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Nondestructive methods in plant biology: an accurate measurement of chlorophyll content by a chlorophyll meter

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Abstract

Nondestructive methods of plant analysis are becoming increasingly popular in plant biology. Optical measurement of leaf chlorophyll content allows to produce reliable results without destructive sampling. The aim of the present investigations was to validate the use of an absorbance-based chlorophyll meter in different fields of plant biology. A near-linear relationship was found between spectrophotometrically determined total chlorophyll content on fresh mass basis and SPAD values measured by a chlorophyll meter for all of the tested plant species. In experiments with galled plant tissues localized changes in chlorophyll content in the infested leaf indicated a significant effect of the gall-former on photosynthesis *Ulmus laevis*. A contrasting effect of the level of gall infestation on photosynthesis-related characteristics was revealed in studies with *Salix fragilis* and *Tilia platyphyllos*. A pronounced rhythmicity of chlorophyll content in leaves of bean seedlings allowed to characterize an endogenous circadian rhythm in photosynthetic activity. Changes in chlorophyll content in leaves of wheat plants were used as an early indicator to predict the effectivity of application of different foliar fertilizers. The obtained results support the potential usage of nondestructive chlorophyll measurement by chorophyll meter in various branches of plant biology, including ecophysiology, plant physiology, and agricultural sciences, when appropriate accurate calibration of SPAD readings against spectrophotometrically determined total chlorophyll content is made for every particular species.

Key words: chlorophyll, nondestructive methods, plant biology.

Introduction

During the last twenty years, nondestructive methods of plant analysis have become increasingly popular in plant biology. These methods allow to characterize important physiological processes of intact plants without injurous sampling of the plant tissues. Chlorophyll *a* fluorescence has been widely used to assess plant adaptation to an environment as well as to measure the stress level experienced by a plant (Oxborough 2004). Nuclear magnetic resonance micro-imaging allows to map plant tissue metabolites using intact plants (Köckenberger 2001). One of the most widely used group of nondestructive methods in plant sciences is optical measurement of leaf chlorophyll content.

A traditional measurement of chlorophyll amount involves extraction of plant tissues

with a solvent with subsequent spectrophotometric measurement of the absorbance (Lichtenthaler, Wellburn 1983). The major drawbacks of the method are destructive sampling and a time-consuming protocol. Several nondestructive optical methods for chlorophyll measurement have been developed in recent years based on absorbance or reflectance of chlorophyll molecules by leaf tissues at particular wavelengths (Richardson et al. 2002). Absorbance-based chlorophyll measurement has become accepted mostly in small-scale ecophysiological experiments (Neufeld et al. 2006). Commercially available portable chlorophyll absorbance meters measure difference in absorbance at two wavelengths: near 660 nm (absorbed by chlorophyll) and near 940 nm (a reference to adjust for differences in leaf structure). However, reflectance method has been used in more specific fields of ecological studies, e.a. remote sensing (Gitelson, Merzlyak 1997).

The absorbance method is fast, easy to use and produce reliable estimates of relative chlorophyll content. It is of special importance in studies where repetitive chlorophyll measurement of the same plant material over prolonged period of time is necessary or when nondestructive methods are preferred. However in the majority of physiological studies traditional methods of chlorophyll measuremet are still used. This can be related to certain possible problems with the absorbance method e.g. non-linearity of the optically measured chlorophyll amount relative to spectrophotometrical measurements (Richardson et al. 2002; Uddling et al. 2007), side effects produced by environmental conditions (Martínez, Guiamet 2004; Neufeld et al. 2006) or others.

The aim of the present experiments was to elaborate a startegy for calibration of chlorophyll meter measurements by means of chemical chlorophyll analysis. Examples for use of the method in ecophysiology, plant physiology, and agriculture are given.

Materials and methods

Plant material

Gall former effects on leaf chlorophyll content were studied using *Ulmus laevis* Pall. infested with *Colopha compressa* Koch. (Homoptera: Aphididae), *Tilia platyphyllos* L. infested with *Eriophyes tiliae* Pgst. (Acarina: Eriophydae), and *Salix fragilis* L. infested with *Pontania vesicator* Br. (Hymenoptera: Tenthredinidae) growing at the National Botanical Garden of Latvia, Salaspils. Leaves of *Ulmus laevis* were analyzed four times during a growth season. Chlorophyll content in leaves of *Tilia platyphyllos* was estimated twice during the season. Leaves of *Salix fragilis* were analyzed in the second half of the growth season in August.

