DOI: 10.32615/ps.2019.161 PHOTOSYNTHETICA 58 (SI): 331-340, 2020

Special issue in honour of Prof. Reto J. Strasser

Seasonal variation and trade-off between frost resistance and photosynthetic performance in woody species

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Abstract

We tested the seasonal variability of frost resistance and photosynthesis characteristics, namely net photosynthetic rate at saturating irradiance and ambient atmospheric CO_2 concentrations, maximum carboxylation rate, and measurements based on the JIP-test, namely maximum quantum yield of PSII and the performance index. Additionally, we measured leaf functional traits, namely specific leaf area, leaf dry matter content, stomatal pore area index, and chlorophyll content. We assessed all traits weekly on two evergreen species *Berberis vulgaris* and *Quercus* × *hispanica* as well as the two summer green species *Betula pendula* and *Quercus pubescens*. We found that there was a high seasonal variability in all traits except stomatal pore area index. In a multivariate analysis, we could show trade-offs between growth rates and leaf thickness on the first axis, as well as resistance and performance on the second axis. The findings help understand ecophysiological processes and fine-tune biosphere models.

Additional key words: *atLeaf*; Botanical Garden; chlorophyll fluorescence; electrolyte leakage; gas exchange; *Pocket PEA*.

Introduction

Frost resistance (FR) and photosynthetic rates (P_N) are the most fundamental processes in plants, which enable them to exist and persist outside the tropics and drive species performance in a given habitat (Sakai and Larcher 1987, Larcher 2000, Körner 2003, Taschler and Neuner 2004, Bucher *et al.* 2018b). Both are adaptive traits which can strongly differ between species as well as between seasons, and are mostly influenced by photoperiod and temperature (Ulmer 1937, Schwarz 1970, Koski 1985, Sakai and Larcher 1987, Bucher *et al.* 2018b). An investment in FR as well as in P_N is energetically costly for plants, and we therefore expect a trade-off between frost resistance, as represented by FR, and performance, represented by P_N. There are two fundamental life history strategies for trees in temperate regions, namely being evergreen or being summer green and shedding the leaves in autumn. This has major implication for longevity of leaves, resource distribution, the necessity of investing in FR, and the overall canopy duration during which photosynthesis takes place (Kadereit *et al.* 2014). We therefore expect this to differ between life forms.

There are several methods for capturing frost resistance and photosynthetic performance. FR can be assessed easily *via* electrolyte leakage (Gurvich *et al.* 2002). Photosynthetic performance, however, can be captured *via* various techniques, such as measuring gas exchange (Farquhar *et al.* 1980, von Caemmerer and Farquhar 1981, Grassi *et al.* 2005, De Kauwe *et al.* 2016, Bucher *et al.* 2018a) or assessing chlorophyll *a* (Chl *a*) fluorescence *via* the JIP-test (Schreiber *et al.* 1995, Strasser and Srivastava 1995, Strasser *et al.* 2000, Bucher *et al.* 2018a). Photosynthetic rates, more specific the CO_2 uptake at saturating light conditions and current atmospheric CO_2 concentration as well as optimal humidity, P_{Nsat}, can be assessed quickly. They can be used to calculate other ecophysiological parameters such as the maximum carboxylation rate, V_{cmax} (Farquhar *et al.* 1980, Wilson *et al.* 2000, Grassi *et al.* 2005, De Kauwe *et al.* 2016, Bucher *et al.* 2018a). Two

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Received 28 August 2019, *accepted* 19 November 2019.

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Abbreviations: Chl – chlorophyll content; F_v/F_m – maximum quantum yield of PSII; PI_{abs} – absorption based performance index; LDMC – leaf dry matter content; PEL_{eff} – effective percentage of electrolyte leakage; P_{Nsat} – net photosynthetic rate at saturating irradiance and ambient atmospheric CO_2 concentrations; SLA – specific leaf area; SPI – stomatal pore area index; V_{cmax} – maximum carboxylation rate.

Acknowledgements: The authors would like to thank the staff of the Botanical Garden of the Friedrich Schiller University, Jena. We would also like to thank Christin Weilandt, Josephine Ulrich, and Amy Milligan for support and conducting measurements and Benjamin Curtis for language editing of the manuscript. We also thank the University of Jena for financial support (DRM/2018-05). We would most of all like to thank Prof. Dr. Reto Strasser for developing the technology of chlorophyll fluorescence measurements for the quick and easy analysis of photosynthesis.

important parameters of Chl fluorescence, the maximum quantum yield of PSII, F_v/F_m, and the absorption-based performance index, PI_{abs}, are a quick method to assess the functionality of PSI and PSII. They are quicker and simpler to describe photosynthetic performance than measuring photosynthetic rates (Strasser and Srivastava 1995, Strasser *et al.* 2000, Bucher *et al.* 2018a). Chl fluorescence has been proven to indicate plant stress, such as ozone stress (Clark *et al.* 2000), water stress (Živčák *et al.* 2008), temperature stress, such as frost or heat (Neuner *et al.* 1999, Maxwell and Johnson 2000, Neuner and Pramsohler 2006, Sharma *et al.* 2015, Bucher *et al.* 2018c), as well as the onset of autumn senescence (Holland *et al.* 2014).

Leaf functional traits capture long-term adaptations of plants to their environment rather than short-term physiological responses as captured by FR and P_N. They are used more and more in ecological research as they can provide further insights into the fundamental mechanisms of a species' adaptation to its environment (Violle *et al.* 2007, de Bello *et al.* 2010, Pérez-Harguindeguy *et al.* 2013, Römermann *et al.* 2016). Traits, such as specific leaf area (SLA), leaf dry matter content (LDMC), and stomatal pore area index (SPI), have been shown to be proxies of photosynthetic performance as well as competitive strength (Rosenzweig 1987, de Bello *et al.* 2010, Pérez-Harguindeguy *et al.* 2013, Bucher *et al.* 2016). SLA, for example, is an indicator of growth rate, indicates competitive strength and stress tolerance (Garnier 1992, Reich *et al.* 1997, Poorter *et al.* 2009, Pérez-Harguindeguy *et al.* 2013). LDMC is a proxy of the investment in structural compounds indicating tougher, long-lived leaves and good defence and resistance especially to herbivory (Pérez-Harguindeguy *et al.* 2013). Stomata are the mediators of gas exchange (Chaerle *et al.* 2005, Roelfsema and Hedrich 2005). The stomatal pore area index (SPI) encompasses both stomatal density and size and thus relates to transpiration and photosynthetic rates (Sack *et al.* 2003, Bucher *et al.* 2016). Chlorophyll content (Chl) can be measured *in situ* with spectroscopical techniques and is closely related to photosynthetic rates (Evans 1989, Zhu *et al.* 2012).

