress, inducing ABA synthesis, which in turn activates signal transduction pathways that increase desiccation tolerance. In the last decade, the role of the plant hormone abscisic acid (ABA) in the induction of antioxidant defense has been the subject of extensive research. Recently, Guan and Scandalios (1998a) reported that two structurally similar cytosolic SOD genes could be regulated in part by ABA during late embryogenesis. It has been documented that ABA can result in the increased generation of ROS (Guan *et al.* 2000, Pei *et al.* 2000, Jiang and Zhang 2001, Murata *et al.* 2001, Zhang *et al.* 2001), induce the expression of antioxidant genes encoding SOD and CAT (Williamson and Scandalios 1992, Anderson *et al.* 1994, Zhu and Scandalios 1994, Sakamoto *et al.* 1995, Bueno *et al.* 1998, Guan and Scandalios 1998a, b, Kaminaka *et al.* 1999, Guan *et al.* 2000), and enhance the activity of antioxidant enzymes such as SOD, CAT, APX and GR and the contents of antioxidant metabolites such as ascorbate, GSH, α-tocopherol and carotenoids (Anderson *et al.* 1994, Prasad *et al.* 1994, Bueno *et al.* 1998, Bellaire *et al.* 2000, Jiang and Zhang 2001). It has been proposed that ABA-dependent and independent signal pathways are

involved in the expression of some antioxidant genes or up-regulation of antioxidant enzymes under osmotic stress or NaCl stress (Bellaire *et al.* 2000, Guan *et al.* 2000). The changes in the transcripts of SOD and CAT and the enzyme activities of their isozymes in response to ABA may be caused, in part, by the altered metabolic activities of cells that lead to changes in ROS levels (Guan and Scandalios 1998a, b, Guan *et al.* 2000). ABA treated cells maintain the ability to scavenge ROS and remain viable (Fath *et al.* 2001). However, details about the interaction between ABA, ROS and antioxidant defence response remain to be determined. It was therefore, hypothesized that ABA up-regulates the free radical scavenging enzymes, thus reducing ROS damage. The aim of this section was to measure the changes in the activity of the antioxidant enzymes CAT and SOD during desiccation and rehydration in *A. androgynum.*

## 5.2 Materials and Methods

Initially, mosses were desiccated over silica gel for 16 h and the enzyme activities were strongly reduced by desiccation and did not recover following rehydration (results not shown). To determine the appropriate conditions to desiccate the moss without damaging the plant severely, mosses were slowly desiccated by placing them at different relative humidity's; 100, 78 and 52% for 3 d while for the control, mosses were stored hydrated. To determine the RWC during the 3 d storage water content was measured every day. Results showed that storage at 52% RH caused a greater increase in the activity of SOD in the moss (Figure 5.3). In the next set of experiments, enzyme activity was assayed shortly after collection, and again after storage at 52% RH and again after hardening by partial dehydration and/or after storage for 3 d following treatment with ABA or distilled water, and during dehydration and rehydration unless specified otherwise. Mosses were either pretreated with 100 µM ABA or distilled water for 1 h and stored hydrated for 3 d at 15 - 20°C and 75 µmoles photons $m^{-2}s^{-1}$ or hardened by partial dehydration. Partial dehydration was achieved by placing the mosses on dry filter paper for 3 d, then on wet filter paper for 1 d at 100% RH and stored at the above conditions. Mosses were then slowly desiccated at 52% RH for 4, 8, and 32 h and rehydrated for 2, 4, 6, 8 and 24 h. Relative water content was also measured in untreated and ABA treated mosses during drying.

## 5.3 Results

The RWC of the plant material following 3 d storage was unaffected in the material that was stored hydrated. In material stored at 100%, 78% and 52% relative humidity, the RWC declined to 0.39, 0.18 and 0.08 (Figure 5.1). Figure 1b presents a detailed drying curve for plant material stored at 52% RH for 32 h. Material pretreated with ABA and distilled water dried at similar rates (Figure 5.2). Desiccation to progressively lower water contents increased SOD (Figure 5.4) but reduced CAT activity (Figure 5.3).

Hardening treatments by partial desiccation slightly reduced CAT activity (Figure 5.5). When mosses were dehydrated at 52% RH for 32 h, CAT activity decreased during the first 2 h in distilled water and ABA treated material, and increased slightly in material hardened by partial dehydration. In the next 6 h, CAT increased slightly in all the treatments and then slightly declined to below original levels after 32 h desiccation. However, no significant differences existed between the treatments (Appendix 4A). During rehydration, CAT activity remained almost constant in distilled water and ABA treated material, and slightly declined in material hardened by partial dehydration then increase after 40 h to a similar level in all treatments (Figure 5.5). No significant differences existed between the treatments (Appendix 4B).

SOD activity increased progressively during dehydration in all treatments and rapidly declined during the first few minutes of rehydration after which a steady decline was observed (Figure 5.6). However, there were no significant differences between the treatments during desiccation (Appendix 5A) and rehydration (Appendix 5B). Hardening by partial dehydration and ABA treatment tended to reduce both CAT activity and the induction of SOD activity, although these effects were not significant.

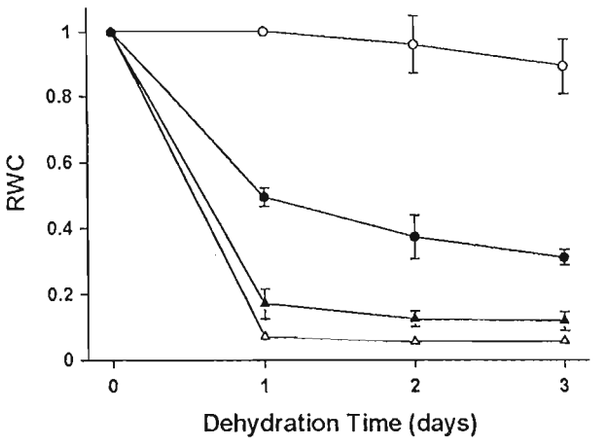



**Figure 5.1:** The effect of desiccation on RWC in the moss *A. androgynum*. In this figure and subsequent, the points represent the means and the error bars the standard deviation. Overlapping error bars were removed, (n=3). Symbols: open circles, hydrated; solid circles, 100% RH; solid triangles, 78% RH and open triangles, 52% RH.

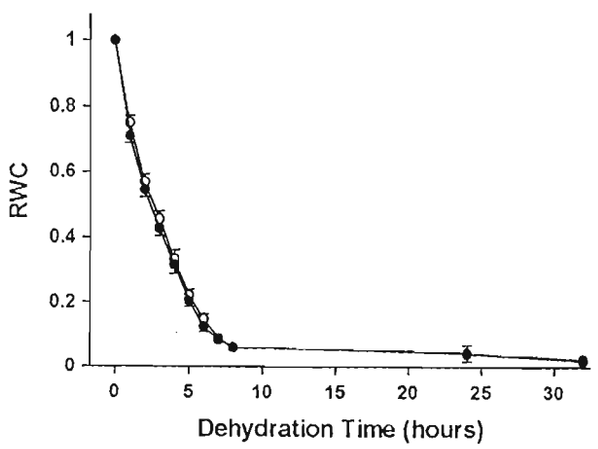



**Figure 5.2:** The effect of desiccation time on RWC in the moss *A. androgynum*. Symbols: solid circles, ABA treated; open circles, distilled water.

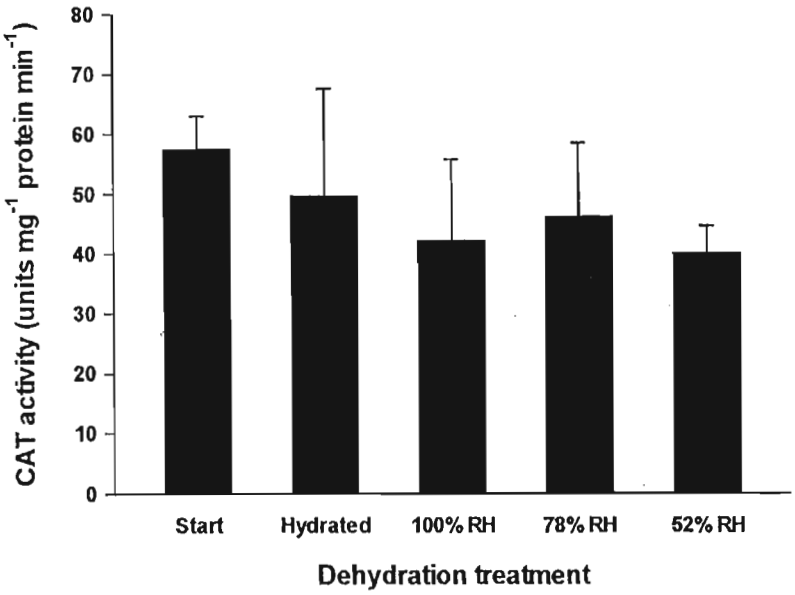



**Figure 5.3:** The effect of desiccation in air of progressively lower relative humidities on CAT activity in the moss *A. androgynum*

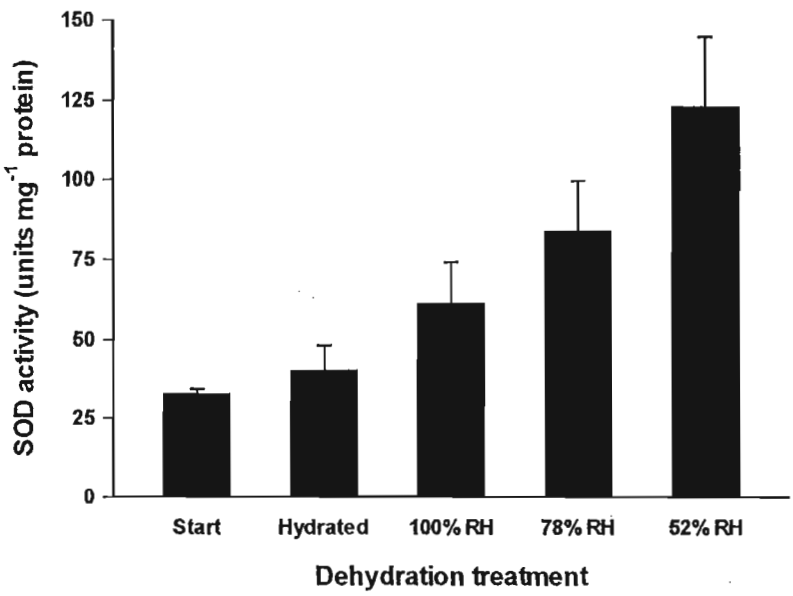



**Figure 5.4:** The effect of desiccation in air of progressively lower relative humidities on SOD activity in the moss *A. androgynum*.

