Technical communication

Calibration of the Minolta SPAD-502 leaf chlorophyll meter

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Abstract

Use of leaf meters to provide an instantaneous assessment of leaf chlorophyll has become common, but calibration of meter output into direct units of leaf chlorophyll concentration has been difficult and an understanding of the relationship between these two parameters has remained elusive. We examined the correlation of soybean (*Glycine max*) and maize (*Zea mays* L.) leaf chlorophyll concentration, as measured by organic extraction and spectrophotometric analysis, with output (M) of the Minolta SPAD-502 leaf chlorophyll meter. The relationship is non-linear and can be described by the equation chlorophyll (μmol m^{-2}) = $10^{(M^{0.265})}$, $r^2 = 0.94$. Use of such an exponential equation is theoretically justified and forces a more appropriate fit to a limited data set than polynomial equations. The exact relationship will vary from meter to meter, but will be similar and can be readily determined by empirical methods. The ability to rapidly determine leaf chlorophyll concentrations by use of the calibration method reported herein should be useful in studies on photosynthesis and crop physiology.

Abbreviations: Chl – chlorophyll; M – SPAD-502 meter value

Introduction

One of the more common measurements made by plant scientists is the determination of Chl concentration. Such measurements have traditionally been made by extraction of leaf materials and spectrophotometric determination (Arnon 1949; Porra et al. 1989) using wavelengths in the red region of the visible spectrum where Chls are the primary absorbing species. The spectrophotometric determination of Chl concentration is not, however, entirely straightforward, and many modifications of these techniques have been developed (Holden 1976)

The desirability of a more rapid and straightforward method for estimation of leaf Chl has also attracted the attention of other plant researchers. There have been reports from a number of laboratories (Inada 1965; McClure 1969; Hardwick and Baker 1973; MacNicol et al. 1976) on attempts to develop instruments capable of rapid Chl measurement in whole leaves, but the com-plex optical properties of leaves have been a serious problem which limited commercial applications.

A Chl meter has recently been developed by Minolta Corporation for determining the nitrogen status of crops. The SPAD-502 meter utilizes two light-emitting diodes (650 and 940 nm) and a photodiode detector to sequentially measure transmission through leaves of red and infrared light. The relationship between the output of the SPAD-502 meter and leaf Chl concentration is nonlinear (Monje and Bugbee 1992), and similar relationships were obtained with leaves of wheat, rice and soybean. To date, the primary application for the Chl meter has been to determine the potential efficacy of additional nitrogen treatments to crop plants (Turner and Jund 1991; Kaakeh et al. 1992; Piekielek and Fox 1992; Wood et al. 1992a; Wood et al. 1992b; Blackmer et al. 1993; Fox et al. 1994). These reports, however, present results in terms of meter output rather than in terms of leaf chlorophyll concentration. Therefore, the objective of this study was to determine the relation-

ship between meter output and leaf Chl concentration and provide a rational method for calibration so that a direct estimate of leaf Chl may be facilitated.

Materials and methods

A Minolta SPAD-502 meter was purchased from Spectrum Technologies, Inc., Plainfield, IL. The meter was supplied with a calibration filter stated to produce a meter reading of 87.1. Analysis of this filter with a Milton Roy Spectronic 601 spectrophotometer indicated that it transmitted 0.4% of light at 650 nm and 89.2% of light at 940 nm.

Plants used for this study were grown in nursery-field plots on the East Campus of the University of Nebraska-Lincoln and measured during July of 1994. The moderately fertile soil at the site is a Zook silty clay loam (fine, montmorillonitic, mesic Cumulic Haplaquoll). Soybean plants received no supplemental nitrogen, whereas maize plants received 100 kg N ha^{-1}; plots of both plants were irrigated as needed. Rather than attempt to replicate individual measurements, individual plants of various genotypes were selected to provide a large number of SPAD-502 meter readings with a wide range of values. Measurements on soybean leaves avoided placing the meter over major leaf veins. Measurements on maize involved placement of the meter on the fifth leaf from the bottom, midway between the midrib and the leaf margin about 20 cm from the stalk; plants were measured prior to emergence of tassels.

To demonstrate application of the SPAD-502 meter for Chl determination within a single genotype, two specific soybean strains were used in this study from the USDA–ARS Genetic Type Collection maintained at the University of Illinois, Urbana, IL (R.L. Nelson, Curator). Those strains were T315, a cytoplasmic Chl-deficient type (cyt-Y5), and T275, also a cytoplasmic Chl-deficient type (cyt-Y2). Both genetic types exhibited yellowish young leaves that become green as they mature.

