New Forests (2019) 50:377–388
https://doi.org/10.1007/s11056-018-9662-4

Relationships between reflectance and absorbance chlorophyll indices with RGB (Red, Green, Blue) image components in seedlings of tropical tree species at nursery stage

Elizabeth Santos do Amaral[1] · **Daniela Vieira Silva**[2] · **Letícia Dos Anjos**[3] · **Ana Cristina Schilling**[4] · **Ândrea Carla Dalmolin**[1] · **Marcelo Schramm Mielke**[1]

Received: 21 December 2017 / Accepted: 4 July 2018 / Published online: 6 July 2018

Abstract
Methods based on RGB (Red, Green, Blue) image segmentation may emerge as a new and low-cost method for estimation the quality of tree seedlings. However, the vast number of indexes based on the use of the RGB image segmentation and the lack of references in the literature still hinder the widespread use of this technology. Thus, we conducted a study aiming to test the relationships between methods based on absorbance and reflectance, widely used for the estimation of chlorophyll contents and physiological status of trees, and ten indexes based on RGB component analysis. We used leaves of five tropical tree species, belonging to different botanical families. Leaf absorbance was measured using the handheld chlorophyll meter SPAD-502, reflectance was measured using a spectrometer and the RGB indices were obtained from digitalized images of the leaves using a flatbed scanner. Modified linear regression models including all five species were used to relate RGB indices to absorbance and reflectance indices. Data collected from leaves of seedlings of five tropical tree species indicated that digital image processing technology can be a useful and rapid nondestructive method for assessment of physiological status of tree seedlings at nursery stage. Among the RGB indexes tested in this study the R, 2R*(G − B)/(G + B) and 2G*(G − B)/(G + B) are the most promising for analysis the tropical seedlings physiological status and quality.

Keywords Leaf image analysis · Leaf reflectance · Multispecies regression model · Portable chlorophyll meters

Introduction

Tropical forests around the world have been degraded by deforestation caused by logging and conversion of forest remnants into pasture and agricultural land (Wright 2005; Giam 2017). The forest restoration and conservation of remnants appear as alternatives for the

✉ Marcelo Schramm Mielke
msmielke@uesc.br

Extended author information available on the last page of the article

maintenance of areas covered by forests and has gained increased focus of attention in the tropics (Lamb 2002; Rodrigues et al. 2009; Calmon et al. 2011). Even though different procedures are being tested, the plantation of high quality seedlings is often successful for the restoration of areas previously covered by forests (Holl et al. 2011; Corbin and Holl 2012; Grossnickle 2012; Liu et al. 2012). In this way, the development of simple and accurate methodologies for assessment of seedling quality is an important component for the procedures of seedling production in forest nurseries, that can help the successful of forest restoration programs.

The forest nurseries are places designed for the multiplication of seedlings, which aims to provide superior quality plant material for the forest plantations. Thus, the evaluation of seedling quality and prediction of its performance in the field are relevant criteria for directing the operational activities in the forest nurseries (Mattsson 1996; Mexal et al. 2002; Esen et al. 2012; Villar-Salvador et al. 2012; Tsakaldimi et al. 2013; Villalobos et al. 2014). Methodologies for evaluating the quality of seedlings have been discussed in the literature for a long time (Grossnickle 2012) and different techniques have been proposed, for instance the evaluation of the Dickson quality index at the whole plant scale (Dickson et al. 1960). Currently, assessments of the physiological status of seedlings, through measurements of leaf water potential, chlorophyll fluorescence, infrared thermography, chlorophyll content, among others, have been increasingly used and provide accurate evaluations of seedlings quality (Esen et al. 2012; Liu et al. 2012; Villar-Salvador et al. 2012; Villalobos et al. 2014). Despite the accuracy of the methods based on the physiological performance, these techniques require the use of sophisticated and costly equipment. This is particularly critical in tropical regions, where the cost of the equipment is always a limiting factor.

Chlorophylls are organic molecules responsible for capturing light during the photosynthetic process, being essential for the conversion of light radiation into chemical energy (Nobel 2009). The chlorophylls are found in all photosynthetic organisms and their abundance varies according to species and environment (Coste et al. 2010; Mielke et al. 2010; Campoe et al. 2014). Therefore, chlorophyll contents can provide important information about the physiological status of plants (Sims and Gamon 2002; Blackburn 2007). Monitoring chlorophyll content in tree seedlings provides an accurate and direct measure of light acclimation (Naidu and DeLucia 1998; Naramoto et al. 2006; dos Anjos et al. 2015), nutritional status (Van den Berg and Perkins 2004), senescence (Adamsen et al. 1999; Junker and Ensminger 2016) and stress physiology (Mielke et al. 2010; dos Anjos et al. 2012). Because nitrogen is a component of chlorophyll molecules, the estimation of chlorophyll content is a useful indicator of nutritional status in plants (Van den Berg and Perkins 2004; Mercado-Luna et al. 2010; Vollmann et al. 2011; Yuzhu et al. 2011; Vibhute and Bodhe 2013).

