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Rapid, nondestructive measurement of chlorophyll content in leaves with nonuniform chlorophyll distribution

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Abstract. A practical microcomputerized video image analysis method is described for quantifying leaf chlorophyll content without extraction. Chlorophyll concentration is estimated from densimetric measurements of whole, intact leaves. Direct comparison with conventional extraction measurements on *Epipremnum aureum*, a variegated species, verified the image analysis technique's accuracy. The inherent advantages with regard to the nondestructive and convenient nature of the measurement, and suitability for leaves with irregular chlorophyll distribution, are discussed.

Introduction

Many plant physiological, ecological, and horticultural studies require comparative analysis of leaf chlorophyll density (Devlin and Barker 1971). Conventional chlorophyll measurement, which extracts the pigment from the tissue, is often slow and tedious to accomplish and precludes monitoring photosynthesis on individual leaves because the tissue is destroyed by the extraction (Bruinsma 1961, Inskeep and Bloom 1985, Singh and Anantrao 1937, White et al. 1960). A convenient method of estimating intact leaf chlorophyll content has been reported (Yadava 1986); however, this method is incapable of observing more than a very limited area (0.126 cm^2) per measurement. It is therefore inappropriate for plants with nonuniform leaf chlorophyll distribution (variegation, chlorotic patterns, necrotic patterns) which might result from genetic, physiological, or pathological effects.

This paper describes a unique adaptation of the newly-emerging technology of microcomputer video image analysis to the rapid, non-destructive measurement of whole leaf chlorophyll content. The validity of the method is demonstrated by direct comparison of image analysis measurements with those obtained by conventional extraction from the same leaves of Pothos

(*Epipremnum aureum*), a species characterized by variegation caused by irregular leaf chlorophyll distribution. This inexpensive system was assembled around a common microcomputer from readily available hardware and software components. In addition to the featured application, this system has demonstrated the versatility for numerous other measurement, experimental, and general laboratory applications (Smith and Spomer 1987, Smith et al. 1986).

Materials and methods

Plants used in this study were vegetatively propagated from rooted cuttings and grown in a 1–1–1 (by volume) soil–peat–perlite medium contained in 0.25 m dia standard plastic pots (hanging baskets) in full sun in a glasshouse with temperatures set at 30/20°C (day/night). Irrigation, liquid fertilization, and pesticide treatments were applied as required to maintain rapidly growing, healthy plants.

Fully expanded leaves exhibiting different degrees of variegation were selected for chlorophyll measurements. Each leaf was carefully (to avoid injury) held flat for imaging by placing it on a plane white, frosted plastic diffuser and covering it with a 120 cm^2 rectangle of 3 mm thick clear window glass (Fig. 1). The diffuser, mounted over a convoluted white neon lamp tube (Aristo Grid Lamp Products, Inc., Port Washington, NY; model V-56), provided a surface with a uniform photosynthetic photon flux density of

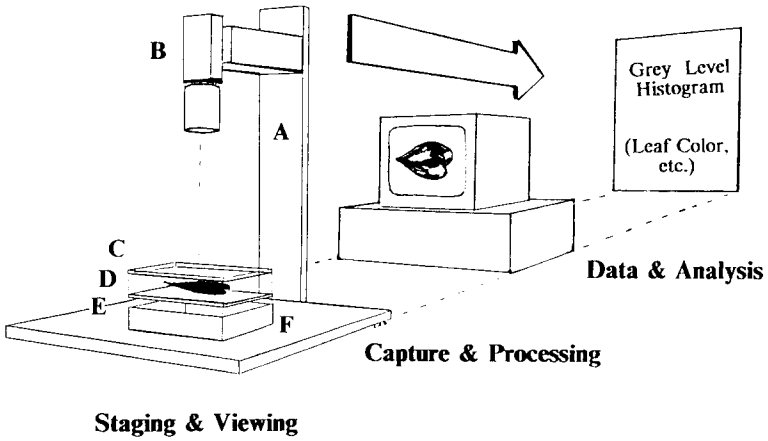

Fig. 1. Overview of leaf chlorophyll measurement system including details of leaf staging and viewing set up. a. copystand. b. video camera. c. clear glass cover. d. leaf sample. e. plastic diffuser. f. neon lamp housing.

