DOI: 10.4025/actasciagron.v33i4.10926

Non-destructive analysis of photosynthetic pigments in cotton plants

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ABSTRACT. Analytical techniques used to extract chlorophyll from plant leaves are destructive and based on the use of organic solvents. This study proposes a non-destructive quantification of the photosynthetic pigment concentration in cotton leaves using two portable chlorophyll meters, the SPAD-502 and the CLOROFILOG 1030. After obtaining 200 leaf discs, each with an area of 113 mm^2, the greening rate in each disc was determined by the average of five readings from both meters. Immediately after measurement, 5 mL of dimethyl sulfoxide (DMSO) was added, and the samples were kept in a water bath at 70ºC for 30 min. After cooling, 3 mL of the liquid extract was used for analyses by spectrophotometry at 470, 646 and 663 nm. Mathematical models were adjusted from analytical results using the reading index obtained from both devices to predict the contents of chlorophyll a, chlorophyll b, total chlorophyll and carotenoids. Based on these results, it was concluded that both portable chlorophyll meters are an effective way to estimate the concentration of photosynthetic pigments in cotton leaves, thus saving time, space and the resources that are often required for these analyses.

Keywords: *Gossypium hirsutum*, chlorophyll, carotenoids, cotton.

RESUMO. Análise não destrutiva dos pigmentos fotossintéticos em plantas de algodoeiro. Técnicas analíticas empregadas na extração de clorofila em plantas são destrutivas e fundamentam-se no uso de solventes orgânicos. Este estudo propõe a quantificação não destrutiva da concentração de pigmentos fotossintéticos em folhas de algodoeiro utilizando os medidores portáteis de clorofila SPAD-502 e CLOROFILOG 1030. Com as folhas coletadas foram elaborados 200 discos foliares com área de 113 mm^2. A determinação do índice de esverdeamento em cada disco foi realizada por meio da média de cinco leituras com ambos clorofilômetros portáteis e imediatamente após a determinação, adicionaram-se 5 mL de Dimetil sulfóxido (DMSO). Os discos foram mantidos em banho-maria a temperatura de 70ºC por um período de 30 min. Após o resfriamento do extrato líquido, uma alíquota de 3,0 mL foi utilizada para leitura utilizando espectrofotometria a 470, 646 e 663 nm. A partir dos resultados analíticos obtidos foram ajustados modelos matemáticos utilizando-se o índice das leituras efetuadas por ambos os equipamentos para estimar os teores de clorofila a, clorofila b, clorofila total e carotenóides. Considerando os resultados obtidos conclui-se que ambos medidores portáteis de clorofila poderão ser utilizados para estimar a concentração dos pigmentos fotossintéticos em folhas de algodoeiro, economizando tempo, espaço e recursos comumente demandados nessas análises.

Palavras-chave: *Gossypium hirsutum*, clorofila, carotenóides, algodão.

Introduction

There are important factors related to photosynthetic efficiency in plants, such as the concentration and composition of chloroplast pigments that affect plant growth and their adaptability to environments with different luminosities (DAI et al., 2009).

The production of dry matter by crop species and their ability for abiotic stress tolerance has been influenced by the amount of chlorophyll (Chl) present, due to the vital relationship of this pigment with the photosynthetic process (DAWSON et al., 2003; LIETH; WHITTAKER, 1975). Losses in chlorophyll content are associated with damaging environmental factors, such that variations in the total chlorophyll/carotenoids ratio are good indicators of plant injury (HENDRY; PRICE, 1993; KARA; MUJDECI, 2010).

The determination of the leaf chlorophyll content is a common procedure for plant scientists. Destructive techniques have been traditionally used for the determination of chlorophyll content in stands of vegetation. In

general, these techniques involve very laborious and destructive sampling plus various analytical protocols (LIETH; WHITTAKER, 1975; TUCKER, 1977). These methods use organic solvents that include acetone (BRUISNA, 1961; MAcKINNEY, 1941), dimethyl sulfoxide (DMSO) (HISCOX; ISRAELSTAM, 1979), methanol, N, N-dimethyl formamide and petroleum ether (INSKEEP; BLOOM, 1985; LICHTENTHALER; WELLBURN, 1983). During the extraction and dilution processes, significant pigment loss can take place, leading to a high variability in the results. Shoaf and Lium (1976) modified the extraction methodology using DMSO, thus eliminating the squashing and centrifuging stages. This method allowed for the extension of the storage period for the extracted pigment, so that spectrophotometric analyses need not be performed immediately after extraction.

