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Predictive capability of a leaf optical meter for determining leaf pigment status during senescence

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Abstract

We conducted an experiment to assess the predictive capability of a leaf optical meter for determining leaf pigment status of *Acer mono* Maxim., *A. ginnala* Maxim., *Quercus mongolica* Fisch., and *Cornus alba* displaying a range of visually different leaf colors during senescence. Concentrations of chlorophyll (Chl) *a*, Chl *b*, and total Chl [*i.e.*, Chl (*a*+*b*)] decreased while the concentration of carotenoids (Car) remained relatively static for all species as leaf development continued from maturity to senescence. *C. alba* exhibited the lowest average concentration of Chl (*a*+*b*), Chl *a*, and Car, but the highest relative anthocyanin concentration, while *Q. mongolica* exhibited the highest Chl (*a*+*b*), Chl *b*, and the lowest relative anthocyanin concentration. *A. mono* exhibited the highest Chl *a* and Car concentrations. The relationships between leaf pigments and the values measured by the optical meter generally followed an exponential function. The strongest relationships between leaf pigments and optical measurements were for *A. mono*, *A. ginnala*, and *Q. mongolica* (R^2 ranged from 0.64 to 0.95), and the weakest relationships were for *C. alba* (R^2 ranged from 0.13 to 0.67). Moreover, optical measurements were more strongly related to Chl *a* than to Chl *b* or Chl (*a*+*b*). Optical measurements were not related to Car or relative anthocyanin concentrations. We predicted that weak relationships between leaf pigments and optical measurements would occur under very low Chl concentrations or under very high anthocyanin concentrations; however, these factors could not explain the weak relationship between Chl and optical measurements observed in *C. alba*. Overall, our results indicated that an optical meter can accurately estimate leaf pigment concentrations during leaf senescence — a time when pigment concentrations are dynamically changing — but that the accuracy of the estimate varies across species. Future research should investigate how species-specific leaf traits may influence the accuracy of pigment estimates derived from optical meters.

Additional key words: absorption; anthocyanin; calibration; carotenoid; chlorophyll; leaf properties; reflectance; SPAD; transmittance.

Introduction

Chlorophyll (Chl) is the primary pigment responsible for absorbing the light energy that drives photosynthesis. The concentration of Chl in a leaf can directly limit photosynthetic potential. Likewise, since the amount of solar radiation absorbed by a leaf is a function of the foliar quantity of photosynthetic pigments (Richardson *et al*. 2002), total canopy Chl content is linearly related to gross primary production (Gitelson *et al*. 2006). In addition, Chl concentration can provide an indirect estimate of foliar nutrient status because much of the nitrogen in leaves is incorporated in Chl (van den Berg and Perkins 2004). Further, foliar Chl content is a good indicator of various biotic and abiotic stresses, such as one-year old apple trees infested with spirea aphid (*Aphis spiraecola*) (Kaakeh *et*

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Abbreviations: Ag – Acer ginnala; *Am – Acer mono*; Ant – anthocyanin; *Ca – Cornus alba*; C_{Ant} – relative anthocyanin concentration; Car – carotenoids; Chl – chlorophyll; DMSO – dimethylsulphoxide; DOY – day of year; FM – fresh mass; MAT – minimum air temperature; OD – optical density readings; *Qm – Quercus mongolica*; R^2 – coefficient of determination; SPAD – unitless value obtained with the *SPAD502* optical meter.
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al. 1992), fluttering elm (*Ulmus laevis* Pall.) infested with *Colopha compressa* Koch. (Homoptera: Aphididae), large-leaved linden (*Tilia platyphyllos* L.) infested with *Eriophyes tiliae* Pgst. (Acarina: Eriophydae), and crack willow (*Salix fragilis* L.) infested with *Pontania vesicator* Br. (Hymenoptera: Tenthredinidae) (Samsone *et al*. 2007), ozone-affected leaves of cutleaf coneflower (*Rudbeckia laciniata* var. *digitata*) (Neufeld *et al*. 2006), nitrogen stress in foliar tissue of sycamore (*Acer pseudoplatanus*), English oak (*Quercus robur*), European beech (*Fagus sylvatica*) (Percival *et al*. 2008), citrus rootstock for tolerance to bicarbonate-induced iron chlorosis (Sudahono *et al*. 1994), and senescence process (Biber 2007). Therefore, the indirect, timely and nondestructive detection of leaf Chl concentration is of great importance for a variety of plant studies.

Traditionally, foliar Chl concentration [Chl *a*, Chl *b*, and Chl (*a*+*b*)] has been measured after extraction of leaf tissue with acetone, N,N-dimethylformamide (DMF), or dimethylsulphoxide (DMSO), followed by spectrophotometric measurements. Although the extraction method and other similar techniques are still widely employed (Minocha *et al*. 2009), these approaches have a number of drawbacks. Specifically, the extraction method is destructive, time consuming, and expensive. Further, substantial pigment losses from the sample tissue may occur during transport from the field to the laboratory, sample preparation, the extraction and dilution, thus introducing a potentially large amount of errors into the results (Torres Netto *et al*. 2005).

