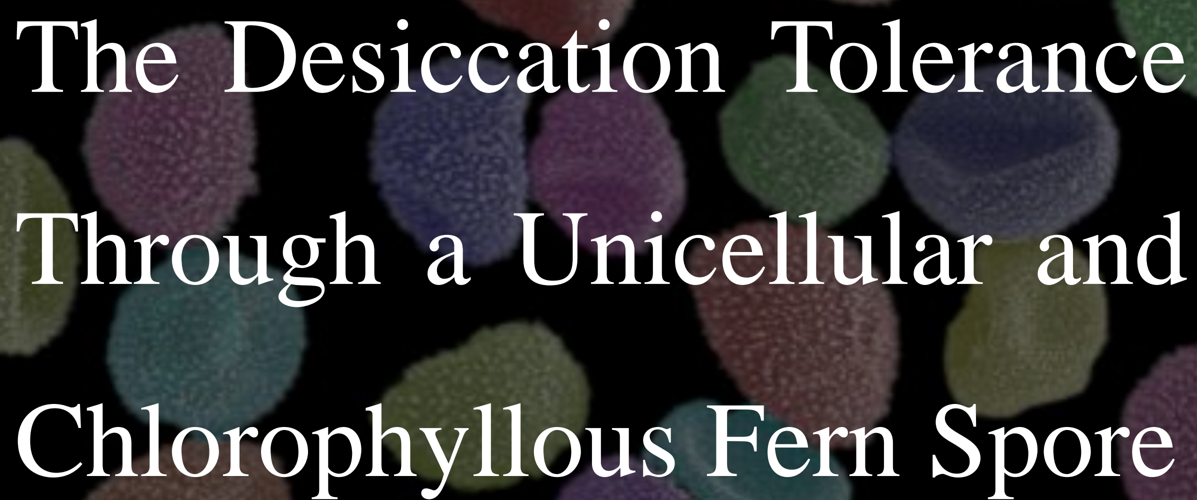

The Desiccation Tolerance Through a Unicellular and Chlorophyllous Fern Spore

Marina López-Pozo

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CAMPUS OF
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EXCELLENCE

The Desiccation Tolerance Through a Unicellular and Chlorophyllous Fern Spore

DOCTORAL THESIS

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Departament Of Plant Biology and Ecology
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Preface

Structure and format of the thesis

This thesis contains eleven chapters divided in six main sections as follows: a general Introduction, main objectives, general material and methods, four chapters of results, general discussion, conclusions, general bibliography and appendixes. Results have been written in the format of manuscripts that have already been published, have been submitted or are almost ready for it, and consequently, each chapter contains each own specific introduction, methodology, and discussion of results, as an individual entry.

Marina López-Pozo is the author of this thesis and was the main responsible for the experimental design, sample collection, analytical and physiological measurements, data analysis and paper report. All the authors of each paper contributed in the experimental designs and in the discussion of the results.

A mi madre,

A mi padre,

A mí.

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SUMMARY

The present thesis first establishes a universal protocol to define the tolerance to desiccation in photosynthetic tissues and subsequently focuses on investigating the ecophysiological characteristics that enable this strategy using fern chlorophyllous spores as the model of study. These spores have a fully functional photosynthetic apparatus in mature state. Through experimentation based on chlorophyllous spores, some of the key aspects in the physiology of desiccation have been defined. Thus, it has been concluded that there is a tolerance gradient from those photosynthetic tissues unable to tolerate water loss (considered as sensitive to desiccation) and those that can tolerate several cycles of severe water loss. This greater or lesser tolerance to desiccation has been related to specific physiological processes and to the ecological requirements of the species. A first and main finding derived from this thesis has been the observation of the change in the lipid composition occurring in the chloroplast membranes in parallel to the loss of tolerance during germination of chlorophyllous spores. A downregulation of those galactolipids, that are responsible for membrane stability, was observed within 72 hours. In addition, galactolipids responsible for greater membrane fluidity and a major photochemical activity suffered a considerable increase when desiccation tolerance was lost. Second, the activation of thermal energy dissipation in dry chlorophyllous spores was evidenced for the first time in this thesis, being this mechanism of photoprotection positively related to desiccation tolerance. Third, it was found that light and oxygen are the main determinants of viability loss in dry chlorophyllous spores. Finally, it is concluded that the use of chlorophyllous spores offers a unique opportunity to study the infrequent phenomenon of desiccation tolerance in photosynthetic tissues. Its unicellular and simple character facilitates (in comparison with other more complex tissues) the study of physiological and physicochemical mechanisms that allow a cell to survive extreme water loss.

RESUMEN

La tolerancia a la desecación (TD) representa una estrategia ecofisiológica que muy probablemente se desarrolló durante la conquista del medio terrestre por los primeros organismos fotosintéticos. Esta característica se ha ido perdiendo progresivamente a lo largo de la evolución, probablemente debido al desarrollo de tejidos complejos. A medida que aumentaba el control del contenido de agua por medio de la cutícula o los estomas, la capacidad de tolerar la pérdida de agua, fue desapareciendo. A día de hoy, la TD de los organismos fotosintéticos sigue siendo común en las algas, líquenes y briofitos, pero extremadamente rara en los traqueófitos, especialmente en angiospermas. Las estructuras dispersivas (polen, esporas, semillas) siguen manteniendo un alto grado de TD, pero usualmente, éstas no contienen cloroplastos desarrollados y lidiar con la pérdida de agua resulta más sencillo.

El objetivo principal de esta tesis es caracterizar los mecanismos fisiológicos y bioquímicos que intervienen en la tolerancia a la desecación, con un énfasis particular en los eventos metabólicos que tienen lugar durante la pérdida de agua. Para ello se utilizaron esporas clorofílicas de helecho como modelo. Este objetivo principal se desarrolló a través de cuatro objetivos específicos, que corresponden a uno por capítulo.

1.- Desarrollo de un procedimiento para la evaluación de la tolerancia a la desecación en tejidos fotosintéticos.

El "Falcon method" desarrollado durante la tesis, representa un procedimiento útil para la estimación semicuantitativa de la tolerancia a la desecación en tejidos fotosintéticos. Se trata de un protocolo universal, simple, estandarizado y portátil que permite evaluar la TD en una amplia variedad de tejidos y organismos fotosintéticos, requiriendo requisitos instrumentales mínimos y siendo particularmente útil en ubicaciones remotas. El método, probado en briófitos y angiospermas, se basa en generar diferentes tasas de desecación en varias humedades relativas, proporcionando un índice comparativo, con un umbral de recuperación del 30% de Fv/Fm como indicador de tolerancia a la desecación.

2.- Caracterización del grado de TD y los mecanismos bioquímicos y fisiológicos que se activan en esporas clorofílicas de helechos:

El primer análisis bioquímico y fisiológico de estas estructuras nos permitió determinar que las esporas clorofílicas de helecho no difieren fisiológicamente de las células fotosintéticas de las plantas verdaderas. De hecho, los pigmentos lipofílicos, los tocoferoles, la composición de la membrana y su respuesta a los factores ambientales son los mismos que se observan en otras plantas. Posteriormente, se definió su grado de TD concluyendo que efectivamente son capaces de tolerar la perdida de agua y que, además, activan los mecanismos protectores propios de organismos fotosintéticos TD. Las principales conclusiones a las que este objetivo nos permitió llegar, son las siguientes:

- Cuando las esporas clorofílicas del helecho *O. regalis* se liberan del esporangio son TD.
- Mientras las esporas son TD, existe una fuerte atenuación no fotoquímica de la fluorescencia de la clorofila, indicativa de disipación térmica. Una vez se pierde la TD, este mecanismo de fotoproteccion, desaparece.
- Una vez las esporas se colocan en situación de germinación (presencia de luz y agua), pierden la TD en unas 72 horas, pudiéndose observar importantes cambios en las membranas del cloroplasto. Tanto la composición como la función cambian incluso antes de la emergencia del rizoide. Básicamente, los trigalactolípidos disminuyen a favor de los monogalactolípidos, bajando paralelamente el grado de saturación de los ácidos grasos. Por último, los lípidos de reserva, como el triacilglicerol, se agotan rápidamente.

Estos resultados destacan la importancia de la estructura de los lípidos de las membranas de los tilacoides como punto clave para proteger la integridad de la membrana durante la desecación, junto con el grado de saturación de ácidos grasos y la disipación térmica como mecanismo para prevenir el daño oxidativo. Además, el cambio de TD observado dentro de la misma célula en tan solo 72h, ofrece un modelo ideal para estudiar los mecanismos que permiten la supervivencia a la pérdida de agua.

3.- Interpretación de la TD en función de los requerimientos ecológicos.

Tras haber determinado la extensión de la TD en las esporas clorofílicas de *O. regalis* y los mecanismos que la permiten en el helecho modelo, analizamos esporas clorofílicas de otros helechos en relación a la época del año en la que estas esporas son liberadas. La

viabilidad de las esporas clorofílicas en estado seco es muy variable entre las especies y los mecanismos fisiológicos, bioquímicos y biofísicos que sustentan esta variabilidad permanecen poco estudiados y su interpretación desde un enfoque ecofisiológico, es prácticamente nula. Para este objetivo se seleccionaron otras dos especies más. *Matteuccia struthiopteris* y *Equisetum telmateia*. Ambos helechos son productores de este tipo de esporas, pero su esporulación se da en estaciones del año diferentes. *M. struthiopteris* desarrolla los esporofilos en invierno, manteniéndolos incluso bajo la nieve. Sus esporas no son liberadas hasta pasado el invierno. *E. telmateia* en cambio, esporula en primavera cuando las temperaturas son más cálidas y la precipitación abundante. Por último, *O. regalis* libera las esporas en verano, donde la disponibilidad hídrica es más intermitente y las temperaturas mayores. Sabiendo los diferentes requerimientos ecológicos de cada especie, nuestro objetivo fue desentrañar si la TD de estas esporas se relacionaba con esas características. Para ello se compararon varios parámetros hídricos y bioquímicos de las esporas justo en el momento en el que se liberan (grado de TD, curvas presión-volumen, humectabilidad, movilidad molecular…). Las principales conclusiones fueron las siguientes:

- *Equisetum telmateia*: De entre las tres especies evaluadas, sus esporas son las más sensibles a desecación. Su humectabilidad, contenido de agua y Fv/Fm alcanzan valores proporcionalmente muy altos. No activan la disipación térmica de la energía una vez secas y su estado vítreo es inestable, pudiendo haber pequeños movimientos moleculares que comprometen la integridad celular. La pérdida de turgencia se da a contenidos de agua muy altos; y el potencial en el punto de perdida de turgor también es alto. Dado que maduración y liberación de sus esporas se da en primavera, no es necesaria una alta TD ya que las condiciones ambientales son favorables a una germinación inmediata.
- *Osmunda regalis*: desarrolla una alta TD, capaz de superar varios ciclos de hidratación-desecación. Sus paredes celulares son muy flexibles y la perdida de turgencia se da a potenciales de agua muy bajos; la espora es liberada en un estado no turgente. Presenta una mayor cantidad de antioxidantes que la especie anterior. Es capaz de activar la disipación térmica durante el proceso de desecación y su estado vítreo es estable. Las

condiciones de disponibilidad hídrica intermitente del verano, se relacionan con el momento de la maduración y liberación de estas esporas.

- *Matteuccia struthiopteris*: Presenta el mayor grado de TD, pudiendo superar varios ciclos de hidratación-desecación incluso en mayor grado que *O. regalis*. La espora se libera turgente, pero muestra los valores más bajos de contenido de agua cuando se pierde la turgencia. Además, su cápsula es extremadamente hidrofóbica. Sus paredes celulares son las más rígidas de las tres especies estudiadas. A su vez, presenta la concentración más alta de prolina, así como otros antioxidantes. Al igual que *O. regalis*, la activación de la disipación térmica se da en el proceso de secado y su estado vítreo también es estable. Todas estas características probablemente evitan la germinación bajos las desfavorables condiciones invernales.

Las relaciones hídricas que mostró cada especie de espora mostraron fuertes relaciones con la estacionalidad y la TD. En general, nuestros datos revelaron un síndrome de TD relacionado con la temporada de esporulación que se caracterizó por un mayor potencial fotoprotector, propiedades hídricas específicas y una menor movilidad molecular en estado seco.

4.- Efecto de las condiciones de almacenamiento en la longevidad.

Por último, y sabiendo cómo respondían las especies a la perdida de agua, analizamos si estos mecanismos mantienen la viabilidad a largo plazo. La desecación se considera la perdida de agua y recuperación del metabolismo por debajo de 0.1 gH_2O gDW^{-1}, pero la longevidad se refiere a cuánto tiempo pueden permanecer un organismo seco y a los daños que se pueden dar durante esos periodos. Así, dos especies pueden ser TD al mismo nivel, pero no tener la misma longevidad.

El objetivo fue identificar qué mecanismos se asocian al envejecimiento de esporas clorofílicas, mediante la comparación de dos especies *(O. regalis* y *M. struthiopteris)* almacenadas bajo diferentes regímenes de luz y oxígeno. Ambas presentan diferentes viabilidades en función de las condiciones de almacenamiento. Para la consecución de este objetivo se analizó su pérdida de viabilidad a lo largo del tiempo, los cambios en la composición de antioxidantes hidrófilicos (glutatión) y lipofílicos de los tilacoides (tococromanoles, carotenoides libres y otros) y el análisis de especies reactivas de oxígeno relacionadas con el envejecimiento.

Las conclusiones a las que este objetivo nos permitió llegar son las siguientes:

- El tratamiento que más acelera el envejecimiento en las esporas fue el que combina la presencia de luz y oxígeno. La pérdida de viabilidad en ambas especies bajo estas condiciones fue mucho mayor que cuando se almacenaron bajo oscuridad y ausencia de oxígeno. Para el almacenamiento a largo plazo y el mantenimiento de la integridad de esporas clorofílicas de helecho, estas últimas condiciones retrasan el envejecimiento, lo que indica que existe una interacción negativa entre la luz, el oxígeno y la viabilidad.
- La presencia de especies reactivas de oxigeno se relaciona ni con el tiempo transcurrido durante el almacenado en seco ni de la presencia o ausencia de luz y oxígeno.
- Los principales carotenoides consumidos durante el envejecimiento son la luteína y el β-caroteno, seguidos por el α-tocoferol. *M. struthiopteris* presentó mayor capacidad antioxidante tanto en términos cuantitativos como cualitativos. Sólo se detectó plastocromanol-8 en esta especie, que además mostró una alta correlación positiva con la germinación.
- Los cambios en la potencial redox de antioxidantes hidrofílicos (glutatión) se relaciona con el envejecimiento de las esporas clorofílicas de helecho. Valores más positivos de $E_{GSSG/2GSH}$ corresponden a tasas de germinación más bajas. El rango de reducción de $E_{GSSG/2GSH}$ en el que las esporas pierden viabilidad fue el mismo que el encontrado para otros organismos.

Tras la finalización de esta tesis, se puede concluir que el uso de esporas clorofílicas de helecho como modelo para estudiar la tolerancia a la desecación proporciona una interpretación directa del fenómeno. En una sola célula, se pueden observar todos los mecanismos que permiten la tolerancia a la desecación. Además, los cambios fisiológicos realizados cuando se pierde esta tolerancia también se pueden estudiar, debido al comportamiento sensible a la desecación del gametofito.

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CHAPTER I

INTRODUCTION

"DESICCATION TOLERANCE IN FERNS: FROM THE UNICELLULAR SPORE TO THE MULTI-TISSULAR SPOROPHYTE"

PUBLISHED CURRENT ADVANCES IN FERN RESEARCH.

Desiccation tolerance in ferns: from the unicellular spore to the multi-tissular sporophyte

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Abstract

Desiccation tolerance (DT) represents an ecophysiological strategy probably developed during land conquest by primitive plants that has been progressively lost in photosynthetic tissues throughout evolution, in parallel to the development of mechanisms for the control of water content (i.e. vascular system, cuticle, stomata). Currently, DT of photosynthetic organisms is common in algae, but extremely rare in tracheophytes, especially in angiosperms. Moreover, when considering the different developmental stages of a photosynthetic organism, not all of them present the same response to desiccation. Ferns and lycophytes have three different life forms: (1) the unicellular spore -chlorophyllic or non-chlorophyllic-, (2) the simple multicellular gametophyte, and (3) the complex multicellular and multi-tissular sporophyte. The three life forms comprise a wide extent of DT and particular adaptations to cope with the stresses produced by desiccation and life in the dry state. The intermediate position of ferns between bryophytes and spermatophytes in an evolutionary context, and the variation of responses to desiccation of the different life forms of ferns, allow us to use them as diverse models to study plant adaptations to terrestrial environment, as well as the evolution of the mechanisms of DT in land plants. The current knowledge on fern spore, gametophyte and sporophyte DT and the mechanisms to survive in the dry state are reviewed in this chapter.

Key words: spore, gametophyte, sporophyte, desiccation tolerance, reactive oxygen species (ROS), antioxidants, chlorophyll, glass, Late Embryogenesis Abundant (LEA) proteins

1.1 Desiccation tolerance

1.1.1 General background about plant desiccation tolerance

Plants are able to cope with environments in which the availability of liquid water within their tissues is severely restricted. Three main strategies are employed by plants to cope with this stress: escape, avoidance and tolerance. In the first, plants rapidly complete their life cycle, so that reproductive structures are developed before the plant dehydrates. The second one implies that the plant delays water loss and prioritizes the maintenance of cell turgor by imposing structural barriers to dehydration (cuticles, stomatal closure, enhanced boundary layer, etc.). For the third strategy, tolerance, the plant equilibrates its water content with that of the air by an organized restructuration of cell that prevents irreparable damage and allows resumption of normal metabolic activity after rewatering (Fernández-Marín *et al.,* 2016).

Desiccation-tolerant (DT) plants can lose more than 90 % of their relative water content and then resume normal functions after rehydration (Rascio and La Rocca, 2005). Desiccation-tolerance (DT) can be defined as the ability of some organisms to survive drying to below the absolute water content of around 0.08 to 0.10 g of water per g of dry mass (H_2O g^{-1} DW) or lower, without suffering irreparable damage (Vertucci and Farrant, 1995; Hong and Ellis, 1996; Fernández-Marín *et al.,* 2016). Nevertheless, one should be aware that any benchmark is relatively flexible after several factors have been considered. For example, these water content benchmarks can encompass drying at a wide range of relative humidity (RH) depending on the internal cell composition of the organisms (Ballesteros *et al.,* 2017). All this considered, survival to the water contents achieved after equilibration with air at 50% RH at 20 °C is also used as reference for definition of a DT organism. This extent of drying can correspond to a drop in the water potential down to –100 MPa or even lower (Alpert, 2006), although again these values are dependent on different cell composition. Finally, another proposed benchmark for DT is in the vicinity of the glass transition (Walters, 2015). Among plants, DT is widespread in organs and structures related with dispersion and reproduction (Franchi *et al.,* 2011; Gaff and Oliver, 2013; Hoekstra, 2005). Almost all photosynthetic species possess the capability to survive dehydration in their pollen, spores, or special organs such as rhizomes, seeds, etc. Indeed, desiccation-sensitivity (DS) is the exception more than the rule in the seeds of angiosperm species. It is estimated that around 92 % of seeds are tolerant to desiccation after complete maturation (orthodox seeds) (Wyse and Dickie, 2017).

The presence of DT in photosynthetic tissues has a much more restricted distribution, being more common among species of algae, lichens and bryophytes. It has been proposed that the initial development of DT in photosynthetic tissues was probably a crucial step in the colonization of land by early plants (Oliver *et al.,* 2000). Within Pteridophytes and Angiosperms this ability is rare (no gymnosperms are known to date) (Gaff, 1980, Alpert, 2000, Oliver *et al.,* 2000). Two main groups of DT photosynthetic organisms can be distinguished according to the rate of water loss they can endure: "fully DT organisms" withstand very fast drying and base their tolerance mostly on constitutive mechanisms (i.e. most DT algae, lichen and bryophytes); "Modified DT organisms" or "Resurrection plants" refer to DT tracheophytes which relies mostly on inducible mechanisms, and are thought to not be directly derived from primitive DT-plants but from DS-plants that had already lost DT in their photosynthetic tissues (i.e.: the genetic information for DT was preserved in their genome although only expressed in reproductive organs, such as seeds(Oliver and Bewley, 1997).

1.1.2 General mechanisms of plant desiccation tolerance

During desiccation, but also in the dry state and throughout rehydration, DT cells must deal with two major threats: (1) mechanical stress due to changes in cell turgor and (2) chemical stress, which regards, on the one hand, the loss of native conformation of macromolecules, and on the other hand, an increase in oxidative stress that is particularly exacerbated in photosynthetic tissues (i.e. tissues containing chlorophyll) (Fernández-Marín *et al.,* 2016). Coping with mechanical stress during dehydration implies three not mutually exclusive options (Rascio and La Rocca, 2005): (1) to keep cell volume (i.e. in some species water is replaced by low-molecular-weight osmoprotectants that allow preservation of cell volume), (2) to divide the vacuole into smaller vacuoles (or synthesize new small vacuoles –Georgieva *et al.,* 2017–) that increase mechanical stability of the cell, or (3) to produce flexible cell-walls than can then follow bending of plasma membrane during desiccation without disruption of membrane-wall interactions. The accumulation of specific types of proteins, and small sugars and polyols seems to play indispensable roles in the maintenance of cellular and macromolecular structures upon desiccation.

Proteins and amino acids

The accumulation of high levels of Late Embryogenesis Abundant proteins (LEA) is a common mechanism among DT-plants also found in orthodox seeds. LEA proteins

are highly hydrophilic and unstructured in aqueous solution but structured upon desiccation. Although their biochemical functions are still not completely understood, these proteins are thought to have important roles in the protection of nucleic acids, preservation of macromolecular structure (Close, 1997) and glass formation (Farrant *et al.,* 2015). LEA proteins are localized in the cytosol, the nucleoplasm and the chloroplast and are synthesized in response to desiccation and abscisic acid (ABA) in vegetative tissues of DT plants (Bartels and Sukar, 2005). Although genes encoding LEA proteins are present also in DS plants, DT plants seem to accumulate higher amounts of LEA transcripts during dehydration (Juszczak and Bartels, 2017).

A second important group of proteins are the Early Light Inducible Proteins (ELIP) and ELIP-like proteins. These are thylakoidal pigment-binding proteins that have been found in DT angiosperms and bryophytes and that seem to play a key role in the protection and/or repair of the photosynthetic apparatus (Neale *et al.,* 2000, Alamillo and Bartels, 2001, Zeng *et al.,* 2002).

The function of some amino acids as compatible solutes or as mobile nitrogen reserves for rehydrating tissues have also been suggested (revised in Dinakar and Bartels, 2013).

Sugars

Non-reducing sugars have a very important role as stabilizers of membranes and proteins in the dry state (Crowe *et al.,* 1992). Due to their propensity to form a glass through hydrogen-bonding interactions, they are thought to play a fundamental role in cytoplasm vitrification (see Sect. 19.1.2.3) (Hoekstra *et al.,* 2001). Currently, it is acknowledged that besides sugars, other molecules such as proteins (including LEA proteins) play a pivotal role in intracellular glass formation (Butink and Leprince, 2008, Walters *et al.,* 2010). Non-reducing sugars allow the removal of the closely associated water from proteins by satisfying the hydrogen-bonding requirement of polar groups preserving their functionally correct folding (Hoekstra *et al.,* 2001). Thus, sugars act as water substitutes on the surface of dried proteins. Soluble sugars seem to play important additional roles as a rapid source of energy during rehydration (Georgieva *et al.,* 2017). The major sugar involved in these protective mechanisms is species-specific although sucrose is the most frequently found among DT plants (Farrant *et al.,* 2007). Sucrose and raffinose accumulate during desiccation mainly in angiosperms (Scott, 2000, Peters *et al.,*

2007, Georgieva *et al.,* 2017) whereas trehalose occurs predominantly in non-flowering plants (Liu *et al.,* 2008, Pampurova and Dijck, 2014). Constitutively high amounts of trehalose, sucrose and glucose have been found in DT species of spike mosses (Yobi *et al.,* 2013).

Slowing down of metabolism under desiccation: the vitrification of tissues and the glassy state

Upon dehydration, the viscosity of the internal cellular compartments increases and solutes are concentrated within the tissues. At RH ranges between 30 and 60%, the the aqueous mixtures, such as cells, become more viscous and molecules become densely packed, reducing drastically molecular mobility up to a point that the cytoplasm resembles a solid (Butink and Leprince, 2008; Fernández-Marín *et al.,* 2013; Leprince and Buitink, 2015; Walters, 2015; Ballesteros *et al.,* 2017). Such types of solids are known as amorphous solids and have different thermodynamic properties than crystalline solids (Walters *et al.,* 2010). The change from fluid to amorphous solid is known as vitrification (glass transition when the change is from solid to fluid), and the resulting amorphous solid is often called a glass. Glasses are known to decrease detrimental reactions, to increase the stability of enzymes, and to prevent conformational changes of proteins (Butink and Leprince, 2008). The formation of intracellular glasses is indispensable to survive the dry state, but glass formation itself is not a mechanism that initially confers the tolerance to desiccation during drying (Butink and Leprince, 2008; Walters *et al.,* 2010).

Plant cellular glasses have been studied through different calorimetric and spectroscopic techniques (e.g. differential scanning calorimetry (DSC) or electron paramagnetic resonance (EPR), and mainly in seeds because of its importance in longevity during storage (Walters *et al.,* 2010). Recently, a different approach using dynamic mechanical thermal analysis (DMTA) has been used for the characterization of the mechanical properties and molecular mobility within dry seed glasses (Walters *et al.,* 2010; Ballesteros and Walters, 2011) and has provided first evidence for the absence of enzymatic reactions in dry photosynthetic tissues of bryophytes (Fernández-Marín *et al.,* 2013). Still, many questions remain open about intracellular glasses and how (and for how long) they confer cell stability and longevity in dry plant tissues. This represents a gap of knowledge to be filled that could be of noticeable relevance to understanding DT and longevity in the dry state of plant germplasm.

Coping with oxidative stress

The chloroplast is the main source of reactive oxygen species (ROS) upon desiccation. ROS production can be exacerbated when desiccation occurs under strong irradiance. Consequently, the first barrier to prevent oxidative stress is the reduction of light energy reaching the photosynthetic apparatus. The presence of chlorophyll during desiccation represents a difference against DS plants in which damage upon desiccation includes the irreparable loss of photosynthetic pigments and thylakoid structure (Juszczak and Bartels, 2017). During the loss of turgor, many DT species curl or fold their leaves exposing the abaxial surface which is frequently covered with hairs, scales or increased content of anthocyanins that, in sum, efficiently reduce the interception of light by chlorophyll (Lebkuecher and Eickmeier, 1991, Alpert, 2000). The next step in photoprotection relies on the switching off of the photosynthetic apparatus and the efficient dissipation of light energy still absorbed by chlorophylls (revised in García-Plazaola *et al.,* 2012, Fernández-Marín *et al.,* 2016). Desiccation induced deactivation of PSII seems to be an intrinsic protective mechanism of DT species since DS plants maintain activity even at very low water content, after photosynthetic electron transport has already been stopped (Nabe *et al.,* 2007).

Antioxidant mechanisms play such a fundamental role in DT plants, that the survival after long storage in the desiccated state and after subsequent rehydration depends on their efficiency to counteract ROS production (Kranner *et al.,* 2002). Despite the general increase in the antioxidant response upon desiccation (Yahubian *et al.,* 2009), two molecules play outstanding roles in the DT strategy: the hydrophilic and ubiquitous glutathione (GSH_{tot}) and the lipophilic and chloroplast-located zeaxanthin (Z). The relevance of these two molecules is evidenced by their conserved response: their synthesis and accumulation are triggered by desiccation among species of phylogenetically diverse taxa, e.g. including lichens, bryophytes, and angiosperms. The proportion of reduced glutathione to GSH_{tot} is related to the survival of DT organs, including seeds (Kranner *et al.,* 2002, Nagel *et al.,* 2015). Besides its role as an antioxidant, reduced glutathione is thought to interact with proteins and protect them from irreversible formation of disulphide bonds upon water loss (Kranner *et al.,* 2008). Synthesis of Z is activated in DT plants by desiccation, even in the absence of incident irradiance (Fernández-Marín *et al.,* 2009, 2010, 2011, 2013). Z can play up to three different protective roles in DT photosynthetic tissues: (1) it is involved in the thermal dissipation of energy, (2) it

efficiently quenches triplet chlorophyll and singlet oxygen and (3) it is thought to stabilize thylakoid membranes during desiccation and enhance the recovery of photochemical efficiency during rehydration (Alamillo and Bartels, 2001; Deltoro *et al.,* 1998, Gill and Tuteja, 2010, Havaux *et al.,* 2007, Kranner *et al.,* 2002, 2008, Fernandez-Marin *et al.,* 2009, 2010, 2011, 2013).

1.1.3 Why study tolerance to desiccation in Pteridophytes?

Ferns represent a highly valuable model group for the study of plant desiccation tolerance, as they have unique characteristics to study plant adaptations for the conquest of land and the mechanisms that allowed this colonization. Ferns have a strategic evolutionary position in the evolution to land, between bryophytes and seed-plants, and their mechanisms of DT seem to be intermediate between those of “fully DT plants” (i.e. bryophytes) and those of flowering plants (Oliver *et al.,* 2000). This intermediate position is, for example expressed in terms of the vascular system and stomatal regulation, which present two different ways in the independence of plants from water (McElwain, 2011, Cai *et al.,* 2017). In this context, ferns have recently been used to demonstrate that ABA regulation of stomata evolved much earlier than the divergence of ferns and seed plants, as different fern species have shown ABA-induced closure of stomata, pointing to alterative and complex pathways for ABA-induced stomatal closure in plants (Cai *et al.,* 2017, Hõrak *et al.,* 2017). This characteristic makes ferns valuable models for the study of plant regulation of DT-responses.

Another interesting characteristic of ferns is that they present three different life forms in their life cycle that can be used as diverse models: the unicellular spore (chlorophyllous or non-chlorophyllous), the simple multicellular gametophyte, and the complex multicellular and multissular sporophyte. These life forms not only have different cellular and tissue complexity, but also present different DT responses and mechanisms (described in the following sections). Sporophyte and gametophyte could resemble the behavior of both vascular and non-vascular plants, respectively. The lack of complexity, cuticle and stomata in gametophyte tissues could allow an adaptation to water loss, due to the inability to control the exit and entry of water that is totally dependent on the ambient humidity. In turn, the sporophyte has full control over water loss because of the development of complex tissues, cuticle and stomata.

In addition, spores offer a simple model to understand the mechanisms responsible of seed DT and longevity in the dry state, and in turn, what changes are necessary at the cellular level to lose this property. Both propagules, seeds and fern spores, differ in their structure and ontogeny but they carry out the same function: they are survival units, suitable for dispersion, acting as population backup in soil banks, and often exposed to extreme environmental conditions but being able to survive them (Page, 2002). Moreover, they are related in many physiological aspects, such as the induction or inhibition of germination by red or far-red light, the loss of water during maturation, or the role of gibberellins in germination (e.g. Hoekstra *et al.,* 2001). Given the unicellular character of spores, the study of these is simpler than that of seeds (that contain a hypocotyl, cotyledon(s), embryo with plumule and radicle) where each part will act or have some mechanism of DT. Additionally, chlorophyllous spores can be understood as a miniature photosynthetic tissue whose compression would aid in the comprehension of more complex tissues.

1.2 Desiccation tolerance in fern spores

The ability to survive extreme desiccation in vascular plants is generally expressed in reproductive cells, such as fern spores, pollen and seeds (see Sect. 19.1.1). However, the extent of DT in fern spores, the stage when this tolerance is acquired, as well as the physical and biochemical mechanisms that confer tolerance to desiccation have been understudied in comparison to other plant propagules (e.g. seeds and pollen). This section will review the current knowledge in DT of fern spores and will discuss possible directions for future research.

1.2.1. Sporogenesis, spore dispersal, and fern spore water content

Ferns produce spores by meiosis in the sporangia in a process known as sporogenesis. During sporogenesis, after meiosis is completed, the fern spore goes through a maturation stage where plastids and storage reserves accumulate, including the precursors of proteins analogous to seed LEA proteins (Raghavan and Kamalay, 1993; see Sects. 19.1.2 and 19.2.4). The characteristics of this phase suggest that the maturation stage of the fern spores could be similar to that occurring in DT seeds (Angelovici *et al.,* 2010). However, most research published on fern spores has focused in describing the ultrastructural changes during the early stages of sporogenesis (e.g. Marengo, 1979; Brown and Lemmon, 2001a,b), and little is known about the maturation phase of the

spore, the changes in water content that may occur in the spores, and if this is actually the stage where fern spores acquire any tolerance to desiccation. After sporogenesis, when the sporangia is mature and the environment is dry enough, evaporation of water from the annular cells of the sporangia induces sufficient stress to cause an abrupt dehiscence, which ends up in the release and dispersal of the spores (Noblin *et al.,* 2012).

Fern spores are classified as green (chlorophyllous) or non-green (non-chlorophyllous) depending on their color at maturity (Lloyd and Klekowsky, 1970). However, this traditional naming is not accurate since some chlorophyll-containing spores can visually appear as non-green when the cover of the spore is not transparent (Sundue *et al.,* 2011). Green spores usually have higher water contents than non-green spores (Lloyd and Klekowsky, 1970). For example, according to water sorption isotherms calculated for several species at 25°C, water contents of spores equilibrated at 50 % RH vary from ca. 0.09 to ca. 0.04 g water/g dry mass for green spores and non-green spores, respectively (Ballesteros and Walters, 2007a; Ballesteros *et al.,* 2017). The causes for the difference of water content between green and non-green spores are not completely known, but it is likely that it is related to their chemical composition. For example, it is known in seeds that the moisture content at a particular RH varies according to the lipid composition (Vertucci and Roos, 1990), and non-green spores have 10 to 40 times larger lipid contents than green spores (Ballesteros *et al.,* 2007b; Ballesteros *et al.,* unpublished).

Measurements of water content for 'freshly harvested' spores have usually been made in green spores collected 1 or 2 days after sporangia dehiscence or in non-green spores collected about 1 to 5 days after sporangia dehiscence (e.g. Farrant *et al.,* 2009, Li *et al.,* 2010, Li and Shi 2014, 2015; Mikula *et al.,* 2015, Lopez-Pozo *et al.,* 2019). Freshly harvested spores are viable and water contents range from 0.11 to 0.14 g water/ g fresh mass in green spores of *Osmunda* sp. and from 0.04 to 0.07 g water/ g fresh mass in diverse non-green spores or in the green spores of *Matteuccia struthiopteris*. These water contents correspond to those of spores equilibrated at 30-80 % RH and 25 °C (Ballesteros and Walters, 2007a; Ballesteros *et al.,* 2017, Fig. 1.1). The exception is the spores of *Equisetum* sp. for which water contents above 0.50 g water/g dry mass (which correspond to the water contents of *Equisetum* sp. spores equilibrated at >96 % RH at 25 °C, Fig. 1.1) have been measured in diverse species (Gabriel y Galan and Prada, 2010; Lopez-Pozo *et al.,* 2019).

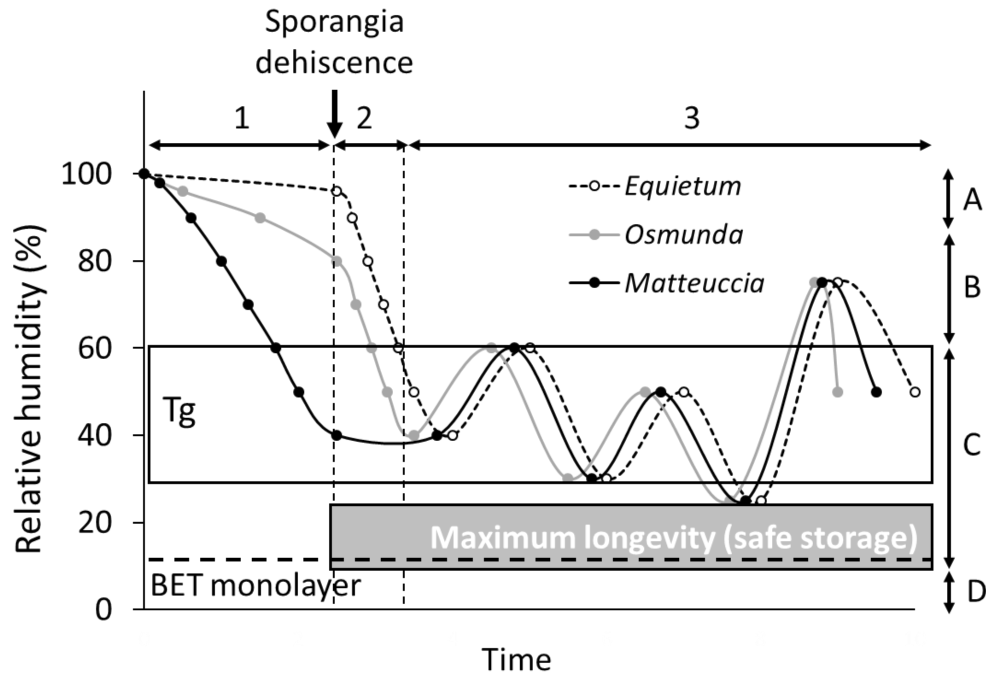

Figure 1.1. Moisture changes (in terms of relative humidity, RH) of diverse green or chlorophyllous fern spores during spore maturation in the sporangia (1), during the initial hours after sporangia dehiscence (2), and during a hypothetical scenario of long-term dispersal with environmental RH changes (3). The moisture contents at which fern spore desiccation sensitivity would be expressed (A), or they are subjected to accelerated aging at high RH (B), aging (C), and aging at low RH or 'over-drying' (D) are also indicated as suggested by Ballesteros *et al.,* (2017).

After sporangia dehiscence and spore dispersal, both green and non-green spores can remain in the sporangia for several days or months, fall around the mother plant, or travel by air very large distances (Farrar, 1976, Parris, 2001, Wolf *et al.,* 2001, Page, 2002, Geiger *et al.,* 2007). In these scenarios they may be exposed (and tolerate) highly stressful conditions, including low and high RH (Fig. 1.1), freezing temperatures, fast winds at high altitudes, and UV exposure (Page, 1979; 2002). After dispersal, it is likely that spores equilibrate their internal water content rapidly, in a few hours, depending on the ambient RH (Ballesteros and Walters, 2007a). This capacity of fern spores to quickly modulate their internal water content to low levels and their tolerance to these low water

contents seems to be key in their ability to disperse and tolerate the environmental stressful conditions that they may experience (Page, 2002).

1.2.2. Variation of desiccation tolerance and longevity among fern spores

DT of non-green spores has typically been described as similar to that of orthodox seeds and other DT organisms (Dyer, 1979; Ballesteros, 2010; Walters *et al.,* 2005; Ballesteros *et al.,* 2012). In contrast, there was a widely-held belief that green spores had limited DT (Lloyd and Klekowsky, 1970, Perez-Garcia *et al.,* 1994, Gabriel y Galan and Prada, 2010, Ibars and Estrelles, 2012). This belief was based on conclusions of pioneer studies that suggested that the short longevity of green spores was related to their high water content, respiration rate and catalase levels (Okada, 1929; Hauke, 1978), as well as to the lack of a spore wall protecting against drying (Wollersheim, 1957). However, conflicting evidence suggests that green spores can tolerate substantial water loss, as they tolerate drying at low RHs (1 – 20 %) to moisture contents < 0.08 water/g fresh weight (Lebkuecher, 1997, Pence, 2000, Hoekstra, 2005, Li and Shi, 2014, 2015; Mikula *et al.,* 2015). Recently, in agreement with these late reports, it has been shown that both green and non-green fern spores possess a similar and remarkable level of DT (Ballesteros *et al.,* 2017). Fern spores from diverse unrelated species (3 green spore and 2 non-green spore species) survived the immediate effects of extreme desiccation (i.e. initial drying to 1 % RH). However, survival in the desiccated state was temporary, especially under the most severe desiccation treatments (RH < 5 %). Longest survival times were observed in spores placed at about 10 – 25 % RH, independently of the water contents that the spores had at such conditions. This response is similar to what has been observed for other fern spores, orthodox seeds, DT pollen, and various DT organisms (e.g Vertucci and Roos, 1990, Buitink *et al.,* 1998, Walters *et al.,* 2005).

Though response to RH during storage is similar among fern species (Ballesteros *et al.,* 2017), the kinetics of deterioration in the dry state vary considerably among species. Generally green spores present shorter life-spans than non-green spores, ranging from a few days to one year for green spores and from a few months to several decades in non-green spores (Lloyd and Klekowsky, 1970). It is likely that the short longevity of green spores is related to their particular chemical and cellular composition (e.g. thin and transparent walls and the presence of chloroplasts in the dry cytoplasm). Analogously, the chlorophyllous seeds of *Salix nigra* deteriorate fast in the dry state due to photo-oxidative damage of the thylakoid membranes mediated by free radicals and ROS

(Roqueiro *et al.,* 2010). However, we may find non-green spores of particular species with shorter longevity than the green spores of some species. For example, the non-green spores of *Culcita macrocarpa* show shorter longevity at the optimal RH for storage than the green spores of *Matteuccia struthiopteris* (Ballesteros *et al.,* 2017), indicating that diverse physical and biochemical aspects of the fern spore may also be playing an important role in the differences in longevity among spores.

1.2.3. Structural and biophysical aspects of desiccation tolerance and longevity in fern spores

Diverse definitions can be found to describe the extent of the desiccation stress that defines "desiccation tolerant" organisms (see Sect. 19.1.1; Ballesteros *et al.,* 2017). However, what seems to be common to all of the definitions is that DT organisms survive the compressive forces and cell volume reduction before glasses (see Sect. 19.1.2.3) are formed (Leprince and Buitink, 2015; Walters, 2015; Ballesteros *et al.,* 2017). If green spores had limited DT as initially thought (see Sect. 19.2.2), one would expect that the most sensitive green spores, which are released at high water contents (e.g. *Equisetum* sp., see Sect. 19.2.1), would die before glasses are formed (A in Fig. 1.1). However, the cytoplasm of non-green and green fern spores enter into a glassy state between 30 and 60 % RH (Hoekstra, 2005; Ballesteros *et al.,* 2017), and all the species tested (including *Equisetum* sp.) survived this transition (Hoekstra, 2005, Ballesteros *et al.,* 2017).

Longest survival times for fern spores are observed when they are placed at about 10 –25 % RH, hence when they are in the glassy state (Walters *et al.,* 2005, Ballesteros *et al.,* 2017). At moisture contents above the RH that provides longest longevity, a timed response damage is expressed in fern spores, which is faster as the RH increases (Ballesteros *et al.,* 2017; Fig. 1.1). This type of deterioration is classically referred to as ageing (C in Fig. 1.1) and accelerated ageing (B in Fig. 1.1) in the literature on seed longevity, and deterioration kinetics in fern spores are consistent with modelled behavior in seeds (Ellis and Roberts, 1980, Ballesteros *et al.,*, 2017). Below the RH that provides longest longevity, desiccation damage occurs in DT cells, and this is also expressed as a time-dependent response (D in Fig. 1.1). Although in this case deterioration is faster as spores are moved to lower RH, it has been suggested that this deterioration could also be considered as ageing (Ballesteros *et al.,* 2017). However, as the aging kinetics at water contents above and below the RH that provides longest longevity have opposite responses

to water content, it is suggested that the mechanisms of damage and protection involved in these conditions may be different (Ballesteros *et al.,* 2017).

One of the explanations for the damage that occurs in fern spores at < 10 – 25 % RH, is that they are dried below the BET monolayer (Ballesteros and Walters, 2007a; Ballesteros *et al.,* 2017). The BET monolayer is a parameter calculated from the Brunauer–Emmett–Teller (BET) model which is used to explain the physical adsorption of water on the solid surface of biomolecules of the cell cytoplasm of diverse organisms, including fern spores (Ballesteros and Walters, 2007a). The BET monolayer describes the water content at which all sites at the adsorbent surface are filled, and generally has been indicated as a good marker of maximum stability during dry storage (Passot *et al.,* 2012; Rahman, 2009; Roos and Drusch, 2015; Roussenova *et al.,* 2010). It could be possible that the removal of the water that forms the BET monolayer destabilizes the physical structure of the cytoplasm, allowing also a greater attack of free radicals and ROS. These hypothetical situations could lead to death over the time as suggested for orthodox seeds (Mira *et al.,* 2010; Walters *et al.,* 2010). However, the mechanisms of fern spore deterioration in the dry state are still conjectural, and the complexity in structure and motion within the glass of fern spores conferring different responses to desiccation and longevity still need to be ascertained (Ballesteros *et al.,* 2017)

1.2.4. Accumulation of proteins

LEA proteins

Studies carried out in *Onoclea sensibilis* determined that there is an accumulation of mRNAs related to survival, growth maturation and germination during the sporogenesis (Raghavan and Kamalay, 1992). These mRNAs detected in fern spores by Raghavan and Kamalay (1992) were related to members of group-3 LEA sequences described by Dure *et al.,* (1989) from angiosperm embryos during seed maturation and desiccation. A similar process has also been shown during pollen development in angiosperms (Stinson *et al.,* 1987). The sequences in *O. sensibilis* were spore-specific, and the accumulation was initiated only after meiosis and as long as the spores were mature (desiccated), but were absent in the gametophyte. For the case of seeds during the maturation phase there is a decrease in cell water potential and a synthesis of LEA proteins that protect embryonic organs from this desiccation (Curry *et al.,* 1991, Angelovici *et al.,* 2010). Thus, it could be expected that spore maturation could suffer similar process and LEA proteins would play the same or an analogous role.

During spore germination, many other proteins are degraded in the cytoplasm and in thylakoid membranes, some of which are related to their maintenance. This is the case of 22-kDa protein of thylakoid membranes isolated from spores of *Osmunda japonica*. Before germination, high levels of this protein are found, but when germination starts the amount of protein decreases. Something similar was observed in spores of *Adiantum capillus-veneris* (non-chlorophyllous spores) (Minamikawa *et al.,* 1984) whose spore proteins may be a homolog of ABA-inducible, desiccation-related, or LEA proteins. Several studies have reported degradation of proteins that could be related to DT in fern spores (Raghavan 1991, DeMaggio and Stetler, 1980, Paless *et al.,* 1984). Currently, the specific role of the 22-kDa protein in chloroplasts remains unknown (Inoue et *al.,* 2000).

Other proteins

Within gymnosperms and angiosperms seeds, vicilins and legumins are the two major classes of storage proteins, which have the function of nutrition once the seed germinates (Shutov et *al.,* 2003). Shutov *et al.,* (1998) concluded that these seed-storage proteins presented by phanerogamic species were involved in cellular processes of desiccation and rehydration and *Physarum polycephalum* presents similar proteins that allowed the acquisition of cellular desiccation tolerance (Lane *et al.,* 1991). Thus, it may be possible that they are involved in desiccation tolerance in tissues. *Matteuccia struthiopteris* presents an intermediate vicilin-like protein (Kakhovskaya *et al.,* 2003, Shutov *et al.,* 1998), with features of both vicilins and legumins. These structural features resemble those of a non-spermatophyte (cryptogamic) 'primitive' storage protein, thus, this protein probably represents an ancestor of the spermatophyte storage globulins (Shutov *et al.,* 1998). The question exists whether this protein is related with storage or with desiccation tolerance in fern spores. On the one hand, it has been proposed that fern spores may also function as a storage tissue (for more details see Schallau *et al.,* 2008), but the idea that this type of protein descended from germin- and spherulin-like ancestors involved in various cellular desiccation processes (Shutov and Baumlein, 1999, Shutov *et al.,* 1998, Wohlfarth *et al.,* 1998) supports that it is related with DT and not with storage functions.

It seems that the role of these proteins is similar to LEA proteins (Baker *et al.,* 1988). A similar role has been proposed for a variety of other glycine-rich proteins in mature seed-embryos (Galau *et al.,* 1987, Mundy and Chua, 1988).

Osmotic adjustment: Proline accumulation in cells exposed to stress conditions

As occurs in tissues of higher plants exposed to osmotic stress, dry chlorophyllous spores of *Equisetum arvense* show high levels of arginine and proline. Also, spores of *O. japonica* present high contents of these amino acids but they disappear in the gametophyte. Besides, it has been described that non-chlorophyllous spores of *Adiantum capillus-veneris* contain proline in the dry state (Minamikawa *et al.,* 1984)

Protection of fern spores against oxidative processes during desiccation and life in the dry state.

During the maturation phase of fern spores and their desiccation after dispersal is likely the production and accumulation of ROS. In the dry state, the repair of the oxidative damage caused by ROS may be limited by the formation of the glass and the reduced molecular mobility allowed in such state (see Sect. 19.1.2.3 and 19.2.3). Decreased or suppression of mitochondrial and chloroplastic activities during desiccation is the best way to reduce ROS generation, even at low water contents (Leprince *et al.,* 2000). Antioxidative defenses play a major role once free radicals have already been formed. Two main antioxidant enzymes have been found in the desiccated green spores of *Equisetum arvense*: ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR). Both enzymes can be localized in the cytoplasm and chloroplast (Zhao *et al.,* 2015) and take part in the glutathione-ascorbate cycle. Furthermore, a chloroplast drought-induced stress protein (CDSP32) has been found in these spores. This protein has two thioredoxin (TRX) modules, which are involved in the protection of the photosynthetic apparatus against oxidative damage. Hence, it could be suggested that these antioxidants play the main role in the ROS homeostasis of the chlorophyllous spores of *E. arvense (*Zhao *et al.,* 2015). Other spores also exhibit enzymes of the glutathione-ascorbate cycle. In the dry green spores of *Osmunda cinnamomea* all the enzymes of this cycle were found (Suo *et al.,* 2015)

Photoprotection of green spores during desiccation

When a chlorophyllous tissue starts to lose water, photosynthesis declines but the total irradiance intercepted by the chlorophyll is essentially the same. In this context, the major threat is the formation of ROS by excited chlorophyll molecules and the consequent oxidative deterioration (Heber and Shuvalov, 2005, Farrant *et al.,* 2007). Thus, the simplest way to protect the photosynthetic tissue is to reduce the amount of light absorbed

by chlorophyll molecules through several mechanisms as have been commented in Sect. 19.1. In green spores, *M. struthiopteris* has a brown coat that filters light and could act as the first barrier to reduce light absorbance, however other spores have a transparent coat. In addition to a dark coat, quenching of chlorophyll could be another photoprotection mechanism in green spores. In this sense, Lebkuecher (1997) showed that the spores of *Equisetum hyemale*, reduced the photochemical efficiency of PSII (Fv/Fm) during the first week of storage at 2% RH without major changes in spore viability and recovered their Fv/Fm after rehydration. This could be considered a type of photoprotection as observed in the DT algae *Trebouxia asymmetrica* (Wieners *et al.,* 2012). The decrease in photochemical activity of PSII can be due to various mechanisms: (1) desiccation-induced detachment of the light-harvesting complex from PS II (LHCII) (Schreiber and Armond, 1978), (2) transference excess of excitation energy from PS II to PS I associated with phosphorylation of LHC II (Williams and Allen, 1987), and (3) thermal energy dissipation mediated by the VAZ cycle (Demmig *et al.,* 1987; Gilmore *et al.,* 1995). All of these mechanisms are reversible (deactivate) after, at least, 24 h of rehydration.

1.3. Desiccation tolerance in gametophytes

1.3.1. Presence and extent of desiccation tolerance in gametophytes

Most fern gametophytes are photosynthetic from the initial steps of development. Lacking a vascular structure, gametophytes are forced to be poikilohydric. However, their structure is not the fragile prothallus as described in the literature, and gametophytes of many species can establish in xeric places. Furthermore, gametophytes are frequently perennial and persist for years (Johnson *et al.,* 2000), encountering successive periods of desiccation and rehydration during their long lifespan. As a consequence of such poikilohydric commitment, it is considered that DT has to be much more frequent among gametophytes than in sporophytes (Pittermann *et al.,* 2013; Watkins *et al.,* 2007). In fact the first experimental studies on DT in gametophytes date back to more than 100 years ago, when Pickett (1913) demonstrated that prothalli of the limestone-colonizing North American fern *Camptosorus rhizophyllus*, were able to completely recover after desiccation. Since then, reports on DT in gametophytes have been scarce, describing its presence in several other species (Kappen, 1965; Ong and Ng, 1998; Kappen and Valladares, 2007). Only recently more extensive surveys on tropical species (Pittermann *et al.,* 2013; Watkins *et al.,* 2007) have confirmed the intuitive believe that DT is probably

widespread among fern gametophytes. These studies concluded that the degree to which gametophytes can withstand desiccation seems to be strongly linked to habitat preferences with species inhabiting the most xeric places also being the most DT. However, this general rule does not imply that gametophytes are necessarily DT, as indicates the extreme sensitivity to desiccation of gametophytes from species growing in moist ecosystems such as the Mexican cloud forest (Riaño and Briones, 2015) or European temperate environments (Kappen, 1965). Examples of genera for which DT has been described in gametophytes can be found across a wide taxonomic diversity that includes most of the phylogenetic fern clades. Being a polyphyletic character, gametophyte habitat becomes the main factor determining DT. Specifically DT has been tested and confirmed in the following families and genera (Diamond *et al.,* 2012, Kappen, 1965; Ong and Ng, 1998; Pickett, 1913; Pittermann et *al.,* 2013; Watkins et *al.,* 2007): Aspleniaceae (*Asplenium*, *Ceterach*), Athyriaceae (*Diplazium*), Cibotiaceae (*Cibotium*), Davalliaceae (*Davallia*), Dennstaedtiaceae (*Dennstaedtia*), Hymenophyllaceae (*Trichomanes*), Lomariopsidaceae (*Nephrolepis*, *Cyclopeltis*), Polypodiaceae (*Campyloneurum*, *Drynaria*, *Pyrossia*, *Phlebodium*, *Microgramma*, *Polypodium*), Pteridaceae (*Adiantum, Cheilanthes*, *Pteris*, *Pellaea*, *Pityrogramma*, *Vittaria*,), Thelypteridaceae (*Thelypteris*).

