Universidad de Concepción
Dirección de Postgrado
Facultad de Ciencias Naturales y Oceanográficas
Programa de Doctorado en Ciencias Biológicas Área Botánica

"Photoprotection mechanisms in desiccation tolerance of two Hymenophyllaceae Family (Pteridophyte) species contrasting in their vertical distribution: morpho-anatomical variations of fronds and structural and physiological changes at the chloroplasts level"

Thesis submitted to obtain the degree of
Doctor in Biological Sciences Botany Area.

Alejandra del Pilar Flores Bavestrello
Concepción-Chile
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"Mecanismos fotoprotectores en la tolerancia a la desecación en dos especies de la Familia Hymenophyllaceae (Pteridophyta) contrastantes en su distribución vertical: variación morfo-anatómica de frondes y cambios estructurales y fisiológicos a nivel de cloroplastos"

Tesis para optar al grado de Doctor en
Ciencias Biológicas Área Botánica.

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A todo quien aportó un granito de arena
para la realización de este estudio.

To all who contributed a sand grain
in the achievement of this work.

Thesis committee

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Contents

Chapter I

Chapter II

hydrated (100 % *RWC*, 1.9 and 2.0 $gH_2O\ gDW^{-1}$, respectively), desiccated (25 % (0.4 $gH_2O\ gDW^{-1}$) and 17 % (0.3 $gH_2O\ gDW^{-1}$) *RWC*, respectively) and rehydrated (>86 % *RWC*, 1.6 and 1.8 $gH_2O\ gDW^{-1}$, respectively) fronds probed with antibodies raised against PsaA. **153**

App2.2. Representative western blots of SDS-PAGE separated polypeptides of thylakoid membranes isolated from (1) *Adiantum tenerum* (Pteridophyta: Pteridaceae), (2) *Pellaea* sp., (Pteridophyta: Pteridaceae) (3) *Davallia fejeensis* (Pteridophyta: Davalliaceae), (4) *Dryopteris* sp., (Pteridophyta: Dryopteridaceae) (5) *Asplenium serratum* (Pteridophyta: Aspleniaceae), (6) *Cibotium* sp., (Pteridophyta: Cibotiaceae) (7) *Polipodium aereum* (Pteridophyta: Polypodiaceae), (8) *Cyrtomium falcatum* (Pteridophyta: Dryopteridaceae), (9) *Nephrolepis exaltata* (Pteridophyta: Lomariopsidaceae), (10) *Sellaginella kraussiana* (Selaginellaceae: Lycopodiophyta), (11) *Hymenophyllum dentatum* Cav. (Pteridophyta: Hymenophyllaceae), (12) *Hymenoglossum cruentum* C. Presl (Pteridophyta: Hymenophyllaceae) and (13) *Arabidopsis thaliana* (Magnoliophyta: Brassicaceae) fronds and leaves probed with antibodies raised against PsaA.

154

General supplementary material **155**

GSM1. Chlorophyll a fluorescence emission spectra at 77 K (-196°C) of thylakoids of (A) *H. dentatum* Cav. and (B) *H. cruentum* C. Presl in hydrated, desiccated and rehydrated state. The Chl concentration of all samples was 10 $\mu g\ ml^{-1}$ and the excitation wavelenght was 436 nm. Spectra represent an average of 3 corrected scans of each hydration state. **155**

GMS2. Ratios of Chlorophyll-protein complexes of *H. dentatum* Cav. and *H. cruentum* C. Presl in hydrated, desiccated and rehydrated states (n=3). **156**

Abbreviations

1	**PSI**	Photosystem I
2	**PSII**	Photosystem II
3	**RC**	Reaction centre
4	**Cyt b_6f**	Cytochrome b_6f complex
5	**Fv/Fm**	PSII maximum quantum yield
6	**(Fv/Fm)h**	PSII maximum quantum yield in the hydrated state
7	**(Fv/Fm)d**	PSII maximum quantum yield in the desiccated state
8	**(Fv/Fm)r**	PSII maximum quantum yield in the re-hydrated state
9	**PI**	Photoinactivation, PI=1-[$(Fv/Fm)_d/(Fv/Fm)_h$].
10	**LHC**	Light harvesting complexes
11	**RWC**	Relative water content
12	**PTT**	Total turgor weight
13	**FW**	Fresh weight
14	**DW**	Dry weight
15	**Chl**	Chlorophyll
16	**Chl *a***	Chlorophyll a
17	**Chl *b***	Chlorophyll b
18	**Car**	Carotene
19	**Vx**	Violaxanthin
20	**Ax**	Anterexanthin
21	**Zx**	Zeaxanthin
22	**L**	Lutein
23	**Lx**	Lutein-epoxide
24	**VDE**	Violaxathin de-epoxidase
25	**ZX**	Zeaxanthin epoxidase
26	**QA**	Quinone A
27	**QB**	Quinone B
28	**$^1O_2$**	Singlet oxygen
29	**OH-**	Hydroxil radical

30	**O_2^-**	Superoxide radical
31	**H_2O_2**	Hydrogen peroxide
32	**^{3}Chl**	Excited triplet state chlorophylls
33	**NPQ**	Non-photochemical quenching
34	**qT**	State transitions
35	**qE**	ΔpH-dependent quenching
36	**qI**	Photoinhibition
37	**OsO4**	Osmium tetroxide
38	**$UO_2(CH_3COO)_2.2H_2O$**	Uranyl acetate
39	**$(PbNO_3)_2$**	Silver nitrate
40	**$Na_3C_6H_5O_7.2H_2O$**	Trisodium citrate

General introduction

The central requirements for autotrophic plant life are the availabilities of light, water, and CO_2 (Heber et al. 2006). However, plants are continuously exposed to changes in temperature, light intensity, humidity, water availability and other environmental factors, which are known to affect their capability to use the energy absorbed from the sun (Pérez-Torres et al. 2006). Water is a major limiting factor in growth and reproduction in plants (Vicré et al. 2004).

1.1. Desiccation tolerance

Drought tolerance can be considered as the tolerance of moderate dehydration, down to a moisture content below which there is no bulk cytoplasmic water present (~23% H_2O FW, or ~0,3 (g H_2O) (g DW)$^{-1}$) (Hoekstra et al. 2001). Desiccation tolerance is the ability of an organism to survive the loss of most (>95%) of its cellular water for extended periods and to recover full metabolic competence upon rehydration (Farrant et al. 2007). The phenomenon of desiccation tolerance is found throughtout the microbial, fungal, animal, and plant kingdoms (Alpert 2006; Tuba 2008). Desiccation tolerance occurs widely in the plant kingdom (Tuba 2008). It is common among bryophytes and lichens, and occurs sporadically among vascular plants of diverse taxonomic affinities (Tuba 2008). Many mosses, lichens, and ferns can survive dehydration of their vegetative organs, whereas this is uncommon in tracheophytes (Oliver et al. 2000). Desiccation-tolerant plants can survive the loss of 80-95% of their cell water, so that the plants appear completely dry and no liquid phase remains in their cells; after a shorter or longer period in the desiccated state, they revive and resume normal metabolism when they are remoistened (Tuba et al. 1998). Most higher plants are able to produce structures such as seeds or pollen, which are tolerant to desiccation, but only a small number of species, termed resurrection plants,

possess desiccation-tolerant vegetative tissues (Vicré et al. 2004). These plants are widespread and found in most taxonomic groups ranging from pteridophytes to angiosperms with the exception of gymnosperms (Gaff 1971). Although some mechanisms are common to all desiccation-tolerant cells, there are also major differences in the strategies developed by these plants to cope with desiccation (Vicré et al. 2004). In general, the more tolerant bryophytes are termed “fully desiccation tolerant” (Oliver & Bewley 1997), as tolerance is constitutive and is not affected by the rate of drying (Vicré et al. 2004). Some of the less tolerant bryophytes and many desiccation-tolerant vascular plants are termed “modified desiccation-tolerant plants” as tolerance is induced in the course of slow drying (Oliver & Bewley 1997). The majority of vegetative desiccation-tolerant plants are found in the less complex clades that constitute the algae, lichens and bryophytes, however, within the larger and more complex groups of vascular land plants there are some 60 to 70 species of ferns, and approximately 60 species of angiosperms that exhibit some degree of vegetative desiccation tolerance (Oliver et al. 2000). Upon dehydration, resurrection plants shrivel up and fold their leaves until water is available, whereupon these plants revive in a remarkable manner (Moore et al. 2008).

Vascular desiccation tolerant plants fall into two groups depending on the degree to which they retain their chlorophyll when dry (Proctor & Tuba 2002). Homoichlorophyllous species retain their photosynthetic apparatus and chlorophylls in a readily recoverable form, while poikilochlorophyllous species dismantle their photosynthetic apparatus and lose all of their chlorophyll during drying, and these must be resynthesised following rehydration (Tuba et al. 1998). The homoichlorophyllous strategy is based on the preservation of the integrity of the photosynthetic apparatus by protective mechanisms (Proctor & Tuba 2002). The poikilochlorophyllous strategy evolved in plants which are anatomically complex and it is based on the dismantling of internal chloroplast structure by an ordered reconstruction process during

drying, and its resynthesis upon rehydration by an ordered reconstruction process (Sherwin & Farrant 1996).

1.2. Photosynthesis and the structure of the photosynthetic apparatus

The absorption of light and the conversion of excitation energy to chemical energy takes place in Photosystem I and II (PSI, PSII) in the thylakoid membrane of chloroplasts (Melis 1999). Photosynthesis is powered by light absorbed by chlorophyll and carotenoid molecules bound to thylacoid membrane proteins (Alboresi et al. 2009; Szabó et al. 2005). Whereas light absorption by photosynthetic pigments is proportional to the incident light intensity, photosynthesis is not (Heber et al. 2006). The excitation energy is transferred from the site of absorption, primarily the light-harvesting complexes (LCHs), to the reaction centres (RC) (Nield et al. 2000), where the excitation is converted into charge separation, which drives the electron flow between PSII and PSI through the Cyt b_6f complex (Szabó et al. 2005). The net result of this process is the oxidation of water molecules, the production of molecular oxygen, the reduction of $NADP^+$ and the generation of a proton gradient (ΔpH), which is exploited for ATP synthesis (Szabó et al. 2005).

These pigment-binding proteins are organized in two supramolecular complexes, the PSI and PSII (Alboresi et al. 2009). Each PS is composed of two different moieties: (i) the core complex, responsible for charge separation and the first steps of electron transport and (ii) the peripheral antenna system, which plays a role in light harvesting and transfer of excitation energy to the reaction centre (Alboresi et al. 2009). The antenna polypeptides in green algae and plants are all members of a multigenic family of proteins called Lhc (Light harvesting complexes) (Jansson 1999). The PSI antenna system (LHCI) comprises four major proteins, Lhca1-4, while PSII antenna comprises six, Lhcb1-6 (Jansson 1999). Additional Lhc sequences have been

identified, namely Lhca5-6 and Lhcb7-8, however they are less expressed and their physiological significance remains unclear (Klimmek et al. 2006). The PSII antenna is a highly dynamic system that is able to adjust the amount of excitation energy delivered to the PSII reaction centre to match the physiological need (Horton et al. 1996). The PSII antenna comprises the main trimeric light-harvesting complex, LHCII, which is composed of the Lhcb1-3 polypeptides, and the minor light-harvesting complexes, CP29, CP26, and CP24, composed of Lhcb4, -5, and -6, respectively (Johnson et al. 2008). In *Arabidopsis thaliana*, four LHCII trimers associate with two copies each of CP24, CP26, and CP29 and a core dimer of PSII (CP43/D1/D2/CP47) to form the $C_2S_2M_2$ LHCII-PSII supercomplex (Dekker & Boekema 2005). Depending upon the growth conditions, two or three extra LHCII trimers per PSII may be present in LHCII-only regions of the the grana, providing additional light-harvesting capacity (Johnson et al. 2008).

The photosynthetic apparatus is highly dynamic and able to respond to several environmental stimuli, including changes in the quality and quantity of incident light and the availability of carbon dioxide (Szabó et al. 2005). There are four main features of the radiation that have ecological and evolutionary significance: the intensity, quality or spectrum, directionality, and distribution in time and space (Canham et al. 1990). A short-term response to light is ensured by non-photochemical quenching (NPQ), a process in which absorbed light energy is dissipated as heat and does not take part in photochemistry (Szabó et al. 2005). The phenomenon involves quenching of Chl*a* fluorescence, which is induced under steady-state illumination and which can be analysed in terms of three components: state transitions (qT), ΔpH-dependent quenching (qE) and photoinhibition (qI). The majority of NPQ is believed to occur through qE in the PSII antenna pigments bound to the light-harvesting proteins (LHCII) (Demmig-Adams & Adams 1992).

1.3. Photoprotection mechanisms

Plants and algae adjust the structure and function of the photosynthetic apparatus in response to change in their growth environment (Jin et al. 2001). Plant have evolved photoadaptive and photoprotective mechanisms at level ranging from the whole plant to leaves and thylakoid membrane of chloroplasts, to avoid excessive radiant energy interception (Choudhury & Behera 2001). Adjustments in pigment content and composition, and the organization of PS are important, because they confer acclimation to prevailing irradiance conditions (Melis 1991). Pigments such as chlorophylls (Chl) and carotenoids (Car) are major components of the photosynthetic apparatus (Jin et al. 2001). Under limiting irradiance conditions, the PS acquire a large Chl antenna size and the thylakoid membranes attain a low Chl*a*/Chl*b* ratio (Jin et al. 2001). Carotenoids participating in the so-called "xanthophylls cycle" (Yamamoto et al. 2008) are found in their epoxidized form with violaxanthin as the dominant species (Jin et al. 2001). Under high irradiance, especially under conditions when the absorbed light is greater than can be utilized by the photosynthetic apparatus, a number of chloroplast acclimation changes are elicited (Jin et al. 2001). These include a smaller Chl antenna size for the PS, a higher Chl*a*/Chl*b* ratio in the thylakoid membrane (les Chl*b*), and conversion of violaxanthin (Vx) via antheraxanthin (Ax) to zeaxanthin (Zx) (Jin et al. 2001).

The most stressful environmental conditions limit the ability of plants to use absorbed light energy, resulting in the over-excitation of the PS, even at a moderate light intensities (Demmig-Adams & Adams 1992). When the rate of transfer of excitation energy from the antennae to RC exceeds the rate of transfer from the RC to the electron transport chain, photoinhibition is resulted (Choudhury & Behera 2001). A typical manifestation of photoinhibition in leaves includes sustained decrease in quantum yield and often a reduction in maximum photosynthetic capacity (Choudhury & Behera 2001). Photoinhibition is often

associated with damage to the photosynthetic apparatus under prolonged high irradiance (Choudhury & Behera 2001). One of the components most frequently suggested to be damaged is the D1 protein of PSII RC (Anderson & Aro 1997). Photooxidative damage, especially to PSII, one of the most sensitive structures to abiotic stress (Choudhury & Behera 2001), seems to be a consequence of photosynthetic activity, and also, the main factor causing sustained reductions in the efficiency of this process (Aro et al. 1993).

The mechanisms that protect the plant against the excess of light are divided into: (a) those that prevent the absorption of excess light, (b) those that dissipate the excess of absorbed light energy, and (c) the mechanisms that eliminate toxic species formed by the excess of light energy (Demmig-Adams & Adams 1992).

Photosynthetic organisms have developed numerous protective mechanisms tended to avoid the effects of excessive light absorption, such as the decrease of light interception through the leaf movement (parahelitropism) and chloroplasts (Moshelion & Moran 2000) and the development of epidermis reflective structures (pubescence, waxes, salts, cuticles) (Lambers et al. 1998); dissipating the excess of absorbed light energy as heat (Demmig-Adams & Adams 1992) and/or eliminating the reactive species that cause damage, such as chlorophylls in excited triplet state, excited singlet oxygen, superoxide, etc. (Cogdell & Frank 1987). Among all these, it is believed that the dissipation of excess excitation energy as heat of the absorbed light energy is the most important in the prevention of photooxidative damage in the photosynthetic apparatus (Demmig-Adams & Adams 1992).

Over all, the evidence suggests that desiccation tolerant plants, generally deal with the potential hazards of oxidative damage during the drying - rewetting cycle by anticipating the problem at source rather than by invoking extravagantly high activity of antioxidant enzymes or antioxidants after the event (Proctor & Tuba 2002). Protective mechanisms include leaf and stem

curling, heavy anthocyanin pigmentation (Farrant 2000), progressive reduction of molecular mobility and controlled down-regulation of metabolism on drying (Hoekstra et al. 2001), and high level of Zx mediated photo-protection (Demmig-Adams & Adams 1992). Cellular damage and metabolic changes during desiccation and rehydration exacerbate the release of reactive oxygen species, and the high antioxidant activity to address it, is essential in the adaptation of desiccation tolerant plants (Navari-Izzo et al. 1997).

Plants depend on non-photochemical and photochemical protections to endure an energy imbalance (Horton & Ruban 2005). The non-photochemical protection is a rapid response mechanism, which involves the dissipation of light energy, mainly at the level of the LHC as heat before photochemical conversion of energy has occurs (Niyogi et al. 2005). Photochemical protection may also occurs after absorbed light energy has been converted into a flux of electrons at the reaction centre. It involves reactions related to the allocation of electrons from the photosynthetic electron transport chain and includes: photorespiration, the water-water cycle, cyclic electron transport around PSI and CO_2 assimilation (Johnson 2005).

1.4. The role of the xanthophyll cycle pigments

The main function of the xanthophylls cycle pigments (Vx, Ax, Zx) in chloroplasts, is to increase non-radiative dissipation of excess excitation energy as heat in the pigment bed of antennae of PSII and protect chloroplasts from high irradiance stress (Demmig-Adams 1990). Induction of an acid lumen pH is the basic requirement for the operation of xanthophylls cycle, which involves the interconversion of Vx to Zx through the formation of an intermediate, Ax (Choudhury & Behera 2001). The enzymes involved for the interconversion are violaxanthin deepoxidase (VDE) and zeaxanthin epoxidase (ZE) (Choudhury & Behera 2001). The VDE enzyme facing the lumen side is responsible for converting Vx to Zx at low lumen pH (pH 5,2) induced by high

irradiance (Choudhury & Behera 2001). The activity of ZE (pH 7,5), facing the stroma, enhances the formation of Vx from Zx (Choudhury & Behera 2001). By the conversion of Vx to Zx, the excess absorbed energy in the PSII antennae is ultimately dissipated as heat, generally referred to as energy dependent quenching (qE) or ΔpH-dependent non-photochemical chlorophyll quenching (NPQ) (Demmig-Adams 1990). Structural analogies between Ax to Zx and Lx to L interconversions by VDE and ZE, suggest the the Lx-cycle could serve as an additional, more slowly relaxing mechanism for sustained energy dissipation in understorey leaves after the occurrence of the first sun-fleck or the formation of gaps within the forest canopy (García-Plazaola et al. 2003).

1.5. Conformational changes in the PSII

An observed alteration, is the separation of the PSII reaction centres (RC) from its peripheral antenna complexes (LHCII), migrating the first at the stroma exposed thylakoids regions and positioned in the PSI vicinities (Timmerhaus & Weis 1990). The PSII, which is in its normal position and condition is called PSIIα, while that one which is separated from its antenna and placed at the stroma exposed thylakoids regions is called PSIIβ (Melis 1989). Another mechanism developed by plants to optimize the utilization of light energy capture is called “state transitions” (González et al. 2001). When the PSII absorbs more energy than the PSI, it creates an imbalance between the electrons flow (Tikkanen et al. 2008). The reduction of the plastiquinone pool and the Cyt b_6f complex activates a kinase which phosphorylates the LHCII proteins, separating the antenna from the PSII RC, which allows the diffusion of the phosphorylated LHCII near the PSI (Allen 2003). This causes the reduction of the functional antenna size of PSII, increasing the distribution of the excitation between the PS, and decreasing the likelihood of photoinhibition (Melis 1996).

1.6. Differences and similarities in photoprotection strategies between PSI and PSII

PSII is known to experience radiation damages even at very low illumination intensities, which is due to the fact that $^1O_2$ is generated by charge recombination from $P680^+$ $Pheo^-$ and that effective protection from carotenoids is lacking (Alboresi et al. 2009). Carotenoid molecules in PSII core structure are located too far from P680 to be able to efficiently quench the Chl triplets generated by charge recombination (Telfer 2002). When light is in excess, the D1 protein is rapidly degraded and resynthesized to reestablish a fully active PSII reaction centre (Baena-González & Aro 2002). A similar mechanism is not known for PSI, because after radiation damages its recovery requires several days (Zhang & Scheller 2004). Furthermore, the repair from photoinhibition does not require the turn-over of a single polypeptide, as in the case of D1, but involves degradation and resynthesis of the whole PSI complex (Alboresi et al. 2009). Despite the similarity between the polypeptides of the PSII antenna complex, it has not been tested a role of the PSI antenna complex polypeptides in photoprotection (Slavov et al. 2008). Evidence suggests that PSI antenna can play a more relevant role, since Lhca deficient plants showed a large reduction in its adaptability in a natural environment (Ganeteg et al. 2004). Furthermore, carotenoids linked to Lhca polypeptides are very efficient in the Chl triplets quenching, suggesting that the pigments bound to these complexes are well protected from radiation damages (Carbonera et al. 2005; Croce et al. 2007).

1.7. Model to study

Epiphytic plants are one of the major components of the plant diversity of the rainforest and the first community to decrease when the fragile ecosystems are threatened (Barthlott et al. 2001). Ferns are the second group of vascular plants in terms of epiphyte diverstity, with 29% of the species occupying this habitat (Kress 1986). The epiphytic habitat is quite constraining because

the lack of relation to the soil implies a high risk of desiccation and the obligatory capacity to quickly and directly use rainwater or moisture (Dubuisson et al. 2009). Hymenophyllaceae consists of a group of herbaceous ferns, generally small with a thin and delicate consistency (Gunckel 1984). Fronds are characterized by a single-cell thick lamina and lack of cuticle and differentiated epidermis and stomata; therefore, they depend upon environmental moisture, because no barrier exists to prevent unregulated loss of water (Dubuisson et al. 2009). They are very abundant and characteristics of the Valdivian temperate rain forest, from the VIII Bío-Bío Region to the south of Chile. Generally Hymenopyllaceae inhabit tropical, subtropical and temperate areas, but always in an atmosphere of high humidity and low light, developing either on the ground and in the epiphyte form (Gunckel 1984). In Chile, the ferns of Hymenophyllaceae are represented by 24 species (Stoll y Hahn 2004) distributed in 4 genera (Stoll y Hahn 2004; Gunckel 1984): *Hymenoglossum*, *Hymenophyllum*, *Serpyllopsis* and *Trichomanes* (Gunckel 1984). Ecophysiological studies with Hymenophyllaceae species are valuable empirical evidence of an evolutionary shift of adaptive strategy from typical vascular plant adaptation to the poikilohydry most typical of bryophytes (Proctor 2012).

Formulation of the research problem

Saldaña et al. (2014) studied the vertical distribution of Hymenophyllaceae, both in young and old trees from different stands in a temperate forest of southern Chile, and observed that there exist habitat preferences among the species. Different species can be distributed either mainly at the trunk base, between 4 and 4.5 m height from the base, or up to 9 m high along the tree trunk. Therefore, it is possible to establish that there are light and humidity gradients from the basal to the top zone of the hosts. Those species that are placed throughout the trunk will undergo light and humidity changes. These changes will be more abrupt in the species which inhabit higher zones than the basal ones. For example, species from the top zone are exposed to higher light intensities, compared to those that are located only in the basal zone. *Hymenophyllum dentatum* and *Hymenoglossum cruentum,* inhabiting up to 9 m high along the trunk, and mainly at the trunk base, respectively, were the selected species to analize the influence of the vertical distribution. This generates the first question: (1) *Are there any differences in relation to desiccation tolerance between species with contrasting vertical distribution?* When we studied the light energy partitioning for PSII in desiccated fronds (Flores-Bavestrello et al. 2016), the quantum yield of the regulated energy dissipation of PSII (Y (NPQ)) did not increase, nor the effective quantum yield of PSII (Y(II)). However, the quantum yield of non-regulated energy dissipation of PSII (Y(NO)) reached values close to 1, which may indicate, that the dissipation mechanism is not due to the activation of the xanthophyll cycle, but to the use of structural changes at the level of chloroplast and photosynthetic apparatus. This prompted us to formulate the following questions: (2) *What are the changes that occur in the chloroplast during the desiccation process that allow these individuals to recover so fast and continue with their photosynthetic activity when water becomes available?* (3) *Which are the photoprotection mechanisms and how they work to let*

individuals of Hymenophyllaceae undergo a desiccation and rehydration process? and finally, (4) *Are there any differences between the photoprotection mechanisms used by Hymenophyllaceae species with contrasting vertical distribution?*

Working hypotheses

Hypothesis 1.

Hymenophyllaceae species subjected to a desiccation and rehydration process, mainly use constitutive photoprotection mechanisms, such as morphological characteristics of the fronds, and structural properties at the level of chloroplast and photosynthetic apparatus.

Hypothesis 2.

The damage caused by desiccation in fronds from Hymenophyllaceae is greater in the species distributed mainly in the trunk base, than in the species distributed along the trunk.

Objectives

General objective:

To study the morpho-anatomical variations at the frond level and the structural and physiological changes at the chloroplasts level associated to photoprotection in two species of Hymenophyllaceae with contrasting vertical distribution during a desiccation and rehydration process.

Specific objectives:

- To study the desiccation tolerance and its recovery ability in two Hymenophyllaceae species contrasting in their vertical distribution.
- To study the morpho-anatomical changes at the frond level in two Hymenophyllaceae speciesduring a desiccation and rehydration process.

- To study the structural and physiological variations in the chloroplast in two Hymenophyllaceae species during a desiccation and rehydration process.

