Universidade de Lisboa
Faculdade de Ciências
Departamento de Biologia Vegetal

Copping with Extreme Dehydration: A Physiological, Biochemical and Molecular Study on the Aquatic Bryophyte *Fontinalis antipyretica*

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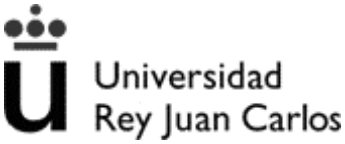

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Publications

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Submissions

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“I am the resurrection.
Anyone who believes in me,
even though that person dies, will live,
and whoever lives and believes in me
will never die.”

John 11, 25-26

Table of Contents

List of Abbreviations

2DE	Two-dimensional electrophoresis
A	Net photosynthesis
ABA	Abscisic acid
Asc	Ascorbic acid
ANOVA	Analysis of variance
APS	Ammonium persulfate
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine 5'-triphosphate synthase
b6f	Cytochrome b6f
Ca; Ca^{2+}	Calcium; calcium cation
$Ca(NO_3)_2$	Calcium nitrate
CHP	Cumene hydroperoxide
CMP	Chloramphenicol
CO_2	Carbon dioxide
CsCl	Caesium chloride
d.f.	Degrees of freedom
DAB	Diaminobenzidine
DCF	2,7-dichlorofluorescein
$DCFH_2$	2,7-dichlorodihydrofluorescein
$DCFH_2$-DA	2,7-dichlorodihydrofluorescein diacetate
DNA	Deoxyribonucleic acid
DT	Desiccation tolerance; desiccation tolerant
DTT	Dithiothreitol
DW	Dry weight
ELIPs	Early light-inducible proteins
ESR	Electron spin resonance
F_0	Dark-adapted minimum fluorescence yield
F_0'	Light-adapted minimum fluorescence yield
FDT	Full desiccation tolerance
F_m	Dark-adapted maximum fluorescence yield

F_m'	Light-adapted maximum fluorescence yield
F_s or F_t	Steady-state value of fluorescence before saturating light pulse
FT	Full turgor
F_v	Difference between F_m and F_0
F_v/F_m	Maximum photochemical efficiency of PSII reaction centres of dark-adapted leaves
FTW	Full turgor weight
FW	Fresh weight
GAPDH	Glyceraldeyde-3-phosphate dehydrogenase
GLO	Glycolate oxidase
GR	Glutathione reductase
GSSG	Oxidized glutathione
h	Hour
H_2O	Water
H_2O_2	Hydrogen peroxide
HCl	Hydrogen chloride
HSP	Heat shock proteins
IEF	Isoelectric focusing
IRGA	Infra-red gas analyser
K; K^+	Potassium; potassium cation
K_2SO_4	Potassium sulphate
$KC_2H_3O_2$	Potassium acetate
KCN	Potassium cyanide
$KHCO_3$	Potassium bicarbonate
$LaCl_3$	Lanthanum (III) chloride
LEA	Late embryogenesis abundant proteins
MALDI	Matrix assisted laser desorption ionization
MDA	Malonyldialdehyde
MDT	Modified desiccation tolerance
Mg; Mg^{2+}	Magnesium; magnesium cation
min	Minute
mRNA	Messenger ribonucleic acid

MS	Mass spectroscopy
N_2	Molecular nitrogen
n.d.	No data
NDPK1	Nucleoside diphosphate kinase 1
NH_4HCO_3	Ammonium bicarbonate
NH_4NO_3	Ammonium nitrate
$NiCl_2$	Nickel (II) chloride
NO	Nitric oxide
NPQ	Non-photochemical quenching coefficient
O_2	Molecular oxygen
$O_2^{\cdot -}$	Superoxide radical
OH·	Hydroxyl radical
$ONOO^-$	Peroxynitrite ion
P	Levels of significance
PAGE	Polyacrylamide gel electrophoresis
PAR	Photosynthetic active radiation
PDA	Piperazin di-acrylamide
pI	Isoelectric point
PSI	Photosystem I
PSII	Photosystem II
PV	Pressure-volume curve
qP	Photochemical quenching coefficient
r	Pearson correlation coefficient
R	Oxygen consumption in the dark
RH	Relative humidity
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rubisco	Ribulose-1,5-bisphosphatase carboxylase/oxygenase
RWC	Relative water content
RWD	Relative water deficit
s	Second
SD	Standard deviation

SDS	Sodium dodecyl sulphate
SE	Standard error
SHAM	Salicylhydroxamic acid
TBARS	Thiobarbituric acid reacting substances
TEMED	Tetramethylendiamine
TIM	Triosephosphate isomerase
TLP	Turgor loss point
TOF	Time of flight
v/v	Volume/volume
w/v	Weight/volume
WC	Water content
ε	Elastic modulus of cell walls
ψ	Water potential
Ψ_p	Turgor potential
$\psi_{\pi s}$	Osmotic potential at full turgor
$\psi_{\pi TLP}$	Osmotic potential at turgor loss point

Summary

Water is the most important element for life. During land invasion, the first plants had to face an extreme dry environment, undergoing desiccation, a process in which tissues virtually lose almost all water. Therefore, plants developed desiccation tolerance (DT) mechanisms through which they could experience the dry state and return to normal function upon rehydration. Nowadays, the organisms that have the ability to tolerate desiccation include members of different taxa such as microbes, lichens, bryophytes, vascular plants and animals. Nevertheless, DT is more frequent in lichens and bryophytes. Researchers have been closely studying DT mechanisms hoping to identify new genes that can be transferred through biotechnology to crop species, increasing their drought tolerance.

In the Mediterranean region, the aquatic species *Fontinalis antipyretica* is periodically exposed to desiccation, in intermittent streams that lose their water during the dry season. Field observations suggest DT mechanisms in this aquatic bryophyte. The main objective of this Thesis is to study DT through the combination of physiological, biochemical and molecular techniques in a bryophyte from a habitat not usually reported as prone to desiccation, comparing with bryophytes already studied and described as desiccation tolerant. Moreover, this integrated study aims to identify DT processes/mechanisms that are common across desiccation tolerant plant species.

Photosynthesis is a very sensitive indicator of desiccation. The measurement of the oxygen production rate, coupled with chlorophyll *a* fluorescence, allowed to follow the photosynthetic response through non-invasive techniques (**Chapter 2**). Examining the metabolic response to desiccation, we aimed to establish if *F. antipyretica* was DT and whether the recovery of this species was mainly determined by the extent or by the rate of dehydration, or by both. Our findings showed that the metabolic response of *F. antipyretica* to desiccation, both under field and laboratory conditions, is consistent with a DT pattern. It was concluded that dehydration must proceed slowly for the bryophyte to regain its pre-desiccation state following rehydration. This was further confirmed in field-desiccated samples which showed a similar recovery pattern as slow dehydration. This physiological study of a widely distributed aquatic bryophyte periodically subjected to desiccation contributed to improve our knowledge about dehydration rate in bryophyte survival.

The metabolic impairment observed during dehydration led to an increase in reactive oxygen species (ROS) production (**Chapter 3**). It was found that managing oxidative stress is one critical aspect for surviving desiccation. Although ROS production in response to desiccation/rehydration has been determined in terrestrial bryophytes it was not investigated in aquatic bryophytes. In addition, there were no published studies examining the impact of dehydration rate on ROS production in bryophytes previously subjected to desiccation and no information on the impact of drying rate on its production. Since it was determined that

dehydration rate is fundamental for surviving DT, we investigated whether this response included an oxidative burst sensitive to dehydration rate using an innovative approach combining ROS-specific probes and confocal microscopy. The response was a very high ROS production under fast dehydration whereas under slow dehydration was almost absent.

ROS react with cellular constituents, such as protein and lipids, leading to damage and, thus, affecting directly cell survival. After observing the effect of dehydration rate on ROS production, the next step was to test if the drying rate affected membrane damage (**Chapter 4**). We measured lipid peroxidation as well as cation dynamics and conductivity measurements to assess membrane damage and permeability. Since NO as also been associated with DT and a possible role in membrane protection, we measure and discuss the possible roles of its production and action. We also found that the increase in ROS and red autofluorescence can be used in future works in desiccation and drought stress as an indicator for detecting membrane damage and cell rupture.

During dehydration, cell water relations change occurring in some cases osmotic adjustment through sucrose accumulation. Sucrose has an important role in DT by preventing denaturation of macromolecules and slowing down damaging reactions with ROS. We investigated how contrasting (fast and slow) dehydration rates change cell water relations and sucrose content in an aquatic bryophyte and if those changes can explain the requirement of slow dehydration to induce DT (**Chapter 5**). The characteristics of the bryophytes cell walls appear to change under fast dehydration, allowing it to become more elastic which probably allows to maintain a functional metabolism to lower water potentials. Sucrose also increases under fast dehydration which can be an attempt to minimize damage when time for a more effective protection is not available.

The theory for DT was based in a constitutive protection mechanism that would allow the bryophyte to tolerate and protect the structures during desiccation, coupled with a repair-based mechanism upon rehydration which would repair damage that accumulated during the dried state. This would be controlled at the transcriptional level by accumulation of mRNA that would be activated during rehydration. However, recently the study of proteomes in bryophytes in response to dehydration suggests another approach to DT. We investigated the effect of fast and slow drying rates on the protein profiles, considering both dehydration and rehydration (**Chapter 6**). After fast dehydration, the proteome profiles of *F. antipyretica* are very similar to control. However, rehydration following fast dehydration leads to loss of almost all proteins, providing evidence that the bryophyte does not have enough time to prepare for desiccation under this dehydration regime. Nevertheless, under slow dehydration there are substantial changes in the proteome profile, both during dehydration and rehydration which might indicate an induction of DT mechanisms under these circumstances. The development of desiccation tolerance mechanisms that involved the accumulation of specific proteins during cycles of dehydration/rehydration allowed land colonization by early

bryophyte ancestors. The basis for such tolerance relies on common patterns of protein expression and metabolic adjustments which are very similar even in bryophytes from very distinct habitats. During dehydration, photosynthesis shuts down, high levels of soluble sugars occur in the cytoplasm, defence proteins increase, cytoskeleton is disassembled and sugar metabolism enzymes are up-regulated. After rehydration, photosynthesis restart, cytoskeleton is re-assembled, high levels of: soluble sugars, sugar metabolism enzymes and defence proteins are maintained.

The main conclusion suggested by this work is that DT at the cellular level, namely at the level of the molecular mechanisms, is similar among bryophytes independently of their preferred habitat. Furthermore, it states that DT is induced by slow dehydration rate being eventually controlled to some point by the morphology, being the determinant factor in the adaptation of bryophytes to each habitat and desiccation conditions.

Keywords: aquatic bryophytes, chlorophyll *a* fluorescence, desiccation tolerance, electrolyte leakage, *Fontinalis*, membrane damage, oxidative stress, photosynthesis, reactive oxygen species

Resumo

A água é o elemento mais importante para a Vida na Terra. Durante a ocupação do meio terrestre, as primeiras plantas enfrentaram um ambiente extremamente árido levando-as à dessecação, um estado no qual os tecidos perdem praticamente toda a água. Desta forma, foram-se desenvolvendo mecanismos de tolerância à dessecação (DT, do inglês *desiccation tolerance*) nas plantas, graças aos quais estas podem submeter-se ao estado de dessecação e regressar a um funcionamento normal após a reidratação. Atualmente, os organismos que apresentam DT incluem membros de taxa muito distintos, tais como micróbios, briófitos, líquenes, plantas vasculares e animais. No entanto, a DT é mais frequente nos líquenes e nos briófitos. Os investigadores têm estudado os mecanismos de DT na esperança de descobrir novos genes que possam ser transferidos por meio da biotecnologia para espécies vegetais de interesse agrícola, aumentando sua tolerância à seca.

Na região do Mediterrâneo, a espécie aquática de briófito *Fontinalis antipyretica* é periodicamente exposta à dessecação, em riachos que perdem a água durante a estação seca. Observações no campo sugerem mecanismos de DT neste briófito aquático. O objetivo principal desta Tese é estudar a DT através da combinação de técnicas fisiológicas, bioquímicas e moleculares num briófito proveniente de um habitat que normalmente não está sujeito à dessecação, comparando com briófitos já estudados e descritos como tolerantes à dessecação. Além disso, este estudo integrado pretende identificar processos/mecanismos de DT que sejam comuns nas diversas espécies vegetais tolerantes à dessecação.

A fotossíntese é um indicador muito sensível à dessecação. A resposta fotossintética foi seguida por meio de técnicas não-invasivas através da medição da taxa de produção de oxigénio, acoplado com a fluorescência da clorofila *a* (**Capítulo 2**), do briófito *F. antipyretica* de modo a determinar o grau de DT. Examinou-se a resposta metabólica à dessecação e de que forma ocorre a recuperação, se é determinada principalmente pela extensão ou a taxa de desidratação, ou por ambas. Os nossos dados mostraram que a resposta metabólica de *F. antipyretica* à dessecação, tanto sob condições de campo como em laboratório, é consistente com um padrão de DT. No entanto, a desidratação deve desenrolar-se lentamente para o briófito recuperar totalmente o seu estado inicial após reidratação. Isto foi confirmado em amostras dessecadas no campo as quais possuem um padrão de recuperação semelhante ao das amostras desidratadas lentamente. Este estudo fisiológico de um briófito aquático com distribuição global contribuiu para melhorar o nosso conhecimento sobre o papel desempenhado pela taxa de dessecação na sobrevivência dos briófitos.

Durante a desidratação, o normal funcionamento metabólico fica comprometido, originando um aumento do stresse oxidativo, especialmente na produção de espécies reativas de oxigénio (ROS, do inglês *reactive oxygen species*) (**Capítulo 3**). Desta forma, a manutenção de níveis aceitáveis de stresse oxidativo que permitam o funcionamento celular é um aspeto crítico na sobrevivência à dessecação. Embora a produção de ROS em resposta à dessecação / reidratação tenha sido observada em briófitos terrestres, o mesmo não sucedeu em briófitos aquáticos. Além disso, não existiam estudos publicados que relacionassem o impacto da taxa de desidratação na produção de ROS em briófitos previamente submetidos a dessecação. Uma vez que a taxa de desidratação é fundamental para sobreviver à dessecação, investigámos se essa resposta incluiu um *burst* oxidativo sensível a taxa de desidratação utilizando uma abordagem inovadora que combina sondas específicas para ROS e microscopia confocal. Após reidratação, observou-se uma elevada produção de ROS em amostras desidratadas rapidamente, enquanto nas desidratadas lentamente essa produção era quase ausente.

As ROS reagem com componentes celulares, tais como proteínas e lípidos, levando a danos celulares, afetando, desta forma, a sobrevivência celular. Depois de observar o efeito da velocidade de desidratação na produção de ROS, o passo seguinte foi testar como a taxa de desidratação afetava a estrutura e a estabilidade membranares (**Capítulo 4**). Através da quantificação de peroxidação lipídica, da dinâmica de catiões e das medições de condutividade, foram avaliados os danos e permeabilidade membranares. Dado que o óxido nítrico também foi associado com DT, tendo um possível papel na proteção da membrana, procedemos à sua quantificação e discutimos os possíveis papéis da sua produção e ação. Após a reidratação, observou-se um aumento de ROS e da autofluorescência vermelha os quais podem ser usados em futuros trabalhos na dessecação e stresse hídrico como um indicador de danos e rutura da membrana celular.

Durante a desidratação, as relações hídricas das células alteram-se ocorrendo, em alguns casos, ajustamento osmótico através da acumulação de sacarose. A sacarose tem um papel importante na DT, Procedeu-se à investigação de como duas taxas de desidratação contrastantes (rápida e lenta) alteram as relações hídricas e o teor de sacarose das células de briófitos aquáticos e se essas alterações podem explicar a necessidade de desidratação lenta para induzir DT (**Capítulo 5**). As características das paredes celulares briófitos parecem sofrer alterações durante a desidratação rápida, permitindo à parede tornar-se mais elástica e que provavelmente permite a manutenção de um metabolismo funcional até potenciais hídricos mais reduzidos. O aumento de sacarose durante a desidratação rápida poderá ser uma tentativa de minimizar os danos quando não existe tempo para estabelecer uma proteção mais efetiva.

A teoria de DT foi baseada num mecanismo de proteção constitutiva que permitiria ao briófito proteger as estruturas celulares durante a dessecação, juntamente com um

mecanismo baseado em reparação após reidratação, que permitiria reparar os danos que se acumularam durante o estado de dessecação. O controlo de síntese proteica seria efetuado ao nível transcripcional através da acumulação de mRNA sendo a síntese de proteínas ativada durante a reidratação. No entanto, recentemente, o estudo de proteomas em briófitos em resposta à desidratação sugere uma nova abordagem para a DT. Procedeu-se à investigação do efeito de taxas de desidratação lenta e rápida para observar os perfis proteicos, considerando ambos os processos de desidratação e reidratação (**Capítulo 6**). Após a desidratação rápida, os perfis de proteoma de *F. antipyretica* são muito semelhantes aos do controlo sem stresse. No entanto, aquando da reidratação após desidratação rápida observou-se a perda quase total das proteínas, fornecendo evidências de que o briófito não tem tempo suficiente para se preparar para a dessecação sob esta taxa de desidratação. Contudo, sob desidratação lenta ocorrem alterações substanciais nos perfis de proteoma, tanto durante a desidratação como na reidratação, o que pode indicar uma indução de mecanismos de DT nestas circunstâncias. O desenvolvimento de mecanismos de tolerância à dessecação, envolvendo a acumulação de proteínas específicas durante os ciclos de desidratação/reidratação, poderá ter permitido a colonização do meio terrestre pelos primeiros briófitos. A base para tal tolerância depende de padrões comuns de expressão de proteínas, bem como de ajustes metabólicos, os quais são muito semelhantes em briófitos de habitats distintos. Durante a desidratação, a fotossíntese é interrompida, ocorre o aumento dos níveis de açúcares solúveis, aumentam as proteínas de defesa, o citoesqueleto é desmontado e enzimas do metabolismo glicolítico aumentam. Após a reidratação, a fotossíntese reinicia, o citoesqueleto é remontado, e são mantidos elevados níveis de açúcares solúveis, enzimas do metabolismo glicolítico e proteínas de defesa.

A principal conclusão sugerida por este trabalho é de que a DT ao nível celular, nomeadamente ao nível dos mecanismos moleculares, é semelhante nos diferentes briófitos independentemente do seu habitat. Além disso, estabelece que a DT é induzida por desidratação lenta sendo esta controlada até certo ponto pela morfologia do briófito, tornando-se um fator determinante na adaptação dos briófitos a cada habitat e às condições de dessecação.

Palavras-chave: briófitos aquáticos, danos membranares, espécies reativas de oxigénio, fluorescência da clorofila *a*, *Fontinalis*, fotossíntese, permeabilidade membranar, stresse oxidativo, tolerância à dessecação

Chapter 1

Introduction

1. Introduction

1.1. Desiccation tolerance

Water is a scarce resource in numerous regions of the world resulting in changes in plant distribution (Maestre *et al.*, 2012). It is the most important element for life, being essential for cellular metabolism. One of the major challenges that living organisms have to face is keeping water inside cells. Upon land invasion in the Devonian period (416-359 million years), the first plants had to face the extreme dryness of this environment which would desiccate any organism (Kenrick and Crane, 1997). Desiccation is the process in which tissues virtually lose almost all water, down to 0.1 g H_2O g^{-1} DW (Alpert, 2005). Therefore, desiccation tolerance (DT) can be defined as going to the desiccated state and returning to normal function upon rehydration (Bewley, 1979). This definition can be broadened according to context, applying DT as the ability to survive desiccation. Desiccation tolerance was a requirement for land transition, as recent phylogenetic studies on land plants suggest (Oliver *et al.*, 2000). On land, plant evolution developed mainly two strategies to survive water deficit: (1) allow cells to lose practically all water equilibrating with the atmosphere (poikilohydric organisms); or (2) prevent water loss to the atmosphere, by waxy layers, internal water conduction through specialized conducting vessels or pores that open or close regulating water content (homeohydric organisms) (Proctor and Tuba, 2002). It appears that both poikilohydric and homeohydric possess a common ancestral that developed DT (Oliver *et al.*, 2000). This common ancestral displayed DT to occupy and thrive in the intertidal space, optimizing the carbon absorption and light capture for photosynthesis. Some species evolved through lines that kept these DT mechanisms functional while others lost the ability to tolerate desiccation in the vegetative state. However, this plants keep the DT potential present in their genome, mainly through seeds (Tweddle *et al.*, 2003), pollen and spores (Hoekstra, 2002) but not anymore in the vegetative tissue.

Nowadays, the organisms that have the ability to dry almost completely and resume normal metabolism upon rehydration are present across all life kingdoms (Crowe *et al.*, 1992), such as microbes (e.g. the cyanobacteria *Nostoc commune* and eukaryotic green algae *Trebouxia erici* Ahmadjian), lichens (e.g. *Ramalina maciformis* [Delise] Bory), bryophytes (e.g. *Syntrichia ruralis* [Hedw.] F. Weber & D. Mohr), vascular plants (e.g. *Craterostigma plantagineum* Hochst.) and animals (e.g. tardigrades, rotifers and nematodes) (Potts, 1994; Proctor, 2001; Alpert, 2005; Kranner *et al.*, 2008; Gasulla *et al.*, 2009; Bartels *et al.*, 2011). In vascular plants, vegetative DT

was virtually lost, despite distinct groups evolved, in several occasions, the ability to tolerate desiccation (Gaff and Oliver, 2013). Amongst flowering vascular plants there are about 300 DT species (Porembski and Barthlott, 2000), that can remain in the desiccated state for several years. However, this corresponds to 0.1% of the known species.

Researchers have been studying the mechanisms behind DT hoping to achieve new products that can be transferred through biotechnology to crop species, increasing their drought tolerance. At present day, genes involved in DT were present in all studied plants, although vegetative DT is rare in vascular plants (Bartels and Salamini, 2001). Yet, it is relatively common in two major groups of organisms, bryophytes and lichens (Kappen and Valladares, 1999). Due to this variety of groups with DT, desiccation-tolerant organisms were broadly classified in two categories by some authors: the “full desiccation tolerant” (FDT) species, and the “modified desiccation tolerant” (MDT) species (Bewley, 1979; Oliver and Bewley, 1997). The first group includes the bryophyte *S. ruralis*, withstanding the loss of almost all tissue water in few minutes and surviving upon rehydration. In the second group, the most studied representative is the resurrection vascular plant *C. plantagineum*, which needs slow dehydration (at least 24 hours) to survive desiccation, possessing morphological and physiological mechanisms to retard water loss, and induce protective mechanisms throughout the drying process (Wood, 2007).

More recently DT is starting to be viewed by several authors not as well-defined types but as a continuous range of variation of DT, since bryophytes and lichens can be found in almost every known habitat and is expected the same diversity in their DT (Proctor *et al.*, 2007a; Veerman *et al.*, 2007). However, since lichens are a symbiotic association between a mycobiont and a photobiont (algae or cyanobacteria) the interactions may be more complex than those found in bryophytes. On the other hand, bryophytes have a more recent common ancestral to vascular plants (Oliver *et al.*, 2000) and any mechanisms that are identified would be easier to replicate in vascular plants through biotechnology techniques, thus justifying their study. Generally, bryophytes possess one-cell thick leaves that remain turgid as long as an external water layer is present. Once this layer disappears, they rapidly equilibrate with the surrounding atmosphere since they lack the structures and/or substances to prevent water loss. In bryophytes, microclimate is of upmost importance in terms of its physiology and ecology (Proctor, 1981) and several parameters such as gradients of humidity, temperature, light exposure and wind speed will determine the rate of dehydration (Dilks and Proctor, 1976; Proctor, 1981; Alpert and Oechel, 1987).

Bryophytes have also other strategies to decrease the rate of water loss. Surface tension is one of the most important forces, being gravity negligible (Oliver *et al.*, 2000). Therefore, morphological characteristics (life forms), such as compact cushions or thick mat growth forms, will also have a crucial role to decrease dehydration rate. Moreover, several parameters are important and have to be accounted for, in order to correctly define DT. These include time in the desiccated state, recovery rate, the number and/or frequency of dry/wet cycles, and the substrate upon they grow that can held different water contents (Alpert, 1988; Tuba *et al.*, 1996; Hernandez-Garcia *et al.*, 1999; Zotz *et al.*, 2000; Cleavitt, 2002). In addition, previous desiccation and/or partial desiccation events can alter DT, leading to hardening (Höfler, 1946; Abel, 1956; Dilks and Proctor, 1976; Schonbeck and Bewley, 1981a; Oliver *et al.*, 1998). This implies an observation in a different space scale that is normally applied to vascular plants, and a multitude of variables that needs to be assessed to correctly determine DT in poikilohydric organisms.

Field observations usually refer forest-floor and mesic grassland bryophytes as exhibiting some DT, being damaged by fast or prolonged desiccation (Abel, 1956). Forest epiphytic bryophytes can tolerate fast, prolonged desiccation and frequent dry/wet cycles (Hosokawa and Kubota, 1957; Tobiessen *et al.*, 1979; Franks and Bergstrom, 2000). The same can be observed in species of open, exposed habitats (e.g. rock surfaces) like *S. ruralis* (Bewley *et al.*, 1978). It is expected in these bryophytes to have the highest DT levels as they face extremely low humidity and high radiance levels (Proctor *et al.*, 2007a). Therefore, the idea that ecology also has some correspondence to the physiology of the organism has led to the expectation that a desert bryophyte is more DT than a bryophyte from habitats with more moisture.

There are several works that tried to classify bryophyte species according to their different DT levels (Abel, 1956; Brown and Buck, 1979). Recently, Wood (2007) attempted to establish a protocol to approach the universality of DT in bryophytes in a more quantitative way, rather than only field observations. In this protocol, modulated chlorophyll *a* fluorescence, combined with different rates of dehydration and recovery times, was used to assess DT in the bryophyte. Most bryophytes can survive mild water stress but not a severe stress (Proctor *et al.*, 2007a). However, there is contradictory information such as the fact that semi-aquatic bryophytes like *Cinclidotus fontinaloides* (Hedw.) P. Beauv. and *Leskea polycarpa* Hedw. are referred as showing DT in the summer when water levels are low and become stranded above water (Dyer and Duckett, 1984). Thus, if a semi-aquatic bryophyte can survive out of water during the dry period and recover after being rewet, it must have some type of DT. However,

this has never been thoroughly explored in semi-aquatic or aquatic bryophytes as in the desert bryophyte *S. ruralis*, in which the physiological, biochemical, and cellular responses have received much attention (Bewley, 1979; Smirnoff, 1992; Oliver and Bewley, 1997; Oliver *et al.*, 2004).

1.2. Physiological, biochemical and molecular responses to desiccation

The effects observed in the physiological, biochemical and molecular responses resulting from either desiccation or rehydration are not easily separated. Most studies focus only in the events upon rehydration almost neglecting what happens during dehydration (Gwózdz *et al.*, 1973; Dilks and Proctor, 1974; Oliver, 1991; Proctor and Smirnoff, 2000; Oliver *et al.*, 2004). However, dehydration rate and duration of the desiccated state will have a high impact in recovery upon rehydration. For example, in *Plagiothecium undulatum* (Hedw.) Schimp., a prolonged period at low water potential increases damage but for *Racomitrium lanuginosum* (Hedw.) Brid., equilibration to low water potentials is best for bryophyte survival (Dilks and Proctor, 1974). Most bryophytes cope better with either full hydration or desiccation, rather than with an intermediate state (Proctor, 2001). Moreover, rehydrated samples show a net carbon loss that increases with desiccation time and, after a certain period, according to species, recovery will take longer and will be less complete due to progressive accumulation of cellular damage, reaching a point of no return that will lead to cell death (Hinshiri and Proctor, 1971). Therefore, this work will analyse both the dehydration and rehydration processes in order to have a clear and correct view of DT.

1.2.1. The drying process

The drying rate is a very important factor in the DT process, although for the more tolerant species it have been claimed to have little effect on recovery (Proctor, 2001). However, some authors showed that even a desert bryophyte like *S. ruralis* benefits from a slow drying rate, presenting less damage after fast drying (Schonbeck and Bewley, 1981b).

After 24 hours recovery following different drying rates provided through equilibration with atmosphere at different water potential (–41, –113, –218 and –412 MPa), the bryophytes *R. lanuginosum* and *S. ruralis* showed small differences in the chlorophyll *a* fluorescence parameter F_v/F_m (maximum quantum efficiency of photosystem II [PSII]) (Proctor, 2001), usually used as an indicator of plant stress. This indicates that the PSII structure remains intact through the dehydration/rehydration cycle and an apparent lack of response to dehydration rate. Moreover, they showed

worst performance after being equilibrated to higher water potentials (–9 to –22 MPa) (Proctor, 2001). These two species are from open, sun-exposed habitats with extreme water potentials scenarios either fully hydrated or with very low water potentials.

In the more shaded habitats like woodlands or north exposed mountain slopes, exposure to high frequent drying rates is less common. Nevertheless, it has been shown that DT can be established even in the less tolerant species through a process of hardening. This results from exposing the bryophytes to a less intense drying rate (e.g. 96% relative humidity [RH] for 24 hours) before a more intense one, although little is known about the underlying mechanisms. Höfler (1946) and Abel (1956) observed this process in a wide range of bryophytes, such as *Bryum capillare* Hedw., *Fissidens adianthoides* Hedw. or *Pohlia elongate* Hedw.. However, in the more sensitive (e.g. *Bryum pseudotriquetrum* [Hedw.] G. Gaertn., B. Mey. & Scherb. or *Mielichhoferia elongata* [Hoppe & Hornsch.] Nees & Hornsch.) and the more tolerant (*S. ruralis* or *Grimmia pulvinata* Bruch & Schimp.) the hardening effect was not so obvious.

During dehydration, primary metabolism declines as lower water potentials are reached and there are no differences regarding the DT level or the group of organisms (bryophytes, lichens or vascular plants) (Tuba *et al.*, 1996; Proctor, 2000; Dinakar *el al.*, 2012). Respiration appears to be more resistant to desiccation than photosynthesis, since oxygen consumption measurements were made at lower water potentials (Dilks and Proctor, 1979). Moreover, oxygen consumption presented steady-state values until 30-40% of the fresh weight. At this point, a high peak of oxygen consumption is reached and afterwards starts to decrease to zero when the bryophyte becomes desiccated. This pattern is similar both in DT (*S. ruralis*) and non-DT (*Cratoneuron filicinum* [Hedw.] Spruce) bryophytes (Krochko *et al.*, 1979).

Throughout desiccation, membranes remain intact, either in the cell, mitochondria or chloroplast (Platt *et al.*, 1994; Pressel *et al.*, 2009). However, the accumulation of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$) and the metabolic intermediate hydrogen peroxide (H_2O_2) will increase damage reacting with proteins and lipids. ROS are generated mainly in the chloroplast, but also in mitochondria, peroxisomes and plasma membrane, during the desiccated state (Scheibe and Beck, 2011). Recently, there has also been some discussion over the importance of cytoskeleton disassembly during dehydration in maintaining organelle and cell shape by association with membranes, increasing flexibility and allowing to withstand volume variations during the dehydration/rehydration cycle (Proctor *et al.*, 2007b; Wang *et al.*, 2009).

Late Embryogenesis Abundant (LEA) proteins accumulate in response to

abiotic stress, in particular dehydration. Their function is unclear but they may function as antioxidants and as membrane and proteins stabilisers (Tunnacliffe and Wise, 2007). This LEA accumulation has been observed both in vascular resurrection plants (Bartels and Salamini, 2001) and in *Physcomitrella patens* (Hedw.) Bruch & Schimp. (Wang *et al.*, 2009; Cui *et al.*, 2012). However, in *S. ruralis* apparently there is no protein synthesis during drying (Oliver, 1991) and LEA proteins synthesis seem to be rather rehydration induced (Oliver *et al.*, 2004). In this particular bryophyte species there is an accumulation of mRNA during dehydration that appear to be translated only during rehydration (Wood and Oliver, 1999). However, recently Wang *et al.* (2009) and Cui *et al.* (2012) presented evidence of protein differential expression during dehydration in bryophytes.

The increase of soluble sugars is one of the major metabolic changes during dehydration, being associated with DT (Alpert and Oliver, 2002; Buitink and Leprince, 2004). Substantial increase in soluble sugars changes the cytoplasm into a biological glass with high viscosity (vitrification) that is thought, combined with the increase of LEA proteins, to protect macromolecules and membranes by maintaining their structure intact (Buitink and Leprince, 2004; Goyal *et al.*, 2005; Shih *et al.*, 2010). It also slows-down the damaging reactions of proteins, lipids and DNA with ROS (Scheibe and Beck, 2011). In vascular resurrection plants, sucrose increases in response to rehydration (Buitink and Leprince, 2004) but in bryophytes the high sucrose content remains constant throughout the desiccation/rehydration cycle (Bewley *et al.*, 1978; Oldenhof *et al.*, 2006).

1.2.2. Recovery upon rehydration

Recovery from desiccation is not immediate. Recovery can be divided in two phases, an initial fast recovery phase (within a few minutes) and a second longer and slower recovery phase (from minutes to days). In the first phase, most metabolic systems return to its hydrated state through a physical process and depending on the damage degree will resume function (Proctor and Pence, 2002). The time for the second phase to be concluded depends essentially on damage level. This phase will determine the time for full recovery to be attained, due to the fact that the different metabolic systems have different recovery times. During rehydration, most metabolic pathways such as photosynthesis, respiration and protein synthesis return to its function, being membrane integrity also restored, as seen in *Anomodon viticulosus* (Hedw.) Hook. & Taylor, *R. lanuginosum* and *S. ruralis* (Gwózdz *et al.*, 1973; Oliver, 1991).

Chloroplast membranes seem to be rather independent of drying rate since, upon rehydration, F_v/F_m returns to normal values within minutes with apparent photosynthetic function recovered (Csintalan *et al.*, 1999). However, some proteins need to be repaired since the presence of the protein synthesis inhibitor chloramphenicol (CMP) leads to loss of recovery in the light, indicating a very important role of chloroplast-encoded protein synthesis in the first stage of recovery (Proctor and Smirnoff, 2000; Proctor, 2001). In addition, gas exchange measurements showed that positive net photosynthesis takes longer to recover than the photosystems (Hinshiri and Proctor, 1971; Dilks and Proctor, 1974, 1976; Tuba *et al.*, 1996). Since electrons keep reaching the photosystems but photosynthesis is not fully recovered, ROS are highly produced upon rehydration (Minibayeva and Beckett, 2001; Beckett *et al.*, 2004), increasing damage during the initial phase, especially at the chloroplast level. Additionally, this ROS burst can also act, to a certain level, as a defence against pathogenic fungi and bacteria that can attack cells upon rehydration (Minibayeva and Beckett, 2001).

Respiration resumes almost immediately upon rehydration at higher rates than the pre-desiccation dark respiration (Dilks and Proctor, 1976; Tuba *et al.*, 1996), probably due to other oxygen consuming metabolic systems that not only mitochondrial respiration. Moreover, with increasing dehydration rate and desiccation time, oxygen consumption also increased upon rehydration (Krochko *et al.*, 1979). *A. viticulosus* and *Rhytidiadelphus loreus* (Hedw.) Warnst., took 5–10 hours to return to steady state respiration following several days of desiccation (Dilks and Proctor, 1976), whereas *S. ruralis* took 2 hours after a few hours in the desiccated state (Tuba *et al.*, 1996). Therefore, resuming normal respiration rates will depend both on the rate of dehydration and time spent in the desiccated state.

Cells recover their form very rapidly after rehydration (from 30 seconds to one minute) (Glime, 2007). In the first moments, membrane leakage occurs. In the more DT species, this leakage is transient, probably due to lipid-phase transitions occurring in the plasma membrane (Crowe *et al.*, 1992). In the more sensitive species, this leakage is more substantial (Brown and Buck, 1979; Bewley and Krochko, 1982; Crowe *et al.*, 1992), eventually leading to complete loss of all intracellular content and cell death.

Protein synthesis is fully operational after 24 hours (Oliver, 1991). Although only novel proteins were found during the rehydration phase of *S. ruralis* (Oliver, 1991), recent works in *P. patens* (Frank *et al.*, 2005; Wang *et al.*, 2009; Cui *et al.*, 2012) have shown protein synthesis during dehydration, as well as, after rehydration. In the latter phase, there is an increase of LEA proteins and heat shock proteins (HSP) both

probably functioning as cell structure protection mechanisms (Cui *et al.*, 2012). On one hand, data indicates that LEA proteins may be able to stabilize membranes by establishing hydrogen bounds with macromolecules (Goyal *et al.*, 2005; Shih *et al.*, 2010). On the other hand, HSP have been correlated to DT in orthodox (survive drying and/or freezing) seeds (DeRocher and Vierling, 1994; Wehmeyer *et al.*, 1996), in *C. plantagineum* (Alamillo *et al.*, 1995), in *P. patens* (Wang *et al.*, 2009; Cui *et al.*, 2012) and in *S. ruralis* (Oliver *et al.*, 2004), preventing proteins from irreversible aggregation by an energy-independent process.

In vascular resurrection plants, the sucrose accumulated during dehydration is rapidly metabolized upon rehydration, insuring a source of energy for repairing damage from desiccation/rehydration (Scott, 2000). In bryophytes, sucrose levels remain unchanged after rehydration (Bewley *et al.*, 1978; Oldenhof *et al.*, 2006) but it may function as well as a source of energy during cell protection/repair.

1.2.3. Constitutive and induced desiccation tolerance

Being poikilohydric, cells in bryophytes equilibrate with the surrounding atmosphere. Therefore, a constitutive DT would be expected, especially in bryophyte of more sun-exposed habitats (Oliver *et al.*, 2005). Indeed some of the data collected from studies in *S. ruralis* (Oliver, 1991; Oliver and Bewley, 1997; Oliver *et al.*, 1998), *A. viticulosus* and *R. lanuginosum* (Proctor and Smirnoff, 2000) pointed towards the perspective of a constitutive protection with an induced repair mechanism upon rehydration. This is based on results produced studying just one unique system based on accumulation of specific mRNA during dehydration that will be required for protein synthesis and damage repair upon rehydration (Oliver, 1991; Wood and Oliver, 1999; Oliver *et al.*, 2004). However, that did not explain why *S. ruralis* submitted to fast drying would present extensive damage after rehydration (Schonbeck and Bewley, 1981b). In addition, recent proteomic studies, showed the importance of protein synthesis during dehydration (Wang *et al.*, 2009; Cui *et al.*, 2012), especially LEA proteins, defence enzymes and HSP necessary for cell protection against desiccation. Furthermore, recent works (Pressel *et al.*, 2006; Proctor *et al.*, 2007b) demonstrated the importance of cytoskeleton disassembly for organelle protection, a mechanism that requires time and appears to be dehydration induced.

Therefore, it seems that DT is composed of a gradient of combinations between constitutive and induced mechanisms according to the species specifications in its habitat and life form. Moreover, it is not clear if even a highly DT bryophyte like *S. ruralis* might have some induced mechanisms during dehydration, including protein synthesis, since it prefers slow drying rates (Schonbeck and Bewley, 1981b).

