DOI: 10.1007/s11099-015-0172-8 PHOTOSYNTHETICA 54 (1): 130-137, 2016

Use of the *atLEAF+* chlorophyll meter for a nondestructive estimate of chlorophyll content

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Abstract

At present, chlorophyll meters are widely used for a quick and nondestructive estimate of chlorophyll (Chl) contents in plant leaves. Chl meters allow to estimate the Chl content in relative units – the Chl index (CI). However, using such meters, one can face a problem of converting CI into absolute values of the pigment content and comparing data acquired with different devices and for different plant species. Many Chl meters (*SPAD-502, CL-01, CCM-200*) demonstrated a high degree of correlation between the CI and the absolute pigment content. A number of formulas have been deduced for different plant species to convert the CI into the absolute value of the photosynthetic pigment content. However, such data have not been yet acquired for the *atLEAF+* Chl meter. The purpose of the present study was to assess the applicability of the *atLEAF+* Chl meter for estimating the Chl content. A significant species-specific exponential relationships between the atLEAF value (corresponding to CI) and extractable Chl *a*, Chl *b*, Chl (*a*+*b*) for *Calamus dioicus* and *Cleistanthus* sp. were shown. The correlations between the atLEAF values and the content of Chl *a*, Chl *b*, and Chl (*a*+*b*) per unit of leaf area was stronger than that per unit of dry leaf mass. The atLEAF value–Chl *b* correlation was weaker than that of atLEAF value–Chl *a* and atLEAF value–Chl (*a*+*b*) correlations. The influence of light conditions (Chl *a*/*b* ratio) on the atLEAF value has been also shown. The obtained results indicated that the *atLEAF+* Chl meter is a cheap and convenient tool for a quick nondestructive estimate of the Chl content, if properly calibrated, and can be used for this purpose along with other Chl meters.

Additional key words: absorption; adaptation; light; leaf water content; morphological trait; reflection; transmission.

Introduction

The photosynthetic pigment content and pigment ratio vary over a wide range in plants of different species and growing in different latitudinal zones (Murchie and Horton 1997, Shmakova and Markovskaya 2010). Estimating their content is of great practical and theoretical importance, since photosynthetic pigments participate in the absorption and transformation of light energy into chemical bound energy, and their content influences the photosynthetic rate and the plant productivity (Šesták 1966, Buttery and Buzzell 1977, Murchie and Horton 1997, Ghosh *et al.* 2004). The photosynthetic pigment content depends on abiotic factors (light, soil moisture, soil fertility, salinity, *etc*.), as well as biological factors (competition, the presence or absence of herbivorous organisms) (Murchie and Horton 1997, Wang and Nii 2000, Carter and Knapp 2001, Amujoyegbe *et al.* 2007, Dai *et al.* 2009, Guerfel *et al.* 2009, Nikolaeva *et al.* 2010). In this respect, monitoring changes in the photosynthetic pigment content enables to estimate plant interactions with the environment and the influence of stress factors.

The spectrophotometric method is traditionally used

Received 7 April 2015, *accepted* 3 July 2015, *published as online-first* 15 August 2015.
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Abbreviations: atLEAF value – CI obtained with the *atLEAF+* chlorophyll meter; Chl – chlorophyll; CI – chlorophyll index; LWC – leaf water content; SLA – specific leaf area.
Acknowledgements: This research was carried out with the support of the Joint Russian–Vietnam Tropical Research and Technological Centre. We thank the administration and staff of the Tropical Centre for the possibility of working in Vietnam, as well as the administration of the Cát Tiên National Park (South Vietnam), where all the experimental studies were executed. The work of J. Kurbatova was supported by Russian Science Foundation (Project № 14-27-00065). Also this study was partially supported by the Ministry of Education and Science of Russian Federation (project № 6.724.2014/k).