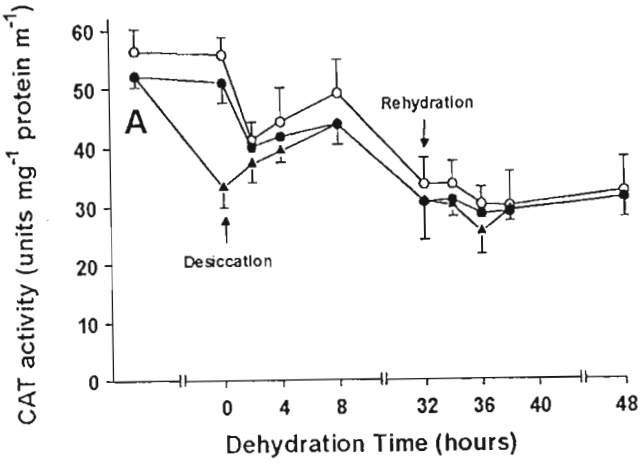



**Figure 5.5:** The effect of hardening by partial dehydration and treatment with distilled water or 100 μM ABA for 1 h on catalase activity during dehydration at 52% RH and rehydration in the moss *A. androgynum*. Symbols: solid circles, ABA treated material; open circles, distilled water; solid triangle, hardening by partial dehydration. Letter: A, start of the experiment.

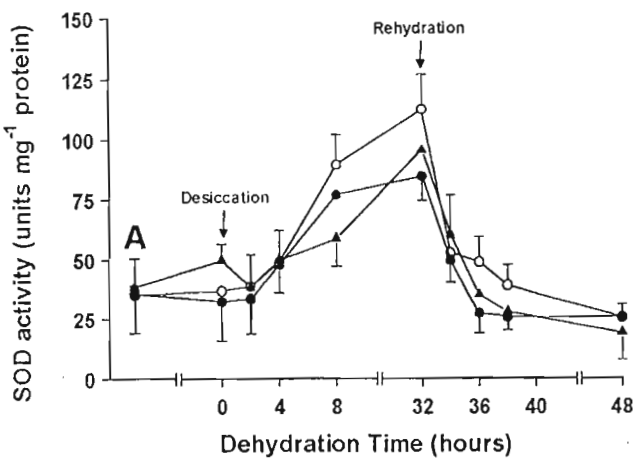



**Figure 5.6:** The effect of hardening by partial dehydration and treatment with distilled water or 100 μM ABA for 1 h on superoxide dismutase activity during dehydration at 52% RH and rehydration in the moss *A. androgynum* during desiccation at 52% RH and rehydration. Symbols: solid circles, ABA treated material; open circles, distilled water; solid triangle, hardening by partial dehydration. Letter: A, start of the experiment.

## 5.3 Discussion

Results presented here clearly illustrates that antioxidant enzyme activities of CAT and SOD are considerably affected by desiccation and subsequent rehydration in the moss *A. androgynum* depending on the rate at which the mosses were dried. These results agree with the literature (Dhindsa and Matowe 1981, Werner *et al.* 1991, Seel *et al.* 1992a) that slow drying can induce SOD in mosses. The rate of drying greatly affects desiccation tolerance in mosses (Proctor 1990). This effect is presumably related to two different stress vectors: direct mechanical or physical stress because of the loss of water and physicochemical damage of tissues as a result of metabolic alterations during drying. It was predicted that partial desiccation and ABA treatments, known to harden mosses to subsequent desiccation, could increase the activities of free radical scavenging enzymes thus increasing the potential to detoxify cells. Surprisingly, although not significant, results showed that ABA and partial desiccation actually reduced the induction of SOD during the first 8 h of desiccation. Furthermore, during subsequent rehydration, SOD activities were always lower in 'hardened' mosses. This may be due to the fact that during desiccation hardened mosses form less free radical therefore SOD was not induced because it was not needed.

The similarities between ABA induced response, and the response induced by partial desiccation suggests in part that ABA may be genuinely involved in desiccation tolerance in *Atrichum*. Beckett (1999) used a simple ion leakage assay to test if specific hardening treatments can increase tolerance in the moss *A. androgynum*. Results showed that this species could be hardened to desiccation stress under controlled conditions. Reducing the RWC to approx. 0.6 for 3 d increased tolerance and exogenous application of ABA followed by storage for 3 d can fully substitute for partial dehydration. These results suggest that ABA play an essential role in the induction of genes that harden *A. androgynum* and at least some other bryophytes, to desiccation stress (Hartung *et al.* 1998). In bryophytes, Seel *et al.* (1992a) found that the main difference between desiccation tolerant and sensitive mosses was more the ability of tolerant species to maintain the levels of α-tocopherol and GSH rather than activities of antioxidant enzymes. Interestingly, in the resurrection angiosperm *Myrothamnus flabellifolia* Sherwin and Farrant (1998) only found limited evidence that APX and SOD are involved in desiccation tolerance,

while Kranner *et al.* (2002) has shown that non-enzymic antioxidants play an important role in tolerance in *Myrothamnus flabellifolia*. Results presented here indicate that, at least when the stress is moderate, the enzymes CAT and SOD may be unaffected during desiccation and maintained during subsequent rehydration suggesting that they play an important role safe guarding the overproduction of free radicals produced during normal metabolism. Furthermore, various mechanisms other than the induction of SOD may be responsible for ABA-induced increase in desiccation tolerance. These include high NPQ in desiccation tolerant bryophytes, particularly at low water contents (Deltoro *et al.* 1998a, b, Csintalan *et al.* 1999). As shown previously in Chapter 3, pretreatment with ABA increased NPQ, which will reduce free radical formation around the photosystems. Increased NPQ in *A. androgynum* may explain in part how ABA hardens the moss to desiccation stress.

Results presented here suggest that ABA is not involved in the induction of antioxidants SOD and CAT, and that these enzymes may not be important in desiccation tolerance in *A. androgynum*. There are several ways that cells can protect themselves from metabolic imbalance and ROS-mediated damage. At higher moisture levels, free radical scavenging enzymes efficiently detoxify ROS (Bewley 1979, Dhindsa 1987, Hendry 1993, Smirnoff 1993, Foyer *et al.* 1994, Kranner and Grill 1997, Sherwin and Farrant 1998, Pammenter and Berjak 1999, Farrant 2000). Probably one reason that ABA does not induce SOD could be that SOD is not active at low RWCs (Walters *et al.* 2002). In such cases some mechanisms of desiccation tolerance may be involved other than enzymic antioxidants, molecules like tocopherols, glutathione and ascorbate may be more effective at low RWCs.

# CHAPTER 6

# 6. The effect of desiccation on the activities of free radical scavenging enzymes in lichens from contrasting habitats

## 6.1 Introduction

Although desiccation tolerance in lichens is fairly well documented, the role of enzymic antioxidants in removing ROS from lichens during desiccation has received very little attention. Foyer and Halliwell (1976) were the first to suggest that healthy plants have a well-balanced interplay of antioxidants and enzymes that scavenges cytotoxic oxygen species. The objective of this section was therefore to measure changes in the activities of the antioxidant enzymes APX, CAT and SOD during desiccation and rehydration in three lichen species from contrasting habitats. The species chosen were *Peltigera polydactyla*, a desiccation sensitive species that grows in moist microhabitats, *Ramalina celastri*, an intermediate species from tree trunks and *Teloschistes capensis*, lichen from the most xeric microhabitats. It has been hypothesized that if these enzymes are involved in conferring desiccation tolerance, during water stress or during recovery following water stress their activities should be higher in species from xeric microhabitats.

## 6.2 Materials and Methods

Once collected, lichens were gradually rehydrated using air at a relative humidity of 100% for 24 h (over distilled water) at 20°C and a light intensity of 135 μmol $m^{-2}s^{-1}$, followed by contact with wet filter paper for a further 24 h. Enzyme activity was assayed shortly after collection, and again after hydration. Lichens were then desiccated over silica gel as described by Kranner and Lutzoni (1999), although the desiccation period was 28 rather than 60 d. Samples were taken after desiccation for 14 d. After 28 d material remaining was rapidly rehydrated by incubation in deionized distilled water. Samples were taken at intervals for 30 minutes during rehydration.

## 6.3 Results

Freshly collected material of *T. capensis* from the Namib Desert consistently displayed the highest activities of APX, CAT and SOD (Figures 6.1, 6.2 and 6.3). *R. celastri* from a moderately xeric habitat had lower activities of APX than *P. polydactyla* from a moist habitat, higher activities of CAT and similar activities of SOD. In all species, enzyme activities tended to rise or stay the same following rehydration for 48 h. After desiccation for 14 d, enzyme activities decreased, and decreased further to low values after 28 d desiccation. In all cases, enzyme activities tended to remain constant or decline further during the 30 min following rehydration.

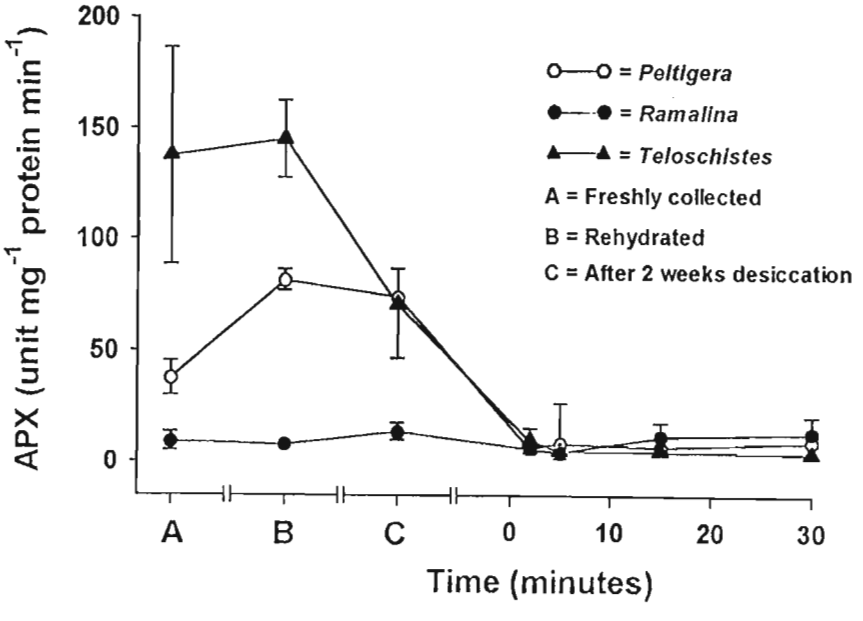



**Figure 6.1:** The effect of desiccation and rehydration on the activities of ascorbate peroxidase in *P. polydactyla*, *R. celastri* and *T. capensis*. In this and subsequent figures, points represent fitted values with 95% confidence limits calculated using the "Spline" program of Hunt and Parsons (1974). Overlapping error bars have been removed.