1.3.2. Mechanisms that confer tolerance to desiccation in gametophytes

DT is common in gametophytes of mosses and liverworts, and presumably the same mechanisms employed by bryophytes (see Sect. 19.1) can be used by ferns to withstand desiccation. However, there is an almost complete absence of physiological studies on DT in fern gametophytes. Among the few studied mechanisms, morphology seems to play a prominent role on slowing the rate of water loss, with the more complex structures retaining water for longer (Watkins *et al.,* 2007). Regulation by ABA is also involved in some species, as demonstrated by the increase on survival rate after desiccation when gametophytes were cultured on medium containing ABA (Pence, 2000). Interestingly, once desiccated, gametophytes can survive liquid nitrogen, allowing the development of protocols for cryopreservation (Pence, 2000, chapter 11). Furthermore, in the field, overwintering gametophytes can survive much lower temperatures that the respective sporophyte (Sato, 1982).

1.3.3. Photoprotection of gametophytes during desiccation

From the initial steps of development most fern gametophytes are photosynthetic but very little is known about their photosynthetic responses (Hagar and Freeberg, 1980, Johnson *et al.,* 2000, Watkins *et al.,* 2007). Gametophytes, in comparison with their sporophytic counterparts, can be considered as shade-plants, with a limited capacity for light acclimation and a pigment composition typical of low light grown plants (Fernández-Marín *et al.,* 2012). For those species in which Fv/Fm has been measured (Johnson *et al.,* 2000, Watkins *et al.,* 2007, Fig. 1.2), control values are in the range 0.6 to 0.75, similar to gametophytes of mosses and liverworts, indicating that no major photoinhibition occurs under non-stress conditions. In addition, our lab has recently measured that some species, such as *Davallia canariensis*, are able to recover Fv/Fm after a dehydration-hydration cycle (Fig. 1.2, based in Lopez-Pozo et al, unpublished)

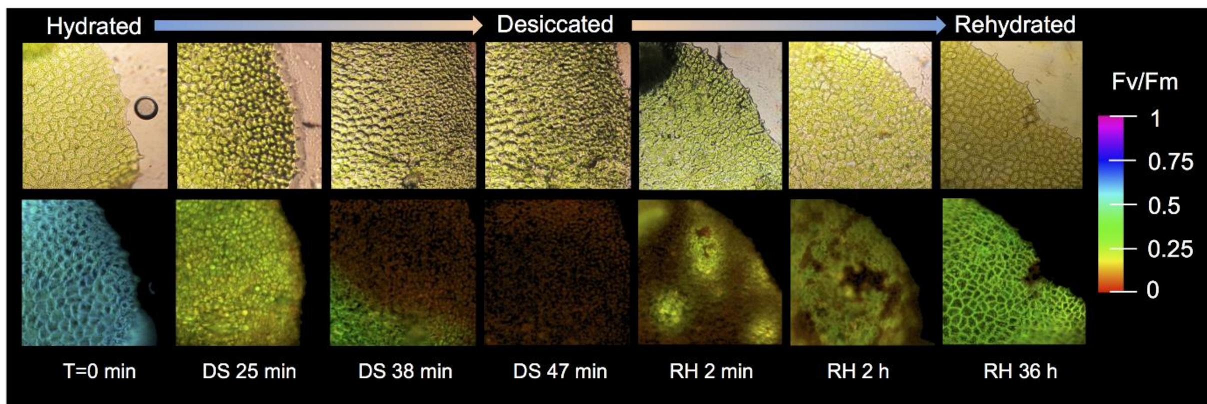

Figure 1.2. Deactivation/activation of photochemical efficiency (measured as Fv/Fm) during a cycle of desiccation and rehydration in gametophytes of *Davallia canariensis*. Upper panels show images taken with the optical microscope and bottom panels the equivalent images of Fv/Fm captured with the Imaging PAM microscopy fluorometer.

1.4. Desiccation tolerance in sporophytes

1.4.1. Presence and extent of desiccation tolerance in the leaves or whole plants in some species

In hydric terms, most fern sporophytes, with well-defined vascular tissue, cuticle and stomata can be considered as homoiohydric. Some of them are DT, but function as typical vascular plants when water is available. Others, such as *Mohria cafforum*, produce two types of fronds: DT in the dry and DS in the rainy season (Farrant *et al.,* 2009). The exception are the filmy ferns (family Hymenophyllaceae), whose leaves are formed by

one layer of cells, lacking epidermis and stomata. This family, which comprises more than 600 species (Dubuisson *et al.,* 2009), in some way has returned to the poikilohydric strategy exemplified by bryophytes (Proctor and Tuba, 2002). In the absence of a specific evaluation for each species, it seems likely that most, if not all, members of this family are to some extent DT (Proctor 2003, 2012). Based on this assumption, Porembski (2011) estimated that the number of DT fern species should be between 200 and 1200, with the latter being more likely. Given that c.a. 12,000 fern and lycopod species have been described and accepted (Christenhusz and Byng 2016), the presence of vegetative DT in fern sporophytes could be as frequent as 10 %, a proportion at least 10-fold higher than in angiosperms and much higher than previous estimations of DT frequency around 1 % for fern sporophytes (Oliver *et al.,* 2000, Proctor and Pence, 2002). Apart from Hymenophyllaceae (genera *Hymenophyllum, Hymenoglossum* and *Trichomanes*), DT has been described mostly in epiphytic and rock-colonizing species including the following families and genera (Gaff, 1977, 1987; Hietz, 2010, Porembski, 2011, Tuba and Lichtenthaller, 2011): Aspleniaceae (*Asplenium*, *Ceterach*, *Pleurosorus*), Dryopteridaceae (*Dryopteris*, *Elaphoglossum*), Polypodiaceae (*Ctenopteris*, *Drynaria*, *Loxogramme*, *Melpomene*, *Pecluma*, *Polypodium*, *Phymatosorus, Platycerium, Pleopeltis, Pyrrosia*), Pteridaceae (*Actiniopteris*, *Adiantum*, *Cheilanthes*, *Doryopteris*, *Hemionitis*, *Notholaena*, *Pellaea, Paraceterach*, *Vittaria*), Schizaeaceae (*Anemia*, *Mohria*, *Schizaea*), Selaginellaceae (*Selaginella*), Tecatariaceae (*Arthropteris*) and Woodsiaceae (*Woodsia*).

1.4.2. Mechanisms of desiccation tolerance in fern sporophytes

Mechanisms of DT in ferns are sometimes constitutively expressed, while others require a certain time for their activation, differentiating between constitutive DT (CDT) and inducible DT (IDT) (Stark *et al.,* 2013). Most fern sporophytes behave as IDT, in the sense that, in general, a rapid desiccation causes more extensive damage than a slower one. An exception are filmy ferns that lacking epidermis display a DT strategy (see Sect. 19.4.1), being exposed to rapid and continuous cycles of desiccation and rehydration. The importance of CDT mechanisms is evidenced in this group by the existence of a little variation (3-4 %) on proteome upon desiccation (Garces *et al.,* 2014). In species with IDT, ABA plays a fundamental role in the regulation of the expression of desiccation responsive genes (Wang *et al.,* 2010), and the external application of this hormone increases DT as has been shown in *Polypodium virginianum* (Reynolds and Bewley,

1993). To allow enough time for the activation of IDT, ferns employ mechanisms that slow down water loss, this is, for example, the role of the epidermal scales in *Pleopeltis polypodioides* that prevent rapid water loss from desiccating fronds (John and Hasenstein, 2017).

An unavoidable consequence of water loss is cell and tissue shrinkage. To prevent mechanical damage, DT sporophytes have to be able to fold and unfold fronds in a plastic and organized way. The presence of arabinose-rich pectins and proteins makes cell walls more plastic (Moore *et al.,* 2013). Besides, it has been described in *Polypodium polypodioides* that the rapid synthesis and degradation of dehydrins in response to desiccation is probably involved in the mechanical properties of cell walls (Layton *et al.,* 2010).

Fern sporophytes also respond to dehydration by osmotic adjustment, that in *Pleopeltis pleopeltifolia* is achieved by an increase in solute content (sugars) (Voytena *et al.,* 2014) while in *Selaginella tamariscina* sugars and proline contribute to the osmotic adjustment (Wang *et al.,* 2010). A more detailed characterization of carbohydrates accumulation in *Mohria cafforum* reported the presence of high amounts of sucrose, raffinose family oligosaccharides and cyclitols (Farrant *et al.,* 2009)

1.4.3. Role of photoprotection mechanisms in desiccation tolerance in fern sporophytes

Most fern species show a certain chlorophyll loss (Voytena *et al.,* 2014, Wang *et al.,* 2010, Tausz *et al.,* 2001, Kavitha and Murugan, 2016) that can be as high as 75% in the case of *Pleopeltis pleopeltifolia,* (Voytena *et al.,* 2014). Even in this kind of species that down-regulate chlorophyll content one of the main sources of stress in the desiccated state is the generation of oxidative damage by photoactivated chlorophyll. Thus, DT ferns need to activate a set of photoprotective responses to prevent damage to photosynthetic tissues during desiccation. The simplest and most effective way to prevent such photooxidative stress is to reduce photosynthetic surface exposed to illumination. In most DT fern sporophytes this is simply achieved by the frond curling or shrinkage, as has been characterized in a wide representation of species such as *Selaginella lepidophylla* (Brighigna *et al.,* 2002, Lebkuecher and Eickmeier, 1991), *Hymenophyllum dentatum* and *Hymenoglossum cruentum* (Flores-Bavestrello *et al.,* 2016), *Ceterach officinarum* (Fernández-Marín *et al.,* 2009), *Pleopeltis pleopeltifolia* (Voytena *et al.,* 2014) or

Polypodium polypoides (Helseth and Fischer, 2005; Muslin and Homann, 1992). In some of these species, such as *M. cafforum* and *C. officinarum*, this strategy is reinforced with chlorophyll masking by highly reflective abaxial scales (Farrant et *al.,* 2009)

As a second line of defense, in the desiccated state fern photosynthetic apparatus enter into a highly dissipative condition with a maximum quenching capacity (Flores-Bavestrello *et al.,* 2016), which prevents the formation of ROS by excited chlorophyll molecules. In fact, the desiccation-induced production of H_2O_2 has been demonstrated in *Adiantum latifolium* (Lubaina et *al.,* 2016). The quenching of excited chlorophyll and ROS formation can be compensated by the enhanced synthesis of Z, a xanthophyll with important antioxidant activity that regulates the level of energy dissipation in the antenna (Demmig-Adams and Adams, 1996, Fernández-Marín *et al.,* 2013). Interestingly it has been described in the DT fern *Ceterach officinarum* that the sole process of desiccation is able to trigger the synthesis of Z even in complete darkness (see Sect. 19.1.2.4) (Fernández-Marín *et al.,* 2009). The synthesis or activities of other antioxidants are also up-regulated during desiccation, as is the case of tocopherol in epiphytic ferns (Tausz *et al.,* 2001) and superoxide dismutase, peroxidase, catalase, and glutathione reductase in *Selaginella tamariscina* (Wang *et al.,* 2010).

1.5 Conclusions

DT is a trait that is relatively extended among ferns in all their life forms, being more common in the spores (all spores appear to be DT), followed by gametophytes (widespread but habitat dependent), and then sporophytes (10 % of species may show vegetative DT). Gametophytes and sporophytes have mechanisms to cope with DT analogous to those present in poikilohydric and homoiohydric plants, respectively (except for Hymenophyllaceae sporophytes that show poikilohydric strategies). Fern spores appear to show comparable mechanism to seeds, which are likely developed during the maturation phase of the spore and rely in the formation of a glassy cytoplasm for long-term survival in the dry state. For all three life forms protection against oxidative stress seems to be a common and important feature, and diverse strategies with some commonalities (e.g. accumulation of antioxidants, protection against photo-oxidation from the chloroplasts during desiccation and while in the dry state) are found depending on the life form. The intermediate position of ferns between bryophytes and spermatophytes in an evolutionary context, and the variation of responses to desiccation

of the different life forms of ferns, can allow us to use them as diverse models to study plant adaptations to terrestrial environment, as well as the evolution of the mechanisms of DT in land plants.

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CHAPTER II

OBJECTIVES

2.1. General objective

The main objective of the present work was to characterize, using chlorophyllous fern spores as model system, the physiological and biochemical mechanisms that are involved in desiccation tolerance, with a particular emphasis on the metabolic events that take place during drying. This main target was developed through four specific objectives, one on each chapter.

2.2. Specific objectives

Objective 1 *(Chapter 4)*:

- **To develop a procedure for the evaluation of desiccation tolerance in photosynthetic tissues.**

  We aimed to develop a simple, reliable, standardized and portable method to assess the level of desiccation tolerance of a wide variety of photosynthetic tissues and organisms.

Objective 2 *(Chapter 5):*

- **To characterise which are the main protection processes in fern chlorophyllous spores tolerant to desiccation.**

  By the characterisation of the loss of DT during imbibition and germination in spores of *Osmunda regalis*, we aimed to find out which are the key mechanisms involved in DT.. This involved the analysis of the activation/deactivation kinetics of photoprotective thermal dissipation, the analysis of the chlorophyll fluorescence in the dried state, the characterization of the changes in pigments involved in photoprotective thermal dissipation, the characterization of thylakoids membranes under dry conditions and the quantification of changes in thylakoid lipid composition.

Objective 3 *(Chapter 6)*:

- **To assess the mechanisms of desiccation tolerance in chlorophyllous fern spores from an ecophysiological perspective.**

  We aimed to unravel the role of DT on the ecological requirements of each species by the comparison of hydric and biochemical parameters in spores from three species with contrasting ecological habits. This involved the determination of the extent of DT in chlorophyllous spores of several taxa, the analysis of pressure-volume curves and wettability during drying and the analysis of the molecular mobility with special emphasis on the temperatures of transition to glassy state in dehydrated spores.

Objective 4 *(Chapter 7)*:

- **To study, in the long-term, the effects that storage conditions have in desiccated chlorophyllous spores**

  We aimed to identify the mechanisms of ageing-associated damage, by the comparison of two species with different viabilities under different storage conditions, with particular focus on the effects of oxygen and light in chlorophyllous spores. This involved the analysis of the loss of viability along time in chlorophyllous spores, the analysis of the antioxidant system and redox balance maintained during desiccation and long-term storage, the characterization of the composition of lipophilic antioxidants of thylakoids (tocochromanols, free carotenoids and others), the changes in the redox ratio of hydrophilic antioxidants (glutathione) and the analysis of reactive oxygen species related to ageing and viability in dried systems.

CHAPTER III

GENERAL MATERIALS AND METHODS

3.1. Plant material

3.1.1 Main features of the studied species

Osmunda regalis

The fern Osmunda regalis belongs to one of the most ancient families in the order Filicales, the Osmundaceae. This family has around 20 species included in the genera Osmunda. *O. regalis* is a deciduous fern, native to Europe, Africa and Asia. It is extensively distributed in warm and tropical areas with high ambient moisture, due to the fact that rhizoids require high humidity to develop. It has two reproductive forms, vegetative and sexual (by spores). Fronds are developed in spring and the fertile fronds in summer. In autumn, fronds become yellow and fall down. The stem is short, upright or ascendant and is attached to the substrate by adventitious, coarse and hard dark-coloured roots. The terminal fronds are fertile and develop the sporangia, which is not grouped in sori and do not present indusium. In *O. regalis* there is not dehiscence ring and the sporangium opens vertically when spores are mature (Figure 3.1a). These are trilets, spherical, and measure around 50 µm in diameter (Figure 3.1b). The wall of the spores is composed by three layers; endospore, perispore and exospore (containing this last one small protuberances or irregular projections) (Figure 3.1c). The nucleus of the ripe spore is prominent and many chloroplasts can be seen. The spores were collected when the sporangium was green in the top and brown in the bottom (Figure 3.1a).

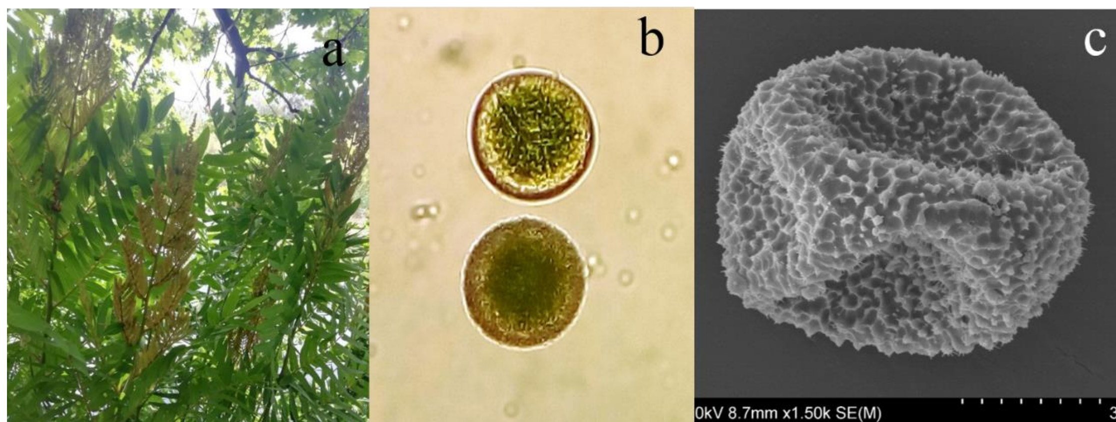


Figure 3.1: (a) *Osmunda regalis* fertile and sterile sporophyte. (b) *O. regalis* spores. Optical microscopy (c) *O. regalis* spores. Scanning electron microscopy.

Equisetum telmateia

The horsetail Equisetum telmateia belong to the order Equisetales. The only extant genus is Equisetum, made up of 15 species. The group is one of the most ancient among

pteridophytes. Is native to European Southern-temperate zone and also in western north America. It grows in open habitats and is particularly frequent on eroding sea- and river-cliffs, but also grows on roadsides and railway embankments. Is well adapted to base-rich clay soils. This pteridophyte is perennial and develops fertile and sterile fronds independently. The vegetative fronds are produced in late spring until autumn. Before them, fertile fronds are produced where spores are developed. Strobilus is produced in early spring. The stem of the fertile frond lack branches and is not photosynthetic, having when mature a green colour due to the presence of spores. Spores growth inside the strobilus until mid-spring, when they are released. When these are released, strobilus became yellow. Each strobilus consists in lines of 5 to 10 rings of sporangia, each one opening and shedding spores by a longitudinal slit without dehiscence ring and they are not grouped in sori. Spores size is about 30 µm, they are chlorophyllous with a circular aperture and four paddle-shaped eleators. Walls are composed of the three typical layers (see description given for *O. regalis*). (Figure 3.2).

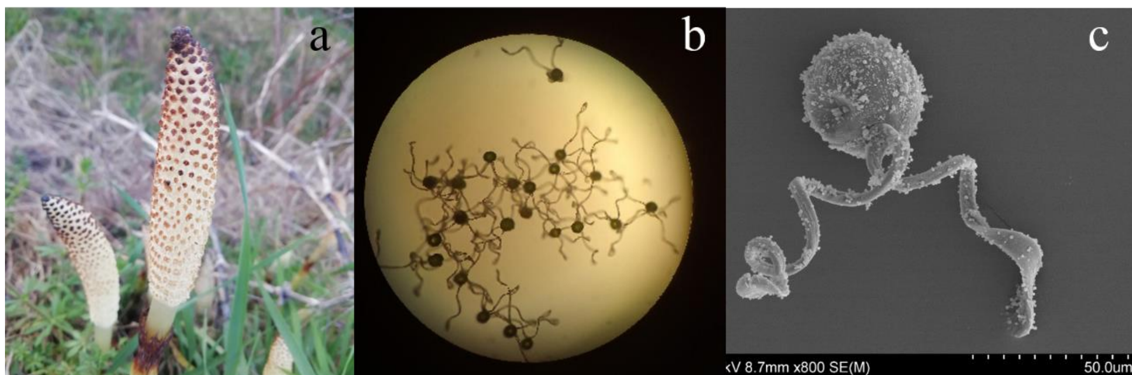


Figure 3.2: (a) *Equisetum telmateia* fertile sporophyte. (b) *E. telmateia* spores. Optical microscopy (c) *E. telmateia.* Scanning electron microscopy.

Matteuccia struthiopteris

Matteuccia is a distinctive temperate and boreal genus of two or three species belong to the family Onocleaceae. Matteuccia Struthiopteris specifically grows in the circumboreal region. It prefers wet habitats or in moist, shady places, sometimes in periodically inundated streams valleys where the erect stems may be nearly covered by alluvial deposition. At mid-summer, shorter fertile fronds emerge. They are stiff and indurated and, as they mature, turn dark brown. While sterile fronds will die in winter with the first cold, snow, and ice, fertile frond remains functional and erect until mid- later-spring when finally they released the spores. They may persist for about two years. This species is the

most evolved compared to the others. Is the only fern that presents dehiscence ring, sori and indusium. Spores are chlorophyllous and have few, coarse folds and obscure, minute, cristate to echinate surface elements with a size around 40µm (Figure 3.3).

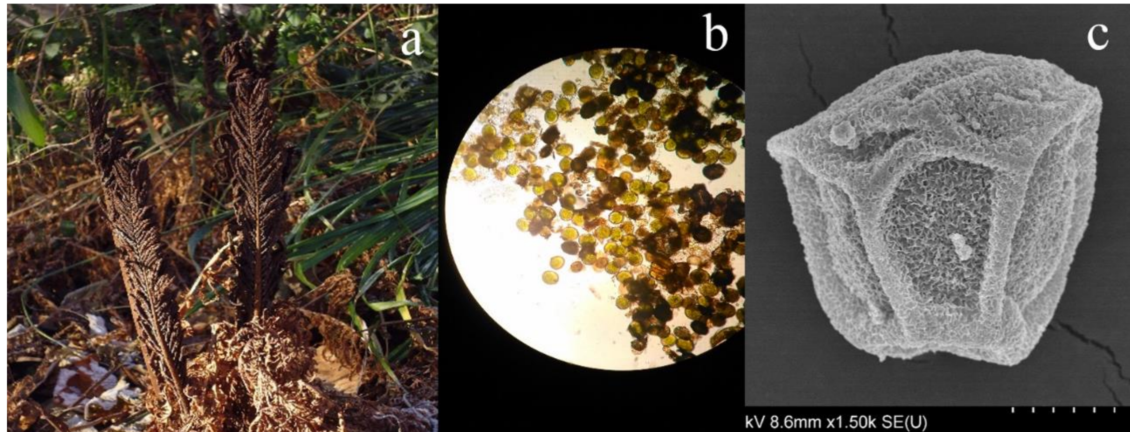
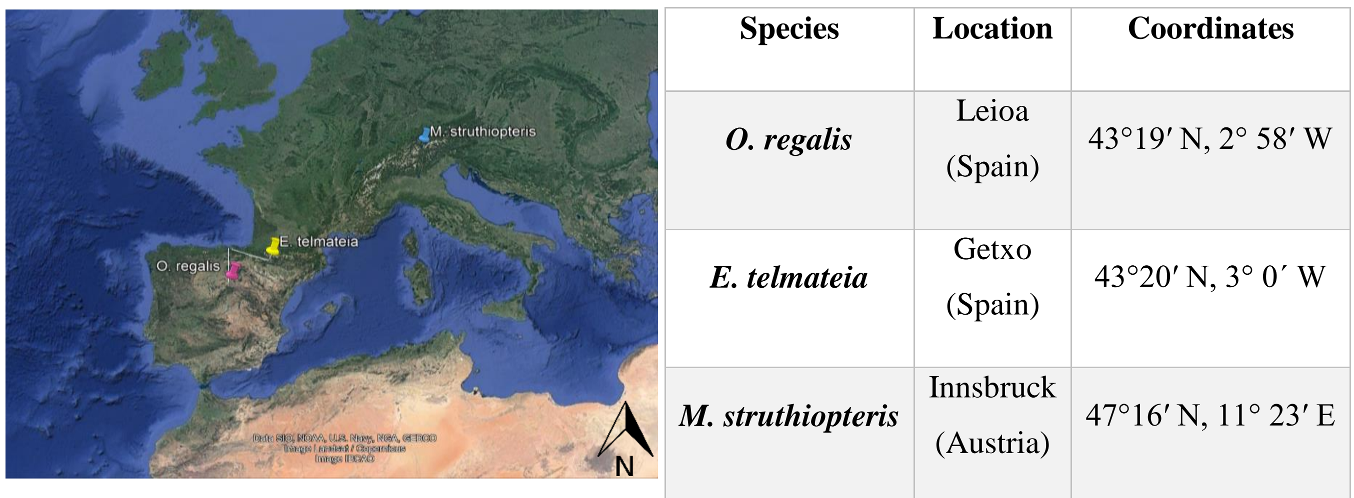


Figure 3.3: (a) *Matteuccia struthiopteris* fertile sporophyte. (b) *M. struthiopteris* spores. Optical microscopy (c) *M. struthiopteris.* Scanning electron microscopy.

3.1.2. Sampling sites

In the present work, three sampling sites were used for plant collection. The three sampling sites have in common the presence of bodies of water. The *O. regalis* plants used grew on the banks of a swamp surrounded by pedunculate oaks. *E. Telmateia* was developing on the banks of a river in a flood plain. *M. struthiopteris* plants were collected on the banks of a pond (Figure 3.4).



Species	Location	Coordinates
O. regalis	Leioa (Spain)	43°19′ N, 2° 58′ W
E. telmateia	Getxo (Spain)	43°20′ N, 3° 0´ W
M. struthiopteris	Innsbruck (Austria)	47°16′ N, 11° 23′ E

Figure 3.4: Sampling sites for plant collection of the three species used

3.1. 3. After collection; Sporangia dehiscence

After collection, fertile fronds of all ferns species were kept in laboratory at 60% relative humidity (RH) for 24 h to allow the dehiscence of the sporangia and the release of the spores. The spores were then immersed in liquid N_2 and stored at −80 °C until the beginning of the experiments. Before use, spores were defrosted in a water bath at 35 °C during 5 min, following the method described in Pence (2008).

3.2. Desiccation treatments.

Chapter 4 corresponds to the development of a semi-quantitative protocol to study DT in photosynthetic tissues known as “Falcon method”. The section “General Material and Methods” does not explain the procedure, since the chapter itself offers a detailed description.

Desiccation treatments were performed inside hermetically closed chambers equilibrating the atmospheres to various relative humidities (RH). These RHs were of 80%, 50% RH and 10 %. Saturated solutions of NaCl and $MgCl_2$ were used for the two higher RHs (80% and 50% respectively). Silica gel was used to achieve 10%RH. During desiccations, a sensor was placed inside the chambers to ensure the RH. After each desiccation, a rehydration was done adding distilled water to the spores and placing them in a saturated atmosphere (100% RH) to prevent desiccation (Figure 3.5).

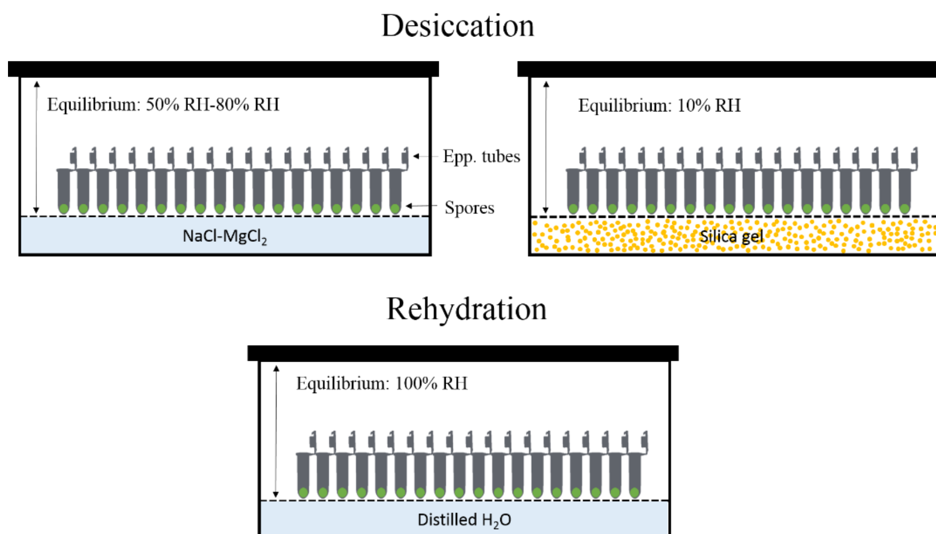


Figure 3.5: Schematic representation of desiccation and rehydration treatments.

3.3 Spores germination

Viability of spores before and after dehydration cycles was assessed by germination test in which spores were sown in 5.5 cm diameter Petri dishes with the mineral culture medium described by Dyer (1979) (with modifications by Quintana *et al.,* 2000) solidified with agar at 1% and containing the fungicide Nystatin (100 U mL^{-1}) (Sigma-Aldrich, Madrid, Spain) (Table 3.1). Due to the loss of viability that sterilization treatment can produce, spores were not sterilized. Petri dishes were sealed with Parafilm and placed in a germination chamber at 20 ± 2 °C with 12 h light photoperiod and a photosynthetic photon flux density of 70 µmol m^{-2} s^{-1}. Spore germination was considered as the first emergence of the rhizoid, when the spore coat breaks (Ballesteros, 2011) (Figure 3.6). The germination was examined microscopically 15 days after the sown. Inside the Petri dishes, a part of it was randomly selected and the germinated spores were counted over a total of 100 and expressed as the percentage of germinating (Figure 3.6, 3.7).

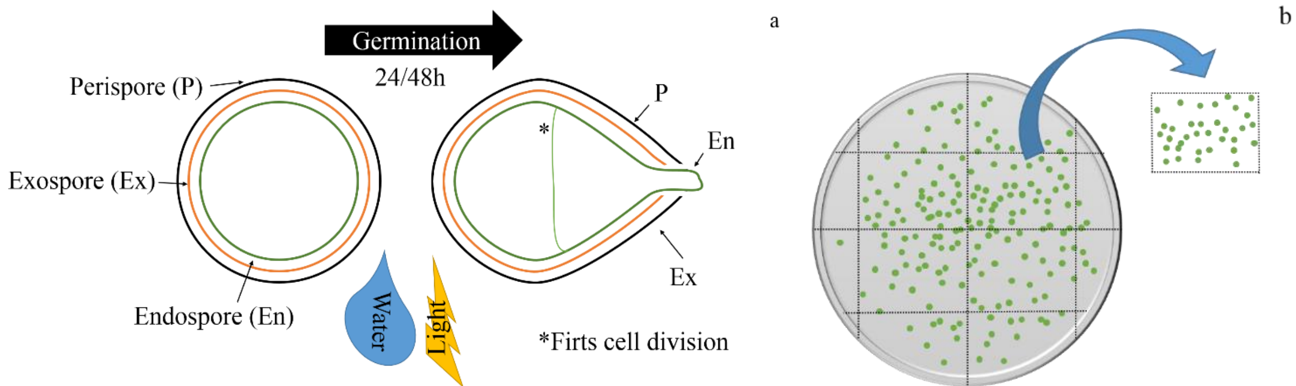


Figure 3.6: (a) Spore germination. (b) Estimation of germination percentage.

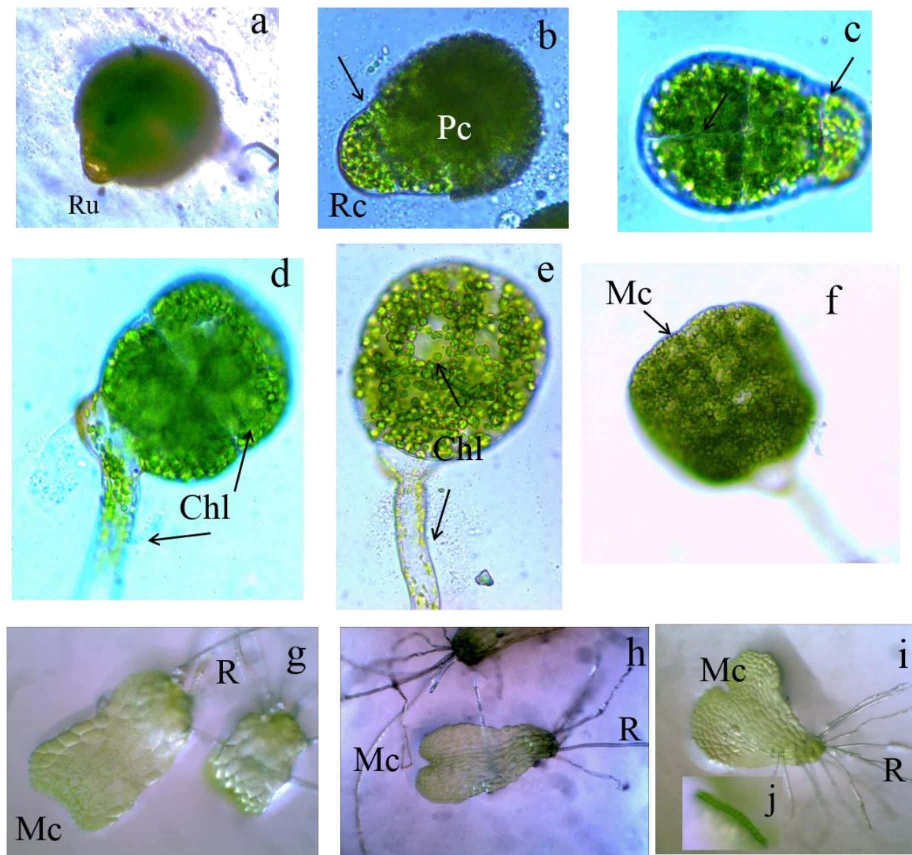


Figure 3.7: Germination and development of freshly collected chlorophyllous spores of *O. regalis*. (a) Ruptured spore coat 2-days after sowing. (b) Appearance of rhizoidal and protonemal cells 3-days after sowing. (c) Several divisions in protonemal cells after 5-days after sowing. (d) Multicellular gametophyte with elongated rhizoid. See divisions inside the protallus. Gametophyte with cells containing abundant chloroplasts. Rizoids also present chloroplasts, 7-days after sowing. (e) More division of protallus cells, chloroplasts change place and placed in the periphery after 8-day of sowing. (f) Initiation of gametophyte meristematic zone formation 13-days after sowing. (g) young gametophyte. (h) Gametophyte. (i) Adult gametophyte. (j) Meristematic zone detail. C, spore coat; Chl; chloroplasts; Mc, meristematic cells; Rc, Rhizoidal cell; Pc, Protonemal cell; R, rhizoid; Ru, rupture coat.

Table 3.1: Dyer (1979) medium for growing ferns Spores.

NUTRIENTS	**[] (µM)**
MG SO_4 7·H_2O	149

K NO3	92.9
FE $C1_3$ 6·H_2O	5.31
CA 2·(NO_3) 4·H_2O	610
KH_2PO_4	109
K_2HPO_4	93.6
NYSTATIN (SIGMA)	100.000 units/mL

3.4 Fluorescence

3.4.1 PAM-2500

The maximum fluorescence of the chlorophyll in fronds and spores was measured using a Pulse Amplitude-Modulated Fluorimeter (PAM 2500, Walz, Effeltrich, Germany). Frond discs were measured using an oblique clamp. Spores were measured entering the optical fiber inside the eppendorf tube. This method allows maintaining the same distance between fiber and sample in all measurements. Initial (F0) and maximal fluorescence (Fm) were measured in dark adapted samples. The maximal photochemical efficiency of photosystem II (PSII) was estimated by the ratio Fv/Fm=(Fm-Fo)/Fm. The saturating pulse in each system was required to reduce the plastoquinone pool and obtain Fm value that was measured during the pulse.

3.4.2 Imaging fluorescence-Microscope

For the microscopic imaging measurements whole gametophytes were placed between holder and covers measuring in one of the two “ears” of the prothallus chlorophyll fluorescence. For fern chlorophyllous spores measurements, these were placed in wet paper above a holder for avoid dehydration. Samples were prepared under low-green-illumination (<1µmol m^{-2} s^{-1}, provided by LEDs), over wet-white paper to avoid sample desiccation, and maintained in the dark until examination. Measures were made with a Microscopy Pulse Amplitude-Modulated (PAM) fluorimeter (WALZ, Effeltrich, Germany), coupled to Axiostar plus microscope (Carl Zeiss, Gottingen, Germany) with WinControl program for image processing software used to control the timing, settings and trigger signals for the saturating pulse light resources and special detector-ocular microscope. The epi-iluminator features contain a MICRO-head blue single Luxeon LED

measuring light source with a peak emission at 470nm, with short-pass filter, diachronic beam splitter and collimators optic which provides pulse-modulated measuring light and actinic and saturation pulse illumination. It was integrated with a CCD camera Imag-K4 (1392 x 1040 pixel) and Zeiss fluor objective 20X/0.75. Fluorescence was detected by the photomultiplier attached to the photo port of the Axiostar plus microscope. Measure of fluorescence was made using a saturating light pulse of 600 μE m^{-2} s during 0.8 s. To process the images captured, false colours images of prothallus or spore chlorophyll fluorescence yield (Fv/Fm) were established based on the assumption that pixel intensity values can be related to the physiological process (Aldea *et al.,* 2006). Data analysis was made integrating Fv/Fm results over the surface of the ear area or the whole spores (Figure 3.8).

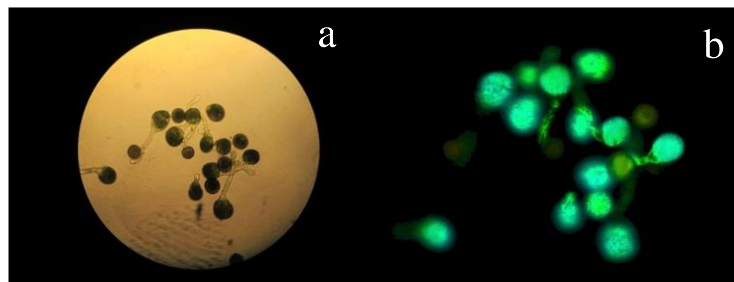


Figure 3.8: (a) images taken with the optical microscope of chlorophyllous Spores of *O. regalis*. (b) Equivalent images of Fv/Fm captured with the Imaging fluorescence-Microscope

3.5 Biochemical methods

3.5.1 Pigments and tocochromanols extraction

Lipophilic antioxidants (tocochromanols) and photosynthetic pigments (chlorophyll and carotenoids) from approximately 10 mg of frond, 15 mg of spores or 20mg of gametophyte were homogenized at the temperature of liquid N_2 with a muller in pure acetone (100%) solution buffered with CaCO3, centrifuged at 16.000g for 20 minutes, and supernatans filtered with 0.2 μm PEFT filters (Teknokroma, Barcelona, Spain). During all the extraction samples were maintained at 4 ºC to avoid pigments alterations.

3.5.2 Pigment and tocochromanol analysis

Photosynthetic pigments (chlorophyll and carotenoids) as well as tocopherol contents, were determined by High Pressure Liquid Chromatography (HPLC) with a reverse-phase

column (250 x 4.6 mm 5 μm particle size) protected by a Nova-Pak C-18 guard column (20 x 3.9 mm 4 μm). The system was equipped with two pumps (Water model 150), a diode array detector (PDA) (Walter model 996, Massachusetts, USA) and a scanning fluorescence detector (Waters model 474). The instrument was controlled by Empower Pro software package (Waters Corporation). Extracts were injected (15 μL) in Waters (Milford, MA, USA) HPLC system following the method of García-Plazaola and Becerril (1999) with the modifications described in García-Plazaola and Becerril (2001).

Column temperature was maintained at 35ºC with a Waters temperature control Module. The mobile phase is composed by two solvents; Solvent A, acetonitrile:methanol:tris 0.1M pH8 (84:2:14); and solvent B, methanol:ethyl acetate (68:32). Before use, solvent were filtered through a nylon membrane of 0.2 um pore size. The elution of these solvents is as follow: linear gradient from 100% solvent A to 100% solvent B for the first 10 min. Isocratic elution with 100% solvent for 5 min. A linear gradient from 100% solvent B to 100% solvent A during 2 mins and finally a isocratic elution with 100% solvent A for 2 at least mins prior the next injection.

Identification and quantification of carotenoids and chlorophylls

Photosynthetic pigments were measured with a PDA detector (Waters model 996) in the range 250-700 nm. Peaks were detected and integrated at 445 nm for carotenoid and chlorophyll content. Pigments were identified and quantified by the method described by García-Plazaola and Becerril (1999). Retention times and conversion factors for pigments were the same as described by García-Plazaola and Becerril (1999, 2001). The tocopherols that this method allows to separate are: Neoxanthin (N), violaxanthin (V), lutein epoxide (Lx), antheraxanthin (A), lutein (L), zeaxanthin (Z), chlorophyll b (Chl b), chlorophyll a (Chl a), α-carotene (α-car) and β-carotene (β-car). No Lx was found in the samples inyected (Figure 3.9).

Identification and quantification of tocopherols

Tocopherol contents (toc) were detected using the Scanning fluorescence detector (SFD Waters 474) with an excitation λ of 295 and an emission λ of 340. Tocopherols were identified and quantified by the method described by García-Plazaola & Becerril (1999). Retention times and conversion factors were the same as described by García-Plazaola & Becerril (1999, 2001). The tocopherols that this method allows to separate are: α-toc, (β+γ)-toc, and δ-toc (Figure 3.9).

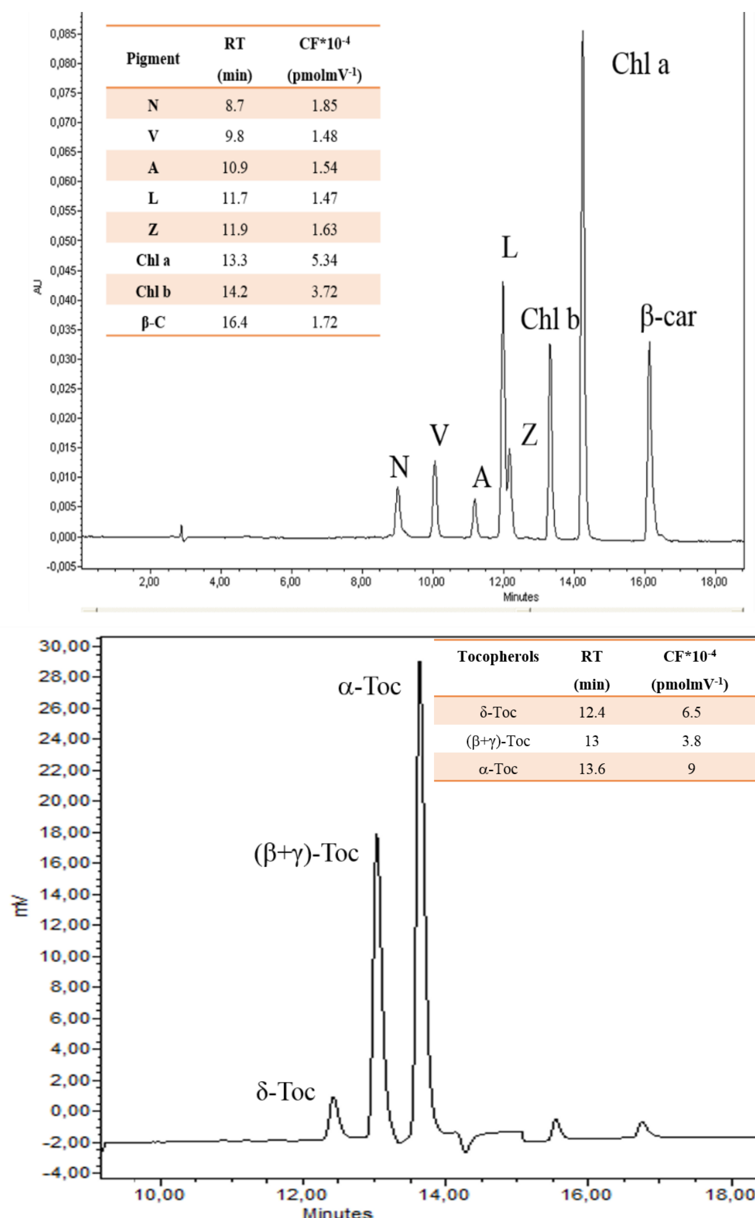


Figure 3.9: (a) HPLC chromatograms showing the typical pattern of lipophilic pigments found in fern chlorophyllous spores. Retentions times and conversion factors for lipophilic pigments are included. (b) HPLC chromatograms showing the typical pattern of tocopherols detected in chlorophyllous spores of *M. struthiopteris*. Retentions times and conversion factors for tocopherols are included

3.5.3 Lipid extraction and quantification

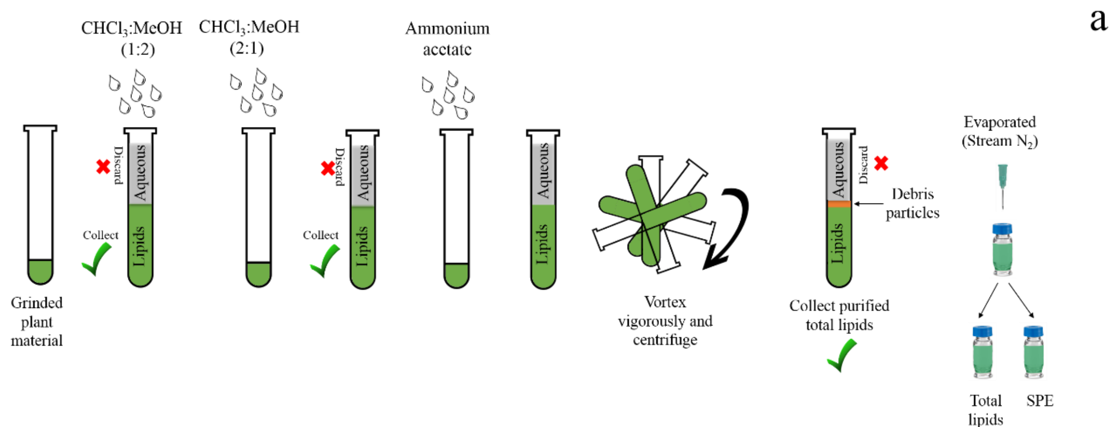
Lipids for mass spectrometry were extracted from fern spores and gametophytes according to Roughan *et al.,* (1978) and Gasulla *et al.,* (2013) with some modifications. Briefly, 15 mg of freeze-dried plant material per replicate were grinded with a Mixer Mill MM 200 (Retsch, Germany) with liquid nitrogen, at a frequency of 23Hz, during 5 min. A first extraction was done with 1 mL of $CHCl_3$: MeOH (1:2, v/v) and the organic phase collected. The lipid extraction was repeated two times with 1 mL $CHCl_3$: MeOH (2:1, v/v) and the organic phases were combined. One volume of $CHCl_3$ and 0.75 volumes of aqueous 300 mM ammonium acetate were added to the combined chloroform extracts. Samples were vortexed and centrifuged (2000 g, 5 min). The organic phase was

harvested. The solvent of the lipid extract was evaporated under a stream of N_2. Total lipids were dissolved in 1 mL of $CHCl_3$/MeOH (2:1, v/v), divided into two 500 µL aliquots and concentrated again by evaporation (Figure 3.10). Solid phase extraction of complex lipid extract on silica column as follows:

Solid phase extraction (SPE) of complex lipid extract on silica column.

Silica columns were equilibrated previously with 100% chloroform. The evaporated aliquot of 500 µL is resuspended in 100 ul of 100% chloroform. That 100ul were placed in the silica columns and 6 mL of chloroform:acetone (4:1) were added. The step is done twice. This first fraction contains chlorophylls, neutral lipids, MGDG and DGDG SQDG. Next fraction contains TGDG and TeGDG. Column was eluted with 6 mL of acetone:methanol. This step is done 5 times. Last fraction contains TeGDG and is eluteded with methanol 100%. Finally, all fractions were evaporated and if needed resuspended in chloroformo:methanol (2:1) (Figure 3.10).

Lipid quantification by Q-TOF MS/MS measurements was carried out according to Gasulla *et al.,* (2013).



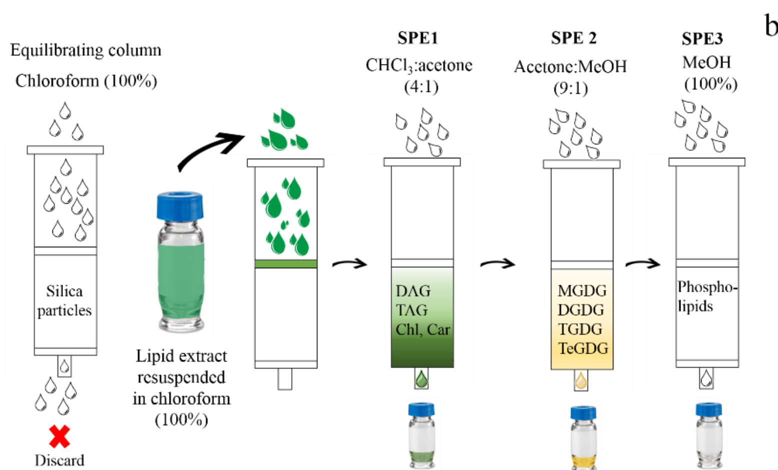


Figure 3.10: Schematic procedure for lipids extraction (a) and separation (b)

3.5.4 Proline determination

Proline content was extracted from ~10 mg of spores in their water (MCW) (12:5:1) according to Borsani *et al.,* (1999). The homogenized was centrifuged for 2 min at 2.000 g and another milliliter of MCW added. After vortexing vigorously, 1 mL chloroform and 1.5 mL water were added. Two phases were generated where the upper phase corresponds to free proline. This phase was mixed with ninhydrin reagent, warmed at 100°C for 1 h and left at room temperature to cool down. Finally, 2 mL of toluene was added, vortexed, and centrifuged at maximum speed during 10 min. Proline determination was measured by spectrophotometry at 515 nm (Troll and Lindsley, 1955).

3.5.5 Glutathione determination

Spores (approximately 10 mg) were ground as described above, extracted in 1mL of 0.1M HCl with 10mg polyvinylpolypyrrolidone (PVPP) and centrifuged at 20 min at 20,000 × g at 4°C. The PVPP was imbibed in 1ml of 0.1M the day before the extraction. The supernatant was then used to determine both GSH and GSSG as described by Bailly and Kranner (2011). Briefly, this procedure uses fluorescence labelling of thiols with monobromobimane (mBBr). GSH+GSSG were determined after reduction of disulphides by dithiothreitol (DTT). For disulphides determination, thiol groups were blocked with N-ethylmaleimide (NEM). As well as glutathione, this method can also measure the low-molecular-weight thiols cysteine, cysteinylglycine and γ-glutamyl-cysteinyl. However, only glutathione was detected in the spores samples. Standards of glutathione at different concentrations were prepared to construct calibration curves. GSH and GSSG were

separated by reversed-phase HPLC on an HiQsil RP18 column (150x2.1mm i.d., 3µm particle size; KyaTech), and detected fluorimetrically (excitation λ: 380 nm; emission λ: 480 nm) with a gradient elution of 0.25% (v/v) acetic acid in distilled water at pH 3.9/methanol (Figure 3.11). Calculation of $E_{GSSG/2GSH}$ followed the formulas given in Schafer and Buettner (2001) and Kranner *et al.,* (2006) using the Nernst equation:

$$E_{GSSG/2GSH} = E^{O'} - \frac{RT}{nF} ln \frac{(GSH)^2}{(GSSG)}$$

Where R is the gas constant (8.314 JK^{-1} mol^{-1}); T, temperature in K; N, number of transferred electrons; F, Faraday constant (9.6485x104$Cmol^{-1}$); Eo´, standard half-cell reduction potential at pH 7 [$Eo´_{GSSG/2GSH}$ = -240mV]; [GSH] and [GSSG] are molar concentrations of GSH and GSSG, estimated using the different water contents WCs.

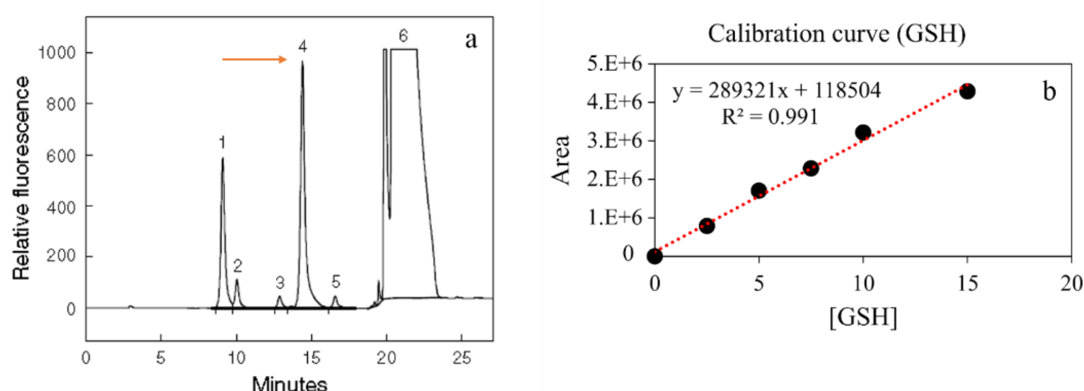


Figure 3.11: (a) HPLC chromatograms showing the typical pattern of GSH and other low-molecular-weight thiols of *Pisum sativum*. 1, cysteine; 2, γ-glutamyl-cysteine; 3, cysteinyl-glycine; 4, GSH (Bailly and Kranner, 2011). Peak 5 is the reductant DTT, and peak 6 corresponds to mBBr rests. In chlorophyllous spores samples only GSH (peak 4) was found. (b) Calibration curve for GSH calculation.