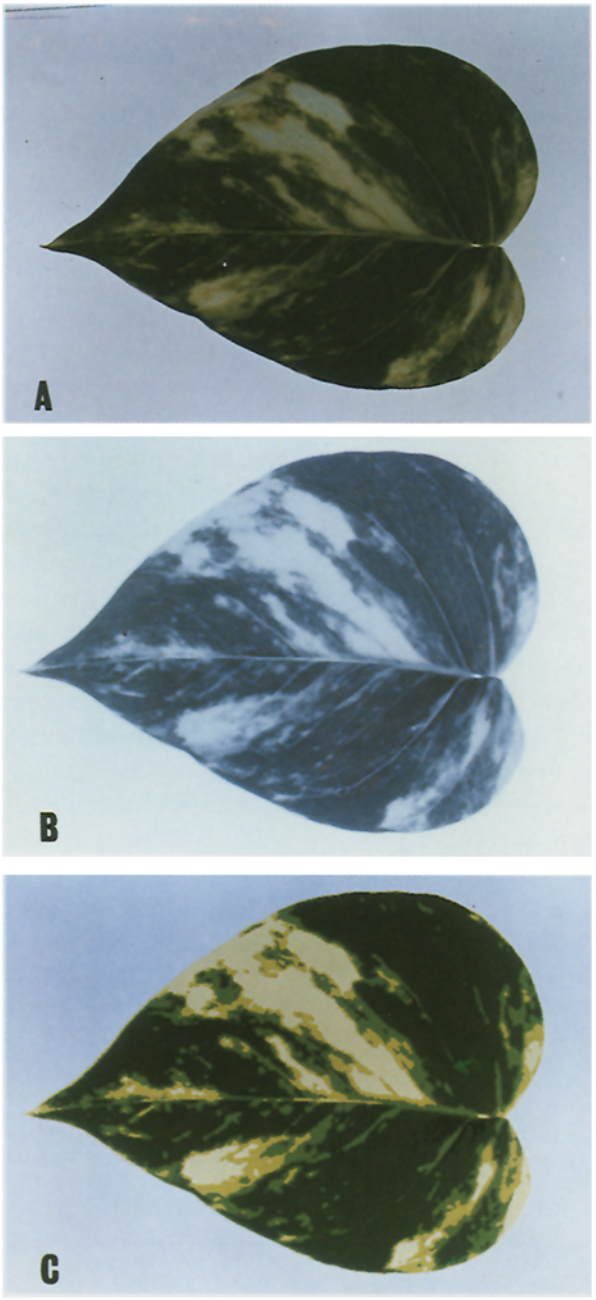

Fig. 2. a. Light transmission photopraph of a leaf of *Epipremnum aureum*, as viewed by the video camera. b. Digitized, black and white video image of the same leaf. c. The digitized leaf after posterization and assignment of lifelike pseudocolors.

56–57 micro-moles m^{-2} s^{-1} (measured with a Li-Cor Li-190SB Quantum sensor [Li-Cor, Inc., Lincoln, NE] and LI-1776 Solar Monitor). The leaf's video image was viewed (Fig. 2a) through a 0.28–0.85 m focal length Tokina lens from a distance of about 0.4 m with a Sony AVC-D1 CCD camera rigidly mounted on a copy stand (Fig. 1). A light transmission image of each leaf was captured from analog video output and digitized within 0.33 s with an Imaging Technology FG-100-AT digitizer (Woburn, MA) housed in an IBM PC/AT micro-computer and operated with Image Pro software (Media Cybernetics, Silver Spring, MD). The entire image capture process required only 15 to 30 seconds, so the leaf experienced minimal disturbance. The digitized image had an absolute morphometric resolution of about 4.07×10^{-4} cm^2 $pixel^{-1}$ (512×480 pixels for a total viewing area of about 100 cm^2) and photometric resolution of 256 brightness or grey level indices (Fig. 2b). The lens aperture was set between f/11 and f/16 where the best image detail was retained and a linear camera photometric response was ensured (a doubling of leaf optical density was reflected by a change of 32 grey level index steps). The greater the chlorophyll concentration within an area on a leaf, the darker that area in the image. The number of leaf grey levels (or optical density values) was automatically simplified from about 150 to six by a software 'posterization' routine and subsequently assigned a pseudocolor resembling its actual color (Fig. 2c). This facilitated indentification and sampling of leaf zones having distinctly different chlorophyll concentrations and also eliminated background distortions. The area of each zone was determined from the sum of pixels having that color (Table 1). Following image capture, processing, and analysis, ten 0.5 cm^2 disks were cut from each leaf color zone and immersed in 5 cm^3 of 80% aqueous acetone in an ice bath. The content of each vial was homogenized for 40 s at 20,000 rpm in a Virtis homogenizer, brought to a standard volume of

