SPECIES SPECIFIC CALIBRATION FOR THE CHLOROPHYLL METER FOR EIGHT TREE SPECIES IN NORTHEAST CHINA

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Abstract

We assessed the predictive capability of the optical meter for determining leaf pigment status of Quercus mongolica, Betula platyphylla, Phellodendron amurense, Juglans mandshurica, Fraxinus mandshurica, Acer mono, Tilia amurensis, and Ulmus propinqua during the growing season. The relationships between extractable Chl (a+b) concentration per unit of leaf area/content per unit of fresh leaf mass and the SPAD values generally followed a nonlinear function, and variations in the calibration line slope were markedly evident for eight tree species across sampling dates. Moreover, the relationships between the SPAD values and the concentration of Chl (a+b) were stronger than content both within and across dates in all tree species in most cases. Even though R^2 values between Chl (a+b) content and SPAD meter readings were improved by incorporating leaf mass area (LMA) and leaf water content (LWC) as the independent variables into regression compared with those values showed in simple regression equations of eight tree species, which the content of Chl (a+b) were significantly negatively correlated with LMA, and mostly positively with LWC. Overall, our results indicated that the optical meter can accurately estimate leaf pigment during the growing season-but that the accuracy of the estimate varies across species throughout development. Precautions for using the meter are prescribed if unhealthy leaves are to be measured.

Keywords: *concentration/content of leaf chlorophyll, SPAD-502 chlorophyll meter, calibration, temperate broadleaved species, Northeast China*

INTRODUCTION

Chlorophyll (*Chl*) is the basic pigment responsible for absorbing the light energy that drives photosynthesis. The amount of *Chl* in a leaf can directly limit photosynthetic potential (Richardson *et al*., 2002) and total canopy GPP (Gitelson *et al*., 2006). In addition, foliar *Chl* quantity is a good indicator of various biotic and abiotic stresses, such as 1 year old apple trees infested with *Aphis spiraecola* (Kaakeh *et al*., 1992), *Ulmus laevis* infested with *Colopha compressa* (Homoptera: Aphididae), *Tilia platyphyllos* infested with *Eriophyes tiliae* (Acarina: Eriophydae), and *Salix fragilis* infested with *Pontania vesicator* (Hymenoptera: Tenthredinidae) (Samsone *et al*., 2007), disease severity of cassava plants from *Bemisia tabaci* (Sims *et al*., 2018), ozone-affected leaves of *Rudbeckia laciniata* var. *digitata* (Neufeld *et al*., 2006), N stress in foliar tissue of *Acer pseudoplatanus*, *Quercus robur*, and *Fagus sylvatica* (Percival *et al*., 2008), citrus rootstock for tolerance to bicarbonate-induced iron chlorosis (Sudahono *et al*., 1994), and senescence process (Biber, 2007; Li *et al*., 2017; Primka IV & Smith, 2019; Donnelly *et al*., 2020). Therefore, an indirect, rapid, reliable and nondestructive detection of leaf *Chl* status is of great importance for related studies.

Traditionally, foliar *Chl* quantity (total *Chl*- *Chl* (*a+b*), *Chl a*, and *Chl b*) has been measured by extraction of leaf tissue obtained with acetone, methanol, ethanol, N, N-dimethylformamide (DMF), or dimethylsulphoxide (DMSO), followed by spectrophotometric measurements. Although the extraction method is still widely employed (Minocha *et al*., 2009), these approaches have a number of drawbacks such as being destructive, labor-intensive, time consuming, and costly. Especially substantial pigment losses from the sample tissue may occur during sample collection, transport from the field to the laboratory, sample preparation, the extraction and dilution, thus leading to a potentially large amount of error in the results when handling a large-scale sampling of *Chl* (Torres Netto *et al*., 2005). The

most frequently used commercially handheld optical meters appears to be the SPAD-502 (Soil-Plant Analyses Development, Minolta Camera Co. Ltd., Japan) among a number of optical meters currently available. The optical meter can instantaneously assess the *Chl* status of intact leaves and eliminate the errors associated with pigment loss that may potentially occur and compound at each step of the extraction process. Briefly, the SPAD-502 meter records the difference in absorbance of red (650 nm, the peak absorbance wavelength for *Chl*) and near infrared (940 nm, a reference wavelength for compensating for the differences in leaf internal structure such as leaf thickness, water status, and other factors that may affect photon passage through the leaf) radiation through an intact or detached leaf. The meter converts these absorbance relationships into a unitless value simply referred to as "SPAD", which ranges from 0 to 100 as an expression of the relative values of *Chl* quantity in a leaf. Although the SPAD values cannot be directly translated to foliar *Chl* quantity, they can be indirectly related to the concentration or content of *Chl* measured via the classic chemical methods of pigment extraction, thereby providing a fast and convenient estimate of foliar *Chl* quantity.

A few studies have observed that a single calibration between the optical readings and *Chl* concentration/content can be used for different species (Yadava, 1986; Gratani, 1992; Markwell *et al*., 1995; Coste *et al*., 2010) and for cultivars of the same species (Uddling *et al*., 2007), but this is not always the case. The utility of the optical meter is diminished because the relationship between the optical meter value and the actual *Chl* concentration/content does not necessarily hold up across species. For example, a number of researchers have found that the relationship between SPAD and foliar *Chl* concentration/content are species-dependent (Richardson *et al*., 2002; Wang *et al*., 2005; Pinkard *et al*., 2006; Biber, 2007; Samsone *et al*., 2007; Marenco *et al*., 2009; Bielinis *et al*., 2015; Liu *et al*., 2019; Primka IV & Smith, 2019). Furthermore, the calibration may not be always consistent throughout developmental stages in four *Quercus* species (Silla *et al*., 2010) or stable regardless of the leaf development stage (Pinkard *et al*., 2006; Mizusaki *et al*., 2013). These inconsistent relationships suggest that species-specific calibration curves should be generated to ensure that the optical meter provides an accurate estimate of *Chl* quantity for a certain growth stage.

The optical meter readings vs *Chl* quantity calibration equations, varied among species, and growth stage, can probably be attributed to differences in leaf traits (*i.e*. leaf thickness - LT, and leaf water content - LWC), and the associated effects of these on leaf optical properties, in addition to leaf *Chl* quantity. But the results were inconsistent in the literature. The correlation direction between optical meter readings and one of indicators of leaf thickness – leaf mass area (LMA, the reciprocal of specific leaf area (SLA)) was either positive (Jifon *et al*., 2005; Marenco *et al*., 2009), negative (Demotes-Mainard *et al*., 2008), or even no significant effects (Fanizza *et al*., 1991a; Wang *et al*., 2005; Silla *et al*., 2010; Coste *et al*., 2010; Fu *et al*., 2013; Bielinis *et al*., 2015). Positive (Niu *et al*., 2007), negative (Martínez & Guiamet, 2004; Marenco *et al*., 2009), and no relationship (Li *et al*., 1998; Mielke *et al*., 2010; Silla *et al*., 2010) between LWC and optical meter readings were also co-existed. How these factors affect optical meter readings are still to be confirmed.

In the present study, we did experiments to assess the possible applicability of the SPAD-502 meter for estimating the *Chl* concentration/content in leaves of eight broad-leaved tree species in Northeastern China, and to our knowledge, no calibrations have been established previously. The purposes of the study were to find out: (1) how effectively the SPAD-502 meter allows to estimate the foliar concentration/content of *Chl* (*a*+*b*); (2) whether the accuracy of this estimation varies for different tree species; (3) whether the leaf traits (LMA and LWC) influence the accuracy of the *Chl* concentration/content estimate with the SPAD-502 meter.

MATERIALS AND METHODS

Site description and plant material

The study was conducted at the Maoershan Forest Ecosystem Research Station of Northeast Forestry University in Heilongjiang Province, northeastern China (127°30'-34'E, 45°20'-25'N). It represents typical forest types and landscapes in Northeastern China with an average altitude of 300 m above sea level and an average slope of 10-15°. The parent material is granite bedrock, and the soil is

Haplumbrepts or Eutroboralfs. The climate is a continental monsoon climate with a strong monsoon windy spring, a warm and humid summer, and a dry and cold winter. Annual precipitation is in the range of 600-800 mm, of which 80% falls in July and August. Annual evaporation is 884 mm. The mean annual air temperature is 2.8 ℃, and average January and July air temperature are -18.5 ℃ and 22.0 ℃, respectively. The annual accumulated temperature above 5 ℃ is 2897 ℃. The frost-free period is between 120 and 140 days with an early frost in September and late frost in May. The mean annual solar period is 1850 h. The mean wind speed is 1.5 m s^{-1}.

