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Light distribution in leaf chambers and its consequences for photosynthesis measurements

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Abstract

The impact of a heterogeneous distribution of actinic light within a leaf chamber for photosynthetic measurements by gas exchange on the photosynthesis-irradiance relationship was investigated. High-resolution light distributions were measured over the area of a commercially available clamp-on leaf chamber equipped with build-in red and blue LEDs, as well as over the area of a custom-made leaf chamber with external light source, using a low-cost digital camera and freely available software. The impact of the measured heterogeneity on the photosynthesis-irradiance response curve was calculated for two realistic scenarios. When the average light intensity over the leaf chamber area was estimated accurately, heterogeneity had minor effects on the photosynthesis-irradiance response curve. However, when the irradiance was measured in the chamber centre, which is common practice, and assumed to be homogeneous, for both leaf chambers the photosynthesis-irradiance response curve was subject to considerable error and led to serious underestimation of the light-limited quantum yield of photosynthesis. Additionally, mixed light sources with different heterogeneity patterns per light source, such as in the clamp-on leaf chamber, potentially increase errors due to heterogeneous physiological responses to light spectrum. High-resolution quantification of the leaf-chamber light distribution enables calculation of the correct average light intensity and already resolves the most pressing problems associated with heterogeneity. To exclude any light-distribution related errors in gas-exchange measurements a leaf chamber and actinic irradiance source design with a homogeneous light distribution is an absolute requirement.

Additional key words: gas exchange; heterogeneity; leaf chamber; light distribution; photosynthesis; photosynthetic quantum yield.

Introduction

Leaf chambers are widely used for photosynthetic measurements by gas exchange. For a correct presentation of the photosynthesis-irradiance relationship, the correct light intensity needs to be known and the distribution of the light projected on the leaf area in the chamber should be homogeneous. Heterogeneity of light distribution in a leaf chamber is undesirable for a number of reasons. In the first place, heterogeneity in light distribution easily leads to a wrong estimation of the average light intensity over the leaf chamber area. Many leaf chambers have an area of only a few square centimeters. It is common practice to calibrate the average light intensity over a leaf area in the chamber by measuring light intensity in the center of the leaf chamber using a well calibrated device (*e.g.* thermopile or PAR-sensor). In case of a heterogeneous light distribution over the leaf chamber area this will inevitably lead to an erroneous estimation of the actual average light intensity which the leaf area in the chamber is subjected to. An important problem that arises from such an error is a wrong estimation of maximal quantum yield for CO_2 fixation (α), which has been suggested to have frequently occurred in the past (Singsaas *et al.* 2001).

Second, a heterogeneous light distribution has consequences for the interpretation of photosynthetic measurements, even when the average amount of light over the leaf surface is known. When measuring at an irradiance which is strictly light-limited for all chloro-

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Abbreviations: P_{max} – light saturated gross assimilation rate; P_N – net assimilation rate; R_D – dark respiration; SD – standard deviation; α – quantum yield for CO_2 fixation; θ – scaling constant for curvature light-response curve.
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plasts in the leaf so that α is maximal, a correct photosynthesis-irradiance relationship can still be calculated from data measured using a chamber with a heterogeneous light distribution. This requires that heterogeneity has been accounted for so that the average light intensity is correct and nowhere exceeds the light-limited range. Beyond the light-limited irradiance range photosynthesis measurements will also be erroneous when the correct average light intensity over a heterogeneously illuminated leaf area is used, as α will also become heterogeneous. The significance of such errors for the interpretation of photosynthesis-irradiance response data has not yet been explored.

In systems using combined light sources, such as mixed red and blue LEDs, the situation is more complicated, as the light distribution for the different light sources can be different. This may result in spatial differences in spectral composition of incident light, which can contribute to heterogeneous photosynthesis rates over the measured leaf area, caused by spectral effects on *e.g.* stomatal opening (*e.g.* Zeiger 1990, Willmer and Fricker 1996) and photosynthetic quantum yield (*e.g.* McCree 1972, Inada 1976, Evans, 1987).

Several recent studies have contributed to the improvement in accuracy of photosynthetic measurement and its consequent calculations: Pons and Welschen (2002) studied effects of respiration rates under the seal of leaf chambers on net photosynthesis, Flexas *et al.* (2007) and Rodeghiero *et al.* (2007) analyzed the effect of diffusion leakage in clamp-on leaf cuvettes and Dubois *et al.* (2007) optimized the statistical estimation of the parameters of the Farquhar, von Caemmerer and Berry model (Farquhar *et al.* 1980). We present an example of a relatively easy, low-cost method for measuring the light distribution in a leaf chamber, and show the light distribution over the area of a custom-made leaf chamber and a widely used commercially available clamp-on chamber. The difference between the photosynthesis-irradiance relationship associated with the measured and an ideal, homogeneous light distribution is explored *via* a photosynthesis-irradiance response simulation. The consequences of a heterogeneous light distribution for the interpretation of measured photosynthesis data are discussed.

