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Non-destructive determination of maize leaf and canopy chlorophyll content

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Model;
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Summary

The objective of this study was to develop a rapid non-destructive technique to estimate total chlorophyll (Chl) content in a maize canopy using Chl content in a single leaf. The approach was (1) to calibrate and validate a reflectance-based non-destructive technique to estimate leaf Chl in maize; (2) to quantify the relative contribution of each leaf Chl to the total Chl in the canopy; and (3) to establish a relationship between leaf Chl content and total Chl in a maize canopy. The Red Edge Chlorophyll Index $CI_{red\ edge} = (R_{NIR}/R_{red\ edge})-1$ based on reflectances, R, in the red edge (720–730 nm) and near infrared (770–800 nm) was found to be an accurate measure of maize leaf Chl. It was able to predict leaf Chl ranging from 10 to 805 mg Chl m^{-2} with root mean-square error less than 38 mg Chl m^{-2}. Relationships between Chl content in each maize leaf and total canopy Chl content were established and showed that Chl in the collar leaf before silking or ear leaves explained more than 80% and 87% of the variation in total Chl in a maize canopy, respectively. Thus, non-destructive measurements of both reflectance and area of a single leaf (either collar or ear) can be used to accurately estimate total Chl content in a maize canopy.

Introduction

The production of dry matter by pasture and crop species has been demonstrated to be ultimately limited by the amount of chlorophyll (Chl) due to the strong relationship of this pigment with the

Abbreviations: Chl, chlorophyll; CI, chlorophyll index; RMSE, root mean-squared error.

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photosynthetic processes (Sprague and Curtis, 1933; Brougham, 1960; Lieth and Whittaker, 1975; Dawson et al., 2003). Irrespective of the efficiency with which the various tissues and organs of the plant may function, a deficient supply of Chl or its inefficient operation limits plant growth (Sprague and Curtis, 1933). In turn, canopy biophysical parameters such as N content (Evans, 1989), above-ground biomass, green leaf area index, net ecosystem CO_2 exchange (Lieth and Whittaker, 1975; Gitelson et al., 2006a), absorbed photosynthetic active radiation (Viña and Gitelson, 2005), and yield (Walters, 2003) have been related to canopy Chl content. Chl content has been suggested as the community property most directly relevant to the prediction of productivity (Lieth and Whittaker, 1975; Dawson et al., 2003). Foyer et al. (1982) further affirmed that "...all quantitative means for expressing photosynthetic rate in current use (for example, ground area, fresh weight) carry inescapable disadvantages. Chl is likely to remain the universal basis for expressing photosynthetic rate."

Destructive techniques have been traditionally used for the determination of Chl content in vegetation stands. In general, they involve very laborious and destructive sampling plus various analytical protocols (e.g., Brougham, 1960; Lieth and Whittaker, 1975; Tucker, 1977). These techniques implicitly assume (1) a homogeneous contribution of Chl from the different canopy components, (2) a linear and consistent relationship between Chl content in the sample and total Chl in the canopy, or (3) both. However, current knowledge does not provide quantitative and precise descriptions of the distribution of Chl in a canopy for different vegetation stands. In addition, there are no reported relationships either among or between different canopy components and total Chl content of a canopy. On the contrary, it is well known that the distribution of Chl among leaves ultimately depends on the canopy acclimation to light penetration (e.g., Kull, 2002), characteristics of each canopy species, and the environment. Further, the distribution of Chl within a canopy can vary considerably as a function of time and space, making the estimation of canopy Chl content through destructive sampling a labor-intensive and expensive process (e.g., Coops et al., 2003).

The distribution of Chl within maize leaves is, in general, quite homogeneous at a specific growth stage. However, either biotic or abiotic factors can induce stress in a plant affecting specific processes on individual leaves resulting in both a loss of Chl and a change in its distribution pattern (Barton, 2000). Consequently, methods are required for accurate, non-destructive, and simple estimates of Chl content at canopy scales, rather than for individual leaves (Curran et al., 1990). These methods should improve the accuracy of Chl estimation by taking into account the variability in Chl content *within* and *among* leaves in the canopy.

The use of portable Chl meters (e.g., Minolta SPAD) has been proposed as a non-destructive technique to estimate Chl content by means of absorbance/transmittance measurements (e.g., Piekielek and Fox, 1992; Markwell et al., 1995). Richardson et al. (2002) evaluated the performance of optical methods that are based on the absorbance/transmittance and reflectance of certain wavelengths of light by intact leaves. They concluded that non-invasive optical methods all provided reliable estimates of leaf Chl. However, some reflectance indices (Gitelson and Merzlyak, 1994) consistently out-performed two commercially available hand-held Chl absorbance meters CCM-200 and the SPAD-502. Steele et al. (2008) further showed that the SPAD-502 has adequate sensitivity to Chl content below 300 mg m^{-2}. Above that level, however, the accuracy of the instrument considerably diminished. This decrease in sensitivity takes place in the range of Chl that is typical for green vegetation, which prevents using SPAD for accurate measurement of Chl in healthy vegetation and indication of early (pre-visual) stages of plant stress.

Non-destructive techniques based on leaf reflectance have been proposed as alternative, robust, and simple methods for pigment quantification in leaves (Collins, 1978; Curran and Milton, 1983; Buschmann and Nagel, 1993; Gitelson and Merzlyak, 1994, 1996; Richardson et al., 2002; Sims and Gamon, 2002; Gitelson et al., 2003; Hu et al., 2004; Le Maire et al., 2004) and in canopies (e.g., Barton, 2000; Gitelson et al., 2005). However, an important uncertainty remains when Chl content values for individual leaves are used to represent the Chl content in the canopy. Gitelson et al. (2005) estimated total Chl in maize canopies during the growing season as $Chl = Chl_{upper} \times$ green LAI, where Chl_{upper} is the Chl content of the upper leaf and green LAI is the green leaf area index of the canopy. This approach markedly improved current techniques proposed for Chl quantification in the canopy. However, the major assumption of this approach – Chl content of the uppermost expanded leaf represents the Chl content of the plant – was not proved in the cited paper.

