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Evaluation of Optical Chlorophyll Meters as Proxy for Nitrogen in *Theobroma cacao* L.

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Abstract. Leaf nitrogen (N) and leaf chlorophyll concentrations are key indicators of a plant's nutritional status. For many crops, readings from simple optical methods for estimating chlorophyll correlate well with laboratory-based chlorophyll extraction methods. Yet, in cacao (*Theobroma cacao* L.), an important tree commodity crop, optical measuring devices for chlorophyll are widely used but are not yet validated against actual leaf chlorophyll concentrations. Moreover, cacao leaf chlorophyll has not been demonstrated to be a good proxy for cacao tree N status. Closing these knowledge gaps to support cacao farmers in determining the N status of their trees was the goal of this study. The experiment was conducted with leaves from mature trees raised under five N fertigation treatments ranging from 10 to 150 mg·L^{-1} N (10, 25, 50, 75, and 150 mg·L^{-1} N). The two tested optical meters (SPAD-502 and MC-100) were found to be suitable for chlorophyll content determination, showing good linear correlations with in vitro chlorophyll concentrations (R^2 = 0.87 for the SPAD-502 and R^2 = 0.83 for the MC-100). Leaf chlorophyll concentrations by all three types of measurement increased as leaf N increased up to a value of 3.2% leaf N (75 mg·L^{-1} N fertigation). None of the chlorophyll measurement techniques were sensitive to higher levels of leaf N (3.4%, 150 mg·L^{-1} N fertigation). Nitrogen concentrations in the leaves of the two lowest N treatments (N10 and N25) are considered deficient and low, respectively. Leaf N concentrations in the N50 and N75 treatments are in the normal to high ranges. Leaf N in the N150 treatment is considered excessive. Trees in the different treatments indeed displayed many signs characteristic of their nutritional status, including influence on timing and intensity of flowering, and fruit set. These findings confirm that optical meters can be a dependable and easy-to-use tool for assessing the nutritional status of cacao trees, and can guide fertilization reliably to minimize N deficiencies.

Chlorophyll plays a crucial role in the process of photosynthesis. Light energy is captured by chlorophyll and converted into chemical energy in the form of adenosine triphosphate and nicotinamide adenine dinucleotide phosphate. This chemical energy, along with carbon dioxide and water, is used by plants, algae, and cyanobacteria to produce glucose through a series of complex biochemical reactions known as the Calvin cycle (Taiz and Zeiger 2016). The concentration of chlorophyll in leaves is affected by nutrition, light, temperature, genotype, and leaf age. Chlorophyll determination has long been known as a useful proxy for assessing plant nutritional status and leaf nitrogen (N) concentration in various crops (Kalaji et al. 2017; Padilla et al. 2018; Smith and Benitez 1955).

There are several ways to measure chlorophyll concentration in leaves. Portable optical devices are easy to use and provide results quickly (Cate and Perkins 2003; Cerovic et al. 2012; Coste

et al. 2010; Marenco et al. 2009; Schaper and Chacko 1991). They measure the absorbance of the leaf at different wavelengths to evaluate the pigment concentration. There are also in vitro measurements, which are more accurate, in which chlorophyll is extracted from leaves using organic solvents (Bruuinsma 1963; Lichtenthaler 1987). These methods are more time-consuming and require destructive sampling and analysis.

Studies of many crops show good correlations between optical methods and chlorophyll extraction methods (Parry et al. 2014). In cacao (*Theobroma cacao* L.), an important tree commodity crop, optical measuring devices for chlorophyll are widely used (Arévalo-Gardini et al. 2021; Baligar et al. 2021; Daymond and Hadley 2004; Khoddamzadeh and Souza Costa 2023; Posse et al. 2018; Suárez-Parra et al. 2022), but are not yet validated against actual leaf chlorophyll concentrations. Furthermore, cacao leaf chlorophyll has not yet been demonstrated to be a good proxy for cacao tree N status.