Materials and methods

Description of leaf chambers: The custom-made leaf chamber is of a conventional design: It is comprised of two separate round chamber parts (upper and lower) made from nickel-plated brass, mounted in a lab stand (No. 1, 2, and 8 in Fig. 1). The 5.2 cm^2 chamber area is covered with a quartz window for the upper chamber half. For the lower chamber half a perspex window is used, through which an infrared leaf temperature sensor is mounted. A leaf can be clamped between the two parts by lowering the upper chamber part onto the lower, so that the leaf is sealed gas-tight between two rings of white, flexible foam. Light was provided using a randomized optical fiber which was split into four fibers (*Heinz Walz GmbH*, Effeltrich, Germany; No. 5 in Fig. 1) allowing four different light sources to be used for leaf illumination simultaneously. The single fiber end rested in the upper chamber part at 4.5 cm from the leaf and had an effective diameter of 1.2 cm. The light sources used were two projector lamps equipped with 250 W halogen lamps. One lamp was used to obtain narrow-band light using a near-infrared cut-off filter and bandpass filters (10 nm width at half maximum, range 400–740 nm, every 20 nm; *Thorlabs*, Newton NJ, USA). The other lamp provided a broad-band spectrum, using a near-infrared cut-off filter in combination with a tungsten-to-day-light conversion filter (Full C.T. Blue; *Lee Filters*, Hampshire, UK). Light intensity was monitored using a computing multimeter (*Thurlby Thandar Instruments Ltd.*, Huntingdon, Cambs, UK), connected to a photodiode (*OSD15-5T, Centronic*) in a light-proof box mounted on the outside of the upper leaf chamber part (No. 7 in Fig. 1). The photodiode was in contact with a light-guiding pipe (3 mm in diameter, *Mentor GmbH & CO*, Erkrath, Germany) which was mounted in an opening drilled through the chamber wall, picking up light *via* a 45º cut, polished end above the upper chamber window. The light pipe did not interfere with the actinic light beam. The multimeter output was calibrated using a thermopile (*PS10Q, Molectron Detector Inc.*, Portland, USA), which was calibrated using a quantum sensor (*LI-COR*, Lincoln, Nebraska USA). The calibration was repeated with a spectroradiometer (*USB2000* spectrometer, *Ocean Optics*, Duiven, The Netherlands), which produced identical results. The sensors used for calibration were placed at the position where the leaf would be clamped in, in the centre of the chamber.

The commercial leaf chamber tested was a *LI-6400-40* Leaf Chamber Fluorometer (*LI-COR*, Lincoln, Nebraska, USA) equipped with independently controllable red (27) and blue (3) LEDs as actinic light source, with peak wavelengths of 640 and 464 nm, respectively. This chamber has a 1.59 cm diameter (2.0 cm^2 area), black foam as a seal for the lower chamber part and white foam for the upper chamber part.

Light-distribution imaging procedure: For determining the light distribution in the leaf chambers, the upper parts of the leaf chambers were placed upside-down, placing a piece of thin white filter paper (*Whatman 589/1*) in place of the leaf. This type of filter paper does not produce much scattering which can alter the light distribution pattern of the incident light. The filter paper was illuminated by the actinic-light source and imaged in a dark room using a digital camera (*P&S Canon 590IS*)