Daily changes in chlorophyll content were studied in primary leaves of *Phaseolus vulgaris* L. Plants were sown in individual 250 ml containers with commercial neutralized peat moss and watered with tap water. Plants were kept in laboratory in natural light conditions with average photosynthetic photon flux density of 150 μmol s^{-1} m^{-2}. Simultaneously ten 8-day-old plants were measured. Chlorophyll content was analyzed repetitively every hour within 24 h in both secondary leaves. The experiment was repeated after four days using the same plants.

Effect of foliar fertilizers on leaf chlorophyll content was studied with *Triticum aestivum* L. cv. Jasna plants grown in containers with soil at photosynthetic photon flux density of 150 μmol s^{-1} m^{-2} at the plant level, 16-h-photoperiod. As a substrate, a humus podzolic glay soil was used. Plants were grown in 2-l-containers, with 10 plants per container, four containers per treatment. Plants were sprayed with micronutrient solution

DDMn (Kemira GrowHow) or phosphite-containing foliar macronutrient solution *Phosfik* (Kemira GrowHow) at the stage of fully grown secondary leaves (21 days after sowing). Chlorophyll content was measured repetitively in secondary leaves.

Chlorophyll measurement by a chlorophyll meter

For individual leaves or leaf parts, five to ten successive readings (depending on the area) in SPAD units were taken by a chlorophyll meter SPAD-502 (Konica Minolta, Osaka, Japan) across the whole surface of leaves. The mean of the measurement was calculated using the internal function of the chlrophyll meter.

Spectrophotometric chlorophyll analysis and calibration

Calibration was performed with leaves of an appropriate plant species with different content of chlorophyll. First, chlorophyll amount of the particular leaf was tested by a chlorophyll meter performing ten measurements throughout the leaf. Second, leaves with the known SPAD values were analyzed for total chlorophyll content by means of spectrophotometric analysis.

Ten leaf discs were prepared by a cork borer from a particular leaf. Pigments were

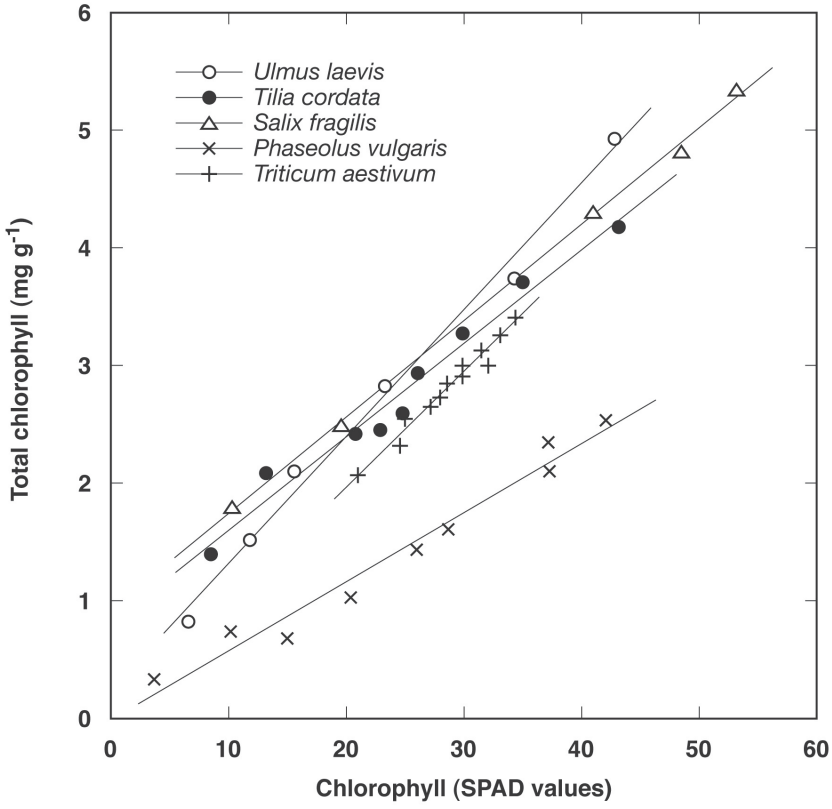

Fig. 1. Calibration of relative chlorophyll units (SPAD values) against spectrophotometrically measured total amount of chlorophyll of the same material. For every data point, SPAD was mesured five to ten times and chlorophyll content was analyzed in triplicate. Mean values are shown.

extracted from a fresh plant material with 96 % ethanol in the presence of $CaCO_3$. Spectrophotometrical readings were made in filtered solutions at 663 and 646 nm (Lichtenthaler, Wellburn, 1983). Data were expressed on a fresh mass basis.