Chlorophyll meters are extensively used in agriculture; they quickly estimate the chlorophyll content of leaves with a hand-held device that measures the leaf absorbance in two different wavelength regions using two light emitting diodes (LEDs). The chlorophyll meter Soil Plant Analysis Development (SPAD-502) is a simple and portable diagnostic tool that measures the greenness or the relative chlorophyll concentration of leaves (KARIYA et al., 1982; TORRES-NETTO et al., 2005). It provides instantaneous and non-destructive readings on plants based on the quantification of the intensity of absorbed light by the tissue sample using a red LED (wavelength peak is ~650 nm) as a source. An infrared LED, with a central wavelength emission of approximately 940 nm, acts simultaneously with the red LED to compensate for the leaf thickness (MINOLTA CAMERA Co. Ltd., 1989).

Another device used to estimate chlorophyll concentration is the Clorofilog 1030 chlorophyll meter, which has three LEDs working at 635, 660 and 850 nm, with the last wavelength used for the normalization of readings. This device might provide a substantial savings in time, space and resources. To determine the amount of chlorophyll in a sample, the mathematical relationship between the meter readings and the chlorophyll concentration in the tissue sample must be made.

However, to determine the chlorophyll concentration of a sample with a chlorophyll meter, the mathematical relationship between meter readings and the chlorophyll concentration in the tissue sample must be ascertained. The chlorophyll concentration, or leaf greenness, is affected by many factors. One such factor is the status of nitrogen (N) in the leaves (KARA; MUJDECI, 2010). A positive correlation between leaf N or the N fertilization rate and chlorophyll content has been well documented for a large number of plant species, and it has been investigated for a rapid determination of the N status using Chl meters in most major crops, including corn (*Zea mays* L.), rice (*Oryza sat*iva L.), wheat (*Triticum aestivum* L.) and cotton (*Gossypium hirsutum* L.), as well as numerous other plant species (BULLOCK; ANDERSON, 1998; CHANG; ROBISON, 2003; EVANS, 1989; LIN et al., 2010; MAUROMICALE et al., 2006; NAGESWARA RAO et al., 2001; NTAMATUNGIRO et al., 1999; PENG et al., 1993; REEVES et al., 1993; WU et al., 1998).

Carotenoids are integral constituents of the thylakoid membrane and are usually well associated with many of the proteins that constitute the photosynthetic apparatus (SIKUKU et al., 2010). They represent an important role in the light-harvesting complex, as well as in the photoprotection of the photosystems (PSs). Some reports show that these compounds are very important in the preservation of the photosynthetic apparatus against photodamage by their interconversion with xanthophyll molecules (ORT, 2001; YOUNG et al., 1997). In the xanthophyll cycle, violaxanthin undergoes a de-epoxidation to give rise to anteroxanthin and, finally, zeaxanthin (HAVAUX, 1988). Zeaxanthin participates in the regulation of the heat dissipation of PSII energy when this photosystem has an energetic overload (ORT, 2001). Therefore, an indirect and non-destructive quantification of the total content of carotenoids is of great importance for many related studies.

The main objectives of this study were the following: to assess the cotton chlorophyll composition and establish a possible correlation between the photosynthetic pigments extracted in DMSO with readings obtained by both chlorophyll meters; and to verify the relationship between these characteristics in the leaf tissue of Upland Cotton cultivated under field conditions.

Material and methods

Plant material and growth conditions

Cotton plant leaves (BRS 187 8H) were collected when the crop was at the height of the flowering period, characterized as the F4 cotton development stage (MARUR; RUANO, 2001; ROSOLEM, 2007). The experiment was carried out at the experimental station of the National Center of Cotton Research, located in Apodi, Rio Grande do

Norte State, Brazil (5º37'22" S; 37º48'58" W; 131 m of altitude). The region climate is characterized as warm tropical and semi-arid, with a predomination of BSw´h´ type (KÖPPEN; GEIGER, 1928). The soil is classified as Eutrophic Cambisol. The planting date was September 23, 2008. The chlorophyll extraction and greenness reading indexes were obtained from leaves harvested at the base of the petiole and placed in plastic zip-loc bags that were kept in the dark and cool until arrival at the laboratory. All samples were processed within approximately 2 hours after being gathered in the field.