Study perspective

The relevance of the present study mainly provides a contribution in the following four aspects: (a) the physiology of poikilohydric ferns, (b) biotechnology, (c) the ecology of vascular plants, and (d) the conservation of endangered plants species.

The main contribution of this thesis is the knowledge of the biology *of the poikilohydric ferns*, specifically the interaction of the effects of desiccation and light stress through the characterization of photoprotective mechanisms in desiccation tolerant filmy ferns. The potential contribution of *biotechnology* is that this study could be the basis for gene selection involved in the protection and maintenance of cell stability during desiccation. These genes could be incorporated in an economically important species to create a genetically modified organism that, resists, without significant yield decrease, a period of severe water stress. In relation to *ecology*, Hymenophyllaceae belongs to the Pteridophyte Division, a primitive group of vascular plants with extinct representatives, whereby the obtained information will be an input for the characterization of this group. There are many species that inhabit contrasting light and humidity environments of the forest. This distribution may be determined by a differential tolerance to this combination of factors. Therefore, this study could provide physiological basis to explain the distribution and abundance of these species in the temperate rainforest. Finally, an important number of Chilean ferns are placed in the *endangered conservation status*, specially the Hymenophyllaceae, because as they are epiphytes, they are one of the main components of the plant diversity of the rainforest and the first community to decrease when the fragile ecosystems are threatened. These has been clearly established in the recent eruptions of the Puyehue-Cordón del Caulle volcanic complex in 2011, where ashes from the eruption destroyed a significant part

of the Hymenophyllaceae community in Puyehue National Park. Another important threat for these filmy ferns is the yet unpredictable impact of climate change in precipitations in southern Chile. This ferns study will cause a wake-up call according to their importance in plant diversity, creating a greater concern in the intervention of its natural environment.

Chapter I

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Morphological frond changes in *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C. Presl (Pteridophyta: Hymenophyllaceae) in different hydration states

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Abstract

Hymenophyllum dentatum Cav. and *Hymenoglossum cruentum* C. Presl (Pteridophyta: Hymenophyllaceae) are desiccation tolerant ferns. They are poikilohydric epiphytes, with fronds composed by a single layer of cells and without true mesophyll cells and stomata. In Chile, these species belong to the temperate rain forest, and they usually inhabit shady and humid environments. However, as epiphytes, their vertical distribution varies along the trunk of the host plant. Some species inhabit drier sides with higher irradiance, as *H. dentatum* Cav., while others inhabit wetter and shadier sides. The aim of this work was to characterize the morphological variations in *H. dentatum* Cav. and *H. cruentum* C.Presl fronds during hydrated, desiccated, and rehydrated states. Both species were subjected to desiccation and rehydration kinetics to analyze the frond morphological variations in different hydration states by a scanning electron microscope. It was observed in both species, a change in the shape of desiccated cells from convex to concave. Fully hydrated fronds showed cells with an irregular polygon shape; a disorganized disposition of cells, and different sizes of them. Glandular multicellular hairs with a distal secretory cell were observed only in *H. dentatum* Cav. throughout the vascular system of the lamina. We conclude that, although cells decreased their sizes, there was not cells collapse in the desiccated state of both species, which helps to their fast recovery and functioning. The presence of wax glands in *H. dentatum* Cav., appears to be a different strategy to cope with desiccation tolerance in the higher zones of the host trunk.

***Keywords*:** *Hymenophyllum dentatum* Cav.; *Hymenoglossum cruentum* C.Presl; frond morphological changes; Hymenophyllaceae, desiccation tolerance.

Introduction

Fern leaves are typically envisioned as compound (dissected or divided) with pinnae or pinnules arranged along a central axis (the rachis or costa) (Vasco et al. 2013). These leaves are called fronds (Rodríguez et al. 2009). The stalk of the leaf is called the stipe or petiole. Distal to this, the laterally expanded portion of the leaf is termed blade or lamina, whose central midrib is referred to as rachis (Vasco et al. 2013). Since leaves are so crucial to a plant, features such as their arrangement, shape, size, and other aspects are highly significant for their survival (Raven & Johnson 1989).

Plants are composed mainly of water. The actual water content will vary according to tissue and cell type. It is dependent to some extent on environmental and physiological conditions, but water typically accounts for more than 70% by weight of non-woody plant parts (Hopkins & Hüner 2009). Protoplasm displays signs of life only when provided with water. If it dries out, it does not necessarily die, but it must at least enter an inactive (anabiotic) state, in which vital processes are suspended (Larcher 2003). The highly dissected leaves of a fern loose water much more rapidly than other species. Therefore, leaves features differ greatly in plants that grow in different environments (Raven & Johnson 1989).

Filmy ferns (Pteridophyta: Hymenophyllaceae) are desiccation tolerant epiphytes characterized by fronds with a single-cell thick lamina; they lack cuticle, differentiated epidermis, and stomata (Dubuisson et al. 2009). They are poikilohydric, because no barrier exists to prevent water loss (Kappen & Valladares 1999; Dubuisson et al. 2009). Although they are associated with humid and shade environments (Krömer & Kessler 2006), their vertical distribution varies throughout the trunks (Parra et al. 2009; Gehrig-Downie et al. 2012). Filmy ferns undergo light and humidity variability in the vertical gradient, with more abrupt changes in the higher zones

(Saldaña et al. 2014). Species from the top zone are exposed in a greater proportion to higher light intensities and lower humidity than those located in the basal zone.

One of the first and foremost problems that desiccation tolerant plants must face in order to survive desiccation is the noteworthy reduction of cell volume that occurs in drying tissues (Larcher 2003). In desiccation tolerant plants, the dehydration process highlights the dramatic morphological changes that accompany desiccation (Vicré et al. 2004). In our previous studies, we have observed in the desiccated state of *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C.Presl (Pteridophyta: Hymenophyllaceae) a reduction of the total projected area of 78% and 68%, respectively (Flores-Bavestrello et al. 2016). According to their vertical distribution, the first one is associated with the upper 9 m of the host trunk, while the latter is associated mainly at the trunk base (Saldaña et al. 2014). Due to these differences, we wanted to observe the morphological variations between these two species in hydrated, desiccated, and rehydrated states. We hypothesized that cells of *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C.Presl in the desiccated state will show an area reduction, which will be-restored after a rehydration period. In addition, no cells collapse will be observed in the desiccated state, helping them to a quick recovery of their functioning. To test this hypothesis, we characterized the morphological variations in *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C.Presl fronds during the hydrated, desiccated and rehydrated states.

Materials and methods

Study site and plant material

Hymenophyllum dentatum Cav. and *Hymenoglossum cruentum* C.Presl or *Hymenophyllum cruentum* Cav. (Larsen et al. 2013) (Pteridophyta: Hymenophyllaceae) consisting in frond, rhizome, and roots growing on dead trunks were collected in a second-growth forest located in Katalapi Park (41°31′8″ S, 72°45′2″ W, elevation ca. 80 m a.s.l.) in Pichiquillaipe, 18 km south-east from Puerto Montt, Región de Los Lagos, Chile. The weather in this zone is considered highly humid and temperate rainy (Di Castri & Hajek 1976). Samples were transferred to shaded experimental nursery gardens at Universidad de Concepción with controlled humidity. The pieces of tree trunks which supported the growth of *H. dentatum* Cav. and *H. cruentum* C.Presl were wrapped and maintained in burlap and remained moist by periodically sprinkling them tap water during until use. Since Hymenophyllaceae present a modular development from a rhizome, it is difficult to isolate a complete individual. For this reason, the experimental unit was an isolated module composed by a frond, rhizome, and roots.

Samples preparation and treatments

Isolated, fully hydrated control modules of *H. dentatum* Cav. and *H. cruentum* C.Presl were exposed to a desiccation and rehydration regime in a controlled environment in the dark at 15 ± 2 °C and 80% relative humidity. Nine isolated modules from each species were placed in a glass container filled with distilled water for a 12 h period in dark. After that time, the isolated modules were dried with absorbent paper. Subsequently, no water was supplied to the isolated modules which initiated the desiccation process. After 24 h of desiccation, the rehydration process was

initiated by placing the same fronds in a glass container filled with distilled water for 24 h period in dark. Three fronds of each species were collected in each hydration state.

Superficial view of epidermis and its variations

Fronds sections from isolated modules of *H. dentatum* Cav. and *H. cruentum* C.Presl in hydrated, desiccated, and rehydrated states were collected. The samples were immediately fixed in glutaraldehyde (2.5%) in 0.1 M Phosphate Buffer (pH 7.2) for 24 h at 4 °C. Leaves were sectioned in 2-3 mm fragments and fixed again in glutaraldehyde (2.5%) in 0.1 M Phosphate Buffer (pH 7.2) for 24 h at 4 °C. After 2 rinses of 10 min each with 0.1 M Phosphate Buffer (pH 7.2), the plant material was fixed in OsO4 (1%) in 0.1 M Sodium Cacodylate Buffer (pH 7.2) for 2 h at 4 °C. The fixed tissue was rinsed 2 times per 10 min each in 0.1 M Phosphate Buffer (pH 7.2); dehydrated in a graded acetone series (30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%); dried at critical point with liquid carbon dioxide (CO_2), and coated with gold.

Visualization

The samples were observed with a JEOL JSM6380 nLV, Tokyo, Japan scanning electron microscope. Three fragments of three different isolated modules of each species in each hydration state were used in this study.

Results

Characterization of cells of the frond

By achieving the morphological characterization of frond cells in *H. dentatum* Cav. and *H. cruentum* C.Presl (Fig. 1), the first thing we noticed was the variation in the shape of the cell from a convex shape in the hydrated (Fig. 1A-B) and rehydrated (Fig. 1E-F) states to a concave shape in the desiccated (Fig. 1C-D) state in both species. Both *H. dentatum* Cav. and *H. cruentum* C.Presl control (fully hydrated) fronds showed cells with an irregular polygon shape (Fig. 2). Lamina cells are of different sizes and show an irregular disposition in both species (Fig. 2). As shown by the numbers inside the cells, it was possible to observe different number of faces of the cells (Fig. 2A-B). Folded concave cells were more evident in *H. dentatum* Cav. in the desiccated state than in *H. cruentum* C.Presl (Fig. 3), although both species reached 0.4 g H_2O g DW^{-1} after a desiccated period (results not shown). Sizes of cells were around 1397.9 ± 94.7 and 2043.9 ± 361.0 μm^2 for *H. dentatum* Cav. and *H. cruentum* C.Presl, respectively (results not shown).

Presence of wax glands in the fronds

Wax glands were observed in the abaxial face of *H. dentatum* Cav. fronds (Fig. 4) throughout the vascular system of the lamina. Rachis, costa, and costule, the terms for the midrib of the frond, pinna, and pinnules (Vasco et al. 2013), respectively, presented cells with pores or holes from which there was secretion of waxes of a membraneous platelets type (according to Barthlott et al. 1998). In a transverse section of a pinnule analyzed in the microscope, it was possible to observe xylem cells (tracheids) surrounded by phloem cells. However, besides these phloem cells, there were two big compact cells in opposite sites with a different composition, which gives the impression to contain a denser compound. This structure could be defined as a “glandular

multicellular hair with a distal secretory cell" (Bower 1963). This hair has a cylindrical segmented shape, and is composed by 8.6 ± 0.5 segments. Each segment has an area of 2221.2 ± 135.6 μm^2, and in the last segment, which has an area of 1233.2 ± 163.3 μm^2, there is a pore or hole of 350.19 ± 112.9 μm^2, which is facing up. The discharging distal cells can be together, but in general these are spaced by 491.2 ± 31.8 µm. On the contrary, wax glands were not observed in *H. cruentum* C.Presl (Fig. 5).

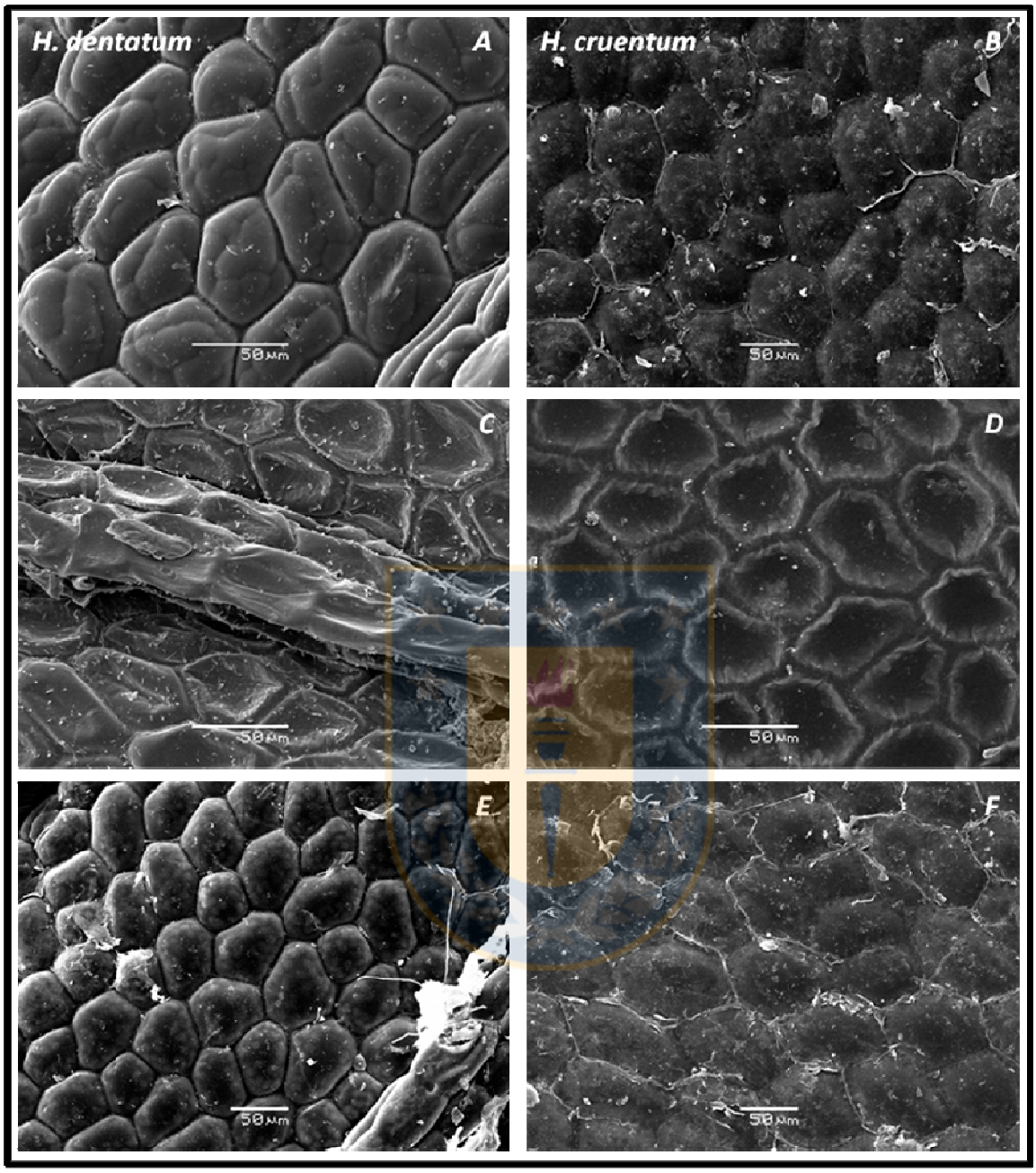

Figure 1 *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C.Presl frond cells in different hydration states. A, Fully hydrated cells of *H. dentatum* Cav. B, Fully hydrated cells of *H. cruentum* C.Presl. C, Desiccated cells of *H. dentatum* Cav. D, Desiccated cells of *H. cruentum* C.Presl. E, Rehydrated cells of *H. dentatum* Cav. F, Rehydrated cells of *H. cruentum* C.Presl. The bar indicates 50 µm.

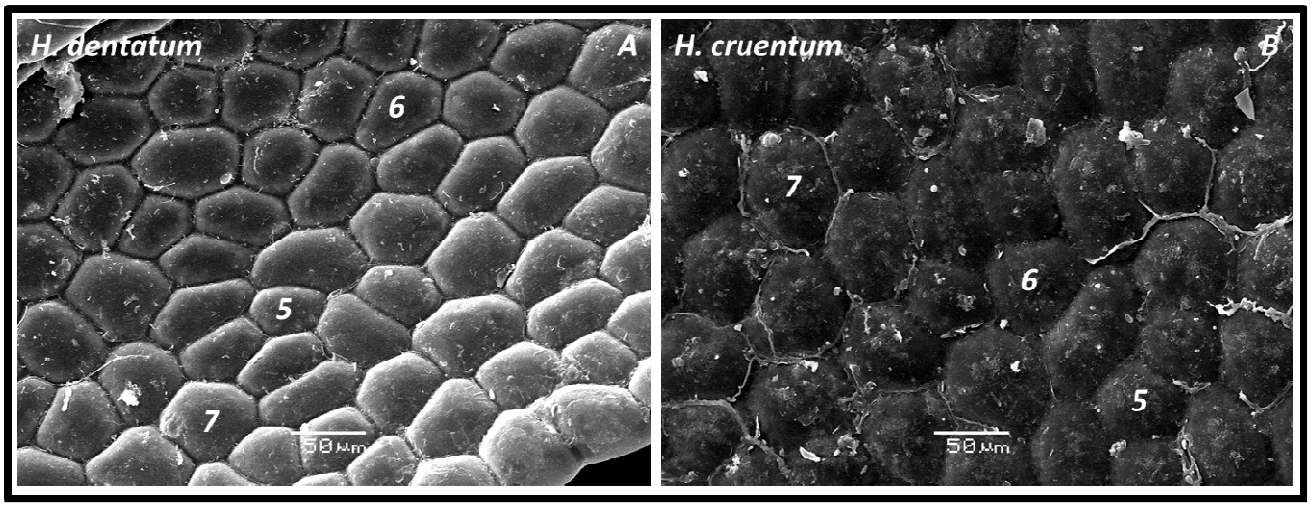

Figure 2 General view of *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C.Presl hydrated cells. A, Fully hydrated cells of *H. dentatum* Cav. B, Fully hydrated cells of *H. cruentum* C.Presl. Numbers inside the cells indicate different numbers of faces in each cell. The bar indicates 50 µm.

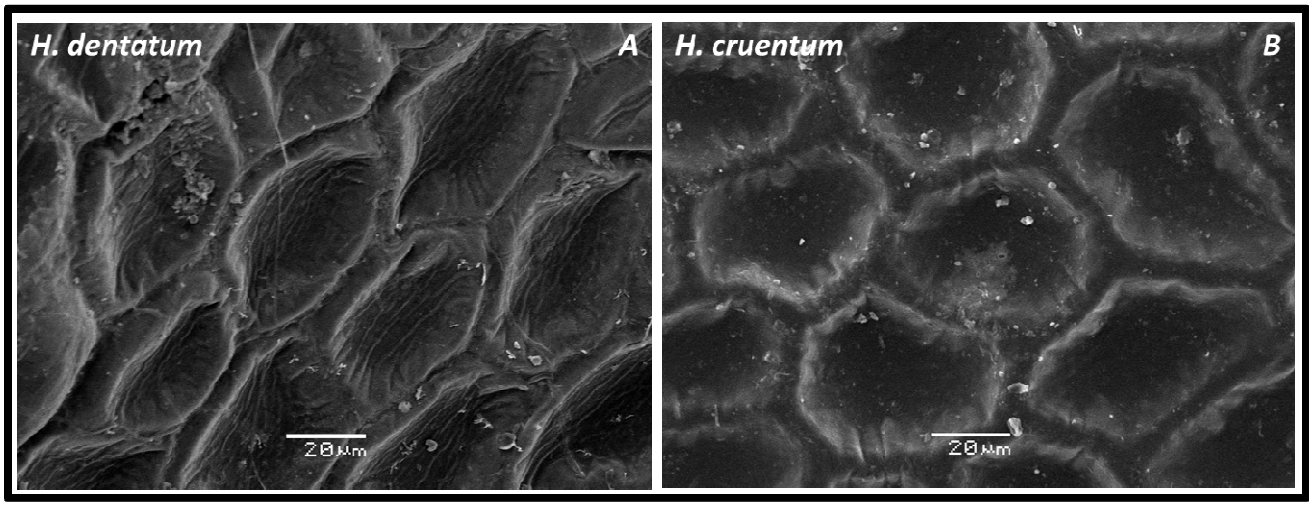

Figure 3 General view of *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C.Presl desiccated cells. A, Folded cells in the desiccated state of *H. dentatum* Cav. B, Folded cells in the desiccated state of *H. cruentum* C.Presl. The bar indicates 20 µm.

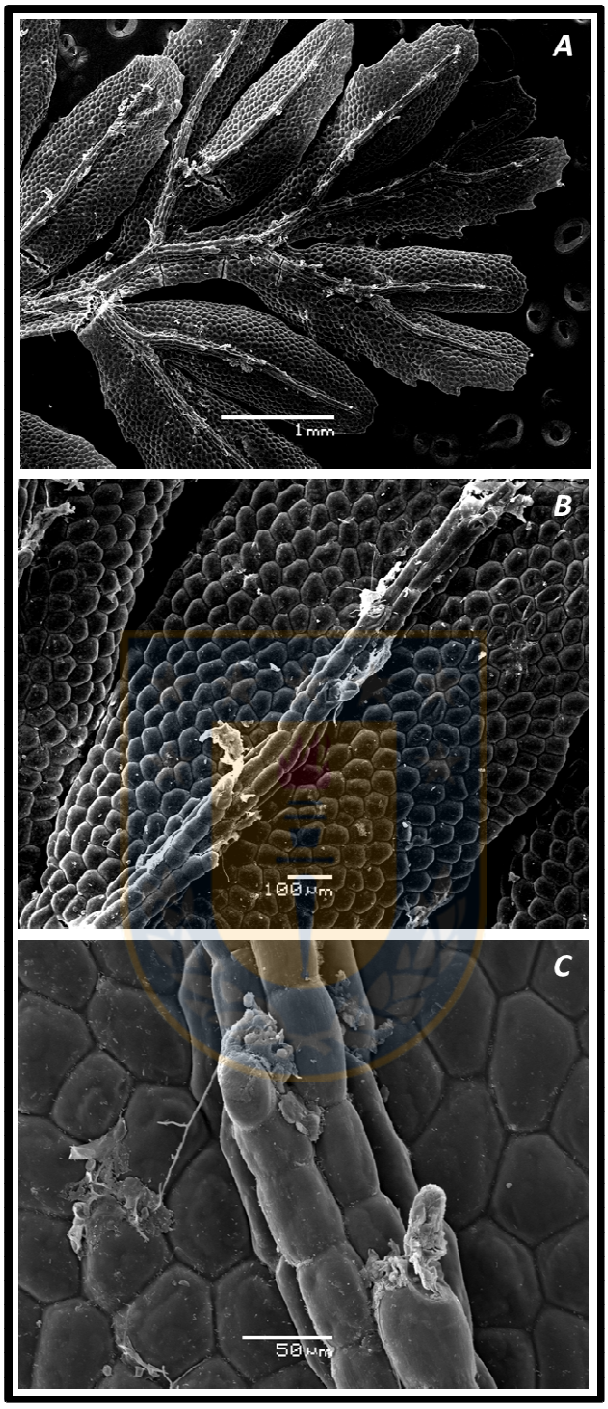

Figure 4 Wax glands in fully hydrated fronds of *Hymenophyllum dentatum* Cav. A, View of pinna. The bar indicates 1 mm. B, View of pinnule. The bar indicates 100 µm. C, View of costule (midrib of the pinnule). The bar indicates 50 µm.

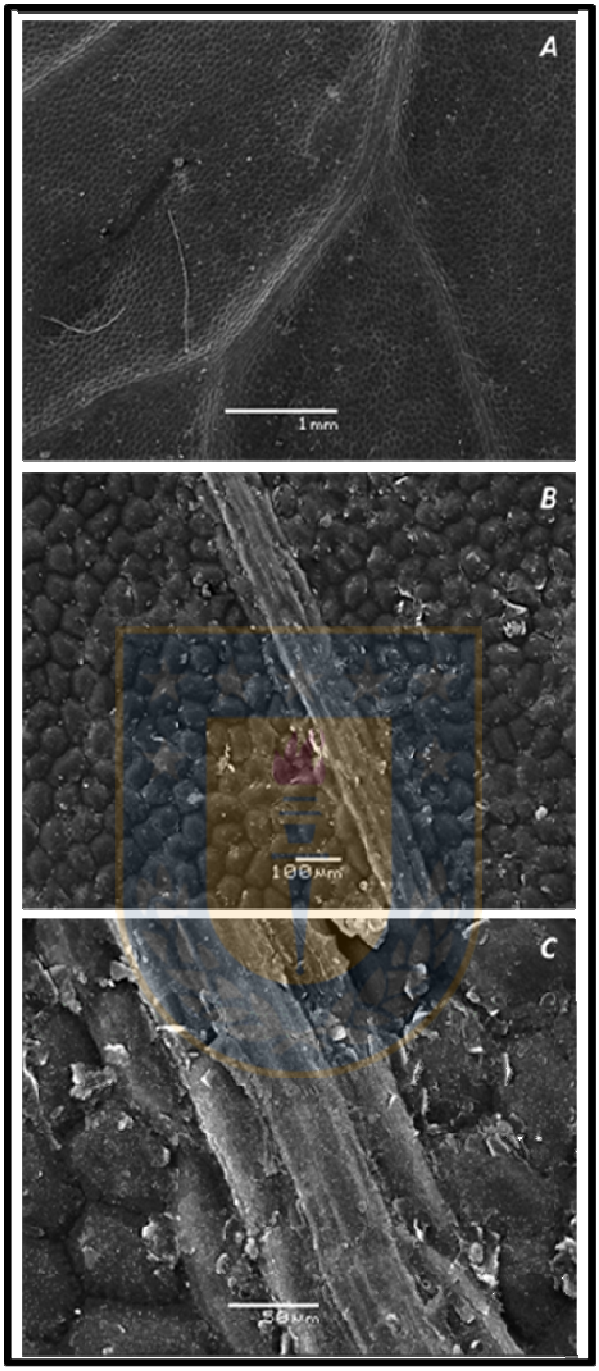

Figure 5 Fully hydrated fronds of *Hymenoglossum cruentum* C.Presl. A, View of lamina and rachis. The bar indicates 1 mm. B, View of lamina and rachis. The bar indicates 100 µm. C, View of lamina and rachis. The bar indicates 50 µm.