Most of the drawbacks inherent to the extraction method, especially the errors associated with pigment loss, which may potentially occur, and compound at each step of the extraction process, can be eliminated through the use of commercially available portable optical meters that can instantaneously assess the Chl status of intact leaves. Indeed, there are several optical meters currently available, such as the *CCM200* meter (*Opti-Sciences*, Tyngsboro, MA, USA), *CL01* (*Hansatech*, King's Lynn, Norfolk, England), and the *atLEAF* meter (*FT Green LLC*, Wilmington, DE, USA). The most commonly used hand-held optical meter for research appears to be the *SPAD502* meter (*Soil Plant Analyses Development*, *Minolta Camera Co. Ltd*., Japan). Briefly, the meter records the difference in absorbance of radiation through an intact or detached leaf at 650 nm (*i.e.*, the peak absorbance wavelength for Chl) and 940 nm (*i.e.*, a reference wavelength for adjusting the differences in leaf internal structure, such as leaf thickness, water status, and other factors that may affect photon passage through the leaf). The meter converts such absorbance relationships into a unitless value simply referred to as "SPAD", which ranges from 0 to 100 as an expression of the relative Chl quantity in a leaf. Although the values derived from optical meters, such as SPAD, cannot be directly translated to foliar Chl concentration, they can be indirectly related to Chl concentrations measured *via* traditional extraction methods, thereby providing a quick and efficient estimate of foliar Chl concentration once these relationships have been established. However, there is not broad utility in this approach as the relationship between SPAD and foliar Chl concentration appears to be species-dependent (Schaper and Chacko 1991, Richardson *et al*. 2002, Wang *et al*. 2005, Pinkard *et al*. 2006, Biber 2007, Samsone *et al*. 2007, Marenco *et al*. 2009) and does not necessarily hold up across cultivars within the same species (Jifon *et al*. 2005, Hawkins *et al*. 2009). Further, the relationship between SPAD and foliar Chl concentration may also change throughout development within a single species (Anand and Byju 2008).

Senescence represents the final stage of leaf development and the absolute and relative changes in leaf pigment concentrations, which are associated with the observable color changes during senescence, may influence the relationship between SPAD and foliar Chl concentration. In general, the degradation of Chl occurs at a greater rate than that of Car, resulting in yellow coloration (Moy *et al*. 2015). Conversely, anthocyanin (Ant) concentration increases during senescence in some species, resulting in red coloration (Feild *et al*. 2001, Landi *et al*. 2015). It is this increase in Ant concentration that may influence the relationship between SPAD and the foliar Chl concentration. For example, Hlavinka *et al*. (2013) demonstrated that relatively high Ant concentrations substantially affected the relationship between Chl content and SPAD values measured from mature to partly senescent leaves of tomato (*Solanum lycopersicum* L.) with low Chl content (about 0–100 mg m^{-2}, SPAD < 20) compared to mature green leaves. Dwyer *et al.* (1991) found that the negative Chl concentrations for fully senesced leaves of corn hybrid Pride 5 indicated that the relationship between the *SPAD 501* reading and Chl concentration was poor at very low Chl concentrations. They advised caution in using the calibration at very low Chl concentrations such as those occurring as a plant approaches senescence. However, Manetas *et al*. (1998) found that relationships between SPAD and actual Chl content were not affected by the amount of Ant in young leaves of eucalypt (*Eucalyptus* sp.), rose (*Rosa* sp.), and castor bean (*Rixnus communis*). Similarly, Cate and Perkins (2003) found that leaves of sugar maple (*Acer saccharum*) expressing a range of colors exhibited a strong relationship between foliar Chl concentration and the Chl status index provided by an optical meter regardless of Ant concentrations. Despite these inconsistencies regarding the effect of Ant concentration on the relationship between SPAD and foliar Chl concentration, we are aware of only one study (Cate and Perkins 2003) that has investigated this relationship during senescence – the developmental stage when absolute and relative Ant concentrations should exert the largest effect on healthy leaves as Chl concentrations decrease markedly.

Here, we examined the relationship between SPAD and foliar Chl concentration in leaves of four tree species

expressing a range of colors during leaf senescence. We hypothesized that the relationship between analytical and optical measurements of leaf Chl status would be influenced by changes in pigment concentrations that occur during senescence. Specifically, we expected that optical measurements would fail to predict leaf Chl status at very low Chl concentrations and at high Ant concentrations. A second objective of our study was to understand how the form of our predictive models would change as a result of the potential changing relationship between analytical and optical measures of leaf Chl status.

Materials and methods

Site description and plant material: The experiment was conducted on the campus of Northeast Forestry University, Harbin, Heilongjiang Province, northeast China (45°42′N, 127°35′E). The regional climate is described as temperate monsoon which is characterized by warm summers, cold winters, a short growing season, and abundant precipitation with the annual average temperature and annual precipitation of 3.5°C and 534 mm, respectively. The zonal vegetation is mixed coniferous and broadleaved forest. We investigated four deciduous tree species: mono maple (*Acer mono* Maxim.; hereafter *Am*), amur maple (*A. ginnala* Maxim.; hereafter *Ag*), Mongolian oak (*Quercus mongolica* Fisch.; hereafter *Qm*), and Siberian dogwood (*Cornus alba*; hereafter *Ca*). We chose these species for our experiment because they exhibit a range of leaf colors during senescence.