1.3. Studying desiccation tolerance

1.3.1. Primary metabolism: photosynthesis and respiration

Photosynthesis and respiration have been assessed through infra-red gas analysers (IRGA) and gas- or liquid-phase oxygen analysers (Hinshiri and Proctor, 1971; Dilks and Proctor, 1974, 1976; Schwab *et al.*, 1989; Nash *et al.*, 1990; Scheidegger *et al.*, 1995; Tuba *et al.*, 1996). However, there are some limitations to these techniques especially in IRGA due to the very low respiration/photosynthesis in drying/dried material and the fast drying rate that occurs inside the chamber (Cruz de Carvalho, personal observation). Therefore, during this work the primary metabolism was only measured before dehydration and upon rehydration following desiccation through liquid-phase oxygen electrode.

Coupled with the oxygen evolution, we measured chlorophyll *a* fluorescence, a non-destructive technique to assess photosynthesis, leading a wide methodological application range and allowing to compare responses across different DT photosynthetic organisms (Csintalan *et al.*, 1999; Proctor and Smirnoff, 2000; Beckett *et al.*, 2005; Gasulla *et al.*, 2009; Proctor and Smirnoff, 2011). This technique assesses PSII electron transport efficiency as well as nonphotochemical quenching processes (Marques da Silva *et al.*, 2007). After a dark adaptation period, a saturating light pulse is applied over the measuring light to determine the F_o (dark-adapted minimum fluorescence yield) and F_m (dark-adapted maximum fluorescence yield). These parameters allow to calculate the maximum quantum efficiency of PSII (F_v/F_m), i.e., when all reaction centres are open (Baker and Oxborough, 2004), a ratio that is usually used as a vitality index that responds to stress (Cruz de Carvalho *et al.*, 2011). Once measured these parameters, photosynthetic organisms are illuminated for a period of time at the end of which another saturating pulse is applied to determine F_o' (light-adapted minimum fluorescence yield, measured after the actinic light switches off and a pulse of infra-red light applied), F_m' (light-adapted maximum fluorescence yield) and F_s or F_t (steady-state value of fluorescence before the saturating light pulse is applied). These light-adapted parameters allow the determination of the photochemical quenching coefficient (qP) (Schreiber *et al.*, 1986) and non-photochemical quenching (NPQ) (Bilger and Björkman, 1994) and further explore the photosynthetic process.

1.3.2. Oxidative stress

The production of ROS by organelles, such as chloroplasts and mitochondria, is a major source of cell damage in desiccation events (Smirnoff, 1993). However, it is not

clear if ROS production is the cause of desiccation sensitivity or rather it is an effect. It is necessary to evaluate its production carefully because even dead cells can produce ROS (Hendry, 1993). The number of techniques to assess oxidative stress is limited, being most results from the thiobarbituric acid reacting substances (TBARS) assay and the electron spin resonance (ESR) spectroscopy technique. The first measures malonyldialdehyde (MDA) equivalents which result from the breakdown of lipid peroxidation (Heath and Packer, 1968), while the latter is a non-invasive technique which estimates an organic free radical linked to respiration, oxidative stress and desiccation tolerance (Hendry, 1993; Leprince *et al.*, 1995). The TBARS assay has some limitations including a lack of sensitivity and specificity, reacting also with sugars, oligosaccharides and anthocyanins, all compounds that usually accumulate in large concentrations in DT organisms (Gutteridge and Halliwell, 1990; Hodges *et al.*, 1999). On the other hand, in ESR, the problem is that the signal is sensitive to water and, in dry tissues, it is difficult to use (Hendry, 1993).

More recently, the successful use of ROS-specific fluorescent probes in lichens associated with confocal microscopy has open a new range of possibilities to study oxidative stress *in vivo* preserving the tissue integrity (Catalá *et al.*, 2010). One of such epifluorescent probes is 2,7-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), used to detect intracellular ROS production. This chemical compound easily penetrates cell membrane and is hydrolysed by cellular esterases to 2,7-dichlorodihydrofluorescein ($DCFH_2$), becoming trapped within the cell. In the presence of cellular ROS, $DCFH_2$ is oxidized to fluorescent 2,7-dichlorofluorescein (DCF), which is observable by confocal microscopy (λ_{exc} = 504 nm, λ_{em} = 524 nm). However, this method does not allow its use in desiccated tissues since the probe as to be in a soluble form.

1.3.3. Membrane leakage

As mentioned previously, membrane damage is one of the major problems associated with desiccation. One technique that has been used to assess membrane leakage is the measure of the release of intracellular electrolytes (Beckett and Hoddinott, 1997; Shakya *et al.*, 2008). Sequential elution is a technique used to determine specific cations such as Ca^{2+}, K^{+} or Mg^{2+}, allowing the evaluation of the physiological status of the cells and the integrity of cell membranes (Brown and Wells, 1988; Branquinho, 1997). In conjunction with commercially available electrical conductivity meters, the degree of membrane damage can easily be determined (Bramlage *et al.*, 1978; Brown and Buck, 1979; McKersie and Tomes, 1980). However, caution should be taken if samples are collected in the field since it is difficult to determine whether the electrolytes are of cell origin or others such as dust or pollution.

1.3.4. Water content and dehydration rates

Determination of tissue water content (WC) is crucial in the study of the mechanisms of plant drought and desiccation tolerance (Sun, 2002), allowing the correlation of water deficit limits and underlying mechanisms and metabolisms.

The determination of WC based on mass is widely used to measure tissue hydration, being a simple easy method to compare values between plant species. In plant tissues, water can be found in three fractions: symplast (inside the cells), apoplast (spaces in the cell wall), and intercellular spaces. In lichens and bryophytes, this latter fraction may be as high as 35% of the total water (Beckett, 1997; Proctor, 1999) and may have a very important role in DT, functioning as a source of water and/or barrier to delay cell desiccation (Proctor, 2008).

However, in determining WC there are several precautions to account for, in order to ensure valid conclusions. A widely used parameter is relative water content (RWC) related to tissue water content at full turgor. It presents linear expression being a good indicator for dehydration rate and presenting good correlations with the physiological responses to water stress (Sinclair and Ludlow, 1985; Marques da Silva and Arrabaça, 2004; Cruz de Carvalho *et al.*, 2011). However, in bryophytes this parameter has some particular challenges due to the fact that they possess high levels of external water, making it difficult to assess the full turgor weight (FTW). To bypass this difficulty it has been used gravimetric methods or blotting with paper towels to remove external water. The first method is less efficient due to the leaf structure which difficult the removal of excess water by gravity (Santarius, 1994). Blotting the material dry with paper towels has been a method applied to remove the excess water. However, applying too much pressure to samples will give an underestimation of FTW. On the other hand, less pressure would not remove entirely external water and would give an overestimation of the same measurement. Nevertheless, this latter method has been considered the best and more reproducible for RWC determination in bryophytes together with psychrometric measurements of the associated water potential (Santarius, 1994; Beckett, 1997; Proctor *et al.*, 1998). The correct determination of WC is one of the major aspects for validating any conclusions in DT studies and monitoring this parameter has been carefully assessed throughout this work.

A good measurement of dehydration rate is also crucial for comparative studies, and sometimes this information is not very explicit. Water loss is dependent on the water potential differential between the tissue and the atmosphere (usually RH) and the hydraulic conductivity of the tissue (Sun, 2002). The decrease in WC is exponential over time (if RH is kept constant): in the first phase water loss is fast; and in the second

phase variation is very low as it reaches equilibrium (Tompsett and Pritchard, 1998). Usually, saturated salt solutions have been used to obtain different RH atmospheres inside small containers in DT studies (Dilks and Proctor, 1979; Schonbeck and Bewley, 1981a, b; Oliver, 1991; Pressel *et al.*, 2009).

1.3.5. Differential proteomics

The development of protein isolation and identification technology, mainly through two dimensional gel electrophoresis and mass spectrometry, led to the development of proteomics. This recent study field allows to integrate information about the concentrations, interactions, and functions of proteins, which are the major functional agents of cells (Baginsky, 2009).

In DT, recent studies using transcriptomics have been used both in bryophytes (Oliver *et al.*, 2004) and vascular plants (Rodriguez *et al.*, 2010; Gechev *et al.*, 2013) identifying many genes that are differentially transcribed during drought events. DT must be evaluated through a quantitative approach of protein expression and regulation in order to understand the role of each protein that is operating in different physiological status. Recent works in bryophyte desiccation using this approach demonstrated the induction of several proteins either by dehydration or rehydration (Wang *et al.*, 2009; Cui *et al.*, 2012).

1.4. The aquatic bryophyte *Fontinalis antipyretica*

The *Fontinalis* genus comprises 35 species belonging to Division Bryophyta, commonly known as mosses (Stech and Frey, 2008). *Fontinalis antipyretica* Hedw. has been characterised through molecular studies (nuclear ribosomal DNA and chloroplast DNA) (Shaw and Allen, 2000), presenting itself in the form of dark green lawns mostly in running waters of the temperate or cold regions in the northern hemisphere (Jenkins and Proctor, 1985). Mosses are approximately 10–20 cm long and develop rhizoids that anchor to stones and wood. Leaves are one cell thick through which the elements uptake occurs, and can be oval to lance-shaped and keeled, concave or nearly flat (Bleuel *et al.*, 2005).

As *F. antipyretica* is an aquatic bryophyte, authors were initially lead to think that it would not had DT mechanisms, and in previous studies was classified as desiccation intolerant solely based in electrolyte leakage (Brown and Buck, 1979). Until recently, the belief that the habitat was indicative of the DT degree prevailed (Kimmerer and Allen, 1982; Seel *et al.*, 1992; Franks and Bergstrom, 2000). However, bryophytes have some plasticity in adapting the DT mechanisms to their habitat characteristics

(Green *et al.*, 2011). In nature, especially in Mediterranean climate, the small streams, where this particular species grows, can be subjected to seasonal drying cycles, when water can be completely absent. The aquatic bryophyte *F. antipyretica* has two options: (1) shoots must tolerate the desiccation period or (2) regrow new shots from protected cells in the cauloid/rhizoid. It is known that close species (*F. novae-angliae* Sull. and *F. dalecarlica* Bruch & Schimp.) can survive being stranded out of water for one year and show new growth from these protected buds (Glime, 1971) being this strategy more appropriated to extended periods of desiccation (several months). However, the first strategy may be applicable to the shoots in periods of short/medium duration (days to several weeks) and was the object of study of this work.

1.5. Aims and Outline of the Thesis

The climate changes expected in the near future imply an increase in desertification (IPCC, 2012). The conjunction of high temperatures with scarce water resources will increase soil erosion due to changes in vegetation distribution (Maestre *et al.*, 2012), particularly the proportion of DT species all over the world. Therefore, the study of DT mechanisms is of crucial importance to understand worldwide future changes in plant communities. Another possible strategy is to apply these organisms to prevent/slow soil erosion, and/or transfer these DT mechanisms to species of agronomic importance to increase drought tolerance, allowing to survive in more arid environments.

The main objective of this Thesis is to study DT through the combination of physiological, biochemical and molecular techniques in a bryophyte from a habitat not usually reported as prone to desiccation, comparing with bryophytes already studied and described as desiccation tolerant. This integrated study starts from the field observations that described what appeared to be vegetative DT in an aquatic bryophyte. Therefore, if even a bryophyte of this particular habitat is able to develop DT, there may be underlying mechanisms that are common to all bryophyte species and can be induced. Moreover, this work aims to identify DT processes/mechanisms that are common across desiccation tolerant plant species.

In order to attain this integrated view, after the Introduction (**Chapter 1**), this Thesis is divided into 6 chapters (**Chapters 2** to **7**).

In **Chapter 2**, the aim is to examine primary metabolism (photosynthesis and respiration) in order to determine the response to desiccation of an aquatic bryophyte and evaluate if recovery is mainly determined by the extent or rate of dehydration, or by both. The initial hypothesis was that *F. antipyretica* had some degree of DT and that

recovery would be affected by both parameters. The study of photosynthesis and respiration through oxygen production/consumption, coupled with chlorophyll *a* fluorescence, showed that the response of *F. antipyretica* to desiccation is consistent with a DT pattern observed in other bryophytes. However, dehydration rate must be slow in order to regain its pre-desiccation function following rehydration. This was further confirmed in field-desiccated samples which have been stranded out of water and showed a full recovery of these parameters.

During desiccation, metabolism becomes impaired and, thus, it is expected an increase in oxidative damage. However, there was no information of the impact of dehydration rate on this subject. In **Chapter 3**, the importance of dehydration rate to ROS production and cellular location upon rehydration is explored, being hypothesized its production would be greater subjected to a fast dehydration rate. Combining the use of confocal microscopy and a ROS-specific chemical probe, this response was assessed and related to induction of cell protection mechanisms during dehydration with increased cell survival upon rehydration.

Desiccation affects, amongst others, membrane integrity leading to intracellular solute leakage. In **Chapter 4**, using sequential elution techniques, conductivity measurements, lipid peroxidation, fluorescence techniques and nitric oxide (NO) end-product quantification, the hypothesis that fast dehydration increases membrane damage is explored. Increases in ROS and red autofluorescence emission in cells are discussed in relation with membrane permeability and the action of NO in inducing/preventing cellular damage.

In **Chapter 5**, water relations are assessed through psychrometric measurements, and sucrose quantification is analysed, under different dehydration rates. The starting hypothesis was that drying rates would induce differences in water relation parameters, especially osmotic potential at turgor loss point and cell wall elasticity modulus, and in sucrose content.

At this point, the importance of dehydration rate in the DT of an aquatic bryophyte is established. In **Chapter 6**, it is shown that the dehydration rate plays a very important role allowing the establishment of mechanisms of protection/preparation to repair. The resulting differential proteome, the first to be evaluated in an aquatic bryophyte, led to the identification of patterns of variation of major protein functional groups that were compared to terrestrial bryophytes both during dehydration and rehydration, identifying common processes that occur throughout the entire cycle. The common elements for DT to be induced are analysed and further hypothesis are advanced in order to explain the differential DT across bryophyte species, including life

form/morphology in determining dehydration rate and the ability to withstand drought.

The final Chapter (**Chapter 7**) is a general discussion, where all information of the previous chapters is interpreted and integrated in the current knowledge, highlighting the advances to the state of the art in DT in plants resulting from this work and future perspectives.

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Chapter 2

Physiological consequences of desiccation in *Fontinalis antipyretica*

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2. Physiological consequences of desiccation in *Fontinalis antipyretica*

2.1. Abstract

Fontinalis antipyretica Hedw., an aquatic bryophyte previously described as desiccation-intolerant, is known to survive intermittent desiccation events in Mediterranean rivers. To better understand the mechanisms of desiccation tolerance (DT) in this species and to reconcile the apparently conflicting evidence between DT classifications and field observations, gross photosynthesis and chlorophyll *a* fluorescence were measured in field-desiccated bryophyte tips and in bryophyte tips subjected in the laboratory to slow, fast, and very fast drying followed by either a short (30 min) or prolonged (5 days) recovery. The results show, for the first time, that the metabolic response of *F. antipyretica* to desiccation, both under field and laboratory conditions, is consistent with a DT pattern; however, drying must proceed slowly for the bryophyte to regain its pre-desiccation state following rehydration. In addition, the extent of dehydration was found to influence metabolism whereas the drying rate determined the degree of recovery. Photosystem II regulation and structural maintenance may be part of the induced DT mechanism allowing this bryophyte to recover from slow drying. The decrease in the photochemical quenching coefficient (qP) immediately following rehydration may serve to alleviate the effects of excess energy on photosystem I, while low-level non-photochemical quenching (NPQ) would allow an energy shift enabling recovery subsequent to extended periods of desiccation. The findings were confirmed in field-desiccated samples, whose behaviour was similar to that of samples slowly dried in the laboratory.

Keywords: aquatic bryophytes, Bryophyta, chlorophyll *a* fluorescence, desiccation tolerance, *Fontinalis*, recovery.

2.2. Introduction

Vegetative desiccation tolerance (DT) can be classified into two types (Toldi *et al.*, 2009): (1) full desiccation tolerance (FDT), which allows plants to survive rapid drying due to constitutive tolerance, and (2) modified desiccation tolerance (MDT), in which plants possess inducible tolerance and are able to survive slow drying. In general, the more desiccation-tolerant bryophytes belong to the first group (Oliver and Bewley, 1997).

Genetic and evolutionary evidence indicates that DT is a primitive character lost in those lineages that evolved mechanisms to resist desiccation or that adapted to habitats where they were not subject to desiccation (Oliver *et al.*, 2005; Alpert, 2006). Present-day evidence of these tolerance mechanisms in bryophytes are based on intensive cellular and molecular studies in *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr and *Physcomitrella patens* (Hedw.) Bruch & Schimp., both of which are adapted to desert or semi-arid habitats (Oliver *et al.*, 2005; Wood, 2007). However, different degrees of DT are also found in bryophyte species exhibiting other habitat preferences (Davey, 1997), including fully aquatic environments. Despite the apparent advantage conferred by DT, these species are poor competitors and there is a tendency for their replacement by desiccation-sensitive species along gradients of increasing water availability, probably due to faster growth and reproduction by the latter (Alpert, 2006).

Desiccation-tolerant bryophytes are characterized by their ability to survive desiccation, recovering their metabolic activity upon rehydration (Bewley, 1979; Crowe *et al.*, 1998; Alpert and Oliver, 2002; Proctor *et al.*, 2007b). A more quantitative definition establishes as desiccation-sensitive those species unable to survive drying to 20% water content (WC) and as DT those surviving drying to 10% WC or less (Alpert, 2006). However, “recovery” is less clear and, depending on the context, has been defined as the return to a normal rate of carbon fixation (positive net photosynthesis) or the full restoration of all metabolic systems (Proctor and Pence, 2002).

Desiccation time, rate of water loss, and relative water content (RWC) have been examined in numerous desiccation-tolerant/-sensitive species. However, since the conditions of those experiments ranged from several years of uncontrolled desiccation to 30 min, with different rates, temperatures, and light regimes (see review in Proctor and Pence, 2002), it is difficult to compare the results and thus to fully understand DT. In general, the extent and rate of water loss (Krochko *et al.*, 1978; Schonbeck and Bewley, 1981) as well as the desiccation time (Hinshiri and Proctor, 1971; Proctor, 2001) have been shown to be important factors controlling the impact of desiccation on bryophyte physiology, including that of DT species (Glime, 2007).

Most studies on the effects of desiccation and rehydration in bryophytes have involved terrestrial species (recently reviewed by Oliver *et al.*, 2005 and Proctor *et al.*, 2007b) as it is assumed that all aquatic bryophytes, due to their habitat preferences, are intolerant to desiccation (Kimmerer and Allen, 1982; Seel *et al.*, 1992; Franks and Bergstrom, 2000). However, that conclusion does not have substantial support from physiological experimental data (see review in Glime and Vitt, 1984) except for few particular cases (Lee and Stewart, 1971; Brown and Buck, 1979; Šinžar-Sekulić *et al.*, 2005). For example, after 90 min rehydration, *Fontinalis antipyretica* Hedw. and *Brachythecium rivulare* Schimp. photosynthesis was completely inhibited when these species were previously dried under zero relative humidity (RH). When submitted to 50% and 98% RH, *F. antipyretica* showed, respectively, 50% and 100% photosynthesis compared to a control sample (Lee and Stewart, 1971). However, Proctor (2000) found that the primary metabolic response to desiccation by tolerant and intolerant bryophytes was similar, such that it was not possible to draw any conclusions regarding the nature of DT based only on this parameter.

The aquatic bryophyte *F. antipyretica* has long branches with many ramifications, with free distal extremities and rhizoids that attach to the substrate (Glime, 1980). It is commonly used as biomonitor for heavy-metal pollution (Sérgio *et al.*, 1992; Martins *et al.*, 2004) but little is known about its physiology, especially its ability to tolerate desiccation. This bryophyte was classified as desiccation-intolerant, due to intracellular potassium leakage in response to desiccation (Brown and Buck, 1979). This was the only criterion used by these authors to classify bryophytes as tolerant or intolerant to desiccation. By contrast, Irmscher (1912) showed that *F. antipyretica* can survive 3 weeks of desiccation, although survival was due to new growth originating in the highly protected apical bud.

The term “rheophytic” applies to species that mainly, but not exclusively, inhabit the flooded areas of rivers and streams (Akiyama, 1995). In Portugal, the Iberian Peninsula, and generally in the Mediterranean region, the species *F. antipyretica* is periodically exposed to desiccation, in intermittent streams that lose their water during the dry season (Vieira, 2008). However, uncertainty remains concerning the ability of *F. antipyretica* to withstand the seasonal desiccation imposed by its habitat. Therefore, based on field observations suggesting desiccation tolerance by *F. antipyretica*, the main objectives of this work were (i) to examine metabolic indicators in order to establish DT by *F. antipyretica* and (ii) to test whether the recovery of this species is mainly determined by the extent or rate of dehydration, or by both. The starting hypothesis was that *F. antipyretica* had some degree of desiccation tolerance and that

recovery would be affected by both parameters. Specifically, photosynthesis was studied in *F. antipyretica* by monitoring the oxygen production rate, a very sensitive indicator of desiccation stress effects (Tuba *et al.*, 1996). In addition, chlorophyll *a* fluorescence was measured, as a non-invasive technique to follow photosynthesis in organisms subjected to stress conditions (Maxwell and Johnson, 2000).

2.3. Material and Methods

2.3.1. Plant material and culture conditions

Submerged and emerged *F. antipyretica* samples were collected from *Serra de S. Mamede Natural Park* (central Portugal; 39°16'N, 7°19'W) and then transported under cool conditions (about 5 ºC) to the laboratory, where they were rinsed in distilled water, transferred to a modified Knop culture medium (Traubenberg and Ah-Peng, 2004), and grown under controlled conditions (17 ºC day/13 ºC night, 20–30 µmol m^{-2} s^{-1} photosynthetic active radiation [PAR], and a 16-h photoperiod). Emerged and dry samples of *F. antipyretica* were collected on April 4th, 2009 and kept dry between several sheets of paper. Although we were unable to determine when these bryophytes had emerged, the cumulative precipitation between January 1st, 2009 and the time of collection, as recorded at the Alegrete climatic station (São Mamede, Portugal), was 5.2 mm, with no measurable precipitation in March. Moreover, the maximum daily precipitation during this period was 0.8 mm (National Information System of Water Resources, 2010). Accordingly, these samples had probably emerged more than 1 month prior to the collection date. These so-called field-desiccated samples were cleaned in the laboratory using a flux of N_2 gas before being used in the studies described below.

Ten shoot tips of 1 cm each were selected for the three to six replicates used for each measurement. Relative water content (RWC) was calculated according to Deltoro *et al.* (1998). Full turgor weight was determined before drying treatment and after blotting any external water away from the tips. Preliminary data of pressure-volume curves confirmed the removal of external water after blotting (data not shown). Fresh weight (stress weight) was determined at the end of the stress period and before oxygen-electrode measurements. Dry weight was determined at the end of the assays by placing the samples at 80 ºC for 48 h. In field-desiccated samples, the weight after rehydration was defined as the full turgor weight in RWC determinations. According to this method, the RWC for the field-desiccated samples was 15%.

2.3.2. Dehydration and recovery treatments

Dehydration was induced in the laboratory by placing the samples in small containers over saturated salt solutions of $KC_2H_3O_2$ (23% RH, –202 MPa), $Ca(NO_3)_2.4H_2O$ (50% RH, –100 MPa), or K_2SO_4 (95% RH, –6 MPa), which resulted in very fast, fast, and slow drying rates, respectively. These samples were incubated at ambient temperature (20–23°C) at low PAR (2–5 µmol m^{-2} s^{-1}) for stress times of 0.5, 1, 2, 24, 168, 336, and 960 h for short-term recovery assays. Additionally, long-term recovery assays were carried out by incubating the fast dried for stress times of 0.5, 1, 2, and 3 h, and the slow dried samples for 2, 4, 12, 24, 48, 96, and 168 h. In all cases, rehydration was achieved by direct immersion of the samples in an oxygen-electrode solution (0.1 mM $KHCO_3$) at 17 ºC. In the long-term recovery assays, the bryophyte tips were placed in culture medium under the previously described conditions.

2.3.3. Gross photosynthesis and chlorophyll *a* fluorescence analysis

Oxygen consumption and production and chlorophyll *a* fluorescence were measured prior to desiccation in order to determine the control values, and then either 30 min or 5 days after rehydration for short-term and long-term recovery, respectively. All samples were rehydrated using a Clark-type liquid-phase oxygen electrode (DW2/2 electrode chamber, Hansatech Instruments Ltd., Norfolk, UK) coupled to a PAM 101 chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany) connected to a PAM data acquisition system PDA 100 (Heinz Walz GmbH) adapted to the electrode chamber by fiber optics and controlled by the software WinControl v2.08 (2003) (Heinz Walz GmbH). Control and desiccated bryophyte samples were placed inside the electrode chamber containing 0.1 mM $KHCO_3$ solution for 10 min in the dark, allowing respiration (as oxygen consumption) to be measured in the absence of light (OxConsump1). Immediately before the end of the dark period, a saturating light pulse (approximately 4000 µmol m^{-2} s^{-1}) (KL 2500 LCD, Schott AG, Mainz, Germany) was applied over the measuring light to determine the maximum quantum efficiency of PSII (F_v/F_m), i.e., when all reaction centres are open (Baker and Oxborough, 2005). Subsequently, a light source (KL 1500 LCD, Schott AG) was switched on for 10 min to determine net photosynthesis, measured as the oxygen production rate. Previous photosynthesis vs. irradiation response curves indicated an optimum PAR of 46 µmol m^{-2} s^{-1}, at which oxygen (O_2) production is maximal (results not shown). Another saturating pulse was administered immediately prior to the end of the light period to determine the photochemical quenching coefficient (qP) (Schreiber *et al.*, 1986) and non-photochemical quenching (NPQ) (Bilger and Björkman 1994). Subsequently, the

light was switched off and respiration again measured for 5 min (OxConsump2). Photorespiration, resulting from the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) using O_2 instead of carbon dioxide (CO_2) as a substrate, with associated energy losses, was minimized by the addition of $KHCO_3$ (final concentration of 0.1 mM) as a non-limiting inorganic carbon source. Gross photosynthesis was calculated as A + |R| (A = net photosynthesis; R = OxConsump2).

2.3.4. Statistical analysis

Relationships between variables/parameters and RWC and stress time were investigated by linear and non-linear regression analyses. Pearson correlation coefficients (r) and degrees of freedom (d.f.) were used to determine the levels of significance (*P*) between observed and predicted data.

Gross photosynthesis values varied greatly, depending on the collection time, previous weather conditions (Vieira *et al.*, 2009), time in the laboratory, etc. Accordingly, a pool of 174 replicate samples from the control population, corresponding to four different collection periods, were used to establish the control value for gross photosynthesis and chlorophyll *a* fluorescence. All previous values of all the assays were used to create box-and-whiskers plots. The horizontal line in those plots represents the median, boxes the 25% and 75% quartiles, and whiskers the 5% and 95% quantiles.

Whenever necessary, significant differences between groups were determined using ANOVA, with the Tukey post-test (significance level $\alpha = 0.05$). All statistical analyses were performed with GraphPad Prism 5.02 for Windows (2008) (GraphPad Software, San Diego California USA).

2.4. Results

2.4.1. Relative water content and relative water loss

Samples of bryophyte tips differed in their drying rates when submitted to the three different RHs, reaching the same RWC at different times and with half-desiccation times of about 35 min (23% RH), 1 h (50% RH), or 10 h (95%) (Fig. 2.1). Values below 15% RWC were discarded as they mainly reflected the effects of storage time at low RWC.

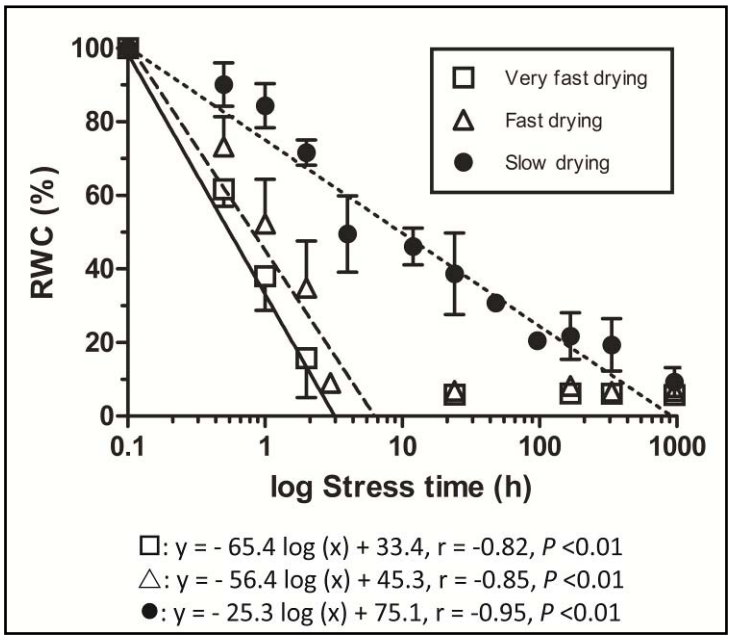

Figure 2.1. Relative water content (RWC) variation as a function of stress time in the aquatic bryophyte *Fontinalis antipyretica*, determined at different relative humidity (RH) values (□ - very fast drying rate, 23% RH; △ - fast drying rate, 50% RH; ● - slow drying rate, 95% RH). Symbols are means of 3–6 replicates in which RWC was > 15%; bars are the standard deviation.

2.4.2. Effects of desiccation on the recovery of gross photosynthesis and chlorophyll *a* fluorescence

2.4.2.1. Short-term recovery

To evaluate the physiological performance of samples submitted to different drying rates (slow, fast, and very fast) in the laboratory, four photosynthetic indicators measured after short-term recovery were analysed with respect to RWC and stress time (Table 2.1). In samples submitted to slow drying rates, all indicators changed significantly, with positive correlations to RWC and negative correlations to stress time (Table 2.1). However, in samples submitted to faster drying rates gross photosynthesis, F_v/F_m, and NPQ correlated only with RWC (Table 2.1). Samples dried at intermediate rates showed a correspondingly intermediate behaviour. Although photosynthetic indicators still correlated significantly with RWC and stress time, for most of them a decrease in Pearson *r* values with increasing stress time was observed (Table 2.1).

Table 2.1. Pearson correlation coefficient *r* between short-term recovery (30 min) of gross photosynthesis and chlorophyll *a* fluorescence parameters (F_v/F_m, qP, NPQ), and RWC and stress time in samples of the aquatic bryophyte *Fontinalis antipyretica* lab-desiccated at different rates. Only samples with RWC > 15% were considered.

	23% RH (*n* = 17)		**50% RH (*n* = 19)**		**95% RH (*n* = 26)**	
	RWC	**Stress time**	**RWC**	**Stress time**	**RWC**	**Stress time**
Gross photosynthesis	0.85**	–0.48	0.67**	–0.53*	0.79**	–0.52**
F_v/F_m	0.79**	–0.25	0.81**	–0.63**	0.77**	–0.91**
qP	0.46	–0.30	0.40	–0.62**	0.53**	–0.89**
NPQ	0.62**	–0.38	0.81**	–0.60**	0.62**	–0.64**

* $P < 0.05$; ** $P < 0.01$

Under short-term recovery, gross photosynthesis in lab-desiccated bryophyte samples showed consistently higher correlation coefficients with RWC than with stress time, regardless of the drying rate (Table 2.1). Indeed, gross photosynthesis *in F. antipyretica* samples decreased linearly with declining RWC (Fig. 2.2a) but did not differ in response to the different drying rates (Fig. 2.2a).

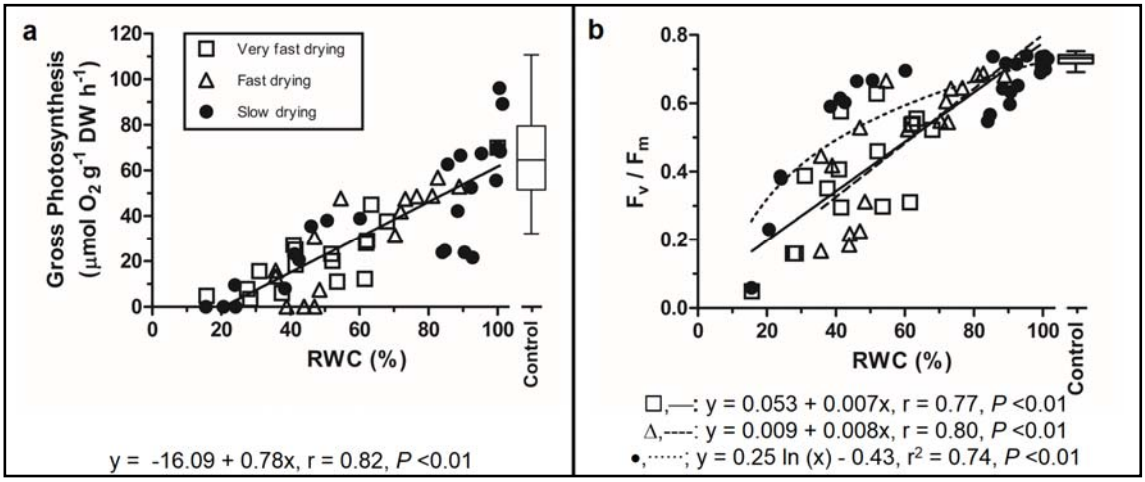

Figure 2.2. Short-term recovery of gross photosynthesis (**a**) and maximum quantum efficiency of photosystem II (F_v/F_m) (**b**) with changes in relative water content (RWC) at very fast (□ - 23% RH; *n* = 17), fast (△ - 50% RH; *n* = 19), and slow (● - 95% RH; *n* = 26) drying rates in lab-desiccated samples of the aquatic bryophyte *Fontinalis antipyretica.* Only samples with RWC > 15% were considered. In the box-and-whiskers plots, the horizontal line represents the median, boxes the 25% and 75% quartiles, and whiskers the 5% and 95% quantiles.

The F_v/F_m correlated with variations in RWC irrespective of the drying rate. However, when a function was fitted to the data, F_v/F_m decreased linearly with RWC decline in bryophyte tips submitted to 23% and 50% RH. At 95% RH, the behaviour of the bryophyte tips better fitted a logarithmic function, being F_v/F_m relatively constant and showing no significant effect of desiccation between 100% and 40% RWC (Fig. 2.2b). For F_v/F_m, correlations with stress time increased with decreasing drying rates (Table 2.1).

To evaluate the effect of desiccation on the energy captured by PSII and used for photochemical and non-photochemical processes, qP and NPQ were measured (Table 2.2). In lab-desiccated samples, qP (with a pre-desiccated value of ~0.85) correlated significantly with stress time for slow and fast drying rates whereas for RWC only a correlation with slowly dried samples was found (Table 2.1). NPQ (with a pre-desiccated value of ~1.3) correlated significantly with RWC for all three treatments and with stress time in slow- and fast-dried bryophyte samples (Table 2.1).

Table 2.2. Photochemical quenching coefficient (qP) and non-photochemical quenching (NPQ) in lab-desiccated samples of the aquatic bryophyte *Fontinalis antipyretica* following short-term recovery (30 min). ANOVA analysis (Tukey post-test) was performed to identify those values statistically different from the control. Only samples with RWC > 15% were considered.

	Stress time	qP	NPQ
Lab-desiccated (*n* = 174)	Non-stressed control	0.85±0.11	1.29±0.34
Very fast drying (23% RH) (*n* = 3-6)	0.5 h	0.85±0.12	0.37±0.10 [a]
	1 h	0.78±0.15	0.44±0.08 [a]
	2 h	0.88±0.13	0.43±0.07 [a]
Fast drying (50% RH) (*n* = 3-6)	0.5 h	0.97±0.03	0.76±0.15 [a]
	1 h	0.92±0.10	0.66±0.37 [a]
	2 h	0.80±0.11	0.46±0.08 [a]
Slow drying (95% RH) (*n* = 3-6)	0.5 h	0.83±0.08	0.74±0.17 [a]
	1 h	0.83±0.05	0.61±0.07 [a]
	2 h	0.89±0.03	0.76±0.18
	24 h	0.83±0.06	0.71±0.14 [a]
	336 h	0.72±0.11	0.23±0.08 [a]

[a] statistically different from control

Field and lab-desiccated samples were compared based on their RWC, since it was not known for how long and at what rate the field-desiccated samples had become dehydrated. Field-desiccated bryophyte samples had a low RWC, about 10–15%. When rehydrated in the laboratory, their median values of gross photosynthesis were

within the range determined for control samples (Fig. 2.3a) but their F_v/F_m values were lower (Fig. 2.3b). Field-desiccated bryophyte tips also had qP values slightly lower than those of controls and NPQ values about half those of pre-desiccated samples (Table 2.3).

Table 2.3. Photochemical quenching coefficient (qP) and non-photochemical quenching (NPQ) in field-desiccated samples of the aquatic bryophyte *Fontinalis antipyretica* following short-term (30 min) and long-term recovery (day 5). ANOVA analysis (Tukey post-test) was performed to identify those values statistically different from the control.

		qP	NPQ
Lab-desiccated (*n* = 174)	Non-stressed control	0.85±0.11	1.29±0.34
Field-desiccated (*n* = 5)	Short-term recovery (30 min)	0.56±0.04 [a]	0.59±0.17 [a]
	Long-term recovery (5th day)	0.86±0.02	0.52±0.13 [a]

[a] statistically different from control

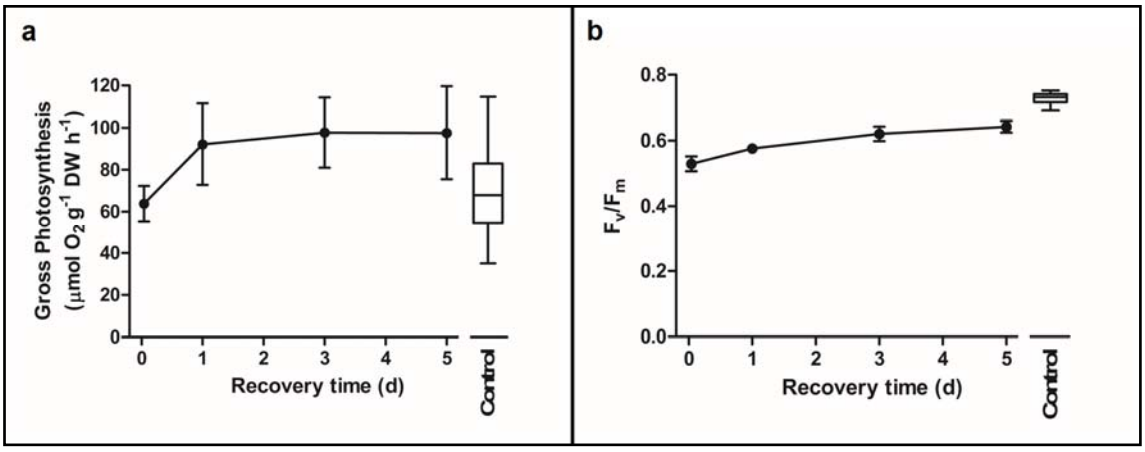

Figure 2.3. Short-term (10–20 min) and long-term (5 days) recovery of (**a**) gross photosynthesis and (**b**) the maximum quantum efficiency of photosystem II (F_v/F_m) in field-desiccated (*n* = 5) vs. control samples of the aquatic bryophyte *Fontinalis antipyretica* (box-and-whiskers plots). In the box-and-whiskers plots, the horizontal line represents the median, boxes the 25% and 75% quartiles, and whiskers the 5% and 95% quantiles.