3.5.6 Reactive Oxygen Species (ROS)

Quantification of superoxide radical ($O^{\cdot-}_2$)

Superoxide ($O^{\cdot-}_2$) was measured at the beginning and the end of the storage time, as described by Bailly and Kranner (2011), with some modifications. Ten mg of spores were grounded and homogenized with 2 mL of 50 mM potassium phosphate buffer (pH 7.8) and centrifuged at 16.000 g for 15 min at 4ºC. 1 mL of the supernatant was mixed with 1 mM hydroxylamine hydrochloride and incubated at 25°C for 30 min. Then, 0.5 mL of 17

mM sulphanilamide and 0.5 mL of 7 mM α-naphtylamine were added to 0.5 mL of the reaction mixture and incubated at 25°C for 30 min. After incubation and centrifugation at 13.000 g for 10 min, absorbance was measured at 540 nm. O2- concentrations were calculated using a calibration curve obtained with solutions of various concentrations of sodium nitrite (Figure 3.12).

Quantification of hydrogen peroxide (H_2O_2)

Hydrogen peroxide was measured as in Junglee *et al.,* (2014). Grounded spores (±10 mg FW) were homogenized with 1 mL of solution containing 0.25 mL Trichloroacetic acid (TCA) 0.1% (w:v), 0.5 mL KI (1 M) and 0.25 mL potassium phosphate buffer (10 mM) at 4˚C. Good care was taken to protect samples and standards from light and heat. The homogenate was centrifuged at 12,000 × g for 15 min at 4ºC. The absorbance of the supernatant was measured spectrophotometrically at a λ of 350 nm. H_2O_2 content of the samples was estimated using a calibration curve obtained with solutions of H_2O_2 concentrations prepared in 0.1% TCA (Figure 3.12).

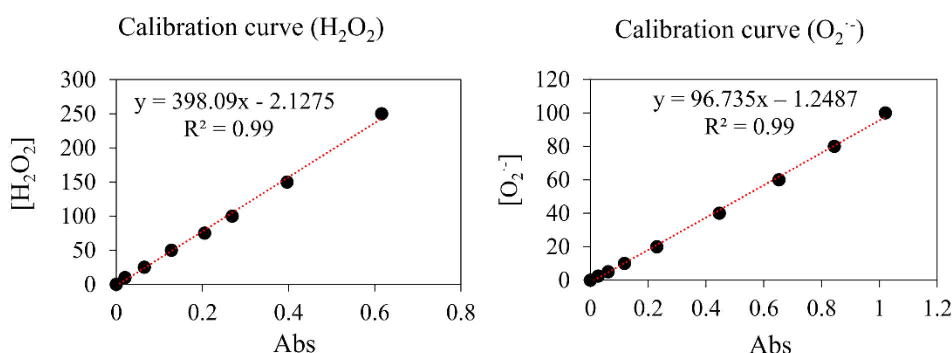


Figure 3.12: example of calibration curves for ROS determination.

3.5.7 Water relations

Pressure–Volume Curves

Water potentials of CS were measured using a dewpoint hygrometer (WP4, Decagon Devices, Pullmann, WA, USA). Around 200 mg of spores was placed in the psychrometer cuvette and held at 100% RH for 48 h in darkness (for germination avoidance) in closed chambers to allow rehydration. After this period, the samples were considered fully hydrated and ready for water potentials measurements. Before the first measurement, the instrument was calibrated with a standard solution of KCl (0.5 M) and temperature was set at 20ºC.The first measurement corresponded to the initial water potential. Once the first reading was complete, the sample was weighed on a balance, corresponding to total

fresh weight. Consecutive measurements were made on each replicate, weighing them after each reading. Between readings, the samples were kept in the lab at 50% RH, allowing the loss of water. Samples for last readings were kept over silica gel between measurements to force the loss of water. After the measurements, samples were then oven dried at 70°C for 24 h and their DW determined.

The results were analyzed by two graphs: the linear plot of water content against water potential and the pressure–volume (P–V) curve relating the reciprocal of the water potential to water content. In the first measurements, a high amount of water loss could be observed but not a decline in water potential (Ψ). As the samples were losing water, the water potentials began to fall, at first slowly without much change, until reaching a point of abrupt change, considered the water potential at turgor loss point (Ψ_{TLP}). The experiment was concluded when the relationship between 1/Ψ and the cumulative amount of water loss by samples became linear ($r^2 > 0.98$). From P-V curves, several parameters were obtained, such as relative water content at turgor loss point (RWC_{TLP}), saturated water content (SWC), water potential at full turgor (Ψ_O), water potential at turgor loss point (Ψ_{TLP}), water potential in the sporangial state (Ψ_{SPO}), and the modulus of elasticity, known as epsilon (ε) (Sack *et al.,* 2011). Water potential in the sporangial state was inferred through the adjustment of the trend line in the regression curve between Ψ and WC. The curve of each replicate was adjusted to a polynomial of degree 4. Sporangial WC values were substituted in the equation (Figure 3.13).

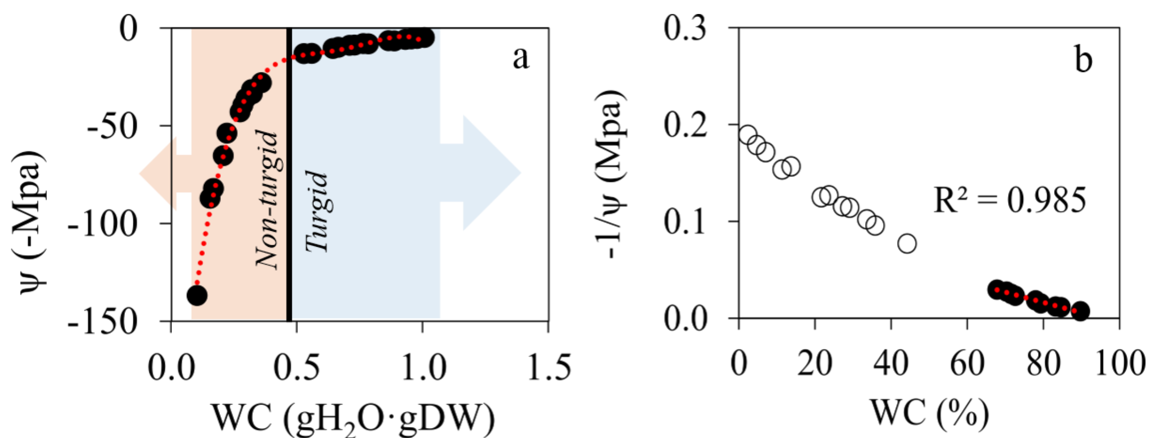


Figure 3.13: Example of typical pressure-volume curve. (Sack et *al.,* 2011) (a) Changes in Ψ during the loss of water. (b) Relationship between 1/Ψ and WC ($r^2 > 0.98$).

Analysis of rehydration kinetics: coat surface hydrophobicity

Static contact angle (CA) and its change during time were used for the interpretation of the rehydration capacity of chlorophyllous spores in their sporangial state (e.g., mature spores at the stage in which they are naturally released from sporangia). For each replicate, a monolayer of spores was mounted over a microscope slide and fixed with a double-sided cellophane (Figure 3.14, c). An optical CA measuring instrument (OCA 15EC, from Data Physics Instruments GmbH, Filderstadt, Germany) was used to define the hydrophobicity of the spore surface. A sessile droplet of 6 µl distilled water was released over the spores (Figure 3.14, b) and changes in its CA recorded at a speed of 30 frames s^{-1}, during 10 s, with a charge-coupled device equipped camera (Figure 3.14, a). CAs were measured with the SCA software for OCA, v.4.4.3., as the mean from the optically left and right margins of the water droplet in each frame. The measuring precision was of ±0.1°. Measurements were performed in 10 different replicates per species.

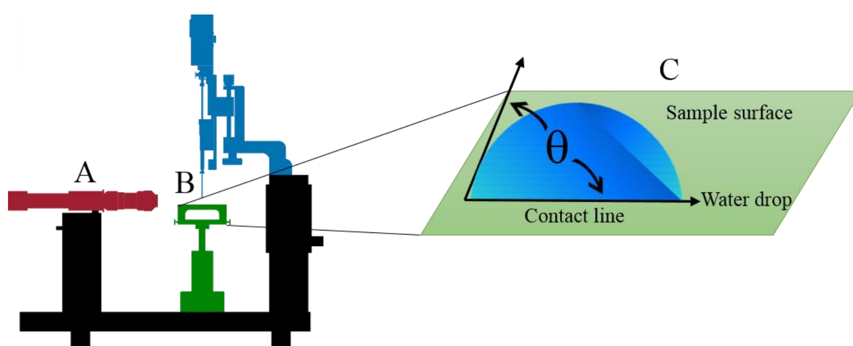


Figure 3.14: Schematic representation of wettability measurements. (a) Camera recording changes in CA, (b) syringe to place the drop of water over the sample, (c) mathematical basis of the measure.

3.5.8 Estimation of spore molecular mobility through Dynamic Mechanical Thermal Analysis (DMTA)

Mechanical properties of spores equilibrated at 10% RH for 48 h were measured using a dynamic mechanical thermal analyzer (DMA/SDTA861e, Mettler Toledo, Greifensee, Switzerland DMTA). Shear tests required the production of circular samples ≤2 mm thick and 13 mm in diameter (Figure 3.16). For that purpose, 300 mg (150 mg per circular sample) of spores dehydrated at 10% RH were compressed in a hydraulic press using a maximum pressure of 10 t. All tests were carried out in the dynamic mode, from -50 to 150°C at a heating rate of 2°C min^{-1} (Fernández-Marín *et al.,* 2013). The analysis of molecular mobility provides information about the potentiality of enzymatic activity at a

wide range of temperature. Shear storage modulus (G´) and the loss tangent (tan δ) were calculated using the Mettler Toledo STARTe software during dynamic mechanical thermal analysis (DMTA) scans. For each biological replicate, the temperature value at the maximum tan δ coincident with the α-relaxation measured at 1 Hz was considered for glass transition temperature (Tg) estimation. The Tg indicates that the spore cytoplasm changes from a solid state (known as "glassy state") to a more fluid state upon warming, which allows diffusive motion (Buitink *et al.,* 1996; Ballesteros and Walters, 2011). The extent to which relaxations released restricted motion was approximated by the size of the relaxation signal and was calculated from the difference in value of the tan δ at the onset and peak of α-relaxation (Ballesteros and Walters, 2011) (Figure 3.15, 3.16).

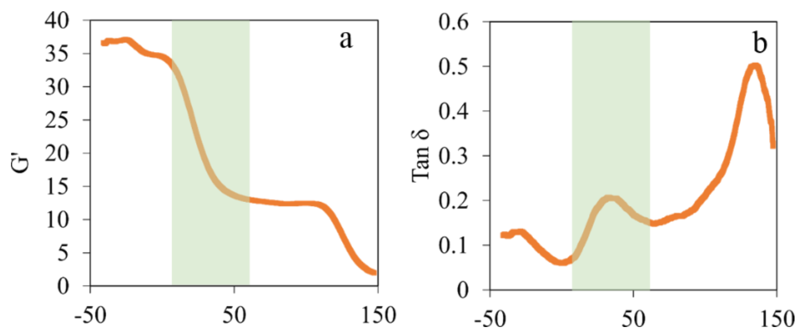


Figure 3.15: Example of typical curves of storage modulus (G') (a) and Tg (Tan δ).

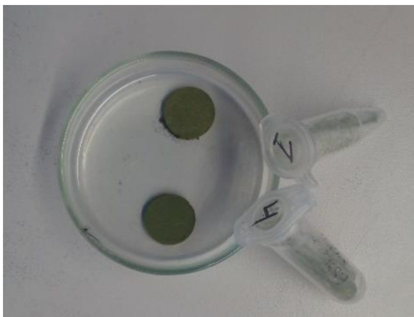


Figure 3.16: Detail of the circular samples of chlorophyllous spores of *O. regalis* prepared for DMTA analysis.

3.5.9 Scanning Electron Microscopy (SEM)

Spores in their sporangial state without any desiccation treatment were used for scanning electron microscopy (SEM) analysis, placing them on adhesive disks pasted on stubs (Agar Scientific Ltd, Essex, UK), and gold coated for 10 min with a fine coat ion sputter (JFC-1100, Tokyo, Japan). Spores were observed using a scanning electron microscope (Hitachi S-4800, Hitachi Ltd., Tokyo, Japan) with an average working distance of 8 mm and a voltage of 10 kV.

CHAPTER IV

A FIELD PORTABLE METHOD FOR THE SEMI-QUANTITATIVE ESTIMATION OF DEHYDRATION TOLERANCE OF PHOTOSYNTHETIC TISSUES ACROSS DISTANTLY RELATED LAND PLANTS

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A field portable method for the semi-quantitative estimation of dehydration tolerance of photosynthetic tissues across distantly related land plants

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Abstract

Desiccation tolerant (DT) plants withstand complete cellular dehydration, reaching relative water contents (RWC) below 30% in their photosynthetic tissues. Desiccation sensitive (DS) plants exhibit different degrees of dehydration tolerance (DHT), never surviving water loss >70%. To date, no procedure for the quantitative evaluation of DHT extent exists that is able to discriminate DS species with differing degrees of DHT from truly DT plants.

We developed a simple, feasible, and portable protocol to differentiate between DT and different degrees of DHT in the photosynthetic tissues of seed plants and between fast desiccation (<24 h) tolerant (FDT) and sensitive (FDS) bryophytes. The protocol is based on (1) controlled desiccation inside Falcon tubes equilibrated at three different relative humidities that, consequently, induce three different speeds and extents of dehydration and (2) an evaluation of the average percentage of maximal photochemical efficiency of PSII (F_v/F_m) recovery after rehydration.

Applying the method to 10 bryophytes and 28 tracheophytes from various locations, we found that (1) imbibition of absorbent material with concentrated salt-solutions inside the tubes provides stable relative humidity and avoids direct contact with samples; (2) for 50 ml capacity tubes, the optimal plant amount is 50–200 mg fresh weight; (3) the method is useful in remote locations due to minimal instrumental requirements; and (4) a threshold of 30% recovery of the initial F_v/F_m upon reaching RWC ≤30% correctly categorises DT-species, with three exceptions: two poikilochlorophyllous species and one gymnosperm.

The protocol provides a semi-quantitative expression of DHT that facilitates comparisons of species with different morpho-physiological traits and/or ecological attributes.

Abbreviations – DHT, dehydration tolerance; DS, desiccation sensitive; DT, desiccation tolerant; DW, dry weight; FDT, fast desiccation (≤24h) tolerant; FDS, fast desiccation sensitive; F_v/F_m, maximal photochemical efficiency of photosystem II; FW, fresh weight; RH, relative humidity; RWC, relative water content; TW, turgor weight; WC, water content.

4.1 Introduction

Desiccation tolerance (DT) is the ability of some organisms to resume normal metabolic activity upon rehydration after being dehydrated to an absolute water content below 0.1 g H_2O g^{-1} dry weight (DW; Bewley 1979, Alpert 2005, Fernández-Marín *et al.,* 2016, Farrant *et al.,* 2017), a water potential ≤-100 MPa, or to a relative water content (RWC) ≤30% (Zhang and Bartels 2018). It is frequently considered as a qualitative trait, and plants are accordingly classified as desiccation tolerant (DT) or desiccation sensitive (DS). DT is common in reproductive structures such as seeds or pollen (i.e., it is estimated that around 90–95% of seeds are DT; Hong *et al.,* 1998, Gaff and Oliver 2013), but its occurrence among photosynthetic tissues has a much more restricted distribution,

particularly among tracheophytes (Alpert 2006, Fernández-Marín *et al.,* 2016, López-Pozo *et al.,* 2018). DT in photosynthetic tissues is quite frequent among lichens and bryophytes, uncommon among pteridophytes, not reported in gymnosperms, and is very rare (135 species) among angiosperms, the latter referred to as "resurrection plants" (Gaff 1977, 1989, Porembski 2011, Gaff and Oliver 2013, Fernández-Marín *et al.,* 2016, Farrant *et al.,* 2017, López-Pozo *et al.,* 2018). Only a few monocots among DT plants degrade chlorophyll and dismantle the photosynthetic apparatus in a reversible manner during dehydration, the so-called poikilochlorophyllous species (Tuba and Lichtenthaler 2011, Fernández-Marín *et al.,* 2016). Upon rehydration, poikilochlorophyllous plants have to reconstruct the photosynthetic apparatus and thus naturally take longer to regain full photosynthetic capacity (Sherwin and Farrant 1996, Farrant *et al.,* 2015).

The mechanism of DT relies mostly on three aspects: (1) the ability to withstand mechanical stress (i.e., preservation of plasmalemmae, cell wall interaction, cell to cell connections through the plasmodesmata, and intracellular compartmentalization into organelles), as well as the ability to preserve ultrastructure and function of macromolecules (i.e., enzymes) at very low intracellular water contents; (2) the ability to reversibly slow down metabolism to the equivalent of a quiescent state; and (3) the ability to cope with high oxidative stress exacerbated during desiccation and rehydration processes (Fernández-Marín *et al.,* 2016). Protection of the subcellular organization is proposed to occur through complex interactions of stress-associated molecules such as LATE EMBRYOGENESIS ABUNDANT (LEAs) and HEAT SHOCK PROTEINS (HSPs), sucrose and osmoprotectants such as raffinose family oligosaccharides (RFOs), as well as the presence of extensive and robust antioxidant systems (Sherwin and Farrant 1998, Hoekstra *et al.,* 2001, Dinakar and Bartels 2012, Farrant *et al.,* 2017, Giarola *et al.,* 2017, Fernández-Marín *et al.,* 2018, Verhoeven *et al.,* 2018). Conversely, DS species may show different degrees of dehydration tolerance (DHT), ranging from the loss of viability after loss of 1% total water content (e.g., as in many succulents) to a maximum of 69% in a few species (Höfler 1941). In this sense, DHT can be defined as a continuum from DS plants with very little tolerance, through to plants with intermediate tolerances, to truly DT plants. This continuum has not been characterized thus far.

The process of rehydration is potentially more damaging than desiccation. It can exacerbate mechanical and metabolic stresses and result in the elevated production of reactive oxygen species (ROS; Kranner *et al.,* 2008). In DT species, rehydration is

accompanied by the repair of damage incurred on drying, regulated cell wall unfolding, and the re-establishment of cellular integrity and metabolic activity (Moore *et al.,* 2013, Fernández-Marín *et al.,* 2016). Consequently, rewatering of desiccated tissues is also a critical component of the experimental protocols developed to characterise the responses of DHT in plants (Slate *et al.,* 2018).

The attainment of tolerance to desiccation depends on both internal (biological) and external (environmental) factors. For example, leaf age or collection season are among the biological factors that influence DT capability in a few species (Farrant *et al.,* 2009, Griffiths *et al.,* 2014), while irradiance and dehydration recurrence as external factors can enhance antioxidant concentration and protective mechanisms in photosynthetic tissues (Lizarazo *et al.,* 2010, Fernández-Marín *et al.,* 2010). Among the external factors, the rate of dehydration (speed), the extent (final water content) and the length of time the tissue is maintained in the desiccated state are particularly relevant (Hoekstra 2005, Koster *et al.,* 2010, and very nicely reviewed recently by Stark 2017a). Thus, even among DT-species, rapid drying can be more damaging as it precludes sufficient time to furnish adequate protection. This general rule applies for most groups of photosynthetic DT organisms, including mosses (Fernández-Marín *et al.,* 2013, Cruz de Carvalho *et al.,* 2012, 2017), green algae (Gasulla *et al.,* 2009, Guéra 2009), lichens (Fernández-Marín *et al.,* 2010, Gauslaa *et al.,* 2012), angiosperms (Farrant *et al.,* 1999, Fernández-Marín *et al.,* 2011, 2018), and even for DT animals, such as tardigrades (Boothby *et al.,* 2017). From this perspective, some differences can also be found within DT plants, with some species being able to withstand drying <30% RWC only when it is reached slowly and/or after acclimation processes (Farrant *et al.,* 1999, Cruz de Carvalho *et al.,* 2011, 2012, 2014). This is particularly true for several bryophyte species that do not survive rapid (typically ≤24 h) desiccation to ≤30% RWC, but will survive if the drying process is much slower (e.g. from several days to up to 1–2 weeks; Stark *et al.,* 2013, Cruz de Carvalho *et al.,* 2014, Hajek and Vicherová 2014, Xiao *et al.,* 2018). Acclimation/deacclimation or hardening/dehardening processes can partially shift the survival response of a species to a certain desiccation regime. For instance, typical DT taxa such as *Syntrichia* species can lose their ability to survive dehydration after long-term dehardening treatment (e.g. several weeks) by incubation under controlled hydrated conditions (Schonbeck and Bewly 1981, Stark *et al.,* 2016). Similarly, traditional DS taxa such as some *Spahgnum* species can survive desiccation provided that controlled hardening (partial dehydration)

is applied (Hájek and Vicherova 2014). Depending on the species ecology, these pre-treatments may not, however, reflect what the plants actually experience in nature (Hellwege *et al.,* 1994). While these artificial pre-treatments can be very useful for addressing specific physiological questions (Cruz de Carvalho *et al.,* 2014, Stark *et al.,* 2013b), it is also true that under the same conditions, some bryophyte taxa are more tolerant to fast desiccation than others. As an example, gametophores that previously experienced dehydration events (either in the field or in the lab) are able to survive rapid desiccation, as in the case of *Syntrichia ruralis* (Schonbeck and Bewley 1981). In contrast, *Physcomitrella patens* requires a slow process of desiccation in order to ensure survival (Xiao *et al.,* 2018).

Based on this differential sensitivity, the so-called "Austin protocol" was proposed as a method to systematically characterise DT among bryophytes (Wood 2007). This protocol was based on the assessment of the recovery of maximal photochemical efficiency (F_v/F_m) after the rehydration of bryophyte samples previously desiccated and equilibrated under two different atmospheric relative humidities (RH): 67–75% and 20–30%. In this protocol, the tested bryophytes were classified into two groups according to their survival at equilibration at either 67 or 23% RH (Wood 2007). With regards to other groups of DT organisms, different desiccation procedures have been used as a method to discriminate recovery levels, including in lichens (Gauslaa *et al.,* 2012, Fernández-Marín *et al.,* 2010), free-living microalgae (Candotto-Carniel *et al.,* 2015), fern gametophytes (Riaño and Briones 2015), and angiosperm pollen (Marks *et al.,* 2014).

As indicated above, vegetative tissues of DS species display different degrees of tolerance to cell dehydration (DHT). We propose that there is a continuous gradient of DHT in such species, while true "DT" can be defined as the ability to recover metabolic activity after severe dehydration to RWC <30%. This distinction is of relevance for the correct interpretation of physiological and –omics data (Zhang and Bartels 2018). Therefore, although the terms dehydration- and desiccation-tolerance have been used synonymously with respect to vegetative tissues (Blum and Tuberosa 2018), here we use DHT as a quantitative continuum trait and DT as a qualitative absolute term, the latter referring only to truly tolerant plants. It is necessary to mention that while this threshold of RWC <30% seems to be clearly defined for tracheophytes (Zhang and Bartels 2018), it may be not that accurate for bryophytes where a much higher proportion of species are known to tolerate this water content if the drying speed is sufficiently slow (>24 h and typically up

to several days). Regardless, no simple screening technique for DHT is available that allows for comparisons of plants within a wide phylogenetic range, neither is there a portable protocol reliable for use in remote areas in the field. With this aim, we have developed a standard, portable, and simple procedure for the semi-quantitative evaluation of tolerance to dehydration in a wide range of embryophyte species and photosynthetic tissues in the field, providing evidence of its usefulness from two contrasting case studies with different levels of technical support and difficulty: (1) an assay of bryophytes with contrasting habitat conditions in the field in Spain, and (2) a survey of tracheophytes comprising plants from controlled growing conditions, botanical gardens and from the field in South Africa, Chile, United Kingdom and Spain.

4.2 Methods

4.2.1 Protocol set up

General description of the procedure

Only green photosynthetic tissues were used. Individual sample weight and F_v/F_m were recorded at three time points along the protocol, i.e., that of fully hydrated material prior to dehydration ($t_{Control}$), upon full dehydration (t_{Dh}), and upon rehydration (t_{Rh}). After collection in the field, leaf material was maintained in darkness in a saturated atmosphere for 24 h in order to assess maximal F_v/F_m and to obtain turgor weight (TW) at $t_{Control}$ (Fig. 4.1). Afterwards, plant samples were placed inside 50 ml-Falcon® tubes under three desiccating regimes (RHs: =80%, =50%, and $<10\%$) and were allowed to equilibrate with their respective atmospheres for 24 h (in the case of bryophyte samples) or 48 h (in the case of tracheophytes). Three replicates were used per treatment. After desiccation, samples were removed from the tubes, weighed, and F_v/F_m was again measured (t_{Dh}). Plant samples were finally rehydrated in a saturated atmosphere (≈100% RH) and in darkness for 24 h; a time considered sufficient to obtain an almost complete recovery of F_v/F_m in many organisms (Pandey *et al.,* 2010, Proctor 2010) and for small leaf pieces to recover turgor. F_v/F_m and weight were again measured (represented as t_{Rh}). This allowed the calculation of relative recovery from each dehydration treatment with respect to control values [e.g. for each species and desiccation treatment, we expressed Fv/Fm as the % of the initial value (Hájek and Vicherová 2014)]. Then the average F_v/F_m recovery following the three desiccating treatments was used as a proxy of overall recovery and thus as a quantitative indicator of DHT. Consequently, the majority of DT species should

yield higher values (i.e., closer to 100%), while the values in most DS species are expected to be low (closer to 0%). All incubations were conducted in darkness and within a temperature range of 20±5ºC. The complete protocol is summarised in Fig. 4.1.

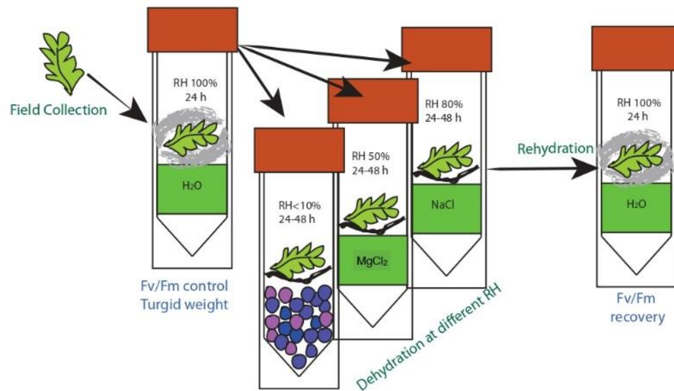

Fig. 4.1. Diagram illustrating the proposed protocol for the comparative estimation of DT in the photosynthetic tissues of plants belonging to different clades or functional groups (see Methods for details). Samples are surrounded with wet paper tissue during hydration and rehydration. In addition, the sponge (represented as a green square in the picture) is moistened with distilled water to allow for an atmosphere at RH≈100 %.

Desiccation procedure

Desiccation treatments were performed inside 50 ml-Falcon tubes (Fig. 4.2A) – hereafter referred as the "Falcon test"– in equilibrium with approximately 12 g of silica gel [RH <10%, which at 20ºC corresponds to a water potential (Ψ) of -310 MPa (Gaff and Oliver 2013)] or with 10 ml of concentrated solution of $MgCl_2$ (280 g of $MgCl_2$ hexa-hydrated were dissolved in 150 ml of Milli-Q water; 50% RH, Ψ≈-94 MPa) or of NaCl supersaturated solution (80% RH, Ψ ≈-30 MPa). The concentrated solution of $MgCl_2$ allowed for a relatively constant ≈50% RH in the temperature range between 15 and 30ºC (Fig. S4.1, Supporting information). RH is kept constant as soon as the Falcon tubes are incubated in darkness (e.g. to avoid overheating due to a greenhouse effect). One of the main problems of using salt solutions in the field is that they can easily enter into contact with the samples, irreversibly affecting the measurements. In the present protocol, we have prevented sample moistening by salt solutions (and facilitated handling and transport) by simply absorbing the solutions to coiled pieces (3 x 17 cm) of commercial kitchen sponge (Spontex Natura ®) separated from the samples by plastic mesh (65 mm x 65 mm, 1 mm ø). Relative humidity in the atmosphere of the empty tube and in the tube containing samples was assessed with a RH probe (HMP45C, Campbell Scientific,

Loughborough, UK) and recorded with a data logger (CR10, Campbell Scientific, NL; Fig. 4.2B). Each sensor was placed inside the tube through a hole in the lid of the tube, conveniently sealed once the wire was placed through it. Data were recorded until stabilization every 30 s and averaged every hour.

Optimization of biomass load inside the test tubes and of the time required to reach desiccation equilibrium was performed using different initial amounts (25, 50, 100, 200, or 400 mg of FW) of the moss *Syntrichia ruralis* (Hedw.) F.Weber & D.Mohr (Fig. 4.2). Once optimised, the same amount of plant material was used for bryophyte and tracheophyte analyses, with the tracheophytes being subjected to a longer desiccation time (48 h).

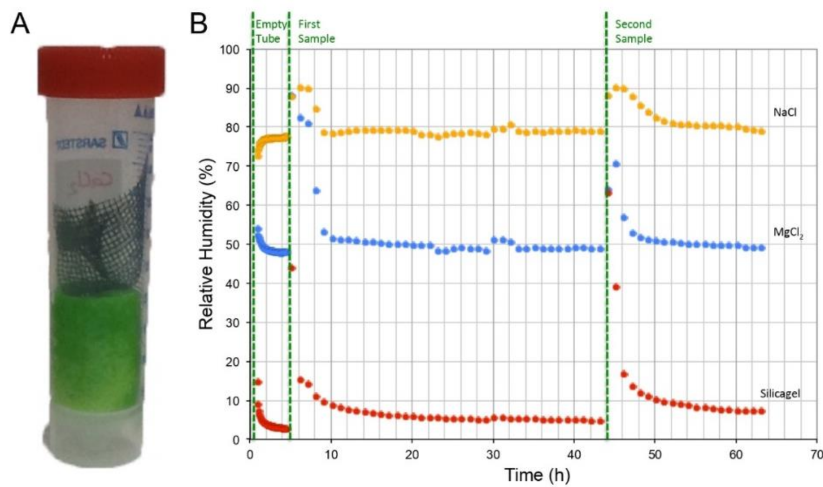

Fig. 4.2. Description of the Falcon tubes and monitoring of the RH inside them. (A) Falcon tube prepared for desiccation tests: in the bottom of the tube, a coiled kitchen sponge absorbs 10 ml of a supersaturated salt solution of NaCl (80% RH) or a solution of $MgCl_2$ (280 g of $MgCl_2$ hexa-hydrated dissolved in 150 ml of Milli-Q water; 50% RH), preventing leaks of liquid droplets. No sponge is used in the case of silica gel treatment. A mesh prevents direct contact between the sample and the desiccant. (B) Time course of changes in RH inside the desiccating tubes. First, RH was monitored during 5 h in empty tubes, and then in two consecutive desiccation cycles of 48 h (containing a first plant sample) and 24 h (containing a second plant sample).

Rehydration procedure

Rehydration of samples was conducted by contact with wet tissue paper (using distilled water) and incubation in a saturated atmosphere (RH≈100%) for 24 h in darkness. The

rehydration procedure was selected after three alternative procedures were tested in a representative group of bryophyte and tracheophyte species, including several bryophytes (*Bryum* sp., *Porella canariensis* (F.Weber) Underw., *Hypnum cupressiforme* Hedw., *Pseudoscleropodium purum* (Hedw.) M.Fleisch., *Plagiomnium undulatum* (Hedw.) T.J.Kop., *Polytrichastrum formosum* (Hedw.) G.L.Sm.), ferns (*Nephrolepis exaltata* (L.) Schott, *Phlebodium aureum* (L.) J.Sm., *Davallia canariensis* (L.) Sm.) and angiosperms (*Amaranthus* sp., *Olea europaea* L., *Fraxinus* sp.), collected from the experimental field of the Universitat de les Illes Balears (UIB; Figs S4.1 and S4.2). The first method involved the direct immersion of samples in distilled water. The second procedure involved the maintenance of samples in a Petri dish in contact with moistened tissue paper. The third procedure was as the second, except that the Petri dishes were maintained in a closed chamber with 100% RH. After testing a desiccation-rehydration cycle under the three described methods, it was concluded that moistening with wet tissue paper in a saturated atmosphere was the best protocol that yielded the highest recovery, and hence constituted the adopted methodology. The first procedure (water immersion) was discarded, as it tended to overestimate water content and underestimate recovery after rehydration and also caused visible necrotic lesions, particularly on the excised tissues of vascular plants, which may affect the results. The other two methods yielded similar results in some species, but in many others the third method allowed for a better recovery.

4.2.2 Determination of absolute water content and relative water content

Plant material was weighed with a Mettler Toledo scale (AB104, Mettler Toledo, Barcelona, Spain) with a precision of 0.1 mg in the case study I (bryophytes), and with a COBOS scale (JT-120M, Balanzas COBOS Precision, Barcelona, Spain) with 1 mg precision for the case study II (tracheophytes). To obtain the dry weight (DW), plant material was dried at 70°C in an oven for 24 h and maintained in a closed box with silica gel until weighing. Water content (WC) was calculated as: WC=(FW-DW)/DW and expressed as g H_2O g^{-1}DW.

Relative water content (RWC) was estimated as the percentage of water content at any time, referred to the maximum (turgid) water content: RWC=(FW-DW)/(TW-DW) × 100, where TW was turgid weight (after 24 h incubation over wet tissue paper and 100% RH in darkness. Note that excess water was blotted with tissue paper before weighing).

4.2.3 Chlorophyll fluorescence

Maximal photochemical efficiency of photosystem (PS) II (F_v/F_m) was used as a proxy for the photosynthetic integrity of the tissue and was measured with a portable modulated PAM fluorometer (Walz, Effeltrich, Germany): PAM 2500 was used in the case study I (bryophytes) and in the protocol setup, and a Junior PAM in the case study II (tracheophytes). The maximum Chl *a* fluorescence yield (F_m) was induced with a saturating pulse, while minimum fluorescence (F_o) was recorded with low measuring light intensities after several hours of dark acclimation. The maximal photochemical efficiency of PSII (F_v/F_m) was then calculated as $(F_m-F_o)/F_m$. The relative rate of recovery of F_v/F_m after desiccation-rehydration (t_{Rh}) with respect to the initial values ($t_{Control}$) was used as an estimator of tolerance to the reached RH, in each case similar to Hájek & Vicherová (2014). The average F_v/F_m recovery of the three desiccating treatments at t_{Rh} was used as a proxy for the DT level of each species.

4.2.4 Studied species and collection sites

Case study I: Bryophytes

Here, 10 bryophyte species that had been previously well-characterised in their responses to desiccation were analysed, including five liverworts and five mosses, half of which are described as fast desiccation tolerant (FDT; e.g. tolerant to desiccation below 30% RWC within a time extent ≤24 h) and the other half described as fast desiccation sensitive (FDS; see Table 1 for specific references to each species). Specimens were collected in the field in different locations along a climatic and elevational gradient (400–1300 m a.s.l.) in La Rioja (Northern Spain) in November 2015 (autumn) in the hydrated state (specifications on meteorological conditions the week before sampling are shown in Table S1). Immediately after collection, samples were stored in darkness for 24 h at a moderately variable temperature of 20±5ºC and in a 100% RH atmosphere.

Case study II: Tracheophytes

For this case study, 14 DS (e.g. not previously described as DT) and 14 known DT species were evaluated, the latter including some of the model DT-plants (i.e., *Xerophyta viscosa*, *Craterostigma plantagineum* and *Myrothamnus flabellifolius*; Table 1). The 28 evaluated species included different functional groups (angiosperms, gymnosperms, ferns) and

were collected from different sites, during the summers of 2016 and 2017, as specified in Tables 1 and S1. For a number of species, both field-grown and potted plants were compared, with no significant differences in the recovery levels (in agreement with the fact that DT is an inherent character of some species); thus, data from field and potted species were pooled together in the results. The potted plants were grown with horticultural substrate Prohumin® (Projar SA, Valencia, Spain) and perlite (3:1) with additional fertilization (Multigreen®, Haifa Chemicals, Madrid, Spain): 5 g per litre of substrate. The plants were watered daily to field capacity and maintained under optimal conditions. Further details on plant conditions during the 30 days before sampling are shown in Table S1. Only photosynthetic tissue (i.e., leaf blade) of 100–200 mg initial FW, avoiding main venations, was used.

Table 1: Species list of the analysed bryophytes (Case study I) and tracheophytes (Case study II), their functional groups, collection site, and tolerance to desiccation (FDS: fast desiccation (≤24h) sensitive and FDT: fast desiccation-tolerant) as described in the bibliography. Species nomenclature follows the Tropicos database (http://www.tropicos.org/). Royal Botanic Garden Edinburgh (UK) is abbreviated as RBGE, and University of the Balearic Islands (Spain) as UIB.

Case study	***Species***	***Functional group***	***Site***	***Tolerance***	***References***
I	*Marchantia polymorpha* L.	Liverwort	Spain	FDS	Pence *et al.,* 2005
I	*Scapania undulata* (L.) Dumort.	Liverwort	Spain	FDS	Gupta 1977
I	*Lunularia cruciata* (L.) Lindb	Liverwort	Spain	FDS	Deltoro *et al.,* 1998
I	*Fontinalis antipyretica* Hedw	Moss	Spain	FDS	Cruz de Carvalho *et al.,* 2011, Cruz de Cargalho *et al.,* 2014
I	*Hookeria lucens* (Hedw.) Sm	Moss	Spain	FDS	Proctor *et al.,* 2007b
I	*Porella platyphylla* (L.) Pfeiff.	Liverwort	Spain	FDT	Gupta 1977
I	*Frullania tamarisci* (L.) Dumort.	Liverwort	Spain	FDT	Proctor *et al.,* 2007b
I	*Rhizomnium punctatum* (Hedw.) T.J.Kop	Moss	Spain	FDT	Bartoskova *et al.,* 1999

I	Polytrichastrum formosum (Hedw.) G.L.Sm.	Moss	Spain	FDT	Proctor *et al.,* 2007a
I	Syntrichia ruralis (Hedw.) F.Weber & I D.Mohr	Moss	Spain	FDT	Tuba *et al.,* 1996
II	*Vachellia erioloba* (E.Mey.) P.J.H.Hurter	Angiosperm	South Africa	DS	
II	*Angiopteris lygodiifolia* Rosenst	Fern	RBGE	DS	
II	*Blechnum magellanicum* (Desv.) Mett	Fern	Chile	DS	
II	*Boscia albitrunca* (Burch.) Gilg & Benedict	Angiosperm	South Africa	DS	
II	*Davallia canariensis* (L.) Sm.	Fern	UIB	DS	
II	*Diospyros austroafricana* De Winter	Angiosperm	South Africa	DS	
II	*Enneapogon desvauxii* P.Beauv.	Angiosperm	South Africa	DS	
II	*Helianthus annuus* L.	Angiosperm	UIB	DS	
II	*Juniperus oxycedrus* L.	Gymnosperm	UIB	DS	
II	*Maytenus oleoides* (Lam.) Loes.	Angiosperm	South Africa	DS	
II	*Montinia caryophyllacea* Thunb	Angiosperm	South Africa	DS	
II	*Oedera squarrosa* (L.) Anderb. & K.Bremer	Angiosperm	South Africa	DS	
II	*Quercus ilex* L.	Angiosperm	UIB	DS	

II	*Triticum aestivum* L.	Angiosperm	UIB	DS	
II	*Ceterach officinarum* L.	Fern	RBGE	DT	Fernández-Marín *et al.,* 2009, Proctor 2009
II	*Astrolepis sinuata* (Lag. ex Sw.) D.M.Benham & Windham	Fern	RBGE	DT	RBGE (pers. communication)
II	*Barbacenia purpurea* Hook	Angiosperm	UIB2	DT	Suguiyama *et al.,* 2014
II	*Bommeria hispida* (Mett. ex Kuhn) Underw	Fern	RBGE	DT	RBGE (pers. communication)
II	*Cheilanthes eatonii* Baker	Fern	RBGE	DT	Proctor 2009
II	*Craterostigma plantagineum* Hochst.	Angiosperm	South Africa	DT	Gaff 1977
II	*Eragrostis nindensis* Ficalho & Hiern	Angiosperm	South Africa	DT	Gaff 1977
II	*Haberlea rhodopensis* Friv.	Angiosperm	RBGE	DT	Rakic *et al.,* 2014
II	*Hymenoglossum cruentum* (Cav.) C.Presl	Fern	Chile	DT	Saldaña *et al.,* 2013
II	*Hymenophyllum dentatum* Cav.	Fern	Chile	DT	Saldaña *et al.,* 2013
II	*Mohria caffrorum* (L.) Desv.	Fern	South Africa	DT	Gaff 1977, Farrant *et al.,* 2009
II	*Myrothamnus flabellifolius* Welw.	Angiosperm	South Africa	DT	Gaff 1977, Sherwin *et al.,* 1998
II	*Ramonda myconi* (L.) Rchb	Angiosperm	Spain	DT	Rakic *et al.,* 2014
II	*Xerophyta viscosa* Baker	Angiosperm	UIB	DT	Rakic *et al.,* 2014

4.2.5 Statistical analyses

Statistical differences among treatments with respect to the WC, RWC, and F_v/F_m of the samples were analysed by one-way analysis of variance (ANOVA) after assessing data

homoscedasticity. Alternatively, the Kruskal-Wallis test was used for heteroscedastic data. All statistical analyses were assessed at $\alpha = 0.05$. The SPSS v20 package (IBM Corp., Armonk, NY) was used for the statistical analyses.

4.3 Results

4.3.1 Protocol setup

Falcon tubes of 50 ml constituted excellent hermetic containers for individual replicates, allowing for the handling of a relatively high number of samples in a relatively small space. Sponge and mesh efficiently prevented direct contact between the saturated salt solutions and the samples (Figs. 4.1 and 4.2A), concurrently allowing for a relatively quick equilibration of the atmosphere (Fig. 4.2): i.e., RH inside the tubes reached 95% of the expected equilibrium value in less than 5 h (Fig. 4.2B). When samples were placed inside the tubes, the equilibrium took longer, but was achieved in less than 24 h (Fig. 4.2B). When the desiccating tubes were recycled for a second use, equilibrium RH was maintained within the expected range. Samples incubated over silica gel did exhibit an initial slight increase in RH within the tube, but this was nevertheless maintained at < 10% RH during the second cycle.

As expected, equilibrium was reached more rapidly with the strongest desiccant. Thus, silica gel was faster than the $MgCl_2$ and NaCl solutions. After 12 h, at least 99% potential water loss had occurred in the silica gel tubes when the sample mass was ≤100 mg (Fig. 4.3). In contrast, after 12 h, only the smallest sample (25 mg) had reached 99% potential water loss in the NaCl treatment (Fig. 4.3A). After 24 h, with the only exception of 400 mg FW in NaCl, 99% potential water loss was reached for all desiccants and masses tested (Fig. 4.3A). Thus, a FW between 50–200 mg of hydrated bryophyte tissue and a desiccation time of at least 24 h were appropriate to obtain desiccated tissues in equilibrium with the tube atmosphere (Fig. 4.3B). As the desiccation treatments dried the bryophyte samples in ≤24 h, and given that recent literature indicates that slow desiccation in bryophytes occurs over several days or even weeks (Buda *et al.,* 2013, Stark *et al.,* 2013b, Xiao *et al.,* 2018, Cruz de Carvalho *et al.,* 2017), we used the nomenclature fast DT (FDT) and fast DS (FDS) to refer to the bryophyte species tested. The three RHs used successfully induced three different rates of dehydration and final water contents after 24 h (Fig. 4.3B). Same amount of initial FW was also suitable for photosynthetic tissue of tracheophytes (Figs S4.1 and S4.2).

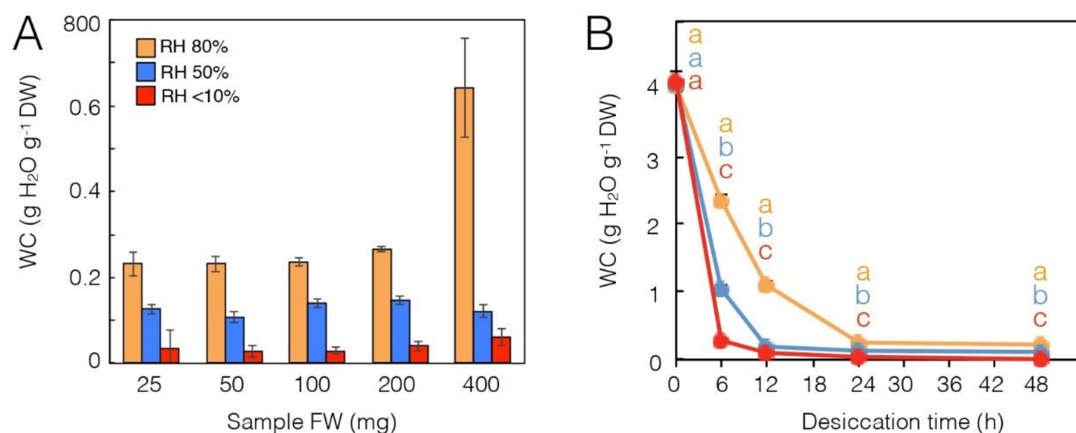

Fig. 4.3. (A) Effect of the initial amount of plant material (*Syntrichia ruralis*) placed in the tube (in mg FW) on the final tissue water content (g H_2O g^1 DW) after 24 h of desiccation under the three RHs tested. (B) Effect of the time of desiccation on tissue water content (g H_2O g^{-1} DW) under the three RHs tested for a sample with initial FW of 200 mg. Means ± SE are shown (n=3).

4.3.2 Case study I: bryophytes with contrasting responses to desiccation

Initial values of F_v/F_m were close to or higher than 0.7 in most of the bryophyte species, except in two FDS species (*Marchantia polymorpha* and *Hookeria lucens*; Table 2). Initial F_v/F_m values were, on average, slightly and significantly lower in FDS than in FDT species: 0.659 ± 0.026 and 0.722 ± 0.013 (average ± SE, respectively). The final WC reached after desiccation treatments did not differ significantly when comparing FDT and FDS species (Fig.4. 4A) being, on average (± SE), 0.22 ± 0.06 g H_2O g^{-1} DW at RH ≈ 80%, 0.08 ± 0.00 at RH ≈ 50%, and 0.02 ± 0.00 at RH <10%. In contrast, final RWC was slightly but significantly higher in the FDT than in the FDS species at the end of the dehydration treatments (Fig. 4.4B). The F_v/F_m recovery rate after rehydration clearly discriminated between FDS and FDT species at all RHs, being on average 18% for DS and 96% for DT species (Fig. 4.5). FDS bryophytes only showed an intermediate recovery (49%) under the mildest dehydration treatment (RH≈80%; Fig. 4.5). When considered individually, and for the RH≈80% only, some of the FDS species showed a $\%Fv/Fm_{NaCl}$ higher than 30%: *F. antipyretica*, *H. lucens*, *L. cruciata*, *S. undulata* (Table S4.2).

4.3.3 Case study II: tracheophytes from different worldwide origins

Initial values of F_v/F_m showed no significant differences between DT (0.736 ± 0.025 on

average) and DS (0.757 ± 0.050) tracheophyte species (Table 4.2). The strength of the different desiccants was strongly related with both WC and RWC (Fig. 4.4). No differences in WC or RWC were observed between DS and DT species (Fig. 4.4A). On average, dehydration treatments resulted in 29.6% RWC at 80%RH, 16.4% at 50%RH and 8.4% at RH <10% (Fig. 4.4B). DT species yielded higher F_v/F_m recoveries after dehydration than DS species, independently of the treatment applied (Fig. 4.5). On average, recovery was 58% in DT and 24% in DS species.

Table 4.2: Control values of Fv/Fm (after 24 h hydration) in the studied bryophytes (Case study I) and tracheophytes (Case study II). Means ± SE (n = 9).

Bryophytes, *Case study I*			
DS species	**Fv/Fm**	**DT species**	**Fv/Fm**
F. antipyretica	0.69±0.01	*P. formosum*	0.77±0.01
M. polymorpha	0.57±0.01	*P. platyphylla*	0.72±0.01
H. lucens	0.64±0.01	*S. ruralis*	0.71±0.01
L. cruciata	0.70±0.01	*R. punctatum*	0.70±0.01
S. undulata	0.69±0.01	*F. tamarisci*	0.70±0.01

Tracheophytes, *Case study II*			
DS species	**Fv/Fm**	**DT species**	**Fv/Fm**
V. erioloba	0.81±0.00	*A. sinuata*	0.83±0.00
A. lygodiifolia	0.76±0.00	*B. purpurea*	0.61±0.00
B. magellanicum	0.79±0.01	*B. hispida*	0.83±0.01
B. albitrunca	0.71±0.03	*C. eatonii*	0.83±0.00
D. canariensis	0.65±0.04	C. officinarum	0.83±0.00
D. austroafricana	0.75±0.01	*C. plantagineum*	0.70±0.01
E. desvauxii	0.61±0.02	*E. nindensis*	0.64±0.01
H. annuus	0.80±0.00	*H. rhodopensis*	0.77±0.01
J. oxycedrus	0.75±0.01	*H. cruentum*	0.77±0.01
M. oleoides	0.74±0.01	*H. dentatum*	0.73±0.01
M. caryophyllacea	0.65±0.04	*M. caffrorum*	0.53±0.04
O. squarrosa	0.66±0.03	*M. flabellifolius*	0.42±0.03
Q. ilex	0.77±0.01	*R. myconi*	0.77±0.01
T. aestivum	0.75±0.01	*X. viscosa*	0.61±0.01

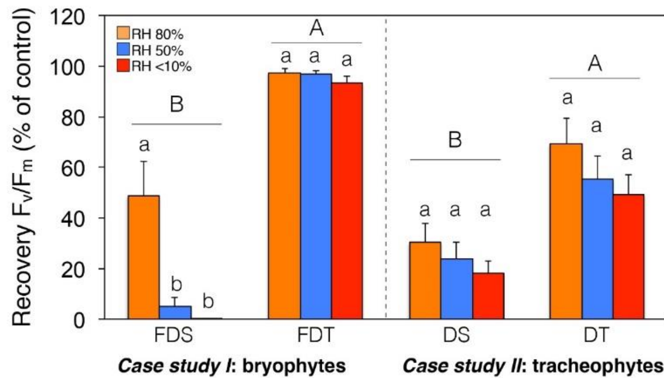

Fig. 4.5. Average recovery of F_v/F_m (% of control values) under the three RHs tested in the FDT and FDS bryophyte (Case study I) and in the DT and DS tracheophyte (Case study II) species (see Table 1). Each bar shows the mean ± SE (n=5 species for bryophytes and n=14 for tracheophytes). Lowercase letters above the bars indicate significant differences among treatments. When significant, differences between FDT and FDS (Case study I) or between DS and DT (Case study II) are depicted with capital letters (P<0.05).

4.3.4 Overall evaluation of DT

The average value of F_v/F_m after rehydration (used as a proxy for DHT) was calculated for each species. A value ≥30% F_v/F_m recovery was the threshold that best discriminated between FDT and FDS in the case study I and between DT and DS in the case study II (highlighted in Fig. 4.6), and this was improved further when only samples that had desiccated at RWC≤30% were included in the F_v/F_m average calculation (Fig. 4.6b, Table S4.3). The difference between DS and DT was clearly delineated by a gap of between 25 and 92% among bryophytes (Fig. 4.6). In contrast, recovery values represented a continuum among tracheophytes, ranging from 0% in *Helianthus annuus* to 96% in the filmy fern *Hymenophyllum dentatum* (Fig. 4.6). Interestingly, several DT-ferns yielded the highest recoveries, whereas two angiosperm 'resurrection plants' (*X. viscosa* and *Barbacenia purpurea* Hook.) presented very low recovery values within the DT group. This was particularly evident in *B. purpurea* (with 9.3% F_v/F_m recovery, which was similar to the lowest values obtained in DS species) when compared to the average of the remainder of DT angiosperms studied (37.9% F_v/F_m recovery). This is likely due to the fact that these two species are poikilochlorophyllous and thus take longer to recover photosynthesis. Conversely, four DS species, namely the angiosperm *Triticum aestivum*, the ferns *Davallia canariensis* and *Blechnum magallanicum,* and the gymnosperm *Juniperus oxycedrus* presented recoveries slightly above 30% (Fig. 4.6A). Only *J.*

oxycedrus maintained this trend, when samples that dried at ≥30% RWC were not included in the F_v/F_m recovery calculation (Fig. 4.6B).

