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Relationship between specific leaf area, leaf thickness, leaf water content and *SPAD-502* readings in six Amazonian tree species

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Abstract

The aim of this work was to assess the effect of leaf thickness, leaf succulence (L_S), specific leaf area (SLA), specific leaf mass (W_s) and leaf water content (LWC) on chlorophyll (Chl) meter values in six Amazonian tree species (*Carapa guianensis, Ceiba pentandra, Cynometra spruceana, Pithecolobium inaequale, Scleronema micranthum* and *Swietenia macrophylla*). We also tested the accuracy of a general calibration equation to convert *Minolta* Chl meter (*SPAD-502*) readings into absolute Chl content. On average, SPAD values (x) increased with fresh leaf thickness (FLT [μm] = 153.9 + 0.98 x, $r^2 = 0.06^{**}$), dry leaf thickness (DLT [μm] = 49.50 + 1.28 x, $r^2 = 0.16^{**}$), specific leaf mass (W_s [g (DM) m^{-2}] = 6.73 + 1.31 x, $r^2 = 0.43^{**}$), and leaf succulence (L_S [g(FM)] m^{-2} = 94.2 + 1.58 x, $r^2 = 0.19^{**}$). However, a negative relationship was found between SPAD values and either specific leaf area [SLA (m^2 kg^{-1}) = 35.1 – 0.37 x, $r^2 = 0.38^{**}$] or the leaf water content (LWC [%]= 80.0 – 0.42 x, $r^2 = 0.58^{**}$). Leaf Chl contents predicted by the general calibration equation significantly differed ($p<0.01$) from those estimated by species-specific calibration equations. We conclude that to improve the accuracy of the *SPAD-502* leaf thickness and LWC should be taken into account when calibration equations are to be obtained to convert SPAD values into absolute Chl content.

Additional key words: Carapa guianensis, Ceiba pentandra, Pithecolobium inaequale, Scleronema micranthum, Swietenia macrophylla.

Introduction

Chlorophylls (Chl) are the main pigments involved in light capture for photosynthesis and other photochemical and non-photochemical reactions; therefore the amount of light absorbed by a leaf is related to Chl content. Besides its importance in light capture, leaf Chl content may be used as an indicator of the light environment during plant growth. For example, the Chl *a*/*b* relationship is lower in shade than in sun leaves (Boardman 1977). Most common methods for determining leaf Chl content involve leaf destruction, which precludes assessing leaf Chl content over time. In the 1990s *Minolta Corporation* developed a portable Chl meter (*SPAD-502*) which measures an index related to the Chl content of a leaf. It allows accurate and rapid measurements of relative leaf Chl content in the field. As there is a close relationship between leaf nitrogen and leaf Chl content, the *SPAD-502* Chl meter has been used as a tool to assess leaf nitrogen content in crops (*e.g.* Gáborcik 2003, Fritschi and Ray 2007). Also, it could be used to assess specific leaf area in peanut (Nageswara Rao *et al.* 2001) and transpiration efficiency in wheat (Fotovat *et al.* 2007).

In general, it has been considered that calibration equations for the *SPAD-502* are species-specific (Richardson *et al.* 2002, Uddling *et al.* 2007). Nevertheless, Markwell *et al.* (1995) found that a single calibration equation could be used for soybean (a C_3 plant) and maize (a C_4 plant). Several factors may affect *SPAD-502* values including cultivar, plant growth stage, and any edaphic or climatic factor causing leaf chlorosis (Turner and Jund 1994; Chapman and Barreto 1997). In addition, the importance of some leaf traits, such as leaf thickness, leaf succulence and specific leaf mass (the reciprocal of

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Abbreviations: Chl – chlorophyll; DLT – dry leaf thickness; DM – dry matter; FLT – fresh leaf thickness; FM – fresh matter; L_S – leaf succulence; LWC – leaf water content; SLA – specific leaf area; *SPAD-502* – *Minolta* chlorophyll meter; W_S – specific leaf mass.