Seasonal variation in FR, P_N, and leaf functional traits are of major importance for the economy of the plants, as the balance of the trade-off between the maintenance of FR *vs*. the investment in photosynthesis-related processes is important to minimise energy investments and maximise performance (Polgar and Primack 2011, Römermann *et al.* 2016, Bucher *et al.* 2018b). Plants harden in autumn and gradually deharden in spring, reducing the investment in FR during the summer (Aulitzky 1961, Sakai and Larcher 1987, Körner 2003, Neuner and Pramsohler 2006, Bucher *et al.* 2018b). Photosynthetic rates usually show higher values in summer and lower in winter (Larcher 1994, Öquist and Huner 2003, Adams *et al.* 2004, Neuner and Pramsohler 2006, Holland *et al.* 2014, Bucher *et al.* 2018b). Chloroplasts break down very early during leaf senescence, so metabolically, carbon assimilation is replaced by catabolism of the Chl and other macromolecules (Lim *et al.* 2007). For the summer green western larch (*Larix occidentalis*), Chl content declines by 11% before photosynthetic rates decline, whereas F_v/F_m does not decline until late during senescence (Rosenthal and Camm 1997). For evergreen species, both leaves, which are newly formed and leaves, which overwintered and were formed the previous year, contribute to the overall functionality of the organism. Analysing and quantifying the differences between leaf types is crucial to capture physiological processes on an organism scale and also to help establish sampling procedures and avoid systematic errors when only sampling one leaf type.

To study the trade-off between frost resistance and photosynthetic performance as well as the seasonal variation in these parameters, we monitored FR and P_N (measured *via* gas exchange and Chl *a* fluorescence) as well as leaf functional traits, namely SLA, LDMC, SPI, and Chl, on two evergreen and two summer green species in the Botanical Garden of the Friedrich Schiller University Jena, Germany. To investigate the seasonal variation in these parameters as well as the trade-off between FR and P_N, we measured all parameters on a weekly basis. More specifically, we asked the following research questions: (*1*) Is there a seasonal variation in FR, P_N, and leaf functional traits in summer green and evergreen species? (*2*) Do leaves, which are newly formed, differ from leaves, which overwintered? (*3*) Is there a trade-off between frost resistance and photosynthetic performance and how do leaf functional traits help understand the functional mechanisms behind it?

Understanding these processes and seasonal variations help us predict species behaviour under changing climate conditions and broaden our knowledge of the use of traits to capture trade-offs between resistance and performance.

Materials and methods

Plant material: We selected four woody species, namely two evergreen species *Berberis vulgaris* and *Quercus* × *hispanica* as well as two summer green species *Betula pendula* and *Quercus pubescens*. There was only one individual per species in the Botanical Garden, so we chose to measure multiple leaves per individual and week to capture the variability within the individuals (Fig. 1). To avoid midday depression of photosynthesis, we did not perform measurements (neither gas exchange nor Chl *a* fluorescence measurements) after 12:00 h (Demmig-Adams *et al.* 1989, Lichtenthaler *et al.* 2005). We selected sun leaves for all analyses. Plants in the Botanical Garden did not suffer from nutrient deficiencies as fertilizer was applied. Plants were irrigated during the extremely dry summer of 2018. For each species, we selected and studied ten leaves per week (Fig. 1). On five leaves, we assessed FR, P_{Nsat}, V_{cmax}, Chl *a* fluorescence, SPI, and Chl. On the remaining five leaves, we recorded Chl *a* fluorescence, SLA, and LDMC as well as Chl. The measurements could not be carried out on the same leaves, as some of the measurements were destructive (FR) or left traces on the leaves (SPI). The measurements were carried out from 9 April till 19 June 2018 and again from 3 September till 28 November 2018 as we wanted to capture the phases

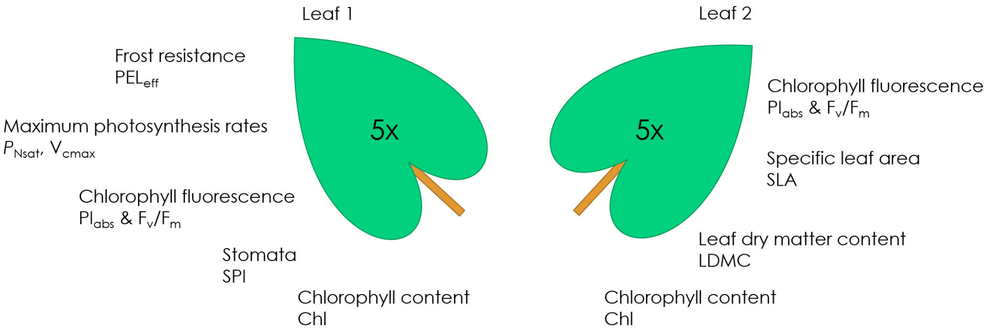

Fig. 1. Study design to extract trait data on a set of two leaves. On leaf one, frost resistance, gas exchange, chlorophyll fluorescence, stomata pore area index, and chlorophyll content were assessed. On leaf two, chlorophyll fluorescence, specific leaf area, dry matter content as well as chlorophyll content were recorded. On each sampling event, there were five replicates of leaf one and leaf two per species, so ten leaves were analysed per species.

with major change, *i.e.*, the dehardening and hardening of the plants after and just before the winter. Evergreen species could be measured more frequently, as summer green species formed their leaves later in the season (Table 1). In sampling weeks 6–9, evergreen species started to form new leaves as well and it was possible to differentiate visually between newly formed leaves and the leaves formed in previous years. After week 9, no difference could be visually detected anymore. We therefore collected data on both new and old leaves during that time to get an estimate about the overall resistance and performance of the plant and to analyse the functional differences between the leaf types.