Conventional methodology for determination of chlorophyll content requires destruction of leaves (Wellburn 1994). Although destructive methods for chlorophyll content analysis are quite accurate, they also use expensive equipment and are time consuming. Currently, there are several technologies for non-destructive leaf pigments estimates, most of them are based on reflectance (Richardson et al. 2002; Sims and Gamon 2002) and absorbance (Richardson et al. 2002; Coste et al. 2010). In the first, reflectance measurements are done using portable spectroradiometers, which allow the application of remote sensing technology in levels of leaves or branches (Gamon and Surfus 1999; Sims and Gamon 2002; Blackburn 2007; Steele et al. 2008; Mielke et al. 2012; Vieira Silva et al. 2016). In the second, portable chlorophyll meters perform measurements of the absorption of electromagnetic radiation in regions of the red and near infrared (Markwell et al. 1995; Uddling et al. 2007). Several handheld chlorophyll meters are available on the market, which have been used to estimate leaf chlorophyll in a large number of temperate and tropical trees (Richardson et al. 2002; Torres Netto et al. 2005; Coste et al. 2010; Mielke et al. 2010, 2012; Vieira Silva et al. 2016).

Increased focus has been placed on testing the use of digital image technology to estimate chlorophyll content (Kawashima and Nakatani 1998; Ali et al. 2012; Riccardi et al. 2014; Rigon et al. 2016; Putra and Soni 2018). Methodologies using image processing techniques are based on the segmentation of leaf color image in RGB (Red, Green, Blue), and RGB indexes have highly significant relationships with chlorophyll (Yadav et al. 2010; Ali et al. 2012; Riccardi et al. 2014) and nitrogen contents (Mercado-Luna et al. 2010; Vollmann et al. 2011; Yuzhu et al. 2011; Vibhute and Bodhe 2013). The use of RGB indexes requires digital images, which are taken from digital cameras (Vollmann et al. 2011; Li et al. 2010; Riccardi et al. 2014; Wang et al. 2014; Junker and Ensminger 2016; Putra and Soni 2018), smartphones (Gong et al. 2013; Rigon et al. 2016) and scanners (Ali et al. 2012; Murakami et al. 2005; Dey et al. 2016). In addition, the segmentation of images in RGB indexes can be done using softwares available for free download on the internet (Abramoff et al. 2004). The great advantage of the use of RGB indexes in comparison with methods based on absorbance and reflectance is the low cost and flexibility, since the instruments (digital cameras, smartphones and scanners) can be used for other purposes. Nonetheless, the variety of RGB indexes available in literature makes the comparison among data in literature difficult. Moreover, most of the published articles on the use of RGB image segmentation method are focused on agronomic plants (Kawashima and Nakatani 1998; Yuzhu et al. 2011; Ali et al. 2012; Li et al. 2010; Vibhute and Bodhe 2013; Riccardi et al. 2014; Wang et al. 2014; Dey et al. 2016; Rigon et al. 2016), which limits the use of this method and data comparison in the study of tropical tree species.

Based on these advantages, digital image processing may emerge as a new method that can be used to estimate chlorophyll contents in tree seedlings at the nursery stage and to monitor seedling performance following planting. However, the vast number of indexes based on the use of the RGB image segmentation method and the lack of references in the literature still hinder the widespread use of this technology. Thus, we conducted a study aiming to test the relationships between methods based on absorbance and reflectance, widely used for the estimation of chlorophyll contents, and ten indexes based on RGB component analysis. Additionally, aiming the application of this technology for monitoring the quality of tree seedlings in tropical nurseries, we used leaves of five tropical tree species, belonging to different botanical families. In this study we did not directly relate chlorophyll contents to RGB indices. Instead, we used as reference the methods based on reflectance and absorbance, which are already well established and widely used for estimating the contents of chlorophyll and nitrogen in leaves of temperate and tropical trees (Richardson et al. 2002; Torres Netto et al. 2005; Coste et al. 2010; Mielke et al. 2010, 2012; Vieira Silva et al. 2016).

Materials and methods

Data was collected from the seedlings of five Neotropical rainforest tree species belonging to different botanical families: *Brosimum rubescens* Taub. (Moraceae), *Cytharexyllum myrianthum* Chamiáo (Verbenaceae), *Eriotheca macrophylla* (K. Schum.) A. Robyns (Bombacaceae), *Inga capitata* Desv. (Fabaceae) and *Tapirira guianensis* Aublet. (Anacardiaceae). The seedlings were between 2 and 4 months-old and were grown under 50%

artificial shading, at the nursery of Floresta Viva Institute (www.florestaviva.org.br), Serra Grande, Uruçuca, Bahia, Brazil. For this study, we used 28–30 leaves from each species, with a total of 145 leaves. Fully expanded leaves, with preserved edges and without herbivore damage or disease symptoms were used. Leaf absorbance was measured using the handheld chlorophyll absorbance meter SPAD-502 (Minolta Inc., Osaka, Japan). Leaf reflectance of the adaxial leaf surface was measured with an USB4000-UV-VIS spectrometer using a LS-1 tungsten-halogen light source (Ocean Optics Inc., Dunedim, FL, USA). Based on the values of reflectance at 705 nm (R_{705}) and 750 nm (R_{750}), normalized difference reflectance index (ND_{750}) were calculated as $ND_{705} = (R_{750} - R_{705})/(R_{750} + R_{705})$, in according with Gitelson et al. (2003). We performed one measurement per leaf for each device, always in the same place, at the middle of the leaf blade. Just after the measurements described above, the leaves were digitalized with a HP 2400 scanner. The digitalized images were saved in the TIFF format and segmentation of each image in R, G and B were done using the software ImageJ (Abramoff et al. 2004). From the R, G and B values ten RGB indexes available in the literature were calculated (Table 1).

