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 (<250 words)

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 activate a photoprotective process which consists on the down-regulation of photosynthetic efficiency, referred to as winter photoinhibition (WPI). WPI has been classified in dynamic and chronic depending on the rapid (<14 h) or slower (>14 h) recovery kinetics. With the aim of characterize whether WPI is a general trait in evergreen high elevation photosynthetic organisms and its dependence to the deepoxidation state of xanthophyll cycle, 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 show that high levels of WPI are much more frequent (but not exclusive) among woody plants than in any other group. Changes in AZ/VAZ were not related to the activation/deactivation of WPI in the field and do not follow changes in photochemical efficiency during recovery treatments. Thylakoid proteins seasonal changes differ among different functional groups. The obtained results highlight the diversity of physiological solutions to winter stress.

ABBREVIATIONS

ΔpH: Transthylakoid proton gradient**; A:** Antheraxanthin; **AZ/VAZ:** De-epoxidation degree of xanthophylls cycle pigments (antheraxanthin+zeaxanthin)/(violaxanthin+antheraxanthin+zeaxanthin); **Chl:** Chlorophyll; **F_m:** Maximum chlorophyll fluorescence; **F_0:** Minimum chlorophyll fluorescence; **F_v/F_m:** Maximum quantum yield of PSII; **L:** Lutein; **Lhc:** Light harvesting complex of photosystem; **NPQ:** Non-photochemical quenching of chlorophyll fluorescence; **$^1O_2$:** Singlet oxygen; **PS:** Photosystem; **ROS:** Reactive oxygen species; **V**: violaxanthin; **V-cycle:** Xanthophyll cycle; **WPI_{all}:** Total winter photoinhibition; **$WPI_{>12h}$:** Chronic Winter Photoinhibition; **$WPI_{0.5h}$:** Dynamic Winter Photoinhibition; **WPI:** Winter photoinhibition; **Z:** Zeaxanthin.

1. INTRODUCTION

High mountain climates are characterized by low temperatures, 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)). On the contrary, other species are exposed, at least periodically, to the 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 and low temperature, known as "photochilling" (Huner et al. 2003; Ivanov et al. 2003).

Photochilling stress is due to the fact that low temperature slow down enzymatic carbon assimilation (Falk et al. 1996), whereas the absorption of light by the photosynthetic apparatus remains constant because 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 WW III 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 (ΔpH), PsbS protein (Li et al. 2002) and activation of violaxanthin cycle (V-cycle) (Niyogi et al. 1997; Niyogi et al. 1998). Depending on the maintenance or not of the activation this mechanism 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. The consequence of sustained (also refereed to as chronic) thermal dissipation is a concomitant reduction of photochemical efficiency. Hence, this process results in a depression of maximal photochemical efficiency and as a consequence can be considered as a type of photoinhibition (Demmig-Adams and Adams WW III 2006). Contrasting with other processes that generate an

uncontrolled damage in photosynthetic machinery, particularly of reaction centers (photodamage), chronic thermal dissipation is a highly regulated protective mechanism.

Severe winter stress activates sustained thermal dissipation, which involves the slow reversion from a dissipative state in the photosynthetic apparatus of evergreen plants (Verhoeven 2014). This is also termed as a sustained/chronic winter photoinhibition (WPI) when recovery is extremely slow (more than one night) even after incubation under optimal conditions. WPI is apparently independent on ΔpH (Verhoeven et al. 1998; Gilmore and Ball 2000; Demmig-Adams et al. 2006) and PsbS protein (Öquist and Huner 2003; Adams WW III 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 WW III 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 e.g. (Demmig-Adams and Adams WW III 2006; Zarter et al. 2006; 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 characterized in a few woody species e.g. (Demmig-adams and Adams III 2014), it is still unknown how widespread this character is among other evergreen species, specially mosses and lichens. What is more, 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 well-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 frequence 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 different functional groups (iii) elucidating the role of pigment and thylakoid proteins in photoprotection and along recovery process.

2. MATERIALS AND METHODS

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) due to the complete coverage by snow on previous months. Besides, according to a recent study of (Verhoeven 2013), the slowest rates of photosynthesis recovery are observed in late winter, indicating that in this period, at least conifers, present the deepest downregulation of photosynthesis. In both seasons, samples were collected at noon. The temperatures at that time in the field oscillated between 3 and 7^{o}C in winter and between 19 and 25^{o}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 1). Immediately after collection, samples were incubated under darkness at 100% relative humidity (in plastic bags with wet paper) and at room temperature (20ºC) during 14h to allow their recovery from any kind of dynamic WPI (here termed $WPI_{0.5h}$). Chl fluorescence measurements were taken after 30min and after 14h 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 -80ºC until pigment and protein analysis.

To calculate WPI, it has been considered that the photochemical efficiency of PSII (F_v/F_m) measured in late spring after 14h of recovery in darkness is the highest value that each species can reach. Taking this assumption into consideration, the percentages of dynamic winter photoinhibition ($WPI_{0.5h}$) and chronic winter photoinhibition (here termed $WPI_{>12h}$) were calculated for each species as follows:

$$WPI_{0.5h}=(F_v/F_{m\ 14h\ winter}- F_v/F_{m\ 30min\ winter})/(F_v/F_{m\ 14h\ spring}) \times 100$$

$$WPI_{>12h}=(F_v/F_{m\ 14h\ spring} - F_v/F_{m\ 14h\ winter})/(F_v/F_{m\ 14h\ spring}) \times 100.$$

$$WPI_{all}=WPI_{>12h} + WPI_{0.5h}$$

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

Experiment 2: To study in deep which are the photosynthetic recovery responses of each functional group (woody species, herbs, bryophytes and lichens), a species representative of each group was chosen and sampled in 2013: *Cytisus cantabricus* (Wilk.) Rchb. F., *Hieracium pilosella* L., *Syntrichia muralis* (Hedw.) Raab and *Lasallia hispanica* (Frey) Sancho & Crespo, respectively (Table 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 extensive representation in the sampling area. For this manipulative approach, 10 individuals of each species were directly sampled in the field in winter. 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 Fv/Fm in the field before starting the process of recovery, all samples were placed over an ice bath at a temperature of 4-7ºC (simulating field conditions) and under darkness conditions during 30 min. After this period, Chl fluorescence measurements were performed. Then, 5 samples per species were frozen in liquid nitrogen for pigment and thylakoid protein determination (t_o). 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. During all the recovery process, samples were located in containers with nutritive solution or in petri dishes to avoid desiccation. Samples (100mg approximately) were frozen in liquid nitrogen after 30 min in darkness and low temperature (t_0), after 0.5 and 14h 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^2 size and (iv) in *S. muralis* the apical part of each caulid (shoot) containing photosynthetically active phyllids (leaves).

2.2. Fluorescence measurements

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 µmol photons $m^{-2}s^{-1}$) while minimum fluorescence (F_o) was recorded with low measuring light intensities. The maximal photochemical efficiency of PSII (F_v/F_m) was calculated as $(F_m-F_o)/F_m$. 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.

2.3. Pigment and tocopherol analysis

The frozen samples, stored at -80ºC, were homogenized with a mortar in pure acetone solution buffered with $CaCO_3$. The extracts were centrifuged at 16100 g and 4ºC for 20 min, and supernatants

were filtered with 0.2 μm PTFE filters (Teknokroma, Spain). Pigment separation was performed by HPLC with a reverse phase C18 column (Waters Spherisorb ODS1, 4.6 x 250 mm, Mildord, MA, USA) with a photodiode array (PDA) detector, following the method of (García-Plazaola and Becerril 1999) with modifications (Garcia-Plazaola and Esteban 2012).

2.4. Protein extraction and characterization

The proteins examined in this study were D1 (photosystem II (PSII) core complex protein), D1-P (D1 protein phosphorilated), Lhca2 (antenna protein from PSI), Lhcb2 (antenna protein from PSII), PsbS (essential protein for thermal dissipation) and ELIP (Early Light Induction Protein), closely related to stress. All the antibodies were from Agrisera AB (Vännäs, Sweeden). 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. Inmuno-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.

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 WPI0.5h, 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.).

3. RESULTS

3.1. Chronic and dynamic winter photoinhibition: Differences among functional groups

The presence of chronic ($WPI_{>12h}$) and/or dynamic photoinhibition ($WPI_{0.5h}$) was studied in 50 representative species of the following functional groups: woody species, herbs, mosses and lichens. The highest total photoinhibition (WPI_{all}) occurred in woody plants (Fig. 1A). They presented not only the highest $WPI_{>12h}$ but also the most relevant $WPI_{0.5h}$. Altogether, $WPI_{0.5h}$ and $WPI_{>12h}$ reached more

than 30% (Fig.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. 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. 1B). The highest variability was present in woody species, in both $WPI_{>12h}$ and $WPI_{0.5h}$. 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 $WPI_{>12h}$, comparable to those of woody plants (Appendix S2). Horizontal line in each bar represents the median value. Interestingly, in herbs, mosses and lichens, a half of species did not present any $WPI_{>12h}$ as the median for $WPI_{>12h}$ was 0% (for more detail, see Appendix S2). In reference to $WPI_{0.5h}$, the same occurred for mosses and lichens while woody and herbaceous species presented a median of 13 and 5 % respectively (Fig. 1B).