Chlorophyll meter readings

Leaf disks were randomly sampled from leaves using a borer with a diameter of 12.0 mm. Five readings obtained by both portable chlorophyll meters (SPAD-502 by Minolta, Japan and CLOROFILOG 1030 by Falker, Brazil) on each disc from individual leaves were averaged. Approximately 200 leaf discs were used, and the values obtained by the meters varied from 4 to 60, making the maximum amplitudes between value readings.

Photosynthetic pigment analysis

After obtaining the meter readings, the chlorophyll was extracted from the leaf disks using the Hiscox and Israelstam (1979) procedure. Each disc was cut into smaller pieces and placed in a test tube containing 5 mL of dimethyl sulfoxide (DMSO). All samples were incubated at 70ºC for 30 min. (HISCOX; ISRAELSTAM, 1979) until all of the visible green pigmentation disappeared. After cooling, a 3-mL aliquot of the chlorophyll extract was transferred to a cuvette for the determination of the chlorophyll absorbance using a spectrophotometer at 470, 646 and 663 nm. Absorption measurements were used to quantify the chlorophyll a, chlorophyll b, and total chlorophyll concentrations, based on the equations reported by Wellburn (1994).

Data analyses

Analysis of variance (ANOVA) ($p < 0.05$) was applied to the data, and linear regression analyses were made. The mathematical equations were adjusted with a high coefficient of determination. The readings for the greenness indexes were used as the dependent variable, while the pigment concentrations extracted by the classical method were used as the independent variable. Data analyses were conducted using SigmaPlot 10.0 software in order to fit the suitable mathematical equations to all of the analyzed variables. A Pearson correlation analysis between the two portable chlorophyll meters readings was conducted.

Results and discussion

Despite the fact that the two portable meters provided different values for the chlorophyll measurements, we observed a high correlation between their data (Figure 1). The Clorofilog 1030 (Falker Agricultural Automation) chlorophyll meter showed higher values than the SPAD-502 (MINOLTA CAMERA Co. Ltd., 1989) meter. It was observed that this difference was higher in leaves that presented less chlorophyll content spectrophotometrically, namely, with the lower readings with the devices. Consequently, it was necessary to perform distinct adjustments in the mathematical models for the prediction of chlorophyll and carotenoid content by each portable meter.

We suggest that it is likely that such differences occurred because these two devices operate in different wavelength ranges. According to Markwell et al. (1995), the chlorophyll meter developed by Minolta uses two LEDs in the bands of 650 and 940 nm and a photodiode detector to measure sequentially the transmittance of red and infrared light through the leaves. In contrast, the Clorofilog 1030 functions with LEDs in wavelengths of 635, 660 and 880 nm.

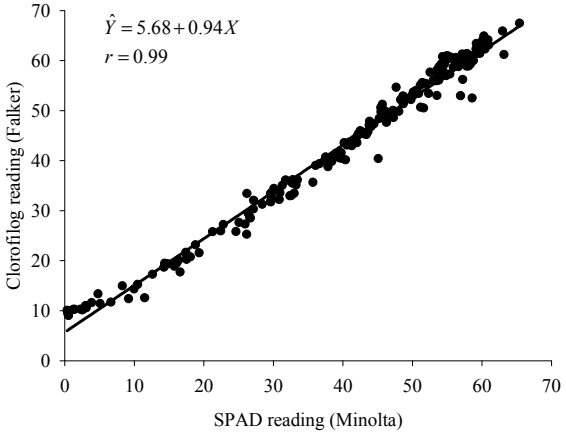

Figure 1. The relationship between SPAD-502 readings (MINOLTA CAMERA Co. Ltd., 1989) and Clorofilog 1030 readings (Falker Automation) in *Gossypim hirsutum* leaves.

Figures 2 and 3 show the relationships between the readings obtained in cotton leaves by the two chlorophyll meters and the concentrations of chlorophylls a and b, respectively. The relationship between the chlorophyll readings from both portable meters and the contents of chlorophyll a

and chlorophyll b was more readily expressed with a quadratic model. Determination coefficients of the adjusted models were 0.90 and 0.91 for chlorophyll a and 0.82 and 0.80 for chlorophyll b using the SPAD 502 and the Clorofilog 1030 meters, respectively.