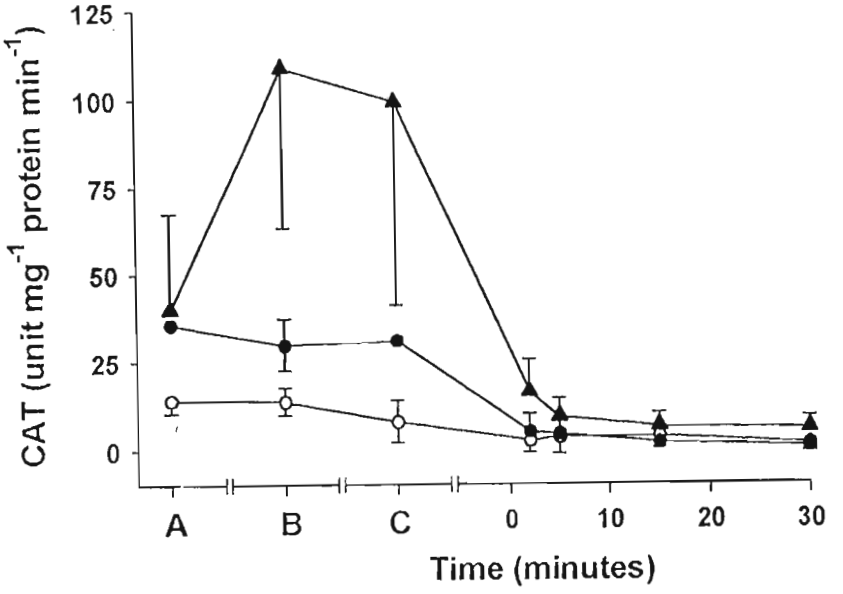



**Figure 6.2:** The effect of desiccation and rehydration on the activities of catalase in *P. polydactyla, R. celastri and T. capensis.* Legend as for Figure 6.1

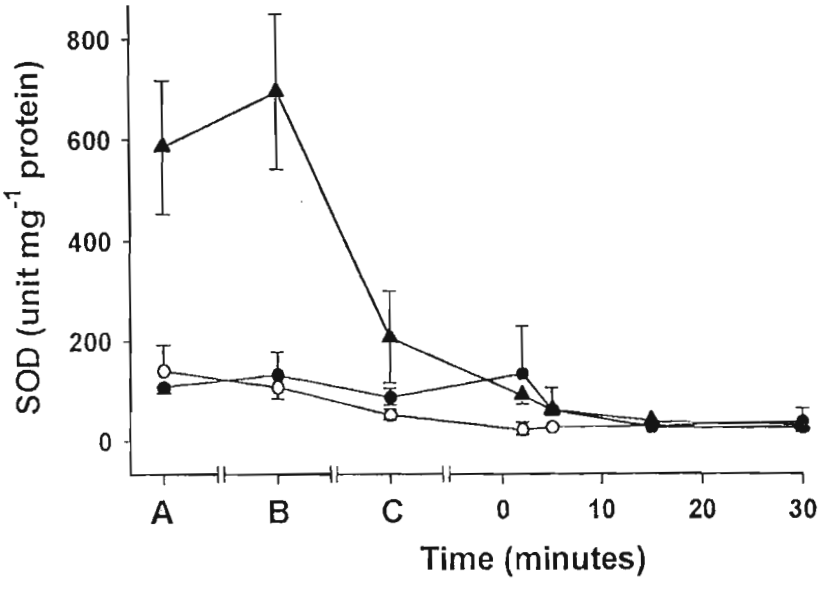



**Figure 6.3:** The effect of desiccation and rehydration on the superoxide dismutase activities of *P. polydactyla, R. celastri* and *T. capensis.* Legend as for Figure 6.1

## 6.4 Discussion

Results presented here clearly show that the activities of the enzymes APX, CAT and SOD are very low in lichens during rehydration following desiccation, even in species from xeric habitats. Enzymic antioxidants will be unavailable to remove ROS accumulating in lichen tissues as a result of desiccation (Smirnoff 1993). Taken with the limited data available in the literature, these results suggest that non-enzymic antioxidants are more important than enzymic antioxidants in protecting lichens from desiccation-induced ROS. Kranner (2002) provided good evidence that GSH in particular has an important role in desiccation tolerance in lichens. Two enzymes involved in GSH cycling, glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (Glc-6-PDH), became active within minutes of rehydration in lichens desiccated under similar conditions to those used in the present experiment. Calatayud *et al.* (1997) and Chakir and Jensen (1999) provided evidence that the xanthophyll cycle may reduce ROS formation during desiccation in lichens. High levels of non-photochemical quenching (NPQ) occurred in desiccation-stressed lichens, and in the study of Calatayud *et al.* (1997), increased NPQ was accompanied by the conversion of the xanthophyll cycle compounds violaxanthin to zeaxanthin. Operation of the xanthophyll cycle can prevent excess excitation energy from being passed to oxygen from photo-excited chlorophyll pigments, forming singlet oxygen, and also the production of superoxide and hydrogen peroxide at photosystem II (McKersie and Lesham 1994). In bryophytes, Seel *et al.* (1992a) found that the main difference between desiccation tolerant and sensitive mosses was more the ability of tolerant species to maintain levels of α-tocopherol and glutathione rather than maintain the activities of antioxidant enzymes. Interestingly, in the resurrection angiosperm *Myrothamnus flabellifolia* Sherwin and Farrant (1998) only found limited evidence that APX and SOD are involved in desiccation tolerance, while Kranner *et al.* (2002) has shown that non-enzymic antioxidants play an important role in tolerance in this species. Results presented here indicate that, at least when desiccation stress is severe, the enzymes APX, CAT and SOD play only a minor role in the removal of desiccation-induced ROS in lichens.

Possibly the enzymes APX, CAT and SOD are more involved in removing ROS produced during the normal metabolic functioning of poikilohydric plants. In plant and animal tissues, the production of ROS occurs naturally as a result of the side

reactions of metabolism. In a healthy, normally functioning organism a multilevel antioxidant system, including APX, CAT and SOD, keeps the levels of ROS at safe levels (Fridovich 1984). However, it is worth noting that unstressed material of the desert lichen *Teloschistes capensis* had higher activities of all three enzymes than the other species. Similarly, Seel *et al.* (1992a) found that unstressed material of the desiccation tolerant moss *Tortula ruralis* had much higher activities of SOD and CAT, but not APX, than the sensitive moss *Dicranella palustris*. Further work is needed, but a general correlation seems to exist between the stressfulness of the environment a cryptogam grows in and the levels of antioxidant enzymes. The most important conclusion of our results is that the activities of these enzymes during rehydration following desiccation are very low, even in the species from the xeric microhabitats. In lichens, APX, CAT and SOD may be more involved with the removal of ROS produced during processes like photosynthesis and respiration than the detoxification of ROS produced by desiccation.

# CHAPTER 7

## 7. The effect of rehydration on oxidative burst in response to stress in a moss *Atrichum androgynum*

### 7.1 Introduction

The production of ROS not only acts as toxic by-products of stress, but also as an important component of the plant defence response to pathogenic infections (Wojtaszek 1997, Allan and Fluhr 1997, Bolwell 1999, Bolwell *et al.* 1999). Within the last 15 years it has become apparent that the rapid production of extracellular ROS, often called the "oxidative burst", also plays an important role in plant defence against pathogenic infections. Here it is shown that in the moss *A. androgynum* an oxidative burst of $H_2O_2$ rather than $O_2^{\cdot-}$ occurs during rehydration following desiccation. Basic kinetics of oxidative burst is reported in this investigation, and preliminary findings are described on the nature of the enzymes responsible for $H_2O_2$ formation. Minibayeva and Beckett (2001) recently studied extracellular $O_2^{\cdot-}$ production in a range of bryophytes and lichens. Results showed that $O_2^{\cdot-}$ production varies greatly among different species, and is often related to the ability of a species to tolerate desiccation stress. Some desiccation sensitive lichens, hornworts and liverworts that occupy wet, shaded microhabitats produce $O_2^{\cdot-}$ at high rates, even when they are not stressed. Furthermore, some of these species display a powerful oxidative burst during rehydration following desiccation. In contrast, both unstressed and stressed desiccation tolerant lichens produce $O_2^{\cdot-}$ at extremely low rates. Among the lichens and bryophytes surveyed by Minibayeva and Beckett (2001), as a group, the mosses had surprisingly low rates of $O_2^{\cdot-}$ production. Even desiccation sensitive species from wet habitats produced almost no $O_2^{\cdot-}$ before or after desiccation stress. Asakawa (1998) suggested that mosses, like many lichens, have compounds with antibiotic and anti-fungal properties. However, the ecological significance of these compounds in bryophytes is poorly understood (During and Van Tooren, 1990). It was hypothesised that in mosses, desiccation may stimulate the production of ROS other than $O_2^{\cdot-}$. It is known that in some higher plants production of $H_2O_2$ but not $O_2^{\cdot-}$ occurs following a challenge by pathogens (Wojtaszek 1997, Bolwell *et al.* 1998).

## 7.2 Materials and Methods

Mosses were desiccated by placing the stem segments in 2 X 5 cm specimen bottles in a desiccator over silica gel in a controlled environment chamber at 15°C and PPFR of 75 μmol photons $m^{-2}s^{-1}$ of continuous fluorescent light. Water contents at full turgor were determined by weighing thoroughly blotted material that had been stored in contact with wet filter paper for at least 24 h (turgid mass), then subtracting the dry mass, obtained by drying for 48 h at 80 °C.

### 7.2.1 Effect of light intensity on the rate of $H_2O_2$ production

The effect of light intensity on the rate of $H_2O_2$ production was determined by desiccating mosses for 16 h then rehydrating them in complete darkness, and at a PPFR of 10 μmol photons $m^{-2}s^{-1}$ and 150 μmol photons $m^{-2}s^{-1}$. Production of $H_2O_2$ was measured at selected 15 min intervals over which $H_2O_2$ production was measured. Results were expressed as μmol $g^{-1}$ dry mass $h^{-1}$. Rehydrating mosses at a PPFR of 10 μmol photons $m^{-2}s^{-1}$ significantly increased the rate of $H_2O_2$ production during the first 15 min of rehydration following desiccation (Figure 7.2), and therefore 10 μmol photons $m^{-2}s^{-1}$ was used in all subsequent experiments.

### 7.2.2 Effect of desiccation on the amount of $H_2O_2$ produced

To determine the maximum concentration of $H_2O_2$ production that can occur in rehydration solutions, mosses were desiccated for 16 h, rehydrated, and the amount of $H_2O_2$ present in the rehydration solutions of different, replicated, samples rehydrated for 0.25, 0.75, 1 and 4 h were determined. For this experiment only, results were expressed as μmol $g^{-1}$ dry mass.

### 7.2.3 Effect of desiccation time on the rate of $H_2O_2$ production

To determine the effect of desiccation time on the rate of $H_2O_2$ production during rehydration, mosses were desiccated for 2, 4, 8, 16, 24 and 32 h. Following desiccation, mosses were rehydrated immediately in 5 ml of distilled water, and the rate of $H_2O_2$ production was measured over selected 15 min intervals for 2.5 h. The rehydration solution was changed at the start and end of each 15 min interval over which $H_2O_2$ production was measured. In this, and all subsequent experiments, the rate of $H_2O_2$ production was expressed as μmol $g^{-1}$ dry mass $h^{-1}$.