for the analysis of the photosynthetic pigment content in plant leaves (Brougham 1960, Terborgh and Thimann 1964, Einhellig and Rasmussen 1979, Baziramakenga *et al.* 1994, Zhao *et al.* 2001, Hallik *et al.* 2012). This method is based on the absorption spectrum of pigment extracts (Mac Kinney 1941, Comar and Zscheile 1942, Vernon 1960, Wintermans and De Mots 1965, Hiscox and Israelstam 1979, Lichtenthaler 1987). The spectrophotometric method allows us to acquire the data on the content of Chl *a* and Chl *b*, as well as carotenoids without their prior separation. However, this method has some limitations. It is labour- and time-consuming; it brings certain problems when a large number of samples is involved. When using this method, minimal time lapses between the sample collection and their analysis should be ensured, because plant pigments break down very quickly. Besides, the pigment extraction leads to destruction of the plant; it makes impossible to study a dynamic pattern of the pigment content in the same sample or to study rare and endangered plant species.

All of those limitations can be overcome by using nondestructive methods for the estimation of the photosynthetic pigment contents. Currently, various devices were used for a quick estimate of the relative pigment content without destructing plant samples: the *SPAD-502* Chl meter (*Spectrum Technologies, Inc*., Plainfield, IL, USA), the *CL-01* Chl meter (*Hansatech Instruments, Ltd*., United Kingdom), the *CCM-200* Chl content meter (*Opti-Sciences, Inc.*, Hudson, NH, USA), the *atLEAF+* Chl meter (*FT Green LLC*, Wilmington, DE, USA). The estimation of the photosynthetic pigment content with these devices is based on the measurement of radiation absorption by Chl at different wavelengths. As a rule, one wavelength corresponds to the spectral range with the maximum Chl activity, another wavelength – the infrared region (where the Chl absorption is very low) – is used for the compensation of the leaf water content and leaf thickness (Hawkins *et al.* 2007). For example, the *SPAD-502, atLEAF+*, and *CL-01* Chl meters use the wavelengths of 650, 660, and 620 nm, respectively, as the region of maximum Chl absorption, while they use 940 nm in the infrared region (*SPAD-502 Plus*: product manual; *atLEAF* specification; *CL-01* specification). The *CCM-200* Chl meter uses the the wavelengths of 653 and 931 nm (*CCM-200 plus* brochure).

Chl meters estimate the Chl content in relative units – the Chl index (CI), which is not directly comparable among different Chl meters. As a result, many problems arise in this connection with the conversion of CI into absolute values of the pigment content and the comparison of data acquired with different devices and for different plant species. For many Chl meters (*SPAD-502, CL-01, CCM-200*), a high degree of correlation between the CI and the absolute values of the pigment content has been confirmed and various formulas for the conversion of the CI into the absolute pigment content have been deduced (Van den Berg and Perkins 2004, Pinkard *et al.* 2006, Cassol *et al.* 2008, Mielke *et al.* 2010). However, many researchers point out that the CI may depend on a variety of factors, such as a leaf water content, leaf thickness, anatomical traits, and peculiarities of a Chl distribution in the leaf (Giunta *et al.* 2002, Marenco *et al.* 2009, Songsri *et al.* 2009, Wang *et al.* 2009). In this respect for each particular plant species and environment, individual equations ought to be formulatted for dependence between the CI and the absolute photosynthetic pigment content.

At present, the most popular Chl meter is the *SPAD-502*. It is widely used for estimation of the Chl content in plant leaves (Ranganathan *et al.* 2006, Fotovat *et al.* 2007, Senger *et al.* 2014). However, similar instrument – the *atLEAF+* Chl meter – is a cheaper and more accessible instrument than the *SPAD-502*. Although the *SPAD-502* and the *atLEAF+* Chl meters use the same principle, the data on the Chl content acquired with these devices differ, because the radiation of different wavelengths is used (Zhu *et al.* 2012). There are practically no information on the dependence between the atLEAF value and the absolute Chl content in leaves as well as on the influence of leaf traits on this dependence.

