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A novel method for the estimation of soybean chlorophyll content using a smartphone and image analysis

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Abstract

The development of smartphones, specifically their cameras, and imaging technologies has enabled their use as sensors/measurement tools. Here we aimed to evaluate the applicability of a fast and noninvasive method for the estimation of total chlorophyll (Chl), Chl *a*, Chl *b,* and carotenoids (Car) content of soybean plants using a smartphone camera. Single leaf disc images were obtained using a smartphone camera. Subsequently, for the same leaf discs, a Chl meter was used to obtain the relative index of Chl and the photosynthetic pigments were then determined using a classic method. The RGB, HSB and CIELab color models were extracted from the smartphone images and correlated to Chl values obtained using a Chl meter and by a standard laboratory protocol. The smartphone camera was sensitive enough to capture successfully a broad range of Chl and Car contents seen in soybean leaves. Although there was a variation between color models, some of the proposed regressions (*e.g*., the S and b index from HSB and Lab color models and NRI [RGB model]) were very close to the Chl meter values. Based on our findings, smartphones can be used for rapid and accurate estimation of soybean and Car contents in soybean leaves.

Additional key words: camera, color model, mathematical models, nondestructive, photosynthetic pigments, portable equipment.

Introduction

The amount of solar radiation absorbed by a leaf is a function of the photosynthetic pigment content (Steele *et al*. 2008). Thus, the contents of chlorophyll (Chl) *a* and Chl *b* are linked directly to a photosynthetic potential and primary production (Curran *et al*. 1990). Carotenoids (Car) are also very important as a mechanism for attenuating the stress caused by excess irradiation (Sikuku *et al*. 2010). Thus, monitoring changes in the Chl content enables assertions about the plant interaction and the influence of stress factors (Vesali *et al*. 2015).

Nondestructive techniques have been investigated for estimating the Chl content of plants (Rigon *et al*. 2012a). However, with the development of remote-sensing technology in recent years, image technologies have been used for potential real-time estimation of Chl content and subsequent analysis of photosynthetic properties (Williams *et al*. 2010). Among the techniques, digital cameras are widely used together with the segmentation of images and color models (Li *et al*. 2010) and are becoming a new quantitative tool in agriculture (Riccardi *et al*. 2014). Smart phones and their cameras have provided an opportunity for using their sensors as measurement tools, and computation and analysis can be done without any additional attachments. Digital image processing is increasingly used to estimate variables of interest for agronomic activities (Confalonieri *et al*. 2013).

Smartphone images have already been used to estimate crop water requirements and photosynthetic primary production (Confalonieri *et al*. 2013). They have also been used to estimate a citrus yield (Gong *et al*. 2013), and a colorimetric analyzer based on a smart phone can determine the available phosphorus content in soil (Moonrungsee et *al*. 2015). A recent study created an android application to estimate the Chl content of a corn leaf (Vesali *et al*. 2015). The image acquisition is reliable for real-time estimation of Chl content. The objectives of the present study were (*1*) to evaluate the applicability of a fast and nondestructive method to estimate the contents of Chl *a* and *b*, total Chl, and Car content and (*2*) to determine several color models for analysis of digital images acquired with a smart phone camera and validate their applicability for quick estimation.

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Abbreviations: B – mean values of blue; Car – carotenoids; Chl – chlorophyll; DGCI – dark green color index; G – mean values of green; R – mean values of red; R1 – flovering stage; R3 – pod formation stage; VI_{green}– vegetation index.

Materials and methods

Field data acquisition: An experiment was conducted at São Paulo State University, UNESP, Botucatu, SP, Brazil. Were collected 80 disc-shaped samples of soybean leaves in the middle third of the plant with 113-mm diameter and different physiological stages (flowering, R1, and pod formation, R3, respectively). The Chl relative rates were individually determined by a portable Chl meter *ClorofiLOG-1030* (*Falker Agricultural Automation*, Brazil).

Digital images: Single images were obtained through a smartphone camera (8.0 MP) from the leaf discs. For the acquisition of images, the phone was mounted on a support in the nadir position relative to the leaf discs under identical illumination conditions, as proposed by Riccardi *et al*. (2014). Munsell images were used (2.5 G 5/5 yellowish green; 5.0 G 5/5 green; 7.5 GY 5/5 green greenish-yellow) for further calibration with known RGB values.