Modified linear regression models were used to relate RGB with absorbance and reflectance indices. 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). We used the 'boxcox' function of the MASS library (Venables and Ripley 2002) available in statistical environment R (R Development Core Team 2010). The predictive performance of the regression models was validated using the cross-validation method (Efron and Tibshirani 1993). The data was randomly divided into $k = 5$ groups and the model was adjusted k times, so that every time a different k group was used as test set for the model, which was adjusted with corresponding training sets (the remaining $n - k$ data division of each data set). The relative difference between the error of the full model and the estimated error in cross-validation, the prediction error (PE), was used as a measure of the predictive accuracy of the fitted model (Maindonald and Braun 2003). The best RGB indexes were selected based on the highest R^2 values and lower values of RMSE and PE.

Results

The lowest and highest values of RGB components (R, G and B) were measured in *B. rubescens* and *C. myriantum*, respectively (Fig. 1). Considering average values including all five species, the great contrasts among RGB components were observed for R, followed by G and B. The values of R ranged 84 units (Fig. 1a), G ranged 72 units (Fig. 1b) and B ranged 29 units (Fig. 1c). The values of SPAD index and ND_{705} varied from 10.3 and 0.089, for leaves of *E. macrophylla*, to 75.8 and 0.708, for leaves of *B. rubescens*, respectively (data not shown).

The highest R^2 values for the regressions of RGB with SPAD (Table 2) and ND_{705} (Table 3) were obtained for the indexes $2R^*(G - B)/(G + B)$, $2G^*(R - B)/(R + B)$ and R, whereas the lowest R^2 values were obtained for B. In general, the highest values of R^2 and PE were obtained for the relationships between RGB indexes and ND_{705}, with

Table 1 List of RGB indexes tested in this study

RGB indexes	Instrument	Estimation	Species	References
R	Video camera	Chlorophyll	Wheat, rye	Kawashima and Nakatani (1998)
	Digital camera	Chlorophyll	Barley	Hu et al. (2010)
	Digital camera	Chlorophyll	Quinoa, amaranth	Riccardi et al. (2014)
G	Video camera	Chlorophyll	Wheat, rye	Kawashima and Nakatani (1998)
	Digital camera	Chlorophyll	Barley	Hu et al. (2010)
	Digital camera	Nitrogen	Pepper	Yuzhu et al. (2011)
	Digital camera	Chlorophyll	Quinoa, amaranth	Riccardi et al. (2014)
B	Video camera	Chlorophyll	Wheat, rye	Kawashima and Nakatani (1998)
	Digital camera	Chlorophyll	Barley	Hu et al. (2010)
	Digital camera	Chlorophyll	Quinoa, amaranth	Riccardi et al. (2014)
(R – B)/(R + B)	Video camera	Chlorophyll	Wheat, rye	Kawashima and Nakatani (1998)
	Portable scanner	Chlorophyll	Tomato, lettuce, broccoli	Ali et al. (2012)
	Digital camera	Nitrogen	Grape	Vibhute and Bodhe (2013)
	Digital camera	Chlorophyll	Quinoa, amaranth	Riccardi et al. (2014)
(G – R)/(G + R)	Smartphone	Chlorophyll	Soybean	Rigon et al. (2016)
R/(R + G + B)	Video camera	Chlorophyll	Wheat, rye	Kawashima and Nakatani (1998)
	Scanner	Chlorophyll	Betel vine	Dey et al. (2016)
	Smartphone	Chlorophyll	Soybean	Rigon et al. (2016)
G/(R + G + B)	Video camera	Chlorophyll	Wheat, rye	Kawashima and Nakatani (1998)
	Digital camera	Nitrogen	Pepper	Yuzhu et al. (2011)
	Scanner	Chlorophyll	Betel vine	Dey et al. (2016)
	Smartphone	Chlorophyll	Soybean	Rigon et al. (2016)
B/(R + G + B)	Video camera	Chlorophyll	Wheat, rye	Kawashima and Nakatani (1998)
	Digital camera	Nitrogen	Pepper	Yuzhu et al. (2011)
	Scanner	Chlorophyll	Betel vine	Dey et al. (2016)
	Smartphone	Chlorophyll	Soybean	Rigon et al. (2016)
2G*(R – B)/(R + B)	Digital camera	Nitrogen	Grape	Vibhute and Bodhe (2013)
2R*(G – B)/(G + B)	Digital camera	Nitrogen	Grape	Vibhute and Bodhe (2013)

exception of R and G (Table 3). Contrary, the highest values of RMSE were always found for the regressions between RGB indexes and handheld chlorophyll meter (Table 2). Also, for regressions between RGB and SPAD index the highest and lowest values of RMSE were obtained for G/(R + G + B) and R, respectively. For regressions between RGB and ND_{705} the highest and lowest values of RMSE were obtained for (G – R)/(G + R), and for both 2G*(R – B)/(R + B) and 2G*(G – B)/(G + B), respectively. Considering all RGB indexes the best relationships with SPAD and ND_{705} were found for R, 2G*(R – B)/(R + B) and 2G*(G – B)/(G + B) (Fig. 2).