When all species among each group were considered together, the Falcon test was able to discriminate between FDT and FDS among bryophytes and between DT and DS species among tracheophytes (Fig. 4.5). Nevertheless, the average F_v/F_m recovery was much higher in FDT bryophytes than in DT tracheophytes (Figs 4.5 and 4.6). Even the highest values of recovery among tracheophytes, observed in ferns, were lower than the lowest values in FDT bryophytes (Fig. 4.6). This occurred despite that WC and RWC values at the end of the desiccation treatments were remarkably lower (around 4-fold) in the bryophytes for all RHs (Fig.4. 4, Tables S4.2 and S4.3).

4.4 Discussion

The method described here, based on different rates of desiccation and equilibrium at different water contents, was able to (1) quantitatively categorise species based on their photosynthetic tissue tolerance to dehydration and (2) discriminate DT species by the threshold value of 30% recovery of initial F_v/F_m, with a few exceptions, notably those DT angiosperms displaying a poikilochlorophyllous strategy. This is very likely because poikilochlorophyllous species take a prolonged time, some 48 h beyond full hydration, to restore the photosynthetic apparatus. Such species break down thylakoid membranes and chlorophyll during dehydration and thus require some time to reconstitute this apparatus to reflect full photosynthetic capability (Farrant *et al.,* 2017). Recovery times longer than 48 h might have thus increased the accuracy of the Falcon test in detecting DT-poikilochlorophyllous species. Nevertheless, poikilochlorophylly represents a minority strategy among DT plants, and the use of much longer incubation times of the detached tissues may have also increased the rate of false negatives among homoiochlorophyllous species. Homoiochlorophyllous plants are able to reinstate photosynthesis more rapidly upon rehydration, as they retain and protect their photosynthetic apparatus (i.e. all DT-bryophytes evaluated in this study). Thus, the Falcon test overall presents a good balance between feasibility, simplicity, portability, broad screening possibilities, and accuracy.

When only two desiccants (NaCl and silica gel) were included in the DT evaluation (data not shown), the results closely matched those using three desiccants. From this perspective, the protocol could be simplified by removing the intermediate desiccant ($MgCl_2$, RH=50%). However, this third desiccant provides a “fine-tuning” that allows for

the discrimination of "intermediate" species, that is, with different DHT within the DT group. As an example, some FDT species, such as *Rhizomnium punctatum*, failed to show a complete recovery (under the rehydration conditions of the test) after desiccation at RH <10%, while some FDS species showed a certain degree of recovery after being equilibrated at RH = 80% (i.e., *F. antipyretica*, *H. lucens*, *L. cruciata*, *S. undulata*; Table S2).

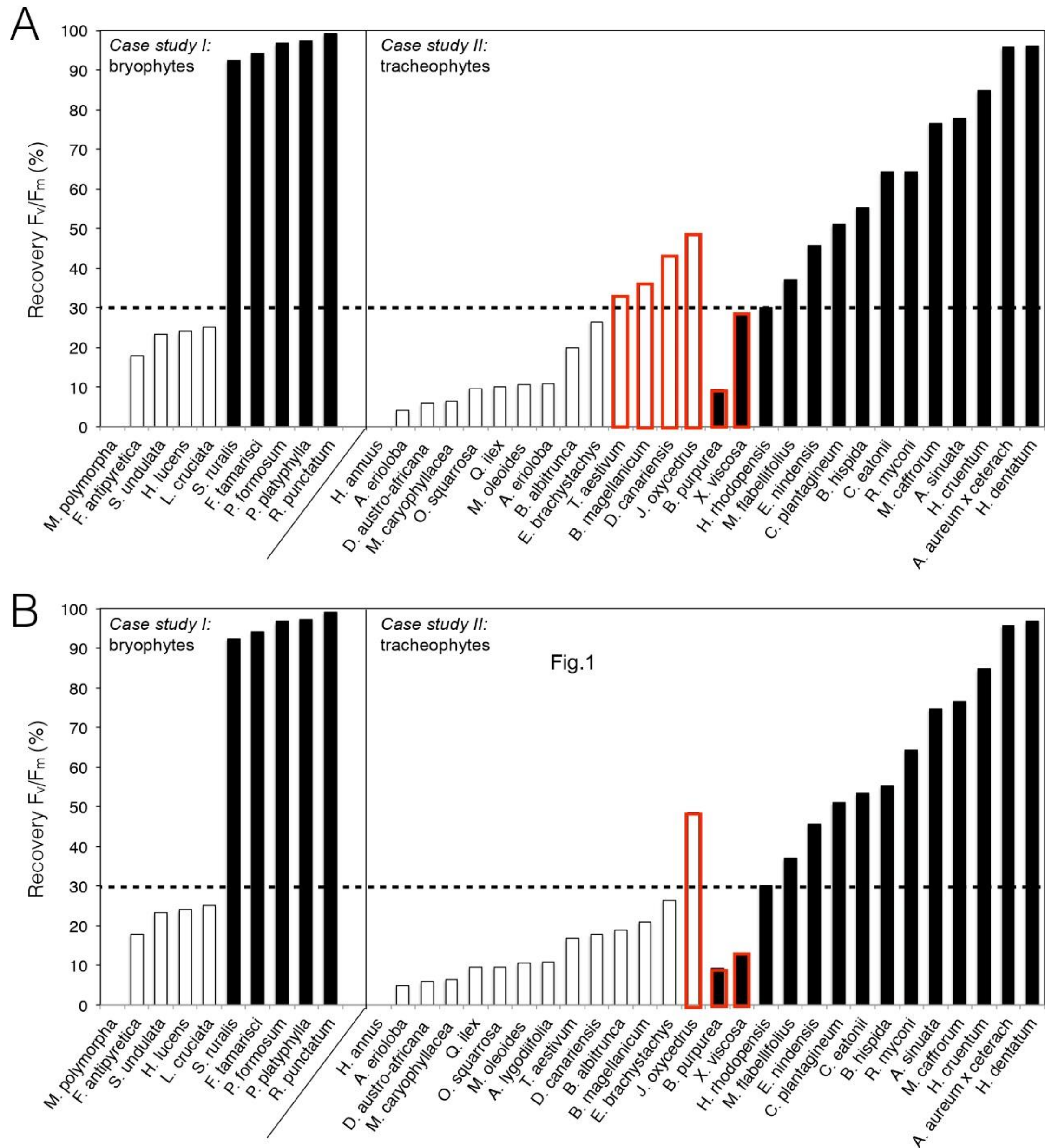

Fig. 4.6. (A) Average recovery of F_v/F_m after rehydration of each species. (B) Average recovery of F_v/F_m after rehydration of each species estimated by using only those samples that achieved RWC ≤30% upon desiccation. (F)DS species are depicted as white bars and (F)DT as solid bars. For further details regarding

the species see Table 1. Each bar shows the mean of the three desiccation treatments for each species (n=9). F_v/F_m recovery for *M. polymorpha* and *H. annuus* was 0%. Bars highlighted in red depict the species erroneously classified by the Falcon test according to the currently available literature (see Table 1).

4.4.1 The effects of acclimation

The environmental conditions prior to sampling may create a “historical stress memory” that can persist for several days, thereby shifting the speed or extent of the desiccation that a species can tolerate (Stark *et al.,* 2013a). Thus, some DS species, typically bryophytes and ferns, may activate inducible DT mechanisms in response to dry conditions (Cruz de Carvalho *et al.,* 2011), while typical DT species may suffer a de-acclimation to DT when artificially kept under conditions of suprasaturation (Stark *et al.,* 2017b). Conversely, time-lags for the reestablishment of photochemical activity after moistening can be much longer for DT angiosperm species collected from arid habitats or during dry periods (Lidén *et al.,* 2010, Proctor 2010). To minimize such effects, in the present study, bryophytes were collected in autumn during a rainy period. However, this is a factor that should be always taken into account in field studies. Regardless, the preliminary tests in repeated species under both field and laboratory conditions suggest that the results of the Falcon test reasonably reflect the inherent and species-specific character of DT. As an example, despite the initial F_v/F_m value of *M. caffrorum* collected from the field (0.53, Table 2) being below the optimum recorded in the bibliography for this species (around 0.75 was reported in Farrant *et al.,* 2009), it was correctly categorised as DT by the Falcon test (Fig. 4.6). Thus, a comparison of the final F_v/F_m values recorded for each species with those obtained initially provides a relatively reasonable buffering for slight field-acclimation effects. In addition, the comparison of initial F_v/F_m values to those found in the literature for the same species (when available) can provide an indication of the potential acclimation factors involved in the photochemical stage of the studied specimen. A more complex version of the test that focuses on species-specific plasticity in response to environmental conditions could be developed in future studies.

4.4.2 False negative and false positive exceptions

While the protocol described here was able to discriminate, without exception, between FDT and FDS bryophytes, there was some degree of overlap in the case of tracheophytes. In this group, two main error types may affect the sensitivity of the method to discriminate

between DT and DS species: (1) the occurrence of false positives (i.e., species that show apparent recovery of F_v/F_m being severely damaged by desiccation) and (2) the occurrence of false negatives (i.e., species that do not recover F_v/F_m after the experimental desiccation/rehydration described here, but that are able to survive desiccation under natural conditions). False positives may occur in those species, such as spinach, that once dehydrated are able to maintain charge separation in PSII and a reduction in the primary electron acceptor of PS II (Q_A), but not the functionality of the complete electron transport chain (Heber and Shuvalov 2005, Kopecky *et al.,* 2005). Another source of false positives would be xerophytic species (sclerophyllous or succulent plants) that, because of their leaf architecture, would require more time to achieve equilibrium with the desiccating atmosphere. This error can be avoided by including only species that have been desiccated below a certain threshold of RWC around 30%, which corresponds to the boundary between dehydration and desiccation described by Zhang and Bartels (2018) based on physiological and molecular changes in the tissues, and indirectly suggested in previous works (Farrant and Moore 2011, Ginbot and Farrant 2011, Farrant *et al.,* 2015). Indeed, when we applied this criterion to our results, we obtained 90% success within tracheophytes and 93% overall success in the classification of DT within the analysed species (Fig. 4.6B). Tracheophytes assessed in our study were dried out ≤ 30% on average (Fig. 4.4). However, some of the analysed species, such as *Blechnum magellanicum*, *Davallia canariensis,* and *Triticum aestivum,* maintained RWC ≥ 30% under the NaCl desiccation treatment. This may explain their average F_v/F_m values being over 30% (Fig. 4.6) and reinforces the usefulness of a threshold of 30% RWC to truly distinguish DT from merely DHT plants (Fig. 4.6B). This boundary RWC, however, may be slightly wider or different among species and for DT photosynthetic organisms other than angiosperms (Zhang and Bartels, 2018).

False negatives can occur in species that require a method (or time) of rehydration different to that reported here for a complete recovery. This can be the case in tracheophytes that need to regain water through the xylem to achieve a safe and organised leaf unfolding (Vicré *et al.,* 2004). In agreement with this, *M. flabellifolius*, one of the DT angiosperms with a lower recovery in our study (37%, Fig. 4.6), is a woody plant with a more complex requirement for xylem refilling and leaf unfolding than herbaceous plants (Wagner *et al.,* 2000). A specific rehydration method would also be needed in the case of poikilochlorophyllous species, where a longer time or even a proper light/dark

photoperiod might be required for a full restoration of the photosynthetic capacity even after full rehydration is achieved (Sherwin and Farrant 1996, Pérez *et al.,* 2011). This is in agreement with the low recovery values found under the "Falcon test" experimental conditions in the poikilochlorophyllous species *X. viscosa and B. purpurea* (Fig. 4.6). The last case of false negatives related to an inappropriate rehydration method could be relevant for species, such as *Sporobolus stapfianus*, in which only intact but not detached leaves are tolerant to desiccation (Gaff and Loveys 1992, Whittaker *et al.,* 2004). However, this is not known to be a common phenomenon. Despite the limitations of our method, no false negatives were encountered in bryophytes, where all species previously described as FDT recovered to values higher than 90% F_v/F_m. We did not detect any false positives in bryophytes, as all species described as FDS recovered less than 25% of the control F_v/F_m (Fig. 4.6). Additionally, the low recovery values obtained at 80% RH can indicate DS in species such as *M. polymorpha,* which, in contrast to others (e.g. *F. antipyretica*), was unable to recover even following less severe dehydration (Table S4.2).

4.4.3 Is the method useful for tracheophytes?

For tracheophytes, F_v/F_m recovery values showed a continuum from DS species belonging to different phylogenetic lineages to DT ferns. Some DS angiosperms gave values close to those of DT angiosperms, implying the risk of finding false positives, while a couple of resurrection plants gave false negatives, as the abovementioned case of *M. flabellifolius*. Overall, the thresholds of ≤ 30% RWC achieved upon desiccation and ≥ 30% recovery of average F_v/F_m upon rehydration allowed for the establishment of a clear separation between DT and DS. As originally proposed, this method is not fully precise, but the proportion of false positives or negatives appears to be very low (3 species out of 38≈ 7% error, Fig. 4.6B), which favours its use as a course, portable method for rapid and wide screening under remote field conditions.

If we assume that DT is a complex phenomenon resulting from the interaction of constitutive and inducible processes (Carvalho *et al.,* 2014), the recovery rates observed using the proposed test are mostly reflecting the constitutive component of the DT strategy. Interestingly, when focusing on tracheophytes only, some of the highest F_v/F_m recovery values for DS plants were obtained in gymnosperms, and in ferns for DT plants. High resistance to cavitation in both groups (Pittermann *et al.,* 2011) may be part of their distinguishable response to dehydration/rehydration cycles.

In addition to the abovementioned advantages, the method can be used with comparable precision both in the laboratory and in the field, as demonstrated with the case study II, provided some precautions are taken. Two limitations of biological field studies include (1) the maintenance of experimental conditions within the desired ranges and (2) the availability of scientific instruments. Here, we were able to generate desired RHs inside the Falcon tube atmospheres by absorbing saturated salt solutions in sponges while at the same time preventing accidental moistening of samples during transportation and handling. Falcon tubes prepared in this manner can be re-used at least three times without changes in the RHs. Additionally, the method can be adapted for use in remote locations due to minimal instrumentation requirements: a scale with at least three decimal digits (mg range), a box with some Falcon tubes (that must be incubated in darkness), a portable chlorophyll fluorometer, and an enclosed space, such as a building or car, where temperature can be maintained within a reasonable range of 20±5ºC (or even ±10ºC). Provided these instrumental requirements are met, this method allows for the exploration of remote biomes in instances where technical facilities of any type are unavailable. Furthermore, once back in the laboratory, biological data can be completed by biochemical analyses of samples collected in the field and preserved in silica gel (Esteban *et al.,* 2009), or by morphological studies of tissues fixed in situ as previously described for several species of different groups (Tosens *et al.,* 2012, Carriquí *et al.,* 2015).

4.5 Conclusion

The protocol described here was able to discriminate between DT and DS bryophytes and quantitatively classify DHT within tracheophytes. Awarding that RWC ≤ 30% is reached during the three desiccation treatments and an average recovery value of $F_v/F_m \geq 30\%$ is achieved upon rehydration, the Falcon method appears as a relatively coarse but reliable and highly portable procedure for the rapid and wide screening of DHT, which could be used even under remote field conditions in both non-vascular and tracheophyte species.

Author contributions

J.I.G-P. and B.F-M. conceptualised the study and initiated the protocol set up. JMF advised on choice of DT angiosperm plants. M.L-P., M.C., A.P-C., E.N-O., JMA, A.H., U.A., J.I.G-P. and AV conducted the bryophyte experiment. M.L-P. was responsible for the bryophyte experiment. J.F., Ja.G., M.N., J.B., Jo.G., M.J.C-M. conducted the

tracheophyte experiment. B.F-M., A.P-C. and M.N. performed the statistical analyses. B.F-M. and J.I.G-P. drafted the manuscript. All co-authors contributed to the final version of the work.

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CHAPTER V

UNRAVELING METABOLIC MECHANISMS BEHIND CHLOROPLAST DESICCATION TOLERANCE: CHLOROPHYLLOUS FERN SPORE AS A NEW PROMISING UNICELLULAR MODEL

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Unraveling metabolic mechanisms behind chloroplast desiccation tolerance: chlorophyllous fern spore as a new promising unicellular model

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Abstract

Fern spores are unicellular structures produced by the sporophyte generation that give rise to the haploid gametophyte. When released from the sporangium, spores are desiccation tolerant (DT) in the royal fern (*Osmunda regalis*) and contain fully developed chloroplasts. As a consequence, this type of spores is called chlorophyllous spores (CS). Upon transfer to germination conditions, CS initiate a process of imbibition that suppresses DT in 72h, before the germination starts. In parallel to such change in DT, thylakoids undergo a profound remodelling in composition and function. Firstly, sustained quenching of chlorophyll fluorescence is relaxed, giving rise to photochemically active CS, while lipid composition shifts from that of a resting structure to a metabolically active cell. Basically trigalactolipids decreased in favour of monogalactolipids, with a parallel desaturation of fatty acids. Storage lipids such as triacylglycerol were quickly depleted. These results highlight the importance of the structure of thylakoids lipid as a key to protect membrane integrity during desiccation, together with the saturation of fatty acids and the constitutive chlorophyll quenching to prevent oxidative damage. The CS used here, in which the same cell shifts from DT to sensitive strategy in 72h, reveal their potential as unicellular models for future studies on DT.

Key words: galactolipids, germination, green spores, fatty acid, non-photochemical quenching, *Osmunda*, pteridophyte, photoprotection, thylakoid

Abbreviations

A, antheraxanthin; AZ//VAZ, de-epoxidation state of the xanthophyll cycle; β-Car, β-carotene; C, control; Chl, chlorophyll; CS, chlorophyllous spores; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; D, desiccation; D-H, dehydration-rehydration; DS. Desiccation sensitive; DT, desiccation tolerance; DW, dry weight; EW; equilibrium weight, FFAs, Free fatty acids; Fm, maximum chlorophyll fluorescence; Fo, minimum chlorophyll fluorescence; Fv, variable chlorophyll fluorescence; Fv/Fm, maximum photochemical efficiency of photosystem II; L, lutein; MGDG, monogalactosyldiacylglycerol; N, neoxanthin NPQ, non-photochemical quenching; NPQd, desiccation-induced quenching of chlorophyll fluorescence; PA, phosphatidic acid; PC, phospatidylcholine; PE, phospatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, total polar lipids; PSII, photosystem II; R, rehydration; RH, relative humidity; ROS, reactive oxygen species; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglycerol; TeGDG, tetragalactosyldiacylglycerol; TGDG, trigalactosyldiacylglycerol; V, violaxanthin; VAZ, violaxanthin+anteraxanthin+zeaxanthin; WC, water content; Z, zeaxanthin;

5.1 Introduction

Plant desiccation tolerance (DT) represents the ability of cells to re-establish physiological functions after drying below 0.1 g H_2O g^{-1}DW water content, or -100 MPa water potential (Alpert, 2005). This remarkable physiological strategy requires dealing with two major sources of damage linked to desiccation: mechanical and oxidative, being the latter particularly relevant in photosynthetic tissues (i.e. since photooxidation represents an additional source of injure). DT was probably essential for the water to land transition of plants (Oliver *et al.,* 2000). In fact, DT in photosynthetic tissues is much more frequent among basal plant lineages (mosses, liverworts) than in recently evolved groups (ferns) and is very rare among angiosperms (Gaff, 1977, Poremski, 2011, Gaff and Oliver, 2013, Fernández-Marín *et al.,* 2016, López-Pozo *et al.,* 2018). DT of photosynthetic tissues was progressively lost in parallel to the development of efficient mechanisms to deal with drought such as cuticles, stomata and vascular tissue (Gaff and Oliver, 2013). In parallel, the evolution of land plants was also characterised by a progressive substitution of the haploid gametophyte as the dominant generation (as is the case in mosses) by the sporophyte-dominated life-cycle that occurs in seed plants. Ferns

occupy an intermediate evolutionary position, with the co-existence of two independent generations: a multicellular gametophyte and a multitissular sporophyte. Fern gametophytes, resemble mosses in the sense that they lack mechanisms to prevent water losses and thus, are considered as poikilohydric (Kappen and Valladares 2007). On the other hand, fern sporophytes are homoiohydric (i.e. possess vascular tissue, cuticle and stomata with the exception of filmy ferns, Fam. Hymenophyllaceae, which lack stomata). Thus, to some extents, ferns summarize the evolution of land plants, and occupy an interesting intermediate phylogenetic position between mosses (with gametophyte-dominated generation and frequently DT) and angiosperms (with a sporophyte-dominated generation and only exceptionally DT).

The continuity between both fern generations occurs through the meiotic production of spores, which are released from the sporangia (in the sporophyte) and give rise to the new gametophytes. Fern spores are unicellular propagules, usually covered by a thick coat of cellulose and sporopollenin (Banks, 1999) and, frequently, DT (Ballesteros *et al.,* 2017). The spores of some fern species (i.e.: *Matteucia*, *Osmunda*, *Equisetum*, *Onoclea, Grammitis, Todea…*) contain chlorophyll (Chl) and well-developed chloroplasts in their mature stage, these are the so-called green fern spores or, more appropriately: chlorophyllous spores (CS) (Sundue *et al.,* 2011). It has been generally assumed that CS retain higher moisture content upon release from the sporangia and are overall more sensitive to desiccation than non-chlorophyllous fern spores (Pence, 2000). However, this distinction is not clear and recent studies have demonstrated that CS can also be remarkably DT (Ballesteros *et al.,* 2017, 2018). As germination is generally much faster in CS than in non chlorophyllous fern spores, it has been hypothesised that the presence of chlorophyll and operative chloroplasts may represent and ecological advantage during spore germination and initial stages of gametophyte development, due to autotrophic metabolism (Pence, 2000). The payback is a risk of photooxidative damage whenever light absorption is not properly coupled to energy use (Demmig-Adams, 1990). This is typically the case of desiccated photosynthetic tissues, when Chl molecules continue to harvest light but carbon assimilation is completely uncoupled (Farrant, 2007). Under these conditions, the absorbed energy can potentially activate atmospheric oxygen, acting as a continuous source of reactive oxygen species (ROS) (Heber *et al.,* 2006). In this context, fern CS that are tolerant to desiccation could be particularly vulnerable to photooxidative stress (Ballesteros *et al.,* 2018).

Nevertheless, photosynthetic tissues of DT plants display a complete set of photoprotection mechanisms that efficiently counteract photooxidative stress, playing thermal energy dissipation a central role among them (Heber *et al.,* 2006, García-Plazaola *et al.,* 2013, Fernández-Marín *et al.,* 2016, Verhoeven *et al.,* 2018). This mechanism consists on the reemission of the exceeded light energy as heat in the antennae complexes of the photosynthetic apparatus, and is associated with a decrease of Chl fluorescence yield, the so-called non-photochemical quenching (NPQ). Thermal energy dissipation also relates with the de-epoxidation of the so-called xanthophyll cycle (i.e. synthesis of the carotenoids antheraxanthin (A) and zeaxanthin (Z) from violaxanthin (V)) (Demmig-Adams *et al.,* 1995). The accumulation of zeaxanthin upon drying occurs in many taxonomically diverse DT organisms (Kranner *et al.,* 2002, Fernández-Marín *et al.,* 2009, 2010, 2011, 2013, 2018). Nevertheless, its relationship with desiccation-induced thermal dissipation (NPQd) is not clear and strong quenching of fluorescence independent of zeaxanthin has been described in DT-cryptogams (Bilger, 2014). All things considered, the strong and reversible quenching of chlorophyll fluorescence upon desiccation, seems to be an important protective strategy related to DT and absent in DS organisms (Bilger, 2014, Rakic *et al.,* 2015).

In addition to dissipation of excess energy, maintenance of membrane functionality (i.e. avoidance of adjacent membrane fusion) represents another fundamental process for survival upon desiccation (Hoekstra *et al.,* 2001). The polarity, the degree of unsaturation and the length of the fatty acid chains that constitute cell membranes, determine their biophysical properties, and thus, their capability to undergo reversible loss of water without irreparable injury to the cell (Hoekstra and Golovina, 1999). Thylakoids represent the vast majority of photosynthetic cell membranes and are mainly composed of galactolipids. Among them, mono- and di-galactosyldiacylglycerols (MGDG and DGDG, respectively) dominate in photosynthetically active membranes (Dörman, 2013). While the presence of MGDG and DGDG is essential for photosynthesis in vascular plants, it has been recently shown that their ratio to phospholipids and to oligogalactolipids (tri- and tetra: TGDG and TeGDG) declines upon dehydration in DT angiosperms (Gasulla *et al.,* 2013). In addition, the degree of fatty acid unsaturation increases under desiccation and seems to be higher in DT than in DS species (Koonjul *et al.,* 2000, Georgieva *et al.,* 2011, Tshabuse *et al.,* 2018).

These processes have been described in seeds or leaves of angiosperms. However, most studies on fern spores and their DT have been focused in terms of germplasm preservation, longevity and loss of viability (Mikula *et al.,* 2015, Ballesteros *et al.,* 2017, 2018), while the biochemical mechanisms behind DT of fern spores in general and of CS in particular are far from being understood (López-Pozo *et al.,* 2018). We used the royal fern (*Osmunda regalis* L.), which produces CS able to survive low moisture content (Mikula *et al.,* 2015), as model species. Specifically we aimed (i) to determine the extent of DT, and (ii) to underpin the physiological and biochemical mechanisms behind DT of fern CS which particular focus on photoprotection and lipid changes at thylakoid level. Additionally, and based on the fact that fern spores are frequently DT while most fern sporophytes are not (López-Pozo *et al.,* 2018), we evaluated biochemical changes occurring in the thylakoids during the initial stages of spore imbibition and gametophyte development, and their potential relationship to the loss of DT. As recently proposed, their unicellular structure that contains the genetic information of a whole tracheophyte organism, make fern spores a very interesting model system to study multiple cellular processes such as polar differentiation and molecular networks (Suo *et al.,* 2015), cell ageing processes (Ballesteros *et al.,* 2017), gravity responses (Salmi *et al.,* 2011) or cellular toxicity (Catalá *et al.,* 2009). In this work we add a step further and propose fern CS as a unique model to study physiological and biochemical mechanisms of acquisition and or loss of tolerance to desiccation in photosynthetic organs.

5.2 Materials and methods

5.2.1 Plant material

Plant material (mature fertile fronds) was obtained in spring 2016 and 2017 from adult individuals of *O. regalis* growing in the surroundings of the University Campus (UPV/EHU, Leioa, Bizkaia, País Vasco, 43°19'48.8"N 2°58'08.5"W).

After collection, fertile fronds were kept in laboratory at 60% relative humidity (RH) for 24 h to allow the dehiscence of the sporangia and the release of the spores. The spores were then immersed in liquid N_2 and stored at -80 °C until the beginning of the experiment. Before use, spores were defrosted in a water bath at 35 °C during 5 minutes (Pence, 2008).

5.2.1.1. Growing conditions for gametophytes

Mature spores of *O. regalis* were sown in 5.5 cm diameter Petri plates with 10 mL Dyer medium (1979) (with modifications described by Quintanilla *et al.,* 2000) solidified with agar at 1% and containing the fungicide Nystatin (100 U mL^{-1}) (Sigma-Aldrich, Madrid, Spain). Plates were maintained at 20 ± 2 °C with 12 h light /12 h dark cycles and a photosynthetic photon flux density (PPFD) of 70 µmol m^{-2} s^{-1} during 6 months, until fully developed gametophytes were observed. Gametophytes were carefully removed from the agar medium before starting the experiment mentioned above.

5.2.2. Experimental design

Two different experiments were performed as follows (Fig. 5.1):

Experiment 1: Desiccation tolerance in spores and gametophytes

To estimate the extent of DT in spores and gametophytes and find the most appropriate RH to work with for the following experiments, a desiccation-rehydration (D-H) cycle was performed under controlled conditions. 50 mg of spores directly obtained from mature sporangia and nine individuals (three per treatment) of cultivated gametophytes (see below), were equilibrated during 24 h inside hermetically closed chambers at 80%RH, 50%RH and 10%RH (see Fig. 5.1 for details). The big volume of the chambers (3L), in comparison to the small amount of plant tissue, prevented O_2 limitation during the incubation. Spores released from the sporangium without any desiccation treatment were considered as controls. Afterwards, rehydration was conducted by adding distilled water (1.3 µL mg^{-1} for spores) or by placing the gametophytes above wet paper. Then, both structures were maintained hydrated during 24 h. To estimate the effects of recurrent desiccation events, a second desiccation in silica gel (10%RH) was subsequently applied during 72 h to the spores only, due to the inability of the gametophytes to survive the first desiccation treatment (see results). Finally, a second rehydration was done with the same procedure mentioned above only for spores. All the treatments were performed in darkness. Chlorophyll fluorescence, pigments, water content (WC) and germination were measured after desiccation and rehydration in both cycles. The degree of DT was estimated by comparing Fv/Fm values (see details on Section 2.5.) of desiccation treatments versus controls.

Experiment 2: DT loss during spore germination

A second experiment was conducted to determine when the spores loose DT during the germination. For this, spores were placed over moistened Whatman paper (1825047, England) and kept in a growth chamber under appropriate conditions for germination (i.e.: at 100%RH to prevent desiccation, at 20 ± 2 °C and under a 12 h photoperiod at PPFD of 70 µmol m^{-2} s^{-1}). Spores were harvested at different times during germination (12 h, 24 h, 36 h, 48 h, 72 h, 120 h and 144 h) and then desiccated over silica gel for 24 h in darkness. To test their viability after desiccation spores were rehydrated again with distilled water in the same Whatman paper during 24h in darkness (Fig. 5.1). The percentage of germination and the Fv/Fm were assessed as estimators of viability after initial period of hydration (control) and after desiccation-rehydration times.

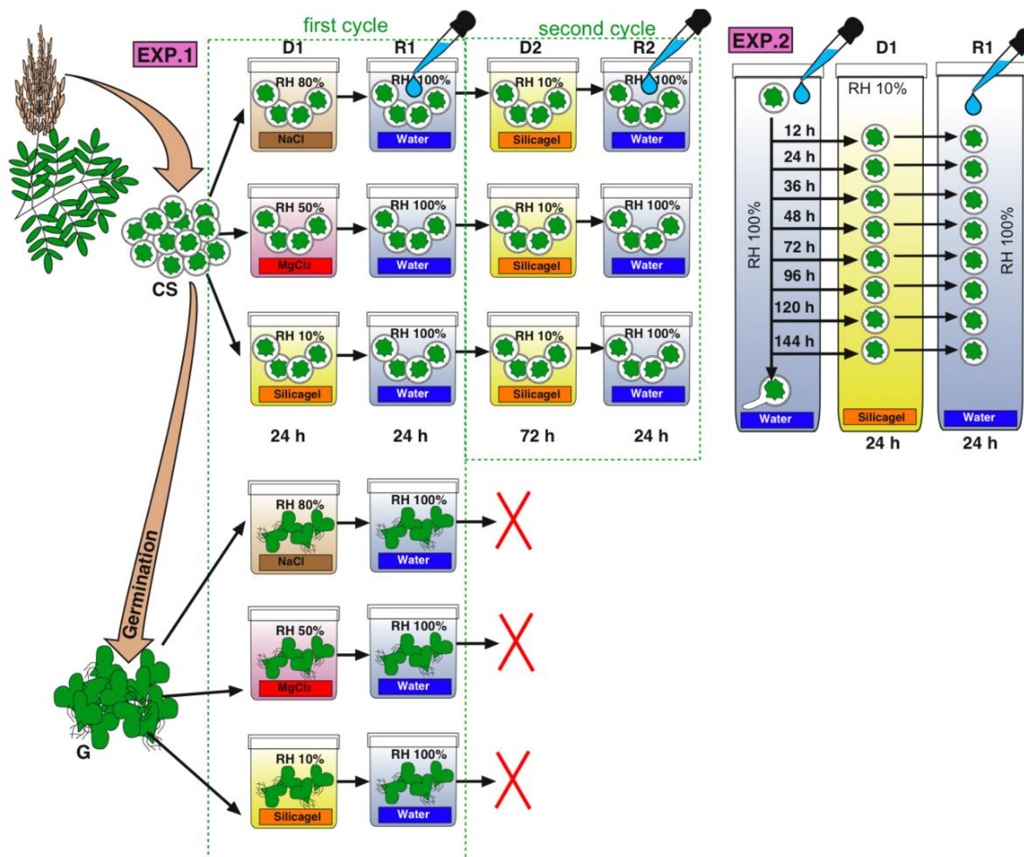

Figure 5.1: Experimental design: Experiment 1) desiccation tolerance in spores and gametophytes and Experiment 2) loss of desiccation tolerance in spores upon germination. Desiccations and rehydrations were conducted in darkness, while gametophyte growth and germination of spores were conducted under 12 h photoperiod. First desiccation (D1), first rehydration (R1), second desiccation (D2), second rehydration (R2), relative humidity (RH), magnesium chloride ($MgCl_2$), sodium chloride (NaCl), spores (S), gametophytes (G) (see Methods for further details).

5.2.3 Germination test

Viability of spores before and after dehydration cycles in experiments 1 and 2 was assessed testing the percentage of germination under the same conditions used for growing gametophytes. Germination percentage was examined microscopically 20 days after sowing in 100 randomly selected spores, being expressed as percentage of germination. The criteria for considering a positive spore germination was the first emergence of the rhizoid, when the spore coat breaks (Ballesteros *et al.,* 2011).

5.2.4 Desiccation methods

Desiccation was performed by equilibrating samples in hermetically closed chambers at three RHs. The lowest RH (10%) was achieved by equilibrating the chamber atmosphere with silica gel. Relative humidities of 80% and 50% were generated by equilibrating the atmospheres with salt solutions of NaCl and $MgCl_2$, respectively.

The WC of samples was estimated as follows:

WC=(EW-DW)/DW

Where EW is the weight at the equilibrium with RHs; DW is the weight after drying for 24-48 h in an oven at 70 ºC. Samples were weighed using a balance (AB104, Mettler Toledo, Barcelona, Spain) with 0.1 mg accuracy.

5.2.5 Chlorophyll fluorescence

Chlorophyll fluorescence was measured by two different techniques. Prior to measurements CS were always hydrated for 24 hours in darkness, so that NPQd was completely relaxed.

Experiments 1

Chlorophyll fluorescence in spores and gametophytes was measured using a Pulse Amplitude-Modulated Fluorimeter (PAM 2500, Walz, Effeltrich, Germany). For spores, measurements were done by entering the optical fiber inside the Eppendorf tube and for gametophytes an oblique leaf clip was used. This method allows maintaining the same distance (15 mm) between the optical fiber and the sample in all measurements. The minimum chlorophyll fluorescence (Fo) was determined in dark-adapted (30 min) gametophytes and spores. The maximum chlorophyll fluorescence (Fm) was induced with a saturating pulse for 500 ms. The variable chlorophyll fluorescence (Fv) was

calculated as Fm–Fo. The ratio Fv/Fm represents the maximum photochemical efficiency of photosystem II (PSII).

Experiment 2

To estimate the rate of recovery of individual spores the Imaging fluorescence-Microscope was used for experiment 2, which allows measuring individual spores. Pieces of Whatman paper containing spores were placed onto a microscope slide and acclimated to darkness during 30 minutes. Measurements were made with a Microscopy Pulse Amplitude-Modulated (PAM) imaging fluorimeter (WALZ, Effeltrich, Germany), coupled to Axiostar plus microscope (Carl Zeiss, Gottingen, Germany) with WinControl program for image processing software to control the timing, settings and trigger signals for the saturating pulse light resources and special detector-ocular microscope (Fernández-Marín *et al.,* 2012)

5.2.6 Determination of photosynthetic pigments

For analyses of chlorophylls, ~15 mg FW per replicate of plant material was used. Frozen samples were homogenized with a mortar in 1 mL of pure acetone solution buffered with $CaCO_3$. The extracts were centrifuged at 13 200 rpm for 20 minutes and syringe-filtered the supernatants. Extracts were injected (15 µL) in a reverse-phase Waters (Milford, MA, USA) HPLC system following the method of García-Plazaola and Becerril (1999) with the modifications described in García-Plazaola and Becerril (2001). PDA detector (Waters model 996) was used for measuring photosynthetic pigments in the range 250-700 nm, and peaks were detected and integrated at 445 nm for chlorophyll content. Pigments were identified and quantified by the method described by García-Plazaola and Becerril (1999). Retention times and conversion factors for pigments were the same as described by García-Plazaola & Becerril (1999, 2001). The de-epoxidation state of the xanthophyll cycle was estimated as (A+Z)/(V+A+Z) and abbreviated as AZ/VAZ.

5.2.7 Lipid extraction and quantification

Lipids for mass spectrometry were extracted according to Roughan *et al.,* (1978) and Gasulla *et al.,* (2013) with some modifications. Briefly, 15 mg of freeze-dried plant material per replicate were grinded with a Mixer Mill MM 200 (Retsch, Germany) with liquid nitrogen, at a frequency of 23Hz, during 5 min. A first extraction was done with 1 mL of $CHCl_3$: MeOH (1:2, v/v) and the organic phase collected. The lipid extraction was repeated two times with 1 mL $CHCl_3$: MeOH (2:1, v/v) and the organic phases were

combined. One volume of $CHCl_3$ and 0.75 volumes of aqueous 300 mM ammonium acetate were added to the combined chloroform extracts. Samples were vortexed and centrifuged (2000 g, 5 min). The organic phase was harvested. The solvent of the lipid extract was evaporated under a stream of N_2. Total lipids were dissolved in 1 mL of $CHCl_3$/MeOH (2:1, v/v), divided into two 500 µL aliquots and concentrated again by evaporation. Solid phase extraction of complex lipid extract on silica column and lipid quantification by Q-TOF MS/MS measurements was carried out according to Gasulla *et al.,* (2013).

5.2.8 Statistical analysis

Kolmogorov-Smirnov and Levene tests were used respectively to check for normal distribution and homogeneity of variance of the data. One-way analysis of variance (ANOVA), with Tukey as post-hoc, was used to check for significant differences among treatments. For non-normal data, Kruskal-Wallis test was applied. Statistical differences were considered at $p<0.05$. All analyses were performed using the SPSS 17.0 statistical package.

5.3 Results

5.3.1 Exp1: Desiccation tolerance in spores and gametophytes

Tolerance to desiccation at three different RHs of spores and gametophytes of *O. regalis* was estimated by measuring Fv/Fm (Fig. 5.2a,b). Spores equilibrated at 80 and 50%RH during 24h did not show differences on Fv/Fm respect to control, whereas at 10%RH Fv/Fm was significantly lower than the rest (Fig. 5.2a). On the other hand, gametophytes did not recover control values (0.515 ± 0.014) after any desiccation treatments at any RH (Fig. 5.2b), with no differences between treatments. Thus, the spores, but not the gametophytes, were able to recover from desiccation. Consequently, a second cycle of desiccation was conducted in spores only, at the most severe condition (10%RH), to evaluate the effect of recurrent desiccation cycles as it may happen in nature.

After second dehydration (D2), recovery of Fv/Fm fell significantly in all spores, accentuating differences among treatments. When all spores were kept in 10%RH, those previously exposed to the stronger desiccation (10%RH) showed the highest decline in Fv/Fm and germination percentage (0.378 ± 0.147 and 41.49 ± 2.77%, respectively),

although maintained the highest WC after this second desiccation. Thus, the lower RH was in D1, the worse was the recovery of Fv/Fm after D2.

Water content (WC) of spores equilibrated at 80%RH during 24 h was very similar to that of spores in their sporangial state, this is, control values (Fig. 5.2c). Thus, after 24 hours of desiccation under different RHs, WC decreased only in those spores exposed to 50%RH and 10%RH, reaching a final WC of 0.117±0.007 and 0.110±0.020 $gH_2O\ g^{-1}DW$, respectively. When the spores equilibrated at 80%RH and 50%RH during D1 were exposed to D2, WC values were even lower than after the first cycle. Interestingly, this was not the case of spores desiccated twice at 10%RH in silica gel, which presented the highest WC (Fig. 5.2c). In spite of the significant differences in WC between desiccation treatments, the Fv/Fm recovery did not show the same differences in mature spores.

Germination rate as a second estimate of spore viability did not decrease significantly after D1, regardless the final WC (Fig. 5.2d). Thus, this first desiccation process did not affect the germination percentage of mature spores or the Fv/Fm recovery (except for the 10%RH treatment) (Fig. 5.2a, c). Germination percentage showed the same tendency as Fv/Fm recovery data (Fig. 5.2a, d).

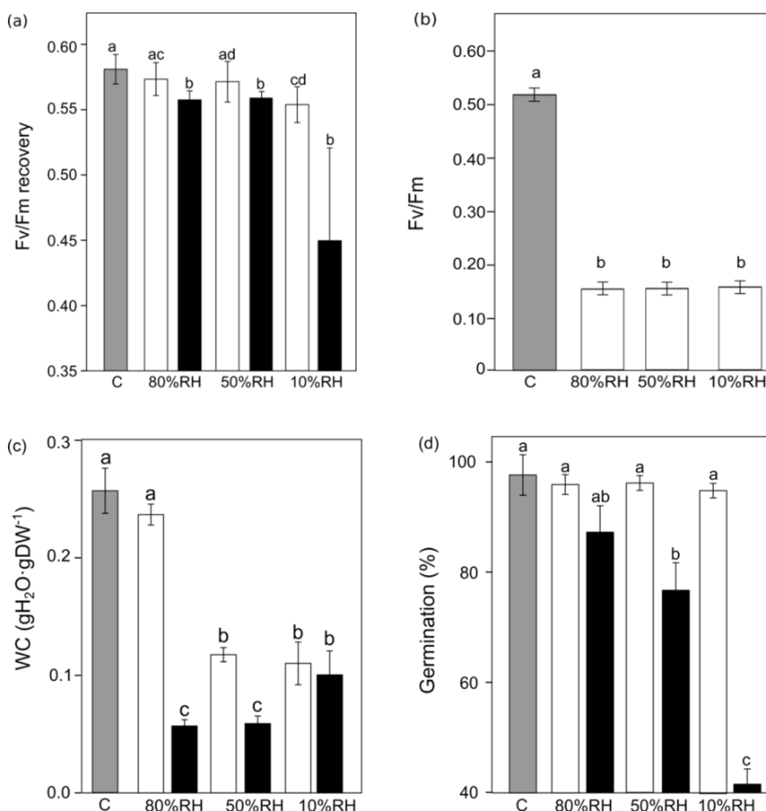

Figure 5.2: Estimation of tolerance to desiccation in spores and gametophytes of *O. regalis*. (a and b) maximum photochemical efficiency of PSII (Fv/Fm) spores and gametophytes respectively; (c) water content (WC), and (d) germination percentage of spores. The WC was measured after desiccation. The Fv/Fm values were obtained after rehydration: 24h of incubation under wet and dark conditions. Germination was estimated after 20 days of incubation under wet and 12h photoperiod. Two desiccation/rehydration cycles were performed in spores. First desiccation was performed at the indicated RHs while second desiccation was performed at 10%RH in all sample sets. Control values are indicated in grey bars. Measurements after first desiccation treatment (white bars) and after second desiccation (black bars) are shown. Each bar represents the mean±SE (n=5 for spores and n=3 for gametophytes). Different letters within each panel indicate significant differences among treatments ($P < 0.05$).

In parallel with the above mentioned changes in Fv/Fm of spores, the minimal fluorescence (Fo) changed dramatically during the course of the desiccation and rehydration treatments (Fig. 5.3). Thus, during the first desiccation, Fo was quenched with no significant differences among treatments and control values of spores. Similarly, Fo increased again in the first hydrated state, without differences between RHs. However, during the second desiccation Fo of the spores previously desiccated at 10%RH was not quenched to the same extent as the other treatments (Fig. 5.3). Notice the significant differences between D1 and D2 in panel (c), while no significant differences are found between D1 and D2 in panels (a) and (b).

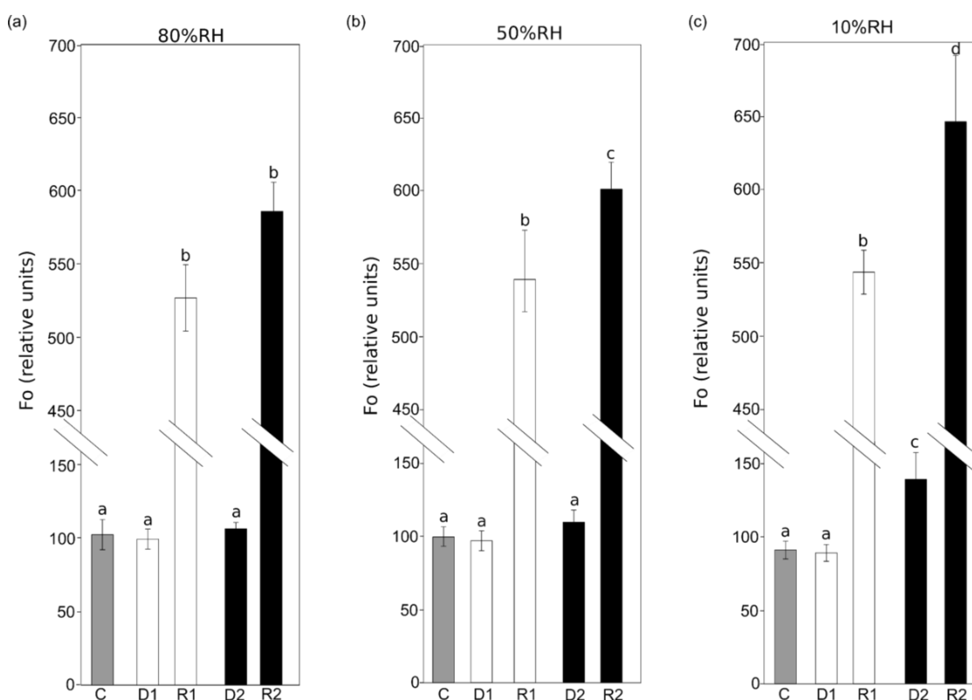

Figure 5.3: Fo of *O. regalis* spores at different points during two consecutive desiccation-rehydration cycles under different RHs. First desiccation was performed at the indicated RHs (80% in panel (a), 50% in panel (b) and 10% in panel (c)) while second desiccation was performed at 10%RH in all sample sets. Grey bars represent Fo control values (C). White bars represent Fo after first desiccation (D1) and rehydration (R1). Black bars represent Fo after second desiccation (D2) and rehydration (R2). Each bar represents the mean±SE (n=5). Different letters indicate significant differences among treatments (P < 0.05).

To evaluate whether fluorescence quenching was related with the operation of the xanthophyll cycle, the de-epoxidation state (AZ/VAZ) was assessed (Fig 4). Mature spores, in the control stage, showed an elevated AZ/VAZ ratio 0.597 ± 0.003 (Fig. 4). During desiccation treatments, no further de-epoxidation was induced in neither D1 or D2. Indeed, AZ/VAZ decreased in the subsequent cycles of dehydration-hydration performed in darkness, regardless of the extent of desiccation (Fig.5.4, compare panels a, b and c.)

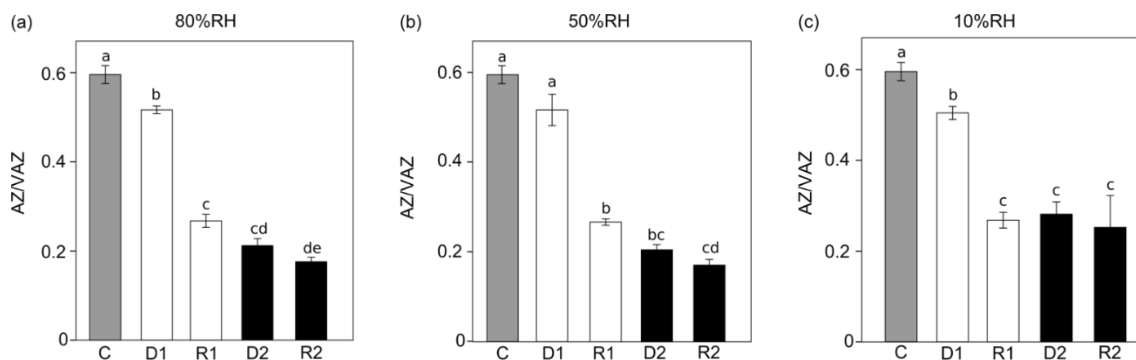

Figure 5.4: AZ/VAZ of *O.regalis* spores at different points during two consecutive desiccation-rehydration cycles under different RHs. Grey bars represent AZ/VAZ control values (C). White bars represent AZ/VAZ after first desiccation (D1) and rehydration (R1). Black bars represent AZ/VAZ after second desiccation (D2) and rehydration (R2). Each bar represents the mean±SE (n=5). Different letters indicate significant differences between treatments (P < 0.05).

Photosynthetic pigment composition was compared in spores and gametophytes of *O. regalis* (Table 1). Both generations contained the same six major carotenoids (neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin and β-carotene) but their ratios to chlorophyll were significantly higher in the gametophyte. The relative proportions among

some carotenoids also differed. Thus, the de-epoxidation level of xanthophyll cycle (AZ/VAZ) was higher in spores compared with gametophytes. The ratio Chl a/b was slightly, but significantly lower, in gametophytes than in spores (mean ±SE, Table5.1).

Table 5.1

Pigment composition (mmol mol^{-1} Chl) of mature spores of *O. regalis* in the control state (C) (non-manipulated) and of the 6-month old gametophyte (G). Values represent the mean±SE (n=5). Neoxanthin (N), violaxanthin (V), antheraxanthin (A), lutein (L), zeaxanthin (Z), β-carotene (β-Car), chlorophyll a/b ratio (Chl a/b). Values in the same row line with different letters are significantly different (P < 0.05). Values are means ± SE (n = 5).

	S	**G**
N	45.2 ± 0.6 a	54.0 ± 1.4 b
V	21.0 ± 0.4 a	30.2± 0.5 b
A	7.5 ± 0.2 a	5.2 ± 0.2 b
L	203.4 ± 1.9 a	118.9 ± 2.5 b
Z	23.5 ± 0.5 a	9.6 ± 0.3 b
β-C	54.8 ± 2.5 a	62.4 ± 0.6 b
VAZ	52.1 ± 0.8 a	45.0 ± 0.6 b
Chl a/b	3.1 ± 0.0 a	2.2 ± 0.0 b
AZ/VAZ	0.6 ± 0.0 a	0.3 ± 0.0 b

5.3.2 Exp 2: DT loss during spore germination

Based on the observation that the spores are DT but not the gametophyte that is formed after germination, it is obvious that the ability of surviving water loss, has to disappear at some point of this development. With the aim to determine when this capacity is lost, and which major changes in thylakoid membranes are associated to it, spores were incubated under germination conditions (to trigger germination and initial stages of gametophyte development) and their capability to withstand desiccation was assessed at different times

during 144 h. The response to a cycle of desiccation and rehydration of Fv/Fm and germination percentage along time (Figure 5.5) showed that spores of *O. regalis* became sensitive to desiccation 72h after incubation under germination conditions (e.g. at this time point: no spore was able to germinate after a desiccation treatment). Thus, 3 days of illumination and imbibition was the shortest time needed for loss of DT in 100% of *O. regalis* spores. No recovery after desiccation was possible beyond this time point (e.g. at ≥3d, Fig. 5.5).

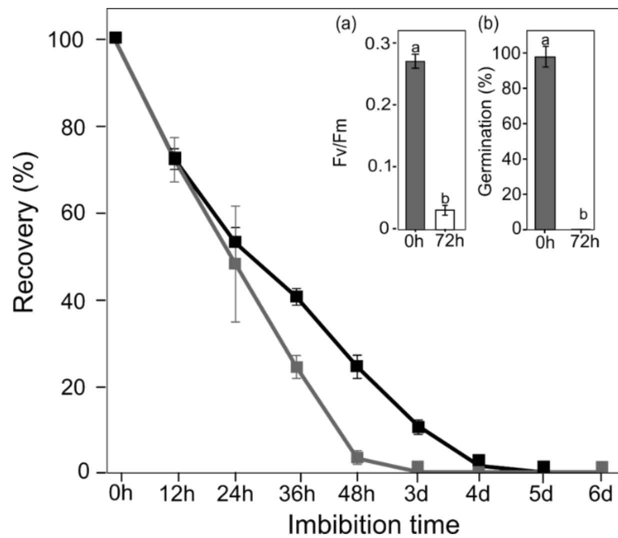

Figure 5.5: Fv/Fm recovery percentage (black line) and germination percentage (grey line) after a cycle of desiccation and rehydration in spores exposed for various times to germinating conditions (hydrated and illuminated) during 6 days. Fv/Fm (a) and germination percentage (b) after 72h of light and hydration. Control and recovery values. Each bar represents the mean±SE (n=3). Different letters indicate significant differences between treatments ($P < 0.05$).

To study whether changes in chloroplast lipid composition were related with DT, it was characterized in gametophytes and spores before and after DT loss. Total lipid amount, and composition varied greatly during germination of the spore and gametophyte development (Fig. 6). The content of TAG, the most abundant lipid (120 nmol mg^{-1} DW), decreased about 30 % after three days of germination and gametophytes only had 6 % of the initial amount. The analysis of TAG by Q-TOF MS/MS showed that 52:x (16:x/18:x/18:x) and 54:x (18:x/18:x/18:x) were the most abundant molecular species in

the spores, after hydration the amount of all of them started to decrease (Fig. S5.1). The absolute content of DAG increased from 7.9 nmol mg^{-1} DW in the spores to 16.9 nmol mg^{-1} DW 72 h after hydration, decreasing to 13.6 nmol mg^{-1} DW in the gametophyes (Fig. 5.6A). The slight accumulation of DAG was due to a raise in the proportion of 34:x (16:x/18:x) and 36:x (18:x/18:x) molecular species (Figure S5.1), which might be the result of the early TAG degradation. The total amount of polar lipids showed a 20 % raise, from 74.5 to 89.5 nmol mg-1 DW, after 72 h of hydration, and returned to spore levels in the gametophytes (Fig. 6a, b).

