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Assessment of Photosynthetic Pigment and Water Contents in Intact Sunflower Plants from Spectral Indices

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Abstract: Under water-limited conditions, monitoring water and chlorophyll status is essential to avoid restrictions in crop growth and yield. This study was carried out to assess water and chlorophyll contents from spectral indices in sunflower plants. The hybrid Sunbright Supreme was cultivated inside a non-acclimatized greenhouse until the start of the flowering stage, and later was maintained in a growth chamber with the purpose of submitting the plants to a slow and progressive dehydration rate for 12 consecutive days. Spectral (reflectance and transmittance), leaf masses (fresh and dry), and total chlorophyll measurements were accomplished in sunflower plants. The water stress caused a reduction in the water and chlorophyll contents, resulting in linear and nonlinear decreases for the spectral indicators Water Index (WI) and Chlorophyll Content Index (CCI), respectively. The low scattering of the average values around the fitted models indicates that WI and CCI were effective in representing changes in water and chlorophyll status for sunflowers (R^2 = 0.912 and R^2 = 0.905). The benefits of using hand-held optical meters for reflectance and transmittance are that they enable rapid, accurate, and nondestructive assessments of water and chlorophyll contents in sunflower plants from radiometric indicators.

Keywords: *Helianthus annuus* L.; reflectance; transmittance; chlorophyll; water stress

1. Introduction

The increasing worldwide shortages of water is leading to an emphasis on developing innovative technologies that make it possible to maximize the water use efficiency and to improve the productivity of crops.

Water is a fundamental constituent of plants for maintaining leaf structure and shape, photosynthesis, and thermal regulation [1]. Chlorophylls are the most important photosynthetic pigments in plants [2]. The amount of chlorophyll per unit leaf area is an indicator of the overall condition of plants. According to Wu et al. [3] and Hawkins et al. [4], the determination of the water and chlorophyll contents can be used to detect and study photosynthetic activity, stress conditions, nutritional status, and physiological changes in crops over time. Furthermore, they can be applied to improve the efficiency of fertilization and irrigation managements.

The comprehension of the physiological mechanisms of plants under drought conditions from the assessment of water and chlorophyll status is very important in terms of developing selection and breeding strategies [5], since water stress is one of the most common limitations of crop growth and yield.

Although the traditional methods of measuring water and chlorophyll contents in leaves are reliable, they are destructive, time-consuming, labor-demanding, and expensive. Alternatively, chlorophyll and water contents can be nondestructively estimated in leaves through optical methods. Spectroscopy has become popular in ecophysiological studies due to its simplicity, sensitivity, rapidity, and nondestructive nature [6].

Spectral indices have been developed with the purpose of reducing complex spectra to a single value, combining reflectance, absorbance, or transmittance in specific wavelengths that are more sensitive to the biochemical components in leaves. Several spectral indices have been developed for the prediction of chlorophyll and water contents in different plant species [3,7–9]. However, some spectral indices have been presented low correlations when related to the chlorophyll and water contents measured by traditional methods.

Optical equipment with high spectral resolution allow examining adaptation physiological mechanisms of plants under stress conditions, which occur on fine temporal and spatial scales. Beyond predicting water and chlorophyll contents, it is also possible to identify composition of single seeds [10], differences and similarities between plants of same species [11], nutritional levels of plants [12], senescence stages in flowers [13], soil properties related to mineral deficiency in plants [14], and bruises caused by impact or mechanical damage in fruits [15] based on spectral measurements.

In this context, the present study was carried out to assess water and chlorophyll contents from spectral indices in sunflower plants under drought conditions.

2. Materials and Methods

2.1. Greenhouse and Growth Chamber for Sunflower Cultivation

The hybrid Sunbright Supreme was chosen for this study because it is uniform and vigorous in growth, offering earlier flowering, shorter and stronger stems, and pollenless flowers with bright golden petals.