The relationship between the chlorophyll readings in both portable meters and the concentrations of carotenoids were fit in a quadratic model, and an R^2 value of 0.79 was obtained for both meters (Figures 4a and 5a). The relationship between the readings and total chlorophyll are presented in Figures 4b and 5b, and a high R^2 value was obtained. The coefficient of determination for the adjusted models of total chlorophyll content was 0.91 for both of the chlorophyll meters.

The relationship between the photosynthetic pigment concentration and the chlorophyll readings have been established for several species of plants, such as the total chlorophyll in *Glycine max* and *Zea mays* (MARKWELL et al., 1995) and Chl a, b, total Chl and carotenoids in *Carica papaya* L. (TORRES NETO et al., 2002) and *Coffea canephora* Pierre (TORRES NETO et al., 2005). The relationship between the chlorophyll meter readings obtained by the devices and the concentrations of photosynthetic pigments was adequately represented for cotton by the quadratic mathematical model, suggesting that this species has a similar relationship as the leaves of wheat, rice, soybean (MONJE; BUGBEE, 1992) and coffee (TORRES NETO et al., 2005). In some species, the linear and exponential models have also been adjusted to express these relationships (TORRES NETO et al., 2002).

Figures 6a and 7a show the relationship between total chlorophyll/carotenoids and the chlorophyll meter readings obtained by the SPAD 502 and Clorofilog 1030 devices, respectively. The readings obtained by both chlorophyll meters allowed for the estimation of these relationships between chlorophyll and carotenoids via an indirect, though highly precise method. The quadratic mathematical model provided a better representation, with determination coefficients of 0.91 and 0.92 for the SPAD-502 and Clorofilog 1030 meters, respectively.

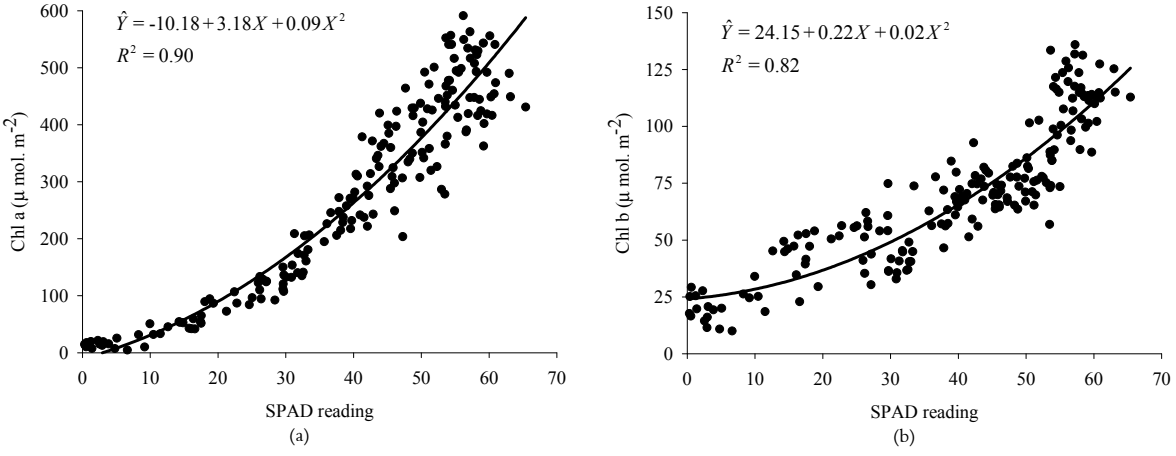

Figure 2. The relationships between SPAD-502 readings, chlorophyll *a* (Chl a) (a) and chlorophyll *b* (Chl b) (b) in *Gossypim hirsutum* leaves.

