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CHLOROPHYLL CONTENT ESTIMATION IN OREGANO LEAVES USING A PORTABLE CHLOROPHYLL METER: RELATIONSHIP WITH MESOPHYLL THICKNESS AND LEAF AGE

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SUMMARY

Chlorophyll meter CCM 200 plus capacity to estimate total leaf chlorophyll content (*Chl*) in *Origanum vulgare* ssp. *hirtum* was evaluated. This study aimed to elaborate a calibration model adapted to the species and evaluate the effect of mesophyll thickness and leaf age on the readings. This research included a wide range of chlorophyll concentrations, mesophyll thicknesses and leaves from different layers within the plant. Thus good estimates of total chlorophyll content were obtained with this meter, although it was less accurate in mature leaves, which have higher values of chlorophyll content and mesophyll thickness. As mesophyll thickness increases, spongy parenchyma develops more than other anatomical structures, modifying the leaf optical properties and increasing the variability of the readings. The best fit was obtained with a logistic equation [$Chl=0.343/(1+1.925^{(-0.069x)})$], adapted to a broad data ranges and any leaf typology, according to ontogenetic age and position in the plant.

Key words. *Origanum vulgare*, CCM 200 plus, thickness index, spongy parenchyma.

ESTIMACIÓN DEL CONTENIDO DE CLOROFILA EN HOJAS DE ORÉGANO USANDO UN CLOROFILÓMETRO PORTÁTIL: RELACIÓN CON EL ESPESOR DEL MESÓFILO Y EDAD FOLIAR

RESUMEN

Se evaluó la capacidad del medidor portátil de clorofila CCM 200 plus para estimar el contenido de clorofila total (*Chl*) en hojas de *Origanum vulgare* ssp. *hirtum*. Este estudio tuvo como objetivos elaborar un modelo de calibración ajustado a la especie, y evaluar el efecto del espesor del mesófilo y la edad foliar sobre las lecturas. Se incluyó en el estudio un amplio rango de concentraciones de clorofila, grosores de mesófilo y hojas de distintos estratos dentro de la planta. Se consiguieron buenas estimaciones de *Chl* con el medidor, aunque pierde precisión en hojas maduras, que poseen mayores valores de contenido de clorofila y espesor del mesófilo. A medida que se incrementa el espesor del mesófilo, se genera mayor desarrollo del parénquima esponjoso con respecto a las demás estructuras anatómicas, modificando las propiedades ópticas foliares e incrementando la variabilidad de las lecturas. El mejor ajuste se obtuvo con una ecuación de tipo logística [$Chl=0.343/(1+1.925^{(-0.069X)})$], la cual se adaptó a un amplio rango de datos y tipología foliar según edad ontogénica y posición en la planta.

Palabras clave. *Origanum vulgare*; CCM 200 plus; índice de espesor; parénquima esponjoso.

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INTRODUCTION

Chlorophyll content measurement yields direct information on photosynthesis potential and primary productivity. Together with associated parameters, it provides an indirect measure of plant's physiological stress (Arunyanark *et al.,* 2008) and nutritional status (Gáborcík, 2003; Neilsen *et al.,* 1995). There are different methods to estimate the pigment content, and most of these are destructive. In contrast, there are new methods based on the leaf optical properties turn out to be more rapid, inexpensive and practical, providing direct measurement in the field without destroying the sample (Richardson *et al.,* 2002). There are chlorophyll meters that use absorbance to calculate leaf chlorophyll content, using the Beer-Lambert law. These yield a chlorophyll content index (CCI) calculated by the percentage ratio of transmittance at two wavelengths: 653 nm (Red), efficiently absorbed and correlated with pigment content; and 931 nm (NIR), which serves for fitting to structural differences. Therefore, the CCI is a dimensionless relative value. However, CCI values were positively correlated with total chlorophyll content in a wide range of studies in different species (Ruiz-Espinoza *et al*., 2010; Silla *et al*., 2010; Jifon *et al*., 2005; van den Berg and Perkins, 2004; Loh *et al.*, 2002; Castelli *et al.*, 1996; Dwyer *et al.*, 1991) with mathematical models emerging for the calibration of instruments for converting CCI values to absolute chlorophyll content. The models obtained vary depending on the leaf optical properties and the meter employed (Jifon *et al*., 2005; Richardson *et al*., 2002; Monje and Bugbee, 1992).