Sunflower seeds were germinated in commercial substrate (pine bark, vermiculite, and peat) contained in plastic pots with a capacity of 905 cm^3. The pots were disposed in a non-acclimatized greenhouse (arch type) with a density of 0.25 plant m^{-2}. The greenhouse was constructed at the experimental area of the Federal University of São João del-Rei, Sete Lagoas, Minas Gerais, Brazil, with the following geographical coordinates: 19°28′34″ S latitude, 44°11′44″ W longitude, and 796 m elevation. According to Köppen's climate classification [16], the local climate is humid subtropical with dry winter and hot summer (Cwa).

Suitable agronomic practices of management, fertilization, and irrigation were followed for sunflower cultivation. The plants were fertigated with a nutrient solution prepared from granulated fertilizer (Peters Excel, Scotts, Marysville, OH, USA), with a formulation of 15–5–15–5–2 (N–P–K–Ca–Mg), containing 200 mg of nitrogen per liter of water and resulting in a 1·dS·m^{-1} electrical conductivity. The irrigation water (0.07 dS·m^{-1} electrical conductivity) was automatically supplied to plants by a time-controlled drip system.

The strategy of fertigation and irrigation events was pre-established so that two daily fertigations or irrigations (50 mL·$event^{-1}$) per plant were performed at 10:00 and 15:00 h. Thus, the fertigation and irrigation events were always accomplished on alternate days. This procedure provided a proper nutrient balance in the substrate and prevented root salinity [17].

At the start of the sunflower flowering stage, 60 days after seeding occurred in January 2016 (summer season), a final irrigation event was accomplished at the early morning with the purpose of maximizing the substrate moisture. From this day, no more fertigation events were applied. Sunflower plants were transported to a reach-in grown chamber equipped with actuators (heating, cooling, fogging, and lighting systems) and sensors (photometric, pyranometer, air temperature, and relative humidity) with the purpose of submitting the plants to a slow and progressive dehydration rate for 12 consecutive days. The chamber was programmed to control internal conditions for 1600 fc (foot-candle, 17.2 klux) illumination, 12 h photoperiod, 30 to 25 °C (day–night) temperature, and 40% humidity.

2.2. Sunflower Measurements under Drought Conditions

The plant water content was associated with spectral reflectance and leaf mass (fresh and dry) measurements. On the other hand, the plant chlorophyll content was related to spectral transmittance and total chlorophyll measurements. Four sunflower plants (healthy, vigorous, and well-formed) were randomly selected from day to day during the water stress period. Spectral measurements were made in eight leaves of each plant, four for reflectance and another four for transmittance. Three separate measurements on adaxial surface were performed in each leaf, avoiding its veins and boundaries.

The measuring equipment of the spectral reflectance was a miniature and hand-held spectrometer (JAZ-EL350, Ocean Optics, Dunedin, FL, USA) coupled to a tungsten-halogen light source. This equipment was preconfigured to acquire and store reflectance data in visible and near-infrared wavelength range (500–1000 nm), with a spectral resolution of 1.3 nm. A specific clip probe to collect reflected light from leaves (SpectroClip-R, Ocean Optics, Dunedin, FL, USA) was used for the nondestructive measurements. This probe contains an integrating sphere that captures diffuse reflected light more efficiently than lens-based collection optics. Two premium fibers (600 μm diameter) interconnected the spectrometer and the light source to the clip probe. A diffuse reflectance standard with Spectralon™ was used as a reference to measure spectral reflectance.

Daily, the warm-up time of the light source was waited and the reference standard measurement was made prior to the spectral reflectance measurements in sunflower leaves. The values were calibrated by means of the software SpectraSuite™ (Ocean Optics, Dunedin, FL, USA) and expressed as a relative percentage of the reference standard [18]:

$$\rho_\lambda = \left(\frac{S_\lambda^{\mathrm{leaf}} - D_\lambda^{\mathrm{dark}}}{\rho_\lambda^{\mathrm{reference}} - D_\lambda^{\mathrm{dark}}} \right) 100 \tag{1}$$

where ρ_λ is the spectral reflectance of the leaf (%), $S_\lambda^{\mathrm{leaf}}$ is the intensity of the reflected radiation by the leaf (dimensionless), $D_\lambda^{\mathrm{dark}}$ is the intensity of the reflected radiation considering light absence (dimensionless), and $\rho_\lambda^{\mathrm{reference}}$ is the spectral reflectance of the reference standard (dimensionless). All these parameters were applied considering a wavelength λ. Average spectral reflectance curves were generated per plant to analyze these measurements.