The eight main broad leaved tree species chosen for this study included Mongolian oak (*Quercus mongolica* Fisch.; hereafter *Qm*), white birch (*Betula platyphylla* Suk.; hereafter *Bp*), Amur cork-tree (*Phellodendron amurense* Rupr.; hereafter *Pa*), Manchurian walnut (*Juglans mandshurica* Maxim.; hereafter *Jm*), Manchurian ash (*Fraxinus mandshurica* Rupr.; hereafter *Fm*), maple (*Acer mono* Maxim.; hereafter *Am*), Amur linden (*Tilia amurensis* Rupr.; hereafter *Ta*), and elm (*Ulmus propinqua* Koidz.; hereafter *Up*). Leaf samples were collected from trees located at three different natural light irradiance environments (open sites, the forest edge, and understory) to obtain the widest possible range of the pigment values. The mean height range (±SE) and mean DBH of the sample trees were 3.84±0.37-4.74±0.66 m, 2.89±0.20-4.82±0.55 cm, respectively. Since the year of 2001, leaves for *Bp* were healthy in spring, then had yellow spot in the leaves and the area of spot increased gradually at the beginning of August, and premature leaf senescence occurred. In addition, leaves left on the trees appeared lighter green than foliage before August, indicating reductions in the tree vitality at the Station.

SPAD measurement and chlorophyll extraction

Leaves were sampled across different developmental stages consisting of spring (end of May, leaves just leafed-out, fully young leaves), summer (from June to July, mature leaves), and autumn (mid-September, leaves turned yellow-pre-senescent leaves). On a sunny morning around 0800 h (Nauš *et al*., 2010) during each stage of development, we selected 5 fully expanded leaves from the middle of the south facing side of the crown from each of ten trees per species. The *Chl* index of these leaves was measured using a SPAD-502 meter (Soil-Plant Analyses Development, Minolta Camera Co. Ltd., Japan). In brief, the SPAD-502 measures the difference in absorbance of light through the sample leaf at two wavelengths: near 650 nm (strongly absorbed by *Chl*) and 940 nm (a reference wavelength for adjusting the differences in leaf internal structure that may affect photon passage through the leaf), converts them into digital signals, and then into SPAD unit. The measurement was repeated eight times in different locations in the inter-venal area of each leaf to generate an average SPAD value for each leaf. Following optical measurements, leaves were placed in labeled plastic bags in coolers with ice, and immediately transported to the laboratory for *Chl* extraction.

Chl concentration/content was quantified following the method of Wellburn (1994). Briefly, 4-6 discs per leaf were punched from the areas measured by the SPAD, which discs' diameter were 0.845 cm, 1.070 cm, and 1.264 cm depending on leaf size. Fresh mass (FM) of leaf discs was determined by an analytical balance (Sartorius BT224S, Sartorius Scientific Instruments (Beijing) Co., Ltd., China). Samples were ground in 10 ml DMSO, then were incubated at 65 ℃ until the leaf disks were completely colorless. Absorbance in the wavelengths of 665 nm (chlorophyll *a* – *Chl a*), 649 nm (chlorophyll *b* - *Chl b*), and 480 nm (carotenoids - *Car*) was determined using a spectrophotometer (722 N, INESA Analytical Instrument Co., LTD, Shanghai, China). Values obtained by spectrophotometer were used to calculate the concentrations per unit leaf area (*Chl-area*, μg cm^{-2}) or contents per unit leaf mass (*Chl-mass*, mg g^{-1}) of *Chl a*, *Chl b*, and *Car* according to the equations proposed by Wellburn (1994). Total chlorophyll (*Chl* (*a*+*b*)) was calculated as the sum of *Chl a* and *Chl b*.

Leaf traits measurement

An additional 50 leaf samples per ten trees of each species were collected at each development stage to determine leaf traits including leaf mass area (LMA) and leaf water content (LWC). The fresh mass of each leaf without petioles was determined by an analytical balance and then the leaves were scanned (Model T210, Founder Technology Instrument Co. Ltd., China) to obtain high-resolution

images to measure leaf area by ImageJ software. Finally, Leaf samples were dried at 85 ℃ for at least 24 h and the dry mass was recorded. LMA (mg cm^{-2}) was calculated as the ratio of leaf dry mass to leaf area. LWC (%) was calculated as the ratio of the difference between the fresh mass and dry mass to the fresh mass.

Statistical analysis

We analyzed species and temporal effects on *Chl* and *Car* concentration/content, SPAD values, the ratio of *Chl* (*a*+*b*)/*Car*, and leaf traits 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/content or SPAD value among all leaves within an individual tree and measurement period was used in the analysis (i.e., n = 10 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 & Anderson, 1998) to determine which candidate covariance structure best fit each model. Denominator degrees of freedom were estimated according to the Kenward-Roger method (Kenward & Roger, 1997). Treatment means were compared using Fisher's Least Significant Difference test. Coefficients of variations (CV) for *Chl a*, *Chl b*, *Chl* (*a*+*b*), and SPAD were calculated for each species during the specific season. Above analyses were performed using a mixed model procedure (PROC MIXED) of SAS (SAS, Version 9. 3, SAS Inc., Cary, NC, USA) with α=0.05.

We fit the relationships of SPAD values as the independent variable against *Chl* concentration/content as the dependent variable through regression analysis for each season of each species and eight species combined at certain season, the whole data set for three seasons of the each species and eight species using the curve-fitting procedure by various functions—polynomial quadratic, logarithmic, exponential, power, and simple linear models—and chose the function that provided the highest coefficient of determination (R^2) by SPSS18.0 (SPSS Inc., Chicago, IL, USA). The ordinary least squares regression (OLS) techniques were performed to test growing season and species on SPAD versus *Chl* (*a*+*b*) relationships (Warton *et al*., 2006).

RESULTS

Differences between means of eight tree species for SPAD readings, *Chl* and *Car* concentration / content, *Chl* (*a*+*b*) to *Car* ratios, and leaf traits (LMA, LWC) in leaves were significant in season, species, and interaction between season and species (P<0.05) except for LWC for the two-term season×species interaction through seasons and *Chl a-mass* in spring. The differences between means of the same tree species across seasons for the above index were also significant except for *Chl b-mass* in *Bp* and *Pa*, and *Chl b-area* in *Jm*. For a full description of species trends through time and species comparisons within temporal periods see Table 1. In general, concentrations/contents of pigments significantly higher in summer, lower in spring and autumn, as did the corresponding SPAD values. LMA was higher in summer and autumn, lower in spring whereas LWC was higher in spring than those in summer and autumn. *Pa* and *Up* had the highest average SPAD values and the lowest in *Fm* and *Bp* while *Qm* had the highest average *Chl* (*a*+*b*) concentration/content and the lowest in *Fm*. There were *Fm* with the highest average LWC, the lowest in *Bp*, and *Bp* with the highest LMA and the lowest in *Am* among the eight tree species.

A highly significant positive species-specific relationships including power, linear, polynomial quadratic, and exponential mathematical models were observed between the SPAD values and extractable *Chl* (*a*+*b*) concentration/content for eight tree species across sampling dates (Table 2). For example, the slope of the regression equation in *Bp* combined through three seasons based on leaf area displayed the lowest values and highest in *Jm*, *Am*, *Fm*, and *Pa* compared with the eight species pooled through three seasons for the whole data set (P<0.01) whereas the remaining three tree species including *Qm*, *Ta*, and *Up* had similar slopes with the whole data set (P>0.05).

