ACTA SCIENTIARUM POLONORUM
Acta Sci. Pol. Hortorum Cultus, 20(6) 2021, 105–115
https://czasopisma.up.lublin.pl/index.php/asphc ISSN 1644-0692 e-ISSN 2545-1405 https://doi.org/10.24326/asphc.2021.6.11
ORIGINAL PAPER
Accepted: 1.04.2021

POSSIBILITIES OF USING RGB-BASED IMAGE ANALYSIS TO ESTIMATE THE CHLOROPHYLL CONTENT OF MICROPROPAGATED STRAWBERRY PLANTS

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ABSTRACT

An image analysis method based on RGB features for rapid estimation of the chlorophyll content of leaves of micropropagated strawberry plants (2 cultivars) was presented in the study. An algorithm describing the relationship between the absolute values of chlorophyll content and the colour components of a leaf image captured with a conventional scanner was developed and tested. The accuracy of the proposed method was compared with that of an optical greenness meters designed for assessing leaf chlorophyll content. The chlorophyll content in the strawberry leaves was correlated with the results of measurements recorded by the two optical meters (SPAD-502, CCM-200) and with RGB values of scans of these leaves. The highest values of correlation coefficients were obtained between the chemical analysis results and mean values of the red colour (R) of the scans. However, varietal differences were evident here, which indicates the need for individual calibrations. In the case of the green colour (G), the accuracy was slightly lower; however, no varietal differences were found, thus one calibration can be used for both cultivars. Three formulas: (R–G)/(R+G), (R–G)/(R+G+B), and R/(R+G+B) were selected and their relationship with the changes in chlorophyll content was tested. These variables did not explain the changes in chlorophyll content better than the variable R. The study confirmed the possibility of using the image capture method for the detection of chlorophyll status in strawberry plantlets cultured *in vitro*.

Key words: tissue cultures, scanner, chlorophyll meter, precision farming

INTRODUCTION

In recent years, tissue cultures have become a tool used for plant propagation, commonly employed in horticultural production around the world. Micropropagation has found application in the protection of biodiversity (protection of genetic resources of rare, endangered or valuable species), production of virus-free material and research on the genetic transformation of plants Thanks to the *in vitro* method, it is also possible to study the developmental cycle of a given species, its breeding biology and nutritional requirements [Ticha et al. 1998, Rybczyński and Mikuła 2006].

Due to the special conditions prevailing in *in vitro* cultures: high humidity, low irradiance, limited gas exchange, the presence of sugars at high concentrations and growth regulators in the medium, plants may exhibit abnormal morphology, anatomy and physiology [Valero-Aracama et al. 2006]. This is often the cause of problems during plant acclimatization after transfer to *ex vitro* conditions [Borkowska 2001, Pospísilová et al. 2007]. Many papers have reported on the influence of culture conditions (e.g. presence of sugars, light intensity) on the concentration of pig-

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ments, photosynthetic activity and growth [Tichá et al. 1998, Valero-Aracama et al. 2006]. According to some authors, the physiological state of micropropagated shoots is of crucial importance for the progress of their development (multiplication, rooting) and acclimatization after the transfer of micropropagated plants to natural conditions [Magyar-Tábori et al. 2011]. An improvement in the parameters related to photosynthesis, such as chlorophyll content or the structure of stomata, may increase the survival of plants in *ex vitro* conditions [Van Huylenbroeck et al. 2000, Pospísilová et al. 2007].

The environmental conditions inside the culture container cannot be controlled directly; they are regulated by modifying the parameters on the outside of the container and by the development of the micro-plant itself. It is important to understand the relationships between culture conditions, growth and physiological state of shoots in order to optimize the micropropagation conditions. Assessment of the physiological state of micropropagated shoots can help to understand these relationships.

Analysis of the physiological state of micro-plants, in particular assessment of the activity of the photosynthetic apparatus during *in vitro* culture, has been the subject of some research work [Van Huyelenbroeck et al. 2000]. Shoots propagated *in vitro* are potentially capable of developing a functioning photosynthetic apparatus [Arigita et al. 2002]; however, the unique conditions cause technical problems with the measurement of photosynthesis by means of gas exchange analysis. Attempts to assess the functionality of the photosynthetic apparatus with the use of chlorophyll *a* fluorescence have been more frequent [Borkowska 2001]; however, using this method is not easy and can be burdened with measurement errors due to the small leaf area making measurements difficult [Ibaraki and Matsumura 2005]. For these reasons, simple methods suited for micropropagated plants are needed to be developed.

Assimilatory pigments are some of the most important chemical compounds in a plant that influence the rate of photosynthesis and biomass production. An assessment of the chlorophyll content of tissues can provide useful information about the physiological state of shoots during micropropagation. Normally, the chlorophyll content is measured by spectrophotometric and chromatographic methods. These methods are expensive and often time and labour-intensive. Several indirect, optical methods for determining foliar chlorophyll status have become available in the last decade, and portable leaf greenness meters have become popular in agricultural studies and crop production systems (e.g. SPAD-502, CCM-200) [Uddling et al. 2007, Pagola et al. 2009, Treder et al. 2016]. As reported in many papers, the relationship between relative chlorophyll values (measured with optical meters) and absolute chlorophyll content is variable and depends on the plant species and cultivar, and may be influenced by environmental and measurement conditions, and therefore must be standardized for these parameters [Treder and Cieśliński 2003].