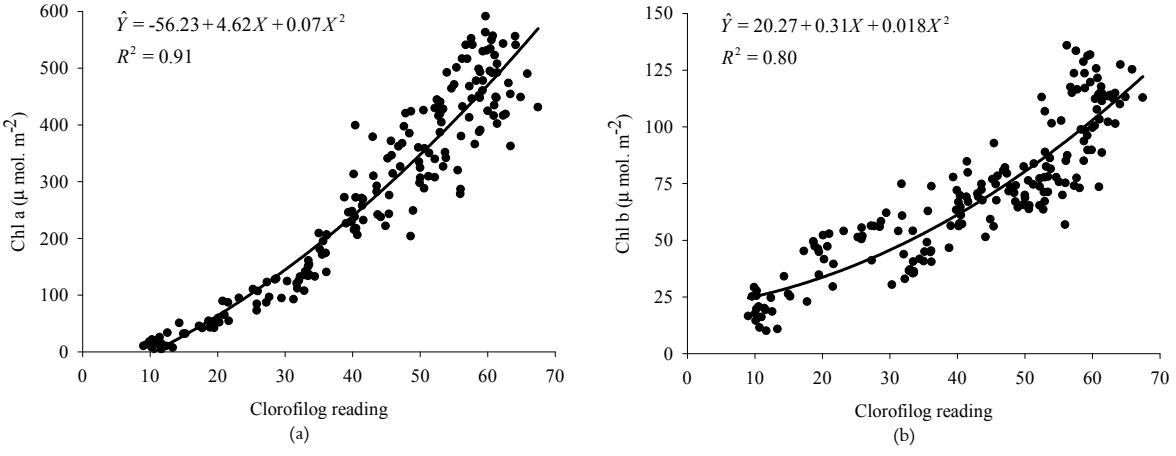

Figure 3. The relationships between Clorofilog 1030 readings, chlorophyll *a* (c) and chlorophyll *b* (Chl b) in *Gossypim hirsutum* leaves.

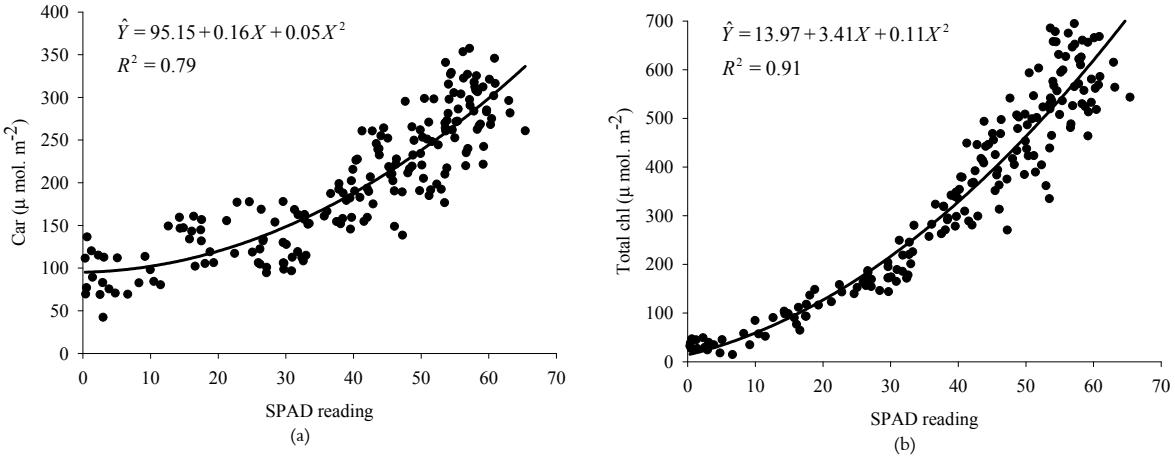

Figure 4. The relationships between SPAD-502 readings, carotenoid (Car) concentration (a) and total chlorophyll (total Chl) (b) in *Gossypim hirsutum* leaves.