Regarding galactolipids, the percentage of MGDG in relation to total polar lipids increased from 35 % in spores to 43 % in hydrated spores and 47 % in gametophytes, whereas the percentage of DGDG remained constant around 24 % (Figure 5.6b). On the contrary, the amount of TGDG decreased rapidly during the first three days of germination, from 1.2 to 0.6 % (Figure 5.6d), its presence was residual in the *O. regalis* gametophyte.

The most abundant phospholipid in *O. regalis* spores was PC, representing the 14 % of total polar lipids, during germination it decreased to 12 %, dropping to 8 % in gametophytes (Fig. 5.6b). The proportion of PI also decayed significantly after 72 h of hydration, from 9 % to 5 %, and in gametophytes the proportion of PI declined to 3 % (Fig. 5.6b). The amount of PE was reduced 40 % in gametophytes respect to dried and hydrated spores (Fig. 5.6c). In these three phospholipids, the decreased was due to a decay in 34:x molecular species, 34:2 in PE and PI, and 34:3 in PC (Fig. S5.2 and S5.3). The percentage of PG remained constant during germination and gametophyte development (Fig. 5.6c). The only phospholipid which concentration increased was PA, its concentration was about two-fold higher in hydrated spores and gametophytes than in dried spores (Fig. 5.6c). The percentages of the different PA molecular species remained constant indicating that the increase in PA was caused by the accumulation of all molecular species. Finally, the proportion of SQDG did not change during *O. regalis* germination and gametophyte development (Fig. 5.6b).

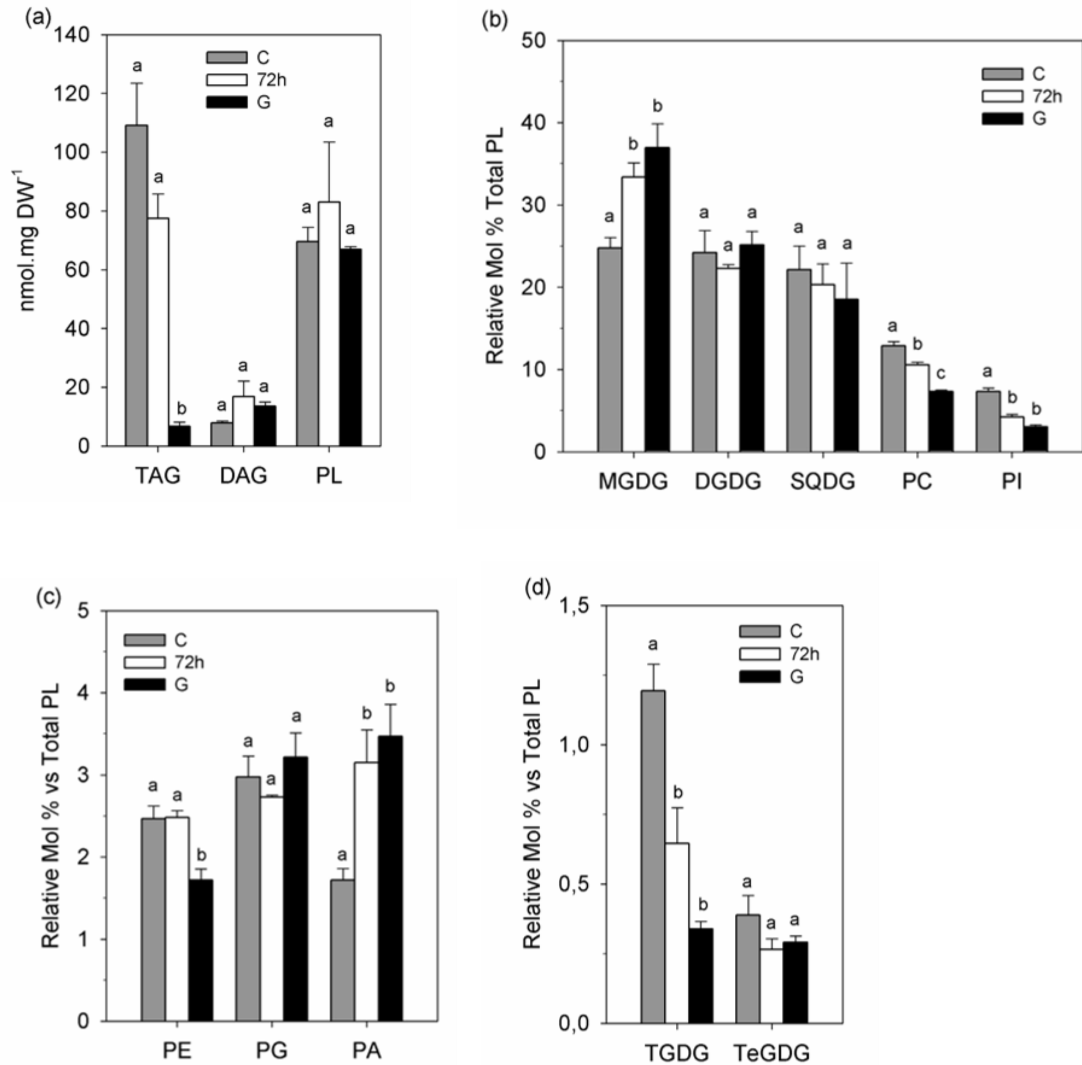

Figure 5.6. Changes in lipid composition during germination and development of *Osmunda regalis*: C) control values; 72h) spores after 3 days of light and hydration; G) 6 months gametophyte. (a) Amounts of the neutral lipids (TAG and DAG) and total polar lipids are expressed in nanomols per milligram of dry weight (a). Major (b), minor polar lipid (c) and oligogalactolipids (d) expressed as molar percentage relative to the total polar lipid amount. DW, dry weight. DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phospatidylcholine; PE, phospatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, total polar lipids; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglycerol; TeGDG, tetragalactosyldiacylglycerol; TGDG, trigalactosyldiacylglycerol. Values in the same row line with different letters are significantly different ($P < 0.05$). Values are means ± SE ($n = 3$).

The analysis of the molecular species of each glycolipid showed that there was a general replacement of low unsaturated by high unsaturated phospholipids and galactolipids during germination and, specially, in the gametophytes (Fig. S5.2 and S5.3). The calculation of the double bound index (DBI) confirmed this observation (Table 2). The

degree of unsaturation was higher in all phospholipids and galactolipids, with the exception of TGDG and TeGDG, which abundance was negligible in the gametophytes. In average, the DBI of polar lipids was 35 % higher in the gametophytes than in the spores. For neutral lipids, the unsaturation level remained constant in TAG, while the DBI decreased in DAG, being 30 % lower in gametophytes than in spores.

Table 2. Double bond index (DBI) of *Osmunda regalis* lipids in the initial control state (C), in spores after 72 h of light and hydration (72h) and in 6 months gametophytes (G). DBI = (Σ[N × mol% lipid])/2, where N is the total number of double bonds in the two fatty acid chains of each glycerolipid molecule. Values in the same row line with different letters are significantly different ($P < 0.05$). Values are means ± SE (n = 3).

	C	**72h**	**G**
MGDG	240.3 ± 1.8 a	235.7 ± 0.5 a	312.9 ± 26.4 b
DGDG	177.9 ± 10.1 a	189.6 ± 3.6 a	220.2 ± 3.6 b
SQDG	155.0 ± 1.4 a	165.1 ± 9.3 ab	198.6 ± 13.9 b
PC	156.4 ± 0.2 a	158.8 ± 0.8 a	200.8 ± 4.3 b
PI	120.9 ± 2.0 a	107.9 ± 1.3 a	142.9 ± 1.6 a
TGDG	229.4 ± 1.2 a	218.2 ± 3.4 b	225.2 ± 3.6 ab
TeGDG	229.6 ± 3.7 a	226.1 ± 3.9 a	226.7 ± 3.4 a
PE	156.4 ± 0.2 a	158.8 ± 0.8 a	200.8 ± 4.3 b
PG	115.6 ± 0.9 a	114.2 ± 3.6 a	161.7 ± 16.9 b
PA	167.2 ± 1.5 ab	156.0 ± 3.9 a	170.8 ± 3.9 b
PL	180.1 ± 3.1 a	189.5 ± 4.0 a	243.3 ± 11.0 b
TAG	140.7 ± 2.7 a	145.7 ± 1.0 a	166.5 ± 26.3 a
DAG	191.1 ± 4.1 c	156.7 ± 9.3 b	130.6 ± 1.9 a

5.4 Discussion

Pteridophytes occupy a key evolutionary position between bryophytes and angiosperms, showing an intermediate DT behavior (Oliver *et al.,* 2000). Thus, while bryophytes are considered "fully" DT plants, angiosperms are referred to as "modified" DT plants (Oliver *et al.,* 2000) with ferns showing characteristics of both groups. In addition, both DS and DT generations are observed in their life cycle, as a mixture between vascular and non-vascular plants (Farrar *et al.,* 2008). Thus, DT is considered to be common in fern gametophytes whereas the sporophyte is DT only in 10% of species (López-Pozo *et al.,* 2018). Additionally, ferns produce unicellular spores, which are DT in most, but not all, species (López-Pozo *et al.,* 2018). Then, they represent an excellent model to understand the mechanisms that allow plants to be DT. The species studied here, *O. regalis*, produces chlorophyllous spores that are DT but give rise to a DS gametophyte. This implies that, at a certain point of development, DT has to be lost. Overall, the spores of *O. regalis* represent a unique opportunity for the study of physiological processes behind the loss of DT in photosynthetic tissues.

The occurrence of DT within tracheophytes is widespread in reproductive structures, including non-green spores, while in vegetative tissues is much more uncommon (Bewley and Krochko 1982, Oliver and Bewley 1997). This is probably because chloroplasts are the main source of reactive oxygen species (ROS) upon desiccation, as light energy absorbed by chlorophyll greatly exceeds the capacity for photosynthetic use, leading to an unavoidable oxidative damage (Smirnoff, 1993). In the case of *O. regalis*, spore pigment composition was qualitatively undistinguishable from that of angiosperm leaves. In fact, the Chla/b ratio in spores was closer to that of tracheophytes (Esteban et al 2015) than in the gametophyte, which it resembled that of bryophytes (Table 5.1) (Marschall and Proctor 2004). When mature and hydrated, these spores were able to activate photochemical processes as shown by Fv/Fm values (Table 5.1, Fig. 5.2) which is in agreement with well-developed chloroplasts shown in other *Osmunda* species (*O. japonica*, *O. cinnamomea*, Takano and Inoue, 1992, Suo *et al.,* 2015).

Mature spores of *O. regalis* behaved as DT photosynthetic organs, since they were able to recover Fv/Fm after D-H cycles in which the WC (in % to DW) fell bellow 10 % (Fig 5.2). Not only the final water content, but also the drying speed, influence the recovery of photosynthesis, being generally fast drying more damaging (Proctor, 2003; Gasulla *et al.,* 2009; Pressel and Duckett, 2010; Stark 2017). This is probably the reason for the

reduced recovery when the spores were dried at the lowest RH (10%), and for the greater decrease on photochemical efficiency of PS II after the second D (Fig. 5.2). Interestingly, Fv/Fm recovery values correlated with germination percentage of spores (data not shown), which suggests Fv/Fm as a potential non-invasive marker of spore viability. This could represent an additional interesting advantage for monitoring of fern CS during storage, as it would avoid unnecessary use of samples for germination tests.

DT organisms possess a set of physiological tools to limit oxidative risk due to desiccation. One of the most widespread among DT organisms is a kind of sustained thermal dissipation of excess energy that results on a desiccation-induced quenching of chlorophyll fluorescence (NPQd) (Bilger, 2014). This mechanism has been reported in bryophytes, algae, cyanobacteria and lichens (Bilger, 2014), and until now was suggested to be absent in DT tracheophytes (Georgieva *et al.,* 2005). The NPQd mechanism consist in a strong decline in Fo in those DT organisms, whereas in DS Fo increases upon desiccation probably due to injury in PSII (Bilger, 2014). Our results with *O. regalis* spores, though, have revealed its occurrence in ferns too (Fig. 5.3). Interestingly, when the survival was affected by desiccation, the capacity of Fo quenching was lower (Figs 5.2, 5.3). In this study, the almost complete quenching of Fo that takes place upon drying in green spores of *O. regalis*, supports the activation of NPQd probably playing a fundamental role in the dissipation of light that, in absence of water, is always excessive (Fig 5.3). In agreement with such strong photoprotection capacity, the AZ/VAZ ratio is considerably higher in spores before hydrating them for the first time than in non-stressed vascular plants (Demmig-Adams, 1990). Although the presence of Z is not a prerequisite to the NPQd as shown by Herber *et al.,* (2010) in experiments in which its formation was inhibited, it has been demonstrated that Z contributes by NPQ-independent mechanisms to decrease the damaging effects of desiccation (Fernández-Marín *et al.,* 2013). Similarly, *O. regalis* spores activated NPQd without Z accumulation (Fig 5.4). Very likely the free fraction of Z molecules located within the thylakoid membrane (and not directly associated with photosynthetic protein complexes) play key roles in the stabilization and antioxidant protection of the photosynthetic membranes (Havaux and García-Plazaola, 2014; Dall'Osto *et al.*, 2010).

One of the most remarkable physiological changes described in this work is the loss of DT occurred within a single cell (i.e. CS of *O. regalis*) in the impressively fast of 72h during imbibition. (Individual spore lost DT in 12 hours but if we understand the spores

as a population, after 12 hours, the 75% of the population remains DT that is why we used the time 72h, where almost all the population is DS). Taking advantage of such a fast transition lipid composition was assessed before and after the DT-loss in *O. regalis* spores and in gametophytes too. Among the thylakoid lipids, the proportion of MGDG increased rapidly since the beginning of germination, while the concentration of DGDG, PG and SQDG remained constant (Fig. 5.6b). Several studies carried out with Arabidopsis mutants containing significantly less MGDG have demonstrated that this lipid is essential for the proper organization of the thylakoid membranes, for the optimal PS activity and for the correct function of the photoprotection mechanisms (Kobayashi *et al.,* 2007; Aronsson, 2008; Aronsson *et al.,* 2008). On other hand, a decrease in the concentration of MGDG is a common response of plants to different kind of dehydration stress like salt, freezing or desiccation (Moellering *et al.,* 2010; Gasulla *et al.,* 2013; Wang *et al.,* 2016). MGDG are cone-shaped lipids and trend to form inverted hexagonal II structures during water loss destabilizing the thylakoid membranes and causing their fusion or fission (Sprague, 1987; Webb and Green, 1991). The chloroplast of the spores also contained a relative high proportion of oligogalactolipids, especially TGDG. These galactolipids have been associated with the acquisition of DT because of their large polar-head that can enhance the repulsive forces between membranes avoiding their fusion when the intracellular water is lost (Gasulla *et al.,* 2013). In vascular plants growing under optimal conditions oligogalactolipids are completely degraded, like in *O. regalis* gametophyes, because their presence might result in inappropriate repulsive forces between neighboring membranes affecting the photosynthesis activity (to be demonstrated) (Chow *et al.,* 2005). Thus, the accumulation of MGDG and the degradation of TGDG in *O. regalis* during the gametophyte growth might be beneficial for improving the photosynthesis activity but, on the contrary, might compromise its survival in case of dehydration. In parallel, there was a decrease in the concentration of PI in the extraplastidial membranes during germination. The accumulation of PI has been related with the acquisition of desiccation-tolerance in photosynthetic organisms (Gasulla *et al.,* 2013; Gasulla *et al.,* 2016). Due to their sugar alcohol polar-head PI could play the same protective role that oligogalactolipids do in thylakoids, i.e., avoiding the fusion of extraplastidial membranes in dried cells. Consequently, the gradual decrease of PI concentration in *O. regalis* during germination might also reduce its resistance to desiccation.

The most abundant lipid in *O. regalis* spores was TAG but after 72 h of germination it started to be degraded and gametophytes only contained about 6 % of the initial concentration. In plants, TAG is accumulated forming lipid droplets, like those observed in *O. regalis* spores (Ballesteros *et al,* 2011). When needed, the cytosolic lipase SUGARDEPENDENT1 (SDP1) hydrolyzes the TAG into DAG and free fatty acids (Kelly *et al.,* 2013), which are broken down via β-oxidation in the glyoxisome into acetyl-CoA, a key metabolite for energy production via mitochondrial respiration and for the synthesis of carbohydrates (Graham, 2008).The mobilization and sequential hydrolysis of storage reserves has been found to be crucial in both chlorophyllous and non-chlorophyllous spores for the spore germination (DeMaggio and Stetler, 1985; Raghavan, 1992). The important decay of PC concentration after germination may also be related with the mobilization of the TAG reservoir. PC is the main phospholipid in the glyoxisomes and lipid bodies membranes, representing 58 % and 37 %, respectively (Moreau *et al.,* 1998). Thus a reduction in the size and number of the lipid droplets, and probably the associated glyoxysomes, might explain the decline in PC abundance during the development of *O. regalis* gametophytes. Another indirect consequence of TAG degradation may be the accumulation of PA. The DAG derived from SP1-mediated TAG hydrolysis can be phosphorylated by DAG kinase to synthesize PA (Katagiri *et al.,* 1996).

The spores of *O. regalis* showed a higher degree of lipid saturation than gametophytes, being the DBI of polar lipids 35 % higher in gametophytes than in the spores. The proportion of polyunsaturated fatty acids in membranes has been negatively related with the longevity of dried plant propagules (Hoekstra, 2005). Double bonds of fatty acid can be easily oxidized by free radicals because of their reactive hydrogen atoms (North *et al.,* 1994). Lipid peroxidation can result in membrane disruption or the oxidation can be propagated to other lipids or enzymes (Stark, 2005). In organisms containing chloroplast the risk of generation of free radicals is enhanced during drying in presence of illumination. Thus, in *O. regalis*, the low proportion of poly-unsaturated fatty acids could reduce the possibility of membrane damages caused by oxidation. Another advantage of having polar lipids with highly saturated acyl chains is that they decrease the fluidity of the membranes limiting the mobility of their components. Avoiding the access of enzymes -especially those with hydrolytic activity, like PLD- to the membranes is critical for the protection of membranes during desiccation (Chen *et al.,* 2018).

5.5 Concluding remarks

In the fate of climate change and the compromise with food security, the community of plant researchers have the duty to develop a new generation of stress tolerant crops. Resurrection plants have been considered for a long time as a promising resource for biotechnological applications (Zhang and Bartels 2017). However we are still far from understanding the intricate network of mechanisms involved in plant desiccation tolerance. With the present work we propose a new model for the study of these mechanisms in vascular plants: the chlorophyllous fern spores. With the advantage of using a unicellular tracheophyte system bearing fully developed chloroplasts, here we show the importance of thylakoid lipid remodelling and photochemical processes in the fast transition from a desiccation-tolerant to a desiccation-sensitive state in a same single cell. But this is just one among the many aspects of plant stress physiology that can be unravelled by the use of this unique structure.

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CHAPTER VI

DESICCATION TOLERANCE IN CHLOROPHYLLOUS FERN SPORES: ARE ECOPHYSIOLOGICAL FEATURES RELATED TO ENVIRONMENTAL CONDITIONS?

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Desiccation tolerance in chlorophyllous fern spores: Are ecophysiological features related to environmental conditions?

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Abstract

Fern spores of most species are desiccation tolerant (DT) and in some cases are photosynthetic at maturation, the so-called chlorophyllous spores (CS). The lifespan of CS in the dry state is very variable among species. The physiological, biochemical and biophysical mechanisms underpinning this variability remain understudied and their interpretation from an ecophysiological approach, virtually unexplored. In this study, we aimed at fulfilling this gap by assessing photochemical, hydric and biophysical properties of CS from three temperate species with contrasting biological strategies and longevity in the dry state: *Equisetum telmateia* (spore maturation and release in spring, ultra-short lifespan), *Osmunda regalis* (spore maturation and release in summer, medium lifespan), *Matteuccia struthiopteris* (spore maturation and release in winter, medium-long lifespan).

After subjection of CS to controlled drying treatments, results showed that the three species displayed different extents of DT. CS of *E. telmateia* rapidly lost viability after desiccation, while the other two withstood several dehydration-rehydration cycles without compromising viability. The extent of DT was in concordance with water availability in the sporulation season of each species. CS of *O. regalis* and *M. struthiopteris* carried out the characteristic quenching of chlorophyll fluorescence, widely displayed by other DT cryptogams during drying, and had higher tocopherol and proline contents. The turgor loss point of CS is also related to the extent of DT and to the

sporulation season: lowest values were found in CS of *M. struthiopteris* and *O. regalis*. The hydrophobicity of spores in these two species was higher and probably related to the prevention of water absorption under unfavourable conditions. Molecular mobility, estimated by DMTA, confirmed an unstable glassy state in the spores of *E. telmateia*, directly related to the low DT while the DT species entered in a stable glassy state when dried. Overall, our data revealed a DT syndrome related to the season of sporulation that was characterised by higher photoprotective potential, specific hydric properties and lower molecular mobility in the dry state. Being unicellular haploid structures, CS represent a challenge for germplasm preservation (e.g. as these spores are prone to photooxidation) but also an excellent opportunity for studying mechanisms of DT in photosynthetic cells.

Keywords

Desiccation tolerance, dynamic mechanical analysis, environmental conditions, glassy state, green spores, molecular mobility, tocopherol, water relations.

Abbreviations

AZ/VAZ, de-epoxidation state of the xanthophyll cycle; β-C, β-carotene; Chl, chlorophyll; CS, chlorophyllous spores; D1, first dehydration; D2, second dehydration; D, desiccation; DMTA, Dynamic Mechanical Thermal Analyser; D-R, dehydration-rehydration; DS, Desiccation sensitive; DT, desiccation tolerance; DW, dry weight; ε, elasticity modulus; *ETe, Equisetum telmateia*; EW; equilibrium weight, Fm, maximum chlorophyll fluorescence; Fo, minimum chlorophyll fluorescence; Fv, variable chlorophyll fluorescence; Fv/Fm, maximum photochemical efficiency of photosystem II; G´, storage modulus; *MSt, Matteuccia struthiopteris*; NPQd, desiccation-induced quenching of chlorophyll fluorescence; *ORe, Osmunda regalis*; PSII, photosystem II; P-V, Pressure-Volumen; R1, first rehydration; R2, second rehydration; R, rehydration; RH, relative humidity; ROS, reactive oxygen species; RWC_{tlp}, relative water content at turgor lost point; SWC, saturated water content; Tan δ, loss tangent; Tg, glass transition temperature; VAZ, violaxanthin+anteraxanthin+zeaxanthin; Ψ, water potential; Ψ_{o}, osmotic water potential; Ψ_{spo}, sporangial water potential; Ψ_{tlp} water potential at turgor lost point.

6.1 Introduction

The terrestrial atmosphere is, in general, an ambient desiccant and therefore, the organisms that inhabit here run the risk of drying out if they do not control water loss (Oliver and Bewley, 1997). To face this challenge, some plants have developed a survival strategy, known as "desiccation tolerance" (DT). This allows the recovery of full metabolism when the organisms are rehydrated after being desiccated to low water contents (WC), usually those below 0.1 g H_2O g^{-1} DW or those in equilibrium at relative humidity (RH) <50% (Proctor and Tuba, 2002, Leprince and Buitink, 2010; Fernández-Marín et al., 2016).

DT is frequent in reproductive structures (seeds, pollen and spores) (Gaff and Oliver, 2013), but its occurrence among vegetative photosynthetic tissues is much more limited (Fernandez-Marin et al., 2016). There is increasing recognition of variation in the responses of diverse plant reproductive structures to desiccation (e.g., Berjak and Pammenter, 2008; Franchi et al., 2011; Walters, 2015; López-Pozo et al., 2019). This variation has been mainly studied in seeds, where distinction between "fully" DT and desiccation sensitive (DS) seeds (commonly expressed in the duality orthodox vs. recalcitrant seeds) is complemented with the presence of seeds showing an intermediate tolerance to drying (Walters, 2015). In the case of other reproductive structures, such as spores, the same intermediate tolerance may be found. Ferns produce two different types of spores; chlorophyllous spores (CS) and non-CS. The former have fully developed photosynthetic machinery once mature, being able to start photosynthesis immediately once hydrated (Sundue et al., 2011, López-Pozo et al., 2019). The presence of chloroplasts, and hence, of chlorophyll, makes CS particularly sensitive to photooxidative damage (Ballesteros et al., 2018). During desiccation and in the dry state, CS must prevent light-chlorophyll interaction avoiding the production of reactive oxygen species (ROS) (Kranner et al., 2002; Ballesteros et al., 2018).

Fern spores are generally DT, but their lifespan in the dry state may vary among species, probably due to different extents of DT (Ballesteros et al., 2017). Notwithstanding the above, most of them are non-CS (López-Pozo et al., 2018). Spores are the main dispersal structure in ferns. Upon germination, spores give rise to the gametophytic generation where sexual reproduction occurs. Therefore, the ecological success of the pteridophytes will depend on the ability of their spores to disperse and survive–under various environmental conditions, with controlling water loss one of the most critical factors

(Page, 2002). To date, there are no studies on how fern spores survive natural environmental fluctuations (particularly moisture fluctuations), and less is known about the importance that some features can have in the temporal or spatial distribution of species.

The mechanisms that confer DT and longevity in fern CS (or other chlorophyllous reproductive structures such as chlorophyllous seeds) have been rarely studied and large gaps of knowledge still exist (Roqueiro et al., 2010; Ballesteros et al., 2018). For example, to our knowledge, only one study has investigated some molecular mechanisms of DT in CS. This study discovered the expression of late embryogenesis abundant (LEA) proteins during the maturation stage of the CS of *Onoclea sensibilis* in the sporangia and highlighted the role that these LEA proteins may have in the acquisition of DT and the survival of the desiccation stage (Raghavan and Kamalay, 1992). Most of the studies available have focused on the interaction of temperature and moisture content once spores are dried and how they control longevity of the CS, often for *ex situ* conservation purposes (Pence 2000; Ballesteros et al., 2011, 2017, 2018, 2019; Li and Shi, 2014, 2015; Magrini and Scoppola, 2012; Mikula et al., 2015). However, the degree of DT in CS can be inferred from them since, in the great majority of studies, the spores are dehydrated for later storage. Even so, some experiments focused on the direct response to dehydration, freezing or light (Kato 1976; Lebkuecher 1997; Ballesteros et al., 2018; López-Pozo et al., 2019). All studies showed to some extent that the damage to spores was exacerbated according to the severity of desiccation, and that deterioration was greater in CS than in non-CS. This variation suggests that the structural and physicochemical stability of the dry state differs according to the species and the type of spore e.g. CS or non-CS (e.g. Ballesteros et al., 2017, 2019). The unicellular structure of CS makes them a good unicellular model to investigate DT and longevity in the dry state. The knowledge generated can be used to understand what happen in more complex systems, such as seeds and photosynthetic vegetative tissues, in which the presence of multiple cells and tissues can confound the interpretation of the results obtained (Ballesteros et al., 2017, 2019; López-Pozo et al., 2018, 2019). In addition, the intermediate position of ferns in the plant phylogeny (between the bryophytes and the seeded plants) can be useful to understand the evolution of DT across different plant lineages (López-Pozo et al., 2018).

Available literature on DT (non-chlorophyllous) seeds and in leaves of resurrection plants has already revealed some common mechanism among DT tissues. When desiccation is completed, cell content is stabilized by solidification and it enters into a glassy state (Walters et al., 2010). At these conditions, differences in the properties of the solid formed (i.e. differences in the glassy state) and the short range molecular mobility allowed within the solid matrix (including enzymatic activity) will regulate life in the dry state and will be responsible for longevity (Buitink et al., 1998, 2000; Walters et al., 2010; Ballesteros and Walters, 2011; Ballesteros et al., 2019; Ballesteros and Walters, 2019). Two major divisions can be observed in DT organisms; those who dismantle the photosynthetic apparatus upon desiccation, known as poikilochlorophyllyllous, and those who maintain its integrity, known as homoiochlorophyllyllous (reviewed in Fernandez-Marin et al 2016). The first is rarely found among DT plants, requires more time to reactivate metabolism during rewatering and it is restricted to vascular plants. Basically, poikilochlorophyllyllous plants degrade chlorophyll and the chloroplast ultrastructure when water availability starts to be limited. Homoiochlorophyllyllous DT plants, instead, survive desiccation without this machinery degradation and have to cope with the oxidative damage occurred during the absence of water (Fernández-Marín et al 2016). Based on these characteristics, it has been suggested that CS spores may act as homoiochlorophyllyllous organisms and non-CS spores as poikilochlorophyllyllous (Ballesteros et al., 2018). The mechanisms that allow DT in homoiochlorophyllyllous organisms preserve the cellular integrity during drying and in the dry state by avoiding the disruption of ultrastructures, and by counteracting the oxidative damage that results from the generation of ROS and free radicals (Dinakar et al., 2012; Walters, 2015). Among these mechanisms, some of the most universal responses are: the accumulation of compatible osmolytes (Bartels and Sunkar, 2007), the accumulation of specific compounds such as LEA proteins or heat shock proteins (HSP), and an efficient thermal energy dissipation (in the case of dry chlorophyllous cells). The osmotic adjustment and changes in membrane lipids composition are also essential mechanisms to cope with water loss (Gasulla et al., 2013; Fernández-Marin et al., 2016). When desiccation begins, a decrease of chlorophyll fluorescence emitted by photosystem II (PSII) has been reported in several DT organisms with chlorophyllous cells (Lebkuecher 1997; Heber, 2008; López-Pozo et al., 2019). This mechanism, the so-called non-photochemical quenching induced by desiccation (NPQd), plays a central photoprotective role by reemitting heat asexcess light energy absorbed by chlorophyll (Bilger, 2014). It is often correlated with

the synthesis of zeaxanthin (Fernández-Marín et al., 2011), but can also be induced in the absence of activation of the xanthophyll (VAZ) cycle (Herber et al., 2010). Regarding mechanical stress, osmotic adjustment is essential for the proper functioning of the cell (Dinakar et al., 2016). Maintaining turgor prevents mechanical and biochemical damage at the cellular level, preserving cellular integrity (Fernández-Marin et al., 2016). An important molecule in cellular osmotic adjustment is proline, which has a double function: preservation of cell turgor (Bartlett et al., 2014; Fernández-Marin et al., 2016) and removal of ROS (Borsani et al., 1999).

Our aim in this multidisciplinary work is to study the biochemical and physiological features that may be involved in the variation of responses to desiccation of CS of three different fern species, as well as in relation to their ecophysiological significance. The three CS studied (*Matteuccia struthiopteris, Osmunda regalis,* and *Equisetum telmateia*) differ in the extent of DT and longevity in the dry state as well as in the ecological niche that they occupy (Ballesteros et al., 2017). We hypothesize that CS with higher DT will show more stable physicochemical features. In addition, we hypothesize that those that are more DT, will be released in the more unfavourable environmental conditions, whereas, those less DT, will mature in more favourable environmental conditions.

6.2 Materials and methods

6.2.1Plant material

CS of the three fern species studied were obtained from mature fertile fronds. *Matteuccia struthiopteris (MSt)* CS were obtained from outdoor individuals from the Botanical Garden of the University of Innsbruck, Austria (47°16′N,11°23′ E, 600m a.s.l.) in December 2016. *Equisetum telmateia (ETe)* horsetails growing along Gobelas river, Getxo, Spain (43°20’32.31’’ N, 3°0’0.20’’W) were sampled in March 2017. *Osmunda regalis (ORe)* CS were obtained from the surroundings of the University of the Basque Country, Leioa, Spain (43°19'48.8"N 2°58'08.5"W) in June 2017.

After collection, *ORe, MSt* and *ETe* fronds were kept in the laboratory at 60% relative humidity (RH) for 24 h allowing the dehiscence of the sporangia and the release of the spores. Once the spores were released, they were separated in aliquots of 50 mg, immersed in liquid N_2 and stored at -80 °C until the beginning of the experiment. Before use, spores were defrosted in a water bath at 35 °C for 5 minutes (Pence, 2008).

6.2.2 Determination of photosynthetic pigments and tocopherols

Approximately 15 mg of frozen spores per replicate (exact weight recorded) were freeze-dried and then used for the characterization of photosynthetic pigments and tocopherols in the sporangial state. Samples were first extracted in acetone:water (95:5) and second in pure acetone, both buffered with $CaCO_3$. Freeze-dried samples were homogenized with a mill and the extracts were centrifuged at 16100 g for 20 min. Finally, supernatants were filtered through a 0.2 µm polytetrafluoroethylene filter (Teknokroma, Barcelona, Spain) before being analysed by HPLC. Extracts were injected (15 µL) into a reverse-phase Waters (Milford, MA, USA) HPLC system following the method of García-Plazaola & Becerril (1999) with the modifications described in García-Plazaola & Becerril (2001). PDA detector (Waters model 996) was used to measure photosynthetic pigments in the range 250-700 nm, and peaks were detected and integrated at 445 nm for chlorophyll content. Pigments were identified and quantified by the method described by García-Plazaola and Becerril (1999). Retention times and conversion factors for pigments were the same as described by García-Plazaola and Becerril (1999, 2001).

6.2.3 Proline determination

Proline content was extracted from approximately 10 mg of spores in their sporangial state with a mixture of methanol:chloroform:water (MCW) (12:5:1) according to Borsani et al., (1999). The homogenized was centrifuged for 2 min at 2000g and another mL of MCW added. After vortexing vigorously, 1 mL chloroform and 1.5 mL water were added. Two phases were generated where the upper phase corresponds to free proline. This phase was mixed with ninhydrin reagent, warmed at 100ºC for 1 h and left at room temperature to cool down. Finally, 2 mL of toluene was added, vortexed and centrifuged at maximum speed during 10 min. Proline determination was measured by spectrophotometry at 515 nm (Troll and Lindsley, 1955).

6.2.4 Extent of desiccation tolerance in CS

To estimate the extent of desiccation tolerance (DT), two consecutive desiccation-rehydration cycles (D-R) were conducted in aliquots of 50 mg spores under 10% RH and under 100% RH. Treatments were performed in hermetically closed chambers of 5 L volume; 10% RH was achieved placing 400 g of silica gel (Labkem, SGE0-002-3K0) in the bottom of the hermetically closed chambers. The large volume of the chambers

compared to the amount of spores avoided O_2 limitation. Spores released from the sporangium and hydrated during 24 hours in darkness were considered as "controls". Spores from mature fronds of each fern species (not subjected to any previous hydration) were placed in Eppendorf tubes of 2mL and kept under 10% RH during 36 hours in darkness. After this first dehydration (D1), distilled water was added to the spores and they were incubated under 100%RH for 24 hours, considering this as the first rehydration (R1). Once this period of 24 hours was finished, second dehydration (D2) was performed at 10% RH in darkness. Due to the higher amount of water in spores than in the first desiccation, 72 hours were needed for the complete dehydration. Finally, the second hydration (R2) was carried out with the same procedure as the first one.

All the treatments for all species were performed in darkness. Chlorophyll fluorescence was measured after both desiccations (D1 and D2) and rehydrations (R1 and R2) and water content (WC) after both D1 and D2

Chlorophyll fluorescence

Chlorophyll fluorescence in spores was measured using a Pulse Amplitude-Modulated Fluorimeter (PAM 2500, Walz, Effeltrich, Germany). The optical fibre was fixed off to maintain the same distance to the samples in all measurements. The minimum chlorophyll fluorescence (Fo) was determined in dark-adapted (≤30 min) spores. The maximum chlorophyll fluorescence (Fm) was induced with a saturating pulse for 500 ms. The variable chlorophyll fluorescence (Fv) was calculated as Fm–Fo. The ratio Fv/Fm represents the maximum photochemical efficiency of photosystem II (PSII).

Water content

To determine the amount of water in spores after being released from the frond (sporangial state) and after each desiccation (D1 and D2), the WC of samples was estimated as follows:

$$WC = \frac{(EW - DW)}{DW}$$

Where EW is the weight at the equilibrium with RH; DW is the weight after drying for 24 h in an oven at 70 ºC. Samples were weighed using a balance (AB104, Mettler Toledo, Barcelona, Spain) with 0.1 mg accuracy.

Germination percentage

To determine the viability of spores after the D-R cycles, spores were sown on Dyer culture medium (Dyer, 1979). Percentage of germination was examined 10 days after sowing. The estimation of germination rate was carried out by counting random samples of around 100 spores per Petri dish. Mature spores without treatment sown on Dyer medium during 10 days at 20 ± 2ºC, with 12h/12h of photoperiod and a photosynthetic photon flux density (PPFD) of 70 µmol m^{-2} s^{-1} were germination control values. Germination was considered complete once the spore coat had broken and the rhizoids had emerged (Ballesteros, 2012).

6.2.5 Molecular mobility

Mechanical properties of spores equilibrated at 10 % RH for 48 hours were measured using a Dynamic Mechanical Thermal Analyser (DMA/SDTA861e, Mettler Toledo, Greifensee, Switzerland DMTA). Shear tests required the production of circular samples ≤2 mm thick and 13 mm in diameter. For that purpose, 300 mg (150 mg per circular sample) of spores dehydrated at 10% RH were compressed in a hydraulic press using a maximum pressure of 10 t. All tests were carried out in the dynamic mode, from –50 °C to 150 °C at a heating rate of 2 °C min^{-1} (Fernández-Marín et al., 2013). The analysis of molecular mobility provides information about the potentiality of enzymatic activity at a wide range of temperature. Shear storage modulus (G´) and the loss tangent (tan δ) were calculated using the Mettler Toledo STARTe software during dynamic mechanical thermal analysis (DMTA) scans. For each biological replicate, the temperature value at the maximum tan δ coincident with the α- relaxation measured at 1Hz, was considered for glass transition temperature (Tg) estimation. The Tg indicates that the spore cytoplasm changes from a solid state (known as “glassy state”) to a more fluid state upon warming that allows diffusive motion (Buitink et al., 1996; Ballesteros and Walters, 2011). The extent to which relaxations released restricted motion was approximated by the size of the relaxation signal and was calculated from the difference in value of the tan δ at the onset and peak of α relaxation (Ballesteros and Walters, 2011).

6.2.6 Water relations

Pressure -Volume curves

Water potentials of CS were measured using a dewpoint hygrometer (WP4, Decagon Devices, Pullmann, WA, USA). Around 200 mg of spores were placed in the psychrometer cuvette and held at 100% RH for 48 hours in darkness (for germination avoidance) in closed chambers to allow rehydration. After this period, the samples were considered fully hydrated and ready for water potentials measurements. Previous to the first measurement, the instrument was calibrated with a standard solution of KCl (0.5 M). The first measurement corresponded to the initial water potential. Once the first reading was complete, the sample was weighed on a balance, corresponding to total fresh weight. Consecutive measurements were made on each replicate, weighing them after each reading. Between readings, the samples were kept in the lab at 50 % RH allowing the loss of water. Samples for last readings were kept over silica gel between measurements to force the loss of water. After the measurements, samples were then oven dried at 70 ºC for 24h and their DW determined.

The results were analysed by two graphs; the linear plot of water content against water potential, and the P–V curve relating the reciprocal of the water potential to water content. In the first measurements, a high amount of water loss could be observed, but not a decline in water potential (Ψ). As the samples were losing water, the water potentials began to fall, at first slowly without much change, until reaching a point of abrupt change, considered the water potential at turgor loss point (Ψ_{TLP}). The experiment was concluded when the relationship between 1/ Ψ and the cumulative amount of water loss by samples became linear ($r^2 > 0.98$). From P-V curves, several parameters were obtained, such as relative water content at turgor loss point (RWC_{TLP}), saturated water content (SWC), water potential at full turgor (Ψ_O), water potential at turgor loss point (Ψ_{TLP}), water potential in the sporangial state (Ψ_{SPO}) and the modulus of elasticity, known as epsilon (ε) (Sack et al., 2011). Water potential in the sporangial state was inferred through the adjustment of the trend line in the regression curve between Ψ and WC. The curve of each replicate was adjusted to a polynomial of degree 4. Sporangial WC values were substituted in the equation.

Analysis of rehydration kinetics via the coat surface

Static contact angle (CA) and it's change during time was used for the interpretation of the rehydration capacity of CS in their sporangial state, (e.g. mature spores at the stage in which they are naturally released from sporangia). For each replicate, a monolayer of spores was mounted over a microscope slide and fixed with a double-sided cellophane. An Optical Contact Angle measuring instrument (OCA 15EC, from DataPhysics Instruments GmbH, Filderstadt, Germany) was used to define the hydrophobicity of the spore surface. A sessile droplet of 6 µL distilled water was released over the spores of *ORe*, *ETe* and *MSt* and changes in its contact angle recorded at a speed of 30 frames sec^{-1}, during 10 seconds, with a charge-coupled device equipped camera. Contact angles were measured with the SCA software for OCA, v.4.4.3., as the mean from the optically left and right margins of the water droplet in each frame. The measuring precision was of ± 0.1°. Measurements were performed in 10 different replicates per species.

6.2.7 Scanning electron microscopy (SEM)

Spores in their sporangial state without any desiccation treatment were used for SEM analysis, placing them on adhesive disks pasted on stubs (Agar Scientific Ltd, Essex, UK), and gold coated for 10 min with Fine Coat Ion Sputter (JFC- 1100, Tokyo, Japan). Spores were observed using a Scanning Electron Microscope (Hitachi S-4800, Hitachi Ltd., Tokyo, Japan) with an average working distance of 8mm and a voltage of 10 kV.

6.2.7 Statistical analysis

Kolmogorov-Smirnov and Levene tests were used respectively to check for normal distribution and homogeneity of variance of the data. One-way analysis of variance (ANOVA), with Tukey as post-hoc, was used to check for significant differences among treatments. For non-normal data, the Kruskal-Wallis test was applied. Statistical differences were considered at $p<0.05$. All analyses were performed using the SPSS 17.0 statistical package.

6.3 Results

6.3.1 Characterization of CS

Lipophilic pigments and antioxidant composition were analyzed in the CS of the three ferns species in their sporangial state. All contained the same six major carotenoids

(neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin and β-carotene) as typical for photosynthetic tissues, but their proportion differed among the three species. Carotenoids to Chl ratios showed a general pattern, being higher in *ETe* than in the other two species. The VAZ pigments, β-C concentrations, and the ratio AZ/VAZ was highest in *ETe.* The highest ratio Chla/b was found again in *ETe* with significant differences with *ORe* and *MSt* (Table 1).

Table 6.1: Pigment composition and maximum photochemical efficiency of PSII (Fv/Fm) at the sporangial state in CS of the three studied ferns. Results are expressed as follows: chlorophyll a/b ratio (Chl a/b) (mol mol^{-1}). Neoxanthin (N), violaxanthin (V), antheraxanthin (A), lutein (L), zeaxanthin (Z), β-carotene (β-C) and VAZ (mmol mol^{-1} Chl a+b). Each value represents the mean±SE (n=5 for pigments and n=4 for Fv/Fm). Different letters indicate significant differences between species ($P < 0.05$). The term "n.d" means that the fluorescence signal is below the detection threshold.

	ORe	***MSt***	***ETe***
Chl a/b	2.80 ± 0.07 a	2.71 ± 0.02 a	3.18 ± 0.01 b
AZ/VAZ	0.21 ± 0.01 a	0.16 ± 0.0 b	0.25 ± 0.01 c
N	52.5 ± 2.2 a	52.2 ± 1.4 a	49.9 ± 1.4 a
V	60.1 ± 6.4 a	81.8 ± 2.8 b	79.9 ± 2.8 b
A	5.26 ± 0.49 a	5.74 ± 0.29 a	14.3 ± 0.9 b
L	226.1 ±13.3 a	226.1 ±3.97 a	283.1 ± 6.1 b
Z	10.5 ± 0.5 a	9.6 ± 0.3 a	12.5 ± 1.0 b
β-C	96.7 ± 11.7ab	95.4 ± 3.2a	105.8 ± 2.2b
VAZ	75.9 ± 7.3 a	97.2 ± 3.1 b	106.8 ± 4.1 b
Fv/Fm	n.d	n.d.	0.131 ± 0.066

To investigate the antioxidant capacity of the CS studied, the composition of the tocopherol pool and proline contents were examined in their sporangial state when they are released from sporangium. α-Tocopherol was ubiquitous among all the species. The lowest concentration of this antioxidant was found in *ORe*, while *MSt* had almost 3-fold higher values. Additionally, only the latter contained the other isoforms (β-, δ- and γ-) that represented 25% of the total tocopherol pool.

The highest proline contents were also found in *MSt* CS. The amount of this amino acid showed great differences among the species. *MSt* CS accumulated proline in the highest

proportion once mature having two-fold higher proline contents than *ORe* and seven-fold higher than *ETe* (Table 2). In summary, *MSt* presented the highest capacity of the antioxidant system.

Table 6.2: Tocopherol and proline contents at the sporangial state in the spores of the three studied ferns. Results are expressed as follows: α- tocopherol (α-T); β- + γ-tocopherol (β+γ-T); δ- tocopherol (δ-T) and proline (μmol g DW^{-1}). Each value represents the mean±SE (n=5). Different letters indicate significant differences between species ($P < 0.05$). The term "n.d." means that the concentration is below the detection threshold.

	ORe	***MSt***	***ETe***
α-T	0.30 ± 0.01 a	0.83± 0.01 b	0.43 ± 0.02 c
(β+γ)-T	n.d.	0.25 ± 0.00	n.d.
δ -T	n.d.	0.04 ± 0.00	n.d.
Proline	17.5 ± 2.7 a	35.7 ± 1.3 b	4.9 ± 0.1 c

6.3.2 Extent of desiccation tolerance

Mature CS of the three fern species were subjected twice to a D-R cycle at 10% RH in darkness. After D1, the WC greatly differed among species, with *ETe* able to retain the highest amount of water, while the other two species did not show significant differences (Fig. 6.1A). After D2, no differences were observed among species, and WC values were below 0.1 gH_2O gDW^{-1} for all the species studied.

Regarding the capacity to tolerate desiccation (estimated by the recovery of Fv/Fm), *ORe* showed the highest values of Fv/Fm recovery after D1 and D2. Curiously, significant differences were found between both desiccations, being Fv/Fm recovery and germination percentage even higher after D2 than after D1 (Fig. 6.1B, C). The next species that showed the highest recovery capacity were *MSt* CS. After the first drying, a recovery of Fv/Fm of 92% was possible, against the 87% recovery that occurred after D2. Finally, in the case of *ETe*, a reduction in almost 43% of Fv/Fm control values was observed after D1 and no recovery was possible after D2 (Fig. 6.1B). In conclusion, all species were able to recover a certain percentage of Fv/Fm, at least after D1, but to a different extent. On the other hand, D2 treatment allowed the recovery of Fv/Fm only in

ORe and *MSt*. Germination percentage, although with little differences with Fv/Fm recovery values, followed the same tendency. *ETe* showed a severe decrease in viability after D1, and no CS germinated after D2 (Fig. 6.1C).

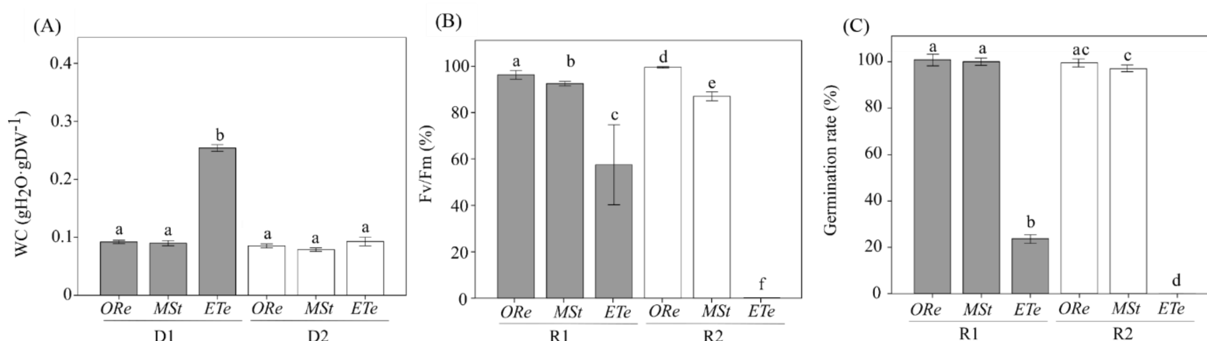

Figure 6.1: Water content (WC) (A), percentage of recovery of Fv/Fm (B) and percentage of germination (C) relative to the respective control values after first rehydration (R1) (grey bars) and second rehydration (R2) (white bars) at 10% RH in darkness in the spores of the three studied ferns. Control values of Fv/Fm in non-treated spores were obtained after 24 h of hydration in darkness. Control values of germination in non-treated spores were obtained 10 days after sowing on Dyer medium. Each bar represents the mean±SE (n=4). Different letters indicate significant differences among species and dehydration-rehydration (D-H) cycles (P < 0.05).

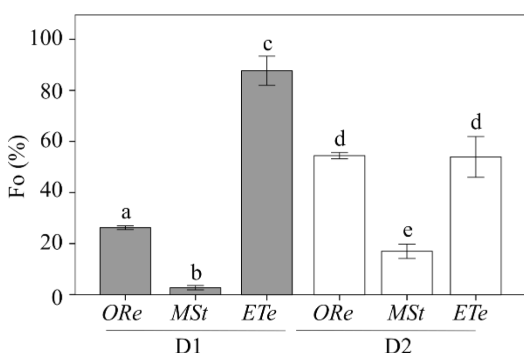

Figure 6.2: Percentage of Fo relative to the respective control values in the CS of the three studied ferns after desiccation (D1) (gray bars) and second desiccation (D2) (white bars). Control values were obtained as in Figure 6.1. Each bar represents the mean ± SE (n = 4). Different letters indicate significant differences among species and dehydration–rehydration (D–R) cycles (P < 0.05).

Concomitant with Fv/Fm changes, the basal fluorescence of chlorophyll (Fo) also underwent important changes during the D-R cycles and completely different behaviours were observed among species. CS of *MSt* showed the highest quenching of chlorophyll upon desiccation with a reduction of 98% of the Fo in D1 and around 83% in D2. On the contrary, *ETe* CS did not show such attenuation of chlorophyll fluorescence, neither in D1 or D2, although a greater quenching was observed during the D2. Finally, *ORe*

increased the quenching of Fo much more during D1. After D2, Fo emission increased to values 50% lower than the controls (Fig. 6.2).

6.3.3 Molecular mobility of CS in the dry state

Molecular mobility in CS was estimated by DMTA. Spores were equilibrated for 48 hours at 10% RH in darkness. Water contents achieved after this treatment were 0.055 ± 0.004, 0.051 ± 0.002 and 0.057 ±0.00 g H_2O g^{-1} DW for *ORe, MSt* and *ETe* CS respectively, with no significant differences between species. At these water contents, diverse structural relaxations were observed in the DMTA scans that were characterised by peaks in the Tan δ and step-wise changes in the storage modulus (G') (Fig. 6.3). A relatively large relaxation was observed in the scans of all three species between 0 and 50 °C (Fig. 6.3). This relaxation was named as α-relaxation and it was characterised by a relatively large peak in the Tan δ (upper panel, Fig. 6.3) that coincided with a large decrease in storage modulus (G') (lower panel, Fig. 6.3). α-relaxation coincides in temperature with the glass transition temperature (Tg) measured by Differential Scanning Calorimetry in these three species at equivalent water contents (Ballesteros et al., 2017) and was considered the Tg of the CS in the three species studied. α-relaxation or Tg occurred at 39.8 ± 2.3, 43.5 ± 1.8 and 42.2 ± 5.3 ºC for *ETe, MSt* and *ORe* CS respectively, with no significant differences between species (Fig. 6.3). The size of the α-relaxation or Tg in the Tan δ (value related to the molecular mobility released during the relaxation, Ballesteros and Walters, 2011) was 0.08 ± 0.03, 0.17 ± 0.03 and 0.19 ± 0.09 for *ETe, MSt* and *ORe* CS respectively, being significantly different in the *ETe* CS when compared to those of *MSt* and *ORe*.

Below Tg (lower temperatures), all CS are in the glassy state, but some molecular mobility was detected in the Tan δ as small peaks between -40 and -20 °C coincided with a decreases of G' (in *MSt* and *Ore* CS only). Interestingly, right before the α-relaxation or Tg starts, the G' of *ETe* increased dramatically (in all replicates performed), an event that did not occur in the other two species.

As the temperature increases above the Tg, the spore cytoplasm enters in a fluid state where molecular mobility increases greatly and chemical reactions are possible, including gelation and melting events of the starch and proteins that are characterised by large peaks of tan δ > 100°C, accompanied sometimes (e.g. *ETe*) with increases of G' (detailed explanations of these events in Ballesteros and Walters, 2011).