### 7.2.4 Effect of Polyethylene glycol on the rate of $H_2O_2$ production

To test whether the moss produces $H_2O_2$ during desiccation, mosses were incubated in deionized distilled water for 15 min, then in polyethylene glycol (PEG) solution (600 g polyethylene glycol 6000 added to 1 l distilled water, water potential approximately -3.7 MPa was measured using thermocouple psychometry) for 4 h, and then in deionized distilled water for 45 min. The rate of $H_2O_2$ production was measured during selected 15 min intervals throughout, changing the solution at the start and end of each 15 min interval.

### 7.2.5 Effect of peroxidase inhibitor, a NAD(P)H-oxidase inhibitor and exogenously supplied reductants for peroxidase on the rate of $H_2O_2$ production

The effect of the peroxidase inhibitor azide (supplied as 1 mM $NaN_3$) and the flavoprotein inhibitor diphenylene iodonium (DPI; supplied at 0.25 mM; Sigma) on $H_2O_2$ production was measured during the first 15 min of rehydration following desiccation for 8 h and 32 h. Mosses were rehydrated in solutions containing the inhibitors. The effect on $H_2O_2$ production of exogenously supplied reductants was tested by desiccating mosses for 8 h and 32 h, and then rehydrating them in deionized distilled water, 0.1 mM cysteine or 0.5 mM NADH. Production of $H_2O_2$ was measured at selected 15 min intervals during the first 1 h of rehydration, changing the solution at the start and end of each 15 min interval.

### 7.2.6 Effect of pretreatment with abscisic acid on the rate of $H_2O_2$ production

The effect of ABA treatment on $H_2O_2$ production was determined as follows. Abscisic acid (± cis, trans; Sigma) was dissolved in a drop of 1 M NaOH, and the pH of the resulting solution adjusted to 5.6 with HCl. Mosses were pretreated by gently shaking them in 10 ml of 100 µM ABA or distilled water for 1 h, and then storing them hydrated for 3 d at 15°C and a PPFR of 75 µmol photons $m^{-2}s^{-1}$. They were then desiccated for 8 h and 32 h, and the rate of $H_2O_2$ production was measured over selected 15 min intervals during the first 60 min of rehydration, changing the solution at the start and end of each 15 min interval.

## 7.3 Results

Figure 7.1a illustrates the decline in RWC of *A. androgynum* during desiccation for varying periods of time. RWC declined following desiccation for 2, 4, 8 and 16 h of desiccation from a RWC of 1 to *c.* 0.65, 0.45, 0.2 and 0.1 respectively. However the RWC remained constant following further desiccation for 24 and 32 h (*c.* 0.1). Figure 7.1b and 7.1c illustrate the effects of desiccation on the physiological processes of *A. androgynum*. Desiccation for 8 h had little effect on photosynthesis, but after 16, 24 and 32 h the moss needed *c.* 1, 4 and 6 h respectively to recover net carbon fixation (Figure 7.1b). Similarly, while desiccation for 8 h had little effect on $K^+$ leakage, leakage progressively increased with increasing desiccation time (Figure 7.1c).

Desiccated *A. androgynum* displays an oxidative burst of $H_2O_2$ production during rehydration (Figure 7.2). The highest rates of $H_2O_2$ production occurred during the first 15 min of rehydration, and rates then progressively declined with time. Light significantly increased the size of the oxidative burst (Figure 7.2). Even a PPFD of 10 μmoles photon $m^{-2}s^{-1}$, well below the light compensation point for this species, increased $H_2O_2$ production. A PPFD of 150 μmoles photon $m^{-2}s^{-1}$, equivalent to the PPFD that saturates photosynthesis, increased $H_2O_2$ production, but slightly less than 10 μmoles photon $m^{-2}s^{-1}$, and rates of production rapidly declined following further rehydration. In all subsequent experiments plants were rehydrated at a PPFD of 10 μmoles photon $m^{-2}s^{-1}$.

Figure 7.3 illustrates the effect of time on the amount of $H_2O_2$ in the rehydration solution of *A. androgynum* previously desiccated for 16 h. In this experiment the concentration of $H_2O_2$ was measured in the rehydration solution of different samples rehydrated for various times. The rehydration solution was never changed. Undesiccated plants produced no $H_2O_2$, while in desiccated material the amount produced, increased to *c.* 0.8 μmol $g^{-1}$ dry mass in the first 30 min of rehydration. After 4 h the amount of $H_2O_2$ in the rehydration solution declined to *c.* 0.42 μmol $g^{-1}$ dry mass.

Figure 7.4 illustrates the effect of desiccation time on the rate of $H_2O_2$ production in *A. androgynum*. By contrast to the previous experiment, the rehydration solution was changed at the start and end of each 15 min interval over which $H_2O_2$

production was measured. While desiccation for 2 h did not stimulate $H_2O_2$ production, production during rehydration was stimulated when mosses were desiccated for 4 h or more to *c.* 0.8, 1.2, 1.6 and 1.8 μmol $g^{-1}$ dry mass $h^{-1}$ respectively (Figure 7.4). Rates of $H_2O_2$ production were highest in the first 15 min of rehydration and then progressively declined. The rate of $H_2O_2$ production during the first 15 min of rehydration progressively increased with increasing duration of desiccation.

Figure 7.5 illustrates the effect of desiccation for 4 h induced by PEG 6000 at -3.7 MPa on $H_2O_2$ production in *A. androgynum*. Rates of $H_2O_2$ production were very low during incubation in deionized distilled water, and then increased progressively for 1 h when material was transferred to PEG. During the next 3 h in PEG, rates remained almost constant, then rapidly declined when the moss was transferred back to deionized distilled water.

The presence of peroxidase inhibitor sodium azide ($NaN_3$) in the rehydration solution almost completely abolished the oxidative burst of *A. androgynum* desiccated for 8 h (Table 1). However, azide increased the rates of $H_2O_2$ production in material desiccated for 32 h. Addition of the reductants NADH and cysteine to the rehydration solutions considerably increased the rates of $H_2O_2$ production in *A. androgynum* desiccated for 8 and 32 h (Figure 7.6a, b).

Figure 7.7a, b illustrates the effect of ABA treatment on $H_2O_2$ production. At the start, the rate of $H_2O_2$ production in undesiccated material was *c.* 1.0 μmol $g^{-1}$ dry mass $h^{-1}$ and this did not change following 3 d storage after treatment with ABA or distilled water. However, the rate of $H_2O_2$ production in untreated material increased during the first 15 min of rehydration following desiccation for 8 (Figure 10a) and 32 h (Figure 10b) to *c.* 11 and 26 μmol $g^{-1}$ dry mass $h^{-1}$ respectively. In ABA treated material the rate of $H_2O_2$ production following desiccation for 8 and 32 h did not increase during rehydration.

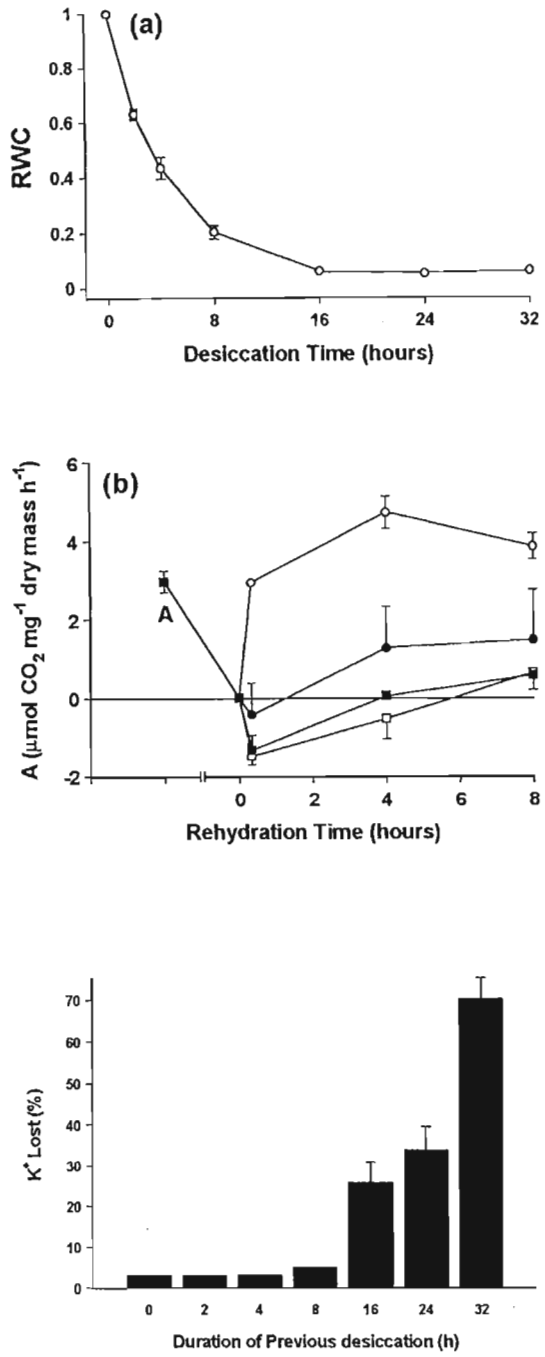



**Figure 7.1:** (a) The effect of desiccation time on the RWC (b) rate of photosynthesis, symbols: open circles, 8 h; closed circles, 16 h; closed squares, 24 h; open squares, 32 h and (c) K+ leakage during rehydration of the moss *Atrichum androgynum*. In this figure, and in all subsequent figures unless specified otherwise, points represent the means and the error bars represent the standard deviation, n = 5.

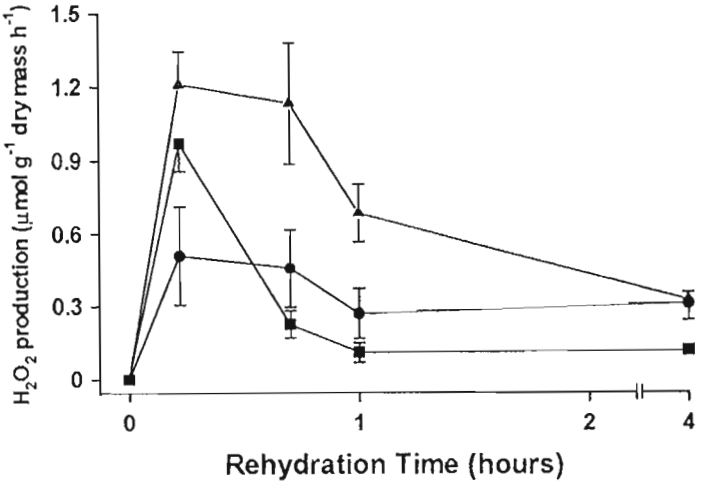



**Figure 7.2:** The effect of light intensity on the rate of $H_2O_2$ production during rehydration following desiccation for 16 h in the moss *Atrichum androgynum*. Symbols: Triangles, 10 µmoles photon $m^{-2}s^{-1}$; squares 150 µmoles photon $m^{-2}s^{-1}$ and circles, darkness.