There is still a lack of accurate, rapid, and practical methodologies available to quantify Chl content in the canopy per unit of ground area.

The general objective of this study is to find a way to accurately and quantitatively characterize canopy Chl content using Chl content in a single leaf. Specific objectives were (1) to calibrate and validate a reflectance-based non-destructive technique (Gitelson and Merzlyak, 1994; Gitelson et al., 2003, 2006b) to estimate leaf Chl in maize; (2) to quantify the relative contribution of each leaf Chl to the total Chl in the canopy; and (3) to establish a relationship between leaf Chl content and total Chl in a maize canopy.

Materials and methods

This study took advantage of an established research facility, which is part of the Carbon Sequestration Program at the University of Nebraska-Lincoln. The research facility consists of three agricultural fields of approximately 65 ha each, located in the vicinity of Lat. 41.175N, Long. 96.425W. The cropping system was established in 2001 and differs among the three fields: field 1 is under continuous sprinkler-irrigated maize; field 2 is a sprinkler-irrigated maize–soybean rotation; and field 3 is a rain-fed maize–soybean rotation. The study took place in the 2004 and 2005 growing seasons. In 2004, field 1 was planted with maize hybrid Pioneer brand 33B51. In 2005, fields 1 and 2 were planted with maize hybrids Dekalb 6375 (D-6375) and Pioneer brand 33B51 (P-33B51), respectively, and field 3 was planted with maize hybrid Pioneer brand 31G68 (P-31G68).

Sampling and labeling procedures

Three plants from each field were sampled weekly or biweekly during the reproductive period after tasseling of the 2004 growing season and during the entire 2005 growing season: from the early vegetative growth stage beginning with the third leaf developed through late reproductive stages.

A total of 26 plants in 2004 and 128 in 2005 were sampled resulting in approximately 300 and 2000 leaves measured in the first and second years, respectively. Once the plants were selected, the position of the collar or ear leaf was identified. The collar leaf was defined as the uppermost leaf whose leaf collar is visible (Ritchie et al., 1992), while the ear leaf was defined as the leaf next to the maize ear. Positions of the other leaves on each plant were numerically labeled with respect to the collar or the ear leaf position during vegetative or reproductive stages, respectively. The position of the collar or ear leaf was labeled as leaf position 0. The leaves above or below leaf 0, were identified with a "+" or a "−" sign, respectively, followed by the corresponding position number. For example, the first leaf *above* the ear/collar leaf was identified as +1, the second one as +2, the third one +3, etc., up to the top leaf. In contrast, the first leaf *below* the ear/collar leaf was identified as −1, the second as −2, the third one as −3 until the closest leaf to the ground was reached. After labeling, the leaves were cut from the stem, placed in a sealed plastic bag, and brought to the laboratory inside a cooler.

Non-destructive estimation of leaf chlorophyll content

Leaf Chl content was measured using a recently developed technique based on models that relate leaf reflectance with pigment content (Gitelson et al., 2003). One of the models, so-called Red Edge Chlorophyll Index, $CI_{red\ edge}$, was suggested for Chl determination in both anthocyanin-containing and anthocyanin-free leaves (Gitelson et al., 2006b). $CI_{red\ edge}$ was tested in this study; it is based on reflectances in the red edge ($R_{red\ edge}$) and near infrared (R_{NIR}) wavebands and defined as:

$$CI_{red\ edge} = (R_{NIR}/R_{red\ edge}) - 1 \quad (1)$$

where R_{NIR} is average reflectance in the range from 770 to 800 nm and $R_{red\ edge}$ is the average reflectance in the range from 720 to 730 nm.

Once during the growing season, maize leaves within a wide range of greenness were collected from the crop fields in 2004 (20 leaves) and 2005 (61 leaves). Reflectance of each leaf was measured in the spectral range from 400 to 900 nm using a leaf clip, with a 2.3-mm-diameter bifurcated fiber-optic cable attached to both an Ocean Optics USB2000 spectroradiometer and to an Ocean Optics LS-1 tungsten halogen light source. The leaf clip allows individual leaves to be held with a 60° angle relative to the bifurcated fiber-optic. The software CDAP (CALMIT, University of Nebraska-Lincoln Data Management Program) was used to acquire and process the data from the sensor. A Spectralon reflectance standard (99% reflectance) was scanned before each leaf measurement. The reflectance at each wavelength was calculated as the ratio of upwelling leaf radiance to the upwelling radiance of the standard. The average reflectance obtained from 10 scans was used to compute the $CI_{red\ edge}$ defined in Eq. (1). Once these measurements were completed, two to four circular disks (1 cm diameter) were punched from each leaf for analytical extraction of Chl and quantification using absorption spectroscopy. The extraction of Chl was done using 10 mL of 80% acetone. The extinction absorption coefficients published by Porra et al. (1989) were used for final calculations of total Chl content.