Electrolyte leakage: Frost resistance was assessed *via* electrolyte leakage as described by Gurvich *et al.* (2002). For each leaf, six discs with a diameter of 0.5 cm were cut out and partitioned into two treatments (with three replicates per treatment): a frost treatment at –18°C and a control at room temperature. Plant tissues were exposed to these temperature treatments for 12 h and then gradually warmed up again to room temperature. Conductivity was measured after warming up with a *LAQUAtwin B-771* (*HORIBA Instruments*, Piscataway, New Jersey, USA) and again after boiling the samples for 15 min, where approximately 100% of the cells in a tissue died and the membranes leaked, which lead to maximum conductivity. The percentage of electrolyte leakage (PEL) was then calculated for each treatment as the quotient between the conductivity before and after the boiling of the samples × 100. The effective PEL (PEL$_{eff}$) was then calculated as the difference between the PEL of the samples stored in the freezer and the PEL of the control treatment. High values of PEL$_{eff}$ thus indicate low FR.

The net photosynthetic rate at saturating irradiance and at ambient atmospheric CO_2 concentrations (P_{Nsat}) was measured using the *Li-6400XT* (*LI-COR Bioscience*, Lincoln, Nebraska, USA). Irradiance was kept constant at 1,500 μmol(photon) m^{-2} s^{-1} and CO_2 concentration at 400 ppm. Block temperature was set to 20°C to make the measurements comparable throughout the season. We used the 'one-point method' to calculate V_{cmax} from P_{Nsat} measurements (De Kauwe *et al.* 2016, Bucher *et al.* 2018a).

Chl *a* fluorescence was determined using a Plant Efficiency Analyser, a portable continuous excitation time resolved Chl fluorimeter (*PocketPEA*, *Hansatech Instruments*, King's Lynn, UK). Leaves were dark-adapted for 30 min using black leaf clips prior to measurements. The maximum PSII quantum yield (F$_v$/F$_m$) and the Chl *a* fluorescence performance index (PI$_{abs}$; Strasser and Srivastava 1995, Clark *et al.* 2000) were recorded.

Specific leaf area and leaf dry matter content were determined *via* assessing the dry and fresh mass using a fine scale (*ABJ*, *Kern & Sohn GmbH*, Balingen, Germany), leaf area was recorded *via* scanning the fresh leaves (*CanoScan LiDE110*, *Canon*, Tokyo, Japan). Both parameters were then calculated in *R* using the package *LeafTraits* (Bernhardt-Römermann, unpublished).

Stomatal pore area index: Stomatal imprints from the adaxial and abaxial leaf side were taken using the clear nail polish method as described in Hilu and Randall (1984). Stomata were counted and guard cell length was measured with a light microscope (*Olympus CH20*, *Olympus*, Shinjuku, Japan). From these values, the stomatal pore area index, SPI, was calculated as proposed by Sack *et al.* (2003). None of the species studied showed stomata on the upper leaf side, which was checked on every sample.

The relative Chl concentration was measured using an *atLeaf PLUS* device (*atLeaf*, Wilmington, Delaware, USA) and then converted to total Chl concentration using the coversions on the homepage (https://www.atleaf.com/SPAD).

Statistical analysis: To test differences of the parameters between species, we performed analysis of variance (*ANOVA*) followed by *Tukey*'s multiple comparisons of means tests. To assess the seasonal variation in the parameters recorded (PEL$_{eff}$, P_{Nsat}, V_{cmax}, F$_v$/F$_m$, PI$_{abs}$, SLA, LDMC, SPI, and Chl), we performed linear models using the parameters measured as dependent variables and day of the year of the measurements (doy) as well as doy^2 as the explanatory variable to account for nonlinear relationships. To account for species-specific responses, species identity was included in the model as a covariate as well as the twofold interactions with doy (doy:species and doy^2:species). These full models were then simplified backwards, until the minimum adequate model was found as described in Crawley (2013).

To analyse the differences between newly formed leaves and leaves which overwintered in the two evergreen species, we performed *Welch*'s two sample *t*-test or *Wilcoxon* rank sum test, depending on data distribution tested with the *Shapiro-Wilk* normality test in all parameters (PEL$_{\text{eff}}$, P_{Nsat}, V$_{\text{cmax}}$, F$_v$/F$_m$, PI$_{\text{abs}}$, SLA, LDMC, SPI, and Chl).

To analyse trade-offs between frost resistance and photosynthetic performance and the association with leaf functional traits, we performed linear models with species and the interaction of species and traits as explanatory variables for each trait separately. If possible, these were then simplified as described above. This was done using leaf-level data of the replicate measurements each week, or mean values per species and sampling date, depending whether the traits had been measured on the same leaf or on a different one (Fig. 1). In a second step, we analysed the relationships in the multivariate trait space and performed a principal component analysis (PCA) with the species-wise mean values per sampling date for all parameters. This was done as some parameters were not measured on the same leaves (Fig. 1). Furthermore, the mean values in between old and new leaves within the first weeks of the measurement period represent the total resistance and performance better than the individual values. Confidence ellipses are drawn in the plot for every species.

All statistical analyses were performed using *R* (*R Core Team* 2017), the PCA was computed using the ‘vegan’ package (Oksanen *et al.* 2007) and displayed graphically using ‘ggplot2’ (Wickham 2009), ‘devtools’ (Wickham and Chang 2017) and ‘ggbiplot’ (Vu 2011).