Results

Calibration of SPAD measurements by spectrophometrical chlorophyll analysis

A near-linear relationship was found between spectrophotometrically determined total chlorophyll content on fresh mass basis and SPAD values measured by a chlorophyll meter for all of the tested plant species (Fig. 1). However, for the individual species, characteristic features of the relationship were found. Thus, a characteristic shift of the chlorophyll calibration line off zero was evident for tree leaves, especially, *Tilia platyphyllos* and *Salix fragilis*, indicating that at extremely low leaf chlorophyll levels (below 1.5 mg g^{-1}) the optical chlorophyll measurement method could give uncorrect results. In addition variations in the calibration line slope for different species were evident.

Application of nondestructive chlorophyll measurement in plant ecophysiology

Galls on leaves of *Ulmus laevis* induced by *Colopha compressa* started to develop on May 13. Already two weeks later (May 27) chlorophyll content in the infested leaf below the gall was significantly lower than that above it or in non-infested leaves (Fig. 2). The difference in chlorophyll content due to the gall activity remained significant throughout the vegetation season.

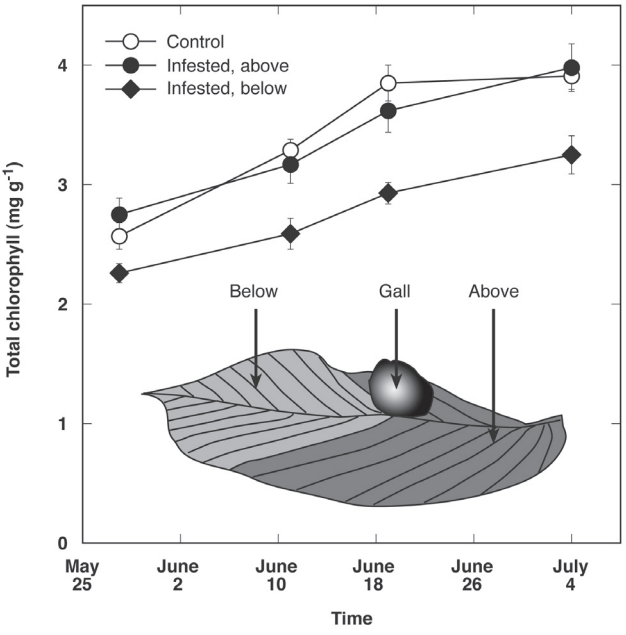

Fig. 2. Time course of chlorophyll content in leaves of *Ulmus laevis* infested with a gall-forming aphid *Colopha compressa*. Five leaves for every data point were measured, with seven SPAD measurements per leaf section. Mean values ±SE are shown.

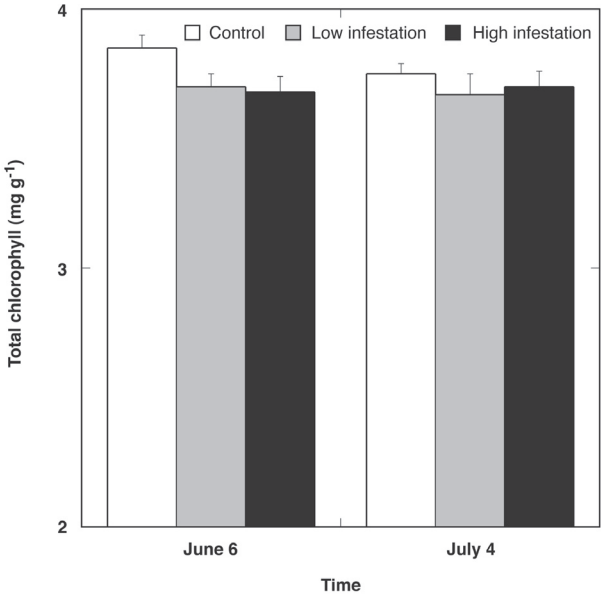

Fig. 3. Chlorophyll content in leaves of *Tilia platyphyllos* with a different level of infestation with a gall-forming mite *Eriophyes tiliae*. Low infestation, 5 to 10 galls per leaf; high infestation, 20 to 30 galls per leaf. Five leaves for every data point were measured, with ten SPAD measurements per leaf. Mean values ±SE are shown.