3.2. Variable responses of lipophilic antioxidant composition during the year in different functional groups.

Concomitantly with high WPI, all woody species without exception, showed the highest content of lipophilic antioxidants (tocopherols and carotenoids) during winter (see Appendix S1, S2). The most relevant changes between both seasons occurred in: *Pinus sylvestris* and *Pinus uncinata*, whose β-carotene/Chl was 2-fold higher in winter than in spring; *Vaccinium myrtillus*, which had a 5 fold increase in AZ/Chl in winter and the shrubs *Daphne cneorum*, *Erica aragonensis*, *Erica vagans* and *Globularia repens* which doubled the VAZ/Chl ratio in winter compared to spring. Interestingly, in this group the highest difference between winter and spring were in α-tocopherol/Chl content except for *Calluna vulgaris* of site 3 and *Cytisus cantabricus.* In the rest of woody plants, α-tocopherol/Chl showed an increase of at least 2 or 3 times, becoming even 12 times in the case of *Daphne cneorum*.

Contrasting with woody plants, the opposite tendency was observed in mosses, which presented the highest amount of photoprotective and antioxidative molecules in spring, in parallel with a higher photoinhibition (Appendix S3), indicating that possibly, for mosses, spring conditions were more stressful than winter. This pattern occurred for all species and antioxidants except for *Polytrichum piliferum* which showed a bigger amount of β-carotene in the winter.

In the case of herbaceous plants there was not a unique pattern of response to seasonality in the antioxidant content. Thus, although for lutein/Chl and VAZ/Chl all the species showed slightly higher amounts in winter, in the case of AZ/Chl and α-tocopherol/Chl, there was not a consistent seasonal pattern among herbaceous species. The most remarkable changes were observed in *Asperula hirta* which presented the triple amount of AZ/Chl in winter than in spring and in *Scilla* sp. with the

opposite tendency. Regarding α-tocopherol/Chl, the most relevant response was observed in *Digitalis parviflora*, which in winter reached a value (1973 mmol mol Chl^{-1}) almost 30 times higher than in spring (70 mmol mol Chl^{-1}). The opposite tendency, but not so marked, was detected in *Scilla* sp.

Within lichens, half of the studied species, showed an increase in AZ/VAZ during winter while the others did not show any difference with spring values. The α-tocopherol/Chl ratio did not show any consistent pattern among species, being in some species higher in winter, in others higher in spring and in some of them similar among seasonal.

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 2). Thus, not only V-cycle components (V/Chl, A/Chl and total VAZ/Chl) and total carotenoids correlated linearly with WPI, but it was also the case of tocopherols. Surprisingly, in woody species, which presented the highest values of $WPI_{>12h}$, none significant linear regression with V-cycle components or other pigments was found. On the contrary, $WPI_{0.5h}$ and WPI_{all} of woody plants are correlated with V-cycle depoxidation activity (Table 2). In mosses and lichens, only $WPI_{0.5h}$ showed significant correlation with lipophilic antioxidants per Chl ratios. $WPI_{0.5h}$ was correlated with A/Chl in mosses and with L/Chl and with Neoxanthin/Chl (N/Chl) in lichens.

3.4. Recovery kinetics: Which are the differences between functional groups?

Recovery for winter conditions was studied in four model species representative of each major functional groups (the shrub *C. cantabricus*, the perennial herb *Hieracium pilosella*, the moss *Syntrichia muralis* and the lichen *Lasallia hispanica*). For that purpose, thalli, branches or leaves were transferred to the laboratory and their recovery at 20ºC and dim light was followed by measuring F_v/F_m and AZ/VAZ content. Interestingly, under spring conditions all functional groups presented similar value of de-epoxidation index (0.3-0.37) and during winter conditions it increased moderately in the herb, the moss and the lichen (0.45-0.5) but dramatically in the woody plant (0.75) (Fig. 2). Additionally, the recovery kinetics detected in model species could be classified following three major patterns: (i) the woody species (*C. cantabricus*), which showed mainly the slow component (in 92 hours only is recovered the 30% of control F_v/F_m) (Fig. 2A)) (ii) the moss *S. muralis* and the herbaceous *H. pilosella* that did not present any recovery because they were not photoinhibited and (iii) 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. 2D).

3.5. Changes in photosynthetic apparatus protein composition

Thylakoid protein composition was studied by western blot in samples of the four model species collected in the field in spring and winter and also after recovery from winter conditions in the laboratory. Our results show that even after relatively short periods of time, in comparison with seasonal changes, there were changes in protein composition. Thus in *C. cantabricus*, phosphorylated D1 protein (D1-P), PsbS, Elip and Lhca diminished significantly after 140h of recovery in the laboratory (Fig. 3A). *S. muralis* presented also a reduction in PsbS and Lhca for the same period of time (Fig. 3C). Contrasting with those species, *H. pilosella* showed a diminution of PsbS in 41h, as well as an augmentation of D1-P (Fig. 3B). In *L. hispanica*, the only significant change after recovery was the increase in D1 content (Fig. 3D).

As observed during the recovery experiments under laboratory conditions, in the field, the protein composition of each species showed a characteristic seasonal pattern. Thus, in the woody species *C. cantabricus* the amount of Elip and PsbS proteins was higher in winter, while D1 protein was more abundant in spring (Fig. 3A). Unlike *C. cantabricus*, the moss and the lichen had higher amounts of PsbS and Elip in spring than in winter (Fig. 3C, D). On the contrary, the only variations observed in *H. pilosella* were a higher content of Lhca in spring (Fig 3B). In *L. hispanica*, there was a higher abundance of Lhcb2 in winter (Fig. 3D).

4. DISCUSSION

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 WW III et al. 1995; Ottander et al. 1995; Verhoeven et al. 1998; Öquist and Huner 2003; Taulavuori et al. 2011). By contrast, herbs are thought to respond to winter stress by a process of dynamically reversible modulation of PSII ($WPI_{0.5h}$) (Öquist and Huner 1993; Li et al. 2000). To determine the winter strategy in other functional groups (mosses and lichens) in comparison to woody plants, we analyzed 50 different taxa from different functional groups. 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.) presented a $WPI_{>12h}$ around 20%. These species were neither ecologically nor phylogenetically related.

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 favour their coverage by protective snow mantle and 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 WW III et al. 2004).

4.2. Role of carotenoids and tocopherols on WPI

Around late 1980s and early 1990s, Demmig et al. (Demmig et al. 1987; Demmig et al. 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 WW III 2006), more concretely 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 2). The results indicated that both factors are not always correlated. Woody species only presented a significant linear correlation between (i) $WPI_{0.5h}$ and AZ/Chl, and (ii) WPI_{all} with Z/Chl and AZ/VAZ. 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 $WPI_{0.5h}$ and other xanthophylls such as L and Neo in this group.

Contrary to our expectations, in herbs, $WPI_{>12h}$ was more strongly correlated with V-cycle content than in woody species (table 2). This could be due to the fact that in each species, the components which affect to sustained 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.

Several 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. In the present study, β-carotene, which is able to quench singlet oxygen ($^1O_2$) (Burton and Ingold 1984), was higher in woody plants during winter, especially in *Pinus* species (Appendix S2, S3). 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 plants (herbaceous and woody species) was the increase of α-tocopherol in winter with respect to spring (Appendix S1, S2). This trend was maintained even in the cases in which WPI was close to 0. This suggests that the role of α-tocopherol 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 low temperature 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 S3). In fact, in mosses and some lichens, the amount of antioxidants was higher during spring, suggesting that low temperatures do not represent a severe stress. But during spring, other stresses as episodes of drying cause the accumulation of antioxidant components.

4.3. Recovery kinetics: 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 (Verhoeven et al. 1998; Verhoeven et al. 2009; 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 woody plants (*Cytisus cantabricus*) but also to representatives of the other functional groups studied: herbs (*Hieracium pilosella*), bryophytes (*Syntrichia muralis*) and lichens (*Lasallia hispanica*) (Fig. 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 (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).