In the present study, we assessed the possibility of the *atLEAF+* Chl meter application for estimating the Chl content. The study objectives were to find out: (1) how effectively the *atLEAF+* Chl meter allows to estimate the content of Chl *a*, Chl *b*, and Chl (*a*+*b*); (2) whether the accuracy of this estimation varies for different plant species; (3) whether light conditions (which influence Chl *a*/*b*) and the leaf traits [specific leaf area (SLA) and leaf water content (LWC)] influence the accuracy of the Chl content estimate with the *atLEAF+* Chl meter.

Materials and methods

This study was carried out in the Cát Tiên National Park, situated in South Vietnam (11º21'–11º48'N; 107º10'–107º34'E). The climate of South Vietnam is tropical monsoon (McKnight and Hess 2000). The average yearly air temperature is 26–27 ºC, the annual precipitation is about 2450 mm. There are two distinct seasons during the year: the dry (November–April) and the wet season (May–October) (Blanc *et al.* 2000, Deshcherevskaya *et al.* 2013).

For the study purposes, two plant species were chosen, *Calamus dioicus (Arecaceae)* and *Cleistanthus sp.* (*Phyllanthaceae*). The choice of the species for the study was determined by our concern about the fact that the data obtained for one species do not fully reflect the correlation between the atLEAF value and the absolute Chl content and thus they cannot be applied for other plant species. The selected plant species vary highly in their morphological characteristics. For *Calamus sp.*,

xerophilous leaves are typical, and even in young leaves, SLA quickly reaches low values. At the same time, in *Cleistanthus sp.,* the young leaves with the low Chl content contain a larger amount of water and are thinner (high SLA). These leaf features of the selected species enabled us to explore the influence of the SLA and LWC on correlations between the atLEAF value and the absolute Chl content.

For the analysis, 60 leaves of each species were collected. The leaves were taken from plants growing under different light conditions, such as unbroken forest cover (under the canopy) and in gaps. The leaves differed in their age (from young to old), and, consequently, in the photosynthetic pigment content and SLA (Fig. 1). The leaves were collected at 11–12:00 h of local time (UTC+7), which corresponded to the local midday. The leaves were placed into black plastic bags with ice and then taken to the laboratory. For our study, the central part of the leaf was used, avoiding the midribs. In the laboratory, the atLEAF values were measured 5 times for each leaf, and then an average value for each leaf was calculated. Immediately after this procedure, the absolute photosynthetic pigment content was measured in the same samples and SLA and LWC were estimated. The absolute pigment content was determined spectrophotometrically. The pigments were extracted with 96% ethanol. The absorption was measured at wavelengths of 665, 649, and 470 nm by a spectrophotometer (*PD-303, APEL*, Japan). The content of Chl *a* and Chl *b* (per unit of leaf dry mass) in the extract was calculated by equations of Wintermans and De Mots (1965). The total Chl content [Chl (*a*+*b*)] was calculated as the sum of Chl *a* and Ch *b*. The Chl content per unit of leaf area was calculated with regard to the SLA. In order to estimate the SLA and LWC, the leaf parts (without the midribs) of the known area were weighed to determine fresh mass (FM). Then, these parts were dried until oven-dry mass at 80°C and then weighed again to determine the dry mass (DM). The SLA was calculated as a ratio of leaf area to dry leaf mass. The LWC was calculated using the following formula:

$$\mathrm{LWC} = \frac{(\mathrm{FM} - \mathrm{DM})}{\mathrm{FM}} * 100$$

The regression analysis was performed in order to determine relationships between the atLEAF values and the Chl content, SLA, and LWC for each species. Also a general equation for the relationship between the atLEAF value and the Chl content was deduced for the whole data set (for both species). *Student´*s *t*-test for paired observations was used to test differences between the general and the species-specific equations. To estimate the influence of the Chl *a*/*b* ratio on the relationship between the Chl (*a*+*b*) and atLEAF values, two sets of data were formed, differing in the Chl *a*/*b* ratio; in the first one, the Chl *a*/*b* ratio varied from 1.8 to 2.1, while in the second one, the Chl *a*/*b* ratio varied from 2.11 to 2.40. For each variant, the relationship between the Chl (*a*+*b*) and atLEAF values was analysed separately with the aid of the regression analysis. For significance testing of the regression equations and comparison of different models, the data were modified to linearity. The reliability of the regression equations was estimated by means of the *F*-test. The *STATISTICA*, *version 10* (*StatSoft Inc*.) was used for the data analysis.

