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Modelling of the relationship between the SPAD values and photosynthetic pigments content in *Quercus petraea* and *Prunus serotina* leaves

Abstract: In forest research and nursery practice there is often a need to monitor the condition and responses of trees to different stressors. Chlorophyll content in leaf is a good indicator of plant health and can be measured rapidly in many repetitions using the chlorophyll meter SPAD-502Plus. This practical tool provides the values of chlorophyll content in relative units (SPAD values), therefore it should be calibrated for each species to determine chlorophyll content in physiological units. In this study, the chlorophyll meter SPAD-502Plus was calibrated to be used for total chlorophyll (Chl), chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids (Car) contents determination in leaves of *Quercus petraea* and *Prunus serotina* seedlings growing in different light environments. In the same leaf, SPAD values were measured with the Chl meter, and then photosynthetic pigments content (PP; chlorophyll and carotenoids) was consistently assessed using a conventional extraction method. The measurements were conducted once a month from May to November in three light treatments to obtain the widest possible range of the PP content values. To estimate total Chl content in leaves using the chlorophyll meter the quadratic polynomial functions: $y = 0.0374x^2 + 0.5345x + 0.5137$ and $y = 0.024x^2 + 2.1998x – 32.7866$ were obtained from the relationship between the Chl meter SPAD readings and total Chl determined spectrophotometrically for *P. serotina* and *Q. petraea*, respectively. Chl was higher under shade compared with full light regime and Car were linearly correlated with Chl. PP content was positively correlated with air temperature except for Car in *P. serotina* leaves. It was concluded that at the same soil conditions chlorophyll content in leaves of *Q. petraea* and *P. serotina* depended on species, light regimes and temperature of growth. The chlorophyll meter can be used as a practical tool to monitor and compare photosynthetic pigments content in leaves between tree species or populations acclimated to different environments together with a control of abiotic and biotic factors affecting pigments content and leaf optical properties.

Additional key words: black cherry; carotenoids; chlorophyll content; chlorophyll meter; sessile oak

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Abbreviations: A – absorbance; Car – carotenoids; Chl – chlorophyll; Chl *a* – chlorophyll *a*; Chl *b* – chlorophyll *b*; Chl (*a* + *b*) – total chlorophyll; PP – photosynthetic pigments (chlorophylls and carotenoids); SPAD-502Plus – *Minolta* chlorophyll meter

Introduction

Leaf chlorophyll (Chl) content is associated with the physiological status of a plant. Low Chl content can limit the potential of photosynthesis, thereby reducing the primary production of organic compounds (Niyogi 1999; Valladares et al. 2002). In ecophysiological studies quantity of Chl reflects responses of plants to different stressors such as: nitrogen deficit (van den Berg and Perkins 2004, Zhao et al. 2005, Percival et al. 2008), drought (Anderson and Tomlinson 1998; Peñuelas and Filella 1998; Schlemmer et al. 2005) or high irradiance (Merzlyak and Gitelson 1995; Valladares et al. 2002; Adams et al. 2004, Main et al. 2011). Carotenoids (Car) play a role of "screening pigments" protecting the photosynthetic apparatus against excess energy and their high content in leaf may indicate a photoinhibitory stress (Demmig-Adams et al. 1989; Adams and Demmig-Adams 1994; Niyogi 1999).

Usually, the photosynthetic pigments (PP) content in leaves is determined in laboratory with methods based on pigment extraction and spectrophotometry (e.g. Porra *et al.* 1989; Wellburn 1994). However, not only conventional extraction methods lead to disintegration of plant material, but also need more time, specialist equipment and solvents toxic for human health and environment. Therefore, non-invasive, inexpensive and rapid methods of measuring Chl have been developed based on optical leaf properties. For this purpose a portable Chl meter (SPAD-502Plus, Minolta Camera Co., Osaka, Japan) has been often used in plant physiology, agriculture and less frequently in forestry research and practice (Gratani 1993; Markwell et al. 1995; Gáborčik 2003; Pinkard et al. 2006; Uddling et al. 2007; Marenco et al. 2009). This handy hold device allows a convenient estimation of total Chl content in leaves, especially with a great number of measurement repetitions on the same leaf ahead (Bauerle et al. 2004; Hawkins et al. 2009; Coste et al. 2010).

Markwell et al. (1995) described the physical principles and equations on which the functioning of the SPAD Chl meter was based. In brief, this device measures the transmittance of light by leaves at two different wavelengths: red (650–660 nm) and near-infrared (930–940 nm). Red light is absorbed by chlorophylls, and its absorption is correlated with the Chl content. The peak absorbance areas of chlorophyll are in blue and red regions. The wavelength ranges chosen to be used for measurements are the red area where chlorophylls *a* and *b* absorbance is high and unaffected by Car (Chlorophyll Meter SPAD-502Plus; Instruction Manual 2009). Near-infrared absorption is used as a "reference value" for adjusting the differences in leaf structure. However, the values given by the Chl meter are in SPAD units which have to be converted into physiological units (pigment concentration: mg g^{-1} fresh mass or dry mass and pigment content: mg m^{-2}). Thus, the calibration curve between the Chl meter readings and chlorophyll content determined with an extraction method should be generated before an attempt to assess physiological responses of plants to environmental factors.

The objects of this study were seedlings of two forest tree species: *Quercus petraea* (Matt.) Liebl. (sessile oak) and *Prunus serotina* (Ehrh.) Borkh. (black cherry). To our knowledge, calibration curves for these species have not yet been published. *Q. petraea* is a native for Europe deciduous broadleaf tree of great economic importance occurring between the latitudes 60°N to 30°N (Robakowski and Stachnowicz 2005). *P. serotina* is native to North America and invasive to European forests deciduous broadleaf tree or understory shrub occurring between the latitudes 49°N to 30°N (Marquis 1990). In Europe, *P. serotina* invaded many types of plant communities and has become a threat to biodiversity. It has been observed that seedlings of sessile oak and black cherry co-occur in different light environments under the Scots pine canopy of European forests and compete for space and resources (Koutika et al. 2007). Therefore, we addressed the question about the importance of PP content in leaves of *Q. petraea* and *P. serotina* seedlings growing in different microclimate conditions to between-species competition.