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specific leaf area) on SPAD values has been recognized (Fanizza *et al.* 1991, Chapman and Barreto 1997; Chang and Robison 2003), but how these leaf characteristics actually affect SPAD readings are still to be confirmed. In *Lupinus havardii*, for example, at mild water stress (–0.7 to –1.2 MPa), *SPAD-502* readings increased with leaf water potential (Niu *et al.* 2007). On the contrary, Martínez and Guiamet (2004) observed that the SPAD index tends to be greater on leaves with low water content (LWC). Campbell *et al.* (1990) suggested that differences in leaf thickness (a factor that can vary among species, stage of development and environmental conditions) may cause variation in the SPAD-leaf N content relationship. However, Wang *et al.* (2005) did not observe any significant effect of leaf thickness (on a fresh mass basis) on the SPAD index. Although a negative W_s-SPAD relationship was reported for barley (Bort *et al.* 1998), a positive correlation between the SPAD index and specific leaf mass was observed in durum wheat (Araus *et al.* 1997). Thus, the major aim of this work is to determine the relationship between leaf thickness, leaf succulence, specific leaf traits (area and mass), and LWC (percent of fresh mass) on SPAD readings. Also to generate calibration equations to convert relative SPAD units into absolute Chl content in six Amazonian trees.

Materials and methods

The study was conducted at the V8 Campus of the National Institute for Research in the Amazon (03° 05' 30" S; 59° 59' 35" W), Manaus, AM, Brazil. The city of Manaus has a mean temperature of 27 °C, average rainfall of 2240 mm, and a relative humidity of 78 %. In this work six tropical tree species growing at the open were used. The studied species were: *Carapa guianensis* Aubl. (Meliaceae), *Ceiba pentandra* L. Gaertn. (Bombacaceae), *Cynometra spruceana* Benth. (Leguminosae), *Pithecolobium inaequale* (Willd.) Benth. (Leguminosae), *Scleronema micranthum* Ducke (Bombacaceae) and *Swietenia macrophylla* King (Meliaceae). On each species the relative Chl content was measured with a *SPAD-502* Chl meter (*Minolta Camera Co.*, Osaka, Japan). Ten readings were taken from the widest portion of the leaf blade at about 15 mm from the leaf margin. We positioned the adaxial leaf surface toward the emitting window of the instrument while avoiding major veins.

To determine dry leaf thickness (DLT), fresh leaf thickness (FLT), specific leaf mass (W_S), specific leaf area (SLA), and leaf water content (LWC) four circles, 17.2 mm in diameter, were punched early in the morning from fresh leaves of the studied tree species. Leaf thickness was measured between major leaf veins with a digital caliper, both on fresh leaves and after oven-drying at 72 °C until reaching constant mass. W_S was calculated as the leaf mass to leaf area ratio, and SLA as the inverse of W_S. The leaf water content (LWC), as a percentage of fresh mass, was calculated according to the following formula: LWC = 100 (FM – DM)/FM, where DM and FM denote dry matter and fresh matter, respectively. Besides W_S, we also calculated the fresh mass to leaf area ratio (L_S, succulence). In this study 30 to 50 leaves per species were used.

To generate calibration equations, SPAD readings were taken early in the morning, then the leaves were covered with a plastic bag, detached and immediately taken to the laboratory for Chl extraction. To obtain a wide range of Chl content, leaves of different stages of development, from very young to fully expanded sun leaves, were used. For Chl extraction, five to ten 5-mm-circles (depend on leaf greenness) were punched with a cork borer from the same portion of the leaf previously used for SPAD measurements weighed and immediately ground in 10 ml of 80 % aqueous acetone (v/v) added of 10 mg of $MgCO_3$ and 20 mg of quartz crystals and absorbance determined at 645 and 663 nm with a spectrophotometer (*SP-2000 UV*, *Shanghai Spectrum,* Shanghai, China). The SP-2000 UV has a spectral slit-width of 5 nm. Chl contents were calculated using Arnon´s equations (Arnon 1949).

Regression analyses were carried out to determine the relationship between SPAD values and Chl content for each species (species-specific equation). In addition, we fitted a prediction equation for all data across the studied species (general equation). Leaf thickness, W_S, L_S, SLA, and LWC as a function of SPAD readings were examined by regression analysis. Student's *t*-test for paired observations was used to test differences between Chl contents predicted by the species-specific equation and the general equation (Steel and Torrie 1981). The *SAEG 9.0* statistical package from the Federal University of Viçosa-Brazil was used for statistical analysis.

