Evaluation of reflectance spectroscopy indices for estimation of chlorophyll content in leaves of a tropical tree species

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Abstract

The effectiveness of eight spectral reflectance indices for estimating chlorophyll (Chl) content in leaves of *Eugenia uniflora* L., a tropical tree species widely distributed throughout the world and a key species for ecosystem restoration projects, was evaluated. Spectral reflectance indices were tested using sun and shade leaves with a broad variation in leaf mass per area (LMA). Shortly after plants were exposed to chilling temperatures, there was a dramatic visible change in some sun leaves from green to red. Prior to testing Chl-related reflectance indices, the green and red leaves were separated according to the anthocyanin reflectance index (ARI). Slightly green to dark green leaves corresponded to an ARI value less than 0.11 (n = 107), whereas slightly red to red leaves corresponded to an ARI value greater than 0.11 (n = 35). To estimate leaf Chl, two simple reflectance indices (SR_{680} and SR_{705}), two normalized difference indices (ND_{680} and ND_{705}), two modified reflectance indices (mSR_{705} and mND_{705}), a modified Chl absorption ratio index ($mCARI_{705}$) and an index insensitive to the presence of anthocyanins (CI_{re}) were evaluated. Good estimates of leaf Chl content were obtained using the reflectance indices tested regardless of the presence of anthocyanins and changes in LMA. Based on the coefficients of determination (r^2) and the root mean square errors ($RMS\varepsilon_c$) the best results were obtained with reflectance indices measured at wavelengths of 750 and 705 nm. Considering the performance of the models the best reflectance indices to estimate Chl contents in *E. uniflora* leaves with a broad variation in LMA and anthocyanin contents was SR_{705} and $mCARI_{705}$.

Additional key words: anthocyanins; ecosystem restoration; *Eugenia uniflora*; leaf mass per area; *SPAD-502*.

Introduction

Changes in leaf pigment content and composition provide indications of tree stress due to abiotic or biotic factors. Leaf pigments can be quantified by destructive or non-destructive methods (Hendry and Price 1993, Markwell *et al*. 1995, Gamon and Surfus 1999, Blackburn 2007). Destructive methods are accurate but expensive and time-consuming. Additionally, destructive methods require transport of leaves from the field to the laboratory, which in addition to preventing pigment determination under natural conditions can interfere with the accuracy of results. Nondestructive methods are more rapid and allow for analyses of considerably more leaves in a shorter time than destructive methods. Nondestructive methods also allow for analyses of the same leaf over time (Cate and Perkins 2003, Mielke *et al*. 2010) and facilitate collecting data in areas that are difficult to access.

One of the most useful nondestructive methods to estimate leaf Chl content is the use of hand-held Chl

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Abbreviations: ARI – anthocyanin reflectance index; Chl – chlorophyll; LMA – leaf mass per area; R – reflectance.
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absorbance meters (Markwell *et al.* 1995) such as the *SPAD-502* (*Minolta Inc.*, Osaka, Japan), which calculates an index based on absorbance at 650 and 940 nm. Such instruments are lightweight, easy to operate and have been successfully used to estimate Chl content in several woody plant species (Campbell *et al.* 1990, Cate and Perkins 2003, Torres-Netto *et al.* 2005, Pinkard *et al.* 2006, Hawkins *et al.* 2009, Mielke *et al.* 2010). The simple indices provided by hand-held Chl meters are often highly correlated with leaf Chl content. However, they are not useful for analyzing changes in anthocyanin or carotenoid contents.

In leaves, Chl and carotenoids accumulate inside the chloroplasts and are directly related to the absorption of photosynthetic light, whereas anthocyanins, the pigments responsible for red pigmentation (Close and Beadle 2003), are located inside the vacuoles of the epidermal cells or just below the adaxial epidermis, although they can also be found in cells of the palisade and spongy parenchyma (Merzlyak *et al.* 2008). Anthocyanins can accumulate in leaves in response to environmental stresses (Close and Beadle 2003) such as excess light (Close and Beadle 2005), UV light (Mendez *et al.* 1999), chilling (Wen *et al.* 2010) and protection against herbivory (Karageorgou and Manetas 2006). Thus, estimating anthocyanin content and the interaction between anthocyanins and Chls in leaves could be a useful tool for diagnosing stress of individual trees and ecosystems.

