Romanian Journal of Ecology & Environmental Chemistry, 4(2), 2022

https://doi.org/10.21698/rjeec.2022.202

Article

Comparative methods for chlorophyll detection in leaves

LAURA NOVAC[1#], CATALINA STOICA[1#*], ANDA-GABRIELA TENEA[1,2], GABRIELA GEANINA VASILE[1], MIHAI NITA-LAZAR[1]

[1]*National Research and Development Institute for Industrial Ecology – ECOIND, 57-73 Drumul Podu Dambovitei, 060652, Bucharest, Romania, laura.novac@ecoind.ro; gabriela.vasile@incdecoind.ro; mihai.nita@incdecoind.ro*
[2]*University of Craiova, science Faculty, Department of Chemistry, 107i Bucharest Road, 200585, Craiova, Romania, anda.tenea@incdecoind.ro*
[#] *Laura Novac and Catalina Stoica have contributed equally to this work.*
[*] *Correspondence: catalina.stoica@incdecoind.ro*

Received:	*Accepted:*	*Published:*
14.12.2022	*19.12.2022*	*28.12.2022*

Abstract

Population growth puts an increasing pressure on the agriculture, so progressive field crops practice requires modern, reliable and cost efficient methods to monitor crop development and yield. The chlorophyll plant leaves content is the most common and used biochemical parameter for the plant physiological monitoring such as, the dynamic processes of growth, metabolism, reproduction and defense. Our paper aims to analyze the chlorophyll content by comparing two methods, optical method vs classical spectrometric detection method. A comparison between a quick and cost-effective screening method using portable CCM-200 plus (Opti-Science, Inc) and a more laborious and time consuming biochemical chlorophyll extraction method.

The chlorophyll concentration data was obtained from terrestrial plants such as marigold (Calendula officinalis) and wheat (Triticum sp.). Chlorophyll pigments have had a non-uniform distribution on the leaves surface, thus the concentration differs during the day lengthy, and this induces variation for the optical measurements. In this study, we optimized the optical measurement of chlorophyll pigments by a mathematical correlation of the optical chlorophyll content conversion to absolute chlorophyll. The relationships between the CCM-200 plus measurements and the values obtained for the absolute chlorophyll concentration, as well as the chlorophyll a/b ratio, suggested a wide range of relationships between the two species.

Keywords: *Chlorophyll, CCM-200 plus, Chl a/ Chl b ratio, Calendula officinalis, leaves, Triticum sp.*

INTRODUCTION

Modern agricultural farming requires precise, quick and non-destructive methods for detection of plant physiological parameters [1]. One of the commonly used parameters is the chlorophyll content in plant leaves. Chlorophyll concentration varies in plant leaves depending on plant species background, environmental mineral elements content and different stress factors [2-4]. Leaf chlorophyll concentration is most accurately measured by chlorophyll extraction in a solvent followed by spectrometric measurement [3]. However, lately, attention has been directed towards non-destructive, optical techniques which have become widely used to provide a relative indication of leaf chlorophyll concentration [3].

Over 30 studies were conducted to quantify the optical/ absolute chlorophyll relationship, but neither biochemical chlorophyll extraction nor optical measurement techniques have been consistent among studies [1, 5-7]. Three commercially available Chlorophyll content detection meters are widely used such as SPAD-502 (Spectrum Technologies, Illinois, USA), CCM-200 (Opti-Sciences, Inc., Hudson, USA) and Dualex-4 (Force, Orsay, France).

Although Dong et al. (2019) [2] reported good performance of Dualex-4, for leaves chlorophyll measurement compared with SPAD-502 and CCM-200, using a general conversion function for all crops tested, Kalaji et al. [8] showed that all devices, mentioned above, proved to be suitable to reasonably estimate chlorophyll content under optimal nutrient conditions. For SPAD-502 and CCM-200, the readings error increases with increasing leave chlorophyll content. The sensitivity analysis reveals that deviations from the calibration functions are more induced by non-uniform leaves chlorophyll distribution than leaf architectures [2].

Moreover, a comparison study between SPAD-502, Dualex and CCM-200 devices was done to estimate chlorophyll content in maize and tomato plants. The studied highlighted that under nutrient deficiency conditions, tested devices showed different values for the same plant. This suggested that these devices should be validated by a sampling destructive method under such conditions [8].

Nevertheless, according to Parry et al. (2014) [3] none of these portable optical meters had a linear relationship with chlorophyll concentration, and the reported optical/absolute chlorophyll concentration relationship has varied widely, sometimes even within the same species.