Discussion and conclusions

The primary goal of this study was to characterize the morphological variations in *H. dentatum* Cav. and *H. cruentum* C.Presl fronds during the desiccated state, and to compare them with the hydrated and rehydrated states.

The reduction of cell volume in the desiccated state of both species shown by the concave shape of the cells reveals the extreme tissues dehydration, and it allows to perceive the significant changes that each cell must face to struggle with a desiccation process. The basic cell curvatures are tabular (flat), convex (arced to the outside) and concave (arced to the inside), whereby the most common cell shape is the convex form (Koch and Barthlott 2009). The cell changes include cell wall folding, roundish chloroplasts, and vacuole fragmentation (results not shown). This massive loss of water from cells led to a drawing inward of the cell contents, causing tension between the plasmalemma and the cell wall, which generally exhibits limited elasticity (Levitt 1980). However, despite all these strong changes during the desiccated state, the same cells recover in the rehydrated state. *H. dentatum* Cav. and *H. cruentum* C.Presl reached maximum photochemical efficiency of PSII (Fv/Fm) values around 0.6 and over 0.7 after 30 and 60 min of a rehydration process, respectively (results not shown). This shows a recovery, compared to the hydrated state, of 91% and 88%, respectively (Saldaña et al. 2014).

An unexpected result was the discovery of wax glands in the midrib of the frond in one of the species, *H. dentatum* Cav. Waxes are an essential structural element of the surface and of fundamental functional and ecological importance for the interaction between plants and their environment (Barthlott et al. 1998). A waxy surface indicates that the surfaces are covered by wax crystalloids which can be recognized as clear protrusions under SEM. Non-waxy surfaces are also covered by wax, but it is deposited as a film that it is often hardly distinguishable by

SEM (Neinhuis and Barthlott 1997). Waxes are esters of long-chain acids and long-chain primary alcohols. However, plant surface lipids, usually called epicuticular waxes are composed of a large mixture of different chemical compounds, and they comprise cyclic and long-chain aliphatic components that can be further classified according to their structure, functional groups, and by the distribution of their homologues (Barthlott et al. 1998). It has been suggested that, during development, minute cuticular pores (often called microchannels) facilitate the transfer of wax to the surface of the cuticle (Beck 2010). However, Neinhuis et al. (2001) provided evidence that molecules of wax and water simply move together through the cuticle under the influence of cuticular transpiration. Raven & Johnson (1989) pointed out that trichomes play an important role in regulating the heat and water balance of the leaf. Some trichomes are glandular, often secreting sticky or toxic substances that may deter potential herbivores. As Hymenophyllaceae typically do not present cuticule, perhaps there is transfer of wax through the movement of water from the cells of the vascular system through glandular trichomes. On the other hand, in our previous studies of chlorophyll *a* imaging fluorescence of PSII in fronds of the species previously mentioned, it was observed that the rachis maintained a higher quantum efficiency of PSII photochemistry (Fv/Fm) compared to the lamina during desiccation, and recovers faster than lamina during rehydration (results not published). Therefore, these waxes may be covering and protecting more the vascular system than the lamina, then slowing the loss of Fv/Fm. However, if we emphasize that our models of study are poikilohydric species, it is likely that waxes would be only covering the vascular system, making its surface hydrophobic; meanwhile the lamina, as it is not covered by waxes, or it has a very thin film of wax, it would be hydrophilic. Therefore, this would allow the movement of water from the inside to the outside and vice versa as in a poikilohydric species. As a consequence, *H. dentatum* Cav. and perhaps the other pinnate species of this family would need the presence of wax glands. Therefore, the presence of wax glands in

one species (*H. dentatum* Cav.) and not in the other (*H. cruentum* C.Presl) may be due to the highly dissected fronds of *H. dentatum* Cav., which loose water more rapidly than *H. cruentum* C.Presl.

Another interesting aspect is the vertical distribution of Hymenophyllaceae species. It was recently reported that they exhibit habitat preferences in young and old trees from different stands in a temperate forest of southern Chile. Hymenophyllaceae species can be distributed either mainly at the trunk base or between 4 and 4.5 m height or even up to 9 m high along the tree trunk (Saldaña et al. 2014). Therefore, those individuals that are found at different heights along the trunk of the host will be exposed to different light and humidity conditions. Thus, Hymenophyllaceae species found near the top of trunk of the host should exhibit different strategies to cope with the desiccation tolerance and light than those species located near the base of the host. In this way, one of these strategies could be the presence of wax glands in the species which inhabits the top of the trunk to increase their desiccation tolerance, as in *H. dentatum* Cav. Another aspect to analyze is that *H. dentatum* Cav., and maybe the other species with similar vertical distribution, are more exposed to rain in the temperate rain forest. For this reason they need a higher production of wax glands to face with the mechanical abrasion due to rain, because waxes have a low mechanical stability and are easily destroyed by erosion through rain (Neinhuis and Barthlott 1997).

As a future step, it would be interesting to study the presence or absence of wax glands in other species from Hymenophyllaceae with contrasting vertical distribution to see if this pattern repeats itself.

Finally, as it was predicted, folded cells were observed in the desiccated state of both lamina and rachis. After rehydration, the folding of cells disappeared, without observable cell collapse. The presence of a glandular multicellular hair with a distal secretory cell was observed

in the vascular system of *H. dentatum* Cav., but not in *H. cruentum* C.Presl. This probably indicates a protection of the midrib from desiccation in pinnate leaves, and perhaps an ecological key feature which allows the colonization of higher zones of the trunk of the host tree.

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Chapter II

Manuscript in preparation.

Qualitative and quantitative variations in cell ultrastructure of *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C. Presl (Pteridophyta: Hymenophyllaceae) fronds during desiccation and rehydration

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Abstract

Hymenophyllum dentatum Cav. and *Hymenoglossum cruentum* C. Presl (Pteridophyta: Hymenophyllaceae) are desiccation tolerant and poikilohydric epiphytic ferns that inhabit the Southern temperate rainforest of Chile. These ferns have unilaminar fronds which lack stomata. Although these species are associated with damp and dark environments, their distribution along trunks varies. There are some species that inhabit more illuminated and drier sites. *H. dentatum* and *H. cruentum* were the chosen species because of their contrasting vertical distribution. Due to the different shrinkage degree that these species present during the desiccated state, and to the fast recovery that they reach after rehydration, it would be interesting to characterize the changes inside the cells during these states. The aim of this work was to study the qualitative and quantitative variations in cell ultrastructure of *H. dentatum* and *H. cruentum* fronds during desiccation and rehydration. Both species were subjected to desiccation and rehydration kinetics to observe the variations inside the cells with a transmission electron microscope. In the desiccated state of both species, we observed folded cell walls, a disbanding of the vacuole, and chloroplasts with a roundish shape, which was reversed after rehydration. *H. cruentum* presented a higher number of chloroplasts per cell, a higher number of starch granules per chloroplasts, and a higher starch area compared to *H. dentatum*. Chloroplasts area decreased in *H. dentatum*, but in *H. cruentum* remains the same. Chloroplasts width was the characteristic that most changed in both species. We conclude that both species, despite of their vertical distribution, present similar qualitative and quantitative changes, conditions that reverse upon rewatering.

Keywords: Hymenophyllaceae, morpho-anatomy, ultrastructure, desiccation tolerance.

Introduction

Hymenophyllaceae Family consists of desiccation tolerant and poikilohydric epiphytic ferns that inhabit the temperate rainforest of Chile. This group of herbaceous ferns, small and of delicate and fine consistency, have fronds composed by a lamina that is characterized by having one or few cell thick, absence of stomates, and lack of roots (Tryon & Tryon 1982). They depend on the ambient humidity, as there is no barrier to prevent water loss (Dubuisson et al. 2009). Despite being vascular plants, they have similar characteristics to bryophytes presenting quick drying and rehydration properties (Shreve 1911). Although associated with humid and dark environments, their distributions in the trunk vary. Thus, some species are able to live in more illuminated and drier sites than others (Zotz & Büche 2000; Parra et al. 2009; Krömer & Kessler 2006; Gehrig-Downie et al. 2012; Saldaña et al. 2014).

Desiccation tolerance is the ability of plants to recover from low cellular water content (5%-15%) (Wang et al. 2009). Fully mature leaves can lose up to 95% of their water content; upon rewatering, the leaves rehydrate and are fully photosynthetically active within 24 h (Bernacchia et al. 1996). Desiccation tolerant plants are also called resurrection plants, and one of the first problems that these must face in order to survive desiccation is the reduction of cell volume that occurs in drying tissues. Resurrection plants shrivel up and fold their leaves until water is available, whereupon these plants revive in a remarkable manner (Moore et al. 2009). A decrease in cellular volume causes crowding of cytoplasmic components and the cell contents become increasingly viscous, increasing the chance for molecular interactions that cause protein denaturation and membrane fusion (Hoeskstra et al. 2001). Sugars can stabilize membranes and proteins in the dry state by maintaining hydrogen bonding within and between macromolecules (Allisson et al. 1999); sugars could also vitrify the cell content and stabilize internal cell

structures (Crowe et al. 1996). During desiccation, chloroplasts appeared more spherical compared with their usual ellipsolidal shape, consistent with morphologies reported previously to occur in vascular plants subjected to drought and salt stresses (Abdelkader et al. 2007; Barhoumi et al. 2007). Controlled fragmentation of the vacuole into multiple smaller vacuoles may act to facilitate mechanical stabilization (Quartacci et al. 1997; Vander Willigen et al. 2004). Starch grains, which filled this organelle prior to desiccation, are lost. Grana gradually became less prominent, and plastoglobuli-like structures appeared. Besides the vacuole and chloroplast, other organelles were not obviously disrupted (Wang et al. 2009).

The ability to fold as the protoplast volume lessens apparently relies on an uncommon flexibility of cell walls. This is a vital feature of resurrection plants, and can be attributed to the specific composition of leaf cell walls and to biochemical events that take place inside them during dehydration (Rascio & La Rocca 2005). Arabinose-rich polymers have been implicated in maintenance of cell wall flexibility in several resurrection plants (Moore et al. 2013). Various resurrection plants may also employ drought-inducible cell wall modifications including calcium ion deposition, xyloglucan remodeling, and elevated cell wall expansions which can act to increase cell wall flexibility and allow cells to contract and fold without collapsing (Quartacci et al. 1997; Wu et al. 2001; Jones & MacQueen-Mason 2004; Vicré et al. 2004). Maintenance of cellular volume during dehydration, via water replacement in vacuoles with substances such as sugars, proline, polyphenols, and glycerol can also occur (Vander Willigen et al. 2004; Moore et al. 2007; Farrant et al. 2009).

In the later stages of dehydration, after photosynthesis ceases and the starch reserves are exhausted, carbon flux is directed to sucrose and amino acid biosynthesis. However, this increase in amino acid may also be attributable to insoluble protein breakdown (Whittaker et al. 2007). Amino acid accumulation has been associated with the stabilization of cytoplasmic constituents,

ion sequestration, and water retention (Chen & Murata 2002). The increase in sucrose is associated with a process known as vitrification, where the formation of biological glasses in the drying cell protects organelles from damage. The interaction between accumulating sugars and dehydration induced late embryogenesis abundant (LEA) proteins (Ingram and Bartels 1996), which are commonly associated with embryo development at the later stages of seed maturation (Dure 1993), is thought to be important for the protection of cellular components. Therefore, we hypothesized that during the dramatic shrinkage of the frond upon desiccation, *H. dentatum* and *H. cruentum* generate changes in chloroplast shape, vacuole fragmentation, and cell wall folding, which will be rapidly restored during the rehydration process. To test this hypothesis, we study the qualitative and quantitative variations in cell ultrastructure of *H. dentatum* and *H. cruentum* fronds during desiccation and rehydration.

Materials and methods

Study site and plant material

Hymenophyllum dentatum Cav. and *Hymenoglossum cruentum* C. Presl or *Hymenophyllum cruentum* Cav. (Larsen et al. 2013) (Pteridophyta: Hymenophyllaceae) consisting in frond, rhizome, and roots growing on dead trunks were collected in a second-growth forest located in Katalapi Park (41°31′8″ S, 72°45′2″ W, elevation ca. 80 m a.s.l.) in Pichiquillaipe, 18 km south-east from Puerto Montt, Región de Los Lagos, Chile. The climate in this zone is considered highly humid and temperate rainy (Di Castri & Hajek 1976). Samples were transferred to shaded experimental nursery gardens at Universidad de Concepción with controlled humidity. The pieces of tree trunks which supported the growth of *H. dentatum* and *H. cruentum* were wrapped and maintained in burlap and remained moist by periodically sprinkling them tap water during until use. Since Hymenophyllaceae present a modular development from a rhizome, it is difficult to isolate a complete individual. For this reason, the experimental unit was an isolated module composed by a frond, rhizome, and roots.

Samples preparation and treatments

Isolated, fully hydrated, control modules of *H. dentatum* and *H. cruentum* were exposed to a desiccation and rehydration regime in a controlled environment in the dark at 15 ± 2 °C and 80% relative humidity. Nine isolated modules from each species were placed in a glass container filled with distilled water for a 12 h period in dark. After that time, the isolated modules were dried with absorbent paper. Subsequently, no water was supplied to the isolated modules which initiated the desiccation process. After 24 h of desiccation, the rehydration process was initiated

by placing the same fronds in a glass container filled with distilled water for 24 h period in dark. Three fronds of each species were collected in each hydration state.

Variations in cell ultrastructure

Fronds sections from isolated modules of *H. dentatum* and *H. cruentum* in hydrated, desiccated, and rehydrated states were collected. The samples were immediately fixed in glutaraldehyde: paraformaldehyde (2.5%: 2%) in 0.1 M Phosphate Buffer (pH 7.2) for 24 h. Leaves were sectioned in 2-3 mm fragments and fixed again in glutaraldehyde: paraformaldehyde (2.5%: 2%) in 0.1 M Phosphate Buffer (pH 7.2) for 24 h at 4 °C. After 3 rinses with 0.1 M Phosphate Buffer, the plant material was fixed in OsO4 (1%) in 0.1 M Phosphate Buffer (pH 7.2) in a vacuum pump for 2 h. The fixed tissue was rinsed in 0.1 M Phosphate Buffer (pH 7.2); dehydrated in a graded acetone series (30%, 50%, 70%, 80%, 90%, 100%); transferred to mixtures with increasing ratios of Acetone: Araldite Resin (3: 1; 2: 2; 1: 3); settled to pure Araldite Resin for 24 h in a vacuum pump, and finally embedded in pure Araldite Resin for 48 h at 60 °C. Both semi-thin (150-200 nm) and ultra-thin (≤50 nm) sections were prepared on a Sorvall MT2-B ultramicrotome (Dupont-Sorvall, Newtown, CT). Semi-thin sections were stained with toluidine blue and observed with a microscope to analyze morphological attributes of the frond. Ultra-thin sections were cut with a diamond knife; placed on grids; stained with a saturated solution of ($UO_2(CH_3COO)_2.2H_2O$); rinsed with ultrapure water; dried, and stained with Reynolds' stain (($(PbNO_3)_2$: ($Na_3C_6H_5O_7.2H_2O$)).

Visualization

The samples were observed with a JEOL JEM-1200-EX II, Tokyo, Japan transmission electron microscope. Three fragments of three different isolated modules of each species in each hydration state were used to study the ultrastructure of *H. dentatum* and *H. cruentum*.

For the qualitative and quantitative cells characterization, each micrograph was analyzed with ImageJ 1.48v image processing program (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/).

Statistical analysis

The data were processed throughout parametric tests, due to the normality and homoscedasticity of the collected data. One-way analysis of variance (ANOVA) was applied, being the hydration state (hydrated, desiccated and rehydrated) the analyzed factor. When the F value showed significance difference ($p \leq 0.05$), the mean values were compared according to the Tukey Test ($\alpha=0.05$) using Statistica software (StatSoft Inc.).

Results

Qualitative variations of cells frond

In hydrated fronds (Fig. 1A and 1B), a large central vacuole; chloroplasts with a lenticular shape, and a stretch cell wall were observed. Also, a thickening (similar to the union of two semi circles) in both extremes of the lateral walls with thin wall in the center was observed (Fig. 1D and 1F and supplementary material). In contrast, in fronds subjected to a desiccation process for 24 h (Fig. 1C and 1D), a folded cell wall (principally the thin portion of the cell wall in the center of the lateral walls) and many small vacuoles and chloroplasts with a roundish form were observed. A better appreciation of folded cells walls in the desiccated state can be seen in Fig. 4A-C in supplementary material. After a rehydration process of 24 h (Fig. 1E and 1F), chloroplasts resumed the lenticular form and the thylakoid organization; the large vacuole was reformed from the numerous small ones; however, the cell wall still showed some folding.

The change in chloroplasts morphology (Fig. 2 and 3) in the desiccated state (Fig. 2C, 2D, 3C and 3D) was dramatic with respect to the hydrated (Fig. 2A, 2B, 3A and 3B) and rehydrated states (Fig. 2E, 2F, 3E and 3F). It appears that the thylakoid system in the desiccated state was preserved (Fig. 2D and 3D); however it appears that there is a lesser quantity of grana, which appeared again after rehydration.

Quantitative variations of cells frond

In the hydrated control state, *H. dentatum* had cells with lower widths and lengths than *H. cruentum* (areas of 1397.9 μm^2 and 2043.9 μm^2, respectively) (Table 1). *H. dentatum* had a higher cell wall width than *H. cruentum* (Table 1). The number of chloroplasts per cell was 8.4 in *H. dentatum* and 10.9 in *H. cruentum*. Inside the chloroplasts of *H. cruentum*, there were more

starch granules with a bigger area than in *H. dentatum*. Also the area of plastoglobuli was higher in *H. cruentum*. Chloroplasts size had a great change because of a decrease in the width. However, the height remained the same in *H. dentatum*, but in *H. cruentum* increased during the desiccated state (Table 2). The area continued being the same in *H. cruentum* at all hydration states, but in *H. dentatum* the area decreased 35% in the desiccated state, and recovered to 85% in the rehydrated state with respect to the initial hydrated state (Table 2).

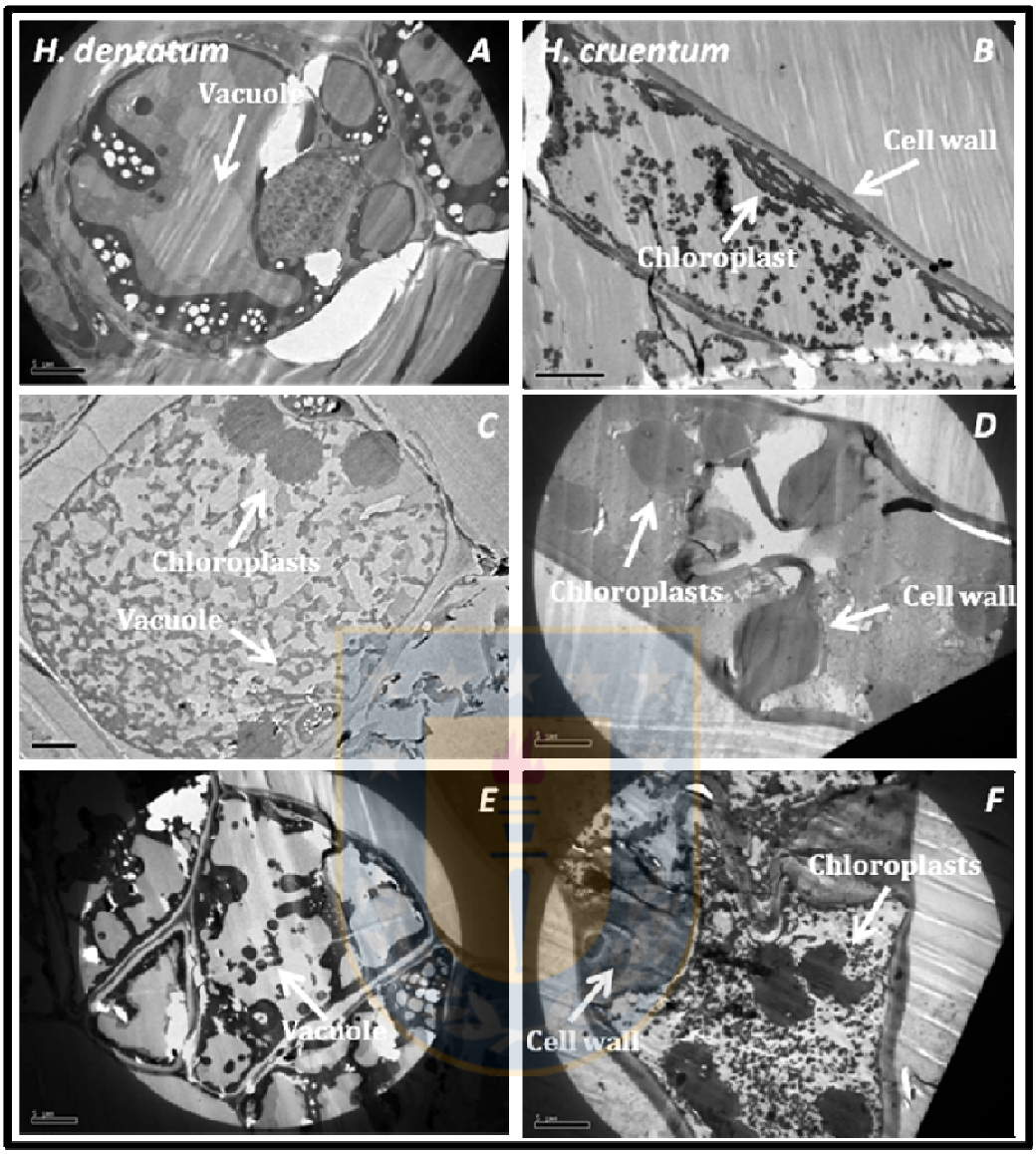

Figure 1 Transmission electron microscope micrographs from fronds cells of *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C. Presl in different hydration states. A, Micrograph of *H. dentatum* Cav. hydrated cell. B, Micrograph of *H. cruentum* C. Presl hydrated cell. C, Micrograph of *H. dentatum* Cav. desiccated cell. D, Micrograph of *H. cruentum* C. Presl desiccated cell. E, Micrograph of *H. dentatum* Cav. rehydrated cell. F, Micrograph of *H. cruentum* C. Presl rehydrated cell. The bar indicates 5 µm.

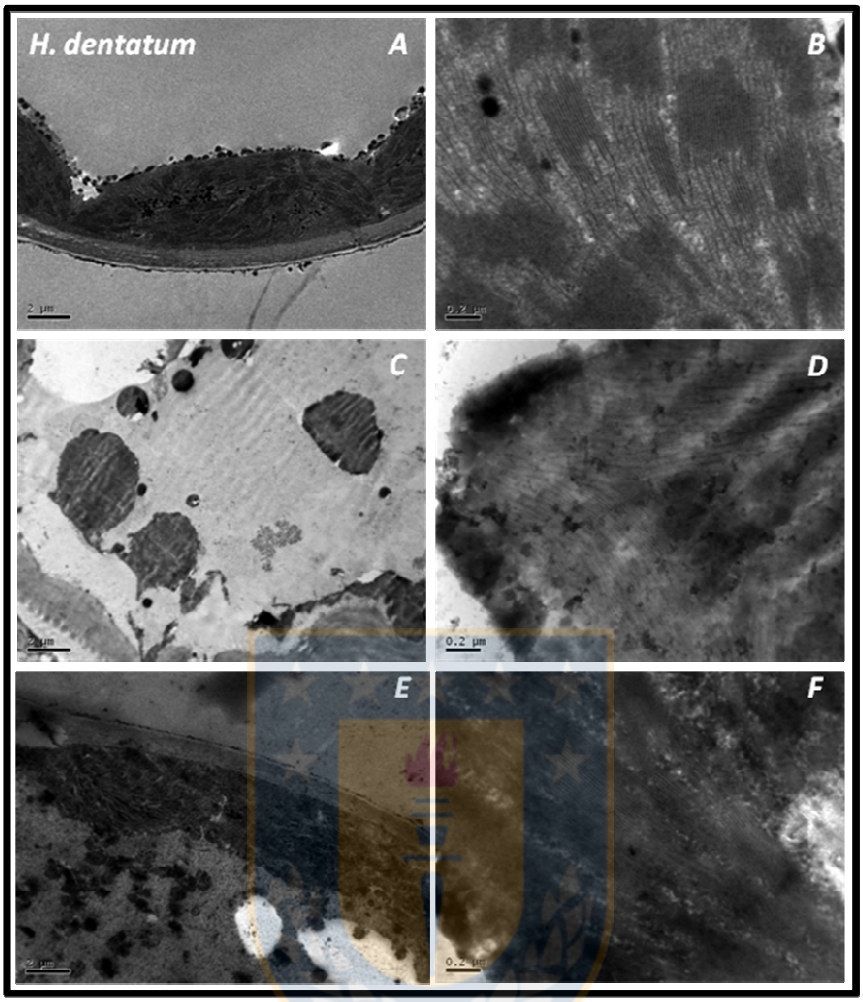

Figure 2 Transmission electron microscope micrographs from chloroplasts from frond cells of *Hymenophyllum dentatum* Cav. in different hydration states. A, Micrograph of *H. dentatum* Cav. hydrated chloroplasts with lenticular shape. The bar indicates 2 µm. B, Micrograph of internal structures of hydrated chloroplasts of *H. dentatum* Cav. The bar indicates 0.2 µm. C, Micrograph of *H. dentatum* Cav. desiccated chloroplasts with roundish shape. The bar indicates 2 µm. D, Micrograph of internal structures of desiccated chloroplasts of *H. dentatum* Cav. The bar indicates 0.2 µm. E, Micrograph of *H. dentatum* Cav. rehydrated chloroplasts resuming the lenticular shape. The bar indicates 2 µm. B, Micrograph of internal structures of rehydrated chloroplasts of *H. dentatum* Cav. The bar indicates 0.2 µm.