In recent years, there have been several published reports about pigment content and composition in leaves using indices derived from spectral reflectance measurements (Gamon and Surfus 1999, Carter and Spiering 2002, Sims and Gamon 2002, Gitelson *et al.* 2006, Blackburn 2007, Steele *et al.* 2008, Wu *et al.* 2008, Gitelson *et al.* 2009, Steele *et al.* 2009). Information gathered in the electromagnetic spectrum between approximately 400 to 1,000 nm by reflectance spectroscopy, provides the basis for mathematical indices used to predict leaf Chl (Gitelson and Merzliak 1994, Gamon and Surfus 1999, Carter and Spiering 2002, Gitelson *et al.* 2006, Steele *et al.* 2008, Ding *et al.* 2009), carotenoid (Gamon and Surfus 1999) and anthocyanin contents (Gamon and Surfus 1999, Gitelson *et al.* 2006, 2009; Steele *et al.* 2009). Recent studies have shown that some reflectance-based indices are more accurate than hand-held Chl absorbance meters for nondestructive determination of leaf Chl content (Richardson *et al.* 2002, Steele *et al.* 2008) because hand-held Chl meters tend to lose sensitivity at high Chl concentrations (Steele *et al.* 2008). Estimating leaf pigment content with portable reflectometers is also beneficial because the same indices are used in remote sensing applications (Peñuelas and Filella 1998, Le Maire *et al.* 2004, Pettorelli *et al.* 2005, Grace *et al.* 2007). Thus, individual leaf measurements can be used to calibrate measurements at canopy (Perry and Davenport 2007) or ecosystem (Hashemi 2010) levels. Despite attempts to relate spectral reflectance indices to leaf Chl content for several different plant species simultaneously (Sims and Gamon 2002), the best results were obtained when reflectance indices were calibrated for an individual species (Carter and Spiering 2002, Richardson *et al.* 2002). This is because at the same wavelengths, leaf reflectance can differ among species due to the presence of other pigments and differences in leaf internal structure (Slaton *et al.* 2001, Blackburn 2007).

Leaf mass per unit of leaf area (LMA) is an important leaf morphological trait relating leaf dry mass to leaf area and associated with phenotypic plasticity to light (Valladares and Niinemets 2008). Sun and shade leaves are easily distinguished by their morphological and anatomical characteristics such as differences in leaf area, lamina thickness, the amount of palisade and spongy parenchyma and the presence of lignified tissues, which directly affects changes in LMA (Castro-Díez *et al.* 2000, Poorter *et al.* 2009). Sun leaves often have higher LMA than shade leaves (Valladares and Niinemets 2008) which is related to greater leaf thickness and density (Poorter *et al.* 2009). Thus, sun leaves have higher net photosynthetic rates per unit leaf area than shade leaves as a result of a higher proportion of photosynthetic cells per unit area (Evans and Poorter 2001). We are aware of no studies published to date, addressing the performance of reflectance indices to estimate Chl content in leaves of a single tree species with varying leaf structure as a result of the light environment during leaf development.

Eugenia uniflora L. (Myrtaceae) is a small tree native to South America ranging from Surinam to southern Brazil and Uruguay (Margis *et al.* 2002). It grows in almost any type of soil and can tolerate waterlogging for a time (Mielke and Shaffer 2010). Studies with young plants have shown that this species has the highest growth rate in full sun, but leaves have a great deal of plasticity allowing them to adapt to sun or shade environments (Mielke and Shaffer 2010). *E. uniflora* produces a red fruit that is attractive to birds and is therefore very useful in ecosystem restoration programs. Moreover, similar to temperate broad-leaf trees (Close and Beadle 2003, Merzlyak *et al.* 2008), *E. uniflora* accumulates large quantities of anthocyanins in senescing leaves under environmental stress conditions.

Considering the importance of this species for ecosystem restoration programs, spectroscopy reflectance indices can be a tool to rapidly and nondestructively assess Chl and anthocyanin contents and evaluate physiological stress under field conditions. The *SPAD* meter was previously determined to be a good tool for estimation of Chl content in *E. uniflora* leaves (Mielke *et al.* 2010). This provides a basis for calibrating leaf Chl measurements determined by reflectance indices, with values obtained with a *SPAD* meter. An advantage of nondestructive estimation of leaf Chl content from reflectance indices is the ability to scale up to selectively monitor vigor of this species by remote sensing of the canopy, which may be particularly useful in reforestation applications. The objective of this study was to analyze the effectiveness of eight spectral reflectance indices for estimation of Chl content in sun- and shade leaves of *E. uniflora*, varying in color from green to red and with a broad variation in LMA.

Materials and methods

Study site and plant material: The study was conducted at the Tropical Research and Education Center, University of Florida (TREC/UF) in Homestead, Florida, USA (25.5ºN, 80.5ºW). Seedlings of *Eugenia uniflora* L. were acquired from a commercial nursery in Homestead and cultivated in 10-L plastic containers in a standard nursery substrate of 65% pine bark, 25% Florida peat and 10% coarse sand by volume. Details of the experimental conditions were described by Mielke *et al*. (2010). In November 2008, after the first autumn cold front in which the minimum air temperature dropped to below 7ºC, some of the leaves changed color from green to red, providing a gradient of pigmentation. Measurements were made on three to five leaves per plant. A total of 142 leaves from 30 plants were used and all measurements were made in the morning. Among the measured leaves, 36 leaves were collected from shade-grown plants (about 30% of full sun) and 69 leaves from sun-grown plants. Also, 35 sun leaves that clearly exhibited a reddish color were intentionally collected.