Reliable estimation of plant water status by spectral reflectance is dependent upon knowledge of most sensitive wavelengths. According to Claudio et al. [19], the ideal wavelengths for predicting water content are those with weak absorption, which allow the radiation to penetrate far into the leaves. Considering the wavelength range preconfigured in spectrometer, the reflectance at 970 nm (%) is the light absorption band more sensitive to leaf water content variations. The reflectance at 900 nm (%) is a reference, where there is no absorption by water but that is affected in the same way with respect to leaf structure [20]. For this reason, the radiometric indicator Water Index (WI) was chosen:

$$\mathrm{WI} = \frac{R_{900}}{R_{970}}. \tag{2}$$

Past studies have presented that WI provides a good indicator of the water content in the fine tissues of the canopy [21]. Additionally, this spectral index was used to estimate water vapor and carbon dioxide fluxes in chaparral vegetation [19], as well as to irrigation scheduling and to monitor vineyard performance [22].

Daily during the dehydration period, after the reflectance measurements, each leaf was cut at the base of the petiole and its fresh mass was obtained on an analytical balance (AL-500, Marte, São Paulo, SP, Brazil). Later, the leaves were individually placed in paper bags, dried at 70 °C in an oven (MA-035/1, Marconi, São Paulo, SP, Brazil) until a constant mass. Again, each leaf was weighed and its dry mass was obtained. The leaf water content (dry basis) was calculated dividing the difference of leaf fresh and dry masses by the leaf dry mass. Finally, the average water status per plant was determined from water content of leaves of the same plant.

Beyond the loss of turgidity, the water stress induces the chlorophyll degradation in leaves. For evaluating this injury, spectral transmittance measurements (%) in two distinct wavelengths (653 and 931 nm) were accomplished by the hand-held photometer (CCM-200, Opti-Sciences, Tyngsboro, MA, USA).

The red light at 653 nm is absorbed by chlorophyll, and its absorption is evidently correlated with this pigment content, whereas the near-infrared absorption at 931 nm is used as a reference to compensate for mechanical differences between leaves [2]. Thus, the radiometric indicator Chlorophyll Content Index (CCI) was computed:

$$\mathrm{CCI} = \frac{\mathrm{T}_{931}}{\mathrm{T}_{653}}. \tag{3}$$

The arithmetic averages of CCI measurements were calculated per plant for subsequent analysis.

The total chlorophyll content of the sunflower leaves was extracted with dimethyl sulphoxide (DMSO). The procedure was accomplished using glass vials containing 7 mL of solvent preheated to 65 °C in a water bath. Daily, the chlorophyll was extracted from four leaf disks (unit area 1.15 cm^2), equivalent to 100 mg of fresh tissue from each of the four leaves. When the extractions were completed (in the dark), the leaf disks were removed from the water bath, and each graduated vial was topped up to exactly 10 mL with DMSO. Later, 3 mL of each extract were transferred to disposable quartz cuvettes (path length of 10 mm). The absorbance at 649 and 665 nm wavelengths was measured using a workbench spectrophotometer (700S, Femto, São Paulo, SP, Brazil), with a resolution of ±1 nm and previously calibrated to zero absorbance using a blank of pure DMSO. Based on the measurements, total chlorophyll content was calculated using the equation proposed by Wellburn [23]:

$$\mathrm{C} = (18.54\mathrm{A}_{649} + 6.87\mathrm{A}_{665})\mathrm{V}/(1000\mathrm{M}) \tag{4}$$

where C is the total chlorophyll content ($\mathrm{mg{\cdot}g^{-1}}$), A_{649} is the absorbance at 649 nm (dimensionless), A_{665} is the absorbance at 665 nm (dimensionless), V is the volume of the extract (mL), and M is the mass of fresh tissue (g). The total chlorophyll content obtained from this equation was converted into mass per unit area ($\mathrm{mg{\cdot}cm^{-2}}$). Average values were calculated per plant to analyze the measurements.