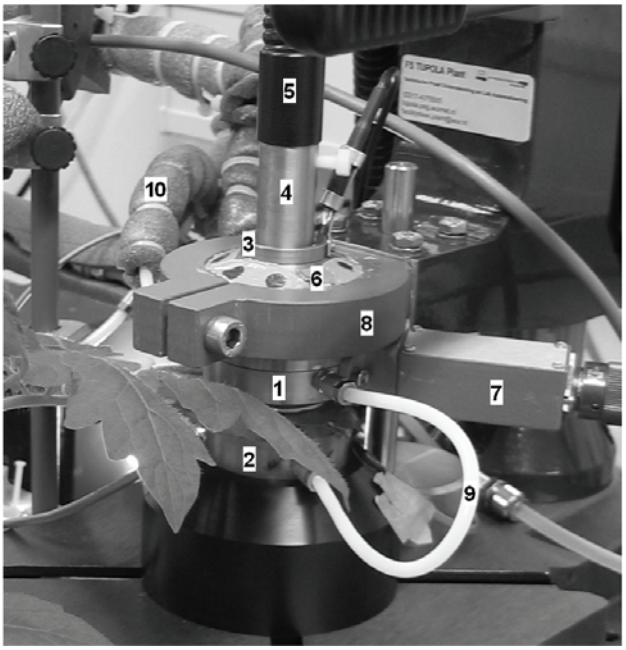

Fig. 1. Custom-made leaf chamber with enclosed tomato leaf. The numbers correspond to different parts of the leaf chamber and accessories: Upper leaf chamber part (1), lower leaf chamber part (2), brass ring equipped with 16 LEDs (640 nm peak wavelength, *Luxeon Rebel, Philips Lumileds Lighting Company*, San Jose, CA, USA) which can provide a 10,000 µmol m^{-2} s^{-1} light pulse to obtain a maximum fluorescence signal (3), brass holder allowing the fiber to be positioned at different distances from the leaf (4), randomized optical fiber providing actinic light (5), one of the nine apertures for an additional fiber (6), light-proof box with photodiode in contact with a light pipe picking up actinic irradiance in the chamber, connected to a multimeter (7), lab stand holding the upper leaf chamber in position (8), tube leading gas from the lower to the upper chamber part (9), and insulated tube through which water is pumped into channels in both chamber part walls to control the leaf chamber temperature (10).

expanded with software allowing RAW-format imaging (Appendix). Shutter time and aperture were set manually, so that no pixels were saturated or underexposed. The illuminated filter paper circle was imaged in the middle of the image (Fig. 2), to prevent a reduction in brightness or saturation at the periphery compared to the image centre (*i.e*. vignetting). Some images were made closer to the margin of the image, to be able to test whether vignetting effects were significant. No vignetting was observed in the image area used for analysis.

When using a common digital camera as we did, the option to use a RAW-image-format is required to obtain quantitative data on light-intensity. A usual jpg format does not represent light intensity linearly, as the camera software tends to dim bright spots and make dark spots brighter. Imaging methods using more specialized equipment can also be used (*e.g*. a technical camera), providing no information is lost due to image-processing by the camera software.

For the custom-made leaf chamber, images were made using blue (445 nm), green (560 nm), red (620 nm), and broad-band ("white") light, at different light intensities for each color (n = 4). For the *LI-6400* leaf chamber, images were made using the red and the blue LEDs at different light intensities for both colors ($n \geq 5$). For each different color, shutter time and aperture were optimized and kept unchanged for the different light intensities imaged for each color. An image was also made with a reference object with known area placed in the centre of both leaf chambers to allow the dimensions to be scaled to millimeters.

Quantification of the light distribution in a leaf chamber: The images of the illuminated filter paper placed in the leaf chamber were processed such that the intensity value of the pixels corresponded linearly with light intensity (Appendix). The reliability of the procedure was determined by comparing the different light intensities as measured by the measuring device (µmol m^{-2} s^{-1}) per color of actinic light imaged, with the mean pixel intensity of the corresponding images after processing. The relationships were always perfectly linear (Fig. 3), hence proving that the relative light intensities obtained from the image analysis procedure (Appendix) are representative for the real light intensity in the leaf chamber.

The mean pixel intensity was measured for the centre of both leaf chambers, which is usually used for light-intensity calibration, and the whole area, representative for the light intensity that a clamped leaf would receive.

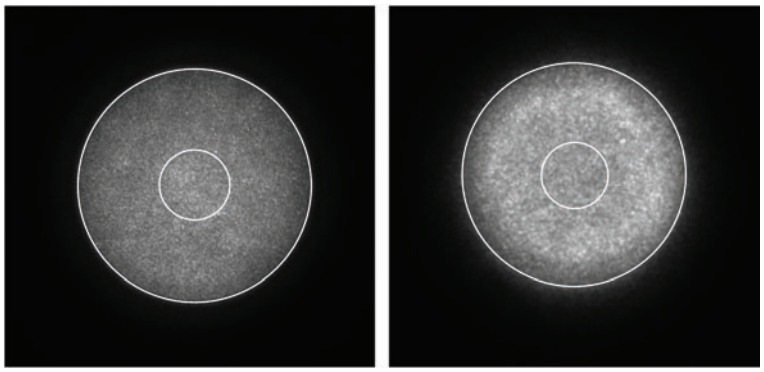

Fig. 2. Gray-scale images of custom-made (white light, *left*) and *LI-6400* leaf chamber (red LEDs, *right*). The outer white circles represent the whole-leaf chamber area, the inner white circles the "centre-area", representative for the area commonly used for light intensity calibration, as used in Table 1.