2.4.2.2. Long-term recovery

In bryophyte samples left to recover for up to 5 days (Table 2.4 and Fig. 2.4), RWC values were similar in slow- and fast-dried samples, but the response patterns of several physiological parameters differed (Table 2.4).

Table 2.4. Pearson correlation coefficient *r* between gross photosynthesis and chlorophyll *a* fluorescence parameters (F_v/F_m, qP, NPQ), and RWC and stress time for samples of the aquatic bryophyte *Fontinalis antipyretica* lab-desiccated at fast (50% RH) and slow (95% RH) drying rates and then allowed to recover for 5 days (long-term recovery). Only samples with RWC > 15% were considered.

	Fast drying (50% RH) (*n* = 15)		**Slow drying (95% RH) (*n* = 30)**	
	RWC	**Stress time**	**RWC**	**Stress time**
Gross photosynthesis	0.84**	–0.75**	0.46**	–0.67**
F_v/F_m	0.75**	–0.79**	0.60**	–0.82**
qP	–0.22	0.12	–0.12	0.19
NPQ	0.46	-0.36	0.33	–0.54**

** $P < 0.01$

Gross photosynthesis and F_v/F_m correlations with RWC and stress time were similar in short-term and long-term recovery samples but the changes showed opposing signs (Tables 2.1 and 2.4). Different response patterns were observed for qP and NPQ with respect to RWC and stress time in samples dried at different rates and then allowed either a short or a long-term recovery (Table 2.1 and 2.4). After 5 days of recovery, there was no significant correlation between either qP or NPQ and RWC in samples subjected to fast or slow drying (Table 2.4). However, in slowly dried samples qP did not correlate with stress time whereas a significant correlation was found for NPQ (Table 2.4).

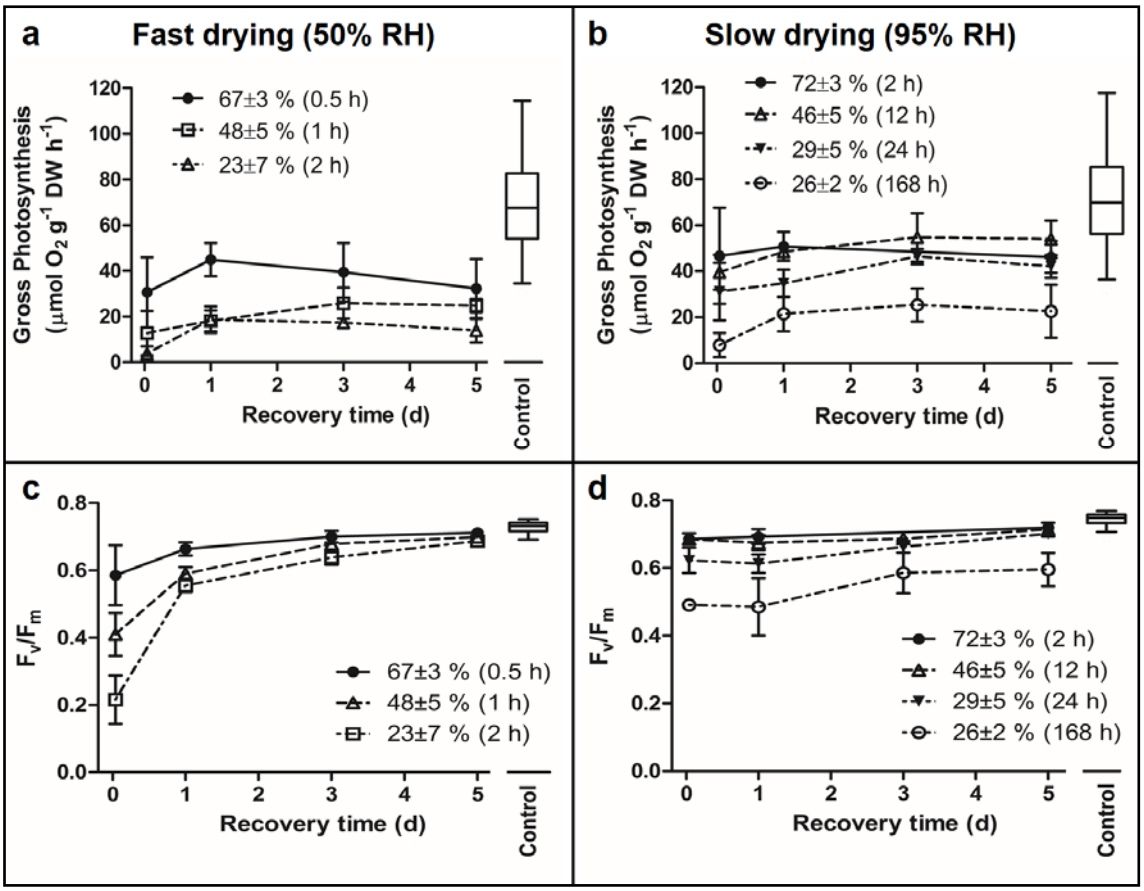

Figure 2.4. Long-term recovery (5 days) of gross photosynthesis (**a**, **b**) and the maximum quantum efficiency of photosystem II (F_v/F_m) (**c**, **d**) for samples of the aquatic bryophyte *Fontinalis antipyretica* lab-desiccated at fast (50% RH) and slow (95% RH) drying rates at different stress times. Symbols are the means of 5 replicates, and bars the standard deviation. In the box-and-whiskers plots, the horizontal line represents the median, boxes the 25% and 75% quartiles, and whiskers the 5% and 95% quantiles.

Gross photosynthesis levels were lower in bryophyte samples that recovered from fast drying than from slow drying (Fig. 2.4a and 2.4b). The gross photosynthesis values of slowly desiccated samples subjected to long-term recovery were closer to pre-desiccation ones and always higher than those of fast dried samples for a similar RWC, with the exception of bryophyte tips dried at 95% RH for 7 days (Fig. 2.4b). Although in fast-dried samples there was an initial recovery (1 day) of gross photosynthesis, the levels subsequently remained unchanged even after 5 days (Fig. 2.4a).

Immediately after rehydration, F_v/F_m values were always higher in slow- than in fast-dried samples (Fig. 2.4c and 2.4d). In slow-dried samples, F_v/F_m recovery was slower than in fast-dried samples but in both cases most of the samples had reached pre-desiccation values after 5 days (Fig. 2.4c and 2.4d). Despite this general pattern, it

is interesting to note that slow-dried samples did not completely recover control values of F_v/F_m when the stress time was more than 1 day (Fig. 2.4d).

After 5 days of recovery, NPQ was almost fully restored in slow-dried samples (with the exception of 7-day dried samples) while in bryophytes subjected to fast drying lower values were recorded (Table 2.5). By contrast, qP recovered fully and recovery was not influenced by the drying rate or the stress time (Table 2.5).

Table 2.5. Photochemical quenching coefficient (qP) and non-photochemical quenching (NPQ) in lab-desiccated samples of the aquatic bryophyte *Fontinalis antipyretica* following long-term recovery (day 5). ANOVA analysis (Tukey post-test) was performed to identify those values statistically different from the control.

	Stress time	qP	NPQ
Lab-desiccated (*n* = 174)	Non-stressed control	0.85±0.11	1.23±0.34
Fast drying (50% RH) (*n* = 4-5)	0.5 h	0.83±0.03	0.76±0.04 [a]
	1 h	0.85±0.03	0.75±0.06 [a]
	2 h	0.85±0.04	0.81±0.08
Slow drying (95% RH) (*n* = 4-5)	2 h	0.74±0.03	0.90±0.03
	12 h	0.82±0.02	0.98±0.15
	24 h	0.78±0.02	0.91±0.13
	168 h	0.80±0.02	0.59±0.26 [a]

[a] statistically different from control

In field-desiccated bryophyte samples, gross photosynthesis fully recovered after 1 day of rehydration but F_v/F_m values were lower than pre-desiccation ones (Fig. 2.3a), with a pattern similar to that of the slow lab-desiccated samples. Despite this difference, F_v/F_m progressively recovered over the 5-day period (Fig. 2.3b). The pattern observed in field-desiccated bryophytes for both gross photosynthesis and F_v/F_m more closely resembled that of slow lab-desiccated samples (Fig. 2.4b and 2.4d). Finally, rehydration of field-desiccated samples resulted in the recovery of qP but not of NPQ to pre-desiccation values (Table 2.3).

2.5. Discussion

2.5.1. *Fontinalis antipyretica* shows partial desiccation tolerance

Although *F. antipyretica* is an aquatic bryophyte, its metabolic responses to desiccation, both under field and lab conditions, were consistent with a DT pattern. As shown in the present work, this species was able to resume photosynthesis after both fast and slow drying, at RWC levels as low as ~20%. Previous classifications of *F.*

antipyretica as desiccation-intolerant were based mostly on habitat-preference data (Kimmerer and Allen, 1982; Seel *et al.*, 1992; Franks and Bergstrom, 2000) since habitat is related to DT. However, in ecological studies, several confounding factors can modify the expected response. Davey (1997) reported that for most bryophytes in Antarctica the rates of carbon exchange and recovery following dehydration were related to habitat water availability, but many of the responses measured were either not correlated with habitat or showed a wide spread around the general trend. Thus, physiological studies are needed to obtain a detailed understanding of the limits of a species' response to a particular environmental factor. In the few studies in which the degree of DT was tested under controlled conditions, *F. antipyretica* was classified as desiccation sensitive since photosynthesis did not recover after dehydration (Lee and Stewart, 1971) and intracellular K leakage under desiccation conditions was substantially higher than in other species (Brown and Buck, 1979). However, none of these works followed the recovery of this species over the course of several days. It is known that even FDT bryophytes suffer desiccation-related membrane damage, but as metabolism is recovered quickly, membrane damage alone (as previous plasmolysis assays) is not an accurate indicator of bryophyte desiccation tolerance.

If a DT bryophyte is defined as one that survives desiccation and recovers its metabolic activity upon rehydration (Bewley, 1979; Crowe *et al.*, 1998; Alpert and Oliver, 2002; Proctor *et al.*, 2007b), then *F. antipyretica* is undoubtedly a member of this group. However, recovery can be defined as a return (within few days) to a normal rate of carbon fixation (positive net photosynthesis) or as the full restoration of metabolic systems, and thus to a pre-desiccation state (Proctor and Pence, 2002). According to these criteria, the recovery of *F. antipyretica*, measured as a return of photosynthesis to pre-desiccation values, requires a slow drying process (more than 2 h). Under conditions of fast drying (< 2 h) and 5 days of recovery, the photosynthetic rate in this aquatic bryophyte was 25–50% of pre-desiccation values. Thus, *F. antipyretica* cannot be classified as exhibiting MDT (a category that includes DT vascular plants) since, unlike DT angiosperms, it is able to survive desiccation if water loss occurs in less than 12 h (Oliver *et al.*, 2000).

Oliver (2008) reported that even DT species require a relatively slow drying process in order to allow cells to survive desiccation, implying that the rate of water loss also plays an important role in subsequent recovery. Since not all previous works provided a detailed accounting of the experimental conditions to which the studied bryophytes were submitted during desiccation, namely, the rate of water loss, temperature, and atmospheric RH, comparisons of our data with the results of those

studies are difficult. In *Atrichum androgynum* (Müll. Hal.) A. Jaeger dried over silica gel (0% RH), 25% RWC was reached in about 8 h (Mayaba *et al.*, 2002) whereas in *F. antipyretica* the same RWC was achieved in 2 h at 50% RH. This difference is related with bryophyte morphology and cell organization as determinants of the rate of water loss in different bryophytes, as recently suggested by Pressel and Ducket (2010). *Fontinalis antipyretica* is an aquatic bryophyte with only a single layer of cells; this simple structure is the probable explanation for its very high rate of water loss. Furthermore, *F. antipyretica* cannot be compared with desert bryophytes such as *S. ruralis* and *Syntrichia ruraliformis* (Besch.) Cardot, both of which are able to survive desiccation occurring in less than 3 h and fully recover their photosynthetic rate (Schonbeck and Bewley, 1981; Seel *et al.*, 1992). Nevertheless, even for *S. ruralis* the rate of water loss is important since it suffers less damage if desiccation occurs in 3 h rather than 1 h (Schonbeck and Bewley, 1981). *F. antipyretica* is, however, similar to *Plagiothecium succulentum* (Wilson) Lindb. and *Mnium stellare* Hedw., as these species only recover from slow, and not fast drying (Abel, 1956), by a process referred to as hardening (Wood, 2007). The aeroterrestrial green alga *Klebsormidium crenulatum* (Kütz.) Lokhorst. retrieved from alpine regions, where water availability frequently fluctuates (between precipitation, condensation, and water vapour), also presents some similarities to *F. antipyretica* since even after fast dehydration some of the cells are able to survive and recover (Karsten *et al.*, 2010).

Although the extent of dehydration is clearly important, the rate at which it occurred appears to be the key factor determining the recovery response. Regardless of the RWC reached by the studied bryophytes during desiccation, they were able to recover to their pre-desiccation state if the drying process took more than 2 h. This finding is in accordance with the observations of other authors (Oliver and Bewley, 1997; Oliver *et al.*, 2005).

This work is one of the few studies (Glime, 1971) comparing lab-desiccated samples with field-desiccated ones. We found that the physiological response of the latter was consistent with a slow drying process since the recovery response resembled that of the slow-dried laboratory sample. Indeed, in both field- and lab-desiccated *F. antipyretica*, photosynthesis and fluorescence completely recovered to control values after 24 h of rehydration. It should be noted that under field conditions fast drying most probably does not occur, as *F. antipyretica* is aquatic, with a habitat in streams that, at least in Mediterranean areas, do not dry out in less than 2 h.

2.5.2. PSII as a target for DT protection mechanisms

PSII reaction centres are obvious targets for protection by DT mechanisms (Proctor, 2008). In FDT bryophytes such as *Racomitrium lanuginosum* (Hedw.) Brid. (Proctor and Smirnoff, 2000) and *Polytrichum formosum* Hedw. (Proctor *et al.*, 2007a), full recovery (10–20 min) of F_v/F_m is extremely fast (10 and 18 d post-desiccation, respectively), although the authors of that study did not specify the drying rate. Basal fluorescence, F_o, one of the parameters used to calculate F_v/F_m ($F_v = F_m – F_o$), is very sensitive to changes in the spatial organization of supramolecular thylakoid complexes (Havaux and Strasser, 1992), which, according to Proctor *et al.* (2007a) is related to the maintenance of grana-stroma thylakoid networks in chloroplasts. Rapid recovery of F_v/F_m also occurred in our samples of slowly dried *F. antipyretica*. Although F_v/F_m also recovered in fast-dried samples, recovery was much slower and was achieved only after 24–48 h, underlining the importance of drying rate in maintaining an intact chloroplast membrane structure. Nevertheless, after 5 days recovery these particular F_v/F_m presented lower F_0 and F_m values relatively to control, indicating fewer cells contributing to the ratio. However, in the same slowly dried samples, photosynthesis did not recover as quickly as F_v/F_m, with normal levels restored only after 3 days. This delay may have been due to the involvement of a later step in photosynthetic electron transport or photosynthetic carbon assimilation process, since both are mediated by enzymes, which may take more time to be repaired. After a few days of rehydration, F_v/F_m fully recovered to pre-desiccation values in both slow- and fast-dried bryophytes, in contrast to photosynthesis, which in the latter samples did not fully reached pre-desiccation values. The decrease in photosynthesis in absolute terms was due to the presence of damaged cells, whereas photosynthesis was functional and F_v/F_m restored to normal pre-desiccation values in the remaining active cells. The death of some of the cells especially in the fast-dried samples was confirmed by microscopy observations (**Chapter 3**) and is consistent with their simple structure, which results in rapid water loss and bryophyte cells more vulnerable to irreversible injury.

We also observed that in slow-dried samples the storage time at low RWC influences F_v/F_m recovery, perhaps reflecting damage to the D1 protein of PSII (Smirnoff, 1993). Alternatively, there may be down-regulation of PSII itself (Deltoro *et al.*, 1998; Hamerlynck *et al.*, 2002) in order to protect the complex while the cell maintains energy levels sufficient to counteract ROS formation during recovery. This would explain why in field-desiccated bryophytes (drying time and rate unknown) low F_v/F_m values persisted after a few days of recovery. According to this scenario, PSII regulation and structural maintenance might be part of the induced, protective

mechanism of desiccation tolerance that allows slowly dried *F. antipyretica* to eventually recover.

2.5.3. Energy-flow regulation as a DT mechanism

As discussed above, the regulation of energy flow may contribute to the mechanism of DT. The decrease in qP with dehydration extent during the initial moments of recovery, especially in the slowly dried samples, indicated that the fraction of absorbed energy used in photochemistry is smaller than in unstressed samples and the plastoquinone pool more reduced (Schreiber *et al.*, 1986), resulting in excess energy production at PSII compared to its consumption downstream. In tracheophytes, photosynthetic electron flux is controlled in the interchain between PSII and PSI, during electron transfer either between plastoquinol and cytochrome b6/f (Foyer, 2002) or, as more recently proposed, between cytochrome b6/f and plastocyanin (Schöttler *et al.*, 2004). A hypothetical time-dependent decrease of qP combined with slow drying may be a short-term adaptation response directed at avoiding excessive electron pressure at PSI and the concomitant production of superoxide by the Mehler reaction. Nevertheless, a few days after rehydration, qP had fully recovered in both fast- and slow-dried samples, implying that energy was being channelled to photosynthesis.

In the first few minutes of *F. antipyretica* recovery, NPQ values were lower than those measured in the pre-desiccation stage. Deltoro *et al.* (1998) observed that, in *Frullania dilatata* (L.) Dumort., NPQ recovered to pre-desiccation levels rather quickly while in *Pellia endiviifolia* (Dicks.) Dumort. a large increase was registered. Low NPQ values have been positively correlated with DT (Deltoro *et al.*, 1998; Hamerlynck *et al.*, 2002). However, more recently Proctor and Smirnoff (2011) observed in bryophytes from unshaded more exposed to desiccation habitats, like *Andreaea rothii* F. Weber & D. Mohr or *Schistidium apocarpum* (Hedw.) Bruch & Schimp., that protection against high radiation involves high photosynthetic electron transport to oxygen and high NPQ. NPQ is an indicator of photosynthetic electron-transport-chain protection mechanisms in response to excessive light energy in the PSII antenna system (Demmig-Adams, 1990). The main component of NPQ is usually qE (for review see Horton *et al.*, 1996), the high-energy-state quenching that is dependent on the transthylakoidal pH gradient. The decrease in NPQ during the initial moments of rehydration was paralleled by a decrease in the activity of the PSII reaction centre, as shown by the decrease in F_v/F_m. According to this scenario, there may have been an increase in energy pressure over the PSII antenna that could not be dissipated by NPQ mechanisms. This would suggest that *F. antipyretica* is more susceptible to light damage within the first

moments of rehydration. Thus may be due to xanthophyll degradation but also to a lack of membrane organization at this early stage, when it is difficult to properly quench the excess energy. However, in nature, water acts as a film and thus as a light filter (Glime, 2007) as does the riparian vegetation bordering the small streams, in both cases reducing the incident light (Cruz de Carvalho, personal observation). Therefore, under field conditions, the energy pressure over the PSII antenna may be less than expected. After a few days of recovery, NPQ was almost fully restored in slow dried samples, with the exception of the 7-day dried samples. The lack of full NPQ recovery following fast drying supports the hypothesis that xanthophyll integrity and/or its functionality can only be restored following slow drying. In field-desiccated samples, the NPQ did not show any recovery signs, reaching only about half of the value in the unstressed control bryophytes and values similar to those determined in 7-day dried samples. This might reflect the effect of stress time on non-photochemical energy dissipation protection mechanisms or on energy flow and thus on the shift to photochemical processes mediating recovery. We suggest that the decrease in qP alleviates the effects of excess energy on PSI that occurs during the initial moments of rehydration and that low NPQ allows an energy shift that enables recovery over the following days. This process becomes increasingly important as desiccation continues and it contributes to the partial desiccation tolerance displayed by *F. antipyretica*.

2.6. Conclusion

In summary, this work demonstrates, for the first time, that *F. antipyretica* exhibits partial desiccation tolerance, in contrast to what has been reported previously. Being aquatic, this bryophyte is able to induce DT mechanisms, mainly the protection of the PSII reaction centres, and to fully recover only if drying is slow. While the extent of dehydration affects metabolism, the rate at which it occurs determines the degree of recovery. Our findings were confirmed in field-desiccated samples, whose behaviour more closely resembled slow rather than fast lab-desiccated samples. Bryophytes have been of utmost importance in the study of desiccation tolerance. This physiological study of a widely distributed aquatic bryophyte periodically subjected to desiccation contributes to improving our knowledge of the role played by desiccation rate in bryophyte survival. Moreover, aquatic bryophytes such as *F. antipyretica* allows us to gain insight into both the existence of DT mechanisms in bryophytes presently adapted to very humid habitats and the evolutionary implications of these processes.

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Chapter 3

The impact of dehydration rate on the production and cellular location of reactive oxygen species in *Fontinalis antipyretica*

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3. The impact of dehydration rate on the production and cellular location of reactive oxygen species in *Fontinalis antipyretica*

3.1. Abstract

The aquatic bryophyte *Fontinalis antipyretica* L. ex Hedw. requires a slow rate of dehydration to survive a desiccation event. The present work examined whether differences in the dehydration rate resulted in corresponding differences in the production of reactive oxygen species (ROS) and therefore in the amount of cell damage. Intracellular ROS production by the aquatic bryophyte was assessed with confocal laser microscopy and the ROS-specific chemical probe 2,7-dichlorodihydrofluorescein diacetate. The production of hydrogen peroxide was also quantified and its cellular location was assessed. The rehydration of slowly dried cells was associated with lower ROS production, thereby reducing the amount of cellular damage and increasing cell survival. A high oxygen consumption burst accompanied the initial stages of rehydration, perhaps due to the burst of ROS production. A slow dehydration rate may induce cell protection mechanisms that serve to limit ROS production and reduce the oxidative burst, decreasing the number of damaged and dead cells upon rehydration.

Keywords: cell survival, confocal microscopy, dehydration rate, desiccation, diaminobenzidine, dichlorodihydrofluorescein diacetate, *Fontinalis antipyretica*, oxygen consumption, reactive oxygen species.

3.2. Introduction

Bryophytes are poikilohydric organisms lacking the structures needed to prevent water loss. Some bryophytes can tolerate as little as 0.1 g H_2O g^{-1} DW and recover fully on rehydration (Alpert and Oliver, 2002). Among the factors that influence desiccation tolerance (DT) are the dehydration rate and duration, temperature, and light (Alpert and Oliver, 2002). Desiccation-tolerant bryophytes differ in their abilities to withstand drying and to recover from desiccation (Abel, 1956). Recovery depends, to some degree, on the habitat, i.e., on the circadian/seasonal variations in water availability (Tuba *et al.*, 1998). Even the semi-aquatic bryophytes like *Cinclidotus fontinaloides* (Hedw.) P. Beauv. and *Leskea polycarpa* Hedw. can behave like terrestrial bryophytes displaying DT (Dyer and Duckett, 1984). Thus, in aquatic bryophytes of the Mediterranean region, seasonal variations may have a greater influence than the daily variations to which terrestrial bryophytes are subjected. In species belonging to the latter, such as *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr, constitutive protection mechanisms are thought to confer DT, while inducible repair mechanisms come into play during rehydration (Bewley *et al.*, 1978, 1993; Oliver, 1991). However, Beckett (1999) showed that the mesic bryophyte *Atrichum androgynum* (Müll. Hal.) A. Jaeger was able to tolerate desiccation if there was a period of partial dehydration prior to a desiccation event or if the bryophyte was subjected to abscisic acid (ABA) treatment. Werner *et al.* (1991) also showed in *Funaria hygrometrica* Hedw. that ABA increased during slow dehydration. These works indicate that an induced protection system operates to develop DT, under certain circumstances. Therefore, most bryophytes of dry, sun-exposed sites that have constitutive DT can withstand fast dehydration rates. On the other hand, bryophytes of moist or shady habitats, usually more sensitive to fast dehydration, require a period of sub-critical water stress during which metabolic changes occur (commonly associated with sugar metabolism and/or synthesis of specific proteins) which confer DT, similar to the induced mechanisms observed in the desiccation tolerant vascular plants (Farrant *et al.*, 1999; Bartels and Salamini, 2001). Recent molecular-cladistic research shows that bryophytes have continued to evolve in response to the evolving environment during the Mesozoic and Cenozoic (Goffinet *et al.*, 2004). As the development of DT has inherent energetic and metabolic costs, during evolution mesic/hydrophytic bryophytes might have forego the constitutive cellular protection in favour of an inducible system allowing them to better compete in a mesic habitat (Oliver, 2008).

Aquatic bryophytes are not usually the object of desiccation studies, although they are clearly thought to be desiccation sensitive (Brown and Buck, 1979; Seel *et al.*,

1992*a*; Franks and Bergstrom, 2000; Robinson *et al.*, 2000). This finding is consistent with the expectation that desiccation-protection mechanisms are unnecessary and therefore absent or less well developed in desiccation-sensitive bryophytes of humid/aquatic habitats. However, **Chapter 2** showed that the aquatic bryophyte *Fontinalis antipyretica* L. ex Hedw., previously described as desiccation intolerant (Brown and Buck, 1979), is indeed able to tolerate desiccation if it dehydrates at a slow rate. A similar effect was observed in the lichen photobiont *Trebouxia erici* Ahmadjian (Gasulla *et al.*, 2009). Moreover, a slow dehydration rate was reported to be crucial in desiccation-tolerant vascular plants, such as *Xerophyta humilis* (Baker) T. Durand & Schinz and *Myrothamnus flabellifolius* Welw., in which tolerance is induced through slow drying (Farrant *et al.*, 1999).

Desiccation induces an increase in oxidative stress (Smirnoff, 1993), such that as cellular water content decreases, organelles with high rates of electron flow, i.e., chloroplasts, mitochondria, and peroxisomes, together with membrane oxidases and peroxidases (Mittler, 2002; Scheibe and Beck, 2011) up-regulate the production of superoxide ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2). These reactive oxygen species (ROS) react with proteins, lipids, and nucleic acids, thereby causing damage to enzymes (Wolff *et al.*, 1986; Halliwell and Gutteridge, 1999), membranes (Senaratna and McKersie, 1983; McKersie *et al.*, 1989; Halliwell and Gutteridge, 1999; Leprince *et al.*, 2000), and chromosomes (Dizdaroglu, 1994). Proteomic studies carried out in resurrection plants have shown that during dehydration there may be an increase in ROS scavenging enzymes (Ingle *et al.*, 2007; Jiang *et al.*, 2007). On the other hand, since the photosynthetic system is blocked during desiccation, there is generally a decrease in proteins related with photosynthetic activity to avoid ROS formation (Ingle *et al.*, 2007; Oliver *et al.*, 2010). The decrease in the fluidity of membranes (McKersie *et al.*, 1989) caused by lipid peroxidation leads to their fusion and interferes with their permeability upon rehydration. By contrast, in vascular plants extracellular ROS production has been shown to play an important defensive role, acting directly on pathogenic bacteria and fungi at the site of infection (Wojtaszek, 1997; Murphy *et al.*, 1998), or by stimulating defence mechanisms in neighbouring cells (Miller *et al.*, 2008). In terrestrial bryophytes, the H_2O_2 burst has different functions, acting as: i) a toxic compound leading to cell death (Apel and Hirt, 2004); ii) a signal that induces the expression of protective genes; iii) a cell-to-cell communication system, inducing protection in neighbouring cells (Apel and Hirt, 2004; Miller *et al.*, 2008); and iv) a defence against fungi and bacteria (Mayaba *et al.*, 2002) especially in the moist environments where *F. antipyretica* grows.

Although ROS production in response to desiccation/rehydration has been determined in terrestrial bryophytes (Minibayeva and Beckett, 2001; Mayaba *et al.*, 2002; Beckett *et al.*, 2004), to our knowledge, it has not been investigated in aquatic bryophytes. In addition, there are no published studies examining the impact of dehydration rate on ROS production in bryophytes previously subjected to desiccation, although large decreases in pools of the antioxidants ascorbate and glutathione were shown to decrease sharply in *Syntrichia ruraliformis* (Besch.) Cardot (Seel *et al.*, 1992*b*) and *S. ruralis* (Dhindsa, 1987) under conditions of rapid dehydration. Therefore, the aim of the present work was to investigate the response to dehydration of *F. antipyretica*. Specifically we asked whether this response includes an oxidative burst that is sensitive to dehydration rate in terms of intensity and tissue location. We hypothesized that ROS production, and thus oxidative damage, is greater in bryophytes subjected to a fast *vs.* a slow drying rate.

3.3. Material and Methods

3.3.1. Plant material and culture conditions

Samples of the bryophyte *F. antipyretica* were collected at the *Serra de S. Mamede Natural Park*, central Portugal, from a natural and well preserved stream with no observable human impact. Samples were transported under cooling conditions (about 5 ºC) to the laboratory, where they were cleaned of debris and sediments in distilled water. The bryophytes were grown in a modified Knop culture medium (Traubenberg and Ah-Peng, 2004) under controlled conditions (17 ºC day/13 ºC night, photosynthetic active radiation [PAR] of 20–30 µmol m^{-2} s^{-1} and a photoperiod of 16 h) and maintained under the same conditions for no more than 60 days before being used in the analyses. Five replicates of ten shoot tips (1 cm) were selected for each treatment. The relative water content (RWC) was calculated according to Deltoro *et al.* (1998) using the procedures described in **Chapter 2**. After the external water had been blotted from the tips using lab paper, the samples were first weighed to determine the full turgor weight. They were subsequently dried and the fresh weight (stress weight) was determined. At the end of the assays, the samples were dried at 80 ºC for 48 h and then weighed to determine the dry weight.

3.3.2. Dehydration induction and recovery

Dehydration was carried out by placing samples in small containers over saturated salt solutions. In the slow dehydration experiments, the bryophytes were incubated for 24 h over a saturated solution of K_2SO_4 (corresponding to 95% relative humidity [RH], and a dehydration rate of 1.2 ± 0.3 mg H_2O h^{-1}); for fast dehydration, the bryophytes were incubated for 3 h over a saturated solution of NH_4NO_3 in the confocal laser microscopy assays (65% RH) and over saturated $Ca(NO_3)_2.4H_2O$ in the remaining assays (50% RH) (a dehydration rate of 6.9 ± 0.9 mg H_2O h^{-1}), in both cases under ambient temperature (20–23 ºC) and at low PAR (2–5 µmol m^{-2} s^{-1}). Under the described conditions, the RWC of the four replicates at the end of the treatment was 13 ± 3% and 18 ± 3% for the fast- and slow-dehydration samples, respectively. The rates and patterns of dehydration in *F. antipyretica* are described in detail in **Chapter 2**. For confocal microscopy, the bryophytes were rehydrated directly on the microscopy slides. For oxygen exchange measurements, the bryophytes were subjected to fast and slow dehydration for different lengths of time (0.5, 1, 2, 24, 168, 336, and 960 h) and then rehydrated through immersion in the oxygen electrode solution (0.1 mM $KHCO_3$).

3.3.3. Epifluorescence probes and confocal microscopy imaging analysis

The epifluorescent probe 2,7-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) was used to detect intracellular ROS production. $DCFH_2$-DA easily penetrates the cell membrane and is then hydrolysed by cellular esterases to dichlorodihydrofluorescein ($DCFH_2$), which is trapped within the cell. In the presence of cellular free radicals, $DCFH_2$ is oxidized to fluorescent dichlorofluorescein, which is observable by confocal laser microscopy (DCF, λ_{exc} = 504 nm, λ_{em} = 524 nm). The oxidative air pollutant cumene hydroperoxide (CHP) (10 µM) and the antioxidant ascorbic acid (Asc) (10 mM) were used as controls to stimulate (Catalá *et al.*, 2010) and counteract ROS production, respectively. For confocal microscopy assays, the leaves of the bryophyte were detached from the stem after slow (95% RH) and fast (65% RH) dehydration, disposed individually on slides, and rehydrated for 1 h according to Table 3.1.

Table 3.1. Rehydration treatments after slow (95% RH) and fast (65% RH) dehydration of individual leaves of the aquatic bryophyte *Fontinalis antipyretica* disposed on slides for confocal laser scanning microscopy.

Rehydration treatment
1. Deionized water
2. 50 μM 2,7- dichlorodihydrofluorescein diacetate ($DCFH_2$-DA)
3. 10 μM cumene hydroperoxide (CHP) + 50 μM $DCFH_2$-DA
4. 10 mM ascorbic acid (Asc) + 50 μM $DCFH_2$-DA
5. 10 mM Asc + 10 μM CHP + 50 μM $DCFH_2$-DA

To evaluate the RWC of these samples, they were compared with other leaves submitted to the same dehydration conditions and used for dry weight determination. Samples were observed by confocal laser scanning microscopy (TCS Leica SP confocal laser scanner microscope, Leica, Heidelberg, Germany) at the Servei Central de Suport a la Investigació Experimental (Universitat de València, Valencia) using an Ar excitation laser (λ_{exc} = 488 nm) for $DCFH_2$-DA (λ_{em} = 543 nm). Chlorophyll autofluorescence was also evaluated (λ_{em} = 633 nm). The magnification is indicated in each figure. For each treatment, four leaves were examined resulting in four images. Both positive ROS and chlorophyll autofluorescence signals were measured by quantifying the amount of green and red signal, respectively. Thus, in the 8-bit images of the $DCFH_2$-DA treatments the number of pixels in the intensity range of 44–255, corresponding to the green signal, was quantified using ImageJ 1.43 (available from http://rsbweb.nih.gov/ij/; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Pixels in the 0–43 range were considered to indicate the absence of signal, based on the observation that 95–99% of the pixels of images from unstressed and untreated samples were mostly in this range.

3.3.4. Hydrogen peroxide production and localization

Four replicates of the aquatic bryophyte were submitted to either slow (95% RH) for 24 h or fast (50% RH) dehydration for 3 h, followed by a 24-h recovery in culture medium. The samples were then boiled in ethanol for 10 min, left overnight in cold ethanol, and observed using an optical microscope (Leica DM2500, Leica Microsystems, Wetzlar, Germany). Images were acquired with a digital camera (Leica DFC500, Leica Microsystems). In two independent assays (three replicates each), one for measuring and the other for localizing H_2O_2 production, the samples were submitted to the same slow and fast dehydration treatments. Hydrogen peroxide production was quantified using the xylenol orange assay of Gay and Gebicki (2000) while the cellular

location of H_2O_2 was determined using diaminobenzidine (DAB) staining (5 mM, 3 h) before and after dehydration and 24 h after recovery.

3.3.5. Oxygen consumption analysis

Samples of the aquatic bryophyte were submitted to either slow (95% RH, 24 replicates) or fast (50% RH, 18 replicates) dehydration for 0.5, 1, 2, 24, 168, 336, and 960 h. Oxygen consumption was measured in each sample before and after dehydration using a Clark-type liquid phase oxygen electrode (DW2/2 electrode chamber, Hansatech Instruments Ltd., Norfolk, UK) in a 0.1 mM $KHCO_3$ solution. Respiration was measured for 10 min in the dark. Rehydration was achieved by immersing the samples in the oxygen electrode solution. Another set of three samples each was submitted to either non-stressed conditions, slow dehydration (95% RH) for 24 h, or fast dehydration (50% RH) for 3 h and then assayed to determine the effects of the inhibitors potassium cyanide (KCN) (1 mM) and salicylhydroxamic acid (SHAM) (5 mM) on oxygen consumption in the first 5–7 minutes following rehydration. KCN inhibits cytochrome *c* oxidase in the mitochondria electron transport chain as well as metallo-enzymes, such as catalases (Allen and Whatley, 1978), peroxidases (Choi *et al.*, 2007), and superoxide dismutases (Chen *et al.*, 2001), while SHAM inhibits the mitochondrial alternative oxidase (Vanlerberghe and McIntosh, 1997).

3.3.6. Statistical analysis

Relationships between variables/parameters and RWC were investigated using linear regression analysis. Pearson correlation coefficients (r) and the degrees of freedom (d.f.) were used to determine the levels of significance (P) between observed vs. predicted data. A pool of 174 replicate samples from controls corresponding to four different collection periods was used to establish the control value for oxygen consumption. All previous values of all the assays were used to create box-and-whiskers plots, in which the horizontal line represented the median, the boxes the 25% and 75% quartiles, and the whiskers the 5% and 95% quantiles. Whenever necessary, significant differences between groups were determined through ANOVA with a Tukey post-hoc test (significance level $\alpha = 0.05$). All statistical analyses were performed with GraphPad Prism 5.03 for Windows (2009) (GraphPad Software, San Diego California USA).

3.4. Results

3.4.1. ROS production depends on the dehydration rate

Intracellular ROS production during the rehydration of *F. antipyretica* tips was determined by immersing the dehydrated samples in deionized water containing the probe $DFCH_2$-DA (50 μM) and then measuring the amount of the green fluorescent oxidation product 2',7'-dichlorofluorescein (DCF), which reflects reaction of the probe with intracellular free radicals.

In the control hydrated leaves, elongated cells containing peripheral elongated chloroplasts were observed (Fig. 3.1a, red autofluorescence). Green autofluorescence that could interfere with DCF localization was absent. Following slow dehydration, there was a remarkable morphological change in the leaves, in that the cell walls and cytoplasm took on a condensed appearance and chloroplasts or other structures were no longer visible (Fig. 3.1c,d). Confocal images showed a generalized red autofluorescence not due to chlorophyll because the location did not match that of chloroplasts (Fig. 3.1c). Moreover, the red fluorescence appeared first at one site in the cell and then spread across the cytoplasm. In addition, some cells showed a very uniform and light inner space, as if they were empty, but they also emitted a weak DFC green fluorescence, presumably due to free radical production (Fig. 3.1c, arrowheads). This pattern was not related to the position of these cells in the leaves, as they were scattered individually rather than forming clusters.

In cells subjected to fast rehydration, there was a burst of free radicals and all cells of the leaves assumed the generalized appearance of empty cells (Fig. 3.1e). Small rounded chloroplasts were observed in some cells and some areas emitted intense non-chloroplastic red autofluorescence. None of the cells contained the condensed structures seen in the slowly dehydrated leaves. However, the photosynthetic tissues of rapidly dehydrated leaves were brownish in colour and, to a lesser extent, in slowly dehydrated leaves (data not shown).

In cells rehydrated with the oxidant CHP, high-level ROS production was determined in the rapidly dehydrated leaves whereas there was no detectable production in slowly dehydrated samples (data not shown). The appearance of rapidly dehydrated leaves rehydrated with the antioxidant Asc was similar to that of slowly dehydrated cells with no detectable ROS production. This pattern also occurred in leaves rehydrated with both Asc and CHP (data not shown).

