NOTE

The Assessment of Leaf Nitrogen in Corn from Digital Images

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ABSTRACT

Environmental concerns of nitrate pollution coupled with the cost of N fertilizers have led to increased interest in assessing plant N status. Our objective was to use a digital camera and image-analysis software to assess leaf N concentration in corn (*Zea mays* L.) leaves from the association between leaf N and green color of chlorophyll. In greenhouse experiments conducted at Fayetteville, AR, in 2008 and 2009, digital photographs of the uppermost collared leaf of 3- to 5-leaf corn plants grown over a range of soil N treatments were processed into a dark green color index (DGCI), which combines the hue, saturation, and brightness into one composite number. Soil plant analysis development (SPAD) and DGCI values agreed closely across both years with $r^2 \geq 0.91$. There was a close relationship (r^2 ranged from 0.80 to 0.89) between DGCI and leaf N concentration. Yellow and green disks of known DGCI values were successfully used as internal standards to correct for differences in color sensitivity among cameras. Similarly, DGCI standard disks were able to correct for differences in lighting conditions for corn grown in the field. Determination of leaf N concentration in corn by digital image analysis offers a potential new tool for assessing corn N status.

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Abbreviations: DGCI, dark green color index; HSB, hue, saturation, and brightness; NDVI, normalized difference vegetation index; RGB, red, green, and blue; SPAD, soil plant analysis development.

Increased N use and cost have produced a need to increase fertilizer use efficiency in row crops. Nitrate runoff and leaching have become important issues around farmlands where nonleguminous crops are grown. Researchers have begun to implement indirect sensing tools such as chlorophyll meters, reflectance measurements, and color analysis to try to achieve near-optimal yields while reducing N inputs and minimizing N loss.

Hand-held chlorophyll meters are devices that clamp to leaves and instantly measure absorbance in the red (around 660 nm) and in the near-infrared (around 940 nm) regions of the spectrum. Chlorophyll strongly absorbs in the red region, and absorbance in the near-infrared region serves as a reference wavelength that is used to correct for differences in leaf thickness, water content, etc. The readings from these meters are closely related to the relative chlorophyll concentration (Richardson et al., 2002).

Values from the chlorophyll soil plant analysis development (SPAD) meter (Minolta Camera Co., Osaka, Japan) are closely related to the N status of crops such as corn (*Zea mays* L.) (Blackmer and Schepers, 1995; Varvel et al., 1997; Fox et al., 2001) and wheat (*Triticum aestivum* L.) (Jia et al., 2007). Most research for these devices has used strip-calibration techniques, in which

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there is a well fertilized strip within a field, and SPAD values from the rest of the field are expressed relative to the N-rich strip; however, the chlorophyll meter is not effective in estimating N concentrations above the optimum N concentration (Zhang et al., 2008). Chlorophyll meters are also costly (~$1500), have a small sampling area (6 mm^2), and are subject to operator bias (Blackmer and Schepers, 1995). These problems have slowed their adoption by growers. Nevertheless, the SPAD meter has been used to diagnose N deficiencies and to help make in-season predictions of yield losses (Scharf and Lory, 2002).

Reflectance measurements are also used to evaluate crop N status by determining the ratio of the amount of radiation reflected by an individual leaf or canopy at specific wavelengths to the amount of incident radiation (Schroeder et al., 2000). This method gives the option of hand-held (Graeff and Claupein, 2003; Ma et al., 1996), on-the-go, vehicle-mounted sensors (Bausch and Duke, 1996; Scharf and Lory, 2009) and aerial measurements (Blackmer et al., 1996; Scharf and Lory, 2002). Schroeder et al. (2000) found that parameters in which the reflectance of red (or green) and near-infrared light was combined appeared to be the best integrator of leaf greenness and leaf mass. The most commonly used measurement of this type is the normalized difference vegetation index (NDVI). Similar to SPAD techniques, some NDVI methods require a high N reference strip for calibration. The main negative issue when dealing with reflectance-measuring tools is their high cost.

