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Relation between Changes in Photosynthetic Rate and Changes in Canopy Level Chlorophyll Fluorescence Generated by Light Excitation of Different Led Colours in Various Background Light

Linnéa Ahlman [1], Daniel Bånkestad [2] and Torsten Wik [1,*]

[1] Department of Electrical Engineering, Chalmers University of Technology, SE-41293 Gothenburg, Sweden; linnea.ahlman@chalmers.se

[2] Heliospectra AB, SE-41458 Gothenburg, Sweden; daniel.bankestad@heliospectra.onmicrosoft.com

* Correspondence: torsten.wik@chalmers.se; Tel.: +46-31-772-51-46

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Abstract: Using light emitting diodes (LEDs) for greenhouse illumination enables the use of automatic control, since both light quality and quantity can be tuned. Potential candidate signals when using biological feedback for light optimisation are steady-state chlorophyll a fluorescence gains at 740 nm, defined as the difference in steady-state fluorescence at 740 nm divided by the difference in incident light quanta caused by (a small) excitation of different LED colours. In this study, experiments were conducted under various background light (quality and quantity) to evaluate if these fluorescence gains change relative to each other. The light regimes investigated were intensities in the range 160–1000 µmol m^{-2} s^{-1}, and a spectral distribution ranging from 50% to 100% red light. No significant changes in the mutual relation of the fluorescence gains for the investigated LED colours (400, 420, 450, 530, 630 and 660 nm), could be observed when the background light quality was changed. However, changes were noticed as function of light quantity. When passing the photosynthesis saturate intensity level, no further changes in the mutual fluorescence gains could be observed.

Keywords: optimal light spectrum; greenhouse illumination; light emitting diode; chlorophyll fluorescence; fluorescence gain; photosynthesis

1. Introduction

Today, greenhouses are to a large extent automated [1]. There is climate control, stepwise movement of the plants from sowing area to the harvesting area, and automated irrigation and fertilisation. Traditionally though, high pressure sodium (HPS) lamps that are only controlled by switching them on or off are used. However, an increasing amount of growers are interested in using light emitting diodes (LEDs) for greenhouse illumination instead. One benefit with such a transition is the enabling of tuning both the light quality (spectrum) and quantity (intensity). The quality and quantity of light affect plant growth in many ways; it affects growth, such as biomass, leaf elongation and chlorophyll concentration, as well as metabolic effects, such as antioxidant potential or vitamin and nitrate concentrations. In general, UV-light (280–400 nm) inhibits cell elongation and can cause sunburn, blue light (400–520 nm) enhances the photosynthesis, green light (520–610 nm) enhances photosynthesis but is to a larger extent (relative to blue and red) transmitted through, and reflected by, the leaves, red light (610–750 nm) enhances the photosynthesis and impacts the photoperiodism while far-red (750–1000 nm) stimulates cell elongation and influences flowering and germination (Table 8.1 in [2]). For a review of studies on light quality effects on plant growth we refer to Olle and Viršile [3] and Ouzounis et al. [4]. However, the question, "what specific spectrum and photon flux density is

optimal for maximal productivity?", is still not easy to answer, since it depends on plant species, the ontogenesis stage, surrounding environment and how "maximal productivity" is defined. As an example, Snowden et al. [5] measured the sensitivity to blue light for a number of species including cucumber and lettuce. Increasing the amount of blue light did not significantly affect plant growth for lettuce. For cucumber on the other hand, the increased amount of blue light reduced growth (by 22%), but only if the total light intensity was high (500 $\mu mol\, m^{-2}\, s^{-1}$) and not if it was low (200 $\mu mol\, m^{-2}\, s^{-1}$).

One possible way of defining optimal light spectrum is the spectrum that increase photosynthesis the most. Already in 1937, Hoover [6] determined the rate of photosynthesis as a function of wavelength of the light (measured on a wheat plant), called the action spectrum, and concluded that there is a peak in red light wavelengths and a secondary one in blue. In the 1970s two large studies were conducted on leaves from 22 and 33 plant species, respectively, with the conclusion that the action spectra were similar (but not identical) for all species tested [7,8]. However, the early action spectra were conducted on cut leaves illuminated with light with a wavelength span of 25 nm at a time. We propose firstly, that it cannot be ruled out that the results would differ if the measurements are made remotely on a canopy level. Secondly, it cannot be taken for granted that the results are identical in presence of background light. One example of the opposite is the "Emerson effect" [9]. He showed that the photosynthetic rate was higher when illuminating with red and far-red light (wavelengths lower and higher than 680 nm, respectively) in combination, compared to the sum of the photosynthetic rate when illuminate with either red or far-red light. This is due to the two photosystems in plants, which are activated by light of different wavelength.

If the plants have different spectral preferences depending on the current growth environment, one could in theory build a self-optimising controller, to find the optimal light spectrum for the current state, by using feedback control [10]. For such a scenario one needs a non-destructive biological growth signal that can be measured fast, remotely, and preferably without interacting with the plants. We suggest chlorophyll fluorescence to be such a candidate. The main tools used for measuring and analyzing chlorophyll fluorescence are the pulse amplitude modulation fluorometer (PAM) developed by Schreiber [11] and the photosynthetic efficiency plant analyzer (PEA) developed by Strasser et al. [12]. The PAM measures the fluorescence response to short duration (micro second) light pulses and discriminates chlorophyll fluorescence induced by the excitation pulse from chlorophyll fluorescence induced by ambient light through synchronous detection. The actual yields of photochemistry and heat dissipation, respectively, are sorted out from the fluorescence signal by the so-called fluorescence quenching analysis [11]. The PEA measures the fluorescence rise during the first second of saturating illumination, and analyzes photosynthesis through inflection points on the resulting fluorescence curve [12]. Both these methods require a precise measurement of the fluorescence from fully dark adapted plants, to compare with the fluorescence at fully saturating light. These requirements limit the use of the methods for remote sensing during day time and imply a restriction of their use to mainly on-leaf measurements.

There are methods developed for remote sensing applications. The best example is the passive solar-induced fluorescence (SIF) methodology, facilitating satellite measurements, based on fluorescence detection within the Fraunhofer lines, the dark lines in the solar spectrum caused by absorption by chemical elements in the Earth's atmosphere [13,14]. There are ongoing efforts in remote sensing research to quantify relationships between steady-state chlorophyll fluorescence (e.g., using SIF) and photosynthesis [15]. Although promising, the new SIF methodology, as well as other remote sensing methodologies, introduces many scientific questions that remain to be answered. The main questions are related to temporal, spatial and mechanistic up-scaling when going from traditional leaf-level measurements using the PAM-technique [15] to SIF. For example, how do we handle differences in canopy structure and geometry, and what type of information can be derived from the signal when the quantum yield of photochemistry cannot be directly resolved? We are trying to expand that knowledge base, by employing an active proximal remote sensing system, measuring

chlorophyll fluorescence on canopy level. The measure we propose, is a relative quantity of how much the fluorescence (in a wavelength span around 740 nm) increases for a certain intensity change. We define the steady-state chlorophyll a fluorescence gain (dF740/dq) as the difference in fluorescence at 740 nm divided by the difference in incident light quanta caused by (a small) excitation of one LED colour. This work is an expansion of earlier research [16,17], where we proposed the use of the fluorescence gain as an measure of the photosynthetic efficiency increase, to be used as a feedback signal in a (future) self-optimising controller to find the optimal light spectrum.

The relations between the amount of absorbed light, the amount of fluorescent light and the photosynthetic rate have been studied on both leaf level [18] and canopy level [19], and are dependent on plant health. For example, the fraction of fluorescence and photosynthesis is negatively correlated at low light intensity while it is positively correlated at high light intensity and stress [20–22]. However, the absolute quantities of both fluorescence and photosynthesis are expected to increase with an increased incident light intensity, and the absolute values (not to be confused with the fraction) will hence be positively correlated. We have seen that there is indeed a strong correlation between steady-state chlorophyll a fluorescence at 740 nm (F740) and photosynthetic rate (measurements on basil, in absence of background light, [16]), which indicates that the F740 could be a useful remotely measured signal to estimate the photosynthetic efficiency. The maximum photosynthetic rate for a predefined total power corresponds to the spectrum that maximies F740 [10]. It also means that the LED colour that has the highest fluorescence gain, is the one that increases photosynthesis the most (per incident quanta) at the current settings.