Chl contents and leaf reflectance: Leaf Chl content was estimated with a *SPAD-502* meter (*Minolta Inc*., Osaka, Japan). *SPAD* values were converted to Chl content using the equation described by Mielke *et al*. (2010) developed for *E. uniflora* of the same age as those used in this study. A previous study by Manetas *et al*. (1998) demonstrated that the estimation of leaf Chl content with *SPAD* in green or red leaves is insensitive to the presence of anthocyanins. Immediately after *SPAD* measurements, leaf reflectance on the adaxial leaf surface was measured with a portable spectrometer (*Unispec, PP Systems*, Amesbury, Massachusetts, USA) using a bifurcated fiber optics cable *UNI400* and a standard leaf clip *UNI500* that held the fiber at 60º to the adaxial leaf surface. The *Unispec* detects a wavelength range from 310 to 1,100 nm and the average spectral slit-width in our study was about 3.3 nm. To separate the red and green leaves, the anthocyanin reflectance index (ARI) was calculated as described by Gitelson *et al*. (2006) (Table 1). Within the spectral range from 350 to 1,100 nm, simple reflectance indices (SR), normalized difference indices (ND), modified reflectance indices (mSR and mND), a modified Chl absorption ratio index ($mCARI_{705}$) and the Red Edge Chl Index (CI_{re}), an index for Chl determination in both anthocyanin-containing and anthocyanin-free leaves, were calculated (Table 1).

LMA: Immediately after reflectance measurements, leaf area was determined with a leaf area meter (model *LI-3000*, *Li-Cor Inc*., Lincoln, NE, USA) and leaf dry mass was obtained after oven-drying leaves at 75°C until a constant mass was reached. LMA was calculated by dividing the leaf dry mass by the leaf area.

Data analysis: Modified linear regression models were used to relate reflectance indices to leaf pigment content. The models were selected according to the best quality of fit measured by the coefficient of determination (r^2) and the diagnostic graphics provided by the routine lm of the statistical environment R (R Development Core Team 2010). When no suitable fits were obtained using the variables in their original scale, transformations of variables and predictors were tested. When the most common transformations (Sokal and Rohlf 1995) were ineffective, a Box-Cox transformation was used (Box and Cox 1964). The Box-Cox family of power transformations is an empirical solution to make data behave according to a linear regression model (Marazzi *et al*. 2009, Marazzi and Yohai 2004). The procedure is to find a power transformation, λ, that maximizes the likelihood when a predictive variable is fitted to $(y^{\lambda} - 1)/\lambda$ as the response.

Table 1. List of the spectral reflectance indices and references. ARI – the anthocyanin reflectance index, R_{NIR}, $R_{red\ edge}$, and R_{Green} – the average of reflectance values within: R_{NIR} = 770.0–799.8 nm (*n* = 10), $R_{red\ edge}$ = 690.2–710.2 nm (*n* = 7), and R_{Green} = 540.0–560.0 nm (*n* = 7), respectively. For all indices R is the reflectance and subscripts indicate the wavelengths in nm.

Index	Reference
$ARI = R_{Green}^{-1} - R_{red\ edge}^{-1}$	Gitelson *et al.* (2006)
$SR_{680} = R_{800}/R_{680}$	Jordan (1969)
$SR_{705} = R_{750}/R_{705}$	Gitelson and Merzlyak (1994)
$ND_{680} = (R_{800} - R_{680})/(R_{800} + R_{680})$	Rouse *et al*. (1974)
$ND_{705} = (R_{750} - R_{705})/(R_{750} + R_{705})$	Gitelson and Merzlyak (1994)
$mSR_{705} = (R_{750} - R_{445})/(R_{705} - R_{445})$	Sims and Gamon (2002)
$mND_{705} = (R_{750} - R_{705})/(R_{750} + R_{705} - 2R_{445})$	Sims and Gamon (2002)
$mCARI_{705} = [(R_{750} - R_{705}) - 0.2(R_{750} - R_{550})](R_{750}/R_{705})$	Wu *et al.* (2008)
$CI_{re} = (R_{NIR}/R_{red\ edge}) - 1$	Gitelson *et al.* (2003)

When $\lambda = 0$, the Box–Cox transformation is defined as ln(y) (Crawley 2007). The regression coefficients and the parameter λ are generally estimated by maximum likelihood. We used the 'boxcox' function of the MASS library (Venables and Ripley 2002) available in statistical environment R (R Development Core Team 2010). For all models, the calibration error (ε_c) was calculated by the quotients between fitted and observed values. The root mean square calibration error ($RMS\varepsilon_c$) was calculated following Richardson *et al*. (2002). The indices were ranked from the lowest to the highest values of $RMS\varepsilon_c$, expressed as a percentage of mean Chl contents. To test the performance of the models associated with each reflectance index, the observed and estimated values of Chl contents were plotted from the perspectives of 1:1 correspondence lines. In this case, the reflectance indices were ranked according to the linear regression of the observed and estimated Chl values, in which the best indices were those with regression slope values closer to 1 and the lowest intercept values.

