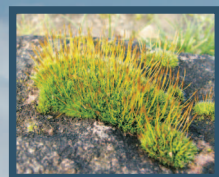
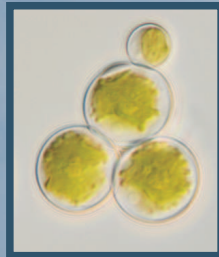


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Our data show that WPI was stronger in woody than in any other group of photosynthetic organisms. However, some herbs and lichens also presented sustained levels of dissipation. Responses differed among functional groups and among species within each group; nevertheless, some trends can be established, such as the enhancement of antioxidants, the diminution of antenna size and the deactivation of reaction centres of photosystem II.

Present work highlights the importance of studying natural processes in non-model organisms, that can corroborate or refute the general patterns described in model species. From the ecological point of view, and taking into consideration the future climatic scenarios of warmer winters, the observed patterns of WPI suggest changes in the ecological relationships in subalpine environments that probably will be in detriment of woody species in relation to the rest of functional groups.



*Functional diversity of the mechanism of winter photoinhibition across the Plant Kingdom*

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Fátima Míguez Cano  
Tesis doctoral, 2016

Fátima Míguez



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Universidad del País Vasco Euskal Herriko Unibertsitatea



## **FUNCTIONAL DIVERSITY OF THE MECHANISMS OF WINTER PHOTOINHIBITION ACROSS THE PLANT KINGDOM**

Memoria presentada por Fátima Míguez Cano, bajo la dirección del Dr. José Ignacio García Plazaola para optar al Grado de Doctora en Ciencias.

Fátima Míguez Cano

Leioa, Diciembre 2015



*A mis padres,  
mi hermano  
y Jaime*





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## SUMMARY

Plants survival depends on the ability to regulate and adjust the utilization of light with the aim to prevent damage in the photosynthetic tissues, especially during winter, when usually there is a combination of high light and low temperatures. Under this situation, evergreens suffer an imbalance between light collection and energy use. As a response they activate a photoprotective dissipative process which consists on the down-regulation of photosynthetic efficiency, referred to as winter photoinhibition (WPI).

Throughout this thesis we aimed to gain light in the characteristics of WPI process, describing the physiological and biochemical responses during winter, or under artificially modified temperature conditions. We analyzed 59 evergreens, including woody species, herbs, mosses, lichens and algae; as well as different photosynthetic organs (leaves and stems).

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Present work highlights the importance of studying natural processes in non-model organisms, that can corroborate or refute the general patterns described in model species. From the ecological point of view, and taking into consideration the future climatic scenarios of warmer winters, the observed patterns of WPI suggest changes in the ecological relationships in subalpine environments that probably will be in detriment of woody species in relation to the rest of functional groups.

## RESUMEN

Las plantas son capaces de regular la utilización de la luz en función de las condiciones ambientales, con el fin de prevenir daños en los tejidos fotosintéticos. En invierno, esta regulación adquiere mayor relevancia debido a que habitualmente se da la combinación de bajas temperaturas y alta irradiación. Bajo estas condiciones, los procesos enzimáticos se ven ralentizados mientras que la captación de energía lumínica permanece constante. Como consecuencia, el exceso lumínico puede dañar las estructuras fotosintéticas mediante la formación de radicales libres de oxígeno. Para evitar esto, las plantas deben de presentar sistemas antioxidantes, así como procesos que disipen el exceso lumínico. Entre estos destaca la disipación térmica que consiste en la reemisión del exceso lumínico que llega a la superficie de la planta en forma de calor. Un aumento en la disipación térmica conlleva a una disminución de la eficiencia fotosintética. Este proceso es conocido como fotoinhibición, en invierno, más concretamente se la denomina fotoinhibición invernal (WPI). Para que se dé la disipación térmica de la energía, es necesaria la presencia de ciertos componentes celulares entre los que cabe destacar el antioxidante denominado zeaxantina, la proteína PsbS, el gradiente de protones a través de la membrana tilacoidal, así como una conformación específica de las antenas de los fotosistemas. La WPI puede ser de dos tipos dependiendo la velocidad de su cinética de recuperación. Si la recuperación es rápida (menos de 12 horas), se la denomina fotoinhibición dinámica mientras que si para una recuperación total de las funciones fotosintéticas se requieren más de 12 horas bajo condiciones óptimas (20°C y luz tenue), a la fotoinhibición se la considera crónica.

Hasta ahora, la WPI ha sido caracterizada principalmente en leñosas y en algunos cultivos, pero poco se sabe de lo que ocurre en otras especies que también viven de forma natural en zonas con condiciones invernales extremas como son algunas herbáceas, helechos, líquenes, briófitos y algas terrestres. Por ello, en esta tesis, el objetivo principal ha sido conocer la extensión adaptativa de

la WPI en el Reino Vegetal. Para responder a este objetivo principal, en este trabajo se han usado tres aproximaciones diferentes: (i) la recopilación de datos mediante un meta-análisis, (ii) el estudio de las respuestas que los organismos vegetales presentan en su hábitat natural y (iii) el análisis de las respuestas que presentan dichos organismos ante condiciones artificiales de adaptación al frío o de recuperación bajo condiciones control. Así mismo, los objetivos específicos fueron:

**1. ¿Hay respuestas generales en la WPI?**

Mediante un meta-análisis, considerando toda la literatura disponible sobre la WPI, se establecieron tendencias generales de comportamiento en diferentes organismos fotosintéticos. Se incluyeron tanto experimentos llevados a cabo en campo como en condiciones de laboratorio. Mediante esta compilación de literatura, también se descubrieron cuales eran los puntos débiles en los que la información era insuficiente.

**2. ¿Cuál es la extensión de la WPI en el Reino Vegetal?**

Para responder a esta pregunta se estudiaron las respuestas fisiológicas y bioquímicas de los organismos fotosintéticos en condiciones invernales de campo así como bajo condiciones controladas en el laboratorio. En este objetivo se incluyeron especies de todos los grupos funcionales que conviven bajo estrés invernal en la alta montaña como son las leñosas, herbáceas, musgos y líquenes.

**3. ¿Cuál es el efecto de la WPI en los tallos fotosintéticos en comparación a las hojas? ¿La edad del órgano intensifica o reduce la WPI?**

A lo largo de esta tesis, aparte de considerar diferentes grupos funcionales, también se analizó la respuesta de los tallos, en comparación a las hojas, así como los efectos de la edad bajo condiciones invernales. Para ello se utilizó el muérdago (*Viscum album*) como planta modelo.

**4. ¿Cómo es la foto-protección en algas de vida libre y en líquenes sometidos a condiciones de exceso lumínico y bajas temperaturas?  
¿Beneficia la liquenización a las algas en términos de WPI?**

La exposición a bajas temperaturas y alta irradiación se estudió en algas de vida libre y en fotobiontes de líquenes.

Para responder a estos objetivos, se analizaron un total de 59 especies diferentes provenientes todas ellas de áreas con al menos una estación fría. La metodología usada consistió principalmente en: (i) la medición de la fluorescencia para conocer la eficiencia fotoquímica del fotosistema 2 ( $F_v/F_m$ ) (ii) la determinación de pigmentos y de azúcares mediante cromatografía líquida de alta presión (iii) la caracterización de proteínas tilacoidales del aparato fotosintético mediante la técnica del western blot. Para el cálculo de los porcentajes de fotoinhibición dinámica y crónica se utilizaron los valores de la  $F_v/F_m$  tanto control como bajo condiciones invernales y se aplicaron las siguientes fórmulas:

$$\text{WPI dinámica} = (F_v/F_{m \text{ 14h invierno}} - F_v/F_{m \text{ 0.5h in invierno}}) / (F_v/F_{m \text{ 14h primavera}}) \times 100$$

$$\text{WPI crónica} = (F_v/F_{m \text{ 14h invierno}} - F_v/F_{m \text{ 14h invierno}}) / (F_v/F_{m \text{ 14h primavera}}) \times 100$$

Donde el número de horas indica el tiempo que la muestra estuvo en condiciones control antes de hacerse la medida.

Las conclusiones principales obtenidas a lo largo de esta tesis doctoral fueron:

- ❁ La WPI es un proceso muy variable en el Reino Vegetal y, aunque las plantas leñosas son aquellas que presentan unos niveles más altos de fotoinhibición, este proceso de fotoprotección no es exclusivo de este grupo funcional ya que a lo largo de esta tesis ha sido observado en algunas especies herbáceas y en algunos líquenes.

- ❁ Una característica común a todas las especies fotoinhibidas crónicamente es que la activación de este mecanismo de fotoprotección se da a 0°C, independientemente del bioma o del grupo funcional analizado.
- ❁ Cuando una planta es transferida desde sus condiciones ambientales naturales a condiciones óptimas controladas, se observa que la recuperación de la actividad fotosintética se da en dos fases. Una primera fase rápida y una segunda fase lenta. Este proceso de recuperación es más lento en especie leñosas que en el resto de grupos funcionales considerados.
- ❁ A temperaturas por debajo de los 2°C, se da una retención sostenida de zeaxantina. Por otro lado, la transformación de zeaxantina a violaxantina es más rápida que la disminución de la WPI bajo condiciones controladas de recuperación.
- ❁ La zeaxantina no sólo es acumulada en aquellas especies fotoinhibidas, sino que durante el invierno parece un antioxidante esencial en prácticamente todas las especies analizadas, independientemente de su grado de fotoinhibición. Todo esto sugiere que la zeaxantina tiene un importante rol, a parte del relacionado con la disipación térmica.
- ❁ En plantas leñosas, los patrones de recuperación de los tallos fotosintéticos son iguales a los descritos para el caso de las hojas, presentando ambos el componente lento y rápido. Sin embargo, los tallos contribuyen a mantener un balance de carbono positivo durante el invierno, gracias a sus bajos niveles WPI y al efecto positivo de la edad en la eficiencia fotosintética. Estos resultados apuntan a que los tallos del muérdago son un buen ejemplo de senescencia negativa.
- ❁ Las algas de vida libre están generalmente más afectadas por las bajas temperaturas y la alta irradiación que los fotobiontes de la misma especie cuando forman simbiosis líquénicas. Lo que es más, la

protección por el cortex del liquen va más allá de un simple sombreamiento y parece estar implicada en cambios bioquímicos.

- ❁ La gran diversidad de respuestas del mecanismo de la disipación térmica en algas, no depende de la posición filogenética, sino en la presión de selección de los diferentes ambientes.
- ❁ La acumulación de azúcares en algas y líquenes no tiene ninguna relación causa-efecto con el proceso de la fotoinhibición bajo condiciones de bajas temperaturas.
- ❁ Los cambios de las proteínas tilacoidales bajo condiciones de bajas temperaturas no siguen un patrón común en las especies estudiadas, a excepción de la proteína D1, que disminuye en respuesta al frío en todas las especies estudiadas excepto para el caso de los tallos fotosintéticos.

Este trabajo subraya la importancia de estudiar los procesos naturales en organismos no modelo, cuyos patrones de comportamiento bajo determinadas condiciones no siempre coinciden con aquellos presentes en los organismos modelo. Desde el punto de vista ecológico, y teniendo en consideración los futuros escenarios de cambio climático, los patrones observados bajo condiciones invernales en los diferentes grupos funcionales estudiados, indican que con la disminución de la crudeza invernal, parece inevitable que las especies leñosas se vean en desventaja respecto al resto de grupos funcionales debido a que la WPI es un método de fotoprotección muy útil bajo condiciones invernales estresantes pero que evita el aprovechamiento de la luz cuando a lo largo del invierno se intercalan días de temperaturas agradables.

## ABBREVIATIONS

<b>ΔpH:</b> Transthylakoid proton gradient	<b>PQ:</b> Plastoquinone
<b>A:</b> Antheraxanthin	<b>PS:</b> Photosystem
<b>AE:</b> <i>Asterochloris erici</i>	<b>RP:</b> <i>Ramalina pollinaria</i>
<b>AL:</b> <i>Apatococcus lobatus</i>	<b>ROS:</b> Reactive Oxygen Species
<b>AZ/VAZ:</b> De-epoxidation state of the violaxanthin cycle pigments	<b>Toc:</b> Tocopherol
<b>BR:</b> <i>Baeomyces rufus</i>	<b>TA:</b> <i>Trebouxia arboricola</i>
<b>CC:</b> Core Complex	<b>V:</b> Violaxanthin
<b>Chl:</b> Chlorophyll	<b>V-cycle:</b> Violaxanthin cycle
<b>CS:</b> <i>Cladonia squamosa</i>	<b>WPI:</b> Winter photoinhibition
<b>Cyt b<sub>6</sub>f:</b> Cytochrome b <sub>6</sub> f	<b>WPI<sub>&lt;12h</sub>:</b> Dynamic Winter Photoinhibition
<b>EB:</b> <i>Elliptochloris bilobata</i>	<b>WPI<sub>&gt;12h</sub>:</b> Chronic Winter Photoinhibition
<b>Elip:</b> Early light inducible proteins	<b>WPI<sub>all</sub>:</b> Total winter photoinhibition
<b>F<sub>m</sub>:</b> Maximum chlorophyll fluorescence	<b>Z:</b> Zeaxanthin
<b>F<sub>0</sub>:</b> Minimum chlorophyll fluorescence	<b>β-car:</b> β-carotene
<b>HL:</b> High light, high irradiance	
<b>H<sup>+</sup>:</b> Protons	
<b>H<sub>2</sub>O<sub>2</sub>:</b> Hydrogen Peroxide	
<b>L:</b> Lutein	
<b>LL:</b> Low light, low irradiance	
<b>LT:</b> Low temperature	
<b>L<sub>x</sub>-cycle:</b> Lutein epoxide cycle	
<b>LHC:</b> Light Harvesting Complex	
<b>Lhca1-4:</b> Antennae PSI	
<b>Lhcb1-6:</b> Antennae PSII	
<b>N:</b> Neoxanthin	
<b>O<sub>2</sub><sup>-</sup>:</b> Superoxide Radical	
<b><sup>1</sup>O<sub>2</sub>:</b> Singlet oxygen	
<b>OEC:</b> Oxygen evolving complex	
<b>OH<sup>•</sup>:</b> Hydroxyl radical	
<b>PI:</b> Photoinhibition	





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# 1. Introduction

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## 1.1 THE BIRTH AND EVOLUTION OF PHOTOSYNTHESIS

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Photosynthetic organisms, from the smallest bacteria to the biggest tree, are everywhere in our planet. Nevertheless, they cannot be considered decorative pieces in a landscape or a garden. They are our “live-mates” as they have been contributing enormously to the Earth surface development since their appearance. They have shaped current earth ecosystems, nutrient cycles and atmosphere composition. Even today they are indispensable as they are used in the production of medicines and fuels as well as in the maintenance of atmosphere composition, among others.

It is estimated that oxygenic photosynthesis appeared around 3.4 billion years ago (Palmer and Barret 2010). Initially photosynthesis was developed only by cyanobacteria but this character spread out when, around one billion years ago, primary endosymbiosis occurred. Primary endosymbiosis was one of the most important events in the history of the life on Earth. It consisted on the incorporation of a cyanobacterium into the cytoplasm of a heterotroph eukaryotic organism. With the passing of time, the integrated cyanobacterium was transformed into a chloroplast, losing its capacity to live free. From this initial endosymbiosis, three lineages evolved: the green (Chlorophyta or Viridiplantae), the red (Rhodophyta) and the Glaucophyta. Then, secondary and tertiary endosymbiosis provoked the expansion of photosynthesis with the appearance of the other algal groups. These were the first steps for the formation of all the algae and plants that nowadays co-habit in the Earth (Fig. 1.1 A).

Viridiplantae is a monophyletic clade that includes all green algae and plants, as well as mosses. Currently, Viridiplantae is grouped into two divisions: Chlorophyta and Streptophyta (Lewis and McCourt 2004). Streptophyta comprises all embryophyte plants (land plants) and a diverse outfit of freshwater green algae (*Klebsormidium* sp., *Chara* sp., *Spirogyra* sp....) (Becker and Marin 2009) while Chlorophyta comprises the majority of green algae classes, most of

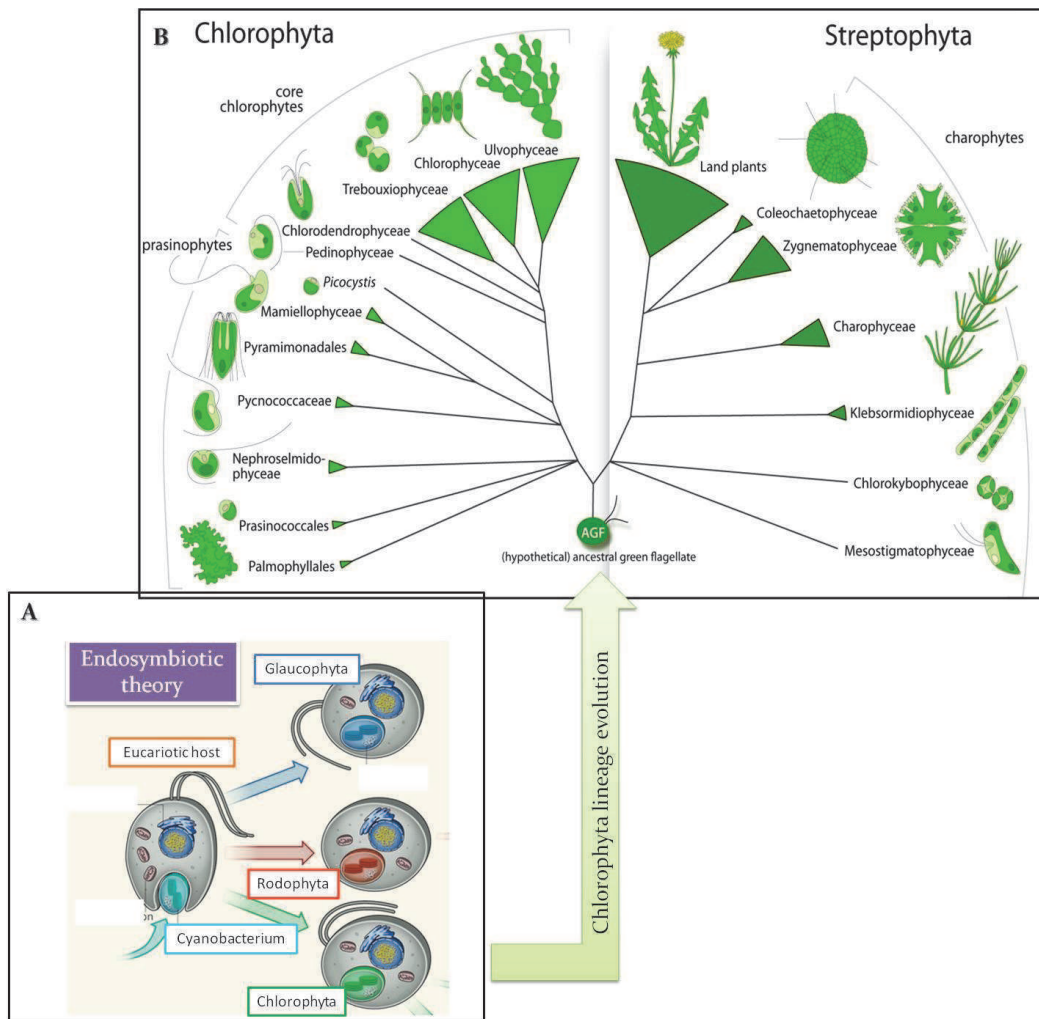


them being seawater species. (Guiry 2012) (Fig. 1.1 B). It is remarkable that some of them have evolved to an aeroterrestrial habitat

The colonization of the terrestrial habitat was a major event in plant evolution, transforming our planet once and forever (Kenrick et al. 2012). It took place in the Paleozoic era, approximately 400-490 millions of years ago (Becker and Marin 2009). It was carried out by a group of Streptophyta, which was initially a freshwater group. This early adaptation to freshwater condition was probably the key for the later colonization of land (Becker 2013). The expansion from moist habitats in the proximity of water, to dry land was fast due to the low competition in that inhabited environment (Becker and Marin 2009). Nowadays terrestrial algae habit in all land ecosystems from desserts to forests, from tropics to polar areas and in all types of soils.

There are other algae species, from Chlorophyta and Cyanophyta phyllums, that were also able to colonize terrestrial environments trough the formation of symbiosis with a fungus, known as lichen symbiosis. As lichen symbiosis is an ecological concept, and it appeared several times along the evolution, it is not possible to establish a date or a period for its appearance (Gargas et al. 1995).

So, even at first glance green algae, bryophytes, gymnosperms and angiosperms seem to have little in common, they are close relatives, that share a common ancestor and have evolved, diversified and adapted to the terrestrial relatively recently in evolutionary terms.



**Fig. 1.1** (A) Endosymbiotic theory. Modified from Gould (2012) (B) Phylogenetic overview of the green algal evolution, posing the early divergence of two discrete lineages known as Chlorophyta and Streptophyta. Modified from Leliaert et al. (2012)





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## 1.2 PHOTOSYNTHETIC APPARATUS

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Chloroplasts are the organelles where photosynthesis takes place. Within these organelles, photosynthetic apparatus is composed of an extensive system of internal membranes (thylakoids) with proteins embedded on it, and soluble proteins located in the chloroplast stroma. Membrane proteins are typically involved in light harvesting and energy conversion (light reactions) binding all chlorophyll (Chl) molecules, while soluble proteins constitute the carbon assimilation system, being Rubisco the most abundant. In photosynthetic apparatus there are also associated pigments (Table 1.1)

Thylakoid photosynthetic apparatus is composed of 4 protein complexes: Photosystem I (PSI), Photosystem II (PSII), Cytochrome *b<sub>6</sub>f* (Cyt *b<sub>6</sub>f*) and ATP synthase (Fig. 1.3). Almost all photosynthetic pigments are bound to these protein complexes (PSI and PSII).

**1.2.1. PSII** is a complex formed by 21 polypeptides located in thylakoid grana membranes. It is composed by two central proteins (D1 and D2) forming a dimer. Then, this complex presents major antenna proteins which form trimers (Lhcb1, Lhcb2, Lhcb3) and minor antennae (Lhcb4, Lhcb5, Lhcb6) which are disposed around the core complex (Fig. 1.2). The functions are the oxidation of water and consequently, the reduction of plastoquinone (PQ). In PSII, trimeric LHCII<sub>s</sub>, are able to move along the thylakoids independently of the core complex, in response to environmental factors, being detached of reaction centers of bound to PSI (the so-called state transitions).

**1.2.2. PSI** composition is very conservative in all Plant Kingdom and similar to that of PSII. It presents 15 polypeptides of which, the two biggest proteins, known as PsaA and PsaB, form the core complex. The PSI antenna is formed by 4 proteins: Lhca1, Lhca2, Lhca3 and Lhca4 arranged as dimers around the core complex. PSI takes part in the last part of electron transport. It transfers electrons



from plastocyanin (PC) (located in the lumen) to the ferredoxin (Fd) (located in the stroma). PSI is located in grana membranes of thylakoids.

In both photosystems, the function of antennae (LHC) is the collection of light. The main pigments associated to LHC are Chl but there are also some carotenoids (Table 1.1).

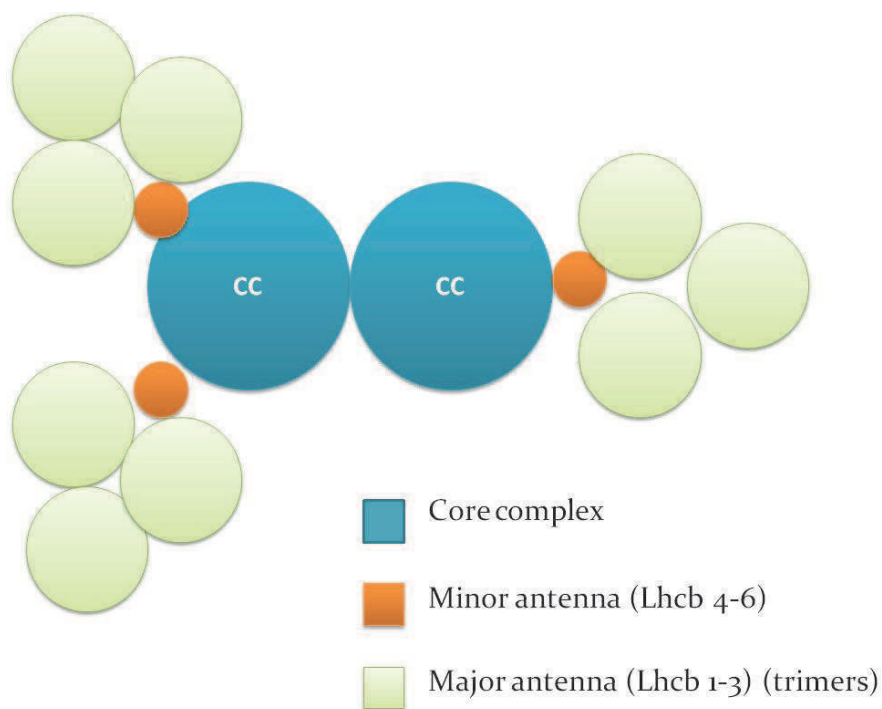
**1.2.3 Cyt *b<sub>6</sub>f*** is the link of union between PSII and PSI. It regulates the transference of electrons between both complexes. It oxidizes PQ with the consequent reduction of PC. It is formed by 4 main polypeptides: cytochrome *f*, cytochrome *b<sub>6</sub>*, subunit IV and Rieske protein.

**1.2.4 ATP-synthase** is an enzymatic complex with a domain inserted in the membrane and with extremes located in the lumen and in the stroma respectively. It is able to use the energy previously accumulated in the electron transport chain in form of protons ( $H^+$ ), to produce energy. In the electron transport along the photosynthetic apparatus, this electron flux is coupled with the proton movement through thylakoid membrane from stroma to lumen that becomes acid. Proton gradient occurs through ATP-synthases allowing ATP synthesis in the stromal side using ADP and protons.

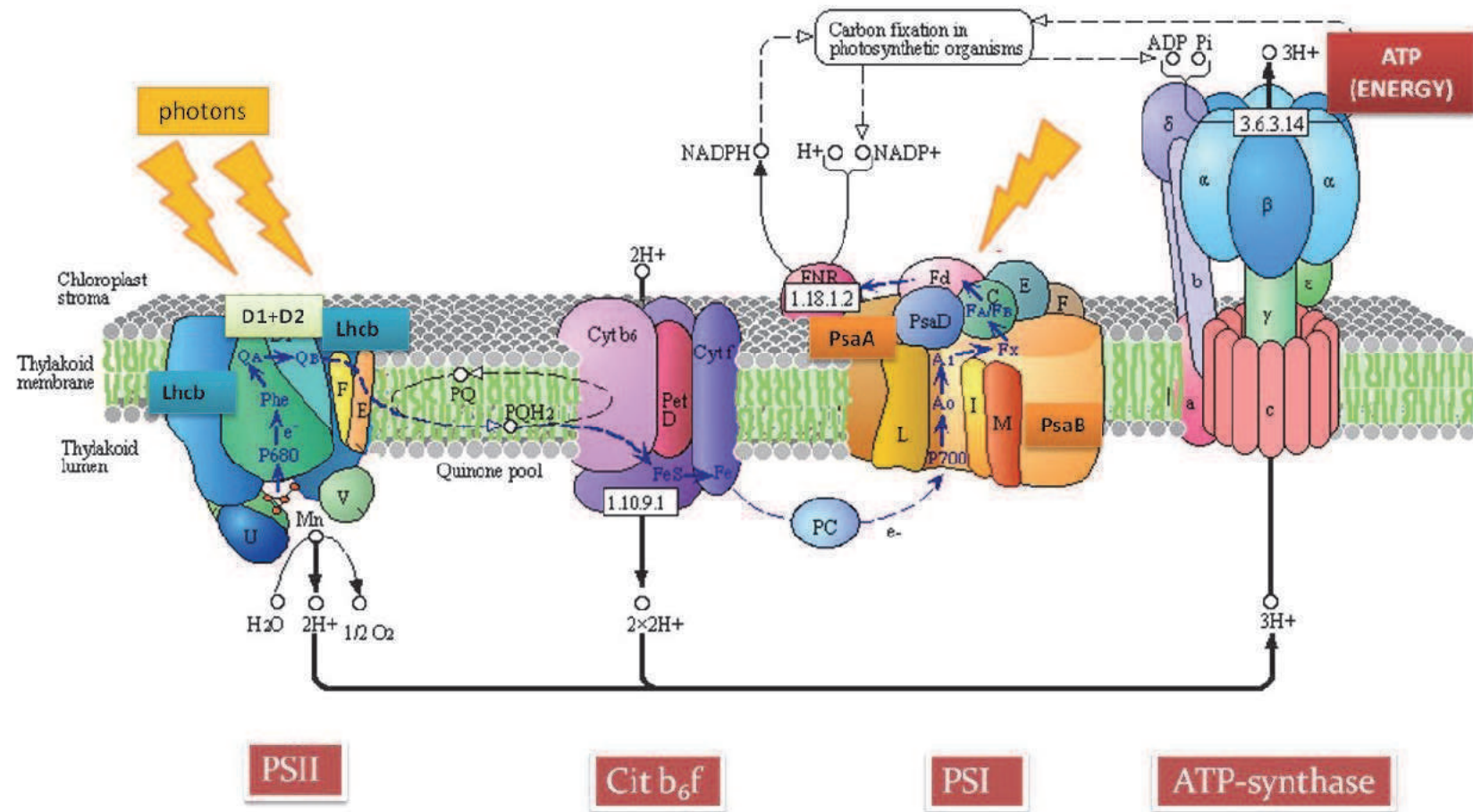


**Table 1.1:** Stoichiometric pigment composition of photosystem I (PSI) and photosystem II (PSII) constituents. Chl (Chlorophyll),  $\beta$ -car ( $\beta$ -carotene), VAZ (Total pool of xanthophylls: violaxanthin, antheraxanthin and zeaxanthin), Neo (Neoxanthin), L (lutein) Modified from Esteban et al. (2014).

		Chla	Chlb	$\beta$ -car	VAZ	Neo	L
<b>PSI:</b>	Core complex	96		22			
	Antenna (LhcI)	48	13	0-2	2-4		4-8
	Gaps and linkers	17					
<b>PSII:</b>	Core complex	37		11			
	Minor antenna	12-17	5-10		0-3	2	2-5
	Major antenna (trimer)	24	18		0-6	3	3-9



**Fig. 1.2:** Idealized representation of the photosystem II (PSII) composition



**Fig. 1.3:** Schematic representation of the PSII, PSI, Cit  $b_6f$  and ATP-synthase in the thylakoid membrane. The movement of electrons is represented along all the photosynthetic apparatus components with a discontinuous line.



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### 1.3 LOW-TEMPERATURE ALONG EARTH'S BIOSPHERE HISTORY

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Climate has been changing along the Earth's history. Photosynthetic organisms that inhabit in emerged areas of the planet have been subjected to continuous environmental changes. Since the end of Tertiary Age (Pliocene) several cold episodes, known as glaciations have occurred. These cold phases alternated with other periods in which the climate was similar to the present one, called interglacials. These thermal oscillations, which encompass 2 million of years, took place in a world where: (i) mountain chains had been already formed so; most of the alpine plants were present; (ii) the disposition of oceans and continents was very similar to the current; (iii) angiosperm species had evolved to the present diversity and they were widespread along almost all the continents.

This means that, almost all the plants that nowadays are cohabiting in Earth's surface, had been subjected in the past to glaciations. These past conditions influenced in their evolution, leaving physiological and/or morphological remnants.

During glaciations, northern and central Europe was totally covered by an ice layer which rose a thickness of 2-3 km. Under these conditions there was a migration of forests to the south and southeast peninsulas, more concretely to the coastal zones (where the sea moderated the harsh environmental conditions) or to the summits of mountainous areas (where wind removed the snow). These peculiar zones acted as flora refuges. Thanks to these areas of protection, now we have the diversity of plants we know. Nevertheless, during those glacial periods a lot of species faced extinction (i) due to the environmental conditions were too hard for them or (ii) because the parallel disposition of mountain chains and the presence of Mediterranean Sea, acted as traps in the migration of



species, making impossible the flight of a lot of species which finally became extinct (Loidi et al. 2011).

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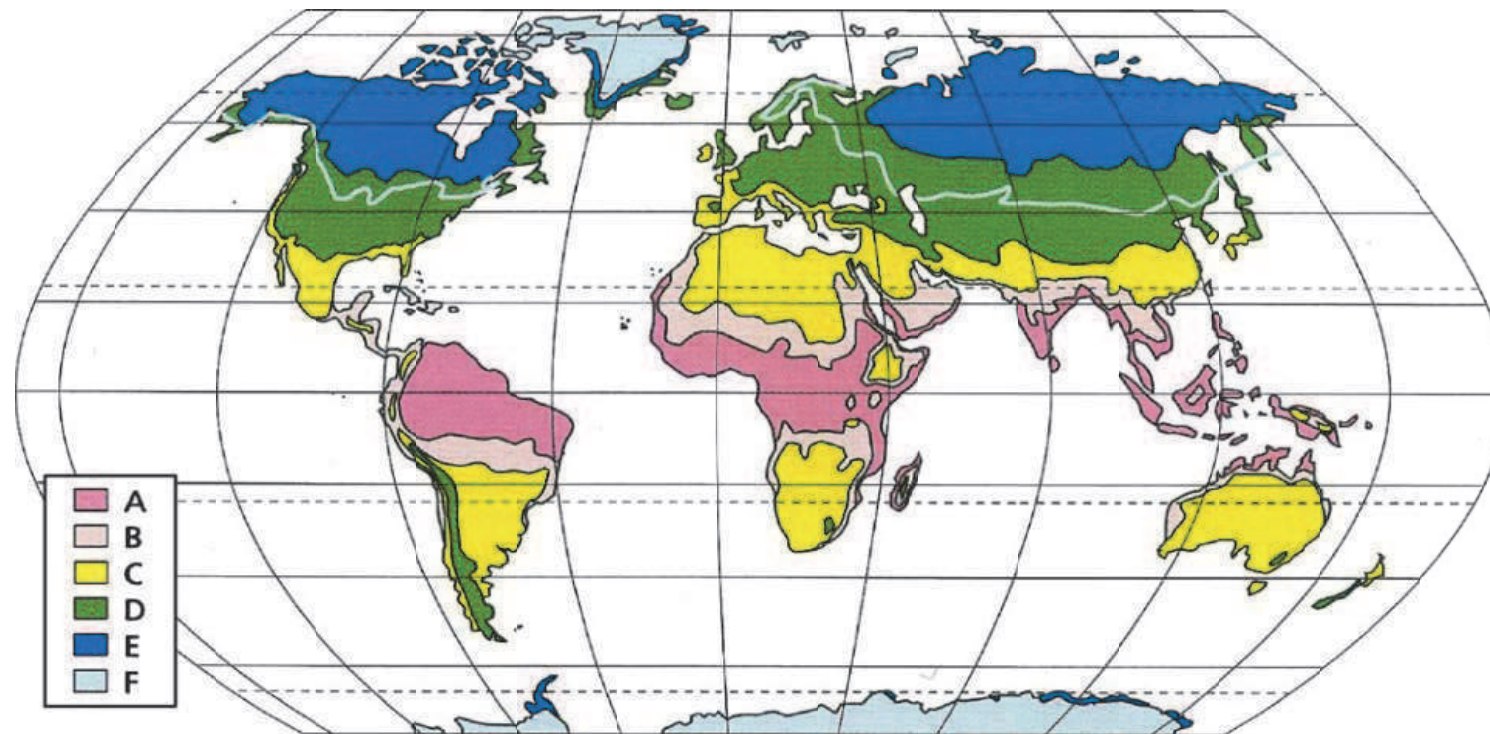
#### 1.4 COLD AREAS THESE DAYS... WHO ARE LIVING THERE?

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Cold intensity variations not only occur at so long time scales such as glaciations. Shorter periods, as seasonal changes, also affect to the plant performance due to temperature changes. Cold adapted organisms are abundant in Earth's biosphere, being their study of paramount importance. These organisms include unicellular organisms (prokaryotes and eukaryotes), plants and ectothermic animals (Margesin et al. 2007). The behavior and physiology of them is influenced by these hard conditions. The interest in the study of the survival of organisms in cold environments has increased recently, driven in part by the desire to ascertain whether life can exist elsewhere in our solar system (Miteva et al. 2004). It is also remarkable the interest of these studies to predict how climate change will affect to these species which will be in principle, at a disadvantage under new scenarios of global warming. In terms of cold, not only the temperature is important, but also the frequency, duration, intensity, as well as the date which the cold event starts or finishes (Margesin et al. 2007).

In the Earth there are different climate zones but most of them present at least a cold season. In Fig. 1.4 there is a representation of the occurrence of low temperatures (LT) and frost on Earth.





**Fig. 1.4:** Occurrence of low temperatures and frost on the Earth. **A** annual minimum temperatures above +5°C, **B** annual minimum temperature above 0°C, **C** episodic frosts with temperatures down to -10°C, **D** regions with cold winters and minimum temperatures between -10 and -40°C (white lines -30°C minimum isotherm), **E** minimum temperatures below -40°C, **F** polar ice. The above zones correspond to the areas of plant species with different types of frost resistance: **Zone A** chilling-sensitive plants of the equatorial tropics, **zone B** extremely freezing-sensitive plants, **zone C** plants protected by effective supercooling and depression of the FP, **zone D** plants with limited freezing tolerance and trees with wood capable of deep supercooling, **zone E** completely freezing-tolerant plants (from Larcher (2003))



## Winter season in mountainous areas, how are plants living there?

Approximately 20% of the Earth's land surface is covered by mountains ranging from poles to the tropics. These areas present a very special climate characterized by LT, frequent and intense wind, high visible and UV radiation an irregular distribution of precipitation and a small growing and reproductive period. Besides, mountainous climate is also characterized by short-term changeability (Larcher 2012).

This hard climate makes photosynthetic organisms to present special morphological and physiological features. Plant species living in mountainous areas are mostly **perennial** evergreens, due to the period with optimum conditions for reproduction is very short to fully complete a life-cycle in a single growing season, so, they have not time to produce their entire foliage every year.

On the other hand, they are usually **small**, and grow forming cushions, tussocks or rosettes. The reason of this is not only that they do not have a lot of resources available in those areas (soils are very poor due to the erosion caused by wind and snow) but also because being small has advantages. Thus, the more the plants attach to the ground, the more they decouple their microclimate from the ambient (Körner 1999). For example, near to the soil, temperatures are not so variable and usually are relatively higher than above. Also the abrasive effects of wind are softer and the amount and the intensity of light energy absorbed are generally lower. Furthermore, during winter, they are easily covered by snow. Snow mantle acts as an effective protection, buffering temperature oscillations, protecting from severe frosts, decreasing light intensity and avoiding the mechanical stress provoked by strong wind (Körner 1999).





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## 1.5 NEGATIVE EFFECTS OF COLD IN PHOTOSYNTHETIC ORGANISMS.

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Mean maximum rates of photosynthesis measured under standard conditions are similar in alpine plants compared with lowland plants (Körner 1999). Nevertheless, optimum temperature to photosynthesize is shifted to lower values compared with lowland and tropical plants. This reflects the acclimation to the general lower mean air temperature measured in mountain areas (Körner 1999).

LT affect plant survival by three processes: loss of metabolic activities (chilling stress), ice formation within the tissues (freezing stress) and the over-excitation of the photosynthetic apparatus (photochilling stress). These effects influence from cell to ecosystem scales (Loik et al. 2004). Nevertheless, a lot of species are totally adapted to live in cold environments. They love cold and as they are evergreens it is a prerequisite for them to present photoprotective mechanisms to protect their green tissues from the adversities of winter.

Resistance to LT changes seasonally, being higher during the coldest months. This process is known as **cold acclimation (CA)**. CA is a long term response whereby plants, in response to cold, undergo a range of biological changes in order to increase their frost tolerance and prepare themselves to winter season (Sandve et al. 2011). Generally, CA is induced under LT (Levitt 1980). In the case of woody plants, CA also starts when the days become shorter, indicating the winter arrival (Sakai and Larcher 1987). In the following section, the most frequent effects and responses under cold conditions in Plant Kingdom will be described.



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## 1.6 EFFECTS OF LOW TEMPERATURES AT CELLULAR AND PLANT LEVEL

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### 1.6.1 CELLULAR MEMBRANE MODIFICATIONS

It is a consensus that the primary cause of freezing injury is the dysfunction of the plasma membrane as a consequence of the dehydration generated by the formation of extracellular ice. When the extracellular water is frozen, the osmotic effect induces the releases of water from the intracellular to the extracellular media provoking the cellular dehydration (Levitt 1980).

Under LT, there is also a loss of fluidity due to the increase of saturated fatty acids. This leads into an increase of membrane rigidity and the consequent reduction of their functionality, affecting all physiological processes of the plant (Los et al. 2013).

### 1.6.2 PHOTOINHIBITION

Metabolic reactions, with no exception, are temperature-dependent. One of the primary reasons is that enzymatic processes are slowed down under LT. Among other processes, these conditions reduce the capacity of the photosynthetic enzymatic machinery in terms of carbon fixation. Light harvesting, however, is a physical process, basically temperature-independent. As a consequence, the imbalance between the amount of light which is absorbed by the photosynthetic surface and the quantity of light which is used by photosynthetic machinery, generates, even at low light regimes, a reduction of photosynthetic activity, that is referred to as photoinhibition (PI) (Osmond and Grace 1995). As a consequence, the photosynthetic electron transport chain becomes over-reduced and the excess excitation energy potentially leads to the formation of reactive oxygen species (ROS).



Other authors relate PI with PSII damage. PSII is vulnerable to light excess due to singlet oxygen ( $^1\text{O}_2$ ) by triplet excited Chls (highly produced under high irradiance conditions). The CC of PSII (D1 protein) is extremely sensitive to the generation of oxidative damage by  $^1\text{O}_2$  (Vass and Cser 2009). This sensitivity means that PSII has to be rebuilt every 30 minutes even under relatively low irradiances (LL) (Foyer and Shigeoka 2011). The degradation and the repair of PSII usually occur at the same velocity but, when the repair is slower than the degradation, PI appears. In the present work, this phenomenon will be defined as **photodamage**.

### 1.6.3 FORMATION OF REACTIVE OXYGEN SPECIES

Under LT, in a photoinhibited organism, whereas the photosynthetic apparatus is overexcited, the absorption of light by the photosynthetic apparatus remains constant because it is not temperature-dependent. Under these conditions, the excited Chls cannot donate the electrons to the electron chain because it is blocked (acceptors reduced). These conditions are a hazardous source of ROS that can damage cell structures and even induce cell death. In plant cells, ROS are preferentially formed in mitochondria and chloroplasts. The main ROS generated are  $^1\text{O}_2$ , hydroxyl radical ( $\text{OH}^\cdot$ ), superoxide radical ( $\text{O}_2^{\cdot-}$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). These molecules apart from being harmful in high amounts, are also essential for cell signaling and the protection against biotic stresses (Foyer and Noctor 2005).

### 1.6.4 THYLAKOID PROTEIN CHANGES

Protein synthesis and degradation pathways are essential processes in plant responses to changing environment (Vierstra 1996). Almost all the proteins suffer conformation, composition, concentration and/or location changes depending on the environmental conditions. As a result, changes in the metabolic activity at different scales in the plant occur. In the present work, we will focus on **photosynthetic apparatus proteins** (for more detail about



proteins responsible for photosynthesis see Introduction Section 1.2) that typically change their conformation, arrangement or concentration, depending on the season and therefore depending on light-temperature conditions.

### A) Phosphorylation

Phosphorylation is the first step for degradation of several proteins as for instance D1 protein. Phosphorylation of thylakoid proteins is dependent on the delicate cooperation of the kinases STN7 and STN8 (Bonardi et al. 2005) and at least two phosphatases TAP38/PPH1 (Shapiguzov et al. 2010) and PBCP (Samol et al. 2012). Phosphorylation of PSII core proteins increases upon an increase in light intensity (Tikkanen et al. 2010), while the phosphorylation of LHCII presents the opposite pattern. It was postulated by Tikkanen and Aro (2014) that the phosphorylation process is as follows:

Under **moderate light intensities**, when light energy is effectively captured by LHCII and there is not potential damage of PSII, both LHCII and PSII are moderately phosphorylated. This status provides sufficient fluidity to the tightly packed grana membranes for fluent PSII turnover. At the same time, moderate phosphorylation of LHCII attracts PSI complexes to the grana margins, enabling sufficient excitation energy transfer to PSI, allowing photosynthesis (Goral et al. 2010).

Under **excess light** conditions, PSII core proteins (D1 and D2) become increasingly phosphorylated. This is essential for the controlled neutralization of photodamaged PSII centers. Upon this process the packing of the PSII-LHCII complexes opens up, increasing the amount of PSII-LHCII to interact with PSI. Upon continued HL illumination, gradual de-phosphorylation of LHCII starts, preventing the excess excitation energy transfer from PSII to PSI, avoiding PSI photoinhibition (Rintamäki et al. 2000).



Some studies (Ebbert et al. 2005; Chen et al. 2012) show in woody species that, during winter, when there is a **combination of high light and low temperatures**, D1 is arrested in a phosphorylated state. It means that even during night, phosphorylation is not reverted. It is supposed, that sustained phosphorylation, changes the conformation of PSII playing a role in the inactivation of photochemistry and/or up-regulation of thermal dissipation itself (Ebbert et al. 2005). It was speculated that sustained D1 phosphorylation may prevent the turnover of PSII CC and it is a prerequisite for D1 degradation (Ebbert et al. 2005). Correlations between sustained energy dissipation and thylakoid protein phosphorylation have been demonstrated in rice leaves under chilling conditions (Kim et al. 1997), in photoinhibited leaves of the shade plant *Monstera deliciosa* Liebm. (Ebbert et al. 2001) and in the evergreen Douglas fir on subfreezing winter nights (Ebbert et al. 2005).

### **B) Changes in thylakoid protein concentrations:**

It is totally accepted that modifications in proteins under winter stress go beyond phosphorylation. Hence, there are also changes in the relative abundance of the photosynthetic proteins (Ensminger et al. 2004; Zarter et al. 2006; Verhoeven et al. 2009). Nevertheless, until now there is not a consensus about the common tendencies of some proteins, in part, regarding the different behavior between shade and sun exposed leaves or different patterns among species.

It was demonstrated in a wide range of species (mostly conifers) that the last consequence of **D1** phosphorylation is the reduction of D1 content during winter (Zarter et al. 2006; Verhoeven et al. 2009). The reduction of D1 content in conifers occurs in response to both LT and shorter day-length. This indicates that D1 decrease is part of the process of cold hardening that occurs during winter acclimation and is independent of excitation pressure. Table 1.2 (based on



Verhoeven (2014)), is a compilation of the most recent studies, showing the most frequent protein changes during winter.

**LHCs** are the antenna proteins. They act as light collectors. In winter, when the excess of light is harmful, plants reduce the LHCs content, leading into smaller antennae to reduce the light capture surface to the minimum size (Savitch et al. 2002; Verhoeven et al. 2009).

**PsbS** is a four-helix protein that belongs to LHC protein superfamily. It is integrated in thylakoid membrane and it presents two pairs of glutamate residues located within or close to the lumen-exposed loops of the protein (Li et al. 2002). These residues act as pH sensors. When lumen is acid (which means HL conditions), glutamate residues are protonated and flexible thermal energy dissipation (qE) is active, being, PsbS an essential component of qE (Li et al. 2000).

**Early light inducible proteins (Elip)** is a group of three-helix proteins inserted in the thylakoid membranes that belong to the LHC protein superfamily. Proposed roles for these proteins include: (i) the protection of Chl complexes during turnover, preventing the transference of excitation energy to oxygen and (ii) the dissipation of excess energy from Chl preventing photooxidation in sustained dissipation (Montané and Kloppstech 2000; Zarter et al. 2006; Demmig-Adams et al. 2006). The upregulation of Elips has been observed during winter in a numerous species (Ensminger et al. 2004; Zarter et al. 2006).

**Oxygen evolving complex (OEC)** is the responsible of water splitting and the donator of electrons for transport through the photosynthetic electron transport chain. It is degraded under photoinhibitory conditions (Adams et al. 2004).



**Table 1.2:** Compilation of thylakoid protein response to winter conditions. Arrows indicate if the protein amount decreases or increases in winter in reference to summer. Modified from (Verhoeven 2014)

Reference	Species	PSII		Other	
		CC (D1)	Antenna (Lhcb)	PsbS	Elip
Ottander et al. 1995	<i>Pinus sylvestris</i>	↓	↓	↑	
Ensminger et al. 2004	<i>Pinus sylvestris</i>	↓	=	↓	↑
Zarter et al. 2006	<i>Arctostaphylos uva-ursi</i>	↓		↑	↑
Verhoeven et al. 2009	<i>Abies balsamea</i>	↓	=	=	
Verhoeven et al. 2009	<i>Pinus strobus</i>	↓	↓	↓	
Savitch et al. 2002	<i>Pinus concorta</i>	↓	↓	↑	
Savitch et al. 2002	<i>Triticum aestivum</i>	=	=	=	
Busch et al. 2007	<i>Pinus banksiana</i>	↓	=	↓	
Busch et al. 2008	<i>Pinus banksiana</i>	↓	↑		
Savitch et al. 2010	<i>Pinus concorta</i>	↓	↑	↑	↑
Ebbert et al. 2005	<i>Pseudotsuga menziesii</i>	=		↑	

### 1.6.5 TRANSCRIPTOMICS OF COLD ACCLIMATION AND FROST TOLERANCE

The majority of the previous changes induced by cold, including physiological, biochemical and molecular alterations, have a genetic basis (Houde et al. 1992; Crosatti et al. 1996). Until now, the research on transcriptional modulation during CA has been based on *Arabidopsis*, some cold tolerant crops (Jaglo-Ottosen et al. 1998; Thomashow 1999), some conifers (Holliday et al. 2008; Dauwe et al. 2012) and broadleaf trees (Druart et al. 2007; Ruttink et al. 2007). The results show that, generally, genes involved in signaling, regulation of transcription, cellular transport, cell membrane and genes with the role of protection during freezing-related stress are up-regulated during winter while genes involved in metabolism, respiration and photosynthesis are down-regulated (Svensson et al. 2006; Winfield et al. 2010).



These expression changes are induced by LT or lengthening of nights in autumn or in a CA period (Dauwe et al. 2012).

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## 1.7 PLANTS AND LIGHT... LOVE-HATE STORY: MECHANISMS OF PHOTOPROTECTION

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Photosynthetic organisms have to cope with the lethal effects of light excess, but at the same time the light is necessary for their life. Thus, among the evolution, plants have developed several mechanisms, which allow them to achieve an efficient, and safe use of light, and to simultaneously protect photosynthetic tissues from the damaging effects of light excess. These mechanisms can be grouped in four types: (i) the avoidance of light absorption, (ii) the dissipation of energy absorbed in excess, (iii) the scavenging of highly oxidative molecules and, (iv) the repair of damage.

### 1.7.1 HOW PLANTS REDUCE THE ABSORPTION OF LIGHT?

In order to reduce the absorption of light under stress, plants are able to increase the amount of **waxes** in the adaxial leaf surface. Epicuticular waxes act as reflective compounds (Shepherd and Griffiths 2006) decreasing light penetration towards photosynthetic layers.

Other method to decrease light absorption by the leaf mesophyll is the **movement of leaves**. Under an excess of irradiance, leaves can orientate parallel to sun rays reducing interception (Kao and Forseth 1991). This response is known as paraheliotropism. Also **chloroplast** can change their position inside the cell to increase or reduce the light energy capture (Suetsugu and Wada 2007; Wada 2013). When there is an excess of light, chloroplast form piles gathering at cell walls parallel to the direction of the light to shade each other (Tholen et al. 2008).





**Leaf reddening** in winter is also a photoprotection mechanism used by a huge number of evergreen species. In angiosperms, anthocyanins are the most common pigments reported in this process while in gymnosperms red carotenoids are. Because of the vacuolar location of anthocyanins, chloroplasts of anthocyanic cells generally remain intact and functional throughout the winter (Hacker and Neuner 2006). By contrast, synthesis of red carotenoids corresponds with conversion of chloroplasts to chromoplasts which loss their photosynthetic capacity. Hence, only the abaxial cells with functional chloroplast persist active. Red pigments are thought to alleviate these stress factors by intercepting green sunlight and/or neutralizing ROS directly as antioxidants (Han et al. 2004).

#### 1.7.2 WHEN AN EXCESS OF LIGHT IS ABSORBED...

##### HOW DO PLANTS DIMINISH THE LIGH EXCESS DAMAGE?

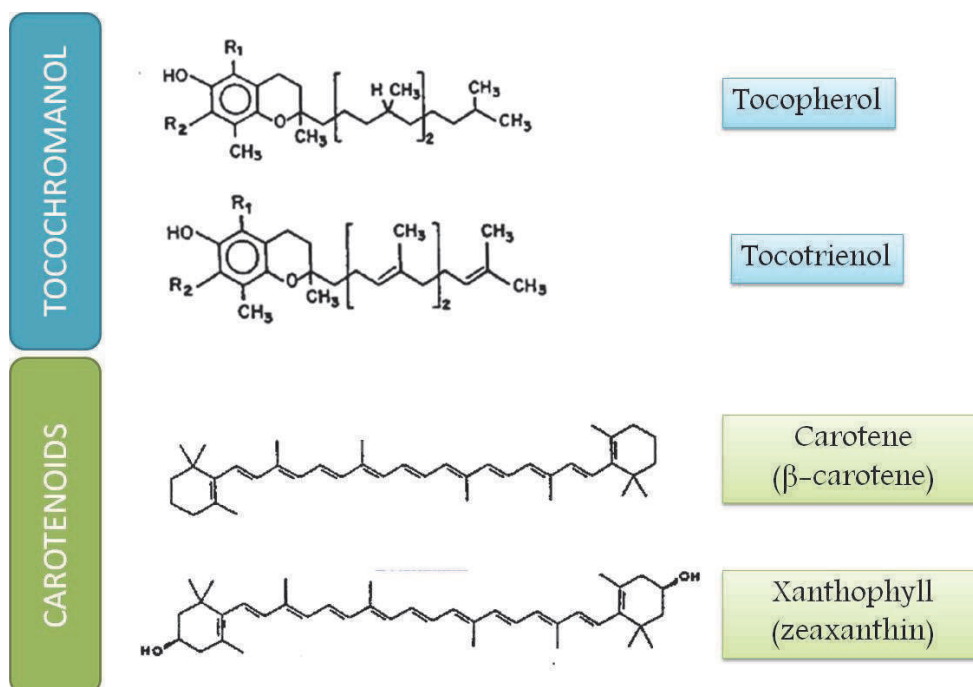
When the physical barriers mentioned before (Section 1.7.1) are not enough prevention for avoiding an excess of light absorption, plants face the challenge of dissipating the excess of energy, that otherwise will trigger the generation of oxidative damage (Mittler et al. 2004; Foyer and Noctor 2005). Four main strategies are employed to prevent photo-oxidations: (i) upregulation of the metabolic sinks, (ii) deactivation of ROS by the antioxidant system, (iii) thermal dissipation of the excess of energy and the (iv) repair of oxidative damage.

#### **A) Antioxidant activity**

Very different stresses induce the formation of ROS. In fact, photosynthesis itself is the main source of ROS in plant tissues. Thus, even under optimal conditions,  $H_2O_2$  is produced in chloroplasts as a product of photosynthesis (Foyer and Noctor 2003). So, the balance between ROS production and ROS scavenging by **antioxidans** must be constantly and strictly controlled in chloroplasts. There are two types of antioxidants: enzymatic and



non-enzymatic molecules. They are traditionally classified, depending on their polarity, on: hydrophilic and lipophilic. In this work we will focus on lipophilic non-enzymatic antioxidants, particularly in **tocochromanols** and **carotenoids**.