In previous experiments we compared the fluorescence gains caused by an excitation of six different LED colours (in the absence of background light), for six different plant species [17], including basil, cucumber, and lettuce. Differences in the mutual relation of the fluorescence gains (caused by the different LED colours) were noticed between different plant species. However, red LEDs caused the highest fluorescence gains per incident quanta for all species tested. Experiments on basil have also been conducted under various background light [16]. In contrast to the working hypothesis, that the mutual relations of the fluorescence gains would vary with various background light, the variation of individual fluorescence gains were minor for the background lights studied. Red LEDs remained the most efficient per quanta for all investigated background light. In this article we present experiments on cucumber and lettuce, conducted in order to answer the following research questions:

- Is there a strong correlation between photosynthetic rate (PN) and steady-state fluorescence (F740) also in the presence of background light?
- Do the mutual fluorescence gains, caused by excitation of different LED colours, change as the background light spectrum approaches only red light at different intensity levels?

2. Materials and Methods

Experiments were conducted on cucumber (*Cucumis sativus* F1 'Max') and lettuce (*Lactuva sativa* 'Black seeded Simpson'), with all settings summarized in Table 1. Two experimental sets are presented, set A and set B, as we wanted to repeat the experiments, initially only performed at background light intensity 160 µmol m^{-2} s^{-1} (set A), also for higher background light intensity levels (200, 350, 500 and 1000 µmol m^{-2} s^{-1}, set B). The slightly different settings in the two setups were a consequence of the available resources at the time.

Table 1. Summary of the experimental conditions.

	Set A	**Set B**				
Growth conditions	160 µmol m^{-2} s^{-1}	195 µmol m^{-2} s^{-1}				
Plant age when experiments start	7 weeks	5 weeks				
Intensity of background light	160 µmol m^{-2} s^{-1}	200, 350, 500, 1000[c] µmol m^{-2} s^{-1}				
Spectra (% red light)	50, 70, 85, 95, 100	50, 70, 85, 95, 100				
LEDs used for excitation (nm)	400, 420, 450, 530, 630, 660	400, 420, 450, 530, 630, 660				
Time at each level	3 min	long setting: 3 min short setting: 20 s				
Repetitions	2 *[i]	Intensity	200	350	500	1000
		3 min/lev	**c**[i]**l**	**c**[i]	**c**[i]**ccl**	**cc**
		20 s/lev *	**ccll**	**ccll**	**ccll**	**cc**
Infrared gas analyzer (IRGA) settings						
Flow rate	400 µmol/s	300 µmol/s				
CO_2 content	400 ppm	400 ppm				
Temperature	23 °C	21 °C				
Relative humidity	50%	50%				
Irga used (see *i*) during exp.	two machines at each run	one machine at a time, only for cucumber at three different intensity levels				
Light curves	one (increasing intensity)	three (decreasing intensity)				

c = cucumber, **l** = lettuce, [c] = intensity level only for cucumber. *i* = irga used in this setup, * = switching between increased and decreased wavelength order.

2.1. Conditions during Plant Growth

Five days after seeding, the plants were placed under LED lamps (LX60, Heliospectra, Sweden) in a plant lab with a photoperiod of 16 h and 23/17 °C day/night temperatures. The photosynthetic photon flux at plant level was approximately 160/195 µmol m^{-2} s^{-1} (set A/set B), with a spectral distribution of 20% blue, 10% green and 70% red light. Due to the different growth light intensities (which were a result of the available resources at the time), the experiments were conducted seven and five weeks after seeding for sets A and B, respectively, to have plants at approximately the same size.

2.2. Experimental Setup

The experiments were performed in a plant lab, in an experimental unit, delimited with reflective curtains (silver/white Diamond Diffusion Foil, Easy Grow, UK) giving a closed, controlled environment. In set A, two RX30 lamps (Heliospectra, Sweden) generated both background light and excitation light. Six different LED colours with peak wavelengths at 400, 420, 450, 530, 630 and 660 nm were used. These were all the available LEDs in the lamp that where assumed not to have any significant overlap with the spectrum of the fluorescence signal in the span 735–745 nm. In set B, the same type of lamp was used for the excitation light, but the background light was generated by two LX (Heliospectra, Sweden) lamps. Three LED groups in the LX60 lamps were used; blue, white, and red. The white LED produce light also in the fluorescence region. However, as the intensity of the white LED remained constant at each background light level and the experiments study variations in fluorescence, this overlap should have no effect. Figure 1 shows the spectrum for each LED colour in the LX lamp and RX lamp, respectively.

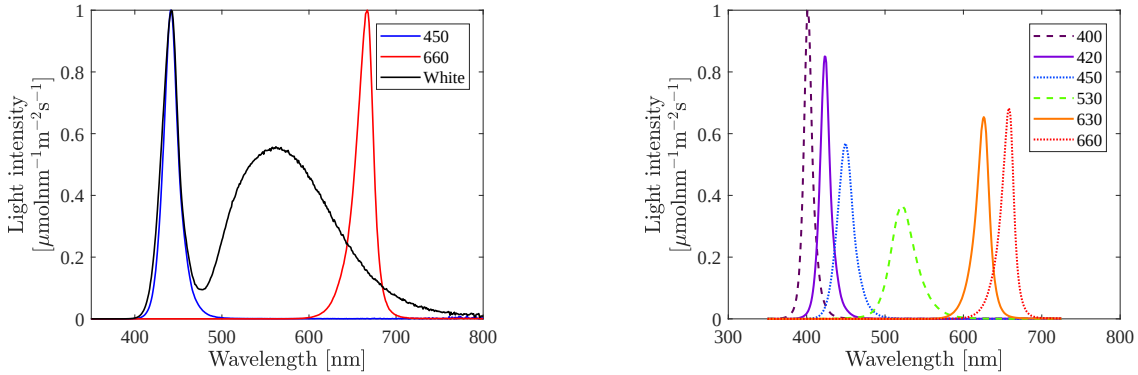


Figure 1. (**a**) Spectrum from the LX lamp for the blue, white and red LEDs, used to generate background light. (**b**) Spectrum from the RX lamp for each of the six LEDs used to generate excitation light.

2.3. Spectrometer Measurements

To detect the incident light and the reflected/fluorescent light two spectrometers were used, one facing the plants and one facing the lamps. In set A we used two Maya2000 Pro spectrometers (Ocean Optics, USA), each equipped with a 600 µm optical fibre, and in set B one Maya2000 Pro (equipped with a 600 µm optical fibre) and one STS-VIS (Ocean Optics, USA; no fiber needed). The incident light detector (Maya/STS, in set A/B) was placed at the canopy level with a cosine diffuser providing a field of view of 180°. The distance from the canopy level to the lamp was 40–50 cm (depending on plant height). The spectrometer detecting the reflected/fluorescent light (Maya in both sets) was placed on the side of the lamps with a field of view limited to 20° to only measure green area.

The input signal in this system is the light intensity reaching the canopy level. The wavelength span for photons that is normally considered important for photosynthesis is 400–700 nm, which is commonly referred to as photosynthetically active radiation (PAR). However, the cutoff wavelength is slightly different for different species [7]. Throughout this article, the photon flux in the range 350–700 nm has been included in the input light intensity, in order to capture all light, including the light from the LEDs having peak wavelength at 400 nm. Furthermore, blue light is defined as photons in the range 350–500 nm, green light in the range 500–600 nm and red light in the range 600–700 nm.

The primary output signal of the system is the fluorescence. The reflected and fluorescent light were measured with a spectrometer covering the whole wavelength span (see Figure 2a). Earlier work did not show satisfactory results when analysing reflected light, and therefore we have focused on analysing the data in the fluorescence band (having a first peak at 685 nm and a second at 740 nm). Furthermore, previous research [16] found a better correlation between photosynthetic rate and the fluorescence at 740 nm, compared to the peak at 685 nm. In addition, LED 660 significantly overlaps with the fluorescence spectrum at 685 nm though it was assumed not to overlap at 740 nm at the time of the experiments (later it was, however, found to have a small overlap affecting the results to some extent, see Section 3.2.3). However, this motivates our choice of only presenting the fluorescence at 740 nm.