Results

The green and red leaves showed distinct patterns of reflectance (Fig. 1*A*) and can be easily separated considering ARI > 0.11. With increasing Chl content, reflectance between 500 and 600 nm decreased, whereas with increasing ARI, reflectance between 600 and 650 nm increased (Fig. 1*B*). There was a clear association between Chl content and reflectance at 600–699 nm ($\sum R_{Red}$) for all values of ARI (Fig. 2*A*), whereas a decrease in reflectance at 500–599 nm ($\sum R_{green}$) was observed after ARI > 0.1 (Fig. 2*B*). Therefore, we used the ARI to separate green (ARI < 0.11, *n* = 107) and red (ARI > 0.11, *n* = 35) leaves.

The LMA for all measured leaves varied from 75.5 to 145.4 g m^{-2} (Fig. 3). For shade leaves, the LMA ranged from 78.2 to 130.6 g m^{-2}. For sun leaves, the LMA ranged from 75.5 to 131.6 g m^{-2} for green leaves and from 92.4 to 145.4 g m^{-2} for red leaves. Only the red, sun leaves had LMA above 131 g m^{-2}.

The regression models for the data relating each reflectance index to leaf Chl content (shown in Figs. 4, 5) are shown in Table 2. The r^2 values for all regression models were above 0.80. The root mean square error, expressed as the percentage of mean Chl content of all leaves ($RMS\varepsilon_c$, %), followed the same tendency observed for r^2 and varied from 14.6% for mND_{705} ($r^2 = 0.93$) to 29.2% for ND_{680} ($r^2 = 0.80$).

All indices tested showed tendencies to underestimate Chl contents above 600 µmol m^{-2} (Fig. 6). The tendency to underestimate the Chl contents were more pronounced for SR_{680} (Fig. 6*A*) and ND_{680} (Fig. 6*C*), followed by ND_{705} (Fig. 6*D*) and mND_{705} (Fig. 6*F*). Considering the analysis showed in Fig. 6, the best estimates of Chl contents were observed for SR_{705} (Fig. 6*B*) and $mCARI_{705}$ (Fig. 6*G*).

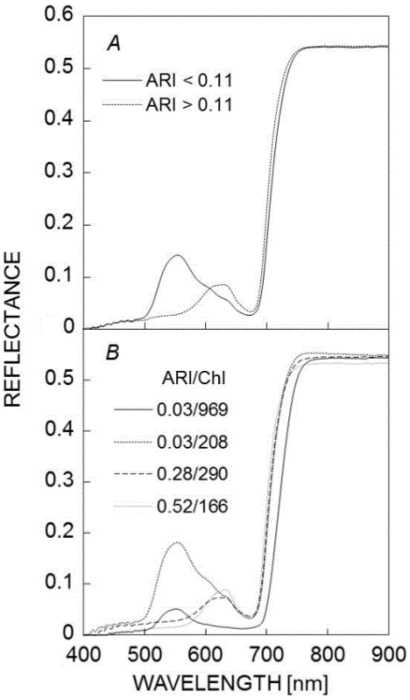

Fig. 1. Spectral properties of *E. uniflora* leaves with anthocyanin reflectance index (ARI) above and below 0.1 (*A*), and changes in reflectance spectra in individual leaves according to ARI values (0.5, 0.5, 2.0, and 3.5) and total chlorophyll content (969, 208, 290, and 166 µmol m^{-2}) (*B*). For *A*, each line represents the mean of 15 leaves.

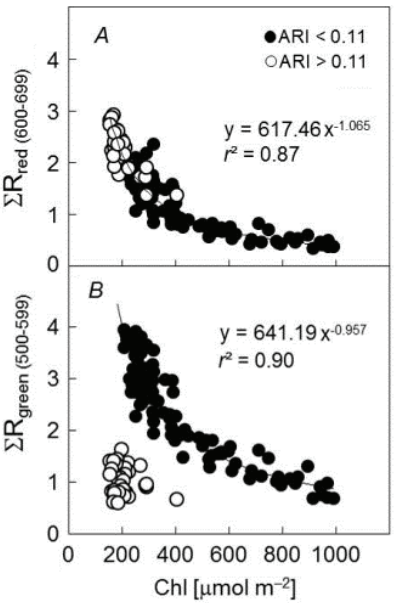

Fig. 2. Relationships between red (*A*) and green (*B*) spectral bands and chlorophyll (Chl) content in *E. uniflora* leaves with ARI above or below 0.1 (*n* = 142).

Discussion

The spectral reflectance properties of leaves with ARI < 0.11, as shown in Fig. 1*A*, is typical of green leaves of different plant species from different ecosystem types (Billings and Morris 1951, Richardson *et al*. 2002, Ding *et al*. 2009). In the present study, all data from reddish leaves were collected in November, eleven days after one of the first cold fronts of the year, typical of the transition from autumn to winter in southern Florida. Although anthocyanin content was not measured directly, the ARI has been demonstrated to be good indicator of the presence of anthocyanins in leaves (Gitelson *et al*. 2006). Also, the spectral properties of green (ARI < 0.11) and red (ARI > 0.11) leaves were very similar to those reported by Karageorgou and Manetas (2006) for *Quercus coccifera*. An increase in leaf reflectance between 500 and 600 nm with a decrease in Chl content was also reported by Richardson *et al*. (2002) for *Betula papyrifera* and Ding *et al*. (2009) for *Prunus dulcis*, *Populus trichocarpa* × *P. deltoides* and *Malus domestica*.