Chl extraction: After obtaining the images, the circular samples were placed in test tubes and wrapped in aluminum foil to protect against degradation by light. We used 5 ml of the reagent extractor dimethylsulfoxide, and the tubes were incubated at 70ºC for 30 min in accordance with the methods described by Arnon (1949) and Hiscox and Israelstam (1979). A 3-ml portion was transferred to a quartz cuvette, and then the absorbance was measured using a UV-VIS spectrophotometer (*UV mini-1240, Shimadzu*, Japan) at wavelengths of 480, 646, and 663 nm, simultaneously. The equations described by Wellburn (1994) were used for the quantification of the Chl *a*, Chl *b*, and Car contents:

$$\text{Chl } a = 12.47\, A_{665} - 3.62\, A_{649} \tag{1}$$
$$\text{Chl } b = 25.06\, A_{649} - 6.5\, A_{665} \tag{2}$$
$$\text{Car} = \frac{1000\,\text{A}_{480} - 1.29_{\text{Ca}} - 53.78_{\text{Cb}}}{220} \tag{3}$$

Image segmentation and calculation of color indices: The images were transferred to a computer and analyzed in *Corel Photopaint* software (*version X4*, *Corel Corporation*, 2008). Using the software histogram function, the center of the circular image was selected, and the size was automatically adjusted to 25 square pixels. Values were obtained for the RGB color model of each image, which is the most commonly used for the representation of digital images. R, G, and B are the mean values of red, green, and blue, while r, g, and b are the RGB values normalized by the following equations:

$$\text{r} = \frac{\text{R}}{(\text{R+G+B})} \tag{4}$$
$$\text{g} = \frac{\text{G}}{(\text{R+G+B})} \tag{5}$$
$$\text{b} = \frac{\text{B}}{(\text{R+G+B})} \tag{6}$$

The green vegetation index (VI_{green}) was obtained as follows (Gitelson *et al*. 2002):

$$\text{VI}_{green} = \frac{\text{G}-\text{R}}{\text{G+R}} \tag{7}$$

RGB values were converted to the HSB model through algorithms reported by Karcher and Richardson (2003). This color model is represented by cylindrical coordinates in which the angle around the central vertical axis corresponds to the hue (H). The HSB calculation was performed as follows:

$$\text{H} = \begin{cases} 60 * \left\{\frac{\text{G}-\text{B}}{[\max(\text{R,G,B}) - \min(\text{R,G,B})]}\right\}, \max(\text{R, G, B}) = \text{R} \\ 60 * \left\{\frac{\text{B}-\text{R}}{[\max(\text{R,G,B}) - \min(\text{R,G,B})]}\right\} \max(\text{R, G, B}) = \text{G} \\ 60 * \left\{\frac{\text{R}-\text{G}}{[\max(\text{R,G,B}) - \min(\text{R,G,B})]}\right\} \max(\text{R, G, B}) = \text{B} \end{cases} \tag{8}$$

$$\text{S} = \frac{[\max(\text{R,G,B}) - \min(\text{R,G,B})]}{\max(\text{R,G,B})} \tag{9}$$

$$\text{B} = \max(\text{R, G, B}) \tag{10}$$

The values were then converted to model X, Y, Z and subjected to color model CIE L* a* b* (Robertson 1977) by means of Eq. (11–13):

$$\text{L} * = \left(\frac{\text{Y}}{\text{Yn}}\right)^{\frac{1}{3}} - 16 \tag{11}$$
$$\text{a} * = 500\left[\left(\frac{\text{X}}{\text{Xn}}\right)^{1/3} - \left(\frac{\text{Y}}{\text{Yn}}\right)^{1/3}\right] \tag{12}$$
$$\text{b} * = 200\left[\left(\frac{\text{Y}}{\text{Yn}}\right)^{1/3} - \left(\frac{\text{Z}}{\text{Zn}}\right)^{1/3}\right] \tag{13}$$

This model describes all visible colors using L* as the lightness of the color, while a* represents green (a* negative) and red (a* positive), and b* represents the blue (b* negative) and yellow (b* positive).

Based on the HSB values, the dark green color index (DGCI) was obtained by Eq. (14) proposed by Karcher and Richardson (2003):

$$\text{DGCI} = \frac{\left[\frac{(\text{H}-60)}{60} + (1-\text{S}) + (1-\text{B})\right]}{3} \tag{14}$$

Data were subjected to *Pearson*'s correlation analysis. The significant data was subjected to linear regression and the *Student*'s *t*-test at 95% confidence. Data analysis was performed using *Origin 8.6* software and the graphics were obtained using *SigmaPlot*.