Figure 2b shows the fluorescence spectra for lettuce at background light 500 µmol m^{-2} s^{-1}, and when an excitation step is added with LED 400 and with LED 660. The vertical lines points out the wavelength span 735–745 nm, which is were we have integrated the intensity to get the fluorescence signal, i.e.,

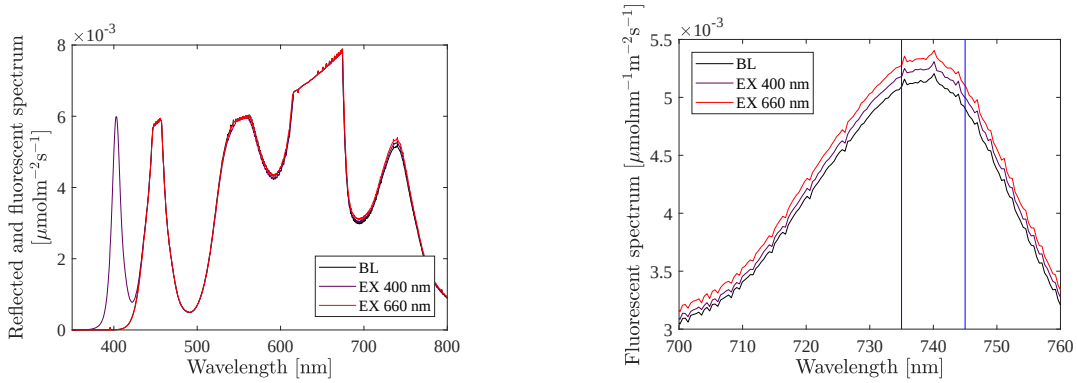
$$F740 = \int_{\lambda=735\,nm}^{745\,nm} \text{Photon flux}\, d\lambda. \quad (1)$$

The absolute value of the fluorescence was affected by the chlorophyll content in the canopy but also by the background light level (intensity and spectrum) and the distance between the spectrometer and the plants. Notice that in Figure 2a, the white LED (having one peak at 450 nm and a second one at 560 nm) have a tail in the red and far-red region and hence overlaps with the fluorescence

signal. To cope with this, we studied the relative increase in fluorescence, more precisely the increase in fluorescence, caused by one specific LED colour at a time, divided by the increase in incident light caused by that LED. This is referred to as the fluorescence gain, i.e.,

$$\text{Fluorescence gain } = dF740/dq \approx \frac{\Delta F740}{\Delta q} = \frac{F740_{EX} - F740_{BL}}{q_{EX} - q_{BL}}, \quad (2)$$

where F740 is the fluorescence signal according to Equation (1) at background light (BL) and when additional excitation light (EX) is added, and q is the quantum flux (µmol m^{-2} s^{-1}) of the incident light in the wavelength span 350–700 nm.



(**a**) Reflected/fluorescent spectra for whole wavelength span

(**b**) Details of the fluorescence region

Figure 2. Reflected/fluorescent spectra for lettuce at background light intensity 500 µmol m^{-2} s^{-1} and spectrum with 85% red light. Three spectra are shown; for background light and with an additional excitation signal with light emitting diode (LED) 400 and LED 660 respectively. Vertical lines in plot (**b**), indicate the region where the spectrum is integrated to get the F740 signal according to Equation (1). Notice that the spectrometer is light saturated in wavelengths 630–670 nm, however this is outside the wavelength of interest (i.e., the span 735–745 nm).

2.4. Reference Measurements

As a reference measurement an infrared gas analyzer (IRGA, Li-Cor 6400XT, USA) was used (in set A, two IRGA systems were used in parallel) to determine the photosynthetic rate as CO_2 uptake per leaf area and time. The calculations were based on measurements of the carbon dioxide and water content of a controlled air flow over a leaf sealed in a chamber, called the leaf cuvette. The cuvette had a transparent window, allowing measurements to be made under the desired light quality and quantity. The air flow, carbon dioxide content and the leaf temperature were automatically controlled, details are found in Table 1. The plants have been illuminated with the light settings similar to the first light level prior to the experiment in order for the photosynthesis to reach a steady value. However, no dark adaption was done, since we were primarily interested in changes and not absolute values. Four light curves were also performed, one with the same settings as in set A, and three with the same settings as in set B.

Differences in photosynthetic rate between species may be partly attributed to foliar pigment content and composition. Relative chlorophyll and flavonoid content were therefore estimated using Dualex Scientific® (Force-A, France), a leaf-clip optical sensor designed to non-destructively evaluate the content of chlorophyll and epidermal flavonoids [23,24]. We collected 32 point measurements for the analysis (four measurements on each of four leaves from two plants).

2.5. Data Processing

The spectrometers took a sample every second. A mean value of the 4 or 10 last measurements at each intensity step (that was held for 20 s or 3 min respectively) was used as the measure for that

setting. Figure 3 shows the raw data of the incident light and the fluorescence signal as a function of time, and the large dots indicates the mean value of the last points at each step. The fluorescence at background light (BL) level was measured between each excitation (in total 6 or 7 times/BL), and these data were used to identify possible trends in the fluorescence that were not an effect of the excitation light. The integration times (IT) were set in advance. The same IT was used for all excitations at one specific BL level, but were adjusted when changing BL, to get as strong a signal as possible of the relatively weak fluorescence. As a consequence, a large part of the reflection spectrum became saturated, which can be seen in Figure 2a (in the range 630–670 nm). However, these wavelengths are not included in this analysis.

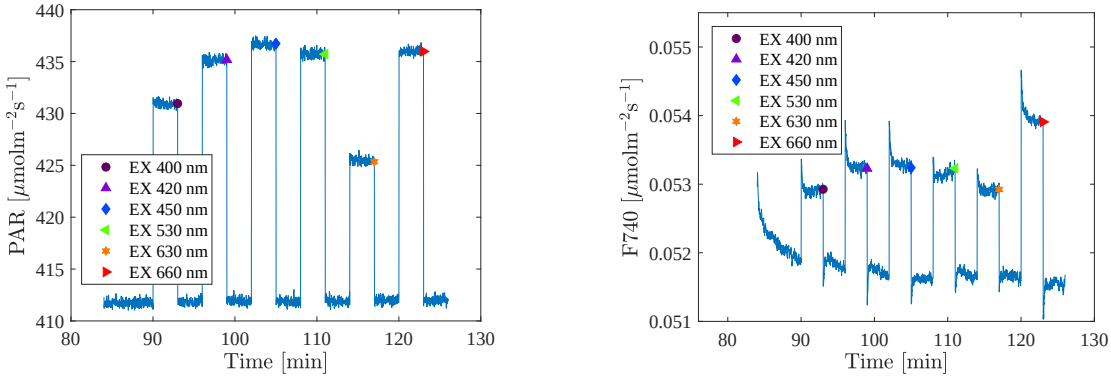
Under the assumption that the variance is the same for all LEDs used, even though they have different means, the pooled (or combined) variance can be estimated by [25]

$$s_p^2 = \frac{\sum_i \sum_{j=1}^{n_i} (y_j - \bar{y}_i)^2}{\sum_i (n_i - 1)}, \tag{3}$$

where $\bar{y}_i$ is the mean value of the fluorescence gain for each LED colour at each intensity level, and n_i is the total number of measurements of that state i ($n_i = 5$ different background spectra times 2–5 repetitions). Further, the standard error of the mean (s_m), can be estimated by

$$s_m = \frac{s_p}{\sqrt{n_i}}. \tag{4}$$

The IRGA measurements (on leaf level) had a much higher noise to signal ratio, compared to the fluorescence (canopy level) measurements. The measurement frequency is approximately 3 measures/min, and the mean value of the five last measurements at each light level were used to get an estimate of the photosynthetic rate (PN) at the current light level. The mean value of the photosynthetic rate at the BL level before and after an excitation step was used to determine the PN for the background light level. This was a way of compensating for trends in the data (i.e., for not being at a true steady-state). There has been no dark adaption, since only relative values, i.e., increase in PN due to excitation, were of interest.