Species	Season	SPAD	Content (mg g^{-1})				Concentration (μg cm^{-2})				*Chl* (*a*+*b*)/*Car*	LMA (mg cm^{-2})	LWC (%)
			Chl a	*Chl b*	*Chl* (*a*+*b*)	*Car*	*Chl a*	*Chl b*	*Chl* (*a*+*b*)	*Car*			
Qm	Spring	26.9(0.6)Cc	1.7(0.0)Ca	1.2(0.0)Ca	2.9(0.1)Ca	0.5(0.0)Ba	21.9(0.7)Cab	14.4(0.4)Ca	36.3(1.1)Ca	6.3(0.2)Bbc	5.9(0.1)Cb	3.0(0.1)Bb	78(0.6)Aab
	Summer	41.1(0.4)Aa	2.9(0.1)Aab	1.9(0.0)Aa	4.8(0.1)Aa	0.7(0.0)Aab	34.1(0.5)Aa	23.3(0.6)Aa	57.4(1.0)Aa	8.2(0.2)Ab	7.0(0.1)Bcd	4.3(0.1)Abcd	64(0.4)Bc
	Autumn	34.5(0.7)Bb	2.4(0.1)Ba	1.7(0.1)Ba	4.1(0.2)Ba	0.6(0.0)Ba	26.7(0.8)Bb	19.3(0.7)Ba	46.0(1.5)Ba	6.3(0.2)Bab	7.3(0.1)Abc	4.1(0.1)Abcd	64(0.4)Bb
	Total	34.2(4.1)bc	2.3(0.3)b	1.6(0.2)a	3.9(0.6)a	0.6(0.0)a	27.5(3.6)a	19.0(2.6)a	46.6(6.1)a	6.9(0.1)b	6.7(0.1)a	3.8(0.4)c	68.7(4.5)d
Bp	Spring	28.4(0.4)Bbc	1.5(0.0)ABa	0.6(0.0)Abc	2.1(0.0)ABb	0.4(0.0)Ab	19.8(0.3)Bbc	7.8(0.1)Bbc	27.6(0.4)Bbc	5.3(0.1)Bcd	5.2(0.1)Bb	4.0(0.1)Ba	68.2(0.4)Ac
	Summer	36.2(0.5)Ab	1.8(0.0)Ad	0.7(0.0)Ac	2.5(0.1)Ade	0.4(0.0)Ad	28.5(0.5)Ab	11.1(0.3)Ad	39.6(0.8)Ab	7.0(0.1)Ac	5.6(0.1)Ad	6.1(0.1)Aa	60.2(0.6)Bd
	Autumn	28.4(1.0)Bcd	1.4(0.1)Bb	0.5(0.0)Ae	1.9(0.1)Bd	0.4(0.0)Ac	22.7(1.1)Bbc	8.6(0.4)Bcd	31.3(1.5)Bc	6.9(0.2)Aab	4.5(0.1)Cd	5.7(0.1)Aa	58.3(0.1)Bc
	Total	31.0(2.6)d	1.6(0.1)d	0.6(0.1)e	2.2(0.2)f	0.4(0.0)c	23.7(2.6)bc	9.2(1.0)d	32.8(3.6)c	6.4(0.1)c	5.1(0.1)d	5.3(0.6)a	62.2(3.1)e
Pa	Spring	34.5(0.7)Ba	1.6(0.1)Ba	0.8(0.0)Abc	2.4(0.1)Bab	0.6(0.0)Ba	19.5(0.5)Bbc	9.3(0.3)Bb	28.7(0.7)Bb	7.2(0.3)Bab	4.5(0.3)Ac	2.7(0.1)Bb	79(0.7)Aa
	Summer	42.2(1.0)Aa	2.0(0.1)Acd	0.8(0.0)Ac	2.8(0.1)Acd	0.7(0.0)Ab	29.1(1.2) Ab	11.9(0.4)Acd	41.0(1.5)Ab	10.0(0.5)Aa	4.6(0.3)Ae	4.6(0.2)Abc	71(0.5)Bab
	Autumn	33.8(1.3)Bb	1.2(0.1)Cb	0.8(0.1)Acd	2.0(0.1)Bd	0.4(0.0)Cc	18.7(1.3)Bcd	12.1(0.8)Ab	30.8(1.7)Bc	6.2(0.4)Cb	5.3(0.4)Ac	4.5(0.2)Abc	65(0.2)Cb
	Total	36.9(2.7)a	1.6(0.2)d	0.8(0.0)c	2.4(0.3)e	0.5(0.0)b	22.4(3.3)c	11.1(0.9)c	33.5(3.8)c	7.8(0.3)a	4.8(0.2)de	3.9(0.6)c	71.5(3.8)bc
Jm	Spring	33.1(0.5)Bab	1.9(0.0)Ba	1.0(0.0)Bab	2.9(0.1)Ba	0.6(0.0)Ba	26.3(0.5)Aa	13.2(0.4)Aa	39.5(0.9)Aa	7.8(0.1)Ba	5.1(0.1)Ab	3.1(0.1)Bab	76.0(0.4)Ab
	Summer	41.3(0.8)Aa	2.6(0.1)Abc	1.3(0.1)Ab	3.9(0.2)Ab	0.8(0.0)Aa	29.4(0.8)Ab	14.3(0.5)Abc	43.7(1.3)Ab	8.8(0.1)Ab	5.0(0.1)Ae	4.2(0.2)Abcd	67.8(0.6)Bbc
	Autumn	32.9(1.2)Bbc	1.5(0.1)Cb	1.1(0.1)Bbc	2.6(0.1)Bcd	0.6(0.0)Ba	17.6(0.9)Bd	12.6(0.7)Ab	30.2(1.6)Bc	7.4(0.2)Ba	4.0(0.2)Bd	4.8(0.2)Aabc	64.3(0.6)Bb
	Total	35.8(2.8)ab	2.0(0.3)c	1.1(0.1)b	3.1(0.4)c	0.6(0.0)a	24.4(3.5)b	13.4(0.5)b	37.8(4.0)b	8.0(0.1)a	4.7(0.1)e	4.0(0.5)c	69.4(3.5)d
Fm	Spring	30.3(0.5)Babc	1.6(0.1)Aa	0.5(0.0)Cc	2.1(0.1)Ab	0.4(0.0)Ab	21.3(0.5)Ab	7.1(0.2)Cbc	28.4(0.7)Ab	5.2(0.1)Acd	5.5(0.1)Bb	2.9(0.1)Cb	81(0.5)Aa
	Summer	35.7(1.0)Ab	1.0(0.0)Be	1.2(0.1)Ab	2.2(0.1)Ae	0.2(0.0)Be	14.5(0.6)Bc	16.2(0.8)Ab	30.7(1.2)Ac	3.9(0.1)Be	8.4(0.4)Aa	5.0(0.3)Ab	73(0.7)Ba
	Autumn	27.8(1.2)Bd	0.8(0.1)Bc	0.8(0.1)Bcd	1.6(0.1)Bde	0.2(0.0)Bd	10.8(0.6)Ce	11.1(0.9)Bbc	21.8(1.3)Bd	3.8(0.1)Bd	6.3(0.5)Bb	4.1(0.2)Bbcd	72(0.6)Ba
	Total	31.3(2.3)d	1.1(0.2)e	0.8(0.2)c	1.9(0.2)g	0.3(0.0)c	15.5(3.1)d	11.4(2.7)c	27.0(2.7)d	4.3(0.1)f	6.7(0.2)a	4.0(0.6)c	74.9(2.9)a
Am	Spring	25.4(0.6)Ccd	1.8(0.1)Ca	0.6(0.0)Cbc	2.4(0.1)Cab	0.4(0.0)Cb	16.1(0.5)Cc	5.1(0.2)Bc	21.1(0.6)Cc	3.6(0.1)Be	5.9(0.1)Ba	2.1(0.1)Bb	76(0.9)Ab
	Summer	41.2(0.5)Aa	3.2(0.1)Aa	1.1(0.0)Abc	4.3(0.1)Aab	0.7(0.0)Aab	31.4(0.7)Aab	10.9(0.2)Ad	42.4(0.9)Ab	6.7(0.2)Acd	6.3(0.0)Ac	3.4(0.1)Ad	67(0.4)Bbc
	Autumn	37.3(0.6)Bab	2.6(0.1)Ba	0.9(0.0)Bcd	3.5(0.1)Bab	0.6(0.0)Ba	26.5(0.6)Bb	9.2(0.3)Acd	35.6(0.8)Bb	6.0(0.1)Abc	6.0(0.1)Bbc	3.1(0.1)Ad	67(0.5)Bb
	Total	34.6(4.8)b	2.5(0.4)a	0.9(0.2)c	3.4(0.6)b	0.6(0.0)a	24.7(4.5)b	8.4(1.7)d	33.0(6.3)c	5.5(0.1)e	6.1(0.1)b	2.9(0.4)e	70.1(3.2)cd
Ta	Spring	31.5(0.8)Babc	1.8(0.1)Ba	0.7(0.0)ABbc	2.5(0.1)Bab	0.4(0.0)Bb	20.5(0.5)Bbc	8.4(0.3)Bb	28.9(0.8)Bb	4.7(0.1)Cde	6.2(0.1)Aa	2.3(0.1b)Bb	78(0.4)Aab
	Summer	38.6(0.9)Aab	2.5(0.1)Abc	0.9(0.0)Ac	3.3(0.2)Abc	0.6(0.0)Ac	31.6(1.0)Aab	11.1(0.4)Ad	42.6(1.4)Ab	7.4(0.2)Abc	5.8(0.1)Ad	3.9(0.2)Acd	69(0.6)Bab
	Autumn	28.2(1.0)Bcd	1.6(0.1)Bb	0.6(0.0)Be	2.2(0.1)Bd	0.5(0.0)Bb	19.3(0.9)Bcd	7.1(0.3)Bd	26.4(1.2)Bcd	5.8(0.2)Bc	4.6(0.2)Bd	3.8(0.2)Acd	70(0.6)Bab
	Total	32.8(3.1)c	2.0(0.3)c	0.7(0.1)d	2.7(0.3)d	0.5(0.0)b	23.8(3.9)bc	8.9(1.2)d	32.7(5.1)c	6.0(0.1)d	5.5(0.1)c	3.3(0.5)d	72.2(2.7)b
Up	Spring	27.3(0.5)Bc	1.6(0.1)Ba	0.5(0.0)Bd	2.1(0.1)Bb	0.4(0.0)Bb	19.5(0.4)Bbc	5.8(0.1)Cc	25.2(0.5)Bbc	4.8(0.1)Bde	5.3(0.1)Cb	3.0(0.1)Cb	74(0.6)Ab
	Summer	42.4(0.6)Aa	2.2(0.1)Ac	0.8(0.0)Ac	3.1(0.1)Acd	0.5(0.0)Acd	32.6(0.7)Aab	12.3(0.3)Acd	44.9(1.0)Ab	7.7(0.2)Abc	5.9(0.1)Acd	6.0(0.4)Aa	66(0.5)Bc
	Autumn	41.1(0.9)Aa	2.3(0.1)Aa	0.7(0.0)ABde	3.0(0.1)Abc	0.5(0.0)Ab	31.3(1.0)Aa	9.2(0.3)Bcd	40.5(1.2)Aab	7.2(0.2)Aab	5.6(0.0)Bbc	4.1(0.2)Bbcd	67(0.4)Bb
	Total	36.9(4.8)a	2.0(0.2)c	0.7(0.1)de	2.7(0.3)d	0.5(0.0)b	27.8(4.2)a	9.1(1.9)d	36.9(6.0)b	6.5(0.1)bc	5.6(0.1)c	4.4(0.9)b	68.9(2.7)d