Results

In almost all species, we found a significant relationship ($p<0.05$) between leaf traits (leaf thickness, W_S, SLA, L_S) and LWC and the output of the Chl meter (Fig. 1,2). In *S. micranthum* no correlation was found between leaf succulence (L_S) and SPAD readings. In addition, it is worth noting, that in this species W_S had a significant relationship with the SPAD index although no effect of leaf thickness on SPAD values was observed, as could be expected.

For the pool of species, the goodness of the fit for the FLT-SPAD relationship was lower than for measurements made on dry leaves, as inferred from the values of

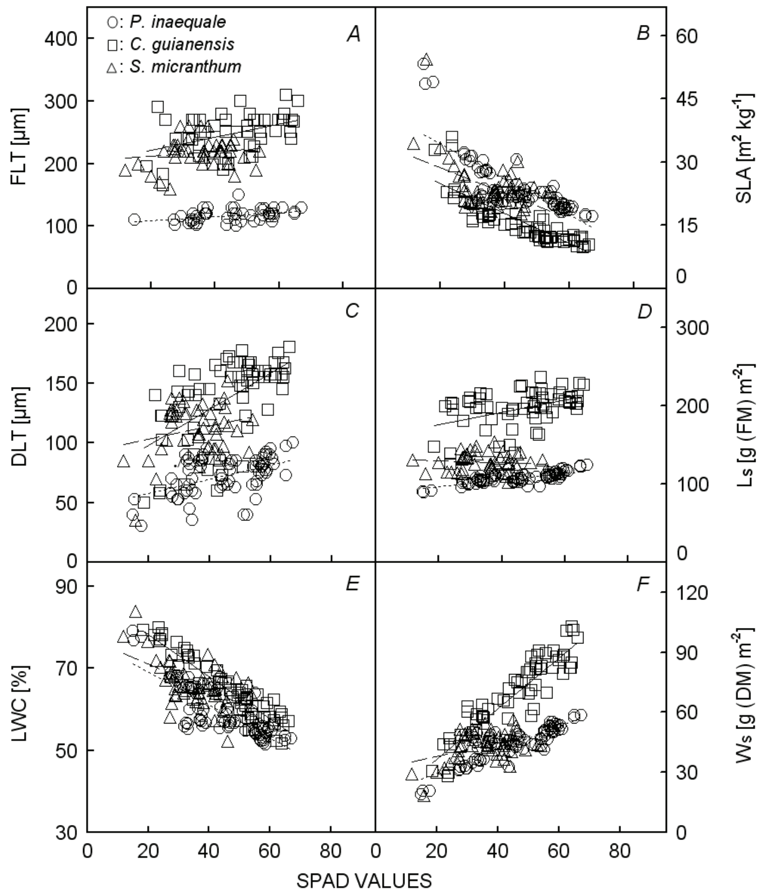

Fig. 1. Relationship between fresh leaf thickness (FLT, *A*), specific leaf area (SLA, *B*), dry leaf thickness (DLT, *C*), leaf succulence (L_S, *D*), leaf water content (LWC, *E*), and specific leaf mass (W_S, *F*), and SPAD values in *P. inaequale* (○, *dotted line*), *C. guianensis* (□, *solid line*), *S. micranthum* (Δ, *dashed line*).

determination coefficients, r^2 of 0.16 for dry leaves *versus* 0.06 in fresh leaves (all species, Table 1), which suggests some effect of LWC on SPAD values (Fig. 1*E*, 2*E*).

The SPAD-Chl relationship was better described by an exponential function with two parameters (Fig. 3, Table 2) than by a linear model, as the slope of the regression model increased with SPAD values. In almost all species, equations fitted for data of the six species combined (general equation) predicted Chl contents different from those estimated for the species-specific model, as inferred from Student's *t*-test for paired observation (Fig. 3). The exception was found in *S. macrophylla* on which the general model did not differ from the species-specific one (p>0.05). Chl(a+b) content varied from 600 mg m^{-2} for *S. micranthum* to 900 mg m^{-2} for *S. macrophylla* and *C. pentandra*, whereas on a mass basis total Chl concentration oscillated from 2.5 (*C. guianensis*) to 7.5 mg g^{-1} in *P. inaequale* (Fig. 3).