The Chl content in red leaves was low (*i.e*., less than 300 μmol m^{-2}). According to Feild *et al*. (2001) and Hoch *et al*. (2001) the increases in anthocyanin contents in senescing leaves of temperate trees during autumn has been suggested as a protection mechanism for photoinhibitory damage during the process of leaf nutrient resorption. Because Chl contains nitrogen, the decrease in Chl content associated with the increase in the ARI associated with cold stress corroborates the hypothesis that these pigments serve a photoprotective function during leaf senescence (Lee 2002). Moreover, there seems to be a threshold in Chl content near 300 μmol m^{-2}, after which the synthesis of anthocyanins begins. It is also important to note the possibility of clearly separating green and red leaves using the ARI. In this case, a value

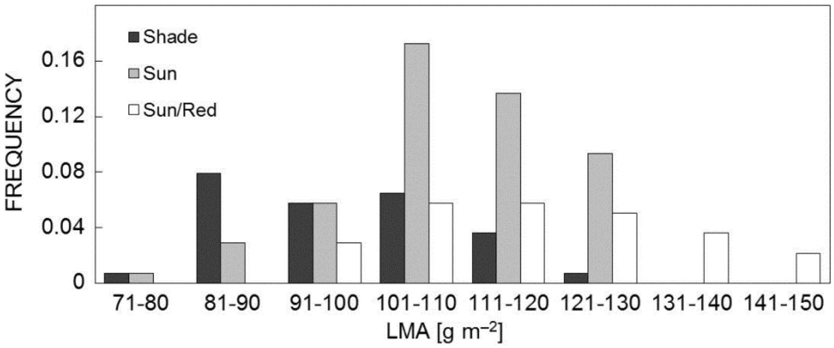

Fig. 3. Frequency distribution for the leaf mass per area (LMA) of 142 *E. uniflora* leaves. Shade (n = 36), sun (n = 71), and sun/shade (n = 35).

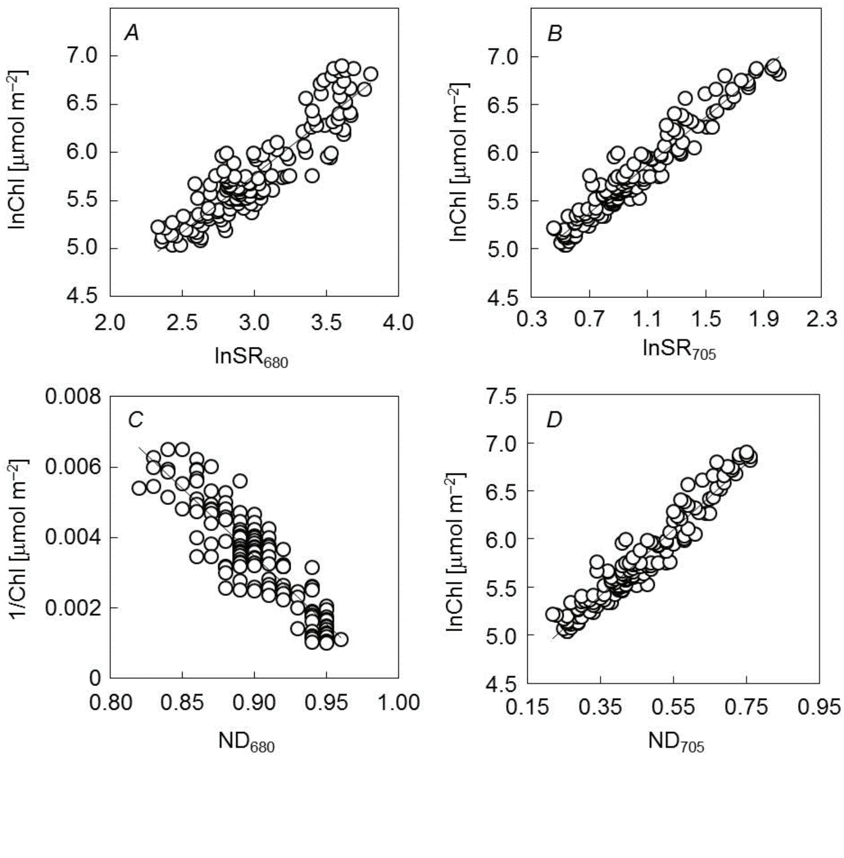

Fig. 4. Relationships between two simple reflectance indices (SR_{680} and SR_{705}) and two normalized difference indices (ND_{680} and ND_{705}) and total chlorophyll (Chl) content in *E.uniflora* leaves (n = 142). The regression models and coefficient of determinations are shown in Table 1.

