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Photosynthetic properties of *Quercus* × *hispanica* Lam. and *Q. suber* L. under harsh Central European winter conditions

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Abstract

In search for new forestation tree species for future Central European climate conditions, Mediterranean evergreen oak taxa are investigated for their summer drought- and winter frost-hardiness. Here we report on the winter performance of the photosynthetic apparatus of *Quercus* × *hispanica* Lam. and its evergreen parental species *Q. suber* L. under extraordinary harsh winter conditions. Both taxa showed a strong decline of photosystem II (PSII) quantum efficiency (F_v/F_m) with a concomitant increase in the deepoxidation state (DES) of the xanthophyll pigments depending on (severe) frost events during winter, and these parameters significantly correlated with minimum air temperatures during periods of chronic photoinhibition at mid-winter, but not at the onset of winter in response to the first frost nights. F_v/F_m and DES correlated with each other in both taxa throughout the winter.

Additional key words: evergreen; frost stress; oak; photoinhibition; xanthophyll cycle.

Introduction

Global climate change is expected to lead to increased average summer temperatures and an increase of the frequency of summer drought and heat periods in Central Europe (Schär *et al.* 2004). Dryer Central European forest habitats are frequently colonized by *Quercus robur* L. and *Q. petraea* L. On such dry stands, these tree species may experience increasingly less favourable growth conditions in the future. We therefore analyze various drought-tolerant *Quercus* species from Southern Europe for their potential as putative future forestation trees under warmer and dryer climate conditions in Central Europe. This program includes several evergreen or semideciduous taxa with scleromorphic leaves, like *Q. ilex* L. or *Q. suber* L. and their hybrids to deciduous oak species. In particular, the hybrid of *Q. suber* with *Q. cerris* L. (*Q.* × *hispanica* Lam.) is of interest, since it occurs naturally as hybrid populations named *Q. crenata* Lam. as a synonym in the Central Mediterranean basin (Bellarosa *et al.* 1996).

In addition to summer drought tolerance, forest trees in Central Europe will also in the future be subjected to (less frequent) winter conditions with subzero periods. Therefore, winter hardiness of the taxon in question is a crucial prerequisite for future forestation attempts. Frost hardiness of *Q. ilex* and *Q. suber* tissues has been reported to reach significantly below –20°C (Larcher 2000); however, severe frost-induced xylem embolism may occur at much milder frost degrees and may significantly affect the plants during springtime until either refilling of the vessels or new vessel formation is possible (Nardini *et al.* 2000, Cavender-Baras *et al.* 2005), with *Q. suber* being regarded as slightly less frost-tolerant than *Q. ilex*. Under severe winter conditions, the evergreen Mediterranean species *Q. ilex* and *Q. suber* showed transient strong decreases of the efficiency of PSII, as evaluated by the gross annual courses of the chlorophyll (Chl) fluorescence parameter F_v/F_m (Aranda *et al.* 2005, Brüggemann *et al.* 2009). At subzero temperatures, winter-acclimated *Q. suber* and *Q.* × *hispanica* showed increases of the deepoxidation state (DES) of the

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Abbreviations: A – antheraxanthin; CFLSI5 – cumulative frost- and light stress index of 5 preceding days; DES – deepoxidation state; F_0 – minimal fluorescence of the dark-adapted state; F_m – maximal fluorescence of the dark-adapted state; F_v – variable fluorescence; PSII – photosytem II; q_E – energy-dependent chlorophyll fluorescence quenching; T_{min} – minimum temperature; V – violaxanthin; Z – zeaxanthin.

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xanthophyll pigments, *i.e.* a conversion of viola- (V) to anthera- (A) and zeaxanthin (Z) (Brüggemann *et al.* 2009). Similar punctual findings have been reported for *Q. ilex* (*e.g.* Martinez-Ferri *et al.* 2004). Anthera- and zeaxanthin presumably play several direct and indirect roles in protective heat dissipation from PSII, *i.e.* they are involved in heat dissipation by the transthylakoid pH-gradient-dependent fluorescence quenching (energy-dependent quenching, q_E) mechanism and in chronic photoinhibition (Adams *et al.* 2004). Recently, Weng *et al.* (2009) published several year cycles of predawn F_v/F_m in *Pinus taiwanensis* and showed that with winter temperatures decreasing down to minima of –2°C, a moderate F_v/F_m decline (by 0.2 units) could be observed. Similar results were observed for the Asian oak species *Q. myrsinaefolia* (Yamazaki *et al.* 2011). For the evergreen shrub *Rhododendron ferrugineum*, a correlation of the extent of (chronic) winter photoinhibition under subzero conditions with the average minimum temperature of the preceding (five) days had been reported (Neuner *et al.* 1999).