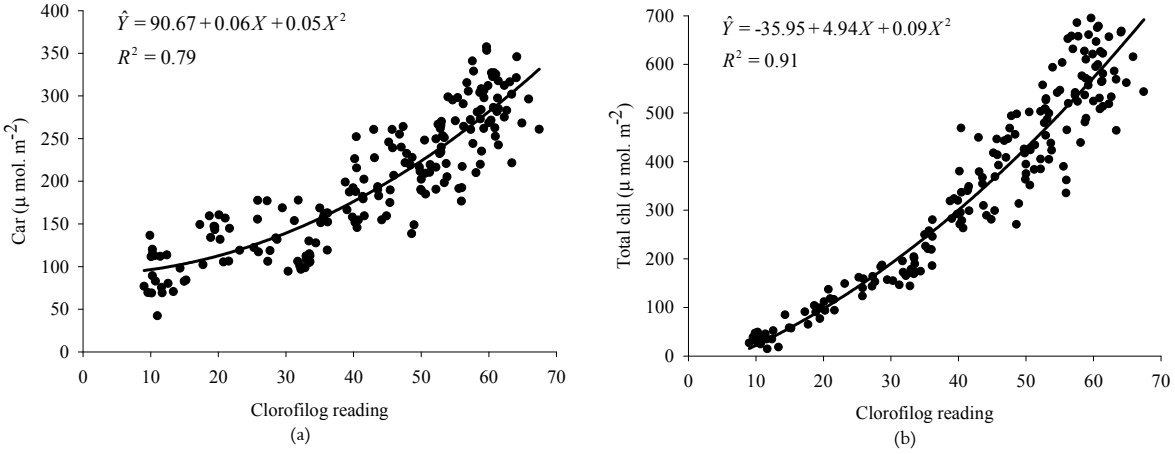

Figure 5. The relationships between Clorofilog 1030 readings, carotenoid (Car) concentration (a) and total chlorophyll (total Chl) (b) in *Gossypim hirsutum* leaves.

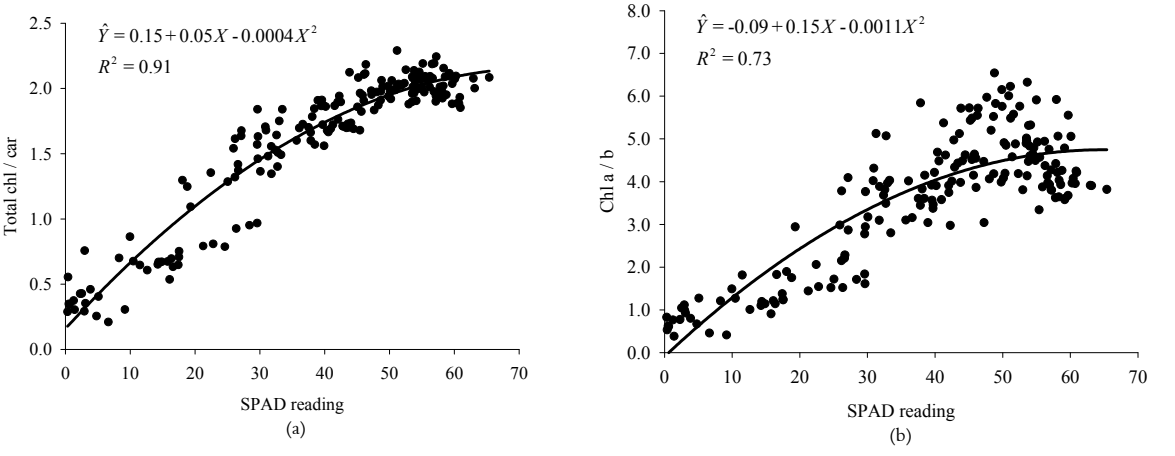

Figure 6. The relationships between SPAD- 502 readings, the total Chl/Car ratio (a) and the Chl *a/b* ratio (b) in *Gossypim hirsutum* leaves.

Plants with SPAD readings lower than 40 presented reduction of the relationship total chlorophyll/carotenoids. The same effect was also observed when a SPAD 502, with values lower than 40, was used for leaves of *Carica papaya* L. (TORRES NETO et al., 2002) and *Coffea canephora* Pierre (TORRES NETO et al., 2005). This may be due to the onset of leaf senescence (BUCKLAND et al.,

1991). The relationship between chlorophyll and carotenoids has been much less used, although this ratio may be considered a good indicator to distinguish between natural senescence and senescence as a result of environmental injuries, such as desiccation in mosses (BUCKLAND et al., 1991) and the occurrence of water deficit in plants in bloom (SEEL et al., 1992; SIKUKU et al., 2010).

Measurements of less than 40 indicate the beginning of a possible reduction in photosynthetic processes. This effect was also observed by Torres Neto et al. (2005) in coffee plants. Additionally, this relationship has been considered a good indicator of disturbances in plants that have been caused by environmental factors (HENDRY; PRICE, 1993).

Figures 6b and 7b show the relationship between chlorophyll a/b and the chlorophyll meter readings from the SPAD 502 and Clorofilog 1030 meters, respectively. Similar to the other characteristics analyzed, the quadratic mathematical model best fit the data, exhibiting determination coefficients above 0.73 and 0.76 for the SPAD and Clorofilog meters, respectively. Analogous with the behavior of the total chlorophyll/carotenoids ratio, a dramatic reduction in values was observed when readings were below 40 for the chlorophyll a/b ratio.