Regression analyses were made by using the SigmaPlot software (Systat Software Inc., San Jose, CA, USA). Moreover, predicting equations and coefficients of determination were obtained. Statistical analyses (*t*-test statistic and *p*-value) were accomplished to check the significance of each equation parameter.

3. Results and Discussion

The response surface shows the average variations occurred in the spectral reflectance signatures of sunflower plants during 12 consecutive days of dehydration (Figure 1). Two continuous lines were detached in this figure, emphasizing the spectral reflectance fluctuations for the wavelengths of WI (900 and 970 nm). The spectrum sensitivity at 970 nm is due to the great penetration of radiation into the leaves at this wavelength.

The water stress caused by interrupting the irrigation events turns the internal leaf structure less capable of absorbing the electromagnetic radiation, propitiating greater radiation path deviations comparatively with what occurred with the turgid leaves [24]. Consequently, increases on the spectral reflectance values were observed as the leaf water loss was intensified. This occurred mainly at the 970 nm wavelength (more sensitive to leaf water content variations) in relation to the 900 nm wavelength (reference). Similar results were obtained by Peñuelas and Inoue [25] when evaluating reflectance indices associated with water and pigment contents of peanut and wheat leaves. Ullah et al. [1] proposed three indices for the retrieval of leaf water content in nine different plant species and verified that spectral reflectance increased as drought conditions were intensified.

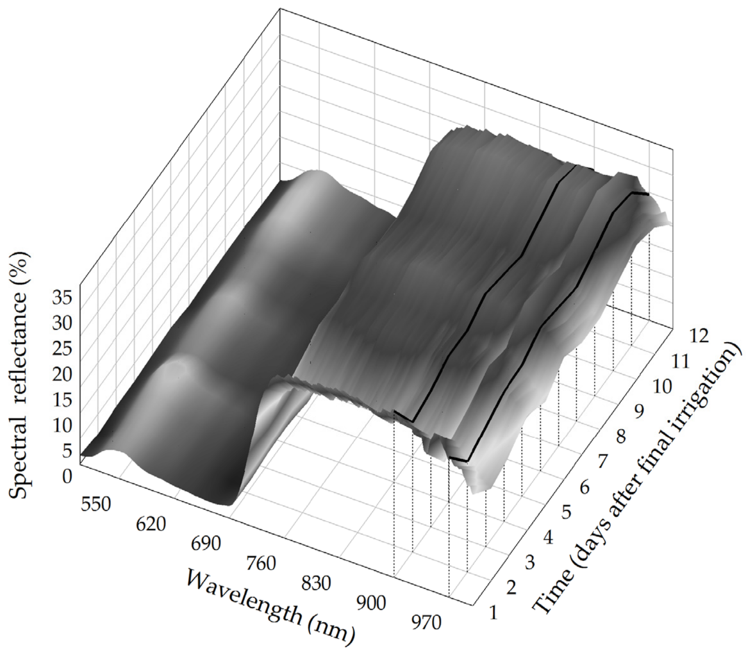

Figure 1. Spectral reflectance signatures of sunflower plants submitted to water stress for 12 consecutive days without irrigation, emphasizing the wavelengths associated with water index.