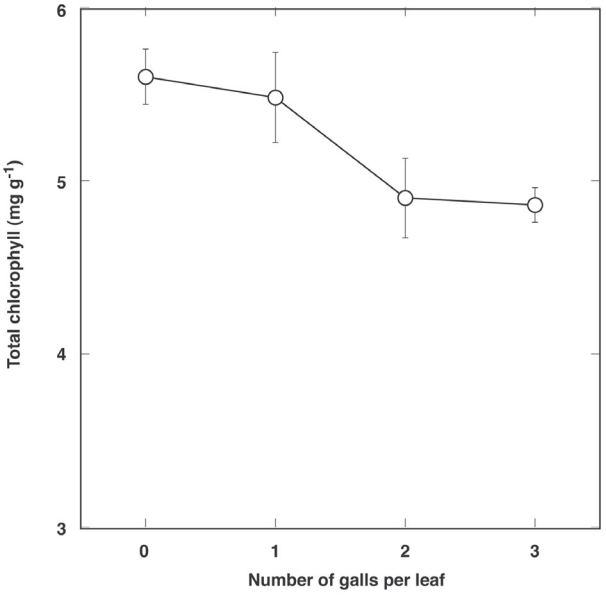

Fig. 4. Effect of number of galls per leaf on chlorophyll content in leaves of *Salix fragilis* infested by gall-wasp *Pontania vesicator*. For every data point, five appropriate leaves were measured, with five SPAD measurements per leaf. Mean values ±SE are shown.

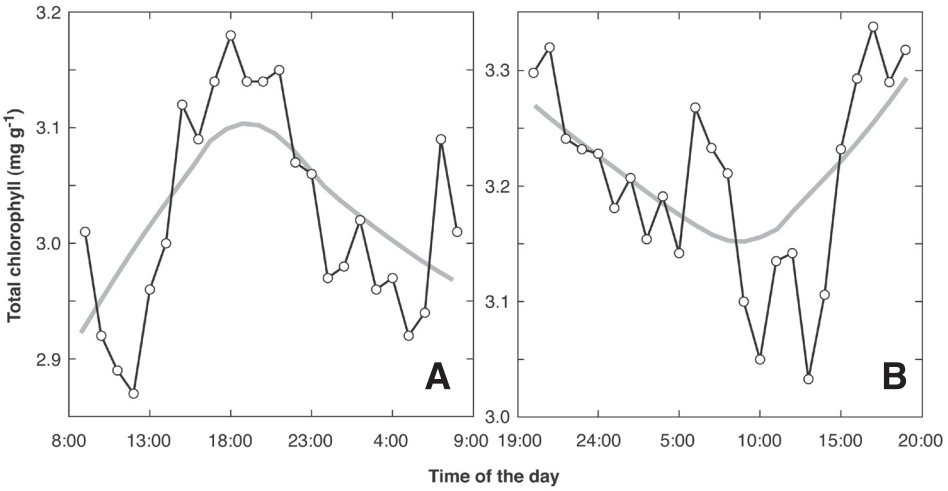

Fig. 5. Daily rhythm of chlorophyll content of *Phaseolus vulgaris* plants. A, 8-day-old plants; B, 12-day-old plants. For every data point, a pair of primary leaves from ten plants was measured, with five SPAD measurements per leaf. Mean values are shown. The gray line corresponds to the mathematically weighed curve (smoothing factor 65 %).