Results and discussion

Chl pigments by Chl meter: The analytical laboratory extraction of the leaves resulted in a broad range of photosynthetic pigments, ranging from 75 to 600 µmol m^{-2} for Chl *a*, from 8 to 120 µmol m^{-2} for Chl *b*, and from 50 to 350 µmol m^{-2} for Car (Fig. 1). These ranges were necessary for prediction of several conditions of leaf status.

Using the classical method, a close relationship was found between the measurement using the portable Chl meter and the pigment contents (Fig. 1). The leaf discs indicated that there was a similar relationship between the degree of leaf greenness, as determined by the portable Chl meter, and the Chl *a* content, which was extracted by the classical method. The relationship between the methods was best expressed by a quadratic model with a high range of Chl contents and nearly linear relationships. Most scientists agree that nonlinear equations are more appropriate for indicating the relationship between portable meter measurements of Chl and the actual Chl content (Marenco *et al*. 2009, Mielke *et al*. 2010, Rigon *et al*. 2013).

The adjusted coefficients of determination of the model were 0.96, 0.92, 0.96, and 0.94 for Chl *a* and *b*, total Chl, and Car, respectively. These results were nearly identical to those observed in a recent study for Chl (Novichonok *et al*. 2016). Another study with soybean plants found similar results with a close relationship between portable Chl meter measurements and Chl measured by the classical method (Markwell *et al*. 1995). The Chl meter is one of the most used hand-held instruments for a rapid and nondestructive assessment of Chl contents in many crops species, such as quinoa (Riccardi *et al*. 2014), castor oil, sesame (Rigon *et al*. 2012a, 2012b), and Surinam cherry (Mielke *et al*. 2010). However, the reflectance depends on leaf anatomical features, such as surface relief, and/or external architecture, such as trichome density and waxy cuticle (Levizou *et al*. 2005). Consequently, this relation changes in accordance with the intrinsic characteristics of each species (Marenco *et al*. 2009). Furthermore, it was observed that in some cases, the determination of Chl *b* was inaccurate (Rigon *et al*. 2013). This resulted from the fact that the absorption peak of Chl *a* was similar to the wavelength emitted by the device, making it more difficult to separate (Neves *et al*. 2005).

Torres-Netto *et al*. (2005) demonstrated SPAD to be the method with the best performance in terms of precision and the best metrics for both repeatability and reproducibility. Thus, the generated regression models in Fig. 1 may be used to estimate the content of Chl pigments in the leaves of soybean plants with great precision in a fast and efficient way and without any added cost for reagents.