The chlorophyll content in the leaves can also be determined by analysing the distribution of colour components of the image of a single leaf or group of plants [Spomer et al. 1988, Mohan and Dutta Gupta 2019]. Any colour can be decomposed into the three primary components (red, green, and blue), and the determination of colour component values can be performed in a digital image [Yadav et al. 2010, Treder et al. 2016]. Taking this into consideration, there is a possibility to develop mathematical models to describe the relationship between leaf chlorophyll content and the distribution of colour components of leaf blade surface. Such approach was tested on several crop species [Özreçberoğlu and Kahramanoğlu 2020, Kahramanoğlu et al. 2021]. Kahramanoğlu and coauthors [2021] found that the analysis of RGB values obtained by using contact imaging is a useful tool for quick estimation of the chlorophyll contents of strawberry leaves.

In the recent years artificial neural network (ANN) technique has been applied to estimate chlorophyll content of plants. This technique can identify and learn from correlated patterns between input data sets and corresponding target values, even when the underlying data relationship is unknown [Odabas et al. 2017 a]. The study performed by Caliskan and coauthors [2020] demonstrated that the ANN could be used to predict the chlorophyll level of sugar beet by analyzing leaf images using the image-processing techniques with reasonable accuracy. A similar approach was applied by Odabas and coauthors [2017 a, b] on lettuce and some medicinal and aromatic plants.

Plant leaf colour analysis has been applied in agriculture in managing plant nutrition (determination of nitrogen content in leaves), assessing water availability, plant disease identification, and analysis of senescence [Dutta Gupta et al. 2013, Treder et al. 2016]. In plant tissue culture, image analysis has been adopted to estimate shoot length [Honda et al. 1997] and to evaluate the health condition of *in vitro*-stored tissue-cultured plantlets [Aynalem et al. 2006]. Also, first attempts to use this method for assessing the chlorophyll content of regenerated plantlets have been made [Yadav et al. 2010, Dutta Gupta et al. 2013].

The present work describes an image analysis method based on RGB features, for rapid estimation of the chlorophyll content of leaves of micropropagated plants. Strawberry was used as the test species (2 cultivars). An algorithm describing the relationship between the absolute values of chlorophyll content and the colour components of a leaf image captured with a conventional scanner was developed and tested. Additionally, the accuracy of the proposed method was compared with that of an optical greenness meter designed for assessing leaf chlorophyll content.

MATERIAL AND METHODS

Plant material and culture conditions. The study was conducted at the Research Institute of Horticulture (currently The National Institute of Horticultural Research), Skierniewice Poland. The experiments were performed on 2 genotypes of strawberry (*Fragaria* × *ananassa* Duchesne): 'Pink Rosa' and 'Grandarosa'. The *in vitro* strawberry shoot cultures were established from apical meristems of runners taken from virus-free mother plants grown in a greenhouse. The basal medium developed by Boxus [1974; modified by Borkowska 2001] was applied, which contained macroelements [Knop 1965], microelements and vitamins [Murashige and Skoog 1962], supplemented with glycine (2 mg l^{-1}), inositol (100 mg l^{-1}), glucose (40 g l^{-1}), indole-3--butyric acid (IBA, 0.1 mg l^{-1}), 6-benzylaminopurine (BA, 0.1 mg l^{-1}), gibberellic acid (GA3, 0.1 mg l^{-1}), and solidified with Difco Bacto Agar (8 g l^{-1}). The pH of the medium was adjusted to 5.6–5.7 prior to autoclaving. The medium for shoot proliferation contained higher concentrations of IBA (0.5 mg l^{-1}) and BA (0.5 mg l^{-1}). The cultures were maintained in a growth chamber at 23°C/18°C (day/night), under white light (Philips fluorescent tubes 'TL'D 36W; Philips Lighting, the Netherlands) with quantum irradiancy of 55 µmol m^{-2} s^{-1} and 16 h photoperiod. Every six weeks, the shoots were transplanted onto a fresh medium. The last six-week propagation passage was carried out on media modified to vary the chlorophyll content in the tested plant material. The modified media contained different concentrations of calcium nitrate $Ca(NO_3)_2 \cdot 4H_2O$ (0.36, 0.72, 1.44 g l^{-1}), potassium nitrate KNO_3 (0.0625, 0.125, 0.25 g l^{-1}) and sugar (10, 20, 40 g l^{-1}).

Leaf greenness measurements. After the last passage, the leaves (separately from each nutrition treatment) were collected and digital recording of the adaxial surface was performed using an Epson Perfection 3170 PHOTO scanner. The leaves *were scanned* against a *black background at 3*00 DPI. Images were saved as JPEG (joint photographic experts group) files. The acquired images were analysed to obtain average values of the basic colours: red, green, and blue (RGB). After the imaging sequence was completed, greenness level measurements (on all sampled leaves) were performed using SPAD-502 (Minolta, Japan) and CCM-200 (Opti-Sciences, USA) meters.

Image analysis and leaf colour evaluation. A dedicated application had been created that determined the colour component values individually for each pixel of leaf image without the background (Fig. 1). A threshold was established (RGB values below 30 corresponding to a dark, blackish colour) to distinguish leaf blade colours from the background. To build the application, Hypertext Preprocessor (PHP), HyperText Markup Language (HTML), Cascading Style Sheets (CSS), JavaScript and jQuery programming tools were used. To generate and analyse graphics in PHP, the appropriate Graphics Library (GD) was applied. This library provides a number of functions for creating and manipulating graphics. The application uses the functions: 'imagecreatefromjpeg', 'imagecolorat' and 'imagecolorsforindex'.