(**a**) Incident light intensity in the wavelength span 350–700 nm

(**b**) Fluorescence light intensity in the wavelength span 735–745 nm

Figure 3. Measurements on lettuce, holding each intensity level at three minutes. (**a**) Incident and (**b**) fluorescence light intensity (µmol m^{-2} s^{-1}) vs. time for one background light level (background intensity 500 µmol m^{-2} s^{-1}and spectrum having 85% red light). An excitation step pulse is sequentially added by each LED colour, one at a time. Note that the intensity level measured during the experiment was only about 412 and not 500 µmol m^{-2} s^{-1}. This is because when the light level was determined, no plants were present in the experimental unit and another type of spectrometer was used.

2.6. Experimental Light Scheme

The background light intensities (within the PAR region) that were investigated were 160, 200, 350, 500 and 1000 µmol m^{-2} s^{-1} and for each intensity level five different spectra were investigated, having 50, 70, 85, 95 and 100% red light and a constant blue to green ratio, B:G, of 2:1. Figure 4 shows the spectral distribution for spectra having 50% and 85% red light respectively.

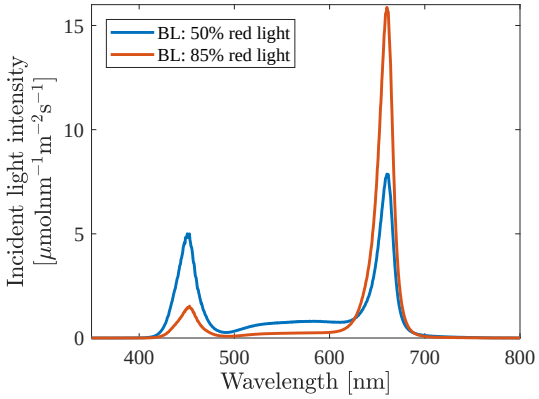


Figure 4. Spectra of background light. Intensity level 500 µmol m^{-2} s^{-1} and spectrum distribution 50% and 85% red light respectively.

At each background light (for all combinations of intensity and spectra) an excitation signal (a step of about 20 µmol m^{-2} s^{-1}) was added. This was sequentially done with all six different LEDs that were included in the investigation, i.e., LEDs having peak wavelength at 400, 420, 450, 530, 630 and 660 nm. Figure 3 shows the incident light (a) and fluorescence signal (b) for one (out of five) background spectrum at background intensity 500 µmol m^{-2} s^{-1}. Notice that the measured background intensity level shown in the figure is about 412 µmol m^{-2} s^{-1}. The reason is that the background light levels were determined using another spectrometer. In addition, the light was measured in an empty room, but when inserting the plants (for the experiments) the light environment is changed even though the lamp settings are constant. In Appendix A additional Figures A1 and A2 for other setups are presented. In one setup, the scheme in Figure 3a was repeated five times, once for each background spectrum, starting with 50% red light and finishing with only red light. In some setups the wavelength order was reversed, i.e., starting with background light spectrum with only red light and then decreasing the amount, and also starting the excitation with LEDs of longest wavelengths and continue in shorter wavelength order (that is reading Figure 3a from right to left). All details of the different settings are summarized in Table 1. In set A, the light was held constant at each light level for three minutes (six minutes at the first background light step when changing to a new background spectrum), while in set B an additional setup, staying at each light level only 20 s (60 s at the first BL), was also used. Another change from set A to set B was that an additional step of background light was added after the last excitation. This was done in order to more accurately capture any trend in the fluorescence that does not depend on the excitation step.

3. Results and Discussion

This section is divided into four subsections. In the first one, Section 3.1, the normalized absolute values of fluorescence (F740) and photosynthetic rate (PN) from set A are presented, which demonstrates a clear positive correlation between F740 measured at canopy level and PN measured at leaf level in the presence of various background light. In Section 3.2 the results of the F740 gains are presented, as a function of background light spectra and as a function of background light intensity. The mutual relation of the F740 gains do not change significantly with background light spectra. However, changes were noticed when light intensity increased up to a certain point. In Section 3.2.3 we discuss the fact that LED 660 is likely overestimated in our analysis. In Section 3.3

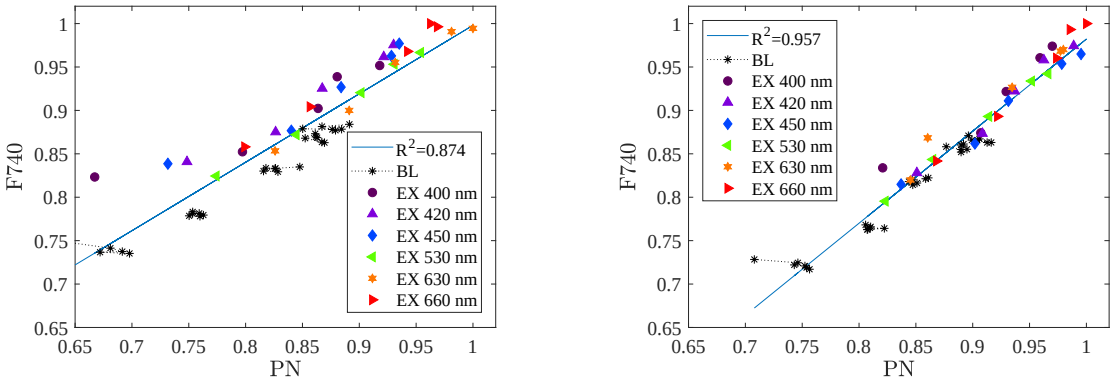
the results from the infrared gas analyzer are presented. Here we found that the light saturation level in the light curves, coincides with the light intensity where the mutual relation of the F740 gains do not change any more. Furthermore, the PN gains are presented and shown to correlate with the F740 gains. Finally in Section 3.4, we discuss our results of the F740 gains and PN gains, compared to data from McCree and our dualex measurements.

3.1. Absolute Values of Fluorescence vs. Photosynthetic Rate

The normalized absolute value of fluorescence as a function of the normalized absolute value of the photosynthetic rate (i.e., all values is divided by the maximum measured value to a normalized value being one or less), is shown in Figure 5 for (a) cucumber and (b) lettuce. The background light intensity is 160 µmol m^{-2} s^{-1}, and background spectra ranging from 50% to 100% red light (i.e., set A). The black stars in the plot correspond to these background light levels, clustered in five groups. It corresponds to the five different spectral distribution of the background light, with higher amounts of red light resulting in both higher fluorescence and higher photosynthetic rate. The coloured dots correspond to the measurements when an excitation step (of about 20 µmol m^{-2} s^{-1}) is added by one of the six available LED colours.

The measurements clearly show a strong positive correlation between fluorescence (F740) measured at canopy level and photosynthetic rate (PN) measured at leaf level in the presence of various background light. For cucumber (Figure 5a), one reasonable approximation is to view the relation as two different linear regressions, one for the lower background intensity level (black stars, PAR 160 µmol m^{-2} s^{-1}) and one for the slightly higher (coloured dots, PAR 180 µmol m^{-2} s^{-1}). This indicates that the relation between F740 and PN is close to, but not exactly linear, changing slightly with the intensity. However, for the purpose of optimizing the spectrum for short term photosynthesis only a positive correlation between F740 and PN is required (it does not have to be linear). It should be noted that the time at each light level (three minutes) was enough to get a useful signal of the fluorescence, but hardly enough for the photosynthetic rate signal due to the relatively high noise magnitude. Furthermore, the variation in the strength of the correlation (R^2 = 0.87 and 0.96 for cucumber and lettuce respectively) could be attributed to mainly the variation in the photosynthetic rate signal. At this stage no conclusions about differences due to crop species can be drawn.

In set B, the background light consists of light from white LEDs (among others), which produce light also in the region where the fluorescence signal is measured. The white LEDs are held constant at each background light level, but the higher the intensity levels and the less red light in the spectrum, the more light is emitted from the white LEDs. That is why no data of the absolute value of the fluorescence in set B can be presented here.