However, one of the first devices used to estimate the chlorophyll and nitrogen in leaves was CCM-200, a valuable tool for researchers with great advantages of time-saving and very cost-effective [4]. The CCM-200 plus Chlorophyll Content Meter is a hand-held, battery-operated instrument which had been designed for a rapid, non-destructive, determination of foliar chlorophyll content in leaf samples in order to estimate the plant health and condition [9].

Monitoring chlorophyll content via a non-destructive analysis gives researchers, agronomists and farmers valuable diagnostic information. This data could then be applied to a multitude of crop production and research initiatives such as nutrient and irrigation management, pest control, environmental stress evaluation and crop breeding [10].

Chlorophyll concentration data obtained by CCM-200 device was correlated with data obtained by dimethyl sulfoxide (DMSO) and Kjeldahl methods and they were in a similar range [4, 11]. The regression models were developed with destructively measured parameters as the dependent variable and a parameter derived from CCM-200 as the independent variable (chlorophyll content index, CCI) to estimate chlorophyll. In most species, the slope of the CCI–chlorophyll concentration varied greatly during leaf development, thus great caution is needed when using the CCM-200, as the interpretation of CCI readings should be limited to samples of similar leaves age. The data also indicate that the models should also be species-specific due to differences in the equations' intercept and/or slope [12].

Chlorophyll (Chl) is an important photosynthetic pigment of a plant, largely determining photosynthetic capacity and hence the plant growth [13].

Chlorophyll a (Chl *a*) and chlorophyll b (Chl *b*) are considered two of the most important leaf pigments, as they are accountable for the majority of the conversion of light energy into stored chemical energy within plants [14]. Chl *a* is essential in the energy phase of photosynthesis, whereas Chl *b* captures light at a slightly different wavelength. Moreover, the pigment content variation in total chlorophyll (i.e., TChl = Chl *a* + Chl *b*) between and within species is important for several reasons such as: photosynthetic activity and primary production [15].

On the other hand, pigmentation can be directly related of the plant's physiological stress because chlorophyll concentrations tend to fall below stress and with the aging of the plant. Consequently, quantifying these proportions can provide important information regarding relationships between plants and their environment [15]. Moreover, it is suggested that chlorophyll content can be adopted as a very useful in vivo indicator of heavy metal toxicity [16].

Considering the fact that chlorophyll content represents a definitory indicator for plants growth and health, this paper aimed to compare different chlorophyll content results obtained by the biochemical extraction method, followed by spectrometric reading, with the one resulted from CCM-200 readings from two terrestrial plants, marigold (*Calendula officinalis*) and wheat (*Triticum sp.*).

EXPERIMENTAL PART

Biological models

Two terrestrial plants of great importance for human food and health, marigold (*Calendula officinalis*) and wheat (*Triticum* sp.) were selected for the study. Both marigold and wheat seeds were purchased from a vegetable producer (Florian LTD, Bulgaria). The seeds were grown in a greenhouse in experimental pots with universal soil obtained from Agro CS (Bucharest, Romania) at the beginning of March. The chemical composition of the soil was according to the producer, as follows N (50-400 mg/L); P_2O_5 (50-200 mg/L); K_2O (50-200 mg/L); pH (5-7,5); organic substances (min. 67%); humidity (60%); KCl < 3 g/L. The seedlings were watered twice a week, with tap water. Ten different experimental pots for each of the studied plant were grown in similar conditions. The quantification of chlorophyll content was done on young leaves of marigold and wheat harvested in April. The plants leaves were collected randomly for the detection of chlorophyll concentration.

Chlorophyll content detection

Same leaf samples were analyzed by *i)* optical non-destructive screening method using CCM-200 plus (Opti-Science, Inc. USA) and *ii)* biochemical chlorophyll extraction method. To reduce variation due to leaf age, the samples were collected mostly from the plant apex, since those were the newest fully developed leaves.

Optical chlorophyll content screening method

Fresh leaf samples were subjected, firstly, for chlorophyll content index (CCI) measurement with CCM-200 plus (Opti-Science, Inc. USA). CCI was defined as the ratio between transmission of radiation from a light emitting diode (LED) at 931 nm and the transmission of radiation from a LED at 653 nm [9]. Chlorophyll content meter was calibrated with a blank chamber prior to each series of measurements, following the manufacturer's instructions [9]. The CCI measurements of plants leaves were done from both upper and lower sides. Secondly, after CCI values readings, the same leaf samples were processed for total chlorophyll (TChl), chlorophyll a (Chl *a*), and chlorophyll b (Chl *b*) estimation using spectrophotometric method.