Determination of chlorophyll content. Chemical analyses were performed on the same leaves that had been used for greenness measurements described above. The material for the determination of chlorophyll pigments consisted of fragments of leaf blades of the test plants. From each group, previously used

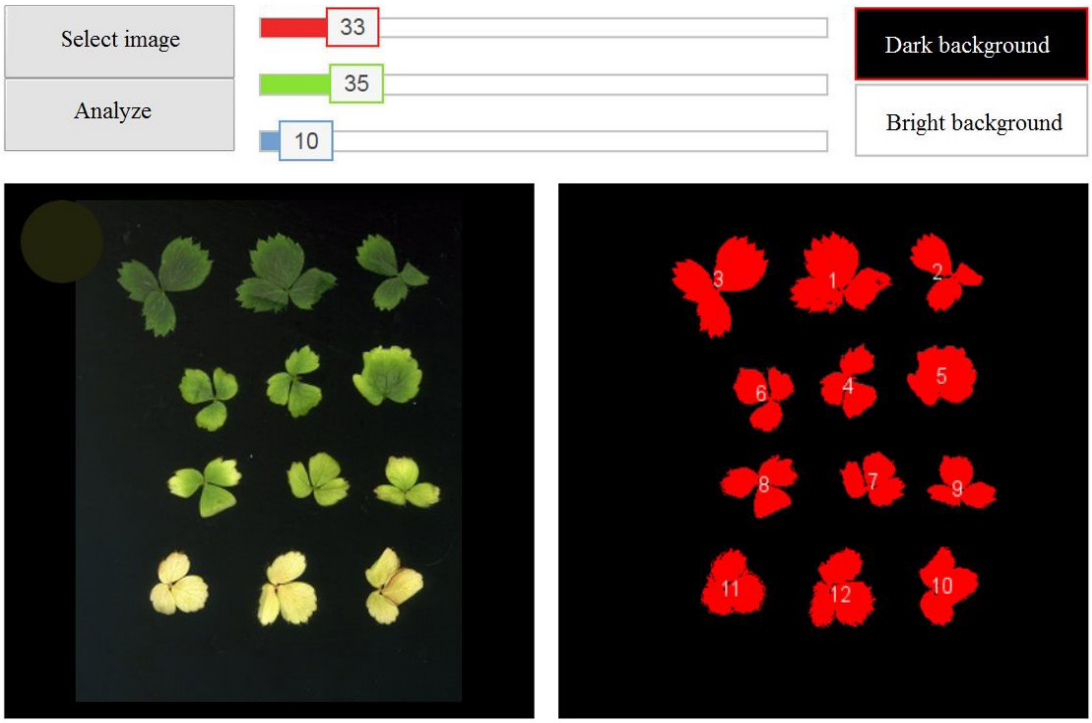

Fig. 1. A screenshot of the image analysis software (example)

for RGB and greenness analyses, a weighed fragment of leaf blade (about 100 mg, what corresponded to 3–4 leaf blades) was sampled, ground in a mortar in a solution of acetone (80%) with the addition of anhydrous $CaCO_3$. Leaf sampling was repeated 9 times. The ground material was quantitatively transferred to graduated test tubes and made up with 80% acetone to a volume of 10 ml. A sample prepared in this way was then agitated in darkness for 3 hours at +4ºC (WU-4, Premed, Poland). From each sample, 1.3 ml of the solution was taken three times for separate determinations. The solution was placed in Eppendorf tubes and centrifuged at 11,000 rpm for 10 minutes. Absorbance of the extract was determined spectrophotometrically at 663 and 645 nm wavelengths (NICOLET EVOLUTION 300, Thermo Electron Corporation, USA). The chlorophyll content was calculated according to the formulas given by Bruisma [1963]:

$$\text{Chl } a = \frac{[12.7\times(A663) - 2.7\times(A645)] \times V}{1000 \times W}$$

$$\text{Chl } b = \frac{[22.7\times(A645) - 42.7\ (A663)] \times V}{1000 \times W}$$

V equals the volume of chlorophyll extract (mL) and W is the fresh weight of leaf samples used for the analysis. The chlorophyll *a* and *b* values were summed to reach the total chlorophyll contents of the samples.

In order to assess the relationship between the actual chlorophyll content and the measurements made by means of chlorophyll meters and image analysis components, regression analysis was used. Relationships were evaluated using simple linear, second polynomial, exponential and logarithmic models. Correlation coefficients calculated after model linearization were used as a measure of the model's fit. The assessment of the universality of the method depending on the genotype was verified by comparing the coefficients of the obtained equations using the Student test. The influence of particular components of the image on chlorophyll content was evaluated using the multiple

step-wise regression procedure. All calculations were performed using STATISTICA v. 11 package (Dell Inc., 2016).

RESULTS AND DISCUSSION

Assessment of the chlorophyll content in plant tissue is important for the selection of cultivars and phenotyping. It is the basis for the expression of the rate of photosynthesis and plant productivity [Cerovic et al. 2012]. For large-scale canopy monitoring, chlorophyll estimation is often performed through remote sensing, whereas the use of portable hand-held meters is useful for ground-based (open field and greenhouse) chlorophyll assessment.