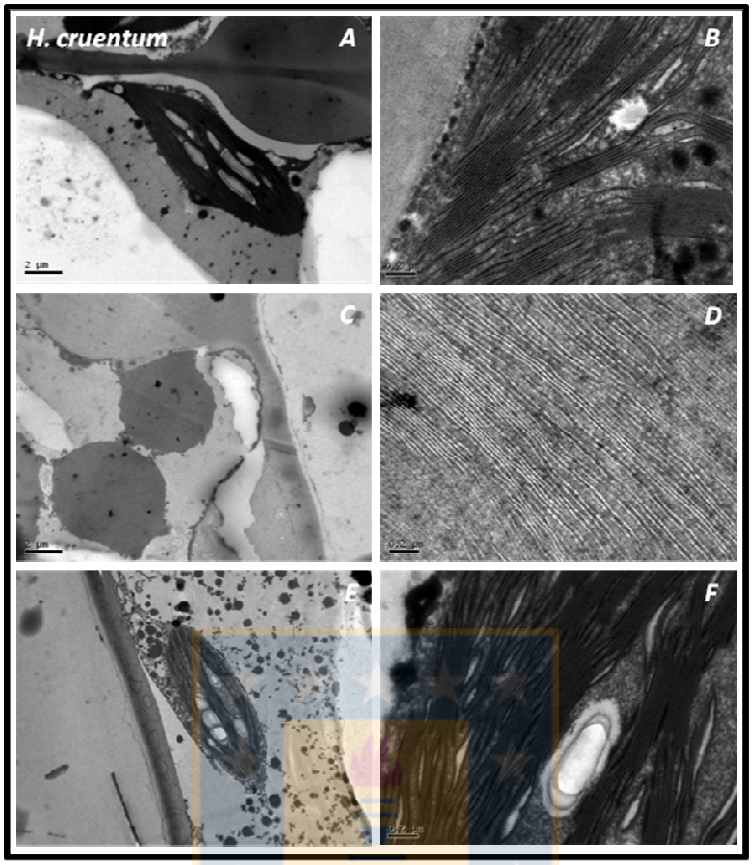

Figure 3 Transmission electron microscope micrographs from chloroplasts from frond cells of *Hymenoglossum cruentum* C. Presl in different hydration states. A, Micrograph of *H. cruentum* C. Presl hydrated chloroplasts with lenticular shape and starch granules inside. The bar indicates 2 µm. B, Micrograph of internal structures of hydrated chloroplasts of *H. cruentum* C.Presl. Granum (g), starch granule (g) and plastoglobuli (p) are indicated. The bar indicates 0.2 µm. C, Micrograph of *H. cruentum* C. Presl desiccated chloroplasts with roundish shape. The bar indicates 2 µm. D, Micrograph of internal structures of desiccated chloroplasts of *H. cruentum* C. Presl. The bar indicates 0.2 µm. E, Micrograph of *H. cruentum* C. Presl rehydrated chloroplasts resuming the lenticular shape and starch granules inside. The bar indicates 2 µm. B, Micrograph of internal structures of rehydrated chloroplasts of *H. cruentum* C. Presl. Granum (g), starch granule (s) and plastoglobuli (p). The bar indicates 0.2 µm.

Table 1

Cell characterization in *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C. Presl hydrated fronds. The values are means ± standard error. Different letters following values indicate statistical difference between species ($P \leq 0.05$, Tukey test). n indicates the number of replicas of each factor by species.

	Species	
Characteristics	***H. dentatum* Cav.**	***H. cruentum* C. Presl**
Cell width (µm) (n=5/n=3)	36.7 ± 4.0 a	41.9 ± 1.8 a
Cell length (µm) (n=4/n=3)	43.9 ± 2.1 a	54.7 ± 7.0 a
Cell area (µm^2) (n=4/n=3)	1397.9 ± 94.7 a	2043.9 ± 361.0 a
Cell wall width (µm) (n=8/n=3)	1.21 ± 0.12 a	0.98 ± 0.1 a
Number of chloroplasts per cell (n=5/n=7)	8.4 ± 0.4 b	10.9 ± 0.8 a
Starch area (µm^2) (n=29/n=55)	0.3 ± 0.1 b	0.5 ± 0.0 a
Number of starch granules per chloroplasts (n=14/n=9=)	3.2 ± 0.5 b	6.4 ± 0.6 a
Plastoglobuli area (µm^2) (n=26/n=18)	0.013 ± 0.001 a	0.017 ± 0.002 a

Table 2

Changes in chloroplasts dimension of *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C. Presl fronds in hydrated, desiccated and rehydrated states. The values are means ± standard error. Different letters following values indicate statistical difference by hydration state in each species ($P \leq 0.05$, Tukey test). n is the number of analyzed chloroplasts in each hydration state.

Species	**Hydration state**	**Chloroplasts dimensions**		
		Width (µm)	**Heigth (µm)**	**Area (µm^2)**
***H. dentatum* Cav.**	Hydrated (n=16)	9.7 ± 0.8 a	4.3 ± 0.2 a	35.3 ± 3.2 a
	Desiccated (n=14)	5.6 ± 0.3 b	4.9 ± 0.3 a	23.0 ± 2.4 b
	Rehydrated (n=15)	9.6 ± 0.6 a	4.3 ± 0.2 a	30.0 ± 1.9 ab
***H. cruentum* C. Presl**	Hydrated (n=14)	10.61 ± 0.6 a	4.4 ± 0.2 b	35.4 ± 1.5 a
	Desiccated (n=11)	7.0 ± 0.4 b	6.2 ± 0.3 a	36.1 ± 3.1 a
	Rehydrated (n=11)	9.6 ± 0.7 a	5.1 ± 0.5 ab	41.5 ± 5.9 a

Discussion and conclusions

The purpose of this research study was to determine the qualitative and quantitative variations in cell ultrastructure of *H. dentatum* and *H. cruentum* fronds during desiccation and rehydration.

Based on our data of cell characterization, *H. cruentum* has bigger cells than *H. dentatum* with a higher number of chloroplasts per cell, and a higher number of starch granules per chloroplasts. These three features could be related with the shade habitat of *H. cruentum* (Larcher 2003). On the other hand, the qualitative variations showed that the desiccation process generates morphological changes as frond shrinkage; cell wall folding; roundish chloroplasts, and vacuole fragmentation. In a previous study, *H. dentatum* and *H. cruentum* desiccated fronds presented a reduction of the total projected area of 78% and 68%, respectively (Flores-Bavestrello et al. 2016), which allows us to observe the changes mentioned above. *Craterostigma plantagineum* leaves, a resurrection plant, drop to around 15% of their original area (Hartung et al. 1998). In *Chamaegigas intrepidus*, another resurrection plant, leaves shrink to 25% of their original size (Schiller et al. 1999). The change in chloroplasts shape from a lenticular to a roundish shape in the desiccated states has been reported before (Cooper & Farrant 2002; Rascio & La Rocca 2005; Fernández-Marín 2016), but to our knowledge it is the first time that a study shows a quantitative analysis of the modification in chloroplasts size in resurrection plants from Hymenophyllaceae. In this study, we observed that *H. dentatum* chloroplasts decreased their area in the desiccated state; however, *H. cruentum* chloroplasts conserved its area. *Physcomitrella patens*, a moss, belongs to the homoiochlorophyllous group (Wang et al. 2009), as *H. dentatum* and *H. cruentum* (Flores-Bavestrello et al. 2016). In this moss, chlorophyll levels remained high and chloroplast structure was preserved, although it was changed to a spherical form with less well-stacked thylakoids (Wang et al. 2009). *H. dentatum* and *H. cruentum* presented similar characteristics,

which according to Tuba et al. (1993), is typical of a homoiochlorophyllous strategy. In contrast, the chloroplasts in poikilochlorophyllous species degrade chlorophylls and thylakoid membranes yielding desiccoplasts, devoided of any internal structures (Rascio & La Rocca 2005), which, as it was mentioned above, is not the case of these Hymenophyllaceae. Other changes that these species presented in the desiccated state are the fragmentation of the vacuole and the folding of the cell walls. *Craterostigma wilmsii*, a resurrection plant, maintained small vacuoles and prevented plasmolysis through invagination and folding of the cell wall (Farrant 2000). *Myrothamnus flabellifolius*, a resurrection shrub, revealed folded cell walls in the majority of desiccated leaf cells, implicating wall folding as the major mechanisms by which minimizes mechanical damage (Moore et al. 2006). A study of the leaf cell wall composition of the same species suggested that constitutive protection was afforded through the presence of highly flexible pectin-associated arabinans and arabinogalactan proteins (Moore et al. 2013). The ability of resurrection plants to endure very rapid drying indicates that the protective mechanism relies on constitutive systems rather than inductive mechanisms, consisting of the presence of large amounts of free sugars and proteins (sucrose and dehydrins) in hydrated tissues known to maintain the cellular order upon desiccation (Oliver et al. 1998). In drying leaves of *Craterostigma wilmsii*, a significant increase in unesterified pectins and xyloglucans occurs that is accompanied by changes in the hemicellulose sugars (Vicré et al. 1999, 2004). These alterations in polysaccharides have been suggested to modulate the mechanical properties of cell walls, leading to a more tensile strength, which permits folding of the cell wall, but avoids its total inward collapse in the dry state (Vicré et al. 2004). Expansins have been recently implicated in increasing the flexibility of the cell wall (Jones & McQueen-Mason 2004). They have a role in loosening of the cell wall, and are involved in several important plant growth and development processes (Chen & Bradford 2000). At present four expansin groups (α-δ) have been

characterized (Li et al. 2003). In *Craterostigma plantagineum* desiccated leaves, the marked increase in cell wall flexibility is paralleled by a rise in the expression and activity of several α-expansins (Jones & McQueen-Mason 2004). In addition, the peculiar thickening in both extremes of the lateral walls with thin wall in the center presented by *H. dentatum* and *H. cruentum* was reported by Shreve (1911) in *Trichomanes rigidum* Sw., but with the thickening in the center of the lateral wall with areas of thin wall above and below this structure. According to Shreve (1911), this structure could provide the leaf with a rigid meshwork, and could serve to hinder the transfer of water from cell to cell, and could be accountable for the inability of this species to secure root-absorbed water quickly enough to meet even the demands of transpiration in a nearly saturated atmosphere. We disagree with the indicated at the end, because we have seen that these species are not rehydrated by providing water to the rhizome and roots. They rehydrate just when water is sprinkled to fronds or relative humidity is high (results not shown). On the other hand, Hartung et al. (1998) reported preformed folding sites of cell wall in poikilohydric plants. Thus the ability of cell walls to fold by changing their chemistry and texture is an adaptive strategy against desiccation that is essential for the survival of resurrection plants. Not all resurrection plants adopt this mechanism to mitigate the potential mechanical stress during dehydration. Some species, especially among monocotyledons, utilize a strategy of water substitution, where the original cell volume is maintained and vacuole shrinkage is hindered by replacing water with nonaqueous substances such as amino acids, small proteins, and sugars (Farrant 2000). Ions are also removed from the cytoplasm and sequestered into the vacuole, an activity that might correlate with the upregulation of a V-ATPase found in drying leaves of *Sporobolus stapfianus* (Blomstedt et al. 1998) and *Xerophyta viscosa* (Marais et al. 2004). Concomitantly to filling with water substitutes, the large central vacuole is subdivided into a number of smaller units (Quartacci et al. 1997; Dalla Vecchia et al. 1998; Vander Willigen et al. 2004). This event

requires the *ex novo* synthesis of tonoplast, since the total length of the vacuolar membrane increases (Vander Willigen et al. 2004). This fragmentation of the vacuole also comes into desiccation adaptive mechanisms, since in dried tissues several small vacuoles having an increased surface/volume ratio show a mechanical stability higher than that of one large vacuole (Iljin 1957).

In hydrated and rehydrated *H. dentatum* and *H. cruentum* micrographs, starch grains filled chloroplasts, however these were lost during desiccation as Hoesktra et al. (2001) and Wang et al. (2009) reported. Starch accumulates in the chloroplast stroma during illumination, forming large granules, but during darkness it is remobilized and consumed in respiration (Lawlor 1993). The catabolism of polysaccharides, such as starch, is enhanced at the early stage of desiccation. This is shown by the α-glucan water dikinase, which catalyzes starch phosphorylation, which is required for starch degradation (Mikkelsen et al. 2004). The accumulation of this protein is consistent with the degradation of starch grains under desiccation. Starch degradation provides energy for the cell; it provides carbon skeletons for osmotic adjustment, stabilizing the membrane system and preventing the crystallization of cellular solutes (Hoekstra et al. 2001).

We conclude that both species, despite of their vertical distribution, exhibit similar morpho-anatomical reversible changes during the desiccation-rehydration cycle. As our hypothesis state, both species present in the desiccated state similar qualitative and quantitative changes, as cell wall folding; roundish chloroplasts, and vacuole fragmentation. After rehydration, cell walls recover their normal shape and chloroplasts resume a lenticular shape with a correct organization and composition of thylakoids. This capacity indicates that there is a protective mechanism based on constitutive systems which minimized mechanical damage.

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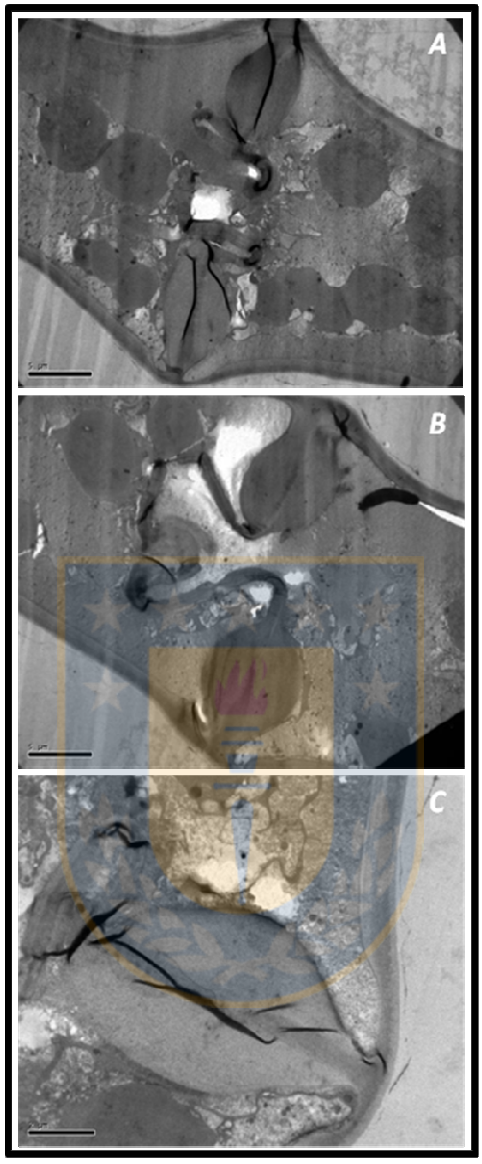
Supplementary material



Transmission electron microscope micrographs of cells of desiccated fronds of *Hymenoglossum cruentum* C. Presl. A, Desiccated cell of *H. cruentum* C.Presl with folded cell walls and roundish chloroplasts. The bar indicates 5 µm. B, Cell wall with it foldings in desiccated cells of *H. cruentum* C.Presl. The bar indicates 5 µm. C, Close-up of a cell wall folding of a desiccated cell of *H. cruentum* C.Presl. The bar indicates 5 µm.

Chapter III

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Two Hymenophyllaceae species from contrasting natural environments exhibit a homoiochlorophyllous strategy in response to desiccation stress.

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Abstract

Hymenophyllaceae is a desiccation tolerant family of Pteridophytes which are poikilohydric epiphytes. Their fronds are composed by a single layer of cells and lack true mesophyll cells and stomata. Although they are associated with humid and shady environments, their vertical distribution varies along the trunk of the host plant with some species inhabiting the drier sides with a higher irradiance. The aim of this work was to compare the structure and function of the photosynthetic apparatus during desiccation and rehydration in two species, *Hymenophyllum dentatum* and *Hymenoglossum cruentum*, isolated from a contrasting vertical distribution along the trunk of their hosts. Both species were subjected to desiccation and rehydration kinetics to analyze frond phenotypic plasticity, as well as the structure, composition and function of the photosynthetic apparatus. Minimal differences in photosynthetic pigments were observed upon dehydration. Measurements of ϕPSII (effective quantum yield of PSII), ϕNPQ (quantum yield of the regulated energy dissipation of PSII), ϕNO (quantum yield of non-regulated energy dissipation of PSII), and TL (thermoluminescence) indicate that both species convert a functional photochemical apparatus into a structure which exhibits maximum quenching capacity in the dehydrated state with minimal changes in photosynthetic pigments and polypeptide compositions. This dehydration-induced conversion in the photosynthetic apparatus is completely reversible upon rehydration. We conclude that *H. dentatum* and *H. cruentum* are homoiochlorophyllous with respect to desiccation stress and exhibited no correlation between inherent desiccation tolerance and the vertical distribution along the host tree trunk.

Keywords: Desiccation tolerance; Filmy ferns; Homoiochlorophyllous; Hymenophyllaceae; Poikilohydric.

Abbreviations:

ϕPSII, effective quantum yield of PSII; ***ϕNPQ***, quantum yield of the regulated energy dissipation of PSII; ***ϕNO***, quantum yield of non-regulated energy dissipation of PSII; $\mathbf{\Delta A_{820\text{-}860}}$, extent of the absorbance decrease at 820-860 nm; **MT**, multiple turnover saturating flash, **ST**, single turnover saturating flash; $\mathbf{A_{MT}/A_{ST}}$, functional pool size of intersystem electrons per reaction centre; $\mathbf{t_{½}}$, cyclic electron flow around PSI; ***PI***, photoinactivation; ***PA***, photoactivation; **Chl*a***, chlorophyll *a*; **Chl*b***, chlorophyll *b*; **α-car**, α-carotene; **β-car**, β-carotene; ***N***, neoxanthin; ***L***, lutein; **TL**, thermoluminescence; $\boldsymbol{T_M}$, the temperature maxima of the TL peaks. ***FW***, fresh weigth; ***DW***, dry weight.

Introduction

Species that can survive exposure to severe dehydration are called desiccation tolerant. During desiccation, the hydration shell of molecules is gradually lost (<0.3 (g H_2O) (g dry weight)$^{-1}$). Desiccation tolerant species maintain the ability to rehydrate successfully (Hoekstra et al., 2001). “Poikilohydry” is the ability of an organism to equilibrate its internal water potential with that of the environment (Rascio and La Rocca, 2005). The majority of desiccation tolerant terrestrial plants are found in the clades that constitute the algae, lichens, and bryophytes (Oliver et al., 2000). However, within the larger groups of vascular land plants there are ca. 70 species of ferns, and approximately 60 species of angiosperms that exhibit some degree of vegetative desiccation tolerance (Oliver et al., 2000).

Desiccation tolerant vascular plants fall into two groups depending on the degree to which they retain their chlorophyll when desiccated (Proctor and Tuba, 2002). Homoiochlorophyllous species retain their photosynthetic apparatus and chlorophylls in a readily recoverable form, while poikilochlorophyllous species dismantle their photosynthetic apparatus and lose all of their chlorophylls during desiccation. Consequently, these pigments must be resynthesized following rehydration (Tuba et al., 1998). In addition, the poikilochlorophyllous leaves cannot resurrect when they are detached from the plant in contrast to homoiochlorophyllous resurrection plants (Dinakar et al., 2012).

The photosynthetic apparatus comprises two photosystems. Photosystem II (PSII) catalyses the solar-energy driven conversion of water to O_2 through the photochemical generation of a strong oxidant, $P680^+$, while Photosystem I (PSI) catalyses the final step of photosynthetic electron transport, the oxidation of plastocyanin in the thylakoid lumen, and the photochemical generation of a strong reductant, reduced ferredoxin, in the chloroplast stroma. Both

photosystems are interconnected by the cytochrome-b_6f complex via two diffusible redox carriers: plastoquinone and plastocyanin (Schöttler et al., 2011). PsbA (D1) and PsbD (D2) are the polypeptides that constitute the photochemical reaction centre of PSII which bind P680 and a series of redox components which catalyze charge separation and primary electron transfer reactions (Dekker and Van Grondelle, 2000). The PSI reaction centre consists of the protein subunits PsaA and PsaB, which bind 80 chlorophylls and the redox-active cofactors required for PSI photochemistry and charge separation (Schöttler et al., 2011). The physical properties of the photosynthetic apparatus are crucial for the survival of desiccation-tolerant plants since it is very sensitive to photoinhibition and photodamage. Consequently, the photosynthetic apparatus must either be maintained during desiccation or quickly repaired upon rehydration (Georgieva et al., 2009). There are several studies that explain some of the mechanisms involved in desiccation tolerance (Proctor, 2003; 2012; Heber et al., 2006a; b; 2007; Heber, 2008; Georgieva et al., 2009; Charuvi et al., 2015). The present study shows the structural and functional mechanisms of the photosynthetic apparatus involved in the desiccation tolerance and subsequent rehydration of two Hymenophyllaceae species contrasting in their vertical distribution along tree trunks.

The Hymenophyllaceae is a family of ferns that, despite belonging to the vascular plants, possesses characteristics of the less complex clades of the plant kingdom (Proctor, 2003; 2012). The Hymenophyllaceae Family is a desiccation tolerant group of Pteridophytes, called also “filmy ferns” because of their transparent and delicate fronds. These species are poikilohydric epiphytes composed of lamina that are only one cell-thick. Their fronds typically lack a cuticle and true mesophyll cells as well as differentiated epidermal cells and stomata. Hymenophyllaceae have distinctive marginal sori and varying pigmentation and textures of the rachis. Their rhizomes are long and filiform making a net that covers the ritidome of the phorophytes. The main characteristics of this family are the denticulation of the margins of segments, and the sori

are often positioned at the tips of acroscopic segments (Hennequin et al., 2010). Hymenophyllaceae comprises more than 600 species (Dubuisson et al., 2009) of very small to middle-sized ferns. This family extends into temperate latitudes in high-rainfall oceanic regions, such as the Macaronesian islands and the Atlantic coastal regions of western Europe, southern Chile, New Zealand, south-east Australia, Trinidad, Venezuela (Proctor, 2012), Panama (Zotz and Büche, 2000), Bolivia (Krömer and Kessler, 2006), Argentina (Fernández et al., 2012), South Africa (Dinakar et al., 2012), and Jamaica (Shreve, 1911).

In the temperate rainforest of southern Chile, the dominant host tree species for Hymenophyllaceae are *Amomyrtus luma*, *Rhaphythamnus spinosus*, *Laurelia philippiana* and *Drimys winteri* (Saldaña et al., 2014). Phorophyte specificity is more pronounced in dry forest habitats, where the epiphytes grow in direct contact with the bark of the host tree (Ibish, 1996). In humid forests, mats of bryophytes, lichens, and detritus lead to similar growth conditions on different trees (Krömer and Kessler, 2006), even where canopy openness ranges from 0.1 to 31 % full sunlight (Parra et al., 2009). The vertical distribution of several Hymenophyllaceae species, both in young and old trees from different stands in a temperate forest of southern Chile, was reported to exhibit habitat preferences. Hymenophyllaceae species can be distributed either mainly at the trunk base, between 4 and 4.5 m height from the base or up to 9 m high along the tree trunk (Saldaña et al., 2014). Therefore, those individuals that are found at different heights along the trunk of the host will be exposed to different light and humidity conditions. Thus, we predicted that Hymenophyllaceae species found near the top of trunk of the host should exhibit different strategies to cope with the desiccation tolerance and light than those species located near the base of the host. We hypothesized first, that Hymenophyllaceae species which inhabit the top zone will be more tolerant to a desiccation process and exhibit a faster recovery during the rehydration process than those that inhabit the basal zone. Second, we hypothesized that the

Hymenophyllaceae are homoiochlorophyllous in their response to desiccation stress. To test these hypotheses, we compared the structure and function of the photosynthetic apparatus during desiccation-rehydration in *H. dentatum* and *H. cruentum*, isolated from contrasting vertical distribution along the trunk of their hosts.

Materials and methods

Study site and plant material

Hymenophyllaceae (Pteridophyte) species were collected in a second-growth forest located in Katalapi Park (41°31′8″ S, 72°45′2″ W, elevation ca. 90 m a.s.l.) in Pichiquillaipe, 18 km south-east from Puerto Montt, Región de Los Lagos, Chile. The climate in this zone is considered highly humid and temperate rainy (Di Castri and Hajek, 1976). Two species were sampled, *Hymenophyllum dentatum* and *Hymenoglossum cruentum*, consisting of frond, rhizome, and roots growing on dead trunks. Samples were transferred to shaded experimental nursery gardens at Universidad de La Frontera and Universidad de Concepción with controlled humidity. The pieces of tree trunks which supported the growth of *H. dentatum* and *H. cruentum* were wrapped and maintained in burlap and remained moist by periodically sprinkling them tap water during until use. A second set of plant material was cleaned of debris and sediments with distilled water and subsequently wrapped in moist paper towels and transported in plastic boxes to Western University, London, Ontario, Canada. The plant material was kept under low light (7 µmol photons m^{-2} s^{-1}), at 4 °C and sprayed with distilled water several times a day until use. Each experimental plant or intact module consisted of a frond, rhizome, and roots.