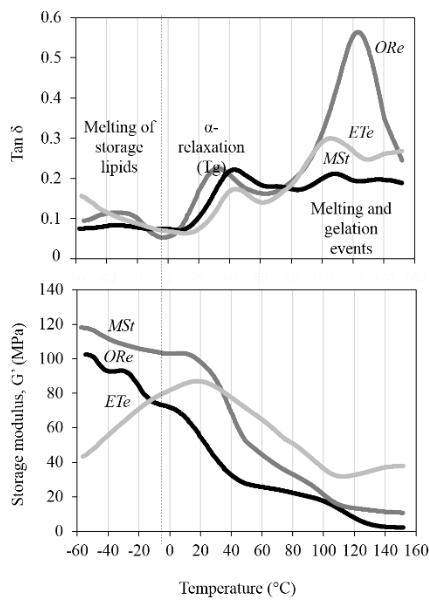

Figure 6.3: Dynamic mechanical thermal analysis (DMTA) scans of *ORe*, *MSt* and *ETe* CS desiccated at 10% RH for 48 hours. α-relaxation is characterised by a peak in the Tan δ between 30-50 °C (upper panel) that coincides with a large decrease in storage modulus (G') (lower panel). α-relaxation coincides in temperature with the glass transition temperature (Tg) measured by Differential Scanning Calorimetry (Ballesteros et al., 2017). Other molecular relaxations are observed above and below the Tg that are associated to melting and gelation events (as from Ballesteros and Walters, 2011) and melting of the storage lipids (Ballesteros et al., 2017), respectively. One representative curve (from n=3 independent biological replicates) is shown for each spore species.

6.3.4 P-V curves

Water potentials were measured during the loss of water in the CS of the three fern species equilibrated for 48 hours at 100% RH. When spores were released from the sporangium there were important differences with respect to their water potentials (Ψ_{SPO}). *ORe* and *MSt* CS were released with a Ψ_{SPO} of - 123±10 MPa and - 67±3, respectively. In contrast Ψ_{SPO} was much higher in *ETe* (- 28±4 MPa). Considering that the water potential at turgor loss point (Ψ_{TLP}) was - 44±5, - 92±6 and - 33±2 MPa, in *Ore*, *MSt* and *ETe* CS, respectively, it can be concluded that spores of *ORe* are released from the sporangium in a "non-turgid" state (Fig. 6.4 A). As can be observed in Fig.6.4 A, in the case of CS of *ETe*, the range between Ψ_{SPO} and Ψ_{TLP} is very narrow but enough to release the spore in a turgid state. When spores are at full turgor, the water potential did not reflect these differences.

Regarding the amount of water at full turgor (SWC), *MSt* showed the highest SWC, as well as the lowest WC at the turgor lost point (WC_{TLP}). At the other extreme, *ETe* had the narrowest range of WC between the full turgor and the loss of turgor (Fig. 6.4 B). *ORe* spores, with an average of the modulus of elasticity (Ɛ) of 3.4±0.9, showed the most flexible wall, and was significantly different from the other two species. In the case of *MSt* and *ETe*, the wall was more rigid with Ɛ of 7.8±0.6 and 6.3±0.2, respectively, and even some cracks were observed in the coat of *MSt* (Fig. 6.6).

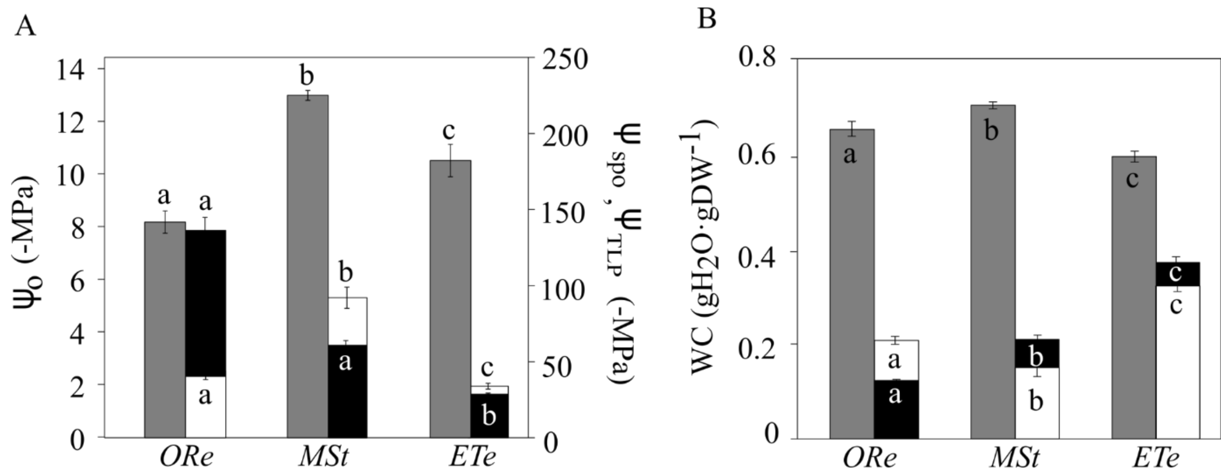

Figure 6.4: Water potential (A) and water content (WC) (B) in the spores of the three studied ferns at full turgor, at turgor loss point and in the sporangial state. Grey bars correspond to water potentials at full turgor (Ψ_O) (left axis), white bars correspond to water potential at turgor loss point (TLP) (Ψ_{TLP}) and black bars correspond to water potential in the sporangial state (Ψ_{SPO}) (right axis). The same colour code is used for water content (WC). Each bar represents the mean±SE (n=3). Different letters indicate significant differences among species for the same parameter ($P < 0.05$).

6.3.5 Water uptake capacity

The wettability of the spores was measured in their sporangial state with the aim of knowing their ability to absorb water once they are released from the sporangium.

Static contact angles and how it changed with time are shown in Fig. 6.5. The species that underwent the greatest changes in contact angle during 10 seconds (e.g. was more hydrophilic) was *ETe*. Drop contact angle changed from 127±3 degrees in the second 1, to 79±7 in the second 5 and 60±6 in the second 10. At the other extreme, no change was observed in *MSt* (Fig. 6.5 A). This species presented a hydrophobic behaviour without significant differences in contact angles between the three-time points (Fig. 6.5 B). It should be noted that *ORe* had a lower rate of water absorption than *ETe*, despite starting with similar contact angle degrees.

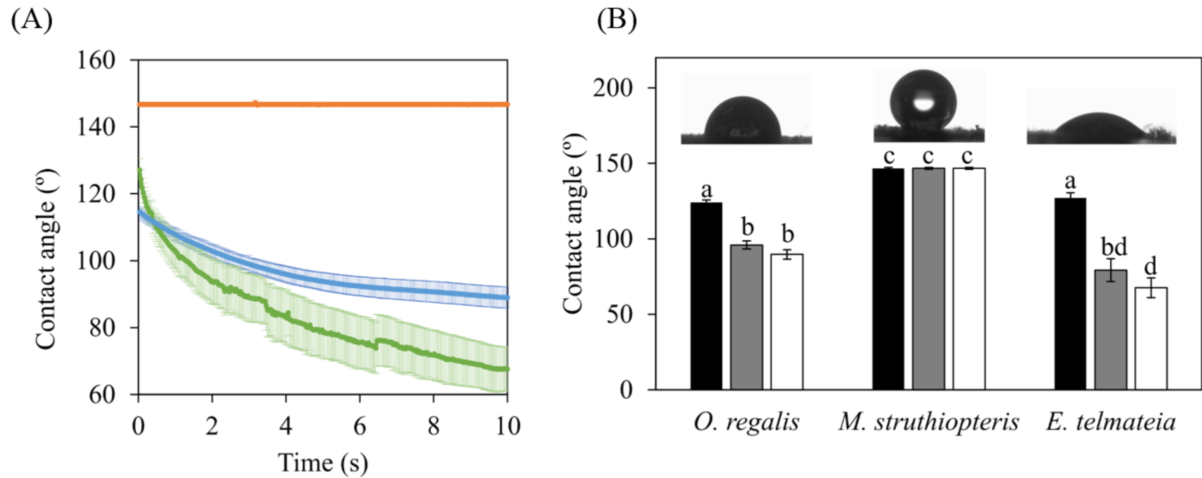

Figure 6.5: Static contact angles of 6 µl distilled water drops over the spores of the three studied species in their sporangial state. (A): Time course of the change in the contact angle during 10 seconds. Colour lines represent the average ± SE (n=10) for each of the three species evaluated *MSt* (orange line), *ORe* (blue line) *and ETe* (green line). Contact angles at selected timepoints during the recording: 1 second (black bars), 5 seconds (grey bars) and 10 seconds (white bars) after water drop contacted with the spore surface (B). Each bar represents the mean±SE (n=10). Different letters indicate significant differences among species and time points ($P < 0.05$). Details of a representative distilled water drop in contact with spores surface at time 1 second are shown as insets.

6.3.6 Outer structure of the spores

The outer structure of the spores was studied by SEM. The *ORe* CS had a diameter of approximately 40 µm and a rugose perispore with echinate structures (Fig. 6.6 A). These were homogeneously distributed along the entire spore surface. Several undulations were also apparent in the spore coat. *MSt* CS measured around 30µm, had coarse folds, prominent, and minutely echinate-rugose surface structures fused at the perispore. A break in the perispore can be observed in Fig.6.6 B. *ETe* CS presented fewer amounts of peripheral structures associated with the perispore, but spherical structures could be observed (Fig.6.6 C) both in spore surface and in elaters. Each spore measured around 30 µm and had four paddle-shaped elaters. Only one pair is presented in Fig.6.6 C.

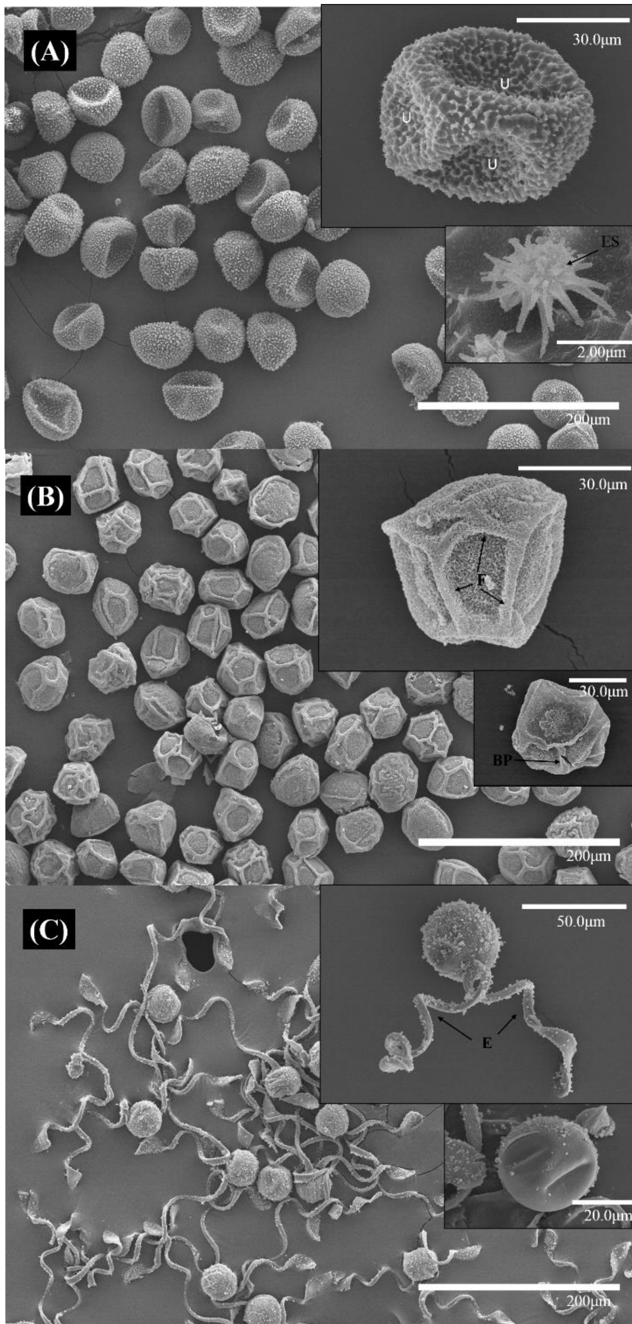

Figure 6.6: SEM micrographs of spores of *ORe (A), MSt (B) and ETe* (C) dried in silica gel during 24 hours. The scale of each micrograph is indicated in the lower part. Abbreviations: broken perispore (BP), elaters (E), folds (F), undulations (U).

6.4 Discussion

In this study, we detected significant variation in the physiological and physicochemical properties of CS of three ferns species when subjected to D-R cycles, suggesting diverse extents of DT among CS. Based on the different ecological niches that these species occupy, this variation in DT may represent different adaptive strategies in terms of spore dispersal and subsequent sporophyte development. To date, no studies have investigated how CS respond to environmental conditions.

6.4.1 Extent of DT among CS

All mature CS of the three fern species studied showed a certain extent of DT since they were able to recover Fv/Fm and germinate once rehydrated. This feature, in more or less extent, has been commonly found in diverse CS (Ballesteros 2011, 2017; López-Pozo et al., 2019). However, despite the ability to recover metabolism after desiccation, several differences were observed between species that suggest different levels of DT. For example, the viability of *ETe* spores significantly decreased during D1, whereas *ORe* and *MSt* spores maintained their high initial viability (Fig. 6.1). This initial reduction in viability occurred at higher water contents in *ETe* spores (c. 0.25 gH_2O gDW^{-1}) than those reached in *ORe* and *MSt* spores (<0.10 gH_2O gDW^{-1}) (Fig. 6.1). It is well known that the genus *Equisetum* presents short longevity in the dry state, whereas *ORe* and *MSt* can persist for months or years, respectively (Lloyd and Klekowski, 1970; Whittier, 1996; Lebkuecher, 1997; Ballesteros et al., 2017). However, the extremely fast viability loss in *ETe* and the high-water contents at which it occurred, contrast with the relatively low water contents that other *Equisetum* species can tolerate for longer periods. In this sense, *E. hyemale* can tolerate desiccation between 0.03 and 0.08 gH_2O gDW^{-1} during one week without significant viability losses (Lebkuecher, 1997; Ballesteros et al., 2017). Our results showing large viability decrease in 36 hours at 0.25 gH_2O gDW^{-1} suggest a deficient DT of the spores of *ETe*, that resembles the DS found in recalcitrant seeds (Berjak and Pammenter, 2008; Walters, 2015).

Although all fern spores studied presented certain recovery of Fv/Fm, (at least in R1), only spores of *MSt* and *ORe* carried out the characteristic NPQd of DT cryptogams. This phenomenon has been described widely in algae, cyanobacteria, lichens and bryophytes (Bilger, 2014), but it has only been observed once in CS (López-Pozo et al., 2019). During desiccation, DT organisms that activate NPQd mechanism experience a strong decline in Fo. Instead, in DS organisms, an increment in Fo is noted. This process allows the dissipation of light energy in the dried state helping to limit oxidative risk. The poor (or the lack of) NPQd activation in *ETe* spores is another characteristic supporting the low DT or DS that they display (Fig. 6.2).

6.4.2 Extent of DT of CS based on pigments and antioxidant system

The analysis of the pigment composition showed that all CS studied consist of the photosynthetic pigments characteristic of embryophytes (Esteban et al., 2015). *ETe* CS

showed a higher Chl a/b ratio, and were only photochemically active when released from sporangium (Table 6.1). At the other extreme, *MSt* had the lowest Chl a/b and AZ/VAZ ratio, as well as β-C. Carotenoids are effective quenchers of ROS, thereby they play an important role in photosynthetic machinery protection (Demmig-Adams, 1990). The loss of viability of CS has always been related to the presence of chlorophyll, the absence of DT, the high metabolic rates, and the lack of dormancy (Kato 1976). As CS possess fully developed photosynthetic apparatus, and chlorophyll absorbs light even in the dry state, the risk of photodamage is exacerbated under these conditions. In this sense, CS have the behaviour of any photosynthetic tissue and must cope with the high probability of photodamage (Ballesteros et al., 2018; López-Pozo et al., 2019). Not only pigments but also antioxidant system (tocopherols and proline) showed significant differences between species (Table 6.2). *ETe* and *ORe* contained only α-tocopherol, whereas *MSt* had additionally the other isoforms (β+γ)-tocopherol and δ- tocopherol. The photoprotective role of α-tocopherol is well documented (Munné-Bosch and Alegre 2002) and is very common in photosynthetic tissues. The other isoforms β and δ are quite rare in most plant species and γ-tocopherol is more common in seeds (Grusak and DellaPenna, 1999; Fernández-Marín et al., 2017). Abassi et al., (2007) found that tobacco seeds increased the amounts of α-tocopherol as DT was acquired during maturation. Proline has also been described as a good ROS scavenger and compatible solute under stress conditions (Borsani et al., 1999). This amino acid could increase the antioxidant enzymatic capacity (e.g. APX, SOD and CAT enzymes) under water stress conditions (de Campos et al., 2011). Proline would avoid or reduce lipids peroxidation and the production of free radicals. Besides, it would help in the scavenging of singlet oxygen and hydroxyl radicals (Kishor and Sreenivasulu, 2014). In this sense, *MTs* spores presented the highest concentration of this antioxidant, while *ETe* almost did not accumulate it (Table 6.2), supporting the DT difference between these species from an antioxidant point of view.

Overall, the results on pigments and antioxidant system indicate that while *MSt* and *ORe* photoprotection relies mostly on antioxidant systems, *ETe* shows a chloroplast composition more prone to imminent photochemical activation. In all likelihood, *MSt* and *ORe* displayed a trade-off between lower photosynthesis and higher DT.

6.4.3 Variation of the stability of the glassy state and its relation to the extent of DT in CS

All spores equilibrated at 10%RH for 48 hours were below the Tg and entered in a glassy state at ambient temperature (20 ºC) (Fig. 6.3), where it is considered that there are no chemical reactions due to the low molecular mobility (Ballesteros and Walters, 2011; Fernandez-Marin et al., 2013). However, some molecular mobility was observed below the Tg. For example, some broad peaks in the Tan δ were observed between -40 and -20 ºC. These peaks can be attributed to the melting of the spore storage lipids, as previously described and measured by Differential Scanning Calorimetry (DSC) in these three species (Ballesteros et al., 2017). While the molecular mobility attributed to storage lipids may not be relevant to the variation of DT, there are other mechanical properties that may be important. For example, G' of *ETe* increased dramatically right before the α-relaxation (Tg) occurs, an event that was not observed in the other two species (Fig. 6.3). Increments of G' before the Tg have been related to inter- and intramolecular rearrangements that can lead to microstructural changes and are considered important in the stability of other dry systems (Herrera-Kao and Aguilar-Vega, 1999; Menard, 1999; Champion et al., 2000; Roudaut et al., 2004; Ballesteros and Walters, 2011). The presence of these inter- and intramolecular rearrangements in *ETe* could be interpreted as a consequence of the formation of an unstable glassy state upon drying and cooling, that could lead to large pores and a quick relaxation of the glassy state once formed (Walters et al., 2010). The significantly smaller size of the α-relaxation in *ETe* when compared to *ORe* and *MSt,* could also be indicative of a glassy state in *ETe* that has collapsed and compacted during the inter- and intramolecular rearrangements. In the case of *MSt* it is interesting to consider its stable glassy state together with the release of the spores during wintertime. Freezing produces dehydration at the cellular level, so the development of DT mechanisms and the formation of stable intracellular glasses are necessary for their long-term survival. Overall, these results are in agreement with a lower DT and shorter lifespan of *ETe* spores at low water content.

6.4.4 The loss of turgor as a parameter of discrimination between species and habitats

To understand how organisms respond to changes in water availability, the study of pressure volume-curves (P-V) offers a series of physiological parameters about water

relations (Tyree and Hammel, 1972; Kikuta et al., 1985). These parameters have been widely studied in vascular plants, but little is known about poikilohydric organisms (Holzinger and Karsten, 2013, Nardini et al., 2013; Petruzzellis et al., 2018). Several parameters inferred from P-V curves of *MSt, ORe* and *ETe* showed important differences that could be related to their ecological requirements (water availability) and physiological features (DT) (Fig. 6.4). One of the greatest risks to the cell during water loss is the reduction of its volume (Walters, 2015). When the protoplast begins to dehydrate, this reduction in volume can threaten the integrity of the membrane-wall junctions. Therefore, the correct folding of the wall during desiccation is of vital importance to overcome the mechanical stress that occurs, avoiding the disruption of these connections (Fernandez-Marin et al., 2016). The parameters that best describe the ability of the cells to maintain turgor are Ψ_O, Ψ_{TLP} and Ɛ (Lenz et al., 2006; Ding et al., 2014). Of plants that tolerate drought, some of the most characteristic features within water relations are that they have lower Ψ_O, which in turn is related to lower Ψ_{TLP} (Bartlett et al., 2012). *ETe*, the least DT, showed the highest Ψ_{TLP}, whereas *ORe*, the most DT, showed the lowest Ψ_{TLP} (Fig. 6.4). Not only was drought tolerance correlated with low Ψ_{TLP}, but environmental water availability in the sporulation moment also correlated with this parameter. *ORe*, followed by *MSt*, had the lowest Ψ_{TLP}, coinciding with times of the year with lower water availability. Spores of *ETe* had the highest value of Ψ_{TLP}, coinciding with the fact that this species releases its spores in the spring (Fig.6.4). The relationship between Ψ_{TLP} and environmental conditions has been demonstrated in vascular plants, where those that grow in climates with water deficit (those adapted to live in xeric climates) present lower Ψ_{TLP} values (Aranda et al., 1996, Lenz et al., 2006, Bartlett et al., 2012, Nardini et al., 2013).

Regarding Ɛ, there is still no consensus to clarify whether low values are related to xeric climates or not (Abrams and Kubiske, 1994, Anderson and Helms, 1994, Saito and Terashima, 2004). It has been suggested that low values of Ɛ can help to maintain turgidity even when large amounts of water are lost (Schulte 1992), but, on the other hand, high values could help a rapid recovery of the turgidity. In organisms subjected to daily hydration-dehydration cycles, this could be a great advantage (Bidussi et al., 2013; Alam et al., 2015). Therefore, *ORe* could maintain the turgor through the high elasticity of the walls, whereas *MSt* may have a greater osmotic adjustment capacity. The maintenance of turgor could be based on compatible solutes (e.g. proline, Table 2) instead of Ɛ. By

observing SEM images, the capacity of *ORe* to fold their wall can be appreciated (Fig.6.6). These undulations were not found in the *MSt* spores.

6.4.5 Wettability. Dispersal function or unfavourable conditions avoidance?

How CS surfaces absorb water will determine to a great extent the physiological responses when released to the environment. Haines et al., (1985) found that environmental factors would more greatly affect the plant surface if the wettability was high, so the species would be more exposed to environmental factors. In agreement with this, the CS of *ETe* would be more affected by environmental conditions, due to their higher wettability (Fig. 6.5). On the other hand, *ORe* and *MSt* developed CS that were remarkably hydrophobic and thus with significantly lower wettability (Fig. 6.5). Low wettability in elm samaras has been related to high floatability, which would help to a dispersal mechanism based in hydrochory (Guzmán-Delgado et al., 2017). Although a similar advantage for hydrochory could be argued in the case of *MSt,* the low permeability of its CS may also relate to a delay of spore germination in the field. As hydrating during winter could induce germination under unfavourable conditions, a delay in the absorption of water, activation of metabolism and finally, germination, may increase survival probabilities of developing gametophytes during late-winter/early spring. *ORe* and *ETe* are released in more favourable atmospheric conditions, so their relatively faster capacity to take up water may be advantageous according to their phenologies.

6.5 Concluding remarks

In view of the results, several conclusions can be achieved. CS of *ETe* had the highest capacity to absorb water, the lowest DT and presented the most unstable glassy state when dried. Its pigment composition revealed effective photosynthetic machinery and WC and Fv/Fm were higher than in the two other species. Since these spores are released in spring, when water availability is high and spores will germinate immediately after dispersal, this species does not require the development of DT strategies to survive for long periods in the dry state (Fig. 6.7). These spores displayed a trade-off between photosynthesis and DT. In summer, when water availability fluctuations are more frequent, *ORe* releases its spores. Because of this, *ORe* is more likely to suffer various cycles of D-R. Its spores had the highest DT and wall flexibility and the lowest Ψ_{TLP} and Ψ_{SPO}. All of these features enable *ORe* to dehydrate and rehydrate in their natural habitat once released without

compromising viability (Fig. 6.7). Finally, *MSt* had the highest hydrophobicity, highest antioxidant system and high DT. The release of this spore occurs in winter, under very unfavourable environmental conditions. The high hydrophobicity could prevent the entry of water under these conditions (Fig. 6.7).

Overall, the coordination among several hydric, mechanical, physical and physiological traits, represents a syndrome that confer CS the level of desiccation tolerance required to survive under the conditions prevailing during the natural dispersion of each species.

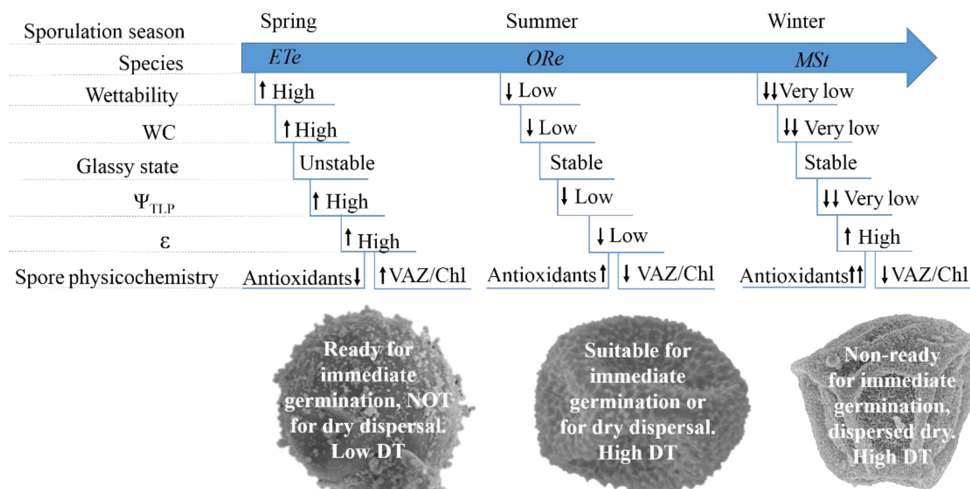

Figure 6.7: Summary view of the main trends on the physiological and physicochemical features in CS of the three ferns studied. Arrows indicate higher or lower values when each parameter is compared among species.

Authors contribution

MLP: designed the experimental protocol, conducted the experimental phase and statistical analyses and drafted the manuscript with support from BFM and JIGP. DB: contributed to the interpretation of DMA data and in the discussion of overall results. JML: performed the DMA analyses with contributions of BFM and MLP. JIGP: designed the experimental protocol and supervised the writing of the manuscript. BFM: designed the experimental protocol and supervised the writing of the manuscript. All co-authors contributed to the final version of the work.

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CHAPTER VII

LIGHT AND OXYGEN AS AGEING PROMOTERS IN FERN CHLOROPHYLLOUS SPORES DURING DRY LONG-TERM STORAGE

TO BE SUBMITTED TO PLANT CELL AND ENVIRONMENT.

7.1 Introduction

Tissue desiccation is one of the major challenges that a living organism can deal with. Coping with desiccation implies the activation of a sophisticate array of protection mechanisms that allow the recovery of metabolism after the removal of all, or almost all the cellular water without irreversible damage. In practical terms, desiccation tolerance (DT) is considered when drying to water contents (WC) below 0.1 g H_2O g^{-1} dry weight (DW) or to equilibrium under relative humidity (RH) lower than 70% (Vertucci & Farrant, 1995; Leprince & Buitink, 2010; Fernández-Marín Holzinger, & García-Plazaola, 2016). Among tracheophytes, DT is a rare trait in photosynthetic tissues (Gaff, 1980; Alpert, 2000; Oliver, Tuba, & Mishler, 2000) but is widely expressed in reproductive structures (pollen, seeds and spores). Therefore the majority of reproductive or dispersal structures are able to dry to low WC for a short period (e.g. minutes to hours) and recover full metabolism upon rehydration (Franchi, Nepi, Matthews, & Pacini, 2011). In addition, most seeds and spores can survive in the dry state for periods ranging from days to centuries, or even millennia, depending on the species and the environmental conditions during storage (Priestley, Cullinan, & Wolfe, 1985; Shen-Miller, Mudgett, Schopf, Clarke, & Berger, 1995; Buitink, Walters, Hoekstra, & Crane, 1998; Walters, Wheeler, & Stanwood, 2004; Walters, Wheeler, & Grotenhuis, 2005a,b; Nagel & Borner, 2010; Ballesteros, Hill, Lynch, Pritchard & Walters, 2019, Colville & Pritchard, 2019). Although the acquisition of DT and longevity in reproductive structures are related and are consecutively acquired during maturation, they do not relate to the same traits (Verdier et al., 2013; Pereira-Lima et al., 2017). DT implies the ability to cope with a high loss of water at the cellular level, while longevity refers to survival over time while in the dry state (Verdier et al., 2013; Walters, 2015; Pereira-Lima et al., 2017; Ballesteros, Hill, Walters, 2017). Longevity in the dry state requires dealing with the stress produced by initial desiccation, by that produced during deterioration over time (i.e. ageing reactions) and the subsequent stress during rehydration, when also the mechanisms of repair of damages acquired during drying and while dry are activated (Kranner, Minibayeva, Beckett & Seal, 2010; Farrant, 2017).

DT plants have a series of strategies to face water loss and to be able to survive in an ambient where water is scarce (Wood, 2005). The function of these mechanisms is usually to preserve the cellular and molecular integrity, avoiding the disruption of ultrastructures during drying and over time, and to counteract the oxidative damage that results from the

depletion of the antioxidant machinery and, usually, the presence of reactive oxygen species (ROS) and free radicals (Kranner, Birtić, Anderson & Pritchard., 2006; Dinakar, Djilianov & Bartels, 2012; Walters, Ballesteros & Vertucci, 2010; Walters, 2015; Pereira-Lima et al., 2017). This ROS pressure is exacerbated in photosynthetic DT-tissues, since the chlorophyll present in the dry state will still absorb light energy that cannot be used for electrochemistry and will thus led to an additional source of oxidative pressure (e.g.: singlet oxygen). Ageing and loss of viability over time in dry reproductive structures has been mainly associated with oxidative damage (Bailly, 2004; Kranner & Birtic 2005; Mira, González-Benito, Hill & Walters, 2010, Mira, Hill, González-Benito, Ibáñez & Walters, 2016; Fleming, Richards & Walters, 2017; Fleming, 2018) and in photosynthetic tissues, it has also been related with the break down of the antioxidant system (Kranner, Beckett, Wornik, Zorn, & Pfeifhofer, 2002a). In non-photosynthetic tissues, oxidative damage has been related to lipid peroxidation, RNA fragmentation and the activation of a signalling cascade by ROS that initiates programmed cell death (PCD) leading to inter-nucleosomal DNA fragmentation in the final (Bailly, 2004; Kranner & Birtic 2005; Kranner et al., 2006; Mira et al., 2010, 2016; Fleming et al., 2017, 2018). During dry storage, autoxidation of lipids may also occur, increasing the production of ROS (McDonald, 1999). In photosynthetic tissues, oxidative stress has also been related to lipid peroxidation in the thylakoid membranes (Roquiero et al., 2010), but in this case the presence of chlorophyll, which still interacts with light even in the dried state, is a fundamental catalyser of this oxidative reaction (Roquiero et al., 2010). This is possible because during dehydration and while in the dry state, chlorophyll molecules excited by light photons produce oxidative deterioration through the generation of ROS (Heber & Shuvalov, 2005; Roquiero et al., 2010; Ballesteros, Narayan, Varghese, Sershen, 2018). This production of ROS is the consequence of the metabolic imbalance between the received and the used light energy in the photosynthetic apparatus (Vertucci & Farrant 1995; Verhoeven, García-Plazaola, Fernández-Marín, 2018). In addition, in the dry state, enzymatic activities and metabolism are virtually absent due to the vitrification (i.e. solidification) of the cytoplasm (Fernandez-Marin et al., 2013). Since antioxidant protection is not possible across enzymatic antioxidants, plants must use non-enzymatic ROS scavengers or antioxidant systems. Therefore, the balance between ROS production and efficient antioxidant machinery in both, photosynthetic and non-photosynthetic tissues, seems to be an imperative requisite to tolerate desiccation and to maintain cellular

integrity during long-term storage (Hendry, 1993; Leprince et al., 1993; Vertucci & Farrant, 1995; Kranner et al., 2006; Nagel et al., 2016).

Among non-enzymatic antioxidant defences, two main groups can be found: lipid- and water-soluble antioxidants. One of the most efficient antioxidants is glutathione (GSH), water-soluble molecule and responsible for the removal of H_2O_2 (Kranner et al., 2006). Under non-stressed conditions, GSH can be found in the reduced state, able to donate an electron to several ROS (e.g.H_2O_2) with glutathione disulphide (GSSG) as oxidation product. The reduction of GSSG to GSH follows an enzymatic pathway by the enzyme glutathione reductase and, as commented, this is an unviable reaction when the glass is formed inside cells. In this situation, the unique direction is the oxidation of GSH pool. The decrease in GSH has been often related to various stresses (Kranner & Birtić, 2005), including seeds ageing (Kranner et al., 2006). Within lipid-soluble antioxidants, α-tocopherol (α-toc) and some carotenoids seem to play a central role in ROS scavenging (Krinsky, 1994) and membrane stability (Munné-Bosch & Alegre, 2002). On the one hand, the presence of tocopherols (specifically α-toc) has been correlated with seeds viability (Seal, Zammit, Scott, Flowers & 2010). On the other hand, carotenoids (mostly lutein and zeaxanthin) can act as radical quenchers. Since both (tocopherols and carotenoids) can be immersed in plastid membranes, they could act as stabilizers during the loss of water (Francini et al., 2006; Fernández-Marín et al., 2013). Both antioxidants decline during seeds storage (Francini et al., 2006; Seal et al., 2010) and tocopherol, but not zeaxanthin, also declines during dry-storage of DT-plants and lichens (Kranner et al., 2002a; Kranner, 2002b; Fernandez-Marin, Míguez, Becerril & García-Plazaola, 2011; Fernandez-Marin et al., 2013) and its content is affected by ageing in photosynthetic tissues (Lizarazo, Fernández-Marín, Becerril & García-Plazaola, 2010). These antioxidants systems, very likely working together (Foyer & Noctor, 2005), may maintain the physiological integrity of seeds during long-term storage, needed to survival and ex-situ conservation (Fernandez-Marin et al., 2017). Finally, in photosynthetic tissues, ROS formation can be “controlled” or “prevented” in the light-harvesting complexes by the dissipation of light energy as heat through specific carotenoids (VAZ cycle) (Demmig-Adams, 1990). The reduction in the pool of all these antioxidants has been associated with injury and ageing, and therefore, loss of viability in DT tissues and seeds (Berjak, Sershen, Varghese & Pammenter, 2001; Illing, Denby, Collett, Shen & Farrant, 2005)

while their content is positively correlated with that of chlorophyll in dry seeds (Fernandez-Marin et al., 2017).

The capacity to survive in the dry state over the time, in addition to the development, biochemistry and physiology of the tissue, also depends on environmental factors (Walters et al., 2005; Hoekstra, 2005; Ballesteros et al., 2017). Moisture, temperature, light and gas environment (i.e. the presence of oxygen) are the more determinant factors in the ageing of DT seeds, pollen and fern spores and photosynthetic tissues in the dry state (Harrington, 1963; Roberts, 1960; Buitink, Walters, Hoekstra, & Crane, 1998; Walters et al., 2005; Groot, Surki, de Vos & Kodde, 2012; Steiner and Ruckenbauer, 1995; Telewski & Zeevaart, 2002; Gasulla et al., 2009; Roquiero et al., 2010; Fernandez-Marin et al., 2013; Ballesteros et al., 2018, 2019; Fernandez-Marin et al., 2019). When a DT tissue dries, its cytoplasm solidifies entering in a physical state known as amorphous solid or glass (Leprince & Buitink, 2010; Walters et al., 2010). When a glass is formed, molecules and cell components are entrapped in a solid matrix that suppresses most molecular diffusion and mobility, hence impeding most chemical reactions (Leprince & Buitink, 2010; Walters et al., 2010; Fernández-Marín et al., 2013, 2016). But glasses are not thermodynamically stable over time and tend to relax depending on the molecular mobility allowed in a process known as physical ageing (Roudaut, Simatos, Champion, Contreras-López & Le Meste, 2004; Walters et al., 2010). Intermolecular porous along with heterogeneities are created during the glass formation (Roudaut et al., 2004; Walters et al., 2010). In addition, some vibrational and rotational mobility is still possible in atoms, bonds and groups of atoms (Roudaut et al., 2004; Ballesteros & Walters, 2011). The type and amount of porous and the molecular mobility within the glass is strictly dependent on moisture and temperature. When moisture and temperature increase, the free volume of the glass increases, incrementing the molecular mobility allowed and expanding the intermolecular porous (Ballesteros & Walters, 2011). Besides, translational diffusion of gases and small solutes (such as oxygen and ROS) is still possible and will likely increment with moisture and temperature increase (Roudaut et al., 2004). Furthermore, as mentioned above, the excitation of macromolecules such as chlorophyll by light and their change in redox status is also possible. Hence, in these solid but dynamic structures, the increment of molecular mobility among the different types of substrates and the diffusion of gases within the glass due to environmental changes will be key in the deterioration rate of the diverse dry tissues.

Understanding the diversity of mechanisms that allow long-term survival in the dry state is important to understand the resilience of DT plants to environmental changes, and hence their ecological distribution and tolerance to events such as climate change. In addition, this knowledge is key to improve genebank procedures (particularly for short-lived germplasm such as photosynthetic seeds and fern spores) and expand plant *ex situ* conservation options for diverse DT tissues (Li & Pritchard, 2009; Roquiero et al., 2010; Ballesteros et al., 2018, 2019; Colville & Pritchard, 2019). The diversity of behaviours in plant reproductive structures brings also an opportunity to discover molecules and mechanisms that regulate survival to desiccation and life in the dry state in DT tissues, opening the doors to new biotechnological applications.

In this work we have focused on fern spores as the model study. Among ferns, two kinds of spores may be found: chlorophyllous spores (CS) which have fully developed chloroplasts after maturation and dispersion and usually short longevity, and non-chlorophyllous spores, without chloroplasts at maturity also characterised by their longer longevity (Lloyd & Klekowshy, 1970). We have previously investigated the eco-physiological diversity of DT among CS (Lopez-Pozo, Ballesteros, Laza, García-Plazaola & Fernández-Marín, 2019a), as well as the influence of moisture and temperature in the variation of longevity of CS (Ballesteros et al., 2017, 2019) and have found that preservation of plastid integrity plays a key role in the survival of chlorophyllous fern spores when dry (Lopez-Pozo, Gasulla, García-Plazaola & Fernández-Marín, 2019b). Understanding what the role of the photosynthetic system is across CS species showing diverse longevity, can be key to understand what modulates DT, ageing and loss of viability along time in the dry state on these and other photosynthetic tissues. Based on the biochemical and biophysical mechanisms exposed above and since the spores used in this work are chlorophyllous, we hypothesize that light exposure will determine viability loss during storage conditions and that this cell deterioration will be greater in the presence of oxygen. We also hypothesize that the faster deterioration will be related to an increase of ROS and a decrease in the antioxidant machinery, which will ultimately shape photochemical functioning and germination. For this purpose, CS of two fern species with different behaviour in the dry state (Lopez-Pozo et al., 2019) were subjected to four different storage regimes: light + normoxia, light + hypoxia, dark + normoxia, and dark + hypoxia. Lipophilic and hydrophilic antioxidants, ROS, photochemical performance and photosynthetic pigments were assessed in parallel to the loss of viability over a

remarkably long storage-period of up to 22 months. Our results support the relevant role of the antioxidant system for long-term preservation in the dry state, depict oxygen as key factor and reveal important interspecific differences for storability.

7.2. Material and methods

7.2.1 Plant material

Fertile fronds of *Osmunda regalis* were obtained from the surroundings of the Millennium Seed Bank, Wakehurst, UK, (51°03′57"N, 0°05′17"W) in summer of 2017. *Matteuccia struthiopteris* fertile fronds were received from the Botanical Garden of the University of Innsbruck Austria (47°16′N, 11°23′ E, 600 m a.s.l.) in December of 2016. *Equisetum telmateia* horsetails were growing along Gobelas river, Getxo, Spain (43°20′32 ″N, 3°0′0 ″W), sampling was done in March of 2017.

After collection, fronds were kept in the laboratory allowing the dehiscence of the sporangia and the release of the spores. Once the spores were released, they were stored under different conditions explained below.

7.2.2 Experimental design

After sporangia dehiscence and prior the beginning of the experiment, spores were dried at 15% RH (with saturated LiCl salt solution) and 4ºC for 24 hours (Ballesteros and Walters 2007a). Under this RH, spores are expected to enter in the glassy state where chemical and enzymatic reactions are highly limited (Ballesteros and Walters 2007a, Fernandez-Marin et al., 2013). After this first 24 h, spores were placed on open Petri dishes (85 mm diameter) covering the entire surface and kept inside hermetic chambers at 25 ± 2 °C for the long-term storage (note that RH inside the chambers decreased from 15% RH to 11% RH due to the increase of temperature from 4ºC to 25ºC (Ballesteros and Walters 2007a). The spores were finally long-term stored at four different regimes, resulting from the combination of oxygen and light conditions, until germination dropped below 50% in all of them. This happened after 659 d in *M. struthiopteris* and after 302 d in *O. regalis*. Long-term storage conditions were:

A: Light and normoxia | B: Light and hypoxia | C: Darkness and normoxia |D: Darkness and hypoxia

The photosynthetic photon flux density (PPFD) under the light conditions was 200 µmol m^{-2} s^{-1}, with a photoperiod of 16 h. Hypoxia was achieved by replacing the oxygen inside the chambers with gas N_2. For dark conditions chambers were placed inside cover bags that totally blocked light penetration. Relative humidity and temperature were constantly monitored inside the chamber.

Viability (as % of total germination and as recovery of the maximum photochemical efficiency of photosystem II (PSII): Fv/Fm), ROS production, (O_2^- and H_2O_2), hydrophilic antioxidant content (glutathione, GSH+GSSG) and photosynthetic pigment and tocopherol contents were measured before and at different storage times. Three parallel sets of samples were collected at each timepoint: one for germination tests, a second for Fv/Fm assessment, and a third one was immediately immersed in liquid N_2, preserved at –80ºC.

7.2.3 Water content determination

Water content (WC) of spores was determined gravimetrically during the first week until this was stable. After the spores achieved a stable WC no more samples were taken with this aim. Three samples of 5–10 mg of spores from each treatment were taken for WC determination. After fresh mass determination, spores were dried in an oven at 80 °C for 72 h for dry mass (DW) determination; water contents were expressed on a DW basis (g H_2O g^{-1} DW).

7.2.4 Viability

Viability of the CS was assessed along the storage period at each sampling time by two approaches: (i) through the evaluation of their ability to germinate (a 'gold standard' technique for assessing health and viability of preserved plant propagules including fern spores (Ballesteros et al., 2019) and (ii) through the assessment of photochemical efficiency of PSII (Fv/Fm) upon imbibition, which represents a promising new procedure for assessing viability of chlorophyllous spores (Lopez-Pozo et al., 2019).

Germination assay

In order to obtain samples with viability ranging from maximum (i.e. that obtained when spores were freshly harvested) to zero, the sampling period of the experiment was adjusted according to the expected ageing rate for the species in the temperature used in our experiments (e.g. Ballesteros et al., 2017, 2019).

Spores were sown into four 60 mm diameter Petri dishes filled to half depth with Dyer's culture medium for fern spores solidified with 1.3% agar and prepared with the fungicide nystatin (100 U mL^{-1}) (Ballesteros et al., 2017). Petri dishes were sealed with Parafilm (American National Can) and placed in a germination chamber set at 20 ± 2°C with 12 h light/dark cycles (white light with photon flux density ca. 50 W m2) (Ballesteros et al., 2017, 2019). Germination was scored when the outer wall of the spore ruptured the spore wall and the rhizoid or the first chlorophyllic cell emerged (Ballesteros et al., 2017, 2019; Lopez-Pozo et al., 2019). This was observed using a dissecting microscope at x40 magnification. Between 100-200 spores were located and scored per Petri dish, and the number of germinated spores per observed spores per dish was recorded throughout the germination period. Spores were selected randomly in each Petri dish, often using several fields of view within each dish. Viability was usually assessed near days 7, 10 and 15 (Ballesteros et al., 2017, 2019). When viability was similar among days assessed for each storage treatment and Petri dish, the average of the two largest numbers was considered total germination, which usually occurred between days 10 and 15 (Ballesteros et al., 2017). If viability at day 15 was significantly higher that that found at days 10 and 7, only values from 15 days were used as the viability for that storage treatment and Petri dish (Ballesteros et al. 2019). After verifying no effect among Petri dishes in a treatment (usually arising from contamination), data among Petri plates within a treatment were pooled, to calculate the proportion of germinating spores (Crawley, 2007) for each storage duration and treatment.

Chlorophyll fluorescence

Pulse Amplitude-Modulated Fluorimeter (PAM 2500, Walz, Effeltrich, Germany) was used for chlorophyll fluorescence measurements. 10 mg of spores were placed in petri dishes with agar medium and allowed to rehydrate under the same conditions used for germination. The optical fibre was fixed off to keep the same distance to the samples in all measurements as described in Lopez-Pozo et al., (2018, 2019). The minimum chlorophyll fluorescence (Fo) was determined in dark-adapted (≥30 min) spores. The maximum chlorophyll fluorescence (Fm) was induced with a saturating pulse for 500 ms. The variable chlorophyll fluorescence (Fv) was calculated as Fm–Fo. The ratio Fv/Fm represents the maximum photochemical efficiency of photosystem II (PSII). Measurements were done at 1h, 6h, 12h and 24 h after rehydration.

7.2.5 Biochemical analyses.

Approximately 10 mg of fresh material was used for GSH+GSSG, O_2^- and H_2O_2 analyses, samples were ground on ice using a pestle and mortar and due to the high resistance of the spore coat, a glass cover slide was used as an abrasive to facilitate grinding. All of these biochemical assays were performed in triplicate for each species, treatment and time-point storage. Methods used to measure each parameter are described below.

HPLC analysis of glutathione and glutathione disulphide

Spores were ground as described above, extracted in 1ml of 0.1M HCl with 10mg polyvinylpolypyrrolidone (PVPP) and centrifuged at 20 min at 20,000 × g at 4°C. The PVPP was imbibed in 1ml of 0.1M the day before the extraction. The supernatant was then used to determine both GSH and GSSG as described by Bailly and Kranner (2011). Briefly, this procedure uses fluorescence labelling of thiols with monobromobimane (mBBr). GSH+GSSG were determined after reduction of disulphides by dithiothreitol (DTT). For disulphides determination, thiol groups were blocked with N-ethylmaleimide (NEM). As well as glutathione, this method can also measure the low-molecular-weight thiols cysteine, cysteinylglycine and γ-glutamyl-cysteinyl. However, only glutathione was detected in the spores. Standards of glutathione at different concentrations were prepared to construct calibration curves.

GSH and GSSG were separated by reversed-phase HPLC on an HiQsil RP18 column (150x2.1mm i.d., 3µm particle size; KyaTech), and detected fluorimetrically (excitation λ: 380 nm; emission λ: 480 nm) with a gradient elution of 0.25% (v/v) acetic acid in distilled water at pH 3.9/methanol. Calculation of $E_{GSSG/2GSH}$ followed the formulas given in Schafer & Buettner (2001) and Kranner et al., (2006) using the Nernst equation:

$$E_{GSSG/2GSH} = E^{O'} - \frac{RT}{nF} ln \frac{(GSH)^2}{(GSSG)}$$

Where R is the gas constant (8.314 JK-1 mol-1); T, temperature in K; N, number of transferred electrons; F, Faraday constant ($9.6485x10^4 Cmol^{-1}$); $E^{o'}$, standard half-cell reduction potential at pH 7 [$E^{o'}_{GSSG/2GSH}$ = -240mV]; [GSH] and [GSSG] are molar concentrations of GSH and GSSG, estimated using the different water contents WCs.

Quantification of superoxide radical

Superoxide (O_2^-) was measured at the beginning and the end of the storage time, as described by Bailly and Kranner (2011), with some modifications. Ten mg of spores were grounded and homogenized with 2 mL of 50 mM potassium phosphate buffer (pH 7.8) and centrifuged at 16.000 g for 15 min at 4ºC. 1 mL of the supernatant was mixed with 1 mM hydroxylamine hydrochloride and incubated at 25°C for 30 min. Then, 0.5 mL of 17 mM sulphanilamide and 0.5 mL of 7 mM α-naphtylamine were added to 0.5 mL of the reaction mixture and incubated at 25°C for 30 min. After incubation and centrifugation at 13.000 g for 10 min, absorbance was measured at 540 nm. O_2^- concentrations were calculated using a calibration curve obtained with solutions of various concentrations of sodium nitrite.

Quantification of hydrogen peroxide

Hydrogen peroxide was measured as in Junglee et al., (2014). Grounded spores were homogenized with 1 mL of solution containing 0.25 mL Trichloroacetic acid (TCA) 0.1% (w:v), 0.5 mL KI (1 M) and 0.25 mL potassium phosphate buffer (10 mM) at 4˚C. Good care was taken to protect samples and standards from light and heat. The homogenate was centrifuged at 12,000 × g for 15 min at 4ºC. The absorbance of the supernatant was measured spectrophotometrically at a λ of 350 nm. H_2O_2 content of the samples was estimated using a calibration curve obtained with solutions of H_2O_2 concentrations prepared in 0.1% TCA.

Determination of photosynthetic pigments and tocopherols

Approximately ±10 mg of freeze-dried spores per replicate were used for the quantification of photosynthetic pigments and tocopherols at the beginning of the experiment and during long-term storage. Freeze-dried samples were homogenized with a mill and were doubly extracted, first in acetone:water (95:5) and second in pure acetone, both buffered with $CaCO_3$. Finally, extracts were centrifuged at 16100 g for 20 min and supernatants were filtered through a 0.2 µm polytetrafluoroethylene filter (Teknokroma, Barcelona, Spain) before being analysed by HPLC. Extracts were injected (15 µL) in a reverse-phase Waters (Milford, MA, USA) HPLC system following the method of García-Plazaola & Becerril (1999) with the modifications described in García-Plazaola & Becerril (2001). PDA detector (Waters model 996) was used to measure photosynthetic pigments in the range 250-700 nm, and peaks were detected and integrated at 445 nm.

Pigments were identified and quantified by the method described by García-Plazaola & Becerril (1999). Retention times and conversion factors for pigments were the same as described by García-Plazaola & Becerril (1999, 2001).

2.6 Statistical analyses

Statistical differences among pigments, Fv/Fm, germination and glutathione with respect to storage conditions and time were analysed by one-way analysis of variance (ANOVA) after assessing data homoscedasticity. Alternatively, the Kruskal-Wallis test was used for heteroscedastic data. All statistical analyses were assessed at $p<0.05$. The SPSS v23 statistical package was used for the statistical analyses.

7.3-. Results

7.3.1-. Loss of viability

Initial germination percentages were 94±1% and 99±0% for CS of *Osmunda regalis* and *Matteuccia struthiopteris,* respectively. Germination over time was adjusted to a GLM, with logic function for germination (Fig.7.1). During long-term storage at 11%RH and 25ºC, total germination, as well as Fv/Fm, decreased significantly in all storage conditions and between both species (Fig 7.1). The loss of viability was much faster in *O. regalis* than in *M. struthiopteris* in all treatments. Within treatments, "A" led to the faster deterioration of CS of both species. Thus in this treatment, the total loss of viability was complete after just 125 days for *O. regalis* and 225 days for *M. struthiopteris*. On the other extreme, and according to the germinability of the spores treatment D appeared as the less severe. Hypoxia and darkness maintained spores viability during more than 302 days in *O. regalis* and 659 days in *M. struthiopteris*. After this period, both species still maintained a germinability of 25%±2 and 43±3% respectively. Treatments B and C showed the highest variability, both in Fv/Fm and in germination values, but with significant differences at the end of the experiment in *O. regalis* only. The trends observed in the recovery of Fv/Fm mimicked those of germination. Interestingly, the drop in Fv/Fm occurred earlier than in germination in both species.