Foliar Chl content was measured by removing samples with a leaf punch. The 1.0 cm leaf disks were ground in 5 ml aqueous 80% acetone with a Ten Broek tissue homogenizer. Extracts were clarified by centrifugation in a clinical centrifuge for two minutes and then the absorbance determined at 646.6, 663.6 and 750.0 nm using a Varian DMS-70 spectrophotometer with a 2 nm bandpass. Chl concentrations were determined using published equations (Porra et al. 1989).

The relationship between the SPAD-502 meter values and leaf Chl concentration was determined using the curve fitting routines of KaleidaGraph (Synergy Software, Reading, PA) on a Macintosh microcomputer.

Theoretical considerations

Upon initial calibration, the Minolta SPAD 502 processor converts a current produced by the red (I_{650}) and infrared (I_{940}) light beams into a voltage and stores the digital values in the unit's memory. When a leaf is subsequently measured, the Chl meter successively measures the transmission of red (I'_{650}) and infrared (I'_{940}) light and outputs a processed value based on the ratio of the measured voltage produced by each wavelength relative to the values stored in the memory. Thus, the values produced by the meter (M) are nontrivial ratios (Eq. (1)).

$$M = \log \frac{I'_{940}/I_{940}}{I'_{650}/I_{650}} = \log \frac{I'_{940} \cdot I_{650}}{I'_{650} \cdot I_{940}} \quad (1)$$

If a leaf were a perfect optical system containing no molecules which absorb light at 940 nm and only the absorption of Chl would attenuate the red beam, the system would simplify to Eq. (2) which would approximate the absorbance.

$$M_{ideal} = \log \frac{I_{650}}{I'_{650}} \quad (2)$$

Because Chls a and b are the dominant pigments absorbing at 650 nm, these ideal meter values would be proportional to the Chl concentration in the leaf.

However, like most biological materials, leaves are not perfect optical systems (Vogelmann 1993). Because collimated light is refracted and focused by epidermal cells near the surface of the leaf (Myers et al. 1994), light intensity within the leaf is not uniform. Similarly, the Chl pigments are localized within chloroplasts, which are not uniformly distributed within the leaf, and different rays of light within a collimated beam may pass through microenvironments with different apparent Chl content; this is the so-called 'sieve effect' (Rabinowitch 1951). Portions of the incident radiant flux will be subject to absorptance (A), scatterance (S) and reflectance (R), all of which will contribute to a decrease in the apparent transmission

(Kirk 1994). The attenuation of radiant flux by a leaf will be an additive function of these three factors (Eq. 3).

$$I' = I - I_A - I_S - I_R = I - (I_A + I_S + I_R) \quad (3)$$

The relationship between absorptance, scatterance and reflectance is complex and their individual contributions are difficult to assess (McClendon and Fukshansky 1990a; McClendon and Fukshansky 1990b; Vogelmann 1993). If significant amounts of scatterance and reflectance occur, and their values cannot be assessed, they may simultaneously decrease the transmission through the leaf, increase the path length for absorption (McClendon and Fukshansky 1990a; Uz and Saygin 1994), and lead to an overestimation of the Chl present. Also, the proportion of the initial radiant flux subject to scatterance and internal reflectance will be decreased as the absorptance increases and would attenuate the radiation within the leaf. Furthermore, the meter reading contains a measure of the attenuation of the 940 nm radiant flux (Eq. 4) which will vary in a manner different from that of the 650 nm light (e.g. Rayleigh scattering is a function of λ^{-4}). It should also be noted that the above discussion has ignored possible contributions from Chl fluorescence, which may equal 1 to 3% of the light absorbed by Chl (Nobel 1991). In spite of the above possible sources of error, one might predict, using a model (McClendon and Fukshansky 1990a) in which the difference between the expected transmittance due to the pigments within a leaf and the actual transmittance through the leaf are exponentially related, that there exists an exponential relationship between the Chl present in the leaf and the SPAD-502 meter output.

$$M = \log \frac{I_{650} \cdot [I_{940} - (I_A + I_S + I_R)_{940}]}{(I_{940} - [I_{650} - (I_A + I_S + I_R)_{650}]} \quad (4)$$