Morpho-anatomical structure changes produce modifications in leaf optical properties (Castro and Sanchez-Azofeifa, 2008) and also reflect plant growth conditions. As a leaf develops, changes occur in leaf anatomical structure, chlorophyll concentration and photosynthetic activity. Thus, specific adjustments and measurement protocols are required for each species, apparatus (Richardson *et al.*, 2002; Markwell *et al.*, 1995) and growth conditions (Monje and Bugbee, 1992).

Despite numerous studies related to chlorophyll determination and manual meter calibration, there is no previous information on the subject in oregano leaves. Aspects such as chlorophyll content, its variation by leaf age and its relation to morpho-anatomical parameters are very useful for understanding the physiology of the species.

The aims of this work were: i) to assess the ability of the CCM 200 plus handheld chlorophyll meter to estimate chlorophyll content in *Origanum vulgare* ssp. *hirtum* lestw. leaves, establishing a correlation with laboratory analytical methods, and to obtain a calibration model for the device adapted to the species and growth conditions; ii) to determine the influence of the mesophyll anatomical structure and leaf age on estimated values.

MATERIALS AND METHODS

Plant material and study site

This study was conducted in the experimental farm of the Faculty of Agricultural Sciences, National University of Córdoba (31º30´S; 64º00´O). The cultivar sampled was *Origanum vulgare* ssp. *hirtum* lestw. (Criollo ecotype), in its second year of production. Six plots were randomly distributed and their size was 21 m^2, planted with 0.70 m between rows and 0.20 m between plants. During the growing season, supplementary irrigation was applied with a drip irrigation system.

Sampling design

The study was based on leaf age to achieve a more accurate determination of chlorophyll content and improve the fitting of the meter. Three types of leaves were taken into account: Young (Y), Mature (M) and Senescent (S) leaves. They were differentiated in the field by their coloration: deep green (Y), dark green to grey (M) and light green and/or chlorotic symptoms (S). The aim was to analyze a wide range of chlorophyll concentrations and both upper and lower leaf layers of the plant. Leaves were randomly extracted from plants of each plot. Sampling was performed from floral branch beginning and up

to full blooming phenological stages (Davidenco *et al.,* 2012), where both layers and the different kind of leaves by age can be found. The young leaves (Y) are associated with lower layers in initial phenological phases and lateral ramifications throughout the crop cycle. These leaves (Y) without adequate light intensity rapidly begin to senesce (S) by shading. Finally, mature leaves (M) are located in upper layers and they were harvested before reaching senescence, according to the agronomic management of the crop.

From the beginning of the assay two harvests at full blooming were made (December and March), including three foliar sampling in each period. All leaf types according leaf age were collected at each sampling.

Measurements

CHLOROPHYLL: five leaves from each leaf age were recollected (group) and measured with CCM 200 plus chlorophyll meter (Opti-Sciences Inc., Tyngsboro, MA, USA), obtaining an average value for each leaf type (CCI). For sampling, five repetitions were made. All measurements were performed early in the morning (9.00-10.00 am) to avoid variations caused by the movement of chloroplasts during the course of the day.

The same leaves were removed from the plant and quickly subjected to the laboratory chlorophyll content measuring process (Chl). Chlorophyll passive extraction was determined based on a buffer solution of ethanol 80% and CO_3Na_2 (1.12 mM). For this, five disks were obtained 1.04 cm in diameter (similar to meter measuring area) by a punch (one per leaf). Then, five disks in test tubes with 10 ml of solution were placed and subjected to boiling (70 °C) in a water bath to discoloration (20 min). In addition, measured leaves group with chlorophyll meter was respected to establish an appropriate relationship with the laboratory determination.