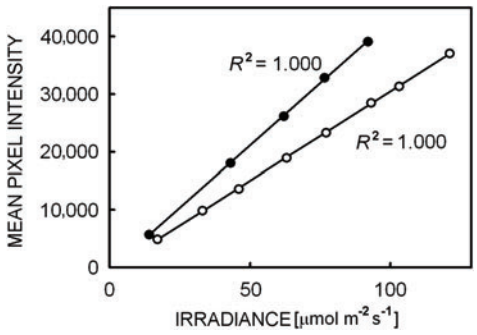

Fig. 3. Light intensity in the *LI-6400* leaf chamber as indicated by the *LI-6400* system *vs*. the mean pixel intensity of the centre circle (3.4 mm width) in the image, as analyzed in ImageJ (Appendix). *Closed circles*: red LEDs; *open circles*: blue LEDs. For the custom-made chamber a similar linearity was observed (not shown).

The standard deviation (SD) of the mean light intensity was also determined for the total leaf chamber area. Furthermore, the mean pixel intensity was measured for the area of nine circular bands around the centre, up to the margin of the chamber area. As the width of each band was equal, the area of the bands analyzed increased from the centre towards the margin of the chamber area. For each band the relative light intensity was calculated separately, which allowed the light distribution from the centre to the margin of the leaf chamber to be mapped in 10 intervals. This approach requires a centrally symmetrical distribution of the light intensity, as was the case for the two chambers tested. In the case that a distribution is not centrally symmetrical, a map of isophots would be more appropriate (*see e.g*. Laisk and Oja 1998, p. 24).

Quantification of the impact of a heterogeneous light distribution on the photosynthesis-irradiance response curve: To assess the impact of the measured light distribution in both leaf chambers on the validity of photosynthetic measurements the response curve was simulated for three situations. Photosynthesis-irradiance response curves were produced for (*1*) an ideal, homogeneous light distribution, for (*2*) the measured light distribution assuming the light intensity in the chamber centre to be representative for the entire chamber area and for (*3*) the measured light distribution using the correct average light intensity over the entire chamber area (*see* Appendix for procedure). Note that the maximum intensity of the blue LEDs (*LI-6400*) is <300 µmol m^{-2} s^{-1} and, therefore, insufficient to measure a complete photosynthesis-irradiance response curve on leaves of most plant species. The irradiance provided by the blue LEDs is often mixed with irradiance provided by red LEDs when measuring photosynthesis-irradiance response curves.

Results

Light distribution in the leaf chambers: For both leaf chambers, the mean light intensity in the centre of the leaf chamber (Fig. 2, *inner circles*) was different from the mean light intensity over the entire chamber area (Table 1). Over the entire area of the custom-made chamber, the light intensity was only 77% of that in the centre of the chamber. Differences for the different colors used were negligible. In the *LI-6400-40* leaf chamber the light intensity over the entire area compared to the centre deviated much more for the blue LEDs than for the red LEDs (respectively 80% and 94% of the centre intensity).

The SD from the mean light intensity over the entire chamber area was in a much closer range (22–27%) than the ratios of light intensity over the entire area compared to the centre (77–94%; Table 1). The granularity of the filter paper is visible in the image (Fig. 2), but does not obstruct the analysis of the light distribution pattern over the chamber area.

Table 1. Relative light intensity of total leaf chamber area compared to the chamber centre (as represented by the *inner circles* in Fig. 2) and standard deviation (SD) of mean light intensity for the total chamber area (mean intensity total area = 1).

	Custom-made chamber				*LI-6400* chamber	
	red	blue	green	"white"	red	blue
Relative light intensity	0.78	0.77	0.77	0.77	0.94	0.80
SD	0.23	0.25	0.24	0.24	0.22	0.27

The more detailed analysis of light distribution over the leaf chambers makes clear why the SD of the light intensity produced by the red LEDs was relatively large compared with the ratio of light intensity over the entire chamber area and that in the centre (Table 1). Whereas the light intensity over the entire area produced by the red LEDs (*LI-6400*) was only slightly smaller than in the centre, local differences over the area were considerable (Fig. 4). The light intensity was >30% lower in the outer margin of the chamber, compared to the centre, whereas slightly higher in the area between the centre and the margin (about 10%). The blue LEDs (*LI-6400*) produced the highest light intensity in the chambre centre, gradually decreasing away from the centre and dropping rapidly close to the chamber margin. A comparable pattern as for the blue LEDs (*LI-6400*) was observed for the light distribution in the custom-made leaf chamber (Fig. 4: white, red, blue, and green light had a similar distribution). The relative light distribution was similar for the different light intensities tested per colour/leaf chamber combination, as indicated by the very small standard errors in Fig. 4.