There is evidence that the calibration of the chlorophyll meter is needed to determine Chl content expressed in physiological units for all individual species, ecotype or even cultivar (Yamamoto et al. 2002; Fritschi and Ray 2007; Marenco et al. 2009). Most curves were obtained for various crops, but much less for forest tree species (Cate and Perkins 2003; Pinkard et al. 2006; Samsone et al. 2007; Percival et al. 2008; Coste et al. 2010). To determine the physiological condition or responses of trees to different abiotic and biotic factors, PP content should often be measured in a great number of leaves. Therefore, the SPAD Chl meter could be a useful tool for forestry research and practice, especially in nurseries where broadleaved tree species are grown.

The objective of the present study was to develop the mathematical models of the relationship between the SPAD values and spectrophotometrically determined total Chl and Car contents in leaves of *Q. petraea* and *P. serotina* seedlings growing under different light environments. To incorporate changes depending on sampling time and microclimate conditions (light, temperature and relative humidity) into the models, the PP content was monitored monthly with both methods during the whole vegetative season in three light regimes.

Materials and methods

Plant material

In the experiment, current year seedlings of *P. serotina* and *Q. petraea* were used. Oak seedlings were germinated from acorns collected in the permanent seed stand situated in 'Jarocin' Forest Inspectorate (western Poland), whereas black cherry seedlings were germinated from stone fruits harvested from trees growing in the 'Zielonka' Experimental Forest (27 km from Poznan, western Poland). Seedlings of both species were grown from October 2010 to May 2011 in containers, in the local nurseries.

The plants were transported to the Dendrological Garden of the Poznan University of Life Sciences and transplanted into 7-litre pots filled with neutral peat, humus and sand mixed in 1:1:1 ratio (v/v/v). Then, potted seedlings were randomly distributed to one of three light treatments: 6, 25 or 100% of full irradiance. In May, the seedlings were fertilized using 15 g of slow-releasing fertilizer 'Osmocote Exact Standard' (N, P, K, Mg – 15:9:12:2) per pot. The plants were watered every two days using an automatic irrigation system up to the field capacity. Fifteen seedlings of *P. serotina* (five per light treatment) and 24 seedlings (eight per light treatment) of *Q. petraea* were randomly taken for the greenness (SPAD) and PP content measurements once a month from May to November 2011.

Meteorological conditions

Light treatments were established using a shading cloth. The determination of the light regimes and the spectral proprieties of the shading cloth were described earlier in Wyka et al. (2007). In brief, on cloudy days photosynthetic photon flux (*PPF*) was simultaneously measured with two light meters calibrated in μmol m^{-2} s^{-1} (*Spectrum Technologies, Inc., USA*) 80 cm above each pot under shade and in the open. The *PPF* measurements were repeated on three occasions: in May, July and September. Three light treatments were established: high light (HL, 100 % of full sun light), moderate light (ML, 25 %) and low light (LL, 6%). Air temperature and relative humidity (*RH*) were monitored with Hobo Pro v2 (*OnSet Computers*, Pocasset, MA, USA) throughout the growing season. Six hobos (two per light treatment) registered meteorological data each twenty minutes. The light environments differed in *PPF*, monthly mean temperature (T_{mean}), monthly maximal temperature (T_{max}), monthly minimal temperature (T_{min}) and, importantly, in monthly amplitudes. The microclimatic differences were the most noticeable between both shade treatments and HL. Shading decreased monthly mean temperatures, monthly amplitudes and increased *RH* compared with HL (Table 1).

Table 1. Meteorological conditions in three experimental light environments: low light (LL – low light, 6%; ML – moderate light, 25%; HL – high light, 100% of full light). The data were registered each twenty minutes from May to November 2011. T_{mean} – monthly mean temperature, T_{max} – monthly maximal temperature, T_{min} – monthly minimal temperature, $T_{max} - T_{min}$ – temperature amplitude, *RH* – relative humidity

Month	Light	T_{mean} (°C)	T_{max} (°C)	T_{min} (°C)	$T_{max} - T_{min}$ (°C)	*RH* (%)
May		15.1±0.1	31.4	0.7	30.7	70.4±0.4
June		18.0±2.4	30.7	7.4	23.3	70.9±10.3
July		16.8±2.0	27.6	7.5	20.1	88.2±8.2
August	LL	17.5±2.1	28.9	7.4	21.5	86.6±7.5
September		13.9±2.3	27.9	4.0	23.9	87.5±7.7
October		8.4±2.5	22.3	−1.3	23.7	93.1±5.4
November		2.6±2.0	14.8	−4.6	19.4	95.9±3.4
May		15.0±0.1	32.1	0.9	31.2	71.5±0.5
June		18.5±2.8	33.7	7.4	26.3	71.5±11.4
July		17.1±2.2	30.9	7.4	23.4	88.0±8.8
August	ML	17.7±2.4	30.9	7.3	23.6	86.4±8.2
September		14.0±2.5	29.6	3.9	25.7	88.5±8.1
October		8.3±2.6	24.5	−1.8	26.3	94.0±5.3
November		2.3±1.9	14.9	−4.8	19.7	97.0±3.1
May		15.9±0.2	35.1	−0.23	35.3	68.7±0.4
June		20.0±6	36.6	6.0	30.6	67.8±11.3
July		18±2.7	34.9	6.4	28.5	87.1±8.6
August	HL	18.9±2.9	36.8	5.9	30.9	84.5±8.4
September		14.9±3.2	36.4	2.4	33.9	90.3±6.9
October		9.1±3.0	33.3	−2.2	35.5	90.3±6.9
November		3.3±2.4	19.2	−5.4	24.6	92.2±5.4

Measurements with SPAD Chl meter

Leaf greenness was measured on fully expanded leaves *in situ* using the Chl meter SPAD-502Plus. To reduce the impact of daily chloroplasts movement on SPAD values, the measurements in each individual sampling time were conducted at the same day time from 9:00 to 12:00 (Nauš et al. 2010). The meter head was placed at adaxial leaf surface avoiding a mid-vein. Five measurements were taken and averaged per leaf in each seedling. Afterwards a leaf was cut and put immediately into a plastic tube and a vacuum flask prior to the transport to the laboratory.