For establishing a relationship between chlorophyll index $CI_{red\ edge}$ and Chl content, the dataset collected in 2005 was used. A linear relationship between $CI_{red\ edge}$ and Chl was established in the form

$$Chl\ (mg\,m^{-2}) = a \times CI_{red\ edge} + b \quad (2)$$

Validation of the technique was performed on an independent dataset of 20 leaves collected in 2004. Reflectance and Chl content (Chl_{meas}) of these leaves were measured using the procedures described above. Calibration Eq. (2) was used to predict Chl in leaves (Chl_{pred}) of this dataset. The accuracy of Chl prediction was quantified by root mean-square error (RMSE) of Chl_{pred}.

Estimation of chlorophyll content in canopy

In the laboratory, each leaf of the canopy was visually examined to identify and separate sections that were different in color. Leaf sections were marked, labeled, and cut for further measurements. Ten reflectance scans were recorded from each leaf or leaf section with different colors. In the case of a leaf that was considered homogeneous in color, ten randomly distributed scans were made along the leaf margin (both sides of midrib). However, in the case of a leaf with a heterogeneous distribution of color, sections that appeared homogeneous in color were treated independently and ten randomly distributed scans were taken on each such leaf section.

The mean of the reflectance obtained from each set of ten scans was used to compute the $CI_{red\ edge}$ defined in Eq. (1). Then, Chl content (in $mg\,m^{-2}$) of each leaf (Chl_{leaf}) or leaf section (Chl_{sect}) was estimated using Eq. (2).

Once the reflectance measurements were completed, the area of each leaf, S_{leaf}, or the area of each leaf section, S_{sect} (in the case of heterogeneous leaves) was measured with a leaf area meter (Model LI-3100A, Li-Cor, Inc., Lincoln, NE). Total weight of $Chl_{wt/leaf}$ (in g) in individual leaves was calculated as a product of leaf area S_{leaf} (in m^2) and its Chl content (Chl_{leaf} in $mg\,m^{-2}$). In the case of leaves with "m" sections (i.e., with "m" areas of different "greenness"), the sum of the products of each section area (in m^2) and each section Chl content (Chl_{sect}, in $g\,m^{-2}$) resulted in the amount of Chl of the entire leaf ($Chl_{wt/leaf}$). This was calculated using following equation:

$$Chl_{wt/leaf}\,(g) = \sum_{i=1}^{m} Chl^{i}_{sect} \times S^{i}_{sect} \quad (3)$$

Total amount of Chl in the canopy (Chl_{canopy}), expressed as the amount of Chl per unit of ground area (i.e., $g\,Chl\,m^{-2}$ ground), was calculated as the sum of Chl of individual leaves ($Chl_{wt/leaf}$) of each plant normalized to ground area, S_{ground}:

$$Chl_{canopy} = \sum_{i=1}^{n} (Chl_{wt/leaf})_i / S_{ground} \quad (4)$$

where n is number of leaves in each plant, $Chl_{wt/leaf}$ is chlorophyll (in g) of each leaf, calculated from Eq. (3), and S_{ground} (in m^2) was calculated as a product of the average distance between plants in the row and the distance between rows. The relationship between leaf Chl and canopy Chl defined in Eq. (4) was established using data collected in 2005 ($n = 128$) and validated with an independent dataset collected in 2004 ($n = 26$).

Results and discussion

Non-destructive leaf Chl estimation

Chl content determined analytically in the dataset consisting of 61 maize leaves acquired in 2005 varied widely from 22 to 886 $mg\,Chl\,m^{-2}$. The relationship between analytical Chl and the reflectance-based $CI_{red\ edge}$ obtained for these leaves was described by a linear best-fit function with a coefficient of determination of $r^2 > 0.94$ and RMSE of less than 51 $mg\,Chl\,m^{-2}$ (Figure 1):

$$Chl\,(mg\,m^{-2}) = 37.904 + 1353.7 \times CI_{red\ edge} \quad (5)$$

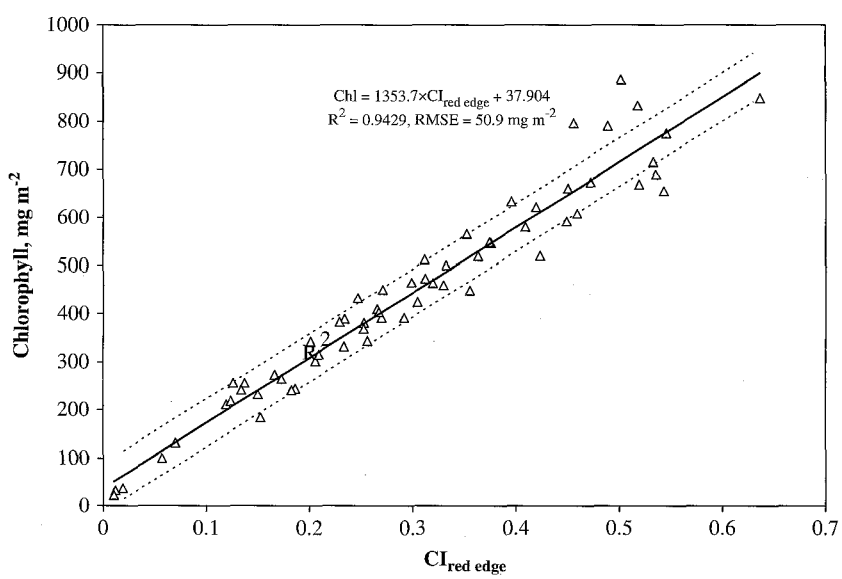

Figure 1. Relationship between Chl content in leaves and Red Edge Chlorophyll Index $CI_{red\ edge} = (R_{NIR}/R_{red\ edge}) - 1$ for the 2005 dataset. This relationship was used for calibration of the non-destructive determination of Chl from leaf reflectance. Solid line is the best-fit function; dotted lines correspond to one standard error of chlorophyll estimation. RMSE is root mean-square error of leaf Chl estimation.