In recent years, digital cameras and image-analysis programs have been used to quantify the "greenness" of foliage as indirect measurements of crop N status. Digital images record information as amounts of red, green, and blue (RGB) light emitted; however, the intensity of red and blue will often alter how green an image appears (Karcher and Richardson, 2003). To simplify the interpretation of digital color data, Karcher and Richardson (2003) suggested converting RGB values to the more intuitive hue, saturation, and brightness (HSB) color spectrum, which is based on human perception of color.

Karcher and Richardson (2003), working with quality of turfgrass in response to N fertilizer, processed HSB values into a single measure of dark green color, the dark green color index (DGCI). Dark green color index values were a more consistent measure of green color than were individual RGB values across all turf varieties and N treatments. Dark green color index was calculated as:

$$\text{DGCI value} = [(\text{Hue} - 60)/60 + (1 - \text{Saturation}) + (1 - \text{brightness})]/3, \quad [1]$$

Plant color has potential to indicate N status of corn and other nonleguminous row crops accurately when the correct methods are applied. Differences in lighting conditions, camera quality, and available camera settings could affect DGCI values and limit their utility in diagnosing N deficiencies; furthermore, factors such as disease, water status, nutritional deficiencies other than N, or differences in hybrid may affect greenness regardless of N status.

The main objectives of our research were to (i) evaluate the relationship among chlorophyll content (SPAD), DGCI, and leaf N concentration and (ii) develop a technique to correct for differences in camera quality and lighting conditions by using internal standards of known color. An underlying goal of this research was to ensure that specialized skills or an expensive camera was not required to make the digital images.

MATERIALS AND METHODS

Greenhouse Experiment 2008

A greenhouse study was conducted to compare SPAD, leaf N concentration, and DGCI in corn. Seeds of G90 sweet corn were sown on 8 Jan. 2008 in 25-cm-diameter pots with an approximate soil volume of 3 L. The potting medium was a mixture of two-thirds LB2 potting mix (a vermiculite, perlite, and peat mix; Sun Gro Co., Bellvue, WA.) and one-third sifted Captina Silt Loam soil (fine-silty, siliceous, active, mesic Typic Fragiudults) obtained from the Arkansas Agriculture and Extension center in Fayetteville, AR. Overhead lamps on a 16 h photoperiod provided approximately 200 μmol cm^{-2} s^{-1} photosynthetically active radiation at plant height and supplemented natural illumination. Day and night temperatures were 26 ± 4°C and 20 ± 4°C, respectively. Four seeds were planted per pot and thinned to two plants after emergence. Pots were supplied N (as NO_3^-) from 2 L of a modified Hoagland solution (de Silva et al., 1996) containing 0, 168, 336, 504, 672, or 840 mg N. Solution pH was adjusted to 6.8 before being applied to a 30-cm saucer underneath the pots. All pots were kept well watered using deionized water. The experimental design was a randomized complete block with two replications.

Sampling Procedures

The upper-most collared leaf (youngest leaf with a ligule) was removed from 3- to 5-leaf plants. Leaves were placed against a black felt background (under fluorescent lighting) and photographed from a stationary camera of 58 cm and at an angle of approximately 40° offset from vertical. An Olympus C-3030 (Olympus America Inc., Melville, NY) digital camera with an image size of 1280 × 960 pixels was set to an ISO of 100, a shutter speed of 1/15 s, an aperture of 2.0, exposure compensation of 0, and to fluorescent white balance with the flash turned off.

Images were saved as JPEG (joint photographic experts group) files, renumbered using Faststone Image Viewer (FastStone Soft, 2011), and processed using Sigma Scan Pro (v. 5.0; SPSS Science, 1999) and a macro similar to that described by Karcher and Richardson (2003). Threshold ranges in Sigma Scan were set at 30 to 120 for hue and 27 to 100 for saturation. The macro scanned each image and analyzed individual objects within the threshold values to give the average weighted RGB values for each image. Using the procedure described by Karcher and Richardson (2003), the RGB values were then converted to HSB values. The HSB values were used to calculate DGCI values (Eq. [1]), which encompass dark green color on a scale of zero to one with values closer to one representing a darker green (Karcher and Richardson, 2003).