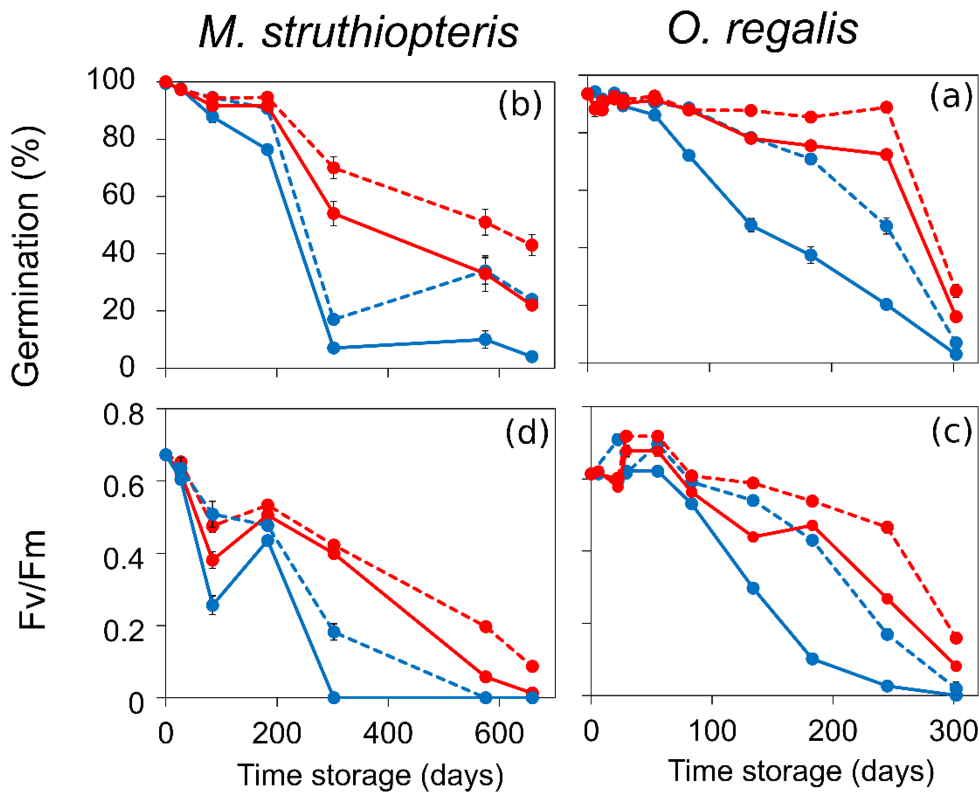

Fig. 7.1: Changes in germination percentage (a, b) and Fv/Fm (c, d) of CS spores stored at 25ºC and 11%RH. Storage conditions are represented as follow: Blue colour represents a normoxia atmosphere. Red colour represents hypoxia atmosphere. Solid lines represent light conditions. Dotted lines, dark conditions. Each point represents the proportion of germinating spores in a sample of 100–400 observed spores ± SE for panels a and b and mean±SE for panels c and d (n=3).

Not only germination capacity was lost during the course of the experiment, also a progressive slowing in the reactivation of photosynthesis after rehydration was observed in CS (Fig 7.2, Fig S7.1). Thus, during the first days of dry storage, *O. regalis* did not show significant differences in T_{50}. With the progression of the long-term storage differences among treatments became more marked and at the end of the experiment T_{50} of the spores stored in condition "A" was 19.5 hours while it was 10.2 hours for treatment D (Fig. 7.2, b). *M. struthiopteris*, on the other hand, showed a totally different behaviour. The ability to absorb water in this species declined and it was impossible to measure Fv/Fm except for the spores under D treatment. Because of this, the analysis of the reactivation rate of Fv/Fm at all the time-points was possible only in the "D" treatment. (Fig 7.2). In general, the CS of this species required longer reactivation times of Fv/Fm.

In control samples T_{50} was 3.2 ± 0.2 hours, while *O. regalis* required only 1.8 ± 0.2 hours to achieve 50% of Fv/Fm.

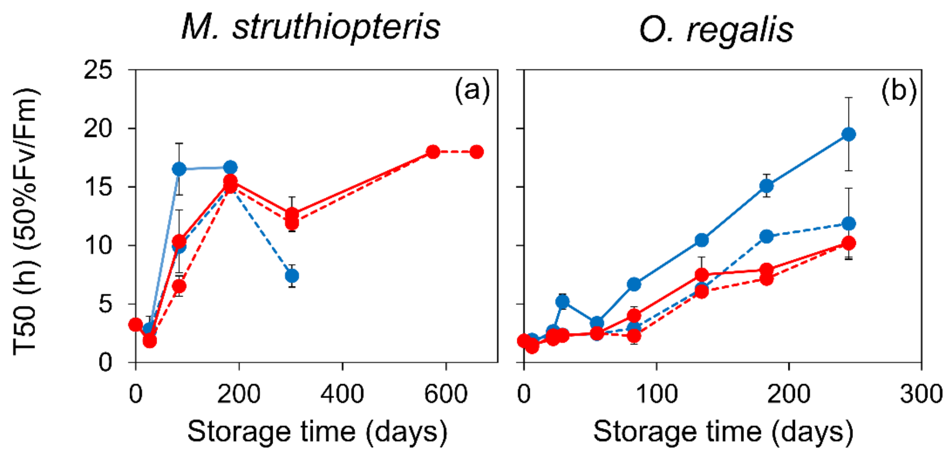

Fig.7.2: Estimated t50 values (hours) for CS of *M. struthiopteris* and *O. regalis* during dry storage at 25ºC and 11%RH. Storage conditions are represented as follow: Blue lines represent a normoxia atmosphere. Red lines represent hypoxia atmosphere. Solid lines represent light conditions. Dotted lines, dark conditions. Each point represents mean±SE (n=3).

ROS levels and antioxidants activity during dry storage of CS

Oxidative status of CS of the two fern species just after drying and at the end of the dry storage was measured in terms of $O_2^{\cdot-}$ and H_2O_2 production. Any of the ROS analysed under each storage condition showed significant differences between the beginning and the end of the experiment ($P < 0.05$). This means that ROS production was not treatment dependent, being the presence of these oxidants independent from light or oxygen or the combination of both.

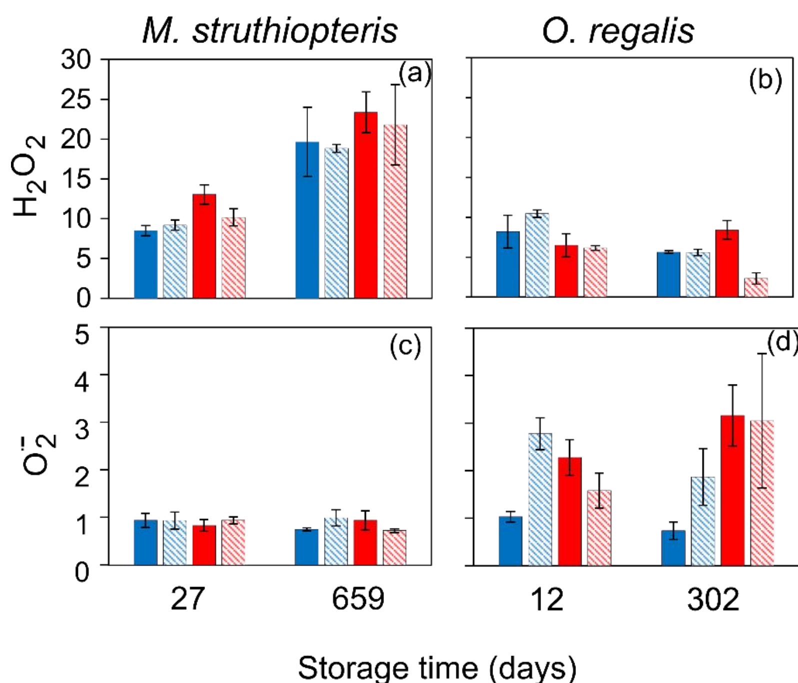

Fig 7.3: Hydrogen peroxide (H_2O_2) (µmol gDW^{-1}) (a,b) and superoxide ($O_2^{\cdot-}$)(µmol gDW^{-1}) (c,d) levels of *M. struthiopteris* and *O. regalis* spores. First and last time points are represented. Storage conditions are represented as follow: Blue bars represent a normoxia atmosphere. Red bars represent hypoxia atmosphere. Solid bars represent light conditions. Dotted bars represent dark conditions. Each point represents mean±SE (n=3).

During dry storage the amounts of GSH in *M. struthiopteris* did not show any pattern consistent with the storage condition ($P<0.05$) (Fig. 7.4). Even so, there was a downward trend in the amount of GSH compared to control values along time. The control values of the CS *of M. struthiopteris* were three-fold higher than the values at the end of the experiment. In the case of *O. regalis*, it seems that two trends can be observed. Those treatments with oxygen showed fewer amounts of GSSG, especially from day 183. After this point, the CS stored in nitrogen suffered a slight increase before falling again. There were no significant differences between treatment A, B and C, instead, treatment D showed significant differences.

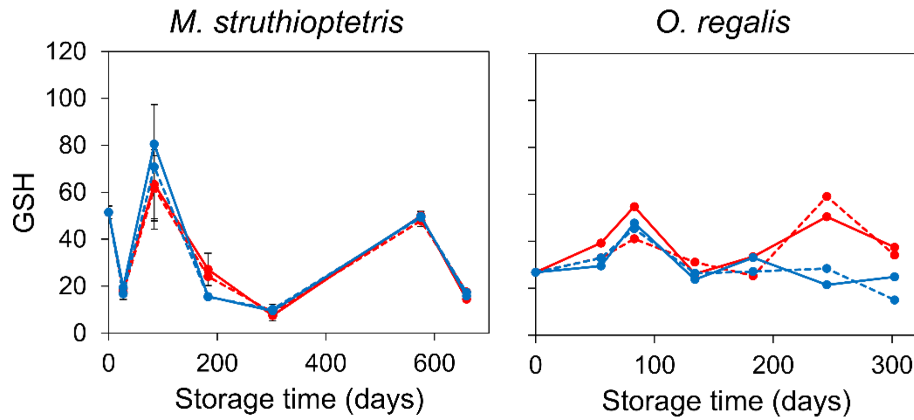

Fig 7.4: Changes in GSH (nmol GSH gDW^{-1}). Storage conditions are represented as follow: Blue lines represent a normoxia atmosphere. Red lines represent hypoxia atmosphere. Solid bars represent light conditions. Dotted lines represent dark conditions. Each point represent mean±SE (n=3)

The lipophilic pigments and antioxidants were measured before storage and during the course of the experiment. Initially, both species presented differences with respect to the concentrations of carotenoids, chlorophylls and tocopherols, with CS of *M. struthiopteris* showing the highest proportions. Over the dry storage time, there was a degradation of most of the lipophilic pigments and antioxidants in both species, although the degradation rates were different (Fig 7.5).

The amount of total chlorophyll under the different storage conditions was not significantly different from the control ones in any of the species studied. Amount of chlorophyll a+b remained constant in both species during dry storage in all treatments (Fig 7.5, a, b). Instead, total carotenoids (A,Z,L,V,a-car,b-car, N) suffered a decrease along time and showed significant differences between all treatments in both species. In chlorophyllous spores of *M. struthiopteris* significant differences were found after 302 days of storage, while only 83 days were needed in *O. regalis* to differ in total carotenoids. This decrease was greater in light and oxygen conditions (A), and also in *M. struthiopteris* CS (Fig 7.5. c, d). Among total carotenoids, L suffered the greatest decrease during the course of the experiment. This carotenoid showed, once again, a treatment effect, being D treatment the one that maintained the highest proportion of this carotenoid (Fig. 7.5 e,f). The same time was necessary to see differences in lutein contents with respect to the storage condition. α-tocopherol instead, needed more storage time to show an influence of the treatment. Significant differences between treatment A and D were found in this antioxidant contents for both species, although these differences were manifested at

different days of storage. *M. struthiopteris* needed 302 days to suffer a treatment effect, whereas only 245 days were necessary for *O. regalis*.
It is worth noting that in *M. struthiopteris*, in addition to the most common tocopherols (α, δ, γ, β) plastochromanol-8 (PC-8) was detected in considerable amounts. PC-8 suffered a significant decrease in light and oxygen conditions and in dark and oxygen conditions. Not only that, but also the differences observed in this antioxidant, were manifested immediately after starting dry storage. PC-8 was the fastest antioxidant that responds to the storage condition. Whit only 56 days of storage significant differences could be observed (Fig. 7.5, i).

Several relations were found between germination and other parameters. In the case of Fv/Fm, this correlation was statistically significant and well correlated (Fig 7.6,a), indicating that both parameters can be used as an indicator of viability in CS. Besides, Fv/Fm shows a faster decline during dry storage (Fig 7.1), what could be interpreted as damage in photosystems before the germination is affected. Glutathione half-cell reduction potential and germination also showed a clear negative sigmoid correlation. The less negative these potentials were, CS presented the less germinability. Between -175 and -165 mV was the decrease in germination for both species. Values above -180mV were related to healthy CS, while at -140mV germination was not possible. Finally, it seems that *O. regalis* CS were in a grater "stressed state" both, at the beginning of the experiment (-140mV; 94% germination for *O. regalis*, and -202mV;100% germination for *M. struthiopteris*) and during dry storage (compare open and closed points) (Fig. 7.6, b). Total carotenoids also correlated very well with germination percentage, with a positive slope, between both parameters (Fig 7.6, c). Finally, the positive correlation between PC-8 and the viability of *M. struthiopteris* CS was clear. As germination was compromised, the amount of PC-8 dropped significantly (Fig. 7.6, d)

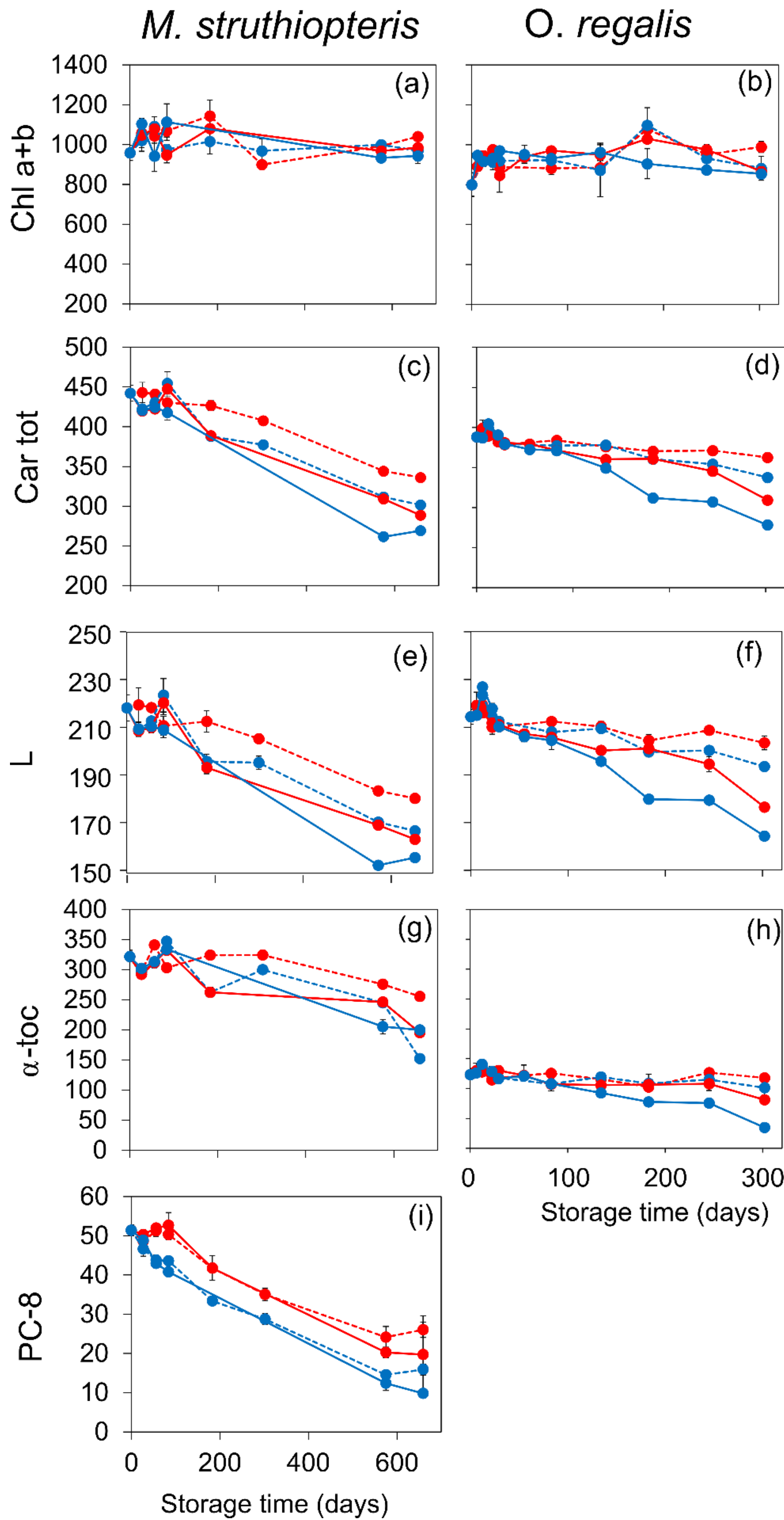

Fig 7.5: Changes in lipophilic pigments and antioxidants during storage at 25ºC and 11%RH. Chla+b (nmol g DW^{-1}) total carotenoids (car tot), lutein (L), a-tocopherol (a-toc) and plastochromanol-8 (PC-8) (mmol mol^{-1} Chla+b). Storage conditions are represented as follow: Blue lines represent a normoxia atmosphere.

Red lines represent hypoxia atmosphere. Solid bars represent light conditions. Dotted bars represent dark conditions. Each point represent mean±SE (n=3)

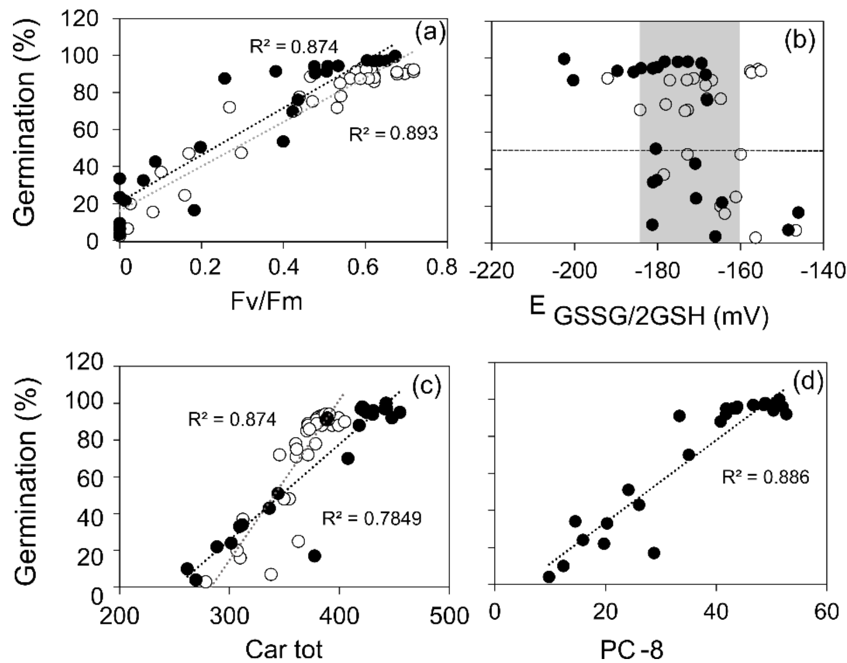

Fig 7.6: Correlations between germination and Fv/Fm (a), germination and GSH potential redox (b) , germination and total carotenoids (mmol mol^{-1} Chla+b) (c) and germination and pastocromanol-8 (mmol mol^{-1} Chla+b) (d). Open points correspond to *O. regalis*. Closed points represent *M. struthiopteris*. All correlations were significant at p<0.001.

7.4 Discussion

Seeds and spores are dispersal units, and because of this they should be able to persist extended periods under unfavourable growing conditions (Mène-Saffranéa, Jonesa & DellaPennaa, 2010). The oxidative stress occurred during drying and during long-term storage, cause seed ageing what will finish in seed deterioration and death (Chen et al., 2016). In the present work, we elucidated those mechanisms that enable the viability maintenance during dry storage of CS under different light and oxygen regimes.

Results showed that when stored dry, CS of *O. regalis* aged more rapidly than *M. struthiopteris* CS (Fig.7.1a, b). The viability observed in these two types of CS were in concordance with previous works (Lopez-Pozo et al., 2019, Ballesteros et al., 2017, 2018, 2019, Lloyd & Klekowski, 1970). Moreover, the storage condition affected CS viability

to a great extent, being oxygen and light the elements that most compromise the capacity of CS to germinate (Fig 7.1). When green seeds of *Salix nigra* were exposed to light, also a greater deterioration was done (Roqueiro et al., 2010). The decrease observed in germinability of both species was accompanied with a decline in Fv/Fm along time (Fig.7.1c,d) and it was done slightly earlier than in germination, which can be due to the damage in PSII. Due to the presence of a photosynthetic apparatus in CS the oxidative stress derived from the interaction with light could be exacerbated. Similar results were found in CS of Equisetum hyemale (Lebkuecher, 1997) and CS compared to non-CS (Ballesteros et al., 2018)

When the viability begins to be compromised, not only Fv/Fm values decreased, but it also, slowed down. As seen in Fig. 7.2, the longer CS were kept in dry storage conditions, the more time they needed to recover T_{50}. In addition, from treatment a to D, the time for Fv/Fm recovery also increased. This delay in metabolism reactivation has been widely observed in DT photosynthetic tissues (Protcor, 2003, 2010). In this works, bryophytes dried for different periods recovered Fv/Fm at different rates, taking more time those that were desiccated for longer times. Two theories explain this phenomenon: it seems that the surfaces of ferns and bryophytes in the dry state increase their hydrophobicity, which would lower the speed of absorbing water. On the other hand, the damage produced in the photosystems during long-term storage would compromise Fv/Fm recovery (Proctor, 2009). With our results, it can be concluded that both events occurred during the course of the experiment; the increase in hydrophobicity and the decrease in viability. Since Fv/Fm and germination show a strong correlation (Fig 7.6), the fall in T_{50} over time (Suplementary) is due to the loss of viability. But, on the other hand, the increase in hydrophobicity compromises and lengthens the recovery of Fv/Fm. In the case of *M. struthiopteris* CS, the hydrophobicity is very high, almost impermeable, and much higher than in *O. regalis* (Lopez-Pozo et al., 2019). Therefore, we can expect that during dry storage the surface of CS of this species will become more water repellent. Finally, no water uptake was possible (Fig 7.2, b).
This ageing is the result of many injuries based on non-enzymatic reactions. Among these reactions the most common are the glycosylation of proteins, generation of ROS and other free radicals and lipids peroxidation. Even in the glassy state, these reactions take place, causing oxidative damage to membranes, proteins and DNA (Smolikova et al., 2011). The ability to persist during long time without compromising the viability will depend on

the antioxidant defence. The defence plays the central role and the inefficient antioxidant system will finish in oxidative damage accumulation, ageing, and finally the death of the organism (Kranner, 2010). The decrease in its activity has been widely associated with loss of viability in several seeds (Franchicni, 2006). The differential ageing observed in treatments was not related to ROS production (Fig. 7.3) an these did not show significant differences with the storage condition, which suggests that there is no ROS production control or that these ROS were being scavenged. In contrast, strong differences were observed regarding the antioxidant activity. The reduction in the major antioxidants during dry storage was associated to the loss of viability especially under light and oxygen conditions.

The chloroplast is considered one of the major sources of ROS due to the imbalance done between the energy absorbed and used by the photosynthetic apparatus when water is absent (Kranner et al., 2002, 2008; Illing et al., 2005; Heber et al., 2006; Farrant, 2007). Chlorophyll may, direct or indirect, enhance the ROS production compromising viability (Roqueiro et al., 2010). In the case of CS of *M. struthiopteris* and *O. regalis*, there was not detected a degradation in this pigment during dry storage not even under light conditions (Fig. 7.1 a, b), what gives the idea that chlorophylls (or photosystems) immersed in the membranes are heavy protected from photoxidation. It has been proposed that some carotenoids may be quenchers of excited pigments when these are in triplet state (Krinsky, 1994) and they seem to be particularly relevant in seeds with presence of chlorophyll (Smolikova et al., 2011). Before placing spores under the different storage conditions, both species differed in their lipophilic pigment and antioxidant composition. The higher amount of carotenoids and tocopherols in *M. struthiopteris* CS stablish bigger photoprotective status, as was observed in López-Pozo et al., (2019). In contrast to chlorophyll behaviour, the concentration of total carotenoids was significantly different under the storage conditions used in this work for *M. struthiopteris* and *O. regalis* (Fig 7.5). The major difference in the consumption of carotenoids was found under light and oxygen conditions. Not only that, but also a significant correlation with germination was found (Fig. 7.6) in agreement with the role of carotenoids in stabilizing the photosynthetic membranes and scavenging ROS (Seal et al., 2010). Among all carotenoids, several studies have related the consumption of lutein to an increase in oxidative stress and its fundamental role as an antioxidant in seeds (Franchini, 2006) as we have observed in CS of both species (Pinzino et al., 1999). Besides, is also the major carotenoid, consistent

with the data obtained by other authors (Smolikova et al., 2011). Not only some lipophilic pigments play a central role as antioxidants, but tocotrienols and tocopherols are also known to be indispensables to survive desiccation and long-term storage (Kranner, Seal, Ballesteros). It has been demonstrated that the absence or decrease of tocochromanols increases lipid oxidation. Lipid oxidations are initiated during seed desiccation and are amplified during seed dry storage compromising the viability (Mène-Saffrané, 2010). In several seeds, a strong decrease in α-tocopherol resulted in poor viability, concluding that it is responsible for the viability maintenance along time in dry conditions (Seal et al., 2010). Instead, other works showed a constant level of α-tocopherol during both, desiccation and ageing in seeds. Our data showed consumption of this antioxidant during seeds ageing, but other antioxidants were probably more responsible for this defence than α-tocopherol.

Besides, only CS of *M. struthiopteris*, which also showed the highest viability in the dry state, displayed PC-8 (Fig 7.5) and a very strong correlation between this antioxidant and germination percentage (Fig. 7.6). Instead, we did not detect PC-8 in *O. regalis* CS. As commented, the protection functions of tocopherols and tocotrienols are well documented but little is known about PC-8 functions in vivo. Few studies have detected this antioxidant in plants (Olejnik, Gogolewski & Kalucka, 1997) and only one has established the importance of PC-8 in seed ageing and germination (Mène-Saffrané et al., 2010). The decrease in α-tocopherol and total carotenoids (especially lutein) in both species, and the decrease in PC-8 in *M. struthiopteris* support the conclusion that an effective lipid antioxidant system is necessary for CS viability along time.
The antioxidant capacity through water-soluble antioxidants did not show significant differences with the storage condition in terms of GSSG (Fig. 7.4). There is a strong controversy about the behaviour of glutathione in the dried state. While in some studies it increases, in others it decreases, not being an accurate marker for viability (Kranner et al., 2006). On the other hand, the redox potential of glutathione does not seem to present as much variability. The correlation between redox and germination in CS resembled those values found in other studies, demonstrating once again its value as a feasibility marker. E GSSG/2GSH was well correlated with germination (Fig 7.6) showed a range where the viability of CS was lost. This range was between -175 and -165 mV and the same transition zone have been proposed for plant, human and fungal cells (Kranner, 2006, Seal et al., 2010). Due to this antioxidant is not membrane immersed, likely its

function in the stabilization of the membrane is not important, and lipid-soluble antioxidants displayed this function in CS. These decrease in the majority antioxidant compounds were also observed in green spores under regimes of light and dark conditions, being once again, the light condition decreased more these compounds (Ballesteros et al., 2018).

7.5 Conclusions

Our results support the hypothesis that an efficient antioxidant system would prevent loss of viability, we demonstrated that both oxygen and light conditions are ageing promoters in CS. In order to ensure the greater long-term viability of CS, conditions of darkness and hypoxia would be necessary for both *M. struthiopteris* and *O. regalis*.

Acknowledgements

CHAPTER VIII

GENERAL

DISCUSSION

Desiccation tolerance in the Plant Kingdom

The conquest of emerged land took place around 500 m.y.a. when a clade of freshwater green algae started the colonization of terrestrial habitats (Niklas, 2010) and continued with the development of the early land plants like bryophytes, pteridophytes and finally, gymnosperms and angiosperms. With the progressive increase in complexity, the capacity of plants to retain water also improved. Consequently, most terrestrial plant and animal life has escaped to desiccation stress by imposing the physical barriers to water loss. However, there are species of animals, plants and microbes that do tolerate complete desiccation. These are known as desiccation tolerant (DT) species (Alpert, 2005).

Vegetative desiccation tolerance (DT, also meaning 'desiccation tolerant') is formally defined as the ability to recover biological functions from an air-dry state in equilibrium with an atmosphere at or below 50 % relative humidity (RH) (Alpert, 2005; Proctor *et al.,* 2007). Drying to equilibrium with even moderately dry air is instantly lethal to most species of animals and plants, making water availability one of the most important ecological factors and evolutionary pressures on terrestrial life. Many of the DT plants are found in the phylogenetically basal clades that constitute the algae, bryophytes and lichens (Proctor and Tuba, 2002; Oliver *et al.,* 2005). However in vascular plants (pteridophytes, gymnosperms and angiosperms) is a rare trait (Porembski and Barthlott, 2000) in their vegetative tissues, but their spores, seeds, and pollen still maintain DT feature (Dickie and Prichard, 2002; Tweddle *et al.,* 2003; Illing *et al.,* 2005). The basal DT species (ie, algae, bryophytes and lichens) exhibit a type of desiccation tolerance, believed to be primitive that allows for the survival of even rapid rates of drying (Oliver *et al.,* 2000). Because of their early evolution, these organisms have very limited capacity of withdrawing water from substrate, and therefore the water required for their metabolic processes is mostly derived from rainfall or from water flowing over the plants. These are known as poikilohydric plants and the main difference with vascular plants is the lack of control over water loss (Proctor and Tuba, 2002). Many poikilohydric organisms are subjected to large variations in their water content, and they must be able to withstand desiccation, nevertheless, DT is not inherent to poikilohydric organisms (Green *et al.,* 1991), and a considerable number of mosses, liverworts, and algae (Bewley and Krochko 1982) die if desiccate (Chapter 4).

This capacity to "drying without dying" depends on internal and external factors. External factors can be summarized in the speed of desiccation and the length of these conditions. Organisms require a certain time to activate protection mechanisms so the cellular damage is high when the water loss is fast (Proctor 2003; Pressel and Duckett, 2010; Stark *et al.,* 2013). Similarly DT mechanisms cannot be active for ever, requiring hydration periods to regenerate, thus the longer is the period in the dry state, the higher is the damage and only the more tolerant species are able to survive long desiccation periods.

From this perspective, bryophytes is one of the most tolerant group, being most species highly DT (Cruz de Carvalho *et al.,* 2012). However is difficult to compare the magnitude of DT among different species. To solve this limitation we develop a method was developed for the quantitative estimation of DT in different types of organisms, the so-called "Falcon Method". For its development, more than 38 species of bryophytes and angiosperms were tested, and actually, their DT was high for most cases (18% of the tested bryophytes were DS, in contrast to the 96% that were DT) (Chapter 4). This method was based on desiccations at three different humidities that induce three speeds of desiccation (RHs: =80%, =50% and<10%). The relative humidities are achieved by equilibrating the atmospheric humidity with different salts solutions absorbed by an absorbent material that avoids the contact of sample with the liquid. The degree of desiccation tolerance is established using the percentage of recovery of the maximal photochemical efficiency of PSII (Fv/Fm) after rehydration. The whole procedure is shown in Figure 8.1. The amount of plant material can vary from 50 to 200 mg without changing the results, and minimal instrumentation is required. These features, make the protocol particularly suitable for its use in remote locations. This procedure of desiccation allowed us to discriminate between fast desiccation tolerant and fast desiccation sensitive among bryophytes and between DT and DS species among tracheophytes (Fig 4.5)

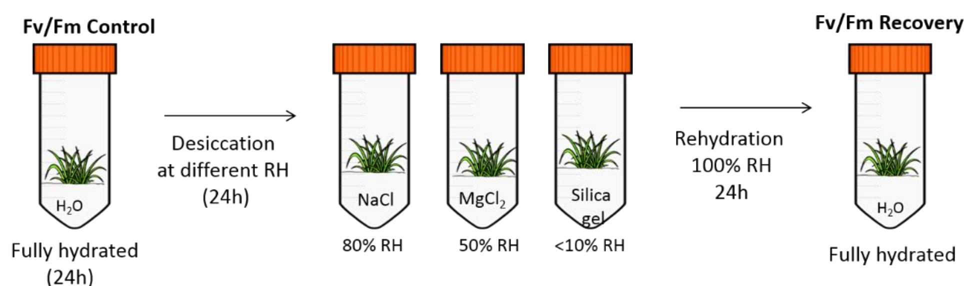

Figure 8.1: Schematic representation of the "Falcon method"

Fern spores as model for desiccation tolerance study

Pteridophytes (ferns) represent an intermediate evolutionary position that encompasses several features that make them an ideal model to study DT. Their life cycle is formed by three stages of development with different hydric behaviours. Gametophytes are poikilohydric and resembled the physiology of a non-vascular plant. The lack of cuticle, stomata or hydraulic tissues limits the control of water loss. On the contrary, the sporophyte is vascular, develops complex tissues and has mechanisms to regulate water loss.

In general, both gametophyte and sporophyte, display the characteristic DT of non-vascular plants and the desiccation sensitive (DS) of vascular plants, respectively. The life cycle is closed by the production of spores by the sporophyte that once germinated give rise to the gametophyte generation. In these unicellular spores, the mechanisms of DT can be studied in a simpler model than in the case of seeds or complex vegetative tissues (Fig. 8.2).

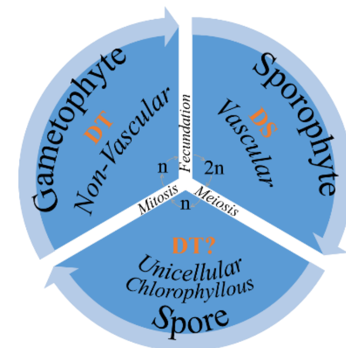

Figure 8.2: “Ferns as model to study DT in photosynthetic cells”

Ferns spores can be classified into two types according to the presence or absence of chlorophyll: non-chlorophyllous and chlorophyllous spores, respectively. The first are common, highly DT, lack chloroplasts and are unable to photosynthesize. On the other hand, chlorophyllous spores (CS) have a fully developed photosynthetic apparatus and can perform photosynthesis once they are hydrated (Sundue *et al.,* 2011). This type of spores is rare among ferns although more frequent in ancestral fern groups, while among bryophytes is more common. Nevertheless, their DT is considered to be very low. Instead, the most evolved ferns almost do not develop CS, but if do it, they are highly DT (Lloyd and Klekowsky, 1972). In somehow, the DT of chlorophyllous spores follows the opposite phylogenetically way than in vegetative tissues (Lloyd and Klekowsky, 1972; Whittier, 1996; Lebkuecher, 1997; Berjak and Pammenter, 2008). As bryophytes, they can tolerate rapid and slow dehydration (Fig 5.2) and they can be considered poikilohydric, due to the lack of control over water loss. Finally, they display the common mechanisms developed by both, vascular and non-vascular plants DT plants as this thesis has demonstrated.

Are fern chlorophyllous spores truly DT?

Despite the poikilohydric behaviour of fern CS and its relation with DT, among the studied species there were not only DT, but also DS. Thus, the CS of *E. telmateia* developed a very low capacity to tolerate desiccation, being classified as low DT even a DS species (Fig 6..1). The sensitive behaviour to desiccation observed in *E. telmateia* resembles that found in recalcitrant seeds (Berjak and Pammenter, 2008; Walters, 2015). Other species of the genus *Equisetum* also develop lesser degree of DT compared to other fern CS (Ballesteros *et al.,* 2011). In fact, this species did not achieved during desiccation, the WC to consider it DT (WC<0.1gH_2O gDW^{-1}). Instead, chlorophyllous spores of *O. regalis* and *M. struthiopteris* were both DT and achieved WC values below 0.1gH_2O gDW^{-1} (Chapter 5, Chapter 6, Chapter 7). During the recovery of Fv/Fm significant differences were found, being in agreement with literature (Ballesteros *et al.,* 2018, 2017), the CS of *M. struthiopteris* more DT than the CS of *O. regalis*. Overall, given the variable degree of DT among fern CS, they not only provide valuable information on the mechanisms of DT in photosynthetic tissues, but also can be used as models to study the processes underlying DS.

OK, you are DT, but for how long?

The ability to tolerate desiccation is also affected by the duration of the desiccation period itself. Thus, the longer is this period, the more compromised the survival will be (Ellis and Roberts, 1980: Ellis and Hong, 2007). The capacity to dry without dying is a common feature in orthodox seeds and non-chlorophyllous spores, but its presence in chlorophyllous propagules is much more unusual (Ballesteros *et al.,* 2007; Berjak and Pammenter, 2008). Under the premise that the spores of the three studied species are

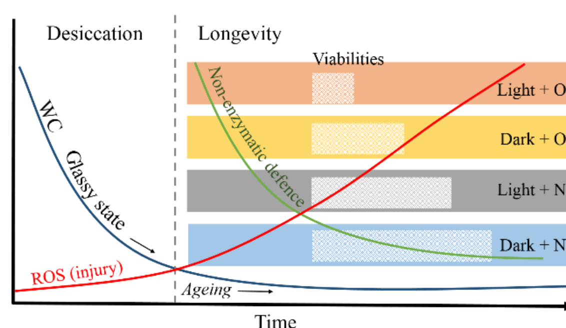

Figure 8.3: Schematic representation of the physiological processes happening during desiccation and storage of studied fern spores, and of the changes in longevity along time, under four different storage conditions.

chlorophyllous, we hypothesised that storage with light and oxygen would induce a faster ageing than that under conditions of hypoxia and darkness, because of the generation of light induced oxidative damage (Hendry, 1993) (Fig. 8.3). To assess how these factors could compromise the viability of CS of *O. regalis* and *M. struthiopteris* in a long-term storage an experiment was designed combining conditions of normoxia/hypoxia and light/darkness (Fig. 7.3). As we had established before (Chapter 5, 6), both species showed a **different response to desiccation and we tested if their longevity** also differed under different storage conditions over the time. During the dry storage, CS of *O. regalis*, lost viability much faster than in *M. struthiopteris* in all treatments. Specifically, the treatment of light and normoxia was the most damaging for both species (Fig.7.1). Thus, faster ageing was observed when spores were stored under these conditions, probably due to the presence of photodynamic chlorophyll that interacts with light even when desiccated.

It is important to point out that during these long-term treatments, not only viability decreased with the storage time, but also the speed of metabolism reactivation after rehydration was affected. This delay in the reactivation time has been observed in several DT taxa (Protcor 2003, 2010) and typically increases with the duration of storage time. Proctor (2009) suggested two theories to explain these changes in the recovery of metabolisms rates; an increase in the hydrophobicity that reduces the speed of absorbing water, and the damage suffered by the photosynthetic apparatus during dry storage. In agreement with the first theory, the hydrophobicity of *M. struthiopteris* increased during dry storage, particularly under light and oxygen conditions, to so high extent that we the spores were not able to imbibe in 48 h time window (Fig. 7.2).

The hydrophobicity of spores in their sporangial state was also quite different between species and increased along time. The capacity of *O. regalis* to absorb water was two-fold higher than in *M. struthiopteris* when spores were released from sporangia as evidenced with the water droplet contact angles measured (Fig 6.5). Presumably, spores decreased their wettability during the course of the experiment, but in parallel the cumulative chloroplast damage compromised the recovery of Fv/Fm to a greater extent than the hydrophobicity. Finally the two species differed greatly in the time required for a complete loss of viability: 659 days in *M. struthiopteris* and 302 days in *O. regalis*. For both of them, dark and hypoxia conditions preserved the viability for longer periods.

Chlorophyllous and DT: advantageous in which ecological context?

Desiccation tolerance is strongly related to environmental and habitat conditions. Thus, in xeric habitats, where water availability is erratic and dehydration is a common phenomenon, plants might have evolved a larger capacity to dissipate excess energy and deal with water stress. On the other extreme, the species that have evolved in wet habitats, where water is always available, will become severely damaged because the capacity of recovery after desiccation is not a requirement for living in such habitats. In between both extremes, mesic habitats include species that could or not survive to the desiccation, especially if it is fast (Deltoro *et al.,* 1998). For example, among ferns, the gametophyte phase is considered to be DT (Watkins, 2007). They can be considered as poikilohydric plants due to the lack to control water loss, they are usually exposed to wide changes in their water contents (Pittermann *et al.,* 2013; Watkins *et al.,* 2007). Despite this assumption, we found that the gametophytes of *O. regalis* were not able to withstand desiccation (Fig. 5.2, b), although those of the fern *Davallia canariensis* were DT (Fig. 1.2), as shown by the fact that during a cycle of desiccation and rehydration, Fv/Fm was able to fully recover once the water was again available. Interestingly these two ferns differ in their ecological requirements of their sporophyte phase: *O. regalis* grows in wet habitats while *D. canariensis* is an epiphytic fern whose gametophytes will develop in a xeric ambient and it will be exposed to different degrees of desiccation. Consequently while the gametophytes of *D. canariensis* must develop DT, those of *O. regalis* will grow in an environment where water is abundant, making DT an unnecessary adaptation. In general, although DT is in principle quite common in the gametophytic phase of ferns, its frequency in the sporophyte is rare (Watkins *et al.,* 2007) as occurs in the case of *O. regalis* whose sporophytes live in the river banks of temperate climates, where DT is completely unuseful. On the contrary, DT is known for sporophytes of several species (Fernández-Marín et al. 2008, Eicmeier, 1979, Stuart, 1968, Oppenheimer and Halevy, 1962) and it was demonstrated in several ferns from xeric habitats (ie. South Africa) during the development of the Falcon method (Chapter 4).

The relationship between DT and environmental conditions in the natural habitat was also observed in the CS (Chapter 6). Thus, apparently the capacity to withstand desiccation was consistent with the water availability at the time of the sporulation. The three species studied here grow in mesic habitats, but the sporulation season differed among them. Thus, *E. telmateia* releases its spores in early spring, when precipitation is abundant, *O.*

regalis does it in late spring when the temperature is higher and precipitation lower. Finally, *M. struthiopteris* sporulates in winter, in latitudes were snows are common and where usually the sporophyll can be covered by snow. In conclusion, the three types of spores developed a different degree of DT according to the prevailing climatic conditions of each microhabitat and season (Fig. 6.1).

And, what do you need to be DT?

DT plants have a series of strategies to face water loss and to be able to survive in an ambient where water is scarce (Wood, 2005). The function of these mechanisms is usually to preserve the cellular integrity, preventing the disruption of ultrastructures (particularly preventing the loss of membrane integrity and changes in configuration of macromolecules such as enzymes) and to counteract the oxidative damage that results from the generation of reactive oxygen species (ROS) and free radicals (Dinakar *et al.,* 2012). To deepen into the operation of such mechanisms we have explored their role in the unicellular CS.

Turn-off the metabolism…

Upon dehydration the cell viscosity increases and the tissues enter to the so-called "glassy state" which is a highly viscous liquid that resembles a solid but maintains the disorder and physical properties of the liquid state (Buitink and Leprince 2008; Fernández-Marín *et al.,* 2013; Leprince and Buitink 2015; Walters 2015; Ballesteros *et al.,* 2017). The process through which a fluid becomes an amorphous solid is known as vitrification (Buitink and Leprince 2008). The development of this state depends on water content, temperature and chemical composition. When cytoplasm enters in the glassy state, there are not enough water molecules to form a monolayer around macromolecules. It has been suggested that glasses increase the stability of enzymes and proteins and decrease detrimental reactions inside the cell (Leprince and Buitink, 2007). Furthermore, Fernández-Marín *et al.,* (2013) showed that enzymatic reactions do not take place during the glassy state. Because of that, the metabolism is close to zero, preventing damage caused by the generation of ROS linked to electron transport chains and by the damaging interactions between molecules, which are immobilized (Leprince and Buitink, 2007).

Consequently, the glassy state could be understood as an adaptive strategy to face desiccation in anhydrobiotic organisms.

Most of DT organisms are able to deal with the mechanical forces that appear during the loss of water and the subsequent formation of the glassy state (Buitink and Leprince, 2008), but it is not clear how this situation helps in the overcome of DT. In this sense, we observed that CS differed among species in their degree of stability once the glassy state was reached (Fig 6.3). The species with the most prominent differences with the other two was *E. telmateia*. Thus, its CS had lower α-relaxation (Tg) (39.8 for *E. telmateia*, compared to the 43.5, and 42.2 for *M. struthiopteris* and *O. regalis* respectively). Even though, all spores studied were in the glassy state (below their respective Tg) at moderate temperatures (below 40 ºC). This implied that under most conditions, no chemical reactions were expected, due to the restriction of molecular mobility (Ballesteros and Walters, 2011; Fernandez-Marin *et al.,* 2013). However it should be noted that an increase in G` just a couple of degrees above the Tg was observed and this has been related to molecular mobility even in the glassy state (Ballesteros and Walters, 2011). Several inter- and intramolecular rearrangements may be happening inducing microstructural changes and changing the stability of dry systems (Herrera-Kao and Aguilar-Vega, 1999; Menard, 1999; Champion *et al.,* 2000; Roudaut *et al.,* 2004; Ballesteros and Walters, 2011). Several authors evidenced that the formation of a stable glass is a prerequisite to DT (Buitink and Leprince 2008; Walters *et al.,* 2010), but the capacity to enter in a stable glass does not imply the DT feature. On the light of our own results, we concluded that the unstable glassy state of CS of *E. telmateia* is related to its DS (Fig. 7.3). Instead, the CS of the other two DT species have a very stable glassy state where no molecular mobility is expected. All these results suggest that this vitrification is crucial in the long-term preservation of CS.

Cell walls and membranes walking together…

Another consequence of the progressive loss of water is the loss of turgor (Fernández-Marín *et al.,* 2016). As the cells begin to dehydrate, both membrane and cell wall start to shrink, and the more water is lost, the risk of rupture of the membrane-wall cell interactions increases. Therefore, the correct folding of the cell wall avoids the mechanical and biochemical damage that can occur during desiccation, thereby

preserving cellular integrity in many species (Rascio and La Rocca, 2005). For this purpose, cell walls must be extremely flexible, as most of DT plants displayed compared to sensitive ones (Moore *et al.,* 2008). The parameters that best describe the ability of the cells to maintain turgor are water potential at turgor loss point (Ψ_{TLP}), water potential at full turgor (Ψ_O), and the modulus of elasticity (ε) (Lenz *et al.,* 2006; Ding *et al.,* 2014). It seems clear that those species able to tolerate, at least, drought, have lower values of Ψ_O and Ψ_{TLP} values than those sensitive to water deficit (Bartlett *et al.,* 2012). This means that at lower water contents, they can maintain higher turgor pressure and in fact, higher values of Ψ_{TLP} were found in the DS species (Fig 6.4). By inferring the value of water potential at the time of sporulation (Ψ_{SPO}) it was shown that *O. regalis* is the only CS that was released in a non-turgid state and also presented the most flexible walls (ε=3.4 ± 0.9) (Fig 6.4). Curiously, *M. struthiopteris* presented the opposite cell wall behaviour, with an ε of 7.8 ± 0.6. Regarding ε, there is no consensus; some authors have concluded that more flexible walls can help in the maintenance of turgor with lower water contents (Schulte, 1992). But also, more rigid walls would help to recover the turgor faster in organisms subjected to desiccation cycles (Bidussi *et al.,* 2013; Alam *et al.,* 2015). These differences in the degree of walls elasticity can be seen in the SEM photographs (Fig. 6.6). While CS of *O. regalis* has rounded and smooth undulations, cracks and abrupt shapes appear on CS of *M. struthiopteris*. Probably both spores are using different strategies; the maintenance of turgor is done through the fold of the wall in *O. regalis* CS and through compatible solutes (osmotic adjustment) in *M. struthiopteris* CS. *E. telmateia* and *O. regalis* showed 2-fold and 7-fold lower proline contents, respectively, when compared to *M. struthiopteris* (Table 6.3) .Finally, with the aim to known how spores will respond to water absorption once released from the sporangium, we analysed the wettability of the spores. The most hydrophobic CS were those of *M. struthiopteris*, followed by *O. regalis* and *E. telmateia* (Fig. 6.5). The latter changed the drop contact angle from 127° ± 3° to 60° ± 6 in only 10 seconds. No changes could be appreciated in CS of *M. struthiopteris*. These differences among species were related to the environmental conditions in the sporulation season. Thus, it seems that water absorption is allowed only in those ideal situations, which will eventually result in spore germination. Consequently, germination under unfavourable conditions is prevented by a high hydrophobicity.

Not only the above-mentioned membrane-cell wall interactions are important to preserve cellular structures, but also membranes itself have to maintain its integrity. Since these spores contain a photosynthetic apparatus, which is largely a membrane structure, maintaining the integrity of the chloroplast thylakoids will be a requirement to tolerate desiccation. Three types of membranes, outer and inner envelopes and thylakoids, compose these organelles. In thylakoids, where photosystems are immersed, the main galactolipid is the monogalactosyldiacylglycerol (MGDG) (Gounaris and Barber, 1983). This molecule has an unstable conic-shape that can compromise membrane stability as it does not form bilayer structures (Garab *et al.,* 2016). The second most abundant galactolipid is the bilayer-forming digalactosyldiacylglycerol (DGDG), with a cylinder-shape that contributes to membrane stability (Gasulla et *al.,* 2019). These two galactolipids interact with all light-harvesting and electron transport proteins and the MGDG/DGDG ratio has been positively related to photosynthesis, chlorophyll content and photoassimilates (Jarvis *et al.,* 2000; Dörmann *et al.,* 1995; Steffen *et al.,* 2005). The risk of MGDG is that when water is scarce it tends to form inverted hexagonal II structures that finally lead to the fusion or fission of membranes (Sprague *et al.,* 1987; Webb *et al.,* 1991). Instead, DGDG shape avoids these disruptions due to the major stability. Another two galactolipids have been proposed to contribute to the maintenance of the integrity and stability of chloroplast membranes under stress conditions: trigalactosyldiacylglycerol (TGDG) and tetragalactosyldiacylglycerol (TeGDG), with three and four galactose residues, respectively (Gasulla et *al.,* 2009). These compounds are derived from the glycosydation of DGDG and commonly known as oligogalactolipids (OGLs) (Heemskerk *et al.,* 1983).

Under stress conditions, thylakoids composition is drastically remodelled. Thus, the ratio MGDG/DGDG decreases, and this decrease is paralleled by an increase in the amounts of TGDG and TeGDG. Besides the unstable situation that MGDG may provoke, the downregulation of this galactolipid is associated to a lower photochemical activity, which ultimately may alleviate generation of oxygen reactive species. In other words, will reduce the oxidative risk. It has been proposed that the synthesis of OGLs occurs via the SFR2 enzyme (Moellering *et al.,* 2010), that catalyses the transgalactosyation from MGDG to other galactolipid to form OGLs. This reaction was first found in Arabidopsis in response to cold (Moellering *et al.,* 2010) and subsequently found in many other

organisms exposed to different stresses, including desiccation, salt, and freezing (Moellering *et al.,* 2010; Gasulla *et al.,* 2013; Wang *et al.,* 2016; López-Pozo *et al.,* 2019). As mentioned above, of the three phases of the development of the fern *O. regalis*, only the spore is DT. Therefore, we perform a lipid analysis to investigate the galactolipids changes associated to the rapid loss of DT during germination (Fig 5.6). This analysis revealed that CS of *O. regalis* constitutively contained high proportions of TGDG and TeGDG. However, when their DT was abruptly lost during germination, a decrease in both OGLs accompanied by an increase in major galactolipids was observed, supporting a role of OGLs in the stabilisation of thylakoids in the dry state.

Besides, the degree of fatty acid saturation seems to be another key feature for membranes stability during drying and has been negatively related to life in the dried state (Hoekstra, 2005). The double bonds interact more easily with reactive oxygen species causing lipid peroxidation and thereby polyunsaturated fatty acids are more prone to oxidative damage (North *et al.,* 1994; Stark *et al.,* 2005). In the case of CS of *O. regalis* the unsaturation degree increased with germination and the development of gametophyte, which could alleviate lipid oxidation in CS, especially in desiccated photosynthetic tissues. Besides, membranes with low proportion of polyunsaturated fatty acids have low molecular mobility, which could be an additional advantage in the dry state. Regarding the non-chloroplastic membranes, the accumulation of phosphatidylinositol (PI) seems to contribute to desiccation tolerance feature (Gasulla *et al.,* 2013; Gasulla *et al.,* 2016). Chapter 5 concludes that the germination of the spores and the subsequent loss of DT, was accompanied by a profound lipid remodelling, characterised by a decrease in PI, TGDG and TeGDG and an increase in MGDG. All these changes favour a greater photosynthetic capacity to the gametophyte, but compromise their viability if water is not available.

Dissipating energy…

Once the tissue is dry, even in the glassy state, several reactions that compromise its viability can occur. Autoxidation of lipids is one of the most common reactions that can take place during drying, and it is also the main sources of ROS (McDonald, 1999). Besides, this damage can be exacerbated by the presence of light as chlorophyll molecules can be excited even in the dry state (Roquiero *et al.,* 2010). Therefore, the first defence is to avoid the formation of ROS through an efficient photoprotective system. The mechanism of photoprotection consists in the thermal dissipation of the absorbed light

energy by chlorophyll, counteracting in this way photooxidative stress before the damage is generated (Herber *et al.,* 2006, García-Plazaola *et al.,* 2012). This process can be easily monitored by studying changes in chlorophyll fluorescence. Thus, once dried, variable fluorescence of DT non-vascular cryptogams decreases to values close to zero, while is maintained or even increases in DS organisms (Lange *et al.,* 1989; Harel *et al.,* 2004 ; Nabe *et al.,* 2007). In parallel, there is a strong decline in F_0 upon desiccation, while in DS species F_0 in the desiccated state reach values even higher than when water is present. The first process is due to the activation of quenching mechanisms while the second is caused by the generation of damage in the reaction centre of PSII (Fig. 8.4).