The algorithm (Eq. (5)) was validated by an independent dataset of 20 maize leaves taken in 2004. Predicted Chl content (Chl_{pred}) was closely linearly related to Chl content measured analytically (Chl_{meas}) with RMSE $<38\,mg\,Chl\,m^{-2}$ and coefficient of variation (CV) less than 10.3% (Figure 2):

$$Chl_{pred} = 16.53 + 0.89 \times Chl_{meas} \quad (6)$$

Total Chl in canopy and its relation to leaf Chl

Total Chl in canopy increased during the vegetative growth period, reaching a maximum close to tasseling (VT) and then decreased during reproductive and senescence periods (Figure 3). Hybrid P-31G68, grown under rain-fed conditions showed lower values of total Chl content through the entire growing season. However, the three hybrids followed the same pattern of Chl changes over time.

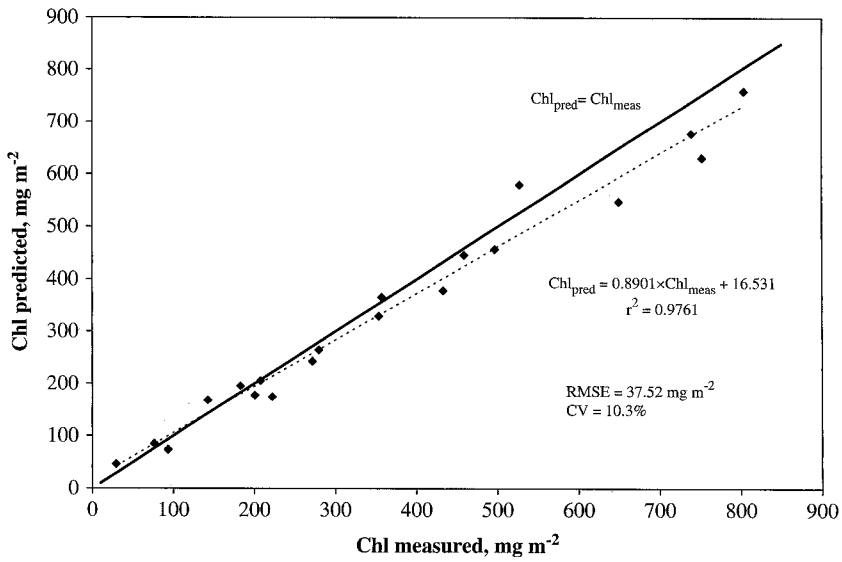

Figure 2. Chlorophyll predicted by the Red Edge Chlorophyll Index plotted vs. measured analytically. Solid line is $Chl_{pred} = Chl_{meas}$; dotted line is best-fit function Chl_{pred} vs. Chl_{meas}. RMSE is root mean-square error of leaf Chl prediction.

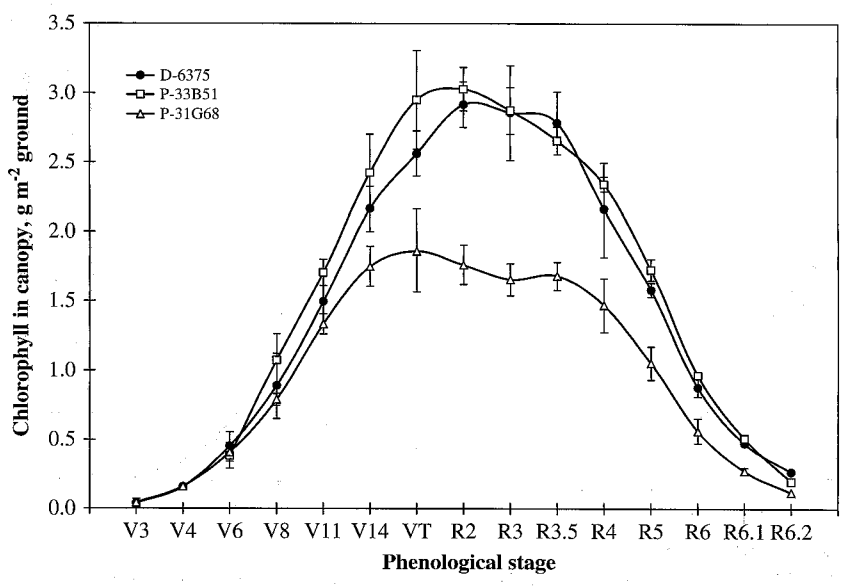

Figure 3. Total chlorophyll content in canopy (per ground area) of three maize hybrids during the growing season. Each point represents the average chlorophyll content in three plants and the vertical bars represent the standard error.