Biochemical chlorophyll extraction method

The chlorophyll pigments (Chl *a* and Chl *b*) of both marigold and wheat samples were analyzed considering the methods described in [17-19]. The chlorophyll from the leaves samples was extracted in 80% acetone at 1:2.5 [mass (g)/volume (mL)] ratio. The leaves were cut into 1–2 cm pieces and homogenized. The leaves tissues were incubated in 80% buffered acetone on a mixing tube revolver Rotator D6050 (neoLabLine, Heidelgerg, Germany) for 12 h at 4°C. The extract was centrifuged for 3 min at 2545 g (centrifuge 5702R, Eppendorf AG Hamburg, Germany), and subsequently, the absorbance at 662 nm and 645 nm was measured using UV-VIS spectrometer Specord 205BU (Analytik Jena, Jena, Germany). Chlorophyll concentrations were calculated using the following equations:

Chlorophyll concentration a (Chl a) (mg/mL) = $(12.25 \times A_{662\ nm}) - (2.79 \times A_{645\ nm})$ (1)

Chlorophyll concentration b (Chl b) (mg/mL) = $(21.5 \times A_{645\ nm}) - (5.1 \times A_{662\ nm})$. (2)

The sum of the two types of chlorophyll pigments represented the total chlorophyll concentration (mg/mL).

Statistical analysis

Two independent sets of fresh leaf samples were collected from each experimental pots in order to have a statistical accuracy. The average (Avg) and standard deviation (SD) values were calculated for all chlorophyll concentration (n = 2) detected in leaf experimental samples (n=10). The interpretation of the obtained experimental data was performed using ANOVA ONE-WAY, depending on the *p*-value obtained. The *p*-value was considered insignificant for $p > 0.05$ (ns), significant for $p < 0.05$ (*), and very significant for $p < 0.01$ (**). Tukey HSD (Honestly Significant

Difference) test was used to highlight if there are statistical correlations between the sets of measurements. In addition, for a direct comparison of CCI with conventional spectrophotometric measurements, all values of chlorophyll content mg/ml were plotted against the CCI values for regression analysis.

RESULTS AND DISCUSSION

A comparative chlorophyll content study between non-destructive optical and biochemical extraction methods was performed on *Calendula officinalis* and *Triticum sp.*

Calendula officinalis belongs to Asteraceae family a well-known medicinal perennial plant, about 80 cm tall, with sparsely branched lax or erect stems and oblong-lance leaves. The importance of *Calendula officinalis* in phytopharmaceutical studies relies on its anti-viral activity, anti-HIV properties of therapeutic interest, and anti-genotoxic properties [20, 21].

Triticum sp. is one of the most cultivated plant crops with the highest monetary yield. Genus *Triticum*, are annual or biennial grasses cultivated mainly for their grains. Wheat can reach 1.2 m in height and, like other cereals, has been developed into different varieties that are adapted to planting at different times of the year [22].

The chlorophyll content index (CCI) detected for marigold in all ten experimental tests ranged between 1.2 and 2.6. The Chl *a* concentration from marigold leaves detected by a biochemical chlorophyll extraction method showed a concentration range between 5.94 mg/ml and 10.60 mg/ml Chl *a*. The Chl *b* values were 2-fold lower than Chl *a* values, ranging from 3.40 mg/ml to 5.03 mg/ml. Total chlorophyll concentration (mg/ml) calculated as the sum of Chl *a* and Chl *b*, in case of *Calendula officinalis* leaves varied between 9.34 mg/ml and 15.62 mg/ml (Table 1).

Table 1. The chlorophyll content detected by non-destructive optical and UV-Vis spectrometric methods in *Calendula officinalis*. CCI and chlorophyll concentration (mg/ml) were expressed as average ± SD. Chl *a* and Chl *b* values represents the average of two independent tests

Experimental test number	**Non-destructive optical method**	**Spectrometric method**			
	CCI (Avg ± SD)	Chl *a* (mg/ml)	Chl *b* (mg/ml)	TChl (mg/ml)	Ratio Chl *a*/*b*
1	1.4 ± 0.2	8.43	4.24	12.67 ±1.7	1.99
2	1.4 ± 0.3	8.04	4.28	12.32 ± 0.1	1.88
3	1.2 ± 0.1	10.60	5.02	15.62 ± 1.0	2.11
4	2.0 ± 0.2	5.94	3.40	9.34 ± 0.7	1.75
5	2.1 ± 0.4	6.64	3.69	10.33 ±0.8	1.80
6	2.6 ± 0.5	8.30	4.62	12.92 ± 0.9	1.80
7	1.7 ± 0.3	9.82	5.03	14.84 ± 0.5	1.95
8	1.4 ± 0.1	7.73	3.82	11.56 ± 0.5	2.02
9	1.4 ± 0.2	9.03	4.33	13.37 ± 0.3	2.09
10	1.8 ± 0.3	9.48	4.54	14.02 ± 0.3	2.09

The ratio between Chl *a*/Chl *b* calculated for marigold leaf tests ranged from 1.80 to 2.11 with an average of 1.95.