Our study had two specific objectives: 1) determine whether chlorophyll measurements made by portable optical in situ devices correlate with in vitro measurements of leaf chlorophyll in cacao and 2) examine whether chlorophyll concentrations represent the N nutritional status of cacao trees. Differences in leaf chlorophyll concentration in response to different levels of N fertilization are documented, and the results demonstrate how simple handheld meters can be used to guide farmers in N fertilization needs of their cacao crop.

Materials and Methods

Experimental site. A cacao nutrition experiment was conducted in a controlled drainage lysimeter experiment in the Cocoa Cure Center greenhouse in Ben Shemen, Israel (lat. 31°57′41″N, long. 34°55′43″E). The greenhouse and experiment were fully described in Weinstein et al. (2024). The lysimeter experiment examined the influence of five different N levels on growth, development, water use, and reproduction of F1 individuals from manually self-pollinated pods of the CCN-51 cultivar. This experiment ran for > 3 years (May 2021–Dec 2024). Nitrogen levels were fixed at concentrations of 10, 25, 50, 75, and 150 mg·L^{-1} in the fertigation water (designated N10, N25, N50, N75, and N150, respectively). All the trees (seven replicate trees per treatment) received the same doses of phosphorus (20 mg·L^{-1}), potassium (70 mg·L^{-1}), and microelements (iron, 1 mg·L^{-1}; manganese, 0.45 mg·L^{-1}; zinc, 0.3 mg·L^{-1}; copper, 0.08 mg·L^{-1}; and molybdenum, 0.03 mg·L^{-1}). The water supply included calcium, magnesium, sulfate, and boron at approximate concentrations of 60, 20, 7, and 0.16 mg·L^{-1}, respectively.

Chlorophyll determination. Six replicate trees from each of the N treatments were chosen randomly for sampling for our study. From each tree, five diagnostic leaves, defined as the most mature leaf of the youngest leaf flushes, were examined, for a total of 150 leaves (five N levels, six replicate trees per N level, five leaves per tree). Sampling took place on four sampling dates between Jul and Aug 2024.

Chlorophyll determination with portable meters. Two portable devices were used to determine chlorophyll concentration in the leaves: the MC-100 Chlorophyll Concentration meter (Apogee Instruments Inc., Logan, UT, USA) and the SPAD-502 (Konica Minolta, Inc., Tokyo, Japan). Both devices measure the absorbance at two wavelengths by which they calculate the chlorophyll concentration of the sample: the MC-100 at 653 and 931 nm, and the SPAD-502 at 650 and 940 nm. The output is a dimensionless value referred to as chlorophyll content index (CCI) units for the MC-100 instrument, and soil plant analysis development (SPAD) units for the SPAD-502 instrument.

Sensors of the optical instruments were clipped onto each leaf in five premarked circles. The readings of all five circles per leaf were averaged into one value per leaf. Every circle on each leaf was measured with both portable devices, one after the other.

In vitro chlorophyll determination. Immediately after completing the optical measurements, disks were punched from the premarked circles on each leaf using a 10-mm-diameter cork borer (Parry et al. 2014). Chlorophyll was extracted from the leaf samples according to Ben-Yaakov et al. (2006). The five leaf punches from each leaf were placed together into 20-mL amber glass vials containing 5 mL dimethyl sulfoxide and were sealed with screw caps lined by Teflon-faced silicon septa. The vials were maintained at 25 °C in the dark for 72 h. The chlorophyll concentration of the extracts was measured with a spectro photometer (GENESYS™ 10 UV-Vis; Thermo Fisher Scientific, Waltham, MA, USA). Chlorophyll (Chl) was calculated according to Bruuinsma (1963) using the following equation: Chla+b (measured in micrograms chlorophyll per milliliter) = $27.8A_{652}$, where A_{652} is the value obtained from the spectrophotometric reading at a wavelength of 652 nm.

Leaf N concentration determination. Leaves were sampled monthly during 2023. At every leaf sampling, four physiologically mature leaves per tree were picked from four sides of the tree (the youngest leaf flush from each side, eighth leaf from the apex). Leaves were oven-dried at 65 °C for at least 4 d and were then finely ground in a ball mill (Mixer Mill MM 400; Retsch GmbH, Haan, Germany). The fine powders were analyzed for N concentration by a Fourier transform–near infrared spectrometer (Antaris™ II FT-NIR Analyzer; Thermo Fisher Scientific).