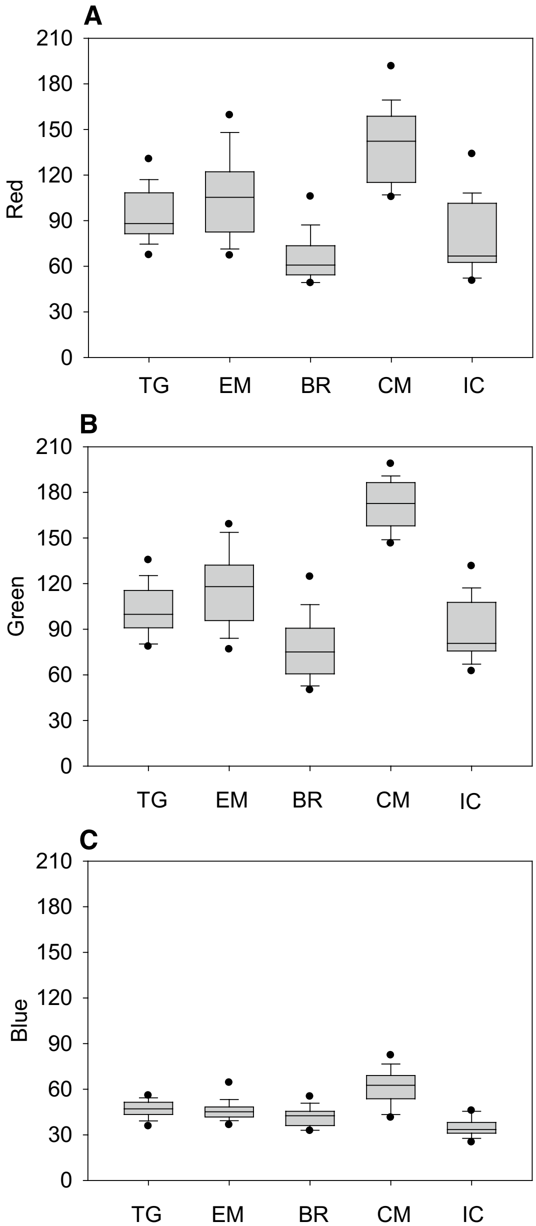

Fig. 1 Red (**a**), Green (**b**) and Blue (**c**) components of leaves of seedlings of five Neotropical rainforest tree species: TG, *Tapirira guianensis;* EM, *Eriotheca macrophylla;* BR, *Brosimum rubescens;* CM, *Cytharexyllum myrianthum;* IC, *Inga capitata*. Continuous lines inside box plots represent the medians. The points above and below the boxes represent the maximum and minimum values for each category, respectively

Discussion

The values of R, G and B in our study are between the values reported for other plant species (Hu et al. 2010; Riccardi et al. 2014). Likewise, the values of SPAD index and ND_{705} correspond to the range of values reported for tropical and temperate tree species (Richardson et al. 2002; Torres Netto et al. 2005; Coste et al. 2010; Mielke et al. 2010, 2012; Vieira

Table 2 Regression equations, coefficients of determination (R^2), relative root mean square errors (RMSE) and prediction errors (PE) for models relating SPAD-502 index (SPAD) with ten RGB indexes (n = 145)

RGB indexes	Model	R^2	P	RMSE	PE (%)
R	$\ln(y)=8.72998-1.1685\ln(x)$	0.80	0.000	0.040	0.98
G	$y^{0.3}=7.1928-0.93364\ln(x)$	0.70	0.000	0.044	0.68
B	$\ln(y)=6.121-0.703\ln(x)$	0.14	0.000	0.177	1.67
(R − B)/(R + B)	$\ln(y)=4.4198-2.8904x$	0.62	0.000	0.079	4.36
(G − R)/(G + R)	$y=30.217+62.014x$	0.03	0.030	231	1.03
R/(R + G + B)	$\ln(y)=7.989-12.051x$	0.55	0.000	0.093	3.12
G/(R + G + B)	$y^2=-8482-11870\ln(x)$	0.35	0.000	1,052,846	0.71
B/(R + G + B)	$y^{0.5}=1.1313+24.4669x$	0.62	0.000	0.633	3.36
2G*(R − B)/(R + B)	$\ln(y)=5.9007-0.5852\ln(x)$	0.79	0.000	0.044	2.87
2R*(G − B)/(G + B)	$\ln(y)=6.1886-0.6459\ln(x)$	0.79	0.000	0.043	2.71

Table 3 Regression equations, coefficients of determination (R^2), relative root mean square errors (RMSE) and prediction errors (PE) for models relating the normalized difference reflectance index (ND_{750}) with ten RGB indexes (n = 145)

RGB indexes	Model	R^2	P	RMSE	PE (%)
R	$y^{0.5}=2.08-0.33\ln(x)$	0.77	0.000	0.004	2.26
G	$y^{0.5}=1.997-0.306\ln(x)$	0.63	0.000	0.006	0.78
B	$y^{0.5}=1.13-0.15\ln(x)$	0.07	0.001	0.016	2.42
(R − B)/(R + B)	$y=-0.032-0.323\ln(x)$	0.76	0.000	0.005	2.18
(G − R)/(G + R)	$\ln(x)=-1.5+4.6x$	0.18	0.000	0.196	3.25
R/(R + G + B)	$\ln(y)=4.346-14.646x$	0.70	0.000	0.073	2.16
G/(R + G + B)	$y=-0.632-1.168\ln(x)$	0.24	0.000	0.017	0.00
B/(R + G + B)	$y=-0.2367+3.0805x$	0.71	0.000	0.007	1.80
2G*(R − B)/(R + B)	$y=1.197-0.203\ln(x)$	0.86	0.000	0.003	0.91
2R*(G − B)/(G + B)	$y=1.294-0.224\ln(x)$	0.86	0.000	0.003	0.91

Silva et al. 2016). Values of SPAD index between 10 and 80 correspond to chlorophyll contents between 80 and 1500 mg m^{-2}, respectively (Coste et al. 2010; Vieira Silva et al. 2016).