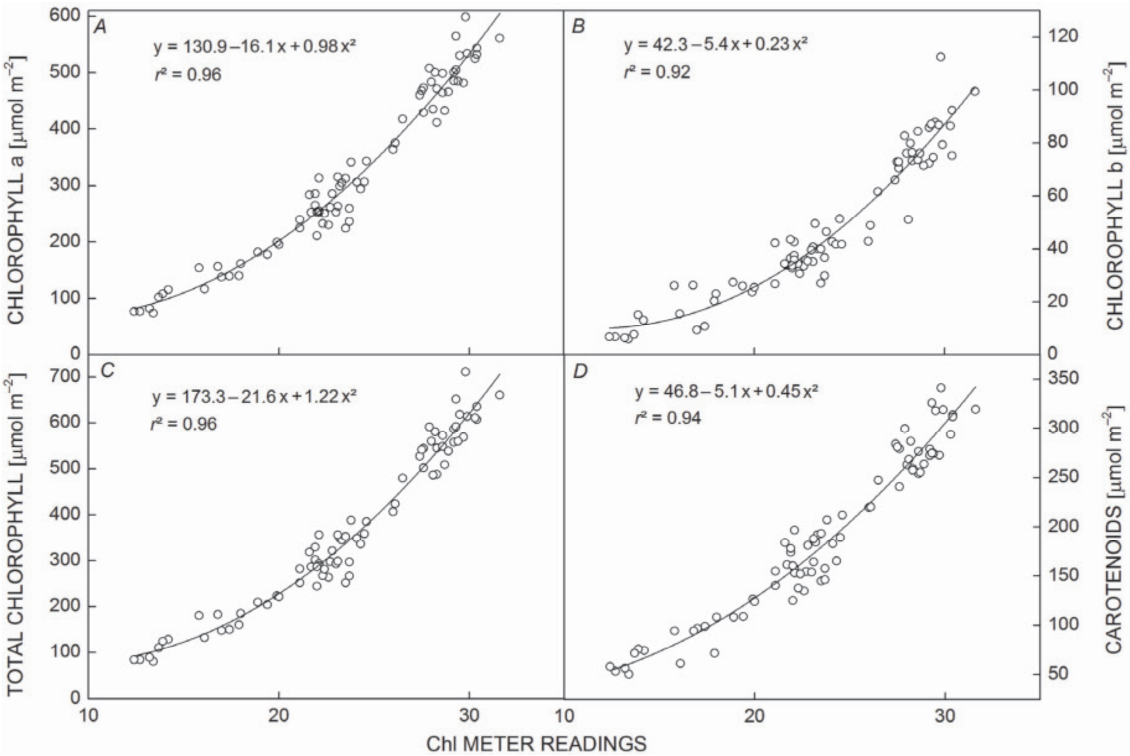

Fig 1. Relationship between the readings of the portable chlorophyll meter and the contents of chlorophyll *a* (*A*), *b* (*B*), total chlorophyll (*C*), and carotenoids (*D*) measured analytically in the laboratory for soybean leaves.

RGB color model: A color in the RGB model is described by indicating how much of each of the red (R), green (G), and blue (B) is included, and each color had 256 graduations. The index B was positively correlated with Chl and Car, whereas R and G were negatively correlated (Fig. 2). This was similar to the findings of Yadav *et al.* (2010), Gupta *et al.* (2013), and Riccardi *et al.* (2014). G values were bigger than R and B values in all the images measured, and the R index was almost double of the B index. Thus, the best fit regression models for the single color component index G were about 0.82, 0.79, 0.82, and 0.82 for Chl *a*, *b*, total Chl, and Car. Studies have reported that the G index had a better relationship with the Chl content (Vesali *et al.* 2015). This better correlation can be explained by higher rates.

The correlation coefficients for Chl *a* were similar to those observed by Yadav *et al.* (2010) for each index in the model RGB content in a study with potato. The possibility of using all the three indices RGB to estimate the Chl content has been demonstrated (Su *et al.* 2008). Vollmann *et al.* (2011) analyzed the state of nodulation and soybean Chl content with the use of SPAD and digital image analysis. These investigations of nondestructive methods using RGB image analysis are a relatively new area of research (Gupta *et al.* 2013).

The CIE L*a*b color model: Although it is not common to use the CIE L*a*b color system, a significant linear relationship was observed in all photosynthetic pigments with indices and L*, with ratios for each pigment of about 0.80 and 0.62, respectively. For the correlation with the index b*, the coefficient of determination was about 0.90 for each pigment (Fig. 3). These results were also observed by Wang *et al.* (2014) in rice, where the b* index had the highest correlation coefficients. In a study of soybean crops, the Chl content in seeds was also observed, and the CIE L*a*b model had excellent correlation with the Chl content, especially the a* index (Sinnecker *et al.* 2002). In the CIE L*a*b color model, the L* coordinates represent the brightness, while the dimensions a* and b* are close to red–green and yellow–blue, respectively (Fairchild 2005). Interestingly, the best results were with the index b*,