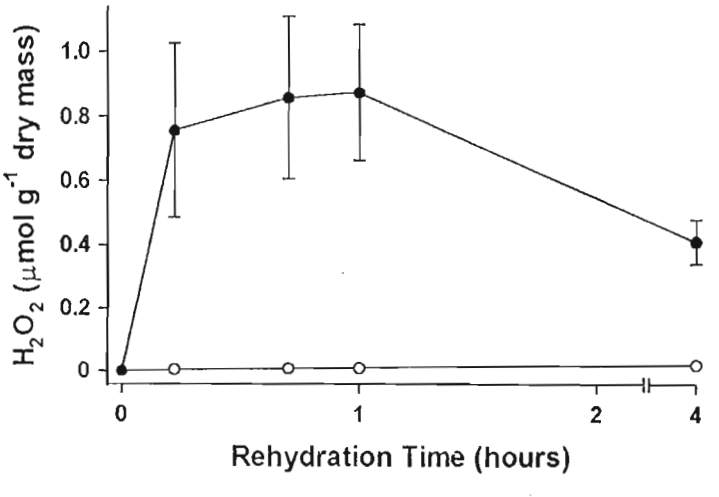



**Figure 7.3:** The effect of rehydration following desiccation for 16 h on the amount of $H_2O_2$ produced by the moss *Atrichum androgynum*. Symbols: solid circles, desiccated material and open circles, undesiccated material.

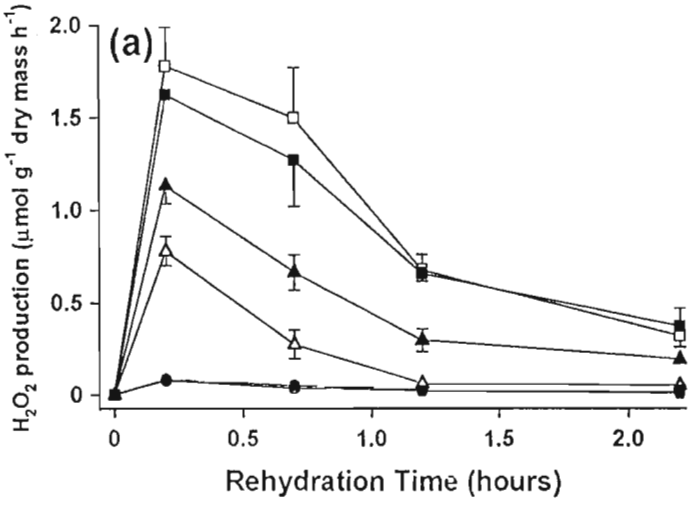



**Figure 7.4:** The effect of desiccation time on the rate of $H_2O_2$ production during rehydration following desiccation in the moss *Atrichum androgynum*. Symbols: open circles, undesiccated; closed circles, 2 h; open triangles, 4 h; closed triangles, 8 h; closed squares, 16 h; open squares, 32 h. 2, 4, 8 and 16 h represent desiccation period.

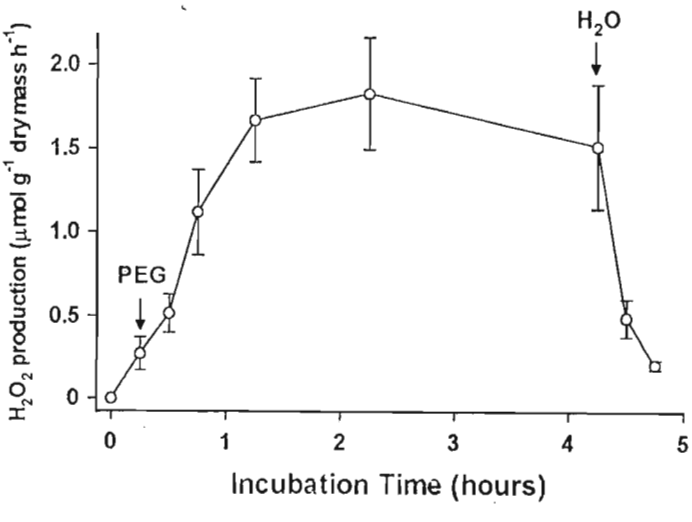



**Figure 7.5:** $H_2O_2$ production in the moss *Atrichum androgynum*, following incubation in 6000 g $l^{-1}$ PEG 6000 (B = -3.7 MPa) and after transfer back to distilled water.

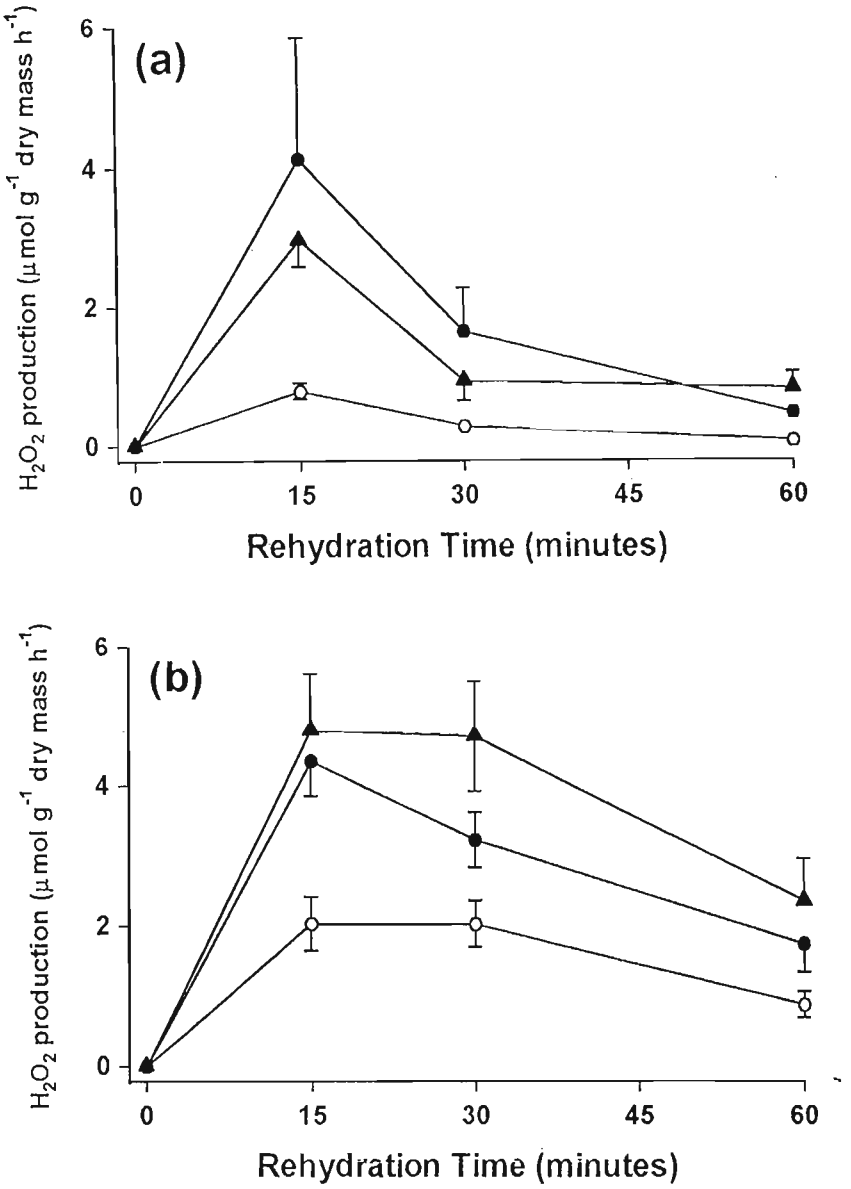



**Figure 7.6:** The effect of rehydration in 0.1 mM cysteine and 0.5 mM NADH follows desiccation for 8 h (a) and 32 h (b) on the rate of $H_2O_2$ production in the moss *Atrichum androgynum*. Symbols: open circles, distilled water, closed circles, NADH and closed triangles, cysteine.

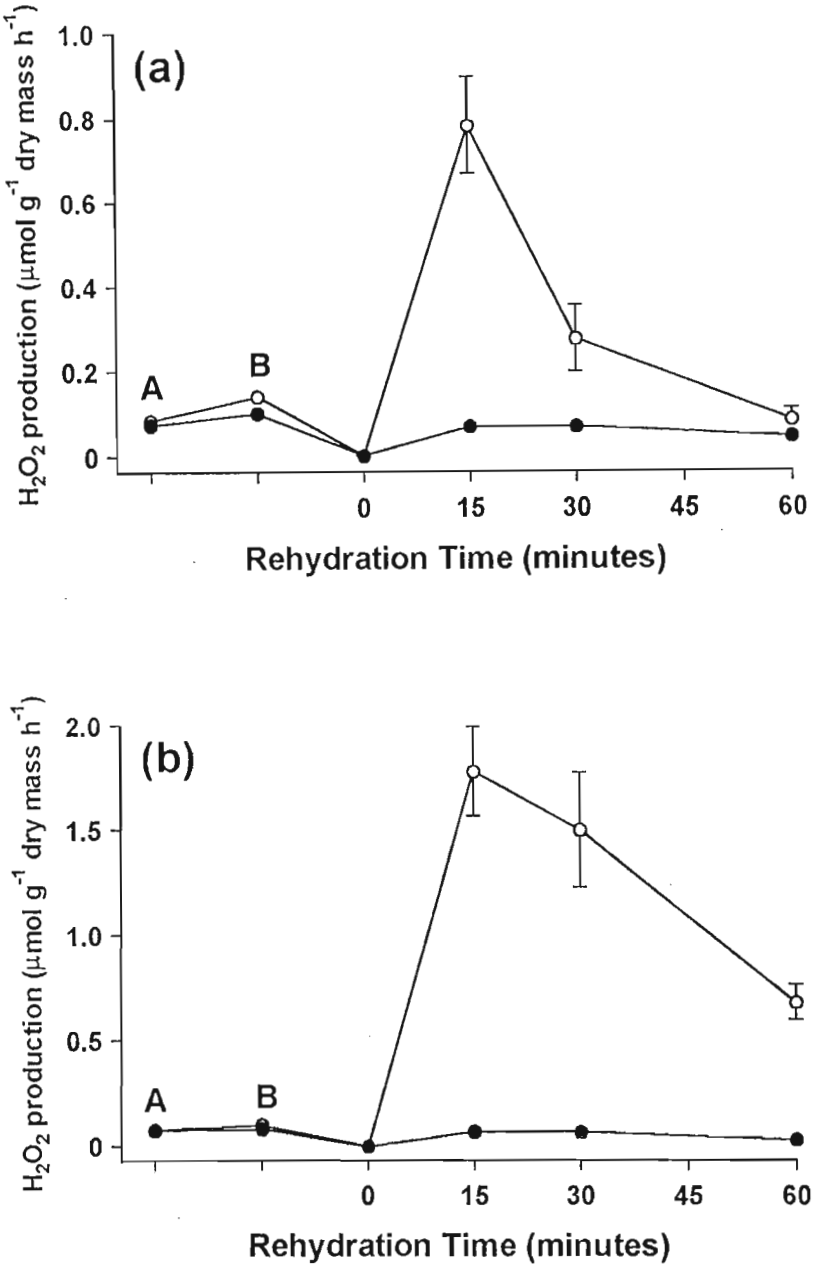



**Figure 7.7:** The effect of ABA treatment on the rate of $H_2O_2$ production following desiccation for 8 h (a) and 32 h (b) in the moss *Atrichum androgynum*. Symbols: open circles, distilled water and closed circles, ABA treated material. Letters: A, start of the experiment; B, after 3 d storage following treatment.