After leaves were photographed, three SPAD (Spectrum Technologies. Inc., Plainfield, IL) readings were taken from each leaf and averaged. Leaves were dried at 85°C until weight was constant, ground through a 20 mesh screen, and analyzed for total N via Micro-Dumas combustion by the Soil Test and Plant Analysis Laboratory at the University of Arkansas (Fayetteville, AR). Relationships among DGCI, leaf N concentration, and SPAD were evaluated by linear regression (PROC GLM; SAS Institute. 2003) and segmented linear regression (PROC NLIN).

The segmented linear regression occurred in two steps. In the first step, the regression procedure tested if there was a change in slope over the range of values and determined at what point (*x*-value) a change in slope occurred. The procedure also tested if the slope and intercept values were significant above and below the break point. If the slope above the breakpoint was not significant in the first step, the procedure was repeated, but in the second step the slope above the breakpoint was assigned a value of 0 and the regression procedure determined new coefficients and a new breakpoint value.

Greenhouse Experiment 2009

An additional greenhouse study was sown 5 Feb. 2009 to further compare DGCI values with SPAD and leaf N concentration as well as to evaluate the ability to calibrate cameras of varying quality using standardized color disks within images. Experimental design, treatment structure, and sampling procedure were similar to 2008 except for number of cameras used, background color, threshold settings and calibration procedure.

Leaves were photographed against a pink felt background under normal fluorescent lighting. When leaves were photographed against a black background, small reflections were detected within the threshold settings of the imaging software. Changing the image background to pink allowed us to expand threshold ranges and eliminate the false-positive reflections. Three cameras were used to evaluate internal standards for camera calibration: a Canon Power Shot S51S (Canon U.S.A., Inc., Lake Success, NY.) with an image size of 3264 × 2448 pixels (Camera A), a Fuji Fine Pix A400 (Fuji Photo Film Co., Ltd., Minato-Ku Tokyo, Japan) with an image size of 2304 × 1536 pixels (Camera B), and a Canon Power Shot A20 (Canon U.S.A., Inc. Lake Success, NY.) with an image size of 1600 × 1200 pixels (Camera C). Threshold ranges in Sigma Scan were 7 to 126 for hue and 0 to 100 for saturation, which allowed for complete analysis of the entire leaf without including any of the background in the final leaf color value.

All of the cameras that we used had sensors based on the widely-used CCD (charged coupled device) platform. Sensor size for each camera was 21.7 mm^2 (Canon Power Shot A20), 24.7 mm^2 (Fuji Fine Pix A400 and Canon Power Shot S51S), and 38.2 mm^2 (Olympus C-3030).

Evaluation of Standards to Correct for Camera Differences

Internal standards were included in each image to account for differences in lighting and camera quality. Standards consisted of green and yellow 9-cm-diameter circular disks that were cut from X-Rite color paper (X-Rite Inc., Kentwood, MI). Standards had known Munsell color values of 6.7GY 4.2/4.1 for the green disk and 5Y 8/11.1 for yellow, which gave HSB values of 91, 38, and 42 and 66, 88, and 100, respectively, for the disks. Munsell notation was converted to HSB values using the online color basic conversion package (Van Aken, 2006). Values of HSB resulted in disk DGCI values of 0.0733 (yellow) and 0.5722 (green).

Disks were placed a minimum of 4 cm from any leaf because if the disks were closer than 4 cm the software would not consistently separate the calibration disks from leaves. Disks were placed the field of view closest to the camera to minimize distortion of the disk shape from circular to oval. This was important because the macro recognized the disks by their circular shape. An axis ratio of the largest and smallest section of the object was used to determine shape. The macro used in the experiment was developed to analyze each object in the image separately and give individual values on a separate line in an Excel (Microsoft Corporation, 2007) spreadsheet. After all the objects were scanned, the macro excluded values that were near circular (length to width ratio ≥ 0.8) and averaged all other values, giving one final leaf DGCI value. A separate DGCI value was calculated for each of the calibration disks. Corrected DGCI values for each image assumed that the relationship between observed and known DGCI values from the cameras was a simple linear response. The first step in calibration was to determine the difference between observed DGCI values and the known DGCI values for the green and yellow standard disks. The slope of this relationship was:

$$\text{Slope} = (\text{Known Green DGCI} - \text{Known Yellow DGCI}) / (\text{Observed Green DGCI} - \text{Observed Yellow DGCI}), \quad [2]$$