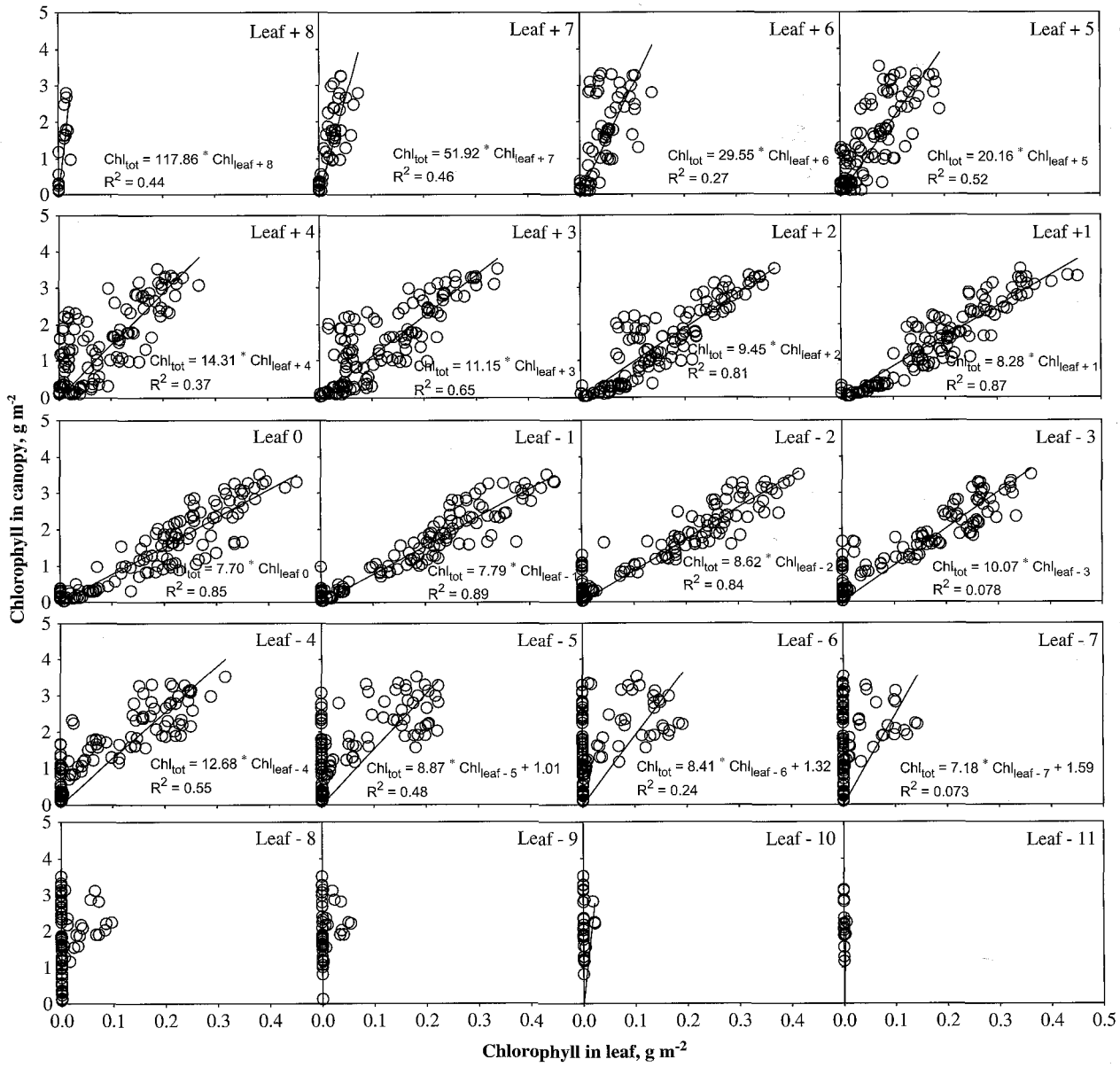

Figure 4. Relationships between chlorophyll content in canopy and chlorophyll content in individual leaves (both calculated per ground area). Data from three hybrids (D-6375, P-31G68, and P-33B51) are pooled together. Leaf in position 0, 0 leaf, corresponds to the collar or ear leaf during vegetative and reproductive periods, respectively. Positive and negative numbered leaves correspond to leaves positioned above or below 0 leaf, respectively.

The relationships between Chl content in individual leaves (Chl_{leaf}) located at different plant positions and total Chl content in the canopy (Chl_{canopy}) are shown in Figure 4. Leaf in position 0 corresponds to the collar or ear leaf during vegetative and reproductive periods, respectively. Positive and negative numbered leaves correspond to leaves positioned above or below 0, respectively. The parameters of the linear relationships between Chl_{leaf} and Chl_{canopy} varied with the position of the leaf. From the top to the middle leaf positions, down to leaf position −1 the slope of these relationships decreased while the coefficient of determination (r^2) increased from 0.44 to 0.89. Chl_{leaf} also increased from top to middle positioned leaves. From middle to bottom positioned leaves the relation Chl_{leaf} vs. Chl_{canopy} becomes weaker, showing a higher dispersion of the points and lower r^2.

The highest correlation between Chl_{leaf} and Chl_{canopy} was found among +1, 0, and −1 leaves with r^2 of 0.87, 0.85, and 0.89, respectively. The relationships Chl_{leaf} vs. Chl_{canopy} were weaker for both above and below +1 and −1 leaves. It is important to note that for leaves positioned below −4 the relationship was markedly weaker. This

phenomenon was so intense for the last four leaf positions, −8 through −11, that it was not possible to fit a model (bottom row in Figure 4).

The relationship between Chl_{leaf} and Chl_{canopy} is governed by both the leaf Chl content and the leaf area (Figure 5). The r^2 of these relationships followed a bell shape distribution: highest r^2 values were for leaves in the middle of canopy and gradually decreased to both top and bottom leaves (Figure 5). Chl_{leaf} of the upper leaves, +8 and +7, could explain only about 45% of the variability of total Chl_{canopy}. On the other hand, Chl_{leaf} of the leaves positioned in the middle of the canopy, −1, 0, and +1, were closely related to Chl_{canopy} and each of them could explain more than 85% of the variability in Chl_{canopy}.