To evaluate the suitability of indirect methods for assessing the chlorophyll content of strawberry leaves, the results of measurements with the indirect methods were compared with the data of chemical analyses. Table 1 presents the correlation coefficients between the actual chlorophyll content in strawberry leaves, on the one hand, and the indices of chlorophyll content (SPAD and CCM) and various RGB formulas, on the other hand. The chlorophyll content of the leaves of strawberry plants was correlated with the results of measurements recorded by the two optical meters and with RGB values of scans of these leaves. The highest values of correlation coefficients were obtained between the chemical analysis results and mean values of the red (R) and green (G) colours of the scans.

Generally, the results obtained indicate a close correlation between the results of the actual chlorophyll content (chemical analysis) and the measurements taken with the optical meters in comparison with the literature data pertaining to plants cultivated in field or glasshouse conditions [Cerovic et al. 2012, Jiang et al. 2017]. The literature results also show smaller errors in forecasting the chlorophyll content for the SPAD meter compared with CCM [Richardson et al. 2002]. Our study yielded higher values of the correlation coefficient for the SPAD meter than the CCM-200 meter.

Many papers have shown that readings of optical meters may suffer from interference by environmental conditions and measurement conditions [Treder and Cieśliński 2003, Naus et al. 2010]. The variability observed between different species or even cultivars has been explained by the differences in leaf structure causing differences in light reflection, or by the scattering effect [Richardson et al. 2002]. Significant differences found between different instruments have led to the recommendation that each instrument should be calibrated individually [Markwell et al. 1995, Treder et al. 2016].

The best calibration model for the CCM meter was the second-degree polynomial model (Fig. 2). For the SPAD meter, it was the exponential model (Fig. 3). Other researchers have reported different models to describe the relationship between optical values and the actual chlorophyll concentration [Richardson et al. 2002, Jifon et al. 2005, Hawkins et al. 2009]. In most studies, linear relationships between the leaf chlorophyll and the SPAD values have usually been proposed for the calibration of SPAD meters [e.g. Fanizza et al. 1991, Wang et al. 2004], although reports showing non-linear relationships between chlorophyll and SPAD readings also exist [Jifon et al. 2005, Uddling et al. 2007]. Some authors have suggested that the observed non-linearity can be caused by a non-uniform distribution of chlorophyll or radiation across the leaf surface [Monje and Bugbee 1992, Uddling et al. 2007].

The study did not show any significant effect of the cultivar on the results obtained with the use of the optical meters. The lack of such differentiation means that the same calibration can be used for a specific meter (for the cultivars being evaluated). There are reports available in the literature proposing a single optical/absolute chlorophyll relationship for multiple cultivars of different crop species, e.g. soybean, maize, wheat [Markwell et al. 1995, Uddling et al. 2007]. However, in other studies, significantly different relationships have been observed among cultivars [Fanizza et al. 1991, Jifon et al. 2005]. Generally, it is suggested that separate models should be tested for each cultivar to maximize the accuracy of estimating leaf chlorophyll content from optical measurements [Richardson et al. 2002]. The fact that a single prediction equation cannot be applied across a wide range of cultivars to determine chlorophyll content is considered a major limitation of indirect methods.

The experiment showed that, compared with the optical meters, the chlorophyll content of strawberry leaves is better described by the measurements of the mean green or red colour values of leaf scans. The highest accuracy was found for the red colour values

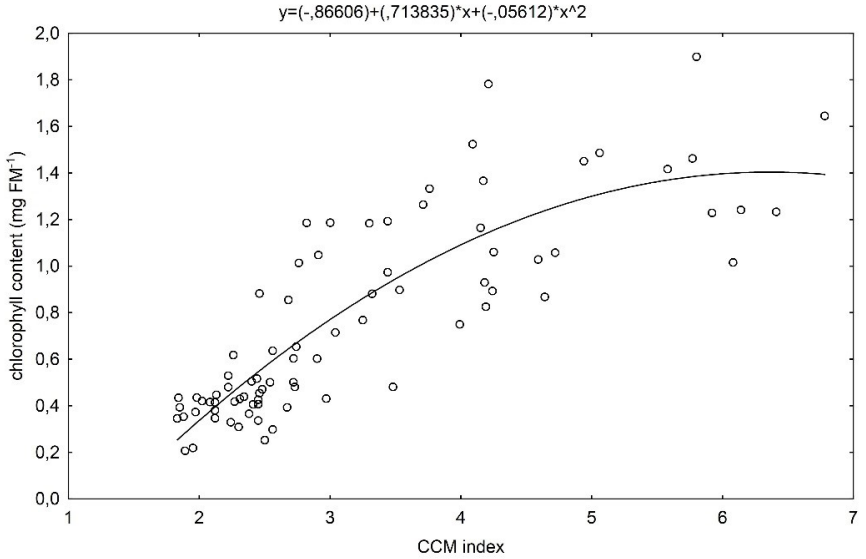

Fig. 2. Relationship between CCM indices and chlorophyll content in strawberry leaves