Table 1. Mean (± SE) SPAD, concentration/content of total chlorophyll *Chl* (*a*+*b*), *Chl a*, *Chl b*, *Car*, the ratio of *Chl* (*a*+*b*)/*Car*, leaf mass area (LMA), and leaf water content (LWC) values for *Q. mongolica* (*Qm*), *B. platyphylla* (*Bp*), *P. amurense* (*Pa*), *J. mandshurica* (*Jm*), *F. mandshurica* (*Fm*), *A. mono* (*Am*), *T. amurensis* (*Ta*), and *U. propinqua* (*Up*) (n = 50 for each season, and n = 150 for three seasons) measured during the growing season in Northeast China. Different lowercase letters in columns indicate significant differences in a parameter for the same season between species at P<0.05 using Fisher's Least Significant Difference test. Significant differences between season within a species are indicated by different capital letters at P<0.05.

Species	Season		*Chl* (*a*+*b*)	R^2	*Chl a*	R^2	*Chl b*	R^2
Qm	Spring	concentration (μg cm^{-2})	$y=0.70x^{1.20}$	0.89b	$y=0.38x^{1.23}$	0.92	y=-38.00+15.99lnx	0.78
		content (mg g^{-1})	$y=0.36x^{0.64}$	0.53b	$y=0.19x^{0.67}$	0.52	$y=0.16x^{0.59}$	0.47
	Summer	concentration(μg cm^{-2})	$y=0.32x^{1.39}$	0.76b	$y=0.47x^{1.15}$	0.82	$y=0.03x^{1.76}$	0.60
		content (mg g^{-1})	$y=0.29x^{0.76}$	0.24b	$y=0.42x^{0.52}$	0.11	$y=0.03x^{1.12}$	0.36
	Autumn	concentration(μg cm^{-2})	$y=0.25x^{1.46}$	0.72a	$y=0.11x^{1.55}$	0.83	$y=0.17x^{1.33}$	0.48
		content (mg g^{-1})	$y=0.04x^{1.31}$	0.48a	$y=0.02x^{1.39}$	0.52	$y=0.03x^{1.17}$	0.35
	Total	concentration(μg cm^{-2})	$y=0.75x^{1.17}$	0.87bB	y=-3.67+0.91x	0.91	$y=0.29x^{1.18}$	0.76
		content (mg g^{-1})	$y=0.09x^{1.08x}$	0.72bCD	$y=0.71e^{0.03x}$	0.71	$y=0.47e^{0.03x}$	0.67
Bp	Spring	concentration(μg cm^{-2})	$y=2.25x^{0.75}$	0.70b	$y=1.02x^{0.89}$	0.67	$y=3.69x^{0.22}$	ns
		content (mg g^{-1})	$y=0.21x^{0.69}$	0.41b	$y=0.09x^{0.86}$	0.53	$y=0.31x^{0.19}$	ns
	Summer	concentration(μg cm^{-2})	$y=0.22x^{1.45}$	0.80a	$y=0.22x^{1.35}$	0.80	$y=0.03x^{1.70}$	0.72
		content (mg g^{-1})	$y=0.03x^{1.23}$	0.30a	$y=0.03x^{1.13}$	0.28	$y=0.003x^{1.47}$	0.32
	Autumn	concentration(μg cm^{-2})	$y=150.49x^{0.49}$	0.13c	$y=51.34-1.82x+0.03x^2$	0.14	$y=18.07-0.57x+0.008x^2$	0.21
		content (mg g^{-1})	$y=7.76x^{0.43}$	0.11c	$y=3.56-0.14x+0.002x^2$	0.15	$y=1.25-0.05x+0.001x^2$	0.22
	Total	concentration(μg cm^{-2})	$y=90.76-4.67x+0.09x^2$	0.34dC	$y=64.47-3.29x+0.06x^2$	0.32	$y=26.29-1.37x+0.03x^2$	0.38
		content (mg g^{-1})	$y=1.25x^{0.16}$	0.15dE	$y=3.22-0.13x+0.002x^2$	0.14	$y=1.32-0.06x+0.001x^2$	0.16
Pa	Spring	concentration(μg cm^{-2})	$y=-0.64x^{1.08}$	0.76b	$y=0.30x^{1.18}$	0.71	$y=0.53x^{0.80}$	0.27
		content (mg g^{-1})	$y=0.04x^{1.15}$	0.40b	$y=-8.80+0.58x-0.008x^2$	0.55	$y=-4.60+0.30x-0.004x^2$	0.21
	Summer	concentration(μg cm^{-2})	$y=0.13x^{1.53}$	0.89a	$y=0.05x^{1.71}$	0.90	$y=0.21x^{1.08}$	0.57
		content (mg g^{-1})	$y=0.007x^{1.62}$	0.60a	$y=0.002x^{1.80}$	0.66	$y=0.01x^{1.17}$	0.35
	Autumn	concentration(μg cm^{-2})	$y=0.22x^{1.39}$	0.96a	$y=2.89e^{0.05x}$	0.84	$y=0.09x^{1.35}$	0.55
		content (mg g^{-1})	$y=0.07x^{0.96}$	0.58bB	$y=0.31e^{0.04x}$	0.59	$y=0.03x^{0.92}$	0.25
	Total	concentration(μg cm^{-2})	$y=0.22x^{1.39}$	0.93aA	$y=3.35e^{0.05x}$	0.86	$y=0.18x^{1.13}$	0.49
		content (mg g^{-1})	$y=0.04x^{1.14}$	0.57bCD	$y=0.32e^{0.04x}$	0.59	$y=0.03x^{0.88}$	0.25
Jm	Spring	concentration(μg cm^{-2})	y=13.66+0.58x y=0.90$e^{0.03x}$	0.24b	$y=30.28-1.46x+0.04x^2$	0.74	$y=47.74-2.64x+0.05x^2$	ns
		content (mg g^{-1})		0.47b	$y=2.36-0.14x+0.004x^2$	0.80	y=0.62+0.01x	ns
	Summer	concentration(μg cm^{-2})	$y=0.21x^{1.44}$	0.84a	$y=0.16x^{1.40}$	0.85	$y=0.05x^{1.50}$	0.64
		content (mg g^{-1})	$y=0.01x^{1.58}$	0.43a	$y=0.01x^{1.54}$	0.47	$y=0.003x^{1.64}$	0.35
	Autumn	concentration(μg cm^{-2})	$y=0.35x^{1.27}$	0.91a	$y=0.25x^{1.21}$	0.92	$y=2.30e^{0.05x}$	0.85
		content (mg g^{-1})	$y=0.06x^{1.08}$	0.69a	$y=0.42e^{0.04x}$	0.68	$y=0.24e^{0.04x}$	0.65
	Total	concentration(μg cm^{-2})	y=-5.98+1.19x	0.83aAB	$y=0.29x^{1.23}$	0.79	$y=0.24x^{1.10}$	0.63
		content (mg g^{-1})	$y=0.04x^{1.18}$	0.66aCD	$y=0.45e^{0.04x}$	0.67	$y=0.28e^{0.04x}$	0.51
Fm	Spring	concentration(μg cm^{-2})	$y=0.27x^{1.36}$	0.87a	$y=5.57e^{0.04x}$	0.82	$y=-13.77+1.05x-0.01x^2$	0.80
		content (mg g^{-1})	$y=0.001x^{2.21}$	0.69a	$y=6.06-0.43x+0.009x^2$	0.70	$y=1.08-0.08x+0.002x^2$	0.72
	Summer	concentration(μg cm^{-2})	$y=0.31x^{1.29}$	0.78a	$y=12.77-0.26x+0.01x^2$	0.26	$y=0.02x^{1.91}$	0.88
		content (mg g^{-1})	$y=0.001x^{2.09}$	0.84a	$y=0.01x^{1.48}$	0.88	$y=0.06e^{0.08x}$	0.74
	Autumn	concentration(μg cm^{-2})	$y=0.20x^{1.40}$	0.96a	$y=0.26x^{1.12}$	0.76	$y=1.31e^{0.07x}$	0.80
		content (mg g^{-1})	$y=0.002x^{1.97}$	0.90a	$y=0.003x^{1.69}$	0.90	$y=0.04e^{0.09x}$	0.79
	Total	concentration(μg cm^{-2})	$y=0.23x^{1.37}$	0.89aA	$y=0.41x^{1.04}$	0.39	$y=1.25e^{0.07x}$	0.73
		content (mg g^{-1})	$y=0.003x^{1.90}$	0.82aA	$y=0.004x^{1.57}$	0.57	$y=0.05e^{0.09x}$	0.74
Am	Spring	concentration(μg cm^{-2})	$y=0.76x^{1.03}$	0.79b	$y=0.52x^{1.06}$	0.81	$y=-9.06+0.99x-0.02x^2$	0.37
		content (mg g^{-1})	$y=0.02x^{1.54}$	0.75a	$y=0.01x^{1.57}$	0.79	$y=-1.22+0.12x-0.002x^2$	0.41
	Summer	concentration(μg cm^{-2})	$y=0.18x^{1.48}$	0.82a	$y=0.11x^{1.51}$	0.79	$y=-18.71+1.07x-0.01x^2$	0.65
		content (mg g^{-1})	$y=-43.68+2.28x-0.03x^2$	0.58b	$y=-32.90+1.71x-0.02x^2$	0.53	$y=-10.78+0.57x-0.01x^2$	0.42
	Autumn	concentration(μg cm^{-2})	y=-9.86+1.22x	0.70b	$y=27.91-0.94x+0.02x^2$	0.68	$y=6.04-0.18x+0.01x^2$	0.55
		content (mg g^{-1})	y=-0.16+0.10x	0.18c	$y=3.40-0.11x+0.002x^2$	0.17	$y=0.99-0.03x+0.001x^2$	0.20