We assumed that the photosynthetic capacity of marigold plants could be influenced by the Chl *a*/ Chl *b* ratio. This statement was sustained by the fact that Chl *a* and Chl *b* absorb sunlight at different wavelengths (e.g. red-orange light Chl *a* and blue-purple light Chl *b*), leading to the assumption that the total amount of leaf chlorophyll content and allocated ratio directly influenced the production capacity of plants [23]. Moreover, Zhang et al. (2020) [24] reported that Chl *a*/ Chl *b* ratio is an indication of the plant functional balance between the efficiency of light capture and electron transport. Castro and Sanchez-Azofeifa (2008) [25] showed that Chlorophyll *a/b* ratios are known to decrease during leaf senescence, while other studies have found that drought stress has no

effect on the Chlorophyll *a/b* ratio [26]. By contrast, Mulero et al. (2022) [27], Tian et al. (2017) [28] and Guo et al. (2016) [29] have stated that Chl *a/b* ratio can be affected under water stress or nutrient status.
An inversely proportional relationship between CCI and Chl *a* was observed in case of marigold results, but not in case of wheat chlorophyll content results (Table 3).

The Tukey HSD analysis ("honestly significant difference") for both *Calendula officinalis* and *Triticum sp.* chlorophyll content was performed to pinpoint statistical correlations or significant differences between CCI and Chl *a*, Chl *b* and total chlorophyll measurements sets (Table 2 and Table 4). Six measurements pairs were analysed by Tukey HSD (Table 2).

Table 2. Tukey HSD results for *Calendula officinalis* measurements sets

Measurement pairs	**Tukey HSD Q statistic**	**Tukey HSD p-value**	**Tukey HSD inference**
CCI vs TChl	27.8468	0.0010053	** $p<0.01$
CCI vs Chl *a*	16.9653	0.0010053	** $p<0.01$
CCI vs Chl *b*	6.5750	0.0010053	** $p<0.01$
TChl vs Chl *a*	10.8815	0.0010053	** $p<0.01$
TChl vs Chl *b*	21.2718	0.0010053	** $p<0.01$
Chl *a* vs Chl *b*	10.3903	0.0010053	** $p<0.01$

The results showed that the *p*-value corresponding to the F-statistic of one-way ANOVA was lower than 0.01 which strongly suggests that all the measurements pairs were significantly different between each of the six measurements pairs.
CCI measured using CCM-200 plus chlorophyll meter for *Triticum sp*. leaf samples varied between 3.1 and 6.9 (Table 3). Chl *a* concentration detected by biochemical extraction method ranged from 26.29 mg/ml to 29.13 mg/ml. Chl *b* performed in wheat leaves was of about 2.5 lower than Chl *a* concentration, from 9.19 mg/ml to 13.82 mg/ml, respectively (Table 3). Chl *a* / Chl *b* ratio in *Triticum sp*. leaves varied between 2.1 and 2.9, of aprox. 20% higher than the Chl *a* / Chl *b* ratio calculated for *Calendula officinalis*.

Table 3. The chlorophyll content detected by non-destructive optical and UV-Vis spectrometric methods in *Triticum sp.* CCI and total chlorophyll concentration (mg/ml) were expressed as average ± SD. Chl *a* and Chl *b* values represents the average of two independent tests.

Experimental test number	**Non-destructive optical method**	**Spectrometric method**			
	CCI (Avg ± SD)	Chl *a* (mg/ml)	Chl *b* (mg/ml)	TChl (mg/ml)	Ratio Chl *a*/*b*
1	3.1 ± 0.1	28.03	10.82	38.85 ±0.9	2.6
2	4 ± 0.1	26.79	10.23	37.02 ± 0.9	2.6
3	4.1 ± 0.1	27.83	10.69	38.52 ± 0.1	2.6
4	4.95 ± 0.2	27.62	10.67	38.29 ± 0.7	2.6
5	3.95 ± 0.4	27.21	9.82	37.03 ±0.1	2.8
6	6 ± 0.6	27.04	10.06	37.10 ± 0.5	2.7
7	4.35 ± 0.1	26.29	9.19	35.48 ± 1.4	2.9
8	4.5 ± 0.4	28.95	12.79	41.74 ± 1.6	2.3
9	6.3 ± 0.8	29.11	13.82	42.93 ± 1.9	2.1
10	5.15 ± 0.1	29.13	13.62	42.75 ± 0.2	2.1