Sample preparation and treatments

Isolated, fully hydrated, control modules of *H. dentatum* and *H. cruentum* were exposed to a desiccation and rehydration regime in a controlled environment in the dark at 15 ± 2 °C and 80 % relative humidity. Treatments were performed intentionally in the dark to simplify the task of giving interpretations to the physiological responses related to desiccation and rehydration. Eight isolated modules from each species were placed in a glass container filled with distilled water for

a 12 h period in dark. After that time, the isolated modules were dried with absorbent paper and weighed to obtain the total turgor weight. Maximum photochemical efficiency of PSII (*Fv/Fm*) was measured in these control samples. Subsequently, no water was supplied to the isolated modules which initiated the desiccation process. Fresh weight and the *Fv/Fm* were measured every 30 min until a constant desiccated weight was reached. After 24 h of desiccation, the rehydration process was initiated by sprinkling the same fronds with distilled water inside a plastic box. Every 30 min these modules were surface dried, *Fv/Fm* measured and weighed until a constant rehydrated weight was reached. The final measurements were made after 24 h of rehydration. As *H. dentatum* and *H. cruentum* have one cell thick layer fronds, 24 h in each state is enough to reach equilibrium with air.

Frond shrinkage

Fronds from isolated modules of *H. dentatum* and *H. cruentum* were scanned in the hydrated, desiccated, and rehydrated states (24 h in each state). The area was quantified with ImageJ 1.48v image processing program (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/) from which the projected frond area was obtained. Frond shrinkage in the desiccated state was calculated as the difference between the projected area of the hydrated and rehydrated states.

Photosynthetic pigment content

Frond samples were collected and immediately frozen in liquid nitrogen and kept in the dark. All samples were extracted in acetone (100 %). The pigment extracts were filtered throughout a 0.45 µm membrane filter and separated and quantified by HPLC according to the protocol of García-

Plazaola and Becerril (1999) with the modifications described in García-Plazaola and Becerril (2001).

Water content (WC) and relative water content (RWC)

WC and *RWC* was determined gravimetrically by weighing the isolated modules in the hydrated, desiccated, and rehydrated process, and after oven drying at 72 °C for 48 h for dry weight determinations. *WC* (dry weight basis) and *RWC* was calculated as WC=*[(FW-DW)/DW]* and *RWC=[(FW-DW)/(TTW-DW)]x100*, respectively, where *FW* is the fresh weight, *DW* is the dry weight, and *TTW* is the total turgor weight of the sample. The weight was measured every 30 min.

Photoinactivation (PI) and photoactivation (PA) kinetics

The maximum photochemical efficiency of PSII (*Fv/Fm*) in the hydrated state $(Fv/Fm)_h$ and after some degree of dehydration $(Fv/Fm)_d$, was measured in dark-adapted fronds with a modulated fluorometer (FMS 2, Hansatech Instruments, UK) every 30 min at 15 °C. Minimal fluorescence (*Fo*) was determined by applying a weak modulated light of 650 nm (0.4 µmol photons m^{-2} s^{-1}), and maximal fluorescence (*Fm*) was induced by a 0.8 s saturating pulse of white light (9000 µmol photons m^{-2} s^{-1}) (van Kooten and Snel, 1990). Photoinactivation (*PI*) during desiccation was calculated as $PI=1-[(Fv/Fm)_d/(Fv/Fm)_h]$ and photoactivation (*PA*) during rehydration was calculated as $PA=[(Fv/Fm)_d/(Fv/Fm)_h]$ (adapted from Ögren and Rosenqvist, 1992). *PI* and *PA* were used to assess the functional integrity of photosystem II.

Modulated chlorophyll fluorescence

Chlorophyll *a* fluorescence of *H. dentatum* and *H. cruentum* fronds in hydrated, desiccated, and rehydrated states was measured at 15 °C under ambient CO_2 conditions using a pulse amplitude modulated imaging fluorometer (IMAGING-PAM, Heinz Walz GmbH, Effeltrich, Germany) as described in Ivanov et al., (2006a). Before measurements, fronds were dark adapted for 30 min and the fluorescence images were captured by a CCD camera (IMAG-K, Allied Vision Technologies) featuring 640x480 pixel CCD chip size and CCTV camera lens (Cosmicar/Pentax F1.2, f=12 mm). Light emitting diode ring array (IMAG-L) consisting of 96 blue LEDs (470 nm) provided standard modulated excitation intensity of 0.5 µmol quanta m^{-2} s^{-1} (modulation frequency 4 Hz) for measuring the basal (*Fo*) chlorophyll fluorescence and a saturation pulse of 2400 µmol quanta m^{-2} s^{-1} PAR for measuring the maximal chlorophyll fluorescence (*Fm*), both from a dark adapted leaf. The maximal photochemical efficiency of PSII, which represents the maximum efficiency at which light absorbed by PSII is used for reduction of Q_A was estimated as *Fv/Fm=(Fm-Fo)/Fm*. Partitioning of absorbed light energy was estimated when the steady state *Fs* level was reached according to the model proposed by Kramer et al., (2004). The allocation of photons absorbed by the PSII antennae to photosynthetic electron transport and PSII photochemistry was estimated as *ϕPSII=(Fm'-Fs)/Fm'*, where *Fm'* is the maximal fluorescence from a light adapted leaf. The quantum efficiencies of regulated ΔpH- and/or xanthophylls-dependent non-photochemical dissipation processes within the PSII antennae (*ϕNPQ*) were calculated as: *ϕNPQ=1-ϕPSII-ϕNO*. Constitutive non-photochemical energy dissipation and fluorescence were calculated as *ϕNO=1/[NPQ+1+qL(Fm/Fo-1)]*. qL, which estimates the fraction of open PSII centres based on a lake model (PSII reaction centres in a common pigment bed), was calculated as *qL=(Fm'-Fs)/(Fm'-Fo')xFo'/Fs*, where *Fo'* is the minimal fluorescence from light adapted leaf. *ϕPSII*, *ϕNPQ* and *ϕNO* are defined as effective photochemical yield of

PSII; quantum yield of light-induced non-photochemical fluorescence quenching, and quantum yield of non-light-induced non-photochemical fluorescence quenching, respectively (Pfündel et al., 2008). Electron transport rates (*ETR*) were calculated as: $ETR=PARx0.5x\phi PSIIxA$ (Genty et al., 1989), where *A* is the actual measured leaf absorbance before performing the fluorescence measurement.

Thermoluminescence measurements

Thermoluminescence (TL) measurements of *H. dentatum* and *H. cruentum* fronds in hydrated, desiccated and rehydrated states were performed on a personal-computer-based TL data acquisition and analysis system as described earlier (Ivanov et al., 2001; 2006b). A photomultiplier tube (Hamamatsu R943-02, Hamamatsu Photonics, Shizuoka-ken, Japan) equipped with a photomultiplier power supply (Model C3350, Hamamatsu Photonics, Shizuoka-ken, Japan), was used as a radiation measuring set. The heating rate was 0.6 °C s^{-1}. For identifying the $S_{2/3}Q_B^-$ charge recombination peaks, dark-adapted fronds were subjected to two consecutive saturating microsecond flashes of white light (1.5 μs peak width at 50 % of maximum) applied by a xenon-discharge flash lamp (XST103, Heinz Walz GmbH, Effeltrich, Germany). Dark-adapted fronds of *H. dentatum* and *H. cruentum* (30 min at 20 °C) were cooled to 2 °C prior to exposing to the flashes. The nomenclature of Sane et al. (2012) was used for characterization of the TL glow peaks.

PSI photochemistry

The reduction-oxidation (redox) state of P700 was determined *in vivo* in hydrated, desiccated and rehydrated fronds of *H. dentatum* and *H. cruentum* under ambient O_2 and CO_2 conditions using a

PAM-101 modulated fluorometer (Heinz Walz GmbH, Effeltrich, Germany) equipped with a dual wavelength emitter-detector ED-P700DW unit and PAM-102 units (Klughammer and Schreiber 1991) as described in detail by (Ivanov et al., 1998). Far red light (FR; max =715 nm, 10 W m^{-2}) equipped with a Schott filter (RG 715) was provided by a FL-101 light source. The redox state of P700 was evaluated as the absorbance change around 820 nm ($\Delta A_{820-860}$) in a custom designed, thermoregulated cuvette at 15 °C. Multiple turnover (MT, 50 ms) and single turnover (ST, half peak 14 µs) saturating flashes were applied with XMT-103 and XST-103 (Heinz Walz GmbH, Effeltrich, Germany) power/control units, respectively. The transient reduction of the $P700^{+}$ signal after application of single (ST) and multiple (MT) turnover flashes of white saturating light was used for the estimation of the intersystem electron (e^{-}) pool size (Asada et al., 1992; Ivanov et al., 1998). Each frond was measured twice.

SDS-PAGE and immunoblotting

Thylakoid membranes from hydrated, desiccated, and rehydrated fronds of *H. dentatum* and *H. cruentum* were isolated according to the method of Król et al., (1999). SDS-PAGE was performed according to Laemmli (1970) using 15 % (w/v) polyacrylamide in the resolving gel and 8 % (w/v) in the stacking gel. Thylakoids membranes were solubilized with SDS (SDS:protein ratio of 4:1). Protein concentration was determined using the Pierce BCA Protein Assay Kit-Reducing Agent Compatible (Thermo Scientific) and protein was loaded on an equal dry weight basis of 0.08 mg $lane^{-1}$. Specific antibodies against PsaA, Lhca1-4, PsbA, and Lhcb1-6 were obtained from Agrisera (Sweden) and used for immunoblotting as described in detail previously (Król et al., 1999). Thylakoid proteins were detected with specific antibodies at the following dilutions: PsaA, 1:1000; Lhca1-4, 1:2000; PsbA (D1), 1:4000; Lhcb1, 1:2000; Lhcb2, 1:5000; Lhcb3, 1:2000; Lhcb4, 1:7000; Lhcb5, 1:1000; Lhcb6, 1:2000. Densitometric scanning

and analysis of X-ray films from each replicate immunoblot was performed with a Hewlett Packard Scanjet 4200c desktop scanner and ImageJ 1.48v image processing program (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/).

Statistical analysis

The data were processed throughout parametric tests, due to the normality and homoscedasticity of the collected data. One-way analysis of variance (ANOVA) was applied, being the hydration state (hydrated, desiccated and rehydrated) the analyzed factor. When the F value showed significance difference ($p \leq 0.05$), the mean values were compared according to the Tukey Test ($\alpha=0.05$) using Statistica software (StatSoft Inc.).

Results

Frond morphology

There was a pronounced change in the projected frond area between hydrated, desiccated and rehydrated states, showing statistical differences between the desiccated state versus the hydrated and rehydrated states in both *H. dentatum* and *H. cruentum* species (Fig. 1). The greatest frond shrinkage was observed in *H. dentatum*, showing a decrease of 78 % in its projected area whereas *H. cruentum* showed a decrease of 68 %. After 24 h of rehydration, both *H. dentatum* and *H. cruentum* reached 92 to 95 % of the original projected frond area in the hydrated state. Thus, the desiccation-induced shrinkage was reversible in both species.

Structure and composition of the photosynthetic apparatus

Pigment concentration and composition of fronds for both *H. dentatum* and *H. cruentum* on a dry weight basis showed, in general, little changes between the hydrated, desiccated, and rehydrated states (Table 1). The total Chl*a* and Chl*b* as well as the Chl*a/b* ratios remained constant in both species irrespective of treatment. However, a decrease in the Chl*a/b* ratio in the rehydrated state of *H. dentatum* was observed. *H. cruentum* presented higher values of Chl*a* and Chl*b*, as well as β-carotene, neoxanthin and lutein. α-carotene was not detected in *H. dentatum* irrespective of the treatments, but it was in *H. cruentum*. The N/β-carotene ratio was higher in *H. cruentum* irrespective of the treatment, however in *H. dentatum* was higher in the desiccated state.

Photosynthetic pigments are associated with the major pigment-protein complexes associated with PSII and PSI. In general, the reaction centre polypeptides of PSII (PsbA) and PSI (PsaA) as well as the polypeptides associated and the light harvesting complexes of PSII (Lhcb1-6) and PSI (Lhca1-4) did not exhibit significant differences in the relative abundance when the

different hydration states were compared in both *H. dentatum* and *H. cruentum* species (Fig. 2). Thus, based on pigment and polypeptide analyses, we conclude that the structure and composition of the photosynthetic apparatus of both *H. dentatum* and *H. cruentum* appeared to be stable to desiccation stress and rehydration.

PSII function

The maximum photochemical efficiency of PSII, measured as *Fv/Fm*, of *H. dentatum* (Fig. 3A, closed symbols) associated with the upper 9 m of the host trunk, appeared to be more sensitive to a decrease in relative water content (*RWC*) than that of *H. cruentum* (Fig. 3A, open symbols). At 30 % *RWC* (0.6 gH_2O gDW^{-1}), *Fv/Fm* was about 3-fold lower in *H. dentatum* than in *H. cruentum*. However, upon rehydration, both species exhibited fully recovery of *Fv/Fm* at 60 % *RWC* (1.13 and 1.22 gH_2O gDW^{-1}, respectively) (Fig. 3B). Although *H. dentatum* is more sensitive to desiccation and exhibits a greater photoinactivation response than *H. cruentum* at 30 % *RWC* (0.6 gH_2O gDW^{-1}) (Fig. 3C), both species exhibited comparable, maximum photoactivation levels at 60 % *RWC* (1.13 and 1.22 gH_2O gDW^{-1}, respectively) (Fig. 3D). Chl fluorescence was also used to estimate energy partitioning within PSII as a function of desiccation and rehydration. Fig. 4 illustrates light response curves for $\phi PSII$, ϕNPQ, and ϕNO under control (hydrated) as well as desiccated and rehydrated states for *H. dentatum* and *H. cruentum*. In the hydrated state (100 % *RWC*, 1.9 and 2.0 gH_2O gDW^{-1}, respectively) (Fig. 4A and D), both species showed a decrease in the effective quantum yield of PSII ($\phi PSII$) as a function of increased irradiance. However, the quantum requirement to inhibit $\phi PSII$ was much lower in *H. cruentum* than in *H. dentatum* as estimated by the inverse of maximum initial slopes of the light response curves for $\phi PSII$. Concomitantly, the inhibition of $\phi PSII$ was associated with an increase in ϕNPQ as a function of irradiance in both species (Fig. 4A and D). Furthermore, the

quantum requirement to generate a unit of NPQ was much lower in *H. cruentum* than in *H. dentatum* as indicated by the differences in the initial slopes of the light response curves for *ϕNPQ* (Fig. 4A and D). Thus, light intensity at which the *ϕNPQ* equals *ϕPSII* is distinctive between these two species. While *H. cruentum* showed the light interception point between yield of PSII and yield of NPQ at about 120 µmol photons m^{-2} s^{-1}, *H. dentatum* reached this condition at about 300 µmol photons m^{-2} s^{-1} (Fig. 4A and D). Although energy-dependent, non-photochemical quenching (*ϕNPQ*) increased dramatically as a function of irradiance, constitutive quenching (*ϕNO*) decreased by about 30 % as a function of increased irradiance in both species (Fig. 4A and D).

In the desiccated state (25 % (0.4 gH_2O gDW^{-1}) and 17 % (0.3 gH_2O gDW^{-1}) *RWC*, respectively) (Fig. 4B and E), *ϕNO* reached values closed to 1, while *ϕNPQ* and *ϕPSII* were essentially undetectable indicating that in the desiccated state all energy quenching was proceeding through constitutive pathways rather than through regulated, energy-dependent quenching pathways. However, rehydration of *H. dentatum* (Fig. 3C) and *H. cruentum* (Fig. 3F) (>86 % *RWC*, 1.6 and 1.8 gH_2O gDW^{-1}, respectively) re-established the original patterns for the light response curves for *ϕPSII*, *ϕNPQ* and *ϕNO* observed in the control, hydrated states (Fig. 3A and D).

Thermoluminescence (TL) measurements were used as an alternative approach for assessing the effects of desiccation on the photosynthetic PSII-associated electron transfer reactions (Vass and Govindjee, 1996; Sane et al., 2012). Since most of the photosynthetic TL components have been assigned to arise from the reversal of light-driven charge separation in PSII, TL properties of photosynthetic apparatus provide information on the activation energies associated with the back reactions of electron acceptors (QA and QB) with the electron donors (S2 and S3) of PSII (Vass and Govindjee, 1996; Sane, 2004; Sane et al., 2012). The temperature

maxima (T_M) of the TL peaks related to the recombination of these charge pairs reflect the activation energies and hence a measure of the redox potentials of the participating oxidized and reduced donors (DeVault and Govindjee, 1990). Typical TL emission glow curves representing $S_{2/3}Q_B^-$ charge recombinations of hydrated, desiccated (50 % of *FW*) and rehydrated fronds of *H. dentatum* and *H. cruentum* obtained following excitation with two consecutive saturating flashes are presented in Fig. 5. The TL glow curves of hydrated fronds of both *H. dentatum* (Fig. 5A) and *H. cruentum* (Fig. 5D) exhibited typical for $S_{2/3}Q_B^-$ charge recombination TL peaks (Sane, 2004; Sane et al., 2012) with characteristic temperatures (T_M) of 31.1 °C and 27.5 °C, respectively (Fig. 5, Table 2). Desiccation of both samples yielded $S_{2/3}Q_B^-$ peaks with slightly higher temperatures. In addition, the amplitudes and the integrated areas of the TL peaks representing the $S_{2/3}Q_B^-$ charge recombination used for assessing the PSII photochemistry were significantly reduced in desiccated samples (Table 2). It should be noted that while the decrease in the overall TL area in desiccated *H. cruentum* was only 12 % (Fig. 5E, Table 2), desiccated *H. dentatum* exhibited much stronger (45 %) reduction of TL area (Fig. 5B, Table 2). Interestingly, while *Fv/Fm* values recovered in rehydrated samples (Fig. 3) the overall TL area in rehydrated *H. cruentum* decreased even further to about 40 % and remained steady lower in *H. dentatum* (Table 2). More strikingly, rehydrated samples exhibited massive low temperature shifts of $S_{2/3}Q_B^-$ peak T_M to 20.2 °C and 21.7 °C in *H. dentatum* and *H. cruentum*, respectively.

PSI function

The extent of far-red (FR) light-induced absorbance transients at 820 nm ($\Delta A_{820-860}$), which reflects the oxidation of P700 to $P700^+$ (Klughammer and Schreiber, 1991; Ivanov et al., 1998; 2006a), were used to estimate the functional performance of PSI in hydrated, desiccated and rehydrated fronds of *H. dentatum* and *H. cruentum in vivo*. The typical traces of *in vivo* measurements

illustrating the FR light-induced oxidation of P700 to $P700^+$ presented in Fig. 6 and the data summarized in Fig. 7 indicate a much lower (40 %) capacity for P700 photo-oxidation ($P700^+$) measured as $\Delta A_{820-860}$ in *H. dentatum* compared with the $P700^+$ values in *H. cruentum* under control hydrated conditions (Fig. 7). Desiccation caused a strong decrease (80-90 %) of $\Delta A_{820-860}$ values in both species (Fig. 7A and D). Interestingly, rehydration of both samples did not induce complete recovery of the capacity for P700 oxidation. In addition the apparent electron donor pool size to PSI was assessed by measuring single (ST) and multiple (MT)-turnover flash induced $\Delta A_{820-860}$ transients under steady state oxidation of PSI by FR light (Asada et al., 1992; 1993; Ivanov et al., 1998). The intersystem electron pool size, estimated as the ratio between the area of MT and ST induced $P700^+$ transients and (A_{MT}/A_{ST}) was undetectable upon desiccation of both species and this effect was reversible upon rehydration (Fig. 6B and E; Fig. 7B and E). Concomitantly, kinetic measurements of dark re-reduction of $P700^+$ after turning off the FR light, which is thought to reflect the capacity for cyclic electron flow (CEF) around PSI (Maxwell and Biggins 1976; Ravenel et al., 1994; Ivanov et al., 1998) and/or the interaction of stromal components with the intersystem electron transport chain (Asada et al., 1992) revealed much slower re-reduction of $P700^+$ in desiccated state and reversed to control levels upon rehydration in both *H. dentatum* and *H. cruentum* (Fig. 7C and F). These results clearly indicate that the capacity for PSI-dependent CEF is strongly restricted in desiccated plants.

Electron transport rate

The maximum *ETR* (Fig. 8) in the hydrated state was 58 µmol electrons m^{-2} s^{-1} in *H. dentatum* (Fig. 8A) and 28 µmol electrons m^{-2} s^{-1} in *H. cruentum* (Fig. 8B). ETR light saturation points were around 400 µmol m^{-2} s^{-1} and 250 µmol m^{-2} s^{-1} for *H. dentatum* and *H. cruentum*, respectively. The *ETR* initial slope was higher in *H. dentatum* by about 56 % than *H. cruentum*.

Although in the desiccated state, *ETR* was almost completely inhibited (Fig. 8A and B), *ETR* in both *H. dentatum* and *H. cruentum* in the rehydrated state recovered close to the original control values (Fig. 8A and B).

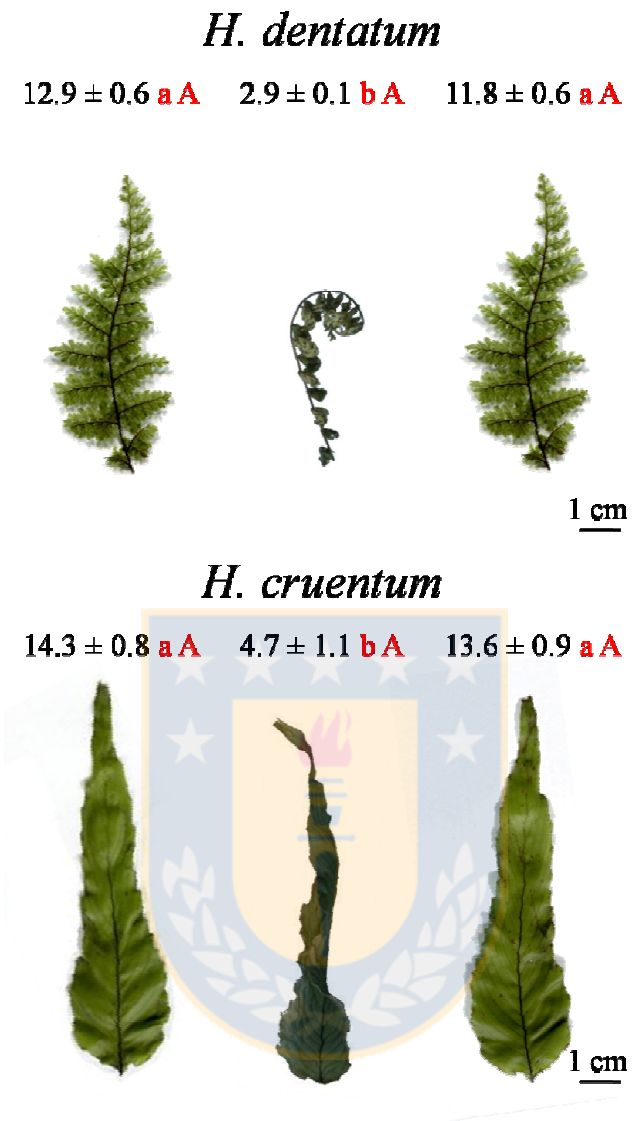

Fig. 1. Fronds shrinkage degree in *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C. Presl subjected to a process of desiccation and subsequent rehydration. Values represent the area (cm^2) ± standard errors. Different lowercase letters following values indicate statistical difference by hydration state for each species, and different capital letters indicate statistical difference between species in each hydration state ($P \leq 0.05$, Tukey test; n=4).