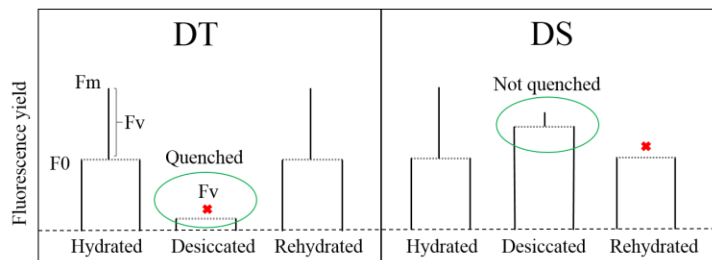

Figure 8.4: Schematic representation of NPQd in DT and DS species during a cycle of hydration-desiccation.(Based on Bilger et *al.,* 2014)

This mechanism is known as Non-photochemical Quenching due to desiccation (NPQd) and consists in the safely reemission of excess light from photosystems as heat during desiccation. This NPQd is associated with a quenching in basal chlorophyll fluorescence (F_0) and has been observed in most DT non-vascular cryptograms like algae, cyanobacteria, lichens, and bryophytes (Bilger, 2014). Apparently, DS organisms are not able to activate this NPQd and there is a general consensus in that NPQd is a key mechanism of photoprotection in DT plants (Rakic *et al.,* 2015). Thermal energy dissipation is usually related to the synthesis of zeaxanthin (Z) in the so-called xanthophyll cycle, where Z is formed from violaxanthin by the enzyme violaxanthin de-epoxidase through the intermediate antheranxanthin (Fernandez-Marin *et al.,* 2012). Nevertheless, the synthesis of Z and the activation of xanthophyll cycle is not an obligate requirement for the activation of NPQd (Heber 2008; Heber et al. 2010). This quenching of basal chlorophyll fluorescence during the loss of water is reversed once water is available again and Fv/Fm restored.

There are no reports about this kind of energy dissipation among DT vascular plants (Georgieva *et al.,* 2005), such as ferns or angiosperms. Our work may thus represent first

report of NPQd in reproductive structures of a tracheophyte (Fig.5.3). When the chlorophyllous spores of *O. regalis* were subjected to desiccation, a strong decline in F_0 was observed. Furthermore, the importance of this mechanism is shown by the fact that when the viability of the spores was compromised by desiccation, the attenuation of the chlorophyll fluorescence lowered. Interestingly NPQd was achieved in absence of Z synthesis. This is likely due to desiccation-induced conformational protein changes in the light-harvesting complexes (Coughlan and Schreiber, 1984). In agreement with the absence of NPQd in DS species, chlorophyll fluorescence was attenuated to a much lower extent in the CS of *E. telmateia* that are considered as "low DT" or even "DS" (Fig 6.2), while both DT species (*M. struthiopteris, and O. regalis)* displayed NPQd. All this together suggests that the thermal energy dissipation through NPQd in the absence of Z is activated in CS of tracheophytes dissipating a major fraction of the excess of light energy in the dried state. Interestingly NPQd has not been observed in other DT tracheophytes (ferns or angiosperms) such as *Polypodium vulgare* and *Haberlea rhodopensis* (Kopecky *et al.,* 2005; Georgieva *et al.,* 2005) that maintain a certain capacity of charge separation ($Fv/Fm>0$) even in the dried state. Several authors have pointed out that early land plants were pohikilohidric and DT, requisite indispensable for land conquest (Oliver *et al.,* 2000; Proctor *et al.,* 2007). It is likely that this ancient trait was lost during the course of the evolution and later other secondary mechanisms to deal with desiccation evolved. Fern chlorophyllous spores, small (unicellular) and poikilohydric, might represent an intermediate evolutionary state maintaining some of those ancestral mechanisms.

Coping with stress…

Finally, when an imbalance in the ROS production/consumption has already occurred, it is necessary to eliminate their excess. The overaccumulation of ROS compromises the viability during both desiccation and during the time that the organism remains desiccated. Consequently, an efficient antioxidant system is required to compensate this (Kranner *et al.,* 2010; Farrant, 2017). Considering that spores during dry-storage are in the glassy state, the antioxidant defences must be of non-enzymatic nature, including lipophilic and hydrophilic antioxidants (Fig. 7.4,7.5,7.6).

Curiously, no chlorophyll degradation was observed during long-term storage, what probably means that this pigment is strongly protected from photo-oxidative damage.

Some carotenoids have been proposed as quenchers of chlorophylls triplets, so in all likelihood, these molecules are the responsible for chlorophyll stabilization. In agreement with this pivotal role of antioxidants, their constitutive pools are higher in *M. struthiopteris* in terms of total carotenoids and tocopherols (Table 6.1, Figure 7.5). Not only in quantitative terms, but also a qualitative difference was found among these compounds: *O. regalis* only contains α-tocopherol, while *M. struthiopteris* possesses a more diverse set of lipophilic antioxidants including α, β, γ and δ, and plastocromanol 8 (PC-8) (Table 6.2, Fig. 7.5). The relevant role of PC-8 in preventing the accelerated ageing has been previously described in seeds (Mène-Saffrané *et al.,* 2010) and it seems that plays a central role in the antioxidant protection during long-term storage. In CS seems to play a similar role, at least in *M. struthiopteris* as indicated by the strong correlation between PC-8 and viability of CS. During the course of the experiment, the degradation of the major carotenoids and lipophilic antioxidants mimicked that of Fv/Fm and viability, being greater under light and normoxic conditions. The protective action of lipophilic antioxidants is complemented with the protection given by the hydrophilic antioxidants, among which, glutathione is the most important in fern CS. As CS lost viability, the glutathione half-cell reduction potential ($E_{GSSG/2GSH}$) becomes more positive (Chapter 7, Fig. 6). We found a range of $E_{GSSG/2GSH}$ were viability started to decrease (Between -175 and -185 mV). Similar results (values are in the range -180 and -160 mV) have been observed for other plants, animal and human cells (Kranner *et al.,* 2006). It has been suggested that above –160mV the antioxidant capacity of glutathione is lost, leading ROS attacking randomly macromolecules and causing generalised oxidative damage (Schafer *et al.,* 2001).

Desiccation tolerance, ferns chlorophyllous spores and true plants…

The present study concludes that fern CS are an ideal model to study DT. The three studied species (*Osmunda regalis*, *Equisetum telmateia* and *Matteuccia struthiopteris*) can be understood as unicellular green tissues in all the aspects. The physiology of these CS is in essence the same as that of any photosynthetic plant. They present the characteristic pigment composition of vascular plants, with CS of *E. telmateia* displaying the highest Chl a/b ratio. It was also the only CS with Fv/Fm values higher than zero once it was released from the sporangium, which means it was potentially able

to perform photosynthetic electron transport. CS of *O. regalis* and *M. struthiopteris*, instead, contained a high amount of photoprotective pigments, as β-carotene and, tocopherols and proline. All these compounds are considered ROS scavengers, which means that these two species have more photoprotective capacity compared to *E. telmateia* that was able to activate more rapidly its metabolism (Table 6.1, 6.2). The physical-chemical changes given at the cellular level during desiccation (e.g. loss of turgidity, vitrification, remodelling of membranes...) are the same as those in DT vascular or non-vascular plants. In addition, its antioxidant systems (lipophilic and hydrophilic) also respond in the same way to oxidative stress. Finally, the selected species of CS displayed different degrees of DT, as can be observed in plants "*sensu stricto*". Everything mentioned above is given in a single cell. The changes observed are not interfered with by other tissues, so the results can be more clearly interpreted (that for instance in seeds).

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Future perspectives: Winter is coming…

Overall, fern CS have probed to be a useful tool to simplify the study of metabolic processes associated to the "miracle" of desiccation tolerance. Thus, after four years of research, there are many uncertainties that we have been able to resolve, but many new questions have also appeared. One of the most attractive discoveries of this work was the behaviour of the CS of *Matteuccia struthiopteris*. This fern develops the spores in winter and keeps them inside the sporophylls until the snow melts. During winter, the spores are exposed to temperatures below zero and the risk of freezing is really high. As freezing causes dehydration at the cellular level, we could wonder whether the spores of this fern, in addition to being desiccation tolerant, are also freezing tolerant. In a small assay conducted in collaboration with the Universidad de la República (Uruguay) we were able to determine that these spores contain both freezing and desiccation related proteins (data not shown). This cross-tolerance has been observed in other desiccation tolerant species such as the angiosperm *Ramonda myconi* (Fernandez-Marín *et al.,* 2018), and in all likelihood, this phenomenon also exists in fern chlorophyllous spores. So, the next step will be to define what shared or unique mechanisms allow the survival under a combination of stresses…

CHAPTER IX

GENERAL

CONCLUSIONS

The main findings provided by this work can be summarised as follows:

- **The "Falcon method" represents a useful procedure for the semiquantitative estimation of desiccation-tolerance in photosynthetic tissues.** The method, tested in bryophytes and angiosperms, is based on different rates of desiccation at several relative humidities and provides a comparative index, with a threshold of 30% recovery of Fv/Fm as indicator of desiccation tolerance. With minimal instrumental requirements is particularly useful in remote locations.

- **Fern chlorophyllous spores do not differ physiologically from photosynthetic cells of true plants.** Lipophilic pigments, tocopherols, membrane composition and their response to environmental factors are the same that observed in other vascular or non-vascular plants.

- **Fern chlorophyllous spores can be desiccation tolerant** photosynthetic organs, displaying several degrees of tolerance among different species. Once the spore germinates, desiccation tolerance is lost.

- **The vitrification is achieved during desiccation.** Desiccation tolerant fern chlorophyllous spores have a stable glassy state while desiccation sensitive species have intracellular movements even after vitrification.

- **Desiccation triggers a remodelling in thylakoid lipids that is needed to maintain membrane integrity in fern chlorophyllous spores.** The role of oligogalactolipids seems to be particularly relevant. Most of this remodelling is reversible upon rehydration, and subsequent loss of desiccation tolerance, during imbibition of spores.

- **Thermal energy dissipation in the dry state, through the activation of a specific Non-Photochemical Quenching, induced by desiccation plays a central photoprotective role in chlorophyllous spores.** This mechanism is only

activated in desiccation tolerant fern chlorophyllous spores but not in the desiccation sensitive ones.

- **Water relations in fern chlorophyllous spores follow the same pattern as in desiccation tolerant plants**. DT fern CSs show lower water potential at turgor loss point (Ψ_{TLP}) than DS ones. The turgor lost point in these species is reached at very low water contents.

- **For long-term storage, darkness and hypoxia delay ageing, indicating that the loss of viability in fern chlorophyllous spores is exacerbated by the presence of light and oxygen**. The parallel degradation of antioxidant compounds confirms the (photo)oxidative nature of ageing-related damage. Among these compounds, α-tocopherol, plastochromanol-8 and several carotenoids are particularly essential for the prevention of oxidative damage during long-term storage. The range of glutathione half-cell reduction potential in which the spores lose viability was the same as that found for other organisms.

- **Fv/Fm is a good non-invasive viability reporter** correlating significantly to germination. This finding is of particular interest for germplasm banks as it reduces the need to use plant material for germination tests to estimate viability.

- **The use of fern chlorophyllous spores as model to study desiccation tolerance provides a direct interpretation of the phenomenon**. In a single cell, all the mechanisms that allow the desiccation tolerance can be observed. Besides, the physiological changes done when this tolerance is lost also can be studied, due to the desiccation sensitive behaviour of the gametophyte.

CHAPTER X

GENERAL BIBLIOGRAPHY

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CHAPTER XI

SUPPLEMENTARY MATERIAL

Chapter 4:

Table S4.1: Meteorological information on the collecting sites. For *Case study I* all samples were collected in the same date November 2015 and data correspond to the 5 days prior to the sampling. For the *Case study II*, specific dates and locations are detailed: average temperature, accumulated precipitation and, if available, average RH of the 30 days prior to the sampling are specified. Royal Botanic Garden Edinburgh (UK) is abbreviated as RBGE, and University of the Balearic Islands (Spain) as UIB.

(1) *Note: For South Africa, the average of the month of sampling (January) over the last hundred years is provided.*

(2) *Potted plants: grown in the university garden and daily watered to field capacity*

(3) *Potted plants: grown in a growth chamber and daily watered to field capacity*

(4) *Plants in a glasshouse: well watered*

Case study	Site	Date	T (ºC)	Prec. (mm)	RH (%)	Species
I	**La Rioja**	**Nov-2015**	**3**	**15**	**87**	**All bryophytes**
II	**South Africa**	**Jan-2016**	**20.5**(1)	**11.4**(1)		**All tracheophytes from SouthAfrica**
II	**UIB**	**June-2016**	**22.83**	**Potted plants**(2)	**55.2**	***Quercus ilex***
		July-2016	**25.6**	**Potted plants**(2)	**53.2**	***Helianthus annuus*, *Juniperus oxicedrus***
		Sept-2016	**22.7**	**Potted plants**(2)	**69.8**	***Davalia canariensis***
		May-2017	**22.5**	**Potted plants**(3)	**40**	***Triticum aestivum, Barbacenia purpurea***
II	**Chile**	**Jan-2016**	**14.8**	**54.3**	**89.9**	**All tracheophytes from Chile**
II	**RBGE**	**July 2017**		**Glasshouse plants** (4)		**All tracheophytes from the RBGE (except for *Haberlea rhodopensis***
		July 2017	**14.9**	**181**		***Haberlea rhodopensis***

Table S4.2. Data per species and desiccation treatment of *Case Study I* regarding final absolute water content (WC) and relative water content (RWC) after dehydration plus average % of Fv/Fm recovery after rehydration. Note that all RWC achieved after desiccation fell below the threshold of 30%. The Fv/Fm percentages highlighted in green depict those values above the threshold of 30% considered in this work as to represent a significant recovery.

(1) FDS or FDT assigned according to previous literature as specified in Table 1

FDT or FDS [(1)]	*Especie*	Treatm.	WC (gH_2O g^{-1}DW)	RWC (%)	%Fv/Fm per treatment	Av %Fv/Fm
FDS	*Marchantia polymorpha*	NaCl	0,54	5	0	0
		MgCl2	0,09	1	0	
		Silica gel	0,04	0	0	
	Fontinalis antipyretica	NaCl	0,14	2	45	18
		MgCl2	0,06	1	7	
		Silica gel	0,00	0	1	
	Scapania undulata	NaCl	0,13	3	70	23
		MgCl2	0,07	2	0	
		Silica gel	0,03	1	0	
	Hookeria lucens	NaCl	0,19	2	54	24
		MgCl2	0,10	1	18	
		Silica gel	0,02	0	0	
	Lunularia cruciata	NaCl	0,18	2	75	25
		MgCl2	0,09	1	0	
		Silica gel	0,02	0	0	
FDT	*Syntrichia ruralis*	NaCl	0,13	5	94	92
		MgCl2	0,11	4	94	
		Silica gel	0,01	0	89	
	Frullania tamarisci	NaCl	0,19	13	94	94
		MgCl2	0,11	8	94	
		Silica gel	0,03	2	94	
	Polytrichastrum formosum	NaCl	0,38	26	101	97
		MgCl2	0,09	6	101	
		Silica gel	0,00	0	88	
	Porella platyphylla	NaCl	0,13	6	98	97
		MgCl2	0,06	3	97	
		Silica gel	0,00	0	97	
	Rhizomnium punctatum	NaCl	0,26	10	100	99
		MgCl2	0,10	4	98	
		Silica gel	0,02	1	99	

Table S4.3. Details on final water content after desiccation treatments and recovery of Fv/Fm after rehydration for each species in *Case Study II*. The Fv/Fm percentages highlighted in green depict those values above the threshold of 30% considered in this work as to represent a significant recovery. Note: in Case Study II, if only those samples with RWC below 30% are considered (last column) the number of false positives and negatives in the DT assignation is significantly reduced.

DT or DS [(1)]	*Especie*	Treatm.	WC (gH_2O $g^{-1}DW$)	RWC (%)	%Fv/Fm per treatment	Av %Fv/Fm	Av %Fv/Fm WhenRWC<30
DS	*Helianthus annus*	NaCl	0,51	23	0	0	0
		MgCl2	0,31	14	0		
		Silica gel	0,25	11	0		
	Acacia erioloba	NaCl	0,29	49	2	4	5
		MgCl2	0,17	22	4		
		Silica gel	0,01	1	6		
	Diospyros austro-africana	NaCl	0,22	19	4	6	6
		MgCl2	0,15	13	8		
		Silica gel	0,09	8	5		
	Montinia caryophyllacea	NaCl	0,45	10	2	7	7
		MgCl2	0,31	7	11		
		Silica gel	0,17	4	6		
	Oedera squarrosa	NaCl	0,36	21	10	10	10
		MgCl2	0,39	21	11		
		Silica gel	0,16	9	8		
	Quercus ilex	NaCl	0,17	19	11	10	10
		MgCl2	0,09	9	9		
		Silica gel	0,01	2	10		
	Maytenus oleoides	NaCl	0,32	13	7	11	11
		MgCl2	0,29	12	9		
		Silica gel	0,21	9	16		
	Angiopteris lygodiifolia	NaCl	0,78	18	16	11	11
		MgCl2	0,42	11	9		
		Silica gel	0,42	9	8		
	Boscia albitrunca	NaCl	0,92	51	22	20	19
		MgCl2	0,14	14	19		
		Silica gel	0,10	9	19		
	Enneapogon brachystachy	NaCl	0,83	27	26	27	27
		MgCl2	0,60	20	26		
		Silica gel	0,61	18	28		
	Triticum aestivum	NaCl	0,93	34	64	33	17
		MgCl2	0,59	14	23		
		Silica gel	0,18	2	10		
	Blechnum magellanicum	NaCl	1,04	48	66	36	21
		MgCl2	0,36	14	24		
		Silica gel	0,03	1	18		
	Davallia canariensis	NaCl	1,24	77	93	43	18
		MgCl2	0,32	29	23		
		Silica gel	0,17	12	12		
	Juniperus oxycedrus	NaCl	0,35	21	64	49	49
		MgCl2	0,14	9	42		
		Silica gel	0,12	7	39		
DT	*Barbacenia purpurea*	NaCl	0,39	24	4	9	9
		MgCl2	0,26	28	15		
		Silica gel	0,11	19	10		
	Xerophyta viscosa	NaCl	1,00	68	38	28	13
		MgCl2	0,44	35	13		
		Silica gel	0,11	10	33		
	Haberlea rhodopensis	NaCl	1,33	23	29	30	30
		MgCl2	0,56	13	37		
		Silica gel	0,47	11	25		
	Myrothamnus flabellifolius	NaCl	0,44	20	23	37	37
		MgCl2	0,26	14	37		
		Silica gel	0,39	13	50		
	Eragrostis nindensis	NaCl	0,98	20	49	46	46
		MgCl2	0,52	15	65		
		Silica gel	0,47	12	23		
	Craterostigma plantagineu	NaCl	0,30	4	95	51	51
		MgCl2	1,02	11	35		
		Silica gel	0,83	10	23		
	Bommeria hispida	NaCl	0,62	29	92	55	55
		MgCl2	0,35	12	33		
		Silica gel	0,17	8	41		
	Cheilanthes eatonii	NaCl	0,50	36	86	64	53
		MgCl2	0,36	22	52		
		Silica gel	0,22	11	55		
	Ramonda myconi	NaCl	0,48	10	84	64	64
		MgCl2	0,35	8	75		
		Silica gel	0,33	9	34		
	Mohria caffrorum	NaCl	0,32	13	49	77	77
		MgCl2	0,23	9	100		
		Silica gel	0,19	8	81		
	Astrolepis sinuata	NaCl	0,60	55	84	78	75
		MgCl2	0,26	29	77		
		Silica gel	0,06	5	72		
	Hymenoglossum cruentum	NaCl	0,35	21	97	85	85
		MgCl2	0,21	12	95		
		Silica gel	0,10	6	61		
	Asplenium aureum x ceter	NaCl	0,33	17	97	96	96
		MgCl2	0,13	8	95		
		Silica gel	0,15	6	95		
	Hymenophyllum dentatum	NaCl	1,34	59	100	97	97
		MgCl2	0,86	37	98		
		Silica gel	0,09	5	92		

(1) DS or DT assigned according to previous literature as specified in Table 1

Figure S4.1. F_v/F_m kinetic of 12-studied species during the "Falcon test" using three different rehydration procedures, as described in the text box. $t_{Control}$: turgor state after 24 h of incubation at one of the rehydration procedures. t_{Dh}: dehydrated state after 24-48 h of desiccation in a 50 mL Falcon tube with 12 g of silica gel. t_{Rh}: second rehydration state after 24 h of incubation at one of the rehydration procedures. Lower case letter: significant differences of F_v/F_m between rehydration procedures (n = 3; $P < 0.05$, one way-ANOVA with Duncan test as post-hoc, or Kruskal-Wallis in the case of heteroscedastic data, after testing normality with Shapiro-Wilk and equal variance with Levene tests). Full species names are depicted in the "Rehydration procedure" section of the Methods.

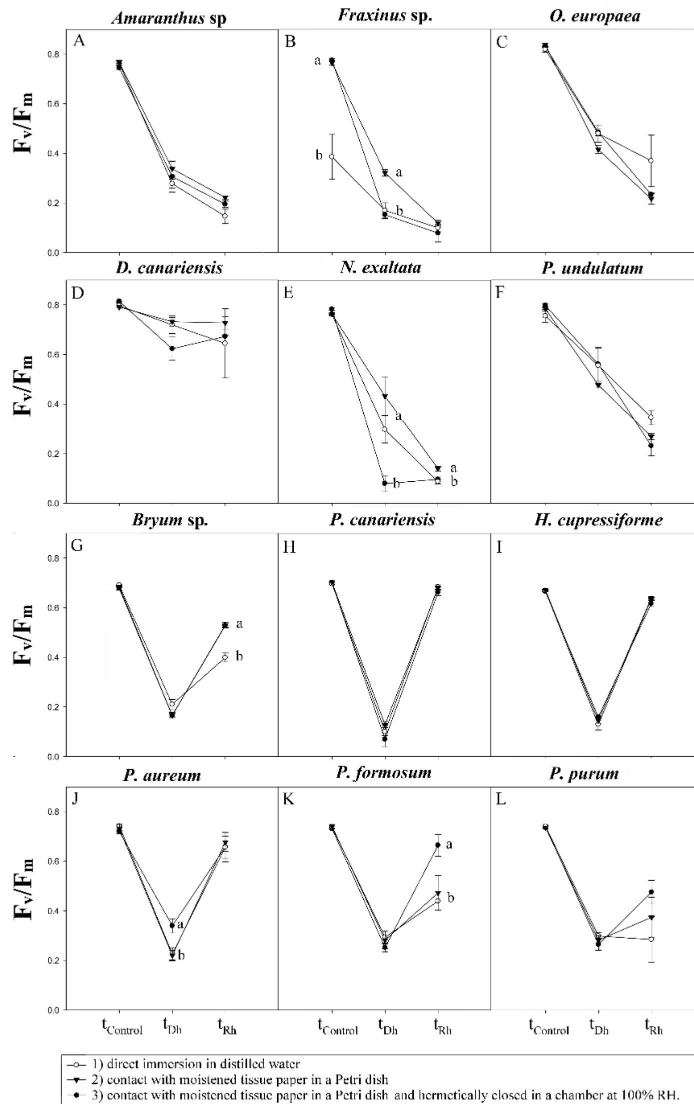
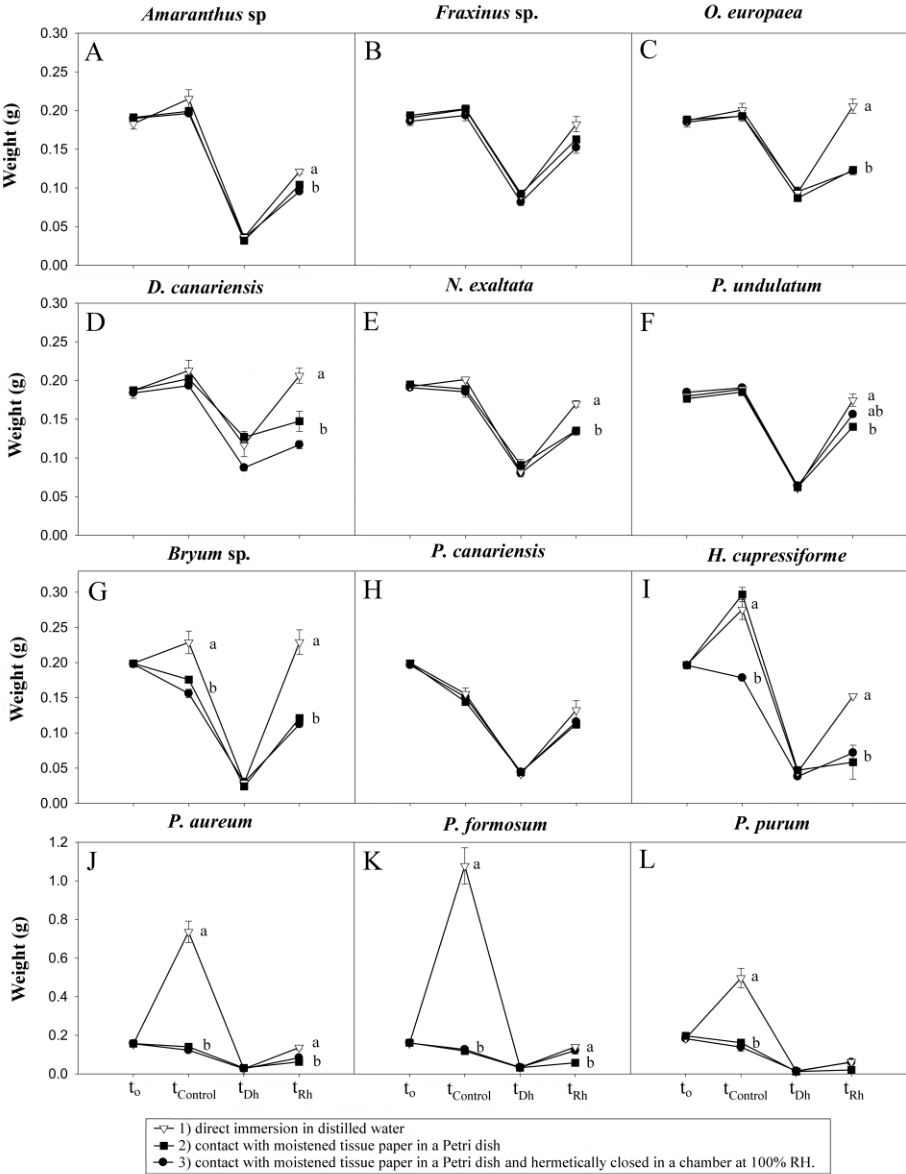


Figure S4.2. Weight kinetic of 12-studied species during the set up of the "Falcon test" procedure using three different rehydration treatments, as described in the text box. $t_{Control}$: turgor state after 24 h of incubation at one of the rehydration procedures. t_{Dh}: dehydrated state after 24-48 h of desiccation in a 50 mL Falcon tube with 12 g of silica gel. t_{Rh}: second rehydration state after 24 h of incubation at one of the rehydration procedures. Note that rehydration by immersion could increase the recorded weight of tissues, especially in bryophytes (panels G, I, J, K, L), probably due to the difficulty in removing high amount of interstitial liquid, overestimating water content of the tissues. Lower case letter: significant differences of weight between rehydration procedures ($n = 3$; $P < 0.05$). Statistical analyses as in Figure S1.



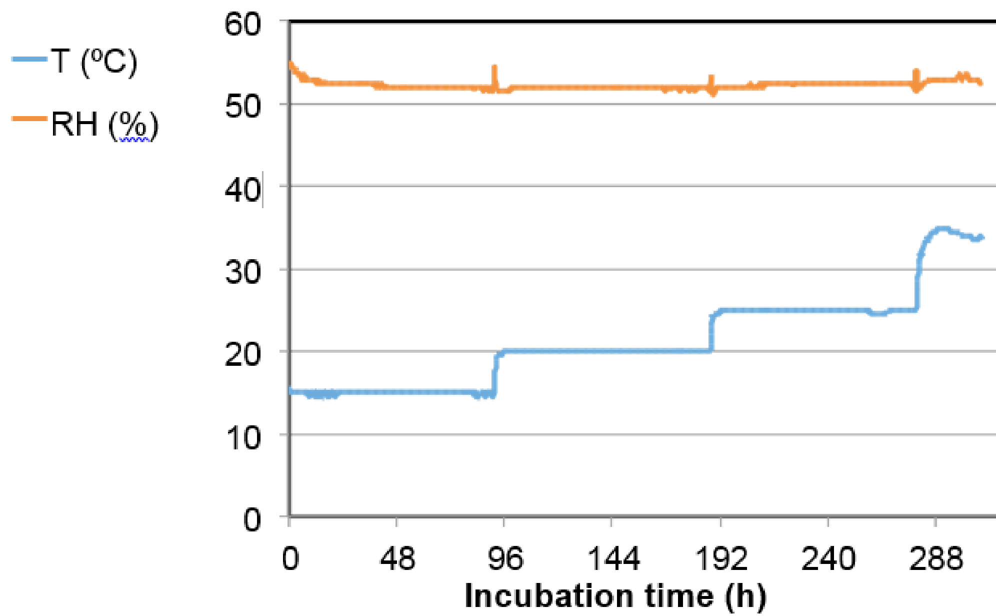


Figure S4.1 RH achieved by the concentrated $MgCl_2$ solution with regards to temperature (T). As it is appreciated in the figure, RH was kept pretty constant from 15 to 30ºC. RH and T were recorded with a sensor and data logger EL-USB-2-LCD+ (LASCAR electronics, UK) with ±2% accuracy in RH and ±0.3ºC in T.

Chapter 5:

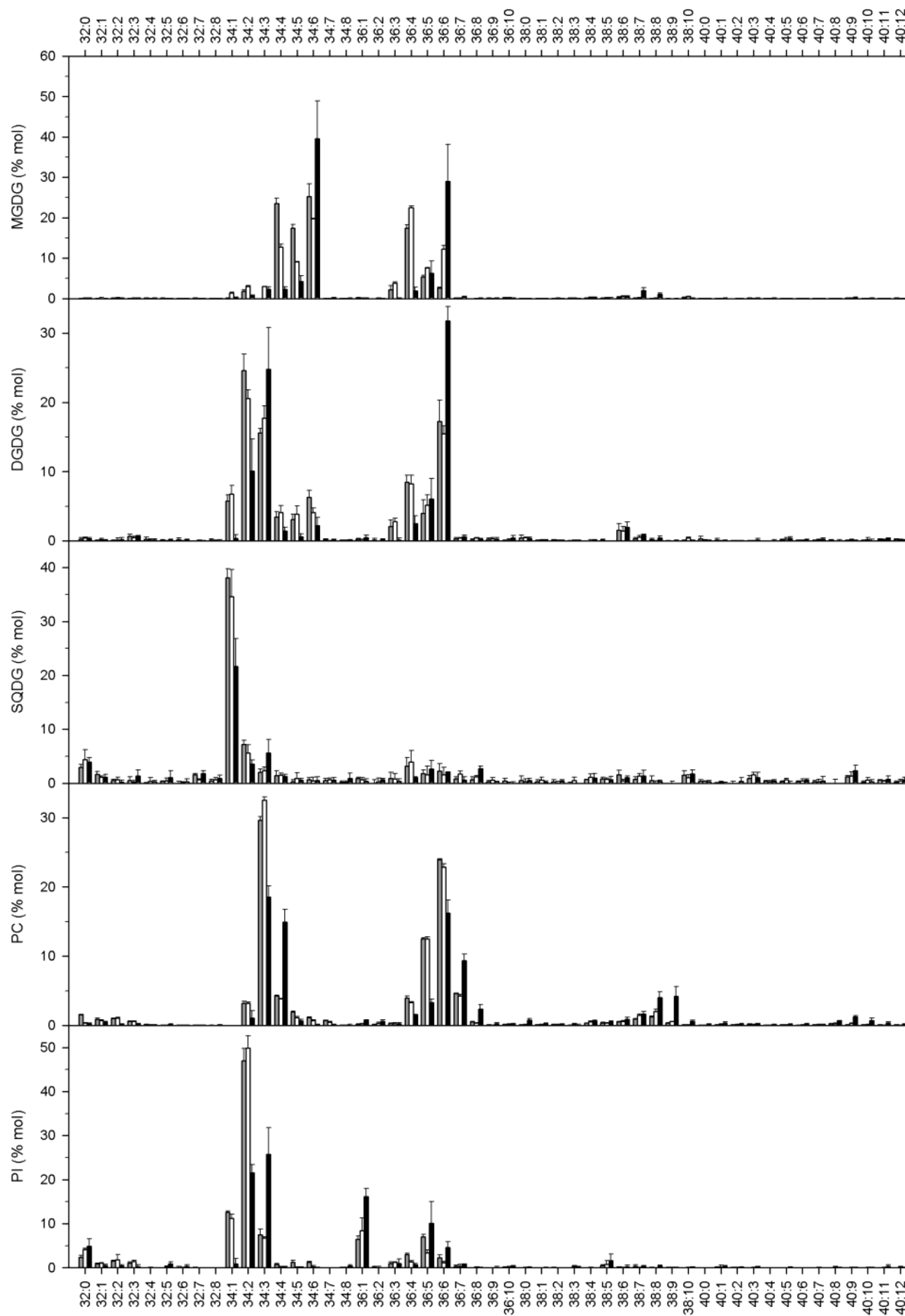


Figure S5.1. Molar percentages of molecular species of each major polar lipids measured in *Osmunda regalis* in sporangial state (grey bars), in spores after 3 days of light and hydration (white bars), and in 6 months gametophytes (black bars). MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiaylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol. Values are means ± SE (n = 3)

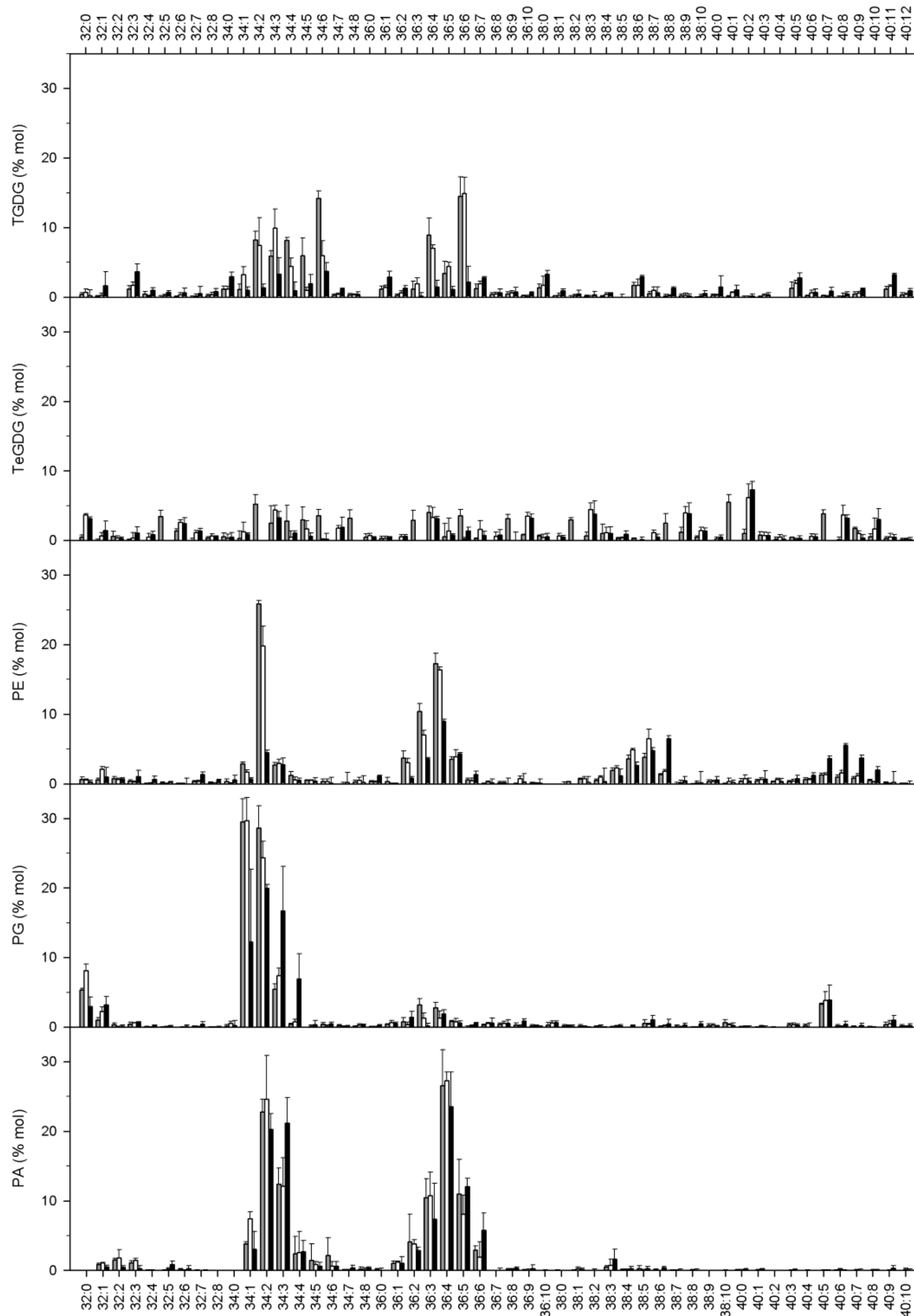


Figure S5.2. Molar percentages of molecular species of each minor polar lipids measured in *Osmunda regalis* in sporangial state (grey bars), in spores after 3 days of light and hydration (white bars), and in 6 months gametophytes (black bars). TGDG, trigalactosyldiacylglycerol; TeGDG, tetragalactosyldiacylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid. Values are means ± SE (n = 3).

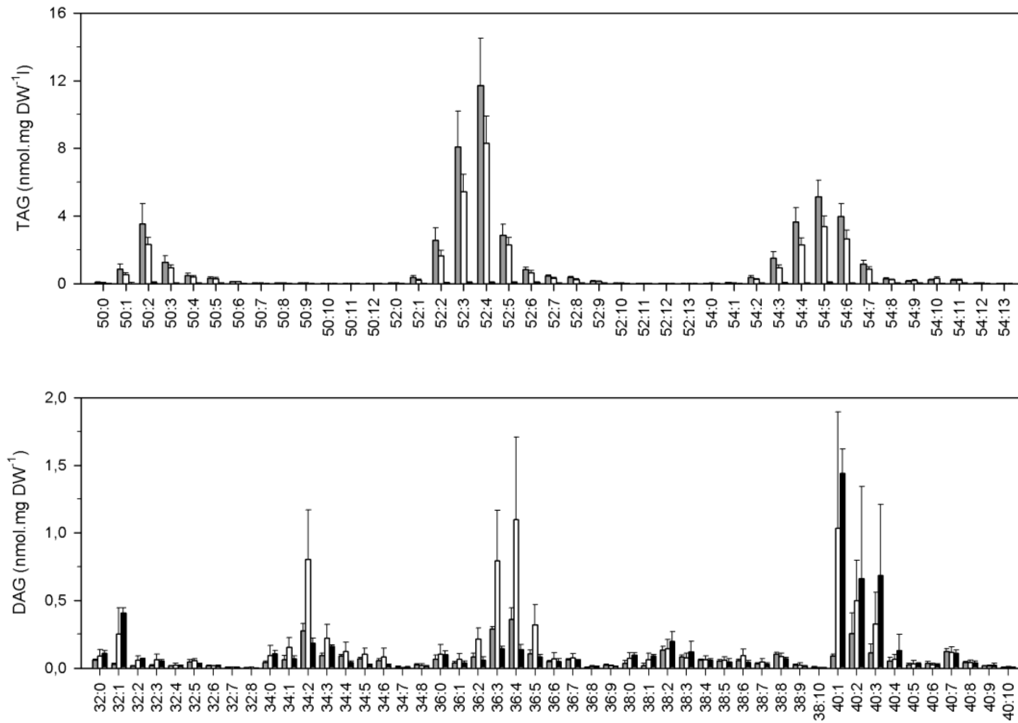


Figure S3. Molar percentages of molecular species of each neutral polar lipids measured in *Osmunda regalis* in sporangial state (grey bars), in spores after 3 days of light and hydration (white bars), and in 6 months gametophytes (black bars). TAG, triacylglycerol; DAG, diacylglycerol. Values are means ± SE (n = 3).

Chapter 7:

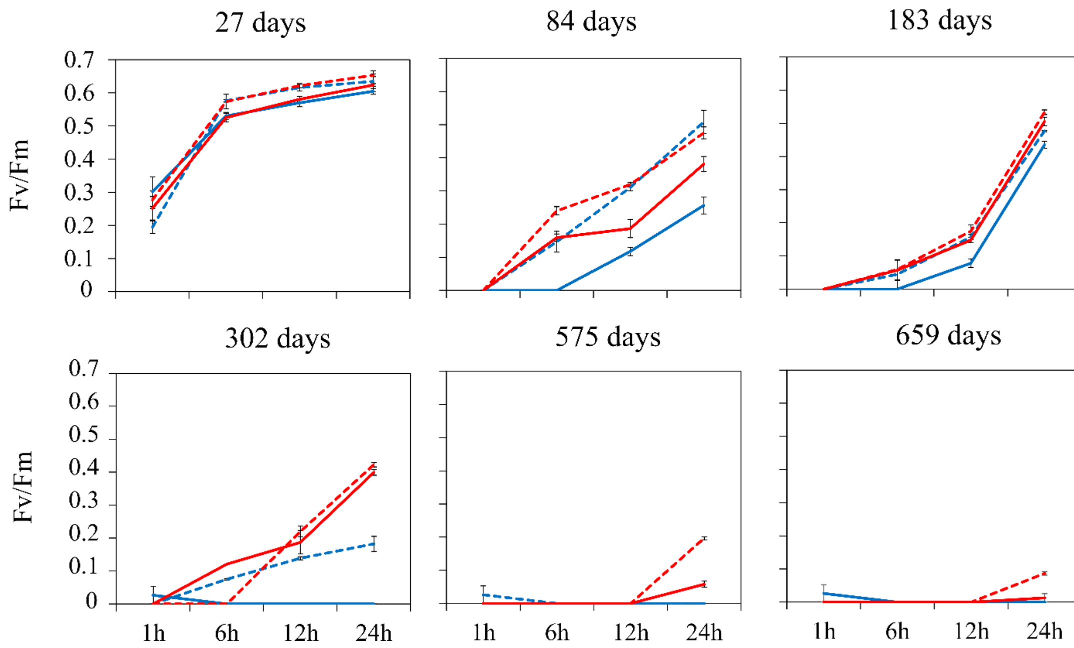


Figure S7.1: Changes in recovery rates of Fv/Fm during imbibition of CS of *M. struthiopteris* along time. Each point represent mean±SE (n=3)

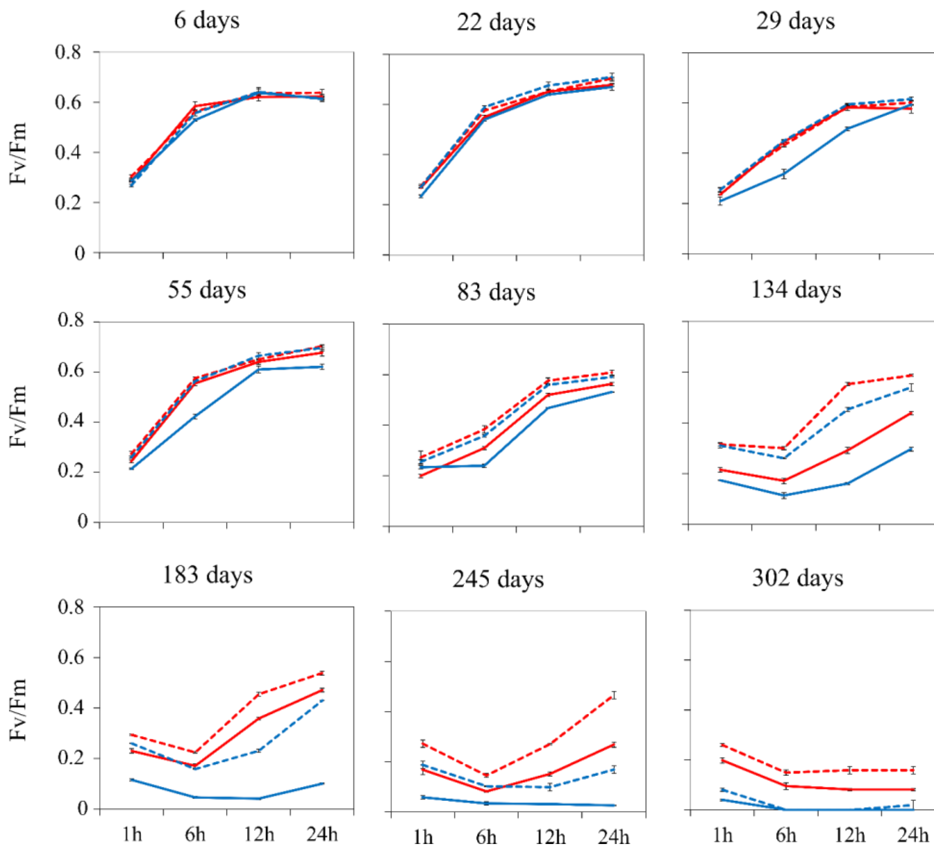


Figure S7.2: Changes in recovery rates of Fv/Fm during imbibition of CS of *O. regalis* along time. Each point represent mean±SE (n=3)

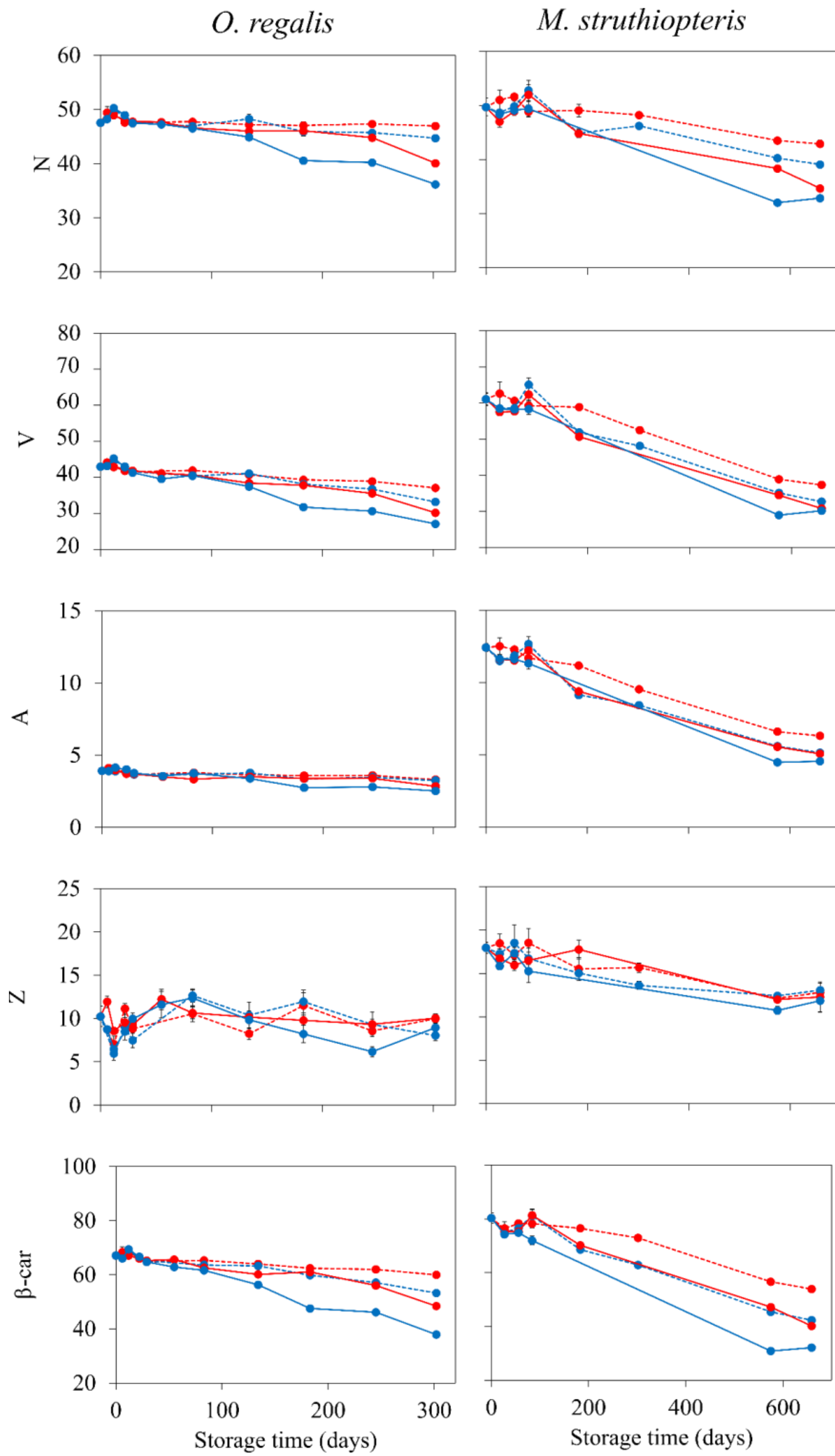
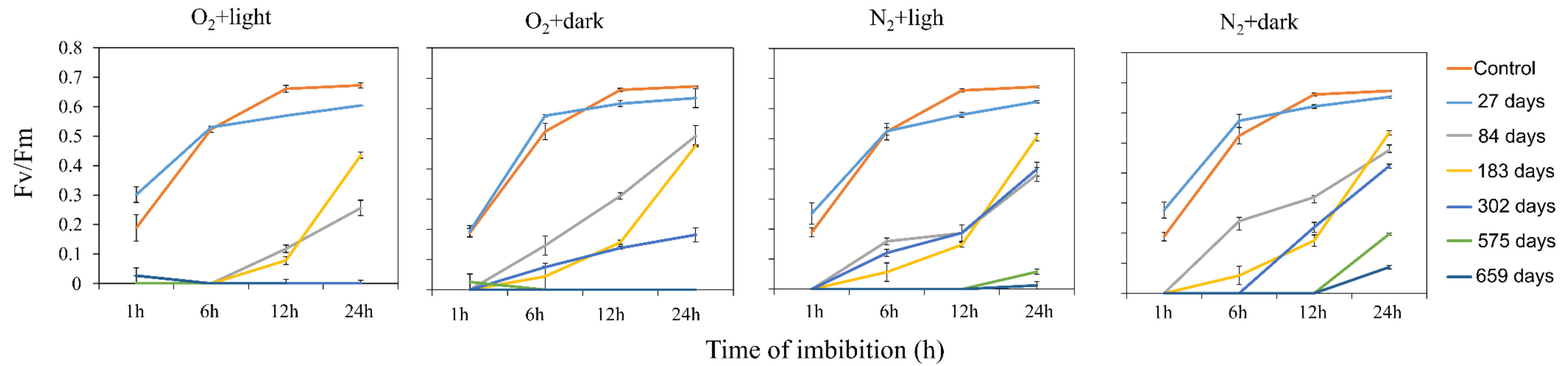


Figure S7.3: Fig 5: Changes in lipophilic pigments and antioxidants during storage at 25ºC and 11%RH. Chla+b (nmol g DW^{-1}) β- carotene (β-car), neoxanthin (N), violaxanthin (V) antheraxanthin (A), zeaxanthin (Z) (mmol mol^{-1} Chla+b). Storage conditions are represented as follow: Blue lines represent a normoxia atmosphere. Red lines represent hypoxia atmosphere. Solid bars represent light conditions. Dotted bars represent dark conditions. Each point represent mean±SE (n=3)



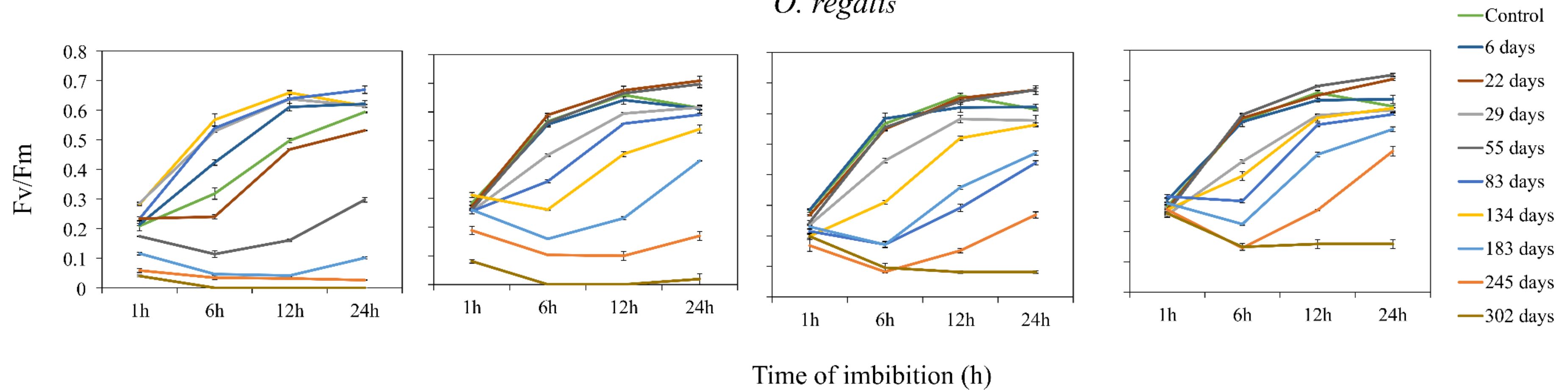


Figure S7.4: changes in Fv/Fm recovery during imbibition of CS under different storage conditions.