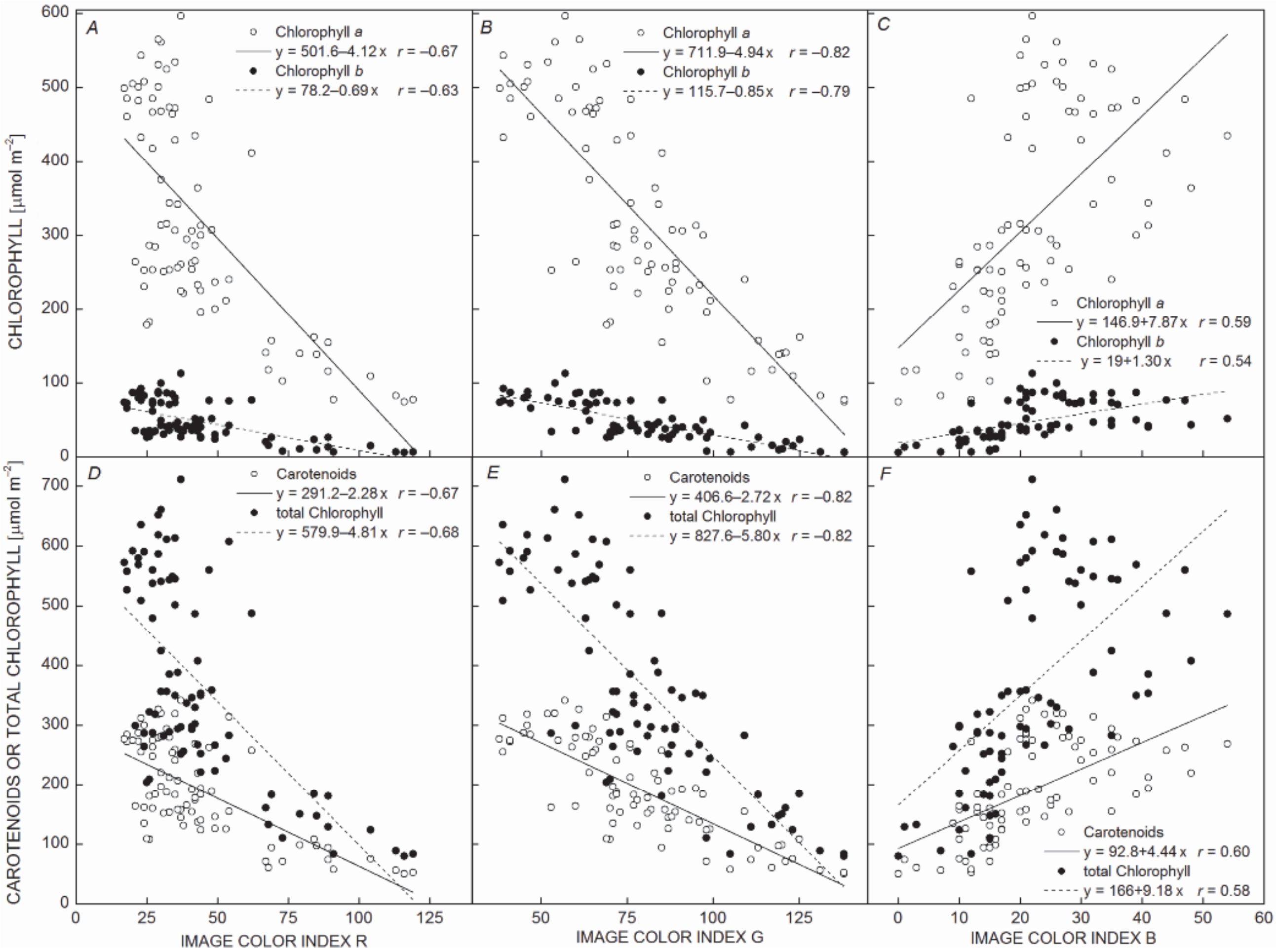

Fig. 2. Relationships between chlorophyll and carotenoids contents measured analytically in the laboratory and from an image based on the RGB color model with color indices R (*A,D*), G (*B,E*), and B (*C,F*) for soybean leaves.