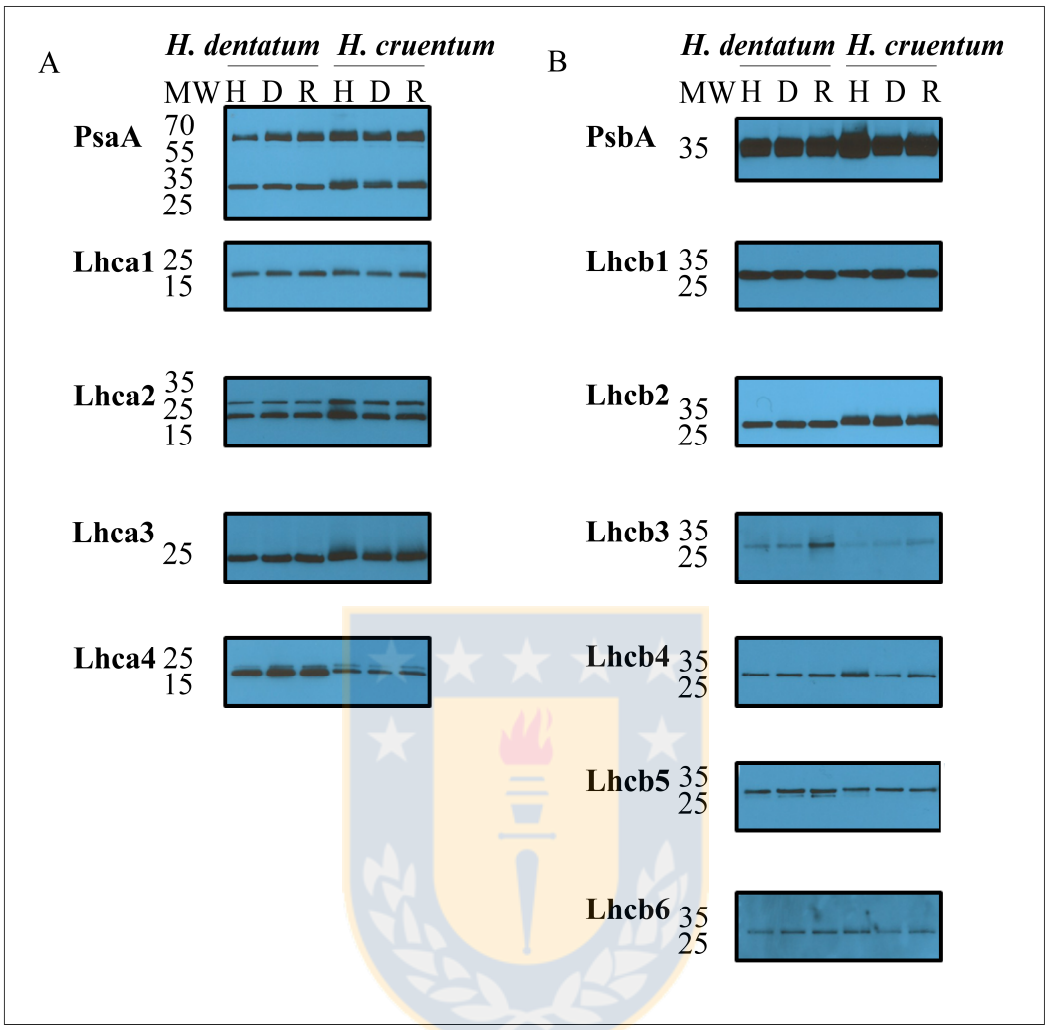

Fig. 2. Representative western blots of SDS-PAGE separated polypeptides of thylakoid membranes isolated from *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C. Presl hydrated (100 % *RWC*, 1.9 and 2.0 $gH_2O\ gDW^{-1}$, respectively) (H), desiccated (25 % (0.4 $gH_2O\ gDW^{-1}$) and 17 % (0.3 $gH_2O\ gDW^{-1}$) *RWC*, respectively) (D) and rehydrated (>86 % *RWC*, 1.6 and 1.8 $gH_2O\ gDW^{-1}$, respectively) (R) fronds probed with antibodies raised against PSI (A) and PSII (B) associated proteins. PSI proteins: PsaA and Lhca1-Lhca4 polypeptides. PSII proteins: PsbA (D1) and Lhcb1-Lhcb6.

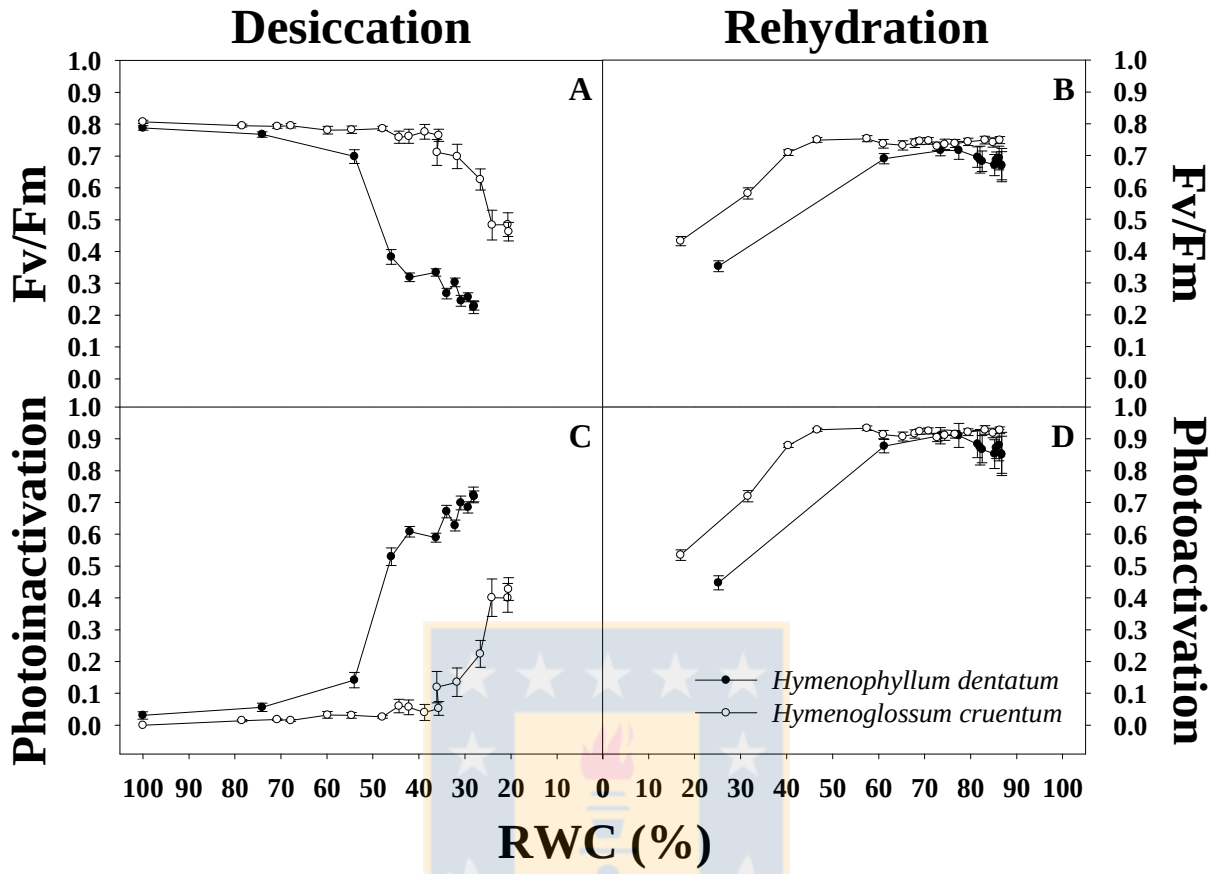

Fig. 3. The maximum photochemical efficiency of PSII (*Fv/Fm*) of *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C. Presl during a desiccation (A) and rehydration (B) process. Photoinactivation (*PI*) (C) and photoactivation (*PA*) (D) of PSII of *Hymenophyllum dentatum* and *Hymenoglossum cruentum* during a desiccation and rehydration process. *PI* and *PA* were calculated as $PI=1-[(Fv/Fm)_d/(Fv/Fm)_h]$ and $PA=[(Fv/Fm)_d/(Fv/Fm)_h]$, respectively. Each value is the average of 8 fronds.

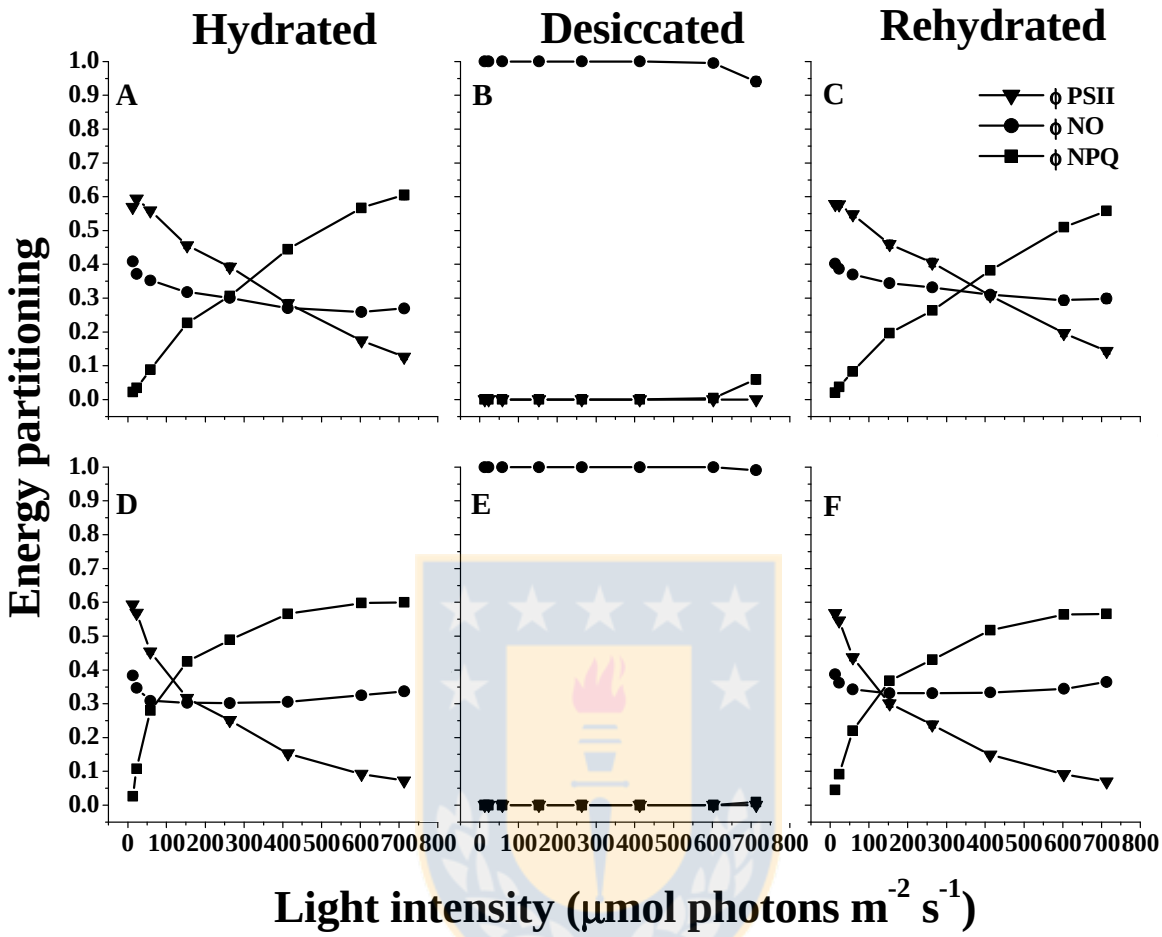

Fig. 4. Light energy partitioning for PSII. Effective quantum yield of PSII (*ϕPSII*), quantum yield of non-regulated energy dissipation of PSII (*ϕNO*), and quantum yield of the regulated energy dissipation of PSII (*ϕNPQ*) during the course of a light curve in *Hymenophyllum dentatum* Cav. (A, B and C) and *Hymenoglossum cruentum* C. Presl (D, E and F) fronds in the hydrated (100 % *RWC*, 1.9 and 2.0 $gH_2O\ gDW^{-1}$, respectively) (A and D), desiccated (25 % (0.4 $gH_2O\ gDW^{-1}$) and 17 % (0.3 $gH_2O\ gDW^{-1}$) *RWC*, respectively) (B and E), and rehydrated (>86 % *RWC*, 1.6 and 1.8 $gH_2O\ gDW^{-1}$, respectively) (C and F) states (n=3).

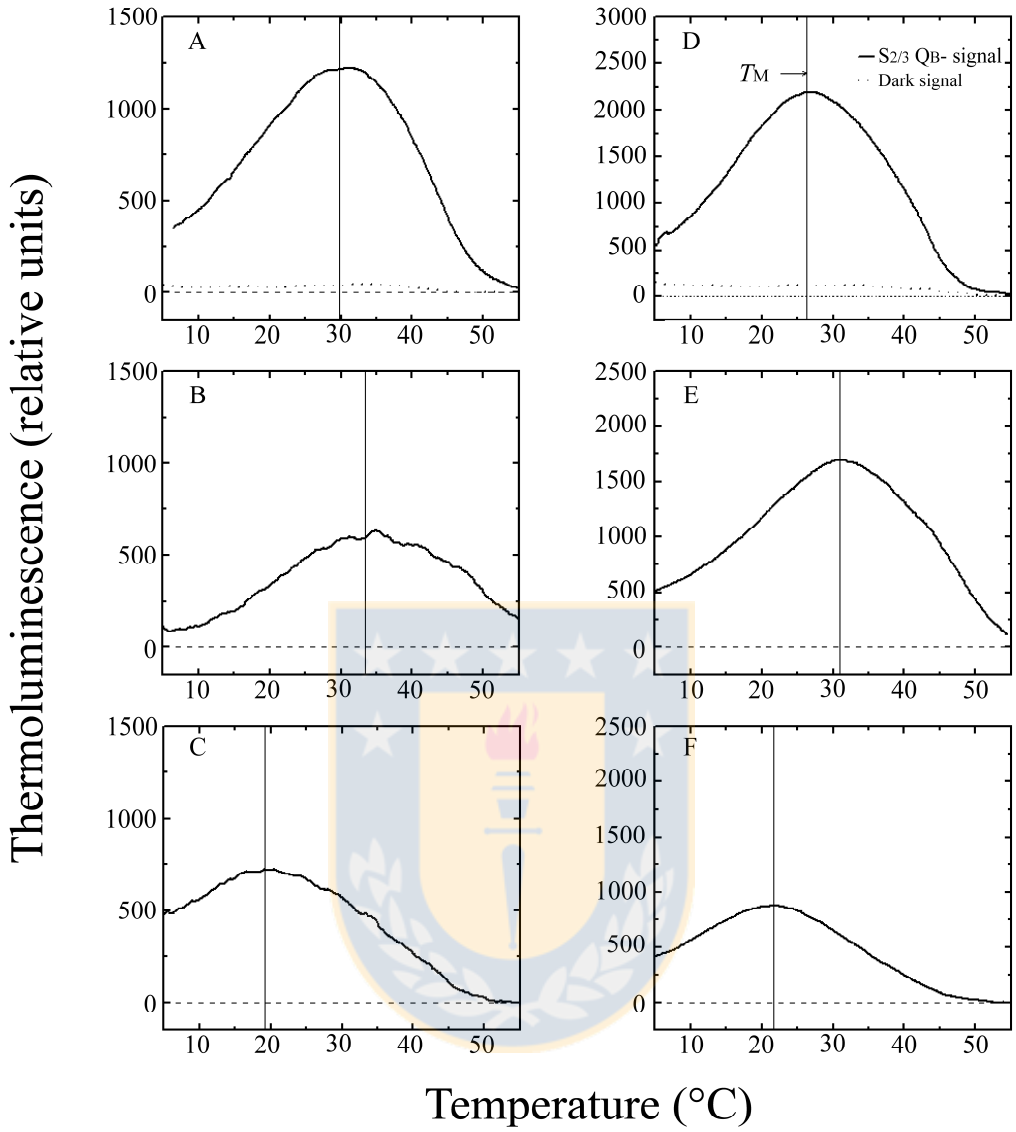

Fig. 5. Thermoluminescence (TL) glow curves of $S_{2/3}Q_B^-$ charge recombinations in hydrated (100 % *RWC*, 1.9 and 2.0 gH_2O gDW^{-1}, respectively) (A, D), desiccated (50% of *FW*) (B, E) and rehydrated (>86 % *RWC*, 1.6 and 1.8 gH_2O gDW^{-1}, respectively) (C, F) fronds of *Hymenophyllum dentatum* Cav. (A, B, C) and *Hymenoglossum cruentum* C. Presl (D, E, F) after illumination with two single turnover flashes. TL glow curves were recorded immediately after illumination. The presented glow curves are averages from 3 independent measurements.

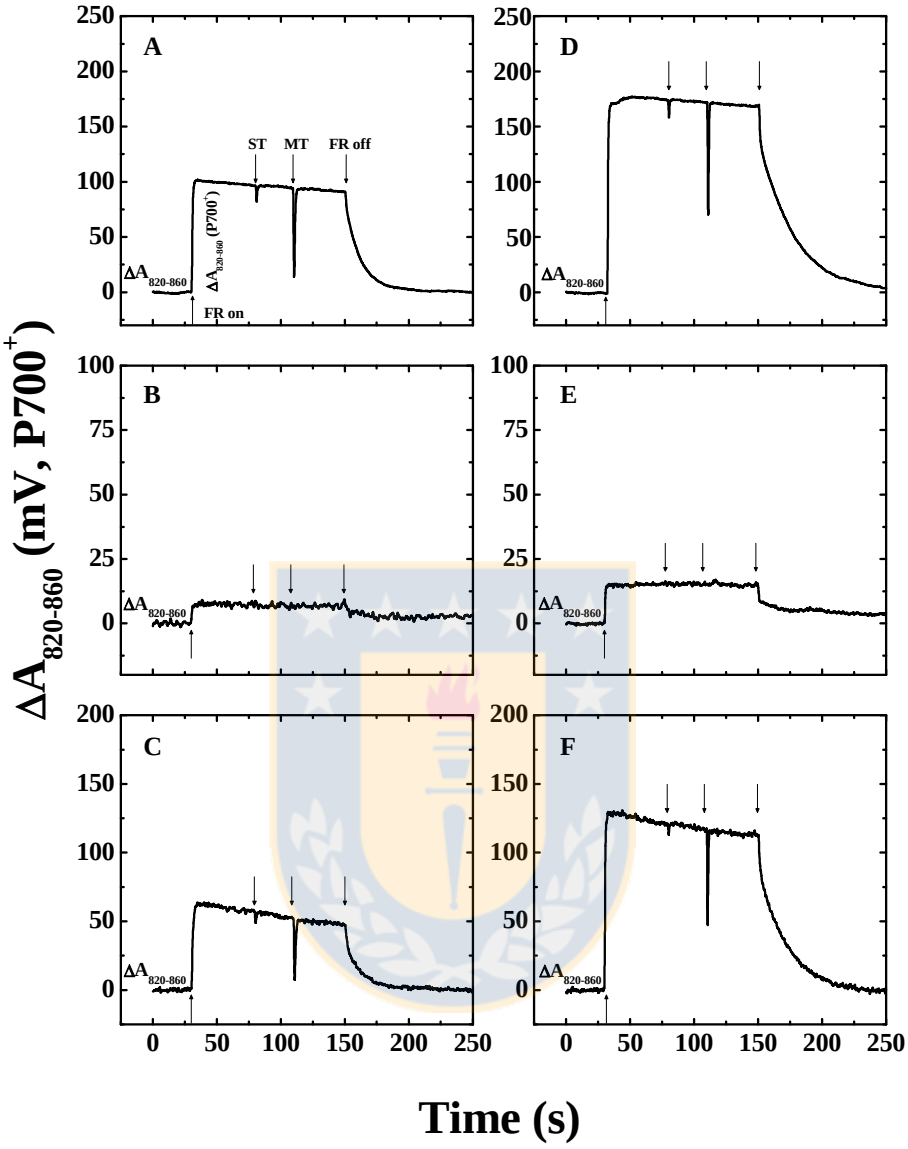

Fig. 6. Typical traces of *in vivo* measurements of P700 oxidation by far-red light of *Hymenophyllum dentatum* Cav. (A, B and C) and *Hymenoglossum cruentum* C. Presl (D, E and F) in hydrated (100 % *RWC*, 1.9 and 2.0 $gH_2O\ gDW^{-1}$, respectively) (A and D), desiccated (25 % (0.4 $gH_2O\ gDW^{-1}$) and 17 % (0.3 $gH_2O\ gDW^{-1}$) *RWC*, respectively) (B and E) and rehydrated (>86 % *RWC*, 1.6 and 1.8 $gH_2O\ gDW^{-1}$, respectively) (C and F) states. The measurements were performed at 15 °C.

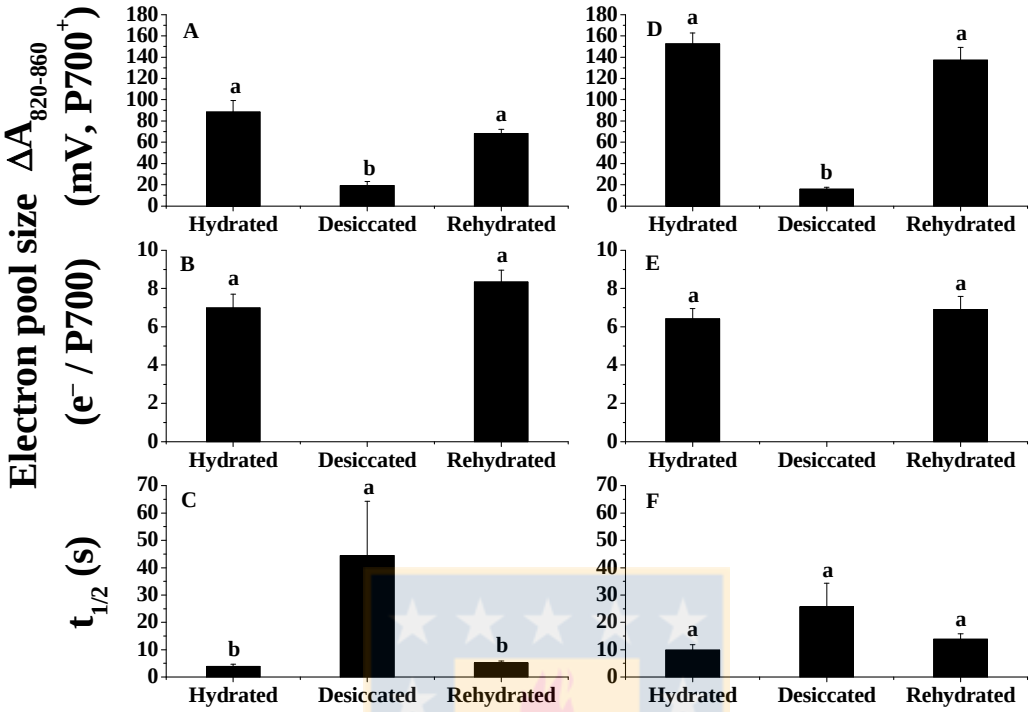

Fig. 7. P700 parameters of *Hymenophyllum dentatum* Cav. (A, B and C) and *Hymenoglossum cruentum* C. Presl (D, E and F) hydrated (100 % *RWC*, 1.9 and 2.0 $gH_2O\ gDW^{-1}$, respectively), desiccated (25 % (0.4 $gH_2O\ gDW^{-1}$) and 17 % (0.3 $gH_2O\ gDW^{-1}$) *RWC*, respectively) and rehydrated (>86 % *RWC*, 1.6 and 1.8 $gH_2O\ gDW^{-1}$, respectively) fronds (n=8). $\Delta A_{820\text{-}860}$ is the absorbance decrease at 820-860 nm. The electron pool size is obtained from A_{MT}/A_{ST}, which is the ratio between the area of the multiple turnover flash (AMT) and the area of the single turnover flash (AST), and represents the functional pool size of intersystem electron per reaction centre, $t_{½}$ (s) represents the cyclic electrons flow around P700.

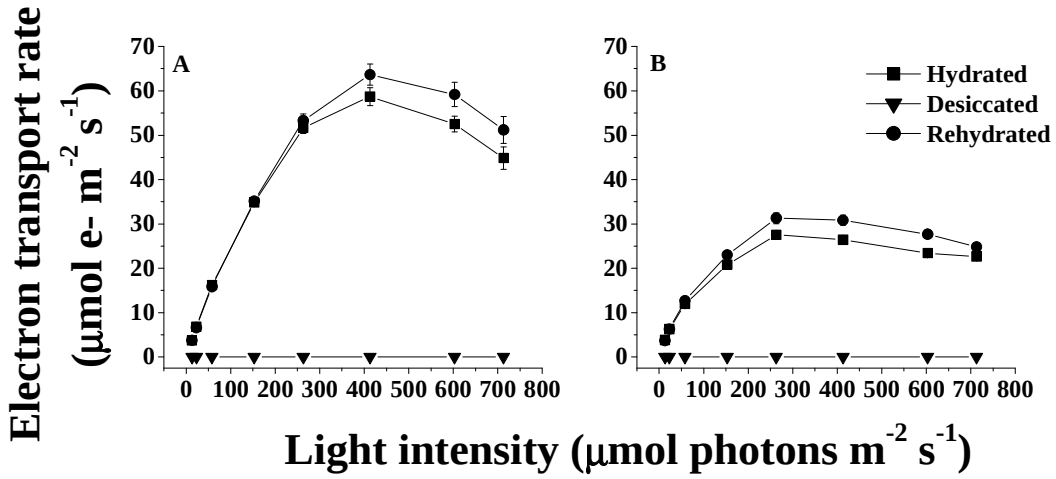

Fig. 8. Electron transport rate (*ETR*) during the course of a light curve of *Hymenophyllum dentatum* Cav. (A) and *Hymenoglossum cruentum* C. Presl (B) hydrated (100 % *RWC*, 1.9 and 2.0 $gH_2O\ gDW^{-1}$, respectively), desiccated (25 % (0.4 $gH_2O\ gDW^{-1}$) and 17 % (0.3 $gH_2O\ gDW^{-1}$) *RWC*, respectively) and rehydrated (>86 % *RWC*, 1.6 and 1.8 $gH_2O\ gDW^{-1}$, respectively) fronds (n=3).

Table 1

Photosynthetic pigments (nmol gDW^{-1}) isolated from whole-cell extracts of *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C. Presl in hydrated (H) (100 % *RWC*, 1.9 and 2.0 $gH_2O\ gDW^{-1}$, respectively), desiccated (D) (25 % (0.4 $gH_2O\ gDW^{-1}$) and 17 % (0.3 $gH_2O\ gDW^{-1}$) *RWC*, respectively) and rehydrated (R) (>86 % *RWC*, 1.6 and 1.8 $gH_2O\ gDW^{-1}$, respectively) states. Chl*a*, chlorophyll *a*; Chl*b*, chlorophyll *b*; α-car, α-carotene; β-car, β-carotene; *N*, neoxanthin; *L*, lutein. The values are means ± standard error. Different letters following values indicate statistical difference by hydration state between both species ($P \leq 0.05$, Tukey test; n=4).