**Fig. 1.5.** Structure of lipophilic non-enzymatic antioxidants.

### TOCOCRHOMANOLS

Tocopherols and tocotrienols are collectively known as tocochromanols. They are formed by a chromanol head group and a phenyl side chain in plastids. Tocopherols differ from tocotrienols in the degree of saturation of their hydrophobic tails (Fig. 1.5). Both act as antioxidants in thylakoid membranes, where they scavenge ROS and lipid radicals, avoiding the auto-oxidation and taking part in membrane stabilization (Holländer-Czytko et al. 2005). Tocopherols also play a key role as antioxidants, scavenging  $^1\text{O}_2$  (Munné-Bosch and Alegre 2010). In photosynthetic organisms there are four tocopherol isoforms:  $\alpha$ ,  $\gamma$ ,  $\beta$ ,  $\delta$ , being  $\alpha$ -toc the most abundant. It has been demonstrated that oxidative stress activates the expression of genes responsible for the synthesis of tocopherols in higher plants (Gang et al. 2007).



**$\alpha$ -toc** is the isoform with the higher antioxidant activity due to the presence of three methyl groups in its molecular structure (Kamal-Eldin and Appelqvist 1996). It is synthesized from the less abundant form  $\gamma$ -tocopherol. A single molecule can scavenge up to 120 molecules of  $^1\text{O}_2$  (Munné-Bosch 2005). Apart from its antioxidant activity,  $\alpha$ -toc regulates thylakoid rigidity (Munné-Bosch and Alegre 2010) and it may also affect to intracellular signalling. Several works have shown that  $\alpha$ -toc is induced under stress conditions such as HL (Niinemets et al. 2003) and/or LT (Hodges et al. 1997).  $\alpha$ -toc also shares a photoprotective function with V-cycle that leads to some degree of inter-compensation between both mechanisms. It means that in *npq1 Arabidopsis* mutants, in which V-cycle is absent, the absence of functional xanthophylls can be compensated by the accumulation of  $\alpha$ -toc and *vice versa* in *vt1* mutants that are tocopherol deficient (Havaux et al. 2000, 2005, 2007).

#### CAROTENOIDS: CAROTENES AND XANTOPHYLLS

**Carotenoids** are terpenoids with C40 linear backbone. They are divided in two groups, depending if they have oxygen or not in their structure (Fig. 1.5). They are lipid soluble antioxidants and also play a multitude functions in metabolism. Their main functions related to photosynthesis are:

- **Accessory light harvesting role:** Carotenoids absorb at wavelengths between 400 and 550 nm and transfer the energy to Chl for photosynthesis (Siefermann-Harms 1987).
- **Antioxidant function:** Carotenoids protect photosynthetic apparatus by quenching  $^3\text{Chl}$ ,  $^1\text{O}_2$  and other harmful free radicals which are naturally formed during photosynthesis. The carotenoids involved in this protective role, are located in the lipid phase of the membrane or bound to thylakoid proteins (Collins 2001).



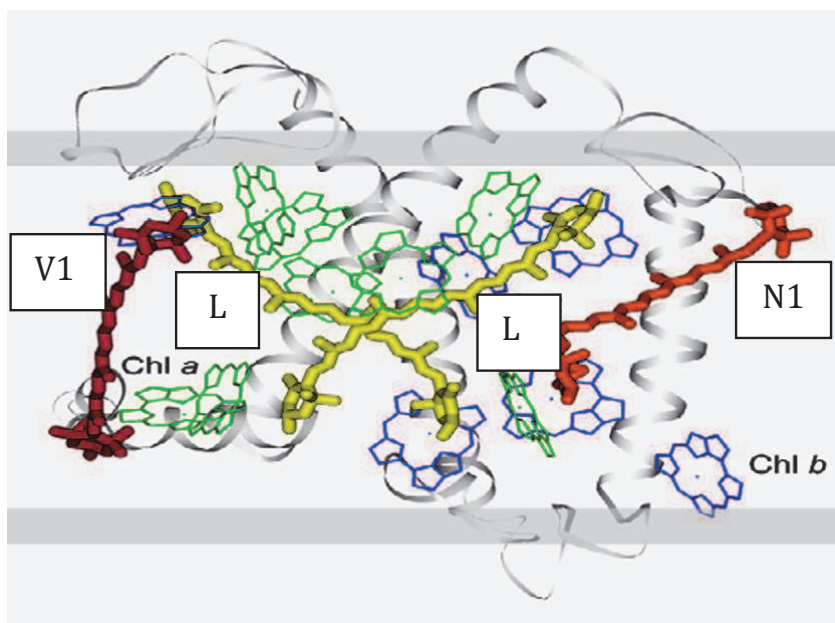
- **Thermal energy dissipation:** Carotenoids are implied in regulation of the dissipation as heat of the energy excess absorbed by Chls (for more detail see Introduction Section 1.8)
- **Structural role:** several xanthophylls and  $\beta$ -car serve as constituents of functional multiprotein complexes such PSI, PSII and Citochrome *b<sub>6</sub>f* as well as LHCs. They are essential for their assembly and stability. They also contribute to the thylakoid membrane stabilization through the generation of carotene-lipid-protein interactions (Niyogi et al. 2001).

**Xanthophylls** are the oxygenated derivatives of carotenoids (Fig. 1.5). Five xanthophylls (Violaxanthin (V), anteraxanthin (A), zeaxanthin (Z), neoxanthin (Neo), lutein (L)) are bound to Chl a/b binding antenna proteins of both photosystems (Lhca 1-4 and Lhcb 4-6) in higher plants, while only a fraction of the VAZ pool is inserted in the lipid phase of the membrane (Havaux 1998). In general, each carotenoid presents a specific binding site in the PSs. Nevertheless, it was elucidated using carotenoid mutants that when one carotenoid is absent, other specific carotenoid can replace it (Niyogi et al. 1998). Nevertheless, in these cases, the photoprotective capacity differs from the wild-type. These carotenoids bind at specific sites of Lhc proteins known as N1, L1, L2 and V1 (for more details see Fig. 1.6 and Table 1.3).



**Table 1.3:** Binding sites of xanthophylls in Lhc proteins. Modified from Jahns et al. (2009).

Protein	Binding site
<b>LHCII</b>	V1
<b>Lhcb4</b>	L2 (N1)
<b>Lhcb5</b>	L2 (V1)
<b>Lhcb6</b>	L2
<b>Lhca1</b>	L2 (N1)
<b>Lhca2</b>	L2
<b>Lhca3</b>	V1
<b>Lhca4</b>	L2

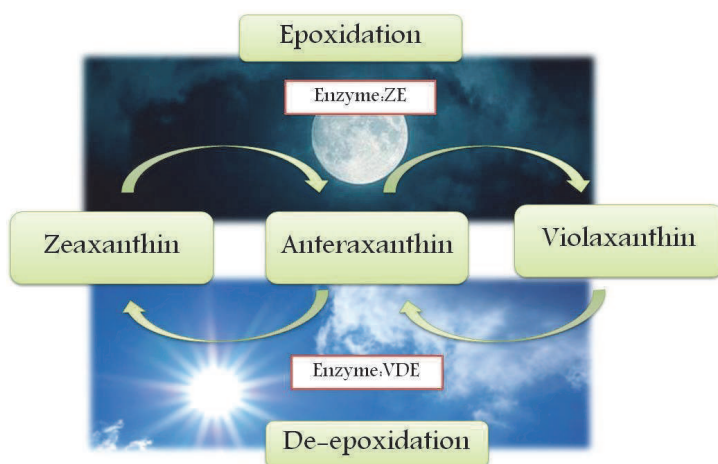


**Fig. 1.6.** Structure of LHCII. Modified from Liu et al. (2004), Jahns and Holzwarth (2012). The protein backbone with the three transmembrane helices (gray) is shown in the background, the pigments are shown in color. Blue represents Chl a and green is Chl b. Four bound xanthophylls were identified in the structure: Two central luteins (yellow, binding sites L1 and L2), a xanthophyll cycle pigment (red, V1 site) and neoxanthin (orange, N1 site).



## B) Violaxanthin cycle

Three different molecules are involved in violaxanthin cycle (V-cycle): Z, A and V. It consists on a forward reaction in which the di-epoxy xanthophyll V is de-epoxidized to the epoxy-free xanthophyll Z. The intermediate between V and Z is the xanthophyll anteraxanthin (A) which contains only one epoxy group. The transformation from V to Z usually occurs under light conditions and it is catalyzed by the enzyme V de-epoxidase (VDE) that is activated by the light-driven acidification of the lumenal space. The contrary reaction is catalysed in darkness by the Z-epoxidase (ZE) when proton gradient is dissipated. This cycle is a paramount importance due to Z plays an essential role in thermal dissipation (see Introduction section 1.8.1) (Niyogi et al. 1998) and as an antioxidant in the lipid phase of the thylakoid membrane (Havaux 1998) (Fig. 1.7).



**Fig. 1.7.** Scheme of the reactions of xanthophyll cycle (V-cycle) where ZE is the zeaxanthin epoxidase and VDE is the violaxanthin de-epoxidase.

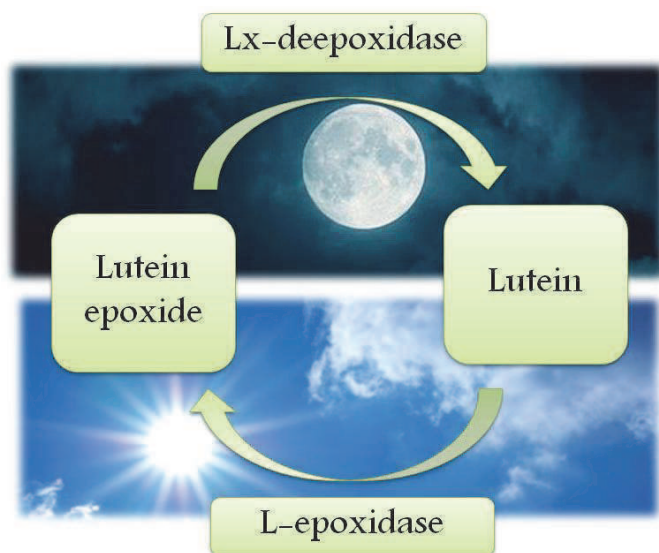
## C) Lutein epoxide cycle

Whereas V-cycle is widespread in all photosynthetic organisms belonging to green lineage of the plant kingdom, lutein epoxide cycle (Lx-cycle) is restricted to some taxonomic groups (Esteban et al. 2009). Lx-cycle consists on the transformation of Lx to L under illumination by Lx-deepoxidase (García-Plazaola et al. 2007). In a lot of species, the reversion from L to Lx, do not occur



even under darkness (Truncated Lx-cycle) (García-Plazaola et al. 2003; Esteban et al. 2007, 2008). Nevertheless, in other species as parasites and tropical and subtropical trees, the recovery of Lx-pool occurs, although at very slow rates (Bungard et al. 1999; Matsubara et al. 2001, 2009; Esteban et al. 2010).

Together with V-cycle, Lx-cycle is also a paramount importance in thermal dissipation for example in response to chilling stress (Matsubara et al. 2002; García-Plazaola et al. 2004). In fact, it has been demonstrated a relationship between L formation from Lx and the velocity of NPQ development (García-Plazaola et al. 2003). Hence, both cycles act in parallel complementing to each other in the regulation of NPQ.



**Fig. 1.8.** Scheme of the reactions of Lutein epoxide cycle (Lx-cycle).

#### **D) Metabolic dissipation.**

Despite the operation of the photoprotective mechanisms described before, alternative sinks for the electron excess, such as metabolic dissipation are needed to avoid PI, are sometimes required to dissipate the excess of energy:

**Photochemical mechanisms (qP):** this term include all the processes which imply the electron transport such as photosynthesis, carbon and nitrate assimilation, photorespiration, chlororespiration, Halliwell-Asada pathway and





cyclic electron transport. The objective of this routes under light excess is to consume the energy excess to avoid damage (Huner et al. 1993; Öquist and Huner 2003).

Carbon assimilation: photosynthetic reduction of CO<sub>2</sub> to form sugars. It is the main energy sink in plastids. Under particular stress conditions (e.g.: LT) it is up-regulated in some species.

Nitrate assimilation: is the reduction of nitrate (NO<sub>3</sub><sup>-</sup>) to ammonia (NH<sub>4</sub><sup>+</sup>). Reducing power (NA(D)PH) is consumed in plastids (Tischner 2000).

Photorespiration: utilizes NADPH and ATP to oxygenate the ribulose-1,5-biphosphate. These reactions occur in chloroplasts, mitochondria and peroxisomes to recover fixed carbon (Osmond and Grace 1995).

Chlororespiration: removes electrons from the PQ by an alternative oxidase. This oxidase activity is critical for an efficient carotenoid biosynthesis. Under intense light this oxidase allows carotenoid biosynthesis (Bennoun 2002).

Water-water cycle / Halliwell Asada pathway: uses the electrons generated from the oxidation of water at PSII to reduce O<sub>2</sub> to water at PSI. Univalent electron transfer to O<sub>2</sub> results in the generation of O<sub>2</sub><sup>-</sup>, which can subsequently be converted to H<sub>2</sub>O<sub>2</sub> by superoxide dismutase. Ascorbate peroxidase activity in the chloroplast is responsible for the reduction of H<sub>2</sub>O<sub>2</sub> to water (Asada 1992, 1999).

Cyclic electron transport: Ferredoxin (Fd) acts as an electron carrier when it is reduced. When NADP<sup>+</sup> is not available for Fd-NADP-reductase, reduced Fd returns the electrons to cyt *b<sub>6</sub>f*, generating a cyclic electron transport around PSI. The operation of this flow provides a mechanism whereby ATP production can be increased relative to NADPH. It prevents the overreduction of the acceptor side of PSI (Munekage et al. 2008). This is important because O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> are generated on the acceptor side of PSI. Cyclic electron flow may





also help to limit the  $^1\text{O}_2$  production at PSII because it enhances protonation in the lumen, which triggers protective NPQ mechanisms (Rumeau et al. 2007).

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## 1.8 THERMAL ENERGY DISSIPATION

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One of the most important strategies that photosynthetic organisms employ to protect their photosynthetic machinery under conditions of over-reduction of PSII is the safe dissipation of the excess of excitation energy absorbed by Chl *a* as heat, the process of thermal dissipation (Niyogi et al. 1998). The operation of this mechanism provokes the quenching of Chl fluorescence that can be easily measured with a fluorometer (Krause and Jahns 2004). This quenching is expressed by the so-called non-photochemical quenching (NPQ), defined by Bilger and Björkman (1990) as:

$$\text{Eq. 1: NPQ} = (F_m - F_m') / F_m'$$

Nowadays, in order to simplify, NPQ is used as a synonym of thermal dissipation by most authors. Nevertheless, this is not totally true due to the existence of other factors which affect also fluorescence quenching and contribute to NPQ, but *sensu stricto* are not mechanisms of thermal dissipation. This is the case of the variations of Chl content, the chloroplast movements or the state transitions (qT) that allow energy redistribution between PSII and PSI. For simplicity, in this thesis the term NPQ will be used hereafter as a synonym of thermal energy dissipation (García-Plazaola et al. 2012; Cazzaniga et al. 2013).

### 1.8.1 FACTORS IMPLICATED IN NPQ

It is considered that NPQ is a sum of processes which requires the participation of different interactive factors, whose physiological role cannot be considered separately from the others (Goss and Lepetit 2015) (Fig. 1.10).



### A) V-cycle:

The first correlative evidence for an involvement of V-cycle in NPQ emerged during the late 80's by Demmig et al. (1987). These authors found that three species (*Populus balsamifera*, *Hedera helix* and *Monstera deliciosa*) exposed to HL and air containing 2% O<sub>2</sub>, 98% N<sub>2</sub> and 0% CO<sub>2</sub> formed a huge amount of Z. In this experiment they concluded that the operation of the V-cycle plays a specific role in the protection of photochemical apparatus against HL. For more information about V-cycle see Section 1.7.2B.

Now, Z implication in NPQ of higher plants has been demonstrated by the absence of NPQ in (i) plants incubated in the presence of inhibitors of VDE such as dithiothreitol (DTT), which inhibits Z synthesis (Yamamoto and Kamite 1972), and (ii) in *npq1* mutants, which lack VDE (Niyogi et al. 1998). When the photosynthetic apparatus absorbs light energy in excess, the de-epoxidation of V to Z is proportional to such excess, enhancing the dissipation of energy in the antenna system of the PSII (Horton et al. 2008). By contrast, it must be highlighted that in green algae, not always Z synthesis is coupled to NPQ. For instance, *Clamydomonas reinhardtii* does not present de-epoxidase gene (Grossman et al. 2010).

### B) Importance of grana and LHCII structure

As well as V-cycle, NPQ takes place in grana membranes of higher plants (Dekker and Boekema 2005). When there are changes in the stacking of membranes, V-cycle continues functioning at the same rates, but NPQ values differ considerably. A plausible explanation is that high NPQ is only detected in stacked grana. In fact, Goss et al. (2007) pointed out that NPQ depends on the organization of LHCII in stacked grana regions. What is more, Horton et al. (2008) assured that NPQ is dependent, not only in one specific component (LHCII), but on the assembly of PSII-LHCII super-complexes in the grana membranes (Li et al. 2000). The functional quenching site is formed by the



interaction between the major LHCII and the minor PSII antenna proteins Lhcb4-6. Low pH and the conversion of V to Z are involved in the aggregation of LHCII (Phillip et al. 1996; Ruban et al. 1997).

### **C) PsbS protein**

With the study of *npq4* mutants, which lack PsbS protein, under HL stress conditions, it was elucidated that PsbS protein is essential for NPQ. Nevertheless, there are contradicting beliefs concerning the exact function of PsbS in NPQ process. Some studies confirm that PsbS is an “antenna organizer”, which facilitates the structural changes that are needed in LHCII to transform from a light-harvesting to the heat dissipating state (Teardo et al. 2007). This theory is supported by the mobility of this protein in the thylakoid membrane. Hence, it can be a union link between PSII, CC and LHCII.

Other point of view proposed by Demmig-Adams and Adams (2006) hypothesizes that PsbS is the link between the  $\Delta\text{pH}$  changes, and the de-epoxidation activity of V-cycle. In fact PsbS possesses functional groups suitable for early protonation upon increases in  $\Delta\text{pH}$ , when light absorption begins to exceed the utilization of ATP.

In spite of the importance of PsbS, NPQ also can occur in the absence of this protein, but only at very low pH in the luminal side of thylakoid resulting on a slower NPQ activation (Johnson and Ruban 2011).

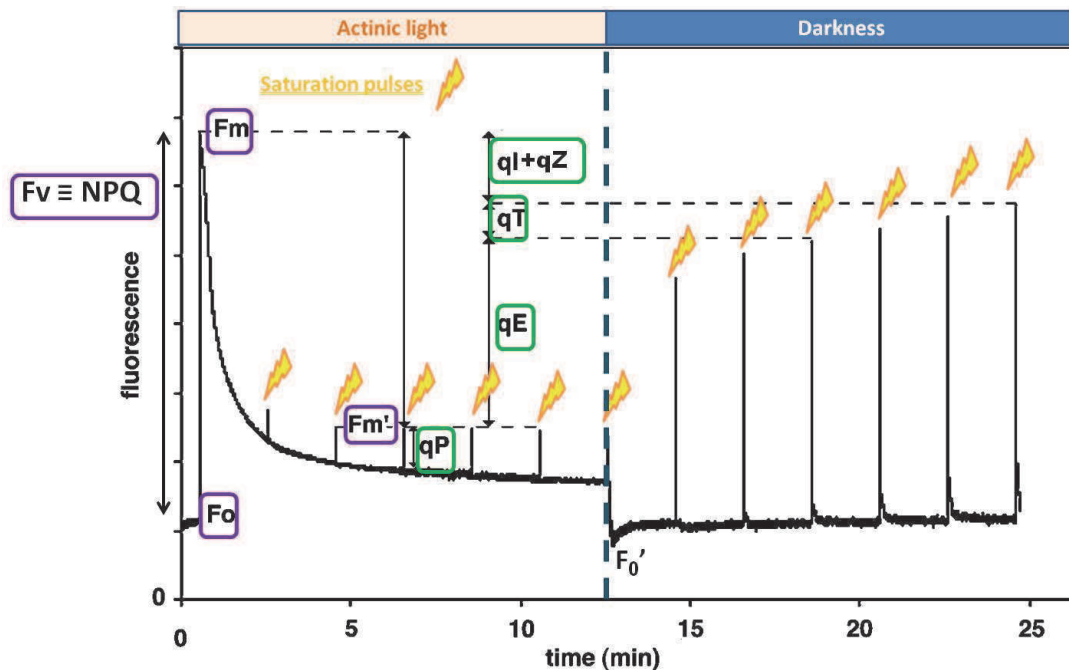
### **D) Proton gradient across the thylakoid membrane:**

The magnitude of proton gradient ( $\Delta\text{pH}$ ) across the thylakoid membrane increases with irradiance intensity. Hence,  $\Delta\text{pH}$  indicates the state of the photosynthetic apparatus. This state influences in the rest of factors affecting NPQ.



## 1.8.2 NPQ COMPONENTS

NPQ consists of different components (Quick and Stitt 1989). The proportion of each component in total NPQ depends on the level of stress and on the species (Fig. 1.9 and 1.10).



**Fig 1.9.** Chl fluorescence quenching analysis of PSII in a dark adapted leaf. Upon continuous illumination a combination of qP and NPQ lowers the fluorescence yield. The four components of NPQ: qE, qT, qZ and qI are shown. After switching off the light, recovery of  $F_m'$  reflects relaxation of the qE component of NPQ. Modified by Müller et al. (2001).

### A) High energy state quenching (qE) or flexible thermal energy dissipation

qE is the faster component of NPQ. It is characterized by its reversibility under darkness or LL conditions. The induction of qE is generated in 9 to 100 seconds and requires the presence of  $\Delta pH$  and PsbS protein (Li et al. 2000). In presence of Z, qE is fully expressed (Li et al. 2002; Johnson et al. 2009). When Z is absent, L can take its place as a quencher, but natural xanthophyll



composition and Lhcb protein content within the PSII antenna are necessary for a fully functional NPQ (Li et al. 2000).

Although inherent differences exist between species, apparently, all plants present qE under moderate stress or in response to environmentally light fluctuations under favorable conditions. Short-lived, fast growing crops have lower maximal capacity for qE than long-lived slow growing tropical evergreens. This is logic because fast-growing crops are able to invest a much greater fraction of absorbed sunlight in photosynthesis (and growth), than slow growing species (Demmig-Adams and Adams 2006).

The exact biophysical mechanism of qE generation has not been well characterized and several models have been proposed: electron transfer from Z to Chl (Holt et al. 2005), energy transfer from Chl to L (Ruban et al. 2007) and Chl-Chl charge transfer (Miloslavina et al. 2008).

Holzwarth et al. (2009) and Jahns and Holzwarth (2012) proposed a qE model with two different quenching sites: Q1 and Q2. According to them, Q1 is located in major LHCII that during HL are detached from the PSII core complex. Q1 requires the interaction with a protonated PsbS protein. It reflects the fast part of qE. On the other hand, Q2 is located in minor LHCII proteins (Lhcb4-6) which stay attached to the PSII CC. The qE induction is slower than for Q1, (10-15 minutes approximately), which corresponds with the kinetics of the conversion from V to Z. Hence, Q2 strongly depends on V-cycle.

## **B) Fluorescence quenching of state transition (qT)**

State transitions are the transference of energy from PSII to PSI in order to allow the distribution of energy between both PS (Bellafiore et al. 2005). This process implies the phosphorylation and the lateral migration of major light harvesting complex (LHCII) from PSII to PSI. This movement diminishes the energy in PSII, reducing its emission of fluorescence (Müller et al. 2001). This



process occurs only under LL conditions (Walters and Horton 1991) and it does not contribute significantly to NPQ at saturating light intensities in higher plants (Müller et al. 2001; Nilkens et al. 2010). Although it is included in NPQ, in the sense that it implies a decrease on fluorescence yield, it does not involve any thermal energy dissipation. The time for turning on and off is 10 min.

### **C) Zeaxanthin dependent component (qZ)**

It is a slowly developing (10-30 min) and slowly relaxing (10-60 min) component of NPQ (Nilkens et al. 2010). It depends on synthesis and accumulation of Z. Due to its dependence on Z, the formation and the relaxation of qZ are indirectly dependent on  $\Delta\text{pH}$ . However, once established, qZ maintains the heat dissipation after long periods of HL stress, independently of  $\Delta\text{pH}$  (Jahns and Holzwarth 2012).

Some authors consider qZ as part of qI since in physiological terms, this mechanism can be understood as a kind of “memory state” under HL, allowing the reactivation of quenching under re-illumination (Jahns and Mische 1996; Thiele et al. 1996). Nevertheless, other studies affirm that qZ is independent of qI due to it is fully development after 30 minutes of pre-illumination at moderate light intensities, being consequently unrelated with photoinhibitory processes (Betterle et al. 2009).

### **D) Chloroplast movements component (qM)**

The triggering of qM requires uniquely the formation of a transmembrane proton gradient. qM presents a fluorescence decay rate intermediate between qE and qI (photoinhibitory quenching) (20-35min). The relaxation kinetics for nearly 50% of qM amplitude is based on the mechanisms of chloroplast photo-relocation (in response to light, chloroplasts move parallel to incident light) (Dall’Osto et al. 2014).

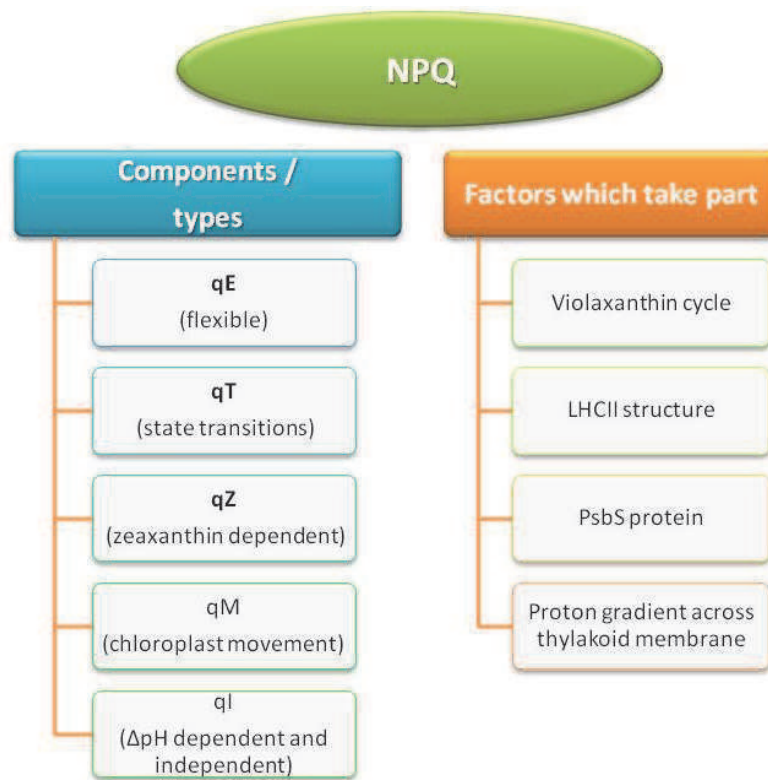


### **E) Photoinhibitory quenching (qI) or sustained thermal dissipation**

This type of quenching is the slowest in terms of recovery. It needs more than one hour to recover. In fact, this dissipation process does not relax upon darkening of leaves. To maintain in darkness this mode of sustained thermal dissipation there are different mechanisms:

**qI  $\Delta$ pH-dependent:** Some species, when are subjected to LT, maintain the  $\Delta$ pH in darkness. Under these conditions, ATP-ase acidifies the lumen though the ATP consumption. As this mechanism is  $\Delta$ pH-dependent, it is easily reversible if temperature increases (Verhoeven et al. 1998; Demmig-Adams and Adams 2006).

**qI  $\Delta$ pH-independent:** Some overwintering evergreens maintain this type of thermal dissipation that is characterized by PSII core degradation and Z accumulation. V-cycle is arrested in a photoprotective form and it is not necessary the generation of a pH gradient for its maintenance (Demmig-Adams et al. 2006). In fact PSII CC proteins may be degraded so plants are not able to build-up the  $\Delta$ pH (Li et al. 2000). This type of qI is typical under more stressful conditions than the  $\Delta$ pH dependent qI, qZ and qE and usually is maintained along the entire unfavorable season as a mechanisms of photoprotection (Demmig-Adams and Adams 2006).



**Fig. 1.10** Schematic representation of the components of non photochemical quenching and the factors implicated in its formation.





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## 1.9 PHOTOINHIBITION AS A MECHANISM OF PHOTOPROTECTION

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Photoinhibition (PI) is a drastic reduction of photosynthesis when there is an imbalance between the light absorbed by photosynthetic apparatus and the light which is used by photosynthetic machinery (Osmond and Grace 1995).

Nowadays, PI is a term full of complexity. There are different standpoints depending on the authors. Some authors consider PI as a negative **process related to photodamage**. For them, PI is a downregulation of photosynthesis and growth determined by the balance between the rate of degradation and repair of PSII (Ögren and Sjöström 1990; Takahashi and Murata 2008; Reynolds et al. 2012). This point of view is based on the oxidation and degradation processes that occur in photosynthetic apparatus in response to exposure to excess light. Recent investigations suggest that exposure to environmental stresses, such as salt, cold, moderate heat and oxidative stress, do not affect photodamage but inhibit the repair of PSII through suppression of the synthesis of PSII proteins (Takahashi and Murata 2008).

Other theory, based mainly on ecophysiological studies considers photoinhibitory responses as **photoprotective mechanisms** (Adams and Demmig-Adams 1995; Gilmore and Ball 2000; Adams et al. 2001; Savitch et al. 2002; Huner et al. 2003; Adams et al. 2004, 2006; Ensminger et al. 2004; Demmig-Adams and Adams 2006). This is based largely on the ubiquitous involvement of xanthophylls on sustained thermal dissipation. When there is an overexcitation of PSII, plants enter in a controlled state of PI through the activation of several mechanisms, such as (i) the upregulation of antioxidant metabolism, (ii) the repair of oxidative damage (Noctor and Foyer 1998; Mullineaux and Rausch 2005) and (iii) thermal dissipation (Siefermann-Harms 1987), among others. In this theory, PI avoids PSII photodamage and that is the



reason why it is considered as a photoprotection mechanisms (Adams et al. 1995).

It has been also recently proposed that the term PI can be placed in the **context of whole-plant source-sink regulation** of photosynthesis. PI may represent downregulation of the photosynthesis in response to excess light when the production of sugars is bigger than their consumption by the plant (Adams et al. 2013).

**Different approximations to photoinhibition:**

**Photoinhibition → photodamage**

**Photoinhibition → photoprotection**

**Photoinhibition → whole plant source-sink regulation**

*In this work, photoinhibition (PI) is understood as an adaptive photoprotective process not involving uncontrolled damage to the photosynthetic apparatus. When PI is generated by a combination of low temperatures and high light (winter conditions), it is called winter photoinhibition (WPI).*

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## 1.10 HOW DO COLD LOVERS SOLVE THE COMBINATION OF COLD AND LIGHT STRESS?

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Plants in cold climates are frequently exposed to sub-zero temperatures. Besides, when LT are combined with HL, the consequences can be even worse and faster. To survive under these stressful conditions, plants present a wide range of molecular and physiological adaptations as well as different mechanisms of photoprotection at different levels. Apart from those mechanisms described previously in Section 1.7.2, they use other specific mechanisms to prevent or reduce cold-induced damage.



### 1.10.1 LEAF REDDENING

As it was discussed in the recent review about winter leaf reddening published by Hughes (2011), the adaptive function of winter leaf color changes are still a matter of debate. In winter leaves red color is provided by the accumulation of anthocyanins and some unusual red carotenoids. These pigments can act as sunlight attenuators and as antioxidant protectors (Gould 2004). Red pigments are thought to alleviate light stress in winter by intercepting green sunlight and/or by deactivating ROS directly (Hughes et al. 2005). The role of this antioxidant protectors acquires a bigger importance in winter, since enzymatic antioxidant processes are limited by LT (Bienert et al. 2006).

Apart from the light-screening effect increased absorption of green light by red pigments might also alleviate LT stress by increasing leaf temperature during the day (Lee et al. 2003). At the same time, they can act as an ‘energy escape valve’ by consuming ATP and NADPH during their biosynthesis (Hernández and Van Breusegem 2010).

### 1.10.2 SUGARS CONTENT

Products of photosynthesis, typically sugars, synthesized by leaves, are exported to the plant’s sinks for growth, storage, and/or respiration maintenance. When the rate of sugar production in source leaves exceeds the rate of export (often as a result of insufficient sink activity), sugars are accumulated in source leaves. In a feedback response, photosynthetic rates decrease. A wide range of overwintering evergreens, present this strategy (Kozlowski and Pallardy 2002; Welling and Palva 2006), that can be viewed in a context of increased freezing tolerance as well as in a context of source-sink imbalances (Demmig-Adams and Adams 2014).

The role of soluble sugars on LT acclimation is not completely understood, however, several functions have been attributed to these compounds: (i) to act as compatible osmolytes or cryoprotectants (ii) to



scavenge ROS (iii) to act as signaling molecules (Janská et al. 2010; Theocharis et al. 2012) (iv) to improve the water up-take (Huber and Huber 1992), (v) to stabilize cellular membranes (Livingston et al. 2006) and (vi) to act as reserves for plant survival and spring growth.

PI affects to the proportion of different sugar forms. The general trend is the accumulation of sucrose during LT photosynthesis in most species that results in a high disaccharide (sucrose) to monosaccharide (glucose and fructose) ratio (Grace 1987). Variation in the proportion of different sugars can be attributed to changes in invertases activity, involving hydrolyzation of sucrose into monosaccharides. Apparently, ice formation in tissues activates various hydrolytic enzymes including invertases, which are also considered as an important step in plant adaptation to stress.

Sugar accumulation can be also an active process resulting from the activation of the gluconeogenesis pathway (Livingston and Henson 1998). Not only sucrose, but also raffinose is accumulated in alfafa and pea under LT and it was determined that this accumulation is involved in freezing tolerance and even involved in the stabilization of PSII during cold exposure (Grimaud et al. 2013).

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### 1.11 WINTER PHOTOINHIBITION: HOW DEEP ARE PLANTS SLEEPING IN WINTER?

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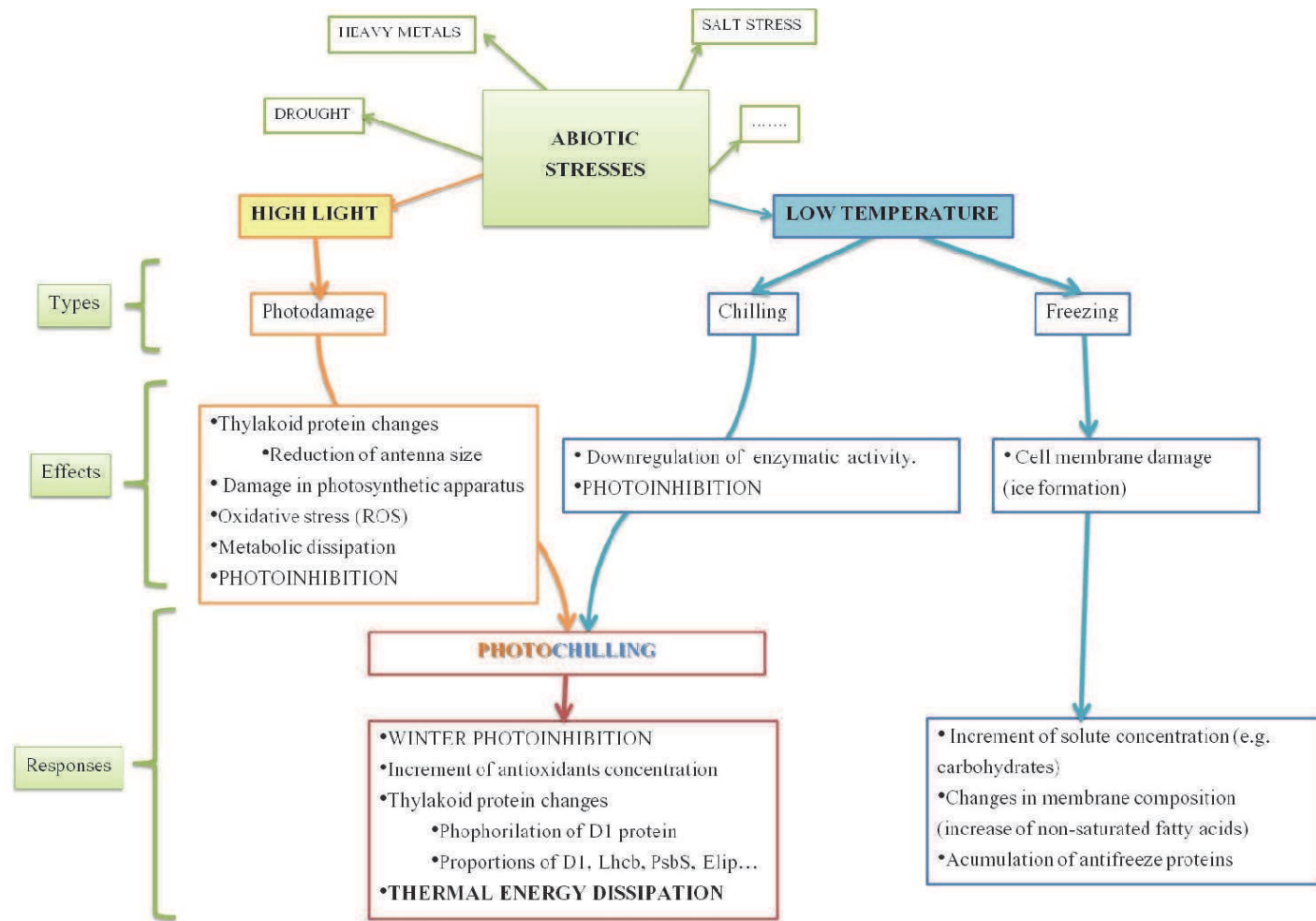
During winter, evergreen species present a special type of PI known as winter photoinhibition (WPI) (Nishiyama et al. 2006). Usually, WPI is more pronounced than PI due to the combination of HL with LT. Such stress combination drastically limits the enzymatic activity and consequently CO<sub>2</sub> fixation activating WPI even under relatively LL (Takahashi and Murata 2008). Depending on the rate of recovery, two different types of WPI can be distinguished:

**Chronic photoinhibition** (WPI<sub>>12h</sub>) that is characterized by an extremely slow rate of recovery even after long incubation under optimal



conditions. This process is apparently independent of  $\Delta pH$  (Verhoeven et al. 1998; Gilmore and Ball 2000; Demmig-Adams et al. 2006) and PsbS protein (Öquist and Huner 2003; Adams et al. 2004), but most studies show that  $WPI_{>12h}$  requires the presence of Z. Thus, in species chronically photoinhibited, V-cycle is maintained in a photoprotective state, which means that Z is retained and persistently engaged in thermal dissipation. Under these conditions, the photochemical system is in a dissipative state and consequently, photosynthesis is down-regulated (Adams et al. 1995, 2004).  $WPI_{>12h}$  process is typically present in evergreen woody species living under winter stress.

**Dynamic photoinhibition** ( $WPI_{<12h}$ ), contrasting with  $WPI_{>12h}$ ,  $WPI_{<12h}$  is easily reversible under darkness and optimal conditions in the time lapse of a night (12h). It involves the building of transthylakoid  $\Delta pH$ , the operation of V-cycle, the protonation of PSII proteins and the presence of PsbS protein (Eskling et al. 1997; Gilmore 1997; Müller et al. 2001; Demmig-Adams and Adams 2006).



**Fig 1.11:** Effects of high light stress (photodamage) and low temperature stresses (chilling and freezing) in plants as well as the effects provoked by the combination of both stresses (high light and low temperature), known as photochilling. In the lower part of the figure, the most typical ways of reaction/response to those stresses are numbered.



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## 1.12 CLIMATE CHANGE IS COMING... HOW WILL IT AFFECT TO PLANTS LIVING IN HIGH MOUNTAIN AREAS?

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Predictions from the Intergovernmental Panel on Climate Change (IPCC) indicate that increased levels of CO<sub>2</sub> and other greenhouse gases will likely result in a temperature rise between 1.4°C and 5.8°C in the next century (Schneider 1989). Such warming might result in dramatic changes on ecosystems, due to the alteration of the establishment, survival, and reproduction of plants. At higher levels of organization, atmospheric warming will enhance even the extinction and/or geographic migration of certain species, changing communities composition and affecting the rates of ecosystem processes (Bachelet et al. 2003).

At high latitudes and altitudes, where plants are adapted to survive hard wintry conditions, global warming could cause variations in critical climatic indexes such as snow depth, time of snow appearance and melting, duration of snow cover and mean daily temperatures in snow-free periods (Larcher 2003). As snow provides insulation under episodic freezing temperatures, early snow melt coupled with changes in soil surface temperature, water content and light conditions may alter vegetative growth timing. Hence, the earlier end of dormancy state in the season could induce the premature emergence of reproduction structures, new buds and/or new leaf production, inducing irreversible damages in plants (Loik et al. 2004). On the other hand, physiologically active plants are more vulnerable than dormant plants to a transient LT event. Hence, spring frosts can cause massive damage in plant tissues, reducing functional leaf surface area and productivity (Stuart-Chapin et al. 1991). Therefore, earlier acclimation to warmer temperatures in spring may make plants more susceptible to late frost damage (Loik et al. 2004).



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## 2. Objectives

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## THE NEED OF STUDING WINTER PHOTOINHIBITION

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Until now, the process of winter photoinhibition (WPI) has been characterized in woody species and some crops. Nevertheless, little is known about the consequences of winter stressful conditions in other photosynthetic organisms, such as lichens, bryophytes, terrestrial algae and ferns, which paradoxically dominate in many boreal and alpine ecosystems. Thus, in the present work, the main aim has been to fill this gap of knowledge by studying the responses to winter acclimation in different species representative of the main groups of terrestrial photosynthetic eukaryotes. This central aim has been developed following three different conceptual approaches: data mining (literature compilation), observational (responses under field conditions), and manipulative (responses to artificially imposed conditions). The specific objectives, summarised in Fig. 2.1, were:

***Objective 1: Are there general trends on WPI responses?***

Data mining: A literature compilation, based on the available published studies on WPI was developed to establish general trends in the responses of plants to winter conditions in the field or under laboratory controlled conditions, as well as to diagnose the existence of gaps of knowledge in this topic (Chapter 4).

***Objective 2: What is the extension of WPI in Plant Kingdom?***

Observational and manipulative: In chapter 5, we aimed to determine the presence and magnitude of WPI across a wide representation of Plant Kingdom, including species from all representative functional groups of plants co-occurring in subalpine areas: woody species, herbs, mosses and lichens.

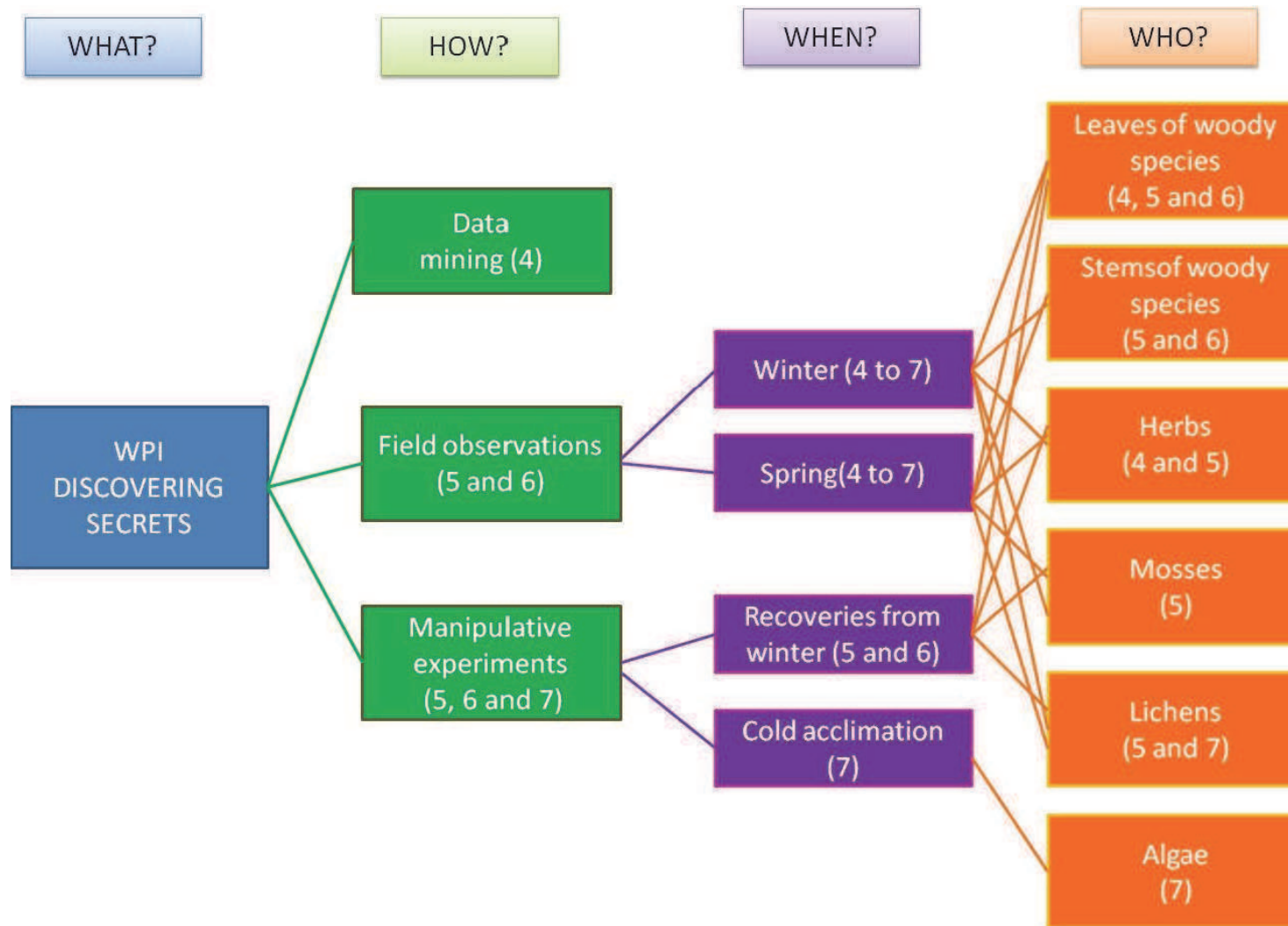


**Objective 3. What is the effect of WPI in green photosynthetic stems in comparison to leaves? Does ageing intensify or reduce winter photoinhibition in green stems?**

Observational and manipulative: We expand the diversity of photosynthetic tissues studies using mistletoe as a model. We verified whether green stems present also WPI and to what extent ageing enhance or reduce the photoprotection under winter conditions (Chapter 6).

**Objective 4. How is the photoprotective response of free-living algae and lichens to photochilling stress? Does lichenization benefit to algae?**

Manipulative: Along the exposition of different species of algae and lichens to photochilling controlled conditions (low temperature and high light), pigments, low molecular weight carbohydrates and energy partitioning were analyzed to characterise their different photoprotective responses (Chapter 7).



**Fig. 2.1.** Scheme summarizing the main objectives of this work. The numbers in brackets represent the chapter where the results, answering the specific question are.



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# 3. Materials and methods

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### 3.1 THE ENORMOUS VARIABILITY OF PLANT KINGDOM AND PECULIARITIES OF MODEL SPECIES

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The huge variability of Plant Kingdom forces scientists to search for model species to really understand the physiological processes in photosynthetic organisms. Model plants are very useful to understand the most common responses under certain conditions but not all the species face up to different stresses in the same way as model plants do. Winter photoinhibition (WPI) is not an exception so we considered that a suitable way to evaluate the extension of WPI in Plant Kingdom is the study of this phenomenon in a huge range of species, belonging to different functional groups. Thus, in the present study, physiological responses under winter conditions in comparison to the same responses observed in spring, have been characterised in 59 different species, including 6 terrestrial green algae, 12 lichens, 5 mosses, 20 herbs and 15 woody plants (See Appendix 1). Among them, here, the most intensively studied species are described in more detail.

#### 3.1.1 GREEN ALGAE

In table 3.1 the algae species used in this work are described. Three of them are able to form lichenic symbiosis and the other three only exist as free-living organisms in terrestrial environments. All of them belong to Chlorophyta division. The origins of all these species are very diverse but all of them come from areas with at least a cold season, indicating they need to be able to withstand periods of LT. For example, there have been included one species isolated from alpine ecosystem. For more detail about the algae phylogenetic position and morphology see Fig. 3.1 and Fig. 3.2 respectively.





**Table 3.1.** Summary of the characteristics of algae species used in this thesis, their taxonomic assignments, habitat and origins. Algal strains (prefix SAG) are available from the culture collections of algae, University of Göttingen (Germany).

Species	Taxonomic assignment	Habitat and characteristics	Origins	Strain number
<i>Apatococcus lobatus</i> (Chodat) J.B.Petersen (AL)	Chlorophyta Trebouxiophyceae, <i>Apatococcus</i> -clade	Bark of <i>Platanus</i> sp. Terrestrial free- living green algae	Basel, Switzerland	SAG 21.45
<i>Asterochloris erici</i> (Ahmadjian) Skaloud et Peksa (AE)	Chlorophyta Trebouxiophyceae, <i>Trebouxia</i> -clade	Soil	Photobiont of lichen <i>Cladonia cristatella</i> . Whitensville village, Massachusetts, United States	SAG 32.85
<i>Elliptochloris bilobata</i> Tschermak-Woess (EB)	Chlorophyta Trebouxiophyceae <i>Elliptochloris</i> -clade	Unknown	Phycobiont of <i>Catolechia</i> <i>wahlenbergii</i> Kärnten (southernmost Austrian State) 2200 m	SAG 245.80
<i>Trebouxia</i> <i>arboricola</i> Puymaly (TA)	Chlorophyta Trebouxiophyceae	Unknown	Typically photobiont of <i>Ramallina</i> sp. (Associated lichen not documented)	SAG 219.1a

### 3.1.2 LICHENS

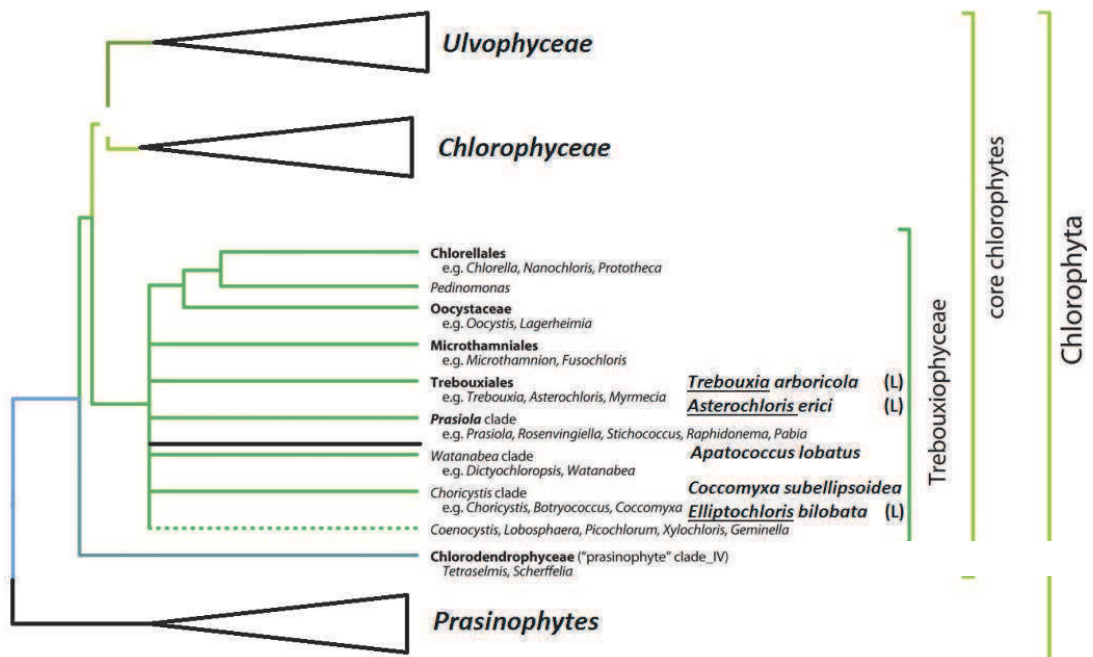
***Baeomyces rufus* (Hudson) Rebert (BR)** is a crustose green to dull greenish grey lichen, belonging to Baeomycetaceae family. The apothecias are dark red-brown or pale dull pink (Fig. 3.3). The thickness of the thallus is usually 2-3 cell layers. The photobiont is *EB*. *BR* grows typically on soils of conifer forests and particularly in recently disturbed moist sites. It is widely distributed across temperate and boreal zones of the Northern Hemisphere. (Nash et al. 2004). The thallus of this lichen was collected in a soil of a steep slope at the forest edge in the Isle of Usedom, Mecklenburg-Western Pomerania, Germany (Fig. 3.8). Reproductive structures were not considered in the sampling.



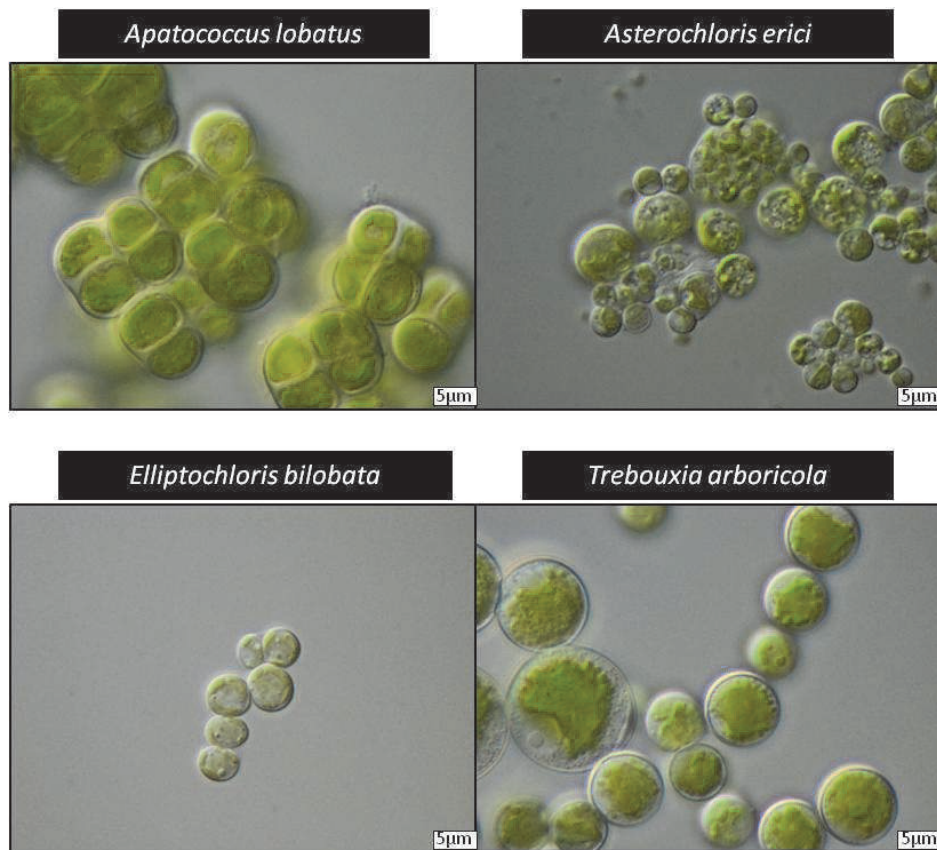
***Cladonia squamosa* Hoffm.** (CS) is a squamulose lichen from Cladoniaceae family. Thallus width is 1-3 mm, being deeply incised, with a shape from narrow-lober to coralloid. Its upper surface is light greyish-green and the lower is white (Fig. 3.3). It lives in mossy rocks or rotting woody or tree bases, primarily in temperate regions. Its photobiont is *AE* (Nash et al. 2004). Thalli of this lichen was collected in a strongly degraded peat bog, on rotten pine stump in Central Mecklenburg, Western Pomerania, Germany (Fig. 3.8). The reproductive structures were always rejected in the sampling.