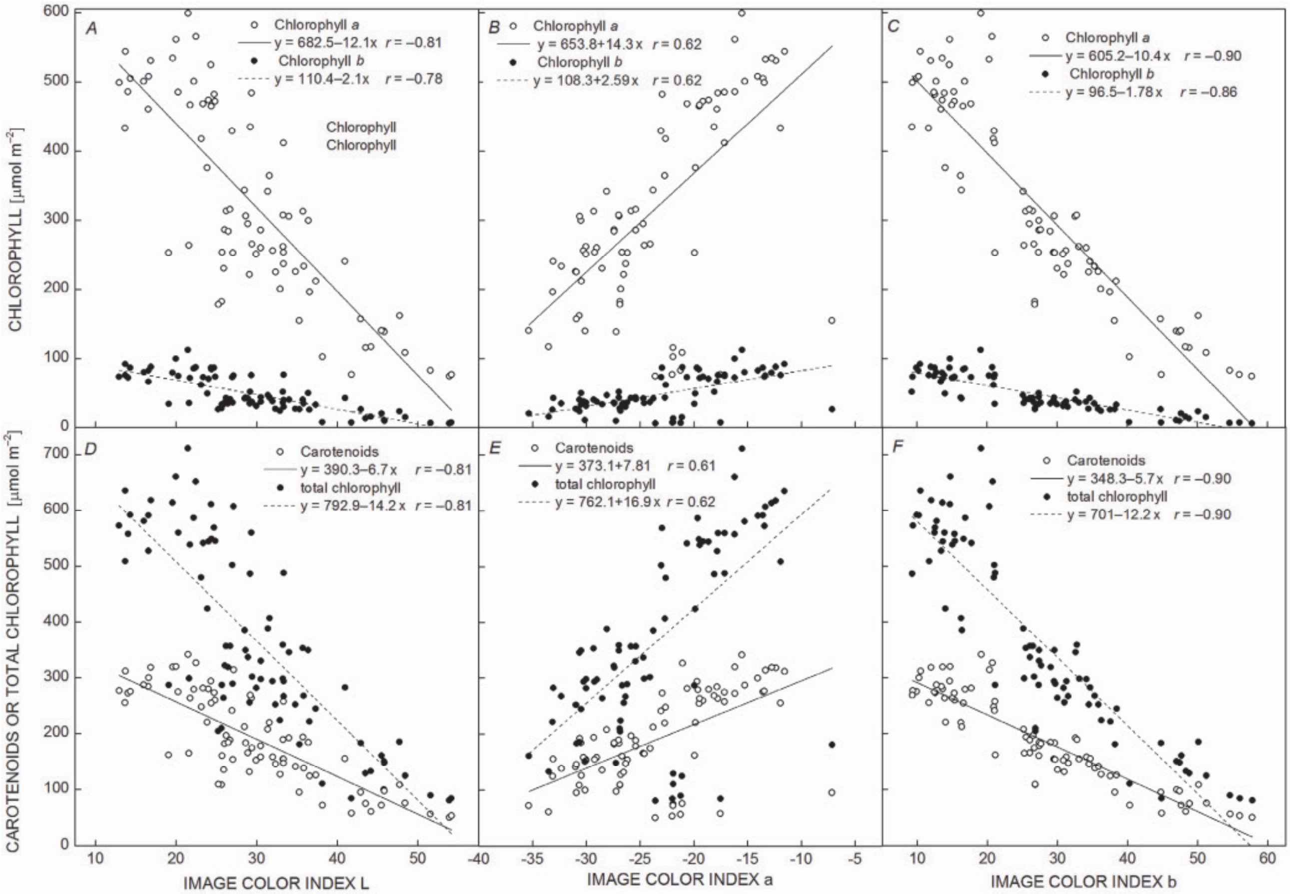

Fig. 3. Relationships between chlorophyll and Car content measured analytically in the lab and from an image based on the CIE L*a*b color model with indices L (*A,D*), a (*B,E*), and b (*C,F*) in soybean leaves.

which represents the yellow–blue color, whereas the Chls are determined by the leaf greening.

HSB color model: Karcher and Richardson (2003) observed that the green of vegetation does not appear or represent the green value in the RGB color space exactly. They suggested converting the RGB values to a more intuitive hue, saturation, and brightness (HSB) color spectrum, which is based on the human perception of color. The H index (Hue) that means the pure color (Fairchild 2005), and was the only positively correlated with Chl and Car, while S and B were negatively correlated with photosynthetic pigments (Fig. 4). A higher relationship in the S index was observed with a coefficient of determination of about 0.87. Although the color is the same during the day, the saturation changes. That means the light was better than the pure color from the image captured, and the B index (brightness) was higher than the H index (correlation coefficients of 0.81 and 0.61, respectively). Another study using a smart phone found a relationship between Hue (H index) and SPAD (0.76), but the other indices (S and B) also fitted well (Vesali *et al*. 2015), as observed by Wang *et al*. (2014). These variations can be explained by the different species studied and the influence of image accuracy, which is subject to climatic factors and illumination intensity apart from the influences of the sensors, photometric system, and processing system of the camera (Pagola *et al*. 2009, Wang *et al*. 2013).

DGCI, VI_{green}, and NRI indices: Figures were obtained using the described color models. There was a good linear relation (0.84) between DGCI and the Chl and Car contents (Fig. 5). Values near 1 represent a darker green. The advantage of this model is that the amount of red and blue can change the way of how often a green image appears in the RGB model (Karcher and Richardson 2003). DGCI has been among the most widely used indices to estimate the Chl content and SPAD index with high correlations ($r > 0.85$) in corn (Rorie *et al*. 2011) and grass (de Lima *et al*. 2012). The VI_{green} index is often used for images above the vegetation covering various plants. Perhaps for this reason, there was no significant correlation to the Chl content in this study. However, in a study of rice, Wang *et al*. (2014) observed correlations between VI_{green} index and both the SPAD and the content of nitrogen, but the coefficients observed were low (0.67) and not effective enough to be used. In this study, however, the NRI index satisfactorily correlated to the SPAD index (0.85), and the results were higher than those obtained by Wang *et al*. (2014).

Vesali *et al*. (2015) could not find good results for the relationship between SPAD and leaf N status in maize using this index with a smartphone camera. However, when using the hue index (HSB model), they had the strongest linear relationship with the SPAD value, although the SPAD value was analyzed rather than the Chl and Car content.

These differences between species may be related to the nonuniform distribution of Chl in leaves as an effect of the clustered structural organization of Chl molecules in chloroplasts, chloroplasts in cells, and cells in leaves (Fukshansky *et al*. 1993).