Species	**State**	**Pigment concentration (nmol gDW^{-1})**							
		Chl*a*	**Chl*b***	***a/b***	***α*-car**	**β-car**	***N***	***L***	***N*/ β-car**
H. dentatum	**H**	4044±461 a	1516±180 a	2.7±0.0 a	0±0 b	268±28 a	374±47 a	990±117 a	1.4±0.04 a
	D	4237±243 a	1584±114 a	2.7±0.0 a	0±0 b	233±56 a	377±24 a	1040±87 a	2.1±0.75 a
	R	3990±330 a	1774±130 a	2.2±0.1 b	0±0 b	276±20 a	392±32 a	1092±85 a	1.4±0.05 a
H. cruentum	**H**	6007±769 a	2245±289 a	2.7±0.0 a	167.7±38.4ab	285±29 a	528±67 a	1439±176 a	1.8±0.08 a
	D	6045±710 a	2286±297 a	2.7±0.1 a	147.6±37.7ab	309±18 a	536±74 a	1492±185 a	1.7±0.14 a
	R	6185±1864 a	2329±700 a	2.6±0.0 a	289.5±50.7a	301±85 a	510±161 a	1485±428 a	1.7±0.08 a

Table 2. Characteristic thermoluminescence (TL) peak emission temperatures (T_M) and the overall TL emission area (A) of S_2/S_3Q_B- glow peaks of fronds of *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C. Presl in hydrated (H) (100 % *RWC*, 1.9 and 2.0 $gH_2O\ gDW^{-1}$, respectively), desiccated (D) (50 % of *FW*) and rehydrated (R) (>86 % *RWC*, 1.6 and 1.8 $gH_2O\ gDW^{-1}$, respectively) states. The samples were dark adapted for 30 min, then cooled to 2 °C and subsequently illuminated with two single turnover flashes of white light. The peak areas are presented as a percentage of the total TL light emission in hydrated fronds. Mean values ± SE were calculated from 3 independent measurements.

Hydration state	***H. dentatum***		***H. cruentum***	
	T_M (°C)	**A (%)**	**T_M (°C)**	**A (%)**
H	31.1 ± 1.5 a	100 a	27.5 ± 0.9 a	100 a
D	33.6 ± 2.9 a	54.7 ± 7.3 b	30.6 ± 0.7 a	88.2 ± 13.7 ab
R	20.2 ± 1.7 b	62.7 ± 11.2 ab	21.7 ± 1.0 b	39.5 ± 5.8 b

Discussion

A primary goal of this study was to assess whether *Hymenophyllum dentatum* and *Hymenoglossum cruentum*, members of the Hymenophyllaceae, exhibited a homoiochlorophyllous or a poikilochlorophyllous strategy in response to dehydration stress. Based on our data for the stability of the complement of photosynthetic pigments (Table 1), the rapid recovery of *Fv/Fm* and of photoactivation of PSII (Fig. 3) during rehydration coupled with the maintenance of the composition and the relative abundance of the photosynthetic polypeptides (Fig. 2), we conclude that *H. dentatum* and *H. cruentum* have evolved a homoiochlorophyllous strategy to respond to desiccation stress and rehydration. This is consistent with the capacity of these species to exhibit resurrection as detached fronds (Dinakar et al., 2012). On the other hand, the poikilochlorophyllous strategy evolved in anatomically complex plants, which include the biggest in size of all desiccation tolerant species. These species lose their chlorophylls and dismantle the internal structures of the chloroplast during desiccation. Chlorophyll synthesis starts 8-10 h after rehydration, and the regeneration of the photosynthetic apparatus is complete within 72 h (Tuba 2008).

Despite the fact that both *H. dentatum* and *H. cruentum* are homoiochlorophyllous, the two species did exhibit significant differences in their sensitivities to dehydration stress. First, *H. dentatum* exhibited much greater sensitivity to PSII inactivation than *H. cruentum* as a function of *RWC* (Fig. 3C) during desiccation. On the other hand, *H. cruentum* exhibited much greater sensitivity than *H. dentatum* during rehydration as a function of *RWC* (Fig. 3D), as well as an increased light sensitivity of $\phi PSII$ than *H. dentatum* (Fig. 4). The latter is reflected in a lower quantum requirement for $\phi PSII$ as well as ϕNPQ in *H. cruentum* compared to *H. dentatum* (Fig. 4A and D) which is re-established upon rehydration (Fig. 4C and F). *H. dentatum* and *H.*

cruentum present high values of *Fv/Fm* in the desiccated state (Fig. 3A) compared with other desiccation tolerant species as *Pelvetia canaliculata* (Phaeophyceae, brown algae); *Blidingia* sp. (Chlorophyceae, green algae); *Frullania dilatata* (Marchantiopsida, liverworts); *Syntrichia ruralis* (Bryopsida, mosses); *Ceterach officinarum* (Filicopsida, ferns); *Ramonda nathaliae* (Magnoliopsida, flowering plants) (Fernández-Marín et al., 2011); *Haberlea rhodopensis* (Magnoliopsida, flowering plants) (Georgieva et al., 2007); *Rhytidium rugosum* (Bryopsida, mosses) (Heber et al., 2006b); *Cladonia rangiformis* (Cladoniaceae, fruticose lichen); *Parmelia sulcata* (Parmeliaceae, foliose chlorolichen); *Peltigera neckeri* (Peltigeraceae, cyanolichen) (Heber 2008), which show values between 0 and around 0.2. However, the values obtained of *Fv/Fm* in *H. dentatum* and *H. cruentum* are consistent within the Hymenophyllaceae (Saldaña et al., 2014). This difference can be attributed to the higher value of the water content in *H. dentatum* and *H. cruentum* desiccated fronds (25 % (0.4 gH_2O gDW^{-1}) and 17 % (0.3 gH_2O gDW^{-1}) *RWC*, respectively) compared for example with *Haberlea rhodopensis* (5% *RWC*, 0.16 gH_2O gDW^{-1}). Light saturated rates of *ETR* in *H. dentatum* were double than those of *H. cruentum* (Fig. 8). TL results strongly support the stronger desiccation induced reduction of PSII photochemistry measured as *Fv/Fm* in *H. dentatum* compared to *H. cruentum* fronds (Fig. 3). In contrast, *H. cruentum* exhibited a 1.7-fold greater P700 signal (Fig. 7A and D) which was consistent with a higher PsaA polypeptide content than *H. dentatum* (Fig. 2A).

Desiccation completely inhibited $\phi PSII$, ϕNPQ (Fig. 4B and E) as well as *ETR* (Fig. 8), but maximized ϕNO in both species (Fig. 4B and E). This indicates that the photosynthetic apparatus is completely reorganized upon dehydration and is converted from an energy transformer to an energy quencher with absorbed energy being dissipated through the constitutive pathway for nonphotochemical quenching. The reorganization process induced by desiccation is completely reversible with the relaxation of the highly quenched state and its conversion into a

functional state as an energy transformer upon rehydration (Fig. 4C and F). In hydrated and rehydrated fronds, light intensity induced a reduction in $\phi PSII$ representing the saturation of electron transport processes with light (Hendrickson et al., 2004). The quantum yield of the regulated energy dissipation of PSII (ϕNPQ) was present in hydrated and rehydrated fronds as light intensity increased, but it was absent in the desiccated state. NPQ can be subdivided into three components. The major and most rapid component is the pH-or energy dependent component, qE. A second component, qT, relaxes within minutes, and is due to the phenomenon of state transition, the uncoupling of light harvesting complexes from PSII. The third component shows the slowest relaxation, it is related to photoinhibition of photosynthesis, and is called qI (Müller et al., 2001). qE is regulated by the magnitude of the transmembrane pH gradient (ΔpH) across the thylakoid membrane, therefore making the quenching rapidly reversible. When the magnitude of ΔpH surpasses the value present when the amount of light does not exceed the photosynthetic capacity, the violaxanthin de-epoxidase enzyme is activated and triggers the interconversion of violaxanthin to zeaxanthin, via the xanthophyll cycle. qE requires the presence of the PsbS protein (Holt et al., 2004; Niyogi 1999). On the contrary, the quantum yield of non-regulated energy dissipation of PSII (ϕNO) was present in hydrated, desiccated, and rehydrated fronds, but in a different order of magnitude. In hydrated and rehydrated fronds, ϕNO, the constitutive quenching, remained constant or decreased slightly with light intensity, when ϕNPQ is more important due to the fast function of the xanthophyll cycle, perhaps because of its photoprotective effectiveness. This result is showing that ϕNO, as a constitutive quenching, is always present, even when there is no presence of the stressor, in this case light. ϕNO is constitutive in the sense that short-term changes in light intensity do not alter its efficiency (Hendrickson et al., 2004); however, Klughammer and Schreiber (2008) do not agree with this definition, because ϕNO can display large changes, complementary to simultaneous changes in

ϕPSII and *ϕNPQ*. In desiccated state, *ϕNPQ* is not present, because NPQ has been known to operate only under wet conditions (Horton et al, 1994); therefore, *ϕNO* is extremely high, showing blocked PSII reaction centres, inhibiting a transthylakoidal proton gradient, which is a prerequisite for regulated energy dissipation (*ϕNPQ*). Absorption of sunlight that exceeds the capacity of a plant for CO_2 fixation results in a buildup of the thylakoid ΔpH that is generated by photosynthetic electron transfer. As a consequence, a decrease in lumen pH induces qE through protonation of PSII proteins and activation of the xanthophyll cycle (Müller et al., 2001). The ΔpH is required to cause the change in light harvesting complexes organization needed to bring chlorophyll and zeaxanthin close enough for energy transfer. However, a very low pH would inhibit the electron flow at the level of the cytochrome-b_6f complex, inhibiting qE (Horton et al., 1994). Slavov et al., (2013) suggest a drying-induced reorganization of the thylakoid membrane, which changes the relative association and distance of PSII and PSI particles, and this was also demonstrated in the studies by Veerman et al., (2007) and Komura et al., (2010) in *Parmelia sulcata* and *Physciella melanchla* lichens, respectively. Chlorophyll and carotenoids are lipophilic and occur as pigment-protein complexes within the thylakoid membrane (Hüner et al., 2005). Loss of water during desiccation is thought to alter the position of pigments to one another by altering the conformation of a specific pigment-protein complex. This is thought to lead to the formation of quenching centres (Heber et al., 2007). In desiccation-sensitive plants, chlorophyll-protein complexes are destroyed after severe drought stress (Georgieva et al., 2009). On the other hand, in desiccation tolerant plants, the integrity of thylakoids and the amount and ratio of pigment-protein complexes are maintained, as in dehydrated *Boea hygrometrica* and *Haberlea rhodopensis*, both homoiochlorophyllous desiccation tolerant plants (Deng et al., 2003; Georgieva et al., 2007; Mitra et al., 2013). Thus, we suggest that the highly quenched state

induced by dehydration in *H. dentatum* and *H. cruentum* reflects a mechanism to protect the photosynthetic apparatus from potential photodamage in the desiccated state.

Light energy partitioning in PSII among the hydrated, desiccated, and rehydrated states, showed that in the desiccated state, the quantum yield of non-regulated energy dissipation of PSII (ϕNO) reached values of 1 (Fig. 4B and E). If $\phi PSII$ value is zero at high light intensity, a high value of ϕNPQ represents a high photoprotective capacity. In contrast, a high value of ϕNO reflects the inability of a plant to protect itself against damage by excess illumination (Klughammer and Schreiber, 2008), and this would indicate the use of structural changes at the level of chloroplast and photosynthetic apparatus to overcome the desiccated state. Photosystem I is only marginally functional and exhibits lower capacity for cyclic electron flow (CEF) around PSI in the desiccated state, compared to the hydrated and rehydrated states. Ivanov et al., (1998) support the thesis that one role of the down regulation of PSII may be to protect PSI from photoinhibition, which is shown by our results of photoinactivation. *ETR* was undetectable in the desiccated state, indicating no flow of electrons through the intersystem in both species, as it was also shown by the functional pool size of intersystem electrons per reaction centre (A_{MT}/A_{ST}) in the study of PSI. On the other hand, light energy partitioning within PSII, PSI evaluated parameters, and *ETR* in hydrated and rehydrated fronds show the differences between *H. dentatum* and *H. cruentum* to cope with light, and this is strongly linked to their natural habitat on the host tree trunk. It is consistent that *H. cruentum*, the species which inhabits mainly at the trunk base, has lower light requirements to activate the quantum yield of the regulated energy dissipation of PSII (ϕNPQ). Pigment concentration showed small changes between treatments for both *H. dentatum* and *H. cruentum*. However, *H. cruentum* showed a big difference, presenting accumulation of α-carotene, which is accumulated in larger amounts in the shade (Demmig-Adams and Adams, 1992), in contrast to *H. dentatum*, where there was not detection of α-

carotene. Also, *H. cruentum* presented higher values of Chl*a* and Chl*b*, as well as β-carotene, neoxanthin, and lutein. The higher content of pigments could create an efficient system for capturing light in a low light environment, such as the understory. The N/β-carotene ratio was higher in *H. cruentum* in the three hydration states, but in *H. dentatum* was higher in the desiccated state. Xanthophylls are restricted to the outer antenna and light harvesting proteins, while β-carotene is a component of the photosystem reaction centres and core antenna (Trebst 2003); therefore, this ratio is showing that *H. cruentum* has bigger light harvesting complexes to capture photons. The decrease in the N/β-carotene ratio in the desiccated state of *H. dentatum* may be reflecting some injury in the photosystem reaction centres. Additionally, the extent of PSI photochemistry ($\Delta A_{820\text{-}860}$) shows a higher number of P700 reaction centres in *H. cruentum* for an efficient use of light compared to *H. dentatum*, being the first the most shade tolerant. In addition, the functional pool size of intersystem electrons per reaction centre (A_{MT}/A_{ST}) was also lower in *H. cruentum*, as it is shown by the *ETR* values.

As expected, the desiccation process strongly reduced the projected frond area of *H. dentatum* and *H. cruentum* in a reversible manner. This process generates morphological changes as cell wall folding, roundish chloroplasts, and vacuole fragmentation (results not shown). In the temperate rainforest of Chile, the Hymenophyllaceae species occupy shaded areas. Perhaps, this shrinkage is an adaptive feature to avoid light-induced damage due to sunflecks. Another explanation could be that shrinkage during the desiccated state helps with spore release in mature leptosporangia (results not shown) due to water evaporation from the cells of the annulus, causing their dehiscence (Simpson, 2010).

In summary, *H. dentatum* and *H. cruentum* are homoiochlorophyllous with respect to dehydration stress, which reflects the ability to reversibly convert a photochemically active photosynthetic apparatus into a stable, highly quenched structure. This, coupled with a

significant reduction in light exposed photosynthetic surface area, provides protection against potential photodamage during dehydration.

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Supplementary Data

Densitometric analysis (%) of PSI and PSII associated proteins from thylakoid membranes isolated from *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C. Presl hydrated (100 % *RWC*, 1.9 and 2.0 $gH_2O\ gDW^{-1}$, respectively), desiccated (25 % (0.4 $gH_2O\ gDW^{-1}$) and 17 % (0.3 $gH_2O\ gDW^{-1}$) *RWC*, respectively) and rehydrated (>86 % *RWC*, 1.6 and 1.8 $gH_2O\ gDW^{-1}$, respectively) fronds. The numerical data for the relative protein abundance were normalized to the maximal values of the hydrated state of each species. Values are means ± standard error. Different letters following values indicate statistical differences among hydration states ($P \leq 0.05$, Tukey test; n=3).

Protein	***Hymenophyllum dentatum***			***Hymenoglossum cruentum***		
	Hydrated	**Desiccated**	**Rehydrated**	**Hydrated**	**Desiccated**	**Rehydrated**
PsaA	1.00 ± 0.00 b	1.37 ± 0.09 ab	1.43 ± 0.12 a	1.00 ± 0.00 a	0.80 ± 0.06 a	0.90 ± 0.12 a
PsaA*	1.00 ± 0.00 a	1.17 ± 0.03 a	1.13 ± 0.07 a	1.00 ± 0.00 a	0.83 ± 0.07 a	1.00 ± 0.06 a
Lhca1	1.00 ± 0.00 a	1.13 ± 0.13 a	1.00 ± 0.06 a	1.00 ± 0.00 a	0.67 ± 0.09 b	0.83 ± 0.07 ab
Lhca2	1.00 ± 0.00 a	1.33 ± 0.29 a	1.50 ± 0.35 a	1.00 ± 0.00 a	0.80 ± 0.10 a	0.73 ± 0.12 a
Lhca2*	1.00 ± 0.00 a	1.07 ± 0.15 a	1.13 ± 0.17 a	1.00 ± 0.00 a	0.73 ± 0.12 a	0.73 ± 0.07 a
Lhca3	1.00 ± 0.00 a	1.17 ± 0.12 a	1.13 ± 0.07 a	1.00 ± 0.00 a	0.80 ± 0.00 b	0.93 ± 0.03 a
Lhca4	1.00 ± 0.00 a	1.23 ± 0.07 a	1.23 ± 0.07 a	1.00 ± 0.00 a	0.56 ± 0.03 a	0.93 ± 0.23 a
PsbA	1.00 ± 0.00 a	1.00 ± 0.06 a	1.03 ± 0.07 a	1.00 ± 0.00 a	0.77 ± 0.03 a	0.93 ± 0.09 a
Lhcb1	1.00 ± 0.00 a	0.93 ± 0.09 a	1.03 ± 0.09 a	1.00 ± 0.00 a	0.97 ± 0.24 a	1.00 ± 0.06 a
Lhcb2	1.00 ± 0.00 a	1.33 ± 0.13 a	1.10 ± 0.06 a	1.00 ± 0.00 a	1.27 ± 0.07 a	1.53 ± 0.23 a
Lhcb3	1.00 ± 0.00 a	1.07 ± 0.03 a	1.57 ± 0.32 a	1.00 ± 0.00 a	1.03 ± 0.26 a	1.30 ± 0.21 a
Lhcb4	1.00 ± 0.00 c	1.10 ±0.00 b	1.23 ± 0.03 a	1.00 ± 0.00 a	0.33 ± 0.07 c	0.63 ± 0.03 b
Lchb5	1.00 ± 0.00 b	1.37 ± 0.07 a	1.40 ± 0.10 a	1.00 ± 0.00 b	1.20 ± 0.00 a	1.03 ± 0.03 b
Lhcb6	1.00 ± 0.00 b	1.33 ± 0.07 a	1.23 ± 0.07 ab	1.00 ± 0.00 a	0.53 ± 0.13 b	0.70 ± 0.10 ab

*It indicates the second band.

General discussion

Phenotypically, the rehydration process of *H. dentatum* and *H. cruentum* fronds resulted in a complete recovery. The energy partitioning of PSII and the measurements of the redox state of PSI functionally corroborated this result. Although, PSII and PSI are not functional in the desiccated state, fronds rehydrated for 24 h exhibited values that were close to hydrated fronds in both species. A surprising effect of the desiccated fronds is the high value of *ΦNO* compared to the *ΦNPQ*. This is showing that all the quenching of PSII is occurring through non-light induced processes. Non-photochemical quenching (NPQ) of excess excitation energy in the antenna pigment bed of PSII is considered to be the major PSII photoprotective mechanism (Demmig-Adams and Adams, 1992). The models based on chlorophyll fluorescence parameters for quantifying the partitioning of total absorbed light energy by PSII ("puddle" (Demig-Adams et al. 1996) or "lake" (Kramer et al. 2014; Hendrickson et al. 2004) antenna models) recognize two thermal dissipation processes. The major one originating from the light harvesting antenna complexes (LHCII) and defined as regulated NPQ (*ϕNPQ*), and an additional one defined as constitutive thermal dissipation (*ϕNO*) or "excess excitation energy" (Demmig-Adams et al. 1996), defined as the fraction of absorbed light neither going to photochemistry nor regulated NPQ. It is suggested that energy in excess that is not quenched by either qP, the down regulation of PSII through NPQ, or state transitions is quenched by an additional constitutive process (Ivanov et al. 2008). This additional quenching process is consistent with the fact that significant levels of NPQ can occur independent of zeaxanthin (Hurry et al. 1997; Demmig-Adams et al. 1999; Finazzi et al. 2004), and cannot accounted for by antenna quenching (Kramer et al. 2004). State transition, in which energy absorbed by PSII is redistributed to PSI after phosphorylation of the LHCII is obtained by the ratio of emission at 688-689 nm compared with that of 733-734 nn

at 77 K, and is used to examine variations in the redistribution of excitation energy between both photosystem (Horton and Black 1981). Our results showed (see supplementary material) that there were minimal changes in the energy distribution between PSII and PSI when the hydrated, desiccated and rehydrated states of *H. dentatum* and *H. cruentum* were compared. Therefore, regulated thermal dissipation (ϕNPQ) that was observed in the photochemical energy partition of PSII it is not related with a change in the energy distribution between PSII and PSI.

It has been demonstrated that fluorescence quenching might result from a conversion of PSIIα-centres (dimers) to PSIIβ-centres (monomers) in a low fluorescence state (Delrieu 1998), and this process can be triggered by high light (Kruse et al. 1997). This mechanism have been observed in beans exposed to high temperatures (Pastenes and Horton 1996). The increased population of Q_A^- due to the altered redox potentials of Q_A and Q_B during the shift and acclimation to low temperature may enhance the dissipation of excess light within the reaction centre of PSII via non-radiative $P680^+Q_A^-$ recombination, protecting the Q_A site from excessive excitation pressure (Hüner et al. 1998; Öquist and Hüner 2003). Since either low temperature or high light induced a comparable reduction state of Q_A (Hüner et al. 1996), this implies that reaction centre quenching is correlated with the excitation pressure. Thus, photoprotection of PSII through reaction centre quenching can complement photoprotection through antenna quenching (Krause and Weis 1991). Irrespective of the photoautotrophic species, the condition used to induce reaction centre quenching, the accumulation of closed PSII reaction centres (accumulation of reduced Q_A), predisposes the induction of reaction centre quenching (Ivanov et al. 2008). Reaction centre quenching may represent a mechanism that accounts for the dissipation of excess energy through constitutive energy quenching, and since evolution of reaction centres preceded that of ligh harvesting systems, reaction centre quenching may represent the oldest photoprotective mechanism (Ivanov et al. 2008). Öquist and Hüner (2003) studied the

photosynthesis of overwintering evergreen plants during frost desiccation. Their evidence indicates that the PSII reaction centres that remain intact during the winter may also function to dissipate absorbed energy as heat through charge separation and recomination. On the other hand, PSI is largely preserved during winter probably because and enhanced capacity of the oxygen scavenging system on the reducing side of PSI and the capacity for cyclic electron flow. Therefore, PSI has the potencial to quench absorbed light photochemically and nonphotochemically throughout the winter. In addition, the intersystem electron transport chain is kept largely reduced during winter by electrons of metabolic rather than photochemical origin. This reduced level also protects against oxidative damage during winter. They suggest that cyclic electron transport aroun PSI and a proposed chlororespiratory pathway may both contribute to the maintenance of the functional integrity of the winter-green foliage upon occasional thawing during winter and throughout recovery of photosynthesis during spring. Although in the desiccated state of *H. dentatum* and *H. cruentum*, reaction centre quenching of PSII is induced, PSI also is not functional, contrary of what happens in overwintering evergreen plants. This was shown with the results of P700 parameters ($\Delta A_{820\text{-}860,}$ absorbance decrease at 820-860 nm; electron pool size, which represents the functional pool size of intersystem electron per reaction centre; and $t_{½}$ (s), which represents the cyclic electrons flow around P700). In the desiccated state, the cyclic electron flow is extremely low. Therefore, contrary to what happens in overwintering evergreen plants, the major contribution to cope with the desiccated state is the quenching of the reaction centre of PSII. PSI was not functional during the desiccated state in these ferns, as oppose to overwintering evergreens. It is probable that both reaction centre and antenna quenching function *in vivo* to different extents, depending on the environmental conditions, to protect PSII from photodamage (Ivanov et al. 2008b). It has been proposed that the conversion of photochemically active, fluorescent, closed PSII reaction centres into

photochemically inactive, nonfluorescent PSII reaction centres may serve as an effective mechanisms for energy dissipation (Krause 1988; Krause and Weis 1991), and prevent further damage, not only to the photoinactivated reaction centres themselves, but also to neighbouring active PSII reaction centres (Lee et al. 2001; Matsubara and Chow 2004).