Results

Seasonal variation: An overview of all parameters measured including mean values and ranges is presented in Table 1. All parameters measured showed a species-specific seasonal variation. FR showed a strong species-specific seasonal variation with lower values, *i.e.*, higher resistance in early spring and late autumn (R^2 = 0.55, F$_{12,\,392}$ = 40.3, p<0.001; Fig. 2*A*). *Quercus pubescens* showed almost a linear decrease in PEL$_{\text{eff}}$, thus an increase in FR during the course of the year. The relationship was defined by doy, doy^2 and species as well as the two-fold interactions thereof. Net photosynthetic rates also showed a pronounced seasonal variation with a strong species-specific pattern. Again, the relationship was defined by doy, doy^2 and species as well as the two-fold interactions thereof (R^2 = 0.61, F$_{12,\,389}$ = 50.8, p<0.001; Fig. 2*B*). The two summer green species showed overall higher values in

Table 1. Characterisation of four woody species with respect to their mean and range in measured trait values. Given is the mean value for each species as well as the range, *i.e.*, minimum and maximum values measured, of frost resistance measured as effective percentage of electrolyte leakage (PEL$_{\text{eff}}$), net photosynthetic rate at saturating irradiance and ambient atmospheric CO$_2$ concentrations (P_{Nsat}), maximum carboxylation rate (V$_{\text{cmax}}$), maximum quantum yield of PSII (F$_v$/F$_m$), absorption-based performance index (PI$_{\text{abs}}$), specific leaf area (SLA), leaf dry matter content (LDMC), stomatal pore area index (SPI), as well as chlorophyll content (Chl). Differences between species were assessed with *ANOVA* and followed by a *Tukey*'s multiple comparisons of means test. The *ANOVA* results are reported, the statistical results are given as n.s. – not significant, ‘.’ – p<0.1, ‘*’ – p<0.05, ‘**’ – p<0.01, and ‘***’ – p<0.001. *Letters* indicate significant differences at the p<0.05 level. The canopy duration indicates, during which period leaves were displayed on summer green trees.

Species	Life form	Canopy duration [d]	PEL$_{\text{eff}}$ [%]	P_{Nsat} [µmol(CO$_2$) m^{-2} s^{-1}]	V$_{\text{cmax}}$ [µmol(CO$_2$) m^{-2} s^{-1}]	F$_v$/F$_m$	PI$_{\text{abs}}$	SLA [kg m^{-2}]	LDMC [g kg^{-1}]	SPI	Chl [mg cm^{-2}]
ANOVA			F$_{3,\,401}$ = 23.5***	F$_{3,\,398}$ = 51.4***	F$_{3,\,392}$ = 32.1***	F$_{3,\,775}$ = 42.7***	F$_{3,\,775}$ = 160.0***	F$_{3,\,371}$ = 227.0***	F$_{3,\,394}$ = 34.2***	F$_{3,\,401}$ = 455.7***	F$_{3,\,753}$ = 325.1***
Berberis vulgaris	evergreen		54.0$^{\text{b}}$ [-4.5; 89.6]	6.5$^{\text{c}}$ [0.1; 14.5]	18.6$^{\text{c}}$ [0.3; 41.7]	0.83$^{\text{a}}$ [0.71; 0.86]	12.4$^{\text{a}}$ [1.5; 23.6]	5.7$^{\text{c}}$ [4.0; 13.6]	477.1$^{\text{a}}$ [242.2; 561.0]	0.231$^{\text{a}}$ [0.141; 0.299]	0.0491$^{\text{a}}$ [0.0142; 0.0695]
Quercus × *hispanica*	evergreen		42.8$^{\text{c}}$ [10.8; 85.9]	4.1$^{\text{d}}$ [0.1; 9.0]	13.7$^{\text{d}}$ [0.3; 44.6]	0.82$^{\text{b}}$ [0.69; 0.84]	12.2$^{\text{a}}$ [0.4; 23.4]	13.2$^{\text{b}}$ [6.3; 30.6]	441.9$^{\text{b}}$ [227.3; 529.1]	0.139$^{\text{c}}$ [0.074; 0.203]	0.0383$^{\text{b}}$ [0.0090; 0.0814]
Betula pendula	summer green	115–305	60.3$^{\text{a}}$ [29.3; 106.0]	10.0$^{\text{a}}$ [1.9; 19.1]	29.8$^{\text{a}}$ [5.3; 64.6]	0.82$^{\text{b}}$ [0.60; 0.89]	6.6$^{\text{b}}$ [0.2; 21.7]	15.6$^{\text{a}}$ [11.0; 27.8]	396.4$^{\text{d}}$ [256.6; 476.7]	0.081$^{\text{d}}$ [0.037; 0.123]	0.0277$^{\text{c}}$ [0.0075; 0.0406]
Quercus pubescens	summer green	122–310	54.4$^{\text{ab}}$ [27.6; 77.6]	8.3$^{\text{b}}$ [1.0; 16.7]	22.9$^{\text{b}}$ [1.8; 51.0]	0.80$^{\text{c}}$ [0.52; 0.85]	4.5$^{\text{c}}$ [0.1; 12.5]	14.7$^{\text{a}}$ [10.7; 28.4]	420.5$^{\text{c}}$ [256.5; 479.4]	0.162$^{\text{b}}$ [0.089; 0.265]	0.0253$^{\text{c}}$ [0.0055; 0.0555]