(**a**) Cucumber (**b**) Lettuce

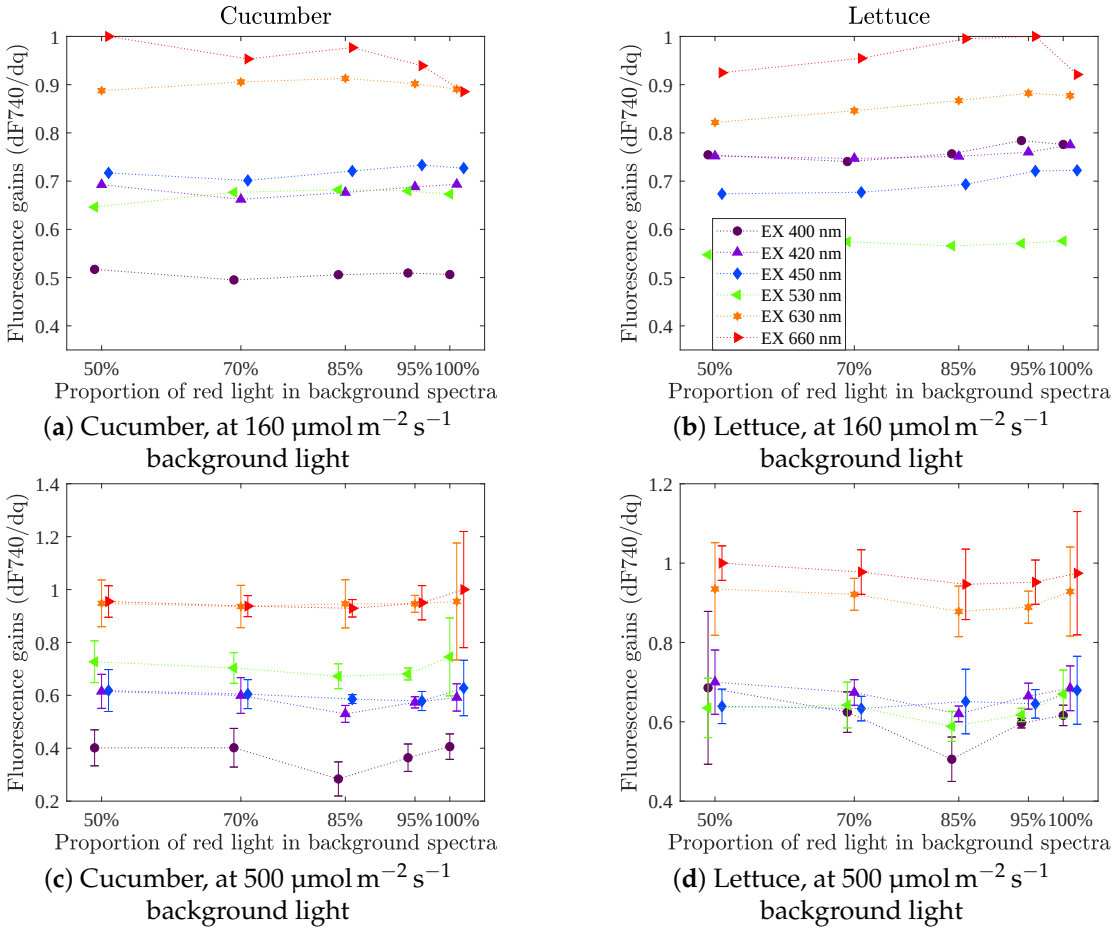
Figure 5. Normalized fluorescence at 740 nm versus normalized photosynthetic rate. The black stars correspond to measurements at background light, i.e., intensity 160 µmol m^{-2} s^{-1} and the five clusters corresponds to five different spectral distribution from 50% up to 100% red light. The coloured dots correspond to measurements with additional excitation light with one LED at a time.

3.2. Fluorescence Gains, dF740/dq

The fluorescence gains (defined by Equation (2)), i.e., the change in fluorescence (integrated over 735–745 nm) divided by the change in incident light caused by an (small) excitation of one specific LED colour, were studied for excitations of every LED colour at different light qualities (50–100% red light) and quantities (160–1000 µmol m^{-2} s^{-1}).

3.2.1. dF740/dq vs. Incident Light Quality

Figure 6 shows the fluorescence gains vs. the proportion of red light in the background spectra for intensities 160 µmol m^{-2} s^{-1} (first row, from set A) and 500 µmol m^{-2} s^{-1} (second row, from set B). The figures are representative also for the other intensity levels tested. In set A the experiments were only repeated once (giving two measurements per level). The results presented are the mean values of these two measurements. In set B, at 500 µmol m^{-2} s^{-1}, the fluorescence gains were measured three times for lettuce and five times for cucumber. The error bars (in the second row) indicate the spread (one standard deviation) of the measurements at each point. As the background light approaches only red light, a small decrease in fluorescence gain for LED 660 was noticed for both cucumber and lettuce, at background intensity 160 µmol m^{-2} s^{-1}. However, this was not observed for higher light intensities. Considering the one standard deviation error bars, no significant change in fluorescence gain as a function of background spectrum can be concluded.



(**a**) Cucumber, at 160 µmol m^{-2} s^{-1} background light

(**b**) Lettuce, at 160 µmol m^{-2} s^{-1} background light

(**c**) Cucumber, at 500 µmol m^{-2} s^{-1} background light

(**d**) Lettuce, at 500 µmol m^{-2} s^{-1} background light

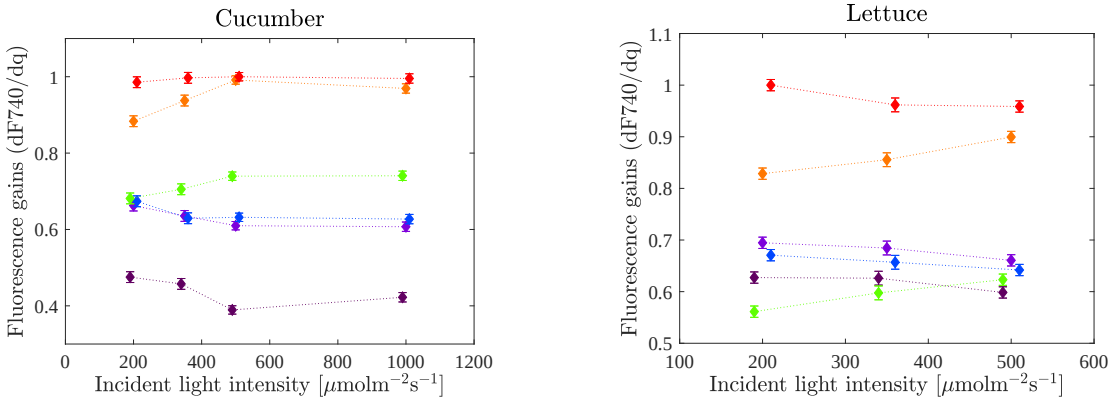
Figure 6. Fluorescence gains for each of the six LED colours when background light quality varies from 50 to 100% red light. The upper row is data from set A, and the lower row is for parts of set B. Left column is measurements on cucumber and right column is on lettuce. The bars in the lower plots represent ±one standard deviation calculated from the measurements at the current light setting.

The fluorescence gain is always highest for an excitation in red light (highest for LED 660 followed by LED 630). For the others it differs slightly depending on species and intensity level. LED 400 gives the lowest gain for cucumber, whereas all three blue LEDs (400, 420 and 450 nm) are about equal for lettuce. LED 530 gives a relatively higher gain for cucumber than for lettuce.

Our experiments do not indicate that the steady-state fluorescence gains (or the short term photosynthetic rate gain, since they correlate) would change significantly with background light quality. That means, for a given incident intensity there is no need for an online controller to work continuously to find the optimal spectrum. On the other hand, due to the strong correlation between photosynthetic rate and fluorescence, and the fact that the relative fluorescence gains are consistent at each incident intensity level, the fluorescence gains could be an interesting signal to monitor, for example daily through the growth cycle, in order to detect if something unpredicted happens. If the input signal is the current to the lamp, instead of the measured incident light quanta, it would be possible to detect if any of the LED colours have degraded more than the others, and in such case reconsider the power distribution over the different LEDs.