Estimation of canopy Chl from a single leaf Chl

The Chl content of three single leaves, 0, +1, or −1, was found to be the best proxy of Chl_{canopy} (Figures 4 and 5). Each leaf could explain more than 85% of the total canopy Chl variability. In practical terms, however, 0 leaf is the easiest leaf to identify in the plant under field conditions and its contribution to Chl_{canopy} was one of the highest during the growing season. Thus, the relationship of Chl in 0 leaf vs. Chl_{canopy} was analyzed in detail to develop a simple technique for the estimation of Chl_{canopy}. Note that 0 leaf represents the *collar leaf* during the vegetative period and the *ear leaf* in the reproductive period. Therefore, the relationship

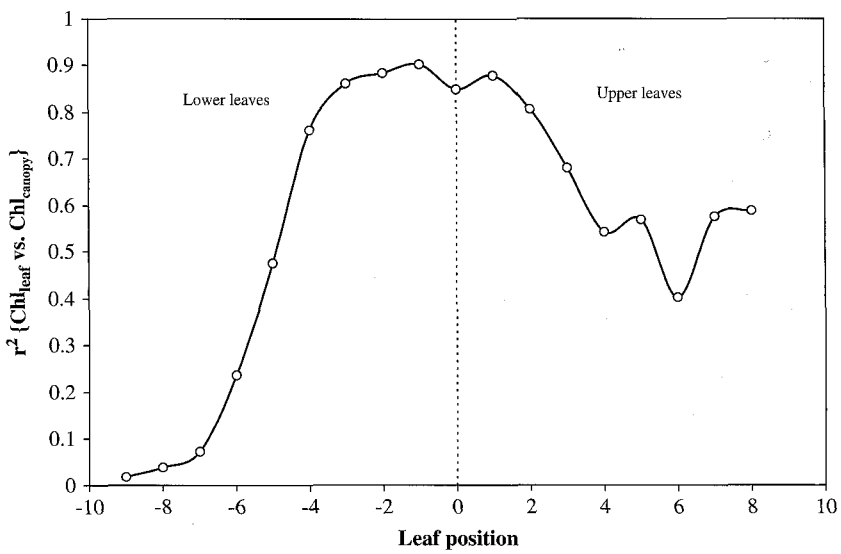

Figure 5. The coefficient of determination, r^2, of the linear relationship Chl_{canopy} vs. Chl_{leaf} plotted vs. leaf position. Leaf 0 corresponds to the collar or ear leaf during vegetative and reproductive periods, respectively. Positive and negative numbered leaves correspond to leaves positioned above or below 0 leaf, respectively.

Table 1. Intercept, slope, RMSE, and coefficient of determination (r^2) of the linear relationship between chlorophyll content in the collar or ear leaf and chlorophyll content in canopy, Chl_{canopy} vs. Chl_{leaf}, for three hybrids: D-6375, P-33B51, and P-31G68, and all samples together

Leaf	Hybrid	n	r^2	Intercept (g Chl m^{-2})	Slope	RMSE (g Chl m^{-2})
Collar	D-6375	18	0.813	−0.076a	7.712b	0.350
	P-33B51	12	0.731	−0.148a	6.484b	0.399
	P-31G68	18	0.835	−0.125a	7.875b	0.294
	Altogether	48	0.795	0.000	6.562	0.352
Ear	D-6375	27	0.935	0.129c	8.689d	0.297
	P-33B51	27	0.851	0.024c	7.353d	0.465
	P-31G68	26	0.934	0.095c	7.858d	0.183
	Altogether	80	0.875	0.000	8.120	0.375

n is number of samples. Numbers followed by the same letter are not significantly different at $\alpha = 0.001$. Both Chl_{canopy} vs. Chl_{leaf} were calculated per ground area.

Chl_{leaf} with Chl_{canopy} was analyzed within the two growth periods: vegetative and reproductive. In addition, the possible differences among the three hybrids (D-6375, P-33B51, and P-31G68) grown under different cropping systems were taken into account by fitting a linear model for each of them within each growth period (Table 1).

No significant differences were found among hybrids during the vegetative growth period: the slopes and intercepts of the three linear regressions of Chl content in the collar leaf Chl_{collar} vs. Chl_{canopy} were not statistically different. Also, during the reproductive period no significant differences were found for the linear relationship Chl_{ear} vs. Chl_{canopy} among the three hybrids (Table 1). These results revealed that the relationships Chl_{collar} vs. Chl_{canopy} and Chl_{ear} vs. Chl_{canopy} are consistent and can be used for Chl_{canopy} retrieval regardless of hybrid, cropping system, and plant density.

Relationships Chl_{collar} vs. Chl_{canopy} and Chl_{ear} vs. Chl_{canopy} were linear across hybrids (Table 1, Figure 6). Thus, two algorithms for Chl_{canopy} estimation were proposed:

Vegetative period:

$$Chl_{canopy} = 6.56 \times Chl_{collar} \quad (7)$$

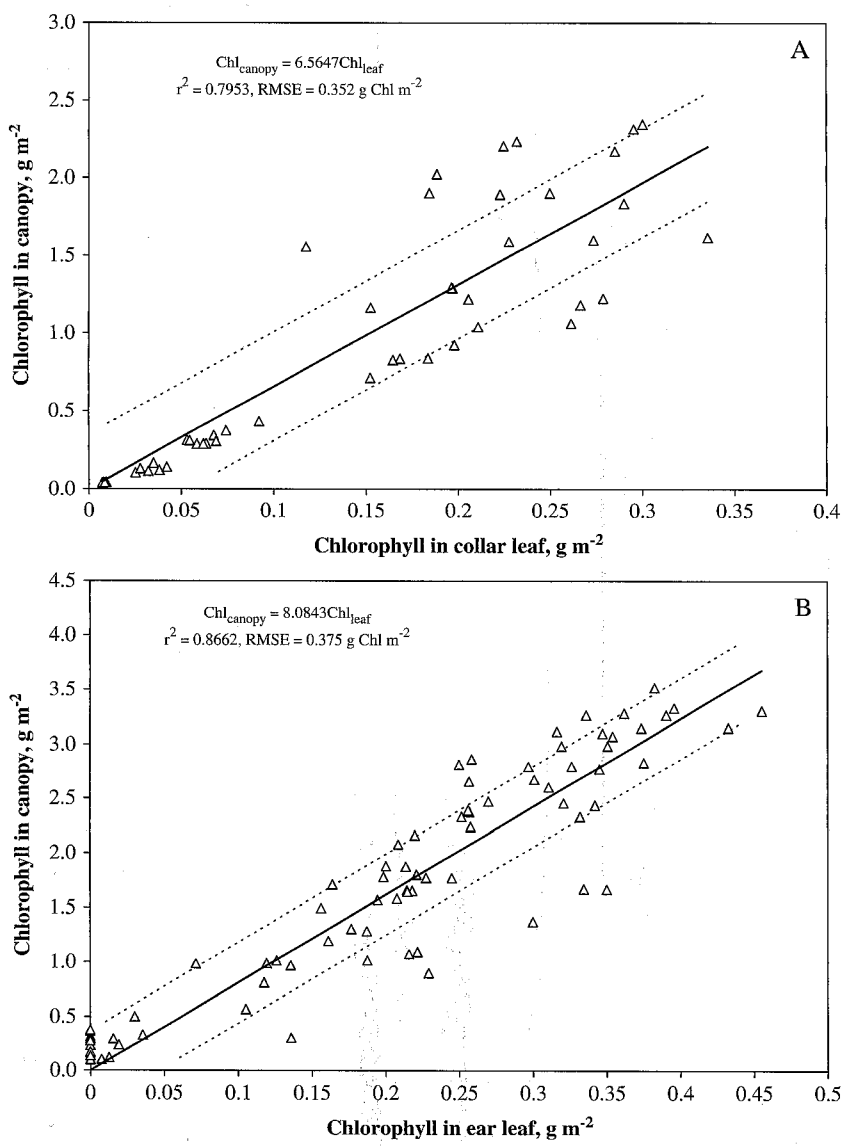

Figure 6. Relationship between chlorophyll content (both calculated per ground area) in (A) the collar leaf and (B) the ear leaf and total chlorophyll content in canopy. The solid line represents the linear fit function; the dotted lines represent one standard error of chlorophyll in canopy estimation. RMSE is root mean-square error of canopy Chl estimation.

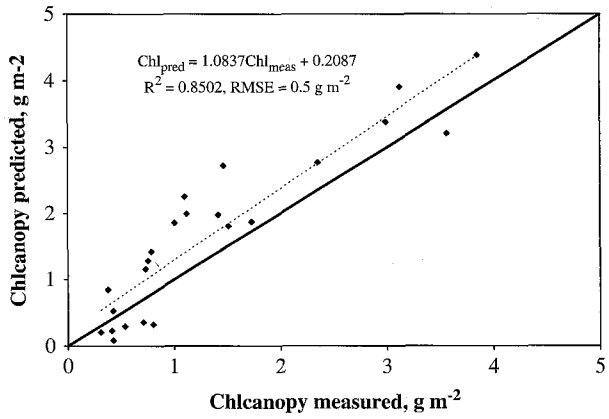

Figure 7. Validation of the model for the estimation of Chl content in canopy from Chl content of a single leaf. The measured Chl content of an independent dataset (2004) was compared with Chl content predicted by Eq. (8) developed with 2005 data. Solid line is $Chl_{pred} = Chl_{meas}$; dotted line is best-fit function Chl_{pred} vs. Chl_{meas}. RMSE is root mean-square error of canopy Chl prediction.

Reproductive period:

$$Chl_{canopy} = 8.08 \times Chl_{ear} \quad (8)$$

During the vegetative period, Chl_{collar} explained around 80% of the total Chl variability in the canopy (Table 1). During the reproductive period, Chl_{ear} explained more than 87% of Chl_{canopy} (Table 1, Figure 6).

For validation of the algorithm for Chl_{canopy} retrieval (Eq. (8)), the independent dataset collected during the reproductive period of 2004 was used. The results of the validation are presented in Figure 7. The algorithm predicted Chl content in a canopy with a RMSE of less than 0.5 g Chl m^{-2} for Chl_{canopy} that ranged from 0.3 to 4 g Chl m^{-2}:

$$(Chl_{canopy})_{pred} = 1.0837 \times (Chl_{canopy})_{meas} + 0.2087 \quad (9)$$

Thus, estimation of canopy Chl per ground area can be done via either the collar or ear leaf Chl content (per ground area) using the following procedure: (1) measure reflectance in two spectral bands 720–730 and 770–800 nm; (2) calculate $CI_{red\ edge}$: Eq. (1); (3) calculate Chl content of an entire leaf or leaf section: Eq. (5); (4) measure the area of the collar or ear leaf using either portable leaf area meters (e.g., LI-3000C Portable Area Meter http://www.licor.com/env/Products/AreaMeters/LI-3000C/3000C_intro.jsp) or applying the empirical formula developed by Montgomery (1911) and widely used (e.g., Sprague and Curtis, 1933; Muchow and Davis, 1988): individual leaf area = 0.75 × leaf length × maximal leaf width; (5) calculate Chl_{leaf}^{weight}: Eq. (3); and (6) calculate Chl_{canopy}: Eqs. (7) or (8).