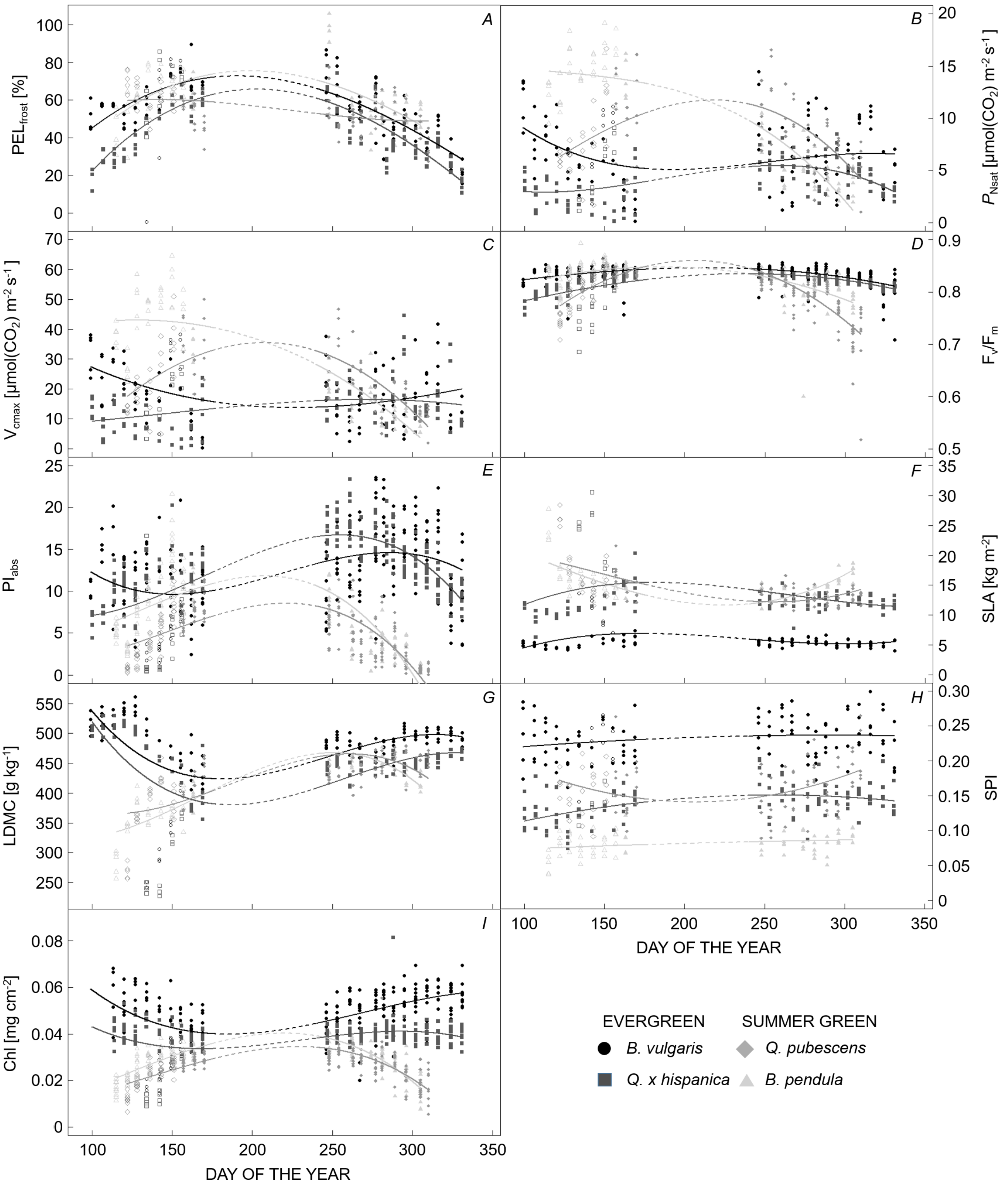

Fig. 2. Seasonal variation of parameters recorded, namely (*A*) frost resistance as effective percentage of electrolyte leakage (PEL_{eff}), (*B*) photosynthetic rates (P_{Nsat}), (*C*) maximum carboxylation capacity (V_{cmax}), (*D*) efficiency of PSII ($\text{F}_{\text{v}}/\text{F}_{\text{m}}$), (*E*) performance index (PI_{abs}), (*F*) specific leaf area (SLA), (*G*) leaf dry matter content (LDMC), (*H*) stomatal pore area index (SPI), and (*I*) chlorophyll content (Chl). Species are represented by colour, the evergreen species *Berberis vulgaris* and *Quercus* × *hispanica* are displayed in *black circles* and *dark grey squares*, respectively. The two summer green species are *Betula pendula* (*pale grey triangles*) and *Q. pubescens* (*light grey diamonds*). Leaves, which were visually newly formed and not fully grown, are indicated by open symbols.

P_{Nsat} and a stronger seasonal variation with a pronounced decline of P_{Nsat} in autumn, whereas the two evergreen species showed more stable values throughout the season. A similar pattern could be observed in $\mathrm{V_{cmax}}$ ($R^2 = 0.55$, $\mathrm{F}_{12,\,383} = 39.8$, $p<0.001$; Fig. 2*C*). $\mathrm{F_v/F_m}$ also showed a slight seasonal variation. Again, evergreen species showed less seasonal variation, whereas summer green species showed lower $\mathrm{F_v/F_m}$ values early and late in the season and higher values in mid-season ($R^2 = 0.54$, $\mathrm{F}_{12,\,766} = 74.4$, $p<0.001$; Fig. 2*D*). The pattern of the seasonal variation in $\mathrm{PI_{abs}}$ was, however, comparable between evergreen and summer green species. Especially in the second half of the measurement period, the differences between evergreen and summer green species were high, with evergreen species having higher values. For both life forms, there was a decline in $\mathrm{PI_{abs}}$ in autumn ($R^2 = 0.64$, $\mathrm{F}_{12,\,766} = 111.2$, $p<0.001$; Fig. 2*E*). The seasonal variation in SLA, however, was much smaller within evergreen species, which showed rather constant values, than in summer green species. Summer green species showed higher values early and late in the season, whereas evergreen species showed slightly higher values in mid-season than early and late ($R^2 = 0.74$, $\mathrm{F}_{12,\,362} = 86.4$, $p<0.001$; Fig. 2*F*). The seasonal variation in LDMC, however, was much stronger than that in SLA, with evergreen species showing higher values in early spring and summer green species showing lower values in spring and again a pronounced decrease in autumn ($R^2 = 0.52$, $\mathrm{F}_{12,\,385} = 34.8$, $p<0.001$; Fig. 2*G*). The seasonal variation in SPI was very low. The interaction between doy:doy^2 was not significant and could be removed from the full model. Only *Q. pubescens* showed a noticeable difference in SPI values throughout the season with higher values in spring and autumn than in summer ($R^2 = 0.80$, $\mathrm{F}_{11,\,393} = 140.0$, $p<0.001$; Fig. 2*H*). There was a strong difference in Chl content between evergreen and summer green species. While evergreen species had higher contents in early spring and in late autumn, the summer green species had the opposite tendency with lower values in spring and autumn ($R^2 = 0.70$, $\mathrm{F}_{11,\,744} = 145.1$, $p<0.001$; Fig. 2*I*).