Discussion

In this work, the effect of W_S, SLA and leaf thickness on SPAD values concurs with those reported by others, either for *Citrus* (Jifon *et al.* 2005) or *Sorghum* sp. and *Cajanus cajan* (Yamamoto *et al.* 2002). A negative relationship between the SPAD index and SLA was also found in peanut by others (Nageswara Rao *et al.* 2001; Nigam and Aruna 2008). Our results, however, do not confirm those reported by Wang *et al.* (2005), who found no effect of leaf thickness on SPAD values. This discrepancy may be attributed to differences in leaf thickness between the leaves they used (FLT of 0.19 to 0.66 mm) and the leaves we used in this experiment (0.1 to 0.3 mm for fresh leaves). Thicker leaves (often related to high W_S and low SLA) tend to have a lower transmittance due to an increased multiple scattering and optical pathlength (detour effect) through the leaf as compared to thinner leaves (Vogelmann 1993). Photon pathlength through the leaf increases with leaf thickness, which enhances multiple scattering and as a result the SPAD index increases.

Here we show that, on average, a drop in LWC from 70 to 55 % leads to an increase of 35 SPAD units (Fig. 1,2), thereby confirming that leaf water content may interfere with the accuracy of the *SPAD-502* (Schlemmer *et al.* 2005).The increase in SPAD values in response to a decline in leaf turgor is consistent with the findings of Martínez and Guiamet (2004). In contrast to the effect of leaf thickness, increasing LWC reduces photon path

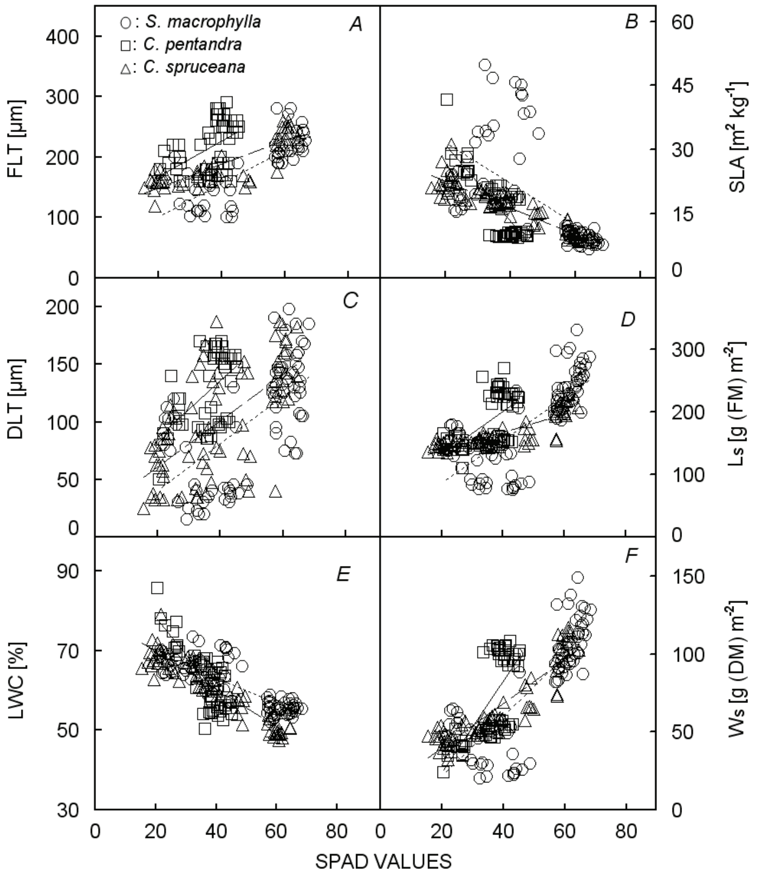

Fig. 2. Relationship between fresh leaf thickness (FLT, *A*), specific leaf area (SLA, *B*), Dry leaf thickness (DLT, *C*), leaf succulence (L_S, *D*), leaf water content (LWC, *E*), and specific leaf mass (W_S, *F*), and SPAD values in *S. macrophylla* (○, *dotted line*), *C. pentandra* (□, *solid line*), *C. spruceana* (Δ, *dashed line*).

length through the leaf, which may lead to a reduced light absorptance (Vogelmann 1993).