The extracts were stored at 4 °C in darkness to prevent degradation until spectrophotometer study (Shimadzu UV-120-02). Chlorophyll content was determined spectrophotometrically at 654 nm according to Wintermans and De Mots (1965). The results were converted and expressed per unit area (mg cm^{-2}).

MESOPHYLL: For these measurements, the techniques proposed by D'Ambrogio de Argüeso (1986) were followed. After chlorophyll extraction, the disks were removed from the solution and preserved in FAA fixing solution (Formaldehyde, Alcohol and Acetic). Above them, temporary, semipermanent and permanent slides of cross sections of leaves were made. Then, they were observed under light microscope with a slide graded for thickness measurement (µm). The variables analyzed were: mesophyll thickness; spongy parenchyma thickness; thickness index calculated by the ratio between spongy parenchyma thickness and total thickness; and the number of palisade parenchyma layers.

Data analysis

For data analysis the InfoStat software was used (Di Rienzo *et al.*, 2011). ANOVA and DGC test were performed with a significance level of $p \leq 0.05$. Correlation coefficients (r) were obtained ($P \leq 0.0001$). Similarly, simple linear and non-linear type regressions were performed ($P < 0.0001$), between chlorophyll determination in laboratory as dependent variable and meter readings as independent variable to determine which calibration model achieved best fit. For this, regression coefficients (R^2) and error tests were determined. Additionally, assumptions of normality and homogeneity of variances on statistical residue of the fitted model were determined, verifying compliance.

RESULTS AND DISCUSSION

The readings obtained with the chlorophyll meter (CCI) and chlorophyll content (Chl) obtained by the destructive method, yield significant differences for all treatments ($p \leq 0.05$) reflecting that the chlorophyll content increases with leaf development, and then decreases with the senescence phenomenon (Fig. 1). The overall mean for the crop was 0.238 mg cm^{-2} (±0.61). Measurement *in situ* was positively correlated with the laboratory extraction, expressed in function of area (r=0.88; $P \leq 0.0001$) through all ages, indicating a strong relationship between both variables. Therefore, the CCM 200 plus chlorophyll meter provides a satisfactory estimate

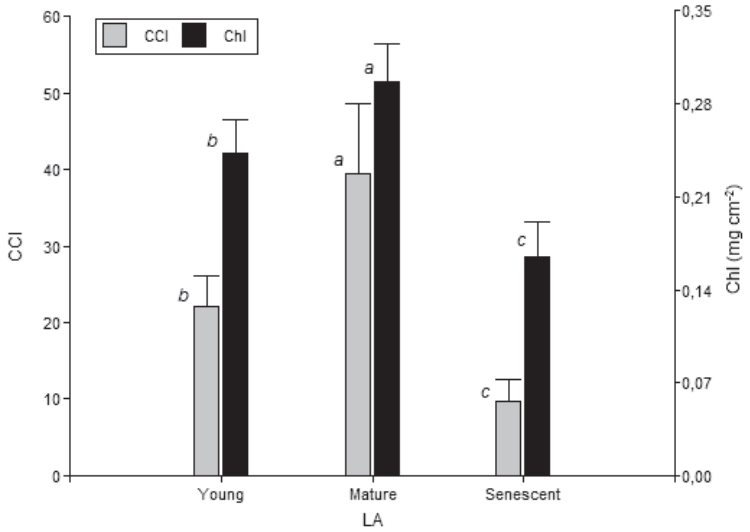

Figure 1. Chlorophyll concentration (mg cm^{-2}) through laboratory determination (Chl) and non-destructive chlorophyll meter readings (CCI) with corresponding standard deviation. Different letters indicate significant differences between leaf ages (LA) ($p \leq 0.05$).

of chlorophyll content of *Origanum vulgare* ssp. *hirtum* leaves, consistent with the findings of Silla *et al*. (2010) on leaves of *Quercus* sp., Jifon *et al*. (2005) in *Citrus* sp., van der Berg and Perkins (2004) in sugar maple, all using the same meter.