Overall, the results showed that the color index can be used to estimate the content of photosynthetic pigments, with the advantage of allowing rapid and repeated measures in the same leaves over time for ontogenetic studies. The results can be used to build applications to work with different color models efficiently using smart phones.

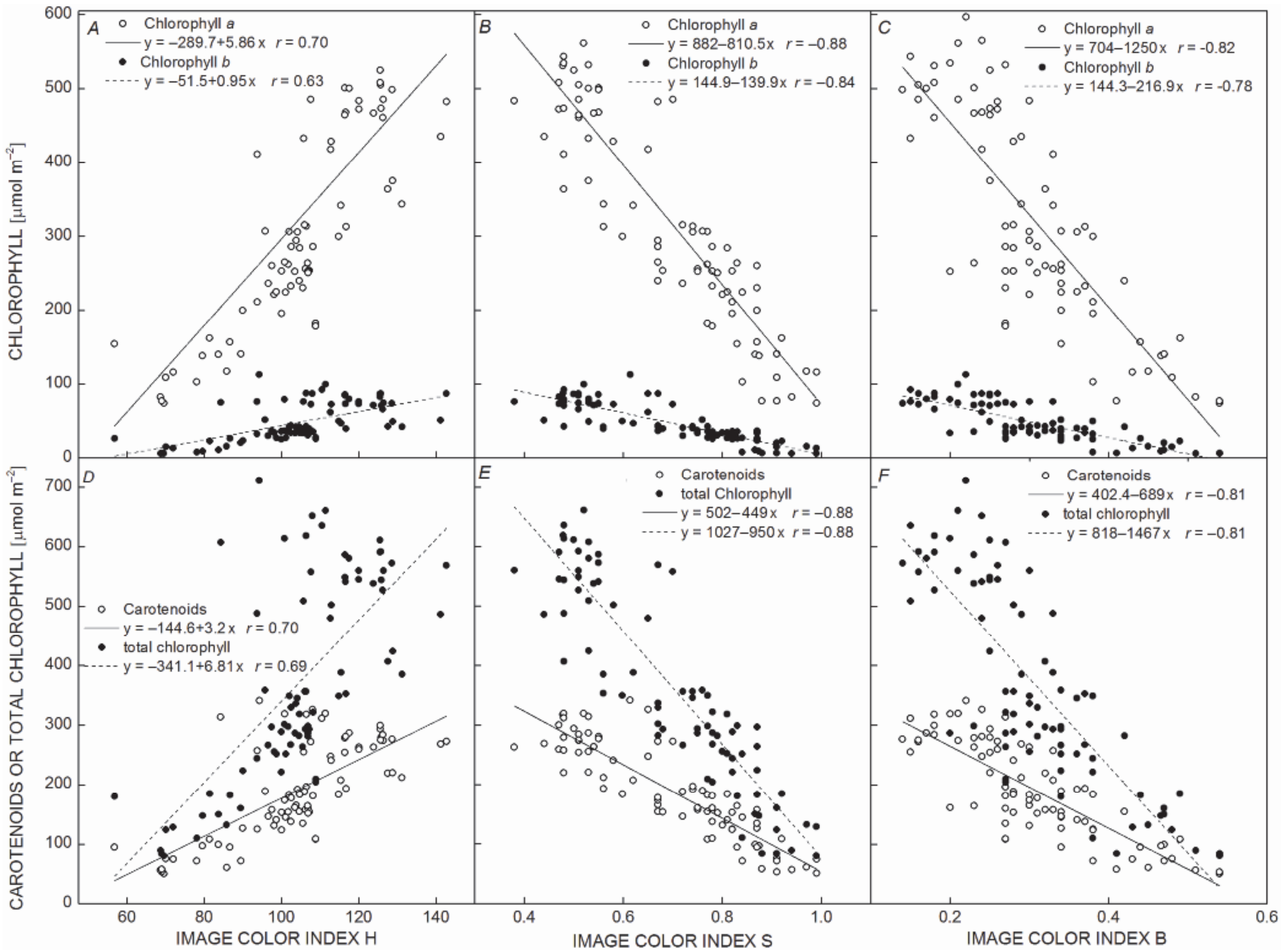

Fig. 4. Relationships between chlorophyll and carotenoids content measured analytically in lab and the image based in HSB component model with index H (*A,D*), S (*B,E*), and B (*C,F*) in the leaves of the soybean.