3.2.2. dF740/dq vs. Incident Light Quantity

The difference in relative fluorescence gain as a function of incident light intensity was studied. In Figure 7 the mean values of the fluorescence gains of each LED colour at each intensity level are plotted with one standard error of the mean (according to Equation (4)). A couple of statistically significant changes can be noted as the intensity increases. The difference in gain between LED 660 and LED 630 decreases. Also, the gain of LED 530 increases relative to the gains of the blue LEDs. The starting point though, differs for the two species. For cucumber, the gain of LED 530 is about equal to the gain of LED 420 and LED 450 at 200 µmol m^{-2} s^{-1}, while LED 530 has a higher gain than the blue LEDs at higher intensities. For lettuce on the other hand, the gain of the green LED is lower than the blues' at 200 µmol m^{-2} s^{-1}, while they are in the same region for higher intensities.



(**a**) Cucumber (**b**) Lettuce

Figure 7. Mean values (and standard errors of the mean) of the fluorescence gains at each intensity level, independent of the background spectra, for (**a**) cucumber and (**b**) lettuce.

For cucumber an additionally high intensity level was included in the experiments, at 1000 µmol m^{-2} s^{-1}. As the intensity increases from 500 to 1000 µmol m^{-2} s^{-1}, no change in relative fluorescence gain could be observed. One possible reason for this is that the light curve has reached saturation (as can be seen in Figure 8 in the next section), which means that even though more light is added, the photosynthesis rate cannot increase further.

3.2.3. Overestimated Fluorescence Gain from LED 660

The white LED has a significant illumination power in the fluorescence region (i.e., 735–745 nm), which can clearly be seen by observing its spectrum (see Figure 1a). For all other LEDs, a potential

overlap is very hard to detect since it is very small or even zero. At the time when the experiments were conducted it was assumed that none of the LEDs in the excitation lamp had any significant overlap with the fluorescence at 740 nm, based on observations of their spectra (see Figure 1b). However, even a very small overlap can have a relatively large impact since, (i) a large proportion of the light at 740 nm will be reflected and (ii) the fluorescence signal is weak compared to the incident light.

When integrating the incident light in the range 735–745 nm, and time-averaging the noisy signal, a slight increase can be observed when adding an excitation with LED 660, compared to when only illuminating with the background light or adding an excitation with any other LED colour. This indicates that the signal $\Delta F740 = F740_{EX} - F740_{BL}$ (see Equation (2)) that is measured with the spectrometer facing the plants, contains both fluorescence and reflectance when adding an excitation with LED 660, but not when adding an excitation with LED 400, 420, 450, 530 or 630. Since in all reported results, this signal is assumed to only consist of fluorescence, and no reflectance, the fluorescence gain for LED 660 is likely overestimated.

Several attempts were made to estimate the reflectance to estimate the errors caused by the reflected light from LED 660. However, we have not been able to get an estimate with a reasonable confidence interval, and thereby we are not able to report a reasonable estimate of how much the reflectance affect the measurements. This is partly, or fully, attributed to the low signal to noise ratio in the incident light spectrometer measurements in this waveband (signal level similar to noise level). Measurements with longer integration time to increase the signal level, or a spectrometer with a lower noise level, e.g., with a cooled detector, seems to be necessary for doing this estimation. If indeed LED 660 has a significant overlap with the chlorophyll fluorescence waveband it may be possible to mitigate it by moving the waveband towards longer wavelengths, or compensate for the overlap, or more drastically, using optical filters in the lamp to block light in the fluorescence region.

3.3. Photosynthetic Rate

3.3.1. Light Curve

The light curve, i.e., the photosynthetic rate as a function of incident light intensity, was measured for four different cucumber leaves using an infrared gas analyzer (IRGA), see Figure 8. One time the external LED lamp was used as the light source (starting with high intensity and stepwise decreasing), and the other three times the internal LED lamp in the IRGA-machine was used, once with increasing light intensity and twice with decreasing intensity. The black dots in the figure are from the three different background intensity levels of the experiments in set B.

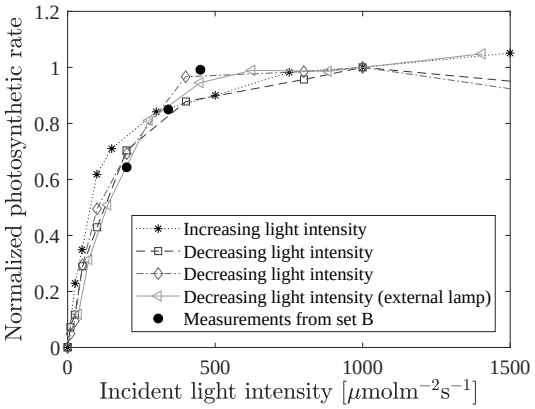


Figure 8. Normalized light curve for cucumber, i.e., the photosynthetic rate vs. light intensity.

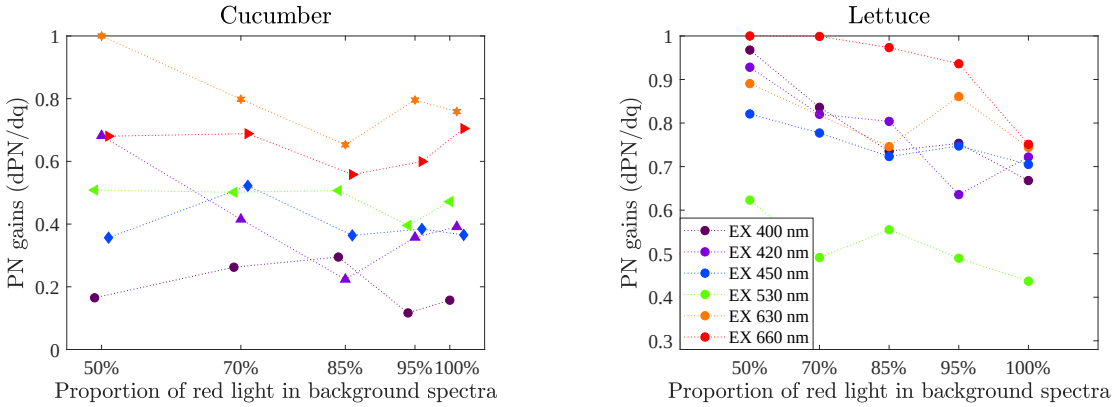
At low light the relation between incident light and photosynthetic rate is almost linear, but the dependency of the rate on the light intensity decreases, and for intensities higher than about 500 µmol m^{-2} s^{-1}, there is almost no increase in photosynthetic rate. This saturation phenomena

is not seen for the absolute value of the steady-state fluorescence, which continues to increase with increasing light intensity (data not shown). On the other hand, the mutual relation of the fluorescence gains seems to be unchanged after reaching light saturation, as was discussed in the previous section (see Figure 7a). Hence, one possible application of fluorescence gain measurements is to identify where light saturation occurs. This could, for example, be of interest in stress detection due to light inhibition, as an alternative to the method presented in [26].

3.3.2. Photosynthetic Rate Gains, dPN/dq

Figure 9 shows the photosynthetic rate gains as a function of incident light quality (50–100% red light) when the incident intensity was 160 µmol m^{-2} s^{-1} (set A). The measurements were repeated on four different leaves (for each species), of which we show their mean value. The variability was notably larger than in the fluorescence data because of the nature of the method, but shows similar behavior to the fluorescence gains, compare with Figure 6 upper row (in Appendix A Figure A3, a scatter plot of PN gain vs. F740 gain are shown). The red light gave the highest gains. For cucumber LED 630 had a slightly higher gain than LED 660, which was not the case for the fluorescence gain. However, the fluorescence gain for LED 660 was most likely overestimated (see Section 3.2.3). Further, LED 530 gave the lowest gain for lettuce whilst LED 400 gives the lowest gain for cucumber. No significant change in photosynthetic rate gain as a function of background spectrum could be proven.

