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Changes in thermic limits and acclimation assessment for an alpine plant by chlorophyll fluorescence analysis: F_v/F_m *vs.* R_{fd}

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Abstract

A comparison between maximum quantum yield of PSII photochemistry (F_v/F_m) and chlorophyll fluorescence decrease ratio (R_{fd}) for low and high temperature resistance was assessed in a seasonal study of the acclimation in *Pterocephalus lasiospermus*. Analyzing the regression adjustment of both parameters and the lethal temperatures (LT_{50}), R_{fd} resulted in being a more sensitive indicator for low and high temperature treatments, since the thermic resistance estimated with R_{fd} parameter was never higher than those estimated with F_v/F_m. Furthermore, the use of F_v/F_m led to an overestimation of the acclimation phenomena, with 6ºC of a maximum difference between both parameters. Using R_{fd} as the indicator parameter, *P. lasiospermus* acclimated to low temperatures but it kept on being a sensitive species (the lowest LT_{50} values only achieved –9.9 ± 0.3ºC). However, no heat acclimation was observed (LT_{50} around 43.5ºC). Thus, according to R_{fd} evaluation of the thermic threshold, this species could be in risk of damage at low temperatures in this alpine ecosystem.

Additional key words: freezing acclimation; heat; high mountain; Teide National Park; temperature resistance; vitality index.

Introduction

Chl *a* fluorescence has been widely recognized as a noninvasive technique for monitoring photosynthetic activity and for assessing plant responses to a variety of environmental stresses (Baker 2008). High and low temperatures have been shown to be two of those stressful factors (Neuner and Pramsohler 2006, Ehlert and Hincha 2008), since both membrane fluidity and protein conformation changes are affected (Ruelland and Zachowski 2010).

Several methods have been developed to assess the extreme temperature tolerance. Generally, the calculation of LT_{50} values, the lethal temperature at which irreversible damage is provoked, is of interest, since it delimits the resistance to thermic stress and allows comparisons between species. Probably, the most common methods used for the calculation of LT_{50} values are visual damage, electrolyte leakage, and chlorophyll (Chl) fluorescence. However, in some species, necrotic damage is not clearly observed (*e.g.* Sierra-Almeida *et al.* 2010) or perceptible electrolyte release is not detected (*e.g.* Cieraad *et al.* 2012). Thus, the choice of the suitable Chl fluorescence parameter as an indicator of thermic damage is of a great importance. F_v/F_m is one of the most known Chl fluorescence parameters and it reflects the state of the initial part of photosynthesis, PSII and the primary acceptor. Several other Chl parameters have been also described. One of them is the Chl fluorescence decrease ratio or the vitality index (R_{fd}), introduced in the 1980s (Lichtenthaler *et al.* 1986, Haitz and Lichtenthaler 1988, Lichtenthaler and Rinderle 1988). R_{fd} is a physiological straight-forward indicator of the photosynthetic quantum conversion of leaves (*i.e.* the capacity of using the potential photochemical activity of PSII under continuous irradiation), which is not really registered with F_v/F_m (Haitz and Lichtenthaler 1988, Lichtenthaler *et al.* 2005a). R_{fd} depends on the outcome of the whole electron transport

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Abbreviations: Chl – chlorophyll; F_0 – minimal fluorescence yield of the dark-adapted state; F_0' – minimal fluorescence yield of the light-adapted state; F_m – maximal fluorescence yield of the dark-adapted state; F_m' – maximal fluorescence yield of the light-adapted state; F_s – steady-state fluorescence yield; F_v – variable fluorescence; F_v/F_m – maximal quantum yield of PSII photochemistry; LT_{50} – temperature which cause a 50% of damage; R_{fd} – Chl fluorescence decrease ratio or vitality index.

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chain and the final synthesis of ATP and NADPH, furthermore it has been demonstrated to be highly correlated with CO_2 fixation (Haitz and Lichtenthaler 1988, Flexas *et al.* 2002, Lichtenthaler *et al.* 2005a). Thus, theoretically both parameters must show a different response to a thermic stress if different photosynthesis processes are involved. However, the thermic damage studies by Chl fluorescence focus mainly on F_v/F_m (*e.g.* Fisker *et al.* 1995, Germino and Smith 2000, Bannister and Lord 2006, Ehlert and Hincha 2008, Martínez-Peñalver *et al.* 2011, Sierra-Almeida and Cavieres 2012, Venn *et al.* 2013, Briceño *et al.* 2014, Pescador *et al.* 2016), and few studies consider R_{fd} as an indicator of damage (Balota and Lichtenthaler 1999, Murkowski 2001, Mishra *et al.* 2014). Although contrasting Chl fluorescence and electrolyte leakage (Ehlert and Hincha 2008) and visual damage methods (Bannister *et al.* 2005) have been conducted, to our knowledge, studies demonstrating simultaneously the variability in LT_{50} values obtained by F_v/F_m and R_{fd} are absent.