Three sample trees of each species were repeatedly sampled on ten occasions between 22 August and 13 October 2014. At each measuring date, six fully developed, outermost leaves from the top third of the south-oriented crown per tree were randomly chosen to measure SPAD and pigment concentrations.

SPAD measurement, extraction of Chl and Ant: Four non-overlapping measurements (two on each side of the midrib) were recorded in the inter-veinal area of each individual leaf (3 trees per species × 6 leaves per tree = 18 leaves per species) using a portable optical meter (*SPAD 502*, *Konica-Minolta*, Osaka, Japan). Major veins were avoided. The mean of the four nonoverlapping measurements was used as the leaf-level SPAD value. Immediately following optical measurements, leaves were removed from trees and placed in plastic bags on ice and then transported to the laboratory for analysis. First, the leaves were scanned (*Model T210*, *Founder Technology Instrument Co. Ltd*., China) to obtain high-resolution images for color analysis. Four leaf discs (diameter = 0.8 cm) were removed from the corresponding locations of the SPAD measurements on each leaf. Leaf discs from nine leaves were used for Chl extraction and nine were used for Ant extraction. All leaf samples were processed within approximately 2 h after being removed from trees.

The Chl extraction followed the protocol described by Wellburn (1994). Briefly, leaf discs were cut into fine strips and 0.1 g of leaf tissue was placed in 10 ml of DMSO and incubated in a water bath at 65°C until leaf tissue lost all color. The absorbance of the solution was measured at 665 (Chl *a*), 649 (Chl *b*), and 480 nm (Car) using a spectrophotometer (*722 N*, *INESA Analytical Instrument Co. LTD*., Shanghai, China). Chl and Car concentrations were determined by applying the absorbance values to the equations reported by Wellburn (1994). Chl (*a*+*b*) was calculated as the sum of Chl *a* and Chl *b*.

The Ant extraction was modified from Pirie and Mullins (1976). Briefly, leaf discs were ground and 0.5 g of leaf tissue was placed in 1% (w/v) HCl in an ethanol and incubated in a water bath at 32°C for 4 h and semiquantitatively measured as Ant equivalents by quantification at 520 nm (C_{Ant}) by optical density readings (OD) with a visible spectrophotometer (*722 N, INESA Analytical Instrument Co. LTD*., Shanghai, China) blanked with an extracting solution.

Statistical analysis: We analyzed species and temporal effects on pigment concentrations and SPAD values using repeated measures analysis of variance (*ANOVA*). Species was treated as a fixed factor, sampling date was treated as the fixed repeated factor, and individual tree – the experimental unit – was treated as a random factor. The mean pigment concentration or SPAD value of all leaves within an individual tree and measurement period was used in the analysis (*i.e*., $n = 3$ experimental units per species). To model the correlation within experimental units over time, we analyzed each response using appropriate candidate covariance structures and used AICC (Burnham and Anderson 1998) to determine which candidate covariance structure best fit each model. Denominator degrees of freedom were estimated according to the *Kenward-Roger*'s method (Kenward and Roger 1997). Treatment means were compared using *Fisher*'s least significant difference test. All analyses were performed using a mixed model procedure (PROC MIXED) of *SAS* (*SAS*, *Version 9.3, SAS Inc*., Cary, NC, USA) with $\alpha = 0.05$.

We explored relationships between SPAD values and leaf pigments [*i.e*., Chl (*a*+*b*), Chl *a*, Chl *b*, Chl (*a*+*b*)/Car ratio, and C_{Ant}] through regression analysis using the curve-fitting procedure of *SPSS 18.0* (*SPSS Inc*., Chicago, IL, USA). We fit relationships using various functions – quadratic polynomial, logarithmic, exponential growth, and simple linear models – and chose the function that provided the highest coefficient of determination (R^2).

Results

The daily minimum air temperature in Harbin decreased from 18 to –2°C during our observation period from 22 August to 13 October (Fig. 1; obtained from China Meteorological Administration). Leaf color changed during the observation period as leaf development continued from maturity to senescence – leaves of *Am* and *Ag* changed from dark green to red, *Qm* from green to yellow, and *Ca* from green to dark red (Fig. 1).

Leaf pigment concentrations changed as leaf development continued from maturity to senescence and often differed among species. For a full description of species trends through time and species comparisons within temporal periods see Table 1S (*supplement available online*). In general, concentrations of Chl *a*, Chl *b*, and Chl (*a*+*b*) significantly decreased during our observation period, as did the corresponding SPAD values (Fig. 2). The