4.4. Thylakoid protein composition during winter acclimation

Winter changes in thylakoid protein composition during deacclimation have been precisely characterised in conifers (Verhoeven 2013), but little is known in 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. In the present study, the process of deacclimation 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 4). In the shrub *C. cantabricus* the degradation of D1 was accompanied by an upregulation of PsbS and Elip protein as well as *de novo* synthesis of Z. 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 during winter but maintains its stems photosynthetically active during winter. Hence, the protein analysis was performed in green stems. A previous study analyzing the stems of *Viscum album* in winter and spring (Míguez et al. 2014), come to the same conclusions so, presumably, photosynthetic stems and leaves follow the same seasonal pattern in terms of thylakoid protein changes. As occurred in woody plants, in *L. hispanica* D1 content was lower in winter but it was not accompanied by an increase in PsbS. Besides, while *C. cantabricus* presented the highest D1 content in winter, in *L. hispanica* it was higher after recovery under controlled conditions. This demonstrated that *L. hispanica* growing in the field is not under optimum conditions in winter and also not in spring. 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. On the other hand, *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 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 low temperature treatment in a growth chamber (Bascuñán-Godoy et al. 2012).

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 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 recently demonstrated that PsbS protein was absent in algae, where Lhcsr protein played similar roles (Li et al. 2000; Peers et al. 2009). By contrast, in bryophytes, such as *Physcomitrella patens*, both PsbS and Lhcsr coexist (Alboresi et al. 2010). In this study, PsbS was detected in all species, diminishing after recovery except in the case of lichen *L. hispanica*, where PsbS was independent from stress. This lichen is formed by a fungus and a green alga of *Trebouxia* genus. It was surprising

that PsbS appeared in a green algae because it has been recently demonstrated that in Chlorophyta, PsbS gene can also be expressed under stress but as far as it was known, the protein was suppose to be absent (Gerotto and Morosinotto 2013). This could indicate that *L. hispanica*, and probably many other lichens have evolved a NPQ system different from that of green algae, presumably more adapted to the terrestrial environment.

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 high levels of WPI are much more frequent (but not exclusive) among woody plants than in any other group. Why the strategy is so widespread in trees and shrubs independently of their phylogenetic position or growth form remains to be understood, but a relationship with the prevention of xylem cavitation or growth out of 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 physiological solutions 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.

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.

ACKNOWLEDGEMENTS

FM received a fellowship from the Department of Education, University and Research of the Basque Government. BFM received two post-doctoral fellowships from the Research Vicerrectorate of the UPV/EHU (Grant for recent PhDs and Grant for specialization of researchers) and a Marie Curie IEF grant (328370 MELISSA) from the European FP7-PEOPLE. The work was also supported by the project BFU 2010–15021 from the Spanish Ministry of Economy and Competitiveness (MINECO) and the European Regional Development Fund ERDF (FEDER), and by the Basque Government (UPV/EHU-GV IT-624–13).

REFERENCES

Adams WW III, Demmig-Adams B, Verhoeven A, Barker D (1995) "Photoinhibition" during winter stress: Involvement of sustained xanthophyll cycle-dependent energy dissipation. Aust J Plant Physiol 22:261–276.

Adams WW III, Zarter CR, Ebbert V, Demmig-adams B (2004) Photoprotective strategies of overwintering evergreens. Bioscience 54:41–49.

Alboresi A, Gerotto C, Giacometti GM, et al (2010) Physcomitrella patens mutants affected on heat dissipation clarify the evolution of photoprotection mechanisms upon land colonization. Proc Natl Acad Sci U S A 107:11128–33.

Aro EM, Kettunen R, Tyystjärvi E (1992) ATP and light regulate D1 protein modification and degradation Role of D1* in photoinhibition. FEBS Lett 297:29–33.

Asada K (1999) The water-water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons. Annu Rev Plant Physiol Plant Mol Biol 50:601–639.

Barták M, Vráblíková H, Hájek J (2003) Sensitivity of photosystem 2 of Antarctic lichens to high irradiance stress: Fluorometric study of fruticose (Usnea antarctica) and foliose (Umbilicaria decussata) species. Photosynthetica 41:497–504.

Bascuñán-Godoy L, Sanhueza C, Cuba M, et al (2012) Cold-acclimation limits low temperature induced photoinhibition by promoting a higher photochemical quantum yield and a more effective PSII restoration in darkness in the Antarctic rather than the Andean ecotype of Colobanthus quitensis Kunt Bartl. BMC Plant Biol 12:114.

Burton GW, Ingold KU (1984) Beta-Carotene: an unusual type of lipid antioxidant. Science 224:569–573.

Chen L, Jia H, Tian Q, et al (2012) Protecting effect of phosphorylation on oxidative damage of D1 protein by down-regulating the production of superoxide anion in photosystem II membranes under high light. Photosynth Res 112:141–148.

Corcuera L, Morales F, Abadía A, Gil-Pelegrín E (2005) Seasonal changes in photosynthesis and photoprotection in a Quercus ilex subsp. ballota woodland located in its upper altitudinal extreme in the Iberian Peninsula. Tree Physiol 25:599–608.

Dall'Osto L, Cazzaniga S, Havaux M, Bassi R (2010) Enhanced photoprotection by protein-bound vs free xanthophyll pools: a comparative analysis of chlorophyll b and xanthophyll biosynthesis mutants. Mol Plant 3:576–93.

DellaPenna D, Pogson BJ (2006) Vitamin synthesis in plants: tocopherols and carotenoids. Annu Rev Plant Biol 57:711–738.

Demmig B, Winter K, Krüger A, Czygan F (1987) Photoinhibition and zeaxanthin formation in intact leaves : A possible role of the xanthophyll cycle in the dissipation of excess light energy. Plant Physiol 84:218–224.

Demmig B, Winter K, Krüger A, Czygan F-C (1988) Zeaxanthin and the heat dissipation of excess light energy in Nerium oleander exposed to a combination of high light and water stress. Plant Physiol 87:17–24.

Demmig-adams B, Adams III W (2014) Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria. Springer Netherlands, Dordrecht

Demmig-Adams B, Adams WW III (2006) Photoprotection in an ecological context : the remarkable complexity of thermal energy dissipation. New Phytol 172:11–21.

Demmig-Adams B, Ebbert V, Mellman DL, et al (2006) Modulation of PsbS and flexible vs sustained energy dissipation by light environment in different species. Physiol Plant 127:670–680.

Doulis AG, Hausladen A, Mondy B, et al (1993) Antioxidant response and winter hardiness in red spruce (Picea rubens Sarg.). New Phytol 123:365–374.

Ensminger I, Sveshnikov D, Campbell DA, et al (2004) Intermittent low temperatures constrain spring recovery of photosynthesis in boreal Scots pine forests. Glob Chang Biol 10:995–1008.

Eskling M, Arvidsson PO, Åkerlund HE (1997) The xanthophyll cycle, its regulation and components. Physiol Plant 100:806–816.

Esterbauer H, Grill D (1978) Seasonal variation of glutathione and glutathione reductase in needles of Picea abies. Plant Physiol 61:119–121.

Falk S, Maxwell DP, Laudenbach DE, Huner NPA (1996) Photosynthetic adjustment to temperature. In: Baker NR (ed) Photosynthesis and the environment. Dordrecht: Kluger academic publishers, pp 367–385

García-Plazaola JI, Becerril JM (1999) A rapid high-performance liquid chromatography method to measure lipophilic antioxidants in stressed plants: Simultaneous determination of carotenoids and tocopherols. Phytochem Anal 10:307–313.

Garcia-Plazaola JI, Esteban R (2012) Determination of chlorophylls and carotenoids by HPLC. In: PrometheusWiki.

Gerotto C, Morosinotto T (2013) Evolution of photoprotection mechanisms upon land colonization: evidence of PsbS-dependent NPQ in late Streptophyte algae. Physiol Plant 583–598.

Gilmore AM (1997) Mechanistic aspects of xanthophyll cycle-dependent photoprotection in higher plant chloroplasts and leaves. Physiol Plant 99:197–209.

Gilmore AM, Ball MC (2000) Protection and storage of chlorophyll in overwintering evergreens. Proc Natl Acad Sci U S A 97:11098–11101.

Havaux M, Dall'osto L, Bassi R (2007) Zeaxanthin has enhanced antioxidant capacity with respect to all other xanthophylls in Arabidopsis leaves and functions independent of binding to PSII antennae. Plant Physiol 145:1506–20.

Heber U, Bilger W, Bligny R, Lange OL (2000) Phototolerance of lichens, mosses and higher plants in an alpine environment: analysis of photoreactions. Planta 211:770–780.

Huner N, Öquist G, Melis A (2003) Photostasis in plants, green algae and cyanobacteria: The role of light harvesting antenna complexes. In: Green BR, Parson WW (eds) Advances in Photosynthesis and Respiration. Light-harvesting antennas in photosynthesis. Springer Netherlands, Dordrecht, pp 402–421

Ivanov AG, Sane P, Hurry V, et al (2003) Low-temperature modulation of the redox properties of the acceptor side of photosystem II: photoprotection through reaction centre quenching of excess energy. Physiol Plant 119:376–383.

Johnson MP, Goral TK, Duffy CDP, et al (2011) Photoprotective energy dissipation involves the reorganization of photosystem II light-harvesting complexes in the grana membranes of spinach chloroplasts. Plant Cell 23:1468–79.