In *S. micranthum*, specific leaf mass (W_s) had a significant relationship with SPAD readings although no effect of DLT on SPAD values was observed, which suggests that other parameters intrinsically related to leaf optical properties also interfere with SPAD readings. W_s is often but not always closely related to DLT. For example, Garnier and Laurent (1994) found no correlation between DLT and W_s in several grass species. Also, it is remarkable that the lack of an effect of DLT on SPAD values was only apparent in *S. micranthum,* a species with the thinnest leaves and the highest SLA (mean of 24 $m^2 kg^{-1}$) among examined species.

In this study, Chl data as a function of SPAD values were best described by an exponential function which is in agreement with results reported by others (Markwell *et al.* 1995; Uddling *et al.* 2007). In general, regression parameters were species-specific which concurs with the findings of others (Marquard and Tipton 1987, Castelli *et al.* 1996, Yamamoto *et al.* 2002). Indeed, calibration equations can even be specific for cultivars within the same species, as observed in *Citrus* sp. (Jifon *et al.* 2005). Although in some other studies similar calibrations have been obtained for different species (Gratani 1992; Markwell *et al.* 1995), hybrids or cultivars within the same species (Dwyer *et al.* 1991, Uddling *et al.* 2007). It is not clearly understood what determines the species-specific variations in the SPAD-Chl relationship. It is likely that besides variations in LWC (Martinez and Guiamet 2004, Schlemmer *et al.* 2005), growing conditions may also affect the relationship between Chl content and SPAD values, as found in *Malus domestica* (Campbell *et al.* 1990) or *Citrus* sp. (Jifon *et al.* 2005). Growth environment may lead to changes in leaf thickness (Boardman 1977, Jifon *et al.* 2005), which can contribute to variation in the SPAD-Chl relationship (Chang and Robison 2003). Loss of linearity in the SPAD-Chl relationship may result from several factors, including multiple scattering, LWC, leaf thickness, and non-uniform Chl distribution across the leaf lamina. Compared to leaves with Chl molecules uniformly distributed across the leaf lamina, a non-uniform Chl distribution leads to lower SPAD values, due to high transmittance of the leaves (at 650 nm) in those areas when light passes without encountering an absorber, the so called sieve effect (Rabinowitch 1951*)*. Multiple scattering (the reflection and refraction of absorbed radiation that occurs in the intercellular spaces), a function of the photon pathlength through the leaf, may also contribute to the SPAD-Chl deviation from linearity, as it increases light absorptance (Vogelmann 1993). Thus, non-linearity

Table 1. Relationship between fresh leaf thickness (FLT), dry leaf thickness (DLT), leaf succulence (L_S), specific leaf mass (W_S), specific leaf area (SLA) and leaf water content (LWC) and SPAD readings in six Amazonian tree species. ns: not significant ($p>0.05$), *: $p<0.05$ and **: $p<0.01$).