In the present contribution, we analyze the response of the photosynthetic apparatus of *Q. suber* and *Q.* × *hispanica* var. Lucombeana throughout a complete winter cycle to analyze, whether the so far only punctually observed simultaneous occurrences of high DES and low F_v/F_m values in field studies correlated with the prevailing night temperatures.

Materials and methods

Established specimens, one individual per species, of *Q. suber* (*ca.* 30 years old), *Q. cerris* (*ca.* 40 years old) and *Q.* × *hispanica* var. Lucombeana (*ca.* 70 years old) are growing in the Palmengarten of the city of Frankfurt (50°07,4`N, 08°39,4`E) as solitary trees. *Q.* × *hispanica* has been produced in an Essex tree nursery in the 18th century by William Lucombe by crossing *Q. cerris* L. and *Q. suber* L.

Temperature data were collected at the Botanical Garden in Frankfurt, 200–300 m N of the analysed trees. For all analysis, one-year-old, south-exposed sun leaves growing at 1.5–2 m height were used, which received several hours of direct sunlight every day around midday and in the afternoon. All Chl fluorescence measurements were performed between 10 and 11 h (winter time) after 30 min of predarkening with a *Mini-PAM* (*Walz*, Effeltrich, Germany) only on undamaged leaves (n = 10, 3-μs pulses at 600 or 20,000 Hz with 0.4 and 7 μmol (quantum) m^{-2} s^{-1} measuring light intensities before and during the 1-s saturating blue flash of 2,000 μmol (quantum) m^{-2} s^{-1}, respectively. Morning irradiation on the measured leaves was mostly indirect sunlight due to the shading of the lower tree parts by other plants and structures. To account for a possible memory effect of combined (noon and afternoon) irradiation and frost stress during the days preceding to the measurement, and in view of a lack of ability to measure the actual radiation at the site of the analysed leaves, a parameter "cumulative frost-light stress index 5" (CFLSI5) was introduced and depicted in Figs. 1 and 2. This parameter was calculated by multiplying the (negative) daily temperature minima ($-T_{min\ day\ n}$) below 0°C with the sunshine hours of the respective day [frost-light stress index, $FLSI_{day\ n}$ = – ($T_{min\ day\ n}$ h sunshine $_{day\ n}$)] and accumulating this value for the preceding 5 days prior to the respective measuring day. Daily sunshine hours were taken from Deutscher Wetterdienst (Offenbach, www.dwd.de) for the weather station Frankfurt-Airport. By definition, $FLSI_{day\ n}$ is zero for all days with $T_{min} \geq 0$°C.

Leaf pigment analyses were performed by HPLC according to Färber *et al.* (1997) and Beyel and Brüggemann (2006) on extracts from 18-mm leaf (n = 5) discs stored in the dark in liquid N_2, excised immediately after the F_v/F_m measurements from the nonilluminated part of the predarkened leaves. The discs were pulverized under liquid N_2 and extracted in 1.2 ml of solvent A (87% acetonitril, 10% methanol, 3% 100 mM TRIS-HCl, pH 8.0) in the presence of some solid $CaCO_3$. The xanthophylls were identified by their retention times and their deepoxidation state (DES) was then calculated using the molar concentrations V, A, and Z according to DES = (A + Z)/(V + A + Z).

Samples with the *Mini-PAM* (n = 10) and for HPLC analysis (n = 5) were taken on 22 and 29 September; 10, 13 and 16 October; 3, 10, 17 and 25 November; 4 and 18 December 2008; and on 10, 20 and 29 January; 12, 23, and 28 February; 16, 20, and 21 March; 7 and 20 April, and 1 May 2009.

Photosynthetic capacity was measured in Clark-type electrodes (*Oxylab 32, Hansatech*, Norfolk, UK) under saturating CO_2 (*i.e.* 2% O_2, 4.5% CO_2 in N_2, Delieu and Walker 1981). Illumination was provided by red photodiodes at 900 μmol(quantum) m^{-2} s^{-1}, temperature was controlled at 20°C. During winter, punctual additional measurements were performed *in situ* under ambient CO_2 conditions with a *GFS 3000* gas-exchange analyser (*Walz*, Effeltrich, Germany), set at 1,000 μmol(quantum) m^{-2} s^{-1} actinic light and 10°C to assess, whether photoinhibition, as estimated from F_v/F_m measurements, was reflected in declining photosynthetic rates.