Results

We estimated leaves with a wide range of photosynthetic pigment contents (Table 1). Consequently, the atLEAF values varied over a wide range: 1.2–51.0 (*Cleistanthus sp.*) and 12.5–51.2 (*Calamus sp.*) (Table 1). The relationships between the atLEAF values and the content of Chl *a*, Chl *b*, Chl (*a*+*b*) were better expressed by means of an exponential function, not the linear one. We found out relationships between the atLEAF values and contents of Chl *a*, Chl *b*, Chl (*a*+*b*) calculated both per unit of leaf area and per unit of DM for *Cleistanthus sp.* and *Calamus sp.* (Table 2, Fig. 1). General equations differed from the equations deduced separately for each species. The relationship between the atLEAF values and Chl content was stronger when calculated as the Chl content per unit of leaf area than that per unit of DM. The atLEAF value–Chl *b* relationship was weaker than that of the atLEAF–Chl *a* and atLEAF–Chl (*a*+*b*) relationships (Table 2, Fig. 1).

The strength of the relationship between the atLEAF value and content of Chl (*a*+*b*) depended on the Chl *a*/*b* ratio. Moreover, the significant relationship between these values was observed when calculating the content of Chl (*a*+*b*) both per unit of leaf area (Fig. 2), and per unit of DM (data not shown). R^2 for the relationship

Table 1. Range of the atLEAF value, the contents of chlorophyll (Chl) *a*, Chl *b*, and total Chl (*a*+*b*) (determined spectrophotometrically), the specific leaf area (SLA), and leaf water content (LWC), estimated in *Cleistanthus sp.*and *Calamus sp.*

Species	atLEAF	Chl *a*		Chl *b*		Chl (*a*+*b*)		SLA [cm^2 g^{-1}]	LWC [%]
		[mg g^{-1}]	[mg m^{-2}]	[mg g^{-1}]	[mg m^{-2}]	[mg g^{-1}]	[mg g^{-1}]		
Cleistanthus sp.	1.2–51.0	0.6–8.8	12.3–339.6	0.3–4.2	6.3–151.5	0.9–13.0	19.5–468.8	136.4–607.9	22.2–87.6
Calamus sp.	12.5–51.2	0.7–8.7	54.3–428.6	0.3–3.8	14.1–209.4	1.1–12.5	68.4–638.0	97.4–267.0	30.5–67.1

Table 2. The relationships between the atLEAF value and the content of chlorophyll (Chl) *a*, Chl *b*, and total Chl (*a*+*b*). For all the equation $p<0.001$. The regression equations marked with *different letters* differ significantly between species-specific equations and general equation ($p<0.05$).