Many studies have been developed in order to determine low and high temperature resistance of many tropical, temperate, and polar species (*e.g.* Sakai and Larcher 1987, Larcher 2003b). As a result, it has been reported that some species, including many plants from temperate climates, are able to increase their thermic resistance as a result of a previous exposition in a process termed acclimation, both to low temperatures (Thomashow 1999, Xin and Browse 2000, Pflug and Brüggerman 2012) and high temperatures (Dat *et al.* 1998, Larkindale and Huang 2004, Xu *et al.* 2006, Wahid *et al.* 2007). Furthermore, most plants follow a variable annual cycle of acclimation and deacclimation (Larcher 2003c) and even diurnal variations of LT_{50} values in terms of heat-tolerance have been also reported (Buchner and Neuner 2003).

Temperature thresholds and the acclimation phenomena are of a special interest, since thermic stress is one of the most important limiting factors that determinate the plant survival, especially in alpine ecosystems, where freezing stress is the first environmental "filter" that a species has to pass through in order to become "alpine" and where heat stress may appear every time solar radiation peaks and top soils dry (Körner 2003, Bannister *et al.* 2005, Larcher *et al.* 2010, Sierra-Almeida and Cavieres 2012). It is precisely in alpine ecosystems, where climate change acts more quickly than in other terrestrial habitats, causing biodiversity loss, habitat degradation, and landscape modifications (Chersich *et al.* 2015), depending on the rate of climatic change, the species pool, and the biogeographical region (Beniston 2003, Pauli *et al.* 2012). Among these high mountain habitats, the Mediterranean alpine ecosystems deserve particular attention for hosting a high number of endemic and rare plants (Pauli *et al.* 2003). In these privileged areas, little detailed studies have been done (Cabrera 2002, Evangelista *et al.* 2016), including in the Mediterranean alpine ecosystem of the Canary Islands (Spain), which is inside a hot spot of biodiversity in the Macaronesia region (Myers *et al.* 2000). The most representative alpine ecosystem of the archipelago is harbored in Tenerife and preserved in Teide National Park, where a total of 168 alpine species with a 32% of endemics have been listed (Acebes *et al.* 2010).

Teide National Park has a typically cold winter with minimum air temperatures of –5 to –2ºC and maximum temperatures of 4 to 8ºC (measured 1.5 m above ground level). Although during the summer maximum temperatures, temperature can reach up to 30ºC, annual mean temperatures maintain around 8ºC, which reflects a strong diurnal and seasonal oscillation in temperature, with 14.8ºC of difference between the minimum and maximum temperature mean of the coldest and the warmest month of the year (Del-Arco *et al.* 2006). The high radiation (the highest one in Spain according to Meteorology State Agency, AEMET) and the very hard edaphic conditions with leptosols as predominant soils (Rodríguez *et al.* 2014) are also characteristic factors of this singular ecosystem.

In this ecosystem, climate change studies have denoted an increase of 0.31 ± 0.12ºC each decade in the mean temperature since 1970 (Martín *et al.* 2012). Since 1944, the minimum and maximum mean temperatures have increased by 1.18 ± 0.4ºC and 0.66 ± 0.52ºC, respectively, and taking into account that extreme temperatures are considered one of the most important limiting factor in this ecosystem, its vegetal structure could change radically, involving therefore a loss of biodiversity (Del-Arco *et al.* 2008).

Currently, one of the most abundant species in Teide National Park is the endemic alpine shrub *Pterocephalus lasiospermus* Link *ex* Buch, which distribution area is restricted to the highest part of Tenerife from 2,000 to 2,500 m a.s.l. In the 1960s, this species practically disappeared, but during the recent decades, it has over-expanded at the expense of other nearby species (Martín and Wildpret 1999). These changes in the abundance and distribution of this species remain undefined and they are now a priority for Teide National Park managers.

Given the above, the present study aimed to (*1*) evaluate sensitivity of both F_v/F_m and R_{fd} parameters for low and high temperature stress detection, and (*2*) determine the thermic limits and the annual tolerance of the endemic alpine evergreen shrub *P. lasiospermus* by Chl fluorescence in order to contribute for elucidating reasons of its recent expansion.