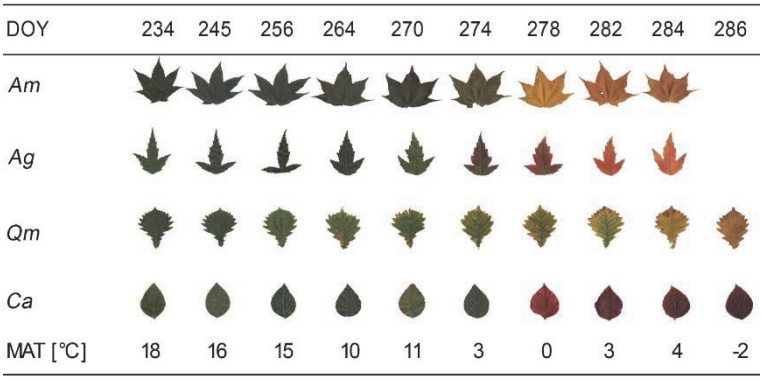

Fig. 1. Leaf color and minimum air temperature (MAT) dynamics during our observation period from day of year (DOY) 234 to 286 for *Acer mono* (*Am*), *A. ginnala* (*Ag*), *Quercus mongolica* (*Qm*), and *Cornus alba* (*Ca*). No leaves remained on trees of *A. mono* or *A. ginnala* by DOY 286 (13 October).

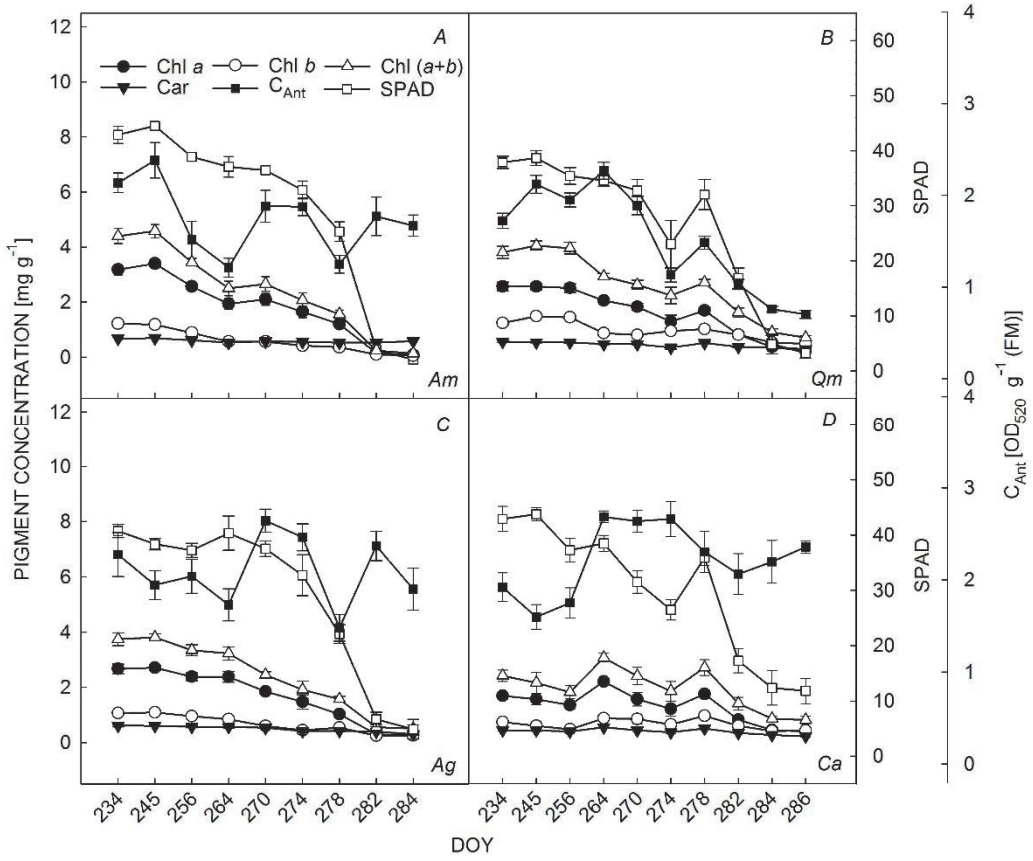

Fig. 2. Mean ± SE chlorophyll (Chl) *a*, Chl *b*, Chl (*a*+*b*), carotenoids (Car) concentrations, relative anthocyanin concentrations (C_{Ant}), and SPAD values measured throughout leaf senescence for *Acer mono* (*Am*), *A. ginnala* (*Ag*), *Quercus mongolica* (*Qm*), and *Cornus alba* (*Ca*) from day of year (DOY) 234 to 286.

Table 1. Mean (± SE) total chlorophyll [Chl (*a*+*b*)], Chl *a*, Chl *b*, carotenoids (Car), relative anthocyanin concentrations (C_{Ant}), the ratio of Chl (*a*+*b*)/Car, and SPAD values for *Acer mono* (*Am*), *A. ginnala* (*Ag*), *Quercus mongolica* (*Qm*), and *Cornus alba* (*Ca*) (*n* = 90 for *Q. mongolica* and *C. alba*, and *n* = 81 for *A. mono* and *A. ginnala*) measured throughout senescence. *Different letters* in columns indicate significant differences at $P \leq 0.05$ using *Tamhane* and *Duncan*'s multiple range test.