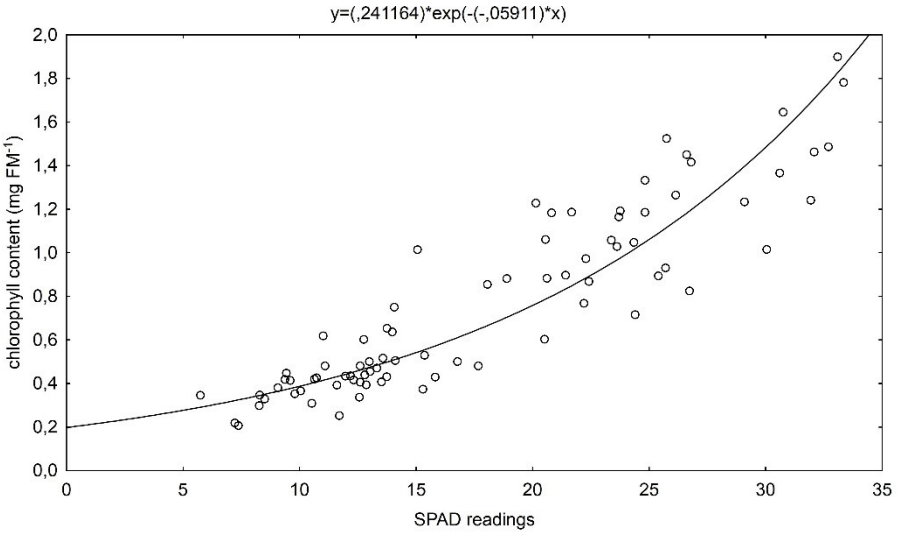

Fig. 3. Relationship between SPAD indices and chlorophyll content in strawberry leaves

using the exponential model (Fig. 4). However, varietal differences were evident here, which indicates the need for individual calibrations. In the case of the green colour (Fig. 5), the accuracy was slightly lower; however, no varietal differences were found, thus one calibration can be used for both cultivars.

Possibility of estimating the leaf chlorophyll content from the G or R values in strawberry was noted also by other authors [Kahramanoğlu et al. 2021]. Similar to our findings, Kahramanoğlu and coauthors [2021] found a poor correlation between the B value and leaf chlorophyll content in strawberry leaves.

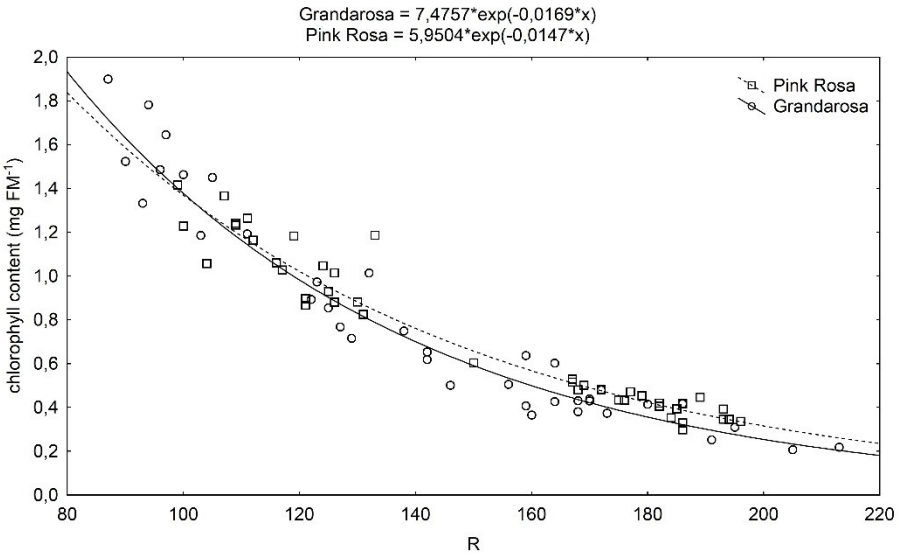

Fig. 4. Relationship between the average values of the red colour (R) and chlorophyll content in strawberry leaves

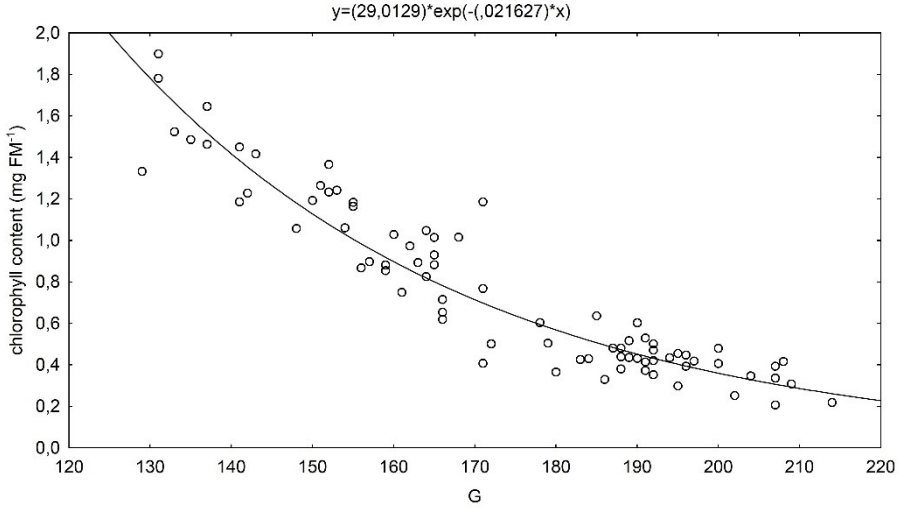

Fig. 5. Final model describing the relationship between the average values of the green colour (G) and chlorophyll content in strawberry leaves