Materials and methods

Study site and meteorological conditions: The study site was located at 2,070 m a.s.l. in the northeastern-exposed slope of Teide National Park (Tenerife, Canary Islands, Spain), close to El Portillo (28°18'16.25"N, 16°34'0.01"W). An automatic weather station (*MiniCube VV/VX16*, *EMS*, Brno, CR) was installed and connected to a data logger programmed to record 30-min averages of the environmental measurements taken every minute from 12 December 2013 to 24 July 2014. Air temperature (*EMS33*, *EMS*, Brno, CR) was monitored in order to record all freezing events and to know the temperature context of each measurement. Four measurements were made during the end of 2013 and 2014: E1 (17 December), E2 (14 February), E3 (25 February), and E4 (24 July). Winter measurements were carried out according to the unpredictable freezing events, which took place on the study site. The last test was made during the warm and dry period for comparisons of contrasting seasons.

Plant material and sampling: The sampling was done early in the morning (around 7:00 and 9:00 h), since the light exposure has been reported to increase LT_{50} values at heat temperatures (Buchner *et al.* 2013). The apical parts of the stems of *P. lasiospermus* were randomly collected from plants growing naturally in the study site. Each plume was chosen from the upper part of the shrub in order to avoid variations due to the different microclimate. After collecting, plumes were immediately introduced into plastic bags with a hydrated atmosphere in order to prevent samples from drying out and transported to the laboratory in boxes that maintained the ambient temperature, avoiding extra heating of the samples. All samples were stored overnight until the beginning of the measurements and treatments the next day morning. Then, single, healthy leaves were selected, always belonging to 3rd–5th node and carefully removed from the plume immediately before the temperature treatments.

Temperature treatment: In each temperature experiment, leaves were put into plastic hermetic bags with a hydrated atmosphere. Both cooling and heating treatments were carried out in a circulated water bath (*Hetofrig CB11E*, *Heto*, Birkerød, Denmark). Samples were immediately exposed to the target temperature for an exposure time of 30 min (Larcher 2003b, González-Rodríguez *et al.* 2005). We were aware that the exposure of leaves to freezing temperatures without a slow decrease in temperature could affect their freezing resistance, however, this procedure has been reported by others authors (Sierra-Almeida and Cavieres 2012). A thermocouple fixed to the leaves indicated the exact temperature of the leaves during the temperature treatment in order to avoid the thermo-protective effect of the bag. In the case of low temperature treatments, extracellular ice formation was confirmed by the observation of freezing exotherm. A total of 24 temperatures were selected (from –20 to 0 and 30 to 50°C in 2ºC steps, and 20 and 25ºC as nondamaging temperatures), except in E3, where only low temperatures were evaluated. For each temperature, a set of 5–9 leaves was used.

Chl *a* fluorescence parameters, F_v/F_m and R_{fd}, were obtained with *IMAGING-PAM* fluorometer (*Mini blue version*, *Walz GmbH*, Effeltrich, Germany). Each fluorescence data was obtained by typical integration of all the pixel values of the whole leaf area, avoiding errors derivated from the heterogeneity of the damage patterns (Lichtenthaler *et al.* 2005b). F_v/F_m was calculated by applying a saturating light pulse [>3,000 µmol(photon) m^{-2} s^{-1}] both before (control) and after the temperature treatment on the same leaf, with a delay of ½–1 h and after 24 h. The thermal baths were placed in a dark room, so that an additional 30 min for dark adaptation after the thermal bath was not required. Once obtained F_v/F_m after the temperature treatment, a saturating and continuous actinic light [blue light of 470 nm, 923 µmol(photon) m^{-2} s^{-1}, previously tested according to the F_m' and F' values] was switched on and the decrease of the fluorescence was recorded for 5 min until achieving to a steady state (F_s) (Lichtenthaler *et al.* 2005a), allowing R_{fd} calculation. Control R_{fd} values were calculated for control (not subjected to bath with different temperatures) leaves (n = 5–9). All Chl fluorescence measurements were done at room temperature. In E1, measurement of F_s was not possible due to a technical mishap.

Data and statistical analysis: Linear or nonlinear regression between F_v/F_m and R_{fd} values was done fitting the data to a line or a curve by the Solver function of *Microsoft Excel*. The method employed to fit the regression curves was the least squares estimation (Seber and Wild 2003, De Lobo *et al.* 2013). The resulted model was tested with *IBM SPSS Statistics* 21 (*IBM*, USA.).