Variable		*Cleistanthus sp.*	*Calamus sp.*	Both species (general)
Chl *a*	[mg g^{-1}]	$y = 1.015e^{0.034x}$ ($R^2 = 0.61$)^a	$y = 0.905e^{0.035x}$ ($R^2 = 0.52$)^b	$y = 0.994e^{0.033x}$ ($R^2 = 0.60$)^c
	[mg m^{-2}]	$y = 22.03e^{0.059x}$ ($R^2 = 0.84$)^a	$y = 47.65e^{0.038x}$ ($R^2 = 0.75$)^b	$y = 27.31e^{0.052x}$ ($R^2 = 0.82$)^c
Chl *b*	[mg g^{-1}]	$y = 0.524e^{0.029x}$ ($R^2 = 0.54$)^a	$y = 0.393e^{0.035x}$ ($R^2 = 0.50$)^b	$y = 0.490e^{0.030x}$ ($R^2 = 0.53$)^c
	[mg m^{-2}]	$y = 11.36e^{0.055x}$ ($R^2 = 0.81$)^a	$y = 20.57e^{0.038x}$ ($R^2 = 0.67$)^b	$y = 13.46e^{0.049x}$ ($R^2 = 0.79$)^c
Chl (*a*+*b*)	[mg g^{-1}]	$y = 1.540e^{0.032x}$ ($R^2 = 0.60$)^a	$y = 1.304e^{0.035x}$ ($R^2 = 0.52$)^b	$y = 1.488e^{0.032x}$ ($R^2 = 0.59$)^c
	[mg m^{-2}]	$y = 33.40e^{0.058x}$ ($R^2 = 0.84$)^a	$y = 68.71e^{0.038x}$ ($R^2 = 0.73$)^b	$y = 40.88e^{0.051x}$ ($R^2 = 0.82$)^c

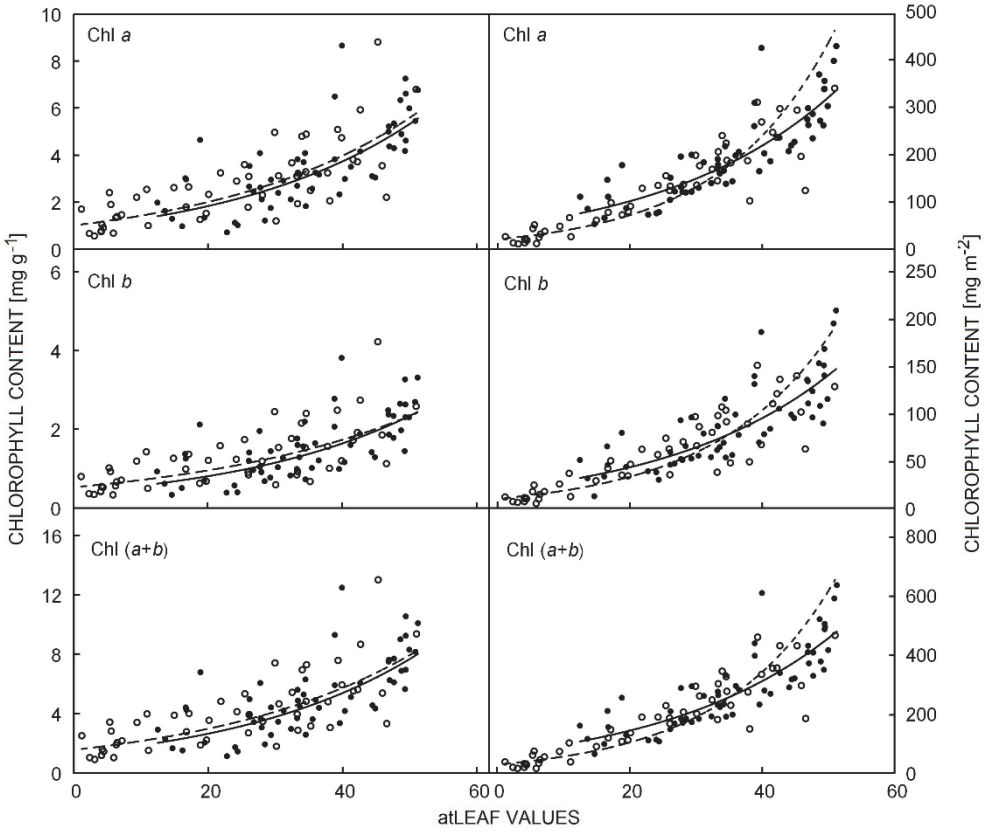

Fig. 1. Relationships between the atLEAF value and the content of chlorophyll (Chl) *a*, Chl *b*, and total Chl (*a*+*b*) in the leaves of *Cleistanthus* sp. (○, *dotted line*) and *Calamus* sp.(●, *solid line*).