***Lasallia hispanica* (Frey) Sancho & A. Crespo,** (*L. hispanica*) is a foliose green lichen from Umbilicaceae family. It presents pustulous thalli. The upper surface is grey to white and the lower part is brown. It is a strongly photophilous species (Fig. 3.3). Its photobiont is *Trebouxia* sp. being present in habitats where it is plentiful are wind and dry exposed siliceous surfaces. It is well represented in Mediterranean and Eurosiberian regions. In the latter, it is found from the montane to subalpine zones (Sancho and Crespo 1989). For the present study this lichen was collected at 1750m a.s.l in the south of Cantabria (Spain) (Fig. 3.8).

***Ramalina pollinaria* (Westr.) Ach.** (RP) is fruticose lichen from Ramalinaceae family. It presents long branches and it is very variable in morphology, specially, depending on the substrate (rocky surface, wood...). Its surface is greenish yellow (Fig. 3.3). Its photobiont is *TA*. It is a cosmopolitan species (Nash et al. 2004). In the case of this study, *RP* was sampled in a North-facing granitic wall of a rural medieval church in the north of Germany (Central Mecklenburg, Western Pomerania) (Fig. 3.8).



**Fig. 3.1.** Phylogenetic classification of the four algae used in the present thesis. (L) means that the algae can live as a lichen photobiont. The other species are exclusively free-living.



**Fig.3.2 :** Algae used for this experiment

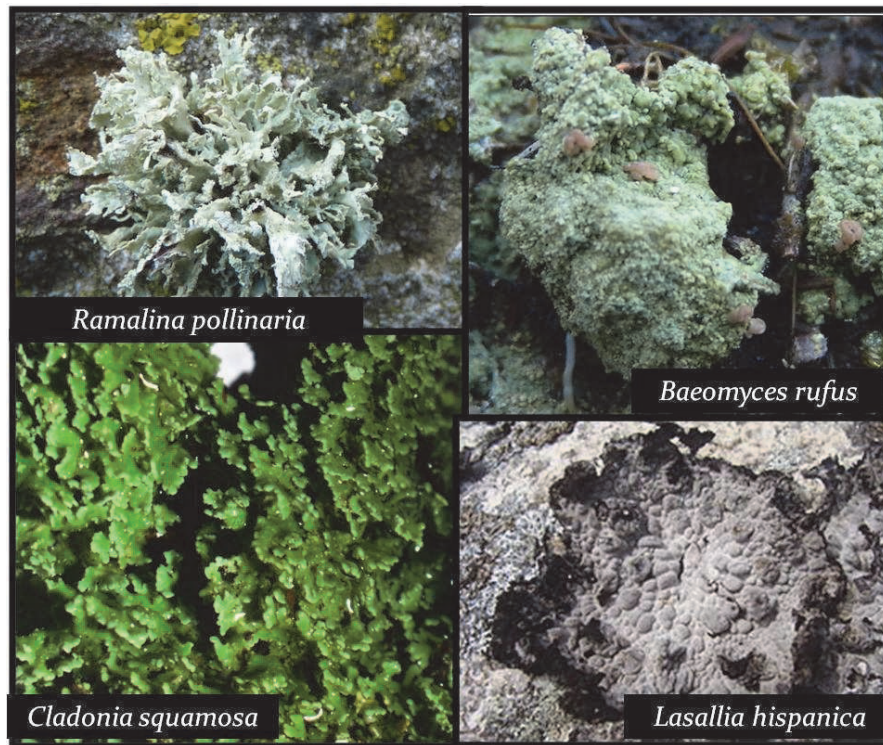


Fig. 3.3. Lichen species used for experiments in this work

### 3.1.3 MOSSES

*Syntrichia muralis* (Hedw.) Raab (*S. muralis*) (synonyms: *Desmatodun muralis* (Hedw.) Jur. and *Tortula muralis* Hedw.). It is a cosmopolitan moss growing in patches, tufts and neat cushions less than 1 cm tall. A long, smooth, silvery, excurrent nerve projects from the rounded leaf tip, making the moss look hoary grey when dry (Fig. 3.4). It is very common in bricks and stones but not in wood (Atherton et al. 2010). The old phyllids (leaves) become brown with ageing, being the green apical section the only photosynthetically active. It was collected in the same location of the Cantabrian mountains as *L. hispanica* (Fig. 3.8).

### 3.1.4 HERBS

*Hieracium pilosella* L. (synonymous: *Pilosella officinarum* Vaill.) is a perennial plant, that belongs to the Asteraceae Family (Fig. 3.4). It prefers dry



and sunny areas. It is an allelopathic plant, avoiding the growth of other species in its surroundings. It is native to Europe and northern Asia. It shows tremendous variation and it is a complex species with several dozen subspecies and hundreds of varieties and forms (Coworkers of Wikipedia 2015). The leaves of this species were sampled in the same as *L. hispanica* (Fig. 3.8).



**Fig. 3.4.** Herbaceous species (left) and moss species (right) used as model species in this work.

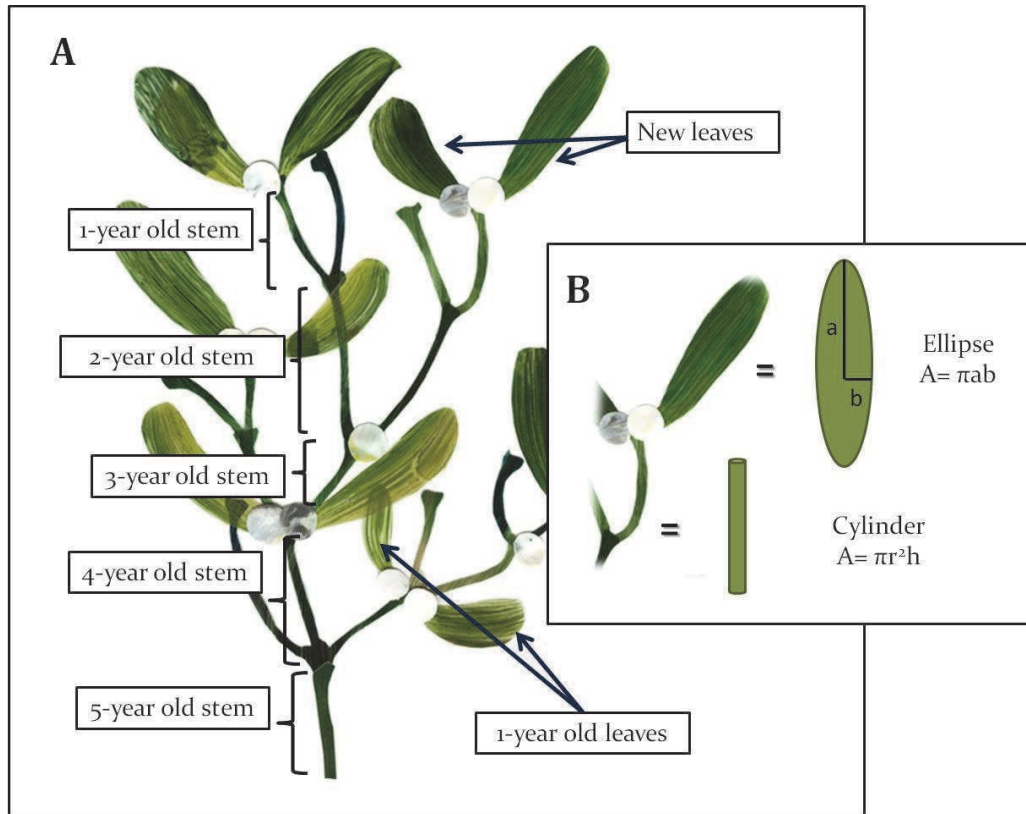
### 3.1.5 WOODY PLANTS

***Cytisus cantabricus* (Willk.) Rehb. F.** (*C. cantabricus*) is a shrub belonging to Leguminosae Family. It is a perennial plant that loses its leaves during winter, while its stems remain green permanently throughout the year (Fig. 3.5). As in other species of this group (Nilsen et al. 1993; Haase et al. 1999) under adequate conditions stems are able to photosynthesize, even during winter (Valladares et al. 2003). It is located always in sunny areas and typically in dry siliceous soils. Its presence is a sign of acid substrate. Its geographical distribution includes the N of Spain and the SW of France. It can grow from sea level to 1300m (1800m) a.s.l, typically in montane zone (Talavera 1999) (Fig. 3.7). In the present study, stems of *C. cantabricus* were collected in a shrubland located in the same area as *L. hispanica* (Fig. 3.8).

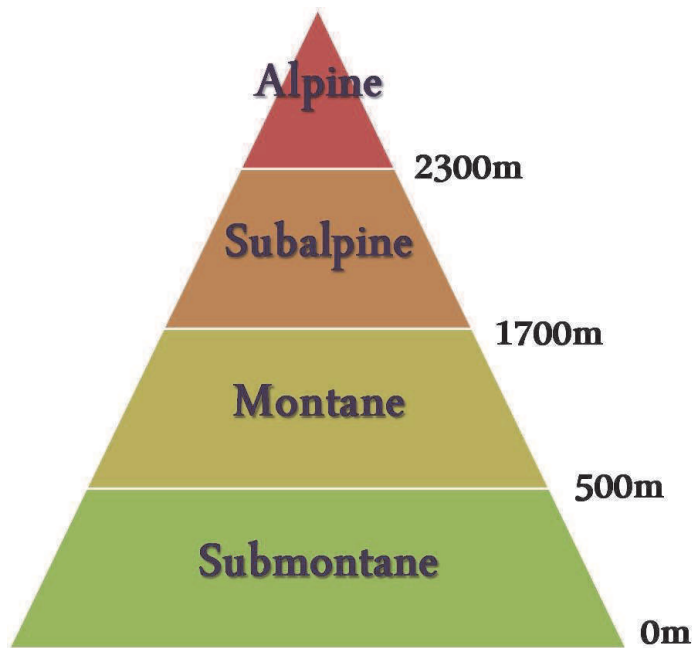


Fig. 3.5. Woody species used in this work.

*Viscum album* L. (*V. album*) commonly known as European mistletoe is an dioecius evergreen, perennial, epiphytic and hemiparasitic shrub that lives on a wide range of woody plant species (Fig. 3.5). It belongs to the Viscaceae family. Its leaves remain two years in the plant so, at the same time there are leaves from the current and from the previous year. Besides, it is also an appropriate tool for analyzing the effect of ageing as the age of each segment of the stem is easily discernible (Fig 3.6A). It presents photosynthetically active stems with stomata which maintain their CO<sub>2</sub> fixation activity during several years. It is widely distributed across Eurasia, from 10° W to 80° E and from about 60° N to 35° S. Normally it occurs in the montane and submontane region below 1000 m altitude (Fig. 3.7). Nevertheless, at warmer and sunnier sites, such as, southern Spain, it may also be found at higher altitudes (Zuber 2004). In this study, stems and leaves of *V. album*, growing as a parasitic with hawthorn (*Crataegus monogyna*) as host, were collected in Monte Santiago Natural Monument, Burgos, Spain at an altitude of 950m asl (Fig. 3.8).



**Fig. 3.6.** Morphology of *V. Album*. A: Representation of the age of each stem segment and leaves in a branch of *V. album*. B: Formula to calculate the total photosynthetic surface of *V. album* stems and the photosynthetic area of leaves.



**Fig. 3.7.** Altitudinal zonation in the European temperate region according to (Alcaraz et al. 1999)



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### 3.2 WHERE WERE COLLECTED THE PLANTS USED IN THIS THESIS?

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All sampling sites are located in areas where at least there is a cold season. Here, general characteristics of each area of study are presented:

**Mecklenburg** is a region located in the North of Germany. All lichen specimens used in chapter 7 were sampled in rocky surfaces of old buildings of this site, except *BR* that was sampled in a forest soil. The climate of this area is temperate oceanic, characterized by a mean temperature of -0.1 and 17.1°C the coldest and the hottest month respectively. The highest precipitation occurs in July while the driest month is February with a total annual rainfall of 550-600mm (<http://en.climate-data.org>). For map location see Fig. 3.8.

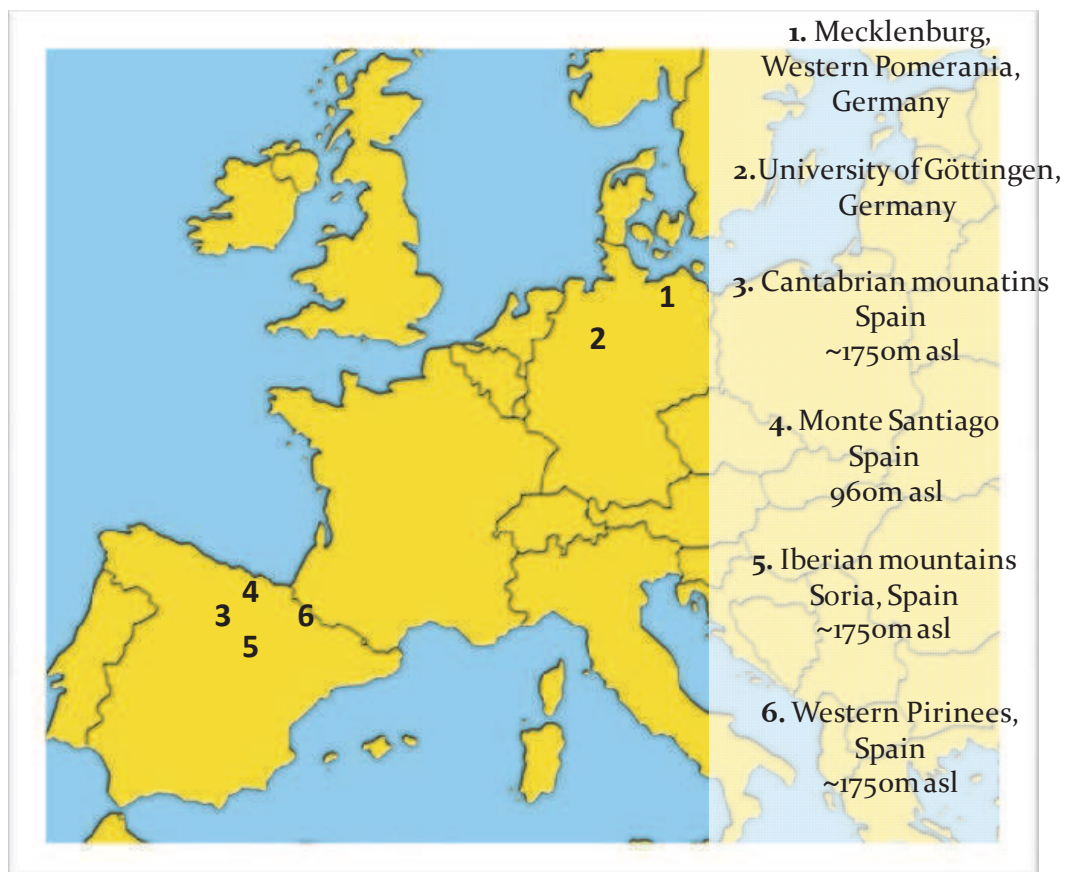
**Monte Santiago** Natural Monument is a protected area located in the north of Spain at submontane zone (944m a.s.l) (Fig 3.7 and 3.8). The vegetation of this site is dominated by two main ecosystems: beech forest and brushwood. The samplings of *V. album* were carried out in a brushwood where tree stratum is formed almost exclusively by *Crataegus monogyna*, the host of *V. album* (known commonly as hawthorn). This site is characterised by a temperate oceanic climate, with annual rainfall 1116mm and mean temperatures ranging from 16°C in August to 2.7°C in February (Míguez et al. 2014) being the average annual temperature is 11.5°C. The precipitation is abundant all year round being the annual average around 1000mm (<http://es.climate-data.org/location/360641/>).

**Cantabrian Mountains, Iberian Mountains and Western Pyrenees** (Fig. 3.8) were three subalpine (Fig. 3.7) sampling sites in which the species screening shown in chapter 5 was performed (See appendix 1 for species). These three sites are located at an altitude of ~1750m a.s.l in the north of Spain. They present mountain climate. Winters are characterised by LT, strong wind, fog and persistent snow above 1600m a.s.l. being, perpetual snow uncommon. There is



not dry season and the summer is cool. The average precipitation rise 1000mm in Cantabrian mountains and even 2000mm in Western Pyrenees. The average temperature is 7-10°C. (“<http://www.climaynievepirineos.com/c/c6/c6.htm>”; Ancell Trueba et al. 2011)

Most of the algae strains used for the chapter 7 were provided by the Culture Collection of Algae (SAG) of the **University of Göttingen**, There, there is a living resource of culture material of microalgae which allows to scientific community to have access to different algae strains for experimentation.



**Fig. 3.8.** Map showing the sites where plant material was collected.



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### 3.3 FIELD SAMPLING PROCEDURES

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Field experiments were carried out in late winter (March), when the most stressful combination of HL and LT occurs, and during late spring (June), after snow melt but before the occurrence of any summer drought.

#### 3.3.1 SPECIES SCREENING FOR EVALUATION OF WPI (Chapter 5):

The species sampled for this experiment are listed in appendix 1. Five replicates per species were collected in sunny days at noon during late winter and spring. After collection, they were kept in darkness in closed bags under an atmosphere of 100% relative humidity (RH) to avoid desiccation. After 30 minutes and 12 hours under these conditions, Chl fluorescence was measured as described in section 3.5. Photoinhibition (PI) was calculated using the photochemical efficiency of PSII ( $F_v/F_m$ ), as it is indicated in section 3.5.2 After fluorescence measurements, samples were frozen for pigment and protein determination.

#### 3.3.2 STUDIES ON *V. album* (Chapter 6):

South facing *V. album* individuals, growing as hemiparasitic plants in branches of *Crataegus monogyna* trees, were randomly selected. First of all, 5 replicates of their leaves and stems (from 1 to 7 years old) were dark adapted during 30 minutes prior to  $F_v/F_m$  determinations. After, entire leaves and stems chlorenchyma were cut from the plant, frozen in liquid nitrogen and subsequently stored at -80 °C until pigment and protein determinations.

Other set of samples composed by first-year twigs (stem and pair of leaves), were cut under nutrient solution (Murashige & Skoog Medium) in the field to avoid cavitation and transferred to the lab immersed into the same solution. Kinetics of photosynthesis recovery were followed under controlled conditions (dim light and room temperature) (See section 3.4.1 for recovery kinetic description).

**Estimation of leaf and stem photosynthetic area**

In order to calculate the contribution of leaves and stems to the total photosynthetic surfaces, the number of leaves and stems of each age class, was determined in each *V. album* individual. In one representative branch per individual, the length and the width were measured with a gauging device. To estimate the photosynthetic area per plant, cylinder and ellipse formula were used as models of stems and leaves area respectively (Fig. 3.6B).



**Fig. 3.9.** Photos showing the sites where plant material was collected.



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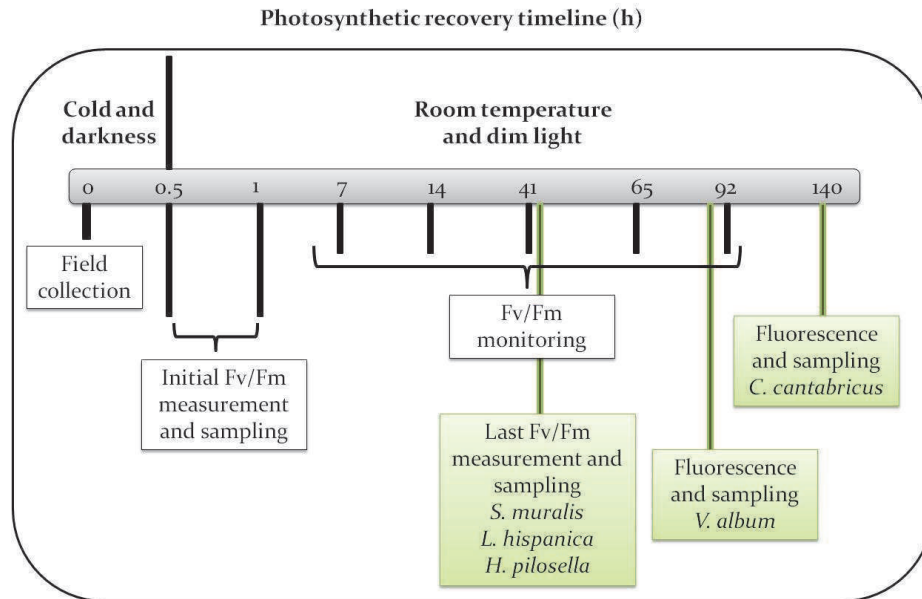
### 3.4 DESIGN OF LABORATORY EXPERIMENTS

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#### 3.4.1 RECOVERY KINETICS FROM WPI IN MODEL SPECIES

Four species, each one representative of one functional group, were used as models to characterise photosynthetic recovery kinetics upon transfer to lab conditions (Chapter 5). These experiments were carried out in stems of *C. cantabricus*, leaves of *H. pilosella*, phyllids of *S. muralis* and thalli of *L. hispanica*. All of them were collected in the field during winter. In order to avoid cavitation, *C. cantabricus* stems were cut under nutrient solution (Murashige & Skoog Medium) and consecutively maintained into the solution along all the recovery process. The rest of species were preserved in Petri dishes over wet paper to avoid desiccation. Once collected in the field,  $F_v/F_m$  was measured after 30 min of dark acclimation. This initial incubation was performed at 4°C (ice bath), to estimate the actual value of  $F_v/F_m$  in the field. Then, a set of 5 samples per species was frozen in liquid nitrogen and stored at -80°C for pigment and thylakoid protein determination. A second set of samples was transferred to optimum conditions (20°C, dim light and saturating humidity) to allow the recovery from WPI during 42h (for *H. pilosella*, *S. muralis* and *L. hispanica*) or 140h (in *C. cantabricus*) (Fig. 3.10).

*V. album* experiment (Chapter 6): Kinetics of recovery from winter stress were analyzed in leaves and one-year-old stems of *V. album* during winter and spring. Branches were cut under nutrient solution (Murashige & Skoog Medium) to avoid cavitation and placed under dim light and at 20°C until complete recovery of  $F_v/F_m$  (90h) (Fig. 3.10).



**Fig. 3.10.** Schematic representation of recovery kinetics indicating when the fluorescence was measured and when the samplings were carried out in each species.

### 3.4.2 ALGAE CULTIVATION

Unicellular algae were grown in sterile media at 20°C and PPFD 30  $\mu\text{molphotons m}^{-2}\text{s}^{-1}$  until enough biomass for the experiment was obtained. For more detail about media composition see Table 3.2 and Appendix 2.

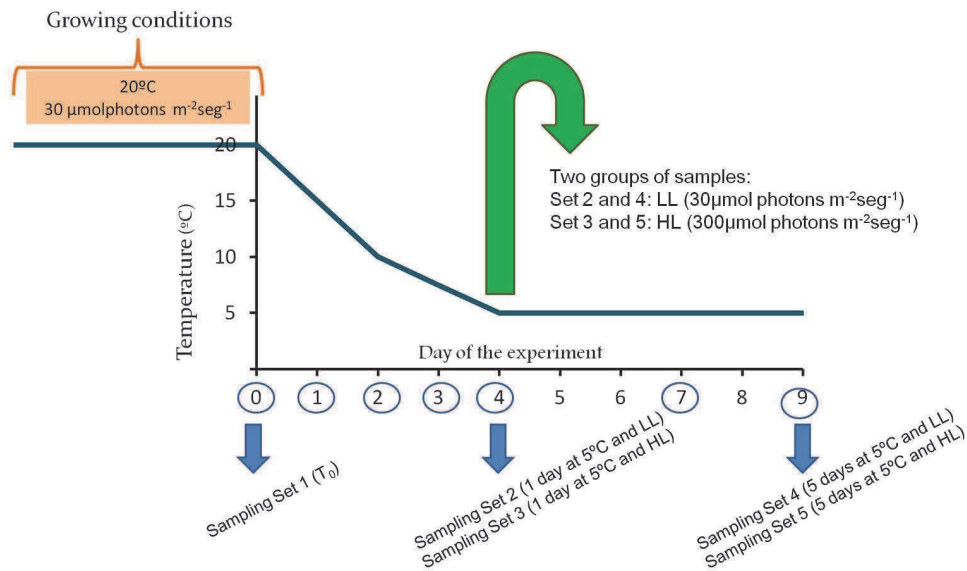
**Table 3.2:** Culture media where algae were growth. The media used were (i) bold basal medium with 3-fold nitrogen and vitamins (3N-BBM) and (ii) modified bold's basal medium for heterotrophs known as TOM (Trebouxia Organic Media) according to Ahmadjian (1967) modified after by Friedl (1989) by the addition of 1.5 % glucose, 2 % proteose-peptone and to 3N-BBM+V.

Species	Media	Solid (S)/ Liquid (L)
<i>Apatococcus lobatus</i> (AL)	TOM	S
<i>Asterochloris erici</i> (AE)	TOM	S
<i>Elliptochloris bilobata</i> (EB)	3N-BBM	S
<i>Trebouxia arboricola</i> (TA)	TOM	S



### Cold acclimation experiment in algae and lichen

Four algae species (*AL*, *TA*, *EB*, *AE*) and three lichens (*RP*, *BR*, *CS*) were used to characterise the process of LT acclimation. Once algae density and biomass were enough for the experiment, cultures were transferred to small sterile bottles containing liquid 3N-BBM medium (for media composition see Appendix 2). Algae were acclimated to that new medium and the incubator conditions during 2-3 days before starting the experiment. After that, temperature of the incubators was gradually shifted from 20°C to 5°C as it is indicated in Fig. 3.11. The fifth day, when the lowest temperature (5°C) was reached, the samples were divided in two different sets. Both of them were maintained at 5°C but the first was maintained under low light (LL = 30µmolm<sup>-2</sup>s<sup>-1</sup>) and the other set (Set 2) was subjected to the combination of high light (HL = 30µmolm<sup>-2</sup>s<sup>-1</sup>) and LT (5 °C) (Fig. 3.11). Light curves were measured daily (except on days 6,7 and 9) in three replicates of each species as is described in section 3.5.3.



**Fig. 3.11:** Schematic representation of the changes on incubation conditions during experimental cold acclimation of algae and lichens. Fluorescence measurements are indicated by blue circles and the sampling days are indicated with blue arrows. The day-length was 16 hours the first day, 10 hours the second day and from the third to the last day it was 8 hours.



### 3.5 CHLA FLUORESCENCE MEASUREMENTS

Prior to Chl fluorescence measurements, plant or algae samples were always dark adapted during 30 minutes in a water vapour saturated atmosphere. Measurements were performed with a portable pulse amplitude modulation fluorometer (PAM-2500 (Walz, Effeltrich, Germany)) with dark leaf clips.

#### 3.5.1 CALCULATION OF MAXIMAL PHOTOCHEMICAL EFFICIENCY OF PSII ( $F_v/F_m$ ):

The parameters used were:

- The maximum Chl a fluorescence ( $F_m$ ), which was induced with a saturating pulse ( $7795 \text{ mmol photons m}^{-2} \text{ s}^{-1}$ ).
- The minimum fluorescence ( $F_o$ ), which was recorded at LL intensities.
- The maximal photochemical efficiency of PSII ( $F_v/F_m$ ) which was calculated as follows (Kitajima and Butler 1975):

$$\text{Eq. 2: } F_v/F_m = (F_m - F_o)/F_m.$$

#### 3.5.2 CALCULATION OF WINTER PHOTOINHIBITION (WPI)

To calculate WPI, it was considered that the  $F_v/F_m$  measured in late spring after 14h in darkness is the highest value that each species can reach because they were under optimal environmental conditions for photochemistry. Taking this assumption into consideration, the percentages of dynamic winter photoinhibition ( $WPI_{<12h}$ ) and chronic winter photoinhibition ( $WPI_{>12h}$ ) were calculated for each species as follows:

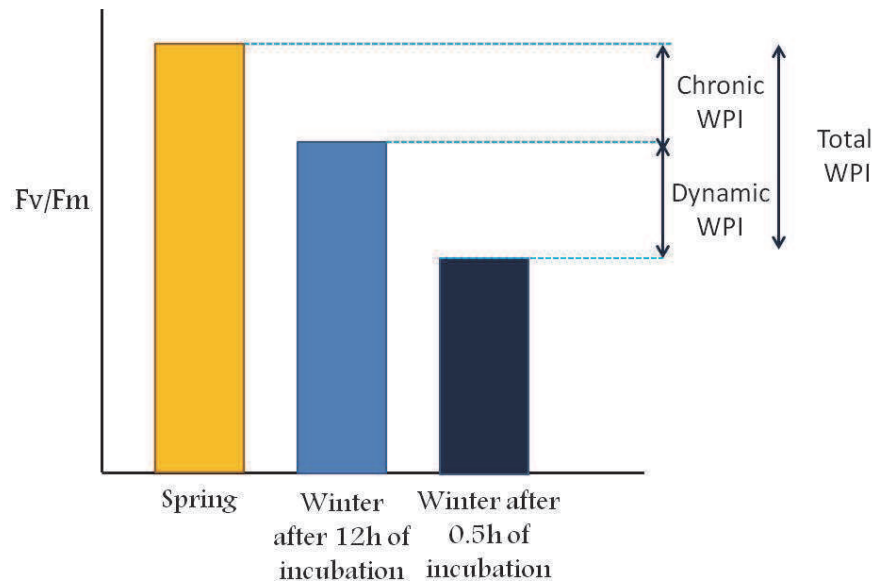
$$\text{Eq. 3: } WPI_{<12h} = (F_v/F_m \text{ 14h winter} - F_v/F_m \text{ 0.5h in winter}) / (F_v/F_m \text{ 14h spring}) \times 100$$

$$\text{Eq. 4: } WPI_{>12h} = (F_v/F_m \text{ 14h spring} - F_v/F_m \text{ 14h winter}) / (F_v/F_m \text{ 14h spring}) \times 100.$$

Where: 14h and 30min indicate the time that plants were incubated in darkness and 20°C before the  $F_v/F_m$  measurement. This approach is in agreement with the kinetics of  $F_v/F_m$  recovery described by Verhoeven (2013), where a rapid component, (which lasts less than 2h) and a slow component were



described in wintry photoinhibited conifers.  $WPI_{all}$  is the total photoinhibition and it was calculated as a sum of  $WPI_{>12h}$  and  $WPI_{<12h}$  (Fig. 3.12).



**Fig. 3.12.** Schematic representation of  $F_v/F_m$  tendency along the year and the magnitude of dynamic and chronic winter photoinhibition ( $WPI_{<12h}$  and  $WPI_{>12h}$ , respectively).

The use of  $F_v/F_m$  as an estimator of changes in PSII efficiency offers several advantages compared with other parameters, such as NPQ (as was described by Demmig-Adams et al. (2012) and Verhoeven (2013)). In many winter stressed leaves is not possible to obtain a precise  $F_m$  value (necessary for NPQ calculation) since they remain in a quenched state even when measured at predawn. Furthermore,  $F_m$  can be affected by factors other than thermal dissipation, such as chloroplast movements (Cazzaniga et al. 2013) and changes in Chl content. Furthermore,  $F_v/F_m$  possesses a defined maximal value, so it is easy comparable between species.

### 3.5.3 LIGHT CURVES IN UNICELLULAR ALGAE

To determine the dependence of NPQ on PPFD in algae samples, a PAM-2500 fluorometer (Walz, Effeltrich, Germany) was used. Prior to measurements, algae cultures were vacuum filtered and then dark adapted during





30 min. To prevent sample desiccation, filters with algae samples were placed above other filter with liquid medium during the dark adaptation period and during the fluorescence measurement. Light ramps were composed of thirteen successive steps (1 min each) starting at  $12 \mu\text{mol photons m}^{-2}\text{s}^{-1}$  and ending at  $489 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ . After each light step, a saturating pulse ( $7795 \text{ mmol photons m}^{-2}\text{s}^{-1}$ ) was given to obtain the parameters explained in Table 3.3.

#### Platt equation model

Photosynthesis/Irradiance curves (P/I curves) were fitted in the mathematical model proposed by (Platt et al. 1980). This model was used for this study as it provides a quantitative estimation of the photoinhibition of photosynthesis. The parameters obtained from this model are explained and represented in Table 3.4 and Fig. 3.13 respectively.



**Table 3.3.** Definition of fluorescence parameters and equations used for its calculation

<b>Parameter and definition</b>	<b>Abreviation (and formula)</b>
<b>Effective photochemical quantum yield of photosystem</b> (Genty et al. 1989).	<b>Eq. 5:</b> $\Phi_{PSII}=(F_m'-F)/F_m'$
<b>Quantum yield of non-regulated heat dissipation and fluorescence emission</b> $\Delta pH$ and $Z$ independent (Genty et al. 1996)	<b>Eq. 6:</b> $\Phi_{f,d}=F/F_m$
<b>Quantum yield of light induced non-photochemical energy conversion</b> $\Delta pH$ and $Z$ dependent (Genty et al. 1996)	<b>Eq. 7:</b> $\Phi_{NPQ}=(F/F_m')-(F/F_m)$
<b>The electron transport rate of PSII</b> (Genty et al. 1989)	<b>Eq. 8:</b> $ETR=P=PF D \times \Phi_{PSII}$
<b>Theoretical minimum fluorescence</b> in a photosynthetic organism under illumination. For its measurement, actinic light is switched off and “PSI light” is on during 5 second after each saturation pulse.	<b>Eq. 9:</b> $F_o'=F_m' \times (1 - \Phi_{PSII})$
<b>Maximum fluorescence levels during a treatment</b> is induced by saturating light pulses which temporarily close all PS II reactions centres. $F_m'$ decreases with respect to $F_m$ by non-photochemical quenching.	$F_m'$ (obtained by fluorometer measurement)
<b>Non photochemical quenching</b> represents the amount of energy which is dissipated as heat.	<b>Eq. 10:</b> $NPQ=(F_m-F_m')/F_m$
<b>Maximum Chl fluorescence</b> after a dark adapted period, when all the reaction centres are open	$F_m$ (obtained by fluorometer measurement)
<b>Minimum Chl fluorescence</b> is the minimum fluorescence of a dark-acclimated sample. For $F_o$ determination, the sample is not subjected to a saturated flash	$F_o$ (obtained by fluorometer measurement)



Table 3.4. Platt parameters definition and equation for its calculation

Definition	Abbreviation (and formula)
Relative electron transport rate of PSII	<b>Eq. 11:</b> $P = P_{max} * (1 - \exp(-\alpha * PFD / P_m)) * \exp(-\beta * PFD / P_m)$
Saturating light intensity (Henley 1993)	<b>Eq. 12:</b> $I_k = P_{max} / \alpha$
Photosynthetic efficiency	$\alpha$
Photoinhibition	$\beta$
Maximum capacity of photosynthesis	$P_m$

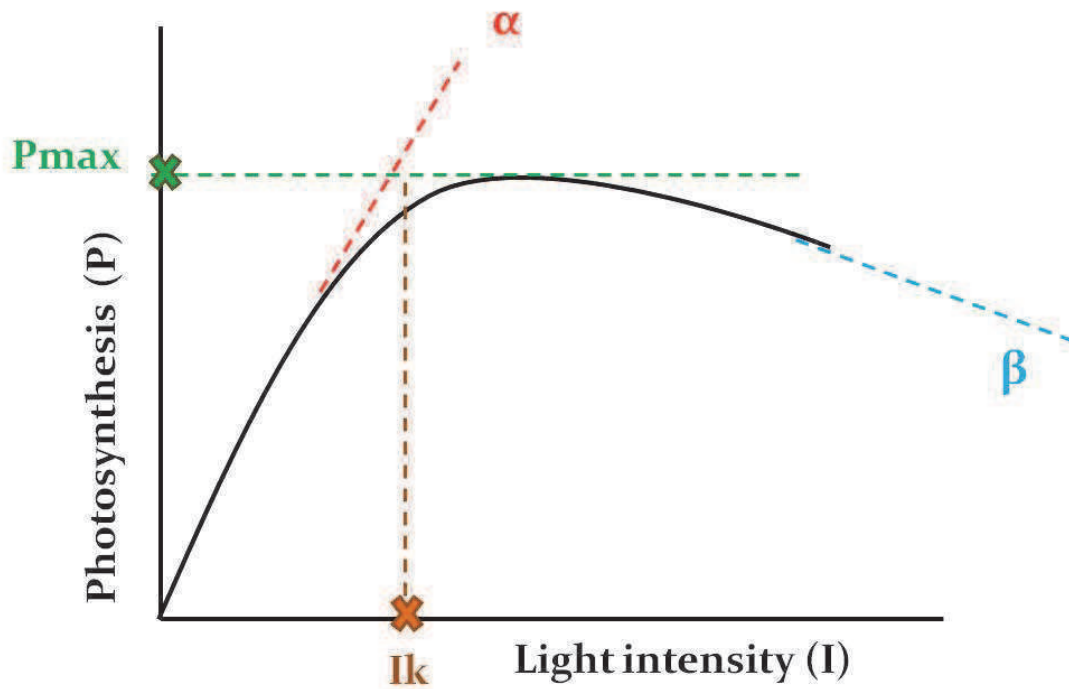


Fig. 3.13. Platt equation parameters representation according to Platt et al. (1980)

## 3.6 ANALYTICAL METHODS

### 3.6.1 PIGMENT DETERMINATION

Photosynthetic pigments (carotenoids and chlorophylls) together with tocopherols were determined by High Pressure Liquid Chromatography (HPLC) with a reverse phase C18 column (Spherisorb ODS1, 4.6 x 250mm, Waters, Milford, MA, USA) with a photodiode array (PDA) detector and a scanning fluorescence detector, following the method described by García-Plazaola and Becerril (1999, 2001) (Fig. 3.14).



**Fig. 3.14.** HPLC system from UPV/EHU. Each number indicates one component:

- 1: Temperature control module: it maintains the column temperature at 35°C (Waters).
- 2: Guard column and column: The column is Spherisorb ODS-1 with reversed phase column (5µm particle size; 4.6 x 250 mm) (Waters Ireland). The guard column is a Nova-Pak C-18 with a size of 4µm (3.9 x 20mm) (Waters Ireland). The column was preconditioned before using by flushing with methanol:water (50:50) at flow rate of 0.5-1 ml min<sup>-1</sup> for 24h.
- 3: Scanning fluorescence detector: (Waters 474) allows the detection of tocopherols (Toc) and pheophytins. The method used for Toc separation, detection and quantification uses an excitation  $\lambda$  of 295nm and an emission  $\lambda$  of 340 nm. The retention times and conversion factors are showed in Fig. 3.15.
- 4: UV-VIS diode array detector (PDA) (Waters model 996, Massachusetts, USA): the absorption spectra for identification and quantification of photosynthetic pigments was detected from 250 to 700 nm. The retention times and conversion factors are showed in Fig. 3.16.

5: Autosampler 717 plus is equipped with a thermostat which maintains temperature constantly at 4°C during analysis, avoiding pigment degradation or alteration. The injection volume was 15µl.

6: Pumps: This system is equipped with two pumps (Waters model 510), one per solvent.

7: Solvents: The mobile phase consisted on two components:

**Solvent A**: acetonitrile:methanol:H<sub>2</sub>O:tris 0.1M pH 8 (84:2:12.6:1.4)

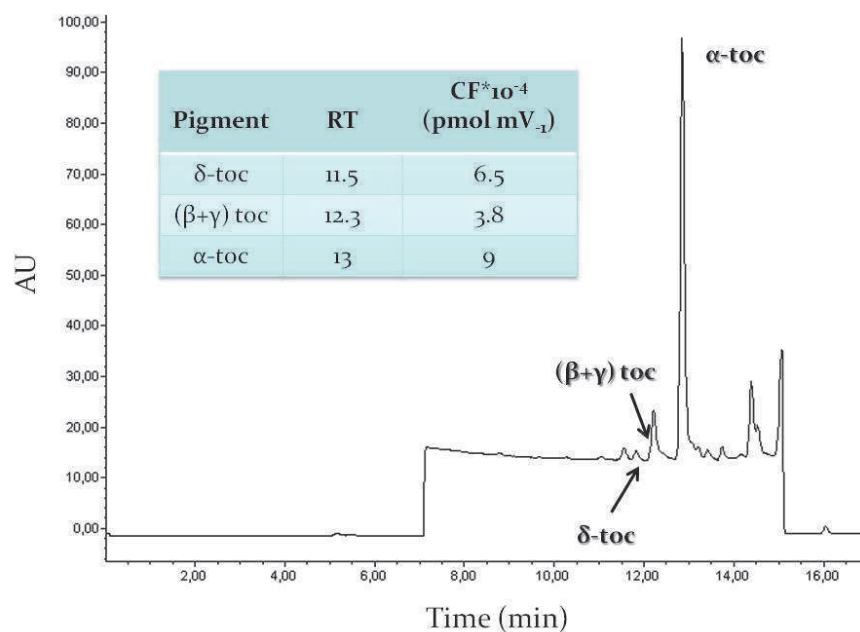
**Solvent B**: methanol:ethyl acetate (68:32)

Both solvents were vacuum filtered through a nylon membrane of 0.2 µm pore size before use.

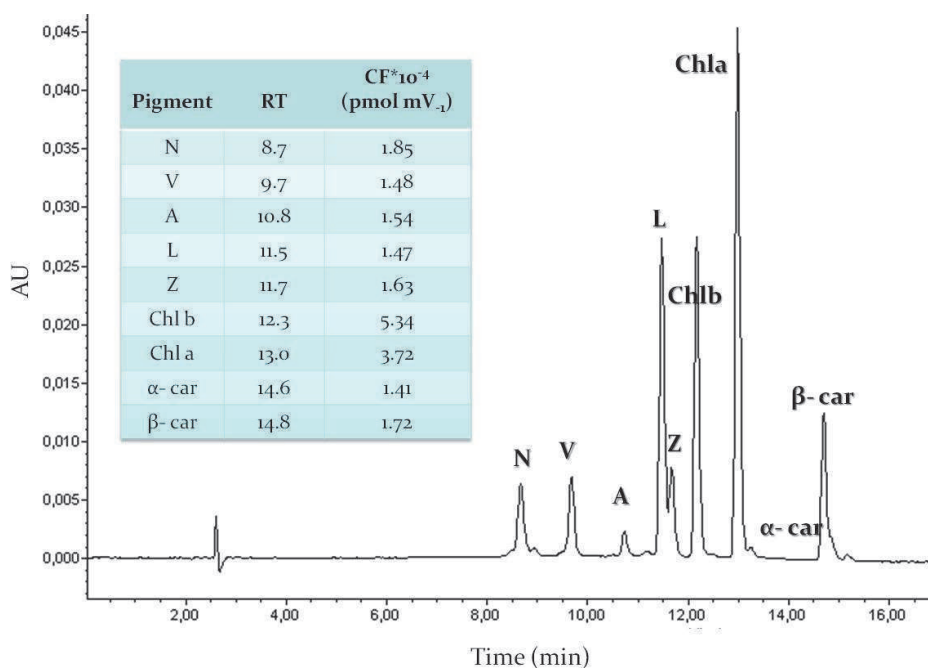
The evolution of solvents elution (flow 1.2 ml min<sup>-1</sup>) is as follows.

- 10 min: linear gradient from 100% solvent A to 100 % solvent B
- 5 min: isocratic elution with 100% solvent B
- 2 min: linear gradient from 100% B to 100% A.
- 8 min: Isocratic elution with 100% A prior to the next injection.

8: Software package: Empower Pro 2000, provided by Waters Corporation. Integration of the chromatograms was made at 445nm.



**Fig 3.15.** HPLC chromatogram showing the typical tocopherols pattern in a green leaf extract. In the inset, the retention times (RT) and the conversion factors (CF) for each tocopherol are shown.



**Fig. 3.16.** Chromatogram of a green leaf showing the main photosynthetic pigments: neoxanthin (N), violaxanthin (V), antheraxanthin (A), lutein (L), zeaxanthin (Z), chlorophyll a (Chl a), chlorophyll b (Chl b),  $\alpha$ -carotene ( $\alpha$ -car) and  $\beta$ -carotene ( $\beta$ -car). In the inset, the retention times (RT) and the conversion factors (CF) are shown for each pigment.

## **Pigment extraction**

### **General pigment extraction method**

Samples were frozen in liquid nitrogen immediately after each treatment and maintained at  $-80^{\circ}\text{C}$  until extraction. Hydrated frozen samples (leaves, photosynthetic surface of stems, phyllids, or the thalli of the lichens listed in Appendix 1) were homogenized with a mortar in pure acetone solution buffered with  $\text{CaCO}_3$  (0.5g/l). The extracts were centrifuged at 16,100 g for 20min, and supernatans were filtered with 0.2  $\mu\text{m}$  PTFE filters (Tecknokroma, Barcelona, Spain). During the whole process, samples were maintained at a temperature around  $4^{\circ}\text{C}$  to avoid pigments degradation. This extraction method was used for most of species except in the case of algae and lichens from chapter 7 in which specific extraction protocols were used (see bellow).

### Vacuum-evaporated algae samples

Algal cultures growing in liquid media were centrifuged after the corresponding treatment in 40 ml tubes. Then, the pellet was suspended in few ml of remaining medium and the resulting thick suspension was transferred to pre-weighted Eppendorf tubes. After centrifugation and discard of the clear supernatant, the algal pellets were subsequently frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until extraction. To obtain the dry weight of algal pellets, they were vacuum-evaporated in a Savant SpeedVac (SPD 111V) linked to a Lyovac GT2 Freezedryer (Steris, Germany) (Fig 3.17). To obtain an extract for pigment and tocopherol detection two consecutive extractions were performed: in the first they were homogenized with a mortar in acetone (95%) buffered with  $\text{CaCO}_3$ . In the second, the extracts were centrifuged at 16100g for 5 min. Then the pellet was re-suspended in pure acetone buffered with  $\text{CaCO}_3$  with a Tissue Tearor Homogenizer (Model 395, Dremel, Mexico). The extracts were centrifuged again at 16100g for 5 min. Both supernatants were mixed and were filtered with  $0.2\mu\text{m}$  PTFE filters (Tecknokroma, Barcelona, Spain). During the whole process, samples were maintained at a temperature around  $4^{\circ}\text{C}$  to avoid pigment degradations.





**Fig. 3.17.** Vacuum evaporator: Savant SpeedVac (SPD 111V) linked to a Lyovac GT2 Freezedryer.

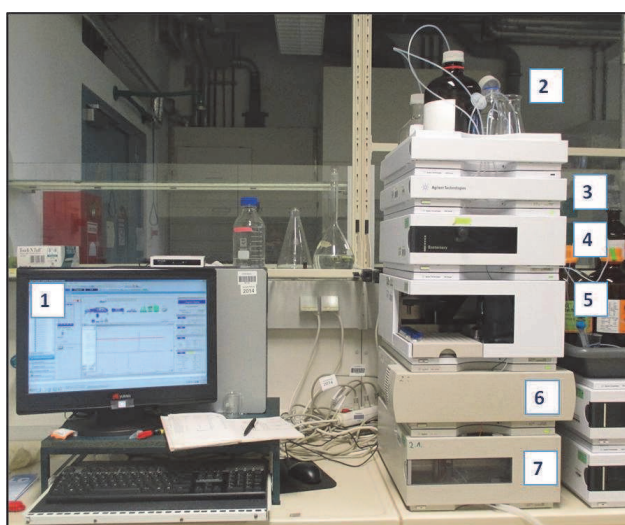
### Lichen evaporated samples extraction

Some lichen species accumulate large amounts of acid metabolites in their tissues. During the extraction, these acids induce the replacement of  $Mg^{++}$  by  $H^+$  in Chl molecules, leading to the formation of pheophytin. To avoid this artefact, the extraction medium was buffered with N-ethyl diisopropylamine (NEDPA) (Kranmer et al. 2003)

Hence, the extractions of lichens (Chapter 7) were carried out in a mortar using acetone 95% buffered with 0.5% NEDPA and the same amount of  $CaCO_3$  than sample. The extracts were centrifuged at 16100g for 20min, and supernatans were filtered with 0.2 $\mu$ m PTFE filters (Tecknokroma, Barcelona, Spain). During the whole process, samples were maintained at a temperature around 4°C to avoid pigment degradations.

### 3.6.2 LOW MOLECULAR WEIGHT CARBOHYDRATES DETERMINATION

Low molecular weight carbohydrates (LMWC) were determined by an Agilent HPLC system equipped with a refractive index detector (RID G1362A, Agilent, Santa Clara, USA) (Fig. 3.18).







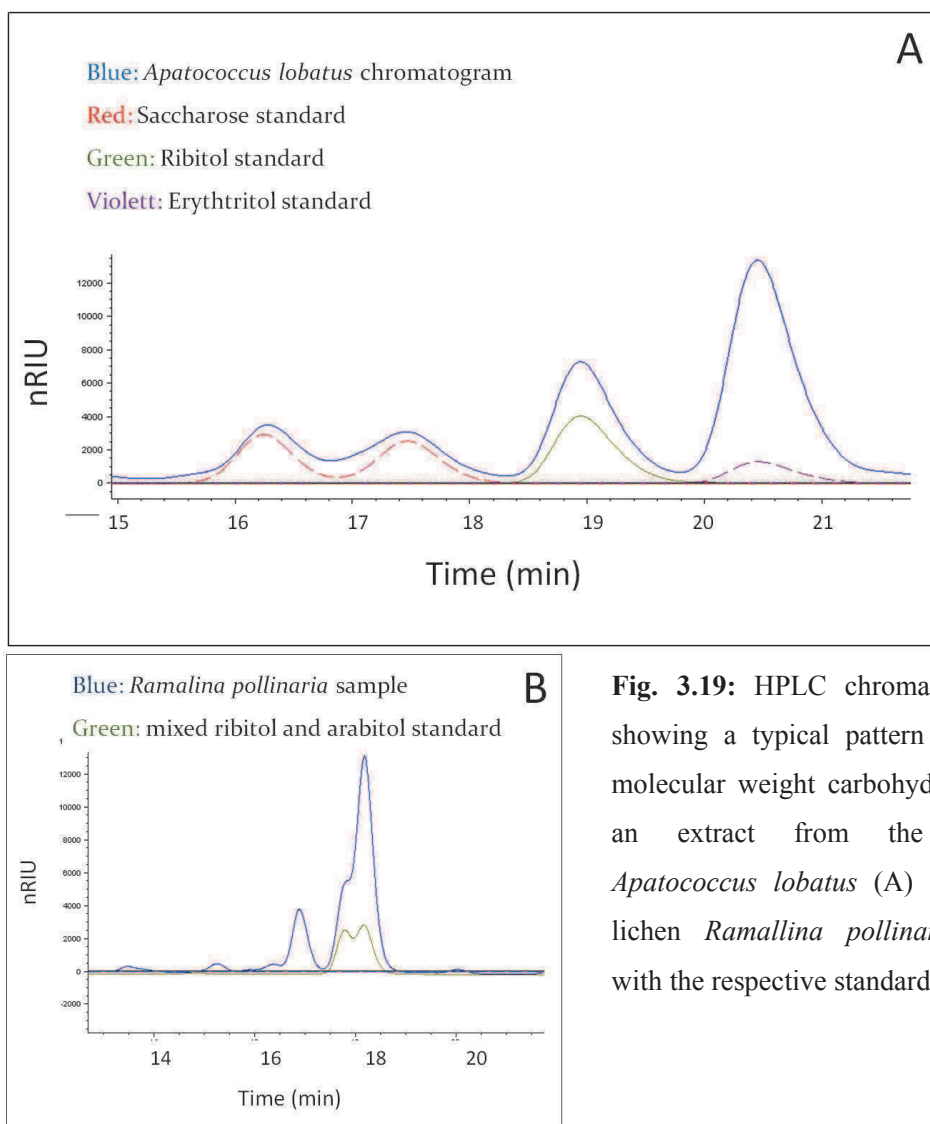
**Fig 3.18.** HPLC system (University of Rostock) for the detection of low molecular weight carbohydrates. Each number indicates one of the components of the system:

- 1: Software package: Agilent ChemStation
- 2: Eluents: Water 100% pure for HPLC or 5 mM H<sub>2</sub>SO<sub>4</sub>, depending on the method.
- 3: Degasser (G1322A) for the elimination of all the bubbles inside the system to avoid embolism in the column
- 4: Pump: QuatPump (G1311A)
- 5: Autosampler ALS (G1313A)
- 6: Column Thermostat: ColCom (G1316A) to maintain the column at 70°C.
- 7: Differential refractive index detector: RID G1362A

Two different isocratic methods were carried out depending on the detected carbohydrate:

The separation of **sucrose and ribitol**, was performed on a Bio Rad resin based column (Aminex Fast Carbohydrate Analysis, 100×7.8 mm) using a Phenomenex Carbo-Pb<sup>2+</sup> (4×3 mm) guard cartridge. These carbohydrates were eluted with 100% HPLC grade water at a flow rate of 1ml min<sup>-1</sup> at 70°C (Karsten et al. 1991).

On the other hand, the separation of **polyols** was performed on a Phenomenex resin-based column Rezex ROA-Organic Acid (300×7.8 mm) protected with a Phenomenex Carbo-H<sup>+</sup> guard cartridge (4×3 mm). On the latter column, carbohydrates were eluted with 5 mM H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.4ml min<sup>-1</sup> at 75°C (Karsten et al. 2005). Different carbohydrates were identified by comparison of retention times with those of the commercial standard compounds sucrose, arabitol, erythritol and ribitol (Roth, Karlsruhe, Germany). Quantification was achieved through respective calibration curves calculated from a 10 step dilution series with concentrations between 0.5 and 5mM. Carbohydrate concentrations within the algal samples were calculated through integration of peak area in relation to extracted dry weigh. An example of the chromatograms obtained is represented in Fig. 3.19.



**Fig. 3.19:** HPLC chromatograms showing a typical pattern of low molecular weight carbohydrates in an extract from the algae *Apatococcus lobatus* (A) and the lichen *Ramalina pollinaria* (B) with the respective standards.

### Low molecular weight carbohydrates extraction

Samples stored at  $-80^{\circ}\text{C}$  were vacuum-evaporated overnight in a Speed Vac Concentrator SVC 100H (Fig 3.17). Then, the samples were incubated during 4 hours in 1ml of 70% ethanol in a bath at  $70^{\circ}\text{C}$  (Karsten et al. 1991). Further, the samples were centrifuged during 5min at 13000 rpm at room temperature. 0.7ml of the clear supernatant was taken for vacuum-evaporation. Dry pellets were dissolved in the equal volume of HPLC-grade water in a ultrasonic bath during 30 minutes. Afterwards, they were centrifuged again with the same settings than before. The supernatant was transferred to HPLC vials for analysis.



## 3.7 THYLAKOID PROTEIN DETERMINATION

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### 3.7.1 PROTEIN EXTRACTION

Vegetal tissues were pulverized in a mortar with liquid nitrogen in darkness. Then, with the objective of obtaining isolated chloroplasts, extraction buffer was added. The sample was centrifuged during 10min at 10000rpm and 4°C. The pellet was mixed with washing buffer and then centrifuged again with the same settings. The pellet was washed with washing buffer until the supernatant was transparent. The final pellet was mixed with the solubility buffer to separate the proteins from lipid bilayer and the thylakoid membranes. This sample was stored at -20°C until western blot (WB) analysis was performed (For buffer detailed composition see appendix 3).

### 3.7.2 ELECTROPHORESIS

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel) was the method used for the determination of protein relative concentration. Gel and buffers composition is detailed in appendix 3.

Extracted samples, mixed with a 20% of running buffer and after subjecting them to 99°C during 5min and ice, were loaded in the gel. They were running during 2.5h at 110V. Then, proteins were transferred to nitrocellulose membranes during 1h at 75 V.

After that, membranes were:

- Blocked during 20 min in T-TBS (Tris Buffered Saline-Tween 20) with 5 g l<sup>-1</sup> of powder milk (for T-TBS composition see appendix 3).
- Washed three times with T-TBS
- Incubated with primary antibody during 1h.
- Washed three times (10min each) with T-TBS.



- Incubated with secondary antibody during 1h.
- Washed three times (10min each) with T-TBS.