Species	Chl (*a*+*b*) [mg g^{-1}]	Chl *a* [mg g^{-1}]	Chl *b* [mg g^{-1}]	Car [mg g^{-1}]	C_{Ant} [OD_{520} g^{-1} (FM)]	Chl (*a*+*b*)/Car	SPAD
Am	2.4 ± 0.2^{ab}	1.8 ± 0.1^{a}	0.6 ± 0.1^{b}	0.6 ± 0.01^{a}	1.8 ± 0.1^{b}	3.97 ± 0.3^{c}	29.7 ± 1.7^{a}
Ag	2.3 ± 0.2^{ab}	1.7 ± 0.1^{ab}	0.7 ± 0.1^{b}	0.5 ± 0.02^{b}	2.1 ± 0.08^{a}	4.4 ± 0.2^{bc}	29.0 ± 1.6^{a}
Qm	2.6 ± 0.1^{a}	1.6 ± 0.1^{ab}	1.0 ± 0.1^{a}	0.5 ± 0.01^{b}	1.5 ± 0.1^{b}	5.5 ± 0.2^{a}	25.9 ± 1.5^{a}
Ca	2.0 ± 0.1^{b}	1.3 ± 0.1^{b}	0.6 ± 0.1^{b}	0.4 ± 0.01^{c}	2.2 ± 0.1^{a}	4.8 ± 0.1^{b}	29.8 ± 1.4^{a}

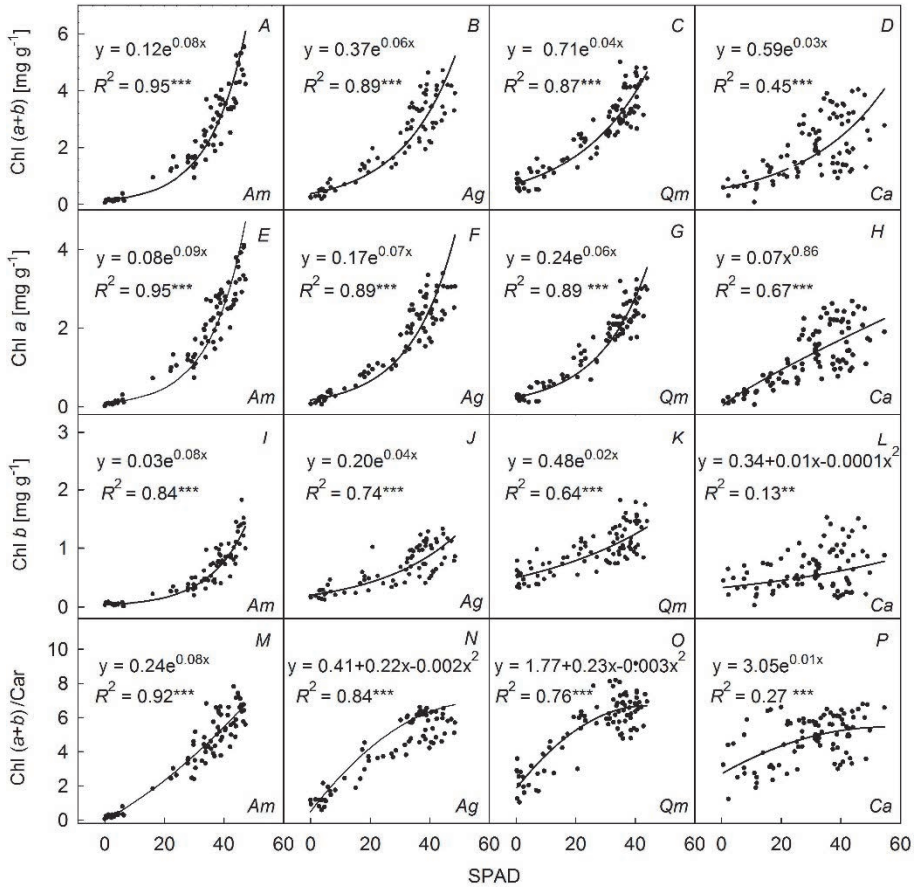

Fig. 3. Relationship between total chlorophyll [Chl (*a*+*b*)], Chl *a*, Chl *b*, the ratio of Chl (*a*+*b*) to carotenoids (Car), and SPAD values for *Acer mono* (*Am*), *A. ginnala* (*Ag*), *Quercus mongolica* (*Qm*), and *Cornus alba* (*Ca*) (*n* = 90 for *Q. mongolica* and *C. alba*, and *n* = 81 for *A. mono* and *A. ginnala*) measured throughout leaf senescence. All lines were significantly different from zero ($P<0.01$).

mean, maximum, and minimum ratio of these different Chl concentrations and SPAD values experienced 22–44, 4–11, 3–12, and 2–6-fold changes for *Am*, *Ag*, *Qm*, and *Ca*, respectively. Car concentrations remained relatively similar during leaf senescence for all tree species. *Ca* had the lowest average concentration of Chl (*a*+*b*), Chl *a*, and Car, the highest C_{Ant} while *Qm* had the highest Chl (*a*+*b*) and Chl *b*, the lowest C_{Ant}, and *Am* had the highest Chl *a* and Car among the four tree species (Table 1).