Regression analysis indicated that the calibration model with the greatest fit in determining total leaf chlorophyll content is logistic (Fig. 2A) and responds to the equation [Chl (mg cm^{-2})=0.343/(1+1.925$^{(-0.069x)}$)]. However, other authors such as Ruiz Espinoza *et al.* (2010) in basil; Loh *et al.* (2002) in rubber plant and poplar, and Dwyer *et al.* (1991) in corn; using another meter (SPAD) described linear fit models. In addition, van der Berg and Perkins (2004) with the CCM 200 plus obtained a linear fit model.

In oregano, the relationship between both variables showed a clear curvilinear component, tending to stabilize when readings values rise. With regard to model fit and leaf age, a linear type relationship (Y=0.145+0.004x; R^2=0.77; *P*<0.0001) overestimates low chlorophyll values (senescent or chlorotic leaves) as well as leaves that have high chlorophyll concentrations (Castelli *et al.*, 1996). Working in corn, Dwyer *et al.* (1991) obtained a linear model, because they disregarded basal leaves with scant amounts of chlorophyll, in a state of senescence or close to it, obtaining negative values for this kind of leaves when making the conversion to absolute values. In contrast, this work included leaves with a wide range of photosynthetic pigment content and from different layers to get a calibration model with greater fit and representation.

A second-order polynomial model (Y=0.11+0.0073x-0.0001x^2), like that obtained by Monje and Bugbee (1992) in rice, wheat and soybean makes a more accurate statistical fit, improving the regression coefficient (R^2=0.82; *P*<0.0001). However, a logistic model (Fig. 2B) was more accurate in a wider range of data, improving the representation of the chlorophyll content. Conversely, second-order polynomial curve did not follow the shape of the relationship observed between destructive and non-destructive methods. Thus, the accuracy of the chlorophyll estimation diminishes, even when the apparent arrangement of the data was statistically superior (loss of accuracy above 40 CCI).