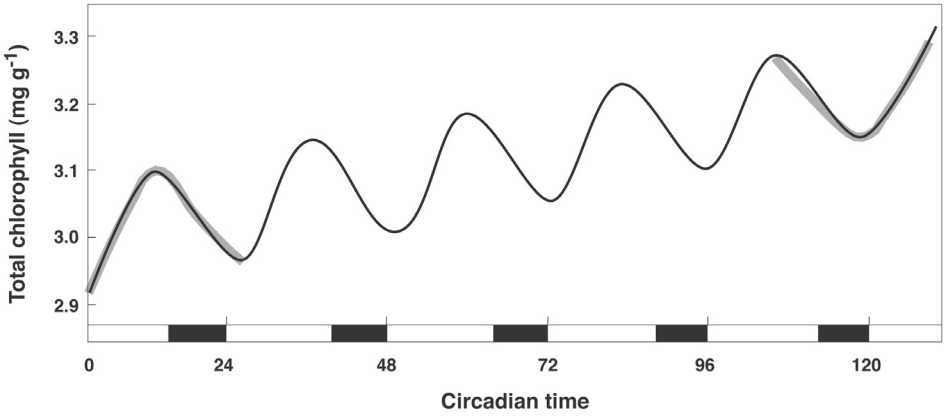

Fig. 6. Interpolated circadian rhythm of chlorophyll content in primary leaves of *Phaseolus vulgaris* plants. Gray line corresponds to the experimental data from Fig. 5. Solid bars at the bottom of the graph indicate the dark period.

In leaves of *Tilia platyphyllos* chlorophyll content was not significantly affected by infestation with a gall-forming mite *Eriophyes tiliae* (Fig. 3). However, there was a tendency to have a statistically significant ($P > 0.05$) lower content of total chlorophyll in the infested leaves early in the season.

Relatively high level of infestation (two to three galls per leaf) with a gall-wasp *Pontania vesicator* resulted in decreased content of chlorophyll in leaves of *Salix fragilis* when measured late in the growth season (Fig. 4).

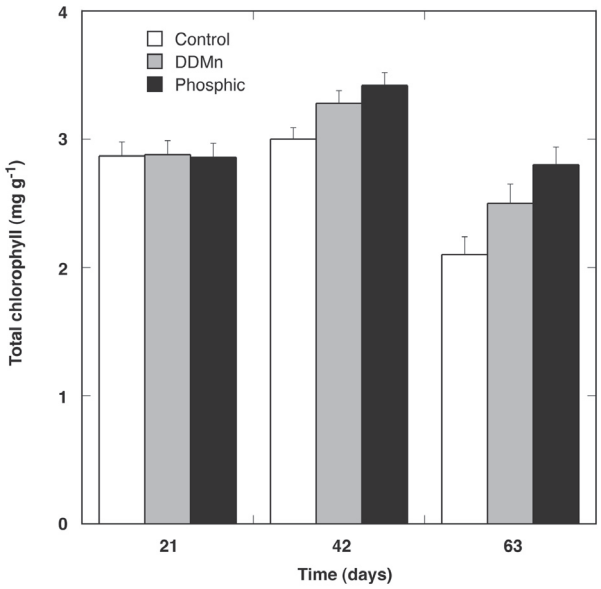

Fig. 7. Effect of foliar fertilizers *DDMn* and *Phosfik* on chlorophyll content in secondary leaves of *Triticum aestivum* plants at different times after sowing. Plants were sprayed with appropriate fertilizer 21 days after sowing. For every data value, five leaves from five plants were measured, with five SPAD measurements per leaf. Mean values ±SE are shown.

Application of nondestrucrive chlorophyll measurement in plant physiology

When a chlorophyll level was measured repetitively every hour within 24 h in primary leaves of 8-day-old bean plants, a pronounced rhythmicity in cholrophyll content was found (Fig. 5A). When the measurement was repeated four days later, a similar rhythm in chlorophyll content was evident, although with a higher absolute level (Fig. 5B). Interpolating both sets of data in time resulted in a pronounced circadian rhythm in chlorophyll content with a daily minimum corresponding to the beginning of a light period and a maximum towards the end (Fig. 6).

Application of nondestrucrive chlorophyll measurement in agricultural sciences

Effect of mineral supply through leaves on changes of chlorophyll content in wheat plants was studied in controlled conditions as an addition to the appropriate field tests. Both micronutrient (*DDMn*) and macronutrient (*Phosfik*) foliar fertilizers applied 21 days after sowing effectively increased total chlorophyll content in the second leaves of both 42- and 63-day-old wheat plants (Fig. 7).