In set B, the IRGA was used in three experiments on cucumber, once at each intensity level: 200, 350 and 500 µmol m^{-2} s^{-1} and with spectra of 50–100% red light. In Figure 10 the mean values of the PN gains at different intensity levels are plotted with the standard error of the mean (according to Equation (4)). The general picture is the same as for the cucumber in set A (recall set A had PAR 160 µmol m^{-2} s^{-1}, see Figure 9a), i.e., the gain was highest for red LEDs, followed by green and lowest for blue LEDs. Only in one out of the three intensity levels the PN gain of LED 400 was significantly lower than the other blue, which was the case for all sets of the fluorescence gains (see Figure 7a). The PN gain of LED 660 was lower (or equal) to that of LED 630, which is not the case for the fluorescence gains at lower light intensities (recall again though, that the fluorescence gain for LED 660 was most likely overestimated). However in this setup, measurements were only conducted on one leaf per intensity level. To draw any conclusion about differences in relative PN gains as a function of light intensity level, more repetitions would be needed and also on different leaves.



(**a**) Cucumber (**b**) Lettuce

Figure 9. Photosynthetic rate gains for each of the six LED colours when the background light quality varies from 50–100% red light, for (**a**) cucumber and (**b**) lettuce, normalized to the highest gain. Background intensity level was 160 µmol m^{-2} s^{-1} (set A). The plot shows the mean value of measurements on 4 different leaves.

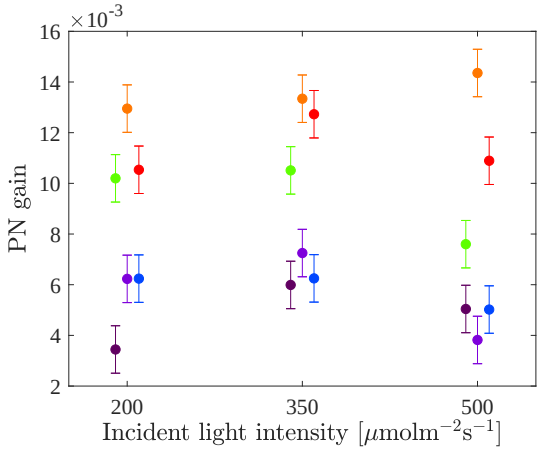


Figure 10. Photosynthetic rate gains vs. incident light intensity for cucumber (set B). Mean values of PN gains and one standard error of the mean are depicted. Note that different leaves have been used for the different intensity levels.

3.4. Comparisons

3.4.1. Comparison with McCree Data

Among many other species, McCree [7] measured the quantum yield (CO_2 uptake per absorbed photon) and absorbance rate for different wavelengths, for both cucumber and lettuce. Figure 11 shows the relative action spectra for these two (CO_2 uptake per incident photon), derived from data from McCree. Multiplying this action spectra and with the spectra of each individual LED colour from the excitation lamp (Heliospectra RX lamp, see spectra in Figure 1b), and calculating the integral, one gets the PN gain for each LED colour that would have been expected based on McCree1972's experiments. The outcome is shown in Figure 12a. Comparing these with the PN gains that we measured (Figures 9 and 10), they do match very well, despite of all the different settings. For cucumber, McCree also found a slightly higher gain for LED 630, compared to LED 660, and also the lowest gain for LED 400. For lettuce on the other hand, LED 660 were slightly higher than LED 630, the blue LEDs were all close to each other, and LED 530 had the lowest gain. Also this is in complete agreement with our experimental results of the PN gains.

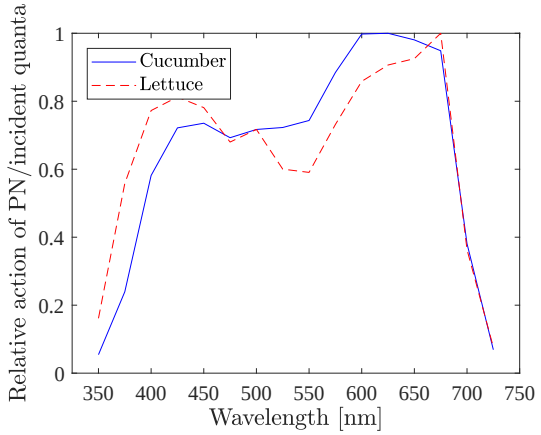


Figure 11. Relative action spectra, i.e., CO_2 uptake per incident quanta, derived from the experimental data given by McCree [7].

Figure 12b, is our experimentally measured fluorescence gains at the lowest background light that was used in this setup (160 µmol m^{-2} s^{-1}), i.e., the mean values of the gain for each colour in Figure 6a,b. It is remarkable how well these remotely measured steady-state fluorescence gains,

caused by excitation of different colours, fit with the old photosynthetic rate data from McCree. This comparison also supports the assumption that the fluorescence gain for LED 660 is overestimated (see Section 3.2.3).

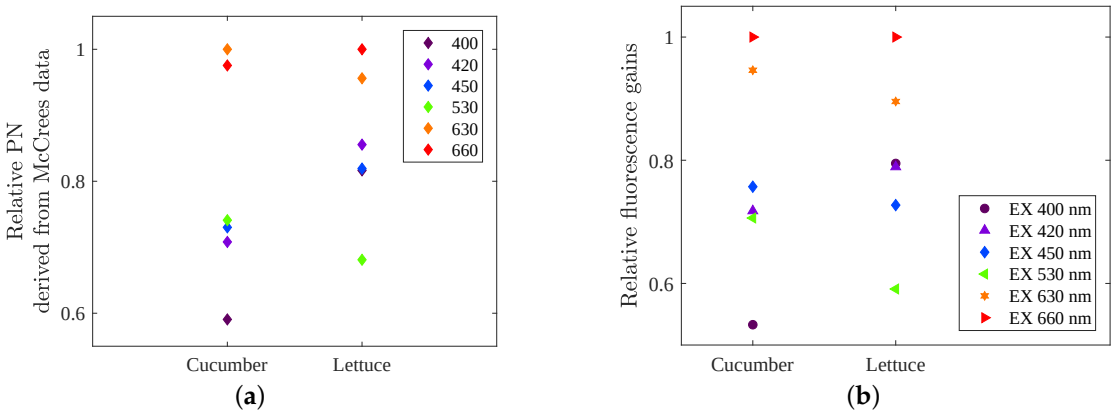


Figure 12. (**a**) Relative PN gain of each LED colour for cucumber and lettuce, calculated from data from McCree. (**b**) Experimentally derived fluorescence gains at background light intensity 160 µmol m^{-2} s^{-1} (set A).

3.4.2. Comparison with Dualex Measurements

The estimations of chlorophyll and flavonoid content, using Dualex Scientific's Chl and Flv indices (relative measurement), showed that cucumber has a significantly higher concentrations of both pigments compared to lettuce; twice higher chlorophyll content (35.0 $\pm$ 7.0 vs. 16.5 $\pm$ 4.2) and three times higher flavonoid content (1.04 $\pm$ 0.24 vs. 0.36 $\pm$ 0.08). Notice, the instrument has not been calibrated, hence only relative values are reported.

Flavonoids are concentrated on the surface structures and tissues of plants (cuticle and epidermis) and exerts some of its protection functions by screening incoming light in the UV but also the blue-violet part of the spectrum [27] the expected effect of a higher flavonoid content is a lower photosynthesis efficiency [28]. The effect is likely most distinct for the wavelengths where the flavonoids have the highest absorbance [27], it is expected to be highest for LED 400 and 420 in our experiment. Hence, the higher flavonoid content in cucumber could partly explain the lower fluorescence gains for LED 400 and somewhat also for LED 420, compared to the gains for lettuce.

A high photosynthetic rate for LED 630 and a low for LED 400 for cucumber (Figure 9a), could be a indicator of the relation between the content of chlorophyll *a* and *b*, in favor for chlorophyll *b*. The absorbtion spectrum for chlorophyll *b* in solvent has two peaks, at approximately 450 nm and 640 nm [29]. The absorbtion peaks for chlorophyll *a* on the other hand are slightly shifted, to around 430 nm and 660 nm. The blue peak for chlorophyll *a* is also essentially broader towards lower wavelengths, which means that chlorophyll *a* absorbs a considerably higher amount of light from LED 400, in comparison to chlorophyll *b*. Hence, a low photosynthetic rate gain for LED 400 together with a higher gain for LED 630 compared to LED 660, indicate that cucumber has a high content of chlorophyll *b* compared to chlorophyll *a*. However, it must be noted that the absorption in vivo and in a solvent differs [15], partly due to vibrational energy and the fact that the pigments are tightly packed and orbitals of the molecules overlaps.