Koivuniemi A, Aro E-M, Andersson B (1995) Degradation of the D1 and D2 proteins of photosystem II in higher plants is regulated by reversible phosphorylation. Biochemistry 34:16022–16029.

Körner C (1999) Alpine plant life: functional plant ecology of high mountain ecosystems. Springer Berlin, Germany

Leipner J, Fracheboud Y, Stamp P (1997) Acclimation by suboptimal growth temperature diminishes photooxidative damage in maize leaves. Plant, Cell Environ 20:366–372.

Lenné T, Bryant G, Hocart CH, et al (2010) Freeze avoidance: A dehydrating moss gathers no ice. Plant, Cell Environ 33:1731–1741.

Levy H, Tal T, Shaish A, Zamir A (1993) Cbr, an algal homolog of plant early light-induced proteins, is a putative zeaxanthin binding protein. J Biol Chem 268:20892–20896.

Li XP, Björkman O, Shih C, et al (2000) A pigment-binding protein essential for regulation of photosynthetic light harvesting. Nature 403:391–395.

Li XP, Phippard A, Pasari J, Niyogi KK (2002) Structure-function analysis of photosystem II subunit S (PsbS) in vivo. Funct Plant Biol 29:1131–1139.

Logan BA, Grace SC, Adams III WW, Demmig-Adams B (1998) Seasonal differences in xanthophyll cycle characteristics and antioxidants in Mahonia repens growing in different light environments. Oecologia 116:9–17.

Mayr S, Wolfschwenger M, Bauer H (2002) Winter-drought induced embolism in Norway spruce (Picea abies) at the Alpine timberline. Physiol Plant 115:74–80.

Míguez F, Fernández-Marín B, Becerril JM, Garcia-Plazaola JI (2015) Activation of photoprotective winter photoinhibition in plants from different environments: a literature compilation and meta-analysis. Physiol Plant. doi: 10.1111/ppl.12329

Míguez F, Fernández-Marín B, Hernández A, et al (2014) Does age matter under winter photoinhibitory conditions ? A case study in stems and leaves of European mistletoe (Viscum album). Funct Plant Biol 42:175–185.

Müller P, Li XP, Niyogi KK (2001) Non-photochemical quenching. A response to excess light energy. Plant Physiol 125:1558–1566.

Mullineaux PM, Rausch T (2005) Glutathione, photosynthesis and the redox regulation of stress-responsive gene expression. Photosynth. Res. 86:459–474.

Niyogi KK (2000) Safety valves for photosynthesis. Curr Opin Plant Biol 3:455–560.

Niyogi KK, Björkman O, Grossman AR (1997) The roles of specific xanthophylls in photoprotection. Proc Natl Acad Sci U S A 94:14162–14167.

Niyogi KK, Grossman AR, Björkman O (1998) Arabidopsis mutants define a central role for the xanthophyll cycle in the regulation of photosynthetic energy conversion. Plant Cell 10:1121–1134.

Noctor G, Foyer CH (1998) Ascorbate and glutathione: Keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 49:249–279.

Öquist G, Huner NA (1993) Cold-hardening-induced resistance to photoinhibition of photosynthesis in winter rye is dependent upon an increased capacity for photosynthesis. Planta 189:150–156.

Öquist G, Huner NPA (2003) Photosynthesis of overwintering evergreen plants. Annu Rev Plant Biol 54:329–355.

Østrem L, Rapacz M, Jørgensen M, Höglind M (2011) Effect of developmental stage on carbohydrate accumulation patterns during winter of timothy and perennial ryegrass. Acta Agric Scand Sect B - Plant Soil Sci 61:153–163. doi: 10.1080/09064711003652522

Ottander C, Campbell D, Oquist G (1995) Seasonal changes in photosystem-II organization and pigment composition in Pinus sylvestris. Planta 197:176–183.

Peers G, Truong TB, Ostendorf E, et al (2009) An ancient light-harvesting protein is critical for the regulation of algal photosynthesis. Nature 462:518–521.

Polle A, Rennenberg H (1992) Field studies on Norway spruce trees at high altitudes: II. Defence systems against oxidative stress in needles. New Phytol 121:635–642.

Porcar-castell A, Juurola E, Nikinmaa E, et al (2008) Seasonal acclimation of photosystem II in Pinus sylvestris . I . Estimating photochemistry †. 1475–1482.

Powles SB, Critchley C (1980) Effect of light intensity during growth on photoinhibition of intact attached bean leaflets. Plant Physiol 65:1181–1187.

Ruban A V, Pascal A, Lee PJ, et al (2002) Molecular configuration of xanthophyll cycle carotenoids in photosystem II antenna complexes. J Biol Chem 277:42937–42942.

Sáez PL, Bravo LA, Latsague MI, et al (2013) Light energy management in micropropagated plants of Castanea sativa, effects of photoinhibition. Plant Sci 201-202:12–24.

Sanchez A, Smith WK (2015) No evidence for photoinhibition of photosynthesis in alpine Caltha leptosepala DC. Alp Bot 125:41–50. doi: 10.1007/s00035-015-0146-2

Sperry JS, Sullivan JE (1992) Xylem embolism in response to freeze-thaw cycles and water stress in ring-porous, diffuse-porous, and conifer species. Plant Physiol 100:605–613.

Strand M, Öquist G (1985) Inhibition of photosynthesis by freezing temperatures and high light Levels in cold-acclimated seedlings of Scots pine (Pinus sylvestris). - I. Effects on the light-limited and light-saturated rates of CO2 assimilation. Physiol Plant 64:425–430.

Streb P, Aubert S, Gout E, Bligny R (2003) Reversibility of cold- and light-stress tolerance and accompanying changes of metabolite and antioxidant levels in the two high mountain plant

species Soldanella alpina and Ranunculus glacialis. J Exp Bot 54:405–418. doi: 10.1093/jxb/erg048

Streb P, Cornic H (2012) Photosynthesis and antioxidative protection in alpine herbs. In: Lütz C (ed) Plants in Alpine Regions, Springer. Verlag-Wien, pp 75–97

Sui N (2015) Photoinhibition of Suaeda salsa to chilling stress is related to energy dissipation and water-water cycle. Photosynthetica 53:207–212. doi: 10.1007/s11099-015-0080-y

Sutinen M, Arora R, Wisniewski M, et al (2001) Mechansims of frost survival and freezing damage in nature. In: Bigras FJ, Colombo SJ (eds) Conifer Cold Hardiness. Springer Netherlands, Dordrecht, pp 89–120

Szymańska R, Kruk J (2008) Gamma-tocopherol dominates in young leaves of runner bean (Phaseolus coccineus) under a variety of growing conditions: the possible functions of gamma-tocopherol. Phytochemistry 69:2142–2148.

Taulavuori K, Bauer E, Taulavuori E (2011) Overwintering stress of Vaccinium vitis-idaea in the absence of snow cover. Environ Exp Bot 72:397–403.

Turkina M V, Kargul J, Blanco-Rivero A, et al (2006) Environmentally modulated phosphoproteome of photosynthetic membranes in the green alga Chlamydomonas reinhardtii. Mol Cell Proteomics 5:1412–1425.

Vener A V, Ohad I, Andersson B (1998) Protein phosphorylation and redox sensing in chloroplast thylakoids. Curr Opin Plant Biol 1:217–223.

Verhoeven AS (2014) Sustained energy dissipation in winter evergreens. New Phytol 201:57–65.

Verhoeven AS (2013) Recovery kinetics of photochemical efficiency in winter stressed conifers: the effects of growth light environment, extent of the season and species. Physiol Plant 147:147–58.

Verhoeven AS, Adams WW III, Demmig-Adams B (1998) Two forms of sustained xanthophyll cycle-dependent energy dissipation in overwintering Euonymus kiautschovicus. Plant, Cell Environ 21:893–903.

Verhoeven AS, Osmolak A, Morales P, Crow J (2009) Seasonal changes in abundance and phosphorylation status of photosynthetic proteins in eastern white pine and balsam fir. Tree Physiol 29:361–374.

Verhoeven AS, Swanberg A, Thao M, Whiteman J (2005) Seasonal changes in leaf antioxidant systems and xanthophyll cycle characteristics in Taxus x media growing in sun and shade environments. Physiol Plant 123:428–434.

Zarter CR, Demmig-Adams B, Ebbert V, et al (2006) Photosynthetic capacity and light harvesting efficiency during the winter-to-spring transition in subalpine conifers. New Phytol 172:283–292. doi: 10.1111/j.1469-8137.2006.01816.x

SUPPORTING INFORMATION

Appendix S1. Photochemical efficiency of PSII (F_v/F_m) and pigment composition in different functional groups in winter. Results show mean values (14 woody plants n≥5; 20 herbaceous plants n≥5; 5 mosses n=5; 9 lichens n=5).