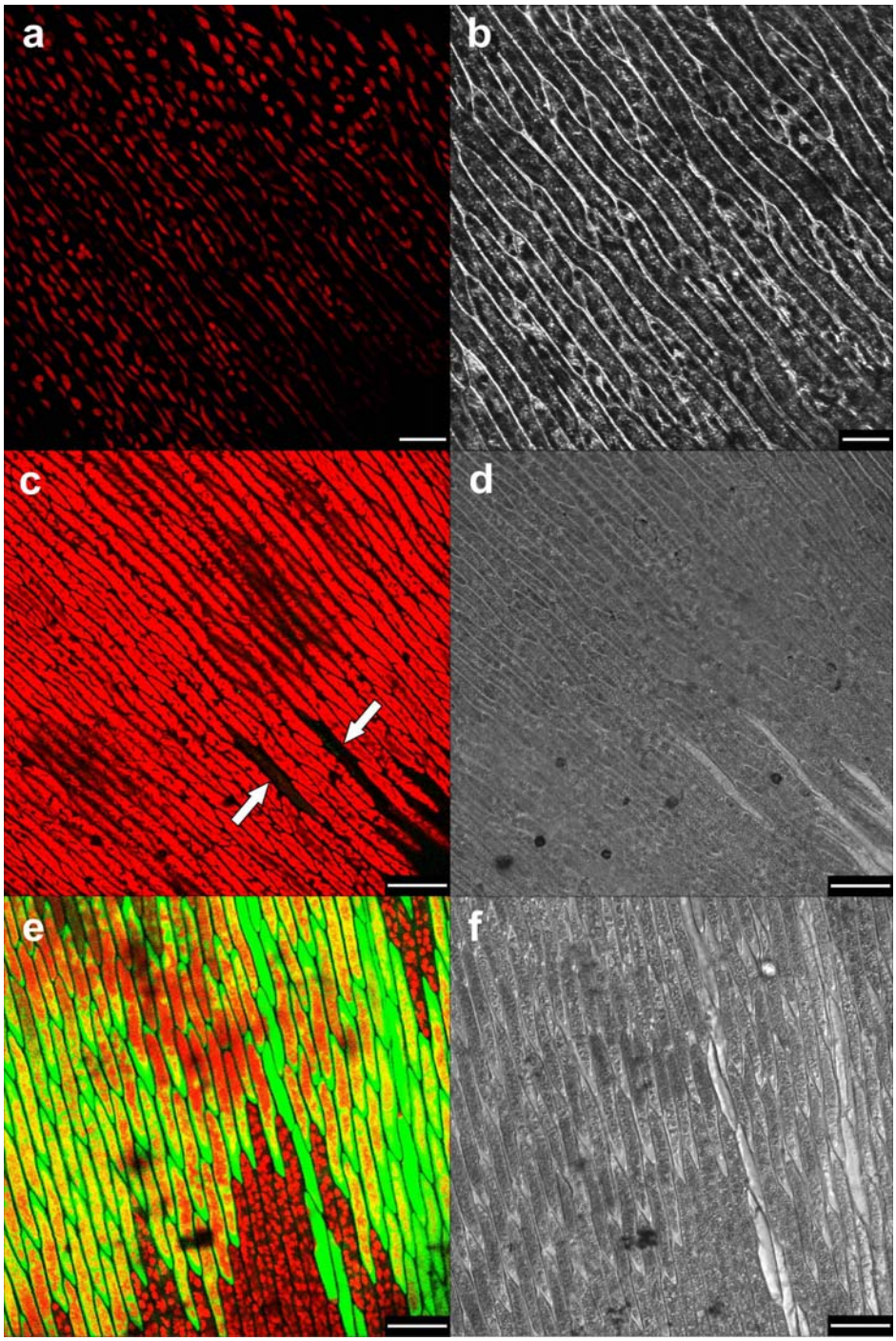

Figure 3.1. Leaves of the aquatic bryophyte *Fontinalis antipyretica* incubated with 50 µM (final concentration) of the probe $DFCH_2$-DA before dehydration (**a-b**) and 1 h after rehydration (**c-f**). Samples were dehydrated either slowly (95% RH, **c-d**) or rapidly (65% RH, **e-f**). Green ROS fluorescence and red autofluorescence are shown. Arrows indicate the only cells with ROS fluorescence in slowly dried leaves. White scale bar: 25 µm (**a-b**), 50 µm (**c-f**). **a**,**c**,**e**: confocal images; **b**,**d**,**f**: bright-field images.

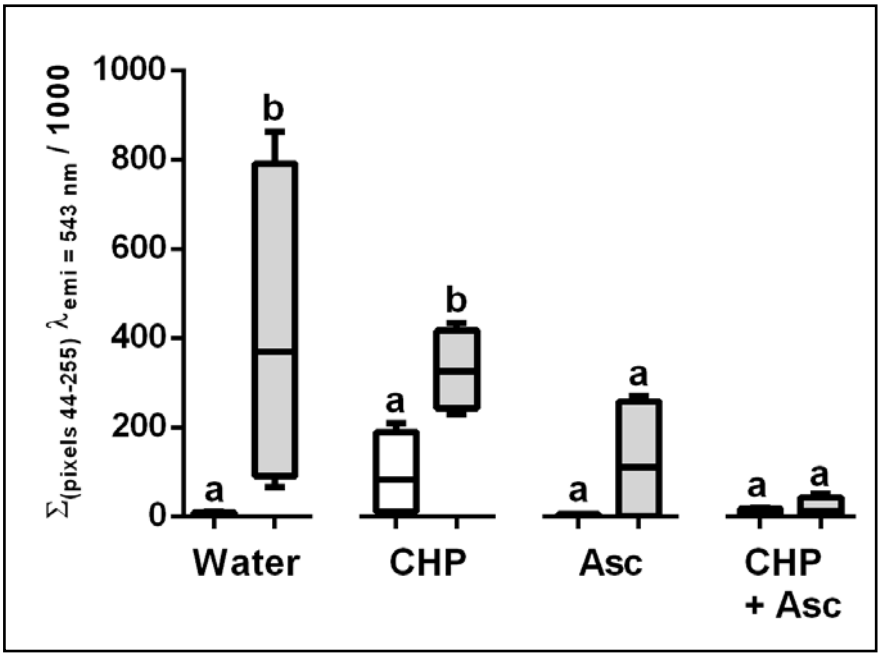

Figure 3.2. Confocal microscopy image analysis based on the quantification of pixels with intensities between 44 and 255 as seen on 8-bit images viewed at 543 nm. Slowly (95% RH; white bars) and rapidly (65% RH; grey bars) dehydrated leaves of the aquatic bryophyte *Fontinalis antipyretica* rehydrated with different treatments with cumene hydroperoxide (CHP) and ascorbic acid (Asc) in the presence of the ROS-specific probe $DFCH_2$-DA (for details see Table 3.1). In the box-and-whiskers plots, the horizontal line represents the median, the boxes the 25% and 75% quartiles, and whiskers the minimum and maximum values of four independent leaves. Values with different letters in the rehydration treatments between slow and fast dehydration are statistically different.

As shown in Fig. 3.2, the number of green pixels was much higher in rapidly dehydrated than in slowly dehydrated leaves. In a semi-quantitative analysis based on the number of pixels, the addition of CHP increased the number of green pixels in slowly dehydrated leaves but did not cause a further increment in rapidly dehydrated leaves. By contrast, Asc treatment strongly decreased the number of green pixels in rapidly dehydrated leaves, an effect that was maintained following the combined treatment with Asc and CHP, as no green pixels were present during rehydration.

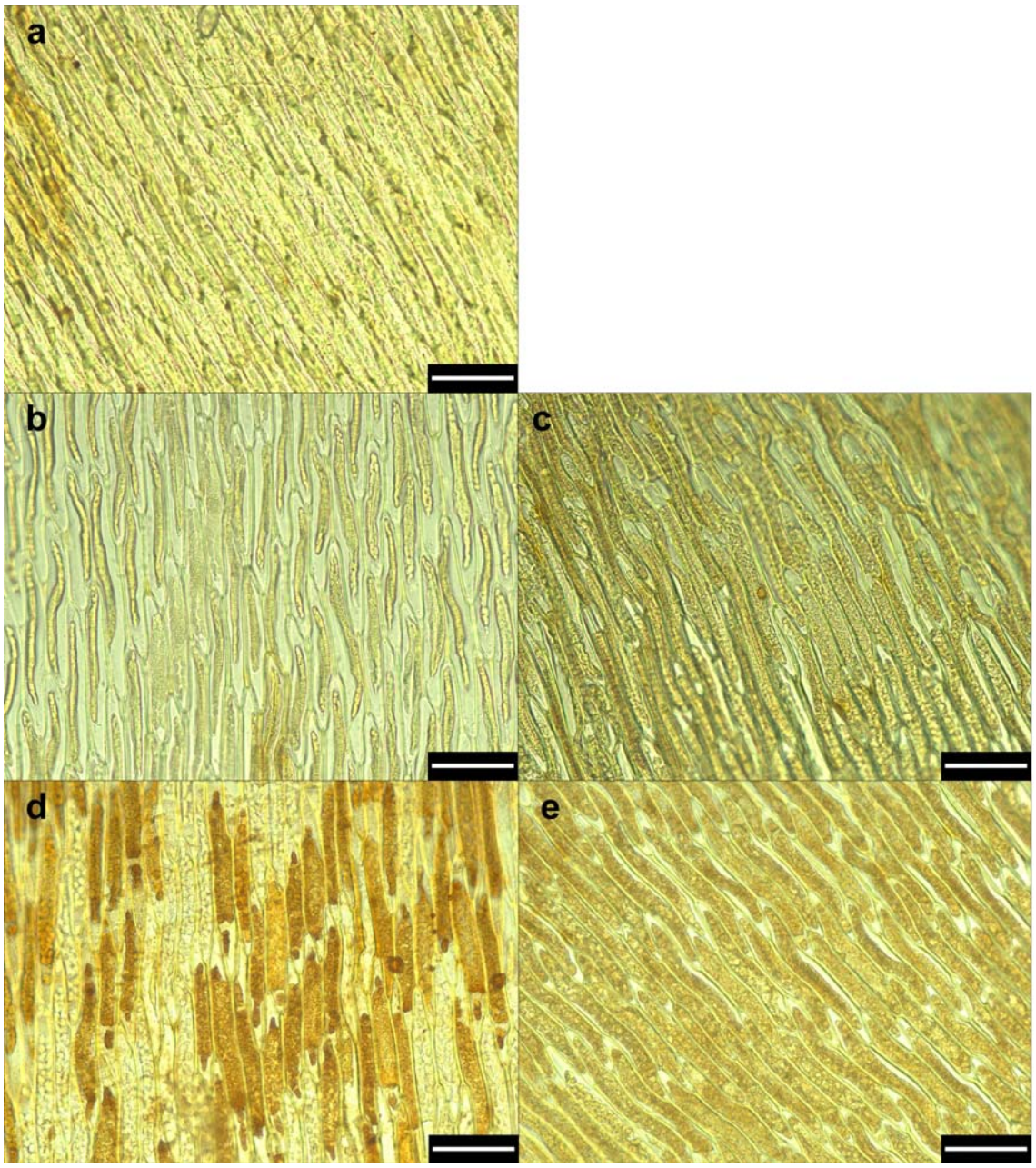

Figure 3.3. Hydrogen peroxide (H_2O_2) localization by diaminobenzidine (DAB) staining (5 mM, 3 h) in the non-stressed control (**a**), after slow (95% RH, **b**) and fast (50% RH, **d**) dehydration, and after a 24-h recovery from slow (**c**) and fast (**e**) dehydration, in leaves of the aquatic bryophyte *Fontinalis antipyretica*. White scale bar: 50 µm.

Intra- and extracellular H_2O_2 production was localized based on the dark brown product generated by DAB staining (Fig. 3.3). In slowly dehydrated samples (Fig. 3.3b,c), H_2O_2 production was minimal, similar to that of non-stressed control samples (Fig. 3.3a), whereas in rapidly dehydrated samples H_2O_2 production was marked (Fig. 3.3d) and continued during recovery (Fig. 3.3e).

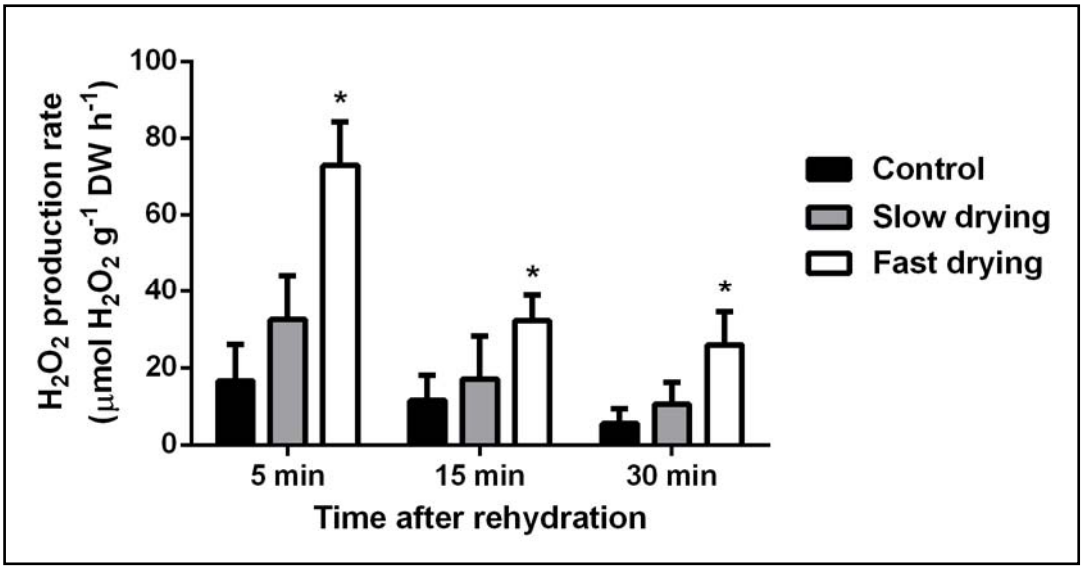

Figure 3.4. Extracellular hydrogen peroxide (H_2O_2) production rate in non-stressed control and in slowly (95% RH) and rapidly (50% RH) dehydrated leaves of the aquatic bryophyte *Fontinalis antipyretica* after 5, 15, and 30 min of rehydration. Columns indicate the Mean and the bars the SD of four replicates. An asterisk indicates a column value statistically different from the control value.

The H_2O_2 production was monitored throughout the first 30 min of rehydration (Fig. 3.4). While slow drying resulted in an increase in H_2O_2 as early as after 5 min of rehydration (33±12 μmol H_2O_2 g^{-1} DW h^{-1}), the amount was not statistically different from that of the non-stressed control (17±9 μmol H_2O_2 g^{-1} DW h^{-1}). However, rapid drying resulted in a large peak of H_2O_2 production (73±11 μmol H_2O_2 g^{-1} DW h^{-1}), four-fold higher than control values, 5 min after rehydration. In fact, H_2O_2 levels were always higher in rapidly dried than in slowly dried bryophyte, although, over time, the rate of H_2O_2 production decreased in both treatments.

3.4.2. Oxygen consumption peaks in the early stages of rehydration

Prior to dehydration, oxygen consumption was 33±11 μmol O_2 g^{-1} DW h^{-1}. In non-stressed samples, the addition of KCN largely (70%) inhibited oxygen consumption, probably by inhibiting cytochrome c oxidase. Under the same conditions, the alternative oxidase pathway, accounts for ~16% of the respiratory electron flux. The residual oxygen consumption is relatively high (~14%). Rehydration induced a high rate of oxygen consumption in the first 5–7 min: up to 15-fold higher than the pre-desiccation levels in slowly dehydrated leaves but 40 times higher in rapidly dehydrated leaves. This result suggested that the amount of oxygen consumption during rehydration was dependent on the drying rate (Fig. 3.5). However, regardless of

the drying rate, oxygen consumption was fully inhibited by KCN but not by SHAM (Table 3.2), suggesting the involvement of the cytochrome *c* pathway but not the oxidase pathway in the recovery from dehydration.

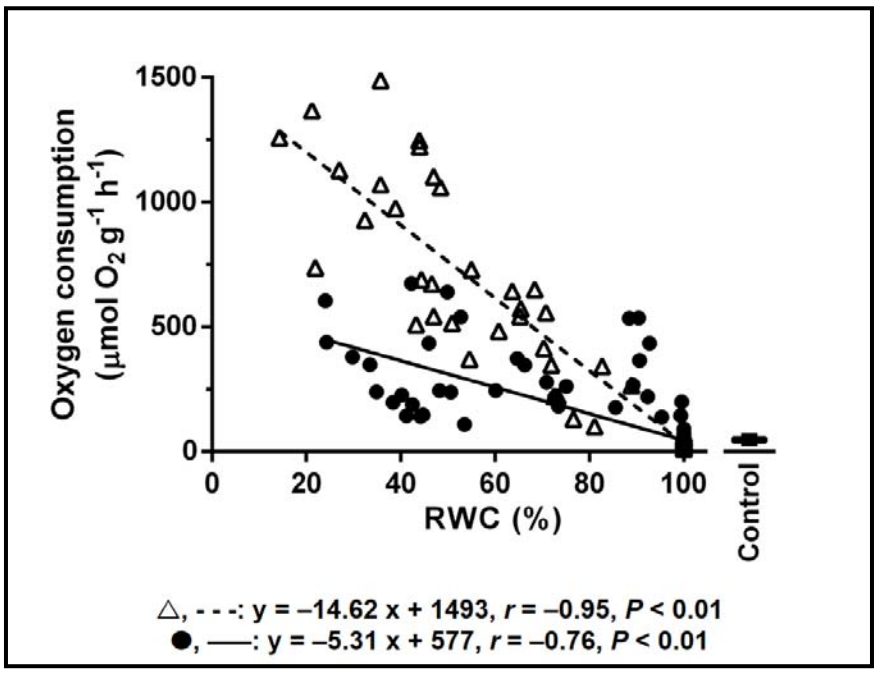

Figure 3.5. Oxygen consumption rate in the first 5–7 min of rehydration in samples dehydrated to different relative water contents (RWCs) at slow (95% RH: ●, ––––) and fast (50% RH: △, - - - - -) dehydration rates, in leaves of the aquatic bryophyte *Fontinalis antipyretica*.

Table 3.2. Oxygen consumption and inhibition by potassium cyanide (KCN) and salicylhydroxamic acid (SHAM) in leaves of the aquatic bryophyte *Fontinalis antipyretica* rehydrated from 20% RWC. Values are Mean and SD (n = 3).

	Oxygen consumption rate (μmol O_2 g^{-1} DW h^{-1})	**Inhibition (%)**		
		+ KCN (1 mM)	**+ SHAM (5 mM)**	**+ KCN (1 mM) + SHAM (5 mM)**
Control	17 ± 1	70 ± 1	16 ± 2	77 ± 7
Slow dehydration	192 ± 14	100 ± 0	0 ± 0	100 ± 0
Fast dehydration	859 ± 58	100 ± 0	0 ± 0	100 ± 0

3.5. Discussion

3.5.1. ROS production and damaged cells

In this work, ROS production in cells of the aquatic bryophyte *F. antipyretica* was shown to be related to the dehydration rate, with low levels of ROS production determined in leaves that were slowly dehydrated and significantly higher levels in those that were rapidly dehydrated. The 70-fold difference in the production of ROS between rapidly and slowly dehydrated samples is clearly relevant to the impact of oxidative damage on cell survival, since these compounds are lethal to bryophytes.

While there are no previous reports showing a relationship in bryophytes between the rate of water loss and ROS production, similar results were obtained with *S. ruralis* with respect to the activity of glutathione reductase (GR), an antioxidant enzyme (Dhindsa, 1987). In that study, slow dehydration of the bryophyte correlated with an increase in GR and thus with low cellular levels of oxidized glutathione (GSSG), whereas during fast dehydration there was no change in GR activity and GSSG levels were higher (Dhindsa, 1987). In a later study, these high levels of GSSG were shown to account for the inhibition of protein synthesis observed immediately after rehydration (Dhindsa, 1991), an effect that was suggested to be a carry-over from the post-desiccation repair mechanisms. According to Proctor and co-workers, the lack of recovery from rapid dehydration occurring in the light may be due to photodamage since in bryophytes the recovery after desiccation was shown to be independent of *de novo* protein synthesis in the dark, suggesting that organelles need time to resume their original shapes and functions (Proctor, 2000; Proctor and Smirnoff, 2000; Proctor *et al.*, 2007*a*, 2007*b*; Pressel *et al.*, 2009*)*. Oxidative stress increased directly with the dehydration rate in *S. ruralis* (Dhindsa, 1987), as was also the case in *F. antipyretica.* Thus, despite their completely different ecological preferences, the former occurring in the desert the latter in streams, the physiological responses of these two species to desiccation are similar. Moreover, in both species, slowly dried samples were shown to completely recover after a desiccation event (Dhindsa, 1987; **Chapter 2**). However, in rapidly dehydrated samples, photosynthesis and respiration returned to control levels after a few hours in *S. ruralis* (Schonbeck and Bewley, 1981) whereas recovery was shown to be only partial (25–50%) in *F. antipyretica*, perhaps due to the death of some of the cells (**Chapter 2**). This conclusion is supported by the present work, in which some of the dehydrated cells were observed to be completely empty, especially those subjected to fast dehydration.

The death of some rapidly dehydrated cells and the disrupted aspect of others were likely related to the high-level production of ROS, as determined using the $DCFH_2$-DA probe and confocal microscopy. These morphological changes were also seen in *Fontinalis hypnoides* Hartm., in which exposure to pollution and other stresses (Glime and Keen, 1984; Gimeno and Puche, 1999) ultimately led to cell death. Our finding that empty cells also produced high levels of ROS suggested that these compounds are involved in the cell-damaging effects. The increase in red fluorescence following rehydration, with its spread from one point in the cell across the cytoplasm, was likely due to the leakage of phenolic compounds from membrane-damaged vacuoles (Hura *et al.*, 2009).

Regarding the spatial pattern of ROS production with respect to cell injury the marginal parts of the leaves would seem to be more susceptible to the negative effects of drying since they are in closer contact with the dry atmosphere, however this was not the case. In slowly dried leaves, the cells that produced ROS and showed evidence of injury were scattered across the leaves rather than clustered along the margins. This pattern resembles that of plant signalling cells in response to pathogenic infection (Morel and Dangl, 1997). In rapidly dried leaves, the majority of the cells produced ROS and were injured.

Among the ROS produced in *F. antipyretica* is H_2O_2. Indeed, there was a large burst in H_2O_2 production immediately after rehydration, analogous to what occurs in desiccation-tolerant *A. androgynum* (Mayaba *et al.*, 2002) and in the less tolerant *Dumortiera hirsuta* (Sw.) Nees (Beckett *et al.*, 2004). As shown by the DAB assay, H_2O_2 production is associated not only with chloroplasts but also with plasma membranes, especially in rapidly dried cells. However, since H_2O_2 is able to permeate membranes and aquaporins (Henzler and Steudle, 2000) it was difficult to determine its relative proportions in the different cellular fractions.

Bryophytes subjected to slow drying had sufficient time (more than 3 h) to adapt to the desiccation event and their limited damage compared to rapidly dried samples suggests the induction of protection mechanisms involving protein synthesis. According to this scenario, bryophytes subjected to fast dehydration (3 h) would lack the time needed to induce protein synthesis and cellular anti-oxidative systems, resulting in enhanced ROS production and cell death. Bryophytes were shown to have both constitutive and inducible mechanisms to cope with desiccation (Oliver and Bewley, 1997; Mayaba *et al.*, 2001). Since rapidly dehydrated samples of *S. ruralis* but not of *F. antipyretica* recover after a few hours, the relative importance of such protection mechanisms would seem to differ in the two species. Alternatively, their temporal

regulation may be different, with *F. antipyretica* more dependent on long-term inducible protection mechanisms. In desiccation-tolerant vascular plants such as *Craterostigma plantagineum* Hochst., a desiccation event that allows a slow rate of dehydration will lead to the induction of desiccation tolerance mechanisms allowing survival. These mechanisms involve sucrose and Late Embryogenesis Abundant (LEA) proteins (Bianchi *et al.*, 1991; Bartels and Salamini, 2001), both of which are also present in bryophytes (Oliver *et al.*, 2004).

In most studies in which bryophytes were subjected to desiccation neither the rate of water loss nor the drying method used was reported (e.g. Sun, 2002). For example, in the various studies, drying has been carried out at constant RH in silica gel, or at the same RH but at different temperatures (10 and 22 ºC), presumably resulting in different rates of water loss that make it difficult to compare species performance. In bryophytes grown in the field, morphological features and habitat variations can be expected to dramatically and specifically alter water loss conditions. Our results indicate the need to consider the rate of water loss at the cellular level if bryophytes are to be correctly classified in terms of their physiological tolerance of desiccation.

3.5.2. The oxygen consumption burst during rehydration

Oxygen consumption and inhibitor effects in control samples of *F. antipyretica* were similar (slightly less KCN inhibition) to those reported by other authors (Maberly, 1985; Azcón-Bieto *et al.*, 1987). However, in this work, despite a similar RWC, oxygen consumption was lower in slowly than in rapidly dehydrated samples, suggesting that there is a critical limit to the water loss rate above which a large burst in oxygen consumption occurs. Yet, regardless to the dehydration rate, the oxygen consumption burst was insensitive to SHAM, an inhibitor of the mitochondrial alternative oxidase, but was fully inhibited by KCN, an inhibitor of the mitochondrial cytochrome *c* oxidase, as well as several metallo-enzymes, such as catalases (Allen and Whatley, 1978), peroxidases (Choi *et al.*, 2007) and superoxide dismutases (Chen *et al.*, 2001) involved in ROS detoxification. A burst in oxygen consumption has also been observed in other bryophytes (Dilks and Proctor, 1974; Krochko *et al.*, 1979) and lichens (Smith and Molesworth, 1973; Farrar and Smith, 1976), in which an uncoupling of mitochondrial respiration (Krochko *et al.*, 1979) and a breakdown of cell compartmentalization (Farrar and Smith, 1976) were, respectively, proposed as the underlying mechanisms. However, the magnitude of oxygen consumption is not compatible with the amount of mitochondrial respiration, suggesting the additional involvement of superoxide ($O_2^{\bullet -}$)

production, with the consumption of molecular oxygen, in a reaction catalysed by KCN-sensitive extracellular peroxidases (Bestwick *et al.*, 1997). The loss of tonoplast integrity may also explain the high level of oxygen consumption since increased ROS production can account for the burst in oxygen consumption and may bring the vacuolar content, enriched in phenols, in contact with cytosolic polyphenol oxidases (Thipyapong *et al.*, 2004) that catalyse the oxidation of these compounds. An increase in red fluorescence under conditions of dehydration stress has been correlated with a high content of phenolic compounds (Hura *et al.*, 2009). In *F. antipyretica*, with its high phenol content (Glime, 2006), this would explain the browning of the photosynthetic tissues.

3.6. Conclusion

In summary, the slow dehydration rate in *F. antipyretica* seems crucial for the establishment of desiccation tolerance, as demonstrated in **Chapter 2**. This may allow the induction of cell protection mechanisms similar to what happens to mesic bryophytes in opposition to the highly desiccation tolerant terrestrial bryophytes. These protection mechanisms limit ROS production and reduce the oxidative burst, increasing the survival rate of cells upon rehydration.

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Chapter 4

Nitric oxide production alleviates membrane damage in bryophyte cells following desiccation

4. Nitric oxide production alleviates membrane damage in bryophyte cells following desiccation

4.1. Abstract

Desiccation affects, amongst others, membrane integrity leading to intracellular solute leakage. We test the hypothesis that fast drying at cellular level increases membrane damage (measured through lipid peroxidation) permeability (measured through sequential elution techniques and conductivity), reactive oxygen species (ROS) (measured through ROS specific probe) and nitric oxide (NO) (measured through NO end-product quantification). Our results confirm this hypothesis since following rehydration, the increase in ROS and NO production correlates with membrane leakage. We found a very strong signal of autofluorescence that was interpreted as resulting from the release of phenolic substances from the vacuole reacting with cytosolic components. On the other hand, slow dehydration rate showed lower leakage rate of intracellular cations. Moreover, upon rehydration we observe a very high increase of NO that could be related to ROS scavenger activity and/or activating signal pathways to induce cellular protection/repair mechanisms. The membrane damage associated with ROS production may lead to cell death and prevent recovery following a desiccation event.

Keywords: bryophytes, fluorescence, lipid peroxidation, membrane damage, nitric oxide, oxygen consumption

4.2. Introduction

The cell membrane is an obvious place for desiccation damage, suggested by cytoplasmic solutes leakage (Oldenhof *et al.*, 2006). During desiccation, poikilohydric organisms lose most of its water leading to a reduction of the surface area of the membrane. In order to accommodate the reduction in cell volume, membranes fold or form vesicles. It is important to maintain total membrane surface area so that upon rehydration, when volume increases, cells maintain their integrity, avoiding burst (Steponkus, 1979; Steponkus and Lynch, 1989; Steponkus *et al.*, 1995). Bryophytes, which usually have small vacuoles, suffer less physical damage throughout this process than bryophytes with big vacuoles (Bewley and Krochko, 1982) maintaining a high surface area/volume ratio in cells and vacuoles, avoiding membrane fusion (Iljin, 1957; Bewley, 1979).

Throughout dehydration, membranes change from a liquid crystalline to a gel phase that may lead to membrane fusion and loss of cell compartmentation (Crowe *et al.*, 1986; Crowe and Crowe, 1992; Steponkus *et al.*, 1995). However, the desiccation tolerant bryophyte *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr can retain normal organelle organization in the dried state (Platt *et al.*, 1994) and regain normal function during rehydration (Tuba *et al.*, 1996). Nevertheless, even the desiccation tolerant bryophytes go through some leakage upon rehydration (Brown and Buck, 1979; Bewley and Krochko, 1982) that could result from the lipid-phase transitions occurring in the membrane upon rehydration (Crowe *et al.*, 1992). The study of pollen membranes and liposome-based experiments (Golovina *et al.*, 1998) lead to the hypothesis that amphiphiles that are mobilized from the cytoplasm into membranes may function as antioxidants protecting membranes from damage by reactive oxygen species (ROS) generated during desiccation and rehydration. These membrane amphiphiles may explain the initial leakage upon rehydration that occurs through an intact membrane. However, this could be an acceptable loss in exchange for protection against oxidative stress.

Amongst the electrolytes that leak after rehydration due to membrane transitions from gel to liquid phase, the loss of ions, particularly potassium (K), from the cell interior has been used as an indication of membrane integrity (Beckett and Hoddinott, 1997; Shakya *et al.*, 2008). This is based on the fact that most of the K is located intracellularly. An accumulation of magnesium (Mg) is also present within the cell, and extracellular levels of this cation may also be used as a marker of severe membrane damage (Branquinho *et al.*, 2011). The recovery of soluble elements from incubation media or washing solutions, measured by conductivity, has also been used

to indicate cellular damage in bryophytes (Niemi *et al.*, 2002). Although some terrestrial bryophytes such as *S. ruralis* are able to protect their cell membranes from oxidative destruction, a group of desiccation-induced effects may still be observed, such as increase in lipid peroxidation and transient K loss (Dhindsa and Matowe, 1981).

Recent works in lichens (Catalá *et al.*, 2010) and seeds (Bai *et al.*, 2011) suggest that nitric oxide (NO) increases desiccation tolerance (DT). In fact, NO has chemical properties that provoke both beneficial and harmful effects in plant cells. It is a very small molecule with hydrophobic properties diffusing freely through membranes (Arasimowicz and Floryszak-Wieczorek, 2007). It can react with the anion superoxide ($O_2^{\bullet-}$) forming the peroxynitrite ion ($ONOO^-$) which is unstable in the physiological pH range. However, the peroxynitrite ion reacts with thiol groups of proteins and polyunsaturated radicals of fatty acid lipids of membrane, causing damage to cell structures (Wendehenne *et al.*, 2001). This ion can also be seen as an antioxidant acting as a ROS scavenging molecule, if the level of ROS production remains relatively low (Lamattina *et al.*, 2003). On the other hand, NO as also been identified as a signal molecule inducing pathways involving abscisic acid (ABA), salicylic acid, jasmonic acid and ethylene that translate into acclimation and/or defence responses (Arasimowicz and Floryszak-Wieczorek, 2007).

Chlorophyll as an emission peak in the red/far-red wavelength when excited by either UV or blue radiation (Lang and Lichtenthaler, 1991). In damaged cells with a high content of phenolic compounds, the loss of tonoplast integrity can bring the vacuolar content in contact with cytosolic polyphenol oxidases (Thipyapong *et al.*, 2004), increasing red autofluorescence of non-chloroplastic origin (Koga *et al.*, 1988; Hura *et al.*, 2009).

Our hypothesis is that fast dehydration leads to higher membrane leakage due to higher oxidative stress upon rehydration, as observed in **Chapter 3**. For this purpose, we used fluorescence techniques to measure both the production of ROS (with a ROS specific probe) and the autofluorescence in the first moments following rehydration. To assess membrane damage we measured lipid peroxidation as well as membrane permeability through sequential elution techniques and conductivity measurements. Since NO has also been associated with DT we assess its production, investigating the correlation with the level of membrane leakage in the different treatments, and discuss possible roles in DT mechanisms.

4.3. Materials and Methods

4.3.1. Plant material and culture conditions

To assure a minimal impact of human activity, samples of *Fontinalis antipyretica* Hedw. were collected at *Serra de S. Mamede National Park*, central Portugal, transported under cooling conditions (about 5 ºC) to the laboratory and cleaned in distilled water. The aquatic bryophyte was kept in a modified Knop culture medium (Traubenberg and Ah-Peng, 2004) under controlled conditions (17 ºC day / 13 ºC night, 20-30 µmol m^{-2} s^{-1} PAR and photoperiod of 16 hours). Each replicate consisted of ten shoot tips with 1 cm each. Relative water content (RWC) was calculated according to Deltoro *et al.* (1998) and as described in **Chapter 2**.

4.3.2. Dehydration induction and recovery

Slow and fast dehydration was attained by placing shoots in small containers over saturated salt solutions of K_2SO_4 (95% RH, –6 MPa) and $Ca(NO_3)_2.4H_2O$ (50% RH, –100 MPa), respectively. Throughout dehydration, samples where maintained under controlled temperature (*circa* 20–23 ºC) and at low photosynthetic active radiation (PAR) (2–5 µmol m^{-2} s^{-1}). Rehydration was made through either immersion in the oxygen electrode solution for 20 min (10 minutes in the dark and 10 minutes in the light) or in culture medium for 72 h (long-term recovery) under similar growth conditions.

4.3.3. Fluorescence emission during rehydration

The autofluorescence of non-stressed, slow and fast dehydrated and rehydrated shoots was analysed with a fluorescence microscope Olympus Provis AX 70. Samples were excited with ultraviolet light using a 330–385 nm excitation filter (Olympus fluorescence filter cube U-MWU2) and the fluorescence emitted was analysed through a 635 nm barrier filter. Images were captured with a digital camera (Canon PowerShot A650 IS).

In an independent assay, fluorescence emission in green (λ_{emi} = 535 nm) and red (λ_{emi} = 635 nm) wavelengths after excitation (λ_{exc} = 485 nm) was measured in a microplate reader (SPECTRAFluor Plus with XFLUOR4 v4.50, Tecan, Switzerland) during 30 minutes in single shoots of non-stressed samples, fast dehydrated samples, and following rehydration of fast dehydrated samples with the epifluorescent probe 2,7-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), used to detect intracellular ROS production (for more details see **Chapter 3**). DNA amount was estimated in the same

samples through fluorescence emission (λ_{exc} = 360 nm; λ_{emi} = 465 nm) of bisbenzimide (Hoechst 33258) (Sigma-Aldrich), a DNA-specific stain (Cesarone *et al.*, 1979).

4.3.4. Cation leakage

Slow and fast dehydrated samples were allowed to reach different RWC at different times (0.5, 1, 2, 24, and 168 h). Intra and extracellular potassium (K), magnesium (Mg) and calcium (Ca) were quantified using a sequential elution procedure developed by Brown and Wells (1988) and modified by Branquinho (1997) for cation location. For the extracellular fraction, each replicate, *circa* 30 mg fresh weight, was shaken for 60 min with 5 ml of 20 mM $NiCl_2$. After removing the extracellular elements, samples were dried overnight at 80 °C and weighed. Intracellular cations (K, Mg and Ca) were then extracted by shaking the samples in 10 ml of 20 mM $NiCl_2$ for 60 minutes. Bryophyte element concentrations were expressed on a total dry weight basis. Each fraction was analysed by atomic absorption spectrophotometry (Varian Techtron AA6, UK) using an air/acetylene flame with added CsCl and $LaCl_3$ (1 g L^{-1}) to both samples and standards, as suppressants of ionization and refractory compound formation, respectively.

4.3.5. Oxygen consumption and conductivity measurements

Slow and fast dehydrated samples were allowed to reach different RWC at different times (0.5, 1, 2, 24, 168, 336, and 960 h). Oxygen consumption was measured prior to dehydration, to determine control values, and in the first moments following rehydration of slow and fast dehydrated shoots using a Clark-type liquid-phase oxygen electrode (DW2/2 electrode chamber, Hansatech Instruments Ltd., Norfolk, UK). Shoots were placed inside the electrode chamber containing 0.1 mM $KHCO_3$ solution for 10 min in the dark, allowing oxygen consumption to be measured in the absence of light. About 30 minutes after rehydration, the electrode chamber solution was collected, diluted in 3 mL deionised water and conductivity was measured with a conductimeter (Con 5 – EcoScan, Eutech Instruments, Singapore). Conductivity measures were also performed 72h after rehydration.

4.3.6. Lipid peroxidation and nitric oxide end-products determination

Non-stressed control shoots, and slow (72 hours) and fast (3 hours) dehydrated shoots (*circa* 20% RWC) were used. Five replicates per treatment weighting approximately 500 mg each were powdered with an ice-cooled mortar and pestle in deionised water. Lipid peroxidation was quantified in the pellet by the TBARS method

according to Catalá *et al.* (2010). To estimate nitric oxide (NO) production, NO oxidation end-product (nitrite) were measured in the soluble fraction of the samples with a colorimetric Nitric Oxide assay kit by Griess reaction (Innoprot Derio, Bizkaia, Spain). The absorbance of the end coloured azo dye was measured at 540 nm.

4.3.7. Statistical analysis

Linear regression and non-linear regression analyses were used to investigate relationships between variables/parameters. The levels of significance (*P*) between observed vs. predicted data were determined from the Pearson correlation coefficients (r) and the degrees of freedom (d.f.). Whenever necessary, significant differences between groups were determined through ANOVA with Tukey post-test (significance level $\alpha = 0.05$). All statistical analyses were performed with GraphPad Prism 5.03 for Windows (2009) (GraphPad Software, San Diego California USA).

4.4. Results

4.4.1. Free radical release and red autofluorescence increases in the first moments of rehydration

After 30 minutes of rehydration following fast dehydration we observed that red autofluorescence (λ_{emi} = 635 nm) not associated to chloroplasts increased (Fig. 4.1d), showing higher fluorescence than control (Fig. 4.1a), dehydrated (Fig. 4.1b) or rehydrated samples following slow dehydration (Fig. 4.1c).

In an independent assay in which fluorescence was measured in a single shoot (4 replicates per treatment) with a microplate reader, fast dried samples showed an increased fluorescence yield both in the green (intracellular ROS) and red (autofluorescence) wavelengths in the first 30 minutes after rehydration with water (Fig. 4.2a,b) when compared with either non-stressed control or dehydrated samples. Non-stressed and dehydrated samples showed no fluorescence variation in the same time period for both wavelengths (Fig. 4.2a,b).