	Total	concentration(μg cm^{-2})	$y=0.29x^{1.33}$	0.93aA	$y=0.24x^{1.30}$	0.92	$y=0.93+0.06x+0.004x^2$	0.84
		content (mg g^{-1})	$y=0.05x^{1.21}$	0.64cBC	$y=0.04x^{1.17}$	0.63	$y=-0.78+0.07x-0.001x^2$	0.59
Ta	Spring	concentration(μg cm^{-2})	$y=10.17e^{0.03x}$	0.89b	$y=9.29+0.12x+0.01x^2$	0.90	$y=2.10e^{0.04x}$	0.81
		content (mg g^{-1})	$y=0.04x^{1.18}$	0.62b	$y=0.05x^{1.06}$	0.59	$y=0.15e^{0.05x}$	0.64
	Summer	concentration(μg cm^{-2})	$y=9.78e^{0.04x}$	0.96b	$y=7.14e^{0.04x}$	0.96	$y=2.63e^{0.04x}$	0.91
		content (mg g^{-1})	$y=0.02x^{1.41}$	0.47a	$y=0.01x^{1.43}$	0.47	$y=0.006x^{1.37}$	0.46
	Autumn	concentration(μg cm^{-2})	$y=0.70x^{1.09}$	0.89b	$y=0.33x^{1.21}$	0.90	$y=2.23e^{0.04x}$	0.76
		content (mg g^{-1})	$y=0.05x^{1.14}$	0.70b	$y=0.02x^{1.27}$	0.74	$y=0.16e^{0.04x}$	0.62
	Total	concentration(μg cm^{-2})	$y=0.50x^{1.20}$	0.90aB	$y=0.29x^{1.26}$	0.90	$y=2.22e^{0.04x}$	0.87
		content (mg g^{-1})	$y=0.04x^{1.23}$	0.67bBC	$y=0.02x^{1.29}$	0.69	$y=0.17e^{0.04x}$	0.60
Up	Spring	concentration(μg cm^{-2})	$y=0.66x^{1.10}$	0.91b	$y=0.64x^{1.03}$	0.90	$y=0.07x^{1.36}$	0.85
		content (mg g^{-1})	$y=0.01x^{1.53}$	0.59a	$y=0.01x^{1.46}$	0.59	$y=0.001x^{1.78}$	0.59
	Summer	concentration(μg cm^{-2})	$y=0.32x^{1.35}$	0.61a	$y=0.25x^{1.30}$	0.61	$y=0.08x^{1.35}$	0.60
		content (mg g^{-1})	$y=0.03x^{1.19}$	0.17b	$y=10.84-0.48x+0.01x^2$	0.20	$y=3.91-0.17x+0.002x^2$	0.21
	Autumn	concentration(μg cm^{-2})	$y=0.30x^{1.32}$	0.88a	$y=0.18x^{1.39}$	0.91	$y=0.15x^{1.10}$	0.66
		content (mg g^{-1})	$y=0.03x^{1.23}$	0.56a	$y=0.02x^{1.30}$	0.57	$y=0.02x^{1.01}$	0.48
	Total	concentration(μg cm^{-2})	$y=0.42x^{1.23}$	0.93aB	$y=0.39x^{1.18}$	0.93	$y=0.06x^{1.41}$	0.84
		content (mg g^{-1})	$y=0.08x^{0.97}$	0.52bD	$y=0.07x^{0.91}$	0.48	$y=0.01x^{1.14}$	0.56
All species	Spring	concentration(μg cm^{-2})	$y=1.43x^{0.88}$	0.46c	$y=1.05x^{0.87}$	0.61	$y=0.32x^{0.96}$	0.19
		content (mg g^{-1})	$y=0.13x^{0.87}$	0.31c	$y=0.09x^{0.85}$	0.35	$y=0.03x^{0.97}$	0.16
	Summer	concentration(μg cm^{-2})	$y=0.22x^{1.43}$	0.68a	$y=-11.44+1.01x$	0.54	$y=0.13x^{1.26}$	0.33
		content (mg g^{-1})	$y=0.005x^{1.77}$	0.44a	$y=0.002x^{1.89}$	0.40	$y=0.003x^{1.61}$	0.29
	Autumn	concentration(μg cm^{-2})	$y=0.63x^{1.12}$	0.60b	$y=0.32x^{1.19}$	0.52	$y=0.37x^{0.95}$	0.34
		content (mg g^{-1})	$y=0.03x^{1.23}$	0.47b	$y=0.02x^{1.30}$	0.45	$y=0.02x^{1.06}$	0.29
	Total	concentration(μg cm^{-2})	$y=0.55x^{1.17}$	0.67B	$y=0.36x^{1.18}$	0.57	$y=0.18x^{1.15}$	0.41
		content (mg g^{-1})	$y=0.04x^{1.19}$	0.47BC	$y=0.03x^{1.20}$	0.42	$y=0.01x^{1.17}$	0.33

Table 2. The best equations between leaf total chlorophyll (*Chl* (*a*+*b*)), *Chl a*, and *Chl b* based on leaf area basis (μg cm^{-2}), fresh mass (mg g^{-1}) and SPAD readings for *Q. mongolica* (*Qm*), *B. platyphylla* (*Bp*), *P. amurense* (*Pa*), *J. mandshurica* (*Jm*), *F. mandshurica* (*Fm*), *A. mono* (*Am*), *T. amurensis* (*Ta*), and *U. propinqua* (*Up*) in each of three stages (n=50) and the three stages combined (n=150), all species in each of three stages (n=400) and the three stages as a whole (n=1200) for Spring (end-May, leaves just expanded fully- young leaves), Summer (June to July, mature leaves), and Autumn (mid-September, leaves turned yellow- pre-senescence leaves). For all the equation $P<0.05$. Different lowercase and capital letters in the regression equations differ significantly between same species among sampling dates, and eight species throughout growing season with the general equation for all species combined across sampling dates, respectively ($P<0.05$).

For the same species, different equations were also found both for concentration and content during the different growing seasons. Generally slopes in concentration/content during spring were significantly lower ($P<0.05$) due to the lower data range and CV values for *Chl* and SPAD, similar in summer and autumn ($P>0.05$) compared to that of combined data through the three seasons for the same species except that slopes for *Bp* in spring, summer, and autumn were significantly higher than the combined data ($P<0.05$), and values for *Qm* and *Fm* in concentration/content and *Ta* in content were similar among seasons ($P>0.05$).