Proteins were revealed by chemiluminescence ECL Plus (GE Healthcare) through CHEMIDOC XRS system (Bio-Rad). Densitometric measurements for the quantification of band intensity were carried out by using Quantity One (Bio-Rad) software.

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## 3.8 META-ANALYSIS

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### 3.8.1 LITERATURE COMPILATION

Literature from peer-reviewed journals of the period 1991–2011 was compiled using the “ISI Web of Knowledge” database (Thomson-ISI, Philadelphia, USA). Review-type articles were excluded in the search criterion and the areas considered were: Plant Science, Biochemistry, Ecology, Forestry, Biophysics, Cell Biology, Agronomy, Environmental Sciences, Horticulture and Agriculture. Keywords used for searching were:

**(winter OR chilling OR low\_temperature\*) AND (F<sub>v</sub>/F<sub>m</sub> OR photoinhibition OR Zeaxanthin OR down\_regulation).**

The criterion for the incorporation of data in the final dataset were the existence of F<sub>v</sub>/F<sub>m</sub> values for a cold period and a corresponding reference from a warm period. Treatment/exposures to cold conditions were only considered if they lasted for more than 24 hours.

### 3.8.2 PARAMETERS CALCULATION

In the present literature compilation, winter photoinhibition (WPI) was estimated by comparing F<sub>v</sub>/F<sub>m</sub> values of plants exposed to LT in the field (winter) or in growth chambers (long term treatments) with those of their respective unstressed controls.



**Eq. 13: % Winter photoinhibition**

$$\text{WPI} = (F_v/F_{\text{mctrl}} - F_v/F_{\text{mcold}})/(F_v/F_{\text{mctrl}}) \times 100$$

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4. Activation of photoprotective winter photoinhibition  
in plants from different environments:  
A literature compilation and meta-analysis

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ACTIVATION OF PHOTOPROTECTIVE WINTER  
PHOTOINHIBITION IN PLANTS FROM DIFFERENT  
ENVIRONMENTS: A LITERATURE COMPILATION AND  
META-ANALYSIS.

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**Abstract:** Overwintering plants face a pronounced imbalance between light capture and use of that excitation for photosynthesis. In response, plants up-regulate thermal dissipation, with concomitant reductions in photochemical efficiency, in a process characterized by a slow recovery upon warming. These sustained depressions of photochemical efficiency are termed winter photoinhibition (WPI) here. WPI has been extensively studied in conifers and in few overwintering crops, but other plant species have received less attention. Furthermore, the literature shows some controversies about the association of WPI with xanthophylls and the environmental conditions that control xanthophylls conversion. To overview current knowledge and identify knowledge gaps on WPI mechanisms, we performed a comprehensive meta-analysis of literature published over the period 1991–2011. All publications containing measurements of  $F_v/F_m$  for a cold period and a corresponding warm control were included in our final database of 190 studies on 162 species. WPI was estimated as the relative decrease in  $F_v/F_m$ . High WPI was always accompanied by a high AZ/VAZ. Activation of lasting WPI was directly related to air temperature, with a threshold of around 0°C. Tropical plants presented earlier (at a temperature of >0°C) and higher WPI than non-tropical plants. We conclude that (1) activation of a xanthophyll-dependent mechanism of WPI is a requisite for maintaining photosynthetic structures at sub-zero temperatures, while (2) absence (or low levels) of WPI is not necessarily related to low AZ/VAZ; and (3) the air temperature that triggers lasting WPI, and the maximum level of WPI, do not depend on plant growth habit or bioclimatic origin of species.





## 4.1 INTRODUCTION

Low temperatures (LT) are one of the main constraints for plant growth in all temperate and mountain climates (Ensminger et al. 2012). Not only wild plants, but also crops, such as cereals, are limited by the occurrence of cold periods (Colton-Gagnon et al. 2014). LT affect plants by three processes: lowering of enzymatic activity and loss of membrane function (chilling stress); ice formation and mechanical damage within tissues (freezing stress); and the over-excitation of the photosynthetic apparatus (photochilling stress). The latter phenomenon results from the combination of LT and light, and is considered one of the most challenging stresses in plants (Tausz et al. 2004). This is owing to the fact that this combination generates a potentially pronounced imbalance between light-energy absorption, which is largely temperature independent, and use of excitation energy through photosynthetic metabolism, which is impaired by LT. Plants whose photosynthetic tissues are periodically (seasonally or daily) exposed to such unfavorable conditions have developed metabolic adaptations that allow maintenance of a balance between energy input and utilization, a concept referred to as ‘photostasis’ (Öquist and Huner 2003). Photostasis can be achieved by several means, which are not mutually excluding and frequently occurring simultaneously: (1) decrease in the efficiency of light absorption, (2) increase in the rate of thermal dissipation of absorbed energy, and (3) upregulation of the metabolic use of energy. When these mechanisms are not sufficient to restore photostasis, damage is counteracted by deactivation of reactive-oxygen species generated as by-products of this energy imbalance and/or by the removal of PSII reaction centers and oxygen-evolving centers (Zarter, Adams WW III, Ebbert, Cuthbertson, et al. 2006; Zarter, Demmig-Adams, et al. 2006). Among protective mechanisms, the upregulation of thermal energy dissipation has traditionally been one of the most deeply studied, thanks to its easy assessment through the measurement of the non-photochemical quenching



of chlorophyll fluorescence (NPQ). In fact, this parameter is frequently employed as a synonym of thermal dissipation (García-Plazaola et al. 2012), even when other factors independent of energy dissipation might also affect NPQ (Cazzaniga et al. 2013).

NPQ represents a sum of processes with different biochemical basis, rather than a single mechanism, which have in common the generation of a dissipative state in the photosynthetic apparatus. In this sense, one of the most dramatic expressions of photochilling acclimation in winter is the activation of a ‘sustained’ or ‘chronic’ version of NPQ that is reversible only over hours or days upon warming (Verhoeven 2013). This sustained NPQ is in contrast with ‘dynamic NPQ’ that represents a fully reversible diurnal decline in the photochemical efficiency of light-energy conversion (Werner et al. 2002; Demmig-Adams et al. 2012). Overnight retention of NPQ can also be caused by the maintenance of a transthylakoid pH gradient, but this form of NPQ is also easily distinguishable as it relaxes rapidly upon warming (Verhoeven et al. 1998). Thus, sustained enhancement of NPQ in winter typically involves a reduction in  $F_v/F_m$  upon warming of the leaves. Owing to the associated depression in the efficiency and maximal rate of photosynthesis, this mechanism is also referred to as ‘photoinhibition’ (Demmig-Adams and Adams WW III 2006) – here termed WPI. WPI is now thought to be an adaptive photoprotective process not involving uncontrolled damage to the photosynthetic apparatus. Apart from WPI, other forms of photoinhibition also involve a protective downregulation of PSII function (Demmig-Adams et al. 2006, 2012, 2014; Adams et al. 2013), as is the case of the decrease in light absorption via chlorophyll (Chl) loss (Adams WW III et al. 2014). WPI is also typically associated with the accumulation of de-epoxidized xanthophylls: zeaxanthin (Z) and antheraxanthin (A) (Demmig-Adams and Adams 1996; Demmig-Adams and Adams WW III 2006), deactivation (Ensminger et al. 2004) and/or removal of PSII reaction centers and oxygen-evolving complex



(OEC) (Zarter, Adams WW III, Ebbert, Cuthbertson, et al. 2006; Zarter, Adams WW III, Ebbert, Adamska, et al. 2006), structural aggregation of light-harvesting complexes (LHCII) (Gilmore et al. 2003) and downregulation of antenna pigments and proteins (Ottander et al. 1995) and accumulation of specific stress-related proteins such as Elips (early-light-inducible proteins) and Hlips (high-light-inducible proteins) that may contribute to stabilizing or enhancing thermal dissipation (Zarter, Adams WW III, Ebbert, Cuthbertson, et al. 2006). Contrasting with such a conservative strategy in some overwintering species, the response of other plant groups to photochilling is to up-regulate the metabolic use of energy through carbon assimilation (Huner et al. 1998; Demmig-Adams et al. 2012; Cohu et al. 2014) and other processes that avoid over-reduction of the electron transport chain such as Mehler reaction or plastid terminal oxidase (Streb and Cornic 2012a; Laureau et al. 2013). The existence of a trade-off between contrasting strategies is well established: the ‘photochemical mechanism’ represented by plants that respond to photochilling by increasing metabolic energy demand and the ‘non-photochemical mechanism’ represented by species that become less metabolically efficient by a decrease in light capture and an increase in NPQ (for a detailed description of the two strategies, see (Savitch et al. 2002; Demmig-Adams and Adams WW III 2006; Sandve et al. 2011; Demmig-Adams et al. 2012, 2014; Cohu et al. 2014). These two strategies are typically exemplified by winter crops (such as wheat and spinach) that up-regulate photosynthesis in winter *vs* boreal conifers that arrest growth over the winter season, respectively. However, there is much in-between these two extremes, and a systematic overview integrating the functional diversity of photosynthetic organisms that remain green during cold seasons is lacking.

Thus, in this study, we aim to establish some general patterns by compiling a literature data set on the effects of photochilling on activation of protective thermal dissipation. For this purpose, we have surveyed



existing literature on both field and laboratory-based measurements, covering all terrestrial photosynthetic organisms except algae. Literature compilation and meta-analysis are robust strategies increasingly employed in the study of photosynthesis, as they allow to condensate and integrate information from many independent studies. In fact, several previous studies have employed this strategy to address other general trends in photosynthesis and photoprotection (e.g.: (Thomas and Winner 2002; Wujeska et al. 2013; Galmés et al. 2013; Esteban et al. 2014). Here, we specifically address the questions, (1) is there a temperature threshold for activation of WPI? (2) is WPI always linked to overnight retention of xanthophyll cycle pigments in their de-epoxidised state? and (3) are there differences in WPI employment between plants of different functional groups or climatic and biome origin?

## 4.2 MATERIALS AND METHODS

### 4.2.1 Literature compilation

Literature from peer-reviewed journals of the period 1991–2011 was compiled using the ‘ISI Web of Knowledge’ database (Thomson-ISI, Philadelphia). Review-type articles were excluded in the search criteria and the following areas were considered: Plant Science, Biochemistry, Ecology, Forestry, Biophysics, Cell Biology, Agronomy, Environmental Sciences, Horticulture and Agriculture. Keywords strings used for searching were the following:

(winter OR chilling OR low-temperature\*) AND ( $F_v/F_m$  OR photoinhibition OR Zeaxanthin OR down-regulation).

A total of 1131 articles were initially identified with this search formula. From there, the criterion for the incorporation of data in the final dataset was existence of  $F_v/F_m$  values for a cold period and a corresponding reference from a warm period. Treatment/exposures were considered only if they lasted for more than 24h. After direct examination of the preselected



references, a total of 190 studies (Appendix 4), representing 162 species (Appendix 4B), were selected. Data were extracted from text, tables or figures within these sources. When more than one replicate was reported, the average of all of them was calculated. The resulting data set was organized according to the criteria of growth conditions (natural habitat, cultivated plants in the field, botanical gardens or growth chambers), climatic conditions (tropical, alpine tropical, Mediterranean, temperate, boreal and alpine), functional group (herbs, shrubs, broad-leaf trees, palms, conifers and bryophytes). To calculate temperature ranges, averages for winter and control conditions were included. For field experiments, temperature values used were averages of the month previous to the experiment where possible.

#### **4.2.2 Calculation of parameters**

In this literature compilation, winter photoinhibition (WPI), caused by a inactivation/removal of PSII reaction centers/OEC and/or activation of thermal dissipation, was estimated by comparing  $F_v/F_m$  values of plants exposed to low temperatures (LT) in the field (winter) or in growth chambers (long-term treatments) with those of their respective unstressed controls. The use of  $F_v/F_m$  as an estimator of changes in PSII efficiency (that is a measure of any inactivation of PSII photochemistry as well as of increased thermal dissipation, and thus represents PSII efficiency depressions irrespective of their mechanism) offers several advantages compared with other parameters, such as NPQ (as described by (Demmig-Adams et al. 2012; Logan et al. 2014; Verhoeven 2014)). In many winter stressed leaves, it is not possible to obtain a precise  $F_m$  value (necessary for NPQ calculation) since it remains in a quenched state even when measured at predawn. Furthermore,  $F_m$  can be affected by factors other than thermal dissipation, such as chloroplast movements (Cazzaniga et al. 2013) and changes in chlorophyll content. Furthermore,  $F_v/F_m$  possesses an absolute and comparable range of values, while NPQ does not have a defined



maximal value. Besides,  $F_v/F_m$  is a very convenient parameter for the comparison of distant taxonomic groups growing in their natural ecosystems, while  $F_m$  values can be dissimilar among plants even under the same environmental conditions.

To calculate WPI,  $F_v/F_m$  values obtained in winter or under low temperature ( $F_v/F_{m\text{cold}}$ ) were compared with their respective non-stressed control ( $F_v/F_{m\text{ctrl}}$ ) using the formula:

$$\% \text{ WPI} = (F_v/F_{m\text{ctrl}} - F_v/F_{m\text{cold}}) / (F_v/F_{m\text{ctrl}}) \times 100$$

when the length of the period of dark-adaptation before  $F_v/F_m$  determination was longer than 2h ( $F_v/F_{m>2h}$ ), values of WPI obtained were considered as indicative of sustained/chronic photoinhibition, and thus denoted as  $\text{WPI}_{>2h}$ . Thus, the term photoinhibition was restricted to non-reversible changes in  $F_v/F_m$ .

When the length of the dark-adaptation period was not indicated in the original source, derived WPI were only used for comparative purposes among functional groups and climatic origins (Fig 4.3 and 4.4). In this case, these data with undefined dark-adaptation period were pooled together with  $\text{WPI}_{>2h}$  as the sum of both and denoted as  $\text{WPI}_{\text{all}}$ . When xanthophylls cycle (V-cycle) components were described (37 of 190 studies), the parameter AZ/VAZ was also extracted from the references.

### 4.2.3 Statistics

Differences in pigment content among functional groups and biomes were evaluated using one-way ANOVA and Duncan test as post hoc (C-Dunnet post hoc in the case of data showing heterogeneity of variances). Log-transformation was performed when necessary to meet the criteria of homoscedasticity (Levene's test) and normality (Kolmogorov–Smirnov test). When normality was not met even after data transformation, non-parametric Mann–Whitney U-test was used. Significant differences were considered at  $\alpha=0.05$ . Statistical analyses were performed with the SPSS 19.0 statistical package (SPSS, Armonk, NY).

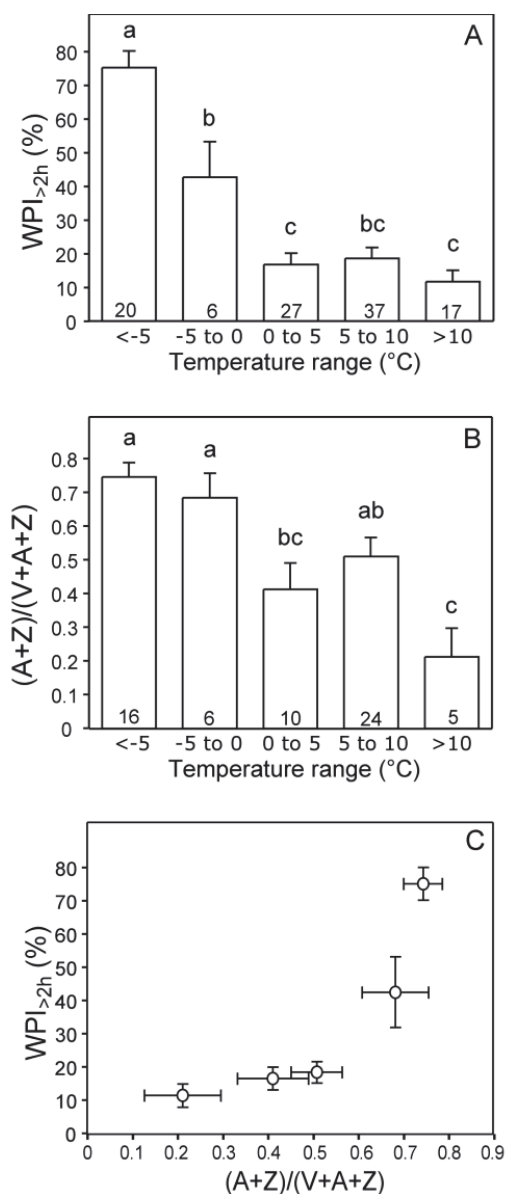


## 4.3 RESULTS

### 4.3.1 Responses of photoinhibition to low temperatures

Based on the times of dark acclimation reported in the literature included in the compilation, the value of 2h of recovery was considered a sufficient threshold to discriminate chronic PSII depressions ( $WPI_{>2h}$ ) from dynamic, reversible modulation of PSII efficiency ( $WPI_{<2h}$ ). Fig. 4.1 shows the relationships between  $WPI_{>2h}$ , the de-epoxidation index of the xanthophyll cycle  $AZ/VAZ$ , and winter/low temperature ranges, irrespective of whether the measurements were taken in field or from controlled environments. With this general analysis, we aimed to establish general patterns of relationship among these three factors. Mean values of  $WPI_{>2h}$  were below 20% at positive temperatures, but increased abruptly at temperatures lower than 0°C (Fig. 4.1A). On the other hand,  $AZ/VAZ$  increased progressively across the temperature range and was, on average, higher than 0.7 below 0°C (Fig. 4.1B). As a consequence, when the parameters (de-epoxidation *vs*  $WPI_{>2h}$ ) were plotted against each other, a positive, but non-linear relationship was found (Fig. 4.1C).  $WPI_{>2h}$  increased more strongly at  $AZ/VAZ$  values exceeding 0.5.





**Fig. 4.1.** Effect of low temperature on the activation of WPI. For this representation, plants grown in their natural habitat or cultivated in the field, botanical gardens or growth chambers data were included and temperatures were grouped into five ranges. Panel A shows temperature dependence of chronic/sustained winter photoinhibition (WPI<sub>>2h</sub>). Panel B shows the relationship between mean temperatures and levels of xanthophyll cycle de-epoxidation AZ/VAZ. Panel C shows the relationship between AZ/VAZ and WPI<sub>>2h</sub>. The number of reported data (n) is shown in the bottom of each box. Error bars represent SE.

#### 4.3.2 Field studies: winter mean temperature and WPI

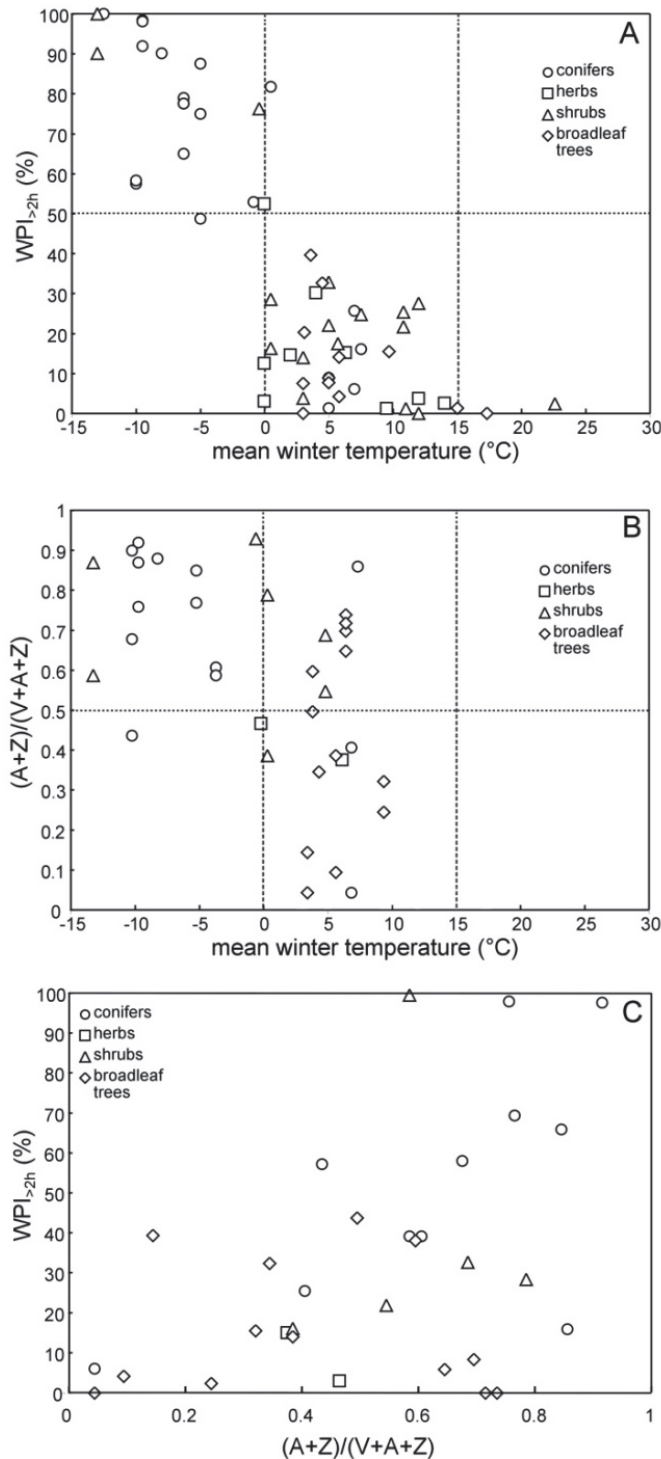
When only field data were considered and all individual fluorescence and pigment data were plotted as a function of mean winter temperature (Fig. 4.2), the same general trends as described in Fig. 4.1 emerged. In particular, WPI<sub>>2h</sub> increased progressively with decreasing temperature (Fig. 4.2A). At mean temperatures below 0°C, WPI<sub>>2h</sub> was greater than 50% for all data points. Conversely, WPI<sub>>2h</sub> was negligible at temperatures higher than 15°C (Fig. 4.2A). Conifers and shrubs exhibited the highest WPI levels (Fig. 4.2A). As most of the data from sub-zero



temperatures do come from woody plants (shrubs and conifers), these trends may be somewhat biased. When the same field data set was used to describe the responses of the AZ/VAZ ratio, the number of eligible individual studies decreased substantially. However, enough data remained to obtain clear response patterns (Fig. 4.2B, C). There was a clear transition at 0–3°C; below this temperature, AZ/VAZ was always greater than 0.4, while above this temperature AZ/VAZ varied considerably (Fig. 4.2B). When this pigment ratio was plotted vs WPI<sub>>2h</sub> (Fig. 4.2C), it was noticeable that at AZ/VAZ ratios below 0.4, WPI<sub>>2h</sub> was never higher than 40%, thus demonstrating that high AZ levels were always present when WPI was fully active, and that in some functional plant groups (mostly broad-leaf trees) a high AZ/VAZ ratio does not have to be associated with high WPI.

### 4.3.3 Functional and ecological factors

To compare the extent of photoinhibition in plants from different functional groups or climatic origins, data were pooled into groups by temperature increments of 5°C ranges. Within each range, data were classified according to several criteria. First, we tested whether, at comparable temperatures, woody plants were more likely to experience WPI than herbs, and whether tropical plants experience more WPI than non-tropical ones (Fig. 4.3). As shown in Fig. 4.3A, tropical plants showed significantly stronger WPI<sub>all</sub> in the positive temperature range between 0 and 15°C. It should be noted that all data from tropical species corresponding to the sub-zero range correspond to high-mountain tropical plants. On the other hand, no differences were observed in WPI between woody plants and herbs at any temperature range (Fig. 4.3B).

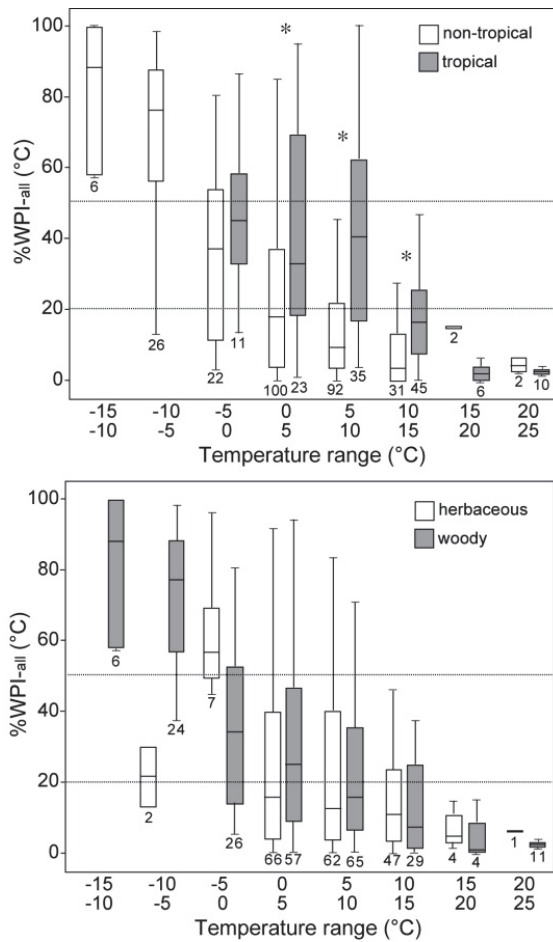


**Fig. 4.2** Effect of low temperature on WPI activation in the field. Panel A shows the relationship between mean winter temperature for plants grown in their natural habitat and chronic/sustained  $\text{WPI}_{>2h}$  ( $n=36$  data). Dashed lines represent critical temperature thresholds ( $0^{\circ}\text{C}$  and  $15^{\circ}\text{C}$ ) and the  $\text{WPI}=50\%$ . Panel B represents the relationship between mean winter temperatures in the field and the level of xanthophyll cycle de-poxidation  $AZ/VAZ$  for plants grown in their natural habitat ( $n=62$  data). Dashed lines represent critical temperature thresholds ( $0^{\circ}\text{C}$  and  $15^{\circ}\text{C}$ ) and the  $AZ/VAZ=0.5$ . Panel C represents the relationship between the level of  $AZ/VAZ$  and  $\text{WPI}_{>2h}$ . Each plant functional group is depicted by a different symbol (see key).

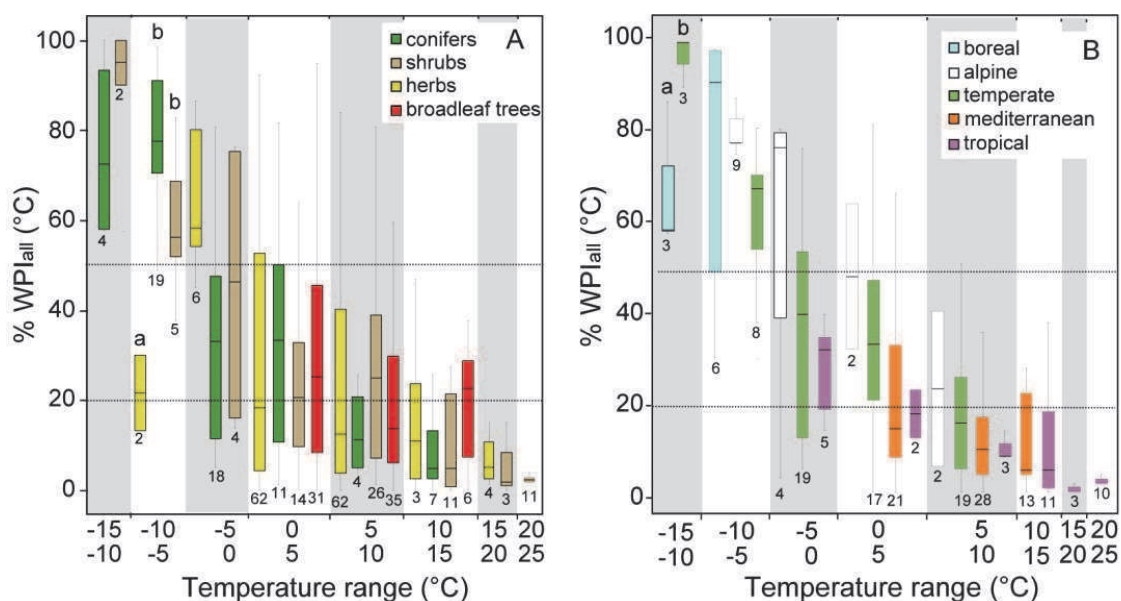
In a second approach, field data were classified within each temperature range according to plant functional group and biome (Fig. 4.4). Very few significant differences were observed and all them occurred below  $5^{\circ}\text{C}$ , with herbs being less photoinhibited than conifers and shrubs



between  $-10$  and  $-5^{\circ}\text{C}$  (Fig. 4.4A). Furthermore, using a threshold of 20% WPI demonstrates that WPI becomes activated at  $0$ – $5^{\circ}\text{C}$  for conifers and between  $5$  and  $15^{\circ}\text{C}$  for the other groups, such as broad-leaf trees as the group most sensitive to low temperature (Fig. 4.4A). On the other hand, when classified by biome, groups were even more uniform, and the only effect observed was greater WPI in plants from temperate biomes as compared with those from boreal regions in the coldest temperature range between  $-10$  and  $-15^{\circ}\text{C}$  (Fig. 4.4B). However, it should be noted here that these differences correspond to groups with low sample size, and this fact greatly limits any generalization to habitats (Fig. 4.4A) or functional groups (Fig. 4.4B).



**Fig. 4.3** Box plots showing chronic/sustained winter photoinhibition ( $\text{WPI}_{\text{all}}$ ) for different ranges of mean winter temperatures. In panel A, plants were separated for each temperature range according to their climatic origin as tropical and non-tropical. In panel B, plants were separated according to their functional group as woody and herbaceous. Boxes represent 50% of the data, central lines represent medians, and whiskers represent minimum and maximum values among non-atypical data. Horizontal dashed lines denote threshold values of 20 and 50% WPI. The number of reported data ( $n$ ) is shown below each box. Asterisks above boxes denote significant differences among groups within each temperature range ( $n \geq 3$ ).



**Fig. 4.4.** Boxplots showing total winter photoinhibition ( $WPI_{all}$ ) for various ranges of mean winter temperatures. In panel A, data were separated according to plant functional type; in the panel B, according to biome. Boxes represent 50% of the data, central lines represent medians, and whiskers represent minimum and maximum values among non-atypical data.

Horizontal lines denote threshold values of 20 and 50% WPI. Bryophytes, palms and succulents were removed because of their low representation in the survey. The number of reported data ( $n$ ) is shown below each box. Letters above boxes indicate significant differences among functional groups (panel A) or biomes (panel B) within each temperature range.

## 4.4 DISCUSSION

With the present literature compilation, we aimed to reveal general patterns of response in the extent of WPI. As described in the methods, we used  $F_v/F_m$  changes as an indicator of WPI activation, classifying them WPI chronic/sustained when  $F_v/F_m$  depression was not reversible over a period of at least 2h. This recovery threshold is shorter than a period of roughly 12h and corresponding to a night period, which is often employed to characterize chronic photoinhibition (Werner et al. 2002; Martínez-Ferri et al. 2004; Grant and Incoll 2005). In most of the compiled literature, the dark-acclimation periods were not as long as 12h. Thus, in order to include



a wider range of studies in our analysis, we used a period of 2h to distinguish between chronic/sustained WPI and reversible PSII efficiency modulation. Our approach is in agreement with the kinetics of  $F_v/F_m$  recovery in low light described by (Verhoeven 2013), where a rapid component lasts less than 2h. However, it should be considered here that ambient temperature has a profound influence in the time required for de-epoxidation of V-cycle and this might be related to the higher variation observed in AZ/VAZ relative to WPI (Reinhold et al. 2008). While one may argue that a limitation of this approach is that it is not possible to distinguish between inactivation of PSII centers and sustained NPQ, one can also argue that this is an advantage of our approach since we assess the combination of PSII core inactivation/removal and dissipation in the antennae. At the same time, our data suggest that we were able to distinguish two different response types, i.e. (1) one type of response, where very high AZ levels are associated with very high WPI levels (as assessed via high levels of sustained  $F_v/F_m$  depressions) and a separate response type (2) where intermediate AZ levels are associated with low WPI (low levels of  $F_v/F_m$  depression) (Fig. 1). Two such response types were distinguished by (Adams and Demmig-Adams 1995; Verhoeven et al. 1998) for an overnight evergreen shrub. At the beginning of the winter season, the shrub exhibited overnight retention of AZ by virtue of overnight trans-thylakoidal  $\Delta pH$  maintenance without apparent PSII core removal, which led to relatively rapid recovery of  $F_v/F_m$  and a slower removal of AZ upon warming of the leaves. Later in the winter season, this shrub continuously exhibited high levels of WPI associated with continuously high levels of AZ that were likely associated with PSII core degradation as demonstrated for conifers (Zarter, Adams WW III, Ebbert, Cuthbertson, et al. 2006; Zarter, Demmig-Adams, et al. 2006) and a broad-leafed evergreen (Zarter, Adams WW III, Ebbert, Adamska, et al. 2006).



Considering the wide range of species and studies included in the present compilation, and despite the limitations discussed above, our survey reveals clear, general trends in the temperature dependence of the WPI process. Despite variability in WPI values for given temperatures, 12°C was the point at which  $WPI_{>2h}$  started to be detected in some of the species, and 0°C was the temperature threshold that uniformly activated a  $WPI_{>2h}$  to greater than 50% in all species groups (Fig. 4.2). There was thus a clear and uniform general trend for  $WPI_{>2h}$  to be lower than 50% at above-zero temperatures and higher than 50% at sub-zero temperatures. This threshold reflects the point at which photosynthesis for most plant species is negligible (Öquist and Huner 2003). Even in alpine plants well adapted to seasonally very cold environments, the limit for net photosynthesis ranges from  $-3$  to  $-7^\circ\text{C}$  (Streb and Cornic 2012b). At positive temperatures, in the  $0$ – $12^\circ\text{C}$  range, the wider range of responses largely reflects inter-specific differences in the temperature optimum for photosynthesis, and these species' strategies of response to low temperature, i.e. some species upregulate carbonassimilation by virtue of increasing Rubisco concentrations (Galmés et al. 2013) and the number of palisade layers (Cohu et al. 2014), while others solely enhance thermal dissipation (Demmig-Adams and Adams WW III 2006). Above this temperature range, no WPI is observed in any species.

Similar temperature thresholds as those for  $F_v/F_m$  depressions were also observed for the xanthophyll cycle de-epoxidation index  $AZ/VAZ$ . Thus, at temperatures below  $2^\circ\text{C}$ , the xanthophyll pool was mostly de-epoxidised with  $AZ/VAZ > 0.5$ , while above  $7^\circ\text{C}$ , this ratio was always below 0.5 (Fig. 4.2). This response pattern, parallel to that of  $WPI_{>2h}$ , suggests a connection between the two parameters even though it was curvilinear rather than linear. These observations indicate that in the sub-zero temperature range, WPI activation involves maintenance of a sustained de-epoxidation of the xanthophyll cycle (Verhoeven 2014), while a high de-





epoxidation state does not necessarily mean a high WPI. As discussed above, these features could be accounted for by a difference in the involvement of cold-sustained trans-thylakoidal  $\Delta\text{pH}$  maintenance vs PSII core inactivation/removal. Alternatively, additional functions of Z such as antioxidant and membrane stabilization (Havaux et al. 2007; Dall'Osto et al. 2010) might contribute to the existence of two response ranges. In any case, Fig. 4.2C shows an important difference in between chronic WPI and AZ/VAZ correlation among functional groups of plants, being conifers the group that better fit a linear correlation over all ranges of values analyzed. On the other hand, broad-leaf trees showed a wider range of variation in the relationship between WPI and AZ/VAZ, likely due to inter-specific differences in the strategies to cope with winter stress.

The third and last objective of the present literature analysis was to address any possible intrinsic differences in WPI activation among different plant functional groups or as dependent on plant climatic origin. The similarity of the responses of all species included shows that the relationship between WPI and xanthophyll epoxidation state is the same irrespective of the fact that herbaceous plants up-regulate photosynthesis in response to decreasing temperatures while woody plants do not (Savitch et al. 2002; Demmig-Adams et al. 2012; Cohu et al. 2014). Additional clear trends were the well-known lower sensitivity of conifers to LT (Verhoeven 2014) and the higher  $\text{WPI}_{\text{all}}$  in tropical vs non-tropical plants compared in the positive temperature range between 0–15°C. These differences in the range of chilling temperatures probably reflect the intrinsic high susceptibility of tropical plants to low temperatures (Xin and Browse 2000). However, this difference is abolished at below-zero temperatures, as all data from such conditions are derived from tropical alpine species that experience tremendous daily fluctuations in temperature in their natural habitats and are well equipped to cope with freezing temperatures (Körner 2003).



While functional groups, such as trees and crops, were well represented in the present literature survey, others (lichens, bryophytes, terrestrial algae, ferns) were not, even though the latter groups are dominant in many boreal and alpine ecosystems. Future studies should address this gap and provide more information on the photosynthetic performance of the latter groups under low temperatures.

**Author contributions:** F. M. carried out the literature survey. J. I. García-Plazaola and J. M. B. were responsible for the experimental design and coordination of the project. B. F.-M. performed the statistical analyses. All authors contributed equally to the preparation of the manuscript.

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5. Diversity of winter photoinhibitory responses: A case study in co-occurring lichens, mosses, herbs and woody plants from subalpine environments

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## 5. DIVERSITY OF WINTER PHOTOINHIBITORY RESPONSES: A CASE STUDY IN CO-OCCURRING LICHENS, MOSSES, HERBS AND WOODY PLANTS FROM SUBALPINE ENVIRONMENTS

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**Abstract:** Evergreens living in high mountainous areas have to cope with hard conditions in winter as there is a combination of high light and low temperatures. Under this situation, there is an imbalance between light collection and energy use. As a response evergreens can activate a photoprotective process which consists on the down-regulation of photosynthetic efficiency, referred to as winter photoinhibition (WPI). WPI has been mainly studied in woody evergreens and crops, even when other functional groups such as lichens or bryophytes dominate alpine and boreal habitats. Additionally, it is still unclear to what extent WPI depends on specific proteins and carotenoids of the photosynthetic apparatus. With the aim of characterizing whether WPI is a general trait in overwintering evergreens and its dependence on the de-epoxidation state of violaxanthin cycle (AZ/VAZ), WPI was analyzed in the field in 50 species including woody species, herbs, lichens and mosses. Recovery kinetics were studied in detail in one model species from each group. Results showed that high levels of WPI were much more frequent among woody plants than in any other group, but were also present in some herbs, lichens and mosses. Winter conditions almost always led to higher AZ/VAZ levels. Nevertheless, changes in AZ/VAZ were not related to the activation/deactivation of WPI in the field and did not follow always changes in photochemical efficiency during recovery treatments. Seasonal changes on thylakoid proteins (mainly D1 and PsbS) were dependent on the functional group. The obtained results highlight the diversity of physiological solutions to winter photochilling.



## 5.1. INTRODUCTION

High mountain climates are characterized by low temperatures (LT), low atmospheric pressure and high proportion of short wavelength radiation (Körner 1999). Photosynthetic organisms acclimated to these extreme conditions need to complete their life cycle within a short vegetative period and to accumulate sufficient reserves for a long-lasting winter (Streb and Cornic 2012). Among perennial alpine plants, there are some species which require snow cover to overwinter successfully because the conditions below the snow are milder, mainly due to the amelioration of extreme temperatures and to the decrease of light intensity (e.g. Strand and Öquist 1985). By contrast, other species are exposed, at least periodically, to adverse conditions out of snow banks. The major stresses that these plants with evergreen foliage have to cope with are the freezing of apoplastic water (Sutinen et al. 2001) and the combined effect of high light (HL) and LT, known as “photochilling” (Huner et al. 2003; Ivanov et al. 2003).

Photochilling stress occurs when LT slows down enzymatic carbon assimilation (Falk et al. 1996), whereas the absorption of light by the photosynthetic apparatus remains constant since it is temperature-independent. As a consequence, light energy absorption by antennae is much higher than its potential use by the photosynthetic machinery, so the photosynthetic apparatus remains overexcited. This situation greatly increases the risk of photooxidative damage, and plants must up-regulate photoprotection mechanisms to counteract these effects. Apart from the reduction of light absorption through morphological modifications or the adjustments in photosystems (PS) antenna size, plants employ other physiological photoprotection mechanisms that can be grouped in three main strategies: (i) the up-regulation of alternative energy emission pathways such as the dissipation of exceeded light energy as heat (thermal

dissipation) (Öquist and Huner 2003; Demmig-Adams and Adams 2006) (ii) the increase of metabolic activity of energy sinks (Asada 1999; Niyogi 2000) and (iii) the deactivation of reactive oxygen species (ROS) through the antioxidant metabolism, and/or the repair of oxidative damage (Noctor and Foyer 1998; Mullineaux and Rausch 2005).

Regulated thermal dissipation is associated with a decrease in fluorescence yield, which is estimated by the fluorescence parameter called non-photochemical quenching (NPQ). For its activation, NPQ requires three different components: transthylakoidal proton gradient ( $\Delta pH$ ), PsbS protein (Li et al. 2002) and activation of violaxanthin cycle (V-cycle) (Niyogi et al. 1997, 1998). Depending on its permanence in darkness, NPQ can be considered dynamic, when is completely reversed after one winter night (12 hours), or sustained, when it needs more time (even several days of low light and optimal temperature) for a complete recovery (Verhoeven 2014). The consequence of sustained (also referred to as chronic) thermal dissipation is a reduction of photochemical efficiency. As this process results in a depression of maximal photochemical efficiency it can be considered as a type of photoinhibition (Demmig-Adams and Adams 2006). More concretely, it is termed as chronic or sustained winter photoinhibition ( $WPI_{>12h}$ ). Contrasting with other processes that generate an uncontrolled damage in photosynthetic machinery, particularly of reaction centers (photodamage),  $WPI_{>12h}$  is a highly regulated protective mechanism. In the cases in which the recovery is extremely slow (more than one night), even after incubation under optimal conditions, WPI is apparently independent on  $\Delta pH$  (Verhoeven et al. 1998; Gilmore and Ball 2000; Demmig-Adams et al. 2006) and PsbS protein (Öquist and Huner 2003; Adams et al. 2004), but it has been demonstrated that it requires the presence of zeaxanthin (Z). Thus, when WPI is activated, Z is retained and persistently engaged in thermal dissipation (Demmig-Adams and Adams 2006). A unified view of





winter downregulation of photosynthesis in woody species, integrating the roles of pigments and proteins, and different types of “quenching” that occur simultaneously, has been recently proposed by (Verhoeven 2014).

In temperate alpine ecosystems, the mechanism of WPI was well characterized in woody plants (Demmig-Adams and Adams 2006; Zarter et al. 2006; Porcar-Castell et al. 2008; Verhoeven 2014) and some herbs (Streb et al. 2003; Østrem et al. 2011; Sanchez and Smith 2015; Sui 2015). These studies showed that a wide range of species use the downregulation of photosynthesis as a photoprotective mechanism under wintry conditions. Although metabolic and protein changes involved on WPI have been well identified in some woody species (Demmig-Adams and Adams 2014). A recent literature compilation (Míguez et al. 2015) has revealed that very scarce number of works have studied WPI in lichens, bryophytes, terrestrial algae or ferns, even though these groups are dominant in many boreal and alpine ecosystems. Hence, in the present work we aimed to fulfill these gaps by comparing the better-known response of woody species with the rest of alpine flora (herbs, lichens and mosses) at three different levels: (i) performing a survey on the frequency of this character under field winter conditions in mosses, lichens and herbs; (ii) analyzing the potential for photosynthetic recovery under the simulation of a period of warm temperatures in winter, in selected species from the four functional groups; and (iii) elucidating the role that specific photosynthetic pigments and thylakoid proteins play in winter photoprotection and along the recovery processes in all these functional groups (mosses, lichens, herbs and woody species).

## 5.2 MATERIALS AND METHODS

### 5.2.1. Site description, plant material and experimental design

Field experiments were carried out (i) during late spring (June), after snow melt but before the occurrence of any summer drought and (ii) in late winter (March), when plants are exposed to strong photochilling conditions (moderate to HL intensities combined with still LT), and when, as it has been described by (Verhoeven 2013), the slowest photosynthesis recovery is observed. In both seasons, samples were collected at noon. The temperatures at that time in the field oscillated between 3 and 7°C in winter and between 19 and 25°C in spring. Photosynthetic organisms collected in winter were not covered by snow. The altitude of sampling sites was between 1750 and 1850m corresponding to the subalpine bioclimatic level. Two approaches, the first observational (experiment 1) and the second manipulative (experiment 2), were carried out:

Experiment 1: In order to encompass a wide range of different species, a screening comprising 50 subalpine species representative of the main functional groups (woody plants, herbaceous species, mosses and lichens) was carried out in 2012. The samplings were performed in winter and spring in three different mountainous areas in the north of Spain (Table 5.1). Immediately after collection, samples were incubated under darkness at 100% relative humidity (in plastic bags with wet paper) and at room temperature (20°C) during 12h to allow their recovery from any kind of dynamic WPI (here termed WPI<sub><12h</sub>). Chlorophyll (Chl) fluorescence measurements were taken after 30min and after 12h under those optimal conditions in 5 individuals of each species. After the second measurement, 5 replicates per species (100 mg approximately) were sampled and immediately frozen into liquid nitrogen and thereafter preserved at -80°C until pigment and protein analysis.

**Table 5.1:** List including all plant species for screening (experiment 1) and the locations in which they were collected. Species in bold are the model species used in the Experiment 2.

Functional group	Site 1 43°2'33"N 4°22'14"W	Site 2 42°3'38"N	Site 3 42°56'26"N
Woody species	<i>Calluna vulgaris</i>	<i>Calluna vulgaris</i>	<i>Calluna vulgaris</i>
	<i>Cytisus cantabricus</i>	<i>Erica aragonensis</i>	<i>Daphne cneorum</i>
	<i>Erica cinerea</i>	<i>Pinus sylvestris</i>	<i>Erica vagans</i>
	<i>Juniperus communis</i> subsp.	<i>Vaccinium myrtillus</i>	<i>Erica cinerea</i>
	<i>alpina</i>		<i>Genista hispanica</i> <i>Globularia repens</i> <i>Pinus uncinata</i>
Herbaceous	<i>Arabis alpina</i>	<i>Armeria</i> sp.	<i>Asperula hirta</i>
	<i>Cerastium fontanum</i>	<i>Digitalis parviflora</i>	<i>Hieracium pilosella</i>
	<i>Hieracium pilosella</i>	<i>Festuca</i> sp.	<i>Poacea</i>
	<i>Scilla</i> sp.	<i>Polypodium</i> sp.	<i>Saxifraga paniculata</i>
	<i>Plantago lanceolata</i>	<i>Sedum brevifolium</i>	<i>Teucrium</i>
	<i>Ranunculus repens</i>	<i>Senecio</i> sp.	<i>Thymelaea</i> sp.
	<i>Sedum album</i>		
	<i>Sedum</i> sp.		
Mosses	<i>Dicranum scoparium</i>	<i>Syntrichia muralis</i>	
	<i>Didymodon</i> sp.		
	<i>Grimmia pulvinata</i>		
	<i>Syntrichia muralis</i>		
	<i>Polytrichum piliferum</i>		
Lichens	<i>Dermatocarpon</i> sp.	<i>Rhizocarpon</i> sp.	
	<i>Lasallia hispanica</i>		
	<i>Lichinella stipatula</i>		
	<i>Parmelia saxatilis</i>		
	<i>Physcia</i> sp.		
	<i>Ramalina</i> sp.		
	<i>Umbilicaria cylindrica</i> <i>Umbilicaria polyphylla</i>		

Experiment 2: To study in deep which are the photosynthetic recovery kinetics under the simulation of a period of warm temperatures in winter in each functional group (woody species, herbs, bryophytes and lichens), one

species representative of each group was chosen: *Cytisus cantabricus* (Wilk.) Rechb. F., *Hieracium pilosella* L., *Syntrichia muralis* (Hedw.) Raab and *Lasallia hispanica* (Frey) Sancho & Crespo, respectively (Table 5.1, species with names in bold). The criteria for selecting these model species were: the easy identification in winter in the absence of flowers or fruits and the extensive representation in the sampling area. For this manipulative approach, 10 individuals per each species were directly sampled in the field in the late winter in 2013. In order to avoid cavitation, *C. cantabricus* stems were cut under nutritive solution and maintained into the solution along all the recovery process. The rest of species were preserved in Petri dishes over wet paper to avoid desiccation. To estimate the actual  $F_v/F_m$  in the field before starting the process of recovery, all samples were placed over an ice bath at a temperature of 4-7°C (simulating field conditions) and under darkness conditions during 30 min immediately after collection. After this period, Chl fluorescence measurements were performed. A second set of samples was transferred to optimum conditions to allow the recovery from WPI during 42h (for *H. pilosella*, *S. muralis* and *L. hispanica*) or 140h (in *C. cantabricus*) in a chamber at 20°C, dim light and saturating humidity. The maximal photochemical efficiency of PSII ( $F_v/F_m$ ) (see below) was monitored in these organisms at different times until  $F_v/F_m$  stabilization. Samples (100mg approximately) were frozen in liquid nitrogen after 0.5h in darkness and LT ( $t_0$ ), after 0.5 and 12h in darkness and optimal temperature and after the last fluorescence measurement, for pigment and protein analysis. Due to their different morphologies, the sampling of each species was as follows: (i) in *C. cantabricus*, the apical part of green stems was sampled (ii) in *H. pilosella*, whole green leaves (iii) in *L. hispanica*, thallus pieces of around 2 cm<sup>2</sup> size and (iv) in *S. muralis* the apical part of each caulid (shoot) containing photosynthetically active phyllids (leaves).



### 5.2.2. Fluorescence measurements

Chla fluorescence was measured using a portable modulated fluorometer PAM 2500 (Walz, Effeltrich, Germany). The maximum Chla fluorescence yield ( $F_m$ ) was induced with a saturating pulse ( $7795 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ ) while minimum fluorescence ( $F_0$ ) was recorded with low measuring light intensities. The maximal photochemical efficiency of PSII ( $F_v/F_m$ ) was calculated as  $(F_m - F_0)/F_m$ . In this study, the comparison of  $F_v/F_m$  values in spring and winter were used as an estimator of thermal dissipation because it offers several advantages (Verhoeven 2013; Míguez et al. 2015), especially when species from very different functional groups are being compared. To calculate WPI, Eq. 3 and 4 were used (Chapter 3).

### 5.2.3. Pigment and tocopherol analysis

The frozen samples, stored at  $-80^\circ\text{C}$ , were homogenized with a mortar in pure acetone solution buffered with  $\text{CaCO}_3$ . The extracts were centrifuged at 16100 g and  $4^\circ\text{C}$  for 20 min, and supernatants were filtered with  $0.2 \mu\text{m}$  PTFE filters (Teknokroma, Spain). Pigment separation was performed by HPLC with a reverse phase C18 column (Waters Spherisorb ODS1,  $4.6 \times 250 \text{ mm}$ , Mildred, MA, USA) with a photodiode array (PDA) detector, following the method of García-Plazaola and Becerril (1999) with modifications (García-Plazaola and Esteban 2012). The de-epoxidation rate of violaxanthin cycle pigments was estimated as  $\text{AZ}/\text{VAZ}$ , abbreviated as  $\text{AZ}/\text{VAZ}$ .

### 5.2.4 Protein extraction and characterization

The proteins examined in this study were D1 (PSII core complex protein), D1-P (phosphorylated D1 protein), Lhca2 (antenna protein from PSI), Lhcb2 (antenna protein from PSII), PsbS (essential protein for thermal dissipation) and Elip (Early Light Inducible Protein), closely related to stress. All the antibodies were from Agrisera AB (Vännäs, Sweden). The extraction, thylakoid isolation and SDS-page were carried out as (Sáez et al.

2013) with the modifications of (Míguez et al. 2014). Total protein content was determined by DC protein assay commercial kit (BioRad) to elucidate the quantity of protein in each extract. This method was used because it is compatible with the solvents used for the sample extraction. Immuno-detected proteins were detected by enhanced chemiluminescence ECL Plus (GE Healthcare) through CHEMIDOC XRS system (Bio-Rad). Densitometric measurements for the quantification of band intensity were carried out by using Quantity One (Bio-Rad) software.

#### **5.2.5. Statistics**

Kolmogorov-Smirnov and Levene tests were used to test for the normality of data and homogeneity of variances respectively. To analyze the presence of significant correlations between different types of photoinhibition and different pigment concentration, Pearson and Spearman tests were used with normal data and no normal data respectively. To check for differences in the percentage of WPI, non parametric Mann-Whitney U test was used. In the case of  $WPI_{<12h}$ , as the data were distributed normally but the variances were no homogeneous, one way ANOVA was applied with Dunnet C test as post-hoc. In order to look for significant differences in AZ/VAZ content along the recovery, one way ANOVA test was used. Post-hoc test used were Duncan, when there was variance homogeneity and Dunnet C test, when variances where no homogeneous. When necessary, data were log transformed. Significant differences were assumed at  $P < 0.05$ . All analyses were performed using the SPSS 17.0 statistical package (Chicago, SPSS Inc.).



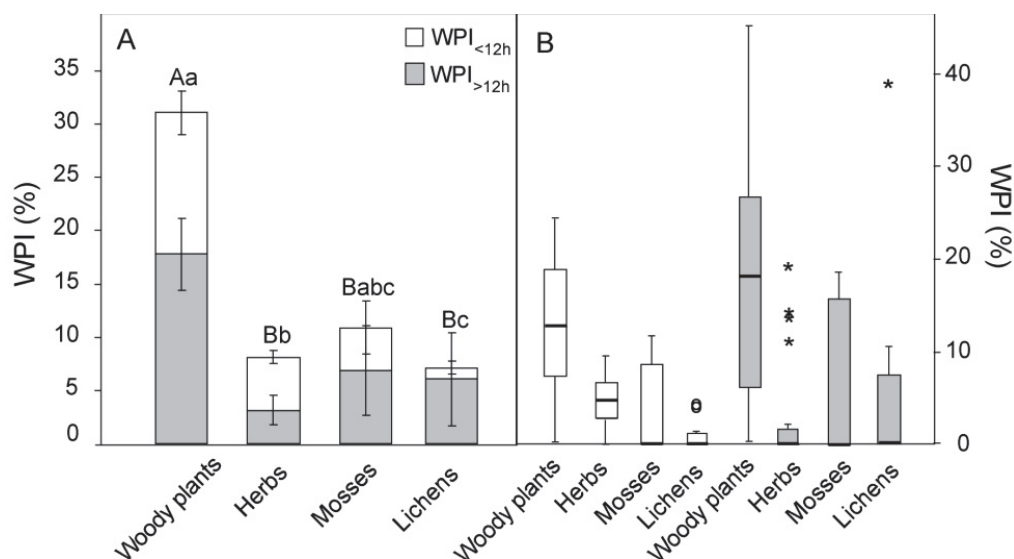
## 5.3 RESULTS

### 5.3.1. Chronic and dynamic winter photoinhibition: Differences among functional groups

The presence of winter photoinhibition (WPI) and the contribution of chronic ( $WPI_{>12h}$ ) and/or dynamic photoinhibition ( $WPI_{<12h}$ ) to it, were studied in 50 species comprising the main functional groups of photosynthetic macroorganisms that inhabit in subalpine areas. The highest total photoinhibition ( $WPI_{all}$ ) occurred in woody plants (Fig. 5.1A). They presented not only the highest  $WPI_{>12h}$  but also the most relevant  $WPI_{<12h}$ . Altogether,  $WPI_{<12h}$  and  $WPI_{>12h}$  reached more than 30% (Fig. 5.1A), representing  $WPI_{>12h}$  the 57% of the  $WPI_{all}$ . In contrast with woody species, in the rest of functional groups,  $WPI_{all}$  was on average lower than 11% (Fig. 5.1A). In the case of lichens virtually all the WPI was  $WPI_{>12h}$ .