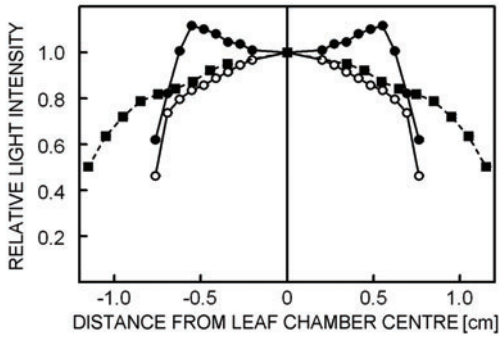

Fig. 4. Relative light distribution over the custom-made leaf chamber area (*squares, dotted line*) and the *LI-6400* leaf chamber area (red light: *closed circles*; blue light: *open circles*). For the custom-made chamber, the distribution of the white light is shown. The other three colors analyzed (blue, green, red) had similar distributions. Zero on the x-axis represents the centre of the chamber, each following point indicates the relative light intensity of bands with an equal width, so the outer bands have a greater area than those closer to the chamber centre. Error bars indicate SE of the mean relative light intensity over the images taken at different light intensities ($n \geq 4$) of actinic light per colour/leaf chamber combination (not visible in graphs as SE was always ≤ 0.007).

Consequenses of heterogeneous light distribution: The simulated photosynthesis-irradiance response curves show a considerable difference between an ideal homogeneous light distribution and the measured actual light distribution assuming the light intensity in the leaf chamber centre is representative for the entire chamber area, especially for the custom-made chamber and the blue light in the *LI-6400* chamber (Fig. 5). A reduction in the slope of the light-limited part of the photosynthesis-irradiance response curve (*i.e*. the maximum quantum yield for CO_2 fixation) of 23%, 6%, and 20% was found for, respectively, the custom-made leaf chamber, the *LI-6400* red LEDs and the blue LEDs (Fig. 5, *insets*). These simulated differences in α are proportional to the ratio of average light intensity in the leaf chamber centre versus that of the entire chamber area (Table 1). The curve resulting from the correct light intensity distribution over the entire chamber area is also different from the curve representing a homogeneous light distribution, however, the difference is small. In this case there is no significant effect of the heterogeneity in light distribution on the light-limited part of the curve (Fig. 5, *insets*). Clearly, a heterogeneous light distribution has a considerable effect on the validity of photosynthesis-irradiance measurements which can largely be resolved by measuring and correcting for heterogeneity.

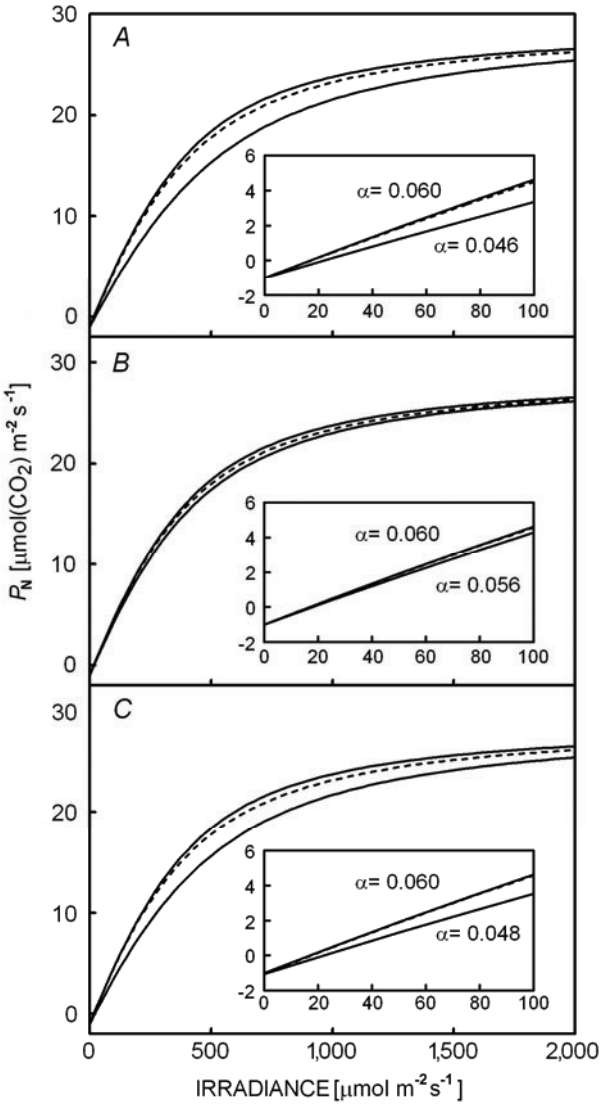

Fig. 5. Simulated photosynthesis-irradiance response curves for the custom leaf chamber (*A*) and the red (*B*) and blue (*C*) light in the *LI-6400* leaf chamber. The *upper solid lines* represent an ideal, homogeneous light distribution, the *lower solid lines* the real light distribution assuming the chamber centre light intensity to be representative for the entire chamber area and the *dashed lines* the real light distribution using the correct average light intensity over the entire chamber area. The same graphs are shown on a different scale in the insets, where α indicates the maximum quantum yield for CO_2 fixation. Note that the maximum intensity of the blue light in the *LI-6400* chamber (*C*) is <300 µmol m^{-2} s^{-1} and, therefore, insufficient to measure a complete photosynthesis-irradiance response curve on leaves of most plant species.