In this species, F_v/F_m and R_{fd} reached zero values in dead leaves, so the percentage of leaf damage was equivalent to (*1*) the percentage of reduction of F_v/F_m after treatment with respect to the measurements before the treatment of the same leaf (control), and (*2*) the percentage of reduction of R_{fd} after treatment with respect to the control leaves. It is considered that a 50% of damage corresponds to a lethal or irreversible damage, so that the temperatures which cause this percentage of damage are termed LT_{50} (Larcher 2003b). For the calculation of LT_{50}, the percentage of damage was plotted against the treatment temperatures, so that the temperature at which a 50% of damage was produced could be calculated by linear regression fitted to the central (linear) part of the sigmoid relationship between the damaging temperature and the percentage of damage, using at least three temperatures (Bannister *et al.* 2005; Bannister 2007). The regression models were obtained with *StatGraphics* (*Centurion XVI.I*, *Manugistic*s, Rockville, USA) once tested normality and homoscedastic hypothesis. Apart from the mean value of LT_{50}, the upper and lower limits of the intervals were estimated with a confident interval of 95% (α = 0.05) and the intercepts and slopes of the regression models were compared by *F*-test.

Results

Meteorological conditions: Air temperatures and freezing events recorded during the studied period are shown in Fig. 1. The first freezing event occurred on 12 December. It was short and the absolute minimum temperature hardly dropped at –0.7ºC, with daily means of 2.1ºC. E1 measurement was realized after this first freezing event. During January, the temperatures dropped below zero several times, reaching to –1.8ºC. From 15 to 17 February, temperatures below zero were kept. It was the strongest freezing event of the year with absolute minimum of –3.1ºC and maximum of 0.8ºC. E2 and E3 measurements occurred before and after this event, respectively, with a difference of 11 d. The last freezing event occurred on 25 May, when absolute minimum reached –1.3ºC. Finally, the last measurements – E4 – were done in a summer context, when temperatures gradually increased to absolute maximum around 28ºC and absolute minimum around 10ºC.

F_v/F_m vs. R_{fd}: All shown data were referred to the measurements taken after thermic treatments with a delay of 0,5–1 h. The measurement after 24 h of thermic treatment are not shown, since there was neither additional damage nor recuperation values after this time.

Different behavior was observed for both parameters depending on the period of the year and the temperatures, except in E1, where a comparison of F_v/F_m and R_{fd} values was not possible due to a technical problem with the R_{fd} measurements. At high temperatures, the correlation between F_v/F_m and R_{fd} was linear (Fig. 2*A*). On the contrary, at low temperatures, the data were fitted to a curve with an asymptote at the highest F_v/F_m values (Fig. 2*B*), only perturbed by data of E4 (Fig. 2*B*), which showed a similar behavior to high temperatures. Thus, at low temperatures during the winter, the F_v/F_m values below 0.6 corresponded to R_{fd} values below 1, while when the damage was due to high temperatures, the F_v/F_m values of 0.6 equated to R_{fd} values around 3.

This dissimilar sensitivity of both parameters was coherent with the differences which were found in the evaluation of the percentage of thermic damage obtained for each parameter (Fig. 3). In E2 and E3, the regression model, fitted to low damage temperature data, resulted in a significantly different intercept and slope from F_v/F_m to R_{fd} as indicator parameters (Fig. 3*B,D* compared with *C,E*). It means that in February tests (E2 and E3), low temperature treatments evaluated with F_v/F_m provoked damage earlier and more slowly (0.43 and 0.63 times more slowly in E2 and E3, respectively) than when they were evaluated with R_{fd} (the rise of 1ºC corresponded to 3.25 and 10.27% of damage more with R_{fd} than with F_v/F_m in E2 and E3, respectively). These differences were not significant at low-temperature treatments in E4 (Fig. 3*F,G)*.

On the contrary, at the high-temperature treatment, the slope of the regression models did not significantly change between F_v/F_m and R_{fd} in both E2 and E4 (Fig. 3*B,F vs. C,G*). Only in E4, the intercept of the regression model was different depending on the parameter used, which did not compromise the linear relation between both parameters at high temperatures already described (Fig. 2*A*).