Photosynthetic pigments content

PP were extracted from the same leaves which were used for the SPAD determination. Sampled leaves, sheltered from light, were transported to the laboratory and all plant material was prepared within 2 hours after the collection. First, fresh leaf tissue for the pigments extraction was obtained using a circular punch (cork borer). Excised discs had an area of 0.76984 cm^2. Then, leaves were scanned and their area was determined with DigiShape software (*Cortex Nova*, Bydgoszcz, Poland). Leaves were dried for 48 h at 65°C and weighted to calculate leaf mass-to-area ratio (LMA).

Chlorophylls *a* and *b,* and Car concentration was measured by dimethylsulfoxide (DMSO) extraction technique of Hiscox and Israelstam (1979). Sampled discs were cut in fine strips and placed in a glass tube containing 5 cm^3 DMSO. Each sample was incubated in a water bath at 65°C in the dark for one hour. After cooling the absorbance of extract (3 cm^3 aliquot) was measured spectrophotometrically (Jasco V-530, *Jasco,* Tokyo, Japan). The absorbance was measured at 480, 649 and 665 nm, relative to a DMSO blank. The Chl *a*, *b* and Car concentrations were determined following the equations proposed by Wellburn (1994):

$$\text{Chl } a \ (\mu g \cdot cm^{-3}) = 12.19 \cdot A_{665} - 3.45 \cdot A_{649} \ (1)$$
$$\text{Chl } b \ (\mu g \cdot cm^{-3}) = 21.99 \cdot A_{649} - 5.32 \cdot A_{665} \ (2)$$
$$\text{Car } (\mu g \cdot cm^{-3}) = (1000 \cdot A_{480} - 2.14 \cdot \text{Chl } a - 70.16 \cdot \text{Chl } b)/220 \ (3)$$

The PP concentrations calculated from Eqns. 1, 2 and 3 were converted into leaf PP content per unit of leaf area using leaf mass-to-area ratio (LMA).

Data analysis

Relationships between the SPAD values and PP content were determined for *P. serotina* and *Q. petraea* with the curve fitting routines of Statistica 9.0 software package (*StatSoft,* Tulsa, OK, USA). The non-linear regression analysis using the Levenberg-Marquardt al-

gorithm was applied to test the relations between: (i) Chl (*a* + *b*) and SPAD; (ii) Chl *a* and SPAD; (iii) Chl *b* and SPAD; (iv) Car and SPAD. The functions found in literature (Table 3) were applied in curve fitting procedure using the data set obtained for each species. The next step was an exclusion of the equations with the lowest coefficients of determination (r^2) to generate the models with the highest goodness of fit.

The models were fitted to the data pooled from May to November (for *P. serotina*) and from May to October (for *Q. petraea*). This difference was due to species-specific responses to the length of growing season (Robakowski and Bielinis 2011). In November, there were observed only necrotic brown leaves of *Q. petraea* with low Chl content and high SPAD values. Necrotic leaves were not included in the statistical analysis. To compare the slopes of linear regression between Chl (*a* + *b*) and Car contents for the study species, the analysis of covariance (ANCOVA) with "species" as a categorical predictor, "Chl (*a* + *b*)" as a continuous predictor and "Car" as a response was conducted. For each study species, separately, PP in leaves acclimated to one of three light regimes was compared among the sampling dates and light treatments with the two-factorial analysis of variance with interaction where "sampling time", "light treatment" and interaction were the sources of variance. To exclude an effect of varying leaf mass to area ratio on the relationship between the SPAD values and chlorophyll content in leaf, the relation between the normalized SPAD values (SPAD to leaf mass to area (LMA) ratio) and chlorophyll content in leaf was tested.

Differences between the light treatments were tested with Tukey's *a posteriori* test at *a* < 0.05. All graphs were prepared using Sigmaplot 12.0 (Systat Sofware, Inc., San Jose, CA, USA).

Results

Changes in SPAD and pigments content in leaves during the vegetative season

Greenness measured with the Chl meter in SPAD units followed the similar seasonal trend as total Chl content (Fig. 1). In leaves of both study species total Chl and Car increased from May to July and stabilized at the highest level from July to September in *P. serotina* (Fig. 1a) and from July to August in *Q. petraea* (Fig. 1b). An abrupt decrease of PP content in *Q. petraea* leaves began already in September and in *P. serotina* one month later. PP content in *Q. petraea* was higher than in *P. serotina* leaves at all sampling dates, except for October (0.116±0.003 and 0.066±0.003 g total Chl m^{-2}; 0.0190±0.0004 and 0.0120± 0.0004 g Car m^{-2} in *Q. petraea* and *P. serotina* leaves, respectively, Fig. 1, 3).
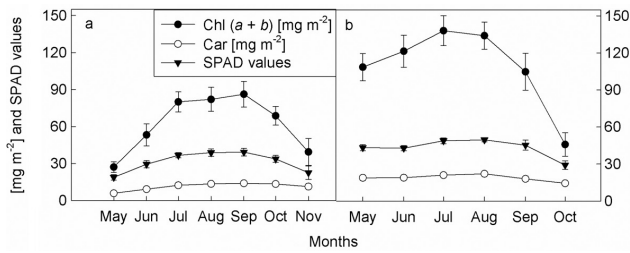
Fig. 1 Changes in Chl ($a + b$) content, Car content and SPAD values in leaves of *Prunus serotina* (a) and *Quercus petraea* (b) seedlings growing in pots in full light during the vegetative season 2011 (means ± 95% confidence interval, $n = 15$ for *Prunus serotina* and 24 for *Quercus petraea* per sampling date). The upper and lower 95% confidence intervals for carotenoids were not more than 2.1062 [mg m^{-2}]