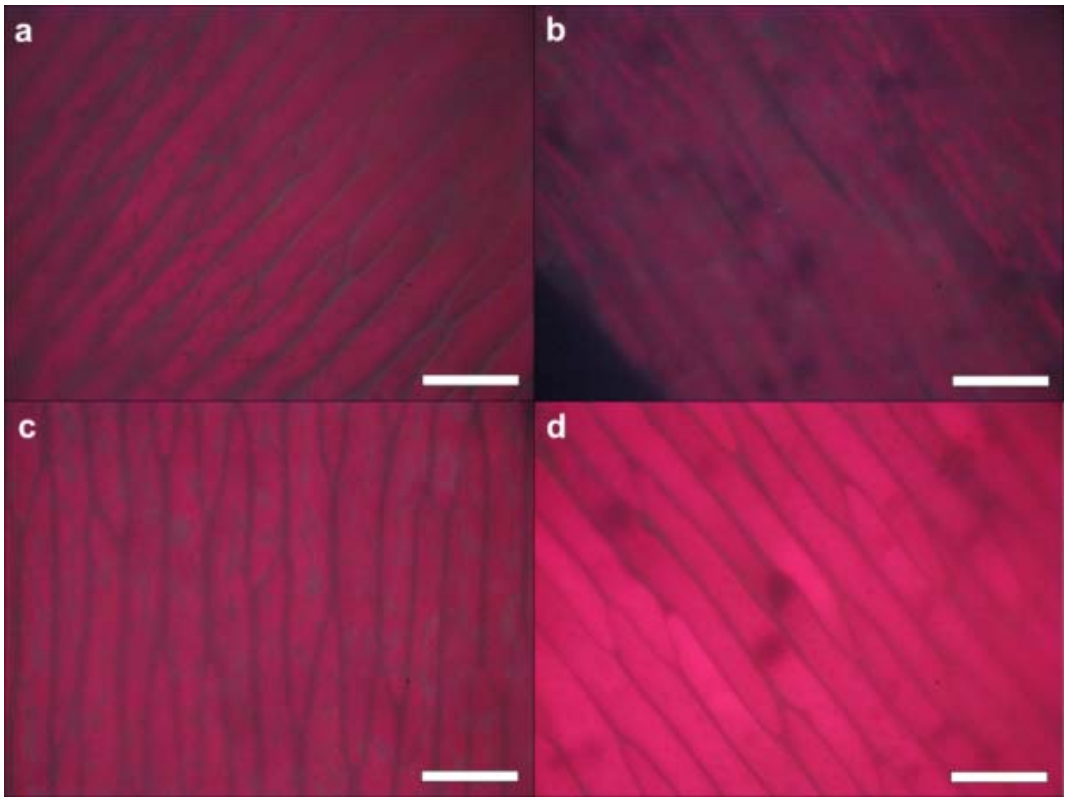

Figure 4.1. Autofluorescence (λ_{exc} = 330-385 nm, λ_{emi} = 635 nm) of the aquatic bryophyte *Fontinalis antipyretica* phyllids (**a**) non-stressed, (**b**) dehydrated, and 30 minutes after rehydration with water following (**c**) slow and (**d**) fast dehydration. White scale bar: 25 µm.

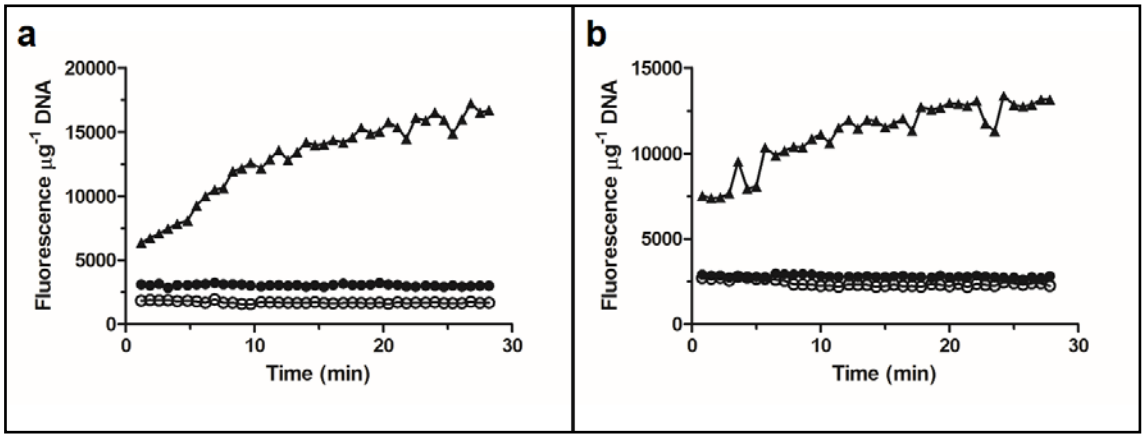

Figure 4.2. Fluorescence quantification (λ_{exc} = 485 nm) in the (**a**) green (λ_{emi} = 535 nm) and (**b**) red (λ_{emi} = 635 nm) wavelength was measured during 30 minutes in non-stressed (O), dehydrated (●), and rehydrated with the epifluorescent probe 2,7-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) following fast dehydration (▲) samples of the aquatic bryophyte *Fontinalis antipyretica*. Each point is the mean of 4 replicates. Standard deviation bars were not included for visualization purposes.

4.4.2. Higher intracellular cations leakage upon rehydration following fast dehydration

Membrane permeability upon rehydration was evaluated using the loss of intracellular K and Mg from the cell and conductivity as indicators (Fig. 4.3) using a sequential elution for cation cellular location. On the other hand, conductivity measurements were also performed, in order to validate its use in future experiments since it is more practical and less time consuming than the sequential elution. At full turgor K was mainly intracellular (95%) (Fig. 4.3a) while Mg was evenly distributed inside (60%) and outside (40%) the cells (Fig. 4.3b), as expected.

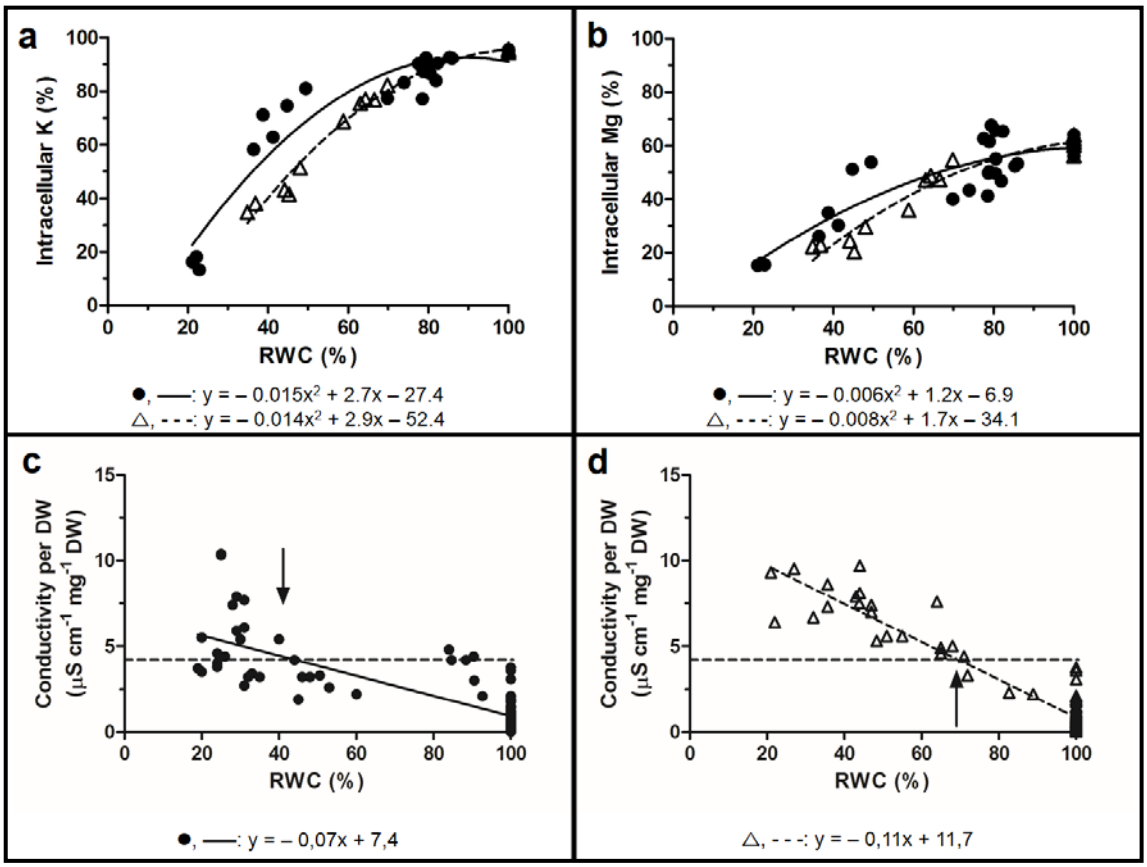

Figure 4.3. Intracellular (**a**) potassium and (**b**) magnesium variation with end-stress RWC in slow (●, ––––) and fast (△, - - - - -) dried samples of the aquatic bryophyte *Fontinalis antipyretica*. Conductivity variation with end-stress RWC in (**c**) slow and (**d**) fast dried samples was also measured. Arrows indicate RWC at which samples begin to present most conductivity values higher than non-stressed control samples.

In slow drying samples, K loss mainly occurred after reaching lower values than *circa* 30% RWC, while in fast drying K was continuously lost throughout dehydration. Magnesium leakage showed similar patterns in both dehydration rates as lower RWC were reached. As ion leakage occurred, the conductivity of the surrounding medium

increased (Fig. 4.3c-d), showing a good correlation with cation loss quantification. However, the values of RWC at which conductivity surpassed non-stressed samples was different, being higher in fast dehydrated samples (60-70%) (Fig. 4.3d) than in slow dehydrated ones (30-40%) (Fig. 4.3c).

A very high oxygen consumption in the first moments of rehydration, already observed in **Chapter 3**, also presented a good positive linear correlation (r = 0.94) with conductivity measurements (Fig. 4.4). However, 72h after rehydration conductivity was at pre-desiccation values in both dehydration treatments (data not shown).

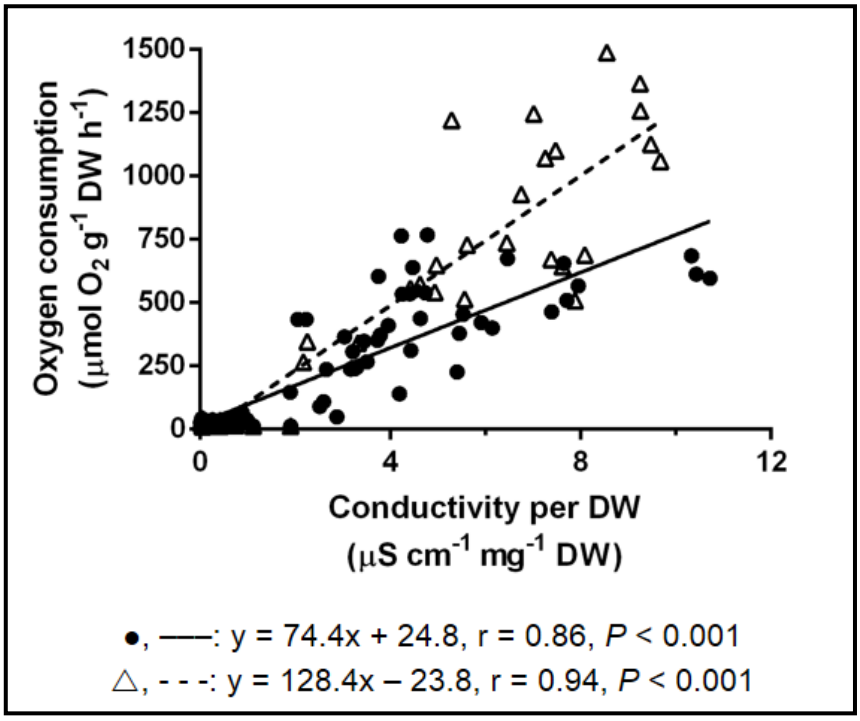

Figure 4.4. Oxygen consumption variation with conductivity in slow (●, ––––) and fast (△, - - - - -) dehydration samples of the aquatic bryophyte *Fontinalis antipyretica*.

4.4.3. Lipid peroxidation remains unaltered

In non-stressed samples, lipid peroxidation (11.4 ± 1.1 nmol MDA g^{-1} FW) was not statistically different from the other treatments (Fig. 4.5). The only exception was the recovery after slow dehydration treatment in which lipid peroxidation was statistically lower than the other treatments (9.2 ± 0.4 nmol MDA g^{-1} FW) but not statistically different from non-stressed samples.

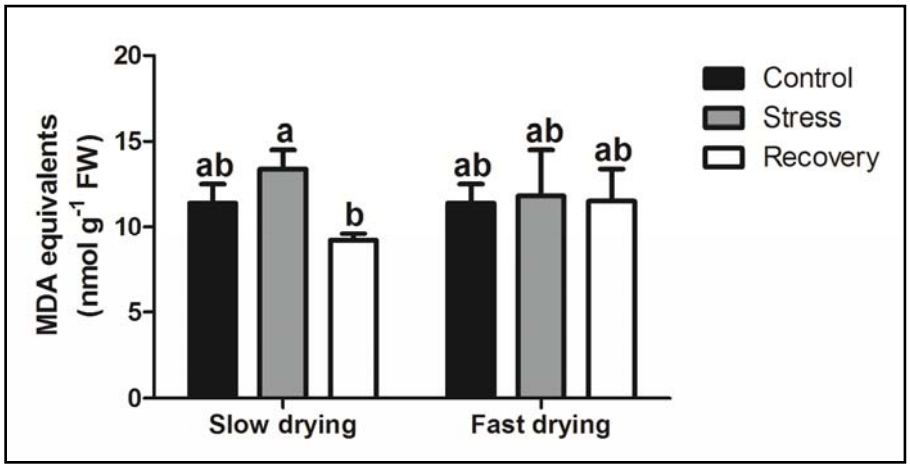

Figure 4.5. Lipid peroxidation quantification in non-stressed (control), fast and slow dehydrated samples (stress) and after rehydration (recovery) in the aquatic bryophyte *Fontinalis antipyretica* shoots. Bars indicate Mean and SD of 5 replicates.

4.4.4. Nitric oxide end-products increased after recovery from desiccation

To observe nitric oxide production in *F. antipyretica*, nitrites as NO end-products (NO_x) were quantified by the Griess method. Values showed significant differences among non-stressed, dehydrated and recovery treatments (Fig. 4.6). Before dehydration, NO_x levels were 15 ± 15 µmol NO_x g^{-1} FW (Fig. 4.6). At the end of the dehydration process NOx levels were not statistically different from non-stressed samples, in either dehydration treatment (Fig. 4.6). However, after rehydration, both dehydration treatments showed an increase in NO_x levels higher in fast dehydrated samples (363 ± 84 µmol NO_x g^{-1} FW) than in slow dehydrated ones (263 ± 77 µmol NO_x g^{-1} FW) (Fig. 4.6).

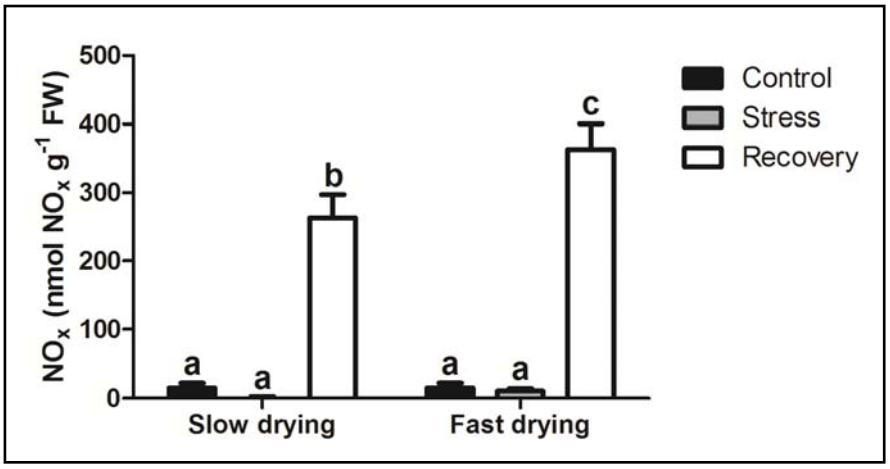

Figure 4.6. Nitric oxide end-products (NO_x) quantification in non-stressed (control), fast and slow dried samples (stress) and after rehydration (recovery) in the aquatic bryophyte *Fontinalis antipyretica*. Bars indicate Mean and SD of 5 replicates.

4.5. Discussion

In the aquatic bryophyte *F. antipyretica* slow drying is essential in order to re-establish quickly the normal metabolism after a desiccation event. All bryophytes show a transient leakage of intracellular contents upon rehydration but only the slow dehydrated were able to prevent high membrane damage. It seems that slow drying enables *F. antipyretica* to engage mechanisms to minimize cell damage, maintaining membrane integrity through establishing low levels of ROS and increasing signal molecules like nitric oxide.

4.5.1. Higher ROS production and red autofluorescence correlates with higher membrane damage

The increase in intracellular ROS (green fluorescence) agrees with the increased oxygen consumption during rehydration of dehydrated samples observed in *F. antipyretica* (**Chapter 3**). In accordance with several authors (Crowe *et al*., 1986; Crowe and Crowe, 1992; Steponkus *et al.*, 1995), this oxygen consumption may result from loss of cell compartmentation, and specifically the release of phenolic compounds into the cytoplasm which react with other cellular contents.

We observed an increase in this type of red autofluorescence of non-chloroplastic origin after fast dehydration which might be associated with membrane damage. Koga *et al.* (1988) observed that following cell death, the release of phenolic compounds from vacuoles after membrane damage resulted in increased red autofluorescence in barley leaf epidermal. Hura *et al.* (2009) also observed an increase in red autofluorescence correlated with the leakage of phenolic compounds from the vacuole due to membrane damage in genotypes of triticale. This may be the case of *F. antipyretica* since it possesses a high phenolic content (Glime, 2006).

4.5.2. Slow dehydration delays membrane leakage

The sequential elution procedure was shown to be a valuable technique for distinguishing between elements acquired from the environment and those located intracellularly, as it was already found for lichens (Branquinho *et al.*, 1999; Branquinho *et al.*, 2011) and bryophytes (Vieira *et al.*, 2009). Both intracellular K and Mg concentrations were significantly correlated with conductivity measurements from the assay solutions, showing that the measurement of conductivity, which is a less time consuming procedure, might be used in this aquatic bryophyte as a truly indication of membrane damage. The results in *F. antipyretica* are in accordance to those reported by other authors (Brown and Buck, 1979; Platt *et al.*, 1994) which showed that one of

the major effects of desiccation is membrane damage, with leakage of important cations upon rehydration. Brown and Buck (1979) had already demonstrated that when subjected to 52% RH for 48 h, some bryophyte species more tolerant to desiccation like *S. ruralis* or *Anomodon viticulosus* (Hedw.) Hook. & Taylor lose only 20-25% of K. In *Atrichum androgynum* (Müll. Hal.) A. Jaeger, dried under silica gel (0% RH; reaching 20% RWC after 8h dehydration), little intracellular K loss was observed during the first 15 minutes rehydration (Mayaba *et al.*, 2002). During dehydration, leakage may be very limited in *F. antipyretica* since no increase in red/green fluorescence was measured when compared to non-stressed ones. Most of the variation in conductivity in *F. antipyretica* was explained by RWC, being almost all K lost upon rehydration after reaching 20% RWC. In slow dried samples conductivity values are only higher than the control after being dehydrated to less than 40% RWC, whereas in fast dried samples this occurs immediately after being dehydrated to 60-70% RWC. This suggests the existence of different threshold of dehydration rate in this species, depending on the rate of water loss.

As the extent of water loss increases, the loss of membrane integrity may lead to impaired cell homeostasis as intracellular K decreases (Walker *et al.*, 1996). Also Mg leakage might have a deleterious effect on the function of the porphyrinic ring of the chlorophyll molecules, leading to their pheophitinization and decreasing the efficiency of light harvesting and primary photochemical reaction. The regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity is dependent of the presence of Mg (Parry *et al.*, 2003) and, thus, the leakage of this cation might be associated with the loss of photosynthetic activity after dehydration, especially after fast dehydration (**Chapter 2**). Moreover, the pattern of Mg loss resembles the F_v/F_m variation with RWC (**Chapter 2**). Several antioxidant enzymes (peroxidase, ascorbate peroxidase, superoxide dismutase) are also dependent of the presence of Mg (Tewari *et al.*, 2006) and, as suggested in **Chapter 3**, the impairment of their activity can lead to higher ROS production and increased cell damage.

Lipid peroxidation of membrane fatty acids is prevented in *S. ruralis* through suppression of lipoxygenase activity in part by oil droplets present which acts as a buffer for peroxidation (Stewart and Bewley, 1982). However another DT bryophyte, *A. androgynum*, showed significant membrane lipid peroxidation (Guschina *et al.*, 2002). It has also been observed that amphiphilic substances like phenolic compounds relocate from the aqueous cytoplasm to the membranes as lower water contents are reached, acting as strong antioxidants and stabilizing membranes (Beckett and Minibayeva, 2007). It seems that despite high ROS in fast dehydration (**Chapter 3**) *F. antipyretica*

antioxidant defences such as the high phenolic content may be enough to prevent significant lipid peroxidation but not enough to avoid membrane leakage and prevent cell death. This may be related to other forms of membrane protection that fast dehydration does not allow to be established.

4.5.3. Nitric oxide increases after rehydration

During rehydration an increase in NO production has been shown in lichen species (Piccotto, 2009; Catalá *et al.*, 2010). The measurement of nitrites through the Griess reaction is a method used to estimate NO production (Nagano, 1999). NO is known to modulate the toxic effects of ROS, acting either as a scavenger or further increase lipid peroxidation, depending on its concentration (Kröncke *et al.*, 1997; Darley-Usmar *et al.*, 2000; Miranda *et al.*, 2000). Lipid peroxidation did not change dramatically in *F. antipyretica* and that could be related, at least in part, to the fact that NO terminates lipid peroxidation in aqueous medium (Hiramoto *et al.*, 2003). Guschina *et al.* (2002) suggested that ABA can mediate these mechanisms, since a putative role of NO was proposed in signalling for dehydration protection mechanisms. This suggests that *F. antipyretica* is adapted to dehydration/rehydration cycles if dehydration is slow. In higher plants, NO reversibly binds to PSII (Petrouleas and Diner, 1990; Diner and Petrouleas, 1990; Goussias *et al.*, 1995; Sanakis *et al.*, 1999) also modulating electron transfer and quenching processes (Wodala *et al.*, 2008). Our results suggests the importance of NO in antioxidant systems in the first moments of rehydration, as was already seen in lichens (Weissman *et al.*, 2005; Catalá *et al.*, 2010). NO confers chlorophyll stability and prevents lipid peroxidation, allowing photosynthesis to resume its normal function upon rehydration (**Chapter 2**).

4.6. Conclusion

In this work we observed the effect of NO production in alleviating membrane damage as long as low levels of ROS are maintained, either by direct action or through signal pathways. The increase in ROS and red autofluorescence can be used as an indicator for detecting membrane damage and cell rupture in future works in desiccation and drought stress. However, future work will be necessary to identify which substances are responsible for these changes in fluorescence.

4.7. Acknowledgements

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Chapter 5

The influence of fast and slow dehydration on elasticity of bryophytes cell wall

5. The influence of fast and slow dehydration on elasticity of bryophytes cell wall

5.1. Abstract

During desiccation, very low water potentials are reached in the cells. Osmoregulation through increase of soluble materials (e.g. soluble sugars) is a response to the increasing osmotic pressure in the cells. In bryophytes, sucrose acts as an osmolyte also stabilizing membranes and proteins through vitrification. We used psychrometric measurements in the aquatic bryophyte *Fontinalis antipyretica* Hedw. to construct pressure-volume isotherms and determine the water relations parameters under fast and slow dehydration rates. Sucrose was also extracted and quantified. The starting hypothesis was that a slow dehydration rate would increase sucrose concentration and change water relation parameters, especially osmotic potential at turgor loss point and cell wall elasticity modulus, leading to differences in osmoregulation between drying rates as a response to decreasing water potential in the cells. When dehydrated, *F. antipyretica* lost their turgidity at relative water content (RWC) of 50-60%, independently of drying rate. However, fast dehydrated samples present more elastic cell walls allowing cells to shrink and slow water loss acting as an attempt of desiccation tolerance (DT) strategy. In addition, sucrose also increased in fast dehydrated samples increasing the osmotic potential at turgor loss point which led to higher osmoregulation. However, upon rehydration, fast dried samples lost 50% of the sucrose through leakage. On the other hand, slow dehydrated cells maintain their sucrose levels constant, producing sucrose during recovery to compensate the small parcel that is lost through leakage upon rehydration. Nevertheless, DT appears to be achieved through slow dehydration and, at this drying rate, it does not require significant changes in sucrose concentration to survive desiccation. On the other hand, external water is able to be maintained at very high levels due to the life form of *F. antipyretica* allowing a slow dehydration rate required for induction of other DT mechanisms.

Keywords: bryophytes, desiccation, *Fontinalis*, sucrose, turgor, cell wall elasticity, water relations components

5.2. Introduction

Bryophytes are typically poikilohydric plants, with water content depending on the humidity of the surrounding atmosphere, exhibiting, however, different mechanisms to delay water loss and often being able to tolerate extreme desiccation (Proctor and Pence, 2002). These organisms lack conducting tissues like vascular plants, absorbing directly external water. Thereby, in addition to the symplast water fraction (within the protoplasts) and the apoplast water fraction (cell walls and spaces between cells) also present in vascular plants, a third fraction, the external capillary water, must be taken into account (Dilks and Proctor, 1979; Beckett, 1997; Proctor *et al.*, 1998; Proctor and Tuba, 2002).

When exposed to a drying atmosphere, the water and osmotic potential inside cells change, occurring osmoregulation by increasing soluble molecules to maintain homeostasis and preserve cell structure and functionality. The construction of pressure-volume (PV) curves allows the determination of several water relations parameters (Santarius, 1994; Beckett, 1997; Proctor *et al.*, 1998; Hájek and Beckett, 2008). Therefore, water potential (ψ) is plotted as a function of RWC and then re-plotted as ($-1/\psi$) against relative water deficit (RWD = 100 – RWC). This allows a linear relation at lower water potentials, where turgor does not act, as well as the determination of several water relations parameters. The osmotic potential at full turgor ($\psi_{\pi s}$) and at turgor loss point ($\psi_{\pi TLP}$) allows identifying if osmoregulation occurs. On the other hand, the apoplastic water fraction, and the elastic modulus of cell walls (ε) allows the identification of adaptive mechanisms to dehydration, measuring water and the elasticity/rigidity of cell walls (Hájek and Beckett, 2008).

Osmotic adjustment enables water potential to be maintained during mild dehydration, involving compatible solutes and sugars, such as sucrose (Ingram and Bartels, 1996). Moreover, sucrose has an important role in desiccation tolerance (DT), acting as an osmoregulator and preventing denaturation of important macromolecules and membranes, contributing to biological vitrification of the cytoplasm of dried cells, slowing down damaging reactions with reactive oxygen species and maintaining cell structure intact (Crowe *et al.*, 1992; Smirnoff, 1992). High concentrations of sucrose were found in dry tissues of DT bryophytes and higher plants (Buitink *et al.*, 2002). In the DT terrestrial bryophyte *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr, sucrose makes up approximately 10% of the dry mass and its concentration is maintained during desiccation and rehydration (Bewley *et al.*, 1978).

Drying rate is crucial for DT induction in the aquatic bryophyte *Fontinalis antipyretica* Hedw. as seen in the previous Chapters. The aim of the present work was

to investigate how contrasting (fast and slow) dehydration rates change cell water relations in an aquatic bryophyte and if those changes can explain the requirement of slow dehydration to induce DT. Sucrose content was also evaluated as well as the role of this sugar on cell osmoregulation. The initial hypothesis was that a slow dehydration rate allows an increase in sucrose content and change water relation parameters, especially osmotic potential at turgor loss point, leading to differences in osmoregulation between drying rates.

5.3. Material and Methods

5.3.1. Plant material and culture conditions

Bryophyte samples of *F. antipyretica* were collected at the *Serra de S. Mamede Natural Park*, central Portugal in a clean stream, away from human impact. Samples were transported to the laboratory under cooling conditions (about 5 ºC) and cleaned of debris and sediments in distilled water. Bryophyte were grown in a modified Knop culture medium (Traubenberg and Ah-Peng, 2004) under controlled conditions (17 ºC day / 13 ºC night, 20-30 µmol m^{-2} s^{-1} PAR and photoperiod of 16 hours). Samples were left in the lab for no more than 60 days before analysis. In the water relations assay, each sample consisted of a single shoot tip with 1 cm, while in the sucrose assay samples consisted of ten shoot tips bundled together. Relative water content (RWC) was calculated according to methodology in **Chapter 1**. After blotting any external water from the tips and before drying, samples where weighted to determine full turgor weight (FTW). After the dehydration treatment, fresh weight (stress weight) was determined. At the end of the assays, dry weight (DW) was determined by placing the samples at 80 ºC for 48 h.

5.3.2. Dehydration induction

Different dehydration rates were attained by placing shoots in small containers over saturated salt solutions of K_2SO_4 (slow dehydration, 95% RH, –6 MPa) and $Ca(NO_3)_2.4H_2O$ (fast dehydration, 50% RH, –100 MPa). During this period, conditions where maintained under controlled temperature (*circa* 20–23 ºC) and at low photosynthetic active radiation (PAR) (2–5 µmol m^{-2} s^{-1}). In the water relations assay, slow dehydration was attained by air drying bryophytes at lab conditions (*circa* 80% relative humidity) a value lower than the one used in the other Chapters, but still high enough to allow slow dehydration. Rehydration was made through immersion in culture medium for 72 h under similar growth conditions.

5.3.3. Water potential determination and water relation components calculation

Slow dehydrated (blotted and non-blotted) and fast dehydrated (blotted) samples were placed in a small steel cup and introduced in a C-52 chamber (Wescor Electronics, Logan, USA) linked to a PsyPro microvoltmeter (Water Potential System, Wescor Electronics, Logan, USA) for an equilibration period of 30 minutes before water potential (ψ) measurement. Between measurements, samples were allowed to lose water inside small containers containing a saturated salt solution (see previous description of dehydration induction). Five standard solutions of NaCl (0, 0.05, 0.25, 0.5 and 1 molal) where used to establish a calibration curve. After RWC determination, PV curves where plotted as $-1/\psi$ versus RWD (100 – RWC). Water relations components where calculated according to Hájek and Beckett (2008). At low ψ, turgor does not contribute to ψ and the PV curve is linear. Apoplastic water was determined as the *x*-intercept of the linear portion of the PV curve. Osmotic potential (ψ_π) at full turgor ($\psi_{\pi s}$) was calculated as the *y*-intercept of the same linear portion of the PV curve. Turgor potential (ψ_p) was determined as the difference of the extrapolated linear portion and the real curve. For simplification of visualization, it was not plotted on the graph. Turgor loss point (TLP) was determined at the point where ψ_p reached zero, where it was also determined the osmotic potential at turgor loss point ($\psi_{\pi TLP}$). Above TLP, ψ_p increased linearly until full turgor was achieved and cell walls elasticity modulus (ε) was determined as the slope of this line.

5.3.4. Sucrose quantification

Soluble sugars extraction was performed as described by Arrabaça (1981). Shoots with approximately 0.1 g were quickly grinded with 500 µl of 80% (v/v) ethanol in a mortar with pestle at room temperature. The homogenate was quickly transferred to tubes with 1 ml of 80% (v/v) ethanol and placed for 5 min at 80 ºC. Extracts were cooled on ice, and then centrifuged at 16,000 *g*, for 5 min in a microcentrifuge (Centrifuge 5415 C, Eppendorf). The supernatant was transferred to another tube, evaporated in nitrogen (N_2) flow, resuspended in 1 ml of ultra-pure water and filtered through 0.45 µm filters (Dinistrat Filter RC 25, Sartorius). Sucrose quantification was determined following the resorcinol (1,3-hidroxybenzene) method (Roe, 1934). Briefly, 100 µl of the extract or the recovery solution were added to 0.25 ml of resorcinol solution (1% diluted in absolute ethanol (w/v)), and to 0.75 ml of a solution of 30% HCl (w/v), mixed and incubated at 80 ºC for 8 min. Samples were cooled on ice and absorbance at 405 nm was read in the microplate reader (ELx800, BioTek, USA). A regression trendline was determined through the application of this method to solutions with pre-determined concentrations, allowing the sucrose quantification in the sample.

5.3.5. Statistical analysis

All statistical analyses were performed with GraphPad Prism 5.03 for Windows (2009) (GraphPad Software, San Diego California USA). Relationships between variables/parameters were investigated running linear regression analysis. Three replicates of slow dehydrated and fast dehydrated samples where used in the measurements of water relation components. Whenever necessary, significant differences between groups were determined through ANOVA with Tukey post-test (significance level α = 0.05).

5.4. Results

5.4.1. Cell water relations

Pressure-volume (PV) curves for slow and fast dehydrated samples where constructed (Fig. 5.1) allowing the determination of several parameters of water relations (Table 5.1) and the distribution of water in *F. antipyretica* in the different dehydration rates.

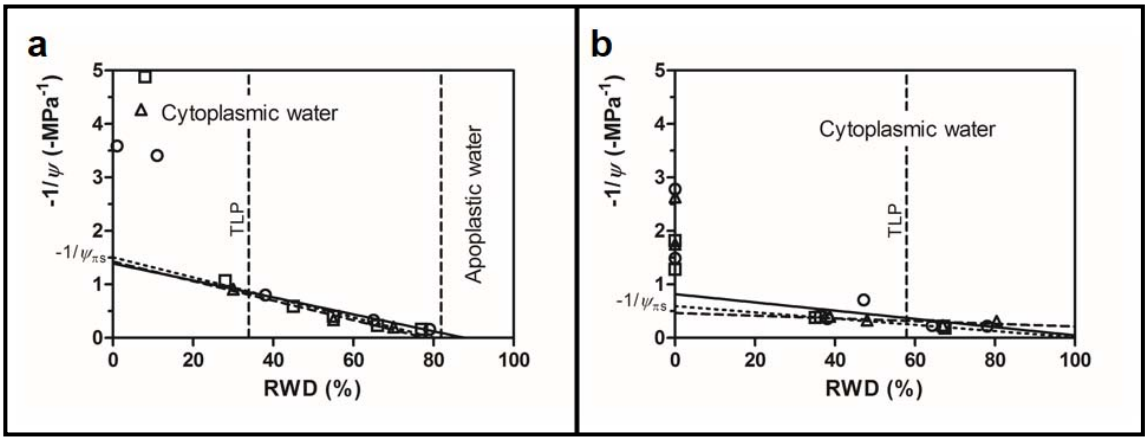

Figure 5.1. Reciprocal of water potential (–1/ψ) plotted against relative water deficit (RWD = 100–RWC) (pressure–volume curves) of the aquatic bryophyte *Fontinalis antipyretica* (three independent samples, ○, △, □) in (**a**) slow dehydrated and (**b**) fast dehydrated. Turgor loss point (TLP) was determined when turgor pressure (ψ_P) reached zero (not plotted) calculated as the difference of the extrapolated linear portion and the actual pressure–volume curves. At low ψ, turgor does not contribute to ψ and the PV curve is linear (——, - - - -, ·····), corresponding the *y*-intercept of the extrapolated line at RWC = 100% to the reciprocal of osmotic potential at full turgor ($1/\psi_{\pi s}$) and the *x*-intercept to the apoplastic water in the shoots. In fast dehydrated samples, no apoplastic water was present.

Table 5.1. Parameters of water relations in slow and fast dehydrated samples of the aquatic bryophyte *Fontinalis antipyretica*. Values are Mean ± SD of three replicates. Different letters indicate significant differences between treatments ($P < 0.05$; Tukey's HSD test). For parameter explanation, see text.

	Slow dehydration	Fast dehydration
RWC_{TLP} (%)	58 ± 4[a]	42 ± 30[a]
ε (MPa)	7.54 ± 2.09[a]	1.45 ± 1.05[b]
$-\psi_{\pi s}$ (MPa)	0.69 ± 0.03[a]	1.68 ± 0.46[b]
$-\psi_{\pi TLP}$ (MPa)	1.44 ± 0.24[a]	3.68 ± 2.83[a]
Apoplastic water (%)	18 ± 5[a]	0 ± 0[b]

Although, RWC at turgor loss (RWC_{TLP}) is not statistically different in both treatments (*circa* 50–60% RWC), the elasticity modulus of cell walls (ε) changes with drying rate being much higher (more rigid) in slow dehydrated (ε = 7.54 ± 2.09) than in fast dehydrated samples (more elastic) (ε = 1.45 ± 1.05). The osmotic potential at full turgor ($-\psi_{\pi s}$) also differs with treatment being higher in samples submitted to fast dehydration (–1.68 ± 0.46 MPa) than to slow dehydration (–0.69 ± 0.03 MPa). The osmotic potential at turgor loss point ($\psi_{\pi TLP}$) is higher in fast dehydrated samples (–3.68 ± 2.83 MPa) but not statistically different from slow dehydrated ones (–1.44 ± 0.24 MPa), although the first present a higher variance. Regarding the content of apoplastic water, in slow dehydrated samples is *circa* 20% but in fast dehydrated samples it was absent.

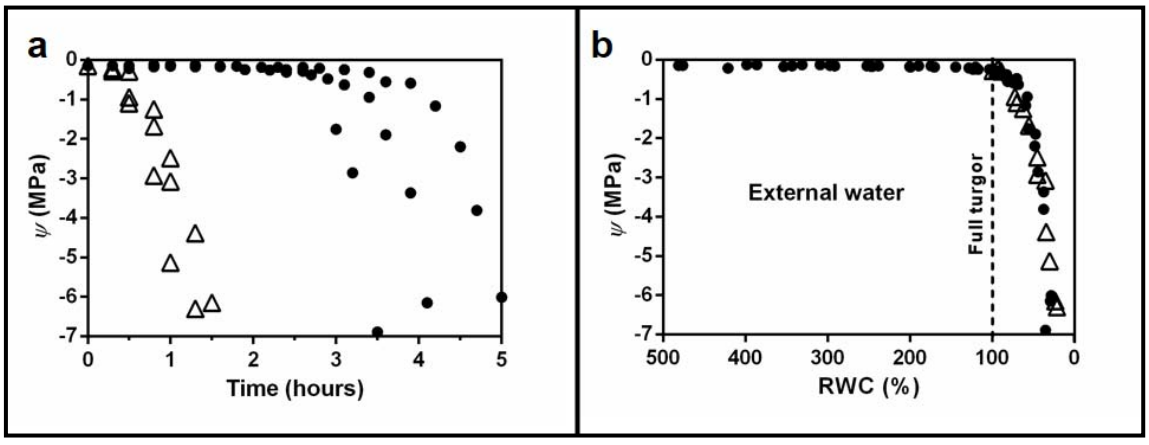

Figure 5.2. Water potential (ψ) plotted against (**a**) time (hours) and against (**b**) decreasing relative water content (RWC) in one shoot of the aquatic bryophyte *Fontinalis antipyretica* non-blotted (●) and blotted free from water (△) (three independent samples). In (**b**), dashed line limits the full turgor water content (RWC = 100%), with all water content on the left of the line corresponding to external water.