To characterise the variability of WPI within each group, WPI was represented in a box plot (Fig. 5.1B). The highest variability was present in woody species, in both  $WPI_{>12h}$  and  $WPI_{<12h}$ . Although herbaceous species and lichens presented the lowest  $WPI_{>12h}$ , it must be highlighted that there were four herbaceous species (*Digitalis parviflora*, *Festuca* sp., *Saxifraga paniculata* and *Thymelaea* sp.) and one lichen (*Rhizocarpon* sp.) that did not follow this pattern, showing high values of  $WPI_{>12h}$  (outliers of Fig. 5.1B) comparable to those of woody plants. Interestingly, in herbs, mosses and lichens, a half of species did not present any  $WPI_{>12h}$  and, consequently, the median for  $WPI_{>12h}$  (represented by a horizontal line within the boxes of Fig. 5.1B) was 0% (see Appendix 6 for individual values of each species). In reference to  $WPI_{<12h}$ , mosses and lichens showed again median values of zero, while woody and herbaceous species presented a median of 13 and 5 % respectively (Fig. 5.1B).





**Fig. 5.1:** Extent of winter photoinhibition (WPI) and the contribution of chronic and dynamic components ( $WPI_{>12h}$  and  $WPI_{<12h}$ , respectively) in different functional groups. (A) Percentage of chronic (grey bars), dynamic (white bars) and total winter photoinhibition ( $WPI_{all}$ ) (maximum value of combined bars) within each functional group. Each bar represents the mean  $\pm$  SE. The number of species included were: 14 woody species, 20 herbs, 5 mosses and 9 lichens ( $n=5$  for each species). Letters above bars indicate significant differences for  $WPI_{>12h}$  (A and B) and  $WPI_{<12h}$  (a, b and c) (B) Box plot showing the variability in the percentage of  $WPI_{<12h}$  (white boxes) and  $WPI_{>12h}$  (grey bars) within each functional group. Each box encloses the middle half of the data between the first and third quartiles. Horizontal line represents the median; vertical line shows the range of data values. Outliers are shown as '\*' and atypical data are represented as 'o'. As in A panel, data showed in panel B comprises 14 woody species, 20 herbs, 5 mosses and 9 lichens ( $n=5$  for each species).

### 5.3.2. Yearly lipophilic antioxidant composition during the year in different functional groups.

HPLC analysis of antioxidant composition was performed in winter and spring in the four functional groups studied. In all woody species, concomitantly with high WPI, they showed the highest content of all lipophilic antioxidants (tocopherols and carotenoids) during winter, except for 6 species which presented slightly high amounts of  $\beta$ -carotene ( $\beta$ -car) in spring (Appendixes 6 and 7). Contrasting with woody plants, the response



to seasonality, in terms of antioxidant content, was much more heterogeneous among mosses and lichens. The most consistent responses among lichen species were the decrease of  $\beta$ -car and the increase of V-cycle de-epoxidation index (AZ/VAZ) during winter. In the case of mosses, the general trend observed indicate that AZ/VAZ is higher in spring, while  $\alpha$ -tocopherol rises the highest values in winter. Nevertheless, in both groups, antioxidant responses presented enormous variability depending on the species (Appendixes 6 and 7). Within herbaceous species, the most remarkable result was that both xanthophyll cycles present on them (V-cycle and lutein epoxide cycle (Lx-cycle)), showed an opposite pattern. Hence, while lutein (L) was accumulated in almost all the species in winter, V-cycle, presented a high de-epoxidation index (AZ/VAZ) in spring (Appendixes 6 and 7).  $\alpha$ -tocopherol ( $\alpha$ -toc) and L/Chl were the components which showed the most consistent pattern in all species, independently on the functional group, being higher in winter (Appendixes 6 and 7).

### **5.3.3. Winter photoinhibition: To what extent is it related with the lipophilic antioxidant composition?**

To clarify the possible inter-relationship between antioxidant composition and WPI, correlations between both parameters were assessed. Despite their low WPI, herbaceous species showed significant correlations between lipophilic antioxidants and  $WPI_{all}$  or  $WPI_{>12h}$  (Table 5.2). Thus, not only V-cycle components (V/Chl, A/Chl and total VAZ/Chl) and total carotenoids correlated with WPI, but it was also the case of tocopherols. Surprisingly, in woody species, which presented the highest values of  $WPI_{>12h}$ , none significant correlation with V-cycle components or other pigments was found. On the contrary,  $WPI_{<12h}$  and  $WPI_{all}$  of woody plants were correlated with V-cycle de-epoxidation ratio (Table 5.2). In mosses and lichens, only  $WPI_{<12h}$  showed significant correlation with lipophilic

antioxidants per Chl ratios.  $WPI_{<12h}$  was correlated with A/Chl in mosses and with L/Chl and Neo/Chl (N/Chl) in lichens.

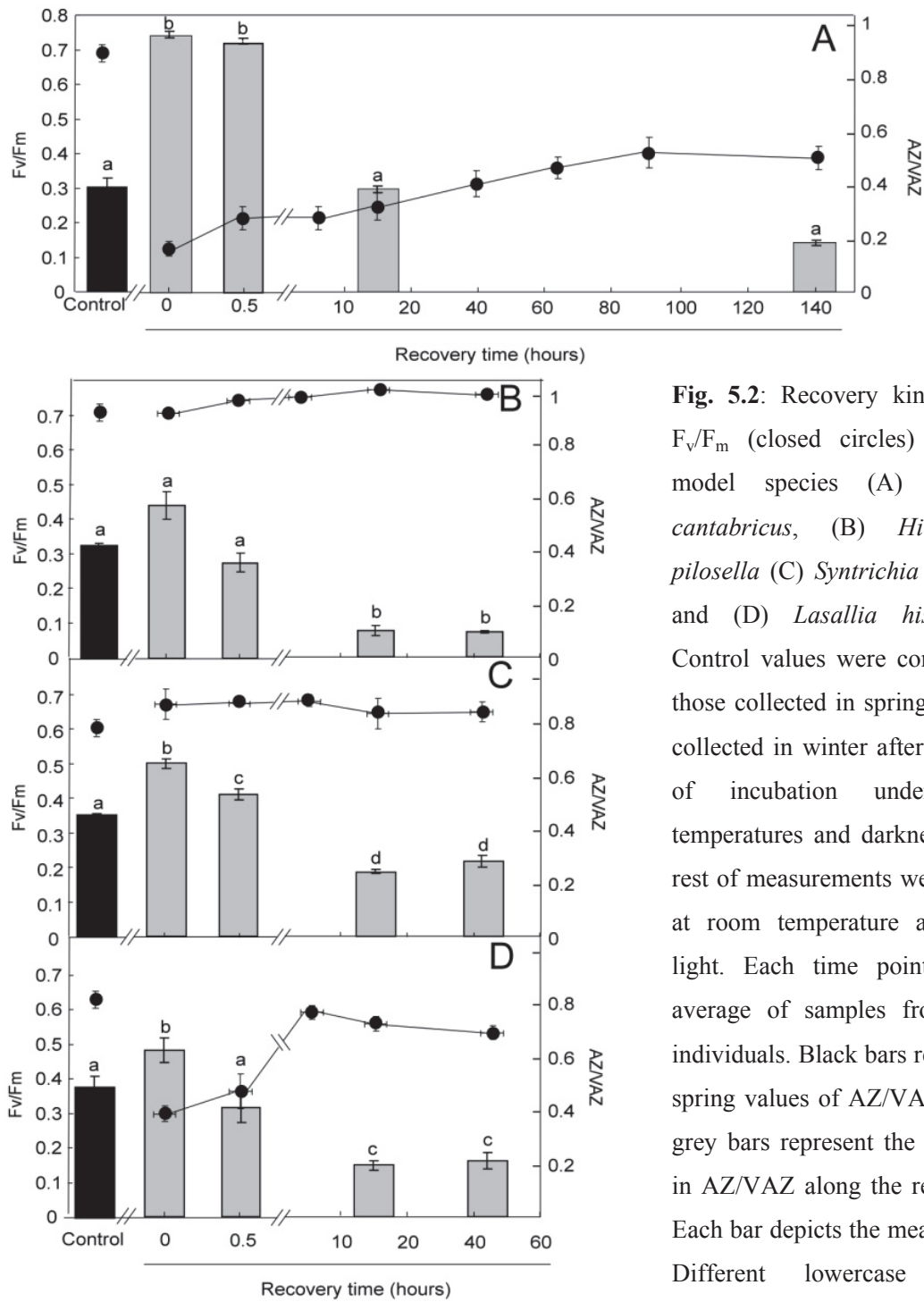
#### **5.3.4 Recovery kinetics: Which are the differences between functional groups?**

Recovery from winter conditions was studied in four model species representative of each major functional group (the shrub *Cytisus cantabricus*, the perennial herb *Hieracium pilosella*, the moss *Syntrichia muralis* and the lichen *Lasallia hispanica*). For that purpose, thalli, branches or leaves (depending on the species) were transferred to the laboratory. Then, their recoveries at 20°C and dim light were followed by measuring  $F_v/F_m$  and AZ/VAZ content. Interestingly, under spring conditions, the model species of all functional groups presented similar values of de-epoxidation index (0.3-0.37) and, during winter conditions, this value increased moderately in the herb, the moss and the lichen (0.45-0.5) but dramatically in the woody plant (0.75) (Fig. 5.2). Additionally, the recovery kinetics detected in model species could be classified following three major patterns: (i) that observed in the woody species (*C. cantabricus*), which showed mainly the slow component (in 92 hours they only recovered the 30% of control  $F_v/F_m$ ) (Fig. 5.2A) (ii) that found in the moss *S. muralis* and the herbaceous *H. pilosella* that did not present any recovery because they were not photoinhibited (Fig. 5.2B and C) and (iii) the pattern shown by the lichen *L. hispanica* which displayed only the rapid component, being the 50% of control  $F_v/F_m$  value raised in 7 hours under recovery conditions (Fig. 5.2D).



**Table 5.2:** Correlation coefficients (r) between the different types of photoinhibition (chronic photoinhibition (WPI<sub>>12h</sub>), dynamic photoinhibition (WPI<sub><12h</sub>) and total photoinhibition (WPI<sub>all</sub>)) and the ratios of carotenoids and tocopherols per chlorophyll. The number of species included in calculations was 14 for woody plants, 20 for herbaceous plants, 5 for mosses and 9 for lichens. Asterisks denote significant correlations (hyphen '-': P ≥ 0.05, \*: P < 0.05, \*\*: P < 0.01, \*\*\*: P ≤ 0.001).

	Woody			Herbaceous			Mosses			Lichens		
	WPI all	WPI >12h	WPI <12h	WPI all	WPI >12h	WPI <12h	WPI all	WPI >12h	WPI <12h	WPI all	WPI >12h	WPI <12h
V/Chl	-	-	-	0.456 ***	0.332 **	-	-	-	-	-	-	-
A/Chl	-	-	-	0.398 **	0.286 *	-	-	-	0.779 *	-	-	-
Z/Chl	0.296 *	-	-	-	-	-	-	-	-	-	-	-
AZ/Chl	-	-	0.293 *	0.241 *	-	-	-	-	-	-	-	-
VAZ/Chl	-	-	-	0.471 ***	0.335 **	-	-	-	-	-	-	-
AZ/VAZ	0.416 *	-	-	-	-	-	-	-	-	-	-	-
Neo/Chl	-	-	-	-	-	0.204 *	-	-	-	-	-	0.499 *
<i>Trans</i> Neo/Chl	-	-	-	-	-	-	-	-	-	-	-	0.666 **
L/Chl	-	-	-	-	-	-	-	-	-	-	-	0.480 *
Total car/Chl	-	-	-	0.33 **	0.258 *	-	-	-	-	-	-	-
Total toc/Chl	-	-	-	0.253 *	0.222 *	-	-	-	-	-	-	-

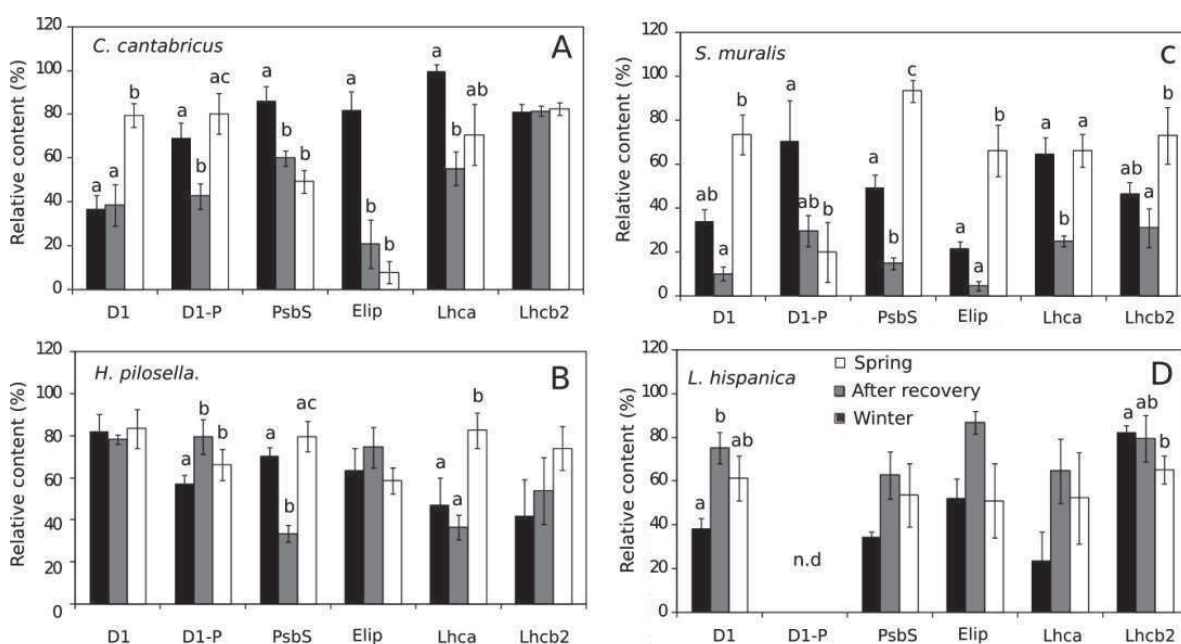


**Fig. 5.2:** Recovery kinetics of  $F_v/F_m$  (closed circles) for all model species (A) *Cytisus cantabricus*, (B) *Hieracium pilosella* (C) *Syntrichia muralis* and (D) *Lasallia hispanica*. Control values were considered those collected in spring.  $t_0$  was collected in winter after 30 min of incubation under low temperatures and darkness. The rest of measurements were done at room temperature and low light. Each time point is an average of samples from five individuals. Black bars represent spring values of AZ/VAZ while grey bars represent the changes in AZ/VAZ along the recovery. Each bar depicts the mean  $\pm$  SE. Different lowercase letters indicate significant differences at  $P < 0.05$  for each species and protein ( $n=5$ ). Different lowercase letters indicate significant differences at  $P < 0.05$ .



### 5.3.5 Changes in photosynthetic apparatus protein composition

Thylakoid protein composition was studied by western blot in the four model species collected in the field in spring and winter and also after recovery from winter conditions in the laboratory. In *C. cantabrigicus*, phosphorylated D1 protein (D1-P), PsbS, Elip and Lhca diminished significantly after 140h of recovery conditions (Fig. 5.3A). Contrasting with woody species, *H. pilosella* showed a diminution of PsbS in 41h, as well as an augmentation of D1-P (Fig. 5.3B). *S. muralis* presented also a reduction in PsbS and Lhca for the same period of time (Fig. 5.3C). In *L. hispanica*, the only significant change after recovery was the increase in D1 content (Fig. 5.3D).



**Fig. 5.3:** Relative thylakoid proteins content D1, D1-P, PsbS, Elip, Lhca, Lhcb2 in (A) *Cytisus cantabrigicus*; (B) *Hieracium pilosella*; (C) *Syntrichia muralis* and (D) *Lasallia hispanica* in winter (solid bars), after recovery treatment (grey bars) and in spring (open bars). Recovery treatment refers to optimum conditions at room temperature of 20°C and dim light during 40h for *S. muralis*, *L. hispanica* and *H. pilosella* and during 140h for *C. cantabrigicus*. Values correspond to mean  $\pm$  SE ( $n \geq 3$ ). “n.d” indicates that the protein was not detected. Different lowercase letters indicate significant differences at  $P < 0.05$  for each species and protein.

As observed during the recovery experiments under laboratory conditions, in the field, the protein composition of each species showed a characteristic seasonal pattern. In *C. cantabrigicus* the amount of Elip and PsbS proteins was higher in winter, while D1 protein was more abundant in spring (Fig. 5.3A). Unlike *C. cantabrigicus*, the moss presented the opposite trend, having higher amounts of PsbS and Elip in spring than in winter (Fig. 5.3C).

## 5.4 DISCUSSION

### 5.4.1. Diversity of photosynthetic responses to winter conditions

Photoinhibition is a frequent phenomenon whenever intense light exceeds the capacity for energy use by photosynthetic organisms (Powles and Critchley 1980). However, despite its ubiquity, photoinhibition is a term full of complexity and with a wide variability in Plant Kingdom. One of the most outstanding representations of this process is observed in winter when the so-called “chronic winter photoinhibition” ( $WPI_{>12h}$ ) restricts carbon assimilation in woody species (Adams et al. 1995; Ottander et al. 1995; Verhoeven et al. 1998; Öquist and Huner 2003; Taulavuori et al. 2011). By contrast, herbs (mainly represented in the bibliography by studies on crops) are thought to respond to winter stress by the process of dynamically reversible downregulation of PSII photochemical efficiency ( $WPI_{<12h}$ ) (Öquist and Huner 1993; Li et al. 2000). To determine the winter behavior of photosynthesis in other functional groups (mosses and lichens) in comparison to woody plants, we analyzed 50 different species comprising woody species, herbs, mosses and lichens that naturally inhabit in subalpine areas. In agreement with previous studies,  $WPI_{>12h}$  was higher in woody species than in the rest of functional groups. Nevertheless, it is noticeable that 4 herbaceous species (*Digitalis parviflora*, *Festuca* sp., *Saxifraga panaliculata* and *Thymelaea* sp.), which interestingly are not ecologically or phylogenetically related, presented a  $WPI_{>12h}$  around 20%.





Contrasting with woody species and the herbs mentioned above, lichens, bryophytes and most herbaceous species presented very low WPI. It is difficult to ascribe this strategy to a defined pattern since, despite their substantial contribution to the primary production in boreal and alpine ecosystems, these groups are not well represented in WPI studies, as was shown in a recent literature compilation (Míguez et al. 2015). It should be noted that most mosses and lichens present the peculiarity of drying out regularly, precluding metabolic activities (Heber et al. 2000). Their capacity to tolerate desiccation when they are frozen, is an advantage under winter conditions (Lenné et al. 2010). Furthermore, most of the mosses and lichens are comparatively small and commonly grow in cushions, tussocks or rosettes. These growth forms reduce the mechanical effects of wind and temperature stress, creating an appropriate microclimate (Körner 1999). By contrast, the majority of woody plants are taller, thus, they are exposed to much lower temperatures and higher irradiances. Furthermore, in air-exposed organs, such as tree branches, air embolisms in the xylem are very common due to freeze-thaw cycles (Sperry and Sullivan 1992; Mayr et al. 2002) leading to tissue injury or even death. To prevent this damage, evergreen woody species enter in a state of reduced photosynthetic activity and stomatal closure associated with cessation of water and carbohydrate transport (Adams et al. 2004).

#### **5.4.2. Role of carotenoids and tocopherols on WPI**

Around late 1980s and early 1990s, Demmig et al. (1987, 1988) provided the first evidence of an involvement of the V-cycle in NPQ, particularly in its sustained forms ( $WPI_{>12h}$ ) (Demmig-Adams and Adams 2006), in plants living in montane and subalpine areas in Colorado. Later, many other studies have corroborated these observations (for a recent compilation see Míguez et al. (2015)). In the present study, taking advantage of the large number of species analyzed (50), we aimed to verify

whether there is a correlation between V-cycle de-epoxidation and WPI (Table 5.2).

Contrary to our expectations, in herbs,  $WPI_{>12h}$  was more strongly correlated with the content of V-cycle xanthophylls than in woody species (Table 5.2). This could be due to the fact that in each species, the components which affect NPQ (Z concentration, reduction in antenna cross-sections, aggregation of light harvesting proteins, the photoinhibition of reaction centres or the accumulation of specific families of proteins such as Elip or Ohps (Ensminger et al. 2004)), may have different quantitative importance. Alternatively, this low correlation in woody plants could be explained by the fact that few Z molecules are enough to generate the maximum induction of NPQ (Ruban et al. 2002). Hence, one species could be strongly chronically photoinhibited with low concentration of Z. In summary, our results indicate that WPI of herbaceous species relies more on the total content of V-cycle xanthophylls, while WPI of woody species correlates better with the de-epoxidation state of V-cycle (Table 5.2). On the other hand, contrary to the results of Barták et al. (2003), we did not find any correlation between WPI and the de-epoxidation state of V-cycle pigment pool in lichens. Nevertheless, we detected a correlation connecting  $WPI_{<12h}$  and other xanthophylls such as L and Neo in this group.

Previous studies have examined seasonal changes in leaf antioxidant systems in different woody evergreens growing in seasonally cold environments (Esterbauer and Grill 1978; Demmig et al. 1988; Polle and Rennenberg 1992; Doulis et al. 1993; Logan et al. 1998). There is a considerable variation between species, but most of them increase the activities of at least some of the antioxidant enzymes and metabolites during winter. This assumption is supported by this study. Hence,  $\beta$ -car, which is able to quench singlet oxygen ( $^1O_2$ ) (Burton and Ingold 1984), was higher in most of herbs and woody plants during winter, especially in *Pinus*



species (Appendix 6, 7). The same trend has been described previously in broadleaf evergreens such as *Quercus ilex* (Corcuera et al. 2005). One of the most general trends observed among vascular and non vascular plants included in this study, was the increase of  $\alpha$ -toc in winter with respect to spring (Appendix 5, 6, 7). This tendency was maintained even in the species where WPI was close to 0. This suggests that the role of  $\alpha$ -toc in the stabilization of the thylakoid membrane and the prevention of lipid peroxidation (Verhoeven et al. 2005; DellaPenna and Pogson 2006) could be important in LT acclimation, as has been shown in non-alpine model species (Leipner et al. 1997; Szymańska and Kruk 2008). In non vascular plants, there is not a general trend in carotenoids and tocopherols accumulation during winter season (Appendix 7). In fact, in mosses and some lichens, the amount of antioxidants was higher during spring, suggesting that LT do not represent a severe stress. But during spring, other stresses as episodes of drying cause the accumulation of antioxidant components.

#### **5.4.3 How do different functional groups recover from winter photoinhibition?**

The kinetics of recovery of photosynthetic activity during winter deacclimation have been characterized in conifers by Verhoeven et al. (1998, 2009) and Verhoeven (2013) but little is known about other groups. In those studies, it was shown the existence of two phases in the recovery process when plants are transferred from field winter conditions to optimal chamber environment: a fast component that appears in leaves in early winter or in shade acclimated organisms, and a slow component. The fast phase is reversible within minutes to hours while the slow component needs several days and involves the retention of AZ/VAZ and a thylakoidal protein reorganization (Verhoeven 2013). In the present study a similar recovery protocol was applied not only to a woody plant but also to

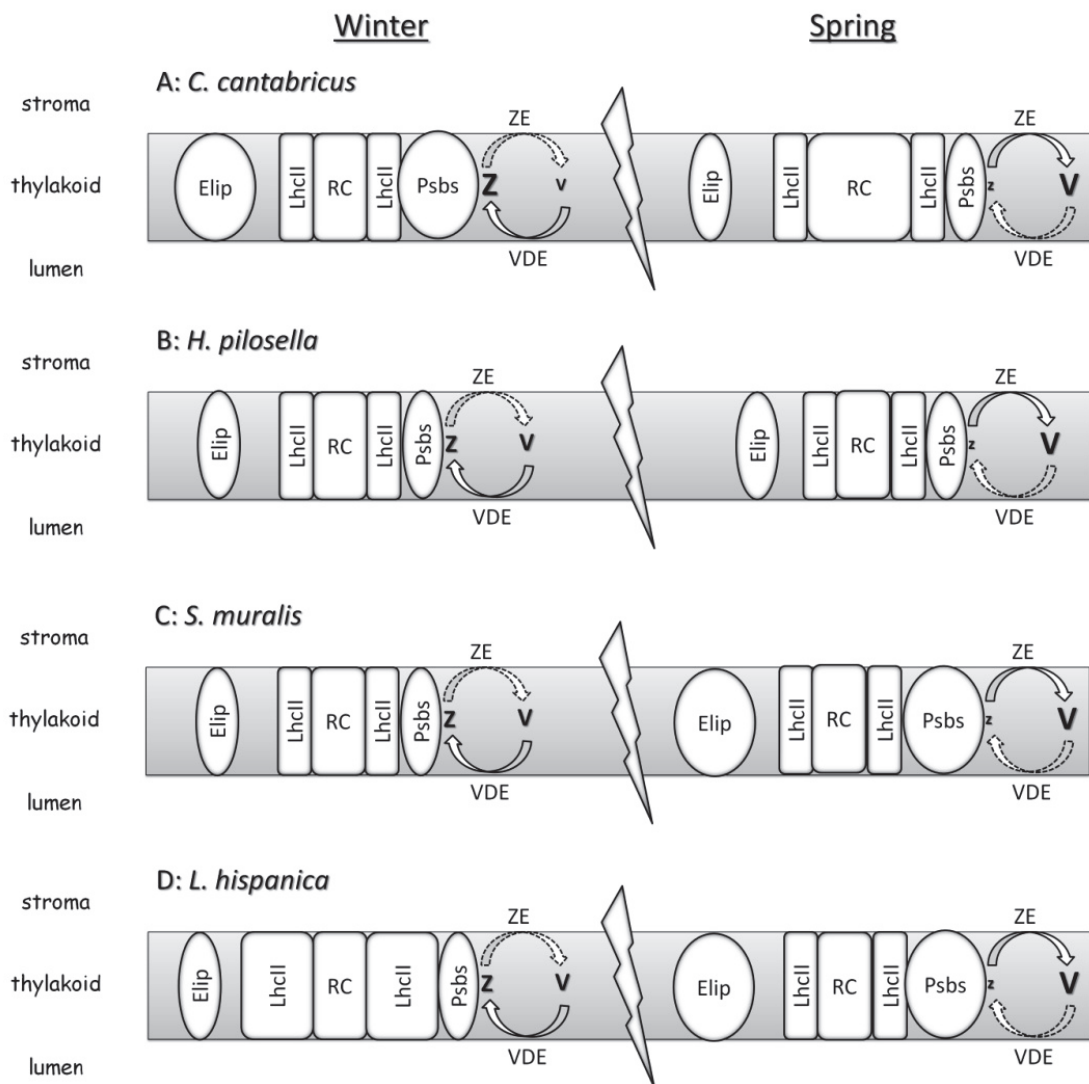
representatives of the other functional groups (Fig. 5.2). As described in conifers, *C. cantabricus*, a shrub that maintains photosynthetic stems in winter, showed both a rapid and a slow component during the recovery process on its stems. Despite the long incubation period (140 hours) recovery rate was so slow that restoration of photosynthetic activity was not complete, even when the relaxation of V-cycle was much earlier completed. These results are comparable with those of the study of Verhoeven (2013) carried out in conifers during late winter season. Contrastingly, the other studied species presented only the fast component (*L. hispanica*) or no WPI at all (*H. pilosella* and *S. muralis*). Irrespective of their level of WPI, all these species showed initially a high AZ/VAZ that recovered at the same rate in these three species. Hence, only *L. hispanica* presents a rapid recovery of  $F_v/F_m$  and AZ/VAZ. The existence of such uncoupling between AZ/VAZ and WPI in *H. pilosella* and *S. muralis*, agrees with a recent meta-analytic study (Míguez et al. 2015) that showed that in absence of WPI, the values of AZ/VAZ can vary from 0 to 0.9. So, although low values of AZ/VAZ indicate the absence of WPI, the presence of high AZ/VAZ content does not assure WPI. Other protective roles of Z different from the modulation of NPQ, such as antioxidant or membrane stabilizer might justify this discrepancy and remark the importance of de-epoxidised xanthophylls under winter conditions (Havaux et al. 2007; Dall'Osto et al. 2010).

#### 5.4.4 Thylakoid protein composition during winter acclimation

Winter changes in thylakoid protein composition during deacclimation have been precisely characterised in conifers, but little is known about other groups. The main changes described in those woody plants, involve a diminution of D1 protein and an increase of Elips during winter. Nevertheless, in the case of Lhcb, Lhca and PsaA, when different studies are considered, there is not a consistent pattern (Verhoeven 2013).



In the present study, the process of de-acclimation was characterized by the study of some key thylakoid proteins: D1 and Lhcb2 which are proteins from the reaction center and the antennae of PSII respectively (Vener et al. 1998), PsbS which is directly involved in the regulation of NPQ (Alboresi et al. 2010) and Elips which are a family of stress related proteins (Levy et al. 1993) (Fig. 5.4). In the shrub *C. cantabricus* the degradation of D1 in winter was accompanied by an upregulation of PsbS and Elip protein as well as *de novo* synthesis of Z (Fig. 5.4 and Appendix 6,7). This is consistent with previous results (Demmig-Adams et al. 2006; Verhoeven 2014), and it indicates the development of a protective sustained down regulation of photosynthesis as well as the deactivation of reaction centres and the upregulation of stress related proteins. It must be considered that *C. cantabricus* is a woody plant which loss their leaves but maintains its stems photosynthetically active during winter. So, this protein analysis was performed in green stems. A previous study analyzing the stems of *Viscum album* in winter and spring (Míguez et al. 2014), came to the same conclusions so, presumably, photosynthetic stems and leaves follow the same seasonal pattern in terms of thylakoid protein changes. *L. hispanica* growing in the field seemed not to be under the most optimal conditions, as it was deduced from the fact that the highest D1 content occurs after recovery under controlled conditions instead on the field. The most plausible reason is that in the field, the hydration-dehydration cycles are constant while in the laboratory conditions, they were constantly maintained at 100% of humidity. Besides, *L. hispanica* presented higher Elip and PsbS content in spring indicating that this season is more harmful than winter. In *H. pilosella*, consistently with the absence of WPI, protein content was seasonally stable. However, this is not the case of all herbaceous species, as has been shown in *Colobanthus quitensis*, where D1 degradation was detected when it was subjected to LT treatment in a growth chamber (Bascuñán-Godoy et al. 2012).



**Fig. 5.4:** Schematic representation of thylakoid protein composition and V-cycle for model species (A) *Cytisus cantabricus*, (B) *Hieracium pilosella*, (C) *Syntrichia muralis* and (D) *Lasallia hispanica* in winter and spring. The size of V-cycle letters and of protein boxes illustrates the amount of each component in winter or spring.

Not only the amount of each protein is relevant, but also their structural organization in thylakoid membrane (Johnson et al. 2011) as well as their post-translational modifications. For example, it is known that D1 is subjected to phosphorylation under stress (Koivuniemi et al. 1995). This small biochemical change provokes modifications in structure of photosynthetic apparatus that are of paramount importance because: (i) induce the reduction of oxidative damage in membrane proteins; (ii) cause



the diminution of ROS generation (Chen et al. 2012) and (iii) prevent the degradation of D1 protein (Aro et al. 1992; Koivuniemi et al. 1995). In this study, D1-P was detected in all model species except in the lichen. As far as we know, there are not studies which analyze D1-P in lichens, but there are for algae (Turkina et al. 2006). Algae also present phosphorylation of D1 under stress, so more studies in lichen photosynthetic proteins under different scenarios are needed to determinate if lichenized algae behave in the same way than free living algae.

It was thought until recently that PsbS protein was absent in algae, where Lhcsr protein plays similar roles (Li et al. 2000; Peers et al. 2009) while in bryophytes, such as *Physcomitrella patens*, both PsbS and Lhcsr coexist (Alboresi et al. 2010). In our study, PsbS was detected in all species, diminishing after recovery, except in the case of lichen *L. hispanica*, where PsbS was detected but it was independent on stress. This lichen is formed by a fungus and a streptophycean green alga of *Trebouxia* genus. Although unexpected, the detection of PsbS protein in this algal photobiont, agrees with more recent studies in which PsbS protein has been found in the tidal chlorophycean macroalgae *Ulva prolifera* (Mou et al. 2013) and in other streptophycean species (Gerotto and Morosinotto 2013).

#### **5.4.5. Concluding remarks**

After studying chronic and dynamic winter photoinhibition in 50 species (woody plants, herbs, mosses and lichens) co-occurring in three subalpine locations, as well as the recovery process from winter photoinhibition on selected species representative of each group, it is confirmed that, although much more frequent than in any other group, high levels of WPI are not exclusive of woody plants. Why the strategy is so widespread in trees and shrubs independently of their phylogenetic position or growth form remains to be understood, but a physic-mechanical reason



related with the prevention of xylem cavitation or with the growth out of the protective snow layer can be hypothesised as underlying explanations. Whatever the functional reason for the uneven distribution of WPI in each functional group, the detailed analysis provided by the present study has highlighted that the diversity of photosynthetic acclimation strategies to the unfavourable conditions generated in winter is wider than previously described. Since WPI represents a conservative strategy that lowers photosynthetic efficiency to prevent damage, future climatic scenarios of warmer winters could limit the effectiveness of these mechanisms, altering the ecological relationships in mountain and subalpine ecosystems in detriment of woody species.

**Author contributions:** JIGP, FM and JMB originally formulated the idea. BFM, JIGP and FM conceived the experiments. BFM and FM developed methodology. FM analyzed the data. FM, JIGP, BFM and JMB wrote the manuscript.

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## 6. DOES AGE MATTER UNDER WINTER PHOTOINHIBITORY CONDITIONS? A CASE STUDY IN STEMS AND LEAVES OF EUROPEAN MISTLETOE (*VISCUM ALBUM*)

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**Abstract:** European mistletoe (*Viscum album* L.) is a hemiparasitic plant with perennial leaves and photosynthetic stems easily discernible according to their age. These properties make *V. album* the perfect species to (i) compare the mechanisms of seasonal acclimation of photosynthetic stems with those of leaves, and (ii) evaluate the influence of ageing in the efficiency of photosynthetic tissues. To achieve these general objectives, photosynthetic pigments, maximal photochemical efficiency of PSII ( $F_v/F_m$ ), recovery kinetics and key thylakoidal proteins were analysed during winter and spring in leaves and at different age stems. During winter, some woody species are able to maintain photosynthetic activity, but at lower rates than during spring. In the case of *V. album*, photosynthetic relevance of green stems appears equal to leaves in terms of total area. Besides, mistletoe stems are able to maintain higher  $F_v/F_m$  and lower level of antioxidants than leaves, especially during winter season. The recovery from winter photoinhibition is also faster in stems than in leaves. Thylakoid protein composition (mainly high levels of D1) also supports the idea of stems as main photosynthetic organs in *V. album* during winter. Further, in winter, the level of photoinhibition of *V. album* stems decreased concomitantly with ageing. This work highlights the importance of stem photosynthesis in plant carbon balance and demonstrates that ageing does not necessarily imply a loss of vitality in stems.



## 6.1 INTRODUCTION

European mistletoe (*Viscum album* L.) is a hemiparasitic plant widely distributed across the European continent, with a latitudinal range between 35 and 60°N (Zuber 2004). Low winter temperature is the main factor which limits the distribution of mistletoe in latitude and altitude (Jeffree and Jeffree 1996). Indeed, climate warming studies predict an upward shift in altitude limit that, for example, has been documented in Switzerland (Dobbertin et al. 2005). Mistletoes obtain water from host trees (Escher et al. 2004), maintaining a high rate of transpiration. At the same time, their photosynthetic rates are typically similar or lower than those of their hosts (Lüttge et al. 1998; Strong et al. 2001). These traits partly resemble the syndrome of shade acclimation when mistletoe leaves are compared with sun leaves from their hosts (Strong et al. 2000). In mistletoes, not only leaves but the entire surface is photosynthetically active, with functional chloroplasts being present in unusual locations such as the haustorium (Fineran 1995), stem bark and leaf epidermis (Zuber 2004). Several subspecies of *V. album* have been described, being *V. album* subsp. *album* L. (*V. album*) the only growing in deciduous angiosperms (Zuber 2004), whereas the others parasite conifers. In contrast with their evergreen-parasiting relatives, subspecies *album* is exposed to dramatic changes in light environment, in parallel with the phenological leaf cycles of their hosts. Thus, in spite of the lower sun zenith angle in winter, leading into a light intensity decrease, *V. album* has to tolerate a drastic increase in irradiance, after leaf shedding. Concomitant with this, there is a decrease in temperature. This combination of environmental factors induces a reduction in photochemical efficiency, referred to as winter photoinhibition (WPI). This mechanism results from the slowing down of enzymatic processes under low temperatures (Falk et al. 1996). This reduction in enzymatic activity provokes that part of the light that arrives at the photosynthetic apparatus cannot be used for CO<sub>2</sub> fixation. This situation leads to photo-oxidative damage or cell death (Adams et al. 2004). To cope with this light excess, plants increase the level of thermal energy dissipation during



winter. Two modes of thermal energy dissipation, which differ in their kinetics of recovery upon transfer to optimal conditions, have been described (Verhoeven 2014). One is rapid, and reverses in minutes to hours; the other is sustained, and requires several days to recover. The latter is frequent among conifers and other winter evergreens, and implies dramatic changes on thylakoid proteins and photosynthetic pigments composition (Ottander and Oquist 1991; Ottander et al. 1995; Verhoeven et al. 1999).

Despite being exposed to this combination of stressful conditions during winter, mistletoes remain physiologically active, maintaining transpiration even when the host has shed its leaves (Ziegler et al. 2009). During the coldest periods, the activation of protective mechanisms, such as the accumulation of cryoprotective lectins (Hincha and Pfu 1997) or the sustained activation of thermal energy dissipation, coupled to the activity of violaxanthin (V-cycle) and lutein epoxide cycles (Lx-cycle). These processes allow the maintenance of photosynthetic activity in *V. album* leaves (Matsubara et al. 2001). However, whether these mechanisms operate also in photosynthetic stems, which are also exposed to the same harmful combination of high light (HL) and low temperatures (LT) during winter has not been previously reported. In addition, photosynthetic stems are also exposed to additive ageing effect, as they can survive for more than 30 years.

There are two main theories that try to explain the effects of ageing in perennial species. Some authors propose that in perennials, ageing is a synonym of downregulation of photosynthesis and other physiological rates that lead to an increase in photo-oxidative damage as well as in a diminution of growth (Day et al. 2002; Munné-Bosch and Lalueza 2007). The other current of thought supports the theory of negative senescence, in which the main idea is that survival increases with age (Vaupel et al. 2004; García et al. 2011). It is also known that ageing induces a plethora of modifications in foliage structural and physiological characteristics. Some of them are changes in cell size, tissue composition and leaf thickness (Yamashita et al. 2002; Fleming 2005), as well





as changes in internal architecture including cell-wall porosity, among others. These changes also affect photosynthetic rates and are of particular importance in evergreen species which need to maintain photosynthetic tissues for several growing seasons (Niinemets et al. 2004).

Since mistletoe stems are divided in segments that correspond with the growing years, their age can be easily estimated, making them a perfect model for the study of ageing in photosynthetically active tissues. Furthermore, as a parasitic plant on deciduous hosts, its light environment changes dramatically with sprout or fall of its host leaves. So, in the case of *V. album*, winter implies changes not only in temperature but also in light intensity and quality. Therefore, studies with *V. album* are able to provide additional insights into the process of seasonal acclimation of photosynthetic tissues. Overall, the aims of the present work were to (i) study in depth the mechanisms of seasonal acclimation and recovery in stems; (ii) check whether seasonal acclimation mechanisms are the same in stems than in leaves, and (iii) verify whether ageing has influence on the ability of mistletoe plants to cope with winter conditions, in terms of photochemical efficiency.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Site description, plant material and experimental design

We investigated naturally occurring mistletoes (*Viscum album* subsp. *album* L.) growing on hawthorn trees (*Crataegus monogyna* L.). The host is a deciduous shrub or tree (4–12m) whose leaves sprout in April/May and fall in October/November. The experimental site was located in Monte Santiago Natural Monument (Burgos, northern Spain, latitude 42°56'27.69''N long 3°0'3.18''W, altitude 900m above sea level). The site is characterised by a temperate oceanic climate, with annual rainfall 1116mm and mean temperatures ranging from 16°C in August to 2.7°C in February. Field experiments were conducted in late winter (when there is a combination of HL and LT) and late



spring (before the occurrence of any episode of summer drought) during the year 2013.

To analyse the influence of winter photoinhibition (WPI), 7 south facing individuals of *V. album*, were randomly selected for measurement/sampling. In each individual, leaves and stems were sampled during winter and spring. Entire leaves and chlorophyllous tissue of stems (~100 mg) were sampled in the field or in the growth chamber, and immediately frozen in liquid nitrogen and stored at -80°C until their analysis of pigments, tocopherols and proteins.

To evaluate which mode of thermal dissipation (rapidly reversible or sustained) is developed by *V. album*, recovery kinetics were studied in 1-year-old branches and 1-year-old leaves of at least 10 individuals. During winter and spring, branches were cut under nutritive solution to avoid cavitation and placed in the same solution under dim light ( $30 \text{ mmolm}^{-2}\text{s}^{-1}$ ) and room temperature (20°C) until they were totally recovered (90h in winter and 120 h in spring). During recovery, photochemical efficiency of PSII ( $F_v/F_m$ ) was monitored at different times. Leaves and 1-year-old stems for pigments and protein analysis, were sampled after the first (30min) and the last  $F_v/F_m$  measurement (90h in winter and 120h in spring).

To analyse the effect of ageing in photosynthetic apparatus performance, leaves from different cohorts and stems at different ages (1–8 years old) were also measured *in situ* after 30 min under dark conditions.

### 6.2.2 Estimation of stem and leaf photosynthetic area

To estimate the photosynthetic area per plant, cylinder and ellipse formula were used as models of stems and leaves area respectively (See materials and methods section Fig.3.6). In the field, the diameter and the length of different age stems, as well as the width and length of leaves of two cohorts, were measured in eight individuals with a gauging device in one representative branch per individual. The total number of leaves and stems of each age class were also counted in each individual, and the total photosynthetic area was estimated as the sum of the areas of all leaves and all stems.



### 6.2.3 Fluorescence measurements

To analyse the excitation state of PSII, before the collection from the host branch, samples (leaves and stems) were dark adapted *in situ* with leaf clips during at least 30 min. Afterwards, chlorophyll a (Chl *a*) fluorescence was measured using a portable modulated fluorometer PAM 2500 (Walz, Effeltrich, Germany). The maximum Chl *a* fluorescence yield ( $F_m$ ) was induced with a saturating pulse (7795 mmol photons  $m^{-2}s^{-1}$ ) while minimum fluorescence ( $F_o$ ) was recorded at low light intensities. The maximal photochemical efficiency of PSII ( $F_v/F_m$ ) was calculated as  $(F_m - F_o)/F_m$ .

### 6.2.4 Gas exchange measurements

Gas exchange was measured *in situ* in five *V. album* individuals, using a portable photosynthesis system (LCA-4, ADC BioScientific Ltd, Kirrawee, NSW, Australia) in winter and spring. Photosynthetic light response was recorded at 300–350 mmol photons  $m^{-2}s^{-1}$  of active radiation. The air temperature during the measurement was around 23°C in spring and 11°C in winter.

### 6.2.5 Pigment and tocopherol analysis

Frozen samples were homogenised with a mortar in pure acetone solution buffered with  $CaCO_3$ . The extracts were centrifuged at 16100g for 20min, and supernatants were filtered with 0.2mm PTFE filters (Teknokroma, Barcelona, Spain). Chlorophylls *a* + *b* (Chl), carotenoids and tocopherols separation were performed by HPLC with a reverse phase C18 column (Spherisorb ODS1, 4.6x250 mm, Waters, Milford, MA, USA) with a photodiode array (PDA) detector, following the method by (García-Plazaola and Becerril 1999, 2001).

### 6.2.6 Protein extraction and characterisation

The proteins examined in this study were D1 (PSII core complex protein), Lhcb2 (antenna protein from PSII), PsbS (essential protein for thermal dissipation), early light induction protein (Elip, closely related to stress). All the antibodies were from Agrisera AB (Vännäs, Sweeden). The extraction, thylakoid



isolation and SDS-page were conducted as in work by (Sáez et al. 2013). Immuno-detected proteins were developed by enhanced chemiluminescence ECL Plus (GE Healthcare, Uppsala, Sweden) through CHEMIDOC XRS system (Bio-Rad, Hercules, CA, USA). Densitometric measurements for the quantification of band intensity were conducted using Quantity One (Bio-Rad) software.

### 6.2.7 Statistics

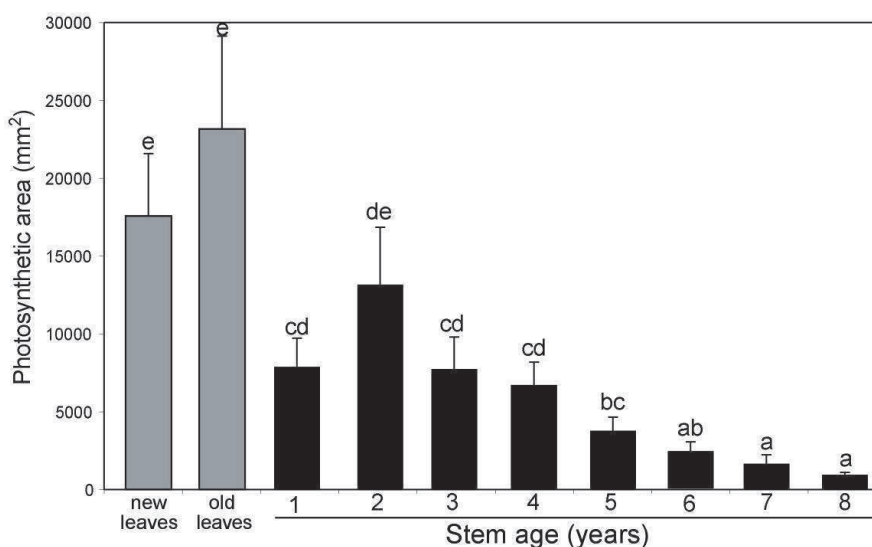
Kolmogorov–Smirnov and Levene tests were used to test for the normality of data and homogeneity of variances respectively. One-way ANOVA test was used to check for differences in  $F_v/F_m$  and pigment contents. In the case of pigments, tocopherols and protein data, *t*-student was used to elucidate possible significant differences. Duncan *post-hoc* test was performed to discriminate  $F_v/F_m$  values among different organs and ages. When necessary, data were log or root transformed. A linear regression was used to analyse the relationship between  $F_v/F_m$  and age. Significant differences were assumed at  $P < 0.05$ . All analyses were performed using the SPSS 17.0 statistical package (SPSS, Armonk, NY, USA).



## 6.3 RESULTS

### 6.3.1 Stems/leaves partitions of photosynthetic area in *Viscum album*.

Both leaves and stems of *V. album* contain Chl and present stomata in the epidermis (data not shown). Fig. 6.1 shows the relative contribution of leaves and stems to the overall photosynthetic area of the plant. We noted that the photosynthetic area of stems, measured in 1- to 8-year-old plants is almost the same (47% of total photosynthetic area) as that of the leaves (53%). The number of old and new leaves was  $59 \pm 14$  and  $118 \pm 25$  respectively.



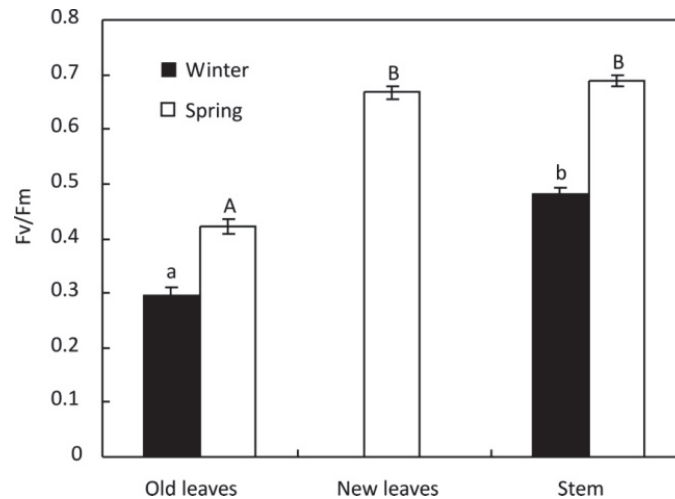
**Fig. 6.1.** Total photosynthetic area of leaves (from two different cohorts) and stems of different age in 8 years old mistletoe plants in spring. Each bar represents the average  $\pm$  s.e. ( $n = 8$  individuals). The letters above the columns indicate significant differences among ages ( $P < 0.05$ ).

### 6.3.2 Effects of seasonality in photochemical efficiency of leaves and stems

In Table 6.1, leaf net  $\text{CO}_2$  assimilation during winter and spring is shown. Net  $\text{CO}_2$  assimilation was active during winter and spring, although spring values were more than 2-fold higher than those of winter. In agreement with this, photochemical efficiency of PSII ( $F_v/F_m$ ) decreased during winter in leaves and stems (Fig. 6.2). Nevertheless,  $F_v/F_m$  was higher in stems than in



leaves, independently of the season. Overall, leaves and stems behaved differently mainly when both organs were examined under the perspective of the seasonal acclimation of the photosynthetic apparatus.



**Fig. 6.2.** Maximum photochemical efficiency of PSII ( $F_v/F_m$ ) in old leaves (1-year-old leaves), new leaves and stems during spring and winter. Each bar represents the mean  $\pm$  s.e. (in leaves  $n \geq 17$ ; in stems  $n \geq 84$ ). In the case of stems, the average of segments of different ages is shown. Upper case letters and lower case letters above the columns indicate significant differences among organs in spring and in winter respectively ( $P < 0.05$ ).

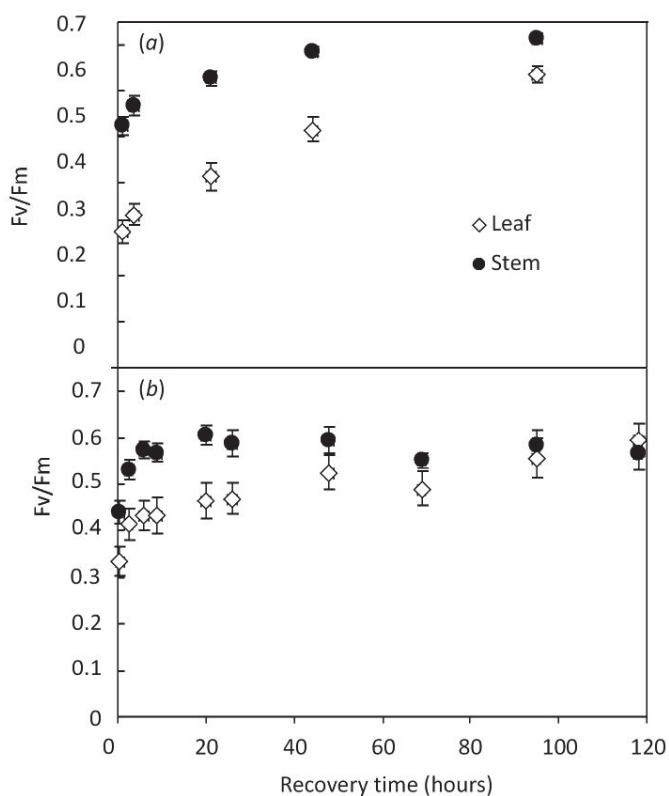
**Table 6.1:** Photosynthetic assimilation in spring and winter in sun leaves of *Viscum album* and its host (*Crataegus monogyna*) ( $n=5$ ).

Season	Organ	CO <sub>2</sub> assimilation ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )
Spring	Host leaves	6.41 $\pm$ 0.50
Spring	New leaves	3.99 $\pm$ 0.14
Spring	Old leaves	4.86 $\pm$ 0.39
Winter	Old leaves	1.81 $\pm$ 0.21



### 6.3.3. Effects of recovery on chlorophyll fluorescence parameters, pigment composition and photosynthetic apparatus structure

With regards to recovery kinetics, 1-year-old branches and leaves were recovered at room temperature and dim light from winter stress. Along all recovery time, stems presented higher  $F_v/F_m$  values than leaves (Fig. 6.3). At the same time, Fig. 6.3 shows that recovery patterns differed between winter and spring (Fig. 6.3 a, b respectively). Thus, the rapid component of recovery (see ‘Introduction’) was more conspicuous during spring, while the slow component persisted for longer in winter. As a result of the additive effect of both components, final  $F_v/F_m$  after last measurement was higher in winter than in spring. It was also higher in stems when they are compared with leaves.



**Fig. 6.3.** Recovery kinetics at room temperature of photochemical efficiency of PSII ( $F_v/F_m$ ) in 1-year-old stems (closed symbols) and leaves (open symbols) collected in the field in (a) winter and (b) spring.  $t = 0$  was measured in the field after at least 30 min of dark acclimation. Each time point is an average of samples ( $n=10-18$ ).





In parallel with recovery of photochemical efficiency, pigment composition also changed in leaves and stems. Thus, VAZ pool (violaxanthin (V) + anteraxanthin (A) + zeaxanthin (Z)) shifted from a highly deepoxidised state to an almost complete epoxidation during the incubation (Fig. 6.4 a–d). What is more, after 90–120h of recovery, Z content totally disappeared in leaves whilst in stems, the content was lower than 10 mmol mol<sup>-1</sup> Chl (Fig. 6.4 c). In parallel to Z decrease, there was a rise in lutein epoxide (Lx) content (Fig. 6.4 e, f).

Thylakoidal proteins composition was also analysed in the same samples, as depicted in Fig. 6.5. The relative amount of D1 protein increased significantly in leaves although on stems the increment was almost null throughout the recovery (Fig. 6.5a). In the rest of analysed proteins (PsbS, Fig. 6.5b, Lhcb2, Fig. 6.5c, and Elip, Fig. 6.5d), leaves and stems presented a reverse trend: all these proteins increased in leaves after recovery but the contrary occurred in stems.

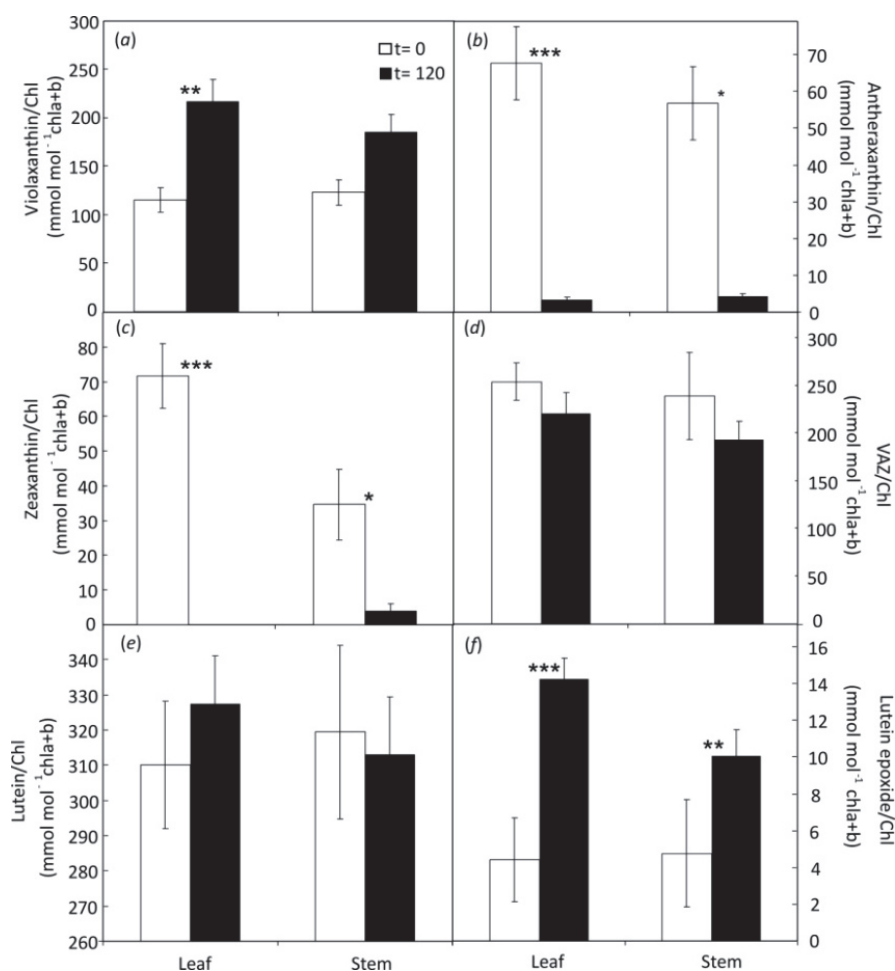
#### **6.3.4 Effects of ageing in photochemical efficiency and pigment composition of stems and leaves**

Results in Fig. 6.6 show that age had a significant influence in  $F_v/F_m$  of photosynthetic stems. Thus, this parameter increased with stem age during winter. Nevertheless, in spring, this tendency along stems age was attenuated (Fig. 6.6a). To highlight these patterns, in Fig. 6.6b data from one representative individual are depicted showing clearly the positive trend during winter, that is minimized in spring.