We found strong positive relationships between many leaf pigment concentrations and SPAD values for all species; however, the strength of the relationship varied among species and pigments (Fig. 3, Table 2). We observed the strongest relationships between SPAD values and pigment concentrations in *Am* (R^2 ranged from 0.84 to 0.95), intermediate relationships in *Ag* and *Qm* (R^2 ranged from 0.64 to 0.89), and the weakest relationships in *Ca* (R^2 ranged from 0.13 to 0.67). We observed some strong positive relationships between relative Ant concentrations and SPAD values for *Am* and *Qm* ($P<0.01$, R^2 ranged from 0.18 to 0.84), but not for *Ag* or *Ca* (Table 2).

Discussion

Our observation period spanned the latter part of leaf development – from maturity to senescence – and thus provided a range of pigment concentrations. This range of pigment concentrations allowed us to explore relationships between analytical and optical measures of pigment concentrations and to further investigate the potential impact of Ant concentration on the capability of optical measurements to accurately estimate Chl concentration. We found that the relationship between the values produced by the *SPAD 502* and the analytical extraction of Chl were very strong for three of the four species we tested. Although previous studies have demonstrated that *SPAD 502* performs poorly at low Chl concentrations (Monje and Bugbee 1992, Uddling *et al*. 2007), we observed that it performed well throughout senescence as Chl concentrations decreased substantially. The weaker relationship between SPAD and Chl concentration in the fourth species (*Ca*) may be due to the higher Ant concentrations measured in this study or, perhaps, due to some other chemical or physical leaf characteristic, which were not measured in our study. Regardless of the ultimate reason for the weaker relationship between SPAD and Chl concentration in the *Ca*, SPAD values were generally much higher (*i.e.*, 3–6 times) in *Ca* than in the other three species which suggests that there was something about those leaves that differed from the others.

Ant concentrations, averaged across the entire observation period, were higher in *Ca* than in two of the other species (*Am* and *Qm*), but not higher than that in *Ag*. This is particularly interesting because *Ag* exhibited a very strong relationship between analytical and optical measures of Chl concentrations even with high Ant concentrations. Given this inconsistency, it is difficult to attribute the poor relationship exhibited by *Ca* entirely to the Ant content. However, a closer look at Fig. 3 suggests that either relative Ant concentration peaked at approximately 3.0 for all of the species or that perhaps our approach to measuring Ant concentration was not able to measure anything above that level. If our approach to measuring Ant failed to account for values above this threshold, then it remains possible that *Ca* could have had higher Ant concentrations than that of *Ag*. Using methods identical to ours, the relative Ant content for *Acer mandshuricum*, *A. triflorum*, *A. ginnala*, and *A. mono* were 3.5, 3.3, 3.5, and 1.0, respectively, during the early October in Harbin (Pang and Zhuo 2007). Moreover, relative Ant contents for three types of fringe flower (*Loropetalum chinense* var. *rubrum*), black purple, red purple, and red ochre were about 4.5 (Tang *et al*. 2006), suggesting that the values observed in our study were below any reported maxima. Therefore, it appears that Ant concentration cannot explain the poor relationship between analytical and optical measures of Chl concentrations.

Table 2. The coefficient of determination R^2 of the relationship between the total chlorophyll [Chl (*a*+*b*)], Chl *a*, Chl *b*, carotenoids (Car), the ratio of Chl (*a*+*b*)/Car, relative anthocyanin concentrations (C_{Ant}), and SPAD values for *Acer mono* (*Am*), *A. ginnala* (*Ag*), *Quercus mongolica* (*Qm*), and *Cornus alba* (*Ca*) during leaf color changing period (ns – not significant, $P>0.05$).

Species	Model	Chl (*a*+*b*)	Chl *a*	Chl *b*	Car	Chl (*a*+*b*)/Car	C_{Ant}
Am	Linear	0.83	0.85	0.71	0.13	0.89	ns
	Quadratic	0.91	0.92	0.85	0.57	0.89	0.18
	Exponential	0.95	0.95	0.84	0.11	0.92	ns
	Power	0.79	0.84	0.63	ns	0.83	ns
	Logarithmic	0.60	0.62	0.39	ns	0.60	ns
Ag	Linear	0.82	0.86	0.64	0.62	0.80	ns
	Quadratic	0.83	0.87	0.64	0.70	0.84	ns
	Exponential	0.89	0.89	0.74	0.63	0.81	ns
	Power	0.61	0.67	0.46	0.44	0.54	ns
	Logarithmic	0.41	0.43	0.34	0.36	0.48	ns
Qm	Linear	0.84	0.89	0.58	0.63	0.72	0.78
	Quadratic	0.85	0.89	0.58	0.71	0.76	0.79
	Exponential	0.87	0.89	0.64	0.59	0.71	0.84
	Power	0.71	0.76	0.50	0.33	0.65	0.69
	Logarithmic	0.59	0.62	0.42	0.35	0.62	0.59
Ca	Linear	0.39	0.50	0.12	0.38	0.25	ns
	Quadratic	0.40	0.52	0.13	0.44	0.26	ns
	Exponential	0.45	0.63	0.09	0.37	0.27	ns
	Power	0.42	0.67	0.07	0.35	0.27	ns
	Logarithmic	0.40	0.44	0.09	0.34	0.22	ns