The differences between chlorophyll content detected in marigold compared with wheat could be explained by temperature dependent-enzymatic reactions, although similar growth condition for both plants were applied. Moreover, Li et al. (2018) [12] reported that the lack of water in leaves

could influence the synthesis of chlorophyll, while accelerating leaf yellowing and consequently, decomposition of chlorophyll. Recent studies have suggested that Chl *b* is more resistant to the degradation during high temperature treatment [30]. Microgreens growth in daylight having low Chl *a/b* ratio are more bioaccessible than those in sunlight that have higher Chl *a/b* ratio [31]. Another explanation of chlorophyll content differences between the tested plants could be associated to the growth stage of the plants, although young leaves were selected for the tests. Considering that Chl *a* and Chl *b* can be easily distinguished in vitro, there has been a surprising lack of literature reporting differences among species.

Similarly, as in case of *Calendula officinalis*, the Tukey HSD analysis for *Triticum sp.* showed significant differences between the measurements pairs (Table 4). The *p*-value corresponding to the F-statistic one-way ANOVA was lower than 0.01, suggesting that the four treatments were significantly different.

Table 4. Tukey HSD results for *Triticum sp.* measurements sets

Measurement pairs	**Tukey HSD Q statistic**	**Tukey HSD *p*-value**	**Tukey HSD inference**
CCI vs TChl	64.0213	0.0010053	** $p<0.01$
CCI vs Chl *a*	43.1772	0.0010053	** $p<0.01$
CCI vs Chl *b*	12.1490	0.0010053	** $p<0.01$
TChl vs Chl *a*	20.8441	0.0010053	** $p<0.01$
TChl vs Chl *b*	51.8724	0.0010053	** $p<0.01$
Chl *a* vs Chl *b*	31.0283	0.0010053	** $p<0.01$

Moreover, to emphasise the possible relationship between the chlorophyll content index obtained with CCM-200 plus chlorophyll meter and chlorophyll content in young leaves of *Calendula officinalis* and *Triticum sp.* obtained with spectrometric methods, the regression analysis was performed.

For each of the studied plants such as marigold and wheat, the linear equation (95% confidence limits), the coefficient of determination (R^2) and the analysis of variance were used to examine the linear association between pigment chemical analysis (Chl *a*, Chl *b* and TChl). All values of chlorophyll content mg/ml were plotted against the CCI values for regression analysis and graphics were represented in Figures 1 and 2.

The coefficient of determination R^2 varied between 0.028 and 0.17 for *Calendula officinalis*, while for *Triticum sp.* R^2 varied between 0.094 and 0.21, suggesting a weak association between all three measurement sets.

The r values for *Triticum sp*. was between 0.3 and 0.46 and the best correlation was identified between Chl *b* and CCI. Weak positive correlations between CCI, Chl *a*, Chl *b* and TChl were found for *Triticum* sp., while for *Calendula officinalis* the direction was negative, however, the robustness of the correlations varied (Figures 1 and 2).

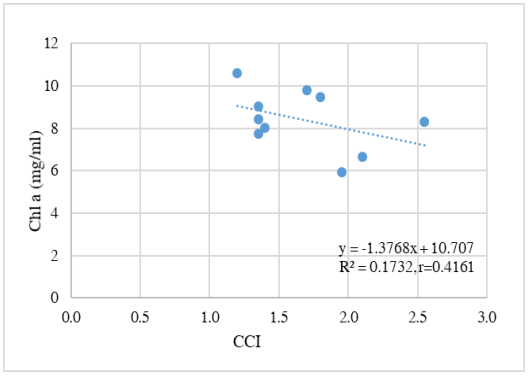
The size, orientation and type of leaves surface could influence the chlorophyll measurements. The flat and smooth surface serve better at reflecting and transmitting the LED light to the detector in CCM-200 meter [32], this can be a reason for the differences in obtaining a positive correlation for *Triticum sp*. and negative correlation for *Calendula officinalis.*