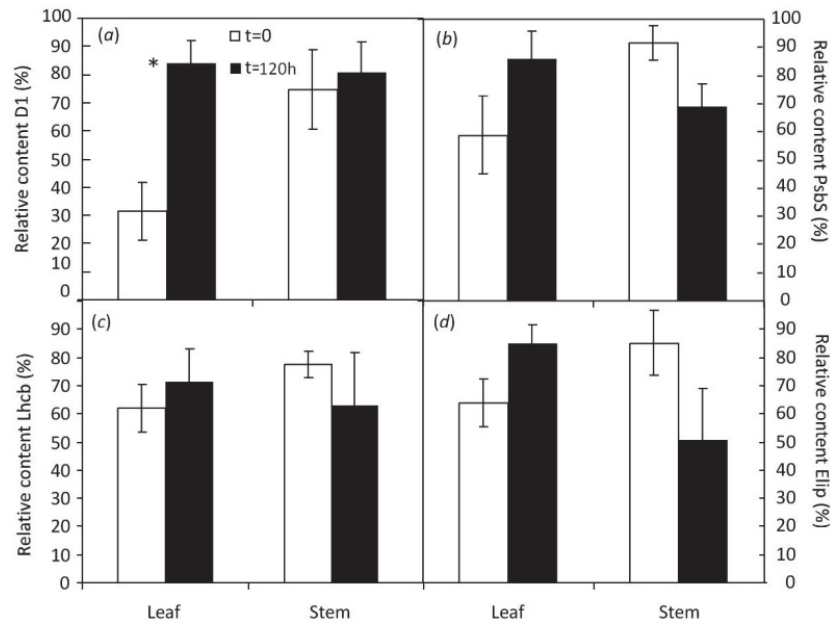
To understand the biochemical mechanism underlying these patterns of  $F_v/F_m$  changes, pigment composition was analysed in old and new leaves as well as in different stem ages. The results show that total Chl content (mmol g<sup>-1</sup> FW) was much higher in new leaves than in old leaves and stems (Appendix 9). In contrast,  $\beta$ -carotene amount (Appendix 9) was larger in stems but it did not present a clear age-dependent trend. Total neoxanthin (Neo) content was similar irrespective of organ or age in winter but it was higher in stems during spring



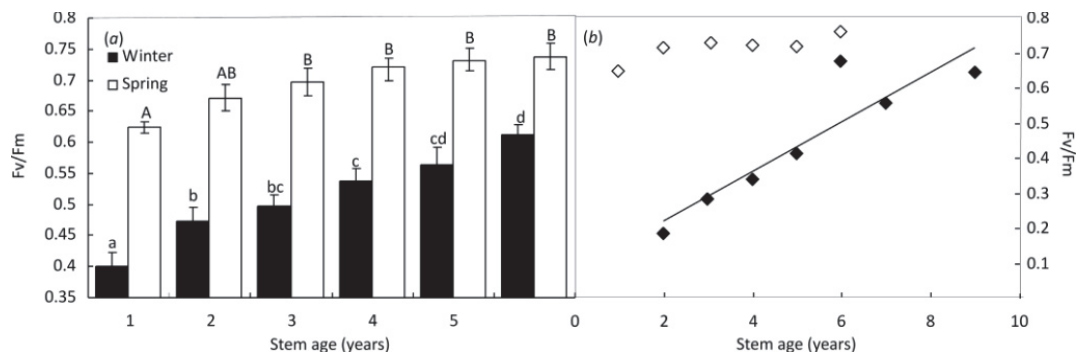
(Appendix 10). In winter, *cis*-Neo (Appendix 10) increased with age, whereas *trans*-Neo (Appendix 10) remained almost constant independently of the age. V-cycle carotenoids pool size (VAZ) and the content of deepoxidised xanthophylls (A+Z) did not differ between leaves and 1-year-old stems (Fig. 6.7a–c). Nevertheless, an increase in stem age, led to a diminution of total VAZ pool (Fig. 6.7d). Unlike V-cycle, Lx-cycle did not present a clear tendency with the age (Fig. 6.7e, f).



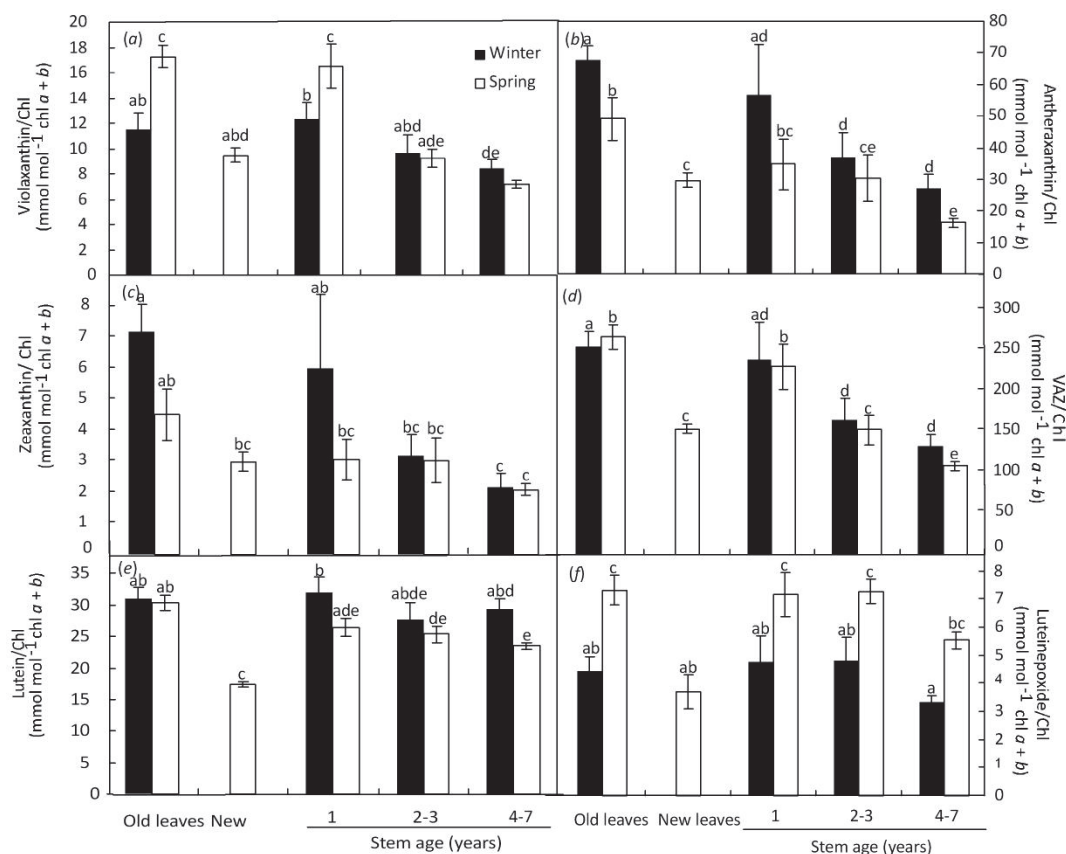
**Fig. 6.4:** Analysis of the conversion state of the V and Lx cycle in leaves and 1-year-old stems of mistletoe individuals collected in the field in winter. White bars represent samples collected at t=0 (after 30 min in darkness). Closed bars after 120 h of recovery at room temperature: (a–d) V-cycle and (e, f) Lx cycle. Each bar represents the mean  $\pm$  s.e. ( $n \geq 6$ ). Significant differences between t=0 and t=120 are indicated: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .



**Fig. 6.5:** Relative content of thylakoidal proteins (a) D1, (b) PsbS, (c) Lhcb2 and (d) Elip in leaves and 1-year-old stems of mistletoe individuals, analysed by Western Blot. Open bars represent values of samples subjected to winter photoinhibitory conditions and solid bars are values after 120 h of recovery at room temperature. Values correspond to mean  $\pm$  s.e. ( $n = 4$  except for Elip in 120 h recovery stems where  $n = 2$ ). Data are normalised values to the value in which higher intensity was obtained. Significant differences for leaves or stems between  $t=0$  and  $t=120$  are indicated: \*,  $P < 0.05$



**Fig. 6.6:** Effects of ageing on maximum photochemical efficiency of PSII ( $F_v/F_m$ ) in spring and winter. (a) Each bar represents the mean  $\pm$  s.e. of at least nine different individuals. The capital and lowercase letters above the columns denote significant differences among different age groups in spring and winter, respectively ( $P < 0.05$ ). (b) Example of  $F_v/F_m$  changes along the different age segments of the stem of a representative individual in winter (closed symbols) and spring (open symbols). The fit to a linear regression model (solid line) is shown. Correlation between age and  $F_v/F_m$  was significant only in winter (Pearson's correlation coefficient;  $R^2 = 0.825$ )



**Fig. 6.7:** Components of photoprotective cycles in leaves and stems of mistletoe during winter and spring: (a–d) V-cycle, and (e, f) Lx-cycle. Each bar represents the mean  $\pm$  s.e. (in winter,  $n \geq 4$ ; in spring  $n \geq 9$ ). The letters above the columns are indicative of significant differences among organs and different stem ages in both seasons ( $P < 0.05$ ).

When spring and winter are compared, the analyses show that the concentrations of  $\beta$ -car and total Chl were higher in spring (Appendix 9), although Neo content was higher during winter (Appendix 10). Neo has two different isoforms that are *cis*- and *trans*- in *V. album*, and the increment of Neo during winter was mainly due to the increase of the *cis*- isoform. As a consequence, *cis*-isoform (Appendix 10) is predominant in winter whereas during spring *trans*- (Appendix 10) becomes more abundant.

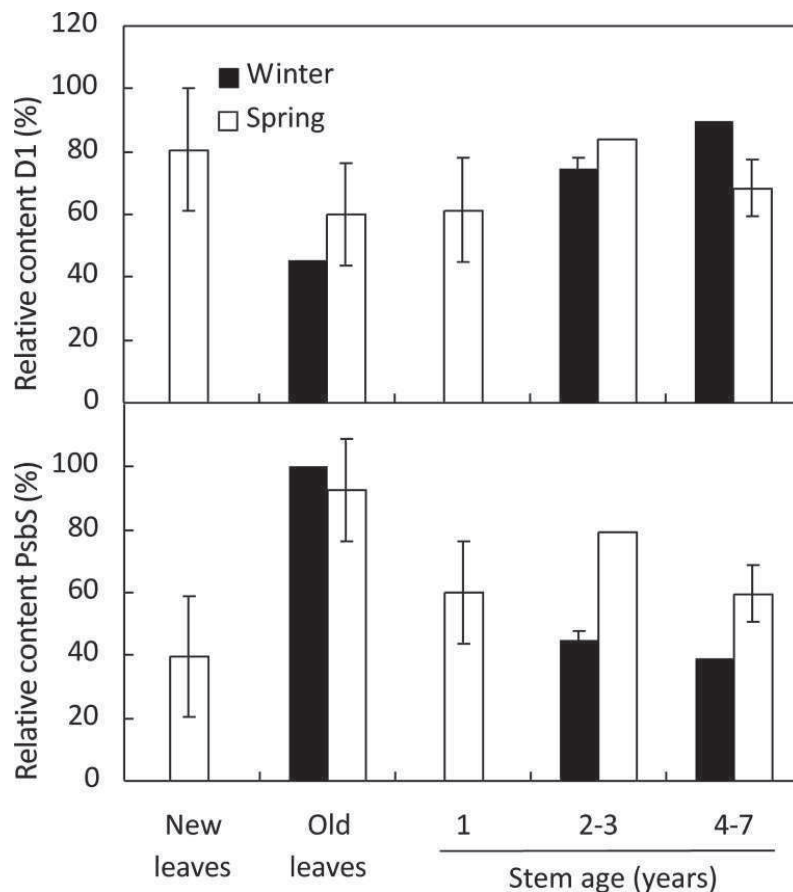
The de-epoxidation state of the V-cycle was higher during winter than in spring (Fig. 6.7b, c). These differences were markedly higher in old leaves and 1-year-old stems than in the rest of the photosynthetic tissues. In parallel with V-cycle, Lx-cycle was also active to deal with winter stress. Thus, Fig. 6.7



(e, f) shows that during winter, lutein (L) concentration was higher than in spring whereas Lx displayed the opposite pattern.

### 6.3.5 Effects of ageing in thylakoid protein composition

To characterize in deep structural changes that regulate the activation of photoinhibitory processes, D1 and PsbS levels were studied during winter and spring (Fig. 6.8). In spring D1 content (Fig. 6.8a) was constant, independently of age or organ location. Nevertheless, in winter it increased concomitantly with the stem age. PsbS content was higher in new leaves than in old leaves and stems in both seasons. There was no a clear age-related trend in PsbS content but in all stem ages the highest concentrations were observed in spring (Fig. 6.8b).



**Fig. 6.8:** Relative content of thylakoidal proteins (analysed by Western Blot) in leaves and stems of mistletoe during winter and spring: (a) D1 and (b) PsbS. Values correspond to mean $\pm$ s.e. ( $n \geq 3$ ). One-year-old stems were not included for winter season. Data are normalised values to the value in which higher intensity was obtained.



## 6.4 DISCUSSION

### 6.4.1 The importance of stem photosynthesis in *Viscum album*

Photosynthesis studies are usually restricted to green leaves. However, non-foliar photosynthesis is an ancient, widespread characteristic that evolved before leaves as specialized photosynthetic organs (Osborne et al. 2004). Non-foliar photosynthesis has been retained throughout evolution and nowadays can be observed in petioles or pedicels of most plants. Two main groups of non-foliar photosynthesis can be differentiated. The first group, known as green stem photosynthesis, is formed by organs that lack a well-developed periderm and present abundant and functional stomata. With this type of photosynthesis, a plant obtains a high carbon gain with minimal water losses because the transpiration surface of the plant is reduced due to the spherical or cylindrical shape of the stem (Pfanz et al. 2002; Wittmann and Pfanz 2007; Filippou et al. 2007). The second group, known as bark photosynthesis, is represented by chlorophyll-containing bark and woody tissues in which chlorenchyma layers are located under a well-developed stomata-free periderm. This type of photosynthesis is involved in the internal recycling of CO<sub>2</sub> (Aschan et al. 2001). Green stems, compared with photosynthetic barks, present the advantage of being more efficient when they are under optimal conditions because their photosynthesis is not limited by the periderm opposition to gas diffusion or by the light attenuation along the different layers of cells. Nevertheless, when the light is in excess, green stems can be easily damaged (Aschan and Pfanz 2003).

Stem photosynthesis has been widely studied in leafless or drought-deciduous xerophytic perennials growing in permanently or periodically dry habitats, but, green stems are not a peculiarity of this type of organisms (Osmond and Smith 1987; Yiotis et al. 2008). This is for example the case of European mistletoe (*V. album*) in which leaves and green stems coexist all year round. For this species, we have determined that the photosynthetic area of stems (47%) is almost the same than that of leaves (53%), suggesting the essential contribution of stems to plant carbon balance.



### 6.4.2 Winter acclimation in *Viscum album*

It has been reported that stress responses can differ between leaves and stems, increasing the relative importance of the latter during favourable periods, when leaves are more affected (Yiotis et al. 2008). There are species with fully developed leaves, such as *Justicia californica*, which present photosynthetic stems whose contribution to local carbon gain during stress periods is 75% (Tinoco-Ojanguren 2008). In the case of *V. album*, the most severe stress period occurs in winter, when it is exposed to LT and HL levels after leaf shedding from its deciduous host. As a consequence, the photochemical efficiency of PSII ( $F_v/F_m$ ) decreased in winter (Fig. 6.2) in whole photosynthetic area, including leaves and stems. The decrease in  $F_v/F_m$  can be interpreted as a process of photoprotective downregulation of photosynthesis, similar to that described in boreal conifers (Adams et al. 2004; Verhoeven 2014). However,  $F_v/F_m$  in winter was 40% higher in stems compared with leaves, indicating that thanks to stem photosynthesis, the carbon assimilation is maintained during the whole year.

Cold acclimation is a process whereby plants undergo a range of biological changes in order to increase their frost tolerance and prepare for the winter season in response to low but nonfreezing temperatures (Thomashow 1999). It includes very different processes as, for example, changes in protein composition as well as the enhancement of antioxidative mechanisms, among others. In evergreens, it typically also involves the sustained increase in thermal energy dissipation (Verhoeven 2014). In our study, it is shown that in *V. album*, downregulation of photosynthesis was accompanied by a deep restructuration of the photosynthetic apparatus, especially in leaves. Thus, in leaves, the capture of light was reduced thanks to a decrease on antenna size evidenced by the higher Chl *a/b* ratio and lower total Chl content in winter. On the other hand, energy dissipation increased as it is indicated by the higher level of de-epoxidation of the V-cycle as well as the Lx-cycle, which has been previously described as a protective mechanism in the Viscaceae family (Matsubara et al. 2001). With regards to protein composition, a reduction in photochemical efficiency is





usually characterised by a pronounced decrease on D1 protein during winter (Verhoeven 2014). In *V. album*, this was the case of leaves, but not of stems, which did not present differences in D1 protein content between winter acclimated and recovered samples. Thus, under winter conditions the higher efficiency of the stems would be able to compensate the downregulation of photosynthesis that take place in leaves.

Other biochemical changes were observed in the proportion of the two isomers of the carotenoid neoxanthin (Neo). During winter the isomer *cis* was more abundant while the contrary occurred during spring, when *trans* isoform was present in higher concentration in both leaves and stems. Neo is closely related to the synthesis of stress-related hormone abscisic acid (ABA), being *cis*-Neo one of its precursors while *trans*-Neo acts as an intermediary. A plausible explanation for these changes is that during spring, due to higher rates of transpiration, all the *cis*-Neo would be converted into ABA and consequently *trans*-Neo becomes more abundant (North et al. 2007).

#### **6.4.3 Recovery kinetics and its implications in photosynthetic apparatus composition and functionality**

Recovery from the stage of sustained energy dissipation in winter was a biphasic process (an initial fast rate followed by a slower one) in both leaves and stems (Fig. 6.3). This behaviour is similar to that described for overwintering conifers. Nevertheless, recovery process presents differences, depending on the season. When it took place in spring, the fast component is more prominent. This is similar to the pattern described in conifers by Verhoeven (2014) in which the rapid phase is almost nil in late winter. Despite significant recovery of  $F_v/F_m$  in both organs, it is remarkably that stems recovery was always faster and raised higher  $F_v/F_m$  values. Thus, it is demonstrated the huge importance of stem photosynthesis in the carbon assimilation rate at whole-plant level. Besides, although leaves presented changes in D1 protein composition, which increase after recovery, stems are able to maintain D1 concentration at high values even





under winter conditions. Thus, stems are not as susceptible as leaves to the combination of high light and low temperatures and are able to maintain the PSII activity during winter to take advantage of episodes of optimal conditions that occur during winter. The absence of changes in other important proteins (PsbS, ELIP and Lhcb) agrees with the variable range of responses reported in a recent literature compilation (Verhoeven 2014).

Recovery kinetics are also accompanied by a progressive epoxidation of VAZ pool and Lx-cycle, showing the relevance of some carotenoids as antioxidants under winter conditions.

#### 6.4.4 Does age matter?

Ageing causes a downturn in animal physiology, but the negative impacts in plants are still under discussion. It has been described that, along the development, leaves experience continuous morphological transformations which have a strong influence in whole-plant physiology (Niinemets et al. 2004). Leaf expansion along development leads into an increase in photosynthetic area as well as in surface temperature and transpiration rates (Stokes et al. 2006; Niinemets and Anten 2009; Niinemets et al. 2012). Niinemets (1999) showed that thicker leaves commonly contain more photosynthetic mesophyll while dense leaves contain more structural and less photosynthetic biomass. In the case of *V. album* stems, we observe that the older the stems are, the lower proportion of photosynthetic surface they represent (Fig. 6.1). More precisely, this is due to the fact that the number of old stems is lower than that of young stems because of the dichotomous branching of *V. album* (Zuber 2004). This (growth/branching) pattern provokes that each segment of the stem presents also a different light interception angle. However, this is unlike the reason for higher  $F_v/F_m$  of old stems since branching pattern generates randomised distribution of stem orientation. Furthermore, the fact that the host is a shrub or small tree with a sparse crown reinforces the interpretation of the age-dependent  $F_v/F_m$  enhancement as a genuine ageing response. An initial



enhancement of photosynthesis before leaves reach maturity is a typical developmental response which may last more than 1 year in leaves with long lifespan (Niinemets et al. 2012). After reaching the maximum of photosynthesis, a progressive decrease occurs during leaf ageing. This is clearly not the case of *V. album* stems where photochemical efficiency increase continuously until at least 8 years old.

At present, there are two opposite points of view with respect to the effects of ageing in photosynthesis: (i) ageing induces a progressive decline of photosynthesis and growth in parallel with an increase in photo-oxidative damage due to the enhancement of water and nutrient demands (Niinemets et al. 2012); (ii) support of the theory of negative senescence, whose main statement is that survival increases with ageing (García et al. 2011). Negative senescence is intimately related with extrinsic factors, which play a critical role in the physiological process of ageing (Morales et al. 2013). In fact, most of the studies in which negative senescence was tested, were conducted under stressful conditions, typically drought (Bond 2000). In these cases, age-related changes were attributed to deeper roots and therefore increased water availability when they are older. Apart from the environment, in some species, the increment of age and size has been correlated with increased stomatal aperture and net photosynthetic rates (Munné-Bosch and Lalueza 2007). Since *V. album* is a parasitic plant which obtains water through the haustoria from the xylem of the host, ageing effects cannot be explained by the development of deep roots able to fulfill plant water demands. Therefore, it could be concluded that stomatal conductance and photosynthesis generally decrease as plant ages with the exception on those species which are able to maintain a continuous access to water, such as *V. album* (Bond 2000).

In agreement with negative senescence theory, our results show that photochemical efficiency increases concomitantly with the age in the stems of *V. album*, mainly in winter. In parallel with this, V-cycle and Lx-cycle are more de-epoxidised in young stems and leaves. This means that young organs need a



higher amount of photoprotectants to avoid oxidative stress. Changes in other molecules, such as thylakoidal proteins (D1 and PsbS) appeared as age-independent factors.

It has been demonstrated that Lx-cycle acts in parallel with V-cycle in *V. album* under stressful conditions (Matsubara et al. 2001; García-Plazaola et al. 2003). In this work, both cycles act in parallel under winter conditions, being the Z and L contents higher in winter than in spring (Fig. 6.7). Contrasting with this pattern, Lx-cycle was independent from ageing (Fig. 6.7 e, f), whereas V-cycle presents a age dependent behavior (Fig. 6.7 a–d), the younger the stems are, the higher level of V-cycle deepoxidised they present. This tendency was not only relevant under winter conditions but also during spring, demonstrating that age dependent differences are intrinsic and not always dependent on external factors. Although in spring  $F_v/F_m$  did not present any significant increase in stems  $\geq 2$  years old, total VAZ pool and de-epoxidation index of V-cycle were significantly lower in old than in young stems. This lead us to conclude that *V. album* presents a negative senescence since youngest stems present lower photochemical efficiency and higher antioxidant content, especially under winter conditions.

#### 6.4.5 The strengthened eldership of *V. album* stems

Very few studies, have considered age as an intrinsic factor triggering physiological degenerative processes at the organism level in perennial plants (Munné-Bosch and Lalueza 2007). Higher photochemical efficiency in older stems could be explained as a consequence of the replacement of their epidermal tissues by protective cork layers that reduce light transmission to photosynthetic cells (Aschan et al. 2001), preventing an excess of light arriving to the Chl. This protection of old stems from photo-oxidative conditions through a cork layer, occurs in many mistletoe species, but is unlikely to be the case in the temperate European mistletoe (*V. album*) plants since their stems remain green throughout their lifespan. More probably, the enhancement of photosynthesis with age in



this species reflects an intrinsic trait which compensates for the higher photoinhibition of leaves in winter. As observed in other species (Yiotis et al. 2008), the lower stress sensitivity of green stems would be able to outcompete leaf photosynthesis on an annual basis.

#### **6.4.6 Concluding remarks**

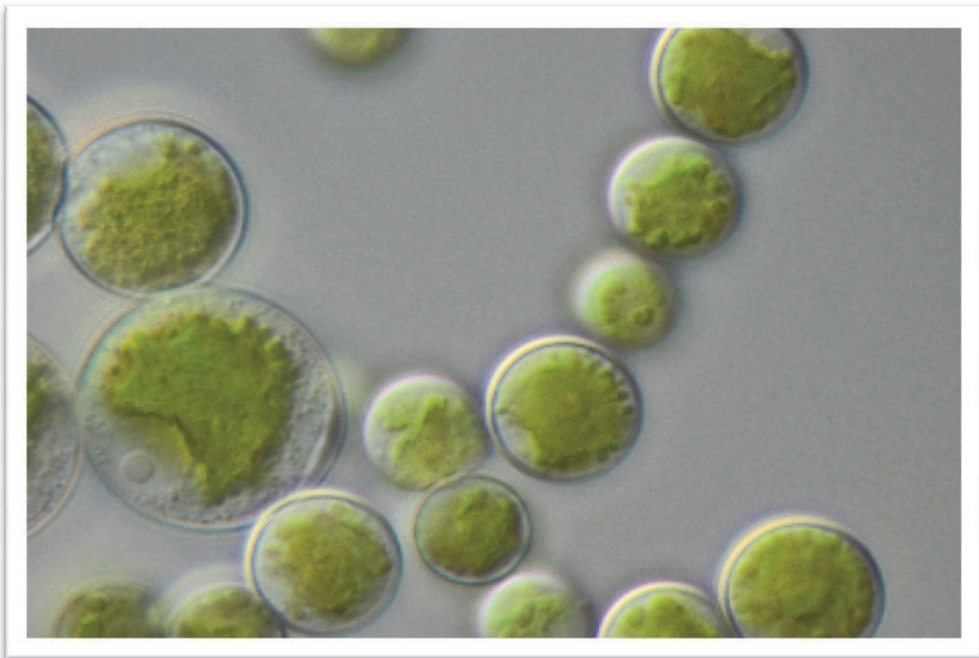
In summary, our results support the ideas that (i) young photosynthetic stems develop mechanisms of seasonal acclimation comparable to those of leaves; (ii) ageing is a synonym of enhancement of photosynthetic efficiency in winter; and (iii) the presence of photosynthetic stems in European mistletoe represents the clue to maintain photosynthetic activity during whole year, especially under winter stress.

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7. Unravelling the photoprotective response of  
lichenized and free-living Trebouxiophyceae  
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## UNRAVELLING THE PHOTOPROTECTIVE RESPONSE OF LICHENIZED AND FREE-LIVING TREBOUXIOPHYCEAE ALGAE TO PHOTOCHILLING STRESS

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**Abstract:** Algae and lichens are two groups of photosynthetic organisms hugely widespread in the Earth surface, including areas with very hard winters where they overwinter successfully. Here, the photoprotective responses under photochilling (low temperatures and high light) stress were studied in three lichens and four algae species (being 3 of them photobionts of the selected lichens, and the other an aposymbiotic alga). With the aims of (i) characterizing the photoprotective response of different algae genus as well as (ii) seeing if algae present a similar pattern of response than lichens or if by contrast, the photobionts respond better when they are protected by the fungus, fluorescence measurements as well as pigment and low molecular weight carbohydrates were analyzed along a cold and high light exposition. We found that in most algae, photochemical efficiency decreased with the increase of the stress but it was not the response of the majority of lichens which maintained their activity unchangeable. Nevertheless, this cannot be generalized due to the algae *Trebouxia arboricola* and the lichen *Ramalina pollinaria* (associated with *Trebouxia* genus), presented similar decrease in photochemical efficiency. This study also highlighted the importance of NPQ in the response of these species to photochilling stress in both lichens and algae. It was also demonstrated that V-cycle was one of the main photoprotective mechanisms in both groups while the accumulation of sugars was no so relevant, with the exception of the alga *Elliptochloris bilobata*. The differences detected among species can be explained taking into consideration the origin of the species. That from alpine ecosystems (*Elliptochloris bilobata*) presented the better physiological performance, showing an increase in photochemistry under cold stress and even an increase in sucrose content, not present in the rest of species.

## 7.1 INTRODUCTION

Overwintering plants face up a pronounced imbalance between light capture and use under low temperatures (LT). LT affect plants by three processes: decrease of enzymatic activity and loss of membrane function (chilling stress); ice formation and mechanical damage within tissues (freezing stress); and the over-excitation of the photosynthetic apparatus (photochilling stress). These third phenomenon results from the combination of LT and high light (HL) stresses, and it is considered one of the most challenging stresses for plants living in temperate regions (Huner et al. 2003; Ivanov et al. 2003). The reason is that the combination of HL and LT, results in an imbalance between the light absorbed and used. This situation provokes the over-excitation of the photosynthetic apparatus and increases the risk of photo-oxidative damage. Hence, all photosynthetic organisms need strategies for maintaining the balance between efficient light harvesting, photochemistry and photoprotection to avoid damage by excess of light. This is particularly the case for terrestrial algae, which face steeper temperature and radiation gradients in comparison to their aquatic relatives. These algae can colonize a range of biotic and abiotic surfaces (Rindi 2011) and together with lichens, can form soil crusts in hostile environments such as polar and alpine habitats and cold and hot deserts (Gray et al. 2007; Büdel and Colesie 2014; Quaas et al. 2015). In these terrestrial environments where desiccation, HL and LT strongly limit photosynthesis, dissipation of excess absorbed radiation is critical to protect photosynthetic structures (Lunch et al. 2013).

Cold acclimation (CA) is a complex multi-step process involving a series of concerted physiological and biochemical changes (Guy 1990; Thomashow 1999). In a lot of species, from conifers (Adams et al. 2013) to algae (Bohnert and Sheveleva 1998), the CA process is associated with the accumulation of soluble sugars, particularly sucrose. Current models propose that sugars contribute to the acquisition of freezing tolerance, acting as





compatible osmolytes, cryoprotectants, scavengers of reactive oxygen species (ROS) and signalling molecules (Janská et al. 2010; Theocharis et al. 2012). CA also induces an increase in the activity of antioxidant enzymes as well as an augmentation of the pools of non-enzymatic antioxidants content such as tocopherols and carotenoids (Thiele et al. 1996; Verhoeven et al. 1996). The xanthophyll cycle (V-cycle) is one of the most important antioxidant photoprotective mechanisms in photosynthetic organisms. It consists on a forward reaction comprising two de-epoxidation steps, in which the di-epoxy xanthophyll violaxanthin (V) is converted to the epoxy-free zeaxanthin (Z). The intermediate product of this reaction sequence is antheraxanthin (A), which contains one epoxy group. The conversion from A to Z occurs especially under HL conditions. This cycle plays a very important role in the photoprotection mechanisms of vascular plants as well as in green and brown algae (Yamamoto et al. 1962; Stransky and Hager 1970). Z takes part in the dissipation of excess excitation energy as heat, preventing the inactivation and the damage of the photosynthetic apparatus (Demmig-Adams et al. 1990). This process is known as non-photochemical quenching (NPQ) and seems crucial for the survival of biofilm-forming algae (Quaas et al. 2015).

Terrestrial green algae represent a heterogeneous assemblage of microscopic organisms belonging primarily to the Chlorophyta or the Streptophyta division, the latter ones including the first land plants (Rindi 2011). Within the Chlorophyta, the class Trebouxiophyceae comprises the majority of terrestrial algae, including certain genera which predominantly occur forming lichen symbiosis with fungi. Lichens are symbiotic associations consisting of a fungus (the mycobiont), a photosynthetic partner (the photobiont) and a diverse bacterial community (Grube et al. 2012). The algal genera *Trebouxia* and *Asterochloris* are the most common eukaryotic photobionts (Tschermak-Woess 1988) and they rarely occur in the aposymbiotic (non-lichenized) state (Skaloud et al. 2015, Ahmadjian 2002), probably due to their dependency on the

symbiotic partner. In contrast, other terrestrial algae, such as the genus *Apatococcus*, are predominantly free-living (Gustavs et al. 2015) and although they live in close associations with fungi, a true lichenization is at best extremely rare (Voytsekhovich 2013). The situation for representatives of the *Elliptochloris*-clade, namely the genera *Coccomyxa* and *Elliptochloris*, is somehow less distinct as they are considered facultative photobionts, which also occur frequently free-living in terrestrial and aquatic habitats. Although *Elliptochloris* is less frequent than *Coccomyxa*, it displays a conspicuous versatility in its choice of host species and can associate with ascomycetes (Tschermak-Woess 1980) and even with aquatic invertebrates (Letsch et al. 2009, for review see Gustavs et al. 2015).

While most terrestrial green algae have developed several adaptations to the harsh terrestrial habitat, the symbiotic association with lichen enables them to colonize an even wider range of hostile habitats where most vascular plants are at their physiological limits, such as high alpine, arctic, antarctic and desert ecosystems (Honegger 2009). It is considered that, in general, the lichen thallus provides a growth chamber to the photobiont cells, buffering the daily and seasonal fluctuations in radiation and providing an effective water retention system to keep the alga metabolically active, being the isolated symbionts more susceptible to extreme conditions than the lichen (De Vera et al. 2008). Furthermore, the exchange of nutrients, vitamins and trace elements between the two symbiotic partners is postulated (Richardson et al. 1968; Ahmadjian 2002; Alam et al. 2015), explaining the ecological success of this symbiotic association. Nevertheless, it is controversially discussed if terrestrial algae adapted to extreme environmental conditions benefit from the lichenized state in contrast to an aposymbiotic lifestyle (Lange et al. 1990; Kranner et al. 2005; Candotto Carniel et al. 2015). A recent detailed study on the facultative photobiont *Coccomyxa* comparing 35 strains from the mentioned different lifestyles revealed a wide range of physiological plasticity, characterizing this



genus as robust generalists able to cope with a wide range of abiotic stresses (Darienko et al. 2015). The situation for the genera *Asterochloris* and *Trebouxia* might be comparable, as they are as well world-wide distributed (Blanc et al. 2012, Ettl and Gärtner 2014). In contrast, *Elliptochloris* is far less frequent (Gustavs et al. in press) and might be more sensitive to variable abiotic conditions. Consequently, the plasticity of photobiont physiology in response to certain abiotic stresses has to be defined for the addressed genera, and if possible on the species level, to evaluate the benefit that photobionts might gain from the lichen symbiosis (Sadowsky and Ott 2015).

As it has been pointed out recently (Valledor et al. 2013; Míguez et al. 2015), the process of winter photoinhibition (WPI) and CA has been widely described in higher plants but little is known about photosynthetic responses of free-living and lichenized green algae. In this study, we analyzed the response of three lichens and four algae (one obligate free-living and three, which typically or facultatively live as symbionts in lichens) to chilling in combination with excessive radiation. The objectives of this study were (i) to determine the photoprotective response in lichens and free-living algae during their acclimation to LT or to the combination of LT and HL (ii) to evaluate if lichenization is a benefit for the investigated organisms or if they perform equally well in a free-living state, (iii) to compare this response with the better-known response in higher plants under these conditions (iv) to analyze if the response of sugars and pigments concurs with the state of photoprotection in this organisms as documented for higher plants.

## 7.2 MATERIALS AND METHODS

### 7.2.1 Organisms and culture conditions

Four aeroterrestrial unicellular green algae and three lichens were investigated in this study (for details, see Table 7.1). *Elliptochloris bilobata* (EB)



was grown in modified Bolds Basal Medium (3N-BBM+V; medium 26a in (Schlösser 1997). *Apatococcus lobatus* (AL), *Asterochloris erici* (AE) and *Trebouxia arboricola* (TA) were grown at solid (1.5% DIFCO agar) TOM medium (Trebouxia Organic Medium) according to (Ahmadjian 1967) modified after (Friedl 1989) by the addition of 1.5 % glucose, 2 % proteose-peptone to 3N-BBM+V. For more detail about media composition see Appendix 2.

**Table 7.1:** Species studied, their taxonomic assignments, habitat and origin. Algal strains (prefix SAG) were obtained from the culture collection of algae, University of Göttingen (Germany).

Species	Taxonomic assignment	Habitat and characteristics	Origins
<i>Cladonia squamosa</i> (Scop.) Hoffm. (CS)	Class Lecanoramycetes	strongly degraded peat bog, on rotten pine stump	Germany, Mecklenburg-Western Pomerania, Central Mecklenburg
<i>Baeomyces rufus</i> (Hudson) Rebert (BR)	Class Lecanoramycetes	steep slope at the forest edge, on soil	Germany, Mecklenburg-Western Pomerania, Isle of Usedom
<i>Ramalina pollinaria</i> (Westr.) Ach.(RP)	Class Lecanoramycetes	northern side of rural medieval church, on granite	Germany, Mecklenburg-Western Pomerania, Central Mecklenburg, Rövershagen
<i>Apatococcus lobatus</i> SAG 2145 (AL)	Trebouxiophyceae, <i>Apatococcus</i> -clade	bark of <i>Platanus</i> sp. Terrestrial free-living green algae.	Switzerland, Basel, Rheinweg,
<i>Asterochloris erici</i> SAG 32.85 (AE)	Trebouxiophyceae <i>Trebouxia</i> -clade	soil	Photobiont of lichen <i>Cladonia cristatella</i> . United States, MA, Whitensville
<i>Trebouxia arboricola</i> SAG 219.1 (TA)	Trebouxiophyceae <i>Trebouxia</i> -clade	Unknown	Typically photobiont of <i>Ramalina</i> sp. (associated lichen not documented)
<i>Elliptochloris bilobata</i> SAG 245.80 (EB)	Trebouxiophyceae- <i>Elliptochloris</i> clade.	Unknown	phycobiont of <i>Catolechia wahlenbergii</i> . Austria, Kärnten, Kreuzeckgruppe, 2200 m



The lichens studied were *Ramalina pollinaria* (RP) associated with *Trebouxia*, *Cladonia squamosa* (CS) associated with *Asterochloris* and *Baeomyces rufus* (BR) associated with *Ellitpochloris*. They were sampled in October 2014 in different localities in the North-East of Germany (see table 7.1 for more details). While *Ramalina* and *Cladonia* are rather common and widely distributed genera, *Baeomyces* is less frequent (Wirth et al. 2013). The habitat of *Ramalina* is rather light exposed and it is typical occurring at free-standing trees or building walls. In contrast, *Cladonia* prefers humid and shaded habitats while *Baeomyces* can cope with both situations but is sensitive to eutrophication. After sampling, in order to avoid the dehydration of lichen thalli and to maintain them metabolically active, they were placed in transparent boxes hermetically closed with water in the base to obtain 100% humidity inside.

Both algae and lichens were incubated at 20°C and 30  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  at a light/dark cycle of 16:8 h during approximately 5 weeks. Osram Daylight Lumilux Deluxe lamps were used as light sources. Radiation measurements were carried out with a Li-Cor LI-190-SB cosine corrected sensor connected to a Li-Cor LI-1000 datalogger (Lambda Instruments, Lincoln, USA, sensor error <2%).

### 7.2.2 Cold and high light treatment experiment

Prior to the cold treatment, algal cultures were transferred to sterile cell culture flasks (Corning, NY, USA; size 25 cm<sup>2</sup>) filled with 3-N BBM + V. The flasks were placed into purpose-built incubators (Kunststoff-Technik Rostock, Rostock, Germany) which guaranteed constant temperatures ( $\pm 0.1^\circ\text{C}$ ) and are equipped with LED arrays (LED neutral white Ediline III 3.5 W COB Modul, Edison Opto Corp., Taipei City, Taiwan) ensuring an efficient regulation of a wide range of photon flux densities (PFDs). The transfer of the cell culture flasks inside the incubator was carried out 3 days prior the start of the experiment to ensure acclimation to the given abiotic conditions (Gustavs et al.

2009). Lichen thalli maintained in the previously described plastic containers were placed in a modified wine storage cabinet (Liebherr, Biberach an der Riss, Germany) which is illuminated by a set of 6 Lumitronix LED strips (Nichia, Tokushima, Japan). The first 5 days of the experiment, temperature was down regulated from 20°C to 5°C while day-length decreased in parallel from 16h to 8h. The next 5 days, algae and lichens were maintained under cold and short-day conditions (5°C and 8h of light), facing either a low light (LL: 30  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ ) or high light (HL: 300  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ ) treatment. The exact time course of temperature, irradiation and day-length changes along the experiment is illustrated in Fig. 7.1. Additionally, the dates of fluorescence measurements and samplings are indicated. The samples for biochemical analyses were taken (i) the first day of the experiment, before any cold acclimation (CA) process (control), (ii) the fifth day of the experiment (after four-days under gradual transfer from 20 to 5°C and one day at 5°C) and (iii) the last day of the experiment (when the samples were at 5°C during 5 consecutive days). Both the second and third sample points were performed over lichens and algae exposed to LL and HL regimes.

### 7.2.3 Fluorescence analysis

Photosynthesis irradiance curves (ETR/I curves) were determined by PAM-2500 fluorometer (Walz, Effeltrich, Germany). The algae cultures were filtered in filter discs (diameter 25 mm, pore size 1-3  $\mu\text{m}$ , Whatman GmbH, Dassel, Germany) which were dark adapted during 20-30 min. To avoid drought stress, the filter with the algal sample was placed above another filter with medium during dark adaptation period and fluorescence measurements. Then, they were exposed to a light ramp consisting of 13 steps of 1 min each. Photon flux densities (PFDs), ranged from 12 up to 489  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ . After each illumination step, a saturating pulse was applied to obtain the maximum chlorophyll fluorescence under illumination ( $F_m'$ ). In table 3.3 and 3.4 (Materials



and methods section), the formulas to calculate fluorescence parameters are indicated.

#### **7.2.4 Sample preparation**

Algal cells were harvested by two consecutive centrifugations during 5 minutes each at LT (5°C). The first centrifugation reduced the complete culture volume harvested from the cell culture flasks (5000g, Heraeus Megafuge 1.0 R, Heraeus GmbH, Dassel, Germany) while the second centrifugation allowed the partition of highly concentrated biomass in several aliquots for subsequent extractions (14 000g, Heraeus Biofuge primo R, Heraeus GmbH, Dassel, Germany). After centrifugation, the pellets were frozen in liquid nitrogen and stored at -80°C until extraction. To obtain the dry weight of algal pellets, they were vacuum-evaporated in a Savant SpeedVac (SPD 111V, Thermofisher Scientific, Waltham, USA) connected to a Lyovac GT2 Freezedryer (Steris, Cologne, Germany).

#### **7.2.5 Low-molecular weight carbohydrates (LMWC) determination.**

Dry algal samples (7–12 mg dry weight) were extracted with 70% aqueous ethanol (v/v) in capped centrifuge tubes at 70°C in a water bath for 4h according to (Karsten et al. 1991). After centrifugation for 5min at 5000g, 700µl of the supernatant were evaporated to dryness under vacuum (Speed Vac Concentrator SVC 100H). Dried extracts were dissolved again in 700 µl distilled water, vortexed for 30s and treated in an ultrasonic bath for 5 min (Bandelin Sonorex, Berlin, Germany). After centrifugation, the clear supernatant was pipetted to HPLC vials closed with a membrane-equipped lid.

The detection of LMWCs was carried out with an Agilent HPLC system equipped with a refractive index detector (RID G1362A, Agilent, Santa Clara, USA) following two isocratic methods, depending on the investigated species.



The determination of saccharose and ribitol was performed on a Bio Rad resin based column (Aminex Fast Carbohydrate Analysis, 100×7.8 mm) using a Phenomenex Carbo-Pb<sup>2+</sup> (4×3 mm) guard cartridge. These carbohydrates were eluted with 100% HPLC grade water at a flow rate of 1ml min<sup>-1</sup> at 70°C (Karsten et al. 1991). To separate various polyols from each other, LMWC, analysis was performed on a Phenomenex resin-based column Rezex ROA-Organic Acid (300×7.8 mm) protected with a Phenomenex Carbo-H<sup>+</sup> guard cartridge (4×3 mm). On the latter column, carbohydrates were eluted with 5 mM H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.4ml min<sup>-1</sup> at 75°C (Karsten et al. 2005). All carbohydrates were identified by comparison of retention times with those of the commercial standard compounds saccharose, arabitol, erythritol and ribitol (Roth, Karlsruhe, Germany). Quantification was achieved through respective calibration curves calculated from a 10-step dilution series with concentrations between 0.5 and 5 mM. Carbohydrate concentrations within the algal samples were calculated through integration of peak area in relation to extracted dry weight.

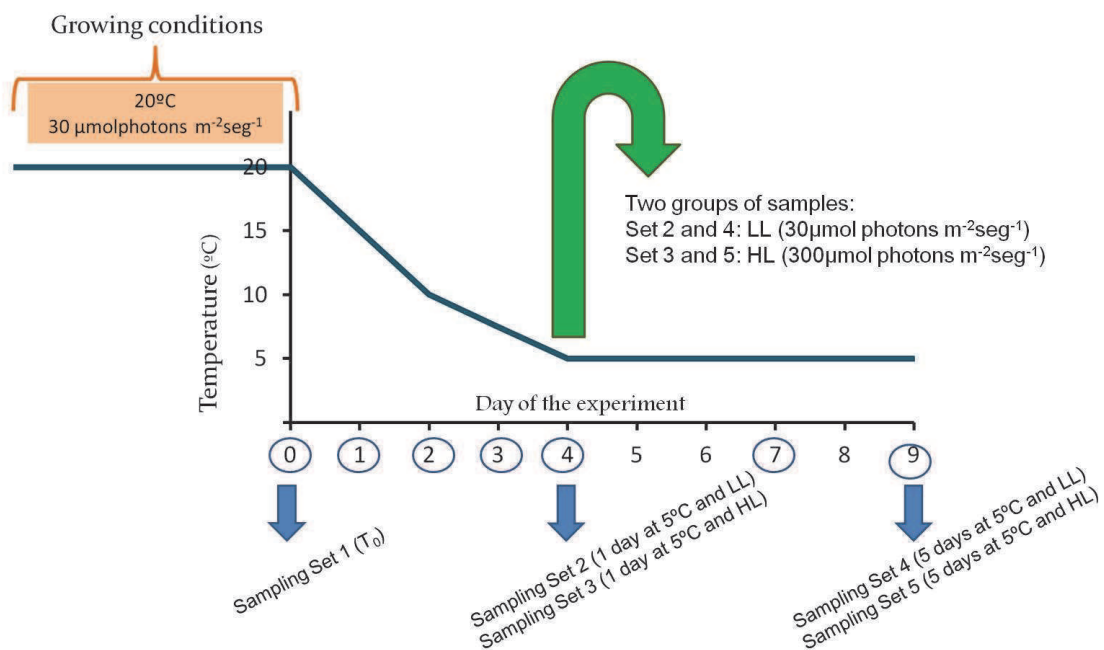
### 7.2.6 Pigments analysis

Dry algal samples were doubly extracted: Firstly, they were homogenized with a mortar in acetone (95%) buffered with CaCO<sub>3</sub>. After that, the extracts were centrifuged at 16,100g for 5 min. Then the pellet was re-suspended in pure acetone buffered with CaCO<sub>3</sub> with a Tissue Tearor Homogenizer (Model 395, Dremel, Mexico). The extract was centrifuged again at 16,100g for 5 min and both supernatants were mixed and were filtered with 0.2µm PTFE filters (Tecknokroma, Barcelona, Spain). In the case of lichens, to avoid chlorophyll (Chl) degradation, the extraction was carried out using acetone 95% buffered with 0.5% NEDPA and the same amount of CaCO<sub>3</sub> than sample. Lichen extracts were centrifuged at 16100 g and 4°C for 10 min, and supernatants were also filtered with 0.2 µm PTFE filters (Teknokroma, Spain).





During the whole process, samples were maintained at a temperature around 4°C to avoid pigment degradations. Pigment separation was performed by HPLC with a reverse phase C18 column (Waters Spherisorb ODS1, 4.6 x 250 mm, Mildred, MA, USA) with a photodiode array (PDA) detector, following the method of (García-Plazaola and Becerril 1999) modified after by (García-Plazaola and Esteban 2012). The de-epoxidation rate of violaxanthin cycle pigments was estimated as  $(A+Z)/(V+A+Z)$ , abbreviated as AZ/VAZ.



**Fig. 7.1.** Experimental design of the temperature/light treatments applied to algae and lichens.

Fluorescence is indicated by blue circles and sampling for pigment and low molecular weight carbohydrates analysis are indicated by arrows. Photoperiod decreased from 16 hours on day 1 to 10 hours on day 2 and 8 hours from day 3 onwards.



### **7.2.7 Light microscopy**

For the morphological investigations, an Olympus BX-51 light microscope was used. The micrographs were taken with an Olympus UC 30 camera using the cellSens Entry imaging system (Olympus, Tokyo, Japan).

### **7.2.8 Statistics**

Kolmogorov–Smirnov and Cochran tests were used to test for the normality of data and homogeneity of variances respectively. One-way ANOVA tests were applied to check for differences in pigment, and LMWC content when data were normal. For no normal data, the Kruskal-Wallis test was used with the same objective. Duncan (when variances were homogeneous) or Dunnett (when they were not homogeneous) post-hoc tests were performed to discriminate changes in pigments content along the treatment in each species. In the case of no normal data, Mann Whitney U non parametric test was carried out. In order to analyze the existence of any correlation between AZ/VAZ and non photochemical quenching (NPQ), Pearson and Spearman tests were used for normal and no normal data, respectively. All analyses were performed using the SPSS 17.0 statistical package (SPSS, Armonk, NY, USA).



## 7.3 RESULTS

### 7.3.1 Photochemical efficiency and energy partitioning during cold acclimation in algae and lichen species

Physiological changes associated with the process of cold adaptation (CA) were monitored during 10 days in four isolates of terrestrial green algae (Chlorophyta, Trebouxiophyceae) and three lichens (Ascomycota, Lecanoramycetes, with trebouxiophyte photobiont) (Table 7.1). The morphological responses of the algal cultures were documented by microscopic observations while the physiological and biochemical responses of algae and lichens were followed by fluorescence measurements and the documentation of carbohydrate and pigment pools.

During the course of the experiment, no significant morphological changes have been observed (See Appendix 11). The study of Chl fluorescence revealed the existence of species-specific response pattern within the investigated algae. Thus, while in *Trebouxia arboricola* (TA) and *Asterochloris erici* (AE) (Fig. 7.2 A, B)  $F_v/F_m$  was affected by LT even under LL, *Elliptochloris bilobata* (EB) and *Apatococcus lobatus* (AL) (Fig. 7.2 C, D) downregulated their photosynthetic efficiency only under HL, independently on the temperature. The effects of HL and LT also affected the energy partitioning in a species-dependent mode. Thus, AE and AL (Fig. 7.2B, D) increased the fraction of regulated thermal dissipation ( $\Phi_{NPQ}$ ), after HL and LT stresses. In contrast, TA only increased  $\Phi_{NPQ}$  when the two stresses occurred in combination (Fig. 7.2A). Responses of EB were more distinctive with an increased allocation of energy towards photochemistry ( $\Phi_{PSII}$ ) when it was subjected to LT and towards non-regulated dissipation ( $\Phi_{f,D}$ ) when it was exposed to the combination of HL and LT (Fig. 7.2C). What is more, EB was the only algae analyzed which maintained the relative  $ETR_{max}$  along all the treatment.  $\alpha$ , the photosynthetic efficiency calculated using the (Platt et al. 1980) model was also maintained in

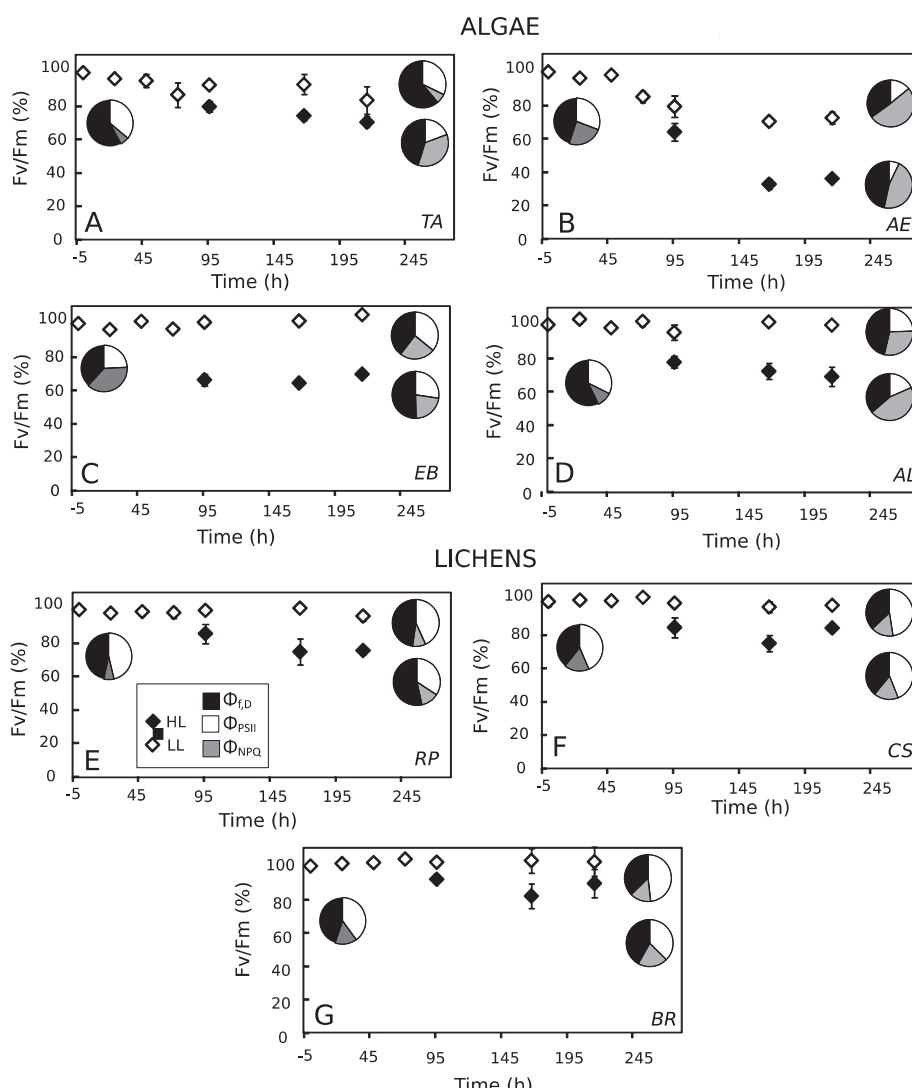


*EB* under HL, and even it increased when LL is combined with LT also in this species (See Appendix 12).