Table 1. Grey level index breakdown and chlorophyll concentrations recorded by image analysis for a single leaf of *Epipremnum aureum* (from Fig 2).

Index number	Pixels in range	Index area (cm^2)	Chlorophyll concentration (μMole cm^{-2})[a]	Chlorophyll content (μMole)
85	295	0.12	57.01	6.84
106	53598	21.81	40.96	893.34
128	10733	6.93	20.38	141.23
149	10084	4.10	12.13	49.73
170	13011	5.30	5.17	64.29
Total	94021	38.26	--	1155.43

[a] Chlorophyll concentration calculated from grey level index as shown in Fig. 2.

10 cm^3 by adding more acetone, and centrifuged for 10 min at 5000 g to sediment the solids from solution. Chlorophyll concentration was estimated to a precision of 5×10^{-8} grams cm^{-3} from absorbance at 645 and 663 nm with a (Beckman DU-7) spectrophotometer according to Arnon (Arnon 1949). Each treatment was replicated eight times. Leaf grey level index was correlated to measured chlorophyll concentration by linear regression analysis.

An additional confirmation of the technique involved direct comparison of image analysis with extraction measurement of whole leaf chlorophyll content. Individual leaves were imaged to determine the area represented by each color (chlorophyll concentration). Total chlorophyll content was calculated by summing the products of measured leaf color areas and their characteristic chlorophyll contentration. The values obtained by imaging and extraction were compared by linear regression analysis.

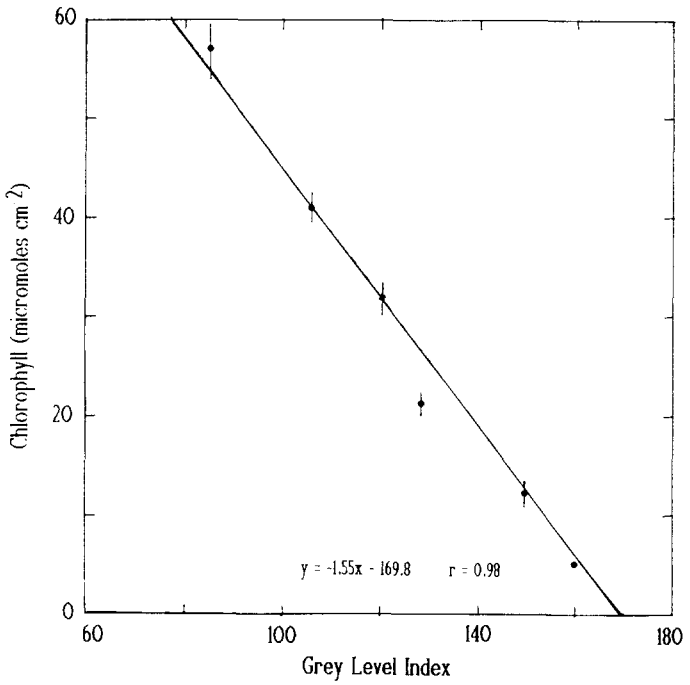

Fig. 3. Comparison of densimetric and extraction measurements of chlorophyll content from distinct image grey level indices (color zones) on leaves of *Epipremnum aureum*. The mean and standard deviation are indicated for each index value (line drawn by linear regression analysis).