In general, the relationships between the SPAD values and the concentration of *Chl a*, *Chl b*, and *Chl* (*a*+*b*) per unit of leaf area were stronger than that per unit of fresh leaf mass both within and across dates in all tree species which corresponding with CV of SPAD and *Chl* concentration/content (Fig. 1). R^2 values for the regressions between foliar *Chl* per unit of fresh leaf mass and SPAD meter readings were improved for eight tree species by incorporating LMA and LWC into the regression equations which the content of *Chl* for eight tree species were significantly negatively correlated with LMA in most cases, and mostly positively with LWC for same species across sampling dates (data not shown), and still lower than those of *Chl* concentration. *Chl a* and *Chl b* per unit of leaf area can eliminate LMA effects such as *Am* in summer and autumn, and *Bp* in summer which one or two trees had the highest LMA, higher SPAD, and relatively lower *Chl* content among the sampling trees (Fig. 2). SPAD and *Chl* concentrations/contents for *Bp* in autumn showed a substantial degree of scatter throughout the wide range of data, and relationship between *Chl* concentration/content and SPAD were significant but with a lower R^2 due to leaf disease (Fig. 2).

In all species most of *Chl* present was *Chl a*, and R^2 between *Chl* concentration/content and SPAD was similar for *Chl* (*a*+*b*) and *Chl a*, and higher than those of *Chl b* especially that *Chl b* concentration/content for *Bp* and *Jm* in spring were not significantly correlated with the SPAD due to small variations for *Chl b* and SPAD (e.g., for *Bp* see Fig. 2).

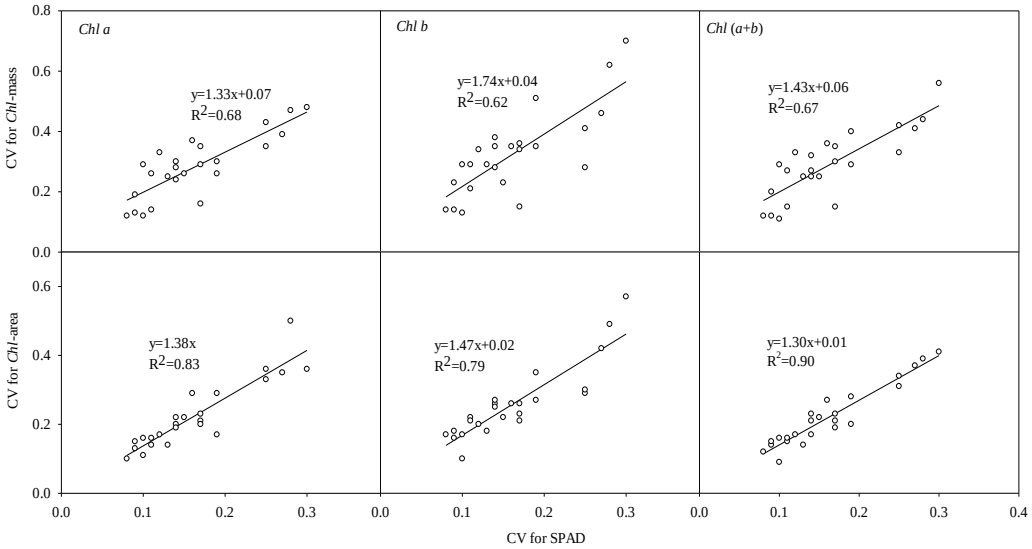

Figure 1. Linear relationship between coefficients of variation (CV) values for SPAD and quantity of *Chl a*, *Chl b*, and *Chl* (*a*+*b*) per unit leaf mass (*Chl-mass*) and on a leaf area basis (*Chl-area*) for eight species measured throughout growing season. All lines were significantly different from zero (P<0.01).

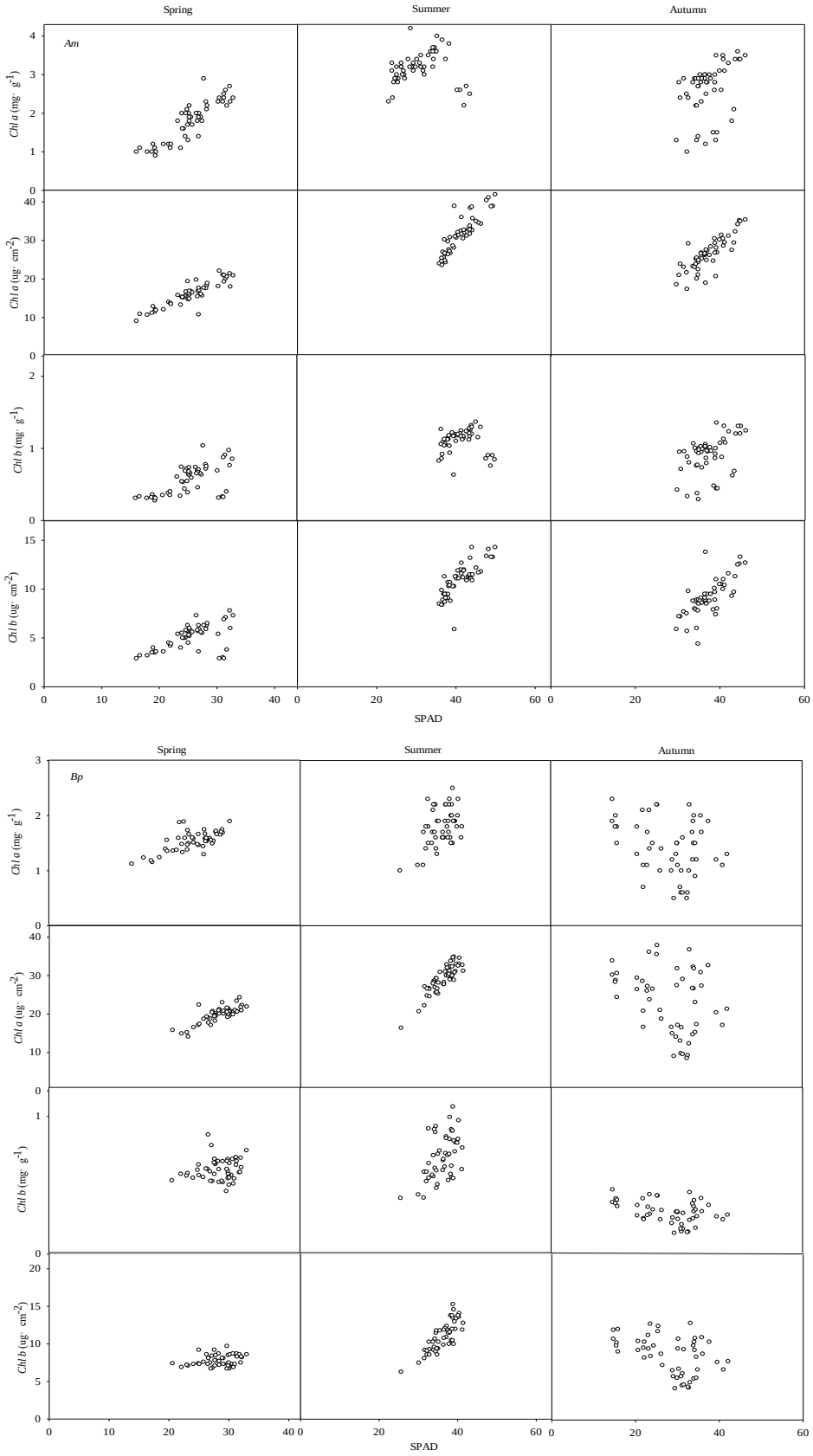

Figure 2. Scatter plots of SPAD values versus extractable *Chl a*, *Chl b* concentration per leaf area (μg cm^{-2}) and content per leaf mass (mg g^{-1}) in *A. mono* (*Am*) and *B. platyphylla* (*Bp*) for three seasons (n = 50 for each season). For regression equations and R^2, see Table 2.

DISCUSSION

Our observation period spanned the whole leaf development under different light environments such as open sites, the forest edge, and understory– from young to pre-senescent – and thus provided a broad range of pigment status. This range of pigment values allowed us to explore relationships between analytical and optical measures of pigment concentration/content and to further investigate the potential impact of leaf mass area and leaf water content on the capability of optical measurements to accurately estimate *Chl* concentration/content. We found that the relationship between the values produced by the SPAD-502 and the analytical extraction of *Chl* were very strong for the eight species we tested except for *Chl b* content/concentration in *Bp* and *Jm* in spring. No relationship between SPAD and *Chl b* content/concentration in *Bp* and *Jm* during spring may be due to a narrow range of the data resulting in a tight cluster of points. The weaker relationship between SPAD and *Chl* content for *Am* and *Up* in certain season and *Chl* content/concentration for *Bp* in autumn was due to LMA and leaf disease, respectively.