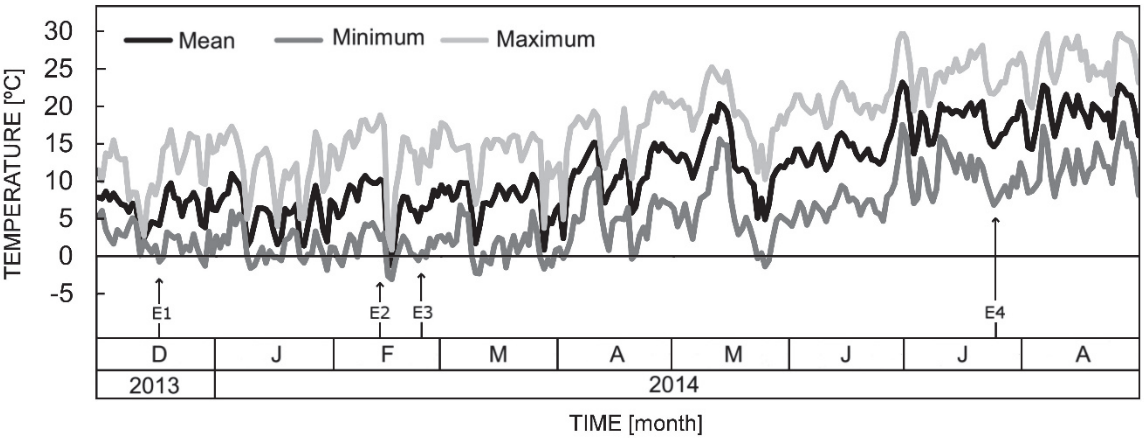

Fig. 1. Daily minimum absolute, maximum absolute, and mean air temperatures of the studied area. Experiments: E1 (17 December), E2 (14 February), E3 (25 February), and E4 (24 July).

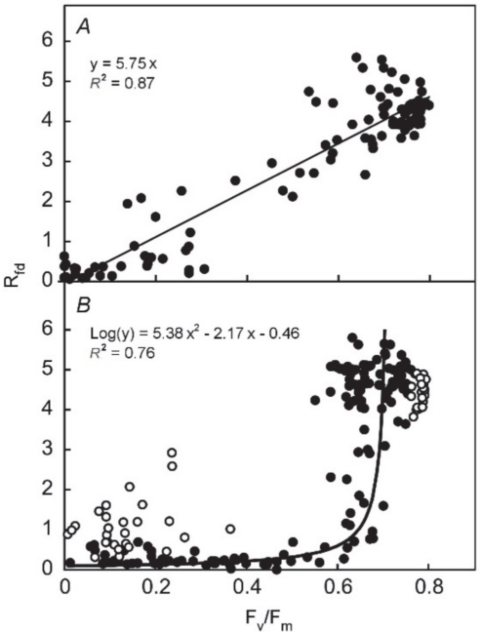

Fig. 2. F_v/F_m and R_{fd} values correlation in *Pterocephalus lasiospermus*. High temperatures (*A*) include data from 20ºC to 50ºC treatments. Low temperatures (*B*) include data from 0ºC to –20ºC treatments (E4 data excluded). Open circles: low temperature data of E4 (24 July). $P<0.05$ for both regression models.

LT_{50} values and acclimation phenomena. The differences detected in intercepts and slopes of the correlation models for both F_v/F_m and R_{fd} mentioned above were reflected in calculation of LT_{50} values.

At high temperatures (Fig. 4*A*), LT_{50} values obtained with F_v/F_m showed an increase of 1.9 ± 1.6ºC in E4 compared to E1 and 1.55 ± 1.05ºC compared to E2. On the contrary, LT_{50} values obtained with R_{fd} did not show significant variation between E2 and E4. It is clear that from the winter to the summer (from E2 to E4), the F_v/F_m parameter registered an apparent acclimation to high temperatures that R_{fd} did not show.

At low temperatures (Fig. 4*B*), LT_{50} values obtained with F_v/F_m showed a decrease of 3.5 ± 1.5ºC between E2 to E3, which involved an acclimation phenomenon developed within 11 d. In E4, a loss of acclimation was observed in a range of 7 ± 1ºC from E3, reaching LT_{50} values of 4.25 ± 1.25ºC higher than that in E1. LT_{50} values obtained with R_{fd} showed similar pattern but not so accentuated. In this case, the acclimation observed was 1.38 ± 1.12ºC in E3 with respect to E2, while LT_{50} of E3 and E4 did not differ, not showing the deacclimation observed with the F_v/F_m values.

Regarding the differences between LT_{50} values obtained with both parameters, variable tendencies were observed depending on the temperature treatments. So, at high temperatures, the divergences of both indicators were only significant in E4 with differences of 1.8 ± 0.9ºC. At low temperatures, LT_{50} values from R_{fd} were significantly higher than those of F_v/F_m, except in July, when both indicators became equal. The highest difference between the LT_{50} values of both parameters was found in E3 with values close to 6ºC.