		F_v/F_m (30 min recovery)	F_v/F_m (12h recovery)	V/Chl (mmol mol^{-1})	A/Chl (mmol mol^{-1})	Z/Chl (mmol mol^{-1})	A+Z/VAZ	VAZ/Chl (mmol mol^{-1})
Winter	Woody plant	0.43	0.53	114.60	33.08	32.82	0.36	180.51
	Herb	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	59.76	3.04	13.48	0.23	76.28
Spring	Woody plant	0.75	0.77	87.87	5.36	8.59	0.14	101.83
	Herb	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	0.57	64.38	2.20	16.32	0.23	82.90

Appendix S1. Continuation

		clorf a/b	Neo/Chl mmol mol^{-1}	L/Chl mmol mol^{-1}	α-Car/Chl mmol mol^{-1}	β-Car/Chl mmol mol^{-1}	α-Car/β-Car	Total Car/Chl mmol mol^{-1}	α-Toc/Chl mmol mol^{-1}	Total Toc/Chl mmol mol^{-1}
Winter	Woody plant	3.33	46.56	219.63	1.11	113.15	0.01	560.97	626.41	654.16
	Herb	3.08	46.73	181.89	0.10	105.39	0.00	462.56	331.52	340.78
	Mosses	2.56	51.92	185.23	1.68	144.41	0.01	483.96	220.29	242.56
	Lichens	3.42	70.71	214.68	5.60	77.23	0.33	444.51	65.90	67.65
Spring	Woody plant	3.15	38.57	141.20	1.80	95.13	0.02	378.52	148.14	158.29
	Herb	2.96	40.19	148.40	0.17	102.37	0.00	394.53	173.52	191.44
	Mosses	2.26	60.43	205.15	7.82	156.97	0.21	561.58	103.35	252.40
	Lichens	3.06	83.50	315.23	3.35	137.77	0.04	622.75	82.59	93.70

Appendix S2. Photoprotective parameters: dynamic and chronic photoinhibition ($WPI_{0.5h}$ and $WPI_{>12h}$ respectively) of each analyzed species and pigment concentrations in winter. Results are the average of 5 replicates.

		% $WPI_{>12h}$	% $WPI_{0.5h}$	A+Z/VAZ	AZ/Chl (mmolmol Chl^{-1})	L/Chl (mmolmol Chl^{-1})	β-Car/Chl (mmolmol Chl^{-1})	VAZ/Chl (mmolmol Chl^{-1})	α-Toc/Chl (mmol mol Chl^{-1})
Woody species	*Cytisus cantabricus*	31,50	6,45	0,32	54,90	232,72	133,42	166,07	274,01
	Calluna vulgaris 1º site	17,02	10,15	0,40	57,63	195,70	78,78	143,25	470,38
	Calluna vulgaris 2º site	0,00	23,88	0,54	85,48	199,06	73,30	155,78	757,49
	Calluna vulgaris 3º site	27,67	0,00	0,24	28,15	163,54	81,74	119,94	368,38
	Daphne cneorum	15,79	12,75	0,26	82,23	343,07	121,66	305,18	1809,97
	Erica aragonensis	22,72	13,69	0,36	99,30	226,48	109,32	269,60	1220,56
	Erica cinerea	4,50	4,58	0,18	28,49	143,10	90,92	155,81	202,00
	Erica vagans	6,39	23,95	0,33	86,72	231,35	90,57	255,96	671,61
	Genista hispanica	21,32	9,39	0,31	50,55	198,18	119,50	157,05	459,66
	Globularia repens	19,43	7,53	0,29	54,38	232,61	111,21	177,03	1124,88
	*Juniperu scommunis*subsp. *alpina*	3,08	24,47	0,32	54,88	220,15	90,54	158,57	294,74
	Pinus sylvestris	7,73	18,75	0,45	85,45	271,96	256,96	184,59	442,59
	Pinus uncinata	26,69	18,13	0,36	48,09	234,90	149,75	133,21	212,98
	Vaccinium myrtilus	45,11	12,82	0,71	109,92	157,76	49,90	147,45	761,27
Herbaceous species	*Arabis alpina*	0,00	7,07	0,15	16,64	201,06	103,53	109,28	191,20
	Armeria cantabrica	0,00	2,55	0,12	11,88	141,95	103,21	103,09	76,96
	Asperula hirta	1,87	2,27	0,22	32,88	213,91	109,80	146,52	249,12
	Cerastium fontanum	0,00	5,92	0,13	21,07	158,56	118,22	162,71	60,98
	Digitalis parviflora	14,44	3,76	0,12	24,09	240,44	139,22	200,32	1973,21
	Festuca sp.	11,38	0,00	0,09	12,12	177,99	116,06	127,12	121,59
	Hieracium pilosella 1º site	0,00	6,70	0,14	15,45	155,33	119,20	111,33	248,99
	Hieracium pilosella 3º site	0,00	9,73	0,14	19,93	167,21	104,03	138,52	530,96
	Plantago lanceolata	0,00	5,72	0,10	11,95	165,61	107,39	119,89	164,34

Appendix S2: Continuation.

		% $WPI_{>12h}$	% $WPI_{0.5h}$	A+Z/VAZ	AZ/Chl (mmol mol Chl^{-1})	L/Chl (mmol mol Chl^{-1})	β-Car/Chl (mmol mol Chl^{-1})	VAZ/Chl (mmol mol Chl^{-1})	α-Toc/Chl (mmol mol Chl^{-1})
Herbaceous species	*Poacea* sp.	0,00	2,75	0,10	8,71	145,55	96,42	85,65	73,50
	Polypodium sp.	0,00	5,95	0,14	14,96	164,52	98,83	102,33	174,44
	Ranunculus repens	0,00	9,37	0,12	16,91	165,63	104,78	138,02	80,16
	Saxifraga paniculata	19,46	3,68	0,19	30,38	175,97	104,37	158,29	253,71
	Scilla sp.	0,01	3,65	0,17	13,07	205,30	101,96	78,87	111,83
	Sedum album	0,63	2,11	0,16	17,74	162,42	84,41	105,49	147,84
	Sedum brevifolium	0,00	6,48	0,17	19,13	189,38	98,45	114,34	328,90
	Sedum sp.	0,00	4,65	0,13	15,53	181,04	98,64	119,21	207,76
	Senecio sp.	0,58	2,85	0,13	18,17	196,08	117,62	136,92	609,46
	Teucrium pyrenaicum	0,27	9,64	0,19	30,01	216,37	97,31	157,10	434,78
	Thymelaea sp.	13,93	5,04	0,14	20,81	213,40	84,28	154,13	590,61
Mosses	*Dicranum scoparium*	0,00	0,00	0,24	21,47	165,70	82,69	87,50	192,23
	Didymodon sp.	0,00	11,70	0,40	40,38	186,08	196,86	100,60	306,89
	Grimmia pulvinata	18,60	0,00	0,27	23,90	166,63	89,43	86,71	100,94
	Polytrichum piliferum	15,69	0,00	0,34	39,78	187,07	231,52	116,01	293,67
	Syntrichia muralis	0,00	8,56	0,31	35,33	220,70	121,57	112,69	207,73
Lichens	*Dermatocarpon* sp.	0,00	0,00	0,25	19,14	171,92	53,64	71,03	202,36
	Lasallia hispanica	0,00	1,32	0,18	14,72	193,81	69,94	80,66	55,92
	Lichinella stipatula	0,00	0,00	0,19	11,94	188,25	51,09	62,18	22,55
	Parmelia saxatilis	0,00	0,00	0,20	17,40	214,25	122,33	89,11	31,17
	Physcia sp.	0,00	4,41	0,34	22,31	387,12	120,06	82,46	108,94
	Ramalina sp.	7,32	0,00	0,21	17,95	171,28	64,33	85,06	62,96
	Rhizocarpon sp.	39,15	0,00	0,14	11,64	182,74	70,63	84,10	59,36
	Umbilicaria cylindrica	0,00	3,94	0,23	17,10	189,00	54,36	71,64	42,14
	Umbilicariapolyphylla	10,70	0,00	0,27	14,82	187,24	66,17	61,04	48,86

Appendix S3. Photoprotective parameters: dynamic photoinhibition ($WPI_{0.5h}$) and pigment concentration in spring. Results are the average of 5 replicates.