The regression between plant water content (dry basis) and water index is presented in Figure 2 for Sunbright Supreme variety under progressive dehydration. The plant water status reduction caused by hydric stress promoted a linear decrease of WI values. The low scattering of the average values around the fitted line (R^2 = 0.912) indicates that WI is effective to represent changes in sunflower water content. Rodríguez-Pérez et al. [7] used this spectral index for detecting the water content of grapevines in vineyards, founding R^2 of 0.810 and 0.725 for wet and dry basis, respectively.

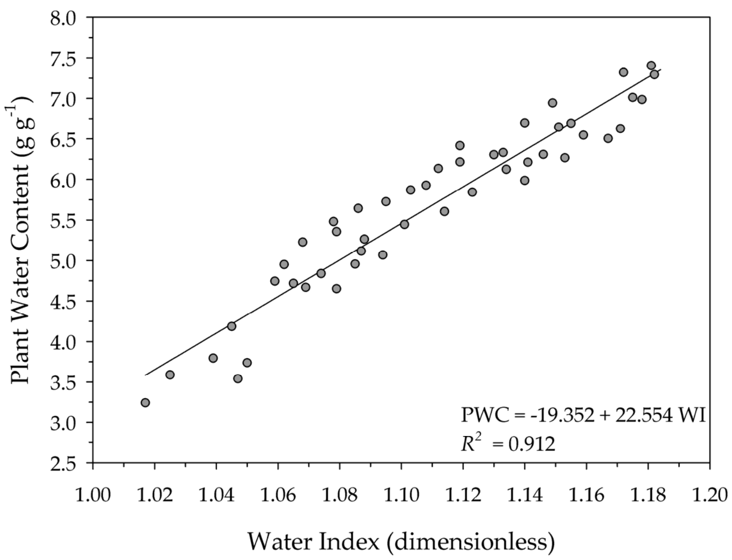

Figure 2. Linear regression between plant water content and water index obtained from measurements in sunflower leaves (Sunbright Supreme variety) under water stress conditions. The parameters of the regression model were significantly greater than zero ($p < 0.0001$).

After 12 days without irrigation under controlled climatic conditions in the grown chamber, the plant water content decreased by 56%, which represent severe water stress level.

Past scientific studies also evaluated plant water status from WI for distinct vegetation species under drought conditions (Table 1), agreeing with the variation range obtained for sunflower plants in this study.

Table 1. Water index range determined for different vegetation species.

Water Index Range	Vegetation	Reference
0.88–1.15	Trees, shrubs, and grasses	Peñuelas et al. [20]
0.99–1.03	Wheat and peanut	Peñuelas and Inoue [25]
0.96–1.23	Annual crops, vines, trees, and shrubs	Sims and Gamon [21]
0.93–1.10	Semiarid shrubland ecosystem (chaparral)	Claudio et al. [19]
0.97–1.03	Grapevine	Rodríguez-Pérez et al. [7]
0.96–1.04	Species of tropical forests	Cheng et al. [26]
1.01–1.18	Sunflower	In this study

Figure 3 presents the regression between total chlorophyll content and CCI for Sunbright Supreme variety under progressive dehydration. Similarly to WI, the plant chlorophyll status reduction caused by water stress promoted decrease of the CCI values. However, this decline followed a nonlinear and slightly convex trend, as reported in previous scientific articles [27–30].