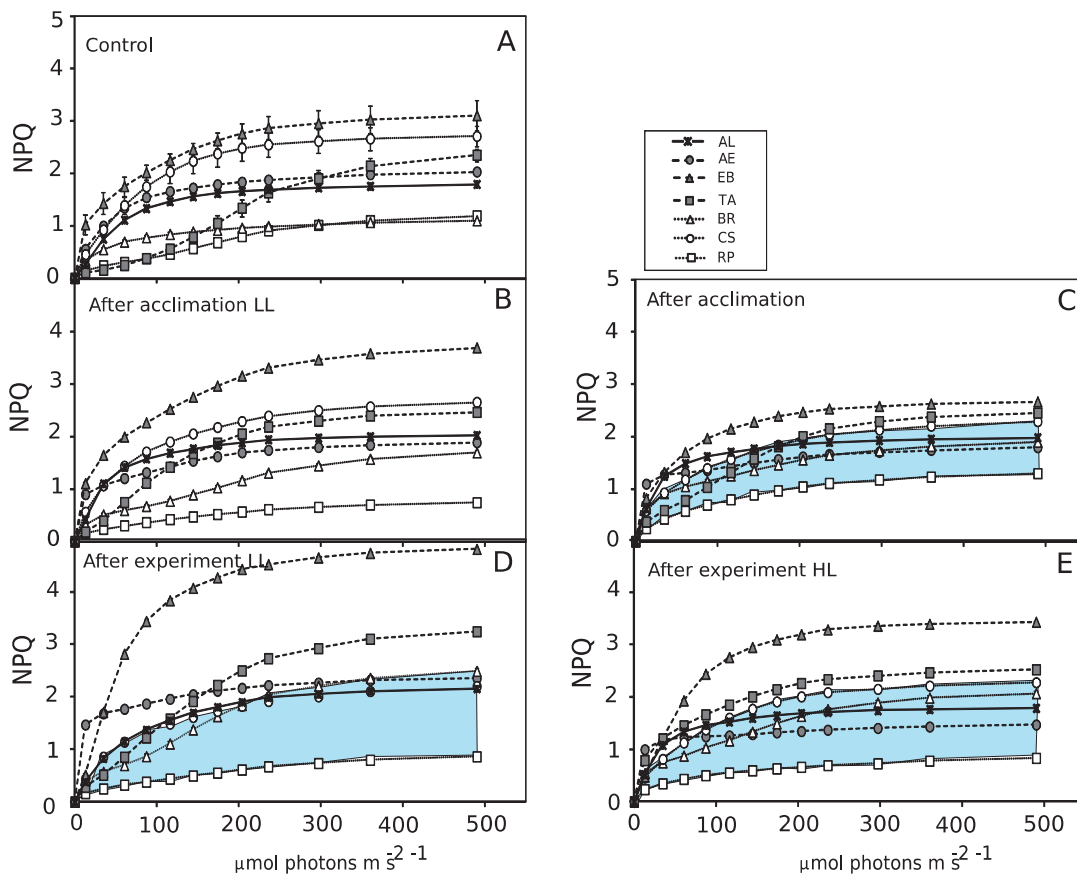
In contrast with the diversity observed in free-living algae, all lichen species (*Ramalina pollinaria* (RP), *Cladonia squamosa* (CS) and *Baeomyces rufus* (BR)) followed basically the same pattern of response under stress conditions (Fig. 7.2E, F, G). Temperature decrease itself, did not affect photosynthetic efficiency, but HL triggered a decrease of  $F_v/F_m$  of 24-33% in all of them. No significant differences in energy partitioning were observed throughout the experiment in lichens, except for the case of RP, which showed an increase in  $\Phi_{fD}$  under HL conditions. Regarding the parameters calculated using the (Platt et al. 1980) model, the response along the treatment of  $ETR_{max}$  and  $\alpha$  was more attenuated in lichens than in algae (See Appendix 12). Overall, present results showed that free-living algae were more affected by the combination of HL and LT than their lichenized counterparts.

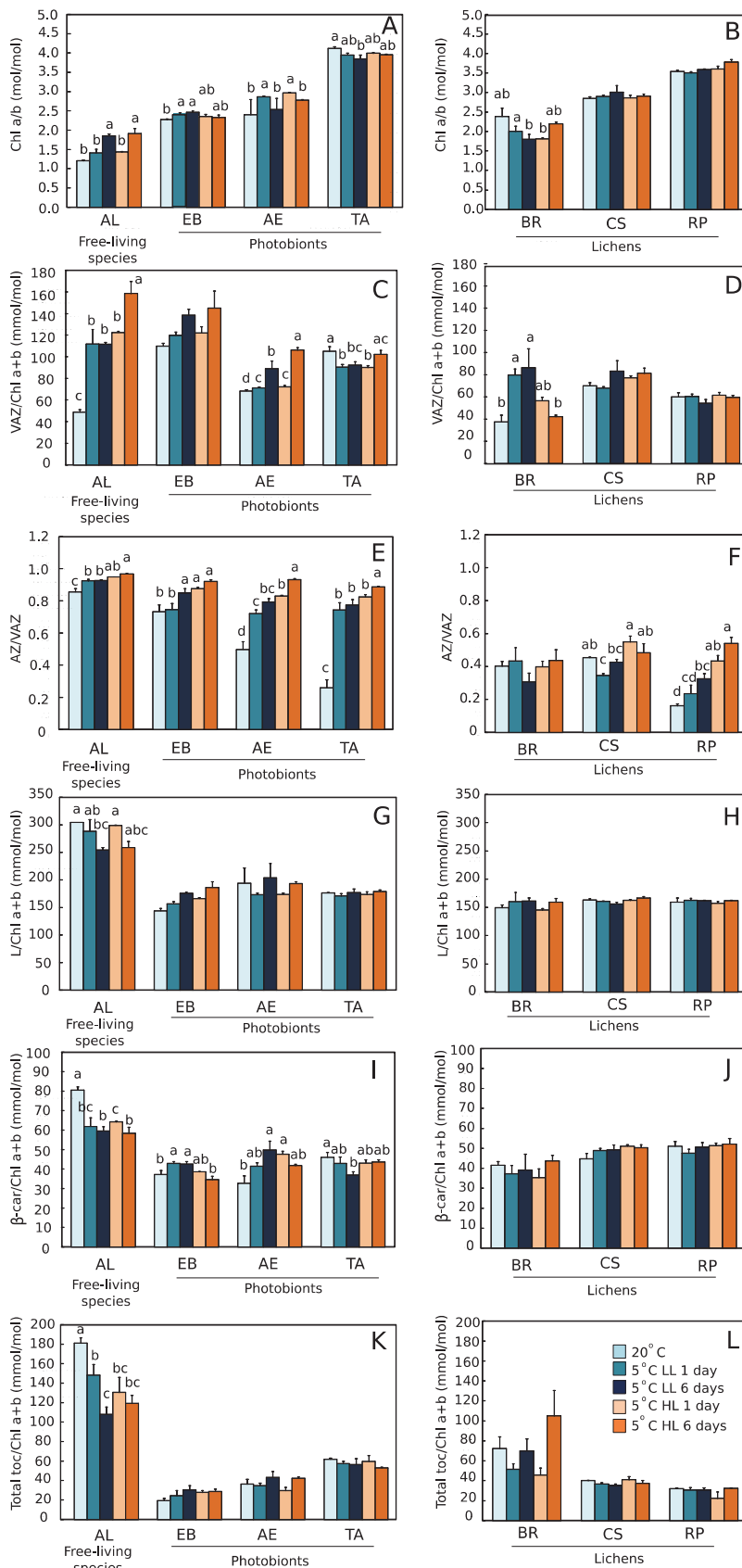


**Fig. 7.2:** Chlorophyll fluorescence measurements during the cold acclimation treatment. Diamonds represent the percentage of  $F_v/F_m$  with respect to control conditions. Open symbols indicate LL (low light) treatment and close symbols HL (high light). (A) *TA: Trebouxia arboricola*; (B) *AE: Asterochloris erici*; (C) *EB: Elliptochloris bilobata*; (D) *AL: Apatococcus lobatus*; (E) *RP: Ramalina pollinaria*; (F) *CS: Cladonia squamosa* and (G) *BR: Baeomyces rufus*. Control values of  $F_v/F_m$  for each species were: 0.619 for *TA*; 0.565 for *AE*; 0.570 for *EB*; 0.565 for *AL*; 0.721 for *RP*; 0.709 for *CS* and 0.705 for *BR*. Pie charts show the energy partitioning into  $\Phi_{\text{PSII}}$  (white),  $\Phi_{\text{NPQ}}$  (grey) and  $\Phi_{\text{f,D}}$  (black) under control conditions (left side of each panel) and at the end of experimental treatments (right side of each panel: LL up, HL down) Data are means  $\pm$  SE (n=3).



**Fig. 7.3:** Irradiance responses of NPQ along the experimental treatments. *TA*: *Trebouxia arboricola*; *AE*: *Asterochloris erici*; *EB*: *Elliptochloris bilobata*; *AL*: *Apatococcus lobatus*; *RP*: *Ramalina pollinaria*; *CS*: *Cladonia squamosa* and *BR*: *Baeomyces rufus*. NPQ values depicted in this figure were calculated from the  $F_m$  in dark acclimated samples and  $F_m'$  values obtained at each light intensity step. (A) the measurements were done under control conditions, (B) after 5 days under cold acclimation period and low light (LL), (C) after 5 days under cold acclimation period but the last 24hours under high light (HL), (D and E) after 10 days at low temperatures under LL (D) or HL (E). Each point represents the mean  $\pm$  SE (n=3).





**Fig. 7.4:** Effects of experimental treatments on pigment composition in free algae (left panels) and lichens (right panels). (AL: *Apatococcus lobatus*; EB: *Elliptochloris bilobata*; AE: *Asterochloris erici*; TA: *Trebouxia arboricola*; BR: *Baeomyces rufus*; CS: *Cladonia squamosa*; RP: *Ramalina pollinaria*). Panels A and B show Chl a/b in algae and lichens respectively; panels C and D show VAZ/Chl in algae and lichens respectively; panels E and F show AZ/VAZ in algae and lichens respectively; panels G and H show L/Chl in algae and lichens respectively; panels I and J show β-car/Chl in algae and lichens respectively and panels K and L: Total toc/Chl in algae and lichens respectively. Blue colors refer to LL (low light) treatment and orange colors refer to HL (high light) treatment. Data are mean ± SE (n=3). Letters indicate significant differences for each species at P<0.05. The absence of letters means no significant differences.





### 7.3.2 Light curves: Comparison between lichens and free-living algae.

Figure 7.3 shows light curves of NPQ during the process of cold and HL acclimation. The NPQ response to PFD in all species exhibited a comparable response to increasing light intensities with maximum NPQ values (achieved at the highest light intensities) ranging from 1.7 to 3.5 in algae and from 0.5 to 2.5 in lichens after one day of acclimation. Under control conditions (Fig. 7.3A), the response of each alga was species-dependent, but under HL-LT or after 5 days of LT treatments, two patterns were easily discernible (blue and white areas). In the case of lichens (Fig. 7.3 C, D, E blue area), NPQ raised lower values than in algae at each PFD intensity, except for the case of *AE* whose NPQ values were similar to those obtained in its lichenized form. The case of *AL* was peculiar as it occupied always an intermediate position compared to the other species, being the fastest in terms of NPQ response to PFD. By contrast, in *EB* the singularity was that NPQ was the highest for all the treatments even under control conditions.

### 7.3.3 Biochemical response under HL and LT treatment

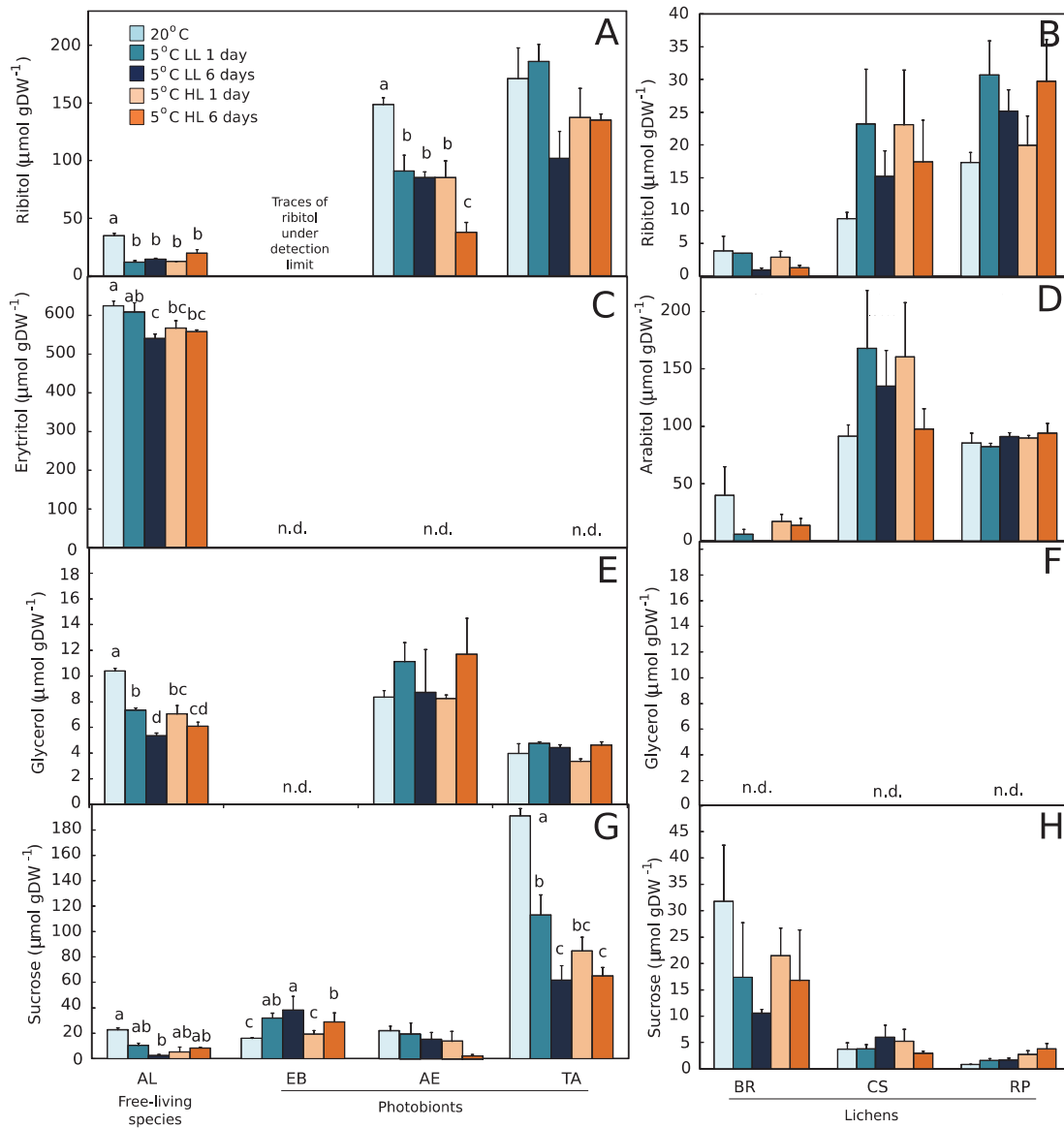
To understand the biochemical mechanisms underlying the photochemical responses associated to the process of CA, pigments and low molecular weight carbohydrates (LMWC) content were measured along stress treatment (Fig. 7.4 and 7.5, respectively).

Overall, some photosynthetic pigment profiles in these species followed changes along the temperature and light treatment. The Chl a/b ratio increased significantly in *AL* and *EB* (Fig. 7.4A) while in lichen *BR* followed the opposite pattern (Fig. 7.4B). The V-cycle pool also increased influenced by HL and LT exposure in most algal species (*AL*, *EB*, *AE*) (Fig 7.4C) and also in the lichen *BR* (Fig 7.4D). The de-epoxidation state of pigments in the xanthophyll cycle (AZ/VAZ) was higher after the HL and LT treatment in all species except for *BR*



and *CS*. These increases were higher for HL-LT than for only LT treatments (Fig. 7.4 E, F). By contrast, no response was observed in the components of the lutein epoxide cycle for any of the species (Fig. 7.3 G,H).  $\beta$ -car and tocopherol display very similar pattern. Both metabolites/pigments decreased in *AL* but remained stable in the rest of the species (Fig. 7.3 I,J,K,L). The only case in which  $\beta$ -car increased under HL and LT was in the algae *AE* (Fig. 7.3I).

The basic composition of LMWC (ribitol, erytritol, arabitol, glycerol and sucrose) differed among species (Fig. 7.5), sucrose and ribitol were ubiquitous, while the other LMWC analysed were irregularly distributed. The patterns of response to the experimental treatment can be basically described as a net decrease or maintenance of the pools of these compounds along the stress period. The only exception is displayed by *EB* which showed an increase in sucrose content for both, LT and the combination of LT and HL (Fig. 7.4D).



**Fig. 7.5:** Effects of experimental treatments on low molecular weight carbohydrates (LMWC) in free algae (left panels) and lichens (right panels). (*AL*: *Apatococcus lobatus*; *EB*: *Elliptochloris bilobata*; *AE*: *Asterochloris erici*; *TA*: *Trebouxia arboricola*; *BR*: *Baeomyces rufus*; *CS*: *Cladonia squamosa*; *RP*: *Ramalina pollinaria*). Panels A and B show ribitol content in algae and lichens respectively; panel C shows erytritol content; panel D shows arabitol content; panels E and F show glycerol content in algae and lichens respectively; panels G and H show sucrose content in algae and lichens respectively. Blue colors refer to LL (low light) treatment and orange colors refer to HL (high light) treatment. Data are mean  $\pm$  SE (n=3). Different small letters indicate significant differences for each species at  $P < 0.05$ . The absence of letters means no significant differences.



## 7.4 DISCUSSION

### 7.4.1 Diversity of photoprotective strategies during cold acclimation

In the present study, we investigated the response to photochilling stress of three different lichen specimens associated with different trebouxiophyte photobionts and representing differently irradiation-influenced habitats in northern Germany. The lichens were sampled in autumn and incubated under controlled laboratory conditions to a continuous decrease in temperature and day-length, simulating winter conditions. Additionally to the cold stress, high irradiance (HL) has been applied to determine the intensity and kinetics of photoprotective mechanisms. Besides the lichens, four green algae have been investigated in pure culture, three of them representing the typical photobiont genus associating with the target lichens, namely *Trebouxia*, *Asterochloris* and *Elliptochloris*. The fourth investigated genera were (i) *Apatococcus* which represents the most common free-living trebouxiophyte in the study area, (ii) *Trebouxia* and (iii) *Asterochloris* which are the most frequent photobiont found in lichen thalli (Nash 2008), and although they are world-wide distributed, they only occur rarely in the aposymbiotic state and (iv) *Elliptochloris* that is less frequent and is considered a facultative photobiont, able to live in both ways. However, we did not isolate the associated photobiont from the here investigated lichen thalli but accessed the most suitable algal strain available from public culture conditions (Table 7.1). Besides, the three strains considered here as “associated photobiont” have all been isolated from lichen thalli. They are native from areas with a hard winter season; therefore they are naturally exposed to the stresses associated to low temperatures (LT). Among these environments, the hardest was the alpine ecosystem where *Elliptochloris bilobata* (*EB*) was isolated (Table 7.1) (Eliáš et al. 2008). Consistently with this origin, *EB* presented the better physiological performance under LT, compared to the other species. It was evidenced by several physiological responses along cold



acclimation (CA) treatment. Thus, in *EB*, LT acclimation did not affect  $F_v/F_m$  (Fig. 7.2). At the same time it caused an increase of sucrose, while in the other free-living algae,  $F_v/F_m$  decreased and not only sucrose, but also other of low molecular weight carbohydrates (LMWC) (ribitol, erytritol and glycerol) were consumed. The accumulation of LMWC is a typical response to LT in higher plants (e.g. Adams et al. 2013; Trischuk et al. 2014) and some algae (Nagao et al. 2008). These molecules contribute to the acquisition of freezing tolerance, acting as compatible osmolytes and cryoprotectants and also acting as scavengers of reactive oxygen species (ROS) as well as signalling molecules (Janská et al. 2010; Theocharis et al. 2012). What is even more striking a recently postulated hypothesis that affirms that some LMWC play an important role in the NPQ formation under drought conditions in *Trebouxia* sp. (Kosugi et al. 2013). That study highlighted the importance of arabitol in NPQ formation. It is believed that this sugar acts as a modulator of gene expression or changes the protein conformation during dehydration. Obviously, this was not the case for algae species analysed in this study for which NPQ and LMWC variations induced by LT and HL were not related. By contrast, *EB*, the only species in which sucrose content increased after LT treatments, showed a higher partition of absorbed energy towards photochemistry and lower towards NPQ, suggesting that it was able to maintain photosynthetic activity under the experimental conditions. The same response has been described in different genotypes of two herbaceous angiosperms subjected to a CA period during 3 weeks at 2°C (Pociecha et al. 2010a). The decline in LMWC at LT and HL stress, observed in the rest of species, may have been associated with increased fructan synthesis as occurred in other studies with some herbaceous species (Martinez-Carrasco et al. 1993; Pociecha et al. 2010b).

The physiological responses to CA of *Trebouxia arboricola* (*TA*), a genus also adapted to LT in its natural environment, illustrated by the fact that it is the dominant photobiont in continental Antarctic macrolichens (Helms et al.



2001a), can be also related to habitat specific adaptations. Nevertheless, in contrast with the responses observed in *EB*, NPQ followed the opposite pattern, increasing in *TA* as a response to *CA*. The same response was observed in the alga *Asterochloris erici* (*AE*), which is phylogenetically closely related to *TA* (Helms et al. 2001b; Piercey-Normore and Depriest 2001). The increase of NPQ is a frequent response to LT and even algal species living in ice achieve photoprotection against HL through thermal dissipation (Alou-Font et al. 2013; Katayama and Taguchi 2013). Other studies also observed that, under constant light, the decrease in temperature also provokes an increment in NPQ (Mock and Hoch 2005).

In contrast with the three species mentioned before, *Apatococcus lobatus* (*AL*) is predominantly free-living and although it occurs in close association with certain fungi, a true lichenization is questionable (Voytsekhovich 2013). Hence, it is never protected from the external environment by a fungal layer as occurs in the other species. It implies that its photoprotection mechanisms need to be sufficient to counteract the environmental hazards by themselves without the protection given by the lichen cortex. However, it grows in sarcinoid colonies (Appendix 11), sometimes building up thick cell walls and even forming three-dimensional structures when cell density increases (Gustavs et al. 2015). Thus, its macroscopic appearance as well provides a certain protection against the harsh terrestrial environment. In agreement with this model, *AL* presented the highest amount of lipophilic antioxidants (tocopherol and  $\beta$ -car) during all the experimental treatment, even under initial control conditions (Fig. 7.4). As has been elucidated using model organisms such as the green algae *Chlamydomonas reinhardtii*, antioxidants act as efficient protectors of thylakoids (Baroli and Niyogi 2000). The accumulation of these protective compounds may help to maintain the balance between efficient light harvesting, photochemistry and photoprotection in *AL* biofilms.

Considering the specific irradiation conditions in the lichens natural habitats, *CS* was considered the most irradiation-sensitive candidate, while *BR* occupied an intermediate position and *RP* was expected to cope well with the combined LT and HL stress. Unfortunately, we did not consider the protective potential of the mycobiont which can accumulate pigments and crystals of secondary metabolites in the upper cortex (Gasulla et al. 2012) in response to variable abiotic conditions. However, regarding the close phylogenetic relationship between the *CS* and *RP* photobionts, the here revealed contrasting pattern, points again to the hypothesis of Quaas et al. (2015) that the environment is more decisive than close phylogenetic relations.

Overall, the present study highlights the importance of NPQ in most algae species (all studied species except for *EB*) as a protective response to both LT and the combination of LT with HL. Besides, it demonstrates that the different responses of *EB*, *TA* and *AE*, were in any case sufficient to avoid strong photoinhibition under CA treatment and being essential to subsist in their respective hard environments.

Since the discovery of the operation of V-cycle (Yamamoto et al. 1962), it has been considered as one of the main components of the dissipation of excess energy as heat. By contrast, a more recent NPQ model proposed by Holzwarth et al. (2009) and Jahns and Holzwarth (2012) suggests the existence of two quenching sites. The quenching site known as Q1 is located in the major light harvesting complex, and does not require *Z* for its activation but it is amplified by the presence of *Z*. In contrast, Q2 is strictly *Z*-dependent and it is located in the minor antenna proteins. This model, originally based on observations of higher plants, has been used also to understand the diversity of NPQ observed in algae (Goss and Lepetit 2015). Quaas et al. (2015) observed these two trends in terms of the dependence between NPQ and *Z* in a study about the activation of NPQ process under HL in 6 different green algae. Based on those observations these authors gave mechanistic support to the high



diversity of NPQ mechanisms in algae. This variability was not dependent on phylogenetic position but on the selection pressure of different environments. This is logic as NPQ does not necessarily rely on a uniform mechanistic basis but can be realized by different processes at the molecular level (Quaas et al. 2015). In agreement with such observations, our results showed that in all algae species as well as in RP, there was an increase of AZ/VAZ along the treatment, but this index was not necessarily related to NPQ.

#### **7.4.2 Photobionts and lichens: How do they behave under photochilling stress conditions?**

The photobionts have often been considered as the more sensitive partner within the lichen symbiosis (De Vera and Ott 2010). The present study, comparing three photobionts in their free-living and symbiotic states, does not support the generalization of such assumption. Thus, two out of three photobionts, namely *AE* and *EB*, presented lower values of photochemical efficiency than their respective lichens along all the treatment. In contrast, the *TA* culture presented a decrease in  $F_v/F_m$  similar to *RP* (which is associated with a *Trebouxia* photobiont).

One of the main benefits that the algae obtains from the lichen symbiosis is the light shielding caused by the accumulation of fungal secondary metabolites in the upper surface of the cortex (Lawrey 1986). Nevertheless, the interaction between both organisms (fungus and alga) goes beyond this passive protection, inducing the accumulation of antioxidants and the activation of photoprotective mechanisms under stresses such as desiccation (Kranter et al. 2005). Sadowsky and Ott (2012) demonstrated that freezing temperatures do not cause a severe long-term stress reaction in different species of *Trebouxia* isolated from arctic lichen genera, indicating that the algae *per se* is robust towards such stress. In fact Kranter et al. (2005) described that both the mycobiont and the photobiont suffered oxidative damage during desiccation if



they were separated, but, in the form of lichen, each partner up-regulates the photoprotective system of the other. This effect is evidenced in *CS* and *AE*, which are the lichen and its respective photobiont. In this pair of organisms the sole effect of LT on  $F_v/F_m$  was higher in the alga than in the lichen, even when occurred under LL in the first and was combined with HL in the latter. Apart from this protective effect, there were other pairs, such as *TA* and *RP* in which  $AZ/VAZ$  and NPQ were correlated in both free alga and lichen, evidencing the existence of a common mode of NPQ regulation (Appendix 13).

**Fig. 7.6:** Summary of the physiological and biochemical responses of studied species during the experimental cold acclimation period. Blue and orange arrows represent the trends of each parameter under low temperature and low ( $30\mu\text{mol photons m}^{-2}\text{s}^{-1}$ ) high light ( $300\mu\text{mol photons m}^{-2}\text{s}^{-1}$ ) respectively (see Fig. 1). When no arrows trends were shown the parameter remained stable along the experimental treatment.

	AL	EB	AE	TA	BR	CS	RP
$AZ/VAZ$	↑ ↑	↑ ↑	↑ ↑	↑ ↑			↑ ↑
L/Chl	↓						
B-car/Chl	↓ ↓	↑	↑	↓			
Total toc/Chl	↓ ↓						
Sucrose	↓	↑ ↑		↓ ↓			
Sugar-alcohols	↓ ↓		↓ ↓				
$\Phi_{PSII}$	↓ ↓	↑	↓ ↓	↓ ↓	↑		↓
$\Phi_{NPQ}$	↑ ↑	↓ ↓	↑ ↑	↑			



### 7.4.3 Concluding remarks

In the present laboratory experiment, we aimed to give light on winter photoinhibition (WPI) process by further comparing the response to photochilling stress in algae living in a lichenized and in an aposymbiotic state. In conclusion, and regarding the main statement of Fig. 7.6 which summarizes the regulation potential within the variety of investigated processes across the investigated taxa, we state that “lichenization matters”. The up or down-regulation as indicated by the arrows is only occasionally found in the lichenized specimens, in contrast the algal strains regulate the majority of investigated traits, although not in a uniformly pattern. From previous studies on generalist green algal taxa, it is known that their physiological plasticity is tremendous. Thus, we have to consider that our results cannot be discussed as genus-wide characters, but have to be carefully interpreted as case studies confirming again the diversity and plasticity of these ecologically successful organisms. Further, the results on lichen are influenced by the absence of mycobiont-related data. Moreover, algal biofilms or soil crust are characterized by a diverse mixture of the most different components, thus being more similar to lichen thalli than to the here investigated unialgal cultures. To overcome this problem, and to finally figure out if the macroscopic appearance of biofilms might also have a protective function, we strongly recommend to include natural biofilms in follow-up studies.



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# 8. Discussion

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## SHEDDING LIGHT ON WPI AND NPQ: TWO INTIMATELY RELATED TERMS

Most of the Earth surface presents at least one cold season along the year. These periods of **low temperatures** (LT), can occur in combination with **high light** (HL), particularly under conditions of high atmospheric pressures and clear skies (Körner 1999). Under this harmful combination of stresses, photosynthetic organisms must compensate the imbalance between the light absorption and light utilization (Levitt 1980). One of the mechanisms that maintains this balance within optimal limits, is the dissipation of the excess of energy absorbed by chlorophylls (Chls) as heat, the so-called **non photochemical quenching** (NPQ) (Adams et al. 1995; Niyogi et al. 1998). An inherent consequence of this process is the existence of a downregulation of photochemical efficiency, here termed as **winter photoinhibition** (WPI), which results from the above mentioned stresses combination. The study and characterisation of the diversity of WPI, is the main objective in the present work. Throughout this study, **three conceptual approaches** have been used to achieve this objective: (i) data mining through the analysis of available literature, (ii) observational, through the study of physiological responses of natural communities and (iii) manipulative, through the modification of environmental conditions.

As stated before, WPI and NPQ are closely related concepts. However, the term NPQ is full of controversy. Although it is used as a synonym of thermal dissipation, it *sensu stricto* represents a fluorescence parameter. Besides, there are **other factors**, different from a genuine dissipative process, which may affect it. This is the case of the chloroplast movements (Dall'Osto et al. 2014), the Chl content or the state transitions (Brugnoli and Björkman 1992). On the other hand, the **traditional calculation of NPQ** (See Eq.1 in the introduction section) is also not



appropriate to estimate the magnitude of WPI, as  $F_m$  remains quenched even under predawn conditions (Ensminger et al. 2004; Porcar-Castell 2011; Verhoeven 2014). This was the reason why in this work, WPI was estimated from the depressions in the photochemical efficiency of PSII (measured as decreases of  $F_v/F_m$ ) (See Eq. 2, 3 and 4 in materials and methods section) (Verhoeven 2014). This approach presents some advantages, such as the fact that  $F_v/F_m$  has a maximum well-known value, that can be easily compared among very different species and environmental conditions.

### WPI IN PLANT KINGDOM. HOW? WHO? WHEN? DIVERSITY!

It is a fact well established by numerous studies that **woody species** are able to develop a strong **chronic** (slowly reversible) WPI (Demmig-Adams et al. 2006) while **herbs** present a **dynamic** WPI, characterized by a rapid reversion when temperature increases (Huner et al. 1993; Verhoeven et al. 1998; Öquist and Huner 2003). Furthermore, present study (Chapter 5, Fig. 5.1 and 5.2) shows that not only herbs (angiosperms) but also mosses and lichens, activate exclusively a mechanism of dynamic photoinhibition with little or no sustained downregulation of photosynthesis during winter. These observations raise immediately the question of what is the *raison d'être* for such differential response between woody plants and the rest of photosynthetic groups, which includes not only herbs (angiosperms), but also **mosses and lichens**, (both characterized along this thesis as dynamically photoinhibited organisms or even with no downregulation of photosynthesis during winter). One of the most plausible explanations could be a **physical-mechanical reason**. As woody species are taller than the rest of photosynthetic organisms, if they do not stop their physiological activity, they become exposed to the risk of xylem cavitation. Besides, their morphology does not assure to live under microclimate formed near to the soil, usually more favorable than in higher layers (Scherrer and Körner

2010; Larcher 2012). Moreover, in areas dominated by LT, plants become victims of their own evolutionary success since complex live cycles need more days of warm temperatures to develop leaves, roots and flowers, while, lichens and mosses require less time to amortize carbon costs (Pannewitz et al. 2003; Pointing et al. 2015). Nevertheless, the existence of these two distinct patterns of behavior which divide woody and herbaceous species in two separate groups is **not so strict** and in chapter 5 we described four herbs (*Digitalis parviflora*, *Festuca* sp., *Saxifraga paniculata* and *Thymelaea* sp.) and one lichen (*Rhizocarpon* sp) showing chronic WPI (Fig. 5.1).

The downregulation of photosynthesis is activated by the decrease in **temperature and/or photoperiod** (Krol et al. 1995). Moreover, the analysis of literature data (chapter 4 Fig. 4.4), revealed that, independently of the biome or functional types, the temperature threshold necessary for the activation of chronic WPI is 0°C. Hence, at freezing temperatures, chronic WPI is higher than 50% for all the analyzed species. However, it should be noted that this trend is biased, as most of the information about the regulation and nature of WPI comes from a few species, basically conifers and some crops (chapter 4, Fig. 4.2). In the **recovery** of those chronically photoinhibited species, there can be two phases (Verhoeven 2014): one rapid, followed by a slower one. The first is the main component at above-freezing temperatures, particularly in shaded environments (Verhoeven et al. 1998; Verhoeven et al. 2009) while the slow phase of recovery occurs only after several days of warming, being more pronounced at HL (Ottander and Oquist 1991; Ottander et al. 1995). Furthermore, Verhoeven (2014) observed that in conifers seasonality also influences the type of recovery. Our results were consistent with this observation as leaves and green stems of *V. album* presented the slow component in winter and the rapid in spring (chapter 6, Fig. 6.3).





The study of WPI in photosynthetic **organs** different to leaves carried out in this thesis, also adds a new dimension to the complexity of the phenomenon. The **green stems** of *Cytisus* sp. (chapter 5 Fig. 5.2) supported the notion that the slow component is the dominant in temperate woody species during winter, not only in leaves (Verhoeven 2013) but also in stems. Moreover, the variability of WPI is high even within the different photosynthetic tissues of the same individual. This issue has been addressed in chapter 6 by the study of the photosynthetic responses of stems and leaves of *V. album*. Interestingly, WPI was lower in stems than in leaves during winter, helping to maintain the positive carbon balance of the whole plant during this season. Furthermore, even inside stems, there are significant differences in the degree of photoinhibition depending on the age of each stem portion. Thus, WPI decreases with stems **ageing**. This counterintuitive pattern, which represents an increase of vigor with age, has been observed in other species and processes, and has been postulated as the negative senescence theory (Morales et al. 2013; Jones et al. 2014). **Negative senescence** is a process described for most of perennial plants that consists on the increase of efficiency with the ageing of tissues. The main statement is that the hormones responsible of growth, cytokinins, prevent the senescence in cells and leaves and consequently in the whole organisms (Munné-Bosch 2015).

## WPI AND Z: TWO CLOSE FRIENDS

Around late 1980s and early 1990s, Demmig et al. (1987, 1988) provided the first evidence of an involvement of the **V-cycle as an important factor in the activation and regulation of NPQ**, particularly in its sustained forms (chronic WPI) in plants living in montane and subalpine areas in Colorado. These authors found that what makes different the V-cycle response in winter woody evergreens, is that, once V is transformed into Z, the latter is retained overnight (Adams and Demmig-Adams 1995;

Adams et al. 1995; Watson et al. 2004), correlating its level with the predawn inhibition of  $F_v/F_m$  (equivalent to WPI). The literature survey (chapter 4) shows that, as occurred with the activation of WPI, there is a **temperature threshold** for the overnight Z retention. Thus, when the ambient temperature is below 2°C, the AZ/VAZ ratio is always higher than 0.5, while the opposite trend is observed at temperatures higher than 7°C. It implies that the activation of xanthophyll dependent mechanism of WPI is a requisite for maintaining photosynthetic structures particularly at freezing temperatures. Furthermore, irrespective of the WPI level all the species studied on chapters 5 and 6 also showed an enhanced Z content in winter compared to that of spring. This concurs with the Fig. 4.2C (chapter 4) in which it was clarified that the presence of Z does not assure the presence of WPI while the **absence of Z means no winter stress**. Present work also highlights that when species from chapter 5 and 6, were artificially forced to recover from LT stress, changes on epoxidation index did not match the recovery of  $F_v/F_m$  (chapter 5, Fig. 5.2). The fact that **no photoinhibited species**, particularly mosses and lichens, showed also high concentrations of Z in winter, agrees with the specific functions of Z elucidated using the *npq1* and *npq2* mutants, which are V de-epoxidase and Z epoxidase deficient, respectively (Niyogi et al. 1998; Kalituhu et al. 2007; Havaux et al. 2007). These studies have revealed that besides being an essential molecule in the process of heat dissipation (Jahns and Holzwarth 2012), where it acts as a direct quencher or a modulator in NPQ, Z has a central role in the deactivation of excited  $^1\text{Chl}^*$  in the antenna of PSII (Niyogi et al. 1998) and an antioxidant function as non-protein bound xanthophyll in the lipid phase of the thylakoid membrane (Havaux et al. 2007).

A more **recent NPQ model** (Holzwarth et al. 2009; Jahns and Holzwarth 2012) suggests the existence of two quenching sites. One, known as Q1, located in major light harvesting complexes, which does not



require Z for its activation but it is amplified by the presence of Z. And the second one, known as Q2, which is strictly Z-dependent and it is located in the minor antenna proteins. Under this mechanistic model, the rapidly reversible component would represent a form of the Q1 type of quenching and the slowly reversible component would be a sustained form of the Q2 type of quenching, as indicated Verhoeven (2014).

## ROLE OF THYLAKOID PROTEIN CHANGES ON WPI: A DIFFICULT MATTER

The downregulation of photosynthesis in winter is also intimately linked with a deep restructuring of PSII. This restructuring implies changes on all thylakoid constituents, including proteins. In conifers, the best characterised group, a pronounced decrease in abundance of the PSII **core complex protein (D1)** occurs in winter (Zarter et al. 2006; Verhoeven et al. 2009). Nevertheless, for the rest of thylakoid proteins, a uniform pattern of changes cannot be concluded. Hence, their responses depend on the species or the specific conditions of the experiment (chapter 1 table 1.2) (Verhoeven 2014). Despite this variability, some general trends are usually observed, such as the decrease of **minor and major antenna proteins (Lhcb1-6)** with the aim of reducing the capture of light (Yang et al. 1998; Niyogi 2000). Other proteins that show seasonal changes and play important roles under stress are the **Elips** (early light induced proteins) and **PsbS** (Zarter et al. 2006). It is supposed that, Elips bind at least Chla and L molecules (Adamska et al. 1999), taking part in the Chl turnover or storage. Probably, they also bind to Z as was evidenced by parallel changes in the upregulation of Elip synthesis and Z retention in overwintering bearberry plants (Zarter et al. 2006). The PsbS protein, takes part in the process of NPQ, acting as a sensor of the lumen acidification (Li et al. 2004), and as an antenna organizer (Teardo et al. 2007). This variability of responses

observed among conifer species becomes more evident when other functional groups are considered (chapter 5, Fig. 5.3). By contrast, proteins from different organs of the same species showed similar patterns of response. Hence, thylakoid proteins cited before followed the same wintry patterns in leaves than in stems of *V. album* with no differences among seasons except for D1, as was described in conifers (Chapter 6, Fig. 6.5).

The **aggregation of antenna system** (LHCs) located in the grana membrane of higher plants (Johnson et al. 2011) influences NPQ, particularly in its sustained forms. LHCs are the site where the majority of VAZ cycle pigments are located and where the NPQ takes place (Goss and Lepetit 2015). High proton concentration and the presence of Z are beneficial for LHC aggregation and the consequent thermal dissipation process (Ruban et al. 1997). Studies examining individual LHCs have demonstrated that once Z is bound to LHC, the proton gradient is no longer necessary for maintaining thermal dissipation (Dall'Osto et al. 2005), suggesting that sustained forms of thermal dissipation can persist in darkness if Z is maintained.

Some post-translational changes in thylakoid proteins such as the **phosphorylation of D1 protein** were detected in *S. muralis* and *C. cantabricus* (Chapter 5, Fig. 5.3). This response is also implicated in the response to stress (Ebbert et al. 2005) as it: (i) reduces the oxidative damage of membrane proteins and the generation of ROS (Chen et al. 2012), (ii) avoids the proteolysis of core complexes (Aro et al. 1992; Kato and Sakamoto 2014) and (iii) maintains the integrity of PSII. All these protective effects could justify why the phosphorylation of D1 protein protects PSII during winter acclimation in so diverse systems such as the stems of *C. cantabricus* or the phyllids of *S. muralis* described here. An observation, also corroborated in conifer needles by Adams et al. (2001).



## ALGAE: GOING BEYOND THE GENERAL PATTERNS

Contrasting with the monophyletic terrestrial plants, green algae are a much more complex and diverse group. However the characterisation of their photosynthetic responses has been performed basically using a single model species: *Chlamydomonas reinhardtii*. These studies have shown that in *C. reinhardtii*, the NPQ is only formed under HL conditions. Nevertheless, among algae, physiological diversity goes beyond this particular case. In chapter 7, in two of the four studied algae species from **Trebouxiophyceae** family (*Elliptochloris bilobata* and *Trebouxia arboricola*), the sole exposition to LT was enough to induce an increment of NPQ, independently of the radiation. In algae, also the antenna structure required for the formation of NPQ is different since Lhcb3 and Lhcb6 are missing (Gunning and Schwartz 2000). However, the most prominent difference is that in several green algae, the PsbS protein is in principle not present and is replaced by the **LHCSR** protein (Peers et al. 2009). This generalization has been recently questioned by the finding of the presence of PsbS protein in the chlorophyta macroalgae *Ulva prolifera* (Mou et al. 2013) and in some species integrated in the Streptophyta division (ancestors of high plants) (Gerotto and Morosinotto 2013). This could justify the presence of PsbS also in algae living as photobionts of lichens that is reported in Chapter 5 (Fig. 5.3), assuring PsbS is also present in algae species, at least, when they are lichenized.

**SUGARS AND WPI:** An alternative point of view in the study of photoinhibition (including WPI), states that the phenomenon should be placed in the context of **whole-plant source-sink regulation of photosynthesis** (Adams et al. 2013). Hence, a plant becomes photoinhibited when the consumption of sugars is lesser than their synthesis or when all the light energy harvested cannot be utilized by the chloroplast to fixate CO<sub>2</sub>. To study whether this is the case in **algae and lichens, low**



**molecular weight carbohydrates** (LMWC) were analysed in algae cultures and lichens exposed to LT. This experimental approach did not support this theory as their LMWC content did not change, or even decreased, along the CA treatment, in combination or not with HL (Chapter 7, Fig. 7.5). The only exception was alpine species *Elliptochloris bilobata* in which sucrose content increased in response to CA. In this particular case, photochemical activity was maintained in response to CA, rejecting any feedback effect on photosynthesis caused by sucrose accumulation.

**LICHENIZED VS APOSYMBIOTIC:** Algae usually dominate in stressful environments such as Antarctica in both lichenized and aposymbiotic states (Bartak et al. 2004; Sadowsky and Ott 2012). The photobionts have often been considered as the more **sensitive partner** within the lichen symbiosis (De Vera and Ott 2010). But in the present work, it has been demonstrated that although the interaction between both organisms (the algae and the fungus) help to face winter stress, there are species from genus *Trebouxia* that respond in the same way when they are lichenized or in a free-living state (chapter 7, Fig. 7.2). In general, one of the main benefits that the algae obtain from the lichen symbiosis is the light **shielding** (Lawrey 1986) but, the interaction between both organisms also induces the accumulation of **antioxidants** and the activation of **photoprotective** mechanisms under stress.

#### PHYSIOLOGICAL DIVERSITY: MORE AND MORE BEHAVIORS IN RESPONSE TO WINTER

*The more I learn, the more I realize how much I don't know (Einstein)*

Diversity is intrinsically linked to all physiological processes in the Plant Kingdom, and WPI is not an exception. The formation of species implies evolution, and evolution cannot be understood without diversification. Each species, living on Earth, is a particular case shaped by



time and influenced mainly by the environmental conditions. This is the reason why model species are useful tools to understand complex biological processes but at the same time, they represent only one strategy among the many that the course of evolution has generated (García-Plazaola et al. 2012). Hence, it is necessary expand the experimentation beyond *Arabidopsis thaliana* for the case of herbs (Meinke 1998), *Physcomitrella patens* for bryophytes or *Chlamydomonas reinhardtii* for green algae.

Throughout this thesis we have aimed to gain light in the WPI process, describing different physiological behaviours within the Plant Kingdom, in response to winter stress. The study has been conducted over 59 species including 6 terrestrial green algae, 12 lichens, 5 mosses, 20 herbs and 15 woody plants (See Appendix 1). In table 8.1, there is a summary of the general responses observed in this thesis. It is difficult to establish a universal response for all the photosynthetic groups. The target of this thesis, WPI, was mostly observed in stems and leaves of woody species, while in the rest was uncommon. Nevertheless, some trends can be established for most parameters associated to winter acclimation: (i) a enhancement of antioxidants, including Z and tocopherols, (ii) a diminution of antenna size, indicated by the increase of Chla/b and (iii) a deactivation of RC indicated by the decrease of D1. However, it should be noticed that even within each group there are exceptions to these general trends. This gives importance to studies that involve the use of non-model organisms with the aim of knowing the world we are surrounded by and to quench bit by bit with the thirst of knowledge inherent in human beings.

From the ecological point of view, and taking into consideration the compromise that science has to provide information about current ecological changes in our planet, these results highlight that future climatic scenarios of warmer winters could alter the ecological relationships in



mountain and subalpine ecosystems in detriment of woody species as seems that WPI is a conservative strategy mainly present in this group.

**Table 8.1:** Summary of the physiological and biochemical responses of studied functional groups during winter. Upward arrows indicate enhancement in comparison to spring, downward arrows indicate decrease or degradation with respect to spring, “-” means absence of changes, “V” means that the response is variable depending on the species and “?” means no data analyzed.

	Green stems	Leaves		Mosses	Lichens	Algae
		Woody	Herbs			
<b>Downregulation of photosynthesis</b>	Yes Chronic	Yes Chronic	No	No	Yes dynamic	Yes
<b>Recovery components in winter</b>	Fast + Slow	Fast + Slow	-	-	Fast	?
<b>Chla/b</b>	↑	↑	↑	↑	↑	-
<b>β-carotene</b>	V	↑	-	↓	↓	V
<b>α-tocopherol</b>	V	↑	↑	↑	↓	-
<b>VAZ/Chl</b>	V	↑	↑	↓	↓	↑
<b>AZ/VAZ</b>	↑	↑	-	↓	-	↑
<b>D1</b>	V	↓	-	↓	↓	?
<b>PsbS</b>	V	-	-	↓	-	?
<b>Elip</b>	V	-	-	↓	-	?
<b>LHC</b>	-	-	V	V	V	?





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# 9. Conclusions

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

- ❁ Winter photoinhibition (WPI) is a process highly variable within the Plant Kingdom with woody plants showing much higher levels of it than any other functional group. However this photoprotective process is not exclusive of this group, and some herbs and lichens also show sustained levels of dissipation.
- ❁ A common characteristic of chronic WPI, independently of the biome and the functional group is that the temperature threshold for its activation is around 0°C.
- ❁ When plants are transferred from the field to a warmer environment, recovery from WPI occurs in two phases, the first fast and the second slow, lasting the process in trees and shrubs several days or even weeks.
- ❁ At temperatures below 2°C, there is a sustained zeaxanthin accumulation. Nevertheless, the epoxidation rate is usually faster than the increase in the photochemical efficiency under recovery treatments. Z is also accumulated in plants not showing WPI. All these observations suggest that Z may play roles other than WPI or NPQ regulation.
- ❁ In woody species, recovery pattern of photosynthetic stems is equal to those of leaves, presenting both, the slow and the rapid component. However, stems contribute to maintain a positive carbon balance during winter season thanks to their lowest levels of WPI and the positive effect of ageing on photosynthetic efficiency, which at the same time supports the existence of a negative senescence in mistletoe stems.
- ❁ Free-living algae are usually more affected by photochilling stress than photobionts of the same species when forming lichenic symbioses. Furthermore, protection by the lichen cortex goes beyond a light shielding effect, inducing also biochemical changes.

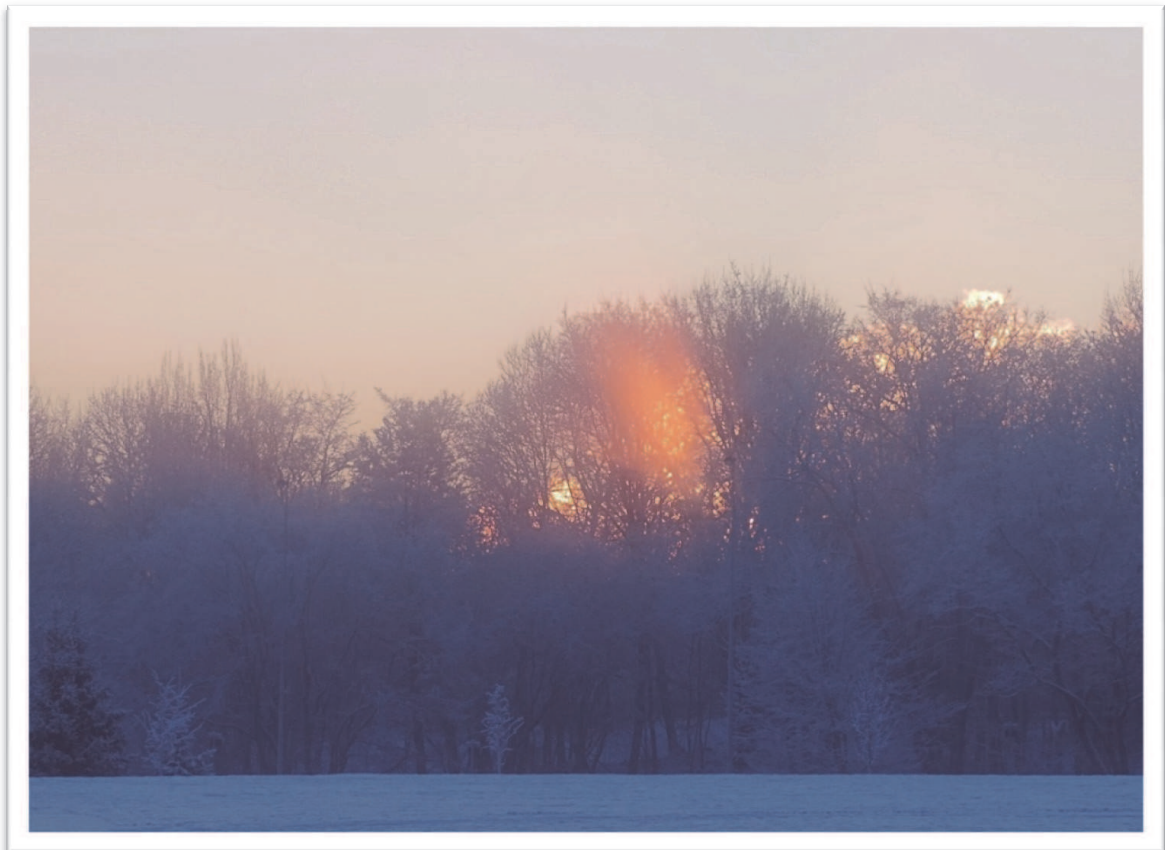


- ❁ The high diversity of NPQ mechanisms in algae, were not dependent on phylogenetic position but on the selection pressure of different environments.
- ❁ The accumulation of sugars in algae and lichens does not have any cause-effect relationship with the process of photoinhibition under cold stress.
- ❁ Thylakoid protein changes under low temperatures did not followed consistent patterns in the different functional groups, with the exception of D1 protein, which decreases in response to cold in all the species studied with the exception of green stems.

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# 10. General *bibliography*

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# 11. Appendixes

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**APPENDIX 1: LIST OF SPECIES USED IN THIS THESIS.  
IN BRACKETS THERE IS THE NUMBER OF THE CHAPTER WHERE  
THEY APPEAR.**

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<b>Woody species</b>		
<i>Calluna vulgaris</i> (5)	<i>Erica vagans</i> (5)	<i>Pinus sylvestris</i> (5)
<i>Cytisus cantabricus</i> (5)	<i>Ericacinerea</i> (5)	<i>Pinus uncinata</i> (5)
<i>Daphne cneorum</i> (5)	<i>Genista hispanica</i> (5)	<i>Vaccinium myrtillus</i> (5)
<i>Erica aragonensis</i> (5)	<i>Globularia repens</i> (5)	<i>Viscum album</i> (6)
<i>Erica cinerea</i> (5)	<i>Juniperus communis</i> subsp. <i>alpine</i> (5)	
<b>Herbaceous species</b>		
<i>Arabis alpine</i> (5)	<i>Plantago lanceolata</i> (5)	<i>Sedum album</i> (5)
<i>Armeria</i> sp. (5)	<i>Poacea</i> (5)	<i>Sedum brevifolium</i> (5)
<i>Asperula hirta</i> (5)	<i>Polypodium</i> sp (5).	<i>Sedum</i> sp. (5)
<i>Cerastium fontanum</i> (5)	<i>Ranunculus repens</i> (5)	<i>Senecio</i> sp. (5)
<i>Digitalis parviflora</i> (5)	<i>Saxifraga paniculata</i> (5)	<i>Teucrium pyrenaicum</i> (5)
<i>Festuca</i> sp. (5)	<i>Scilla</i> sp. (5)	<i>Thymelaea</i> sp. (5)
<i>Hieracium pilosella</i> (5)		
<b>Mosses</b>		
<i>Dicranum scoparium</i> (5)	<i>Grimmia pulvinata</i> (5)	<i>Polytrichum piliferum</i> (5)
<i>Didymodon</i> sp. (5)	<i>Syntrichia muralis</i> (5)	
<b>Lichens</b>		
<i>Baeomyces rufus</i> (7)	<i>Parmelia saxatilis</i> (5)	<i>Rhizocarpon</i> sp. (5)
<i>Cladonia squamosa</i> (5)	<i>Physcia</i> sp. (5)	<i>Umbilicaria cylindrical</i> (5)
<i>Dermatocarpon</i> sp. (5)	<i>Pseudevernia furfuracea</i> (5)	<i>Umbilicaria polyphylla</i> (5)
<i>Lasallia hispanica</i> (5)	<i>Ramalina</i> sp. (7)	<i>Usnea</i> sp. (5)
<i>Lichinella stipatula</i> (5)	<i>Ramallina pollinaria</i> (4)	
<b>Algae</b>		
<i>Apatococcus lobatus</i> (7)	<i>Elliptochloris bilobata</i> (7)	<i>Trebouxia arboricola</i> (7)
<i>Asterochloris erici</i> (7)	<i>Klebsormidium flaccidum</i> (7)	

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APPENDIX 2: MEDIUMS FOR GROWING ALGAE CULTURES

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**Bold basal medium with 3-fold nitrogen and vitamins (3N-BBM+V)**

This media is a BBM modified by the addition of triple nitrate concentration and vitamins.