Old and newly formed leaves in evergreen species: When looking at the differences between leaves formed in the previous year(s) and leaves from the same growth period, we found that some traits differed significantly between leaves of different age whereas others did not (Fig. 1S, *supplement*). In *B. vulgaris,* there was no difference in $\mathrm{PEL_{eff}}$, $\mathrm{V_{cmax}}$, and SPI between old and new leaves, a marginal one in P_{Nsat} (t = 1.85, $p=0.08$) and $\mathrm{F_v/F_m}$ (W = 132.5, $p=0.07$) and a significant one in $\mathrm{PI_{abs}}$ (t = –8.7, $p<0.001$), SLA (W = 64, $p<0.001$), LDMC (t = –7.6, $p<0.001$), and Chl (t = –9.3, $p<0.001$). Similar results could be seen in *Q.* × *hispanica*, where we found a marginal difference in SPI (t = 1.8, $p=0.09$) and significant differences in $\mathrm{PEL_{eff}}$ (t = 2.3, $p<0.05$), P_{Nsat} (W = 88, $p<0.05$), $\mathrm{V_{cmax}}$ (t = 3.8, $p<0.01$), $\mathrm{PI_{abs}}$ (W = 18, $p<0.001$), $\mathrm{F_v/F_m}$ (W = 51, $p<0.001$), SLA (W = 70, $p<0.001$), LDMC (t = –9.1, $p<0.001$), and Chl (W = 0, $p<0.001$) between leaves of this year and the previous year(s). For a graphical display of the differences between old and new leaves, please *see* Fig. 1S.

Trade-off between resistance and performance: The pairwise trait-trait relationships are summarised in Table 2 and graphically represented in Fig. 2S (*supplement*). There are positive relationships between $\mathrm{PEL_{eff}}$ and all parameters related to photosynthesis (P_{Nsat}, $\mathrm{V_{cmax}}$, $\mathrm{F_v/F_m}$, $\mathrm{PI_{abs}}$), indicating that the higher the performance, the lower the investment in FR. For some trait-trait relationships, there

Table 2. Pairwise relationships between all functional traits measured. Given is the frost resistance measured as effective percentage of electrolyte leakage ($\mathrm{PEL_{eff}}$), the net photosynthetic rate at saturating irradiance and ambient atmospheric $\mathrm{CO_2}$ concentrations (P_{Nsat}), the maximum carboxylation rate ($\mathrm{V_{cmax}}$), the maximum quantum yield of PSII ($\mathrm{F_v/F_m}$), the absorption based performance index ($\mathrm{PI_{abs}}$), the specific leaf area (SLA), the leaf dry matter content (LDMC), the stomatal pore area index (SPI), as well as the chlorophyll content (Chl). Statistical results are given as n.s. – not significant, ‘·’ – $p<0.1$, ‘*’ – $p<0.05$, ‘**’ – $p<0.01$, and ‘***’ – $p<0.001$. For graphical representation, please *see* Fig. 2S.

	$\mathrm{PEL_{eff}}$	P_{Nsat}	$\mathrm{V_{cmax}}$	$\mathrm{F_v/F_m}$	$\mathrm{PI_{abs}}$	SLA	LDMC	SPI
P_{Nsat}	$R^2 = 0.17$ $\mathrm{F}_{4,\,397} = 21.1^{***}$							
$\mathrm{V_{cmax}}$	$R^2 = 0.18$ $\mathrm{F}_{4,\,391} = 20.9^{***}$	$R^2 = 0.17$ $\mathrm{F}_{7,\,388} = 540.6^{***}$						
$\mathrm{F_v/F_m}$	$R^2 = 0.23$ $\mathrm{F}_{7,\,397} = 16.6^{***}$	$R^2 = 0.43$ $\mathrm{F}_{7,\,394} = 42.6^{***}$	$R^2 = 0.38$ $\mathrm{F}_{7,\,388} = 33.3^{***}$					
$\mathrm{PI_{abs}}$	$R^2 = 0.18$ $\mathrm{F}_{7,\,397} = 12.7^{***}$	$R^2 = 0.55$ $\mathrm{F}_{7,\,394} = 68.0^{***}$	$R^2 = 0.52$ $\mathrm{F}_{7,\,388} = 59.8^{***}$	$R^2 = 0.60$ $\mathrm{F}_{7,\,771} = 161.9^{***}$				
SLA	$R^2 = 0.30$ $\mathrm{F}_{7,\,80} = 4.9^{***}$	n.s.	n.s.	$R^2 = 0.25$ $\mathrm{F}_{7,\,345} = 16.4^{***}$	$R^2 = 0.55$ $\mathrm{F}_{7,\,345} = 59.1^{***}$			
LDMC	$R^2 = 0.30$ $\mathrm{F}_{7,\,80} = 5.0^{***}$	$R^2 = 0.51$ $\mathrm{F}_{7,\,80} = 11.9^{***}$	$R^2 = 0.41$ $\mathrm{F}_{7,\,80} = 7.8^{***}$	$R^2 = 0.22$ $\mathrm{F}_{7,\,364} = 15.0^{***}$	$R^2 = 0.50$ $\mathrm{F}_{7,\,364} = 52.9^{***}$	$R^2 = 0.93$ $\mathrm{F}_{7,\,366} = 748.1^{***}$		
SPI	$R^2 = 0.17$ $\mathrm{F}_{7,\,397} = 11.4^{***}$	$R^2 = 0.30$ $\mathrm{F}_{7,\,394} = 23.9^{***}$	$R^2 = 0.22$ $\mathrm{F}_{7,\,388} = 15.9^{***}$	$R^2 = 0.15$ $\mathrm{F}_{7,\,397} = 10.1^{***}$	$R^2 = 0.42$ $\mathrm{F}_{7,\,397} = 40.0^{***}$	n.s.	n.s.	
Chl	$R^2 = 0.21$ $\mathrm{F}_{7,\,346} = 13.9^{***}$	$R^2 = 0.35$ $\mathrm{F}_{7,\,373} = 28.2^{***}$	$R^2 = 0.27$ $\mathrm{F}_{7,\,367} = 19.9^{***}$	$R^2 = 0.36$ $\mathrm{F}_{7,\,748} = 60.4^{***}$	$R^2 = 0.59$ $\mathrm{F}_{7,\,348} = 153.4^{***}$	$R^2 = 0.82$ $\mathrm{F}_{7,\,344} = 229.9^{***}$	$R^2 = 0.56$ $\mathrm{F}_{7,\,363} = 65.4^{***}$	$R^2 = 0.78$ $\mathrm{F}_{7,\,376} = 190.6^{***}$