Results

Time course of PSII quantum efficiency and photosynthesis of overwintered leaves: In contrast to the preceding mild winters 2005–2008, the winter 2008/09 was much harsher with temperature minima down

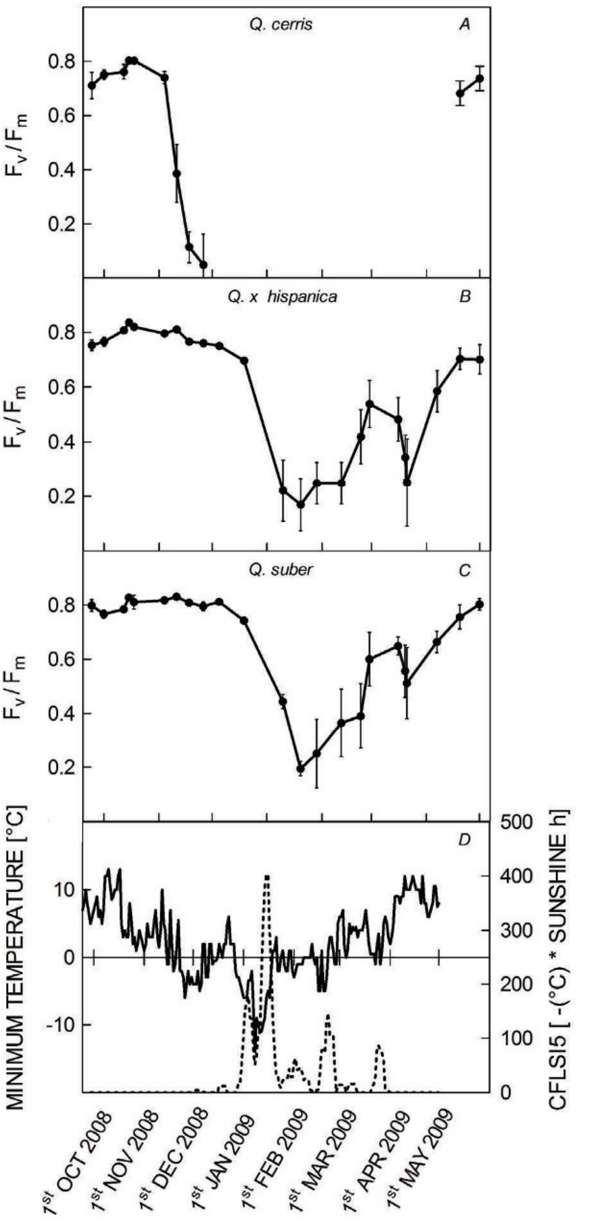

Fig. 1. Maximum quantum yield (F_v/F_m) of *Q. cerris* (*A*), *Q. × hispanica* (*B*) and *Q. suber* (*C*) leaves and morning minimum temperatures (*full line*, left y-axis) and CFLI5 (*dotted line*, right y-axis) (*D*) between 22 September 2008 and 1 May 2009. means ± SD, *n* = 10.