Discussion

Chlorophyll content of leaves is a useful indicator of both potential photosynthetic productivity and general plant vigour (Alonso et al. 2002; Zarco-Tejada et al. 2002). In addition, changes in the amount of chlorophyll may be a part of adaptive responses (Morales et al. 2002). Consequently, the use of non-destructive methods of chlorophyll measurement

provides reliable and effective means of plant analysis in a wide range of biological context. In the present experiments, measurement of leaf chlorophyll content was used for different purposes in the context of various problems of plant biology. First, during studies of gall-forming aphid-host tree relationship localized changes in chlorophyll content in the infested leaf indicated a significant effect of the gall-former on photosynthesis (Fig. 2). Different effect of the level of gall infestation on photosynthesis-related characteristics was revealed in studies with *Salix fragilis* (Fig. 3) and *Tilia platyphyllos* (Fig. 4). Second, a pronounced rhythmicity of chlorophyll content in leaves of bean seedlings (Fig. 5) allowed to characterize an endogenous circadian rhythm in photosynthetic activity (Fig. 6). Third, changes in chlorophyll content in leaves of wheat plants were used as an early indicator to predict the effectivity of application of different foliar fertilizers (Fig. 7).

Changes in leaf chlorophyll content often has been regarded as a relatively late mechanism of photosynthetic adaptation (Anderson et al. 1995). Other mechanisms e.a. regulation of CO_2 supply by stomatal limitation and shifts in photochemistry of photosynthesis are thought to be the primary responses to a changing environment. In the present experiments, change of leaf chlorophyll content was evident in plants in different time scales under the effect of various factors including endogenous rhythm (Fig. 5), mineral nutrition (Fig. 7), and biotic interactions (Fig. 2 and 4). The data collectively indicate that changes in chlorophyll content are a part of an adaptive regulative system of photosynthesis to changes in internal and external environment, presumably acting together with the other means of photosynthesis regulation.

As the relationship between SPAD measurements and extractable chlorophyll concentration had species-specific characteristics, individual calibration for every particular species should be performed. In addition it appears that the SPAD measurements on a plant material with relatively low total chlorophyll content (less than 1.5 mg g^{-1}) should be interpreted with precaution do to a possible nonlinearity and a low accuracy of the measurement, especially for tree leaves. Similar observations have been described earlier for various plant species (Gratani 1992; Monje, Bugbee 1992).

Deviation from linearity in the high and low SPAD range was shown to be caused by a non-uniform distribution of chlorophyll across the leaf surface and multiple scatering, respectively (Uddling et al. 2007). The first can be easily overcome by appropriate multiple SPAD measurements across the leaf and by sampling of an adequate number of leaves. It was shown also that irradiance, leaf water status and time of the day when the measurements were performed may affect a correlation between SPAD readings and extractable leaf chlorophyll content (Martinez, Guiamet 2004). However, it is difficult to generalize the above findings as only two crop plant species (e.g., wheat and maize) were used. In addition a shift by about 2 to 4 SPAD units was usually found caused by the above-mentioned factors, which is within the ordinar biological variability of chlorophyll content within and between individual leaves for most plant species (Ievinsh et al., unpublished data).

Still, the optical method does not allow for a direct comparison of chlorophyll content between different plant species. Most importantly, species-specific leaf traits affecting a correlation between SPAD measurements and extractable chlorophyll content should be taken into account. Leaf surface characteristics (wax, trichomes etc.) as well as presence of microbial agents on leaves and inside them are among important factors in this respect. Thus, age-dependent development of wax layer on leaves of dune xerophyte *Eryngium*

maritimum leads to significant overestimation of chlorophyll content measured as SPAD units (Ievinsh et al., unpublished data).

Our results support the usage of nondestructive chlorophyll measurement by chorophyll meter in various branches of plant biology, including ecophysiology, plant physiology, and agriculture, when appropriate accurate calibration of SPAD readings against spectrophotometrically determined total chlorophyll content is made for every particular species. Together with the other nondestructive methods, e.a. chlorophyll a fluorescence measurements, chlorophyll analysis represents a valuable tool for studies in natural habitats or field experiments allowing continuous measurement of the same plant material. These methods have a special importance in biodiversity studies e.a. ecophysiology of wild plants in native habitats and investigation of genetic resources of agricultural plants.

References

Alonso M., Rozados M.J., Vega J.A., Pérez-Gorostiaga P., Cuiñas P., Fontúrbel M.T., Férnandes C. 2002. Biochemical responses of *Pinus pinaster* trees to fire-induced trunk girdling and crown scorch: secondary metabolites and pigments as needle chemical indicators. *J. Chem. Ecol.* 28: 687–700.