Estimation of canopy Chl from multiple leaves.

To establish relationships between Chl_{canopy} and Chl in multiple leaves, the Chl content of leaves above or below 0 leaf were added successively to Chl content in 0 leaf until the topmost or the lowermost leaf was included. The r^2 of the relationship Chl_{leaves} vs. Chl_{canopy} plotted vs. number of leaves added is shown in Figure 8. The addition of Chl in leaves positioned below or above the collar leaf to Chl_{collar} into the regression analysis had very different effects on the accuracy of Chl_{canopy} estimation (Figure 8A). Adding leaves below the collar leaf increased the statistical significance considerably: r^2 grew from 0.79 to 0.97 up to the point when the −5 leaf was added. Adding additional leaves did not change the relationship. Thus, Chl in leaves positioned below the collar leaf contributed noticeably to total Chl_{canopy} and measuring Chl in three leaves instead of one collar leaf made a difference in Chl_{canopy} estimation: the r^2 increased from 0.79 to 0.94.

Just the opposite effect on Chl_{canopy} estimation was seen when Chl in leaves positioned above the collar leaf was added into the analysis. The accuracy of Chl_{canopy} estimation decreased after adding only one leaf. It shows that Chl in leaves positioned above the collar leaf were not representative of total Chl_{canopy}. Thus, Chl in leaves positioned below the collar leaf is recommended for estimation of Chl_{canopy}. The decision to use more than one leaf for canopy Chl estimation should balance the gain in accuracy in the estimation with the extra labor that comes with the estimation of Chl content of more than just one leaf.

The significance of adding leaves to the regression analysis for the ear leaf (Figure 8B) was conspicuously different than for the collar leaf (Figure 8A). The initial r^2 for the ear leaf was higher than for the collar leaf (0.87 vs. 0.79), but the addition of leaves to the ear leaf analysis was less pronounced than in the case of the collar leaf (Figure 8B). Adding Chl in leaves above the ear leaf slightly increased r^2 (opposite that of for the collar leaf). Thus, Chl in leaves positioned below the ear leaf is recommended to determine canopy Chl with two to four leaves being optimal. The latter brought an increase in r^2 from 0.87 to more than 0.95.

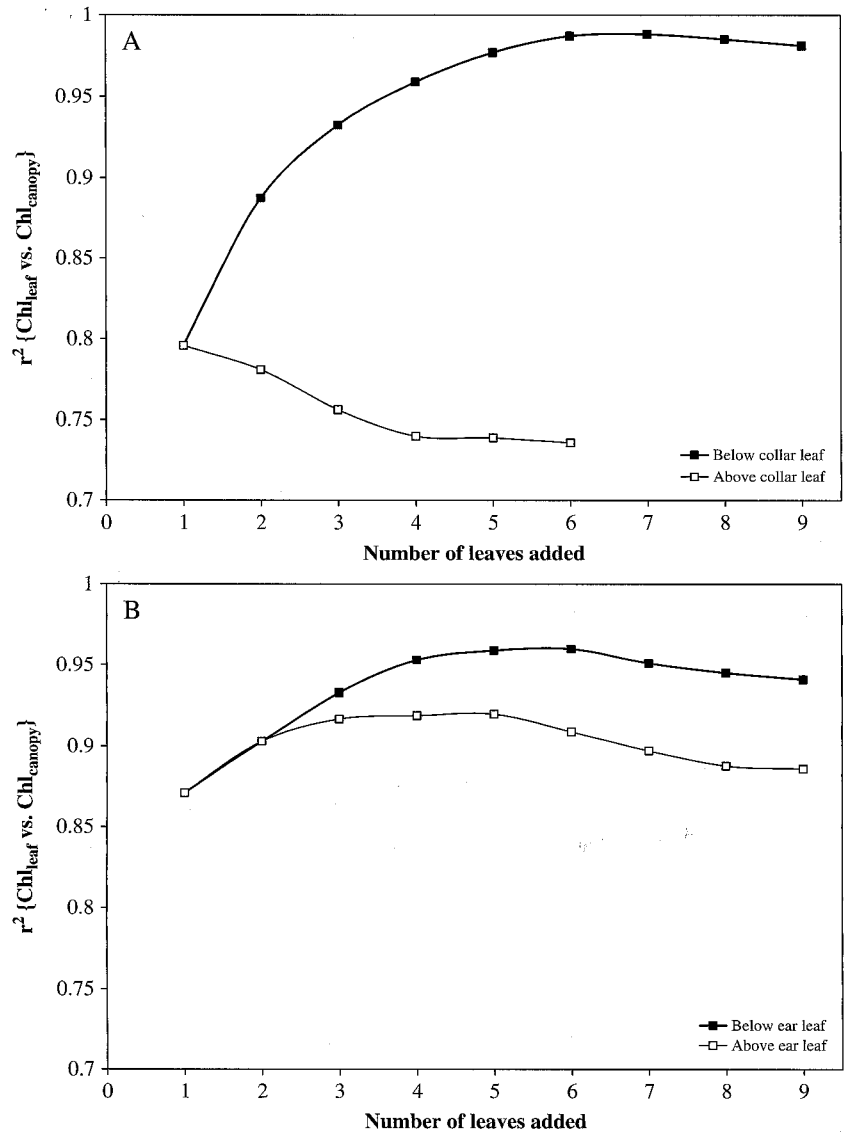

Figure 8. The coefficient of determination, r^2, of the linear relationship between chlorophyll content in leaves and total chlorophyll in canopy (both calculated per ground area) with successive addition of leaves, either below or above the collar (A) and ear (B) leaf.

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