There are a number of methods available to quantify Ant that rely on either chromatography or spectrophotometry. Chromatography requires expensive instrumentation and time-consuming sample preparation, whereas spectrophotometry is relatively cheaper, faster, and easier to use than chromatography. Thus, methods based on spectrophotometry are most commonly employed to quantify Ant. Ant are polar molecules, thus the most common solvents used for the extractions are hydrochloric acid in ethanol (Lazcano *et al*. 2001, Zhang *et al*. 2010, Hlavinka *et al*. 2013, Pelletier *et al*. 2015), methanol (Cai *et al*. 2005, Diaz *et al*. 2006, Wen *et al*. 2010, Yang *et al.* 2012), N,N-dimethylformamide (Feild *et al*. 2001) or acetic acid (Shiraishi *et al*. 2007, Ban *et al.* 2014). The extraction occurs in the dark at 4–5°C for 12 h, 24 h, or 48 h (Diaz *et al*. 2006, Shiraishi *et al*. 2007, Wen *et al*. 2010, Hlavinka *et al*. 2013, Ban *et al*. 2014), with incubation at room temperature for 2 h (Cai *et al*. 2005), followed by a water bath at 30°C until the green color disappeared (Zhang *et al*. 2010) or at 65°C for 30 min (Yang *et al*. 2012). Total Ant content has been determined spectrophotometrically as $OD_{530} - 0.24\ OD_{653}$ according to Murray and Hackett (1991), $OD_{530} - 0.25\ OD_{657}$ (Mancinelli 1984), $10 \times (OD_{530} - OD_{600})$ (Zhang *et al*. 2010), $OD_{529} - 0.288\ OD_{650}$ (Novak and Short 2011), $OD_{535} - 1.15\ OD_{640}$ after correction for interference by Chl pigment (Hlavinka *et al*. 2013) or as $(OD_{max} - OD_{700})_{pH\ 1.0} - (OD_{max} - OD_{700})_{pH\ 4.5.}$ OD_{max} was 520 nm (Lee *et al*. 2008, Hosseinian *et al*. 2008), 510 nm (Fuleki and Francis 1968, Pelletier *et al*. 2015) based on the pH differential method or semiquantitatively measured as Ant equivalents by quantification by OD at 520 nm (Shiraishi *et al*. 2007, Ban *et al*. 2014), 525 nm (Diaz *et al*. 2006), 530 nm (Cai *et al*. 2005, Wen *et al*. 2010), 535 nm (Lazcano *et al*. 2001), and 525–535 nm depending on plant species (Manetas *et al*. 1998), where absorption peak wavelength for Ant is 510–550 nm. Based on the subtle differences in methodology, comparison of total Ant values across studies should be conducted with caution because values reported may be highly dependent upon the extraction and quantitative method used.

The *SPAD 502* is designed to measure at a peak absorption wavelength of 650 nm (red), assuming that only the Chl complex absorbs within that wavelength, thus eliminating any potential interference from Car (which has a peak absorption at 450 nm) and Ant (which has a peak absorption at 520–550 nm) pigments. In support of this assumption, Manetas *et al*. (1998) concluded that SPAD estimates were not affected by the amount of Ant in young leaves of eucalypt (*Eucalyptus* sp.), rose (*Rosa* sp.), and castor bean (*R. communis*). However, Ant content was relatively low in the young leaves of *Eucalyptus* sp., *Rosa* sp., and *R. communis* even though the ratio of SPAD to total Chl ranged from 3 to 5 and the ratio of SPAD to Ant ranged from 5 to 14. Similarly, Cate and Perkins (2003), working with sugar maple (*A. saccharum*) leaves expressing a range of colors on four fall foliage color dates, observed a strong linear relationship (R^2=0.72, P<0.001) between analytical and optical measures of Chl concentrations with high maximum and minimum ratios of 48 and 22, respectively, that was not influenced by the amount of Ant. In contrast, Hlavinka *et al*. (2013) showed that the quantity of Ant influenced the relationship between analytical and optical measures of Chl concentrations in mature to partly senescent violet to blue leaves of tomato (*S. lycopersicum*) with low Chl content (about 5–65 mg m^{-2}, SPAD < 17) and relatively high Ant content, but not in mature green leaves with high Chl content (about 5–270 mg m^{-2}, 0 ≤ SPAD ≤35). In our study, we observed both strong and weak relationships between analytical and optical measures of Chl in partly senescent leaves, and were unable to attribute the differences to Ant concentrations.

It is also possible that multiple chemical and physical leaf properties may interact to influence the ability of optical measurements to provide accurate estimates of leaf Chl status. For example, in addition to other leaf pigments, optical measurements may be influenced by leaf thickness, pubescence, or moisture content. For example, reflectance increases and transmittance decrease with an increase in leaf thickness (Knapp and Carter 1998) and different measures of leaf thickness have previously been implicated in the poor accuracy of optical estimates of leaf Chl status (Fanizza *et al*. 1991, Chang and Robison 2003, Jifon *et al*. 2005, Marenco *et al*. 2009). However, other studies suggest that leaf thickness does not influence the accuracy of optical estimates of leaf Chl status (Wang *et al*. 2005, Coste *et al*. 2010, Fu *et al*. 2013). While it has been shown that accounting for leaf water content can improve estimates of absolute Chl content derived from optical measurements (Marenco *et al*. 2009), previous studies have also reported positive (Chang and Robison 2003), negative (Martínez and Guiamet 2004, Marenco *et al*. 2009), and no relationship (Mielke *et al*. 2010, Silla *et al*. 2010) between leaf water content and optical estimates of leaf Chl status.