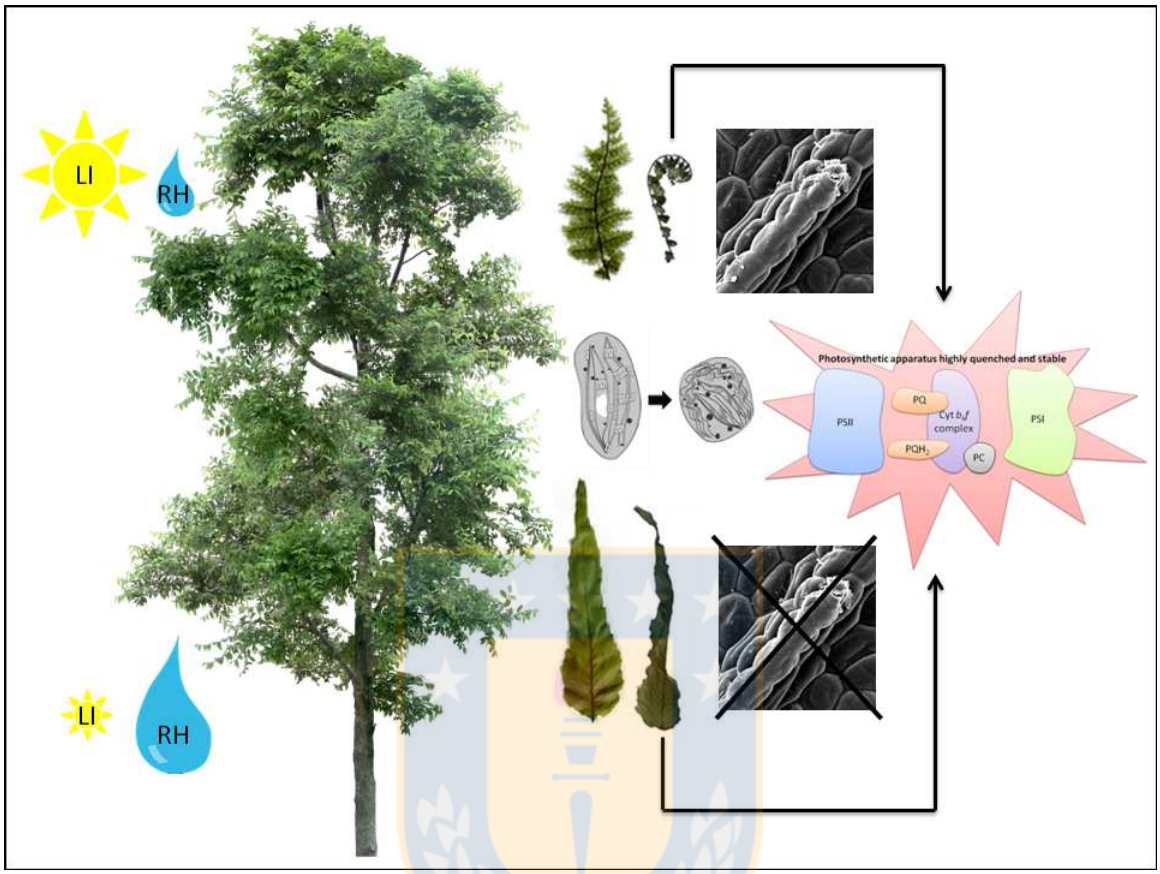
On the other hand, the structure of the photosynthetic apparatus and chlorophyll content were maintained under the different states of hydration, which means that this species present the Homoiochlorophyllous strategy. In addition, during the desiccated state, the changes observed as cell wall folding; roundish chloroplasts, and vacuole fragmentation were recovered after rehydration. Thefore, our study also showed that *H. dentatum*. and *H. cruentum* were totally reactivated post-rehydration, regardless of the presence of a glandular multicellular hair with a distal secretory cell of wax in *H. dentatum*, which could be one feature that allows this species to colonize higher zones of the trunk of the host tree. Surface properties in plants include the provision of mechanical stability; provision of an efficient water transpiration barrier and minimizing the leaching of molecules from the inside of the living cells; formation of low-adhesive surfaces (e.g. sliding of insects); disease resistance; increase in the reflection of visible light or absorption of harmful UV radiation; surface wettability, and self-cleaning properties of plant surfaces (Koch and Barthlott 2009). Finally, there was no relation between the desiccation tolerance and the vertical distribution of the studied species, although *H. dentatum* has a higher velocity of reaction to quench the photosynthetic apparatus in function of a decresease in the *RWC*.

Then, we proceed to answer the questions proposed in the formulation of the research problem: (1) *Are there any differences in relation to desiccation tolerance between species with contrasting vertical distribution?* No, there are not. Both species reached 0.4 g H_2O g DW^{-1} after a desiccated period, and recovered perfectly well after rehydration. *H. dentatum* appeared to be

more sensitive to a decrease in the relative water content (*RWC*) than *H. cruentum.* However, upon rehydration, both species exhibited full recovery of the maximum photochemical efficiency of PSII (*Fv/Fm*). (2) *What are the changes that occur in the chloroplast during the desiccation process that allow these individuals to recover so fast and continue with their photosynthetic activity when water becomes available?* In the desiccated state, cell shapes changed from convex to concave. Although cells decreased their sizes, there was not cells collapse of both species, which helps to their fast recovery and functioning. The qualitative study of cell ultrastructure showed in the desiccated state of both species folded cell walls, a fragmentation of the vacuole, and chloroplasts with a roundish shape. All these changes were reversed after rehydration. On the other hand, the quantitative study of cell ultrastructure showed that *H. cruentum* presented a higher number of chloroplasts per cell, a higher number of starch granules per chloroplasts, and a higher starch area compared to *H. dentatum* which could be related to its condition of a shade species. (3) *Which are the photoprotection mechanisms and how they work to let individuals of Hymenophyllaceae undergo a desiccation and rehydration process?* The energy partitioning within PSII showed that in the hydrated state, *H. dentatum* and *H. cruentum* showed a decrease in the effective quantum yield of PSII ($\phi PSII$) as a function of increased irradiance. The inhibition of $\phi PSII$ was associated with an increase in ϕNPQ as a function of irradiance in both species. *H. cruentum* was the species with higher ϕNPQ across the entire experimental light curve during hydration and rehydration, which coincides with the results of Parra et al. (2015). Constitutive quenching (ϕNO) decreased as a function of increased irradiance in both species in fully hydrated state. In the desiccated state, ϕNO reached values closed to 1, while ϕNPQ and $\phi PSII$ were essentially undetectable indicating that in the desiccated state all energy quenching was proceeding through constitutive pathways. This suggest that desiccation may cause structural reversible changes at the photosynthetic apparatus, which consists in the conversion of PSII

reaction centres from a photochemical energy transducers that convert light into ATP and NADPH to an efficient, non-photochemical energy quenchers that protect the photosynthetic apparatus from photodamage (Ivanov et al. 2008). However, rehydration of *H. dentatum* and *H. cruentum* re-established the original patterns for the light response curves for $\phi PSII$, ϕNPQ and ϕNO observed in the control, hydrated states. (4) *Are there any differences between the photoprotection mechanisms used by Hymenophyllaceae species with contrasting vertical distribution?* No, there are not. Both species use the same photoprotection mechanisms. There is just a difference related to the velocity of reaction in *H. dentatum* compared to *H. cruentum* as a function of the decrease in *RWC*. However, the presence of wax glands in *H. dentatum* may maintain the vascular system protected in enviroments with high light intensities and lower humidities. Nonetheless, wax presence is not the trait responsable for the colonization of higher zones of the tree trunk. Thus, wax may not be a trait determining the distribution pattern. It would be interesting to study if this trait is an adaptation in other species with the same contrasting vertical distribution.

Thesis model



Model description: *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C. Presl, present contrasting vertical distribution on tree trunks. *H. dentatum* is associated with the upper 9 m of the host trunk, while *H. cruentum* is associated mainly at the trunk base (See Saldaña et al. 2014). This generates light and humidity gradients. *H. dentatum* is exposed to higher light intensities and lower humidity than *H. cruentum*. Phenotipically, *H. dentatum* presents a higher shrinkage of its fronds in the desiccated state and the presence of a glandular multicellular hair with a distal secretory cell (according to the description of Bower 1963). The ultrastructure study showed the same changes is both species, as cell wall folding, roundish chloroplasts and vacuole fragmentation, characteristics that are restored after a rehydration process. If there are not big differences in the desiccation tolerance of both species, regardless of their vertical distribution,

how do these species face a desiccation period? *H. dentatum* and *H. cruentum* reversibly convert a photochemically active photosynthetic apparatus into a stable, highly quenched structure.

General conclusions

- *H. dentatum* and *H. cruentum* presented the same constitutive photoprotection mechanisms. Both species shrink their leaves to avoid light interception during the desiccated state. Structural pigments and proteins of the photosynthetic apparatus remain stable during desiccation. *H. dentatum* and *H. cruentum* reversibly convert a photochemically active photosynthetic apparatus into a stable, highly quenched structure.

- There is not damage caused by desiccation in *H. dentatum* neither in *H. cruentum*. Both species recovered with the same dependence of the photoprotection mechanisms from the desiccated state.

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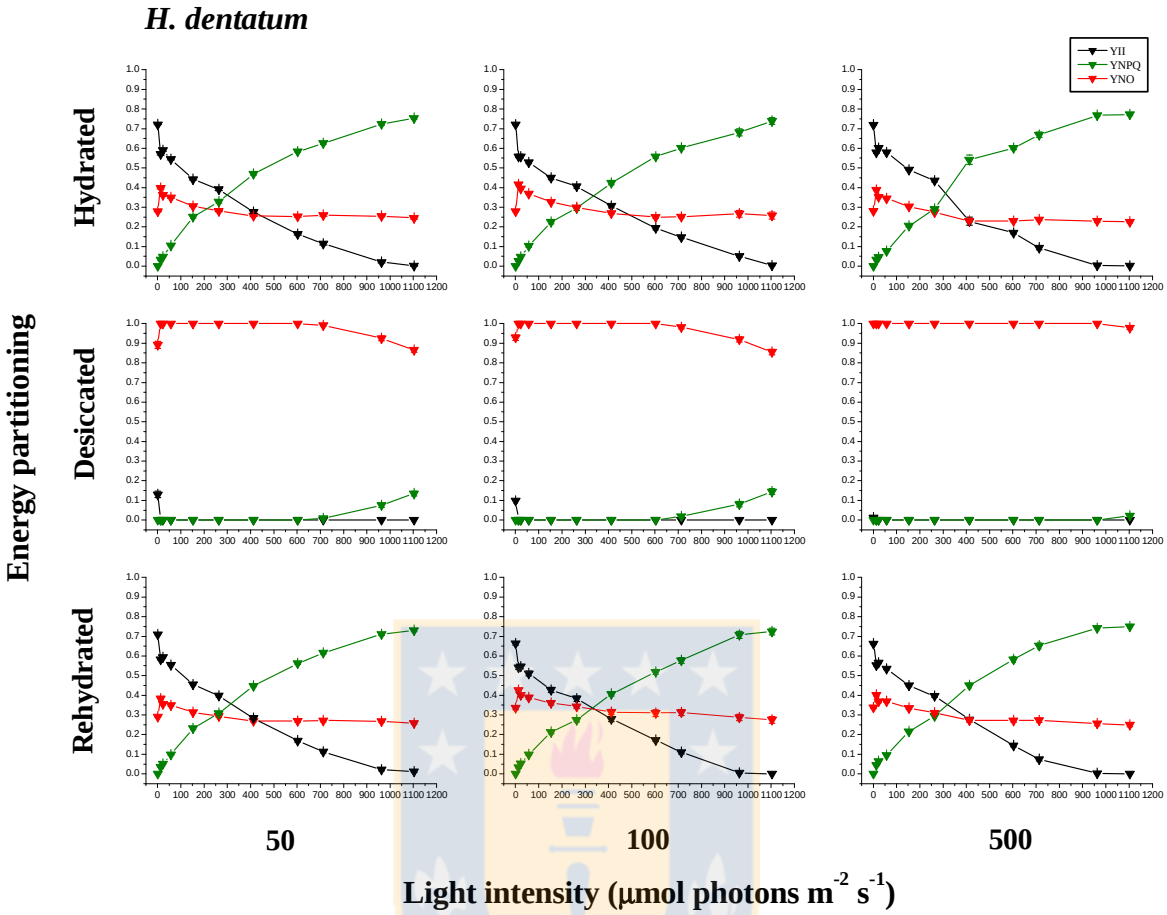
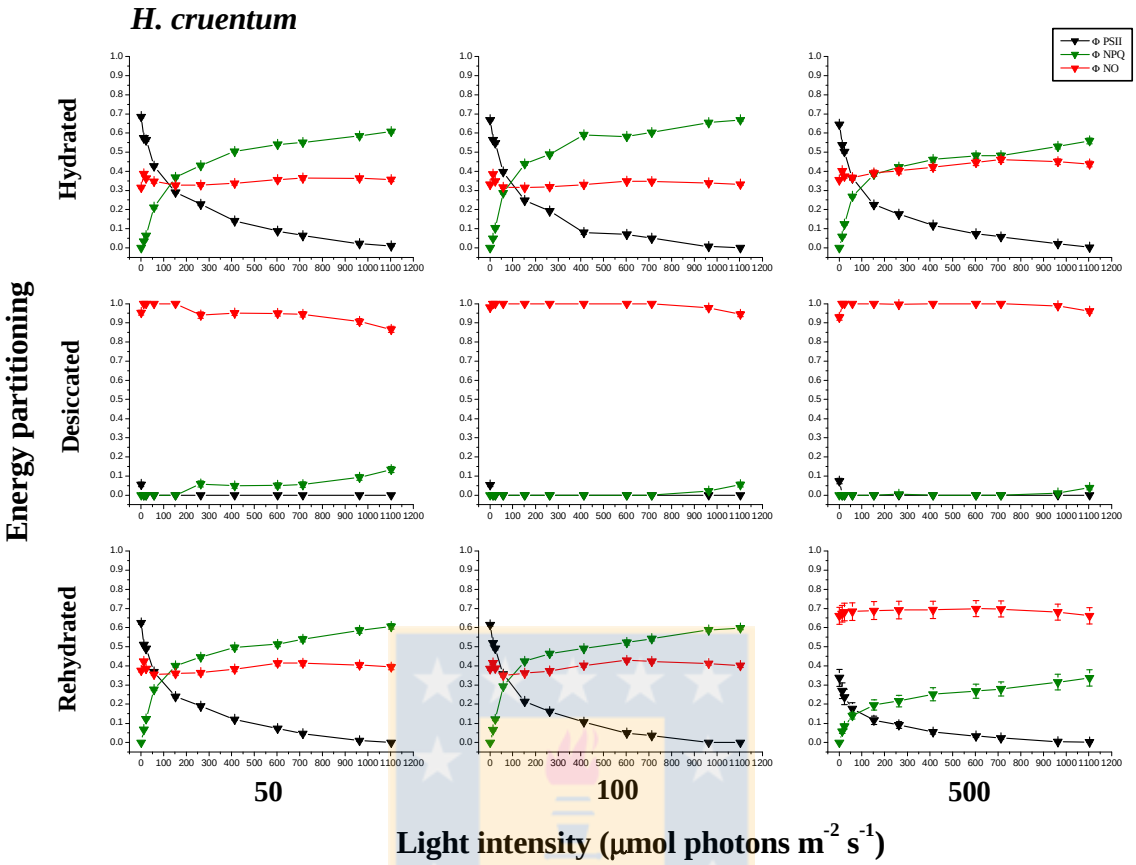
Appendices

Appendix 1. Hymenophyllum dentatum Cav. and *Hymenoglossum cruentum* C. Presl fronds subjected to different light intensities.

Light energy partitioning for PSII was studied in *H. dentatum* Cav. and *H. cruentum* C. Presl after the application of 45 min of 3 different light intensities (50, 100 and 500 µmol photons m^{-2} s^{-1}) in hydrated, desiccated and rehydrated fronds. Both species showed a decrease in the effective quantum yield of PSII (*ϕPSII*) as a function of increased irradiance in hydration and rehydration in the 3 different light intensities. The regulated energy dissipation of PSII (*ϕNPQ*) increased as a function of irradiance in both species in the 3 different light intensities. Constitutive quenching (*ϕNO*) decreased as a function of increased irradiance in both species in the 3 different light intensities. In the desiccated state, *ϕNO* reached values closed to 1, while *ϕNPQ* and *ϕPSII* were essentially undetectable indicating that in the desiccated state all energy quenching was proceeding through constitutive pathways rather than through regulated, energy-dependent quenching pathways in the 3 different light intensities. Rehydration of *H. dentatum* Cav. re-established the original patterns for the light response curves for *ϕPSII*, *ϕNPQ* and *ϕNO* observed in the control, hydrated states in the 3 different light intensities. However in *H. cruentum* C. Presl fronds subjected to 45 min of 500 µmol photons m^{-2} s^{-1} of light intensity, *ϕNPQ* and *ϕNO* are complementary in the protection of PSII, being more important *ϕNO*. In the hydration state of the same light intensity, *ϕNPQ* and *ϕNO* had the same importance.



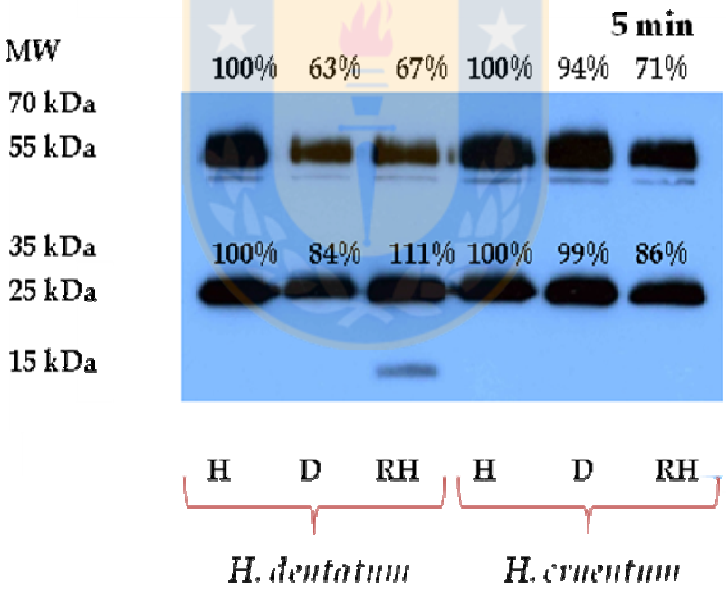
App1.1. Light energy partitioning for PSII at 50, 100 and 500 µmol photons m^{-2} s^{-1}. Effective quantum yield of PSII (*ϕPSII*), quantum yield of non-regulated energy dissipation of PSII (*ϕNO*), and quantum yield of the regulated energy dissipation of PSII (*ϕNPQ*) during the course of a light curve in *Hymenophyllum dentatum* Cav. fronds in the hydrated (100 % *RWC*, 1.9 gH_2O gDW^{-1}), desiccated (25 %, 0.4 gH_2O gDW^{-1}), and rehydrated (>86 % *RWC*, 1.6 gH_2O gDW^{-1}) states. Samples were subjected to that light intensity for 45 min in each hydration state before the light curve (n=3).



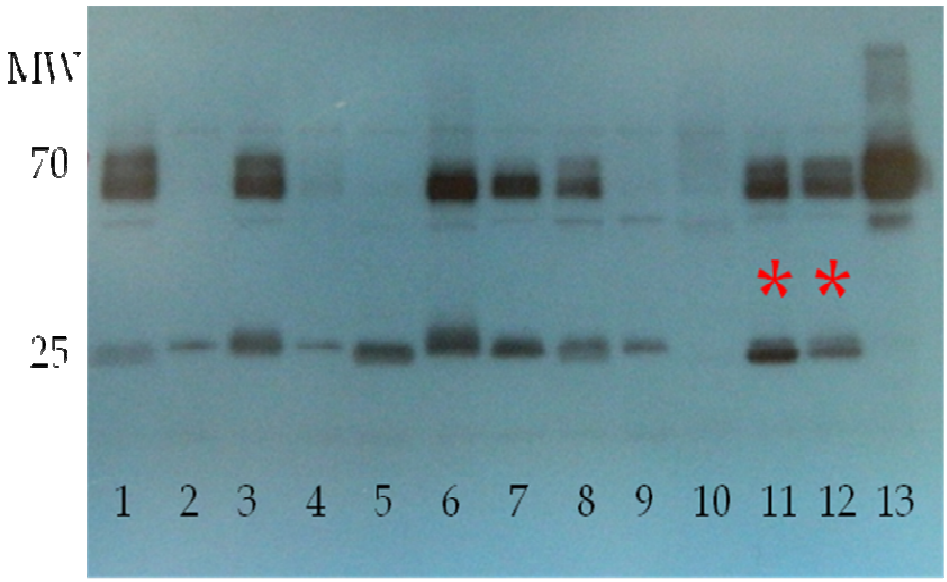
App1.2. Light energy partitioning for PSII at 50, 100 and 500 µmol photons m^{-2} s^{-1}. Effective quantum yield of PSII (*ϕPSII*), quantum yield of non-regulated energy dissipation of PSII (*ϕNO*), and quantum yield of the regulated energy dissipation of PSII (*ϕNPQ*) during the course of a light curve in *Hymenoglossum cruentum* C. Presl fronds in the hydrated (100 % *RWC*, 2.0 gH_2O gDW^{-1}), desiccated (17 % *RWC*, 0.3 gH_2O gDW^{-1}), and rehydrated (>86 % *RWC*, 1.8 gH_2O gDW^{-1}) states. Samples were subjected to that light intensity for 45 min in each hydration state before the light curve (n=3).

Appendix 2. Study of the reaction centre PsaA protein in ferns.

PsaA is a core protein of photosystem I. The expected molecular weight is 82/ 55-60 kDa. When we studied the thylakoid membranes from hydrated, desiccated, and rehydrated fronds of *H. dentatum* Cav. and *H. cruentum* C. Presl through SDS-PAGE, two bands were detected. The first one, situated between 70 and 55 kDa, and the second one situated between 35 and 25 kDa (App2.1). To evaluate if this characteristic is typical from ferns, we included 9 species from Pteridophyta, 1 species from Lycopodiophyta, and 1 species from Magnoliophyta (App2.2). This result suggested that the double band from PsaA is a treat typical from ferns (Pteridophyta), suggesting that ferns apparently could present a structurally different PSI.

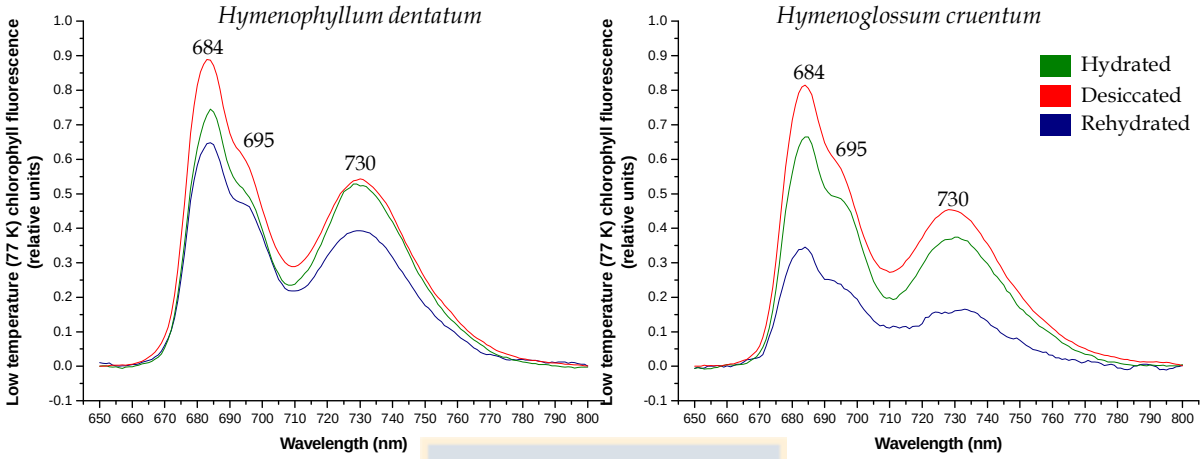


App2.1. Representative western blots of SDS-PAGE separated polypeptides of thylakoid membranes isolated from *Hymenophyllum dentatum* Cav. and *Hymenoglossum cruentum* C. Presl hydrated (100 % *RWC*, 1.9 and 2.0 $gH_2O\ gDW^{-1}$, respectively), desiccated (25 % (0.4 $gH_2O\ gDW^{-1}$) and 17 % (0.3 $gH_2O\ gDW^{-1}$) *RWC*, respectively) and rehydrated (>86 % *RWC*, 1.6 and 1.8 $gH_2O\ gDW^{-1}$, respectively) fronds probed with antibodies raised against PsaA.



App2.2. Representative western blots of SDS-PAGE separated polypeptides of thylakoid membranes isolated from (1) *Adiantum tenerum* (Pteridophyta: Pteridaceae), (2) *Pellaea* sp., (Pteridophyta: Pteridaceae) (3) *Davallia fejeensis* (Pteridophyta: Davalliaceae), (4) *Dryopteris* sp., (Pteridophyta: Dryopteridaceae) (5) *Asplenium serratum* (Pteridophyta: Aspleniaceae), (6) *Cibotium* sp., (Pteridophyta: Cibotiaceae) (7) *Polipodium aereum* (Pteridophyta: Polypodiaceae), (8) *Cyrtomium falcatum* (Pteridophyta: Dryopteridaceae), (9) *Nephrolepis exaltata* (Pteridophyta: Lomariopsidaceae), (10) *Sellaginella kraussiana* (Selaginellaceae: Lycopodiophyta), (11) *Hymenophyllum dentatum* Cav. (Pteridophyta: Hymenophyllaceae), (12) *Hymenoglossum cruentum* C. Presl (Pteridophyta: Hymenophyllaceae) and (13) *Arabidopsis thaliana* (Magnoliophyta: Brassicaceae) fronds and leaves probed with antibodies raised against PsaA.

General supplementary material



GSM1. Chlorophyll a fluorescence emission spectra at 77 K (-196°C) of thylakoids of (A) *H. dentatum* Cav. and (B) *H. cruentum* C. Presl in hydrated, desiccated and rehydrated state. The Chl concentration of all samples was 10 µg ml^{-1} and the excitation wavelenght was 436 nm. Spectra represent an average of 3 corrected scans of each hydration state.

GSM2. Ratios of Chlorophyll-protein complexes of *H. dentatum* Cav. and *H. cruentum* C. Presl in hydrated, desiccated and rehydrated states (n=3).

Species	Hydration state	PSI/LHCHII	PSI/PSII	LHCII/PSII
H. dentatum	Hydrated	0.719 ± 0.101 a	1.035 ± 0.055 a	1.482 ± 0.156 a
H. dentatum	Desiccated	0.609 ± 0.039 a	0.911 ± 0.048 a	1.499 ± 0.035 a
H. dentatum	Rehydrated	0.629 ± 0.082 a	0.844 ± 0.041 a	1.368 ± 0.108 a
H. cruentum	Hydrated	0.567 ± 0.053 a	0.769 ± 0.012 a	1.377 ± 0.116 a
H. cruentum	Desiccated	0.553 ± 0.018 a	0.775 ± 0.018 a	1.403 ± 0.046 a
H. cruentum	Rehydrated	0.504 ± 0.085 a	0.733 ± 0.063 a	1.516 ± 0.199 a