A number of researchers have found that each of the different species (Richardson *et al*., 2002; Wang *et al*., 2005; Pinkard *et al*., 2006; Biber, 2007; Samsone *et al*., 2007; Marenco *et al*., 2009; Primka IV & Smith, 2019; Donnelly *et al*., 2020) or cultivars of that species (Fanizza *et al*., 1991a; Jifon *et al*., 2005; Hawkins *et al*., 2009) has its own equations for *Chl* (*a*+*b*) for both calculations per unit of leaf area and per unit of leaf mass, suggesting that SPAD readings require calibrations by chemical analysis to determine *Chl* amount even though a few studies have shown that different species (Gratani, 1992; Markwell *et al*., 1995; Coste *et al*., 2010) and cultivars within the same species (Uddling *et al*., 2007) have similar optical/absolute *Chl* amount relationships. For example, Wang *et al*. (2005) derived that relatively poor coefficients of simple regression when data of the 10 species were pooled in a single regression model, where R^2 value for SPAD and *Chl* (*a*+*b*) concentrations was only 0.65. This finding was also true when SPAD readings from 22 species were pooled against their total extracted *Chl* concentrations (Yadava, 1986). In addition, Fanizza *et al*. (1991a) showed the similar relationships between SPAD and total *Chl* concentrations for the cvs Montepulciano, Negro Amaro and Teroldego were different from those of the cvs Bombino Bianco and Barbera, as well as from those of the cvs Trebbiano and Verdeca although a single regression for data pooled from the 12 cultivars accounted for 89% of the variability. We also found that the models should also be species-specific owing to differences in the slope of the equations, and R^2 values for SPAD and *Chl* (*a*+*b*) concentration and content were 0.67 and 0.47 from data of eight species pooled in a general regression equations. Above results suggested that separate equations should be developed for each species so as to maximize the accuracy of estimating leaf *Chl* concentration/content as a function of SPAD readings.

Furthermore, the calibration may not always hold across developmental stages for the same species. Growth stage with different growth environment, which induced variation of leaf structural parameters that affect absorption, transmission and scattering of light across the leaf, could be expected that the relationship between optical readings and *Chl* will be affected by the factor even though the *Chl* concentration/content has not changed. Thus, the relationships between optical estimates and leaf *Chl* amount may change with growth stage. To date, inconsistent results have been obtained regarding growth stage in these relationships. It has demonstrated that the relationships between the changes in optical estimates and *Chl* concentration was stable regardless of the leaf development stage (Pinkard *et al*., 2006), and the best models did not include parameters with leaf age adjustments for five warm-temperate, evergreen broadleaved tree species to estimate *Chl* concentration (Mizusaki *et al*., 2013). In contrast, Silla *et al*. (2010) observed the optical estimates - *Chl* concentration relationship for four *Quercus* species depends on leaf age, since the interaction between optical estimates and leaf stage was significant in all species. Above finding was in agreement with our work in which the relationships between optical estimates and leaf *Chl* concentration/content for eight tree species showed a seasonal change in most cases, especially for *Chl* content. This indicated that the extent of seasonal change in the relationship was also species-specific. Therefore, calibrations should be growth-stage-specific owing to differences in the slopes of the optical estimates -*Chl* concentration/content relationship; if not, interpretation of the data should be taken with caution when leaf samples of different developmental stages are used.

It should be noted that R^2 for the regression models relating optical readings to *Chl* were either in the order of *Chl a* ≥*Chl* (*a*+*b*)>*Chl b* (Torres Netto *et al*., 2005; Pinkard *et al*., 2006; de Jesus & Marenco, 2008; Amarante *et al*., 2009; Mielke *et al*., 2010; Nascimento & Marenco, 2010; Bielinis *et al*., 2015; Liu *et al*., 2019; Shibaeva *et al*., 2020), similar R^2 values among the relationship between optical readings and *Chl a*, *Chl* (*a*+*b*), or *Chl b* (Richardson *et al*., 2002; Wang *et al*., 2005), a relatively low R^2 for optical readings – *Chl a* relation than for equations fitted for *Chl b* and *Chl* (*a*+*b*) (de Jesus & Marenco, 2008; Reis *et al*., 2009; Fu *et al*., 2013), or even non-significant among optical readings – *Chl a* or *Chl b* except for *Chl* (*a*+*b*) (Anand & Byju, 2008). We derived that R^2 for all trees in most cases was in the order of *Chl a* ≥*Chl* (*a*+*b*)>*Chl b*. It supported the suggestions by Nascimento & Marenco (2010) that the SPAD-502 meter more accurately reflected the *Chl a* quantity of a leaf rather than *Chl b* or *Chl* (*a*+*b*), and used the chlorophyll meters to estimate *Chl a*.

It is possible that multiple chemical and physical leaf properties determined by environmental conditions may interact to influence the ability of optical measurements to provide accurate estimates of leaf *Chl* status for tree species through different leaf development stages. For example, in addition to leaf *Chl*, optical measurements may be influenced by leaf thickness, moisture content, or leaf development stage which affected the leaf thickness, leaf water content, and other properties for leaves. Reference wavelength for optical estimates were the use of the near infrared light beam at 940 nm which tends to compensate for differential scattering by different leaf thicknesses. However, several studies have reported that variation in the optical meters-leaf *Chl* amount relationship might have been partly due to the differences in LMA (one of indicators of leaf thickness) (Campbell *et al*., 1990; Fanizza *et al*., 1991ba; Jifon *et al*., 2005; Demotes-Mainard *et al*., 2008; Marenco *et al*., 2009). Nevertheless, the correlation direction between optical readings and LMA was either positive (Jifon *et al*., 2005; Marenco *et al*., 2009; Novichonok *et al*., 2016) or negative (Demotes-Mainard *et al*., 2008). For example, Novichonok *et al*. (2016) showed that the LMA values in the studied leaves varied over a wide range, 16.5- 73.3 g m^{-2}, which were strong significant positive relationships between the optical value and LMA for *Cleistanthus sp.* As LMA as the independent variable was added into regression between optical readings and *Chl* relationship increased R^2 values compared with those values showed in simple regression equations in leaves of six Amazonian tree species (Marenco *et al*., 2009), *Q. pyrenaica* and *Q. suber* (Silla *et al*., 2010). We also found that there was a strong negatively correlated relationship between *Chl* content and LMA values for eight tree species in most cases whereas the correlations between *Chl* concentration and LMA were either positive-, negative significantly or non-significantly for same species across sampling dates. The addition of LMA as a complementary parameter improved the accuracy of *Chl* content estimation in species-specific equations to a considerable extent when leaf samples of similar development were considered.

Indeed, foliar *Chl* quantity is expressed on a leaf area basis in most cases can be eliminated the confounding effect of leaf thickness. Relationships between optical estimates and extractable *Chl* was stronger when it was expressed on a leaf area basis than a fresh mass basis (Marquard & Tipton, 1987; Campbell *et al*., 1990; Fanizza *et al*., 1991a; Cate & Perkins, 2003; Esposti *et al*., 2003; Berg & Perkins, 2004; Uddling *et al*., 2007; Marenco *et al*., 2009; Amarante *et al*., 2009; Hawkins *et al*., 2009; Novichonok *et al*., 2016) though several studies showed the relationship between optical estimates and foliar *Chl* cannot be improved if foliar *Chl* is expressed per leaf area compared with per unit mass basis (Cassol *et al*., 2008 with floss-silk tree; Bielinis *et al*., 2015). We observed strong relationships between analytical *Chl* on an area basis and optical measures for eight tree species especially for *Qm*, *Bp*, *Am*, and *Up* in certain season. It is known that the SPAD readings increases as leaves with higher LMA tend to have a lower transmittance due to an increased multiple scattering and optical path length through the leaf as compared to leaves with lower LMA (Jifon *et al*., 2005; Uddling *et al*., 2007). Indeed, it happened in tree samples species among *Qm*, *Bp*, *Am*, and *Up*. It confirmed that differences in LMA among leaves can considerably interfere the correlation between the SPAD readings and foliar *Chl* content. This suggests that the optical estimates might be useful with greater certainty in predicting *Chl* status in leaves of woody species if both variables are expressed on a leaf area basis.