Chlorophyll a is more strongly degraded than chlorophyll b (WOLF, 1956), which may explain the reduction of the chlorophyll a/b ratio when the chlorophyll meter readings were below 40 (Figure 2b). These low readings may occur in shaded leaves; because the total chlorophyll content per unit of leaf area is lower in leaves that are exposed to high irradiance, while the ratio between chlorophyll a/b is larger, when compared with leaves grown under shaded conditions. The effect of the photon flux density on the chlorophyll a/b ratio is one of the most striking features between plants growing under sunny or shady conditions (ANDERSON, 1986; BOARDMAN, 1977). Because of this response to different light intensities, the chlorophyll a/b ratio has been proposed as a bioassay to analyze the irradiance level to which a plant was subjected (DALE; CAUSTON, 1992). In fact, the chlorophyll content and the chlorophyll a/b ratio are responsive to changes within the mesophyll of individual leaves (CUI et al., 1991; TERASHIMA et al., 1986).

The chloroplasts of leaves grown in the shade develop a higher proportion of thylakoids compared to the volume of the stroma, with both larger thylakoids and more thylakoids per granum (ANDERSON, 1986; BOARDMAN, 1977). While the relative proportion of chlorophyll associated with the compound complex of Photosystem I and the reaction center of Photosystem II decreases with a reduction in the a/b ratio, the relative proportion of chlorophyll associated with the a/b light-collecting protein complex increases (LEONG; ANDERSON, 1984). The complex light collector (LHC2) has a lower chlorophyll a/b ratio than the other proteins linked to the chlorophyll molecules associated with Photosystem II, because LHC2 contains most of the chlorophyll b (GREEN; DURNFORD, 1996). Therefore, the chlorophyll a/b ratio may be useful as an indicator of the chloroplast composition. Some authors have used this ratio as an indicator of leaf N partitioning, based on the positive relationship between chlorophyll a/b and the rate of light collected by the chlorophyll-protein complex of Photosystem II (KITAJIMA; HOGAN, 2003; TERASHIMA et al. 1986).

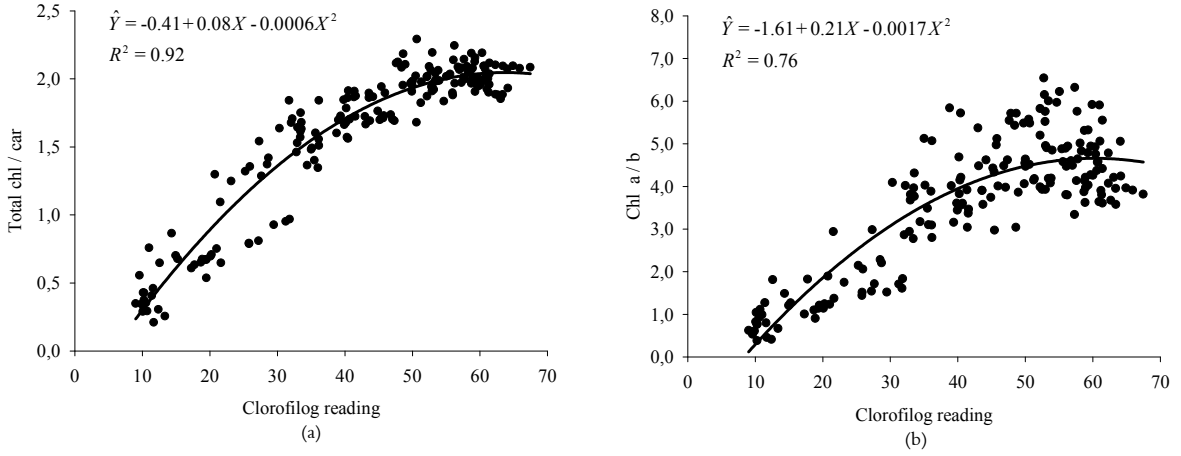

Figure 7. The relationships between Clorofilog 1030 readings, the total Chl/Car ratios (a) and Chl *a/b* ratios (b) in *Gossypim hirsutum* leaves.

Conclusion

In general, it was observed that the use of the portable chlorophyll meters, SPAD-502 and 1030 Clorofilog, produced results associated with empirical models and allowed for a quick prediction of the concentration of photosynthetic pigments in the leaves of cotton, with high accuracy and without the use of chemical reagents and extensive laboratory protocols.

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Received on August 18, 2010.
Accepted on November 5, 2010.