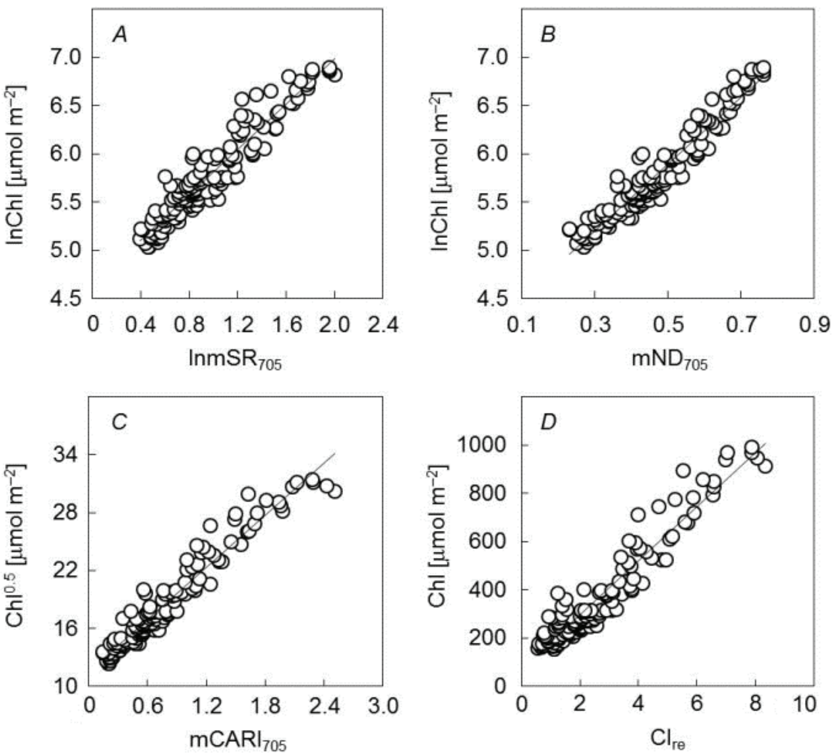

Fig. 5. Relationships between two modified reflectance indices (mSR_{705} and mND_{705}) a modified chlorophyll (Chl) absorption ratio index ($mCARI_{705}$) and the red edge Chl Index (CI_{re}) and total Chl content in *E.uniflora* leaves (n = 142). The regression models and coefficient of determinations are shown in Table 1.

Table 2. Regressions equations, coefficients of determination, and root mean square errors ($RMS\varepsilon_c$) for models relating leaf chlorophyll contents with eight spectral reflectance indices. For the coefficients, ** p<0.01, * p<0.05, ns p>0.05. Data for each index are plotted in Figs. 5 and 6.

Index	Equation	r^2	$RMS\varepsilon_c$ [μmol m^{-2}]	$RMS\varepsilon_c$ [%]
mND_{705}	ln(y) = 4.1598** + 3.4729** x	0.93	54.0	14.6
$mCARI_{705}$	$y^{0.5}$ = 11.5026** + 9.0142**x	0.94	55.3	15.0
SR_{705}	ln(y) = 4.5105** + 1.2416** ln(x)	0.93	55.9	15.1
ND_{705}	ln(y) = 4.2128** + 3.424**x	0.92	57.2	15.5
CI_{re}	y = 70.665** + 112.299**x	0.91	61.6	16.7
mSR_{705}	ln(y) = 4.6397** + 1.1726** ln(x)	0.90	64.8	17.6
SR_{680}	ln(y) = 2.2142*** + 1.1837*** ln(x)	0.82	100.5	27.2
ND_{680}	1/y = 0.0383** – 0.0387**x	0.80	107.7	29.2

of ARI above 0.11 seems to be an indicator of environmental stress.

Although the SR_{705} and CI_{re} indices were calculated from different formulas, the response patterns of these indices in relation to Chl content were very similar. Also, the indices SR_{705} and CI_{re} were highly correlated between them (r^2 = 0.98, data not shown). The CI_{re} was developed by Gitelson *et al*. (2006) to estimate Chl content of anthocyanin-containing and anthocyanin-free leaves. This index uses a conceptual framework that relates the content of the pigment of interest to reflectance at three wavelengths, with R_1 the band most sensitive to the pigment of interest, R_2 the band most sensitive to pigments other than the pigment of interest and R_3 closely related to leaf scattering. For the CI_{re}, index, R_1 is the reflectance in the red edge position ($R_{red\ edge}$) and $R_2 = R_3$ the reflectance in the near infrared region (R_{NIR}). The CI_{re} was successfully used by Gitelson *et al*. (2009) for estimating leaf Chl content in *Corylus avellana*, *Cornus alba*, *Acer platanoides* and *Parthenocissus quinquefolia*, which accumulate high concentrations of anthocyanins in juvenile and senescing leaves, and by Steele *et al*. (2008) for *Vitis labrusca*. The relationship between SR_{705} and CI_{re} indicates that the estimated Chl content in *E. uniflora* leaves is relatively insensitive to variations in anthocyanin content. Also, the main difference between SR_{705} and CI_{re} is the width of the spectral bands used. Whereas SR_{705} uses only two single reflectance points within the spectra, CI_{re} uses the average values from 770 to 800 nm (n = 10) and from 690 to 710 (n = 7). Although CI_{re} is very useful for estimation of Chl in leaves that accumulate high concentrations of anthocyanin, SR_{705} should be indicated in the case of *E. uniflora*.