Results and discussion

Due to the complex nature of the relationship between the Chl concentration of a leaf and the output value determined by the SPAD-502 meter, we took an empirical approach. Most nonsenescent plants display a narrow distribution of Chl concentrations under field conditions. To study the relationship between the SPAD-502 meter reading and the leaf Chl concentration, it was desirable to have as diverse a range of Chl concentrations as possible. We were also concerned that
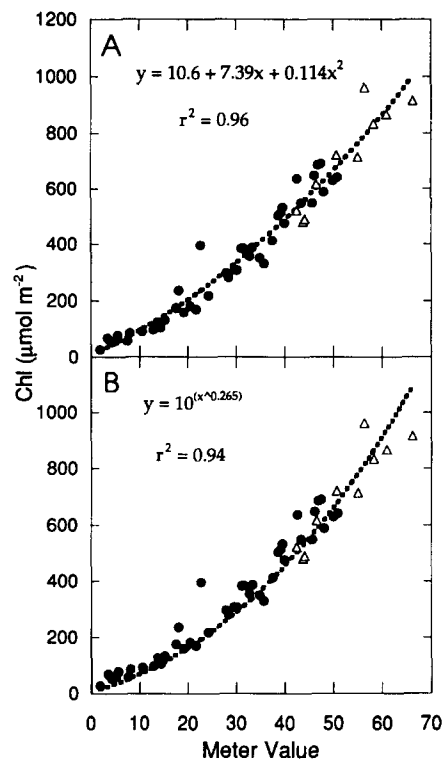

Fig. 1. Correlation of leaf Chl concentration with SPAD-502 meter values for a variety of soybean (●) and maize (△) genotypes selected from nursery plots to give a wide range of chlorophyll contents. The data are shown with dashed lines representing fits provided by polynomial (A) or exponential (B) equations.

restriction to data from one species or one genotype within a species would not provide a generally applicable understanding of the meter response to differences in leaf Chl concentration. Consequently, we selected leaves from nursery field plots representing a variety of lines of soybean and maize to give a diverse range of SPAD-502 meter readings.

The data for correlation between the SPAD-502 meter value and the leaf Chl concentration (Fig. 1) suggested that both soybean and maize follow the same relationship. As previously noted in a study of leaves from wheat, rice and soybean plants (Monje and Bugbee 1992) , the relationship between the SPAD-502 meter value and leaf Chl concentration is nonlinear. These authors were able to fit the curve as a second-order polynomial equation. Although we similarly found (Fig. 1A) a response that fit a second order polynomial, we were also able to fit the data to an exponential function (Fig. 1B): Chl (μmol m^{-2}) = $10^{(M^{0.265})}$ ($r^2 = 0.94$). The slight underestimation of leaf Chl concentration at low meter values was likely

due to the reliance on a measuring wavelength of 650 nm. This wavelength will be preferentially absorbed by Chl b and higher plants generally deficient in Chl tend to be more deficient in Chl b than Chl a. Fortunately, increasing error at lower meter values appears to have a minimal effect on fitting the meter response to the exponential equation in relation to the value of the plants in the normal range of Chl concentration. For studies in which accurate determination of Chl concentration in leaves of plants with extreme Chl deficiency is essential, leaves should be extracted and Chl determined by spectrophotometric methods.

For the remainder of this work, we utilized the exponential equation to convert SPAD-502 meter readings into Chl concentrations of leaves. A practical consideration for use of the exponential relationship was that this equation format forced a fit to the shape defined by a complete set of data as shown in Fig. 1. For example, utilizing only the maize data in Fig. 1 and fitting curves to this data as a second order polynomial or exponential equations gives different results. The second order polynomial fit gave the equation Chl (μmol m^{-2}) $= -2366 + 97.5 \cdot M - 0.7237 \cdot M^2$ with a correlation $r^2 = 0.886$, whereas the exponential fit gave the equation Chl (μmol m^{-2}) $= 10^{(M^{\wedge}0.264)}$, almost identical to the equation for the complete set of data, with $r^2 = 0.785$. Use of the polynomial fit for data within a limited range of meter readings gave an equation that did not follow the shape of the relationship over a broader range of Chl concentrations and would have decreased accuracy of calculated Chl concentrations outside of the narrow calibration range even thought the apparent fit to the data was statistically better. Since most plant researchers will not have access to the range of leaf chlorophyll contents which we utilized from the nursery plots, we feel use of the exponential fit for meter calibration will be superior in a practical sense to the polynomial method because it consistently represents the appropriate relationship of meter output to leaf chlorophyll concentration.