Discussion

Our results show that whenever accurate quantitative data on the relationship between irradiance and photosynthesis are required, the light distribution in a leaf chamber becomes important to know. For a custom-made leaf chamber, knowing the light distribution is crucial for the reliability of quantitative measurements. When a usual

light intensity calibration in the centre of the chamber deviates from the light intensity over the entire chamber area, the error of calculated quantum yields for CO_2 fixation (α) in the light-limited range will be proportional. In our example that would imply measurements of α of only 77% of those obtained using the correct light intensity the leaf received (Table 1). In commercial systems heterogeneity may have been corrected for by the manufacturer, so that the read-out indicates a correct average light intensity. This is the case for the *LI-6400* system tested (*LI-COR*, pers. comm.). A disadvantage of measuring α using a chamber with a heterogeneous light distribution which is corrected for so that the average intensity is correct is that the strictly light-limited irradiance range will be smaller. This implies that irradiance levels that chloroplasts in the leaf receiving the highest light intensity are subjected to may already start becoming non-light-limited, whereas the average light intensity would be well within the light-limited range for an individual chloroplast. Especially shade-plants can become non-light-limited at low irradiances, even below 20 μmol m^{-2} s^{-1} (Singsaas *et al.* 2001). In such leaves a heterogeneous light distribution further limits the already narrow range of average irradiances low enough to measure the light-limited part of the photosynthesis-irradiance response curve. Quantum yield measurements made with an Ulbricht sphere leaf chamber on 11 diverse C_3 species showed a high quantum yield compared to many earlier studies and low variance among species (Long *et al.* 1993). Singsaas *et al.* (2001) studied variation in the light-limited quantum yields for photosynthesis found in literature and concluded that in numerous studies the presented quantum yields were too low due to methodological errors. They concluded that photosynthetic measurements beyond the strictly light-limited range are a main source of error. We conclude that a heterogeneous light distribution in the leaf chamber used can also easily be a potential source of error in quantum yield studies. A heterogeneous light distribution indeed does greatly limit the strictly light-limited measuring range, so that already at relatively low irradiances an error as concluded by Singsaas *et al.* (2001) is made.

At irradiances beyond the light-limited range quantum yields remain heterogeneous when the correct average light intensity over a heterogeneously illuminated leaf chamber area is used. For example, when red light becomes saturating in the chamber centre of the *LI-6400* chamber, the light intensity in the outer margin of the chamber is only 62% of that required for saturation (Fig. 4). In fact, all chloroplasts, both over the leaf surface and in the leaf cross-section, need to be subjected to a saturating irradiance for a correct measurement of light-saturated photosynthesis. Simultaneous illumination of both leaf sides greatly reduces inhomogeneous illumination of chloroplasts through the leaf cross-section, as applied by *e.g.* Oya and Laisk (1976) and Terahshima (1986). Nevertheless, in contrast to heterogeneity in light distribution over the surface without making a correction for the average light intensity, the overall effect of a heterogeneous light distribution on the photosynthesis-irradiance response curve using the correct average light intensity value was small in the examples we presented (Fig. 5). An error of such magnitude may be acceptable for the majority of users and therefore a correction for heterogeneity using a method as we presented may be sufficient in most cases.

Physiological effects of differences in light distribution of different light sources used for mixed actinic light will further complicate the accuracy of photosynthesis-irradiance relationship measurements. Especially under conditions where the internal leaf CO_2 concentration is limiting for photosynthetic rate, blue-light-induced stomatal opening (*e.g.* Sharkey and Raschke 1981, Zeiger 1990) will directly affect photosynthesis. A heterogeneous photosynthetic rate associated with heterogeneous stomatal conductance has been shown making use of chlorophyll fluorescence images (Morison *et al.* 2005, Nejad *et al.* 2006). Blue light which is heterogeneously distributed over a leaf may therefore affect the photosynthesis-irradiance response curve not only by the distribution of irradiance intensity (as simulated in Fig. 5*C*), but also by other physiological responses of the leaf. The most commonly used blue/red ratio in the *LI-6400* chamber is 0.1. Based on the distribution of the two colors as presented in Fig. 4, this ratio will deviate up to 24% over the leaf area. Chen *et al.* (2008) showed that heterogeneity of photosynthetic parameters in leaves during gas-exchange measurements affects biochemical parameter estimates using the Farquhar model (Farquhar *et al.* 1980). An increased heterogeneity of photosynthetic parameters due to a heterogeneous light distribution will make biochemical parameter estimates less reliable. Note that parameters other than those from gas exchange measured in relatively large leaf chambers with a heterogeneous light distribution may also be susceptible to errors when measurements are made from a part of the clamped leaf area. Parameters often measured simultaneously with gas exchange involve photosystem I- and photosystem II electron transport, measured respectively by 820 nm absorbance changes at P_{700} and chlorophyll fluorescence (Baker *et al.* 2007).