It was reported that the use of natural light or white LED (e.g. scanner light source) provides a good correlation for the G and R values, and a poor correlation for the B value, for the verification of the leaf chlorophyll content [Özreçberoğlu and Kahramanoğlu 2020]. R, G were found to be the most closely related with SPAD readings in rice [Tewari et al. 2013]. The results of the present study are in accordance with this literature. In contrast, Vesali and coauthors [2017] found that the green component of RGB colour space was not an appropriate index to estimate the Chl content in maize. The results obtained by Özreçberoğlu and Kahramanoğlu [2020] showed that the best estimation of the leaf chlorophyll content of

Table 1. Linear correlation coefficients between leaf colour parameters (RGB), SPAD, CCM indices and the actual chlorophyll content in strawberry leaves, and multiple linear regression analysis to test the dependence of chlorophyll content on the colour parameters. Data averaged for two test cultivars

Linear correlation coefficient							
Chlorophyll content	Covariates						
	CCM	SPAD	R	G	B	(R–G)/(R+G)	(R–G)/(R+G+B)
	0.821***	0.912***	–0.940***	–0.930***	–0.693***	–0.938***	–0.928***
Multiple step-wise regression analysis							
Covariates (xi)	*b*	S_b	*t*	*p*	*F*	*p*	R^2
Model I							
Intercept	2.62	0.3041	8.61	<0.0001	202.7	<0.0001	0.885
R	–0.0112	0.0029	3.88	<0.0001			
G	0.0018	0.0039	0.46	0.648			
B	0.0030	0.0023	1.33	0.187			
Model II							
Intercept	2.48	0.0720	34.5	<0.0001	307.1	<0.0001	0.886
R	–0.0125	0.0007	16.8	<0.0001			
B	0.0034	0.0020	1.69	0.0945			
Model III							
Intercept	2.46	0.0711	34.6	<0.0001	597.1	<0.0001	0.883
R	−0.0115	0.00047	24.4	<0.0001			

b – estimated value of model parameter, S_b – standard error of coefficient, *t* – value of Student test statistic, *p* – probability of statistic, *F* – Fisher statistic, R^2 – corrected determination coefficient (n = 80, *** – p < 0.001)

pomegranate trees was possible by using both G and B values.

Multiple regression analysis indicates that the variables G and B have an insignificant effect on the dependent variable, and the loss of information by eliminating these variables from the model is small, and the changes in chlorophyll content are only explained by the changes in the variable R (Tab. 1). After the initial selection between the standardized variables, three formulas: (R–G)/(R+G), (R–G)/(R+G+B), and R/(R+G+B) were selected and their relationship with the changes in chlorophyll content was tested. It became evident that these variables did not explain the changes in chlorophyll content better than the variable R.

The image analysis method has been used to successfully assess leaf chlorophyll content in some crop species. For instance, Pagola et al. [2009] calculated a greenness index using the RGB components of the colour image in barley. Ali et al. [2012] achieved better performance than SPAD in chlorophyll measurements using a leaf-colour-based algorithm developed for lettuce, broccoli and tomato plants.

Recently, we have demonstrated the potential of the RGB image analysis to assess the nitrogen content of apple trees using scanned images of the leaves [Treder et al. 2016]. Higher accuracy was found in determining foliar N content using image analysis compared with the accuracy of chlorophyll meters designed for this purpose. Portable chlorophyll meters have limited resolution, and measure only small samples, which precludes them from being used for leaves with a non-uniform chlorophyll distribution [Spomer et al. 1988]. Rorie and co-authors [2011], after analysing images of corn, concluded that digital colour

analysis had the potential to be used in the same way as chlorophyll meters, but at a fraction of the cost. Because of its own (constant) light source, the scanner makes it possible to obtain the same intensity of colours regardless of the external lighting conditions.

There have been some attempts to apply the image analysis method to estimating chlorophyll in tissue cultures. Yadav et al. [2010] tested the possibility of measuring the chlorophyll content of regenerated leaves (micropropagated potato plantlets) using their scanned images. They found a significant correlation between the mean brightness of R and G with the chlorophyll content measured in regenerated plants with a SPAD meter (using a single colour component approach). The same team proposed an RGB-based image analysis method using a digital camera for estimating chlorophyll content during micropropagation of potato plants [Dutta Gupta et al. 2013].

In conclusion, the present study confirmed the possibility of using the image capture method for the detection of chlorophyll status in plantlets cultured *in vitro*. Considering the quality of the prediction using the developed model, this simple system, consisting of a conventional scanner and a colour analysis algorithm, might be utilized for estimating the chlorophyll content in strawberry plantlets in order to optimize the culture conditions for efficient micropropagation and to characterize the physiological status of the regenerated plants for better *ex vitro* acclimatization and further development.

ACKNOWLEDGEMENTS

This research was financially supported by the Ministry of Science and Higher Education of the Republic of Poland under statutory funds.

REFERENCES

Ali, M.M., Al-Ani, A., Eamus, D., Tan, D.K.Y. (2012). A new image processing based technique to determine chlorophyll in plants. Am. Euras. J. Agric. Environ. Sc., 12, 1323–1328. https://doi.org/10.5829/idosi.aejaes.2012.12.10.1917

Arigita, L., Gonzalez, A., Sanchez Tames, R. (2002). Influence of CO_2 and sucrose on photosynthesis and transpiration of *Actidinia deliciosa* explants cultured *in vitro*. Physiol. Plant., 115, 166–173.