The poor fits for *Ca* is that SPAD–Chl *b* is the lowest among four species, which decreases the R^2 for Chl (*a*+*b*). It should be noted that R^2 for the regression models relating SPAD to Chl were either in the order of Chl *a* ≥ Chl (*a*+*b*) > Chl *b* (Torres Netto *et al*. 2002, 2005; Pinkard *et al*. 2006, de Jesus and Marenco 2008, Mielke *et al*. 2010, Nascimento and Marenco 2010), similar R^2 values among the relationship between SPAD and Chl *a*, Chl (*a*+*b*), or Chl *b* (Richardson *et al*. 2002, Wang *et al*. 2005), a relatively low R^2 for SPAD–Chl *a* relation than for equations fitted for Chl *b* and Chl (*a*+*b*) (Jesus and Marenco 2008, Reis *et al*. 2009, Fu *et al*. 2013), or even insignificant among SPAD–Chl *a* or Chl *b* except for Chl (*a*+*b*) (Anand and Byju 2008). We derived that R^2 for all trees was in the order of Chl *a* ≥ Chl (*a*+*b*) > Chl *b*. The results supported the suggestions by Nascimento and Marenco (2010) that the *SPAD 502* more accurately reflected the Chl *a* content of a leaf rather than Chl (*a*+*b*) or Chl *b*.

Best fit models between Chl content and SPAD have been achieved using linear, polynomial quadratic, log transformed, and exponential models at low Chl contents in the literature. Linear regression models consistently over- or underestimated Chl concentration at low Chl contents (Monje and Bugbee 1992, Samsone *et al*. 2007, Uddling *et al*. 2007, Hawkins *et al.* 2009), especially predicted an unrealistic negative Chl content at SPAD = 0 [Schaper and Chacko 1991 (some species), Uddling *et al*. 2007, Nascimento and Marenco 2010 (some species)], but not in all cases. For example, Richardson *et al*. (2002) concluded that SPAD was linearly related to Chl of paper birch (*Betula papyrifera*) across Chl contents ranging from 4 to 455 mg m^{-2}, and Jifon *et al*. (2005) showed that the regression models between total Chl and SPAD were generally more linear and stronger at lower Chl contents (< 0.5 mmol m^{-2}) than at a higher Chl contents in intact leaves of six citrus cultivars.

Ling *et al*. (2011) observed a much stronger fit using second order polynomial functions between SPAD values and Chl concentrations derived using a series of *Arabidopsis* chloroplast biogenesis mutants, which exhibit Chl deficiencies of varying severity and were verified by the subsequent analysis of dark-induced senescent leaves. R^2 values for linear and exponential relationships were significantly lower than the selected polynomial functions. Others have similarly reported that such polynomial functions describe best the relationship between SPAD values and Chl concentration (Hawkins *et al*. 2009). However, Percival *et al*. (2008) found that quadratic polynomial regression models were not adequate in explaining the relationships between SPAD and Chl (*a*+*b*)/Car ratio with R^2 of 0.49 (*A. pseudoplatanus*), 0.54 (*F. sylvatica*), and 0.13 (*Q. robur*).

Markwell *et al*. (1995) considered use of the exponential fit for meter calibration is theoretically justified and forces a more appropriate fit to a limited data set than polynomial method. Uddling *et al*. (2007), Marenco *et al*. (2009), Nascimento and Marenco (2010) also suggested that the exponential equation is more reliable than the linear equation, particularly when SPAD values are relatively low. In our study, there was also a strong exponential relationship between Chl concentration and SPAD values for four tree species with the exception that quadratic polynomial regression models better described the relationship between SPAD and Chl *b* (*Ca*) and Chl (*a*+*b*)/Car ratio for *Ag* and *Qm*.

Conclusions: The amount of Chl in the foliage of woody species is a basic physiological parameter for forest research and management. Compared to conventional destructive methods, the use of hand-held, nondestructive, self-calibrating optical meters, such as the *SPAD 502*, provides a simple, inexpensive, and reliable estimate of the relative amount of Chl in leaves across a wide range of different tree species under different conditions. Here we showed that the *SPAD 502* optical meter remains an effective tool for evaluating leaf Chl concentration during leaf senescence for some – but not all – species. We were unable to attribute the poor relationship between analytical and optical measures of Chl directly to any single factor and therefore we conclude that multiple chemical and physical leaf properties may interact to influence the ability of optical measurements to provide accurate estimates of leaf Chl status. Further research should investigate species-specific leaf traits, such as pigment status and distribution, leaf thickness, leaf water content, and specific leaf area, which may be influenced by various environmental and morphological factors.

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