The most pressing problems associated with a heterogeneous light distribution in a leaf chamber can be resolved by using the correct average light intensity over the leaf area (Fig. 5). The method we presented offers the advantage over more specialized equipment (*e.g.* a technical camera) that the investment in a simple digital camera suffices. However, heterogeneous light distributions in leaf chambers can affect the reliability of photosynthetic measurements in numerous ways, even when using the correct average light intensity. Calculations from crop- and canopy growth models

which make use of photosynthesis-irradiance relations will also be affected by such errors. Therefore the use of leaf chambers with a well distributed light intensity would be the simplest way to improve the accuracy of such data. Laisk and Oja (1998) described a leaf chamber design with a notably homogeneous distribution of irradiance (± 10%) and provided an exemplary mapping of the distribution. Recent technological developments resulting in smaller, more powerful LEDs offer opportunities to improve the configuration of light sources for leaf chambers. We consider that the distribution of light intensity in a leaf chamber deserves more attention in research using photosynthetic measurements.

References

Baker, N.R., Harbinson, J., Kramer, D.M.: Determining the limitations and regulation of photosynthetic energy transduction in leaves. – Plant Cell Environ. **30**: 1107-1125, 2007.

Chen, C.P., Zhu, X.G., Long, S.P.: The effect of leaf-level spatial variability in photosynthetic capacity on biochemical parameter estimates using the farquhar model: A theoretical analysis. – Plant Physiol. **148**: 1139-1147, 2008.

Dubois, J.J.B., Fiscus, E.L., Booker, F.L., Flowers, M.D., Reid, C.D.: Optimizing the statistical estimation of the parameters of the Farquhar-von Caemmerer-Berry model of photosynthesis. – New Phytol. **176**: 402-414, 2007.

Evans, J.R.: The dependence of quantum yield on wavelength and growth irradiance. – Aust. J. Plant Physiol. **14**: 69-79, 1987.

Farquhar, G.D., von Caemmerer, S., Berry, J.A.: A Biochemical model of photosynthetic CO2 assimilation in leaves of C_3 species. – Planta **149**: 78-90, 1980.

Flexas, J., Diaz-Espejo, A., Berry, J.A., Cifre, J., Galmes, J., Kaidenhoff, R., Medrano, H., Ribas-Carbo, M.: Analysis of leakage in IRGA's leaf chambers of open gas exchange systems: quantification and its effects in photosynthesis parameterization. – J. Exp. Bot. **58**: 1533-1543, 2007.

Inada, K.: Action spectra for photosynthesis in higher plants. – Plant Cell Physiol. **17**: 355-365, 1976.

Laisk, A., Oja, V.: Dynamics of leaf photosynthesis: Rapid response measurements and their interpretations. – CSIRO Publishing, Collingwood 1998.

Long, S.P., Postl, W.F., Bolharnordenkampf, H.R.: Quantum yields for uptake of carbon dioxide in C_3 vascular plants of contrasting habitats and taxonomic groupings. – Planta **189**: 226-234, 1993.

McCree, K.J.: Action spectrum, absorptance and quantum yield of photosynthesis in crop plants. – Agr. Meteorol. **9**: 191-216, 1972.

Morison, J.I.L., Gallouet, E., Lawson, T., Cornic, G., Herbin, R., Baker, N.R.: Lateral diffusion of CO_2 in leaves is not sufficient to support photosynthesis. – Plant Phys. **139**: 254-266, 2005.

Nejad, A.R., Harbinson, J., van Meeteren, U.: Dynamics of spatial heterogeneity of stomatal closure in Tradescantia virginiana altered by growth at high relative air humidity. – J. Exp. Bot. **57**: 3669-3678, 2006.

Oya, V., Laisk, A.: Adaptation of the photosynthesis apparatus to the light profile in leaf. – Soviet Plant Phys. **23**: 381-386, 1976.