Once the slope was determined, the Y intercept was calculated as:

$$\text{Y intercept} = \text{Known Yellow DGCI} - (\text{Slope} \times \text{Observed Yellow DGCI}), \quad [3]$$

The corrected DGCI, based on the internal standards, was then calculated as follows:

$$\text{Corrected leaf DGCI} = (\text{Slope} \times \text{Observed Leaf DGCI}) + \text{Y intercept}, \quad [4]$$

Differences in DGCI response to leaf N among cameras before and after calibration with internal standards were evaluated by covariate analysis in which individual cameras served as the covariate. The covariate analysis allowed for a statistical test among cameras for differences in the slope and intercept of DGCI versus leaf N concentration.

Evaluation of Standards to Correct for Lighting

To evaluate the effectiveness of the DGCI color disks for correcting for differences among lighting conditions, corn leaves were collected from plots of a field experiment evaluating a wide range of N treatments. Seeds of Pioneer hybrid 33D49 were sown on 29 Apr. 2010 on a Captina Silt Loam soil (fine-silty, siliceous, active, mesic Typic Fragiudults) in four-row plots 0.95 m apart and 7.6 m in length. Urea was hand applied (0, 85, and 170 kg N ha^{-1}) to plots of 3-leaf corn and immediately sprinkle irrigated (~12 mm). At the 5-leaf stage, an uppermost collared leaf was removed from five replicated plots of each treatment, placed in plastic bags on ice, and

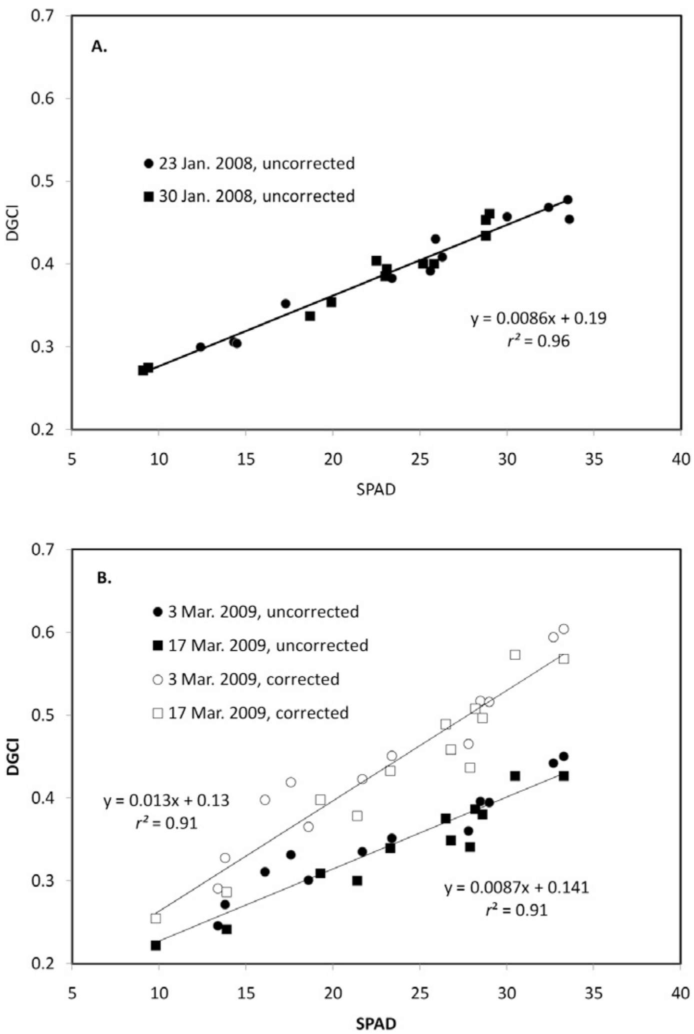

Figure 1. The relationship between dark green color index (DGCI) and soil plant analysis development (SPAD) for two sampling dates in 2008 (Panel A) and in 2009 (Panel B). In 2008, DGCI values were not corrected using standard color disks that served as internal standards. In 2009, values are shown before and after correction using calibration disks.

photographed indoors under fluorescent and incandescent lighting and then photographed outdoors under full sun and shaded conditions. All photographs were taken between 1000 and 1300 h on a day with few clouds. Included within each image were the green and yellow calibration disks of known color. The camera (Canon Power Shot S51S, Canon U.S.A., Inc., Lake Success, NY) was set to the auto function, which automatically adjusts focus and lighting. Simple linear regression was used before and after correction with the standard disks to assess the effectiveness of the internal standards for correcting differences in lighting.