There are many indexes based on the color segmentation in the literature. In this work we opted to test only the ten most used RGB indexes, that have been successfully applied by other authors to estimate chlorophyll (Kawashima and Nakatani 1998; Hu et al. 2010; Ali et al. 2012; Riccardi et al. 2014; Rigon et al. 2016) or nitrogen (Yuzhu et al. 2011; Vibhute and Bodhe 2013) in leaves of different plant species. The results published in the literature are highly variable in relation to the performance of different RGB indexes. For example, Hu et al. (2010), obtained R^2 values of 0.727 and 0.851 for regressions between R and G with the total chlorophyll content in leaves of three barley cultivars, but low Pearson correlation coefficients (r) when the indexes R/(R + B + G), G/(R + B + G) and B/(R + B + G) were tested. Ali et al. (2012) obtained high r values for the relationship between the index (R − B)/(R + B) and chlorophyll content in leaves of tomato, but low r values for lettuce. Riccardi et al. (2014), on the other hand, reported values of R^2 around 0.62 and 0.42 for the

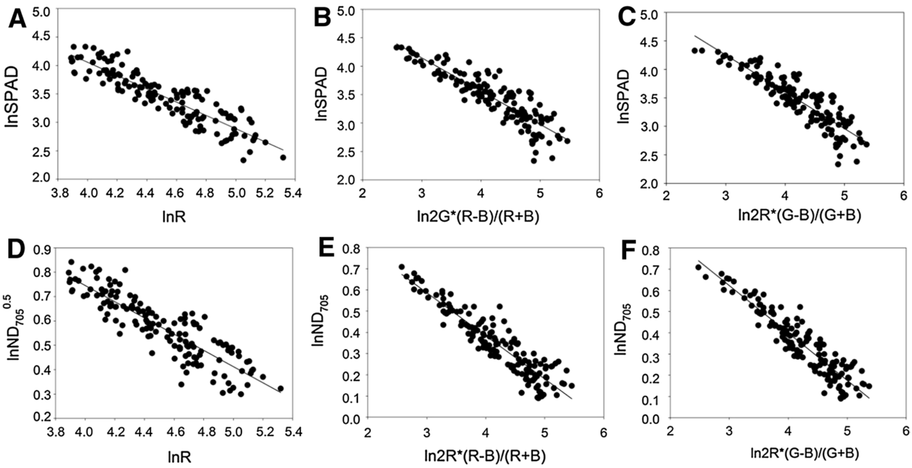

Fig. 2 Regressions between SPAD-502 index (SPAD) (**a**, **b**, **c**) and normalized difference reflectance index (ND_{750}) (**d**, **e**, **f**) with RGB indexes for leaves of seedlings of five tropical rainforest tree species (n = 145). Equations are shown in Tables 2 and 3

relationship between the index (R − B)/(R + B) and chlorophyll contents in leaves of amaranth and quinoa, respectively. In our work, the best results were obtained using the simple index R, as well as with the modified indexes 2R*(G − B)/(G + B) and 2G*(R − B)/(R + B), which were successfully used by Vibhute and Bodhe (2013), for estimates of N content in grape leaves.

Chlorophylls are the most abundant pigments in leaves and directly involved in capturing photons for the photosynthetic process (Nobel 2009). Although the chlorophylls *a* and *b* are very abundant in leaves, the contents and the ratio of these leaf pigments in different species vary widely (Sims and Gamon 2002). The presence of other pigments, such as carotenoids and anthocyanins affects the reflectance in different wavelength bands (Gamon and Surfus 1999; Blackburn 2007), providing changes in the color of leaves (Lee 2007) and, consequently, in the values of R, G and B. Accordingly, possible discrepancies in the results described in the literature can be assigned to the pigment composition and spectral characteristics of the leaves of different species. Nevertheless, in our study we obtained optimal adjustments for regressions between indices derived from RGB components for a set of five different tropical tree species. These results indicate that this is a precise, practical and inexpensive technique, which deserves more attention.

The use of RGB indexes requires digital images, which are taken from digital cameras (Riccardi et al. 2014; Li et al. 2010; Wang et al. 2014; Putra and Soni 2018), smartphones (Gong et al. 2013; Rigon et al. 2016) and scanners (Murakami et al. 2005; Ali et al. 2012; Dey et al. 2016). It is important to maintain similar light conditions when take the pictures, since light quality and intensity, as well as light reflection on the surface of the leaf, will directly affect the color properties of the image and, thus, the estimation of RGB parameters. Different light conditions can significantly hamper comparison among images and among data in literature. It is difficult to evaluate whether the differences in RGB indexes are due to distinct species, different growth conditions or due to the different light conditions in which the images were taken. Therefore, in our study we chose to use scanned images, obtained from a flatbed low cost scanner. Flatbed scanners in general have the same type of light source and flatten the leaves, avoiding inclination and reflections bias. Although this method requires detachment and destruction of the leaf, it is a good way to obtain homogeneous and comparable data.