		% $WPI_{0.5h}$	AZ/VAZ (mmolmolChl^{-1})	AZ/Chl (mmol mol Chl^{-1})	L/Chl (mmol mol Chl^{-1})	β-car/Chl (mmol mol Chl^{-1})	VAZ/Chl (mmol mol Chl^{-1})	α-Toc/Chl (mmol mol Chl^{-1})
Woody species	*Cytisus cantabricus*	5.47	0.14	10.22	137.59	97.89	72.63	209.51
	Calluna vulgaris 1º site	1.27	0.14	13.69	137.65	93.49	98.44	106.98
	Calluna vulgaris 2º site	0.46	0.24	24.64	152.71	99.71	106.63	141.95
	Calluna vulgaris 3ºsite	0	0.11	9.57	137.85	89.47	84.24	379.85
	Daphne cneorum	6.68	0.12	17.65	159.99	108.99	142.60	157.26
	Erica aragonensis	1.93	0.11	13.64	147.25	104.55	130.02	157.72
	Erica cinerea	1.87	0.15	13.20	125.90	95.09	92.58	55.14
	Erica vagans	0.19	0.12	10.85	117.60	90.62	86.89	92.72
	Genista hispanica	0.00	0.11	11.53	123.30	107.08	105.02	44.92
	Globularia repens	4.32	0.12	11.10	122.36	98.56	88.06	200.38
	Juniperus communis subsp. *alpina*	4.64	0.15	14.28	147.58	76.27	97.16	85.30
	Pinus sylvestris	0.85	0.09	8.94	167.61	94.61	121.77	129.99
	Pinus uncinata	2.08	0.09	6.96	128.19	75.74	83.93	82.98
	Vaccinium myrtilus	3.51	0.25	22.08	136.13	93.57	91.36	281.03
Herbaceous species	*Arabis alpina*	0.00	0.15	14.31	166.36	100.19	98.12	264.22
	Armeria cantabrica	1.26	0.21	17.27	135.06	106.32	83.82	117.83
	Asperula hirta	0.00	0.12	10.76	120.34	104.38	87.24	58.88
	Cerastium fontanum	2.91	0.23	13.25	146.44	104.97	59.53	108.85
	Digitalis parviflora	1.70	0.12	9.54	143.55	97.44	90.27	70.39
	Hieracium pilosella 1º site	4.55	0.11	8.72	120.48	109.84	83.47	133.53
	Hieracium pilosella 3º site	4.08	0.11	8.72	119.90	111.49	81.94	98.78
	Plantago lanceolata	1.28	0.13	9.52	125.52	102.99	73.68	122.91

Appendix S3. Continuation

		% $WPI_{0.5h}$	AZ/VAZ (mmol mol Chl -1)	AZ/Chl (mmol mol Chl -1)	L/Chl (mmol mol Chl -1)	β-car/Chl (mmol mol Chl -1)	VAZ/Chl (mmol mol Chl -1)	α-Toc/Chl (mmol mol Chl -1)
Herbaceous species	*Poacea*	0.00	0.19	24.57	140.81	108.81	123.72	74.36
	Polypodium sp.	3.72	0.13	12.15	140.89	82.45	95.86	64.93
	Ranunculus repens	3.53	0.14	17.17	133.67	98.88	125.77	48.25
	Saxifraga paniculata	1.79	0.28	28.35	132.13	102.89	107.10	145.09
	Scilla sp.	2.90	0.42	49.48	154.67	127.85	100.26	328.71
	Sedum album	8.90	0.23	18.83	138.19	84.37	83.16	168.18
	Sedum brevifolium	0.00	0.25	43.23	238.04	83.24	174.78	871.90
	Sedum sp.	10.14	0.15	26.01	205.06	109.90	172.83	206.56
	Senecio sp.	6.87	0.11	9.60	121.77	102.65	90.23	76.12
	Teucrium sp.	0.00	0.14	14.32	125.33	98.67	100.79	111.43
	Thymelaea sp.	4.62	0.11	9.08	141.73	99.65	83.75	318.71
Mosses	*Dicranum scoparium*	0	0.49	60.00	221.77	238.08	121.50	390.69
	Didymodon sp.	14.92	0.45	52.71	200.27	260.41	107.25	442.90
	Grimmia pulvinata	26.07	0.41	42.06	194.07	115.31	103.22	315.23
	Polytrichum piliferum	0.25	0.38	39.07	173.14	199.63	113.50	308.63
	Syntrichia muralis	0.00	0.57	90.78	255.78	167.32	161.15	203.89
Lichens	*Dermatocarpo* sp.	13.17	0.24	17.11	208.26	66.89	72.89	45.36
	Lasallia hispanica	11.11	0.19	13.75	182.63	66.92	75.18	57.38
	Lichinella stipatula	9.77	0.18	12.98	185.71	55.95	72.99	48.15
	Parmelia saxatilis	9.97	0.21	18.72	198.93	106.58	89.94	39.24
	Physcia sp.	11.14	0.22	36.23	571.69	202.87	188.40	62.82
	Ramalina sp.	3.97	0.17	18.31	183.13	76.28	106.64	59.79
	Umbilicaria cylindrica	11.71	0.17	10.24	184.46	59.87	61.58	29.26
	Umbilicaria polyphylla	6.36	0.14	10.27	187.81	74.38	72.95	35.69

Table 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 43º2'33"N 4º22'14"W	Site 2 42º3'38"N 2º32'4"W	Site 3 42º56'26"N 0º46'7"W
Woody species	*Calluna vulgaris*	*Calluna vulgaris*	*Calluna vulgaris*
	C. cantabricus	*Erica aragonensis*	*Daphne cneorum*
	Ericacinerea	*Pinus sylvestris*	*Erica vagans*
	Juniperus communis subsp. *alpina*	*Vaccinium myrtilus*	*Erica cinerea*
			Genista hispanica
			Globularia repens
			Pinus uncinata
Herbaceous	*Arabis alpina*	*Armeria* sp.	*Asperula hirta*
	Cerastium fontanum	*Digitalis parviflora*	*Hieracium pilosella*
	Hieracium pilosella	*Festuca* sp.	*Poacea*
	Scilla sp.	*Polypodium* sp.	*Saxifraga paniculata*
	Plantago lanceolata	*Sedum brevifolium*	*Teucrium pyrenaicum*
	Ranunculus repens	*Senecio* sp.	*Thymelaea* sp.
	Sedum album		
	Sedum sp.		
Mosses	*Dicranum scoparium*	*Syntrichia muralis*	
	Didymodon sp.		
	Grimmia pulvinata		
	Syntrichia muralis		
	Polytrichum piliferum		
Lichens	*Dermatocarpon* sp.	*Pseudevernia furfuracea*	
	Lasallia hispanica	*Rhizocarpon* sp.	
	Lichinella stipatula	*Usnea* sp.	
	Parmelia saxatilis		
	Physcia sp.		
	Ramalina sp.		
	Umbilicaria cylindrica		
	Umbilicaria polyphylla		

Table 2: Correlation coefficients (r) between the different types of photoinhibition (chronic photoinhibition ($WPI_{>12h}$), dynamic photoinhibition ($WPI_{0.5h}$) and total photoinhibition (WPI_{all})) and the ratios of carotenoids and tocopherols per chlorophyll. The number of species included in calculations was 14 woody plants, 20 herbaceous plants, 5 mosses and 9 lichens. Asterisks denote significant correlations (hyphen "-" $P \geq 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P \leq 0.001$).