RESULTS

Response of Soil Plant Analysis Development and Dark Green Color Index to Leaf Nitrogen Concentration

When data for the two measurement dates within a year were combined, there was a close linear relationship ($r^2 \geq 0.91$) between DGCI and SPAD values for both the 2008 and 2009 experiments (Fig. 1a and 1b). In 2008, DGCI values were not corrected using the internal color standards and slope and intercept values from the regression of DGCI and SPAD (Fig. 1a) were similar to those for the uncorrected DGCI regression coefficients in 2009 (Fig. 1b). Regression of corrected DGCI versus SPAD values in 2009 increased responsiveness (i.e., slope) compared to the uncorrected DGCI versus SPAD values (Fig. 1b).

In 2008, the response of DGCI to leaf N concentration reached a plateau at 2.06 (SE = 0.19; 23 January) and 1.72 (SE = 0.14; 30 January) g N 100 g^{-1} and could no longer differentiate treatment differences (Fig. 2). Segmented linear regression predicted a linear increase in DGCI (Fig. 2a) as N concentration increased to these values, but constant DGCI values (0.42 and 0.45) were observed above this N concentration. Similar responses were observed for the relationship between SPAD and leaf N concentration (data not shown). In 2009, DGCI values were linearly associated with leaf N concentration for the first ($r^2 = 0.80$) and second ($r^2 = 0.88$) sampling dates (Fig. 2b). From the first sampling date to the second, there was a decrease in leaf N concentration. As the leaf N concentration decreased from the 3 March to the 17 March sampling dates, the responsiveness (i.e., slope) of DGCI approximately doubled. Similar responses were observed for the relationship between SPAD and leaf N concentration (data not shown).

Camera Calibration

Covariate analysis was used to evaluate the ability of the color standards to adjust DGCI values for differences among cameras. By using camera as a covariate, we were able to test for differences among cameras with and without correction of the standard disks. Uncorrected DGCI values among different cameras regressed against leaf N concentration showed large differences in Y intercept values ($P < 0.01$), but DGCI response (i.e., slope) did not differ (Fig. 3a; $P < 0.69$). When corrected DGCI values were evaluated, there were no differences in Y intercept ($P < 0.79$) or slope (Fig. 3b; $P < 0.86$). Similar results were obtained for a second sampling date (data not shown). These analyses show that the calibration standards were able to remove differences in the responsiveness of DGCI values to leaf N from different cameras.

After correction of DGCI values using the calibration disks, there were slight differences in DGCI values that were consistent for the individual cameras, even though statistically there was no difference in response of DGCI regressed against leaf N concentration (Fig. 3b). A direct comparison of the effectiveness of the calibration procedure was to evaluate DGCI values from camera B and C against DGCI values of camera A. A simple linear regression of uncorrected DGCI values of camera B and C plotted against uncorrected DGCI values of camera A had an