Anderson J.M., Chow W.S., Park Y.-I. 1995. The grand design of photosynthesis: Acclimation of the photosynthetic apparatus to environmental cues. *Photosynth. Res.* 46: 129–139.

Gitelson A.A., Merzlyak M.N. 1997. Remote estimation of chlorophyll content in higher plant leaves. *Int. J. Remote Sens.* 18: 2691–2697.

Gratani L. 1992. A non-destructive method to determine chlorophyll content of leaves. *Photosynthetica* 26: 469–473.

Köckenberger W. 2001. Nuclear magnetic resonance micro-imaging in the investigation of plant cell metabolism. *J. Exp. Bot.* 52: 641–652.

Lichtenthaler H.K., Wellburn A.R. 1983. Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. *Biochem. Soc. Trans.* 603: 591–592.

Martínez D.E., Guiamet J.J. 2004. Distortion of the SPAD 502 chlorophyll meter readings by changes in irradiance and leaf water status. *Agronomie* 24: 41–46.

Monje O.A., Bugbee B. 1992. Inherent limitations of nondestructive chlorophyll meters: a comparison of two types of meters. *HortScience* 27: 69–70.

Morales F., Abadia A., Abadia J., Montserrat G., Gil-Pelegrin E. 2002. Trichomes and photosynthetic pigment composition changes: responses of *Quercus ilex* subsp. *ballota* (Desf.) Samp. and *Quercus coccifera* L. to Mediterranean stress conditions. *Trees* 16: 504–510.

Neufeld H.S., Chappelka A.H., Somers G.L., Burkey K.O., Davison A.W., Finkelstein P.L. (2006) Visible foliar injury caused by ozone alters the relationship between SPAD meter readings and chlorophyll concentrations in cutleaf coneflower. *Photosynth. Res.* 87:.281–286.

Oxborough K. 2004. Imaging of chlorophyll a fluorescence: theoretical and practical aspects of an emerging technique for the monitoring of photosynthetic performance. *J. Exp. Bot.* 55: 1195–1205.

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.

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.

Zarco-Tejada P.J., Miller J.R., Mohammed GH, Noland TL, Sampson PH. 2002. Vegetation stress detection through chlorophyll *a*+*b* estimation and fluorescence effects on hyperspectral imagery. *J. Env. Qual.* 31: 1433–1441.

Nedestruktīvās metodes augu bioloģijā: precīza hlorofila satura noteikšana ar hlorofilmetru

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Kopsavilkums

Nedestruktīvās augu analīzes metodes augu bioloģijā kļūst arvien populārākas. Lapu hlorofila satura optiskā moteikšana ļauj iegūt ticamus rezultātus bez destruktīvas paraugu ievākšanas. Aprakstīto pētījumu mērķis bija apstiprināt uz absorbcijas pamata izveidota hlorofila mērītāja izmantošanas iespējas dažādās augu bioloģijas nozarēs. Visām analizētajām sugām varēja novērot sakarību starp spektrofotometriski noteiktu kopējā hlorofila daudzumu uz dzīvās masas vienību un ar hlorofila mērītāju noteiktām SPAD mērvienībām, kas bija tuvu lineārai. Pētījumos ar augu pangām lokālas hlorofila izmaiņas infestētajā lapā norādīja uz pangu veidotāja būtisku ietekmi uz saimniekauga *Ulmus laevis* fizioloģiju. Pretrunīga pangu infestācijas pakāpes ietekme uz fotosintēzes īpašībām atklāta pētījumos ar *Salix fragilis* un *Tilia platyphyllos*. Izteikts hlorofila daudzuma ritms pupiņu dīgstu lapās deva iespēju raksturot fotosintēzes aktivitātes endogēno cirkādo ritmu. Hlorofila satura izmaiņas kviešu lapās izmantoja kā agrīnu indikatoru, lai paredzētu dažādu foliāro mēslošanas līdzekļu lietošanas efektivitāti. Iegūtie rezultāti apstiprina iespēju dažādās augu bioloģijas apakšnozarēs (ekofizioloģijā, augu fizioloģijā, lauksaimniecības zinātnēs) izmantot nedestruktīvo hlorofila analīzes metodi, katrai konkrētajai sugai veicot atbilstošu SPAD mērvienību kalibrēšanu attiecībā pret spektrofotometriski noteiktu kopējo hlorofila daudzumu.