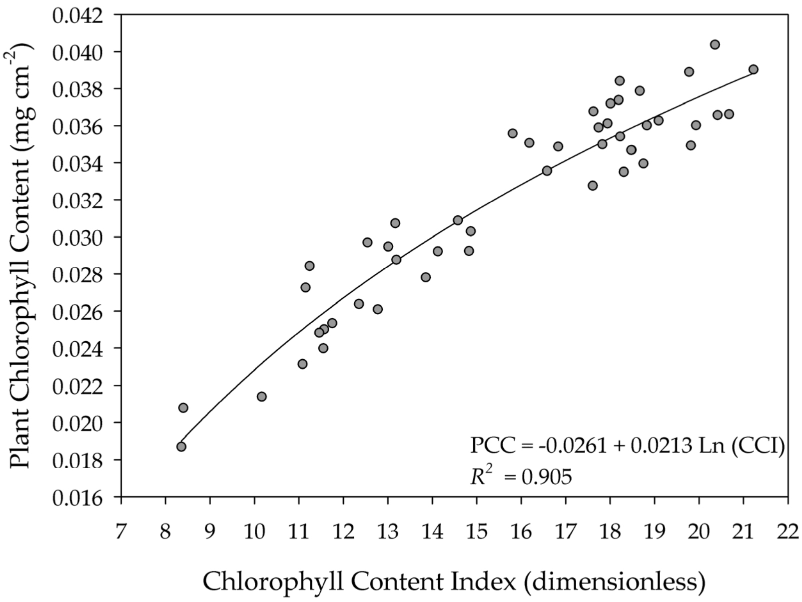

Figure 3. Nonlinear regression between plant total chlorophyll content and chlorophyll content index obtained from measurements in sunflower leaves (Sunbright Supreme variety) under water stress conditions. The parameters of the regression model were significantly greater than zero ($p < 0.0001$).

De Maria et al. [31] evaluated the effects of soil cadmium contamination on accumulation and distribution, growth and physiological responses of sunflower plants, obtaining total chlorophyll contents varying from 0.022 to 0.034 mg·cm^{-2}. On the other hand, Moschen et al. [32] characterized the leaf senescence process in sunflower plants and reported that the maximum chlorophyll content was 0.030 mg·cm^{-2} under field conditions. These results corroborated with the total chlorophyll values shown in Figure 3.

Silla et al. [2] evaluated the potential of a hand-held meter (CCM-200) for estimating total chlorophyll content of *Quercus* leaves at different development stages (young, fully expanded, mature, and pre-senescent). These authors tested linear, exponential, logarithmic, and second-order polynomial functions, obtaining the best correlations (R^2 varying from 0.370 to 0.880) when fitting the logarithmic model to the data. This result corroborated with the model proposed in the present study (R^2 = 0.905).

Table 2 shows the CCI ranges obtained from nondestructive leaf measurements in distinct plant species. Comparatively, the chlorophyll content index range obtained for sunflower plants in this study is close to those of other agricultural crops.

Table 2. Chlorophyll content index range determined for different vegetation species.

Chlorophyll Content Index Range	Vegetation	Reference
1.0–23.0	Paper birch	Richardson et al. [27]
2.4–23.7	Sugar maple	Van den Berg and Perkins [33]
3.0–34.0	Lemon	Jifon et al. [28]
7.3–38.0	Physic nut	Yong et al. [34]
5.0–27.5	Maize	Dalil et al. [35]
1.0–36.0	Kiwi	Cerovic et al. [29]
4.4–25.5	Sunflower	In this study

The results of the present study are in agreement with Kiani et al. [36] who verified a reduction in the plant chlorophyll status to a significant level at higher water deficits in sunflower plants.

The progressive dehydration of sunflower plants promoted an oxidative stress, inducing to a chlorophyll degradation and deficiency of this pigment synthesis in leaves. Additionally, the photosynthetic activity was altered by the biochemical damages of enzymes. Bannari et al. [37] mentioned that the photosynthetic pigments are strongly related to the physiological condition of the plant and its productivity.

4. Conclusions

The water index (WI) was significantly correlated with plant water content in the fine tissues of the sunflower leaves and might effectively be used as an irrigation management tool in order to estimate changes in leaf water content under moderate to severe water stress by spectral reflectance measurements.

The specific calibration model for sunflower plants proposed in this study is important to accurately estimate the total chlorophyll status from chlorophyll content index (CCI), since this relationship is variable among agricultural crops mainly due to differences in leaf structure.

The main benefit of using hand-held optical meters for reflectance and transmittance is that they enable rapid and accurate assessment of water and chlorophyll contents in sunflower plants from spectral indices. Furthermore, these equipments avoid the need of destroying the plant samples and using hazardous solvents, in turn reducing environmental contamination and human health hazards.

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Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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