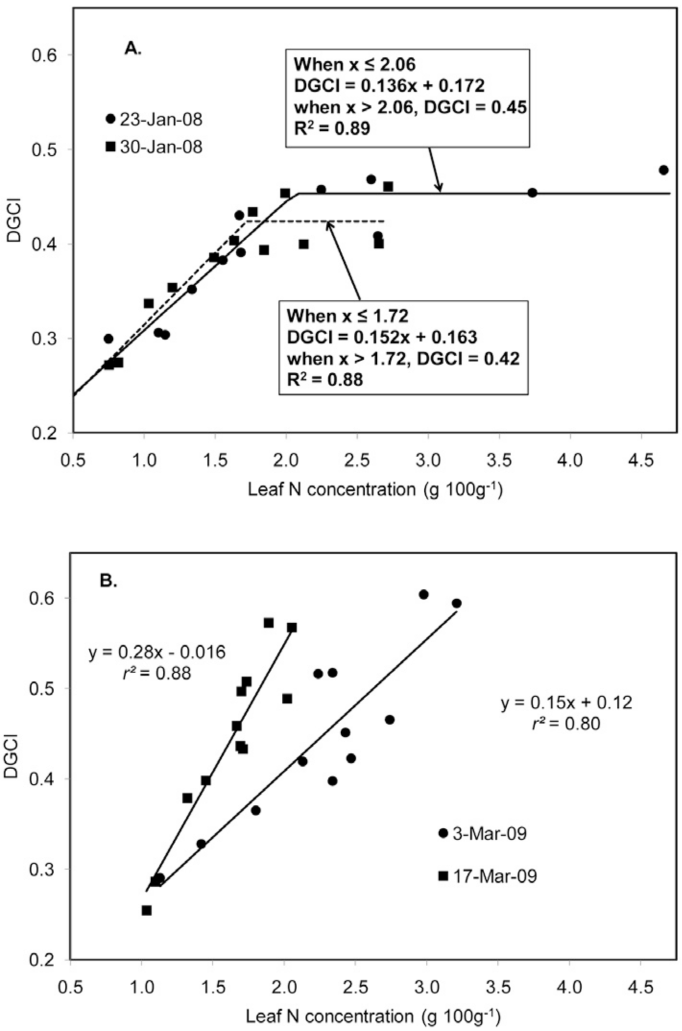

Figure 2. Dark green color index (DGCI) versus leaf N concentration for plants sampled 23 Jan 2008 (Panel A). Segmented linear regression was used to determine the breakpoint at which the slope changed. Panel B shows the response of dark green color index to leaf N concentration for sampling dates 3 and 17 Mar. 2009.

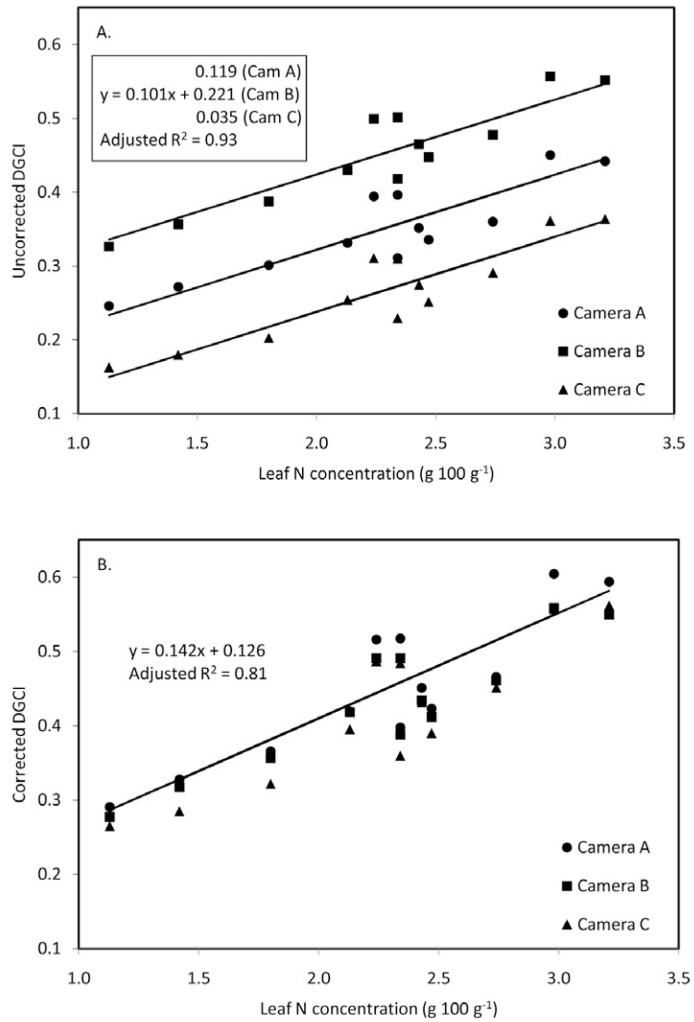

Figure 3. Uncorrected dark green color index (DGCI) values for three cameras versus leaf N concentration (Panel A). Corrected DGCI values versus leaf N concentration (Panel B). Cameras were used as covariates to determine if there were statistical differences in slope and intercept before and after correction with calibration disks.

r^2 value of 0.33 and a RMSE of 0.055 (Fig. 4a). After correction, the r^2 value increased to 0.98 and the RMSE was decreased to 0.025 (Fig. 4b). These comparisons of corrected and uncorrected linear regressions indicate that, although there were slight differences among cameras following correction, the error was greatly reduced and the precision was greatly increased.