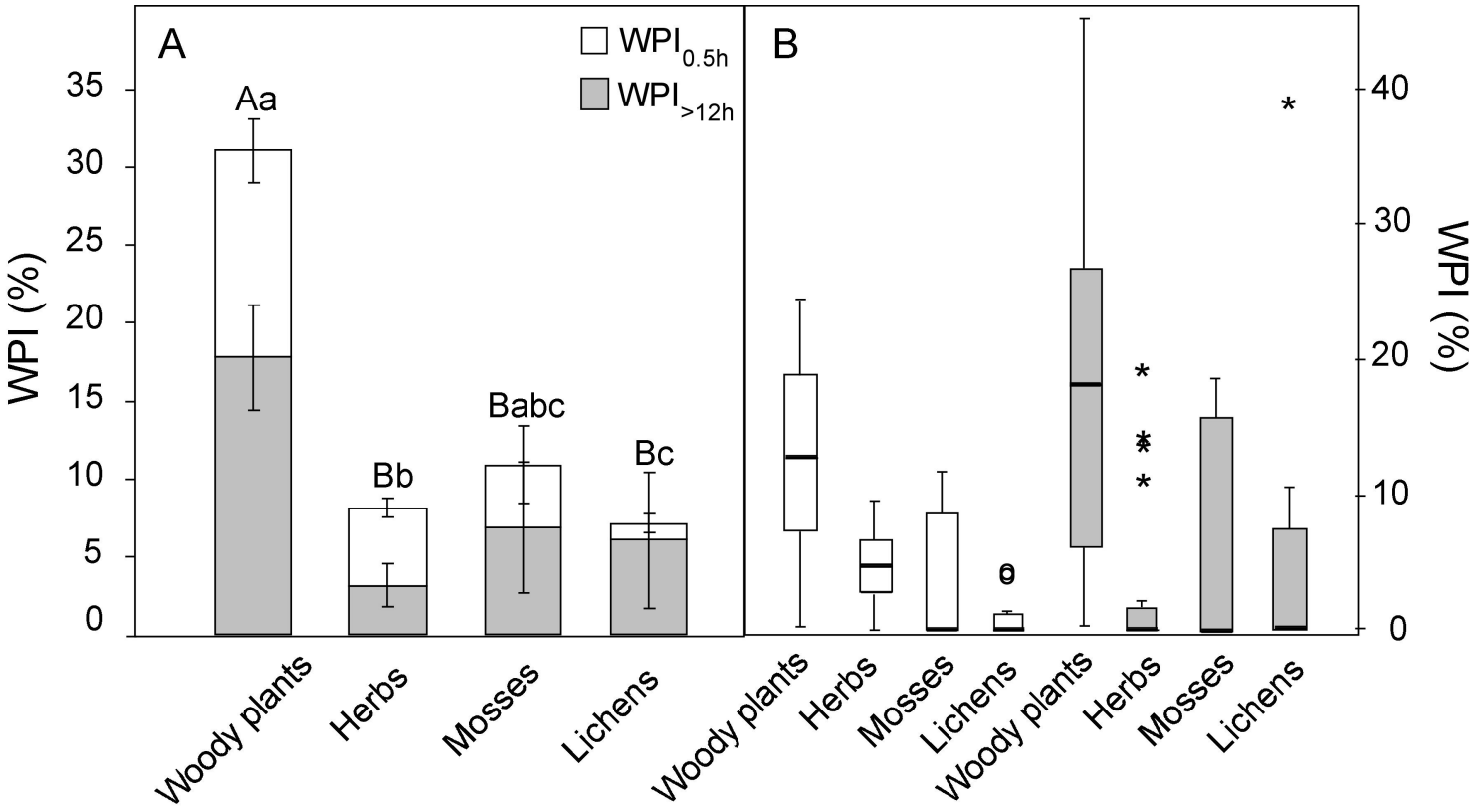
	Woody			Herbaceous			Mosses			Lichens		
	WPI_{all}	$WPI_{>12h}$	$WPI_{0.5h}$	WPI_{all}	$WPI_{>12h}$	$WPI_{0.5h}$	WPI_{all}	$WPI_{>12h}$	$WPI_{0.5h}$	WPI_{all}	$WPI_{>12h}$	$WPI_{0.5h}$
V/Chl	-	-	-	0.456***	0.332**	-	-	-	-	-	-	-
A/Chl	-	-	-	0.398**	0.286*	-	-	-	0.779*	-	-	-
Z/Chl	0.296*	-	-	-	-	-	-	-	-	-	-	-
AZ/Chl	-	-	0.293*	0.241*	-	-	-	-	-	-	-	-
VAZ/Chl	-	-	-	0.471***	0.335**	-	-	-	-	-	-	-
AZ/VAZ	0.416*	-	-	-	-	-	-	-	-	-	-	-
Neo/Chl	-	-	-	-	-	0.204*	-	-	-	-	-	0.499*
Trans Neo/Chl	-	-	-	-	-	-	-	-	-	-	-	0.666**
L/Chl	-	-	-	-	-	-	-	-	-	-	-	0.480*
Total car/Chl	-	-	-	0.339**	0.258*	-	-	-	-	-	-	-
Total toc/Chl	-	-	-	0.253*	0.222*	-	-	-	-	-	-	-

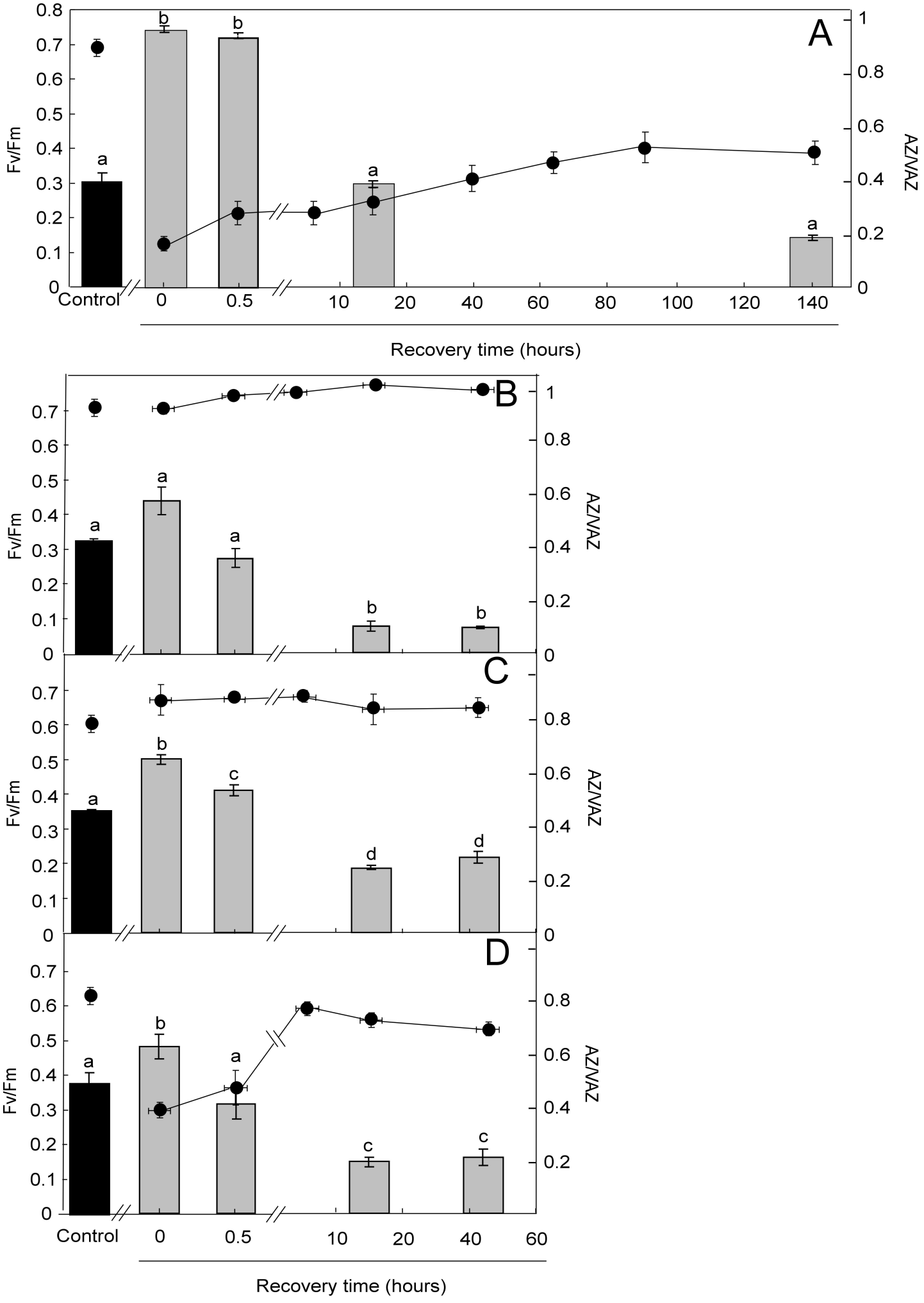
Fig. 1: Chronic and dynamic winter photoinhibition ($WPI_{>12h}$ and $WPI_{0.5h}$, respectively) in different functional groups. (A) Grey bars represent $WPI_{>12h}$ and white bars are the $WPI_{0.5h}$. Each bar represents the mean ± SE. The number of species included were: 14 woody species, 20 herbs, 5 mosses and 9 lichens (n=5 for each species). (B) Box plot of $WPI_{0.5h}$ (white bars) and $WPI_{>12h}$ (black bars) in all functional groups. For each functional group the percentage of each type of photoinhibition is plotted for functional group (horizontal axis). 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´. The number of species included in each bar in A and B figures was 14 in woody, 20 in herbaceous, 5 in mosses and 9 in lichens (n=5 for each species).