Alternatively, many 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). For example, Wang

et al. (2005) did not observe any significant effect of leaf thickness on either optical readings or *Chl* concentration among ten green-leaved foliage plant species which leaf thickness ranged from 0.19–0.66 mm. This finding concurs with work by Fanizza *et al*. (1991a) in which other factors must have been involved when cultivars with different regression equations had essentially the same range of LMA. Moreover, Silla *et al*. (2010) found that the accurateness of *Chl* concentration predictions for *Q. faginea* and *Q. ilex* did not show any improvement when fresh LMA was added as an independent variable to the optical readings in the multiple regression equations. Coste *et al*. (2010) also suggested no effect of LMA per se which LMA varied between 38.2 and 119 g m^{-2} of 13 tropical tree species representing a three-fold gradient in specific leaf area. In addition, Fu *et al*. (2013) found LMA was poorly correlated with SPAD values which LMA values varied between 192 and 222 g m^{-2} among the 4 tested litchi cultivars (*Litchi chinensis* Sonn.). Novichonok *et al*. (2016) also showed that the LMA values in the studied leaves varied over a wide range 37.5-102.7 g m^{-2} for *Calamus sp*, the relationships between the optical values and LMA were not significant. Above results suggested that optical estimates were not related to leaf thickness, and hence the difference in leaf greenness among species was not due to leaf thickness but to *Chl* status. Therefore, differences in models between optical estimates and *Chl* among leaf sets with similar or wide ranges of LMA may be influenced by other factors.

While it has been shown that accounting for LWC can improve estimates of absolute *Chl* quantity derived from optical measurements (Marenco *et al*., 2009), previous studies have also reported positive (Niu *et al*., 2007), negative (Martínez & Guiamet, 2004; Marenco *et al*., 2009), and no relationship (Li *et al*., 1998; Mielke *et al*., 2010, Silla *et al*., 2010) between LWC and optical estimates of leaf *Chl* status. For example, Novichonok *et al*. (2016) showed that the LWC values in the studied leaves varied over a wide range from 22.2% to 87.6% for *Cleistanthus sp* and 30.5-67.1% for *Calamus sp.*. There were strong significant negative relationships between the optical value and LWC for *Cleistanthus sp.* For *Calamus sp.*, the relationships between the optical values and LWC were not significant. In the study, we observed the content of *Chl* (*a*+*b*) for eight tree species were mostly positively correlated with LWC whereas the correlations between SPAD, concentration of *Chl* (*a*+*b*) and LWC values were either positive, negative significantly or non-significantly for same species across sampling dates, and were unable to attribute the differences to LWC.

Foliar *Chl* is a good indicator of various plant biotic and abiotic stresses. The indirect, time-saving and nondestructive detection of leaf *Chl* status is of great importance for plant health and physiological performance. For example, SPAD readings are strongly and significantly correlated with *Chl* content as measurements of tolerance to bicarbonate-induced iron chlorosis in citrus rootstock (Sudahono *et al*., 1994), and disease severity scores of cassava leaves caused by whitefly (*B. tabaci*) (Sims *et al*., 2018). Close linear correlations between SPAD values and extractable *Chl* concentration/content have been reported for several species under biotic or abiotic stress, including 1 year old apple trees infested with *A. spiraecola* based on a narrow range of measurements (Kaakeh *et al*., 1992), relatively uninjured leaves of cutleaf coneflower (*R. laciniata* var. *digitata*) by ozone (Neufeld *et al*., 2006), species-specific linear equations for *U. laevis*, *S. fragilis*, and *T. platyphyllo* infested with gall (Samsone *et al*., 2007), and mature urban severely and moderately declining trees of *T. vulgaris* with separate slopes and intercepts (Scattolin *et al*., 2013). A high polynomial quadratic mathematical models were fitted between SPAD readings and total *Chl* content in foliar tissue of *A. pseudoplatanus*, *F. sylvatica*, and *Q. robur* displaying visual symptoms of N deficiency (Percival *et al*., 2008), exponential equations relating SPAD to *Chl* content/concentration was best fit in *Solanum lycopersicum* leaves with mottled, interveinal chlorosis induced by continuous lighting (Shibaeva *et al*., 2020), and even at very low *Chl* concentration/content during leaf senescence process with a broad range of data (Biber, 2007; Li *et al*., 2017; Primka IV & Smith, 2019; Donnelly *et al*., 2020).

However, Neufeld *et al*. (2006) showed that SPAD meter became unreliable for estimating *Chl* concentrations in visibly ozone-injured leaves in cutleaf coneflower. Liu *et al*. (2019) also found low measurement accuracy for healthy lichen species with a coarse blade surface. In the current study, SPAD was also correlated with foliar *Chl* concentration/content for *Bp* in autumn with the low R^2. SPAD values in *Bp* decreased significantly as the onset and time development of foliar injury from summer to autumn, but values were similar to those of healthy trees in spring. Carotenoids contents

remained constant, and content of *Chl* (*a*+*b*) was the lowest and concentration decreased significantly compared with summer. Thus, the *Chl* (*a*+*b*)/*Car* ratio had been decreased from spring to autumn, which may indicate a diminished photosynthetic capability or a response to *Bp* stresses. However, R^2 between SPAD and *Chl* (*a*+*b*)/*Car* ratio for *Bp* in autumn was only 0.17, and the percentage of the degree of fit between *Chl* (*a*+*b*)/*Car* and SPAD below 0.50 was more than 67% for eight species in each season. Percival *et al*. (2008) also found a poor correlation between SPAD values and *Chl* (*a*+*b*)/*Car* ratios, which are widely used for diagnosing the physiological state of plants, for three tree species which R^2 ranged from 0.13 to 0.54.

The reason for the meter becoming unreliable in the face of *Bp* in autumn is not yet fully understood. However, there are several alternative explanations for the result. One possible explanation for the poorer performance of SPAD for *Bp* in autumn is that severely infected leaves may simply have too little *Chl* for the SPAD meter to read accurately. Several studies indicated that the SPAD-502 became less accurate when *Chl* concentration/content in leaves was below 100 $mg \cdot m^{-2}$ (Monje & Bugbee, 1992) or 1.5 mg g^{-1} (Samsone *et al*., 2007). However, *Chl* amounts for *Bp* in autumn, with the exception of one tree, were consistently above the 120 $mg \cdot m^{-2}$ or 1.5 mg g^{-1} minimum, and some values were in the same range as leaves in summer. Thus, *Chl* deficiency was not the primary factor for leaves within the operational range of the meter.

Alternatively, perhaps highly uneven distribution of *Chl* within the infested leaves introduces additional scatter in the SPAD/*Chl* relationship for *Bp* in autumn. To cope with this possibility, we selected tree samples under different habitats trying to have as diverse a range of *Chl* status as possible, and did try to measure eight different places on each leaf to generate a mean SPAD value where we obtained the *Chl* from the same area and took an adequate number of leaf samples to minimize the variability due to *Chl* distribution within and between leaves. The procedure was followed for averaging heterogeneity of *Chl* distribution in the leaf surface to have a representative estimation of the overall leaf *Chl* to derive accurate conclusions (Dwyer *et al*., 1991; Chang & Robison, 2003). The ranges of SPAD and *Chl* content (the differences between the maximum and the minimum) for *Bp* in autumn were the greatest among seasons. The poor result may not be due to non-uniform distribution of *Chl* content in leaves.

The most plausible explanation is that the presence of necrotic tissue, which changed leaf physical (e.g., surface roughness, color, LMA, and LWC) and chemical properties, probably modifies leaf optical traits. Neufeld *et al*. (2006) suspected that visibly ozone-injured leaves would reduce absorption, enhance light transmission, alter light scattering properties, and explain why the SPAD readings are higher in injured leaves at a given *Chl* content than in uninjured leaves. Bielinis *et al*. (2015) also found the necrotic brown leaves of *Q. petraea* had low *Chl* concentration and high SPAD values in November. In fact, the SPAD readings for *Bp* were not lower in autumn at a given *Chl* content compared to leaves in spring and summer. Thus, the sensitivity decrease may be the result of light transmission changed by certain leaf surface and inside characteristics influenced by various environmental and ontogenic stresses, all of which may have important effects on leaf optical properties. This may be an inherent limitation of the meter that cannot be overcome, even with a large sampling area (Monje & Bugbee, 1992). Further studies with the role of necrotic lesions in affecting light scattering, reflectance, absorption, and transmittance must be taken into account to precisely monitor the *Chl* status of trees with the SPAD-502 meter.

CONCLUSION

The amount of *Chl* in the woody species is the basic physiological parameters both for forest research and management. Compared with conventional methods, employment of a useful hand-held, non-destructive optical meter is a very simple, quick and direct procedure to aid in the evaluation of *Chl* status in the leaves of a wide range of different tree species. The obtained results support the potential usage of nondestructive *Chl* measurement by SPAD in providing a direct conversion of optical readings to foliar absolute *Chl* amount, when appropriate individual calibration of SPAD readings against *Chl* concentration/content is established for each particular tree species accurately and

reliably at a certain growth stage because of the differences in leaf surface and inside characteristics. The maximum accuracy of the estimate was achieved when the SPAD-502 meter was used for the determination of the *Chl* concentration per unit of leaf area, especially for *Chl a* and *Chl* (*a*+b). Precautions for using the meter are prescribed if unhealthy leaves are to be measured.

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