Stock solutions in g / 1000 ml water	for 1 litre final medium
75.0 g NaNO <sub>3</sub>	10 ml
2.5 g CaCl <sub>2</sub> .2H <sub>2</sub> O	10 ml
7.5 g MgSO <sub>4</sub> .7H <sub>2</sub> O	10 ml
7.5 g K <sub>2</sub> HPO <sub>4</sub> .3H <sub>2</sub> O	10 ml
17.5 g KH <sub>2</sub> PO <sub>4</sub>	10 ml
2.5 g NaCl	10 ml
Trace element solution *	6 ml
vitamin B <sub>1</sub> (0.12g Thiaminhydrochloride in 100 ml distilled water)	1 ml
vitamin B <sub>12</sub> (0.1 g Cyanocobalamin in 100 ml distilled water, take 1 ml of this solution and add 99 ml distilled water)	1 ml

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\*Trace element solution:

Add to 1000 ml of distilled water 0.75 g Na<sub>2</sub>EDTA and the minerals in exactly the following sequence:

FeCl <sub>3</sub> .6H <sub>2</sub> O	97.0 mg
MnCl <sub>2</sub> .4H <sub>2</sub> O	41.0 mg
ZnCl <sub>2</sub>	5.0 mg
CoCl <sub>2</sub> .6H <sub>2</sub> O	2.0 mg
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	4.0 mg

Make up to 1 liter with distilled water. For agar add 15 g per litre Bacterial Agar. Autoclave at 15 psi for 15 minutes. ([www.epsag.uni-goettingen.de](http://www.epsag.uni-goettingen.de))

**Modified bold's basal medium for heterotrophs TOM (Trebouxia Organic Medium)**

Trebouxia Organic Media (TOM) is a solid 1.5% DIFCO agar according to Ahmadjian (1967) and modified after by Friedl (1989) by the addition of 1.5 % glucose, 2 % proteose-peptone to 3N-BBM+V media.

([www.epsag.uni-goettingen.de](http://www.epsag.uni-goettingen.de)).

APPENDIX 3: COMPOSITION OF BUFFERS AND GELS USED FOR ELECTROPHORESIS

<p><b><u>Extraction buffer:</u></b>            Tricine → 8.96 g/l            Sorbitol → 72.87 g/l            Anhydrous NaCl → 0.058 g/l            Anhydrous MgCl<sub>2</sub> → 1.02 g/l            Adjust the pH to 7.8 and storage at 4°C</p>	<p><b><u>Washing buffer:</u></b>            Tricine → 8.96 g/l            Anhydrous NaCl → 0.58 g/l            Anhydrous MgCl<sub>2</sub> → 1.02 g/l            Adjust the pH to 7.8 and storage at 4°C</p>
<p><b><u>Solubilization buffer</u></b>            Tricine → 7.2 g/l            SDS (sodium dodecyl sulfate) → 40g/l            EDTA (Ethylenediaminetetraacetic acid disodium salt dehydrate) → 0.336 g/l            Adjust the pH to 7.8</p>	<p><b><u>Solubility buffer</u></b>            DTT (dithiothreitol) → 0.1 gr            Protease inhibitor cocktail → 2µl            Solubilization buffer → 10 ml            Not storage</p>
<p><b><u>Loading buffer</u></b>            200 mM Tris-HCl pH 6.8            400 mM DTT            8% SDS (sodium dodecyl sulfate)            0.4% Bromophenol-blue            40% Glicerol            Storage at -20°C</p>	<p><b><u>Electrophoresis buffer 10x</u></b>            Tris → 30.2 g/l            Glicine → 144 g/l            SDS (sodium dodecyl sulfate) 20% → 50 ml/l            Storage at 4°C</p>
<p><b><u>Transference buffer</u></b>            Absolut ethanol → 200ml/l            Electrophoresis buffer 10x → 100ml            Deionized water → 700ml</p>	<p><b><u>TBS (Tris Buffered Saline) 10x</u></b>            Tris → 24.22 g/l            Anhydrous NaCl → 87.66 g/l  <b><u>T-TBS (Tris Buffered Saline-Tween 20)</u></b>            TBS 10x → 100ml/l            Tween 20 → 1ml/l  <b>Block buffer</b> → 5% T-TBS in skimmed powder milk</p>

**Resolving or running gel 10% (for proteins around 25-30Kda)**

H<sub>2</sub>O → 0.90ml/10ml

Tris pH 8.8 1M → 3.90ml/10ml

SDS 10% (sodium dodecyl sulfate) → 0.10ml/10ml

Acrilamide 30% → 5ml/10ml

TEMED (N,N,N',N'-Tetramethylethylenediamine) → 0.004ml/10ml

APS 10% (ammonium persulfate) → 0.10ml/10ml

**Stacking gel 5% (for proteins around 30Kda)**

H<sub>2</sub>O → 6.17 ml/10ml

Tris pH68.8 1M → 1.95 ml/10ml

SDS 10% (sodium dodecyl sulfate) → 0.10 ml/10ml

Acrilamide 30% → 1.67 ml/10ml

TEMED (N,N,N',N'-Tetramethylethylenediamine) → 0.01 ml/10ml

APS 10% (ammonium persulfate) → 0.10 ml/10ml

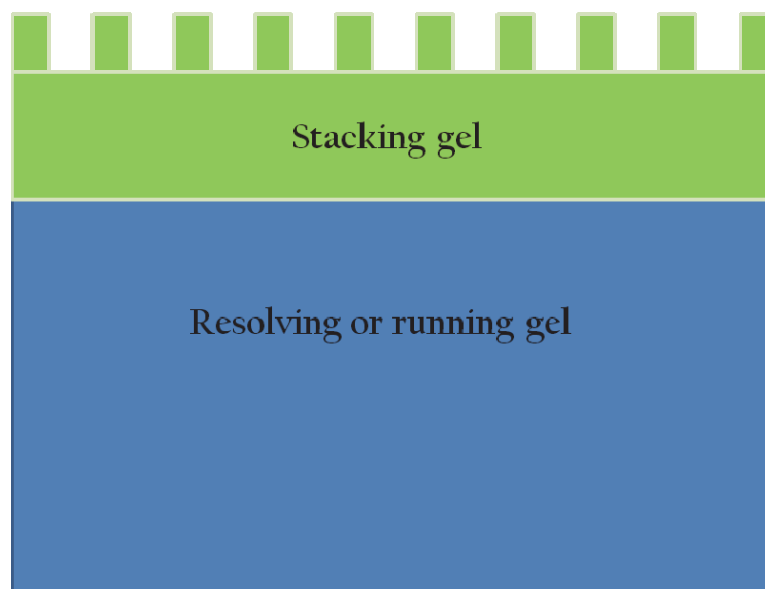


Fig. 11.1: Electrophoresis gel composition

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## APPENDIX 4: LIST OF REFERENCES INCLUDED IN THE META-ANALYSIS (CHAPTER 4).

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**APPENDIX 4B: LIST OF SPECIES INCLUDED IN THE META-ANALYSIS (CHAPTER 4).**

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<b>SPECIES</b>			
<i>Abies alba</i>	<i>Erythrophleum guineense</i>	<i>Myrtus communis</i>	<i>Sabina przewalskii</i>
<i>Abies balsamea</i>	<i>Eucaliptus pauciflora</i>	<i>Nicotiana tabacum</i>	<i>Saxifraga canaliculata</i>
<i>Abies lasiocarpa</i>	<i>Eucaliptus urnigera</i>	<i>Olea europaea</i>	<i>Secale cereale</i>
<i>Acacia melanoxylon</i>	<i>Eucalyptus nitens</i>	<i>Olea grandiflora</i>	<i>Solanum commersonii</i>
<i>Achnatherum splendens</i>	<i>Eucalyptus pauciflora</i>	<i>Oryza sativa</i>	<i>Solanum sogarandinum</i>
<i>Arabidopsis thaliana</i>	<i>Euonymos kiautschovicus</i>	<i>Pennisetum clandestinum</i>	<i>Solanum tuberosum</i>
<i>Arctostaphyllum uva-ursi</i>	<i>Eupatorium adenophorum</i>	<i>Pennisetum setaceum</i>	<i>Spinacea oleracea</i>
<i>Avena sativa</i>	<i>Festuca pratensis</i>	<i>Phaseolus vulgaris</i>	<i>Stranvaesia niitakayamensis</i>
<i>Avicennia marina</i>	<i>Fontinalis antipyretica</i>	<i>Phyllirea latifolia</i>	<i>Taxus baccata</i>
<i>Bactris gasipaes</i>	<i>Globularia alypum</i>	<i>Picea abies</i>	<i>Trachycarpus fortunei</i>
<i>Bohemeria rugulosa</i>	<i>Glycine max</i>	<i>Picea engelmannii</i>	<i>Tripholium michelianum</i>
<i>Brassica napus</i>	<i>Gossiphium hirsutum</i>	<i>Picea glehnii</i>	<i>Tripholium subterraneum</i>
<i>Brassica rapa</i>	<i>Halimium calycinum</i>	<i>Picea mariana</i>	<i>Tripsacum dactyloides</i>
<i>Bromus inermis</i>	<i>Halimium halimifolium</i>	<i>Picea morrisonicola</i>	<i>Triticum aestivum</i>
<i>Buugainvillea spectabilis</i>	<i>Hedera helix</i>	<i>Pinus armandii</i>	<i>Trochodendron aralioides</i>
<i>Calathea makoyana</i>	<i>Heuchera americana</i>	<i>Pinus banksiana</i>	<i>Tuberaria gutata</i>
<i>Calistemon lanceolatus</i>	<i>Hevea brasiliensis</i>	<i>Pinus cembra</i>	<i>Vaccinium vitis-idaea</i>
<i>Calophyllum polyanthum</i>	<i>Hexastylis arifolia</i>	<i>Pinus contorta</i>	<i>Valonia utricularis</i>
<i>Camellia japonica</i>	<i>Hordeum vulgare</i>	<i>Pinus halepensis</i>	<i>Vigna unguiculata</i>
<i>Camellia sinensis</i>	<i>Ilex aquifolium</i>	<i>Pinus morrisonicola</i>	<i>Vinca minor</i>
<i>Ceratonia silicua</i>	<i>Ipomoea batatas</i>	<i>Pinus mugo</i>	<i>Vitis vinifera</i>
<i>Chamaemulum novile</i>	<i>Jatropha curcas</i>	<i>Pinus pinea</i>	<i>Xanthoria parietina</i>
<i>Cichorium intybus</i>	<i>Jungermannia exsertifolia</i>	<i>Pinus ponderosa</i>	<i>Yucca brevifolia</i>
<i>Cistus albidus</i>	<i>Junniperus oxycedrus</i>	<i>Pinus strobus</i>	<i>Yucca glauca</i>
<i>Cistus creticus</i>	<i>Junniperus phoenicea</i>	<i>Pinus sylvestris</i>	<i>Yucca schidigera</i>
<i>Cistus salvifolius</i>	<i>Kandelia candel</i>	<i>Pinus taiwanensis</i>	<i>Yushana niitakayamensis</i>
<i>Citrus sinensis</i>	<i>Khaya ivorensis</i>	<i>Pistacia lentiscus</i>	<i>Zea mays</i>
	<i>Leymus chinensis</i>	<i>Pisum sativum</i>	<i>Ziziphus attopensis</i>

<i>Cleistogenes squarrosa</i>	<i>Linociera insignis</i>	<i>Poa pratensis</i>
<i>Coffea arabica</i>	<i>Lolium perenne</i>	<i>Pometia tomentosa</i>
<i>Coffea canephora</i>	<i>Lomatia ferruginea</i>	<i>Prosopis juliflora</i>
<i>Coffea dewevrei</i>	<i>Lycopersicon esculentum</i>	<i>Pseudotsuga menziesii</i>
<i>Colobanthus quitensis</i>	<i>Lycopersicon hirsutum</i>	<i>Quercus coccifera</i>
<i>Cornus sericea</i>	<i>Lycopersicon peruvianum</i>	<i>Quercus glauca</i>
<i>Cryptomeria japonica</i>	<i>Machilus thunbergii</i>	<i>Quercus ilex</i>
<i>Cucumis sativus</i>	<i>malva neglecta</i>	<i>Quercus myrsinaefolia</i>
<i>Dalbergia odorifera</i>	<i>Manglifer indica</i>	<i>Quercus oleoides</i>
<i>Daphniphyllum humile</i>	<i>Medicago polymorpha</i>	<i>Quercus suber</i>
<i>Dendranthema grandiflora</i>	<i>Medicago truncatula</i>	<i>Quercus virginiana</i>
<i>Deschampsia antarctica</i>	<i>Miscanthus floridulus</i>	<i>Rhododendron ferrugineum</i>
<i>Doritaenopsis</i>	<i>Miscanthus transmorrisonensis</i>	<i>Rhododendron mori</i>
<i>Embothrium coccineum</i>	<i>Miscanthus x giganteus</i>	<i>Rhododendron maximum</i>
<i>Eragrostis minor</i>	<i>Momordica charantia</i>	<i>Rosa hybrida</i>
<i>Erica multiflora</i>	<i>Mussa acuminata</i>	<i>Rosmarinum officinalis</i>
<i>Erica scoparia</i>	<i>Mussa paradisiaca</i>	<i>Sabina chinensis</i>



APPENDIX 5: AVERAGE PHOTOCHEMICAL EFFICIENCY OF PSII ( $F_v/F_m$ ) AND PIGMENT COMPOSITION OF THE DIFFERENT FUNCTIONAL GROUPS IN WINTER.

The number of species included where: 14 woody plants; 20 herbaceous plants; 5 mosses and 9 lichens (n=5 for each species).

		$F_v/F_m$ (30 min recovery)	$F_v/F_m$ (12h recovery)	V/Chl (mmol mol <sup>-1</sup> )	A/Chl (mmol mol <sup>-1</sup> )	Z/Chl (mmol mol <sup>-1</sup> )	AZ/VAZ	VAZ/Chl (mmol mol <sup>-1</sup> )
Winter	Woody plants	0.43	0.53	114.60	33.08	32.82	0.36	180.51
	Herbs	0.67	0.71	109.88	6.77	11.80	0.14	128.46
	Mosses	0.61	0.58	68.53	6.27	25.90	0.31	100.70
	Lichens	0.58	0.56	60.43	2.52	13.77	0.23	76.73
Spring	Woody plants	0.75	0.77	87.87	5.36	8.59	0.14	101.83
	Herbs	0.72	0.74	85.95	6.40	11.05	0.17	103.40
	Mosses	0.50	0.56	59.63	26.39	45.19	0.44	131.21
	Lichens	0.50	0.58	65.74	2.10	17.06	0.24	84.91

Continuation Appendix 5

		Chl a/b	Neo/Chl mmol mol <sup>-1</sup>	L/Chl mmol mol <sup>-1</sup>	$\alpha$ -Car/Chl mmol mol <sup>-1</sup>	$\beta$ -Car/Chl mmol mol <sup>-1</sup>	Total Car/Chl mmol mol <sup>-1</sup>	$\alpha$ -Toc/Chl mmol mol <sup>-1</sup>	Total Toc/Chl mmol mol <sup>-1</sup>
Winter	Woody plants	3.33	46.56	219.63	1.11	113.15	560.97	626.41	654.16
	Herbs	3.08	46.73	181.89	0.10	105.39	462.56	331.52	340.78
	Mosses	2.56	51.92	185.23	1.68	144.41	483.96	220.29	242.56
	Lichens	3.39	71.40	218.34	5.94	79.26	451.68	54.20	54.20
Spring	Woody plants	3.15	38.57	141.20	1.80	95.13	378.52	148.14	158.29
	Herbs	2.96	40.19	148.40	0.17	102.37	394.53	173.52	191.44
	Mosses	2.26	60.43	205.15	7.82	157.08	561.58	103.34	252.40
	Lichens	3.00	88.15	329.31	3.35	146.09	652.15	90.26	100

APPENDIX 6: PHOTOPROTECTIVE PARAMETERS: DYNAMIC AND CHRONIC PHOTOINHIBITION (WPI<sub><12H</sub> AND WPI<sub>>12H</sub> RESPECTIVELY) OF EACH ANALYZED SPECIES AND PIGMENT CONCENTRATIONS IN WINTER.

Results are the average of 5 replicates.

	% WPI <sub>&gt;12h</sub>	% WPI <sub>&lt;12h</sub>	AZ/VAZ	AZ/Chl	L/Chl	β-Car/Chl	VAZ/Chl	α-Toc/Chl
				(mmol molChl <sup>-1</sup> )	(mmol molChl <sup>-1</sup> )	(mmol molChl <sup>-1</sup> )	(mmol molChl <sup>-1</sup> )	(mmol molChl <sup>-1</sup> )
<i>Cytisus cantabricus</i>	31,50	6,45	0,32	54,90	232,72	133,42	166,07	274,01
<i>Calluna vulgaris</i> 1° site	17,02	10,15	0,40	57,63	195,70	78,78	143,25	470,38
<i>Calluna vulgaris</i> 2° site	0,00	23,88	0,54	85,48	199,06	73,30	155,78	757,49
<i>Calluna vulgaris</i> 3° site	27,67	0,00	0,24	28,15	163,54	81,74	119,94	368,38
<i>Daphne cneorum</i>	15,79	12,75	0,26	82,23	343,07	121,66	305,18	1809,97
<i>Erica aragonensis</i>	22,72	13,69	0,36	99,30	226,48	109,32	269,60	1220,56
<i>Erica cinerea</i>	4,50	4,58	0,18	28,49	143,10	90,92	155,81	202,00
<i>Erica vagans</i>	6,39	23,95	0,33	86,72	231,35	90,57	255,96	671,61
<i>Genista hispanica</i>	21,32	9,39	0,31	50,55	198,18	119,50	157,05	459,66
<i>Globularia repens</i>	19,43	7,53	0,29	54,38	232,61	111,21	177,03	1124,88

		% WPI <sub>&gt;12h</sub>	% WPI <sub>&lt;12h</sub>	AZ/VAZ	AZ/Chl (mmol molChl <sup>-1</sup> )	L/Chl (mmol molChl <sup>-1</sup> )	β-Car/Chl (mmol molChl <sup>-1</sup> )	VAZ/Chl (mmol molChl <sup>-1</sup> )	α-Toc/Chl (mmol molChl <sup>-1</sup> )
Woody species	<i>Juniperus communis</i> subsp. <i>alpina</i>	3,08	24,47	0,32	54,88	220,15	90,54	158,57	294,74
	<i>Pinus sylvestris</i>	7,73	18,75	0,45	85,45	271,96	256,96	184,59	442,59
	<i>Pinus uncinata</i>	26,69	18,13	0,36	48,09	234,90	149,75	133,21	212,98
	<i>Vaccinium myrtillus</i>	45,11	12,82	0,71	109,92	157,76	49,90	147,45	761,27
Herbaceous species	<i>Arabis alpina</i>	0,00	7,07	0,15	16,64	201,06	103,53	109,28	191,20
	<i>Armeria cantabrica</i>	0,00	2,55	0,12	11,88	141,95	103,21	103,09	76,96
	<i>Asperula hirta</i>	1,87	2,27	0,22	32,88	213,91	109,80	146,52	249,12
	<i>Cerastium fontanum</i>	0,00	5,92	0,13	21,07	158,56	118,22	162,71	60,98
	<i>Digitalis parviflora</i>	14,44	3,76	0,12	24,09	240,44	139,22	200,32	1973,21
	<i>Festuca</i> sp.	11,38	0,00	0,09	12,12	177,99	116,06	127,12	121,59
	<i>Hieracium pilosella</i> 1° site	0,00	6,70	0,14	15,45	155,33	119,20	111,33	248,99
	<i>Hieracium pilosella</i> 3° site	0,00	9,73	0,14	19,93	167,21	104,03	138,52	530,96
<i>Plantago lanceolata</i>	0,00	5,72	0,10	11,95	165,61	107,39	119,89	164,34	

	% WPI <sub>&gt;12h</sub>	% WPI <sub>&lt;12h</sub>	AZ/VAZ	AZ/Chl (mmol mol Chl <sup>-1</sup> )	L/Chl (mmolmol Chl <sup>-1</sup> )	β-Car/Chl (mmol mol Chl <sup>-1</sup> )	VAZ/Chl (mmol mol Chl <sup>-1</sup> )	α-Toc/Chl (mmol mol Chl <sup>-1</sup> )
<i>Poacea</i> sp.	0,00	2,75	0,10	8,71	145,55	96,42	85,65	73,50
<i>Polypodium</i> sp.	0,00	5,95	0,14	14,96	164,52	98,83	102,33	174,44
<i>Ranunculus repens</i>	0,00	9,37	0,12	16,91	165,63	104,78	138,02	80,16
<i>Saxifraga paniculata</i>	19,46	3,68	0,19	30,38	175,97	104,37	158,29	253,71
<i>Scilla</i> sp.	0,01	3,65	0,17	13,07	205,30	101,96	78,87	111,83
<i>Sedum album</i>	0,63	2,11	0,16	17,74	162,42	84,41	105,49	147,84
<i>Sedum brevifolium</i>	0,00	6,48	0,17	19,13	189,38	98,45	114,34	328,90
<i>Sedum</i> sp.	0,00	4,65	0,13	15,53	181,04	98,64	119,21	207,76
<i>Senecio</i> sp.	0,58	2,85	0,13	18,17	196,08	117,62	136,92	609,46
<i>Teucrium pyrenaicum</i>	0,27	9,64	0,19	30,01	216,37	97,31	157,10	434,78
<i>Thymelaea</i> sp.	13,93	5,04	0,14	20,81	213,40	84,28	154,13	590,61

Herbaceous species

		% WPI <sub>&gt;12h</sub>	% WPI <sub>&lt;12h</sub>	AZ/VAZ	AZ/Chl (mmolmol Chl <sup>-1</sup> )	L/Chl (mmolmol Chl <sup>-1</sup> )	β-Car/Chl (mmolmol Chl <sup>-1</sup> )	VAZ/Chl (mmolmol Chl <sup>-1</sup> )	α-Toc/Chl (mmol mol Chl <sup>-1</sup> )
Mosses	<i>Dicranum scoparium</i>	0,00	0,00	0,24	21,47	165,70	82,69	87,50	192,23
	<i>Didymodon</i> sp.	0,00	11,70	0,40	40,38	186,08	196,86	100,60	306,89
	<i>Grimmia pulvinata</i>	18,60	0,00	0,27	23,90	166,63	89,43	86,71	100,94
	<i>Polytrichum piliferum</i>	15,69	0,00	0,34	39,78	187,07	231,52	116,01	293,67
	<i>Syntrichia muralis</i>	0,00	8,56	0,31	35,33	220,70	121,57	112,69	207,73

	% WPI <sub>&gt;12h</sub>	% WPI <sub>&lt;12h</sub>	AZ/ VAZ	AZ/Chl (mmol molChl <sup>-1</sup> )	L/Chl (mmol molChl <sup>-1</sup> )	β-Car/Chl (mmol mol Chl <sup>-1</sup> )	VAZ/Chl (mmol mol Chl <sup>-1</sup> )	α-Toc/Chl (mmol mol Chl <sup>-1</sup> )
<i>Dermatocarpon</i> sp.	0,00	0,00	0,25	19,14	171,92	53,64	71,03	202,36
<i>Lasallia hispanica</i>	0,00	1,32	0,18	14,72	193,81	69,94	80,66	55,92
<i>Lichinella stipatula</i>	0,00	0,00	0,19	11,94	188,25	51,09	62,18	22,55
<i>Parmelia saxatilis</i>	0,00	0,00	0,20	17,40	214,25	122,33	89,11	31,17
<i>Physcia</i> sp.	0,00	4,41	0,34	22,31	387,12	120,06	82,46	108,94
<i>Ramalina</i> sp.	7,32	0,00	0,21	17,95	171,28	64,33	85,06	62,96
<i>Rhizocarpon</i> sp.	39,15	0,00	0,14	11,64	182,74	70,63	84,10	59,36
<i>Umbilicaria cylindrica</i>	0,00	3,94	0,23	17,10	189,00	54,36	71,64	42,14
<i>Umbilicaria polyphylla</i>	10,70	0,00	0,27	14,82	187,24	66,17	61,04	48,86

Lichens



APPENDIX 7: PHOTOPROTECTIVE PARAMETERS: DYNAMIC AND CHRONIC PHOTOINHIBITION (WPI<sub><12H</sub> AND WPI<sub>>12H</sub> RESPECTIVELY) OF EACH ANALYZED SPECIES AND PIGMENT CONCENTRATIONS IN SPRING.

Results are the average of 5 replicates.

	% WPI <sub>&lt;12h</sub>	AZ/VAZ	AZ/Chl (mmol molChl <sup>-1</sup> )	L/Chl (mmol molChl <sup>-1</sup> )	β-Car/Chl (mmol molChl <sup>-1</sup> )	VAZ/Chl (mmol molChl <sup>-1</sup> )	α-Toc/Chl (mmol molChl <sup>-1</sup> )
<i>Cytisus cantabricus</i>	5.47	0.14	10.22	137.59	97.89	72.63	209.51
<i>Calluna vulgaris</i> 1° site	1.27	0.11	9.6	137.65	89.47	84.2	285
<i>Calluna vulgaris</i> 2° site	0	0.24	24.64	152.71	99.71	106.63	141.95
<i>Calluna vulgaris</i> 3° site	0.46	0.14	13.56	137.85	93.49	98.4	106.98
<i>Daphne cneorum</i>	6.68	0.12	17.65	159.99	108.99	142.60	157.26
<i>Erica aragonensis</i>	1.93	0.11	13.64	147.25	104.55	130.02	157.72
<i>Erica cinerea</i>	1.87	0.15	13.20	125.90	95.09	92.58	55.14
<i>Erica vagans</i>	4.79	0.14	15.70	152.26	95.13	113.4	150.96
<i>Genista hispanica</i>	0.00	0.11	11.40	123.30	107.08	105.02	44.92
<i>Globularia repens</i>	4.32	0.12	10.63	122.36	98.56	88.06	200.38

	% WPI <sub>&lt;12h</sub>	AZ/VAZ	AZ/Chl (mmol molChl <sup>-1</sup> )	L/Chl (mmol molChl <sup>-1</sup> )	β-Car/Chl (mmol molChl <sup>-1</sup> )	VAZ/Chl (mmol molChl <sup>-1</sup> )	α-Toc/Chl (mmol molChl <sup>-1</sup> )	
Woody species	<i>Juniperus communis</i> subsp. <i>alpina</i>	4.64	0.15	14.28	147.58	76.27	97.16	85.30
	<i>Pinus sylvestris</i>	0.85	0.09	10.42	167.61	94.61	121.77	129.99
	<i>Pinus uncinata</i>	2.08	0.09	7.12	128.19	75.74	83.93	82.98
	<i>Vaccinium myrtillus</i>	3.51	0.25	22.08	136.13	93.57	91.36	281.03
Herbaceous species	<i>Arabis alpina</i>	0.00	0.15	14.31	166.36	100.19	98.12	211.38
	<i>Armeria cantabrica</i>	1.26	0.21	17.27	135.06	106.32	83.82	117.83
	<i>Asperula hirta</i>	0.00	0.12	10.76	120.34	104.38	87.24	58.88
	<i>Cerastium fontanum</i>	2.91	0.23	13.25	146.44	104.97	59.53	108.85
	<i>Digitalis parviflora</i>	1.70	0.12	11.18	143.55	97.44	90.27	70.39
	<i>Festuca</i> sp.	0.82	0.18	8.84	180.41	118.09	134.4	183.65
	<i>Hieracium pilosella</i> 1° site	4.55	0.10	14.51	120.48	111.49	81.9	98.78
	<i>Hieracium pilosella</i> 3° site	4.08	0.13	8.72	114.32	109.14	108.5	98.78
<i>Plantago lanceolata</i>	1.28	0.13	9.52	125.52	102.99	73.68	122.91	

	% WPI <sub>&lt;12h</sub>	AZ/VAZ	AZ/Chl	L/Chl	$\beta$ -Car/Chl	VAZ/Chl	$\alpha$ -Toc/Chl
			(mmol molChl <sup>-1</sup> )	(mmol molChl <sup>-1</sup> )	(mmol molChl <sup>-1</sup> )	(mmol molChl <sup>-1</sup> )	(mmol molChl <sup>-1</sup> )
<i>Poacea</i> sp.	0.00	0.19	23.8	140.81	108.81	123.72	74.36
<i>Polypodium</i> sp.	3.72	0.13	12.56	140.89	82.45	95.86	64.93
<i>Ranunculus repens</i>	3.53	0.14	17.33	133.67	98.88	125.77	48.25
<i>Saxifraga paniculata</i>	1.79	0.28	30.48	132.13	102.89	107.10	145.09
<i>Scilla</i> sp.	2.90	0.20	18.48	204	127.85	84.4	328.71
<i>Sedum album</i>	8.90	0.23	19.12	138.19	84.37	83.16	168.18
<i>Sedum brevifolium</i>	0.00	0.25	43.09	238.04	83.24	174.78	871.90
<i>Sedum</i> sp.	10.14	0.15	25.58	205.06	109.90	172.83	206.56
<i>Senecio</i> sp.	6.87	0.11	9.60	121.77	102.65	90.23	76.12
<i>Teucrium pyrenaicum</i>	0.00	0.14	14.32	125.33	98.67	100.79	111.43
<i>Thymelaea</i> sp.	4.62	0.11	9.25	141.73	99.65	83.75	318.71

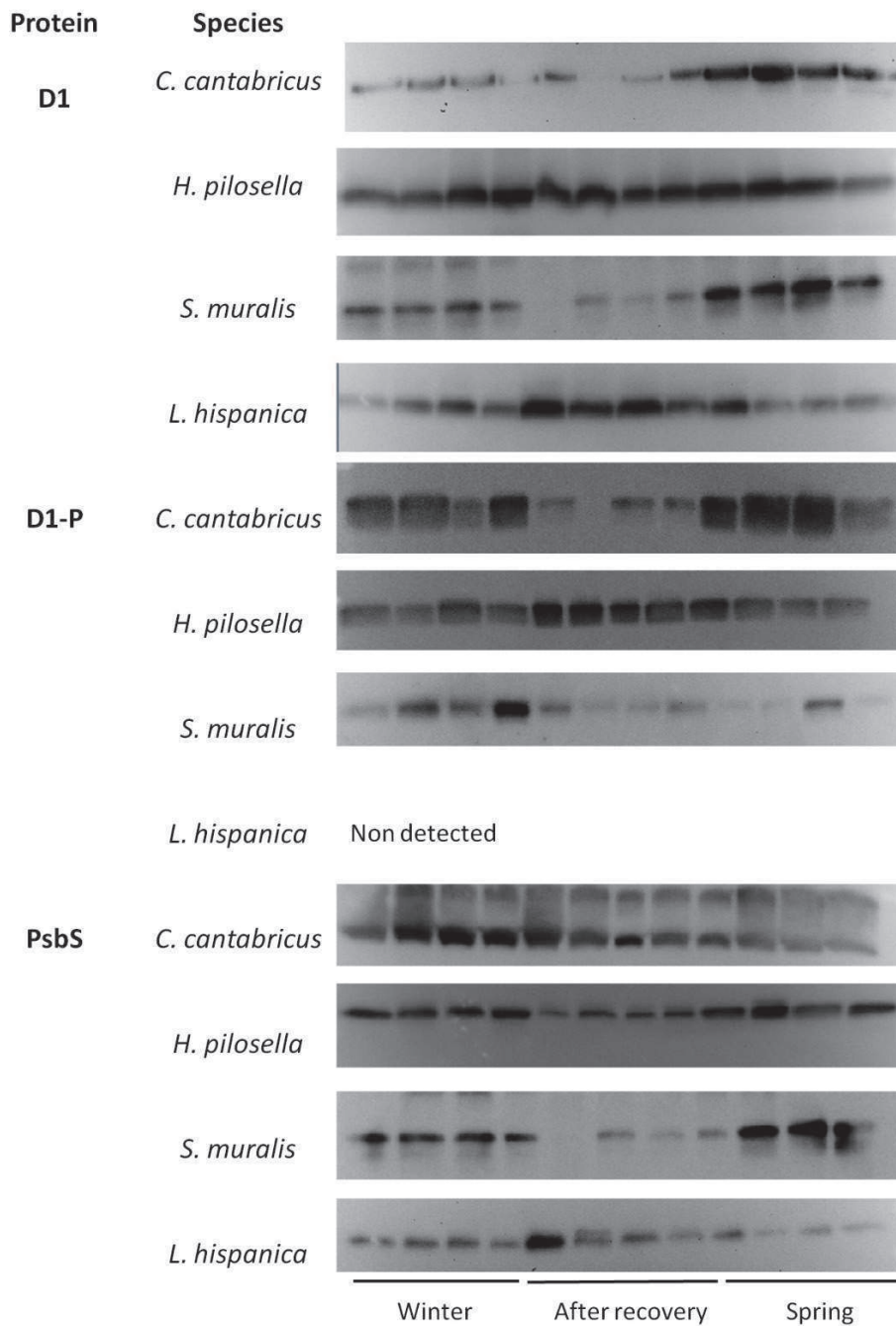
Herbaceous species

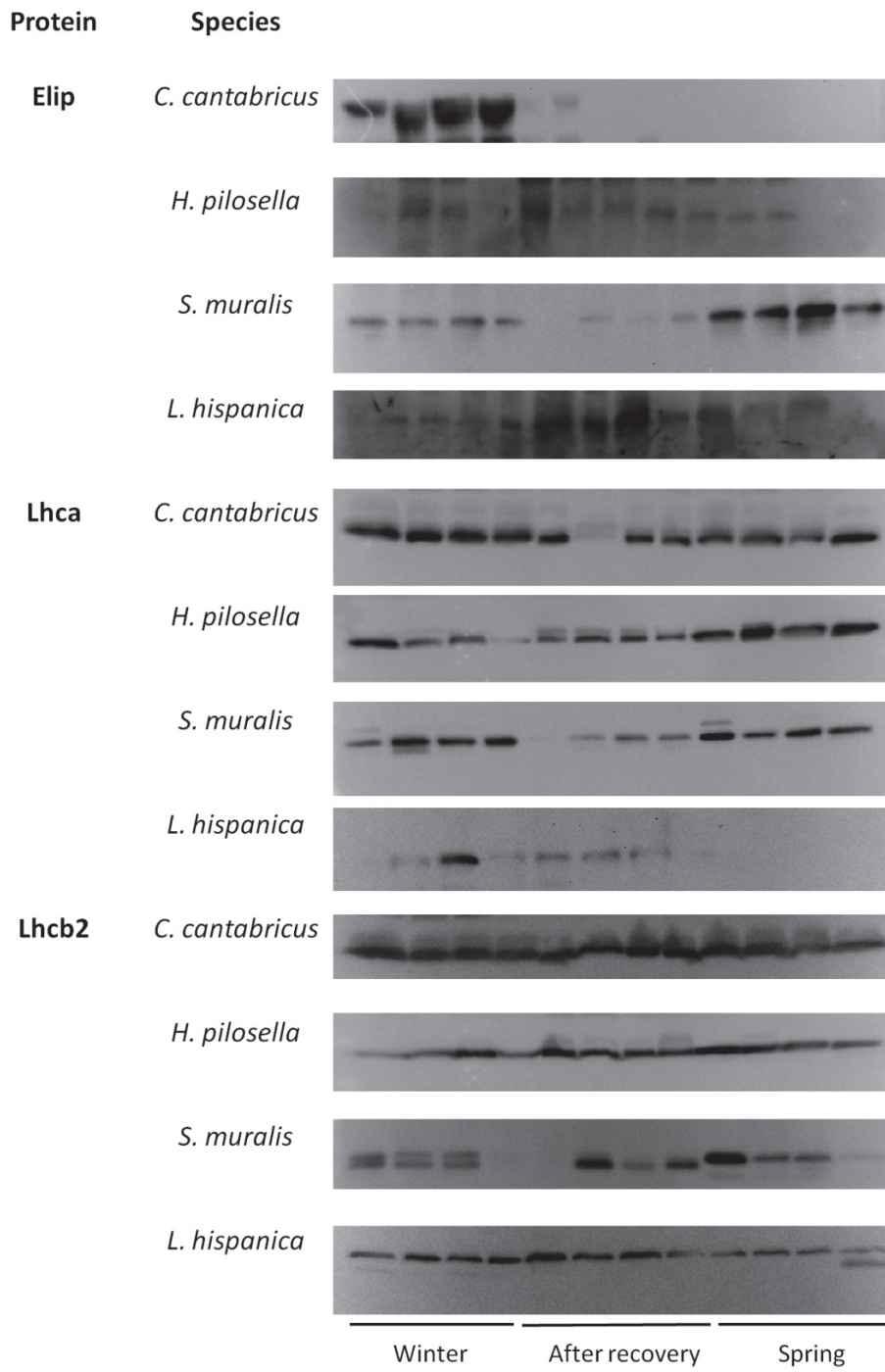
		% WPI <sub>&lt;12h</sub>	AZ/VAZ	AZ/Chl (mmol mol Chl <sup>-1</sup> )	L/Chl (mmol mol Chl <sup>-1</sup> )	β-Car/Chl (mmol mol Chl <sup>-1</sup> )	VAZ/Chl (mmol mol Chl <sup>-1</sup> )	α-Toc/Chl (mmol mol Chl <sup>-1</sup> )
Mosses	<i>Dicranum scoparium</i>	0	0.43	146.7	146.5	72.62	208.4	178.95
	<i>Didymodon</i> sp.	14.92	0.46	39.45	199.31	278.08	85.8	111.34
	<i>Grimmia pulvinata</i>	26.07	0.57	76.4	273.31	237.88	134.5	122.79
	<i>Polytrichum piliferum</i>	0.25	0.23	21.4	171.41	97.84	95.1	26.52
	<i>Syntrichia muralis</i>	0.00	0.44	47.34	220.46	99.02	108.7	47.99

	% WPI <sub>&lt;12h</sub>	AZ/VAZ	AZ/Chl (mmol molChl <sup>-1</sup> )	L/Chl (mmol molChl <sup>-1</sup> )	$\beta$ -Car/Chl (mmol molChl <sup>-1</sup> )	VAZ/Chl (mmol molChl <sup>-1</sup> )	$\alpha$ -Toc/Chl (mmol molChl <sup>-1</sup> )
<i>Dermatocarpon</i> sp.	13.17	0.21	13.96	217	79.49	68.8	28.89
<i>Lasallia hispanica</i>	11.11	0.30	21.05	383.94	148.40	67	59.36
<i>Lichinella stipatula</i>	9.77	0.18	12.86	186	55.95	72.99	48.15
<i>Parmelia saxatilis</i>	9.97	0.14	13.30	180.13	68.08	94.6	37.54
<i>Phyiscia</i> sp.	11.14	0.31	55.33	1169.27	694.01	174.4	57.98
<i>Ramalina</i> sp.	3.97	0.17	18.22	183.13	76.28	106.64	59.79
<i>Rhizocarpon</i> sp.	33.92	0.29	13.74	175.28	38.27	59.2	363.9
<i>Umbilicaria cylindrica</i>	6.36	0.26	7.66	190	61.49	53.5	39.70
<i>Umbilicaria polyphylla</i>	11.71	0.20	13.81	215.05	67.48	69.6	72.28

Lichens

APPENDIX 8: EXAMPLES OF WESTERN BLOT FOR MODEL SPECIES OF CHAPTER 5

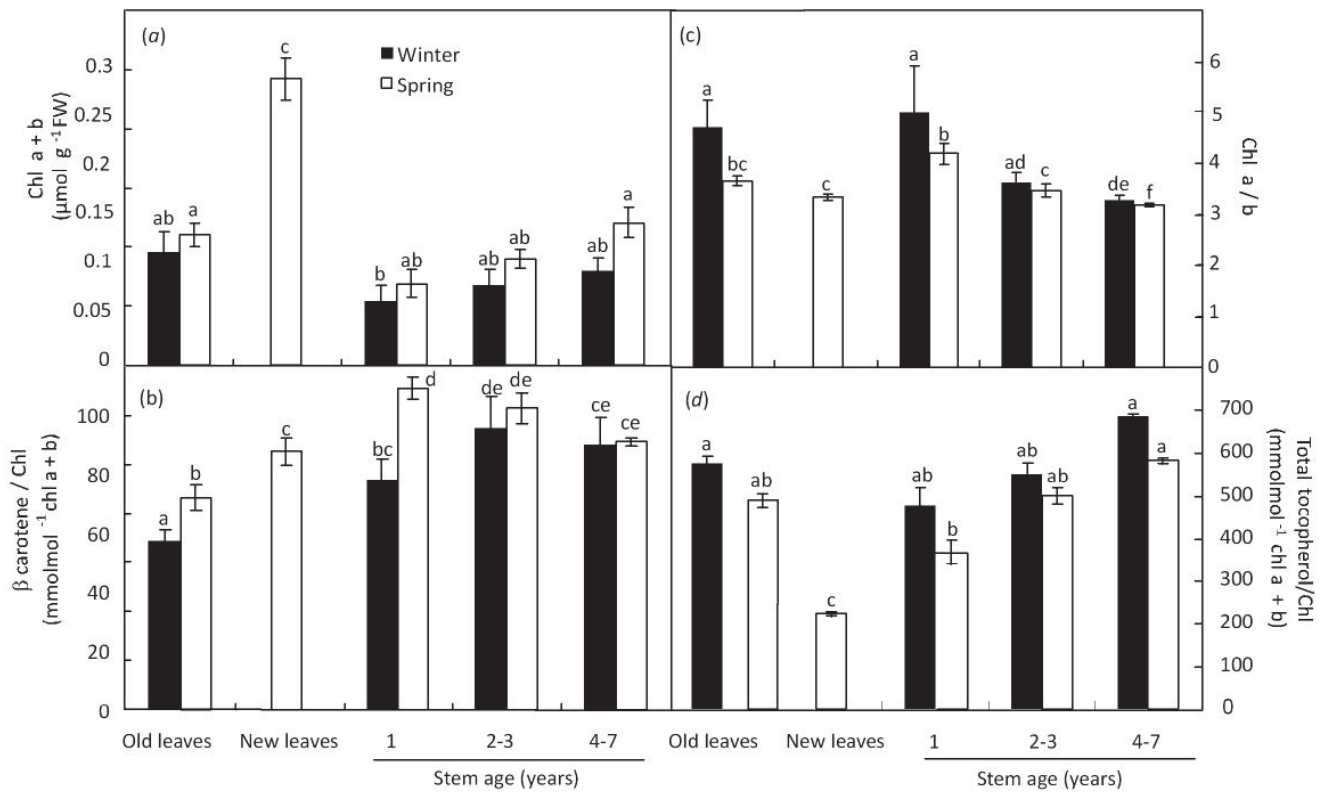






APPENDIX 9: PIGMENT AND TOCOPHEROL COMPOSITION IN LEAVES AND STEMS OF MISTLETOE DURING WINTER AND SPRING

(a) Chl a+b (b)  $\beta$ -carotene/Chl (c) Chl a/b (d) Total tocopherol/Chl. Each bar represents the mean  $\pm$  SE (in winter,  $n \geq 4$ ; in spring  $n \geq 9$ ). The letters above the columns are indicative of significant differences among organs and different stem ages in both seasons ( $p < 0.05$ ).

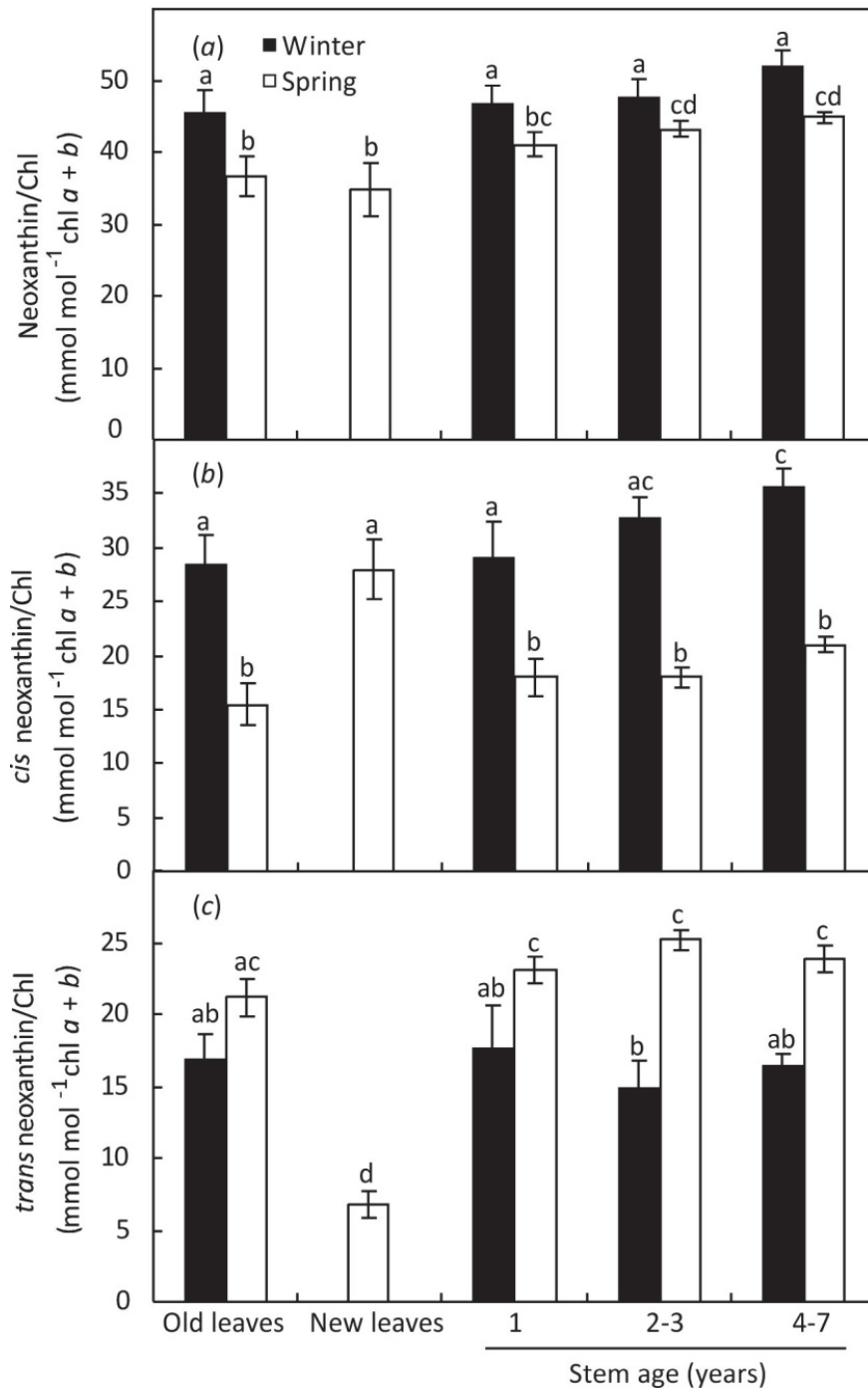


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**APPENDIX 10: NEOXANTHIN CONTENT IN LEAVES AND STEMS OF MISTLETOE DURING WINTER AND SPRING**

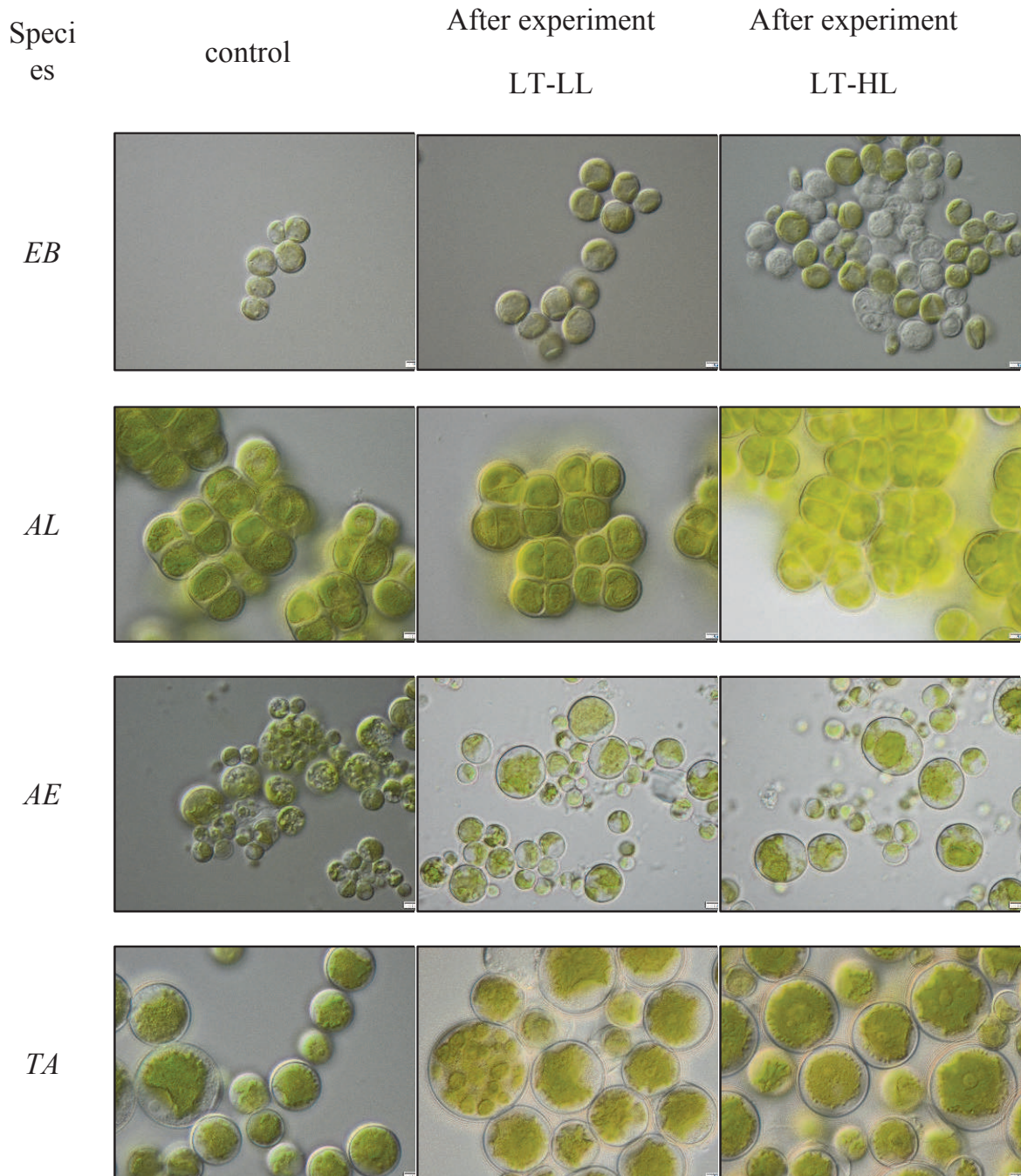
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(a) Total neoxanthin/Chl (b) *cis*-neoxanthin/Chl (c) *trans*-neoxanthin/Chl. Values are the mean  $\pm$  SE (in winter,  $n \geq 4$ ; in spring  $n \geq 9$ ). The letters above the columns are indicative of significant differences among organs and different stem ages in both seasons ( $p < 0.05$ ).



APPENDIX 11: LIGHT MICROGRAPHS

*Elliptochloris bilobata*, *Apatococcus lobatus*, *Asterochloris erici* and *Trebouxia arboricola*, under control conditions and after the 10 days of the experiment (5 days of cold acclimation and 5 days under low temperature conditions (lt) and low light (ll) or (hl)).



APPENDIX 12: PLATT PARAMETERS IN ALGAE AND LICHENS

Maximum capacity of photosynthesis (Pm) and the photosynthetic efficiency ( $\alpha$ ) calculated using the Platt et al. (1980) model in all the species: *Asterochloris erici*, *Elliptochloris bilobata*, *Trebouxia arboricola*, *Apatococcus lobatus*, *Cladonia squamosa*, *Baeomyces rufus* and *Ramalina pollinaria*.

Treatment	Time (h)	<i>AE</i>		<i>EB</i>		<i>TA</i>		<i>AL</i>		
		Pm	$\alpha$	Pm	$\alpha$	Pm	$\alpha$	Pm	$\alpha$	
LL	20°C	0	17.74 ± 1.68	0.34 ± 0.01	14.62 ± 0.78	0.28 ± 0.03	10.63 ± 0.56	0.50 ± 0.05	14.84 ± 0.93	0.42 ± 0.06
	15°C	24	14.51 ± 0.75	0.26 ± 0.02	8.53 ± 1.67	0.33 ± 0.03	12.05 ± 3.69	0.52 ± 0.04	13.54 ± 1.81	0.42 ± 0.01
	10°C	48	17.47 ± 4.21	0.19 ± 0.04	10.20 ± 1.17	0.28 ± 0.02	5.95 ± 1.07	0.69 ± 0.12	8.94 ± 0.70	0.57 ± 0.11
	7.5°C	72	11.97 ± 4.42	0.23 ± 0.04	10.16 ± 0.45	0.31 ± 0.03	6.22 ± 1.46	0.53 ± 0.06	8.03 ± 0.48	0.39 ± 0.03
	5°C	96	9.35 ± 1.83	0.23 ± 0.05	10.32 ± 0.00	0.29 ± 0.07	7.49 ± 1.31	0.62 ± 0.09	7.16 ± 1.52	0.48 ± 0.12
	5°C	168	10.13 ± 0.51	0.11 ± 0.02	9.86 ± 1.07	0.23 ± 0.11	5.48 ± 1.37	0.98 ± 0.18	6.95 ± 0.86	0.55 ± 0.21
	5°C	216	7.63 ± 1.85	0.15 ± 0.02	14.53 ± 1.51	0.39 ± 0.04	4.41 ± 0.77	0.68 ± 0.00	5.77 ± 0.99	0.38 ± 0.1
HL	5°C	96	8.64 ± 2.37	0.13 ± 0.03	11.80 ± 1.67	0.21 ± 0.07	4.75 ± 0.57	0.30 ± 0.04	5.96 ± 0.87	0.42 ± 0.01
	5°C	168	10.81 ± 2	0.07 ± 0.01	9.34 ± 2.22	0.16 ± 0.04	7.15 ± 0.18	0.37 ± 0.05	6.86 ± 0.56	0.39 ± 0.07
	5°C	216	7.51 ± 1.86	0.07 ± 0.00	12.63 ± 2.21	0.25 ± 0.03	8.86 ± 2.18	0.21 ± 0.02	7.52 ± 1.24	0.25 ± 0.01

Treatment	Time (h)	<i>CS</i>		<i>BR</i>		<i>RP</i>		
		Pm	$\alpha$	Pm	$\alpha$	Pm	$\alpha$	
<b>LL</b>	20°C	0	33.75* ± 5.97	0.48375 ± 0.03	32.10 ± 6.10	0.39 ± 0.06	37.31 ± 0.39	0.55 ± 0.06
	15°C	24	46.17 ± 4.34	0.5545 ± 0.01	30.98 ± 4.05	0.54 ± 0.05	38.6 ± 2.48	0.54 ± 0.01
	10°C	48	42.9 ± 8.21	0.54 ± 0.01	21.88 ± 3.06	0.43 ± 0.09	24.76 ± 0.37	0.53 ± 0.03
	7.5°C	72	34.73 ± 3.19	0.57 ± 0.06	20.26 ± 3.30	0.44 ± 0.02	40.32 ± 8.6	0.65 ± 0.05
	5°C	96	25.73 ± 3.42	0.44 ± 0.06	14.18 ± 1.21	0.39 ± 0.06	37.59 ± 3.83	0.49 ± 0.01
	5°C	168	22.14 ± 4.33	0.49 ± 0.05	17.84 ± 1.96	0.36 ± 0.04	16.5 ± 3.96	0.19 ± 0.01
	5°C	216	29.06 ± 5.15	0.48 ± 0.06	23.53 ± 3.88	0.53 ± 0.02	30.98 ± 9.42	0.48 ± 0.09
<b>HL</b>	5°C	96	24.76 ± 1.17	0.43 ± 0.04	21.14 ± 6.41	0.34 ± 0.05	24.12 ± 3.54	0.47 ± 0.1
	5°C	168	20.04 ± 4.22	0.38 ± 0.03	19.77 ± 3.86	0.46 ± 0.03	27.75 ± 1.48	0.43 ± 0.07
	5°C	216	20.54 ± 2.62	0.48 ± 0.03	22.00 ± 9.43	0.46 ± 0.05	27.32 ± 4.19	0.33 ± 0.03

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APPENDIX 13: CORRELATION COEFFICIENTS (R)  
BETWEEN DE-EPOXIDATION STATE OF VIOLAXANTHIN CYCLE  
(AZ/VAZ) AND NON PHOTOCHEMICAL QUENCHING (NPQ).  
Asterisks denote significant correlations (\*: < 0.05; \*\*: p < 0.01) and  
hyphen '-' means no correlation.

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<b>Algae</b>	TA	0.707**
	AE	-
	EB	-
	AL	-
<b>Lichens</b>	RP	0.557*
	CS	-
	BR	-