Statistical analysis. Data were analyzed using JMP® Pro Statistical Software v. 17.0 (SAS Institute Inc., Cary, NC, USA). We analyzed the effects of the five N treatments on leaf chlorophyll concentration and leaf N using analysis of variance with the Tukey-Kramer honestly significant difference post hoc test, with a significance threshold of $P < 0.05$.

Results

A wide range of leaf Chl_{a+b} concentrations were found, from 41 to 397 μmol Chl/m^2. Accordingly, both optical methods revealed wide ranges of results. SPAD values ranged between 18.1 and 70.8; CCI values ranged between 4.8 and 76.3. Correlations for both optical methods with leaf Chl_{a+b} were best described by linear models (Fig. 1), with SPAD demonstrating a somewhat better fit (Fig. 1A; $P < 0.0001$, $R^2 = 0.87$) to the

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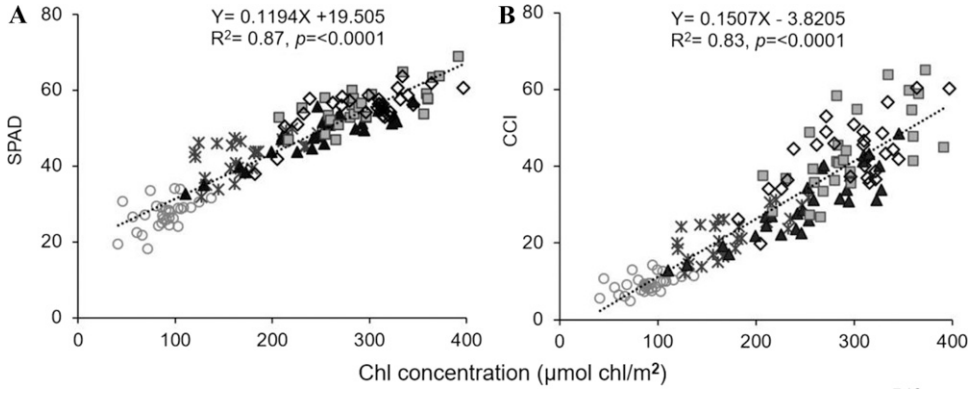

Fig. 1. Relationship between chlorophyll (Chl) a and b concentrations and soil plant analysis development (SPAD) units (A) ($P < 0.0001$, $R^2 = 0.87$) and chlorophyll content index (CCI) units (**B**) ($P < 0.0001$, $R^2 = 0.83$). The different symbols represent the five nitrogen (N) treatments designated as N10 (open circles), N25 (X-type shape), N50 (closed triangles), N75 (closed squares), N150 (open diamonds), with N levels of 10, 25, 50, 75, and 150 mg·L^{-1} in the fertigation water, respectively. All 30 replicate leaves per treatment are presented.

in vitro chlorophyll concentration data than CCI (Fig. 1B; $P < 0.0001$, $R^2 = 0.83$). The correlation between the two instruments is well described by a polynomial curve: (SPAD = –0.0114 × CCI^2 + 1.4294 × CCI +16.104; $R^2 = 0.92$).

Results for the three chlorophyll determination methods (SPAD, CCI, Chl_{a+b}) and for leaf N concentrations are compared with irrigation water N in Fig. 2. SPAD, CCI, and Chl_{a+b} all revealed a significant increase in value with the increase in irrigation N concentration from 10 to 75 mg·L^{-1}, and a plateau between an irrigation N concentration of 75 and 150 mg·L^{-1} (Fig. 2A–C). Leaf N concentration determined over a full year (2023) ranged between 1.6% and 3.4%, with the most variation in leaf N occurring as irrigation N concentration increased between 10 and 75 mg·L^{-1}. An additional small, yet significant, increase in leaf N occurred between 75 and 150 mg·L^{-1} N in the irrigation solution (Fig. 2D). None of the chlorophyll methods detected the small increase in leaf N (from 3.2% to 3.4%) that occurred between irrigation N of 75 and 150 mg·L^{-1}, which is a large increase in the irrigation N load. A regression of chlorophyll concentration vs. leaf N concentration demonstrates this feature very clearly (Fig. 3).