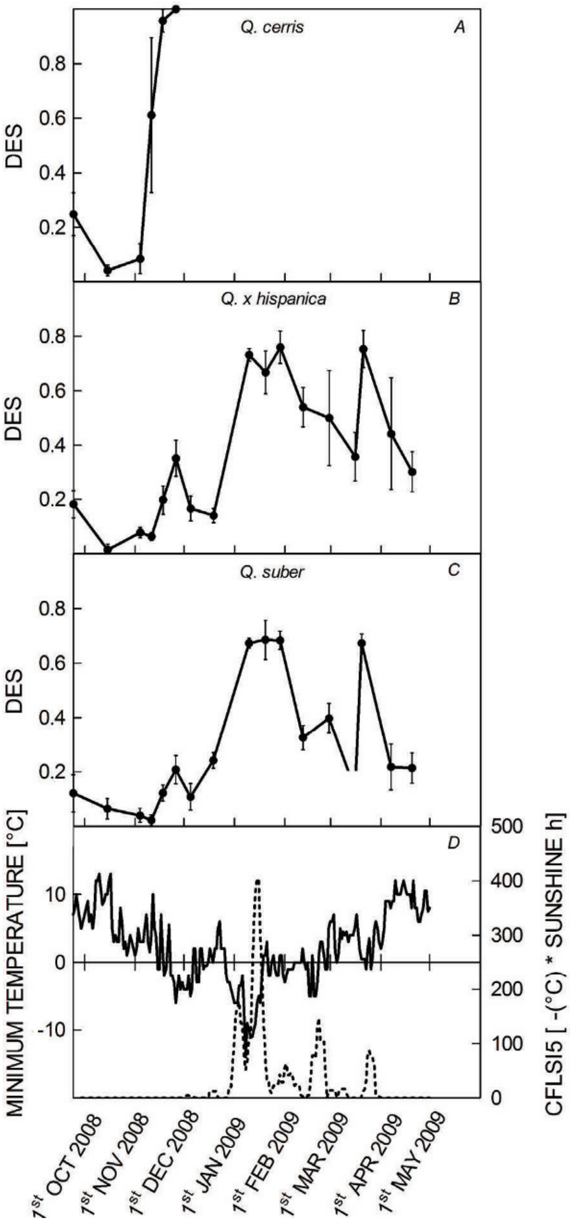

Fig. 2. Time course of the deepoxidation state (DES) of the xanthophyll cycle pigments in *Q. cerris* (*A*), *Q. × hispanica* (*B*) and *Q. suber* (*C*) and morning minimum temperatures (*full line*, left y-axis) and CFLI5 (*dotted* line, right y-axis) (*D*) between 22 September 2008 and 1 May 2009. means ± SD, *n* = 5.

to –16°C (Fig. 1). This led to partial leaf shedding in *Q. × hispanica* (estimated 75% over the whole winter) and visible leaf damage in an estimated 60% of the *Q. suber* leaves. However, this did not damage the trees persistently, since spring flush was normal in both taxa.

Fig. 1 depicts the courses of late morning F_v/F_m of undamaged leaves throughout the winter half year including the morning minimum temperatures. *Q. cerris* lost its leaves by mid November; leaf senescence was accompanied by a sharp loss of PSII efficiency. By April 10th of 2009, new leaves had emerged, which revealed normal F_v/F_m values immediately. While the first frost period around Dec 1st, which was not accompanied by sunshine hours (*i.e.* CFLSI5 remained at zero), had no significant effect on F_v/F_m in the evergreen taxa, the severe frost period at the beginning of January resulted in strong photoinhibition and depressed *in situ* photosynthesis under ambient CO_2 from 5 µmol m^{-2} s^{-1} (both taxa, *n* = 5) in September to 1.3 (*Q. suber*) and 0.2 µmol m^{-2} s^{-1} (*Q. × hispanica*) under 1,000 µmol(quantum) m^{-2} s^{-1} and at 10°C in January (mean values, *n* = 2) as measured with the *GFS 3000*. The slight intermediate frost period at the end of March, again accompanied by sunshine hours, led to an intermediate depression of the F_v/F_m values again, before they returned to normal values of unstressed leaves at the end of April. In *Q. suber*, dynamic photoinhibition of the part of the tree, where samples were collected, was negligible between sunrise and the measuring time, but did occur in the afternoon on bright, cold days, as visible in reversible F_v/F_m declines by 0.2 units or more. In *Q.* × *hispanica*, it accounted for less than 0.15 units of F_v/F_m on bright frosty mornings in late March with the sun standing much higher than in December/January, but was significant around noon and in the afternoon (*i.e.* 0.3 units). In view of the much stronger F_v/F_m changes observed in the course of the winter, we therefore conclude that the contribution of pre-measurement dynamic photoinhibition to the morning F_v/F_m values reported here was negligible.

After recovery, the overwintered leaves revealed photosynthetic capacities of 7.4 ± 1.2 (*Q.* × *hispanica*) and 5.6 ± 1.0 (*Q. suber*) μmol(O_2) m^{-2} s^{-1} under optimum conditions [saturating CO_2, 900 μmol(quantum) m^{-2} s^{-1}, 20°C] measured in Clarck-type electrodes in May 2009, respectively. For comparison, newly emerged leaves of *Q. cerris*, *Q.* × *hispanica* and *Q. suber* showed photosynthetic capacities of 20.1 ± 2.1, 15.8 ± 2.3 and 20.0 ± 1.6 μmol(O_2) m^{-2} s^{-1} in May 2009 (means ± SD, *n* = 3).