As a tree species of the Myrtaceae family, *E. uniflora*

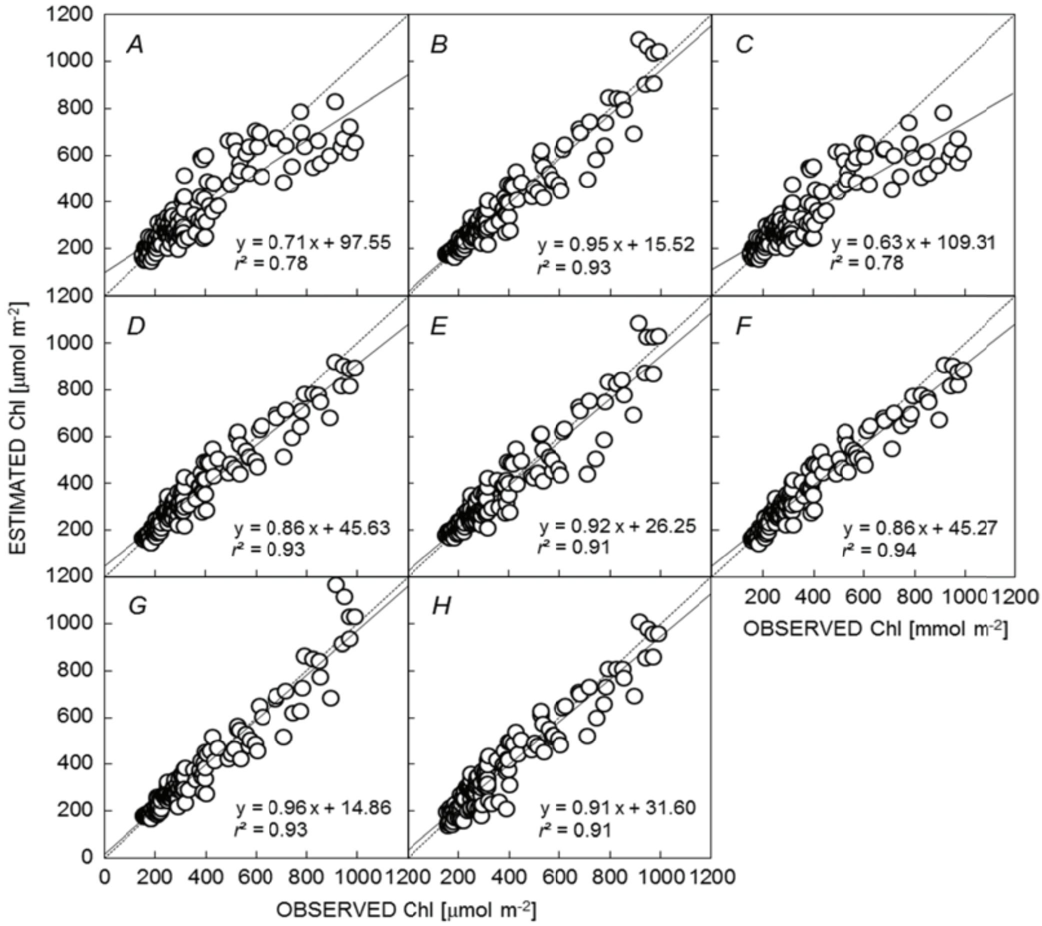

Fig. 6. Plots of observed and estimated chlorophyll (Chl) contents by eight spectral reflectance indices: (*A*) SR_{680}; (*B*) SR_{705}; (*C*) ND_{680}; (*D*) ND_{705}; (*E*) mSR_{705}; (*F*) mND_{705}; (*G*) $mCARI_{705}$; and (*H*) CI_{re}. *Dotted lines* represent 1:1 relationships.

has simple leaves with typical anatomy of a C_3 species. According to Alves *et al*. (2008) *E. uniflora* leaves are dorsiventral, glabrous and hypostomatic with paracytic type stomata. The epidermis is uniseriate and the palisade parenchyma is arranged in a single layer and conspicuous spongy parenchyma formed by seven to nine layers of cells. In both palisade and spongy parenchyma cells the presence of idioblasts containing clusters and prismatic crystals are observed. Also, a number of glands can be observed in the adaxial and abaxial surfaces of the vascular system. In another experiment using the same sun and shade treatments used in the present study, Mielke and Schaffer (2010) observed a significant difference between LMA of sun and shade grown plants. In the present study, a change of LMA from 75.5 to 145.4 g m^{-2} in shade and sun leaves is an indicator that leaves of *E. uniflora* are highly plastic with relation to changes in the light environment. Also, in the case of the anatomy of *E. uniflora* leaves, different reflectance spectra for the adaxial and abaxial surfaces are detected by the *Unispec*, whereas the estimates of Chl content made by the *SPAD* include the entire Chl content of the leaf. Thus, considering that the *Unispec* measurements were made on the adaxial surface of the leaves, it could cause an overestimation of the Chl contents.