Pons, T.L., Welschen, R.A.M.: Overestimation of respiration rates in commercially available clamp-on leaf chambers. Complications with measurement of net photosynthesis. – Plant Cell Environ. **25**: 1367-1372, 2002.

Rodeghiero, M., Niinemets, U., Cescatti, A.: Major diffusion leaks of clamp-on leaf cuvettes still unaccounted: how erroneous are the estimates of Farquhar *et al.* model parameters? – Plant Cell Environ. **30**: 1006-1022, 2007.

Sharkey, T.D., Raschke, K.: Effect of light quality on stomatal opening in leaves of *Xanthium strumarium* L. – Plant Phys. **68**: 1170-1174, 1981.

Singsaas, E.L., Ort, D.R., DeLucia, E.H.: Variation in measured values of photosynthetic quantum yield in ecophysiological studies. – Oecologia **128**: 15-23, 2001.

Terashima, I.: Dorsoventrality in photosynthetic light response curves of a leaf. – J. Exp. Bot. **37**: 399-405, 1986.

Thornley, J.H.M.: Mathematical models in plant physiology: a quantitative approach to problems in plant and crop physiology. – Acad. Press, London 1976.

Willmer, C., Fricker, M.: Stomata. – Chapmann and Hall, London 1996.

Zeiger, E.: Light perception in guard cells. – Plant Cell Environ. **13**: 739-744, 1990.

Appendix

Image analysis procedure: Installation of the free software "*Canon Hack Development Kit*" (*CHDK*) on the camera enabled us to use the RAW-format modus. When using a simple digital camera other lossless image formats than RAW, such as TIF, can not be used for a quantitative analysis of light intensity *via* imaging. As the original 10-bit RAW image is processed by the camera into a readable 8-bit format, information necessary for quantitative analysis will be lost. Note that a home-use digital camera as we used is equipped with a Bayer filter, which inherently leads to a loss of genuinely recorded data. The Bayer filter records 25% in blue, 50% in green and 25% in red. Therefore, when imaging *e.g.* red light, 75% of the pixels are acquired *via* interpolation. To be able to process the RAW-format (CRW-format) images produced, we made use of the open source software "dcraw", which converted the images in a PPM image format.

The PPM-format images (10-bit) were processed in ImageJ (http://rsbweb.nih.gov/ij/) including a plug-in allowing 16-bit images to be read. Images were split into three channels (RGB) and the brightest channel was used for analysis. Knowing the size of the reference object and chamber diameter, the exact area corresponding with the chamber could easily be determined. The pixel intensity over the image represented the light intensity over the imaged area. ImageJ also allows the SD of the mean pixel intensity over an image area to be calculated.

Procedure of photosynthesis-irradiance response curve simulation: Photosynthesis-irradiance response curves were simulated for (*1*) an ideal, homogeneous light distribution, (*2*) a heterogeneous distribution assuming the light intensity in the chamber centre is representative for the entire chamber and (*3*) a heterogeneous distribution using the correct average light intensity over the entire chamber area. The leaf chamber area on images processed in ImageJ as described above was exported as a numerical histogram consisting of 256-pixel intensity classes with corresponding occurrence counts. For the heterogeneous distribution simulations, the different classes of pixel intensities were set as relative pixel intensities compared to the intensity in the chamber centre (simulation 2; chamber centre intensity = 1) or as relative pixel intensities compared to the correct average intensity of the entire chamber area (simulation 3; average entire chamber area intensity = 1). The relative pixel (or light) intensity classes with corresponding occurrence counts were imported in *SAS* (release *9.1.3*; *SAS Institute*, Cary, NC, USA). Both relative pixel intensity classes (256) were multiplied by light-intensity steps needed to simulate a photosynthesis-irradiance response curve (range 0–2,000 μmol m^{-2} s^{-1} using 25 μmol m^{-2} s^{-1} intervals, *i.e.* 80 light steps). This produced two data sets, each consisting of 20,480 light intensities. For all these light intensities the net assimilation rate (P_N) was calculated *via* Eq. 1 (Thornley 1976; input parameters: $\alpha = 0.06$, $R_D = 1$, $\theta = 0.7$ and $P_{max} = 30$, where α is the light-limited slope, R_D dark respiration, θ scaling constant for curvature, and P_{max} the light-saturated gross assimilation rate).

$$P_N = -R_D + \frac{\alpha \cdot PPF + P_{max} - \sqrt{(\alpha \cdot PPF + P_{max})^2 - 4\theta \cdot PPF \cdot P_{max}}}{2\theta} \quad (1)$$

For each of the 80 light steps a weighed mean of P_N associated with the real light distribution was calculated and the nonrectangular hyperbola (Eq. 1) was fitted to these weighed means by nonlinear fitting (PROC NLIN) in *SAS*.