Violaxanthin deepoxidation: In Fig. 2, the time courses of late morning DES over the winter half year are depicted. Most obviously, the curves are inverted to the F_v/F_m curves – whenever F_v/F_m drops, regardless of the cause, DES increases. The only exception from this rule was during the first frost episode around Dec 1st, when F_v/F_m remained unaffected, but DES responded with an intermittent increase. In *Q. cerris*, leaf senescence was accompanied by a sharp DES increase in autumn.

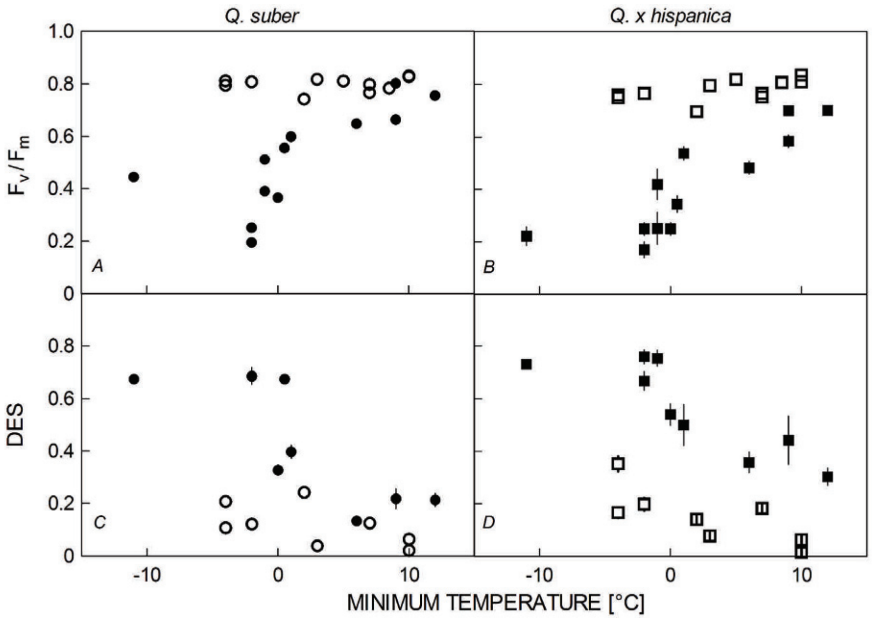

Fig. 3. Correlation analyses of F_v/F_m (*A,B*) and DES (*C,D*) *vs*. morning minimum temperatures in *Q. suber* (*A,C*) and *Q.* × *hispanica* (*B,D*). Data from Figs. 1 and 2 were used. (○, □): values before 1 January 2009; (●, ■): values after 1 January 2009. Spearman correlation coefficients were *A* (●): 0.875***; *B* (■): 0.907***; *C* (●): –0.815**; *D* (■): –0.879**. Levels of significance were: **: $p<0.01$; ***: $p<0.001$.

Correlation between leaf properties and abiotic stress: We analysed various abiotic stress parameters for possible statistically significant correlations with the measured leaf properties (*i.e.* F_v/F_m and DES). While the newly introduced cumulative frost-light stress indices for 3,5, or 7 preceding days showed no statistically significant correlation with F_v/F_m or DES (data not shown), night minimum temperature did, provided the data before the first sharp period were excluded from the analysis (Fig. 3*A-D*). In addition, a significant correlation was observed between DES and F_v/F_m values over the whole winter period in both taxa (Fig. 4*A,B*).

Discussion

During winter, evergreen tree species may react differently to low temperatures, depending on their genetic and geographical background. While, on the one hand, tree species from subtropical origin show moderate F_v/F_m declines (by 0.2 units) upon exposure to light frosts and recover from this during warming periods (Weng *et al*. 2009, Yamazaki *et al*. 2011), species from arctic-alpine environments reveal the phenomenon of chronic