was a clear divide in between evergreen and summer green species, such as in the relationships between P_{Nsat} and V_{cmax}, respectively, and PI_{abs} and Chl, respectively, where summer green species showed a stronger relationship with a much steeper slope. There was a strong positive relationship between Chl and PI_{abs} and LDMC, respectively. The PCA showed a clear separation between the species and the two life forms indicated by ellipses mainly on the first axis (Fig. 3). The first axis represented the trade-off between SLA on one side and LDMC, SPI, Chl, and PI_{abs} on the other, whereas the second axis represented the trade-off between FR (PEL_{eff}) and P_{Nsat}, V_{cmax}, and F_v/F_m. Summer green species tended to show higher growth rates and gas-exchange rates, whereas evergreen species invested more in leaf thickness and resistance as indicated by high LDMC, Chl, LDMC, and PI_{abs} and experienced less stress as indicated by F_v/F_m.

Discussion

In this study, we found that PEL_{eff}, P_{Nsat}, V_{cmax}, F_v/F_m, PI_{abs}, SLA, LDMC, SPI, and Chl varied during the growth period in a species-specific manner and that there was a trade-off between performance and growth rates on one hand and leaf thickness and resistance on the other hand. There was a profound difference between evergreen and summer green species in many parameters.

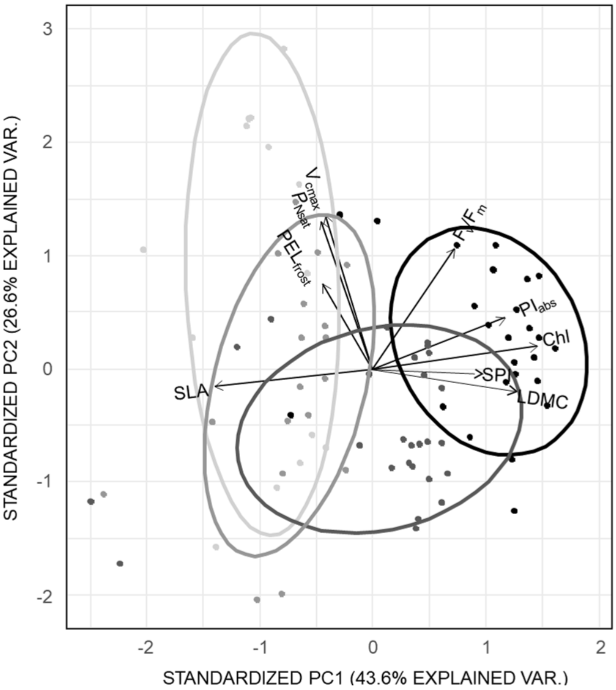

Fig. 3. Principal component analysis of all traits selected. Colours represent species. *Berberis vulgaris* is displayed in *dark green*, *Quercus* × *hispanica* in *light green*, both being evergreen species. The two summer green species are *Betula pendula* (*yellow*) and *Q. pubescens* (*orange*). Given is the frost resistance measured as effective percentage of electrolyte leakage (PEL_{eff}), the net photosynthetic rate at saturating irradiance and ambient atmospheric CO_2 concentrations (P_{Nsat}), the maximum carboxylation rate (V_{cmax}), the maximum quantum yield of PSII (F_v/F_m), the absorption based performance index (PI_{abs}), the specific leaf area (SLA), the leaf dry matter content (LDMC), the stomatal pore area index (SPI), as well as the chlorophyll content (Chl).