Species	FLT [µm]	DLT [µm]	L_S [g (FM) m^{-2}]	W_S [g (DM) m^{-2}]	SLA [m^2 kg^{-1}]	LWC [%]
S. macrophylla	$y = 45.76+2.77\,x$ ($r^2 = 0.60^{**}$)	$y = -1.04 + 2.05\,x$ ($r^2 = 0.40^{**}$)	$y = 18.66 + 3.41\,x$ ($r^2 = 0.56^{**}$)	$y = -16.63 + 1.91\,x$ ($r^2 = 0.65^{**}$)	$y = 41.42 - 0.48\,x$ ($r^2 = 0.39^{**}$)	$y = 75.94 - 0.32\,x$ ($r^2 = 0.66^{**}$)
C. guianensis	$y = 200.98 + 1.03\,x$ ($r^2 = 0.17^{**}$)	$y = 67.12 + 1.51\,x$ ($r^2 = 0.33^{**}$)	$y = 160.69 + 0.79\,x$ ($r^2 = 0.18^{**}$)	$y = 11.16 + 1.26\,x$ ($r^2 = 0.80^{**}$)	$y = 32.001 -0.35\,x$ ($r^2 = 0.68^{**}$)	$y = 88.67 - 0.516\,x$ ($r^2 = 0.85^{**}$)
C. pentandra	$y = 109.93 +2.92\,x$ ($r^2 = 0.21^{**}$)	$y = 35.34 + 2.51\,x$ ($r^2 = 0.27^{**}$)	$y = 62.45 + 3.46\,x$ ($r^2 = 0.30^{**}$)	$y = - 34.92 + 2.94\,x$ ($r^2 = 0.48^{**}$)	$y = 49.04 - 0.89\,x$ ($r^2 = 0.64^{**}$)	$y = 97.19 - 0.94\,x$ ($r^2 = 0.64^{**}$)
P. inaequale	$y = 101.92 + 0.33\,x$ ($r^2 = 0.16^{**}$)	$y = 45.77 + 0.60\,x$ ($r^2 = 0.23^{**}$)	$y = 87.99 + 0.43\,x$ ($r^2 = 0.60^{**}$)	$y = 17.86 + 0.58\,x$ ($r^2 = 0.73^{**}$)	$y = 42.67 - 0.41\,x$ ($r^2 = 0.62$)	$y = 77.14 - 0.40\,x$ ($r^2 = 0.66^{**}$)
C. spruceana	$y = 110.85 + 1.96\,x$ ($r^2 = 0.71^{**}$)	$y = 21.31 + 1.99\,x$ ($r^2 = 0.40^{**}$)	$y = 108.34 + 1.47\,x$ ($r^2 = 0.69^{**}$)	$y = 10.94 + 1.43\,x$ ($r^2 = 0.83^{**}$)	$y = 28.80 - 0.30\,x$ ($r^2 = 0.84^{**}$)	$y = 79.25 - 0.47\,x$ ($r^2 = 0.87^{**}$)
S. micranthum	$y = 204.87 + 0.37\,x$ ($r^2 = 0.02^{\text{ns}}$)	$y = 92.73 + 0.54\,x$ ($r^2 = 0.05^{\text{ns}}$)	$y = 132.64 - 0.14\,x$ ($r^2 = 0.01^{\text{ns}}$)	$y = 31.29 + 0.33\,x$ ($r^2 = 0.18^{**}$)	$y = 34.69 - 0.30\,x$ ($r^2 = 0.22^{**}$)	$y = 77.31 - 0.31\,x$ ($r^2 = 0.30^{**}$)
All species	$y = 153.88 + 0.98\,x$ ($r^2 = 0.06^{**}$)	$y = 49.50 + 1.28\,x$ ($r^2 = 0.16^{**}$)	$y = 94.23 + 1.58\,x$ ($r^2 = 0.19^{**}$)	$y = 6.73 + 1.31\,x$ ($r^2 = 0.43^{**}$)	$y = 35.06 - 0.37\,x$ ($r^2 = 0.38^{**}$)	$y = 80.03 - 0.42\,x$ ($r^2 = 0.58^{**}$)