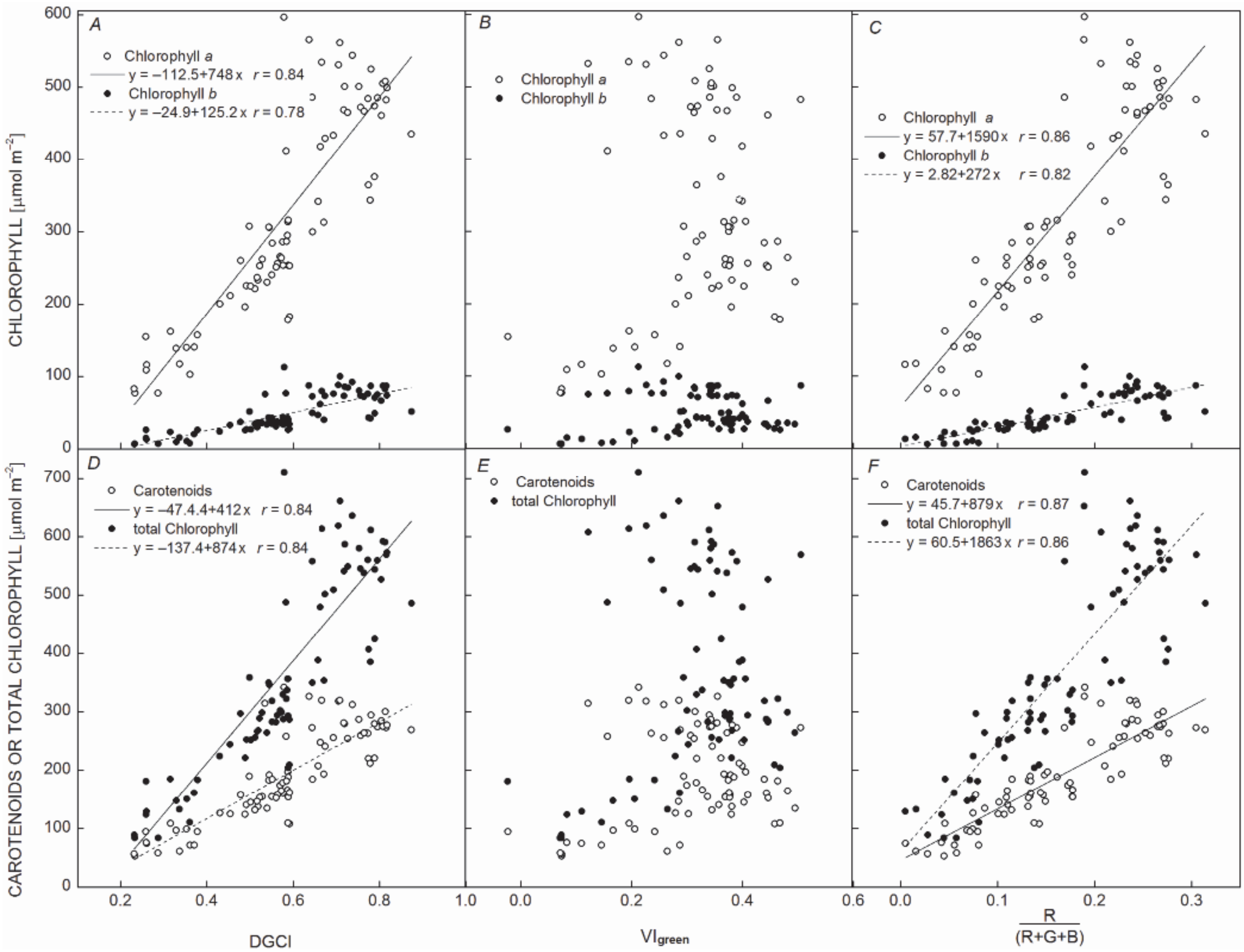

Fig. 5. Relationships between chlorophyll and carotenoids contents measured analytically in laboratory and the image based on DGCI index (*A,D*), VI_{green} (*B,E*), and NRI (*C,F*) in the leaves of the soybean.

Conclusion: The chlorophyll meter showed a proper reliability and may be used to estimate Chl and Car contents accurately, thus saving time and chemical reagents typically used in conventional procedures. The smart phone was accurate and sensitive with good representativeness, which was not diminished even in a broad range of Chl or Car contents in soybean leaves. Although there was variation between color models, some of the proposed methods achieved very close values to the Chl meter values, like the S and B index from the HSB and LAB models and NRI (RGB model). Using these models gave sufficient accuracy and robustness towards use for rapid and accurate estimation.

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