Lighting Calibration

In a similar manner, simple linear regression was used to evaluate the ability of color standards to adjust for different light sources. Uncorrected DGCI values for all light sources were plotted against uncorrected DGCI values for fluorescent light (considered our standard), and the regression had an r^2 of 0.38 (Fig. 5a). Corrected DGCI values for all light sources were then plotted against corrected fluorescent DGCI values, and the r^2 value was 0.85 (Fig. 5b). Using calibration standards did not fully account for all of the variation introduced by the different light sources, but the variability among light sources was decreased substantially.

DISCUSSION AND CONCLUSIONS

The close association between DGCI and leaf N provides an additional tool for the assessment of leaf N concentration in corn. Our research is consistent with previous work by Karcher and Richardson (2003) who found that DGCI values were able to differentiate among turf cultivars receiving various N treatments. Karcher and Richardson (2003) largely eliminated differences among cameras and differences in lighting conditions by measuring with the same camera under full illumination between 1300 and 1500 h. In the present research, the inclusion of disks of known color largely eliminated variation due to camera and lighting conditions.

Values of SPAD, which were closely related to leaf N in previous research (Blackmer and Schepers, 1995; Fox et al., 2001), were also closely associated with DGCI

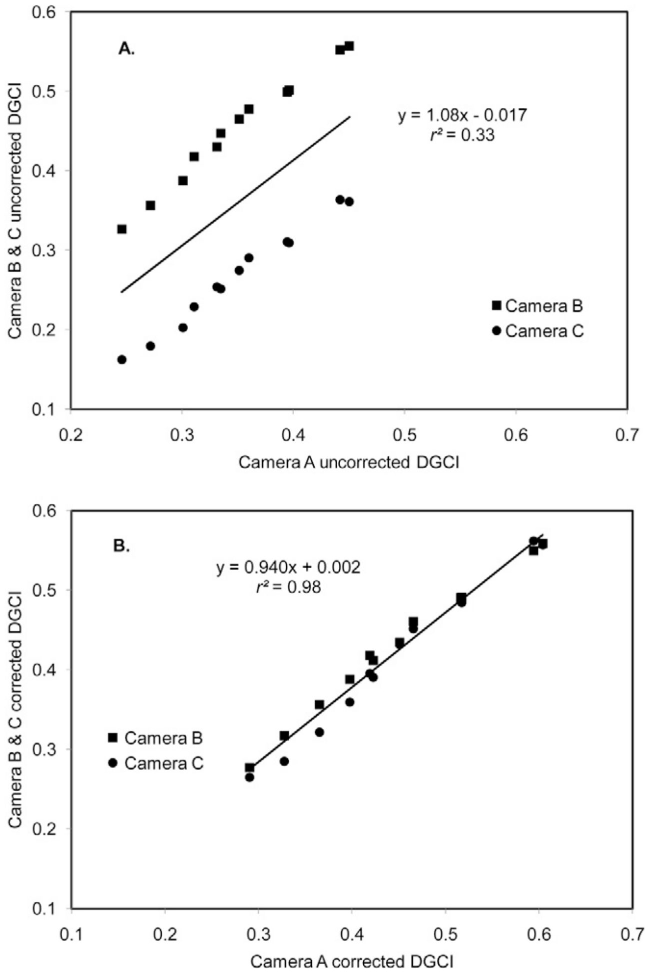

Figure 4. Uncorrected dark green color index (DGCI) values for cameras B and C versus uncorrected DGCI values of camera A (Panel A). Corrected DGCI values for cameras B and C versus corrected DGCI values of camera A (Panel B).