The results of water potential variation with time show that according to the level of external water present in the shoot, cells start to decrease their water potential at different moments in time (Fig. 5.2a). However, samples blotted free from external water start to lose water at exactly the same moment (Fig. 5.2a). On the other hand, water potential is near zero before it reaches full turgor (RWC = 100%) after which it starts to decline, with or without blotting (Fig. 5.2b). Moreover, the bryophyte can store high amounts of external water, up to five times the value at full turgor and once the external water is removed (blotting), cell water potential starts to decrease.

5.4.2. Sucrose quantification

Sucrose content was measured in non-stressed control samples, at the end of slow and fast dehydration, and after rehydration of slow and fast dehydrated samples (Fig. 5.3a). Sucrose leakage was also quantified after retrieving the recovery solution (Fig. 5.3b). The results show that the sucrose content is *circa* 170 µmol g^{-1} DW (6% of DW) in unstressed conditions and in slow dehydrated samples but increases to *circa* 250 µmol g^{-1} DW (9% of DW) after being submitted to fast dehydration. After recovery, sucrose content remains unchanged in slow dehydration, but decreases to *circa* 120 µmol g^{-1} DW (4% of DW) in fast dehydrated samples. From the recovery medium collected after rehydration, almost 50% is lost in fast dehydration samples, whereas slow dehydration about 20-30% was recovered from the medium although not statistically different from control (Fig. 5.3b).

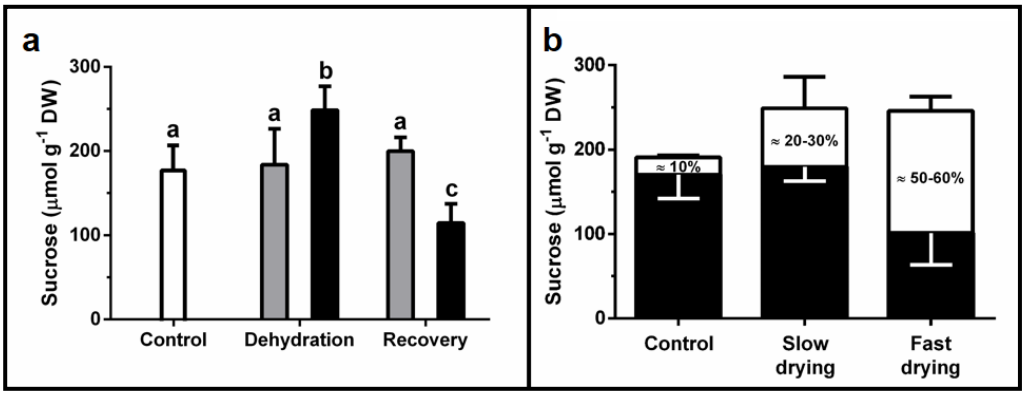

Figure 5.3. Sucrose content of the aquatic bryophyte *Fontinalis antipyretica* samples (**a**) non-stressed (white bar) and slow (grey bars) and fast (black bars) dehydrated at the end of dehydration and after recovery (72 hours). Sucrose leakage upon rehydration (**b**) was also determined measuring sucrose content in *F. antipyretica* samples (black bars) and in the recovery medium (white bars) in slow and fast dehydrated samples (percentages of sucrose leakage are shown). Values are Mean and SD. Different letters indicate significant differences between treatments.

5.5. Discussion

This study compared the water relations parameters (such as cell wall modulus of elasticity, turgor loss point, osmotic potential and external water) of the aquatic bryophyte *F. antipyretica* under contrasting drying rates, allowing to further analysing its DT. When compared with other bryophytes, most of the parameters are very similar, independently of habitat. However, there are differences between samples exposed to different dehydration rates, and the mechanisms behind the DT tolerance of *F. antipyretica*.

5.5.1. Osmoregulation alone does not contribute to desiccation tolerance

The RWC_{TLP} *in F. antipyretica* (*circa* 50-60%) is similar to those found for *Plagiomnium rhynchophorum* (Harv.) T.J. Kop. (*circa* 55%) (Beckett, 1997), *Conocephalum conicum* (L.) Underw. (*circa* 45%) (Proctor *et al.*, 1998) and *Sphagnum* spp. (35-60%) (Hájek and Beckett, 2008). These values are lower than the ones observed in vascular plants (*circa* 75%) (Bartlett *et al.*, 2012) allowing bryophytes to maintain their metabolism active at lower RWC.

Differences were observed in $\psi_{\pi s}$ between slow (–0.69 ± 0.03 MPa) and fast (–1.68 ± 0.46 MPa) dehydration treatments. Nevertheless, these values are very similar to those found in other bryophytes (Beckett, 1997; Proctor *et al.*, 1998; Hájek and Beckett, 2008). In vascular plants $\psi_{\pi s}$ appears to have a good correlation with water in the habitat (Bartlett *et al.*, 2012), but in bryophytes no correlation was found (Proctor *et al.*, 1998). In fact, in opposition to what was seen in vascular plants, in bryophytes cell wall modulus of elasticity (ε) seems to be the predictor for dehydration tolerance, as was pointed out by Hájek and Beckett (2008). *Fontinalis antipyretica* has low ε meaning more elastic cell walls which contribute to maintain RWC_{TLP} at a lower value than vascular plants. This is a characteristic of desiccation tolerant organisms, such as lichens (2–4 MPa) (Beckett, 1995), liverworts such as *C. conicum* (*circa* 2 MPa) (Proctor *et al.*, 1998) and *Dumortiera hirsuta* (Sw.) Nees (*circa* 7 MPa) (Proctor, 1999), and bryophytes such as *Syntrichia ruraliformis* (Besch.) Cardot (*circa* 6 MPa) (Proctor, 1999), *Sphagnum girgensohnii* Russow and *Sphagnum tenellum* (Brid.) Brid. (*circa* 1.5–4 MPa) (Hájek and Beckett, 2008). However, there are differences between slow (7.54 ± 2.09 MPa) and fast (1.45 ± 1.05 MPa) dehydrated samples of *F. antipyretica*. The lower values of ε in the latter treatment may be a short-term adaptation to the fast drying rate, resulting from early perception in cell walls (Kohorn, 2001) and induction of some tolerance mechanisms. These mechanisms may involve alterations in cell wall structure and composition to avoid mechanical stress allowing cells to shrink as water loss increases, as seen in lichens (Beckett, 1995) and the vascular resurrection plant

Craterostigma plantagineum Hochst. (Jones and McQueen-Mason, 2004). However, the full induction of the DT mechanisms is not complete under fast dehydration, as seen in previous Chapters.

The lower $\psi_{\pi TLP}$ in fast dehydration (–3.68 ± 2.83 MPa) compared with slow dehydration samples (–1.44 ± 0.24 MPa) reflects the higher sucrose production in the first treatment. The accumulation of sucrose in fast dehydrated samples may be an attempt to protect membranes against the high concentrations of ions (Bewley and Krochko, 1981; Gaff, 1989), compensating for the quick loss of water, since this rate may not allow time for the induction of other DT mechanisms, including synthesis of proteins to protect cellular structure (**Chapter 6**). However, the lack of protection is evident upon rehydration, since most of the sucrose was detected in the rehydration solution of fast dehydrated samples, probably due to membrane leakage phenomena already observed in **Chapter 4**. After slow dehydration, sucrose content was not statistically different form control. However, after 30 minutes rehydration some sucrose leakage was detected in slow dehydration, although in the cells it remained constant. Therefore, in this brief period some sucrose must have been synthetized allowing to maintaining a stable sucrose concentration inside the cells and the consequent protection of cell membranes. The impact of drying rate in sucrose concentration has been poorly studied in bryophytes. The only information available for bryophytes is that sucrose content is high and remains unaltered throughout the dehydration/rehydration cycle in other studied bryophytes, such as *S. ruralis* (10% of DW) (Bewley *et al.*, 1978) and *Physcomitrella patens* (Hedw.) Bruch & Schimp. (4% of DW) (Oldenhof *et al.*, 2006). In vascular DT plants such as *C. plantagineum*, the sugar 2-octulose is present at high levels in well-watered conditions. During dehydration is converted to sucrose which can increase up to 40% of DW during dehydration (Ingram and Bartels, 1996). Therefore, our work, in conjunction with these data, suggests that high sucrose concentration or an increase in sucrose concentration is an important mechanism for DT achievement, regardless of habitat or plant group.

Combining the high increase in sucrose and the consequent increase in $\psi_{\pi TLP}$, results in osmotic adjustment in an attempt to maintain the turgor loss point and prevent further damage to the cells during fast dehydration (Radin, 1983; DaMatta *et al.*, 2003). On the other hand, slow dehydrated samples were able to maintain a steady level of sucrose throughout the dehydration/rehydration cycle. Combined with changes at the proteome level (**Chapter 6**), they might contribute to form a more stable vitrified cytoplasm during slow dehydration that can improve cells DT, reducing the metabolism and hazardous ROS production (Crowe *et al.*, 1992; Smirnoff, 1992).

5.5.2. Life form as an induction agent of desiccation tolerance

As in vascular plants, RWC can be used to determine water potentials in bryophytes (Powell and Blanchard, 1976; Kirkham, 2004). However, one of the main problems in RWC determination in bryophytes is the fact that they can hold a large amount of external water since they do not have (or exist in only certain parts) a layer of hydrophobic substances in the contact area with the atmosphere. As such, external water does not allow an accurate measurement of FTW. Blotting the material dry with paper towels has been a method applied to remove the excess water (Beckett, 1997; Proctor *et al.*, 1998). However, there is always a problem of applying too much pressure to the samples when blotting which would give an underestimation of FTW. Nevertheless, in *F. antipyretica* blotting before weighting is a good method to eliminate external water since afterwards ψ starts to decrease. Therefore, RWC will be accurate if the weight value after blotting is used as a measurement of FTW. This method had already been considered for RWC determination in bryophytes (Santarius, 1994; Beckett, 1997; Proctor *et al.*, 1998).

External water was very high, about five times that of full turgor. Unblotted shoots start to lose their turgidity at different moments, contrary to what happens in blotted shoots, which start to lose water at the exact same time. The morphology of the leaves and the chemical properties of the cell walls keep the external water layer that slows dehydration. If one small shoot with external water starts to lose their turgidity only after 2-3 hours, a colony with a clump structure would take longer to dry, leading to a slow dehydration rate when the shoots are exposed to the atmosphere. A similar situation occurs in *S. ruralis*, which forms clumps in nature allowing a slower dehydration rate (Oliver, 1991). This demonstrates the importance of the life form of the bryophyte in establishing a slow dehydration rate that allows DT induction, as shown in the previous Chapters.

5.6. Conclusion

Fast dehydration appear to change the characteristics of the bryophytes cell walls, allowing it to become more elastic and acting as a DT mechanism. However, this alone does not allow DT to be established, although it might allow it to survive short and intense periods of dehydration. From an ecological point of view, this has tremendous importance in survival during the drying season. Moreover, slow dehydration provided by the life form may allow the induction of full DT mechanisms, which are probably seen across most bryophyte species.

5.7. Acknowledgements

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Chapter 6

Differential proteomics of dehydration and rehydration in bryophytes: evidence towards a common desiccation tolerance mechanism

Submitted to Plant, Cell and Environment

6. Differential proteomics of dehydration and rehydration in bryophytes: evidence towards a common desiccation tolerance mechanism

6.1. Abstract

All bryophytes evolved desiccation tolerance (DT) mechanisms during the invasion of terrestrial habitats by early land plants. Are these desiccation tolerance mechanisms still present in bryophytes that colonize aquatic habitats? We subjected the aquatic bryophyte *Fontinalis antipyretica* Hedw. to two drying regimes and investigated alterations in protein profiles and sucrose accumulation during dehydration and rehydration. Our results show that during fast dehydration there is very little variation in protein profiles and upon rehydration proteins are leaked. On the other hand, slow dehydration induces changes in both dehydration and rehydration protein profiles, being similar to the protein profiles displayed by the terrestrial bryophytes *Physcomitrella patens* (Hedw.) Bruch & Schimp. And, to what is comparable, to *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr. During dehydration there was a reduction in proteins associated with photosynthesis and the cytoskeleton, and an associated accumulation of proteins involved in sugar metabolism and plant defence mechanisms. Upon rehydration, protein accumulation patterns return to control values for both photosynthesis and cytoskeleton whereas proteins associated with sugar metabolism and defence proteins remain high. These results suggest that bryophytes from different ecological preferences may share a common DT mechanism.

Keywords: bryophytes, desiccation tolerance, photosynthesis, proteomics, water stress.

6.2. Introduction

Water is a scarce resource in numerous regions of the world, many of the consequences of which results in alterations in the distribution of plant species (Maestre *et al.*, 2012). In the last decades, researchers have directed their efforts towards the study of plants with improved water use efficiency (Khana and Hanjra, 2009) and increased resistance to water deficits (Araújo *et al.*, 2013), aiming to apply this knowledge to a biotechnology solution for crop plant improvement. Many traits that contribute to water deficit tolerance in plants (e.g. deeper roots, reduced leaf area) are primarily constitutive (Chaves *et al.*, 2003). Other mechanisms are mainly stress induced leading to changes at the protein synthesis or osmotic adjustment level. These changes are induced by changes in environmental conditions (e.g. drought, excessive radiation, extreme temperatures).

Desiccation may be defined as an extreme form of water loss, where virtually all free water in the organism is removed (Alpert, 2005). Desiccation tolerant organisms such as resurrection vascular plants and some of the poikilohydric photosynthetic organisms (algae, bryophytes and lichens) are able to cope with this extreme dehydration. Within the poikilohydric group, bryophytes were amongst the first organisms to occupy the terrestrial environment. Bryophytes have a simple structure consisting of one-cell layer thick with very low differentiation and without cuticle, losing water very easily. Therefore, they evolved coping with drought and extreme water deficits. The water potential of the cells of bryophytes are, by necessity, in equilibrium with the surrounding environment (poikilohydric), and in most cases subjected to cyclical desiccation-rehydration events (unless they are aquatic or in very mesic environments where free water is always available). The survival of these organisms was only possible because of the development of desiccation tolerance (DT) mechanisms (Oliver *et al.*, 2000). To be considered desiccation tolerant, a plant must be able to protect the cellular integrity of the vegetative tissues in the desiccated state and resume its normal metabolism upon rehydration (Bewley, 1979).

Throughout evolution, bryophytes developed mechanisms to tolerate desiccation occupying different habitats from small water streams subjected to seasonal drying to open sun rock slopes in desert areas (Pharo and Zartman, 2007). A major part of the studies in DT bryophytes was performed with the desert bryophyte *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr. Some authors suggested that the strategy to overcome desiccation appears to be revolving around the polysomal retention of transcripts during dehydration suggesting no *de novo* proteins during dehydration (Wood and Oliver, 1999). Upon rehydration, these transcripts are

translated to proteins, through activation of a repair-based mechanism (Oliver, 1991). Even though there were studies on protein synthesis during dehydration on *S. ruralis* (Bewley, 1973; Oliver and Bewley, 1984a, b, c), samples were collected from the field, and therefore protection may have been already in place. Moreover, the drying regime used in those studies (3-4 h to reach 20% original fresh weight) may have been too fast for protein synthesis induction. Thus, studies never observed the effects of a natural drying rate on protein synthesis but always after rehydration. On the other hand, the number of proteins that change in a dehydration/rehydration event is relatively small compared to the total protein content, as observed in *Physcomitrella patens* (Hedw.) Bruch & Schimp. (5-7 %) (Wang *et al.*, 2009; Cui *et al.*, 2012). Therefore, a quantitative approach of each of the proteins would allow a better understanding of protein expression and its regulation. Recent molecular approaches using transcriptomics have been used both in bryophytes (Oliver *et al.*, 2004) and vascular plants (Rodriguez *et al.*, 2010; Gechev *et al.*, 2013) identifying many genes that are differentially transcribed during dehydration events. However, the proteins that are indeed present have only recently been analysed through a proteome approach in the terrestrial bryophyte *P. patens* (Wang *et al.*, 2009; Cui *et al.*, 2012), based on its recent sequenced genome (Rensing *et al.*, 2008), and in DT grass *Sporobolus stapfianus* Gand. (Oliver *et al.*, 2010).

In **Chapters 2** and **3**, we have shown that if aquatic bryophytes dry slowly they are able to cope with desiccation, allowing photosynthesis to resume its normal function upon rehydration, and maintain low levels of reactive oxygen species (ROS). In fact, those bryophytes in the Mediterranean area are able to survive for more than 1 month out of water with very low RH (**Chapter 2**). DT is relatively common in bryophytes. Therefore, if the ecology or habitat are not determinant factors for having a DT mechanism, then even aquatic bryophytes may display a protein pattern similar to other terrestrial DT bryophytes namely *P. patens* if they share a common DT mechanism. In DT vascular plants like *Craterostigma plantagineum* Hochst., dehydration has to be slow (normally 2-3 days) to allow the establishment of DT mechanisms (Bartels and Salamini, 2001). Recent proteomic studies in *P. patens* (Wang *et al.*, 2009; Cui *et al.*, 2012) also show inducible protein accumulation during dehydration, with a particular increase in seed maturation proteins similar to Late Embryogenesis Abundant (LEA) proteins found in orthodox seeds. Despite the fact that their role is still unclear, data indicates that they may have a role in stabilization and reconstitution of membranes (Oliver *et al.*, 2004). In *P. patens*, 5% of the total proteins were rehydration-responsive (Cui *et al.*, 2012), with the majority of differentially

accumulating proteins in the seed maturation (LEA proteins) and defence categories.

Besides proteins, sugars have also been hypothesized to have a very important role in DT mechanisms, such as sucrose that has been particularly observed in high concentrations in dry tissues of DT bryophytes and all resurrection angiosperms (Buitink *et al.*, 2002). Sucrose is thought to prevent denaturation of important macromolecules and contribute to biological vitrification of the cytoplasm of dried cells, slowing down damaging reactions and maintaining cell structural integrity (Crowe *et al.*, 1992; Smirnoff, 1992). In *S. ruralis*, sucrose makes up approximately 10% of the dry mass and its concentration is maintained during desiccation and rehydration (Bewley *et al.*, 1978). In *P. patens* sucrose is lower in unstressed conditions (3-5% dry mass) (Oldenhof *et al.*, 2006). Recently, it has been proposed a possible interaction between sucrose and LEA proteins in the vitrification process (Goyal *et al.*, 2005).

To investigate if there is a common mechanism of DT in bryophytes we chose the aquatic bryophyte *Fontinalis antipyretica* Hedw. that inhabits Mediterranean areas and that was able to survive 1 month of desiccation under field conditions (**Chapter 2**). We also compare it with results from other studies based on terrestrial bryophytes (Oliver, 1991; Wang *et al.*, 2009; Cui *et al.*, 2012). Since we know that the rate of dehydration is important for DT (Schonbeck and Bewley 1981; **Chapters 2** and **3**), we investigated the effect of two drying rates, fast (3 hours) and slow (72 hours). We also consider both processes, dehydration and rehydration. Our hypothesis is that the differential proteome of an aquatic bryophyte is similar to those observed in terrestrial bryophytes and that the function of the group of proteins that accumulate or decline in the different bryophytes, although can be individually different, assume similar roles in protecting cell structure, showing that they share common mechanisms of tolerance. This is the first investigation into the proteome of an aquatic bryophyte.

6.3. Material and Methods

6.3.1. Plant material and culture conditions

Bryophyte samples of *F. antipyretica* were collected at the *Serra de S. Mamede Natural Park*, central Portugal (N 39° 16.155' W 7° 19.020') in a non-polluted stream, with minimal human impact, involved by *Quercus pyrenaica* Steven and *Castanea sativa* Mill., as well as *Cistus* sp. woodlands. Samples were transported at 5 ºC to the laboratory where they were cleaned of debris and sediments with distilled water. Bryophytes were grown in a modified Knop culture medium (Traubenberg and Ah-Peng, 2004) and under controlled conditions (17 ºC day / 13 ºC night, 20-30 µmol m^{-2} s^{-}

^{1} PAR and photoperiod of 16 hours). Samples were left in the laboratory for at least 60 days before analysis. A pool of seven replicates, containing ten leafy shoot tips (1 cm long), was collected for each treatment. Relative water content (RWC) was calculated according to **Chapter 2** using independent samples and was assumed that the assayed samples had the same RWC. After blotting any external water from the tips and before drying, samples where weighted to determine full turgor weight. At the end of the drying treatment, fresh weight (stress weight) was determined. At the end of the assays, dry weight was determined by placing the samples at 80 ºC for 48 h.

6.3.2. Dehydration induction and recovery

Fast and slow dehydration was attained by placing samples in small containers over saturated salt solutions. Slow dehydration was attained using a saturated salt solution of K_2SO_4 for 72 hours creating an atmosphere with 95% relative humidity (RH) (–6 MPa). Fast dehydration was attained using a saturated salt solution of $Ca(NO_3)_2.4H_2O$ for 3 hours creating an atmosphere with 50% RH (–100 MPa). The containers were kept under ambient temperature (20–23 ºC) at low photosynthetic active radiation (PAR) (2–5 mol m^{-2} s^{-1}), reaching RWC of *circa* 25% (*circa* 20% fresh weight WC). Recovery was achieved through rehydration in the growth culture medium for 72 hours.

6.3.3. Sample preparation for two dimensional gel electrophoresis

Sample preparation was performed according to Proteome Factory's two dimensional gel electrophoresis (2DE) sample preparation protocol for plants (Klose and Kobalz, 1995). Briefly, 300 mg of sample was powdered in liquid N_2 with mortar and pestle and mixed with six volumes sample preparation buffer (9M urea, 2% [w/v] ampholytes [PF 2021, Proteome Factory] and 70 mM dithiothreitol, 1% [v/v] Triton X100). Afterwards, the extracts were disrupted by glass beads (3x 1s) followed by three frozen/thaw cycles. After incubation for 30 min at room temperature and centrifugation for 45 min at 15,000 *g,* the supernatant was removed and frozen in new tubes at –80 ºC.

6.3.4. Two dimensional gel electrophoresis and gel analysis

From the pool of seven biological replicates of each treatment, three technical replicates of 2DE were performed at the Proteome Factory (Berlin, Germany) according to the 2D electrophoresis protocol by Klose and Kobatz (1995). A total of 100 µg protein sample was applied to 20 x 0.1 cm vertical rod gels (9 M urea, 4% [w/v]

acrylamide, 0.3% [w/v] piperazin-di-acrylamide [PDA], 5% [v/v] glycerol, 0.06% [v/v] tetramethylethylendiamine [TEMED] and 2% [w/v] carrier ampholytes, 0.02% [w/v] ammonium persulfate [APS]) for isoelectric focusing (IEF) (*pI* 3–11) and started with 100 V for 1 h followed by 1 h at 200 V, 17.5 h at 400 V, 1 h at 600 V, 30 min at 1,000 V, 10 min at 1,500 V, 5 min at 2,000 V and finally reaching 8,820 V h^{-1} in the first dimension. After focusing, the IEF gels were incubated in equilibration buffer, containing 125 mM Tris-Phosphate buffer (pH 6.8), 40% (v/v) glycerol, 65 mM dithiothreitol, and 3% (w/v) sodium dodecyl sulphate (SDS) for 10 minutes and subsequently frozen at –80°C. The second dimension SDS-PAGE gels (20 cm x 30 cm x 0.1 cm) were prepared, containing 375 mM Tris-HCl buffer (pH 8.8), 15% (w/v) acrylamide, 0.2% (w/v) Bis-acrylamide, 0.1% (w/v) SDS and 0.03% (v/v) TEMED. After thawing, the equilibrated IEF gels were immediately applied to SDS-PAGE gels and sealed with 0.5% (w/v) agarose (low melt). Electrophoresis was performed using 140 V for 5 h 15 min until the dye front reached the end of the gel. After 2DE separation, the gels were stained with FireSilver (Proteome Factory, PS-2001, Berlin, Germany). The 2DE gels were digitized at a resolution of 150 dpi using a PowerLook 2100XL scanner (UMAX Systems GmbH, Willich, Germany) with transparency adapter. After scanning all gels, the images were analysed using Progenesis SameSpots™ version 3.3 (NonLinear Dynamics, Newcastle, UK) image analysis software. All gels were aligned to the reference gel and then the spots with an area less than or equal to 350 and an average normalized volume of less than or equal to 2000 were removed from the spot analysis. The volume of each spot over the volume of all spots in the gel was used for comparison of all groups by an analysis of variance (ANOVA) test for *n* observations ($n = 3$) to assess quantitative differences. All spots with a power over 0.8 and a minimum global variation throughout the dehydration/rehydration cycle of 1.5 fold were manually excised for gel digestion and subsequent identification by Matrix-Assisted Laser Desorption Ionization-Time Of Flight/Time Of Flight (MALDI-TOF/TOF). We also selected for MALDI-TOF/TOF analysis the top 125 spots that showed the highest expression throughout the dehydration/rehydration cycle but did not vary significantly.

6.3.5. *In gel* digestion and MALDI-TOF/TOF MS

For *in gel* digestion, spots were processed as previously described (Roxo-Rosa *et al.*, 2006; Almeida *et al.*, 2010). Briefly, protein spots were excised from the gel, distained with 200 µL of a 1:1 solution of 30 mM potassium hexacyanoferrate (III) and 100 mM sodium thiosulphate, reduced with dithiothreitol, alkylated with iodoacetamide, and dried in speed vacuum. Gel pieces were rehydrated with digestion buffer (50 mM

NH_4HCO_3) containing trypsin (6.7 ng/µl) (Promega, Madison, WI, USA) and incubated overnight at 37 ºC. The buffered peptides were acidified with formic acid, desalted and concentrated using home-made reversed phase microcolumns (POROS R2, Applied Biosystems, Foster City, CA, USA). The peptides were eluted onto a MALDI plate using a matrix solution containing 5 mg/ml α-cyano-4-hydroxycinnamic acid dissolved in 70% (v/v) acetonitrile 0.1% (v/v) trifluoroacetic acid. The mixture was allowed to air-dry. Protein identification was done by MALDI-TOF–TOF (4800*Plus MALDI-TOF-TOF,* Applied Biosystems, Foster City, CA, USA) in both MS and MS/MS modes, according to Marcelino *et al.* (2012). Positively charged ions were analysed in the reflectron MS mode over the *m/z* range of 800–3500 Da. Each MS spectrum was obtained in a result independent acquisition mode with a total of 800 laser shots per spectra and a fixed laser intensity of 3500 V, being externally calibrated using des-Arg-Bradykinin (904.468 Da), angiotensin 1 (1296.685 Da), Glu-Fibrinopeptide B (1570.677 Da), ACTH (1–17) (2093.087 Da), and ACTH (18–39) (2465.199) (Calibration Mix from Applied Biosystems). The ten most intense precursor ions from each MS spectrum were selected for MS/MS analysis. MS/MS analyses were performed using CID (Collision Induced Dissociation) assisted with air, using a collision energy of 1 kV and a gas pressure of 1×10^6 Torr. Two thousand laser shots were collected for each MS/MS spectrum using a fixed laser intensity of 4500 V. Raw data were generated by the 4000 Series Explorer Software v3.0 RC1 (Applied Biosystems, Foster City, CA, USA) and all contaminant *m/z* peaks from human keratin, trypsin autodigestion, or MALDI matrix were included in the exclusion list used to generate the peptide mass list used in database search.

The generated mass spectra were used to search against the NCBI (January 14th 2013, 53,167,621 entries), SwissProt (January 14th 2013, 538,824 entries), Uniref100 (January 14th 2013, 18,057,118 entries) and *Medicago truncatula* 3.5 DB (January 14th 2013, 47,529 entries) protein databases. Searches were conducted using the algorithm Mowse from MASCOT Daemon 2.1.0 Software (Matrix-Science). Protein identifications were accepted if the protein score was above a threshold of 95% ($P < 0.05$) for Mowse. A combined MS+MS/MS search was also performed using the GPS Explorer Software platform (Version 3.5, Applied Biosystems, Foster City, CA, USA) using Mascot as the search engine, with the following parameters: missed-cleavage, one; peptide tolerance, 50 ppm; fragment mass tolerance, 0.25 Da; fixed modification, carbamidomethylation of cysteine; and variable modification, methionine oxidation. From the predicted protein database, the theoretical molecular mass and *pI* of the identified proteins was obtained using the Expasy Mw/*pI* Tool (available from

http://www.expasy.org/tools/pi_tool.html). The identified proteins were only considered if obtained a MASCOT protein score above 84 ($P < 0.05$) for NCBI and Uniref100 databases, above 70 ($P < 0.05$) for SwissProt database, and above 67 ($P < 0.05$) for *Medicago truncatula* 3.5 database, and a minimum of 1 peptide in MS/MS identification.

In addition, we have also used the Peaks® 5.3 software (Peaks studio, Bioinformatics solutions, Waterloo, ON, Canada) for protein identification. Following manufacturer's instructions (Peaks Team, 2011), XTandem, Peaks and OMSSA search algorithms over NCBI database were used and Inchorus as result integration. A cut-off level of one unique peptide matched with MS/MS identification was considered for confident protein identification.

Proteins classified as predicted, without a clear described function, were further analysed through protein-protein searches against NCBI database using Basic Local Alignment Search Tool (BLAST) (available at NCBI: http://blast.ncbi.nlm.nih.gov/) with green plants taxonomical restriction and an acceptance value of $P < 0.05$.

6.3.6. Sucrose quantification

Soluble sugars extraction was performed as described by Arrabaça (1981). Samples subjected to the same treatments as for protein identification with approximately 0.1 g were quickly powdered with 500 µl of 80% (v/v) ethanol in a mortar with pestle at room temperature. The homogenate was quickly transferred to tubes with 1 ml of 80% (v/v) ethanol and placed for 5 min at 80 ºC. Extracts were cooled on ice and then centrifuged at 16,000 *g* for 5 min in a microcentrifuge (Centrifuge 5415 C, Eppendorf). The supernatant was transferred to another tube, evaporated in nitrogen (N_2) flow, resuspended in 1 mL of ultra-pure water and filtered through 0.45 µm filters (Dinistrat Filter RC 25, Sartorius). Sucrose quantification was determined following the resorcinol (1,3-hidroxybenzene) method (Roe, 1934). Briefly, 100 µl of the extract were added to 0.25 mL of resorcinol solution (1% [w/v] diluted in absolute ethanol) and to 0.75 mL of a solution of 30% HCl (w/v), mixed and incubated at 80 ºC for 8 min. Samples were cooled on ice and absorbance at 405 nm was read in a microplate reader (ELx800, BioTek, USA). A regression trend line was determined through the application of this method to sucrose solutions with pre-determined concentrations.

Significant differences between groups were determined through ANOVA with Tukey post-test (significance level $\alpha = 0.05$). Statistical analyses were performed with GraphPad Prism 5.03 for Windows (2009) (GraphPad Software, San Diego, California USA).

6.4. Results

6.4.1. Proteome profiles during dehydration and rehydration

Two-dimensional gel electrophoresis (2-DE) of slow and fast dehydrated, post-rehydration and control (non-stressed) bryophyte samples were performed to further understand the molecular mechanisms involved in DT of *F. antipyretica*.

After dehydration, the gels of the fast-dehydrated samples were very similar to control and, upon rehydration, most of the proteins were lost being this drying rate apparently lethal for the bryophyte (data not shown). Thus, we decided to further analyse and identify only the proteins that presented a statistically significant differential expression in a slow dehydration/rehydration cycle. Total protein extracts were separated in all samples in a *pI* range of 3 to 11 and a molecular mass range of 10 to 150 kDa (Fig. 6.1).

After comparing the gels, a clear separation is observed among the three experimental groups, non-stressed conditions (control), after slow dehydration (stress) and recovery from slow dehydration (recovery) (Fig. 6.2). The following gel analysis revealed a total of 1015 protein spots from which 138 protein spots presented changes equal or higher than 1.5 fold the control value (either accumulated or declined) in dehydrated and/or rehydrated samples. Among the 138 differential spots (Fig. 6.3a), we were able to identify 28 spots using mass spectrometry analysis and these are reported in Table 6.1. The majority (16 spots) exhibit strong sequence similarity with proteins previously identified in *P. patens*, a terrestrial bryophyte. Proteins were classified according to their functional role (Bevan *et al.*, 1998) based in the functional catalogues established for *Escherichia coli* (Riley, 1993) and yeast (Mewes *et al.*, 1997). Within the 28 proteins identified that exhibit variations in quantity throughout the dehydration/rehydration cycle, 12 declined in abundance, 8 accumulated and 8 remained unchanged during dehydration, relatively to unstressed control treatment (Table 6.2).

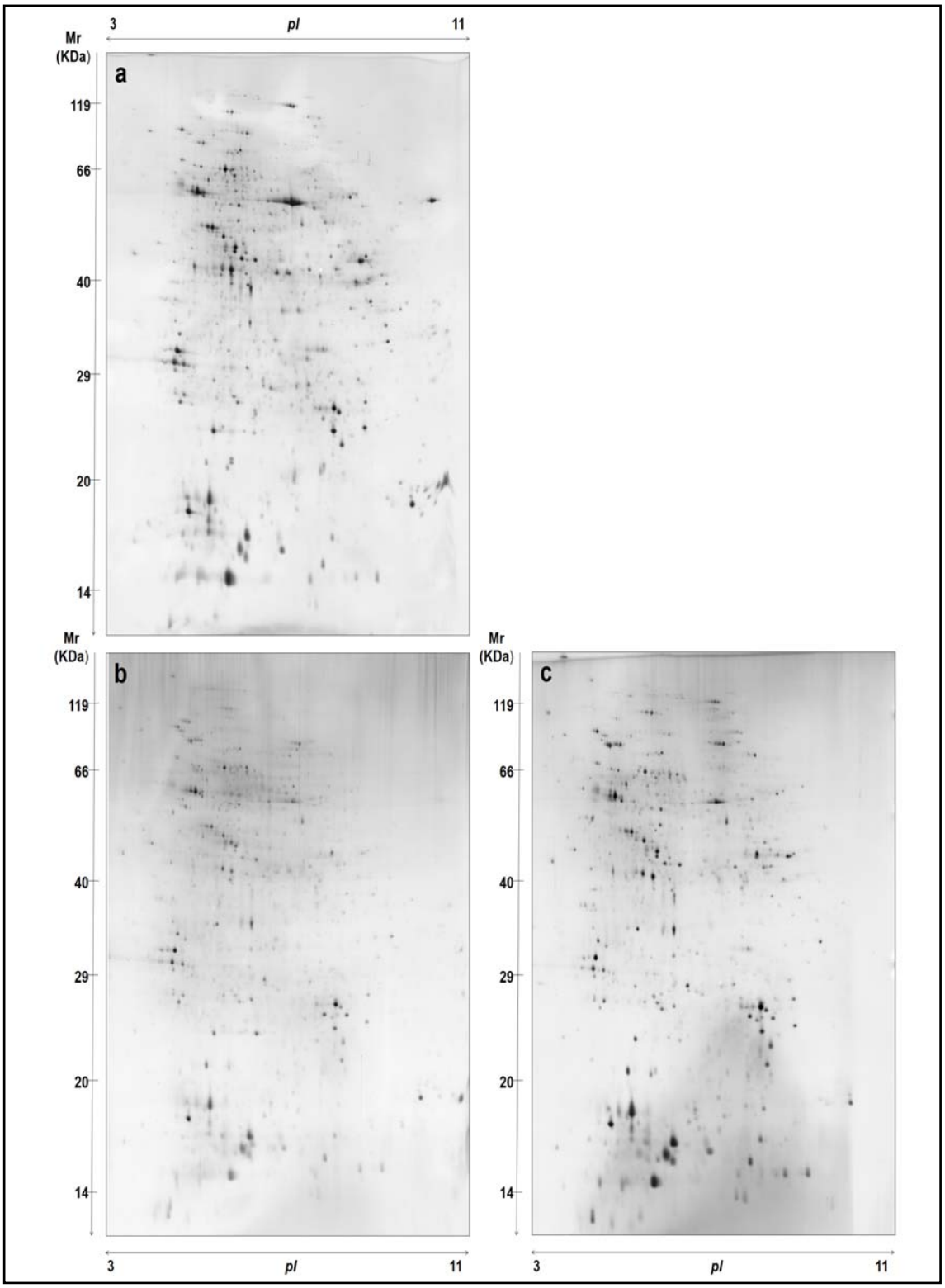

Figure 6.1. Representative images of two-dimensional gel electrophoresis of total protein extracts of the aquatic bryophyte *Fontinalis antipyretica*. (**a**) Non-stressed control; (**b**), slow dehydrated, and (**c**) recovery from slow dehydration. Gels were silver stained. Numbers on the left indicate molecular mass markers (kDa), while those on top and bottom indicate the *pI* range.

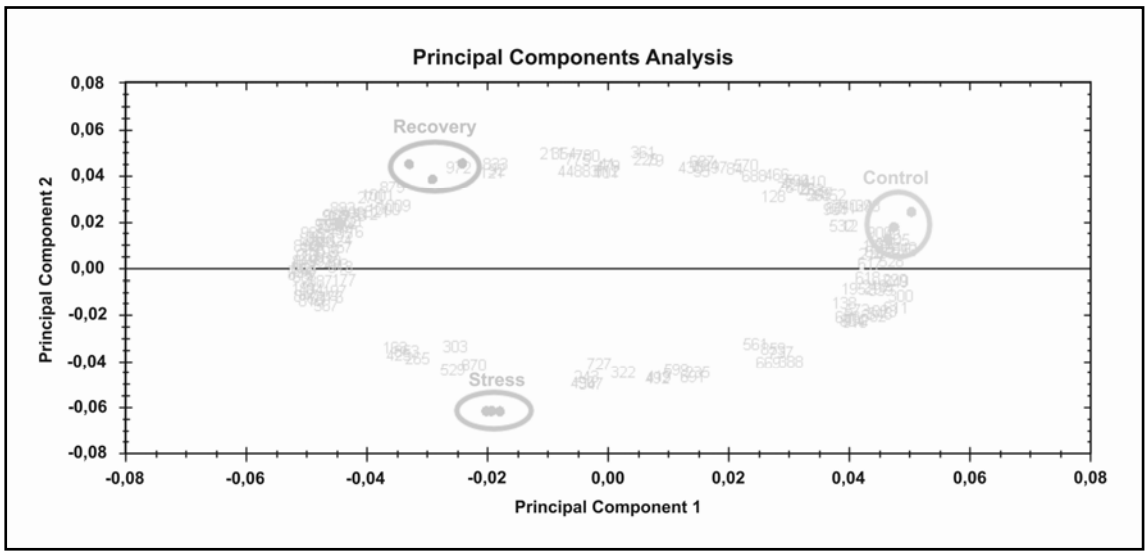

Figure 6.2. Principal components analysis (PCA) of the technical replicates of the pool of seven biological replicates of the aquatic bryophyte *Fontinalis antipyretica* subjected to non-stressed conditions (control), after slow dehydration (stress) and recovery from slow dehydration (recovery). Numbers in grey are protein spots that suffered differential expression.