Discussion

Damage assessment by F_v/F_m and R_{fd} showed different responses at the high and low temperatures. At the high temperature damage, the linear regression between both parameters could be explained by the equal sensitivity of both PSII and thylakoid lamellae showing damage. Consistently with our data, the temperature threshold of PSII has been reported to be one degree under the net photosynthesis limit at high temperatures (Enami *et al.* 1994, Larcher 2003a). Other studies using the determination of the critical temperature (T_c) by Chl fluorescence reported that T_c of PSII and LT_{50} assessed by visual damage had a good linear correlation (Bilger *et al.* 1984), suggesting once again that PSII photochemistry may be the most heat-sensitive process of photosynthesis. On the other hand, photochemical reactions in thylakoid membranes and carbon metabolism in the stroma of chloroplast have been also suggested as the primary sites of injury at high temperatures (Wahid *et al.* 2007).

On the contrary, it is recognized that thylakoid membranes are the primary site of freezing damage in plant cells, thus affecting photosynthesis and CO_2 fixation (Hincha *et al.* 1996, Devacht *et al.* 2011). Low temperatures always tend to inhibit electron transport *via* increased membrane viscosity through alterations in the biophysical properties of thylakoid lipids. This process is more strongly affected than the processes involved in the energy transfer and its photochemical transformation (Huner *et al.* 1998, Hendrickson *et al.* 2006). This is the reason why R_{fd} was more sensitive for estimating the photosynthesis damage and was able to register damage faster than F_v/F_m at low temperatures. The cause of the loss of the asymptotic relation between both parameters at low temperature in the summer remains unknown. We speculate that the differences of high radiation between the summer and winter could be involved. It has been reported that the inhibitory effects of low temperature treatment can be greater after a period of illumination (Sassenrath *et al.* 1990, Flexas *et al.* 1999, Allen *et al.* 2000). According to our meteorological data, irradiation increased by 66% between the summer and winter. Although chronic photoinhibition occurred in both nontreated controls, F_v/F_m and R_{fd} did not show any seasonal variation (data not shown). PSII could be more prone to inhibition and it could equate the limiting character of PSII and electron transport at low temperature treatment in the summer. Anyway, R_{fd} kept on being the more sensitive parameter in all experiments,