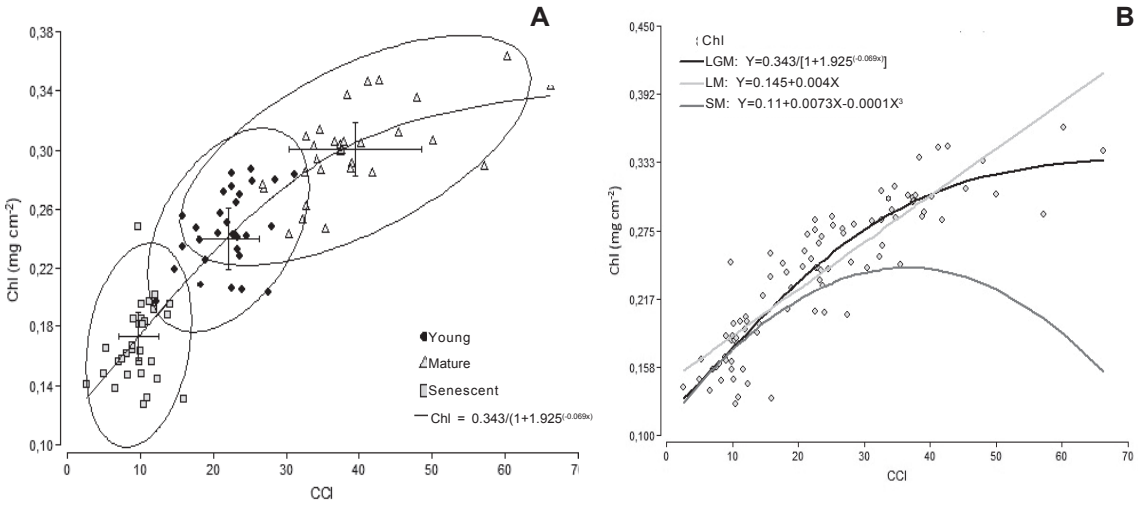

Figure 2. A**)** Relationship between total leaf chlorophyll concentration (**Chl** mg cm^{-2}) and meter readings (**CCI**) from *Origanum vulgare* ssp. *hirtum* leaves with standard deviations. Each subset of readings represents different leaf ages delimited by prediction ellipse. B**)** Logistic model (LGM) compared with linear (LM) and second-order polynomial models (SM).

In the analysis of the leaf anatomical structure (Fig. 3), significant differences were found for the three types of leaves in Spongy Parenchyma Thickness and Thickness Index. In the case of Mesophyll Thickness, only the mature leaves group was differentiated from the other leaf ages. Conversely, the number of palisade parenchyma layers remained constant throughout the different leaf ages, only one palisade cell layer (was uncommon a second layer) and so was removed from subsequent analyses. These values may indicate that leaves from upper layers increase Mesophyll Thickness due to increased Spongy Parenchyma Thickness (r=0.94; P≤0.0001).

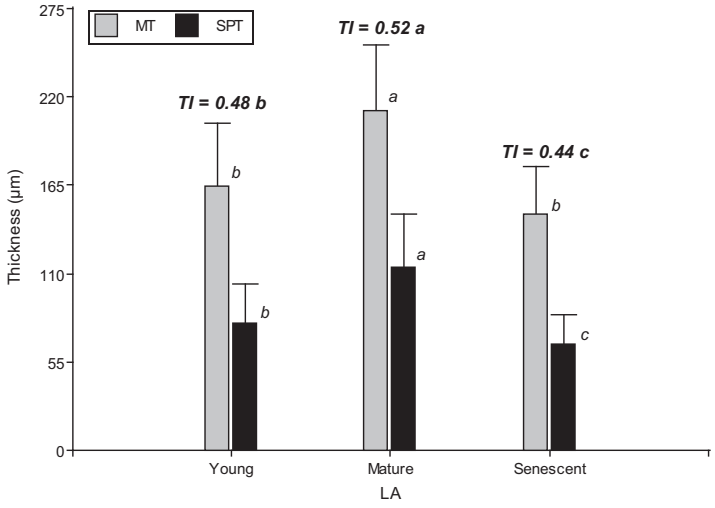

Figure 3. Mesophyll thickness **MT** (µm) and spongy parenchyma thickness **SPT** (µm) in *O. vulgare* ssp. *hirtum*, according leaf age (**LA**) with corresponding thickness index (**TI**) and standard deviation. Different letters indicate significant differences between leaf ages (**LA**) (p≤0.05).

The leaves continue to thicken with age (White and Montes-Rand, 2005). In addition, Gausman *et al.* (1971) demonstrated in cotton a gradient in which the thickness increases from the leaves of apical to basal nodes. In oregano, physiologically mature leaves correspond to the upper layer near the inflorescence, emerging chronologically after those that were taken as senescent. Leaves classified as mature do not reach senescence because they were harvested previously that stage, according to the agronomic management of the crop. For this reason, the leaves of lower layer, from the effect of shade, rapidly begin the process of senescence. This would explain the lower value of Mesophyll Thickness achieved by senescent leaves.

Bosabalidis (2002) mentioned that the spongy parenchyma relative volume was 43.9% of the leaf histological components in *O. vulgare* ssp. *hirtum*, which matches the data in this study (Thickness Index =0.44 to 0.53). Although the author makes no mention of how this parameter varies in leaves of different ages. Gönüz and Özörgücü (1999) also describe a greater share of the spongy parenchyma on *Origanum onites* L.