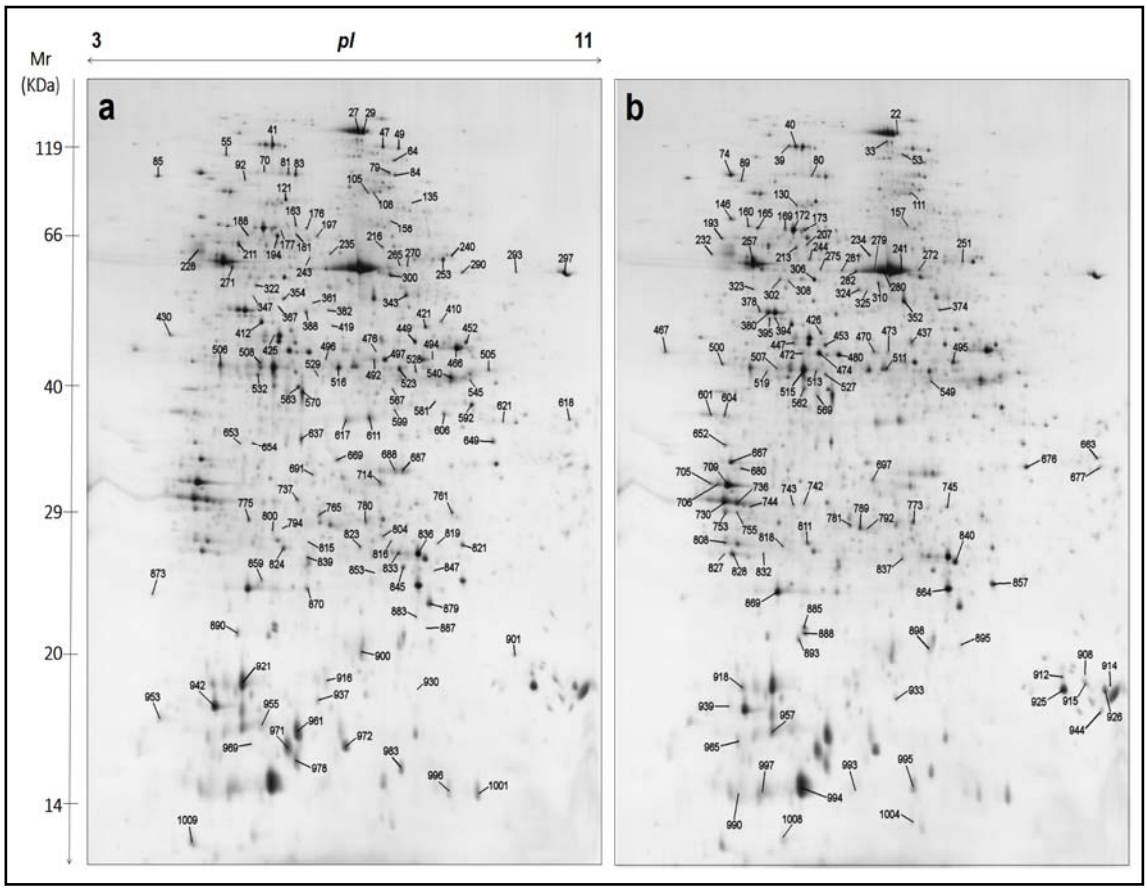

Figure 6.3. Protein spots that (**a**) suffered and (**b**) did not suffer differential expression throughout dehydration/rehydration of the aquatic bryophyte *Fontinalis antipyretica*.

Table 6.1. Identification of dehydration and rehydration responsive proteins of the aquatic bryophyte *Fontinalis antipyretica.*

Protein group	Spot No.	Accession No.	Database	Protein score	Total ion score	NMP MS (MS/MS)	Peptides (MS/MS)	SC (%)	Description	Species
General metabolism	253	A9SHC0	U	216	126	13 (1)	LIVAGASAYAR	23	Serine hydroxymethyltransferase	*Physcomitrella patens*
	421	A9SLK1	U	196	181	5 (2)	VGFISTGAPAR, AHSEILDLEGK	8	Aminomethyltransferase	*Physcomitrella patens*
	497*	gi|168011949 (gi|209778969)	N	156	107	10 (1)	LPVFLDGGVR (94.207%)	29	Glycolate oxidase (predicted protein)	*Physcomitrella patens* (*Cupressus sempervirens*)
	505	gi|20530992	N	202	166	5 (1)	TFQGPPHGIQV ER	9	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Diphyscium suzukii*
	529	gi|330811753	N	105	101	2 (1)	NVALSYAAGVG GGR	8	Ketol-acid reductoisomerase	*Pseudomonas brassicacearum*
	611	RBL_PTYGA	S	395	324	9 (2)	ETDILAAFR, TFQGPPHGIQV ER	21	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Ptychomitrium gardneri*
	617	RBL_PTYGA	S	256	174	10 (2)	ETDILAAFR, TFQGPPHGIQV ER	21	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Ptychomitrium gardneri*
	669	gi|293634282	N	256	151	6 (1)	TFQGPPHGIQV ER	33	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Pogonatum aloides*
	714	gi|188037953	N	247	149	8 (1)	TFQGPPHGIQV ER	40	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Brachythecium salebrosum*
	839	Q9GE52	U	222	177	7 (1)	TFQGPPHGIQV ER	15	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (fragment)	*Eucamptodon muelleri*
	870	gi|124027737	N	210	181	12 (1)	VIATGFGTAVA AR	35	Glutamate dehydrogenase	*Hyperthermus butylicus*
Energy metabolism	322	B9VH70	U	164	51	14 (2)	FVQAGSEVSAL LGR, VALVYGQMNE PPGAR	38	ATP synthase subunit beta, chloroplastic	*Syntrichia ruralis*
	449	G3PC_PHYPA	S	148	95	9 (1)	VPTTDVSVVDL TVR	15	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	*Physcomitrella patens*
	466	gi|87330988	N	493	422	14 (2)	TFAEEVNQAFR , VVAWYDNEWG	36	Glyceraldehyde-3-phosphate dehydrogenase subunit A1, chloroplastic	*Physcomitrella patens*

							YSQR			
	765*	gi|168032548	N	97	89	3 (1)	VASPQQAQEV HAAIR	13	Triosephosphate isomerase (predicted protein)	*Physcomitrella patens*
	804	ATPB_PHYPA	S	160	101	8 (1)	AHGGVSVFGG VGER	17	ATP synthase subunit beta, chloroplastic	*Physcomitrella patens*
	972*	gi|168031425	N	163	141	5 (1)	VVAVDQAPGV R	42	Photosystem I reaction centre subunit IV (predicted protein)	*Physcomitrella patens*
	983*	gi|168041017	N	153	141	3 (1)	GAITGGAIATAA EFLR	25	Triosephosphate isomerase (predicted protein)	*Physcomitrella patens*
	996*	gi|168041017	N	84	74	3 (1)	GAITGGAIATAA EFLR	19	Triosephosphate isomerase (predicted protein)	*Physcomitrella patens*
	1001*	gi|168050602	N	127	120	2 (1)	VGGVGVAHAA VQDTFR	16	Triosephosphate isomerase (predicted protein)	*Physcomitrella patens*
Transcription and protein synthesis	297	A9SA04	U	376	293	14 (1)	IGGIGTVPVGR	26	Elongation factor 1-α	*Physcomitrella patens*
	354*	gi|168026095 (gi|33772119)	N	493	315	22 (2)	NALRADSIK, VLITTDLLAR	48	Translation initiation factor eIF-4A (predicted protein)	*Physcomitrella patens* (*Malus x domestica*)
	540*	gi|168024677	N	334	271	12 (1)	SDQLPHFEVDA VDPK	33	RNA-binding protein (predicted protein)	*Physcomitrella patens*
Cytoskeleton	228	gi|326486293	N	409	359	13 (1)	INVYYNEASGG R	27	β-tubulin	*Vanilla planifolia*
	412*	A9TBG2 (gi|314998974)	U	123	58	8 (1)	SEYDESGPSIV HR	19	Actin (predicted protein)	*Physcomitrella patens* (*Ignatius tetrasporus*)
Defence	211*	gi|168003742 (gi|413953493)	N	256	175	16 (1)	VVAAGANPVQI TR	28	TCP-1/cpn60 chaperonin (predicted protein)	*Physcomitrella patens* (*Zea mays*)
	930	gi|21068488	N	98	91	2 (1)	DAHAVANTQID WR	15	Small heat-shock protein	*Funaria hygrometrica*
Signaling	961*	gi|302791691 (gi|306755923)	N	119	81	5 (1)	GLVGEIISR	18	Nucleoside diphosphate kinase 1 (predicted protein)	*Selaginella moellendorffii* (*Pseudotsuga menziesii*)

N, NCBI database; NMP, number of matched peptides through MS (no parenthesis) or MS/MS (inside parenthesis); S, SwissProt database; SC, sequence coverage; U, Uniref100 database. Spots marked with an asterisk (*) were identified as predicted proteins and its putative function was assessed through BLAST search with the corresponding accession number, description and species inside parenthesis.

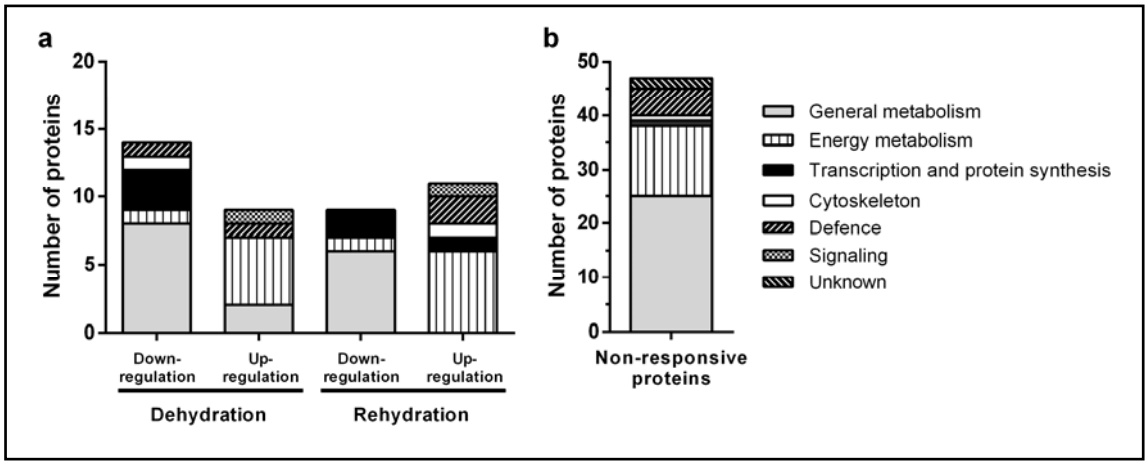

Figure 6.4. Distribution in protein groups of spots that (**a**) suffered and (**b**) did not suffer differential expression throughout dehydration/rehydration of the aquatic bryophyte *Fontinalis antipyretica*.

During dehydration, 57% of the declining proteins belong to the general metabolism, and 21% are proteins involved in the protein synthetic machinery. Proteins associated with energy metabolism are the major group of proteins that accumulate above control levels during dehydration (56%) followed by general metabolism proteins (22%) (Fig. 6.4a). During rehydration, the major groups of declining proteins were, general metabolism (67%) and protein synthesis-related proteins (22%) (Fig. 6.4a). The groups that presented an increase in abundance during this phase are related to energy metabolism (55%) and defence (18%), although some proteins from the protein synthetic machinery, cytoskeleton and signaling were also identified (Fig. 6.4a).

From proteins that did not exhibit statistical differences between treatments, we chose 125 spots based on their high abundance throughout the dehydration/rehydration cycle (Fig. 6.3b). From those 125 protein spots, approximately 40% (48 spots) were identified (Table 6.3). These belong mainly to the general (52%) and energy (27%) metabolism, and the defence proteins group (10%) (Fig. 6.4b).

When comparing the proteome profile during slow dehydration with the proteome of the non-stressed control, we found that proteins that decline in abundance range from 0.7%-2.9% (Table 6.5). The proportion of proteins that increase in abundance ranged from 2.3% to 3.5% (Table 6.5).

During rehydration, the more common process is an increase in abundance of proteins (Table 6.5). We demonstrated that of the 1015 spots present in the gel only 107 spots (11%) exhibit changes higher than 2-fold when comparing proteins throughout the entire dehydration/rehydration cycle (Table 6.5).

Table 6.2. Identification of stress non-responsive proteins of the aquatic bryophyte *Fontinalis antipyretica.*

Protein group	Spot No.	Accession No.	DB	Protein score	Total ion score	Score (%)[a]	NMP MS (MS/MS)[b]	NMP MS/MS (Unique)[c]	Peptides (MS/MS)	SC (%)	Description	Species
General metabolism	193	gi|224079938	N	-	-	99	-	4 (4)	VVLSVTPR, AISVVVETLLH, VIMKLPR, EELNFR	8	Phosphogluconate dehydrogenase	*Populus trichocarpa*
	207	gi|15383637	N	-	-	99	-	9 (2)	FLFVAEAIYK, GGLDFTKDDENVNSQPFMR, TFQGPPHGIQVER, ETDILAAFR, DNGLLLHIHR, VALEACVQAR, LTYYTPDYQTK, QVTLGFVDLLR, AVYECLR	21	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Fontinalis antipyretica*
	232	IMGA|AC233655_47.1	M	74	61	-	3 (1)	-	NNPQFQALR	7	Os02g0465112 protein	*Medicago truncatula*
	241	RBL_MESVI	S	124	74	-	9 (1)	-	TFQGPPHGIQVER		Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Mesostigma viride*
	272	RBL_ILLOL	S	126	105	-	5 (1)	-	TFQGPPHGIQVER	11	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Illicium oligandrum*
	279	RBL_PTYGA	S	592	527	-	11 (5)	-	ETDILAAFR, DNGLLLHIHR, FLFVAEALYK, TFQGPPHGIQVER, GGLDFTKDDENVNSQPFMR	25	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Ptychomitrium gardneri*
	280	RBL_ILLOL	S	149	122	-	6 (1)	-	TFQGPPHGIQVER	11	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Illicium oligandrum*
	281	gi|15224685	N	-	-	54	-	1 (1)	VIMKLPR	2	HXXXD-type acyl-transferase-like protein	*Arabidopsis thaliana*
	284	gi|15383637	N	531	342	-	24 (2)	-	TFQGPPHGIQVER, GGLDFTKDDENVNSQPFMR	48	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Fontinalis antipyretica*
	323*	gi|168029523	N	-	-	62	-	1 (1)	VYHSTANLLPDGR	3	Glyoxal oxidase (predicted protein)	*Physcomitrella patens*
	473	STAD1_OPHSP	S	74	61	-	3 (1)	-	ATFISHGNTAR	7	Acyl-[acyl-carrier-protein] desaturase 1, chloroplastic	*Ophrys sphegodes*

	474	RCA_PHAVU	S	153	136	-	6 (2)	-	FYWAPTR, VYDDEVRK	8	Ribulose-1,5-bisphosphate carboxylase/oxygenase activase, chloroplastic	*Phaseolus vulgaris*
	500	IMGA|Medtr4g079290.1	M	72	51	-	6 (1)	-	VVTVSIPR	7	Acyl-coenzyme A oxidase 3, peroxisomal	*Medicago truncatula*
	507	gi|19988	N	-	-	83	-	4 (4)	VYDDEVR, VYDDEVRK, FYWAPTR, FYWAPTREDR	9	Ribulose-1,5-bisphosphate carboxylase/oxygenase activase	*Nicotiana tabacum*
	511*	gi|326510017	N	-	-	56	-	1 (1)	TSAMVSR	1	NAD kinase (predicted protein)	*Hordeum vulgare*
	604*	gi|168031117	N	-	-	83	-	3 (2)	VLAFEVGR, VLAFEVGRK, GKFDESR	5	Enoyl-ACP reductase (predicted protein)	*Physcomitrella patens*
	697	RBL_ILLOL	S	275	223	-	9 (2)	-	ETDILAAFR, TFQGPPHGIQVER	19	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Illicium oligandrum*
	706	gi|21955978	N	-	-	59	-	1 (1)	TFQGPPHGIQVER	23	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Volvulina compacta*
	730	CB2_PHYPA	S	111	97	-	3 (2)	-	ELEVIHAR, FGEAVWFK	16	Chlorophyll *a-b* binding protein, chloroplastic	*Physcomitrella patens*
	742*	gi|18424049 (gi|9758364)	N	-	-	53	-	1 (1)	VVLSVTPR	3	26S proteasome non-ATPase regulatory subunit 9 (unnamed protein product)	*Arabidopsis thaliana*
	753	gi|334186554	N	-	-	60	-	1 (1)	TTIFSPEGR	4	N-terminal nucleophile aminohydrolases (Ntn hydrolases)-like protein	*Arabidopsis thaliana*
	828*	gi|168031123	N	164	143	-	5 (1)	-	VGLGWATPR	19	Chlorophyll *a-b* binding protein (predicted protein)	*Physcomitrella patens*
	869	RBL_PHYPA	S	163	126	-	4 (1)	-	TFQGPPHGIQVER	8	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Physcomitrella patens*
	994	gi|168020432	N	92	75	-	3 (1)	-	LPMFGCQDSAQVLR	39	Ribulose-1,5-bisphosphate carboxylase/oxygenase	*Physcomitrella patens*

											small subunit (predicted protein)	
	995	gi|48093939	N	-	-	61	-	1 (1)	SPGYYDGR	12	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	*Coffea arabica*
Energy metabolism	172	gi|223931089	N	517	398	-	19 (3)	-	LIESSAPGIISR, IAQIPVSDAYLGR, EAYPGDVFYLHSR	33	ATP synthase CF1 α subunit, chloroplastic	*Syntrichia ruralis*
	213	gi|28202156	N	-	-	58	-	1 (1)	ELIIGDR	7	ATP synthase subunit α, chloroplastic	*Anthoceros formosae*
	257	ATPB_PHYPA	S	374	269	-	13 (2)	-	AHGGVSVFGGVGER, VALVYGQMNEPPGAR	30	ATP synthase subunit beta, chloroplastic	*Physcomitrella patens*
	308	ATPB_NANDO	S	105	78	-	5 (1)	-	AHGGVSVFGGVGER	13	ATP synthase subunit beta, chloroplastic	*Nandina domestica*
	324	gi|28202179	N	-	-	62	-	1 (1)	AHGGVSVFGGVGER	6	ATP synthase CF1 beta subunit	*Anthoceros formosae*
	437	gi|90296193	N	-	-	62	-	1 (1)	LASIGLENTEANR	6	Fructose bisphosphate aldolase, plastidic	*Stevia rebaudiana*
	515	gi|4827253	N	122	107	-	5 (1)	-	LASIGLENTEANR	17	Aldolase, plastidic	*Nicotiana paniculata*
	527	P2SAF_ARATH	S	172	139	-	7 (2)	-	GFGILDVGYR, GFVLGNDGVLLR	16	Photosystem II stability/assembly factor HCF136, chloroplastic	*Arabidopsis thaliana*
	569*	gi|168045659	N	-	-	99	-	6 (6)	GFVLGNDGVLLR, SKDEAWAAGGSGILLR, SIPSAEDEDFNYR, GFGILDVGYR, GFLLGTR, IQNMGWR	18	Photosystem II stability/assembly factor (predicted protein)	*Physcomitrella patens*
	709*	gi|168021458	N	129	115	-	6 (1)	-	AESPFKGGSEGFQNTK	20	Photosystem II oxygen-evolving enhancer protein 1 (predicted protein)	*Physcomitrella patens*
	818*	gi|168032548	N	-	-	62	-	1 (1)	VASPQQAQEVHAAIR	6	Triosephosphate isomerase (predicted protein)	*Physcomitrella patens*
	905*	gi|168065932	N	-	-	61	-	1 (1)	QYFLGLEK	9	Photosystem I light harvesting chlorophyll a/b binding protein 3 (predicted protein)	*Physcomitrella patens*

	908*	gi|168037541	N	-	-	61	-	2 (2)	EKEFPGTVLR, EFPGTVLR	4	Photosystem II oxygen-evolving enhancer protein 2 (predicted protein)	*Physcomitrella patens*
Defence	74	gi|283484351	N	227	156	-	20 (1)	-	IINEPTAASLAYGFDR	27	Chloroplast Hsp70-1	*Physcomitrella patens*
	107*	gi|168067860 (gi|18419633)	N	111	98	-	7 (1)	-	ATAGDTHLGGEDFDNR	15	Hsp70 (predicted protein)	*Physcomitrella patens* (*Narcissus pseudonarcissus*)
	110*	gi|168030657 (gi|18419633)	N	259	214	-	9 (1)	-	ATAGDTHLGGEDFDNR	16	Hsp70 (predicted protein)	*Physcomitrella patens* (*Narcissus pseudonarcissus*)
	157	gi|306755789	N	-	-	62	-	1 (1)	ELLSEINR	100	Putative heat shock protein 2	*Pseudotsuga menziesii*
	160	gi|306755789	N	-	-	60	-	1 (1)	ELLSEINR	100	Putative heat shock protein 2	*Pseudotsuga menziesii*
Cytoskeleton	251	gi|15225947	N	-	-	54	-	1 (1)	LSDRPLR	1	Myosin heavy chain-like protein	*Arabidopsis thaliana*
Transcription and protein synthesis	453	gi|310788246	N	-	-	59	-	1 (1)	FLEKGAQPTETVR	20	Ribosomal protein S16	*Lilaeopsis chinensis*
Unknown function	244	gi|168051134	N	-	-	59	-	1 (1)	SKNMAGSSK	1	Predicted protein with domain of unknown function (DUF3444)	*Physcomitrella patens*
	378	gi|302797300	N	-	-	59	-	1 (1)	DRLIVGIDCK	2	Hypothetical protein SELMODRAFT_419915	*Selaginella moellendorffii*

M, *Medicago truncatula* 3.5 database; N, NCBI database; NMP, number of matched peptides through MS (no parenthesis) or MS/MS (inside parenthesis); S, SwissProt database; SC, sequence coverage; U, Uniref100 database. Spots marked with an asterisk (*) were identified as predicted proteins and its putative function was assessed through BLAST search with the corresponding accession number, description and species inside parenthesis. Columns marked with [a], [b] and [c] were obtained with Peaks® 5.3 software (Peaks studio, Bioinformatics solutions, Waterloo, ON, Canada)

Table 6.3. Variation of the average normalized volumes of protein spots in the different protein groups in control, after dehydration (stress) and recovery after dehydration (recovery) of the aquatic bryophyte *Fontinalis antipyretica*.

Protein group	Spot No.	Average Normalized Volumes (fold in relation to control)			Description
		Control	Stress	Recovery	
General metabolism	714	15500	3767 **(-4.1)****	9288 **(-1.7)****	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
	505	7144	2124 **(-3.4)****	2353 **(-3.0)****	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
	617	22290	9463 **(-2.4)****	8711 **(-2.6)****	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
	253	16010	6605 **(-2.4)****	11240 **(-1.4)**	Serine hydroxymethyltransferase
	611	24030	11020 **(-2.2)****	5169 **(-4.6)****	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
	839	6643	3555 **(-1.9)****	2447 **(-2.7)****	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (fragment)
	421	11230	6248 **(-1.8)****	9070 **(-1.2)**	Aminomethyltransferase
	497*	13770	9141 **(-1.5)****	13460 **(-1.0)**	Glycolate oxidase (predicted protein)
	669	10640	12240 **(1.2)**	3872 **(-2.7)****	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
	529	1282	5504 **(4.3)****	1681 **(1.3)**	Ketol-acid reductoisomerase
	870	5795	25120 **(4.3)****	6481 **(1.1)**	Glutamate dehydrogenase
Energy metabolism	466	63540	25040 **(-2.5)****	49380 **(-1.3)**	Glyceraldehyde-3-phosphate dehydrogenase subunit A1, chloroplastic
	804	5763	4714 **(-1.2)**	3391 **(-1.7)****	ATP synthase subunit beta, chloroplastic

	972*	33160	29570 **(-1.1)**	51780 **(1.6)****	Photosystem I reaction centre subunit IV (predicted protein)
	1001*	23300	26940 **(1.2)**	47040 **(2.0)****	Triosephosphate isomerase (predicted protein)
	322	4404	6659 **(1.5)****	3491 **(-1.3)**	ATP synthase subunit beta, chloroplastic
	983*	19970	33430 **(1.7)****	51730 **(2.6)****	Triosephosphate isomerase (predicted protein)
	996*	13800	23270 **(1.7)****	41950 **(3.0)****	Triosephosphate isomerase (predicted protein)
	765*	9828	23050 **(2.3)****	36190 **(3.7)****	Triosephosphate isomerase (predicted protein)
	449	11520	33600 **(2.9)****	46500 **(4.0)****	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic
Transcription and protein synthesis	297	39980	8538 **(-4.7)****	3981 **(-10.0)****	Elongation factor 1-α
	540*	54410	23820 **(-2.3)****	23980 **(-2.3)****	RNA-binding protein (predicted protein)
	354*	7926	4000 **(-2.0)****	14360 **(1.8)****	Translation initiation factor eIF-4A (predicted protein)
Cytoskeleton	228	44220	28670 **(-1.5)****	49270 **(1.1)**	β-tubulin
	412*	18830	23580 **(1.3)**	36620 **(1.9)****	Actin (predicted protein)
Defence	211*	13570	9414 **(-1.5)****	19590 **(1.5)****	TCP-1/cpn60 chaperonin (predicted protein)
	930	669	3974 **(5.9)****	8981 **(13.4)****	Small heat-shock protein
Signaling	961*	82750	170700 **(2.1)****	236200 **(2.9)****	Nucleoside diphosphate kinase 1 (predicted protein)

Numbers inside parenthesis in bold indicates fold in relation to control values, with positive values indicative of up-regulation and negative values indicative of down-regulation in protein synthesis. Spots marked with an asterisk (*) were identified as predicted proteins and its putative function was assessed through BLAST search. Spots marked with two asterisks (**) have varied significantly in relation to control (fold > 1.5).

Table 6.4. Sucrose accumulation percentage of dry weight (percentage DW) in non-stressed control samples, at the end of the dehydration treatment (stress) and after recovery of the aquatic bryophyte *Fontinalis antipyretica*. Different letters indicate significant differences between values.

	Control	Stress	Recovery
Slow dehydration	6±1[a]	6±1[ab]	7±1[ab]
Rapid dehydration		9±1[b]	4±1[c]

Table 6.5. Comparison between the numbers of protein spots differentially expressed in the aquatic bryophyte *Fontinalis antipyretica* and the terrestrial bryophytes *Physcomitrella patens* and *Syntrichia ruralis* in different stages of the dehydration/rehydration cycle.

				Dehydration		Rehydration		
	Drying conditions	**Rehydration conditions**	**Number of spots**	**Down-regulated spots (> 2 fold decrease)**	**Up-regulated spots (> 2 fold increase)**	**Down-regulated spots (> 2 fold decrease)**	**Up-regulated spots (> 2 fold increase)**	**Reference**
Fontinalis antipyretica	95% RH for 3 days (WC~20%)	KNOPS culture medium for 3 days	1015	29 (2.9%)	23 (2.3%)	24 (2.4%)	31 (3.1%)	This study
Physcomitrella patens	30% RH for 3-4 days (WC~23%)	Solid BCD medium for 6 days	2308	15 (0.7%)	78 (3.4%)	15 (0.7%)	69 (3.0%)	Cui *et al.* (2012)
	0% RH for 30 days (WC~10%)	–	1300	25 (1.9%)	46 (3.5%)	n.d.	n.d.	Wang *et al.* (2009)
Syntrichia ruralis	66% RH for 6 hours (WC~20%)	Distilled water for 2 hours	568	n.d.	n.d.	39 (6.9%)	110 (19.4%)	Oliver (1991)

6.4.2. General metabolism proteins

During dehydration, many proteins of the general metabolism group declined in abundance. Amongst these is the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (spots 505, 611, 617, 714, 839; Table 6.2), an enzyme within the Calvin cycle. Moreover, the decline in abundance of the peroxisomal enzyme glycolate oxidase (spot 497; Table 6.2), along with some mitochondrial enzymes associated with photorespiration, such as serine hydroxymethyltransferase (spot 253; Table 6.2) and aminomethyltransferase (spot 421; Table 6.2), matches the pattern of loss of the large sub-unit of Rubisco.

Nevertheless, other enzymes related to general metabolism accumulated during dehydration, return to pre-desiccation levels after rehydration. These include the ketol-acid reductoisomerase (spot 529; Table 6.2), an enzyme of the amino acid metabolism responsible for the biosynthesis of the hydrophobic branched-chain amino acids valine, leucine and isoleucine; and glutamate dehydrogenase (spot 870; Table 6.2) involved in nitrogen metabolism, including the synthesis of proline.

In general, upon rehydration, the large sub-unit of Rubisco remains lower than the protein levels observed before desiccation, but some isoforms (spots 505, 714; Table 6.2) return to near pre-desiccation levels during this time. Enzymes associated with photorespiration (spots 253, 421, 497; Table 6.2) follow a similar pattern to those associated with photosynthesis.

6.4.3. Energy metabolism associated proteins

The chloroplastic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (spot 466; Table 6.2) is another Calvin cycle enzyme that declines during dehydration, and returns close to pre-desiccation values upon rehydration, similar to what was observed for the large sub-unit of Rubisco.

In contrast, the glycolytic enzymes triosephosphate isomerase (TIM) (spots 765, 818, 983, 996, 1001; Table 6.2) and the cytosolic GAPDH (spot 449; Table 6.2) accumulate above control levels during dehydration and continue to increase following rehydration.

There are two forms of ATP synthase identified in the gels as differentially affected during dehydration, one form declines in abundance throughout the dehydration/rehydration cycle (spot 804; Table 6.2) and one form that increases in abundance during dehydration and returns to pre-desiccation values following rehydration (spot 322; Table 6.2).

Although during dehydration no changes were observed, upon rehydration the

photosystem I reaction centre subunit IV (spot 972; Table 6.2) accumulates above control levels.

6.4.4. Protein synthesis-related proteins

Some protein elements related to the protein synthetic machinery decrease in abundance during dehydration and remain low after rehydration. These include the translation initiation factor eIF-4A (spot 354; Table 6.2), the protein elongation factor 1-α (spot 297; Table 6.2). In contrast the translation initiation factor eIF-4A (spot 354; Table 6.2) although decreases in abundance during dehydration becomes higher after rehydration.

6.4.5. Cytoskeleton, defence and signaling proteins

The cytoskeleton protein actin (spot 412; Table 6.2), which is a component of microfilaments, starts to increase in abundance during dehydration and reaches almost a 2-fold elevation after rehydration. In contrast, the protein β-tubulin (spot 228; Table 6.2), which is a component of cytoskeletal microtubules, declines in abundance during dehydration and recovers to pre-desiccation values upon rehydration, following a pattern similar to TCP-1/cpn60 chaperonin (spot 211; Table 6.2). A defence protein with accumulates significantly during dehydration was a small HSP (sHSP) (spot 930; Table 6.2) which increased 6-fold during dehydration and 13-fold following rehydration.

The ROS-related signaling pathway enzyme, nucleoside diphosphate kinase 1 (NDPK1; spot 961; Table 6.2), doubled in abundance during dehydration and tripled upon dehydration.

6.4.6. Dehydration/rehydration non-responsive proteins

The dehydration/rehydration non-responsive proteins belong mainly to the general and energy metabolism groups. In the first category, some spots were identified as the large (spots 207, 241, 272, 279, 280, 284, 697, 706, 869; Table 6.3) and small (spots 994, 995; Table 6.3) subunits of Rubisco, as well as Rubisco activase (spots 474, 507; Table 6.3).

In the second category, chloroplastic ATP synthase showed increased abundance throughout the dehydration/rehydration cycle (spots 172, 213 257, 308, 324; Table 6.3), even as many proteins associated with the electron transport chain remain unchanged. These include chlorophyll *a*-*b* binding protein (spots 730, 828, 905; Table 6.3), PSII oxygen-evolving enhancer (spots 709; 908; Table 6.3) and PSII stability/assembly factor (spots 527, 569; Table 6.3).

The defence protein group was the third group that accumulate to high abundance, primarily represented by several heat-shock proteins (spots 74, 107, 110, 157, 160; Table 6.3).

6.4.7. Sucrose production

Steady levels of sucrose are maintained throughout the slow dehydration/rehydration cycle (*circa* 6% of DW) (Table 6.4). In fast dehydrated samples, sucrose levels increased (*circa* 9% of DW) but dropped following rehydration (*circa* 4% of DW).

6.5 Discussion

During fast dehydration, very few changes occur in the protein profile. Upon rehydration, there was leakage of almost all intercellular protein content, being the result of cell death (**Chapter 3**). Therefore, fast dehydration does not allow time in preparation for desiccation. This is indicative of the need for dehydration-induced protein synthesis to survive a desiccation event.

During slow dehydration, there is clearly a pattern of progressive reduction in proteins associated with photosynthesis and cytoskeleton disassembly indicating that these processes are inhibited as water is lost from the cells. Sucrose metabolism, however, may increase in activity as several proteins associated with its production increase in abundance indicating a need to maintain sucrose levels in the bryophyte. In contrast, during rehydration proteins associated with protection, energy production and folding processes increase in abundance, reflecting construction and/or the dominance of repair mechanisms. This dominance of repair mechanisms during rehydration has already been postulated for *S. ruralis*, as indicated by mRNA transcript accumulation patterns, which are then translated to proteins (Oliver, 1991). However, *S. ruralis* is a bryophyte known to be extremely DT.

Clearly, we can say that the profiles of proteins during dehydration and rehydration in *F. antipyretica* are similar to those reported for *P. patens*, a terrestrial bryophyte stated as DT by some authors (Wang *et al.*, 2009) but not for all (Koster *et al.*, 2010). This controversial classification depends whether to consider organismal DT (Wang *et al.*, 2009) or cellular DT (Koster *et al.*, 2010). Comparisons with other bryophytes with varying DT properties are not possible since proteomic studies using the most recent technology have not been performed for bryophytes other than *P. patens*. However, based on what is comparable, no major differences were observed between *F. antipyretica* and *S. ruralis*, a bryophyte typical from desert areas and thus

from dramatic contrasting habitats. Although no changes in proteins were observed during dehydration (Oliver, 1991), *S. ruralis* showed different physiological performances depending on the rate of dehydration, being better if slow than fast (Schonbeck and Bewley, 1981). Therefore, molecular studies with desert bryophytes namely with *S. ruralis* are very important to be developed in order to understand the level of preparation needed before drying.

The proportion of proteins that both increased and declined in abundance in *F. antipyretica* during dehydration in comparison to the hydrated control were a small portion of the total protein complement and at a percentage comparable to that reported for *P. patens* (Wang *et al.*, 2009; Cui *et al.*, 2012). During rehydration, the more common response is an increase in abundance of differentially responding proteins for all bryophytes species studied so far: *F. antipyretica*, *P. patens* (Cui *et al.*, 2012) and *S. ruralis* (Oliver, 1991).

6.5.1. Dehydration: metabolism shutdown regulated through protein down-regulation

Photosynthesis is significantly decreased during dehydration in *F. antipyretica* (**Chapter 2**) and this is in part due to a reduction in the abundance of some of the enzymes of the Calvin cycle, such as Rubisco (large sub-unit) and chloroplastic GAPDH. The decrease in photosynthetic rate based on gas exchange measurements has been observed in other bryophytes, such as *Anomodon viticulosus* (Hedw.) Hook. & Taylor (Hinshiri and Proctor, 1971), *Syntrichia ruraliformis* (Besch.) Cardot (Dilks and Proctor, 1974), *S. ruralis* (Bewley *et al.*, 1978) or *Polytrichum formosum* Hedw. (Proctor *et al.*, 2007). However, little information is available on the molecular biology of this process. The exception is the information of ESTs from *S. ruralis* (Wood *et al.*, 1999), in particular the early light-inducible proteins (ELIPs) that appear to bind to chlorophylls (Zeng *et al.*, 2002). Recent studies on *P. patens* showed a similar pattern of decline in the abundance of some Calvin cycle enzymes during dehydration (Wang *et al.*, 2009; Cui *et al.*, 2012). The loss of proteins associated with photosynthesis or shutdown of the photosynthetic process itself during dehydration appears to be a common response for all DT plants, vascular (Bernacchia *et al.*, 1996) or bryophytes (Bewley, 1995). However, once water is available bryophytes rehydrate almost instantaneously (Glime, 2007) while vascular plants can take about 15 hours to reach full turgor (Bernacchia *et al.*, 1996). Therefore, upon rehydration bryophytes resume photosynthetic activity very quickly (Proctor *et al.*, 2007), while vascular plants take more time (Schwab *et al.*, 1989). The present work shows that Rubisco still presents high abundance in cells, as

well as light harvesting, pigment stability and electron transport elements in *F. antipyretica*. This indicates the importance of preservation of these proteins for resuming photosynthesis upon rehydration, even though they are slightly lower than pre-desiccation due to an effective loss of protein either by regulation or degradation. On *P. patens* these elements are rather induced by dehydration (Wang *et al.*, 2009) or up-regulated by rehydration (Cui *et al.*, 2012), being probably a protection mechanism of the photosynthetic machinery.

Photorespiration associated enzymes also follow the same accumulation or decline in abundance pattern of proteins associated with photosynthesis. The photorespiration process is generally associated with protection from photoinhibition, dissipating excess energy through ATP, NADPH and reduced ferredoxin or via alternative oxidase (Takahashi *et al.*, 2007; Voss *et al.*, 2013). Although photorespiration can act as an important process to prevent ROS accumulation, it also leads to the formation of H_2O_2 in the peroxisome by glycolate oxidase (GLO) (Halliwell, 1987; Rea *et al.*, 2002). The down-regulation of GLO during dehydration might prevent an excess H_2O_2 production, since more than 70% of the total H_2O_2 production in plant leaves results from photorespiration via GLO catalysis (Noctor *et al.*, 2002). This might also explain the 2-fold increase in protein of the signaling ROS-related enzyme nucleoside diphosphate kinase 1 (NDPK1) that may regulate this process. These changes reflect the importance of cell oxidative stress regulation to achieve the ideal balance between ROS scavenging and ROS production (Voss *et al.*, 2013) and should be explored in future work.

The increased accumulation of cytosolic GAPDH observed in this study was also observed in leaves of the resurrection plant *C. plantagineum* during dehydration (Velasco *et al.*, 1994) and either during dehydration (Wang *et al.*, 2009) and rehydration (Cui *et al.*, 2012) of *P. patens*. It seems that this is a common pattern across DT species, with this enzyme presumably being involved in the preparation for desiccation by maintaining elevated levels of sucrose. This sucrose accumulation preserves not only cellular structure but also prepares future recovery upon rehydration when a higher demand for energy is needed, providing enough pyruvate for the citric acid cycle in the mitochondria.

From our measurements sucrose was maintained in similar levels throughout a slow dehydration/rehydration cycle, in *F. antipyretica* as it was in *S. ruralis* (Bewley *et al.*, 1978) and *P. patens* (Oldenhof *et al.*, 2006). The amount of sucrose found in *F. antipyretica* was higher (6% DW) than that found in *P. patens* (3-5% DW) (Oldenhof *et al.*, 2006) and lower than that observed in *S. ruralis* (10% DW) (Bewley *et al.*, 1978).