Effects of acclimation to the microclimate conditions

When all the data from the whole vegetative period were pooled, in *Q. petraea* leaves total Chl content was significantly different depending on sampling date (month) and higher in both shade treatments (0.126, 0.128 g m^{-2} in 6 and 25%) compared with HL (0.093) according to two-factorial ANOVA ($F^4_{month}=$ 32.89***, $F^2_{light} = 20.34^{***}$) and post-hoc Tukey's test at $\alpha < 0.05$. In *P. serotina* leaves, total Chl content changed also with time and light regime ($F^5_{month} = 27.60^{***}$, $F^2_{light} = 6.75^{**}$). It was highest in LL (0.074 g m^{-2}±0.003) and did not differ between HL and ML (0.060±0.003 and 0.064±0.003, respectively) in Tukey's test. Car depended on sampling time in both study species and on light regime in *Q. petraea*, but not in *P. serotina* ($F^4_{month} = 15.98^{***}$, $F^2_{light} = 5.78^{**}$; $F^5_{month} = 13.36^{***}$, $F^2_{light} = 1.96$, ns. for *Q. petraea* and *P. serotina*, respectively; ns. – not significant). The mean values of Car in *Q. petraea* leaves were 0.020±0.001, 0.021±0.001, 0.018±0.001 and in *P. serotina* 0.012±0.0004, 0.012±0.0004 and 0.013±0.0004 g m^{-2} in LL, ML and HL.

PP content was correlated with air temperature, but not with *RH* (Table 2). An increase in total Chl content in *Q. petraea* leaf with T_{min} was explained at 82% by linear regression. Similarly, total Chl content and Car increased with $T_{min(5)}$ and T_{mean}. The higher temperature amplitudes, inversely, decreased PP content in *Q. petraea* leaves, but not in *P. serotina* leaves. The latter did not show correlation between temperature and Car. Total Chl content was correlated with T_{min}, $T_{min(5)}$, T_{mean}, $T_{mean(5)}$ and $T_{max(5)}$, but less significantly when compared with *Q. petraea*.

Models for conversion from SPAD into physiological units

The SPAD *vs.* PP content relationship was earlier described by different models depending on the spe-

Table 2. Coefficients of determination (r^2) with associated probabilities (***: $p<0.001$) for the linear regression between the values of different temperatures and total chlorophyll [chl (a + b)] or carotenoids content determined spectrophotometrically in leaves of *Quercus petraea* and *Prunus serotina* ($n = 16$ for *Q. petraea*, $n = 18$ for *P. serotina*). A decreasing trend of the relationship was shown with "(–)". T_{mean} – monthly mean temperature, T_{max} monthly maximal temperature, T_{min} – monthly minimal temperature, $T_{mean(5)}$ – moving average temperature calculated from five days before chlorophyll analysis, $T_{max(5)}$ – moving average maximal temperature, $T_{min(5)}$ – moving average minimal temperature

Species	Temperature [°C]	Chl (a + b) [g m^{-2}]	Car [g m^{-2}]
Quercus petraea	T_{mean}	0.53**	0.52**
	T_{max}	0.01, ns.	0.03, ns.
	T_{min}	0.82***	0.75***
	$T_{max}-T_{min}$	0.40* (–)	0.23* (–)
	$T_{mean(5)}$	0.56**	0.50**
	$T_{max(5)}$	0.34*	0.34*
	$T_{min(5)}$	0.69***	0.70***
Prunus serotina	T_{mean}	0.31*	0.00, ns.
	T_{max}	0.22, ns.	0.00, ns.
	T_{min}	0.36**	0.00, ns.
	$T_{max}-T_{min}$	0.00, ns.	0.00, ns.
	$T_{mean(5)}$	0.31*	0.00, ns.
	$T_{max(5)}$	0.24*	0.00, ns.
	$T_{min(5)}$	0.42**	0.00, ns.

cies and experimental conditions (Table 3). In this study these models have been tested using the data gathered in the present experiment and compared with new models. The relationship between Chl ($a + b$), Chl a, Chl b, Car and SPAD units for *P. serotina* and *Q. petraea* was best fitted with the quadratic equation (y = ax^2 + bx + c) (Fig. 2). In Table 4 the new models together with the coefficients of determination (r^2) and the prediction errors (RMSE) describing relationships between SPAD measurements and PP content in leaves of the study species are shown. All the relationships were very highly significant ($p<0.001$). The highest values of r^2 were obtained for Chl ($a + b$) and for Chl a in both study species. The stronger relationship between the SPAD values and chlorophylls content was shown for *P. serotina* than for *Q. petraea* ($r^2=0.95$ and $r^2=0.84$, respectively). For both species there was a less significant relationship between SPAD and Car ($r^2=0.70$ for *P. serotina*, $r^2=0.76$ for *Q. petraea*) compared with the SPAD-chlorophylls relationship. This difference may partially result from the linear regressions between
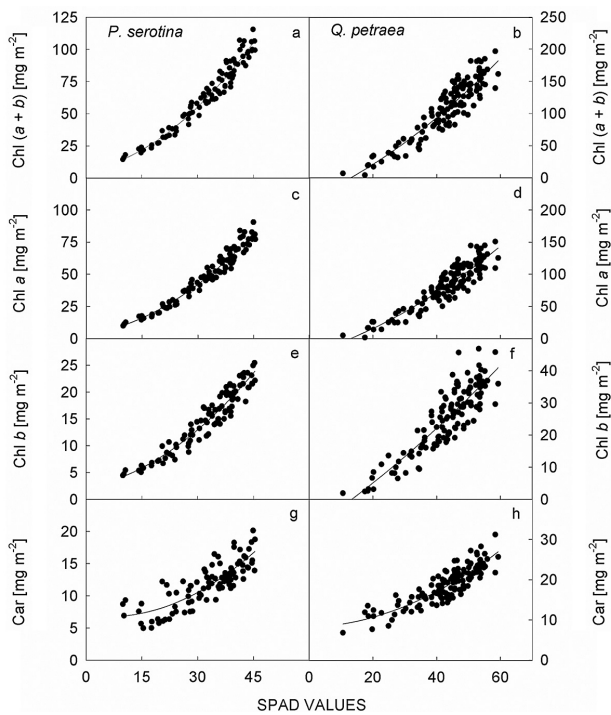
Fig. 2 Relationship between SPAD values and Chl (a + b) (a, b), Chl a (c, d), Chl b (e, f) and Car (g, h) in leaves of two tree species: *Prunus serotina* (a, c, e, g) and *Quercus petraea* (b, d, f, h). The second-order polynomial functions were found to fit the data most closely (for equations, corresponding r^2 and RMSE values, see Table 2)