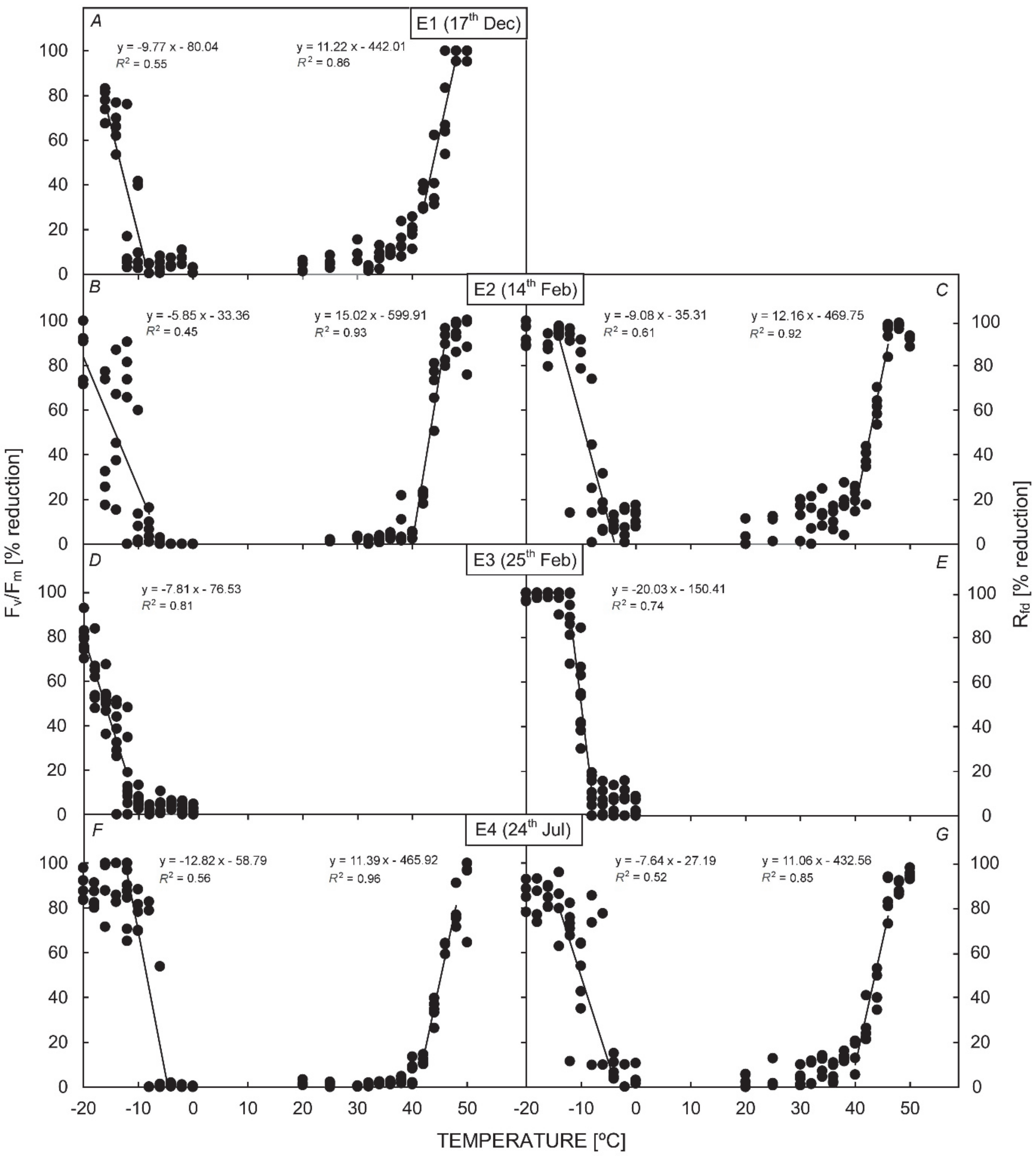

Fig. 3. Percentage of damage at high and low temperature treatments in *Pterocephalus lasiospermus* using F_v/F_m and R_{fd} percentage of control reduction as indicators of damage in four different moments during the studied period. Data are the mean values ± standard error (*n* = 5–9).

since the thermic resistance estimated with R_{fd} parameter was never higher than those estimated with F_v/F_m.

Visual damage evaluation has been considered as the method to obtain the reference LT_{50} values (Buchner *et al.* 2013). In this case, an underestimation of heat tolerance assessed by F_v/F_m compared to visual damage method was reported. Unfortunately, this could not be assessed in our species because of an unclear colour difference between necrotic and healthy tissues mainly due to a high density of hairs. At low temperatures, Neuner and Buchner (1999) found that indices of damage derived from F_v/F_m overestimated winter-frost resistance compared to estimation of LT_{50} by digital image analysis, indicating a higher sensitivity of visual damage method.

In addition to R_{fd} values, a complete analysis of the slow phase of Chl fluorescence transients (OPSMT) can be potentially used as indicators of freezing stress in photosynthetic apparatus (Mishra *et al.* 2015). Apart from the

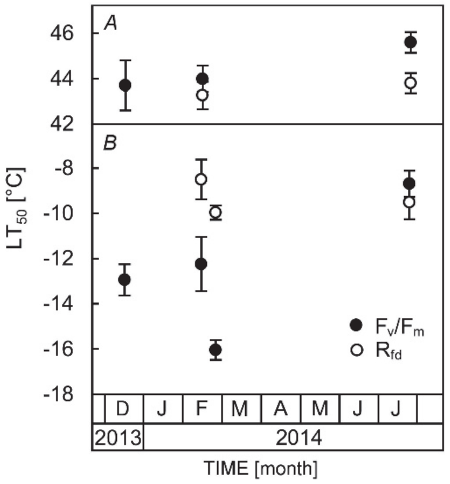

Fig. 4. Changes in LT_{50} values obtained from F_v/F_m (close circles) and R_{fd} (open circles) through the year at high (*A*) and low (*B*) temperatures. Estimated mean values ± confidence interval obtained by the regression model. $P<0.05$.

thermic stress, it has been previously reported that R_{fd} is more sensitive to a variety of stress factors compared to F_v/F_m. Regarding water stress, Schreiber and Bilger (1985) showed that even at a very low relative water content of 36%, where electron transport is severely inhibited, variable fluorescence is almost unchanged, suggesting that the PSII donor side and the primary reaction were not affected. Horgan and Zabkiewicz (2008) proved that R_{fd} was more sensitive and a better indicator than F_v/F_m for herbicide tolerance assessment.