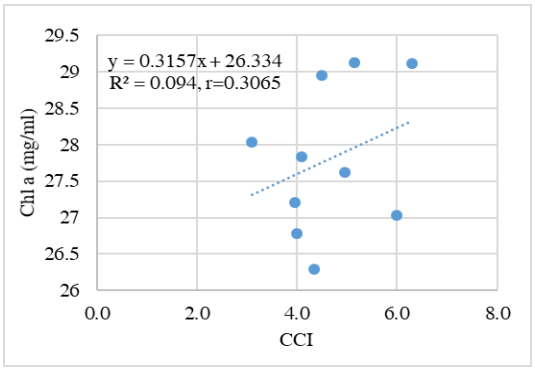
CCI and other non-destructive optical indices of Chl *a* content are important diagnostic tools to repeatedly or rapidly sample a population of plants. For instance, tracking the condition of a population in the field or in an experiment would be much simplified if a rapid and repeatable optical measure of chlorophyll content can be made.

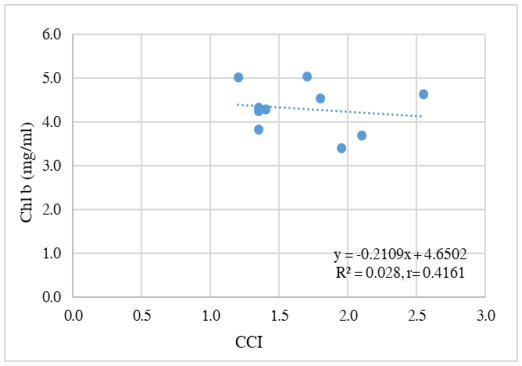
As most studies using CCM and other reflectance indices have focused on crop or forest species, there is still a need to determine the application of these techniques to other plants. Species-specific relationships between CCI and Chl *a* content need to be established before this approach can be more widely adopted in ecological studies. Furthermore, the range of CCI values should be correlated with leaves development. A species-specific library of CCI: Chl *a* relationships should be

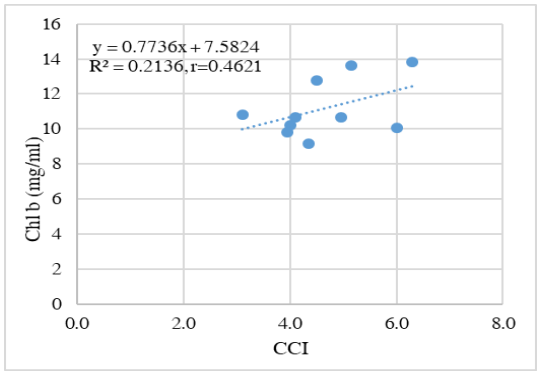
developed by testing plant leaves at different developmental stage, physiological condition and environmental status [32, 33].

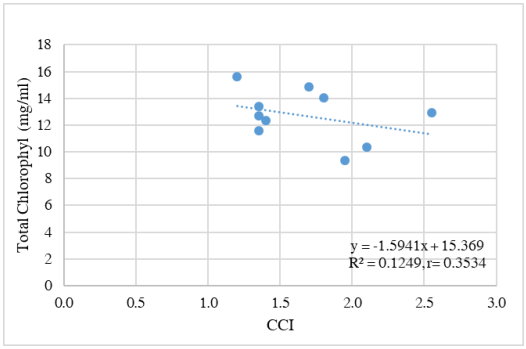
Overall, there are many studies that have established statistical correlations between the values obtained through the non-destructive method obtained through CCM-200 and the biochemical extraction method followed by spectrometric measurements, but there is almost the same amount of studies that do not find a strong correlation between the two measurements, especially a correlation between the value of CCI and chlorophyll obtained by the classic method.











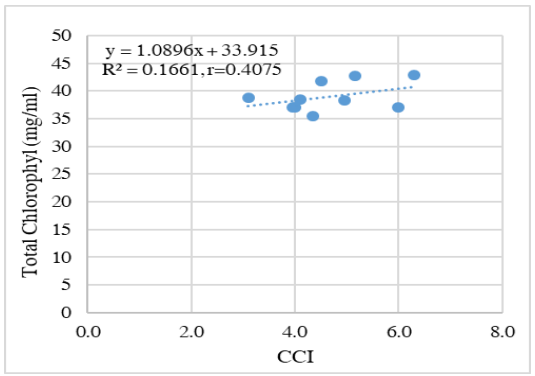


Fig. 1. Scatter plot and linear regression lines of of Chlorophyll Content Index (CCI) using CCM-200 vs. chlorophyll concentration detected by spectrometry method for marigold (*Calendula officinalis)* leaves

Fig. 2. Scatter plot and linear regression lines of of Chlorophyll Content Index (CCI) using CCM-200 vs. chlorophyll concentration detected by spectrometry method for wheat (*Triticum sp.)* leaves

CONCLUSIONS

The present paper focused on comparison between two non-destructive and classical, destructive, methods for the detection of chlorophyll content in higher plants. It was established an appropriate mathematical relationship between the chlorophyll content index (CCI) value resulted from the non-destructive method and chlorophyll contents obtained with spectrometric method in leaf samples of marigold (*Calendula officinalis)* and wheat (*Triticum sp*.).