4. Conclusions

A series of experiments have been conducted in order to evaluate whether fluorescence gains (see Equation (2)) could be used as feedback signals for energy optimising the spectrum of a LED lamp. The experiments did show a strong correlation between photosynthesis rate and steady state fluorescence even in the presence of background light, which entails the possibility of using the fluorescence gain as an indication of the strength of the photosynthetic rate. The advantage is that the

fluorescence is measured remotely, on canopy level, fast and without moving the plants. However, the conducted experiments did not indicate that the mutual relation of the fluorescence gains would change depending on the background light quality. That means, in contrast to our working hypothesis, there is no need for an online controller to work continuously to find the optimal spectrum for a given incident intensity. On the other hand, the experiments showed that for increasing light intensities, the mutual relation of the fluorescence gains do slightly change. However, as the intensity increases from 500 to 1000 µmol m^{-2} s^{-1} for cucumber, no further changes are obtained in the mutual relation of the fluorescence gains.

Due to the strong correlation between photosynthetic rate and fluorescence, and the fact that the mutual relation of the fluorescence gains are consistent at each incident intensity level, the fluorescence gains could be an interesting signal to monitor throughout the growth cycle. One possible application is to identify the light saturation level for photosynthesis, since the changes in the mutual relation of the fluorescence gains saturates at about the same intensity level as where light saturation for photosynthesis occurs. Another application, which is the aim of our further research, is to evaluate if changes in mutual relation of the fluorescence gains might be indication of an induced stress in the plant. Yet another application, is to use the current to the lamp as the input signal (instead of measured incident light quanta), and thereby detect if any specific LED group have degraded more than the others, and in such case reconsider the power distribution over the different LEDs.

Author Contributions: L.A. and D.B. carried out the experiments. L.A. analyzed the data and wrote the manuscript with support from D.B. T.W. conceived the original idea and supervised the project.

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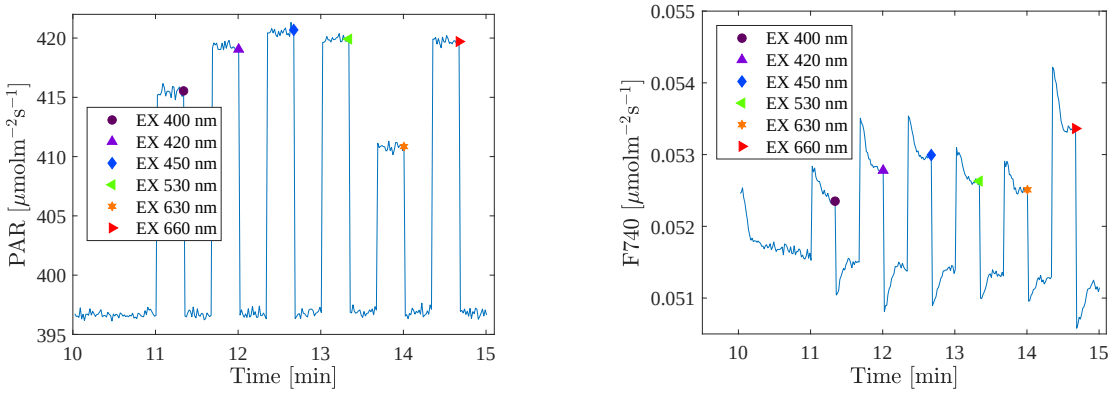
Conflicts of Interest: The authors declare no conflict of interest.

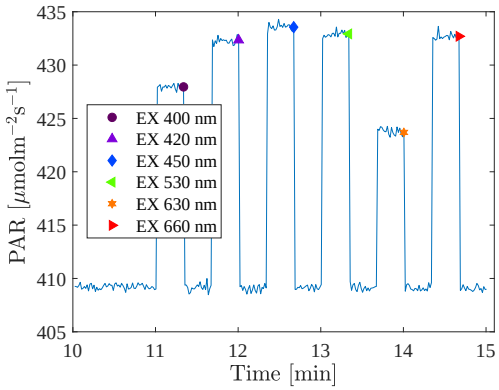
Appendix A



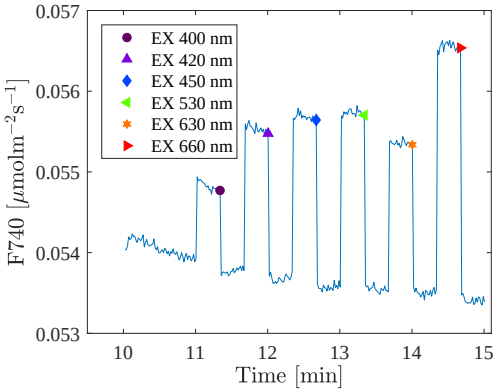
(**a**) Incident light intensity in the wavelength span 350–700 nm

(**b**) Fluorescence light intensity in the wavelength span 735–745 nm

Figure A1. Measurements on lettuce, holding each intensity level at 20 s. (**a**) Incident and (**b**) fluorescence light intensity (µmol m^{-2} s^{-1}) vs. time for one background light level, i.e., background intensity 500 µmol m^{-2} $s^{-1}$and spectrum having 85% red light. An excitation step is sequentially added by each LED colour. Note that the intensity level measured during the experiment was only about 397 and not 500 µmol m^{-2} s^{-1}. This is because when the light level was determined, no plants were present in the experimental unit and another type of spectrometer was used.

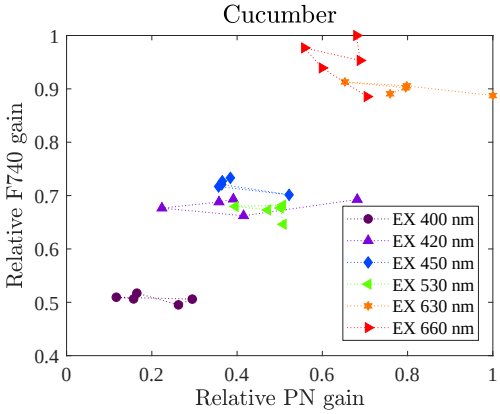


(**a**) Incident light intensity in the wavelength span 350–700 nm

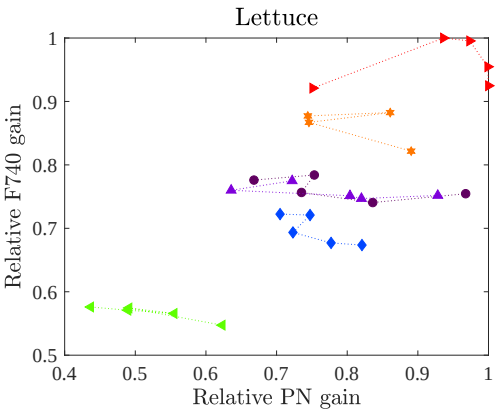


(**b**) Fluorescence light intensity in the wavelength span 735–745 nm

Figure A2. Measurements on cucumber, holding each intensity level at 20 s. (**a**) Incident and (**b**) fluorescence light intensity (µmol m^{-2} s^{-1}) vs. time for one background light level, i.e., background intensity 500 µmol m^{-2} s^{-1}and spectrum having 85% red light. An excitation step is sequentially added by each LED colour. Note that the intensity level measured during the experiment was only about 409 and not 500 µmol m^{-2} s^{-1}. This is because when the light level was determined, no plants were present in the experimental unit and another type of spectrometer was used.



(**a**) Cucumber



(**b**) Lettuce

Figure A3. Fluorescence gain vs. photosynthetic rate gain for background light 160 µmol m^{-2} s^{-1} (set A), i.e., combining Figures 6 and 9. The five points of each colour correspond to the five different background spectra (50–100% red light), and the colour indicates the colour of the added excitation light. The variability in the PN data is higher than in the F740 data.

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