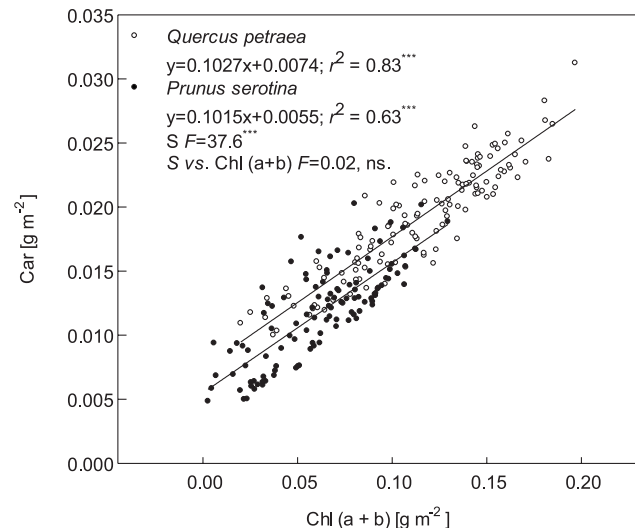Fig. 3 Relationship between leaf-area based content of total chlorophyll and carotenoids in Prunus serotina and Quercus petraea seedlings. The slopes comparison based on the model of the same slopes (S vs. Chl (a + b), ns. – not significant) together with analysis of covariance results for species (S) are shown. Equations of linear regression, coefficients of determinations (r^2) and associated probabilities are given (n = 128 for *Q. petraea*, n = 114 for *P. serotina*)

Chl (*a* + *b*) and Car determined with the extraction method which did not explain 17% of variance in *Q. petraea* and 37% in *P. serotina* (Fig. 3). The slopes of the regression lines did not differ significantly in ANCOVA indicating that an increase in total Chl content was associated with a corresponding increase in Car at the same rate in both study species.

When the values of SPAD normalized with LMA were used for the regression model, the relationship between the SPAD values and chlorophyll content was not improved (Table 4). The significant non-linear relationship was found for *Q. petraea* (Fig. 4), but the values of r^2 were lower by 33% for Chl (*a+b*) and by 57% for Car compared with those obtained with the non-normalized SPAD values (Table 4). The re-

lationship between the PP contents and SPAD/LMA quotient for *P. serotina* was not significant.

Discussion

The chlorophylls and Car contents in leaf of *Q. petraea* and *P. serotina* determined with the conventional extraction method (Hiscox and Israelstam 1979; Wellburn 1994) were significantly correlated with the SPAD values measured using the Chl meter SPAD-502Plus. The relationship between the SPAD values and PP content was best fitted with the polynomial quadratic function. The similar functions (but with different values of parameters) modeling the SPAD *vs*. Chl (*a* + *b*) relationship were used for *Arabidopsis thaliana* (L.) Heynh. (Ling et al. 2011), *Coffea canephora* Pierre (Netto et al. 2005) and *Fagus*

Table 3. A list of models published earlier which were tested for *Q. petraea* and *P. serotina* in the present study

Species	Reference	Model[*]	Correlated parameters	Units
Eucalyptus globulus	Pinkard. *et al.* 2006	y = exp (– 6.49+1.46lnx)	Chl (*a+b*) and SPAD	μg cm^{-2}
Betula pendula	Uddling *et al.* 2007	y = 0.0641 e$^{0.0467x}$	Chl (*a+b*) and SPAD	g m^{-2}
Coffea canephora	Netto *et al.* 2005	y = 0.0933x^2+0.7188x+44.5885	Chl (*a+b*) and SPAD	μmol m^{-2}
Coffea canephora	Netto *et al.* 2005	y = 0.021x^2–0.8595x+42.6458	Car and SPAD	μmol m^{-2}
Malus domestica	Campbell *et al.* 1990	y = 2.37x–83	Chl (*a+b*) and SPAD	μg cm^{-2}
Quercus robur	Percival *et. al.* 2008	y = 1.8159x$^{0.8809}$	Chl (*a+b*) and SPAD	μg g^{-1}
Quercus robur	Percival *et. al.* 2008	y = 0.3096x$^{0.723}$	Car and SPAD	μg g^{-1}
13 neotropical trees	Coste *et al.* 2010	y = 117.1x/(148.84–x)	Chl (*a+b*) and SPAD	μg cm^{-2}

[*]When the models were used for *Q. petraea* and *P. serotina*, the values of r^2 ranged from 0.50 (carotenoids, *P. serotina*) to 0.95 (total chlorophyll, *P. serotina*) depending on the model applied.

Table 4. Relationships between Chl ($a + b$), Chl a, Chl b, Car and SPAD values in leaves of *Quercus petraea* and *Prunus serotina*. RMSE – root-mean-square error; r^2 – coefficient of determination; ***: $p<0.001$

Species	Parameter correlated with SPAD	Equation	r^2	RMSE
P. serotina	Chl ($a+b$)	$y = 0.0374x^2+0.5345x+0.5137$	0.95***	5.66
	Chl a	$y = 0.0297x^2+0.4069x+3.3232$	0.95***	4.46
	Chl b	$y = 0.0077x^2+0.1276x+2.2128$	0.92***	1.64
	Car	$y = 0.0065x^2–0.0752x+6.9603$	0.70***	1.88
Q. petraea	Chl ($a+b$)	$y = 0.024x^2+2.1998x–32.7866$	0.84***	17.87
	Chl a	$y = 0.0207x^2+1.5421x–23.4546$	0.84***	13.92
	Chl b	$y = 0.0032x^2+0.6609x–9.3674$	0.78***	4.85
	Car	$y = 0.0048x^2+0.0296x+8.1290$	0.76***	2.26
	Parameter correlated with SPAD/LMA quotient			
P. serotina	Chl ($a+b$)	–	n.s.	–
	Car	–	n.s.	–
Q. petraea	Chl ($a+b$)	$y = 722.3151^x –553.0973$	0.56***	29.14
	Car	$y = 58.7107^x –34.9605$	0.33***	3.79

sylvatica L. (Percival et al. 2008) (Tables 3, 4). For the most of earlier investigated species the non-linear functions showed the highest goodness of fit. The linear regression was found to be significant for *Sorghum bicolor* (L.) Moench, *Cajanus cajan* (L.) Millsp (Yamamoto et al. 2002) and *Malus domestica* Borkh. (Campbell et al. 1990), and some other tree species (Samsone et al. 2007).