Such a different sensitivity of both parameters is also important in order to describe a real acclimation phenomenon. In this study, a maximum difference of 6ºC between the LT_{50} values calculated with both parameters was reported in the winter. Furthermore, LT_{50} values obtained with F_v/F_m parameter had a higher variation within the studied period than ones obtained with R_{fd}. In the winter, a freezing acclimation was observed with both parameters, but it was more apparent with F_v/F_m than with R_{fd}. In the summer, the F_v/F_m parameter registered an apparent acclimation to high temperatures that R_{fd} did not show. Moreover, we demonstrated with F_v/F_m that the freezing acclimation obtained in E3 was lost in the summer, while it was kept with R_{fd}. It could mean that – at least in *P. lasiospermus* – processes affecting the efficiency of PSII and reduction of first acceptors, Q_A, give a more flexible response to seasonal variations and freezing events, although they are not the main limiting factor of photosynthesis at extreme temperatures. Although the R_{fd} parameter was more sensitive and a better indicator of thermic damage and acclimation phenomena, it does not have a uniquely healthy range defined in contrast to the F_v/F_m parameter, whose interval has been determined within 0.74–0.85 (Lichtenthaler *et al.* 2005a). Even, depending on light exposition, healthy R_{fd} values can strongly vary in the same species, with an interval of 1.3–2.7 for shade leaves, or 3.0–5.5 in sun-exposed leaves (Lichtenthaler *et al.* 2005b). In our case, *P. lasiospermus* is an alpine plant daily exposed to high irradiation, so it is not a surprise that R_{fd} values achieved 5.5 in the controls and a R_{fd} value of 2.75 was considered a 50% of damage. Thus, every time when R_{fd}-LT_{50} values are requested for unknown species and light conditions, a control R_{fd} value must be well defined.

According to all the above mentioned, the thermic limits and acclimation phenomena of *P. lasiospermus* were evaluated by the most sensitive parameter, R_{fd}. Depending on the species, it may take a few days to several weeks to reach maximum freezing tolerance (Xin and Browse 2000). In *P. lasiospermus*, acclimation took place after an exposition to strong freezing events in a short period of time between E2 and E3 (11 d), what is expected for a plant subjected to low temperatures every night due to strong diurnal and seasonal oscillation (Del-Arco *et al.* 2006). Although many researchers showed that heat acclimation might be acquired by a nonlethal heat treatment (Dat *et al.* 1998, Larkindale and Huang 2004, Xu *et al.* 2006, Wahid *et al.* 2007), no heat acclimation was registered in this species in the summer according to LT_{50} calculated with R_{fd}.

Furthermore, even under acclimation, this species is quite sensitive to freezing temperatures compared to other high-mountain species (Larcher 2003b, Taschler and Neuner 2004), since, according to R_{fd} values, *P. lasiospermus* suffered an irreversible damage at –9.9 ± 0.3ºC. This threshold is quite close to the absolute minimum air temperatures registered in Teide National Park (–9.8ºC). As minimal leaf temperatures have been reported to be even lower than air temperature in alpine plants (Braun *et al.* 2002), freezing events could be an important risk factor for this species.

Regarding high temperatures, the threshold at 43.5ºC must be carefully considered since strong diurnal variations of LT_{50} have been reported and underestimation of LT_{50} could be determined in *ex situ* experiments (Buchner *et al.* 2013, Buchner and Neuner 2003). On the other hand, although air temperature registered in Teide National Park reached up to 30.4°C, leaf temperature is probably significantly higher during day time, depending on the exposure to irradiation, wind, and the morphological growth habit of the species (Braun *et al.* 2002). So, the recording of leaf temperatures under high air temperatures should be reported in future studies.

Seventy years ago, *P. lasiospermus* was described as an uncommon endemic species (Sventenius 1946), nevertheless, nowadays is one of the main species of Teide National Park landscape. Even though some authors have pointed out a selective herbivory as one possible explanation of the expansion of *P. lasiospermus* (Martín and Wildpret 1999), the current spread of this species seems to be also in coherence with our data, since the mean temperatures of Teide National Park have increased by 1.3ºC during the last 90 years and the freezing events have been less frequent (Martín *et al.* 2012). However, we cannot confirm with certainty that climate warming is the primary driver of current population changes in *P. lasiospermus*, since photosynthesis is not the only physiological process limited by low temperatures. Additional studies are demanded in order to make reliable predictions about how the increasing temperatures affects the distribution of this species, as well as making the best biodiversity conservation management performance in this unique ecosystem.

Conclusion: In this study, R_{fd} was demonstrated as a more sensitive parameter for thermic damage assessment. The evaluation of F_v/F_m as the unique indicator of thermic damage could lead to an erroneous estimation of both temperature tolerance and acclimation at low and high temperatures. Thus, we proposed the use of R_{fd} instead of F_v/F_m as the indicator of thermic damage, after determining the healthy R_{fd} values for each species and light conditions.

According to R_{fd} as the indicator of thermic damage, low temperature acclimation was not enough to stop considering *P. lasiospermus* as a sensitive species compared to other alpine plants, since –9.9 ± 0.3ºC was the lowest LT_{50} value achieved. On the contrary, heat acclimation was not observed, with LT_{50} values around 43.5ºC during the studied period. Thus, according to R_{fd} evaluation of thermic threshold, this species could be in risk of damage for low temperatures in this ecosystem.

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