The statistical tool Tukey HSD was showed if the relationship between two sets of data was statistically significant as well as the regression analysis were performed to obtain the best mathematical models relating the relationships between portable CCM-200 chlorophyll meter readings and chlorophyll concentration measured in the plants leaf samples.

The Tukey HSD tests identified that all of the measurements pairs were significantly different from each other, and between the measurements pairs. The *p*-value corresponding to the F-statistic of one-way ANOVA was lower than 0.01, suggesting that one or more measurements were significantly different.

The regression equation showed differences among species in the application of the CCI. For this reason, species-specific relationships of CCI to Chl *a* content need to be established for accurate interpretation of data obtained from CCM-200 plus chlorophyll meter, thus more analyses are required further for an appropriate correlation.

Overall, future researches should focus on the investigation on plants belonging to different classes to see if statistical correlations can be observed between the two investigation methods as well as to validate the non-destructive, optical method for chlorophyll content detection in leaves.

ACKNOWLEDGEMENTS

The work was funded by Ministry of Research, Innovation and Digitalization of Romania through Programme Nucleu through contract 20N/2019, Projects no. PN 19 04 02 01 and PN 19 04 01 01.

REFERENCES

[1] ALSINA, I., DUMA, M., DUBOVA, L., SENBERGA, A., DAGIS, S., Agron. Res., **14,** no. 2, 2016, p. 309.

[2] DONG, T., SHANG, J., CHEN, J.M., LIU, J., QIAN, B., MA, B., MORRISON, M.J., ZHANG, C., LIU, Y., SHI, Y., PAN, H., ZHOU, G., Remote Sens., **11**, 2019, https://doi:10.3390/rs11222706.

[3] PARRY, C., BLONQUIST, J.M., BUGBEE, B., Plant, Cell and Environment, **37**, 2014, p. 2508, https://doi.org/10.1111/pce.12324.

[4] GHASEMI, M., ARZANI, K., YADOLLAHI, A., GHASEMI, S., SARIKHANI KHORRAMI, S., Not. Sci. Biol., **3,** no.1, 2011, p.91, https://doi.org/10.15835/nsb315623.

[5] RICHARDSON, A.D., DUIGAN, S.P., BERLYN, G.P., New Phytol., **153**, 2002, p.185.

[6] NAIR, D.B., JACOB, J., Int. J. Plant Sci., **5**, no.1, 2011, p. 47.

[7] CEROVIC, Z.G., MASDOUMIER, G., GHOZLEN, B.N., LATOUCHE, G., Physiol. Plant, **146**, no.3, 2012, p.251, https://doi.org/10.1111/j.1399-3054.2012.01639.x.

[8] KALAJI, H.M., DABROWSKI, P., CETNER, M.D., SAMBORSKA, I.A., LUKASIK, I., BRESTIC, M., ZIVCAK, M., J. Plant Nutr., **40**, no. 7, 2016, p. 1024, https://doi:10.1080/01904167.2016.1263323.

[9] CCM-200 plus manual, 2022, https://www.manualslib.com/manual/1202197/Opti-Sciences-Ccm-200-Plus.html *(accessed in 15.10.2022).*

[10] SONOBE, R., HIRONO, Y., OI, A., Plants, **9**, no. 3, 2020, p. 368, http**s://**doi:10.3390/plants9030368.

[11] PETISCO, C., GARCIA-CRIADO, B., GARCIA-CRIADO, L., VAZQUEZ-DE-ALDANA, B. R., GARCIA – CIUDAD, A., Commun. in Soil Sci. Plant. Ana., **40**, no. 15, 2009, p. 2474, https://doi.org/10.1080/00103620903111350.

[12] SILLA, F., GONZALES-GIL, A., GONZALES-MOLINA, E., MEDIAVILLA, S., ESCUDERO, A., Ann. For. Sci., **67**, no 1, 2010, https://doi.org/10.1051/forest/2009093.