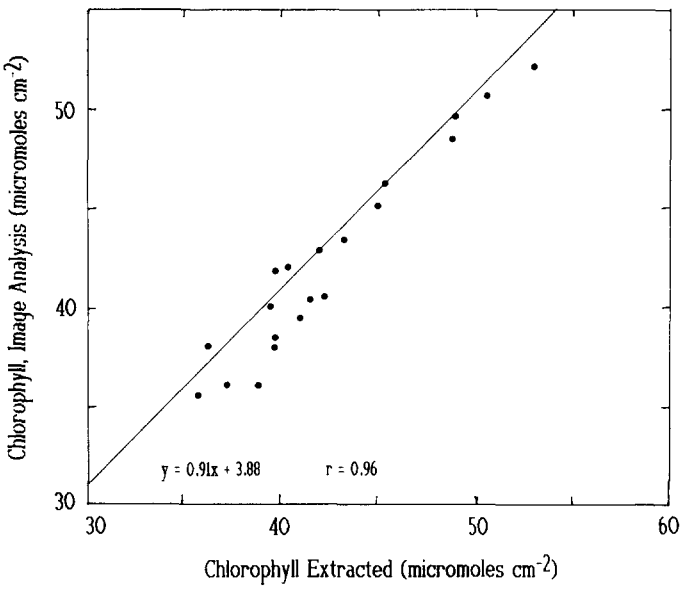

Fig. 4. Comparison of densimetric and extraction measurements of chlorophyll content from entire leaves of *Epipremnum aureum*. All data points are shown (line drawn by linear regression anlysis).

Results

The described image analysis technique proved accurate and practical for making nondisruptive chlorophyll measurements on intact leaves. Densimetric measurements were highly correlated with direct measurements of chlorophyll concentration within distinct color zones on a leaf (Fig. 3). Each grey level step in the digitized image indicated a 5.3×10^{-2} micromole cm^{-2} change in chlorophyll concentration. Image analysis measurements of chlorophyll concentration were also closely correlated with extraction measurements made on a whole leaf basis (Fig. 4).

Discussion

Image analysis for routine measurement of intact leaf chlorophyll concentration clearly offers multiple advantages over earlier techniques. Traditional chlorophyll extraction not only destroys tissue prior to analysis, but involves many steps in sample preparation, which increases the experimental time consumed and contributes to variability in the results (Bruinsma 1961, Yadava 1986). Previously described alternative, nonextractive measure-

ments (based on attenuance rank) have distinct advantages over conventional methods (Daley 1986), but require complex, non-linear mathematical and statistical analysis before estimates can be accomplished. Commercial portable chlorophyll meters have limited resolution, are not reliable for thick leaves and measure only very small sample sizes (Yadava 1986) which precludes their application to leaves with nonuniform chlorophyll distribution.

Image analysis, in contrast, permits objective, nondestructive, noninvasive and quantitative measurement of chlorophyll content in entire, undetached leaves, within a fraction of the time required for other methods. The method demonstrates sufficient sensitivity to detect very small differences in leaf chlorophyll concentration (as demonstrated by parallel extraction measurements), yet each measurement is quickly accomplished through simple, routine sampling once initial calibration is completed for a species. Initial calibration is required to account for differences between species in leaf anatomy and pigmentation (other than chlorophyll). Leaf thickness or surface characteristics (e.g., pubescence) have little or no effect on chlorophyll measurement as long as they are uniform across the leaf's surface. A wide range of leaf sizes can be analyzed at any particular setup; the maximum size is determined by lens focal length and distance between camera and speciman (Fig. 1). Species exhibiting nonuniform distribution of other non-chlorophyll pigments may require the inclusion of filtered lamps or lenses to distinguish the chlorophyll. Although the described measurements were made in the laboratory, an image can be captured *in situ* with an inexpensive portable video recorder for later laboratory analysis as long as the lighting and viewing are consistent. This requires a simple staging system consisting of a hood to exclude reflected light and a portable constant light source or a diffuse background screen and a procedure for adjusting exposure (Smith et al. 1986).

The image analysis system has the added advantage of application to many other laboratory and field measurements. For example, the described procedure can be directly applied to the quantification of leaf necrosis or chlorosis during the development of a disease or physiological disorder, leaf or other surface area, root growth, flower petal pigmentation in genetic studies, or in vitro cell growth (Smith and Spomer 1987).

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