The models of the relationship between the SPAD values and Car developed for *Q. petraea* and *P. serotina* were not so well fitted as those between the SPAD and total Chl content. It resulted mainly from the application of wavelength 650 nm in the Chl meter which was absorbed by chlorophylls *a* and *b*, but not Car. It seems that indirect Car quantification could be obtained for the study species with the Chl meter due to the significant linear relationship between total Chl content and Car determined spectrophotometrically for both species (Fig. 3). However, the statistical significance of this relation was lower for *P. serotina* than *Q. petraea* indicating that determination of Car with the Chl meter can differ in accuracy between the species. A highly significant linear regression between total Chl and Car in coffee leaves
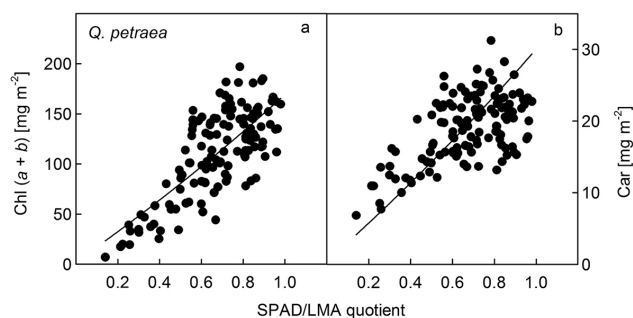

Fig. 4 Relationship between the SPAD/LMA and Chl (a + b) (a) and Car (b) in leaves of *Quercus petraea*. The exponential functions had the highest goodness of fit. For equations, r^2 and RMSE values, see Table 4

($r^2 = 0.91$) was also an argument to use the SPAD-502 meter for an indirect Car estimation (Netto et al. 2005).

Total Chl and Car contents were higher in *Q. petraea* than in *P. serotina* leaves except for October and November (Fig. 1, 3). This difference can result from species-specific genetic traits related to leaf phenology (Uddling et al. 2007), but also from the contrasting life strategies: *P. serotina* is invasive and fast-growing, whereas *Q. petraea* is a conservative and slowly-growing species (Loehle 1988; Closset-Kopp et al. 2007). Chlorophylls in *P. serotina* leaves were better correlated with the SPAD values than that in *Q. petraea* leaves. This result confirms that the Chl meter has to be calibrated for each species, ecotype or even cultivar (Yamamoto et al. 2002; Fritschi and Ray 2007; Marenco et al. 2009). However, the reasons for the inter-specific differences are not clear (Uddling et al. 2007). They may be caused by species-specific leaf structure, adaptation to the local light environments, nutrients availability and other abiotic and biotic factors which may modify leaf optical properties. Additionally, the SPAD readings can be significantly affected by the sieve effect and optical path lengthening caused by high heterogeneity in Chl distribution within a leaf (Uddling et al. 2007; Marenco et al. 2009; Nauš et al. 2010). In our study species, the differences in the relationship between the SPAD readings and real Chl content cannot rather be attributed to the chloroplast movement because the measurements were conducted at the same time on each occasion.

Varying leaf thickness affected the precision of SPAD-502 meter readings (Sardoei et al. 2014). Our results indicated that the correlation between the SPAD values normalized with LMA was contingent on the species. They confirmed also that differences in LMA among leaves may considerably influence the correlation between the SPAD readings and chlorophyll content in leaf.

In the first year of growth, *Q. petraea* seedlings grew slowly in height and produced a few leaves in May and June, especially in LL and ML, whereas more biomass was invested into roots. In contrast, *P. serotina* seedlings were continually growing within the whole vegetative season from May to November and produced a great number of leaves also in July, August and September (Bielinis et al. 2012a; Robakowski et al. 2012). Moreover, already in September, *Q. petraea* leaves showed an abrupt decrease in total Chl content, yellowish and brownish discolouration, whereas those of *P. serotina* decreased total Chl, became yellowish and reddish till in October (Fig. 1). *Q. petraea* leaves appeared 2–3 weeks earlier in spring than *P. serotina* leaves, thus, from the beginning of growth they were older and showed earlier symptoms of senescence. In contrast, it was observed that *P. serotina* was able to maintain some green, photosynthetically functional leaves during the whole winter (Bielinis et al. 2012b). The significant difference in SPAD values were also found between different aged leaves of dominant species in *Castanopsis carlessi* forest (Wang et al. 2009).

In our experiment, plants were grown in pots filled with the uniform substrate and were watered and fertilized, therefore effects of microclimate conditions of growth on PP content were not modified by soil conditions. Shade increased total Chl content in leaves of both species, however, plasticity of the chlorophyll content response to the light regime was higher in *Q. petraea* compared with *P. serotina* (27 and 19%, respectively, plasticity index:

$$\frac{\text{Maximal total Chl} - \text{Minimal total Chl}}{\text{Maximal total Chl}} \times 100$$

(Valladares et al. 2002).

Our results indicated that total Chl content and Car in leaves were positively correlated with air temperature, in particular with T_{min} and $T_{min(5)}$ except for Car in *P. serotina* leaves (Table 2). The reduction of chlorophyll content in leaf induced by low temperatures and light was shown in conifers (Adams and Demmig-Adams 1994; Adams et al. 2004; Robakowski 2005). In the present study, there was evidence that temperature and light influence PP content and the relationship between SPAD values, total Chl and Car. Therefore, when different taxa or experimental treatments are compared with regard to PP content at different dates of measurements, climate conditions should be monitored. Interestingly, PP content decreased in *Q. petraea,* but not in *P. serotina* leaves with increasing monthly temperature amplitudes. This interspecific difference in response to stress of temperature amplitudes may give an advantage to invasive *P. serotina* growing in competition with species that are more sensitive to temperature amplitudes. We cannot exclude a pseudo-correlation between $T_{max} - T_{min}$ and T_{min} or T_{max}, but on the other hand, the stress effect caused by high temperature amplitudes can be more remarkable than that caused by low or high temperature which encouraged us to test the correlation between the temperature amplitudes and leaf chlorophyll content.