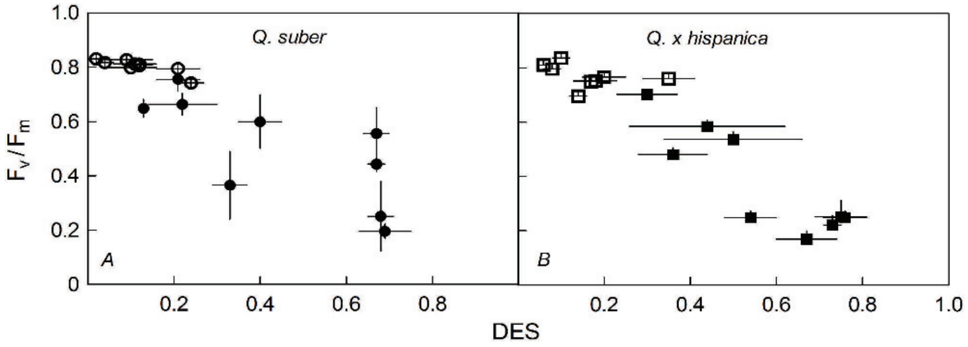

Fig. 4. Correlation analysis of F_v/F_m *vs*. DES in *Q. suber* (*A*) and *Q.* × *hispanica* (*B*). Data from Figs. 1 and 2 were used. (○, □): values before 1 January 2009; (●, ■): values after 1 January 2009. Spearman correlation coefficients were *A* (●,○): –0.947***; *B* (■,□): –0.885*** ($p<0.001$).

photoinhibition, *i.e.* a long-lasting decline of F_v/F_m throughout the winter (Ottander *et al.* 1995, Wieser 1997, Adams *et al.* 2004, Ensminger *et al.* 2004). In Mediterranean evergreen shrub and tree species, various degrees of intermediate behaviour have been reported, *i.e.* during mild winters, most species keep their photosynthetic apparatus functional, but under severe frosts, chronic photoinhibition may occur (*e.g.* Aranda *et al.* 2005, Garcia-Plazaola *et al.* 1997, Martínez-Ferri *et al.* 2004). However, short-term behaviour of the photosynthetic apparatus upon low temperature stress in these species so far has not been in the focus of research. In the present report, we were allowed by the specific weather conditions in winter 2008/09 combined with the (unusual) location of Mediterranean tree species under Central European climate, to study the plasticity of the photosynthetic apparatus of such species in detail.

The first frost period at the beginning of December 2008 had no significant effect on F_v/F_m in both *Q.* × *hispanica* and *Q. suber* (Fig. 1*B,C*). This behavior can be regarded as typical for Mediterranean evergreens, since they obviously are less sensitive to light frost than subtropical species (compare Weng *et al.* 2009, Yamazaki *et al.* 2011), and can make use of any favourable conditions for photosynthesis during winter, as long as they are not chronically photoinhibited (Adams *et al.* 2004). In both taxa, a strong decline of F_v/F_m reflecting photoinhibition was observed at the beginning of January, concomitant with a sharp frost period with minimum temperatures of –16°C, which lasted with moderate frost until February. From Jan 1st on, F_v/F_m values correlated significantly with the preceding minimum night temperatures (Fig. 3*A,C*). During March, F_v/F_m increased again, only to decline again with a second minor frost period at the end of March, after which a complete recovery of F_v/F_m occurred again. Thus, the initial frost period in December did not have the same effect as the much less severe one in March. One possible reason for this differential behavior can be seen, when the combined effect of frost and irradiation stress is taken into account (*i.e.* the CFLSI5 values depicted in Figs. 1*D* and 2*D*). Previously, Neuner *et al.* (1999) reported on a correlation between the minimum average temperature of the preceding five days and (chronic) photoinhibition in *R. ferrugineum* during winter at timberline. Since the reaction of our species towards frost was not solely temperature-, but apparently also light-dependent (*cf.* the lack of reaction towards the first frost period in November/December), we here introduced a combined frost-light stress index, taking both stressful abiotic factors into account. Although this index is only an arbitrary measure derived from meteorological data, due to lack of the possibility to measure the true daily radiation at the site of the analysed leaves, it explains well why the frost in December did not lead to photoinhibition (*i.e.* CFLSI5 was zero, because the weather was cloudy), while the frost period in March did (due to the combination of mild frost with clear weather conditions, CFLSI5 was positive). Apparently, the CFLSIs may give an indication, if the weather conditions are suitable for the occurrence of chronic photoinhibition, but not for explaining quantitative correlations with the F_v/F_m or DES values. For this purpose it would be necessary to assess the true excitation pressure during frost periods at the site of the leaf measured (*cf.* review by Huner *et al.* 1998). This could possibly be done by continuously measuring time-courses of q_P. A similar approach to use a combination of minimum temperature and irradiation intensity to assess their effects on photoinhibition and on DES was used by Ensminger *et al.* (2004), who studied the spring recovery of photosynthesis in boreal Scots pine and found a significant correlation of midday DES with minimum day temperature and (midday) maximum irradiation intensities during the recovery period. However, since the authors performed all their measurements at noon, they included the effects of chronic and dynamic photoinhibition as well as the light-induced xanthophyll deepoxidation necessary for the energy-dependent Chl fluorescence quenching mechanism (q_E). Alternatively to the proposed dependence of chronic photoinhibition on CFLSI, it can be speculated that in March the leaves already started with a dehardening process as a response to increasing daylength and therefore were much more susceptible to photoinhibition than in December, when full winter hardiness had been achieved.