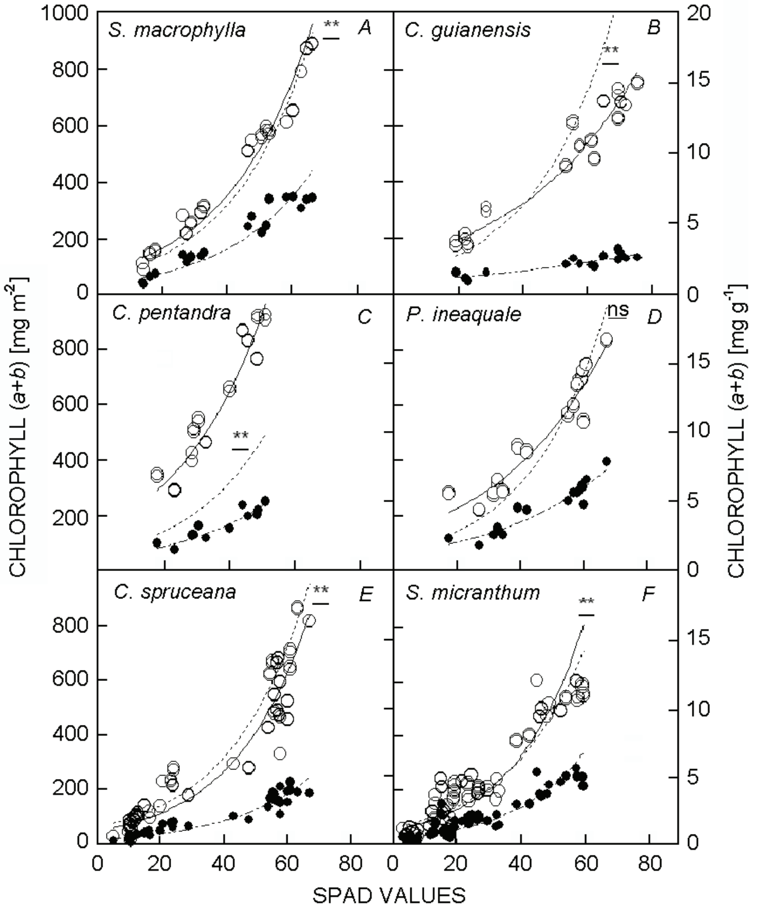

Fig. 3. Relationship between SPAD values and Chl(a+b) content in *S. macrophylla* (*A*), *C. guianensis* (*B*), *C. pentandra* (*C*), *P. inaequale* (*D*), *C. spruceana* (*E*), and *S. micranthum* (*F*), per unit leaf area (○) and on a "fresh mass basis (●). On each panel, statistical significance for the difference between the Chl content predicted (per unit area) by the specific equation (*solid line*) and the general equation (d*ashed line*) is shown. The dash-dotted line represents the output of specific equations on a fresh mass basis. ns: not significant (p>0.05), *: p<0.05 and **: p<0.01).

Table 2. Calibration equations for converting *SPAD-502* readings into Chl (a + b) contents in six Amazonian tree species. **: p<0.01.

Species	Chl (a + b) [mg g^{-1}]	Chl (a+b) [mg m^{-2}]
S. macrophylla	$y = 0.71e^{0.0379x}$ ($r^2 = 0.90^{**}$)	$y = 76.717e^{0.0381x}$ ($r^2 = 0.95^{**}$)
C. guianensis	$y = 0.86e^{0.0158x}$ ($r^2 = 0.80^{**}$)	$y = 117.18e^{0.0253x}$ ($r^2 = 0.94^{**}$)
C. pentandra	$y = 0.87e^{0.0336x}$ ($r^2 = 0.83^{**}$)	$y = 150.22e^{0.0365x}$ ($r^2 = 0.90^{**}$)
P. inaequale.	$y = 1.17e^{0.0273x}$ ($r^2 = 0.88^{**}$)	$y = 128.88e^{0.0276x}$ ($r^2 = 0.90^{**}$)
C. spruceana	$y = 0.37e^{0.0383x}$ ($r^2 = 0.86^{**}$)	$y = 48.591e^{0.0425x}$ ($r^2 = 0.86^{**}$)
S. micranthum	$y = 0.44e^{0.0453x}$ ($r^2 = 0.80^{**}$)	$y = 51.395e^{0.0461x}$ ($r^2 = 0.81^{**}$)
All species	$y = 0.53e^{0.0364x}$ ($r^2 = 0.70^{**}$)	$y = 62.05e^{0.0408x}$ ($r^2 = 0.79^{**}$)

of the SPAD-Chl relationship may result of non-uniform Chl distribution at high Chl content and multiple scattering associated to leaf thickness. Chl contents reported in this study are similar to those reported by others (*e.g.* Björkman and Demmig 1987, Marenco and Santos 1999). Determination coefficients (r^2) were higher for prediction equations describing Chl per unit area than those obtained for regressions predicting Chl content on a mass basis. This indicates that the accuracy of the instrument tends to be higher for estimating Chl content per unit leaf area, which is consistent with the effect of leaf thickness on SPAD indices (Fig. 1,2). In addition, for the same degree of leaf greenness, Chl content per unit area (but not on a mass basis) may remain rather constant irrespective of leaf thickness (McMillen and McClendon 1983).

In almost all species the general model predicted Chl contents different from those estimated for the species-specific model, as inferred from Student´s t-test for paired observations (Fig. 3, *dashed line*), which is consistent with results reported by others (*e.g.* Uddling *et al.* 2007). Thus, we conclude that to improve the accuracy of the *Minolta SPAD-502* Chl meter, leaf thickness and LWC should be taken into account when calibration equations are to be generated to convert SPAD values into absolute Chl content. We also show that the slope of the calibration equation increases with the extent of leaf greenness.

However, it is not possible from this study to unequivocally attribute the loss of linearity of the model solely to non-uniform Chl distributions, as changes in leaf thickness (related to W_S and SLA) or LWC, also seem to interfere with SPAD readings.

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