Aynalem, H.M., Righetti, T.L., Reed, B.M. (2006). Non-destructive evaluation of *in vitro*-stored plants: a comparison of visual and image analysis. In Vitro Cell. Dev. Biol. Plant., 42, 562–567. https://doi.org/10.1079/IVP2006816

Borkowska, B. (2001). Morphological and physiological characteristics of micropropagated strawberry plants rooted *in vitro* and *ex vitro*. Sci. Hortic., 89, 195–206. https://doi.org/10.1016/S0304-4238(00)00230-2

Boxus, P. (1974). The production of strawberry plants by *in vitro* micropropagation. J. Hort. Sci., 49, 209–210.

Bruinsma, J. (1963). The quantitative analysis of chlorophyll *a* and *b* in plant extracts. Phytochem. Phytobiol., 2, 241–249.

Caliskan, O., Kurt, D., Camas, N., Odabas, M.S. (2020). Estimating chlorophyll concentration index in sugar beet leaves using an artificial neural network. Pol. J. Environ. Stud., 29(1), 25–31. https://doi.org/10.15244/pjoes/95031

Cerovic, Z.G., Masdoumier, G., Ghozlen, N.B., Latouche, G. (2012). A new optical leaf-clip meter for simultaneous non-destructive assessment of leaf chlorophyll and epidermal flavonoids. Physiol. Plant., 146, 251–260. https://doi.org/10.1111/j.1399-3054.2012.01639.x

Dutta Gupta, S., Ibaraki, Y., Pattanayak, A.K. (2013). Development of a digital image analysis method for real time estimation of chlorophyll content in micropropagated potato plants. Plant Biotechnol. Rep., 7, 91–97. https://doi.org/10.1007/s11816-012-0240-5

Fanizza, G., Della Gatta, C., Bagnulo, C. (1991). A non-destructive determination of leaf chlorophyll in *Vitis vinifera*. Ann. Appl. Biol., 119, 203–205. https://doi.org/10.1111/j.1744-7348.1991.tb04858.x

Hawkins, T.S. (2009). Modeling the relationship between extractable chlorophyll and SPAD-502 readings for endangered plant species research. J. Nat. Conserv., 17, 123–127. https://doi.org/10.1016/j.jnc.2008.12.007

Honda, H., Takikawa, N., Noguchi, H., Hanai, T., Kobayashi, T. (1997). Image analysis associated with a fuzzy neural network and estimation of shoot length of regenerated rice callus. J. Ferment. Bioeng., 84, 342–347. https://doi.org/10.1016/S0922-338X(97)89256-2

Ibaraki, Y., Matsumura, K. (2005). Non-destructive evaluation of the photosynthetic capacity of PSII in micropropagated plants. J. Agric. Meteorol., 60, 1073–1076. https://doi.org/10.2480/agrmet.1073

Jiang, Ch., Johkan, M., Hohjo, M., Tsukagoshi, S., Maruo, T. (2017). A correlation analysis on chlorophyll content and SPAD value in tomato leaves. HortResearch, 71, 37–42. https://doi.org/10.20776/S18808824-71-P37

Jifon, J.L., Syvertsen J.P., Whaley E. (2005). Growth environment and leaf anatomy affect nondestructive estimates of chlorophyll and nitrogen in *Citrus* sp. leaves. J. Amer. Soc. Hort. Sci., 130, 152–158. https://doi.org/10.21273/JASHS.130.2.152

Kahramanoğlu, İ., Ülker, E.D., Ülker, S. (2021). Integrating support vector machine (SVM) technique and contact imaging for fast estimation of the leaf chlorophyll contents of strawberry plants. IJETT, 69(3), 23–28. https://doi.org/10.14445/22315381/IJETT-V69I3P205

Knop, W. (1865). Quantitative Untersuchungen über die Ernahrungsprozesse der Pflanzen. Landwirtsch. Vers. Stn., 7, 93–107.

Magyar-Tábori, K., Dobránszki, J., Hudák, I. (2011). Effect of cytokinin content of the regeneration media on *in vitro* rooting ability of adventitious apple shoots. Sci. Hort., 129, 910–913. https://doi.org/10.1016/j.scienta.2011.05.011

Markwell, J., Osterman, J. C., Mitchell, J.L. (1995). Calibration of the Minolta SPAD-502 leaf chlorophyll meter. Photosynth. Res., 46, 467–472.

Mohan, P.J., Dutta Gupta, S. (2019). Intelligent image analysis for retrieval of leaf chlorophyll content of rice from digital images of smartphone under natural light. Photosynthetica, 10. https://doi.org/10.32615/ps.2019.046

Monje, O.A., Bugbee, B. (1992). Inherent limitations of nondestructive chlorophyll meters – a comparison of 2 types of meters. HortScience, 27, 69–71.

Murashige, T., Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15, 473–497.