In their natural environments, *Q. suber* and *Q. × hispanica* (= *crenata*) are usually only exposed to mild frost events during winter. In our studies, we could show that *Q. × hispanica* revealed a nearly identical behaviour of the quantum efficiency of PSII during the harsher Central European winter as its parental species, *Q. suber*. In the latter species, genetically fixed differential frost susceptibility has been described. In a common garden experiment, populations from more southern and lower altitudes revealing lower F_v/F_m values (0.1) during frost periods of –5 to –10°C than those from higher altitudes and latitudes (*i.e.* F_v/F_m = 0.25; Aranda *et al.* 2005). Compared to the cited study, our *Q. suber* specimen revealed similar frost susceptibility as the more tolerant ones from their comparative genotype study. The frost hardiness of *Q. suber* and its ability to recover is inherited to the hybrid, with both F_v/F_m of the undamaged leaves approaching pre-winter values after March again and with photosynthetic capacities returning to acceptable rates.

In *Q. × hispanica* and *Q. suber*, the frost-induced drop in F_v/F_m was reflected in increasing DES values, both in January and in March (Fig. 2*B*,*C*). In both evergreen taxa, F_v/F_m and DES data after the start of the severe frost period at the end of December correlated significantly over the measuring period with minimum temperature (Fig. 3*B,D*). DES and F_v/F_m correlated significantly over the whole winter (Fig. 4*A,B*). Contrastingly, Martinez-Ferri *et al.* (2004) reported that they found no significant differences between the (high) predawn DES values of *Q. coccifera* between December and February (after a frost period), while in *Q. ilex* the observed changes in DES were apparently inconclusive with respect to temperature in the two years of observation. However, in their study no direct measurements of the preceding night temperatures for the respective sampling times were given.

Interestingly, in contrast to F_v/F_m, the DES responded to both the moderate frosts at the beginning and at the end of the winter with intermittent increases. Either the increase of morning DES at the end of November was insufficient to result in a measurable downregulation of PSII, which could be supported by the F_v/F_m *vs*. DES curve (Fig. 3*E*,*F*) or the two processes are not coupled at this stage of winter acclimation. It has been shown earlier that in winter-acclimated evergreen *Quercus* and other sclerophyllous species F_v/F_m and DES often are (Verhoeven *et al.* 1999, Brüggemann *et al.* 2009), but need not always be (Fig. 3 in Martinez-Ferri *et al.* 2004) coupled to each other. The latter authors concluded from their data from the winters 1997/98 and 1998/99 that in colder Mediterranean winters strong decreases in late winter morning F_v/F_m values were not correlated with DES, and that the effect of chronic inhibition (as F_v/F_m decline) is not only attributable to the persistence of slowly reversible photoprotective mechanisms (*i.e.* mediated by high DES), but also due to cumulative damage. Contrastingly, our data measured during the intermittent warming and cooling periods in late winter suggest that the DES-mediated energy dissipation probably plays a central role in chronic photoinhibition of fully winter-acclimated leaves. Recently, Peguero-Pina *et al.* (2009) showed that only *Q. ilex* and not *Q. suber* uses high predawn DES as a photoprotective mechanism under summer drought conditions. Our data of short-term frost effects (Brüggemann *et al.* 2009) and the present field study indicate that *Q. suber*, however, may well use high morning DES as a photoprotective mechanism under winter frost stress conditions, and it inherits this trait to the hybrid, *Q. × hispanica*. This interpretation might imply that the two taxa are able to increase their morning DES by the signal night frost, independent of daylight intensities, which independently could modify (increase) DES, provided by additional strong irradiation at daytime (as was the case in December/January and March).

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