The handheld chlorophyll meter and the reflectance index used as a criterion of the performance of RGB indexes for predictions of chlorophyll contents have been largely successfully used for other types of plant species (Gamon and Surfus 1999; Richardson et al. 2002; Sims and Gamon 2002; Torres Netto et al. 2005; Blackburn 2007; Steele et al. 2008; Coste et al. 2010; Mielke et al. 2010, 2012; Vieira Silva et al. 2016). The SPAD-502 is one of the most popular handheld chlorophyll meters and has been used to estimate leaf chlorophyll in a large number of temperate and tropical trees (Richardson et al. 2002; Torres Netto et al. 2005; Coste et al. 2010; Mielke et al. 2010, 2012). The good relationships between SPAD index and the RGB indexes demonstrate the usefulness of using digital image processing technology for estimate chlorophyll content in leaves of tree seedlings and has an important practical application.

Despite the increasing number of information available in the literature on the use of RGB image segmentation method to estimate the chlorophyll content in plants (Kawashima and Nakatani 1998; Yuzhu et al. 2011; Ali et al. 2012; Li et al. 2010; Vibhute and Bodhe 2013; Riccardi et al. 2014; Wang et al. 2014; Rigon et al. 2016), there are no information on using this method for multi-species models. The use of segmentation of digital images in RGB components to estimate chlorophyll content in leaves is still a new technique and has as main obstacles the lack of standardization of the protocols to obtain the images and the selection of the best index to be used. Nevertheless, it is a very promising technique that should receive increased attention for the next years. To our knowledge, this is the first article where the digital image segmentation in RGB components was used to estimate chlorophyll in leaves of tree seedlings. Using five species belonging to different families and with different leaf morphologies we demonstrated that it is possible to use RGB images for estimating chlorophyll content.

The estimation of leaf chlorophyll content is important for studies on tropical tree seedlings ecophysiology, as it is an indicator of physiological performance of plants (Naidu and DeLucia 1998; Adamsen et al. 1999; Van den Berg and Perkins 2004; Naramoto et al. 2006; Mielke et al. 2010; Campoe et al. 2014; Junker and Ensminger 2016). Currently, there are an increasing number of methodologies based on leaf reflectance (Richardson et al. 2002; Sims and Gamon 2002) and absorbance (Richardson et al. 2002; Coste et al. 2010) that permits accurate predictions of chlorophyll contents. Such methods area applied with success in temperate and tropical tree species (Gamon and Surfus 1999; Richardson et al. 2002; Sims and Gamon 2002; Torres Netto et al. 2005; Steele et al. 2008; Coste et al. 2010; Mielke et al. 2010, 2012; Vieira Silva et al. 2016). Despite the accuracy of the methods based on absorbance and reflectance for predictions of chlorophyll contents, these metodologies require the use of sophisticated and costly equipment. This is particularly critical in tropical regions, when the cost of the equipment is always a limiting factor. The use of RGB indexes is a low cost recent technology that has been successfully used for predictions of chlorophyll contents in agricultural plants (Vollmann et al. 2011; Ali et al. 2012; Gong et al. 2013; Li et al. 2010; Riccardi et al. 2014; Wang et al. 2014; Dey et al. Dey et al. 2016; Junker and Ensminger 2016; Rigon et al. 2016; Putra and Soni 2018). Among the RGB indexes tested in this study the R, $2R*(G-B)/(G+B)$ and $2G*(G-B)/(G+B)$ are the most promising for analysis the tropical seedlings physiological status and quality. In summary, our data indicated that good regressions between the widely used methods for estimation of chlorophyll contents and digital image processing technology can be obtained. The segmentation of leaf color image in RGB can be a useful and low costly method for assessment of physiological status of tree seedlings in tropical forest nurseries.

Acknowledgements The authors thank Gerson J. Sales Neto, Nilson A. dos Santos and Rones F. Souza, of Floresta Viva Institute, and M.Sc. Murilo F. C. de Jesus for assistance with data collection. We thank Dr. Fábio P. Gomes of DCB/UESC for providing the SPAD-502 used in this study. Funding for Elizabeth S. Amaral during this study was provided by a scholarship from Capes (Brazilian Higher Education Council). Marcelo S. Mielke gratefully acknowledge CNPq (Brazilian National Council for Scientific and Technological Development) for the award of fellowship of scientific productivity (306531/2015-1). This study was supported by CNPq (561933/2010).