Naus, J., Prokopová, J., Rebíček, J., Spundova, M. (2010). SPAD chlorophyll meter reading can be pronouncedly affected by chloroplast movement. Photosynth. Res., 105, 265–271. https://doi.org/10.1007/s11120-010-9587-z

Odabas, M.S., Simsek, H., Lee, C., İşeri, İ. (2017a). Multilayer perceptron neural network approach to estimate chlorophyll concentration index of lettuce (*Lactuca sativa* L.). Commun. Soil Sci. Plant Anal., 48, 162–169. https://doi.org/10.1080/00103624.2016.1253726

Odabas, M., Senyer, N., Kayhan, G., Ergun, E. (2017b). Estimation of chlorophyll concentration index at leaves using artificial neural networks. J. Circ. Syst. Comput., 26(2), 1750026. https://doi.org/10.1142/S0218126617500268

Özreçberoğlu, N., Kahramanoğlu, İ. 2020. Mathematical models for the estimation of leaf chlorophyll content based on RGB colours of contact imaging with smartphones: a pomegranate example. Folia Hort., 32(1), 57–67. https://doi.org/10.2478/fhort-2020-0006

Pagola, M., Ortiz, R., Irigoyen, I., Bustince, H., Barrenechea, E., Aparicio-Tejo, P., Lamsfus, C., Lasa, B. (2009). New method to assess barley nitrogen nutrition status based on image colour analysis, comparison with SPAD-502. Comput. Electron. Agric., 65, 213–218. https://doi.org/10.1016/j.compag.2008.10.003

Pospísilová, J., Synková, H., Haisel, D., Semorádová, S. (2007). Acclimation of plantlets to *ex vitro* conditions: effects of air humidity, irradiance, CO_2 concentration and abscisic acid (a review). Acta Hort., 748, 29–38. https://doi.org/10.17660/ActaHortic.2007.748.2

Richardson, A.D., Duigan, S.P., Berlyn, G.P. (2002). An evaluation of noninvasive methods to estimate foliar chlorophyll content. New Phytol., 153, 185–194. https://doi.org/10.1046/j.0028-646X.2001.00289.x

Rorie, R.L., Purcell, L.C., Karcher, D.E., King, A.C. (2011). The assessment of leaf nitrogen in corn from digital images. Crop Sci., 51, 2174–2180. https://doi.org/10.2135/cropsci2010.12.0699

Rybczyński, J.J., Mikuła, A. 2006. Engagement of biotechnology in the protection of threatened plant species in Poland. Biodivers. Res. Conserv., 3–4, 361–368.

Spomer, L.A., Smith, M.A.L., Sawwan, J.S. (1988). Rapid nondestructive measurement of chlorophyll content in leaves with nonuniform chlorophyll distribution. Photosynth. Res., 16, 227–284. https://doi.org/10.1007/BF00028845

Tewari, V.K., Arudra, A.K., Kumar, S.P., Pandey, V., Chandel, N.S. (2013). Estimation of plant nitrogen content using digital image processing. Agric. Engineer. Int., CIGR J., 15, 78–86.

Tichá, I., Čáp, F., Pacovská, D., Hofman, P., Haisel, D., Čapková, V., Schäfer, C. (1998). Culture on sugar medium enhances photosynthetic capacity and high light resistance of plantlets grown *in vitro*. Physiol. Plant., 102, 155–162. https://doi.org/10.1034/j.1399-3054.1998.1020201.x

Treder, W., Klamkowski, K., Kowalczyk, W., Sas, D., Wójcik, K. (2016). Possibilities of using image analysis to estimate the nitrogen nutrition status of apple trees. Zemdirbyste-Agriculture, 103, 319–326. https://doi.org/10.13080/z-a.2016.103.041

Treder, W., Cieśliński, G. (2003). Ocena odżywienia jabłoni azotem za pomocą miernika SPAD-502 [Evaluation of apple nitrogen status using SPAD-502]. Folia Hort., suppl. 2, 168–170.

Uddling, J., Gelang-Alfredsson, J., Piikki, K., Pleijel, H. (2007). Evaluating the relationship between leaf chlorophyll concentration and SPAD-502 chlorophyll meter readings. Photosynth. Res., 91, 37–46. https://doi.org/10.1007/s11120-006-9077-5

Valero-Aracama, C., Kane, M.E., Wilson, S.B., Vu, J.C., Anderson, J., Philman, N.L. (2006). Photosynthetic and carbohydrate status of easy-and difficult-to-acclimatize sea oats (*Uniola paniculata* L.) genotypes during *in vitro* culture and *ex vitro* acclimatization. In Vitro Cell. Dev. Biol. Plant., 42, 572–583. https://doi.org/10.1079/IVP2006822

Van Huylenbroeck, J.M., Piqueras, A., Debergh, P.C. (2000). The evolution of photosynthetic capacity and the antioxidant enzymatic system during acclimatization of micropropagagted *Calathea* plants. Plant Sci., 155, 59–66.

Vesali, F., Omid, M., Mobli, H., Kaleita, A. (2017). Feasibility of using smart phones to estimate chlorophyll content in corn plants. Photosynthetica, 55, 603–610. https://doi.org/10.1007/s11099-016-0677-9

Wang, Q.B., Chen, M.J., Li, Y.C. (2004). Nondestructive and rapid estimation of leaf chlorophyll and nitrogen status of peace lily using a chlorophyll meter. J. Plant Nutr., 27, 557–569. https://doi.org/10.1081/PLN-120028878

Yadav, S.P., Ibaraki, Y., Dutta Gupta, S. (2010). Estimation of the chlorophyll content of micropropagated potato plants using RGB based image analysis. Plant Cell Tiss. Org. Cult., 100, 183–188. https://doi.org/10.1007/s11240-009-9635-6