**Table 7.1:** The effect of catalase and enzyme inhibitors on the rate of $H_2O_2$ production during the first 15 min of rehydration following desiccation for 8 and 32 h in the moss *Atrichum androgynum*

| | $H_2O_2$ production (μmol $g^{-1}$ dry mass $h^{-1}$) | |
|---|---|---|
| Treatment | Desiccation for 8 h | Desiccation for 32 h |
| Control (undesiccated) | 0.97 ± 0.21 | 1.89 ± 0.55 |
| Catalase (500 units $ml^{-1}$) | 0.02 ± 0.00 | 0.13 ± 0.06 |
| $NaN_3$ (1 mM) | 0.12 ± 0.04 | 3.42 ± 1.43 |
| DPI (0.25 mM) | 0.91 ± 0.13 | 1.87 ± 0.33 |

## 7.4 Discussion

Results presented here clearly illustrate that the moss *A. androgynum* displays an oxidative burst of $H_2O_2$ during rehydration following desiccation. The burst of $H_2O_2$ appears to be an alternative to the desiccation induced $O_2^{\cdot-}$ based oxidative burst found by Minibayeva and Beckett (2001) in some lichens, thalloid liverworts and hornworts. One difference between these systems is that while some of the lichens and bryophytes surveyed by Minibayeva and Beckett (2001) produce extracellular $O_2^{\cdot-}$ even when unstressed, $H_2O_2$ production in *A. androgynum* is only stress-induced. Both field observations and laboratory experiments indicate that *A. androgynum* can survive moderately severe drying (Beckett 1999, 2001, Beckett and Hodinott 1997, Chapter 5). However, the severe mechanical and physiological damage that accompanies drying and subsequent rehydration will make the moss vulnerable to pathogen attack. Data from higher plant studies indicate that extracellular ROS production can help limit the spread of invading pathogens (Wojtaszek 1997, Bolwell 1999). Work reviewed by Murphy *et al.* (1998) suggests that 5 – 20 μM $H_2O_2$ can inhibit the germination of the spores of pathogenic fungi, while 0.05 – 0.10 nM $H_2O_2$ can inhibit bacterial growth. In the present study, concentrations of $H_2O_2$ ranging from 10 - 50 μM were produced when 100 mg fresh mass of plant material was rehydrated in 5 ml of distilled water. In field situations, even higher concentrations may be formed during rehydration, because the first rainfall of a precipitation event will form a thin film of water on previously desiccated mosses. While it is certainly possible that $H_2O_2$ may also act as a secondary signal that activates defence-related genes, stiffens cell walls, participate in the hypersensitive response or have other

functions (Lamb and Dixon 1997, Levine *et al.* 1994), the oxidative burst may also help *A. androgynum* to protect itself against pathogen attack.

The exact time course of the oxidative burst in *A. androgynum* varied slightly according to how the experiment was carried out. In one protocol (Figure 7.5) plants were rehydrated, and the amount of $H_2O_2$ present in the rehydration solutions determined in different samples rehydrated for progressively longer times, showing how concentrations in the rehydration solution change with time. More usually, however (e.g. Figure 7.4), the rate of $H_2O_2$ production was estimated by changing the rehydration solution at the start and end of each 15 min interval over which $H_2O_2$ production was measured. Results from the first protocol indicate that $H_2O_2$ concentrations do not continue to increase in the rehydration solutions. This is probably because production rates decline with time, and, in addition, $H_2O_2$ spontaneously breaks down. Furthermore, cell walls of the moss, like those of higher plants (Vanacker *et al.* 1998), probably contain endogenous catalases. Results from the second protocol indicate that rates of $H_2O_2$ production are initially high, but then decline rapidly with time. Using this protocol probably exaggerates the decline in the rate of $H_2O_2$ production, because changing the rehydration solutions probably causes the loss of secreted peroxidases (Minibayeva *et al.* 2001) and peroxidase reductants (Bolwell *et al.* 1999). However, both types of experiment indicate that the highest rates of $H_2O_2$ production occur during the first 15 min of rehydration, and then rapidly decline. While some fungal spores may take longer than 15 min to germinate, after this time membrane integrity and metabolic function will be at least partially restored, and the mosses will be less susceptible to pathogen attack.

Desiccating *A. androgynum* for longer times progressively increases rates of $H_2O_2$ production during rehydration (Figure 7.4b). It seems likely that the sources contributing to $H_2O_2$ release change as desiccation time increases. While desiccation for 8 h stimulates $H_2O_2$ production (Figure 7.4b), no $K^+$ release occurs, indicative of an absence of membrane damage (Figure 7.1b). Furthermore, photosynthesis rapidly recovers following rehydration (Figure 7.1c). Production of $H_2O_2$ after 8 h desiccation is azide sensitive (Table 7.1). However, desiccating the moss for longer than 8 h causes more metabolic damage (Figure 7.1b, c). More extensive damage is likely to stimulate $H_2O_2$-producing reactions located in chloroplasts, mitochondria and

peroxisomes. These reactions probably contribute to extracellular ROS release, because any $H_2O_2$ produced can easily penetrate cell membranes (Allan and Fluhr 1997). Production of $H_2O_2$ following desiccation for 32 h is azide insensitive (Table 7.1). Taken together, these results are consistent with the view that extracellular enzymes produce the $H_2O_2$ released by the moss during rehydration after 8 h desiccation, while the $H_2O_2$ released from mosses desiccated for longer originates in addition from a variety of intracellular sources.

Which enzymes are responsible for the release of $H_2O_2$ from *A. androgynum*? As discussed in the Introduction, in higher plants the main sources of ROS produced during the oxidative burst are enzymes localized at the external surface of the plant cells, specifically NAD(P)H-, or other oxidases, and/ or cell wall peroxidases (Bolwell *et al.* 1998, Murphy *et al.* 1998, Bolwell 1999). Sensitivity of ROS production to azide and insensitivity to DPI (Table 7.1) are characteristics of peroxidases (Bestwick *et al.* 1997). While sensitivity to $CN^-$ is also a characteristic of peroxidases, it was rather impossible to test this inhibitor because it interfered with the reagents used to assay $H_2O_2$ (probably the Fe). However, stimulation of $H_2O_2$ production by the reductants cysteine and NADH (Figure 7.6a, b) is also a characteristic of peroxidases. The precise mechanism of $H_2O_2$ production from peroxidases remains unclear. Bolwell *et al.* (1995) have suggested that at pH values of 7 - 9 and in the presence of reducing compounds such as cysteine or glutathione, peroxidase can catalyze the formation of $H_2O_2$ *in vitro*. The reaction is thought to involve Compound III peroxidase, the ferrous-hemeoxygen conjugate, which can be formed when reduced peroxidase binds molecular $O_2$. The release of $H_2O_2$ could result from an exchange of $H_2O$ for $H_2O_2$, or from a direct reduction of Compound III (see Murphy *et al.* 1998 for more details). This model would be consistent with the observation that desiccated or undesiccated *A. androgynum* produces almost no $O_2^{\cdot-}$ radicals (Minibayeva and Beckett 2001). Presumably, the advantage of $O_2^{\cdot-}$ radical generating systems is that can form the highly reactive hydroxyl radical. Hydroxyl ions can be formed by the reaction of $O_2^{\cdot-}$ (formed by a NAD(P)H-oxidase type enzyme in the plasma membrane or from peroxidases different to those present in *Atrichum*) with $H_2O_2$ (originating from the dismutation of $O_2^{\cdot-}$ or from peroxidases as outlined above). Chen and Schopfer (1999) speculate that this is a Fenton-type reaction catalysed by apoplastic peroxidase. These reactions do not appear to take

place in *A. androgynum*, which appears to produce a potentially less toxic oxidative burst comprising peroxidase-derived $H_2O_2$, but very little $O_2^{\cdot-}$.

Very low radiant flux densities stimulate $H_2O_2$ production in *A. androgynum* (Figure 7.7); densities lower even than those that correspond to the light compensation point of photosynthesis in this moss (Chapter 3). Interestingly, radiant flux densities that saturate photosynthesis do not increase the rate of production further, suggesting that light stimulation is not a simply result of the light providing extra energy to produce $H_2O_2$. Rather, light probably initiates signal transduction pathways that increase $H_2O_2$ production. The selective advantage for the light stimulation of $H_2O_2$ production is unclear. However, rehydrating *A. androgynum* in the light is more harmful than in the dark (Chapter 3). This phenomenon has also been observed in other bryophytes (for example Seel *et al.* 1992a, Proctor and Smirnoff 2000). The advantage of a light-stimulated oxidative burst could be that it will provide the moss with greater protection from pathogens that may be needed if plants are rehydated during daylight hours.

Most of the data presented here report on the production of $H_2O_2$ during rehydration following desiccation. In natural, field situations, the moss may also be stressed (and thus susceptible to pathogen attack) during desiccation. Measuring ROS production during desiccation is difficult because of the absence of superficial water. Presumably, under these conditions any extracellular ROS that the moss makes will accumulate in the apoplast. While it is possible to qualitatively visualize $H_2O_2$ in the apoplast using electron microscopy (for example, Bestwick *et al.* 1997), using a PEG solution $H_2O_2$ production could be quantified during desiccation. PEG at –3.7 MPa stimulates sustained production of $H_2O_2$ (Figure 5). From the pressure-volume curve in Beckett and Hodinott (1997) it was estimated that a water potential of –3.7 MPa corresponds to a RWC of *c.* 0.42. Interestingly, mosses only showed an oxidative burst during rehydration after they had been dried for 8 h, after which time they had reached a RWC of *c.* 0.2 (Figures 1 and 4b), corresponding to a water potential of *c.* –10 MPa. Data suggest that plants must dry to lower water potentials to induce $H_2O_2$ production during rehydration than during desiccation, than to cause production during an actual desiccation event, but clearly *A. androgynum* produces $H_2O_2$ during both desiccation and rehydration.

Pretreating *A. androgynum* with ABA for 1 h followed by storage for 3 d before desiccation almost completely suppressed the oxidative burst during rehydration (Figure 7.7). This is almost certain at least in part because ABA treatment increases desiccation tolerance in *A. androgynum* (Beckett 1999, 2001, Beckett *et al.* 2000, Chapter 3). However, it is also possible that ABA is directly involved in signal transduction pathways that control $H_2O_2$ production. Recently, Schopfer *et al.* (2001) demonstrated that ABA treatment suppresses ROS production in germinating radish seeds. However, Lin and Kao (2001) found that ABA treatment increased $H_2O_2$ production by the roots of rice seedling, and also reduced root growth. In addition to the rapid stimulation by light discussed above and stimulation by desiccation, $H_2O_2$ production also appears to be under the control of hormone activated signal transduction pathways.