Frost resistance showed clear seasonal variations with a profound dehardening in spring and hardening in autumn. In woody plants, the main driving forces of frost hardening are typically photoperiod and temperature (Schwarz 1970) which led to the observed increase in FR starting from the end of summer, *i.e.*, the end of August. This effect was much more pronounced in evergreen species, which rely on the reuse of leaves in the following years to come, than in summer green species. Especially in *Q. pubescens*, hardly any hump shaped relationship could be detected, but rather a linear increase of FR over time was found. This response in seasonal variability of *Q. pubescens* confirms findings of Taschler *et al.* (2004), who showed that woody species are more frost susceptible during shoot expansion and regrowth in spring. In *Q.* × *hispanica*, young leaves were also less frost resistant than older leaves, whereas there was no difference in *B. vulgaris* between leaves of different age. Performance, as captured *via* gas exchange and Chl fluorescence, also showed remarkable seasonal variations. In our study, P_{Nsat} and V_{cmax} showed similar patterns in their seasonal variation. Overall, summer green species showed higher P_N than that of evergreen species, yet a strong decline with senescence, which was almost contrary to our findings in evergreen species, which showed low seasonal variation and higher P_N in early spring. *Q. pubescens* showed low P_N in early spring, whereas *B. pendula* started with high values in young leaves. A similar pattern could be observed in PI_{abs}, which has been proven to correlate well with P_{Nsat} and V_{cmax} in previous research (Bucher *et al.* 2018a). However, the seasonal variation in PI_{abs} was much more pronounced than in gas-exchange measurements and evergreen species showed higher values in PI_{abs} than that of summer green species. Differences between gas exchange and Chl *a* fluorescence measurements could be due to the fact, that only the uppermost layers of plant tissue are studied in Chl *a* fluorescence technique. When measuring the adaxial side only, we merely probed the chloroplasts of the palisade parenchyma cells but not the entire leaf (Vogelmann and Evans 2002, Lichtenthaler *et al.* 2005), whereas gas exchange engulfs both sides of the leaves. F_v/F_m was a good indicator of autumn senescence as previously described by Holland *et al.* (2014) in summer green species, but showed little seasonal variation in evergreen species. *Q. pubescens* showed low values in spring, as did *Q.* × *hispanica* in younger leaves, probably hinting to a not fully developed photosynthetic apparatus. The low seasonal variation and the high early-spring values in SLA confirms findings of Römermann *et al.* (2016), who found that SLA peaks in early spring for most of the 16 summer green woody species studied. This can be attributed to the initial period of leaf growth and laminar expansion during which SLA is high (Steer 1971). Once cell expansion has finished, dry matter, especially in the form of nonstructural carbohydrates, accumulates in the cells (Palacio *et al.* 2008, Römermann *et al.* 2016). This is also reflected in LDMC values, which increased over time especially in evergreen species. The leaves of the evergreen species, which overwintered, however, showed an initial decrease in LDMC and later on an increase contrasting summer green and newly formed leaves. Previous studies showed an initial increase of LDMC and a subsequent decrease during the growth period in woody species (Jurik 1986, Palacio *et al.* 2008, Dubey *et al.* 2011, Römermann *et al.* 2016) and in some herbaceous taxa (Bucher *et al.* 2018b) which has been attributed to the accumulation of nonstructural carbohydrates and retranslocation of valuable compounds during senescence. Evergreen species thus seem to accumulate compounds in their overwintering leaves, which could relate to FR during cold periods. We could not observe a strong variation in SPI during the season similar to Römermann *et al.* (2016) and Bucher *et al.* (2018b). In previous research, we found a strong link between SPI and FR (Bucher *et al.* 2018b), which could not be confirmed by this study. However, SPI scaled very well with Chl, which in turn scaled well with LDMC and $\mathrm{PI_{abs}}$. Thus, this relationship could have been masked by leaf thickness. Chl showed strong differences between evergreen and summer green species. Whereas evergreen species showed the highest values in the beginning and end of the measurement period, summer green species showed a mid-season peak. This might explain similar patterns in gas-exchange rates. In *Platanus occidentalis*, leaf nitrogen, which is partially located in Chl, showed a decline during senescence (Adams *et al.* 1990). In this study, the decrease in PSII fluorescence during senescence was attributed to a decreased reabsorption of PSII fluorescence.

There are few studies, which include the difference between newly formed and old leaves. In general, there were stronger differences between new and old leaves in *Q.* × *hispanica* than that in *B. vulgaris*. This might be due to the fact, that *Q.* × *hispanica*, a hybrid between *Q. cerris* and *Q. suber*, is evergreen in our garden, yet there have been reports that this species is only partially wintergreen and senesces its leaves if the winter is too severe. Notably, there were stronger differences in traits associated with leaf morphology, such as SLA, LDMC, and Chl, whereas differences in FR, P_{Nsat}, and $\mathrm{V_{cmax}}$ were less pronounced. In most of these traits, the newly formed leaves of evergreen species were more similar to the newly formed leaves of the summer green species than to the old leaves of the same species. Thus, sampling protocols should take this difference into account, as sampling only one leaf type early in the year would strongly bias the results and not capture the processes on an organism scale.

In our study, there was a profound trade-off between resistance and performance as could be demonstrated with the trade-off of $\mathrm{PEL_{eff}}$ and the two gas-exchange parameters P_{Nsat} and $\mathrm{V_{cmax}}$. However, this pattern was much clearer in the multivariate trait space than in the univariate analysis on the intraspecific scale. Again we could demonstrate that P_{Nsat} is well described by $\mathrm{PI_{abs}}$ (*see* Bucher *et al.* 2018a), yet this link was much stronger in summer green than that in evergreen species. This could be due to differences in morphology and leaf thickness, as summer green species form thinner leaves and less Chl. Additionally, only the uppermost layers of a leaf are captured with the *PocketPEA* (Vogelmann and Evans 2002, Lichtenthaler *et al.* 2005), which might have caused some fundamental differences in between evergreen and summer green plants. Chl seemed to capture resistance and performances very well showing a negative relationship with FR and a positive one with the parameters assessing photosynthetic performance, especially $\mathrm{PI_{abs}}$. We could demonstrate with our multivatiate analysis that the trade-off is rather in between photosynthetic rates and FR than between growth rates and FR, which confirmes work by Larcher (1994), who found that overwintering leaves stayed photosynthetically active but invested their assimilates rather in the accumulation of more carbohydrates and thus higher FR than in growth. In previous studies, FR was linked negatively to SLA and growth rates and positively to SPI, indicating high photosynthetic rates (Sakai and Larcher 1987, Bucher *et al.* 2018b) due to a conflict of resource investment (Larcher 1994, Körner 2003). We could not confirm a relationship between stomatal traits and FR found by Bucher *et al*. (2018b) in six herbaceous plant species. This might have been confounded as mentioned above by high variations in leaf thickness. In our study, there were distinct differences between evergreeen and summer green species, yet the two *Quercus* species were more similar to each other than the other two other species, which hints to an effect of phylogeny.

This study provides valuable insights into the differences between evergreen and summer green species, differences between old and newly formed leaves in evergreen species, and the trade-off between resistance and performance. It also shows how easy to measure plant functional traits can expand our knowledge about ecophysiological processes. Understanding seasonal variations and incorporating them in *e.g.*, terrestrial biosphere models (TBMs) will help to fine-tune the models and increase their reliability. In future studies, the differences in this trade-off as inflicted by changing abiotic conditions and different leaf thicknesses should be tested and the phylogenetic signal as might be seen in the similar patterns of the two oak species should be analysed using higher species numbers.

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