In late summer, in our experiment, translocation of nitrogen and other resources took place from *Q. petraea* leaves to other organs more intensively than from *P. serotina* leaves (Robakowski et al. 2012). The nitrogen translocation from leaves together with discolouration and necrosis affected the SPAD readings and their relationship with the chemically determined pigments content in *Q. petraea* leaves more significantly compared with *P. serotina*. Additionally, in August and September a thin white layer of pathogenic fungus [*Erysiphe alphitoides* (Griffon & Maubl.) U. Braun & S. Takam.] was observed on some *Q. petraea* leaves and less frequently on *P. serotina* leaves (Bielinis et al. 2012a), which also changed leaf transmittance and, as a consequence, affected the SPAD readings and their relation with Chl content.

The relationship between the SPAD values and PP content allows the application of the Chl meter SPAD-502Plus to determine Chl ($a + b$) and Car content in leaves of *Q. petraea* and *P. serotina* seedlings using the equations presented. Car can be determined less precisely using the Chl meter compared with the assessment of Chl ($a + b$). When PP content in leaf is monitored within a time at different climate conditions in a forest nursery or plantation, meteorological parameters need to be simultaneously measured and their effect on PP content determined. Abiotic and biotic factors affecting leaves may change the relationship between Chl ($a + b$) and Car, and leaf optical proprieties independently of the imposed experimental conditions. Therefore, the data obtained from the Chl meter should be interpreted cautiously with respect to the environmental conditions of a plant's growth.

Acknowledgements

References

Adams W.W., Demmig-Adams B. 1994. Carotenoid composition and down regulation of photosystem II in three conifer species during the winter. Physiologia Plantarum 92: 451–458.

Adams W.W., Zarter C.R., Ebbert V., Demmig-Adams B. 2004. Photoprotective strategies of overwintering evergreens. BioScience 54: 41–49.

Anderson P.D., Tomlinson P.T. 1998. Ontogeny affects response of northern red oak seedlings to elevated CO_2 and water stress. I. Carbon assimilation and biomass production. New Phytologist 140: 477–491.

Bauerle W.L., Weston D.J., Bowden J.D., Dudley J.B., Toler J.E. 2004. Leaf absorptance of photosynthetically active radiation in relation to chlorophyll meter estimates among woody plant species. Scientia Horticulturae 101: 169–178.

Bielinis E., Robakowski P., Bułaj B. 2012a. Effects of different light conditions and between-species interactions on photosynthesis, respiration and biomass allocation in *Quercus petraea* and *Prunus serotina* seedlings. In: 9th International Conference "Plant functioning under environmental stress", September 12–15, Cracow, Poland. Book of abstracts. Acta Physiologiae Plantarum 34 Suppl : 1–116.

Bielinis E., Robakowski P., Wyka T., Samardakiewicz S. 2012b. Why does black cherry maintain some leaves during the winter? In: 9th International Conference "Plant functioning under environmental stress", September 12–15, 2012 Cracow, Poland. Book of abstracts. Acta Physiologiae Plantarum 34Suppl 1: 1–116.

Campbell R.J., Mobley K.N., Marini R.P, Pfeiffer D.G. 1990. Growing conditions alter the relationship between SPAD-501 values and apple leaf chlorophyll. HortScience 25: 330–331.

Canham C.D., Kobe R.K., Latty E.F., Chazdon R.L. 1999. Interspecific and intraspecific variation in tree seedling survival: effects of allocation to roots versus carbohydrate reserves. Oecologia 121: 1–11.

Cate T.M., Perkins T.D. 2003. Chlorophyll content monitoring in sugar maple (*Acer saccharum*). Tree Physiology 23: 1077–1079.

Chlorophyll Meter SPAD-502Plus 2009. Instruction Manual, Konica Minolta Sensing, Inc., Japan.

Closset-Kopp D., Chabrerie O., Valentin B., Delachapelle H., Decocq G. 2007. When Oskar meets Alice: Does a lack of trade-off in r/K-strategies make *Prunus serotina* a successful invader of European forests? Forest Ecology and Management 247: 120–130.

Coste S., Baraloto C., Leroy C., Marcon E., Renaud A., Richardson A.D., Roggy J.-Ch., Schimann H., Uddling J., Herault B. 2010. Assessing foliar chlorophyll contents with the SPAD-502 chlorophyll meter: a calibration test with thirteen tree species of tropical rainforest in French Guiana. Annals of Forest Sciences 67: 607p1–607p5.

Demmig-Adams B., Winter K., Krüger A., Czygan F.-C. 1989. Light response of CO_2 assimilation, dissipation of excess excitation energy, and ze-axanthin content of sun and shade leaves. Plant Physiology 90: 881–886.

Fritschi F.B., Ray J.D. 2007. Soybean leaf nitrogen, chlorophyll content, and chlorophyll *a/b* ratio. Photosynthetica 45: 92–98.

Gáborčik N. 2003. Relationship between contents of chlorophyll ($a+b$) (SPAD values) and nitrogen of some temperate grasses. Photosynthetica 41: 285–287.

Gratani L. 1993. A non-destructive method to determine chlorophyll content of leaves. Photosynthetica 26: 469–473.

Hawkins T.S., Gardiner E.S., Comer G.S. 2009. Modeling the relationship between extractable chlorophyll and SPAD-502 readings for endangered plant species research. Journal for Nature Conservation 17: 123–127.

Hiscox J.D., Israelstam G.F. 1979. A method for the extraction of chlorophyll from leaf tissue without maceration. Canadian Journal of Botany 57: 1332–1334.

Koutika L.S., Vanderhoeven S., Chapuis-Lardy L., Dassonville N., Meerts P. 2007. Assessment of changes in soil organic matter after invasion by exotic plant species. Biology and Fertility of Soils 44: 331–341.

Ling Q., Huang W., Jarvis P. 2011. Use of a SPAD-502 meter to measure leaf chlorophyll concentration in *Arabidopsis thaliana*. Photosynthesis Research 107: 209–214.

Loehle C. 1988. Tree life history strategies: the role of defenses. Canadian Journal of Forest Research 18: 209–222.