References

Abramoff MD, Magalhaes PJ, Ram SJ (2004) Image processing with ImageJ. Biophoton Int 11:36–42
Adamsen FJ, Pinter PJ, Barnes EM, LaMorte RL, Wall GW, Leavitt SW, Kimball BA (1999) Measuring wheat senescence with a digital camera. Crop Sci 39:719–724
Ali MM, Al-Ani A, Eamus D, Tan DKY (2012) A new image processing based technique to determine chlorophyll in plants. Am Eurasian J Agric Environ Sci 12:1323–1328
Blackburn GA (2007) Hyperspectral remote sensing of plant pigments. J Exp Bot 58:855–867
Box GEP, Cox DR (1964) An analysis of transformations. J R Stat Soc 26:211–252
Calmon M, Brancalion PHS, Paese A, Aronson J, Castro P, Silva SC, Rodrigues RR (2011) Emerging threats and opportunities for large-scale ecological restoration in the Atlantic Forest of Brazil. Restor Ecol 19:154–158
Campoe OC, Iannelli C, Stape JL, Cook RL, Mendes JCT, Vivian R (2014) Atlantic forest tree species responses to silvicultural practices in a degraded pasture restoration plantation: from leaf physiology to survival and initial growth. For Ecol Manag 313:233–242
Corbin JD, Holl KD (2012) Applied nucleation as a forest restoration strategy. For Ecol Manag 265:37–46
Coste S, Baraloto C, Leroy C, Marcon E, Renaud A, Richardson AD, Roggy JC, Schimann H, Uddling J, Hérault B (2010) Assessing foliar chlorophyll contents with the SPAD-502 chlorophyll meter: a calibration test with thirteen tree species of tropical rainforest in French Guiana. Ann For Sci 67:303–310
Dey AK, Sharma M, Meshram MR (2016) An analysis of leaf chlorophyll measurement method using chlorophyll meter and image processing technique. Procedia Comput Sci 85:286–292
Dickson A, Leaf AL, Hosner JF (1960) Quality appraisal of white spruce and white pine seedling stock in nurseries. For Chron 36:10–13
dos Anjos L, Oliva MA, Kuki KN (2012) Fluorescence imaging of light acclimation of brazilian atlantic forest tree species. Photosynthetica 50:95–108
dos Anjos L, Oliva MA, Kuki KN, Mielke MS, Ventrella MC, Galvão MF, Pinto LRM (2015) Key leaf traits indicative of photosynthetic plasticity in tropical tree species. Trees 29:247–258
Efron B, Tibshirani RJ (1993) An introduction to the bootstrap. Chapman and Hall, New York
Gamon JA, Surfus JS (1999) Assessing leaf pigment content and activity with a reflectometer. New Phytol 143:105–117
Giam X (2017) Global biodiversity loss from tropical deforestation. PNAS 114:5775–5777
Gitelson AA, Gritz Y, Merzlyak MN (2003) Relationships between leaf chlorophyll content and spectral reflectance and algorithms for non-destructive chlorophyll assessment in higher plant leaves. J Plant Physiol 160:271–282
Gong A, Yu J, He Y, Qiu Z (2013) Citrus yield estimation based on images processed by an Android mobile phone. Biosyst Eng 115:162–170
Grossnickle SC (2012) Why seedlings survive: influence of plant attributes. New For 43:711–738
Holl KD, Zahawi RA, Cole RJ, Ostertag R, Cordell S (2011) Planting seedlings in tree islands versus plantations as a large-scale tropical forest restoration strategy. Restor Ecol 19:470–479
Hu H, Liu H, Zhang H, Zhu J, Yao X, Zhang X, Zheng K (2010) Assessment of chlorophyll content based on image color analysis, comparison with SPAD-502. In: 2010 2nd international conference on information engineering and computer science, pp 1–3
Junker LA, Ensminger I (2016) Relationship between leaf optical properties, chlorophyll fluorescence and pigment changes in senescing *Acer saccharum* leaves. Tree Physiol 36:694–711
Kawashima S, Nakatani M (1998) An algorithm for estimating chlorophyll content in leaves using a video camera. Ann Bot 81:49–54