In conclusion, work presented here clearly shows that the moss *A. androgynum* displays an oxidative burst of $H_2O_2$ during rehydration following desiccation. Based on experiments, with inhibitors and exogenously supplied reductants, the burst seems to originate from extracellular peroxidases. Severely stressed plants probably also release $H_2O_2$ from intracellular sites. In the short term, factors like low water potential and light control the size of the burst, while in the longer-term hormones like ABA appear to play a regulatory role. While extracellular ROS production is probably part of the general response of organisms to stress, it seems reasonable to suggest that ROS help protect *Atrichum* from pathogens when its tissues are damaged.

# CHAPTER 8

## 8.1 Conclusions and Recommendations

Among vascular plants, desiccation tolerance of vegetative tissues is restricted to the so-called 'resurrection plants' but dormant seeds and pollen grains can, with some exceptions, survive desiccation. Most organisms that possess desiccation tolerant vegetative tissues are cryptogams, especially members of algae, bryophytes and fungi. The majority of the latter are lichen-forming ascomycetes (Bewley 1979, Bewley and Krochko 1982, Oliver 1996, Oliver and Bewley 1997, Kranner and Lutzoni 1999, Oliver *et al.* 2000). Desiccation tolerant bryophytes and lichens are found world wide and inhabit a variety of habitats, most of which could, during some period of the year, be considered as extreme, either on a macro or micro level (Alpert and Oliver 2002). The contrasting environmental conditions in these macro habitats greatly influence much the metabolism and desiccation tolerance of these organisms. While desiccation tolerance may vary seasonally (Beckett and Hoddinott 1997), desiccation tolerance can be experimentally manipulated using hardening treatments, for example, partial dehydration or pretreatment with ABA. Understanding how such organisms can survive long periods of desiccation may help develop strategies by which drought tolerance can be genetically improved in higher plants. From the results presented in this investigation, it is evident that there is no simple explanation for the mechanism of desiccation tolerance in cryptogams. This was confirmed by determining a wide range of parameters, for example the efficiency of photochemical energy conversion by measuring chlorophyll fluorescence parameters, soluble and insoluble carbohydrates, and enzyme activities and an oxidative burst of hydrogen peroxide during desiccation-rehydration cycles.

### 8.1.1 Mechanisms of desiccation tolerance

Desiccation tolerant plants must meet three criteria to survive desiccation. These include the ability to limit the damage to a repairable level, maintain physiological integrity in the dried state, and mobilize upon repair mechanisms that effect restitution of damage suffered during desiccation and upon rehydration. This is evident from the increased NPQ observed during rehydration. In the moss *A. androgynum* and *R. celastri*, net $CO_2$ fixation recovered much more slowly than PSII activity. If

photophosphorylation recovers faster than C fixation then the plants are absorbing too much light. These conditions are conducive to the formation of ROS in the photosynthetic apparatus (Mckersie and Lesham 1994). Plants can prevent ROS formation in their chloroplasts by removing the energy they have absorbed harmlessly as heat using a process termed non-photochemical quenching (NPQ). Protection by NPQ may however reduce the efficiency of photosynthesis. In *P. polydactyla* PSII activity recovered faster than the $CO_2$ fixation. The rapid recovery of the PSII activity indicates that *P. polydactyla* is tolerant to desiccation conditions in the dark. The resistance of PSII to desiccation stress is of great ecophysiological importance because dehydration is very frequent in the field. However, decreased net photosynthesis and low $F_v$ / $F_m$ could be indicative of either an injurious effect or a regulatory process to rearrange PSII and consequently an expression of a photoprotective energy dissipation. Hardening treatments increased the rate of recovery of photosynthesis and PSII activity and also doubled NPQ in *A. androgynum*. On the contrary, in *P. polydactyla* and *R. celastri* hardening treatments did not significantly increase the recovery of PSII activity but appeared to slightly increase NPQ in *R. celastri* following desiccation. Nevertheless, the variability of the measurements in *R. celastri* was very high due to variation in factors that influence the metabolic activity of the plants e.g. age. In *P. polydactyla* NPQ however, was almost absent, may be not needed because there is very little or no induction of ROS. Protection of photosynthetic tissues from light during desiccation leads to a reduced oxidative damage by preventing absorption of excess excitation energy. In the case of mild desiccation in sensitive plants, exposure of the photosynthetic apparatus to excess excitation energy caused by inhibition of photosynthesis may lead to ROS formation inside the cells. As a result of this, detrimental effects such as photoinhibition and photooxidation could take place. Demming-Adams and co-workers revealed the importance of xanthophylls cycle pigments for scavenging activated oxygen in lichens. Reseachers showed that zeaxanthin frequently occurs in lichens with cyanobacterial photobionts that also contain remarkable amounts of ketocaretenoids (Adams *et al.* 1993). These carotenoids modulate the amount of radiationless dissipation of excess excitation energy, thus avoiding an effective oxidative damage. This modulation appears to be rapidly reversible upon rehydration. Electron flow during desiccation stress can be sustained by increasing consumption pathways other than the Calvin cycle. ABA appears to be involved in improving

desiccation tolerance in cryptogams in more ways than one. Exogenous application of ABA ensures rapid recovery upon rehydration. ABA probably protects the cells by preventing oxidative damage, thus allowing a rapid return to metabolic normality when tissues rehydrate.

### 8.2.2 Protection

As mentioned in chapter 1, among the metabolic changes that take place just prior to or during desiccation is the synthesis of sugars and proteins, these two have long been postulated to form the basis of a series of protective mechanisms that limit cellular damage (Bewley 1979, Leprince *et al.* 1993, Oliver and Bewley 1997). Soluble sugars have been widely implicated as being critical for desiccation tolerance in all plants including vegetative cells (Ingram and Bartels 1996, Scott 2000). Desiccation greatly enhanced the levels of soluble sugars in *R. celastri*, while in *P. polydactyla* remained almost constant, and they seemed to accumulate during desiccation in ABA treated *A. androgynum*. These sugars may protect membranes and macromolecules from irreparable conformational and oxidative damage during desiccation. Sucrose is the only free sugar available for cellular protection in desiccation tolerant mosses, including *T. ruraliformis* and *T. ruralis* (Bewley *et al.* 1978). Interestingly, Smirnoff (1992) reported no increase in soluble sugars in a range of mosses during desiccation.

Over the years it has become clear that the synthesis of antioxidants and enzymes involved in oxidative metabolism also play a critical role in cellular protection and desiccation tolerance (Alpert and Oliver 2002). The evidence in this investigation suggests that desiccation can increase the possibility of ROS formation. ROS have multiple effects in plants as they do in animals (Noctor and Foyer 1998). When they are produced in a controlled manner within specific compartments, ROS have key roles in plant metabolism and molecular biology. When they are produced in excess, the resultant uncontrolled oxidation leads to cellular damage and eventually death (Noctor and Foyer 1998). To prevent damage, yet allow beneficial functions of ROS to continue, the antioxidant defences must keep active oxygen under control. Results presented here clearly illustrate that activities of the antioxidant enzymes APX, CAT and SOD are considerably affected by desiccation in the moss *A. androgynum*, and in lichens, *P. polydactyla*, *R. celastri* and *T. capensis* and in subsequent rehydration in the moss *A. androgynum*. Slow drying greatly induced

SOD in *A. androgynum*. These results agree with the literature (Dhindsa and Matowe 1981, Werner *et al.* 1991, Seel *et al* 1991) that slow drying can induce SOD in mosses. Surprisingly, hardening treatments actually blocked the induction of SOD. Probably, during desiccation hardened mosses form less free radicals therefore, SOD is not induced because it is not needed. This suggests that ABA is not involved in the induction of free radical scavenging enzymes and that these enzymes may not be important in desiccation tolerance in *A. androgynum*. The failure for the induction could be that SOD is not active at low RWCs (Walters *et al.* 2002). In such cases other mechanisms of desiccation tolerance may be involved other than enzymic antioxidants e.g. tocopherols and ascorbate which may be more effective at low RWCs.

In all lichen species, including the *T. capensis* from an extremely xeric habitat, the activities of all enzymes remained at very low levels during rehydration, and were therefore unavailable to remove any ROS accumulating in lichen tissue as a result of desiccation stress. The enzymic antioxidants are more likely to be involved in removing ROS produced during moderate stress or the normal metabolic processes of lichens. Generally, environmental stresses increase the production of superoxide dismutase depending on species, stress period, age of plants, and above all on stress intensity (Navari-Izzo *et al.* 1996). Though both increase and decrease in the superoxide dismutase production have been observed (Sgherri *et al.* 1996), depending on equilibrium between the efficiency of thylakoid electron transport, the antioxidative capacity of membranes, and the leakage of electrons towards oxygen (Navari-Izzo *et al.* 1996) damage may or may not occur. During desiccation, normally encountered by plants, even if ROS formation is increased, the defence mechanisms either have sufficient capacity, or can be induced, with the result that damage does not become apparent. It is interesting that SOD activity increases in the moss, but not in the lichens following desiccation. Probably mosses and lichens use different strategies to cope with stressful conditions. In lichens other mechanisms such as non-enzymic antioxidants, and some secondary compounds may be responsible for the removal of oxygen free radicals.

### 8.3.3 Recovery from desiccation

When the moss *A. androgynum* was rapidly desiccated over silica gel for 16 h, photosynthetic recovery was incomplete even after 8 h rehydration. However, when slowly dried using air at 52% RH photosynthetic recovery was almost complete. The consequence of this may be that many bryophytes which experience rapid drying, when there is insufficient time to induce the protective measures required will suffer an irreparable damage. Cellular repair, as a component of desiccation tolerance mechanisms, has been defined in desiccation tolerant bryophytes (Proctor 1990). Desiccation tolerant bryophytes are thought to employ a mechanism for desiccation tolerance that represents the most primitive form expressed in land plants (Oliver *et al.* 2000). Unlike higher plants, bryophytes have little in the way of adaptations to retain water within the plant and, as a result, the internal water content of these plants rapidly equilibrates to the water potential of the environment (Proctor *et al.* 1998). A consequence of this is that many bryophytes experience drying rates that are extreme and therefore have insufficient time to induce and set in place protective measures (e.g. *Tortula ruralis*). It appears that these bryophytes have constitutive protection mechanisms for desiccation tolerance i.e. mechanisms that are always in place. On the contrary, because lichens and bryophytes dry so rapidly, they are usually found in under the forest canopy where conditions are effectively mesic and may function as shade plants, even in exposed, xeric habitats (Green and Lange 1994, Proctor 2000). The mechanism of desiccation tolerance that has evolved in these plants takes advantage of these conditions by being inducible. As the rate of water loss is relatively slow, there is time to establish the protective measures required, and the plant can thus survive a drying event. If water loss is too rapid, these plants succumb to the damaging effects of water loss and may not recover (Gaff 1989, Bewley and Oliver 1992, Oliver and Bewley 1997). These are the conditions where *A. androgynum* and *P. polydactyla* occur in nature, while *R. celastri* occurs on open tree branches. In the field situations, both *A. androgynum* and *P. polydactyla* may slowly dry, inducing ABA synthesis, which in turn activates signal transduction pathways that increase desiccation tolerance. Results show that *A. androgynum* and *P. polydactyla* possess inducible means of protection mechanism, and *R. celastri*, as in *T. ruralis* possess constitutive protection mechanism. The constitutive protection mechanism appears to be particularly effective in preventing damage to the

photosynthetic apparatus, as evidenced by the rapid recovery of PSII activity (Tuba *et al.* 1996, Csintalan *et al.* 1999, Protcor and Smirnoff 2001).