In summary, the differential protein accumulation patterns during dehydration of *F. antipyretica* suggests a down-regulation of the synthesis of proteins (or an increase in degradation) associated with photosynthesis and photorespiration, an increase the synthesis of proteins associated with ROS signaling and sucrose metabolism. These results are comparable to those reported for *P. patens* (Wang *et al.*, 2009; Cui *et al.*, 2012) in which translation initiation factors increase in abundance (Cui *et al.*, 2012) and a suggested shut-down of metabolism.

6.5.2. Dehydration induced protection mechanisms

The results in this work seem to confirm that cytoskeleton is dismantled during dehydration since cytoskeleton protein β-tubulin, which forms the microtubules of the cytoskeleton, was down-regulated during dehydration. This result is similar to what was observed in *P. patens* during dehydration (Wang *et al.*, 2009). In *F. antipyretica*, the pattern of variation of β-tubulin followed the same pattern of variation of the TCP-1/cpn60 chaperonin, which is known to be responsible for the folding of tubulin (Kubota *et al.*, 1995). Some authors have shown that during dehydration, membranes and organelles maintain their normal configuration (Platt *et al.*, 1994). However, to be able to withstand cell volume variations it has been proposed that the cytoskeleton (namely tubulin) may be deconstructed during dehydration (Pressel *et al.*, 2006; Wang *et al.*, 2009).

Another protective mechanism may involve the increase in ketol-acid reductoisomerase during dehydration, observed in *F. antipyretica* in this work and in *P. patens* (Wang *et al.*, 2009). This enzyme is responsible for the biosynthesis of the hydrophobic branched-chain amino acids valine, leucine and isoleucine. Ferreira *et al.* (2006) suggested that the increase of hydrophobic amino acid might be connected to *de novo* protein synthesis in an oxidative environment, favouring the production of isoforms more tolerant to these stresses. This was observed as a result of stresses such as heat stress in *Populus euphratica* Olivier (Ferreira *et al.*, 2006) and salinity stress in *Avicennia marina* (Forssk.) Vierh. (Tanaka *et al.*, 2002). It can also contribute to osmoregulation by increasing free amino acids (Rhodes *et al.*, 1986). Moreover, in *F. antipyretica* the enzyme glutamate dehydrogenase was also up-regulated and its overexpression has been correlated with higher tolerance to drought in transgenic plants such as *Nicotiana tabacum* L. and *Zea mays* L. (Mungur *et al.*, 2006; Lightfoot *et al.*, 2007), by increasing proline, and other compatible solute contents, also contributing to osmoregulation.

In *F. antipyretica* only one differential expressed HSP increased in abundance during dehydration, in contrast to *P. patens* where several HSP show higher accumulation (Wang *et al.*, 2009; Cui *et al.*, 2012). The high levels of HSP have been correlated with DT in orthodox seeds (DeRocher and Vierling, 1994; Wehmeyer *et al.*, 1996), in *C. plantagineum* (Alamillo *et al.*, 1995), in *P. patens* (Wang *et al.*, 2009; Cui *et al.*, 2012) and in *S. ruralis* (Oliver *et al.*, 2004). These molecular chaperones have a critical role during physiological stress, preventing proteins from irreversible aggregation by an energy-independent process that preserves protein function (Sun and MacRae, 2005). Thus, upon rehydration cells resume normal metabolism much faster with fewer proteins needing to be synthetized.

6.5.3. Rehydration: metabolism slowly returns to pre-desiccation function

Upon rehydration, the large sub-unit of Rubisco and chloroplastic GAPDH contents are still low. This may serve to regulate the photosynthesis process, preventing high ROS production through excess electron accumulation, but allowing time to recover for the different metabolisms. Other explanations of this low content can also be a result of cell death as observed in confocal microscopy of *F. antipyretica* (**Chapter 3**). Nevertheless, the large sub-unit of Rubisco and chloroplastic GAPDH content, as well as proteins involved in photorespiration, slowly return to pre-desiccation values upon rehydration. The increase in the accumulation of photosystem I reaction centre subunit IV, might be a very important mechanism in desiccation tolerance, as recently reported for intertidal macro-algae (Gao *et al.*, 2011; Gao and Wang, 2012), dissipating excess energy during rehydration, producing energy as enzymes from Calvin cycle slowly returning to control levels.

After rehydration, cytosolic GAPDH increases in abundance to levels that exceed those observed during dehydration. This GAPDH elevation was also observed in *P. patens* (Cui *et al.*, 2012), perhaps characterizing a high demand for energy and carbon skeletons required to repair or synthesize new molecules. The enzymes ketol-acid reductoisomerase and glutamate dehydrogenase also return to near pre-desiccation accumulation levels, indicating a reduced requirement for compatible free amino acids after rehydration.

After rehydration, protein synthesis is still impaired in some aspects, since the elongation factor 1- α and RNA-binding protein show lower levels than pre-desiccation. However, the translation initiation factor eIF-4A is elevated which perhaps indicates higher translational initiation. This also occurs in *P. patens* where translation initiation factors are also elevated (Cui *et al.*, 2012). In *S. ruralis*, there was induction or increase

on the synthesis of 74 proteins (Oliver, 1991). These changes show a regulation of protein synthesis after rehydration, which has been pointed to be at the translational level with accumulation of mRNA during the drying process (Wood and Oliver, 1999; Oliver *et al.*, 2004).

6.5.4. Rehydration: preservation of cell and protein structure for cell survival

After rehydration, β-tubulin returns to pre-desiccation values allowing the reconstruction of the cytoskeleton. This process of reconstruction has been observed in the bryophyte *P. formosum* (Pressel *et al.*, 2006) and in desiccation studies of *Inga vera* Willd. seeds (Rocha-Faria *et al.*, 2004). Actin, a protein that is a constituent of the cytoskeletal microfilaments, starts to increase in amount during dehydration and after rehydration reaches almost a 2-fold accumulation above that of the hydrated controls. The elevation of β-tubulin may help to preserve cell structure in *F. antipyretica*. A similar elevation has been documented the aero-terrestrial DT green alga *Klebsormidium crenulatum* (Kütz.) Lokhorst present in biological soil crusts (Holzinger *et al.*, 2011). An additional change occurs for the TCP-1/cpn60 chaperonin which maintains its content during dehydration, but is elevated upon rehydration. This pattern mirrors that of β-tubulin and actin patterns and could be of crucial importance as this protein is involved in the folding process for both proteins (Kubota *et al.*, 1995).

The high accumulation of a small HSP after rehydration (about 13- fold increase above control levels) seems to be an important component in the response of *F. antipyretica*. An increase in HSP is also a common pattern in other DT plants such as *P. patens* (Wang *et al.*, 2009; Cui *et al.*, 2012) and *S. ruralis* (Oliver *et al.*, 2004). After the re-establishment of plant metabolism, HSP are released and proteins undergo refolding by an ATP-dependent process mediated by chaperones (e.g. Hsp70) (Sun and MacRae, 2005). These proteins are involved in the folding and unfolding of other proteins and the response is generally a dramatic increase in accumulation when cells are exposed to different environmental stresses (e.g. temperature, light, drought, etc.) (Hendrick and Hartl, 1993). Some HSP resemble late embryogenesis abundant (LEA) proteins (Sales *et al.*, 2000) which are postulated to play a very important role in DT, protecting protein structure and function in *C. plantagineum* (Bartels, 2005), *P. patens* (Wang *et al.*, 2009; Cui *et al.*, 2012) and *S. ruralis* (Oliver *et al.*, 2004). These proteins are thought to act as chaperones during stress although the mechanism seems to be different, appearing to involve an interaction with soluble sugars, like sucrose or trehalose in a process termed vitrification (Smirnoff, 1992; Goyal *et al.*, 2005). Although we did not see the presence of LEA proteins in this study, they might be present in the spots that we were unable to identify.

Steady levels of sucrose are maintained throughout the slow dehydration/rehydration cycle (*circa* 6% of DW). In fast dehydrated samples, sucrose levels increased (*circa* 9% of DW) but dropped following rehydration (*circa* 4% of DW). In this case, together with the observed loss of proteins upon rehydration of fast dehydrated samples, is the result of extensive membrane disruption and cell content leakage as already observed in **Chapters 2** to **4** confirming that this bryophyte does not survive fast dehydration and time is needed for survival.

6.6. Conclusion

Land colonization by early bryophyte ancestors is thought to require the development of desiccation tolerance mechanisms that involved the accumulation of specific proteins during cycles of dehydration/rehydration. The basis for such tolerance relies on common patterns of protein expression and metabolic adjustments which are very similar even in bryophytes from very distinct habitats, like the aquatic bryophyte *F. antipyretica* and the terrestrial bryophyte *P. patens*. The dynamics of regulation of protein accumulation as well at the level of the protein functional groups seem to have common patterns that point to regulate the decrease of photosynthesis, increase in glycolytic metabolism and a reorganization in the cytoskeleton during dehydration. In addition, the protection of structures and proteins via a high sucrose content, elevation of heat-shock proteins and osmoregulation are common patterns throughout the dehydration/rehydration cycle. Currently, we do not have enough information to compare directly these results with a DT bryophyte like *S. ruralis* and further confirm this hypothesis. However, it is possible that *S. ruralis* functions as it was described in this work. Further investigation is needed to establish these common mechanisms as key components in the desiccation tolerance that can be transferred to other plants of economic interest to further thrive in water shortage environments.

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Chapter 7

General Discussion
and
Future Perspectives

7. General Discussion and Future Perspectives

The main objective of this Thesis was to study the physiological, biochemical and molecular aspects of desiccation of an aquatic bryophyte (*Fontinalis antipyretica* Hedw.) and integrate this information into the state of the art of the Ecophysiology of desiccation in Bryophytes. For the first time, we found that under certain conditions of slow dehydration the physiological, biochemical and molecular responses of an aquatic bryophyte can be similar to those reported for terrestrial bryophytes, known to cope with desiccation. Based on the assumption that the physiology, biochemistry and molecular response of bryophytes are similar it is further discussed what differences might be associated for the different ecologies and habitats preferences of terrestrial and aquatic bryophytes.

7.1. Aquatic bryophytes are desiccation tolerant

Based on several criteria, each bryophyte species has been classified in what concerns desiccation from tolerant to sensitive (reviewed in Wood, 2007). Abel (1956) did one of the first attempts to classify vegetative desiccation tolerance (DT) in a wide range of bryophytes through vitality tests based on the exposure to a very wide range of relative humidity (RH) atmospheres (0% to 96% RH). Since *F. antipyretica* only survived at 96% RH it was classified as desiccation intolerant. It was not the only report classifying this aquatic bryophyte as desiccation intolerant. Other authors reported similar conclusions based on measurements of photosynthesis in desiccated bryophyte tips exposed to fast dehydrating atmospheres (Lee and Stewart, 1971), electrolyte leakage (Brown and Buck, 1979) or habitat preference (Kimmerer and Allen, 1982; Seel *et al.*, 1992; Franks and Bergstrom, 2000). Although, the level of relative humidity at which these experiments were performed could be realistic, the rate of water loss (not monitored in these experiments) could be unrealistic, since these tests were made in isolated leaves or shoots, a condition that does not occur in nature. Most studies did not follow the recovery over a long period, only a few hours after rehydration (Lee and Stewart, 1971; Brown and Buck, 1979). Some authors (Abel, 1956) had already pointed out briefly that some other aquatic bryophytes *could* develop DT under certain conditions; however, this was never the focus of attention of present research in DT.

In this work, we showed that under field conditions *F. antipyretica* can survive the summer periods of Mediterranean drought (2-3 months) when the bryophyte is stranding out of water due to decreased river water flow (**Chapter 2**). Shoots of this bryophyte that were in the field out of water for more than one month, once in the lab and after rehydration photosynthesized at the control level as shown in **Chapter 2**.

***Fontinalis antipyretica* and maybe some other aquatic bryophytes can cope with vegetative desiccation under natural conditions.**

The reasons for the apparent contradiction in the DT classification of *F. antipyretica* in this work compared with the previous ones (Abel, 1956; Lee and Stewart, 1971; Brown and Buck, 1979), could be related to the effect of different rates of water loss in bryophyte physiology. However, this information was mostly never present in previous works.

7.2. The importance of dehydration rate

The fact that dehydration rate is important in DT is not new but its relative importance was never fully evaluated across bryophytes. Some works quoted that only sensitive bryophytes needed slow dehydration prior to desiccation to become DT (Höfler, 1946; Abel, 1956; Krochko *et al.*, 1978). A close look to the data, and not to the conclusions reported on the previews works, evidences that even typical DT bryophytes need to be slow dehydrated to avoid some type of damage. The desert bryophyte, *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr, could avoid displaying visible injury, decrease in total chlorophyll, higher electrolyte leakage and inhibited gross photosynthesis if slow dehydrated (Schonbeck and Bewley, 1981). Another desert bryophyte, *Pterygoneurum lamellatum* (Lindb.) Jur. when subjected to fast dehydration shows slower growth rates, less regeneration and damaged photosynthetic system (Stark *et al.*, 2013).

In this work we showed **that an aquatic bryophyte can cope with desiccation only if the rate of water loss is slow**. Faster rates of water loss (> 6.9 ± 0.9 mg H_2O h^{-1}) led to lower photosynthetic performance in the first moments upon rehydration (**Chapter 2**).

The work in this Thesis, together with one recently published (Stark *et al.*, 2013), allows to suggest that **the rate of water loss is important for all bryophytes independently of the DT level.** If this assumption is acceptable then all bryophytes need time to be prepared for the desiccation, showing that this feature might be common among all bryophytes, different to what was thought until now. Another point that needs to be remarked for the future is the need to evaluate the rate of water loss at each experiment allowing the future comparison among studies.

7.3. Physiological changes during desiccation tolerance

Bryophytes are photosynthetic poikilohydric organisms, and, thus, photosynthesis is one major physiological parameter affected by water loss. According to their poikilohydric nature they equilibrate their water content with the surrounding environment, with photosynthesis shutting down as lower water potentials are reached (Lee and Stewart, 1971). Once rehydrated, bryophytes resume their photosynthetic activity. It was thought that bryophytes should recover completely the pre-desiccation level of photosynthesis immediately after a desiccation event to be considered DT (Seel *et al.*, 1992). However, knowing that the different components of photosynthesis take time to be fully operational (Csintalan *et al.*, 1999; Proctor and Pence, 2002), it is important to follow recovery over time.

We found that aquatic bryophytes subjected to both fast and slow dehydration showed complete recovery of the parameter maximum photochemical efficiency of PSII (F_v/F_m); immediately for slow dehydration and after some days (3-5) for fast dehydration. Photosynthesis was negatively affected by both the extent and rate of water loss but the relative effect of each factor changed along the recovery phases, as previously shown in other bryophytes. This work shows that in the first moments of rehydration (20-30 minutes), there were no differences between dehydration rates, being the response essentially correlated with extent of water stress (**Chapter 2**).

It was not possible to measure photosynthesis in *F. antipyretica* during dehydration. However, it might have decreased with decreasing water content, in accordance to what happened in other bryophytes with different DT levels (Tuba *et al.*, 1996; Proctor, 2000). We found differences in chloroplast organization between slow and fast dehydration conditions, shown through the parameter F_v/F_m. Following fast dehydration, the linear decrease of this parameter with RWC was similar to the gas exchange response. Following slow dehydration, the decrease was not linear presenting a two-phase response: the first was a very slight decrease until RWC of *circa* 30-40% and then a steeper decrease. The chloroplasts appeared to be better preserved after slow dehydration than after fast dehydration, as shown by the photosynthesis recovery over a period of five days. Pre-desiccation photosynthesis was reached only in slow dehydration, even though F_v/F_m was very similar to control in both dehydration rates. This data led us to the hypothesis of cell damage during fast dehydration, and thus investigate oxidative processes in response to different dehydration rates (**Chapters 3** and **4**).

This work shows the **importance of dehydration rate to establish DT mechanism that allow photosynthesis to return to pre-desiccation levels**, as

shown by the analysis of gas exchange and chlorophyll *a* fluorescence, **showing that this aquatic bryophyte as a similar recovery pattern as the one in other DT terrestrial bryophytes**. The reason of this apparent discrepancy with other works and one of the major problems to evaluate is the importance of dehydration rate. In the majority of the studies this information is lacking and, therefore, future works should include a precise description of the dehydration conditions.

7.4. Slow dehydration rate is key to protect / prepare to repair cells in desiccation

It is known that during the process of dehydration metabolism starts to slow-down and the physiological processes associated might generate ROS (Walters *et al.*, 2002). This increase in ROS leads to an increase in reactions with proteins, lipids and nucleic acids (see review in Smirnoff, 1993), causing several types of damage (Hyslop *et al.*, 1988; Kaiser, 1979) including membrane leakage during rehydration (Simon, 1978; Bewley, 1979; Bewley and Krochko, 1982). Until now there was no information of the response of ROS production to dehydration rate in bryophytes, except for some indirect data of the activity of glutathione reductase (GR), an antioxidant enzyme, in *S. ruralis* (Dhindsa, 1987).

In this work we evaluated the cellular damage through analysis of the oxidative status (**Chapter 3**) and membrane damage (**Chapter 4**) upon rehydration following slow and fast dehydration. We found that following rehydration there was a clear difference between the two dehydration rates (**Chapter 3**): slow dehydration showed low ROS production while fast dehydration showed a 70-fold higher ROS production than in slow dehydration. Therefore, it is suggested **that part of the cellular damage was due to this ROS burst that is probably responsible for the membrane damage and solute leakage** (**Chapter 4**), affecting directly cell survival. Moreover, the nitric oxide production upon rehydration seem to benefit membrane protection against damage only in slowly dehydrated cells (**Chapter 4**). During fast dehydration, the increase in sucrose and in cell wall elasticity can be an attempt to minimize damage when there is no time for a more effective protection (**Chapter 5**). The decrease in sucrose content during the recovery period following fast dehydration (**Chapter 5**) can be due to membrane leakage (**Chapter 4**) and the loss of viable cells (**Chapter 3**), evidencing the need of slow dehydration for cell survival.

The different oxidative response to dehydration rate led us to believe in differences of protein expression (**Chapter 6**) that could explain the low ROS production in slow dehydration. Nevertheless, future work should also address the antioxidant systems in response to the dehydration rate.

7.5. Protein regulation to achieve desiccation tolerance

As previously stated, it appears that some important protection elements/mechanisms need to be present before desiccation and only a slow drying rate can enable them to act. Therefore, a molecular analysis through differential proteomics, which revealed some of the protection/repair mechanisms in a dehydration/rehydration cycle that allow the survival of *F. antipyretica* was performed (**Chapter 6**).

Until recently, the few available works on proteins appear to indicate that no protein synthesis occurred during dehydration and DT bryophytes relied on a constitutive protection coupled with a repair-based mechanism upon rehydration (Oliver, 1991; Wood and Oliver, 1999). However, recent works on proteomics in *Physcomitrella patens* (Hedw.) Bruch & Schimp. showed protein regulation during both dehydration and rehydration, being several proteins up-regulated during dehydration, a clear indication of protein synthesis during this phase (Wang *et al.*, 2009; Cui *et al.*, 2012). Moreover, in a very recent work it was demonstrated that desert bryophytes can lose their DT if kept hydrated and DT can be induced only during slow dehydration (Stark *et al.*, 2013), refuting previous statements that DT bryophytes have only a constitutive protection with repair-based system operating following rehydration.

With the work in *F. antipyretica*, we showed that slow dehydration induced changes in patterns during both dehydration and rehydration. These protein patterns resemble the ones found in *P. patens* (Wang *et al.*, 2009; Cui *et al.*, 2012) and also in *S. ruralis* (Oliver, 1991), on what is comparable. The patterns include similar changes in major groups of proteins that show the same variations across bryophytes from distinct habitats throughout the dehydration/rehydration cycle. In the primary metabolism proteins, components from the thylakoid membranes associated to electron transport chains and reaction centres as for most of the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) content are preserved. This is in accordance with the measures for photosynthesis, both through gas exchange and chlorophyll *a* fluorescence, observed during recovery (**Chapter 2**), being a pattern of variation consistent with DT (Proctor, 2001). For Rubisco, there is in fact some down-regulation that can result from damage, controlled regulation or also as a source of amino acids, but it still remains in high levels in cells. Therefore, upon rehydration photosynthesis can be resumed almost immediately, a characteristic of DT bryophytes (Bewley, 1979), occurring with time the repair / synthesis of Rubisco.

Sugars have an important role not only in vitrification (Buitink and Leprince, 2004) but also in osmoregulation (**Chapter 5**) and as a source of energy during

rehydration (Whittaker *et al.*, 2004). Enzymes regulating sugar metabolism are up regulated throughout the dehydration/rehydration cycle, indicating high demands of energy necessary for DT, in preparation for repair upon rehydration. High sucrose levels, a common feature of DT, was also observed in *P. patens* (Oldenhof *et al.*, 2006) and *S. ruralis* (Bewley *et al.*, 1978).

Moreover, the protein NDPK1, related to ROS signalling which interacts with catalases, accumulated throughout the entire dehydration/rehydration cycle and might be one of the mechanisms through which ROS are scavenged during slow dehydration (**Chapter 3**). On the other hand, high levels of heat shock proteins (HSP), defensive proteins associated to protection of proteins and cells, were found to be constitutively expressed and one in particular was found to be dehydration/rehydration responsive increasing up to 13-fold. Other proteins (Late Embryogenesis Abundant, LEA) with similar functions, were found at high levels in *P. patens* (Cui *et al.*, 2012) and in mRNA transcripts of *S. ruralis* (Oliver *et al.*, 2004).

These protein patterns are not only rehydration-induced but also dehydration-induced in both *F. antipyretica* and *P. patens*. In *S. ruralis* there is an accumulation of mRNA during dehydration (Oliver, 1991; Wood and Oliver, 1999), but dehydration-induced protein synthesis and its identification is yet to be analysed. In *F. antipyretica* at the end of fast dehydration, the protein profile is very similar to control and upon rehydration most proteins were lost (**Chapter 6**) most probably due to membrane damage (**Chapter 4**). This clearly demonstrates that fast dehydration rate does not provide enough time for induction of mechanisms of protection / preparation to repair. On the other hand, the protein profile of slow dehydrated samples shows very significant changes relatively to control both during dehydration and rehydration being an evidence of induction of those mechanisms of protection / preparation to repair necessary to survive a desiccation event.

This work shows that in *F. antipyretica* **an aquatic bryophyte, the variations in protein patterns are similar to the ones observed in other DT bryophytes**, such as *P. patens*, and, to what is comparable, in *S. ruralis*, suggesting that bryophytes from different ecological preferences may share a common DT mechanism. If DT mechanisms are similar among bryophytes, what are the differences that make them have different ecological preferences and thrive in contrasting habitats?

7.6. Could morphology be key for explaining ecology?

It is very difficult to compare the rate of dehydration across bryophyte species. One of the problems in their diverse morphological structure (Fig. 7.1) (Alpert and Oliver, 2002).

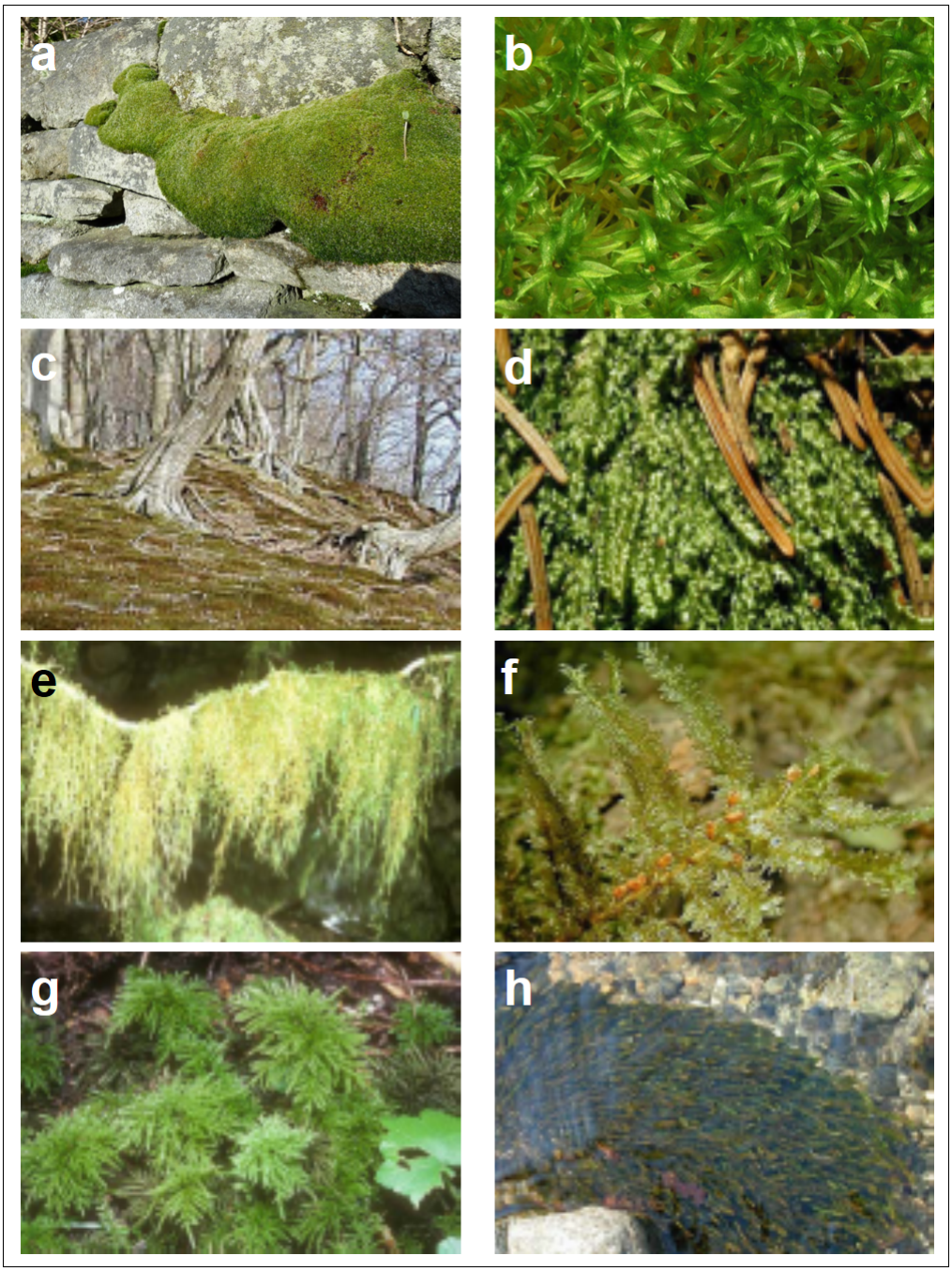

Figure 7.1. Life forms of bryophytes (adapted from Glime, 2007). **a**. Cushion – *Syntrichia ruralis*. **b**. Short turf – *Physcomitrella patens*. **c**. Tall turf – *Polytrichum formosum*. **d**. Mat – *Plagiothecium curvifolium*. **e**. Pendant – *Meteorium* sp.. **f**. Fan – *Neckera urnigera*. **g**. Dendroid – *Pleuroziopsis ruthenica*. **h**. Streamer – *Fontinalis antipyretica*. Photos **c**, **d**, **f** and **h** by Michael Lüth; **a** by Judith Allinson; **b** by Victor Jones; **e** and **g** by Janice Glime.

We can expect that bryophytes with different morphologies, dried in the same conditions and maintaining their initial tissue organization, will dehydrate at completely different rates. Most probably, bryophytes from habitats with higher moisture will dry faster because they have simpler morphologies more adapted to other limiting factors than water. These morphologies or life forms are cushions, short or tall turfs, mats, pendants, fans, dendroids and streamers (Glime, 2007) formed by shoots with different lengths and ramifications with axis supporting the leaves consisting of a single layer of cells (Fig. 7.1).

Bryophytes from dry habitats are organized in more dense forms like cushions (Fig. 7.1a) (Glime, 2007). Thus, they naturally retain more water by capillarity and dehydrate more slowly than the others from damp locations when submitted to the same drying conditions. In denser bryophytes it is expected higher water surface tension and water will be lost at lower rates than the less dense bryophytes. In fact, Proctor (1981), showed that surface tension was the most important factor in maintaining the microclimate (gradients of humidity, temperature and wind speed) within the bryophyte, shaping its physiology and ecology under water deficit.

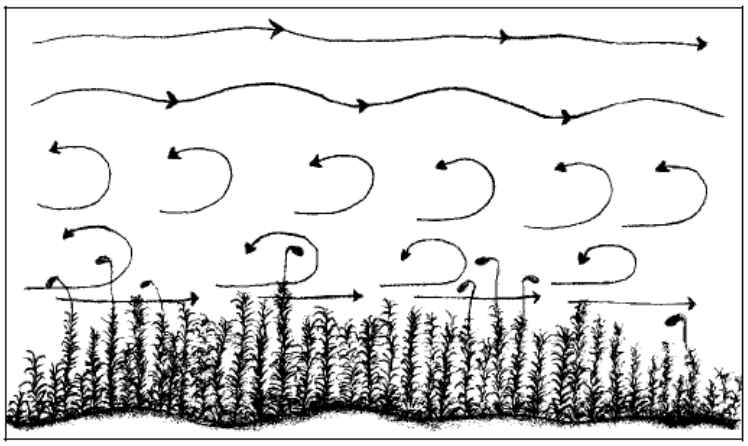

Figure 7.2. Turbulence and boundary layer effect in an irregular surface of a bryophyte weft. In cushions, bryophytes stems have the same height; therefore, turbulence will be significantly lower reducing dehydration rates (Glime, 2007).

It is expected that morphology, life form and colony structure, can be a determinant factor in the adaptation of bryophytes to each habitat and corresponding predicted levels of desiccation (Bates, 1998). More compact life forms (e.g. cushions) will reduce dehydration rate by modifying the microclimate (Fig. 7.2), allowing time to develop DT mechanisms. This hypothesis is in accordance to the information that DT molecular mechanisms, developed early in evolution upon land invasion in the Devonian (Kenrick and Crane, 1997), and were present initially in all bryophytes. Under

this circumstances, morphological adaptations might be the main evolution driver for adaptations to new habitats, according to water availability.

Future works should always address the dehydration rate of the colony and cellular level. This will give some insight in order to compare and understand the thresholds in terms of damage at the cellular level or, on the other hand, on productivity at the organismal/colony level. For example, the classification of the terrestrial bryophyte *P. patens* as DT may depend if one is considering organismal DT (survival and regeneration of the colony) (Wang *et al.*, 2009) or cellular DT (survival of each cell) (Koster *et al.*, 2010). Cellular DT can be induced in *P. patens* if slowly dried (Frank *et al.*, 2005; Wang *et al.*, 2009).

7.7. Why is slow drying less damaging?

It is clear, that fast dehydration has always a more deleterious effect on bryophyte recovery; this effect may result from the induction of protection/repair mechanisms. The initial slow dehydration allows even an aquatic bryophyte like *F. antipyretica* to be as tolerant as any xeric bryophyte (this work). Therefore, the mechanisms are kept active for some time allowing the preparation for drying through a process called hardening. Hardening is a well-known phenomenon in higher plants, a common response to different types of abiotic stress. When plants are subjected to stress, sensing events connected to signalling cascades lead to restitution counter-reactions which, in turn, lead to the phase of resistance to stress. Hardening and de-hardening occur in this latter phase (see Duque *et al.*, 2013).

After field-desiccation, recovery was very similar to slow dehydration in the laboratory (**Chapter 2**), which indicates the strategy used by aquatic bryophytes when subjected to fluctuations of water in the summer. After fast dehydration, even though some cells survive, they never reach the pre-desiccation physiological activity even after five days indicating the failure to prepare for desiccation (no hardening), which is critical for recovery upon rehydration. Fast dehydration is incompatible with cell survival indicating a physiological threshold for DT. Fast drying rates applied in several previous works (Krochko *et al.*, 1978; Oliver, 1991; Deltoro *et al.*, 1998; Wood *et al.*, 1999) may have not reflected what happens in nature and we must be cautious about the interpretation of this data.

The question of whether the DT mechanisms are constitutive or inducible is still under debate (Proctor *et al.*, 2007a). In *S. ruralis*, an accumulation of mRNA during slow dehydration was observed and, apparently, no changes were found in proteins during this phase (Oliver, 1991; Wood and Oliver, 1999). However, the proteomic

profile of this bryophyte during dehydration is still lacking to fully confirm this idea. As seen in **Chapter 6** and other works (Wang *et al.*, 2009; Cui *et al.*, 2012) changes in proteins are very small but appear to be crucial for DT. Fast dehydration (sometimes in periods of only 30 minutes) may not allow the synthesis of new proteins, whereas slower dehydration rates will. The proportion and nature of these mechanisms may be variable, but DT is certainly not a characteristic determined only by constitutive protection mechanisms since some of these mechanisms can be induced by slow dehydration in other bryophytes. Therefore, **dehydration rate is crucial to allow the establishment of DT mechanisms**. The latter idea was confirmed in *F. antipyretica* (in this work), *P. patens* (Wang *et al.*, 2009; Cui *et al.*, 2012) and in a very recent work by Stark *et al.* (2013) in the desert bryophyte *P. lamellatum* that lost DT after being kept hydrated for five days, after which fast drying was lethal. Moreover, DT was restored afterwards only by slow dehydration. Therefore, it appears that bryophytes need time to be prepared or time to induce some molecular synthesis in order to develop DT.

In this work, bryophyte samples used for experiments were never subjected to hardening since they were stored under water with aeration except for the samples directly collected dried under field conditions (**Chapter 2**). However, the imposition of slow dehydration may allow the induction of hardening adapting to the stress conditions. On the other hand, fast dehydration may not allow time for this adaptation to occur. Information about the hardening conditions in this aquatic bryophyte needs to be further explored in future work.

Some bryophytes can be de-hardened over long periods of hydration, as the case of a desert bryophyte like *P. lamellatum* which can be de-hardened after some days of constant hydration and develop DT (Stark *et al.*, 2013). However, there seems to be an exception, the desert bryophyte *Syntrichia caninervis* Mitt. which appears to conserve this protection, even after being hydrated for one month (Stark *et al.*, 2012).

Since this effect of hardening/de-hardening is crucial to make a correct interpretation of the data, future works should describe precisely the methodology regarding the pre-treatment of the samples, in order to understand if there is a protection/repair mechanism already in place or not. Furthermore, future research should focus on comparing all the molecular aspects of these bryophytes to further understand the networks and patterns involved in desiccation tolerance.

7.8. Final remarks and future perspectives

The patterns of variation in the DT mechanisms are very similar across studied bryophyte species. During dehydration, photosynthesis shuts down (Hinshiri and Proctor, 1971; Dilks and Proctor, 1974; Tuba *et al.*, 1996; Proctor *et al.*, 2007b), high levels of soluble sugars occur in the cytoplasm (Bewley *et al.*, 1978; Oldenhof *et al.*, 2006), defence proteins increase (Alamillo *et al.*, 1995; Wang *et al.*, 2009; Cui *et al.*, 2012), cytoskeleton is disassembled (Pressel *et al.*, 2006; Wang *et al.*, 2009) and sugar metabolism enzymes are up-regulated (Velasco *et al.*, 1994; Wang *et al.*, 2009; Cui *et al.*, 2012). After rehydration, photosynthesis restart (Tuba *et al.*, 1996; Proctor and Smirnoff, 2000), cytoskeleton is re-assembled (Pressel *et al.*, 2006) and high levels of soluble sugars (Bewley *et al.*, 1978; Oldenhof *et al.*, 2006), sugar metabolism enzymes and defence proteins (Cui *et al.*, 2012) are maintained.

Therefore, the main (provisional) conclusion suggested by this data and our own work is that DT at the cellular level, namely at the level of the molecular mechanisms, is similar among bryophytes such as *F. antipyretica* (this work), *P. patens* (Wang *et al.*, 2009; Cui *et al.*, 2012) or *S. ruralis* (Oliver, 1991), independently of their preferred habitat. Furthermore, it states that DT is induced by slow dehydration rate. This work, more than just classifying *F. antipyretica* as a DT organism well integrated in its particular habitat, proposes DT as a common feature in bryophytes, with shared molecular patterns that are driven and regulated by dehydration rate, which in turn is strongly modulated by the life form of the bryophyte.

If bryophytes appear to be displaying similar response patterns to desiccation, what is determining the different levels of DT in these organisms?

This work proposes that morphology is very important for DT. It might be the determinant factor in the adaptation of bryophytes to each habitat, leading them to respond in different ways to water availability. DT molecular mechanisms, developed early in evolution upon land invasion in the Devonian (Kenrick and Crane, 1997), are predicted to be present in all bryophytes, even in those living in habitats where water is available most of the time.

Fontinalis antipyretica is under water most of the year, it presents higher vegetative growth rates (higher than 100-fold) compared with other terrestrial bryophytes like *S. caninervis* (Stark *et al.*, 1998; Cruz de Carvalho, personal observation). On other hand, *S. caninervis* does not have much competition in its habitat while *F. antipyretica* has to compete for resources with other aquatic macrophytes and microalgae. Thus, the investment in DT protection mechanisms *F. antipyretica* might be lower than the one in desert bryophytes. While *S. caninervis* has

a DT mechanism derived mostly from hardening due to exposure to daily fluctuations of water availability, *F. antipyretica* may invest more on growth to better colonize the water streams. In fact, this aquatic bryophyte develops DT in the summer (June-September in the Mediterranean) when precipitation is lowest, with streams reaching a very low water level, some of them becoming completely dry. However, water level declines gradually and due to *F. antipyretica* streamer life form (Mägdefrau, 1982), the long shoots overlap each other and dehydration may be slow, allowing time for the induction of DT protective mechanisms. Once DT is established and the bryophyte completely dried, *F. antipyretica* can endure the dry season. Of course the possibility of surviving a desiccation event during the summer, while other macrophytes dry out and die, may have an evolutionary advantage, since once the water becomes available they can start to use the nutrients, while others are still germinating from spores or seeds.

Under global climate change scenarios this might change since an increase in temperature and scarcity of precipitation (IPCC, 2012; Maestre *et al.*, 2012) might increase the rate of water loss. To support this idea recent work in a manipulated experiment showed that a small increase in summer precipitation events coupled with faster evaporation (fast drying rates) under warmer conditions affected dramatically the carbon balance of the desert bryophyte *S. caninervis* and caused its death as recently published by Reed *et al.* (2012).

This work integrated in the present state of the art suggests that the complexity of the physiological, biochemical and molecular response to DT appears to have a higher proportion of induction than of constitutive mechanisms than it was previously expected. Therefore, the partition between constitutive and induced mechanisms needs further research. Future work should address several important questions. One of the aspects would be to apply the same methodologies of the present work to other bryophyte species from a wider range of habitats, but at the colony scale, measuring simultaneously the surrounding microenvironment. Moreover, further analysis of the proteomics and the transcriptomics should also clarify many aspects of the molecular network surrounding DT mechanisms. Furthermore, DT should be addressed from an evolutionary point of view, performing a full phylogenetic study on bryophytes from contrasting habitats to allow the clarification of the nature of DT mechanisms, including the role of life form, and testing if these mechanisms were kept from a primitive ancestor, or, on the other hand, are product of independent evolutionary events.

7.9. References

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