Lamb D (2002) Forest restoration—the third big silvicultural challenge. J Trop For Sci 24:295–299
Lee D (2007) Nature's palette. The science of plant color. The University of Chicago Press, Chicago
Li Y, Chen D, Walker CN, Angus JF (2010) Estimating the nitrogen status of crops using a digital camera. Field Crops Res 118:221–227
Liu Y, Bai SL, Zhu Y, Li GL, Jiang P (2012) Promoting seedling stress resistance through nursery techniques in China. New For 43:639–649
Maindonald J, Braun J (2003) Data analysis and graphics using R—an example-based approach. Cambridge University Press, Cambridge
Markwell J, Osterman JC, Mitchell JL (1995) Calibration of the Minolta SPAD-502 leaf chlorophyll meter. Photosynth Res 46:467–472
Mattsson A (1996) Predicting field performance using seedling quality assessment. New For 13:223–248
Mercado-Luna A, Rico-García E, Lara-Herrera A, Soto-Zarazúa G, Ocampo-Velázquez R, Guevara-González R, Herrera-Ruiz R, Torres-Pacheco I (2010) Nitrogen determination on tomato (*Lycopersicon esculentum* Mill.) seedlings by colour image analysis (RGB). Afr J Biotechnol 33:5326–5332
Mexal JG, Cuevas RRA, Negreros-Castillo P, Paraguirre LC (2002) Nursery production practices affect survival and growth of tropical hardwoods in Quintana Roo, México. For Ecol Manag 168:125–133
Mielke MS, Schaffer B, Li C (2010) Use of a SPAD meter to estimate chlorophyll content in *Eugenia uniflora* L. leaves as affected by contrasting light environments and soil flooding. Photosynthetica 48:332–338
Mielke MS, Schaffer B, Schilling AC (2012) Evaluation of reflectance spectroscopy indices for estimation of chlorophyll content in leaves of a tropical tree species. Photosynthetica 50:343–352
Murakami PF, Turner MR, van den Berg AK, Schaberg PG (2005) An instructional guide for leaf color analysis using digital imaging software. General Technical Report NE-327. U.S. Department of Agriculture, Forest Service, Northeastern Research Station, Newtown Square, PA
Naidu SL, DeLucia EH (1998) Physiological and morphological acclimation of shade-grown tree seedlings to late-season canopy gap formation. Plant Ecol 138:27–40
Naramoto M, Katahata S, Mukai Y, Kakubari Y (2006) Photosynthetic acclimation and photoinhibition on exposure to high light in shade-developed leaves of *Fagus crenata* seedlings. Flora 201:120–126
Nobel P (2009) Physicochemical and environmental plant physiology. Academic Press, New York
Putra BTW, Soni P (2018) Enhanced broadhand greenness in assessing chlorophyll *a* and *b*, carotenoid, and nitrogen in robusta coffee plantations using a digital camera. Precis Agric 19:238–256
R Development Core Team (2010) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna
Riccardi M, Miele G, Pulvento C, Lavini A, D'Andria R, Jacobsen SE (2014) Non-destructive evaluation of chlorophyll content in quinoa and amaranth leaves by simple and multiple regression analysis of RGB image components. Photosynth Res 120:263–272
Richardson AD, Duigan SP, Berlyn GP (2002) An evaluation of noninvasive methods to estimate foliar chlorophyll content. New Phytol 153:185–194
Rigon JPG, Capuani S, Fernandes DM, Guimarães TM (2016) A novel method for the estimation of soybean chlorophyll content using a smartphone and image analysis. Photosynthetica 54:559–566
Rodrigues RR, Lima RAF, Gandolfi S, Nave AG (2009) On the restoration of high diversity forests: 30 years of experiences in the Brazilian Atlantic Forest. Biol Conserv 142:1242–1251
Sims DA, Gamon JA (2002) Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. Remote Sens Environ 81:337–354
Sokal RR, Rohlf FJ (1995) Biometry: the principles and practice of statistics in biological research. WH Freeman & Comp, New York
Steele MR, Gitelson AA, Rundquist DCA (2008) Comparison of two techniques for nondestructive measurement of chlorophyll content in grapevine leaves. Agron J 100:87–92
Torres Netto AT, Campostrini E, Oliveira JG, Smith REB (2005) Photosynthetic pigments, nitrogen, chlorophyll *a* fluorescence and SPAD-502 readings in coffee leaves. Sci Hortic 104:199–209
Tsakaldimi M, Ganatsas P, Jacobs DF (2013) Prediction of planted seedling survival of five Mediterranean species based on initial seedling morphology. New For 44:327–339
Uddling J, Gelang-Alfredsson J, Piikki K, Pleijel H (2007) Evaluating the relationship between leaf chlorophyll concentration and SPAD-502 chlorophyll meter readings. Photosynth Res 91:37–46
Van den Berg AK, Perkins TD (2004) Evaluation of a portable chlorophyll meter to estimate chlorophyll and nitrogen contents in sugar maple (*Acer saccharum* Marsh.) leaves. For Ecol Manag 200:113–117
Venables WN, Ripley BD (2002) Modern applied statistics with S. Springer, New York
Vibhute A, Bodhe SK (2013) Color image processing approach for nitrogen estimation of vineyard. IJASR 3:189–196

Vieira Silva D, Dos Anjos L, Brito-Rocha E, Dalmolin AC, Mielke MS (2016) Calibration of a multi-species model for chlorophyll estimation in seedlings of Neotropical tree species using hand-held leaf absorbance meters and spectral reflectance. iForest 9:829–834

Villalobos EB, Cetina VMA, López MAL, Aldrete A, Paniagua DHV (2014) Nursery practices increase seedling performance on nutrient-poor soils in *Swietenia humilis*. iForest 8:552–557

Villar-Salvador P, Puértolas J, Cuesta B, Peñuelas JL, Uscola M, Heredia-Guerrero N, Rey BJM (2012) Increase in size and nitrogen concentration enhances seedling survival in Mediterranean plantations. Insights from an ecophysiological conceptual model of plant survival. New For 43:755–770

Vollmann J, Walter H, Sato T, Schweiger P (2011) Digital image analysis and chlorophyll metering for phenotyping the effects of nodulation in soybean. Comput Electron Agric 75:190–195

Wang Y, Wang D, Shi P, Omasa K (2014) Estimating rice chlorophyll content and leaf nitrogen concentration with a digital still color camera under natural light. Plant Methods 10:1–11

Wellburn AR (1994) The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J Plant Physiol 144:307–313

Wright SJ (2005) Tropical forests in a changing environment. Trends Ecol Evol 20:553–560

Esen D, Yildiz O, Esen U, Edis S, Çetintas C (2012) Effects of cultural treatments, seedling type and morphological characteristics on survival and growth of wild cherry seedlings in Turkey. iForest 5:283–289

Yadav SP, Ibaraki Y, Dutta Gupta S (2010) Estimation of the chlorophyll content of micropropagated potato plants using RGB based image analysis. Plant Cell, Tissue Organ Cult 100:183–188

Yuzhu H, Xiaomei W, Shuyao S (2011) Nitrogen determination in pepper (*Capsicum frutescens* L.) plants by color image analysis (RGB). Afr J Biotechnol 10:17737–17741

Affiliations

Elizabeth Santos do Amaral[1] · Daniela Vieira Silva[2] · Letícia Dos Anjos[3] · Ana Cristina Schilling[4] · Ândrea Carla Dalmolin[1] · Marcelo Schramm Mielke[1]

[1] Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, Rodovia Jorge Amado km 16, Ilhéus, BA, Brazil

[2] Departamento de Ciências Agrárias e Ambientais, Universidade Estadual de Santa Cruz, Ilhéus, BA, Brazil

[3] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Campus do Pici, Fortaleza, CE, Brazil

[4] Departamento de Ciências Exatas e Tecnológicas, Universidade Estadual de Santa Cruz, Ilhéus, BA, Brazil