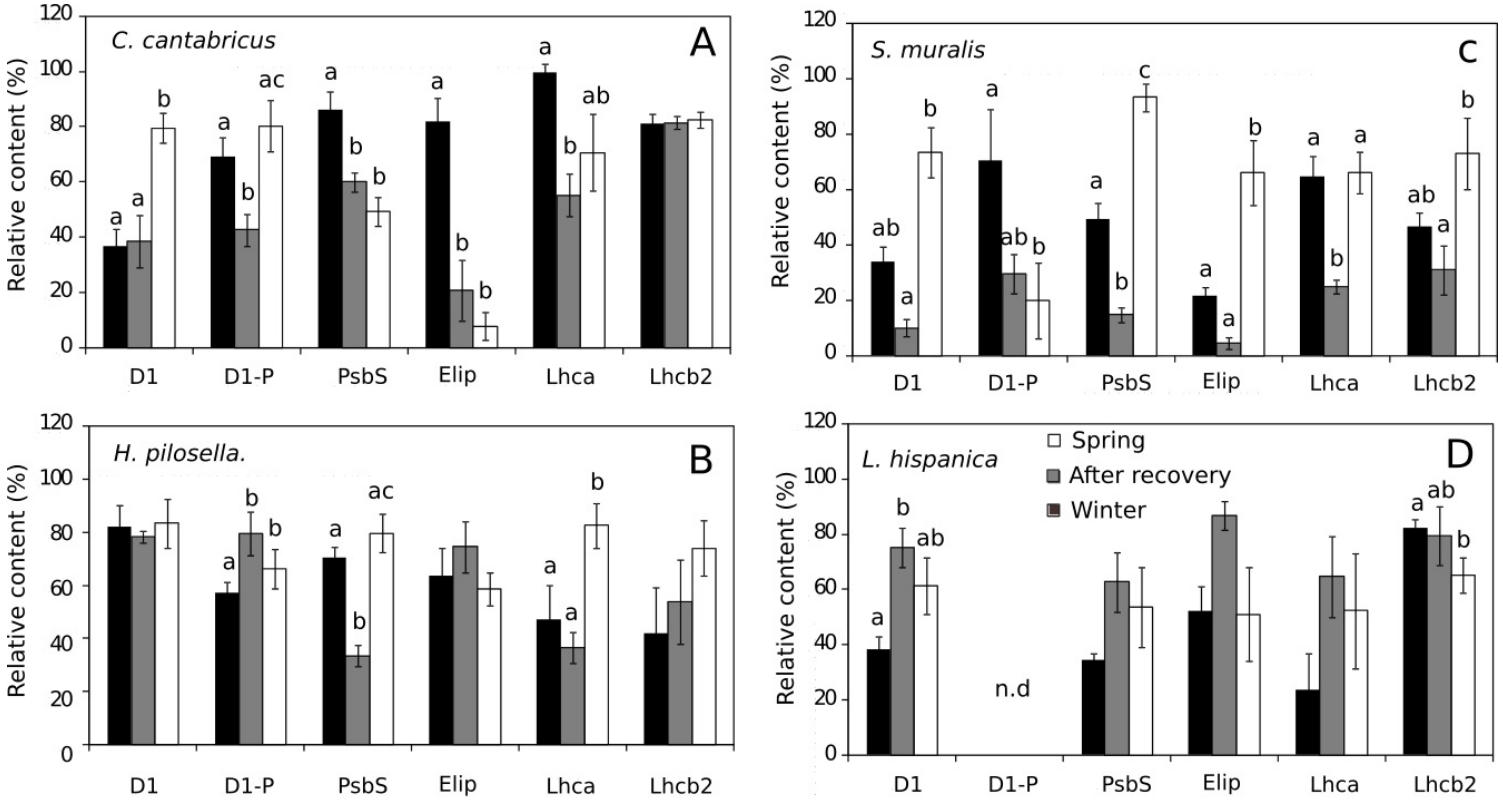
Fig. 2: Recovery kinetics of F_v/F_m (closed circles) for all model species (A) *Cytisus cantabricus*, (B) Hieracium pilosella (C) Syntrichia muralis and (D) Lasallia hispanica. Control valueswere considered those collected in spring. T0 was collected in winter after 30 minutes under low temperatures and darkness. The remaining measurements were done on all species maintained at room temperature and low light. Each time point is an average of samples from five individuals. Black bars (Control) represent spring values of AZ/VAZ while grey bars represent the changes in AZ/VAZ along the recovery. Each bar depicts the mean ± SE (n=5). Different lowercase letters indicate significant differences at $P < 0.05$.

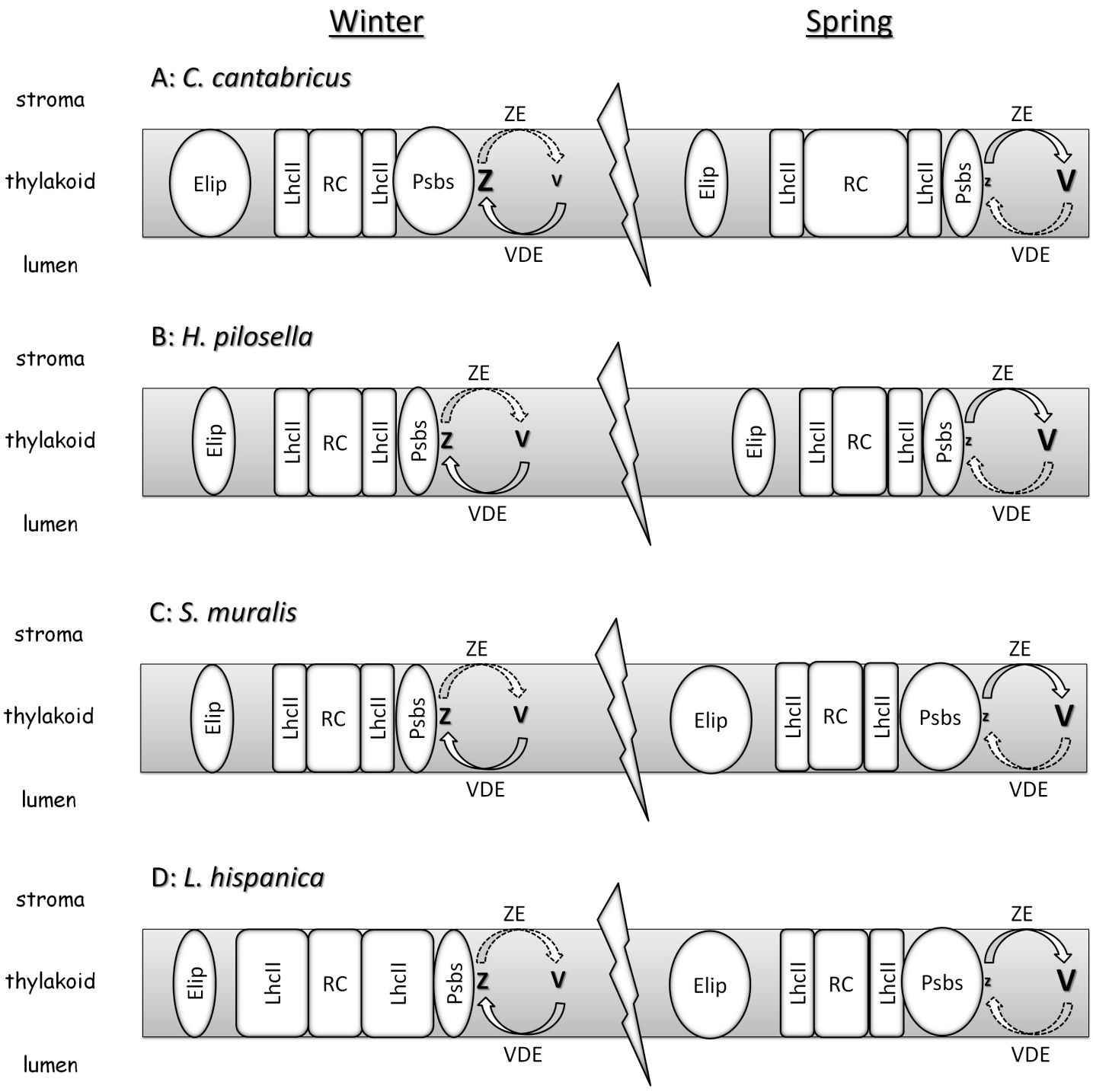
Fig. 3: Relative thylakoid proteins content D1, D1-P, PsbS, Elip, Lhca, Lhcb2 in (A) Cytisus cantabricus; (B) Hieracium pilosella; (C) Syntrichia muralis and (D) Lasallia hispanica in winter (solid bars), after recovery treatment (grey bars) and in spring (open bars). Recovery treatment refers to optimum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

Fig. 4: Schematic representation of thylakoid protein composition and V-cycle for model species (A) Cytisus cantabricus, (B), Hieracium pilosella, (C) Syntrichia muralis and (D) Lasalliahispanica in winter and spring. The size of V-cycle letters and of protein boxes illustrates the amount of each component in winter or spring.


A
B
WPI0.5h
WPI>12h
WPI (%)
Aa
Bb
Babc
Bc
Woody plants
Herbs
Mosses
Lichens


A
B
C
D
Fv/Fm
AZ/VAZ
Control
0
0.5
10
20
40
60
80
100
120
140
Recovery time (hours)


C. cantabricus
A
H. pilosella.
B
S. muralis
C
L. hispanica
D
Relative content (%)
D1
D1-P
PsbS
Elip
Lhca
Lhcb2
Spring
After recovery
Winter
n.d


Winter
Spring
A: C. cantabricus
stroma
thylakoid
lumen
Elip
LhcII
RC
LhcII
Psbs
ZE
Z
V
VDE
B: H. pilosella
C: S. muralis
D: L. hispanica