The physiological result of changes in the morpho-anatomical structure due to leaf age is the modification of the optical properties, inducing differences in calibration models. Thus, Castelli *et al.* (1996) found that young leaves have a more compact mesophyll with few intercellular spaces and rich in protoplasm, while adult leaves have more spongiform tissue, vacuolated cells and higher percentage of intercellular spaces. The meter differentially estimates chlorophyll content between a younger leaf with compact tissue and another more mature leaf with spongy tissue (Castelli *et al.*, 1996). In this way, leaf age contributes to the distortion of the linearity of the relationship between destructive method and meter readings. The results show mean correlation values between chlorophyll meter readings and Spongy Parenchyma Thickness (r=0.61; $P \leq 0.0001$) and slightly lower with Mesophyll Thickness (r=0.58; $P \leq 0.0001$), explaining part of the variability of the readings. For chlorophyll determination in laboratory, the correlations were somewhat weaker: r=0.54 for Spongy Parenchyma Thickness and r=0.53 for Mesophyll Thickness, but equally significant ($P \leq 0.0001$).

The increase effect of Mesophyll Thickness and Spongy Parenchyma Thickness on the variability of the meter readings in mature leaves may be explained by two factors. First, the greater dispersion of the light beam passing through the leaf, dependent on the spatial arrangement of cells in the mesophyll (Monje and Bugbee, 1992). This dispersion reduces the amount of transmitted light in the visible spectrum, as it increases its optical path through the leaf, with more likelihood of being absorbed. The largest component appears to be refraction at the cell-air space interface, which increases the effect with increasing Spongy Parenchyma Thickness (Fig. 3) and consequently Thickness Index (Monje and Bugbee, 1992). Added to the contribution of the dispersion, increased chlorophyll concentration enhances this effect (Santos Nascimento and Marenco, 2010).

Another factor is the pigment distribution (Sieve effect) as indicated Monje and Bugbee (1992). The lower uniformity in the spatial distribution of pigment increases the transmittance and reduces absorption. Thus, this effect is more pronounced in leaves with greater development of spongy parenchyma with an irregular cell distribution, which increases the non-uniformity in the pigment spatial distribution. Likewise, chlorophyll is less evenly distributed as its concentration increases (Uddling *et al.* 2007; Castelli *et al.* 1996; Monje and Bugbee 1992). In summary, light beam dispersion, higher chlorophyll concentration and its uneven distribution explain variability observed in meter readings on leaves related with the increased of Mesophyll Thickness and Spongy Parenchyma Thickness. These factors may denote the loss of accuracy and reliability in the estimates of the chlorophyll meter with increasing chlorophyll concentration.

CONCLUSION

This study provides the first insights into the calibration of the CCM 200 plus chlorophyll meter in oregano leaves under field conditions, and its relationship with leaf age and mesophyll thickness. The CCM 200 plus meter provided good estimates of chlorophyll content for all leaves, independent of leaf age, position within the plant and phenological stage. Nevertheless, the meter loses efficiency with increasing chlorophyll concentration and/or increased leaf thickness. As the leaves develop mesophyll thickness and chlorophyll content increases. The increased thickness given by greater development of spongy parenchyma leads to higher variability in the readings and linear relationship distortion. It is for this reason that the calibration model that responds to a logistic function was the equation that provides the best fit and representation in the ratio between instrument readings and chlorophyll concentration [$(Chl=0.343/(1+1.925^{(-0.069x)}))$]. Furthermore, it adapts to broad data ranges and any leaf typology, according to ontogenetic age and position in the plant, which could compensate for the limitations caused by leaf size and agronomic management. As far as we are aware, this calibration model has not been mentioned in the literature before.

Despite the loss of efficiency mentioned, the CCM 200 plus portable meter is a very useful tool to estimate chlorophyll content, aiming to relate these values with different situations of environmental stress, nutritional management programs and genetic improvement systems.

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