Discussion

Optical chlorophyll concentration measurements were highly correlated to the in vitro analysis. Chlorophyll concentration in the leaves increased significantly between the N10 and N75 treatments, as measured by all methods. Leaf N concentration also increased with increasing N and flattened after N75 treatment, although there was a small but significant increase in leaf N at the highest irrigation N level (the N150 treatment).

In this experiment, not only did we find a significant response of leaf chlorophyll concentration to N fertilization, but also the

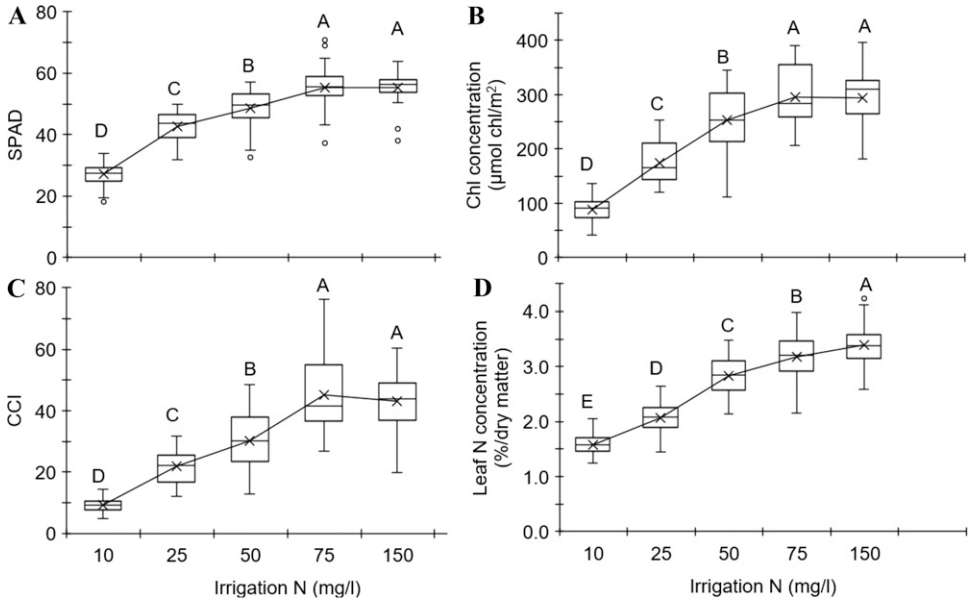

Fig. 2. Effect of irrigation water nitrogen (N) concentration on leaf soil plant analysis development (SPAD) units (**A**); leaf chlorophyll (Chl) a and b concentrations (**B**); leaf chlorophyll content index (CCI) units (**C**); leaf N concentration (**D**) during 2023. For SPAD, Chl_{a+b} and CCI, n = 30 for each treatment. For leaf N, n = 84 for each treatment. X marks the mean value, the horizontal line denotes the median, and the box encloses all measures between the 25th and 75th percentiles. Whiskers represent the full data range and the small circles beyond the whiskers denote outliers. Outliers are defined as 1.5× the interquartile above the third quartile or below the first quartile. Letters denote significant differences using the Tukey–Kramer honestly significant difference test at $P < 0.05$.

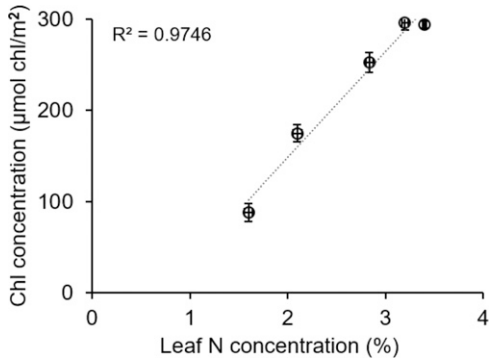

Fig. 3. Leaf nitrogen (N) concentration vs. leaf chlorophyll $(Chl)_{a+b}$ concentration. Error bars represent the standard error along both axes. Linear regression of all five points gives a correlation coefficient $R^2 = 0.9746$.