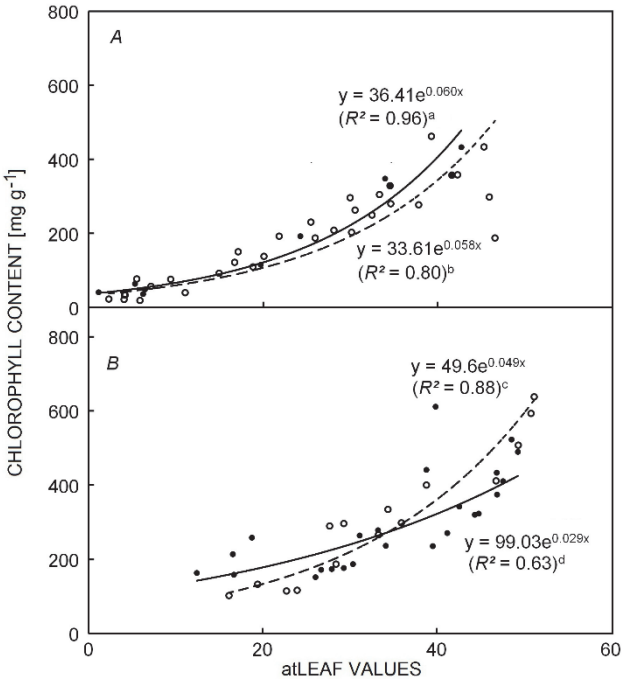

Fig. 2. Influence of chlorophyll (Chl) *a* and Chl *a*/*b* on the relationship between total Chl and the atLEAF value for *Cleistanthus* sp. (*A*) and *Calamus* sp. (*B*). There are two variants: 1 – Chl *a*/*b* = 1.8–2.1 (○, *dotted line*); 2 – Chl *a*/*b* = 2.11–2.4 (●, *solid line*). For all the equations $p<0.001$. The regression equations marked with *different letters* differ significantly ($p<0.05$).

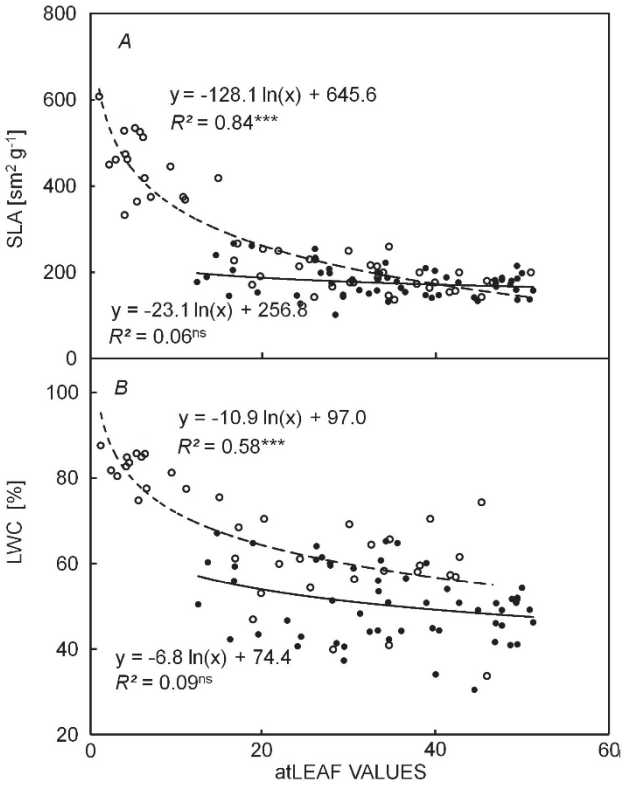

Fig. 3. Relationships between the atLEAF value and *A*: the specific leaf area (SLA), *B*: the leaf water content (LWC) in *Cleistanthus* sp. (○, *dotted line*) and *Calamus* sp. (●, *solid line*). *** – $p<0.001$, ns – not significant.