Main R., Cho M.A., Mathieu R., O'Kennedy M., Ramoelo A., Koch S. 2011. An investigation into robust spectral indices for leaf chlorophyll estimation. ISPRS Journal of Photogrammetry and Remote Sensing 66: 751–761.

Marenco R.A., Antezana-Vera S.A., Nascimento H.C.S. 2009. Relationship between specific leaf area, leaf thickness, leaf water content and SPAD-502 readings in six Amazonian tree species. Photosynthetica 47: 184–190.

Markwell J., Osterman J.C., Mitchell J.L. 1995. Calibration of the Minolta SPAD-502 leaf chlorophyll meter. Photosynthesis Research 46: 467–472.

Marquis A.D. 1990. *Prunus serotina* Ehrh. Black Cherry. In: Silvics of North America: 2. Hardwoods – Agriculture Handbook. Burns R.M., Honkala B.H. (eds.). Department of Agriculture, U.S. Forest Service, Washington, USA.

Merzlyak M.N., Gitelson A.A. 1995. Why and what for the leaves are yellow in autumn? On the interpretation of optical spectra of senescing leaves (*Acer platanoides* L.). Journal of Plant Physiology 145: 315–320.

Moran J.A., Mitchell A.K., Goodmanson G., Stock-burguer K.A. 2000. Differentiation among effects of nitrogen fertilization treatments on conifer seedlings by foliar reflectance: a comparison of methods. Tree Physiology 20: 1113–1120.

Nauš J., Prokopová J., Řebíček J., Špundová M. 2010. SPAD chlorophyll meter reading can be pronouncedly affected by chloroplast movement. Photosynthesis Research 105: 265–271.

Netto A.T., Campostrini E., Oliveira J.G.D., Bressan-Smith R.E. 2005. Photosynthetic pigments, nitrogen, chlorophyll *a* fluorescence and SPAD-502 readings in coffee leaves. Scientia Horticulturae 104: 199–209.

Niyogi K.K. 1999. Photoprotection revisited: genetic and molecular approaches. Annual Review of Plant Physiology and Plant Molecular Biology 50: 333–359.

Öquist G., Huner N.P.A. 2003. Photosynthesis of overwintering evergreen plants. Annual Review of Plant Biology 54: 329–355.

Peñuelas J., Filella I. 1998. Visible and near-infrared reflectance techniques for diagnosing plant physiological status. Trends in Plant Science 3: 151–156.

Percival G.C., Keary I.P., Noviss K. 2008. The Potential of a Chlorophyll Content SPAD Meter to Quantify Nutrient Stress in Foliar Tissue of Sycamore (*Acer pseudoplatanus*), English Oak, (*Quercus robur*), and European Beech (*Fagus sylvatica*). Arboriculture and Urban Forestry 34: 89–100.

Pinkard E.A., Patel V., Mohammed C. 2006. Chlorophyll and nitrogen determination for plantation-grown *Eucalyptus nitens* and *E. globulus* using a non-destructive meter. Forest Ecology and Management 223: 211–217.

Porra R.J., Thompson W.A., Kriedemann P.E. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. BBA Bioenergetics 975: 384–394.

Robakowski P. 2005. Susceptibility to low-temperature photoinhibition in three conifers differing in successional status. Tree Physiology 25: 1151–1160.

Robakowski P., Stachnowicz W. 2005. *Padus serotina*. In: CAB International. Forestry Compendium Global Module, CD ROM edition. Wallingford, Oxon, UK.

Robakowski P., Bielinis E., Stachowiak J., Bułaj B. 2012. Growth and allelochemical interactions among *Quercus petraea* and *Prunus serotina* seedlings in different light conditions. In: 9th International Conference "Plant functioning under environmental stress", September 12–15, 2012 Cracow, Poland. Book of abstracts. Acta Physiologiae Plantarum 34 Suppl : 1–116.

Samsone I., Andersone U., Vikmane M., Ievina B., Pakarna G., Ievinsh G. 2007. Nondestructive methods in plant biology: an accurate measurement of chlorophyll content by a chlorophyll meter. Acta Universitatis Latviensis 723: 145–154.

Sardoei A.S., Rahbarian P., Shahdadneghad M. 2014. Evaluation chlorophyll contents assessment on three indoor ornamental plants with plant growth regulators. European Journal of Experimental Biology 4: 306–310.

Schlemmer M.R., Francis D.D., Shanahan J.F., Schepers J.S. 2005. Remotely measuring chlorophyll content in corn leaves with differing nitrogen levels and relative water content. Agronomy Journal 97: 106–112.

Uddling J., Gelang-Alfredsson J., Piikki K., Pleijel H. 2007. Evaluating the relationship between leaf chlorophyll concentration and SPAD-502 chlorophyll meter readings. Photosynthesis Research 91: 37–46.

Valladares F., Chico J.M., Aranda I., Balaguer L., Dizengremel E.M., Dreyer E. 2002. The greater seedling high-light tolerance of *Quercus robur* over *Fagus sylvatica* is linked to a greater physiological plasticity. Trees 16: 395–403.

Wang H., Gu M., Cui J., Shi K., Zhou Y. 2009. Effects of light quality on CO_2 assimilation, chlorophyll-fluorescence quenching, expression of Calvin cycle genes and carbohydrate accumulation in *Cucumis sativus*. Journal of Photochemistry and Photobiology B-Biology 96: 30–37.

Wellburn A.R. 1994. The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. Journal of Plant Physiology 144: 307–313.

Wyka T., Robakowski P., Żytkowiak R. 2007. Acclimation of leaves to contrasting irradiance in juvenile trees differing in shade tolerance. Tree Physiology 27: 1293–1306.

Yamamoto A., Nakamura T., Adu-Gyamfi J.J., Saigusa M. 2002. Relationship between chlorophyll content in leaves of sorghum and pigeonpea determined by extraction method and by chlorophyll meter (SPAD-502). Journal of Plant Nutrition 25: 2295–2301.

Zhao D.L., Reddy K.R., Kakani V.G., Reddy V.R. 2005. Nitrogen deficiency effects on plant growth, leaf photosynthesis, and hyperspectral reflectance properties of sorghum. European Journal of Agronomy 22: 391–403.