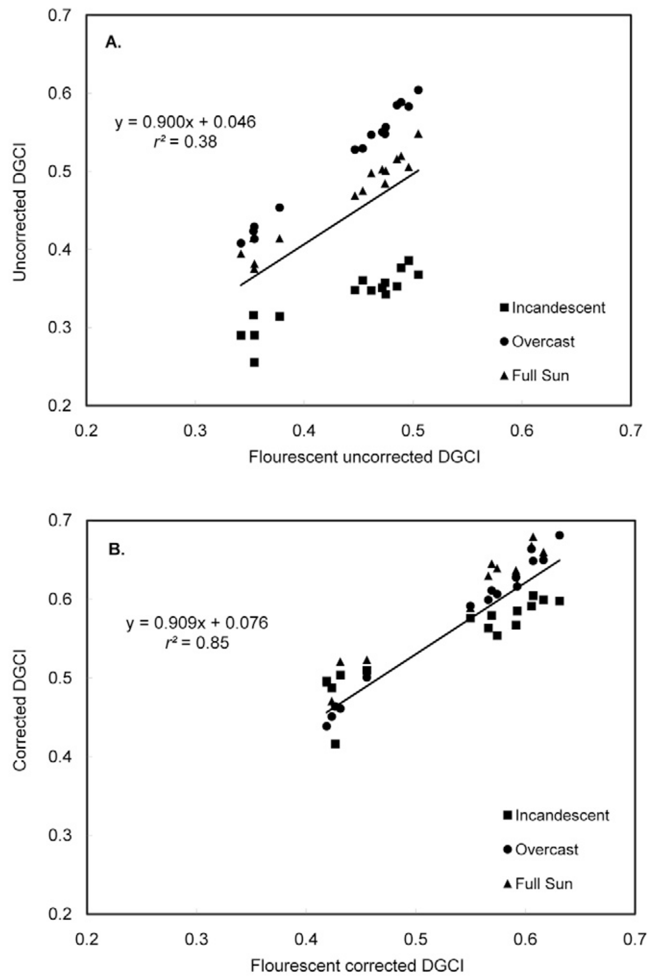

Figure 5. Uncorrected dark green color index (DGCI) values for images taken under different light sources (incandescent, full sun, and shade) versus uncorrected DGCI values for fluorescent lighting (Panel A). Panel B shows corrected DGCI value for the same light sources versus corrected DGCI values of images taken under fluorescent lighting.

($r^2 \geq 0.91$) and leaf N concentration ($r^2 = 0.71$ to 0.85; data not shown). These results show that digital color analysis has the potential to be used in the same way as a chlorophyll meter but at a fraction of the cost.

In the 2008 experiment, the response of both DGCI and SPAD reached a plateau at leaf N concentrations above ~1.7 to 2.1 g N 100 g^{-1}. In 2008, experiments were established in early January (average outside temperature −6.7°C), and in 2009 experiments were established in early and mid March (average outside temperature 10°C). Although the greenhouses were heated, ambient greenhouse temperatures were considerably warmer for the March 2009 experiment. McWilliam and Naylor (1967) showed that a drop in temperature from 28 to 16°C would substantially affect chlorophyll concentrations in corn seedlings. They found that the decrease in chlorophyll at lower temperatures was due to the photo-destruction of chlorophyll before it was stabilized in the membrane structure of the chloroplast lamellae. The drop in chlorophyll at low temperatures could possibly serve as an explanation for the plateau, which was not seen in 2009 greenhouse studies. Additionally, in field experiments in 2008 and 2009, there was no plateau observed in the responses of DGCI and SPAD to leaf N concentration (Rorie et al., 2010).

Rorie et al. (2010) demonstrated that values of DGCI at tasseling were closely associated with corn grain yield, which is similar to relationships others have found (e.g., Scharf et al., 2002) between SPAD and corn grain yield. Digital color analysis may allow an ordinary digital camera to be used as a tool for assessing corn N status. With further refinement, it may be possible to e-mail digital photographs directly from a cell phone to a technician for N assessment or to upload digital photographs to a server for evaluation. More research is required to determine if DGCI values could be used to diagnose N fertilizer needs and to determine the developmental stages at which these measurements would be most useful.

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