This investigation highlighted areas of desiccation tolerance, which require further investigation. Basic physiological processes during desiccation and rehydration require more extensive examination. Synthesis of dehydrin proteins in response to desiccation and rehydration in *A. androgynum*, *P. polydactyla* and *R. celastri*, as well as the specific biochemical pathways involved, should be determined. Endogenous levels of ABA should be analyzed in detail concerning their involvement in desiccation tolerance and hardening. The identification of different isozymes of SOD using polyacrylamide gel electrophoresis would be useful in making conclusions about the possible involvement of these enzymes to desiccation tolerance. Spatial distribution of detoxifying enzymes such as APX and SOD in the membranes could be extremely important in the establishment of desiccation tolerance in these mosses and lichens. A study on the effect and involvement of the non-enzymic antioxidants such as glutathione (GSH) is necessary. Moreover, whether secondary metabolites are produced in response to desiccation stress in lichens, and their role in desiccation tolerance should be detailed.

# CHAPTER 9

## 9. References

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**Appendix: 1A:**

**Summary of the analysis of variance of ABA and the rate of photosythetic recovery of *R. celastri* (15 d desiccation)**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. | s.e.d. | l.s.d |
|---|---|---|---|---|---|---|---|
| ABA | 1 | 0.040129 | 0.040129 | 14.86 | <.001*** | 0.01470 | 0.02970 |
| Time | 4 | 2.092778 | 0.523195 | 193.80 | <.001*** | 0.02324 | 0.04696 |
| ABA.Time | 4 | 0.032181 | 0.008045 | 2.98 | 0.030*** | 0.03286 | 0.06642 |
| Residual | 40 | 0.107988 | 0.002700 | | | | |
| Total | 49 | 2.273076 | | | | | |
| CV % | 13.1 | | | | | | |

Least significant differences of means (5% level)

**Appendix: 1B:**

**Summary of the analysis of variance of ABA and the rate of photosythetic recovery of *R. celastri* (30 d desiccation)**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. | s.e.d. | l.s.d |
|---|---|---|---|---|---|---|---|
| ABA | 1 | 0.032730 | 0.032730 | 6.80 | 0.012*** | 0.01791 | 0.03601 |
| Time | 5 | 1.457104 | 0.291421 | 60.56 | <.001*** | 0.03102 | 0.06238 |
| ABA.Time | 5 | 0.035408 | 0.007082 | 1.47 | 0.217$^{ns}$ | 0.04387 | 0.08821 |
| Residual | 48 | 0.230993 | 0.004812 | | | | |
| Total | 59 | 1.756236 | | | | | |
| CV % | 21.4 | | | | | | |

Least significant differences of means (5% level)

**Appendix: 2A:**

**Summary of the analysis of variance of partial dehydration (PD) and the rate of photosythetic recovery of *R. celastri* (15 d desiccation)**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. | s.e.d. | l.s.d |
|---|---|---|---|---|---|---|---|
| PD | 1 | 0.00049 | 0.00049 | 0.03 | 0.862 $^{n.s}$ | 0.0357 | 0.0722 |
| Time | 4 | 2.10790 | 0.52697 | 33.00 | <.001 $^{***}$ | 0.0565 | 0.1142 |
| PD.Time | 4 | 0.03737 | 0.00934 | 0.59 | 0.675 $^{n.s}$ | 0.0799 | 0.1615 |
| Residual | 40 | 0.63873 | 0.01597 | | | | |
| Total | 49 | 2.78449 | | | | | |
| CV % | 31.2 | | | | | | |

Least significant differences of means (5% level)

**Appendix: 2B:**

**Summary of the analysis of variance of partial dehydration (PD) and the rate of photosythetic recovery of *R. celastri* (30 d desiccation)**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. | s.e.d. | l.s.d |
|---|---|---|---|---|---|---|---|
| PD | 1 | 0.313255 | 0.313255 | 34.77 | <.001 $^{***}$ | 0.0245 | 0.0493 |
| Time | 5 | 1.816124 | 0.363225 | 40.31 | <.001 $^{***}$ | 0.0425 | 0.0854 |
| PD.Time | 5 | 0.114879 | 0.022976 | 2.55 | 0.040 $^{***}$ | 0.0600 | 0.1207 |
| Residual | 48 | 0.432506 | 0.009011 | | | | |
| Total | 59 | 2.676765 | | | | | |
| CV % | 26.5 | | | | | | |

Least significant differences of means (5% level)

**Appendix: 3A:**

**Summary of the analysis of variance of ABA and NPQ activity of *R. celastri* (15 d desiccation)**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. | s.e.d. | l.s.d |
|---|---|---|---|---|---|---|---|
| ABA | 1 | 0.046805 | 0.046805 | 13.09 | <.001*** | 0.01544 | 0.03105 |
| Time | 5 | 0.988834 | 0.197767 | 55.29 | <.001*** | 0.02675 | 0.05378 |
| ABA.Time | 5 | 0.015072 | 0.003014 | 0.84 | 0.526 ns | 0.03782 | 0.07605 |
| Residual | 48 | 0.171685 | 0.003577 | | | | |
| Total | 59 | 1.222396 | | | | | |
| CV % | 21.7 | | | | | | |

Least significant differences of means (5% level)

**Appendix: 3B:**

**Summary of the analysis of variance of ABA and NPQ activity of *R. celastri* (30 d desiccation)**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. | s.e.d. | l.s.d |
|---|---|---|---|---|---|---|---|
| ABA | 1 | 0.056674 | 0.056674 | 35.22 | <.001*** | 0.01036 | 0.02083 |
| Time | 5 | 0.193115 | 0.038623 | 24.00 | <.001*** | 0.01794 | 0.03607 |
| ABA.Time | 5 | 0.033981 | 0.006796 | 4.22 | 0.003*** | 0.02537 | 0.05101 |
| Residual | 48 | 0.077243 | 0.001609 | | | | |
| Total | 59 | 0.361012 | | | | | |
| CV % | 32.7 | | | | | | |

Least significant differences of means (5% level)

**Appendix: 4A:**

**Summary of the analysis of variance of hardening treatments and SOD activity during desiccation in *A. androgynum***

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. | s.e.d. | l.s.d |
|---|---|---|---|---|---|---|---|
| ABA | 2 | 0.03066 | 0.01533 | 0.91 | 0.415 $^{ns}$ | 0.0475 | 0.0970 |
| Time | 4 | 1.37172 | 0.34293 | 20.26 | <.001 $^{***}$ | 0.0613 | 0.1252 |
| ABA.Time | 8 | 0.05471 | 0.00684 | 0.40 | 0.909 $^{ns}$ | 0.1062 | 0.2169 |
| Residual | 30 | 0.50771 | 0.01692 | | | | |
| Total | 44 | 1.96480 | | | | | |
| CV% | 7.5 | | | | | | |

Least significant differences of means (5% level)

**Appendix: 4B:**

**Summary of the analysis of variance of hardening treatments and SOD activity during rehydration in *A. androgynum***

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. | s.e.d. | l.s.d |
|---|---|---|---|---|---|---|---|
| ABA | 2 | 0.11022 | 0.05511 | 1.63 | 0.213 $^{ns}$ | 0.0671 | 0.1371 |
| Time | 4 | 1.52904 | 0.38226 | 11.31 | <.001 $^{***}$ | 0.0867 | 0.1770 |
| ABA.Time | 8 | 0.08285 | 0.01036 | 0.31 | 0.958 $^{ns}$ | 0.1501 | 0.3065 |
| Residual | 30 | 1.01372 | 0.03379 | | | | |
| Total | 44 | 2.73583 | | | | | |
| CV% | 11.7 | | | | | | |

Least significant differences of means (5% level)

**Appendix: 5A:**

**Summary of the analysis of variance of hardening treatments and CAT activity during desiccation in *A. androgynum***

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. | s.e.d. | l.s.d |
|---|---|---|---|---|---|---|---|
| ABA | 2 | 0.00574 | 0.00287 | 0.16 | 0.849 $^{ns}$ | 0.0482 | 0.0985 |
| Time | 4 | 0.47758 | 0.11939 | 6.85 | <.001 $^{***}$ | 0.0622 | 0.1271 |
| ABA.Time | 8 | 0.11299 | 0.01412 | 0.81 | 0.599 $^{ns}$ | 0.1078 | 0.2202 |
| Residual | 30 | 0.52303 | 0.01743 | | | | |
| Total | 44 | 1.11934 | | | | | |
| CV% | 8.2 | | | | | | |

Least significant differences of means (5% level)

**Appendix: 5B:**

**Summary of the analysis of variance of hardening treatments and CAT activity during rehydration in *A. androgynum***

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. | s.e.d. | l.s.d |
|---|---|---|---|---|---|---|---|
| ABA | 2 | 0.008793 | 0.004397 | 1.63 | 0.213 $^{ns}$ | 0.01896 | 0.03871 |
| Time | 4 | 0.017776 | 0.004444 | 1.65 | 0.188 $^{ns}$ | 0.02447 | 0.04998 |
| ABA.Time | 8 | 0.004416 | 0.000552 | 0.20 | 0.988 $^{ns}$ | 0.04239 | 0.08657 |
| Residual | 30 | 0.080849 | 0.002695 | | | | |
| Total | 44 | 0.111834 | | | | | |
| CV% | 3.5 | | | | | | |

Least significant differences of means (5% level)

**Appendix: 6**

**Summary of the analysis of variance of partial dehydration and respiration of *P. polydactyla* (15 d desiccation)**

| Source of variation | d.f. | s.s. | m.s. | v.r. | F pr. | s.e.d. | l.s.d |
|---|---|---|---|---|---|---|---|
| ABA | 2 | 0.04528 | 0.02264 | 2.11 | 0.132 $^{ns}$ | 0.0328 | 0.0659 |
| Time | 3 | 0.22948 | 0.07649 | 7.13 | <.001 $^{***}$ | 0.0378 | 0.0761 |
| ABA.Time | 6 | 0.20603 | 0.03434 | 3.20 | 0.010 $^{***}$ | 0.0655 | 0.1317 |
| Residual | 48 | 0.51504 | 0.01073 | | | | |
| Total | 59 | 0.99583 | | | | | |
| CV % | 32.1 | | | | | | |

Least significant differences of means (5% level)