We further sampled a limited number of leaves from field-grown sorghum (*Sorghum bicolor*) and *Arabidopsis thaliana* plants grown under controlled conditions and found that their response on the SPAD-502 meter were also adequately described by the above exponential equations (data not shown). Thus, it appears that the determination of leaf chlorophyll per unit area is relatively independent of species. We believe that the similar response seen for leaves from five different species in this report and (Monje and Bugbee 1992) is due to the use of the infrared light beam at 940
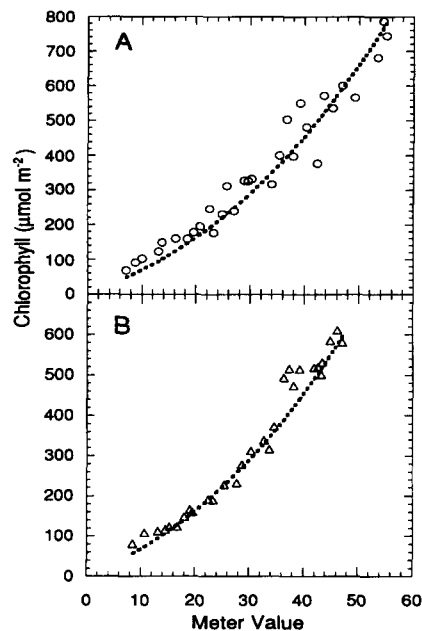

Fig. 2. Correlation of leaf Chl concentration with SPAD-502 meter values for two soybean genotypes. The two soybean mutants are *cyt-Y2* (A) and *cyt-Y5* (B). The fit of the data to the exponential equation derived from Fig. 1 is shown by the dashed lines. The correlation coefficients (r^2) for the data in panels A and B are 0.93 and 0.97, respectively.

nm which tends to compensate for differential scattering by different leaf thicknesses. Whether a different response would be observed for much thicker leaves would have to be empirically determined. An additional consideration is that the pathlength distribution and the absolute amount of transmission and remission are theoretically expected to differ if the light source were changed from the adaxial, to the abaxial, surface of the leaf (Fukshansky et al. 1993). To determine if this would be a significant source of error, we made triplicate determinations in both the adaxial and abaxial orientations on four different leaves of eight different mature maize plants. Such results are at the upper range of normal leaf Chl values and would represent maximal expected differences due to the low amounts of measured transmittance. The results from this study were a mean and standard error of the mean of 880 $\pm$ 15.3 and 891 $\pm$ 16.1 from the adaxial and abaxial surfaces, respectively. We conclude that any differences from measurement of the same leaf from these two orientations is small and within the limits of error.

To test whether the SPAD-502 meter would provide accurate determination of leaf Chl concentration for a single genotype, we conducted further studies

on two cytoplasmic soybean mutants, cyt-Y2 and cyt-Y5, containing leaves with a wide range of pigment concentration (Fig. 2). Both lines were independently found to have leaf Chl concentrations which could be described by application of the exponential equation from Fig. 1B to the meter values. The correlation coefficients (r^2) between the Chl concentration and the meter values for these two soybean lines were 0.93 and 0.97, respectively and corroborate that the response with different genotypes is also valid within single genotypes.

We should emphasize that one should not expect all SPAD-502 meters to give identical data. Each meter is provided with a calibration filter specific for that meter. The response of each meter appears to be slightly different and will give different meter values when checked with the same calibration filter. Thus, data from studies carried out with different SPAD-502 meters should not be directly compared unless calibration procedures are standardized. We recommend that each meter be calibrated independently with parallel extraction and spectrophotometric assay in order to determine the exponential equation to directly convert its output to leaf Chl concentration. We examined two additional SPAD-502 meters available at the University of Nebraska and found that their responses fit the following equations: Chl (μmol m^{-2}) = $10^{(M^{0.256})}$ and $10^{(M^{0.261})}$. Thus, the responses of different SPAD-502 meters follow the same general exponential equation, but are slightly different in their exact response.

We conclude that the SPAD-502 meter is able to provide a rapid and reasonably accurate estimate of leaf Chl. A single determination takes only a few seconds and the leaf area measured (2×3 mm) is small enough to accommodate small plants such as *Arabidopsis*. The use of an exponential equation to fit a data set with a limited range of meter values appears to provide a more accurate approximation of the meter response over a wider range of meter values than the previously suggested (Monje and Bugbee 1992) polynomial equation. The SPAD-502 is currently used primarily to improve N management in crop plants. We suggest that the meter is also a valuable nondestructive tool with which to facilitate studies on photosynthesis and crop physiology.

Acknowledgements

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