[13] LI, Y., HE, N., HOU, J., XU, L., LIU, C., ZHANG, J., WANG, Q., ZHANG, X., WU, X., Front. Ecol. Evol., **6**, no. 64, 2018, p. 1, https://doi.org/10.3389/fevo.2018.00064.
[14] BLACKBURN, G.A., J. Exp. Bot., **58**, 2007, p. 855, https://doi.org/10.1093/jxb/erl123.
[15] FLORES-DE-SANTIAGO, F., KOVACS, J.M., FLORES-VERDUGO, F., Bull. Mar. Sci., **89**, no. 2, 2013, p. 621, https://doi.org/10.5343/bms.2012.1032.
[16] SOUAHI, H., Biosyst. Divers., **29**, no. 3, 2021, https://doi.org/10.15421/012125.
[17] LICHTENTHALER, H., BUSCHMANN, C., Am. J. Plant Sci., **1**, 2001, p. F4.31.
[18] KRISHNAN, P., RAVI, I., Indian J. Exp. Biol., **34**, 1996, p.1030.
[19] DINU, C., GHEORGHE, S., TENEA, A.G., STOICA, C., VASILE, G., POPESCU, R.L., SERBAN, E.A., PASCU, L.F., Int. J. Environ. Res. Public Health**,** **18**, 2021, https://doi.org/10.3390/ ijerph18083904.
[20] PEREZ-CARREON, J.I., CRUZ-JIMENEZ, G., LICEA-VEGA, J.A., ARCE POPOCA, E., FATTEL FAZENDA, S., VILLA-TREVINO, S., Toxicology in Vitro, **16,** 2002, p. 253, https://doi.org/10.1016/S0887-2333(02)00005-X.
[21] ASHWLAYAN, V.D., KUMAR, A., VERMA, M., GARG KUMAR, V., GUPTA, S.K., Pharm Pharmacol. Int. J., **6**, no. 2, 2018, p. 149.
[22] ARZANI, A., ASHRAF, M., Compr. Rev. Food Sci. Food Saf., **16**, no. 3, 2017, p. 477. https://doi.org/10.1111/1541-4337.12262.
[23] CROFT, H., CHEN, J.M., LUO, X., BARTLETT, P., CHEN, B., STAEBLER, R.M., Glob. Change Biol., **23**, 2017, p. 3513, https://doi: 10.1111/gcb.13599.
[24] ZHANG, Y., LI, Y., WANG, R., XU, L., LI, M., LIU, Z., WU, Z., ZHANG, J., YU, G., HE, N., Front. Plant Sci., **11,** no. 1244, 2020, doi:10.3389/fpls.2020.01244.
[25] CASTRO, K.L., SANCHEZ-AZOFEIFA, G.A., Sensors, **8**, 2008, p. 51, https://doi.org/10.3390/s8010051.
[26] MAFAKHERI, A., SIOSEMARDEH, A., BAHRAMNEJAD, B., STRUIK, P.C., SOHRABI, E., Aust. J. Crop Sci., **4**, no. 8, 2010, p. 580.
[27] MULERO, G., BACHER, H., KLEINER, U., PELEG, Z., HERRMANN, I., Remote Sens., **14**, 2022, https:// doi.org/10.3390/rs14112585.
[28] TIAN, Y., SACHARZ, J., WARE, M.A, ZHANG, H., RUBAN, A.V., J. Exp. Bot., **68**, no. 15, 2017, p. 4249, https://doi: 10.1093/jxb/erx213.
[29] GUO, Y.Y., YU, H.Y., KONG, D.S., YAN, F., ZHANG, Y.J., Photosynthetica, **54**, 2016, p. 524, https://doi: 10.1007/s11099-016-0206-x.
[30] OKPALANMA, F.E., OJIMELUKWE, P.C., Asian Food Sci. J., **2**, 2018 https://doi: 10.9734/AFSJ/2018/41603.
[31] NIROULA, A., KHATRI, S., TIMILSINA, R., KHADKA, D., OJHA, P., J. Food Sci. Technol., **56**, no.5, 2019, p.2758, https://doi: 10.1007/s13197-019-03768-9.
[32] BIBER, P.D., JAFES, **1** no. 2, 2007, p.1.
[33] CHEN, K., ROCA, M., Funct Foods., **41**, 2018, p.25, https://doi: 10.1016/j.jff.2017.12.029.

Citation: Novac, L, Stoica, C., Tenea, A.-G., Vasile, G.G., Nita-lazar, M., Comparative methods for chlorophyll detection in leaves, *Rom. J. Ecol. Environ. Chem*., **2022**, 4, no. 2, pp. 23-31.