The root mean square error of predicted values was used by Richardson *et al*. (2002) to determine the effectiveness of ten indices to estimate Chl contents in *Betula papyrifera*. Such indices varied from 12.1% for ND_{705}, to 38.0% for the yellowness index (Adams *et al*. 1999). In the present study, the $RMS\varepsilon_c$ (%) varied from 14.6% for mND_{705} to 29.2% for ND_{680}, and are inside the range of values calculated by Richardson *et al*. (2002). Also, the values of $RMS\varepsilon_c$ (%) calculated for mND_{705}, SR_{705}, ND_{705} and CI_{re} were very close to the most accurate indices described by Richardson *et al*. (2002).

The modified indices mND_{705} and $mCARI_{705}$ were used, respectively by Sims and Gamon (2002), to eliminate effects of leaf reflectance among different plant species, and by Wu *et al*. (2008) to reduce effects of the of nonphotosynthetic materials, such as stalks and senescent leaves, in corn canopies. Although the $mCARI_{705}$ has been developed to estimate Chl contents at a canopy scale, this index appears to be insensitive to changes in leaf internal structure and could be used when plants were exposed to environmental conditions that cause changes in LMA, such as changes in light or nutrient content (Poorter *et al*. 2009). On the other hand, even though the best results were obtained with the modified ND_{705} index (mND_{705}), the differences between the r^2 and $RMS\varepsilon_c$ values of this index and SR_{705} and ND_{705} were very small (Table 2). The modification of the indices SR_{705} and ND_{705} also requires an additional spectral band (R_{445}), increasing the complexity of the formulas (Sims and Gamon 2002). Despite the observation that mND_{705} had the lowest $RMS\varepsilon_c$ among all spectral indexes tested (Table 2), the tendency for underestimation of Chl contents was higher than SR_{705} and $mCARI_{705}$ (Fig. 6). Also, the regression line of observed versus estimated Chl content values for mSR_{705} had the same tendency of mND_{705} and the $RMS\varepsilon_c$ of mSR_{705} was only lower than the $RMS\varepsilon_c$ calculated for SR_{680} and ND_{680} (the two highest $RMS\varepsilon_c$ values among all indices tested). Those results showed that, in the case of *E. uniflora* leaves, the modification (*i.e*. the addition of R_{445} in the formulas) did not improve the accuracy of ND_{705} and SR_{705}.

In the present study, Chl content was estimated from *SPAD* values using the equation reported by Mielke *et al*. (2010) and a linear relationship between *SPAD* values and ND_{705} was found ($SPAD = 8.07 + 77.56ND_{705}$, $r^2 = 0.92$, $n = 142$, data not shown). This linear relationship can be explained by the fact that both *SPAD* and ND_{705} have nonlinear relationships with Chl contents (*see*, *e.g.*, Sims and Gamon 2002 and Mielke *et al*. 2010). The nonlinear relationship between *SPAD* readings and Chl contents is explained by the nonuniform distribution of Chls and the light scattering across the leaf (Udling *et al*. 2007); whereas the nonlinear relationship between ND_{705} and Chl content is caused by the loss of sensitivity as the values of near infrared/red ratio increase (Gitelson 2004).

In addition to the results observed for the *SPAD* and ND_{705} relationship found in our study, other authors have reported positive linear relationships between data obtained from portable Chl meters and the normalized difference vegetation index, when obtained by remote sensing. Perry and Davenport (2007) and Hashemi (2010) found positive linear relationships between *SPAD* values and the normalized difference vegetation index derived from Digitalglobe QuickBird for apple (*Malus domestica*) and MODIS data for hornbeam trees (*Carpinus betulus*), respectively. Good correlations between the *SPAD* index and reflectance indices, both at leaf and canopy scales, have important implications for the use of Chl meters and spectroradiometers as complementary techniques. The *SPAD* is easier to use and less expensive than spectroradiometers and in certain situations could be used to calibrate data obtained by remote sensing (Perry and Davenport 2007, Hashemi 2010) or when a spectroradiometer is not available.

In summary, data collected from *E. uniflora* leaves indicated that good estimates of leaf Chl content can be obtained using the reflectance indices tested, regardless of the presence of anthocyanins in leaves. Based on r^2 and $RMS\varepsilon_c$ values, the best results were obtained with indices with reflectance measured at wavelengths of 750 and 705 nm. Considering the performance of the models, the best reflectance indices to estimate Chl contents in *E. uniflora* leaves with a broad variation in the LMA and anthocyanin contents were SR_{705} and $mCARI_{705}$.

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