between the atLEAF value and content of Chl (*a*+*b*) per unit of DM, when Chl (*a*+*b*) was equal to 1.8–2.1 for *Cleistanthus sp.* amounted to 0.50, for *Calamus sp.* – 0.82. When Chl (*a*+*b*) was equal to 2.11–2.4 R^2 amounted to 0.75 for *Cleistanthus sp. and* 0.30 *Calamus sp.*

The SLA and LWC values in the studied leaves varied over a wide range (Table 1). There were strong significant negative relationships between the atLEAF value and SLA and LWC for *Cleistanthus sp.* For *Calamus sp.,* the relationships between the atLEAF values and SLA, LWC were not significant (Fig. 3).

Discussion

We found a significant relationships between the content of Chl *a*, Chl *b*, and Chl (*a*+*b*) and the atLEAF values (Table 2, Fig. 1). The relationships between the CI values obtained with different Chl meters (*CCI 200, SPAD-502, CL-01*) and contents of Chl *a*, Chl *b,* and Chl (*a*+*b*) were noted by other authors as well (Van den Berg and Perkins 2004, Sheshshayee *et al.* 2006, Cassol *et al.* 2008, Hawkins *et al.* 2009, Mielke *et al.* 2010, Zhu *et al.* 2012).

The relationships between the atLEAF value and content of Chl *a*, Chl *b*, Chl (*a*+*b*) is better described by means of the exponential function. Some researchers describe the relationship between the CI and Chl content by means of linear functions (Cassol *et al.* 2008, Wang *et al.* 2009). However, the majority of scientists assume that nonlinear functions are more suitable for expression of the relationship between the CI and Chl content. Therefore, exponential functions (Marenco *et al.* 2009, Mielke *et al.* 2010), logarithmic functions (Van den Berg and Perkins 2004), and also more complex functions (Netto *et al.* 2002) have been used. The loss of linearity can be related to several factors: the uneven distribution of Chl and chloroplasts in the leaf, different values of SLA (and the leaf thickness, dependent on them) and different leaf water contents (Uddling *et al.* 2007, Marenco *et al.* 2009). These features of the leaf can interfere with its properties for radiation absorption and reflection, which are used for determining the CI (Cassol *et al.* 2008).

We observed strong relationships between the atLEAF value and LWC and SLA in *Cleistanthus sp.* and the absence of such relationships in *Calamus sp.*, which was connected to peculiarities of the leaf development in

this species. For example, xerophilous leaves are typical for *Calamus sp.* and SLA quickly reaches low values even in the young leaves. At the same time, in *Cleistanthus sp.*, the young leaves with the low Chl content contain a larger amount of water and are thinner (a high SLA), which can "strengthen" the relationship. For example, in Fig. 3, one can see that for leaves of *Cleistanthus sp.* with the higher atLEAF values the relatioships of the atLEAF values–SLA and the atLEAF values–LWC was weaker. We separately analysed the relationship between the atLEAF value and LWC and SLA for *Cleistanthus sp.* excluding too young leaves (the atLEAF value lesser than 10). In this case, the atLEAF value–SLA relationship was weak (R^2=0.24, p<0.05), and the atLEAF value–LWC relationship was not found (R^2=0.03, p>0.05) (data not shown). Thus, we could assume that the influence of LWC and SLA on the atLEAF value was not strong when the very young leaves were excluded. But to confirm this hypothesis, more detailed studies of the atLEAF value in mature leaves with different leaf water contents and different SLA are needed. It is expected that the compensation of the influence of the leaf water content and leaf thickness on the atLEAF values is made by using the radiation with a wavelength of 940 nm (Hawkins *et al.* 2007). However, the researchers who were working with the *SPAD-502* Chl meter noted the dependence of CI on SLA and LWC and attributed this dependence to the fact that these values strongly affect the leaf optical properties (Giunta *et al.* 2002, Marenco *et al.* 2009, Songsri *et al.* 2009, Wang *et al.* 2009).