chlorophyll response represented leaf N concentration very well, up to 3.2% N in leaves. Beyond this leaf N concentration, neither the direct chlorophyll determination method nor the proxy optical methods were sensitive to increasing leaf N. This suggests that the extra leaf N accumulated under very high-N content fertilizer inputs is not converted to chlorophyll, which may have a maximum, but to alternative N-containing compounds such as proteins, amino acids, or soluble nitrate. Doubling fertilizer N from 75 to 150 $mg \cdot L^{-1}$ had only a small influence on leaf N, indicating a limit to total N uptake in the leaves at very high N loads.

Nitrogen concentrations in the leaves subjected to the N10 treatment are considered deficient, whereas those of the N25 treatment are defined as low (de Geus 1973; Murray 1957; Wessel 1971). According to the definitions of the authors just listed, leaf N concentrations in the N50 and N75 treatments are in the normal to high range. The trees in those treatments indeed displayed many signs characteristic of good nutritional status, including early flowering, greater flowering intensity, and increased fruit set (Weinstein et al. 2024). In comparison, trees in the low N treatments (N10 and N25) exhibited various signs of physiological stress, including substantially retarded flowering onset and flowering intensity compared with the optimal N treatments (Weinstein et al. 2024). A parallel experiment revealed that the transition to maturity was delayed under these low N fertigation treatments (Weinstein et al. 2025). Cacao chlorophyll followed leaf N in these ranges, and thus can guide fertilization to minimize N deficiencies. Leaf N concentrations of the N150 treatment were very high, and these trees exhibited some symptoms of excess N, such as decreased flowering and decreased fruit set compared with the N75 treatment (Weinstein et al. 2024). Yet in the case of excess N in cacao, leaf chlorophyll did not track with the higher leaf N, such that for precise fertilization in N-abundant environments, traditional leaf N analyses should supplement the chlorophyll methods.

As with other tropical wood species (Coste et al. 2010; Goncalves et al. 2008; Marenco et al. 2009) and other plants, such as sugar maple (Cate and Perkins 2003) and other crops (Parry et al. 2014), we found that the portable methods are a reliable proxy for chlorophyll concentration determination in cacao. The correlations between optical readings and extractable chlorophyll concentrations found here ($R^2 = 0.87$ and 0.83) are in the general range of correlations ($R^2 = 0.72$–0.95) for different tree species in other studies, confirming their overall general reliability for estimating chlorophyll concentrations.

Although this kind of correlation has not yet been published for cacao, portable chlorophyll measuring devices are commonly used in cacao. Daymond and Hadley (2004) found significant differences between chlorophyll concentration of different cacao genotypes using a Hansatech meter. They reported that the chlorophyll concentration exhibited a positive linear relation to temperature and a negative linear (or curvilinear) relation to the daily light integral. Similarly, Arévalo-Gardini et al. (2021) found an increase in chlorophyll concentration with increased shade when examining different cacao genotypes. Marenco et al. (2009) found a negative correlation between specific leaf area and SPAD, which was attributed mainly to differences in water content in different-size leaves. Posse et al. (2018) used a SPAD meter to evaluate the efficacy of different irrigation depths (measured in millimeters per day) in cacao. They reported a decrease in leaf chlorophyll concentrations as the irrigation depth increased, suggesting that more shallow fertigation depths were a more effective N delivery system for cacao seedlings.

Conclusion

The good linear correlations between results for both portable devices, the SPAD-502 and the MC-100, vs. the traditional destructive extraction method for determining leaf chlorophyll concentrations indicate the suitability of these portable devices for estimating chlorophyll in cacao leaves. The similarity between chlorophyll concentrations and leaf N, at least up to a 3.2% leaf N concentration, confirms that the portable devices provide a simple, reliable, and low-budget way for farmers to assess cacao tree N nutritional status. Chlorophyll analyses would be an important step toward precision agriculture in multiple farming regions where N limits productivity and compromises the sustainability of cacao farming.

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