The relationships between the atLEAF values and the content of Chl *a*, Chl *b*, and Chl (*a*+*b*) per unit of leaf area was stronger than that per unit of DM (Table 2). Similar results were achieved for the *SPAD-502* and *CL-1* Chl meter (Cassol *et al.* 2008, Hawkins *et al.* 2009, Marenco *et al.* 2009). Marenco *et al.*(2009) attribute this to the fact that when calculating the photosynthetic pigment content per unit of leaf area, one takes into account SLA, which (as was proven by those scientists) affects Chl meter reading. However, in our study, the SLA showed only a marginal effect on the atLEAF value for *Cleistanthus sp.* and did not affect this value for *Calamus sp.*

The atLEAF value–Chl *b* relationship was weaker than that of the atLEAF value–Chl *a* and atLEAF value–Chl (*a*+*b*) relationships. The strength of the atLEAF value–Chl *a* relationship was similar to the strength of the atLEAF value–Chl (*a*+*b*) relationship (Table 2). Similar results were achieved with the *SPAD-502* Chl meter (Pinkard *et al.* 2006, Mielke *et al.* 2010). The stronger atLEAF value–Chl *a* relationship, compared to the atLEAF value–Chl *b* relationship, was most probably connected with the radiation wavelength used in the *atLEAF+* Chl meter for a Chl content determination. For such determination, the radiation with wavelengths of 660 nm and 940 nm is used. The maximum light absorption in the red region for Chl *a* is closer to 660 nm than that of Chl *b*. For example, the maximum light absorption in the red region for the extract of Chl *a* and Chl *b* in diethyl ether peaks at 661 and 642 nm, respectively, while it is 665 and 649 nm, respectively, in 96% ethanol (Wintermans and De Mots 1965, Lichtenthaler and Buschmann 2001). The variation between the maximums of light absorption in the red region for Chl *a* and *b* can account for the observed dependence of the atLEAF value–Chl (*a*+*b*) relationships on the Chl *a*/*b* ratio (Fig. 2). The Chl *a*/*b* ratio depends, first of all, on the light conditions under which plants are growing (Valladares and Niinemets 2008). Thus, it is necessary to take care when comparing the data acquired with the *atLEAF+* Chl meter for plants growing under different light conditions. For a more accurate estimate of the Chl content, it is important to take into account the Chl *a*/*b* ratio when building calibration models; even the same plant species growing under different light conditions require separate calibration equations.

The relationships between the atLEAF value and the content of Chl *a*, *b*, and Chl (*a*+*b*) were species-specific for both calculations per unit of leaf area and per unit of DM (Table 2), which is consistent with the data acquired by other authors with the *SPAD-502* and *CL-01* Chl meters (Cassol *et al.* 2008, Marenco *et al.* 2009). In summary, the acquired data indicated that despite high accuracy of the Chl content estimation in leaves with the *atLEAF+* Chl meter, it is important to take into account some peculiarities. The maximum accuracy of the estimate was achieved when the *atLEAF+* Chl meter was used for the determination of the Chl content per unit of leaf area. At the same time, it was necessary to use a species-specific equation when converting the atLEAF value into the absolute Chl content for each plant species. When comparing the atLEAF value, one needs to take into account the growth conditions (particularly irradiance), which can affect the Chl *a*/*b* ratio. In case of large discrepancies in the Chl *a*/*b* ratio, the atLEAF value can vary even with the same absolute Chl content in the leaf. Notwithstanding the above, the *atLEAF+* Chl meter could be a convenient tool for a quick, nondestructive estimate of the Chl content along with other chlorophyll meters after the right calibration and taking into account the leaf peculiarities and plant growth conditions.

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